Nitric oxide inhibits ornithine decarboxylase via S-nitrosylation of cysteine 360 in the active site of the enzyme.¹

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Running Title: S-nitrosylation of ornithine decarboxylase by nitric oxide

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SUMMARY

Ornithine decarboxylase is the initial and rate-limiting enzyme in the polyamine biosynthetic pathway. Polyamines are found in all mammalian cells and are required for cell growth. We previously demonstrated that N-hydroxyarginine and nitric oxide inhibit tumor cell proliferation by inhibiting arginase and ornithine decarboxylase, respectively, and, therefore, polyamine synthesis. In addition, we showed that nitric oxide inhibits purified ornithine decarboxylase by S-nitrosylation. Herein, we provide evidence for the chemical mechanism by which nitric oxide and S-nitrosothiols react with cysteine residues in ornithine decarboxylase to form an S-nitrosothiol(s) on the protein. The NONOate nitric oxide donor agent DEA/NO acts through an oxygen-dependent mechanism leading to formation of the nitrosating agents N2O3 and/or N2O4. S-Nitrosoglutathione inhibits ornithine decarboxylase by an oxygen-independent mechanism, likely by S-transnitrosation. In addition, we provide evidence for the S-nitrosylation of four cysteine residues per ornithine decarboxylase monomer including cysteine 360, which is critical for enzyme activity. Finally, S-nitrosylated ornithine decarboxylase was isolated from intact cells treated with nitric oxide, suggesting that nitric oxide may regulate ornithine decarboxylase activity by S-nitrosylation in vivo.
INTRODUCTION

Ornithine decarboxylase (ODC), the rate-limiting enzyme in putrescine synthesis, catalyzes the conversion of ornithine to putrescine, and is essential for polyamine synthesis in mammalian cells. In spite of the fact that polyamines are present in all mammalian cells, extensive research efforts have failed to fully elucidate their physiological functions. That polyamines are required, however, in order to maintain cell growth and function is clearly established (1-3). Many early studies indicate that polyamine synthesis is enhanced during growth and that growth-promoting stimuli lead to increases in polyamine biosynthesis. Direct evidence for this has been provided by experiments in which polyamine synthesis was prevented in mammalian cells in culture by mutations to key enzymes (such as ODC) or by the application of enzyme inhibitors (2, 4-6). This led to cessation of growth unless exogenous polyamines were provided. More recent studies have shown that polyamines stimulate DNA synthesis and increase the transcription of growth related genes (7-8).

Increases in nitric oxide synthase activity and addition of exogenous nitric oxide (NO) have been widely recognized to result in inhibition of cell proliferation (11-15). In many of these studies, cyclic-3′,5′-guanosine monophosphate (cyclic GMP) did not account for the cytostatic effect of NO, and the evidence for cyclic GMP-dependent inhibition of cell proliferation is at best inconsistent and indirect. We have consistently observed cyclic GMP-independent cytostatic effects of NO in a variety of mammalian cell types including, Caco2 human tumor cells, murine macrophages, and rat aortic endothelial and smooth muscle cells (15-16 and unpublished observations). One such mechanism appears to be the inhibition of ODC by NO. In our studies, the cytostatic effect of NO on tumor cells or
rat aortic smooth muscle cells was reversed by the addition of exogenous polyamines but not by ornithine, suggesting that NO inhibited ODC.

ODC is a homodimer and forms two active sites at the interface between the two monomers. It contains two cysteine residues (C70 and C360) in each active site (19) and a total of 12 cysteine residues per monomer. C360 is required for enzymatic activity, as mutating C360 to alanine reduces enzyme activity by 98% (20, 21). Nitrogen oxide species have been shown to readily react with both protein and low molecular weight thiols to form S-nitrosothiols (22, 23). An increasing number of proteins such as albumin, p21ras, caspase 3, glyceraldehyde 3-phosphate dehydrogenase, hemoglobin, and NFκ-B as well as the low molecular weight thiols, cysteine and glutathione, have been found to be S-nitrosylated in vivo (22, 24-28). In addition, many proteins, including ODC, have been shown to be S-nitrosylated in vitro (29).

The objective of this study was three-fold: (1) to provide further evidence for the S-nitrosylation of ODC and the chemical mechanism by which it is S-nitrosylated, (2) to determine if C360, the critical cysteine residue in the active site of ODC, is S-nitrosylated, and (3) to determine if ODC is S-nitrosylated in intact cells.

EXPERIMENTAL PROCEDURES

Chemicals and Solutions: Unless otherwise noted all chemicals and reagents were purchased from Sigma, St. Louis, MO. L-[1-14C]-Ornithine was purchased from Dupont NEN, Boston, MA. Ecolite scintillation cocktail was purchased from ICN, Costa Mesa, CA. S-Nitrosoglutathione (GSNO) (30) and S-nitrosocysteine (CysNO) (31) were synthesized as previously described. 1-Diethyl-2-hydroxy-2-nitroso-hydrazine (DEA/NO)
was a kind gift from David A. Wink, National Institutes of Health, Bethesda, MD. Saturated NO solutions were prepared by bubbling pure NO gas through water under anaerobic conditions.

*Preparation of Plasmids:* pHIS-ODC was prepared as previously described (29). The resulting plasmid codes for an ODC protein with the following amino terminus: MRGSHHHHHHGS. For the pHIS-ODC C360A mutant, cysteine 360 was mutated to alanine in pGEM-ODC using the chameleon mutagenesis kit (Stratagene, La Jolla, CA). The pGEM construct was then digested with *Sph* I and *Sal* I, and the fragment containing the mutation was isolated and inserted into pHIS-ODC. pCMVZeo-ODC was prepared as previously described (32). The plasmid codes for ODC truncated at amino acid residue 425. The truncated protein maintains activity comparable to wild type ODC but is no longer degraded by the proteasome.

*Expression and Purification of HIS-ODC:* pHIS-ODC or pHIS-ODC C360A was expressed and purified from XL1-Blue *E. Coli* (Stratagene, La Jolla, CA) as previously described (33).

*Ornithine Decarboxylase Assay:* ODC activity was determined by monitoring the formation of \([^{14}\text{C}]\) CO₂ from L-[1-\(^{14}\text{C}\)]ornithine (29, 34). Dithiothreitol (DTT) was separated from the enzyme by gel filtration through a sephadex G-25 column in all experiments. For anaerobic experiments all solutions were deoxygenated by either bubbling argon through the solution or by purging the headspace with argon for at least 20 minutes. All anaerobic experiments were performed in a glove bag (I2R, Cheltenham, PA) under positive pressure argon.
**Photolysis-Chemiluminescence:** Samples were injected directly into a photolysis chamber that consists of borosilicate glass capillary tubing coiled around a 200-W high-pressure mercury vapor lamp (Hanovia, Newark, NJ). A carrier stream of nitrogen carries the effluent through the photolysis chamber where the photolabile S-NO bond is broken resulting in the formation of NO and thiol radical (S·). NO, in the gas phase, is then carried through a cold trap (−75ºC) and swept into the chemiluminescence detector. Chemiluminescence was performed as previously described (15). Authentic GSNO was used to make a standard curve and the concentration of the GSNO stock solution was confirmed by spectrophotometry. NO release from GSNO was not detectable when the light source was off.

**Determination of Protein Concentration:** Protein concentrations were determined by the Bradford Coomassie brilliant blue method (Bio-Rad Laboratories, Hercules, CA), using bovine serum albumin as the standard.

**Determination of Protein SNO Content:** Purified ODC or C360A ODC was gel filtered on a G-25 sephadex column pre-equilibrated with 50 mM Tris-HCl, 0.1 mM diethylenetriaminepentaacetic acid, pH 7.5 (assay buffer), to remove DTT. The buffer was then exchanged twice using microcon centrifugal filter devices (Amicon) with a 30,000 kD cut off to further remove DTT and concentrate the protein. ODC, C360A ODC or BSA was incubated for 15 min at room temperature with 1 mM sodium cyanoborohydride, a mild reducing agent to assure that thiols were in the reduced state. The samples were then incubated 1:1 (v/v) with a saturated NO solution (~2 mM NO as determined by spectrophotometry) in the dark at room temperature for 30 min. Excess
NO was removed from the solution by purging the headspace with argon for at least 20 minutes and then analyzed by photolysis-chemiluminescence.

**Cell Culture:** NIH 3T3 cells were grown in DMEM-Hepes medium supplemented with 10% (v/v) FBS, 2 mM L-glutamine, 100 units/ml penicillin, and 100 µg/ml streptomycin. Cell cultures were performed at 37°C in a humidified atmosphere of 5% CO₂-95% air, and were subcultured by trypsinization.

**Plasmid Transfection:** 5 x 10⁵ Cells were seeded in 10 cm plates and transfected the next day with 10 µg of pCMVZeo-ODC or pCMVZeo using SuperFect transfection reagent (Qiagen, Valencia, CA) following methods described by the manufacturer. The cells were then allowed to grow for another 48 hours before use.

**Western Blot Analysis:** Cell lysates from untransfected, pCMVZeo transfected or pCMVZeo-ODC transfected NIH 3T3 cells were prepared using ice cold lysis buffer containing 50 mM Tris HCl (pH 8.0), 150 mM NaCl, 1% NP-40, 1 mM EDTA, 1 µg/ml leupeptin, 1 µg/ml pepstatin, and 1 µg/ml aprotinin. 20 µg total protein from each sample were subjected to SDS-polyacrylamide (10%) gel electrophoresis and proteins were transferred to a nitrocellulose membrane. The membrane was then probed with rabbit anti-ODC antiserum (0.5 µg/ml) followed by goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (1:2000 dilution) (Cell Signaling Technologies, Beverly, MA). Blots were then visualized using LumiGlo chemiluminescent detection reagent (Cell Signaling Technology, Beverly, MA) as described by the manufacturer.

**Immunoprecipitation of ODC:** Cell lysates from 1-2 x 10⁸ pCMVZeo transfected or pCMVZeo-ODC transfected NIH 3T3 cells were prepared as above. The lysates were aliquoted into five tubes so that immunoprecipitations were carried out on lysates from 2-
4 x 10^7 cells. Immunoprecipitations were performed using control rabbit IgG or rabbit anti-ODC antiserum and protein G-Sepharose (Amersham-Pharmacia, Piscataway, NJ) per the manufacturers instructions. Before dissociation of the immunocomplexes, the original 5 aliquots of each sample were again pooled. The antigen-antibody complexes were then dissociated from the beads with 500 μl of 100 mM glycine buffer (pH 2.5). The protein G-Sepharose beads were removed by centrifugation and the supernatant was kept on ice prior to analysis.

RESULTS

The effects of NO donor agents on ODC activity—We previously reported that NO donor agents inhibit purified ODC via S-nitrosylation (29). We show here that the NO donor agents GSNO and DEA/NO inhibit purified ODC in a concentration-dependent manner under aerobic conditions (Fig.1). Furthermore, the inhibition of ODC by both NO donor agents was reversible by addition of 2.5 mM DTT and to a lesser extent by 5 mM glutathione (GSH), providing evidence that NO acts to inhibit the enzyme by S-nitrosylation (Fig.1).

GSNO was more potent than DEA/NO as an inhibitor of ODC despite the fact that GSNO does not readily release NO. DEA/NO, on the other hand, has a half-life of NO release of approximately 2 minutes. This led us to investigate the mechanism of S-nitrosylation by the two NO donor agents. We set forth the hypothesis that GSNO acts by participating in an S-transnitrosation reaction with cysteine residue(s) on ODC, whereas DEA/NO likely acts through the reaction of NO with O2 to form N2O3 and N2O4, both of which are nitrosating agents (Fig.2).
We first tested the capacity of GSNO and DEA/NO to inhibit ODC under anaerobic conditions. According to our hypothesis, GSNO should inhibit ODC under anaerobic conditions whereas DEA/NO should not, since the formation of N_2O_3 or N_2O_4 from NO is oxygen-dependent. As predicted, GSNO was equipotent under aerobic and anaerobic conditions (Fig.3). The inhibition of ODC caused by GSNO under anaerobic conditions was, like under aerobic conditions, reversible by DTT and GSH (Fig.4). This suggests that GSNO acts by the same mechanism under both aerobic and anaerobic conditions.

DEA/NO failed to significantly inhibit ODC under anaerobic conditions at all concentrations tested (Fig.5), demonstrating that NO released from DEA/NO must react with oxygen in order to inhibit ODC. This result lends credence to the hypothesis that DEA/NO acts through the reaction of NO and O_2. In order to provide further evidence for this we took advantage of the reaction between NO and the NO scavenger 2-phenyl-4,4,5,5-tetramethyl-imidazoline-1-oxyl 3-oxide (PTIO) (35). PTIO acts to scavenge NO by oxidizing it to NO_2 (Fig.2). However, NO_2, as shown in figure 2, can react with another molecule of NO or dimerize to form the nitrosating agents N_2O_3 or N_2O_4, respectively. Under anaerobic conditions, 100 µM DEA/NO in the presence of 30 µM PTIO inhibited ODC by approximately 80%, whereas DEA/NO or PTIO alone had no effect (Fig.6). The inhibition of ODC by the combination of DEA/NO and PTIO was reversible by the addition of DTT or GSH, again suggesting that ODC was S-nitrosylated (Fig.7).

Analysis of S-nitrosylated ODC by photolysis-chemiluminescence—Photolysis-chemiluminescence is an established method for determining the S-nitrosothiol content of proteins (22). We, therefore, developed our own apparatus to utilize photolysis-
chemiluminescence to study the S-nitrosylation of ODC by NO. The present method was somewhat less sensitive than that previously described by Stamler et al. (22, 36). This was not a limitation as we were able to repeat Stamler’s earlier work showing that albumin, when in its native state, is S-nitrosylated on a single cysteine residue. 250 µl of a 100 µg/ml solution of albumin was mixed 1:1 (v/v) with a saturated NO solution and incubated for 30 min in the dark at room temperature. Excess NO was removed from the solution by purging the headspace with argon for 20 min. The sample was then analyzed by photolysis-chemiluminescence. A standard curve was prepared using authentic GSNO (Fig.8A). The concentration of GSNO stock solution was verified by spectrophotometry. In agreement with Stamler, albumin was determined to contain 0.91 ± 0.14 mol of SNO per mol of protein (Fig.8B). The same procedure using purified ODC and treatment of ODC with NO resulted in the S-nitrosylation of 3.89 ± 0.23 thiol residues per ODC monomer (Fig.8B), strengthening the hypothesis that NO S-nitrosylates ODC.

As a means of determining if C360 is one of the four residues in ODC S-nitrosylated by NO, C360A ODC, in which C360 is mutated to alanine, was analyzed as above. C360A ODC was determined to contain 2.81 ± 0.17 SNO molecules per ODC monomer (Fig.8B). This is one less SNO molecule per monomer than wild type ODC and was significantly different as determined by the Student’s t-test (p<0.01).

*Isolation of S-nitrosylated ODC from intact cells*– Our final objective was to determine whether S-nitrosylated ODC could be isolated from cells that had been treated with NO. ODC was overexpressed in NIH 3T3 cells by transfection with pCMVZeo-ODC. This plasmid codes for an ODC protein that is truncated at the C-terminus at
amino acid residue 425. Since the C-terminus is required for ODC to be degraded by the proteasome, the truncated form of the protein is stable and can accumulate to very high levels within the cell. pCMVZeo-ODC transfected cells showed a marked increase in ODC protein as determined by Western blot analysis (Fig.9A), and an approximate 200-fold increase in ODC activity (Fig.9B) when cell lysates from untransfected or pCMVZeo transfected cells were compared to pCMVZeo-ODC transfected cells.

Control cells or cells overexpressing ODC were then treated with 1 mM CysNO and incubated for 30 min. The cells were harvested, lysed, and subjected to immunoprecipitation with control rabbit IgG or rabbit anti-ODC polyclonal antibody and protein G-Sepharose as described in Experimental Procedures. The immunocomplexes were then eluted from the protein G-Sepharose and the eluate was analyzed by photolysis-chemiluminescence. S-Nitrosylated protein was not detected in pCMVZeo transfected cells immunoprecipitated with the anti-ODC antibody or pCMVZeo-ODC transfected cells immunoprecipitated with normal rabbit IgG (Fig.10). This suggests that in both cases there was either no S-nitrosylated ODC or that it was below the detectable limit. In pCMVZeo-ODC transfected cells immunoprecipitated with anti-ODC antibody, 36.8 ± 4.5 pmol of S-nitrosylated protein per mg of total cell protein was detected (Fig.10). In addition, there was no detectable S-nitrosylated ODC in immunoprecipitates from pCMVZeo-ODC transfected cells that were not treated with CysNO (data not shown).
DISCUSSION

When ODC is inactivated by the enzyme-activated, irreversible inhibitor, α-difluoromethylornithine (DFMO), a stoichiometric amount of a covalent adduct is formed with the protein, with the major site for this reaction being C360 (37). This suggests that C360 must be located in the active site of ODC, which was recently confirmed by x-ray crystallography (19). The importance of this residue has been established by mutation of this cysteine to serine or alanine (38).

There is ample evidence that NO inhibits ODC. The initial evidence supporting this came from a study in which sodium nitroprusside was shown to inhibit cell proliferation by inhibiting putrescine synthesis (11). Further evidence of this has been provided by studies in our laboratory in which inhibition of Caco-2 cell proliferation or vascular smooth muscle cell proliferation by NO was shown to be reversible by addition of excess putrescine, spermidine, or spermine, but not ornithine (15, 16). It was also shown that NO could inhibit ODC in crude cell lysates of Caco-2 cells or vascular smooth muscle cells. Similar studies have since been published showing inhibition of ODC in crude cell lysates by NO donor agents (17, 18).

We recently demonstrated that NO donor agents inhibit purified ODC by S-nitrosylation (17). In the current study, NO, in the form of GSNO or DEA/NO, inhibited ODC in a concentration-dependent manner. Inhibition of ODC by both NO donor agents was reversible by addition of DTT or GSH, supporting the hypothesis that NO inhibits ODC via S-nitrosylation of a critical cysteine residue(s) on ODC. The present data further support the hypothesis that NO and related species inhibit ODC by S-nitrosylation. Photolysis chemiluminescence is an effective tool for determining not only
whether a protein is S-nitrosylated but also for quantifying the number of residues modified by S-nitrosylation (22). It combines both specificity and sensitivity, since SNOs are photolabile and the chemiluminescence detectors used for detecting NO are both specific for NO and very sensitive. Exposure to ultraviolet light may result in the formation of NO also from nitrite, nitrosamines, and dinitrosyliron complexes and was controlled for in our experiments. Using this method we show that approximately 4 cysteine residues per ODC monomer are S-nitrosylated when the enzyme is treated with a saturated NO solution. In addition, we provide evidence that C360, which is critical for enzyme activity, is S-nitrosylated since the C360A ODC mutant is S-nitrosylated on only 3 cysteine residues.

These findings led us to investigate whether S-nitrosylated ODC could be isolated from cells treated with NO. NIH 3T3 cells transfected with pCMVZeo-ODC and treated with 1 mM CysNO were subjected to immunoprecipitation with anti-ODC antibody. This resulted in the detection of 36.8 ± 4.5 pmol SNO per mg of total cellular protein by photolysis-chemiluminescence. S-nitrosylated protein was not detected in cells transfected with pCMVZeo and immunoprecipitated with anti-ODC antibody. pCMVZeo-ODC transfected cells immunoprecipitated with normal rabbit IgG also resulted in no detection of S-nitrosylated protein, demonstrating that the positive result was not due to non-specific protein interactions or conversion of nitrite, nitrosamines, or dinitrosyliron complexes to NO by exposure of the sample to UV light. These data demonstrate that ODC is S-nitrosylated in intact cells and suggest that the same may occur in vivo.
Both S-nitrosothiol NO donor agents and the NONOate NO donor agents inhibit purified ODC. The S-nitrosothiol GSNO, however, is approximately 6 fold more potent than the NONOate DEA/NO. Not all classes of NO donor agents release NO by common mechanisms. GSNO releases NO only under specific conditions, such as in the presence of transition metals or light. DEA/NO, on the other hand, spontaneously releases NO at pH 7.4 with a half-life of 2 min at 37°C and releases 2 mol of NO per mol of DEA/NO. The mechanisms by which NO donor agents cause S-nitrosylation of ODC are dependent on the class of NO donor agent.

The inhibition of ODC by DEA/NO was O$_2$-dependent, suggesting that the NO released from DEA/NO is autoxidized to N$_2$O$_3$ or N$_2$O$_4$. This was substantiated by experiments in which ODC was incubated under anaerobic conditions in the presence or absence of PTIO. PTIO is an NO scavenger that acts by oxidizing NO to NO$_2$, which is essentially equivalent to the reaction of NO with O$_2$ to form NO$_2$ (35). The reaction of NO with O$_2$ is third order overall and second order with respect to NO, indicating that the rate of formation of NO$_2$ is dependent on the square of the NO concentration. Therefore, as the NO concentration decreases the reaction rate slows dramatically. Under physiological conditions, where the NO concentration is very low (less than 100 nM), the reaction of NO with O$_2$ is very slow, which allows for the diffusion of NO away from its source enabling NO to react with other biological targets. In fact, the suggestion has been made that the reaction between NO and O$_2$ may not be relevant under physiological conditions. However, both NO and O$_2$ are lipophilic molecules and tend to concentrate in biological membranes, perhaps facilitating this reaction. Indeed, this reaction takes place approximately 1500 times faster in micelles as compared to aqueous solution (39).
Therefore, whereas this reaction may not take place in the cell cytosol, it may be important in biological membranes.

The inhibition of ODC, by GSNO, on the other hand was independent of O₂. Furthermore, the enzyme inhibition caused by GSNO was equipotent in the presence and absence of oxygen and was reversible by the addition of DTT or GSH under both conditions. These data suggest that GSNO acts by the same mechanism under aerobic or anaerobic conditions. Furthermore, these data rule out the possibility that GSNO simply acts through release of NO, since NO by itself does not readily react with thiol residues. Besides being able to release NO under the appropriate conditions, S-nitrosothiols can react with thiols resulting in either the release of nitroxy1 (HNO) or donation of nitrosonium (NO⁺) to another thiol via S-transnitrosation. Generation of HNO and its subsequent attack on thiol residues would likely lead to the formation of an N-hydroxysulfenamide, sulfinate, or sulfinic acid (40). S-Transnitrosation reactions result in the transfer of an NO⁺ equivalent from one thiol to another resulting in the formation of a new S-nitrosothiol. S-Transnitrosation is considered to be the predominant mechanism for the biological actions of GSNO (41), and S-transnitrosation has been demonstrated in vivo between S-nitrosylated albumin and low-molecular-weight thiols (42). S-Transnitrosation reactions are relatively fast reactions and are O₂-independent, which is consistent with the higher potency of GSNO as an inhibitor of ODC, and, explains why GSNO inhibits ODC under anaerobic conditions.

Although the presence of low molecular weight thiols such as GSNO and CysNO has been demonstrated in extracellular environments such as plasma, most of the evidence for intracellular low molecular weight thiols is indirect. For example, Mayer et al.
demonstrated that in rat isolated hearts a Cu\(^+\) specific chelator prevented bradykinin-induced cyclic GMP accumulation but did not affect cyclic GMP accumulation due to exogenous NO sources (43). Since Cu\(^+\) is known to catalyze the release of NO from nitrosothiols, it was concluded that an S-nitrosothiol must be involved. However, Stamler et al. recently described the identification of an enzyme, termed ‘GSNO reductase’, which metabolizes GSNO, therefore, regulating intracellular levels of GSNO (44). They demonstrate that deleting the reductase gene abolishes the GSNO-consuming activity of cells and increases intracellular levels of GSNO. The regulation of GSNO within the cell supports the idea that GSNO is an important intracellular molecule and provides evidence for the formation of GSNO intracellularly.

Biologically speaking, however, the problem arises as to how S-transnitrosation reactions are targeted to specific thiols on proteins, when intracellular GSH concentrations can be as high as 5-10 mM and the concentration of total cellular protein thiols is significantly higher than that. Specificity may come from the reactivity of the target thiol, which can vary by several orders of magnitude. For example thiolates, unprotonated thiols, are more reactive than the protonated form. In addition, S-transnitrosation reactions are reversible with the equilibrium favoring the more stable protein nitrosothiols over low molecular weight nitrosothiols (45). Another theory, proposed by Stamler et al., is that basic and acidic amino acids in the vicinity of the target thiol residue catalyze S-transnitrosation reactions by acid-base catalysis, leading to enhanced reactivity of that cysteine residue (46). In fact, Stamler et al. have proposed that (K,R,H)C(D,E) is a consensus motif for the S-nitrosylation of proteins by S-transnitrosation, with the acidic amino acid following the cysteine residue being the most
important. Although ODC does not have a basic amino acid immediately preceding C360, it does have an aspartate residue immediately following C360. The crystal structure of ODC further reveals that the active site contains several acidic and basic amino acids in the vicinity of C360.

We provide evidence herein of the S-nitrosylation of ODC on 4 cysteine residues including C360, a critical thiol residue in the active site of ODC. In addition, we demonstrate the isolation of S-nitrosylated ODC from cells overexpressing ODC, suggesting that ODC may be S-nitrosylated in vivo. We further reveal that low molecular weight S-nitrosothiols inhibit ODC with a greater potency than NO itself and by a different mechanism. The hypothesis is forwarded that low molecular weight S-nitrosothiols may be physiological regulators of ODC activity and, therefore, cell proliferation. We recently demonstrated that NO inhibits vascular smooth muscle cell proliferation by inhibiting ODC. However, low molecular weight S-nitrosothiols and not NO may be the physiological regulator of vascular smooth muscle cell proliferation since the former are found in vivo (41) and are more potent inhibitors of ODC.

REFERENCES


**FOOTNOTES**

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2 The abbreviations used are: ODC, ornithine decarboxylase; NO, nitric oxide; cyclic GMP, cyclic-3',5'-guanosine monophosphate; GSNO, S-nitrosoglutathione; CysNO, S-nitrosocysteine; DEA/NO, 1-diethyl-2-hydroxy-2-nitroso-hydrazine; DTT, dithiothreitol; GSH, glutathione; PTIO, 2-phenyl-4,4,5,5-tetramethyl-imidazoline-1-oxyl 3-oxide; DFMO, α-difluoromethylornithine; HNO, nitroxyl
FIGURE LEGENDS

Fig.1. The inhibitory effects of NO donor agents on purified ODC and its reversibility by thiols. DTT was separated from the enzyme by gel filtration through a sephadex G-25 column in all experiments. ODC was preincubated with various concentrations of GSNO or DEA/NO at 37°C for 15 min. ODC activity was measured after a 30 min incubation at 37°C in the presence or absence of 2.5 mM DTT or 5 mM GSH as described in Experimental Procedures. Data represent the mean ± SE of duplicate determinations from 3 separate experiments.

Fig.2. Reaction mechanisms involving GSNO and NO

Fig.3. The inhibitory effects of GSNO on purified ODC under aerobic vs. anaerobic conditions. Experiments were conducted under aerobic (○) or anaerobic (●) conditions. DTT was separated from the enzyme by gel filtration through a sephadex G-25 column in all experiments. ODC was preincubated with various concentrations of GSNO at 37°C for 15 min and then assayed as described in Experimental Procedures. Data represent the mean ± SE of duplicate determinations from 4 separate experiments.

Fig.4. The inhibition of purified ODC by GSNO under anaerobic conditions is reversible by thiols. Experiments were conducted either under positive pressure argon (anaerobic) or in room air (aerobic). DTT was separated from the enzyme by gel filtration through a sephadex G-25 column in all experiments. ODC was preincubated with 10 µM GSNO at 37°C for 15 min and then assayed in the presence or absence of 2.5 mM DTT or 5 mM GSH as described in Experimental Procedures. Data represent the mean ± SE of duplicate determinations from 4 separate experiments.
Fig.5. The inhibitory effects of DEA/NO on purified ODC under aerobic vs. anaerobic conditions. Experiments were conducted under aerobic (○) or anaerobic (●) conditions. DTT was separated from the enzyme by gel filtration through a sephadex G-25 column in all experiments. ODC was preincubated with various concentrations of DEA/NO for 15 min and then assayed as described in Experimental Procedures. Data represent the mean ± SE of duplicate determinations from 4 separate experiments.

Fig.6. The effects of PTIO and DEA/NO on purified ODC under anaerobic conditions. Experiments were conducted under positive pressure argon. DTT was separated from the enzyme by gel filtration through a sephadex G-25 column in all experiments. ODC was preincubated with or without 100 µM DEA/NO and/or 30 µM PTIO at 37°C for 15 min and then assayed as described in Experimental Procedures. Data represent the mean ± SE of duplicate determinations from 3 separate experiments.

Fig.7. The inhibition of ODC by PTIO and DEA/NO under anaerobic conditions is reversible by thiols. Experiments were conducted under positive pressure argon. DTT was separated from the enzyme by gel filtration through a sephadex G-25 column in all experiments. ODC was preincubated with or without 100 µM DEA/NO and/or 30 µM PTIO at 37°C for 15 min and then assayed in the absence or presence of 2.5 mM DTT or 5 mM GSH as described in Experimental Procedures. Data represent the mean ± SE of duplicate determinations from 4 separate experiments. *Significantly different (p<0.01) from values obtained in the absence of added DTT or GSH.

Fig.8. ODC is S-nitrosylated at C360, a critical thiol in the active site of the enzyme. (A) GSNO standard curve using photolysis-chemiluminescence. The standard curve was linear between 50 pmol and 10 nmol GSNO with 10 nmol being the highest
amount of GSNO tested. The equation for the line was $y = 0.4564x - 12.159$ with a correlation coefficient of $R^2 = .988$. (B) SNO quantitation on albumin and ODC. Albumin, ODC or C360A ODC was incubated 1:1 (v/v) with a saturated NO solution in the dark at room temperature for 30 min and then analyzed by photolysis-chemiluminescence as described in Experimental Procedures. *Significantly different (p<0.01) from values for wild type ODC. Data represent the mean ± SE of duplicate determinations from 5 separate experiments.

**Fig.9. Overexpression of ODC in NIH 3T3 cells.** NIH 3T3 cells were transiently transfected with the pCMVZeo-ODC plasmid or pCMVZeo control vector. 48 hrs after transfection the cells were lysed and subjected to: (A) Western blot analysis for ODC in untreated cells, pCMVZeo transfected cells and pCMVZeo-ODC transfected cells. (B) Assay of ODC activity in cell lysates of untransfected, pCMVZeo transfected, or pCMVZeo-ODC transfected cells. Data represent the mean ± SE of duplicate determinations from 3 separate experiments.

**Fig.10. Isolation of S-nitrosylated ODC from intact cells.** NIH 3T3 cells were transiently transfected with the pCMVZeo-ODC plasmid or pCMVZeo control vector. 48 hrs after transfection the cells were treated with 1 mM CysNO. The supernatant from the lysates of the NO treated cells were subjected to immunoprecipitation with rabbit anti-ODC antiserum or with control rabbit IgG and protein G-Sepharose. Immunoprecipitates were then analyzed by photolysis-chemiluminescence. n.d., not detected. Data represent the mean ± SE of duplicate determinations from 4 separate experiments.
FIGURE 1

ODC Activity (% of Control Activity)

[µM]  |  3  |  10 |  30 |  30 |  30 |  1  |  3  |  10 |  10 |  10

2.5 mM DTT | -   | -   | -   | -   | -   | +   | -   | -   | +   | -
5 mM GSH  | -   | -   | -   | -   | +   | -   | -   | -   | -   | +

DEA/NO    |                  |                  |
GSNO       |                  |                  |
Transnitrosation

\[
\text{GSNO} + \text{RSH} \rightarrow \text{GSH} + \text{RSNO}
\]

Oxygen-dependent S-nitrosylation

\[
\begin{align*}
2\text{NO} + \text{O}_2 & \rightarrow 2\text{NO}_2 \\
\text{NO}_2 + \text{NO}_2 & \rightarrow \text{N}_2\text{O}_4 + \text{RSH} \rightarrow \text{RSNO} + \text{NO}_3^- + \text{H}^+ \\
\text{NO}_2 + \text{NO} & \rightarrow \text{N}_2\text{O}_3 + \text{RSH} \rightarrow \text{RSNO} + \text{NO}_2^- + \text{H}^+
\end{align*}
\]

Reaction of NO with PTIO

\[
\begin{array}{c}
\text{O}^- \\
N^+ \\
\text{N} \\
\text{O}^- \\
\text{N} \\
\text{O}^- \\
\text{N} \\
\text{O}^- \\
\end{array}
+ \text{NO}^* \rightarrow
\begin{array}{c}
\text{O}^- \\
N^+ \\
\text{N} \\
\text{O}^- \\
\text{N} \\
\text{O}^- \\
\text{N} \\
\text{O}^- \\
\end{array}
+ \text{NO}_2
\]
FIGURE 5

ODC Activity (nmol CO$_2$/min/mg protein) vs. DEA/NO [μM]
FIGURE 6

![Graph showing ODC Activity (nmol CO₂/min/mg protein)](image)

- Control
- 100 µM DEA/NO
- 30 µM PTIO
- 100 µM DEA/NO + 30 µM PTIO
FIGURE 7

ODC Activity (% of Control Activity)

+2.5 mM DTT

+5 mM GSH

100 µM DEA/NO + 30 µM PTIO
FIGURE 8B

mol SNO/mol ODC monomer

<table>
<thead>
<tr>
<th></th>
<th>Albumin</th>
<th>Wild Type ODC</th>
<th>C360A ODC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Value</td>
<td>0.8</td>
<td>4.0</td>
<td>2.9</td>
</tr>
</tbody>
</table>

* Significant difference
FIGURE 9A
FIGURE 10

The bar graph shows the pmol SNO/mg Protein for different conditions. The x-axis represents the different treatments: pCMVZeo, pCMVZeo-ODC, and pCMVZeo-ODC α-ODC Ab. The y-axis represents the pmol SNO/mg Protein, ranging from 0 to 40. The data points are n.d. (not detectable) for pCMVZeo and pCMVZeo-ODC, and there is a significant increase for pCMVZeo-ODC α-ODC Ab.
Nitric oxide inhibits ornithine decarboxylase via S-nitrosylation of cysteine 360 in the active site of the enzyme
Philip M. Bauer, Georgette M. Buga, Jon M. Fukuto, Anthony Pegg and Louis J. Ignarro
J. Biol. Chem. published online July 18, 2001

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