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Macrophage Oxidation of L-Arginine to Nitrite and Nitrate: Nitric Oxide Is an Intermediate†

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ABSTRACT: Previous studies have shown that murine macrophages immunostimulated with interferon γ and *Escherichia coli* lipopolysaccharide synthesize NO_2^-, NO_3^-, and citrulline from L-arginine by oxidation of one of the two chemically equivalent guanido nitrogens. The enzymatic activity for this very unusual reaction was found in the 100000*g* supernatant isolated from activated RAW 264.7 cells and was totally absent in unstimulated cells. This activity requires NADPH and L-arginine and is enhanced by Mg^{2+}. When the subcellular fraction containing the enzyme activity was incubated with L-arginine, NADPH, and Mg^{2+}, the formation of nitric oxide was observed. Nitric oxide formation was dependent on the presence of L-arginine and NADPH and was inhibited by the NO_2^-/NO_3^- synthesis inhibitor N^G-monomethyl-L-arginine. Furthermore, when incubated with L-[*guanido*-$^{15}N_2$]arginine, the nitric oxide was ^{15}N-labeled. The results show that nitric oxide is an intermediate in the L-arginine to NO_2^-, NO_3^-, and citrulline pathway. L-Arginine is required for the activation of macrophages to the bactericidal/tumoricidal state and suggests that nitric oxide is serving as an intracellular signal for this activation process in a manner similar to that very recently observed in endothelial cells, where nitric oxide leads to vascular smooth muscle relaxation [Palmer, R. M. J., Ashton, D. S., & Moncada, S. (1988) *Nature (London) 333*, 664-666].

The biosynthesis of NO_2^- and NO_3^- represents an unusual pathway of oxidation in mammals. Initial characterization of this reaction has shown that the pathway is expressed upon immunostimulation of macrophages by the exogenous stimulant *Escherichia coli* lipopolysaccharide (LPS)[1] (Stuehr & Marletta, 1985) and by the endogenous lymphokine IFN-γ (Stuehr & Marletta, 1987a). Murine macrophage cell lines are also capable of carrying out this synthesis after stimulation (Stuehr & Marletta, 1987b). Further characterization with cells in culture revealed that the precursor to NO_2^- and NO_3^- was the amino acid L-arginine (Iyengar et al., 1987; Hibbs et

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[1] Abbreviations: IFN-γ, recombinant murine interferon γ; LPS, *Escherichia coli* lipopolysaccharide; DME, Dulbecco's modified Eagle's media; SMEM, supplemented modified Earle's media; FCS, fetal calf serum; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*'-2-ethanesulfonic acid; EDRF, endothelium-derived relaxing factor.

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al., 1987a). In addition, ^{15}N-labeling studies showed that the N atom of both NO_2^- and NO_3^- was derived exclusively from one of L-arginine's two equivalent guanido nitrogens, and ^{14}C-labeling experiments showed citrulline to be the other product (Iyengar et al., 1987). Thus, only one of the two chemically equivalent guanido nitrogens is oxidized to yield both NO_2^- and NO_3^-. Furthermore, the NO_3^- synthesized is not derived, as was initially expected, from NO_2^- (Stuehr & Marletta, 1987a) but appeared to be partitioned from a common intermediate. Finally, when secondary amines are present in the cell culture medium, the cells will catalyze the formation of carcinogenic *N*-nitrosamines, and thus any proposed pathway must also account for this reaction (Miwa et al., 1987).

The toxicity of NO_2^- to some microorganisms is well established (Castellani & Niven, 1955), and previous studies suggested a link between NO_2^-/NO_3^- synthesis and the acquisition of increased nonspecific bacterial resistance (Stuehr & Marletta, 1985). The true biological role for NO_2^-/NO_3^- synthesis, however, remained unknown. Hibbs et al. (1987b) have reported that L-arginine is required for the selective metabolic inhibition of tumor target cells by macrophages. Concurrently, studies with endothelial cells showed that endothelium-derived relaxing factor (EDRF), a labile humoral agent, was, in fact, ·N=O (Palmer et al., 1987). EDRF is thought to act as a signal by stimulating the formation of cGMP, which in turn leads to smooth muscle relaxation [for a review, see Ignarro and Kadowitz (1985)]. In light of the fact that the only known mammalian pathway to yield any type of nitrogen oxide is the macrophage pathway described above and because the products NO_2^- and NO_3^- can be derived from the solution decomposition of ·N=O, the studies reported here investigated if ·N=O was an intermediate in the L-arginine to NO_2^-/NO_3^- pathway and, if so, was it derived from the same guanido nitrogens as NO_2^- and NO_3^-. Nitric oxide formation from L-arginine in macrophages would suggest the pathway serves as a signal for activation of these cells, in a fashion similar to that observed in endothelial cells.

Experimental Procedures

Materials

L-Arginine, α_2-macroglobulin from human plasma, sodium pyruvate, L-glutamine, penicillin/streptomycin, disodium α-ketoglutarate, bovine liver L-glutamate dehydrogenase, and Dulbecco's modified Eagle's medium (DME) were obtained from Sigma Chemical Co. (St. Louis, MO). DME was supplemented with glucose (4.5 g/L), sodium bicarbonate (3.7 g/L), L-glutamine (584 mg/L), penicillin (50 units/mL), streptomycin (50 μg/mL), and 10% (v/v) heat-inactivated bovine fetal calf serum (FCS). All supplements were of cell culture grade. Supplemented modified Earle's medium (SMEM) was powdered Eagle's minimal essential medium without phenol red (Flow Laboratories, McClean, VA) supplemented with sodium pyruvate (110 mg/L), glucose (3.5 g/L), glutamine (584 mg/L), penicillin (50 units/mL), streptomycin (50 μg/mL), HEPES (15 mM, pH = 7.5), and 10% (v/v) heat-inactivated FCS. *E. coli* LPS (0.111:P4 serotype, Westphal Extract) was obtained from Difco Laboratories (Detroit, MI), and sterile stock solutions with concentrations of 25–250 μg/mL were prepared in 0.9% (w/v) NaCl and stored at −20 °C until use. Recombinant murine interferon γ (IFN-γ) was a generous gift from Genentech, Inc. (South San Francisco, CA), at a specific activity of 0.5×10^7 antiviral units/mg of protein. The preparation also contained 0.032 endotoxin units/mg of protein by the limulus amoebocyte assay. Stock solutions of IFN-γ were prepared in sterile 15 mM HEPES, pH = 7.5, at a concentration of 10^4 units/mL and were stored in liquid nitrogen prior to use. The murine macrophage cell line RAW 264.7 was obtained from American Type Tissue Culture Collection. N^G-Monomethyl-L-arginine [di(*p*-hydroxy)azobenzene-*p'*-sulfonate] was from Calbiochem (San Diego, CA), and L-[*guanido*-$^{15}N_2$]arginine was obtained from ICN Biomedicals, Inc. (Cambridge, MA).

Methods

Detection of [^{15}N]*NO.* Analysis for enrichment of ^{15}N into nitric oxide from L-[*guanido*-$^{15}N_2$]arginine was carried out on a Hewlett-Packard Model 5987 GC/MS using electron ionization. The GC was done on a wide-bore fused silica GS-Q column (J&W Scientific, Folsom, CA) in tandem with a 0.25-mm fused silica column coated with Supelcowax. Samples from the enzymatic reactions were injected directly into the chromatograph without workup, and the column effluent was monitored at m/z = 30 ([^{14}N]NO) and 31 ([^{15}N]NO).

Detection of Nitric Oxide. Nitric oxide was quantitated with a thermal energy analyzer (TEA-502, Thermo Electron Corp.) that specifically detects ·N=O by chemiluminesence following reaction with ozone to produce nitrogen dioxide in an excited state; relaxation of the molecule to the ground state is accompanied by emission of a characteristic energy in the near-infrared region of the spectrum (Fine et al., 1973). Samples were injected through a rubber septum on one neck of a 100-mL three-necked round-bottomed flask containing 40 mL of water. The water was heated to reflux with an oil bath. Helium was passed through another inlet on the flask, and a condenser was attached to the third neck. An ice trap and an acetone–dry ice trap were placed in series between the condenser and the detector. This setup was a modification of the procedure used by Downes et al. (1976). Concentrations of ·N=O in the reaction solution were determined by comparison with standard solutions prepared by dissolving known volumes of ·N=O in degassed argon-saturated water.

Nitrite and Nitrate Analysis. Nitrite and nitrate concentrations in the reaction solutions were determined by using an automated procedure based on the Griess reaction, in which NO_2^- reacts with 1% sulfanilamide in 5% H_3PO_4/0.1% naphthalene–ethylenediamine dihydrochloride forming a chromophore absorbing at 543 nm (Green et al., 1982). Solutions can be passed through a copper-plated cadmium column that reduces all the NO_3^- in the sample to NO_2^-. Nitrite concentrations can be determined independently of NO_3^- by bypassing the cadmium column, and from the difference NO_3^- can be calculated.

IFN/LPS Activation of RAW 264.7 Cell Cultures. Frozen RAW 264.7 cultures, certified to be mycoplasma free at the time of purchase, were plated in 100-mm plastic dishes at a density of 5×10^7 cells/mL in supplemented DME media and incubated for 1 h at 37 °C. Nonadherent cells were removed and fresh DME was added, and then the cells were grown for 3 days, during which time they were passaged once by a 1:5 dilution with DME. DME was removed from log phase cell cultures, which were then activated for 12 h by addition of IFN-γ (1 unit/mL) and LPS (10 ng/mL) in SMEM. All cultures were maintained in a humidified incubator containing 5% CO_2 and 95% air at 37 °C.

Preparation of Macrophage Cytosol. Activated cells were sedimented by centrifugation at 250*g* for 8 min and washed twice with 15 mM HEPES, pH 7.5, before final resuspension to a cell density of 3.5×10^7 cells/mL in HEPES buffer containing α_2-macroglobulin (10 μg/mL). Macrophage suspensions were >98% viable on the basis of exclusion of trypan blue dye (0.4% w/v in saline). Following sonic disruption for

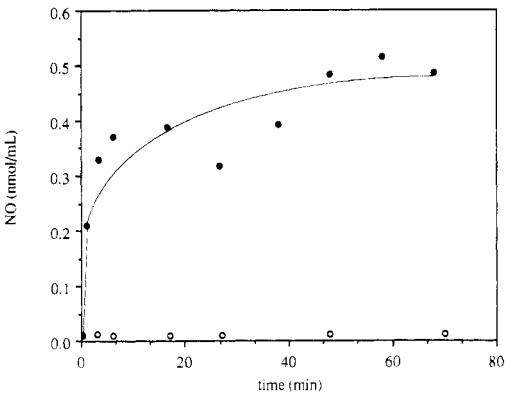


FIGURE 1: Time course of nitric oxide formation. Cytosol isolated from immunostimulated (solid circles) and unstimulated (open circles) RAW 264.7 cells at 1.1 mg/mL was incubated at 25 °C in the presence of L-arginine (2 mM) and $Mg(OAc)_2$ (1 mM), and the reaction was initiated with NADPH (4 mM); 1-mL final volume. All stock solutions were made up in 15 mM HEPES (pH = 7.4). Samples (100 μL) were withdrawn via syringe at the indicated times and analyzed as described under Experimental Procedures.

2 × 10 s with a Heat Systems–Ultrasonics, Inc., sonicator (40% maximal output), suspensions were centrifuged at 102000*g* for 60 min. Supernatant fractions were immediately frozen and stored at −70 °C for up to 2 months with <8% loss of enzyme activity. Control samples were also obtained from unstimulated macrophages by using an identical procedure.

L-Arginine-Dependent NO and NO_2^-/NO_3^- Synthesis. Solutions containing protein levels ranging from 0.3 to 1.1 mg/mL were incubated with 2 mM L-arginine, 4 mM NADPH, 1 mM $Mg(OAc)_2$, and 15 mM HEPES, pH = 7.5. For ·N=O measurement the reactions were carried out at 25 °C in 1-mL vials sealed with a Teflon septum. To analyze for ·N=O, aliquots (100 μL) were withdrawn via syringe and injected directly into the three-necked flask described above. Exact conditions for each experiment are described under Results.

Residual NADPH, which interferes with the Griess reaction, was oxidized to $NADP^+$ by further incubation with 25 mM α-ketoglutarate, 0.15 M NH_4Cl, and 0.52 units of L-glutamate dehydrogenase for 45 min at 25 °C. Nitrite and nitrate levels were then measured by using the procedure described above (Green et al., 1982). Oxidation of NADPH, as determined by the loss of absorbance at 340 nm, was based on a millimolar extinction coefficient of 6.22 (Kaplan, 1960). The Pierce bicinchoninic acid method, as described by Smith et al. (1985), was used to determine protein concentration, with bovine serum albumin as a standard.

RESULTS

·N=O Formation. Experiments were carried out to demonstrate that ·N=O is an intermediate in the L-arginine to NO_2^-/NO_3^- pathway expressed in activated macrophages. The enzyme(s) responsible for this NO_2^-/NO_3^- synthesis is (are) contained in the 100000*g* supernatant derived from macrophages costimulated with LPS and IFN-γ. While the activity is induced by either LPS or IFN-γ alone, costimulation yields a synergistic response (Stuehr & Marletta, 1987a). Supernatant isolated from untreated macrophages had no detectable arginine-dependent NO_2^-/NO_3^- activity. The conditions chosen here [L-arginine, 2 mM; $Mg(OAc)_2$, 1 mM; NADPH, 4 mM] have been found to yield the maximum rate of NO_2^-/NO_3^- synthesis. When activated macrophage supernatant at 1.1 mg/mL was incubated with L-arginine, $Mg(OAc)_2$, and NADPH, the formation of ·N=O was observed (Figure 1). In contrast, when supernatant from unstimulated cells was used, the synthesis of ·N=O was not seen. Furthermore, the formation of ·N=O showed an absolute requirement for L-arginine and NADPH (not shown). Since $Mg(OAc)_2$ enhances the synthesis of NO_2^- and NO_3^-, but is not a requirement for the reaction, the dependence of ·N=O formation on it was not studied. A steady-state concentration of 0.43 nmol/mL ·N=O was reached within 50 min after initiation of the reaction with NADPH.

Table I: Nitrite and Nitrate Synthesis[a]

macrophage supernatant	nitrite[b] (nmol/mL)	nitrate (nmol/mL)
control	5.4	27.4
activated	119.3	281.2

[a] The reactions analyzed for nitrite and nitrate were incubations parallel to those carried out to determine the time course of nitric oxide formation (see Figure 1). The reaction solutions contained 1.1 mg/mL cytosolic protein. The concentrations of the other components were as described under Experimental Procedures. The reaction was run for 19 h at 25 °C. [b] Samples were analyzed in duplicate with the average reported above.

^{15}N Enrichment of Nitrite Oxide from L-Arginine. This reaction was carried out under the same conditions as those for the ·N=O formation experiments described above. It was not possible to calculate the exact enrichment as we had done previously for NO_2^- and NO_3^- (Iyengar et al., 1987) because of interference from other components of the reaction solution in the mass chromatograms at m/z = 30 and 31. The mass chromatograms of m/z = 31 illustrated in Figure 2 clearly show a peak at the retention time of ·N=O with ^{15}N-labeled arginine (A and C), while this peak is absent in the reactions with ^{14}N-labeled arginine (B and D). Also the ratio of 31/30 was higher for the reaction with [^{15}N]arginine than for the reaction containing [^{14}N]arginine for up to 2 h after the addition of NADPH (see Figure 2 legend). In addition, the absolute area under the m/z = 31 peak at the retention time for ·N=O was higher at all time points for the [^{15}N]arginine reaction than at any time for the [^{14}N]arginine reaction.

Dependence on Macrophage Enzyme Activity. The formation of ·N=O was dependent on the concentration of activated macrophage supernatant (Figure 3). Three different concentrations of supernatant isolated from activated macrophages were analyzed after 30-min reaction time under the conditions described above. Supernatant from untreated cells was used throughout as a control. The three concentrations tested showed a linear dependence, while the control supernatant was negative for ·N=O formation at all concentrations. In addition, a second steady-state experiment carried out at 0.55 mg/mL protein showed 50% the level of ·N=O (not shown), when compared to the reaction reported above that was carried out at 1.1 mg/mL.

NO_2^-/NO_3^- Synthesis. We have previously shown that macrophages in culture synthesize NO_2^- and NO_3^- and that the ratio of the two anions is fairly constant at 3:2 (Stuehr & Marletta, 1987a). As is shown in Table I, that ratio changes significantly in cytosol isolated from activated cells. The controls had background levels of NO_2^- and NO_3^- that arise from small amounts of the anions that contaminate the biological solutions. The reaction was carried out at 25 °C for 19 h at a protein concentration of 1.1 mg/mL and the usual concentrations of L-arginine, $Mg(OAc)_2$, and NADPH. NADPH was enzymatically quenched as described above. The experiment gave the following results: NO_2^- = 113.9 nmol/mL and NO_3^- = 253.8 nmol/mL. This corresponds to 0.09 and 0.20 nmol mL^{-1} (mg of protein)$^{-1}$ min^{-1} of NO_2^- and NO_3^-, respectively. The ratio of NO_2^- to NO_3^- was 1:2.2.

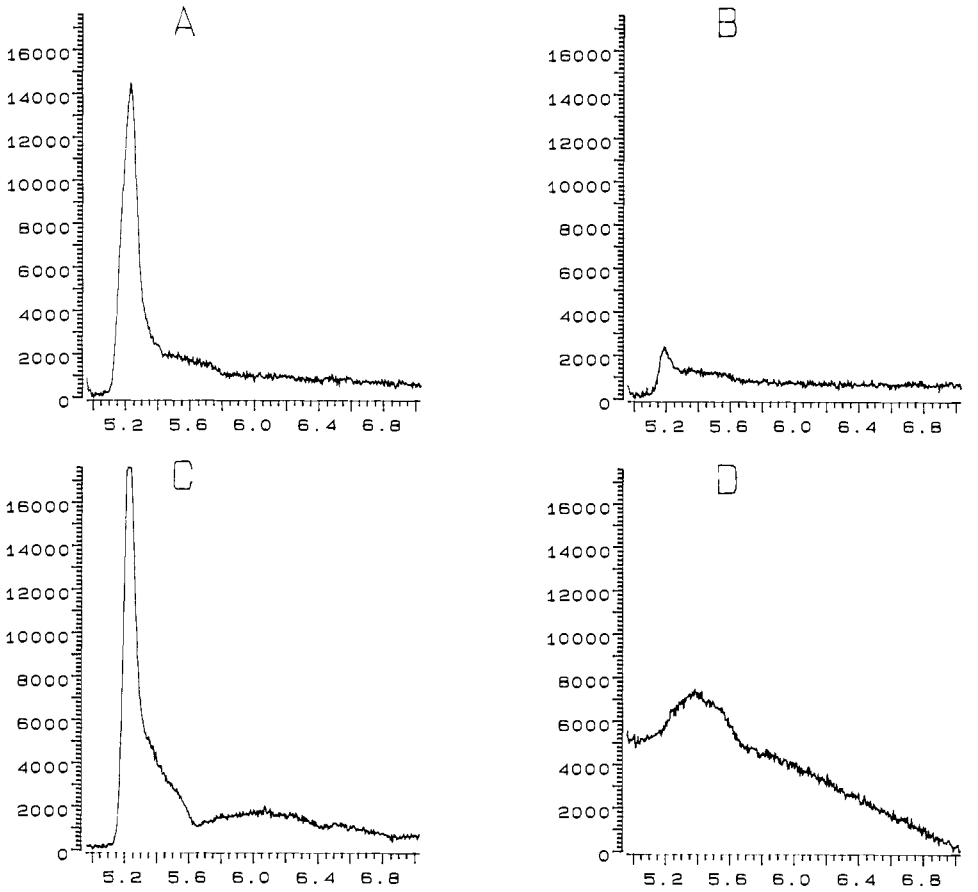


FIGURE 2: Mass chromatograms (m/z = 31) of reactions with [^{15}N]arginine versus [^{14}N]arginine. The mass chromatograms shown are retention time versus area of the peak. The retention time of authentic ·N=O was 5.25 min. Panels A and C are incubations containing L-[*guanido*-$^{15}N_2$]arginine at 20 and 120 min, respectively, after the initiation of the reaction with NADPH. Panels B and D are the corresponding incubations with unlabeled L-arginine at 30 and 120 min. The m/z 31/m/z 30 for the ^{15}N reactions were as follows: 0.03 (104 000) at 20 min; 0.08 (108 000) at 120 min. The ratios for the ^{14}N reactions were 0.01 (10 500) at 30 min and 0.002 (44 000) at 120 min. The numbers in parentheses are the areas obtained from the integration of the m/z = 31 mass chromatograms at the retention time of ·N=O.

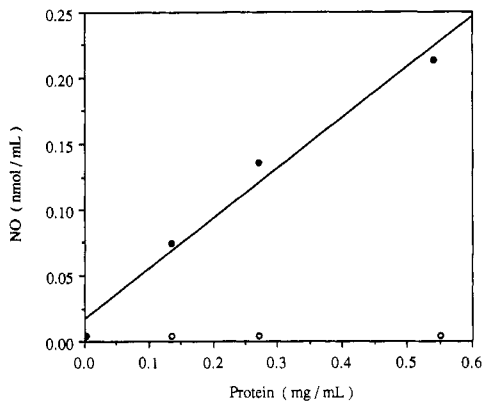


FIGURE 3: Effect of protein concentration on the formation of nitric oxide. Enzyme activity isolated from immunostimulated (solid circles) and unstimulated (open circles) RAW 264.7 cells was incubated at the indicated concentrations under the same conditions as stated in Figure 1. Nitic oxide concentrations were determined after 30 min.

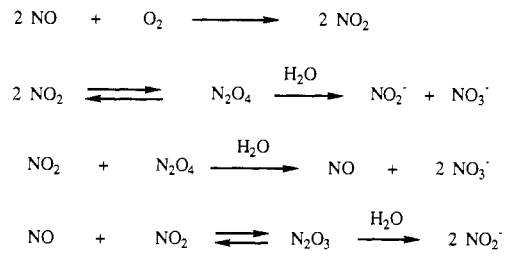
N^G-Monomethyl-L-arginine Inhibition. The arginine analogue N^G-monomethyl-L-arginine has been shown previously, in cultured cells, to be a relatively potent inhibitor of macrophage NO_2^-/NO_3^- synthesis (Hibbs et al., 1987a). Experiments were carried out to determine whether this compound was capable of inhibiting ·N=O synthesis as well. Cytosol from activated macrophages (1.0 mg/mL) was incubated with and without N^G-monomethyl-L-arginine at different concentrations for 20 min at 25 °C. N^G-Monomethyl-L-arginine inhibited the formation of ·N=O in a concentration-dependent manner, with 50% inhibition occurring at 0.13 mM. The inhibition curve was linear (not shown). N^G-Monomethyl-L-arginine alone did not generate ·N=O.

Discussion

Our previous studies have characterized a novel oxidation pathway expressed in immunostimulated macrophages. This oxidation of one of the two chemically equivalent guanido nitrogens of L-arginine to citrulline, NO_2^-, and NO_3^- is an unusual reaction in mammals and quantitatively represents a very important pathway in the activated macrophage, both in vivo and in vitro (Stuehr & Marletta, 1985). The enzymatic activity for this pathway is present in the cytosolic fraction of the cell and is not present in cytosol from unstimulated cells. In addition, protein synthesis is required for expression of the activity (Stuehr and Marletta, unpublished observation). This cytosolic activity is dependent on the addition of L-arginine and NADPH and is enhanced by Mg^{2+}.

With this cytosolic activity, the studies reported here clearly show that ·N=O is an intermediate in this oxidation of L-arginine. Nitric oxide formation is dependent on L-arginine and NADPH, and the activity is found only in the cytosol from activated cells. Furthermore, ·N=O formation reaches a steady-state level that is dependent on the concentration of cytosol. Another key finding is that when L-[^{15}N]arginine is used, the resulting ·N=O is ^{15}N-labeled, consistent with our previous findings for the products NO_2^- and NO_3^- (Iyengar et al., 1987). We have also shown here that N^G-monomethyl-L-arginine, an inhibitor of macrophage NO_2^-/NO_3^- synthesis, is also an inhibitor of ·N=O formation.

Scheme I

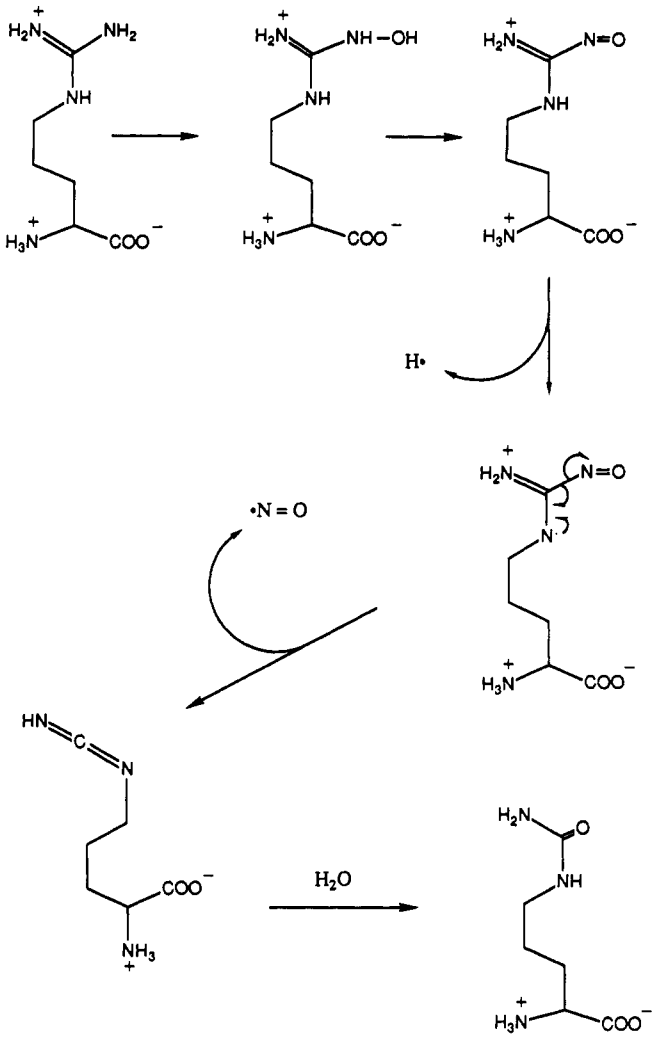


The question arises as to whether the ·N=O formed is responsible for all the NO_2^- and NO_3^- synthesized. The production of ·N=O and its reaction with O_2 to produce NO_2 gas are important industrial processes and have lead to many studies on the chemistry of these molecules (Beattie, 1967; Jones, 1973). While some of these studies have been carried out in solution, most have been gas-phase investigations, and the solution studies are rarely carried out in aqueous solutions under biological conditions. Nevertheless, from the steady-state concentration of ·N=O (0.43 nmol/mL) and reaction constants reported in the literature (Beattie, 1967; Jones, 1973; Redmond & Wayland, 1968), the predicted concentration of NO_2^- and NO_3^- at the end of 19 h would be about 500 μmol/mL, in good agreement with the findings. It must be emphasized that while this calculation strongly supports ·N=O as a kinetically competent intermediate, it is based on reaction constants under different conditions than those employed here.

The demonstration of ·N=O as an intermediate is important both in the interpretation of the chemistry observed so far and in terms of the biological role of this novel reaction. The reactions involved in the solution decomposition of ·N=O are shown in Scheme I. This reaction sequence is consistent with all of our observations. First, the reaction of ·N=O with O_2 is rapid and would account for the low steady-state amount of ·N=O observed. Second, the NO_2^- and NO_3^- synthesized are not derived from one another but result from the hydrolysis of N_2O_4 and N_2O_3 and the other reactions shown. Third, the ratio of NO_2^- to NO_3^- would be expected to vary, dependent on the concentration of ·N=O. For example, in Scheme I a simple doubling of the ·N=O concentration, with no assumptions about equilibria and rate constants, predicts a greater influence on the third reaction compared to the fourth. This would result in an increase in NO_3^- over NO_2^-. This ratio consistently has been 3:2 with cells in culture while here, where the activity of ·N=O formation is greater than that formed in intact cells, it has shifted to 1:2.2. Lastly, the solution decomposition of ·N=O generates N_2O_4 and N_2O_3, both of which are N-nitrosating agents. This would account for our observations that amine nitrosation by macrophages does not require NO_2^- (Miwa et al., 1987) and, when L-[*guanido*-$^{15}N_2$]arginine is used, the nitrosyl is ^{15}N-labeled (Iyengar et al., 1987).

A plausible series of reactions for the enzymatic synthesis of ·N=O from L-arginine are shown in Scheme II. The first step shows a monooxygenase-type reaction occurring at one of the guanido nitrogens generating N^G-hydroxyl-L-arginine. This reaction would require reducing equivalents and is a likely step for the involvement of NADPH. This particular hydroxylated arginine has never been reported; however, the N^5-hydroxy (hydroxylation of the ornithine nitrogen) has been isolated as a fungal metabolite (Maehr & Leach, 1974; Maehr et al., 1973). The next step is a two-electron oxidation to a nitrosoamidine-like intermediate. One-electron oxidation of this intermediate would lead to the fragmentation shown, including the homolytic cleavage of the C–NO bond. This

Scheme II



reaction generates ·N=O and the diimide of ornithine. Reaction of this diimide with water would yield citrulline, the other end product observed. Urea formation from diimides, with dicyclohexylcarbodiimide as a typical example, is a well-known reaction (Smith et al., 1958).

As mentioned above, ·N=O as an intermediate in this reaction also provides the basis for the biological role for this pathway. L-Arginine is required for activation of macrophages to a bactericidal and tumoricidal state (Hibbs et al., 1987b; Granger et al., 1988). Recent studies have shown that EDRF, which leads to vascular smooth muscle relaxation, is ·N=O (Palmer et al., 1987). The relaxation of vascular smooth muscle appears to be a cGMP-dependent process, and the role of ·N=O is to activate a heme-dependent guanylate cyclase (Craven & DeRubertis, 1978, 1983). Immunostimulation of macrophages results in a complex and large number of biochemical changes in the cell, some of which are required for the bactericidal/tumoricidal activity expressed by these activated cells. Macrophages may very well be using ·N=O as a signaling agent in a manner similar to that in endothelial cells. This intracellular signaling could trigger the activation of a number of pathways, some of which may be required for the cell-killing properties of stimulated macrophages. In fact, while this work was under way, it was reported that the source of ·N=O in bradykinin-stimulated endothelial cells is L-arginine with oxidation of the same nitrogen as we observed in the macrophage pathway (Palmer et al., 1988). Therefore, the pathways appear to be identical in both cell types, although

the functions of these cell types are quite different.

In summary, ·N=O is an intermediate in the macrophage pathway of L-arginine to NO_2^- and NO_3^-, and this pathway is required for these cells to attain their essential cell-killing activity. This same pathway, when expressed in endothelial cells, has been reported to control smooth muscle relaxation. Therefore, an understanding of the enzymology and regulation of this pathway may provide a rational basis for the control of vasodilation as well as for control of the immune system.

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