Vol. 157, No. 1, 1988 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
November 30, 1988 Pages 87-94

NITRIC OXIDE: A CYTOTOXIC ACTIVATED MACROPHAGE EFFECTOR MOLECULE

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Received October 3, 1988

The experiments reported here identify nitric oxide as a molecular effector of activated macrophage induced cytotoxicity. Cytotoxic activated macrophages synthesize nitric oxide from a terminal guanidino nitrogen atom of L-arginine which is converted to L-citrulline without loss of the guanidino carbon atom. In addition, authentic nitric oxide gas causes the same pattern of cytotoxicity in L10 hepatoma cells as is induced by cytotoxic activated macrophages (iron loss as well as inhibition of DNA synthesis, mitochondrial respiration, and aconitase activity). The results suggest that nitric oxide is the precursor of nitrite/nitrate synthesized by cytotoxic activated macrophages and, via formation of iron-nitric oxide complexes and subsequent degradation of iron-sulfur prosthetic groups, an effector molecule.

Previous studies show that activated macrophage induced cytotoxicity is L-arginine dependent (1) and that activated macrophages synthesize NO_2^-/NO_3^- (2-5). NO_2^-/NO_3^- are synthesized from the precursor molecule L-arginine which is converted to L-citrulline (3,5,6). CAM induced biochemical changes include intracellular Fe loss (7-9) inhibition of mitochondrial respiration (inhibition of NADH:ubiquinone oxidoreductase and succinate:ubiquinone oxidoreductase of the mitochondrial election transport chain) (5,8-11), inhibition of the citric acid cycle enzyme aconitase (8), and inhibition of DNA synthesis (12, 13). These enzymes and ribonucleotide reductase (the rate limiting enzyme in DNA replication) all have catalytically active non-heme iron coordinated to sulfur atoms that could be a target for a CAM effector molecule. Therefore, we

ABBREVIATIONS: AA, amino aids; BCG, Bacillus Calmette Guerin; CAM, cytotoxic activated macrophages; DMEM, Dulbecco's modified Eagles medium; DMEM-G, DMEM without glucose; DMEM+G, DMEM with glucose; GC/MS, gas chromatography/mass spectrometry; L-[G-^{14}C] arg, L-[guanidino-^{14}C] arginine; L-[G-$^{14}N_2$] arg, L-(guanidino-$^{14}N_2$] arginine; L-[G-$^{15}N_2$], L-[guanidino-$^{15}N_2$] arginine; L-[ueido-^{14}C] cit, L-[ureido-^{14}C] citrulline; LPS, lipopolysaccaride; MEM, modified Eagle's medium; N^GMMA, N^G-monomethyl-L-arginine; NO, nitric oxide; NO_2, nitrogen dioxide; NO_2^-, nitrite; NO_3^-, nitrate; NO_2^-/NO_3^-, NO_2^- + NO_3^-; PEC, peritoneal exudate cells

0006-291X/88 $1.50

performed experiments to determine if authentic NO gas (a molecule with low solubility in water and high solubility in nonpolar solvents which is capable of forming Fe-NO complexes) reproduces the pattern of CAM induced biochemical changes. We also tested for NO synthesis from L-arginine by CAM.

MATERIALS AND METHODS

Culture Medium: Modifications in DMEM were made for these experiments. Stock DMEM (S DMEM) contained: DMEM salts(same concentration as commercial DMEM), 100 U/ml penicillin and 100 μg/ml streptomycin, 1:50 dilution of concentrated (50X) MEM vitamin solution and 4 mM L-glutamine. DMEM 1 contained the components of S DMEM plus 25 mM glucose, 25 mM HEPES, and a 1:25 dilution of essential AA (1:25 AA). To prepare glucose-free second incubation medium (DMEM-G), S DMEM was supplemental with 20 mM HEPES, 1:25 AA, 2.5 gm/l $NaHCO_3$, and 2% dialyzed calf serum. DMEM+G contained all the components of DMEM-G plus 20 mM glucose. To prepare DMEM 2, S DMEM was supplemented with 20 mM glucose, 2.5 gm/l $NaHCO_3$, 20 mM HEPES, and 20 ng/ml LPS (phenol extracted E. coli serotype 0128:B12). To prepare L-arginine-free cultivation medium (DMEM-arg), S DMEM was supplemented with 25 mM glucose, 33 mM HEPES, 5 mM L-ornithine, 0.25 gm/liter $NaHCO_3$ and 20 ng/ml LPS. To prepare L-arginine containing medium (DMEM+arg), DMEM-arg was supplemented with 3.6 mM L-[G-$^{14}N_2$] arg or 3.6 mM L-[G-$^{15}N_2$] arg (99% ^{15}N, ICN Biomedicals, Inc.).

Measurements of Biochemical Effects Induced in L10 Hepatoma Cells by Authentic NO Gas: L10 cells, a nonadherent diethylnitrosamine-induced guinea pig hepatoma line, were added to 20 ml Wheaton bottles in 2 ml of DMEM 1 and sealed with siliconized rubber caps. The Wheaton bottles were deoxygenated at 25°C by flushing with O_2 free N_2 gas for 30 min. via 23 gauge needles inserted through the rubber cap. Control cultures received additional N_2 while other cultures were flushed with 1% NO-99% N_2 (Matheson Gas Products) for 15 min. (1% NO cultures). L10 cells were cultured in the sealed Wheaton bottles at 37°C (first incubation). After 6 h or 24 h of culture [^{55}Fe] release (7), mitochondrial aconitase activity (8), sample protein concentration (14), [^{3}H]TdR uptake (15), and inhibition of mitochondrial respiration (10, 15) were measured as described.

Macrophage Activation: To obtain activated macrophages, C_3H/HeN female mice were infected with BCG intraperitoneally as described (15). Murine peritoneal activated macrophages cultured in vitro in the presence of LPS are termed CAM (15, 16). Stimulated macrophages were removed from uninfected normal mice inoculated with thioglycolate broth as described (1,15).

Preparation of Macrophage Cultures for Measurement of NO: PEC (4.2×10^8) were mixed with 420 mg of cytodex-1 beads (Pharmacia) (1×10^6 PEC/mg) in 50 ml DMEM-arg and added to a 100 ml spinner bottle (rotor speed 60 RPM) for 1.5 h to allow for adherence of CAM to the beads (~60% of PEC were macrophages with ~80% macrophage adherence to the beads). Nonadherent cells were removed by centrifugation (10 g x 1 min) and CAM adherent to beads suspended in DMEM-arg. Centrifugation was repeated and the beads with adherent CAM divided into two equal portions (two spinner cultures performed in parallel) and cultured in 50 ml of DMEM-arg or DMEM+arg with the additives indicated in Table 2. The CAM were cultured in suspension (rotor speed 60 RPM) for 20 h at 37°C in a 75% He-20% N_2-5% O_2 atmosphere prior to measurement of inorganic nitrogen oxides. Final pH of culture medium was always above 6.8 (initial pH 7.4).

Measurement of Inorganic Nitrogen Oxides Synthesized by CAM: NO was detected by modification of methods described by Jacobs for measuring atmospheric nitrogen oxides (17) and illustrated schematically in Fig. 1. NO_2^- and NO_3^- that accumulated in the aqueous phase of macrophage cultures were measured as

described (2,3,5,14). NO and NO_2 emitted into the CAM culture gas phase were trapped as NO_2^- ion in absorbing solutions (Fig. 1 J and L respectively) and measured as described (2,3). ^{15}N enrichment (atom % excess ^{15}N) of NO trapped from the gas phase in the absorbing solution (Fig. 1 J) or NO_2^-/NO_3^- present in the medium (Fig. 1 C) after cultivation of CAM with L-[G-$^{15}N_2$] arg for 20 h, was determined by GC/MS by selective ion monitoring after conversion to nitrobenzene as described (18).

L-[ureido-^{14}C] cit synthesis from L-[G-^{14}C] arg by Macrophages: CAM monolayers were prepared by adding 4 x 10^5 PEC from BCG infected mice to Falcon 3070 microtiter plates and adhered for 1 h at 37°C in a 5% CO_2 atmosphere as described (15). The same procedure was used for culture of stimulated macrophages. CAM and stimulated macrophage monolayers were cultured in DMEM 2 plus 0.4 μCi/ml L-[G-^{14}C] arg (sp act 54.4 mCi/mmols) and, when indicated, 0.1 mM N^GMMA or 5 mM L-ornithine. Following a 20 h incubation, the culture medium was removed and processed for high voltage paper electrophoresis as described (14).

RESULTS

Authentic NO Reproduces the Biochemical Effects of the L-arginine-dependent effector pathway: Authentic 1% NO in 99% nitrogen was added to deoxygenated cultures of guinea pig L10 hepatoma cells and biochemical activity, known from previous work to be altered when L10 cells are cocultivated with CAM (1,3,5, 7-13), was measured. Results in Table 1 show that specific release of [^{55}Fe]

Table 1. Exposure of L10 hepatoma cells to authentic NO causes biochemical changes characteristic of the L-arginine dependent effector pathway

First Incubation Treatments	% L10 Cell Death			L10 Cell [^{55}Fe] Release, DNA Synthesis and Aconitase Activity		
	Immediately After First Incubation	After Second Incubation DMEM-G	DMEM+G	[^{55}Fe] Specific Release	[^{3}H] TdR Uptake (% Control)	Aconitase Activity
6h anaerobic control	5±0.2	4±1.6	2±0.7	---	---	16±2.8
6h 1% NO in 99% N_2	6±0.6	95±1.5	5±0.6	12±1.6	12±2.2	0
24h anaerobic control	8±0.9	11±1.3	6±1.0	---	---	9±1.3
24h 1% NO in 99% N_2	10±1.0	100	13±1.4	27±3.9	12±2.0	0

[^{55}Fe] release from prelabelled L10 cells is expressed as percent specific release as described (7,15) with spontaneous release taken as release of ^{55}Fe from L10 cells from anaerobic control cultures. Uptake of [^{3}H]TdR is expressed as percent anaerobic control L10 cells. L10 mitochondrial aconitase activity is expressed as rate of cis-aconitate disappearance in mmol/min/mg protein. (The aerobic aconitase control was 14.4 ± 2.1 at 6 h, and 12.3 ± 0.9 at 24 h.) Assays were performed immediately after the first 6 h or 24 h incubation. Inhibition of mitochondrial respiration was measured after a second 24 h incubation. L10 cells with inhibited mitochondrial respiration lose viability during a second 24 h incubation in DMEM-G but survive in medium containing substrate for glycolysis (DMEM+G) (10, 15). Values are the mean ± SEM for five separate experiments (three separate experiments for aconitase activity), each performed in duplicate or triplicate. See methods section for experimental details.

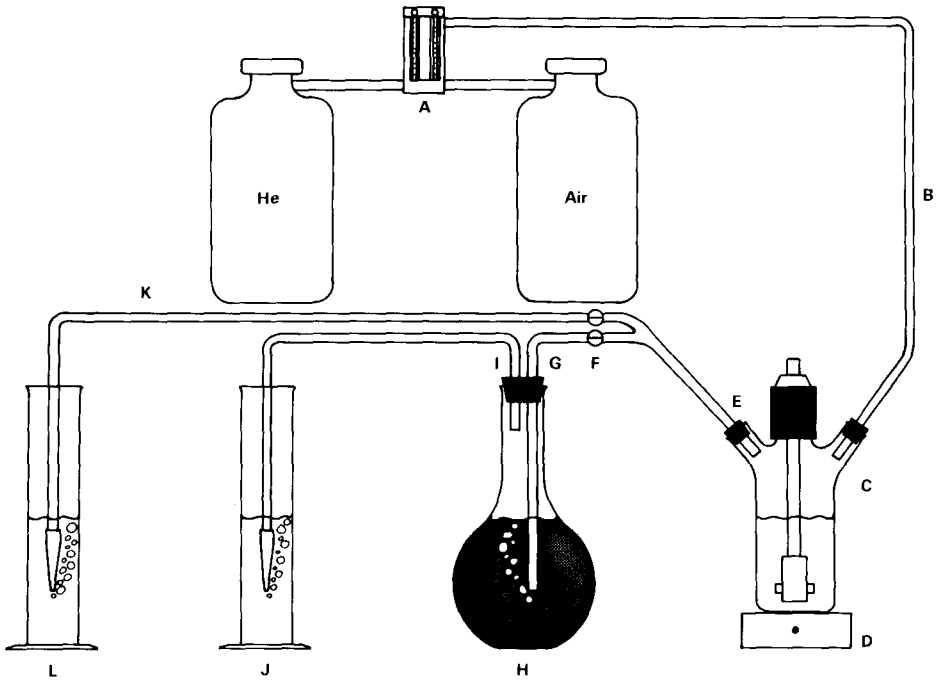


Fig. 1: Schematic diagram of the apparatus used to detect and quantitate NO and NO_2 gases synthesized by CAM. A stream of carrier gas proportioned (A) to a mixture of 75% He-20% N_2-5% O_2 flows (60 ml/min) through teflon tubing (B) and enters a 100 ml spinner bottle (C) containing a suspension of CAM adherent to cytodex-1 beads kept in constant motion by magnetic stirring (D). NO and NO_2 emitted from the aqueous phase to the gas phase of the culture exits the spinner bottle via a glass γ tube (E) and divides into two streams equalized by stainless steel fine metering valves (F) attached to teflon tubing. One stream (G) bubbles through a solution of 0.16 M $KMnO_2$ in 2.5% H_2SO_4 (H) that oxidizes NO to NO_2 which flows via teflon tubing (I) to an absorbing solution of 0.1 N NAOH in 0.2% butanol (J) that converts NO_2 to the stable and nonvolatile NO_2 ion. Both NO and NO_2 produced by CAM are trapped as NO_2^- ions in absorbing solution J. The second stream flows in teflon tubing (K) directly from the spinner flask to absorbing solution (L) where NO_2, but not NO, is trapped. The amount of NO produced by CAM is equal to NO_2^- measured in flask J minus NO_2^- measured in flask L x 2.

from prelabeled L10 cells was 12%±1.6 after 6h and 27%±3.9 after 24h of exposure to 1% NO. In addition, NO treatment of L10 cells induced inhibition of DNA synthesis. Results in Table 1 also show that treatment of L10 cells with 1% NO causes inhibition of mitochondrial respiration (as determined by lysis of L10 cells during a subsequent 24h incubation in culture medium without glucose) as well as inhibition of aconitase activity. These findings demonstrate that authentic NO induces [^{55}Fe] loss and the same pattern of metabolic inhibition in L10 cells as was demonstrated previously to be caused by the L-arginine-dependent effector mechanism.

Detection of NO Synthesis from L-arginine by CAM: The apparatus used to demonstrate NO synthesis by CAM is described in Fig. 1. This is a sensitive method for detecting NO and, when the oxidizing solution is bypassed, for

Table 2. Synthesis of inorganic nitrogen oxides from L-arginine by CAM

Nitrogen Isotope	GAS PHASE nmoles/10^7 cells NO	NO_2	Atom % Excess ^{15}NO	AQUEOUS PHASE nmoles/10^7 cells NO_2^-	NO_3^-	Atom % Excess $^{15}NO_2^-/^{15}NO_3^-$
L-[G-$^{14}N_2$] arg	44± 5.8	1.3±0.3	--	464±64	456±33	--
L-[G-$^{14}N_2$] arg + 50 μM $^{14}NO_2^-$	37± 9.3	1.4±0.2	--	476±89	462±55	--
L-[G-$^{15}N_2$] arg	30± 8.3	1.1±0.1	89±1.1	494±53	483±46	92±2.9
L-[G-$^{15}N_2$] arg + 50 μM $^{14}NO_2^-$	38±13.7	1.0±0.1	86±3.6	458±21	415±23	92±1.2

NO, NO_2, NO_2^-, (correction was made for nanomoles $NO_2^-/10^7$ CAM detected in the culture aqueous phase for groups supplemented with 50 μM $NaNO_2$), and NO_3^- synthesis as well as ^{15}N enrichment (atom % excess ^{15}N) of NO or NO_2^-/NO_3^- was measured as described in the Materials and Methods Section and Fig. 1. CAM were cultured with 3.6 mM L-[G-$^{14}N_2$] arg or 3.6 mM L-[G-$^{15}N_2$] arg for 20h. Values are the mean ± SEM for 3 separate experiments.

detecting NO_2. Compared to CAM cultured in medium without L-arginine (0.9±0.04 nmole NO/10^7 CAM; mean ±SEM of 6 separate experiments), Table 2 shows there was a significant increase in NO synthesis (detected in the culture gas phase) when the culture medium was supplemented with L-arginine. A small amount of NO_2 was also detected which may be generated, entirely or in part, by oxidation of NO (see equation 2 in the Discussion section) during transit from the culture to the absorbing solution (Figure 1, C to L).

When added to the culture medium at a concentration of 0.5mM, N^GMMA, a potent inhibitor of the CAM L-arginine dependent effector system (1,3,5), inhibited NO synthesis by CAM (data not shown). Stimulated macrophages do not synthesize NO_2^-/NO_3^-/L-citrulline from L-arginine (3,5) and are not cytotoxic for target cells (1,19). Likewise, stimulated macrophages did not synthesize NO when they were used in experiments identical to those described above (data not shown).

NO_2^- could be a precursor of NO in the low pH environment of the macrophage vacuolar system (20). To test this possibility CAM were cultured with L-[G-$^{15}N_2$] arg in the presence and absence of supplemental $^{14}NO_2^-$ and the ^{15}NO absorbed from the culture gas phase measured by GC/MS. Results in Table 2 show a similar ^{15}N-enrichment of NO absorbed from the gas phase as well as $^{15}NO_2^-/^{15}NO_3^-$ present in the aqueous phase of cultures with and without supplemental $^{14}NO_2^-$. This demonstrates that NO synthesized by CAM is derived from a terminal guanidino nitrogen atom of L-arginine and that NO is a precursor of and not a product of NO_2^-/NO_3^- synthesized by macrophages.

L-[ureido-14C] cit Synthesis from L-[G-^{14}C] arg by CAM: We obtained further evidence for the identity of the nitrogen atoms oxidized to NO and NO_2^-/NO_3^- by

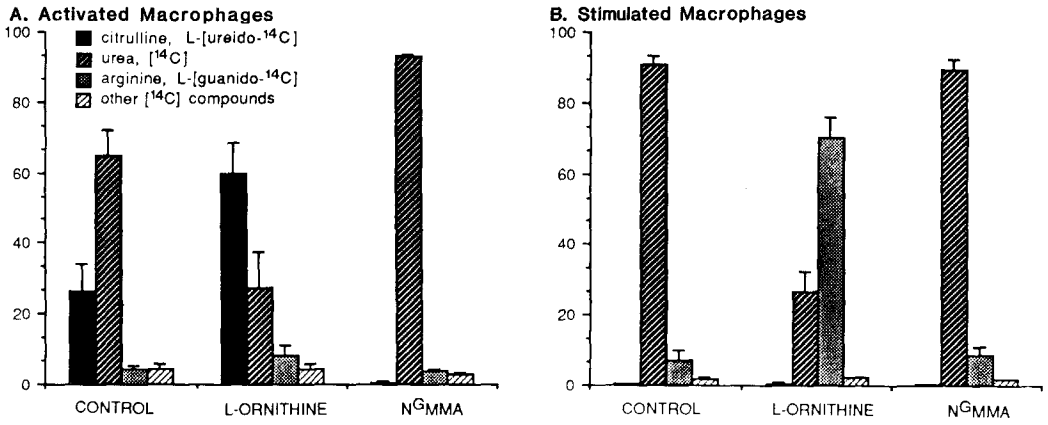


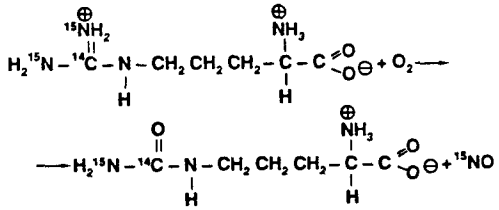
Fig. 2: Synthesis of L-[ureido-^{14}C] cit from L-[G-^{14}C] arg is further evidence that CAM converts one or both of the terminal guanidino atoms of L-arginine to NO and other inorganic nitrogen oxides. Results are expressed as percent of total CPM present as L-[ureido-^{14}C] cit, [^{14}C] urea, or L-[G-^{14}C] arg. Other [^{14}C]-labelled compounds were < 4.4% of total CPM. Values are the mean ± SD for 6-8 separate experiments (CAM) or four separate experiments (stimulated macrophages).

CAM. Figure 2A shows that CAM convert L-[G-^{14}C] arg to L-[ureido-^{14}C] cit (26.5% of CPM) and [^{14}C] urea (64.6% of CPM). L-ornithine, an arginase inhibitor, increased L-[ureido-^{14}C] cit synthesis (60% of CPM) and decreased [^{14}C] urea synthesis (27.9% of CPM). N^{G}MMA, an inhibitor of organic nitrogen oxide synthesis from L-arginine (1,3,5), decreased synthesis of L-[ureido-^{14}C] cit (0.7% of CPM) and increased synthesis of [^{14}C] urea (92.6% of CPM). Figure 1B shows that stimulated macrophages (which hydrolyze L-arginine to urea and L-ornithine via the arginase reaction) (21) do not synthesize L-[ureido-^{14}C] cit (<.8% of CPM) from L-[G ^{14}C] arg. [^{14}C] urea synthesis by stimulated macrophage falls from 90.7% to 26.6% of CPM when arginase was inhibited by L-ornithine. In this case, 70.5% of the CPM remain as unchanged L-[G-^{14}C] arg showing that absence of L-[ureido-^{14}C] cit synthesis by stimulated macrophages is not due to L-arginine depletion by arginase activity. These experiments confirm the origin of the organic nitrogen atom(s) oxidized to NO and that L-arginine is directly converted by this effector pathway to L-citrulline without loss of the guanidino carbon atom. It is possible the initial reaction is an N-oxidation or hydroxylation of one of the terminal guanidino nitrogen atoms of L-arginine (which are biochemically equivalent because of imine-amine tautomerism) rather than a hydrolytic reaction as we originally speculated (3).

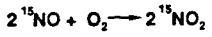
DISCUSSION

These experiments demonstrate that CAM synthesize NO from a terminal guanidino nitrogen atom of L-arginine. The following sequence of reactions are compatible with the experimental results:

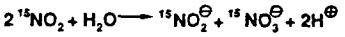
(1)



(2)



(3)



Our results show that authentic NO and CAM cause the same pattern of metabolic inhibition in L10 cells. It is possible that NO_2 produced by oxidation of NO (see equation 2 above) could also contribute to the cytotoxicity. However, earlier work by others demonstrated that Fe-S centers of Fe-S proteins are sensitive targets for formation of Fe-NO complexes (22-25) suggesting a molecular explanation for L-arginine dependent cytotoxicity. NO causes degration of Fe-S centers resulting in release of Fe ions and Fe-nitrosyl complexes (23,25). It is likely that a similar NO-mediated mechanism causes the Fe efflux and enzymatic inhibition observed in CAM and in their target cells (1,3,5,7-13).

It was established in 1981 that a portion of NO_3^- excreted by mammals is endogenously produced (26,27). The metabolic origins, identities and functions of inorganic nitrogen oxides synthesized by mammals are now being elucidated (1-6,14,28-31). The biologically active inorganic nitrogen oxide at physiologic pH is probably NO, but an active organic nitrogen oxide intermediate(s) may also exist. Palmer, et al, demonstrated that NO is synthesized by endothelial cells (28) and we demonstrate in this study that CAM synthesize NO. The insight that synthesis of inorganic nitrogen oxides from L-arginine is part of homeostatic control of cellular proliferation (1,3,14) and vascular tone (29,30) provides new opportunities for better understanding of mammalian physiology as well as for pharmacologic manipulation of certain pathophysiologic events.

ACKNOWLEDGEMENTS: Supported by the Veterans Administration. We are grateful to R. Christensen for assistance as well as S. Henn and J. Heyder for manuscript preparation.

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