ACTIVATED MURINE MACROPHAGES SECRETE A METABOLITE OF ARGININE WITH THE BIOACTIVITY OF ENDOTHELIUM-DERIVED RELAXING FACTOR AND THE CHEMICAL REACTIVITY OF NITRIC OXIDE

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Synthesis of nitrogen oxides is a newly discovered biochemical process in mammals (1) carried out by activated macrophages (2) and endothelial cells (3, 4). In both cell types, these oxides derive from the guanido nitrogens of L-arginine (5–7), but there are marked differences in the agents inducing their synthesis and in the kinetics of their release (8–10). In macrophages, the known products of this class are nitrite (NO_2^-)[1] and nitrate (NO_3^-) (2, 8). Production of NO_2^-/NO_3^- by macrophages is associated with L-arginine–dependent cytostasis of tumor cells (6) and cryptococcus (11). However, NO_2^-/NO_3^- themselves lack cytostatic activity (12), suggesting the involvement of a more reactive compound that may be intermediate in the reaction sequence between arginine and NO_2^-/NO_3^-. Endothelial cells produce nitric oxide (NO·), a highly reactive compound recently identified as a major endothelium-derived relaxing factor (EDRF) (3, 4, 10). EDRF elicits an elevation in cGMP in vascular smooth muscle cells and platelets, leading to vasodilatation (13) and inhibition of platelet aggregation and adhesion (14). We show here that activated mouse macrophages release a molecule derived from the guanido nitrogens of L-arginine, which has both EDRF bioactivity and NO·-like chemical reactivity toward heme-containing and Fe-S proteins.

Materials and Methods

Tissue Preparation and Cell Culture. Rabbits (New Zealand White) were anesthetized with pentobarbital sodium (30 mg/kg) and killed by a blow to the skull. Thoracic aorta was quickly excised, cut into 3–5-mm rings, de-endothelialized, and attached to a transducer (RTO3; Grass Instruments, Quincy, MA) for recording of isometric tension with a Model 7 Polygraph (Grass Instruments). Rings were maintained under 2 g of resting tension and super-

This work was supported by grants CA-43610, HL-34215, HL-37407, and HL-18828 from the National Institutes of Health; by the E. N. Dana Charitable Trust; and by the Pharmaceutical Manufacturer's Association Foundation. D. J. Stuehr is a Fellow of the Leukemia Society of America. Address correspondence to C. Nathan, Box 57, Cornell University Medical College, 1300 York Ave., New York, NY 10021.

[1] *Abbreviations used in this paper:* DMA, N^G,N^G-dimethyl-L-arginine; EDRF, endothelium-derived relaxing factor; Fd, clostridial ferredoxin; KHS, Krebs-Henseleit solution; Mφ, macrophages; NE, norepinephrine; NO·, nitric oxide; NO_2, nitrogen dioxide; NO_2^-, nitrite; NO_3^-, nitrate.

J. EXP. MED. © The Rockefeller University Press · 0022-1007/89/03/1011/10 $2.00
Volume 169 March 1989 1011–1020

fused with Krebs-Henseleit solution (KHS) (pH 7.4, 27°C) saturated with 95% O_2/5% CO_2 at a flow rate of 6.4 ml/min. Tissues were equilibrated for 60 min before contraction with norepinephrine (NE) (10 μM). To avoid oxidation, ascorbic acid (54 μM) was present in the NE-containing perfusion medium. The absence of endothelium was demonstrated by lack of relaxation in response to 10 μM acetylcholine.

Macrophages (Mϕ) were obtained from C3H/HeJ mice (The Jackson Laboratories, Bar Harbor, ME) after intraperitoneal injection of thioglycollate broth 4 d previously, or were the mouse Mϕ-like tumor cell line RAW 264 (American Type Culture Collection, Bethesda, MD) (15). Mϕ beads were prepared by allowing Mϕ (9×10^7 for C3H/HeJ, $1.9-3.4 \times 10^7$ for RAW 264) to adhere in tissue culture flasks to 2.1 ml (packed volume) of Cytodex 3 beads (Pharmacia Fine Chemicals, Piscataway, NJ) for 12–24 h in 35 ml of Minimum Eagle's Medium, α modification, containing 8% calf serum (Hyclone Systems, Logan, UT) at 37°C in 5% CO_2. Mϕ on beads were activated by exposure to rIFN-γ (500 U/ml, murine; Genentech, Inc., South San Francisco, CA) for 12–16 h followed by addition of bacterial LPS (5 μg/ml; *Escherichia coli* 0111:B4, List Biological, Campbell, CA) 5–10 h prior to the experiment. For measurements of NO_2^-/NO_3^- synthesis, aliquots of the cells on beads were removed from the stock cultures and their subsequent NO_2^-/NO_3^- synthesis was measured over 6–12 h as described previously (2). After loading into the columns, the Mϕ-bearing beads were washed with two column volumes of KHS before use. After each experiment, the number of macrophages remaining in the columns was determined by lysing the cells and fixing the nuclei for counting in a hemocytometer as described (16).

Spectrophotometric Assay for NO· and Nitrogen Dioxide (NO_2). Ferredoxin (Fd) from *Clostridium pasteurianum* was dissolved under N_2 in deoxygenated PBS (pH 7.2) to form an 11.3-μM Fd solution (E_{390} = 24 mM/cm; reference 17). This solution (900 μl, 10.2 nmol) was transferred anaerobically to an N_2-flushed anaerobic cuvette. Authentic NO· (Matheson Gas Products, Inc., Rutherford, NJ) that had passed through 1 M KOH was transferred with a gas-tight syringe in sequential 10-μl amounts to the cuvette. After each addition, the cuvette was mixed by several inversions and the spectrum was recorded from 600 to 350 nm in a spectrophotometer (model 557; Hitachi Inc., New York, NY). Reactions of NO_2 were carried out as with NO· except NO_2 was not passed through 1 M KOH. After addition of 70 μl NO· (2.87 μmol) or 30 μl of NO_2 (3.41 μmol), each solution was aerated, the pH was recorded, and NO_2^-/NO_3^- concentrations were measured. The results for NO· were pH = 6.01 ± .08, NO_2^- + NO_3^- = 3.01 ± .17 mM (% NO_2^- = 101 ± 4%). In contrast to the mild acidification caused by NO·, addition of 30 μl NO_2 reduced the pH to 4.05 ± 1.47, sometimes precipitating the Fd. The concentration of NO_2^- + NO_3^- after addition of 30 μl NO_2 was 3.29 ± .71 mM (% NO_2^- = 61 ± 26%). For the reaction of NO_2^- with Fd (n = 3), a 400-mM stock solution of $NaNO_2$ was used and the Fd was dissolved in PBS buffered to pH 5.9 with 20 mM morpholinoethane sulfonate to control for the acidification that occurred in the NO·/Fd reaction.

NO_2^- and NO_3^- Assay. NO_2^- and NO_3^- were measured by an automated colorimetric assay based on the Griess reaction described in detail elsewhere (2). Briefly, samples were reacted with the Griess reagent (1% sulfanilamide, 0.1% naphthylethylene diamine dihydrochloride, 2.5% H_3PO_4) and the NO_2^- concentration was determined by monitoring at 543 nm. NO_3^- in the sample was measured after reduction of NO_3^- to NO_2^-. Data were expressed either in concentration units or as nmoles produced over the indicated time periods. The percentage of NO_2^- in the sample relative to the total NO_2^- plus NO_3^- was also determined.

Other Reagents. N^G,N^G-dimethyl-L-arginine (DMA) was from Chemical Dynamics Corp., South Plainfield, NJ. All other chemicals were from Sigma Chemical Co., St. Louis, MO.

Results

Bioassay for NO·. The bioassay for Mϕ-derived NO· was based on that for EDRF (3, 4, 10). A ring of rabbit thoracic aorta was denuded of endothelial cells and superfused with the admixture of two streams of KHS, one containing 10 μM NE and the other exiting one of two columns: an empty control column, or an experimental

column containing Cytodex beads to which C3H/HeJ mouse peritoneal Mø or RAW 264 mouse Mø-like tumor cells had adhered. The Mø were activated for NO_2^-/NO_3^- production, either by prior exposure to rIFNγ and LPS (8), or to a lesser degree, by exposure to the Cytodex beads themselves. The Mø perfusate elicited a relaxation of the precontracted arterial ring in six of six experiments. Addition of 0.5 mM L-arginine to the solution perfusing the Mø column augmented the vasorelaxation. The degree of augmentation by L-arginine was inversely proportional to the extent of relaxation induced by the Mø perfusate without addition of L-arginine (Fig. 1).

Ferrous hemeproteins react with NO· with high affinity (18), and block vasorelaxation mediated by NO· (19). Similarly, perfusion of the Mø column with ferrous myoglobin (either reduced by ascorbate [Fig. 1, *A* and *B*], or freshly prepared under nonoxidizing conditions [Fig. 1 *C*] completely blocked vasorelaxation. The inhibition specifically required hemeprotein, because ascorbate alone did not reverse the Mø-induced relaxation (not shown). Inhibition of vasorelaxation by myoglobin/ascorbate was not due to toxicity to the Mø, because removal of myoglobin/ascorbate from the perfusion buffer led to immediate restoration of Mø-mediated relaxation in all cases. L-arginine and myoglobin/ascorbate did not act directly on the arterial ring, because L-arginine and myoglobin/ascorbate caused neither contraction nor relaxation when perfused through an empty column directly onto the ring (Fig. 1 *B*).

The vasorelaxing factor released from the Mø was short lived. As shown in Fig. 1, *A* and *B*, collected column effluent had no vasorelaxing effect when it was passed over the aortic ring a second time. Indomethacin had no effect on Mø-mediated vasorelaxation (Fig. 1 *C*), militating against a role for cyclooxygenase metabolites (10). However, vasorelaxation was reversed 50% upon addition of 10 μM methylene blue (Fig. 1 *B*), an inhibitor of guanylate cyclase in vascular smooth muscle (19), suggesting that the Mø-derived vasorelaxant, like NO·, acts through activation of this enzyme (4, 13).

N^G-methylated arginine analogs serve as competitive inhibitors of synthesis of NO_2^-/NO_3^- in Mø (6, 20) and NO· in endothelial cells (21, 22). Perfusion of 0.13 mM N^GN^G-dimethyl-L-arginine (DMA) through the RAW 264 Mø column completely blocked production of the relaxing factor (Fig. 1 *D*). Addition of 2.5 mM L-arginine to the perfusion medium containing DMA reversed the inhibition and potentiated the production of the Mø-derived relaxing factor. Similar results were seen with C3H/He Mø using the related inhibitor N^G-monomethylarginine (not shown). This indicated that generation of the relaxing factor was closely associated with Mø metabolism of L-arginine to NO_2^-/NO_3^-. However, authentic $NaNO_2$ relaxed arterial rings only at concentrations above 50 μM; 50% relaxation required 2.5 mM NO_2^- (n = 3, not shown). Although the Mø used in these experiments were actively synthesizing NO_2^- (see Fig. 1, legend), the NO_2^- concentration in the effluent of Mø columns that relaxed the aortic ring was only 1.8 μM (n = 3, Fig. 1 *C*). Thus, NO_2^- itself was not responsible for the vasorelaxation.

In sum, production of the Mø-derived vasorelaxant was augmented by L-arginine and inhibited by arginine analogs methylated on a guanido nitrogen. NO_2^- was not the active principle. The vasorelaxant was scavenged by ferrous myoglobin. It was highly labile in an aerated aqueous solution at pH 7.4. Its actions were blocked by methylene blue but not indomethacin. These are hallmarks of NO· (3, 4, 7, 19, 21).

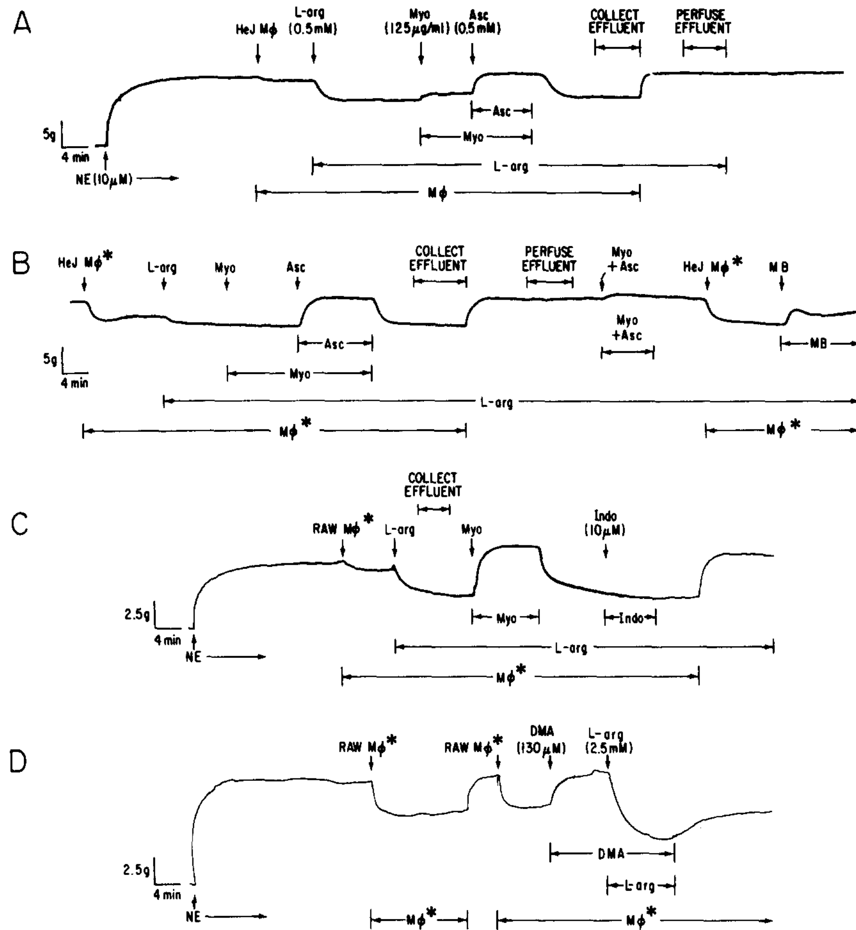

FIGURE 1. Representative tracings demonstrating the secretion of a vasorelaxing factor by activated primary Mø (n = 3) or by the Mø cell line RAW 264 (n = 5). Rings of rabbit aorta were denuded of endothelium and superfused with KHS. The superfusion medium was formed from two streams (each flowing at 3.2 ml/min) mixed just above the aortic ring. One stream contained the vasoconstrictor NE, while the other stream came from one of two columns: an empty control column, or an experimental column containing Mø adherent to Cytodex beads. (*A* and *B*) A single aortic ring was superfused with the effluent from columns containing control (*A*) or activated (*B*) C3H/HeJ Mø (1.1-1.3 ml bed volume; 1.7×10^7 Mø/column). (*C* and *D*) The aortic ring was superfused by a column containing activated Mø-like RAW 264 tumor cells (1.2 and 1.3 ml bed volume; 1.8 and 0.9×10^7 Mø/column). Mø were activated (Mø*) for NO_2^-/NO_3^- synthesis by prior exposure to rIFN-γ and LPS. (*A-D*) Mø generated NO_2^- at 0.80 ± 0.16, 1.34 ± 0.48, 1.62 ± 0.17, and 0.98 ± .14 nmol/h per 10^6 cells, respectively (mean ± SD, n = 3). Thus, control Mø were activated for NO_2^-/NO_3^- synthesis to some extent by cultivation on Cytodex beads alone. Arrows indicate the onset of superfusion with the Mø column effluent or drugs. Mø* effluents (*B* and *C*) relaxed the aortic ring in the presence of NE, whereas control Mø effluents (*A*) had little relaxant effect; L-arginine (L-arg; 0.5 mM) potentiated the relaxation in both cases. Mø- and Mø*-induced relaxation was reversed by reduced myoglobin (Myo; 125 μg/ml) or Myo + ascorbate (Asc; 0.5 mM) and blocked by methylene blue (MB; 10 μM), but not by indomethacin (Indo; 10 μM). DMA completely antagonized the Mø-induced relaxation in the absence of L-arginine; but with addition of L-arginine, production of the Mø-derived relaxing factor was fully restored and even potentiated compared with that produced before addition of DMA or L-arginine (*D*). (*A-C*) An interval is indicated during which the Mø column effluent causing vasorelaxation was collected after its passage over the aortic ring (*COLLECT EFFLUENT*). Reperfusion of the ring with this collected effluent 10 min later (*PERFUSE EFFLUENT*) did not elicit a second vasorelaxant effect. The NO_2^- concentration in the effluent collected in *C* was 1.8 μM and in KHS alone was 1.3 μM (n = 3).

Spectrophotometric Assay for NO· and NO_2. Next, we wished to find a molecule that would react with Mø-derived NO· under aerobic tissue culture conditions and thus serve as an independent assay of NO· production. The cultures had to be aerobic to permit Mø generation of nitrogen oxides. Trapping NO· was necessary, because in aerated solutions, NO· reacts rapidly with O_2 to form nitrogen dioxide (NO_2), which in turn reacts with water to yield NO_2^- and NO_3^-. Thus, if NO· is produced by Mø, it will not accumulate.

Mass spectroscopy has been used to measure NO· in endothelial cell perfusates after their collection into a solution of refluxing potassium iodide/acetic acid (7). However, this method does not discriminate between NO· released by endothelial

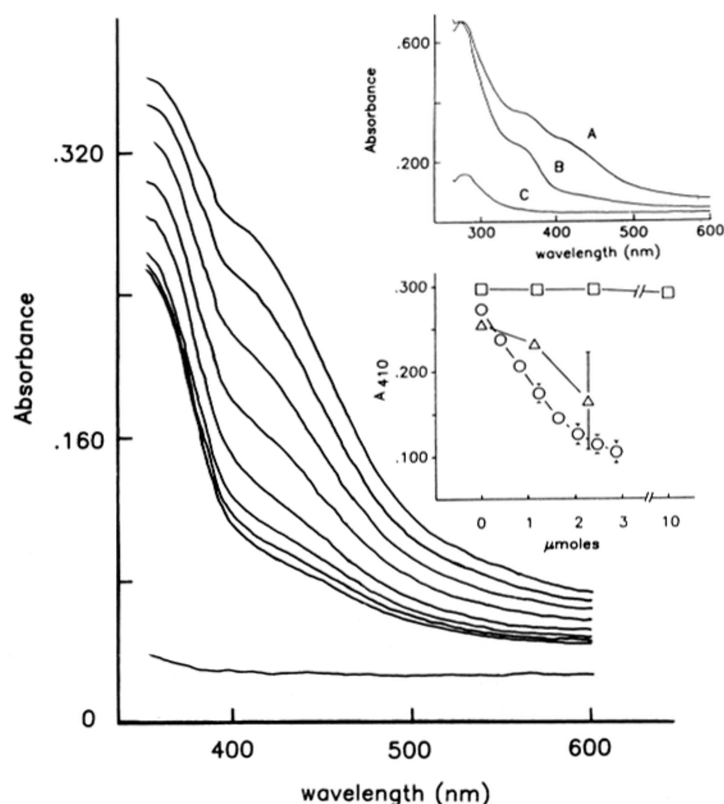

FIGURE 2. Spectroscopic assay of reaction of clostridial ferredoxin (Fd) with three oxides of nitrogen. (*Main figure*) Fd was dissolved under N_2 in deoxygenated PBS (pH 7.2) to form an 11.3-μM Fd solution, of which 900 μl (10.2 nmol) was transferred anaerobically to an N_2-flushed anaerobic cuvette. Authentic NO· that had passed through 1 M KOH was transferred with a gas-tight syringe in sequential 10 μl amounts to the cuvette. After each addition, the cuvette was mixed by several inversions and the spectrum recorded from 600 to 350 nm. The figure represents one of four similar experiments. (*Upper inset*) Spectrum of Fd before (*A*) and after (*B*) reaction with 70 μl (2.87 μmol) NO·, showing lack of change in the UV. Spectrum of buffer control is also shown (*C*). (*Lower inset*) Bleaching of Fd chromophore at 410 nm as a function of added NO· (○), NO_2 (△), or NO_2^- (□). Reactions of NO· (n = 4) and NO_2 (n = 3) gas were carried out as above; reaction of NO_2^- with Fd (n = 3) was done in PBS buffered to pH 5.9 as described in Materials and Methods.

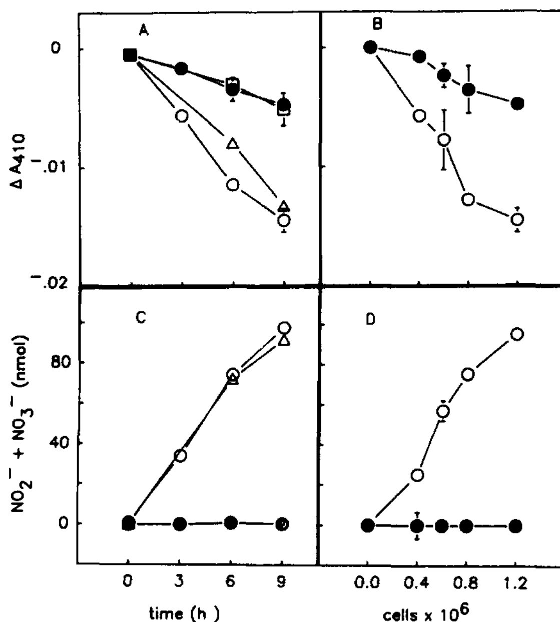

FIGURE 3. Mø-mediated loss of Fd absorbance at 410 nm (*A*, *B*) and production of NO_2^-/NO_3^- (*C*, *D*) as a function of time (*A*, *C*) and number of Mø (*B*, *D*). (*A* and *C*) RAW 264 Mø were plated at 1.2 × 10^6/well and activated for 6 h with rIFNγ and LPS as described in Fig. 1. The monolayers were then washed once with 1 ml of Dulbecco's minimum Eagle's medium with 8% calf serum but without phenol red or L-arginine (DMEM-ARG). This was aspirated and replaced with 0.7 ml DMEM-ARG containing Fd (8.2 μM). The following were then added in 3-5-μl quantities to give the final concentrations indicated: nothing (●); L-arginine (1 mM) (○); NO_2^- (230 μM) (□); L-arginine (1 mM) plus catalase 350 U/ml) (Δ). The wells were incubated at 37°C under 5% CO_2 for the times indicated and the A_{410} of the conditioned medium compared with identically cultured cell-free control media containing Fd or Fd plus catalase. The concentration of NO_2^-/NO_3^- was also determined for each solution and corrected for the value of the cell-free control medium containing catalase (NO_2^-/NO_3^- = 14.5 μM). (*B* and *D*). The experiments were performed as above, varying the cell number and collecting the supernatants at 9 h. The figure shows one of five similar experiments, each in triplicate.

cells and NO· arising in the collection vessel via reduction of NO_2^-. Hemeproteins have been used to trap NO· (4, 23). Preliminary experiments revealed that although nitrosyl-heme complexes were stable in an anaerobic atmosphere, they decomposed rapidly in aerated culture medium (not shown). In view of these drawbacks, we turned to Fe-S proteins. NO· reacts irreversibly with some Fe-S proteins (24), decreasing their absorbance in the visible spectrum. Thus, bleaching of Fe-S chromophores could serve as a cumulative measure of NO· release. Indeed, authentic NO· caused concentration-dependent bleaching of the visible spectrum of clostridial ferredoxin (Fd), an 8-iron, 8-sulphur Fe-S protein (Fig. 2). The absorbance of Fd at 410 decreased in a linear fashion with increasing amounts of NO· up to 2 μmol (Fig. 2, *lower inset*). The absorbance of Fd in the ultraviolet was unchanged under the same conditions (*upper inset*), demonstrating that the decrease in absorbance in the visible region was not due to dilution of the chromophore, and suggesting that the reaction of NO· with Fd was relatively specific for the Fe-S clusters. Authentic NO_2 also bleached Fd (*lower inset*). In this case, acidification caused by hydration of NO_2 complicated interpretation of the dose curve above 2 μmol NO_2. In contrast, NO_2^- did not bleach Fd, even at pH 5.9 (*lower inset*). Finally, the spectrum of Fd was stable over at least 19 h in tissue culture medium at 37°C.

Thus, we used bleaching of Fd to test for NO·/NO_2 production by activated Mø

in culture (Fig. 3). Mø bleached Fd; the magnitude of bleaching was proportional to time, cell number, and the concomitant production of NO_2^-. Only a minor amount of Fd bleaching occurred in Mø cultures in which synthesis of nitrogen oxides was blocked by omitting L-arginine, even when exogenous $NaNO_2$ was added at the start of the coincubation with Fd. Thus, the only Mø product(s) required for substantial bleaching appeared to be those distal to L-arginine and proximal to NO_2^- in the biosynthetic pathway of nitrogen oxides. Hypohalous acids can also bleach Fd (25), but were not involved in this case, because production of H_2O_2, the precursor of hypohalous acids, is not dependent on L-arginine (26), and in any event the addition of catalase to break down H_2O_2 had no effect on Mø bleaching of Fd. Unfortunately, control Mø (those not producing NO_2^-/NO_3^-) could not be tested in this system, because Fd itself partially activated the cells for NO_2^-/NO_3^- synthesis (not shown).

Discussion

The combined evidence from the biological and biochemical assays described above strongly suggests that activated Mø release an L-arginine–derived compound that is either NO· or a closely related substance with similar reactivity, such as NO_2. Compared with agonist-triggered endothelial cells (3, 4, 10), activated Mø secrete the NO·-like compound over much longer periods, and the total amount produced per cell may be far greater. The factors inducing NO· release require more study, but are probably similar to those inducing secretion of NO_2^-/NO_3^-. Inducers of NO_2^-/NO_3^- include LPS and IFN-γ, or synergistic combinations of IFN-γ with TNF, or LPS with IFN-α or IFN-β (8, 9).

Although Mø release of the NO·-like compound was augmented by exogenous L-arginine and completely blocked by DMA, variable amounts were still released by Mø in the absence of exogenous L-arginine (Fig. 1). This suggests existence of sources of L-arginine within the Mø. Such sources are probably short lived, since this metabolic pathway exhibits a strict requirement for exogenous L-arginine when measured over longer time periods (5).

These findings prompt a number of speculations about the role of NO· secretion in the physiology of Mø. First, NO· may be a specific mediator of arginine-dependent antitumor and antimicrobial effects of Mø, including inactivation of the target cell Fe-S enzymes *cis*-aconitase (27), succinate:ubiquinone oxidoreductase, and NADH:ubiquinone oxidoreductase (28). Second, Mø may play a previously unsuspected part in regulating smooth muscle tone during inflammatory or immune responses. Interest in this hypothesis is heightened by the abundance of Mø in the renal juxtaglomerular apparatus and interstitium (29), gastrointestinal mucosa (30), bronchial epithelium (31), pulmonary vasculature, interstitium, and alveoli (31), and sinusoids of liver, spleen, and marrow, as well as in wounds, granulomas, and atheromas. For example, the mononuclear phagocytes associated with blood vessels may affect systemic blood pressure during sepsis, or regional blood flow in inflammation and wound healing. Third, secretion of NO· by vessel-associated Mø or monocytes could influence not only smooth muscle cells and platelets but also endothelial cells. Fourth, in lymphocytes, activation of guanylate cyclase or inactivation of certain Fe-S enzymes by NO· might contribute to Mø-mediated stimulation or suppression of immune responses.

Summary

L-arginine–dependent synthesis of nitrite (NO_2^-) and nitrate (NO_3^-) by macrophages correlates with and is required for their execution of nonspecific cytotoxicity toward some tumor cells and microbes. However, the bioactive L-arginine metabolites responsible for cytotoxicity are unknown. Mammalian endothelial cells have recently been shown to release nitric oxide (NO·); we therefore determined if this reactive metabolite was synthesized by activated murine macrophages. Macrophage-derived NO· was detected by two independent methods: a bioassay for NO·-mediated relaxation of preconstricted rings of rabbit aorta; and a spectroscopic measurement of the reaction of NO· with clostridial ferredoxin, an Fe-S protein. After activation with IFN-γ and LPS, macrophages continuously secreted a substance that relaxed rabbit aortic rings denuded of endothelium. Production of the vasorelaxant was enhanced by 0.5 mM L-arginine and inhibited reversibly by N^G-methylated L-arginine analogs that block macrophage NO_2^-/NO_3^- synthesis. The vasorelaxant was scavenged by ferrous myoglobin, was labile, and was neither NO_2^- nor a cyclooxygenase metabolite. Activated Mø also secreted a substance that bleached Fd, a reaction carried out by NO· and NO_2, but not NO_2^-. Macrophage bleaching of Fd correlated directly with time, cell number, and concomitant NO_2^-/NO_3^- production, required L-arginine, and was independent of reactive oxygen intermediates. Thus, activated murine Mø release NO· and/or a closely related, highly reactive nitrogen oxide such as NO_2, during their conversion of L-arginine to NO_2^-/NO_3^-. NO· and NO_2 may mediate L-arginine–dependent pathologic effects of Mø, as well as physiologic effects not previously considered for this widely distributed cell type.

We thank Drs. Jack Peisach and Ralph Nachman for helpful discussions, and Claudia Morris for expert assistance.

Received for publication 17 October 1988 and in revised form 21 November 1988.

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