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DIRECT MEASUREMENT OF NITRIC OXIDE IN HEADSPACE GAS PRODUCED BY A CHICKEN MACROPHAGE CELL LINE IN A CLOSED CULTURE SYSTEM

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SUMMARY A simple and rapid method was applied for direct measurement of nitric oxide (NO) gas produced by cultured macrophages using a modified chemiluminescence detector, the thermal energy analyzer (TEA). HD11 chicken macrophages (1-3 x 10^6/ml) were cultured on microcarrier beads (100 mg/ml) in 140 ml air-tight glass jars (5 ml cell suspension per jar) containing 0.5 μg/ml of LPS and different concentrations of L-arginine. Headspace gas was sampled at 24 hours of culture via a rubber septum and directly injected into a TEA with a liquid nitrogen trap set at -130 to -140°C. The concentration of NO in the gas sample was quantified using a standard gas mixture of NO (2 μl/L) in nitrogen. Gas samples from L-arginine-supplemented cultures contained NO (0.028 - 0.066 $pl/\mu l$), whereas NO was not detected in samples from controls. These results suggest that chicken macrophages synthesize NO gas in a dose-dependent manner relative to L-arginine concentration. © 1992 Academic Press, Inc.

Nitric oxide (NO), a metabolite of activated macrophages, has been described as a essential cytotoxic effector molecule important in nonspecific immune defense mechanisms in mammalian species (1,2). NO may also play an important role in vascular tone regulation (3,4) and neurotransmission (5). The enzymatic pathway responsible for NO production has been demonstrated to involve oxidation of the guanidino nitrogen of L-arginine, and generate citrulline and NO radical (6,7). NO is then rapidly oxidized, by reaction with oxygen to form N_2O_3 and N_2O_4 or with water to form nitrite (NO_2^-) and nitrate (NO_3^-), the two aqueous products of NO (6,8). Most studies regarding NO production have been conducted by an indirect measurement of NO_2^- using the Griess method (9). We have previously shown that cultured chicken peritoneal exudate cells (> 85% macrophage) and a myelocytomatosis virus MC29-transformed macrophage cell line, HD11, produced NO_2^- in an L-arginine- and endotoxin-dose-dependent manner (10). Since the diazotization reaction (eg. Griess method) involved in the NO_2^- assay sometimes lacks complete specificity (11), a direct measurement of NO is desirable. The existence of NO in aqueous solution has been

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demonstrated by Palmer *et al* using a chemiluminescence technique (12). Hibbs *et al* used an alternative approach by trapping gases from the continuous air flow of a semi-closed culture of murine macrophages and measuring NO as NO_2 after oxidation of NO by passing the gas through an oxidizing solution ($KMnO_2$ in sulfuric acid) (2). Myers *et al* collected NO in effluent from cultured endothelial cells by passing the effluent through a vacuum chamber and delivering the gas directly to a chemiluminescence detector (13). Recently, chemiluminescence detection of NO in headspace gas from a closed system has been described in two studies (14,15); however, neither involved culture of live cells: one used a macrophage lysate (14), the other was applied to measure NO released from nitrovasodilator drugs (15). This report describes a simple and rapid method to directly measure NO gas produced by activated macrophages in a closed culture system. Nitric oxide gas quantitation was accomplished using a chemiluminescence-based detection device, the thermal energy analyzer (TEA; Thermo Electron Corporation, Waltham, MA). Within the analyzer, NO reacts with O_3 in a reaction chamber to form O_2 and excited NO_2. The excited NO_2 decays to ground state and emits a photon. The photon signal is amplified by a photomultiplier and detected by a photon detector.

MATERIALS AND METHODS

Reagents. Glass microcarrier beads (150-210 micron), lipopolysaccharide (LPS) (from *E. coli* strain 0127:B8), L-arginine HCl, N-1-naphthylethylenediamine dihydrochloride, and sulfanilamide were purchased from Sigma Chemical Co., St. Louis, MO. Fetal bovine serum (FBS), Penicillin-Streptomycin solution, L-lysine HCl, D-glucose, regular Dulbecco's Modified Eagle's Medium (DMEM) and Defined Dulbecco's Modified Eagle's Medium (D-DMEM) powder free of D-glucose, L-arginine and L-lysine (without phenol red) were purchased from GIBCO Laboratories, Grand Island, NY. Low-endotoxin fetal bovine serum was obtained from HyClone Laboratories, Logan, Utah. Before each experiment, D-DMEM was reconstituted and supplemented with 4500 mg/L of D-glucose, 146 mg/L of L-lysine, and 3.7 g/L of $NaHCO_3$. L-arginine-containing medium was prepared by adding increasing amounts of L-arginine to D-DMEM. Nitric oxide standard gas (1% v/v in N_2) was purchased from Union Carbide Corporation, Linde Division, Somerset, NJ, and diluted to 2 μl/L (0.0002% or 2 *n*l/ml) at least 24 hrs prior to each experiment by injecting 0.23 ml of NO standard gas into a 1150 ml stainless steel tank containing purified N_2.

Macrophage cell line. The HD11 cell line, an avian myelocytomatosis virus (MC29) transformed macrophage cell line, was originally developed by Dr. Thomas Graf and colleagues (16) and has previous been used in the quantitation of NO_2^- production (10). Cells were maintained in DMEM plus 10% FBS (GIBCO) at 39°C, 5% CO_2. Three days prior to each experiment, cells were passaged in DMEM plus 10% low-endotoxin FBS (HyClone). For the experimental culture, cells were washed twice with D-DMEM prior to further treatment.

Incubation with microcarrier beads. HD11 cells were resuspended at 3-5 x 10^6/ml for HD11) in D-DMEM containing 10% (HD11) low-endotoxin FCS and 200 mg/ml of microcarrier glass beads. Siliconized tubes containing this cell suspension were incubated for 3 hr (39°C, 5% CO_2) on a roller-bottle device rotating at 14 rpm. After incubation, approximately 35% of the cells were adherent to the microcarrier beads. The cell suspension was then centrifuged at 60 *g* for 5 min and non-adherent cells were removed. The glass beads/cells pellet was resuspended with a gentle vortex using the same volume of the above incubation medium plus 20 mM of HEPES and 100 U/100 μg/ml of Penicillin-Streptomycin.

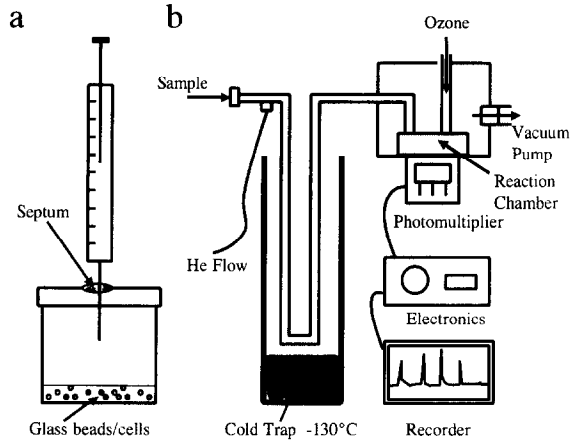

Figure 1. **a**, The physical design of the closed culture system for NO measurement. **b**, Schematic illustration of thermal energy analyzer (TEA).

Culture condition for nitric oxide production and measurement. Five ml of glass beads/cells suspension were transferred to 140 ml sterile Mason jars (Ball Corporation, Muncie, IN) and supplemented with 0.5 μg/ml of LPS and 0.1 mM or 1 mM of L-arginine. The jars were then sealed with gas-tight lids in which thick rubber serum bottle septa were installed (Fig.1a). The closed system culture was continued for 24 hr at 39°C. At the end of the incubation, 200 μl of headspace gas was sampled using a Dynatech gas-tight syringe to measure nitric oxide concentration. The same sampling procedure was also used in measurement of oxygen consumption described below. The gas sample was then directly injected into a modified TEA chemiluminescence detector (17) (Fig.1b). The normal TEA chromatographic system and pyrolyzer were by-passed. Sufficient helium (He) was metered directly into the liquid nitrogen trap such that the combined O_3 and He pressure in the reaction chamber was 2.0 torr (0.9 O_3 and 1.7 He). The liquid nitrogen trap was adjusted to a temperature of -130 to -140 °C. The He line between the gas pressure regulator and the liquid nitrogen trap was fitted with a Swagelock Tee, one side of which was fitted with a silicone septum that allowed samples to be directly introduced into the He stream before the trap. The NO concentration of the headspace was calculated by comparing the area under the response curve (AUC) with responses generated by the authentic NO standard (2 *n*l/ml). A Hewlett-Packard 3390A integrator was used to calculate areas.

Precision of Nitric Oxide Measurement. The precision of the TEA-nitric oxide measurement was assessed by determining the linear dynamic range and the intra-day coefficient of variation (CV) values of each NO standard level (converted to *p*mol as calculated from the ideal gas law, $PV = nRT$). The CV was calculated by analyzing nitric oxide standards in 6 repeated injections.

Measurement of oxygen consumption. Oxygen concentration remaining in the headspace gas was sampled as above (100 μl per injection) and analyzed by gas chromatography (Aerograph 200, Varian Instrument Co., Sunnyvale, CA) with dual thermal conductivity detectors (TCDs) and a Molecular Sieve 5A (3 m x 3.2 mm, stainless steel, 30-40 mesh) column. The operation conditions were: injection port 115°C, detector 135°C, oven 105°C, carrier gas (He) 20 ml/min.

Analysis of *p*H of the culture medium and cell viability after culture. At the end of the cell culture, *p*H of the culture medium in each jar were measured by a *p*H meter (Accumet Model 230A, Fisher Scientific Co., Pittsburgh, PA). Cell viability was estimated by a trypan blue exclusion assay (18).

Nitrite analysis. Nitrite accumulation in the culture medium was measured as described by Green *et al* (9). At the end of cell culture, 1.5 ml of supernatant from each well was added to an equal volume of Griess reagent (1:1, v/v, of N-1-naphthyl-ethylenediamine 0.1% in distilled H_2O and sulfanilamide 1% in 5% H_3PO_4) on a 96-well flat-bottom plate. Absorbance at 550 nm was measured by a spectrophotometer (Ultraspec 4050, Pharmacia LKB Biotechnology Inc., Piscataway, NJ) using dH_2O as blank. Nitrite concentration was calculated from a precalibrated standard curve using $NaNO_2$ as standards.

Statistical analysis. Data were analyzed using one-way analysis of variance (ANOVA) and Fisher's protected LSD test.

RESULTS

The TEA method described in this report detected NO standards ranging from 0.52 *p*mol to 49.20 *p*mol with good linearity (R^2= 0.996) (Fig. 2); the linear dynamic range is therefore 94. The intra-day CVs of NO standards ranged from 1.5 to 9.4% (Table 1), while the limit of detection for a 3:1 signal:noise ratio was 0.09 *p*mol. As shown in Table 2, nitric oxide gas production by chicken macrophages was dependent upon L-arginine supplementation in the culture medium. There was a dose-dependent response relative to L-arginine concentration and the bacterial endotoxin (LPS) signal was required to trigger NO production. Cell viability after culture was between 65% to 80% as determined by trypan blue dye exclusion. Oxygen consumption by macrophages ranged from 4% (16% residual oxygen) to 8% (12% residual oxygen). Cultures containing exogenous nitrite (50 to 150 μM $NaNO_2$ in media at *p*H 5.7 to 6.4) did not give rise to a detectable amount of NO using comparable culture conditions, ruling out the possibility that NO production resulted from the reduction of nitrite under acidic conditions.

DISCUSSION

L-arginine-dependent production of reactive nitrogen intermediates (RNIs: NO, NO_2^-, and NO_3^-) has been shown to be responsible for certain antitumor and antimicrobial activities of

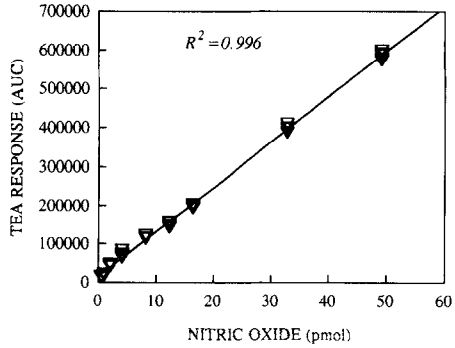

Figure 2. Least-square linear regression analysis of NO standards. The linear regression coefficient (R^2) was 0.996 in this representative measurement. Each triangle represents one measurement. Six measurements were performed for each level of NO standard.

TABLE 1. Intra-day coefficients of variation (CVs)* of the TEA-nitric oxide measurement for nitric oxide standards

Nitric Oxide Standards Injected (*p*mol)	CV (%)
0.52	8.2
1.03	6.3
2.05	5.6
4.10	9.4
8.20	2.5
12.30	3.5
16.40	1.9
32.80	1.7
49.20	1.5

* Coefficients of variation are calculated as follows: (standard deviation/mean) x 100.

mammalian macrophages *in vitro* (2,6,19-23). Chickens represent a useful animal model for the study of this macrophage metabolic pathway, because these uricotelic animals are unable to synthesize the L-arginine substrate (24,25). In a previous study, we reported that activated chicken macrophages produce RNIs in a similar fashion to their mammalian counterparts. This was based on the quantitation of NO_2^- produced by both the HD11 cell line and activated chicken peritoneal macrophages (10).

In the present study, NO was directly measurable in the headspace gas of from the closed cell culture system. A statistically significant dose-dependency for L-arginine concentration was observed. The transformed cell line tolerated the hypoxic culture conditions and produced NO. The possibility that NO was reduced from NO_2^- in the aqueous phase was eliminated based on results with the NO_2^- controls. In addition, gaseous NO_2 does not produce a significant chemiluminescent response (Maragos *et al*, National Cancer Institute, personal communication). This lack of response may be due to the failure of $NO_{2(g)}$ to produce chemiluminescence and the fact that other nitrogen oxides species have boiling points above -90°C, and would be trapped in the liquid nitrogen trapping

TABLE 2. Nitric oxide production by macrophages in different culture conditions. Oxygen consumption, cell viability, *p*H of the medium, and nitrite accumulation at the end of culture were also assessed.

Culture Condition	NO per injection[a] (*p*mol)	NO Concentration (*p*mol/μl)	Total NO in Headspace (*p*mol)	Oxygen Concentration (%)	Nitrite in Medium[d] (*n*mol)	Cell Viability (%)	*p*H in Medium
Air	ND[c]	ND	ND	20	–	–	–
Medium only	ND	ND	ND	19 ± 2	–	–	7.4
LPS 0, Arg 0[b]	ND	ND	ND	16 ± 1	nd[e]	65 ± 4	6.1 ± 0.3
LPS 0.5, Arg 0	ND	ND	ND	13 ± 2	nd	65 ± 4	6.1 ± 0.3
LPS 0.5, Arg 0.1	5.7 ± 0.8	0.028 ± 0.004	3780 ± 540	12 ± 3	393 ± 82	72 ± 8	5.8 ± 0.4
LPS 0.5, Arg 1.0	13.1 ± 2.5 (n=6)	0.066 ± 0.013 (n=6)	8910 ± 1755 (n=6)	13 ± 2 (n=4)	683 ± 93 (n=4)	70 ± 6 (n=3)	5.7 ± 0.3 (n=4)

[a]200 μl per injection. [b]LPS concentration in μg/ml (0, 0.5), arginine concentration in mM (0, 0.1, 1.0). [c]ND = not detectable (< 0.09 *p*mol). [d]Total nitrite in 5 ml of culture medium. Nitrite concentrations of Arg 0.1 and Arg 1.0 treatments are then 78. 6 ± 16.4 μM and 136.6 ± 18.6 μM, respectively. [e]nd = not detectable (< 5 *n*mol in 5 ml or < 1 μM).

loop. Nitric oxide, in contrast, with a boiling point of -151.8°C (26), passes through the cold trap. This indicates that the TEA response was due solely to NO. Direct quantitation of NO from macrophage cultures can be useful in an overall evaluation of RNI production. Because of the possibility of a continuous evaporation of NO in open culture systems, measurement of nitrite alone may underestimate the actual RNI production.

The existence of L-arginine-dependent production of NO by an activated chicken macrophage cell line suggests that the L-arginine/NO pathway may be a universally-utilized defense mechanism of higher vertebrates. This is further suggested by the finding that dietary intake of L-arginine can influence the host-tumor interaction *in vivo* in the chicken (27) as well as in the mouse (28). The opportunity for direct measurement of NO produced by activated macrophages should facilitate an assessment of the factors which can influence this important metabolic pathway.

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