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Differences in Nitric Oxide Synthase Activity in a Macrophage-Like Cell Line, RAW264.7 Cells, Treated with Lipopolysaccharide (LPS) in the Presence or Absence of Interferon-γ (IFN-γ): Possible Heterogeneity of iNOS Activity[1]

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Nitric oxide synthase (NOS) activities were compared in a macrophage-like cell line, RAW 264.7 cells, treated with bacterial lipopolysaccharide (LPS) alone or with LPS and interferon-γ (IFN-γ). An about 5-6-fold higher amount of NO_2^- originating from the nitric oxide radical (·NO) was produced in the culture supernatant of macrophages treated with LPS+IFN-γ than with LPS alone, depending on both the time of incubation and the dose of LPS. However, the difference in the amounts of iNOS protein in these macrophage extracts was much greater than that in the amount of released NO_2^-. To estimate the NOS activity after induction of NOS at 37°C for 8 h, we examined both intact cells and cell extracts. The cells were washed and re-incubated in Hank's balanced salt solution (HBSS) containing 1 mM L-arginine and 100 μM carboxy-PTIO, which enabled us to detect trace amounts of ·NO by means of rapid reaction of carboxy PTIO with ·NO to form $\cdot NO_2$, which was finally converted to NO_2^-. The results showed an about 7-fold difference between the macrophages pretreated with LPS alone and those with LPS+IFN-γ. Using the extracts, the NOS activity was assayed with L-[*U*-^{14}C]arginine as a substrate for NOS *in vitro*, and the results again revealed an about 7-fold difference between the two types of cell extracts. A kinetic study of the NOS activity by means of *in vitro* assay suggested that there was little difference in K_m value for L-arginine between these two iNOSs. However, it revealed two apparent K_ms for β-NADPH, a co-factor of NOS; one was about 0.4 μM, which was common to these two iNOSs, and the other was about 1.5 and 25 μM for LPS-induced NOS and LPS+IFN-γ-induced NOS, respectively. The cellular concentration of β-NADPH was around 14 μM in both LPS- and LPS+IFN-γ-treated macrophages. These results suggest that there is some heterogeneity of iNOSs induced in macrophages with LPS alone and with LPS+IFN-γ, and the heterogeneity seems to be due at least in part to the requirement for β-NADPH.

Key words: inducible nitric oxide synthase, interferon-γ, lipopolysaccharide, macrophage activation, murine macrophage-like cell line.

Nitric oxide (·NO) has been suggested to be involved in the anti-microbial and anti-tumor activities of activated macrophages (*1-4*) treated with several kinds of biological response modifiers, such as LPS and IFN-γ (*2, 5*). Although there have been many reports on the iNOS in macrophages, the differences in and regulatory mechanisms for iNOS activities in activated macrophages treated with different macrophage activators remain largely unknown. In order to elucidate the regulatory mechanism for the induced NOS in activated macrophages, we examined the NOS activities in a macrophage-like cell line, RAW 264.7 cells, treated with either LPS alone or LPS+IFN-γ. NOS activity in intact cells was assayed with an ·NO-scavenger, carboxy-PTIO, which improved the detection of ·NO with Griess reagent (*6*). Another assay was performed *in vitro* with L-[*U*-^{14}C]arginine as a substrate for NOS, using cell extracts of activated macrophages treated with different activators. We also improved the detection method for the reaction products, N^G-hydroxy-L-[^{14}C]-arginine and L-[^{14}C]citrulline, with a thin layer plate, and the subsequent detection of radioactivity with a bioimage analyzer. The results of this study suggest possible heterogeneity of iNOS in LPS- and LPS+IFN-γ-treated macrophages, and also suggest a regulatory mechanism for the NOS activity in activated macrophages based on the concentration of β-NADPH.

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Abbreviations: Carboxy-PTIO, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide; H_4B, (6*R*)-5,6,7,8-tetrahydro-L-biopterin; HBSS, Hank's balanced salt solution; iNOS, inducible nitric oxide synthase; IFN-γ, interferon-γ; LPS, lipopolysaccharide; L-NIO, N^G-(1-iminoethyl)-L-ornithine.

MATERIALS AND METHODS

Materials—L-Arginine, L-citrulline, L-valine, N^G-hydroxy-L-arginine, β-NADPH, FAD, FMN, DTT, and lipopolysaccharide (LPS; *Escherichia coli*, O55:B5, chromatographically purified) were obtained from Sigma (St. Louis, MO). 2-(4-Carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide (Carboxy-PTIO) was obtained from Dojindo Laboratories (Kumamoto), and (6*R*)-5,6,7,8-tetrahydro-L-biopterin (H_4B) was from Alexis (San Diego, CA). ^{125}I-labeled protein A was prepared by iodination of protein A (Sigma) with ^{125}I-labeled NaI (NEN; Du Pont, Lincoln Park, NJ) according to the chloramine-T method, as described previously (*7*). L-[*U*-^{14}C]Arginine was obtained from Amersham International plc (Amersham, England). Recombinant mouse interferon-γ (IFN-γ) was kindly donated by Toray Industries (Tokyo). All other chemicals and reagents were of the purest commercial grade available.

Cell Culture—A murine macrophage-like cell line, RAW 264.7, was obtained from the American Type Culture Collection (Rockville, MD). The cells were maintained by culturing in Ham's F12 medium (Dainippon Pharmaceutical, Osaka), supplemented with 50 U/ml penicillin, 50 μg/ml streptomycin (Flow Laboratories, Irving, Scotland), and 10% (v/v) heat-inactivated fetal bovine serum (GIBCO BRL, Grand Island, NY), in a CO_2 incubator (5% CO_2-95% humidified air) at 37°C. The cells were seeded onto plastic petri dishes (Falcon®, #1001, Deckton Dickinson), and passaged twice a week.

Induction of iNOS in Macrophages—The cells were seeded at 4×10^5 cells/ml onto 60 mm-petri dishes (Falcon®, #1007) in 5 ml of fresh culture medium and then incubated at 37°C overnight. The medium was replaced with 5 ml of fresh medium containing 100 ng/ml LPS with or without 10 U/ml IFN-γ. The cells were further incubated at 37°C, usually for 8 h, and then 1 ml of the culture supernatant was collected in a microfuge tube and centrifuged at 10,000 rpm for 1 min at 4°C. One-half ml of the resultant supernatant was collected and stored at 4°C before assay of NO_2^-. Simultaneously, the cells were chilled on ice, scraped from the dish with a cell scraper (Costar, Cambridge, MA), and then washed twice with phosphate-buffered saline without divalent cations (PBS) by repeated centrifugation at 2,000 rpm for 3 min. The cell pellet was suspended in 0.5 ml of PBS, transferred to a microfuge tube and centrifuged quickly at 7,000 rpm at 4°C. The final cell pellet was extracted with 50 μl of the lysis buffer, composed of 1% (v/v) Triton X-100, 0.1 mM EDTA, and 1% (v/v) Aprotinin (Sigma) in 20 mM HEPES-NaOH buffer, pH 7.5, at 4°C for 30 min. The cell lysate was centrifuged at 10,000 rpm for 1 min at 4°C, and 40 μl of the resultant supernatant was collected in a new microfuge tube. The final cell extract was stored at −80°C until use.

SDS-PAGE and Immunoblotting—Fifty microgram aliquots of the cell extracts were treated with SDS-sample buffer, comprising 1% SDS (Wako Pure Chemicals Industries, Osaka), 5% β-mercaptoethanol (Sigma), 20% glycerol, 1 mM EDTA, and 0.05% bromophenol blue (Sigma), and boiled at 95°C for 5 min. After cooling, the samples were loaded on 7.5% SDS-slab gels and electrophoresed at 20 mA with Fairbank's system (*8*). The gels were rinsed briefly with the transfer buffer, composed of 0.02% SDS and 20% (v/v) methanol in 25 mM Tris-HCl buffer, pH 7.5, and the proteins were electrotransferred to a PVDF membrane (Immobilon-P®; Millipore, Bedford, MA) at 30 V overnight and then at 100 V for 30 min with a Mini-Slab Blotting System® (Bio-rad, Hercules, CA). The blotted membrane was then rinsed with a rinse buffer, composed of 0.1 M NaCl in 25 mM Tris-0.192 M glycine buffer, pH 7.5, and blocked with 30 mg/ml milk casein (Snow Brand Milk Products, Sapporo) at 37°C for 30 min. The proteins on the membrane were reacted with 10 μl of a monoclonal anti-macrophage iNOS antibody (Transduction Laboratories, Lexington, KY) in 10 ml of the rinse buffer containing 3 mg/ml milk casein at 37°C for 2 h. The membrane was rinsed and then reacted with rabbit anti-mouse IgG immunoglobulin (Zymed Laboratories, San Francisco, CA) at 37°C for 1 h. Finally, immune complexes on the membrane were reacted with ^{125}I-labeled protein A at 37°C for 1 h. After repeated washing of the membrane with the rinse buffer containing 1 M NaCl, the filter was air-dried and placed on an imaging plate (Fuji Photo Film, Tokyo). The intensity of the band corresponding to a molecular weight of 130 kDa, *i.e.*, iNOS, was quantitated with a BAS2000 bioimage analyzer (Fuji Film). In order to standardize the iNOS amount in different experiments, we calculated the amounts of iNOS protein from a calibration curve based on various amounts of the cell extracts of the activated macrophages treated with 100 ng/ml LPS and 10 U/ml IFN-γ at 37°C for 8 h, and the results were shown as relative values when the iNOS amount in 50 μg of the cell extract was expressed as 50. Because the calibration curves were similar in different experiments, and because there was a close correlation between the amounts of the cell extracts and the intensities of the bands on bioimage films (*i.e.*, PSL value) in a linear fashion in the range from 1 to 100 μg (data not shown), we used 50 μg of the cell extract from one experiment as a standard of iNOS amount on the gel for immunoblot analysis.

NOS Activity in Intact Cells—To examine the NOS activity in intact macrophages, an improved detection method for ·NO was used for a macrophage culture with carboxy-PTIO, a scavenger of ·NO, and Griess reagent (*6*). This method enabled us to determine NOS activity in intact macrophages after induction of iNOS. In brief, macrophages were treated with LPS or LPS+IFN-γ in the culture medium at 37°C for 8 h as described previously, and then the cells were collected, washed three times with PBS, and suspended at 4×10^5 cells/ml in modified Hank's balanced salt solution, composed of 8.0 g of NaCl per liter, 0.4 g of KCl per liter, 1.0 g of glucose per liter, 0.06 g of $Na_2HPO_4\cdot2H_2O$ per liter, 0.06 g of KH_2PO_4 per liter, 0.35 g of $NaHCO_3$ per liter, 1 mM $CaCl_2$, and 1 mM $MgCl_2$ in 20 mM HEPES-NaOH buffer, pH 7.5 [HBSS(+)]. One-half milliliter of the cells was seeded into a flat-bottomed plate with 24 wells (Sumilon® MS-8024R; Sumitomo Bakelite, Osaka), and then incubated at 37°C for 10 min to allow the cells to adhere to the plate. Then 100 μM carboxy-PTIO and 1 mM L-arginine were added to the cell culture, followed by further incubation for 0-2 h at 37°C. The reaction was terminated by chilling of the cells on ice, and the culture supernatant was collected and analyzed for NO_2^- content. During incubation in HBSS(+), the cells

remained intact without an increase in the number of cells or the amount of iNOS.

NOS Activity in Cell-Free Extracts In Vitro—The 50 μl reaction mixture was composed of 100 μM β-NADPH, 10 μM H_4B, 10 μM FAD, 10 μM FMN, 100 μM DTT, 100 μM L-[*U*-^{14}C]arginine, and 50 mM L-valine in 50 mM HEPES-NaOH buffer, pH 7.5, with 50 μg of cell extract as an enzyme source. The mixture without the enzyme was preincubated at 37°C for 3 min, and then the enzyme in 10 μl of cell extract was added to start the reaction, followed by incubation at 37°C for 10 min. The reaction was terminated by the addition of 200 μl of ice-cold 85% ethanol in water, and the mixture was centrifuged at 15,000 rpm for 10 min at 4°C. Two hundred microliters of the resultant supernatant was collected, transferred to a new tube and dried under vacuum (Speed-Vac®, Savant Instruments, Farmingdale, NY). The reaction products in the dried pellet were dissolved in 40 μl of a mixture of 25 mM L-arginine and L-citrulline, and 10 μl aliquots were spotted onto a 20 cm × 20 cm microfine cellulose thin layer plate (Tokyo Kasei, Tokyo), and the plate was developed with a solvent system of ethanol/diethylamine/acetone/H_2O (30 : 4 : 30 : 15, v/v), which separated L-citrulline and N^G-hydroxy-L-arginine from L-arginine. These amino acids were visualized with vaporized 0.25% (w/v) ninhydrin in acetone, and the TLC plate was air-dried and placed on an imaging plate (Fuji Film). The radioactivity on the plate was quantitated with a BAS2000 bioimage analyzer. Total radioactivity of the reaction products was determined with an Aloka liquid scintillation counter (Aloka, Tokyo), with 10 μl aliquots of the reaction products dispersed in 3 ml of ACS-II liquid scintillation cocktail (Amersham). For estimation of citrulline formation, the % of L-[*U*-^{14}C]citrulline was calculated with respect to all the radioactivity recovered on a lane on a TLC plate, and then normalized to the total radioactivity recovered in the reaction mixture. The net amount of L-[*U*-^{14}C]citrulline produced in the reaction was calculated based on the concentration of 100 μM L-[*U*-^{14}C]-arginine in 50 μl of the reaction mixture.

$$\text{L-[}U\text{-}^{14}\text{C]citrulline formed} = \text{L-[}U\text{-}^{14}\text{C]arginine} \times \frac{\text{L-[}U\text{-}^{14}\text{C]citrulline on a TLC plate}}{\text{[}U\text{-}^{14}\text{C]-labeled spots}} \times \frac{\text{Total radioactivity in the reaction mixture}}{\text{Total radioactivity recovered}}$$

Assaying of Cellular β-NADPH—Cells were treated with LPS or LPS + IFN-γ at 37°C for 8 h, and then washed three times with PBS by centrifugation as described above. The final cell pellet was mixed with 200 μl of 70% (v/v) ethanol in water and then boiled for a few seconds in a water bath. After cooling, the mixture was centrifuged, and 100 μl of the supernatant was collected, and transferred to a new tube dried under vacuum with the Speed-Vac. The dried samples were dissolved in 50 μl of distilled water, and 20 μl aliquots of them were used for assay of β-NADPH with an NADPH assay kit (Bio-Orbit, Turku, Finland), based on the β-NADPH-dependent luciferase reaction, with a Luminoscan plus® luminometer (Labsystems, Helsinki).

Assay of NO_2^-—Assay of NO_2^- was performed as described previously (*6*). In brief, 100 μl aliquots of the culture supernatants were placed in duplicate in a 96-well ELISA plate (Sumitomo Bakelite, Osaka). A standard solution of $NaNO_2$ was also placed in wells on the same plate. To quantitate NO_2^-, Griess Romijin reagent for nitrite assay (Wako Pure Chemicals) was dissolved in distilled water at 6 mg/ml, and a 100 μl aliquot of the reagent was added to the wells of both standards and samples. The reaction products were colorimetrically quantitated at 550 nm with subtraction of background absorbance at 630 nm, using a Multiscan plus® microplate reader (Falcon®, Beckton Dickinson). The concentration of NO_2^- was determined from the calibration curve for the standards.

Other Assays—Protein concentrations were determined according to Bradford (*9*). Cell volume was estimated by measuring of the volume of 5-10 × 10^6 cells, pelleted by centrifugation at 10,000 rpm for 1 min at 4°C, with an Eppendorf micropipette, model 3110 (Eppendorf, Hamburg).

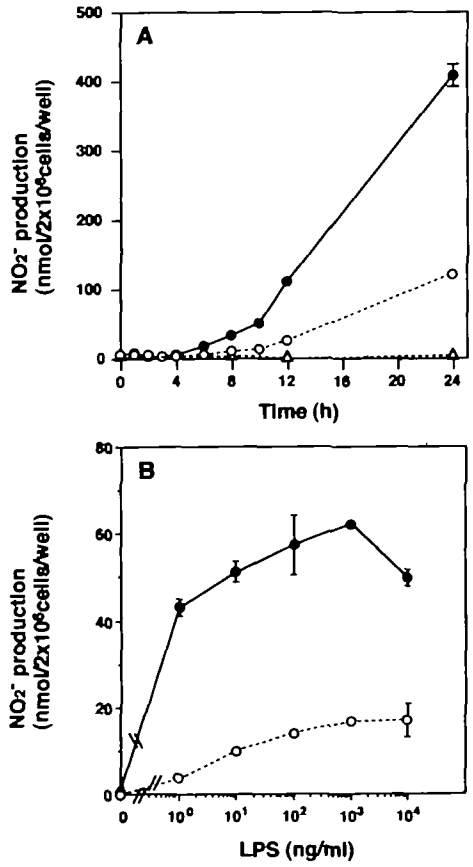

Fig. 1. **Production of NO_2^- by RAW 264.7 cells.** (A) Time-course of NO_2^- accumulation in the culture supernatant of RAW 264.7 cells treated with LPS in the presence or absence of IFN-γ. The cells were preincubated in F12 supplemented with 10% FBS at 37°C overnight. Then the medium was replaced with fresh medium (△) containing 100 ng/ml LPS with (●) or without (○) 10 U/ml IFN-γ. The cells were further incubated at 37°C for 0-24 h. The amount of NO_2^- in the culture supernatant was determined with Griess reagent. (B) Effect of LPS on NO_2^- accumulation in the culture supernatant of cells treated with LPS alone or LPS + IFN-γ. The cells were treated with LPS at 0-1 × 10^4 ng/ml in the presence (●) or absence (○) of 10 U/ml IFN-γ at 37°C for 8 h. The amount of NO_2^- was determined as described above. The results are presented as the means ± SE for two different experiments.

RESULTS

Time-Course of iNOS Induction—After the addition of 100 ng/ml LPS or 100 ng/ml LPS+10 U/ml IFN-γ, the amount of NO_2^- released into the culture supernatant was examined with Griess reagent. As shown in Fig. 1A, both LPS and LPS+IFN-γ induced the release of NO_2^- 4 h after incubation, and the amount of NO_2^- increased with time. However, the amount of NO_2^- was always more in LPS+IFN-γ than in LPS-treated cells throughout the incubation, and the control cells without any addition showed no release. These results suggest that •NO production is induced 4 h after treatment of the macrophages with LPS and LPS+IFN-γ, though the rate of •NO production is always higher in LPS+IFN-γ-treated cells than in LPS-treated ones.

As for induction of the iNOS protein, immunoblotting analysis showed that a 130 kDa band, corresponding to iNOS, appeared 4 h after treatment with LPS and LPS+IFN-γ, and increased with time until 10 h, when it reached the maximal level, and then remained unchanged up to 24 h (Fig. 2A). However, the amount of iNOS protein in LPS+IFN-γ-treated cells was more than 10-fold higher than that in LPS-treated cells 8 h after induction. These results suggest that there are some differences in the NOS activities of these cells in culture, and that iNOS in LPS-treated cells produces relatively more •NO than that in LPS+IFN-γ-treated cells, based on the amount of iNOS protein.

Dependence of iNOS Induction on LPS Dose—To determine the effect of LPS on iNOS induction, various amounts of LPS were added with or without 10 U/ml IFN-γ to macrophage cultures, and the cells were incubated at 37°C for 8 h, by which time considerable amounts of NO_2^- (Fig. 1A) and iNOS protein (Fig. 2A) had appeared. As shown in Fig. 1B, the release of NO_2^- was dependent on the LPS dose; 1 ng/ml LPS induced the release of a significant amount of NO_2^-, and 1 μg/ml LPS induced much greater release in both LPS- and LPS+IFN-γ-treated cells. The difference in the amount of NO_2^- between with and without IFN-γ was about 5-6-fold. IFN-γ alone did not induce NO_2^- production.

Induction of the iNOS protein was also dependent on the LPS dose, and 1 ng/ml LPS was enough to induce a large amount of iNOS protein in the cells treated with IFN-γ, but 10 ng/ml LPS was necessary to induce a significant amount of iNOS protein in the absence of IFN-γ (Fig. 2B). Much higher induction was obtained with 10 μg/ml LPS in both the presence and absence of IFN-γ. However, the difference in the amount of iNOS protein with and without IFN-γ-treatment was about 10-15-fold at each LPS dose. This difference was much greater than that in NO_2^- in Fig. 1B.

NOS Activity in Intact Macrophages Pretreated with

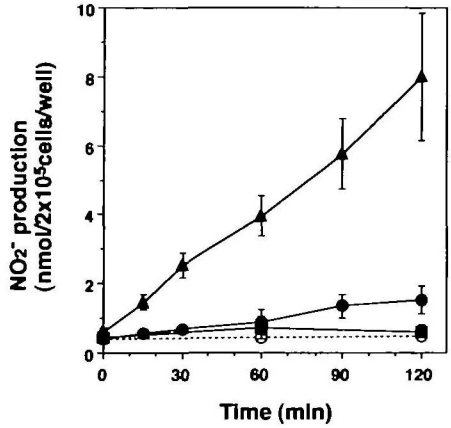

Fig. 3. **Analysis of the initial levels of iNOS activity in activated macrophage cultures with carboxy-PTIO and Griess reagent.** Cells were pretreated with 100 ng/ml LPS or both 100 ng/ml LPS and 10 U/ml IFN-γ at 37°C for 8 h. Then the cells were washed three times with HBSS(+), and incubated in the HBSS(+) containing assay mixture including 100 μM carboxy-PTIO and 1 mM L-arginine in the presence or absence of 1 mM L-NIO. The amount of NO_2^- in the culture supernatant was determined with Griess reagent. The symbols correspond to: +NONE (○), +LPS (●), +LPS+IFN-γ (▲), and LPS+IFN-γ+L-NIO (■). The results are presented as the means±SE for three different experiments.

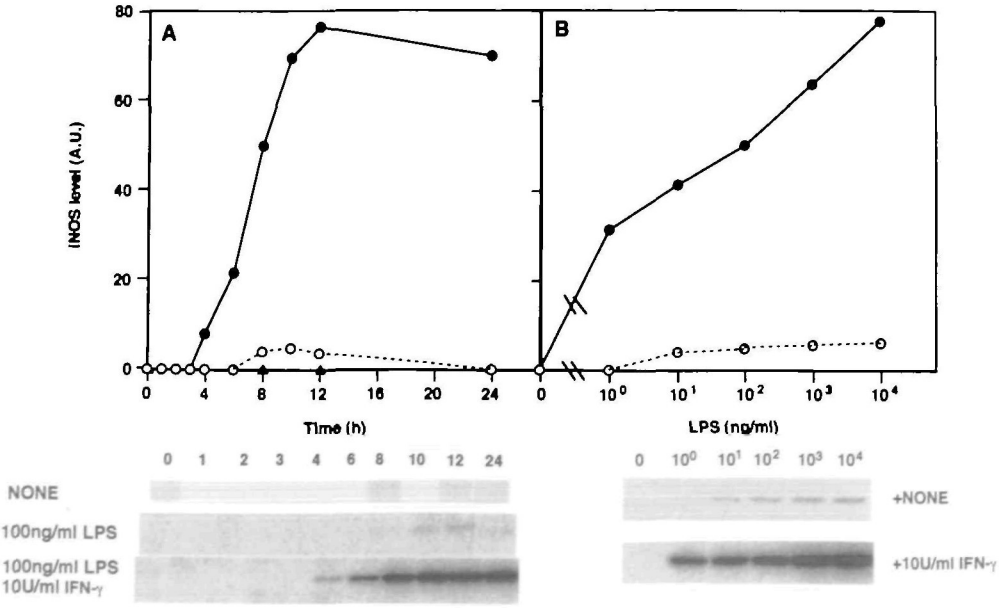

Fig. 2. **Immunoblot analysis of the iNOS protein in RAW 264.7 cells.** (A) Time-course of iNOS induction in RAW 264.7 cells treated with LPS in the presence or absence of IFN-γ. The cells were treated as described in the legend to Fig. 1A. The amount of iNOS in the cell extract was analyzed by Western blotting with monoclonal anti-iNOS as described in the text. (B) Effect of LPS on iNOS induction in cells treated with or without IFN-γ. The cells were treated as in Fig. 1B, and the cell extracts were analyzed as described above. The symbols correspond to: +NONE (△), +LPS (○), and +LPS+IFN-γ (●).

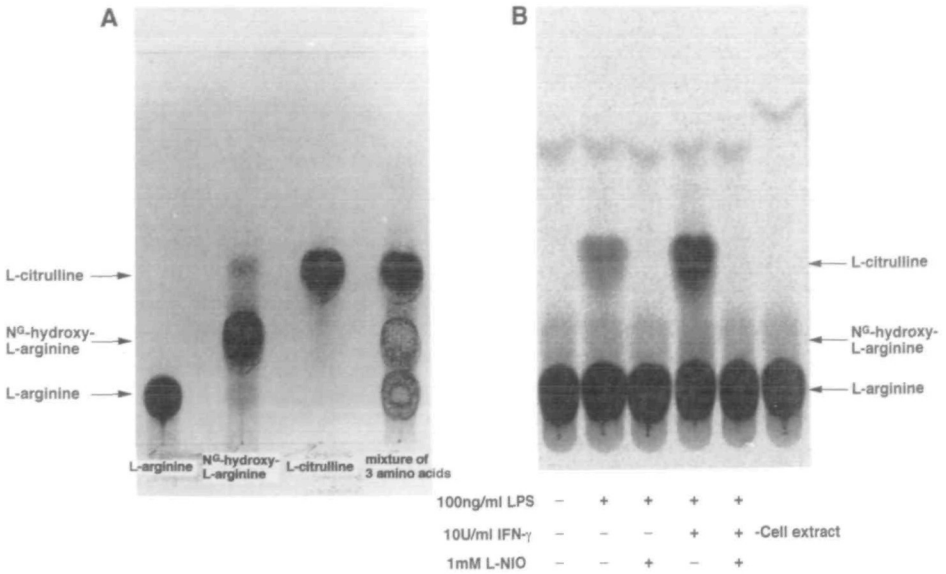

Fig. 4. **Thin layer chromatography for the separation and detection of N^G-hydroxy-L-arginine and L-citrulline produced from L-arginine by iNOS.** (A) The amino acids, L-arginine, N^G-hydroxy-L-arginine and L-citrulline, were separated on a TLC plate. Amino acids, L-arginine, N^G-hydroxy-L-arginine and L-citrulline (250 nmol), were loaded and separated on a microfine cellulose thin layer plate as described under "MATERIALS AND METHODS." The amino acids were visualized with vaporized 0.25% (w/v) ninhydrin in acetone. (B) Separation of L-[U-^{14}C]citrulline and N^G-hydroxy-L-[U-^{14}C]arginine from L-[U-^{14}C]arginine on a TLC plate, and detection of each spot with a bioimage analyzer. Cell extracts of RAW 264.7 cells treated with nothing, LPS alone and LPS+IFN-γ at 37°C for 8 h were used as iNOS sources. The enzyme reaction was performed at 37°C for 10 min in the presence or absence of 1 mM L-NIO.

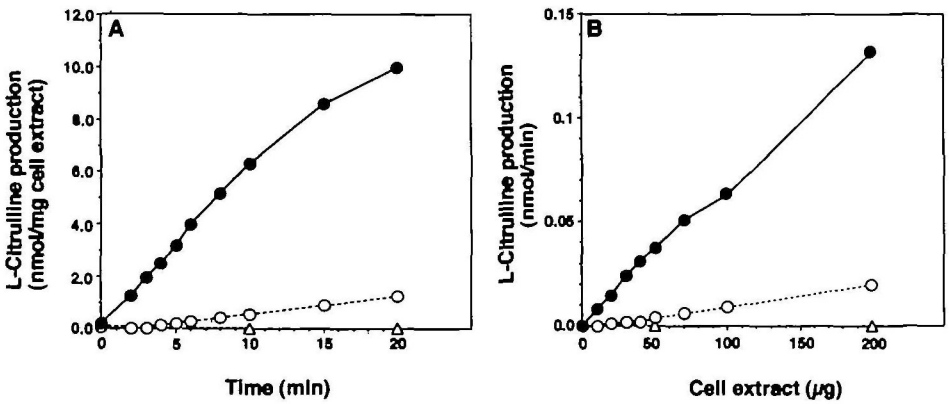

Fig. 5. **Analysis of iNOS activity in cell extracts.** (A) Time course of L-citrulline production by iNOS in cell extracts. Cell extracts of RAW 264.7 cells treated with nothing (△), LPS alone (○), and LPS+IFN-γ (●) at 37°C for 8 h were used as iNOS sources. The enzyme reaction was performed at 37°C with 50 μg of cell extract. The enzyme activity was determined by means of TLC and a bioimage analyzer as described in the legend to Fig. 4. (B) Dose-dependence of the cell extracts. The enzyme reaction was performed at 37°C for 10 min. Symbols are the same as in (A).

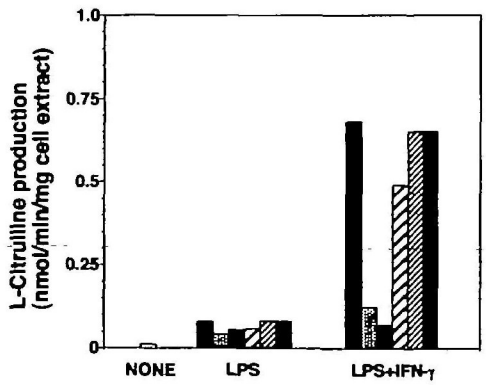

Fig. 6. **Requirement of co-factors for iNOS activity in cell extracts.** Cell extracts of RAW 264.7 cells treated with LPS alone and LPS+IFN-γ at 37°C for 8 h were used as iNOS sources. The enzyme reaction was performed with co-factors in the assay mixture devoid of nothing (■), all (▩), β-NADPH (■), H_4B (▨), FAD (▨), or FMN (■).

TABLE I. **Requirement of co-factors for iNOS activity *in vitro*.** The data presented were calculated from the values in Fig. 6. The results are shown as the amounts of L-citrulline produced by iNOS in cell extracts treated with LPS alone or LPS+IFN-γ, and as a percentage relative to the control with the complete mixture.

Co-factor removed from reaction mixture	100 ng/ml LPS (nmol L-citrulline/min/mg cell extract)	(%)	100 ng/ml LPS+10 U/ml IFN-γ (nmol L-citrulline/min/mg cell extract)	(%)
None	0.154	100	1.36	100
All	0.083	53.8	0.245	18.0
β-NADPH	0.109	70.8	0.138	10.1
H_4B	0.118	76.6	0.944	69.4
FAD	0.160	104	1.30	95.6
FMN	0.161	105	1.30	95.6

LPS and LPS+IFN-γ—To determine ·NO-producing activity in intact macrophages treated with LPS and LPS+IFN-γ, macrophages were reseeded onto a 24 well flat-bottomed plate, and then incubated in HBSS(+) containing 1 mM L-arginine and 100 μM carboxy-PTIO, which converts ·NO to $\cdot NO_2$ and then NO_2^- in a fully stoichiometric

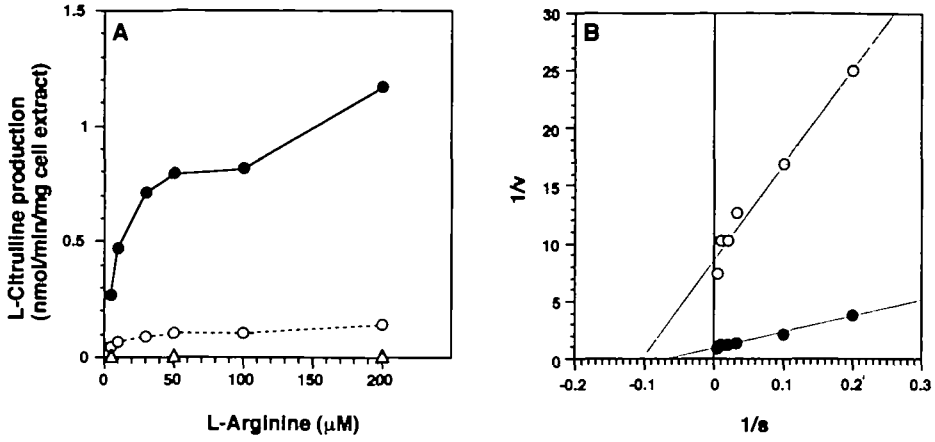

Fig. 7. **Effect of L-arginine concentration on iNOS activity in extracts of RAW 264.7 cells.** (A) The dose-dependence on L-arginine of L-citrulline production by iNOS in cell extracts. Cell extracts of RAW 264.7 cells treated with nothing (△), LPS alone (○), and LPS+IFN-γ (●) at 37°C for 8 h were used as iNOS sources. The enzyme reaction was performed with 5 to 200 μM L-arginine. (B) Double-reciprocal plots of the L-arginine concentrations and amounts of L-citrulline produced by iNOS in Fig. 7A.

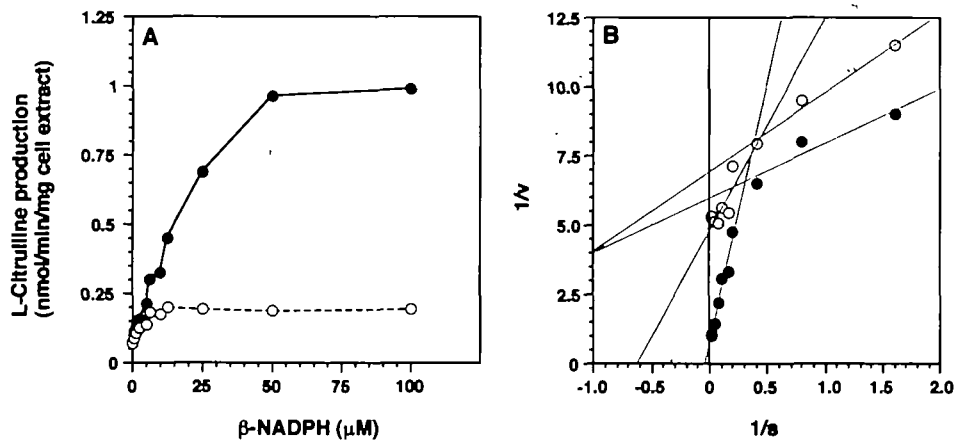

Fig. 8. **Effect of β-NADPH concentration on iNOS activity in cell extracts.** (A) Dependence on the β-NADPH concentration in an iNOS assay. Cell extracts of RAW 264.7 cells treated with LPS alone (○) and LPS+IFN-γ (●) at 37°C for 8 h were used as iNOS sources. The enzyme reaction was performed with 0.625 to 100 μM β-NADPH. (B) Double-reciprocal plots of the β-NADPH concentrations and amounts of L-citrulline produced by iNOS in Fig. 8A.

manner (*10*), as described under "MATERIALS AND METHODS." As shown in Fig. 3, LPS+IFN-γ-treated cells produced 62.5 pmol NO_2^-/min/2×10^5 cells, this production of NO_2^- being completely abolished by 1 mM L-NIO, a strong inhibitor of iNOS (*11*), showing that NO_2^- was produced by an iNOS-mediated reaction (*6*). Control macrophages, pretreated with nothing, did not produce NO_2^-. The LPS-treated cells produced 8.33 pmol NO_2^-/min/2×10^5 cells, which was about 1/7 of the amount of NO_2^- produced in LPS+IFN-γ-treated macrophages. Under these conditions, little O_2^- was generated in any kind of cell culture when it was incubated in HBSS(+) with 0.625 mg/ml cytochrome *c* but without TPA (data not shown). These results suggest that LPS+IFN-γ-treated cells produce an about 7-fold higher amount of •NO than LPS-treated cells, during reincubation of activated macrophages, as distinct from the continuous synthesis of iNOS.

Improvement of the iNOS Assay System In Vitro Using Thin-Layer Chromatography (TLC)—To examine the iNOS activity in cell-free extracts of LPS- and LPS+IFN-γ-treated macrophages, we tried to improve the assay system for iNOS activity with L-[*U*-^{14}C]arginine *in vitro* with TLC. Most of the previously used methods involved Dowex AG 50WX column chromatography to separate L-[*U*-^{14}C]citrulline from L-[*U*-^{14}C]arginine among the reaction products (*12*). We used TLC for separation of the reaction products, L-[*U*-^{14}C]citrulline and N^G-hydroxy-L-[*U*-^{14}C]arginine, from L-[*U*-^{14}C]arginine. As shown in Fig. 4, these amino acids were successfully separated on a TLC plate, and the radioactivity of each spot was visualized and quantitated with a BAS2000 bioimage analyzer. The major product was L-[*U*-^{14}C]citrulline, and the radioactivity of N^G-hydroxy-L-[*U*-^{14}C]arginine, the intermediate of the reaction from L-arginine to L-citrulline with iNOS, was always low. The amount of N^G-hydroxy-L-arginine did not vary among the samples.

TABLE II. **Affinity of iNOS for L-arginine in an *in vitro* assay with cell extracts.** The data presented were calculated from the values in Fig. 7B.

	100 ng/ml LPS	100 ng/ml LPS +10 U/ml IFN-γ
K_m (μM)	9.59	14.7
V_{max} (nmol/min/mg cell extract)	0.116	1.07

We next examined the time-course and dose-dependence of NOS activity in cell extracts (Fig. 5). The reaction proceeded almost linearly for 10 min with the extracts of both LPS- and LPS+IFN-γ-treated cells, although there is an apparent lag-time of 3 min in the extract of LPS-treated cells before the full activity appears (Fig. 5A). However, this lag-time seemed to be due to the radioactivities of L-[^{14}C]citrulline formed by LPS-treated cell extracts being too weak to be detected quantitatively by the BAS 2000 image analyzer. As for the enzyme dose, the reaction was linear with up to 70 μg of the extracts of both LPS- and

TABLE III. **Affinity of iNOS for β-NADPH in an *in vitro* assay with cell extracts.** The data presented were calculated from the values in Fig. 8B.

	100 ng/ml LPS	100 ng/ml LPS +10 U/ml IFN-γ
K_m1 (μM)	0.417	0.331
$V_{max}1$ (nmol/min/mg cell extract)	0.144	0.167
K_m2 (μM)	1.57	24.6
$V_{max}2$ (nmol/min/mg cell extract)	0.204	1.31

TABLE IV. **Concentration of β-NADPH in RAW 264.7 cells treated with nothing, LPS or LPS+IFN-γ.** The cells were treated with nothing, LPS or LPS+IFN-γ at 37°C for 8 h. Then β-NADPH was extracted and quantitated as described under "MATERIALS AND METHODS." The results are the means±SE for three different experiments.

	NONE	LPS	LPS+IFN-γ
β-NADPH concentration (μM)	36.8±10.2	14.8±1.4	13.6±0.7

LPS+IFN-γ-treated cells during incubation at 37°C for 10 min (Fig. 5A). Therefore we set the reaction time as 10 min at 37°C, and the enzyme dose as 50 μg of each extract. The extracts of LPS- and LPS+IFN-γ-treated cells showed iNOS activity levels of 700 and 100 pmol citrulline formed/min/mg cell extract, respectively. It seems important to point out that the differences in iNOS activity between these two types of macrophages were about 7-fold in both intact cells and cell extracts.

Requirement of Co-Factors for iNOS Activity In Vitro—It has been reported that iNOS from activated macrophages requires β-NADPH, H_4B, FAD, and FMN as co-factors to exhibit iNOS activity with L-arginine as a substrate *in vitro* (*13*). We examined the requirement for these co-factors by assaying iNOS activity *in vitro* in extracts of LPS- and LPS+IFN-γ-treated cells. As shown in Fig. 6 and Table I, omission of FAD or FMN from the reaction mixture had little effect on the iNOS activity in both cell extracts. However, omission of β-NADPH decreased the activity below the background level with the LPS+IFN-γ-treated cell extract and also decreased it to about 50% of the level between the complete addition and the background with the LPS-treated cell extract. Omission of H_4B caused moderate inhibition of the reaction with both cell extracts. These results suggested that β-NADPH was of primary importance among the co-factors for the full activity of iNOS *in vitro*, and that the degree of the requirement for β-NADPH was higher with the LPS+IFN-γ-treated cell extract than the LPS-treated one.

Effect of L-Arginine Concentration on iNOS Activity In Vitro—Because NOS requires L-arginine as a substrate, we examined the L-arginine concentration-dependence by NOS assay *in vitro* with LPS- and LPS+IFN-γ-treated cell extracts. As shown in Fig. 7A, both extracts exhibited NOS activity dependent on the L-arginine concentration, and the full activities were obtained with more than 50 μM, although 200 μM L-arginine caused production of a greater amount of L-citrulline than the full activity level when LPS+IFN-γ-treated cell extracts were assayed. The double-reciprocal plots in Fig. 7B revealed similar K_m values for L-arginine: $K_m=9.59$ μM, $V_{max}=0.116$ nmol/min/mg cell extract for the LPS-treated cell extract, and $K_m=14.7$ μM, $V_{max}=1.07$ nmol/min/mg cell extract for the LPS+IFN-γ-treated cell extract (Table II). These results suggest that the difference in iNOS activity *in vitro* between these two extracts is not mainly due to the difference in the affinity of iNOS for the substrate.

Effect of β-NADPH Concentration on iNOS Activity In Vitro—Because β-NADPH is an important co-factor for NOS activity *in vitro* (Fig. 8), and the degrees of loss of activity on omission of β-NADPH from the reaction mixture were different between these cell extracts (Table I), it was of interest to determine the effect of β-NADPH concentration on NOS activity with these enzyme sources. As shown in Fig. 8A, both extracts showed a concentration-dependent increase in NOS activity in the saturation curves for β-NADPH. Double-reciprocal plots revealed two different K_m values for each cell extract, being $K_m1=0.417$ μM, $K_m2=1.57$ mM for the LPS-treated cell extract, and $K_m1=0.331$ μM, $K_m2=24.6$ μM for the LPS+IFN-γ-treated cell extract (Table III). They also showed different V_{max} values (Table III). These results suggest that most of the iNOS molecules in the LPS-treated cell extract have high affinity for β-NADPH, but that most in the LPS+IFN-γ-treated cell extract have low affinity for β-NADPH.

It was therefore important to determine the concentration of β-NADPH in intact macrophages treated with LPS and LPS+IFN-γ at 37°C for 8 h. The results in Table IV show little difference in the β-NADPH concentrations in these cells, although the values were significantly lower than that in the control cells without addition.

DISCUSSION

In this study, we examined NO_2^- production, iNOS induction, and NOS activity both *in vivo* and *in vitro* in a macrophage-like cell line, RAW 264.7, treated with LPS alone and LPS+IFN-γ. It was clear that LPS+IFN-γ was a stronger inducer of iNOS than LPS alone, as has been reported by other laboratories (*5*). However, we examined the quantitative differences among these indicators of iNOS; the amount of released NO_2^- was about 5-6-fold higher for LPS+IFN-γ-treated cells than LPS-treated ones when the cells were incubated at 37°C for 8 h with 100 ng/ml LPS in the presence or absence of 10 U/ml IFN-γ (Fig. 1). There was about 10-15-fold more iNOS protein in LPS+IFN-γ-treated cells than in LPS-treated cells under the same conditions (Fig. 2). Moreover, the differences in iNOS activity between intact cells (Fig. 3) and cell-free extracts (Fig. 5B) were about 7-fold higher for LPS+IFN-γ-treated cells than LPS-treated ones.

The quantitative differences among iNOS indicators, iNOS activity in the cells in culture during induction (NO_2^- production), iNOS activity *in vitro* (L-citrulline production), and the iNOS protein level in LPS- or LPS+IFN-γ-treated cells, are summarized in Table V, the results being from several independent experiments. These results suggest that (1) most of the iNOS protein was in non-active form in LPS+IFN-γ-treated cells, (2) the substrate or some of the co-factors for iNOS were at lower levels in LPS+IFN-γ-treated cells than in LPS-treated ones, or (3) there was heterogeneity of the iNOS protein, and most of the iNOS in LPS+IFN-γ-treated cells was in a low activity form.

A precise kinetic analysis was performed using the new *in vitro* assay method for iNOS activity with cell-free

TABLE V. **Quantitative differences among iNOS indicators, *i.e.*, iNOS activity *in vivo* (NO_2^- production), iNOS activity *in vitro* (L-citrulline production), and the iNOS level.** The results are represented as ratios of the values in LPS-treated cells on the basis of those in LPS+IFN-γ-treated cells, which were calculated in each experiment; the means±SE for at least nine experiments are shown.

	NO_2^- production (8 h culture)	L-Citrulline production	iNOS level
LPS/LPS+IFN-γ (%)	16.8±2.8	13.8±0.42	9.84±2.10

extracts which we established in this study (Figs. 4-6). The K_m values for the substrate, L-[U-^{14}C]arginine, were similar in these two cell extracts (Fig. 7), but different enough to suggest heterogeneity of iNOS (Table II). However, we found two different K_ms for β-NADPH of iNOS from both LPS- and LPS+IFN-γ-treated cells (Fig. 8). The difference between these two K_m values was distinct (Table III). Moreover, the cellular β-NADPH levels were about 10 μM in these two kinds of cell extracts (Table IV). These results suggest that most of the iNOS induced in the LPS+IFN-γ-treated cells was not as active owing to the shortage of β-NADPH.

All these results support possibilities (2) and (3), and the inefficiency of the production of NO_2^- compared with the large amount of iNOS might be explained by the heterogeneity of iNOS with different K_ms for β-NADPH, together with the relatively low level of β-NADPH in LPS+IFN-γ-treated macrophages.

Extensive work on expression of the iNOS gene in activated macrophages has so far been mainly focused on the amount of mRNA (*14, 15*). Recently, the importance of the assembly of dimeric iNOS was pointed out, and it was reported that expression of iNOS activity requires dimerization of iNOS monomers, and that H_4B, heme and L-arginine are associated with the dimerization (*16, 17*).

Although the role of β-NADPH as a co-factor of iNOS has been reported (*13, 18*), little is known about the heterogeneity of iNOS from the viewpoint of different K_m values for β-NADPH, and little attention has been paid to the possible heterogeneity of the mRNA from macrophages exposed to different activators, such as LPS and LPS+IFN-γ. In this context, it seems worth determining whether or not the molecular structure of iNOS changes under reaction conditions with and without β-NADPH using highly purified iNOS protein from LPS+IFN-γ-treated RAW 264.7 cells. It would also be interesting to determine whether the heterogeneity of iNOS with different K_m values for β-NADPH is due to the heterogeneity of the iNOS protein (different amino acid sequences) or to the diversity of the assembly of iNOS with the co-factors, Ca^{2+} and calmodulin. Our preliminary experiments suggest an association of relatively low amounts of calmodulin with 130 kDa bands corresponding to iNOS in an immunoblot of LPS+IFN-γ-treated cell extracts as compared with LPS-treated ones, when the amount of iNOS was normalized between immunoblots of these two extracts (data not shown). In addition, another 135-145 kDa band appeared together with the typical 130 kDa band of iNOS in LPS+IFN-γ-treated cell extracts, but not in LPS-treated cell extracts, when these cell extracts were treated with SDS-sample buffer at 37°C for 10 min but without boiling, and then electrophoresis was performed in a cold room at 6-8°C (*18*) (data not shown).

However, estimation of iNOS dimer contents in these cell extracts by gel filtration might be difficult, because previous authors (*16*) used an elution buffer containing H_4B, L-arginine, β-NADPH, FAD, FMN to separate dimeric iNOS from monomeric iNOS, and all of these components would influence the characteristics of iNOS with different K_ms for β-NADPH. We are now trying to raise antibodies which recognize dimeric iNOS alone.

It is also important to point out the role of β-NADPH as a co-factor of the O_2^--generating enzyme, NADPH oxidase. The apparent K_m value for NADPH oxidase in a cell-free system with neutrophil preparation was about 49.1 μM (*19*), which is higher than the values of iNOS in Table IV.

In this study, we also describe a new assay method for iNOS in both intact cells (Fig. 3) and cell-free extracts (Fig. 4), developed in our laboratory (*6*). Similar kinetic results were obtained for the time-course, and similar differences in NOS activities in the assays were observed between LPS- and LPS+IFN-γ-treated cells and cell extracts. Therefore, the new method is expected to be a good tool for estimating NOS activity in other kinds of cells and macrophages at different activation stages.

We are currently studying such post-translational modifications of the iNOS protein as protein phosphorylation and binding of Ca^{2+}/calmodulin in LPS- and LPS+IFN-γ-treated macrophages.

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