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# Regulation and Subcellular Location of Nitrogen Oxide Synthases in RAW264.7 Macrophages

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## SUMMARY

In nitrinergic signal transduction, nitrogen oxide (NO) synthases (NOS) (EC 1.14.23) catalyze the conversion of L-arginine to L-citrulline and NO, which in turn activates soluble guanylyl cyclase. Macrophages were reported to contain a single isoform of NOS (type II, soluble, $Ca^{2+}$-independent, 130-kDa) and only upon activation of the cells by interferon-$\gamma$ (INF) and lipopolysaccharides (LPS). By a mechanism involving L-type $Ca^{2+}$ channels, calmodulin, and serine proteases, INF/LPS also induce a cytotoxic activation of macrophages. In RAW264.7 macrophages, NO release was detected upon activation of the cells by INF/LPS but also, although at a 20-fold lower level, in control cells. The latter constitutive NOS activity and NO release were $Ca^{2+}$ dependent and were decreased in INF/LPS-activated RAW264.7 cells or with increasing passage number. RAW264.7 cells did not express soluble guanylyl cyclase, suggesting other target molecules for NO. In INF/LPS-activated cells, NOS activities and NO release were $Ca^{2+}$ independent (type II) and coinduced with NADPH-diaphorase activities both in the soluble and in the particulate fractions. The NOS-II activities corresponded to a 130-kDa protein, by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, which was not recognized in a protein immunoblot with anti-NOS-I antibody. The serine protease inhibitor tosyl-lysyl chloromethyl ketone abolished the induction of NOS-II by INF/LPS, by depleting intracellular thiol pools and interfering with protein synthesis. Induction of NOS-II by INF/LPS was transcriptionally based and, for maximal enzyme activity, required increased intracellular tetrahydrobiopterin levels, intracellular $Ca^{2+}$ mobilization, and activation of non-L-type $Ca^{2+}$ channels but, unlike the induction of macrophage-mediated cytotoxicity, neither L-type-$Ca^{2+}$ channels nor calmodulin.

In numerous mammalian cells and tissues, including cytotoxic activated macrophages, the oxidation of a terminal guanidino nitrogen of L-arginine (1–5), yielding NO (6) and L-citrulline, is catalyzed by different NOS (types I–III; Table 1) (7), which are either $Ca^{2+}$ dependent (type I and III) or $Ca^{2+}$ independent (type II). In macrophages, NO has been shown to be identical to the nitric oxide free radical (8–10). In other cells, NO may be a nitroso compound (11). In its target cells and tissues, i.e., smooth muscle (2–4), neural (5, 7, 12–15), and non-neural secretory cells (16, 17), NO functions as the first messenger of nitrinergic signal transduction, activating GC-S [GTP pyrophosphate-lyase (cyclizing), EC 4.6.1.2] (18, 19) and thereby increasing the intracellular concentration of the second messenger molecule cGMP (7, 20, 21). Whether the complete pathway operates in macrophages has not been investigated. In immunologically activated macrophages, NO biosynthesis could also be the mediator of INF/LPS-induced CMC (8–10). CMC, however, is blocked by protease inhibitors (22, 23) and is a $Ca^{2+}$/calmodulin-dependent process (24, 25), whereas only a $Ca^{2+}$-independent NOS-II has been described in macrophages (9). Moreover, the induction of NO release and CMC do not always correlate (26, 27).

We investigated basal and stimulated release of NO in the murine macrophage cell line RAW, regulation of NOS expression by cytokines, LPS, $Ca^{2+}$ and calmodulin, corticosteroids, proteases, thiols, and pterins, and possible post-translational modification of expressed NOS by limited proteolysis and phosphorylation. NOS activity was assayed chemically by measurement of NADPH-d activity (28), formation of L-citrulline, or accumulation of NO (determined as nitrite and nitrate) or by

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**ABBREVIATIONS:** NO, nitrogen oxide(s); BSA, bovine serum albumin; CHAPS, 3-[(3-cholamidopropyl)diethylammonio]-1-propanesulfonate; CMC, cell-mediated cytotoxicity; GAF, guanylyl cyclase-activating factor; GC-S, soluble guanylyl cyclase; $H_4$biopterin, (6*R*)-1-*erythro*-dihydroxypropyl-5,6,7,8-tetrahydropterin; INF, recombinant murine interferon-$\gamma$; LPS, *Escherichia coli* lipopolysaccharide; MeArg, $N^G$-methyl-L-arginine·HCl; NaNP, sodium nitroprusside; NBT, nitroblue tetrazolium; $NO_2$Arg, $N^G$-nitro-L-arginine methyl ester·HCl; NOS, nitrogen oxide synthase; PMSF, phenylmethane sulfonyl fluoride; RAW, cloned murine macrophage cell line RAW264.7; SOD, superoxide dismutase; TLCK, tosyl-lysyl chloromethyl ketone; TMB-8, 8-(*N,N*-diethylamino)-octyl-3,4,5-trimethoxybenzoate; TNF, tumor-necrosis factor-$\alpha$; NBTF, nitroblue tetrazolium formazan; NADPH-d, NADPH-diaphorase; EGTA, ethylene glycol bis ($\beta$-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

TABLE 1
**Isoforms of NOS**

| Type | Regulated by $Ca^{2+}$ | Subcellular location[a] | Molecular mass |
|---|---|---|---|
| | | | kDa |
| NOS-I | Yes[b] | Soluble | 2 × 160 |
| NOS-II[c] | No | Soluble > particulate | 2 × 130 |
| NOS-III | Yes[b] | Particulate > soluble | 135 |

[a] Soluble, 105,000 × *g* supernatant; particulate, 105,000 × *g* particulate fraction.
[b] In brain and endothelial cells, calmodulin-dependent; in neutrophils, calmodulin-independent.
[c] Present in immunologically activated cells.

bioassay of GAF activity, i.e., cGMP increase in RAW cells or reporter cells. Macrophage NOS were partially purified and characterized by cross-reaction with anti-NOS-I antibody (15, 16), subcellular location, and regulation by free $Ca^{2+}$.

## Materials and Methods

**RAW macrophages.** The cloned murine macrophage cell line RAW, from Abelson leukemia virus-induced BALB/c lymphocytic lymphoma (TIB 71; American Type Culture Collection), was cultured as described (1). Cells were usually plated into 6- or 12-well tissue culture plates (surface area, 9.5 $cm^2$ or 4 $cm^2$, respectively). For preparation of crude supernatant and particulate fractions, cells were cultured in spinner bottles (500 ml) under 5% $CO_2$ and were harvested at a density of about $10^6$ cells/ml. Endotoxin levels in the culture media were <0.1 ng/ml (E-Toxate-kit; Sigma).

**Crude supernatant and particulate fractions.** RAW cell suspension cultures were activated with 100 ng/ml LPS and 3 units/ml INF (in 1% BSA), in the absence or presence of 0.1 mM TLCK, or were sham-treated with 1% BSA (control cells). The cells were harvested 14–16 hr later by centrifugation (1200 rpm at 25° for 15 min) and were washed twice with $Ca^{2+}$- and $Mg^{2+}$-free phosphate-buffered saline (Sigma). The cell pellet was homogenized in a Teflon-glass grinder, in 5 ml of ice-cold buffer 1 (50 mM Tris·HCl, pH 7.6, 0.5 mM EDTA, 0.5 mM EGTA, 1 μM leupeptin, 1 μM pepstatin A, 0.2 mM PMSF, 12.5 mM 2-mercaptoethanol). The homogenate was centrifuged at 105,000 × *g* (at 5° for 2 hr), resulting in 4.5 ml of clear supernatant fraction. The 105,000 × *g* pellet was resuspended in buffer 2 (buffer 1 containing 1 M KCl) and centrifuged again at 105,000 × *g* (at 5° for 30 min). The resulting washed pellet was resuspended in 7.5 ml of buffer 1 or solubilized in the same volume of buffer 3 (buffer 1 containing 10 mM CHAPS). Supernatant and particulate fractions were stored at −70° in the presence of glycerol (10%, v/v). In some experiments, crude supernatant fractions from control cells were prepared in the absence of leupeptin, pepstatin, and PMSF and were incubated for different times at 37° with cathepsin B, C (each up to 1 unit/ml), or D (up to 1 μg/ml) or papain (up to 5 μg/ml).

**Partial purification of macrophage NOS-II.** The 105,000 × *g* supernatant fraction or the detergent extract (10 mM CHAPS) of the particulate fraction of RAW cells was applied, at 4°, to a 2′,5′-ADP-Sepharose 4B (Pharmacia) affinity chromatography column, as described (7, 29). The column was subsequently washed with 10 column volumes each of buffer 1, buffer 1 containing 0.5 M NaCl, and buffer 1, and NOS was then eluted with buffer 1 containing 10 mM NADPH. The eluates were concentrated to 75–100 μl and frozen in liquid nitrogen. For purification of particulate NOS, all buffers contained 10 mM CHAPS.

**Incubation of RAW monolayers.** For determination of NO release and protein content of RAW cells, monolayers were incubated at 37° under 5% $CO_2$ for 24 hr, unless otherwise indicated, in phenol red-free culture medium (sterilely filtered and stored in 50-ml aliquots at −70°). For determination of GAF activity of released NO, an aliquot of the culture medium from activated RAW cells was transferred, in the presence of 20 units/ml SOD, onto rat lung fibroblast (RFL-6) reporter cell monolayers and was immediately incubated for 3 min. For determination of cGMP formation or release in RAW cells, monolayers were incubated at 37° for 2 min in Locke's buffer containing 1 mM 3-isobutyl-1-methylxanthine, unless otherwise indicated. cGMP was determined in the cells and in the incubation buffer. Unless otherwise stated, INF, LPS, and A23187 were used at concentrations of 3 units/ml, 100 ng/ml, and 3 μM, respectively. None of the compounds tested had nonspecific or toxic effects in the tested concentration range, unless otherwise stated. Concentrations of ≥0.3 mM TMB-8, which caused cell detachment but did not affect cell viability, were not tested.

**Determination of protein.** For the determination of total protein, RAW macrophage monolayers were washed with Locke's buffer and homogenized in 5 ml of twice distilled water by three freeze-thaw cycles, followed by sonication (on ice, for 3 × 20 sec, dial 35%; Fisher sonic dismembranator model 300; Artek). Protein concentrations were determined spectrophotometrically (microplate reader EL-311; Biotek), according to the method of Bradford (30). BSA was used as standard.

**Determination of nitrite and nitrate.** NO release was determined spectrophotometrically by measuring the accumulation of both nitrite and nitrate (the latter after reduction to nitrite) in the culture medium.

For nitrate reduction, samples were centrifuged at 1000 × *g* (for 15 min at room temperature), to remove cells and particles. All subsequent incubations were performed in red polypropylene tubes. Nitrate was stoichometrically reduced to nitrite (Fig. 1, a and b) by incubation of sample aliquots (up to 150 μl) for 15 min at 37°, in the presence of 0.1 unit/ml nitrate reductase [NAD(P)H:nitrate oxidoreductase, EC 1.6.6.2; *Aspergillus* species; Boehringer-Mannheim),[1] 50 μM NADPH, and 5 μM FAD, in a final volume of 160 μl. When nitrate reduction was complete, NADPH (up to 0.3 mM), which interfered with the following nitrite determination (31), was oxidized with 10 units/ml lactate dehydrogenase (rabbit muscle; Boehringer-Mannheim) (Fig. 1c) and 10 mM sodium pyruvate (Fig. 1d), in a final volume of 170 μl, with incubation for 5 min at 37°.

Nitrite was determined spectrophotometrically by using the Griess reaction (32). Because premixed Griess reagent, exposure to light, and exposure to pH of >1 give incomplete azo dye stuff formation (33), a stepwise procedure was used. Samples were cooled to 4° before 1 mM sulfanilamide and then 0.1 M HCl were added (the order of reagent addition was critical), to a final volume of 200 μl, and samples were then centrifuged at 1000 × *g* (for 15 min at 4°). A 150-μl aliquot of each supernatant fraction was transferred to 96-well microtiter plates (0.16-$cm^2$ surface area/well; Costar). From the absorbance in each well, the averaged absorbance of a row of wells containing 150 μl of water was subtracted, to give absorbance value $A_1$ (blank value, 0.033 ± 0.001). To all wells, including blank wells, 10 μl of naphthylethylenediamine (1 mM) were added, and wells were incubated at room temperature for 10 min before the plates were read again, to give the absorbance value $A_2$ (blank value, 0.034 ± 0.001). $A_2 - A_1$ plotted against μM NO gave superimposable linear calibration curves for nitrite and nitrate ($r^2$ = 0.995; Fig. 1e), with a detection limit of 30 pmol/150-μl sample volume.

**Determination of cGMP.** Cell-associated cGMP levels in monolayers of RAW cells or in RFL-6 cells (bioassay of NO) were determined as described (7, 34).

**Determination of NOS activity.** Crude supernatant or particulate fractions were incubated in the presence of 20 units/ml SOD ($Mn^{2+}$-containing), 300 μM L-arginine-HCl, 100 μM NADPH, and up to 30 nM calmodulin. In some experiments, crude supernatant fractions were treated with various thioproteases (papain and cathepsins A–E and G;

[1] Before use, nitrate reductase stock solutions (10 units/ml; in distilled water) had to be shaken at room temperature for 30 min, to ensure complete solution of the enzyme. *Aspergillus* nitrate reductase from two other vendors gave incomplete recovery of nitrate, especially at nitrate concentrations of <1 μM (Serva), or showed unacceptable variations in activity between different batches (Sigma). *Escherichia coli* formate-nitrate reductase has a relatively high $K_m$ (about 100 μM nitrate), required incubation times of >1 hr, and gave incomplete recovery at concentrations of <10 μM nitrate.

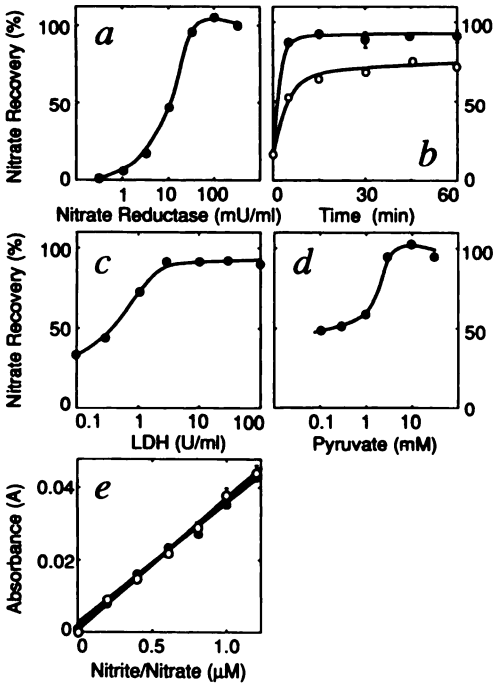


**Fig. 1.** Microdetermination of nitrite and nitrate. Nitrate was reduced by nitrate reductase and remaining NADPH was oxidized by lactate dehydrogenase (*LDH*)/pyruvate. Nitrite was determined spectrophotometrically by a stepwise Griess reaction, as described. The different panels show optimization of various assay conditions, i.e., the concentration (a) and incubation time (b) of nitrate reductase (○, 30 units/ml; ●, 100 units/ml) and the concentrations of lactate dehydrogenase (c) and pyruvate (d). e, Calibration curves for nitrite (○) and nitrate (●). Values are mean ± standard deviation of three determinations.

Sigma). NO formed during 3-min incubations was bioassayed by its GAF activity for RFL-6 cells, as described (7, 34). L-Citrulline formation in the presence of 3 μM L-[2,3,-$^3$H]arginine-HCl (5.5 GBq/mmol), during 10-min incubations, was assayed as described (7, 13). In some experiments, arginine analogs ($NO_2$Arg and MeArg) or thioproteases (papain and cathepsins) were also included in the incubation mixture. Defined $Ca^{2+}$ concentrations were adjusted as described (7).

**Determination of NADPH-d activity.** Diaphorase activity in crude fractions of RAW cells was assayed as described (28), with some modifications. Each sample was incubated in a final volume of 100 μl containing 0.3 mM NBT and 50 mM HEPES (pH 7.6), in the absence or presence of 1 mM NADPH, 1 mM NADH, or 20 units/ml SOD. The reaction was stopped after 30 min by addition of 10 μl of 0.5 N $H_2SO_4$ and 100 μl of dimethyl sulfoxide. The NBTF product was determined spectrophotometrically at 540 nm, in a 150-μl aliquot of the gemisch. Specific diaphorase activity (NBTF formation) was expressed in nmol/min/mg of protein, based on molar extinction coefficient $\epsilon = 12.6 \times 10^{-3} \times M^{-1} \times cm^{-1}$ for NBTF under these conditions (35). Diaphorase activity was defined as NBTF formation in the presence of SOD (36) and was further differentiated into its NADPH-d (28), NADH-diaphorase (37), and DT (i.e., NADPH-NADH)-diaphorase (38) components, according to nucleotide specificity.

**SDS-PAGE.** Discontinuous SDS-PAGE analysis was carried out under reducing conditions using the PhastSystem (Pharmacia), including precast 7.5% polyacrylamide gels bonded to a transparent polyester backing and agarose strips containing the running buffer (0.2 M tricine, 0.2 M Tris, 0.55% SDS, pH 8.1). Gels were developed using a modified silver staining procedure with enhanced uniform sensitivity (39), dried, and then laser scanned (AppleScan).

**Western blot analysis.** For Western blot analysis, aliquots of partially purified NOS-II were separated by 7.5% SDS-PAGE (40) and electroblotted semidry (41) on nitrocellulose membranes (ECL-Hyperbond; Amersham), which were then air dried. All subsequent procedures were performed at room temperature in Tris-buffered saline, containing 0.5% (v/v) Tween-20, and included blocking in 7% BLOTTO (6–12 hr), exposure to rabbit polyclonal antibody to NOS-I (1/1000, for 4 hr (16), and exposure to horseradish peroxidase-conjugated goat polyclonal antibody to rabbit IgG (1/5000, for 1 hr) (Sigma). Between each step, membranes were extensively washed. The immunocomplexes were developed using an enhanced horseradish peroxidase/luminol chemiluminescence reaction, detected with photographic film (Hyperfilm-ECL; Amersham), and laser scanned (AppleScan).

For immunoprecipitation, crude fractions of RAW cells were incubated for 60 min at 4° with ≤30 μg of polyclonal anti-NOS-I antibody (16), followed by the addition of Protein A-Sepharose 4B beads. The incubates were then briefly centrifuged at 1000 × *g* (for 5 min at 4°), and NOS activity was determined in the supernatants and immunoprecipitates.

**Statistical analysis.** Samples were incubated in duplicate and the experiments were repeated *n* times, as indicated. Results were assessed by analysis of variance, followed by the Fisher protected least-significant difference test. Significance was accepted at $p < 0.05$.

## Results

**Basal NO release.** Control RAW cells released small but significant amounts of NO (Figs. 2 and 3), which were unaffected by coincubation with actinomycin D or cycloheximide (data not shown), and contained NOS and NADPH-d activity (see below).

**Cytokines and LPS.** INF (up to 100 units/ml) or LPS (up to 0.1 μg/ml) concentration-dependently increased the basal NO release about 250-fold (Fig. 2, a and b), with $EC_{50}$ values of about 50 units/ml and 70 ng/ml, respectively. In the presence of SOD, NO had GAF activity inasmuch as it stimulated cGMP levels in RFL-6 reporter cells about 200-fold (240 ± 7 pmol of cGMP/$10^6$ RFL-6 cells; $n = 3$). Conversely, cGMP levels in INF/LPS-activated RAW cells were not increased, compared

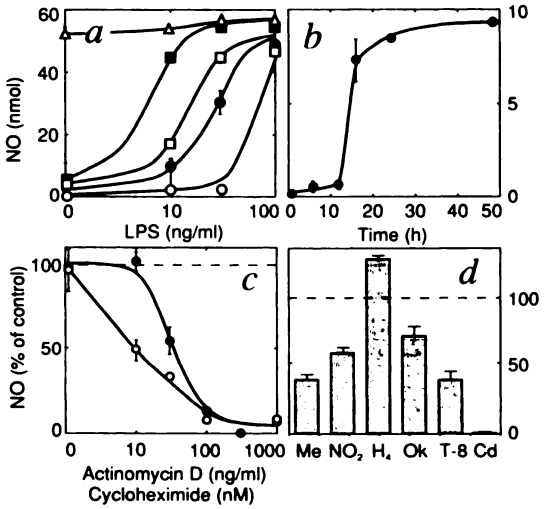


**Fig. 2.** INF/LPS-induced NO release. RAW macrophage monolayers were incubated as described. The different panels show incubations in the presence of increasing concentrations of LPS and different concentrations of INF (○, 0; ●, 1; □, 3; ■, 10; △, 100 units/ml; $n = 6$) (a) or at a constant LPS/INF concentration (10 ng/ml and 1 unit/ml; $n = 9$) but for different times (b), in the presence of increasing concentrations of actinomycin D (○) ($n = 9$) or cycloheximide (●) ($n = 9$) (c), or in the presence of MeArg (*Me*) (1 mM), $NO_2$Arg (*$NO_2$*) (1 mM), $H_4$biopterin (*$H_4$*) (1 μM), okadaic acid (*Ok*) (0.1 μM), TMB-8 (*T-8*) (0.1 mM), or $Cd^{2+}$ (*Cd*) (0.1 mM) (all $n = 3$) (d). NO was determined as described. Values are mean (minus culture medium blank) ± standard deviation.

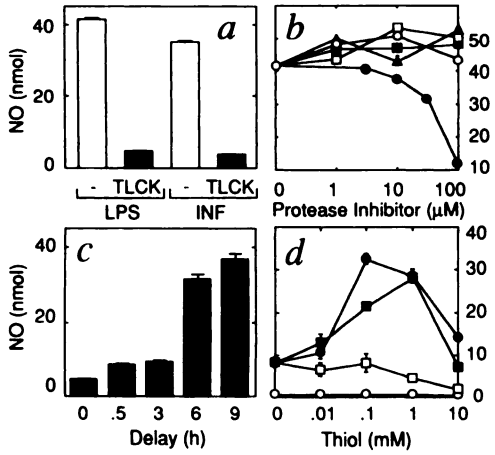


**Fig. 3.** Effects of TLCK on INF- and LPS-induced NO release. RAW macrophage monolayers were incubated in the presence of 1 μg/ml LPS (a–d) or 100 units/ml INF (a), as described. The different panels show incubations in the absence (−) or presence of TLCK (a), in the presence of increasing concentrations of the protease inhibitors TLCK (●), TPCK (○), PMSF (■), diisopropylfluoro phosphanated (□), or pepstatin A (▲) (b), in the presence of TLCK, which was added together with LPS (delay = 0 hr) or at different time points after LPS (delay = 0.5–9 hr) (c), or in the absence (○) or presence (●, □, ■) of 100 μM TLCK and increasing concentrations of glutathione (○, ●, □) or *N*-acetyl-L-cysteine (■), which were added together with LPS (●, ■) or 6 hr after the addition of LPS (□) (d). NO was determined as described. Values are mean (minus culture medium blank) ± standard deviation of four determinations.

with control cells, and were also not stimulated by the NO-donor compound NaNP (see below).

The effects of INF and LPS on NO release were not additive, but each compound potentiated the other, in that INF shifted the concentration-response curve for LPS towards lower LPS concentrations and vice versa (Fig. 2a). When the time dependence of the combined INF/LPS-induced release of NO was monitored, there was a lag period of about 12 hr before a significant stimulation of NO release occured (Fig. 2d). The apparent induction of NO release was maximal 12–18 hr after exposure to INF/LPS and then rapidly declined. Passage number of the cells did not affect the maximal INF/LPS-induced NO release (43.2 ± 0.5 nmol/$10^6$ cells at passage 6 versus 45.3 ± 0.2 nmol/$10^6$ cells at passage 14; $n = 3$). TNF (up to 500 units/ml; $n = 3$) neither induced NO release by itself (1.02 ± 0.3 nmol/$10^6$ cells) nor potentiated NO release induced by INF/LPS (43.0 ± 0.3 nmol/$10^6$ cells in the absence and 41.0 ± 1.2 nmol/$10^6$ cells in the presence of TNF).

**Actinomycin D or cycloheximide.** Actinomycin D or cycloheximide (Fig. 2c) concentration-dependently inhibited the release of NO induced by INF/LPS. After a 24-hr exposure to half-maximally inhibitory concentrations of actinomycin D (10 ng/ml), total protein/well was 128% of control.

**Thiols and pterins.** Glutathione had no effect on basal or INF/LPS-induced NO release but was able to reverse the inhibition by TLCK of INF/LPS-induced NO release (see above). $H_4$Biopterin, an essential cofactor of NOS, or sepiapterin, which is metabolized to $H_4$biopterin via the salvage pathway (42), had no significant effect on basal or A23187 (10 μM)-induced NO release from RAW cells but concentration-dependently ($EC_{50}$ about 0.1 μM) increased INF/LPS-induced NO release 1.3-fold (Fig. 2d). At concentrations of ≥3 μM, $H_4$biopterin interfered with the nitrite/nitrate assay and was not tested.

**$Ca^{2+}$ and calmodulin.** The intracellular $Ca^{2+}$ inhibitor TMB-8 (10 μM) slightly decreased basal and concentration-dependently inhibited INF/LPS-induced NO release (Fig. 2d). $Cd^{2+}$ concentration-dependently ($EC_{50}$ about 30 μM) inhibited the release of NO induced by INF/LPS (Fig. 2d), whereas $Co^{2+}$ and $Ni^{2+}$ (up to 100 μM) were without effect. Calmodulin antagonists (calmidazolium or trifluoperazine; each up to 30 μM), L-type $Ca^{2+}$ channel blockers (verapamil, nifedipin, or diltiazem; each up to 100 μM), or ω-conotoxin GVIA (up to 10 μM) affected neither basal nor INF/LPS-induced NO release.

**cAMP and phosphorylation.** The possible roles of cAMP or kinase or phosphatase activities in NO release were investigated. Forskolin (≤10 μM; $n = 3$), which increases intracellular cAMP by directly activating adenylyl cyclase, had no effect on basal or INF/LPS-induced NO release. Okadaic acid (up to 100 nM; $EC_{50} = 3$ nM; $n = 3$), an inhibitor of protein phosphatases 1 and 2A (43), concentration-dependently inhibited INF/LPS-induced NO release (Fig. 2d).

**Corticosteroids.** Hydrocortisone, dexamethasone, or progesterone (1 μM each; $n = 3$) slightly inhibited INF/LPS-induced NO release, by 13, 24, and 20%, respectively. The rank order of potency was dexamethasone > progesterone > hydrocortisone.

**Protease inhibitors.** Exposure of RAW cells to various protease inhibitors, i.e., tosyl-phenylethyl chloromethyl ketone, PMSF, difluorophosphate, and pepstatin A (Fig. 3), as well i.e., tosyl-L-arginine methyl ester, cystatin, E-64, and acetyl-tryptophane methyl ester (up to 100 μM; $n = 3$; data not shown), had no effect on INF/LPS-induced NO release. However, the serine protease inhibitor TLCK, which specifically inhibits trypsin and papain but not chymotrypsin, abolished the induction of NO release by INF/LPS (Fig. 3, a and b). This effect was partially reversed by addition of glutathione or *N*-acetyl-L-cysteine to the culture medium. To be maximally effective, glutathione had to be present at least 3 hr after exposure of the RAW cells to INF/LPS (Fig. 3c). Addition of the thiols 6 hr after INF/LPS no longer affected the induction by INF/LPS (Fig. 3, c and d). TLCK did not interfere with the NO assay.

**A23187.** The calcium ionophore A23187 concentration-dependently ($EC_{50} = 1.5$ μM) increased basal NO release (Fig. 4). Significant release was detected after 1 hr and was maximal after about 16 hr. Maximal A23187-induced NO release was about 4.6 and 5.2% of the maximal INF- or LPS-induced NO release, respectively. When RAW cells were coexposed to a threshold concentration of INF/LPS and a maximally effective concentration of A23187, the effects of all compounds on NO release were additive but did not potentiate each other. Actinomycin D (up to 1 μg/ml) and cycloheximide (up to 1 μM) did not affect the A23187-induced NO release over 24 hr (1.8 ± 0.3 nmol/well versus 1.4 ± 0.2 and 1.5 ± 0.4 nmol/well, respectively, $n = 3$).

In RAW cells pretreated for 24 hr with submaximally activating concentrations of INF and LPS (1 unit/ml and 10 ng/ml, respectively), A23187 had no effect on NO release, whereas control cells that were sham-treated still responded to A23187 (Fig. 4b). Moreover, with increasing passage number, basal NO release was increased, whereas A23187-induced release (Table 2) and $Ca^{2+}$-dependent NOS activity (see below) were decreased.

**$Ca^{2+}$-dependent NOS.** In control RAW cells of low passage number (<7), both the crude supernatant and particulate frac-

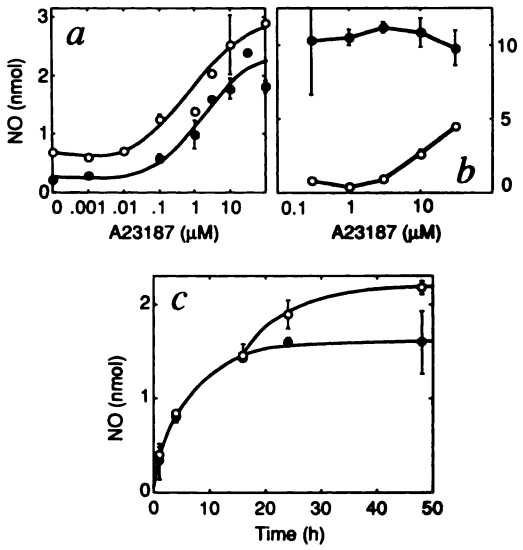


Fig. 4. A23187-induced NO release. RAW macrophage monolayers (passage < 7) were incubated as described. The different panels show incubations in the presence of A23187, in the absence (●) ($n$ = 12) or presence (○) of low concentrations of INF/LPS (10 ng/ml and 1 unit/ml; $n$ = 12) (a), in macrophages either pretreated for 24 hr with INF/LPS (10 ng/ml and 1 unit/ml) (●) ($n$ = 9) or sham-treated (○) ($n$ = 9) (b), or with A23187 (3 μM) for different times in the absence (●) ($n$ = 9) or presence of INF/LPS (○) (10 ng/ml and 1 unit/ml; $n$ = 9) (c). NO was determined as described. Values are mean (minus culture medium blank) ± standard deviation of $n$ determinations.

TABLE 2

**Effects of passage number on NO release**

RAW cell monolayers were incubated in the absence or presence of A23187 (10 μM, for 24 hr). NO release was determined by nitrite/nitrate accumulation, as described. Values are mean ± standard deviation of three to six determinations.

| Passage number | NO release | | |
|---|---|---|---|
| | Basal | A23187 | Difference |
| | *nmol/10⁶ cells* | | |
| 4 | 0.35 ± 0.09 | ND[a] | |
| 7 | 0.65 ± 0.12 | 3.06 ± 0.20 | 2.41 |
| 11 | 0.90 ± 0.16 | 2.68 ± 0.09 | 1.78 |
| 12 | 1.47 ± 0.19 | 2.39 ± 0.21 | 1.62 |
| 14 | 1.70 ± 0.28 | ND | |
| 15 | 2.56 ± 0.20 | 3.40 ± 0.15 | 0.84 |
| 17 | 5.70 ± 0.35 | 6.26 ± 0.05 | 0.56 |

[a] ND, not determined.

tions contained low NOS activity, as determined by citrulline formation (Fig. 5a). Activity was about equally distributed between both fractions and was $Ca^{2+}$ dependent, with 0.5 and 1 μM free $Ca^{2+}$ being maximally stimulatory for the soluble and particulate fractions, respectively.

**Proteases.** To test whether limited proteolysis may activate constitutive NOS, crude supernatant fractions of basal RAW cells were bioassayed for NOS activity in the presence of various thioproteases (for each, $n$ = 3). Coincubation with papain (5 μg/ml) and cathepsin C (1 unit/ml) induced small significant increases in specific activity of constitutive soluble NOS (1.35- and 1.44-fold, respectively), whereas cathepsin D (5 μg/ml, for 3 min) was inhibitory (73% of control) and cathepsin B had no effect. The papain- and cathepsin D-induced increases in specific NOS activity remained at least 1 order of magnitude lower than in crude supernatant fractions of INF/LPS-activated RAW cells (see above).

**$Ca^{2+}$-independent NOS.** In INF/LPS-activated RAW

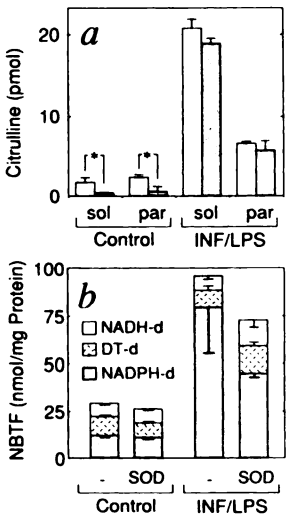


Fig. 5. Subcellular localization and $Ca^{2+}$ dependency of NOS and diaphorases. Aliquots of crude soluble (*sol*) and particulate (*par*) fractions of control or INF/LPS-activated (INF/LPS) RAW cells were incubated for measurement of citrulline formation per 0.1 mg of protein in the presence of 1 μM (□) and <1 nM (▨) free $Ca^{2+}$ (a) or NADPH-, DT-, and NADH-diaphorase activity in the absence or presence of SOD (b), as described. Values are mean ± standard deviation of three determinations. *, Significant difference ($p < 0.05$).

macrophages, both the 105,000 × $g$ supernatant and KCl-washed particulate fraction contained NOS activity, as detected by citrulline formation (Fig. 5a; ratio, 75%:25% of total) or GC-S stimulation (2.74 ± 0.18 versus 0.82 ± 0.06 nmol of cGMP/3 min/mg of protein; ratio, 77%:23% of total). The soluble and the particulate activities showed no dependency on $Ca^{2+}$, classifying them as NOS-II. The latter activity could be solubilized by CHAPS (8% w/v). Apparent recoveries from the 105,000 × $g$ pellet were about 40% after repeated KCl washes and about 25% for CHAPS solubilization. Interestingly, CHAPS and various other detergents stimulated particulate NOS-II 2–3-fold (data not shown).

Both NOS-II activities were partially purified by affinity chromatography on 2′,5′-ADP-Sepharose. Both the 105,000 × $g$ supernatant and particulate fraction contained a 130-kDa protein, in addition to several lower molecular mass proteins (Fig. 6). When cells were activated in the presence of TLCK (100 μM), the lower molecular mass proteins were present in equal amounts but the intensity of the 130-kDa band was greatly reduced (Fig. 7).

By protein immunoblot analysis, three polyclonal antibodies to rat cerebellar NOS-I (15, 16) did not recognize partially purified NOS from supernatant or CHAPS-extracted particulate fractions of INF/LPS-activated RAW cells (Fig. 8). Similar results were obtained with immunoprecipitation experiments with native NOS-II and anti-NOS-I antibody (15, 16). Although 10 μg of anti-NOS-I antibody completely precipitated 0.1 μg of purified rat cerebellar NOS and ≥50% of the original activity was recovered in the immunoprecipitate,[2] up to 30 μg of the same antibody did not significantly reduce soluble NOS-II activity, and <10% of the original activity was recovered in the respective immunoprecipitates.

**NADPH-d.** NADPH-d activity was detected in control

[2] H. H. H. W. Schmidt, G. D. Gagne, M. F. Miller, M. Nakane, and F. Murad, unpublished observation..


**Fig. 6.** SDS-PAGE of partially purified NOS-II. NOS-II from soluble (*lane 1*) and particulate (*lane 2*) fractions of INF/LPS-activated RAW cells was partially purified by 2′,5′-ADP-Sepharose 4B affinity chromatography, as described (7, 15, 29). Protein (10 μg) was separated by SDS-PAGE and silver stained as described. The positions of various molecular mass markers (in kDa) are indicated. *Arrow*, position of the 130-kDa protein staining band of NOS-II.

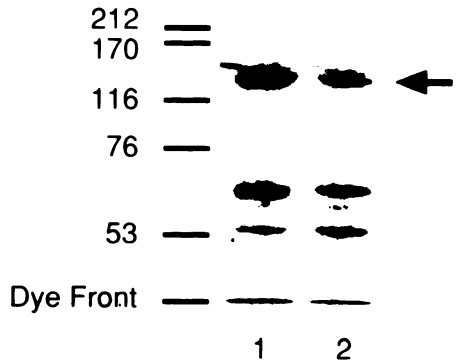


**Fig. 7.** Effect of TLCK on NOS-II expression. NOS-II from soluble fractions of RAW cells that were activated by INF/LPS in the absence (*lane 1*) or presence (*lane 2*) of 100 μM TLCK was partially purified by 2′,5′-ADP-Sepharose 4B affinity chromatography, as described (7, 15, 29). Both eluates were concentrated to 75 μl, and 3 μl were separated by SDS-PAGE and silver stained as described. The positions of various molecular mass markers (in kDa) are indicated. *Arrow*, position of the 130-kDa protein staining band of NOS-II.

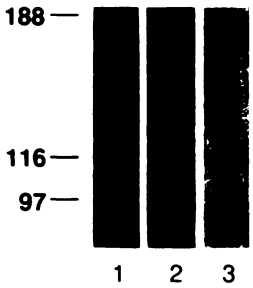


**Fig. 8.** Protein Immunoblot. Samples were separated by SDS-PAGE and transferred to nitrocellulose. Membranes were incubated with polyclonal rabbit antibody to rat cerebellar NOS-I and developed as described. *Lane 1*, supernatant fraction of rat cerebellum (5 μg protein); *lane 2*, soluble fraction of INF/LPS-activated RAW cells; *lane 3*, particulate fraction of INF/LPS-activated RAW cells (each 30 μg of protein). The positions of prestained molecular mass markers (in kDa) are indicated.

RAW cells, where it was equally distributed over the 105,000 × *g* supernatant and particulate fractions (Fig. 5b). NADH-d and DT-diaphorase (nonselective for NADPH or NADH) activities were also observed. In INF/LPS-activated cells, NADPH-d, but not NADH-diaphorase or DT-diaphorase, was about 2–3-fold increased.

**Arginine analogs.** MeArg and $NO_2Arg$ equipotently and in a concentration-dependent fashion inhibited NO release from RAW cells induced by INF and LPS (Fig. 2d) or A23187 (data not shown). In the presence of culture medium (containing 0.4 mM L-arginine), $EC_{50}$ values for MeArg and $NO_2Arg$ were about 0.5 and 1 mM, respectively. When NO release from INF/LPS-activated RAW cells in Locke's buffer (arginine-free) was assayed by transfer onto RFL-6 cells (3-min incubation), MeArg and $NO_2Arg$ were inhibitory, with $EC_{50}$ values of about 30 and 10 μM, respectively.

**cGMP.** When incubated under basal conditions at 37° (2 min), RAW macrophages contained and released small amounts of cGMP (0.68 and 0.23 pmol/$10^6$ cells, respectively). In the presence of the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (0.6 mM), cell-associated cGMP levels increased 1.5-fold (to a maximum of 1.02 pmol/$10^6$ cells; $n = 6$). NaNP (up to 1 mM) and A23187 (up to 30 μM) had no effect ($n = 3$). A 24-hr exposure to INF/LPS ($n = 3$) decreased basal cGMP levels (0.31 pmol/$10^6$ cells), which was prevented by coexposure of the cells to L-cysteine and *N*-acetyl-L-cysteine (5 mM each), which have been shown to reverse tolerance to nitrovasodilators (44). L-Cysteine and *N*-acetyl-L-cysteine also increased basal cGMP levels about 2-fold (2.15 and 2.10 pmol/$10^6$ cells in the absence and presence of INF/LPS, respectively; $n = 6$).

## Discussion

Three isoforms of NOS have been described (7, 14, 29, 45, 46) and classified according to subcellular location and regulation by free $Ca^{2+}$ (Table 1) (7). RAW cells and other macrophages were reported to express only NOS-II, a soluble $Ca^{2+}$-independent homodimer of 130-kDa subunits (47), and to release NO only after immunological stimulation. In the present paper, the formation and release of NO by NOS-II, as well as constitutive isoforms of NOS, and their regulation were investigated in control and immunologically activated RAW cells.

**NOS-II induction.** In macrophages and other cells, NO synthesis and release are apparently induced by cytokines and endotoxins (48–51). In general, exposure to combinations of cytokines (INF and TNF) and endotoxin (LPS; threshold concentration, ≥30 ng/ml) is required (52). However, in RAW macrophages, we observed that LPS or INF alone was sufficient to induce the release of NO. Both compounds potentiated each other but were not additive in their effects, suggesting similar mechanisms of induction. TNF was inactive either alone or in combination with INF/LPS, suggesting a lack of TNF receptors coupled to the induction of NO synthesis in these cells.

Low concentrations of cycloheximide ($EC_{50} \approx 30$ nM), which inhibits eukaryotic protein biosynthesis, or actinomycin D ($EC_{50} \leq 10$ ng/ml), which inhibits transcription without appreciably affecting DNA replication or protein biosynthesis, prevented the INF/LPS-induced NO release. Glucocorticoids have been shown to prevent induction of NOS in J774 macrophages and in mesangial cells (53, 54) but not in peritoneal macrophages (55). Our results in RAW macrophages provide no evidence for glucocorticoid receptors coupled to the induction of NOS. The induction by INF/LPS of NO release in RAW macrophages involved *de novo* protein biosynthesis, which was transcriptionally based and probably not due to increased translation efficiency. Conceivably, INF/LPS induced *de novo* synthesis of the $Ca^{2+}$-independent NOS-II and/or of another

protein, which posttranslationally modified NOS-II or a NOS-II precursor protein, e.g., by dephosphorylation, phosphorylation, or limited proteolysis.

The serine protease inhibitor TLCK (56) abolished the induction of NO release in RAW macrophages, whereas various other serine protease inhibitors related to TLCK did not or did not clearly affect NO release. In good agreement with this observation, TLCK and *N*-acetyl-DL-phenylalanine-$\beta$-naphthyl ester inhibit the induction of NOS-II by interleukin-1$\beta$ in rat pancreatic islets of Langerhans (57) and by INF/LPS in mouse embryonic fibroblasts (58), respectively. These data would suggest that a specific serine-type protease is involved in the induction process of NOS-II. Interestingly, TLCK and several other protease inhibitors have been shown to inhibit CMC of activated leukocytes (22, 23, 59–61). Preincubation of supernatant fractions of control or activated RAW cells with various proteases did not clearly or consistently increase NOS activity in these preparations, making limited proteolysis of a NOS-II precursor protein an unlikely mechanism for INF/LPS-induced NO release. We also observed that TLCK reduced the expression of the NOS-II 130-kDa protein. However, TLCK has been shown to act by mechanisms unrelated to proteases by reducing transcription (62) and macromolecular synthesis (63). TLCK is able to react with glutathione at neutral pH, and TLCK-induced inhibition of macromolecular synthesis is specifically blocked by reduced glutathione (63). The reversal by glutathione or cysteine and the reduction of NOS-II expression (130-kDa band) suggest similar non-protease-related mechanisms of action for TLCK to inhibit NO release in RAW cells.

In various cells, INF/LPS induce both NO formation and biosynthesis of $H_4$biopterin (42), which is an essential cofactor of NOS (14, 35, 64, 65). Nevertheless, the intracellular concentration of $H_4$biopterin may still be rate-limiting, even in the presence of INF/LPS, inasmuch as $H_4$biopterin or sepiapterin, which increases intracellular $H_4$biopterin levels via the salvage pathway, further augmented INF/LPS-induced NO release. Similar results have been reported by Werner *et al.* (42). Although crude supernatant fractions of INF/LPS-activated macrophages contain sufficient amounts of $H_4$biopterin to support NOS-II activity maximally (64), increased availability of this cofactor through induction of the rate-limiting enzyme of its biosynthesis, GTP cyclohydrolase, may represent a potential induction mechanism of INF/LPS in intact cells.

$Cd^{2+}$-sensitive transmembrane $Ca^{2+}$ flux through atypical $Ca^{2+}$ channels may represent an initial event in the process of induction of NO release by INF/LPS. This is indicated by the observations that $Cd^{2+}$ (100 $\mu$M), an inorganic $Ca^{2+}$ antagonist, and TMB-8 ($\leq$10 $\mu$M), an intracellular $Ca^{2+}$ inhibitor (although prone to cause nonspecific effects) (66, 67), inhibited the INF/LPS-induced NO release. However, L-type $Ca^{2+}$ channel blockers (verapamil, nifedipin, and diltiazem) and $\omega$-conotoxin GVIA had no such effect. Conversely, the induction of CMC in macrophages was reported to be blocked effectively by L-type $Ca^{2+}$ channel blockers and calmodulin inhibitors (24, 25). Whether this apparent discrepancy between the role of $Ca^{2+}$/calmodulin in INF/LPS-induced NO release and the INF/LPS-induced cytotoxic response is due to differences in experimental conditions (cultured versus peritoneal macrophages) and whether it is found in other cell lines remains to be investigated. Interestingly, in bone marrow-derived macrophages, another discrepancy between NO release and CMC has been observed. Here, MeArg inhibits NO release and tumoricidal activity with different potencies (26), and several Gram-negative bacteria induce NO release but little tumoricidal activity, whereas Gram-positive bacteria often induce tumoricidal activity but little NO release (27). NO may not be the sole mediator of CMC (9) but, rather, act in concert with reactive oxygen intermediates (68), lysosomal enzymes (22), proteases (69), complement component C3 (70), TNF (71), prostaglandins (72), or myeloperoxidase (73).

The predicted amino acid sequence of the NOS-I cDNA contains consensus sites for phosphorylation (74). Moreover, phosphorylation by protein kinase C and $Ca^{2+}$/calmodulin-dependent (75) but not cAMP-dependent (76) protein kinase regulates NOS-I activity. The phosphatase inhibitor okadaic acid slightly inhibited INF/LPS-induced NO release, suggesting that phosphorylation may down-regulate and phosphatases up-regulate NOS-II activity. However, this phosphorylation would not likely be due to a cAMP-dependent protein kinase, because forskolin-induced increases in intracellular cAMP levels had no effect on NO release in INF/LPS-activated RAW cells and phosphorylation of NOS-I by cAMP-dependent protein kinases has no effect on activity (76). An increase in NOS-I activity has, however, been reported after phosphorylation by protein kinase C (75).

**Subcellular location.** In macrophages, NOS-II has been described only in the supernatant fraction of INF/LPS-activated cells (47). Our results show the presence of an additional $Ca^{2+}$-independent NOS activity (NOS-II) located in the particulate fraction of INF/LPS-activated RAW macrophages. This NOS-II was partially recovered in the KCl-washed, 105,000 $\times$ *g* pellet and could be solubilized with CHAPS and other detergents, clearly characterizing it as a membrane-associated NOS. Upon partial purification, the particulate fraction was shown to contain a 130-kDa protein with chromatographic behavior identical to that of the soluble NOS-II. These findings imply that both NOS-II are identical proteins, possibly containing different posttranslational modifications that lead to different subcellular compartmentalization.

One of three different rabbit polyclonal antibodies to rat cerebellar NOS-I partially inhibited NOS-II activity. However, anti-NOS-I antibodies (as well as monoclonal anti-NOS-III antibodies)[3] did not recognize partially purified NOS-II in protein immunoblot analysis, suggesting INF/LPS-induced NOS-II are immunologically distinct from NOS-I (and NOS-III).

**Constitutive NOS.** Small but significant NO release was observed in control RAW cells. The endotoxin concentration of the culture medium was 300-fold lower than the threshold concentration for LPS to induce NO release and could not account for this basal activity. Thus, RAW cells constitutively expressed NOS. Constitutive NOS were $Ca^{2+}$ dependent and were detected in the supernatant and particulate fractions, which had lower specific activities than the corresponding fractions of activated RAW cells. In agreement with these findings, the $Ca^{2+}$ ionophore A23187 increased basal NO release in control RAW cells. The time courses for A23187- and INF/LPS-induced NO release, i.e., immediate versus a 12-hr lag period, and their susceptibility to inhibition by actinomycin D

[3] J. S. Pollock, M. Nakane, U. Förstermann, and F. Murad, unpublished observations.

and cycloheximide were different, as would be expected from the different mechanisms of action of A23187 and INF/LPS.

Moreover, the $Ca^{2+}$-independent and the $Ca^{2+}$-dependent NOS activities in RAW cells were inversely regulated by INF/LPS; INF/LPS induced the former (Fig. 2) and down-regulated the latter isoform. After treatment with INF/LPS or with higher passage numbers of RAW cells, A23187-induced NO release was decreased and basal NO release was increased. $Ca^{2+}$-dependent NOS were also no longer detected in high-passage control cells. The mechanisms by which INF/LPS or cell passage number down-regulate the $Ca^{2+}$-dependent NOS activity remain unclear. A similar phenomenon has been reported for $Ca^{2+}$-dependent NOS activities in skeletal muscle, aorta, and skin of rats treated with LPS (77).

**NADPH-d.** NOS-I in neural (28) and other (16) tissues is identical to and colocalized with NADPH-d. Macrophage NOS appeared also to be a NADPH-d. In activated RAW cells, we observed soluble and particulate NADPH-d. Specific NADPH-d activity was lower in control versus activated RAW cells, suggesting an increase in NOS protein upon induction by INF/LPS. In the absence of SOD, NADH- and DT-diaphorase were not increased. NBTF formation due to a direct chemical interaction between NBT and cytosolic $H_4$biopterin (35) or NADPH-d different from NOS (78) cannot, however, be excluded until both NOS proteins are available in purified form.

***N*$^G$-Substituted arginine analogs.** Constitutive and inducible isoforms of NOS have been classified according to their different susceptibility to inhibition by MeArg or $NO_2$Arg (79, 80). The former compound may more potently inhibit NOS-II and the latter, NOS-I. However, for both inhibitors, noncompetitive irreversible mechanisms of action have been shown (81, 82).

We observed that MeArg and $NO_2$Arg were about equally potent in inhibiting NOS-II activity in intact RAW cells and crude supernatant fractions thereof. Apparent $IC_{50}$ values for both inhibitors on NO release in intact cells were higher in the presence of arginine-containing culture medium (nitrite/nitrate release) than in the presence of Locke's buffer (RFL-6 bioassay) or in cell-free preparations. This loss in potency did not, however, affect the rank order of potency between MeArg and $NO_2$Arg. Similar observations have been made for other NOS-II-containing cells and tissues.[4] Thus, a consistent classification of NOS isoforms according to the respective apparent $K_i$ values for MeArg or $NO_2$Arg (79, 80) is not possible.

**cGMP.** NO is the most potent activator of GC-S (20, 21) and, thus, induces increases in the second messenger molecule cGMP in various cells and tissues (83, 84). In some macrophage preparations, cGMP was suggested to mediate lymphokine-induced aggregation (85) and parasite internalisation (86).

NO released from activated RAW cells, or formed by fractions thereof, stimulated GC-S in RFL-6 detector cells. cGMP levels in control RAW cells were low and were not increased upon exposure of the cells to INF/LPS and/or the NO-donor compound NaNP. Tolerance of GC-S in INF/LPS-activated cells due to overstimulation by NO can be excluded, because NaNP was equally ineffective in basal cells and *N*-acetylcysteine/cysteine, which have been shown to reverse tolerance of GC-S and augment responses to NO, had no such effect in RAW cells. In this macrophage-type cell line, NO may, therefore, act intracellularly by mechanisms other than stimulation of GC-S (87).

In conclusion, RAW macrophages contained $Ca^{2+}$-dependent and $Ca^{2+}$-independent, soluble and particulate, NOS, which were differentially regulated by INF/LPS and cell passage. Induction of NOS-II by INF/LPS was transcriptionally based, correlated with NADPH-d activity, and appeared to involve intracellular thiol pools, $Ca^{2+}$, and increased $H_4$biopterin biosynthesis. Post-translational modification of NOS by proteases seems unlikely, although phosphorylation may regulate NOS activity to some degree.

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[4] J. A. Mitchell, K. Kolhaas, and F. Murad, unpublished observations.

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