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Nitric Oxide Regulates Interleukin 1 Bioactivity Released from Murine Macrophages*

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Jeanette R. Hill, John A. Corbett‡, Guim Kwon, Connie A. Marshall, and Michael L. McDaniel§

From the Department of Pathology, Washington University School of Medicine, St. Louis, Missouri 63110 and the ‡Department of Biochemistry, St. Louis University, St. Louis, Missouri 63104

The bioactivity of interleukin-1 (IL-1), a major proinflammatory cytokine, can be modulated by a variety of factors including inhibitors of IL-1 production and release and receptor blockade by IL-1 receptor antagonist and by binding to nonsignaling soluble receptors. This study demonstrates that the free radical nitric oxide (NO) is also a regulator of IL-1 bioactivity. Lipopolysaccharide-activated murine macrophage RAW264.7 cells, and lipopolysaccharide plus interferon-γ-activated murine peritoneal macrophages release IL-1 bioactivity, which is increased 10-fold over control levels by 24 h. N^G-Monomethyl L-arginine (NMMA), a nitric oxide synthase (NOS) inhibitor, almost completely inhibits the release of IL-1 bioactivity from activated macrophages in a time- and concentration-dependent manner with an IC_{50} of 50 μM. IL-1 activity was determined by thymocyte proliferation bioassay and by a new spectrophotometric bioassay based on IL-1-specific induction of NOS and NO production by an insulinoma cell line, RINm5F. Neither NO nor NOS inhibitors present in the macrophage supernatant interfere with the bioassays. Aminoguanidine and iodonium diphenyl, mechanistically unrelated NOS inhibitors, also prevent the release of IL-1 activity from RAW 264.7 cells. The addition of the NO donor *S*-nitrosoacetylpenicillamine reconstituted the release of IL-1 bioactivity inhibited by NMMA in a concentration-dependent manner. NO appears to increase the amount of IL-1 protein released by activated macrophages as determined by enzyme-linked immunosorbent assay, but not by mechanisms involving cell death nor modification of IL-1 precursor processing. A cGMP donor, 8-bromo-cGMP, dose-dependently reverses NMMA inhibition of bioactive IL-1 release, suggesting that NO regulates IL-1 release by a cGMP-dependent mechanism. These observations suggest that NO stimulation of the activity of IL-1, a key mediator of the immune response, may be a potentially important mechanism for control of IL-1 activity *in vivo*.

The proinflammatory cytokines, IL-1α[1] and IL-1β, are key mediators in the regulation of the immune response. However, inappropriate expression of IL-1 has been linked to autoimmune diseases such as arthritis and diabetes, and high levels of IL-1 found in the blood during sepsis are associated with hypotension, shock, and mortality (1, 2). Due to the potential deleterious effects of these proinflammatory cytokines, the production and activity of IL-1 are balanced by a number of naturally occurring inhibitors. IL-1 receptor antagonist (IL-1Ra), structurally related to IL-1, specifically blocks IL-1 activity by competitively binding to the surface receptor (IL-1RI) on target cells, preventing signal induction (3). IL-1 activity is also regulated at the levels of synthesis, processing, and release, and by soluble receptors and serum proteins such as α_2-macroglobulin (4–7).

The major *in vivo* sources of IL-1 are macrophages and monocytes, although IL-1 is also produced by other cells such as endothelial, smooth muscle, keratinocytes, and leukemic cells (8). IL-1α and IL-1β are expressed as 33-kDa precursor proteins that are cleaved intracellularly by cysteine protease, calpain (9, 10), and IL-1β converting enzyme (11), respectively. IL-1α is active in both the processed (17 kDa) and precursor (33 kDa) forms, whereas IL-1β is bioactive only as the processed protein (12). IL-1Ra, also expressed as a 33-kDa protein, contains a leader sequence and is cleaved and secreted via the classic ER-Golgi apparatus (13). The mechanism of release of IL-1α and IL-1β, which lack leader sequences, is unknown.

The common methods for measuring IL-1 bioactivity, based on IL-1 co-induced thymocyte proliferation, are complicated by cross-reactivity with other cytokines including IL-2, IL-4, IL-6, and tumor necrosis factor α (14, 15). We have recently developed a specific, nonradioactive bioassay for IL-1 based on IL-1-induced nitric oxide production by an insulinoma cell line, RINm5F (16). RINm5F cells respond specifically and linearly to murine and human IL-1α and IL-1β in the range of 0.1–1 units/ml (10–100 pg) and do not cross-react with IL-2, -4, -6, -9, -11, or -15, tumor necrosis factor α, IFNγ, nor LPS (16).

Factors that induce IL-1 production and release by murine macrophages, including LPS and IFNγ, also induce nitric oxide synthase (NOS) expression and nitric oxide (NO) production. NO, a reactive, free radical gas, is produced by NOS catalyzed conversion of L-arginine to L-citrulline. NO is a mediator in diverse intra- and intercellular processes including roles in neurotransmission, vascular tone, and immune regulation (for recent reviews see Refs. 17–19).

In this study, evidence is presented that NO stimulates the release of IL-1 bioactivity from activated murine macrophages. These results provide the first experimental evidence for a new mechanism of control of IL-1 activity and a new role for NO.

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§ To whom correspondence should be addressed: Dept. of Pathology, Box 8118, Washington University School of Medicine, 660 South Euclid Ave., St. Louis, MO 63110-8118. Tel.: 314-362-7435; Fax: 314-362-4096; E-mail: mcdaniel@pathology.wustl.edu.

[1] The abbreviations used are: IL, interleukin; IFN, interferon; IL-1Ra, IL-1 receptor antagonist; NOS, nitric oxide synthase; LDH, lactate dehydrogenase; LPS, lipopolysaccharide; NMMA, N^G-monomethyl L-arginine; NO, nitric oxide; PEC, peritoneal exudate cells; SNAP, *S*-nitroso-*N*-acetylpenicillamine; ELISA, enzyme-linked immunosorbent assay; MEM, minimum essential medium.

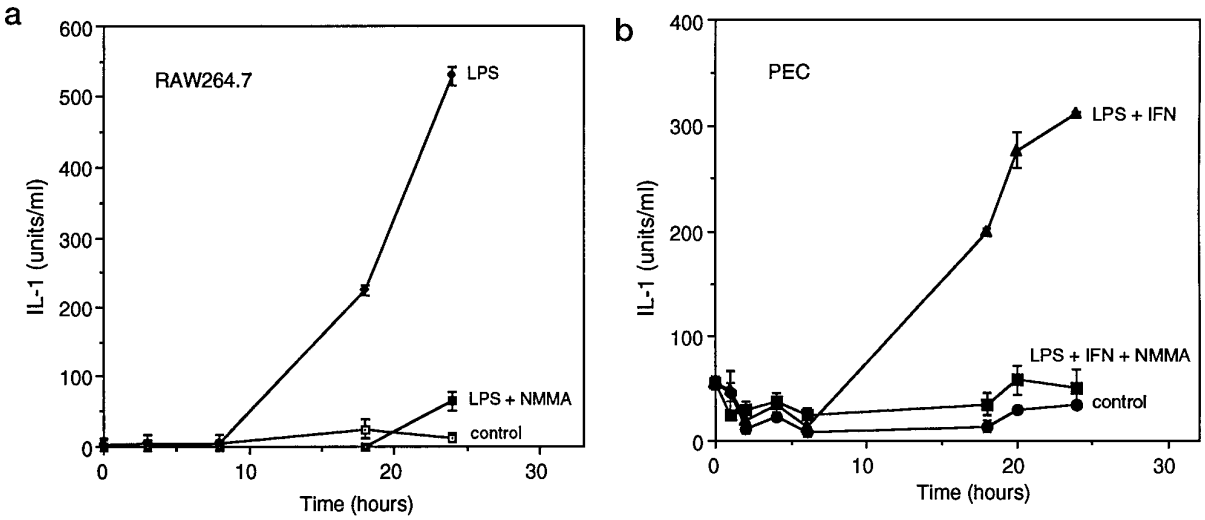

FIG. 1. **NMMA inhibits release of IL-1 bioactivity from activated murine macrophages.** *a*, RAW cells (2×10^5/200 μl) were exposed to 1 μg/ml LPS ± 0.25 mM NMMA. *b*, PEC were exposed to 1 μg/ml LPS plus 150 units/ml IFNγ ± 0.25 mM NMMA. Cell-free medium was removed at the indicated times and stored at −70 °C. IL-1 bioactivity was measured by the RINm5F assay described under "Experimental Procedures." The results are the averages ± S.E. of an individual experiment containing three replicates per condition and are representative of three separate experiments.

EXPERIMENTAL PROCEDURES

Materials

Recombinant human IL-1β and IL-1α were obtained from Cistron (Pine Brook, NJ). Murine IFNγ was from Boehringer Mannheim. Monoclonal hamster anti-murine IL-1α and IL-1β were gifts from Dr. David Chaplin (Washington University). Murine IL-1α and IL-1β ELISA kits were purchased from Genzyme. Anti-human IL-1Ra was from R&D Systems (Minneapolis, MN). CMRL-1066 and MEM tissue media were obtained from Life Technologies, Inc. *N*-Monomethyl L-arginine (NMMA) and (±) *S*-nitroso-*N*-acetylpenicillamine (SNAP) were purchased from Calbiochem (La Jolla, CA), and aminoguanidine and lipopolysaccharide (LPS, *Escherichia coli* serotype 0111:B4) were from Sigma. Iodonium diphenyl was purchased from Aldrich. 8-Bromo-cGMP was from BioMol (Plymouth Meeting, PA). Fetal bovine serum was purchased from Hyclone (Logan, UT), and PHA was from Difco Labs (Detroit, MI). [^{35}S]Methionine trans-label (1000 Ci/mmol) was from Amersham Corp., and [^{3}H]thymidine was from ICN (Costa Mesa, CA). All other reagents were purchased from commercially available sources.

Cell Culture

RINm5F, an insulin secreting cell line purified from a radiation-induced rat islet cell tumor (Refs. 12 and 13; originally a gift from Dr. H. K. Oie, National Cancer Institute), and RAW 264.7, a murine macrophage cell line, (both obtained from the Washington University Tissue Culture Support Center) were removed from T-75 flasks by treatment with 0.05% trypsin/0.02% EDTA at 37 °C. The cells were washed and added to microtiter plates at concentrations of 2×10^5 cells/200 μl (1×10^6 cells/ml) of CMRL-1066 supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin (complete CMRL). The cells were incubated for 24 h after plating at 37 °C in 95% air,5% CO_2, and the medium was replaced prior to initiation of experiments. Peritoneal exudate cells (PEC) were isolated by adherence from male CD1 mice as described previously (20) and plated at concentrations of 2×10^5 cells/200 μl of complete CMRL medium.

IL-1 Bioactivity

RINm5F Assay—IL-1 activity was determined as described previously (16). Briefly, cell-free culture media obtained from activated RAW 264.7 or PEC at a final dilution of 1:100 were added to RINm5F cells (2×10^5 cells/200 μl). Concurrently, a standard curve was performed using hIL-1β (Cistron) at concentrations between 0.1 and 1 units/ml (10–100 pg). After incubation for 24 h at 37 °C in 95% air, 5% CO_2, the culture medium was collected, and nitrite levels were determined. IL-1 concentrations were extrapolated from the standard curve.

Thymocyte Proliferation Assay—Cell-free culture media obtained from activated RAW 264.7 or PEC at a final dilution of 1:100, 1:50 or 1:10 were added to 1×10^6 thymocytes (0.2 ml) isolated from 6–12-week-old C3H/HeJ mice (Jackson Labs) in RPMI medium with 5% fetal bovine serum and 2.5 μM β-mercaptoethanol and costimulated with 1 μg/ml PHA (21). [^{3}H]Thymidine (1 μCi) was added during the last 6 h of a 48-h incubation. The cells were harvested on glass fiber filters, and the incorporated radioactivity was determined by liquid scintillation counting. IL-1 concentrations were extrapolated from a standard curve performed concurrently using hIL-1β.

Nitrite Determination

Nitrite release was determined by mixing 50 μl of culture medium with 50 μl of Griess reagent (1 part 0.1% naphthylethylenediamine dihydrochloride in H_2O plus 1 part 1.32% sulfanilamide in 60% acetic acid (22)) in a 96-well microtiter plate. The absorbance at 540 nm was measured on a Titertek Multiskan MCC/340 plate reader, and nitrite concentrations were calculated from a standard curve using $NaNO_2$ (Fisher) concentrations between 0.1 and 10 nmol.

LDH Activity

100-μl aliquots of cell-free supernatant were assayed for LDH (23) by incubation with 10 mM pyruvate and 0.1 mM NADH (Sigma) in 0.1 M Tris, pH 7.5 (final volume, 1 ml) by measuring the oxidation of NADH at A_{340} for 10 min. Total LDH activity was determined by lysing control cells with 0.1% Triton X-100 for 30 min. LDH release was expressed as the percentage of total LDH activity.

Immunoprecipitation of IL-1

Murine PEC (1×10^6 cells) in MEM methionine-deficient medium (9 parts MEM without methionine:1 part MEM containing methionine) were activated with LPS (1 μg/ml) and IFN-γ (150 units/ml) ± NMMA (0.25 mM) ± SNAP (200 μM) for 3 h. The cells were then pulsed with 150 μCi of [^{35}S]methionine trans-label for 4 h. The cells were washed with MEM to remove label, and NMMA and SNAP were replaced. The cells were incubated for an additional 17 h (total 24 h). The supernatant was collected, and cells were removed by centrifugation at $200 \times g$ for 1 min. The cells were washed in phosphate-buffered saline and lysed in 0.15 M NaCl, 1% Nonidet plus protease inhibitors (aprotinin, 1 μg/ml; leupeptin, 1 μg/ml; phenylmethylsulfonyl fluoride, 0.1 mM; iodoacetamide, 1 mM; EDTA, 0.1 mM) by sonication, and the lysates were clarified by centrifugation at $10{,}000 \times g$ for 30 min at 4 °C. Protease inhibitors were also added to the supernatant. Both lysate and supernatant were precleared with 20 μl protein A-Sepharose (Sigma). Equivalent fractions of each sample were immunoprecipitated for 2 h by addition of anti-murine IL-1α, anti-murine IL-1β, and anti-human IL-1Ra (final dilutions, 1:1000). Protein A-Sepharose was added for 1 h, and the beads were washed three times with phosphate-buffered saline + 1% Nonidet + 0.2% SDS and finally phosphate-buffered saline and then boiled in SDS-polyacrylamide gel electrophoresis sample buffer. Samples were resolved by 15% SDS-polyacrylamide gel electrophoresis and visualized by fluorography.

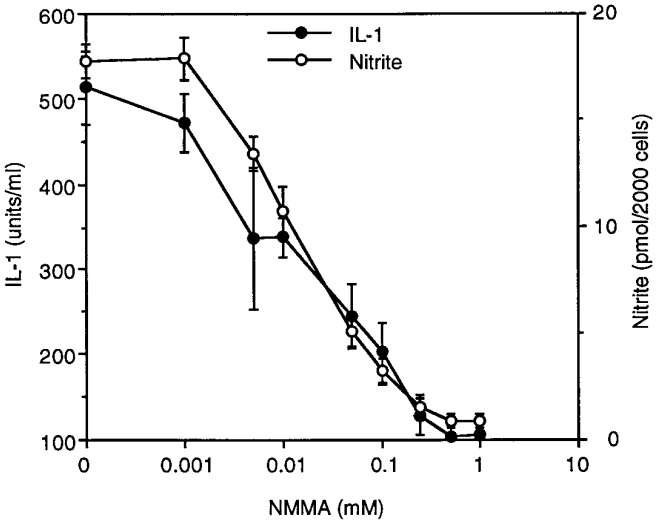

FIG. 2. **Dose-dependent inhibition by NMMA of IL-1 bioactivity released from LPS-stimulated RAW 264.7 cells.** RAW cells (2×10^5/200 μl) were exposed to 1 μg/ml LPS with the indicated concentrations of NMMA for 24 h. IL-1 bioactivity in the medium was determined by the RINm5F bioassay. The results are the averages ± S.E. of three individual experiments containing three replicates per condition.

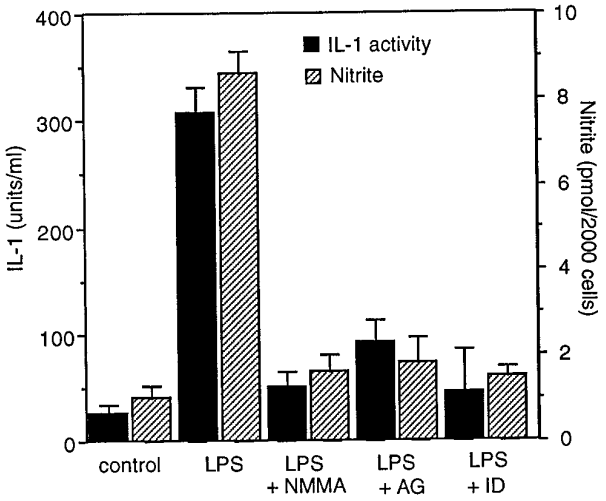

FIG. 3. **LPS-induced IL-1 bioactivity is blocked by NOS inhibitors.** RAW 264.7 cells (2×10^5/200 μl) were exposed to 1 μg/ml LPS with inducible NOS inhibitors for 24 h. Concentrations of inhibitors: NMMA, 0.25 mM; aminoguanidine (*AG*), 0.25 mM, and iodonium diphenyl (*ID*), 0.5 μM. IL-1 bioactivity in the medium was determined by the RINm5F bioassay. The results are the averages ± S.E. of three to four individual experiments containing three replicates per condition.

Statistics

Statistical comparisons were made between groups using a one-way analysis of variance. Significant differences ($p < 0.01$) were evaluated using a Scheffe's F-test posthoc analysis.

RESULTS

Time and Concentration-dependent Inhibition of IL-1 Activity Released from Activated Murine Macrophages by NMMA—LPS-stimulated RAW 264.7 murine macrophage cells release bioactive IL-1, which is first detected at 18 h and increases to levels 10-fold over nonstimulated control levels at 24 h (Fig. 1*a*). Unexpectedly, the NOS inhibitor NMMA almost completely inhibits this time-dependent release of IL-1 bioactivity. The IL-1 activity in the cell-free medium was determined at the indicated times by the RINm5F bioassay as described under "Experimental Procedures." As a control to ensure that carry over of NMMA from RAW 264.7 and PEC media did not interfere with the RINm5F bioassay, NMMA (0.25 mM) was added to the stimulated RAW 264.7 cells at 24 h, immediately before addition to RINm5F cells. The IL-1 activity of media obtained from LPS and LPS + NMMA (at 24 h) stimulated RAW 264.7 cells was identical, indicating that the low concentration (after 1:100 dilution) of NMMA carried over does not affect NO production by RINm5F cells. Murine peritoneal exudate cells (PEC) stimulated with LPS plus IFNγ also release IL-1 activity in a time-dependent manner similar to RAW 264.7 cells, and this activity is significantly inhibited by coincubation with NMMA (Fig. 1*b*).

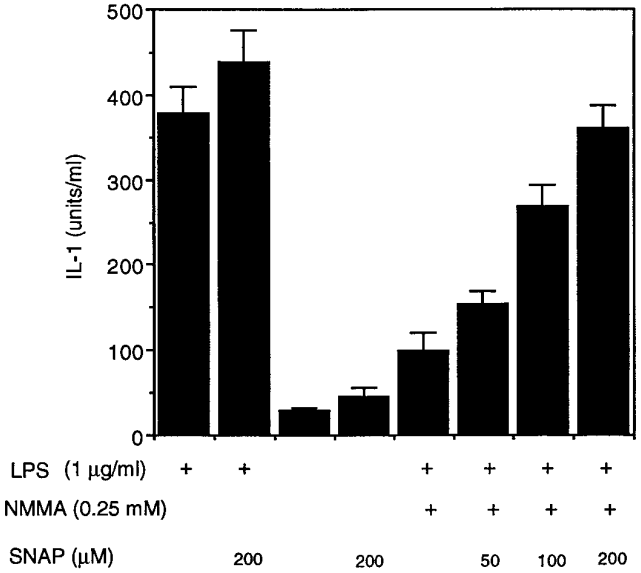

FIG. 4. **NO donors reconstitute LPS-stimulated IL-1 bioactivity inhibited by NMMA.** RAW 264.7 cells were induced with 1 μg/ml LPS in the presence of 0.25 mM NMMA and SNAP as indicated for 24 h. Medium levels of IL-1 bioactivity were measured by the RINm5F bioassay. The results are the averages ± S.E. of four separate experiments containing three replicates per condition.

Inhibition by NMMA of the release of IL-1 bioactivity by LPS-stimulated RAW 264.7 cells is concentration dependent (Fig. 2). RAW cells were coincubated with LPS plus NMMA (concentrations between 0 and 1 mM) for 24 h, and medium levels of nitrite and IL-1 activity were determined. NMMA concentration-dependently inhibits both release of NO (nitrite) and IL-1 activity from RAW 264.7 cells with an IC_{50} of 50 μM.

Effects of other NOS Inhibitors on Release of Bioactive IL-1—To determine whether attenuation of bioactive IL-1 release by NMMA is shared by other NOS inhibitors, LPS-stimulated RAW 264.7 cells were treated with representatives of two families of NOS inhibitors. Aminoguanidine, like NMMA, is a competitive, substrate-based inhibitor whereas iodonium diphenyl inhibits NOS by blocking NADPH and FAD binding sites (24). NMMA (0.25 mM), aminoguanidine (0.25 mM), and iodonium diphenyl (0.5 μM) inhibit both release of nitric oxide and IL-1 bioactivity from LPS activated RAW cells (Fig. 3). Experimental controls were performed concurrently to demonstrate that residual NOS inhibitors in the medium did not interfere with the IL-1 bioassay as described above.

NO Donors Reverse NMMA Inhibition of Bioactive IL-1 Release—To confirm that NO is the mediator that stimulates release of bioactive IL-1 from murine macrophages, we next examined whether NO donors could reconstitute IL-1 release inhibited by NMMA. As shown in Fig. 4, LPS stimulation of RAW 264.7 cells for 24 h results in a 10-fold increase in medium levels of bioactive IL-1 over control levels which is significantly inhibited by 0.25 mM NMMA. SNAP, a NO donor, dose-dependently (50–200 μM) reversed NMMA inhibition of IL-1

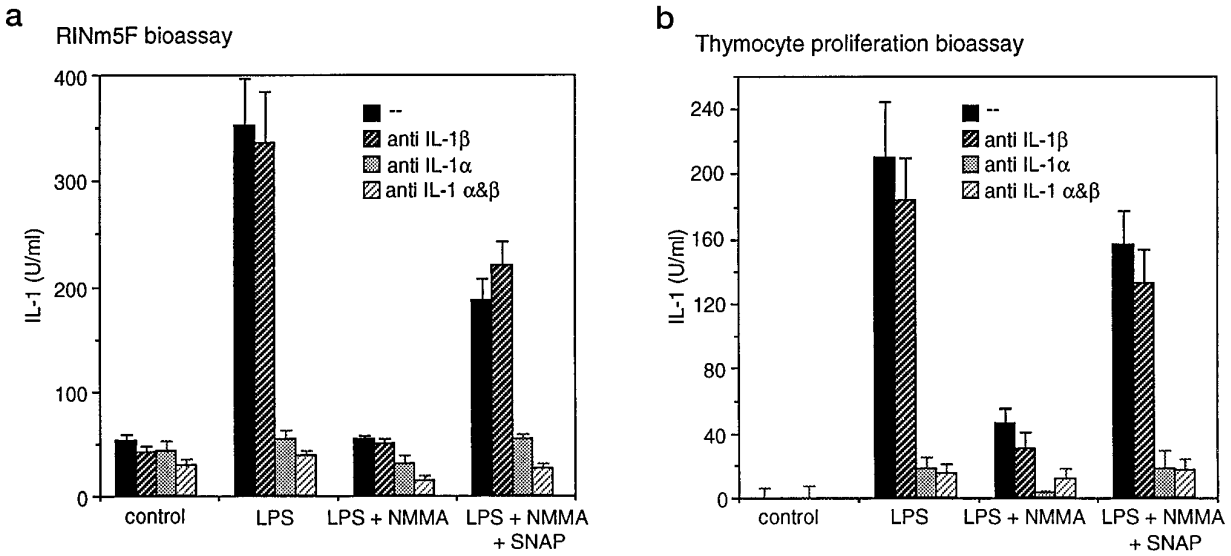

FIG. 5. **IL-1 activity released by LPS-activated RAW 264.7 cells.** RAW 264.7 cells were activated by 1 μg/ml LPS ± 0.25 mM NMMA ± 200 μM SNAP for 24 h, and the medium was assayed for IL-1 activity by RINm5F bioassay (*a*) or thymocyte proliferation bioassay (*b*). The RAW 264.7 medium was preincubated with 1 μg/ml anti IL-1α, anti IL-1β, or both for 30 min at 4 °C. The results are the averages ± S.E. of three separate experiments containing three replicates per condition.

bioactivity release while having little effect on LPS-stimulated and control cells. NO donors diethylamine/NO and spermine/NO also dose-dependently reversed NMMA inhibition of release of bioactive IL-1 when added more than 8 h after initial LPS stimulation (data not shown).

The ability of NO to stimulate IL-1 activity released from activated RAW 264.7 cells was confirmed by a separate bioassay for IL-1 based on thymocyte proliferation. Both the RINm5F bioassay (Fig. 5*a*) and the thymocyte proliferation assay (Fig. 5*b*) show a 10-fold increase in IL-1 activity released by LPS-stimulated RAW 264.7 cells over control levels, which is inhibited by NMMA, and this inhibition is reversed by SNAP. Preincubation of RAW 264.7 medium with antibodies to IL-1α plus IL-1β completely inhibits activity measured by both assays. IL-1α antibodies alone block more than 90% of the measured activity, whereas IL-1β antibody has little effect, indicating that most of the measured IL-1 activity released by RAW 264.7 cells is due to IL-1α.

These findings indicate that inhibitors of endogenous NO formation block the release of bioactive IL-1 from activated macrophages and that exogenous NO donors reverse this inhibition. The following approaches were designed to elucidate the mechanism of NO-induced release of IL-1 bioactivity from murine macrophages.

Effects of NO on LDH Release by RAW 264.7 Cells—The effects of NO on cell death as measured by LDH release were examined to determine whether NO stimulates IL-1 release by causing cell death and membrane permeability. RAW cells were treated under identical conditions as described in Fig. 4 for 24 h and LDH activity. IL-1 activity and nitrite released into the media were determined (Table I). No difference in levels of LDH release was detected in the presence of NO (LPS and LPS + NMMA + SNAP) or in the absence of NO (control and LPS + NMMA). The levels of LDH detected in the media do not correlate with the release of bioactive IL-1 from RAW 264.7 cells. Even though prolonged exposure of RAW 264.7 cells with 200 μM SNAP for 48 h resulted in significant LDH release (54.9%±4.4), NO does not appear to be stimulating nonspecific release of cytosolic proteins via membrane permeabilization at 24 h when release of IL-1 activity is detected (see Fig. 1). The absence of NO-induced cell death at 24 h was also confirmed by trypan blue exclusion and DNA fragmentation experiments (data not shown).

TABLE I

LDH release does not correlate with release of IL-1 activity and NO

RAW 264.7 cells (4×10^5 cells/400 μl) were treated with LPS (1 μg/ml) ± 0.25 mM NMMA ± SNAP for 24 h. The culture medium was removed, and LDH activity, IL-1 activity and nitrite were measured as described under "Experimental Procedures." The results are the averages of three separate experiments containing three replicates per condition.

Treatment	LDH	IL-1 activity	Nitrite
	% total	*units/ml*	*pmol/ 2000 cells*
Control	17.3 ± 1.1	27.8 ± 5.2	1.5 ± 1.6
Control + 200 μM SNAP	20.1 ± 2.3	44.8 ± 11.3	53.7 ± 2.1
LPS	11.5 ± 0.7	378.2 ± 31.3	19.2 ± 1.0
LPS + 200 μM SNAP	21.0 ± 1.8	437.4 ± 38.4	64.9 ± 2.3
LPS + NMMA	11.7 ± 0.9	99.3 ± 21.6	1.4 ± 0.6
LPS + NMMA + 50 μM SNAP	13.0 ± 1.4	152.2 ± 15.8	15.9 ± 0.2
LPS + NMMA + 100 μM SNAP	16.4 ± 1.7	267.5 ± 25.2	28.1 ± 0.6
LPS + NMMA + 200 μM SNAP	19.0 ± 2.0	359.0 ± 29.3	55.4 ± 1.3

Effects of NO on IL-1 Protein Release Determined by ELISA—The concentration of IL-1 protein released from LPS-stimulated RAW 264.7 cells was next measured by ELISA specific for murine IL-1α and IL-1β (Fig. 6). As compared with control levels, IL-1α release by LPS and LPS + NMMA + SNAP ($p < 0.001$) and LPS + NMMA ($p < 0.01$) treated RAW 264.7 cells is significantly increased. The addition of NMMA to activated RAW 264.7 cells significantly decreases ($p < 0.05$) IL-1α release as compared with LPS-activated cells, and this inhibition is reversed by the NO donor, SNAP. Although a trend for attenuation of IL-1β release by NMMA and reversal by the addition of SNAP is observed, no statistically significant differences are found in this experiment.

Effects of NO on IL-1 Precursor Processing—To determine whether NO is affecting IL-1 activity by modifying cysteine proteases that cleave IL-1 precursors, we examined IL-1 precursor cleavage by activated murine PECs in the presence of NO (endogenous and exogenous) and in the absence of NO (NMMA). Murine PECs were stimulated with LPS plus IFNγ ± NMMA ± SNAP and pulse labeled with [^{35}S]methionine. After a 24-h incubation, cells and supernatant (media) were assayed for bioactivity (Fig. 7*a*) and immunoprecipitated with antibod-

ies specific for mIL-1α, mIL-1β, and IL-1Ra (Fig. 7, *b*, *c*, and *d*, respectively). As shown in Fig. 7*a*, IL-1 bioactivity measured in the supernatant of stimulated PECs is inhibited by NMMA and reconstituted with the NO donor, SNAP, whereas NO does not affect activity within the cell measured under these same conditions. Immunoprecipitation shows that the major form of intracellular IL-1α (Fig. 7*b*) and IL-1β (Fig. 7*c*) is the uncleaved 33-kDa precursor protein, whereas the cleaved 17.5-kDa protein is the prevalent form released into the supernatant. IL-1Ra, known to be cleaved within the Golgi, is detected as the 17.5-kDa protein both intracellularly and extracellularly (Fig. 7*d*). NO does not appear to affect the levels of IL-1α, IL-1β, or IL-1Ra protein expressed. Precursor cleavage also is not affected by NO as no difference in the amount of cleaved IL-1α, IL-1β, or IL-1Ra is observed in the absence (LPS/IFN + NMMA) or the presence (LPS/IFN and SNAP) of NO. These data demonstrate that although NO stimulates activity of IL-1 released by activated macrophages, NO affects neither the expression nor precursor processing of IL-1α, IL-1β, or IL-1Ra.

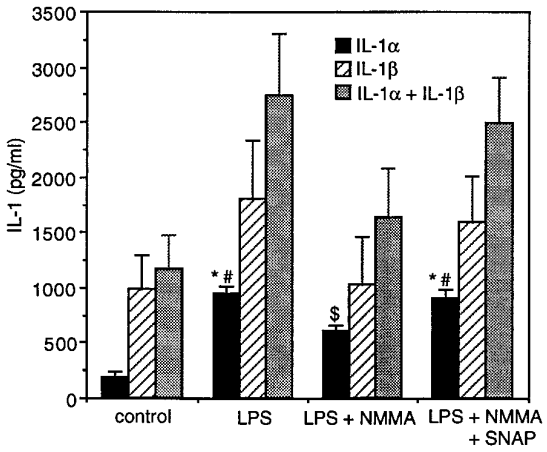

FIG. 6. **Quantitation of IL-1 released from LPS-activated RAW 264.7 cells by ELISA.** RAW 264.7 cells were induced with 1 μg/ml LPS ± 0.25 mM NMMA ± 200 μM SNAP for 24 h. Murine IL-1α and IL-1β ELISA assays were performed on 1:10 and 1:50 dilutions of the cell-free macrophage medium. The results are the averages ± S.E. of six separate experiments containing three replicates per condition. Significant differences to IL-1α control are indicated by * ($p < 0.001$) and by $ ($p < 0.01$), and differences to IL-1α levels of LPS + NMMA samples by # ($p < 0.05$).

8-Bromo-cGMP Reverses NMMA Inhibition of Bioactive IL-1 Release—A cGMP agonist, 8-bromo-cGMP, was used to determine whether NO stimulates IL-1 release by a cGMP-mediated mechanism. Under similar conditions to those shown in Fig. 4, NMMA (0.25 mM) inhibits the release of bioactive IL-1 by LPS-stimulated RAW264.7 cells. 8-Bromo-cGMP, added for the final 3 h of a 24-h incubation, dose-dependently (0.1–1 mM) reversed NMMA inhibition of IL-1 release (Fig. 8) but does not affect control or LPS-stimulated cells. These data suggest that NO may stimulate IL-1 bioactivity release by a cGMP-dependent mechanism.

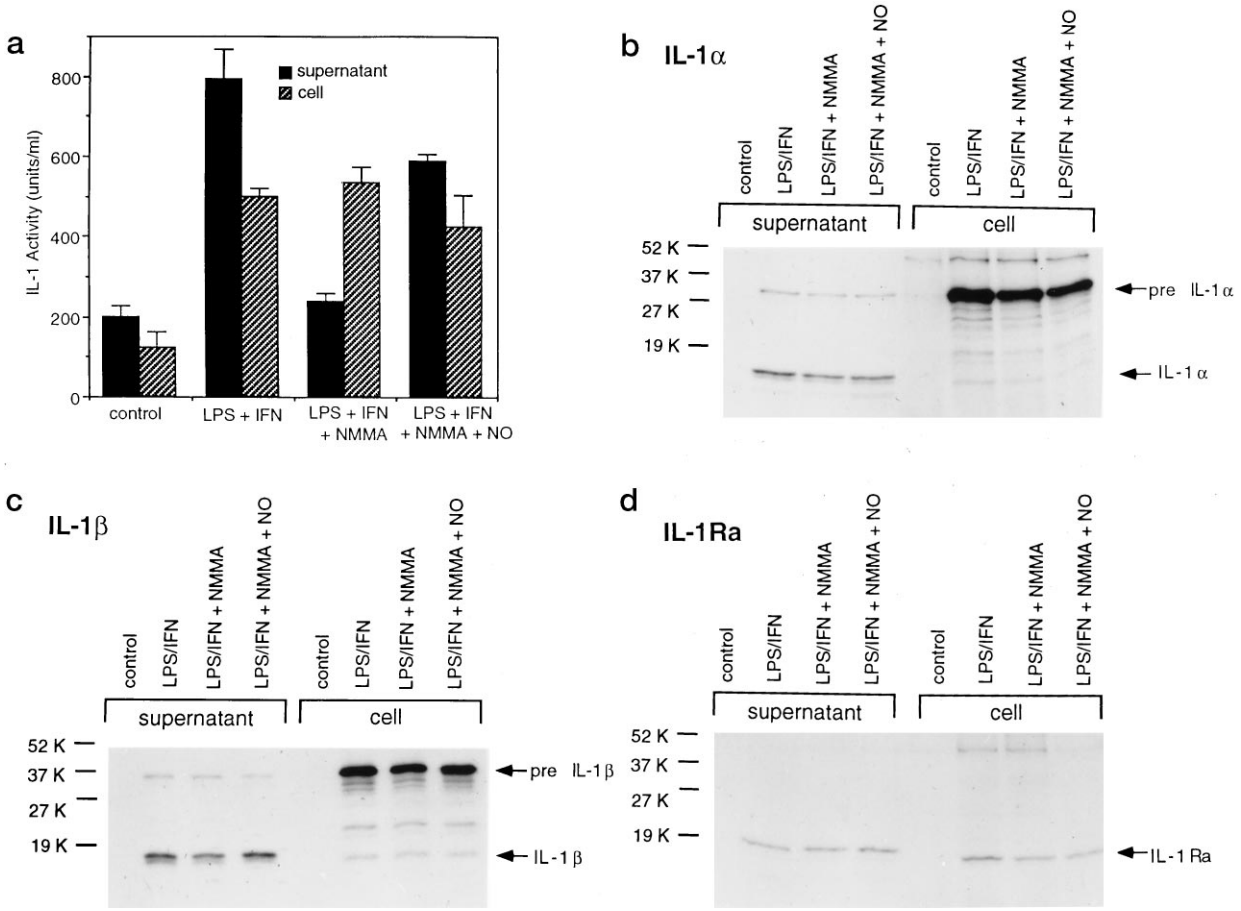

FIG. 7. **Nitric oxide does not affect processing of IL-1.** PECs were incubated for 3 h with 1 μg/ml LPS plus 150 units/ml IFN-γ, 0.25 mM NMMA, and 200 μM SNAP as indicated. [^{35}S]Methionine was added, and the cells were incubated for an additional 4 h. The cells were washed three times to remove label, and NMMA and SNAP were replaced and incubated for a total of 24 h. The medium and cells were collected, and the IL-1 bioactivity (*a*) was measured as described under "Experimental Procedures." The medium and cell fractions were immunoprecipitated with specific antibodies for IL-1α (*b*) IL-1β (*c*), and IL-1Ra (*d*). Immunoprecipitates were separated on 15% SDS-polyacrylamide gels and visualized by fluorography. The results are representative of four to five separate experiments.

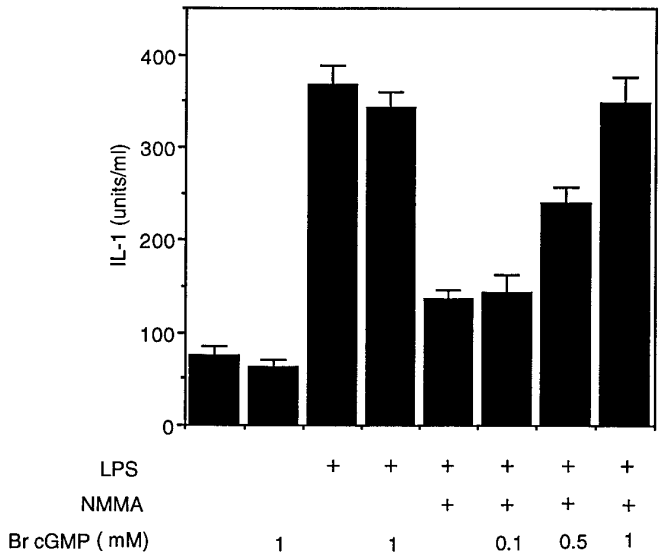

FIG. 8. **8-Bromo-cGMP reconstitutes LPS-stimulated IL-1 bioactivity inhibited by NMMA.** RAW 264.7 cells were induced with 1 μg/ml LPS ± 0.25 mM NMMA for 24 h. 8-Bromo-cGMP was added for the final 3 h of the incubation. Medium levels of IL-1 bioactivity were measured by the RINm5F bioassay. The results are the averages ± S.E. of three separate experiments containing three replicates per condition.

DISCUSSION

Cellular mechanisms have evolved to modulate the deleterious effects of overproduction of interleukin-1 on its target cells during disease states. These processes include control of synthesis and processing of IL-1 (1), neutralization of IL-1 activity by binding to soluble receptors (5) and α_2-macroglobulin (6, 7), and receptor blockade by IL-1Ra (3). In this study we have used a specific IL-1 bioassay (16) to demonstrate that NO regulates IL-1 activity released by activated macrophages, the major *in vivo* source of IL-1.

Studies of the release of bioactive IL-1 from activated macrophages using bioassays based on thymocyte proliferation are difficult to interpret due to cross-reactivity with other cytokines also released by macrophages including tumor necrosis factor α and IL-6 (14, 15). We have recently developed a sensitive IL-1 bioassay based on IL-1-specific induction of NO by the insulinoma cell line, RINm5F (16). RINm5F cells do not cross-react with IL-2, -4, -6, -9, -11, or -15, tumor necrosis factor α, IFNγ, nor LPS. IL-1Ra and antibodies to IL-1α and IL-1β completely block NO production induced by medium derived from LPS-activated RAW 264.7 cells, demonstrating that the measured activity is due solely to IL-1 in the medium (16).

Using this specific assay, we have shown that LPS-activated RAW 264.7 cells and LPS plus IFNγ stimulated murine PECs release bioactive IL-1 in a time-dependent manner that increases to levels almost 10-fold over control levels at 24 h. This increase in release of IL-1 activity is almost completely inhibited in a time- and concentration-dependent manner by the NOS inhibitor, NMMA. Aminoguanidine, a substrate-based inhibitor like NMMA, and iodonium diphenyl, which blocks NADPH and FAD binding sites on NOS (24), also inhibit release of both bioactive IL-1 and NO from RAW 264.7 cells. The ability of these mechanistically unrelated NOS inhibitors to inhibit release of IL-1 bioactivity and the ability of NO donors to reverse this inhibition by NOS inhibitors strongly implicates NO as the mediator responsible for stimulating IL-1 activity.

The mechanism of release of IL-1α and IL-1β, which lack leader sequences and are not secreted via the classic Golgi mechanism, is unknown. Several studies suggest that cell death and membrane permeability are required by some cell types for IL-1 release (25–29). In our system, NO did not cause membrane permeability nor cell death at 24 h as measured by LDH release, trypan blue exclusion, and DNA fragmentation nor did these markers for cell death correlate with IL-1 bioactivity release.

A potential mechanism for NO stimulation of the release of IL-1 activity by murine macrophages is by NO modification of the activity of the cysteine proteases involved in the cleavage of IL-1 precursors. IL-1α and IL-1β are expressed as 33-kDa precursor proteins that are cleaved intracellularly by the cysteine protease, calpain, and IL-1β converting enzyme, respectively. IL-1α is active in both the processed (17 kDa) and precursor (33 kDa) forms, whereas IL-1β is bioactive only as the processed protein. NO readily *S*-nitrosylates intracellular free thiols, which can inhibit or activate affected enzymes (30, 31) and calpain and IL-1β converting enzyme are proteases with essential active-site cysteines that are 10-fold more reactive to labeling than ordinary cellular thiols (13). Recently, Michetti *et al.* (32) demonstrated that *S*-nitrosylation of purified calpain by an NO donor reversibly inactivates proteolytic activity. However, we demonstrate by immunoprecipitation (Fig. 7) that the presence of NO does not affect IL-1α and IL-1β precursor processing in murine PECs nor does NO affect IL-1 protein expression.

These findings suggest that NO does not stimulate the release of IL-1 bioactivity by altering IL-1 expression or precursor processing. Also, NO does not directly alter activity of extracellular IL-1 as demonstrated by lack of effect of NO donors on activity of recombinant IL-1 and of IL-1 derived from activated RAW264.7 media.[2] We are currently investigating whether NO stimulates IL-1 activity by modifying IL-1 intracellularly. A potential mode of action for NO, based on the ability of NO to scavenge superoxide radicals by formation of peroxynitrite (33, 34), is protection of IL-1 and other cellular proteins against superoxide-induced damage. However, exogenous superoxide dismutase and superoxide radicals formed by xanthine and xanthine oxidase have little effect on IL-1 activity released by RAW 264.7 cells,[2] suggesting that the mechanism of NO-stimulated IL-1 release does not involve superoxide or peroxynitrite.

Based on ELISA (Fig. 6), NO appears to stimulate IL-1 release, although due to variability in the assay, only IL-1α levels showed a statistically significant difference. Mechanisms by which NO may modify IL-1 secretion are currently being explored.

Another potential mechanism for NO stimulation of IL-1 activity is by modification of inhibitory factors of IL-1 that are cosecreted from activated macrophages. NO may modify these IL-1 inhibitory factors, which include IL-1Ra, soluble IL-1 receptors, or α_2-macroglobulin (6, 7) by inhibiting production (35, 36), release, or activity of these factors. Immunoprecipitation results (Fig. 7) demonstrate that NO does not affect IL-1Ra production or release, indicating that IL-1Ra does not play a role in NO modification of IL-1 activity.

Nitric oxide stimulates soluble guanylate cyclase (37, 38) and regulates several physiological functions such as smooth muscle relaxation, intestinal ion transport, and platelet function by increasing cellular cGMP levels. 8-Bromo-cGMP, a cGMP agonist, dose-dependently reverses NMMA inhibition of IL-1 release (Fig. 8), suggesting that NO mediates IL-1 release by activation of guanylate cyclase resulting in higher cellular cGMP concentrations. Further studies are in progress to examine the role of cGMP in IL-1 release by activated macrophages.

[2] J. R. Hill, J. A. Corbett, G. Kwon, C. A. Marshall, and M. L. McDaniel, unpublished results.

IL-1 (1) and NO (17, 39) levels are increased in inflammatory disease states such as rheumatoid arthritis, sepsis, atherosclerosis, and asthma. Although the levels of IL-1 protein have been loosely correlated with the severity of disease, the activity of IL-1 is a more essential parameter and cellular mechanisms have evolved to control the activity of IL-1. In this study, we demonstrate that NO stimulates the release of bioactive IL-1 from activated murine macrophages. NO regulation of the activity of IL-1, a key mediator of the immune response, is a potentially important mechanism for control of IL-1 activity *in vivo*.

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Jeanette R. Hill, John A. Corbett, Guim Kwon, Connie A. Marshall and Michael L. McDaniel

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