Regulation of Inducible Nitric Oxide Synthase Expression by Macrophage Purinoreceptors and Calcium*

(Received for publication, October 20, 1995)

Loren C. Denlinger, Philip L. Fisette, Kristen A. Garis, Guim Kwon,
Andres Vazquez-Torres, Andrew D. Simon, Brenda Nguyen, Richard A. Proctor,
Paul J. Bertics, and John A. Corbett

Macrophage activation is central to the progression of multiple diseases via the release of inflammatory mediators such as cytokines and nitric oxide. Despite the recognized overlap in the regulatory mechanisms involved in mediator production, little information exists regarding receptor-initiated signaling pathways that coordinate multiple endpoints, such as tumor necrosis factor-α (TNF-α) and nitric oxide production. In this study, the expression of inducible nitric oxide synthase (iNOS) in macrophages is shown to be regulated by calcium and by a purinoreceptor signaling system. The P2Y purinoreceptor partial agonist, 2-methylthio-ATP (2-MeS-ATP), inhibits the expression of iNOS induced by lipopolysaccharide (LPS) plus interferon-γ (IFN-γ) in primary macrophages. Additionally, 2-MeS-ATP attenuates the expression of iNOS in macrophages isolated from CD-1 mice challenged with LPS, and it inhibits LPS-induced TNF-α and interleukin-1α (IL-1α) release, thereby preventing endotoxic death. Thus, purinoreceptors and calcium are likely to be critical for macrophage activation and the production of inflammatory mediators stimulated by LPS.

The control of macrophage overproduction of inflammatory mediators such as TNF-α, IL-1α, and nitric oxide (NO) should greatly facilitate the treatment of septic shock, rheumatoid arthritis, cerebral malaria, and autoimmune diabetes (1-5). In macrophages, these mediators are regulated primarily at the level of mRNA expression via the involvement of transcription factors such as NF-κB (6-8) and via components that control message stability (9, 10). Given the overlap in the regulatory mechanisms of these genes, it is possible that several receptor-mediated signal transduction pathways exist that can modulate the production of selected mediators in response to an inflammatory stimulus. Several lines of evidence indicate that purinoreceptors may control these regulatory pathways (11-14). For example, we and others have shown that extracellular adenine nucleotides can regulate the circulating levels of TNF-α and IL-1 (12, 14). Specifically, the administration of the ATP analog 2-MeS-ATP to LPS-challenged mice reduces serum levels of TNF-α and IL-1α without affecting the induction of serum levels of IL-6, suggesting that 2-MeS-ATP has an immunomodulatory effect on cytokine production or release (12). This analog also greatly increases the likelihood of survival (>100%) of these mice after an injection of a lethal dose of LPS (12). The effects of 2-MeS-ATP in mice are paralleled by its ability to inhibit a RAW 264.7 macrophage membrane GTPase that is stimulated in the presence of LPS (11, 12). Moreover, previous pharmacologic and kinetic data suggest that the LPS-stimulable GTPase in these membranes is a G-protein associated with a member of the P2 purinoreceptor class (11, 12, 15). This receptor class preferentially binds ATP and ADP but not AMP or adenosine, and many of the P2 purinoreceptors share homology with G-protein-coupled, seven-transmembrane domain receptors (16). Macrophages are known to express P2 purinoreceptors (which preferentially bind AMP and adenosine but not ATP or ADP) as well as several members of the P2 purinoreceptors, namely those from the P2U, P2Y, and P2Z subclasses (17, 18).

We demonstrate here that the LPS-stimulable GTPase in RAW 264.7 macrophage membranes is controlled by P2y purinoreceptor agonists. Earlier studies have shown that this receptor class can mobilize calcium (19), an important intracellular second messenger. Because nitric oxide is an important inflammatory mediator and because extracellular adenosine nucleotides (e.g. 2-MeS-ATP) affect macrophage activation, we have examined the actions of P2y purinoreceptors and calcium on macrophage iNOS expression. We report that calcium can promote LPS-stimulated nitric oxide production and that adenine nucleotides (2-MeS-ATP) can inhibit LPS-induced NO generation by preventing accumulation of iNOS mRNA; these observations represent two previously unidentified pathways regulating iNOS expression.

EXPERIMENTAL PROCEDURES

Cell Culture and Animals—RAW 264.7 cells were obtained from American Type Culture Collection (Rockville, MD) and were grown at 37 °C in a humidified atmosphere containing 5% CO2 using RPMI 1640 medium (BioWhittaker, Walkersville, MD) supplemented with 5% fetal...
The characterization of this GTPase shows that its regulation is P2X receptor involvement. The role of P2Y receptors is also excluded, because adenosine (1–1,000 μM), in the absence of LPS did not stimulate membrane GTPase activity. However, the P2Y receptor partial agonist 2-MeS-ATP dose-dependently blocked the responses to LPS in the presence of ATP or ADP (Table I, Ref. 12, and data not shown). In sum, these results suggest that the LPS-stimulated GTPase activity in RAW 264.7 macrophages is influenced by P2Y-like purinoreceptors.

Ligand-occupied P2Y and other purinoreceptors can modulate G-protein-dependent calcium mobilization (17). Because ADP most potently stimulated the LPS-responsive GTPase in RAW 264.7 cell membranes and has little activity on P2Y and P2Z purinoreceptors, we used this agonist to assess P2Y-like purinoreceptor-induced calcium mobilization in Fura-2-loaded RAW 264.7 macrophages. Indeed, the ratio of Fura-2 fluorescence excited at 340 and 380 nm increased from basal levels of 2.230 ± 0.022 to 2.970 ± 0.160 after RAW cell treatment with 100 μM ADP, a result indicative of increased levels of free calcium due to ADP stimulation of these cells. Of note, previous studies have shown that increased calcium levels can influence various LPS-mediated signal transduction events in this and other systems, including the stimulation of a GTPase in RAW 264.7 macrophages, the phosphorylation of mitogen-activated protein kinases and the translocation of NF-κB (24, 25). Based on these observations, we hypothesized that calcium fluxes are part of the mechanism by which purinoreceptors regulate the production of inflammatory mediators (e.g. TNF-α, IL-1β, NO). Therefore, the role of calcium on LPS- and/or adenosine nucleotide-sensitive end points was assessed, including its effect on NO production. RAW 264.7 cells do not appear to contain a calcium-dependent isoform of NO synthase (Fig. 1 and data not shown); however, the role of calcium in LPS-stimulated expression of iNOS has not been directly investigated. This is particularly relevant because LPS is known to contain large levels of Ca^{2+} and has been reported to mobilize Ca^{2+} in macrophages (26, 27).

To examine the relationship between calcium and LPS or IFN-γ stimulation of nitric oxide production, initial experiments were performed showing that a 20-h incubation of RAW cells with the calcium ionophore A23187 (0.1–10 μM) and LPS had no effect on the stimulation of nitrite production induced by LPS treatment alone. However, when primed with A23187 for 4 h followed by a brief LPS exposure (15 min), RAW 264.7 cells produce 4-fold higher levels of nitrite 20 h later, respectively; these results argue against P2Y and P2Z receptor involvement. The role of P1 purinoreceptors is also excluded, because adenosine (1–1,000 μM), in the presence of bovine serum, 2 mM l-glutamine, 2 mM sodium pyruvate, 100 units/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B. Primary macrophages were obtained by harvesting resident peritoneal exudate cells (PEC) from 5–6-week-old male CD-1 mice, isolated by adherence after red cell lysis (20). These cells were maintained in complete CMRL 1066 (Life Technologies, Inc.) medium supplemented with 10% fetal bovine serum, 2 mM l-glutamine and antibiotics. Phenol-extracted E. coli 0111:B4 was used in experiments containing endotoxin.

**GTPase Activity—Plasma membranes were prepared from RAW 264.7 cells by lysis and differential centrifugation (15). GTPase activity was assayed for 5 min at 30 °C in the presence of 2 μg of membrane protein, 10 μM adenosine nucleotide, 2 μM [γ-32P]GTP, and 5 mM MgCl₂ as described previously (11, 12, 15).**

Intracellular Free Calcium Measurements—RAW 264.7 cells were plated on 1-cm² glass coverslips, allowed to adhere for 1 h at 37 °C in 80 μl of supplemented RPMI 1640, and the coverslips were then incubated overnight in 24-well plates containing an additional 1 ml of medium. Loading of the cells with the fluorescent calcium indicator dye Fura-2AM was initiated by replacing the medium with 1 ml of supplemented RPMI 1640 containing 1 μM Fura-2AM (Molecular Probes, Eugene, OR). 0.02% pluronic detergent (Molecular Probes), and 0.5 mM sulfipyrazone to prevent dye efflux (21). After 20 min of incubation at 37 °C, the medium was replaced with 1 ml of supplemented RPMI 1640 containing only 0.5 mM sulfipyrazone for an additional 20-min incubation. The coverslips were then washed and placed in a cuvette containing 900 μl of a saline solution containing 125 mM NaCl, 5 mM KCl, 1 mM MgSO₄, 1 mM KH₂PO₄, 1.5 mM CaCl₂, 5 mM glucose, 10 mM NaHCO₃, 20 mM HEPES, and 0.5 mM sulfipyrazone at 25 °C and pH 7.4. Fura-2 fluorescence was monitored with continuous stirring for 10 min in a Hitachi model F2000 fluorescence spectrophotometer using excitation wavelengths of 340 and 380 nm and collecting at the emission wavelength of 510 nm. Each measurement was initiated by the addition of 100 μl of saline containing various nucleotides resulting in the indicated final concentrations.

**Nitrergic Oxide Detection—NO production was measured by assessing the nitrite content of 100 μl of tissue culture supernatant using the Griess reagent and reading the absorbance at 550 nm (22). Conversion of nitrite to nitrate was performed at 37 °C using 0.5 mg/ml nitrate reductase and 0.006 unit/ml l-glutamyl dehydrogenase (Sigma) (23).**

**iNOS Northern Blotting—PEC (4 × 10⁶ cells/2.5 ml of medium) were stimulated with LPS (1 μg/ml, IFN-γ (150 units/ml)) and 2-MeS-ATP for 6 h. Total RNA was isolated and probed for cyclophilin and mouse macrophage iNOS by Northern blot analysis. A cDNA probe for murine iNOS was raised and was prepared by reverse transcription-polymerase chain reaction as described previously (22).**

**iNOS Protein Immunoprecipitation and Western Analysis—PEC were incubated for 5 h in methionine-deficient medium in the presence of LPS (1 μg/ml), IFN-γ (150 units/ml), and 2-MeS-ATP. [35S]Methionine (300 μCi) was added, and the cells were incubated for an additional 30 min. The cells were harvested, and iNOS was immunoprecipitated using rabbit antiserum specific for mouse macrophage iNOS. This antiserum was prepared against a C-terminal 27 amino acid peptide from iNOS as described previously (22). The immunoprecipitate was then separated by SDS-polyacrylamide gel electrophoresis followed by visualization using autoradiography. Western analysis of unlabelled cells was performed using a 12,000 dilution of the primary anti-iNOS antibody and ECL reagents (Amersham Corp.) for detection.
relative to that seen in untreated cells (Fig. 1). Unprimed RAW cells stimulated with LPS did not produce nitrite nor did macrophages treated with ionophore alone (Fig. 1). Furthermore, RAW cells treated first with LPS for 15 min followed by 4 h of ionophore exposure also did not produce nitrite (data not shown). These observations are consistent with data showing that simultaneous treatment of RAW cells with LPS and 5 mM EGTA or 30 nmLa³⁺, respectively, produced 70 and 95% inhibition of the amount nitrite measured 18 h later. Similar studies by others show that either simultaneous treatment with LPS and Cd³⁺ or pretreatment with dihydropyridine-type calcium channel blockers followed by LPS stimulation of macrophages can inhibit LPS-induced nitrite production by these cells (23, 28).

We also examined the role of calcium in IFN-γ plus LPS-induced nitrite formation by RAW cells using the same experimental design (Fig. 1). Although 15 min of stimulation with LPS plus IFN-γ is sufficient to induce the production of nitrite by RAW cells, the level of nitrite produced is enhanced 2-fold when the cells are primed with A23187. As is the case for LPS induction, ionophore treatment after the LPS plus IFN-γ stimulus does not produce additional nitrite (data not shown). Following pretreatment with a different calcium ionophore, ionomycin (1 μM), we also observed a 2-fold enhancement of the amount of nitrite produced from LPS plus IFN-γ-stimulated RAW cells relative to similar controls. Ionomophore (A23187) pretreatment followed by an IFN-γ stimulation for 15 min does not induce nitrite formation by RAW cells indicating that ionophore pretreatment cannot mimic the effects of LPS on iNOS expression (Fig. 1). Together, these experiments and the data in Fig. 1 demonstrate a calcium-dependent phase of nitrite production by RAW cells. Additionally, we have observed a lack of stimulation of nitrite production by ionophore alone (Fig. 1), a 9-h lag in ionophore-enhanced nitrite formation after LPS stimulation (data not shown), and the ability of cycloheximide (2.5 μg/ml) to exert >95% inhibition of ionophore-pretreated, LPS-induced nitrite generation. These results implicate a calcium-sensitive factor as an important regulatory molecule for the expression of iNOS by RAW cells.

To assess whether LPS-induced nitric oxide production is regulated by purinoreceptors, we performed initial experiments with RAW cells looking at the ability of adenine nucleotides to stimulate nitrite production in the presence and absence of LPS. These experiments revealed that various adenine nucleotides synergize with low levels of LPS to generate nitrite.

These data have now been confirmed by Tonetti et al. (29), and even though UTP was shown to weakly synergize with LPS in terms of nitrite production, their findings extend this observation to suggest that the P₂x purinoreceptor class on RAW cells is likely to be responsible for this effect. In the present study, we demonstrate that the P₂x purinoreceptor partial agonist...
2-MeS-ATP also influences LPS-stimulated nitrite production in RAW cells and in primary mouse macrophages. As shown in Fig. 2A, concentrations of 2-MeS-ATP of 100 μM and above inhibit LPS-induced nitrite formation in RAW cells by 60% at 0.1 μg of LPS/ml. At higher concentrations of LPS, 2-MeS-ATP was less effective at inhibiting RAW cell production of nitrite. However, in mouse peritoneal macrophages, 2-MeS-ATP inhibits LPS plus IFN-γ-induced nitrite and total nitric oxide production (nitrite plus nitrate) in a concentration-dependent fashion, i.e., inhibition was detectable at 100 μM, and complete inhibition was observed at a concentration of 1 μM 2-MeS-ATP (Fig. 2B). In comparison with the transformed RAW 264.7 cells, the observation that primary macrophages are more responsive to 2-MeS-ATP inhibition of LPS-induced nitric oxide production is consistent with our in vitro and in vivo studies examining the antagonistic effects of 2-MeS-ATP on LPS-stimulated TNF-α production (11, 12). The inhibitory effects of 2-MeS-ATP on nitric oxide production by primary macrophages appears to be specific for LPS signaling events, as 2-MeS-ATP does not inhibit nitrite formation induced by treatment of macrophages with TNF-α and IFN-γ (Fig. 2C). The fact that nitrite production by TNF-α requires IFN-γ and is not affected by IL-1 (data not shown), together with the observation that 2-MeS-ATP inhibits LPS plus IFN-γ-stimulated but not TNF-α plus IFN-γ-induced nitrite generation, suggest that LPS stimulates nitrite production independently of TNF-α and IL-1 generation. Additionally, in a system unrelated to nitric oxide production, 2-MeS-ATP treatment does not affect LPS-induced procoagulant activity in macrophages, suggesting that the inhibitory effects of this adenine nucleotide on LPS-stimulated TNF-α, IL-1, and nitric oxide production are not via a cytotoxic mechanism.

2-MeS-ATP appears to inhibit nitric oxide production by primary mouse macrophages at the level of iNOS gene expression. As shown in Fig. 3A, 2-MeS-ATP does not significantly inhibit the enzymatic activity of iNOS, examined by incubating LPS plus IFN-γ-pretreated primary macrophages for 3 h with this adenine nucleotide. In contrast, an iNOS enzymatic inhibitor, aminoguanidine (30), inhibits nitrite formation in this whole cell assay system. Fig. 3, B and C, demonstrates that 2-MeS-ATP attenuates LPS plus IFN-γ-stimulated induction of iNOS protein and mRNA in a concentration-dependent manner, similar to its effects on nitric oxide production.

2-MeS-ATP also attenuates macrophage expression of iNOS induced by intraperitoneal injections of LPS into CD-1 mice (Fig. 3D). LPS induction in vivo causes a 2.5-fold increase in the amount of nitrite found ex vivo relative to the unstimulated

\[ \text{B. S. Schwartz (University of Wisconsin), personal communication.} \]
Purinoreceptors Regulate iNOS Expression

Fig. 4. ADP- and 2-MeS-ATP-induced calcium mobilization in RAW 264.7 cells. 5 × 10⁴ RAW cells were plated on glass coverslips and loaded with Fura-2 as described under "Experimental Procedures." After obtaining a basal ratio of Fura-2 fluorescence excited at 340 and 380 nm (2.285 ± 0.090 for the entire experiment), adenine nucleotides were added alone or simultaneously at the indicated concentrations. Changes in Fura-2 fluorescence were monitored for 9 min followed by stimulation with 5 mM ionomycin as a positive control. Data represent the mean change in fluorescence and the standard deviation after the basal 340/380 ratio for each individual tracing is subtracted from the peak and averaged with data from two other coverslips. A representative experiment is shown.

control. 2-MeS-ATP treatment in vivo causes a 50% reduction of nitrite levels and iNOS protein associated with these primary macrophages (Fig. 3D, inset). The inhibition of nitrite production by aminoguanidine treatment of the induced macrophages in vitro shows that nitrite production occurs during the in vivo incubation. These data indicate that 2-MeS-ATP is also effective at inhibiting the expression of iNOS induced by LPS under in vivo conditions.

To address whether calcium mobilization is part of the mechanism by which purinoreceptors regulate LPS-induced inflammatory mediator production, 2-MeS-ATP was evaluated for its ability to affect calcium-sensitive Fura-2 fluorescence in RAW cells treated with and without ADP (Fig. 4). Consistent with its well-characterized partial agonist activities in other systems (31), 2-MeS-ATP treatment alone mediated a smaller increase in free calcium levels than did ADP alone, as assessed by the ratio of Fura-2 fluorescence excited at 340 and 380 nm. However, under these conditions, 2-MeS-ATP was unable to block the increase in free calcium levels stimulated by ADP treatment of RAW 264.7 cells; rather, the effect appears to be additive (Fig. 4). These results suggest several possibilities. For example, the inhibitory effects of 2-MeS-ATP on inflammatory mediator production may be through another P₂Y-like or other purinoreceptor class that signals via a mechanism independent of calcium. In this case, ADP stimulation of P₂Y-purinoreceptors may cause calcium level increases that enhance iNOS expression. The occupation of another purinoreceptor class by 2-MeS-ATP could then generate a calcium-independent signal that overrides calcium-dependent effects initiated by ADP-bound P₂Y purinoreceptors. In addition, the additive increase in free calcium by the combination of ADP and 2-MeS-ATP may be cytostatic or cytotoxic to macrophages. However, several observations argue against this possibility, including the enhancement of LPS-induced RAW cell nitric oxide production by calcium ionophore pretreatment, the lack of inhibition by 2-MeS-ATP of LPS-stimulated procoagulant activity in RAW cells² and TNF-α plus IFN-γ generated nitrite in peritoneal macrophages, and trypan blue exclusion studies which indicate that cell viability is unaffected by 2-MeS-ATP. Alternatively, the additive increase in free calcium levels due to ADP and 2-MeS-ATP treatments could be selectively antagonistic to the LPS-stimulated pathway, but the experiments with ionophore pretreatment of RAW cells suggest this is not likely, since the large calcium flux expected with this treatment was not inhibitory to LPS-induced nitrite production.

This study demonstrates that 2-MeS-ATP inhibits LPS-induced iNOS gene expression in vitro and in vivo. Previous findings have shown that this nucleotide can dramatically reduce serum levels of TNF-α and IL-1α in LPS-challenged mice, without affecting LPS-induced IL-6 serum levels in these mice or procoagulant activity in LPS-stimulated macrophages. The prevention of endotoxic death suggests that 2-MeS-ATP has an important immunomodulatory effect on the inflammatory process (12). Our results, in combination with observations that other adenine nucleotides can enhance LPS-stimulated nitrite formation in RAW 264.7 cells (29) and LPS-induced IL-1 production in mice (14), suggest that macrophage purinoreceptors control a signal transduction pathway that intersects with LPS signaling. Because this study demonstrates that calcium is important for iNOS expression, one pathway that can be suggested is that ligand occupation of G-protein-coupled purinoreceptors results in calcium mobilization, ultimately modulating LPS-stimulated iNOS expression by affecting one of the multiple calcium-dependent factors that can be involved in initiating iNOS transcription. 2-MeS-ATP in this model may serve to bound, but not activate, a P₂Y-like purinoreceptor, thereby blocking stimulation of this receptor class by ATP or ADP, but not preventing the ability of ATP or ADP to signal through other receptor classes. As noted above, the role of calcium in the inhibitory mechanism of 2-MeS-ATP action remains to be more fully characterized; however, a role for calcium in iNOS synthesis, and possibly in TNF-α and IL-1 expression (11, 12), is suggested by these data and observations by others showing that calcium channel blockers can prevent sepsis in rats (32).

Because multiple receptor-mediated pathways mobilize calcium in macrophages (e.g. complement, Fcy, and platelet-activating factor receptors, all of which are stimulated during sepsis (1, 18)), the enhancement of mediator expression via elevated calcium is a mechanism that is not exclusive to purinoreceptors. However, purinoreceptors play an important role in the inflammatory response (18). During infection and/or stress, large levels of extracellular ATP and ADP are released locally from cellular damage and from platelet activation, as well as systemically from the adrenal gland (18, 33). Additionally, many cell types have been shown to release ATP through cell surface proteins containing an ATP-binding cassette motif, resulting in autocrine stimulation of purinoreceptors (17, 34). This raises the possibility that LPS may cause ATP release from stimulated macrophages which could then activate purinoreceptors. Regardless of the source of adenine nucleotides, the stimulation of macrophages by ATP, ADP, or other adenine nucleotides present at the inflammatory site and the functional inhibition by 2-MeS-ATP with respect to LPS-induced TNF-α, IL-1, and nitric oxide production strongly indicate that purinoreceptors can control signal transduction pathways that are critically important to macrophage activation.

Acknowledgments—We thank Dr. James Dahlberg, Dr. Paul Lacy, and Dr. Michael McDaniel for their useful discussions and suggestions. We appreciate the technical support and comments provided by Dr. Greg Wiepz, Dorothy Brar, and David Bledorn. We also acknowledge Drs. Ralph Albrecht, Donna Paulnock, Arnold Ruoho, Frank Siegel, Bradford Schwartz, and Erik Forsberg for their helpful suggestions.

REFERENCES

1. Casey, L. C., Balk, R. A., and Bone, R. C. (1993) Ann. Intern. Med. 119, 771–778
2. MadMicking, J. D., Nathan, C., Hom, G., Chartrain, N., Fletcher, D. S., Trumbauer, M., Stevens, M. X., Xie, D., Sokol, K. K., Hutchinson, N., Chen, H., and Mudgett, J. S. (1995) Cell 81, 641–650
3. Roubenoff, R., Roubenoff, R. A., Cannon, J. G., Keyhavias, J. J., Zhuang, H., Dawson-Hughes, B., Dinhara, C. A., and Rosenberg, I. H. (1994) J. Clin. Invest. 93, 2379–2386
4. Kwiatkowski, D., Hill, A. V., Sambou, I., Twumasi, P., Castracane, J.,
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J. Biol. Chem. 1996, 271:337-342.
doi: 10.1074/jbc.271.1.337

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