Purinergic Receptor Modulation of Lipopolysaccharide Signaling and Inducible Nitric-oxide Synthase Expression in RAW 264.7 Macrophages*

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Previous studies have suggested that the P2Z/P2X7 purinergic receptor can participate in nucleotide-induced modulation of lipopolysaccharide (LPS) stimulated inflammatory mediator production. To test this hypothesis, we evaluated whether antagonism of the P2Z/P2X7 receptor can influence LPS signaling and expression of the inducible form of nitric-oxide synthase (iNOS) in RAW 264.7 macrophages. In the present study, we demonstrate that pretreatment of RAW 264.7 macrophages with a P2Z/P2X7 receptor antagonist, periodate oxidized adenosine 5'-triphosphate (o-ATP), substantially inhibits LPS-stimulated NO production and iNOS expression without altering cell viability. This effect on LPS-induced iNOS expression is mimicked by a pyridoxal-phosphate-based antagonist (pyridoxal-phosphate-6-azophenyl-2',4'-disulfonic acid) of the P2Z/P2X7 purinergic receptor, indicating that these results are not unique to o-ATP. Additionally, o-ATP prevents cell death induced by P2Z/P2X7 receptor agonists. To ascertain how P2Z/P2X7 receptor antagonists influence LPS signaling, we evaluated the capacity of o-ATP to regulate LPS-mediated activation of the transcription factor, nuclear factor-kB, and the mitogen-activated protein kinases, extracellular signal-regulated kinase (ERK) 1 and ERK2. These experiments reveal that pretreatment of RAW 264.7 cells with o-ATP attenuates the LPS stimulation of a nuclear factor-kB-like binding activity. Moreover, the activation of ERK1 and ERK2 by LPS, but not by the phorbol ester, phorbol 12-myristate 13-acetate, is also blocked in RAW 264.7 cells by o-ATP pretreatment. In summary, these data suggest that the P2Z/P2X7 receptor modulates LPS-induced macrophage activation as assessed by iNOS expression and NO production. This report implicates the P2Z/P2X7 receptor in the control of protein kinase cascades and transcriptional processes, and these observations are likely to be important for the development of selective purinergic receptor antagonists for the treatment of septic shock.

Macrophage activation by Gram-negative bacterial endotoxin (lipopolysaccharide; LPS) 1 (1) is central to the mammalian inflammatory response to this agent and also has been implicated in endotoxin tolerance (1, 2). Whether an LPS-stimulated macrophage produces a relative surplus of inflammatory or anti-inflammatory mediators is likely to depend on its differentiation state (2). However, little is known about these transitions, and this is reflected by the clinical inability to distinguish between the inflammatory and the immunosuppressive states of septic shock (3). Thus, information regarding the signaling pathways involved in macrophage activation and differentiation could lead to a better understanding of sepsis as well as to the development of therapeutic agents.

Recently, the expression of P2 purinergic receptors, which bind extracellular adenine nucleotides possessing two or more phosphates, has been shown to serve as a marker of macrophage differentiation in response to LPS and various cytokines (4). The P2X subfamily contains at least seven distinct genes that encode proteins with two transmembrane domains that appear to function as multimeric receptors exhibiting ion channel activity (5–8). In addition, the P2Y subfamily has been reported to contain five to seven distinct genes encoding receptors with seven transmembrane domains that are coupled to heterotrimeric G proteins (5, 9). Unstimulated monocytes express relatively high levels of P2Y2 receptor mRNA but low levels of the P2X7 purinergic receptor message (10, 11). In response to LPS, monocyte P2Y2 receptor expression is downregulated with a concomitant induction of P2X7 receptor mRNA levels (10, 11).

The P2X7 receptor in mature macrophages may be the P2X receptor of former classification schemes (8), and it is intrigu-
LPS-induced interleukin-1 that regulates macrophage post-translational processing of tor is also thought to control a signal transduction pathway continuous or repeated application of agonist (14). The P2Z receptor is designated as a P2Z receptor function (8, 12, 13). The P2Z-indirectly generates a pore activity that has been formerly of less than 900 Da in size, which is an activity linked to anion leakage (16, 17). Furthermore, exogenous adenine nucleotides that can interact with various purinergic receptors can synergize with LPS to increase steady state levels of tumor necrosis factor-α and the inducible form of nitric-oxide synthase (iNOS) mRNA (18). These data are consistent with the concept that the P2Z/P2X7 receptor controls a signaling pathway(s) that can influence LPS action distinct from those involved in cell death. In fact, the macrophage signaling events leading to LPS-induced nitric oxide (NO) production may be one such pathway (19).

In terms of the role by which adenine nucleotide may influence LPS-stimulated inflammatory mediator production, LPS-treatment of macrophages is known to release endogenous ATP for subsequent autocrine stimulation (13). Moreover, exogenous ATP and other adenine nucleotides synergize with LPS to induce tumor necrosis factor-α and NO production by the mouse macrophage cell line RAW 264.7 (18), whereas treatment of isolated mouse peritoneal macrophages with the purinergic receptor ligand 2-methylthio-ATP (2-MeS-ATP) profoundly inhibits LPS-stimulated NO generation (19). In addition, the coadministration of 2-MeS-ATP with LPS protects mice from endotoxic death (20). However, because 2-MeS-ATP interacts with multiple P2Y and P2X receptors, including P2Z/P2X7 (5, 8), the molecular mechanism(s) by which ATP and 2-MeS-ATP bring about their divergent responses is unclear but may be due to the differential expression of selected purinergic receptors at various stages of monocyte/macrophage differentiation. Based on these observations along with the finding that there is preferential expression of P2Z/P2X7 receptors by macrophages after LPS-stimulation (11), the goal of the present study was to evaluate the role of this receptor activity in LPS signaling events and NO production.

EXPERIMENTAL PROCEDURES

Materials—RPMI 1640 culture medium, l-glutamine, and sodium pyruvate were all products of Bio Whittaker (Walkersville, MD), whereas chicken serum was obtained from Hyclone (Logan, UT). All antibiotics, ATP, periodate oxidized ATP (o-ATP), 2'- and 3'-O-(4-benzoylbenzoyl) (Bz)-ATP, ATP-γ-S, lipopolysaccharide from Escherichia coli serotype 0111:B4, porcine 12-myristate 13-acetate (PMA), N-1-naphthylthelylenediamine-HCl, sulfanilamide, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), SDS, and 4,4′-diaminobenzidine-HCl were all products of Sigma. The source of 2-methylthio (2-MeS)-2,5-diphenyltetrazolium bromide (MTT), SDS, and 4,4′-diaminobenzidine-HCl were all obtained Sigma. The source of 2-MeS-(2-MeS)-M was blocked overnight with 5% dry milk in Tris-buffered saline with 0.25% Tween 20 (TBST) at 4 °C. The levels of iNOS protein were detected using a 1:4000 dilution of rabbit polyclonal anti-murine iNOS antibody, followed by incubation with a 1:5000 dilution of horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody. The membrane was rinsed with TBST between all hybridizations, and the secondary antibody was visualized using LumiGLO® substrates and luminography.

P2Z′/P2X7 Receptor Effects on LPS Signaling

To determine the level of ERK1/ERK2 (MAP kinase) activation, 5 × 10^6 RAW 264.7 macrophages were plated in six-well culture dishes for 18–24 h prior to treatment. The cells were pretreated for 2 h with either 1 ml of medium alone or o-ATP (250 or 500 μM) and then washed twice with fresh medium. The cells were washed once with lactated Ringer's buffer, washed twice with fresh medium, and incubated at 37 °C for 10 min. The cells were washed once with lactated Ringer's buffer, washed twice with fresh medium, and incubated for 10 min in the dark, as described previously (19). Nitrite concentrations were determined by measuring the absorbance at 595 nm using 50 μl of cell lysate.

Immunoblotting of iNOS and Active MAP Kinases (ERK1/ERK2)—For the determination of iNOS protein expression, RAW 264.7 macrophages were plated at 5 × 10^5 cells/well in 96-well culture dishes and were incubated for 18–24 h before treatment. After pretreatment for 2 h with 100 μM of control FRET (at the indicated concentrations), the medium was removed, and these cells were washed twice with fresh medium prior to the addition of 100 μl of either medium alone or Escherichia coli LPS (10–1000 ng/ml). After the cells were treated for 20 h at 37 °C, 100 μl of the Griess reagent (a 1:1 mix of 0.1% N-1-naphthylethylenediamine-HCl and 1.0% sulfanilamide in 60% acetic acid) were added to each well, and incubated for 10 min in the dark, as described previously (19). Nitrite concentrations were determined by measurement of the optical density at 550 nm. Measurement of Cell Viability—Cell viability was determined by trypan blue exclusion and MTT cytotoxicity assays (22). RAW 264.7 macrophages were treated in a manner similar to that described for the NO assay, with or without 2-h o-ATP pretreatments, followed by 20 h of treatment with either medium alone, LPS, or adenine nucleotides. The cells were washed twice with fresh medium at the end of the treatments, before the addition of either trypan blue or MTT. In the MTT assays, the cells were incubated with 110 μl of 1 mg/ml MTT for 2 h at 37 °C, 5% CO2, followed by an overnight incubation with 100 μl of a solution containing 20% (w/v) SDS and 50% N,N-dimethylformamide. The level of cell viability was determined by measuring the absorbance at 595 nm using 50 μl of cell lysate.
cells were harvested in phosphate-buffered saline containing 2% serum, washed twice with phosphate-buffered saline, 2% serum, and resuspended in 400 μl of buffer A (10 mM Tris, pH 7.8, 5 mM MgCl₂, 10 mM KCl, 0.3 mM EGTA, 0.3 mM sucrose, 10 mM β-glycerol phosphate, 1 mM phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol, 1 μg/ml aprotinin, and 1 μg/ml leupeptin). After the cells were incubated on ice for 15 min and then lysed by the addition of 0.5% Nonidet P-40, the nuclei were harvested by centrifugation at 7,200 × g for 10 s at 4 °C. The resulting pellets were resuspended in 100 μl of buffer B (20 mM Tris, pH 7.8, 5 mM MgCl₂, 320 mM KCl, 0.2 mM EGTA, 0.5 mM dithiothreitol, 1 μg/ml aprotinin, and 1 μg/ml leupeptin), incubated on ice for 15 min, and cleared by centrifugation at 13,500 × g for 15 min at 4 °C. Protein concentration was estimated by the micro-BCA assay (Pierce).

The NF-κB binding protein detection system (Life Technologies, Inc.) was used according to the manufacturer's instructions. Briefly, end labeling of a double-stranded oligonucleotide containing two NF-κB-binding elements (5′-GATCCAAAGGGCTTCCATGGATCCAAGGG-GACTTTCGATG-3′) was performed using [γ-³²P]ATP and T4 polynucleotide kinase. Nuclear extracts (5 μg of protein) were incubated with 1 × 10⁵ cpm of the labeled oligonucleotide at room temperature for 20 min. The samples were separated on a 6% nondenaturing polyacrylamide gel. Gels were dried and exposed to x-ray film.

RESULTS

The Effects of o-ATP on LPS-induced NO Production and iNOS Expression in RAW 264.7 Macrophages—To determine whether the P2Z/P2X₇ purinergic receptor participates in pathways important to LPS signaling, we examined whether agonists selective for P2X receptors can influence LPS-stimulated macrophage inflammatory mediator production. In this regard, NO is known to be produced in response to LPS, and its generation by LPS-treated macrophages has been shown to be modulated by 2-MeS-ATP (19), which is an agent that can bind to multiple P2X and P2Y purinergic receptors (5). To more specifically ascertain the role of P2Z/P2X₇ receptors in this process, we pretreated RAW 264.7 macrophages for 2 h with either 0, 250, or 500 μM o-ATP, which is an irreversible P2Z/P2X₇ inhibitor (23). These cells were then washed, treated with 0, 10, 100 or 1000 ng/ml E. coli LPS for 20 h, and samples of the culture medium were measured for nitrite, a stable metabolite of NO. As shown in Fig. 1, pretreatment of RAW 264.7 cells with increasing concentrations of o-ATP promoted a dose-dependent inhibition in the amount of nitrite produced by these cells in response to LPS. To evaluate whether this effect is due to an inhibition of iNOS expression or activity, we monitored LPS-induced iNOS levels using immunoblotting. The data in Fig. 2 reveal that o-ATP pretreatment reduces the capacity of LPS to stimulate iNOS expression.

The Influence of o-ATP on RAW 264.7 Cell Viability—Given the observation that o-ATP can substantially inhibit iNOS expression and NO production, it was important to determine whether this effect reflected a specific regulation of the inflammatory capacity of the cell or was a result of a nonselective cytotoxic effect. In this respect, several lines of evidence suggest that the effects of o-ATP are not due to altered cell viability. For example, the production of LPS-induced NO after o-ATP pretreatment of RAW 264.7 cells can be partially restored by the presence of interferon-γ during the LPS-stimulation period (data not shown). Second, treatment of cells with o-ATP in the presence or absence of LPS was not toxic to these cells as determined by parallel experiments using the trypan blue exclusion assay (i.e., these cells were greater than 99% viable following all treatments). In addition to these observations and as an additional test of cell viability, we measured the metabolic activity of RAW 264.7 cells following o-ATP pretreatment using an MTT dye-reduction assay (24). As shown in Fig. 3A, o-ATP in the presence or absence of LPS had no measurable effect on RAW 264.7 cell metabolic activity/viability.

The Effect of o-ATP on P2Z/P2X₇ Agonist-mediated Cell Death—Although a known P2Z/P2X₇ receptor antagonist (o-ATP) does not alter cell viability (Fig. 3A), it has been reported that agonists of the P2Z/P2X₇ receptor can mediate cell death in macrophages and other cell types via the stimulation of a pore-forming activity associated with this receptor (14). To demonstrate that RAW 264.7 cells express a P2Z/P2X₇-associated pore, these cells were stimulated with the selective P2Z/P2X₇ agonist, Bz-ATP, as well as with ATP, ATPγS, and 2-MeS-ATP, which are capable of interacting with multiple P2 purinergic receptors. Cell viability was then measured by MTT dye reduction, and as shown in Fig. 3B, Bz-ATP, ATP, ATPγS, and 2-MeS-ATP all caused a decrease in cell viability after 20 h of agonist treatment. In contrast, ATP, a ligand normally present during inflammatory reactions, was less able to kill RAW 264.7 cells at concentrations as high as 1 mM (Fig. 3B). The lower activity of ATP in this system compared with the other ATP analogs is likely due to its more rapid hydrolysis by the high levels of ecto-ATPases that are present on macrophages (25).

To assess whether Bz-ATP, 2-MeS-ATP, and ATPγS were promoting the loss of cell viability via the P2Z/P2X₇ receptor, we pretreated RAW 264.7 cells with 0, 250, and 500 μM o-ATP for 2 h, followed by washing and resimulation with various adenine nucleotides for an additional 20 h. When cell viability was measured by the MTT assay (Fig. 3B), pretreatment with o-ATP was found to prevent cytotoxicity caused by subsequent treatments with Bz-ATP, 2-MeS-ATP, or ATPγS (Fig. 3B). In addition to these pharmacological data, we have confirmed that unstimulated RAW 264.7 cells, a cell line derived from peritoneal macrophages (26), express mRNA encoding the P2X₇ receptor using reverse transcription-polymerase chain reaction
RAW 264.7 macrophages stimulated with LPS or P2Z/P2X7 agonists. A, RAW 264.7 macrophages (5 x 10^4 cells/treatment) were incubated with either control medium or 250 or 500 µM o-ATP for 2 h. These cells were then exposed for 20 h to the indicated concentrations of LPS or distilled water. Following this treatment, the cells were then incubated with 1 mg/ml MTT for 2 h and solubilized overnight, and their viability was determined by measuring the absorbance at 595 nm as described under “Experimental Procedures.” The data are presented as the mean ± S.E. of triplicate measurements, and similar results were obtained in three individual experiments. B, RAW 264.7 macrophages (5 x 10^4 cells/treatment) were incubated with either control medium or 250 or 500 µM o-ATP for 2 h. The cells were then incubated for 20 h with either medium, 1 mM ATP, 1 mM ATP_S, 1 mM 2-MeS-ATP, or 500 µM Bz-ATP. Cell viability was assessed by the MTT assay as described under “Experimental Procedures.” The results presented are the average ± S.E. of triplicate measurements.

(data not shown). Taken together, these data demonstrate that unstimulated RAW 264.7 cells express functional P2Z/P2X7 receptors and that their inhibition by o-ATP can block agonist-induced cytotoxicity and LPS-stimulated NO production.

The Action of PPADS on LPS-stimulated iNOS Expression and Bz-ATP-mediated Cell Death—Although o-ATP has been reported to be a selective antagonist of the P2Z/P2X7 receptor, it is conceivable that o-ATP may block LPS action via a non-P2X7 receptor mechanism(s). Therefore, to assess whether P2X receptors are critical to LPS action and to confirm the specificity of o-ATP, we explored in this system the biological effects of PPADS, which is a structurally unrelated to o-ATP. As shown in Fig. 4A, co-administration of increasing concentrations of PPADS elicited a dose-dependent inhibition of LPS-induced iNOS expression in RAW 264.7 cells. In terms of cell viability, concentrations of PPADS up to 1000 µM were not toxic to these cells and could block the cytotoxicity induced by 500 µM of Bz-ATP (Fig. 4B). In sum, these data provide additional evidence that two distinct P2Z/P2X7 purinergic receptor antagonists can block agonist-induced cell death and LPS-stimulated iNOS expression.

The Influence of o-ATP Pretreatment on LPS-induced NF-κB Activation in RAW 264.7 Macrophages—Although NF-κB is important for LPS action, other signaling pathways activated by LPS also appear to be necessary for NO production and endotoxicity (21). Therefore, it was of interest to explore...

![Figure 3](image3.png)

**Fig. 3.** The effect of o-ATP pretreatment on the viability of RAW 264.7 macrophages stimulated with LPS or P2Z/P2X7 agonists. A, RAW 264.7 macrophages (5 x 10^4 cells/treatment) were incubated with either control medium or 250 or 500 µM o-ATP for 2 h. These cells were then exposed for 20 h to the indicated concentrations of LPS or distilled water. Following this treatment, the cells were then incubated with 1 mg/ml MTT for 2 h and solubilized overnight, and their viability was determined by measuring the absorbance at 595 nm as described under “Experimental Procedures.” The results presented are the average ± S.E. of triplicate measurements. B, RAW 264.7 macrophages (5 x 10^4 cells/treatment) were incubated with either control medium or 250 or 500 µM o-ATP for 2 h. The cells were then incubated for 20 h with either medium, 1 mM ATP, 1 mM ATP_S, 1 mM 2-MeS-ATP, or 500 µM Bz-ATP. Cell viability was assessed by the MTT assay as described under “Experimental Procedures.” The results presented are the average ± S.E. of triplicate measurements.

![Figure 4](image4.png)

**Fig. 4.** The effect of the pyridoxal-phosphate-based P2Z/P2X7 antagonist, PPADS, on LPS-stimulated iNOS expression and viability of RAW 264.7 cells. A, RAW 264.7 macrophages (5 x 10^4 cells/treatment) were cotreated with the indicated concentrations of PPADS and either medium alone or 1 µg/ml LPS for 20 h at 37 °C. The level of iNOS protein was determined by immunoblotting as described under “Experimental Procedures.” Comparable results were obtained in three separate experiments. B, RAW 264.7 macrophages (5 x 10^4 cells/treatment) were cotreated with the indicated concentrations of PPADS and either medium alone or 500 µM Bz-ATP for 20 h at 37 °C. Cell viability was determined using the MTT assay described under “Experimental Procedures.” The percent viability was determined using the following relationship: (absorbance of treated cells – absorbance of untreated control cells) x 100. The data are shown as the average of triplicate measurements ± S.E.
NF-kB nuclear extracts were collected for use in an NF-kB reporter assay in E. coli washed with medium and then treated with medium or increasing concentrations of o-ATP (Fig. 6). The levels of ERK1/ERK2 activation were assessed using antibodies specific for the dually threonine/tyrosine phosphorylated (active) forms of ERK1/ERK2. Similar results were obtained from at least three individual experiments.

To ascertain whether alternative pathways of ERK1/ERK2 activation were still intact after o-ATP pretreatment, we examined the capacity of the phorbol ester PMA to activate ERK1/ERK2 in cells pretreated with o-ATP. We observed that PMA alone dose-dependently activated ERK1/ERK2. Moreover, at all PPS concentrations tested, 2 h of pretreatment with increasing concentrations of o-ATP dose-dependently inhibited the LPS-induced ERK1/ERK2 activation.

To ascertain whether alternative pathways of ERK1/ERK2 activation were still intact after o-ATP pretreatment, we examined the capacity of the phorbol ester PMA to activate ERK1/ERK2 in cells pretreated with o-ATP. We observed that PMA alone could activate ERK1/ERK2 to levels comparable with those seen in response to cells treated with a maximal dose of LPS. Furthermore, consistent with the apparent selectivity of o-ATP on LPS and P2Z/P2X7 agonist-induced effects, the PMA stimulation of ERK1/ERK2 was resistant to the inhibitory actions of o-ATP pretreatment (Fig. 6A). These data also confirm our previous observation that o-ATP pretreatment is not toxic to RAW 264.7 cells. Similar results were obtained with human monocytic THP-1 cells, i.e. ERK1/ERK2 activation by LPS is inhibited by o-ATP pretreatment, whereas PMA stimulation of ERK1/ERK2 was unaffected by o-ATP pretreatment (data not shown).

Although the above studies have shown that the P2Z/P2X7 receptor antagonist o-ATP can block LPS-induced activation of ERK1/ERK2, it was important to ascertain if o-ATP can antagonize signals induced by other purinergic receptors that are expressed by macrophages, i.e. it was necessary to further establish the selectivity of o-ATP in this system. Because the other major purinergic receptor class that is well characterized to be expressed in macrophages is the P2Y2 receptor (9–11), which utilizes UTP as an agonist, we explored the capacity of the P2Z antagonist o-ATP to antagonize UTP-stimulated ERK1/ERK2 activation. We observed that treatment of macrophages with 250 mM o-ATP, which clearly blocks LPS-stimulated MAP kinase activation (Fig. 6, A and B), had little to no detectable effect on the activation of MAP kinases in response to UTP stimulation (Fig. 6B). These results support the hypothesis that it is principally the P2Z/P2X7, receptor class that is o-ATP-sensitive and involved in modulating LPS signaling with respect to ERK1/ERK2 activation.

**DISCUSSION**

Recent investigations have provided evidence that P2 purinergic receptors are likely to be important to the activation of macrophages by LPS (15, 19, 20). These previous studies have demonstrated that the administration of 2-MeS-ATP, which can interact with multiple P2X and P2Y receptors, can prevent death in mice treated with a lethal dose of LPS and results in the concomitant reduction in serum levels of tumor necrosis factor-α and interleukin-1α (20). Likewise, 2-MeS-ATP can inhibit NO production, as well as the production of iNOS protein and the steady-state levels of iNOS mRNA in mouse peritoneal macrophages treated with LPS and interferon-γ (19).
Consistent with these findings, the pretreatment of human monocytes with α-ATP for 2 h, which has been reported to selectively antagonize the P2Z/P2X7 receptor (23), can inhibit interleukin-1β release by cells cotreated with LPS and ATP (15). The results from the present study demonstrate that RAW 264.7 macrophages express a functional P2Z/P2X7 receptor and that P2Z/P2X7 receptor antagonists (α-ATP and PPADS) can have profound effects on both short term and long term LPS-stimulated processes. For example, α-ATP treatment of RAW 264.7 macrophages induces rapid effects on LPS activation of ERK1/ERK2 and the transcription factor NF-κB, as well as long term effects on LPS-induced iNOS expression and NO production. The regulation of LPS action by the P2Z/P2X7 receptor appears to be selective in that two structurally unrelated P2Z/P2X7 receptor antagonists, α-ATP and PPADS, can block LPS signal transduction events without affecting cell viability. Furthermore, both α-ATP and PPADS can inhibit the ability of known P2Z/P2X7 receptor agonists to induce cytotoxicity, demonstrating that α-ATP and PPADS are antagonizing the activity of this receptor.

Relatively little is known about the signal transduction pathways controlled by the P2Z/P2X7, purinergic receptor in macrophages. Earlier studies by el-Moattassim and Dubyak (17) demonstrated that the pore-forming activity of the P2Z/P2X7 receptor, which is thought to be responsible for P2Z/P2X7 receptor-induced cytotoxicity, can be dissociated from a GTP-dependent phospholipase D activity. Additionally, ATP has been found to activate NF-κB (p56) in mouse microglial N9 cells, which is an activity that could be inhibited with a 2-h pretreatment of the cells with α-ATP, although it is not clear whether α-ATP could inhibit LPS-stimulated NF-κB activation in these cells (36). Data generated by the present study reveal that P2Z/P2X7 receptor antagonists can inhibit the LPS-stimulation of ERK1/ERK2, as well as the activation of two NF-κB binding activities by LPS, suggesting that this purinergic receptor regulates both of these signal transduction end points in RAW 264.7 macrophages.

Although several intracellular effectors such as protein kinases and transcription factors are known to be regulated by LPS, it has not been clearly established what processes are important for the initiation of LPS signal transduction at the level of the plasma membrane. Because α-ATP can inhibit many actions of LPS, we postulate that the P2Z/P2X7 receptor can play a role in LPS signaling. It has been reported that LPS can trigger the release of ATP from RAW 264.7 cells (13), perhaps by the activation of an ATP channel or by exocytosis. The released ATP may activate the P2Z/P2X7 receptor in an autocrine or paracrine manner, thereby affecting a cation channel and/or a pore activity. Alternatively, LPS may interact directly or indirectly with the P2X7 receptor itself. This interaction could modulate the P2X7-linked channel activity and thus control the influx of divalent cations (such as Ca2+), which are important in signal transduction. It is also conceivable that α-ATP could be acting to inhibit LPS-induced events through a mechanism independent of the P2Z/P2X7 receptor, e.g. by antagonizing another class of purinergic receptor or LPS-binding protein.

In summary, there are multiple lines of evidence supporting the hypothesis that purinergic receptors are critical for LPS signaling. In the present study, we demonstrate that antagonism of the P2Z/P2X7 receptor can block LPS-induced signaling events leading to the inhibition of iNOS expression and NO production. However, a more precise determination of which aspects of LPS-induced macrophage activation are dependent upon purinergic receptor action will ultimately require the development of cell lines and/or in vivo models with defects in selected purinergic receptor expression or signaling. Regardless, the studies to date support the development of purinergic receptor ligands as novel therapeutics for the treatment of Gram-negative sepsis.

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