Title

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Journal

Journal of Biological Chemistry, 274(51)

ISSN

0021-9258

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Publication Date

1999-12-01

DOI

10.1074/jbc.274.51.36764

Peer reviewed

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 274, No. 51, Issue of December 17, pp. 36764–36768, 1999
Printed in U.S.A.

Regulation of Arachidonic Acid Mobilization in Lipopolysaccharide-activated P388D$_1$ Macrophages by Adenosine Triphosphate*

(Received for publication, July 28, 1999, and in revised form, August 23, 1999)

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Murine P388D$_1$ macrophages exhibit a delayed prostaglandin biosynthetic response when exposed to bacterial lipopolysaccharide (LPS) for prolonged periods of time that is dependent on induction of the genes coding for Group V secretory phospholipase A$_2$ and cyclooxygenase-2. We herein report that LPS-induced arachidonic acid (AA) metabolite release in P388D$_1$ macrophages is strongly attenuated by the P2X$_7$ purinergic receptor antagonists periodate-oxidized ATP and pyridoxal-phosphate-6-azophenyl-2′,4′-disulfonic acid, and this is accompanied by suppression of the expression of both Group V secretory phospholipase A$_2$ and cyclooxygenase-2. The effect appears to be specific for LPS, because the P2 purinergic receptor antagonists do not affect P388D$_1$ cell stimulation by other stimuli such as platelet-activating factor or the Ca^{2+} ionophore A23187. Moreover, extracellular nucleotides are found to stimulate macrophage AA mobilization with a pharmacological profile that implicates the participation of the P2X$_7$ receptor and that is inhibited by periodate-oxidized ATP. Collectively these results demonstrate coupling of the P2X$_7$ receptor to the AA cascade in P388D$_1$ macrophages and implicate the participation of this type of receptor in LPS-induced AA mobilization.

Macrophages respond to a wide variety of extracellular signals by generating large quantities of oxygenated metabolites of AA.[1] This process is central to the mammalian immunoinflammatory responses to bacterial lipopolysaccharide (LPS) and involves two tightly coupled reactions. First, AA is liberated from its phospholipid storage sites by the action of one or more phospholipase A$_2$s (PLA$_2$), and then it is used by cyclooxygenases (COX) to produce different types of prostaglandins such as PGE$_2$ (1).

Activation of macrophages with LPS results in a delayed generation of AA metabolites, which is accompanied by the continuous supply of fatty acid over long periods spanning several hours (2). Despite this process taking place in the absence of intracellular Ca^{2+} elevations, activation of the Group IV Ca^{2+}-dependent cytosolic PLA$_2$ appears to be the critical regulatory step (2). The cytosolic PLA$_2$ appears to serve primarily in a signaling role, *i.e.* it functions as a key step of the intracellular signaling cascade that ultimately leads to the generation of AA metabolites (2). Cytosolic PLA$_2$ activation by LPS enables the cells to synthesize and secrete another PLA$_2$, the Group V secretory PLA$_2$ (sPLA$_2$). The latter plays an augmentative role by providing the bulk of free AA to be converted into prostaglandins (2, 3). Finally, the liberated AA will be oxygenated to form different prostaglandins by the action of COX-2, another enzyme whose expression is dramatically augmented during long term exposure of the macrophages to LPS (2, 3).

Recently, the expression of P2 purinergic receptors that bind extracellular adenine nucleotides containing two or three phosphates has been shown to serve as a marker of macrophage activation and differentiation in response to LPS (4). Macrophages contain two different types of P2 receptors; P2Y and P2X$_7$, formerly known as P2Z (5). Whereas the former are classical G-protein-coupled receptors that activate the phosphoinositide-specific phospholipase C pathway (6), the P2X$_7$ receptor is unique in triggering the formation of large nonselective membrane pores permeable to hydrophilic molecules of molecular mass <0.9 kDa (7).

The P2X$_7$ receptor is known to mediate phospholipase D activation in macrophages (8) and to couple to the transcription factors NFκB (9) and NFAT (10). Other than this one example, despite the fact that the P2X$_7$ receptor has recently been cloned from rats and humans (11, 12), signaling events underlying P2X$_7$ receptor occupancy are almost entirely unknown.

Recent studies (13, 14) have suggested that the P2X$_7$ receptor may be involved in modulating certain macrophage responses to LPS, such as the synthesis of NO or interleukin-1β. These previous reports along with the fact that LPS activation of macrophages preferentially up-regulates the P2X$_7$ receptor (4) have led us to investigate the putative involvement of this receptor on lipid mediator production by LPS-challenged macrophages. Our results suggest a novel pathway for the regulation of AA mobilization that involves ATP as an autocrine mediator of the response.

EXPERIMENTAL PROCEDURES

Materials—ATP, periodate-oxidized ATP (*o*-ATP), ADP, ATPγS, pyridoxal phosphate 6-azophenyl-2′,4′-disulfonic acid (PPADS), BzATP, UTP, and LPS (*Escherichia coli* 0111:B4) were purchased from Sigma). [5,6,8,9,11,12,14,15-^{3}H]arachidonic acid (specific activity, 100 Ci/mmol) was from NEN Life Science Products and 1-palmitoyl-2-[^{14}C]palmitoyl-*sn*-glycero-3-phosphocholine (specific activity, 54 mCi/mmol) was from Amersham Pharmacia Biotech. Anti-mouse CD14 monoclonal antibody rmC5–3 (15) was from Pharmingen (San Diego, CA). COX-2 antibody and murine COX-2 cDNA probe were from Cayman (Ann Arbor, MI).

* This work was supported by Grants HD26171 and GM2051 from the National Institutes of Health, and Grant S96–08 from the University of California BioStar Project/Lilly Research Laboratories. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: AA, arachidonic acid; PLA$_2$, phospholipase A$_2$; sPLA$_2$, secretory PLA$_2$; COX, cyclooxygenase; LPS, lipopolysaccharide; *o*-ATP, periodate-oxidized ATP; BzATP, 2′,3′-*O*-(4-benzoylbenzoyl)-ATP; PPADS, pyridoxal-phosphate-6-azophenyl-2′,4′-disulfonic acid; ATPγS, adenosine 5′-*O*-(thiotriphosphate); PAF, platelet-activating factor.

The cDNA probe for murine glyceraldehyde 3-phosphate dehydrogenase was from Ambion (Austin, TX).

Cell Culture and Labeling Conditions—P388D_1 cells (MAB clone) (2) were maintained at 37 °C in a humidified atmosphere of 90% air and 10% CO_2 in Iscove's modified Dulbecco's medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. P388D_1 cells were plated at 10^6 per well, allowed to adhere overnight, and used for experiments the following day. All experiments were conducted in serum-free Iscove's modified Dulbecco's medium. When required, radiolabeling of the P388D_1 cells with [^{3}H]AA was achieved by including 0.5 μCi/ml [^{3}H]AA during the overnight adherence period (20 h). Labeled AA that had not been incorporated into cellular lipids was removed by washing the cells four times with serum-free medium containing 0.5 mg/ml albumin.

Measurement of Extracellular [^{3}H]AA Release—The cells were placed in serum-free medium for 30 min before the addition of LPS or other stimuli for different periods of time in the presence of 0.5 mg/ml bovine serum albumin. The supernatants were removed, cleared of detached cells by centrifugation, and assayed for radioactivity by liquid scintillation counting. When *o*-ATP was used, it was added to the cells 1 h before the addition of the stimulus. When PPADS was used, the preincubation time was 30 min.

Phospholipase A_2 Assay—Aliquots (100 μl) of supernatants from *o*-ATP-treated cells were assayed for PLA_2 activity as follows. The assay mix (500 μl) consisted of 100 μM 1-palmitoyl-2-[^{14}C]palmitoyl-*sn*-glycero-3-phosphocholine substrate (2,000 cpm/nmol), 10 mM $CaCl_2$, 100 mM KCl, 25 mM Tris-HCl, pH 8.5. Reactions were allowed to proceed at 40 °C for 30 min, after which [^{14}C]palmitate release was determined by a modified Dole procedure (16).

Western Blot Analyses—The cells were overlaid with a buffer consisting of 10 mM Hepes, 0.5% Triton X-100, 1 mM sodium vanadate, 1 mM phenylmethylsulfonyl fluoride, 20 μM leupeptin, 20 μM aprotinin, pH 7.5. Samples from cell extracts (50–100 μg) were separated by SDS-polyacrylamide gel electrophoresis (10% acrylamide gel) and transferred to Immobilon-P (Millipore). Nonspecific binding was blocked by incubating the membranes with 5% nonfat milk in phosphate-buffered saline for 1 h. Membranes were then incubated with anti-COX-2 antisera, and then treated with horseradish peroxidase-conjugated protein A (Amersham Pharmacia Biotech). Bands were detected by enhanced chemiluminescence (Amersham).

Northern Blot Analyses—Total RNA was isolated from unstimulated or LPS-stimulated cells by the TRIZOL reagent method (Life Technologies, Inc.), exactly as indicated by the manufacturer. 15 μg of RNA was electrophoresed in a 1% formaldehyde/agarose gel and transferred to nylon filters (Hybond, Amersham) in 10× SSC buffer. Hybridizations were performed in ExpressHyb solution (CLONTECH) according to the manufacturer's instructions. The ^{32}P-labeled probe for COX-2 was incubated with the filters for 1 h at 66 °C, followed by two washes with 2× SSC containing 0.1% SDS at room temperature for 15 min. Two final washes were carried out at 65 °C for 15 min each with 0.1× SSC containing 0.1% SDS. The ^{32}P-labeled probe for glyceraldehyde 3-phosphate dehydrogenase was incubated with the filters for 1 h at 66 °C, followed by one wash with 0.1× SSC containing 0.1% sodium dodecyl sulfate at 65 °C for 30 min. Bands were visualized by autoradiography.

RESULTS

Inhibition of LPS-induced AA Mobilization by Blockade of the P2X_7 Receptor—Our previous data have revealed that LPS induces a delayed AA mobilization response in the P388D_1 macrophages, with maximal effects being observed at LPS concentrations as low as 10 ng/ml (2). Based on these data, we typically used a LPS concentration of 100 ng/ml for our studies. At these concentrations, the LPS-induced AA mobilization was completely inhibited by cell pretreatment with the anti-mouse CD14 monoclonal antibody rmC5–3 (15), which indicates that CD14 is involved in the response (results not shown).

To determine whether the P2X_7 receptor is involved in LPS signaling in macrophages, we examined whether antagonists selective for that receptor influenced LPS-induced AA release and metabolism. P388D_1 macrophages were pretreated with different concentrations of *o*-ATP, a specific antagonist of the P2X_7 receptor (17), or PPADS, a general P2 purinoreceptor antagonist. The cells were then exposed to LPS for different periods of time, and extracellular AA release and PGE_2 was determined. As shown in Fig. 1, the two P2 receptor antagonists strongly blocked the LPS-induced AA release in a dose-dependent manner.

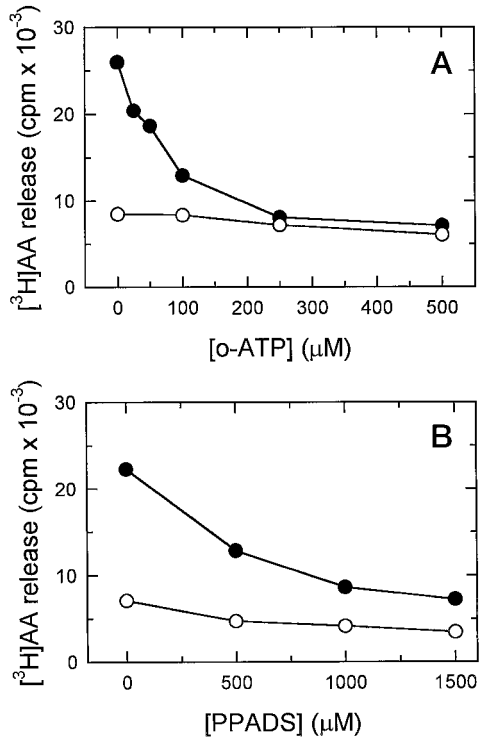

FIG. 1. **Effect of *o*-ATP and PPADS on [^{3}H]AA release from P388D_1 macrophages.** [^{3}H]AA-labeled cells were treated with the indicated concentration of *o*-ATP (*A*) or PPADS (*B*) for 30 min and then incubated without (*open circles*) or with (*closed circles*) 100 ng/ml LPS for 20 h. Supernatants were then collected, and radioactivity was determined by scintillation counting.

Given the above observations suggesting a key role for P2X_7 in LPS signaling, it was important to determine whether this effect reflected a specific regulatory mechanism or was the result of a nonselective inhibitory effect. To investigate this point, we conducted experiments using PAF as the triggering agent. Extensive work from this laboratory has demonstrated that P388D_1 cells display a Ca^{2+}-dependent AA mobilization response to PAF that is entirely dependent on the occupancy of the PAF receptor (18–22). In this response, the cells are first exposed to LPS for 1 h and then challenged by PAF for several minutes. Under these conditions, LPS does not have any effect by itself; it just primes the cells for an enhanced response to PAF (19, 23). Because the PAF-induced response is specifically due to PAF receptor occupancy, P2X_7 receptor antagonists should not affect the AA release and PGE_2 production responses to PAF. Fig. 2 demonstrates that blockade of P2X_7 receptors by *o*-ATP had no measurable effect on the PAF-induced AA release. AA release in response to the Ca^{2+} ionophore A23187, which is a receptor-independent event, also was not affected by *o*-ATP (data not shown). Collectively these data indicate that P2X_7 receptors seem to be specifically coupled to cellular responses to LPS but not to other inflammatory mediators.

Blockade of P2X_7 Receptors Inhibits the Expression of COX-2 and Group V sPLA_2 in LPS-treated Macrophages—Long term AA mobilization and PGE_2 production in P388D_1 macrophages responding to LPS occurs in parallel with induction of Group V sPLA_2 and COX-2. Enhanced expression of these two proteins has been shown to be crucial for the response, because Group V sPLA_2 is responsible for providing most of the AA mobilized, and COX-2 is responsible for all the PGE_2 produced (2, 3). Therefore it was of interest to explore whether the P2X_7 recep-

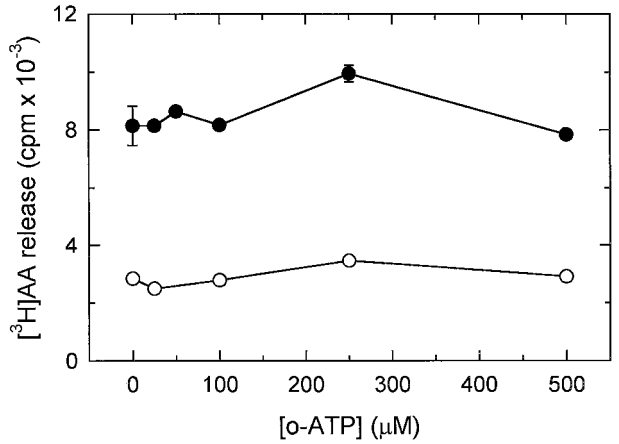

FIG. 2. **Effect of *o*-ATP on [^{3}H]AA release from LPS/PAF-stimulated P388D$_1$ cells.** [^{3}H]AA-labeled cells were preincubated with *o*-ATP for 1 h and then treated with vehicle (*open circles*) or 100 ng/ml LPS for 1 h followed by incubation with 200 nM PAF for 15 min (*closed circles*). Supernatants were then collected, and radioactivity was determined by scintillation counting.

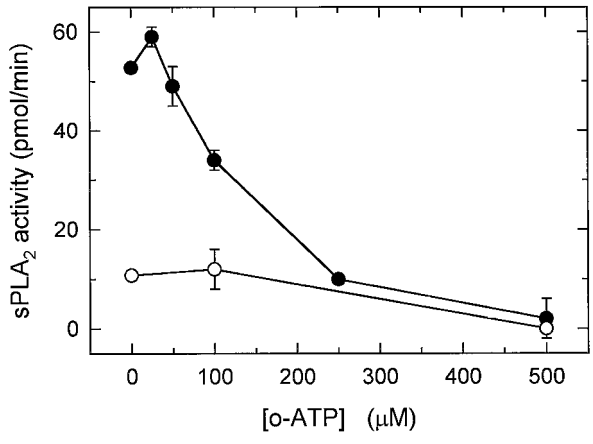

FIG. 3. **Inhibition of sPLA$_2$ activity in supernatants from stimulated P388D$_1$ cells.** Cells were treated without (*open circles*) or with (*closed circles*) 100 ng/ml LPS in the presence of the indicated concentrations of *o*-ATP for 20 h. Supernatants were assayed for sPLA$_2$ activity as described under "Experimental Procedures."

tor participates in the induction of these two proteins by LPS. Unfortunately, an antibody specific for Group V sPLA$_2$ is not available, which prevents us from being able to quantitate Group V sPLA$_2$ protein levels. However, we have shown that induction of the Group V sPLA$_2$ gene by LPS is accompanied by the extracellular accumulation of a sPLA$_2$-like activity, which we have identified to correspond to that of Group V sPLA$_2$ (2). As shown in Fig. 3, LPS-induced increases in extracellular sPLA$_2$ activity were not observed if the cells were pretreated with *o*-ATP. Moreover, pretreatment of the cells with *o*-ATP also inhibited the LPS-induced increases in COX-2 mRNA and protein (Fig. 4). Thus these data support a model whereby up-regulation by LPS of the expression of both Group V sPLA$_2$ and COX-2 in macrophages, and hence AA mobilization and PGE$_2$ production, are modulated by ATP receptors, thus suggesting the involvement of ATP as an autocrine mediator of the LPS effects on macrophage AA signaling.

Extracellular ATP Stimulates AA Release in P388D$_1$ Macrophages—It is well established that macrophages and macrophage cell lines secrete ATP to the extracellular medium in response to LPS (13, 14, 24, 25). To validate the putative autocrine role of ATP on macrophage AA release, it was therefore important to ascertain whether extracellularly applied ATP was able to stimulate the cells for an enhanced release of AA.

Fig. 5 shows that extracellular ATP did trigger an AA release response from the P388D$_1$ macrophages in a time- (Fig. 5*A*) and dose-dependent (Fig. 5*B*) manner. Other ATP analogues that are known to be agonists for the P2X$_7$ receptor, *i.e.* BzATP, ATPγS, and ADP, also stimulated AA release (Fig. 5*B*). The order of potency of the nucleotides matched exactly the one previously shown for the P2X$_7$ receptor (11). UTP, which is not a P2X$_7$ receptor agonist (11), failed to elicit any response. Further proof for the involvement of P2X$_7$ was obtained by the use of *o*-ATP, which strongly inhibited the ATP-induced AA release (Fig. 6).

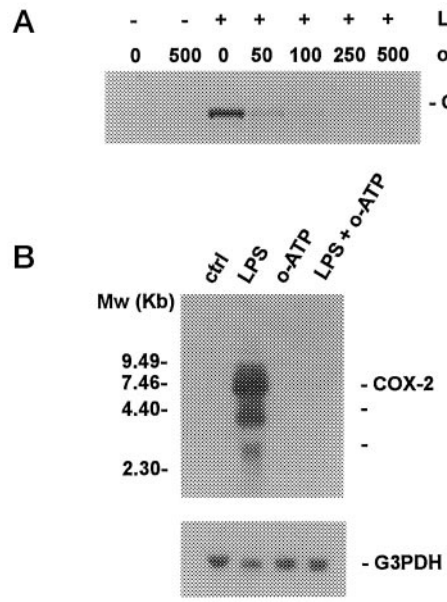

FIG. 4. **COX-2 expression in P388D$_1$ cells.** *A*, cells were preincubated with the indicated concentrations of *o*-ATP for 1 h and then treated without (−) or with (+) 100 ng/ml LPS for 20 h. Homogenates were prepared, and protein was analyzed by immunoblot with an antibody against COX-2 as described under "Experimental Procedures." *B*, the cells were pretreated with 250 μM *o*-ATP for 1 h and then exposed to LPS for 18 h. The RNA was extracted and analyzed by Northern blot with a probe for COX-2.

DISCUSSION

A striking hallmark of the immunoinflammatory response is the generation of oxygenated metabolites of AA such as the prostaglandins. LPS, a major constituent of the outer membrane of Gram-negative bacteria potently induces macrophages to synthesize and release prostaglandins and other inflammatory mediators, which *in vivo* may lead to septic shock and death (26). Signaling mechanisms triggered by LPS on macrophages are initiated by the interaction of the molecule with membrane CD14, in a process that involves participation of the accessory protein LBP (26). Because CD14 does not transverse the membrane, it is not clear how the intracellular signal is initiated.

Importantly, recent results have suggested that P2 purinergic receptors may be involved in some macrophage responses to LPS. Administration of 2-methylthio-ATP, a nonselective antagonist of P2 receptors, was shown to prevent death in mice treated with a lethal dose of LPS and to decrease serum levels of tumor necrosis factor and other cytokines (27).

In this study we show that P388D$_1$ macrophages appear to express functional P2 purinergic receptors because ATP and other P2 receptor agonists are able to trigger AA release and metabolism in these cells in a manner that is blocked by the

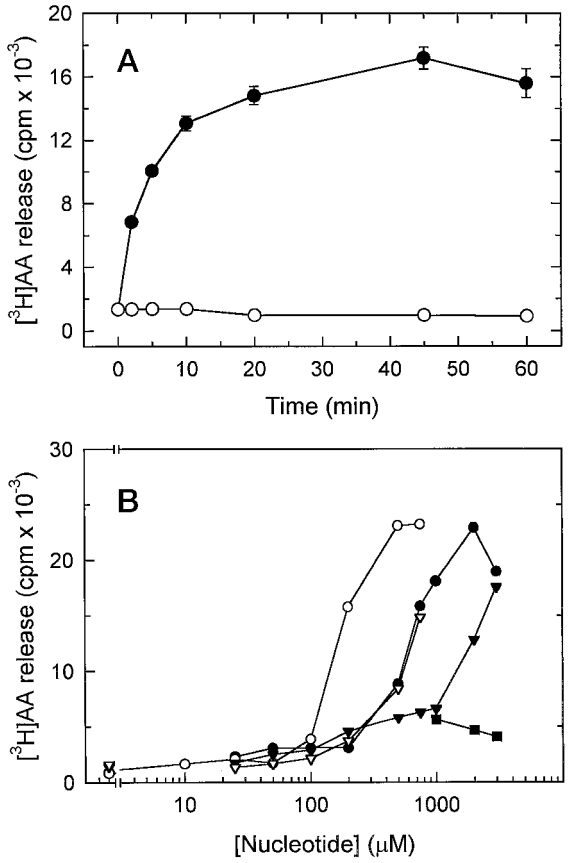

FIG. 5. **[^{3}H]AA release by different nucleotides in P388D$_1$ cells.** *A*, the [^{3}H]AA-labeled cells were treated without (*open circles*) or with (*closed circles*) 2 mM ATP for the indicated times. *B*, the [^{3}H]AA-labeled cells were treated with the indicated concentrations of BzATP (*open circles*), ATP (*closed circles*), ATPγS (*open triangles*), ADP (*closed triangles*), or UTP (*closed squares*) for 1 h. The supernatants were assayed for AA release as described under "Experimental Procedures."

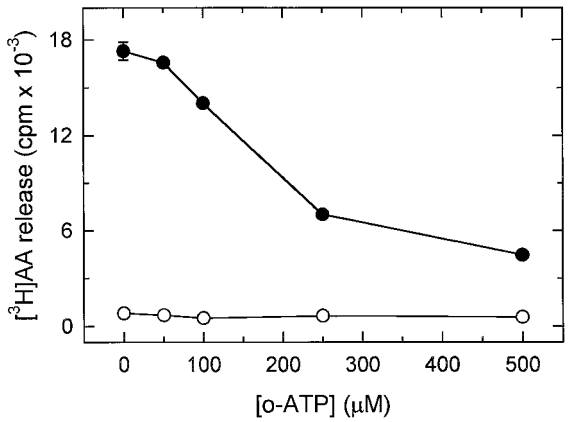

FIG. 6. **Effect of *o*-ATP on [^{3}H]AA release by ATP-stimulated P388D1 macrophages.** The [^{3}H]AA-labeled cells were pretreated with the indicated concentrations of *o*-ATP for 1 h and then incubated without (*open circles*) or with (*closed circles*) 2 mM ATP for 1 h. The supernatants were assayed for AA release described under "Experimental Procedures."

$P2X_7$ purinergic antagonists *o*-ATP and PPADS. Moreover these two receptor blockers have profound inhibitory effects on long term AA release and metabolism stimulated by LPS. Our data clearly suggest that $P2X_7$ purinergic receptor antagonists can block the LPS-induced expression of Group V $sPLA_2$ and COX-2, which leads to inhibited AA metabolism.

Given the central role of both Group V $sPLA_2$ and COX-2 in macrophage AA release and metabolism (2, 3), our results indicate that LPS-induced generation of AA-derived lipid messengers is not an immediate consequence of LPS binding to its membrane receptors (*i.e.* CD14) but to engagement of functional $P2X_7$ purinergic receptors on the surface of the cells. Importantly, the inhibition of macrophage responses to LPS by $P2X_7$ receptor antagonists appears to be selective, because responses triggered by PAF are not affected. Collectively the current results establish that one of the striking biochemical hallmarks of macrophage activation by LPS, *i.e.* the generation of AA-derived mediators, is triggered by a non-LPS receptor, *i.e.* the purinergic receptor $P2X_7$. These findings raise the interesting possibility that purinergic receptor antagonists could be envisioned as drugs for the treatment of pathological conditions triggered by LPS. In support of these observations, recent papers by Ferrari *et al.* (14) and Hu *et al.* (13) have also suggested the involvement of $P2X_7$ receptors in other macrophage responses to LPS such as interleukin-1β secretion and the expression of inducible nitric-oxide synthase. Thus it appears from these observations that an important part of the actions of LPS on macrophages may be mediated by the $P2X_7$ receptor.

Although the possibility of a direct interaction of LPS with the $P2X_7$ receptor cannot be excluded at this time, there is no evidence at present to support such a contention. Instead, the results reported here raise the intriguing hypothesis that this receptor may serve to propagate the LPS signal by responding in an autocrine manner to the ATP that the cell itself produces in response to LPS. It is well known that macrophages secrete ATP in response to LPS (13, 14, 24, 25), and we herein demonstrate that engagement of $P2X_7$ receptors by ATP and its derivatives, BzATP and ATPγS, triggers an AA release response that is inhibited by $P2X_7$ receptor antagonists. In line with the observations by Ferrari *et al.* (14) our results support a model whereby the ATP released by LPS to the extracellular medium may interact with the $P2X_7$ in an autocrine manner and trigger AA release from the macrophages.

At sites of inflammation, the local concentration of ATP is likely to be significant because of the presence in those foci of injured cells that can discard their cytoplasmic ATP content, usually in the 5–10 mM range (7). Thus it can be expected that in pathophysiological settings the macrophages are continuously exposed to ATP concentrations high enough to allow them to orchestrate complex cell responses, such as the induction and expression of Group V $sPLA_2$ and COX-2.

In summary, the results of this study have revealed an unexpected pathway for macrophage activation of the AA cascade by LPS. Our data suggest that the $P2X_7$ receptor participates in the LPS-initiated signaling mechanism leading to induction of Group V $sPLA_2$ and COX-2 gene expression, and hence to the generation of AA-derived lipid mediators. Given the high levels of extracellular ATP at sites of inflammation, the current findings are likely to be of pathophysiological significance. The involvement of the $P2X_7$ receptor in the LPS-induced AA cascade suggests alternative targets for pharmacological intervention in a number of diseases that involve an exacerbated production of AA-derived mediators, such as septic shock and chronic inflammatory states.

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J. Biol. Chem. 1999, 274:36764-36768.
doi: 10.1074/jbc.274.51.36764
