Brief Definitive Report

Purinergic Modulation of Interleukin-1β Release from Microglial Cells Stimulated with Bacterial Endotoxin

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Summary

Microglial cells express a peculiar plasma membrane receptor for extracellular ATP, named $P2Z/P2X_7$ purinergic receptor, that triggers massive transmembrane ion fluxes and a reversible permeabilization of the plasma membrane to hydrophylic molecules of up to 900 dalton molecule weight and eventual cell death (Di Virgilio, F. 1995. *Immunol. Today*. 16:524–528). The physiological role of this newly cloned (Surprenant, A., F. Rassendren, E. Kawashima, R.A. North and G. Buell. 1996. *Science (Wash. DC)*. 272:735–737) cytolytic receptor is unknown. In vitro and in vivo activation of the macrophage and microglial cell $P2Z/P2X_7$ receptor by exogenous ATP causes a large and rapid release of mature IL-1β. In the present report we investigated the role of microglial $P2Z/P2X_7$ receptor in IL-1β release triggered by LPS. Our data suggest that LPS-dependent IL-1β release involves activation of this purinergic receptor as it is inhibited by the selective $P2Z/P2X_7$ blocker oxidized ATP and modulated by ATP-hydrolyzing enzymes such as apyrase or hexokinase. Furthermore, microglial cells release ATP when stimulated with LPS. LPS-dependent release of ATP is also observed in monocyte-derived human macrophages. It is suggested that bacterial endotoxin activates an autocrine/paracrine loop that drives ATP-dependent IL-1β secretion.

With the recent cloning of the macrophage $P2Z/P2X_7$ receptor, purinergic receptors in immune cells are attracting increasing interest (1, 2). Microglial cells are a good model to investigate physiological functions of purinergic receptors in the immune system because neurons are among the few cell types that have been unequivocally shown to release ATP (3). A recent study from our laboratory has shown that exogenous ATP causes a large release of IL-1β from N9 and N13 microglial cell lines and freshly isolated microglial cells by activating the $P2Z/P2X_7$ receptor (2). IL-1β has a pivotal role in several chronic inflammatory diseases and in the pathogenesis of septic shock (4–6). It is synthesized by activated macrophages and microglial cells as an inactive 33-kD precursor (proIL-1β) that is cleaved into the active 17-kD form by a cysteine protease named IL-1β–converting enzyme (ICE) (7, 8). Mature IL-1β is released through an as yet unknown pathway. In macrophages and microglial cells, bacterial endotoxin (LPS), the best characterized stimulant of IL-1β release, causes a rapid and large in vitro accumulation of proIL-1β, followed by slow release of the mature form (9). Release of processed IL-1β can be greatly accelerated by depletion of endogenous K^+ (4, 10). This very recent observation has led to the hypothesis that in vivo an additional stimulus that causes cytoplasmic K^+ depletion must act in synergy with LPS to induce fast IL-1β release (11). Extracellular ATP (ATP_e) is an interesting candidate to serve this role because it causes massive K^+ depletion via activation of the macrophage $P2Z/P2X_7$ receptor (12). The present report suggests that the $P2Z/P2X_7$ receptor is also involved in IL-1β release stimulated by LPS, presumably through an autocrine loop based on LPS-triggered ATP secretion.

Materials and Methods

Cell Culture and Cytokine Measurement. N9 and N13 microglial cell lines were a kind gift of Dr. Paola Ricciardi-Castagnoli (University of Milano, Italy) and were grown in RPMI 1640 medium (PAA, Linz, Austria) supplemented with 2 mM glutamine and 10% (heat-inactivated) FCS (Life Technologies Ltd., Paisley, Scotland), 100 U/ml penicillin, and 100 μg/ml streptomycin as described previously (2). Human monocytes were isolated from buffy coats by one-step gradient (Percoll; Pharmacia Biotech SpA, Cologno Monzese, Italy) or by adherence on plastic Petri dishes. After isolation, cells were kept in culture for 5 d in RPMI medium containing 2 mM glutamine, 5% human serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. IL-1β and IL-6 in the supernatant of LPS (Sigma Chemical Co., St. Louis, MO) treated cells were measured with the Intertext-1βX mouse IL-1β ELISA kit and Intertext-6X mouse IL-6 ELISA kit, respectively (Genzyme srl, Cinisello Balsamo, Italy). All reagents used were dissolved in endotoxin-free water (Sigma) and checked for endotoxin contamination. Periodate-oxidised ATP (oATP) was synthesized by Dr. S. Hanau as described in reference 13.

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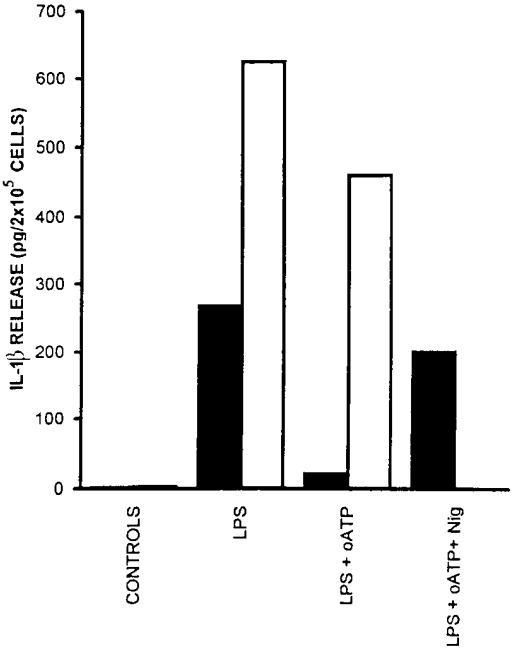

Figure 1. Oxidized ATP inhibits LPS-dependent release of IL-1β. N13 microglial cells were incubated in 24-well plates in RPMI medium supplemented with 10% FCS at a concentration of 2×10^6 and incubated 24 h in the presence or absence (controls) of 10 μg/ml LPS. In the experiments with oATP, cells were treated with this inhibitor (300 μM) for 2 h and then rinsed before addition of LPS. Stimulation with nigericin (20 μM) was performed for 30 min after removal of oATP. *Closed bars*, IL-1β; *open bars*, IL-6. Data are averages of duplicate determinations from a single experiment repeated on three separate occasions. Similar results were obtained with the N9 cell line.

Measurement of Extracellular ATP. Microglial cells (25×10^3/well) were plated in microtiter plastic wells in culture medium and incubated in a CO_2 incubator at 37°C in the absence or presence of LPS for 24 h. At the end of this incubation, the monolayers were thoroughly rinsed with saline solution and supplemented with 100 μl of a special diluent buffer (FireZyme Ltd., San Diego, CA) to stabilize extracellular ATP and directly placed in the test chamber of a luminometer (FireZyme). Then, 100 μl of luciferin-luciferase solution (FireZyme) was added, and light emission was recorded. As a control, total cellular ATP content was also routinely monitored. Under resting conditions, extracellular ATP amounted to ~10–15% of total.

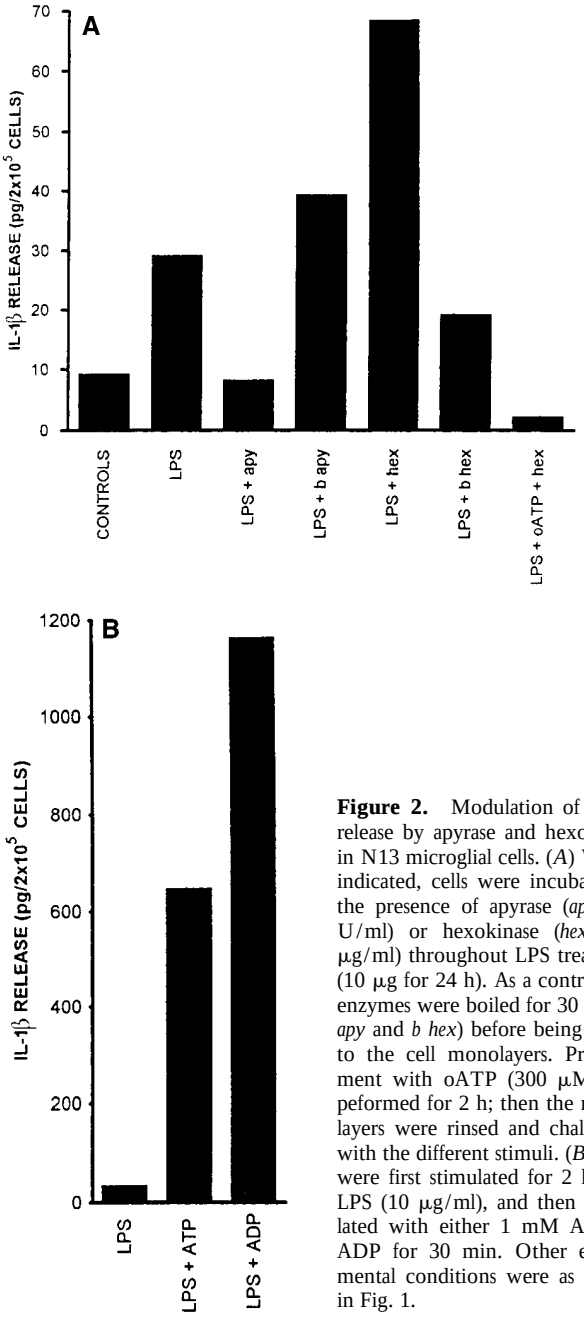

Figure 2. Modulation of IL-1β release by apyrase and hexokinase in N13 microglial cells. (*A*) Where indicated, cells were incubated in the presence of apyrase (*apy*, 0.4 U/ml) or hexokinase (*hex*, 100 μg/ml) throughout LPS treatment (10 μg for 24 h). As a control, the enzymes were boiled for 30 min (*b apy* and *b hex*) before being added to the cell monolayers. Pretreatment with oATP (300 μM) was peformed for 2 h; then the monolayers were rinsed and challenged with the different stimuli. (*B*) Cells were first stimulated for 2 h with LPS (10 μg/ml), and then stimulated with either 1 mM ATP or ADP for 30 min. Other experimental conditions were as shown in Fig. 1.

Results and Discussion

Fig. 1 shows that a 24-h incubation in the presence of 10 μg/ml LPS triggers release of IL-1β and that this is blocked by pretreatment with the selective P2Z/P2X$_7$ inhibitor (13) oATP. To show that the effect of oATP is not due to a nonspecific inhibition of cell responses, we have also monitored IL-6 release, which is much less affected. As further proof that oATP does not have nonspecific effects, we show that IL-1β release is restored in LPS-treated, oATP-inhibited cells by the K^+ ionophore nigericin, an agent known to cause IL-1β release through a receptor-independent pathway (4, 10).

Autocrine/paracrine stimulation of purinergic receptors can also in principle be prevented by exogenously added ATP-consuming enzymes such as apyrase or hexokinase. Fig. 2 *A* shows that apyrase completely inhibits LPS-dependent IL-1β release (the inactivated enzyme has no such effect). Surprisingly, hexokinase does not inhibit but rather stimulates IL-1β release. The main difference between apyrase and hexokinase is that the first hydrolyzes ATP and ADP, thus generating AMP, whereas hexokinase uses ATP as phosphorus donor to phosphorylate glucose, thus generating glucose 6 phosphate and ADP. It is known that ADP is an agonist at P2Z/P2X$_7$ receptor, though less potent than ATP (12). Thus we checked whether the potentiating ef-

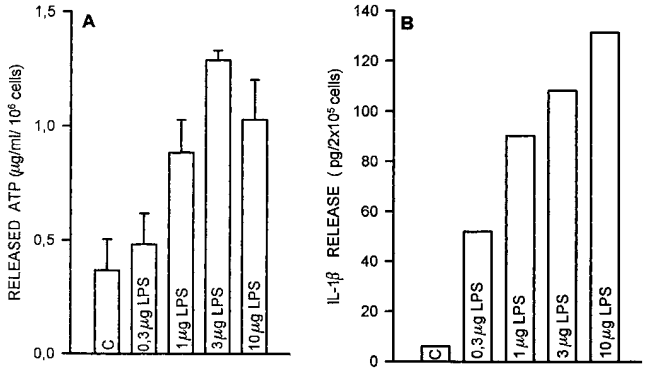

Figure 3. LPS dose response for IL-1β and ATP release. Microglial cells were plated in 24-well plates as described in Fig. 1 for IL-1β secretion or microtiter plastic wells as described in Materials and Methods for ATP release and stimulated with LPS for 24 h in a CO_2 incubator at 37°C. For measurement of ATP, release samples were processed as follows: monolayers were rinsed and 100 μl of diluent buffer (Firezyme) were added (see Materials and Methods). Accumulation of extracellular ATP was measured by the luciferin/luciferase assay. Data for IL-1β release are duplicates from a single experiment repeated with similar results with three different batches of microglial cells. Data for ATP release are means of quadruplicate determinations ± SD from a single experiment repeated in three different occasions.

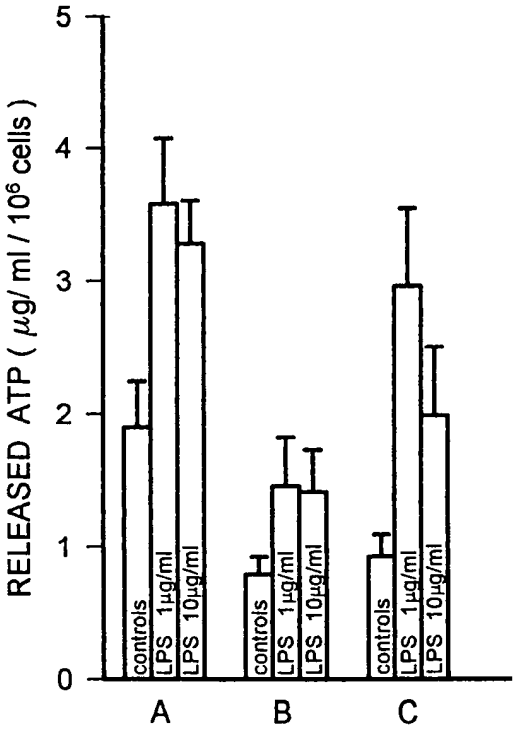

Figure 4. Stimulation with LPS triggers ATP release from human macrophages. Macrophages were isolated from three different donors (*A–C*) as described in Materials and Methods and plated in microtiter plastic wells at a concentration of 50×10^3/well. After plating, cells were stimulated for 24 h with LPS and ATP release measured as detailed in Fig. 3. Data are mean ± SD from quadruplicate determinations.

fect of hexokinase is mediated by stimulation of the P2Z/$P2X_7$ receptor by accumulated ADP. This seems to be the case because pretreatment with oATP blocks IL-1β secretion due to the combined addition of LPS and hexokinase (Fig. 2 *A*), and more importantly, exogenous ADP (ADP_e) is a much more potent stimulus than ATP (Fig. 2 *B*). These experiments suggest that IL-1β release could be modulated by ATP_e and ADP_e, probably released by the inflammatory cells themselves under LPS stimulation.

An obvious sine qua non of this hypothesis is that microglial cells must release ATP in response to LPS. Fig. 3 *A* shows that microglial cells chronically stimulated with LPS release ATP. Because the incubation medium is changed right before ATP determination, extracellular ATP measured in this experiment is very likely not accumulated in the bulk phase but continuously generated by the microglial cells. In support of this interpretation, we consistently found very little extracellular ATP in the cell-free supernatant (not shown). This observation is consistent with that previously reported by Filippini et al. (14) in T lymphocytes. The LPS dose–response curve for ATP release closely matched that for IL-1β release, as shown in Fig. 3 *B*. It has been shown previously that ATP is a powerful stimulus for IL-1β secretion from macrophages (10, 11), thus suggesting that this nucleotide might also have a role in autocrine/paracrine stimulation of these cells. In support of this hypothesis, Fig. 4 shows that ATP is released by human macrophages isolated from three different subjects after stimulation with LPS.

The mechanism of IL-1β processing and release is a key issue in immunology (5–9, 15). Rather surprisingly, recent evidence points to a decrease in cytoplasmic K^+ as a pivotal stimulus for ICE activation and IL-1β maturation (4, 10). However, LPS itself does not directly activate plasma membrane K^+ channels, and mouse microglial cells express inwardly but not outwardly rectifying K^+ channels (16), thus raising the issue of the mechanism responsible for lowering the cytoplasmic K^+ concentration. It has been suggested that this might be achieved by a LPS-dependent increase in the number of voltage-dependent K^+ channels in the macrophage plasma membrane (4), but typical K^+ channel inhibitors blocked IL-1β release only at concentrations far above those necessary to inhibit these channels (4). The P2Z/$P2X_7$ receptor is a good candidate to mediate cytoplasmic K^+ depletion. This receptor is typically expressed in macrophages and macrophage-like cells (2, 17, 18), and it is modulated by inflammatory cytokines (17, 19). A brief stimulation with ATP_e triggers massive K^+ efflux (12) and release of processed IL-1β (2, 10, 11), whereas a sustained activation causes cell death (1, 17, 20). Our data suggest that IL-1β release from microglial cells requires a double stimulation: first, LPS-dependent transcription of the IL-1β gene and cytoplasmic accumulation of proIL-1β; second, paracrine/autocrine activation of the P2Z/$P2X_7$ receptor that causes release of the mature cytokine. Adenine nucleotides can originate from many different sources: (*a*) the microglial cells themselves can release ATP_e, either spontaneously or under LPS stimulation; (*b*) injured or damaged cells certainly release significant amounts of this nucleotide, a process likely to occur in vivo at sites of inflammation; (*c*) in

the central nervous system, ATP_e can be released by neurons that establish close contact with the microglial cells.

It might seem paradoxical that ADP is a better IL-1β–releasing agent than ATP, although notoriously it is a less potent stimulus at the $P2Z/P2X_7$ receptor. However, this is not unexpected because ADP, in contrast with ATP, is devoid of cytotoxic activity, and data from our laboratory show that release of IL-1β is optimal in response to a submaximal, noncytotoxic stimulation of the $P2Z/P2X_7$ receptor, such as that due to ADP_e (D. Ferrari and F. Di Virgilio, manuscript in preparation).

Involvement of the $P2Z/P2X_7$ purinergic receptor in LPS-dependent IL-1β release may allow the development of new pharmacological antagonists (i.e., oATP and derivatives) to modulate the in vivo production of this cytokine in pathological conditions such as septic shock or chronic inflammatory diseases.

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