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EXTRACELLULAR ATP POTENTIATES NITRIC OXIDE SYNTHASE EXPRESSION INDUCED BY LIPOPOLYSACCHARIDE IN RAW 264.7 MURINE MACROPHAGES

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Inducible nitric oxide synthase (iNOS) activity in the murine macrophage cell line RAW 264.7 was increased from two- to four-fold after co-exposure of the cells to low doses of bacterial lipopolysaccharide (LPS) and micromolar ATP, compared to LPS alone. Extracellular ATP and its analogs "per se", i.e. without LPS, were not able to induce iNOS activity. The stimulating effect of UTP too, the concentration range of activity (1-100 mM nucleotides) and the rank of potency (ATP-γ-S = AMP-PNP > ATP = ADP >> AMP-CPP = UTP) seem to indicate an involvement of P_{2Y}-type purinergic receptors. GTP, CTP and adenosine were virtually ineffective. These data suggest that binding of extracellular nucleotides to purinergic receptors may increase nitric oxide production by macrophages. This effect might occur in pathological conditions (i.e. inflammation/infection or trauma) where significant amounts of intracellular ATP can be released due to cellular damage.

Nitric oxide (NO) has been recently identified as a pleiotropic messenger of inter- and intra-cellular signals (1,2). Nitric oxide synthase (NOS), which shows a significant homology with the P_{450} family (3), is able to synthesize NO from L-arginine and molecular oxygen, using NADPH and tetrahydrobiopterin (BH_4) as cofactors (1,4). At least three isoforms of NOS have been identified: two of them, present in endothelial and neuronal cells, appear to be constitutive (cNOS) and are regulated by Ca^{2+} and calmodulin (5-6). A third form, initially described in macrophages, is inducible (iNOS) and Ca^{2+}/calmodulin independent (6-7). Recent evidence suggests that iNOS is expressed in cellular types other than macrophages, as a response to specific stimuli (8).

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Abbreviations used: NO = nitric oxide; cNOS = constitutive nitric oxide synthase; iNOS = inducible nitric oxide synthase; BH_4 = tetrahydrobiopterine; γ-IFN = γ-interferon; ATP-γ-S = adenosine-5'[γ-thio]triphosphate; AMP-PNP = 5'-adenylylimidodiphosphate; AMP-CPP = α,β-methylene ATP; G-protein = GTP binding protein; PLC = phospholipase C; PKC = protein kinase C.

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Considerable interest towards the NO production by activated macrophages has arisen, because this gaseous metabolite seems to play a fundamental role in the killing of microorganisms and tumour cells (9-11) and it has also been implicated in the pathogenesis of septic shock (12). Many agonists able to induce iNOS expression in macrophages have been identified and, among them, bacterial lipopolysaccharide (LPS) and some cytokines, in particular γ-interferon (γ-IFN), are the most characterized (13-14). Specifically, the co-stimulation with LPS and γ-IFN greatly enhances iNOS activity, by increasing the transcription of the iNOS gene (13). Although the precise molecular mechanisms underlying such synergism have not yet been identified, it has been proposed that γ-IFN may induce a priming effect, which makes the cells more responsive to specific activation by LPS (13).

Since extracellular ATP has been shown to promote some peculiar features of macrophage activation, i.e. superoxide release (15), we investigated the effects of this and of other nucleotides on iNOS expression. The results reported in this study indicate that ATP, ATP analogs and also UTP do not induce the enzyme protein "per se", but are able to potentiate the LPS-induced iNOS production significantly.

MATERIALS AND METHODS

The mouse macrophage cell line RAW 264.7 was obtained from the American Type Culture Collection (Rockville, MD, USA) and was cultured in DMEM containing 2 mM glutamine and 4.5 g/l glucose, supplemented with 10% FCS and penicillin/streptomycin. Cells were harvested once a week by scraping and maintained by 1:10 splitting. Endotoxin levels in the media were below 0.5 ng/ml. Cell viability was determined by trypan blue dye exclusion. NOS induction in RAW 264.7 was performed by incubating the cells (1×10^6/ml) for 18 hours in phenol red-free complete medium, either with LPS (from *E. coli*, serotype 0127:88, obtained from Sigma Chemicals, St. Louis, MO, USA), alone or together with adenine nucleotides (Sigma Chemicals). Possible cytotoxic effects of adenine nucleotides at high concentrations were determined by the MTT assay, as previously reported (16).

NOS activity in intact cells was monitored as nitrite release in the culture media after exposure to the inducers for 18 hours. Nitrite concentration was determined spectrophotometrically, using the Griess reagent (1% sulfanilamide, 0.1% N-(1-naphthyl)-ethylendiamine, 5% orthophosphoric acid), as described (13). Briefly, phenol red-free cell supernatants, from 24- or 96-wells plates, were diluted 1:1 with the Griess reagent, incubated for 10 min in the dark at room temperature and the absorbance was determined at 543 nm, using known concentration of nitrite, diluted in medium, as standards.

Assays of NOS activity on cellular extracts were performed after incubation of the cells (1×10^6/ml) with 10 ng/ml LPS or 10 ng/ml LPS + 100 μM ATP. Approximately 60-70 $\times 10^6$ cells were used for each determination. After treatment, the cells were washed three times with ice cold PBS and harvested by scraping in 15 mM Hepes, pH 7.4. After centrifugation at 1,000 x g for 5 min, the pellet was resuspended in 1 ml 15 mM Hepes containing 10 μg/ml α2-macroglobulin. Then, the cells were disrupted by sonication in an ice bath and the resulting lysate was centrifuged at 100,000 x g for 1 hour at 4 °C.

NOS activity in the 100,000 x g soluble fraction was determined spectrophotometrically, as described, using the oxidation of oxyhemoglobin by NO (17). The incubations were performed in 15 mM Hepes, pH 7.4 and contained 2 μM oxyhemoglobin, 1 mM ar-

ginine, 1 mM $MgCl_2$, 0.1 mM NADPH and 60 μM BH_4 (Schirks Laboratories, Jona, Switzerland). N-methylarginine, a specific inhibitor of NOS (18), was used in control experiments.

RESULTS

Extracellular ATP alone was not able to promote iNOS expression in RAW 264.7 cells, but increased from two- to four-fold the iNOS activity induced by LPS (Fig. 1), particularly at low (5-10 ng/ml) LPS dosage (Fig. 1A). The maximum effect was observed with 100 μM ATP (Fig. 1B), while at concentrations above 100 μM a slight reduction in nitrite production was observed, possibly due to cytolytic effects caused by near millimolar ATP levels (19). In the range of concentrations used for these experiments (1-100 μM) no toxic effects, determined by the MTT assay, were observed (not shown). ATP exerted its effect when co-incubated with LPS only. Exposure of the cells to extracellular ATP before or at the end of LPS incubation had no effect (not shown).

Table I reports NOS activity, assayed as nitrite production in intact cells after co-incubation of LPS with 100 μM nucleotides. The maximum effect was obtained with ATP and two non-hydrolyzable analogs of ATP, i.e. ATP-γ-S and AMP-PNP, while ADP and AMP were slightly less effective than ATP. Lower activity was exerted by AMP-CPP and UTP. Adenosine exhibited no effect, thus ruling out a role of A_1 or A_2 purinergic receptors (20), following dephosphorylation of adenine nucleotides by extracellular nucleotidases.

NOS activity was determined also in the soluble fraction from cell lysates (Table II). Co-exposure to 10 ng/ml LPS and 100 mM ATP increased the enzyme activity by approxi-

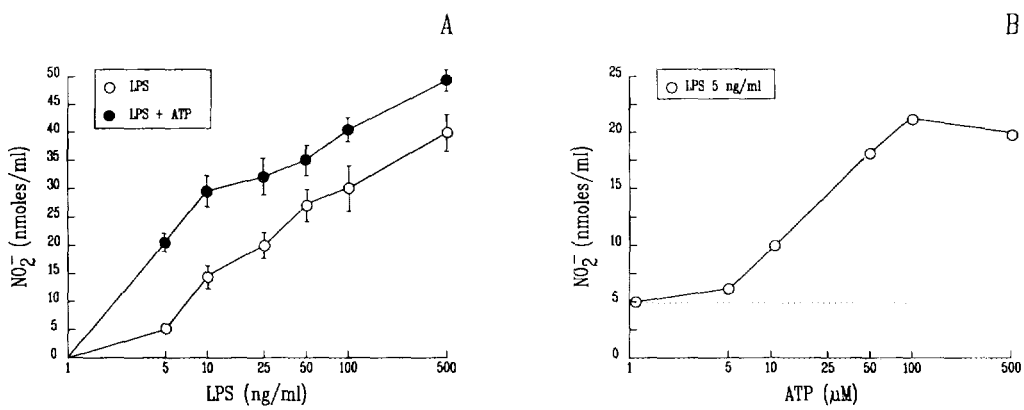

Figure 1. Effects of LPS and extracellular ATP on nitrite release in the culture media, after 18 hours of exposure to the iNOS inducing agents. (A) Effects of 100 μM ATP at increasing concentrations of LPS. ATP alone was ineffective. (B) Effects of increasing concentrations of ATP, co-incubated with 5 ng/ml LPS. The dotted line indicates the NO_2^- production induced by 5 ng/ml LPS alone. The data derive from three independent experiments.

Table I. Effects of ATP and other nucleotides on NO_2^- production induced by LPS

	LPS 5 ng/ml		LPS 10 ng/ml	
	nmoles NO_2^-/ml	%[a]	nmoles NO_2^-/ml	%[a]
LPS	5.6 ± 1.1	100	15.1 ± 2.3	100
+ ATP	20.3 ± 2.3	362	27.8 ± 5.2	180
+ ADP	18.1 ± 6.4	323	28.3 ± 5.1	187
+ AMP	16.3 ± 4.2	286	26.2 ± 4.5	173
+ AMP-PNP	22.8 ± 5.1	393	31.4 ± 8.2	206
+ ATP-γ-S	19.3 ± 2.8	344	30.0 ± 8.5	200
+ AMP-CPP	9.2 ± 2.3	164	21.5 ± 0.1	143
+ UTP	11.5 ± 1.4	197	23.6 ± 4.2	150
+ GTP	6.3 ± 0.7	112	16.5 ± 0.7	110
+ CTP	7.0 ± 1.5	125	16.5 ± 1.5	110
+ Adenosine	6.1± 0.7	109	14.0 ± 2.5	93

Cells ($1x10^6$/ml) were incubated with two different concentrations of LPS together with 100 μM nucleotides for 18 hours in complete, phenol red-free DMEM. Nitrite concentration was determined in the culture media using the Griess reagent. Nucleotides alone had not stimulatory effect. Data are expressed as mean ± SD of three independent experiments.
(a) = percent nitrite production compared to LPS alone.

mately 2.5-fold, compared to LPS alone. This is consistent with the two-fold increase in nitrite production observed in the intact cells in the same conditions (see Table I). No activity was observed in the untreated cells or after treatment with ATP alone (Table II).

DISCUSSION

In recent years considerable evidence has emerged that extracellular ATP and other nucleotides can exert significant biological effects on many cells and tissues (21-22). Pu-

Table II. NOS activity in cell lysates after induction with LPS alone or together with ATP

	nmoles NO/h/mg protein
Untreated	N.D[a]
ATP	N.D.[a]
LPS	1.9 ± 0.2
LPS + ATP	4.5 ± 0.4

NOS activity was determined with the oxyhemoglobin assay (17) on the 100,000 x g soluble fraction, derived from cellular lysates obtained by sonication. Cells were exposed to LPS (10 ng/ml) and/or 100 μM ATP for 18 hours, as reported in "Materials and Methods".
(a) N.D. = not detectable. Data are expressed as mean ± SD of three different experiments.

rinergic receptors have been identified, able to elicit different responses in different cell types. P_{2x} and P_{2t}-type receptors appear to be ligand-gated ion channels, P_{2z} are associated with ATP-induced pore formation and P_{2y}/P_{2u} are coupled with heterotrimeric G-proteins, both pertussin toxin-sensitive and -insensitive (21).

Both P_{2y} and P_{2z}-type purinoceptors have been described on macrophages surface (19). P_{2z}-type receptors, which respond to ATP at millimolar concentrations only, induce the rapid formation of non selective pores in the membrane, which becomes permeable to ions and small M_r organic molecules (19). Their involvement has been related to programmed cell death (23). On the contrary, P_{2y} and P_{2u} respond to micromolar concentrations of a wide range of adenine nucleotides and to UTP, with different degrees of potency (21).

Our experiments indicate that purinoceptors are able to potentiate the inducing effects of LPS on iNOS. The concentration range (μM), the effectiveness of UTP and the low activity of AMP-CPP, a specific agonist of P_{2x} (21), exclude the involvement of either P_{2x} or P2z-type receptors. Furthermore, the relative degree of potency (ATP-γ-S = AMP-PNP > ATP = ADP > > AMP-CPP = UTP) allows to tentatively identify them as the P_{2y}-type (21-22). However, the lack of specific antagonists of this receptor and the frequent reports in the literature of purinoceptors displaying a pharmacology different from the classical type (24), prevent from obtaining conclusive evidence.

The molecular events involved in the activation of macrophages induced by LPS have not yet been completely identified (25). One proposed mechanism postulates that a receptor for LPS can interact with a pertussis toxin-sensitive G-protein (26-28), leading to the sequential activation of PLC and PKC (25, 29). P_{2y}/P_{2u} receptors also are able to activate G-proteins/PLC dependent effectors (21). Thus, extracellular ATP might enhance iNOS expression by acting through the signalling cascade activated by LPS. However, there are not at present experimental data to support this hypothesis. Since macrophage iNOS activity has been shown to be Ca^{2+}/calmodulin independent (6), it seems unlikely that its increased activity could be due directly to the Ca^{2+} mobilization induced by purinergic stimulation.

The physiological role of purinoceptors on the iNOS induction by LPS is still far from being defined. It is now recognized that ATP and other nucleotides can be transiently and locally present at significant concentration in the extracellular fluids in response to physiological and pathological conditions (21). Although exocytotic release through secretory granules or through specific plasma membrane channels can occur in some cell types, the most important source of extracellular ATP is the disruption of intact cells, which might occur during infection, inflammation or trauma (21). Thus, in these conditions extracellular nucleotides could represent a potentiating stimulus for NO production by macrophages.

Experiments are in progress to determine the molecular events involved in the potentiation effects of adenine nucleotides on NO production and in particular to define the role of extracellular ATP in the activation of macrophages for tumour cell killing.

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