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EXTRACELLULAR ATP ENHANCES mRNA LEVELS OF NITRIC OXIDE SYNTHASE AND TNF-α IN LIPOPOLYSACCHARIDE-TREATED RAW 264.7 MURINE MACROPHAGES

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Extracellular ATP potentiates, by activation of P2y-type purinergic receptors, the production of NO induced by low doses of lipopolysaccharide (LPS) in the murine macrophagic cell line RAW 264.7 (Tonetti *et al.* (1994) Biochem. Biophys. Res. Commun. 203, 430-434). Release of TNF-α, known to be an autocrine factor for iNOS expression, was enhanced, too, following exposure of either LPS-induced or uninduced cells to externally added micromolar ATP. Reverse transcription-PCR experiments showed that extracellular ATP increases mRNA levels of both inducible NO synthase (iNOS) and of TNF-α to extents comparable to those of enzymatic and biological activities, respectively. These data demonstrate that activation of purinergic receptors by extracellular ATP results in an enhanced expression of the iNOS and TNF-α genes. © 1995 Academic Press, Inc.

Nitric oxide has been widely recognized to play a fundamental role in many physiological and pathological processes, due to its specific actions as a messenger of inter- and intracellular signals and to its radicalic nature which can lead to potential damaging effects (1,2). In particular, NO represents one of the most important mediators of inflammatory and immune responses (1) and is implicated in the mechanisms of pathogenesis of septic shock induced by bacterial endotoxin (3). Three isoforms of nitric oxide synthase (NOS) have ben described. Two of them are constitutively expressed in brain and endothelial cells and are Ca^{2+}/calmodulin dependent (2). The third form (iNOS), initially described in rodent macrophages (4), is inducible by a variety of different stimuli (in particular bacterial endotoxin and some cytokines, such as IFN-γ and TNF-α (5)) and has been recently described also in cell types other than macrophages and also in human tissues (6). iNOS does not seem to be Ca^{2+} dependent and its activity is mainly regulated by increases in mRNA transcription (5). Thus, an increase of iNOS mRNA levels was observed after treatment with lipopolysaccharide (LPS) both *in vitro* and *in vivo* in animal models (7,8). The many activities of NO

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Abbreviations: LPS, lipopolysaccharide; NO, nitric oxide; iNOS, inducible nitric oxide synthase; TNF-α, tumor necrosis factor α.

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and the potential damaging effects of an uncontrolled NO production suggest that the expression of iNOS must be subjected to very stringent mechanisms of regulation. However, the pathways leading to iNOS induction have not been completely elucidated as yet (6).

We have previously demonstrated that extracellular ATP potentiates the NO production induced by LPS in the murine macrophagic cell line RAW 264.7, while being ineffective in promoting any NO release *per se* (9). This effect seems to be mediated by the activation of P2y-type purinergic receptors (9), which have been described on the surface of both human and murine monocytes and macrophages or macrophage-derived cell lines (10, 11). In an attempt to address the mechanisms of the ATP-induced increase in NO production, we measured the levels of iNOS mRNA in RAW 264.7 cells. Furthermore, since TNF-α represents an important mediator induced by LPS treatment of macrophages and it has been previously reported to be an autocrine cofactor required for NO synthesis (12), the effects of ATP on the production of this cytokine and on its mRNA expression were also investigated.

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 maintained as previously described (9). For activation, cells (1 x 10^6/ml) were incubated in 24 wells plates in phenol red-free complete DMEM with either LPS (from *E. coli*, serotype 0127:88, obtained from Sigma Chemicals, St. Louis, MO, USA) alone or together with extracellular ATP (obtained from Sigma). Endotoxin levels in all culture media and solutions were below detection limits.

NOS activity was monitored as nitrite release in culture media after 24 hours of incubation, by the Griess reagent (9). TNF-α release in culture media was determined after 4 hours of incubation and was measured by the cytotoxicity assay on L929 cells, in the presence of 1 μg/ml actinomycin D (13). The viability of L929 was determined by MTT assay (14). One unit of TNF-α was defined as the amount of TNF-α required to induce lysis of 50% of the cells.

Total RNA from control and treated cells was extracted after 6 hours of treatment, by guanidinium thiocyanate followed by centrifugation in CsCl, as described (15). mRNA levels of iNOS and TNF-α after different treatments were determined by RT-PCR, using β-actin as internal standard. iNOS (or TNF-α) and β-actin reverse transcription and amplification were carried out in the same tube. Oligonucleotide primers were obtained from TIBMOLBIOL, Genova, Italy and were: (i) 5'-CTAGACTTCGAGCAGGAGATGG -3' (sense) and 5'-GAGGTCTTTACGGATGTCAACG-3' (antisense), corresponding to regions 583-604 and 780-801 for mouse β-actin cDNA, respectively (16); (ii) 5'-CTTCTCAGCCACCTTGGTGAAG-3' (sense) and 5'-TTCTGTGCTGTCCCAGTGAGG-3' (antisense), corresponding to regions 106-127 and 416-436 of mouse iNOS (17) and (iii) 5'-TTCTGTCCCTTTCACTCACTGG-3' (sense) and 5'-TTGGTGGTTTGCTACGACGTGG-3' (antisense), corresponding to regions 87-108 and 422-443 of murine TNF-α (18). Total RNA was reverse transcribed with the antisense primers using M-MLV reverse transcriptase (Promega, Madison, WI, USA). PCR amplification was performed in a 100 μl reaction volume containing 1x Thermo Buffer (Promega), 0.2 mM dNTP (Pharmacia, Uppsala, Sweden), 10 pmol primers and 2.5 U Taq polymerase (Promega). $MgCl_2$ concentration was 1 mM for iNOS amplification and 1.5 mM for TNF-α. Prior to addition to the amplification mixture the sense primers were labelled with ATP[γ-^{32}P] (specific activity 5000 Ci/mmol, Amersham, Buckinghamshire, UK), using T4 polynucleotide kinase (Amersham). Analysis of the amplification products was performed after 30 cycles for iNOS and after 23 cycles for TNF-α. For β-actin 20 μl aliquots were withdrawn from the amplification mixtures at 15 cycles.

A 15 µl aliquot of the amplification products was then electrophoresed on a 2% agarose gel and subsequently transferred to a Hybond N^+ membrane (Amersham). Multiple short exposures of the membrane to a Hyperfilm-MP (Amersham) were carried out to ensure linearity of the response and the films were then analysed by densitometry. Band intensities of iNOS and TNF-α cDNAs were then normalized to that observed for β-actin.

RESULTS

As previously demonstrated (9), extracellular ATP was able to potentiate the nitric oxide production, measured as nitrite release, induced by LPS treatment. This was particularly evident at low LPS doses, where approximately a three-fold increase was observed (Table I). No apparent effect was observed on the nitrite production after exposure of the cells to ATP alone. TNF-α production and release, as determined by its cytotoxic effects on L929 cells, took place at shorter time intervals than those of nitrite and were enhanced also following exposure to ATP alone. Combination of LPS and ATP led to increases in the TNF-α release over those recorded with LPS only (Table I).

The relative amounts of mRNAs of iNOS and TNF-a were determined by RT-PCR. The PCR products obtained by amplification as described in Materials and Methods showed clear bands of the predicted size (218, 331 and 356 bps, for β-actin, iNOS and TNF-α, respectively) and their identity was confirmed by cDNA sequencing (data not shown). Preliminary experiments were carried out

Table I. Effects of extracellular ATP on NO_2^- and TNF production induced by LPS treatment

	NO_2^- (nmoles/10^6 cells)		TNF (units/10^6 cells)	
LPS (ng/ml)	-ATP	+ATP	-ATP	+ATP
0	ND	ND	3 ± 1	34 ± 7
0.05	ND	ND	25 ± 5	60 ± 12
0.5	ND	ND	64 ± 7	106 ± 25
1	0.6 ± 0.05	2.0 ± 0.35	196 ± 36	365 ± 54
5	4.5 ± 1.0	12.1 ± 4.5	853 ± 177	1014 ± 203
10	15.8 ± 1.9	28.1 ± 7.7	1234 ± 298	1555 ± 300
50	58.0 ± 14.2	72.3 ± 14.0	2559 ± 562	2340 ± 345

RAW 264.7 cells (1 x10^6 /ml) were treated with a different concentration of LPS in the absence or presence of 100 µM extracellular ATP. TNF activity in the media was determined by the biological assay, as described in Materials and Methods, after 4 hours of incubation. Nitrite production was estimated by Griess reagent after 24 hours.
ND = not detectable. Results are expressed as mean ± SD of at least three independent experiments.

which ruled out any interference due to the co-amplification of β-actin. Furthermore, PCR products were sampled at different cycles throughout amplification, in order to determine the optimal number of cycles ensuring their correct quantitation. By this procedure, analysis was carried out during the exponential phase, before the plateau of amplification was reached. The optimal numbers of cycles for amplificates' analysis were 15 for β-actin and 23 and 30 for TNF-α and iNOS, respectively.

Fig. 1A shows the results of a representative co-amplification of β-actin and iNOS. A basal expression of iNOS in untreated cells was always observed. Amplifications performed without RNA as template or with RNA without reverse transcription excluded the presence of contamination between samples or of residual DNA, respectively. Co-exposure of the cells to both 5 ng/ml LPS and 100 μM ATP determined a significant increase in the iNOS signal, as compared to LPS alone. Relative TNF-α mRNA levels, clearly detectable in the untreated cells, were also slightly increased after treament with ATP alone or in combination with 5 ng/ml LPS, as compared to untreated or LPS-treated cells (Fig. 1B).

The data shown in Fig. 1 were then quantitated by measuring the ratios of optical density of the iNOS and TNF-α amplification bands, respectively, to that of the β-actin amplification band. Fig. 2 demonstrates that iNOS mRNA levels were increased from two to three-fold after treatment of the cells with LPS+ATP, as compared to LPS alone. The potentiating effect of ATP on the TNF-α expression was quantitatively lower (Fig. 2) and showed a good correlation with that on the TNF-α biological activity observed at 5 ng/ml LPS (see Table I).

DISCUSSION

The data obtained in this study confirmed that extracellular ATP is able to potentiate the NO production induced by low doses of LPS on RAW 264.7 macrophages (9) and provided insights into

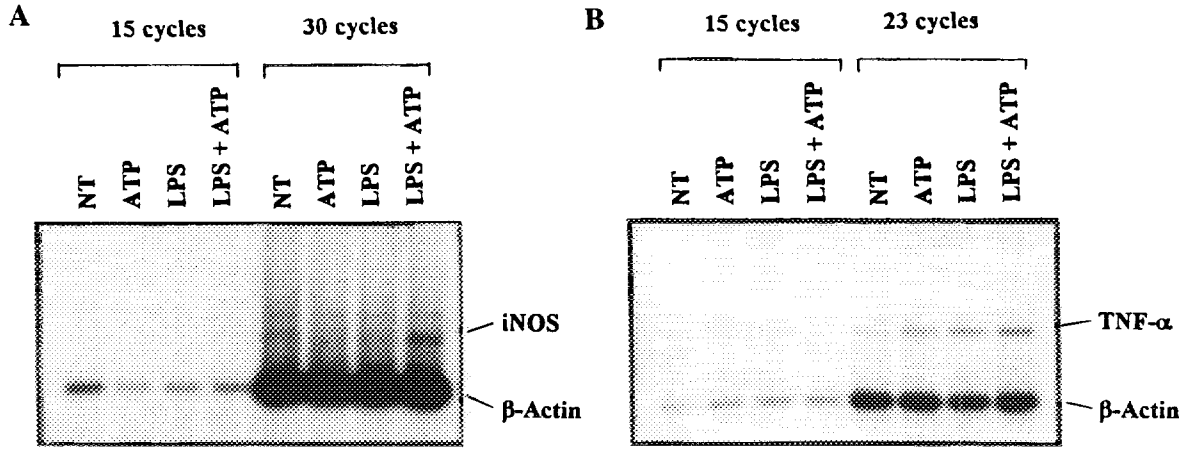

Figure 1. Effects of extracellular ATP on mRNA levels of iNOS (A) and TNF-α (B) after 6 hours of exposure to the inducers, determined by RT-PCR. β-actin was co-amplified as internal control. Data derive from a representative experiment. Similar results were obtained from three amplifications performed on each RNA obtained from two different incubation experiments. For further explanations, see text.
NT = untreated cells; ATP = cells treated with 100 μM ATP; LPS = cells treated with 5 ng/ml LPS; LPS + ATP = cells treated with 100 μM ATP and 5 ng/ml LPS.

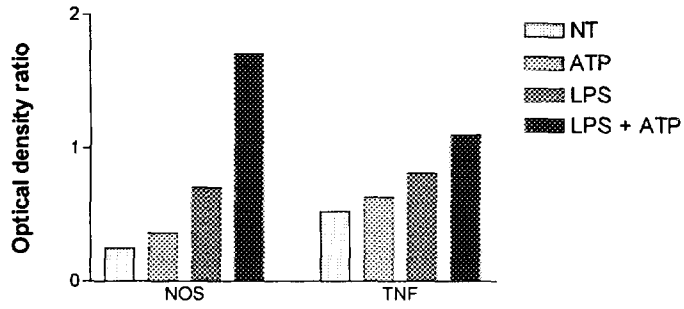

Figure 2. Optical density ratio of iNOS or TNF-α to β-actin amplification bands, obtained from densitometric analysis of Figure 1. β-actin, TNF-α and iNOS bands' intensity was determined at 15, 23 and 30 cycles, respectively.

the molecular mechanisms of this novel ATP effect. Specifically, an increase of iNOS mRNA levels proved to occur, over those recorded after treatment of the cells with LPS alone. This suggests an enhanced transcription of the iNOS gene (5). However, other mechanisms, e.g. mRNA stabilization, may be involved in the regulation of iNOS gene expression triggered by extracellular ATP, through activation of P2y-type purinergic receptors. These possibilities are now open to investigation.

As shown in Figs. 1 and 2, basal levels of iNOS mRNA were observed in untreated RAW 264.7 cells and in cells treated with ATP only, in the absence of detectable NO production (Table I). This discrepancy may either reflect inadequacy of the Griess reagent to detect very low amounts of nitrite, or rather indicate the occurrence of post-trascriptional mechanisms resulting in down-regulation of iNOS activity (6).

Extracellular ATP alone was also able to promote TNF-α release from RAW 264.7 macrophages and to potentiate its production induced by low LPS doses. TNF-α mRNA levels were correspondingly enhanced by treatment of the cells with ATP either alone or in combination with LPS. The extent of the effect afforded by the inducers was detectably lower than that observed for iNOS expression. However, it has been reported that in macrophages the intracellular TNF-α mRNA levels may not be related to the amount of protein secreted (19).

The increase in TNF-α production elicited by extracellular ATP might have a mechanistic role in the process of NOS induction. In fact it has been demostrated that an autocrine stimulation induced by TNF-α, released after LPS treatment, represents a fundamental factor for iNOS expression (5,12,20), as also demonstrated by the finding that the presence of anti-TNF-α antibodies in the culture media can partially inhibit the iNOS expression induced by LPS in macrophages (12). Finally, TNF-α is able to determine the activation of NF-κB (21), which is an important transcription factor involved in the control of iNOS gene expression (22).

Experiments are in progress, based on the present findings, to elucidate the correlation between the enhanced expression of both TNF-α and iNOS genes following activation of purinergic receptors by extracellular ATP.

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