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INTERLEUKIN-10 DECREASES TYROSINE PHOSPHORYLATION OF DISCRETE LIPOPOLYSACCHARIDE-INDUCED PHOSPHOPROTEINS IN HUMAN GRANULOCYTES

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Although Interleukin-10 (IL-10) has been recently shown to modulate lipopolysaccharide (LPS)-induced release of cytokines in human granulocytes, the intracellular signalling pathways of LPS have been only partially defined, while those of IL-10 remain unknown. The present study shows that LPS induces an increase in tyrosine phosphorylation of a discrete number of proteins, in a time- and concentration-dependent manner. In addition, IL-10 negatively influenced protein tyrosine phosphorylation in LPS-treated human polymorphonuclear leukocytes (PMN). The effect of IL-10 was evident only after 60 min LPS-stimulation and was detected by analysing either cell lysates or lysates which were previously immunoprecipitated with anti-phosphotyrosine antibodies. Amongst the tyrosine phosphoproteins mostly affected by IL-10 in LPS-stimulated cells were the species with molecular weights ranging from 46 to 49 kDa. The identity and possible function of these proteins remain unknown. Taken together, our results suggest that tyrosine phosphorylation may constitute one of the intracellular events that mediate LPS and IL-10 responses in granulocytes.

Previous *in vitro* studies have shown that PMN incubated with LPS, can release various cytokines, including Interleukin-1 (IL-1), IL-1 receptor antagonist (IL-1ra), IL-8, Macrophage Inflammatory Protein-1α/β (MIP-1α/β), Tumor Necrosis Factor-α (TNFα), and IL-12/p40 (rewieved in ref. 1). More recently, we (2,3) and others (4-6) showed that induction by LPS of the aforementioned cytokine gene expression and production in PMN, can be

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modulated by IL-10. IL-10 is a newly identified cytokine, primarily produced by T and mononuclear cells, with potent antiinflammatory activity (7). This cytokine has been in fact found to exert suppressive effects on Th1 cytokine production, T cell proliferation, and on macrophage microbicidal activity and cytokine production (7). To elucidate the molecular mechanisms underlying LPS stimulation and the modulatory action by IL-10 in PMN, we investigated whether LPS induces protein tyrosine phosphorylation and whether IL-10 affects this cell response.

MATERIALS AND METHODS

Materials. LPS (from *E.Coli*, serotypes 026B6 and 011B4), were purchased from Sigma (St.Louis, Mo, USA). IL-10 was a kind gift of Dr. K. Moore (DNAX and Schering-Plough Corporation, Palo Alto, CA, USA). Molecular weight standards were from Amersham (Little Chalfont, U.K.). Mouse IgG2b anti-phosphotyrosine monoclonal antibody from hybridoma 4G10 was from UBI (Lake Placid, N.Y., USA). Mouse IgG2b monoclonal anti-phosphotyrosine antibody PY20 was from Transduction Laboratories (Lexington, KY, USA). ERK 1(691), an affinity-purified rabbit polyclonal antibody raised against a peptide which corresponds to amino acids 305 to 327 within the XI subdomain of ERK 1 protein and reacting with both the 43-kDa ERK 1, the 41-kDa ERK 2, and a third ERK protein of 45 kDa, was from Santa Cruz Biotechnology. (Santa Cruz, CA, USA).
Granulocyte preparation and activation. Highly purified human PMN (>99.5%) were isolated under endotoxin-free conditions, as previously described (8). Immediately after purification, PMN were resuspended at 10^7 cells/ml in RPMI-1640 medium supplemented with antibiotics and 10% low-endotoxin FCS (LPS content <0.006 ng/ml, Hyclone, Logan, UT), in the presence or absence of 100 U/ml IL-10 (2,3), and cultured at 37°C/5% CO_2 in 6-well tissue culture plates (Greiner, Frickenhausen, Germany). After 10 min, PMN were stimulated for the times indicated. Incubations were stopped by diluting the cells with a 5-fold excess of ice-cold PBS containing 1 mM PMSF, 2 mM Na_3VO_4, 10 μM phenylarsine oxide (PAO). PMN were then centrifuged (500 x g, 7 min at 4°C), lysed in 0.5 ml of solubilization buffer [25 mM Tris pH 7.5, 150 mM NaCl, 1%(v/v) Triton X-100, 1%(w/v) sodium deoxycholate, 0.1%(w/v) SDS], containing 2 mM Na_3VO_4, 10 μM PAO, 5 μg/ml pepstatin, 5 μg/ml leupeptin, 1 mM EDTA, 1 mM PMSF, and 2 mM Di-isopropyl fluorophosphate, extracted for 30 min on ice, and centrifuged at 12000 x g for 5 min to remove insoluble material. Samples were prepared for electrophoresis by adding concentrated sample buffer [60 mM Tris/HCl, pH 6.8, 20%(v/v) glycerol, 4%(w/v) SDS, 2%(v/v) 2-mercaptoethanol]. Protein content was measured by the method of Bradford (9).

Electrophoresis and immunoblotting. Aliquots of lysates (50 μg) from resting and treated PMN were subjected to SDS/PAGE according to Laemmli (10), and electroblotted as previously described (11). The blots were incubated for 120 min in TBS buffer (50 mM Tris, pH 7.5, 170 mM NaCl) containing 5%(w/v) BSA, and 0.2%(v/v) NP40 (blocking buffer), before an overnight incubation at 4°C with 2 μg/ml 4G10 or ERK 1(691) antibodies in blocking buffer. After various washings with TBS containing 0.2%(v/v) NP40, blots were incubated for 60 min with horseradish peroxidase-labelled anti-rabbit or anti-mouse IgG (Amersham), diluted 1:15000 and 1:2000, respectively, in blocking buffer. After several washes, bound antibodies were revealed by enhanced chemiluminescence detection reagents (Amersham), and stripped as described (11).
Immunoprecipitations. These were performed as previously described (12). Briefly, PMN lysates were incubated for 60 min at 4°C under rotation with 5 μg of PY20 conjugated to trisacryl-protein A (Pierce, Rockford, IL), and washed thrice with 700 μl of ice-cold solubilization buffer. Immunoprecipitated proteins were eluted by boiling the beads for 3 min at 95°C in 50 μl of electrophoresis sample buffer. The beads were pelleted by centrifugation and supernatants were subjected to SDS/PAGE and immunoblotting as described above.

RESULTS AND DISCUSSION

It has been reported that stimulation of PMN with various agonists such as inflammatory microcristals, C5a, fMLP, IL-8, leukotriene B_4, platelet-activating factor, immunocomplexes, TNFα, and GM-CSF leads to tyrosine phosphorylation of a number of proteins (13, and references therein). We therefore examined whether LPS exerted a similar action. Fig. 1 shows a representative experiment in which human PMN were incubated for up to 60 min with 1 μg/ml LPS or its diluent; the corresponding cell lysates were then prepared and analyzed by antiphosphotyrosine immunoblotting. LPS increased PMN protein tyrosine phosphorylation in a time-dependent manner, a maximal effect being observed at 60 min. Specifically, LPS increased the tyrosine phosphorylation of a group of proteins with apparent molecular weights of 200-230, 108-125, 86, 63, 59-60, 52-56, 46-49, as well as that of 40-45 kDa species. Among these proteins, the most prominently phosphorylated in tyrosine were the 63, 59-60, and 46-49 kDa species. Dose response studies performed after 30 and 60 min of LPS stimulation demonstrated that protein tyrosine phosphorylation was optimally induced using 0.1-1 μg/ml LPS (Fig. 2).

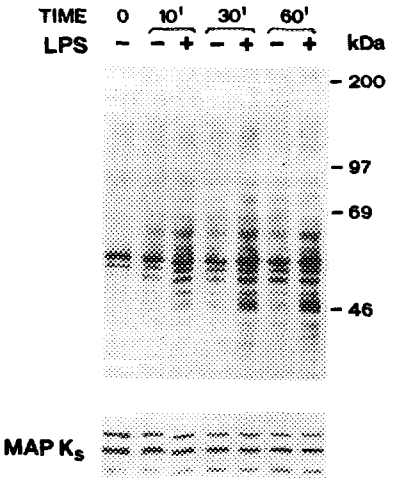

Figure 1. Kinetics of LPS-induced protein tyrosine phosphorylation in human granulocytes.

PMN (10^7/ml) were treated with 1 μg/ml LPS or its diluent for the indicated times. The cells were lysed as described in the Materials and Methods, and the detergent-soluble proteins were separated by SDS-PAGE on 10 % and analyzed by immunoblotting with anti-phosphotyrosine antibody 4G10. The same blot was stripped and reprobed with monoclonal anti-MAP kinase (MAPK) antibodies, to show that equal amounts of protein were loaded onto each lane of the gel. The figure shows one experiment representative of five.

Similar results were obtained with another serotype of LPS (011B4), or by using different antiphosphotyrosine antibodies such as PY20 (not shown).

In untreated granulocytes, the same pattern of tyrosine phosphorylated proteins (Figs. 1 and 2) were observed whether PMN were suspended in HBSS/Hepes containing 0.5 mM $CaCl_2$ and 5 mM glucose or in serum-free RPMI-1640, and then either plated in tissue culture wells precoated or not with FCS, or cultured under continous shaking, i.e. in suspension (data not shown). The latter observations suggested that the basal levels of tyrosine phosphorylation in untreated PMN were neither due to nonspecific cell activation by substances present in the serum, nor to adherence of the cells to plastic. In addition, the pattern of tyrosine phosphorylation induced in PMN by 1 μg/ml LPS was not influenced by the culture conditions or by the absence of serum (not shown). The latter observations are consistent with previous results obtained with human macrophages (14), where similar high

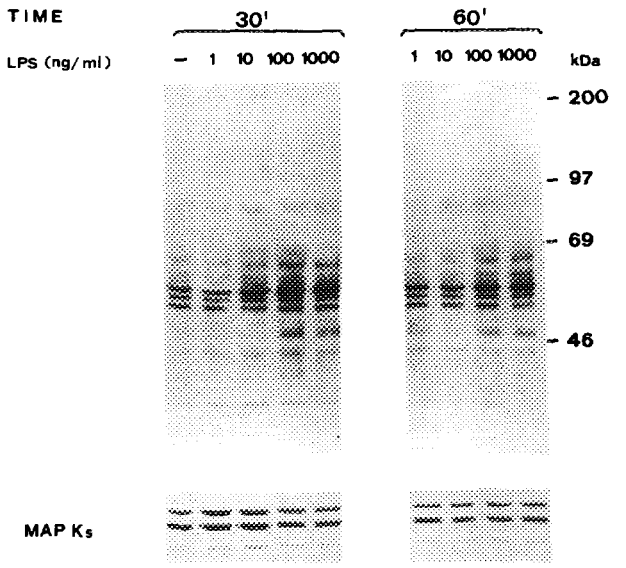

Figure 2. Dose response of LPS-induced protein tyrosine phosphorylation in human granulocytes.

PMN (10^7/ml) were treated for 30 and 60 min with the indicated concentrations of LPS. Whole cell lysates were prepared and analyzed by immunoblotting using anti-phosphotyrosine (4G10) and anti-MAPK antibodies. Similar results were seen in another experiment.

doses of LPS induced phosphorylation independently from CD14. CD14 has been in fact shown to serve as a receptor for complexes of low doses of LPS with LPS binding proteins (15).

Next, we investigated tyrosine phosphorylation patterns in PMN pretreated for 10 min with 100 U/ml IL-10, and then stimulated with LPS. This IL-10 dose was previously found to optimally inhibit cytokine release in PMN (2,3). Fig. 3A shows that tyrosine phosphoproteins were less intensely phosphorylated in IL-10-treated granulocytes than in untreated cells. The extent of this IL-10-mediated decrease was not consistently observed, and varied depending on the donor. In PMN stimulated for 60 min with LPS, however, the increase in protein tyrosine phosphorylation was substantially downregulated by IL-10. The inhibitory effect of IL-10 also varied in amplitude depending on the donor, was significantly only after 60 min of stimulation, and was particularly pronounced for phosphoproteins whose apparent molecular

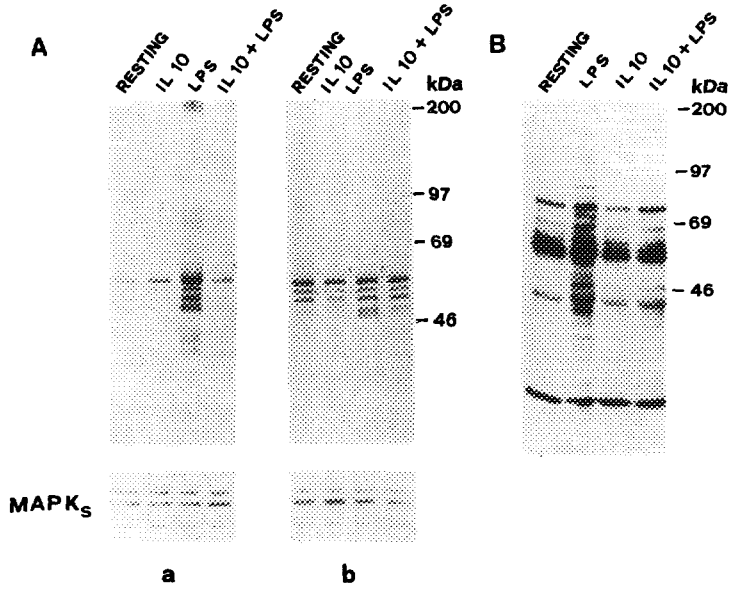

Figure 3. IL-10 inhibition of LPS-induced protein tyrosine phosphorylation in human granulocytes. PMN were incubated with 100 U/ml IL-10 for 10 min and then stimulated with 1 μg/ml LPS for 60 min. (A): whole cell lysates were prepared and analyzed by immunoblotting using anti-phosphotyrosine (4G10) and anti-MAPK antibodies. Two experiments (a and b) representative of four are shown; (B): PMN were extracted in solubilization buffer, immunoprecipitated with antiphosphotyrosine mAb PY20, and then immunoblotted with mAb 4G10. This experiment is representative of three.

weights ranged from 46 to 49 kDa (Fig. 3A). In another series of experiments, we immunoprecipitated PMN lysates with anti-phosphotyrosine antibodies (PY20), and revealed them with 4G10. Fig. 3B shows that, in lysates of either resting or LPS-treated PMN, PY20-immunoprecipitated phosphoproteins were similar to those detected in immunoblot by 4G10 (compare with Figs. 1, 2 and 3A). Some differences in the extent of phosphorylation were noted, which might be attributed to the different affinities of the two antibodies for the aminoacids sequences flanking the tyrosines. These experiments confirmed that pretreatment of PMN with IL-10 caused an inhibition of LPS-induced tyrosine phosphorylation after 60 min, including that of the 46-49 kDa phosphoproteins (Fig. 3B). On the basis of their masses, we exclude that the latter phosphoproteins belong to members of the src family of tyrosine kinases present in PMN (16), such as

$p56^{lyn}$, $p58^{hck}$, $p59^{c-fgr}$, and $p60^{src}$. In human monocytes LPS was found to activate the src family tyrosine kinase $p56^{lyn}$ as well as $p58^{hck}$ and $p59^{c-fgr}$(17-20). Furthermore, LPS-induced activation of $p56^{lyn}$ as well as $p58^{hck}$ in monocytes was found to be inhibited by IL-10 (21).

The above data demonstrate for the first time that LPS increases the tyrosine phosphorylation of several proteins in human granulocytes (22). LPS exerts a number of biological actions towards PMN, such as the potentiation of the respiratory burst (23), the up-regulation of CR3 receptor expression (24), the modulation of NADPH oxidase gene expression (8,25), and the release of cytokines (1). Although we did not attempt to correlate LPS-induced protein tyrosine phosphorylation with any of these responses, our results nonetheless suggest that tyrosine phosphorylation might play an important role in LPS signal transduction in PMN. Interestingly, tyrosine kinase activation appeared to be essential for the LPS induction of cytokine gene expression and protein production in monocytes/ macrophages (21,22,26,27). Thus, further studies are required to verify whether the same events are important in PMN for LPS-induced cyotokine release and for its modulation by IL-10.

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