Effect of lipopolysaccharide on mitogen-activated protein kinases and cytosolic phospholipase A₂

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The addition of platelet-activating factor (PAF) to human neutrophils increases phosphorylation on tyrosine residues and stimulates the activity of p42^{erk2} mitogen-activated protein kinase (MAP kinase). This action is rapid and transient. In contrast, p42^{erk2}, p44^{erk1} and the p40^{hera} MAP kinase isoforms are all not tyrosine phosphorylated or activated in human neutrophils stimulated with low concentrations of lipopolysaccharide (LPS) in combination with serum. In spite of this, the PAF-induced tyrosine phosphorylation and activation of the p42^{erk2} MAP kinase are greatly potentiated in cells pretreated with LPS. More interestingly, although low concentrations of LPS do not affect MAP kinase isoforms in these cells, they cause the

phosphorylation of cytosolic phospholipase A₂ (cPLA₂), as evidenced by a decrease in the electrophoretic mobility of the enzyme. In addition, this stimulus-induced upward shift in the mobility of the enzyme is not inhibited by the tyrosine kinase inhibitor, genistein. Furthermore, LPS increases the release of arachidonic acid in control and PAF-stimulated human neutrophils. These observations clearly show that cPLA₂ can be phosphorylated and activated by kinases other than the currently known MAP kinases. It is proposed that there are MAP kinase-dependent and -independent mechanisms for the phosphorylation of cPLA₂.

INTRODUCTION

Platelet-activating factor (PAF) possesses multiple biological activities that encompass the fields of allergic and inflammatory reactions as well as the regulation of the cardiovascular and haemodynamic systems [1]. The cellular targets of PAF are similarly varied and include, among others, platelets, lung tissue, smooth muscle, hepatocytes, macrophages and neutrophils. In the neutrophil, PAF elicits various responses such as chemotaxis, degranulation, aggregation and the oxidative burst [1–6]. In addition, it elicits several biochemical changes, including hydrolysis of phosphoinositides, a rise in the cytoplasmic level of free Ca²⁺, an increase in tyrosine phosphorylation of several proteins, an increase in cytoskeletal actin, the activation of the Na⁺/H⁺ antiport, the release of arachidonic acid and a change in membrane potential [1–7]. The actions of PAF are mediated through G-protein-coupled receptors [1,8–11]. The cloning and expression of a high-affinity human PAF receptor has been reported [11]. When transfected into COS cells, the cloned DNA confers high-affinity binding sites for PAF, and the ligand-binding properties of this human PAF receptor are similar to those observed in neutrophils [11].

Phosphorylation and dephosphorylation of proteins mediate the actions of many hormones and growth factors. Phosphorylation and dephosphorylation also regulate the activities of many cellular kinases, phospholipases and other enzymes. Most protein phosphorylation occurs on serine and threonine but in some cases tyrosine residues are phosphorylated [12]. Tyrosine phosphorylation plays a crucial role in the control of cell proliferation and differentiation [12,13]. Phosphotyrosine kinases and phosphatases are pivotal enzymes in the control of various cell functions [12,13]. It has been shown that the addition of PAF

to human neutrophils increases the tyrosine phosphorylation of several proteins ranging in molecular mass between 42 and 116 kDa [7]. The identities of these proteins are not known.

Mitogen-activated protein kinase (MAP kinase) is a serine/threonine kinase originally described in 3T3 L1 cells. This kinase system becomes active on stimulation of these cells with various agents [14–18]. There are four forms of this enzyme system (p40^{hera}, p42^{erk2}, p44^{erk1} and p46^{erk4}) in mammals [14–18]. The activation of MAP kinase is an early response to a wide variety of growth factors that mediate their actions through receptors that contain tyrosine kinase domains [14–18]. There are several substrates for the MAP kinase system, including ribosomal S6 kinase (p90^{rsk}), the transcription factors c-Jun and c-Fos and others [14–18]. It has recently been hypothesized that cytoplasmic phospholipase A₂ (cPLA₂) is also a substrate for MAP kinase [19].

Lipopolysaccharide (LPS) from Gram-negative bacteria exerts profound effects on the host immune system [20,21]. The exposure of neutrophils to LPS primes the cells for enhanced release of microbicidal metabolites [20,21]. The increase in the oxidative burst might permit increased resistance to bacterial infection yet predisposes the host to increased oxidative tissue damage and the release of various inflammatory mediators. Although the generated inflammatory mediators are critical to the host defence role of the neutrophils, they can cause severe tissue damage when excessively or inappropriately generated. Neutrophil-induced injury has been implicated in the pathogenesis of many disease processes such as septic shock, myocardial infarction and adult respiratory distress syndrome [22].

The present studies were undertaken to address four questions. First, what is the identity of the 42 kDa protein that undergoes increased phosphorylation on tyrosine during stimulation of

Abbreviations used: LPS, lipopolysaccharide; cPLA₂, cytosolic phospholipase A₂; PAF, platelet-activating factor; MAP kinase, mitogen-activated protein kinase; α-PY, anti-phosphotyrosine antibody; α-p42 and α-p44 MAP kinase antibodies refer to antibodies against p42^{erk2} and p44^{erk1} MAP kinase respectively; HBSS, Hanks balanced-salt solution; PVDF, poly(vinylidene difluoride).

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neutrophils with PAF? Second, does the activity of this protein increase after tyrosine phosphorylation? Third, does the addition of LPS in combination with serum, without a second stimulus, increase the tyrosine phosphorylation and activity of any of the MAP kinase isoforms, and, if not, does it potentiate agonist-induced tyrosine phosphorylation and activity of any of the MAP kinase isoforms? Fourth, can cPLA₂ be phosphorylated independently of the tyrosine phosphorylation and activation of known MAP kinase isoforms?

MATERIALS AND METHODS

Isolation of human neutrophils

Neutrophils were isolated from normal human donors using a Ficoll/Hypaque gradient by the method of English and Andersen [23], and the contaminating red blood cells were lysed by hypotonic shock [24]. The neutrophils were resuspended in modified Hanks balanced-salt solution (HBSS) containing 0.1 % BSA and 10 mM Hepes, pH 7.35.

Materials

LPS and PAF were purchased from Difco Laboratories (Detroit, MI, U.S.A.) and Calbiochem (La Jolla, CA, U.S.A.) respectively. Anti-phosphotyrosine (α -PY), a monoclonal antibody derived from mouse hybridoma 4G10, anti-(MAP kinase R3) (α -p44 MAP kinase), a polyclonal antibody specific for the 44 kDa MAP kinase, anti-(MAP kinase erk 2) (α -p42 MAP kinase), a monoclonal antibody specific for the 42 kDa MAP kinase, anti-cdc2 (α -cdc2), a polyclonal antibody that recognizes the p40^{hera} MAP kinase isoform, were obtained from Upstate Biotechnology Inc. (UBI; Lake Placid, NY, U.S.A.). Poly(vinylidene difluoride) (PVDF) protein-transfer membrane (Immobilon-P) was from Millipore Corp. (Bedford, MA, U.S.A.). Electrophoresis reagents and molecular-mass markers were from Bio-Rad Laboratories (Melville, NY, U.S.A.). Enhanced chemiluminescence (ECL) Western-blotting reagents and [γ -³²P]ATP (3000 Ci/mmol) were purchased from Amersham (Arlington Heights, IL, U.S.A.). All other reagents were from Sigma (St. Louis, MO, U.S.A.). Anti-(phospholipase A₂) (α -cPLA₂) antibody was generously provided by John Knopf (The Genetics Institute, Cambridge, MA, U.S.A.).

Immunoblotting

Immunoblotting was performed as described previously [24,25]. Briefly, cells (1×10^7 /ml) were stimulated with diluent, PAF, LPS or LPS in combination with PAF at 37 °C and the reaction was stopped by rapid centrifugation (5 s using a table-top Eppendorf 3200 centrifuge). Pellets were resuspended in 40 μ l of ice-cold HBSS containing 2 mM sodium orthovanadate, 10 mM sodium pyrophosphate, 1 mM PMSF, 1 mM EGTA, 10 mM NaF, 10 mg/ml leupeptin and 10 mg/ml aprotinin; they were then mixed 1:1 with Laemmli sample buffer (8 % SDS, 10 % 2-mercaptoethanol, 10 % glycerol, 66 mM Tris/HCl, pH 6.8, 0.05 % Bromophenol Blue), heated at 100 °C for 10 min and loaded on to an SDS/8 % polyacrylamide gel. After electrophoresis, proteins were electrophoretically transferred from the gel to PVDF membranes in transfer buffer (20 mM Tris base, 150 mM glycine, 20 % methanol, pH 8.9) at 4 mA/cm² for 3 h at 4 °C. Residual binding sites on the membrane were blocked by incubating the membrane in Tris-buffered saline/Tween (TBS-T) (20 mM Tris base, pH 7.6, 137 mM NaCl, 0.1 % Tween 20) containing 5 % BSA for either 1 h at room temperature or overnight at 4 °C. Blots were washed in TBS-T and then incubated with the appropriate antibody. Incubation time was 2 h at

room temperature for α -PY and α -p44 MAP kinase antibody and 1 h at room temperature for the α -p42 MAP kinase antibody. Blots were again washed with TBS-T and then incubated with the appropriate secondary antibody (either horseradish peroxidase-conjugated anti-mouse IgG or anti-rabbit IgG) for 20 min at room temperature. The enhanced chemiluminescence method was utilized for detection. Where appropriate, blots were stripped of the primary antibody-secondary antibody complex by incubation in stripping buffer (100 mM 2-mercaptoethanol, 2 % SDS, 62.5 mM Tris/HCl, pH 6.7) for 30 min at 50 °C and reprobed with a different antibody by the above procedure.

Immunoprecipitation

Immunoprecipitation was carried out by the method of Campos-Gonzalez and Glenney [26] with slight modifications [24]. Briefly, neutrophils (5×10^7 /ml) were treated with diluent, PAF, LPS or LPS in combination with PAF. Reactions were terminated by rapid centrifugation; 100 μ l of boiling 1 % SDS was added to the pellets and samples were immediately boiled for 15 min. Next 400 μ l of ice-cold water and 500 μ l of 2 $\times$ lysis buffer (20 mM Tris/HCl, 300 mM NaCl, 2.0 % Triton X-100, 1.0 % Nonidet P40, 2 mM EDTA, 2 mM EGTA, 2 mM PMSF, 4 mM sodium orthovanadate, 20 mg/ml leupeptin, 20 mg/ml aprotinin) were then added to the samples. After centrifugation at 15000 *g* for 10 min, supernatants were transferred to tubes containing anti-mouse or anti-rabbit IgG agarose beads to which the appropriate antibody had been conjugated. Briefly, conjugation consisted of the following procedure: anti-mouse or anti-rabbit IgG agarose beads were rinsed with 1 $\times$ lysis buffer and then incubated with 500 μ l of 1 $\times$ lysis buffer containing 10 mg/ml BSA and either α -PY or α -p42 MAP kinase antibody. After overnight incubation at 4 °C, the beads were washed with 1 $\times$ lysis buffer and resuspended in 100 μ l of 1 $\times$ lysis buffer. Lysate-bead-antibody complexes were incubated for 2 h at 4 °C for α -p42 MAP kinase antibody or 4 h at 4 °C for α -PY antibody. After incubation, the complexes were centrifuged at 15000 *g* for 5 min and the supernatants were removed. The immune complexes were then washed with buffer (25 mM Tris/HCl, 150 mM NaCl, 2 mM EDTA, pH 7.4), dissociated by addition of 50 μ l of Laemmli sample buffer, boiled for 5–10 min, and loaded on to an SDS/8 % polyacrylamide gel. Proteins were transferred and analysed by immunoblotting as described above.

Kinase assay

Kinase activity was determined as previously described [27] with minor modifications [25]. Briefly, neutrophils were treated with buffer diluent, PAF, LPS or LPS in combination with PAF; reactions were terminated by rapid centrifugation. Cell pellets were lysed in lysis buffer (50 mM Hepes, 150 mM NaCl, 10 % glycerol, 10 mM EDTA, 1 mM MgCl₂, 2 mM sodium orthovanadate, 10 mM sodium pyrophosphate, 1 mM PMSF, 10 mM NaF, 10 mg/ml aprotinin, 10 mg/ml leupeptin, 1 % Nonidet P40), and equivalent amounts of lysate or immunoprecipitate were electrophoresed on an SDS/8 % polyacrylamide gel containing 0.5 mg/ml myelin basic protein. After electrophoresis, the gel was washed for 1 h at room temperature with three changes of buffer 1 (50 mM Tris/HCl, pH 8.0, and 20 % propan-2-ol). It was then treated for 1 h with buffer 2 (50 mM Tris/HCl, pH 8.0, 6 M guanidinium chloride, 5 mM 2-mercaptoethanol), followed by incubation in buffer 3 (50 mM Tris/HCl, pH 8.0, 0.04 % Tween 40, 5 mM 2-mercaptoethanol) for 16 h at 4 °C, the buffer being changed several times. The gel was then incubated with kinase buffer (40 mM Hepes, 2 mM dithiothreitol, 0.1 mM EGTA, 5 mM magnesium acetate, pH 8.0) for 30 min at room

temperature and then in kinase buffer containing 20 mM ATP and 50 μ Ci of [γ -³²P]ATP for 1 h at room temperature. Finally, the gel was washed for approx. 2–3 h with 5% trichloroacetic acid containing 1% sodium pyrophosphate, dried and autoradiographed at –70 °C using Kodak X-ray film and an intensifying screen.

Cell fractionation

Cell fractionation for determining the upward shift in the molecular mass of cPLA₂ was carried out as described previously [25]. Briefly, 3 ml of cells (10^7 /ml) were treated with diluent or LPS and the reaction terminated by the addition of a 10-fold amount of cold HBSS. The cells were then resuspended at 10^8 /ml in a buffered sucrose solution [10 mM Hepes (pH 7.4), 0.25 M sucrose, 1 mM EGTA, 50 μ g/ml leupeptin, 1 mM di-isopropyl fluorophosphate and 1 mM PMSF], and disrupted by sonication. This homogenate was centrifuged at 8000 *g* for 15 min and the resulting supernatant was then centrifuged at 100 000 *g* for 30 min at 4 °C. Both the pellet (membrane fraction) and the supernatant (cytosol fraction) were saved.

Arachidonic acid release

Labelling the cells with [³H]arachidonic acid and the release of radiolabelled arachidonic acid were carried out as described previously [25]. Briefly, the cells (5×10^7 /ml) were incubated with [³H]arachidonic acid (2.5 mCi/ml; the ethanol in the labelled arachidonic acid was evaporated under a stream of nitrogen) in HBSS containing 0.1 mg/ml fatty acid-free BSA for 1 h at 37 °C. The cells were washed three times, resuspended (1×10^7 /ml), and then stimulated with the appropriate agent. Hexane/propan-2-ol (3:2, v/v) was added to stop the reaction. The samples were mixed and the phases separated by centrifugation. The organic phase was removed and dried under a stream of nitrogen. Neutral lipids were separated on silica-gel-60-precoated TLC plates. A known amount of arachidonic acid (1.75 μ g) was added at the origin with each sample to aid the detection of the lipid with I₂ vapour. The plates were developed in chloroform/acetone (24:1, v/v). After exposure to vapour, the arachidonic acid spot was circled with pencil, the plate was sprayed lightly with water, and the corresponding silica-gel spot carefully scraped from the plate and assayed for radioactivity.

RESULTS

Tyrosine phosphorylation of proteins and identification of the 42 kDa protein in human neutrophils stimulated with PAF

The addition of PAF to human neutrophils induces a rapid increase in tyrosine phosphorylation of several proteins (Figure 1a). The arrows indicate the proteins that are phosphorylated consistently. When the immunoblot from which the data in Figure 1(a) were generated is stripped and reprobed with either α -p42 MAP kinase antibody (Figure 1b) or α -p44 MAP kinase antibody (Figure 1c) a doublet is observed. This upward shift in the molecular mass is due to the phosphorylation of the protein. This suggests that the tyrosine-phosphorylated 42 and 44 kDa proteins are p42^{erk2} and p44^{erk1}, two isoforms of MAP kinase. Note that a protein with a molecular mass of 40 kDa is also heavily phosphorylated in human neutrophils stimulated with PAF. The tyrosine phosphorylation of p44 is much less than that of p42 or p40.

To characterize the tyrosine phosphorylation of the 42 kDa protein further and to determine unequivocally that this protein is p42^{erk2} MAP kinase, we carried out time-course and immuno-

Figure 1 Tyrosine phosphorylation of proteins in human neutrophils stimulated with PAF

Lane 1, control cells; lane 2, cells stimulated with 100 nM PAF for 2 min. After stimulation, the cells were lysed and the lysate was subjected to SDS/PAGE (12% gels). Proteins were then transferred to a Millipore membrane and immunoblotted with α -PY (a). Detection was by the Amersham ECL system. Molecular-mass standards are given in kDa. Arrows indicate the positions of the proteins that were consistently phosphorylated. The amount of protein (100 μ g) loaded on each lane was the same. The immunoblot was then stripped and reprobed with either α -p42 MAP kinase monoclonal antibody (b) or α -p44 MAP kinase polyclonal antibody (c). The lower and upper arrows in (b) refer to the non-phosphorylated and phosphorylated forms of p42^{erk2} MAP kinase respectively. Similar identification for p44^{erk1} MAP kinase is used in (c). The autoradiographs represent a single experiment that was carried out several times with similar results.

Figure 2 Autoradiographs showing the time course of PAF-induced tyrosine phosphorylation of p42^{erk2} MAP kinase in human neutrophils

Lane 1, control cells; lanes 2–4, cells stimulated with 100 nM PAF for 0.5, 1 and 3 min respectively. (a) and (b), immunoblots probed with α -PY and α -p42 MAP kinase antibodies respectively. The amount of protein (100 μ g) loaded on each lane was the same. The autoradiographs represent a single experiment that was carried out several times with similar results.

precipitation experiments. Both the tyrosine phosphorylation and doublet formation of the 42 kDa protein are rapid and transient, reaching maximum responses 1 min after the addition of PAF (Figures 2a and 2b). In the case of immunoprecipitation, neutrophils were stimulated with buffer (HBSS) or 100 nM PAF for 1 min. Reactions were terminated by rapid centrifugation, and p42 MAP kinase was immunoprecipitated utilizing a denaturing protocol as described in the Materials and methods section. The antibody used to immunoprecipitate this protein was α -p42 MAP kinase, a monoclonal antibody specific for the 42 kDa MAP kinase. After SDS/PAGE, proteins were transferred to PVDF membranes and blots were probed with either

Figure 3 Autoradiographs of immunoprecipitation and immunoblotting of neutrophil lysates prepared from control and PAF-stimulated human neutrophils

Cells were incubated with 100 nM PAF (+) or buffer (–) for 1 min at 37 °C. Lysates corresponding to equal numbers of cells were immunoprecipitated with α -p42 MAP kinase antibody (a, b, c) or α -PY antibody (d). The immunoprecipitates were recovered with conjugated agarose beads, subjected to SDS/PAGE, and transferred to PVDF sheets or subjected to an *in situ* gel-kinase assay (c). The blots were probed with α -PY (a) or α -p42 MAP kinase (b, d) antibody. The amount of immunoprecipitate derived from 500 μ l of cell suspension (5×10^7 cells/ml) was loaded on each lane. The autoradiographs represent a single experiment carried out several times with identical results.

Figure 4 Autoradiographs showing that LPS primes the PAF-induced tyrosine phosphorylation of p42^{erk2} MAP kinase

Neutrophils at 37 °C were treated with buffer (lane 1), 10 nM PAF for 1 min (lane 2), 50 ng/ml LPS in combination with 1% serum for 30 min (lane 3) or LPS + serum followed by 10 nM PAF for 1 min (lane 4). Immunoprecipitates prepared using α -p42 MAP kinase (a, b, c) or α -PY (d) antibody were immunoblotted with α -PY (a) and α -p42 MAP kinase (b, d) antibodies or used for an *in situ* gel kinase assay (c). The amount of immunoprecipitate derived from 500 μ l of cell suspension (5×10^7 cells/ml) was loaded on each lane. The autoradiographs are from a single experiment carried out several times.

α -PY or α -p42 MAP kinase antibody. As shown in Figure 3(a), α -p42 MAP kinase antibody immunoprecipitates a 42 kDa protein that can be recognized by α -PY antibody in cells stimulated by PAF. To demonstrate that the p42 MAP kinase was immunoprecipitated, duplicate samples were probed with α -p42 MAP kinase antibody (Figure 3b). The top half of the doublet is aligned with the α -PY spot in the α -PY-probed film.

To substantiate further that this 42 kDa protein is indeed p42^{erk2} MAP kinase, immunoprecipitation with α -PY antibody was performed. Neutrophils were stimulated with buffer (HBSS) or 100 nM PAF for 1 min. Reactions were terminated and tyrosine-phosphorylated proteins were immunoprecipitated by a denaturing protocol as described. After SDS/PAGE and transfer of proteins to PVDF membranes, the blot was probed with α -p42 MAP kinase antibody. As shown in Figure 3(d), α -PY antibody

immunoprecipitates a 42 kDa apotein that can be recognized by α -p42 MAP kinase antibody in neutrophils stimulated with PAF.

In order to show that the tyrosine phosphorylation of p42^{erk2} MAP kinase increases its activity, an *in situ* gel kinase activity assay was utilized. Neutrophils (1×10^7) were treated with buffer (HBSS) or 100 nM PAF for 1 min at 37 °C. After the reaction was terminated by rapid centrifugation, the p42 MAP kinase was immunoprecipitated using α -p42 MAP kinase antibody as described in the Materials and methods section. Known amounts of immunoprecipitates were electrophoresed through an SDS/8% polyacrylamide gel containing 0.5 mg/ml myelin basic protein. After electrophoresis, gels were washed and subjected to a kinase activity assay as described in the Materials and methods section. An identical sample of the immunoprecipitate was electrophoresed under the same conditions, transferred to PVDF membrane and probed with either α -p42 MAP kinase or α -PY antibody. The results were identical with those in Figures 3(a) and 3(b). The data summarized in Figure 3(c) show clearly that α -p42 MAP kinase antibody immunoprecipitates a 42 kDa protein with kinase activity that is increased in human neutrophils stimulated with 100 nM PAF. The data summarized in Figure 3 show clearly that the addition of 100 nM PAF to human neutrophils increases the tyrosine phosphorylation and activity of p42^{erk2} MAP kinase.

LPS involvement in the PAF-induced tyrosine phosphorylation and activation of the p42^{erk2} MAP kinase

It is generally found that the exposure of neutrophils to LPS primes the cells for enhanced chemotactic-factor-induced responses. In order to determine if the priming action of LPS is distal or proximal to the tyrosine phosphorylation of p42^{erk2} MAP kinase, we measured the PAF-induced tyrosine phosphorylation and activation of this protein in cells pretreated with LPS in combination with serum. In these experiments, the cells were incubated with 50 ng/ml LPS + 1% serum [the presence of serum is essential for low concentrations (< 100 ng/ml) of LPS to have any effect] for 30 min at 37 °C and then stimulated with 10 nM PAF for 1 min. This low concentration of PAF was chosen to reduce the effect of PAF by itself. After the reactions were terminated, immunoprecipitates were prepared using either α -p42 MAP kinase or α -PY antibody. The immunoprecipitates were subjected to SDS/PAGE and then probed with either α -p42 MAP kinase or α -PY antibody. The results, which are summarized in Figure 4, clearly show that α -p42 MAP kinase antibody immunoprecipitates a protein with a molecular mass of 42 kDa that can be recognized by α -PY antibody in cells pretreated with LPS and then stimulated with PAF (Figure 4a, lane 4). Conversely, α -PY antibody immunoprecipitates a 42 kDa protein that can be recognized by α -p42 MAP kinase in cells pretreated with LPS and then stimulated with PAF (Figure 4d, lane 4). Moreover, whereas this low concentration of PAF has only a minimal effect (Figure 4a, lane 2) and LPS has no effect (Figure 4a, lane 3) in control cells, the addition of this concentration of PAF to neutrophils pretreated with LPS for 30 min increases greatly the tyrosine phosphorylation of p42^{erk2} MAP kinase. Note that lysates immunoprecipitated and probed with α -p42 MAP kinase show an electrophoretic mobility shift in the LPS-primed PAF-stimulated sample (Figure 4b, lane 4). This priming requires that the cells be incubated with LPS for at least 15 min before stimulation with PAF (results not shown).

In order to show that the priming by LPS of the PAF-induced tyrosine phosphorylation of p42^{erk2} MAP kinase is associated with an increased activity of the enzyme, the activity in immunoprecipitates prepared using α -p42 MAP kinase antibody was

Figure 5 Autoradiographs showing that LPS does not increase the tyrosine phosphorylation of either p42^{erk2} or p44^{erk1} MAP kinase isoforms

Neutrophils at 37 °C were treated with buffer (lane 1) or 200 ng/ml LPS in combination with 1% serum for 30 min (lane 2) or 100 nM PAF for 1 min (lane 3). The effect of PAF is included as a positive control. Immunoprecipitates prepared using α-PY (a, b) or α-p42 MAP kinase (c, d) antibody were subjected to SDS/PAGE and then immunoblotted with α-p42 MAP kinase (a, c) α-p44 MAP kinase (b) or α-PY (d) antibody. The amount of immunoprecipitate derived from 500 μl of cell suspension (5 × 10⁷ cells/ml) was loaded on each lane. The autoradiographs are from a single experiment carried out several times.

measured by the *in situ* gel kinase assay with myelin basic protein as substrate. Immunoprecipitates were prepared from control or LPS-treated (50 ng/ml LPS in combination with serum for 30 min) cells that had been stimulated with 10 nM PAF for 1 min. The results, which are summarized in Figure 4(c), show that, whereas this low concentration of PAF produces little activation of p42^{erk2} MAP kinase in control cells, it increases greatly this activity in LPS-treated cells (lane 4). This priming requires that the cells are incubated with LPS and serum for at least 15 min (results not shown).

Effect of LPS on the tyrosine phosphorylation and activity of p42^{erk2} and p44^{erk1} MAP kinase isoforms

While examining the priming action of LPS on PAF-induced tyrosine phosphorylation and activation of the p42^{erk2} MAP kinase, it was observed that the addition of LPS, without PAF, does not increase the tyrosine phosphorylation of either the 42 or 44 kDa protein. This was quite unexpected as it has been reported that the addition of LPS to macrophages causes a rapid increase in the tyrosine phosphorylation of both the p42^{erk2} and p44^{erk1} MAP kinase isoforms [28]. In order to examine whether the addition of LPS, without a second stimulus, to human neutrophils increases the phosphorylation on tyrosine residues and activity of MAP kinase isoforms, we carried out two sets of experiments. In the first, the neutrophils were stimulated with either diluent or 200 ng/ml LPS in combination with serum for 30 min [higher concentrations up to 500 ng/ml and different times (1–45 min), of stimulation with LPS were examined and the results were the same] or 100 nM PAF for 1 min (this concentration of PAF was used as a positive control). After the reactions were terminated, the cells were lysed and immunoprecipitates were prepared using α-p42 MAP kinase or α-PY antibody. The immunoprecipitates were run on an SDS/polyacrylamide gel and then probed with α-p42 MAP kinase, α-PY or α-p44 MAP kinase antibody. The results are summarized in Figure 5, and the following points can

Figure 6 Autoradiographs showing that LPS does not increase the phosphorylation of myelin basic protein

Neutrophils at 37 °C were treated with buffer (lane 1), 200 ng/ml LPS in combination with 1% serum for 30 min (lane 2) or 100 nM PAF for 1 min (lane 3). The effect of PAF is included as a positive control. The cells were lysed and the lysates were submitted to *in situ* gel kinase assay; myelin basic protein was used as substrate, and the reaction was carried out for 1 h as described in the Materials and methods section. The amount of protein (100 μg) loaded on each lane was the same. The autoradiographs are from a single experiment carried out several times.

be observed. First, α-PY antibody does not immunoprecipitate a 42 or 44 kDa protein that can be recognized by α-p42 MAP kinase (Figure 5a, lane 2) or α-p44 MAP kinase (Figure 5b, lane 2) antibody. Second, α-p42 MAP kinase antibody does not immunoprecipitate a 42 kDa protein that can be recognized by α-PY (Figure 5c, lane 2). Note that LPS, unlike PAF (Figure 5d, lane 3), does not show an electrophoretic mobility shift in p42 MAP kinase immunoprecipitates probed with α-p42 MAP kinase antibody (Figure 5d, lane 2).

In the second set of experiments, the neutrophils were stimulated with either diluent or 200 ng/ml LPS in combination with serum for 30 min [higher concentrations up to 500 ng/ml and different times (1–45 min) of stimulation with LPS were examined and the results were the same] or 100 nM PAF for 1 min (this concentration of PAF was used as a positive control). After the reactions were terminated the cells were lysed, and the total activities (in the region of 39–45 kDa) of MAP kinase isoforms in the lysates were measured by *in situ* gel kinase assay with myelin basic protein as substrate. The data show clearly that, whereas lysates prepared from PAF-treated cells (Figure 6, lane 3) show increased phosphorylation of myelin basic protein, those from LPS-stimulated cells (Figure 6, lane 2) show no detectable increase in the phosphorylation of this protein.

Although the addition of LPS in combination with serum, in the absence of a second stimulus, to human neutrophils does not increase the tyrosine phosphorylation of either p42^{erk2} or p44^{erk1} MAP kinase isoforms, we found that it causes a time- and dose-dependent increase in the tyrosine phosphorylation of a 40 kDa protein (results not shown). This effect is potentiated by okadaic acid (a phosphatase inhibitor), inhibited by herbimycin and genistein (tyrosine kinase inhibitors) and not inhibited in cells pretreated with pertussis toxin (results not shown).

Stimulation of the phosphorylation of cPLA₂ in human neutrophils by LPS

Recently, it has been shown that stimuli that increase the tyrosine phosphorylation of the MAP kinase family of proteins also increase the phosphorylation of cPLA₂, as evidenced by a reduction in the electrophoretic mobility of the enzyme [19,25]. Moreover, incubation of purified cPLA₂ with MAP kinase under phosphorylating conditions leads to the phosphorylation of cPLA₂ on Ser-505 [19]. It has been hypothesized on the basis of those results that activation of cPLA₂ in intact cells is mediated via its phosphorylation by MAP kinase [19,25]. In order to test whether cPLA₂ can be phosphorylated independently of the

Figure 7 Time course of the LPS-stimulated shift in the electrophoretic mobility of cPLA₂ in the cytosolic fraction obtained from human neutrophils stimulated with LPS in combination with serum

The absorbance (densitometric measurement of autoradiograph using α -cPLA₂ antibody) is expressed as the amount of cPLA₂ showing the mobility shift (to the upper band) as a percentage of the total signal (both upper and lower bands). The insert is an actual autoradiograph exhibiting the shift: A, control; B, cells stimulated with LPS (100 ng/ml) and serum for 30 min. No cPLA₂ band was detected in the membrane fraction (results not shown); this is expected as translocation of the enzyme to the membrane fraction requires the presence of Ca²⁺. Neutrophils at 37 °C were treated with buffer or 100 ng/ml LPS in combination with 1% serum for different times. After stimulation the cells were disrupted and fractionated. Samples from both cytoplasm and membrane fractions were subjected to SDS/PAGE, transferred and then probed with α -cPLA₂ antibody. The amount of protein (100 μ g) loaded on to each lane was the same. Each point represents the mean $\pm$ S.E.M. of at least three separate experiments.

phosphorylation on tyrosine residues and activation of the p42^{erk2} and/or p44^{erk1} MAP kinase isoforms, we examined the shift in electrophoretic mobility of cPLA₂ in control and LPS-stimulated human neutrophils. The rationale is that, if the tyrosine phosphorylation and activation of p42^{erk2} and p44^{erk1} MAP kinase isoforms are necessary for the phosphorylation of cPLA₂, then there should be no decrease in the electrophoretic mobility of cPLA₂ in human neutrophils stimulated with LPS, as this stimulus does not activate these isoforms. In these experiments, human neutrophils were stimulated with either diluent or 50 ng/ml LPS (higher concentrations were used and the results were the same) in combination with 1% serum for 5, 15 and 30 min. The reactions were stopped by centrifugation, the cells were disrupted by sonication and fractionated as described in the Materials and methods section. Samples from both the cytosol and membrane fractions were electrophoresed on SDS/polyacrylamide gels, transferred and then immunoblotted using α -cPLA₂ antibody as described in the Materials and methods section. The results summarized in Figure 7 clearly show that stimulation of human neutrophils with LPS in combination with serum, in the absence of a second stimulus, causes a decrease in the electrophoretic mobility of cPLA₂. This shift is slow in that it requires that the cells be stimulated with LPS for at least 15 min. Moreover, this LPS-induced decrease in the electrophoretic mobility of cPLA₂ is not inhibited in human neutrophils incubated for 1 h with genistein, a tyrosine kinase inhibitor (Figure 8). These results show clearly that phosphorylation of cPLA₂ can occur independently of the tyrosine phosphorylation and activation of MAP kinase.

In a separate set of experiments we measured arachidonic acid release in human neutrophils stimulated with LPS, PAF or LPS

Figure 8 Autoradiograph showing that the tyrosine kinase inhibitor, genistein, does not inhibit the decrease in the electrophoretic mobility of cPLA₂ in the cytosolic fraction obtained from human neutrophils stimulated with LPS in combination with serum

No cPLA₂ band was detected in the membrane fraction (results not shown); this is expected as translocation of the enzyme to the membrane fraction requires the presence of Ca²⁺. Control neutrophils or genistein (50 μ g/ml for 1 h)-treated neutrophils at 37 °C were stimulated with buffer or 100 ng/ml LPS in combination with 1% serum for 30 min. After stimulation the cells were disrupted and fractionated. Samples from both cytoplasm and membrane fractions were subjected to SDS/PAGE, transferred and then probed with α -cPLA₂ antibody. The amount of protein (100 μ g) loaded on to each lane was the same. Lane 1, control; lane 2, genistein-treated cells; lane 3, control cells stimulated by LPS in combination with serum; lane 4, cells treated with genistein and then stimulated with LPS and serum. The data are from a single experiment carried out several times.

Table 1 [³H]Arachidonic acid release in human neutrophils under various experimental conditions

The cells were stimulated with PAF for 2 min and with LPS in combination with 1% serum for 30 min. When LPS and PAF were used, the cells were first incubated with LPS and serum for 30 min, and then stimulated with PAF for 2 min. Each value represents the mean $\pm$ S.E.M. for the number of experiments in parentheses. Each experiment was carried out on neutrophils isolated from a different donor.

Conditions	Arachidonic acid release (relative to control)
No addition	1.0
PAF (10 nM, 2 min)	1.3 $\pm$ 0.15 (3)
LPS (50 ng/ml, 30 min)	2.4 $\pm$ 0.28 (5)
LPS followed by PAF	7.9 $\pm$ 0.9 (5)

followed by PAF. The results summarized in Table 1 show two points. First, LPS produces a small but significant increase in arachidonic acid release. Second, LPS in combination with PAF produces a large increase in arachidonic acid release, although the effect of PAF by itself is small. It must be pointed out that PAF, unlike LPS, produces a rise in the intracellular concentration of free Ca²⁺. The data presented are expected on the basis of the current understanding of the mechanisms of arachidonic acid release (see the Discussion section). It was recently found that the stimulated production of leukotriene B₄ is increased in LPS-treated human neutrophils [29].

DISCUSSION

The recently cloned PAF receptor belongs to the family of G-protein-coupled receptors [1]. Most of these receptors lack tyrosine catalytic domains. In spite of this, the data presented here show clearly that the addition of PAF to human neutrophils increases the tyrosine phosphorylation and activity of the p42^{erk2} MAP kinase. This conclusion is based on the following lines of evidence. First, α -PY antibody immunoprecipitates a 42 kDa protein that can be recognized by α -p42 MAP kinase antibody in

neutrophils stimulated with PAF. Second, α -42 MAP kinase antibody immunoprecipitates a 42 kDa protein from cells stimulated with PAF that can be recognized by α -PY antibody. Third, immunoprecipitates and cell lysates prepared from cells stimulated with PAF have increased MAP kinase activity as measured by increased *in situ* phosphorylation of myelin basic protein.

It is generally observed that chemotactic-factor-induced responses are greatly potentiated in neutrophils pretreated with LPS [20,21]. This priming is time-dependent in that a significant effect requires that the neutrophils be treated with LPS for at least 15 min. The present data show that the PAF-induced increase in the tyrosine phosphorylation and activity of p42^{erk2} MAP kinase is greatly enhanced in cells pretreated with low concentrations of LPS in combination with serum. This potentiation is time-dependent and requires that the cells be incubated with LPS for a minimum of 15 min. These data suggest that the part of the event(s) responsible for the commonly observed priming action of LPS on chemotactic-factor-stimulated responses of neutrophils is proximal to the phosphorylation and activation of a MAP kinase step. The mechanism(s) of priming by LPS is totally unknown. We would like to hypothesize that LPS, in the absence of a second stimulus, up-regulates the amount of G-protein associated with the plasma membrane. This up-regulation will increase the number of receptors that are coupled to G-protein. We have shown that the addition of LPS to human neutrophils increases the amount of G₂ α associated with the plasma membrane, and this increase is time-dependent [30].

The data presented in this study show clearly that neither the p42^{erk2} nor the p44^{erk1} MAP kinase is significantly phosphorylated on tyrosine residues or activated in human neutrophils stimulated with LPS in combination with serum. This conclusion is based on the following results. First, α -p42 MAP kinase antibody does not immunoprecipitate a protein that can be recognized by α -PY antibody. Second, α -PY antibody fails to immunoprecipitate proteins that can be recognized by either α -p42 or α -p44 MAP kinase antibody. Third, whole cell lysates of LPS-treated cells are unable to increase the phosphorylation of myelin basic protein. This is unlike the situation in human macrophages in which it has been shown that the addition of LPS to macrophages increases the tyrosine phosphorylation of the p42^{erk2} and p44^{erk1} MAP kinase [28]. This difference in tyrosine phosphorylation of the MAP kinase isoforms between neutrophils and macrophages provides evidence for a distinction between the tyrosine kinase and/or phosphatase regulation of the actions of LPS on these different cell types.

It is generally agreed that MAP kinase phosphorylates cPLA₂ [19,25]. Agents that increase the tyrosine phosphorylation and activity of MAP kinase are known to phosphorylate cPLA₂ as demonstrated by a decrease in the electrophoretic mobility of the enzyme. The data presented here clearly show, for the first time, that tyrosine phosphorylation and activation of p42^{erk2} and/or p44^{erk1} MAP kinase are not necessary for the phosphorylation of cPLA₂ in human neutrophils. This conclusion is based on the fact that, whereas the addition of low concentrations of LPS in combination with serum, in the absence of a second stimulus, to human neutrophils does not increase the tyrosine phosphorylation or activity of any of the known MAP kinase isoforms, it decreases the electrophoretic mobility of cPLA₂. Moreover, the tyrosine kinase inhibitor genistein at concentrations that inhibit PAF-induced tyrosine phosphorylation of MAP kinase isoforms does not inhibit the LPS-induced decrease in the electrophoretic mobility of cPLA₂.

As stated above, the data presented in Table 1 are expected on the basis of the current understanding of the mechanisms of arachidonic acid release. In order to stimulate the release of

arachidonic acid in intact cells, a minimum of two conditions must be satisfied. First, cPLA₂ must be activated, presumably by phosphorylation. Second, the activated enzyme must be translocated to the plasma membrane where the substrate is located. The translocation step requires an increase in intracellular free Ca²⁺. Maximal amounts of arachidonic acid are released when the Ca²⁺ peak occurs at a time when the enzyme is maximally activated. The addition of LPS to human neutrophils does not cause a rise in intracellular free Ca²⁺ concentration. At basal levels of free Ca²⁺ only small amounts of the activated cPLA₂ can be translocated to the plasma where the substrate is located. Accordingly, the LPS-induced arachidonic acid release is small. On the other hand, as PAF induces an increase in intracellular free Ca²⁺, the release of arachidonic acid is very large in cells stimulated with LPS and PAF.

The data presented here make three distinctly important yet related points. First, both the tyrosine phosphorylation and activity of the p42^{erk2} MAP kinase are increased in human neutrophils stimulated with PAF. The PAF-induced increases in these activities are greatly potentiated in cells pretreated with low concentrations of LPS in combination with serum. The event(s) that is responsible for this priming is proximal to the MAP kinase step. Second, the addition of LPS, in the absence of a second stimulus, does not increase the tyrosine phosphorylation or activity of the p42^{erk2} or p44^{erk1} MAP kinase isoforms. Third, LPS phosphorylates cPLA₂, as demonstrated by a decrease in the electrophoretic mobility of the enzyme, and this phosphorylation is not inhibited by the tyrosine kinase inhibitor, genistein. This indicates that cPLA₂ can be phosphorylated and presumably activated by kinases other than the MAP kinase family of proteins. It is proposed that there are MAP kinase-dependent and -independent mechanisms for the phosphorylation of cPLA₂. Identifying the kinase(s), other than the MAP kinase family of proteins, and/or phosphatases that increase the phosphorylation and activity of cPLA₂ is the subject of future studies.

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