Tyrosine phosphorylation and activation of a new mitogen-activated protein (MAP)-kinase cascade in human neutrophils stimulated with various agonists

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The presence of a novel 38 kDa protein that is tyrosine phosphorylated in human neutrophils, a terminally differentiated cell, upon stimulation of these cells with low concentrations of lipopolysaccharide (LPS) in combination with serum has been demonstrated. This 38 kDa protein was identified as the mammalian homologue of HOG1 in yeast, the p38 mitogen-activated protein (MAP) kinase. This conclusion is based on the experimental findings that anti-phosphotyrosine (anti-PY) antibody immunoprecipitates a 38 kDa protein that is recognized by anti-p38 MAP kinase antibody, and conversely, anti-p38 MAP kinase antibody immunoprecipitates a 38 kDa protein that can be recognized by anti-PY antibody. Moreover, this tyrosine phosphorylated protein is found associated entirely with the cytosol. It was also found that this p38 MAP kinase is activated following stimulation of these cells with low concentrations of LPS in combination with serum. This conclusion is based on three experimental findings. First, soluble fractions isolated from LPS-stimulated cells phosphorylate heat shock protein 27 (hsp27) in an *in vitro* assay, and this effect is not inhibited by protein kinase C and protein kinase A inhibitor peptides. This effect is similar to the effect produced by the commercially available phosphorylated and activated MAPKAP kinase-2 (MAP kinase activated protein kinase-2). Secondly, a 27 kDa protein that aligns with a protein recognized by anti-hsp27 antibody is phosphorylated upon LPS stimulation of intact human neutro-

phils prelabelled with radioactive phosphate. Lastly, immune complex protein kinase assays, using [γ - 32 P]ATP and activating transcription factor 2 (ATF2) as substrates, showed increased p38 MAP kinase activity from LPS-stimulated human neutrophils. The phosphorylation and activation of this p38 MAP kinase can be affected by both G-protein-coupled receptors such as platelet-activating factor (PAF) and non-G-protein-coupled receptors such as the cytokine-coupled receptors for granulocyte-macrophage colony-stimulating factor (GM-CSF) and tumour necrosis factor α (TNF- α). The effect of low concentrations of PAF is greatly increased in cells pretreated with LPS. The tyrosine phosphorylation of the p38 MAP kinase is not restricted to stimuli that mediate their actions through membrane-associated receptors, but it can be affected by agents that bypass membrane-associated receptors such as the protein translation blocker anisomycin. While anisomycin is known to increase the tyrosine phosphorylation of the 54 kDa SAPK (stress-activated protein kinase), this is the first report that shows that anisomycin also tyrosine phosphorylates the p38 MAP kinase. Cytokine receptors that increase the tyrosine phosphorylation and activation of the erk1 and erk2 MAP kinases have less effect on this p38 MAP kinase than those that do not affect the erk1 and erk2 MAP kinases. The possible role of the p38 MAP kinase in the phosphorylation of cytosolic phospholipase A_2 is discussed.

INTRODUCTION

Lipopolysaccharide (LPS), tumour necrosis factor α (TNF- α) and granulocyte-macrophage colony-stimulating factor (GM-CSF) exert profound effects on the host immune system [1–3]. *In vivo*, bacterial LPS produces neutrophil sequestration in the vascular bed and promotes neutrophil-mediated vascular injury [4,5]. The exposure of neutrophils to LPS, GM-CSF or TNF- α primes the cells for enhanced release of microbicidal metabolites [1–3]. The increase in the oxidative burst might permit increased resistance to bacterial infection, yet predisposes the host to oxidative tissue damage and the release of various inflammatory mediators. While 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 [6]. The effects of LPS (at low concentrations), GM-CSF and TNF- α are mediated through membrane-associated non-G-protein-coupled receptors [3,7,8]. These receptors do not possess intracellular tyrosine kinase domains.

Platelet-activating factor (PAF) possesses multiple biological activities which encompass the field of allergic and inflammatory reactions as well as the regulation of the cardiovascular and haemodynamic systems [9]. The cellular targets of PAF are similarly varied and include, among others, platelets, lung tissue, smooth muscle, hepatocytes, macrophages and neutrophils. PAF, unlike GM-CSF, LPS and TNF- α , induces its many effects through a G-protein-coupled receptor, which does not possess an intracellular tyrosine kinase domain.

The phosphorylation and dephosphorylation of proteins mediate the actions of many hormones and growth factors. The activities of many cellular kinases, phosphatases, phospholipases

Abbreviations used: LPS, lipopolysaccharide; TNF- α , tumour necrosis factor α ; PAF, platelet-activating factor; MAP kinase, mitogen-activated protein kinase; GM-CSF, granulocyte-macrophage colony-stimulating factor; MAPKAP kinase-2, mitogen-activated protein kinase activated protein kinase-2; hsp, heat shock protein; anti-PY, anti-phosphotyrosine; ECL, enhanced chemiluminescence; RK, reactivating kinase; RKK, reactivating kinase kinase; PKC, protein kinase C; PKA, protein kinase A; ATF2, activating transcription factor 2; HBSS, Hanks balanced salt solution; HRP, horseradish peroxidase; PVDF, polyvinylidene difluoride.

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and other enzymes are regulated by their phosphorylation and dephosphorylation [10]. Most protein phosphorylation occurs on serine and threonine, but in some cases tyrosine residues are phosphorylated. Tyrosine phosphorylation plays a critical role in the control of cell proliferation and differentiation [10]. Thus, phosphotyrosine kinases and phosphatases are pivotal enzymes in the control of various cell functions. Mitogen-activated protein (MAP) kinases are pivotal in signal transduction, resulting in a wide range of effects including gene transduction associated with proliferation and differentiation. Until recently, erk1 and erk2 MAP kinases were the only cloned and well characterized mammalian MAP kinases, and were thought to mediate the actions of many agonists [10].

Recently, we found that the addition of low concentrations of LPS in combination with serum and TNF- α , unlike PAF or GM-CSF, to human neutrophils in suspension does not cause the tyrosine phosphorylation or activation of erk1 or erk2 MAP kinases [11,12]. In these studies we observed that both LPS and TNF- α increase the tyrosine phosphorylation of a 38 kDa protein. The present studies were undertaken to address three questions. First, what is the identity of this 38 kDa protein? Secondly, does this protein have kinase activity, and if so does this activity increase following stimulation of human neutrophils by LPS? Thirdly, can this protein be tyrosine phosphorylated and activated by G-protein-coupled receptors such as PAF and/or cytokine-coupled receptors such as the GM-CSF and TNF- α receptors?

MATERIALS AND METHODS

Isolation of human neutrophils

Neutrophils were isolated from human donors using a Ficoll/Hypaque gradient, and the contaminating red blood cells were lysed by hypotonic shock [11–13]. The neutrophils were resuspended in modified Hanks balanced salt solution (HBSS) containing 0.1% BSA and 10 mM Hepes, pH 7.35.

Fractionation of cells into soluble and particulate fractions

Fractionation of cells into soluble and particulate fractions was carried out as described previously [11,12]. Briefly, 3 ml aliquots of cells (1×10^7 cells/ml) were treated with buffer or the stimulus. Following reaction termination, the cells were resuspended at 10^8 cells/ml in buffered sucrose solution (10 mM Hepes, pH 7.4, 100 mM sucrose, 1 mM EGTA, 50 mg/ml leupeptin, 1 mM diisopropyl fluorophosphate, 1 mM PMSF). Cells were disrupted by sonication, centrifuged at 8000 *g* for 15 min and the supernatant was then centrifuged at 100 000 *g* for 30 min at 4 °C.

Immunoblotting

Immunoblotting was performed as described previously [11,12]. In all experiments, equivalent amounts of protein were loaded on to SDS/10%-polyacrylamide gels. After electrophoresis, proteins were transferred from the gel to polyvinylidene difluoride (PVDF) membranes in transfer buffer [20 mM Tris base, 150 mM glycine, 20% (v/v) methanol, pH 8.9]. Blots were washed in Tris-buffered saline-Tween-20 (TBS-T), incubated with the desired antibody and then probed with the appropriate horseradish peroxidase (HRP)-conjugated antibody. The enhanced chemiluminescence (ECL) method was utilized for detection.

Immunoprecipitation

Immunoprecipitates were prepared as described previously [11,12]. Briefly, cells at 5×10^7 cells/ml in HBSS were treated

with buffer or stimuli. Reactions were terminated by rapid centrifugation; 100 μ l of boiling 1% SDS was added to the pellet and boiled for 15 min. Aliquots (400 μ l) of ice-cold water and (500 μ l) of double-strength lysis buffer (20 mM Tris/HCl, 300 mM NaCl, 2% Triton X-100, 1% Nonidet-P40, 2 mM EGTA, 2 mM EDTA, 1 mM diisopropylfluorophosphate, 20 μ g/ml aprotinin, 4 mM sodium orthovanadate, 2 mM PMSF and 20 μ g/ml leupeptin) were then added to the samples. After centrifugation at 15 000 *g* for 10 min, supernatants were transferred to tubes containing anti-mouse IgG or anti-rabbit IgG agarose beads to which the appropriate antibody had been conjugated. Lysate-bead-antibody complexes were incubated for 4 h at 4 °C. After incubation, complexes were centrifuged at 15 000 *g* for 5 min and the supernatants recovered. The immunocomplexes 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 10 min, and loaded on to SDS/10%-polyacrylamide gels.

In vitro phosphorylation

In vitro phosphorylation of heat shock protein 27 (hsp27) was carried out as described previously [14]. Briefly 15 μ l of soluble fraction containing 2 mg/ml protein prepared from control or stimulated cells was added to 15 μ l of assay buffer which comprised 100 mM Tris (pH 7.4), 20 mM MgCl₂, 40 μ M ATP, 16 μ M okadaic acid, 33 μ g/ml hsp27 and 0.24 μ Ci/ml [³²P]ATP. Reactions were carried out for 10 min at 25 °C, and were stopped by adding 30 μ l of 2 $\times$ SDS/PAGE sample buffer. The samples were boiled for 5 min and electrophoresed on 10% polyacrylamide gels. Phosphorylation of hsp27 protein was measured under conditions where it was linear with respect to the time of the incubation and enzyme dilution.

Phosphorylation of hsp27 in intact cells

Phosphorylation of hsp27 was carried out as follows: human neutrophils in suspension were incubated in phosphate-free buffered isotonic solution containing carrier-free [³²P]orthophosphate (0.5 mCi/ml) at 37 °C for 60 min as described previously [15]. The cells were washed three times, stimulated, disrupted by sonication, and fractionated into soluble and particulate fractions. Aliquots of the soluble fractions were electrophoresed on SDS/10%-PAGE, and the dried gel was autoradiographed.

Protein kinase assay

The p38 MAP kinase activity assay was performed using immunoprecipitates of p38 MAP kinase prepared from the soluble fraction. The immune complexes were washed twice with kinase buffer (25 mM Hepes, pH 7.4, 25 mM β -glycerophosphate, 25 mM MgCl₂, 2 mM dithiothreitol, 0.1 mM orthovanadate). The assays were initiated by the addition of 1 μ g of GST-ATF2 and 3 μ Ci of [γ -³²P]ATP in a final volume of 30 μ l. The reactions were terminated after 20 min at 22 °C by the addition of Laemmli sample buffer. The phosphorylation of activating transcription factor 2 (ATF2) was examined after SDS/PAGE and autoradiography.

Materials

LPS was purchased from Difco Laboratories (Detroit, MI, U.S.A.). Anti-phosphotyrosine (anti-PY), a monoclonal antibody, anti-mouse p38 Hog MAP kinase, a polyclonal antibody, and anti-cdc2, a polyclonal antibody which recognizes the p40^{hara}

MAP kinase isoform were obtained from Upstate Biotechnology Incorporated (UBI, Lake Placid, NY, U.S.A.). Electrophoresis reagents and molecular-mass markers were from Bio-Rad Laboratories (Melville, NY, U.S.A.); PVDF protein transfer membrane (Immobilon-P) was obtained from Millipore Corporation (Bedford, MA, U.S.A.); ECL and Western blotting reagents, and [γ - 32 P]ATP (5000 Ci/mmol) were purchased from Amersham (Arlington Heights, IL, U.S.A.). [32 P]Orthophosphoric acid was from Dupont NEN (Wilmington, DE, U.S.A.). Okadaic acid, protein kinase A (PKA) inhibitor peptide, PKI(6–22)amide, and protein kinase C (PKC) inhibitor peptide, PKC(19–36), were from Gibco BRL (Gaithersburg, MD, U.S.A.). Hsp27 and anti-hsp27 antibody were from Stressgen (Victoria, BC, Canada). PAF and TNF- α were purchased from Calbiochem (San Diego, CA, U.S.A.). Anisomycin was obtained from Sigma (St. Louis, MO, U.S.A.) and GM-CSF was purchased from R and D Systems (Minneapolis, MN, U.S.A.). The anti-p38 MAP kinase (c-20) and the AFT2 used in the immune complex kinase assays were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, U.S.A.).

RESULTS

LPS increases the tyrosine phosphorylation of a 38 kDa protein

While examining the effect of LPS on the erk1 and erk2 MAP kinases, we noted that the addition of a low concentration of LPS in combination with serum to neutrophils in suspension increases the tyrosine phosphorylation of a 38 kDa protein. To characterize this action further, human neutrophils in suspension were stimulated either with diluent or 100 ng/ml LPS (hereafter always used in combination with 1 % serum) for specific periods of time or with different concentrations of LPS for 30 min. Reactions were terminated and soluble fractions were prepared and subjected to SDS/10 % -PAGE, transferred to PVDF membranes, and probed with anti-PY antibody. The results summarized in Figure 1 show clearly that LPS in combination with serum increases the tyrosine phosphorylation of a 38 kDa protein. This stimulation is time- (Figure 1A) and dose- (Figure 1B) dependent. Serum, by itself, has no effect but its presence was necessary to achieve maximal effect when the concentration of LPS was 100 ng/ml or less (results not shown).

As shown previously, LPS does not tyrosine phosphorylate either erk1 or erk2 MAP kinase [11]. It remained possible, however, that the phosphorylated 38 kDa protein was the recently discovered p40^{hera} MAP kinase. This was ruled out using anti-cdc2 antibody which recognizes the p40^{hera} MAP kinase [11,16] and using an *in situ* kinase assay [11,17] employing myelin basic protein as substrate. It was found that neither whole lysates, nor soluble or particulate fractions, nor immunoprecipitates using anti-PY antibody prepared from cells stimulated with LPS were able to phosphorylate myelin basic protein (results not shown). Moreover, in immunoprecipitates prepared using anti-PY antibody, anti-cdc2 antibody did not recognize the tyrosine phosphorylated 38 kDa protein (results not shown).

Identity of this tyrosine phosphorylated 38 kDa protein

Until recently, erk1 and erk2 MAP kinases were the only cloned and well-characterized mammalian MAP kinases. Very recently, two other MAP kinase subtypes, the JNK/SAPK (stress-activated protein kinase) and p38/RK (reactivating kinase; mammalian equivalent of HOG1 in yeast) were discovered [10]. To test whether the 38 kDa protein which is tyrosine phosphorylated upon LPS stimulation is the mammalian equivalent of HOG1 in yeast, we carried out immunoprecipitation

Figure 1 LPS induces a time- and dose-dependent tyrosine phosphorylation of a 38 kDa protein in human neutrophils

Cells were stimulated either with diluent or 100 ng/ml LPS in combination with 1 % serum and sampled at various times (A), or sampled at 30 min using several LPS concentrations (B). After the reactions were stopped, the cells were sonicated, soluble fractions were prepared and electrophoresed on SDS/10 % -PAGE. Immunoblot analysis was performed using anti-PY antibody and the 38 kDa band was quantified by laser scanning densitometry. The integrated absorption levels, representing levels of tyrosine phosphorylation were plotted against time and dose. The insert is an actual immunoblot illustrating the effect of LPS (100 ng/ml with 1 % serum at 30 min) on the tyrosine phosphorylation of a 38 kDa protein. Each point represents the mean $\pm$ S.E.M. of at least three separate experiments.

experiments. Human neutrophils were either stimulated with diluent or 100 ng/ml LPS for 30 min at 37 °C. Reactions were terminated by rapid centrifugation, and immunoprecipitates prepared using either anti-PY or anti-p38 antibody. The use of a denaturing immunoprecipitation protocol was necessary because anti-p38 antibody used in these experiments recognizes only the denatured form of the protein. Aliquots of the immunoprecipitates were subjected to SDS/10 % -PAGE, transferred to PVDF membranes, and blots probed either with anti-p38 or anti-PY antibody. The results summarized in Figure 2 show that anti-PY antibody immunoprecipitates a 38 kDa protein that is recognized by anti-p38 antibody in LPS-stimulated human neutrophils but not in control cells (Figure 2C), and anti-p38 antibody immunoprecipitates a protein that is recognized by anti-PY antibody (Figures 2A and 2B) in LPS-stimulated cells but not in control cells. Note that the time course in Figure 2(A) corresponds well with the time course in Figure 1(A). Anti-p38 antibody immunoprecipitates a 38 kDa protein that is recognized by anti-p38 antibody (results not shown). It was also noted that, unlike erk1 or erk2 MAP kinase, the tyrosine phosphorylated form of p38 does not migrate more slowly than the unphosphorylated form (i.e. tyrosine phosphorylated p38 did not exhibit an electrophoretic mobility shift).

Figure 2 LPS increases the tyrosine phosphorylation of the p38 HOG MAP kinase in human neutrophils

Cells were treated with 100 ng/ml LPS in combination with 1% serum (+) or with buffer (—) for various times (min) (A), or for 30 min (B, C). The cells were lysed and immunoprecipitates using anti-p38 HOG MAP kinase antibody were prepared and the resulting immunoblot probed with anti-PY antibody (A, B) or immunoprecipitates using anti-PY antibody were prepared and the resulting immunoblot probed with anti-p38 HOG MAP kinase antibody (C). The autoradiograph represents the results of a single experiment that was carried out three times.

Figure 3 Soluble fractions from LPS-treated human neutrophils phosphorylate hsp27 *in vitro*

Buffer (lane 1) or soluble fractions prepared from control cells (lane 2) or cells treated with LPS (lane 3) (100 ng/ml, 1% serum, for 30 min), were tested for their ability to phosphorylate hsp27 *in vitro*. The effect of phosphorylated and activated MAPKAP kinase-2 (5 µg/ml) was also tested. The bands represent the degree of hsp27 phosphorylation as evidenced by autoradiography. The results are of one experiment representative of three similar experiments.

Activation of the p38 MAP kinase cascade upon stimulation of human neutrophils with LPS

Next we examined whether the p38 MAP kinase cascade is activated by LPS. In these experiments, human neutrophils in suspension were stimulated either with diluent or 100 ng/ml LPS for 30 min. Reactions were terminated by rapid centrifugation, the cells were disrupted by sonication and the products were fractionated into soluble and particulate fractions. Aliquots of the soluble fractions were tested for their abilities to phosphorylate hsp27 in an *in vitro* kinase assay as described in the Materials and methods section. For comparison, the effect of the phosphorylated and activated commercially obtained MAPKAP kinase-2 on the phosphorylation of hsp27 was also tested (there is no commercially available non-phosphorylated and inactive MAPKAP kinase-2). The results are summarized in Figure 3. These data show several points: (1) soluble fractions prepared from LPS-stimulated neutrophils, but not from control cells, increase the *in vitro* phosphorylation of hsp27; (2) the commercially obtained phosphorylated and activated MAPKAP

Figure 4 The p38 MAP kinase and the LPS-stimulated kinase activity against hsp27 are localized in the cytosolic fraction

Neutrophils were treated either with diluent (—) or 100 ng/ml LPS in combination with 1% serum (+) for 30 min. After the reactions were stopped the cells were sonicated and soluble and particulate fractions were prepared. (A) Samples (100 µg) were subjected to SDS/PAGE, transferred to membrane and probed with anti-(p38 HOG MAP kinase) antibody. (B) Samples (30 µg) were tested for their ability to phosphorylate hsp27 *in vitro*. Samples were subjected to SDS/PAGE and the dried gel autoradiographed. Molecular mass standards are noted in the right-hand column. The results are of one representative experiment.

Figure 5 Effect of PKC and PKA peptide inhibitors on the *in vitro* phosphorylation of hsp27

Soluble fractions from control (A) or LPS- (100 ng/ml, 1% serum, for 30 min) treated (B) neutrophils were prepared and tested for their ability to phosphorylate hsp27 in the presence of PKC (10 µM) or PKA (2.5 µM) peptide inhibitors. The results are from one experiment representative of three similar experiments.

kinase-2 increases the *in vitro* phosphorylation of hsp27; and (3) the inclusion of soluble fractions from either stimulated or control cells with the commercially obtained MAPKAP kinase-2 reduces its activity.

Next we determined the p38 MAP kinase and the LPS-stimulated kinase activity against hsp27 in both the membrane and cytosolic fractions prepared from human neutrophils. The results are summarized in Figure 4. As can be seen in Figure 4(A), the p38 MAP kinase is localized entirely in the cytosolic fraction. Furthermore, the LPS-stimulated activity against hsp27 is also associated entirely with the cytosolic fraction (Figure 4B).

Although, erk1, erk2 and p40^{hera} MAP kinases are not tyrosine phosphorylated or activated upon stimulation of human neutrophils in suspension by LPS, it remained possible that the

Figure 6 LPS activates p38 MAP kinase

Immunoprecipitates of p38 MAP kinase were prepared from neutrophils treated with buffer (**A**) or with LPS (100 ng/ml in combination with 1% serum) (**B**) for 30 min. Protein kinase activity was measured in immune complex protein kinase assays using [γ - 32 P]ATP and ATF2 as substrates. Phosphorylated ATF2 was detected after SDS/PAGE by autoradiography. The results are of one representative experiment.

Figure 7 GM-CSF, TNF- α and anisomycin stimulate the tyrosine phosphorylation of the p38 MAP kinase in human neutrophils

Cells were treated with buffer (lane 1), GM-CSF (500 pM, 5 min; lane 2), TNF- α (50 ng/ml, 5 min; lane 3), and anisomycin (10 μ g/ml, 30 min; lane 4). (**A**) Lysates were immunoprecipitated with anti-p38 antibody and the resulting immunoblot probed with anti-PY antibody. (**B**) Lysates were immunoprecipitated with anti-PY antibody and the resulting immunoblot probed with anti-p38 antibody. One representative experiment among three with similar results is shown.

phosphorylation of hsp27 by soluble fractions obtained from LPS-stimulated cells is mediated by other kinases such as PKC and/or PKA. To test this possibility, the soluble fractions were treated with inhibitor peptides of PKC and PKA before the fractions were used in the assay for the *in vitro* phosphorylation of hsp27. The results summarized in Figure 5 show that neither of these inhibitor peptides had any effect on the phosphorylation of hsp27 by soluble fractions obtained from LPS-stimulated cells.

A more direct measurement of the LPS-stimulated increase in p38 MAP kinase activity is demonstrated using the immune complex protein kinase assay. Immunoprecipitates of p38 MAP kinase using the recently developed anti-p38 antibody (this antibody does not cross-react with other MAP kinases or with JNK1) were prepared. These immunoprecipitates were used in a phosphorylation reaction utilizing [γ - 32 P]ATP and ATF2 as substrates. As can be seen in Figure 6, human neutrophils stimulated with LPS show a marked increase in p38 MAP kinase activity.

Figure 8 Priming effect of LPS on the PAF-stimulated tyrosine phosphorylation of the p38 MAP kinase

Cells were treated with: lane 1, buffer; lane 2, LPS (100 ng/ml, 1% serum, 30 min); lane 3, PAF (10 nM, 1 min); and lane 4, LPS with serum for 30 min and then PAF. Lysates were immunoprecipitated using anti-p38 MAP kinase antibody and the resulting immunoblot probed with anti-PY antibody. One representative experiment among three with similar results is shown.

Table 1 *In vitro* hsp27 phosphorylation activity in soluble fractions prepared from human neutrophils treated with various agonists

Each value represents the mean $\pm$ S.E.M., and the number in parentheses refers to the number of experiments. The mean value of the control absorption value is 0.17 ± 0.04 (14 different experiments). Cells were treated as described then soluble fractions were prepared and tested for their ability to phosphorylate hsp27 *in vitro*. The integrated absorption value of the hsp27 band from an autoradiograph was quantified by laser scanning densitometry.

Condition	Integrated absorption value (relative to control)
No addition	1.0
TNF- α (50 ng/ml, 5 min)	10.0 ± 0.9 (4)
LPS (100 ng/ml, 30 min)	7.0 ± 1.2 (10)
GM-CSF (500 pM, 5 min)	2.9 ± 0.7 (2)
Anisomycin (10 μ g/ml, 30 min)	2.4 ± 0.5 (4)
PAF (500 nM, 1 min)	4.7 ± 1.0 (2)

Tyrosine phosphorylation and activation of this p38 MAP kinase upon stimulation of human neutrophils by other agonists

To determine whether the observed tyrosine phosphorylation and activation of p38 MAP kinase is specific to LPS, we examined the actions of other known agonists on the tyrosine phosphorylation of p38 MAP kinase and the activation of this cascade. Agonists which mediate their actions through G-protein-coupled receptors, non-G-protein-coupled receptors and agonists which bypass membrane-associated receptors were chosen. Human neutrophils in suspension were stimulated with PAF, TNF- α , GM-CSF and anisomycin. PAF was added in combination with LPS to examine the priming ability of LPS on p38 MAP kinase tyrosine phosphorylation. Immunoprecipitates using anti-p38 and anti-PY antibodies were prepared, and aliquots were subjected to SDS/10%-PAGE, transferred to PVDF membranes, and blots probed with either anti-PY or anti-p38 antibody. The results summarized in Figures 7 and 8 show that all the agonists tested increase the tyrosine phosphorylation of the p38 MAP kinase. In comparative experiments using maximal times and concentrations it was found that TNF- α produces the greatest effect followed by LPS followed by GM-CSF. Note also that the stimulatory effect of PAF, at suboptimal concentrations, when added after the cells have been incubated with LPS was greater than the effect of either PAF or LPS added alone (Figure 8). The effect of PAF alone was also examined and was found to be dose- and time-dependent. The ability of soluble fractions isolated from human neutrophils following stimulation with these agonists to increase the phosphorylation of hsp27 in an *in vitro* assay was also examined. The results summarized in Table 1 show that soluble fractions isolated following stimulation of

Figure 9 LPS stimulates the *in vivo* phosphorylation of hsp27 in human neutrophils

³²P-labelled cells were treated with LPS (100 ng/ml, 1% serum). The reactions were stopped at various times, and the cells were solubilized and aliquots were electrophoresed through an SDS/10%-polyacrylamide gel, and the gel was dried and exposed to film. Values to the right are of molecular-mass markers. The hsp27 arrow aligns to an authentic hsp27 stained marker. The autoradiograph is representative of three experiments with similar results.

the cells with all these agonists were able to increase the phosphorylation of hsp27. Again TNF- α produces the strongest effect followed by LPS followed by GM-CSF.

Phosphorylation of hsp27 in intact neutrophils upon stimulation of these cells with LPS

To prove that MAPKAP kinase-2, and by inference the p38 MAP kinase, was indeed activated in intact cells, the phosphorylation of hsp27 following stimulation by LPS of human neutrophils prelabelled with radioactive phosphate was examined. In these experiments, cells prelabelled with radioactive phosphate, as described in the Materials and methods section, were stimulated either with diluent or 100 ng/ml LPS. After the reactions were terminated, the cells were solubilized and aliquots were subjected to SDS/10%-PAGE, and autoradiographed. A second sample was immunoblotted and the blot was probed with anti-hsp27 antibody. The results are summarized in Figure 9. These data show that a 27 kDa protein is phosphorylated in LPS-stimulated intact cells but not in control cells. Moreover, when the blot was probed with anti-hsp27 antibody, a 27 kDa protein was identified which aligned with the phosphorylated protein which also ran identically to added hsp27 standard.

DISCUSSION

In the present studies, we found a novel 38 kDa protein in human neutrophils, a terminally differentiated cell, that was tyrosine phosphorylated upon stimulation with low concentrations of LPS in combination with serum. This effect was dose- and time-dependent. This phosphorylated 38 kDa protein was identified as the mammalian homologue of HOG1 in yeast, the p38 MAP kinase. This conclusion is based on our experimental findings that anti-PY antibody immunoprecipitates a 38 kDa protein that is recognized by anti-p38 antibody, and conversely, anti-p38 antibody immunoprecipitates a 38 kDa protein that can be recognized by anti-PY antibody. Moreover, this protein, like other MAP kinase subtypes, is found entirely in the cytosol.

We also found that stimulation of human neutrophils in suspension by LPS activates the p38 MAP kinase cascade. This conclusion is based on several experimental findings. First, soluble fractions isolated from LPS-stimulated cells, but not from control cells, phosphorylate hsp27 in an *in vitro* assay, and this effect is not inhibited by PKC and PKA inhibitor peptides. In addition, this LPS-induced activation cannot be due to erk1 or

erk2 or p40^{hera} activation since LPS does not tyrosine phosphorylate or activate these MAP kinases. Secondly, immunoprecipitates of p38 kinase from LPS-stimulated human neutrophils show increased activity in an immune complex protein kinase assay. Thirdly, a 27 kDa protein is phosphorylated upon LPS-stimulation of intact human neutrophils prelabelled with radioactive phosphate. Moreover, when samples were immunoblotted and probed with anti-hsp27 antibody, a 27 kDa protein was identified which aligned with the phosphorylated protein. This suggests that hsp27 is present in human neutrophils, and that the 27 kDa phosphorylated protein is the small heat shock protein. Fourthly, commercially available MAPKAP kinase-2, already phosphorylated and activated, increased the phosphorylation of hsp27 *in vitro*. It is interesting to note that when soluble fractions obtained either from control or stimulated cells were included in the assay mixture with the commercially obtained MAPKAP kinase-2, its phosphorylating effect on hsp27 was less than when added alone. This indicates that the soluble fractions may contain an inhibitor(s) of MAPKAP kinase-2 activity. Fifthly, MAPKAP kinase-2 is phosphorylated and activated by some MAP kinase subtypes, and hsp27 is a substrate for MAPKAP kinase-2 [18].

Low concentrations of LPS produce their effects through CD14, a differentiating antigen which is anchored on the surface of leucocytes via a phosphatidylinositol glycan [7,8]. CD14 binds the LPS-serum protein complex, and the blockage of CD14 with monoclonal antibodies prevents the actions of LPS. The tyrosine phosphorylation and activation of the p38 MAP kinase is not limited to LPS, stimulation of human neutrophils in suspension with TNF- α and GM-CSF lead to the phosphorylation and activation of this newly identified kinase. It is interesting to note that while the GM-CSF stimulation of these cells greatly increases the phosphorylation and activation of erk1 and erk2 MAP kinases, unlike LPS and TNF- α , its effect on the p38 MAP kinase tyrosine phosphorylation and activation is much less than that of LPS or TNF- α . The upstream kinases that phosphorylate erk1 and erk2 and p38 MAP kinase subtypes are TEY kinase MEK and TGY kinase RKK respectively [10]. The results presented here suggest very strongly that activation of the GM-CSF receptor stimulates predominantly the TEY kinase MEK, whereas the activation of the LPS and TNF- α receptors stimulates the TGY kinase RKK. Although GM-CSF, LPS and TNF- α behave similarly in potentiating some functions of mature neutrophils, such as adherence, superoxide generation, phagocytosis and arachidonic acid release, these results demonstrate that distinctive signalling pathways are utilized by these agents. Alternatively, these MAP kinases may be involved in mediating responses other than the priming actions of these stimuli.

The tyrosine phosphorylation and activation of the p38 MAP kinase is not limited to non-G-protein-coupled receptors such as the receptors for GM-CSF, LPS and TNF- α . The results presented here show that the stimulation of human neutrophils with PAF, which mediates its effects through a G-protein-coupled receptor [19], leads to the tyrosine phosphorylation and activation of this p38 MAP kinase. The effect of PAF is greatly increased when the cells are preincubated with LPS. This is similar to the effect of LPS on PAF-induced tyrosine phosphorylation and activation of erk1 and erk2 MAP kinases. This suggests that the priming action of LPS is proximal to the steps that lead to the tyrosine phosphorylation and activation of the various MAP kinase subtypes. We have also shown that the tyrosine phosphorylation and activation of this p38 MAP kinase is not restricted to receptor-mediated stimuli. The results presented here show for the first time that stimulation of human neutrophils in suspension with the protein translation blocker

anisomycin, an agent that initiates intracellular signals and mediates early gene expression and superinduction, leads to the tyrosine phosphorylation and activation of this p38 MAP kinase.

In the results presented here we provide evidence for the presence of a novel MAP kinase that is tyrosine phosphorylated and activated following stimulation of human neutrophils with various agonists. This MAP kinase is the mammalian equivalent of the p38 HOG1 in yeast. The tyrosine phosphorylation and activation of this p38 MAP kinase can be affected through both G-protein- and non-G-protein-coupled receptors as well as by agents that bypass membrane-associated receptors. Non-G-protein-coupled receptors that increase the tyrosine phosphorylation and activation of erk1 and erk2 MAP kinases have less effect on this p38 MAP kinase than those that do not affect erk1 and erk2 MAP kinases. This suggests that these receptors activate primarily one of the upstream kinases that lead to the phosphorylation and activation of the various MAP kinase subtypes. Determining the role of this newly identified p38 MAP kinase in mediating the many stimulated responses of human neutrophils, such as superoxide production and arachidonic acid release, is the subject of future studies. In this regard, we have some preliminary results which suggest that the p38 MAPK may mediate the phosphorylation of cytosolic phospholipase A₂ by TNF- α . This view is based on our findings that an inhibitor of the p38 MAP kinase [20,21] abolishes the TNF- α -induced cytosolic phospholipase A₂ phosphorylation but not the phosphorylation of this enzyme by LPS or GM-CSF.

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REFERENCES

- 1 Abbt, J. J. and Johnson, Jr., R. B. (1980) *J. Exp. Med.* **151**, 101–114
- 2 Guthrie, L. A., McPhail, L. C., Henson, P. M. and Johnston, Jr., R. B. (1984) *J. Exp. Med.* **160**, 1656–1671
- 3 Arai, K.-I., Lee, F., Miyajima, A., Miyatake, S., Arai, N. and Yokota, T. (1988) *Annu. Rev. Biochem.* **59**, 783–836
- 4 Morrison, D. C. and Ulevitch, R. J. (1978) *Am. J. Pathol.* **93**, 527–601
- 5 Brigham, K. L., Woolverton, W. C., Blake, L. H. and Staub, N. C. (1974) *J. Clin. Invest.* **54**, 792–804
- 6 Rothstein, J. L. and Schreiber, H. (1988) *Proc. Natl. Acad. Sci. U.S.A.* **85**, 607–611
- 7 Wright, S.D., Ramos, R. A., Tobias, P. S., Ulevitch, R. J. and Mathison, J. C. (1990) *Science* **249**, 1431–1433
- 8 Darby, C., Chien, P., Rossman, M. D. and Schreiber, A. D. (1990) *Blood* **75**, 2396–2400
- 9 Chao, W. and Olson, M. S. (1993) *Biochem. J.* **292**, 617–629
- 10 Cano, E. and Mahadevan, L. C. (1995) *Trends Biochem. Sci.* **20**, 117–122
- 11 Fouda, S. I., Molski, T. F. P., Ashour, S. and Sha'afi, R. I. (1995) *Biochem. J.* **308**, 815–822
- 12 Waterman, W. and Sha'afi, R. I. (1995) *Biochem. J.* **307**, 39–45
- 13 English, D. and Anderson, B. R. (1974) *Immunol. Methods* **5**, 249–252
- 14 White, J. R., Huang, C.-K., Hill, J. M., Naccache, P. H., Becker, E. L. and Sha'afi, R. I. (1984) *J. Biol. Chem.* **259**, 8605–8611
- 15 Freshney, N. W., Rawlinson, L., Guesdon, F., Jones, E., Cowley, S., Hsuan, J. and Saklatvala, J. (1994) *Cell* **78**, 1039–1049
- 16 Williams, R., Sanghera, J., Wu, F., Carboner-Hall, D., Campbell, D. L., Warburton, D., Pelech, S. and Hall, F. J. (1993) *J. Biol. Chem.* **268**, 18213–18217
- 17 Kameshita, I. and Fujisawa, H. (1989) *Anal. Biochem.* **183**, 5966–5969
- 18 Marshall, C. L. (1995) *Cell* **80**, 179–185
- 19 Kunz, D., Gerard, N. P. and Gerard, C. (1992) *J. Biol. Chem.* **267**, 9101–9106
- 20 Cuenda, A., Rouse, J., Doza, Y. N., Meier, P. C., Gallagher, T. F., Young, P. R. and Lee, J. C. (1995) *FEBS Lett.* **364**, 229–233
- 21 Molski, T. F. P., Huang, C.-K., Fernandez, G. A. and Sha'afi, R. I. (1996) *FASEB J.* **10**, A416