RESEARCH COMMUNICATION

Tumour necrosis factor- α -induced phosphorylation and activation of cytosolic phospholipase A_2 are abrogated by an inhibitor of the p38 mitogen-activated protein kinase cascade in human neutrophils

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The role of the newly identified p38 mitogen-activated protein kinase (MAP kinase) in terminally differentiated cells, such as human neutrophils, is totally unknown. In order to examine the possible role of this MAP kinase in the phosphorylation and activation of cytoplasmic phospholipase A_2 (cPLA $_2$), we tested the effect of the recently synthesized inhibitor of p38 MAP kinase, SB 203580, on the phosphorylation and activation of both p38 MAP kinase and cPLA $_2$. We found that while tumour necrosis factor- α (TNF- α)-stimulated tyrosine phosphorylation of p38 MAP kinase is affected only slightly by SB 203580,

its stimulated kinase activity is greatly reduced in human neutrophils in suspension treated with this inhibitor. Furthermore, the TNF- α -stimulated phosphorylation and activation of cPLA $_2$ are completely abolished in cells treated with SB 203580. Based on these data, it is reasonable to conclude that an SB 203580-sensitive kinase, or kinases and/or phosphatases, are involved in the phosphorylation and activation of cPLA $_2$ in intact human neutrophils in suspension stimulated by TNF- α . The possible role of the p38 MAP kinase cascade in the phosphorylation and activation of cPLA $_2$ is discussed.

INTRODUCTION

Many different cell types, including human neutrophils, release arachidonic acid in response to stimulation. This fatty acid is the precursor of biologically active eicosanoids such as prostaglandins, thromboxane and leukotrienes. The rate-limiting step in eicosanoid biosynthesis is the liberation of arachidonic acid. Although the steps involved in the release of arachidonic acid are complex and not understood, the main step involves phospholipase A_2 (PLA $_2$) activation. The PLA $_2$ enzyme system is also important in the generation of platelet-activating factor (PAF). Leukotrienes, prostaglandins, thromboxane, prostacyclin and PAF are important lipid mediators with diverse biological activities. In human neutrophils and other mammalian cells there are at least two forms of PLA $_2$ that lack sequence similarity and differ in their molecular masses and calcium requirements [1,2]. Cytosolic phospholipase A_2 (cPLA $_2$) has an apparent molecular mass of 85 kDa and requires micromolar concentrations of calcium for activity. Secretory phospholipase A_2 (sPLA $_2$) has a molecular mass of 14 kDa and requires millimolar concentrations of calcium for activity. The cytosolic form of PLA $_2$ mediates the production of agonist-induced arachidonic acid release and plays an essential role in the release of PAF [1–7].

Activation of cPLA $_2$ has been shown to require both phosphorylation of the enzyme and an increase in the concentration of intracellular free calcium [6]. Although p42 mitogen-activated kinase (MAP kinase), extracellular signal-related protein kinase 2 (erk2), has been shown to phosphorylate cPLA $_2$ *in vitro*, recent evidence, both in human neutrophils and human platelets, suggests that proline-directed kinases, other than erk1 and erk2, may phosphorylate and activate cPLA $_2$ [8–12]. The identities of these kinases remain to be determined. Moreover, it is not known if stimuli that phosphorylate and

activate cPLA $_2$ in intact cells, mediate their effects through the erk MAP kinase subtype.

Recently, we demonstrated the presence of a novel 38 kDa protein, which is tyrosine phosphorylated and activated in human neutrophils, terminally differentiated cells, upon stimulation of these cells with various agonists [12,13]. This 38 kDa protein was identified as the mammalian homologue of HOG1 in yeast, p38 MAP kinase. The phosphorylation and activation of this p38 MAP kinase can be affected by both G-protein-coupled receptors such as PAF, and non-G-protein-coupled receptors such as lipopolysaccharide (LPS), as well as cytokine-coupled receptors such as tumour necrosis factor- α (TNF- α) and granulocyte-macrophage colony-stimulating factor. The present studies were undertaken to determine the role of this newly identified p38 MAP kinase in the phosphorylation and activation of cPLA $_2$ in human neutrophils stimulated by TNF- α . This was done using the recently developed compound SB 203580, a highly specific inhibitor of p38 MAP kinase activity that does not affect erk1, erk2 or Jun kinase subtypes [14,15].

MATERIALS AND METHODS

Isolation of neutrophils

Neutrophils were isolated from human blood using a Ficoll/Hypaque gradient, and the contaminating red blood cells were lysed by hypotonic shock [16,17]. The neutrophils were resuspended in modified Hanks balanced salt solution containing 0.1% (v/v) 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 [12,13]. Briefly, 3 ml of cells

Abbreviations used: LPS, lipopolysaccharide; TNF- α , tumour necrosis factor- α ; PAF, platelet-activating factor; MAP kinase, mitogen-activated protein kinase; ATF-2, activating transcription factor 2; cPLA $_2$, cytosolic phospholipase A_2 ; anti-PY, anti-phosphotyrosine; ECL, enhanced chemiluminescence; sPLA $_2$, secretory phospholipase A_2 ; erk, extracellular signal-related protein kinase.

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(1×10^7 cells/ml) were treated with SB 203580 or diluent for 30 min followed by the addition of buffer or TNF- α . After termination of the reaction, the cells were resuspended at 10^8 cells/ml in buffered sucrose solution (50 mM Hepes, pH 7.5/250 mM sucrose/1 mM EGTA/1 mM EDTA/100 μ g/ml leupeptin/100 μ g/ml aprotinin/40 μ g/ml pepstatin/5 mM dithiothreitol/2 mM PMSF). Cells were disrupted by sonication; centrifuged at 8000 *g* for 15 min and the supernatant was then centrifuged at 100000 *g* for 30 min at 4 °C. The cytosolic fraction protein was measured, and used in subsequent assays.

Immunoblotting

Immunoblotting was performed as described previously [12,13,17]. In cPLA₂ experiments, equivalent amounts of protein (100 μ g of cytosolic fraction) were loaded on to an 8% (v/v) SDS/polyacrylamide gel. After electrophoresis, proteins were transferred from the gel to poly(vinylidene difluoride) membranes in transfer buffer (20 mM Tris base/150 mM glycine/20% methanol, pH 8.9). Blots were washed in Tris-buffered saline/Tween-20, incubated with the desired antibody and then probed with the appropriate horseradish peroxidase-conjugated antibody. The enhanced chemiluminescence (ECL) method was utilized for detection.

Immunoprecipitation

Aliquots of the cytosolic fraction containing equivalent amounts of protein were added to tubes containing anti-rabbit IgG-agarose beads to which anti-p38 MAP kinase was conjugated. After 2 h of gentle rocking at 4 °C the beads were washed twice with buffer (25 mM Tris/HCl/150 mM NaCl/2 mM EDTA, pH 7.4), dissociated by the addition of 75 μ l of Laemmli sample buffer, boiled for 5 min and loaded on to a 10% (v/v) SDS/polyacrylamide gel. Proteins were transferred to Immobilon-P membrane and analysed by immunoblotting as described.

Protein kinase assay

The p38 MAP kinase activity assay was performed using immunoprecipitates of p38 MAP kinase prepared from the cytosolic fraction [13]. 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 glutathione S-transferase-activating transcription factor 2 (GST-ATF-2) 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 ATF-2 was examined after SDS/PAGE and autoradiography.

Assay for cPLA₂ activity

The cPLA₂ assay was performed as previously described [8,17,18]. Following cell stimulation, cytosolic fractions were prepared as described. The assay was initiated by adding cytosolic extract to the substrate, 1-palmitoyl-2-[¹⁴C]arachidonyl phosphatidylcholine (final concentration 30 μ M). The final concentration of Ca²⁺ in the assay mixture was 2 mM in excess of the chelators. The reaction was stopped after 30 min at 37 °C by addition of ice-cold ethanol containing 2% (v/v) acetic acid and 1 mg/ml arachidonic acid. A 50 μ l volume of this mixture was spotted on Whatman LK6D thin-layer chromatography plates, which were developed in the organic phase of ethyl acetate/iso-octane/water/acetic acid (55/75/100/8, by vol.). Lipids were detected with I₂ vapour. Relevant areas were scraped into scintillation vials and assayed for radioactivity. Results are expressed as pmol

of arachidonic acid released/min per mg of protein, at a substrate concentration of 30 μ M. Results are shown as means $\pm$ S.E.M.

Materials

Anti-phosphotyrosine (anti-PY), a monoclonal antibody, was obtained from Upstate Biotechnology Incorporated (Lake Placid, NY, U.S.A.). Electrophoresis reagents and molecular mass markers were from Bio-Rad Laboratories (Melville, NY, U.S.A.); Poly(vinylidene difluoride) protein transfer membrane (Immobilon-P) was obtained from Millipore Corporation (Bedford, MA, U.S.A.); the ECL kit, Western blotting reagents and [γ -³²P]ATP (5000 Ci/mmol) were purchased from Amersham (Arlington Heights, IL, U.S.A.). TNF- α was purchased from Calbiochem (San Diego, CA, U.S.A.). The recently developed anti-p38 antibody C-20 (this antibody does not cross-react with other MAP kinases or with JNK1) and the ATF-2 were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, U.S.A.). The inhibitor SB 203580 and the inactive analogue SKF 106978 were gifts from SmithKline Beecham Pharmaceuticals (King of Prussia, PA, U.S.A.). 1-Palmitoyl-2-[¹⁴C]arachidonyl phosphatidylcholine was obtained from DuPont NEN (Boston, MA, U.S.A.). Anti-cPLA₂ was generously provided by the Immunology Department of the Genetic Institute Inc. (Cambridge MA, U.S.A.). Anti-rabbit IgG-agarose was from Sigma (St. Louis, MO, U.S.A.). PD 98059 was purchased from New England Biolabs (Beverly, MA, U.S.A.).

RESULTS AND DISCUSSION

Tyrosine phosphorylation and activation of p38 MAP kinase in control and SB 203580-treated human neutrophils

The effect of the inhibitor SB 203580 on the tyrosine phosphorylation of p38 MAP kinase in human neutrophils following stimulation with TNF- α was examined. In these experiments the cells were incubated with diluent or 15 μ M inhibitor for 30 min and then stimulated with TNF- α (10 ng/ml, 5 min). Immunoprecipitates of p38 MAP kinase were prepared from the cytosol fraction using the recently developed anti-(p38 MAP kinase) antibody, C-20 (this antibody does not cross-react with the other members of the MAP kinase family). The results summarized in Figure 1(A) show that TNF- α increases the tyrosine phosphorylation of p38 MAP kinase, as shown previously [13], and this phosphorylation is only slightly reduced by the inhibitor SB 203580, which alone causes a small but reproducible increase in the tyrosine phosphorylation of this protein. The level of TNF- α induced phosphorylation of p38 in the presence of the inhibitor is $85 \pm 10\%$ (three separate experiments) of that in the absence of the inhibitor. The immunoblot (Figure 1A) was stripped and re-probed with anti-p38 MAP kinase antibody. These results, Figure 1(B), show that equivalent amounts of p38 MAP kinase were immunoprecipitated under all conditions (i.e. binding of SB 203580 to p38 MAP kinase does not affect antibody interaction). Several concentrations (5–30 μ M) of the inhibitor, and times of incubation (5–45 min), were tested. It was found that the lower the concentration of the inhibitor the longer was the time of incubation needed to achieve inhibition (results not shown). In our hands, using human neutrophils in suspension, we found that 15 μ M and 30 min of incubation gave the best results, which enable us to draw unambiguous conclusions.

Next we examined the effect of the inhibitor SB 203580, or its inactive analogue SKF 106978, on the basal and stimulated activity of p38 MAP kinase. In these experiments, human neutrophils in suspension were incubated with diluent, 15 μ M SB 203580 or 15 μ M SKF 106978 for 30 min, and then the cells were

Figure 1 Effect of SB 203580 on the TNF- α -stimulated tyrosine phosphorylation of p38 MAP kinase

Human neutrophils were treated with buffer (lane 1), 10 ng/ml TNF- α for 5 min (lane 2), 15 μ M SB 203580 for 35 min (lane 3) or SB 203580 for 30 min followed by TNF- α for 5 min (lane 4). Immunoprecipitates prepared using anti-(p38 MAP kinase) antibody were subjected to SDS/PAGE, transferred to membrane and immunoblotted with anti-PY antibody (A), then stripped and re-probed with anti-(p38 MAP kinase) antibody (B). The band above the 49 kDa mass standard is IgG. The autoradiograph represents the results of a single experiment which was carried out three times.

Figure 2 Effect of SB 203580 and SKF 106978 on TNF- α -stimulated p38 MAP kinase activity

Immunoprecipitates of p38 MAP kinase were prepared from neutrophils treated for 30 min with 15 μ M SB 203580, SKF 106978 or diluent, then stimulated for 5 min with 10 ng/ml TNF- α . Protein kinase activity was measured in an immune-complex kinase assay using [32 P]ATP and ATF-2 as substrates. Phosphorylated ATF-2 was detected after SDS/PAGE and autoradiography. The autoradiograph represents the results of a single experiment which was carried out at least three times.

stimulated with TNF- α (10 ng/ml, 5 min). Immunoprecipitates of p38 MAP kinase were prepared and were used in an immune-complex kinase assay utilizing [γ - 32 P]ATP and ATF-2 as substrates. Treatment of the neutrophils with SB 203580 did not affect the amount of p38 immunoprecipitated (Figure 1B). As can be seen in Figure 2, human neutrophils stimulated with TNF- α show a marked increase in p38 MAP kinase activity, and this activity is reduced in cells treated with the inhibitor SB 203580 but not the analogue SKF 106978.

Phosphorylation and activation of cPLA₂ in control and SB 203580-treated human neutrophils

Recently, we and others [8–12], have presented evidence that suggests the existence of a cPLA₂ phosphorylation pathway

Figure 3 Effect of SB 203580 and SKF 106978 on TNF- α -stimulated cPLA₂ phosphorylation

Human neutrophils were treated for 30 min with 15 μ M SB 203580, SKF 106978 or diluent before stimulation with 10 ng/ml TNF- α , 5 min. After termination of the reaction, cells were sonicated and cytosolic fractions were subjected to 8% (v/v) SDS/PAGE. Immunoblot analysis was performed using anti-cPLA₂ antibody. cPLA₂ phosphorylation was detected as a decrease in the electrophoretic mobility. The autoradiograph represents the results of a single experiment which was carried out at least three times.

Table 1 cPLA₂ activity in control and SB 203580-treated human neutrophils stimulated with TNF- α

The cells were incubated with SB 203580 (15 mM) or its inactive analogue SKF 106978 (15 μ M) for 30 min before addition of TNF- α for 5 min; cPLA₂ activity was determined for the cytosolic fraction as indicated in the Materials and methods section. Each value represents the mean $\pm$ S.E.M. The numbers in parentheses refer to the number of separate experiments. Each experiment was performed in duplicate and carried out on neutrophils isolated from different donors.

Treatment	cPLA ₂ activity (pmol/min per mg of protein)	
	Basal	TNF- α (10 ng/ml)
Control	4.8 $\pm$ 0.6 (7)	9.1 $\pm$ 1.1 (7)
SB 203580	5.7 $\pm$ 1.0 (6)	6.3 $\pm$ 1.2 (5)*
SKF 106978	5.4 $\pm$ 1.0 (2)	9.0 $\pm$ 1.1 (2)

* The effect of SB 203580 on TNF- α -induced stimulation of cPLA₂ activity is statistically significant ($P < 0.001$, using Student's paired sample *t*-test).

independent of erk2 MAP kinase subtype involvement. In order to address the role of p38 MAP kinase in the phosphorylation of cPLA₂, the phosphorylation of cPLA₂ in control and SB 203580-treated human neutrophils upon stimulation with TNF- α was examined. In these experiments, human neutrophils in suspension were incubated with diluent or 15 μ M SB 203580 for 30 min. The cells were then stimulated with TNF- α (10 ng/ml, 5 min). Cytosolic fractions were prepared, electrophoresed and transferred, and the resulting immunoblot was probed with anti-cPLA₂ antibody. Aliquots of these same cytosol fractions were used to measure cPLA₂ activity. The data summarized in Figure 3 clearly show that the phosphorylation of cPLA₂ produced by TNF- α is inhibited in cells pretreated with SB 203580.

Next we measured cPLA₂ activity using aliquots of the cytosol fractions prepared in the previous experiments. Note that samples used for measuring cPLA₂ activity are prepared under reducing conditions (to negate the effect of sPLA₂ enzymes) and in the presence of EGTA and EDTA (to prevent the translocation of the enzyme to particulate fractions). The activity of cPLA₂ in the soluble fractions (under these conditions there was no detectable activity in the particulate fractions) was determined using the substrate 1-palmitoyl-2-[14 C]arachidonyl phosphatidylcholine as described in the Materials and methods section. The results are summarized in Table 1. Note, that while the basal activity from SB 203580-treated cells is slightly increased, the TNF- α -induced

stimulation is completely abolished in cells treated with the inhibitor. These data suggest very strongly that the inhibitor of the p38 MAP kinase cascade has a profound effect on both TNF- α -stimulated cPLA₂ phosphorylation and activation.

It is generally agreed that the erk2 MAP kinase increases the phosphorylation of cPLA₂ under *in vitro* conditions, and that the phosphorylation of cPLA₂ in intact cells by some agonists may be mediated through the tyrosine phosphorylation and activation of the erk2 MAP kinase [6]. Recently, however, several papers have been published suggesting the involvement of an erk2 MAP kinase-independent pathway in the phosphorylation of cPLA₂ in intact cells [8–12]. Recently an inhibitor of p38 MAP kinase, identified as SB 203580, has been developed [14,15]. SB 203580 is a remarkably specific inhibitor of p38 MAP kinase activity *in vitro*, and prevents the activation of MAP kinase-activated protein kinase-2 and the phosphorylation of heat-shock protein 27 in KB cells stimulated by osmotic shock or interleukin-1 [14,15].

Since the TNF- α -induced tyrosine phosphorylation of p38 MAP kinase is only slightly inhibited by SB 203580, the reduction in the TNF- α -stimulated activity of p38 MAP kinase cannot be due to the inhibition of an upstream step(s). It is reasonable to conclude, from the results presented here, that the SB 203580-sensitive kinase or kinases, and/or phosphatases, are involved in the phosphorylation and activation of cPLA₂ in intact human neutrophils stimulated by TNF- α . If the inhibitor is indeed specific only for p38 MAP kinase, then this kinase cascade must be involved in the TNF- α -stimulated phosphorylation and activation of cPLA₂ in these cells. This conclusion is based on several experimental findings. First, TNF- α stimulates the tyrosine phosphorylation and activation of the p38 MAP kinase cascade, but not the erk1 and erk2 MAP kinase subtype, in human neutrophils in suspension [13,17]. Secondly, the activation of the p38 cascade is inhibited by SB 203580 in these cells. Thirdly, TNF- α stimulates the phosphorylation and activation of cPLA₂ in human neutrophils in suspension, and both of these responses are inhibited by SB 203580.

We found that PD 98059, which inhibits the phosphorylation and activation of erk2 MAP kinase [15] by inhibiting the activation of MAP kinase kinase-1, has no effect on the TNF- α -induced phosphorylation of cPLA₂ (results not shown). This result is consistent with our previous finding that TNF- α does not tyrosine phosphorylate or activate erk2 in human neutrophils in suspension [8,17]. More interestingly, we also found that LPS-induced phosphorylation of cPLA₂ is not inhibited in human neutrophils pretreated with SB 203580 (R. I. Sha'afi and T. F. P.

Molski, unpublished work). LPS stimulation of human neutrophils in suspension phosphorylates and activates p38 MAP kinase [13].

Whether p38 MAP kinase phosphorylates cPLA₂ directly or through the activation of one or more kinases cannot be determined from the present data, and is currently being investigated. In this regard, Raingeaud et al. [2] reported that incubation of bacterially expressed p38 MAP kinase with cPLA₂ and [γ -³²P]ATP failed to phosphorylate cPLA₂. This suggests strongly that any involvement of p38 MAP kinase in the phosphorylation and activation of cPLA₂ in intact cells is indirect.

This work was supported in part by NIH grants HL-53786-06 and AI-28810-03 (R.I.S.) and AI-20943 (C.-K.H.).

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