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REGULATION OF EICOSANOID BIOSYNTHESIS IN THE MACROPHAGE

INVOLVEMENT OF PROTEIN TYROSINE PHOSPHORYLATION AND MODULATION BY SELECTIVE PROTEIN TYROSINE KINASE INHIBITORS

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Abstract—The protein tyrosine kinase (PTK) inhibitor genistein has been demonstrated to inhibit platelet-activating factor-stimulated prostaglandin E_2 (PGE_2) production in lipopolysaccharide (LPS)-primed $P388D_1$ macrophage-like cells (Glaser *et al.*, *J Biol Chem* **265**: 8658–8664, 1990). Therefore, the role of PTK in eicosanoid biosynthesis was investigated in murine resident peritoneal macrophages using genistein and tyrphostin-25, selective PTK inhibitors. Genistein, a competitive inhibitor of ATP binding on PTK, inhibited PGE_2 production (IC_{50} = 20 μM) in response to zymosan, calcium ionophore A23187, and phorbol myristate acetate stimulation. Genistein also inhibited leukotriene C_4 (LTC_4) production in response to zymosan and calcium ionophore A23187 (IC_{50} = 10 and 15 μM, respectively) stimulation. Tyrphostin-25, a competitive inhibitor of substrate binding on PTK, inhibited zymosan-stimulated PGE_2 and LTC_4 production, IC_{50} = 20 and 7 μM, respectively. Neither genistein nor tyrphostin-25 had any effect on human synovial fluid phospholipase A_2 (PLA_2) activity *in vitro* or on cyclooxygenase activity in the intact macrophage; however, tyrphostin-25 did affect 5-lipoxygenase activity (determined from the metabolism of exogenously applied arachidonic acid). These results suggest PTK-mediated phosphorylation as a common event in the signal transduction mechanisms of different stimuli which activate PLA_2 for arachidonic acid release and subsequent eicosanoid biosynthesis. Immunoblot analyses of zymosan-stimulated peritoneal exudate cells with the phosphotyrosine monoclonal antibody clone 4G10 demonstrated an increase in protein phosphotyrosine levels in eight major protein bands on sodium dodecyl sulfate–polyacrylamide gel electrophoresis: p59, 71, 76, 90, 100, 112, 125 and 150. Maximal phosphorylation of these protein substrates occurred after 1–2 min stimulation. Zymosan and LPS stimulation of peritoneal exudate cells produced similar patterns of protein tyrosine phosphorylation. Zymosan-stimulated tyrosine phosphorylation was inhibited by tyrphostin-25 in a concentration-dependent manner between 10 and 60 μM, demonstrating a similar concentration response between effects on tyrosine phosphorylation and eicosanoid biosynthesis in the murine peritoneal macrophage. The use of selective PTK inhibitors suggests a common role for PTK and tyrosine phosphorylation in eicosanoid biosynthesis in the murine peritoneal macrophage.

The level of free arachidonate in cells is controlled by many different enzymes, and under resting conditions is maintained at extremely low levels. It is believed that the majority of arachidonate release upon cellular stimulation is the result of the action of phospholipase A_2 (PLA_2†; EC 3.1.1.4). The arachidonate made available is metabolized rapidly into potent inflammatory mediators, the eicosanoids. Our knowledge of the mechanisms which regulate the activity of PLA_2 in the intact cell is minimal. The complexity of the regulation of PLA_2 activity may be associated with the nature of the enzyme–substrate (interfacial) interactions [1] and the multiple cellular mechanisms which may physically modify the enzyme (e.g. phosphorylation or synthesis of accessory proteins).

Regulation of PLA_2 via kinases has been an intriguing and a much implicated part of many signal transduction pathways [2, 3]. Phosphorylation of a high molecular weight PLA_2 (SDS–PAGE, M_r = 110 kDa) [4] on Ser residues has been observed recently [5]. The *in vitro* phosphorylation of a similar 100 kDa PLA_2 from the murine macrophage J744 cell line was demonstrated with exogenous protein kinase C (PKC) [6]. Inhibitors of PKC have been shown to be effective inhibitors of eicosanoid production in many cell types. However, the specificity of these inhibitors does not exclude their effects on several different kinases which may be involved in the signal transduction mechanisms controlling arachidonic acid (AA) release by PLA_2.

More recently, the phosphorylation of tyrosine

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† Abbreviations: PLA_2, phospholipase A_2; PTK, protein tyrosine kinase; PKC, protein kinase C; AA, arachidonic acid; LPS, lipopolysaccharide; HSF-PLA_2, human synovial fluid phospholipase A_2; PGE_2, prostaglandin E_2; TxB_2, thromboxane B_2; 6-keto-$PGF_{1\alpha}$, 6-keto-prostaglandin $F_{1\alpha}$; LTC_4, leukotriene C_4; PMA, phorbol myristate acetate; PBS, phosphate-buffered saline; RP-HPLC, reverse phase high performance liquid chromatography; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; CO, cyclooxygenase; 5-LO, 5-lipoxygenase; PEC, peritoneal exudate cells; PS, phosphoserine; and PY, phosphotyrosine.

residues on cellular proteins has been observed in neutrophils [7–10], platelets [11], RBL cells [12–14], and rat Kupffer cells [15] in response to stimuli which activate the AA cascade. Protein tyrosine kinase (PTK) activity may therefore be an integral part, either independently or in concert with Ser/Thr protein kinases (e.g. PKC), of the signal transduction mechanisms which activate or regulate PLA_2. In lipopolysaccharide (LPS)-primed $P338D_1$ macrophage-like cells, platelet-activating factor (PAF)-induced prostaglandin E_2 (PGE_2) production was inhibited by the PTK inhibitor genistein and was relatively insensitive to inhibitors of PKC (e.g. H-7) or the down-regulation of PKC by phorbol esters [16], suggesting the involvement of PTK and tyrosine phosphorylation in PAF-mediated arachidonic acid release and subsequent PGE_2 production [9, 16].

In this manuscript, the effects of different classes of PTK inhibitors on eicosanoid biosynthesis in murine peritoneal macrophages and on tyrosine phosphorylation in murine peritoneal exudate cells (PEC) are described. The results demonstrate that tyrosine phosphorylation appears to be an integral event in the regulation of eicosanoid biosynthesis (possibly AA release via PLA_2 activation) in the murine macrophage.

MATERIALS AND METHODS

Materials. Zymosan A, calcium ionophore A23187, phorbol myristate acetate (PMA) and AA were obtained from the Sigma Chemical Co. (St. Louis, MO). Zymocel, pyrogen free solution (4 mg/mL), was purchased from Alpha-Beta Technology (Worcester, MA). Hanks' Balanced Salt Solution (HBSS), M 199 cell culture medium, penicillin/streptomycin and glutamine were obtained from Gibco (Grand Island, NY). Genistein and tyrphostin-25 were obtained from Biomol (Plymouth Meeting, PA). [^{3}H]Arachidonic acid-labeled *Escherichia coli*, strain KI2C600 (20,000 cpm/10 nmol phospholipid phosphorus) was supplied by Dr. Franson, Virginia Commonwealth University (Richmond, VA). PGE_2 and leukotriene C_4 (LTC_4) enzyme immunoassay (EIA) kits were obtained from Cayman Chemical (Ann Arbor, MI).

Isolation and culture of resident peritoneal macrophages. CD-1 male mice (Charles River), 8–12 weeks of age, were euthanized by carbon dioxide asphyxiation and the peritoneal cavity was lavaged with 8 mL of ice-cold HBSS (without Ca^{2+} or Mg^{2+}). Lavage fluids were pooled and centrifuged at 400 *g* for 10 min at 4° to pellet cells. Cells were resuspended in 10 mL of M-199 medium (without serum), counted using a hemacytometer and plated in 12-well cluster dishes at 1×10^6 cells/well. Cells were allowed to adhere for 2 hr at 37° in 5% CO_2–95% air and 100% humidity. Following purification by adherence, cells were washed three times with HBSS (with Ca^{2+} and Mg^{2+}) to remove any non-adherent cells. Cells were then preincubated with compounds or vehicle (Me_2SO) for 30 min at 37°. Medium was removed and replaced with fresh medium containing compound or vehicle and the appropriate stimulus [zymosan, 100 μg/mL; A23187, 3 μM; AA, 10 μM; PMA, 0.1 μM; LPS (*E. coli* 0111:B4), 1 μg/mL] and incubated for 2 hr at 37°. After the 2-hr incubation, the medium was removed and frozen at −20° until analysis by EIA. EIA for PGE_2, thromboxane B_2 (TxB_2), 6-keto-$PGF_{1\alpha}$ and LTC_4 were performed according to manufacturer's instructions. Data are presented as the mean of at least two separate experiments each determined in triplicate. Standard error was < 15% and therefore was not presented in the figures.

Exogenous arachidonic acid metabolism. Macrophages were incubated with exogenous AA (2 or 10 μM) for 20 min at 37°. Assay medium was removed and frozen at −20° until analysis. PGE_2 production was used as a crude measure of cellular cyclooxygenase (CO) activity and was quantitated by EIA as described above.

Alternatively, macrophages were incubated with [^{3}H]AA and unlabeled AA (10 μM) as carrier for 20 min at 37°. Assay medium was removed and centrifuged at 13,000 rpm for 5 min in an Eppendorf microcentrifuge to remove any non-adherent cells and debris. An aliquot was transferred to an automated WISP injector and 100 μL was injected onto a C_{18} reverse phase HPLC (RP-HPLC) column. The [^{3}H]AA metabolite profile was analyzed as described by Marshall [17].

Human synovial fluid phospholipase A_2 assay. Human synovial fluid PLA_2 (HSF-PLA_2) was isolated from synovial fluid obtained from rheumatoid arthritis patients as previously described [18]. HSF-PLA_2 activity was assayed using [^{3}H]AA-labeled *E. coli* membranes as previously described [19]. Compounds were preincubated with HSF-PLA_2 for 30 min prior to the addition of substrate to initiate the reaction.

Immunoblot analysis of tyrosine phosphorylated proteins. PEC were prepared as described above for the isolation of macrophages except that the 2-hr adherence was not used. Cells were resuspended at 5×10^6 cells/mL in Iscove's DME (low endotoxin), and 1.0 mL was used per assay in a 2.0-mL microcentrifuge tube. Cells were maintained throughout the experiments in a 37° water bath. Inhibitors or vehicle (Me_2SO) were added 15 min prior to stimulation and cells were stimulated for various times as described for each experiment. After stimulation cells were sedimented at 13,000 rpm in an Eppendorf microcentrifuge, the supernatant was removed and 50 μL of phosphate-buffered saline (PBS) containing 400 μM vanadate and 50 μL sodium dodecyl sulfate-sample buffer (4×) at 100° was added to the cell pellet and vortexed. The samples were boiled for 10 min and then either analyzed immediately or stored at −70° until SDS–PAGE analysis could be performed.

The samples were analyzed using 4–20% gradient SDS–PAGE mini gels (BioRad). Each lane of the gel was loaded with 5×10^5 cell equivalents (10 μL). After electrophoresis the gels were soaked in transfer buffer (192 mM glycine, 25 mM Tris, 15% methanol, pH 8.3 to 8.4) for 30 min. Then the proteins were transferred to Immobilon-P membranes (Millipore) in transfer buffer for 75 min at 100 V at 4°. The Immobilon-P membranes were washed twice in PBS (without Ca^{2+} or Mg^{2+}), blocked in 3% non-fat milk

in PBS (pH 7.4) for 1 hr and incubated with the primary antibody, phosphotyrosine monoclonal antibody (PY mAb clone 4G10) from U.B.I. (Lake Placid, NY) at 1 μg/mL in 3% non-fat milk in PBS (pH 7.4) overnight at 4°. After the primary PY mAb 4G10, the membranes were washed twice with PBS and incubated with the secondary antibody [goat anti-mouse IgG-horseradish peroxidase (HRP) conjugate from Boehringer Mannheim] at a 1:1000 dilution in 3% non-fat milk in PBS (pH 7.4) for 1 hr. Next the membranes were washed twice with PBS and then three times for 5 min each in PBS containing 0.05% Tween-20. Following the Tween-20 wash, the membranes were rinsed ten times in PBS to remove any residual Tween-20.

Development of the HRP-conjugated secondary Ab was performed by blotting the membranes dry and developing with Enzygraphic Web high sensitivity membranes (IBI, Kodak) for 40 sec as per manufacturer's suggestions. Development was terminated by peeling the Immobilon-P membranes off of the Enzygraphic Web and placing them in ice-cold distilled water. Membranes were then dried, photographed and analyzed with an LKB Ultrascan laser densitometer using GelScan software. Immunoblots presented are representative of at least three separate experiments each carried out in duplicate, all showing similar results.

RESULTS

Effect of genistein on macrophage eicosanoid production. Genistein inhibited both PGE_2 and LTC_4 production in normal resident peritoneal macrophages in response to various stimuli in a concentration-dependent manner (Fig. 1). Inhibition of zymosan- (100 μg/mL) stimulated LTC_4 and PGE_2 production occurred in parallel; IC_{50} values of 15 and 20 μM, respectively, were obtained (Fig. 1, top panel). Complete inhibition of eicosanoid biosynthesis was observed at 60 μM and no cellular cytotoxicity (lactate dehydrogenase release) was observed at this concentration. Calcium ionophore A23187-stimulated LTC_4 and PGE_2 production was also inhibited by genistein (IC_{50} = 10 and 20 μM, respectively) (Fig. 1, center panel) in a similar concentration range as zymosan-stimulated eicosanoid production. PMA-stimulated PGE_2 production was inhibited by genistein, also with an IC_{50} of 20 μM (Fig. 1, bottom panel). As PMA only stimulates PGE_2 production in murine macrophages, the effects of genistein on LTC_4 production could not be determined with this stimulus. Due to the similarity of IC_{50} values for each stimulus used, these data suggest that genistein may be inhibiting a common step in the activation mechanism for eicosanoid biosynthesis (possibly arachidonic acid release/PLA_2 activation).

Effect of tyrphostin-25 on macrophage eicosanoid production. Inasmuch as genistein is a competitive inhibitor of ATP binding on PTK [20] and may have effects on other ATP-dependent kinases, tyrphostin-25, a competitive inhibitor of substrate binding on PTK [21], was next evaluated in the macrophage. Tyrphostin-25 inhibited both LTC_4 and PGE_2 production in a concentration-dependent manner in response to zymosan stimulation (Fig. 2). The IC_{50} for inhibition of LTC_4 production was 7 μM, which was less than that for PGE_2, IC_{50} = 20 μM (Fig. 2). Further *in vitro* characterization on selectivity did suggest a direct effect of tyrphostin-25 on the metabolism of exogenously applied AA into 5-lipoxygenase (5-LO) products without any significant effect being observed on CO product formation, indicating a direct effect of tyrphostin-25 on the 5-LO pathway (data not shown).

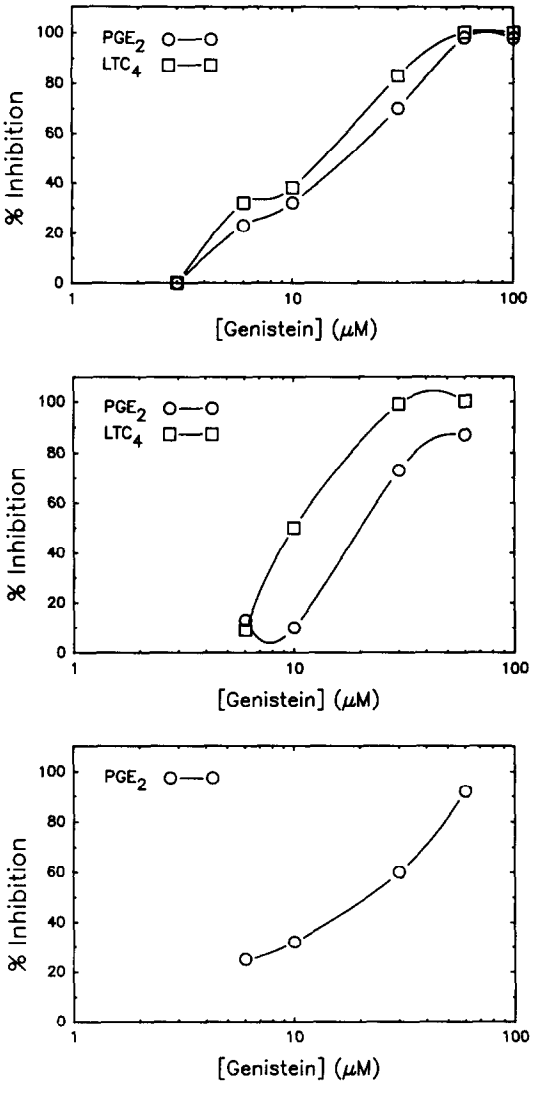


Fig. 1. Effect of genistein on PGE_2 and LTC_4 production in peritoneal macrophages stimulated with (top panel) zymosan (100 μg/mL) (PGE_2 = 12.9 ± 1.7 ng/10^6 cells, LTC_4 = 7.7 ± 0.2 ng/10^6 cells; mean ± SEM, N = 3), (center panel) calcium ionophore A23187 (3 μM) (PGE_2 = 1.4 ± 0.1 ng/10^6 cells, LTC_4 = 6.0 ± 0.2 ng/10^6 cells; mean ± SEM, N = 3), and (bottom panel) PMA (1 μM) (PGE_2 = 10.7 ± 1.7 ng/10^6 cells; mean ± SEM, N = 3). Genistein was preincubated with cells for 30 min prior to addition of respective stimulus. Cells were stimulated for 2 hr at 37° and the supernatants were assayed by EIA.

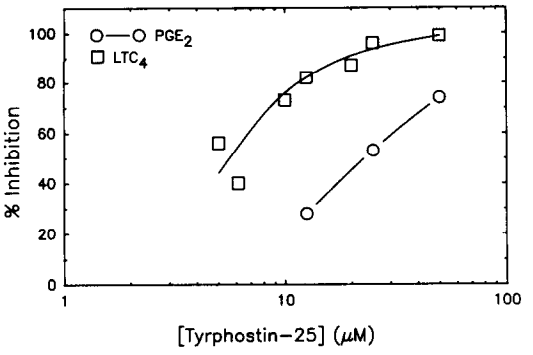


Fig. 2. Effect of tyrphostin-25 on PGE_2 and LTC_4 production in peritoneal macrophages stimulated with zymosan (100 μg/mL). Cells were treated as described in the legend of Fig. 1. ($PGE_2 = 5.6 \pm 0.6$ ng/10^6 cells, $LTC_4 = 7.0 \pm 0.3$ ng/10^6 cells; mean ± SEM, N = 3.)

Effects of genistein and tyrphostin-25 on PLA_2, CO and LO activities. To further evaluate the effects of these PTK inhibitors on other enzymes involved in eicosanoid biosynthesis, these compounds were tested in models of exogenous AA metabolism (general cellular assays for measuring total CO and LO activities). When peritoneal macrophages were given exogenous AA (2–10 μM) in the absence of serum, the AA was rapidly metabolized to cyclooxygenase products [22]. Genistein and tyrphostin-25 had no effect on PGE_2 production (CO activity) in response to exogenous AA, as measured by EIA. Genistein did not affect [^{3}H]AA metabolism significantly, no reduction in CO product (PGE_2, TxB_2 or 6-keto-$PGF_{1\alpha}$) or LO product (LTC_4 or 5-hydroxyeicosatetraenoic acid) formation was observed as measured by RP-HPLC (data not shown). However, tyrphostin-25 did inhibit [^{3}H]5-LO product formation by 69% at 60 μM, suggesting a direct effect of tyrphostin-25 on the metabolism of AA by the 5-LO enzyme (data not shown).

HSF-PLA_2 was used as a model for a general effect on PLA_2 activity. Genistein and tyrphostin-25 had no significant effect on HSF-PLA_2 *in vitro* at a concentration of 100 μM (two times the phospholipid substrate concentration in the assay). These results are similar to the lack of inhibition observed with genistein against the P388D_1 PLA_2 [16].

Immunoblot analysis of zymosan-stimulated macrophages. To study tyrosine phosphorylation in the macrophage under conditions shown in other cells to produce maximal phosphorylation [13], PEC were used with zymocel (a pure form of zymosan A) stimulation. Since PEC are a mixture of lymphocytes and macrophages, tyrosine phosphorylation in response to zymocel would be the result of macrophage and not lymphocyte activation, as lymphocytes do not respond to phagocytic stimuli. As shown in Fig. 3 (inset), stimulation with zymocel (100 μg/mL) for 1 min resulted in the tyrosine phosphorylation of a group of proteins (eight major protein bands) as determined from immunoblot analysis with the PY mAb 4G10. From the laser densitometer analysis of this immunoblot (Fig. 3), the protein bands which are phosphorylated on tyrosine residues in response to zymocel corresponded to M_r values of 59, 71, 76, 90, 100, 112, 125, and 150 kDa. It is apparent from the immunoblot that a constitutive level of tyrosine phosphorylation exists in these PECs as determined by the Enzygraphic Web detection method (Fig. 3, inset). Stimulation with zymocel resulted in the increased tyrosine phosphorylation of these constitutively phosphorylated proteins as shown in the laser densitometer analysis (Fig. 3).

The specificity of the PY mAb 4G10 for tyrosine phosphorylated proteins was evaluated by competition studies with 2.5 mM phosphoserine (PS) and phosphotyrosine (PY) (Sigma) present during the incubation with the PY mAb 4G10 (Fig. 4). The presence of PS (2.5 mM) during the PY mAb 4G10 incubation did not block the reactivity of the mAb for tyrosine phosphorylated proteins (Fig. 4, +PS), and in fact seemed to enhance the detection of tyrosine phosphorylated proteins. In contrast, the presence of PY (2.5 mM) completely blocked the reactivity of the PY mAb 4G10 with tyrosine phosphorylated proteins (Fig. 4, +PY). These experiments demonstrate the specificity of the PY mAb 4G10 for PY in the evaluation of tyrosine phosphorylated proteins from zymocel-stimulated PEC.

Tyrosine phosphorylation time course. PEC were stimulated with zymocel (100 μg/mL) for various times and the level of tyrosine phosphorylation was determined by immunoblot analysis. As shown in Fig. 5, zymocel caused a time-dependent increase in tyrosine phosphorylation with maximal phosphorylation occurring at 1 min. The level of tyrosine phosphorylation apparently decreased from 1–5 min. An increase in the level of tyrosine phosphorylation was observed at 15-min post-stimulation and this was also observed in the non-stimulated control cells (data not shown) and, therefore, may be the result of nutrient depletion or factors other than those specifically due to the stimulation.

Effect of tyrphostin-25 on tyrosine phosphorylation. To evaluate the effects of tyrphostin-25 on protein tyrosine phosphorylation in the PEC, it seemed prudent to demonstrate the inhibition of eicosanoid production in the PEC as was demonstrated for the purified peritoneal macrophage as described in Fig. 2. Tyrphostin-25 concentration-dependently inhibited the production of PGE_2 and LTC_4 in the zymocel-stimulated PEC with IC_{50} values of 30 and 15 μM, respectively (Fig. 6, top panel). These values were not significantly different from those obtained with purified peritoneal macrophages. The center panel of Fig. 6 shows the effect of tyrphostin-25 on protein tyrosine phosphorylation in the PEC. At 6 μM, a slight increase in the level of tyrosine phosphorylation was observed and, as can be seen in Fig. 6 (top panel), this concentration of tyrphostin-25 did not inhibit either PGE_2 or LTC_4 production. In fact, a slight stimulation of eicosanoid production was usually observed. As the concentration of tyrphostin-25 was increased, a concentration-dependent decrease in the level of tyrosine phosphorylation was observed (Fig. 6, center panel).

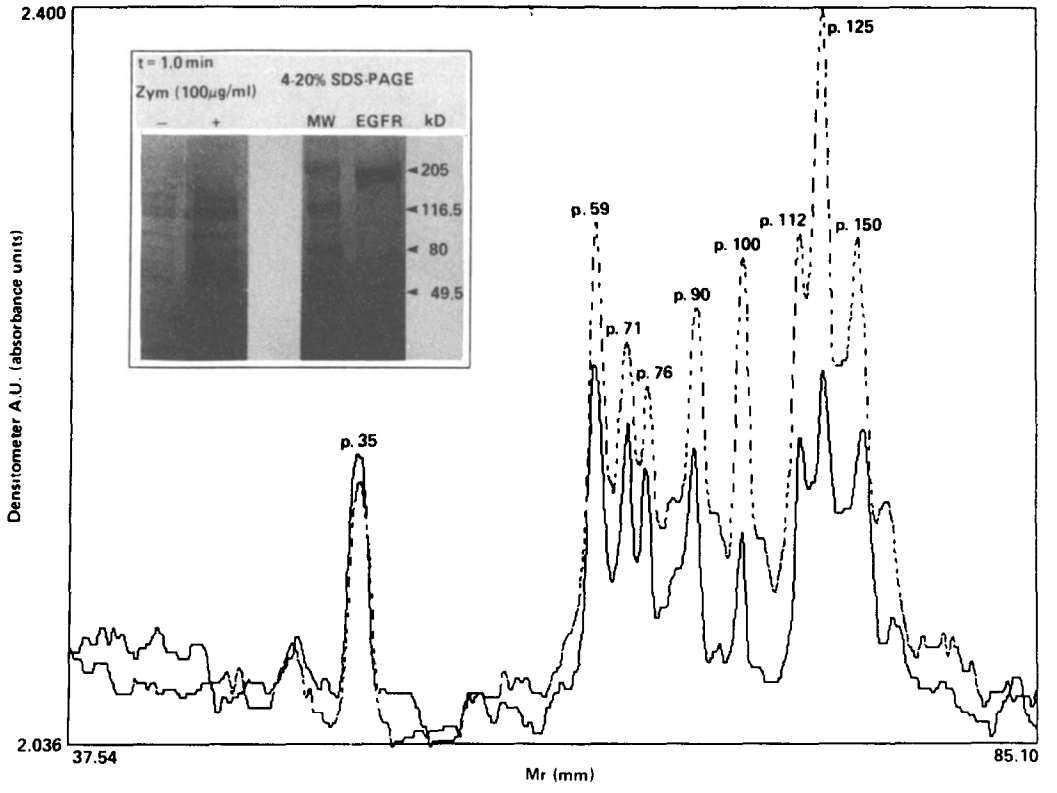


Fig. 3. Protein tyrosine phosphorylation in zymocel-stimulated PEC. PEC were stimulated for 1 min with zymocel (100 μg/mL), immunoblotted with the PY mAb 4G10 for unstimulated (——) and stimulated (– – –) cells and analyzed by laser densitometry. Immunoblot of unstimulated (−) and stimulated (+) PEC is shown in the inset.

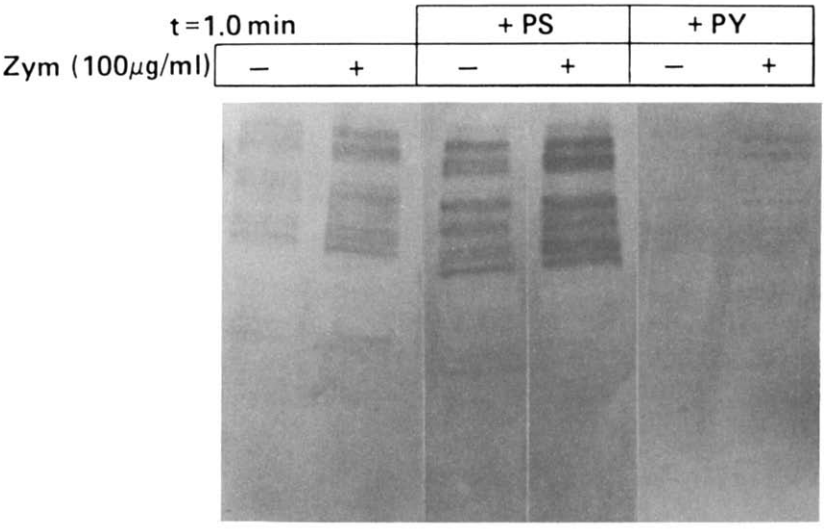


Fig. 4. Competition studies with phosphotyrosine and phosphoserine. PEC were unstimulated (−) or stimulated (+) with zymocel (100 μg/mL). Immunoblot was performed with the PY mAb 4G10 (1 μg/mL) alone, 4G10 + 2.5 mM phosphoserine (+PS), or 4G10 + 2.5 mM phosphotyrosine (+PY).

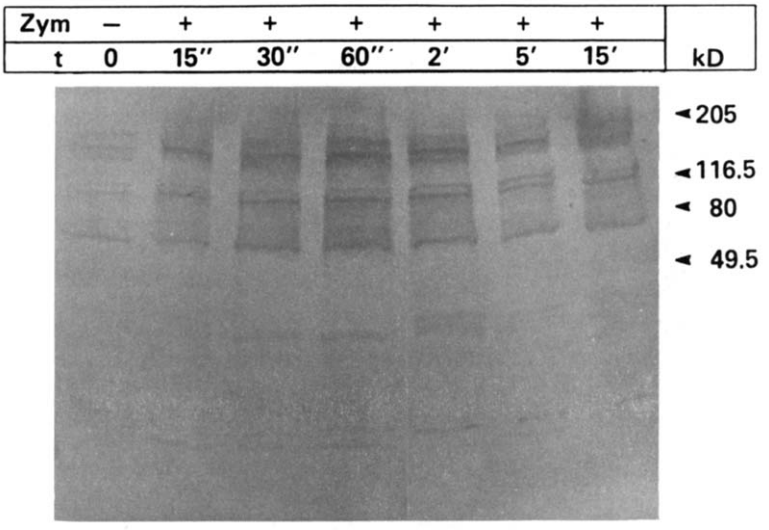


Fig. 5. Tyrosine phosphorylation time course for zymocel-stimulated PEC. PEC were stimulated with zymocel (100 μg/mL) for various time periods (0 = unstimulated, 15 sec to 15 min) at which time the reaction was terminated and quantitated as described in Materials and Methods.

The inhibition of eicosanoid production and tyrosine phosphorylation by tyrphostin-25 occurred in parallel for the p100 band, whereas the tyrosine phosphorylation decreased rather rapidly for the other protein bands (Fig. 6, bottom panel).

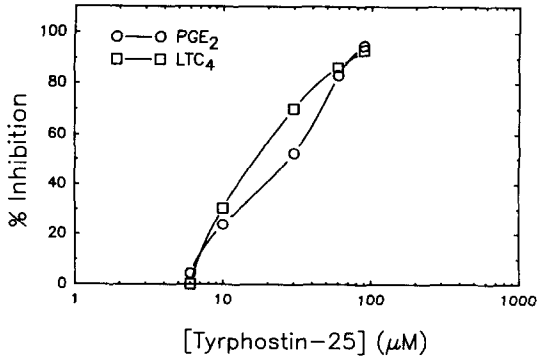
Tyrosine phosphorylation stimulated by LPS. Stimulation of PEC with LPS (lipopolysaccharide from *E. coli* 0111:B4) resulted in PGE_2 (only PGE_2 was produced by PEC in response to LPS) production which was inhibited by tyrphostin-25 (Fig. 7, top panel). LPS also resulted in the phosphorylation of a similar set of proteins as observed with zymocel. A higher molecular weight protein, p166, was observed in the LPS-stimulated PEC. This may be the result of stimulation of other cells in the PEC which may respond to LPS. LPS-stimulated tyrosine phosphorylation was maximal between 1 and 2 min (Fig. 7, center panel) and only tyrosine phosphorylation was detected with the PY mAb 4G10 in LPS-stimulated PEC (Fig. 7, bottom panel). To further validate this observation as due to tyrosine phosphorylation (protein tyrosine kinase activity), vanadate (a non-specific protein tyrosine phosphatase inhibitor) was added to the cells prior to and during stimulation. As shown in Fig. 8, the presence of 100 μM vanadate increased both basal and stimulated levels of tyrosine phosphorylation in the LPS-stimulated PEC which also corresponded to a slight increase in basal and LPS-stimulated PGE_2 production in macrophages (data not shown).

DISCUSSION

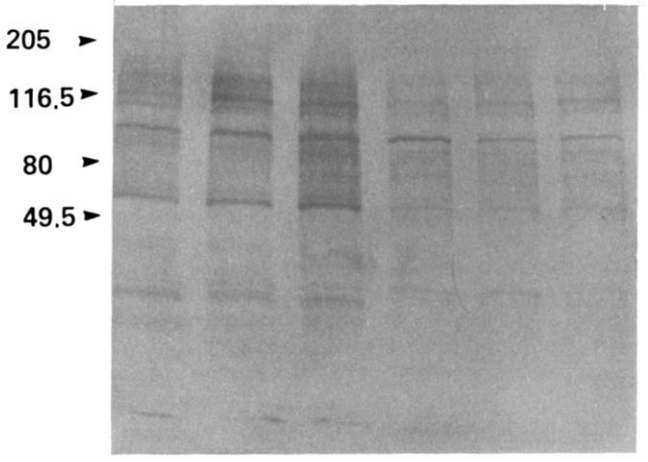
The regulation of AA release from membrane phospholipids is a complex system, as would be expected for a system which is also integral in the maintenance of membrane stability (fluidity). The present study evaluated the possible role of PTK activity (tyrosine phosphorylation) in the regulation of eicosanoid biosynthesis in the macrophage. As the specific PLA_2 responsible for AA release has not been identified yet, this evaluation was based upon the use of selective PTK inhibitors on eicosanoid biosynthesis. Many factors may contribute to the activation of PLA_2 (AA release) [21], and one facet of primary interest has been the regulation of PLA_2 by kinases and specifically PTK [16].

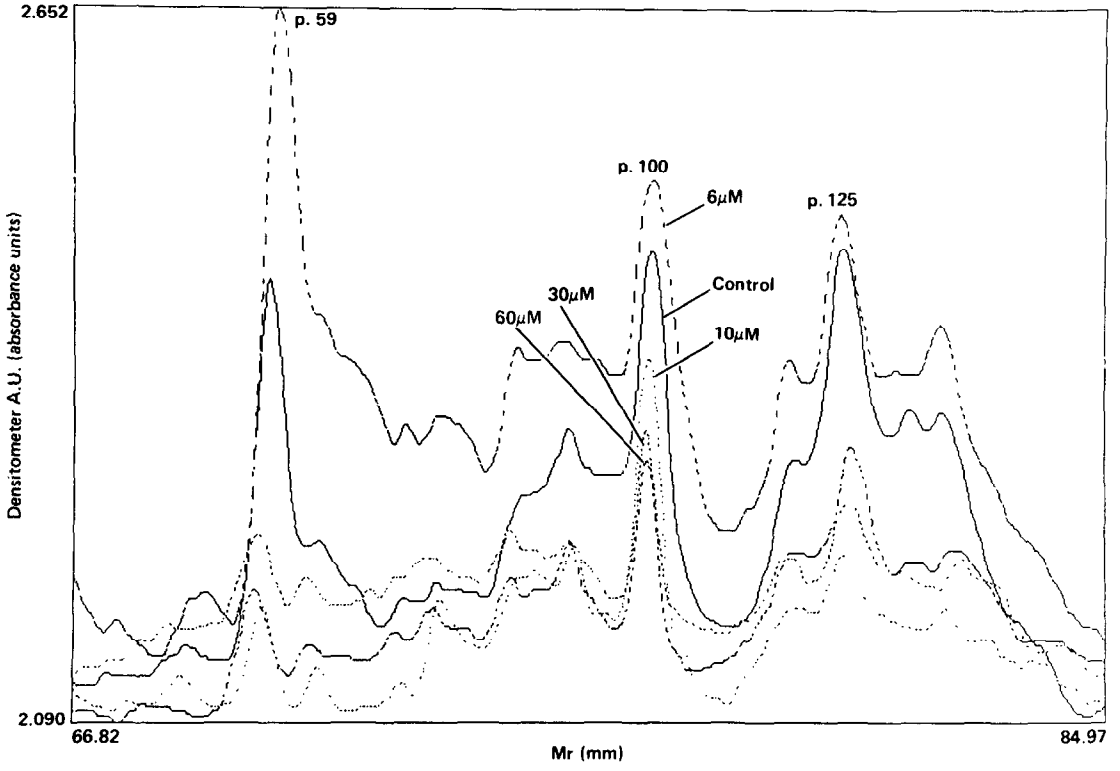
Growth factor receptors contain a PTK domain as an integral part of their structure and activation mechanism [23]. Activation of the epidermal growth factor (EGF) receptor (tyrosine kinase activation) has been shown to be associated with the activation

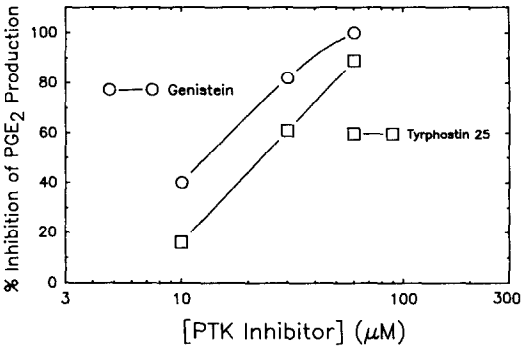
Fig. 6. Effect of tyrphostin-25 on PEC eicosanoid biosynthesis and tyrosine phosphorylation. (Top panel) Concentration–response studies of tyrphostin-25 on zymocel-stimulated PGE_2 and LTC_4 production. PEC were preincubated for 15 min with tyrphostin-25 and stimulated with zymocel (100 μg/mL) for 30 min at 37°. Reactions were terminated by centrifugation to pellet cells; the supernatant was removed, stored at −20° and assayed for PGE_2 and LTC_4 by EIA. ($PGE_2 = 4.3 \pm 1.0$ ng/5 × 10^6 cells, $LTC_4 = 5.9 \pm 0.4$ ng/5 × 10^6 cells; mean ± SEM. N = 3.) (Center panel) Concentration–response studies of tyrphostin-25 on zymocel-stimulated tyrosine phosphorylation. PEC were preincubated with tyrphostin-25 for 15 min prior to stimulation for 1 min with zymocel (100 μg/mL). Reactions were terminated and analyzed as described in Materials and Methods. (Bottom panel) Laser densitometer analysis of immunoblot for tyrphostin-25 inhibition.

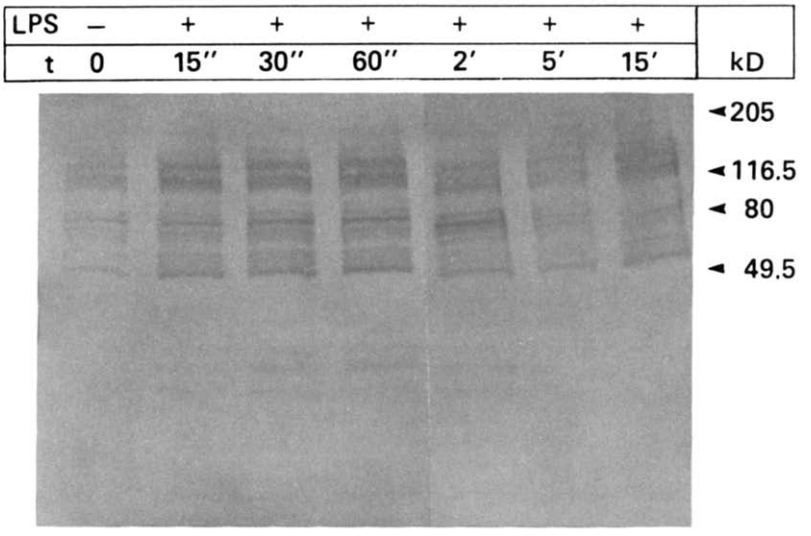


Tyr-25(μM)	–	–	6	10	30	60
Zym (100μg)	–	+	+	+	+	+









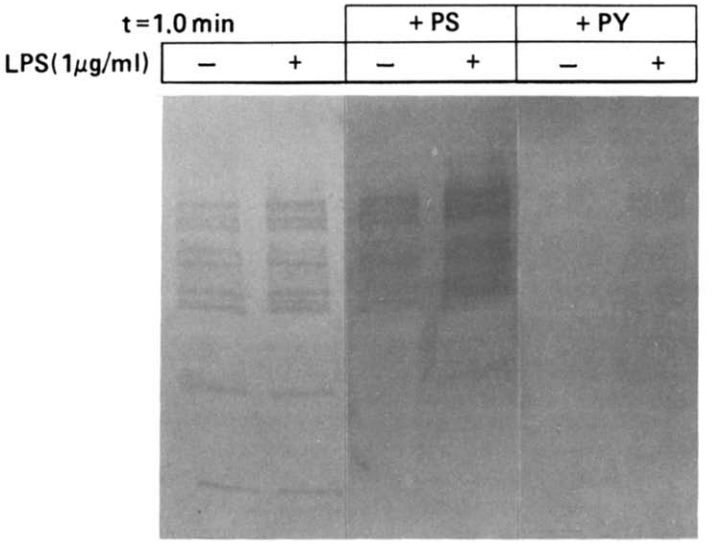


Fig. 7. Effect of LPS on PEC eicosanoid biosynthesis and tyrosine phosphorylation. (Top panel) Effect of tyrphostin-25 on LPS-stimulated PEC. PEC were preincubated with tyrphostin-25 for 15 min prior to stimulation with 1 μg/mL LPS (*E. coli* 0111:B4) for 30 min at 37°. Reactions were terminated by centrifugation to pellet cells and PGE_2 was assayed for by EIA. (PGE_2 = 9.9 ± 3.7 ng/5 × 10^6 cells; mean ± SEM, N = 3.) (Center panel) Tyrosine phosphorylation time course in PEC stimulated with LPS. PEC were either unstimulated (0 time) or stimulated with 1 μg/mL LPS (*E. coli* 0111:B4) for various time periods (15 sec to 15 min). Reactions were terminated and quantitated as described in Materials and Methods. (Bottom panel) Competition studies with LPS-stimulated PEC. Immunoblot performed with PY mAb 4G10 alone, 4G10 + 2.5 mM phosphoserine (+PS), or 4G10 + 2.5 mM phosphotyrosine (+PY).

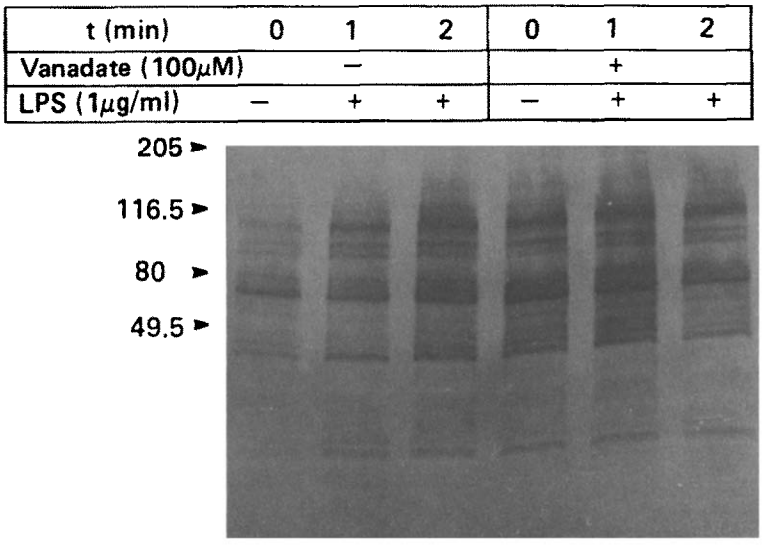


Fig. 8. Effect of vanadate on LPS-stimulated tyrosine phosphorylation. PEC were either preincubated without (−) or with (+) 100 μM sodium ortho-vanadate and stimulated with 1 μg/mL LPS (*E. coli* 0111:B4) for either 1 or 2 min or unstimulated (0 time). Reactions were terminated and analyzed as described in Materials and Methods.

of cellular PLA_2 [24, 25]. Recently, transfection of EGF receptors into CHO cells has been associated with increased PLA_2 activity upon stimulation with EGF [24]. A mutated form of the EGF receptor lacking the PTK autophosphorylation site, when transfected into CHO cells did not cause PLA_2 activation in response to EGF [25]. Thus, for the EGF receptor, PTK activity appears to be required for the activation of PLA_2 and subsequent eicosanoid biosynthesis. Besides the growth factor receptors, other non-receptor linked PTKs have been identified and are being characterized [26]. Signal transduction by non-receptor linked PTKs has been of increased importance as more of these PTKs are identified [23, 27]. Of particular interest are the hematopoietic cell type specific kinases such as $p57^{hck}$ [28] and $p56^{lck}$ [29].

It was demonstrated recently that in the macrophage-like cell line $P388D_1$, PAF-stimulated eicosanoid production was inhibited by the PTK inhibitor genistein [16]. Reports have now shown that PAF-mediated events (PAF receptor) are linked to PTK activation [9]. In the human polymorphonuclear leukocyte, activation by calcium ionophore or Fc receptor has also been shown to induce tyrosine phosphorylation of specific cellular proteins [13]. Phosphorylation by PTK now appears to be a common feature in many signal transduction pathways.

To evaluate the role of cellular PTK activity in the signal transduction pathways which lead to eicosanoid biosynthesis, the murine peritoneal macrophage was selected as a model system. The PTK inhibitors were chosen for their selectivity and also their different binding sites on the PTK. Genistein [20] is a competitive inhibitor of PTK with respect to ATP but not substrate, suggesting that genistein may have effects on other ATP-dependent kinases. Genistein inhibited both PGE_2 and LTC_4 production in the peritoneal macrophage in response to various stimuli, e.g. PMA, calcium ionophore, and zymosan. The IC_{50} values for the different stimuli were all comparable, suggesting a common site or mode of action. This inhibition was not due to any direct effect of genistein on the other enzymes involved in AA metabolism. Tyrphostin-25 is a competitive inhibitor of PTK with respect to the substrate but not ATP [21, 30], suggesting a greater kinase selectivity for this inhibitor. Tyrphostin-25 inhibited both PGE_2 and LTC_4 production in the macrophage; however, tyrphostin-25 appeared to be a more potent inhibitor of LTC_4 production, suggesting a direct effect of tyrphostin-25 on the 5-LO enzyme (metabolism of exogenous AA by the 5-LO pathway was blocked by tyrphostin-25 at comparable concentrations observed for stimulated cells). Overall, these inhibitor data suggest the involvement of PTK (tyrosine phosphorylation) in the activation pathway which leads to the release of AA for eicosanoid biosynthesis.

Direct regulation of phospholipase activity by tyrosine phosphorylation has been demonstrated for phospholipase C-γl [31]. The results in EGF receptor transfected CHO cells demonstrate that PLA_2 may also be directly regulated by tyrosine phosphorylation [24]. The results presented herein demonstrating the rapid tyrosine phosphorylation in response to a phagocytic receptor stimulation and with selective inhibitors of PTK demonstrate involvement of PTK phosphorylation in the regulation of eicosanoid biosynthesis in macrophages. Tyrosine phosphorylation of multiple proteins in the macrophage occurs rapidly (within the first 2 min) when stimulated with agents known to stimulate eicosanoid

biosynthesis. This is similar to the studies performed in human neutrophils and RBL cells where maximum tyrosine phosphorylation was observed at 1–2 min after stimulation [13]. In the macrophage, stimulated tyrosine phosphorylation is inhibited by the selective PTK inhibitor tyrphostin-25, and appears to parallel the inhibition of eicosanoid biosynthesis. It is of interest that inhibition of tyrosine phosphorylation of the p100 band appears to more closely parallel the inhibition of PGE_2 and LTC_4 production by tyrphostin-25. Further identification of this band is necessary to identify it as an accessory protein or a PTK involved in the signal transduction mechanism for the zymosan receptor. As many diverse stimuli activate PLA_2, it is of interest to further identify the protein(s) which is phosphorylated and is involved in the regulation of eicosanoid biosynthesis in the macrophage and other cells. As the macrophage responds to many different stimuli via receptor-mediated events, this is an excellent model to study the involvement of tyrosine phosphorylation and specific PTKs in signal transduction mechanisms.

Further work is necessary to identify the role of tyrosine phosphorylation in the regulation of PLA_2, both the low and high molecular weight forms, in the intact cell. Regulatory mechanisms, such as tyrosine phosphorylation, can occur either directly or more likely through the phosphorylation of accessory (regulatory) proteins involved in eicosanoid biosynthesis. As the phosphorylation of the high molecular weight PLA_2 occurs exclusively on Ser residues [5], it seems unlikely that direct tyrosine phosphorylation is involved in its regulation and implicates the role of tyrosine phosphorylation of an accessory protein(s) in the regulation of this enzyme. The recent cloning and sequence determination for the cytosolic high molecular weight PLA_2 [4], and the *in vitro* demonstration that the high molecular weight PLA_2 can be phosphorylated [6] in the intact cell support the findings of the present study, that phosphorylation mechanisms, probably both Tyr and Ser phosphorylation, regulate PLA_2 and eicosanoid biosynthesis. The regulation of PLA_2 by phosphorylation by kinases [32] or dephosphorylation by phosphatases [33] has been demonstrated in several cell types. Phosphorylation of the 110 kDa PLA_2 *in vitro* results in a slightly greater enzymatic activity [5]. Phosphatase inhibitors such as okadaic acid [34] at low concentrations stimulate eicosanoid biosynthesis in the intact cell [35]. Therefore, both phosphorylation and dephosphorylation appear to play pivotal roles in the regulation of PLA_2 activity (AA release and eicosanoid biosynthesis) and appear to have both negative and positive potential.

Also of recent interest is the communication between Tyr and Ser/Thr kinases in signal transduction pathways [27, 29]. In the macrophage the effects of kinase inhibitors indicate that this is very likely to be involved in the regulation of PLA_2 and eicosanoid biosynthesis. The stimulatory activity of okadaic acid (increased phosphorylation on Ser/Thr residues due to inhibition of protein phosphatases 1 and 2A) and the potent inhibition of eicosanoid biosynthesis by K252a and other Ser/Thr kinase inhibitors demonstrate an important role for Ser/Thr kinase activity (e.g. PKC) in the activation of PLA_2 (eicosanoid biosynthesis). The regulation of PTK by Ser/Thr kinases (PKC and/or cAMP-dependent kinases) has been demonstrated for both growth factor and *src*-like kinases [26, 29]. Therefore, the independent and dependent involvement of both PTK and Ser/Thr kinase activity in AA release is conceivable as a common signal transduction pathway in the macrophage, similar to that described in T cell signal transduction [29].

In the macrophage we have demonstrated that tyrosine phosphorylation of multiple cellular proteins occurs rapidly after phagocytic receptor activation. Selective PTK inhibitors block both the tyrosine phosphorylation events and subsequent eicosanoid generation in these cells. The selectivity of these inhibitors for PTK relative to other enzymes involved in eicosanoid biosynthesis suggests that tyrosine phosphorylation is an essential component in the activation of PLA_2 (AA release) and subsequent eicosanoid biosynthesis. The diversity of stimuli which elicit tyrosine phosphorylation in the macrophage suggests that tyrosine phosphorylation is a common event in macrophage cell activation. As the zymosan receptor has not been shown to be a PTK and tyrosine phosphorylation can occur via non-receptor mediated events (calcium ionophore, PMA, okadaic acid stimulation), it seems likely that the phosphorylation events observed in the macrophage are due to the non-receptor PTK of the *src* family. Identification of the specific PTK involved in these different receptor- and non-receptor-mediated events may be invaluable in the development of specific functional inhibitors against macrophage activation in inflammatory disease states.

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