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A MITOGEN-ACTIVATED PROTEIN KINASE INDEPENDENT PATHWAY INVOLVED IN THE PHOSPHORYLATION AND ACTIVATION OF CYTOSOLIC PHOSPHOLIPASE A_2 IN HUMAN NEUTROPHILS STIMULATED WITH TUMOR NECROSIS FACTOR-α

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SUMMARY: Although tumor necrosis factor (TNF) -α stimulation of human neutrophils does not result in a significant release of arachidonic acid, it primes the cell for arachidonic acid release when cells are further stimulated by agents that induce an intracellular calcium increase. We demonstrate that TNF-α stimulation of neutrophils induces the phosphorylation of cytosolic phospholipase A_2 ($cPLA_2$) and also increases its activity. These results indicate that although TNF-α, by itself, does not cause the release of arachidonic acid in intact cells, it increases the phosphorylation and activation of the enzyme $cPLA_2$. Since we recently found that TNF-α stimulation of neutrophils does not increase the tyrosine phosphorylation or activation of the $p42^{erk2}$ and $p44^{erk1}$ mitogen-activated protein kinases (MAPKs), the present studies demonstrate the involvement of a MAPK independent pathway in the phosphorylation and activation of $cPLA_2$.

Phospholipase A_2 catalyzes the hydrolysis of phospholipids at the sn-2 position yielding free fatty acid and lysophospholipid. These enzymes can be categorized into two major groups, the secretory or low molecular weight (14-18 kDa) forms, $sPLA_2$, and the cytosolic or high molecular weight (31-110 kDa) forms, $cPLA_2$. The recently described and cloned cytosolic phospholipase A_2 ($cPLA_2$) is active at micromolar calcium concentrations, selectively cleaves arachidonic acid from the sn-2 position of membrane phospholipids and is coupled to ligand-stimulated arachidonic acid release (1). Arachidonic acid serves as the rate-limiting precursor for prostaglandins and leukotrienes, two classes of substances that mediate inflammation (2). Activation of $cPLA_2$ has been shown to require an increase in intracellular calcium and phosphorylation of the enzyme (1). Increasing calcium has been shown to translocate recombinant $cPLA_2$ to membrane vesicles suggesting that in response to receptor-

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stimulated increases in Ca^{2+}, $cPLA_2$ translocates from the cytosol to the plasma membrane, where its phospholipid substrate is located (3-6). Recently, it has been demonstrated that mitogen-activated protein kinases (MAPKs) may be involved in the phosphorylation and activation of $cPLA_2$ (7). MAPKs comprise a family of enzymes that are serine/threonine kinases and which become activated by phosphorylation on both a tyrosine and a threonine residue (8). However, while these enzymes are particularly known for their possible roles in cell cycle progession (9), their role in terminally differentiated cells, such as the neutrophil, remains to be elucidated.

Tumor necrosis factor (TNF)-α, a cytokine which can activate or potentiate certain functions of neutrophils such as adherence, superoxide (O_2^-) release and phagocytosis (10), is considered to be one of the major inflammatory cytokines. As such, TNF-α plays an important role in host defense mechanisms against infections by inducing production of prostaglandins, leukotrienes, and platelet-activating factor. The mechanisms involved in TNF-α stimulation of the neutrophil, a terminally differentiated cell, have not been fully explored. Recently, we found that the addition of TNF-α to human neutrophils in suspension does not increase the tyrosine phosphorylation or activation of the $p42^{erk2}$ and $p44^{erk1}$ MAPK isoforms (11). Since activation of $cPLA_2$ has been suggested to be mediated by its phosphorylation by MAPK (7,12), the present studies were undertaken to address three questions. First, does TNF-α cause arachidonic acid release in intact human neutrophils? Second, does TNF-α increase the phosphorylation of $cPLA_2$ in these cells? Third, is the activity of $cPLA_2$ increased in lysates isolated from TNF-α stimulated cells?

MATERIALS AND METHODS

Materials. TNF-α was purchased from R&D Systems, Minneapolis, MN.; Enhanced Chemi-Luminescence (ECL) Western blotting reagents and 1-stearoyl-2-[^{14}C]arachidonyl phosphatidylcholine were purchased from Amersham, Arlington Heights, IL. [^{14}C]-arachidonic acid was from NEN Research Products, Wilmington, DE. Anti-cytosolic phospholipase A_2 (α-$cPLA_2$) antibody was generously provided by Dr. John Knopf (The Genetics Institute, Cambridge, MA).

Isolation of human neutrophils. Neutrophils were isolated from normal human donors utilizing a Ficoll/Hypaque gradient according to the method of English and Andersen (13). Cells were resuspended in modified Hanks' balanced salt solution (HBSS) containing 0.1% bovine serum albumin and 10 mM Hepes, pH 7.35.

Arachidonic acid release. Labeling the cells with [^{3}H]arachidonic acid and the release of radiolabeled arachidonic acid were carried out as described previously (14) with slight modifications. Briefly, the cells (1 x 10^8/ml) were incubated with 2.5 μCi/ml [^{3}H]arachidonic acid (which had been dried under N_2 and resuspended in HBSS containing 0.1 mg/ml fatty acid free BSA) for one hr at 37°C. Cells were then washed three times, resuspended at 5 x 10^6/ml and incubated at 37°C for varying times with the appropriate stimulus. The cells were then vortexed, centrifuged and an aliquot of the supernatant was counted.

Cell fractionation. Cell fractionation was carried out as described previously (12). Briefly, 3 ml of cells (1 x 10^7/ml) were treated with buffer (HBSS) or TNF-α. Following reaction termination, the cells were resuspended at 10^8 cells/ml in a buffered sucrose solution (10 mM Hepes, pH 7.4, 100 mM sucrose, 1 mM EGTA, 50 μg/ml leupeptin, 1

mM DFP, 1 mM PMSF). Cells were disrupted by sonication; centrifuged at 8,000 x g for 15 min; and the resulting supernatant was then centrifuged at 100,000 x g for 30 min at 4°C. Both the pellet (membrane fraction) and the supernatant (cytosol fraction) were saved.

Immunoblotting. Immunoblotting was performed as described (15). Briefly, cells (1 x 10^7/ml) were stimulated and fractionated as described above. Equivalent amounts of protein were loaded onto an 8% SDS polyacrylamide gel. After electrophoresis, proteins were transferred from the gel to polyvinylidine difluoride membranes in transfer buffer (20 mM Tris base, 150 mM glycine, 20% methanol, pH 8.9). Blots were washed in Tris-buffered saline, 20 mM Tris-base, 137 mM NaCl, pH 7.6, containing 0.1% Tween 20 (TBS-T); incubated with α-cPLA$_2$ antibody; washed again with TBS-T and then incubated with the secondary antibody (horseradish-peroxidase-conjugated anti-rabbit IgG). The Enhanced Chemi-Luminescence method was utilized for detection.

cPLA$_2$ activity assay. The cPLA$_2$ assay was performed as previously described (16). Following cell stimulation, cytosol fractions were prepared as described above utilizing sucrose buffer (50 mM Hepes, pH 7.5, 250 mM sucrose, 1 mM EDTA, 1 mM EGTA, 2 mM PMSF, 40 µg/ml leupeptin, 40 µg/ml pepstatin, 80 µg/ml aprotinin, 5 mM DTT). The assay was initiated by adding cytosolic extract to the substrate, 1-stearoyl-2-[^{14}C]arachidonyl phosphatidylcholine (final conc. 30 µM). The final concentration of Ca^{2+} in the assay mixture was 2 mM in excess of the chelators. The reaction was stopped after 15 min at 37°C by addition of ice-cold ethanol containing 2% (v/v) acetic acid and 1 mg of arachidonic acid/ml. 50 µl of this mixture was spotted on Whatman LK6D t.l.c. 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 I_2 vapor. 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 µM. Results are shown as means ± S.E.M.

RESULTS

Arachidonic acid release in human neutrophils stimulated with tumor necrosis factor-α. Since TNF-α does not phosphorylate and activate p42^{erk2} and p44^{erk1} MAPKs in human neutrophils and since these kinases have been implicated in the phosphorylation and activation of cPLA$_2$, we sought to investigate the effect of TNF-α stimulation of human neutrophils on arachidonic acid release. Human neutrophils were stimulated with TNF-α at 37°C and arachidonic acid release was determined as described under Materials and Methods. As shown in Table 1, TNF-α does not produce a significant increase in arachidonic acid release. However, TNF-α in combination with platelet-activating factor (PAF) or the bacterial-derived chemotactic peptide N-formyl-methionyl-leucyl-phenylalanine (FMLP) produces a large increase in arachidonic acid release, although PAF and FMLP by themselves only have a small effect on arachidonic acid release. It must be noted that PAF and FMLP stimulation of human neutrophils, unlike TNF-α, causes a rise in the intracellular concentration of free calcium. Since a rise in intracellular calcium is one of the conditions necessary for cPLA$_2$ activation (satisfied by PAF or FMLP stimulation) and phosphorylation of the enzyme is also required, perhaps TNF-α is involved in phosphorylation of cPLA$_2$.

TNF-α phosphorylation of cPLA$_2$ in human neutrophils. Studies have shown that cPLA$_2$ can be phosphorylated by MAPK both *in vitro* and *in vivo* and phosphorylation of cPLA$_2$

Table 1

[^{3}H]-arachidonic acid release in human neutrophils

Condition*	Arachidonic acid release** (relative to control)
No addition	1.0
TNF-α (20 ng/ml, 30 min)	1.1 ± 0.14 (8)
PAF (100 nM, 2 min)	1.3 ± 0.17 (6)
FMLP (10^{-7} M, 2 min)	1.2 ± 0.25 (2)
TNF-α followed by PAF	4.0 ± 0.42 (6)
TNF-α followed by FMLP	3.3 ± 0.07 (2)

* The cells were stimulated with PAF for 2 min., FMLP for 2 min. and with TNF-α for 30 min. When TNF-α and PAF or FMLP were used together, the cells were first incubated with TNF-α for 30 min and then stimulated with either PAF or FMLP for 2 min.

** Each value represents the mean ± the standard error of the mean (S.E.M.). The numbers in parentheses refer to the numbers of separate experiments.Each experiment was carried out on neutrophils isolated from different donors.

is evidenced by a decrease in electrophoretic mobility. In addition, phosphorylation of $cPLA_2$ is important for its activation and arachidonic acid release (7,17). Therefore, it has been hypothesized that activation of $cPLA_2$ in intact cells is mediated through its phosphorylation by MAPK (7,12). Since we recently demonstrated that TNF-α does not induce the tyrosine phosphorylation or activation of the $p42^{erk2}$ and $p44^{erk1}$ MAPK isoforms in human neutrophils (11), we sought to determine if $cPLA_2$ can be phosphorylated independently of the participation of these MAPK isoforms. In order to test the phosphorylation of $cPLA_2$ we examined the shift in electrophoretic mobility of $cPLA_2$ in control and TNF-α stimulated human neutrophils. If tyrosine phosphorylation and activation of $p42^{erk2}$ and $p44^{erk1}$ MAPK isoforms are necessary for the phosphorylation of $cPLA_2$, then we should not expect to see a decrease in the electrophoretic mobility of $cPLA_2$ in human neutrophils stimulated with TNF-α.

Human neutrophils were stimulated with either buffer (HBSS) or TNF-α at 37°C and plasma membrane and cytosol fractions were evaluated for $cPLA_2$ by immunoblotting with α-$cPLA_2$. Results (not shown) demonstrated, that when neutrophils are fractionated in the presence of a calcium chelator, $cPLA_2$ remains in the cytosol. Subsequent experiments utilized cytosolic fractions to examine the mobility shift of $cPLA_2$. As shown in Fig. 1A and 1B TNF-α induced a time and dose dependent decrease of the electrophoretic mobility of $cPLA_2$. The mobility shift is evident by 5 min., is complete by 10 min., and 2 ng/ml TNF-α is sufficient to induce a complete mobility shift of $cPLA_2$. To confirm that the TNF-α induced mobility shift of $cPLA_2$ was due to phosphorylation of the enzyme, a potato acid phosphatase assay was utilized.

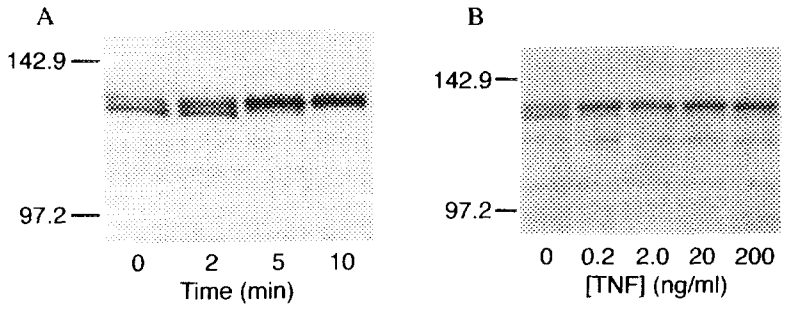

Fig. 1. **Time course and dose response of the TNF-α stimulated shift in the electrophoretic mobility of cPLA$_2$ in human neutrophils.** A, Neutrophils were stimulated with 20 ng/ml TNF-α for the indicated times. B, Neutrophils were stimulated with the indicated concentrations of TNF-α for 10 min. Following stimulation, cells were disrupted and fractionated. Cytosolic samples (100 μg) were subjected to 8% SDS-PAGE; proteins were transferred and blots were probed with α-cPLA$_2$. Detection was determined by the Amersham ECL system. Molecular weight standards are given in kDa. Results are representative of experiments.

Following stimulation of neutrophils with buffer or TNF-α, the cytosolic fraction (75 μg) was incubated with or without 5 μg of potato acid phosphatase at 30°C for 60 min. As shown in Fig. 2 (lane 4), the mobility shift of cPLA$_2$ is sensitive to phosphatase treatment indicating that the decreased electrophoretic mobility is due to phosphorylation of the protein. To determine if genistein-sensitive tyrosine phosphorylation is involved in phosphorylation of cPLA$_2$ induced by TNF-α, neutrophils were treated with genistein, a tyrosine kinase inhibitor, for 1 hr and then stimulated with TNF-α. As shown in Fig. 3 (lane 4), the TNF-α induced mobility shift of cPLA$_2$ is not inhibited in human neutrophils treated with genistein.

Effect of TNF-α stimulation of human neutrophils on the activity of cPLA$_2$. Although TNF-α stimulation of human neutrophils does induce phosphorylation of cPLA$_2$, only a small amount of arachidonic acid is released following TNF-α treatment of neutrophils (Table 1). This is probably because TNF-α does not induce an intracellular rise in calcium which has been shown to be necessary for cPLA$_2$ translocation to the plasma membrane and its subsequent catalysis of phospholipid hydrolysis (3-6). Therefore, to investigate if TNF-α induced phosphorylation of cPLA$_2$ in human neutrophils increases its activity, control and TNF-α stimulated cells were sonicated and then fractionated under reducing conditions (to negate the effect of sPLA$_2$ enzymes) in the presence of EGTA and EDTA to prevent membrane adsorption of the enzyme. The Ca^{2+}-dependent cPLA$_2$ activity of the cytosolic fraction (under these conditions there was no detectable cPLA$_2$ present in the membrane fraction) was determined utilizing the substrate 1-stearoyl-2-[^{14}C]arachidonyl phosphatidylcholine as described under Materials and

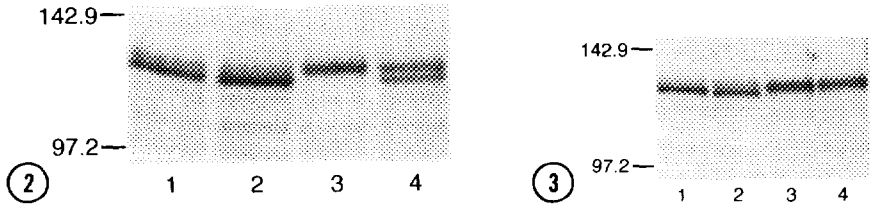

Fig. 2. **Effect of potato acid phosphatase on TNF-α induced mobility shift of cPLA$_2$.** Neutrophils were stimulated with either buffer (HBSS) or TNF-α (20 ng/ml) for 10 min. Following cell disruption and fractionation, cytosolic samples (75 μg) were incubated with 5.0 μg potato acid phosphatase (PAP) for 1 hr at 30°C. Reactions were terminated by addition of 2x Laemmli sample buffer, boiled for 5-10 min, and subjected to 8% SDS-PAGE. Proteins were transferred and blots were probed with α-cPLA$_2$. Detection was determined by the Amersham ECL system. Lane 1, control cells; lane 2, control cells + PAP; lane 3, TNF-α stimulated cells; lane 4, TNF-α stimulated cells + PAP. Molecular weight standards are given in kDa. Results are representative of experiments.

Fig. 3. **Effect of genistein on the TNF-α induced mobility shift of cPLA$_2$.** Neutrophils were treated with either buffer or genistein (50 μg/ml) for 1 hr at 37°C and then stimulated with either buffer or TNF-α (20 ng/ml) for 10 min. Following cell fractionation, cytosolic samples (100 μg) were subjected to 8% SDS-PAGE; proteins were transferred and blots were probed with α-cPLA$_2$. Detection was determined by the Amersham ECL system. Lane 1, control cells; lane 2, genistein treated cells; lane 3, control cells stimulated with TNF-α; lane 4, genistein treated cells stimulated with TNF-α. Molecular weight standards are given in kDa. Results are representative of experiments.

Methods. Under these conditions basal activity remained the same regardless of stimulation times (5 min or 10 min). As shown in Table 2, TNF-α induces a significant increase in cPLA$_2$ activity after 5 min (approx. 60% increase) and 10 min (approx. 66% increase) stimulation.

Table 2

Effect of TNF-α on cPLA$_2$ activity in human neutrophils

Condition*	cPLA$_2$ activity (pmol/mg/min)**
Basal	8.6 ± 0.4 (5)
TNF-α (20 ng/ml, 5 min)	13.8 ± 1.3 (3)
TNF-α (20 ng/ml, 10 min)	14.3 ± 0.8 (3)

* cPLA$_2$ activity was determined as indicated in Materials and Methods in cytosolic fractions of neutrophils following stimulation of the cells with TNF-α (20 ng/ml) for 5 min or TNF-α (20 ng/ml) for 10 min.

** Each value represents the mean ± the standard error of the mean (S.E.M.). The numbers in parentheses refer to the numbers of separate experiments.Each experiment was performed in duplicate and carried out on neutrophils isolated from different donors.

DISCUSSION

Stimulation of human neutrophils with TNF-α induces a wide range of direct and indirect responses that reflect the importance of this cell as a primary defense against bacterial invasion. Among the functions of neutrophils that are activated or potentiated by TNF-α are adherence, superoxide (O_2^-) release and phagocytosis (10). Phospholipase A_2 activity has recently been shown to be required for activation of the assembled NADPH oxidase in human neutrophils (18), thereby suggesting the involvement of PLA_2 in superoxide release. Since neutrophils also release arachidonic acid in response to various stimuli (19), this study was undertaken to investigate the role of TNF-α in arachidonic acid release and phosphorylation and activation of cytosolic phospholipase A_2 in human neutrophils.

The data presented here make three distinctly important yet related points. First, TNF-α stimulation of human neutrophils does not result in a significant increase in arachidonic acid release, but rather primes the cell for increased arachidonic acid release when cells are treated with a second stimulus, such as PAF or FMLP, which induces an increase in intracellular Ca^{2+}. Second, TNF-α induces the phosphorylation of cytosolic PLA_2 ($cPLA_2$) as evidenced by a decrease in the electrophoretic mobility of the enzyme. This phosphorylation is independent of the involvement of the $p42^{erk2}$ and $p44^{erk1}$ MAPK isoforms since we have previously demonstrated that TNF-α stimulation of human neutrophils does not induce the tyrosine phosphorylation or activation of these kinases (11). In addition, the phosphorylation of $cPLA_2$ induced by TNF-α is not inhibited by genistein, a tyrosine kinase inhibitor. Third, TNF-α increases the activity of $cPLA_2$ in human neutrophils. These results clearly show that there is a MAPK independent pathway involved in the phosphorylation and activation of $cPLA_2$ in human neutrophils stimulated by TNF-α. Identification of the kinase(s) and/or phosphatase(s) involved in the regulation of $cPLA_2$ activation is the subject of future studies.

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