THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 273, No. 30, Issue of July 24, pp. 19277–19282, 1998
Printed in U.S.A.

Stimulation of p38 Phosphorylation and Activity by Arachidonic Acid in HeLa Cells, HL60 Promyelocytic Leukemic Cells, and Human Neutrophils

EVIDENCE FOR CELL TYPE-SPECIFIC ACTIVATION OF MITOGEN-ACTIVATED PROTEIN KINASES*

(Received for publication, December 23, 1997, and in revised form, May 14, 1998)

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Although it is well appreciated that arachidonic acid, a second messenger molecule that is released by ligand-stimulated phospholipase A_2, stimulates a wide range of cell types, the mechanisms that mediate the actions of arachidonic acid are still poorly understood. We now report that arachidonic acid stimulated the appearance of dual-phosphorylated (active) p38 mitogen-activated protein kinase as detected by Western blotting in HeLa cells, HL60 cells, human neutrophils, and human umbilical vein endothelial cells but not Jurkat cells. An increase in p38 kinase activity caused by arachidonic acid was also observed. Further studies with neutrophils show that the stimulation of p38 dual phosphorylation by arachidonic acid was transient, peaking at 5 min, and was concentration-dependent. The effect of arachidonic acid was not affected by either nordihydroguaiaretic acid, an inhibitor of the 5-, 12-, and 15-lipoxygenases or by indomethacin, an inhibitor of cyclooxygenase. Arachidonic acid also stimulated the phosphorylation and/or activity of the extracellular signal-regulated protein kinase and of c-jun N-terminal kinase in a cell-type-specific manner. An examination of the mechanisms through which arachidonic acid stimulated the phosphorylation/activity of p38 and extracellular signal-regulated protein kinase in neutrophils revealed an involvement of protein kinase C. Thus, arachidonic acid stimulated the translocation of protein kinase C α, βI, and βII to a particulate fraction, and the effects of arachidonic acid on mitogen-activated protein kinase phosphorylation/activity were partially inhibited by GF109203X, an inhibitor of protein kinase C. This study is the first to demonstrate that a polyunsaturated fatty acid causes the dual phosphorylation and activation of p38.

Arachidonic acid (20:4ω6)[1] is a second messenger that is released by the action of phospholipase A_2 in activated cells (1). In *in vitro* assays, 20:4ω6 and other polyunsaturated fatty acids have been demonstrated to stimulate the activity of protein kinase C (PKC) (2, 3). When added exogenously, 20:4ω6 is biologically active in a wide spectrum of cells. For example, 20:4ω6 has been reported to inhibit gap junctional permeability between adherent cells (4); stimulate superoxide production and degranulation and increase the expression of CD11b/CD18 in human neutrophils (5–7), prime macrophages, and monocytes for enhanced respiratory burst (8); stimulate insulin secretion from isolated islets of Langerhans (9); modulate the permeability of K^+, Na^+, and H^+ channels in a variety of cell types (10–12); stimulate gene transcription (13); and cause differentiation and death (14). However, the molecular mechanisms through which the actions of 20:4ω6 are mediated are poorly understood.

We have previously demonstrated that 20:4ω6 and other polyunsaturated fatty acids stimulate the activity of the extracellular signal-regulated protein kinase (ERK) in WB rat liver epithelial cells (15), suggesting that ERK may mediate some of the biological actions of polyunsaturated fatty acids. Others have reported that arachidonic acid and its metabolites stimulate ERK activity in smooth muscle cells (16). ERK and the closely related p38 and jun N-terminal kinase (JNK) are members of the mitogen-activated protein (MAP) kinase family of kinases (17). These kinases are activated when cells are exposed to growth factors, cytokines, and/or various forms of stress (17, 18). Activation of ERK, JNK, and p38 MAP kinases are achieved through the dual phosphorylation of threonine and tyrosine residues in the TXY motif by upstream MAP kinase kinases. MAP kinases have been proposed to regulate a diverse range of biological functions, including cytokine production and cell growth, differentiation, and death (17, 18). Although 20:4ω6 has recently been reported to stimulate the activity of JNK in proximal tubular epithelial cells and in stromal cells (19, 20), we are not aware of any studies that have investigated whether 20:4ω6 affects the activity of p38. We now report the novel finding that 20:4ω6 stimulated the dual phosphorylation of p38 in HeLa cells, HL60 cells, human umbilical vein endothelial cells, and human neutrophils but not in Jurkat T cells. Further characterization in neutrophils demonstrated that 20:4ω6 also stimulated the phosphorylation/activity of ERK but not of JNK, although the activity of JNK was weakly

* This work was supported in part by the National Heart Foundation and the National Health and Medical Research Council. Portions of this work were presented at the 5th International Conference on Eicosanoids and Other Bioactive Lipids in Cancer, Inflammation, and Other Related Diseases in La Jolla, California in September, 1997. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: 20:4ω-6, arachidonic acid; ERK, extracellular signal-regulated protein kinase; MAP kinase, mitogen-activated protein kinase; JNK, jun N-terminal kinase; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; HBSS, Hanks' balanced salt solution; Pipes, 1,4-piperazinediethanesulfonic acid; GTPγS, guanosine 5′-*O*-(thiotriphosphate).

stimulated by 20:4ω6 in Jurkat cells. 20:4ω6 also stimulated the translocation of a number of PKC isozymes to a particulate fraction in neutrophils. The effect of 20:4ω6 on p38 and ERK dual phosphorylation/activity was partially blocked by GF109203X, a specific inhibitor of PKC. These data demonstrate that the ability of 20:4ω6 to stimulate the activity of p38 and JNK is cell type-dependent and suggest that p38, ERK, JNK, and PKC are potential mediators of the biological actions of 20:4ω6.

EXPERIMENTAL PROCEDURES

Materials

Fatty acids, 20:4ω6, formyl-methionyl-leucyl-phenylalanine, phorbol 12-myristate 13-acetate (PMA), myelin basic protein, kinase A peptide inhibitor, protein A-Sepharose, and general reagents for kinase assays were from Sigma. [γ-^{32}P]ATP (specific activity 4000 Ci/mmol) was obtained from Bresatec, Adelaide, Australia. The anti-ERK antibody, R2, was a kind gift from Dr. S. Pelech, University of British Columbia, or was purchased from Upstate Biotechnology, Inc., Lake Placid, NY. Rabbit anti-p38 and anti-JNK1 antibodies were obtained from Santa Cruz Biotech. The anti-ACTIVE™ ERK and p38 antibodies were obtained from Promega Inc., Santa Cruz, CA. Enhanced chemiluminescence (ECL) solutions and reinforced nitrocellulose were from NEN Life Science Products and Schleicher and Schuell, respectively. Glutathione-Sepharose beads was from Pharmacia Biotech, Australia. Fatty acids, PMA, and formyl-methionyl-leucyl-phenylalanine were dissolved in ethanol, dimethyl sulfoxide (Me_2SO), and Me_2SO, respectively. The final concentrations of the vehicles were: ethanol, 0.1% (v/v) and Me_2SO, 0.1% (v/v). Control cells received vehicle(s) alone.

Cell Culture

HeLa cells were maintained in Dulbeco's modified Eagle's medium in the presence of fetal calf serum (10%) and antibiotics as described previously (21). Cells (0.25×10^6) were plated in 10-cm culture dishes and were used after 4 days. HL60 and Jurkat T cells were maintained in RPMI 1640 supplemented fetal calf serum with and antibiotics at $\leq 1 \times 10^6$/ml. All cells were washed once with Hanks' balanced salts solution (HBSS) 30 min before being incubated with 20:4ω6 or vehicle.

Isolation and Incubation of Neutrophils

Human neutrophils were isolated from the peripheral blood of healthy volunteers by the rapid single-step method of Ferrante and Thong (22). The preparation of neutrophils was of >98% purity and >99% viability as judged by morphological examination of cytospin preparations and the ability of viable cells to exclude trypan blue. Cells in HBSS were incubated in the presence of 20:4ω6 for the times indicated.

Preparation of Cellular Extracts

Incubations were terminated by removing the incubation medium and washing the cells once with HBSS (4 °C).

p38 and JNK—For p38 and JNK assays, cells were lysed in 150 μl of buffer A (20 mM Hepes, pH 7.4, 0.5% (v/v) Nonidet P-40, 100 mM NaCl, 1 mM EDTA, 2 mM, 2 mM Na_3VO_4, 2 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml leupeptin, aprotinin, pepstatin A, and benzamidine) for 2 h at 4 °C. After centrifugation ($16,000 \times g$, 5 min), the supernatants were collected for kinase assay or for Western blotting as described below.

ERK—Activation of ERK was determined by a kinase assay and by Western blotting. Pelleted cells were sonicated (3×10 s, output of 2 units, Soniprobe) in buffer B (25 mM Tris-HCl, pH 7.5, 2 mM EGTA, 25 mM NaCl, 1 mM Na_3VO_4, 38 mM *p*-nitrophenylphosphate, 10 μg/ml pepstatin A, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 0.2 mM phenylmethylsulfonyl fluoride, and 1 mM dithiothreitol) and centrifuged ($100,000 \times g$, 20 min), and the supernatants (termed cytosolic fractions) were collected for Western blotting as described below or for kinase assay. Before the kinase assay, cytosolic fractions were batch-adsorbed onto phenyl-Sepharose CL4B. After washing the beads with 10% (2×) and 35% (2×) ethylene glycol in buffer A (v/v), ERK was eluted with 60% ethylene glycol (23). Previous studies have demonstrated that phenyl-Sepharose-adsorbed ERK1 and ERK2 are eluted between 35 and 60% ethylene glycol (23). The activity of ERK was assayed as described below.

PKC—To study PKC translocation, neutrophils were sonicated in buffer C (25 mM Tris-HCl, pH 7.5, containing 1 mM dithiothreitol, 5 mM EGTA, 2 mM EDTA, 2 mM phenylmethylsulfonyl fluoride, and 10 μg/ml leupeptin, aprotinin, pepstatin A, and benzamidine). After incubating on ice for 30 min, samples were centrifuged ($100,000 \times g$, 30 min), and the pellets were resuspended in Buffer C containing 2% Triton X-100. After a 30-min incubation on ice, samples were centrifuged ($100,000 \times g$, 30 min), the supernatant was mixed with Laemmli buffer, and PKC isozymes were separated by SDS-polyacrylamide gel electrophoresis and immunoblotted with isozyme-specific anti-PKC antibodies as described below.

Kinase Assays

p38—p38 was immunoprecipitated before determination of kinase activity. Briefly, lysates (700 μg of protein) were precleared with protein A-Sepharose (15 μl/sample). Anti-p38 antibody (3 μg/sample) was added, and tubes were incubated with constant mixing for 90 min at 4 °C. The antigen-antibody complexes were precipitated by the addition of protein A-Sepharose (20 μl/sample). The immunoprecipitates were washed once with buffer A and once with assay buffer (20 mM Hepes, pH 7.2, 20 mM β-glycerophosphate, 3.8 mM *p*-nitrophenyl phosphate, 10 mM $MgCl_2$, 1 mM dithiothreitol, 50 μM Na_3VO_4, and 20 μM ATP) at 4 °C. The assay was started by adding 30 μl of assay buffer (30 °C) containing 10 μCi of γ^{32}P-ATP, 3.8 mM *p*-nitrophenylphosphate, and 15 μg of myelin basic protein. After 15 min, the assay was terminated by the addition of Laemmli buffer and boiling the samples for 5 min at 100 °C. Phosphorylated myelin basic protein was resolved by 12% SDS-polyacrylamide gel electrophoresis and was detected and quantitated using an Instant Imager (Packard Instruments).

JNK—A solid phase assay was used to assay JNK activity as described previously (24). Briefly, glutathione *S*-transferase-jun (1–79) fusion protein was purified from bacterial lysates using glutathione-Sepharose beads at 4 °C with gentle rocking. 1 mg of lysate protein, 15 mM $MgCl_2$, and 10 μM ATP were added to 25 μl (packed volume) of glutathione *S*-transferase-jun (1–79) coupled to glutathione-Sepharose beads. The mixtures were incubated for 2 h at 4 °C with gentle rocking. After centrifugation ($16,000 \times g$, 5 min), the beads were washed once with buffer A, once with wash buffer (10 mM Pipes, pH 7, 100 mM NaCl, and the protease inhibitors, which were added to buffer A) and once with assay buffer (see p38 above). The assay was started by adding 35 μl assay buffer containing 8 μCi of γ^{32}P-ATP and bringing the temperature to 30 °C. After 20 min, the assay was terminated by the addition of Laemmli buffer and boiling the samples for 5 min at 100 °C. Samples were resolved by 10% SDS-polyacrylamide gel electrophoresis, and detection and quantitation of phosphorylated glutathione *S*-transferase-jun (1–79) was as described above.

ERK—ERK activity was assayed as described previously (12, 23) by monitoring the incorporation of $^{32}P_i$ into myelin basic protein in the presence of EGTA and protein kinase A peptide inhibitor. The assay mixture did not contain added phospholipids. Assays were terminated by spotting aliquots of the reaction mixture onto P81 filter paper. After 3 washes (5 min each) with 75 mM orthophosphoric acid, radioactivity associated with the paper was determined by liquid scintillation spectrometry. There was no detectable protein kinase A activity in phenyl-Sepharose-purified fractions, since omission of the protein kinase A peptide inhibitor from the assay mixture did not result in increased phosphorylation of myelin basic protein (Ref. 23 and data not shown). Active p38, if present in the fractions, was unlikely to contribute to any significant degree toward the total myelin basic protein kinase activity because the time course of ERK activity did not correlate with the appearance of dual-phosphorylated p38 (see "Results"). Consequently, it is unlikely that PKC, Ca^{2+}/calmodulin-dependent kinases, p38, or protein kinase A were responsible for phosphorylating myelin basic protein in these samples.

Western Blotting

Denatured proteins were separated on either 10 (ERK and p38) or 12% (PKC) polyacrylamide gels and transferred to nitrocellulose (100 V, 1.5 h), and immunoreaction and detection were carried out as described earlier (25). Immediately after transfer, blots were stained with Ponceau S (0.1% in 5% acetic acid) to confirm equal loading of all lanes of the gels. Affinity-purified polyclonal anti-ERK antibody, R2, anti-ACTIVE™ ERK, or anti-ACTIVE™ p38 antibody and anti-PKC isozyme-specific antibodies were used to detect ERK isoforms, dual-phosphorylated ERK, dual phosphorylated p38, and PKC isozymes, respectively. Immunocomplexes were detected by enhanced chemiluminescence (25).

Statistical Analysis

Where appropriate, differences were analyzed by analysis of variance or unpaired Student's *t* test and were considered significant when $p < 0.05$.

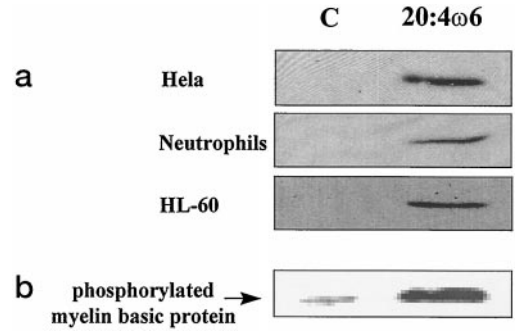

FIG. 1. **Stimulation of p38 dual phosphorylation and activity by 20:4ω6.** HeLa cells (4×10^6 cells), HL60 cells (2×10^7 cells), and neutrophils (3×10^7 cells) were incubated with 20:4ω6 (20 μM) for 4 min at 37 °C and lysed. Fractions were prepared and Western blotted with anti-ACTIVE™ p38 antibody (*a*). In some experiments with neutrophils, kinase activity was also determined using myelin basic protein as a substrate (*b*), as described under "Experimental Procedures." Results are representative of 3–4 separate experiments.

RESULTS

Incubation of HeLa cells, HL60 cells, and human neutrophils with 20:4ω6 (20 μM) for 4 min resulted in the dual phosphorylation of p38 as detected by Western blotting (Fig. 1). Similar results were obtained in human umbilical vein endothelial cells (data not shown). Ponceau S staining confirmed that, within a particular experiment, the individual lanes were loaded with equal amounts of proteins (data not shown). Since the anti-ACTIVE™ p38 antibody only detects p38 that had been dual phosphorylated on the TGY activation motif, these results indicate activation of p38 by 20:4ω6. Kinase activity assays in neutrophils confirmed this (Fig. 1). In contrast to the above data, 20:4ω6 did not enhance p38 dual phosphorylation in Jurkat cells (data not shown) that express p38 (26).

Our previous studies in human neutrophils have demonstrated that 20:4ω6 stimulated superoxide production, degranulation, and adherence to plastic surfaces and increased the expression of CD11b/CD18 (5–7). To elucidate the mechanisms through which 20:4ω6 exerted these actions, the effects of 20:4ω6 on MAP kinases were studied in more detail in the neutrophils. 20:4ω6 stimulated the dual phosphorylation of p38 in a concentration- and time-dependent manner (Fig. 2). Thus, dual-phosphorylated p38 could be detected at 5 μM 20:4ω6, and phosphorylation increased with increasing concentrations of 20:4ω6 up to 20 μM, the maximum concentration tested (Fig. 2*a*). Stimulation of p38 dual phosphorylation, detectable at less than 2 min, was transient, peaking at 5 min after exposure to 20:4ω6 (Fig. 2*b*). Very little dual-phosphorylated p38 was left at 10 min after the addition of 20:4ω6. The ability of 20:4ω6 to stimulate dual phosphorylation of p38 was not diminished by either nordihydroguaiaretic acid, a broad spectrum inhibitor of the 5-, 12-, and 15-lipoxygenase or by indomethacin, an inhibitor of cyclooxygenase (Fig. 3). A small amount of dual-phosphorylated p38 was detected in neutrophils that had been exposed to either nordihydroguaiaretic acid or indomethacin *per se*. This was likely to be due to the accumulation of low levels of endogenous 20:4ω6 in the presence of nordihydroguaiaretic acid or indomethacin.

We next examined whether 20:4ω6 also stimulated the activity of JNK in human neutrophils. Although 20:4ω6 has been reported to stimulate the activity of JNK in stromal and rabbit proximal tubular epithelial cells (19, 20), the fatty acid did not stimulate JNK activity in neutrophils (data not shown). This was not because neutrophils do not express JNK, because the presence of JNK1 was detected in neutrophils (data not shown). However, 20:4ω6 stimulated the activity of JNK in Jurkat T cells (Fig. 4), although the degree of activation was less than that observed with A23187/PMA (Fig. 4). ω3 fatty acids also stimulated JNK activity in Jurkat cells (data not shown).

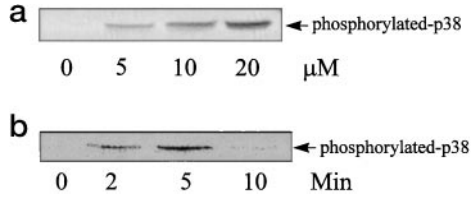

FIG. 2. **Characteristics of 20:4ω6-stimulated p38 dual phosphorylation.** Neutrophils were incubated with 20:4ω6 at the concentrations indicated for 4 min (*a*) or with 20:4ω6 (20 μM) for up to 10 min (*b*). Cells were lysed and the fractions were Western-blotted as described under "Experimental Procedures." The results are representative of at least three experiments.

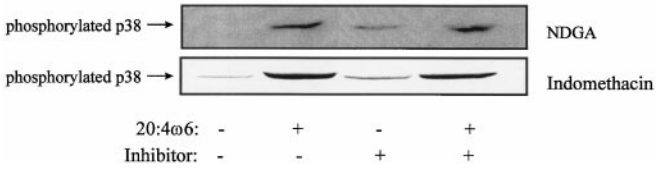

FIG. 3. **Lack of effect of nordihydroguaiaretic acid or indomethacin on the appearance of dual phosphorylated p38.** Neutrophils were preincubated for 10 min with either nordihydroguaiaretic acid (*NDGA*) (10 μM) or indomethacin (100 μM) before being exposed to 20:4ω6. Samples were prepared and Western blotted as described under "Experimental Procedures." The results are representative of three experiments.

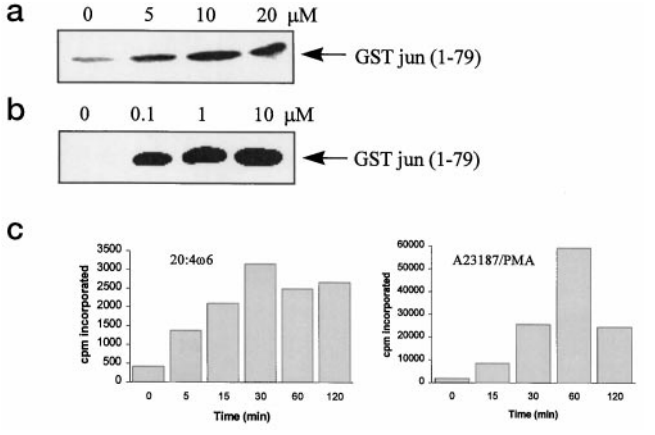

FIG. 4. **20:4ω6 stimulated the activity of JNK in Jurkat cells.** Jurkat cells were stimulated with 0–20 μM 20:4ω6 (*a*), with 0–10 μM A23178 and 35 nM PMA (*b*) for 30 min, or with 20:4ω6 (10 μM) or A23187 (1 μM)/PMA (35 nM) for up to 120 min (*c*). The cells were lysed, and JNK activity was assayed as described under "Experimental Procedures." Results of the time course experiments (*c*) are expressed as cpm $^{32}P_i$ incorporated into glutathione *S*-transferase jun (1–79). Results are representative of three separate experiments.

Although we have previously demonstrated that 20:4ω6 stimulated the activity of ERK in WB rat liver cells (12), the effect of 20:4ω6 on ERK activity in neutrophils has not been reported. Because the above data demonstrate that 20:4ω6 stimulated the dual phosphorylation of p38 and the activity of JNK in a cell type-specific manner, it is, therefore, important to determine whether the activity of ERK in neutrophils is affected by 20:4ω6. Activated ERK isoforms display reduced electrophoretic mobility in SDS-polyacrylamide gels (12, 21, 25, 27) because of the dual phosphorylation of ERK on the TEY activation motif. Incubation of neutrophils with 20:4ω6 caused a retardation in the electrophoretic mobility of the 42- and 43-kDa forms of ERK to give apparent M_r values of 43- and 44-kDa

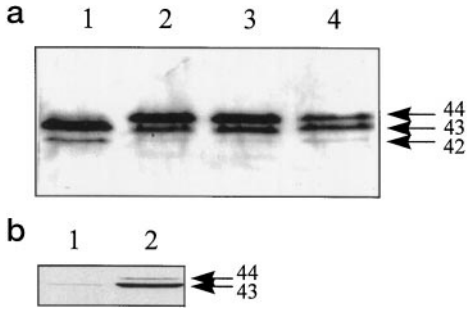

FIG. 5. **Stimulation of ERK phosphorylation by 20:4ω6 in human neutrophils.** Neutrophils (3×10^7 cells in 30 ml of HBSS) were stimulated with 20:4ω6 (20 μM, 10 min), formyl-methionyl-leucyl-phenylalanine (50 nM, 5 min), or PMA (100 nM, 5 min). Cytosolic fractions were prepared and Western-blotted with the anti-ERK antibody, R2 (*a*), or with the anti-ACTIVE™ ERK antibody (*b*) as described under "Experimental Procedures." *a*: *lane 1*, control; *lane 2*, formyl-methionyl-leucyl-phenylalanine; *lane 3*, 20:4ω6; *lane 4*, PMA. *b*: *lane 1*, control; *lane 2*, 20:4ω6. Results are representative of at least three experiments.

(Fig. 5*a*), consistent with their activation by phosphorylation. When the fractions were Western-blotted with anti-ACTIVE™ ERK antibody that detects active, dual-phosphorylated ERK, two bands with M_r values of approximately 43-and 44-kDa were detected predominantly in samples from 20:4ω6-stimulated cells (Fig. 5*b*). Kinase assays demonstrate that the enhancement of ERK activity by 20:4ω6 was concentration-dependent. An increase in kinase activity was detectable at 5 μM 20:4ω6, the lowest concentration tested (Fig. 6*a*). The effect of 20:4ω6 peaked at around 15 min after the addition of 20:4ω6 (Fig. 6*b*) and was longer lasting than stimulation of p38 dual phosphorylation or activity (Fig. 2*b*). This argues strongly against the possibility that contaminating p38, which also phosphorylates myelin basic protein (Fig. 1*b*), was responsible for phosphorylating myelin basic protein in these fractions, since the appearance of dual-phosphorylated p38 peaked at 5 min after the addition of 20:4ω6 and declined rapidly thereafter. 20:4ω6 also stimulated the activity of ERK in human umbilical vein endothelial cells, human mesangial cells, Jurkat cells, HL60 cells, and human monocytes but not in PC12 pheochromocytoma cells (data not shown). The activity of ERK in these cells was also stimulated by the ω3 fatty acids, eicosapentaenoic acid (20:5ω3) and docosahexaenoic acid (22:6ω3) (data not shown).

Since polyunsaturated fatty acids have been demonstrated to activate PKC *in vitro* (2, 3) and to stimulate the translocation of PKC in WB rat liver epithelial cells (12), we investigated whether PKC was involved in the activation of the p38 and ERK cascades by 20:4ω6 in neutrophils. Neutrophils contain PKC α, βI, βII, δ, and ζ (28). Although polyunsaturated fatty acids have been reported to stimulate Ca^{2+} mobilization (29), activate the neutral sphingomyelinase (30), and amplify H^+ ion channel conductance (31) in intact neutrophils and to stimulate GTPγS loading of the heterotrimeric G-proteins in neutrophil membrane fractions (32), the effect of fatty acids on PKC translocation in neutrophils has not been reported. In unstimulated neutrophils, a substantial amount of PKC βII was detected in a particulate fraction, and this was increased after incubation with 20:4ω6 (Fig. 7). The existence of particulate fraction-associated PKC in unstimulated cells has also been observed in the T lymphocyte cell line, CTLL-2 (33). 20:4ω6 also caused a small amount of PKC α and βI to associate with the particulate fraction (Fig. 7). Neither PKC δ nor ζ was detected in the particulate fraction (data not shown). An involvement of PKC in the activation of MAP kinases by 20:4ω6 was confirmed by the observation that GF109203X, an inhibitor of classical PKC isozymes (34), attenuated the stimulatory effect of 20:4ω6 on

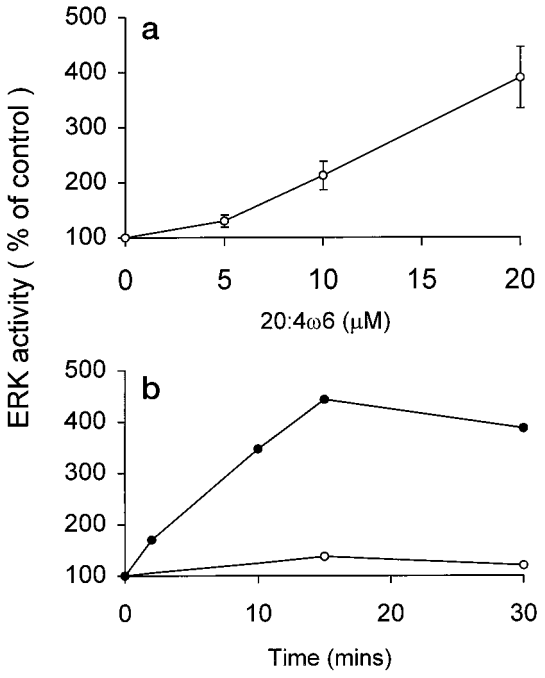

FIG. 6. **The activity of ERK in neutrophils was stimulated in a concentration- and time-dependent manner by 20:4ω6.** Neutrophils were incubated with 20:4ω6 at the concentrations indicated for 10 min (*a*) or for the times indicated (*b*, control (○); 20 μM 20:4ω6 ( )). ERK in cytosolic fractions was partially purified, and the activity was assayed as described under "Experimental Procedures." Results are the mean ± S.E. of three determinations (*a*) or the mean of duplicate determinations (*b*). Results are representative of at least three experiments.

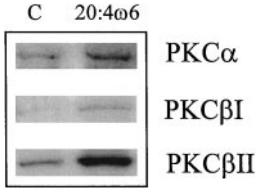

FIG. 7. **20:4ω6 stimulated the translocation of PKCα, βI, and βII.** Neutrophils were stimulated with 20:4ω6 (20 μM) for 3 min, and PKC fractions were prepared and Western-blotted as described under "Experimental Procedures." The results are representative of three experiments.

ERK activity by >85% (Fig. 8*a*). GF109203X also caused a modest reduction in the ability of 20:4ω6 to stimulate the appearance of dual-phosphorylated p38 (Fig. 8*b*).

DISCUSSION

20:4ω6 is a second messenger molecule that is liberated from the sn-2 position of membrane phospholipids by ligand-stimulated activation of the cytosolic phospholipase A_2 (1). The importance of endogenously generated 20:4ω6 in ligand-stimulated responses has been adequately demonstrated using inhibitors of cytosolic phospholipase A_2 and antisense technology (1). The non-esterified 20:4ω6 that is released has been found to be cell-associated as well as being released into the extracellular medium. Thus, many studies have assayed for the appearance of radiolabeled 20:4ω6 in the extracellular medium as a measure of phospholipase A_2 activation (1, 35, 36). Although cell-associated 20:4ω6 can directly serve as an endogenous second messenger and is a substrate for the lipoxygenases and cyclooxygenases, 20:4ω6 that is released into the extracellular fluid has the potential to exert autocrine and paracrine effects. Consistent with this suggestion, exogenously added

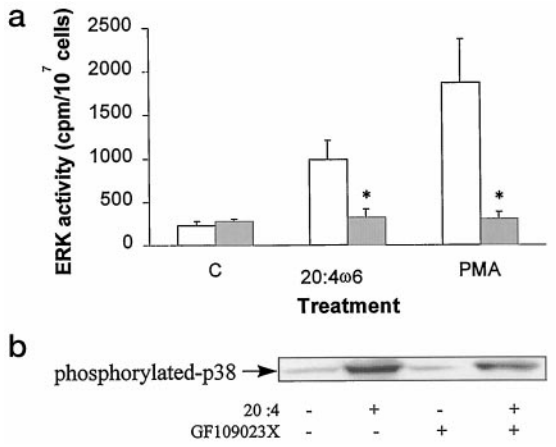

FIG. 8. **Inhibition of ERK activity and p38 phosphorylation by GF109203X.** Neutrophils (1×10^7 in 10 ml HBSS for ERK activity assays, 3×10^7 in 30 ml HBSS for p38 phosphorylation) were preincubated with GF109203X (0.5 μM, *filled bars*) or Me_2SO (0.1% v/v, *open bars*) for 10 min. The cells were then incubated with 20:4ω6 (20 μM) for 10 (ERK activity assays) or 4 (for p38 phosphorylation) min. Determination of ERK activity (*a*) and p38 dual phosphorylation (*b*) were carried out as described under "Experimental Procedures." Data on ERK activity are means ± S.E. of three determinations. Results are representative of three experiments. Significance of difference: * $p < 0.05$.

20:4ω6 has been shown to be biologically active to a wide spectrum of cell types at concentrations (1–20 μM) that have been reported to be present in stimulated cells. Thus, neutrophils have been reported to contain 100–2,200 pmol/10^7 cells of 20:4ω6, and in isolated islets of Langerhans, glucose was found to increase cell-associated nonesterified 20:4ω6 by up to 75 μM (37, 38). However, the mechanisms through which 20:4ω6 act are still poorly understood.

The present study demonstrates that exogenous 20:4ω6 stimulated the dual phosphorylation of p38 in HeLa cells, HL60 cells, human neutrophils, and human umbilical vein endothelial cells but not in Jurkat cells. We demonstrate in neutrophils that this increase in p38 dual phosphorylation was accompanied by an increase in p38 kinase activity. Stimulation of p38 dual phosphorylation by 20:4ω6 in neutrophils was independent of its metabolism by either lipoxygenase or cyclooxygenase since the effect was not affected by nordihydroguaiaretic acid, a broad spectrum inhibitor of the 5-, 12-, and 15-lipoxygenases or by indomethacin, an inhibitor of cyclooxygenase. The fatty acid also stimulated the dual phosphorylation and activity of ERK but not of JNK in neutrophils. This, therefore, excludes an involvement of JNK in the actions of 20:4ω6 in the neutrophils. However, 20:4ω6 stimulated the activity of JNK in Jurkat T cells, an observation that is consistent with reports in proximal tubular epithelial cells and stromal cells that the activity of JNK was stimulated by 20:4ω6 (19, 20). The ability of 20:4ω6 to stimulate the activity of ERK in human neutrophils and a number of other primary cell types and cell lines is consistent with our previous observations in WB rat liver epithelial cells (15). However, 20:4ω6 did not affect the activity of ERK in PC12 cells, although ERK activity in these cells was strongly stimulated by PMA.[2] These data, therefore, demonstrate that 20:4ω6 stimulated the activity of MAP kinases in a cell type/line-specific manner.

The present study demonstrates that PKC may be involved, at least in part, in mediating the effects of 20:4ω6 on p38 and ERK activation. Thus, 20:4ω6 not only stimulated the translocation of PKC α, βI, and βII to a particulate fraction in neutrophils, but the effects of 20:4ω6 on p38 dual phosphorylation and ERK activity were attenuated by the PKC inhibitor, GF109203X. Consistent with a possible involvement of PKC in the p38 and ERK cascades, PMA, a direct activator of PKC, stimulates the activity of ERK in all cell types examined (18) and of p38 in some cell types (39, 40). Recent studies have revealed that at least four members of the p38 family exist. These are p38α (also known as p38, CSBP, RK), p38β, p38γ (ERK6/SAPK3), and p38δ (SAPK4) (41, 42). PMA selectively stimulated the activity of p38γ and p38δ without significantly affecting the activity of p38α or p38β (42), indicating that the γ and δ forms of p38 are regulated by PKC. It remains to be determined whether the PMA-responsive p38 in neutrophils (39) and U937 cells (40) are p38γ and/or p38δ. Although PKC may regulate the ERK cascade by direct phosphorylation of raf-1 (43) or via Shc/Ras (44), it is currently not known how PKC may regulate the p38 cascade.

In contrast to its effect on ERK activity, the effect of GF109203X on p38 dual phosphorylation was partial. This could imply that PKC is not the sole upstream regulator of the p38 cascade. 20:4ω6 has been reported to stimulate the release of rho-GDI from its complex with rac2. guanine nucleotide dissociation inhibitor (45), and constitutively active rac has been found to stimulate the activity of p38 via p21-activated kinase (46). Hence, the fatty acid may also activate p38 via modulation of rac2 and p21-activated kinase, independently of PKC. Alternatively, the partial inhibition could suggest the possibility that neutrophils express both PKC-dependent and independent p38 forms. Until specific antibodies to p38 subtypes become available commercially, it is not possible to determine which p38 form(s) is activated by 20:4ω6.

Although GF109203X has been generally regarded as a specific PKC inhibitor, a recent study has found that GF109203X also inhibited the activity of MAP kinase activated protein kinase-1β (rsk-2) and p70 S6 kinase (47). However, the effects of GF109203X on 20:4ω6-stimulated ERK activity and p38 dual phosphorylation were unlikely to be due to inhibition of rsk-2 or p70 S6 kinase, since neither of these kinases are upstream regulators of ERK or p38.

The inability of 20:4ω6 to stimulate JNK activity in neutrophils is in direct contrast to the observations in stromal (20) and proximal tubular epithelial cells (19). Although 20:4ω6 stimulated the activity of JNK in Jurkat cells, this effect was weak compared with that caused by A21387 and PMA. Our failure to detect JNK activity was not because of a lack of JNK expression in neutrophils. Studies in proximal tubular epithelial cells have shown that stimulation of JNK activity by 20:4ω6 requires activation of the NADPH oxidase (19). Given that 20:4ω6 strongly stimulates the NADPH oxidase in neutrophils, it is, therefore, surprising that the fatty acid failed to stimulate the activity of JNK in neutrophils. This result clearly demonstrates that generation of oxygen radicals *per se* is insufficient to stimulate the JNK cascade.

Many ligands that stimulate the activity of MAP kinases also stimulate the activity of cytosolic phospholipase A_2. It has been widely reported that ERK or p38 directly phosphorylates cytosolic phospholipase A_2 in activated cells and in *in vitro* assays (48, 49) and, with the exception of thrombin-stimulated platelets (50), ERK or p38 has been found to directly regulate the enzymatic activity of cytosolic phospholipase A_2. Our study, therefore, suggests that fatty acids such as 20:4ω6, which are liberated by ligand-stimulated cytosolic phospholipase A_2, may participate in sustaining/amplifying MAP kinase activity and the activity of cytosolic phospholipase A_2. Consistent with this, exogenously added polyunsaturated fatty acids have been

[2] C. S. T. Hii and A. Ferrante, unpublished data.

found to stimulate the activity of cytosolic phospholipase A_2 in intact neutrophils.[3]

It is currently not clear how fatty acids are taken up into cells and exert their effects. There is evidence that indicates that fatty acids enter cells by simple diffusion (51) and/or via a carrier-mediated process. Proteins that function as fatty acid transporters have been reported to exist on the plasma membrane of a number of cell types (52, 53). It is possible that these mechanisms are not mutually exclusive. Clearly, partititioning of a fatty acid into the plasma membrane *per se* is insufficient to exert a biological action (54, 55). A detergent-like action of polyunsaturated fatty acids on the neutrophils has been excluded at concentrations that were used in this study (56). It is also unlikely that the biological activities of a fatty acid are dependent on esterification into membrane phospholipids, since the effects of fatty acids are reversed after the addition of delipidated serum albumin (4, 29) too rapidly to support an esterification-based mechanism of action. A direct agonist-like fatty acid action is therefore likely.

The present study establishes for the first time that 20:4ω6 stimulates the dual phosphorylation of p38 MAP kinase and that this stimulation is cell type-specific. Although this effect was observed in HeLa cells, HL60 cells, human umbilical vein endothelial cells, and human neutrophils, 20:4ω6 did not increase the amount of dual-phosphorylated p38 in Jurkat T cells. Our studies also demonstrate that 20:4ω6 stimulates the activity of ERK and JNK. Again, this effect was cell type-specific. Our data, therefore, suggest that ERK, p38, JNK, and PKC are potential mediators of the biological actions of 20:4ω6. The MAP kinase species that is recruited will depend on the cell type.

Acknowledgment—We thank Dr. S. L. Pelech, University of British Columbia, Canada, for the gift of anti-ERK antibody, R2.

REFERENCES

1. Dennis, E. A. (1997) *Trends Biochem. Sci.* **22,** 1–2
2. Murakami, K., and Routtenberg, A (1985) *FEBS Lett.* **192,** 189–193
3. McPhail, L. C. Clayton, C. C., and Snyderman, R. (1984) *Science* **224,** 622–625
4. Hii, C. S. T., Ferrante, A., Schmidt, S., Rathjen, D. A., Robinson, B. S., Poulos, A and Murray, A. W. (1995) *Carcinogenesis* **16,** 1505–1511
5. Poulos, A., Robinson, B. S., Ferrante, A., Harvey, D. P., Hardy, S. J., and Murray, A. W. (1991) *Immunology* **73,** 102–108
6. Bates, E. J., Ferrante, A., Smithers, L., Poulos, A., and Robinson, B. R. (1995) *Atherosclerosis* **116,** 247–259
7. Bates, E. J., Ferrante, A., Harvey, D. P., and Poulos, A. (1993) *J. Leucocyte Biol.* **53,** 420–426
8. Huang, Z. H., Hii, C. S. T., Rathjen, D. A., Poulos, A., Murray, A. W., and Ferrante, A. (1997) *Biochem. J.* **325,** 553–557
9. Band, A. M., Jones, P. J., and Howell, S. L. (1992) *J. Mol. Endocrinol.* **8,** 95–101
10. Smirnov, S. V., and Aaronson, P. I. (1996) *Circ. Res.* **79,** 20–31
11. Wieland, S. J., Gong, Q., Poblete, H., Fletcher, J. E., Chen, L. Q., and Kallen, R. G. (1996) *J. Biol. Chem.* **271,** 19037–19041
12. DeCoursey, T. E., and Cherny, V. V. (1993) *Biophys. J.* **65,** 1590–1598
13. Clarke, S. D., and Jump, D. B. (1993) *Prog. Lipid Res.* **32,** 139–149
14. Finstad, H. S., Kolset, S. O., Holme, J. A., Wiger, R., Farrants, A. K., Blomhoff, R., and Drevon, C. A. (1994) *Blood* **84,** 3799–3809
15. Hii, C. S. T., Ferrante, A., Edwards, Y., Huang, Z. H., Hartfield, P. J., Rathjen, D. A., Poulos, A., and Murray, A. W. (1995) *J. Biol. Chem.* **270,** 4201–4204
16. Rao, G. N., Baas, A. S., Glascow, W. C., Eling, T. E., Runge, M. S., and Alexander, R. W. (1994) *J. Biol. Chem.* **269,** 32586–32591
17. Kyriakis, J. M., and Avruch, J. (1996) *J. Biol. Chem.* **271,** 24313–24316
18. Seger, R., and Krebs, E. G. (1995) *FASEB J.* **9,** 726–735
19. Cui, X. L., and Douglas, J. G. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94,** 3771–3776
20. Rizzo, M. T., and Carlo-Stella, C. (1996) *Blood* **88,** 3792–3800
21. Hii, C. S. T., Edwards, Y. S., and Murray, A. W. (1991) *J. Biol. Chem.* **266,** 20238–20243
22. Ferrante, A., and Thong, Y. H. (1982) *J. Immunol. Methods* **48,** 81–85
23. Anderson, N. G., Maller, J. L., Tonks, N. K., and Sturgill, T. W. (1990) *Nature* **343,** 651–653
24. Sakata, N., Patel, H. R., Terada, N., Aruffo, A., Johnson, G. L., and Gelfand, E. W. (1995) *J. Biol. Chem.* **270,** 30823–30828
25. Hii, C. S. T., Oh, S. Y., Schmidt, S., Clark, K. J., and Murray, A. W. (1994) *Biochem. J.* **303,** 475–479
26. Crawley, J. B., Rawlinson, L., Lali, F. V., Page, T. H., Saklatvala, J., and Foxwell, B. M. (1997) *J. Biol. Chem.* **272,** 15023–15027
27. Guyton, K. Z., Liu, Y., Gorospe, M., Xu, Q., and Holbrook, N. J. (1996) *J. Biol. Chem.* **271,** 4138–4142
28. Smallwood, J. I., and Malawista, S. E. (1992) *J. Leukocyte Biol.* **51,** 84–92
29. Hardy, S. J., Robinson, B. S., Ferrante. A., Hii, C. S. T., Johnson, D. W., Poulos, A., and Murray, A. W. (1995) *Biochem. J.* **311,** 689–697
30. Robinson, B. S., Hii, C. S. T., and Ferrante, A. (1997) *Immunology* **91,** 274–280
31. Henderson, L. M., Banting, G., and Chappel, J. B. (1995) *J. Biol. Chem.* **270,** 5909–5916
32. Abramson, S. B., Leszczynska-Piziak, J., and Weissman, G. (1991) *J. Immunol.* **147,** 231–236
33. Lu, Y., Tremblay, R., Jouishomme, H., Chakravarthy, B., and Durkin, J. P. (1994) *J. Immunol.* **153,** 1495–1504
34. Toullec, D., Pianetti, D., Coste, H., Bellevergue, P., Grand-Perret, T., Ajakane, M., Baudet, V., Boissin, P., Boursier, E., Lorielle, F., Duhamel, L., Charon, D., and Kirilovsky, J. (1991) *J. Biol. Chem.* **266,** 15771–15781
35. Robinson, B. S., Hii, C. S. T., Poulos, A., and Ferrante, A. (1996) *J. Lipid Res.* **37,** 1234–1245
36. Balsinde, J., and Dennis, E. A. (1996) *J. Biol. Chem.* **271,** 6758–6765
37. Nichols, R. C., and Vanderhoek, J. Y. (1990) *J. Exp. Med.* **171,** 367–375
38. Wolf, B. A., Turk, J., Sherman, W. R., and McDaniel, M. L. (1986) *J. Biol. Chem.* **261,** 3501–3511
39. El Benna, J., Han, J., Park, J. W., Schmid, E., Ulevitch, R. J., and Babior, B. M. (1996) *Arch. Biochem. Biophys.* **334,** 395–400
40. Franklin, C. C., and Kraft, A. S. (1997) *J. Biol. Chem.* **272,** 16917–16923
41. Jiang, Y., Gram, H., Zhao, M., New, L., Gu, J., Feng, L., De Padova, F., Ulevitch, R. J., and Han, J. (1997) *J. Biol. Chem.* **272,** 30122–30128
42. Kumar, S., McDonnell, P. C., Gum, R. J., Hand, A. T., Lee, J. C., and Young, P. R. (1997) *Biochem. Biophys. Res. Commun.* **235,** 533–538
43. Kolch, W., Heidecker, G., Kochs, G., Hummel, R., Vahidi, H., Mischak, H., Finkenzeller, G., Marme, D., and Rapp, U. R (1993) *Nature* **364,** 249–252
44. El-Shemerly, M. Y. M., Besser, D., Nagasawa, M., and Nagamine, Y. (1997) *J. Biol. Chem.* **272,** 30599–30602
45. Chuang, T. H., Bohl, B. P., and Bokoch, G. M. (1993) *J. Biol. Chem.* **268,** 26206–26211
46. Zhang, S., Han, J., Sells, M. A., Chernoff, J., Knaus, U. G., Ulevitch, R. J., and Bokoch, G. M. (1995) *J. Biol. Chem.* **270,** 23934–23936
47. Alessi, D. R. (1997) *FEBS Lett.* **402,** 121–123
48. Lin, L. L., Wartmann, M., Lin, A. Y., Knopf, J. L., Seth, A., and Davis, R. J. (1993) *Cell* **72,** 269–278
49. Borsch-Huabold, A. G., Kramer, R. M., and Watson, S. P. (1997) *Eur. J. Biochem.* **245,** 751–759
50. Kramer, R. M., Roberts, E. F., Um, S. L., Borsch-Haubold, A. G., Watson, S. P., Fisher, M. J., and Jakubowski, J. A. (1996) *J. Biol. Chem.* **271,** 27723–27729
51. Rose, H., Hennecke, T., and Kammermeier, H. (1990) *J. Mol. Cell. Cardiol.* **22,** 883–892
52. Berk, P. D. (1996) *Proc. Soc. Exp. Biol. Med.* **212,** 1–4
53. Vyska, K., Meyer, W., Stremmel, W., Notohamiprodjo, G., Minami, K., Machulla, H. J., Gleichmann, U., Meyer, H., and Korfer, R. (1991) *Circ. Res.* **69,** 857–870
54. Anel, A., Richieri, G. V., and Kleinfeld, A. M. (1993) *Biochemistry* **32,** 530–536
55. Steinbeck, M. J., Hegg, G. G., and Karnovsky, M. J. (1991) *J. Biol. Chem.* **266,** 16336–16342
56. Corey, S. J., and Rosoff, P. M. (1991) *J. Lab. Clin. Med.* **118,** 343–351

[3] B. S. Robinson, C. S. T. Hii, and A. Ferrante, unpublished data.