Evidence for differential activation of arachidonic acid metabolism in formylpeptide- and macrophage-activation-factor-stimulated guinea-pig macrophages

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Alterations of phospholipid and arachidonic acid metabolism were studied by treatment of guinea-pig peritoneal-exudate macrophages with chemotactic peptide, formylmethionyl-leucylphenylalanine (fMet-Leu-Phe) and macrophage activation factor (MAF). The chemotactic peptide caused a rapid rearrangement in inositol phospholipids, including a breakdown of polyphosphoinositides within 30 s, followed by a resultant formation of phosphatidylinositol (PI), diacylglycerol, phosphatidic acid and non-esterified arachidonic acid within 5 min. In addition to these sequential alterations, arachidonic acid was released mainly from PI. On the other hand, MAF induced a slow liberation of arachidonic acid, mainly from phosphatidylethanolamine (PE) and phosphatidylcholine (PC) by phospholipase A₂ after the incubation period of 30 min, but not any rapid changes in phospholipids. Treatment of macrophages for 15 min with fMet-Leu-Phe produced the leukotrienes (LTs) B₄, C₄ and D₄, prostaglandins (PG) E₂ and F_{2α} and thromboxane (TX) B₂. In contrast, MAF could not stimulate the production of arachidonic acid metabolites during the incubation period of 15 min, but could enhance that of PGE₂, PGF_{2α}, TXB₂ and hydroxyeicosatetraenoic acids at 6 h. However, the stimulated formation of LTs was not detected at any time. These results indicate that the effects of fMet-Leu-Phe on both phospholipid and arachidonic acid metabolism are very different from those mediated by MAF.

Macrophages play an important role in the host defence against infectious agents and neoplastic cells, as well as in the removal of antigens,

Abbreviations used: MAF, macrophage activation factor; fMet-, formylmethionyl-; LPS, lipopolysaccharide; MDP, muramyl dipeptide; EMEM, Eagle's Minimum Essential Medium; HBSS, Hanks balanced salt solution; PI, phosphatidylinositol; DPI, phosphatidylinositol 4-monophosphate; TPI, phosphatidylinositol 4,5-bisphosphate; PA, phosphatidic acid; PE, phosphatidylethanolamine; PC, phosphatidylcholine; LPE, lysophosphatidylethanolamine; LPC, lysophosphatidylcholine; MG, monoacylglycerol; DG, diacylglycerol; TG, triacylglycerol; NEFA, non-esterified fatty acid; PG(s), prostaglandin(s); LT(s), leukotriene(s); HETE, hydroxyeicosatetraenoic acid; HPETE, hydroperoxyeicosatetraenoic acid; r.p.-h.p.l.c., reverse-phase h.p.l.c.

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antibody complexes and damaged tissues. Their functions have been known to be modified by many factors or agents, which include MAF, LPS, MDP, fMet-Leu-Phe, some components of complement system and Ca²⁺ ionophore (David & Remold, 1979; Onozaki *et al.*, 1981; Weinberg *et al.*, 1978; Goldstein *et al.*, 1973; Yamamoto *et al.*, 1978; Pick *et al.*, 1979; Homma *et al.*, 1982). Among these substances, fMet-Leu-Phe or complement components induce a metabolic burst accompanied by the increase in respiratory rate, the secretion of lysosomal enzymes and the production of superoxide anion. These effects can be regarded as short-term macrophage activation (Wilkinson, 1976). On the other hand, other substances, which include MAF, LPS and MDP, slowly activate macrophages to a differentiating state in which they resist intracellular parasites and

neoplasms in addition to exhibiting an accelerated glucose consumption and a stimulated incorporation of glucosamine. These effects can be regarded as the long-term macrophage activation.

It has been well known that macrophages actively synthesized PGs and LTs in response to physiological stimuli (Kuehl & Egan, 1980; Bonney *et al.*, 1978; Stenson & Parker, 1980; Samuelsson, 1983). Because arachidonic acid exists primarily in the esterified form in mammalian cells, the liberation of arachidonic acid from phospholipids is considered to be the rate-limiting step for the synthesis and the secretion of these metabolites (Kuehl & Egan, 1980). In the release of arachidonic acid, several possible pathways have been proposed. One of these is associated with phospholipase A₂ activation (Flower & Blackwell, 1976). Phospholipase A₂ acts mainly on PE and PC to liberate arachidonic acid. In addition to this pathway, it has been proposed that the sequential activation of PI-specific phospholipase C and DG lipase can liberate arachidonic acid (Kennerly *et al.*, 1979; Marshall *et al.*, 1980). Alternatively, PA-specific phospholipase A₂ acts on PA which is converted from DG (Billah *et al.*, 1981).

In the present study we chose fMet-Leu-Phe and MAF as typical stimulants associated with the short-term and the long-term macrophage activation respectively, and studied the effects of these stimuli on the metabolism of phospholipids and arachidonic acid.

Materials and methods

Materials

[5,6,8,9,11,12,14,15-³H]Arachidonic acid (120 Ci/mmol) and [³²P]P_i (carrier-free) were obtained from New England Nuclear Corp. (Boston, MA, U.S.A.). Ca²⁺ ionophore A23187 was from Calbiochem-Behring Co. (La Jolla, CA, U.S.A.). PI, PA, LPC, LPE, arachidonic acid and dipalmitoylglycerol were from Sedary Research Laboratories (London, Ontario, Canada). The chemotactic peptide fMet-Leu-Phe was from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Eagle's minimum essential medium (EMEM) and HBSS were from Nissui Co. (Tokyo, Japan). C₁₈ disposable cartridges (SEP-PAK) were purchased from Waters Associates (Milford, MA, U.S.A.). DPI and TPI were prepared by the method of Folch (1949). MAF was isolated from guinea-pig lymphokine, which was obtained by the stimulation of BCG (Bacille Calmette-Guerin)-sensitized spleen lymphocytes with specific antigen, by using affinity column chromatography as described previously (Onozaki *et al.*, 1981). The MAF preparation used in the present paper had the activity of 0.25 of the macrophage activation

index, which was calculated from a ratio of velocity of glucose consumption of MAF-stimulated macrophages to that of unstimulated macrophages.

Macrophages

Female Hartley guinea pigs (300–400 g) were injected with 20 ml of liquid paraffin. The peritoneal-exudate cells were collected 4 days later in HBSS. The cells were washed three times with HBSS, suspended in EMEM containing 15% (v/v) guinea-pig serum, plated in plastic dishes, and incubated for 2 h at 37°C in CO₂/air (1:19). After the removal of non-adherent cells by washing with medium, the remaining cells were collected with a rubber policeman, washed with medium, and used as the macrophage preparation. Over 90% of the cells had phagocytic activity.

Incorporation of [³²P]P_i into phospholipids

Macrophages (2 × 10⁶–5 × 10⁶ cells/0.5 ml) were incubated with 20 μCi of [³²P]P_i in phosphate-free EMEM containing 5% (v/v) guinea-pig serum and 20 mM-Hepes/NaOH buffer, pH 7.2, for 1 h at 37°C. In this case, guinea-pig serum was used after dialysis against phosphate-free EMEM. The cells were then stimulated with fMet-Leu-Phe, Ca²⁺ ionophore A23187 and MAF for the indicated time at 37°C in CO₂/O₂ (1:19). The reaction was terminated by adding 3 ml of chloroform/methanol (1:1, v/v).

[³H]Arachidonic acid redistribution from [³H]arachidonyl-lipids

Macrophages (10⁸ cells/20 ml) were incubated with 25 μCi of [³H]arachidonic acid in EMEM containing 5% guinea-pig serum and 20 mM-Hepes/NaOH buffer, pH 7.2, for 1 h at 37°C. The cells were then sedimented by centrifugation and washed twice with medium to remove free [³H]-arachidonic acid. The washed pellet was resuspended to 2 × 10⁶–5 × 10⁶ cells/0.5 ml in fresh medium, placed in a 37°C water bath, and incubated with each stimulant for the indicated time in CO₂/O₂ (1:19). Reactions were terminated by the addition of 3 ml of chloroform/methanol (1:1, v/v).

Extraction and separation of lipids

Lipids were extracted by adding 3 ml of chloroform/methanol (1:1, v/v) to the reaction mixture, followed by the addition of 0.5 ml of 2M-KCl. The mixture was shaken vigorously and separated into two phases by centrifugation at 1000g for 10 min. After the lower phase was washed with synthetic upper-phase solution, portions of the lower phase were removed for analysis of phospholipids (PE, PC, PI, PA, LPE, LPC) and neutral lipids. For the

analysis of phospholipids, two-dimensional t.l.c. was carried out after the addition of 10 μ g of PA, LPE and LPC as carrier. The plates were developed with a solvent system of chloroform/methanol/aq. 28% (w/v) NH_3 (13:7:1, v/v) for the first dimension and of chloroform/acetone/methanol/acetic acid/water (10:4:2:2:1, v/v) for the second dimension. Lipids on the plates were revealed by exposing the plate to I_2 vapour. Each spot was scraped off and the radioactivity was measured. For the analysis of the neutral lipids, one-dimensional t.l.c. was carried out after the addition of 10 μ g of DG and arachidonic acid as carrier. The plates were developed with a solvent system of light petroleum (b.p. 30–60°C)/diethyl ether/acetic acid (50:50:1, v/v). Each spot was scraped off and the radioactivity was measured.

Extraction and analysis of DPI and TPI

The reaction was stopped by adding 1 ml of ice-cold 10% (w/v) trichloroacetic acid containing 1 mM-EDTA. The mixture was vigorously shaken and centrifuged at 1000g for 10 min. The pellet was extracted twice with 1.5 ml of chloroform/methanol/conc. HCl (100:100:1, v/v) and then with 1 ml of chloroform/methanol/conc. HCl (200:100:1, v/v). The three extracts were pooled and 1.5 ml of chloroform added. The mixture was first washed with 1.5 ml of 0.1 M-HCl and then washed two times with synthetic upper-phase solution. The chloroform phase was evaporated to dryness, and the residue was dissolved in a small amount of chloroform. After Folch's (1949) DPI and TPI fractions were added as carrier, DPI and TPI were separated from other phospholipids by one-dimensional t.l.c. In this case, high-performance thin-layer plates (HPTLC plate, Merck) were used after pretreatment with methanol/water (2:3, v/v) containing 1% potassium oxalate and then activated for 1 h at 110°C by the method of Jolles *et al.* (1981). The plates were developed with chloroform/methanol/4.3 M-aq. NH_3 (18:13:4, v/v).

Extraction of arachidonic acid metabolites

Macrophages (5×10^7 cells/10 ml) were incubated with 25 μ Ci of [^3H]arachidonic acid in EMEM containing 15% guinea-pig serum and 20 mM-Hepes/NaOH buffer, pH 7.2, for 6 h at 37°C in CO_2 /air (1:19). In this case we used 6 h rather than 1 h for the labelling period in order to get macrophages that were highly labelled with [^3H]arachidonic acid. This did not alter the results. The cells were washed twice with EMEM and suspended (10^7 cells/ml) in media containing various stimulants. After incubation for various periods, the medium was removed, and arachidonic acid metabolites were extracted by the following method. To 5 ml of the medium, 25 ml of methanol

and 50 μ l of 90% (w/w) formic acid were added. The mixture was vigorously shaken and then evaporated to about 5 ml. The resulting solution was applied to a C_{18} SEP-PAK cartridge that had been previously swollen with water and then washed with methanol. After washing the cartridge with 20 ml of water, the arachidonic acid metabolites were eluted with 100% ethanol. The eluates were evaporated to dryness under a stream of N_2 and dissolved in 100 μ l of the appropriate starting buffer for r.p.-h.p.l.c.

Separation of arachidonic acid metabolites by r.p.-h.p.l.c.

Arachidonic acid metabolites were separated by the method of Scott and co-workers (Scott *et al.*, 1982; Rouzer *et al.*, 1982a,b). All r.p.-h.p.l.c. analyses were performed with a column (4.6 mm $\times$ 25 cm) of Ultrasphere ODS (Altex Scientific, Beckman Instruments, Berkeley, CA, U.S.A.). After application of a sample, the column was eluted with 60 ml of solvent 1 [methanol/water/acetic acid, 65:34.9:0.1 (by vol.) adjusted to pH 5.4 with aq. NH_3], followed by 40 ml of solvent 2 (methanol/water/acetic acid, 75:25:0.01, by vol.) and 40 ml of solvent 3 (methanol/acetic acid, 100:0.01, v/v) at a flow rate of 1 ml/min (r.p.-h.p.l.c. system I). Each fraction (containing 1 ml) was collected and the radioactivity measured in a liquid-scintillation counter. For the analysis of cyclo-oxygenase metabolites, fractions 4–18 from r.p.-h.p.l.c. system I were pooled, evaporated under reduced pressure, and applied to r.p.-h.p.l.c. using solvent 4 (water/acetonitrile/benzene/acetic acid, 76.3:23.0:0.2:0.1, by vol.) at a flow rate of 1 ml/min (r.p.-h.p.l.c. system II). PGs were eluted with 100 ml of solvent 4, and the column was washed with 40 ml of solvent 3. Sequential 1 ml eluates were counted in a scintillation counter.

Results

Incorporation of [^{32}P]P_i into phospholipids

Phospholipid metabolism is closely related to arachidonic acid metabolism. We first examined the effect of chemotactic peptide fMet-Leu-Phe and MAF on phospholipid metabolism in macrophages (Fig. 1). When [^{32}P]P_i-prelabelled macrophages were cultured in the medium containing guinea-pig MAF, incorporation of [^{32}P]P_i into PE, PC, PI, and PA was slightly increased after the incubation period of 6 h. On the other hand, fMet-Leu-Phe stimulated [^{32}P]P_i incorporation into PA and PI fractions without any significant effect on the incorporation into other phospholipids such as PE and PC. In this case, enhancement of the incorporation into PI was detectable at 5 min and reached a plateau at 1 h. Enhancement of [^{32}P]P_i

Fig. 1. Effect of *f*Met-Leu-Phe and MAF on $[^{32}\text{P}]\text{P}_i$ incorporation into phospholipids

Macrophages (2×10^6 – 5×10^6 cells/0.5 ml) were incubated with $20 \mu\text{Ci}$ of $[^{32}\text{P}]\text{P}_i$ in phosphate-free EMEM containing 5% guinea-pig serum and 20 mM-Hepes/NaOH buffer, pH 7.2, for 1 h at 37°C . After washing, the cells were stimulated by the addition of $1 \mu\text{M}$ -*f*Met-Leu-Phe or -MAF for various periods in CO_2/O_2 (1:19). Other details are as described in the Materials and methods section. ●, Control; ■, *f*Met-Leu-Phe; ▲, MAF. Values are means ($\pm$ S.E.M.) of triplicate determinations. *, Significantly different from control ($P < 0.01$).

labelling into PA reached a maximum at 1 min, and then levelled off to the control level with further incubation. We also studied the effect of *f*Met-Leu-Phe and MAF on polyphosphoinositide metabolism by measuring the redistribution of $[^{32}\text{P}]\text{P}_i$ in the TPI and DPI. Fig. 2 shows the rapid alterations in the TPI and DPI fractions. When macrophages were stimulated by *f*Met-Leu-Phe, a marked loss of labelled TPI was observed within 10 s and that of DPI followed within 30 s. These events were not observed in MAF-stimulated macrophages. These results indicate that *f*Met-Leu-Phe rapidly induces a breakdown of polyphosphoinositides and a compensatory increase of $[^{32}\text{P}]\text{P}_i$ incorporation into PI and PA, and that MAF slowly stimulates a rearrangement of phospholipids.

Effect of *f*Met-Leu-Phe and MAF on the redistribution and liberation of arachidonic acid

Liberation of arachidonic acid from esterified lipids is thought to be strictly regulated, since metabolites derived from arachidonic acid have various and strong biological activities. We examined the effect of *f*Met-Leu-Phe on redistribution of arachidonic acid. Fig. 3 shows that *f*Met-Leu-

Fig. 2. Effect of *f*Met-Leu-Phe and MAF on $[^{32}\text{P}]\text{P}_i$ redistribution in polyphosphoinositides

Macrophages were incubated with $[^{32}\text{P}]\text{P}_i$ in phosphate-free EMEM containing 5% guinea-pig serum for 1 h at 37°C . The reaction was started with the addition of *f*Met-Leu-Phe or MAF. Open and closed symbols indicate TPI and DPI respectively. ○ and ●, Control; □ and ■, *f*Met-Leu-Phe; △ and ▲, MAF. Values are the means $\pm$ S.E.M. of triplicate determinations. *, Significantly different from control ($P < 0.01$).

Fig. 3. Effect of *fMet-Leu-Phe* and *MAF* on the redistribution of [${}^3\text{H}$]arachidonic acid moiety in [${}^3\text{H}$]arachidonyl-lipids. Macrophages were incubated with [${}^3\text{H}$]arachidonic acid for 1 h at 37°C . After washing to remove free arachidonic acid, the cells were stimulated with *fMet-Leu-Phe* or *MAF* for various periods in CO_2/O_2 (1:19). Other details are as described in the Materials and methods section. ●, Control; ■, *fMet-Leu-Phe*; ▲, *MAF*. Values are means $\pm$ S.E.M. of triplicate determinations. *, Significantly different from control ($P < 0.01$).

Phe caused a loss of arachidonic acid in PI fraction and an increase in DG, PA, and NEFA fractions after stimulation of 3 min. These results indicate that *fMet-Leu-Phe* induces the liberation of arachidonic acid from PI. On the other hand, when [${}^3\text{H}$]arachidonic acid-labelled macrophages were cultured with *MAF*, a decrease of [${}^3\text{H}$]arachidonyl-PE and -PC and an increase of free arachidonic acid were demonstrated at 30 min after addition of *MAF*, but no changes were observed at 10 min. Moreover, formation of lysophospholipids, such as LPE and LPC, was slightly enhanced at 30 min (results not shown). These data suggest that *MAF* slowly activates phospholipase A_2 and causes liberation of arachidonic acid mainly from PE and PC. Fig. 3 also shows an increase of [${}^3\text{H}$]arachidonyl-PE and -PI in the control experiment without *fMet-Leu-Phe* or *MAF*. However, the level of free arachidonic acid is low in macrophages washed after the labelling. This would be

due to the rearrangement of arachidonic acid, including transacylation between various lipids.

Released arachidonic acid was immediately converted into PGs and LTs in macrophages, and these metabolites were secreted to their environments (Kuehl & Egan, 1980). Fig. 4 shows the total [${}^3\text{H}$]arachidonic acid metabolites secreted by stimulated macrophages. When macrophages were treated with *fMet-Leu-Phe*, the amount of metabolites increased significantly after 5 min of incubation and reached a plateau within 1 h. On the other hand, in the case of *MAF*, a significant increase in arachidonic acid metabolites was detected only after incubation for 1 h and continued for more than 24 h.

Lipoxygenase metabolites secreted from fMet-Leu-Phe- or MAF-stimulated macrophages

Arachidonic acid metabolites in the culture media of stimulated macrophages were analysed

Fig. 4. Effect of *f*Met-Leu-Phe (a) and MAF (b) on the secretion of ³H-labelled materials from [³H]arachidonic acid-labelled macrophages

The culture conditions were the same as that described in Fig. 3. Radioactivity of samples after each period of incubation was measured by a liquid-scintillation counter. ○, Control; ●, addition of 1 μM-*f*Met-Leu-Phe (a) or MAF (b). Values are means ± S.E.M. of triplicate determinations. *, Significantly different from control ($P < 0.01$).

by using r.p.-h.p.l.c. system equipped with a C-18 reverse-phase column. Lipoxygenase metabolites were eluted from 20 to 100 min in h.p.l.c. system I. Fig. 5 shows r.p.-h.p.l.c. profiles of tritiated materials obtained when macrophages were incubated with *f*Met-Leu-Phe and Ca²⁺ ionophore A23187 for 15 min. Ionophore A23187 was used as a good inducer for the production of both LTs and HETEs (Bokoch & Reed, 1980). *f*Met-Leu-Phe stimulated the production of LTC₄, LTD₄, LTB₄ and other metabolites, including a small amount of HETEs. The chromatographic pattern of arachidonic acid metabolites extracted from the medium in which Ca²⁺ ionophore-stimulated macrophages were cultured was quite similar to those derived from *f*Met-Leu-Phe-activated macrophages. In addition, even when macrophages were stimulated

Fig. 5. R.p.-h.p.l.c. analyses of lipoxygenase acid metabolites produced by non-, *f*Met-Leu-Phe- or Ca²⁺ ionophore A23187-stimulated macrophages

Macrophages were labelled for 6 h with [³H]arachidonic acid at 37°C. After washing to remove free [³H]arachidonic acid, the cells were cultured in medium alone (a), or in medium containing 1 μM-*f*Met-Leu-Phe (b) or 1 μM-A23187 (c) for 15 min in CO₂/O₂ (1:19). After incubation, the medium was removed and the arachidonic acid metabolites were extracted as described in the Materials and methods section. The extracts were subjected to r.p.-h.p.l.c. using r.p.-h.p.l.c. system I. Sequential eluates were collected, and the radioactivity of each fraction was measured in a liquid-scintillation counter. Elution times for each metabolite were as follows: LTC₄, 21–23 min; LTD₄, 32–34 min; LTE₄, 45–46 min; LTB₄, 37–39 min; 15-HETE, 67–70 min; 12-HETE, 73–75 min; 5-HETE, 79–83 min. Recovery of lipoxygenase metabolites through extraction and r.p.-h.p.l.c. was about 50%.

by *f*Met-Leu-Phe or Ca²⁺ ionophore A23187 for 1 h or more, only degradative materials of LTs, but not other kinds of LTs, could be detected.

On the other hand, when macrophages were stimulated with MAF, arachidonic acid metabo-

lites could be detected after a period of 1 h and production of the metabolites continued for more than 24h. We analysed arachidonic acid metabolites after an incubation period of 6h (Fig 6). The r.p.-h.p.l.c. pattern of the metabolites derived from MAF-stimulated macrophages was different from that derived from fMet-Leu-Phe- or Ca^{2+} ionophore A23187-stimulated macrophages. Although production of LTs was very slight, a large amount

of HETEs was produced by MAF-treated macrophages. The amount of 5-HETE produced was about 10 times the control value. Enhanced production of HETEs was observed throughout the 24h incubation, without a significant increase in the production of LTs. This suggests that MAF-induced production of HETEs is not attributable to non-specific oxidation of arachidonic acid metabolites.

Cyclo-oxygenase metabolites secreted by fMet-Leu-Phe- or MAF-stimulated macrophages

In order to analyse cyclo-oxygenase metabolites, the 4–18min eluants were combined, concentrated, and re-chromatographed with r.p.-h.p.l.c. system II. Exudate macrophages actively synthesized PGE_2 , $\text{PGF}_{2\alpha}$, and TXB_2 , but not 6-oxo $\text{PGF}_{1\alpha}$. When macrophages were treated with fMet-Leu-Phe for 15min, production of PGE_2 , $\text{PGF}_{2\alpha}$ and TXB_2 was enhanced about 3-fold compared with the unstimulated control (Table 1). This enhanced production of PGs reached a maximum at the period of 15 or 30min and diminished to the control level on further incubation. Table 1 also shows the enhanced production of cyclo-oxygenase metabolites in MAF-stimulated macrophages. After an incubation period of 6h, unstimulated macrophages also mainly produced PGE_2 , $\text{PGF}_{2\alpha}$ and TXB_2 . In contrast, MAF-stimulated macrophages produced about 2-fold the metabolites produced by the unstimulated control. Enhanced production of PGs continued for more than 6h after the addition of MAF.

Discussion

The present results suggest that phospholipid and arachidonic acid metabolism in the short-term macrophage activation rapidly induced by fMet-Leu-Phe stimulation is very different from that in the long-term activation slowly induced by MAF stimulation. In the case of the short-term activation, alterations in phospholipids could be detected

Fig. 6. R.p.-h.p.l.c. analyses of lipoxygenase metabolites produced by MAF-treated macrophages [3H]Arachidonic acid-labelled macrophages were cultured in medium alone (a) or in medium containing MAF (b) for 6h in CO_2/O_2 (1:19). Extraction methods, r.p.-h.p.l.c. conditions and elution time of lipoxygenase products were the same as those described in Fig. 5.

Table 1. Production of cyclo-oxygenase metabolites synthesized by stimulated macrophages

The PG-containing fractions (4–18) eluted from r.p.-h.p.l.c. system I (Figs. 5 and 6) were pooled, concentrated and subjected to r.p.-h.p.l.c. system II at a flow rate of 1 ml/min. Fractions were collected, and the radioactivity was measured in a liquid-scintillation counter. The recovery of cyclo-oxygenase metabolites through the extraction and r.p.-h.p.l.c. was about 40%.

		Release of radioactivity (d.p.m./10 ⁷ cells)			
Culture time	Additions	6-oxo $\text{PGF}_{1\alpha}$	TXB_2	$\text{PGF}_{2\alpha}$	PGE_2
15 min	None	505	2793	2278	3014
	fMet-Leu-Phe	882	5292	7424	12495
	A23187	1250	2426	12348	12201
6 h	None	1984	2081	5223	10803
	MAF	1224	4277	8996	19640

within 1 min. fMet-Leu-Phe caused a breakdown of TPI and DPI, probably by phospholipase C, within 30 s, and consequently stimulated a resultant formation of PI, DG, PA and free arachidonic acid within 5 min. Michell and other investigators (Michell, 1975; Streb *et al.*, 1983) offered the hypothesis that receptor-linked breakdown of inositol phospholipids (PI response) plays an important role not only in signal transduction, which is closely related with the elevation of intracellular Ca^{2+} concentration, but also in the release of arachidonic acid from esterified phospholipids. In this regard, we previously reported that the liberation of arachidonic acid was completely dependent on exogenous Ca^{2+} , since depletion of Ca^{2+} from culture medium inhibited the release of arachidonic acid (Takenawa *et al.*, 1983). Consistent with this proposal is the observation that DG lipase does not need Ca^{2+} for its activity (Igarashi & Kondo, 1982). Therefore it seems likely that the cleavage of arachidonic acid, which follows the PI response, occurs via the pathway $\text{PI} \rightarrow \text{DG} \rightarrow \text{PA} \rightarrow \text{LPA} + \text{arachidonic acid}$ rather than the pathway $\text{PI} \rightarrow \text{DG} \rightarrow \text{MG} + \text{arachidonic acid}$.

On the other hand, when macrophages were exposed to MAF, we could observe a slight enhancement of incorporation of $[^{32}\text{P}]\text{P}_i$ into phospholipids, but not any rapid changes in phospholipids, including the PI response (Fig. 2). The experiments using $[^3\text{H}]\text{arachidonic acid}$ -labelled macrophages, however, showed that MAF induced degradation of PE and PC and the formation of free arachidonic acid after incubation for 30 min (Fig. 3). Formation of LPE and LPC was slightly stimulated (results not shown). These facts would indicate that MAF induces phospholipase A_2 activation and a resultant liberation of arachidonic acid, but not an activation of the inositol phospholipid-specific phospholipase C. Recently we have demonstrated that MAF stimulation caused the elevation of endogenous Ca^{2+} , because $^{45}\text{Ca}^{2+}$ uptake was stimulated by treatment with MAF for 30 min (K. Onozaki & T. Hashimoto, unpublished work). Thus the elevation of intracellular Ca^{2+} is thought to enhance the activity of non-specific phospholipase A_2 in MAF-stimulated macrophages.

Arachidonic acid metabolites were detected in the culture media in which macrophages were stimulated with fMet-Leu-Phe for 15 min or with MAF for 6 h. fMet-Leu-Phe stimulated the production of some kinds of LTs and PGs, whereas MAF stimulated that of HETEs and PGs. It is uncertain whether the production of HETEs is specific for macrophage activation induced by MAF stimulation, since HETE is formed by non-specific air oxidation of arachidonic acid during the incubation

period. Several observations suggest that non-specific air oxidation of arachidonic acid does not contribute significantly to the amount of HETE. First, HETEs could be detected in the cells incubated with MAF for periods such as 1 h when very little HETE is present in the culture medium. Second, when macrophages were co-cultured with sufficient free arachidonic acid for 6 h, the production of HETEs was not markedly elevated. These results indicate that the lipoxygenase pathway induced in MAF-stimulated macrophages is different from that in fMet-Leu-Phe-stimulated macrophages. It is postulated that fMet-Leu-Phe mainly stimulates the pathway $5\text{-HPETE} \rightarrow \text{LTA}_4 \rightarrow \text{LTB}_4$ and LTC_4 and that MAF does stimulate 5-HPETE peroxidation to 5-HETE. The difference may be explained by the alterations of the enzyme activity induced by MAF stimulation. These include (a) activation or induction of a peroxidase, which causes peroxidation of 5-HPETE to 5-HETE, (b) inhibition of dehydrase of 5-HPETE, which converts 5-HPETE into LTA_4 and (c) different pools of arachidonic acid for each oxygenation (Humes *et al.*, 1982).

Recent work in our laboratory has suggested that macrophages *per se* were regulated by endogenously produced arachidonic acid metabolites, since inhibitors of lipoxygenase or cyclo-oxygenase could affect macrophage activation induced by MAF. In addition, exogenous PGE_2 inhibited macrophage activation and exogenous LTB_4 enhanced it. These results suggest the possibility that each metabolite may act as an effector molecule of the feedback regulation in macrophage activation.

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