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Ligand-Operated Synthesis of 4-Series and 5-Series Leukotrienes in Human Neutrophils: Critical Dependence on Exogenous Free Fatty Acid Supply

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SUMMARY

The influence of exogenously supplied free arachidonic acid (AA) and eicosapentaenoic acid (EPA) on the 5-lipoxygenase metabolism in human neutrophils (PMN) was investigated. Simultaneous application of A23187 with incremental concentrations of free AA caused a dose-dependent augmentation of the ionophore-elicited eicosanoid generation [release of leukotriene B_4 and its ω-oxidation products, nonenzymatic hydrolysis products of leukotriene A_4, and 5-hydroxyeicosatetraeneoic acid (5-HETE)]. A23187 challenge in the presence of free EPA resulted in the dose-dependent appearance of corresponding $n-3$-derived metabolites, parallelled by a decrease in 4-series leukotrienes and 5-HETE. The inflammatory ligands formyl-methionyl-leucyl-phenylalanine and platelet-activating factor evoked no substantial eicosanoid generation in the absence of exogenously supplied polyunsaturated fatty acids (PUFAs). Addition of free AA or EPA in parallel with the ligand challenge evoked exclusive and dose-dependent generation of the respective leukotrienes and 5-HETE or 5-hydroxyeicosapentaenoic acid. Total amounts of 5-lipoxygenase products elicited under these conditions approached those in ionophore-stimulated PMN, with platelet-activating factor challenge surpassing the formyl-methionyl-leucyl-phenylalanine-evoked effect by approximately 50%. Two thirds of the maximum effect was obtained in the presence of only 10 μM free PUFA. Use of labeled fatty acids suggested exclusive origin of the eicosanoids from the exogenously provided precursor PUFA. Critical dependence on timing was noted; maximum response occurred upon simultaneous application of PUFA and ligand, and only 5 min of delay between AA or EPA addition and ligand challenge sufficed to reduce the formation of respective metabolites to <20%. EPA competed with AA and was noted to be the preferred substrate for ligand-evoked eicosanoid synthesis. In contrast to the simultaneous addition of free PUFAs, preloading of PMN with AA or EPA for 60 min revealed only very moderate or even no influence on ionophore- or ligand-evoked eicosanoid synthesis. We conclude that inflammatory ligands induce marked stimulation of PMN eicosanoid synthesis, with critical dependence on the presence of free precursor PUFAs. Preference of EPA over AA is observed under these conditions.

Lipoxygenase products of AA are involved in several inflammatory processes (1–3). LTB_4 appears to be important for chemoattraction and activation of PMN at sites of inflammation (4–6). Recently, dietary enrichment of PMN phospholipid pools with $n-3$ fatty acids, in particular EPA, has attracted attention as a putative therapeutic regimen for dampening PMN-related inflammatory events (7–9). PMN isolated *ex vivo* after intake of $n-3$-supplemented diets exhibited moderately reduced LTB_4 release upon ionophore challenge in several studies (10–15). A variety of responses to inflammatory stimuli, including chemotaxis in the presence of exogenous LTB_4, were noted to be diminished in these PMN (10, 16–18). LTB_5, generated in moderate amounts in EPA-enriched PMN, possesses >10-fold reduced chemotactic and PMN-activating capacity, compared with LTB_4 (19–25). Competition with LTB_4 for receptor occupancy on PMN has been demonstrated (23).

The calcium ionophore A23187 is an established potent activator of PMN LT generation, eliciting mobilization of endogenous AA from different membrane phospholipid pools and stimulation of subsequent 5-lipoxygenase metabolism (26–28). In contrast, natural inflammatory ligands such as FMLP, C_{5a}, LTB_4, or PAF are poor activators of PMN eicosanoid metabolism, with no or only very moderate amounts of LTs being detected after challenge with these stimuli (29–34). Simultaneous exposure of PMN to both FMLP and free AA was, however, recently noted to cause marked LT synthesis (33). This finding may be relevant, in view of substantial levels of free AA known to arise at sites of inflammatory events (35)

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ABBREVIATIONS: AA, arachidonic acid; EPA, eicosapentaenoic acid; PUFA, polyunsaturated fatty acid; LT, leukotriene; HETE, hydroxyeicosatetraenoic acid; HEPE, hydroxyeicosapentaenoic acid; PAF, platelet-activating factor; PMN, polymorphonuclear leukocytes; FMLP, formyl-methionyl-leucyl-phenylalanine; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; RIA, radioimmunoassay; RP, reverse phase; SP, straight phase; HPLC, high performance liquid chromatography; diHETE, dihydroxyeicosatetraenoic acid; diHEPE, dihydroxyeicosapentaenoic acid; 12 HHT, 12-hydroxyhentadecatrienoic acid.

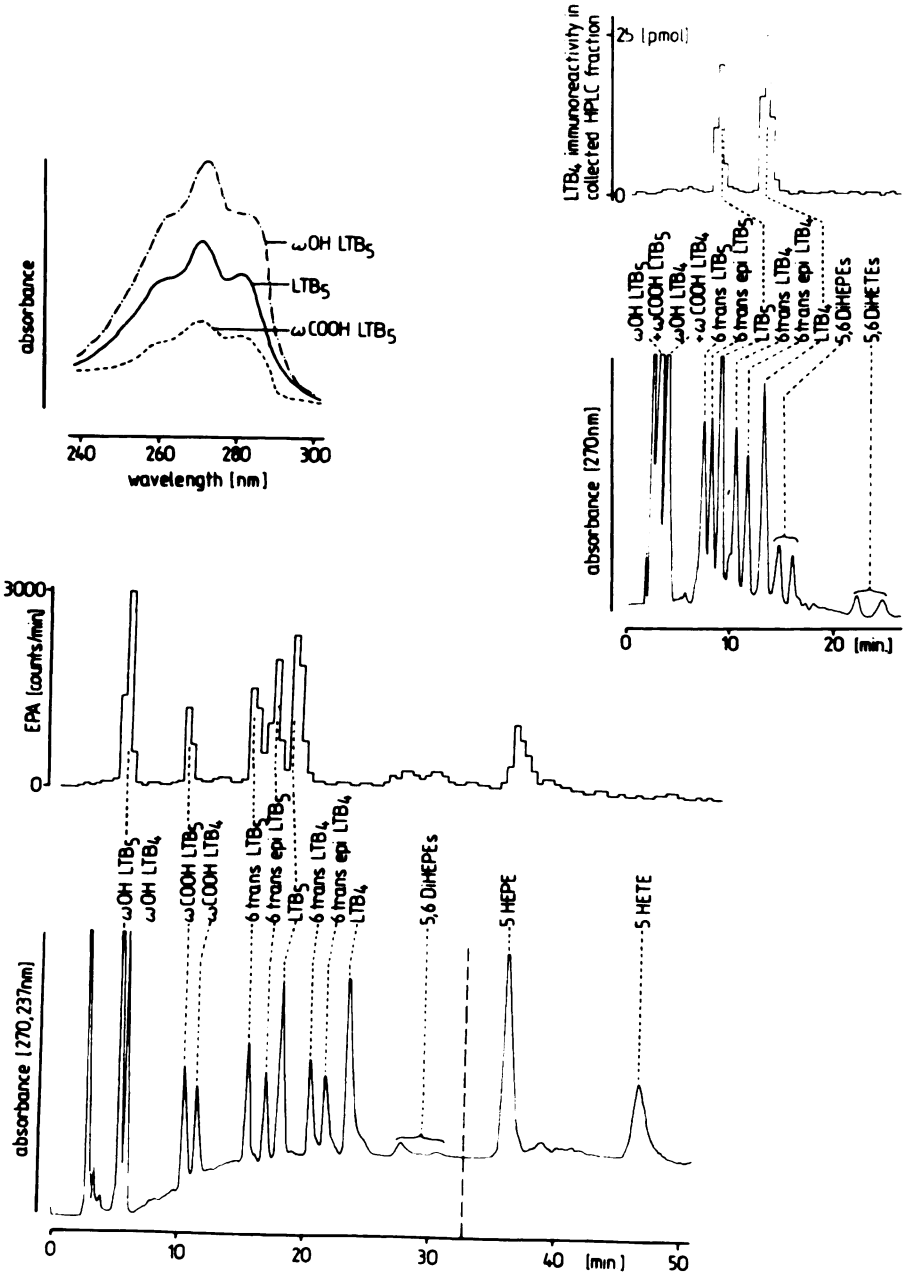

Fig. 1. Profile of lipoxygenase products released from human PMN exposed to 3 μM PAF in the presence of 5 μM EPA and 10 μM AA. PAF, AA, and EPA (labeled at 50 nCi/nmol) were simultaneously admixed with 1.5×10^7 PMN, incubation was stopped after 10 min, and the supernatant was extracted as described. Nonmethylated compounds were separated by RP HPLC and methylated products by RP-HPLC with gradient elution and by SP HPLC. In the case of LTB_5 and its ω-oxidation products, UV spectra obtained by on-line spectrum analysis (photodiode array detector) are displayed. Radioactivity and anti-LTB_4 immunoreactivity of the RP HPLC eluate (collected in 30-sec fractions) are given. The cross-reactivity of the anti-LTB_4 antibody with LTB_5 was determined to be 82.5%, in separate experiments with purified standards.

and in view of recently described PMN-endothelial cell cooperativity in LT synthesis. The latter includes not only a shift of LTA_4 from PMN (feeder cell) to endothelial cell, with subsequent use of the enzymatic equipment of this acceptor cell (36–40), but also a release of free AA from activated endothelial cells, with putative uptake and processing of this precursor by adjacent PMN (36, 41–43). Extracellularly offered $n-3$-free fatty acids, present during such metabolic interactions between PMN and endothelial cells, might thus substantially influence the resultant profile of eicosanoids. Based on this reasoning, we investigated PMN LT generation evoked by inflammatory ligands in the presence of free AA or EPA *in vitro*. Exclusive generation of large amounts of 4- or 5-series LTs, respectively, was noted, and simultaneous application of both precursor PUFAs revealed EPA to be the preferred substrate, capable of suppressing AA-derived product formation.

Experimental Procedures

Materials. LTC_4, LTD_4, LTE_4, and LTB_4, as well as 20-OH- and 20-COOH-LTB_4 and the synthetic LTA_4 methyl ester, were a generous gift from Dr. J. Rokach, Merck Frosst, Canada. Additional LTs were graciously supplied by Dr. Bartmann, Hoechst AG. AA, EPA, 5-, 8-, 9-, 11-, 12-, and 15-HETE, 5*S*,12*S*-diHETE, 5,15-diHETE, and 12-HHT, as well as LTA_5 methyl ester, LTB_5, and 5-HEPE, were obtained from Paesel AG (Frankfurt, FRG). The nonenzymatic hydrolysis products of LTA were prepared by acid hydrolysis of the synthetic LTA. ω-Oxidation products of LTB_5 were prepared for use as reference standards by incubation of LTB_5 and tritiated LTB_5 (3 μM) with 1.5×10^7/ml unstimulated PMN at 37° for 25 min. LTB_5 metabolites were resolved by RP HPLC (methanol/H_2O/acetic acid, 68:32:01, pH 5.0). Using this procedure, 78% of the label eluted with an average retention time of 3.8 min, consistent with that for ω-oxidation products. Separation of 20-OH- and 20-COOH-LTB_5 was achieved by use of SP HPLC, as described below (see Fig. 1), and incorporation of radiolabel.

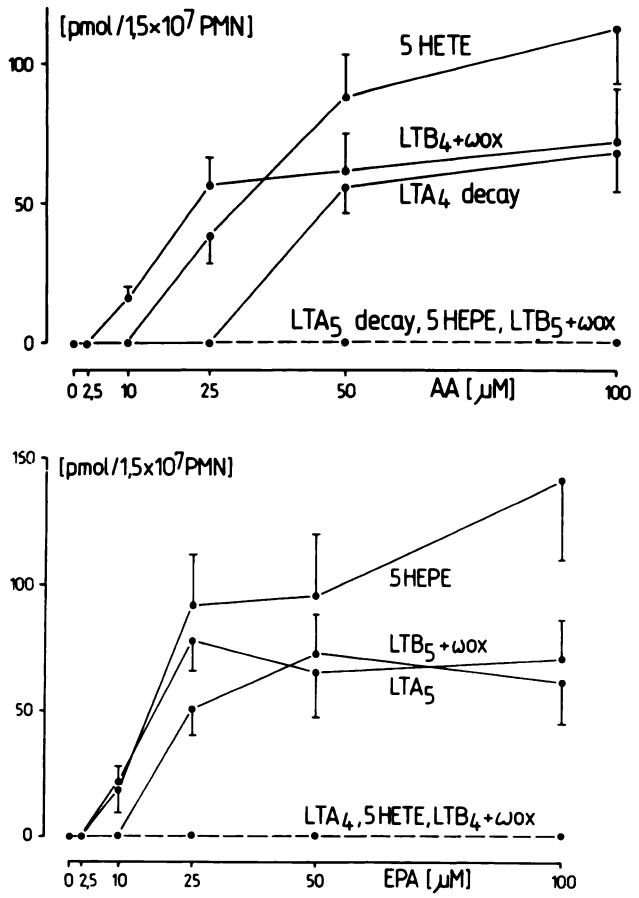

Fig. 2. Dose-dependent generation of 4-series LTs and 5-HETE (*top*) and 5-series LTs and 5-HEPE (*bottom*) in PMN exposed to free AA and free EPA, respectively. PMN (1.5×10^7) were incubated with the respective PUFA for 10 min. $LTB_{4(5)}$ + ωox, $LTB_{4(5)}$, 20-OH-$LTB_{4(5)}$, and 20-COOH-$LTB_{4(5)}$. Nonenzymatic hydrolysis products of $LTA_{4(5)}$ (6-*trans* diastereomeric pair of $LTB_{4(5)}$ and 5,6-diHET(P)Es) are summed as $LTA_{4(5)}$ decay. Means ± standard errors of five independent experiments are given. In the presence of AA, no EPA-derived products were detected, and vice versa.

Identity was further confirmed by UV spectrum analysis. All LTs were checked for purity and quantified spectrophotometrically before use, as described (44). Tritiated LTs used as internal standards, as well as [^{14}C]EPA and [^{3}H]AA, were obtained from NEN (Dreieich, FRG). Anti-LTB_4 antiserum was received from Dr. J. Salmon (Wellcome Research Laboratories, Beckenham, UK). FMLP, PAF, AA, EPA, and palmitic acid were obtained from Paesel AG. Chromatographic supplies included HPLC-grade solvents distilled in glass (Fluka KG, Heidelberg, FRG), octadecylsilyl 5-μm (Hypersil) and silica gel 5-μm column packing (Machery-Nagel, Duren, FRG), and C-18 SepPak cartridges (Waters Associates, Milford, MA). RPMI 1640 medium and fetal calf serum were from Boehringer Mannheim GmbH (Mannheim, FRG), and Percoll was from Pharmacia Fine Chemicals (Uppsala, Sweden). All others biochemicals were obtained from Merck (Munich, FRG).

Preparation of human granulocytes. Heparinized human donor blood was centrifuged in a discontinuous Percoll gradient (27, 45) to yield a PMN fraction of approximately 97% purity. The granulocytes were kept in RPMI 1640 medium with 20% calf serum for 60–90 min. Immediately before experimental use, the cells were washed twice and suspended in Hanks' HEPES buffer. Cell viability, in the absence and presence of stimulus application, was assessed by trypan blue exclusion and lactate dehydrogenase release. Under all experimental conditions, viability ranged above 96% (trypan blue exclusion) and lactate dehydrogenase release was consistently below 3%. In the presence of PUFA concentrations surpassing 50 μM, viability decreased to ≈90%.

Analytical procedures. LTs of the 4- and 5-series, HETEs, and HEPEs were extracted from cell supernatants by octadecylsilyl solid-phase extraction columns, as described (38, 44). Conversion into methyl esters was performed by addition of freshly prepared diazomethane in ice-cold diethyl ether. RP HPLC of nonmethylated compounds was carried out on octadecylsilyl columns (Hypersil, 5-μm particles) with a mobile phase of methanol/water/acetic acid (72:28:0.16, pH 4.9) (44). In addition to the conventional UV detection at 270 nm (LTs) and 237 nm (HETEs and HEPEs), a photodiode-array detector (Waters model 990) was used, which provided full UV spectra (190–600 nm) of eluting compounds and allowed checking for peak purity and subtraction of possible coeluting material. Identity of AA and EPA metabolites was further confirmed by incorporation of radiolabel. For additional verification, samples were collected in 15-sec fractions in selected experiments and subjected to post-HPLC RIA with anti-LTB_4, as described (38, 44). RP HPLC of methylated compounds was performed isocratically (66:34:0.16, pH 4.9) for 5 min, followed by a linear gradient to 90:10:0.16 over 10 min (Gynkothek gradient-former, model 250). SP HPLC of methylated compounds was carried out using a modification of the method of Nadeau *et al.* (46). The mobile phase consisted of hexane/isopropanol/acetate (86:14:0.1), and the column was eluted isocratically at a flow rate of 1.0 ml/min. All data obtained by the different analytical procedures were corrected for the respective recoveries of the overall analytical procedure and are given in pmol/1.5×10^7 PMN throughout the experiments. Recovery was determined by separate recovery experiments using different quantities of the individual compounds in the appropriate concentration range. Factors for recovery were further confirmed by addition of 0.2 [^{3}H]LTB_4 and [^{3}H] 5-HETE to buffer medium, as internal standards, in selected experiments. For quantification of LTs, 5-HETE, and 5-HEPE, correspondence of values calculated from UV absorbance in two different chromatographic procedures was demanded (deviation of <10%). Concerning LTB, quantification was additionally confirmed by the use of post-HPLC RIA.

Results

Ionophore stimulation of PMN in the absence and presence of AA or EPA. Incubation of quiescent PMN with free AA or free EPA resulted in the appearance of moderate amounts of the 4-series and 5-series LTs, respectively, as well as 5-HETE or 5-HEPE (Fig. 2). Plateauing occurred at 25–50 μM concentrations of each PUFA. PMN challenge with the calcium ionophore A23187 evoked a typical profile of lipoxygenase products, corresponding to that repeatedly described (Fig. 3; Table 1) (26, 27, 33, 47, 48). LTB_4 and its ω-oxidation products represented the predominant compounds. Time-dependent 20-hydroxylation and, to a minor extent, further metabolism to 20-COOH-LTB_4 were noted. In addition, substantial amounts of nonenzymatic LTA_4 hydrolysis products and 5-HETE were released in response to the ionophore challenge. 5-HETE level peaked at 5 min, with subsequent rapid decline. This characteristic time course is ascribed to rapid incorporation of the oxygenated AA product into PMN phospholipids (48, 49). LTA_4 decay products plateaued after 5–10 min. No significant amounts of $n-3$-derived products were detected upon ionophore challenge in the absence of exogenous PUFA.

Simultaneous application of A23187 and incremental doses of free AA caused a dose-dependent augmentation of all AA-derived lipoxygenase products, with marked preference for 5-HETE (Fig. 3; Table 1). The generation of LTB_4 and its metabolites and of LTA_4 decay products plateaued at 10–25 μM AA, whereas 5-HETE generation increased slightly further, reaching approximately 6-fold stimulated values at 100 μM AA. Ionophore challenge in parallel with addition of incremental

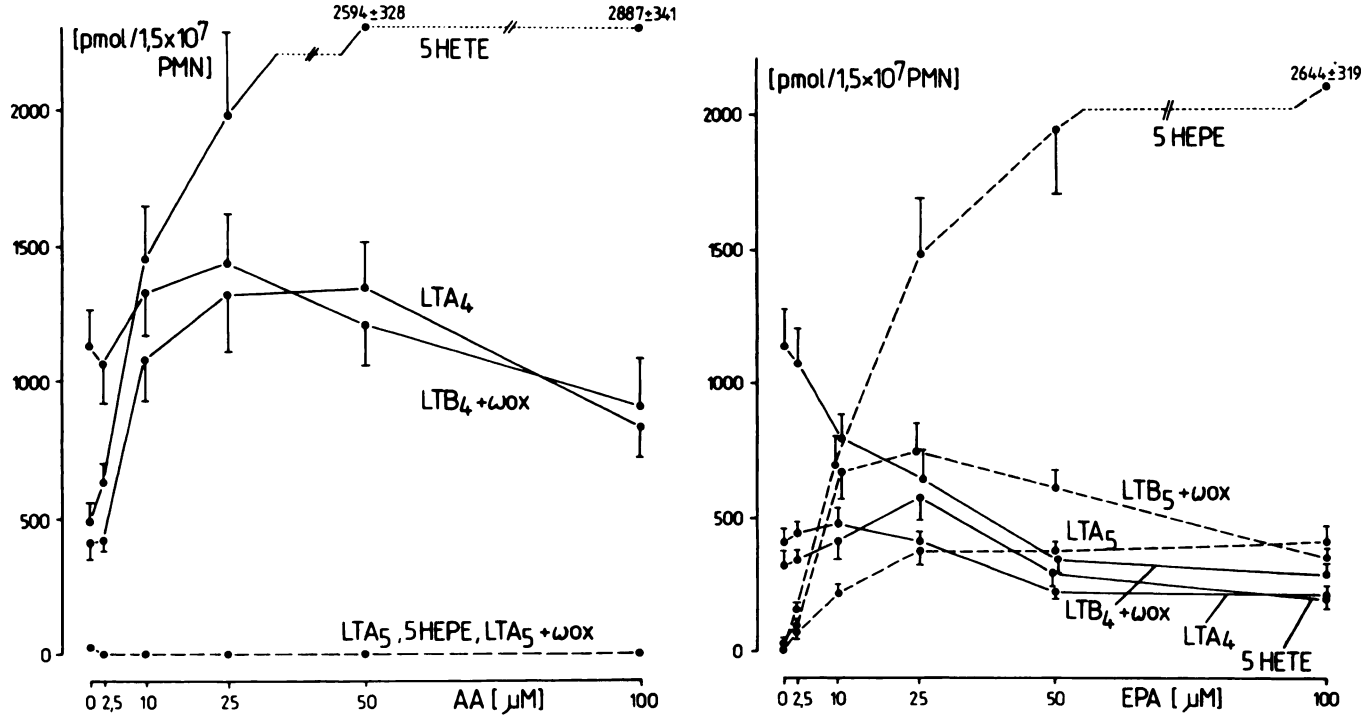

Fig. 3. Dose-dependent generation of 4- and 5-series LTs and 5-HET(P)E in PMN challenged with 1 μM A23187, in the presence of increasing concentrations of AA (*left*) or EPA (*right*). PMN (1.5×10^7) were simultaneously exposed to the ionophore and the respective PUFA, and incubation was terminated after 10 min. *$LTB_{4(5)}$ + ωox*, $LTB_{4(5)}$, 20-OH-$LTB_{4(5)}$, and 20-COOH-$LTB_{4(5)}$. Nonenzymatic hydrolysis products of $LTA_{4(5)}$ (6-*trans* diastereomeric pair of $LTB_{4(5)}$ and 5,6-diHET(P)Es) are summed as $LTA_{4(5)}$ decay. Means ± standard errors of five independent experiments are given.

doses of free EPA provoked a corresponding profile of $n-3$-derived products. In accordance with 5-HETE, levels of 5-HEPE peaked after 5 min, and total amounts approximated those of 5-HETE subsequent to A23187 plus AA stimulation. Substantial amounts of LTB_5 and its ω-oxidation products, as well as nonenzymatic hydrolysis products of LTA_5, were elicited upon simultaneous addition of ionophore and EPA, plateauing at 10–25 μM PUFA. The identities of 20-OH- and 20-COOH-LTB_5, hitherto not characterized in detail, were ascertained by observation of appropriate retention times of methylated and unmethylated compounds in different HPLC procedures, by UV spectrum analysis, and by incorporation of radiolabel (Fig. 1). Time course and extent of ω-oxidation of LTB_5 revealed no major differences from those for LTB_4. The appearance of $n-3$-derived products was paralleled by a 50–75% decrease in 4-series LTs.

Inflammatory ligand-operated stimulation of PMN in the presence of AA or EPA. In the absence of exogenous PUFAs, no $n-3$ product formation was noted in response to FMLP or PAF. AA-derived metabolites ranged at the detection limits of HPLC analysis; small amounts of immunoreactivity were detected by post-HPLC RIA (data not shown). In the presence of AA or EPA, however, marked generation of the respective 5-lipoxygenase products in response to both ligands was noted. First, in order to evaluate optimum doses for FMLP and PAF stimulation, these ligands were added to PMN in increasing concentrations, in parallel with 10 μM AA or EPA (Fig. 4). Maximum generation of AA- or EPA-derived products occurred at 3 μM PAF, and the FMLP response plateaued at 0.1–1 μM. PAF at 3 μM and 1 μM FMLP were thus used in all further studies. Simultaneous application of ligands and incremental doses of free AA evoked dose-dependent generation of 4-series LTs and 5-HETE, with predominance of LTB_4 and its ω-oxidation products (Figs. 5 and 6; Table 2). Plateauing of the response occurred at approximately 25 μM AA upon challenge with both FMLP and PAF. Quantitatively, the PAF-evoked response surpassed that to FMLP by ≈50%. The total amounts of 5-lipoxygenase products, as well as LTB_4 and its metabolites, liberated in the presence of PAF and exogenous AA approximated those in response to A23187. However, in comparison with the ionophore challenge, a more rapid metabolism of 20-OH-LTB_4 to 20-COOH-LTB_4 was noted. The specific radioactivity of all AA-derived products ranged between 35.0 and 52.4 nCi/nmol, which corresponds to that of the exogenously offered AA (50 nCi/nmol). Simultaneous administration of EPA and FMLP or PAF gave rise to the exclusive appearance of the corresponding $n-3$-derived products. Time course of formation and quantities of LTB_5 and its metabolites roughly corresponded to those of LTB_4 and metabolites in the parallel experiments with AA addition. Similarly, accelerated formation of 20-COOH-LTB_5 was noted upon ligand challenge in the presence of EPA. LTA_5 hydrolysis products, and particularly 5-HEPE, were, however, liberated in substantially higher amounts, compared with the $n-6$-derived products in the corresponding experiments (range, 2–4-fold increase). The total amounts of EPA-derived 5-lipoxygenase products in response to PAF challenge were in the same range as those detected upon simultaneous administration of A23187 and EPA. Correspondence of the specific radioactivity of all EPA-derived products to that of the precursor PUFA was again noted. In parallel experiments with FMLP or PAF stimulation of PMN in the presence of 10 μM palmitic acid, no significant effect on mediator release was observed.

TABLE 1

Time course and profile of 4- and 5-series LTs and 5-HET(P)E evoked by calcium ionophore in the presence of AA or EPA

PMN (1.5×10^7) were simultaneously exposed to 1 μM A23187 and 10 μM levels of the respective PUFA. Incubation was terminated after 5, 10, or 15 min. Nonenzymatic hydrolysis products of $LTA_{4(5)}$ (6-*trans* diastereomeric pair of $LTB_{4(5)}$ and 5,6-diHET(P)Es) are summed as $LTA_{4(5)}$ decay. All data are given as pmol/1.5×10^7 PMN (mean ± standard error of five independent experiments). Corresponding experiments were performed in the presence of 2.5, 25, and 50 μM AA. The 10-min values for these experiments are depicted in Fig. 3. Because the kinetics of mediator release in the presence of these different AA concentrations did not differ substantially from those in response to 10 μM AA, the additional data of these experiments are not given.

A23187 (1.0 μM) exposure	$n-6$ Derivatives					$n-3$ Derivatives					
	LTA_4	LTB_4	20-OH-LTB_4	20-COOH-LTB_4	5-HETE	Sum of metabolites	LTA_5	LTB_5	20-OH-LTB_5	20-COOH-LTB_5	5-HETE	Sum of metabolites
min	*pmol/1.5 × 10⁷ PMN*						*pmol/1.5 × 10⁷ PMN*					
AA (10 μM)												
5	919.8 ± 101.3	689.7 ± 69.7	564.6 ± 71.0	61.2 ± 13.3	2232.8 ± 299.7	4468.1 ± 412.7	<30	<10	19.1 ± 4.8	<15	<20	19.1 ± 4.8
10	1084.3 ± 158.4	484.3 ± 59.3	759.3 ± 90.5	94.6 ± 20.4	1445.4 ± 198.4	3867.9 ± 441.7	<30	<10	<10	<15	<20	
15	964.2 ± 114.6	298.6 ± 21.4	1213.4 ± 121.5	98.6 ± 13.8	914.5 ± 111.0	3489.3 ± 269.8	<30	<10	<10	<15	<20	
EPA (10 μM)												
5	429.4 ± 77.3	402.2 ± 60.7	340.7 ± 25.3	21.6 ± 3.1	573.6 ± 83.6	1767.5 ± 191.8	138.6 ± 20.0	301.6 ± 26.8	304.5 ± 24.5	<15	1199.8 ± 135.6	1944.5 ± 158.7
10	468.3 ± 59.8	264.9 ± 30.6	488.6 ± 59.7	46.3 ± 6.0	409.6 ± 59.8	1677.7 ± 145.3	204.5 ± 31.6	211.9 ± 25.9	421.9 ± 57.7	31.4 ± 4.8	689.3 ± 103.6	1559.0 ± 149.4
15	439.7 ± 49.0	203.1 ± 24.3	601.7 ± 79.6	44.2 ± 5.9	175.8 ± 25.4	1464.5 ± 150.9	171.2 ± 24.2	144.7 ± 15.3	534.0 ± 69.7	59.8 ± 8.9	368.7 ± 56.4	1278.4 ± 129.3

Fig. 4. Dose-dependent generation of 4- and 5-series LTs and 5-HET(P)E in PMN challenged with increasing concentrations of PAF and FMLP, in the presence of 10 μM AA or EPA. PMN (1.5×10^7) were simultaneously exposed to the respective ligand and free AA or EPA, and incubation was terminated after 10 min. $LTB_{4(5)}$, 20-OH-$LTB_{4(5)}$, 20-COOH-$LTB_{4(5)}$, nonenzymatic hydrolysis products of $LTA_{4(5)}$, and 5-HET(P)E are summed for each precursor fatty acid. Means ± standard errors of six independent experiments are given.

The marked enhancement of ligand-induced eicosanoid generation by AA or EPA was critically dependent on the temporal relationship between free PUFA addition and ligand application (Fig. 7). Maximum response occurred upon simultaneous administration of both compounds. Delay between PUFA admixture and ligand application resulted in a rapid decrease of eicosanoid generation; a 5-min interval between provision of 10 μM AA or EPA and FMLP or PAF challenge sufficed to reduce the 5-lipoxygenase product formation to <20%.

Simultaneous administration of incremental doses of EPA with AA and ligand resulted in a dose-dependent suppression of 4-series LT and 5-HETE generation. This is depicted in Fig. 8 for cells challenged with FMLP in the presence of 10 μM AA. At equimolar concentrations of both PUFAs, AA-derived product formation was inhibited by ≈60%, and the total amount of 5-series LTs and 5-HEPE surpassed the sum of the corresponding $n-6$-derived products by nearly 3-fold. Corresponding results were obtained in PAF-stimulated PMN (data not shown in detail).

Ionophore- and ligand-operated stimulation of PMN preloaded with AA or EPA. Incubation of quiescent PMN with free AA or EPA resulted in rapid uptake of these PUFAs, plateauing after 10–20 min (Fig. 9). HPLC and gas chromatographic analysis of the nonincorporated material after 60 min suggested that the majority represented decay products of the provided PUFAs (data not shown). In the presence of 10 μM AA or EPA, approximately 5–6 nmol of PUFA were incorporated in 1.5×10^7 PMN after 60 min. This corresponds to a ratio of ≈0.3 in relation to the total neutrophil AA pool (estimated 15–20 nmol/1.5×10^7 PMN) (33, 50). A23187 challenge of PMN preincubated with 10 μM AA or EPA for 60 min resulted in the liberation of 1–2% of the incorporated radioactivity as lipoxygenase products. AA-preloaded PMN displayed typical eicosanoid generation upon ionophore stimulation, which was not different from that of non-AA-enriched cells (data not shown in detail). Similarly, the small amounts of AA-derived products elicited by FMLP or PAF challenge of PMN, detected by post-HPLC immunoreactivity, were not substan-

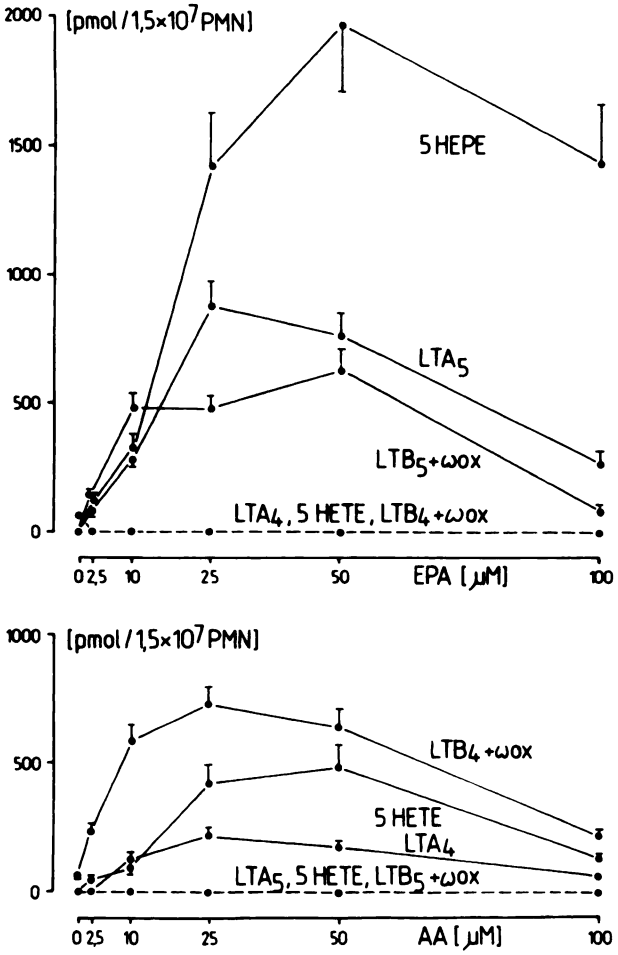

Fig. 5. Dose-related generation of 4- and 5-series LTs and 5-HET(P)E in PMN challenged with 1 μM FMLP, in the presence of increasing concentrations of AA (*bottom*) or EPA (*top*). PMN (1.5×10^7) were simultaneously exposed to the ligand and the respective PUFA, and incubation was terminated after 10 min. *$LTB_{4(5)}$ + ωox*, $LTB_{4(5)}$, 20-OH-$LTB_{4(5)}$, and 20-COOH-$LTB_{4(5)}$. Nonenzymatic hydrolysis products of $LTA_{4(5)}$ (6-*trans* diastereomeric pair of $LTB_{4(5)}$ and 5,6-diHET(P)Es) are summed as $LTA_{4(5)}$ decay. Means ± standard errors of six independent experiments are given.

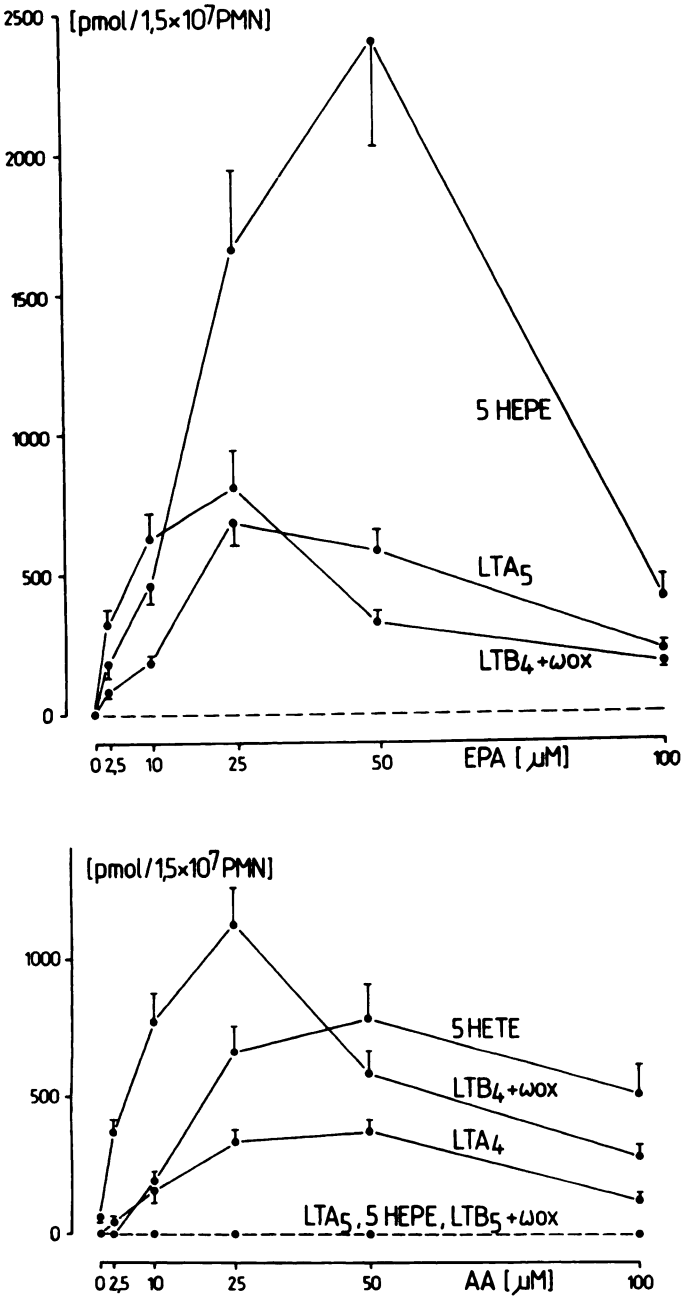

Fig. 6. Dose-related generation of 4- and 5-series LTs and 5-HET(P)E in PMN challenged with 3 μM PAF, in the presence of increasing concentrations of AA (*bottom*) or EPA (*top*). PMN (1.5×10^7) were simultaneously exposed to the ligand and the respective PUFA, and incubation was terminated after 10 min. *$LTB_{4(5)}$ + ωox*, $LTB_{4(5)}$, 20-OH-$LTB_{4(5)}$, and 20-COOH-$LTB_{4(5)}$. Nonenzymatic hydrolysis products of $LTA_{4(5)}$ (6-*trans* diastereomeric pair of $LTB_{4(5)}$ and 5,6-diHET(P)Es) are summed as $LTA_{4(5)}$ decay. Means ± standard errors of six independent experiments are given.

tially influenced by AA preloading. Preincubation of the PMN with 3–25 μM EPA for 60 min caused a moderate reduction in ionophore-evoked AA product formation (maximum suppression, ≈15%; Fig. 10). Small amounts of 5-series LTs and 5-HEPE were liberated under these conditions. The small amounts of immunoreactive AA-derived products elicited by FMLP or PAF challenge of neutrophils were not significantly influenced by preceding EPA enrichment; labeled and nonlabeled EPA-derived products ranged below detection limits in these experiments.

Discussion

Incubation of quiescent human PMN with free AA or EPA resulted in rapid uptake of these PUFAs, with superimposable kinetics. This finding corresponds to previous investigations in PMN of different origin (51–55). Incorporation of exogenously offered PUFAs into the diacyl species of phosphatidylcholine has been noted in these cells, followed by phospholipid remodeling and rapid transfer into different molecular species of phospholipids. Among those, phosphatidylethanolamine with plasmalogen and PAF precursor, and phosphatidylinositol appear to be predominant (50, 56–59). Such mechanisms of phospholipid compartmentalization, as well as processes of subcellular (membrane) compartmentalization after PUFA incorporation (60), are considered responsible for a rapid decline in PUFA availability for release upon agonist stimulation. In accordance with this notion, only a small percentage of incorporated AA or EPA (each ≈1–2%) was released as labeled

TABLE 2

Time course and profile of 4- and 5-series LTs and 5-HET(P)E evoked by FMLP or PAF in the presence of AA or EPA

PMN (1.5×10^7) were simultaneously exposed to 1 μM FMLP and 10 μM AA or EPA or to 3 μM PAF and 10 μM levels of the respective PUFA. Incubation was terminated after 5, 10, or 15 min. Nonenzymatic hydrolysis products of $LTA_{4(5)}$ (6-*trans* diastereomeric pair of $LTB_{4(5)}$ and 5,6-diHET(P)Es) are summed as $LTA_{4(5)}$ decay. All data are given as pmol/1.5×10^7 PMN (mean ± standard error of five independent experiments). Corresponding experiments were performed in the presence of 2.5, 25, and 50 μM AA. The 10-min values for these experiments are given in Figs. 5 and 6. Because the kinetics of mediator release in the presence of these different AA concentrations did not differ substantially from those in response to 10 μM AA, the additional data of these experiments are not given.

Stimulus	Time	Metabolites[a]					
		LTA_4	LTB_4	20-OH-LTB_4	20-COOH-LTB_4	5-HETE	Sum
	min	*pmol/1.5 × 10⁷ PMN*					
FMLP + AA	5	86.7 ± 6.9	325.6 ± 35.8	288.7 ± 36.4	19.1 ± 8.0	144.3 ± 21.4	864.4 ± 82.5
	10	109.5 ± 20.6	211.4 ± 14.3	301.9 ± 39.8	79.8 ± 11.4	84.4 ± 14.1	787.0 ± 80.2
	15	79.6 ± 16.9	155.4 ± 19.7	441.2 ± 58.4	89.7 ± 14.4	39.8 ± 5.6	805.7 ± 89.7
PAF + AA	5	131.4 ± 30.1	366.0 ± 39.1	341.2 ± 35.8	39.6 ± 7.0	256.0 ± 48.5	1134.2 ± 119.6
	10	152.4 ± 18.3	288.4 ± 34.6	404.3 ± 51.9	82.9 ± 10.4	188.5 ± 30.4	1116.5 ± 124.2
	15	139.4 ± 25.6	165.4 ± 21.4	608.3 ± 78.7	80.8 ± 14.3	54.0 ± 10.1	1047.9 ± 110.6
Stimulus	Time	Metabolites[b]					
		LTA_5	LTB_5	20-OH-LTB_5	20-COOH-LTB_5	5-HETE	Sum
	min	*pmol/1.5 × 10⁷ PMN*					
FMLP + EPA	5	271.4 ± 33.2	224.5 ± 28.3	211.3 ± 24.6	18.9 ± 4.2	895.6 ± 95.0	1621.7 ± 148.5
	10	284.8 ± 24.3	142.9 ± 24.4	288.6 ± 36.4	44.3 ± 6.9	301.9 ± 49.1	1062.5 ± 99.7
	15	268.1 ± 44.1	96.7 ± 13.4	361.7 ± 46.2	61.7 ± 14.4	188.6 ± 35.8	976.8 ± 119.7
PAF + EPA	5	235.9 ± 36.3	314.6 ± 39.8	260.2 ± 30.1	29.8 ± 4.9	1114.4 ± 161.4	1954.9 ± 188.2
	10	188.2 ± 24.1	202.4 ± 30.6	344.5 ± 45.8	79.4 ± 10.9	469.6 ± 64.7	1284.1 ± 139.6
	15	220.7 ± 24.8	129.4 ± 16.7	452.4 ± 50.5	91.0 ± 15.8	288.2 ± 40.2	1181.7 ± 121.0

[a] *n* − 3 derivatives not detected.
[b] *n* − 6 derivatives not detected.

lipoxygenase products upon ionophore challenge 60 min after PUFA preloading. In comparison, the total amount of nonlabeled AA products evoked under these conditions corresponded to ≈7.5% of the estimated total PMN AA pool. These data suggest that both exogenous AA and EPA were transferred, within 60 min, to phospholipid pools with reduced availability for release upon ionophore challenge. Not surprisingly, the AA preloading, causing an increase in total PMN AA content of, at most, 30%, did not substantially influence the time course, profile, or quantity of ionophore-evoked LT and HETE generation. Correspondingly, the small amounts of eicosanoids elicited in response to FMLP or PAF challenge were not significantly altered in AA-enriched cells. Sixty minutes of preloading with EPA, resulting in an uptake of ≈5 nmol/1.5×10^7 PMN, which corresponds to ≈30% of the total PMN AA pool, caused a moderate (10–15%) reduction of A23187-evoked generation of 4-series LTs and 5-HETE. This effect was accompanied by the appearance of small amounts of EPA-derived lipoxygenase products, amounting to ≈120 pmol/1.5×10^7 PMN. These data can be related to findings in PMN isolated from humans receiving a fish oil-containing diet for several weeks. Approximately 7.5–10 nmol of EPA were detected in 1.5×10^7 PMN under these conditions, A23187-evoked LTB_4 generation was found to be reduced by 25–40% (10, 11, 13–15) or to be not reduced (61), and minor amounts of 5-series LTs and 5-HEPE were elicited by the ionophore (range, 70–450 pmol of EPA products/1.5×10^7 PMN) (10, 11, 33, 61).

In contrast to PUFA preloading, simultaneous administration of exogenous AA or EPA and A23187 exerted marked influences on the ionophore-evoked lipoxygenase metabolism. The presence of incremental doses of free AA caused an augmentation of LTB_4 (including metabolites), LTA_4 hydrolysis product, and 5-HETE generation, to a maximum of 1.5-, 3-, and 6-fold greater values, respectively. The presence of free EPA in the range between 2.5 and 100 μM induced a dose-dependent suppression of these AA metabolites, by maximally 75% (LTB_4 and products), 50% (LTA_4 hydrolysis products), and 40% (5-HETE). This inhibitory effect on AA metabolism coincided with the generation of large quantities of the corresponding *n* − 3-derived products, with predominance of 5-HEPE, amounting to a total of ≈3400 pmol of EPA metabolites/1.5×10^7 cells at the highest PUFA concentration used. These findings correspond to previous investigations using free EPA, in the range between 5 and 130 μM, to reduce ionophore-evoked PMN AA metabolism, coincident with the generation of EPA-derived products (33, 51, 62). Collectively, these studies and the present one demonstrate a dose-dependent shift from 4-series LT formation to 5-series LT generation in ionophore-challenged PMN, upon addition of free EPA.

The impact of exogenous free PUFAs on eicosanoid generation in activated PMN was particularly evident for the naturally occurring ligands FMLP and PAF. Conflicting data concerning the ability of such ligands to elicite PMN AA metabolism have accumulated. In the majority of studies, FMLP was found to evoke no substantial eicosanoid generation, corresponding to the currently noted virtual absence of LT and 5-HETE release in PMN challenged with FMLP in the absence of exogenous PUFAs (29, 31–33). Simultaneous application of AA or EPA with ligand challenge, however, induced marked dose-dependent lipoxygenase metabolism, with formation of the respective *n* − 6- and *n* − 3-derived products. Total amounts of eicosanoids evoked under these conditions approached (4-series LTs and 5-HETE) or even surpassed those (5-series LTs and 5-HEPE) of ionophore-challenged PMN. Two thirds of the maximum response was evoked by only 10 μM (AA) or 10–25 μM (EPA) as the exogenously provided PUFA. Conformity of the specific radioactivity of the different products with that of administered AA or EPA suggests that the exogenous PUFAs were not involved in preceding acylation-deacylation cycles but served as direct substrates for the 5-lipoxygenase under these

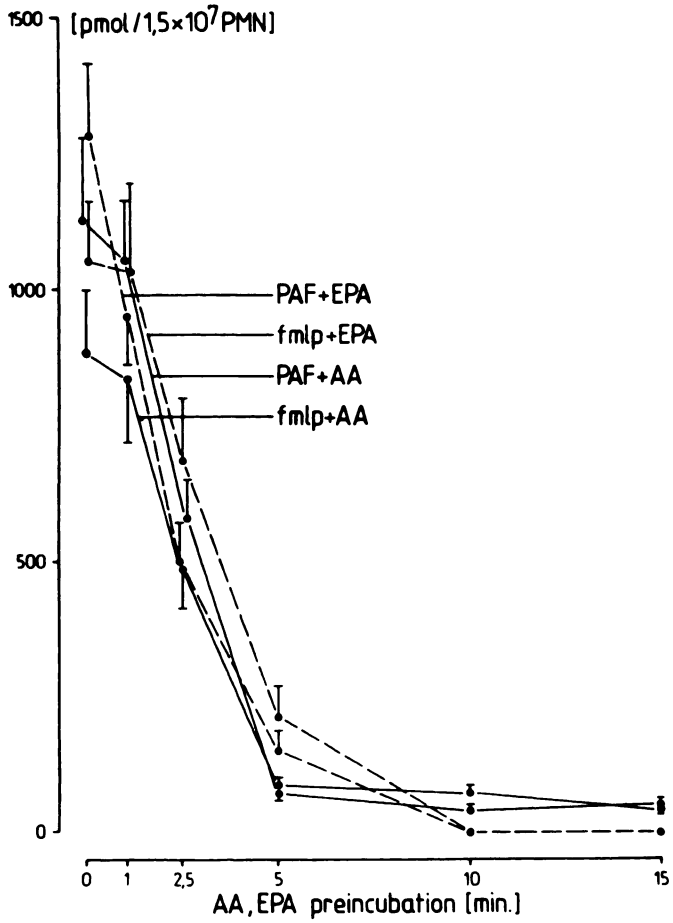

Fig. 7. Influence of time delay between PUFA administration and ligand challenge on the generation of 4- and 5-series LTs and 5-HET(P)E. AA or EPA (10 μM) was admixed with 1.5 × 10^7 PMN. After various time periods, cells were challenged with 1 μM FMLP or 3 μM PAF, and incubation was terminated after 10 min. $LTB_{4(5)}$, 20-OH-$LTB_{4(5)}$, 20-COOH-$LTB_{4(5)}$, nonenzymatic hydrolysis products of $LTA_{4(5)}$, and 5-HET(P)E are summed for each precursor fatty acid. Means ± standard errors of five independent experiments are given.

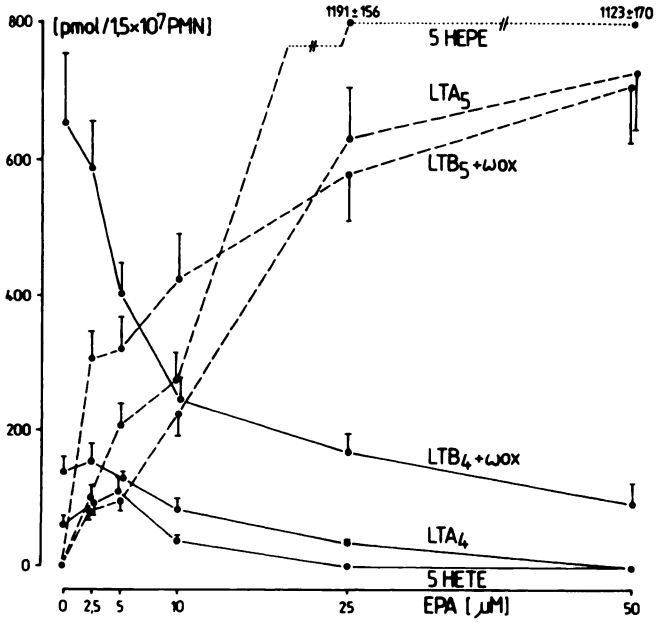

Fig. 8. EPA-dependent suppression of AA product formation in PMN challenged with 1 μM FMLP in the presence of 10 μM AA, coincident with 5-series LT and 5-HEPE generation. PMN (1.5 × 10^7) were simultaneously exposed to the ligand, 10 μM AA, and incremental doses of EPA, and incubation was terminated after 10 min. *$LTB_{4(5)}$ + ωox*, $LTB_{4(5)}$, 20-OH-$LTB_{4(5)}$, and 20-COOH-$LTB_{4(5)}$. Nonenzymatic hydrolysis products of $LTA_{4(5)}$ (6-*trans* diastereomeric pair of $LTB_{4(5)}$ and 5.6-diHET(P)Es) are summed as $LTA_{4(5)}$ decay. Means ± standard errors of five independent experiments are given.

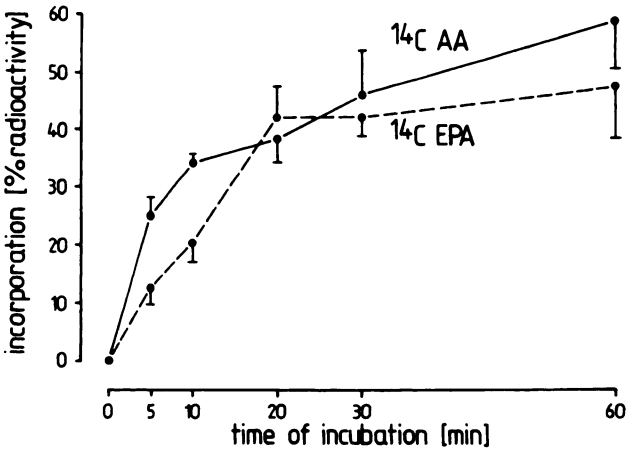

Fig. 9. Time-dependent incorporation of AA (labeled to 100 nCi/nmol) or EPA (labeled to 100 nCi/nmol) in quiescent PMN. PMN (1.5 × 10^7) were exposed to 10 μM AA or EPA, and uptake of radioactivity was determined at various times up to 1 hr. Means ± standard errors of four independent experiments are given.

conditions. These data support the notion that FMLP challenge causes activation of this enzyme without stimulating phospholipolytic pathways, thus failing to elicit substantial eicosanoid generation (1, 2, 33). Exogenous free PUFAs, present at the time of ligand application, compensate for the restricted availability of endogenous precursor PUFAs and are rapidly metabolized via the 5-lipoxygenase pathway. Critical timing of this "dual stimulation" was noted, and lipoxygenase product formation rapidly declined with increasing interval between PUFA admixture and FMLP application. This finding is most probably explained by fast reduction in fatty acid availability for the activated 5-lipoxygenase pathway, due to ongoing PUFA transfer into phospholipid pools not mobilized by FMLP challenge. Notably, only 5 min of delay between PUFA and FMLP application sufficed for reduction in lipoxygenase product formation by >80%. This finding may explain the large variation in eicosanoid formation between different studies with FMLP application 5–15 min after a preceding AA administration (31, 33, 63, 64). The necessity of exogenous PUFA supply for induction of ligand-operated eicosanoid production is corroborated by the corresponding behavior of PAF, similarly investigated in the absence or presence of AA and EPA. As previously described (65), only very small amounts of 5-lipoxygenase products were elicited by PAF challenge alone, whereas simultaneous addition of exogenous PUFAs evoked LTs and 5-HET(P)E in amounts corresponding to those evoked by A23187 stimulation in the presence of exogenous free fatty acids.

Compared with AA, EPA was noted to be the preferred substrate for 5-lipoxygenase metabolism in ligand-activated PMN. In the complete PUFA dose range used, total amounts of EPA-derived products surpassed those of AA metabolites at corresponding concentrations, upon either FMLP or PAF stimulation. Increasing concentrations of EPA dose-dependently

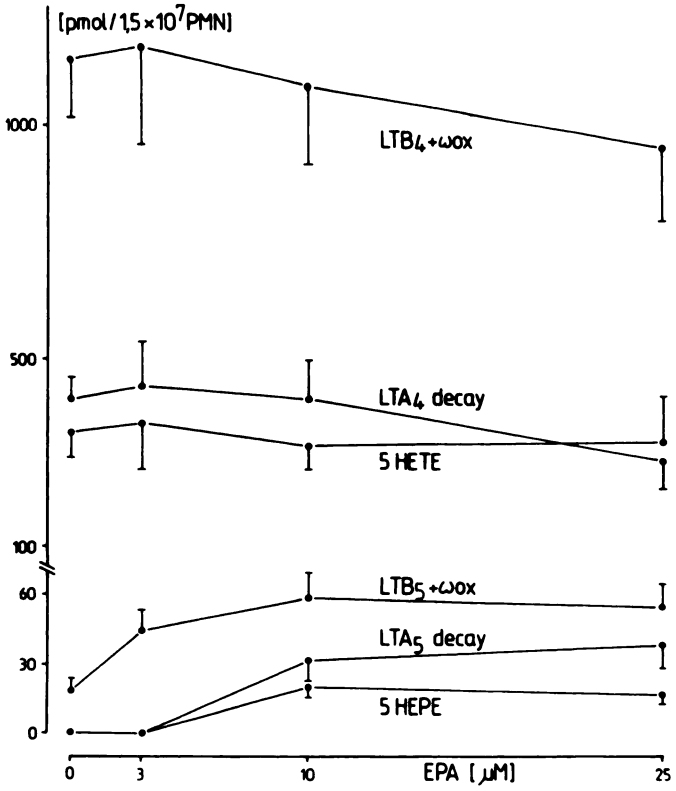

Fig. 10. Influence of short term enrichment of PMN with EPA (labeled to 100 nCi/nmol) on ionophore-evoked eicosanoid generation. PMN (1.5×10^7) were preincubated with 2.5, 10, or 25 μM EPA for 60 min. After cell washing, stimulation with 1 μM A23187 was performed, and incubation was terminated after 10 min. *LTB₄ + ωox*, LTB_4, 20-OH-LTB_4, and 20-COOH-LTB_4. Nonenzymatic hydrolysis products of LTA_4 (6-*trans* diastereomeric pair of LTB_4 and 5,6-diHETEs) are summed as LTA_4 decay. Means ± standard errors of five independent experiments are given.

suppressed AA product formation upon parallel addition of both PUFAs, and equivalent total amounts of $n-3$- and $n-6$-derived products were noted at an EPA/AA ratio of approximately 0.5. These findings are in accordance with the described slightly higher affinity of isolated (purified) PMN 5-lipoxygenase for EPA, compared with AA (66). In contrast, LTA_5 is known to be more slowly converted by the PMN LTA hydrolase than is LTA_4 (54, 67). This characteristic probably underlies the present finding that EPA exposure particularly increased 5-HEPE and LTA_5 hydrolysis product formation, in relation to LTB_5. In this study, the latter was noted to be ω-oxidized to 20-OH- and 20-COOH-LTB_4, with kinetics corresponding to those of the oxidative conversion of LTB_4. Interestingly, the formation of the carboxy product was found to be enhanced in the case of both LTB_4 and LTB_5, in ligand-activated PMN.

In conclusion, the "LTB_4 paradox" that "neutrophils can, but will not, respond to ligand-receptor interactions by forming LTB_4 or its ω-metabolites" (31) is explained by the finding of a critical dependency of 5-lipoxygenase product formation on the simultaneous addition of exogenous free PUFAs. Concentrations of 2.5–10 μM AA or EPA, which may arise at inflammatory foci or under conditions of cell-cell cooperativity, were found to suffice for marked generation of eicosanoids in ligand-activated PMN. Notably, exclusive formation of $n-6$- or $n-3$-derived products, respectively, occurred under these conditions. Administration of free EPA, which competes with AA as preferred substrate, may offer an approach to interfere with PMN-related eicosanoid metabolism under inflammatory circumstances.

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