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Metabolism of leukotriene B_4 by activated human polymorphonuclear granulocytes

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SUMMARY

Human polymorphonuclear granulocytes (PMNs) synthesize leukotriene B_4 (LTB_4) as a response of cell activation. Inactivation of the potent inflammatory mediator proceeds via ω-oxidation, resulting in the formation of 20-hydroxy- and 20-carboxy-LTB_4. The main metabolite after stimulation with the chemotactic peptide *N*-formyl-methionyl-leucyl-phenylalanine (FMLP) is 20-carboxy-LTB_4, and after stimulation with the calcium ionophore A23187 is 20-hydroxy-LTB_4. Differences in the LTB_4 inactivation pathway were also observed when the catabolism of exogenously added LTB_4 was analysed. In contrast to resting cells or cells preactivated with FMLP, prestimulation with the ionophore or with phorbol esters resulted in the inhibition of 20-carboxy-LTB_4-generation. This decrease correlated with the reduction in specific [^{3}H] LTB_4-receptor expression. Studies with the non-penetrating diazonium salt of sulphanilic acid, which is known to interact with ectoenzymes, revealed that LTB_4 is metabolized via receptor-mediated uptake. Our data suggest that the reduction in the amount of LTB_4-receptor sites inhibits the conversion of 20-OH-LTB_4 into 20-COOH-LTB_4.

INTRODUCTION

Human polymorphonuclear granulocytes (PMNs) provide a major defence system against bacterial infection. The transformation of external signals into cellular responses, such as degranulation, activation of the respiratory burst or the generation of arachidonic acid-derived inflammatory mediators (McPhail & Snyderman, 1983; Borgeat & Samuelsson, 1979), is controlled by a complex array of metabolic events. Signal transduction from the cell surface into the interior is initiated by specific ligand–receptor interactions. Mediated by guanine nucleotide regulatory proteins (Cockcroft & Gomperts, 1985), a phosphodiesterase is activated to hydrolyse phosphatidyl-inositol-4, 5-bisphosphate, resulting in the formation of inositol-1,4,5-trisphosphate (IP_3) and 1,2-diacylglycerol (reviewed by Berridge, 1984; Sekar & Hokin, 1986). IP_3 raises the cell calcium concentration by mobilization from internal stores (Prentki, Wollheim & Lew, 1984), especially the endoplasmic reticulum. The burst in the level of free calcium synergizes with transmembraneous 1,2-diacylglycerol to activate a phospholipid-dependent protein kinase *C* (Nishizuka, 1984), which catalyses the phosphorylation of various proteins (Andrews & Babior, 1983). Activation of protein kinase *C* is accompanied by translocation from the cytosol to plasma membranes (Horn & Karnovsky, 1986; O'Flaherty & Nishihira, 1987).

Various stimuli are useful tools in manipulating this transduction system. *N*-formyl-methionyl-leucyl-phenylalanine (FMLP), a synthetic analogue of bacterial peptides, transmits its effects using this pathway. Phorbol 12-myristate 13-acetate (PMA) bypasses the cascade by direct interaction with protein kinase *C* substituting for 1,2-diacylglycerol (Castagna *et al.*, 1982); PMA does not elevate the internal calcium level (Schell-Frederick, 1984). The calcium ionophore A23187 enhances the cytosolic calcium concentration by transporting calcium from the extracellular media into the interior and by the release from internal storages (Schell-Frederick, 1984).

FMLP and, in particular, the ionophore, but not PMA, stimulate the generation of leukotrienes via the 5-lipoxygenase. Leukotrienes are potent mediators induced during inflammatory events, allergic reactions, ischemia and shock (Bray, 1986; Lefer, 1986). Among them leukotriene B_4 (LTB_4, 5S,12R-dihydroxy-Z,E,E,Z-6,8,10,14-eicosatetraenoic acid) leads to the chemotaxis of neutrophils and eosinophils, lysosomal enzyme release and enhances the vascular permeability (Bray, 1983; König, Kunau & Borgeat, 1982). Inactivation of leukotrienes by

Abbreviations: BSA, bovine serum albumin; FMLP, *N*-formyl-methionyl-leucyl-phenylalanine; HPLC, high-performance liquid chromatography; IP_3, inositol-1,4,5-trisphosphate; LDH, lactate dehydrogenase; LTB_4, leukotriene B_4 (5S, 12R-dihydroxy-Z,E,E,Z-6,8,10,14-eicosatetraenoic acid); NADH, β-nicotinamide adenine dinucleotide; NADPH, β-nicotinamide adenine dinucleotide phosphate; PBS, phosphate-buffered saline; PMA, phorbol 12-myristate 13-acetate; PMNs, polymorphonuclear granulocytes; 5S, 12S-DiHETE, 5S,12S-dihydroxy-E,Z,E,Z-6,8,10,14-eicosatetraenoic acid.

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neutrophils seems to be a general principle for the regulation of the mediator level (Bremm *et al.*, 1985; Raulf, Stüning & König, 1985). Human PMNs metabolize LTB_4 by specific hydroxylation at position C_{20}. The ω-oxidation products were identified as 20-hydroxy- and 20-carboxy-LTB_4 (Lindgren, Hansson & Samuelsson, 1981), which reveal diminished biological activity compared with LTB_4 (Hansson *et al.*, 1981). The localization and characterization of the LTB_4-ω-hydroxylase favoured a membrane-associated cytochrome P-450 complex (Shak & Goldstein, 1985; Brom *et al.*, 1987). The significance of these mono-oxygenases for the inactivation of lipoxygenase products was demonstrated recently (Marcus *et al.*, 1987; Örning, 1987). ω-hydroxylation of 12-HETE by human neutrophils and the ω-hydroxylation of *N*-acetyl-leukotriene E_4 by rat liver microsomes, respectively, were reported. The second step during LTB_4-inactivation, the conversion of 20-OH-LTB_4 to 20-COOH-LTB_4, is carried out by the 20-OH-LTB_4-dehydrogenase (Sumimoto, Takeshige & Minakami, 1985). *In vivo* degradation of LTB_4 seems to be the result of β-oxidation, which has been observed in the monkey (Serafin, Oates & Hubbard, 1984) and in isolated hepatocytes (Harper, Garrity & Murphy, 1986). An alternative conversion pathway of LTB_4 starts with an isomerase reaction resulting in the formation of 6-trans-LTB_4 (Breuer & Hammarström, 1987), or with the direct reduction to 5,12-dihydroxy-eicosatrienoic acid followed by ω-hydroxylation (Powell, 1986; Kaever *et al.*, 1987). In human PMNs this pathway seems to play a minor role for LTB_4 inactivation.

It was the purpose of the present investigation to study the interaction between cell activation as well as the release and conversion of LTB_4 and its ω-oxidized metabolites.

MATERIALS AND METHODS

Materials

20-COOH-LTB_4, 20-OH-LTB_4 and LTB_4 were generously provided by Dr Rokach (Merck Frosst, Pointe Claire-Dorval, Canada). [14,15-^{3}H]LTB_4, specific activity 1·11–2·22 TBq/mmol, and [14,15-^{3}H] 20-hydroxy-LTB_4, specific activity 1924 GBq/mmol, were supplied by New England Nuclear, Dreieich. Ficoll 400 was from Pharmacia, Uppsala, Sweden; Macrodex (6%) was from Schiwa, Glandorf; metrizoate (75% w/v) was from Nycomed, Oslo, Norway. Phorbol 12-myristate 13-acetate (PMA), phorbol 12,13-dibutyrate, mezerein, *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine (FMLP), calcium ionophore A23187, propyl gallate, NADH, NAD^+, NADPH, leupeptin, dithiothreitol, thermolysin (protease from *Bacillus thermoproteolyticus*), sulphanilic acid, pyruvate and heparin were purchased from Sigma, Deisenhofen. Bovine serum albumin (BSA) was from Boehringer, Mannheim. Rotiszint was obtained from Roth, Karlsruhe. Organic solvents were from J. T. Baker Chemicals, Deventer, The Netherlands. All other chemicals were from Merck, Darmstadt.

Preparation of cells

Human polymorphonuclear granulocytes (PMNs) were isolated from heparinized (15 U/ml) peripheral blood of healthy donors by the method of Böyum (1968). The leucocytes were separated on a Ficoll–Metrizoate gradient followed by 1 *g* dextran sedimentation. Platelets were removed by repeated centrifugation of the cell suspension at 300 *g*; the amount of platelets did not exceed 2% of total cells within the purified PMN fraction. Contaminating erythrocytes were removed using 0·85% ammonium chloride in 0·015 M Tris buffer (pH 7·4). More than 95% of all cells were identified as PMNs. All experiments, with the exception of studies using cell sonicates, were carried out in Dulbecco's phosphate-buffered saline (PBS) without calcium and magnesium; the pH was adjusted to 7·4.

Cell disruption

PMNs (1×10^8 cells/ml) were suspended in 0·34 M sucrose-0·05 M Tris buffer (pH 7·5). Cells were disrupted by sonication at 30 W performed in six periods over 10 seconds (sonifier W250, Branson Ultrasonics Co., Danbury, CT). The complete cell breakage was assessed by light microscopy. In order to avoid proteolytic inactivation, EDTA (1 mM), dithiothreitol (1 mM) and leupeptin (100 μg/ml) were added.

Incubation conditions

Leukotriene synthesis was determined by incubation of prewarmed PMNs (1×10^7 cells/0·5 ml) with PMA (concentration range: 0·46–230 nM), calcium ionophore A23187 (0·68–27·2 μM) or FMLP (0·4–160 μM) in the presence of calcium (0·9 mM) and magnesium (0·5 mM). In the case of leukotriene conversion, cells were prestimulated as described above or preincubated with thermolysin (concentration 100 μg/ml, for 60 min; described by Paterson, 1984) or the diazonium salt of sulphanilic acid (4·8 mM, for 10 min; described by DePierre & Karnovsky, 1974; the preparation of the diazonium salt is described below), washed twice with PBS followed by the addition of LTB_4 (300 pmol) or 20-hydroxy-LTB_4 (142 pmol), respectively. Experiments using [^{3}H] LTB_4 or [^{3}H] 20-OH-LTB_4 as substrate were carried out using a concentration of 5·6 pmol or 2 pmol, respectively. The incubation time was 15 min using LTB_4 and 45 min using 20-hydroxy-LTB_4 as substrate. In order to determine the LTB_4-20-hydroxylase activity within the cell-free system, the sonicate (0·5 mg) was preincubated with NADPH (1 mM) for 5 min followed by the addition of [^{3}H]LTB_4 (2·8 pmol) and LTB_4 (30 pmol) for 60 min. The 20-OH-LTB_4-dehydrogenase was measured incubating the sonicate (1 mg) with NAD^+ (1 mM) for 5 min, shifting the pH value from 7·5 to 9·0 by the addition of NaOH, and further incubation with [^{3}H] 20-OH-LTB_4 (1 pmol) and 20-OH-LTB_4 (28 pmol) for 60 min. All incubations were performed at 37° with gentle but constant agitation. The reactions were terminated by short-time centrifugation at 9600 *g* (Eppendorf centrifuge 3200) followed by the addition of three volumes of methanol/acetonitrile (50:50, v/v). The resulting supernatants were stored under argon at −20°.

Preparation of the diazonium salt of sulphanilic acid

The salt was synthesized as described by DePierre & Karnovsky (1974). Twenty-three milligrams of $NaNO_2$ and 39 mg of sulphanilic acid were dissolved in 1 ml of water. After the addition of 50 μl of concentrated HCl, the tube sides were scratched and the diazonium salt precipitated out. The suspension was placed on ice for 15 min followed by centrifugation at 1000 *g* for 5 min. The pellet was resuspended in 4 ml of PBS buffer.

Extraction and analysis of leukotrienes

The supernatants were centrifuged at 3000 *g* for 15 min (cryofuge 6–4, Heraeus Christ, Osterode) to remove contaminating proteins. The samples were then evaporated to dryness by lyophilization (Modulyo, Edwards-Kniese, Marburg). After dissolving in 600 μl of methanol/water (30:70, v/v), centrifuga-

tion was performed at 9600 *g* for 4 min (Eppendorf centrifuge 3200) for further purification. Aliquots of 200 µl were subjected to HPLC analysis. The HPLC equipment consisted of a Constametric III pump and a Spectromonitor D detector (Laboratory Data Control/Milton Roy, Hasselroth). Automatic sample injections were carried out using a WISP 710B (Waters, Eschborn). Peak integration was carried out using the chromatography software 2600 system by Nelson Analytical (Cupertino, CA). The column (4·6 × 250 mm) was packed with Nucleosil C_{18} (pore size 5 µM; Macherey–Nagel, Düren). Isocratic elution was carried out using a solvent system consisting of methanol/water/acetonitrile/phosphoric acid (59:33:8:0·03) including 0·016% EDTA. The pH was adjusted to the value of pH 5·1. The flow rate was maintained at 1 ml/min. Detection was carried out at 270 nm. This technique successfully separates 20-COOH-LTB_4 (retention time 6·0 ± 0·1 min; mean ± SEM, $n=7$), 20-OH-LTB_4 (6·6 ± 0·1 min) and LTB_4 (27·1 ± 0·4 min); the respective overall recoveries were 81·1 ± 4·9%, 84·0 ± 4·3% and 86·2 ± 4·0% (mean ± SEM, $n=5$). The LTB_4 peak was contaminated with 5S, 12S-dihydroxy-E,Z,E,Z-6,8,10,14-eicosatetraenoic acid (5S, 12S-DiHETE), the double oxygenation product of 5-lipoxygenase and 12-lipoxygenase (localized within platelets). The amount of 5S, 12S-DiHETE did not exceed more than 20% of the total peak area. Separation of [^{3}H] LTB_4 and its ω-oxidized metabolites were carried out by HPLC using ternary mixtures of methanol, acetonitrile and water, as described previously (Brom *et al.*, 1987). Radioactivity was detected with a continuous-flow monitor (Ramona, Isotopenmeßgeräte GmbH, Straubenhardt). The flow cell was packed with calcium fluoride; the total volume was 400 µl.

Experiments using cell sonicates were screened by thin-layer chromatography. Acidified supernatants were extracted with 5 ml ethyl acetate. The extracts were dried under a stream of nitrogen, reconstituted in 50 µl chloroform/methanol (4:1, v/v) and spotted on silica plates (Kieselgel 60, 250 µm, Merck, Darmstadt). The plates were developed with diethylether/acetic acid/water (100:1:0·5, v/v) as described by O'Flaherty, Kosfeld & Nishihira (1986). The radioactivity was detected with a linear analyser (Raytest, Straubenhardt).

LTB_4-binding assay

LTB_4-binding studies were carried out as has been described by O'Flaherty *et al.* (1986). Samples were prepared as described above. PMNs were incubated with the indicated stimuli for various times at 37°. After the incubation period the cells were washed twice and adjusted to 2×10^7 PMNs/ml. The assays were performed using 96-well filtration plates with 5 µm pore size-polyvinylidene fluoride membranes (Millipore, Eschborn). Each well contained 2·3 nM [^{3}H]LTB_4 (0·9 KBq) and 37·5 µg BSA. The incubation was started by the addition of 4×10^6 stimulated or not stimulated PMNs/200 µl PBS; the total volume was 300 µl. After 45 min at 4°, the reaction was terminated by rapid filtration using a Millititer vacuum holder. The filters were transferred into scintillation vials, 0·5 ml methanol and 9 ml Rotiszint (Roth, Karlsruhe) were added and the radioactivity was measured by liquid scintillation counting (Rack beta 1209, LKB, Turku, Finland). All experiments were carried out in triplicate.

Specific binding was expressed as total binding minus non-specific binding; non-specific binding was determined in the

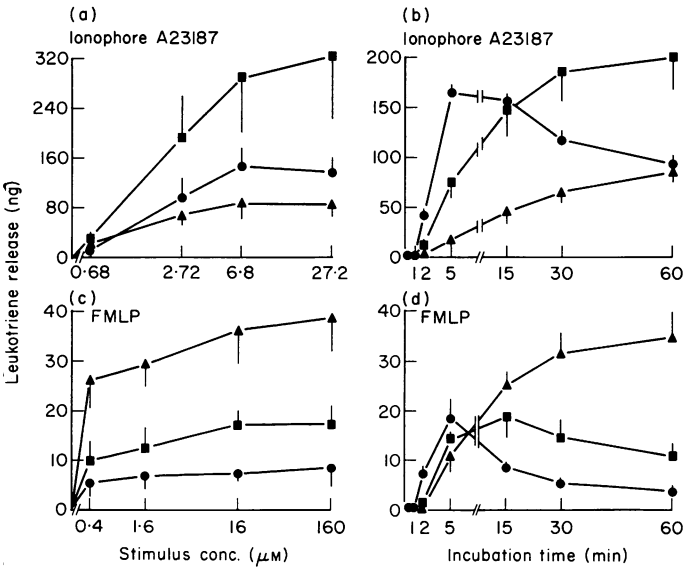

Figure 1. Release of 20-COOH-LTB_4, 20-OH-LTB_4 and LTB_4. PMNs (1×10^7 cells/0·5 ml) were incubated with the calcium ionophore (a,b) or with FMLP (c,d) in the presence of calcium (0·9 mM) and magnesium (0·5 mM) at 37°. In (a) and (c) the release of leukotrienes as a function of increasing stimulus concentrations (using a defined incubation time of 15 min) is shown; (b) and (d) reveal the time–courses (using 6·8 µM of the ionophore or 16 µM FMLP). All samples were analysed by HPLC as described in the Materials and Methods. All values are ng of leukotriene released into the supernatant. (▲), 20-COOH-LTB_4; (■), 20-OH-LTB_4; (●), LTB_4. The results represent mean values calculated from four experiments ± SEM.

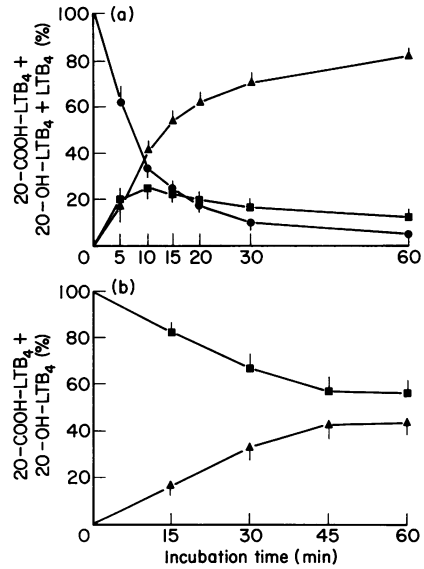

Figure 2. Catabolism of exogenously added LTB_4 and 20-OH-LTB_4 by human neutrophils. PMNs (1×10^7 cells/0·5 ml) were incubated with 300 pmol LTB_4 (a) or 142 pmol 20-OH-LTB_4 (b) in the presence of calcium (0·9 mM) and magnesium (0·5 mM) for various times at 37°. The ordinate represents the percentage of 20-carboxy-LTB_4, 20-hydroxy-LTB_4 and LTB_4. Analysis was carried out using HPLC. (▲), 20-COOH-LTB_4; (■), 20-OH-LTB_4; (●), LTB_4. The figure shows mean values ± SEM ($n=4$).

presence of 220 nM unlabelled LTB_4. The amount of receptor sites and the dissociation constant were calculated by Scatchard analysis.

Toxicity

Cell damage was determined by the release of cytoplasmic lactate dehydrogenase (LDH). The change in absorbance at 365 nm due to the conversion of NADH to NAD^+ in the presence of pyruvate was measured. The conditions and stimuli used did not cause net release of LDH.

Statistical analysis

All data were presented as mean ± standard error of the mean (SEM). Significance was determined using the paired Student's *t*-test.

RESULTS

Generation of leukotrienes by human neutrophils

The formation of LTB_4 and its more polar ω-oxidized metabolites were measured by HPLC (Fig. 1). Purified PMNs (1×10^7 cells) were incubated with the various stimuli in the presence of 0·9 mM Ca^{2+} and 0·5 mM Mg^{2+} at 37°. As is apparent, the calcium ionophore is the most effective stimulus of leukotriene

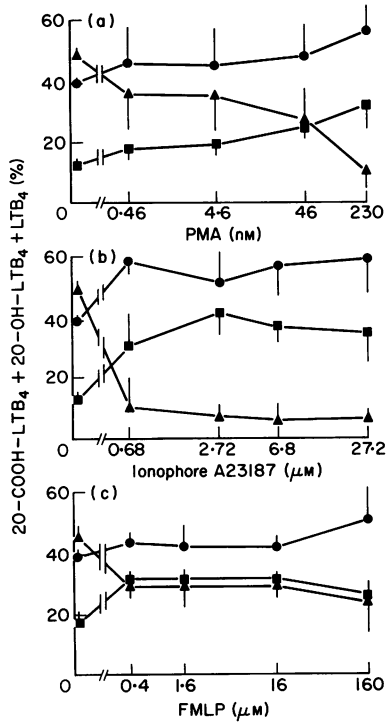

Figure 3. Effects of various concentrations of PMA, ionophore A23187 and FMLP on the catabolism of exogenous LTB_4. PMNs (1×10^7 cells/0·5 ml) were prestimulated with the indicated concentrations of PMA (a), the ionophore A23187 (b) and FMLP (c) in the presence of calcium (0·9 mM) and magnesium (0·5 mM) for 15 min at 37°. After prestimulation the cells were centrifuged and washed in PBS buffer followed by the addition of LTB_4 (300 pmol). The incubation was stopped after 15 min and the samples were analysed by HPLC. The ordinate shows the percentage of LTB_4 and the ω-oxidized products. (▲), 20-COOH-LTB_4; (■), 20-OH-LTB4; (●), LTB4. All data points were mean ± SEM ($n = 4$).

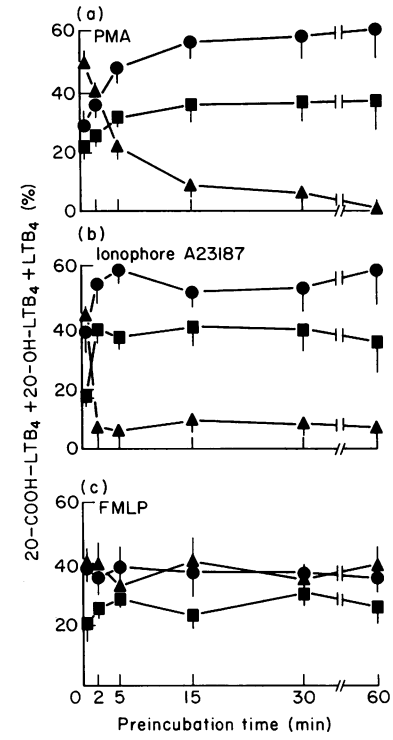

Figure 4. Time-dependent effects of PMA, the calcium ionophore A23187 and FMLP on the catabolism of exogenous LTB_4. PMNs (1×10^7 cells/0·5 ml) were incubated for various times with PMA (a; 0·23 μM), ionophore A23187 (b; 2·72 μM) or FMLP (c; 16 μM) in the presence of calcium (0·9 mM) and magnesium (0·5 mM) at 37°. The cells were washed in PBS buffer and LTB_4 (300 pmol) was added. This incubation period was terminated after 15 min. Analysis was carried out using HPLC. The ordinate shows the percentage of the sum of LTB_4 and the ω-oxidized products. The preincubation time of 0 min represents the controls without prestimulation. (▲), 20-COOH-LTB_4; (■), 20-OH-LTB_4; (●), LTB_4. All values were the mean ± SEM of four individual experiments.

Table 1. Conversion of 20-OH-LTB_4 by prestimulated neutrophils

Prestimulus	Amount of 20-COOH-LTB_4 (% of total leukotrienes) using the following substrate: 20-OH-LTB_4	LTB_4
PBS-buffer	100	100
PMA (0·23 μM)	27·0 ± 5·6	18·4 ± 8·2
A23187 (2·72 μM)	16·8 ± 9·3	21·6 ± 1·5
FMLP (16·0 μM)	42·3 ± 14·5	65·3 ± 6·0

PMNs (1×10^7 cells/0·5 ml) were prestimulated with PMA (0·23 μM), calcium ionophore (2·72 μM) or FMLP (16 μM) in the presence of calcium (0·9 mM) and magnesium (0·5 mM) for 15 min at 37°. After preincubation the cells were washed and resuspended in PBS buffer and 20-OH-LTB_4 (142 pmol) was added. The incubation was terminated after 45 min. Analysis was carried out by HPLC. The amount of 20-COOH-LTB_4 in resting PMNs was defined as 100%. The results were expressed as mean ± SEM, $n = 3$.

synthesis; at an ionophore concentration of 6·8 μM the total sum of 20-COOH-LTB_4, 20-OH-LTB_4 and LTB_4 amounted to 458 ± 50 ng (mean ± SEM, $n=8$). FMLP only generated 12% of the amount produced after ionophore stimulation (55 ± 6 ng; mean ± SEM, $n=8$). In both cases leukotrienes were first detected after an incubation time of 2 min. After this lag phase there was a rapid appearance of LTB_4 within the cellular supernatant, which reached a maximum after 5 min of incubation. During the subsequent incubation period the concentration of LTB_4 decreased as a consequence of LTB_4 conversion into the ω-oxidized products. The predominantly detected metabolite after FMLP stimulation was the 20-carboxy-LTB_4 and after ionophore stimulation the 20-hydroxy-LTB_4, respectively. Suboptimal concentrations of the calcium ionophore (0·68 μM) resulted in marginal release of leukotrienes, but the same distribution pattern of LTB_4 and the ω-oxidized products was observed. Therefore, it was suggested that the high 20-OH-LTB_4 level within the ionophore incubation could not be attributed to a persistent LTB_4 synthesis. In all experiments the amount of leukotrienes generated after exposure of PMA was below the detection level in our assay systems.

Conversion of exogenous leukotriene B_4 and 20-OH-leukotriene B_4

Human PMNs convert endogenously generated as well as exogenously added LTB_4. Purified neutrophils (1×10^7 cells) were incubated with exogenous LTB_4 (300 pmol) at 37°. The incubation results in the formation of 20-hydroxy- and 20-carboxy-LTB_4 (Fig. 2a). Within 7·5 ± 1·5 min (mean ± SEM, $n=4$) 50% of the initial LTB_4 concentration was catabolized. The conversion rate amounted to 22·6 ± 4·5 pmol LTB_4/min × 1 × 10^7 cells (mean ± SEM, $n=4$). As is apparent, 20-COOH-LTB_4 accumulated rapidly; the 20-OH-LTB_4 level did not exceed more than 20% when compared with the total sum of leukotrienes. A similar pattern was observed using [^{3}H]LTB_4 (2·8 pmol) as substrate (data not shown). In order to study the second step in LTB_4 inactivation, PMNs were incubated with exogenous 20-OH-LTB_4 (142 pmol) (Fig. 2b). Under the experimental conditions described, 20-OH-LTB_4 conversion to 20-COOH-LTB_4 was not effective in the presence of intact cells (conversion rate: 1·9 ± 0·4 pmol 20-OH-LTB_4/min × 1 × 10^7 cells; mean ± SEM, $n=4$; $T_{1/2}$ above 60 min). The results demonstrate that the rate of 20-carboxy-LTB_4 formation was dependent on the substrate that was used. The carboxy-metabolite was rapidly generated using exogenous LTB_4; the generation rate was slow when 20-OH-LTB_4 was used as substrate.

Leukotriene B_4 catabolism by prestimulated cells

Stimulation of cells results in a complex array of metabolic activities. Therefore, experiments were performed to study the question to what extent the catabolism of LTB_4 is due to the degree of cellular activation. For this purpose, PMNs were preactivated with different stimuli and subsequently incubated with exogenous LTB_4. In the actual experiment, cells (1×10^7) were pretreated with PMA, the calcium ionophore or FMLP in the presence of calcium (0·9 mM) and magnesium (0·5 mM). In order to minimize the contamination by endogenously generated leukotrienes, the cells were washed in calcium-free buffer after prestimulation and exogenous LTB_4 was added. In order to determine the efficiency of the washing procedure, cells were incubated with the ionophore and then washed by centrifugation, compared with incubations without washing of the cells. The amount of leukotriene was reduced by a factor ranging between 87·6 and 96·7%. Therefore, experiments directed to the catabolism of exogenous LTB_4 with preactivated cells were corrected: the total concentrations of leukotrienes were subtracted by the concentrations of endogenously generated leukotrienes. Experiments were then carried out to analyse to what extent the modulatory effects with regard to the ω-oxidation pathway is dependent on the concentration of the stimulus (Fig. 3) and the time of prestimulation (Fig. 4). The distribution of

Table 2. Effects of prestimulation on the LTB_4-ω-hydroxylase and the 20-OH-LTB_4-dehydrogenase activities of polymorphonuclear granulocytes.

	LTB_4-ω-hydroxylase substrate: LTB_4			20-OH-LTB_4-dehydrogenase substrate: 20-OH-LTB_4	
Prestimulus	20-COOH-LTB_4 (%)	20-OH-LTB_4 (%)	LTB_4 (%)	20-COOH-LTB_4 (%)	20-OH-LTB_4 (%)
PBS buffer	38·5 ± 10·3	54·1 ± 12·8	7·4 ± 6·6	69·8 ± 18·8	30·2 ± 10·0
PMA (0·23 μM)	44·4 ± 9·4	50·9 ± 14·3	4·6 ± 3·7	71·4 ± 21·2	28·6 ± 14·6
A23187 (2·72 μM)	49·4 ± 11·4	42·3 ± 13·5	8·3 ± 5·5	69·1 ± 14·7	30·9 ± 9·6
FMLP (16·0 μM)	37·0 ± 9·9	52·5 ± 16·6	10·5 ± 6·5	67·1 ± 12·5	32·9 ± 11·8

Purified PMNs (1×10^8 cells/ml in 0·34 M sucrose buffer (pH 7·4) were disrupted by sonication, as described in the Material and Methods. The LTB_4-ω-hydroxylase activity was determined by using 0·5 mg protein of the sonicate, which was preincubated with 1 mM NADPH for 5 min and further incubated with [^{3}H] LTB_4 (2·8 pmol) and LTB_4 (30 pmol) for 60 min at 37°. The 20-OH-LTB_4-dehydrogenase activity was determined as followed: 1·0 mg protein was used which was preincubated with 1 mM NAD^+ for 5 min; the pH was shifted from pH 7·5 to 9·0 and subsequently incubated with [^{3}H] 20-OH-LTB_4 (1 pmol) and 20-OH-LTB_4 (28 pmol) for 60 min at 37°. The enzyme preparations were prestimulated with PMA (0·23 μM), calcium ionophore (2·72 μM) or FMLP (16 μM) for 10 min. Analysis was carried out by thin-layer chromatography. The results were expressed as mean ± SEM, $n=2$.

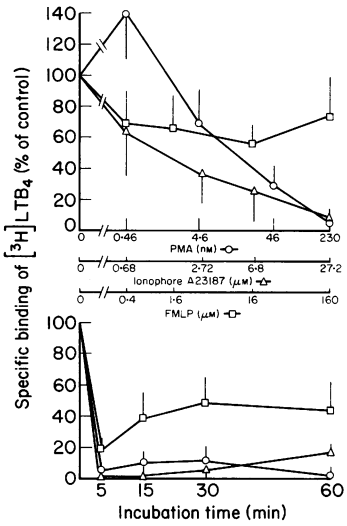

Figure 5. Effects of prestimulation on the specific binding of [^{3}H]LTB$_4$. PMNs ($2{\cdot}4 \times 10^7$ cells/1·2 ml) were prestimulated with the indicated concentrations of PMA, the ionophore A23187 or FMLP for 15 min (a) or for the indicated time periods with defined concentrations of PMA (0·23 μM), the ionophore (2·72 μM) or FMLP (16 μM) (b). The preincubation was carried out in the presence of calcium (0·9 mM) and magnesium (0·5 mM) at 37°. After prestimulation the cells were washed and resuspended in PBS buffer. Samples of 4×10^6 PMNs were added to wells containing 2·3 nM [^{3}H]LTB$_4$ or 2·3 nM [^{3}H]LTB$_4$ plus 220 nM unlabelled LTB$_4$ and 125 μg/ml bovine serum albumin. After 45 min at 4°, the samples were filtrated through 5 μM pore size-polyvinylidene fluoride membranes. The filters were collected, dried and the radioactivity was determined by liquid scintillation counting. The specific binding of cells preincubated with buffer was defined as 100%. (○), specific binding after preactivation with PMA; (△), with the ionophore A23187; (□) with FMLP. Total and non-specific binding were determined in triplicates. The data points represent mean values ± SEM of three individual experiments.

LTB$_4$, 20-OH-LTB$_4$ and 20-COOH-LTB$_4$ was assayed. As is apparent, PMA and the ionophore significantly reduced the formation of 20-carboxy-LTB$_4$ in an irreversible way. Minimal concentrations were 4·6 nM using PMA and 2·7 μM with the ionophore. Inhibition of 20-COOH-LTB$_4$ formation was observed within the first 2 min; during this time no leukotriene synthesis was detected (see above). Parallel with the reduced amount of 20-COOH-LTB$_4$ there was an increase in 20-OH-LTB$_4$ and LTB$_4$. A similar pattern was observed when PMA was replaced by phorbol-myristate-dibutyrate or mezerein. In contrast, prestimulation with FMLP only showed a slight reduction of 20-COOH-LTB$_4$ generation. Experiments were then performed to analyse whether a possible shift of the LTB$_4$-inactivation pathway occurred after prestimulation; for this purpose experiments were carried out using [^{3}H]LTB$_4$ (2·8 pmol) as substrate. As is shown [^{3}H] LTB$_4$ was only metabolized into the ω-oxidized products (data not shown). Therefore, we suggest that prestimulation leads to an inhibition of the ω-oxidation pathway. Similar results were obtained using 20-OH-LTB$_4$ (142 pmol) as exogenous substrate (see Table 1). The most potent stimulus was the calcium ionophore; interestingly, FMLP showed a greater efficiency compared with the experiments using LTB$_4$ as substrate.

Catabolism of leukotriene B$_4$ and 20-OH-leukotriene B$_4$ by cell-free systems

Our experiments were then directed to elucidate the mechanisms resulting in the decreased 20-COOH-LTB$_4$ formation. First, we studied the direct effect of prestimulation with the ionophore (2·72 μM), PMA (0·23 μM) and FMLP (16 μM) on functionally purified LTB$_4$-ω-hydroxylase and 20-OH-LTB$_4$-dehydrogenase activity. Therefore, cells were disrupted by sonication and subsequently analysed to what extent the enzyme activities were affected (see the Material and Methods). Table 2 demonstrates that the prestimulation of the enzyme preparations did not inhibit the enzyme activities compared with unstimulated incubations. Therefore, a direct inhibitory effect of the stimuli could be excluded.

LTB$_4$-binding assay

The existence of specific receptor sites for LTB$_4$ on the surface of human PMNs is well established (Goldman & Goetzl, 1982; Lin, Ruppel & Gorman, 1984). Furthermore, the control of receptor expression by protein kinase *C* is well established for various systems (May *et al.*, 1985; Andersson *et al.*, 1987). In order to determine whether the inhibition of the 20-carboxy-LTB$_4$ generation is due to a possible down-regulation of LTB$_4$-receptor sites, the specific binding of [^{3}H]LTB$_4$ after cell activation was analysed. Purified PMNs (4×10^6 cells) were incubated with [^{3}H]LTB$_4$ (0·9 KBq = 2·3 nM) in the presence of BSA (125 μg/ml) for 45 min at 4°. During this incubation procedure, a metabolization of [^{3}H]LTB$_4$ was not observed (Brom *et al.*, 1987). Total binding amounted to $9{\cdot}7 \pm 1{\cdot}3\%$; specific binding was $55{\cdot}5 \pm 14{\cdot}7\%$ of the amount of total binding (mean ± SEM, $n = 3$). Scatchard analysis revealed a dissociation constant of $4{\cdot}5 \pm 0{\cdot}1$ nM and $1{\cdot}7 \pm 0{\cdot}3 \times 10^4$ receptor sites/cell (mean ± SEM, $n = 3$). [^{3}H]LTB$_4$ binding was antagonized by non-labelled LTB$_4$ and 20-OH-LTB$_4$, but not by 20-COOH-LTB$_4$, indicating that there was no interaction between the specific LTB$_4$-receptor and the carboxy metabolite. The respective IC_{50} values were 29 nM for LTB$_4$ and 380 nM for 20-OH-LTB$_4$ (data not shown). Figure 5 shows that PMA and the ionophore reduced the amount of specific [^{3}H]LTB$_4$-binding in a concentration-dependent fashion. Preincubation of cells with FMLP only showed marginal effects. When cells were stimulated with the ionophore, the occupancy of the receptor sites by endogenously generated leukotrienes could not be excluded. However, preincubation with propyl gallate (0·9 mM) reduced the ionophore-derived leukotriene synthesis by a factor of 95%. In the binding experiments the propyl gallate preincubation only slightly enhanced the amount of specific binding (in the actual experiment 2·8% specific [^{3}H]LTB$_4$ binding was obtained following ionophore stimulation for 15 min, while 13·6% was observed after stimulation of the cells with ionophore and propyl gallate). The data suggest a correlation between the reduced 20-OH-LTB$_4$ conversion and the down-regulation of specific LTB$_4$-binding sites.

Effects of the diazonium salt of sulphanilic acid and thermolysin on the binding and metabolism of LTB$_4$ and 20-OH-LTB$_4$

The following studies were designed to determine whether LTB$_4$/20-OH-LTB$_4$ enters the cell interior via receptor-mediated uptake or by unspecific mechanisms. Therefore, we

Table 3. Effects of diazonium salt and thermolysin on metabolism and binding of [^{3}H]LTB_4 and [^{3}H] 20-OH-LTB_4 by polymorphonuclear granulocytes

	Intact cells					LTB_4-ω-hydroxylase Substrate: LTB_4			20-OH-LTB_4-dehydrogenase Substrate: 20-OH-LTB_4		Binding of [^{3}H]LTB_4 (%)	Release of lactate dehydrogenase (%)
	Substrate: LTB_4			Substrate: 20-OH-LTB_4								
Preincubation	20-COOH-LTB_4 (%)	20-OH-LTB_4 (%)	LTB_4 (%)	20-COOH-LTB_4 (%)	20-OH-LTB_4 (%)	20-COOH-LTB_4 (%)	20-OH-LTB_4 (%)	LTB_4 (%)	20-COOH-LTB_4 (%)	20-OH-LTB_4 (%)		
PBS buffer	49·6 ± 5·7	29·9 ± 1·2	20·3 ± 6·1	40·2 ± 8·4	59·8 ± 10·3	27·0 ± 2·4	65·6 ± 8·9	7·4 ± 2·0	30·3 ± 7·6	69·7 ± 10·4	100	< 2·0
Diazonium salt (4·8 mM)	4·1 ± 2·4	41·0 ± 7·9	54·9 ± 9·8	0	100	0	0	100	0	100	0	< 3·6
Thermolysin (100 μg/ml)	49·3 ± 10·6	26·3 ± 4·3	24·3 ± 7·5	51·5 ± 9·2	48·5 ± 8·6	0	11·1 ± 5·2	88·9 ± 7·8	9·8 ± 1·6	90·2 5·3	88·2 ± 6·7	< 5·0

PMNs (1×10^7 cells/0·5 ml) or the sonicate of the neutrophils (0·5 or 1·0 mg of protein) were preincubated with the diazonium salt of sulphanilic acid (4·8 mM) for 10 min or with thermolysin (100 μg/ml) for 60 min at 37°. Subsequently, the metabolism of tritium-labelled LTB_4 (2·8 pmol) and 20-OH-LTB_4 (1 pmol), the specific binding of LTB_4 to PMNs and the release of lactate dehydrogenase were determined as described (see legends of Fig. 5 and Table 2). All results were expressed as mean ± SEM; $n = 4$ for cell incubations, $n = 2$ for sonicate incubations, $n = 3$ for binding studies and $n = 4$ for the release of lactate dehydrogenase.

treated PMNs with the protease thermolysin or with the diazonium salt of sulphanilic acid. Both reagents are known to interact with ecto-enzymes within the plasma membrane facing the surrounding medium and do not penetrate into the cell any further. The following results were obtained (summarized in Table 3): (i) pretreatment with thermolysin (100 μg/ml, preincubation time: 60 min) and diazonium salt (4·8 mM, preincubation time: 10 min) were not followed by leakage of the cytoplasmic marker lactate dehydrogenase into the medium; (ii) both reagents inhibited LTB_4-ω-hydroxylase and 20-OH-LTB_4-dehydrogenase activities within a cell-free system; (iii) using intact cells, the diazonium salt partly blocked the conversion of LTB_4 and completely blocked the conversion of 20-OH-LTB_4; thermolysin did not have any effects; (iv) in parallel, the diazonium salt completely inhibited the specific binding of [^{3}H]LTB_4 to the LTB_4 receptor; in contrast, thermolysin did not show any effects on LTB_4 binding. Therefore it appears that the LTB_4-ω-hydroxylase and the 20-OH-LTB_4-dehydrogenase are not ecto-enzymes. Since the inhibition of LTB_4 binding and the inhibition of LTB_4 or 20-OH-LTB_4 conversion following diazonium salt pretreatment do correlate, it is likely that (i) LTB_4 is metabolized in part via receptor-mediated uptake and (ii) the inhibition of the 20-carboxy-LTB_4-formation following prestimulation is the result of the decrease in specific LTB_4-binding.

DISCUSSION

Our data demonstrate that LTB_4, which was generated after activation of human PMNs with the ionophore A23187 or FMLP, is metabolized via the ω-oxidation pathway. The main metabolite after FMLP stimulation was the 20-carboxy-LTB_4; ionophore-stimulation inhibited the formation of 20-COOH-LTB_4 and led to the accumulation of 20-OH-LTB_4 and LTB_4. In order to elucidate these differences as to the LTB_4-inactivation mechanism, experiments were directed to analyse the interaction between cell activation and the metabolism of exogenously added LTB_4. Resting neutrophils catabolized exogenous LTB_4 to 20-COOH-LTB_4 via the intermediate metabolite 20-OH-LTB_4, similar to the pattern of metabolism obtained after preexposure of the cells with FMLP. Preactivation of PMNs with the calcium ionophore changed the distribution of LTB_4-derived products. As was shown for the release experiments with the calcium ionophore, the formation of 20-COOH-LTB_4 was inhibited. The same effect was observed after preactivation of the cells with phorbol esters, which did not generate endogenous leukotrienes. Thus the results show a clear difference in LTB_4 metabolism between resting and stimulated cells. The level of LTB_4 is regulated by a balance between the mechanism of generation and the process of conversion. PMA and the ionophore change the biochemical status of the cells in such a way that 20-COOH-LTB_4 was generated to a lesser degree. FMLP showed only slight effects and in this way resembled the LTB_4 conversion by non-stimulated granulocytes.

The transformation of 20-OH-LTB_4 into 20-COOH-LTB_4, the second step of LTB_4 inactivation, is catalysed by the 20-OH-LTB_4-dehydrogenase (Sumimoto *et al.*, 1985). In order to analyse the effect of prestimulation on 20-OH-LTB_4 conversion, the 20-OH-LTB_4 was added exogenously. Similar inhibitory effects were observed when PMNs were prestimulated with the ionophore or PMA. In this system FMLP caused a more pronounced inhibition compared with the experiments using exogenous LTB_4 as substrate. One might suggest that there was only a small tendency for 20-OH-LTB_4 to enter the cell interior compared with LTB_4. In contrast to Clancy, Dahinden & Hugli (1984), we observed that 20-OH-LTB_4 did not interact with the specific [^{3}H]LTB_4 receptor as effectively as LTB_4. These results are in agreement with those observed by Naccache *et al.* (1984). Also, the polar character of the oxidized metabolite impairs an unspecific uptake by diffusion across the plasma membrane. Another explanation for this finding may be that the activation of the LTB_4-hydroxylase further enhances the activity of the subsequent 20-OH-LTB_4-dehydrogenase.

Receptors crucially participate in cellular metabolic regulation. A specific LTB_4 receptor on human neutrophils has been described previously (Goldman & Goetzl, 1982; Lin *et al.*, 1984). However, a great part of LTB_4 was concentrated within the membrane bilayer, resulting in a non-specific binding. It is not established whether LTB_4 metabolization is only induced by receptor-mediated LTB_4 uptake, or whether ω-hydroxylation also occurs via an unspecific binding of leukotriene B_4. Pretreatment of the cells with the diazonium salt of sulphanilic acid blocked in part the conversion of LTB_4 and completely the conversion of 20-OH-LTB_4. Thus it may be suggested that both substrates are partly transferred to the metabolizing enzymes via receptor-mediated uptake. This mechanism may then lead to a permanent recycling of LTB_4 and 20-OH-LTB_4 including generation (or uptake), release into the surrounding medium and re-uptake via specific receptors.

The inhibitory effect with regard to the LTB_4 inactivation is accompanied by a reduction in specific binding. The effects of phorbol esters on receptor functions have been described in various cell systems (May *et al.*, 1985; Andersson *et al.*, 1987). The down-regulation of [^{3}H]LTB_4-binding sites by PMA (also described by O'Flaherty, Redman & Jacobson, 1986) could be the result of protein kinase *C*-mediated phosphorylation of the LTB_4 receptors. However, the effects on lipid metabolism, on membrane fluidity or on the assembly of membrane-associated microfilaments cannot be ruled out. Protein kinase *C* is also activated by the ionophore or FMLP. An activation of the enzyme by non-physiological concentrations of Ca^{2+}, induced by the ionophore A23187, has been described elsewhere (Castagna *et al.*, 1982). Preiss, Bell & Niedel (1987) observed a large and persistent increase in 1,2-diacylglycerol concentration in HL60 cells, which may activate protein kinase *C*. However, with regard to the total down-regulation of receptor sites (e.g. following prestimulation with PMA), LTB_4 is metabolized to 20-OH-LTB_4. The reason for this discrepancy could be because the binding assay was not sensitive enough or that part of the exogenously added LTB_4 unspecifically entered the cell.

Our data demonstrate that the final inactivation product of LTB_4 is the 20-carboxy-metabolite, whereas 20-hydroxy-LTB_4 seems to be an intermediate metabolite. Previously, it was shown clearly that 20-COOH-LTB_4 revealed very weak biological activities compared with LTB_4, but the biological potency of 20-OH-LTB_4 has been debated (Hansson *et al.*, 1981; Clancy *et al.*, 1984). Therefore, the control as to the conversion from 20-OH-LTB_4 into the 20-carboxy product is an important step for the catabolism of LTB_4. Furthermore, our results show a strict dependency of the 20-COOH-LTB_4 generation from the activation status of the cell. We suggest a participation of the expression and re-expression of the specific LTB_4-binding sites, which may be controlled by protein kinase *C*. Recently, it has

been described that various stimuli reveal different capacities for the mobilization of protein kinase *C*, which could be an essential prerequisite for activation (Horn & Karnovsky, 1986; O'Flaherty & Nishihira, 1987).

FMLP, which also activates the protein kinase *C* via the generation of diacylglycerol, could stimulate this enzyme to a much lesser extent or may bypass the phosphorylation system. However, FMLP also enhances the level of cAMP, which may act as a feedback-mechanism within the cell, thus inhibiting the protein kinase *C* (Della Bianca *et al.*, 1986). The differences observed with the various stimuli could also reside at the level of protein kinase *C*-induced phosphorylation.

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