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Research Article

Human polymorphonuclear leukocytes (PMN) not only synthesize and respond to leukotriene B4 (LTB4), but also catabolize this mediator of inflammation rapidly and specifically by omega-oxidation. To characterize the enzyme(s) responsible for omega-oxidation of LTB4, human PMN were disrupted by sonication and subjected to differential centrifugation to yield membrane, granule, and cytosol fractions (identified by biochemical markers). LTB4 omega-hydroxylase activity was concentrated (together with NADPH cytochrome c reductase activity) only in the membrane fraction (specific activity increased 10-fold as compared to whole sonicates, 41% recovery). Negligible activity was detected in granule or cytosol fractions. LTB4 omega-hydroxylase activity in isolated PMN membranes was linear with respect to duration of incubation and protein concentration, was maximal at pH 7.4, had a Km for LTB4 of 0.6 microM, and was dependent on oxygen and on reduced pyridine nucleotides (apparent Km for NADPH = 0.5 microM; apparent Km for NADH = 223 microM). The LTB4 omega-hydroxylase was inhibited significantly by carbon monoxide, ferricytochrome c, SKF-525A, and Triton X-100, but was not affected by alpha-naphthoflavone, azide, cyanide, catalase, and superoxide dismutase. Finally, isolated PMN membranes exhibited a carbon monoxide difference spectrum with a peak at 452 nm. Thus, we have partially purified the LTB4 omega-hydroxylase in human PMN and identified the enzyme as a membrane-associated, NADPH-dependent cytochrome P-450.

Leukotriene B_4 ω-Hydroxylase in Human Polymorphonuclear Leukocytes

Partial Purification and Identification as a Cytochrome P-450

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Abstract

Human polymorphonuclear leukocytes (PMN) not only synthesize and respond to leukotriene B_4 (LTB_4), but also catabolize this mediator of inflammation rapidly and specifically by ω-oxidation. To characterize the enzyme(s) responsible for ω-oxidation of LTB_4, human PMN were disrupted by sonication and subjected to differential centrifugation to yield membrane, granule, and cytosol fractions (identified by biochemical markers). LTB_4 ω-hydroxylase activity was concentrated (together with NADPH cytochrome *c* reductase activity) only in the membrane fraction (specific activity increased 10-fold as compared to whole sonicates, 41% recovery). Negligible activity was detected in granule or cytosol fractions. LTB_4 ω-hydroxylase activity in isolated PMN membranes was linear with respect to duration of incubation and protein concentration, was maximal at pH 7.4, had a K_m for LTB_4 of 0.6 μM, and was dependent on oxygen and on reduced pyridine nucleotides (apparent K_m for NADPH = 0.5 μM; apparent K_m for NADH = 223 μM). The LTB_4 ω-hydroxylase was inhibited significantly by carbon monoxide, ferricytochrome *c*, SKF-525A, and Triton X-100, but was not affected by α-naphthoflavone, azide, cyanide, catalase, and superoxide dismutase. Finally, isolated PMN membranes exhibited a carbon monoxide difference spectrum with a peak at 452 nm. Thus, we have partially purified the LTB_4 ω-hydroxylase in human PMN and identified the enzyme as a membrane-associated, NADPH-dependent cytochrome P-450.

Introduction

Human polymorphonuclear leukocytes (PMN) not only synthesize and respond to leukotriene B_4 (LTB_4)[1] (1–3), but also catabolize this potent mediator of inflammation rapidly and specifically by ω-oxidation (4–8). Human PMN (but not human monocytes, lymphocytes, or platelets) efficiently convert exogenous LTB_4 to the less active products, 20-OH-LTB_4 and 20-COOH-LTB_4 (7, 8). Other dihydroxylated derivatives of arachidonic acid are either not converted to ω-oxidation products or are converted at much slower rates. Because the rate of ω-oxidation of LTB_4 by human PMN is comparable to the maximal rate that PMN are capable of synthesizing LTB_4, ω-oxidation greatly influences the amounts of biologically active LTB_4 that appear in the medium surrounding stimulated PMN (8).

The nature of the enzyme(s) responsible for ω-oxidation of LTB_4 in human PMN is not known. However, considerable information has been obtained concerning the enzyme(s) responsible for ω-oxidation of arachidonic acid metabolites in other tissues (reviewed in reference 9). For example, microsomal monooxygenases in liver, lung, and kidney from a variety of animal species catalyze ω-oxidation of prostaglandins (9–12). Participation of cytochrome P-450 in these reactions has been suggested by results of studies performed with standard enzyme inhibitors and inducers (12). Furthermore, reconstituted hepatic microsomal enzyme systems, consisting of purified cytochrome P-450, cytochrome b_5, NADPH-cytochrome *c* reductase, phosphatidylcholine, and NADPH, catalyze ω-oxidation of prostaglandins E_1 and A_1 (13). Finally, liver microsomes have been reported to hydroxylate LTB_4 by a cytochrome P-450-dependent process, although the products formed from this reaction were not described (14).

We have reported recently that ω-oxidation of LTB_4 in intact human PMN is inhibited specifically and reversibly by carbon monoxide (15). This finding suggested that ω-oxidation of LTB_4 in human PMN is mediated by a heme enzyme which cycles through the ferrous state, as does cytochrome P-450. In addition, we have found that the LTB_4 ω-hydroxylase in intact PMN and in PMN sonicates is inactivated suicidally by long-chain terminal acetylenic fatty acids (16). Thus, the LTB_4 ω-hydroxylase in human PMN also resembles hepatic cytochrome P-450 enzymes with respect to its susceptibility to suicidal inactivation by acetylenic substrates (17).

To directly characterize the LTB_4 ω-hydroxylase in human PMN, we disrupted cells by sonication, and measured ω-oxidation of LTB_4 in granule, cytosol, and membrane fractions isolated by differential centrifugation. In this way, we have partially purified the LTB_4 ω-hydroxylase in human PMN and have identified the enzyme as a membrane-associated, NADPH-dependent cytochrome P-450.

Methods

Materials. Synthetic LTB_4, 20-OH-LTB_4, and 20-COOH-LTB_4 were generously provided by Dr. Joshua Rokach (Merck Frosst, Dorval, Quebec). LTB_4 was stored as a stock solution in methanol under nitrogen at −70°C. Concentrations of LTB_4 were determined by ultraviolet (UV) spectrophotometry (molar extinction coefficient of 50,000 cm^{-1}) (18).

Portions of this work were presented at the annual meeting of the Association of American Physicians, Washington, DC, 4 May 1985, and have been published as an abstract (1985. *Clin. Res.* 33:607A).

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1. *Abbreviations used in this paper:* DFP, diisopropylfluorophosphate; 5,12-diHETE, 5(S),12(S)-dihydroxy-8,14-*cis*-6,10-*trans*-eicosatetraenoic acid; 5,15-diHETE, 5(S),15(S)-dihydroxy-8,11-*cis*-6,13-*trans*-eicosatetraenoic acid; 8,15-diHETE, 8(S),15(S)-dihydroxy-5,11-*cis*-9,13-*trans*-eicosatetraenoic acid; HPLC, high-performance liquid chromatography; LTB_4, leukotriene B_4; P, pellet; S, supernatant.

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For each experiment, aliquots of the stock solution were placed into polypropylene test tubes, the methanol was evaporated under nitrogen, buffer was added, and the tubes were vortexed vigorously. Other dihydroxylated derivatives of arachidonic acid, i.e., 8(S),15(S)-dihydroxy-5,11-*cis*-9,13-*trans*-eicosatetraenoic acid (8,15-diHETE), the two all-*trans* conjugated isomers of 8,15-diHETE, 5(S),15(S)-dihydroxy-8,11-*cis*-6,13-*trans*-eicosatetraenoic acid (5,15-diHETE), 5(S),12(S)-dihydroxy-8,14-*cis*-6,10-*trans*-eicosatetraenoic acid (5,12-diHETE), and the two all-*trans* conjugated isomers of LTB_4, were prepared as described previously (8, 19). Prostaglandin B_2 (PGB_2), NADPH (tetrasodium salt, type X), NADH (disodium salt, grade III), Hepes, EDTA (trisodium salt), EGTA, dithiothreitol, ferricytochrome *c* (horse heart, type III), potassium cyanide, sodium azide, superoxide dismutase, catalase, α-naphthoflavone, phenolphthalein β-glucuronide, and *Micrococcus lysodeikticus* were purchased from Sigma Chemical Co. (St. Louis, MO). Diisopropylfluorophosphate (DFP), sodium dithionite, Triton X-100, and glycerol were obtained from Aldrich Chemical Co., Inc. (Milwaukee, WI), Fisher Scientific Co. (Pittsburgh, PA), J. T. Baker Chemical Co. (Phillipsburg, NJ), and Mallinckrodt, Inc. (St. Louis, MO), respectively. SKF-525A was a gift from Smith, Kline and French Laboratories (Philadelphia, PA). All organic solvents were from Burdick & Jackson Laboratories, Inc. (Muskegon, MI).

Disruption of PMN. Human PMN were isolated from venous blood (anticoagulated with acid-citrate-dextrose) by centrifugation on Hypaque-Ficoll followed by dextran sedimentation (20). Contaminating erythrocytes were removed by hypotonic lysis, and the leukocytes were washed twice with phosphate (10 mM)-buffered 140 mM NaCl, pH 7.4. Cell suspensions contained 95–98% PMN, 2–5% eosinophils, <1% mononuclear leukocytes, and a platelet to leukocyte ratio of <2:1.

PMN (55×10^6 cells/ml) were suspended in disruption buffer (130 mM KCl, 5.0 mM NaCl, 10 mM Hepes, 2.5 mM $MgCl_2$, and 2.0 mM DFP, pH 7.0) at 4°C for 5 min, and then sonicated four times (100 W for 15 s) with a Virsonic Cell Disrupter, model 16-850 (VirTis Co., Inc., Gardiner, NY). The probe was chilled before use, and the cells were maintained at 4°C throughout the procedure. Immediately after sonication, 0.1 vol of disruption buffer, supplemented with EDTA, EGTA, and dithiothreitol (to yield final concentrations of 1.0 mM, 1.25 mM, and 0.5 mM, respectively) was added. Crude sonicates, therefore, reflected a cell concentration of 50×10^6 PMN/ml.

In some experiments, PMN were disrupted by nitrogen cavitation, according to the method described by Klempner et al. (21). Briefly, PMN (55×10^6 cells/ml) were suspended in disruption buffer and equilibrated at 400 psi in a cell disruption bomb (Parr Instrument Co., Moline, IL) for 30 min at 4°C. Upon slow release from the bomb, the cavitate was collected in 0.1 vol of disruption buffer supplemented with EDTA, EGTA, and dithiothreitol (as above).

Subcellular fractionation. Granule, membrane, and cytosol fractions were isolated by differential centrifugation (21). Crude PMN sonicates were centrifuged at 150 *g* for 10 min (TJ-6R centrifuge, Beckman Instruments, Inc., Palo Alto, CA) to yield pellet 1 (P1) (containing nuclei and intact cells) and supernatant 1 (S1). S1 was then centrifuged at 18,000 *g* for 30 min (Beckman J2-21) to yield pellet 2 (P2) (containing granules) and supernatant 2 (S2). S2 was centrifuged at 100,000 *g* for 60 min (Beckman L5-50B) to yield pellet 3 (P3) (containing membranes) and supernatant 3 (S3) (cytosol). All procedures were carried out at 4°C. P1 and P2 were resuspended to original volumes in supplemented disruption buffer. P3 was resuspended in 1.0–3.0 ml of supplemented disruption buffer using a silanized Dounce homogenizer.

Biochemical markers. Activity of the cytosol enzyme, lactate dehydrogenase, was determined according to the method of Wacker et al. (22) using Statzyme LDH (L-P) kits (Worthington Diagnostic Systems, Inc., Freehold, NJ). Results are expressed as international units per milligram of protein, where 1.0 IU reduces 1.0 μmol NAD/min. Two markers of lysosomal granules were assayed. β-Glucuronidase, a marker of azurophil granules, was assayed as described previously (23), using phenolphthalein β-glucuronide as substrate. Activity is expressed as nanomoles of substrate hydrolyzed per hour per milligram of protein. Lysozyme, a marker of specific granules as well as azurophil granules, was assayed by measuring decreases in absorbance at 450 nm of suspensions containing *Micrococcus lysodeikticus* (24). Activity is expressed as units per minute per milligram of protein, where 1.0 U is defined as a change in absorbance of 0.001. Integrity of lysosomal granules (i.e., latency of granule enzymes) was determined by comparing results of enzyme assays performed in the presence and absence of 0.1% (vol/vol) Triton X-100. DNA was measured by the spectrophotometric method of Labarca and Paigen (25). Lipid phosphorus was used as a quantitative marker of total membrane. Extraction was performed by the method of Bligh and Dyer (26), and the phosphorus content of the organic phase was determined by the method of Bartlett (27). Protein was measured using the Bradford reagent (28), with bovine serum albumin as a standard.

Measurements of LTB_4 ω-hydroxylase activity. LTB_4 ω-hydroxylase activity was assayed as described previously (8, 15). Briefly, 0.1-ml aliquots of each fraction were incubated with synthetic LTB_4 (1.0 μM) for 5 min at 37°C. Incubations were terminated by adding 1.5 vol of ice-cold ethanol that contained 334 ng/ml PGB_2 (internal standard), followed by centrifugation at 6,500 *g* for 1 min. LTB_4 and its ω-oxidation products were extracted and partially purified by chromatography on SEP-PAK C18 cartridges (8) (Waters Associates, Millipore Corp., Milford, MA), and then analyzed by reverse-phase high-performance liquid chromatography (HPLC). HPLC was performed using a Beckman model 334 gradient system, a Techsphere-Ultra C18 column (5.0 μm, 5.0 mm × 25 cm) (Phenomenex, Palos Verdes Estates, CA), and an Aquapore RP-300 precolumn cartridge (2.1 mm × 3.0 cm) (Brownlee Labs, Santa Clara, CA). Solvents A and B consisted of methanol/acetonitrile/water/acetic acid (35:25:45:0.02) and methanol/acetonitrile (75:25), respectively. Lipids were eluted at a rate of 1.0 ml/min with continuous monitoring for UV absorbance at 280 nm. The following solvent gradient program was employed to elute LTB_4 and its ω-oxidation products. After equilibrating the column with solvent A, elution was begun at the time of sample injection (0 min) with a linear increase in solvent B (10%/min). From 6 to 12 min, the column was eluted isocratically with 40% solvent A and 60% solvent B. After 12 min, solvent B was increased (20%/min) to 100%. At 30 min, the column was recycled with 100% solvent A. Although some variability was noted from day to day, retention times in consecutive experiments were highly reproducible. Typical retention times for 20-COOH-LTB_4, 20-OH-LTB_4, PGB_2, and LTB_4 were 8.3, 9.0, 15.2, and 17.1 min, respectively. Integrated peak areas were used to quantify LTB_4 and its ω-oxidation products, and the recovery of the internal standard, PGB_2, was used to correct for losses during sample preparation. LTB_4 ω-hydroxylase activity is expressed as 20-OH-LTB_4 plus 20-COOH-LTB_4 generated in picomoles per minute per milligram of protein.

The ω-oxidation products that were generated in fractions of PMN sonicates were identified as 20-OH-LTB_4 and 20-COOH-LTB_4 by cochromatography with synthetic standards after both reverse-phase and normal-phase HPLC, as well as by gas chromatography-mass spectrometry, and thus were identical to the products generated from LTB_4 by intact PMN (8). Briefly, lipids that were purified by reverse-phase HPLC were methylated with ethereal diazomethane (19) and then subjected to normal-phase HPLC on a Techsphere-Ultra Silica column (5.0 μm, 5.0 mm × 25 cm) (Phenomenex) with a Brownlee pre-column cartridge (Si, 2.1 mm × 3.0 cm). Methyl esters were eluted isocratically at a flow rate of 2.0 ml/min with hexane/2-propanol (100:12). For gas chromatography-mass spectrometry, HPLC-purified methyl esters were silylated with *N,O*-bis-(trimethylsilyl)-trifluoroacetamide/1.0% trimethylchlorosilane (Pierce Chemical Co., Rockford, IL). Gas chromatography-mass spectrometry was performed on a Varian 3700 gas chromatograph (Varian Associates, Palo Alto, CA) using a SE-52 fused-silica capillary column and a Kratos MS 25 mass spectrometer (Kratos Analytical Instruments, Ramsay, NJ). Mass spectra were obtained after electron impact with an ionization energy of 25 eV, as previously described (18).

Measurements of cytochrome c reductase activity. Reaction mixtures (0.5 ml) contained 40 μM ferricytochrome *c*, 0.2 mM potassium cyanide, 50 μg/ml superoxide dismutase, and 0.4 mM NADH or NADPH in phosphate (10 mM)-buffered 140 mM NaCl, pH 7.4. To initiate the reaction, 0.05-ml aliquots of subcellular fractions were added, and reduction of cytochrome *c* was monitored spectrophotometrically at 550

Table I. Biochemical Markers in Subcellular Fractions of Human PMN

Fraction	Protein*	DNA*	Lipid phosphorus*	β-Glucuronidase*	Lysozyme*	Lactate dehydrogenase*
	mg	*μg/mg of protein*	*nmol/mg of protein*	*nmol/h · mg of protein*	*U/min · mg of protein*	*IU/mg of protein*
Intact PMN	3.4±0.3	131±13	144±17	201±28	1,130±470	4.8±0.9
S1	3.1±0.5	31±5	99±36	211±37	1,250±310	5.1±0.9
P1	0.8±0.4	538±61	220±120	73±45	870±330	1.6±1.2
S2	2.1±0.4	2±1	60±33	20±7	200±110	7.4±0.9
P2	1.1±0.2	56±23	224±224	493±112	2,970±610	0.2±0.2
S3	2.0±0.3	2±1	0	17±6	160±110	7.6±0.7
P3	0.12±0.4	9±4	727±203	36±15	450±260	0.7±0.6

* PMN were disrupted by sonication and then subjected to differential centrifugation as described in Methods. Results for protein (55×10^6 PMN), and the specific activities of β-glucuronidase, lysozyme, and lactate dehydrogenase are mean values (±standard deviation) obtained in 13 experiments. The specific contents of DNA and lipid phosphorus are mean values (±standard deviation) obtained in five experiments.

nm. Results are expressed as nanomoles of cytochrome *c* reduced per minute per milligram of protein, using a molar extinction coefficient (reduced − oxidized) of 19,500 cm^{-1} (29). Inclusion of potassium cyanide to inhibit mitochondrial heme enzymes was subsequently determined to be unnecessary, in that identical results were obtained in its absence.

Spectrophotometric studies. An Aminco DW-2a spectrophotometer (American Instrument Co., Silver Spring, MD) was used to record difference spectra of the granule (P2) and membrane (P3) fractions (30). Suspensions of each fraction (1.0–3.0 mg protein/ml) were divided into two cuvettes, and a baseline of equal light absorbance from 400 to 600 nm was determined. The sample cuvette was gently bubbled with carbon monoxide, and the difference spectrum was recorded to determine the amount of contamination with oxyhemoglobin. A few grains of sodium dithionite were then added to the sample cuvette, and the carbon monoxide (dithionite-reduced versus oxidized) difference spectrum was recorded. A similar amount of dithionite was then added to the reference cuvette, and the carbon monoxide (dithionite-reduced versus dithionite-reduced) difference spectrum was recorded. Cytochrome P-450 and cytochrome *b* were quantified using molar extinctions coefficients of 91,000 cm^{-1} (31) and 21,600 cm^{-1} (32), respectively.

Results

Subcellular fractionation of human PMN. Human PMN were rapidly and efficiently disrupted by sonication. The degree of cell disruption depended primarily on the power applied to the probe and on the duration of sonication (data not shown). Sonication with 100 W of power for a total duration of 60 s disrupted 93±9% of PMN (mean±SD, $n = 13$) (measured by release of the cytoplasmic enzyme, lactate dehydrogenase).

To prepare granule, membrane, and cytosol fractions, crude PMN sonicates were subjected to differential centrifugation. Low-speed centrifugation (150 *g* for 10 min) of crude PMN sonicates sedimented nuclei and unbroken cells (P1). P1 contained >80% of total cellular DNA, and the specific content of DNA (micrograms per milligram of protein) in P1 was 17-fold greater than that in the supernatant, S1 (Table I, Fig. 1). Medium-speed centrifugation (18,000 *g* for 30 min) of S1 sedimented lysosomal granules (P2). P2 was highly enriched with respect to the marker enzymes of specific and azurophil granules, i.e., P2 contained >77% of total cellular lysozyme and β-glucuronidase (Table I, Fig. 1). The specific activities of these enzymes in P2 were more than 15-fold greater than those observed in S2. The small amounts of DNA in P2 most likely reflected contamination with single nuclear lobes (21). It is likely that the small number of mitochondria in human PMN also sedimented with the lysosomal granules in P2 (21). High-speed centrifugation (100,000 *g* for 60 min) of S2 sedimented membranes (P3). P3 was most enriched with respect to phospholipid, containing 727 nmol of lipid phosphorus/mg of protein (Table I, Fig. 1). Whereas P3 contained almost 20% of total cellular lipid phosphorus, only very small amounts (<1% of total cellular content) of DNA, lactate dehydrogenase, lysozyme, and β-glucuronidase were detected in this fraction (Table I). S3 (cytosol) was most enriched with respect to the cytoplasmic enzyme, lactate dehydrogenase (Table I, Fig. 1). Almost 93% of total cellular lactate dehydrogenase activity was found in S3. In contrast, negligible amounts of lactate dehydrogenase were detected in the granule and membrane fractions.

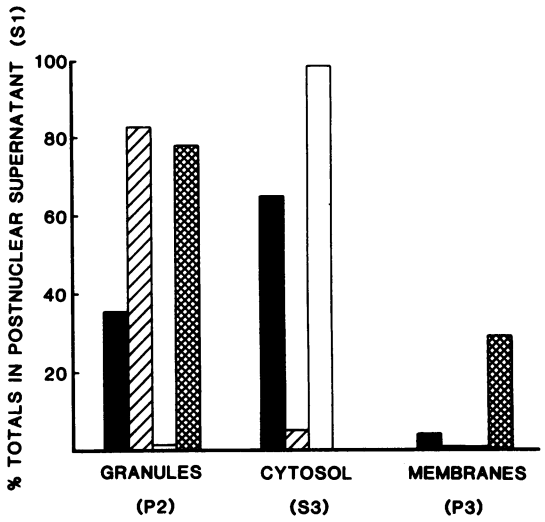

Figure 1. Distribution of biochemical markers in subcellular fractions of human PMN. PMN were disrupted by sonication, and subjected to subcellular fractionation as described in Methods. Aliquots of each fraction were assayed for protein (*solid bars*), β-glucuronidase activity (granule marker) (*hatched bars*), lactate dehydrogenase activity (cytosol marker) (*open bars*), and lipid phosphous (membrane marker) (*cross-hatched bars*). Results are expressed as percentages of totals in postnuclear supernatants (S1) (derived from data in Table I). The distribution of lysozyme was almost identical to that of β-glucuronidase (not shown).

Surprisingly, sonication disrupted only a small percentage of lysosomal granules. Disruption of granules was estimated in two ways. First, the activity of lysozyme in S1 was measured in the presence and absence of Triton X-100 (0.1%, vol/vol). In the absence of detergent, lysozyme activity was reduced 89.6±5.2% (mean±SD). Second, the activities of lysozyme and β-glucuronidase were measured in the cytosol fraction (S3). S3 contained only 8% of the total lysozyme activity and 5% of the total β-glucuronidase activity that were observed in S1. Thus, sonication disrupted >90% of cells, but <10% of lysosomal granules.

Subcellular localization of LTB_4 ω-hydroxylase activity. As we have reported previously (8, 15), intact PMN (25×10^6 cells/ml) in buffer alone rapidly and efficiently catabolized exogenous LTB_4 (1.0 μM) by ω-oxidation (46±14 pmol/min · mg of protein, mean±SD, $n = 13$). In contrast, crude PMN sonicates exhibited only very small amounts of LTB_4 ω-hydroxylase activity (4±4 pmol/min · mg of protein, mean±SD, $n = 5$), and only in some experiments. In the presence of added NADPH (1.0 mM), however, crude sonicates exhibited significant LTB_4 ω-hydroxylase activity (82±12 pmol/min · mg of protein). In fact, the specific activity of the LTB_4 ω-hydroxylase in crude sonicates containing 1.0 mM NADPH was almost twice that observed in intact PMN.

The products generated from LTB_4 in crude PMN sonicates were identified as 20-OH-LTB_4 and 20-COOH-LTB_4 by co-chromatography with synthetic standards after both reverse-phase and normal-phase HPLC, and in the case of 20-OH-LTB_4, by gas chromatography-mass spectrometry. Results of previous studies indicated that intact PMN catabolize LTB_4 to 20-OH-LTB_4, which is then converted to 20-COOH-LTB_4 (8). In experiments with PMN sonicates, however, LTB_4 was converted in the presence of NADPH primarily to 20-OH-LTB_4 (ratio of 20-OH-LTB_4 to 20-COOH-LTB_4 > 6:1).

LTB_4 ω-hydroxylase activity (in the presence of 1.0 mM NADPH) was determined in each of the PMN subcellular fractions isolated by differential centrifugation (Table II, Fig. 2). LTB_4 ω-hydroxylase activity was detected almost exclusively in the membrane fraction (P3). Whereas P3 contained <4% of the total protein in S1, P3 contained 41% of the total LTB_4 ω-hydroxylase activity in S1. In contrast, negligible amounts of LTB_4 ω-hydroxylase activity were detected in the granule (P2) and cytosol (S3) fractions. The specific activity of the LTB_4 ω-hydroxylase in P3 (708±214 pmol/min · mg of protein) was 10-fold greater than that observed in S1. The products of LTB_4 generated during incubation with P3 were identical with those generated during incubation with crude PMN sonicates. Thus, the membrane fraction, P3, represented a partially purified preparation of the LTB_4 ω-hydroxylase in human PMN.

Table II. LTB_4 ω-Hydroxylase and Cytochrome c Reductase Activities in Subcellular Fractions of Human PMN

Fraction	LTB_4 ω-hydroxylase*	NADPH-dependent cytochrome *c* reductase‡	NADH-dependent cytochrome *c* reductase‡
	pmol/min · mg protein	*nmol/min · mg protein*	*nmol/min · mg protein*
Intact PMN	46±14	—	—
S1	70±10	1.9±0.4	12.9±2.2
P1	106±32	2.4±0.5	11.8±5.1
S2	63±12	1.9±0.6	9.5±2.4
P2	2±7	0.5±0.6	4.3±3.3
S3	1±2	0.9±0.5	2.1±1.1
P3	708±214	20.1±6.8	109.8±33.3

* LTB_4 ω-hydroxylase activity was measured in the presence of 1.0 mM NADPH. Results represent mean values (±SD) obtained in 13 experiments.

‡ Results represent mean values (±SD) obtained in nine experiments.

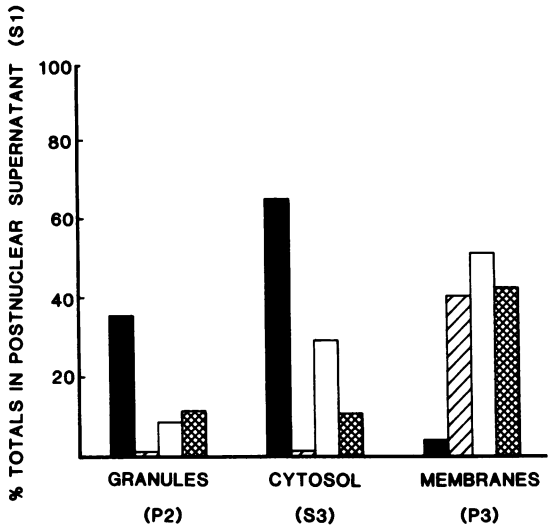

Figure 2. Distribution of LTB_4 ω-hydroxylase and cytochrome *c* reductase activities in subcellular fractions of human PMN. Aliquots of each subcellular fraction were analyzed for protein (*solid bars*), LTB_4 ω-hydroxylase activity (*hatched bars*), NADPH-dependent cytochrome *c* reductase activity (*open bars*), and NADH-dependent cytochrome *c* reductase activity (*cross-hatched bars*). Total enzyme activities in each subcellular fraction are expressed as a percentage of total activities in postnuclear supernatants (S1) (derived from data in Table II).

Measurements of LTB_4 ω-hydroxylase activity in isolated PMN membranes were highly reproducible. For example, the coefficient of variability (standard deviation/mean) for nine consecutive determinations of LTB_4 ω-hydroxylase activity in the membrane fraction prepared from PMN of one donor was 6.5%. In addition, LTB_4 ω-hydroxylase activity in isolated PMN membranes was linear with respect to both duration of incubation and protein concentration (Fig. 3).

In contrast to the nearly complete recoveries of lactate dehydrogenase, lysozyme, and β-glucuronidase, only 42% of the total LTB_4 ω-hydroxylase activity in S1 was recovered in the granule, cytosol, and membrane fractions. No LTB_4 ω-hydroxylase activity was lost during the low-speed (150 *g*) centrifugation. In contrast, LTB_4 ω-hydroxylase activity appeared to be lost during the medium-speed (18,000 *g*) and high-speed (100,000 *g*) centrifugation procedures. Only 63% of the ω-hydroxylase activity in S1 could be accounted for by the sum of the separate activities in S2 and P2. Similarly, only 65% of the ω-hydroxylase activity in S2 could be accounted for by the sum of the separate activities in S3 and P3. However, results of mixing experiments indicated that "enhancing factors" (33) may be present in the inactive fractions. For example, by including P2 in reaction mixtures containing S2 and LTB_4, the rate of ω-oxidation increased by 40±21% (mean±SD, $n = 5$). Similarly, by including S3 in reaction mixtures containing P3 and LTB_4, the rate of ω-oxidation increased by 15±11% (mean±SD, $n = 11$). Thus, losses

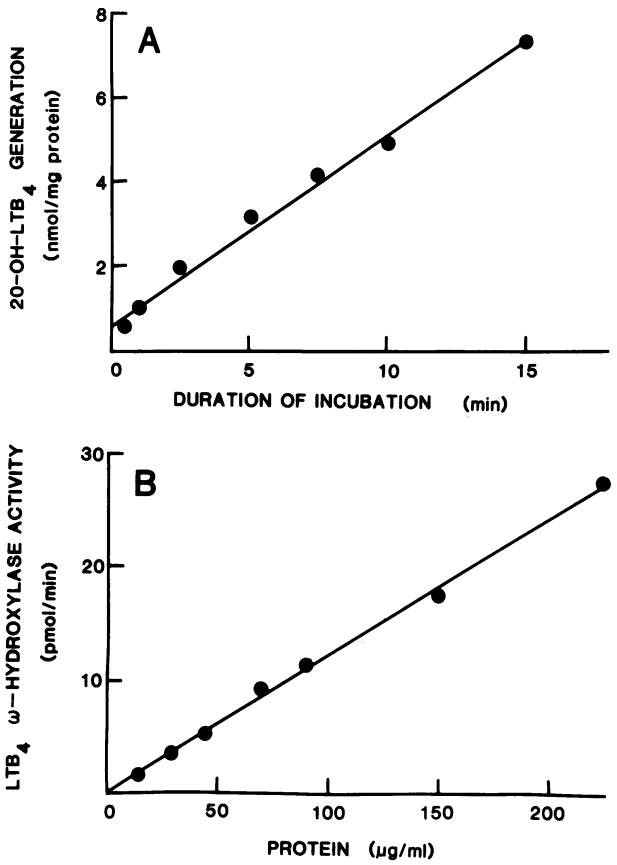

Figure 3. LTB_4 ω-hydroxylase activity in isolated PMN membranes: dependence on duration of incubation and protein concentration. (*A*) LTB_4 (1.0 μM) was incubated with isolated PMN membranes (150 μg protein/ml) and 1.0 mM NADPH for 0.5–15 min at 37°C. (*B*) LTB_4 (1.0 μM) was incubated for 5 min at 37°C with isolated PMN membranes (15–225 μg protein/ml) and 1.0 mM NADPH.

of LTB_4 ω-hydroxylase activity during isolation of membrane fractions actually were small.

Whereas large amounts of LTB_4 ω-hydroxylase activity were recovered after disruption of PMN by sonication, only small amounts were recovered after disruption of PMN by nitrogen cavitation. In each of eight experiments performed using nitrogen cavitation, the specific activity of the LTB_4 ω-hydroxylase in cavitates was <20% of the mean specific activity observed in crude PMN sonicates. In fact, in several of these experiments, LTB_4 ω-hydroxylase activity was undetectable after nitrogen cavitation. Paired experiments were performed in which PMN from the same donor (and in the same buffer) were disrupted either by sonication or by nitrogen cavitation. Although a similar percentage of cells were disrupted by the two procedures, LTB_4 ω-hydroxylase activity in sonicates (90 pmol/min · mg of protein) was much greater than that in cavitates (8 pmol/min · mg of protein) (average of two separate experiments). NADPH-dependent cytochrome *c* reductase activities in the sonicates and cavitates were not significantly different (data not shown). Finally, cavitation did not appear to generate an inhibitor of the ω-hydroxylase. LTB_4 ω-hydroxylase activity in PMN sonicates (90 pmol/min · mg of protein) was not inhibited by the addition of an equal volume of PMN cavitate (94 pmol/min · mg of protein) (average of two separate experiments).

Effects of reduced pyridine nucleotides, oxygen, and pH on LTB_4 ω-hydroxylase activity. As indicated above, the partially purified LTB_4 ω-hydroxylase in P3 required reduced pyridine nucleotides for activity. NADPH, for example, enhanced LTB_4 ω-hydroxylase activity in a concentration-dependent fashion (Fig. 4). LTB_4 ω-hydroxylase activity in the absence of NADPH was only 1±2% of the activity observed in the presence of 1.0 mM NADPH. NADH, on the other hand, was much less effective in enhancing ω-hydroxylase activity (Fig. 4). LTB_4 ω-hydroxylase activity in the presence of 1.0 mM NADH was only 21±4% of the activity observed in the presence of 1.0 mM NADPH. Furthermore, LTB_4 ω-hydroxylase activity in the presence of both 1.0 mM NADH and 1.0 mM NADPH was not significantly different (104±6%) than the activity observed in the presence of 1.0 mM NADPH alone (mean±SD, $n = 5$). The apparent K_m of the LTB_4 ω-hydroxylase for NADPH was 0.50±0.25 μM; the apparent K_m for NADH was 223±53 μM (mean±SD, $n = 3$).

Results of three experiments performed with nitrogen-saturated buffers (15) indicated that LTB_4 ω-hydroxylase activity also was dependent on molecular oxygen. Under anaerobic conditions, LTB_4 ω-hydroxylase activity in isolated PMN membranes was decreased to <5% of that observed in the presence of oxygen.

LTB_4 ω-hydroxylase activity in isolated PMN membranes was greatly influenced by pH. Maximal activity of the enzyme was observed at a pH of 7.4 (Fig. 5). Significantly reduced activity was observed at a pH < 6.7 and at a pH > 8.0.

Dependence of LTB_4 ω-hydroxylase activity on substrate concentration. To examine LTB_4 ω-hydroxylase activity as a function of substrate concentration, membrane fractions (P3) were incubated with various concentrations of LTB_4 (in the presence of 1.0 mM NADPH) (Fig. 6). LTB_4 ω-hydroxylase activity increased with increasing concentrations of LTB_4. Analysis of these data by a Lineweaver–Burk plot (*inset* in Fig. 6) yielded a straight line. Thus, the partially purified ω-hydroxylase had a K_m for LTB_4 of 0.6 μM and a V_{max} of 1.1 nmol/min · mg protein.

Specificity of the PMN ω-hydroxylase. To examine the specificity of the PMN ω-hydroxylase, various dihydroxylated deriv-

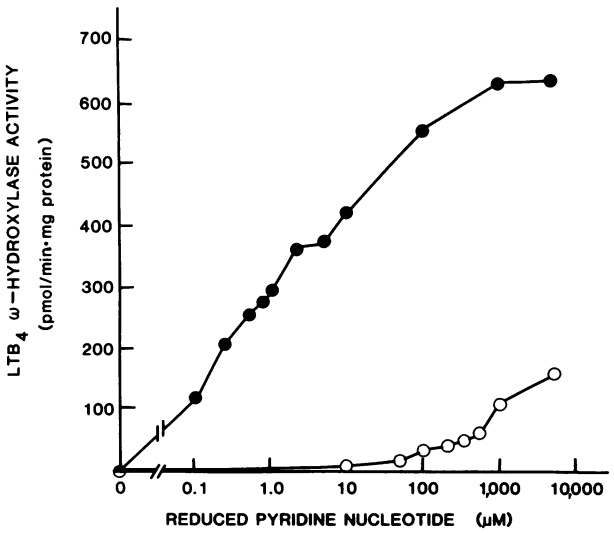

Figure 4. LTB_4 ω-hydroxylase activity in isolated PMN membranes: dependence on reduced pyridine nucleotides. Shown are results of a representative experiment in which LTB_4 (1.0 μM) was incubated with isolated PMN membranes (180 μg protein/ml) for 5 min at 37°C in the presence of either NADPH (●) or NADH (○) (0.1–5,000 μM).

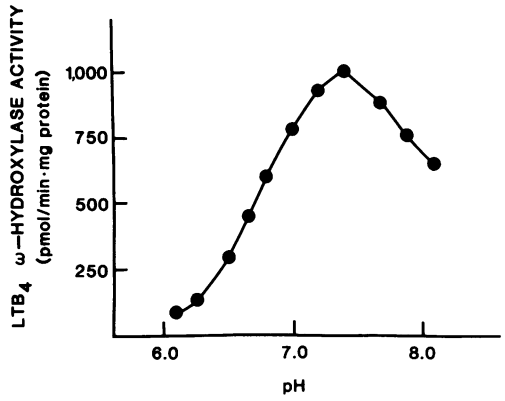

Figure 5. LTB_4 ω-hydroxylase activity in isolated PMN membranes: dependence on pH. LTB_4 (1.0 μM) was incubated with isolated PMN membranes (128 μg protein/ml) and 1.0 mM NADPH for 5 min at 37°C in 50 mM Hepes buffer adjusted to pH 6.0–8.0 with NaOH.

atives of arachidonic acid were incubated at a concentration of 1.0 μM with PMN membrane fractions containing 1.0 mM NADPH. The rates of ω-oxidation of three 5-lipoxygenase products, i.e., 5,12-diHETE, 5(S),12(R)-all-*trans*-LTB_4, and 5(S),-12(S)-all-*trans*-LTB_4 were 45%, 9%, and 8%, respectively, of the rate observed with LTB_4. The rates of ω-oxidation of four 15-lipoxygenase products, i.e., 5,15-diHETE, 8,15-diHETE, and two all-*trans*-conjugated isomers of 8,15-diHETE, were all <1% of the rate observed with LTB_4.

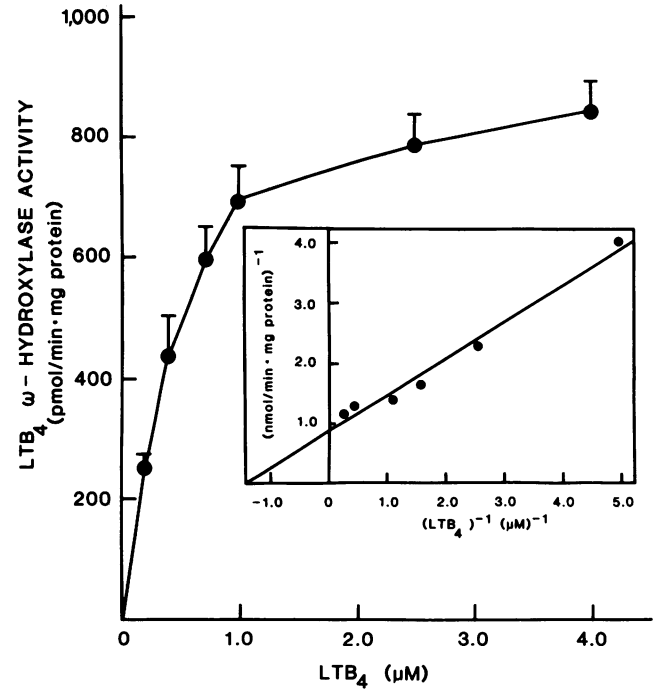

Figure 6. Dependence of LTB_4 ω-hydroxylase activity on substrate concentration. Isolated PMN membranes (127–140 μg protein/ml) were incubated with various concentrations of LTB_4 for 4 min at 37°C in the presence of 1.0 mM NADPH. Results for LTB_4 ω-hydroxylase activity represent the means±SD for three separate experiments. (*Inset*) Lineweaver–Burk plot of this data, and the straight line obtained by linear regression is defined by a slope of 0.59 and a *y*-intercept of 0.93.

Inhibition of LTB_4 ω-hydroxylase activity. LTB_4 ω-hydroxylase activity in isolated PMN membranes was inhibited by a number of agents that characteristically inhibit hepatic cytochrome P-450 enzymes (Table III). Carbon monoxide, ferricytochrome *c*, SKF-525A, and Triton X-100 inhibited the LTB_4 ω-hydroxylase significantly. In contrast, α-naphthoflavone, which inhibits some, but not all, hepatic cytochrome P-450 isoenzymes (12), did not affect LTB_4 ω-hydroxylase activity. Azide and cyanide, which inhibit other heme enzymes, such as myeloperoxidase (34) and cytochrome oxidase (35), also did not inhibit ω-oxidation of LTB_4. Finally, ω-oxidation of LTB_4 was not inhibited by either catalase or superoxide dismutase.

Cytochrome c reductase activities. NADPH- and NADH-dependent cytochrome *c* reductase activities also were concentrated in isolated PMN membranes (P3) (Table II, Fig. 2). As was the case with the NADPH-dependent LTB_4 ω-hydroxylase, the specific activity of the NADPH-dependent cytochrome *c* reductase in the membrane fraction was increased 10-fold as compared to the specific activity in S1. On the other hand, whereas minimal LTB_4 ω-hydroxylase activity was detected in the granule and cytosol fractions, these fractions contained significant cytochrome *c* reductase activity. In all PMN subcellular fractions, the specific activity of cytochrome *c* reductase in the presence of 0.4 mM NADH was ~6-fold greater than that observed in the presence of 0.4 mM NADPH. However, apparent K_m values for NADH- and NADPH-dependent cytochrome *c* reduction in the membrane fraction were similar (~1.0 μM).

Superoxide dismutase was included in the assay for cytochrome *c* reductase in order to prevent reduction of cytochrome *c* by superoxide anion radicals that might be generated by the NADPH-dependent oxidase of PMN (36). However, because PMN were not stimulated before cell disruption, we found no evidence of NADPH oxidase activity in these experiments. Cy-

Table III. LTB_4 ω-Hydroxylase Activity in Isolated PMN Membranes is Inhibited by Carbon Monoxide, Ferricytochrome c, SKF-525A, and Triton X-100

Reagent (strength)	LTB_4 ω-hydroxylase activity*
	% of control
Buffer	100
Carbon monoxide (CO/O_2 ratio = 11)	15
Ferricytochrome *c* (10 μM)	75
Ferricytochrome *c* (100 μM)	4
SKF-525A (0.1 mM)	83
SKF-525A (0.5 mM)	27
SKF-525A (1.0 mM)	2
Triton X-100 (0.02%, vol/vol)	0
α-Naphthoflavone (10 μM)	100
α-Naphthoflavone (50 μM)	97
Sodium azide (1.0 mM)	104
Catalase (20 μg/ml)	99
Potassium cyanide (1.0 mM)	122
Superoxide dismutase (50 μg/ml)	96

* LTB_4 (1.0 μM) was incubated for 5 min at 37°C with aliquots of PMN membrane fractions in the presence of 1.0 mM NADPH and the reagents listed. Control LTB_4 ω-hydroxylase activity was measured in the presence of NADPH alone. Results represent average values obtained in two experiments.

tochrome *c* reduction was exactly the same in the presence and absence of superoxide dismutase (data not shown).

Spectrophotometric studies. Carbon monoxide (dithionite-reduced versus dithionite-reduced) difference spectra of PMN membrane fractions (P3) exhibited a major peak at 452 nm, characteristic of cytochrome P-450 (Fig. 7). A second peak at 420 nm also was observed, characteristic of cytochrome P-420. As described by others (37, 38), both the membrane and granule fractions exhibited dithionite-reduced vs. oxidized difference spectra characteristic of cytochrome *b* (peaks at 429 nm and 559 nm). Granule fractions also exhibited a peak at 474 nm, characteristic of reduced myeloperoxidase (34) (Fig. 8). There was no evidence for contamination with oxyhemoglobin, in that treatment of either the membrane or granule fractions with carbon monoxide alone did not reveal a peak at 420 nm.

The specific content of cytochrome P-450 in isolated PMN membranes (P3) was 0.044±0.017 nmol/mg of protein. The specific content of cytochrome *b* in the membrane and granule fractions was 0.48±0.13 and 0.47±0.31 nmol/mg of protein, respectively (mean±SD, $n = 4$). Thus, 55×10^6 PMN contain ~5.3 pmol of cytochrome P-450 and 570 pmol of cytochrome *b*.

Stability of the LTB_4 ω-hydroxylase. Results of preliminary experiments indicated that the LTB_4 ω-hydroxylase in PMN sonicates was labile (e.g., after 12 h at 24°C and 4°C, sonicates lost 100% and 26% of activity, respectively). Treatment of PMN with DFP, EDTA, and EGTA (to inhibit proteolysis and lipid peroxidation) (39, 40) markedly attenuated losses of activity (data not shown). Because of this observation, we routinely included DFP, EDTA, and EGTA in the buffers used for disrupting PMN and for subcellular fractionation.

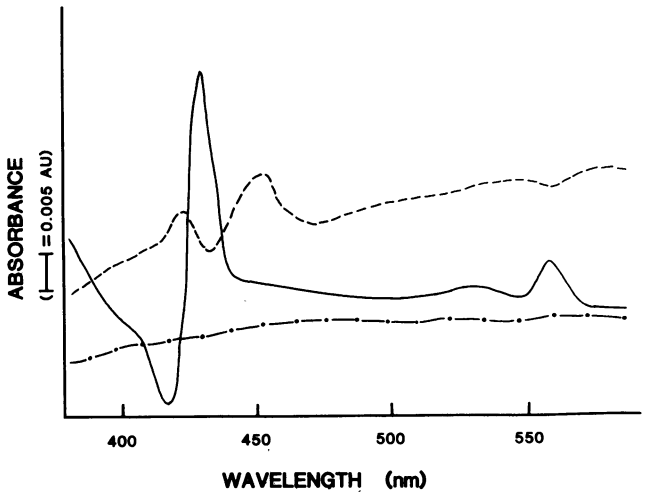

Figure 7. Carbon monoxide difference spectra of isolated PMN membranes. Suspensions of isolated PMN membranes (1.0–3.0 μg of protein) were divided into two cuvettes, and absorbance was measured at wavelengths from 400 to 600 nm. The difference spectrum shown by the interrupted line (-·-·-) was obtained after treating the contents of the sample cuvette with carbon monoxide. The carbon monoxide (dithionite-reduced vs. oxidized) difference spectrum shown by the solid line (——) was obtained after treating the contents of the sample cuvette with sodium dithionite (plotted at half scale). The carbon monoxide (dithionite-reduced vs. dithionite-reduced) difference spectrum shown by the dashed line (– – –) was obtained after treating the contents of the reference cuvette with dithionite. Identical results were obtained in separate experiments performed with membranes isolated from PMN of four normal donors.

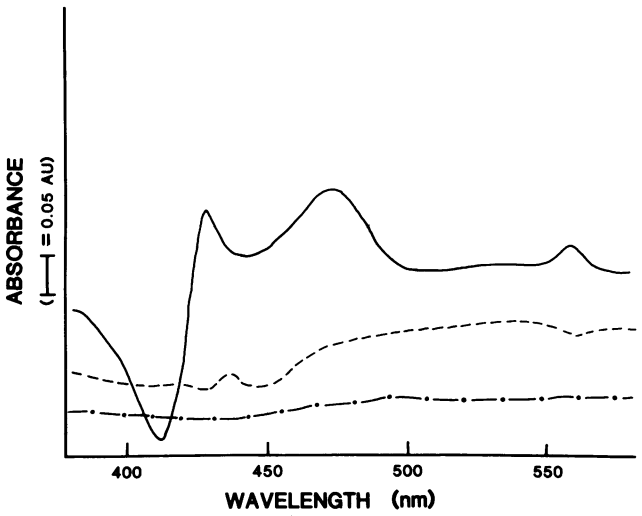

Figure 8. Carbon monoxide difference spectra of isolated PMN granules. Suspensions of granules (1.0–3.0 μg of protein) were divided into two cuvettes and difference spectra were obtained as described in Fig. 7.

LTB_4 ω-hydroxylase activity in membrane fractions was extremely stable when stored at −70°C. >90% of activity was retained after storage for 1 mo. In contrast, significant losses of LTB_4 ω-hydroxylase activity (>50%) were observed when membrane fractions were stored for 1 wk or longer at 4°C. However, addition of 20% (vol/vol) glycerol to membrane fractions stabilized the LTB_4 ω-hydroxylase during storage at 4°C. LTB_4 ω-hydroxylase activity in membrane fractions decreased significantly during storage at 24°C, even with the addition of glycerol.

Discussion

Results of experiments described in this report indicate that the LTB_4 ω-hydroxylase in human PMN is dependent on NADPH and molecular oxygen, is inhibited by ferricytochrome *c*, SKF-525A, carbon monoxide, and Triton X-100, and is associated with a membrane fraction that exhibits a carbon monoxide difference spectrum with a peak at 452 nm. In all respects, therefore, the LTB_4 ω-hydroxylase in human PMN resembles cytochrome P-450 enzymes found in liver.

To partially purify the LTB_4 ω-hydroxylase, human PMN were treated with DFP to inactivate serine proteinases (39), and were disrupted by sonication. Sonication proved to be a simple and efficient method for disrupting cells. Sonication disrupted >90% of PMN, but <10% of their lysosomal granules. These results compare favorably with those obtained by using nitrogen cavitation. For example, in experiments reported by Klempner et al. (21), nitrogen cavitation disrupted 85.4% of PMN, and ~11% of their lysosomal granules. However, for the purpose of purifying the LTB_4 ω-hydroxylase, sonication was greatly superior to nitrogen cavitation. Whereas PMN sonicates (in the presence of NADPH) exhibited large amounts of LTB_4 ω-hydroxylase activity, PMN cavitates (in the presence of NADPH) exhibited only very small amounts or none at all. The mechanism by which nitrogen cavitation reduces the activity of the enzyme(s) responsible for ω-oxidation of LTB_4 was not determined, al-

though differences in NADPH-dependent cytochrome *c* reductase activity and generation of an inhibitor have been excluded. In any case, the specific activity of the LTB_4 ω-hydroxylase in crude PMN sonicates (82 pmol/min · mg of protein) actually exceeded the specific activity in intact PMN (46 pmol/min · mg of protein).

Crude PMN sonicates were subjected to differential centrifugation to yield granule, cytosol, and membrane fractions (identified by biochemical markers) (Table I, Fig. 1). LTB_4 ω-hydroxylase activity was found almost exclusively in the membrane fraction, and was recovered in high yield (Table II, Fig. 2). Almost 42% of the LTB_4 ω-hydroxylase activity in S1 was recovered in the membrane fraction. In contrast, negligible LTB_4 ω-hydroxylase activity was detected in the cytosol and granule fractions. The specific activity of the LTB_4 ω-hydroxylase in the membrane fraction (708 pmol/min · mg of protein) was increased ~10-fold as compared to the specific activity observed in S1. On the other hand, the specific activities of lysozyme, β-glucuronidase, and lactate dehydrogenase in the membrane fraction were decreased six-, three-, and sevenfold, respectively. Thus, by isolating the membrane fraction of PMN, we partially purified the LTB_4 ω-hydroxylase.

Lu et al. (41) resolved and reconstituted the monooxygenase system in liver microsomes that catalyzes ω-hydroxylation of fatty acids. Five components were required for maximal activity, i.e., membrane phospholipid, NADPH, NADPH-cytochrome *c* reductase, molecular oxygen, and cytochrome P-450. Prostaglandins also are substrates for the reconstituted liver microsomal enzyme system, and are hydroxylated at both the (ω-1)- and ω-carbons (13). The LTB_4 ω-hydroxylase that we partially purified from human PMN resembles the monooxygenase system in liver microsomes with respect to its requirement for all five components.

First, LTB_4 ω-hydroxylase activity in human PMN is associated almost exclusively with membranes (Fig. 2). Hepatic cytochrome P-450 has been localized to a specialized membrane compartment, the endoplasmic reticulum (42). Because the membrane fraction of human PMN that we isolated is composed of contributions from the plasma membrane, the endoplasmic reticulum, and the Golgi complex, additional studies will be required to determine which of these membrane components actually contains the LTB_4 ω-hydroxylase. Results of a recent study by Nichols et al. (43) suggest that the endoplasmic reticulum in human PMN is more extensive than previously appreciated. Although mature PMN synthesize little protein, the endoplasmic reticulum may prove to be an important site of other enzymatic processes, such as catabolism of LTB_4 by ω-oxidation.

Second, the LTB_4 ω-hydroxylase in isolated PMN membranes requires reduced pyridine nucleotides for catalytic activity (Fig. 4). Whereas negligible LTB_4 ω-hydroxylase activity was observed in the absence of reduced pyridine nucleotides, large amounts of activity were observed in the presence of extremely low concentrations of NADPH (apparent K_m for NADPH = 0.5 μM). LTB_4 ω-hydroxylase activity also was observed in the presence of NADH (apparent K_m for NADH = 223 μM), but the rate of ω-oxidation of LTB_4 in the presence of 1.0 mM NADH was 20% of the maximal rate observed in the presence of 1.0 mM NADPH. Inasmuch as the concentrations of NADPH and NADH in resting PMN are ~50 μM (44), it is likely that sufficient NADPH is available in resting intact cells to support ω-oxidation of LTB_4. We have reported previously that intact PMN do not require either activation or exogenous NADPH to rapidly catabolize LTB_4 by ω-oxidation (8, 15). Thus, like hepatic cytochrome P-450, the LTB_4 ω-hydroxylase in human PMN is active in resting cells, but is dependent on exogenous NADPH when partially purified.

Third, NADPH-dependent cytochrome *c* reductase activity appears to be an important component of the LTB_4 ω-hydroxylase system in isolated PMN membranes. As was the case for LTB_4 ω-hydroxylase activity, NADPH-dependent cytochrome *c* reductase activity was concentrated ~10-fold in isolated PMN membranes (Table II, Fig. 2). Because very low concentrations of ferricytochrome *c* inhibited LTB_4 ω-hydroxylase activity in isolated PMN membranes (Table III), it is likely that the NADPH-dependent cytochrome *c* reductase plays a role in the pathway leading to ω-oxidation of LTB_4 in intact human PMN.

Fourth, like partially purified hepatic cytochrome P-450 enzymes, the LTB_4 ω-hydroxylase in isolated PMN membranes requires molecular oxygen for catalytic activity. Under anaerobic conditions, LTB_4 ω-hydroxylase activity in isolated PMN membranes was decreased >20-fold.

Finally, it appears that cytochrome P-450 is the terminal oxygenase of the LTB_4 ω-hydroxylase system in isolated PMN membranes. LTB_4 ω-hydroxylase activity in isolated PMN membranes was inhibited significantly by agents that characteristically reduce the activity of cytochrome P-450 enzymes (Table III). As was the case for LTB_4 ω-hydroxylase activity in intact PMN (15), LTB_4 ω-hydroxylase activity in isolated PMN membranes also was inhibited by carbon monoxide. SKF-525A, which inhibits hydroxylation of prostaglandins by liver microsomes (45), and Triton X-100, which inhibits hepatic cytochrome P-450-mediated reactions (46), also significantly inhibited LTB_4 ω-hydroxylase activity in isolated PMN membranes. On the other hand, α-naphthoflavone, which inhibits some, but not all, hepatic cytochrome P-450 isoenzymes (12), did not affect LTB_4 ω-hydroxylase activity. Agents such as azide, cyanide, catalase, and superoxide dismutase, which either inhibit the activities of heme enzymes other than cytochrome P-450 or scavenge toxic oxygen metabolites, also did not influence LTB_4 ω-hydroxylase activity.

In addition to the indirect evidence provided by studies with inhibitors, direct evidence that isolated PMN membranes contain a cytochrome P-450 was obtained spectrophotometrically. Carbon monoxide (dithionite-reduced vs. dithionite-reduced) difference spectra of membrane fractions exhibited a major peak at 452 nm (Fig. 7), characteristic of cytochrome P-450, and a minor peak at 420 nm, characteristic of cytochrome P-420 (an inactive form of P-450) (31, 47). In contrast, carbon monoxide (dithionite-reduced vs. dithionite-reduced) difference spectra of granule fractions exhibited only a very small peak at 438 nm (Fig. 8). Thus, both LTB_4 ω-hydroxylase activity and spectrophotometric evidence of a cytochrome P-450 were found almost exclusively in the membrane fraction of human PMN.

Although spectrophotometric studies have been performed previously to examine the putative role played by cytochrome *b* in the superoxide anion-generating system of human PMN, the presence of a cytochrome P-450 in these cells has not been reported. There are at least four possible explanations to account for previous failures to detect cytochrome P-450 in human PMN. First, the influence of carbon monoxide on difference spectra has been examined in only a few instances (37, 48–50). Second, PMN contain only small amounts of cytochrome P-450 (see below). Third, cytochrome P-450 is relative labile and susceptible to inactivation (e.g., conversion to cytochrome P-420) by proteolytic enzymes (51). Interestingly, a spectrophotometric study

performed previously with PMN membrane fractions isolated without antiproteinases did, in fact, reveal a peak near 420 nm after treatment with carbon monoxide (48). Finally, unless spectrophotometric studies are performed using the procedures that we used, the difference spectrum of cytochrome P-450 in PMN is likely to be obscured by the difference spectrum of cytochrome *b*. For example, we found that the specific content of cytochrome *b* in the membrane fraction was ~10-fold greater than the specific content of cytochrome P-450. Only by reducing the contents of both the sample and reference cuvettes with dithionite, and by treating the sample alone with carbon monoxide, is the prominent spectrum of reduced cytochrome *b* subtracted.

Human PMN contain considerably less cytochrome P-450 than does liver. The specific content of cytochrome P-450 in isolated PMN membranes (0.04 nmol/mg of protein) is ~40-fold less than the specific content of cytochrome P-450 in rat liver microsomes (47). The specific content of cytochrome P-450 in PMN membranes, however, is comparable to the specific content of cytochrome P-450 in membranes isolated from other extrahepatic tissues, such as lung or intestine (52).

Although human PMN contain only small amounts of cytochrome P-450, catabolism of LTB_4 by the ω-hydroxylase is extremely efficient. The K_m for LTB_4 of the partially purified ω-hydroxylase of human PMN is 0.6 μM, and the V_{max} is 1.1 nmol/min · mg of protein (Fig. 6). In comparison, the kinetic constants for the hydroxylation of prostaglandin E_1 by guinea pig liver microsomes were found to be 140 μM and 0.3 nmol/min · mg of protein, respectively (45). Moreover, when rat liver microsomes were incubated with various dihydroxylated derivatives of arachidonic acid (1.0 μM), a marked preference for LTB_4 was not observed, and LTB_4 ω-hydroxylase activity was <4% of the activity measured in membrane fractions of human PMN (Shak, S., unpublished observations). Whereas the physiologic function of hepatic and renal fatty acid hydroxylases are uncertain, results of our experiments strongly suggest that the major function of the ω-hydroxylase in human PMN is to catabolize LTB_4.

Partial purification of the LTB_4 ω-hydroxylase in human PMN has made it possible to examine other biochemical characteristics of the enzyme. For example, LTB_4 ω-hydroxylase activity in isolated PMN membranes was greatly influenced by pH (Fig. 5), in a manner similar to that described for hepatic cytochrome P-450 enzymes (41). The marked decrease in LTB_4 ω-hydroxylase activity at acidic pH may be important in vivo. Sites of inflammation in which PMN accumulate, such as septic pleural effusions, often are acidic environments (pH < 6.8) (53). Consequently, catabolism of LTB_4 in inflammatory exudates may be reduced significantly.

Results of our experiments also indicate that ω-oxidation by the partially purified enzyme in isolated PMN membranes is highly specific. Of the dihydroxylated derivatives of arachidonic acid that we tested, LTB_4 proved to be the preferred substrate. The ω-hydroxylase in intact PMN exhibits similar substrate specificity (7, 8). In fact, the K_m of the LTB_4 ω-hydroxylase in isolated membranes (0.6 μM) is similar to the apparent K_m for ω-oxidation of LTB_4 in intact PMN (1.0 μM) (7). Thus, the specificity of LTB_4 catabolism in intact human PMN appears to be a direct consequence of the substrate specificity of the LTB_4 ω-hydroxylase itself, and not a consequence of receptor-mediated uptake of LTB_4 (54).

The results of our experiments raise questions concerning the relationship between the LTB_4 ω-hydroxylase and the NADPH oxidase in human PMN that is believed to be responsible for superoxide anion generation. We have accumulated substantial evidence that the major pathway for the catabolism of LTB_4 in human PMN involves NADPH-dependent, cytochrome P-450-mediated ω-oxidation. Superoxide anion radicals, generated by the NADPH oxidase in human PMN, increase the rate of catabolism of leukotrienes C_4, D_4, and E_4 by promoting oxidation of thioether bonds (55), but actually reduce the rate of catabolism of LTB_4 (8). However, the LTB_4 ω-hydroxylase and NADPH oxidase systems may be related in several ways. For example, the LTB_4 ω-hydroxylase and the NADPH oxidase both require oxygen and NADPH. In addition, it is possible that both enzyme systems share the same NADPH-dependent cytochrome *c* reductase. It is of interest that a highly specific antibody against guinea pig liver NADPH-cytochrome *c* reductase was found capable of inhibiting superoxide anion generation by the membrane fraction isolated from phorbol myristate acetate-stimulated guinea pig PMN (56).

Badwey et al. (57) recently described the properties of an NADH-cytochrome b_5 reductase in the membrane fraction of human PMN. The function of this enzyme in PMN is not yet known. NADH-cytochrome b_5 reductase and cytochrome b_5 are thought to play a role in hepatic NADH-dependent hydroxylation and in the synergistic effect of NADH on NADPH-dependent hydroxylation (58, 59). However, it is unlikely that the NADH-cytochrome b_5 reductase in human PMN plays a major role in ω-oxidation of LTB_4, in that we found NADH to be significantly less active than NADPH as a cofactor for the LTB_4 ω-hydroxylase (Fig. 4). In addition, NADH did not increase ω-oxidation of LTB_4 in the presence of saturating concentrations of NADPH. Nevertheless, only by purifying these PMN enzymes will it be possible to directly determine whether NADH-cytochrome b_5 reductase and cytochrome b_5 contribute to ω-oxidation of LTB_4 in human PMN.

Sumimoto et al. (60) reported recently that the enzyme in human PMN which converts LTB_4 to a polar product (presumably 20-OH-LTB_4) is located in the 100,000 *g* supernatant of disrupted cells, and noted that this would be unexpected for a eukaryotic cytochrome P-450. We cannot explain this finding. Results of experiments reported here indicate that the LTB_4 ω-hydroxylase in disrupted human PMN is located almost exclusively in the 100,000 *g* pellet (P3) (Fig. 2), and has other properties that are shared by cytochrome P-450 enzymes.

In summary, results of our studies indicate that catabolism of LTB_4 in human PMN is mediated by a membrane-associated, NADPH-dependent cytochrome P-450 enzyme system (LTB_4 ω-hydroxylase). The precise roles played by LTB_4 in regulating PMN function and in mediating inflammation have not yet been determined. Although generation of LTB_4 by PMN might promote inflammation, catabolism of LTB_4 by PMN may be a mechanism whereby inflammatory reactions are modulated. Pharmacologic agents that induce synthesis of the LTB_4 ω-hydroxylase in PMN precursors may augment catabolism of LTB_4 by mature PMN and, consequently, reduce acute inflammation.

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