Studies on the Mechanism of ω-Hydroxylation of Platelet 12-Hydroxyeicosatetraenoic Acid (12-HETE) by Unstimulated Neutrophils

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Abstract

Stimulated platelets, in the presence or absence of aspirin, synthesize significant quantities of 12-hydroxyeicosatetraenoic acid (12-HETE), which is chemotactic and chemokinetic, and enhances mononuclear cell procoagulant activity. During a cell–cell interaction between stimulated platelets and unstimulated neutrophils, platelet 12-HETE is metabolized to 12,20-dihydroxyeicosatetraenoic acid (12,20-DiHETE) by neutrophils. Characteristics of the enzyme system in unstimulated neutrophils responsible for this ω-hydroxylation were investigated. A broad range of cytochrome P-450 inhibitors, as well as leukotriene B₄, blocked formation of 12,20-DiHETE. Owing largely to released proteases, neutrophil homogenization abolished activity. Pretreatment with diisopropylfluorophosphate preserved activity in neutrophil homogenates. ω-Hydroxylation of 12-HETE was confined solely to the microsomal fraction. Specific activity increased 6.6-fold compared with neutrophil sonicates. The electron donor NADPH was a required cofactor. These results indicate that the enzyme in unstimulated human neutrophils, which metabolizes 12-HETE from stimulated platelets to 12,20-DiHETE in this cell–cell interaction, is a cytochrome P-450 monooxygenase.

Introduction

Platelet activation results in release of free arachidonate, the oxygenation of which is catalyzed by two intracellular enzymes. The cyclooxygenase is particle-bound and aspirin-inhibitable and promotes formation of thromboxane A_2 (TXA_2),[1] 12S-hydroxy-5,8,10-heptadecatrienoic acid (HHT), and malonaldehyde (MDA). The platelet 12-lipoxygenase is cytoplasmic and for-mation of its oxygenation product, 12S-hydroxy-5,8-*cis*,10-*trans*,14-*cis*-eicosatetraenoic acid (12-HETE), as measured in washed platelet preparations, continues unabated after as-

pirin ingestion (1). Recently it was demonstrated in animal models that glomerular immune injury is accompanied by increased 12-HETE synthesis (2) and that 12-HETE enhances epidermal cell proliferation (3). Additional autacoid functions of 12-HETE include chemotaxis for human neutrophils in vitro (4) and in vivo (5), chemokinesis for smooth muscle cells (6), and enhancement of mononuclear procoagulant activity (7). Because stimulated platelets synthesize 12-HETE in significant quantities, it is important to determine mechanism(s) of its further metabolism, and whether it interacts with other cells.

We have previously established that neutrophils can metabolize platelet 12-HETE in vitro by two different mechanisms (8, 9). Stimulated neutrophils, in which the 5-lipoxygenase has been activated, convert platelet 12-HETE to 5S,12S-dihydroxy-6-*trans*,8-*cis*,10-*trans*,14-*cis*-eicosatetraenoic acid (5S,12S-DiHETE) (8, 10). In contrast, unstimulated neutrophils metabolize platelet 12-HETE to (12S)-12,20-dihydroxy-5,8,10,14-eicosatetraenoic acid (12,20-DiHETE) (9, 11). The mechanisms underlying this neutrophil ω-hydroxylation of platelet 12-HETE have not been studied.

In many cells and tissues, ω-oxidation of eicosanoids is carried out by cytochrome P-450 enzyme systems such as the one recently described for leukotriene B_4 in neutrophils (12–16). We have investigated whether such an enzyme might be operative in the setting of a cell–cell interaction involving an unstimulated neutrophil and 12-HETE—the product of an activated platelet. We report here, on the basis of inhibition studies, NADPH dependence, and subcellular membrane localization, that the enzyme in unstimulated human neutrophils that ω-hydroxylates released platelet 12-HETE is of the cytochrome P-450 type.

Methods

Preparation of neutrophil suspensions. Cell suspensions containing 95% neutrophils (5% eosinophils) were prepared from 240 ml of whole blood collected by free flow in plastic tubing through a 16-gauge needle (17). The blood was initially centrifuged at 200 *g* and platelet-rich plasma was removed. Remaining leukocytes and erythrocytes were suspended in Dextran T500 (1.5%). Leukocyte-rich plasma was removed and centrifuged at 280 *g* (10 min, 4°C), and the resulting pellets were suspended in cold saline. 15 ml of resuspended cells were layered on each of three 10-ml aliquots of Ficoll-Hypaque (*d* 1.077). After centrifugation at 350 *g* (30 min, 4°C), the interface was discarded and erythrocytes in each neutrophil pellet were lysed with 6 ml of distilled water (25 s). Upon return to isotonicity with 2 ml of 3.5% NaCl, 10 ml of phosphate-buffered saline (pH 7.4) was added. The suspension was centrifuged at 280 *g* for 5 min (4°C), and the neutrophils were suspended in Hepes buffer (pH 7.45) containing calcium and magnesium (8). In experiments involving subcellular fractionation, the Hepes buffer was modified (no calcium, 2.5 mM magnesium). Trypan blue exclusion by the neutrophil preparations averaged 95%, and no platelets were seen on stained smears. After exposure of neutrophils to thrombin or ionophore, 12-HETE, the platelet-derived hydroxy acid could not be identified by high-performance

1. *Abbreviations used in this paper:* DFP, diisopropylfluorophosphate; 5S,12S-DiHETE, 5S,12S-dihydroxy-6-*trans*,8-*cis*,10-*trans*,14-*cis*-eicosatetraenoic acid; 12,20-DiHETE, (12S)-12,20-dihydroxy-5,8,10,14-eicosatetraenoic acid; DTT, dithiothreitol; ETYA, 5,8,11,14-eicosatetraynoic acid; 12-HETE, 12S-hydroxy-5,8-*cis*,10-*trans*,14-*cis*-eicosatetraenoic acid; HHT, 12S-hydroxy-5,8,10-heptadecatrienoic acid; LDH, lactate dehydrogenase; LTB_4, leukotriene B_4; MDA, malonaldehyde; SOD, superoxide dismutase; TXA_2, thromboxane A_2.

liquid chromatography (HPLC), confirming the absence of platelet contamination (9).

HPLC assay for 12,20-DiHETE production by neutrophils. Volumes of 3×10^7 neutrophils or appropriate aliquots of subcellular fractions were adjusted to 1 ml with Hepes buffer. Samples were preincubated in polypropylene tubes for 1 min at 37°C with stirring. Inhibitors or NADPH, when used, were added during the preincubation period. Reactions were started with 4.4 μM 12-HETE (sodium salt) and terminated at 5 min by addition of 1.5 ml of acetone and by placement on ice. In experiments involving subcellular fractionation, the reaction time was increased to 15 or 30 min. In such instances, calcium, which had been eliminated from the buffer, was added to a final concentration of 1.29 mM at the start of preincubation. Intact cells or cell fractions could be assayed without loss of activity after storage at 4°C overnight.

Precipitated protein was removed by centrifugation at 1,400 g for 15 min at 25°C, and acetone evaporated from supernatants under nitrogen at 37°C. After acidification to pH 4.0 with 1 N H_3PO_4, extraction three times with 1-ml aliquots of ethyl acetate, and evaporation to dryness, residues were dissolved in 50 μl of methanol/water (3:1). Tubes were vortexed and centrifuged for 5 min at 225 g, and 5 μl of supernatant was injected into an HPLC column (9). Reversed-phase HPLC was carried out on an 8-mm i.d. Radial-PAK C18 (10 μm) column (Waters Associates, Milford, MA) with methanol/water/acetic acid (75:25:0.01), pH 6.1, as eluting solvent. Flow rate was 0.6 ml/min. Absorbance was monitored at 237 nm.

12,20-DiHETE and 12-HETE were quantified by comparison of peak areas with those of external standards of purified compounds. The 12-HETE standard was synthesized from ionophore-stimulated platelets. 12,20-DiHETE was prepared by incubation of purified 12-HETE with neutrophils or from thrombin-stimulated mixtures of platelets and neutrophils, as previously described (9). Both compounds were purified by reversed-phase HPLC and quantitated spectrophotometrically using an extinction coefficient at 237 nm of 30,500 (18).

For optimal results, assays for 12,20-DiHETE production by broken cell preparations or subcellular fractions were carried out in the presence of an NADPH-regenerating system, consisting of 0.02 M DL-isocitric acid (trisodium salt), 0.1 mg of isocitrate dehydrogenase/ml (grade 1, Boehringer Mannheim, Indianapolis, IN) and 1 mM NADPH (19).

Inhibition of 12,20-DiHETE production by neutrophil homogenates. Neutrophil homogenates derived from $0.9-1.2 \times 10^7$ cells were added to intact neutrophil controls (3×10^7 cells) at the start of the preincubation period and the assay for ω-hydroxylation of 12-HETE performed as above. Reversal of the inhibitory effect of such homogenates by the elastase inhibitor α_1-antitrypsin (Sigma Chemical Co., St. Louis, MO) was demonstrated by incubation of the homogenate with α_1-antitrypsin for 1 h at 0°C prior to addition to intact neutrophils.

Inhibition of ω-hydroxylation of 12-HETE by carbon monoxide. Neutrophils were treated with CO as described by Shak and Goldstein (20), using gas-saturated buffers (21). Controls consisted of neutrophils exposed to nitrogen in the same manner. Hepes buffer was deaerated (vacuum pump) for 15 min and bubbled for 30 min with CO gas or nitrogen. Neutrophil stock suspensions were prepared by resuspension of pelleted neutrophils in the above CO, nitrogen, or air-equilibrated buffers, followed by overlaying with the respective gas. In these specific experiments, reactions were begun by addition of gas-equilibrated neutrophils to 12-HETE (sodium salt) which had been previously dissolved in air-equilibrated buffer. Contents of reaction tubes were overlayed with appropriate gas and capped during incubation. An approximate CO to oxygen ratio of 11:1 was calculated from their solubility in water at 37°C (22). Reoxygenation was accomplished by allowing the gas-equilibrated neutrophil stock solutions to remain uncapped under a hood. The neutrophils were then pelleted, the supernatants were discarded, and the cells were resuspended in air-equilibrated buffer.

Effect of LTB_4 on ω-hydroxylation of 12-HETE. LTB$_4$ (Biomol, Philadelphia, PA) (0.22–4.4 μM, sodium salt) was added to 3×10^7 neutrophils during the preincubation period (15 s before 12-HETE addition). Unlabeled 12-HETE plus [^{3}H]12-HETE (New England Nuclear, Boston, MA) (4.4 μM, 0.16 μCi) was used in this instance and the 5-min incubations terminated with 3.5 ml of methanol/chloroform (5:2). Lipids

were extracted by a modified Bligh and Dyer procedure (17) and chromatographed on activated (1 h, 110°C) silica gel G plates (Analtech, Inc., Newark, DE) along with standards using chloroform/methanol/acetic acid/water (90:8:1:0.8) as solvent. Pertinent zones were scraped and quantitated by scintillation counting as previously described (17).

Diisopropylfluorophosphate (DFP) treatment of neutrophil suspensions. DFP stock solutions (~2.5 M) were prepared by injecting 1 ml of anhydrous isopropanol into a vial containing 1 g of DFP (Sigma Chemical Co.). Aliquots of the DFP stock solution in microliter quantities were added to neutrophil suspensions (averaging 7×10^7 neutrophils/ml, total volume, 5–7 ml) bringing the final DFP concentration to 2 mM. The tube was then vortexed and incubated in ice for 5 min. Excess DFP was washed out twice with 25 ml of cold, modified Hepes buffer, centrifuging at 400 g for 5 min (25°C). Final suspension was made in buffer, to the original volume with a plastic pipette (23). Samples were taken for cell counting. Inactivation of neutrophil serine proteases by DFP pretreatment in individual experiments was monitored by a spectrophotometric elastase assay using the substrate succinyl-alanyl-alanyl-alanine-p-nitroanilide (24) (kindly performed by Dr. Mark Brower, Cornell University Medical College).

Subcellular fractionation experiments. DFP-treated neutrophils in modified Hepes buffer were sonicated in a polypropylene tube at 4°C for five 10-s intervals (Micro-Ultrasonic cell disrupter, Kontes Co., Vineland, NJ; power setting 7). Tubes were cooled on ice between each interval. Dithiothreitol (DTT) was added after sonication to a final concentration of 0.5 mM. Unbroken cells were removed from the sonicate by centrifugation at 150 g for 10 min (4°C). This "sonicate supernatant" constituted the starting material for subsequent subcellular fractionation. Centrifugation was carried out in a L2-65 ultracentrifuge (Beckman Instruments, Inc., Fullerton, CA). The SW-39L rotor was used at 11,000 rpm (10,000 g at center of tube) for 15 min (5°C). The resulting 10,000-g pellet was resuspended in 2 ml of modified Hepes buffer (containing 0.5 mM DTT) with a teflon pestle. The supernatant resulting from the 10,000-g centrifugation was further fractionated by ultracentrifugation at 35,000 rpm (100,000 g at center of tube) for 1 h (5°C). The 100,000-g supernatant was removed and the 100,000-g microsomal pellet was resuspended in 1.35 ml of modified Hepes buffer (0.5 mM DTT).

Carbon monoxide difference spectra. Spectral studies (21) were carried out in a Cary 118 double-beam spectrophotometer (Varian Associates, Palo Alto, CA). Sample and reference cuvettes contained 1 ml of microsomal suspension (1.45 mg of protein) or an identical quantity of granule protein. After the baseline was recorded, the sample cuvette was bubbled gently (3 min) with CO. Another baseline was then obtained to rule out hemoglobin contamination. The latter baseline was subsequently subtracted from succeeding spectra in order to obtain the curves presented in Fig. 6. A CO (dithionite reduced vs. oxidized) difference spectrum was then produced by addition of a few crystals of the reductant sodium dithionite to the sample cuvette. Finally, dithionite was added to the reference cuvette (untreated with CO) to reduce cytochrome b. The resulting difference spectrum of the reduced cytochrome P-450–CO complex was thereby obtained.

Other analytical procedures. Protein was measured by the technique of Lowry et al. (25) as modified by Miller (26), with human serum albumin as standard (27). Cell integrity was monitored by lactate dehydrogenase (LDH) assay (27). The method of Wróblewski and LaDue (28), slightly modified, was employed using Sigma Chemical Co. reagents. One unit of activity equals a decrease in OD of 0.001/min. Cell supernatants for measurement of free LDH were prepared by centrifugation in a Beckman Instruments, Inc. microfuge for 3 min at 4°C. β-Glucuronidase was assayed essentially as previously described (27) using Sigma Diagnostic Procedure 325 at 56°C (1 h), with the addition of 0.15% (vol/vol) Triton X-100. Activity is expressed as microgram of phenolphthalein released per hour per milligram protein.

Results

Effects of cytochrome P-450 inhibitors on ω-hydroxylation of platelet 12-HETE by intact neutrophils. Production of 12,20-
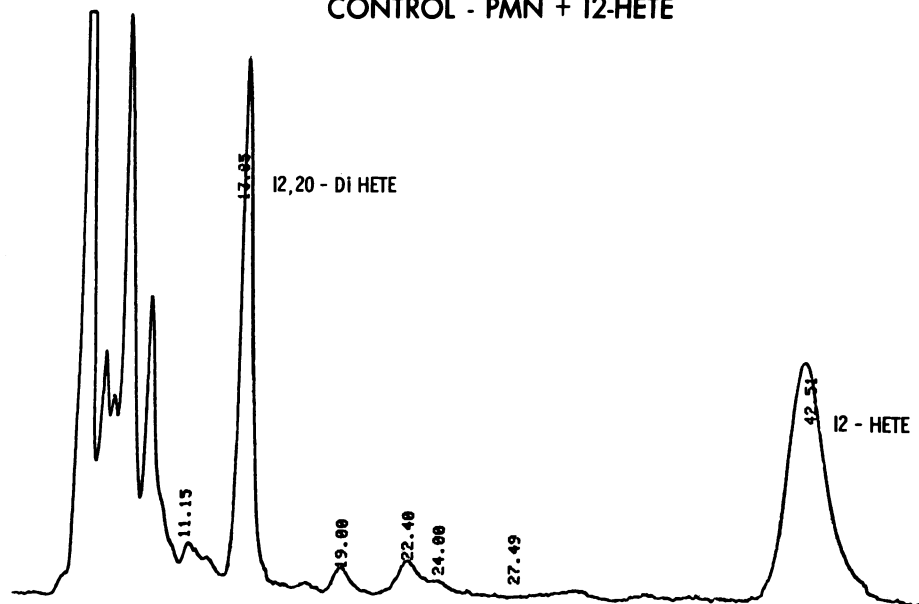
CONTROL - PMN + 12-HETE

12,20 - DI HETE

12 - HETE

Figure 1. HPLC assay for 12,20-DiHETE production by unstimulated neutrophils. 3×10^7 unstimulated neutrophils were incubated with 4.4 μM 12-HETE (5 min). Reactions were terminated with acetone and precipitated protein was removed. Samples were acidified and lipids extracted with ethyl acetate. The lipids were redissolved in methanol/water (3:1) and injected into a reversed-phase HPLC column. Absorbance of effluents was monitored at 237 nm. The 12-HETE peak represents unconverted substrate remaining after the 5-min incubation period.

DiHETE in the presence of known cytochrome P-450 inhibitors was compared with generation of 12,20-DiHETE in the absence of such inhibitors. Fig. 1 depicts results of a control HPLC assay consisting of 3×10^7 unstimulated neutrophils incubated with 4.4 μM 12-HETE for 5 min. Under these experimental conditions, the quantity of 12,20-DiHETE recovered, calculated as a percentage of 12-HETE added to neutrophils, ranged from 24% to 46% in seven donors. When assays were repeated on separate occasions, differences between several individual donors appeared to be consistent. In multiple control assays the standard deviation of 12,20-DiHETE production averaged 4% of mean values. Enzyme activity in intact cells or fractions was stable upon storage at 4°C for at least 24 h.

Compounds that inhibited 12,20-DiHETE production by neutrophils are listed in Table I. Inhibitors of other oxidative processes, such as superoxide dismutase (SOD) and catalase, which had no effect on 12,20-DiHETE formation, are also shown. α-Naphthoflavone at 0.25 mM was a potent inhibitor of 12-HETE ω-hydroxylation. Results of the HPLC assay in the presence of this inhibitor are shown in Fig. 2. Absence of released LDH in the cell supernatant demonstrated that the inhibitory effect observed with α-naphthoflavone was not due to neutrophil lysis. In addition, trypan blue exclusion indicated 96% viability after incubation of cells with 0.05 mM α-naphthoflavone. Furthermore, α-naphthoflavone inhibited ω-hydroxylation of 12-HETE by isolated neutrophil microsomal fractions (see below). In two separate experiments with microsomal preparations, 0.05 mM α-naphthoflavone inhibited 12,20-DiHETE formation by 57% and 87%, respectively. Each of the cytochrome P-450 inhibitors studied interfered with 12,20-DiHETE production in concentrations generally used for this purpose (13, 29). 5,8,11,14-eicosatetraynoic acid (ETYA), at a concentration of 0.03 mM, blocked 12,20-DiHETE formation by 62%. Inhibition of cytochrome P-450 oxidase systems by ETYA has previously been reported (30). ω-Hydroxylation of 12-HETE by neutrophils was also inhibited 79% by 5 mM EDTA. This inhibition occurred despite addition of calcium to the assay system in quantities sufficient to compensate for the chelating effect of EDTA.

Carbon monoxide complexes with cytochrome P-450 en-

zymes and is a potent reversible inhibitor. Treatment of intact neutrophil suspensions with a CO/O_2 mixture (11:1) resulted in almost total inhibition of 12,20-DiHETE production from 12-

Table I. Inhibition of Neutrophil 12,20-DiHETE Formation by Antagonists of Cytochrome P-450 Enzyme Systems

Inhibitor	Percent inhibition of 12,20-DiHETE	Percent increase of unmetabolized 12-HETE
α-Naphthoflavone (0.25 mM)	96	*
α-Naphthoflavone (0.05 mM)	73	*
β-Naphthoflavone (0.05 mM)	20	*
SKF 525-A (0.1 mM)	46	33
SKF 525-A (0.05 mM)	32	16
Imidazole (0.5 mM)	27	25
Cimetidine (1.0 mM)	18	23
Metyrapone (1.0 mM)	19	17
Aminopyrine (5.0 mM)	17	23
Chloramphenicol (1.0 mM)	40	36
Chloramphenicol (0.1 mM)	14	7
ETYA (0.03 mM)	62	172
SOD (15 U/ml)	0	0
Catalase (2,600 U/ml)	0	0

3×10^7 neutrophils/ml were incubated with 4.4 μM 12-HETE at 37°C for 5 min. Inhibitors were added during a 1-min preincubation period. HPLC assay values for 12,20-DiHETE and 12-HETE were compared with those of control measurements made in the absence of inhibitors. Inhibition of 12,20-DiHETE production can be correlated with increases in residual unmetabolized 12-HETE. Results represent the average values obtained in three experiments.
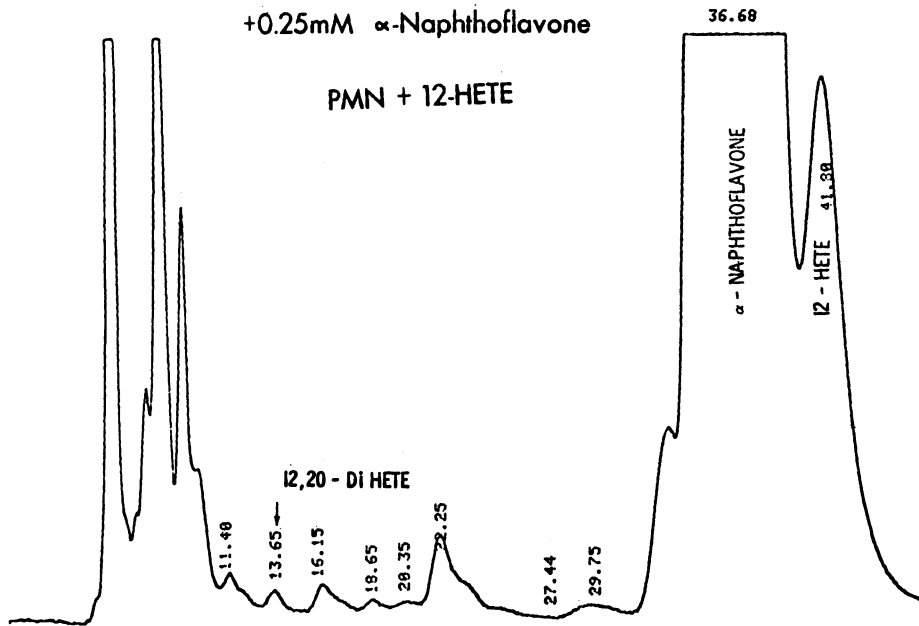* In these instances quantitation was impeded by overlap of inhibitor and 12-HETE peaks on the HPLC chromatogram.

+0.25mM α-Naphthoflavone

PMN + 12-HETE

36.68

α - NAPHTHOFLAVONE

12 - HETE 41.38

12,20 - DI HETE

11.48 13.65 16.15 18.65 20.35 22.25 27.44 29.75

Figure 2. Inhibition of 12,20-DiHETE formation by α-naphthoflavone. The inhibitor was added 30 s prior to exposure of 3×10^7 neutrophils to 12-HETE. Production of 12,20-DiHETE was inhibited by 96% as compared with the control assay shown in Fig. 1.

HETE (Table II). The inhibition was not due to oxygen lack because results with neutrophils treated in parallel with a mixture of nitrogen and oxygen were similar to control values. The carbon monoxide inhibition was completely reversed upon reoxygenation of the neutrophils, and 12-HETE ω-hydroxylation returned to control levels.

Absence of 12-HETE ω-hydroxylation in disrupted neutrophil preparations. After neutrophil sonication, nitrogen cavitation, freeze-thawing, or Polytron homogenization (Brinkmann Instruments, Westbury, NY), ω-hydroxylation of 12-HETE was lost in approximate proportion to the degree of LDH released. For example, in the setting of 90% or more LDH release, ω-hydroxylation of 12-HETE was undetectable despite the presence of NADPH.

Experiments were then carried out to determine whether a proteolytic enzyme such as elastase was released during neutrophil disruption. A neutrophil sonicate (98% lysed according to LDH assay) was prepared, and aliquots equivalent to 1.2×10^7 neutrophils were added to 3×10^7 intact neutrophils at the start of the preincubation period of the standard 12,20-DiHETE assay

Table II. Inhibition of Neutrophil 12,20-DiHETE Production by Carbon Monoxide

Incubation atmosphere	12,20-DiHETE*	12-HETE*
	%	%
Air	34	66
CO/O₂ (11:1)	2	98
N₂/O₂	32	68
Reoxygenation (after CO)	32	68
Reoxygenation (after N₂)	31	69

3×10^7 neutrophils/ml in CO, N₂, or air-equilibrated buffers were incubated with 4.4 μM 12-HETE at 37°C. The results represent averages of triplicate 5-min assays.
* Values shown are percentages of the sum of 12,20-DiHETE and 12-HETE on the HPLC chromatograms.

system (Table III). This resulted in 68% inhibition of control 12,20-DiHETE production. Boiling the sonicate prior to addition to the intact 12,20-DiHETE assay system completely reversed its inhibitory properties. Significant reversal of the inhibitory effect of the neutrophil sonicate was also achieved by pretreatment of the sonicate with the elastase and cathepsin G inhibitor, α₁-antitrypsin. From these results, it was concluded that neutrophil ω-hydroxylation of 12-HETE might be inhibited by a protease(s) released upon cell disruption. Therefore, intact neutrophils were subsequently pretreated with the cell-penetrating

Table III. Inhibition of 12-HETE ω-Hydroxylation by Neutrophil Homogenates is Reversed by Boiling, α_{-1}-Antitrypsin or DFP Pretreatment

Addition to intact neutrophils	Percent inhibition of 12,20-DiHETE production	Percent reversal of inhibition
Neutrophil sonicate	68	—
Boiled sonicate*	0	100
Sonicate + 10 μM α_{-1}-Antitrypsin‡	54	21
Sonicate + 25 μM α_{-1}-Antitrypsin	39	43
Sonicate + 50 μM α_{-1}-Antitrypsin	33	51
Sonicate + 100 μM α_{-1}-Antitrypsin	29	57
Sonicate of DFP-pretreated neutrophils§	0	100

To 3×10^7 intact neutrophils was added a neutrophil sonicate (98% lysed according to LDH assay) equivalent to 1.2×10^7 neutrophils.
* In a parallel experiment, an identical aliquot of sonicate which was preboiled (3 min) was added to the intact assay system.
‡ α_{-1}-Antitrypsin at the indicated concentrations, was preincubated with the sonicate for 1 h (0°C) before addition to intact neutrophils.
§ Intact neutrophil suspensions were incubated with 2 mM DFP (5 min, 0°C), washed, sonicated, and added to the intact neutrophil assay system. 4.4 μM 12-HETE was then added and 12,20-DiHETE was assayed as described. These results were obtained in two separate experiments.

serine protease inhibitor, DFP (23), which permitted preparation of enzymatically active sonicates and subcellular fractions. Elastase activity (24) was not detectable in sonicates of DFP-pretreated neutrophils. When these sonicates were added back to the intact 12,20-DiHETE assay system, no inhibition occurred (Table III).

NADPH dependence of neutrophil 12-HETE ω-hydroxylation. Intact DFP-treated neutrophils were assayed for 12,20-DiHETE production in the presence of an NADPH-regenerating system (isocitric acid, isocitrate dehydrogenase, and NADPH) (Table IV). 12,20-DiHETE was measured 30 min after addition of 12-HETE to neutrophils. Presence of the NADPH regenerating system (or NADPH alone) did not result in an increase in 12,20-DiHETE formation by *intact* neutrophils.

DFP-treated neutrophils were then sonicated (97% disruption) and centrifuged at 150 g for 10 min (4°C). The supernatant was assayed in the presence and absence of the NADPH-regenerating system (Table IV). In the absence of added NADPH, 12-HETE ω-hydroxylation by the sonicate was 4.6% of that noted with intact cells. Addition of the NADPH-regenerating system resulted in a 13-fold increase in activity (Table IV).

Effects of addition of increasing concentrations of NADPH on 12-HETE ω-hydroxylation by a neutrophil microsomal fraction (see below) are depicted in Fig. 3. NADPH (0.1–5,000 μM) was incubated with microsomes (192 μg of protein) and 17.6 μM 12-HETE for 15 min (37°C) in the presence of the NADPH-regenerating system. Low levels of activity were present in the absence of exogenous NADPH, possibly owing to intrinsic residual neutrophil NADPH. However, at a level of 2.5 μM added NADPH, a plateau of activity, 33-fold greater than that observed with no added cofactor, was achieved. Further increases of NADPH to concentrations of 5,000 μM did not enhance 12-HETE ω-hydroxylation.

Subcellular localization of 12-HETE ω-hydroxylase activity. DFP-pretreated neutrophils were sonicated and subcellular fractions were prepared by standard differential ultracentrifugation techniques. The 10,000-g pellet was quantitatively enriched in β-glucuronidase, a granule marker enzyme. In three separate experiments mean values (± standard deviation), expressed as micrograms of phenolphthalein released per hour per milligram of protein, were 361 (±156) for the 10,000-g pellet (granules), 124 (±67) for the 100,000-g pellet (microsomes), and 16 (±1) for the 100,000-g supernatant (soluble protein fraction). Assays

Figure 3. NADPH dependence of neutrophil microsomal 12-HETE ω-hydroxylating activity. 192 μg of microsomal protein was incubated with 12-HETE (17.6 μM) in the presence of increasing concentrations of NADPH for 15 min (37°C). An NADPH-regenerating system was included (see Methods).

of the fractions for 12,20-DiHETE production from 12-HETE were carried out and the activity was correlated with protein content. As summarized in Table V, neutrophil 12-HETE ω-hydroxylating activity was confined exclusively to the microsomal pellet. No activity was detected in the soluble protein or granule fractions of subcellular preparations from neutrophils of three different donors. There was a 6.6-fold increase in specific activity in the microsome pellet as compared with the 150-g "sonicate supernatant" starting material. Total recovery of ω-hydroxylase activity in the microsomal pellet was 72% of that in the "sonicate supernatant."

The rate of conversion of 12-HETE to 12,20-DiHETE by the neutrophil microsomal fraction, as related to substrate con-

Table IV. 12,20-DiHETE Formation from 12-HETE by Neutrophils Requires NADPH

Neutrophil preparation*	12,20-DiHETE production
	ng/mg protein
Intact neutrophils	133
Intact neutrophils + NADPH-regenerating system	129
Neutrophil sonicate	6
Neutrophil sonicate + NADPH-regenerating system	77

Values are averages of duplicate assays and are representative of three separate experiments.
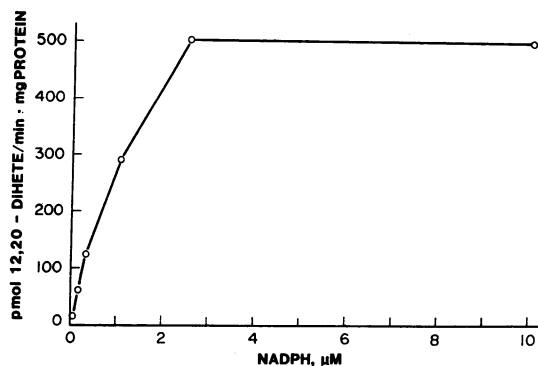* Cells were pretreated with DFP as described in Methods.

Table V. 12-HETE ω-Hydroxylase Activity in Neutrophil Subcellular Fractions

Neutrophil fraction	Protein	Total activity[‡]	Specific activity
	percentage of 150-g supernatant	*ng 12,20-DiHETE per fraction*	*ng 12,20-DiHETE per mg protein*
150-g supernatant	*	405	256
10,000-g supernatant	65.4	434	418
10,000-g pellet (granule fraction)	37.4	0	0
100,000-g supernatant	55.5	0	0
100,000-g pellet (microsome fraction)	10.8	291	1,692

Intact neutrophils were pretreated with DFP, sonicated, and fractionated as described in Methods. Fractions were resuspended in DTT-containing buffer. Assays for conversion of 12-HETE to 12,20-DiHETE were carried out in the presence of an NADPH-regenerating system for 30 min after addition of 12-HETE to the respective fraction. Results are representative of three separate experiments.
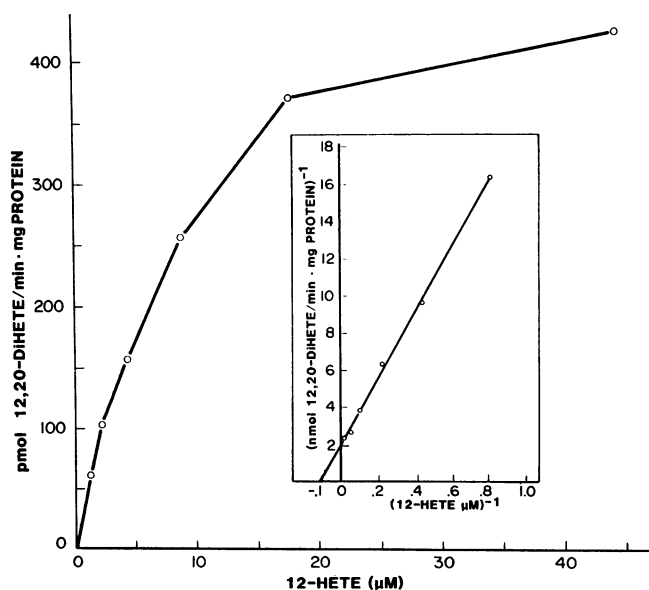* Protein recovery in the 150-g supernatant represented 81% of that present in the intact neutrophils.
[‡] Values for total activity are reported as ng 12,20-DiHETE per fraction derived from 3×10^7 neutrophils.

centration, is shown in Fig. 4. Increasing quantities of 12-HETE were incubated with neutrophil microsomes (350 or 410 μg of protein) for 15 min (37°C). The NADPH regenerating system (1 mM NADPH) was included. Apparent K_m and V_{max} values were determined from Lineweaver–Burk plots of the data and averaged 9.7 μM and 0.4 nmol/min · mg of protein, respectively, in two separate experiments.
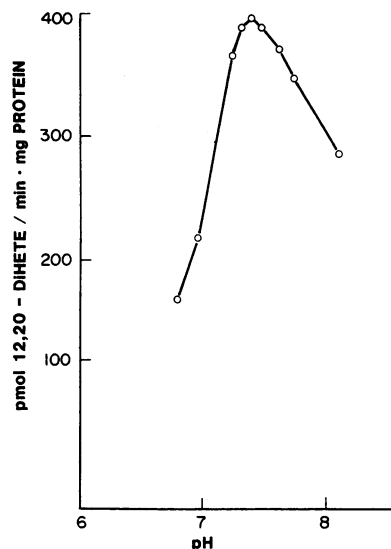
Fig. 5 demonstrates the effect of pH on the 12-HETE ω-hydroxylating activity of the neutrophil microsomal fraction. Optimal activity occurred in the range of pH 7.3–7.5. A decrease in activity to 59% of maximum was noted at pH 7.0 and to 72% at pH 8.1.

A definite peak at 450 nm, indicative of cytochrome P-450 (21) was present in the carbon monoxide (dithionite-reduced vs. dithionite-reduced) difference spectrum of the neutrophil microsomal fraction (16) (Fig. 6). The cytochrome P-450 peak became obvious after attenuation (by addition of dithionite to the reference cuvette) of the large peak representing cytochrome b (426 nm) in the carbon monoxide (dithionite-reduced vs. oxidized) difference spectrum. The CO (dithionite-reduced vs. dithionite-reduced) difference spectrum of the granule fraction (not shown), exhibited a trough rather than a peak at 450 nm.

ω-Hydroxylation of 12-HETE in the presence of LTB₄. LTB₄ is the major eicosanoid synthesized by the *stimulated* neutrophil. Fig. 7 illustrates the effect on 12,20-DiHETE production of the presence of LTB₄ (0.22–4.4 μM), 15 s before [³H]12-HETE (4.4 μM) addition to 3 × 10⁷ neutrophils. LTB₄ induced a progressive inhibition of 12-HETE ω-hydroxylation from 27.1% at a concentration of 0.22 μM to 92.6% when equal amounts (4.4 μM) of 12-HETE and LTB₄ were present. Synthesis of 5,12-DiHETE did not occur at any of the LTB₄ concentrations studied. This

Figure 5. pH dependence of 12-HETE ω-hydroxylating activity in neutrophil microsomes. 175 μg of microsomal protein in DTT-containing buffer was incubated with 17.6 μM 12-HETE in the presence of 1 mM NADPH and an NADPH-regenerating system for 15 min at 37°C. pH of the incubation mixtures in 5 mM Hepes buffer was adjusted with NaOH.

indicated that the levels of exogenous LTB₄ employed did not activate the neutrophil 5-lipoxygenase.

Discussion

Significant quantities of platelet-produced 12-HETE can be processed by neutrophils (8, 9), which are known components of hemostatic platelet plugs and thrombi (31). Unstimulated neutrophils convert 12-HETE from stimulated platelets to 12,20-DiHETE. Our data demonstrate that this occurs via a cytochrome P-450 ω-hydroxylation mechanism. Stimulated platelets can synthesize 12-HETE in abundance (17). This synthesis is unabated after aspirin ingestion as measured in washed platelets

Figure 4. Substrate concentration dependence of 12-HETE ω-hydroxylating activity in the neutrophil microsomal fraction. Results shown are from a representative experiment in which 12-HETE (1.2–44 μM) was incubated with the microsomal fraction (350 μg of protein) for 15 min at 37°C in the presence of 1 mM NADPH and an NADPH-regenerating system. Volumes were adjusted to 1 ml with DTT-containing Hepes buffer (pH 7.45). Lineweaver–Burk analysis of the data from this experiment (*inset*) gave an apparent K_m value of 9.5 μM and a V_{max} of 0.5 nmol/min · mg of protein.

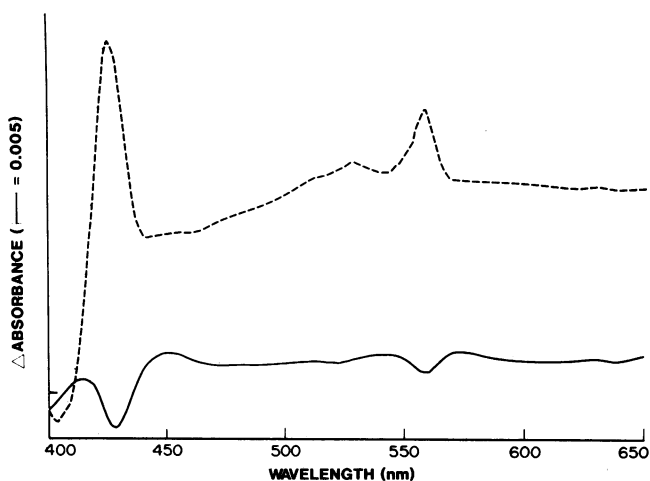Figure 6. Difference spectra of the CO complex of reduced cytochrome P-450 in neutrophil microsomes. 1 ml of microsomal fraction suspension (1.45 mg of protein) was placed in sample and reference cuvettes. A baseline difference spectrum, recorded after bubbling the sample cuvette with CO, has been subtracted from the curves shown. Subsequent addition of sodium dithionite to the sample cuvette produced a CO (dithionite-reduced vs. oxidized) difference spectrum (*dashed line*). Addition of sodium dithionite to the reference cuvette as well, resulted in a CO (dithionite-reduced vs. dithionite-reduced) difference spectrum (*solid line*).
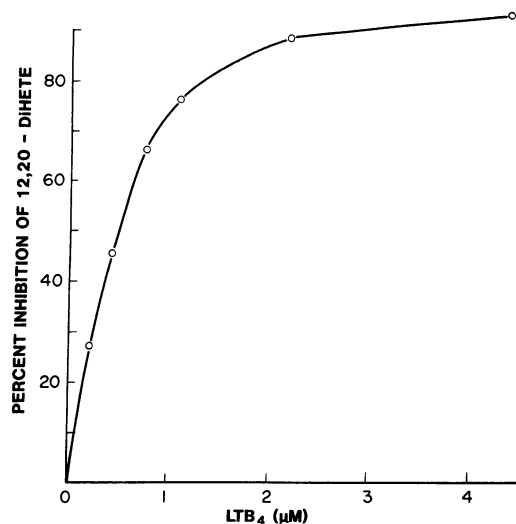
Figure 7. Inhibitory effect of LTB$_4$ on 12,20-DiHETE formation by neutrophils. Increasing concentrations of LTB$_4$ were added to 3×10^7 neutrophils 15 s before addition of [^{3}H]12-HETE (4.4 μM). Mixtures were incubated for 5 min (37°C) after 12-HETE addition, and lipids were extracted, separated by thin-layer chromatography, and quantitated by scintillation counting. Results presented are the averages of duplicate determinations at each concentration of LTB$_4$.

and, as recently reported (32), is only partially and reversibly inhibited by aspirin in platelet-rich plasma as stimulated by collagen (20 μg/ml). Therefore it is important to understand regulation of further metabolism of 12-HETE.

It has been known for some time that eicosanoids are metabolized to ω-oxidation products in vivo (33) and in vitro (13). In tissues such as liver and kidney, the hydroxylation is carried out by typical cytochrome P-450 monooxygenase systems (13). One of the criteria commonly used for characterizing cytochrome P-450 type mechanisms is the action of compounds known to inhibit these enzyme activities. As shown in Table I, an inhibitory effect on 12,20-DiHETE formation from 12-HETE by neutrophils was observed with all "classical" cytochrome P-450 inhibitors studied. Of particular pertinence was α-naphthoflavone which resulted in almost total inhibition in intact neutrophils and in microsomal fractions therefrom. Scavengers of superoxide radicals and hydrogen peroxide, SOD and catalase respectively, had no effect on 12-HETE ω-hydroxylation.

Carbon monoxide interacts with the ferrous ion of the heme moiety of cytochrome P-450 (34). The complex can be reversibly dissociated by reoxygenation. In our experiments, ω-hydroxylation of platelet 12-HETE by neutrophils demonstrated this characteristic property of cytochrome P-450 enzymes. Thus, carbon monoxide exposure of the neutrophil resulted in 94% inhibition of 12,20-DiHETE formation from platelet 12-HETE (Table II). This inhibition was completely reversible when the neutrophils were reoxygenated.

In contrast to cytochrome P-450 enzyme systems in tissues such as kidney (30) and liver (35), mechanical disruption of the neutrophil resulted in loss of 12-HETE ω-hydroxylase activity. This was attributable to release of proteolytic enzymes as demonstrated by experiments in which neutrophil homogenates were added to the 12-HETE ω-hydroxylation assay system containing intact neutrophils. Such homogenates induced inhibition of 12-HETE ω-hydroxylase activity which could be reversed by boiling

or pretreatment of the homogenates with α_1-antitrypsin prior to addition to intact neutrophils.

In an attempt to prepare sonicates and subcellular fractions that were enzymatically active with respect to 12-HETE, neutrophils were sonicated in the presence of α_1-antitrypsin or other protease inhibitors such as phenylmethylsulfonylfluoride (PMSF). However, these sonicates also proved to be inactive. Sonicates capable of metabolizing 12-HETE could only be prepared in the presence of the protease inhibitor DFP, which permeated intact cells and granules and exerted its effect prior to granule disruption (23). It was therefore concluded that neutrophil 12-HETE ω-hydroxylating activity was very sensitive to proteolysis. Protease interference with ω-hydroxylation in tissue homogenates was also observed in rat small intestine: microsomes washed with PMSF demonstrated significant ω-hydroxylating activity, whereas untreated homogenates did not (13). In our experiments no protective effect was observed with PMSF whether added before or after homogenization. With DFP-pretreated neutrophil homogenates or isolated microsomes, it was possible to demonstrate a definite requirement for NADPH as an electron donor (Table IV, Fig. 3). This is another identifying characteristic of cytochrome P-450 hydroxylase systems.

After establishing that 12-HETE ω-hydroxylating activity in neutrophil homogenates could only be preserved by DFP pretreatment, it was possible to carry out subcellular localization experiments. As can be seen in Table V, the capacity to convert 12-HETE to 12,20-DiHETE was localized solely to the neutrophil microsomal fraction. Activity was undetectable in the granule or soluble subcellular compartments. Presence of ω-hydroxylating activity in neutrophil microsomes correlates with cytochrome P-450 localization in other tissues. It is important to mention that "microsomal fractions" as currently isolated contain both plasma membranes and intracellular membranes. As yet it is not known which of these membrane components actually contains the 12-HETE ω-hydroxylase. The neutrophil enzyme we have studied may actually be located at or close to the cell surface and thereby be susceptible to degradation by proteases in the surrounding medium, as observed in this study. Further evidence for possible proximity to the cell surface is that the enzyme seems to be readily available to externally provided lipid substrate (12-HETE) and that the product of the enzymatic reaction is found almost exclusively in the cell supernatant (9). Another explanation for the inhibitory effect of neutrophil homogenates on intact cells is that conformational changes induced by proteolysis at the cell surface might affect components of the enzyme system, resulting in inactivation.

The carbon monoxide difference spectra of the microsomes we studied, demonstrated that 12-HETE ω-hydroxylating activity was associated with a subcellular fraction containing a cytochrome P-450. This was in sharp contrast to the β-glucuronidase-enriched granule fraction. The latter did not convert 12-HETE to 12,20-DiHETE and in fact exhibited a trough rather than a peak at 450 nm in the carbon monoxide difference spectrum.

There is accumulating evidence that cytochrome P-450 systems exist in multiple forms and possess varying substrate specificities (13, 36). Recently, involvement of cytochrome P-450 enzymes in eicosanoid metabolism of cells participating in hemostasis and thrombosis has been demonstrated (37). Thus platelet thromboxane and aortic microsomal prostacyclin synthases as well as neutrophil LTB$_4$ ω-hydroxylase have been characterized as cytochrome P-450 enzymes (16, 37). Several lines of evidence suggest the possibility that, as previously discussed

(9), the neutrophil LTB_4 ω-hydroxylase and the 12-HETE ω-hydroxylase described here may be similar or identical enzymes. LTB_4 strongly inhibited conversion of 12-HETE to 12,20-DiHETE by isolated neutrophils (Fig. 7). As evidenced by the total absence of 5,12-DiHETE production at all concentrations of LTB_4 studied, the inhibition of 12,20-DiHETE formation was not due to diversion of 12-HETE to 5,12-DiHETE by LTB_4 activation of neutrophil 5-lipoxygenase (8, 9). Thus, in all likelihood LTB_4 functioned as a competitive inhibitor of 12,20-DiHETE formation. The neutrophil 12-HETE ω-hydroxylase and the LTB_4 ω-hydroxylase display maximal activity at similar pH ranges and require low concentrations of NADPH as a cofactor (16). The K_m of the 12-HETE ω-hydroxylase for 12-HETE was 9.7 as compared with values reported for the ω-hydroxylation of LTB_4 by the neutrophil LTB_4 ω-hydroxylase of 0.6 (16) for a microsomal preparation, 0.22 (15) for a cytosolic preparation, and 1.0 (12) for intact neutrophils.

If the hydroxylating enzymes are indeed identical, the apparent higher affinity for LTB_4 does not alter the significance of the cell–cell interaction with platelet 12-HETE described here. This reaction has occurred with neutrophils from every donor studied thus far. In situations wherein platelets have been stimulated by thrombin or collagen, neutrophils remain unstimulated and LTB_4 is not formed (9). In such instances, platelet 12-HETE would be the substrate of prime importance.

In our experiments neutrophil ω-hydroxylation of 12-HETE in both intact cells and microsomal preparations was strongly inhibited by α-naphthoflavone (Table I, Fig. 2). This is in contrast to the neutrophil LTB_4 ω-hydroxylase which is not affected by this cytochrome P-450 inhibitor (16). This is significant because α-naphthoflavone, the most potent nonsteroidal inhibitor of human aromatase cytochrome P-450, exerts inhibitory selectivity toward hepatic P-450 isoenzymes (38). The contrasting responses of the 12-HETE ω-hydroxylase and the LTB_4 ω-hydroxylase to α-naphthoflavone inhibition remain to be resolved.

To summarize: 12-HETE is the proinflammatory monohydroxy lipoxygenase product of arachidonic acid, synthesized by stimulated platelets in the presence or absence of aspirin. Properties of the enzyme system in unstimulated neutrophils responsible for 12-HETE metabolism to 12,20-DiHETE have been defined. The enzyme activity involved in this cell–cell interaction was classified as a cytochrome P-450 as indicated by inhibitor studies, NADPH dependence, and subcellular microsomal localization. Neutrophil perturbation by any means inhibited ω-hydroxylation of 12-HETE and this was prevented by the proteolytic enzyme inhibitor DFP. Induction or inhibition of the neutrophil cytochrome P-450 monooxygenase may eventually represent a possible therapeutic approach to modulation of platelet 12-HETE levels in vivo.

Acknowledgments

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