Leukotriene B₄ ω-Hydroxylase in Human Polymorphonuclear Leukocytes

SUICIDAL INACTIVATION BY ACETYLENIC FATTY ACIDS

(Received for publication, February 25, 1985)

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Human polymorphonuclear leukocytes (PMN) not only generate and respond to leukotriene B₄ (LTB₄), but also catabolize this mediator of inflammation rapidly and specifically by ω-oxidation (probably due to the action of a cytochrome P-450 enzyme). To develop pharmacologically useful inhibitors of the LTB₄ ω-hydroxylase in human PMN, we devised a general scheme for synthesizing terminal acetylenic fatty acids based on the "acetylenic zipper" reaction. We found that the LTB₄ ω-hydroxylase in intact PMN and in PMN sonicates is inactivated in a concentration-dependent fashion by terminal acetylenic analogues of lauric, palmitic, and stearic acids (i.e. 11-dodecyenoic, 15-hexadecynoic, and 17-octadecynoic acids). Consistent with a suicidal process, inactivation of the LTB₄ ω-hydroxylase requires molecular oxygen and NADPH, is time-dependent, and follows pseudo-first-order kinetics. Inactivation of the ω-hydroxylase by acetylenic fatty acids also is dependent on the terminal acetylenic moiety and the carbon chain length. Saturated fatty acids lacking a terminal acetylenic moiety do not inactivate the ω-hydroxylase. In addition, the two long-chain (C₁6, C₁₈) acetylenic fatty acids inactivate the ω-hydroxylase at much lower concentrations (<5.0 μM) than those required for inactivation by the short-chain (C₁₂) terminal acetylenic fatty acid (100 μM). Potent suicidal inhibitors of the LTB₄ ω-hydroxylase in human PMN will help elucidate the roles played by LTB₄ and its ω-oxidation products in regulating PMN function and in mediating inflammation.

Human PMN, when appropriately stimulated, convert arachidonic acid to LTB₄ (1). This product of the 5-lipoxygenase pathway possesses potent chemotactic activity for PMN (2-4) and, at high concentrations, provokes cytochalasin B-treated PMN to degranulate (i.e. selectively release lysosomal enzymes) (2, 5). LTB₄ also enhances the adhesiveness of PMN (6) and causes these cells to aggregate (3, 7). Since PMN both generate and respond to LTB₄, it is possible that LTB₄ plays an important role in amplifying inflammatory reactions.

We, and others, have found that human PMN convert LTB₄ to products that are less biologically active (8-13). The major pathway by which human PMN catabolize LTB₄ involves ω-oxidation, that is, oxidation of the terminal methyl group, to yield 20-ΟH-LTB₄ and 20-COOH-LTB₄ (13). Human PMN (but not human monocytes, lymphocytes, or platelets) rapidly convert LTB₄ to 20-ΟH-LTB₄ and 20-COOH-LTB₄; other dihydroxylated derivatives of arachidonic acid are converted either at a much slower rate or not at all. Since the rate of LTB₄ catabolism is comparable to the rate of LTB₄ synthesis, ω-oxidation greatly influences the amounts of biologically active LTB₄ that accumulate in the medium surrounding stimulated human PMN (13).

The precise nature of the enzyme(s) responsible for ω-oxidation of LTB₄ in human PMN is not known. However, we have found that ω-oxidation of LTB₄, in intact human PMN is inhibited specifically and reversibly by carbon monoxide (14). This finding suggests that the LTB₄ ω-hydroxylase in human PMN is a heme enzyme which cycles through the ferrous state, as does cytochrome P-450.

Cytochrome P-450 enzymes in liver and other tissues can be destroyed by certain substrates that contain terminal acetylenic moieties as they are catalytically oxidized (15). This process, an example of mechanism-based or suicidal inactivation, is characterized by irreversible and isoenzyme-specific alkylation of the prosthetic heme group of cytochrome P-450 enzymes. We recently synthesized 11-DDYA, the terminal acetylenic analogue of lauric acid, and demonstrated that it specifically inactivates the lauric acid ω-hydroxylase in rat liver microsomes without affecting other cytochrome P-450 enzymes (16). Thus, the judicious introduction of acetylenic moieties into substrates is a rational strategy for developing specific inhibitors of individual cytochrome P-450 enzymes.

To determine whether the LTB₄ ω-hydroxylase in human PMN is susceptible to suicidal inactivation by acetylenic substrates, we developed a general scheme for synthesizing terminal acetylenic fatty acids. Starting with readily available internal olefinic precursors, compounds with internal acetylenic moieties are prepared by sequential bromination-dehydrazabicyclo[5.4.0]undec-7-ene; HPLC, high-performance liquid chromatography; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.
drobromination (17, 18). Isomerization to terminal acetylenic analogues is then accomplished by means of the "acetylenic zipper" reaction (19). In this report, we describe the synthesis of two long-chain terminal acetylenic fatty acids, 15-HDYA and 17-ODYA, and show that the LTB4 ω-hydroxylase in intact human PMN and in PMN sonicates is inactivated suicidally by low concentrations of both compounds.

EXPERIMENTAL PROCEDURES

Materials—Synthetic LTB4, 20-OH-LTB4, and 20-COOH-LTB4 were generously provided by Dr. Joshua Rokach (Merck Frosst). LTB4 was stored as a stock solution in methanol under nitrogen at −70 °C. Concentrations of LTB4 were determined by UV spectroscopy using a molar extinction coefficient of 50,000 (20). 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. PGB2 was purchased from Sigma; lauric acid, palmitic acid, stearic acid, palmitoleic acid, and oleic acid were from NuChek Prep; 10-UDYA from Farchan Laboratories; and the calcium ionophore, A23187, from Calbiochem-Behring. All solvents were obtained from Burdick and Jackson. The terminal acetylenic analogue of lauric acid (11-DDYA) was synthesized from 11-dodecenoic acid as described previously (16). The synthesis of 15-HDYA—Synthesis of 15-HDYA was accomplished according to the scheme outlined in Fig. 1. Palmitoleic acid (9-hexadecenoic acid) (7.87 g, 31 mmol) in 10 ml of anhydrous diethyl ether was added over a period of 30 min to a cooled (ice-water bath), and the mixture was extracted with diethyl ether (3 X 10 ml), washed and dried (MgSO4). Organic extracts yielded 9-hexadecen-1-ol (93%, 2.03 (m, 4H, -CH2-C=C), 3.63 (t, J = 6 Hz, 2H, -CH2-O-), 4.28 (t, 2H, CH2-CHBr). The dibromide (18.2 g, 40 mmol) was heated at 140 °C for 18 h under nitrogen with 3.0 g (160 mmol) of DBU (Aldrich) in a flask fitted with a reflux condenser. After cooling, 15 ml of H2O was added, and the mixture was extracted with diethyl ether (3 X 10 ml). Filtration through silica gel provided 300 mg of pure 15-hexadecyn-1-ol in 8.0 ml of 1,3-diaminopropane. The dibromide was converted with DBU to the acetylenic derivative (9,10-dibromoheptadecan-1-ol) (50% yield); IR (neat) 3275 cm−1 (CSH), 2135 cm−1 (C=C); 'H NMR 80 MHz: 1.29 (m, 26H, -CH2-), 1.92 (t, J = 8 Hz, 2H, -CH2-O-). To 550 mg (20 mmol) of oil-free NaH under a positive nitrogen atmosphere was added 15 ml of 1,3-dimino propane that had been distilled from BaO (reduced pressure). The mixture was stirred at 70 °C for 1 h and then was cooled to 25 °C before 600 mg (2.5 mmol) of 9-hexadecyn-1-ol in 8.0 ml of 1,3-dimino propane was added. The mixture was cooled overnight at 55 °C. Water (25 ml) was added to the cooled mixture, the product was extracted into diethyl ether (4 X 35 ml), and the extracts were washed successively with 25 ml of H2O, 25 ml of 3.0 M HCl, and 25 ml of saturated NaCl. Solvent removal from the dried extracts (NaSO4) and silica gel chromatography provided 300 mg of pure 15-hexadecyn-1-ol (98% yield); IR (neat) 2920 cm−1 (C=C), 2140 cm−1 (C=C); 'H NMR 80 MHz: 1.29 (m, 26H, -CH2-), 1.92 (t, J = 8 Hz, 2H, -CH2-O-). Oxidation of 15-hexadecyn-1-ol (130 mg, 0.55 mmol) with 3.0 ml of Jones reagent (22) in 15 ml of acetone yielded 135 mg (0.53 mmol) of the methyl ester of 15-HDYA revealed major ions at m/e 266 (M+) and 255 (M− 31). The overall yield of 15-HDYA from 9-hexadecenoic acid was generally 15−25%.

Synthesis of 17-ODYA—Synthesis of 17-ODYA was accomplished essentially as described for the synthesis of 15-HDYA (Fig. 1). The yield of 9-hexadecen-1-ol from oleic acid (9-hexadecenoic acid) was 60-80%; 'H NMR 80 MHz: 0.88 (m, 3H, -CH3), 1.29 (m, 22H, -CH2-), 2.03 (m, 4H, -CH2-C=C-), 3.63 (t, J = 8 Hz, 2H, -CH2-O-), 5.53 (t, J = 5 Hz, 2H, -CH=C-CH2-). The yield of the dibromide (9,10-dibromo-octadecan-1-ol) was 100%; 'H NMR 80 MHz: 0.88 (m, 3H, -CH3), 1.29 (m, 22H, -CH2-), 2.01 (m, 4H, -CH2-CHBr), 3.63 (t, J = 6 Hz, 2H, -CH2-O-), 4.28 (t, 2H, CH2-CHBr). The dibromide was converted with DBU to the acetylenic derivative (9,10-dibromo-octadecan-1-ol) in 50−70% yield; 'H NMR 80 MHz: 0.88 (m, 3H, -CH3), 1.29 (m, 22H, -CH2-), 2.13 (m, 4H, -CH2-C=C-CH2-). The "acetylenic zipper" reaction with 1,3-diaminopropane and NaH produced the terminal acetylenic derivative (17-ODYA) in 50% yield; IR (neat): 3275 cm−1 (C=C), 2135 cm−1 (C=C); 'H NMR 80 MHz: 1.29 (m, 26H, -CH2-), 1.92 (t, J = 8 Hz, 2H, -CH2-O-). The yield of the dibromide (9,10-dibromo-octadecan-1-ol) was 100%; 'H NMR 80 MHz: 0.88 (m, 3H, -CH3), 1.29 (m, 22H, -CH2-), 2.01 (m, 4H, -CH2-CHBr), 3.63 (t, J = 6 Hz, 2H, -CH2-O-). The Jones oxidation generated 17-ODYA in 95−100% yields; IR (neat): 3275 cm−1 (C=C), 2135 cm−1 (C=C), 1700 cm−1 (−C=C−); 'H NMR 80 MHz: 1.29 (m, 24H, -CH2-), 1.92 (m, 1H, C=CH), 2.18 (m, 2H, -CH2-C=CH2-), 3.63 (t, J = 6 Hz, 2H, -CH2-O-). The oxidation of 17-ODYA revealed major ions at m/e 294 (M+) and m/e 283 (M− 1). The overall yield of 17-ODYA from 9-hexadecenoic acid was generally 15−25%.

Preparation of PMN Suspensions—Human PMN were isolated from venous blood by Hypaque-Ficoll centrifugation and dextran sedimentation (23). Contaminating erythrocytes were removed by hypotonic lysis, and leukocytes were washed twice with HEPES (10 mM) (Sigma)-buffered 135 mM NaCl/5.0 mM KCl, pH 7.4. This buffer was used throughout. Cell suspensions contained 95-98% PMN, <4% eosinophils, <1% mononuclear leukocytes, and a platelet:leukocyte ratio of <4.1. Cell viability was consistently greater than 96%, as assessed by vital dye (trypan blue) exclusion.

Preparation of PMN Sonicates—PMN (40 χ 106 cells/ml) were sonicated (100 watts χ 15 s) four times with a Vrsionc Cell Disrupter.
Suicidal Inactivation of LTB₄ ω-Hydroxylase

LTB₄ ω-hydroxylase activity in intact PMN and in PMN sonicates

Table I

<table>
<thead>
<tr>
<th>Protein</th>
<th>LTB₄ ω-hydroxylase activity</th>
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<tr>
<td>PMN</td>
<td>50 ± 12</td>
</tr>
<tr>
<td>PMN sonicates</td>
<td>31 ± 5</td>
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</tbody>
</table>

Fig. 2. Time-dependent inactivation of LTB₄ ω-hydroxylase activity in PMN sonicates by 17-ODYA. LTB₄ ω-hydroxylase activity is plotted in a semilog fashion against the duration of preincubation with 5.0 μM 17-ODYA. The assay for LTB₄ ω-hydroxylase activity was performed as described under "Experimental Procedures." Control LTB₄ ω-hydroxylase activity was 205 pmol/min/mg of protein. Results represent mean values ± SD obtained in seven experiments.

RESULTS

Inactivation of the LTB₄ ω-Hydroxylase in PMN Sonicates—Sonicated suspensions of human PMN exhibited potent LTB₄ ω-hydroxylase activity. In fact, the specific activity of the ω-hydroxylase in PMN sonicates was greater than that observed in suspensions of intact PMN (Table I). Conversion of exogenous LTB₄ to its ω-oxidation products in PMN sonicates was linear with respect to protein concentration and duration of incubation and required the addition of either NADPH or an NADPH-regenerating system (not shown). Only a small amount of conversion was observed (and only in some experiments) in the absence of exogenous NADPH. Interestingly, conversion of 20-OH-LTB₄ to 20-COOH-LTB₄ in PMN sonicates generally was less than 20% of that observed in experiments with intact PMN. Thus, the major product formed from LTB₄ in PMN sonicates was 20-OH-LTB₄.

Although LTB₄ ω-hydroxylase activity in PMN sonicates was labile (sonicates stored for 12 h at 24 and 4 °C lost 100 and 26% of activity, respectively), only small amounts of activity were lost after brief periods of preincubation at 37 °C with 1.0 mM NADPH alone (e.g. 7 ± 5% after 30 min, n = 7). In contrast, after preincubation with 1.0 mM NADPH and 5.0 μM 17-ODYA for 30 min, LTB₄ ω-hydroxylase activity in PMN sonicates decreased 83 ± 6% (mean ± SD, n = 7). LTB₄ ω-hydroxylase activity decreased in a time- and concentration-dependent fashion when PMN sonicates were preincubated with 17-ODYA in the presence of 1.0 mM NADPH (Fig. 2). Virtually no activity was lost, however, if PMN sonicates were preincubated either with 17-ODYA alone or with 17-ODYA and NADPH under anaerobic conditions (i.e. in buffers saturated with nitrogen) (not shown). As shown in Fig. 2, the decrease in LTB₄ ω-hydroxylase activity in PMN sonicates was highly dependent on the duration of preincubation with 17-ODYA. This characteristic is different from the competitive inhibition that occurs at simple competitive inhibition. Preincubation of PMN sonicates at 37 °C for 30 min with 17-ODYA at concentrations of 0.1, 0.5, 1.0, 2.5, 5.0, and 25 μM inactivated the LTB₄ ω-hydroxylase by 20, 53, 66, 73, 83, and 89%, respectively. The duration of preincubation required to inactivate 50% of LTB₄ ω-hydroxylase activity at each 17-ODYA concentration was plotted against the recip-
rocal of the 17-ODYA concentration (Fig. 3). The linear relationship indicates that inactivation of the LTb4 ω-hydroxylase is subject to pseudo-first-order kinetics, as required for a suicidal mechanism (26, 27). Inactivation of the LTb4 ω-hydroxylase is characterized by a binding constant (kι) for 17-ODYA of approximately 1.2 μM, and a rate constant of inactivation (kι) of approximately 1.1 × 10⁻⁵ s⁻¹. Finally, all of the LTb4 ω-hydroxylase in PMN sonicates could be inactivated by 17-ODYA. No LTb4 ω-hydroxylase activity was detected after preincubating PMN sonicates with 50 μM 17-ODYA for 60 min.

Inactivation of the LTb4 ω-hydroxylase in PMN sonicates by acetylenic fatty acids also was dependent on the terminal acetylenic moiety and on the carbon chain-length (Table II). Whereas low concentrations of the two long-chain acetylenic fatty acids (15-HDYA and 17-ODYA) readily inactivated the LTb4 ω-hydroxylase, even very high concentrations of the saturated fatty acid precursors (palmitic acid and stearic acid) had negligible effects. In addition, the short-chain acetylenic fatty acids (10-UDYA and 11-DDYA) were much less effective inactivators of the ω-hydroxylase than were the long-chain acetylenic fatty acids.

Inactivation of the LTb4 ω-Hydroxylase in Intact PMN—Conversion of exogenous LTb4 to its ω-oxidation products in PMN suspensions was inhibited by 17-ODYA in a concentration-dependent fashion (Fig. 4). Higher concentrations of 17-ODYA were required to inactivate the LTb4 ω-hydroxylase in intact PMN than were required to inactivate the LTb4 ω-hydroxylase in PMN sonicates.

Effects of 17-ODYA on ω-oxidation of LTb4 in intact PMN varied with the duration of preincubation. Whereas preincubation of intact PMN with 50 μM 17-ODYA for 0 min at 37 °C reduced ω-oxidation of LTb4 by 8 ± 4%, preincubation of PMN with 50 μM 17-ODYA for 30 min reduced ω-oxidation of LTb4 by 65 ± 13% (mean ± SD, n = 5). Preincubation of intact PMN with 250 μM stearic acid for 30 min had an insignificant effect on the ω-oxidation of exogenous LTb4 (not shown).

The ability of intact PMN to hydroxylate LTb4 that was generated endogenously in response to stimulation with the calcium ionophore, A23187, also was decreased by 17-ODYA. As shown in Fig. 5, significantly greater amounts of LTb4 were detected (and for a longer period of time) in the medium surrounding stimulated PMN after the cells were preincubated with 17-ODYA (50 μM for 30 min) than were detected in the medium surrounding stimulated PMN that had been preincubated with buffer alone. The total amount of LTb4 synthesized (sum of LTb4 and its ω-oxidation products), however, was minimally affected by preincubating PMN with 17-ODYA. Finally, concentrations of 17-ODYA as high as 100 μM were not cytotoxic (i.e., did not cause significant release from PMN of the cytoplasmic enzyme, lactate dehydrogenase) (not shown).

**DISCUSSION**

To develop pharmacologically useful inhibitors of the LTb4, ω-hydroxylase in human PMN and to obtain additional evidence that this enzyme resembles other cytochrome P-450 enzymes by examining its susceptibility to suicidal inactivation, we devised a general scheme for synthesizing terminal acetylenic fatty acids. By combining bromination-dehydrobromination and the "acetylenic zipper" reaction, we expedi-
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Fig. 5. Effects of 17-ODYA on the time course of generation of LTB₄ and its ω-oxidation products by stimulated PMN. Shown are the results of a representative experiment in which PMN (20 x 10⁶ cells/ml) were preincubated for 30 min with either buffer alone (A) or 50 μM 17-ODYA (B) and then stimulated with 10 μM A23187. Aliquots were removed after varying durations of incubation with A23187 and assayed for LTB₄ (O) and total ω-oxidation products (i.e. 20-OH-LTB₄ plus 20-COOH-LTB₄ (O)).

3. Frosst) for generously supplying us with synthetic leukotrienes.

Acknowledgment—We wish to thank Dr. Joshua Rokach (Merck) for generously supplying us with synthetic leukotrienes.

REFERENCES
Suicidal Inactivation of LTB4 ω-Hydroxylase