Metabolism of 12(*S*)-Hydroxy-5,8,10,14-eicosatetraenoic Acid and Other Hydroxylated Fatty Acids by the Reductase Pathway in Porcine Polymorphonuclear Leukocytes†

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ABSTRACT: We have previously shown that porcine polymorphonuclear leukocytes (PMNL) reduce leukotriene B_4 (LTB_4) to 10,11-dihydro-LTB_4, 10,11-dihydro-12-epi-LTB_4, and 10,11-dihydro-12-oxo-LTB_4 [Wainwright et al. (1990) *Biochemistry 29*, 1180–1185]. We have now demonstrated that 12(*S*)-hydroxy-5,8,10,14-eicosatetraenoic acid [12(*S*)-HETE] is metabolized by a similar pathway in porcine PMNL. 12(*S*)-HETE was metabolized to two products that were identified by gas chromatography–mass spectrometry and nuclear magnetic resonance spectroscopy as 12-hydroxy-5,8,14-eicosatrienoic acid (10,11-dihydro-12-HETE) and 12-oxo-5,8,14-eicosatrienoic acid (10,11-dihydro-12-oxo-ETE). Derivatization of 12-hydroxy-5,8,14-eicosatrienoic acid with (*R*)-(+)-α-methoxy-α-(trifluoromethyl)phenylacetic acid, followed by chromatography on a silicic acid column, enabled the resolution of 12*R* and 12*S* stereoisomers, which were identified by cochromatography with synthetic standards. Incubation of 12(*S*)-HETE with PMNL for various times revealed that the stereochemistry of the 12-hydroxyl group of 12-hydroxy-5,8,14-eicosatrienoic acid was initially the same as that of 12(*S*)-HETE. However, after 40 min, 30% of the 12-hydroxy-5,8,14-eicosatrienoic acid had the opposite configuration at C_{12}. 13-Hydroxy-9,11-octadecadienoic acid (13-HODE) was metabolized in a similar fashion by porcine PMNL to 13-hydroxy-9-octadecenoic acid (11,12-dihydro-13-HODE) and 13-oxooctadecenoic acid (11,12-dihydro-13-oxo-ODE). The apparent K_m values for the reduction of 12-HETE, LTB_4, and 13-HODE were 0.21, 0.28, and 2.22 μM, respectively. All three substrates had the same apparent V_{max} [0.029 pmol min^{-1} $(10^6$ cells$)^{-1}$]. Competition experiments between LTB_4 and 12-HETE indicated that they were metabolized by the same pathway. Various structurally related compounds were metabolized by porcine PMNL in the order LTB_4 = 6-*trans*-LTB_4 > 12-epi-6-*trans*,8-*cis*-LTB_4 > 12-epi-6-*trans*-LTB_4 > 12-HETE > LTB_5 > 15-HETE = 13-HODE ≫ 5-HETE > 9-HODE > 20-hydroxy-LTB_4 > 12-hydroxy-5,8,10-heptadecatrienoic acid. Prostaglandins E_2 and $F_{2\alpha}$ were not metabolized to any detectable products by porcine PMNL.

12(*S*)-Hydroxy-5,8,10,14-eicosatetraenoic acid [12(*S*)-HETE][1] is a metabolite of arachidonic acid formed by 12-lipoxygenase (Hamberg & Samuelsson, 1974). In the human, this enzyme is found primarily in the platelet, with lesser amounts in leukocytes (Spector et al., 1988). In the pig, the polymorphonuclear leukocyte is the main source of 12(*S*)-HETE, which is the major product of arachidonic acid metabolism in these cells (Yoshimoto et al., 1982). Although studies into the biological activity of this substance have not revealed a single major physiological role, 12(*S*)-HETE has been shown to have proinflammatory effects (Spector et al., 1988) including chemotactic and chemokinetic actions on neutrophils (Goetzl et al., 1977) and smooth muscle cells (Setty et al., 1987a). It has also been reported to inhibit renin release (Antonipillai et al., 1987) and to modulate cyclooxygenase activity (Setty & Stuart, 1986; Hadjiagapiou & Spector, 1986). 12(*R*)-HETE, which is formed from arachidonic acid by a cytochrome P-450 (Capdevila et al., 1986), has greater chemotactic activity than 12(*S*)-HETE (Cunningham & Woollard, 1987), presumably because it interacts more strongly with the leukotriene B_4 (LTB_4) receptor.

12(*S*)-HETE is metabolized by several pathways. In human PMNL, it is converted by ω-oxidation to 20-hydroxy (Wong et al., 1984; Marcus et al., 1984) and ω-carboxy metabolites (Marcus et al., 1988). 12(*S*)-HETE is also a substrate for 5-lipoxygenase in human PMNL, yielding 5(*S*),12(*S*)-dihydroxy-6*E*,8*Z*,10*E*,14*Z*-eicosatetraenoic acid (12-epi-8-*cis*,6-*trans*-LTB_4) (Borgeat et al., 1981). Finally, it is converted to 8-hydroxy-4,6,10-hexadecatrienoic acid by two successive cycles of β-oxidation in vascular smooth muscle cells (Hadjiagapiou et al., 1987).

We recently reported the existence of another pathway for the metabolism of 5,12-dihydroxy eicosanoids involving reduction of a conjugated double bond. Human PMNL were first reported to convert 6-trans isomers of LTB_4 to 6,11-dihydro and 20-dihydroxy-6,11-dihydro metabolites (Powell,

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[1] Abbreviations: LT, leukotriene; PG, prostaglandin; 5-HETE, 5-hydroxy-6,8,11,14-eicosatetraenoic acid; 12-HETE, 12-hydroxy-5,8,10,14-eicosatetraenoic acid; 15-HETE, 15-hydroxy-5,8,11,13-eicosatetraenoic acid; 9-HODE, 9-hydroxy-10,12-octadecadienoic acid; 13-HODE, 13-hydroxy-9,11-octadecadienoic acid; ODS, octadecylsilyl; PMNL, polymorphonuclear leukocytes; ETYA, 5,8,11,14-eicosatetraynoic acid; MTPA, (+)-α-methoxy-α-(trifluoromethyl)phenylacetic acid; GC–MS, gas chromatography–mass spectrometry; NMR, nuclear magnetic resonance; RP-HPLC, reversed-phase high-pressure liquid chromatography; NP-HPLC, normal-phase high-pressure liquid chromatography; t_R, retention time.

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1986, 1988). Although human PMNL did not appear to metabolize LTB_4 by this pathway, this substance was metabolized to dihydro products by rat (Powell, 1987a) and porcine PMNL (Powell & Gravelle, 1989; Wainwright et al., 1990), rat mesangial cells and fibroblast tumor cells, mouse T-lymphocytes, and macrophages (Kaever et al., 1987), and human monocytes (Fauler et al., 1989) and alveolar macrophages (Schönfeld et al., 1988). However, the nature of the dihydro metabolite formed from LTB_4 by porcine PMNL was different from that formed from the 6-trans isomers of LTB_4 by human PMNL in that it was the 10,11-double bond of LTB_4 which was reduced in the former (Powell & Gravelle, 1989). Porcine PMNL converted 10,11-dihydro-LTB_4 to 10,11-dihydro-12-oxo-LTB_4 (Powell & Gravelle, 1989) as well as to 10,11-dihydro-12-epi-LTB_4 (Wainwright et al., 1990). The dihydro metabolite of LTB_4 produced by rat mesangial cells appears to have little proinflammatory activity (Kaever et al., 1988), although it is not certain whether it is completely identical with the corresponding product formed by porcine PMNL.

Bovine corneal microsomes have recently been shown to convert arachidonic acid to 12(*R*)-hydroxy-5,8,14-eicosatrienoic acid, which was reported to have potent vasodilatory and angiogenic properties (Masferrer et al., 1987; Murphy et al., 1988) and to be a more potent chemotactic agent for human neutrophils than LTB_4 (Rimarachin et al., 1989). The corresponding 12*S* isomer had little biological activity (Murphy et al., 1988; Rimarachin et al., 1989). 12(*R*)-Hydroxy-5,8,14-eicosatrienoic acid was thought to have arisen from arachidonic acid via a cytochrome P-450 pathway not involving 12(*S*)-HETE (Murphy et al., 1988). However, it is possible that this product could also be formed in neutrophils by reduction of the 10,11-double bond of 12(*S*)-HETE in a manner analagous to the formation of 10,11-dihydro-LTB_4.

The major objective of the current study was to determine whether monohydroxy eicosanoids and related substances are metabolized by the 10,11-reductase pathway. In particular, we wanted to determine whether 12(*S*)-HETE could be converted by this pathway to a dihydro metabolite identical with the biologically active cytochrome P-450 product (Murphy et al., 1988) mentioned above. For these studies it was therefore crucial to determine which of the double bonds of 12(*S*)-HETE had been reduced as well as the configuration of the C_{12} hydroxyl group in the dihydro product.

Experimental Procedures

Materials. (*R*)-(+)-α-Methoxy-α-(trifluoromethyl)-phenylacetic acid (MTPA), 1,3-dicyclohexylcarbodiimide, 4-(dimethylamino)pyridine, and Diazald were obtained from Aldrich. A23187 was obtained from Calbiochem-Behring. Arachidonic acid and linoleic acid were purchased from Nuchek Prep Inc. [1-^{14}C]Arachidonic acid, [1-^{14}C]linoleic acid, [5,6,8,11,12,14,15-^{3}H]PGE_2, and [5,6,8,9,11,12,14,15-^{3}H]-$PGF_{2\alpha}$ were obtained from Du Pont-New England Nuclear. 5,8,11,14-Eicosatetraynoic acid (ETYA) was kindly provided by Dr. J. R. Paulsrud of Hoffman-La Roche. PGB_2 was purchased from Sigma. 12(*R*)-Hydroxy-5,8,14-eicosatrienoic acid and 12(*S*)-hydroxy-5,8,14-eicosatrienoic acid were chemically synthesized as previously described (Murphy et al., 1988).

Biosynthesis of Substrates for PMNL Reductase. Unlabeled LTB_4, 6-*trans*-LTB_4, 12-epi-6-*trans*-LTB_4, and 5-HETE were prepared by incubation of arachidonic acid and A23187 (10 μM) with porcine PMNL which were pretreated with ETYA (Powell, 1983; Borgeat et al., 1981). LTB_5 was prepared under identical conditions from 5,8,11,14,17-eicosapentaenoic acid. Unlabeled 12(*S*)-HETE and 12-epi-8-*cis*,6-*trans*-LTB_4 were prepared similarly by incubating arachidonic acid and A23187 (10 μM) with porcine PMNL in the absence of ETYA (Powell, 1983; Borgeat et al., 1981). 1-^{14}C-Labeled LTB_4, 12-epi-8-*cis*, 6-trans-LTB_4, 6-*trans*-LTB_4, 12-epi-6-*trans*-LTB_4, and 5-HETE were prepared by incubation of [1-^{14}C]arachidonic acid with purified human PMNL and A23187 (10 μM) for 5 min at 37 °C (Borgeat & Samuelsson, 1979). 1-^{14}C-Labeled and unlabeled 13-hydroxy-9,11-octadecadienoic acid (13-HODE) and 15-HETE were prepared enzymatically from linoleic acid and arachidonic acid, respectively, with soybean lipoxygenase (Hamberg & Samuelsson, 1967). 1-^{14}C-Labeled and unlabeled 12-hydroxy-5,8,10-heptadecatrienoic acid (HHT) and 12(*S*)-[1-^{14}C]HETE were prepared by incubation of [1-^{14}C]arachidonic acid or unlabeled arachidonic acid with human platelets (Hamberg & Samuelsson, 1974). 1-^{14}C-Labeled and unlabeled 9-HODE were prepared by incubating 1-^{14}C-labeled and unlabeled linoleic acid with a tomato homogenate (Matthew et al., 1977).

Preparation of Porcine PMNL. Porcine peripheral blood was collected in the presence of EDTA (final concentration of 10 mM). Leukocytes were prepared as previously described (Powell, 1984) by treatment of the blood with Dextran T-500 (Pharmacia Fine Chemicals) to sediment the red blood cells. Any remaining red blood cells were lysed with 0.135 M NH_4Cl. This preparation consisted of a mixture of PMNL and mononuclear cells and was used for preparative experiments. Purified PMNL (90–95%), which were used for all of the analytical experiments, were prepared by centrifugation of the crude leukocyte preparation described above over Ficoll-Paque (Pharmacia Fine Chemicals). After being washed with 0.15 mM NaCl, the cells were resuspended in Dulbecco's phosphate-buffered saline, containing 137 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 8.1 mM Na_2HPO_4, 0.5 mM $MgCl_2$, and 0.9 mM $CaCl_2$.

Preparation and Purification of 12-HETE and 13-HODE Metabolites. Unfractionated porcine leukocytes (75×10^6 cells/mL) were incubated at 37 °C with 12(*S*)-HETE (2 μM) for 40 min and 13-HODE (2 μM) for 60 min in separate experiments. The incubations were terminated by the addition of ice-cold methanol (0.15 volume) and immediate cooling to −20 °C. After centrifugation at 400*g* for 10 min, the supernatants were extracted with cartridges of octadecylsilyl silica (ODS-silica) (C_{18} Sep-Paks, Waters-Millipore) as previously described (Powell, 1982). The extract was then purified by reversed-phase (RP) high-pressure liquid chromatography (HPLC) with a Waters solvent delivery system (two Model 510 pumps coupled to a Model 680 gradient controller), a Raytest radioactivity monitor (Ramona 5-LS), and a Waters Model 490 UV detector. The stationary phase was a column (250 × 4.6 mm) of ODS-silica (5-μm Sperisorb ODS-2, Phenomenex). The mobile phase for the purification of the 12(*S*)-HETE metabolites was a linear gradient between acetonitrile/water/acetic acid (28:72:0.02) and acetonitrile/water/acetic acid (52:48:0.02) over 60 min at a flow rate of 2 mL/min. Metabolites of 13-HODE were purified with acetonitrile/water/acetic acid (55:45:0.02) under isocratic conditions at a flow rate of 2 mL/min.

Gas Chromatography–Mass Spectrometry. Products purified by RP-HPLC were methylated with diazomethane unless otherwise indicated. In some cases products were derivatized by treatment with hydroxylamine hydrochloride (1 mg) in pyridine (0.1 mL) overnight at room temperature. Diethyl ether (2 mL) was then added, and the mixture was kept at −20 °C for 30 min. After centrifugation, the diethyl ether was

removed and evaporated under a stream of nitrogen. All products were then converted to their trimethylsilyl derivatives by treatment with *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (30 min, 23 °C). Electron-impact GC-MS was carried out on a VG ZAB instrument located in the Biomedical Mass Spectrometry Unit of McGill University. The stationary phase was a column (20 m × 0.32 mm) of DB-1 (J and W Scientific, Inc.).

Nuclear Magnetic Resonance Spectroscopy. ^{1}H NMR spectroscopy ($CDCl_3$, 500 MHz) was performed on a Varian Model VXR-500S instrument situated in the Department of Molecular Genetics at the University of Texas Southwestern Medical Center.

Steric Analysis of Dihydro Metabolite of 12(S)-HETE. Purified porcine PMNL (20 mL; 50 × 10^6 cells/mL) were incubated at 37 °C with 12-[1-^{14}C]HETE (2 μM; 2 μCi) for various times. The reactions were stopped by the addition of ice-cold methanol (0.5 volume) and immediate cooling to −20 °C. After the addition of PGB_2 (200 ng) as internal standard, the products were extracted with cartridges of ODS-silica as described above. The products were purified by RP-HPLC with a mobile phase of acetonitrile/water/acetic acid (55:45:0.02) and a flow rate of 2 mL/min. The material in the peak corresponding to the 10,11-dihydro metabolite of 12(*S*)-HETE was methylated with diazomethane and then converted to its MTPA derivative by treatment with MTPA (1 mg), 1,3-dicyclohexylcarbodiimide (1 mg), and 4-(dimethylamino)pyridine (0.1 mg) in 20 μL of CCl_4 (20 min, 60 °C). The reaction was terminated by evaporation of the CCl_4 under argon and the addition of 50 μL of methanol. The conversion of 12-hydroxy-5,8,14-eicosatrienoic acid to its MTPA derivative was virtually quantitative, and no other radioactive products were detected. Separation of the MTPA derivatives from nonradioactive contaminants derived from the reagents was accomplished by RP-HPLC on a Phenomenex Ultremex C_6 column (250 × 4.6 mm) with a mobile phase of acetonitrile/water (70:30) at a flow rate of 2 mL/min. The 12*R* and 12*S* isomers of the MTPA derivatives of the dihydro metabolite of 12(*S*)-HETE were not separated under these conditions but were subsequently resolved by NP-HPLC on a 5-μm silicic acid column (RoSil, 250 × 4.6 mm, Alltech Associates) with a mobile phase of 0.08% 2-propanol in hexane at a flow rate of 2 mL/min. The products were quantitated by measuring the radioactivity in fractions collected every 0.5 min by liquid scintillation counting.

Analysis of Reductase Products Formed from Various Hydroxylated Polyunsaturated Fatty Acids (PUFA). Purified porcine PMNL (50 × 10^6 cells/mL) were incubated in 1 mL of PBS at 37 °C with substrates at the concentrations and for the lengths of time indicated in the figure legends. The reactions were terminated by the addition of 0.6 mL of ice-cold methanol and immediate cooling to −20 °C. PGB_2 (200 ng) was added to each sample as an internal standard. The samples were then analyzed by precolumn extraction/RP-HPLC (Powell, 1987b) with a WISP automatic injector and WAVS automated switching valve (Waters-Millipore). The material retained on the precolumn (C_{18} μBondapak Guard-Pak cartridge, Waters-Millipore) was analyzed on a Novapak C_{18} column (3.9 × 150 mm, Waters-Millipore). The mobile phases for each substrate were as follows: LTB_4, 12-epi-8-*cis*,6-*trans*-LTB_4, 6-*trans*-LTB_4, and 12-epi-6-*trans*-LTB_4 [acetonitrile/water/acetic acid (37:63:0.02); isocratic]; 12-HETE, 5-HETE, and 15-HETE [linear gradient between acetonitrile/water/acetic acid (46:54:0.02) and acetonitrile/water/acetic acid (55:45:0.02) over 30 min]; 13-HODE and 9-HODE [linear gradient between acetonitrile/water/acetic acid (42:58:0.02) and acetonitrile/water/acetic acid (48:52:0.02) over 30 min]; 20-hydroxy-LTB_4 [acetonitrile/water/acetic acid (23:77:0.02); isocratic]; LTB_5 [acetonitrile/water/acetic acid (31:69:0.02); isocratic], PGE_2 and $PGF_{2\alpha}$ [linear gradient between acetonitrile/water/acetic acid (26:74:0.02) and acetonitrile/water/acetic acid (46:54:0.02) over 30 min]. The flow rate was 2 mL/min in all cases. The products were quantitated by measuring the radioactivity by liquid scintillation counting, in fractions collected every 0.5 min with the exception of LTB_5 metabolites, which were quantitated by measuring UV absorbance at 235 nm.

Results

Metabolism of 12-HETE by Porcine Leukocytes

Porcine leukocytes were incubated with 12(*S*)-HETE, and the products were analyzed by RP-HPLC as shown in Figure 1A. Two less polar radioactive products (a and b) were formed which did not show any absorbance at 235 nm but were detected at 200 nm. The lack of absorbance at 235 nm of products a and b indicates that they no longer contain conjugated double bonds. The HPLC profile is reminiscent of that observed after incubation of LTB_4 with porcine PMNL (Figure 1C), which also shows two less polar radioactive peaks absorbing at a lower wavelength than the substrate.

Identification of Product a. The mass spectrum of the trimethylsilyl ether, methyl ester derivative of product a (Figure 2A) exhibited intense ions at *m/z* 408 (M), 393 (M − 15), 377 (M − 31), 351 (loss of C_{17}–C_{20}), 318 (M − 90), 297 (C_1–C_{12}), 213 (C_{12}–C_{20}), 207 (297 − 90), 175 [297 − (90 + 32)], 133 (base peak), 129, 121, and 107. This mass spectrum is very similar to that of the corresponding derivative of 12-HETE, except that ions containing the C_1 to C_{12} portion of the molecule, including the molecular ion and the ions at *m/z* 297 and 207 occur 2 mass units higher than the corresponding ions for 12-HETE. This indicates that one of the double bonds between carbons 1 and 12 of 12-HETE has been reduced and that product a is identical with 12-hydroxyeicosatrienoic acid (dihydro-12-HETE).

The absence of UV absorbance at 235 nm by dihydro-12-HETE indicates that one of the two conjugated double bonds betwen carbons 8 and 11 of 12-HETE has been reduced. Since the mass spectrum clearly shows that only one double bond has been reduced, it can be assumed that the double bonds present at positions 5 and 14 of 12-HETE are retained in the dihydro metabolite. The remaining double bond, the position of which could not be determined from the mass spectrum, could theoretically reside in one of three locations: (A) C_8–C_9, (B) C_{10}–C_{11}, or (C) C_9–C_{10} as shown in Figure 3. To ascertain which of these is correct, a sample of dihydro-12-HETE was analyzed by NMR spectroscopy (Figure 4). The NMR spectrum shows a triplet centered at 2.77 ppm, characteristic of the bis-allylic methylene group which is present in structure A but not in either B or C. This clearly indicates that the third double bond of dihydro-12-HETE is located between carbons 8 and 9, identifying the compound as 12-hydroxy-5,8,14-eicosatrienoic acid. Moreover, the NMR spectrum is virtually identical with that of authentic chemically synthesized 12-hydroxy-5,8,14-eicosatrienoic acid. Integration of the relevant peaks revealed that the biological sample was greater than 95% pure with respect to regioisomeric olefins.

Identification of Product b. Product b reacted with hydroxylamine hydrochloride in the presence of pyridine to give an oxime derivative, indicating that it contained an oxo group. The oxime derivative of product b was converted to its trimethylsilyl ether, ester derivative. The mass spectrum of this

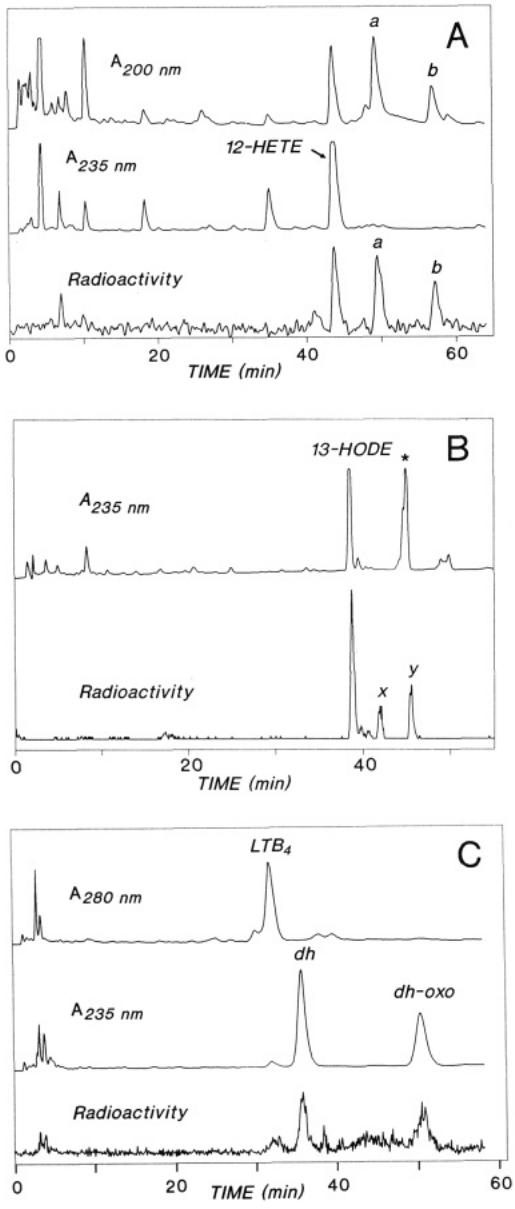

FIGURE 1: Reversed-phase HPLC of metabolites of (A) 12-HETE, (B) 13-HODE, and (C) LTB_4. 12-HETE (2 μM), 13-HODE (2 μM), and LTB_4 (2 μM) were incubated with unfractionated porcine PMNL (75×10^6 cells/mL) at 37 °C for 60, 40, and 60 min, respectively. Products were extracted on a cartridge of ODS-silica and separated by RP-HPLC on a Phenomenex Spherisorb ODS-2 column under the following conditions: (A) linear gradient between water/acetonitrile/acetic acid (72:28:0.02) and water/acetonitrile/acetic acid (48:52:0.02) over 60 min, (B) water/acetonitrile/acetic acid (55:45:0.02) under isocratic conditions, and (C) water/acetonitrile/acetic acid (63:37:0.02) under isocratic conditions. The flow rate was 2 mL/min in all cases. In (B), the peak labeled with an asterisk (*) and absorbing at 235 nm had a retention time shorter than that of product y and was not radioactive. Abbreviations: dh-LTB_4, 10,11-dihydro-LTB_4; dh-oxo, 10,11-dihydro-12-oxo-LTB_4.

compound (Figure 2B) exhibited intense ions at m/z 479 (M), 464 (M − 15), 436 (C_1–C_{17}), 422 (C_1–C_{16}), 368 (C_1–C_{12}), 348 (C_3–C_{20}), 334 (C_4–C_{20}), 320 (C_5–C_{20}), 280 (C_8–C_{20}), 266, 210, 147, and 117. This mass spectrum indicates that product b is a dihydro metabolite of 12-HETE with an oxo group at position 12. The ion at m/z 280 would suggest that there is a double bond in the 8-position, whereas that at m/z 266 would be more consistent with its presence in the 9-position. However, it is possible that the latter ion could have arisen after a rearrangement of the double bonds or that there could be a mixture of products which differ from one another in the

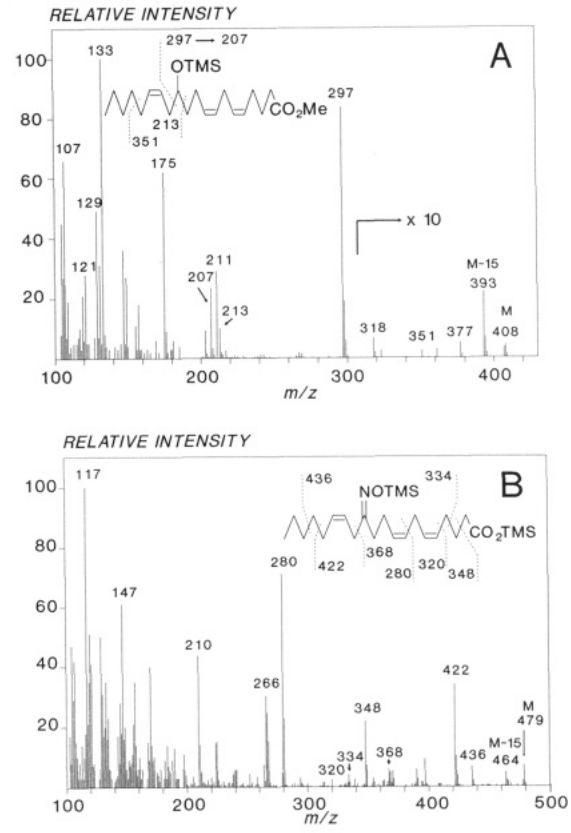

FIGURE 2: Mass spectra of (A) the trimethylsilyl ether, methyl ester derivative of product a formed from 12-HETE and (B) the trimethylsilyl ether, oxime derivative of product b formed from 12-HETE. TMS, trimethylsilyl. Mass spectra were obtained with an ionizing voltage of 70 EV, an accelerating voltage of 6 kV, and a trap current of 100 μA.

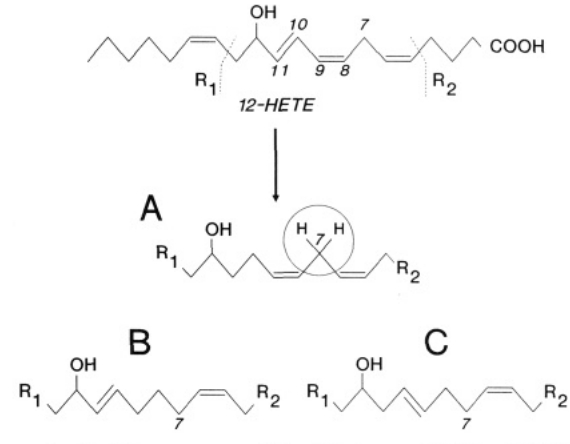

FIGURE 3: Possible structures of the dihydro metabolite of 12-HETE (product a): (A) 12-hydroxy-5,8,14-eicosatrienoic acid; (B) 12-hydroxy-5,10,14-eicosatrienoic acid; (C) 12-hydroxy-5,9,14-eicosatrienoic acid. The circled methylene group is the one giving rise to the triplet centered at 2.77 ppm in the inset to Figure 4.

positions of their double bonds. By analogy with the 10,11-dihydro metabolite of 12(S)-HETE, it would seem more likely that the double bonds are present in the 5-, 8-, and 14-positions, and we would therefore tentatively assign the structure of 12-oxo-5,8,14-eicosatrienoic acid to product b.

Stereochemical Analysis of 12-Hydroxy-5,8,14-eicosatrienoic Acid

The mass spectrum of product a indicates that it is identical with 12-hydroxy-5,8,14-eicosatrienoic acid. Since the 12*R*, but not the 12*S*, isomer of this compound has been reported to have potent proinflammatory properties, it was of consid-

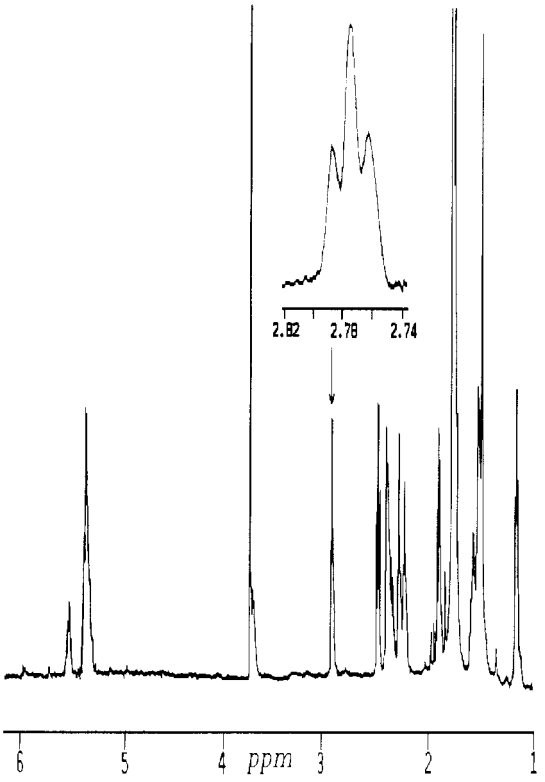

FIGURE 4: Nuclear magnetic resonance spectrum of the dihydro metabolite of 12-HETE (55 μg; product a). The inset is an enlargement of the triplet at 2.77 ppm.

erable interest to determine the configuration of the 12-hydroxyl group of this compound. 12(*S*)-HETE was incubated with PMNL for various times, and the products were purified by RP-HPLC as shown in Figure 1A. The material in the peak corresponding to 12-hydroxy-5,8,14-eicosatrienoic acid was methylated and converted to its MTPA derivative. After removal of contaminants derived from the reagents by RP-HPLC on a C_6 column, the methyl ester, MTPA derivative of the 10,11-dihydro metabolite of 12(*S*)-HETE was subjected to steric analysis by NP-HPLC on a silicic acid column. As shown in Figure 5A, 12-hydroxy-5,8,14-eicosatrienoic acid, formed by incubation of 12(*S*)-HETE with PMNL for 40 min, was resolved into two radioactive components which adsorbed in the UV region at 200 nm. These two products cochromatographed with the methylated MTPA derivatives of authentic 12(*R*)-hydroxy-5,8,14-eicosatrienoic acid (t_R 23 min) and 12(*S*)-hydroxy-5,8,14-eicosatrienoic acid (t_R 25 min) (Figure 5B). The mass spectrum of the unresolved methyl ester, MTPA derivative of 12-hydroxy-5,8,14-eicosatrienoic acid was identical with that of the corresponding derivative of authentic 12(*S*)-hydroxy-5,8,14-eicosatrienoic acid. Although these mass spectra were not particularly informative, intense ions were observed at *m/z* 318 (M − MTPA) and 189 [$C_6H_5 - C(CF_3) = {}^+OCH_3$].

The time courses for the formation of 12(*R*)-hydroxy-5,8,14-eicosatrienoic acid, 12(*S*)-hydroxy-5,8,14-eicosatrienoic acid, and 12-oxo-5,8,14-eicosatrienoic acid are shown in Figure 6. 12(*R*)-Hydroxy-5,8,14-eicosatrienoic acid was the predominant product at all time points, reaching a maximum at 20 min and then declining.[2] 12-Oxo-5,8,14-eicosatrienoic acid reached its maximal level by 10 min and did not change

[2] Due to the priority rules for assigning *R* and *S* configurations, the configuration of the 12-hydroxyl group of 12(*S*)-HETE is identical with that of 12(*R*)-hydroxy-5,8,14-eicosatrienoic acid. Thus, the major product has the same configuration at C_{12} as the precursor.

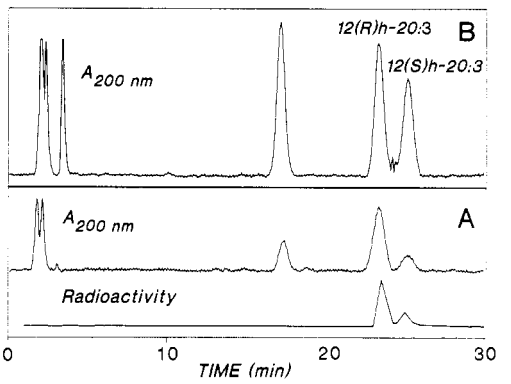

FIGURE 5: Normal-phase HPLC of the MTPA derivatives of 12-hydroxy-5,8,14-eicosatrienoic acid. (A) NP-HPLC of the MTPA derivative of the dihydro product formed after incubation of 12-[1-^{14}C]HETE (2 μM) with purified porcine PMNL (50 × 10^6 cells/mL) for 40 min at 37 °C. The products were extracted on a cartridge of ODS-silica and were purified by RP-HPLC. Dihydro-12-HETE (product a) was converted to the MTPA derivative of its methyl ester as described under Experimental Procedures. The *R* and *S* isomers of the MTPA derivative were separated by NP-HPLC. (B) NP-HPLC of a mixture of the methyl ester, MTPA derivatives of chemically synthesized 12(*R*)-5,8,14-eicosatrienoic acid (t_R = 23 min) and 12-(*S*)-hydroxy-5,8,14-eicosatrienoic acid (t_R = 25 min). Abbreviations: 12(*R*)h-20:3, 12(*R*)-hydroxy-5,8,14-eicosatrienoic acid; 12(*S*)h-20:3, 12(*S*)-hydroxy-5,8,14-eicosatrienoic acid.

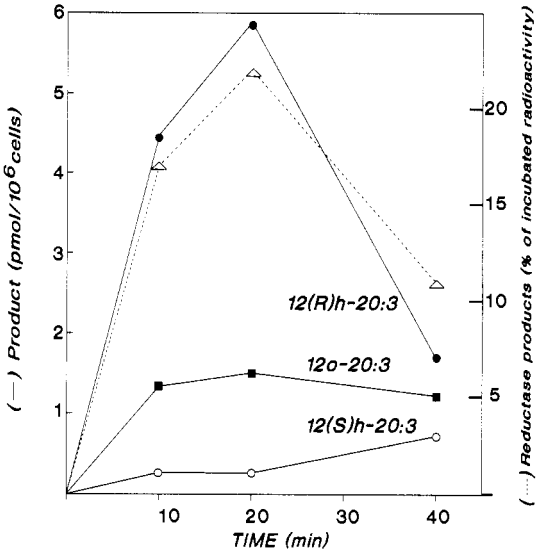

FIGURE 6: Time course for the metabolism of 12(*S*)-HETE to dihydro products. 12-[1-^{14}C]HETE (2 μM) was incubated with purified porcine PMNL (50 × 10^6 cells/mL) for various time periods. 12-(*R*)-Hydroxy-5,8,14-eicosatrienoic acid [12(*R*)h-20:3 (●)], 12(*S*)-hydroxy-5,8,14-eicosatrienoic acid [12(*S*)h-20:3 (○)], and 12-oxo-5,8,14-eicosatrienoic acid [12o-20:3 (■)] were analyzed as described in the legend of Figure 5. Products were quantitated by measurement of radioactivity by liquid scintillation counting in fractions collected every 0.5 min. The percentage of incubated 12(*S*)-HETE recovered as less polar reductase products is also shown (△).

thereafter. The formation of 12(*S*)-hydroxyeicosatrienoic acid exhibited a lag period, followed by an increase to 30% of the total 12-hydroxy-5,8,14-eicosatrienoic acid at 40 min, suggesting that it is not an initial product but that it may be formed from either 12(*R*)-hydroxy-5,8,14-eicosatrienoic acid or 12-oxo-5,8,14-eicosatrienoic acid. The total recovery of the above three 12(*S*)-HETE metabolites was considerably lower after 40 min than after 20 min, suggesting that these products may subsequently be metabolized by other pathways such as β-oxidation. The loss of radioactivity could be substantially lowered by inclusion of ETYA in the incubations (see below), but it was not added in the present experiment to avoid any

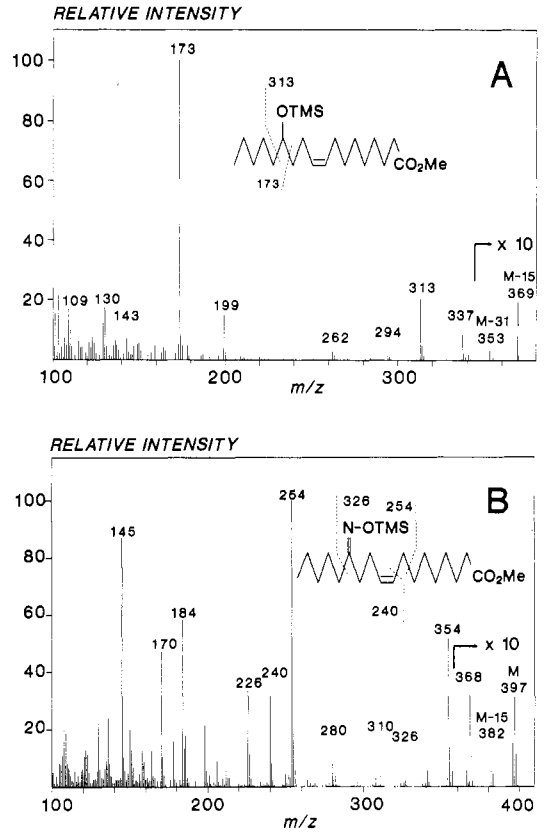

FIGURE 7: Mass spectra of (A) the trimethylsilyl ether, methyl ester derivative of product x and (B) the trimethylsilyl ether, oxime, methyl ester derivative of product y. TMS, trimethylsilyl.

effects it might have on the ratio of the two stereoisomers of dihydro-12-HETE.

Identification of Metabolites of 13-HODE

Porcine PMNL (75×10^6 cells/mL) were incubated with 13-HODE, and the products were analyzed by RP-HPLC (Figure 1B). Two less polar radioactive products (x and y) were detected, neither of which absorbed at 235 nm, indicating that the conjugated diene of the substrate is not present in products x and y.

Identification of Product x. The mass spectrum of the trimethylsilyl ether, methyl ester derivative of product x showed intense ions at m/z 369 (M − 15), 353 (M − 31), 337 (M − 47), 313 (C_1–C_{13}), 294 (M − 90), 262, 199, and 173 (base peak; C_{13}–C_{18}) (Figure 7A). The molecular ion and the ion at m/z 313 (C_1–C_{13}) of this compound are 2 mass units higher than the corresponding ions in the mass spectrum of the trimethylsilyl ether, methyl ester derivative of 13-HODE, indicating that one of the double bonds has been reduced. Although the mass spectrum does not give any information on the location of the remaining double bond, it is probably in the C_9 position, by analogy with the corresponding metabolite of 12-HETE. This product is therefore tentatively identified as 13-hydroxy-9-octadecenoic acid (i.e., 11,12-dihydro-13-HODE).

Identification of Product y. Product y was found to react with hydroxylamine hydrochloride in the presence of pyridine to give an oxime derivative, indicating the presence of an oxo group. The mass spectrum of the trimethylsilyl ether, oxime derivative of the methyl ester of product y (Figure 7B) showed intense ions at m/z 397 (M), 382 (M − 15), 368 (M − 29), 366 (M − 31), 354 (M − 43), 326 (C_1–C_{13}), 310 (C_4–C_{18}), 308, 280, 254 (base peak; C_8–C_{18}), 240 (C_9–C_{18}), 226, 184, 170, and 145. This mass spectrum indicates that this metabolite of 13-HODE has an oxo group at C_{13} and a single double bond. The base peak at m/z 254 would strongly suggest that the double bond is in the 9-position and would not be expected if it were in either the 10- or 11-position. The ion at m/z 240 would support this conclusion, but that at m/z 226 would be more consistent with a double bond in the 10-position. However, as discussed earlier for the dihydrooxo metabolite of 12-HETE, it is possible that the latter ion could have arisen due to a rearrangement or that there could be a mixture of dihydrooxo metabolites which differ from one another in the positions of their double bonds. The most likely structure for product y is 13-oxo-9-octadecenoic acid.

Table I: Effect of ETYA on Recoveries of LTB_4, 12-HETE, 13-HODE, and Their Reductase Products[a]

substrate	products [pmol $(10^7$ cells$)^{-1}$ (10 min$)^{-1}$] reductase[b]	dihydrooxo	recovery of radioactivity (%)[c]
12 HETE	30 (8)	9	40
12-HETE + ETYA	36 (9)	10	51
LTB_4	75 (19)	32	78
LTB_4 + ETYA	70 (17)	37	82
13-HODE	0 (0)	0	9
13-HODE + ETYA	38 (29)	27	69

[a] [1-^{14}C]LTB_4 (2 μM) and 12-[1-^{14}C]HETE (2 μM) were incubated with purified porcine PMNL (50×10^6 cells/mL) for 10 min at 37 °C with and without 5 μM ETYA. 13-[1-^{14}C]HODE (2 μM) was incubated with purified porcine PMNL (50×10^6 cells/mL) for 30 min at 37 °C with and without 10 μM ETYA. Products were quantitated by measurement of radioactivity. [b] The sum of dihydro and dihydrooxo products. The percentages of incubated substrates recovered as less polar reductase products are shown in parentheses. [c] The percentage of incubated radioactivity recovered as the substrate and its less polar dihydro and dihydrooxo products.

Effect of ETYA on Metabolism of Monohydroxy-PUFA's

The data in Figure 6 indicate that there is a reduction in the total amounts of dihydro metabolites of 12-HETE recovered after 40 min. Preliminary experiments indicated that the loss of dihydro products with time was even more pronounced with other 1-^{14}C-labeled monohydroxy-PUFA's including 15-HETE and 13-HODE. This could largely be attributed to β-oxidation, since there was a marked reduction in the total amount of radioactivity recovered. The loss of radioactivity could be reduced by the addition of ETYA (5 μM), which did not affect the formation of dihydro and dihydrooxo products but inhibited their degradation, along with that of the substrate, to other products. Table I shows the percentage recovery of incubated radioactivity by substrates and their dihydro metabolites after incubation of 12-[1-^{14}C]HETE (2 μM), [1-^{14}C]LTB_4 (2 μM), and 13-[1-^{14}C]-HODE (2 μM) with porcine PMNL in the presence and absence of ETYA. The recovery of LTB_4 and its reduced metabolites was not appreciably affected by the addition of ETYA, whereas the recovery of 12-HETE and its reduced products was 11% higher in the presence of ETYA. ETYA had a much more pronounced effect on the recovery of 13-HODE and its dihydro metabolites, increasing it to 69% compared to only 9% recovery in the absence of ETYA. As ETYA (5 μM) was found to enhance the recovery of the substrates and their dihydro metabolites without affecting the formation of the latter, this concentration of ETYA was used in all of the analytical experiments described below.

Effect of Substrate Concentration on Metabolism of LTB_4, 12-HETE, and 13-HODE

Porcine PMNL were incubated with various concentrations of [1-^{14}C]LTB_4, 12-[1-^{14}C]HETE, and 13-[1-^{14}C]HODE for

Table II: Specificity of Porcine Reductase Pathway[a]

substrate	products [pmol (10^7 cells)$^{-1}$ (10 min)$^{-1}$] reductase[b] corrected	reductase[b] uncorrected[c]	dihydrooxo corrected	dihydrooxo uncorrected	recovery of radioactivity (%)[d]
LTB_4	85	70 (17)	46	37	82
6-*trans*-LTB_4	85	63 (16)	49	36	77
12-epi-8-*cis*,6-*trans*-LTB_4	81	51 (13)	29	18	66
12-epi-6-*trans*-LTB_4	77	60 (15)	22	17	82
12-HETE	72	36 (9)	19	10	51
LTB_5	55	42 (10)	38	29	75
15-HETE	47	23 (6)	36	17	48
13-HODE	47	24 (6)	38	20	51
5-HETE	23	8 (2)	9	3	34
9-HODE	15	10 (2)	13	9	67
20-OH-LTB_4	11	7 (2)	6	4	67
HHT	10	7 (2)	8	6	76
PGE_2	0	0 (0)	0	0	91
$PGF_{2\alpha}$	0	0 (0)	0	0	90

[a] 1-^{14}C-Labeled substrates (2 μM) were incubated with porcine PMNL (50×10^6 cells/mL) for 10 min at 37 °C. Products were analyzed by precolumn extraction/RP-HPLC. Products were quantitated by measuring radioactivity by liquid scintillation counting in 0.5-min column fractions. The percent recovery was calculated by comparison with control incubations in which the substrate was incubated with buffer alone. The amounts of reductase products formed were corrected for recovery by dividing the uncorrected amounts by the percent recovery of radioactivity (×100). [b] The total of dihydro and dihydrooxo products. Products formed by the 10,11-reductase and the 6,11-reductase were not distinguished from one another. [c] The values in parentheses are the percentages of incubated substrates which were recovered as less polar reductase products. [d] The percentage of incubated radioactivity recovered as the substrate and its dihydro and dihydrooxo products.

6, 6, and 30 min, respectively. The products were analyzed by precolumn extraction/RP-HPLC, and the apparent K_m and V_{max} values were calculated from Lineweaver–Burk plots. The apparent K_m values for LTB_4 (0.28 μM) and 12-HETE (0.21 μM) were similar, whereas that for 13-HODE (2.2 μM) was much higher. All three substrates shared an apparent V_{max} of approximately 0.029 pmol min^{-1} (10^6 cells)$^{-1}$.

To confirm that 12-HETE and LTB_4 are metabolized by the same enzymes, a competition experiment was performed in which various concentrations of 12-[1-^{14}C]HETE were incubated with PMNL in the presence or absence of a single concentration (2.0 μM) of LTB_4. Lineweaver–Burk analysis revealed that the addition of LTB_4 did not affect the V_{max} but increased the slope of the line, indicative of competitive inhibition. Similar results were obtained when 12-HETE (2.0 μM) was incubated with various concentrations of [1-^{14}C]-LTB_4.

Specificity of Porcine Reductase Pathway

The substrate specificity of the reductase pathway was investigated with various structurally related compounds derived from arachidonic acid and linoleic acid (Table II). To avoid limitations due to lack of substrate availability in the amounts of products formed, the incubation conditions employed (2 μM substrate; 10 min) permitted no more than 25% metabolism of the substrate. The best substrate for the 10,11-reductase pathway is LTB_4. 6-*trans*-LTB_4, and 12-epi-6-*trans*-LTB_4 were metabolized to dihydro products at about the same rate as LTB_4, but these compounds are also converted to 6,11-dihydro products by a second independent reductase pathway in porcine PMNL (Powell & Gravelle, 1990). The two types of dihydro products were not resolved with the conditions used for the present HPLC analysis, and the values in Table II represent their sums. Although the absolute amounts of dihydro metabolites formed from 12-epi-8-*cis*,6-*trans*-LTB_4 and 12-HETE were lower than for LTB_4, after they were corrected for recovery, the amounts were approximately the same as for LTB_4. All of the above eicosanoids share a C_{12} hydroxyl group preceded by two conjugated double bonds and followed by a 2-*cis*-octenyl group. LTB_5, which is a metabolite of 5,8,11,14,17-eicosapentaenoic acid, is metabolized to a lesser extent than LTB_4, probably due to the presence of the C_{17} double bond in the ω-end of the molecule. Introduction of an ω-hydroxyl group, as in 20-hydroxy-LTB_4, nearly eliminates metabolism by the reductase pathway.

Alteration of the position of the hydroxyl group as in 15-HETE and 5-HETE reduced the rate of metabolism to approximately 65 and 21%, respectively, that of 12-HETE. HHT, which is an endoperoxide-derived arachidonic acid metabolite with a 12-hydroxyl group preceded by two double bonds in the 8- and 10-positions but followed by a 2-pentenyl group, was metabolized only slightly, suggesting that the distance of the hydroxyl group to the ω-end of the molecule is also important for metabolism by this pathway. 13-HODE and 9-HODE, in which the positions of the hydroxyl groups are altered and the carbon chain reduced, also exhibited decreased metabolism. PGE_2 and $PGF_{2\alpha}$, which, after oxidation of their 15-hydroxyl groups, are metabolized in many tissues by a 13,14-reductase (Hamberg & Samuelsson, 1971), were not converted to any detectable products by porcine PMNL.

Discussion

We have previously reported that porcine PMNL convert LTB_4 to 10,11-dihydro-LTB_4, 10,11-dihydro-12-epi-LTB_4, and 10,11-dihydro-12-oxo-LTB_4 (Powell & Gravelle, 1989; Wainwright et al., 1990). 10,11-Dihydro-12-epi-LTB_4 was not an initial product but was formed after a lag period, presumably from either 10,11-dihydro-LTB_4 or 10,11-dihydro-12-oxo-LTB_4 (Wainwright et al., 1990). We have now shown that 12(*S*)-HETE is metabolized by porcine PMNL in a similar manner, giving 12(*R*)-hydroxy-5,8,14-eicosatrienoic acid, 12(*S*)-hydroxy-5,8,14-eicosatrienoic acid, and 12-oxo-5,8,14-eicosatrienoic acid, which were identified by mass spectrometry and NMR spectroscopy. 12(*R*)-Hydroxy-5,8,14-eicosatrienoic acid was the major product at all time points investigated. The formation of 12(*S*)-hydroxy-5,8,14-eicosatrienoic acid increased following a lag period and after 40 min amounted to 30% of the total 10,11-dihydro-12-HETE recovered, suggesting that the 12*S* isomer is not an initial product but is probably formed from either 12(*R*)-hydroxy-5,8,14-eicosatrienoic acid or 12-oxo-5,8,14-eicosatrienoic acid. Thus, 12(*S*)-HETE is metabolized to dihydro products by porcine PMNL in a manner quite analogous to that of LTB_4. Murine B lymphocytes have also

been reported to convert 12-HETE to 19- and 20-hydroxylated products in which at least one of the conjugated double bonds has been reduced (Danilowicz et al., 1989). However, the mechanism for the reaction and the positions of the remaining double bonds in the products were not determined (Danilowicz et al., 1989).

It has recently been reported that arachidonic acid is metabolized by a cytochrome P-450 dependent process in bovine corneal microsomes to 12(*R*)-HETE (Schwartzman et al., 1987) and 12(*R*)-hydroxy-5,8,14-eicosatrienoic acid (Murphy et al., 1988). Due to the priority rules in assigning the *R* and *S* configurations, the 12-hydroxyl groups of 12(*R*)-HETE and 12(*R*)-hydroxy-5,8,14-eicosatrienoic acid are both designated *R* but actually have the opposite absolute stereochemistry. Therefore, formation of 12(*R*)-hydroxy-5,8,14-eicosatrienoic acid from 12(*R*)-HETE would require inversion of the stereochemistry at C_{12}. Two pathways for the formation of 12(*R*)-hydroxy-5,8,14-eicosatrienoic acid were hypothesized. First, cytochrome P-450 could oxygenate arachidonic acid to 11,12-epoxy-5,8,14-eicosatrienoic acid, which could then undergo an epoxide rearrangement to 12-oxo-5,8,14-eicosatrienoic acid, followed by reduction to 12(*R*)-hydroxy-5,8,14-eicosatrienoic acid. Alternatively, 12(*R*)-HETE, formed directly from arachidonic acid by cytochrome P-450, could be converted to 12-oxo-5,8,10,14-eicosatetraenoic acid, followed by two stages of reduction first to 12-oxo-5,8,14-eicosatrienoic acid and then to 12(*R*)-hydroxy-5,8,14-eicosatrienoic acid. Both of these hypotheses involve the abstraction of a hydrogen atom from the 12-position, which is consistent with their observation of the formation of only heptadeuterated 12(*R*)-hydroxy-5,8,14-eicosatrienoic acid from octadeuterated [5,6,8,9,11,12,14,15-$^2H_8$]arachidonic acid.

The reductase pathway which we observed in PMNL could account for the conversion of 12(*R*)-HETE to 12(*R*)-hydroxy-5,8,14-eicosatrienoic acid by bovine corneal microsomes. We have shown that LTB_4, which also contains a 12(*R*)-hydroxyl group, is converted by porcine PMNL to 10,11-dihydro-12-epi-LTB_4, which has the opposite configuration at C_{12} (Wainwright et al., 1990). This reaction involves the initial formation of 10,11-dihydro-LTB_4 in which the stereochemistry of the 12-hydroxy group is retained. The latter product is then converted to the corresponding 12-epi metabolite, presumably either by an epimerase or by a combination of a 12-hydroxy dehydrogenase and a 12-keto reductase, involving 10,11-dihydro-12-oxo-LTB_4 as an intermediate (Wainwright et al., 1990). It is possible that 12(*R*)-HETE could be metabolized in a similar manner to 12(*S*)-hydroxy-5,8,14-eicosatrienoic acid, which could then undergo racemization to 12(*R*)-hydroxy-5,8,14-eicosatrienoic acid as described above. This mechanism would be consistent with the loss of a deuterium atom during the formation of the dihydro product from [$^2H_8$]arachidonic acid as discussed above. The production of 12(*R*)-hydroxy-5,8,14-eicosatrienoic acid by corneal microsomes was established by comparing the biological activity of the dihydro product with those of authentic chemically synthesized 12(*R*)-hydroxy-5,8,14-eicosatrienoic acid and 12(*S*)-hydroxy-5,8,14-eicosatrienoic acid. It is possible that 12(*S*)-5,8,14-eicosatrienoic acid was also formed by corneal microsomes, but it would not have been detected by the methods employed, since it is biologically inactive (Murphy et al., 1988; Rimarachin et al., 1989).

13-HODE is a major product of the metabolism of exogenous linoleic acid by the Ω6-lipoxygenase in human PMNL (Reinaud et al., 1989) and by the 12-lipoxygenase in porcine PMNL (Claeys et al., 1985; Yokoyama, 1986). 13-HODE has been reported to stimulate prostacyclin production in endothelial cells (Setty et al., 1987b), to inhibit platelet thromboxane production (Setty et al., 1987c), and to inhibit 5-lipoxygenase in leukocytes (Camp & Fincham, 1985). 13-HODE has also been reported to have chemorepellent properties and has been shown to inhibit the adherence of platelets and other cells to blood vessel walls (Buchanan et al., 1985a,b). We have shown that 13-HODE is metabolized by porcine PMNL to 13-hydroxy-9-octadecenoic acid and 13-oxo-9-octadecenoic acid, the biological activities of which have not yet been investigated. This is the first report of the metabolism of an octadecanoid by the reductase pathway.

The best substrates for the porcine 10,11-reductase are eicosanoids containing a 12(*R*)- or 12(*S*)-hydroxyl group preceded by at least two conjugated bonds and followed by a 2-*cis*-octenyl group. LTB_4, 6-*trans*-LTB_4, 12-epi-6-*trans*-LTB_4, 12-epi-8-*cis*, 6-*trans*-LTB_4, and 12(*S*)-HETE all fall into this category and were very good substrates for this enzyme. Alteration of the position of the hydroxyl group or structural modifications of the ω-end of the molecule decreased the rate of metabolism. We have previously shown that human PMNL metabolize 6-*trans*-LTB_4 and 12-epi-6-*trans*-LTB_4 to 6,11-dihydro products by a different pathway involving initial oxidation of the 5-hydroxyl group followed by reduction of the conjugated triene system (Powell & Gravelle, 1988). A similar 6,11-reductase pathway exists in porcine PMNL which, combined with the 10,11-reductase pathway, results in the formation of tetrahydro products from 6-*trans*-LTB_4 and 12-epi-6-*trans*-LTB_4 and to a lesser extent 12-epi-8-*cis*,6-*trans*-LTB_4 (Powell & Gravelle, 1990). Metabolism by the 6,11-reductase pathway appears to require a 5-hydroxyl group followed by a trans 6,7-double bond in conjugation with one or two additional double bonds (Powell & Gravelle, 1990). In our specificity studies we were not able to distinguish between these two reductase pathways. It is therefore likely that the rates of metabolism of 6-*trans*-LTB_4 and 12-epi-6-*trans*-LTB_4 by the 10,11-reductase pathway are lower than those indicated in Table II. It is also possible that 5-HETE and 9-HODE, which have a hydroxyl group followed by two conjugated double bonds located on the ω-side of the molecule, could be metabolized principally by the 6,11-reductase pathway rather than the 10,11-reductase pathway.

The recoveries of radioactivity in experiments in which 1-^{14}C-labeled HETEs and HODEs were incubated with PMNL were rather low in many cases (Tables I and II), presumably due principally to β-oxidation. Incubation of substrates uniformly labeled with ^{14}C resulted in improved recoveries of total radioactivity, as well as the appearance of a number of more polar products (data not shown). On the basis of their UV absorbance and chromatographic properties, some of the products appeared to have been formed by a combination of the reductase and β-oxidation pathways. Loss of radioactivity, as well as the formation of more polar metabolites from U-^{14}C-labeled substrates, was partially prevented by inclusion of ETYA in the incubation media. Although the latter substance can inhibit a number of arachidonic acid metabolizing enzymes, including lipoxygenases, in this case it appeared to act mainly by inhibiting β-oxidation. 12-HETE is a substrate for 5-lipoxygenase (Borgeat et al., 1981), but we could not detect appreciable amounts of 12-epi-8-*cis*,6-*trans*-LTB_4 with the incubation conditions we used. 15-HETE, on the other hand, was metabolized to 8,15-diHETE by porcine PMNL, presumably via 12-lipoxygenase (Yamamoto et al., 1987), and this reaction was inhibited by ETYA.

Metabolism of eicosanoids by the 10,11-reductase pathway results in the formation of a series of novel compounds, some of which could be biologically active. In porcine PMNL (Wainwright et al., 1990) metabolism by this pathway is accompanied by partial inversion of the stereochemistry of the 12-hydroxyl group. It is clear that the stereochemistry of monohydroxy eicosanoids is critical for their biological activities. 12(*R*)-HETE has more potent proinflammatory effects than 12(*S*)-HETE (Cunningham & Woollard, 1987) and has been implicated in the condition of psoriasis (Woollard, 1986). 5(*S*)-HETE increases the intracellular calcium levels of target cells whereas 5(*R*)-HETE has little activity (Rossi et al., 1988). Finally, 12(*R*)-hydroxy-5,8,14-eicosatrienoic acid, which is the major metabolite formed from 12(*S*)-HETE by porcine PMNL, has potent proinflammatory effects while 12(*S*)-hydroxy-5,8,14-eicosatrienoic acid is inactive (Murphy et al., 1988; Rimarachin et al., 1989). We are currently investigating the possibility that the 10,11-reductase pathway and the associated epimerase activity could result in the formation of other compounds with biological activities.

Acknowledgments

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Registry No. 12(*S*)-HETE, 54397-83-0; 10,11-dihydro-12-HETE, 122210-62-2; 10,11-dihydro-12-oxo-ETE, 129467-51-2; 10,11-dihydro-12(*S*)-HETE, 117346-21-1; 10,11-dihydro-12(*R*)-HETE, 117346-20-0; 13-HODE, 29623-28-7; 11,12-hydro-13-HODE, 129467-52-3; 11,12-dihydro-13-oxo-ODE, 129467-53-4; LTB_4, 71160-24-2; 6-*trans*-LTB_4, 71652-82-9; 12-*epi*-6-*trans*,8-*cis*-LTB_4, 79056-01-2; 12-*epi*-6-*trans*-LTB_4, 71548-19-1; LTB_5, 80445-66-5; 15-HETE, 54845-95-3; 5-HETE, 70608-72-9; 9-HODE, 73543-67-6; 20-OH-LTB_4, 79516-82-8; HHT, 54397-84-1; PGE_2, 363-24-6; $PGF_{2\alpha}$, 551-11-1.

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Room Temperature Characterization of the Dioxygen Intermediates of Cytochrome *c* Oxidase by Resonance Raman Spectroscopy†

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ABSTRACT: Resonance Raman spectroscopy was employed to investigate the heme structures of catalytic intermediates of cytochrome *c* oxidase at room temperature. The high-frequency resonance Raman spectra were obtained for compound C (the two-electron-reduced dioxygen intermediate), ferryl (the three-electron-reduced dioxygen intermediate), and the fully oxidized enzyme. Compound C was formed by photolyzing CO mixed-valence enzyme in the presence of O_2. The ferryl intermediate was formed by reoxidation of the fully reduced enzyme by an excess of H_2O_2. Two forms of the oxidized enzyme were prepared by reoxidizing the fully reduced enzyme with O_2. Our data indicate that, in compound C, cyt a_3 is either intermediate or low spin and is nonphotolabile and its oxidation state marker band, ν_4, appears at a higher frequency than that of the resting form of the enzyme. The ferryl intermediate also displays a low-spin cyt a_3, which is nonphotolabile, and an even higher frequency for the oxidation state marker band, ν_4. The reoxidized form of cytochrome *c* oxidase with a Soret absorption maximum at 420 nm has an oxidation state marker band (ν_4) in a position similar to that of the resting form, while the spin-state region resembles that of compound C. This species subsequently decays to a second oxidized form of the enzyme, which displays a high-frequency resonance Raman spectrum identical with that of the original resting enzyme.

Cytochrome *c* oxidase is a multisubunit, membrane-bound protein that catalyzes the four-electron reduction of dioxygen in mitochondria. The oxygen reduction activity of the enzyme is coupled to proton translocation across the inner mitochondrial membrane during respiration. The enzyme utilizes four redox-active metal centers to perform its catalytic function. These centers include two heme A chromophores and two Cu ions. The dioxygen reduction site consists of a binuclear heme A/Cu cluster (designated cytochrome a_3, Cu_B). The two remaining metal centers (designated cyt a and Cu_A) mediate the electron transfer from ferrocytochrome *c* to cytochrome a_3, Cu_B (Wikstrom et al., 1977, 1981; Palmer et al., 1979). Although the dioxygen reduction kinetics have been studied extensively by a variety of spectroscopic techniques, much less is known about the structure of the intermediates formed during the turnover of the enzyme by O_2 [see Hill et al. (1986) and Chan et al. (1988) for reviews]. Because the turnover rate of the enzyme can be as high as 400 electrons transferred per second, spectroscopic studies of the intermediates have been difficult.

Low-temperature transient absorption studies of the fully reduced CO photolyzed enzyme in the presence of O_2 revealed the existence of at least three distinct species during turnover (Chance et al., 1975). Recent room temperature resonance Raman and transient absorption spectra indicate the possibility of four intermediates (Babcock et al., 1985; Hill & Greenwood, 1984). The first intermediate is believed to be an O_2 adduct bound to the ferrous heme a_3 similar to oxyhemoglobin (compound A) (Babcock et al., 1985; Han et al., 1990; Hill & Greenwood, 1984). Electron transfers from Cu_B and heme a_3 to the bound O_2 produce the second intermediate, generally assumed to be a peroxo heme a_3–Cu_B bridged species (compound C) (Hill & Greenwood, 1984). Blair et al. (1985), using EPR spectroscopy in conjunction with the low-temperature triple trapping technique pioneered by Chance et al. (1975), reported evidence for two distinct intermediates at the three-electron level of dioxygen reduction. It has been proposed that the first of these species is a cupric hydroperoxide coordinated to a ferrous heme a_3 in the binuclear cluster, while the second is an Fe(IV) heme a_3 species resulting from heterolytic cleavage of the O–O bond (Blair et al., 1984).

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