Calcium/Calmodulin-dependent Conversion of 5-Oxoeicosanoids to 6,7-Dihydro Metabolites by a Cytosolic Olefin Reductase in Human Neutrophils*

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Kiflu Berhane‡, Andrew A. Ray‡, Subhash P. Khanapure‡, Joshua Rokach§, and William S. Powell¶

From the §Meakins-Christie Laboratories, Department of Medicine, McGill University, 3626 St. Urbain Street, Montreal, Quebec H2X 2P2, Canada and the ¶Claude Pepper Institute and Department of Chemistry, Florida Institute of Technology, Melbourne, Florida 32901-6988

We previously showed that 6-trans isomers of leukotriene B4 but not leukotriene B4 itself are converted to dihydro metabolites by human neutrophils. The first step in the formation of these metabolites is oxidation of the 5-hydroxyl group by 5-hydroxyeicosanoid dehydrogenase. The objective of the present investigation was to characterize the second step in the formation of the dihydro metabolites, reduction of an olefinic double bond. We found that the olefin reductase reduces the 6,7-double bond of 5-oxoeicosanoids, localized in the cytosolic fraction of neutrophils, and requires NADPH as a cofactor. Neutrophil cytosol converts a variety of both 5-oxo- and 15-oxoeicosanoids to dihydro products. However, conversion of 5-oxoeicosanoids to their 6,7-dihydro metabolites is inhibited by EGTA and a calmodulin antagonist, and stimulated by the addition of calcium and calmodulin, whereas the reduction of 15-oxoeicosanoids to their 13,14-dihydro metabolites is slightly inhibited by calcium. Furthermore, eicosanoid Δ6- and Δ15-reductases could be separated by chromatography on DEAE-Sepharose. 5-Oxo-6,8,11,14-eicosatetraenoic acid (5-oxo-ETE) is converted by the Δ6-reductase to 6,7-dihydro-5-oxo-ETE, which is 1000 times less potent than 5-oxo-ETE in mobilizing calcium in neutrophils. We conclude that neutrophils contain both 5-oxoeicosanoid Δ6-reductase and prostaglandin Δ15-reductase. Metabolism of 5-oxo-ETE by the Δ6-reductase results in loss of its biological activity.

A major pathway in the metabolism of many eicosanoids is initiated by oxidation of one of the hydroxyl groups by an NAD+ - or NADP+-dependent dehydrogenase. This is usually followed by reduction of an adjacent double bond by an olefin reductase in the presence of NADH or NADPH. A number of distinct cytosolic 15-hydroxyprostaglandin dehydrogenases oxidize various prostaglandins (PGs) to their biologically inactive 15-oxo metabolites (1–3). These products can then be reduced by cytosolic PG Δ13-reductases to biologically inactive 13,14-dihydro-15-oxo-PGs (1,4), which in turn can be further reduced to dihydro-PGs by ketoreductases (5).

Lipoxygenase products can be metabolized by analogous pathways. We have shown that leukotriene B4 (LTB4) is converted to 12-oxo-LTB4 by an NAD+-dependent 12-hydroxyeicosanoid dehydrogenase in neutrophils (6). This is followed by reduction of the 10,11-double bond by a cytosolic NADH-dependent Δ12-reductase to give 10,11-dihydro-12-oxo-LTB4, which is then reduced to the corresponding dihydro compound by a ketoreductase (6). Metabolism of the potent neutrophil agonist (7), LTB4, by this pathway results in considerable loss of biological activity (8–10). 12(S)-Hydroxy-5,8,10,14-eicosatetraenoic acid is metabolized in a similar manner by neutrophils (11). However, in this case, the 10,11-dihydro metabolite of 12(S)-hydroxy-5,8,10,14-eicosatetraenoic acid has been reported to be a potent proinflammatory agent (12). LTB4 is metabolized by a similar pathway in monocytes (9, 13) and kidney (14). However, in the latter case, the 12-hydroxy dehydrogenase is clearly distinct from the neutrophil enzyme (14).

We previously showed that neutrophils convert 6-trans isomers of LTB4, which are formed nonenzymatically from LTA4, to dihydro metabolites (15, 16). This reaction proceeds by a sequence analogous to that described above for LTB4, the initial step being oxidation of the 5-hydroxy group, followed by reduction of one of the double bonds and the oxo group (16). We initially speculated that the dihydro products of these reactions might have been 6,11-dihydro metabolites, due to migration of the two remaining double bonds. However, mass spectral evidence subsequently suggested that the products were 6,7-dihydro metabolites (17). The initial step in the formation of these substances is oxidation of the 5-hydroxy group by a microsomal NADP+-dependent dehydrogenase that is highly specific for eicosanoids containing a 5(S)-hydroxy group followed by a 6-trans double bond (18). LTA4, which has a 6-cis double bond, is not metabolized by this pathway. The best substrate for 5-hydroxyeicosanoid dehydrogenase is (5S)-hydroxy-6,8,11,14-eicosatetraenoic acid (5-HETE), which is converted to 5-oxo-ETE (18), a potent activator of neutrophils (19, 20) and eosinophils (21–23).

Relatively little is known about the olefin reductase that converts 5-oxoeicosanoids to their dihydro metabolites. The objectives of this study were to investigate the regulation of 5-HETE, (5S)-hydroxy-6,8,11,13-eicosatetraenoic acid; 15-oxo-5-HETE, (5S)-hydroxy-15-oxo-6,8,11,13-eicosatetraenoic acid; 5,15-diHETE, (5S,15S)-dihydroxy-6,8,11,13-eicosatetraenoic acid; RP-HPLC, reversed-phase high pressure liquid chromatography.
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this enzyme, its substrate specificity, and its subcellular localization. We also wanted to determine whether 5-oxo-ETE could be converted to a dihydro metabolite by this pathway and, if so, how this would affect its biological activity.

EXPERIMENTAL PROCEDURES

Materials—Calmodulin was purchased from Biomol (Plymouth Meeting, PA), whereas the calmodulin inhibitor calmidazolium chloride (R24571) was obtained from Calbiochem. PGF was obtained from Sigma. 6-trans-LTB	extsubscript{4}, 12-epi-6-trans-LTB	extsubscript{4}, 15-oxo-PGF	extsubscript{2a}, and 13,14-dihydro-15-oxo-PGF	extsubscript{2a} were purchased from Cayman Chemical (Ann Arbor, MI).

Preparation of Eicosanoids—A number of the eicosanoids used in this study were prepared by total chemical synthesis. 5-oxo-ETE, 8-trans-5-oxo-ETE, and 6,7-dihydro-5-oxo-ETE were prepared as described previously (24). 5-Oxo-[11,12,14,15-3H]ETE and 8-trans-[5-oxo-[11,12,14,15-3H]ETE were prepared as reduction of an 11,14-diyne Arbor, MI).

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Various eicosanoids were prepared biochemically. 5-Oxo-6-trans-LTB	extsubscript{4} and 5-oxo-12-epi-6-trans-LTB	extsubscript{4} were synthesized by incubation of 5-oxo-LTB	extsubscript{4} (2 μM) and 5-oxo-12-epi-LTB	extsubscript{4} (2 μM) (Cayman Chemical Co., respectively, with a microsomal fraction from human neutrophils for 90 min at 37 °C in the presence of NADPH (1 mM). This product was purified by RP-HPLC as described above and incubated with 5-oxo-5-epi-15-HETE (18). 5-Oxo-15-hydroxy-6E,8Z,11Z,13E-eicosatetraenoic acid (5-oxo-15-HETE) and 5-oxo-15-hydroxy-6E,8Z,11Z,13E-eicosatetraenoic acid (8-trans-5-oxo-15-HETE) were prepared by incubation of 5-oxo-ETE and 8-trans-5-oxo-ETE, respectively, with soybean lipoxigenase (18).

5-HETE was synthesized by incubation of arachidonic acid (NuChem Prep, Inc., Elysian, MN) with potato 5-lipoxygenase (26). 5,15-Dihydroxy-6E,8Z,11Z,13E-eicosatetraenoic acid (5,15-diHETE) was prepared by incubation of 5-HETE with soybean lipoxigenase (27). 5-Hydroxy-15-oxo-6E,8Z,11Z,13E-eicosatetraenoic acid (15-oxo-5-HETE) was prepared by incubation of 5,15-diHETE (2 μM) with the cytosolic fraction obtained from pregnant rabbit lungs in the presence of NAD	extsuperscript{+} (28, 29). Similarly, 15-oxo-[5,6,8,9,11,12,14,15-3H]PGF	extsubscript{2a} was synthesized by incubation of [5,6,8,9,11,12,14,15-3H]PGF	extsubscript{2a} in the original volume) containing calcium and magnesium.

Separation of D- and A-Reductases by Ion Exchange Chromatography—The 200,000 × g supernatant fraction (10 ml) was incubated with 1 ml of DEAE-Sepharose for 30 min at 4 °C with constant mixing. The gel suspension was poured into a column that was eluted with 20 mM phosphate buffer, pH 7.4, containing 0.3 M sucrose, phenylmethylsulfonyl fluoride (1 mM), leupeptin (2 μg/ml), and aprotinin (2 μg/ml). The neutrophils were then disrupted by sonication (model 4710 Ultrasonic Homogenizer; Sonics & Materials, Danbury, CT) in an ice bath for 2 min with a power setting of 1 and for a further 5 s at a power setting of 2. The sonicate was centrifuged successively at 1500 × g for 10 min, 10,000 × g for 10 min, and 200,000 × g. The pellets was the ketoreductase activity, which was monitored using 15-oxo-5-HETE as substrate, whereas the 250 μM NaCl phosphate (10 ml). The reaction was terminated by the addition of methanol (0.5 volumes). Water was added to give a final concentration of methanol of 15%, and the mixture was centrifuged at 1000 × g for 10 min. The supernatant was extracted without acidification on a C	extsubscript{18} Sep-Pak (Waters-Millipore) as described previously (32). The methyl formate fraction was evaporated to dryness under a stream of nitrogen, and the residue (containing 5-oxo-12-epi-6-trans-LTB	extsubscript{4}) was incubated with the 200,000 × g supernatant fraction from human neutrophils for 90 min at 37 °C in the presence of calcium (1 mM) and NADPH (1 mM). The products were extracted using octadeccylsilic silica as described above. RP-HPLC analysis of an aliquot of the methyl formate fraction after the first extraction (material from the incubation with the 1500 × g supernatant) confirmed that the major product was 5-oxo-12-epi-6-trans-LTB	extsubscript{4} (18). Dihydro-5-oxo-12-epi-6-trans-LTB	extsubscript{4}, the major product of the incubation with the 200,000 × g supernatant fraction, was purified by RP-HPLC as described above and incubated at a concentration of 2 μM with the 200,000 × g pellet obtained from porcine neutrophils, prepared as described previously (6), in the presence of NAD	extsuperscript{+} (1 mM). The products of the reaction were analyzed by precolumn extraction/RP-HPLC as described above.

Protein Determination—Protein concentrations were determined as described by Bradford (33).

Results—Cytosolic Calcium Levels—Calcium levels were measured in indo-1-loaded neutrophils as described previously (34), using a Photon Technology International (PTI) Deltatrac II 4000 spectrofluorometer with a temperature-controlled cuvette holder equipped with a magnetic stirrer.

Subcellular Localization of Olefin Reductase Activity in Human Neutrophils—We had previously shown that 5-oxo-6-trans-LTB	extsubscript{4} is converted to dihydro and dihydro-5-oxo metabolites by a 1500 × g supernatant fraction from human neutrophils. To investigate the subcellular localization of the olefin reductase required for the formation of these products, 5-oxo-12-epi-6-trans-LTB	extsubscript{4} was incubated with subcellular fractions from neutrophils in the presence of different cofactors. When 5-oxo-12-epi-6-trans-LTB	extsubscript{4} was incubated with a microsomal fraction from neutrophils in the presence of NADPH, the major metabolite was the ketoreductase product, 12-epi-6-trans-LTB	extsubscript{4} (12-e-6t-B4) (Fig. 1A). Only a small amount of a dihydro product (dh-12-e-B4) was formed under these conditions. In contrast, the major product formed when 5-oxo-12-epi-6-trans-LTB	extsubscript{4} was incubated with the cytosolic fraction from neutrophils in the presence of NADPH was its dihydro metabolite (dh-5o-12-e-B4) (Fig. 1B).

The amounts of the above metabolites of 5-oxo-12-epi-6-trans-LTB	extsubscript{4} formed by different subcellular fractions from neutrophils are shown in Table I. The major product formed by both the 10,000 and 200,000 × g pellets was the ketoreductase product 12-epi-6-trans-LTB	extsubscript{4}. Formation of this product by particular fractions was dependent upon the presence of NADPH, NADH being much less effective. It was formed to a lesser extent by the 200,000 × g supernatant, but in this case its formation was not further affected by the addition of cofactors, perhaps because the level of endogenous cofactor was sufficient. In contrast, the two particular fractions tested displayed relatively little or no olefin reductase activity, whereas the 200,000 × g supernatant was quite active in the formation of dihydro metabolites of 5-oxo-12-epi-6-trans-LTB	extsubscript{4}. The cytosolic...
Legend to Fig. 1. The abbreviations are as described in the legend to Fig. 1. 5-Oxo-12-epi-6-trans-LTB4 was converted to a 6,11-dihydro metabolite by intact neutrophil reductase activity was highly dependent on the presence of NADPH, with much smaller amounts of dihydro metabolites being formed in the absence of exogenous cofactors or in the presence of NADH.

Position of Double Bond Reduced by the Olefin Reductase—We had previously suggested that 12-epi-6-trans-LTB4 was converted to a 6,11-dihydro metabolite by intact neutrophils (16). However, identification of the positions of the double bonds in this product was not very conclusive, because the diagnostic fragment ions in its mass spectrum were not very intense and could possibly have arisen as a result of rearrangements. A recent study employing mass spectral analysis of fragments formed by oxidative ozonolysis provided evidence that it is the 6,7-double bond of 6-trans isomers of LTB4 that is reduced by these cells (17). We used a different approach to investigate the position of the reduced double bond of 12-epi-6-trans-LTB4. As shown in Fig. 2, 5-oxo-12-epi-6-trans-LTB4 could potentially be reduced to three products by an olefin reductase, resulting in 6,7-dihydro, 6,11-dihydro, or 10,11-dihydro metabolites. Reduction of the 9,10-double bond in the middle of the triene chromophore is unlikely, whereas reduction of the 14,15-double bond is theoretically possible but would not result in a change in the UV spectrum of the product. Reduction of the 10,11-double bond of 12-epi-6-trans-LTB4 (Fig. 2) can also be excluded, since the resulting product would have a λmax around 280 nm, which is not observed.

To determine whether 5-oxo-12-epi-6-trans-LTB4 is converted to a 6,7-dihyro or a 6,11-dihydro metabolite, this substance was incubated with microsomal 12-hydroxyeicosanoid dehydrogenase from porcine neutrophils in the presence of NAD+. Oxidation of the 12-hydroxy group of 6,7-dihydro-5-oxo-12-epi-LTB4 by 12-hydroxyeicosanoid dehydrogenase would give a 5,12-dioxo product absorbing at 280 nm, whereas oxidation of the 12-hydroxy group of 6,11-dihydro-5-oxo-12-epi-LTB4 would not result in any change in the λmax of the substrate (Fig. 2). RP-HPLC analysis of the metabolites of dihydro-5-oxo-12-epi-LTB4, formed by porcine microsomes indicated that two fewer polar products were formed, presumably due to oxidation of the 12-hydroxy group (Fig. 3A). The major product had a λmax at 280 nm and was therefore a 6,7-dihydro product (structure I in Fig. 2), whereas a minor product had a λmax at 231 nm and was probably identical to the 6,11-dihydro compound (structure II in Fig. 2) (Fig. 3B). This demonstrates that the cytosolic fraction from human neutrophils reduces 5-oxo-12-epi-6-trans-LTB4 principally by 1,2-addition to the triene chromophore and suggests that 1,6-addition may also occur to some extent. For the purpose of clarity, this activity will be referred to below as Δ6-reductase activity.

Time Course for the Formation of Dihydro Metabolites of 5-oxo-15-HETE—Other 5-oxoeicosanoids were also metabolized by cytosolic fractions from neutrophils in a manner analogous to that shown for 12-epi-6-trans-LTB4 in Fig. 1. 5-Oxo-15-HETE was converted to 5,15-diHETE and two dihydroy products, presumably 6,7-dihydro-5-oxo-15-HETE and 6,7-dihydro-5,15-diHETE. The time course for the formation of these three metabolites is shown in Fig. 4. The initial Δ6-reductase and ketoreductase products (dihydro-5-oxo-15-HETE and 5,15-diHETE) were formed fairly rapidly and reached maximal levels by about 120 min, after which time the amounts declined.

Substrate Specificity for the Formation of Dihydroeicosanoids by Neutrophil Cytosol—To investigate the substrate specificity of the neutrophil cytosolic olefin reductase, a variety of oxoeicosanoids were prepared either chemically or biochemically and incubated with neutrophil cytosol in the presence of NADPH. The products were analyzed by RP-HPLC, and the amounts of dihydro products (dihydro plus dihydro-oxo) were determined. The cytosolic olefin reductase converted a variety of 5-oxoeicosanoids to dihydro metabolites (Table II). Of these, the best substrates were 5-oxo-6-trans-LTB4 and 8-trans-5-oxo-15-HETE. 5-Oxo-ETE and its 8-trans isomer appeared to be
metabolized more slowly, but this may have been due at least in part to the conversion of these substances by a competitive pathway to 15-hydroxy products due to the presence of 15-lipoxygenase in the cytosol. The Δ6-reductase appears to prefer substrates with an 8-trans double bond, since both 8-trans-5-oxo-15-HETE and 8-trans-5-oxo-15-HETE were metabolized more rapidly than the corresponding 8-cis compounds. However, of all of the products tested, 15-oxo-5-HETE was by far the best substrate, being metabolized at a rate at least 3 times that of the 5-oxoeicosanoids tested. This raised the possibility that the cytosolic reductase was actually a Δ13-reductase that was also capable of reducing the 6,7-double bond of 5-oxoeicosanoids.

Effects of Calcium on the Formation of Dihydroeicosanoids by Neutrophil Cytosol—All of the experiments described above were performed in the presence of calcium (1 mM). To determine whether the conversion of oxoeicosanoids to dihydro products was affected by calcium, neutrophil cytosol was incubated with various substrates in calcium-free medium in the presence of EGTA (1 mM). Removal of calcium inhibited the conversion to dihydro metabolites of the three 5-oxoeicosanoids tested (5-oxo-6-trans-LTB4, 5-oxo-15-HETE, and 8-trans-5-oxo-15-HETE) by between 70 and 80% (*p*, 0.01) (Fig. 5). In contrast, conversion of 15-oxo-5-HETE to its dihydro metabolite was stimulated by about 25% (*p* < 0.05) in the presence of EGTA. This experiment thus provides strong evidence that neutrophil cytosol contains at least two distinct olefin reductases and that the activity of one of these is enhanced by calcium.

Effects of Calmodulin on the Metabolism of 5-Oxo-6-trans-LTB4 by Neutrophil Cytosol—To investigate the possibility that the calcium dependence of the Δ6-reductase is mediated by calmodulin, neutrophil cytosol was incubated with various substrates in the presence of calmodulin (1 μM) and calcium (1 mM). Removal of calmodulin inhibited the conversion to dihydro metabolites of the three 5-oxoeicosanoids tested (5-oxo-6-trans-LTB4, 5-oxo-15-HETE, and 8-trans-5-oxo-15-HETE) by between 70 and 80% (*p* < 0.01) (Fig. 6). In contrast, conversion of 15-oxo-5-HETE to its dihydro metabolite was stimulated by about 25% (*p* < 0.05) in the presence of calmodulin. This experiment thus provides strong evidence that neutrophil cytosol contains at least two distinct olefin reductases and that the activity of one of these is enhanced by calmodulin.

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Various oxo-eicosanoids (1 µM) were incubated at 37 °C in the presence of NADPH (1 mM) with neutrophil cytosol (30 min; 1 mM Ca²⁺), the 250 µM NaCl fraction following chromatography on DEAE-Sepharose (60 min; 1 mM Ca²⁺), or the DEAE-Sepharose flow-through fraction (30 min; 1 mM EGTA). The products were analyzed by precolumn extraction/RP-HPLC as described under “Experimental Procedures.” The values are means ± S.E. (n = 3).

| Substrate | Cytosol³ | DEAE bound | DEAE flow-through |
|-----------|----------|------------|-------------------|
| 5-Oxo-6-trans-LTB⁴ | 11.5 ± 0.8 | ND³ | ND³ |
| 5-Oxo-12-epi-6-trans-LTB⁴ | 7.0 ± 0.9 | 4.93 ± 0.27 | ND |
| 8-trans-5-Oxo-15-HETE | 12.6 ± 0.9 | 5.30 ± 0.10 | ND |
| 5-Oxo-ETE (8-cis) | 2.0 ± 0.1¹ | 1.73 ± 0.18 | ND |
| 8-trans-5-Oxo-5-HETE | 5.3 ± 0.5⁵ | 6.13 ± 0.28 | ND |
| 15-Oxo-5-HETE | 38.1 ± 0.8⁴ | ND | 32.67 ± 1.26 |
| 15-Oxo-PGF₂α | ND | 48.53 ± 4.29 | |

² Values are sums of dihydro-oxo (olefin reductase) and dihydro-olefin (olefin reductase plus ketoreductase) products. ³ ND, not detectable. ⁴ These values are underestimates because both 5-oxo-ETE and 8-trans-5-oxo-ETE, but not the other substrates, were converted to 15-hydroxy products by cytosolic 15-lipoxygenase. ⁵ 15-oxo-5-HETE was converted only to a dihydro-oxo product.

calmodulin, the effect of the calmodulin inhibitor calmidazolium chloride on the conversion of 5-oxo-6-trans-LTB₄ to dihydro metabolites was determined. Neutrophil cytosol fractions were incubated with 5-oxo-6-trans-LTB₄ in the presence of calcium (1 mM), NADPH (1 mM), and various concentrations of calmidazolium chloride. Calmidazolium chloride (EC₅₀ = 0.3 µM) strongly inhibited the formation of dihydro metabolites from this substrate by about 85% at the highest concentration tested (Fig. 6). The effects of the addition of calmodulin to neutrophil cytosol fractions on Δ⁶-reductase activity was also investigated. Calmodulin (EC₅₀ = 0.2 µM) stimulated the formation of dihydro metabolites from 5-oxo-6-trans-LTB₄ by 80% above control at the highest concentration tested (Fig. 6, inset).

Separation of Eicosanoid Δ⁶- and Δ¹₅-Reductases by Ion Exchange Chromatography—The experiments described above suggested that neutrophil cytosol contains both Δ⁶- and Δ¹₅-reductases. To attempt to separate these two activities, the cytosol was applied to a column of DEAE-Sepharose, which was washed with 20 mM phosphate buffer, pH 7.4, and eluted with increasing concentrations of NaCl in the same buffer (Fig. 7). The Δ⁶-reductase activity of each of the column fractions was estimated by incubation with 5-oxo-6-trans-LTB₄ in the presence of Ca²⁺ (1 mM) and NADPH (1 mM). Δ¹₅-reductase activity was determined by incubating column fractions with 15-oxo-5-HETE in the presence of EGTA and NADPH. The Δ¹₅-reductase activity was not retained by the DEAE-Sepharose and appeared in the flow-through fraction (Fig. 7). On the other hand, the Δ⁶-reductase was strongly retained by the column and was eluted with 250 mM NaCl. This fraction did not contain significant 15-lipoxygenase activity, which resulted in the metabolism of 5-oxo-ETE and 8-trans-5-oxo-ETE to 15-hydroxy products when they were incubated with neutrophil cytosol fractions (data not shown).

Properties of the Partially Purified Δ⁶-Reductase—The requirements of the DEAE-Sepharose-purified Δ⁶-reductase for cofactors and calcium were investigated using 5-oxo-6-trans-LTB₄ as a substrate. Removal of calcium by chelation with EGTA inhibited Δ⁶-reductase activity in the 250 mM NaCl column fraction by about 40%, whereas the addition of calmodulin resulted in an increase in enzyme activity of about 73% (Table III). The effect of calmodulin was nearly completely inhibited by the addition of EGTA. The reductase reaction was dependent on NADPH. No products could be detected in the absence of cofactors, whereas activity was substantially reduced when NADH was substituted for NADPH.

The substrate specificity of the Δ⁶-reductase was investigated (Table II). These experiments were performed in the presence of calcium and NADPH. However, calmodulin was not included for reasons of cost, and for this reason, the rates of metabolism of most of the substrates were lower in the DEAE-Sepharose fraction than in the cytosol. A variety of 5-oxo-eicosanoids are substrates for the Δ⁶-reductase. As observed for the cytosolic fraction, substrates containing 8-trans double bonds...
Fraction with 15-oxo-5-HETE (1 mM), EGTA (1 mM), calcium (1 mM) or calmodulin (5 mM), were incubated with the 250 mM NaCl DEAE-Sepharose fraction for this experiment because a substantial degree of enzyme activity was lost, probably due to the removal of calmodulin and other components. Unfortunately, we could use calmodulin only sparingly in these experiments because of the cost, and it was not included in the determination of the results.

**Formation of 6,7-Dihydro Metabolites of 5-Oxo-ETE and 8-trans-5-oxo-ETE**—As shown in Table II, both 5-oxo-ETE and 8-trans-5-oxo-ETE are metabolized to dihydro products by the neutrophil Δ^1-reductase. To confirm the identities of these products, 5-oxo-[11,12,14,15-^3H]ETE and 8-trans-5-oxo-[11,12,14,15-^3H]ETE, both in the absence of unlabelled substrates, were incubated with the 250 mM NaCl DEAE-Sepharose fraction in the presence of NADPH and Ca^{2+}. After termination of the reactions, authentic chemically synthesized 6,7-dihydro-5-oxo-ETE was added, and the products were analyzed by RP-HPLC. As shown in Fig. 8A, the major metabolite of 5-oxo-[^3H]ETE cochromatographed with 6,7-dihydro-5-oxo-ETE, which was detected at 200 nm, whereas a smaller amount of 5-HETE was formed. 8-trans-5-Oxo-[11,12,14,15-^3H]ETE was also converted principally to a dihydro metabolite that had a longer retention time than 6,7-dihydro-5-oxo-ETE, presumably because of the different configuration of the 8,9-double bond (Fig. 8B). Although only a small amount of 8-trans-5-HETE was detected, a product with a slightly longer retention time, presumably identical to 6,7-dihydro-8-trans-5-HETE, was present.

To determine the K_m for the Δ^6-reductase, various concentrations of 8-trans-5-oxo-[^3H]ETE were incubated with neutrophil cytosol, after removal of 5-ketoreductase and 15-lipoxygenase activities by precipitation with ammonium sulfate (40%). This was necessary because the latter enzymes competed with the Δ^6-reductase for the substrate. We did not use the DEAE-Sepharose fraction for this experiment because a substantial degree of enzyme activity was lost, probably due to the removal of calmodulin and other components. Unfortunately, we could use calmodulin only sparingly in these experiments because of the cost, and it was not included in the determination of the K_m and V_max values. Lineweaver-Burk analysis (Fig. 9) revealed an apparent K_m for 8-trans-5-oxo-ETE of 130 ± 20 nM and a V_max of 3.0 ± 0.3 pmol/min/mg of protein (n = 3).

**Biological Activity of 6,7-Dihydro-5-oxo-ETE**—Because 5-oxo-ETE is a potent stimulator of calcium mobilization in neutrophils, it was important to determine whether reduction to 6,7-dihydro-5-oxo-ETE affected biological activity. Authentic 6,7-dihydro-5-oxo-ETE was capable of inducing calcium mobilization in neutrophils, but only at very high concentrations, and its potency was about 1000 times lower than that of 5-oxo-ETE (Fig. 10). The effect of 6,7-dihydro-5-oxo-ETE on calcium levels would appear to be mediated by a 5-oxo-ETE receptor,
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Fig. 9. Lineweaver-Burk plots for eicosanoid Δ⁵- and Δ¹⁵-reductases in neutrophil cytosol fractions. Prior to measurement of Δ⁵-reductase activity (●), 5-ketoreductase and 15-lipoxygenase were removed from the cytosol by treatment with ammonium sulfate (final concentration, 40%), followed by centrifugation at 20,000 × g for 10 min. After dialysis against 3 × 2 liters of 20 mM phosphate buffer, pH 7.4, the supernatant was incubated with different concentrations of 15-oxo-5-HETE [11,12,14,15-³H]ETE for 30 min at 37 °C, and the products were quantitated by precolumn extraction/RP-HPLC. Δ¹³-reductase activity (○) was measured by incubating various concentrations of 15-oxo-[³H]PGF₂α with the unpurified cytosol fraction. The results are means of duplicates and are representative of three separate experiments, all with similar results, for each substrate.

Fig. 10. Effects of 6,7-dihydro-5-oxo-ETE on calcium mobilization in neutrophils. Various concentrations of 5-oxo-ETE (●) or 6,7-dihydro-5-oxo-ETE (○) were added to suspensions of indo-1-loaded neutrophils, and changes in fluorescence were monitored as described under “Experimental Procedures.” The data are expressed as percentages of the maximal calcium response to 5-oxo-ETE and are means ± S.E. (n = 3). The inset shows the effects of 6,7-dihydro-5-oxo-ETE (dh-5o; 10 μM) on indo-1 fluorescence and on the subsequent response to 5-oxo-ETE (5o; 10 nM). For comparison, the response to 5-oxo-ETE (10 nM) alone is also shown.

since pretreatment of neutrophils with a high concentration (10 μM) of the former compound desensitized these cells to subsequent addition of 5-oxo-ETE (Fig. 10, inset).

Properties of the Partially Purified Δ¹³-reductase—The effects of cofactors and calcium on Δ¹³-reductase activity in the DEAE-Sepharose flow-through fraction were examined using 15-oxo-5-HETE as a substrate (Table III). As observed for the Δ⁵-reductase, this reaction was dependent upon NADPH, with much lower activity being observed in the presence of NADH. In agreement with the results with cytosol (Fig. 5), calcium inhibited enzyme activity by about 40%.

The specificity of the olefin reductase in the flow-through fraction was also examined by incubating this fraction with various substrates in the presence of NADPH and EGTA (Table II). None of the 5-oxoeicosanoids tested were metabolized to a detectable extent by this enzyme, whereas both 15-oxo-5-HETE and 15-oxo-PGF₂α were excellent substrates. The best substrate for the Δ¹³-reductase was 15-oxo-PGF₂α.

The Kₘ and Vₘₐₓ of the Δ¹³-reductase were determined by incubating neutrophil cytosol fractions with 15-oxo-[³H]PGF₂α in the presence of NADPH. The unfraccionated cytosolic fraction was used in this case because it did not contain any other enzymes that metabolized this substrate to a significant extent. As illustrated by Fig. 9, the Kₘ for conversion of 15-oxo-PGF₂α to its 13,14-dihydro derivative was 221 ± 30 nM, whereas the Vₘₐₓ was 11.8 ± 0.2 pmol/min/mg of protein (n = 3).

DISCUSSION

We previously showed that human neutrophils convert 6-trans isomers of LTB₄ to dihydro metabolites (15, 16). The enzyme responsible for this reaction does not act directly on these substrates but rather requires prior oxidation of the 5-hydroxy group (16, 18). The position of the double bond that was reduced was not clear from our initial studies (16). However, a recent study based on mass spectral analysis of fragments formed by oxidative ozonolysis of the dihydro metabolite of 6-trans-LTB₄ provided evidence that the reduced double bond is in the 6,7-position (17). The present study indicates that the two remaining double bonds in the dihydro metabolite of 12-epi-6-trans-LTB₄ are in the 8,9- and 10,11-positions, since they are conjugated with the 12-oxo group formed upon oxidation of the 12-hydroxy group of 6,7-dihydro-5-oxo-12-epi-LTB₄ by 12-hydroxyeicosanoid dehydrogenase (see Fig. 2). We also obtained evidence suggesting that a small amount of a 6,11-dihydro product may have been formed, raising the possibility that the Δ⁵-reductase may not be completely specific. However, we cannot exclude the possibility that another enzyme is responsible for the formation of the putative 6,11-dihydro product.

Monocytes have been reported to convert lipoxins to dihydro and dihydro-oxo products. Lipoxin A₄ is converted to 15-oxo, 13,14-dihydro-15-oxo, and 13,14-dihydro metabolites by these cells (35), whereas lipoxin B₃ is converted to dihydro products analogues to those formed from 6-trans isomers of LTB₄ (36). It would seem likely that the enzyme responsible for the formation of 13,14-dihydro metabolites of lipoxin A₄ is PG Δ¹³-reductase (35). Although the nature of the enzyme that converts lipoxin B₃ to dihydro products was not investigated further in the above study, it would seem probable that it is identical to the eicosanoid Δ⁶-reductase that we have identified in neutrophils.

In the present study, we investigated the specificity of the olefin reductase in neutrophils by synthesizing a series of substrates that could be converted to dihydro products, which could be detected either by UV absorbance or by radioactivity. Initial studies on the metabolism of these substrates by cytosolic fractions from neutrophils suggested that the olefin reductase that converts 5-oxo-12-epi-6-trans-LTB₄ to its dihydro metabolite may not be specific, since 15-oxo-5-HETE was found to be a better substrate than any of the 5-oxoeicosanoids tested. This raised the possibility that the enzyme responsible for this reaction could be a PG Δ¹³-reductase. However, the markedly different calcium requirements for reduction of 5-oxo- and 15-oxoeicosanoids suggested that this was not the case. This was confirmed when we were able to separate the two activities on a column of DEAE-Sepharose, which retained the Δ⁵-reductase but not the Δ¹³-reductase. Once the two enzymes were separated, it was apparent that the Δ⁵-reductase did not display any Δ¹³-reductase activity and vice versa.
The eicosanoid Δ⁶-reductase has a fairly low $K_m$ (~130 nM) but also a relatively low $V_{max}$ (~3 pmol/min/mg of protein). Thus, this enzyme can efficiently metabolize low, but not high, concentrations of substrate. This is in agreement with our earlier finding that intact neutrophils have only a limited capacity to metabolize 6-trans isomers of LTB₄ to their 6,7-dihydro metabolites (16). The Δ¹₃-reductase has a somewhat higher $K_m$ (~220 nM) than the Δ⁶-reductase but also has a higher $V_{max}$ (~12 pmol/min/mg of protein). The difference in the $V_{max}$ values for the two enzymes may be somewhat greater than this, because the Δ⁶-reductase was partially purified for this experiment to remove enzymes that competed for the substrate, whereas the Δ¹₃-reductase activity was measured using the unfraccionated cytosolic fraction. Thus, the Δ¹₃-reductase has a considerably higher capacity in neutrophils than the Δ⁶-reductase. The $K_m$ of the neutrophil Δ¹₃-reductase is similar to that of a cytosolic NADPH-dependent prostaglandin Δ¹₃-reductase in rat liver, which was reported to be about 280 nM (4).

The dependence of the Δ⁶-reductase on calcium and calmodulin is intriguing and suggests that the activity of this enzyme may be tightly regulated. Other olefin reductases involved in the metabolism of eicosanoids, including PG Δ¹₃-reductase and the Δ¹⁵-reductase responsible for the formation of 10,11-dihydro metabolites of LTB₄, are not known to be affected by calmodulin. Similarly, there is little evidence for the regulation of steroid olefin reductases by calmodulin, with the possible exception of a sterol Δ²₄-reductase present in hepatoma cells and human skin fibroblasts (37). It is not clear whether calmodulin acts directly on the Δ⁶-reductase or whether its actions are mediated by another protein such as a calmodulin-dependent kinase or phosphatase. The relatively low specific activity of the enzyme after chromatography on DEAE-Sepharose suggests that factors other than calmodulin may also be involved. Despite removal of a substantial amount of protein by the chromatographic procedure, the Δ⁶-reductase activity in the presence of calmodulin after DEAE-Sepharose (13.5 ± 0.8 pmol/mg/min; Table III) is no higher than that in the cytosol in the presence of calmodulin (15.20 ± 1.48 pmol/mg of protein/min; Fig. 6, inset).

The 5-hydroxyeicosanoid dehydrogenase/Δ⁶-reductase pathway was first discovered in studies on the metabolism of 6-trans isomers of LTB₄, which are formed nonenzymatically from LTC₄ and have little biological activity (15, 16). The biological significance of this pathway was unclear until we found that the preferred substrate for the first step, catalyzed by the dehydrogenase, is 5-HETE, which is converted into a biologically active product, 5-oxo-ETE (18). A major objective of the present study was to determine whether 5-oxo-ETE is a substrate for the Δ⁶-reductase and, if so, whether the product, 6,7-dihydro-5-oxo-ETE, is more or less potent than its precursor. To accomplish this, we prepared tritium-labeled 5-oxo-ETE (25), which would allow us to monitor the formation of its 6,7-dihydro metabolites, which, unlike other lipoxigenase products, do not absorb significantly in the UV. Furthermore, to enable us to identify the putative 6,7-dihydro metabolite and to test its biological activity, we prepared this compound by chemical synthesis (24).

Our results clearly show that both 5-oxo-ETE and its 8-trans isomer are metabolized by the Δ⁶-reductase to dihydro metabolites. Metabolism of 5-oxo-ETE by this enzyme results in a dramatic loss in biological activity, as 6,7-dihydro-5-oxo-ETE is about 1000 times less potent in stimulating calcium mobilization in neutrophils. This further supports the argument that neutrophils possess a highly specific recognition mechanism for 5-oxo-ETE, since a variety of minor structural modifications cause substantial losses in biological activity (19, 34, 38). It is becoming apparent that metabolism of 5-oxo-ETE by a variety of pathways results in dramatic reductions in biological potency, including metabolism by 20-hydroxylase (100-fold) (34), 12-lipoxigenase (>10,000),² and 5-ketoreductase (100-fold) (19) enzymes. Metabolism of 5-oxo-ETE by the Δ⁶-reductase would result in a permanent loss in biological activity, since this reaction is presumably irreversible, in contrast to reduction of 5-oxo-ETE to 5-HETE by a 5-ketoreductase.

It is interesting that the 8-trans isomer of 5-oxo-ETE appears to be a better substrate for the Δ⁶-reductase than 5-oxo-ETE itself. Indeed, this enzyme shows a preference for substrates with a 5-oxo group followed by two trans double bonds. Metabolism of 8-trans-5-oxo-ETE could be of some significance, since this substance does possess some biological activity, with a potency about one-fifth that of 5-oxo-ETE (34, 38). Moreover, we have detected 8-trans-5-oxo-ETE after stimulation of neutrophils (34), although it is not yet clear whether this compound can be formed enzymatically. However, the results of our specificity studies should be interpreted with some caution, since we do not yet understand completely how this enzyme is regulated, and the conditions employed may not be optimal due to possible requirements for additional factors.

In conclusion, human neutrophils possess two distinct olefin reductases that metabolize eicosanoids, a Δ⁶-reductase that converts 5-oxo-ETE and other 5-oxoeicosanoids to 6,7-dihydro metabolites and a Δ¹³-reductase that converts 15-oxo-PGs and other 15-oxoeicosanoids to 13,14-dihydro metabolites. The Δ⁶-reductase is highly regulated by calmodulin and possibly other factors, whereas the Δ¹³-reductase is, if anything, slightly inhibited by calcium. Metabolism of 5-oxo-ETE by the Δ⁶-reductase results in a dramatic 1000-fold loss in biological potency.

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