Stereoselective carbonyl reductases from rat skin and leukocyte microsomes converting 12-ketoeicosatetraenoic acid to 12(S)-HETE

Jean-Pierre Falgueyret, Yves Leblanc and Denis Riendeau

Merck Frosst Centre for Therapeutic Research, PO Box 1005, Pointe Claire-Dorval, Québec, H9R 4P8 Canada

Cell-free preparations from rat polymorphonuclear leukocytes and skin were found to catalyze the reduction of 12-keto-5,8,10,14-eicosatetraenoic acid (12-KETE) to 12-hydroxyeicosatetraenoic acid (12-HETE). The reductase activity was associated with the microsomal fraction and showed a marked preference for NADH over NADPH as reducing cofactor. Characterization of the reaction product by chiral phase HPLC of the methyl ester derivative indicated that 12-KETE reduction generated almost exclusively 12(S)-HETE. The results demonstrate that rat skin and leukocyte microsomes possess an NADH-dependent 12-KETE reductase activity that forms 12(S)-HETE as a major product. The identification of stereoselective 12-KETE reductases provides a basis for further defining the role these enzymes may play in the regulation of 12-KETE levels and in the protection against degradation of 12-KETE to the pro-inflammatory 12(R)-HETE by selectively generating 12-HETE of the S configuration.

Carbonyl reductase; 12-Ketoeicosatetraenoic acid; 12-Hydroxyeicosatetraenoic acid; Leukocyte; Skin

1. INTRODUCTION

Recent studies have led to the identification of 12-ketoeicosatetraenoic acid (12-KETE) as a metabolite of the 12-lipoxygenase pathway of arachidonic acid oxidation [1–3]. This carbonyl derivative has been shown to be synthesized by human platelets and *Aplysia* nervous tissue after incubation with arachidonic acid [1,2]. In these systems, 12-KETE appears to derive from the 12-HPETE product of 12-lipoxygenase, either by nonenzymatic degradation or subsequent metabolism by lipoxygenases and hemoproteins [3].

With the exception of the neurons of the marine mollusk *Aplysia* where 12-KETE appears to mediate some of the responses to histamine [2], little is known about the biological activity and the metabolism of this eicosanoid. Enzymatic reduction of 12-KETE to a mixture of 12(S)- and 12(R)-HETE has been demonstrated using rat liver microsomes [4]. This result indicates that 12-KETE may be a precursor of a pro-inflammatory mediator since 12(R)-HETE has been shown to function as a neutrophil chemoattractant [5–7]. To further investigate the role 12-KETE reductases may play in the regulation of 12-KETE levels or in the generation of 12(S)- or 12(R)-HETE, we have examined the tissue distribution and the stereochemistry of the reaction

Correspondence address: D. Riendeau, Merck Frosst Centre for Therapeutic Research, PO Box 1005, Pointe Claire-Dorval, Québec H9R 4P8 Canada

Abbreviations: 12-HETE, 12-hydroxy-5,8,10,14-eicosatetraenoic acid; 12-HPETE, 12-hydroperoxy-5,8,10,14-eicosatetraenoic acid; 12-KETE, 12-keto-5(Z),8(Z),10(E),14(Z)-eicosatetraenoic acid

product of these enzymes. In the present communication, we report the identification of 12-KETE reductases which selectively convert 12-KETE to 12(S)-HETE.

2. MATERIALS AND METHODS

2.1. Chemicals
Synthetic 12(S)- and 12(R)-HETE were prepared as previously described [8]. 12-KETE was prepared by oxidation of 12-HETE and purification by silica gel chromatography and RP-HPLC [4].

2.2. Preparation of subcellular fractions
The microsomal fraction from rat peritoneal polymorphonuclear (PMN) leukocytes (100000 × g pellet) was prepared as previously described [9]. Dorsal skin was excised (<1 mm thickness) from shaved adult male rats killed by CO_2 inhalation. The tissue was homogenized (4°C) using a Polytron in 20 mM Tris-HCl, pH 7.5, 0.25 M sucrose, 0.5 mM EDTA, 0.1 mM EGTA and 0.5 mM dithiothreitol (10 ml/g of tissue). Cell debris were removed by centrifugation at 1000 × g for 20 min and the supernatant centrifuged at 10000 × g for 20 min. The resulting supernatant was further centrifuged at 100000 × g (1 h) to pellet the microsomal fraction which was resuspended in Tris-HCl, pH 7.5, containing 5 mM $MgCl_2$. Protein contents were determined using the procedure of Lowry et al. [10], using bovine albumin as standard.

2.3. Assay of 12-KETE reductase activity
The incubation mixture for 12-KETE reductase assays contained 20 μM 12-KETE, 0.5 mM NADH or NADPH, 50 mM Tris-HCl, pH 6.3, and 1–2 mg of protein in a final volume of 200 μl. The mixture was incubated at 32°C for 15 or 30 min and the reaction was stopped by the addition of 800 μl of diethyl ether/methanol/citric acid 1 M (30:4:1). The suspension was clarified by centrifugation and the supernatant was concentrated by evaporation under N_2 and resuspended in methanol for HPLC analysis to resolve 12-KETE from the 12-HETE product [4]. RP-HPLC was performed on a C_{18} NovaPak column (Millipore) in methanol/water/acetic

(75:25:0.01) at a flow rate of 1 ml/min. For the determination of the stereochemistry of the reaction product, the eluting material corresponding to the 12-HETE peak was concentrated under reduced pressure and derivatized to the 12-HETE methyl ester with etheral diazomethane. 12(S)- and 12(R)-HETE methyl esters were resolved by HPLC on a Bakerbond DNPBG (ionic) chiral column [11] in hexane/isopropanol (100:0.5) at a flow rate of 0.5 ml/min.

3. RESULTS

3.1. Enzymatic reduction of 12-KETE by rat leukocyte and skin microsomes

Incubation of the microsomal fraction from PMN leukocytes with 12-KETE (20 μM) and NADH resulted in the formation of a major product that migrates at the position of 12-HETE on RP-HPLC (fig.1). Product formation coincided with a decrease in the amount of 12-KETE and could not be detected in the absence of NADH. A similar reaction was observed using skin microsomes, except that this preparation contained a higher level of endogenous 12-HETE (fig.2). For both systems, the reaction could not be detected using either the cytosolic fractions or heat-inactivated (70°C, 30 min) microsomes.

Further evidence that the reaction product is 12-HETE was obtained by re-analysis of the HPLC-

purified product using a different solvent system. Both the leukocyte and skin microsome reaction products co-eluted with synthetic 12-HETE after chromatography on a C_{18} column in acetonitrile/water/acetic acid (66:33:0.01) (results not shown). The UV spectra of the compounds showed the characteristic absorption for a conjugated diene with a maximum at 235–236 nm.

The 12-KETE reductase activities using 0.5 mM NADH and 20 μM 12-KETE were 2 and 0.4 pmol of 12-HETE/min per mg of protein for the leukocyte and skin microsomal preparations, respectively. NADH was the most efficient reducing cofactor for both reactions, with about 10% of the activity being measured with NADPH (fig.3).

3.2. Stereoselectivity of 12-KETE reduction

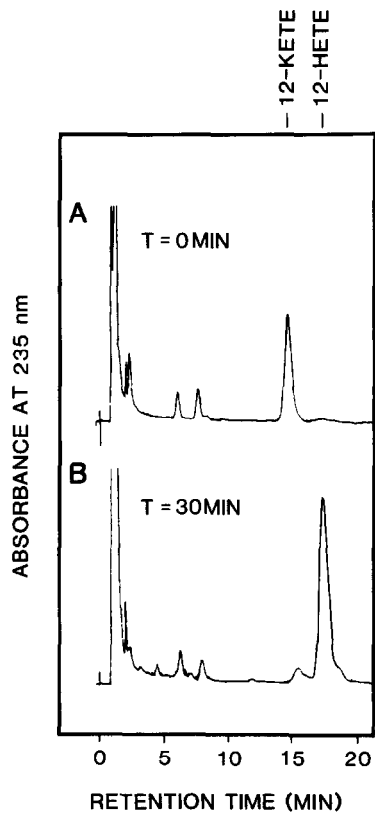The stereoselectivity of 12-KETE reduction was determined by derivatizing the RP-HPLC-purified pro-

Fig.1. Reduction of 12-KETE to 12-HETE by microsomes from PMN leukocytes. 12-KETE (20 μM) was incubated with the microsomal fraction (10 mg of protein/ml) in the presence of NADH under the conditions of the 12-KETE reductase assay for 0 and 30 min, prior to the analysis of the reaction products by RP-HPLC.
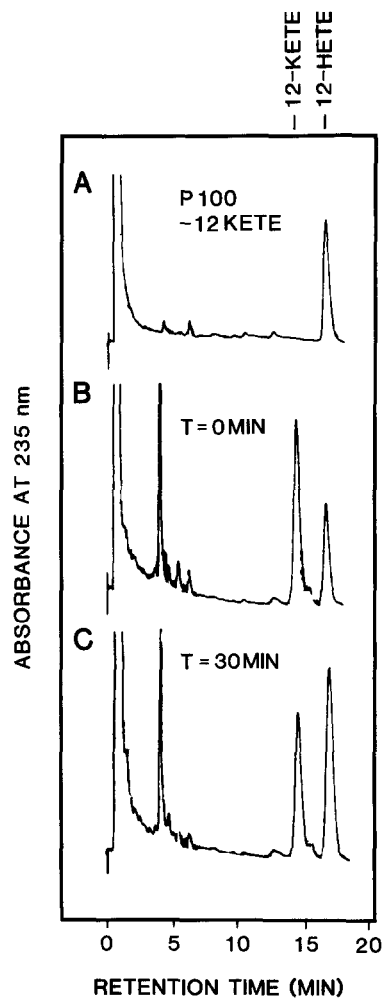

Fig.2. Reduction of 12-KETE to 12-HETE by skin microsomes. 12-KETE reductase activity was measured under standard conditions (10 mg of protein/ml) using NADH as cofactor. RP-HPLC profiles are shown for assays performed in the absence of exogenous 12-KETE (A) or in the presence of 20 μM 12-KETE for reactions stopped at time = 0 (B) and 30 min (C).
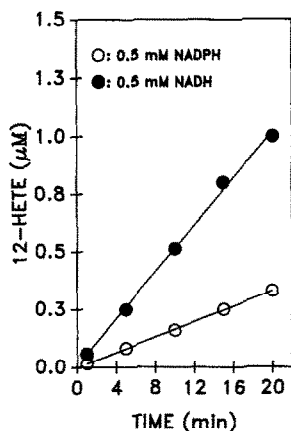
Fig.3. Enzyme cofactor specificity of leukocyte 12-KETE reductase. 12-KETE reductase activity was measured using PMN leukocyte microsomes (5 mg of protein/ml) in the presence of 0.5 mM NADH or NADPH. The formation of 12-HETE was quantitated by RP-HPLC.
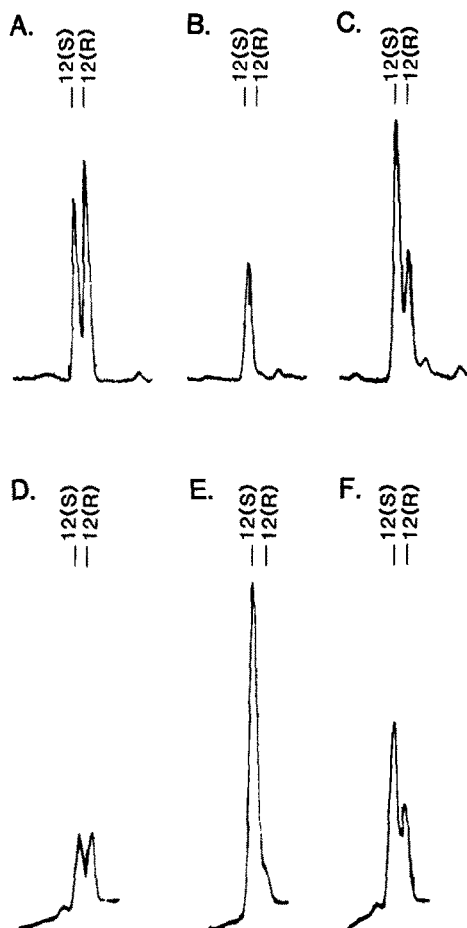

Fig.4. Stereochemical analysis of the 12-HETE product of 12-KETE reductases. Reaction products from the leukocyte (A–C) and skin reductases (D–F) were purified by RP-HPLC, derivatized to the methyl ester and analyzed by chiral phase HPLC. (A) and (D), racemic mixture of synthetic 12(R)- and 12(S)-HETE methyl esters; (B) and (E), 12-HETE reaction products; (C) and (F), co-injection of the racemic mixture of synthetic standards with the derivatized reaction products of 12-KETE reductases (corresponding to B and E, respectively).

duct (see fig.1) to the methyl ester and resolving the 12(R)- and 12(S)-HETE methyl esters by chiral phase HPLC (fig.4). The chromatogram of 12-HETE methyl ester from the reaction product of the leukocyte 12-KETE reductase shows one peak eluting at the position of 12(S)-HETE methyl ester (fig.4B). This result was confirmed by co-injection of the derivatized product with a 1:1 mixture of the R and S isomers (fig.4C). Replacement of NADH by NADPH in the assay did not change the stereoselectivity of reduction (data not shown).

Similar experiments with the 12-HETE reaction product of the 12-KETE reductase from skin microsomes indicated that 12(S)-HETE was the predominant isomer (fig.4E and F). The material analyzed in this case contained a significant proportion of endogenous 12-HETE, which was exclusively of the S configuration (data not shown).

4. DISCUSSION

The characteristics of the various 12-KETE reductase activities reported until now are summarized in table 1. The specific activities of the microsomal preparations from rat PMN leukocytes and skin reported in this study were two to three orders of magnitude lower than that of the liver enzyme [4]. The reactions, however, were more selective both for the utilization of NADH and the generation of 12(S)-HETE, suggesting that they may be catalyzed by different enzymes. In contrast to these 12-KETE reductases, most of the carbonyl reductases are characterized by a cytoplasmic localization and a preference for NADPH [12–14]. The latter properties are also shared by prostaglandin 9-ketoreductases, although a distinct NADH-selective microsomal form has been identified in monkey liver [13]. The relationship between 12-KETE reductase and this enzyme or other aldo-keto reductases, which are known to exhibit a broad substrate specificity [14], remains to be investigated.

The 12-KETE reductase activity could potentially play a role in the regulation of 12-KETE released in response to histamine [2] or arising from degradation

Table 1

Summary of the characteristics of 12-KETE reductases from rat tissues

Source	Cofactor	Specific activity (pmol/min per mg protein)	Isomer product (% 12(S)-HETE)
Liver	NADH	170	70
	NADPH	130	70
PMN leukocyte	NADH	2	>90
	NADPH	0.3	>90
Skin	NADH	0.4	>80
	NADPH	<0.05	–

of the 12-HPETE lipoxygenase product [3]. It is noteworthy that skin and leukocyte 12-KETE reductases generate predominantly 12(S)-HETE, the isomer which has the lower potency in stimulating neutrophil functions [5,6] and in inhibiting ATPase activity in the cornea [15]. 12(R)-HETE is often considered as a pro-inflammatory mediator since it selectively stimulates neutrophil infiltration in the epidermis after topical application to human skin [16] and has been isolated as the major 12-HETE isomer from psoriatic scales [17]. Further characterization of 12-KETE reductase levels and stereoselectivity should contribute to determine if this enzyme plays any role in the modulation of 12(S)- and 12(R)-HETE synthesis in normal or psoriatic skin.

REFERENCES

[1] Fructeau de Laclos, B., Maclouf, J., Poubelle, P. and Borgeat, P. (1987) Prostaglandins 33, 315–337.

[2] Piomelli, D., Feinmark, S.J., Shapiro, E. and Schwartz, J.H. (1988) J. Biol. Chem. 263, 16591–16596.

[3] Fructeau de Laclos, B. and Borgeat, P. (1988) Biochim. Biophys. Acta 958, 424–433.

[4] Falgueyret, J.P., Leblanc, Y., Rokach, J. and Riendeau, D. (1988) Biochem. Biophys. Res. Commun. 156, 1083–1089.

[5] Cunningham, F.M., Greaves, M.W. and Wollard, P.M. (1986) Br. J. Pharmacol. Proc. 87, 107.

[6] Evans, J.F., Leblanc, Y., Fitzsimmons, B.J., Charleson, S., Nathaniel, D. and Léveillé, C. (1987) Biochim. Biophys. Acta 917, 406–410.

[7] Naccache, P.H., Faucher, N., Therrien, S. and Borgeat, P. (1988) Life Sci. 42, 727–733.

[8] Leblanc, Y., Fitzsimmons, B.J., Adams, J., Perez, F. and Rokach, J. (1986) J. Org. Chem. 51, 789–793.

[9] Riendeau, D. and Leblanc, Y. (1986) Biochem. Biophys. Res. Commun. 141, 534–540.

[10] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 263–275.

[11] Kuhn, H., Wiesner, R., Lankin, V.Z., Nekrasov, A., Alder, L. and Sherve, T. (1987) Anal. Biochem. 160, 24–34.

[12] Leibman, K.C. (1971) Xenobiotica 1, 97–104.

[13] Lee, S.-C. and Levine, L. (1974) J. Biol. Chem. 249, 1369–1375.

[14] Wermuth, B. (1985) in: Enzymology of Carbonyl Metabolism. II. Aldehyde Dehydrogenase, Aldo-keto Reductase, and Alcohol Dehydrogenase (Flynn, T.G. and Weiner, H. eds) pp.209–230, Alan R. Liss, New York.

[15] Schwartzman, N.L., Balazy, M., Masferrer, J., Abraham, N.G., McGiff, J.C. and Murphy, R.C. (1987) Proc. Natl. Acad. Sci. USA 84, 8125–8129.

[16] Wollard, P.M., Cunningham, F.M., Murphy, G.M., Camp, R.D.R., Derm, F.F. and Greaves, M.W. (1989) Prostaglandins 38, 465–471.

[17] Wollard, P.M. (1986) Biochem. Biophys. Res. Commun. 136, 169–175.