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12-L-HYDROXY-5,8,10-HEPTADECATRIENOIC ACID (HHT) IS AN EXCELLENT SUBSTRATE FOR NAD$^+$-DEPENDENT 15-HYDROXYPROSTAGLANDIN DEHYDROGENASE

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Summary: 12-L-hydroxy-5,8,10-heptadecatrienoic acid (HHT) was found to be an excellent substrate for NAD$^+$-dependent 15-hydroxyprostaglandin dehydrogenase from porcine kidney. K_{cat}/K_m value of HHT was comparable to that of prostaglandin E although HHT is not a prostanoic acid derivative. Product of enzyme catalyzed oxidation of HHT was identified as 12-keto-5,8,10-heptadecatrienoic acid by gas chromatography-mass spectrometry. The fact that HHT is an excellent substrate for 15-hydroxyprostaglandin dehydrogenase suggest that HHT may have profound unrecognized biological actions and its inactivation may be via oxidation of the hydroxyl group.

The first step in the catabolism of prostaglandins is the oxidation of 15(S)-hydroxyl group to a keto function (1). This reaction is catalyzed by a NAD$^+$-dependent 15-hydroxyprostaglandin dehydrogenase (15-PGDH) which is present in most mammalian tissues (2). This enzyme is considered a key enzyme responsible for biological inactivation of prostaglandins since the oxidized products possess greatly reduced biological activities (3). The enzyme shows specificity toward natural prostaglandins and synthetic analogs having prostanoic acid skeleton (4,5). However, the enzyme does not appear to catalyze the oxidation of TXB_2, a non-prostanoic hydrolyzed product of TXA_2 (6). Although TXA_2 is a biologically active precursor of TXB_2, it is too unstable to be assessed as a substrate. Thromboxane synthase has been suggested to catalyze the synthesis of both TXA_2 and HHT from PGH_2 (7,8). The fate of HHT in the biological systems remains unknown. Here we report that HHT is an excellent substrate for NAD$^+$-dependent 15-hydroxyprostaglandin dehydrogenase although it is not a prostanoic acid derivative. Other hydroxy fatty acids including LTB_4 are poor substrates for the enzyme.

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Abbreviations: HHT, 12-L-hydroxy-5,8,10-heptadecatrienoic acid; KHT, 12-Keto-5,8,10-heptadecatrienoic acid; HETE, hydroxy-eicosatetraenoic acid; LTB_4, leukotriene B_4; TXA_2, thromboxane A_2; TXB_2, thromboxane B_2; PGE, prostaglandin E; PGF, prostaglandin F; PGH_2, prostaglandin H_2.

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MATERIALS AND METHODS

Materials: Arachidonic acid, DL-isoproterenol, NAD^+, methyoxyamine hydrocholoride, and NO-bis(trimethylsilyl)trifluoroacetamide (BSTFA) containing 1% trimethylchlorosilane were purchased from Sigma Chemical Co., St. Louis, MO. [1-^{14}C] Arachidonic acid (55 mCi/mmol.) was obtained from New England Nuclear, Boston, MA. Prostaglandins are a kind gift from the Upjohn Company, Kalamazoo, MI. LTB_4 was kindly supplied by Merck Frost Canada Inc., Montreal, Canada, 12-HETE was synthesized from arachidonic acid by human platelet suspension in the presence of indomethacin and GSH as described by McGuire et al (9). 15-HETE was prepared from arachidonic acid by soybean lipoxygenase in the presence of $NaBH_4$ according to Baldwin et al (10).

Enzyme Assay of 15-PGDH: 15-PGDH activity was determined by following the formation of NADH spectrofluorometrically as described previously (4). The reaction mixture contained: NAD^+, 1 mM; PGE_1, 9.3 μM; and enzyme in a final volume of 1.5 ml of 0.05 M Tris-HCl, pH 7.5. The reaction was initiated by the addition of the enzyme and allowed to proceed at room temperature. The rate of NADH formation was recorded by the increase in fluorescence at 468 nm with excitation at 340 nm using an Aminco-Bowman SPF coupled to a recorder. The instrument was standardized by known concentration of NADH determined by direct measurement of absorbance at 340 nm using $\varepsilon_M = 6.22 \times 10^3\ M^{-1}\ CM^{-1}$ (11).

Purification of 15-PGDH from Porcine Kidney: 15-PGDH from procine kidney was purified to apparent homogeneity according to a previously described procedure for the enzyme from placenta with some modification(s) (13). The specific activity of the purified enzyme was 1.7 U/mg.

Preparation of HHT: Sheep seminal vesicular microsomes and soluble fraction were prepared as described in (13). The soluble fraction was boiled for 5 min and centrifuged at 10,000 x g for 10 min. The supernatant was collected and used for HHT preparation. The incubation mixture contained: arachidonic acid, 0.4 mM; human hemoglobin, 1 μM; isoproterenol, 1 mM; boiled supernatant, 100 ml; and microsomes, 250 mg protein in a final volume of 400 ml of 0.05 M Tris-HCl, pH 7.5. Incubation was carried out for 15 min at 37°C with shaking and terminated by acidification to pH 3.5 with 1 N HCl. The reaction mixture was extracted with 500 ml of ethyl ether twice. The ether extract was dried over Na_2SO_4 and evaporated under vacuum. The residue was chromatographed on precoated silica gel G plates using petroleum ether/ethyl ether/acetic acid (50:50:1) as the developing solvent. The HHT band (R_f = 0.48) was scraped off the plate and the gel was extracted with methanol. About 10% of the arachidonate was converted to HHT at this step. The methanol extract was subjected to HPLC purification using solvent A as described below. [1-^{14}C] HHT was prepared in a similar manner except that a much smaller scale of preparation with [1-^{14}C]arachidonate as a substrate was carried out.

Preparation of KHT: Partially purified 15-PGDH (DEAE-Sephadex A-50 fraction) was used to catalyze the oxidation of HHT to KHT. The incubation mixture contained: HHT, 20 μM; NAD^+, 1 mM; and partially purified enzyme (0.4 Unit) in a final volume of 200 ml of 0.05 M Tris-HCl, pH 7.5. Incubation was carried out for 30 min at 37°C with shaking and terminated by acidification to pH 3.5 with 1 N HCl. The reaction mixture was extracted with 200 ml of ethyl ether twice. The ether extract was dried and chromatographed as described above. The KHT band (R_f = 0.65) was scraped off the plate and the gel was extracted with methanol. Essentially total conversion was observed at this step. The methanol extract was further purified by RP-HPLC using Solvent A as the developing solvent as described below. The HPLC purified KHT was derivatized and subjected to GC-MS analysis.

RP-HPLC Analysis: RP-HPLC was performed on a Varian 5000 Liquid Chromatograph using Varian MCH-10 column (4.6 x 300 mm). Elution was carried out either with Solvent A, methanol/water/acetic acid (70:30:0.05) at a flow rate of 1.0 ml/min

or with Solvent B, acetonitrile/water/85% H_3PO_4 (50:50:0.07) at a flow rate of 1.3 ml/min. HHT and KHT were detected by monitoring the UV absorbance at 232 nm and 276 nm, respectively with UV-50 detector.

GC-MS Analysis: HHT and KHT were methylated by ethereal diazomethane. O-methyl-oxime derivative of KHT methyl ester was prepared by reacting methyl ester with methoxyamine hydrochloride in pyridine (14). Trimethylsilation of methyl esters was carried out with BSTFA contaning 1% trimethylchlorosilane. Immediately before GC-MS analysis, samples were taken down to dryness under nitrogen and redissolved in an appropriate volume of acetone. GC-MS analysis was carried out with a cross-linked methyl silicone column (0.3 mm x 10 m) interfaced with a Hewlett-Packard System 5955 mass spectrometer. The column temperature was programmed from 175-200°C at a rate of 5°C/min. The injector temperature was 185°C. Helium was used as carrier gas. The energy of the electron beam was set at 70 eV.

Spectrophotometric Analysis: The ultraviolet spectrum was recorded on a Varian Cary 219 spectrophotometer.

RESULTS AND DISCUSSION

Sheep seminal vesicular microsomes are known to catalyze the synthesis of HHT from arachidonate and the synthesis can be stimulated by boiled soluble fraction (15). We have employed this biosynthetic route and have isolated HHT first by TLC followed by HPLC as a chromatographically pure compound. The mass spectrum of the trimethylsilyl derivative of the methyl ester of HHT showed ions at m/e 366 (M^+), 335 (M-31; loss of $\cdot OCH_3$), 295 (M-71; loss of $\cdot(CH_2)_4CH_3$), 276 (M-90; loss of Me_3SiOH) and 225 (M-141; loss of $\cdot CH_2 - CH = CH-(CH_2)_3COOCH_3$). The spectrum is in agreement with that expected for trimethylsilyl derivative of the methyl ester of HHT reported previously (16).

Next we examined the ability of HHT to serve as a substrate for a homogeneously purified NAD^+-dependent 15-PGDH from porcine kidney. It is interesting to find that HHT is an excellent substrate for the enzyme as shown in Table I. Other mono- and dihydroxy fatty acids are poor substrates. When HHT was compared with PGEs, the best substrates known so far for 15-PGDH, they were found to be comparable as indicated by the close values in K_{cat}/K_m shown in the same Table. Since the assay monitored the reduction of NAD^+ to NADH by spectrofluorometric measurement, the product of HHT oxidation remained to be identified. When [1-^{14}C]-HHT was incubated with the enzyme in the presence of NAD^+, a time dependent decrease in HHT peak and a concommitant increase in less polar product peak were observed in TLC radiochromatogram as shown in Fig. 1. The retention times in HPLC for isolated HHT and its product in Solvent A were 13.8 min and 12.3 min respectively. However, the retention times for HHT and its metabolite in Solvent B were 11.8 min and 14.3 min respectively. The reverse order of elution was observed in two different solvent system. The reason for this observation is not clear. A small peak near origin was also observed in Fig. 1. This peak might be derived from product reacting with DTT which was present in the enzyme preparation since α,β-unsaturated keto

TABLE I. Substrate Specificity of NAD^+-Dependent 15-Hydroxyprostaglandin Dehydrogenase

A. Hydroxy Fatty Acids As Substrate

Compounds (30 μM)	Initial Rate (pmol/min)
HHT	400
15-HETE	54
5-HETE	19
12-HETE	0
LTB_4	0

B. HHT and Prostaglandins As Substrate

Compounds	K_m(μM)	$K_{cat}/K_m \times 10^{-6}$ ($M^{-1}Sec^{-1}$)
HHT	0.8	3.5
PGE_1	0.5	4.8
PGE_2	1.1	2.2
$PGF_{2\alpha}$	10.0	0.1

Homogeneously purified enzyme was assayed in the presence of the above substrates as described in the section of "Materials and Methods".

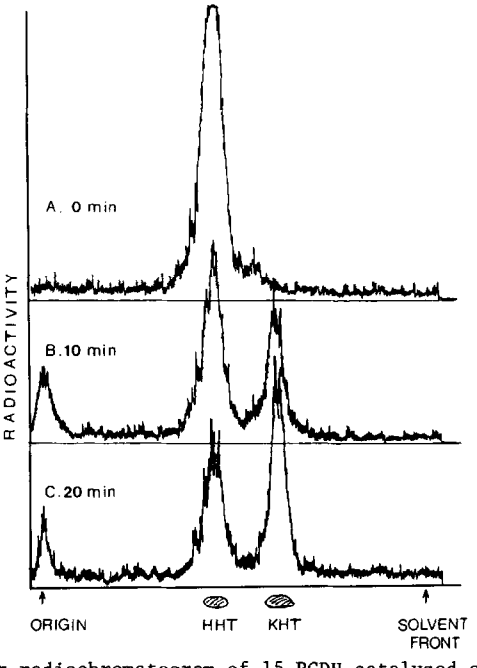

Fig. 1. Thin layer radiochromatogram of 15-PGDH catalyzed oxidation of HHT 15-PGDH (12 ng) was incubated with NAD^+ (1 mM) and [^{14}C]-HHT (1 μM, 20,000 cpm) in a final volume of 1 ml of 0.05 M Tris-HCl, pH 7.5 at 37°C for (A) 0 min, (B) 10 min, (C) 20 min. The reaction was terminated by acidification followed by extraction with ethyl ether. The ether extract was dried under nitrogen and spotted on a precoated silica gel G plate developed in petroleum ether/ethyl ether/acetic acid (50:50:1). Radioactivity on the chromatogram was detected by Berthold radiochromatoscanner.

compound was susceptible to react with sulfhydryl group. Relatively large scale of product preparation was then carried out. The product was first isolated by preparative TLC followed by HPLC. The purified product showed the same retention time as the labeled oxidized product described above. The ultraviolet spectrum of the product exhibited an absorption band with λ_{max}^{MeOH} = 276 nm (ε_m = 34,500) as opposed to that of HHT with λ_{max}^{MeOH} = 232 nm. The spectral characteristics of substrate and product were very similar to those of methyl ester of 12-HETE and its MnO_2 oxidized product described previously (16). GC-MS analysis of the methyl ester of the product indicated ions of high intensity at m/e 292 (M^+), 261 (M-31; loss of $\cdot OCH_3$), 191 (M-101; loss of $\cdot(CH_2)_3COOCH_3$), 177 (M-115; probably loss of $\cdot(CH_2)_4COOCH_3$ formed by rearrangement), 151 (M-141; loss of $\cdot CH_2CH=CH(CH_2)_3COOCH_3$) and 99 (base peak; $\cdot CO(CH_2)_4CH_3$) as shown in Fig. 2. This mass spectrum is consistent with the structure of the methylester of 12-keto-5,8,10-heptadecatrienoic acid (KHT). The gas chromatographic data of O-methyloxime derivative of the methyl ester of product showed two separate peaks presumably due to syn and anti isomers which are characteristic of carbonyl derivatives (data not shown). The mass spectrum of this derivative showed ions of high intensity at m/e 321 (M^+), 290 (M-31; loss of $\cdot OCH_3$), 220 (M-101; loss of $\cdot(CH_2)_3COOCH_3$) and 180 (M-141; loss of $\cdot CH_2CH=CH(CH_2)_3COOCH_3$) as indicated in Fig. 3. This mass spectrum was in agreement with that expected for the O-methyloxime derivative of the methyl ester of KHT. Therefore, the product of HHT oxidation catalyzed by 15-PGDH was conclusively identified as KHT.

HHT has been proposed to derive from PGH_2 by two different mechanisms, an enzymic and a non-enzymic (7,8). We have recently purified thromboxane synthase to apparent homogeneity using immunoaffinity chromatography and have

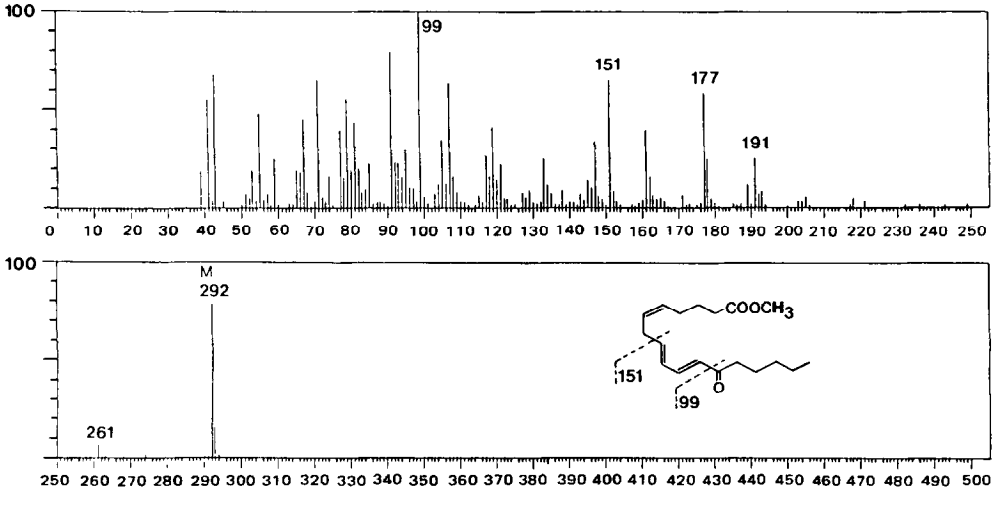

Fig. 2. Mass spectrum of the methyl ester of 12-keto-5,8,10-heptadecatrienoic acid (KHT).

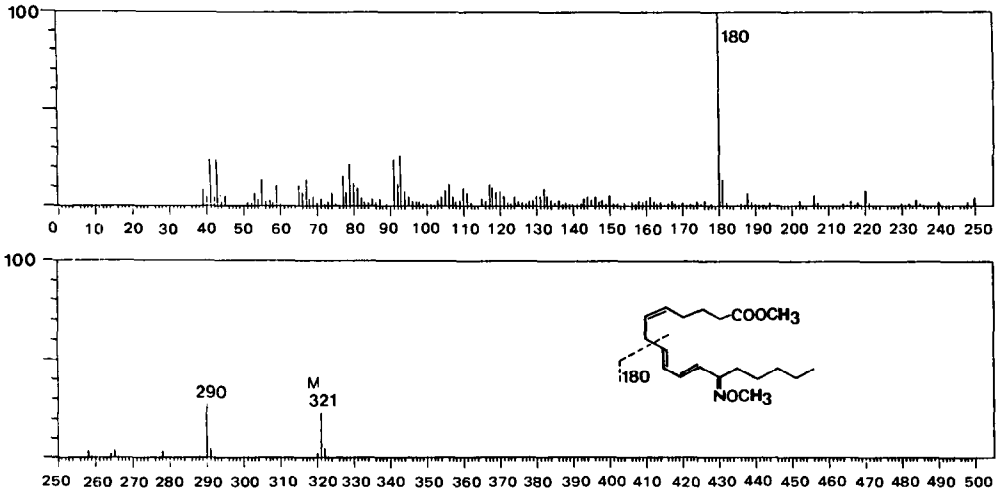

Fig. 3. Mass spectrum of the O-methyloxime derivative of the methyl ester of 12-keto-5,8,10-heptadecatrienoic acid (KHT).

conclusively shown that the enzyme is capable of catalyzing the formation of HHT in addition to TXA_2 (17). HHT can be also resulted from heat stable factor presumably heme mediated decomposition of PGH_2 (8,18). Regardless of its route of synthesis, the biological significance of this hydroxy fatty acid has not been fully recognized. HHT has been shwn to exhibit chemotactic activity for leukocytes although its potency is far less than that of LTB_4 (19,20). The fact that HHT is an excellent substrate for 15-PGDH suggests that it may have other profound biological effects which remain to be determined. It would be important and rewarding to search for the biological activities of HHT and to examine if the enzyme is responsible for its inactivation.

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