Peroxisomal chain-shortening of thromboxane B_2: evidence for impaired degradation of thromboxane B_2 in Zellweger syndrome

Ulf Diczfalusy,[1,*] Ole Vesterqvist,[2,†] Bengt Frode Kase,[**] Erik Lund,[*] and Stefan E. H. Alexson[*]

Department of Clinical Chemistry,[*] Huddinge University Hospital, S-141 86 Huddinge, Sweden; Department of Clinical Chemistry,[†] Karolinska Hospital, S-104 01 Stockholm, Sweden; and Department of Pediatric Research,[**] Rikshospitalet, The National Hospital, N-0027 Oslo, Norway

Abstract We have shown that rat liver peroxisomes can chain-shorten prostaglandins to dinor- and tetranor-metabolites. In a recent in vivo study we could demonstrate that peroxisomes are of major importance for chain-shortening of prostaglandin $F_{2\alpha}$ in humans (1991. Diczfalusy et al. *J. Clin. Invest.* **88:** 978–984). This was shown by identifying the major urinary metabolites of radiolabeled prostaglandin $F_{2\alpha}$ given intravenously to a patient lacking functional peroxisomes (Zellweger syndrome). In the present investigation we have studied the peroxisomal chain-shortening of thromboxane B_2, a compound structurally related to prostaglandins. Isolated rat liver peroxisomes oxidized thromboxane B_2 to a chain-shortened metabolite in an NAD^+-dependent reaction. The metabolite was identified as 9,11,15-trihydroxy-2,3,4,5-tetranor-thromb-13-enoic acid (tetranor-thromboxane B_1). The urinary excretion of the major β-oxidized metabolites of thromboxane B_2 and prostacyclin was determined in three Zellweger patients and six age-matched controls. The controls excreted on an average 1.7 and 1.1 ng/mg creatinine of 2,3-dinor-thromboxane B_2 and 2,3-dinor-6-keto-prostaglandin $F_{1\alpha}$, respectively. In none of the three Zellweger patients could these dinor-metabolites be detected, i.e., the urinary excretion was less than 0.2 ng/mg creatinine. This shows that peroxisomes play an important role in the degradation of the carboxyl side chain of thromboxane B_2 in vivo.—**Diczfalusy, U., O. Vesterqvist, B. F. Kase, E. Lund, and S. E. H. Alexson.** Peroxisomal chain-shortening of thromboxane B_2: evidence for impaired degradation of thromboxane B_2 in Zellweger syndrome. *J. Lipid Res.* 1993. **34:** 1107–1113.

Supplementary key words β-oxidation • rat liver peroxisomes • cerebro-hepato-renal syndrome • urinary metabolites

Prostaglandins are rapidly inactivated and degraded in vivo by a series of enzymatic reactions. These include conversion of the 15-hydroxy group to a ketone and saturation of the 13,14-double bond in the methyl end side chain. The resulting metabolites are subsequently oxidized to more polar metabolites by β- and ω-oxidation. These oxidation products are excreted in the urine and the majority of the excretion products are chain-shortened to dinor- or tetranor-metabolites (1). The chain-shortening of prostaglandins has been reported to be localized to mitochondria (2, 3). We have recently shown that also rat liver peroxisomes can chain-shorten prostaglandin $F_{2\alpha}$ (4), as well as other prostaglandins (5, 6). In vitro experiments indicated that the capacity for peroxisomal chain-shortening appeared to be higher than the corresponding mitochondrial activity (7). To assess the relative importance of the two organelles for chain-shortening of prostaglandins in vivo, radiolabeled prostaglandin $F_{2\alpha}$ was administered to a patient lacking functional peroxisomes (Zellweger syndrome) and urine was collected for analysis of radiolabeled metabolites. We were not able to detect any chain-shortened urinary prostaglandin metabolites in the patient. This in vivo study gave evidence for the importance of peroxisomes for the β-oxidation of prostaglandins in humans (8).

Thromboxane A_2 has potent biological activities. It contracts vascular smooth muscle and induces irreversible platelet aggregation (9, 10). This labile substance quickly adds a water molecule in aqueous media and forms the stable compound thromboxane B_2. Thromboxane B_2 is formed from the same precursor as the prostaglandins (10). Its chemical structure is related to the structure of the prostaglandins and it is also excreted in the urine as chain-shortened metabolites (11). Therefore, a peroxisomal role in the oxidative chain-shortening of thromboxane B_2 was most interesting to investigate. In the present work we have studied chain-shortening of thromboxane B_2 by isolated rat liver peroxisomes. In addition,

Abbreviations: DEHP, di(2-ethylhexyl)phthalate; HPLC, high performance liquid chromatography.

[1]To whom correspondence should be addressed.

[2]Present address: Pharmacology Department, Bristol-Myers Squibb, Princeton, NJ 08543-4000.

the major urinary metabolites of thromboxane B_2 and prostacyclin were quantitated in samples from peroxisome-deficient patients with Zellweger syndrome and healthy age-matched controls.

MATERIALS AND METHODS

Chemicals

[1-^{14}C]arachidonic acid (sp act 58 mCi/mmol) was from Amersham, Buckinghamshire, England. [5,6,8,9,11,12,14,15-^{3}H(N)]thromboxane B_2 (sp act 126.2 Ci/mmol) was purchased from NEN Research Products, DuPont de Nemours, Dreieich, Germany. [18,18,19,19-$^{2}H_4$]thromboxane B_2 and 11-dehydro-thromboxane B_2 were from Biomol Research Laboratories Inc., Plymouth Meeting, PA. [1-^{14}C]Thromboxane B_2 was isolated from incubations of [1-^{14}C]arachidonic acid with human platelet microsomes. Platelet microsomes were prepared as previously described (12).

Preparation of diazoethane

To a stoppered round-bottom flask with two glass tubes through the stopper was added 10 ml diethyl ether, 1 g 1-nitroso-1-ethylurea (Fluka Chemie AG, Buchs, Switzerland) and 3 ml 30% NaOH. The flask was kept on ice during the reaction. A stream of nitrogen was slowly passed through the solution and then bubbled through 10 ml diethyl ether kept on a cooling bath consisting of solid carbon dioxide in ethanol. The reaction was allowed to proceed for 60–75 min until the cold ether solution acquired a dark yellow/orange color.

Preparation of rat liver peroxisomes

Rat liver peroxisomes were isolated from rats that had been treated with di(2-ethylhexyl)phthalate for 1–2 weeks in order to induce peroxisomes. The peroxisomes were obtained by isopycnic gradient centrifugations in Nycodenz of light mitochondrial fractions, prepared as earlier described (6).

Incubation of thromboxane B_2 with isolated peroxisomes

Incubations of thromboxane B_2 with peroxisomes were performed in 0.01 M Tris buffer, pH 8.0, containing 2.5 mM ATP, 0.5 mM coenzyme A, 10 mM $MgCl_2$, and 35 μM FAD. The total incubation volume in preparative incubations was 7.87 ml and 5 mg of peroxisomal protein was included. Thromboxane B_2 (10 μCi [^{3}H]thromboxane B_2 mixed with 100 μg unlabeled thromboxane B_2) was prepared as an α-cyclodextrin solution as previously described (13) and the final substrate concentration was 30 μM. The incubations were started by addition of NAD^+ (200 μM final concentration) and were allowed to proceed for 60 min. At the end of the incubation the reaction mixture was put on ice, acidified to pH 3 with HCl, and applied to a SEP-PAK C_{18} cartridge (Millipore Corporation, Waters Chromatography Division, Milford, MA). The SEP-PAK C_{18} cartridge was eluted with 5 ml portions of water, 15% ethanol in water, petroleum ether, methyl formate, and methanol as previously described (14). Thromboxane B_2 and its metabolites eluted in the methyl formate fraction. For analytical studies, the reaction was scaled down to a total incubation volume of 1 ml and 50–600 μg peroxisomal protein was used.

Separation of reaction products by high performance liquid chromatography (HPLC)

Chromatography of thromboxane B_2 is difficult due to its hemiacetal structure. Therefore, the methyl formate fraction from the SEP-PAK C_{18} fractionation was treated with O-methoxyamine hydrochloride (10 mg/ml in dry pyridine) to lock the ring in its open form as an O-methyl oxime. The derivatized methyl formate fraction was then subjected to reversed phase HPLC. A Waters Nova-Pak C_{18}, 4-μ column (8 mm × 100 mm) was used together with a Nova-Pak Guard-Pak precolumn (Millipore Corporation, Waters Chromatography Division, Milford, MA). The mobile phase was methanol–water–acetic acid 55:45:0.01 (v/v/v) and the flow rate was 1.25 ml/min. The chromatography system was connected in series to a Kontron variable wavelength UV detector, model 432 (Kontron Instruments SpA, Milan, Italy) and a FLO-ONE/Beta Model A-280 radioactivity detector equipped with a 1-ml flow cell (Radiomatic Instruments & Chemical Co. Inc., Meriden, CT). The retention times for the O-methoxyamine derivatives of thromboxane B_2 and 11-dehydrothromboxane B_2 were 27.0 and 20.5 min, respectively.

Gas chromatography–mass spectrometry

Metabolites to be analyzed by gas chromatography were first treated with etheral diazomethane or diazoethane to convert carboxyl groups to methyl esters or ethyl esters, respectively. Hydroxyl groups were derivatized to trimethyl silyl ethers by treatment with trimethylchlorosilane–hexamethyldisilazane–pyridine 1:2:3 (v/v/v). Analyses were performed on a Hewlett-Packard 5890 gas chromatograph connected to a Hewlett-Packard 5970 mass selective detector. The gas chromatograph was used with either a 13 m × 0.18 mm DB-5 column (J & W Scientific, Folsom, CA) or a 12 m × 0.2 mm HP-1 column (Hewlett-Packard Co., Palo Alto, CA).

Measurement of urinary metabolites of thromboxane B_2 and prostacyclin

Urine was collected from six healthy children and three patients with the diagnosis of Zellweger syndrome. Clinical and biochemical data on the Zellweger patients have been published earlier (8, 15, 16). The major urinary metabolites of thromboxane B_2 and prostacyclin,

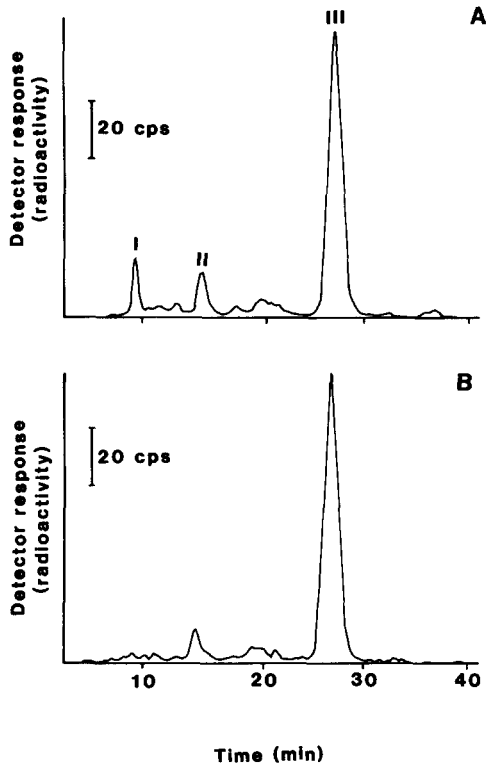

Fig. 1. Reversed phase HPLC chromatograms of O-methoxyamine hydrochloride treated extracts from incubations of thromboxane B_2 with and without peroxisomes. A: Incubation with peroxisomes from DEHP-treated rats. B: Blank incubation without peroxisomes. Peak I corresponds to a polar metabolite; peak II appears in incubations with as well as without peroxisomes; peak III corresponds to unconverted substrate.

2,3-dinor-thromboxane B_2 and 2,3-dinor-6-keto-prostaglandin $F_{1\alpha}$, respectively, were determined by isotope dilution gas chromatography–mass spectrometry using deuterated internal standards as described earlier (17, 18).

RESULTS

Incubations of thromboxane B_2 with isolated peroxisomes

Radio-labeled thromboxane B_2 was incubated with isolated rat liver peroxisomes. After fractionation of the incubation mixture on a SEP-PAK C_{18} cartridge the fraction containing thromboxane and its metabolites was treated with O-methoxyamine hydrochloride to trap the hemiacetal in its open form. The reaction mixture was subsequently subjected to reversed phase HPLC. **Fig. 1A** shows such a HPLC-chromatogram and Fig. 1B shows a chromatogram from a similar incubation of thromboxane B_2 but in the absence of peroxisomes (blank incubation). Peak I in Fig. 1A appeared only when peroxisomes were present in the incubation mixture and its appearance was completely dependent on added NAD^+. Peak II in Fig. 1A was formed also in blank incubations in the absence of peroxisomes. The formation of peak II did not correlate to protein concentration or incubation time. Incubations of [1-^{14}C]thromboxane B_2 with or without peroxisomes resulted in the formation of 1-^{14}C-labeled peak II, showing that it was not chain-shortened from the carboxyl end. This compound was not structurally identified. Peak III in Fig. 1A corresponds to unconverted substrate. Incubation of thromboxane B_2 with increasing amounts of peroxisomal protein resulted in increased production of peak I (Fig. 1A) as shown in **Fig. 2.** Conversion of thromboxane B_2 to peak I increased with incubation time in the presence of a fixed amount (300 μg) of peroxisomal protein as shown in **Fig. 3.** The material corresponding to peak I in Fig. 1A was treated with etheral diazomethane and subjected to trimethylsilylation. The derivatized material was then analyzed by gas chromatography–mass spectrometry. The gas chromatogram showed two major peaks with relative retention times (C-values) of 21.5 and 22.3, indicating chain-shortened metabolites. (The C-

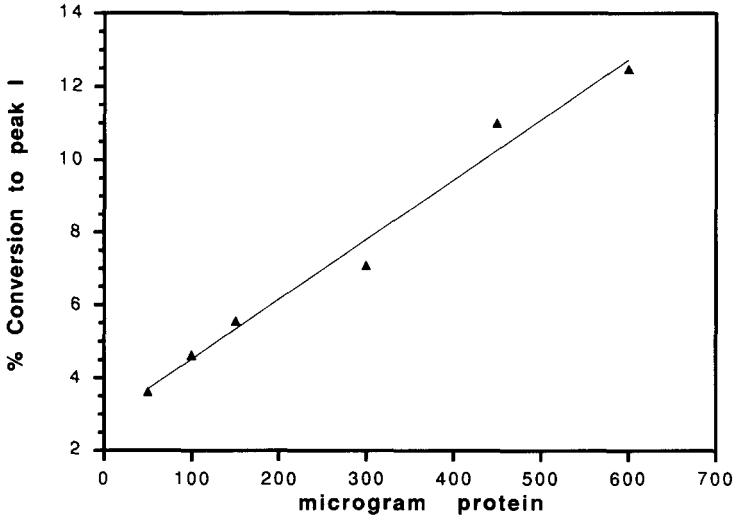

Fig. 2. Peroxisomal conversion of thromboxane B_2 into a polar metabolite (peak I, Fig. 1A) as a function of added peroxisomal protein. Incubation time was 60 min.

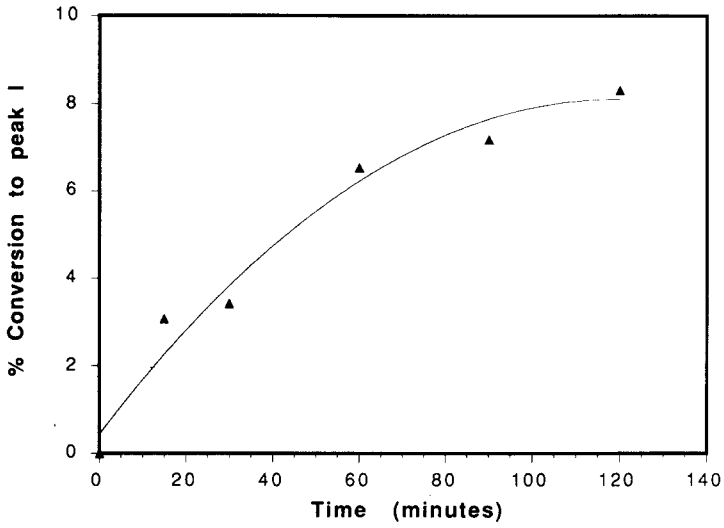

Fig. 3. Peroxisomal conversion of thromboxane B_2 into a polar metabolite (peak I, Fig. 1A) as a function of incubation time. The amount of peroxisomal protein was 300 μg.

value of the corresponding derivative of thromboxane B_2 was 24.9.) The relative amounts of the two gas chromatographic peaks varied considerably in different incubations, but generally they were of the same magnitude. The mass spectrum of the peak with a C-value of 21.5 was in agreement with earlier reported data (11) and corresponds to 9,11,15-trihydroxy-2,3,4,5-tetranor-thromb-13-enoic acid (tetranor-thromboxane B_1). When peak I in Fig. 1 was treated with etheral diazoethane instead of diazomethane and analyzed by gas chromatography after trimethylsilylation, the gas chromatographic peak with a C-value of 21.5 shifted to 22.0 and its mass spectrum showed the expected shifts of 14 mass units in the ions containing the carboxyl ester. Incubation of [18,18,19,19-$^2H_4$]thromboxane B_2 with peroxisomes resulted in the formation of tetradeuterated tetranor-thromboxane B_1 and the mass spectrum of this compound after derivatization to methoximemethyl ester-trimethylsilylether is shown in **Fig. 4.** Ions were present at *m/z* 564 (M–15, loss of $\cdot CH_3$), 548 (M–31, loss of $\cdot OCH_3$), 533 (M–(31 + 15)), 504 (M–75, loss of

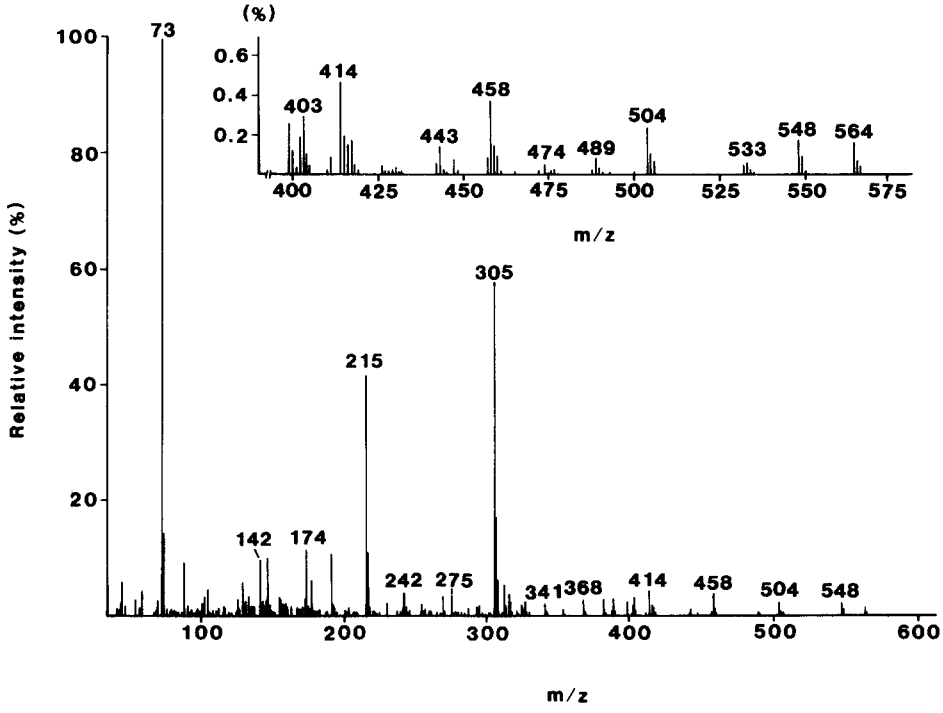

Fig. 4. Mass spectrum of methyl ester–trimethylsilyl ether derivative of material in peak I (Fig. 1A) from an incubation of [18,18,19,19-$^2H_4$]thromboxane B_2 with peroxisomes. Inserted at the top is a magnification of the mass range between *m/z* 395 and 575.

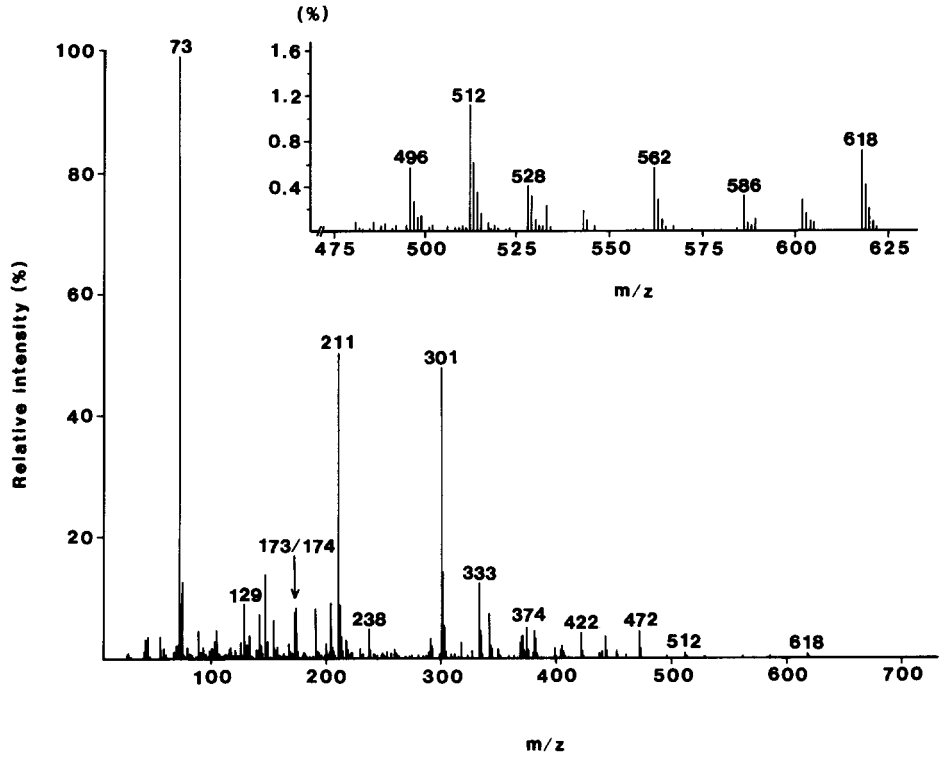

Fig. 5. Mass spectrum of trimethylsilyl ether-trimethylsilyl ester derivative of material in peak I (Fig. 1A) from an incubation of thromboxane B_2 with peroxisomes. Inserted at the top is a magnification of the mass range between *m/z* 475 and 625.

$\cdot(CH_2)_4CH_3$ containing the four deuterium atoms), 489 (M-90, loss of trimethylsilanol), 474 (M-(90 + 15)), 458 (M-(90 + 31)), 443 (M-(90 + 31 + 15)), 414 (M-(90 + 75)), 403, 399 (M-(2 × 90)), 389, 382, 368 (M-(2 × 90 + 31)), 341, 327, 315, 312, 305 ($((CH_3)_3SiO^+=CH-CH=CH-CH(OSi(CH_3)_3(CH_2)_4-CH_3)$), 275, 269, 242, 230, 215 (305-90), 191, 177 ($((CH_3)_3SiO^+=CH(CH_2)_4CH_3$), 174 ($CH_3ON-CH-CH_2-CH(O^+Si(CH_3)_3$), 147, 142, 129, 89 and 73 (base peak). The gas chromatographic peak with a C-value of 22.3 did not change retention time after diazoethane treatment, however. It has been indicated that tetranor-thromboxane B_1 can form a δ-lactone (11) which is blocked for esterification with diazomethane or diazoethane. Subsequent treatment with silylating agents would then result in a trimethylsilyl ester. The mass spectrum of the material with a C-value of 22.3 is shown in **Fig. 5** and is fully compatible with the trimethylsilyl ester of tetranor-thromboxane B_1-methoxime-trimethylsilylether. As in the case of tetranor-thromboxane B_1-methylester-methoxime-trimethylsilylether, the molecular ion (M) is not seen in the mass spectrum. Ions were present at *m/z* 618 (M-15, loss of $\cdot CH_3$), 602 (M-31, loss of $\cdot OCH_3$), 586 (M-(32 + 15)), 562 (M-71, loss of $\cdot(CH_2)_4CH_3$), 543 (M-90, loss of trimethylsilanol), 528 (M-(90 + 15)), 512 (M-(90 + 31)), 496 (M-(90 + 32 + 15), 472 (M-(90 + 71)), 453 (M-2 × 90), 422 (M-(2 × 90 + 31), 374, 333, 301 ($((CH_3)_3SiO^+=CH-CH=CH-CH(OSi(CH_3)_3)(CH_2)_4CH_3$), 238, 211 (301-90), 174 ($CH_3ON-CH-CH_2-CH(O^+Si(CH_3)_3$), 173 ($((CH_3)_3SiO^+=CH(CH_2)_4CH_3$), 129, 73 (base peak).

Measurement of urinary metabolites of thromboxane B_2 and prostacyclin in patients with the Zellweger syndrome and healthy control subjects

The major urinary metabolites of thromboxane B_2 and prostacyclin, 2,3-dinor-thromboxane B_2 and 2,3-dinor-6-keto-prostaglandin $F_{1\alpha}$, respectively, were measured in urine samples from three Zellweger patients and six

TABLE 1. Determination of 2,3-dinor-thromboxane B_2 (TXB2-M) and 2,3-dinor-6-keto-prostaglandin $F_{1\alpha}$ (PGI2-M) in urine from three Zellweger patients and six healthy control subjects

Subject	Age	Sex	TXB2-M	PGI2-M
	months		*ng/mg creatinine*	
Control 1	5	F	0.8	0.6
Control 2	8	F	1.9	3.0
Control 3	9	M	2.1	0.9
Control 4	3.5	M	1.0	0.9
Control 5	4	F	2.5	0.7
Control 6	3	M	1.7	0.7
Zellweger 1	3	F	<0.04	<0.1
Zellweger 2	3	M	<0.2	<0.2
Zellweger 3	1.5	M	<0.3	<0.2

healthy control subjects. The results are presented in **Table 1.** The two chain-shortened metabolites could not be detected in the samples from the Zellweger patients. The values given for the Zellweger patients are the detection limits (1/1000 the amount of added internal standard). The different detection limits in the different samples depend on the volume of urine used for the different determinations.

DISCUSSION

Peroxisomes possess a β-oxidation system that can chain-shorten fatty acids (19–21). We have recently shown that prostaglandins can also be chain-shortened by peroxisomes, and that this reaction is also of importance in vivo (4, 8). In the present work we have studied peroxisomal chain-shortening of thromboxane B_2, a compound structurally similar to the prostaglandins. The in vitro data showed that a chain-shortened metabolite was formed when thromboxane B_2 was incubated with isolated rat liver peroxisomes. The mass spectrum of the methoxime-methyl ester-trimethylsilyl ether derivative of the metabolite showed that the structure of the metabolite (corresponding to peak I in Fig. 1A) was 9,11,15-trihydroxy-2,3,4,5-tetranor-thromb-13-enoic acid (11). The major urinary metabolite of thromboxane B_2 in humans, cynomolgus monkey, and guinea pig has been identified as dinor-thromboxane B_2 (11, 22–26). In rat, however, the major urinary metabolite was identified as tetranor-thromboxane B_1 (27). The chemical structures of these metabolites are shown in **Fig. 6.**

To evaluate the importance of the peroxisomal β-oxidation of thromboxane B_2 in vivo, urine samples from three patients with Zellweger syndrome were analyzed for 2,3-dinor-thromboxane B_2, the major thromboxane metabolite in urine (23). The samples were also analyzed for the dominating prostacyclin metabolite, 2,3-dinor-6-keto-prostaglandin $F_{1\alpha}$ (28). Patients with this rare inherited disease offer a unique possibility to investigate the involvement of peroxisomes in β-oxidation reactions. The results of the present study, presented in Table 1, showed clearly that the Zellweger patients had a seriously impaired capacity to chain-shorten thromboxane B_2 and prostacyclin to the normal urinary metabolites. Recently, we have also studied the metabolism of prostaglandin $F_{2\alpha}$ in a patient with this peroxisomal disorder (8). It was shown that the Zellweger patient apparently could not chain-shorten prostaglandin $F_{2\alpha}$ to the C_{16}-metabolite normally found in the urine. Instead, a non-chain-shortened ω-oxidized metabolite was formed. It was also shown that fibroblasts from the patient oxidized palmitate to a normal extent, indicating an intact mitochondrial β-oxidation. It thus seems that patients with the Zellweger syndrome offer a possibility to discriminate between peroxisomal and mitochondrial β-oxidation. Taken together, our data clearly indicate that functional peroxisomes are essential for the degradation of prostaglandins and thromboxane B_2 in vivo.

In conclusion, we have shown that isolated rat liver peroxisomes can metabolize thromboxane B_2 to tetranor-thromboxane B_1 through two cycles of β-oxidation. Measurement of the major urinary metabolites of thromboxane B_2 and prostacyclin in urine from Zellweger patients showed impaired β-oxidation of these compounds suggesting that peroxisomal β-oxidation of thromboxane B_2 and prostacyclin is of major importance in vivo.

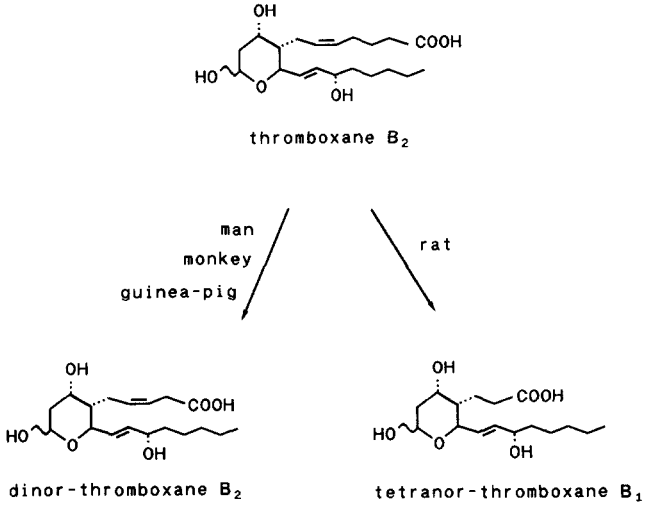

Fig. 6. Metabolic degradation of thromboxane B_2 into major urinary metabolites in different species.

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