methods

Widespread occurrence of glyceryl ether monooxygenase activity in rat tissues detected by a novel assay

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Abstract **An assay was set up for glyceryl ether monooxygenase activity in tissue samples using the novel substrate 1-*O*-pyrenedecyl-*sn*-glycerol and high-performance liquid chromatographic analysis of reaction mixtures with fluorescence detection, allowing robust detection of enzymatic activity in microgram amounts of tissue homogenates. The activity partially purified from rat liver strictly depended on the presence of a tetrahydropteridine. Tetrahydrobiopterin-dependent glyceryl ether monooxygenase activity was observed in all rat tissues tested except female heart, with highest activities in liver, intestine, and cerebellum. Activity was not uniformly distributed in brain: it was higher in cerebellum than in striatum or cortex. These data demonstrate that tetrahydrobiopterin-dependent glyceryl ether monooxygenase is found not only in liver and the gastrointestinal tract but also in brain and other organs of the rat and provide an additional goal for tetrahydrobiopterin biosynthesis in these organs.**—Werner, E. R., A. Hermetter, H. Prast, G. Golderer, and G. Werner-Felmayer. **Widespread occurrence of glyceryl ether monooxygenase activity in rat tissues detected by a novel assay.** ***J. Lipid Res.*** **2007.** 48: **1422–1427.**

Supplementary key words tetrahydrobiopterin • pteridine • ether lipid • alkylglycerol • chemical synthesis • activity assay

The reaction catalyzed by glyceryl ether monooxygenase (EC 1.14.16.5) is shown in **Fig. 1** (1). Glyceryl ether monooxygenase cleaves 1-*O*-alkyl glycerol [I] by tetrahydropteridine-dependent hydroxylation at the carbon adjacent to the alkyl-glycerol ether bond. The primary hydroxylation product [II], a semiacetal, rapidly rearranges to the corresponding aldehyde [III] and glycerol [IV]. In aerobic systems, the aldehyde is then oxidized to the corresponding carboxylic acid [V]. In cells, this reaction is catalyzed by long-chain fatty aldehyde dehydrogenase [Human Genome Organization (HUGO) gene symbol ALDH3A2; EC 1.2.1.48.] with the aid of NAD^+. Long-chain fatty aldehyde dehydrogenase, an enzyme ubiquitously expressed in the body, is defective in Sjögren-Larsson syndrome [Online Mendelian Inheritance in Man (OMIM) number 270200], which is associated with ichthyosis, mental retardation, and spasticity (2). Alternatively, the aldehyde may be enzymatically reduced to the corresponding alcohol [VI] by alcohol dehydrogenase class 4 (HUGO gene symbol ADH4; EC 1.1.1.1.). Despite this potentially multiple fate of the alkyl aldehyde product [III], the corresponding carboxylic acid [V] was always found to be the main product, even when no NAD^+ was added to the assay mixtures in all tissues investigated (3).

Similar to tetrahydropteridine-dependent amino acid hydroxylases, but in contrast to nitric oxide synthases (4), the tetrahydropteridine cofactor of glyceryl ether monooxygenase is thought to leave the reaction as a 4a-hydroxy derivative, which after dehydration results in quinonoid dihydrobiopterin (6,7[8H]-dihydrobiopterin). Quinonoid dihydrobiopterin is then recycled to tetrahydrobiopterin by dihydropteridine reductase (EC 1.6.99.7), which accepts both NADH and NADPH as reductants. Glyceryl ether monooxygenase reacts with substrates carrying 1-*O*-alkyl side chains ranging from 11 to 20 carbon atoms and requires one free hydroxyl group adjacent to the 1-*O*-alkyl ether bond. The third hydroxyl group of glycerol may be coupled to various ligands, including common phospholipid side chains. For example, lyso-platelet-activating factor [1-*O*-hexadecyl-*sn*-glycerophosphocholine (lyso-PAF)], a degradation product of PAF, is a good substrate of glyceryl ether monooxygenase (1). Little is known about the physiological role of glyceryl monoalkyl ethers. They accumulate in proliferating cells and inhibit protein kinase C (5). The physiological significance and sequence of glyceryl ether monooxygenase are unknown. Its tissue distribution was previously thought to be limited to liver and

Manuscript received 20 October 2006 and in revised form 12 February 2007.

Published, JLR Papers in Press, February 15, 2007.
DOI 10.1194/jlr.D600042-JLR200

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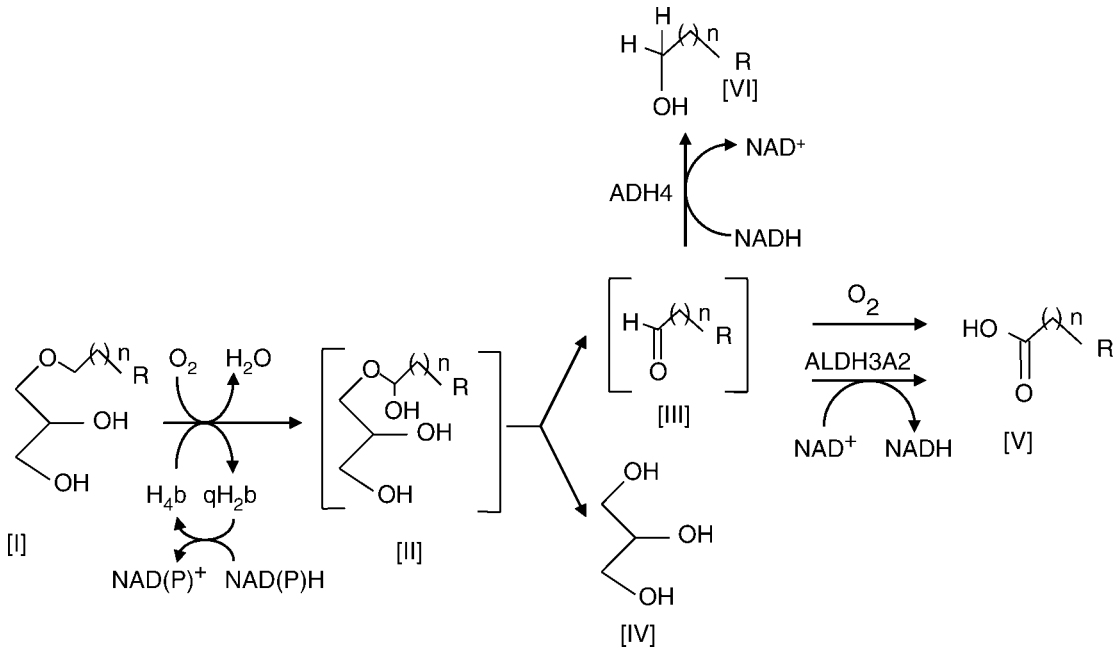

Fig. 1. The glyceryl ether monooxygenase reaction. The glyceryl ether substrate [I] is first hydroxylated in a pteridine-dependent monooxygenase reaction to yield the unstable semiacetal product [II]. The enzyme accepts a range of chain lengths from n = 11 to n = 20 (1). The tetrahydrobiopterin cofactor (H_4b) is first converted to a 4a-hydroxy derivative and leaves the reaction after dehydration as 6,7[8H]-dihydrobiopterin (qH_2b), which is recycled to tetrahydrobiopterin by dihydropteridine reductase with NADH or NADPH. The semiacetal [II] rearranges to the corresponding aldehyde [III] and glycerol [IV]. In the presence of oxygen, the aldehyde [III] is readily oxidized to the corresponding acid [V]. Enzymatically, the aldehyde [III] may be oxidized to the acid [V] by long-chain fatty aldehyde dehydrogenase (ALDH3A2) and NAD^+ or reduced to the alcohol [VI] by alcohol dehydrogenase class 4 (ADH4).

intestine (3). Several investigations characterized its activity in rat liver microsomes (6–11).

Here, we present a sensitive and robust assay of the enzyme based on the use of the novel compound 1-*O*-pyrenedecyl-*sn*-glycerol as substrate, which is converted to 1-pyrenedecanoic acid by the glyceryl ether monooxygenase reaction (Fig. 1, n = 9, R = pyrenyl). We investigated cofactor requirements of glyceryl ether monooxygenase in purified fractions from rat liver microsomes and tissue distribution in rats.

MATERIALS AND METHODS

Preparation of 1-*O*-pyrenedecyl-*sn*-glycerol

Pyrenedecanol was prepared by Vitride reduction of pyrenedecanoic acid (Fluka AG, Buchs, Switzerland) according to standard protocols. Pyrenedecyl mesylate was obtained from pyrenedecanol and methanesulfonylchloride as described for the preparation of long-chain fatty alcohols (12). A mixture of 13 mg (98.4 μmol) of 3,2-isopropylideneglycerol (Bachem, Bubendorf, Switzerland) and 14 mg of KOH in 0.5 ml of dimethyl sulfoxide was stirred at 50°C for 1 h. After the addition of 50 mg (115.1 μmol) of pyrenedecyl mesylate, stirring was continued for an additional 3 h. The reaction was stopped by the addition of 1 ml of water. The reaction mixture was extracted three times with diethylether (once with 5 ml, twice with 3 ml). The combined organic phases were washed three times with 2.5 ml of water and dried over sodium sulfate, followed by removal of the solvent under reduced pressure. The crude 1-*O*-pyrenedecyl-2,3-isopropylidene-*sn*-glycerol product showed one main spot on thin-layer chromatography (relative mobility = 0.4; silica gel; solvent, methylene chloride) and was used without further purification for the next step. After the addition of 800 μl of methanol, 120 μl of 3 N HCl was added under stirring, yielding a precipitate. The reaction mixture was stirred for 3 h, and the solvent was removed under reduced pressure. The crude product was purified by preparative thin-layer chromatography on silica gel (relative mobility = 0.13) using petroleum ether-diethylether (2:8, v/v) as solvent. The yield of pure product was 18 mg (41.8 μmol), which was stored in aliquots at −20°C. While our work was in progress, a report appeared that used a related compound (1-*O*-[9′-(1″-pyrenyl)]nonyl-*sn*-glycerol) to study the metabolic fate of ether lipids in cells (13).

Other materials

DL-α-Hexadecyl glycerol was from Fluka, and lyso-PAF was from Sigma (Vienna, Austria). *R,S*-1-Octadecyl glycerol (+, − batyl alcohol) was a kind gift of Wilf Armarego (Australian National University, Canberra, Australia). Dihydropteridine reductase from *Physarum polycephalum* was expressed in *Escherichia coli*, purified by DEAE chromatography as described (14), and stored at −20°C in the presence of 50% (v/v) glycerol. Pteridine derivatives were obtained from B. Schircks Laboratories (Jona, Switzerland). Methanol for chromatography and buffer salts were from Merck (Darmstadt, Germany). Catalase (from bovine liver) and nucleotides were from Sigma.

Glyceryl ether monooxygenase assay

A typical glyceryl ether monooxygenase assay contained the following components in a total volume of 10 μl: 100 mM Tris-HCl, pH 8.5, 1–12 μg of protein, 0.2 mM NADPH (to promote

recycling by dihydropteridine reductase), 0.2 mM NAD^+ (to promote oxidation of the aldehyde by ALDH3A2), 0.1 mg/ml catalase [purified on NAP-5 Sephadex G-25 columns (Amersham-Pharmacia, Uppsala, Sweden)], 0.5 μmol/min/ml (0.2 μg/ml) dihydropteridine reductase, 0.2 mM 6*R*-5,6,7,8-tetrahydro-L-biopterin, and 0.1 mM 1-*O*-pyrenedecyl-*sn*-glycerol. The reaction was started by the addition of protein, and incubation was performed at 37°C in the dark. After 5–80 min (40 min for rat tissue samples; after 60 min, the reaction was no longer linear in crude homogenates), the reaction was terminated by the addition of 30 μl of methanol. In assays using rat liver microsomes, addition of NAD^+ did not alter the yield of pyrenedecanoic acid and was omitted from the reaction mixtures.

Tissue samples were homogenized in 500 μl of 0.1 M Tris-HCl, pH 7.6, containing 0.25 M sucrose and 1 mM PMSF by means of an Ultra Turrax (IKA, Staufen, Germany). After centrifugation at 16,000 *g* for 2 min at 4°C, supernatants were used for the determination of protein (Bradford assay, BSA standard) and glyceryl ether monooxygenase activity assays. Tissue extracts were diluted to yield <0.4 mg protein/ml final concentration in the assay. Samples were spiked with solubilized rat liver microsomes [10% (v/v) 0.02 mg protein/ml final concentration] to control for interfering substances potentially present in homogenates. The increase in activity in tissues by spiking was compared with the same amount of solubilized rat liver microsomes diluted in assay buffer. A control sample was generated by stopping the reaction immediately after its start, which always yielded <2% pyrenedecanoic acid product compared with the pyrenedecanoic acid levels reached after 40 min of incubation. Reagent blanks (homogenization buffer instead of protein added) were run in parallel and were always negative for pyrenedecanoic acid (<1 nmol/l). Tissues for female and male animals were analyzed in a mixed sequential order to avoid bias by drifting of assay and/or analysis conditions with time.

To test the interference of lipids in the assay, 5 mM stock solutions were prepared in methanol and further diluted in assay buffer before being added to solubilized microsomes. Control samples contained the same amount of methanol but no lipid added.

HPLC analysis of reaction mixtures

A Hewlett-Packard 1050 HPLC system with Chemstation software (Agilent, Vienna, Austria) was used. A sample (4 μl) was injected onto a Zorbax XDB-C8 USP-L7 column (Agilent) eluted with 10 mM potassium phosphate buffer, pH 5.4, containing 79% (v/v) methanol at a flow rate of 1.0 ml/min for 4.5 min, followed by a gradient to 100% (v/v) methanol at 5.0 min. After elution with 100% methanol until 7.5 min, the initial composition (79% methanol) was restored at 8.0 min, and the column was equilibrated until 12.5 min total time to prepare for the next injection. Pyrenedecanoic acid was detected by fluorescence (detector FP-920; Jasco, Vienna, Austria) at 340 nm excitation and 400 nm emission, with a detection limit of 1 nmol/l.

Partial purification of rat liver microsomal glyceryl ether monooxygenase

All procedures were carried out at 4°C. A total of 250 g of rat liver (male Sprague-Dawley) was homogenized in 375 ml of 0.1 M Tris-HCl, pH 7.6, containing 0.25 M sucrose and 1 mM PMSF by means of an Omni Mixer (Sorvall) and centrifuged at 3,000 *g* for 10 min. Supernatants were collected and centrifuged at 13,000 *g* for 20 min. Microsomal pellets were collected after centrifugation at 40,000 *g* for 1 h. After washing with Tris-HCl/sucrose buffer, microsomal pellets were resuspended in 10 ml of assay buffer (100 mM Tris-HCl, pH 8.5) containing 10% (v/v) glycerol, yielding a total protein concentration (Bradford assay) of 11 mg/ml. Microsomes were then solubilized in an equal volume of glycerol, and 3 volumes (referring to the initial volume of microsomes) of water containing 1 mM DTT was added. After centrifugation at 40,000 *g* for 45 min, ethanol was added to the supernatant under stirring to a final concentration of 28% (v/v) and incubated for 30 min, and the pellet was removed after centrifugation at 5,000 *g* for 15 min. Under stirring, ethanol was added to yield a final concentration of 37.5% (v/v), and the pellet was collected by centrifugation at 5,000 *g* for 15 min. The pellet was then resuspended in 10 mM Tris-HCl, pH 7.0, containing 10% (v/v) glycerol and 1 mM DTT. The enrichment in specific activity was ~100-fold compared with crude liver homogenate, with a typical overall yield of 0.5%.

Preparation of rat tissues

All animal work was conducted in conformity with the Public Health Service Policy on Humane Care and Use of Laboratory Animals and approved by the respective committee of the Austrian Ministry of Science and Education. Sprague-Dawley rats (six female, 231–252 g; six male, 244–346 g) were anesthetized with intraperitoneal urethane and decapitated. Tissues were immediately frozen in liquid nitrogen and stored at −75°C until analyzed.

RESULTS

HPLC-based assay of glyceryl ether monooxygenase

Figure 2A shows HPLC chromatograms of typical incubation mixtures of glyceryl ether monooxygenase assays in partially purified fractions of rat liver microsomes. The 1-*O*-pyrenedecyl-*sn*-glycerol substrate is well separated from the product 1-pyrenedecanoic acid. Furthermore, the substrate is virtually free of 1-pyrenedecanoic acid, allowing for a sensitive detection of product in the incubation mixtures (detection limit of 1 nmol/l). Both substrate and product were stable under laboratory light for up to 4 h. Nevertheless, enzyme incubations were performed routinely in the dark to protect the light-sensitive pterin cofactors. Previous work has shown that a small amount of alcoholic product is made in addition to the dominant acid product (3). We cannot detect the alcohol in our HPLC chromatograms, however, because it comigrates with the substrate.

With crude rat liver homogenates as the source of glyceryl ether monooxygenase, we found that enzymatic product formation was linear with protein amount up to 4 μg, which corresponds to 0.4 mg/ml final homogenate protein concentration in our 10 μl total volume assay (Fig. 2B). Similar observations were made with each of the rat tissues tested (data not shown; for a list of tissues tested, see Table 2 below). With purified fractions, the assay is linear up to 12 μg (1.2 mg/ml final protein concentration; data not shown). Product increased linearly up to 60 min in tissue homogenates (Fig. 2C) and up to 80 min using purified fractions (data not shown). Because we found that ammonium sulfate (5 mM) inhibited the reaction already by 80%, we omitted this substance that had been included in previous assay protocols from the assay incubation mixture (see Materials and Methods for details).

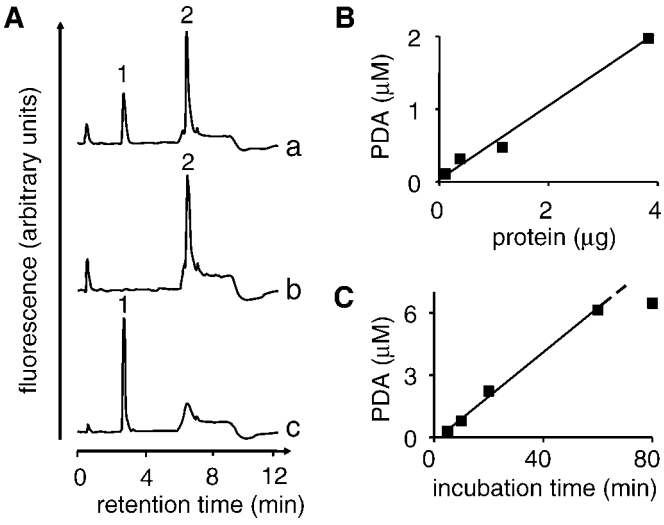

Fig. 2. Properties of the HPLC-based glyceryl ether monooxygenase assay. A: Typical HPLC chromatograms. Partially purified fractions of rat liver microsomes were incubated either in the presence (trace a) or in the absence (trace b) of 6*R*-tetrahydrobiopterin with 1-*O*-pyrenedecyl-*sn*-glycerol in the presence of catalase, dihydropteridine reductase, NAD^+, and NADPH for 1 h at 37°C in a total volume of 10 μl (see Materials and Methods for details). The reaction was stopped by the addition of 30 μl of methanol, and 4 μl of this mixture was injected into the HPLC system. Trace c shows the chromatogram of a synthetic pyrenedecanoic acid standard. Peak 1, pyrenedecanoic acid; peak 2, 1-*O*-pyrenedecyl-*sn*-glycerol. B: Dependence of the assay on protein amount. Male rat liver raw homogenate was incubated in serial dilutions with a fixed incubation time of 40 min. At >4 μg of protein, a lower than proportional increase product occurred (not shown). PDA, pyrenedecanoic acid. C: Dependence of the assay on incubation time in male rat liver raw homogenate (3.1 μg). Panels B and C show one of three independent measurements.

1-*O*-[9′-(1″-Pyrenyl)]nonyl-*sn*-glycerol (13), a compound similar to our substrate with a 9 carbon spacer instead of a 10 carbon spacer between glycerol and the pyrenyl fluorophore, is equally suited for the assay. It exhibits a K_m (6.2 ± 1.9 μM) comparable to that of our 10 carbon spacer ether lipid (8.9 ± 2.0 μM; mean ± SD, n = 3).

Pyrenedecanoic acid formation was competitively inhibited by hexadecylglycerol (K_i = 12.4 μM). Lyso-PAF and octadecyl glycerol also inhibited pyrenedecanoic acid formation. However, >85% of pyrenedecanoic acid was recovered when 20 μM of either of the two compounds was added. To control for the interference of lipids present in tissue extracts, we spiked all tissue extracts with microsomal preparations and found an average of 75% recovery (see below).

Enzymatic properties of glyceryl ether monooxygenase activity in partially purified fractions from rat liver microsomes

With a microsomal fraction further purified by ethanol precipitation, we tested for substrate and pteridine cofactor dependence. The enzyme had an apparent K_m of 8.9 ± 2.0 μM for the substrate and 2.6 ± 1.6 μM for tetrahydrobiopterin (mean ± SD, n = 3). Tetrahydrobiopterin was absolutely required for the reaction. In the absence of tetrahydrobiopterin, we found <1% of the activity observed in the presence of tetrahydrobiopterin (Fig. 2). Tetrahydrobiopterin could not be replaced by any of the following reductants: glutathione (reduced form), flavin adenin dinucleotide, reduced form ($FADH_2$) [formed from flavin adenine dinucleotide (FAD) by reduced glutathione in the presence of glutathione reductase], or L-ascorbic acid (1 mM each), all of which displayed activities of <1% of the full activity observed in the presence of tetrahydrobiopterin (data not shown). The enzyme activity showed clear preferences for the stereochemistry of tetrahydropteridine side chains (**Table 1**). For example, 6*S*-tetrahydrobiopterin was inactive as a cofactor, whereas 6*RS*-tetrahydroneopterin was a slightly better stimulator of activity compared with 6*R*-tetrahydrobiopterin.

Tissue distribution of glyceryl ether monooxygenase activity in rats

Table 2 lists glyceryl ether monooxygenase activities in tissues of female and male rats. The highest activities were observed in liver, followed by intestine, cerebellum, and testes. Activity was not uniformly distributed in the brain but was higher in cerebellum compared with cortex or striatum. Male rats had higher activity in the liver ($P < 0.001$), stomach ($P < 0.05$), and heart ($P < 0.02$; Student's *t*-test) compared with female rats. Tetrahydrobiopterin dependence was observed to a variable degree in all tissues except the female heart. Tetrahydrobiopterin dependence was high in tissues with high activity and declined to ~50% in tissues with low activity (Table 2).

To control for a potential attenuation of the readout of our assay by endogenously present, nonfluorescent substrates, we spiked all tissues with solubilized liver microsomes and observed the recovery of activity when spiking tissue extracts compared with spiking of the same amount to assay buffer. Mean recovery was 75% in both female and male tissues. Recovery did not correlate with the activity

TABLE 1. Relative stimulatory capacity of tetrahydropteridine derivatives for glycerol ether monooxygenase activity

Compound	Activity at 100 μM Pteridine Concentration	Activity at 2 μM Pteridine Concentration
	% of control	
6*R*-Tetrahydrobiopterin	100.0 ± 9.1	23.6 ± 6.1
6*S*-Tetrahydrobiopterin	3.5 ± 0.4	2.5 ± 0.3
6*RS*-Tetrahydrodictyopterin	44.7 ± 6.4	11.7 ± 7.4
6*RS*-6-Methyltetrahydropterin	60.9 ± 5.4	14.7 ± 5.5
6*RS*-6,7-Dimethyltetrahydropterin	94.2 ± 56.6	25.8 ± 7.9
6*RS*-6-Hydroxymethyltetrahydropterin	16.0 ± 1.7	2.7 ± 1.8
6*RS*-Tetrahydroneopterin	124.1 ± 32.0	32.3 ± 9.4

Glycerol ether monooxygenase was partially purified from rat liver as described in Materials and Methods. Glyceryl ether monooxygenase assays were performed in the presence of the indicated concentrations of tetrahydropteridines using 0.7 μg of protein and a 20 min incubation time and were related to the activity observed with 100 μM 6*R*-tetrahydrobiopterin, which was set at 100%. Values shown are means ± SD of three parallel measurements.

TABLE 2. Glyceryl ether monooxygenase activity in rat tissues

Tissue	Female			Male		
	Activity	Tetrahydrobiopterin-Dependent	Recovery	Activity	Tetrahydrobiopterin-Dependent	Recovery
	pmol/mg/min	%		*pmol/mg/min*	%	
Brain, cerebellum	4.83 ± 3.77	91.8 ± 3.4	54.1 ± 15.3	2.63 ± 0.62	86.0 ± 3.5	72.3 ± 21.5
Brain, cortex	1.06 ± 0.17	65.7 ± 4.6	45.2 ± 14.6	0.96 ± 0.24	58.7 ± 7.6	63.1 ± 10.9
Brain, striatum	0.76 ± 0.24	65.2 ± 17.8	60.5 ± 14.4	0.97 ± 0.35	56.1 ± 15.8	74.1 ± 19.6
Heart	0.30 ± 0.09	<5	66.8 ± 14.9	0.54 ± 0.17	48.7 ± 18.9	73.6 ± 15.3
Intestine, small	25.36 ± 21.69	95.5 ± 3.0	93.5 ± 22.0	45.03 ± 18.12	98.0 ± 2.0	73.3 ± 37.1
Intestine, large	15.82 ± 4.96	95.0 ± 2.6	86.7 ± 18.3	18.06 ± 4.73	90.4 ± 3.2	96.9 ± 0.8
Kidney	0.67 ± 0.21	59.5 ± 5.8	89.8 ± 5.2	0.66 ± 0.36	65.6 ± 14.1	76.7 ± 8.2
Liver	346.86 ± 159.94	97.5 ± 0.6	65.6 ± 5.0	1,253.45 ± 92.00	99.5 ± 0.2	66.5 ± 24.7
Lung	2.33 ± 1.33	89.3 ± 6.0	76.8 ± 16.1	2.55 ± 0.85	92.6 ± 3.5	72.8 ± 14.3
Ovaries	1.62 ± 0.81	75.1 ± 5.8	94.8 ± 20.7			
Skeletal muscle	0.57 ± 0.19	46.1 ± 7.2	86.2 ± 5.7	0.67 ± 0.48	50.9 ± 15.5	83.1 ± 9.3
Spleen	0.88 ± 0.48	75.0 ± 6.4	74.5 ± 16.4	0.81 ± 0.34	68.4 ± 13.1	70.6 ± 8.1
Stomach	0.78 ± 0.31	49.0 ± 16.4	73.8 ± 21.5	1.61 ± 0.66	70.9 ± 11.5	88.2 ± 13.7
Testes				3.50 ± 0.67	88.1 ± 4.3	81.6 ± 14.2

Tissues from Sprague-Dawley rats were collected, immediately frozen in liquid nitrogen, and stored at −75°C until assayed for glyceryl ether monooxygenase activity as detailed in Materials and Methods. Values shown are means ± SD of individual activity measurements in tissues derived from six animals.

(i.e., was not higher in liver compared with tissues with much lower activity) (Table 2).

DISCUSSION

To assess glyceryl ether monooxygenase activity in cells and tissues, we developed a novel assay based on HPLC separation of the fluorescent pyrenedecanoic acid product from the novel substrate 1-*O*-pyrenedecyl-*sn*-glycerol, which was chemically synthesized to enable this study. Because of the intense fluorescence of the product, our newly developed assay allows a sensitive, robust detection of glycerylether monooxygenase activity. 1-*O*-[9′-(1″-Pyrenyl)]nonyl-*sn*-glycerol, which had been synthesized for other purposes (13), appears to be equally suited as 1-*O*-pyrenedecyl-*sn*-glycerol as substrate. However, in contrast to pyrenedecanoic acid, the resulting pyrenenonanoic acid is not commercially available for calibration of the HPLC method.

In a previous assay based on the kinetics of ultraviolet light absorption, a detection limit of activity of 0.6 nmol/min was found (9). Our assay, in contrast, had a detection limit 5 orders of magnitude lower (4 fmol/min). This difference arises from our miniaturized assay volume (10 μl instead of 1 ml) and the nanomolar detection limit for the fluorescent substrate compared with the micromolar concentration changes observed by ultraviolet light absorption. Other assays used ^{3}H- or ^{14}C-labeled substrate, thin-layer chromatography, scraping, and counting of spots (6) or gas-liquid chromatography and detection of labeled CO_2 after combustion (3). No minimal detectable activity was mentioned in these reports, but we assume that these assays are sensitive if high radioactive concentrations of substrates are used. However, they appear to be much more laborious and time-consuming than our HPLC-based assay.

Our competition experiments show that our fluorescent substrate has comparable affinity to the enzyme as hexadecylglycerol, the best substrate of the enzyme (1). Thus, ether lipids present in samples would potentially interfere with the reaction when present in comparable amounts to the substrate used. Because of the high sensitivity of the assay, however, dilute tissue extracts were used. We spiked all tissue extract samples with solubilized rat liver microsomes and found a mean recovery of 75%. This recovery was not higher in the liver, which had the highest activity, compared with tissues with much lower activity. These data clearly demonstrate that the differences in glyceryl monooxygenase are not biased by different amounts of interfering compounds potentially present in tissues.

Using a partially purified preparation of glyceryl ether monooxygenase, we demonstrated that among a panel of other reductants or coenzymes tested, the presence of a tetrahydropterin was essentially required for catalysis. The stimulatory effect of tetrahydropteridines depended on the stereochemistry of the side chain. For example, 6*R*-tetrahydrobiopterin was a very good stimulator, whereas 6*S*-tetrahydrobiopterin was not able to stimulate the enzyme. These results extend previous observations (reviewed in Ref. 1). Because the ability of tetrahydropteridines to stimulate glyceryl ether monooxygenase activity does not correlate with the ability of our dihydropteridine reductase preparation to recycle the various pteridine derivatives (14), which was used in large excess of activity, these findings clearly suggest a direct interaction of the tetrahydropteridine cofactor with glyceryl ether monooxygenase. We found micromolar K_m levels for substrate and cofactor, which are up to 10-fold lower than values found in previous investigations (reviewed in Ref. 1). These differences may originate from differences in the scale, setup, and precision of the assay.

In rats, the highest glyceryl ether monooxygenase activities were found in liver and intestine. In accordance

with a previous report, liver of male rats had ~3-fold higher activity than liver of female rats (15). Interestingly, this difference was confined to a few organs (i.e., liver, heart, and stomach) but not reflected in other organs. Activity varied among brain regions and was higher in cerebellum than in striatum or cortex. With the exception of female heart, tetrahydrobiopterin-dependent glyceryl ether monooxygenase activity was detected in all organs investigated. This contrasts with previous widely accepted results that glyceryl ether monooxygenase was only observed in significant activity in liver and intestine (3). We attribute this discrepancy to the better performance of our assay.

Although low residual activity was detected in the tissue homogenates without the addition of tetrahydrobiopterin, glyceryl ether monooxygenase activity in these organs might depend totally on tetrahydrobiopterin if the enzyme activity was partially purified from these tissues, as we have shown here for the liver. The low residual activities may be enabled by the tetrahydrobiopterin cofactor present in the tissues, which was not removed from the homogenates before incubation with the tetrahydrobiopterin-free assay mixture. Alternatively, tissues may contain a low non-tetrahydrobiopterin-dependent glyceryl ether monooxygenase activity similar to the one found in *Leishmania* (16), explaining the decreasing tetrahydrobiopterin dependence we observed with decreasing activity.

The glyceryl ether monooxygenase activity profile we found in the rat indicates that the enzyme may serve a metabolic function attributable to its presence in the gastrointestinal tract and liver. Localization in other areas such as testes and brain indicates that glyceryl ether cleavage is also required in peripheral organs for functions remaining to be investigated. The broad tissue distribution of glyceryl ether monooxygenase should also be considered when interpreting the effects of tetrahydrobiopterin treatment and the correlation of tetrahydrobiopterin biosynthesis with tetrahydrobiopterin-dependent activities in mammalian tissues. It remains to be determined to what extent tetrahydrobiopterin deficiency-related symptoms in mice (17, 18) and humans (19) are caused by the impairment of glyceryl ether monooxgenase activity.

This work was supported by the Austrian Research Funds, zur Förderung der wissenschaftlichen Forschung Project 19764. The authors are indebted to Petra Loitzl, Nina Madl, and Renate Kaus for expert technical assistance. The authors thank Beat Thöny (University Children's Hospital, Zürich, Switzerland) for helpful discussions and Raphael A. Zoeller (Department of Physiology and Biophysics, Boston University School of Medicine) for his kind gift of 1-*O*-[9′-(1″-pyrenyl)]nonyl-*sn*-glycerol.

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