Factors Determining the Intrinsic Lymphatic Partition Rate of Epitiostanol and Mepitiostane

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Substitution of the steroid epitiostanol (EP) at position 17 with methoxycyclopentane yields the extremely lipophilic mepitiostane (MP) with preferential partitioning into the lymph. Most of the MP in the lymph was associated with the core lipids of chylomicrons and very low-density lipoproteins (VLDL), as was also the case for EP. However, the dialysis velocity of EP and MP from lymph to plasma differed greatly; EP, but not MP, was transferred from the lymph to the plasma. This difference was attributed to differences in their unbound fraction in the lymph. Lymphatic transfer and the log*P* value of several tested steroids correlated well. Therefore, the oral EP prodrug, MP, partitioned into the lymph because of its superlipophilicity and resultant retention in the core lipids of chylomicrons and VLDL.

KEY WORDS: mepitiostane; epitiostanol; intrinsic lymphatic partition rate; lipophilicity; chylomicron.

INTRODUCTION

The intestinal lymphatics play an important role in the absorption of nutrients such as triglycerides (1,2), cholesterol (3,4), long-chain fatty acids (5,6), and lipid-soluble vitamins (7,8). The lymphatic transport of drugs and other substances is less well defined, although it has been shown that DDT (9,10), Sudan blue (11), naftifine (12), and mepitiostane (13) are partially absorbed via the lymphatic circulation. While the factors governing the intestinal absorption of drugs have been well defined (14–16), little information is available on the factors determining lymphatic absorption. The intestinal lymphatics are of interest as a route avoiding first-pass metabolism in the liver.

We previously found (17) that following passage through the mucosal cell, 99.6% of epitiostanol (EP) is partitioned into the blood and 0.4% into the lymph, while for mepitiostane (MP), an oral prodrug of EP, 92.4% is partitioned into the lymph. The present study was designed to delineate the factors determining the difference in the intrinsic lymphatic partition ratio between EP and MP.

MATERIALS AND METHODS

Materials

[4-^{14}C]Epitiostanol (^{14}C-EP; 57.5 μCi/mg) and [4-^{14}C]mepitiostane (^{14}C-MP; 10.6 μCi/mg) were synthesized at Shionogi Research Laboratories. The radiochemical purity of the radioactive compounds was confirmed by thin-layer chromatography (TLC) to be higher than 98%. All chemicals and solvents were of analytical or reagent grade.

Animal Experiments

Female Sprague–Dawley rats (11–12 weeks; body weight, 220–280 g) were purchased from CLEA Japan, Inc., and maintained on commercial chow (CA-1 pellets, CLEA Japan, Inc.) and water ad libitum until surgery.

Collection of Lymph. The rats were anesthetized with ethyl urethane (1.4 mg/kg, s.c.) and the thoracic duct was cannulated with dilute heparin-filled polyethylene tubing (PE50, Clay-Adams) by a modification of the method of Bollman *et al.* (18). Two cannulae (PE50) were introduced into the common bile duct. Both ends of the small intestine (from the pylorus to the ileocecal valve) were ligated and a ^{14}C-MP and ^{14}C-EP sesame oil solution (1%) was dispersed in 30 vol of fresh rat bile was instilled into the lumen at a dose of 2 mg/kg. After drug administration, lymph and bile were collected in test tubes for 6 hr; the animals were secured on a warmed plate maintained at 38°C. Bile collected from other rats was instilled into the lumen, through a cannula inserted into the bile duct, at a rate of 0.6 ml/hr from 1 hr after dosing. Control fatty lymph was also collected for 6 hr after the administration of sesame oil (0.2 ml/kg) dispersed in 30 vol of bile into the intestine of rats.

Subcellular Distribution. ^{14}C-EP or ^{14}C-MP sesame oil solution (1%) dispersed in 30 vol of bile was administered into the jejunal loop (the 20 cm length from the Treitz) of rats. The rats were killed 2 hr after dosing, the jejunal loop was removed and rinsed with ice-cold saline. The mucosae were collected by scraping with a glass slide. The mucosal scrapings were homogenized in 4.5 ml of 0.3 *M* sucrose with a Teflon–glass homogenizer. The homogenates were fractionated by differential centrifugation into fractions I (precipitate at 1500*g* for 10 min, brush border plus nuclei), II (precipitate at 22,300*g* for 15 min, mitochondria), III (precipitate at 105,000*g* for 60 min, microsomes), IV (aqueous phase of the final supernatant, soluble), and V (layer of oil floating on top of the final supernatant, lipoproteins) according to the method of Hübscher *et al.* (19) with some modifications.

Lipoprotein Separation

The lymph (0.4 ml) was pipetted into 4-ml polyallomer tubes (Beckman) and layered below 3 ml of 0.195 *M* saline solution (containing 0.01% EDTA-Na_2, pH 7.4). The chylomicron and very low-density lipoprotein [Ch + VLDL] fractions were obtained by ultracentrifugation (114,000*g* for 16 hr) at 4°C using a Beckman L2-65B ultracentrifuge according to the method of Hatch and Lees (20).

For *in vitro* experiments, ^{14}C-MP or ^{14}C-EP dissolved in ethanol (0.01 ml) was added to the control fatty lymph (0.5 ml) and incubated with gentle shaking at 37°C for 30 min. Also, the lymph (0.1 ml) was added to saline (1.9 ml) or fresh rat plasma (1.9 ml) and incubated at 37°C for 5 min. The incubated lymph was subjected to the ultracentrifugation procedure described above.

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The core lipids [Ch + VLDL] were separated from the surface material by dehydration and rehydration in a rotary flash evaporator at 37°C, as described by Zilversmit (21).

Equilibrium Dialysis

^{14}C-MP or ^{14}C-EP dissolved in ethanol (0.07 ml) was added to the control fatty lymph (3.5 ml) and incubated with gentle shaking at 37°C for 30 min. The dialysis cell, which has two chambers (i.d., 25 mm; vol, 2.0 ml), was divided with Visking seamless cellulose tubing (24 Å). The lymph (1.5 ml) was put in one chamber, and the isotonic *M*/15 phosphate buffer (pH 7.4, 1.5 ml) in the other. The cell was gently shaken at 37°C for 24 hr. For experiments to determine the dialysis velocity of drugs from the lymph, the lymph was also dialyzed against fresh rat plasma. At the selected time, the total amount of drugs in both chambers was determined. The binding of drugs to the Visking membrane was negligible.

Determination of Lipophilicity

Shake Flask Method. Ten milliliters each of chloroform containing 0.2 mg of radioactive compounds and *M*/15 phosphate buffer (pH 7.4) was added to the tubes, which were agitated on a mechanical shaker for 60 min at 24°C. The separated aqueous phase and chloroform phase were analyzed.

Fragment Addition Method (*22*). The log*P* (*n*-octanol/water) value was calculated using the computer program CLOGP3 (Pomona College, Medicinal Chemistry, Claremont, CA, Version 3.33).

Analytical Procedures

Radioactivity in various samples was measured with a liquid scintillation counter (Aloka Model LSC-673); and MP and EP in lymph, blood, and other liquids were analyzed by the TLC method reported previously (17).

RESULTS AND DISCUSSION

Incorporation of MP and EP into Chylomicrons and VLDL

During absorption, MP becomes incorporated into the core lipids of chylomicrons and VLDL in the intestinal mucosal cell and the interstitial space of the intestinal mucosa and is transferred into the lymph (13,17). We examined the distribution of MP and EP to the microsomal fraction containing the endoplasmic reticulum of the intestinal mucosal cell, which is the formation site of chylomicrons (23,24), and into chylomicrons and VLDL. Table I shows the distribution of MP and EP in the subcellular fractions of the intestinal mucosa at 2 hr after administration into the jejunal loop of rats. The amount of MP in the homogenate was 20 times higher than that of EP. This seems to be due to the extensive metabolism of EP by the intestine and the accumulation of MP in the intestinal mucosal cell because of the higher lipid solubility of MP than that of EP. The subcellular distributions of MP and EP were compared as percentages of the steroids in the homogenate. Approximately 8% of the total MP of the homogenate was distributed in the microsomal fraction; for EP, this value was 29%. Although MP is incorporated into the chylomicrons and VLDL and transferred to the lymph, the distribution of MP to the microsomal fraction of the intestinal mucosal cell was not higher than that of EP.

Figure 1 shows the distribution of MP and EP between the lymph fractions. When the *in vivo* lymph obtained following the administration of ^{14}C-MP was separated by ultracentrifugation, 95% of the MP in the lymph was localized in the [Ch + VLDL] fraction. Similarly, for the *in vitro* lymph obtained by incubating ^{14}C-MP with the control fatty lymph, most of the MP in the lymph was in the [Ch + VLDL] fraction. Results from the fractionation technique described by Zilversmit (21) showed that most (97–98%) of the MP in the [Ch + VLDL] fraction was associated with the core lipids, both *in vivo* and *in vitro*. For EP, 60% of the EP in lymph was distributed in the [Ch + VLDL] fraction; the *in vivo* and *in vitro* values were similar. Furthermore, most of the EP in the [Ch + VLDL] fraction of the *in vitro* lymph was associated with the core lipids. For the *in vivo* lymph, the exact distribution to the core lipid was not determined because of the low concentration of EP in the [Ch + VLDL] fraction.

Figure 2 shows the distribution of MP and EP between the lymph fractions after the addition of lymph to saline and fresh rat plasma. When lymph containing MP was added to the saline, the distribution of the MP in the lymph was not changed. However, when the lymph was added to fresh rat plasma, the proportion of MP distributed in the [Ch +

Table I. Subcellular Distribution of MP and EP in Intestinal Mucosa 2 hr After Intrajejunal Administration (2.0 mg/kg)[a]

	MP		EP	
Fraction	μg/fraction	% of total	μg/fraction	% of total
Homogenate	36.9 ± 6.1	100	1.95 ± 0.59	100
Brush border plus nuclei	7.8 ± 2.4	21.0 ± 2.8	0.41 ± 0.09	21.4 ± 3.7
Mitochondria	1.6 ± 0.9	4.4 ± 2.9	0.32 ± 0.09	16.6 ± 4.3
Microsomes	2.8 ± 0.4	7.7 ± 2.1	0.59 ± 0.28	28.9 ± 5.9
Soluble	3.2 ± 0.5	8.6 ± 1.0	0.22 ± 0.07	11.6 ± 2.3
Lipoproteins[b]	17.5 ± 3.2	47.3 ± 2.8	0.21 ± 0.12	10.2 ± 2.9

[a] Each value represents the mean ± SD of three rats. Fractions were prepared as described under Materials and Methods.

[b] A layer of oil floated on top of the aqueous phase at 105,000*g* for 60 min.

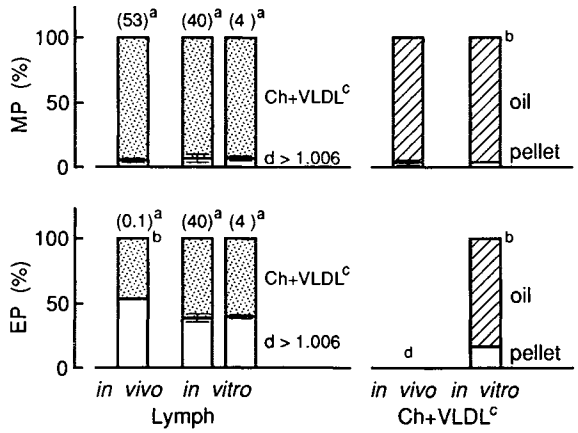

Fig. 1. Distribution of MP and EP between lymph fractions. (a) Drug concentration (μg/ml). (b) The mean of two experiments. (c) Chylomicron and VLDL fraction. (d) The values were not determined due to a low concentration of the lymph EP. The lymph *in vivo* was obtained after intraduodenal administration of ^{14}C-MP or ^{14}C-EP (2 mg/kg) dissolved in sesame oil. The lymph *in vitro* was obtained by incubation of ^{14}C-MP or ^{14}C-EP with control fatty lymph. Each value represents the mean + SD of three experiments, except in b.

VLDL] fraction decreased to 50%. Since MP is highly lipophilic, it was concentrated in the chylomicrons and VLDL when the lymph was added to the saline. However, following addition to plasma, MP was transferred rapidly from the chylomicrons and VLDL to the plasma proteins ($d > 1.006$). This phenomenon was observed both *in vivo* and *in vitro*. Similarly, most of the EP in the [Ch + VLDL] fraction was also transferred to the plasma proteins ($d > 1.006$).

These results showed that the incorporation of MP and EP into chylomicrons and VLDL is nonspecific and reversible. Most of the MP in the lymph was associated with the core lipids of chylomicrons and VLDL, as was also the case for EP. The great difference in the lymphotropic property between MP and EP could not be accounted for by the difference in the incorporation into the chylomicrons and VLDL of both steroids.

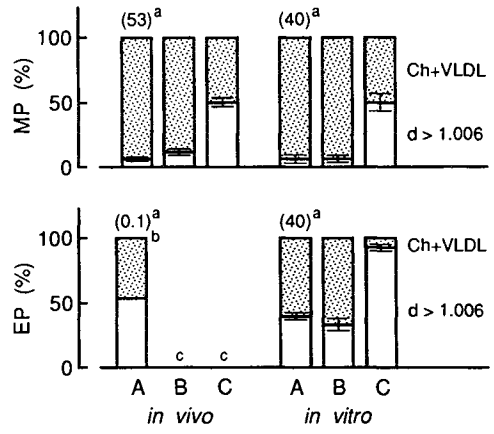

Fig. 2. Reversible incorporation of MP and EP in chylomicron and VLDL. (a) Drug concentration (μg/ml). (b) The mean of two experiments. (c) The values were not determined due to the low concentration of lymph EP. The lymph was obtained after intraduodenal administration of ^{14}C-MP or ^{14}C-EP (*in vivo*) and incubation of ^{14}C-steroids with control fatty lymph (*in vitro*). Samples consisted of lymph (A), lymph mixed with saline (B), and lymph mixed with plasma (C). Each value represents the mean ± SD of three or four experiments.

Release of MP and EP from Chylomicrons and VLDL

Chylomicrons, VLDL, and some proteins in the interstitial fluid of the lamina propria of the villus are transferred to the intestinal lymph and, finally, into the thoracic duct lymph. In general, the composition of tissue fluid is considered to be similar to that of the lymph (25). Therefore, the following experiments were undertaken using control fatty lymph instead of the interstitial fluid. Figure 3 shows the dialysis velocity of MP and EP from the control fatty lymph to fresh rat plasma. EP, but not MP, was transferred from the lymph to the plasma, indicating that, when MP and EP are present in the interstitial fluid of the lamina propria of the villus, only EP, and not MP, incorporated in the chylomicrons and VLDL is rapidly released and transferred into the blood capillaries. This difference seems to account for the different lymphotropic properties between MP and EP.

The results of equilibrium dialysis of MP and EP between the lymph and the isotonic *M*/15 phosphate buffer are shown in Table II. The fraction of unbound EP was 0.1%, but no MP was detected in the buffer. The difference in the dialysis velocity from the lymph to the plasma between MP and EP is attributed to the difference in the unbound fraction of both steroids.

Lipophilicity of MP and EP

Following jejunal administration, a large portion of the lymph MP is associated with the core lipids of the chylomicrons and VLDL (13). Also, MP is transferred into the blood in the terminal ileum and the colon, which do not secrete chylomicrons (26). Proteins such as albumin are present in the interstitial fluids of these regions. Therefore the drug retention in the core lipids of the chylomicrons and VLDL during transfer of these lipoproteins to the lymph, but not

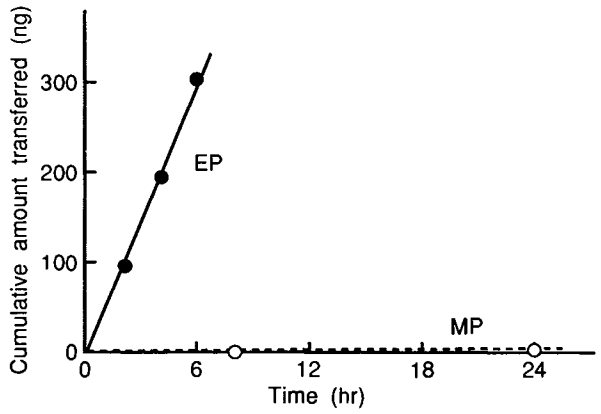

Fig. 3. Dialysis velocity of MP and EP from lymph to plasma. The lymph was obtained after incubation of radioactive compounds with control fatty lymph. The lymph was dialyzed by the use of Visking seamless cellulose tubing (24 Å) against fresh rat plasma at 37°C. Each value represents the mean of two experiments.

Table II. Equilibrium Dialysis of MP and EP Between Lymph and Phosphate Buffer

Compound	No.	Initial (μg/ml)		24 hr (μg/ml)		
		Lymph	Buffer	Lymph	Buffer	Free (%)
MP	1	38.6	0	36.1	ND[a]	$<10^{-3}$
	2	38.6	0	36.2	ND[a]	$<10^{-3}$
	3	9.9	0	9.4	ND[a]	$<10^{-3}$
	4	9.9	0	9.4	ND[a]	$<10^{-3}$
EP	1	39.7	0	37.2	0.023	0.062
	2	39.7	0	37.1	0.023	0.062
	3	7.0	0	6.9	0.0036	0.052
	4	7.0	0	6.8	0.0034	0.050

[a] Not detectable. The lymph was obtained after incubation of radioactive compounds with control fatty lymph and was dialyzed by the use of Visking seamless cellulose tubing (24 Å) against isotonic *M*/15 phosphate buffer (pH 7.4) for 24 hr at 37°C.

protein binding, is essential for the partition to the lymphatics. Consequently, the lipid solubilities of MP and EP were examined (Table III). The chloroform/buffer partition coefficient was 8200 for testosterone and $>10^4$ for EP, MP, and cholesterol. The lipid solubilities of the steroids tested were too high to determine the partition coefficients accurately, but the results are important. Hogben *et al.* (15) have shown that the rate and extent of absorption of weakly acidic and basic drugs from the intestine in rats parallel the lipid/water partition coefficients of their nonionized forms. The absorption of thiopental, a highly lipophilic compound, was the greatest among the compounds tested, yet the chloroform/buffer partition coefficients of EP and MP were 100-fold greater than the partition coefficient (about 300) of thiopental. Thus, the lipophilicity range determining the partition of drugs between blood and lymph is much higher than the lipophilicity related to the membrane permeability.

The log*P* (*n*-octanol/water) value of EP calculated by the fragment addition method was 4.36 and, thus, greater than that of testosterone, 3.35, which is absorbed via the portal system. At this level of the lipophilicity, EP was still absorbed by the portal system. The log*P* value of 5α-androst-2-en-17-one was 5.04 and it tended to be transferred to the lymph. For MP, with a log*P* value of 6.06, 92.4% was partitioned into the lymph, and the log*P* value of cholesterol, which was absorbed almost exclusively by the lymphatics, was 9.46. Thus, lymphatic transfer and the log*P* value correlated well for steroids. MP, an oral prodrug of EP, was partitioned into the lymph because the drug gained lipophilicity, enabling its partition into the lymph by substitution of EP at position 17 with the methoxycyclopentane moiety. We propose that the extremely high lipophilicity, enabling drug retention in the core lipid of chylomicrons and VLDL during the transfer of these lipoproteins into the lymph, be called "superlipophilicity," to discriminate it from the lipophilicity related to membrane permeability.

Table III. Relationship of Lymphatic Transfer and Lipid Solubility

Compound	Lipid solubility		
	Partition coefficient[a]	log*P*[b]	ILPR[c]
Testosterone	8.2×10^3	3.349	Portal[d]
EP	$>10^4$	4.358	0.4 ± 0.1[f]
KO[e]	—	5.036	6.4 ± 0.8[f]
MP	$>10^4$	6.060	92.4 ± 1.9[f]
Cholesterol	$>10^4$	9.460	Lymph[d]

[a] $CHCl_3$/1/15 *M* phosphate buffer (pH 7.4), 24°C, mean ($n = 2$).
[b] The log*P* value was calculated using the program CLOGP3 (Pomona College, Claremont, CO).
[c] Intrinsic lymphatic partition rate: $[D_{lymph}/(D_{lymph} + D_{blood})] \times 100$ (%).
[d] From Ref. 9, p. 265.
[e] 5α-Androst-2-en-17-one.
[f] Results cited from a previous paper (22).

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