Synthesis of phosphatidylethanolamine and ethanolamine plasmalogen by the CDP-ethanolamine and decarboxylase pathways in rat heart, kidney and liver

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INTRODUCTION

Ethanolamineglycerophospholipids (EGP) make up about 20–30% of the total phospholipids in most mammalian tissues and exist in two major subclasses, the diacyl form, phosphatidylethanolamine (PE), and the alkenylacyl form, ethanolamine plasmalogen. A third subclass, the alkylacyl form, exists in very small quantities in most tissues. The synthesis of EGP can occur via (a) the CDP-ethanolamine pathway, (b) the decarboxylation of serineglycerophospholipids (SGP) and (c) a Ca2+-dependent base exchange of ethanolamine with pre-existing phospholipids [1]. Synthesis of ethanolamine plasmalogen by the decarboxylation pathway could occur as a consequence of direct decarboxylation of serine plasmalogen or decarboxylation of 1-alkyl-2-acyl-sn-glycero-3-phosphoserine, followed by desaturation of the C-1 alkyl group. The decarboxylation of SGP to EGP, if followed by base exchange with serine [2], would release ethanolamine that may be utilized for EGP biosynthesis via the CDP-ethanolamine pathway. Thus extracellular serine could indirectly completely satisfy the cellular ethanolamine requirement for EGP synthesis. Although the CDP-ethanolamine pathway has hitherto been thought to be the major pathway for the synthesis of EGP, recent studies with a number of cell lines [3–5] concluded that the decarboxylation of phosphatidylserine (PS) was the sole route for PE synthesis. Such results led to the suggestion that mammalian tissues may derive all their PE exclusively through the decarboxylation of PS and also that the physiological significance of the CDP-ethanolamine pathway was the synthesis of ethanolamine plasmalogens [4]. In a subsequent study using hepatocytes, the decarboxylation pathway was reported to be less significant than the CDP-ethanolamine pathway for the synthesis of PE [6]. The conflicting conclusions between the latter and previous studies were ascribed to inherent differences between continuous and primary cell lines used in the various studies [6]. However, this rationalization cannot be reconciled with an earlier study, also utilizing hepatocytes, which implicated the PS decarboxylase pathway as being quantitatively more significant than the CDP-ethanolamine pathway in PE synthesis [7]. Although there is no doubt that the CDP-ethanolamine pathway exists in mammalian tissues, because most studies on the synthesis of EGP have not distinguished between ethanolamine plasmalogens and PE [8–10], the relative significance of the pathway in the synthesis of the two subclasses in individual tissues has not been clarified. In this study we have examined the synthesis of ethanolamine plasmalogen and PE via the CDP-ethanolamine and decarboxylation pathways in vivo in three rat tissues with different ethanolamine plasmalogen contents.

MATERIALS AND METHODS

Materials

[1,2-14C]Ethanolamine hydrochloride and the scintillant Ecolume were obtained from ICN. l-[3-3H]Serine, 1-acyl-2-[14C]arachidonoyl-glycerophosphoethanolamine and 1-stearoyl-2-[[14C]arachidonoyl-glycerophosphocholine were purchased from Amersham. Adenosyl-l-[methyl-3H]methionine was obtained from Du Pont. Dimethylformamide, phosphatidylmonomethyl-ethanolamine and phosphatidyltrimethylthanolamine were obtained from Sigma. Phospholipase C (Bacillus cereus) was a
product of Boehringer Mannheim. The experimental drug MDL 29350 (2-[3,5-di-(t-butyl-4-hydroxyphenyl)thio]hexanoic acid) was generously provided by Merrell Dow Research Institute. Rats were obtained from the University of Manitoba Animal Breeding facility. Solvents, reagents (ACS grade) and chromatographic material were purchased from Baxter Canlab.

Labelling studies
Rats were weighed and anaesthetized by an intraperitoneal injection of pentobarbitol (60 mg/kg). The trachea was exposed and a tube inserted to allow breathing to occur unimpeded. The right jugular vein was subsequently cannulated. The animals were allowed 30 min to recover from the surgical manipulations before drugs or radiolabelled precursors were introduced. In experiments with MDL 29350, 800 mM stock solution of the drug was prepared as follows: 60 mg of MDL 29350 was dissolved in 50 ml of 4 M-NaOH + 50 ml of dimethylformamide + 150 ml of 1 mM-Tris/HCl, pH 8.5. Portions of the stock solution required to give a final concentration of 1 mM (assuming a blood volume of 65 ml/kg [11]) were diluted to 300 ml with saline. This working drug solution was infused for 10 min periods at a rate of 10 ml/min by using a Sage pump (model 355, Sage Instruments) followed by 10 min rest periods. Radioactive precursors were introduced 10 min after the final infusion by a single injection through the cannula, which was then flushed with three times the dead volume of saline solution. The quantities of labelled compounds used were 5 μCi of [14C]ethanolamine/387 g animal and 50 μCi of [3H]serine/330 g animal. In control experiments the animals were treated in an identical fashion, but were infused with only the vehicle solution. In experiments that did not involve infusion, the labelled precursors were injected 30 min after cannulation.

At selected times after introduction of label, the animals were killed, and the heart, kidney and liver were removed and placed in ice-cold saline. Fat and blood vessels were trimmed off, and the tissues were blotted dry and weighed. Lipids were extracted as previously described [12], and samples of the total lipid extracts were taken for radioactivity determination. The phospholipid classes were isolated on Whatman K6 t.l.c. plates activated at 110 °C for 1 h. The plates were developed in chloroform/methanol/water/acetic acid (100:75:4:7, by vol.) and the bands made visible with dichlorofluorescein. The bands were scraped and eluted by the procedure of Arvidson [13]. Separation of the phospholipids into alkylen and diacyl classes was achieved by acid hydrolysis [12]. The radioactivity associated with phospho-

subcellular fractionation
Rat liver microsomes were prepared by differential centrifugation in accordance with previously published procedures. The protein content was measured by the method of Lowry et al. [16].

PE-N-methyltransferase assay
This was done by a slight modification of the method of Pajares et al. [17]: β-mercaptoethanol was omitted from the assay, and the reaction was stopped with chloroform/methanol (2:1, v/v) and not HCl. The phospholipid products were separated on t.l.c. in the solvent system propionic acid/propan-1-ol/chloroform/water (2:3:2:1, by vol.; [18]).

Synthesis of PS
1-Stearoyl-2-[14C]arachidonoyl-glycero-phosphoserine was synthesized from 1-stearoyl-2-[14C]arachidonoyl-glycero-

phosphocholine by the procedure of Comfurius & Zwaal [19]. PS was purified by silicic acid chromatography.

Phospholipase C hydrolysis
Phospholipase C hydrolysis of SGP and EGP was carried out as described by Vance & Vance [20].

Statistics
Statistical significance of differences was assessed by comparing the means by Student’s t-test. Differences were considered to be significant if P < 0.01.

RESULTS
The EGP and SGP contents in rat heart, kidney and liver and the proportion of plasmalogen in each lipid class are shown in Table 1. The total EGP and SGP contents were in agreement with previously reported values [21,22]. With regard to the proportion of ethanolamine plasmalogen present, the heart and kidney had similar values, which were significantly greater than that in the liver. The serine plasmalogen content in all three tissues was very low, but again the lowest value was found in the liver.

Our studies utilized [14C]ethanolamine to monitor synthesis of EGP via the CDP-ethanolamine pathway, and [3H]serine to monitor synthesis via decarboxylation of SGP. In all experiments, only trace amounts of the radiolabelled precursors were introduced into the animal, in order not to perturb the concentrations of the compounds in vivo. Because EGP may be converted into cholineglycerophospholipid (CGP) via the methylation pathway, it was thought desirable to inhibit this conversion, since it could conceivably lead to a significant underestimation of the amount of label incorporated into the EGP. The incorporations of [14C]ethanolamine into the rat heart, kidney and liver EGP and

Table 1. EGP and SGP contents and subclass composition in rat heart, kidney and liver

Lipids were extracted from rat heart, kidney and liver, and the EGP and SGP were separated by t.l.c. The phospholipid concentration and alkanyl content were determined as described in the Materials and methods section. The values represent means ± s.d. of 5 different determinations each carried out in triplicate.

<table>
<thead>
<tr>
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<th>EGP (μmol/g of tissue)</th>
<th>SGP (μmol/g of tissue)</th>
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<tbody>
<tr>
<td>Heart</td>
<td>9.3 ± 1.0</td>
<td>18.1 ± 3.0</td>
</tr>
<tr>
<td>Kidney</td>
<td>7.0 ± 2.4</td>
<td>16.0 ± 1.6</td>
</tr>
<tr>
<td>Liver</td>
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<td>4.3 ± 1.3</td>
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CGP fractions were therefore monitored in preliminary experiments. The results showed that in the heart and kidney all the radioactivity was associated with the EGP fraction; however, in the liver a substantial amount of label was found with the CGP fraction. These observations are consistent with reports that the methylation pathway is only quantitatively significant in the liver [23]. Attempts were therefore made to inhibit selectively the methylation pathway without affecting the incorporation of label into the EGP fraction. The most effective drug that was identified for this purpose was MDL 29350, an experimental hypolipidaemic drug that inhibits a number of phosphatidylcholine-metabolizing enzymes in vitro [24]. MDL 29350 completely inhibited the rat microsomal PE N-methyltransferase activity at a concentration of 5 μM (Fig. 1). In order to establish if MDL 29350 was capable of inhibiting the methylation of EGP in vivo, the drug was infused into rats, followed by the introduction of [14C]ethanolamine. Animals infused with only the vehicle solution served as controls. MDL 29350 did not affect the quantity of [14C]ethanolamine incorporated in the three tissues examined (results not shown for heart and kidney), and neither did it affect the distribution of label into the PE and ethanolamine plasmalogen fractions. In the liver, where an active methylation pathway is present, MDL 29350 did not inhibit the uptake of the labelled precursor, but clearly inhibited the conversion of EGP into CGP by the methylation pathway (Table 2). No differences in the distribution of [14C]ethanolamine between PE and ethanolamine plasmalogen were found in the control and experimental animals, indicating that the drug did not affect the incorporation of the label into either subclass. The results of the two groups were therefore combined and are reported together below.

Table 3. Specific radioactivity of PE and ethanolamine plasmalogen in rat heart, kidney and liver after injection of [14C]ethanolamine

[14C]Ethanolamine was introduced into rats via a cannula in the jugular vein as described in the Materials and methods section. At selected times after the introduction of label, the heart, liver and kidney were removed and the lipids extracted. The EGP fraction was purified by t.l.c. and extracted from the plates. The quantity of radioactivity associated with the PE and ethanolamine plasmalogen fractions was obtained by selective hydrolysis of the alkenyl bond of the plasmalogen, followed by separation of the hydrolysis products by t.l.c. The results are means ± S.D. for 8–10 (n) rats. Each analysis was carried out in triplicate: *P < 0.005.
tions were also determined, and the specific radioactivities of the subclass were computed (Table 3). At all the experimental times examined, the specific radioactivity of the kidney ethanolamine plasmalogen fraction was greater than that of the PE fraction. In the heart, the specific radioactivity of the ethanolamine plasmalogen fraction was similar to that of the PE fraction. The liver was the only tissue in which the specific radioactivity of PE was greater than its plasmalogen counterpart.

Similar experiments to those described above were conducted with [3H]serine to monitor the synthesis of EGP subclasses by the decarboxylation of SGP. Because serine is incorporated into the diradylglycerol moiety of phospholipids [8], a distinction was made between the radioactivity associated with the base and those associated with the diradylglycerol moiety. In the heart, kidney and liver 92%, 95% and 93% respectively of the [3H]serine in the SGP class was associated with the base at all the times examined. Analysis of the heart and kidney EGP revealed that 91% and 88% of label respectively was associated with the base at all times. In the liver, however, values ranging from 53 to 75% were obtained for different tissues. All the experimental results with [3H]serine reported below represent the radioactivity present in the base of each phospholipid.

[3H]Serine was incorporated into SGP, EGP and CGP in all three tissues. In the presence of MDL 29350 no differences were observed between the experimental and control animals in the quantity of label incorporated into heart and kidney EGP, SGP and CGP. Since we had previously established the absence of methylation in the heart and kidney, and also that MDL 29350 inhibits the methylation pathway, the label associated with CGP in these tissues could not be attributed to the methylation pathway, and probably results from recycling of the labelled groups in serine via the C-1 carbon pathway. In the liver MDL 29350 caused a drastic decrease in label associated with the CGP fraction (63-80% less than that of control animals), but no differences were observed in the label incorporated into the SGP and EGP fractions of animals infused with or without the drug. Further experiments were therefore conducted without MDL 29350.

The quantity of [3H]serine incorporated into the SGP and EGP fractions isolated from rat heart, kidney and liver at each experimental time was determined and expressed as the amount of label incorporated/g of tissue (results not shown). As might be expected, more label was found in the SGP fraction than the EGP fraction in all three tissues. Serine incorporation into SGP was highest in the kidney, followed by the liver and the heart. There was less variation in the amount of label associated with the EGP fraction. The quantity of label associated with the ethanolamine and serine plasmalogen fractions were also determined at each experimental time. In all three tissues, very little label was associated with the plasmalogen fraction, and furthermore, no significant differences were observed in the quantity of label incorporated into the plasmalogen fractions at different times (results not shown). When 1-acyl-2-[14C]lactidglycerophosphoethanolamine and 1-stearylgliceridglycerophosphoserine were subjected to acid hydrolysis, 1.1 ± 0.4% of the total radioactivity was associated with the hydrolytic product. This value did not differ significantly from those obtained for the SGP and EGP fractions isolated from the animals at the different experimental times. We therefore conclude that [3H]serine was not incorporated into the serine and ethanolamine plasmalogen fractions in the three tissues within the time frame of our experiments. Table 4 shows the specific radioactivity of the PE and PS in the three rat tissues. The specific radioactivity of the PS isolated from the heart and kidney was significantly higher than that isolated from the liver at all times, but the specific radioactivity of the liver PE was comparable with that for the kidney and greater than that of PE isolated from the heart.

**DISCUSSION**

The results obtained in this study demonstrate that in rat heart, kidney, and liver both PE and ethanolamine plasmalogen are synthesized by the CDP-ethanolamine pathway, and therefore these tissues do not derive PE exclusively from decarboxylation of PS, as has been suggested [4]. Our results corroborate and extend the studies with freshly isolated hepatocytes [6]. The lack of incorporation of [3H]serine into ethanolamine plasmalogens suggests that, up to 2 h after the introduction of label, the decarboxylation of SGP did not contribute directly to the synthesis of ethanolamine plasmalogens, or indirectly by providing ethanolamine that was utilized for plasmalogen synthesis via the CDP-ethanolamine pathway. These conclusions are similar to those obtained for human Y79 retinoblastoma cells [25], where the incubations with labelled precursors were for as long as 48 h. In view of the very minor contribution of the base-exchange reactions to the synthesis of EGP [26-28], the CDP-ethanolamine pathway can be regarded as the major route for the synthesis of ethanolamine plasmalogens in these tissues, with little contribution by the decarboxylation pathway. These conclusions may not be applicable to all tissues, as there is a report that in C-6 glioma cells both ethanolamine and serine are precursors of ethanolamine plasmalogen [29]. The present study has also demonstrated that serine plasmalogen is not synthesized by base-exchange reactions in the three tissues. Although there was rapid incorporation of serine into PS, there was no incorporation of [3H]serine into serine plasmalogens. A similar conclusion was obtained with cultured rat brain cells [30].

Owing to expected differences in the pool sizes of the phospho-

### Table 4. Specific radioactivity of PS and PE of rat heart, kidney and liver after injection of [3H]serine

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>PS (d.p.m./μmol of lipid)</th>
<th>PE (d.p.m./μmol of lipid)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Heart</td>
<td>Kidney</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>372 ± 846</td>
<td>4950 ± 580</td>
</tr>
<tr>
<td>10</td>
<td>5601 ± 855</td>
<td>7152 ± 1199</td>
</tr>
<tr>
<td>15</td>
<td>6146 ± 1380</td>
<td>7083 ± 1445</td>
</tr>
<tr>
<td>30</td>
<td>8562 ± 1744</td>
<td>7046 ± 1506</td>
</tr>
<tr>
<td>60</td>
<td>11429 ± 2559</td>
<td>9691 ± 1806</td>
</tr>
<tr>
<td>90</td>
<td>12815 ± 1237</td>
<td>10367 ± 1139</td>
</tr>
<tr>
<td>100</td>
<td>13382 ± 1284</td>
<td>9075 ± 1302</td>
</tr>
</tbody>
</table>

At selected intervals after injection of [3H]serine into rats, lipids were extracted from the liver, heart and kidney, and EGP and SGP fractions were purified by t.l.c. The purified lipids were separated into diacyl and plasmalogen fractions as described in Table 3. The results are means ± s.d. for 6 rats. Each analysis was carried out in triplicate.
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lipid precursors in the different tissues, a direct comparison of the specific radioactivity of the final products in the different tissues is not appropriate. However, it is quite legitimate to compare the specific radioactivity of PE and ethanolamine plasmalogen in the same tissue, since the [14C]ethanolamine is introduced via a common precursor, CDP-ethanolamine. This of course assumes that all CDP-ethanolamine pools are equally available for the synthesis of both subclasses. In the heart and kidney, both of which have a relatively high content of plasmalogens compared with the liver, the specific radioactivity of the ethanolamine plasmalogen synthesized from [14C]ethanolamine was either similar to or greater than that of PE. The opposite observation was obtained for the liver. These results raise the possibility that the CDP-ethanolamine pathway may be preferentially utilized for the synthesis of one subclass over the other in different tissues. In the kidney, for example, this appears to be ethanolamine plasmalogen, whereas in the liver it is PE. The key enzyme that would be expected to regulate the relative synthesis of the different subclasses is ethanolaminephosphotransferase. It catalyses the committed step in EGP synthesis. Unfortunately, it is not known if a single or separate ethanolaminephosphotransferases are responsible for the synthesis of ether and diacyl EGP. This information is essential to allow any meaningful speculation on the means by which any co-ordinated regulation of the subclasses is achieved.

We can infer, from comparing the specific radioactivity of PS and PE in the tissues after [3H]serine injection (Table 4), that of the three tissues, the decarboxylation of PS to PE is most active in the liver, followed by the kidney and the heart. The very active synthesis of PE from PS in the liver may be related to the utilization of PE formed by this pathway for lipoprotein synthesis [20]. The kidney and heart are not involved in the synthesis of lipoproteins.

This study has also identified MDL 29350 as a potent inhibitor of PE N-methyltransferase activity in vivo and in vitro. Its mechanism of action is not known, but is probably different from that of other PE N-methyltransferase inhibitors, such as adenosine analogues like deaza-adenosine, which inhibit methylation by increasing cellular S-adenosylhomocysteine [23]. In studies with the drug, no differences were observed in the incorporation of label into liver EGP fraction, even though the conversion of EGP into CGP had been inhibited. This suggests that the rate of EGP synthesis may have been decreased to prevent excessive PE production. It has recently been reported that phospholipids required for lipoprotein formation are not derived from existing stores, but are synthesized and utilized as needed [31]. Because PC synthesized by the methylation pathway is selectively utilized for lipoprotein formation, inhibition of this pathway may be expected to decrease the EGP requirement. Whether the inhibition of methylation by MDL 29350 would result in decreased lipoprotein secretion, unlike inhibition by deaza-adenosine [32], is not known. Studies are needed to answer this question. The results obtained with MDL 29350 also indicate that it does not inhibit EGP-metabolizing enzymes such as ethanolaminephosphotransferase and PS decarboxylase in vivo, even though experiments in vitro with subcellular fractions indicate that at a concentration of 1 mM MDL 29350 inhibits the ethanolaminephosphotransferase and PS decarboxylase activities by 50% and 100% respectively (G. Arthur & L. Page, unpublished work).

We thank Dr. R. Y. K. Man for helping us with the surgical and infusion procedures. This study was supported by funds from the Medical Research Council (MRC). G.A. is an MRC scholar.

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Received 19 July 1990/7 September 1990; accepted 17 September 1990

Vol. 273