動物組織のリン脂質分子種組成の独立性と外的因子による制御

(研究課題番号　06672175)

平成7年度科学研究補助金　(一般研究　C)

研究成果報告書

平成　8年　3月

研究代表者　川嶋洋一
(城西大学　薬学部　教授)

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はしがき

ライフサイエンスの最近の発展のなかで、リン脂質分野の研究はすばらしい進展を続けている。以前は、リン脂質は単なる生体膜の構造体と考えられ、構造と機能との関連性が明確でなかった。その上、リン脂質は生体構成物質としては取り扱いにくい物のひとつであったため、研究対象として必ずしも好んで選ばれる存在ではなかった。

最近の研究で、リン脂質の果たす生理的役割が次第に明らかになってきた。細胞内シグナル伝達に重要な役割を果たす、イノシトールポリリン酸の前駆体はイノシトールリン脂質であり、ジアシルグリセロールの前駆体はホスファチジルコリンやイノシトールリン脂質である。また、エイコサノイドや血小板活性化因子の前駆体はホスファチジルコリンである。この他にも、リン脂質はさまざまな生理活性物質の前駆体となることが明らかにされてきた。これらに共通することは、生理活性物質はリン脂質に内蔵されて生体膜中に存在するという点である。さらに、神経組織におけるドコサヘキサエン酸に見られるように、リン脂質の構成成分として生体二分子膜の分子集合状態に影響を与えることによって生理作用を発揮すると考えられるものもある。

上述した生理活性の発現の多くはリン脂質そのもの、または、前駆体となるリン脂質の有するアシル基の組成に大きく依存することは想像に難くない。これが私達が動物の各臓器・組織がリン脂質のアシル基組成を保持する機構を解明し、その制御に介入する手段を入手すれば、外的因子によって臓器・組織の生理を調節することも可能であると予想した理由である。

本研究に対する文部省からの科学研究補助金の交付に対して感謝する次第である。

研究組織

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本研究は次の研究者の御協力を頂いて行なわれたものです。ここに御氏名を記して感謝の意を表します。

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研 究 発 表

（1） 原 著

1. Mizuguchi, H. and Kawashima, Y.:
   Alterations by clofibric acid of metabolism of phosphatidyl-ethanolamine in rat-liver.
   (submitted)

（2）口 頭 発 表

1．水口博樹，川嶋洋一，狐塚　寛：　クロフィブリン酸によるリン脂質生合成の変動．　第１１３回日本薬学会年会（大阪），１９９３年３月

2．水口博樹，川嶋洋一：　クロフィブリン酸による肝臓のリン脂質分子種組成の変動．　第１１４回日本薬学会年会（東京），１９９４年３月

研究成果

はじめに

生体膜やリポ蛋白質を構成するリン脂質には数種類のリン脂質があり、各々のリン脂質は構成要素である２つのアシル基の組合せの違いによってさらに多くの分子種に別れている。このようにして動物には２千種を超えるリン脂質分子種が存在するが、個々のリン脂質分子種に特異的な生理機能が見出された例はほとんどない。しかしながら、リン脂質の分子種組成は生体膜の物理化学的な性質を規定するし、細胞内のシグナル伝達やエイコサノイドの生成に大きく影響するので、リン脂質の分子種組成がむやみに変化しては臓器・組織の生理にとって不都合であると考えられる。事実、動物の各臓器・組織の個々のリン脂質の分子種組成はよく保持されている。このことは個々の臓器・組織の個々のリン脂質の分子種組成を保つ代謝上の保障機構が存在することを意味する。したがって、この保障機構を解明すれば、外的因子によって特定の臓器・組織の個々のリン脂質の分子種組成を人為的に操作することが可能となり、その臓器・組織の生理に介入してこれを制御することができるようになることを意味する。これが本研究を開始した理由である。

研究の構成

本研究では、まず始めに、肝臓におけるリン脂質分子種生合成を薬物によって変動させることができるかどうか、これが可能であるならば、この変動の機序はどのようなものかについて検討した［Ⅰ］。次に、腎臓のホスファチジルコリンの分子種組成の特殊性について、ホスファチジルコリンの分子種代謝の観点から追求した［Ⅱ］。最後に、肝臓でのリン脂質分子種組成の変化が末梢臓器（脳、膵臓、腎臓）にどの程度波及するか、換言すると、これらの末梢臓器の分子種組成はどの程度独立性を保っているかについて検討を加えた［Ⅲ］。

研 究 成 果

Ⅰ． 肝臓におけるリン脂質分子種組成の薬物による制御

（1）クロフィブリン酸によるラット肝臓のホスファチジルエタノールアミン生合成の変動（発表論文１）

クロフィブリン酸（４－クロロフェノキシ酢酸）は血清脂質改善薬であるクロフィブラート（４－クロロフェノキシ酢酸エチルエステル）の活性本態である。クロフィブリン酸をラットに投与すると、肝臓中のリン脂質、特に、ホスファチジルコリンとホスファチジルエタノールアミンが増加する。ホスファチジルコリンの増加はこの薬物によるホスファチジルコリンの de novo 生合成系の亢進と血液中への分泌抑制に起因することを明らかにした（Kawashima et.al., 1994b）。しかしながらクロフィブリン酸が肝臓のホスファチジルエタノールアミンを増加させる機序はいまだに不明である。クロフィブリン酸を肝臓のリン脂質の分子種組成を制御する外的因子として使用するにあたって、まず、この薬物のホスファチジルエタノールアミン増加作用の機序の解明を試みた。

クロフィブリン酸を投与すると投与量に依存して、肝臓のホスファチジルエタノールアミンは増加した。この増加率は他のリン脂質の場合より顕著であった。ホスファチジルエタノールアミンの de novo 生合成に関与する３種類の酵素に対するクロフィブリン酸の影響を調べたところ、意外にも、ホスファチジルエタノールアミン生成の律速酵素と考えられているＣＴＰ：ホスホコリンシチジリルトランスフェラーゼは著しく抑制されていた。一方、クロフィブリン酸の投与によって、ホスファチジルセリン脱炭酸酵素活性はわずかであるが有意に上昇し、ホスファチジルエタノールアミン Ｎ－メチル化酵素活性は抑制された。さらに、クロフィブリン酸はホスファチジルエタノールアミンの代謝回転を遅くすることが明らかになった。したがって、クロフィブリン酸の影響下では、肝細胞は、通常では副経路であるホスファチジルセリン経由の生合成経路（ホスファチジルコリン → ホスファチジルセリン → ホスファチジルエタノールアミン）を活性化すると同時にホスファチジルコリンへの変換とホスファチジルエタノールアミンそのものの代謝回転を抑制することによって、細胞中のホスファチジルエタノールアミン含量を増加させていることが明らかになった（図１参照）。

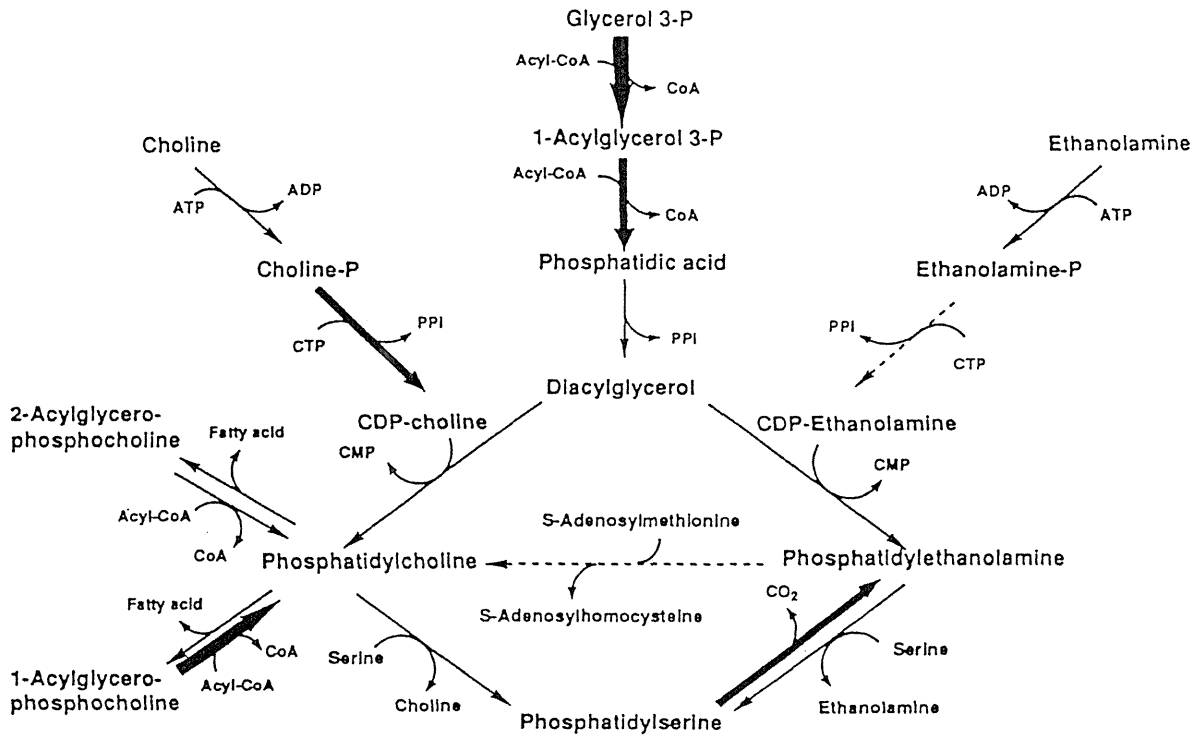

Fig. 1. Pathways phospholipid biosynthesis in rat liver

The broad arrows indicate that the activities of enzymes have been confirmed to be increased by the administration of clofibric acid to rats. The dotted arrows indicate that the activities of enzymes which have been confirmed to be depressed by the treatment of rats with clofibric acid.

（2）クロフィブリン酸による肝臓のホスファチジルコリン分子種組成の改変

クロフィブリン酸をラットに投与すると、肝臓のステアリルーＣｏＡ不飽和化酵素（△９不飽和化酵素）が誘導されてオレイン酸が増加する（Kawashima et.al., 1982）。また、リノール酸からアラキドン酸を生合成する過程に関与する３種の脂肪酸不飽和化酵素（△６、△５及び△８不飽和化酵素）もこの薬物によって誘導される（Kawashima et.al., 1990）。これらの脂肪酸不飽和化酵素の誘導に加えて、クロフィブリン酸はホスファチジルコリン生合成の活性化（Kawashima et.al.,1994b）、ホスファチジルエタノールアミン生合成の改変［Ｉ（１）］、１－アシルグリセロホスホコリンアシルトランスフェラーゼの誘導によるホスファチジルコリン再アシル化系の亢進（Kawashima et.al., 1984）を引き起こす。これらの代謝変動が組合わさった結果、肝臓のホスファチジルコリンの分子種組成はクロフィブリン酸の投与によって著しく変化した(Kawashima et.al., 1992）。肝臓のホスファチジルコリンはリポ蛋白質の成分となって血液中に分泌され、末梢組織へ運ばれてそこで利用される。したがって、肝臓のホスファチジルコリン分子種生合成に対するクロフィブリン酸の作用機序を解明することは、肝臓で起きたリン脂質分子種組成の変化が血液を介して末梢組織に伝達される過程を知る上で必須である。

クロフィブリン酸を投与すると、肝臓のホスファチジルコリンのパルミチルーオレイル（16:0-18:1)分子種が著明に増加し、ステアリルーアラキドニル（18:0-20:4）とステアリルーリノレイル（18:0-18:2)分子種は減少した。一方、ホスファチジルエタノールアミンでは、パルミチルーアラキドニル（16:0-20:4）ならびにステアリルーアラキドニル（18:0-20:4）分子種が増加した（図２）。ホスファチジルコリン分子種の生合成には３経路がある。すなわち、de novo 合成経路、再アシル化経路ならびにホスファチジルエタノールアミンのメチル化経路である（図１）。これらの３経路のホスファチジルコリン分子種の生成に対する寄与がクロフィブリン酸の投与によってどのように変わるかを検討した。

［^{3}H］グリセロールをラットに投与し、肝臓のホスファチジルコリンとホスファチジルエタノールアミンの主な分子種への in vivo でのこの標識の取り込みに対するクロフィブリン酸投与の影響を調べた（図３）。標識の取り込みの速度から判断して、de novo 合成の著しい亢進と再アシル化の亢進によってホスファチジルコリンのパルミチルーオレイル（16:0-18:1)分子種の生成が、また、再アシル化の亢進によってパルミチルーアラキドニル（16:0-20:4）分子種の生成が促進されることを示唆する結果が得られた。一方、ホスファチジルエタノールアミンでは、de novo 合成の亢進によってパルミチルーオレイル（16:0-18:1)分子種の生成が、また、再アシル化の

著しい亢進によってパルミチルーアラキドニル（16:0-20:4）およびステアリルーアラキドニル（18:0-20:4）分子種の生成が促進されているようであった。

de novo 合成によるパルミチルーオレイル（16:0-18:1）分子種の生成促進がジアシルグリセロールの取り込みに関わる酵素の基質特異性の変化によるものか、それとも基質として利用し得るパルミチルーオレイル（16:0-18:1）ジアシルグリセロールの増加によるものかを調べた。クロフィブリン酸を投与しても、肝臓のＣＤＰ－コリン：ジアシルグリセロール コリンホスホトランスフェラーゼとＣＤＰ－コリン：ジアシルグリセロール エタノールアミンホスホトランスフェラーゼの基質特異性になんら変化は認められなかった（表１および２）。一方、クロフィブリン酸の投与によって、肝臓中のパルミチルーオレイル（16:0-18:1）ジアシルグリセロールは増加し（表３）、このジアシルグリセロール分子種への in vivo での［^{3}H］グリセロールの取り込みは著しく増加した（表４）。したがって、ホスファチジルコリンとホスファチジルエタノールアミンにおけるパルミチルーオレイル（16:0-18:1）分子種の増加は主にパルミチルーオレイル（16:0-18:1）ジアシルグリセロールの供給増加に起因するものであると考えられる。

先にⅠ（１）で示したように、ホスファチジルエタノールアミンのＮ－メチル化は動物へのクロフィブリン酸投与によって抑制される。この抑制がメチル化経路で生成するホスファチジルコリン分子種の割合をどのように変化させるかを検討するために、［^{14}C］エタノールアミンをラットに投与してホスファチジルエタノールアミンを標識し、標識されたホスファチジルエタノールアミンから生じるホスファチジルコリン分子種を個々に定量した。パルミチルーオレイル（16:0-18:1）分子種以外のすべてのホスファチジルコリン分子種の生成は抑制されたが、パルミチルードコサヘキサニル（16:0-22:6）、ステアリルードコサヘキサニル（18:0-22:6）およびステアリルーアラキドニル（18:0-20:4）分子種の生成が著しく抑制された（表５、図４）。

これらのリン脂質分子種代謝の結果を総合すると、クロフィブリン酸によって引き起こされるホスファチジルコリン分子種組成の変化（図２）をかなりの程度まで説明することが可能である。

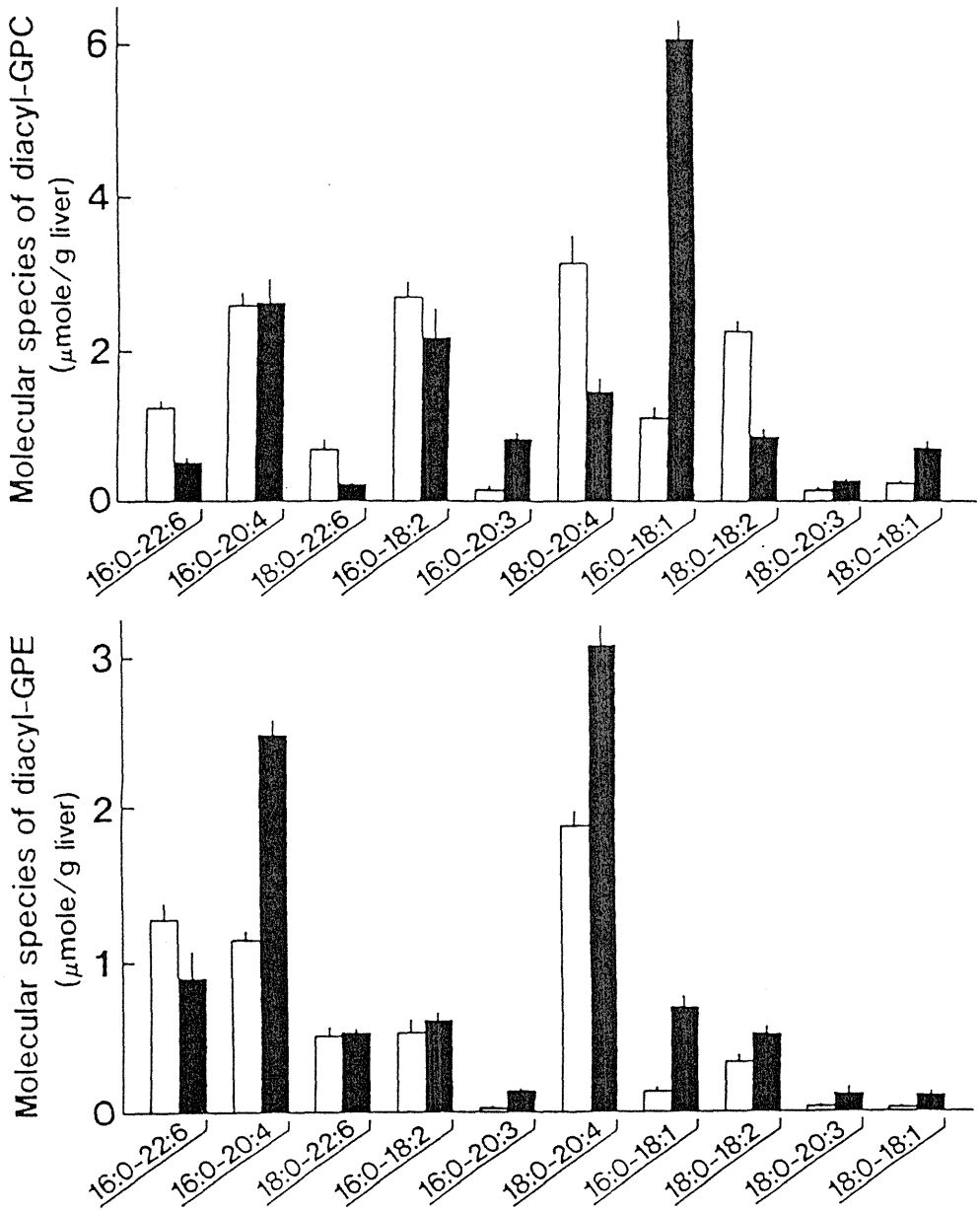

Fig. 2 Changes by clofibric acid in the amounts of molecular species of phosphatidylcholine (PtdCho) and phosphatidylethanolamine (PtdEtn) in rat liver

Rats were fed on a control diet or a diet containing 0.5% (w/w) clofibric acid for 7 days. Liver was isolated, and lipids were extracted; PtdCho and PtdEtn were separated by TLC. Molecular species of PtdCho and PtdEtn were isolated by high performance liquid chromatography. Values are mean ± S.D. for four or five rats. A, PtdCho; B, PtdEtn. □, control rats; ■, clofibric acid-fed rats.

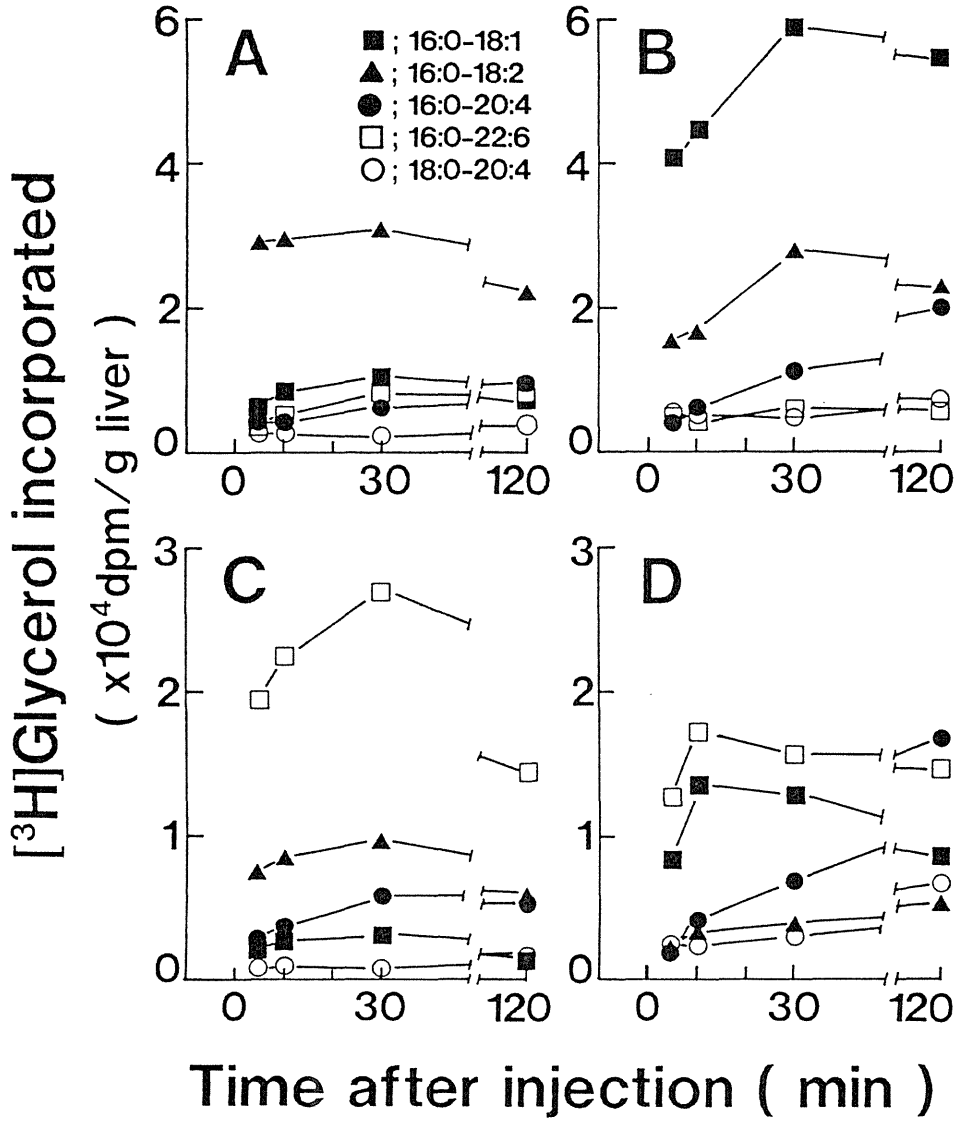

Fig. 3. Effects of clofibric acid on the *in vivo* formation of molecular species of PtdCho and PtdEtn from [³H] glycerol in liver

[³H] Glycerol was injected intraperitoneally to control rats or rats which had been fed on diet containing 0.5%(w/w) clofibric acid for 7 days. A and B, PtdCho; C and D, PtdEtn; A and C, control; B and D, clofibric acid-fed.

Table 1 Effects of clofibric acid on the substrate specificity of CDP-choline : diacylglycerol cholinephosphotransferase in rat liver

Rats were fed on a control diet or a diet containing 0.5% (w/w) clofibric acid for 7 days. CDP-choline:diacylglycerol cholinephosphotransferase in hepatic microsomes was assayed using 16:0-18:1 diacylglycerol (DG) as substrates with various ratio. Phosphatidylcholine (PtdCho) formed was isolated by high performance liquid chromatography. Values are the mean for two different preparations of microsomes.

Substrates DG molecular species	Control	Clofibric acid
	(nmoles/min/mg protein)	
16:0-18:1 (100%)	41.09 (100.0%)	32.95 (100.0%)
16:0-18:2 (0%)	0 (0%)	0 (0%)
16:0-18:1 (67%)	20.61 (55.5%)	17.43 (53.9%)
16:0-18:2 (33%)	16.54 (44.5%)	14.90 (46.1%)
16:0-18:1 (50%)	12.54 (36.0%)	11.69 (36.7%)
16:0-18:2 (50%)	22.18 (64.0%)	20.15 (63.3%)
16:0-18:1 (33%)	8.73 (23.4%)	7.76 (24.4%)
16:0-18:2 (67%)	28.62 (76.6%)	24.10 (75.6%)
16:0-18:1 (0%)	0 (0%)	0 (0%)
16:0-18:2 (100%)	35.43 (100.0%)	33.21 (100.0%)

Table 2 Effects of clofibric acid on the substrate specificity of CDP-ethanolamine:diacylglycerol ethanolaminephosphotransferase in rat liver

Rats were fed on a control diet or a diet containing 0.5% (w/w) clofibric acid for 7 days. CDP-ethanolamine:diacylglycerol ethanolaminephosphotransferase in hepatic microsomes was assayed using 16:0-18:1 diacylglycerol (DG) as substrates with various ratio. Phosphatidylethanolamine (PtdEtn) formed was isolated by high performance liquid chromatography. Values are the mean for two different preparations of microsomes.

Substrates DG molecular species	Control	Clofibric acid
	(nmoles/min/mg protein)	
16:0-18:1 (100%)	7.75 (100.0%)	7.54 (100.0%)
16:0-22:6 (0%)	0 (0%)	0 (0%)
16:0-18:1 (67%)	3.04 (21.5%)	2.90 (18.6%)
16:0-22:6 (33%)	11.05 (78.5%)	12.70 (81.4%)
16:0-18:1 (50%)	1.98 (13.5%)	1.74 (10.8%)
16:0-22:6 (50%)	12.69 (86.5%)	14.43 (89.2%)
16:0-18:1 (33%)	1.35 (8.5%)	1.18 (6.6%)
16:0-22:6 (67%)	14.47 (91.5%)	16.76 (93.4%)
16:0-18:1 (0%)	0 (0%)	0 (0%)
16:0-22:6 (100%)	15.74 (100.0%)	19.33 (100.0%)

Table 3 Effects of clofibric acid on the composition of molecular species of diacylglycerol in liver

Rats were fed on a control diet or a diet containing 0.5% (w/w) clofibric acid for 7 days. Molecularspecies of hepatic diacylglycerol were analyzed by high performance liquid chromatography. Values are the mean ± S.D. for 6 animals.

Molecular species	Control	Clofibric acid
	(nmoles/g liver)	
16:0-22:6	38 ± 2	29 ± 3
16:0-20:4	45 ± 6	57 ± 7
16:0-18:2	210 ± 29	71 ± 13
16:0-20:3	1 ± 0	14 ± 2
18:0-20:4	100 ± 19	65 ± 21
16:0-18:1	146 ± 21	346 ± 58
18:0-18:2	64 ± 11	33 ± 5
18:0-20:3	10 ± 1	10 ± 2
16:0-16:0	10 ± 6	45 ± 28
18:0-18:1	12 ± 4	23 ± 3
Total	1151 ± 94	1171 ± 106

Table 4. Effects of clofibric acid on *in vivo* incorporation of [^{3}H]glycerol into molecular species of diacylglycerol

[^{3}H] Glycerol was injected intraperitoneally to control rats or rats which had been fed on a diet containing 0.5%(w/w) clofibric acid for 7 days. Values are mean ± S.D. for three rats.

Molecular species	Control		Clofibric acid	
	5 min	10 min	5 min	10 min
	(% of radioactivity)		(% of radioactivity)	
16:0-22:6	2.79 ± 1.15	2.68 ± 0.28	0.74 ± 0.08	0.82 ± 0.47
18:2-18:2	4.87 ± 1.28	5.67 ± 1.41	0.30 ± 0.19	0.39 ± 0.20
16:0-20:4	2.39 ± 1.00	2.05 ± 0.77	1.21 ± 0.40	1.33 ± 0.54
16:0-18:2	36.22 ± 2.84	30.10 ± 3.18	5.07 ± 1.69	6.00 ± 2.23
18:0-20:4	0.35 ± 0.13	0.35 ± 0.14	0.17 ± 0.05	0.22 ± 0.21
18:1-18:1	4.49 ± 1.18	6.15 ± 0.95	7.48 ± 0.68	11.83 ± 3.37
16:0-18:1	18.26 ± 1.70	18.00 ± 1.14	60.88 ± 3.78	51.74 ± 5.31
18:0-18:2	1.49 ± 0.28	1.69 ± 0.19	1.53 ± 1.36	1.98 ± 0.68
18:0-18:1	0.55 ± 0.12	0.55 ± 0.05	0.75 ± 0.17	0.86 ± 0.12

Table 5. **Effects of clofibric acid on *in vivo* incorporation of [^{14}C]ethanolamine into molecular species of PtdEtn and PtdCho**

[^{14}C]Ethanolamine was injected intravenously to control rats or rats which had been fed on a diet containing 0.5% (w/w) clofibric acid for 7 days. At 10 min and 120 min after the injections, liver was isolated. Hepatic lipid was extracted; PtdEtn and PtdCho were separated by TLC. Molecular species of PtdEtn and PtdCho were isolated by high performance liquid chromatography and determined as lipid phosphorus. Values were the mean for two rats.

Molecular species	PtdEtn (% of radioactivity)		PtdCho (% of radioactivity)	PtdEtn (dpm/nmol)		PtdCho (dpm/nmol)
	10 min	120 min	120 min	10 min	120 min	120 min
Control						
16:0-22:6	34.52	31.09	33.93	24.35	29.11	8.09
16:0-20:4	9.29	16.16	15.83	7.60	15.62	1.41
16:0-18:2	18.55	18.22	17.47	20.29	21.98	1.58
16:0-18:1	3.54	2.71	7.22	28.03	23.80	1.01
18:0-22:6	7.10	6.34	6.02	12.30	17.72	3.30
18:0-20:4	10.14	13.59	10.38	5.24	7.85	0.76
18:0-18:2	1.53	3.26	3.91	3.80	11.86	0.70
Clofibric acid-fed						
16:0-22:6	20.43	20.88	17.44	11.14	21.07	2.04
16:0-20:4	14.03	30.92	24.40	2.30	10.17	0.68
16:0-18:2	11.07	9.27	14.19	10.84	14.05	0.49
16:0-18:1	21.48	13.71	21.24	16.24	26.40	0.33
18:0-22:6	9.26	3.29	2.37	7.95	10.88	1.29
18:0-20:4	9.26	10.07	5.86	1.64	3.55	0.34
18:0-18:2	3.37	1.49	2.39	4.66	4.87	0.29

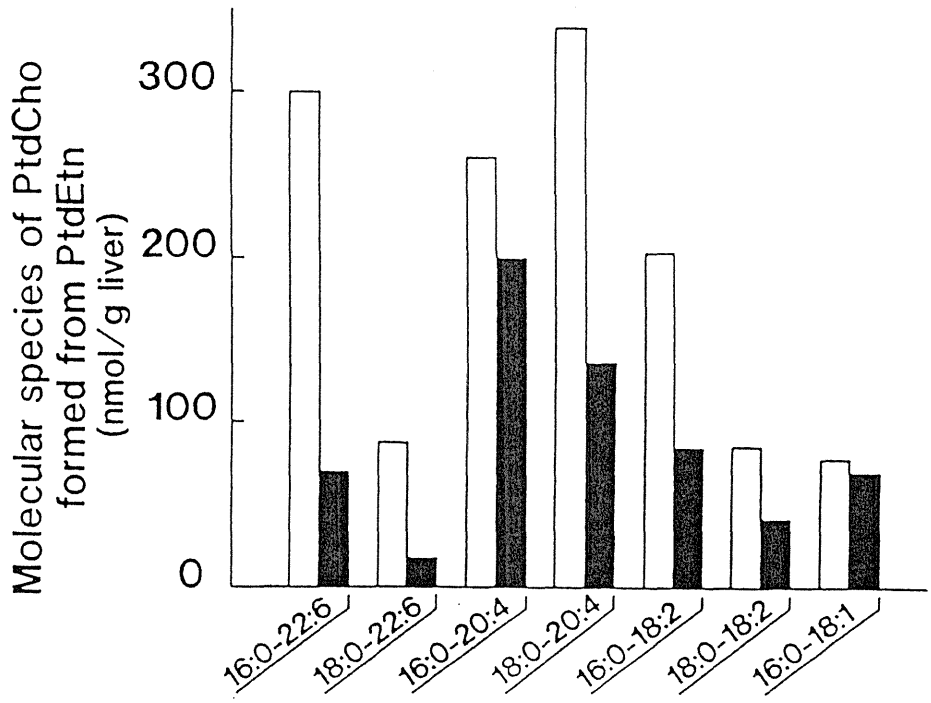

Fig. 4. Effects of clofibric acid on the formation of molecular species of PtdCho from PtdEtn

[^{14}C]Ethanolamine was injected intravenously to control rats or rats which had been fed on a diet containing 0.5% (w/w) clofibric acid for 7 days. At 120 min after the injections, liver was isolated. Hepatic lipid was extracted; PtdEtn and PtdCho were separated by TLC. Molecular species of PtdEtn and PtdCho were isolated by high performance liquid chromatography and determined as lipid phosphorus. The amounts of PtdCho formed from PtdEtn was calculated using the specific radioactivity of PtdEtn. Values are mean for two rats. □, control rats; ■, clofiblic acid-fed rats.

Ⅱ．　腎臓のホスファチジルコリン分子種組成の特殊性

ラットの腎臓は脂肪酸不飽和酵素活性がきわめて低く、脂肪酸の供給の多くを血液に頼っているので、腎臓のホスファチジルコリンの脂肪酸組成は血液中のリン脂質の脂肪酸組成の変化の影響を非常に受けやすい（Kawashima et.al., 1993）。ところが、肝臓や血液のリン脂質中にはほとんど存在しないパルミチルーパルミチル（16:0-16:0）ホスファチジルコリンが多量存在するという特殊性のあることが明らかになった（Kawashima et.al., 1993）。この特殊性が腎臓のホスファチジルコリン分子種に生じる機序について検討した。

ラット、マウスおよびモルモットについて、腎臓のホスファチジルコリンの分子種組成を調べたところ、どの動物種にもジパルミチル（16:0-16:0）分子種がかなりの割合で存在することが明らかになった（表６）。ジパルミチル（16:0-16:0）ホスファチジルコリンは肺胞のサーファクタントの主成分であるが、この分子種が肺胞サーファクタントのホスファチジルコリン中に占める割合はたかだか３０％である。さらに、この分子種の肝臓中の存在割合が０．１％に満たないことを考えると、この分子種の腎臓での含有量はきわめて高いものである。

腎臓のジパルミチル（16:0-16:0）ホスファチジルコリンが どのような代謝経路で合成されるのかを解明するために、［^{3}H］グリセロールをラットに投与して腎臓のホスファチジルコリンの各分子種への取り込みを調べた。［^{3}H］グリセロールはきわめて早い速度でジパルミチル（16:0-16:0）ホスファチジルコリンに取り込まれること、さらに、腎臓にはジパルミチル（16:0-16:0）ジアシルグリセロールが高濃度で存在すること（表７）が明らかになった。したがって、腎臓はジパルミチル（16:0-16:0）ホスファチジルコリンを de novo 合成経路によって合成しているものと考えられる。腎臓が何故にジパルミチル（16:0-16:0）ホスファチジルコリンを大量に合成して保持しているのかについては、現在、探求中である。

Table 6 Composition of molecular spceies of phosphatidylcholine in kidney of rats, mice and guinea-pigs

Molecular species	Rat	Mouse	Guinea-pig
		(mole%)	
16:0-22:6	2.05 ± 0.31	27.06 ± 0.63	0.55 ± 0.06
18:2-18:2	1.32 ± 0.22	0	2.17 ± 0.11
16:0-20:4	14.27 ± 0.95	8.27 ± 1.08	3.52 ± 0.21
18:0-22:6	0.67 ± 0.10	7.06 ± 0.46	0
18:1-18:2	2.19 ± 0.37	2.30 ± 0.08	6.49 ± 0.43
16:0-18:2	15.77 ± 0.64	14.29 ± 1.25	17.78 ± 1.78
18:0-20:4	12.27 ± 0.73	6.18 ± 0.62	5.53 ± 0.43
18:1-18:1	1.05 ± 0.45	0.50 ± 0.06	2.10 ± 0.13
16:0-18:1	14.97 ± 0.27	7.00 ± 0.72	14.55 ± 0.72
18:0-18:2	8.35 ± 0.58	6.45 ± 0.90	32.81 ± 0.64
16:0-16:0	18.56 ± 2.05	11.03 ± 0.76	4.18 ± 0.36
18:0-18:1	2.39 ± 0.11	1.35 ± 0.18	6.33 ± 0.86

Table 7 Compisition of molecular species of diacylglycerol in kidney and liver of rats

Molecular species	Kidney	Liver
	(mole%)	
18:2-18:2	4.84 ± 0.38	10.02 ± 1.78
16:0-20:4	7.81 ± 0.73	3.91 ± 0.67
18:1-18:2	5.66 ± 0.30	11.19 ± 0.82
16:0-18:2	15.07 ± 0.37	18.24 ± 1.87
18:0-20:4	19.60 ± 1.26	8.76 ± 1.87
18:1-18:1	2.34 ± 0.30	6.03 ± 1.05
16:0-18:1	16.37 ± 1.34	12.62 ± 1.04
18:0-18:2	8.43 ± 0.19	5.60 ± 1.11
16:0-16:0	9.32 ± 0.93	0.89 ± 0.53
18:0-18:1	3.16 ± 0.18	1.07 ± 0.34

Ⅲ.　末梢組織のリン脂質アシル基組成の独立性

肝臓で合成されたリン脂質、特にホスファチジルコリン、はリポ蛋白質の構成成分となって血液中へ分泌される。この分泌の過程でホスファチジルコリン分子種に対して選択が働くので、血液中のホスファチジルコリンの分子種組成は肝臓中のホスファチジルコリンの分子種組成とは幾分異なる（Kawashima et.al., 1992）。末梢組織は血液中の脂質を取り込みその脂肪酸を自分の構成要素として利用すると同時に自前である程度の脂肪酸を生合成するが、この２つの脂肪酸供給経路の寄与率については詳しくは調べられていない。腎臓が自前で脂肪酸を供給する能力はきわめて低いため（Kawashima et.al., 1993）、食餌中の脂肪酸の種類を変化させた場合やクロフィブリン酸を投与した場合には、腎臓のホスファチジルコリンのアシル基組成は肝臓と血液中の変化を反映したものに変わる（Kawashima et.al., 1994a）。この事実は腎臓のホスファチジルコリンの分子種組成は血液のホスファチジルコリンに強く依存し、臓器としての独立性は弱いことを示すものである。それでは、すべての末梢組織が腎臓のようにリン脂質のアシル基に関しては血液からの脂肪酸供給に強く依存しているのであろうか？この疑問に答えるために、無脂肪食投与、クロフィブリン酸投与ならびに糖尿病の誘発によって肝臓のホスファチジルコリンのアシル基を変化させ、この変化が血液に波及したとき、脳、膵臓および腎臓のホスファチジルコリンのアシル基はその影響をどの程度受けるかを検討した。

上述した３種類の生理条件はラットの肝臓の脂肪酸不飽和化酵素活性を大きく変動させるものであり、予想どおりに肝臓のホスファチジルコリンのアシル基組成の変化を誘発した（表８）。肝臓の変化に連動して血清リン脂質のアシル基組成も変化したが、両者のアシル基組成の間には高い相関関係のあることが明らかになった（表９）。さらに、主な不飽和脂肪酸に関しては、腎臓のホスファチジルコリンのアシル基と血清のリン脂質のアシル基組成との間には高い相関が認められた（表１０）。腎臓とは対照的に、脳のホスファチジルコリンのアシル基組成は試みたいずれの生理条件でもまったく変化せず、外部の影響を排除して厳密に保持されていた（表１１）。一方、膵臓のホスファチジルコリンのアシル基組成は外因性物質であるクロフィブリン酸の影響をまったく受けず、無脂肪食の投与と糖尿病の誘発によって変化した（表１２）。これは膵臓が脂肪酸の一部を自前で供給する能力を有していることを示唆するものと考えられる。

Table 8 Acyl composition of hepatic phosphatidylcholine of physiologically altered states of rats

Rats were divided into four experimental groups. All groups except for the third group (fat free diet) were fed on a semi-synthetic diet which contained 5% (w/w) soybean oil for 4 weeks. The rats of the second group were fed on the diet for 3 weeks and then fed on the diet containing 0.5% (w/w) clofibric acid for 1 week. The rats of the third group were fed on fat free diet which was prepared by replacing fat in the semi-synthetic diet with starch. The fourth group of rats were made diabetic by an intravenous injection of streptozotocin 3 weeks before killing. Values are the mean ± S.D.

Fatty acid	Control	Clofibric acid	Fat free diet	Diabetic
		(mole %)		
16:0	24.18 ± 1.18	32.82 ± 0.40	23.32 ± 1.14	17.66 ± 0.54
16:1	2.31 ± 0.50	2.14 ± 0.20	5.04 ± 0.33	0
18:0	20.46 ± 1.71	12.23 ± 0.65	20.68 ± 1.94	25.77 ± 0.91
18:1 n-9	9.13 ± 1.08	22.93 ± 1.66	16.36 ± 1.38	7.36 ± 0.13
18:2 n-6	10.31 ± 0.58	11.62 ± 0.84	7.04 ± 0.32	13.32 ± 1.07
20:3 n-9	0.11 ± 0.02	1.48 ± 0.41	2.61 ± 0.54	0.25 ± 0.01
20:3 n-6	1.12 ± 0.26	3.15 ± 0.25	1.53 ± 0.13	0.56 ± 0.12
20:4 n-6	25.78 ± 0.85	11.12 ± 0.94	16.18 ± 0.87	25.92 ± 1.04
20:5 n-3	0.46 ± 0.11	0.36 ± 0.02	0.38 ± 0.09	0
22:5 n-6	0.24 ± 0.04	0	0.62 ± 0.15	0
22:5 n-3	0.49 ± 0.12	0	0	0.70 ± 0.13
22:6 n-3	5.20 ± 0.43	1.08 ± 0.07	5.72 ± 0.62	7.77 ± 0.13

Table 9 Acyl composition of serum phospholipid of physiologically altered states of rats

Fatty acid	Control	Clofibric acid	Fat free diet	Diabetic	Correlation coefficient between liver and serum
			(mole %)		
16:0	23.39 ± 0.83	32.08 ± 0.75	23.68 ± 1.55	19.18 ± 0.75	
16:1	1.65 ± 0.16	2.06 ± 0.04	3.62 ± 0.26	0.37 ± 0.03	
18:0	22.73 ± 1.30	11.34 ± 0.55	23.06 ± 1.29	26.43 ± 0.79	
18:1 n-9	7.90 ± 0.25	19.75 ± 1.13	15.21 ± 0.22	5.38 ± 0.14	0.9937
18:2 n-6	14.43 ± 0.35	20.38 ± 1.41	7.79 ± 0.48	21.10 ± 1.42	0.9724
20:3 n-9	0.24 ± 0.03	1.75 ± 0.88	3.13 ± 0.70	0.19 ± 0.14	
20:3 n-6	0.99 ± 0.14	1.74 ± 0.09	1.48 ± 0.12	0.56 ± 0.07	0.9049
20:4 n-6	22.80 ± 0.55	7.81 ± 0.23	15.56 ± 0.71	20.58 ± 1.01	0.9666
20:5 n-3	0.34 ± 0.07	0.28 ± 0.17	0.33 ± 0.04	0	
22:4	0.18 ± 0.05	0	0.21 ± 0.06	0.27 ± 0.02	
22:5 n-6	0.22 ± 0.06	0	0.68 ± 0.14	0.21 ± 0.03	
22:5 n-3	0.52 ± 0.04	0	0.16 ± 0.02	0.56 ± 0.03	
22:6 n-3	3.90 ± 0.24	0.61 ± 0.20	4.29 ± 0.32	3.95 ± 0.16	0.9048

Table 10 Acyl composition of renal phosphatidylcholine of physiologically altered states of rats

Fatty acid	Control	Clofibric acid	Fat free diet	Diabetic	Correlation coefficient between serum and kidney
			(mole %)		
16:0	35.57 ± 1.57	33.44 ± 1.25	38.91 ± 1.01	35.71 ± 1.36	
16:1	1.62 ± 0.06	3.83 ± 0.55	3.36 ± 0.11	0	
18:0	13.19 ± 0.44	10.55 ± 0.28	11.41 ± 0.35	14.32 ± 0.18	
18:1 n-9	12.22 ± 0.19	22.36 ± 1.67	17.12 ± 0.55	12.11 ± 0.33	0.9755
18:2 n-6	9.86 ± 0.34	14.45 ± 1.10	6.48 ± 0.30	15.83 ± 2.22	0.9889
20:3 n-9	0.12 ± 0.02	0.40 ± 0.19	0.87 ± 0.17	0.29 ± 0.07	
20:3 n-6	0.81 ± 0.05	1.91 ± 0.31	1.08 ± 0.06	0.94 ± 0.26	0.7670
20:4 n-6	24.31 ± 1.02	11.08 ± 0.81	18.40 ± 0.18	18.43 ± 1.61	0.9369
20:5 n-3	0.20 ± 0.05	0.40 ± 0.02	0.43 ± 0.02	0.04 ± 0.07	
22:6 n-3	1.61 ± 0.20	0.73 ± 0.21	1.69 ± 0.03	2.05 ± 0.67	0.9272

Table 11 Acyl composition of brain phosphatidylcholine of physiologically altered states of rats

Fatty acid	Control	Clofibric acid	Fat free diet	Diabetic
		(mole %)		
16:0	45.02 ± 1.99	46.29 ± 1.10	46.42 ± 1.63	44.86 ± 1.94
16:1	0.81 ± 0.04	0.81 ± 0.02	0.93 ± 0.05	0.70 ± 0.02
18:0	13.06 ± 0.15	12.53 ± 0.19	12.77 ± 0.13	12.96 ± 0.27
18:1 n-9	28.21 ± 0.86	28.12 ± 0.66	28.32 ± 0.26	26.90 ± 0.68
18:2 n-6	0.76 ± 0.06	0.72 ± 0.06	0.39 ± 0.03	2.01 ± 0.36
20:0	0.23 ± 0.01	0.23 ± 0.03	0.21 ± 0.02	0.22 ± 0.01
20:1 n-9	1.15 ± 0.03	1.28 ± 0.25	1.10 ± 0.07	0.97 ± 0.06
20:1 n-7	0.48 ± 0.02	0.33 ± 0.22	0.47 ± 0.03	0.37 ± 0.03
20:3 n-6	0.18 ± 0.01	0.22 ± 0.02	0.16 ± 0.01	0.27 ± 0.02
20:4 n-6	5.24 ± 0.60	4.94 ± 0.23	4.76 ± 0.25	5.54 ± 0.52
22:0	0.22 ± 0.02	0.20 ± 0.02	0.20 ± 0.01	0.21 ± 0.00
20:5 n-3	0	0	0	0
22:4	0.65 ± 0.11	0.57 ± 0.04	0.50 ± 0.03	0.54 ± 0.08
22:5 n-6	0.07 ± 0.01	0.09 ± 0.01	0.09 ± 0.01	0.06 ± 0.01
22:5 n-3	0.29 ± 0.02	0.28 ± 0.06	0.24 ± 0.02	0.30 ± 0.03
22:6 n-3	3.35 ± 0.43	3.11 ± 0.22	3.22 ± 0.20	3.67 ± 0.49
24:1 n-9	0.07 ± 0.01	0.04 ± 0.03	0.05 ± 0.01	0.06 ± 0.01

Table 12 Acyl composition of pancreatic phosphatidylcholine of physiologically altered states of rats

Fatty acid	Control	Clofibric acid	Fat free diet	Diabetic	Correlation coefficient between serum and pancreas
		(mole %)			
16:0	43.30 ± 0.92	45.23 ± 1.10	41.53 ± 0.98	37.87 ± 1.82	
16:1	2.48 ± 0.13	2.62 ± 0.14	6.01 ± 0.29	0.33 ± 0.03	
18:0	5.66 ± 0.22	4.01 ± 0.19	4.77 ± 0.25	9.95 ± 0.88	
18:1 n-9	10.42 ± 0.25	9.89 ± 0.34	19.84 ± 0.68	6.40 ± 0.62	0.4600
18:2 n-6	19.61 ± 0.83	18.87 ± 2.06	11.82 ± 0.94	31.62 ± 2.96	0.8009
18:3 n-6	0.84 ± 0.15	1.70 ± 0.23	0.58 ± 0.07	0.15 ± 0.05	
20:3 n-9	0.11 ± 0.02	0.28 ± 0.08	1.75 ± 0.20	0.39 ± 0.10	
20:3 n-6	0.62 ± 0.04	1.04 ± 0.09	0.78 ± 0.06	0.66 ± 0.10	0.8424
20:4 n-6	15.05 ± 1.12	14.56 ± 0.84	10.92 ± 0.89	11.37 ± 1.49	-0.1252
20:5 n-3	1.18 ± 0.09	1.19 ± 0.12	1.07 ± 0.05	0.10 0.02	
22:4	0.11 ± 0.03	0.07 ± 0.02	0.11 ± 0.02	0.24 ± 0.07	
22:5 n-6	0	0	0.11 ± 0.02	0.06 ± 0.04	
22:5 n-3	0.17 ± 0.03	0.12 ± 0.02	0.08 ± 0.02	0.27 ± 0.13	
22:6 n-3	0.28 ± 0.02	0.20 ± 0.03	0.52 ± 0.02	0.42 ± 0.12	0.7811

結　論

本研究では、以下の諸点が明らかになった。

（1）クロフィブリン酸はラット肝臓中のホスファチジルエタノールアミン含量を増加させるが、この増加はホスファチジルエタノールアミン生合成の主経路である de novo 生合成系の亢進ではなく、通常では副経路であるホスファチジルセリン（ホスファチジルコリン → ホスファチジルセリン → ホスファチジルエタノールアミン）を経由する生合成経路の亢進によるものである。

（2）クロフィブリン酸をラットに投与すると、肝臓のホスファチジルコリンとホスファチジルエタノールアミンの分子種組成が大きく変動するが、これは肝臓のホスファチジルコリン de novo 生合成の亢進、ホスファチジルエタノールアミン生合成経路の変化、肝臓で生成するジアシルグリセロール分子種の変化、ホスファチジルコリンとホスファチジルエタノールアミンの再アシル化系の亢進、ホスファチジルエタノールアミンN－メチル化系の抑制が相互に関連しあって生じた結果である。

（3）腎臓はジパルミチル（16:0-16:0）ホスファチジルコリンを多量に含むが、このリン脂質は de novo 生合成経路で生成することを示唆する結果を得た。

（4）肝臓で生じたホスファチジルコリン分子種組成の変化の影響が血液を介して末梢臓器のリン脂質のアシル基組成にどの程度波及するかを、クロフィブリン酸投与、無脂肪食投与、糖尿病ラットの腎臓、膵臓、脳のホスファチジルコリンについて調べた。脳のホスファチジルコリンのアシル基組成は厳密に保持されているが腎臓のホスファチジルコリンは独自性を有しているものの血液中の変化の影響を大きくうけ、膵臓は脳と腎臓の中間的な性質を示す結果が得られた。（ホスファチジルコリン以外のリン脂質についても既に研究成果を得ているが、データが膨大であるため本報告書ではこれらを割愛した。）

本研究の開始時に予想したように、末梢臓器のリン脂質の分子種組成の肝臓・血液のリン脂質分子種組成からの独立性の程度は臓器によってさまざまであった。脳のような独立性の高い臓器は別として多くの末梢臓器のリン脂質の分子種組成は外的因子（クロフィブリン酸または食餌の脂肪酸）によって介入が可能であり、この介入にクロフィブリン酸は有効な手段となり得ることが明らかになった。今後は、さまざまな臓器のリン脂質の分子種生成に対する外的因子による介入の具体的方法についてさらに検討を重ねて行きたいと考えている。

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本研究成果の発表論文

Alterations by Clofibric Acid of Metabolism of Phosphatidylethanolamine in Rat-liver

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(Abstract)

Metabolic changes induced by *p* -chlorophenoxyisobutyric acid (clofibric acid) in hepatic phosphatidylethanolamine (PtdEtn) were studied. The treatments of rats with clofibric acid increased hepatic concentrations of phosphatidylcholine (PtdCho), PtdEtn and phosphatidylinositol (PtdIns), but not phosphatidylserine (PtdSer). Among the phospholipids, an extent of the increase in PtdEtn was the most prominent (1.91-fold on the basis of g liver and 2.73-fold on the basis of whole liver). Of the enzymes which are involved in synthesis *de novo* of PtdEtn, the activity of CTP : phosphoethanolamine cytidylyltransferase was reduced by the administration of clofibric acid to rats. The treatments of rats with the drug significantly decreased serum concentration of free ethanolamine. Clofibric acid enhanced the activity of PtdSer decarboxylase and depressed *N*-methylation *in vivo* of PtdEtn by inhibiting *N* -methytransferase. Moreover, clofibric acid significantly depressed turnover of PtdEtn which was labeled *in vivo* with [^{3}H]glycerol. These results suggest that, under the influence with clofibric acid, hepatocytes facilitate the pathway PtdCho → PtdSer → PtdEtn and reduce turnover of PtdEtn, resulting in the expanded cellular pool of PtdEtn.

Key words phosphatidylethanolamine; metabolism; clofibric acid; peroxisome proliferator; rat liver

(Introduction)

Administration of peroxisome proliferates to rodents caused hepatomegaly[1] and proliferations of organelles including peroxisomes, mitochondria and endoplasmic reticulum.[2-6] These biological changes are considered to require an elevated supply of phospholipids which compose biological membranes. Clofibric acid, a typical peroxisome proliferator, considerably increases hepatic content of phospholipids, especially PtdCho and PtdEtn.[6] To achieve the supply of phospholipids in response to peroxisome proliferators, hepatocytes induce several enzymes which participate in formation of mono- and poly-unsaturated fatty acids[7-11] and in biosynthesis *de novo* of glycerolipid such as glycerol-3-phosphate acyltransferase[7,12,13] and 1-acylglycerophosphate acyltransferase.[14] In addition to the inductions of these enzymes, clofibric acid has been recently demonstrated to enhance synthesis *de novo* of PtdCho by increasing activity of CTP : phosphocholine cytidylyltransferase and by reducing secretion of PtdCho into circulation.[6] Although clofibric acid increased hepatocellular concentration of PtdEtn to a greater extent compared to that of PtdCho, information is lacking about the effects of the drug on metabolism of PtdEtn in liver.

In this context, the present study investigated clofibric acid-induced metabolic changes which expand cellular pool of PtdEtn in liver. To address the problems, we focused on the alterations by clofibric acid of metabolic regulation of cellular level of PtdEtn in liver and demonstrated that the drug lowered synthesis *de novo*, turnover and *N*-methylation of PtdEtn and increased activity of PtdSer decarboxylase. These results suggest that, under the influence of clofibric acid, hepatocytes increase

cellular concentration of PtdEtn by a mechanism completely different from that for PtdCho. We report the results herein.

MATERIALS AND METHODS

Materials [1(3)^{3}H]Glycerol (500 Ci/mol), L-3-phosphatidyl-L-[3-^{14}C]serine (dioleoyl) (55 Ci/mol) were purchased from Amersham (Buckinghamsire, England). [1,2-^{14}C]Ethanolamine (100 Ci/mol) and *S*-adenosyl-L-[methyl-^{14}C]methionine (47 Ci/mol) were from ICN Biochemicals (Mesa,CA,U.S.A.). Cytidine diphospho-[1,2-^{14}C]ethanolamine (45 Ci/mol) was kindly given by Dr. K. Ishidate (Medical Research Institute, Tokyo Medical and Dental University). Clofibric acid, CDP-ethanolamine, ethanolamine phosphate, phospholipase C (from *Cl. welchii*), bovine serum albumin were obtained from Sigma (St.Louis,MO,U.S.A.) ; *N*-methyl-PtdEtn, PtdCho (from egg) and PtdSer (from brain) were from Avanti Polar Lipid; *S*-adenosyl-L-methionine was from Boeringer Mannheim(Mannhein, Germany) ; CTP was from Yamasa Biochemicals (Tokyo, Japan); Tween 20 was from Wako Chemicals (Osaka, Japan) and Triton X-100 (reduced) was from Nakalai Tesque (Kyoto, Japan). Diacylglycerol was prepared enzymatically from egg PtdCho according to Wood and Snyder [15] and purified as described by Ishidate *et al.* [16] [1,2-^{14}C]Ethanolamine phosphate was prepared enzymatically from [1,2-^{14}C]ethanolamine by a procedure of Tijburg *et al.*,[17] in which ethanolamine kinase that was partially purified by ammonium sulfate precipitation according to Ishidate *et al.*[18] was employed. [1,2-^{14}C]Ethanolamine phosphate was purified by anion-exchange chromatography.[19]

Studies on metabolism *in vivo* of glycerolipids For the measurement of incorporation of [^{14}C]ethanolamine into hepatic PtdEtn and PtdCho, [^{14}C]ethanolamine was dissolved in 0.9% NaCl at the concentration of 2.23 mCi/0.2 ml. The experimental animals were male Wistar rats (160 - 200 g) which had been fed on a control diet or a diet containing 0.5% (w/w) clofibric acid for 7 days. Under light anesthesia with diethyl ether, 0.2 ml of the solution containing 2.23 mCi of [^{14}C]ethanolamine was injected into exposed right jugular vein. At the times indicated in Fig. 2, rats were decapitated and livers were excised. Blood remaining in the livers was washed out with ice-cold 0.9% NaCl. Lipids was extracted from livers by the method of Bligh and Dyer.[20]

To estimate turnover rate of PtdEtn in prolonged period up to 72 h following administration of [^{3}H]glycerol, 0.2 ml of [^{3}H]glycerol (100 mCi) was injected intraperitoneally to male Wistar rats (190 - 230 g) which had been fed on a control diet or a diet containing 0.5% (w/w) clofibric acid for 7 days. After the injections, rats were supplied with the same diets and killed at the time indicated in Fig. 3. Turnover time of PtdEtn was determined according to Wise and Elwyn .[21]

Preparation of enzyme sources Male rats were fed on the control diet or the diet that contained 0.5% (w/w) clofibric acid for 7 days. The rats were decapitated. Livers and blood were isolated. Livers were perfused with cold 0.9% NaCl and cut up into three parts. One of them and serum prepared from blood by centrifugation were stored at -80°C until the use for the analyses of lipid and free ethanolamine, respectively.

The second part of liver was homogenized in three volumes of 0.25M sucrose, 1 mM EDTA, 10 mM Tris-HCl buffer (pH 7.4), and microsoemes

and cytosol were prepared as described previously.[6] For the preparation of mitochondrial fraction, the third part of liver was homogenized in nine volumes of 0.25M sucrose, 0.1 mM EDTA, 10 mM Tris-HCl buffer (pH 7.4). The homogenates were centrifuged at 600 x g for 10 min. The resulting supernatant was centrifuged at 5000 x g for 10 min. The pellet was suspended in the original volume of the homogenizing buffer and recentrifuged under the same conditions. The resulting pellet was washed again by the same manner. The pellet obtained was resuspended in a small volume of 0.25 M sucrose, 10 mM Tris-HCl (pH 7.4) and used as mitochondrial fraction.

Enzyme assays Activity of ethanolamine kinase in cytosol was determined by the method of Ishidate *et al.*[16] using [^{14}C]ethanolamine. [^{14}C]Ethanolamine phosphate that was produced by the enzyme was isolated by thin-layer chromatography (TLC) on silica gel G plates (E.Merck,Darmstadt. Germany), which were developed with 96% ethanol : 2% NH_4OH (1:2, v/v).[24] CTP : phosphoethanolamine cytidylyltransferase in cytosol was assayed according to Sundler[22] using [^{14}C]ethanolamine phosphate ; CDP-ethanolamine was separated by TLC on silica gel G plates , which were developed with methanol : 0.5% NaCl : NH_4OH (50:50:1, v/v).[23] The regions on each plate that corresponded to CDP-ethanolamine were scraped off and transferred to vials. To the vial were added 1 ml of water and 10 ml of scintillation fluid. Activity of CDP-ethanolamine : diacylglycerol ethanolamine-phosphotransferase was measured in microcosms by the method of Ishidate *et al.*[16] using CDP-[1,2-^{14}C]ethanolamine and diacylglycerol (from egg) added in Tween-dispersion. PtdSer decarboxylase in mitochondria was assayed employing phosphatidyl-[3-^{14}C]serine according to Houweling *et al.*[24] After the

incubation, lipid was extracted[20] and PtdEtn formed was isolated by TLC according to Holub and Skeaff,[25] and the radioactivity was measured. PtdEtn *N*-methyltransferase in microsomes was assayed according to Audubert and Vance[26] using *S*-adenosyl-L-[^{14}C]methionine in the absence as well as in the presence of 1.2 mM *N*-methyl-PtdEtn. All assays were confirmed to be proportional to the time and the amounts of protein employed. The substrate concentrations employed gave maximal activities.

Analytical Procedures Serum concentrations of free ethanolamine were measured by high performance liquid chromatography according to Baba *et al.*[27] Concentrations of protein were measured by the method of Lowry *et al.*[28] with bovine serum albumin as standard. Lipid was extracted from liver by the method of Bligh and Dyer,[20] and phospholipids were separated by TLC.[25] Lipid phosphorus in scrapes of TLC plates were determined according to Rouser *et al.*[29]

Statistical analysis Analysis of variance was used to test the significance of the difference between the means. Where the difference was significant, the statistical significance of the difference between two means was determined by using a Scheffé's multiple-range test. Statistical significance between two means such as non-treated and clofibric acid-treated groups was determined by Student's *t* test. The significance of the differences in slopes between two graphical lines was tested by a null hypothesis.

RESULTS

Changes in mass of phospholipid Effects of clofibric acid on hepatic content of phospholipids were examined. Rats were fed on a diet that contained the drug at various concentrations ranging from 0.031 to 0.5 % (w/w) for 7 days. Compared on the basis of g liver, hepatic concentrations of PtdCho, PtdEtn and PtdIns significantly increased in a dose-dependent manner (Fig. 1A). The most prominent changes elicited by the drug was a greater increase in cellular concentration of PtdEtn. The dietary treatments of rats with 0.5% (w/w) clofibric acid for 7 days increased substantially a relative proportion of only PtdEtn from 23.5% to 31.3% of lipid phosphorus in liver. It is noteworthy that no significant changes were observed in hepatic concentration of PtdSer. Due to hepatomegaly produced by clofibric acid, the total mass of phospholipid in whole liver was increased. In particular, the content of PtdEtn in whole liver of rats that were treated with clofibric acid at dietary concentration of 0.5% (w/w) was 2.7-times greater than that of control.

Effects on synthesis *de novo* of PtdEtn In order to understand the metabolic alterations by which clofibric acid markedly increased the hepatic content of PtdEtn, effects of the drug on the enzymes participated in biosynthesis *de novo* of PtdEtn were examined. Activity of CTP : phosphoethanolamine cytidylyltransferase was markedly reduced by the treatments of rats with clofibric acid, whereas activities of either ethanolamine kinase or CDP-ethanolamine : diacylglycerol ethanolaminephosphotransferase were unchanged (Table 1). The *in vitro* addition of clofibric acid at the concentrations ranging from 10^{-7} to 10^{-3} M was unable to alter the activity of CTP : phosphoethanolamine cytidylyltransferase. (H. Mizuguchi and Y. Kawashima unpublished results). It should be noted that serum concentration of free

ethanolamine was reduced by 29% following the administration of clofibric acid to rats (Table 2).

Activities of PtdEtn *N*-methylation and PtdSer decarboxylation In accordance with previous findings,[6] the administration of clofibric acid to rats lowered by 20% an activity of PtdEtn *N*-methyltransferase, assayed in the presence of *N*-methyl-PtdEtn as exogenous substrate (Table 1). To confirm whether the decrease in the activity of PtdEtn *N*-methyltransferase is physiologically effective *in vivo*, [^{14}C]ethanolamine was injected intravenously to control rats and rats which had been treated with clofibric acid, and the incorporation of label into PtdCho *via* PtdEtn was measured (Fig. 2). PtdEtn was efficiently labeled with [^{14}C]ethanolamine (Fig. 2A) and the radiolabel initially present in PtdEtn shifted quantitatively to PtdCho during entire period of the time course up to 120 min (Fig. 2B). The rate of formation of radiolabeled PtdCho via PtdEtn in liver of rats treated with clofibric acid was a half that of control rats. The treatments of rats with clofibric acid elevated slightly, but significantly, the activity of PtdSer decarboxylase. The *in vitro* addition of clofibric acid at the concentrations ranging from 10^{-7} to 10^{-3} M did not stimulate the activity of this enzymes (H. Mizuguchi and Y. Kawashima unpublished results).

Effects of clofibric acid on turnover of PtdEtn [^{3}H]Glycerol was injected to rats, and the effects of clofibric acid on rate of disappearance of radioactivity from PtdEtn between 2 and 72 h after the injections were estimated (Fig. 3). Plotting specific radioactivities of PtdEtn on semilogarithmic scale revealed graphically more than one decay slope for the decay of radiolabeled PtdEtn. Turnover times of

rapidly turning-over fractions (from 2 to 10 h for control; from 2 to 24 h for clofibric acid-fed rats) were calculated from the graphically determined half-lives.[21] The turnover time for PtdEtn of clofibric acid-fed rats was approximately 2 times slower than that of control rats, the difference being significant between the two values.

DISCUSSION

Clofibric acid has three biological activities which are related to lipid metabolism, that is, proliferation of organelles, hepatomegaly and hypolipidemic action. In order to attain the proliferation of organelles and enlargement of liver, hepatocytes must be required to increase the supply of membrane phospholipids. The previous study[6] demonstrated that clofibric acid enhanced synthesis *de novo* of PtdCho without changing the rate of PtdCho turnover, so that a larger pool of PtdCho appeared in liver. These findings seem to be consistent with the widely accepted concept for metabolic regulation of cellular level of PtdCho.[30]

In contrast to considerable evidence as to regulation of PtdCho synthesis,[30] information is less available about control of PtdEtn synthesis. PtdEtn can be synthesized by three pathways, namely CDP-ethanolamine pathway, PtdSer decarboxylation and calcium-stimulated exchange of base. Although the relative contribution of each route to total amounts of PtdEtn in liver has not been established, a small portion of hepatic PtdEtn seems to be formed by the exchange of ethanolamine with the base of preexisting phospholipids.[31,32] In baby hamster kidney cells or Chinese hamster ovary mutant cells, PtdSer decarboxylation has been demonstrated to be predominating.[33,34] Compared to these

pathways, however, the contribution of synthesis *de novo via* CDP-ethanolamine pathway is considered to be more important in the PtdEtn synthesis in hepatocytes .[35] Previous studies using hepatocytes provided evidence suggesting that the reaction catalyzed by CTP : phosphoethanolamine cytidylyltransferase is a possible regulatory step in PtdEtn synthesis *de novo* as well as CDP-ethanolamine : diacylglycerol ethanolaminephosphotransferase.[35,36-38] These findings led us to anticipate stimulation by clofibric acid of CTP : phosphoethanolamine cytidylyltransferase and probably CDP-ethanolamine : diacylglycerol ethanolaminephosphotransferase as well. To our surprise, however, the present study revealed that the activity of CTP : phosphoethanolamine cytidylyltransferase was lowered by the treatments of rats with clofibric acid. Moreover, the present study showed that the administration of clofibric acid to rats decreased by 29% serum concentration of free ethanolamine. In regenerating liver of rats after partial hepatectomy, the increased concentration of free ethanolamine in serum was demonstrated to be responsible for the increased biosynthesis of PtdEtn without changing the activities of the enzymes involved in CDP-ethanolamine pathway.[22] These results may suggest the reduced formation of PtdEtn by CDP-ethanolamine pathway in liver of clofibric acid-fed rats.

Another important point addressed by the present study is that, in physiologically altered conditions induced by clofibric acid, hepatocytes reduced turnover and *N*-methylation of PtdEtn and enhanced conversion of PtdSer to PtdEtn. The previous study *in vivo* using [^{3}H]glycerol revealed that specific radioactivity of PtdSer in liver of clofibric acid-fed rats was much higher than that of control rats and that specific radioactivity

of PtdCho was much higher than that of PtdEtn in clofibric acid-treated rats.[6] Moreover, clofibric acid did not alter cellular pool of PtdSer so much as PtdEtn, PtdCho and PtdIns (Fig. 1A). PtdSer has been demonstrated to be synthesized from PtdCho by the exchange of its base with serine in liver.[32] Thus, it seems likely that PtdSer is mainly synthesized from PtdCho, but not from PtdEtn, in clofibric acid-fed rats. Accordingly, one can speculate that, under the influence with clofibric acid, hepatocytes enhance synthesis *de novo* of PtdCho which is subsequently metabolized to PtdSer, and then the conversion of PtdSer to PtdEtn is facilitated, but PtdEtn is accumulated owing to the inhibition of conversion of PtdEtn to PtdCho. Since coordination of synthesis, catabolism and interconversion among glycerophospholipids is expected to maintain a stable pool of PtdEtn, the turnover of PtdEtn for the prolonged period should be taken into consideration to anticipate cellular pool size of PtdEtn. In fact, clofibric acid slowed turnover of PtdEtn, which may lead to expand the cellular pool of hepatic PtdEtn (Fig. 3). In conclusion, the present study revealed that the increase in PtdEtn content was caused by the combined actions of the depression of PtdEtn *N*-methyltransferase activity, the elevation of PtdSer decarboxylase activity and the retardation of turnover of PtdEtn.

It has not been clarified yet the reason why hepatocytes did not stimulate synthesis *de novo* for the increased supply of PtdEtn to the proliferation of biological membranes. Treatments of Nb 2 lymphoma cells with prolactin have been shown to enhance incorporation of choline into PtdCho, which resulted from the retardation of turnover of PtdCho.[39] Accordingly, it could be speculated that the regulation of cellular concentration of phospholipids through the changes in activity of minor

pathway(s) of biosynthesis and/or of turnover is one of the ways for cells to choose for a balanced synthesis of membrane phospholipids that are required for the cells functioning. Consequently, the present results and the previous findings[6] showed that hepatocytes which were treated with clofibric acid expanded cellular pools of PtdEtn and PtdCho by evidently diverse manners.

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Figure legends

Fig. 1. Effects of clofibric acid administration on mass proportion of phospholipids in rat liver

Rats were fed on a diet containing 0, 0.031, 0.063, 0.125, 0.25 or 0.5% (w/w) clofibric acid for 7 days. Hepatic lipid was extracted and determined as described under "Methods". Values are the mean ± S.D. of four or seven separate experiments. ○, PtdCho; ■, PtdEtn; □, PtdIns; ▲, PtdSer. Significantly different from control rats: * $P<0.05$.

Fig. 2. Effects of clofibric acid administration on the formation *in vivo* of PtdCho from PtdEtn by *N*-methylation

[^{14}C]Ethanolamine was injected intravenously to control rats or rats which had been fed on a diet containing 0.5% (w/w) clofibric acid for 7 days. Livers were isolated at the indicated time, and lipid was extracted. PtdEtn and PtdCho were separated by TLC, and radioactivity and lipid phosphorus were determined. Values are the mean ± S.D. for four separate experiments. A, incorporation of [^{14}C]ethanolamine into PtdEtn. B, formation of PtdCho from [^{14}C]PtdEtn. The amounts of PtdCho formed from PtdEtn at each time point were calculated dividing the radioactivity of PtdCho (dpm/g liver) by specific radioactivity of PtdEtn (dpm/μmol). The rates of formation of PtdCho from PtdEtn were caluculated from two-lines, and the values (μmol/min/g liver) for control and clofibric acid-fed rats were 0.91 and 0.51, respectively. Open symbols and closed symbols represent control rats and clofibric acid-fed rats, respectively.

Fig. 3. Effects of clofibric acid administration on turnover of PtdEtn in liver

[^{3}H]Glycerol (100μCi) was injected intraperitoneally to control rats or rats which had been fed on a diet containing 0.5% (w/w) clofibric acid for 7 days. Rats were killed at the time indicated, and livers were isolated. Hepatic lipid was extracted and separated by TLC as described under "Methods". The lines were obtained by regression analyses. Each value represents the mean of two or seven rats; the deviation of each value was within 20% of the mean. ○, control rats; ●, clofibric acid-fed rats. The half-lives ($T_{1/2}$) of PtdEtn in rapidly turning-over fractions (from 2 to 10 hr for control rats; from 2 to 24 hr for clofibric acid-fed rats) were obtained graphically from the lines. Turnover time (T) was calculated[23] by the following equation; $T = T_{1/2} / \ln 2$. The turnover times for control and clofibric acid-fed rats were 11.15 and 20.98 h, respectively. These values were significantly different at $P < 0.05$.

Table 1. Alterations by clofibric acid in activities of enzymes which participate in biosynthesis of PtdEtn

Enzymes	Control	Clofibric acid-fed
	(nmol / min / mg protein)	
Ethanolamine kinase	1.49 ± 0.05	1.43 ± 0.10
CTP:Phosphoethanolamine cytidylyltransferase	4.93 ± 0.32	2.80 ± 0.20***
Diacylglycerol ethanolaminephosphotransferase		
Endogenous substrate	0.31 ± 0.04	0.34 ± 0.04
Exogenous substrate	8.60 ± 0.79	9.72 ± 0.36
PtdEtn N-methyltransferase		
Endogenous substrate	3.17 ± 0.10	3.25 ± 0.16
Exogenous substrate	9.36 ± 1.07	8.00 ± 0.55*
PtdSer decarboxylase	0.60 ± 0.07	0.76 ± 0.08**

Rats were fed on a control diet or a diet containing 0.5% (w/w) clofibric acid for 7 days. Values are the mean ± S.D. for four or ten rats. Significantly different from control: **P<0.01; ***P<0.001.

Table 2. Effects of clofibric acid on free ethanolamine in serum

Treatments	Serum concentration of free ethanolamine (nmol/ml serum)
Control	36.43 ± 7.84
Clofibric acid-fed	25.74 ± 4.82**

Rats were fed on a control diet or a diet containing 0.5%(w/w) clofibric acid for 7 days. Free ethanolamine in serum was determined by high performance liquid chromatography. Values represent the mean ± S.D. for sixteen or seventeen rats. **Significantly different from control at $P < 0.01$.

Fig. 1

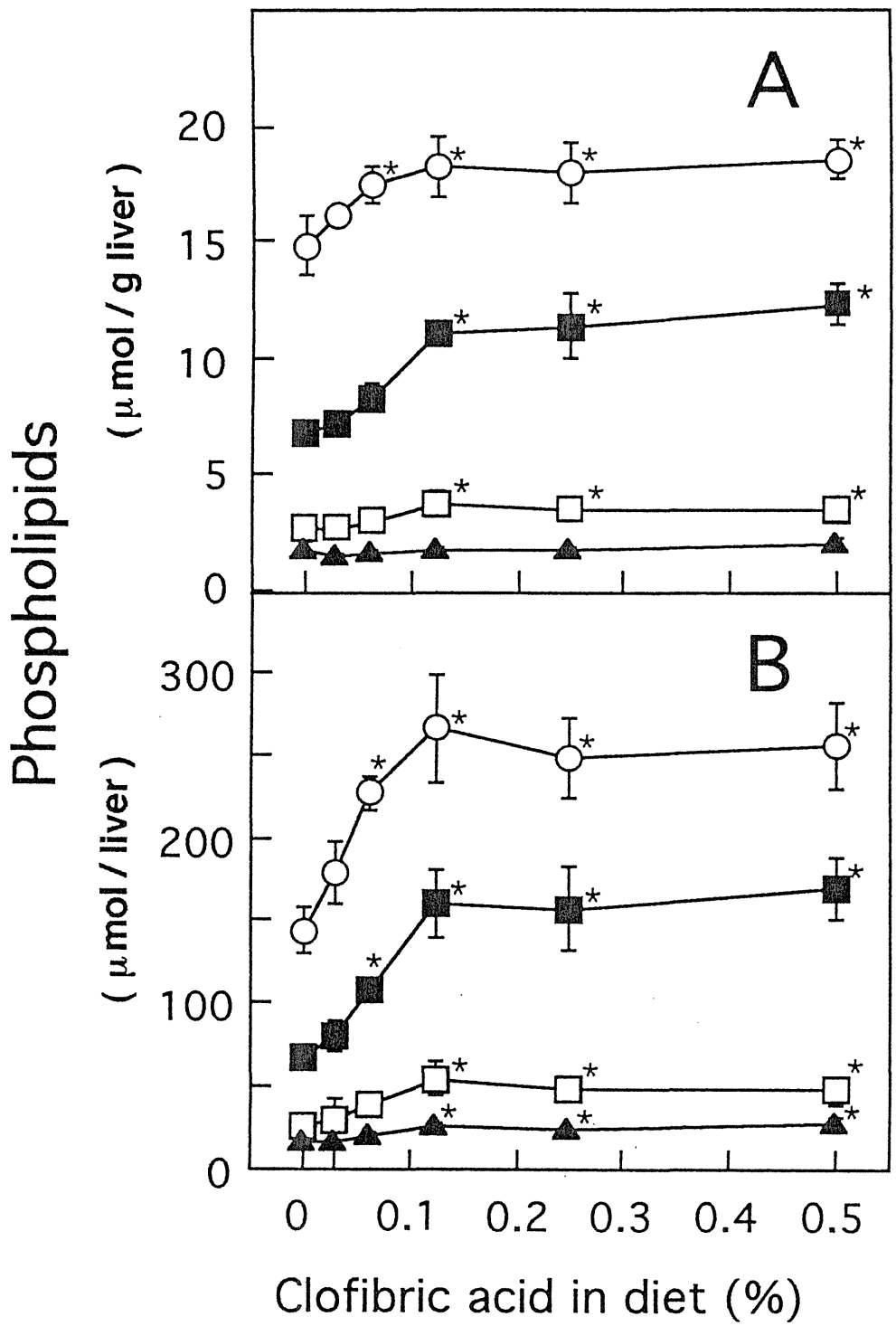

A
B
Phospholipids
(μmol / g liver)
(μmol / liver)
20
15
10
5
0
300
200
100
0
0
0.1
0.2
0.3
0.4
0.5
Clofibric acid in diet (%)

Fig. 2

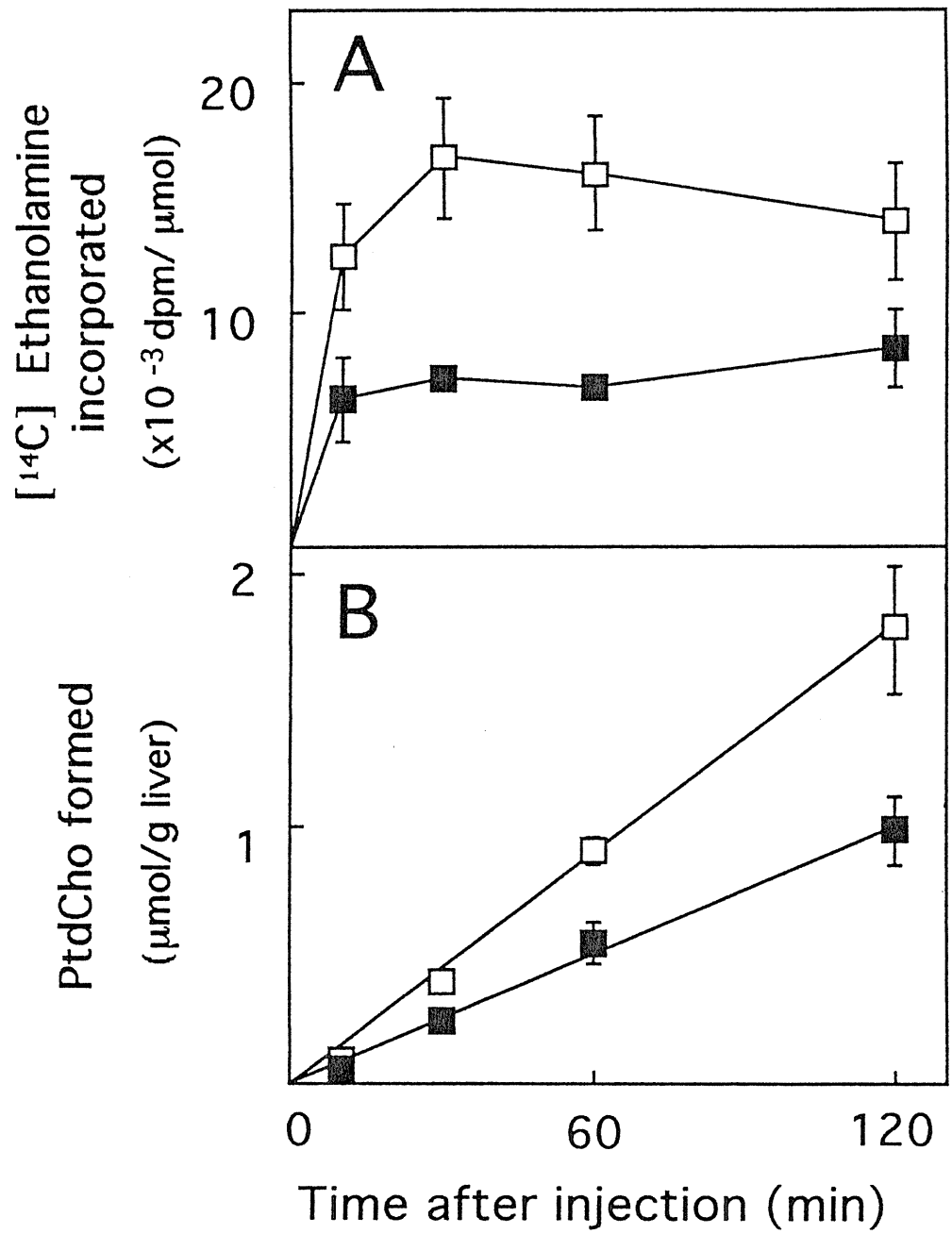
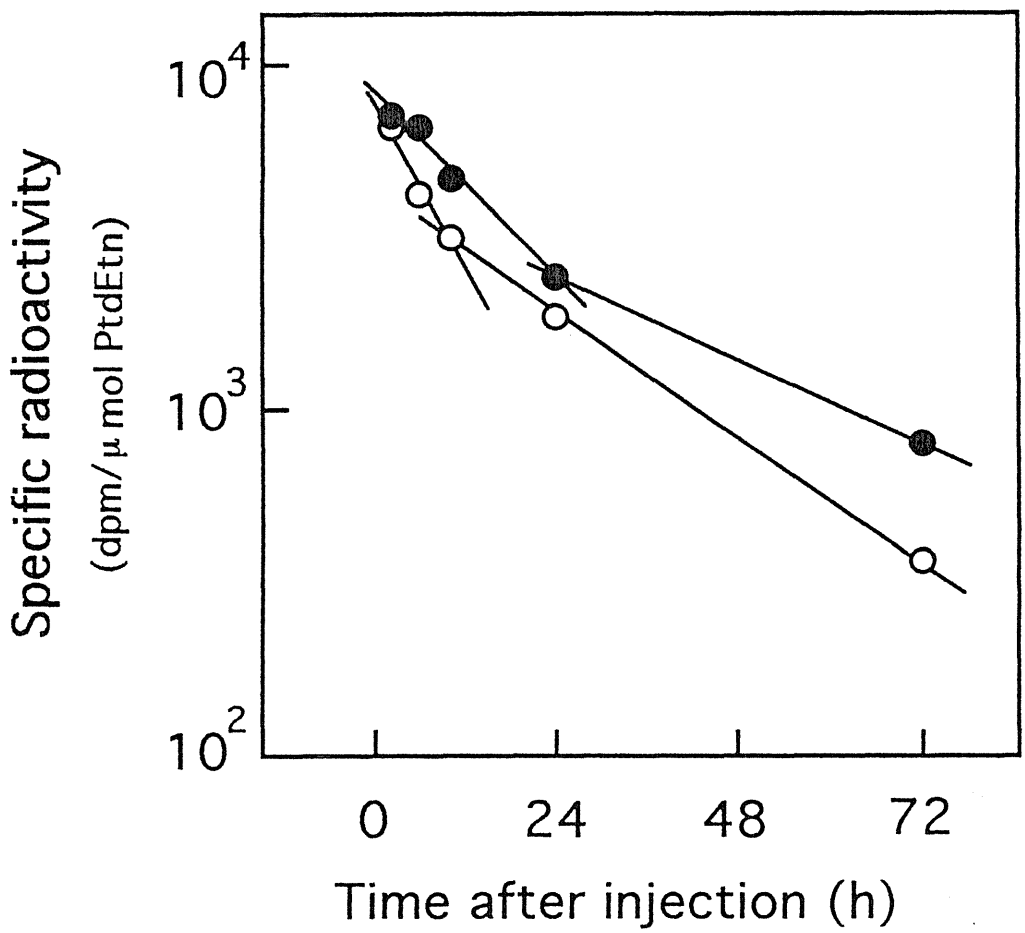

A
B
20
10
2
1
0
60
120
[14C] Ethanolamine incorporated
(x10-3 dpm/ µmol)
PtdCho formed
(µmol/g liver)
Time after injection (min)

Fig. 3


Specific radioactivity
(dpm/μ mol PtdEtn)
10^4
10^3
10^2
0
24
48
72
Time after injection (h)

背景研究の参考論文