難代謝性フッ素化脂肪酸の生体作用の多様性と作用点における分子識別機構の解明

（研究課題番号　09672247）

平成9年度～平成11年度科学研究費補助金
（基盤研究 C(2)）

研究成果報告書

平成12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

ペルフルオロ脂肪酸（PFCA）は脂肪酸の炭素結合水素がすべてフッ素に置換した化合物である。PFCAは通常の脂肪酸にはない特殊な性格をもっている。すなわち、表面張力が極めて弱いため水ばかりでなく、ほとんどの有機溶媒中でも界面活性作用を示す。また、C-F結合はC-H結合に比べて結合エネルギーが大きいため化学的にもきわめて安定である。このような性質を利用して撥水剤、撥油剤、消火剤などに広く利用されている。この化合物の生体毒性は非常に低いと考えられている。しかし、化学工場労働者の血液からPFCAの一種であるペルフルオロオクタン酸が検出されるという報告も出されている。生体に蓄積した微量の化学物質が生体に影響をあたえるという環境ホルモンの例は、目立った毒性がないからといって、蓄積性の高い化学物質を使用することの危険性を示している。

本研究代表者らはこれまでにペルフルオロ脂肪酸の生理作用について研究を重ね、これらの化合物がげっ歯類肝臓の脂質代謝や薬物代謝に広く影響を与えることを明らかにしてきた。その過程で、炭素鎖長の異なるPFCAはときとして生体への作用が異なることが明らかになってきた。このことは炭素１つの違いを生体が何らかの形で認識していることを示している。本研究ではPFCAの炭素鎖長と生体作用の関係を系統的に調べ、生体がPFCAのどのような構造を認識するのかについて知見を得ることを目的とした。さらに、生体作用の差がどのような機構で生ずるのか、すなわち、生体のPFCA分子識別の機構を解明する手がかりを得たいと考えた。本研究によって、PFCAの生体作用として脂質代謝酵素系への影響が新たに明らかになった。さらに、PFCAの生体作用の違いを統一的に理解する上で重要な知見が得られたので、これらを報告させて頂く。

本研究に対する文部省からの化学研究補助金の交付に対して深く感謝する次第である。

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Induction of hepatic triglyceride accumulation by perfluorinated fatty acids in rat liver

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Introduction

Perfluorinated fatty acids (PFCAs), in which all hidrogens are substituted by fluorine, are commercially used as lubricants, weting agents, corrision inhibitors, and fire extinguishers. The effects of PFCAs on biological systems have been studied by several investigators. When rats were treated with perfluorooctanoic acid (PFOA, a PFCA with 8 carbon atoms), hepatomegaly and peroxisomal proliferation were obsereved. The induction of various enzymes involved in lipid metabolism and drug metabolism were also reported in the livers of rats. In addition of the above effects, perfluorodecanoic acid (PFDA), a PFCA having 10 carbon atoms, caused a significant lipid accumulation in rat liver. This raises the question that the induction of hepatic lipid accumulation is a specific effect of PFDA among various PFCAs. There is a sex-related difference of the induction of peroxisomal β-oxidation by PFOA, whereas the effect of PFDA was equally observed in male and female rats. It is not known whether sex-related difference is observed in effect of PFDA on the levels of hepatic lipids. In this context, we compared the effects on helatic level of troglyceride (TG) between PFCAs having 7-10 carbon atoms in both male and female rats.

Materials and Methods

Materials

Perfluoroheptanoic acid (PFHA), perfluorooctanoic acid (PFOA) and perfluorodecanoic acid (PFDA) were purchased from Aldrich Japan (Tokyo, Japan). Perfluorononanoicacid (PFNA) was obtained from Lancastar Synthesis (Lancashire, UK). Methylheptadecanoate and triheptadecanoin was purchased from Nu-Chek-Prep Inc. (MN, USA). Testosterone propionate was purchased from Wako pure Chemicals Inc. (Osaka, Japan). All other chemicals used were of analytical grade.

Animals

Male and female Wistar rats aged 5 weeks old were purchased from SLC Inc. (Hamamatsu, Japan). After acclimatization for 1 week, rats were received intraperitoneal injections with PFHA, PFOA, PFNA or PFDA once a day for 5 days. PFCAs were dissolved into propyleneglycol:water (1:1, v/v) after neutralization with equimolar NaOH. All rats were received a dosing volume of 1 mL/ kg body weight.

Some of male rats (24-26 day-old) were castrated. Four days after castration, rats were administered with testosterone propionate dissolving into corn oil (10 mg/ kg body weight), or vehicle alone once every two days until being killed. These rats were administered with PFNA or PFDA at a dose of 20 mg / kg body weight as described above.

Twentyfour hours after final injection with PFCAs, rats were killed by decapitation under light ether anesthesia. Livers were quickly excised, perflused with

ice-cold 0.9% NaCl, and then homogenized with 9 volumes of 0.25 M sucrose/ 1 mM EDTA / 10 mM Tris-HCl (pH 7.4).

Histopathological examination

Livers were excised under light ether anesthesia from the rats which have been received intraperitoneal injections of PFDA (20 mg/ kg body weight) or vehicle alone once a day for 5 days. Liver was fixed in neutral-buffered 10% formalin. Fixed specimens were enbedded in paraffin, and their sections were stained with Hematoxylin-Eosin forlight microscopy. For discrimination of lipid droplets by Oil Red O staining, frozen sections were made with fixed specimens usinf cryostat (CM-3000, Leica, Germany)

Lipid analysis

Total lipid was extracted from liver homogenates after the addition of triheptadecanoin as an internal standard, according to the method of Bligh and Dyer. Triglyceride was separated by TLC on Silica gel G plates (Merck, Darmstadt, Germany), which were developed with hexane: diethyl ether: acetic acid (80:30:1, v/v/v). The spots corresponding to triglyceride were visualized by saraying 0.005% primulime in 80% acetone (w/v), scraped off from the plates and then transfereed to glass tubes. TG was extrcated from the silica with 5 mL of chlorofrm/ methanol/ 0.1 N HCl (4:4:1, v/v/v) twice. Added 3 mL of 0.1 N HCl to the combined extract and then lower layer was transferred to a new tube. Solvent was dried down under

nitrogen stream, 0.5 M sodium methoxide was added to the tubes to prepare fatty acid methy esters. GLC (Simadzu GC 14A) was employed for the analysis of fatty acid with Spelcowax^{TM} 10 (0.53 mm ID x 30 m).

Determination of perfluorinated fatty acids

After adding an internal standard, PFCAs were extracted from liver homogenates as an ion pair with tetrabutylammonium, derivertized with 3-bromoacetyl-7-methoxycoumarin (BrAMC) and quantified by HPLC with a fluorescent detection according to the method of Ohya, et al. (). PFHA was used as an internal standard for the determination of PFNA and PFDA, and perfluorohexanoic acid was used for the determination of PFHA and PFOA, respectively.

Statistical analysis

Anaysis of variance was used to test the significance of differences between various doses of a PFCA, between different PFCAs at a same dose, and between male and female, castrated male and testosterone –treated male rats which had been castrated. Where Differences were significsant, the statistical significance between any two means was determined using Sheffe's multiple range test. Statistical significance between male and female rats was analyzed by Sudent's t-test or Welch's F-test for two means.

Results and Discussion

Effects of PFCAs on the level of hepatic triglyceride

The level of hepatic TG was compared between the rats being treated with vehicle, PFHA, PFOA, PFNA or PFDA (Figure 1). Treatment with PFDA and PFNA increased hepatic level of TG, whereas PFHA and PFOA did not alter it in male rats. The level of hepatic TG was increased 6-fold by PFDA treatment in male rats (20 mg/ kg) on the basis of g liver. In female rats, only PFDA caused asignificant increase in hepatic level of TG. When rats were administered with PFNA or PFDA at doses over 10 mg / kg body weight, weight loss and less apetite were significantly obsereved. Hepatic TG level of the rats which were restricted in food intake to those consumed by the rats received PFDA (20 mg/ kg) was not significantly different from vehicle treated rats. Therefore, an increase in the level of hepatic TG is not due to restricted food intake in PFCA-treated rats. The accumulation of hepatic TG by PFDA and PFNA (in male rats) was observed in a dose-dependent manner (Figure 2).

Pathological findings

Light microscopy revealed that fine lipid droplets accumulated into hepatocytes, which were found diffusely entire lobule in PFDA-treated male rats (20 mg/ kg B.W., Figure 3). Occasionally, necrotic lesions were recognized focally in lobules by the staining with Hematoxylin and Eosin.

Alterations of fatty acid composition in hepatic lipids

Treatment with PFDA increased all fatty acids except for 20:5(n-3) on the basis of gram liver (Table 1). An increase in 18:1 was greater than those in other fatty acids, therefore, percentage of 18:1 was significantly increased upon PFDA treatment. In phospholipid fraction, composition of oleic acid was also increased. The specific increase in oleic acid is thought to be due to an increase in the production of oleic acid which is catalyzed vy stearoyl-coA desaturase.

Effects of hormonal status on hepatic TG accumulation by PFNA

Whether sex-related difference of PFNA-induced TG accumulation in rat liver correlate with hormonal status was determined. Castration signifcantly reduced hepatic TG level in PFNA-treated male rats, which was close to that of female rats those have been treaed with PFNA at the same condition as male. By contrast, treatment of castrated rats with testosterone restored the level of hepatic TG (Figure 4). By contrast to PFNA, there was no significant difference of hepatic TG levels between male and female rats when treated with PFDA. Castration and testosterone treatment did not affect hepatic TG level in PFDA-treated rats. We and other investigators have reported sex-related difference in the induction of peroxisomal β-oxidation by PFOA in rat liver. Such difference was not observed in the case of PFNA. Therefore, this is the first report that describes sex-related difference in the effects of PFNA in rats.

Correlation between the levels of PFCA and TG in rat liver

To explore the reason why sex-related difference was observed in hepatic TG level but not in the activity oe perxisome b-oxidation, correlation with hepatic concentrations of PFCA was examined. We have previously described that a PFCA with longer carbon chain length more accumulates in rat liver. In this context, it is possible that higher accumulation of PFCA cause higher TG accumulation in rat liver. Figure 3 demonstrates positive correlation between the levels of TG and PFCA in the liver regardless of their carbon chain length. It is noteworthy that correlation coefficient between hepaic PFCA concentration and hepatic TG level was significantly different from that between hepatic PFCA concentration and the activity of peroxisomal β-oxidation. Hepatic PFCA concentration repuired to cause a significant TG accumulation is thought to be 250 μg/ g liver. This concentration is higher than those required to induce peroxisomal β-oxidation. This suggests that PFCA caused the induction of peroxisomal β-oxidation and the accumulation of hepatic TG by independent mechanisms.

In summary, we reported that PFCA caused an increase in hepatic TG level, which was dependent on the concentration of hepatic PFCA regardless of ther carbon chain length. Sex-related difference of the effect was observed in rats. PFCA seems to cause hepatic TG accumulation by the mecanisms independent on the induction of peroxisomal β-oxidation.

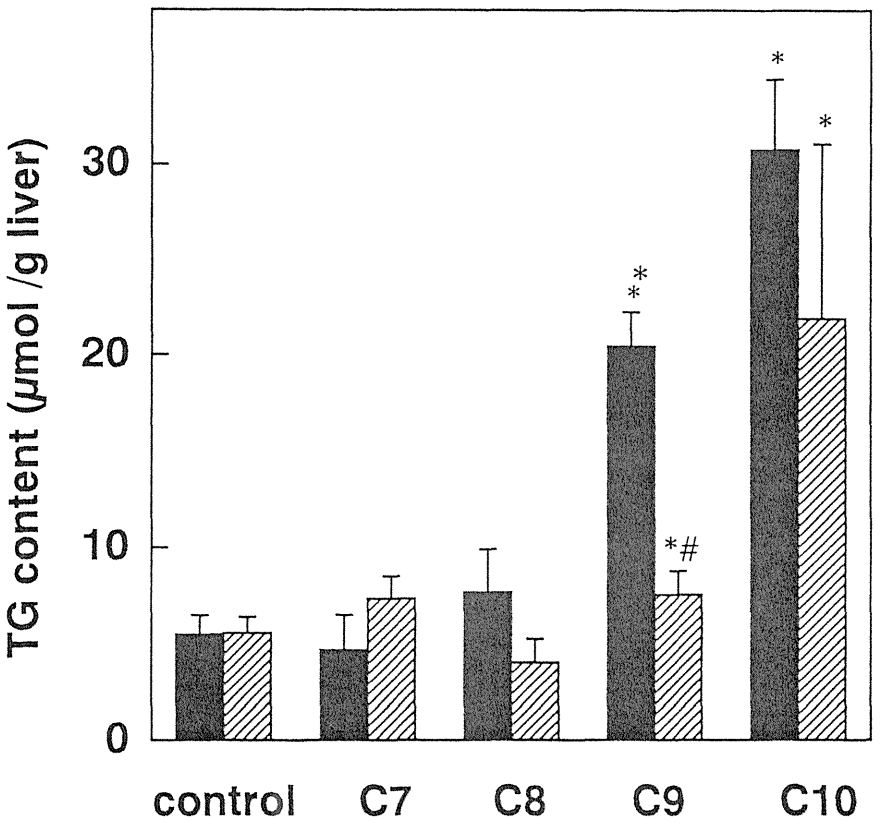


Figure 1 Effects of various PFCAs on the level of hepatic triglyceride in male and female rats

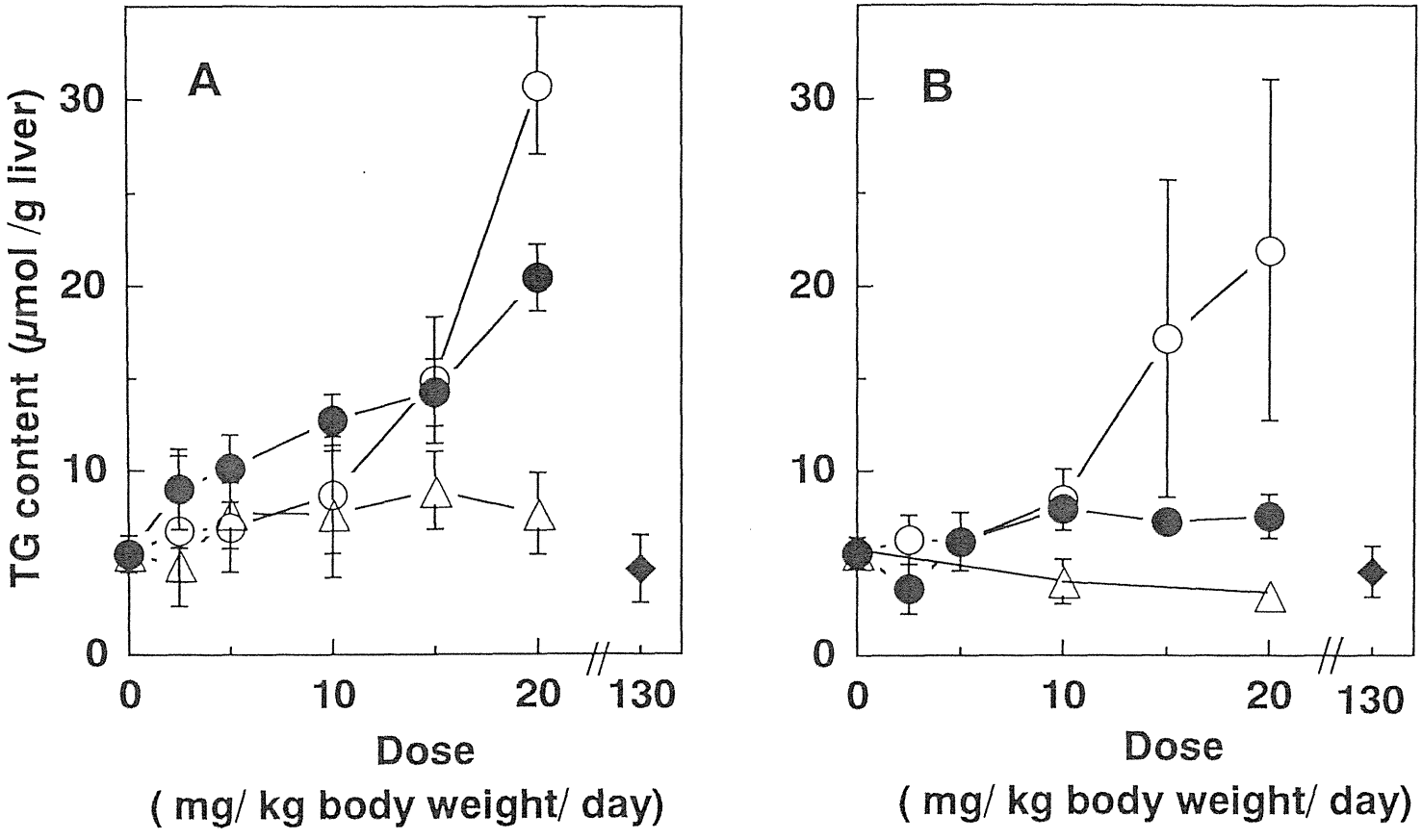
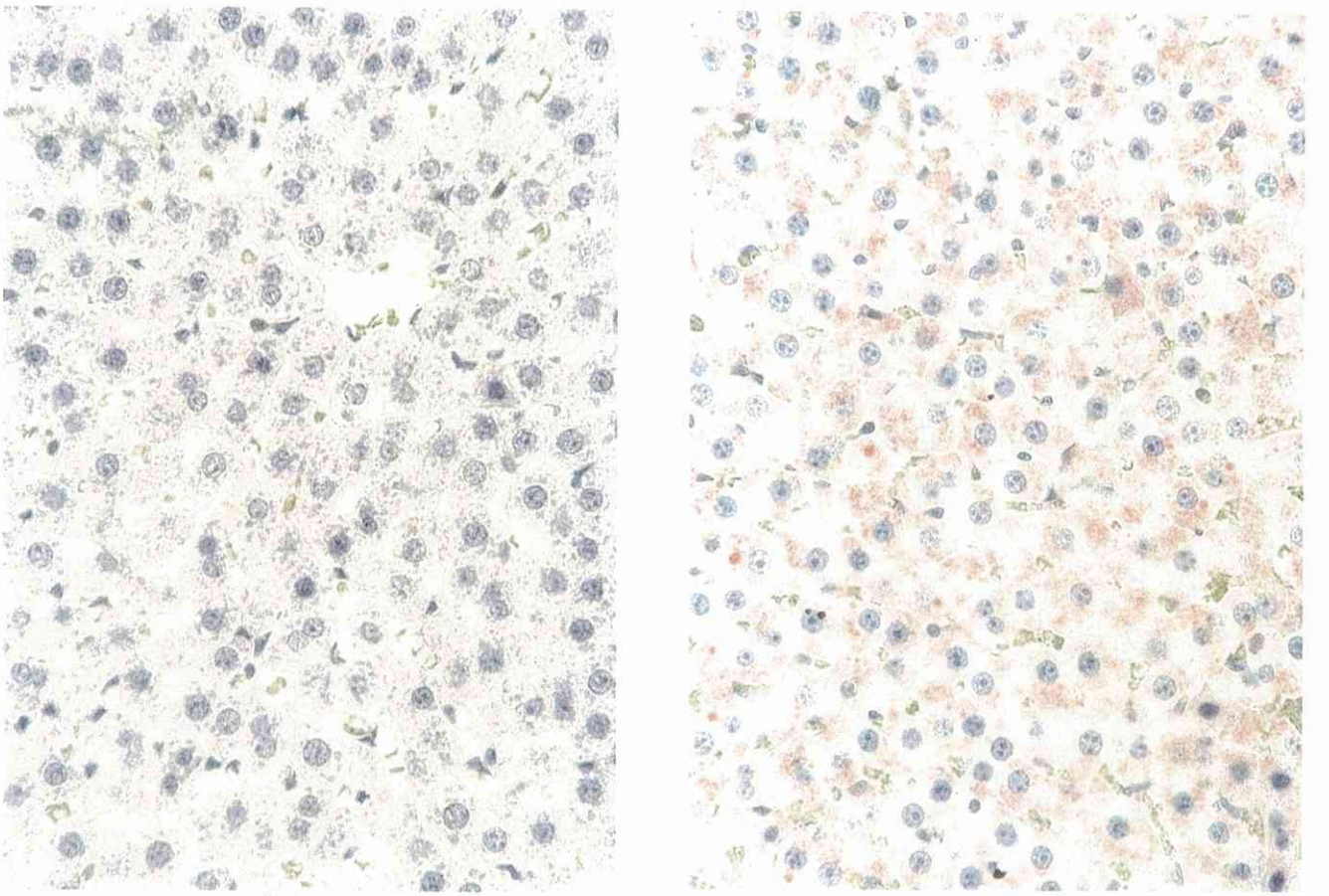


Figure 2 Effects of PFCA on the level of hepatic TG



Control PFDA

Figure 3 Oil red O staining of the liver from PFDA-treated rats.

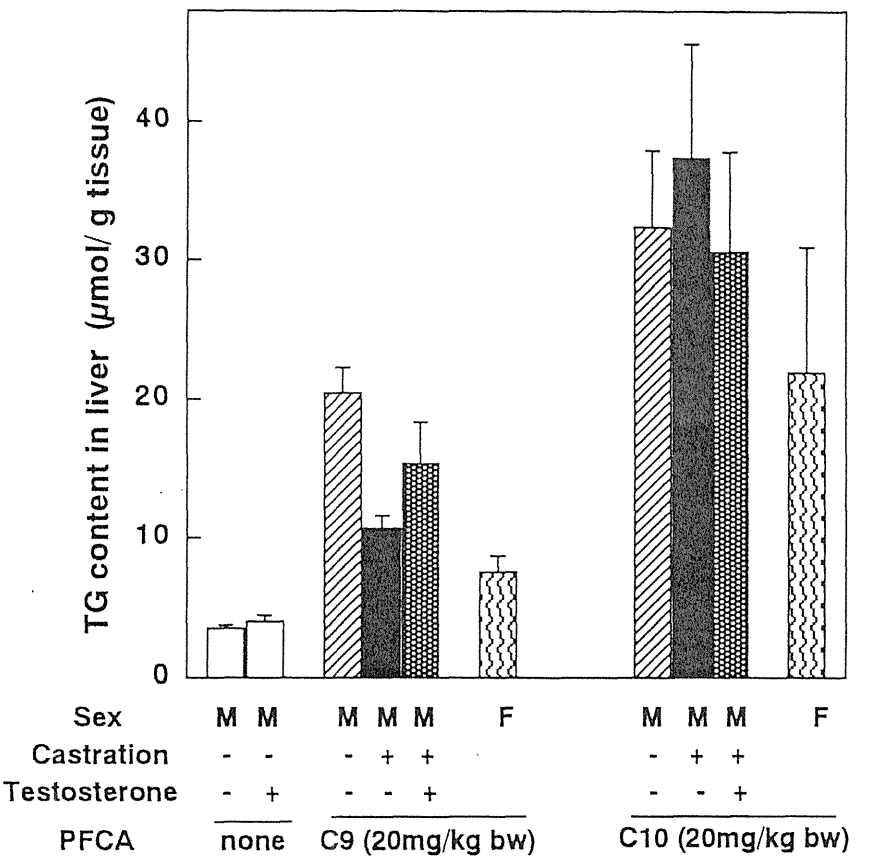


Figure 4 Effects of castration and testosterone on the level of triglyceride in the liver of male rats

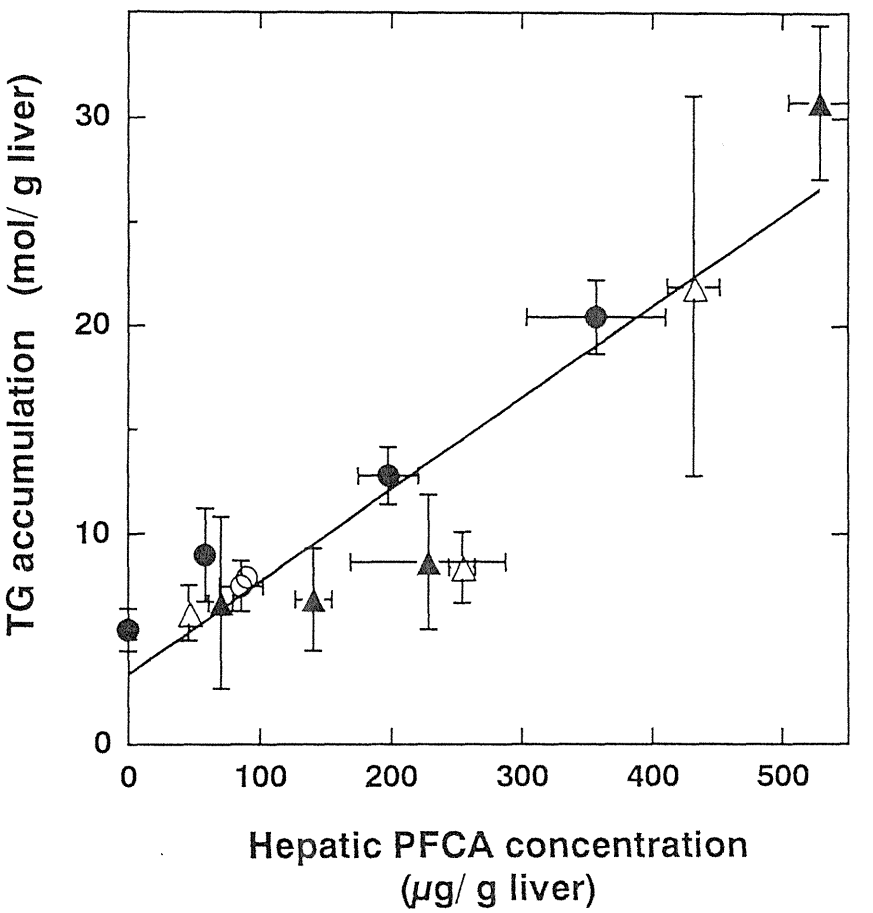


Figure 5 Relationship between PFCA concentration and the level of triglyceride in rat liver

Table 1 Effects of PFDA on fatty acid content of Liver Triglyceride

	% Composition		FA content/ g liver		FA content / whole liver	
	control	PFDA	control	PFDA	control	PFDA
Fatty acid	(% of total)		(μmol/ FA g liver)		(μmol FA/ liver)	
16:0	33.8± 1.7	32.0 ± 0.4	4.9 ± 1.1	29.6 ± 3.4	42.1 ±11.7	244.7 ± 50.2
16:1(n-7)	3.4 ± 0.9	3.2 ± 0.9	0.5 ± 0.2	2.8 ± 0.8	4.4 ± 1.9	25.4 ± 11.5
18:0	1.6 ± 0.2	1.6 ± 0.4	0.2 ± 0.1	1.4 ± 0.1	1.9 ± 0.5	12.3 ± 1.7
18:1(n-9)	22.3 ± 0.7	31.2 ± 1.9	3.3 ± 0.8	28.9 ± 4.5	27.9 ± 7.9	240.1 ± 61.7
18:2(n-6)	28.4 ± 1.9	27.5 ± 2.5	4.1 ± 0.8	25.2 ± 1.5	34.9 ± 8.6	206.8 ± 25.2
18:3(n-3)	1.4 ± 0.3	0.8 ± 0.1	0.2 ± 0.1	0.7 ± 0.1	1.7 ± 0.6	5.7 ± 1.2
20:3(n-6)	0.5 ± 0.1	0.5 ± 0.1	0.5 ± 0.1	0.4 ± 0.0	0.5 ± 0.1	3.7 ± 0.7
20:4(n-6)	1.5 ± 0.3	1.4 ± 0.2	0.2 ± 0.0	1.3 ± 0.1	1.9 ± 0.5	10.5 ± 1.7
20:5(n-3)	1.7 ± 0.3	0.3 ± 0.1	0.2 ± 0.1	0.3 ± 0.1	2.0 ± 0.6	2.2 ± 0.8
22:6(n-3)	3.7 ± 0.7	1.2 ± 0.1	0.5 ± 0.2	1.1 ± 0.1	4.5 ± 1.6	9.2 ± 1.7
Total	100.0 ± 0.0	100.0 ± 0.0	14.5 ± 3.0	92.2 ± 11.0	124.2 ± 32.9	763.7 ± 157.0

Male Wistar rats (6-week old) were intraperitoneally administered with perfluorodecanoic acid (C10) (20 mg/ kg body weight) once a day for 5 days before being killed.

Values represent mean ± SD for 4 rats.