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EFFECTS OF S-8527 (1,1-BIS[4'-(1''-CARBOXY-1''-METHYLPROPOXY)-PHENYL]-CYCLOHEXANE), A NEW HYPOLIPIDEMIC COMPOUND, ON TRIGLYCERIDE METABOLISM IN RATS

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Abstract—The effect of S-8527 (1,1-bis[4'-(1''-carboxy-1''-methylpropoxy)-phenyl]cyclohexane) on triglyceride metabolism was examined and compared with that of clofibrate in rats under various experimental conditions. In rats which were refed either a fat-free diet or a 5% fat diet after a 2-day fast, S-8527 at 30 mg/kg resulted in reductions of serum and liver triglyceride levels. In rats refed a fat-free diet, S-8527 showed a marked decrease in serum and liver triglyceride levels, but when rats were refed a 10% fat diet, the results were not significantly different from control. Clofibrate at 300 mg/kg lowered the liver triglyceride level in rats fed either a fat-free diet or a 5% fat diet but the effect was less than that of S-8527. In rats fed a normal commercial chow *ad lib.*, an oral dose of S-8527 at 30 mg/kg for 8 days decreased the incorporation of [^{14}C]acetate into liver triglyceride by about 50 per cent compared with that of controls, while clofibrate showed a slight increase in the labeled triglycerides in the liver. Addition of S-8527 to the normal rat liver slices at a concentration of 10^{-3} M inhibited the triglyceride and phospholipid formation from [^{14}C]acetate by about 40 and 50 per cent, respectively. S-8527 also inhibited rat liver acetyl-CoA carboxylase activity, but did not affect the activities of glucose 6-phosphate dehydrogenase, NADP-malate dehydrogenase, and citrate cleavage enzyme. These results suggest that the possible mechanism for the decrease in serum and liver triglyceride levels produced by S-8527 is an inhibition of triglyceride synthesis in the liver.

S-8527 (1,1-bis[4'-(1''-carboxy-1''-methylpropoxy)-phenyl]cyclohexane) has been reported to possess pronounced hypolipidemic properties in experimental animals and considered to be more potent in hypolipidemic activity and less potent in hepatomegalic effect than clofibrate [1, 2].

To elucidate the hypolipidemic action of S-8527, we studied the influence of S-8527 on serum and liver triglyceride levels in rats fasted and refed a synthetic diet containing fat at various levels, and its effect on triglyceride synthesis in liver from [^{14}C]acetate *in vivo* and *in vitro*. The effects on the activities of some enzymes associated with fatty acid synthesis were also studied.

MATERIALS AND METHODS

Treatment of animals. Male Wistar rats were used throughout the experiments. In fasted and refed experiments, the rats were maintained on a commercial chow pellet (NIPPON CLEA, CE-2) and water *ad lib.* until they had reached a weight of about 200 g, at which time they were divided into ten groups. Animals of nine groups were first fed a semi-synthetic basal diet containing 5% fat for 4 days, then fed only water for the following 2 days. Finally, groups 1, 2 and 3 were given a fat-free diet, groups 4, 5 and 6 were given the basal diet (5% fat), and groups 7, 8 and 9 were fed a high-fat diet containing 10% fat for 2 days. The rats of group 10 were given a commercial chow pellet *ad lib.* during the experimental period. The composition of the semi-synthetic basal diet was as follows: 68% glucose, 20% casein, 5% corn oil, 4% salt mixture (USP XVII) and 3% vitamin mixture [3]. In a fat-free diet, corn oil was omitted and the glucose content was increased to 73 per cent. In a high-fat diet, the corn oil content was increased to 10 per cent and the glucose content was lowered to 63 per cent. Drugs were suspended in 5% gum arabic solution and given to rats by oral intubation every a.m. during the experimental period (8 days).

For studies on triglyceride synthesis from [^{14}C]acetate and liver enzymes associated with fatty acid synthesis, rats (weighing about 170 g), which were maintained on a commercial laboratory chow and water *ad lib.*, received the drugs by oral intubation every a.m. for 8 days as described above.

Measurement of lipogenesis. In experiments *in vivo*, the rats were injected with [1-^{14}C]sodium acetate (10 μCi/100 g body weight) intraperitoneally 24 hr after the last dose of S-8527 or clofibrate, and 30 min later blood samples were obtained from the inferior vena cava under ether anesthesia. After sacrifice, the livers were removed, washed with ice-cold physiological saline, blotted on a filter paper, and weighed. Serum and liver samples were homogenized with 20 vol. chloroform–methanol (2:1, v/v), and total lipids were extracted as described previously [2]. Triglycerides were isolated by thin-layer chromatography on Silica gel G, using petroleum ether–ethyl ether–acetic acid (80:20:1, v/v). After visualizing the zone by

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means of a few sec exposure to iodine vapor, the triglyceride zone was scraped directly into counting vials, and radioactivity was measured in a liquid scintillation spectrometer.

In experiments *in vitro*, the rats were sacrificed by decapitation and their livers removed immediately, washed in ice-cold saline, and sliced. Samples weighing 500 mg were incubated in 5 ml Krebs–Ringer bicarbonate buffer (pH 7·5) containing 20 mM glucose, 4 mM [U-^{14}C]sodium acetate (5 μCi/20 μmoles), and various concentrations of the drugs at 37° for 3 hr at atmospheric pressure. S-8527 was added as the sodium salt dissolved in saline. Clofibrate (ethyl *p*-chlorophenoxyisobutyrate) is not water soluble. The sodium salt of chlorophenoxyisobutylic acid (CPIB), the non-esterified derivative of the drug, was dissolved in saline and added. After incubation, the slices were blotted and homogenized with 15 ml chloroform–methanol (2:1, v/v) and total lipids were extracted. Incorporation of [^{14}C]acetate into lipids was determined by the method described above.

Determination of enzyme activities. Acetyl-CoA carboxylase determination *in vitro* was based on the method of Nakanishi and Numa [4]. The rats were decapitated, and the liver was removed and homogenized with 3 vol. of 0·25 M sucrose in a Potter–Elvehjem homogenizer. The homogenate was then centrifuged at 105,000 *g* for 45 min at 3°, an aliquot of the soluble supernatant was filtered through a 10-fold volume of Sephadex G-25, and the eluate was used as the enzyme preparation. The Na salt of S-8527 or CPIB was dissolved in saline and added to the incubation mixture. The activity of the enzyme was determined by $H^{14}CO_3^-$ fixation assays.

Glucose 6-phosphate dehydrogenase, NADP-malate dehydrogenase, and citrate cleavage enzyme were assayed as described below. Twenty-four hr after receiving the last dose of the drug on day 8, the rats were decapitated and the livers were immediately removed and homogenized with 8 vol. of 0·25 M sucrose at 3° and centrifuged at 21,600 *g* for 30 min. The supernatant was used for measuring the activity of glucose 6-phosphate dehydrogenase [5] and NADP-malate dehydrogenase [6]. To determine the activity of the citrate cleavage enzyme, a portion of the liver was homogenized with 9 vol. of 0·25 M sucrose and the homogenate was centrifuged at 105,000 *g* for 30 min at 3°. Enzyme activity was measured as described by Srere [7].

Assay of lipid levels. Serum and liver triglyceride levels were determined by a Technicon AutoAnalyzer as previously described [2].

Chemicals. S-8527 and its Na salt were synthesized in this laboratory. Clofibrate (ethyl *p*-chlorophenoxyisobutyrate) was obtained from Imperial Chemical Industries Ltd. in England and Na salt of CPIB (*p*-chlorophenoxyisobutyric acid) was prepared in this laboratory. All radiochemicals were purchased from Daiich Pure Chemicals Ltd., Tokyo, Japan.

RESULTS

Effects on serum and liver triglyceride levels in fasted and refed rats. Table 1 shows the effects of the drugs on serum and liver triglycerides in rats which were refed a semi-synthetic diet containing 0, 5 or 10% fat for 2 days after a 2-day fast period. Fasting and refeeding with fat-free diet produced a 4-fold increase in the liver triglyceride level, when compared with rats on a normal commerical laboratory chow pellet *ad lib.* Feeding either the 5% fat diet or the 10% fat diet also increased the liver triglyceride levels, but the degree was lower than that observed in the fat-free diet group. In contrast to the liver triglyceride level, there was little or no change in the level of serum triglyceride in rats fed any of the three diets. S-8527 at a dose of 30 mg/kg for 8 days lowered serum and liver triglyceride levels in rats fed either the fat-free diet or the 5% fat diet. In rats fed the fat-free diet, S-8527 markedly lowered serum and liver triglycerides by about 60 and 84 per cent, respectively, but in rats treated with the 10% fat diet, the results were not significantly different from control. Clofibrate at 300 mg/kg lowered the liver triglyceride level in rats fed either the fat-free diet or the 5% fat diet, while there was no effect in rats refed the 10% fat diet. Levels of serum triglyceride were not altered significantly by clofibrate in all of the experimental groups.

Effects on incorporation of [^{14}C]acetate into liver lipids in rats in vivo *and* in vitro. In experiments *in vivo*, the rats were given a daily oral dose of 30 mg/kg of S-8527 or 300 mg/kg of clofibrate for 8 days. Table 2 shows the effects of the drugs on the

Table 1. Effects of S-8527 and clofibrate on serum and liver triglyceride levels in rats fasted and refed a semi-synthetic diet containing fat at various levels*

Treatment	Liver triglycerides (mg/100 g)				Serum triglycerides (mg/100 ml)			
	Chow (*ad lib.*)	0% fat	5% fat	10% fat	Chow (*ad lib.*)	0% fat	5% fat	10% fat
		(fasted and refed)				(fasted and refed)		
Control	328·3 (4) ± 12·1	1397·0† (4) ± 214·6	724·6† (5) ± 78·9	772·8 (5) ± 184·0	67·8 (4) ± 5·9	70·1 (4) ± 14·0	52·3 (5) ± 7·7	59·2 (5) ± 3·2
S-8527 (30 mg/kg)		221·4‡ (5) ± 37·0	258·0§ (5) ± 56·9	424·0 (4) ± 42·4		28·3‖ (5) ± 3·8	32·2‖ (5) ± 7·5	52·1 (4) ± 9·4
Clofibrate (300 mg/kg)		308·6§ (5) ± 109·5	287·8§ (4) ± 27·9	686·6 (4) ± 182·7		43·7 (5) ± 3·1	85·9 (4) ± 13·5	63·3 (4) ± 7·5

* Each value represents the mean ± S.E. Figures in parentheses indicate the number of animals.
† Significantly different from chow group ($P < 0·01$).
‡ Significantly different from control ($P < 0·001$).
§ Significantly different from control ($P < 0·01$).
‖ Significantly different from control ($P < 0·05$).

Table 2. Effects of S-8527 and clofibrate on the incorporation of [^{14}C]acetate into liver triglycerides in rats*

Treatment		Body wt Initial (g)	Body wt Gain (g/8 days)	Liver wt (g/100 g body wt)	Content of triglycerides Serum (mg/100 ml)	Content of triglycerides Liver (mg/100 g)	Radioactivity of liver triglycerides (TG) (dis./min/g)	Radioactivity of liver triglycerides (TG) (dis./min/mg/TG)
Control	(5)	165 ± 5	28·0 ± 2·1	4·25 ± 0·19	78·3 ± 12·6	349·0 ± 85·1	7340 ± 1410	2300 ± 400
S-8527 (30 mg/kg)	(7)	167 ± 2	44·0 ± 1·8	4·49 ± 0·23	39·0† ± 6·4	243·0‡ ± 21·7	3560† ± 320	1570‡ ± 221
Clofibrate (300 mg/kg)	(6)	166 ± 3	19·0 ± 9·0	6·61 ± 0·17	32·7† ± 3·1	319·2 ± 86·9	9960 ± 3040	5330 ± 1160

* Each value represents the mean ± S.E. Figures in parentheses indicate the number of animals.
† Significantly different from control ($P < 0.01$).
‡ Significantly different from control ($P < 0.05$).

incorporation of [^{14}C]acetate into liver triglycerides and on triglyceride levels of serum and liver. S-8527 reduced labeled triglycerides in liver by about 50 per cent/g of liver and 30 per cent/mg of triglycerides; serum and liver triglyceride levels were also decreased. On the other hand, when rats were treated with clofibrate, the incorporation of [^{14}C]acetate into liver triglycerides was increased in spite of the decrease in serum triglycerides.

The effects of the drugs on the incorporation of [^{14}C]acetate into triglycerides of normal rat liver slices were measured at various concentrations (Fig. 1). The addition of S-8527 to the incubation mixture at a concentration of 10^{-5} M (4·7 μg/ml) decreased the acetate incorporation by about 40 per cent. This inhibition was more pronounced when the concentration of S-8527 was increased. When CPIB was added to the incubation mixture in concentrations from 10^{-5} M (1·9 μg/ml) to 10^{-4} M, acetate incorporation was not affected, but when the concentration of the drug was increased to 10^{-3} or 10^{-2} M, inhibition (30–50 per cent) was observed. Figure 2 shows that S-8527 had a similar effect on

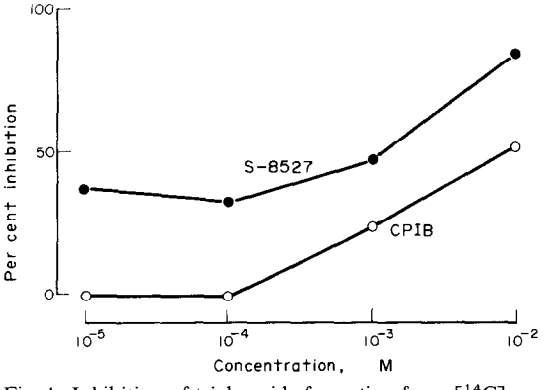

Fig. 1. Inhibition of triglyceride formation from [^{14}C]acetate by S-8527 and CPIB in rat liver slices. Triglyceride formation was assayed by [^{14}C]acetate incorporation into triglycerides. Samples of liver slices weighing 500 mg were incubated in 5 ml Krebs–Ringer bicarbonate buffer (pH 7·5) containing 20 mM glucose, 4 mM [^{14}C]acetate (5 μCi/20 μmoles) and various concentrations of the drugs at 37° for 3 hr. S-8527 was added as Na salt. The Na salt of *p*-chlorophenoxyisobutylic acid (CPIB), the non-esterified derivative of clofibrate, was dissolved in saline and added. Each value represents the mean from five independent determinations.

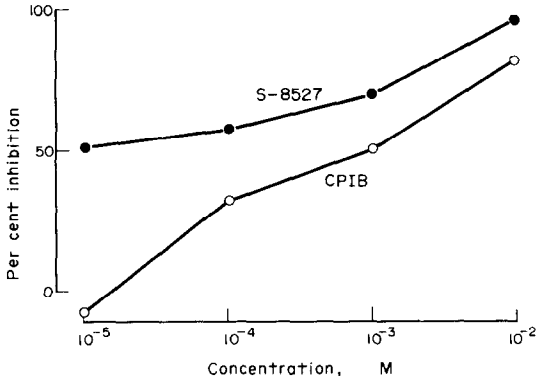

Fig. 2. Inhibition of phospholipid formation from [^{14}C]acetate by S-8527 and CPIB in rat liver slices. Phospholipid formation was assayed by [^{14}C]acetate incorporation into phospholipids. Each value represents the mean from five independent determinations. All other conditions are the same as in Fig. 1.

the incorporation of [^{14}C]acetate into phospholipids in rat liver slices and that the inhibitory effect of S-8527 was more potent than that of CPIB.

Effects on acetyl-CoA carboxylase in vitro. Activity of acetyl-CoA carboxylase was measured when the drugs were added to the incubation mixture. S-8527 caused inhibition of the enzymatic activity (Fig. 3). Maximum inhibition was achieved with a 3×10^{-3} M

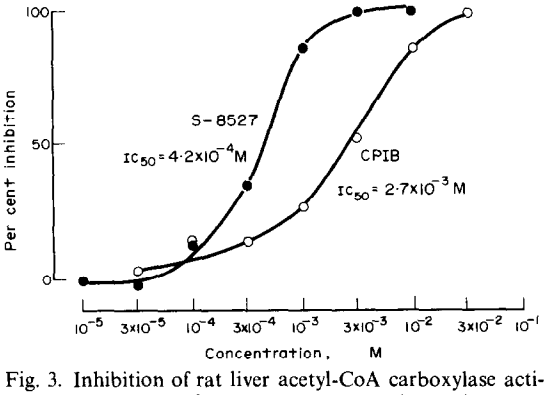

Fig. 3. Inhibition of rat liver acetyl-CoA carboxylase activity by S-8527 and CPIB *in vitro*. The activity of enzyme was determined by $H^{14}CO_3^-$ fixation assays. For other experimental details see the Methods section. Each value represents the mean of three determinations.

Table 3. Effects of S-8527 and clofibrate on liver enzymes associated with fatty acids synthesis in rats*

Treatment		Activity (nmoles/min/mg protein) Glucose 6-phosphate dehydrogenase	NADP-malate dehydrogenase	Citrate cleavage enzyme
Control	(4)	22·8 ± 2·3	24·0 ± 3·2	13·0 ± 1·2
S-8527 (30 mg/kg)	(4)	17·2 ± 1·7	20·5 ± 2·6	9·8 ± 0·7
Clofibrate (300 mg/kg)	(4)	54·4† ± 2·8	136·9† ± 9·1	8·5‡ ± 0·4

* Each value represents the mean ± S.E. Figures in parentheses indicate the number of animals.
† Significantly different from control ($P < 0.001$).
‡ Significantly different from control ($P < 0.01$).

concentration of the drug. A similar effect was observed with the presence of CPIB in the reaction mixture at a much higher concentration. The concentration of S-8527 and CPIB for 50 per cent inhibition of the enzyme activity (IC_{50}) was 4.2×10^{-4} M and 2.7×10^{-3} M, respectively. Therefore, the potency of S-8527 was about 6·5 times that of CPIB.

Effects on liver enzymes associated with fatty acid synthesis. When the drugs were given to rats daily for 8 days by a stomach tube, S-8527 at 30 mg/kg did not affect glucose 6-phosphate dehydrogenase, NADP-malate dehydrogenase, and citrate cleavage enzyme in the liver (Table 3). Clofibrate at 300 mg/kg increased glucose 6-phosphate dehydrogenate and NADP-malate dehydrogenase to about 2-fold and 6-fold, respectively, while citrate cleavage enzyme activity was lowered by about 35 per cent compared with control.

DISCUSSION

When S-8527 was given to rats fasted and refed a fat-free diet, a marked triglyceride-lowering effect was observed, but the activity diminished when the content of fat in the diet was increased. Thus, the effect of S-8527 seems to depend upon the content of fat in the diet during the refeeding period.

In rats refed a fat-free diet, it was reported that fatty acid synthesis in the liver was greatly enhanced and triglyceride content was increased; the liver was considered to be a major source of plasma triglycerides [8–11]. Feeding a high-fat diet depressed fatty acid synthesis in the liver [9, 11, 12]. In view of these results, it is suggested that the decreased rate of triglyceride synthesis in the liver possibly accounts for the reduction of serum triglyceride in S-8527-treated rats.

This possibility of action was further explored by experiments *in vivo* and *in vitro* using a radioactive precursor. When rats were treated with a daily oral dose of 30 mg/kg of S-8527, the incorporation of [^{14}C]acetate into liver triglycerides was decreased with simultaneous reduction of serum and liver triglyceride levels, as shown in Table 2. Also, addition of S-8527 to liver slices of normal rat inhibited the incorporation of [^{14}C]acetate into triglycerides; the concentration of S-8527 showing 40 per cent inhibition was 10^{-5} M, which was found to be nearly equal to the blood levels (5–10 μg/ml) of S-8527 at 24 hr after a single oral dose of S-8527 at 30 mg/kg in rats. The above results support the concept that a possible mechanism for the decrease in serum triglyceride level produced by S-8527 is an inhibition of triglyceride synthesis *de novo* in the liver.

Addition of S-8527 to normal liver slices inhibited the incorporation of [^{14}C]acetate into triglycerides and phospholipids. This can be explained by a reduction of long-chain fatty acid formation. It is generally accepted that acetyl-CoA carboxylase plays a critical role in the regulation of long-chain fatty acid syntheses [4, 9, 11], and some hypolipidemic agents were shown to have an inhibitory effect on the liver acetyl-CoA carboxylase [13, 14]. The results of our present study show that S-8527 and clofibrate inhibit acetyl-CoA carboxylase activity, and that the inhibitory effect of S-8527 is greater than that of clofibrate (Fig. 3). These results suggest that triglyceride synthesis from [^{14}C]acetate is probably reduced, at least in part, by inhibiting the activity of acetyl-CoA carboxylase.

Clofibrate has been reported to have a number of effects on lipid metabolism of rats, including an inhibition of liver triglyceride synthesis [13–16], a decrease in the rate of liver triglyceride release [17–19], a decrease in fatty acid release from adipose tissue [20, 21], and an increased uptake of serum triglyceride by adipose tissue [22]. However, which of these effects is responsible for the lowered levels of plasma lipids remains a matter of speculation [23].

During our experiments *in vitro*, clofibrate inhibited the incorporation of [^{14}C]acetate into liver triglycerides and inhibited the activity of acetyl-CoA carboxylase, as noted previously by others [13, 14]. In these respects, S-8527 seems to possess qualitatively similar effects to clofibrate. But in clofibrate-treated rats, the incorporation of [^{14}C]acetate into liver triglycerides and the triglyceride content were not decreased in spite of the decrease of serum triglycerides, findings in agreement with those of others [17, 18]. As there is little information on liver levels or intracellular distribution of clofibrate after dosage with clofibrate, it is uncertain whether clofibrate actually inhibits the triglyceride synthesis *in vivo*. Our present results show that clofibrate possibly decreases the release of triglycerides from the liver. Clofibrate seems to decrease the rate of liver triglyceride release rather than to diminish the triglyceride synthesis, as reported by others [17–19]. In rats treated with S-8527, however, labeled triglycerides were lower than in control and clofibrate-treated animals after injection of [^{14}C]acetate. Thus, S-8527 does not seem to affect triglyceride release from the liver.

The activities of both the lipogenic and glycolytic enzymes are important determinants of the rate of hepatic fatty acid synthesis [9,11]. Clofibrate increased the activities of NADP-malate dehydrogenase and glucose 6-phosphate dehydrogenase, but decreased citrate cleavage enzyme. On the other hand, S-8527 did not affect the activities of these enzymes. In these respects, the action of S-8527 seems to differ from that of clofibrate.

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