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Effects of KRN4884, a novel pyridinecarboxamidine type K_{ATP} channel opener, on serum triglyceride levels in rats

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1 The effects of KRN4884, a novel pyridinecarboxamidine type K_{ATP} channel opener, on serum triglyceride levels were investigated in Sprague-Dawley rats.

2 Oral administration of KRN4884 (3 mg kg^{-1}) for 10 days caused a significant reduction in serum triglyceride levels, which was comparable to that of clofibrate (160 mg kg^{-1}). Reduction in serum triglyceride levels by KRN4884 and clofibrate were accompanied by a reduction in triglyceride levels both in chylomicron and in very low density lipoprotein. KRN4884 treatment did not affect serum concentrations of total cholesterol and phospholipid, but did increase free fatty acid levels. Clofibrate reduced total cholesterol, phospholipid and free fatty acid levels.

3 Administration of clofibrate significantly decreased triglyceride secretion rate as measured by the Triton WR-1339 injection procedure, while KRN4884 did not.

4 Rats receiving KRN4884 exhibited an increase in lipoprotein lipase (LPL) activity both in adipose tissue and in skeletal muscle. There was an inverse correlation between serum triglyceride levels and tissue LPL activities. KRN4884 did not change hepatic triglyceride lipase (HTGL) activity. Clofibrate affected neither LPL nor HTGL activities.

5 It is concluded that administration of KRN4884 results in reduced serum triglyceride levels which may be due to the enhancement of LPL activity in peripheral tissues.

Keywords: KRN4884; K_{ATP} channel opener; triglyceride; lipoprotein lipase

Introduction

In recent years, more and more evidence has accumulated on the correlation between hypertriglyceridaemia and an increased risk of cardiovascular disease (Austin, 1991). Triglyceride is thought to promote atherosclerosis in concert with other risk factors for ischaemic heart disease, such as hypertension, diabetes and fat accumulation (Fujioka *et al*., 1987; Reaven, 1988; Kaplan, 1989). Thus, a drug which modifies not only hypertriglyceridaemia but also the other risk factors should be of great value for the prevention of atherosclerosis. In earlier studies, a new category of antihypertensive agents termed K_{ATP} channel openers possessing either a cyanoguanidine- or a benzopyran- skeleton in their structure, were shown to lower both blood triglyceride levels and blood pressure in man and rats (Rockhold *et al*., 1989; Maztno *et al*., 1994; Sugo *et al*., 1994). Therefore, it would seem that K_{ATP} channel openers have beneficial effects on serum lipids regarding their clinical utility as a hypotensive agent (Poyser & Hamilton, 1994).

Recently, we found a novel pyridinecarboxamidine type vasodilator, KRN4884, in the course of a screening programme (Figure 1; Nakajima *et al*., 1994). In vascular smooth muscle, this compound increased the efflux of ^{86}Rb, a marker for potassium (Kasai *et al*., 1995). The vasodilating effect of KRN4884 was antagonized by glibenclamide, a blocker of K_{ATP} channels (Izumi *et al*., 1995). From these observations, although KRN4884 has neither a cyanoguanidine nor a benzopyran moiety in its structure, its mechanism of action appears to be based on a K_{ATP} channel opening action. However, whether a pyridinecarboxamidine derivative such as KRN4884 can affect triglyceride metabolism has yet to be determined. We addressed this question in the present study by examining the effects of KRN4884 on serum triglyceride levels in rats, and comparing them with those of clofibrate, a potent hypotriglyceridaemic agent (Capurso, 1991).

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Methods

Experimental animals and treatment

Five-week old male Sprague-Dawley (Crj; CD) rats were obtained from Charles River Japan (Kanagawa, Japan) and housed in a temperature-controlled environment with a 12 h daily light cycle with free access to water and chow (CE-2, Clea, Japan). At 6-weeks old, the animals were assigned to vehicle, clofibrate (160 mg kg^{-1}) or KRN4884 (3 mg kg^{-1}) treatment groups. The drug was given daily, by gavage for 10 days at 2 ml kg^{-1} as a suspension in 0.5% (w/v) carboxymethyl cellulose. Systolic blood pressure was determined 1 h after administration on day 9 with the tail cuff method by use of a sphygmomanometer (PS-100, Riken Kaihatu, Tokyo, Japan). Four hours after the final administration of the drug or vehicle on day 10, non-fasting blood was collected from an abdominal aorta of each rat under ether anaesthesia. Serum was provided for lipid analysis. Epididymal adipose tissue, thigh muscle, and liver were removed and weighed, then immediately immersed in liquid nitrogen and stored at $-80°C$ until use. Epididymal adipose tissue and thigh muscle were used for determining lipoprotein lipase (LPL) activity and the liver was used for determining hepatic triglyceride lipase (HTGL) activity. All protocols concerning animals were approved by the Kirin Brewery Committee for the Humane Care of Laboratory Animals.

Serum analysis

Serum triglyceride, total cholesterol, phospholipid, free fatty acid and glucose levels were measured enzymatically, and insulin levels were determined by EIA by use of commercial kits (Wako Pure Chemical, Osaka, Japan). Residual serum was used to prepare lipoprotein fractions by sequential ultracentrifugation (Hatch & Lee, 1968; Olivier *et al*., 1988). Chylomicron ($d < 0.96$) was separated by centrifugation at 26, $000 \times g$ and 15°C for 30 min in a TLA100.2 fixed angle rotor that holds 10×1.0 ml thick walled polycarbonate tubes. After harvesting chylomicron fractions by aspiration, lipoprotein fractions were separated into very low density lipo-

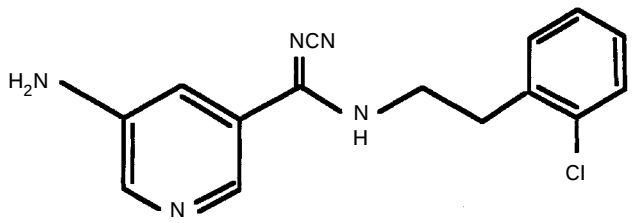

Figure 1 Chemical structure of KRN4884.

protein (VLDL, $0.96 < d < 1.006$), low density lipoprotein (LDL, $1.006 < d < 1.063$) and high density lipoprotein (HDL, $d > 1.063$) by repeated centrifugation ($435{,}680 \times g$, 2 h). Triglyceride concentrations in each lipoprotein fraction were measured as described above.

Measurement of triglyceride secretion rate

Triglyceride secretion rate (TGSR) was determined by measuring the increase in plasma triglyceride concentration according to the method of Steiner *et al.* (1984). Rats were given clofibrate or KRN4884 for 10 days as described above. Four hours after the final administration, Triton WR-1339 (Nakalai, Kyoto, Japan) (400 mg kg^{-1}), a detergent that blocks hepatic and peripheral clearance of triglyceride-rich lipoprotein, was administered intravenously via the tail vein. Blood samples for measurement of triglyceride were obtained from the tail vein before and 90 min after the injection of Triton WR-1339. TGSR was calculated as the increment of plasma triglyceride concentration per minute multiplied by plasma volume (Hirano *et al.*, 1990). Plasma volume was assumed to be 40 ml kg^{-1} (Reaven *et al.*, 1979).

Determination of LPL and HTGL activities

Acetone-ether powders of adipose tissue and homogenates of muscle were prepared for determining LPL activity. LPL activity was measured at pH 8.0 according to the method of Nilsson-Ehle & Schotz (1976), with [^{3}H]triolein (23 Ci $mmol^{-1}$) (Amersham, UK) as a substrate. The assay mixture contained 5.66 mM triolein, 0.35 mM lecithin, 1% (v/v) bovine serum albumin, 8.5% (v/v) heat-inactivated rat serum, 8.5% (v/v) glycerol, 70 mM Tris-HCl (pH 8.0). The oleic acid released was extracted with a mixture of methanol-chloroform-heptane 1.41 : 1.25 : 1. (v/v/v), followed by 0.1 M potassium carbonate buffer (pH 10.5). For measurement of HTGL activity, homogenates of liver were used (Kasim *et al.*, 1992), and the assay was performed at pH 8.8 in the presence of 0.75 M NaCl (Frenkel *et al.*, 1988; Galan *et al.*, 1994). All lipolytic activities were assayed under conditions where activity was linear with time and enzyme source concentrations. The results are expressed as μ mol oleic acid h^{-1} g^{-1} wet tissue weight.

Drugs

KRN4884 was synthesized in our laboratory. Clofibrate was purchased from Wako Pure Chemicals (Osaka, Japan).

Statistical analysis

Differences were considered significant when $P < 0.05$ by Student's t test and analysis of variance for multiple comparisons. When multiple comparisons were made with a single control, Dunnett's test was used to determine significance. All data are presented as mean $\pm$ s.e.mean.

Results

KRN4884 (3 mg kg^{-1} day^{-1}) or clofibrate (160 mg kg^{-1} day^{-1}) was administered for 10 days in Sprague Dawley rats. There was no significant difference in the initial and final body weights between the vehicle and drug-treated groups (data not shown). Systolic blood pressure, assessed by the tail-cuff method, was significantly decreased in the KRN4884-treated group (130.7 ± 4.0 mmHG in the vehicle group and 111.3 ± 2.4 mmHg in the KRN4884-treated group). KRN4884 did not alter the ratio of liver to body weight in rats, whereas clofibrate significantly increased it by 16.4%. The ratio of liver to body weight (%) in each group was as follows: 4.32 ± 0.05 in the vehicle group, 5.03 ± 0.09 in the clofibrate-treated group, and 4.32 ± 0.08 in the KRN4884-treated group. The ratio of epididymal adipose tissue to body weight (%) was not affected by administration of either clofibrate of KRN4884 (0.634 ± 0.021 in the vehicle group, 0.628 ± 0.022 in the clofibrate-treated group, and 0.628 ± 0.022 in the KRN4884-treated group).

Serum lipid levels on day 10 are summarized in Figure 2. The administration of clofibrate resulted in a decrease of 32.6% in serum triglyceride levels. Total cholesterol, phospholipid and free fatty acid levels were also decreased by clofibrate treatment. Serum triglyceride levels in rats receiving KRN4884 were significantly reduced by 41.6%. Total cholesterol and phospholipid levels were unchanged by KRN4884 treatment. Free fatty acid levels were increased by KRN4884 treatment. Neither glucose nor insulin levels were affected by clofibrate or KRN4884 administration. Glucose and insulin levels in each group was as follows: glucose (mg dl^{-1}), 147 ± 3 in the vehicle group, 142 ± 2 in the clofibrate-treated group, and 147 ± 2 in the KRN4884-treated group; insulin (μunits ml^{-1}) 22.5 ± 3.2 in the vehicle group, 14.7 ± 2.7 in the clofibrate-treated group, and 33.8 ± 8.7 in the KRN4884-treated group.

For further serum triglyceride analysis, serum was fractionated by ultracentrifugation, which was followed by the determination of triglyceride concentration in each lipoprotein fraction. The recovery of triglyceride during the fractionation procedure was 103.6 ± 2.3%. As shown in Figure 3, the majority of triglyceride was distributed in the chylomicron and VLDL fractions. Clofibrate administration significantly reduced triglyceride levels in the chylomicron and VLDL fractions by 35.9% and 27.5%, respectively, as compared with those in the vehicle group. Triglyceride levels in the LDL and HDL fractions were not affected by clofibrate administration. Rats receiving KRN4884 showed a significant reduction in triglyceride levels in the chylomicron and VLDL fractions by 46.2% and 40.2%, respectively. Triglyceride levels in the LDL and HDL fractions were unchanged by KRN4884 treatment.

TGSR was determined by the increase in serum triglyceride concentration after injection with Triton WR-1339. As shown in Figure 4, administration of clofibrate reduced TGSR significantly by 19.5% as compared to vehicle. KRN4884-treatment appeared to decrease TGSR in rats, but the difference between vehicle and KRN4884 treatment was not statistically significant.

Figure 5a and b show LPL activity in adipose tissue and skeletal muscle, respectively. Clofibrate administration did not alter LPL activity in adipose tissue or skeletal muscle, whereas KRN4884 administration significantly increased LPL activity in both tissues. The relationship between serum triglyceride levels and LPL activity was assessed in rats given vehicle or KRN4884. As shown in Figure 6a, serum triglyceride levels were inversely correlated with LPL activity in adipose tissue ($r = -0.636$, $P < 0.01$, $n = 22$). A similar inverse correlation was observed between serum triglyceride levels and muscle LPL activity ($r = -0.05$, $P < 0.05$, $n = 25$) (Figure 6b). When KRN4884 was added to adipose tissue LPL samples from a normal rat in concentrations of 1 μM – 100 μM *in vitro*, KRN4884 failed to produce any enhancement in LPL activity (data not shown). While administration of clofibrate reduced HTGL activity, KRN4884 did not (Figure 7). However, since the liver to body weight ratio was increased by clofibrate treatment as described above, we compared HTGL activity per liver in each group. When HTGL activity was expressed as μmol h^{-1} per liver instead of μmol h^{-1} g^{-1} liver weight, HTGL

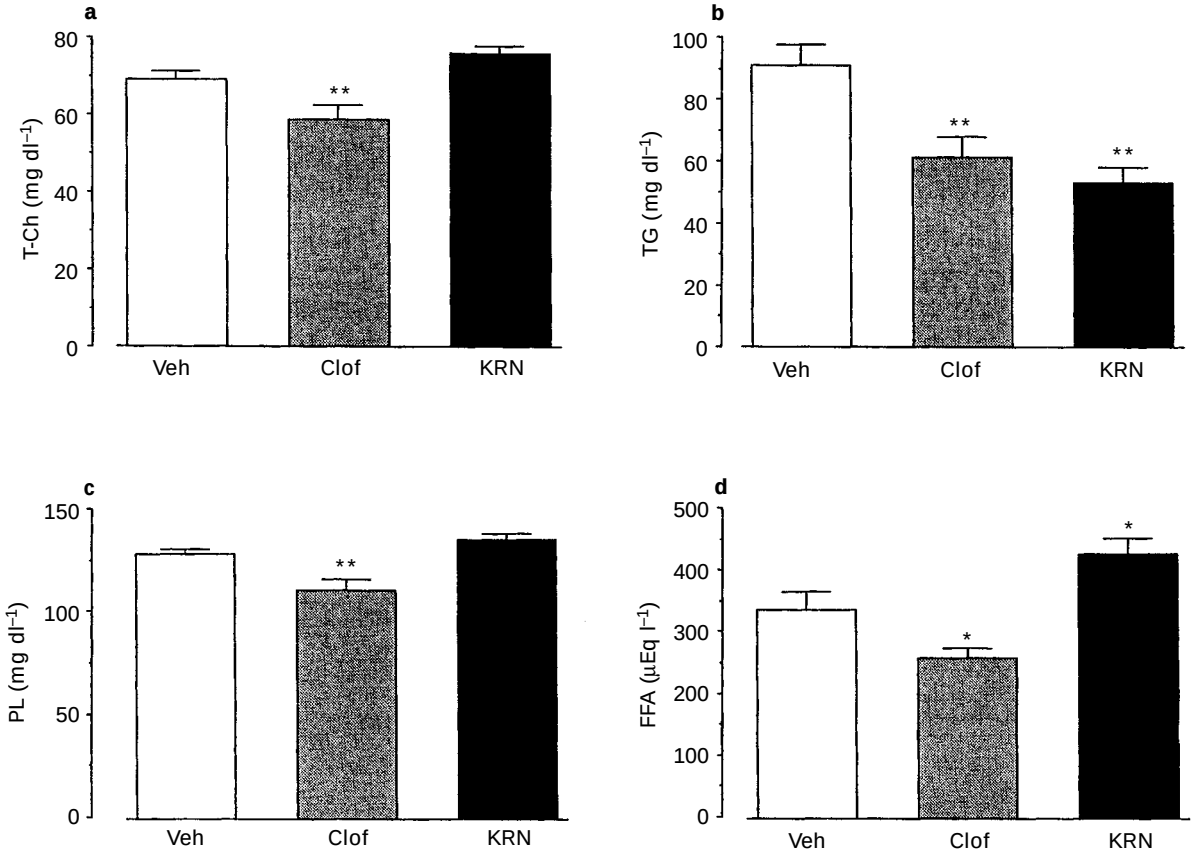

Figure 2 Effects of clofibrate (Clof) and KRN4884 (KRN) on serum lipid levels in Sprague-Dawley rats. Compounds were administered orally for 10 days. Serum (a) total cholesterol (T-Ch), (b) triglyceride (TG), (c) phospholipid (PL) and (d) free fatty acid (FFA) levels are shown ±s.e.mean ($n=11-13$). *$P<0.05$, **$P<0.01$, compared with vehicle groups (Veh).

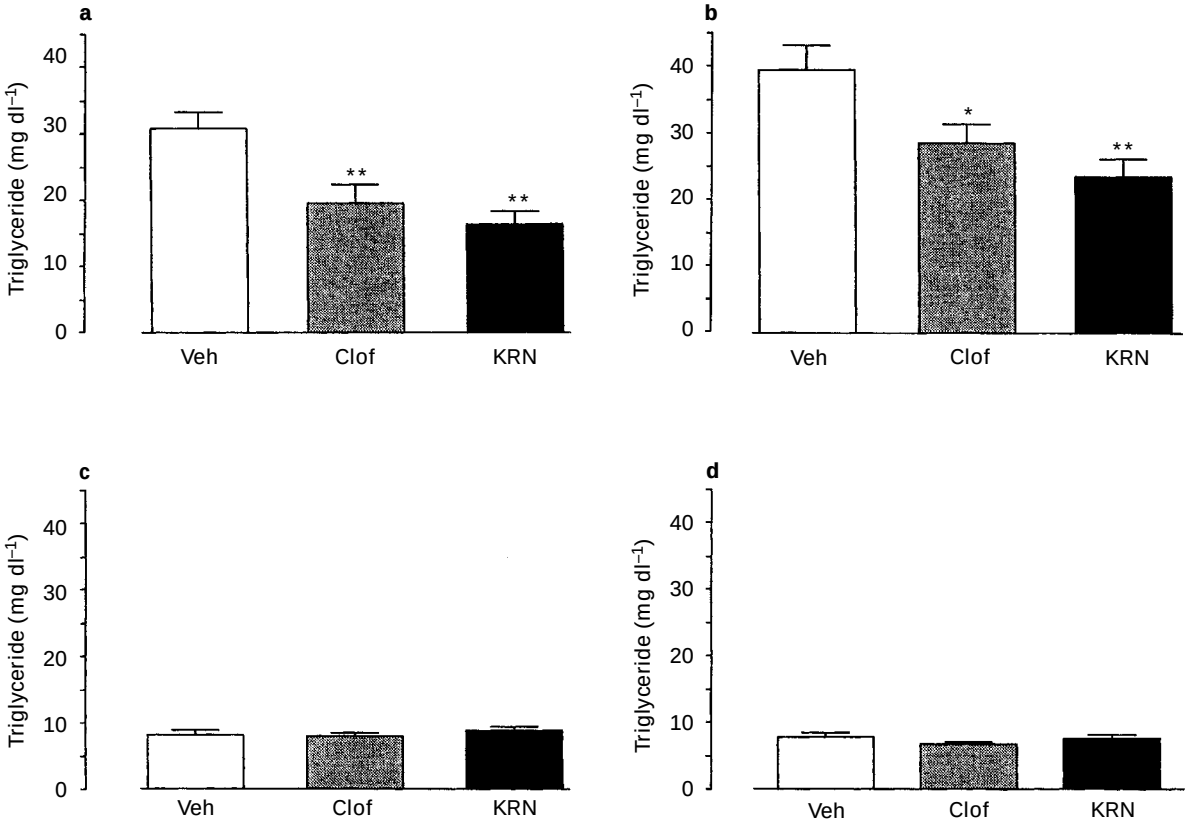

Figure 3 Effects of clofibrate (Clof) and KRN4884 (KRN) on serum lipoprotein triglyceride levels in Sprague-Dawley rats. Compounds were administered orally for 10 days. Each lipoprotein fraction ((a) chylomicron, (b) VLDL, (c) LDL and (d) HDL) was prepared by sequential ultracentrifugation. Triglyceride levels in each lipoprotein fraction are shown ±s.e.mean ($n=11-13$). *$P<0.05$, **$P<0.01$, compared with vehicle groups (Veh).

activities in the clofibrate-treated group and KRN4884-treated group remained unchanged. HTGL activity (μmol h^{-1} per liver) in each group was as follows: 84.5 ± 2.3 in the vehicle group, 87.6 ± 1.7 in the clofibrate-treated group, 82.4 ± 2.9 in the KRN4884-treated group.

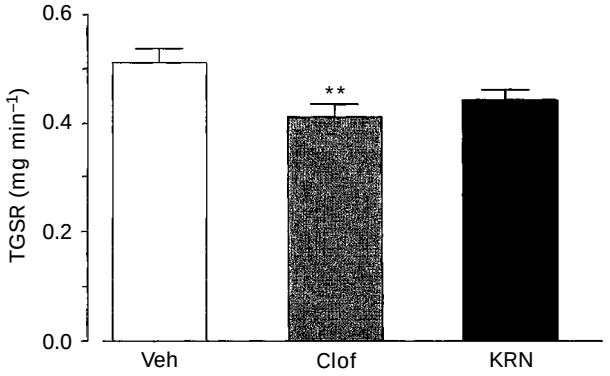

Figure 4 Effects of clofibrate (Clof) and KRN4884 (KRN) on triglyceride secretion rate (TGSR) in Sprague-Dawley rats. Compounds were administered orally for 10 days. TGSR was determined by the method of Triton WR-1339 injection. TGSR in each group is shown $\pm$s.e.mean ($n = 8-9$). $^{**}P < 0.01$, compared with vehicle group (Veh).

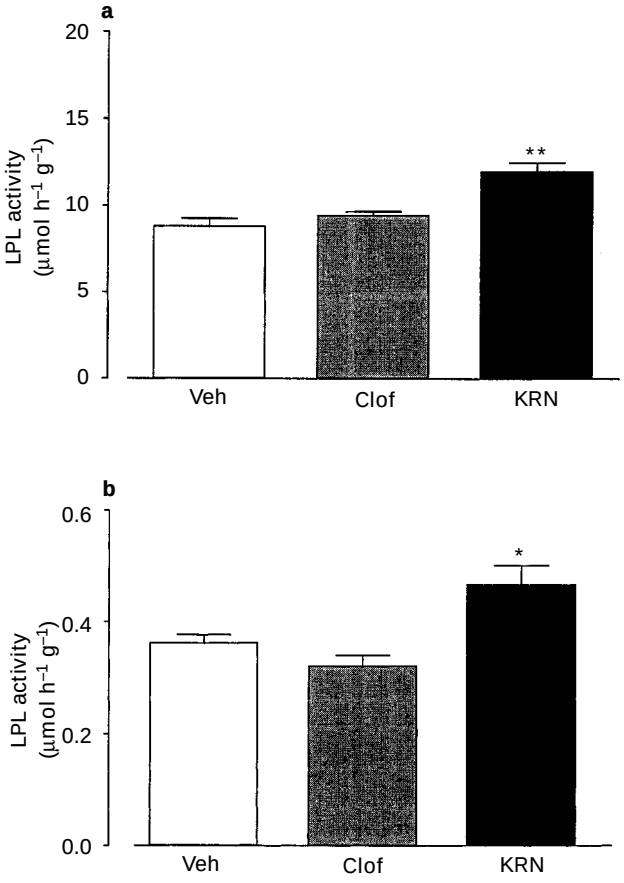

Figure 5 Effects of clofibrate (Clof) and KRN4884 (KRN) on lipoprotein lipase (LPL) activity in adipose tissue (a), and in skeletal muscle (b) in Sprague-Dawley rats. Compounds were administered orally for 10 days. LPL activity was measured by using [^{3}H]triolein as a substrate. LPL activity in each group is shown as mean $\pm$s.e.mean ($n = 9-13$). $^{**}P < 0.01$, compared with vehicle group (Veh).

Discussion

The purpose of the present study was to define the effects of KRN4884, a novel pyridinecarboxamidine type K_{ATP} channel opener, on lipid metabolism in rats. KRN4884 was administered to rats in a dose (3 mg kg^{-1}) that produced significant reduction in systolic blood pressure. Although total cholesterol and phospholipid levels were not affected by KRN4884 treatment, serum triglyceride levels were significantly de-

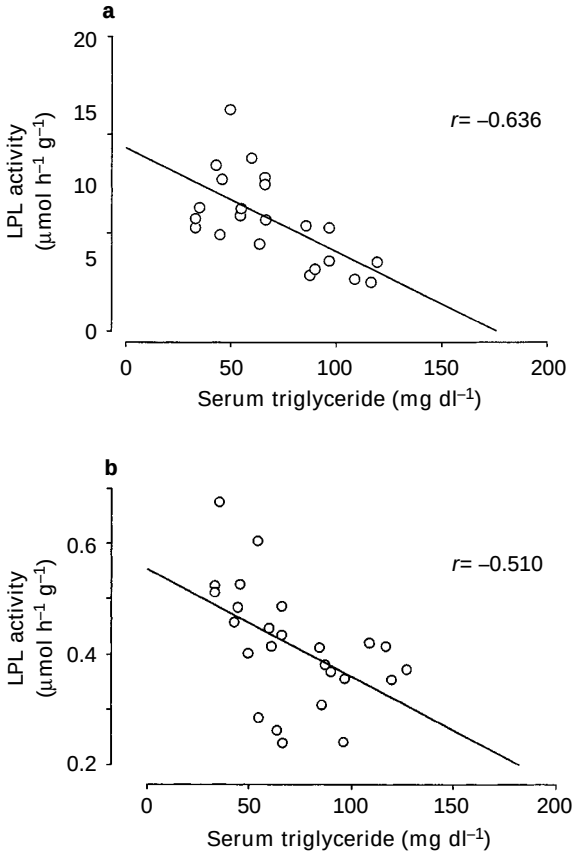

Figure 6 Relationship between tissue LPL activity and serum triglyceride levels in vehicle-, clofibrate (Clof)- and KRN4884-treated rats. (a) Adipose tissue LPL activity vs. serum triglyceride levels, (b) muscle LPL activity vs. serum triglyceride levels.

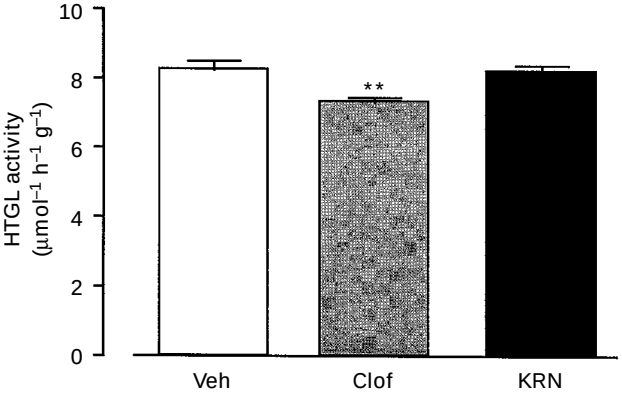

Figure 7 Effects of clofibrate (Clof) and KRN4884 on hepatic triglyceride lipase (HTGL) activity in Sprague-Dawley rats. Compounds were administered orally for 10 days. HTGL activity was measured by using [^{3}H]triolein as a substrate. HTGL activity in each group is shown as mean $\pm$s.e.mean($n = 12-13$). $^{**}P < 0.01$, compared with vehicle group.

creased by 41.6% as compared to those in the vehicle-treated group. The reduction in triglyceride levels induced by KRN4884 was comparable to that induced by clofibrate (160 mg kg^{-1}), which is a known hypotriglyceridaemic agent (Capurso, 1991). Cyanoguanidine- and benzopyran-derivatives, which possess K_{ATP} channel opening action, have recently been shown to produce hypotriglyceridaemic effects in rats (Matzno *et al*., 1994; Sugo *et al*., 1994) and in man (Rockhold *et al*., 1989; Poyser & Hamilton, 1994). Thus, it is suggested that the ability to lower triglyceride levels is a common pharmacological feature shared by structurally different K_{ATP} channel openers.

Because K_{ATP} channels in pancreatic β cells regulate insulin secretion (Ashcroft & Ashcroft, 1990), it can be assumed that a K_{ATP} channel opener, such as KRN4884 may affect insulin secretion. Earlier studies by Pratz *et al*. (1991) investigated the effects of several K_{ATP} channel openers on insulin secretion and showed that diazoxide decreased insulin secretion, while cromakalim and nicorandil did not. As shown in our study, KRN4884 did not produce any significant change in serum insulin or glucose levels. Thus, it is unlikely that KRN4884 affects K_{ATP} channel activity in pancreatic β cells.

Clofibrate treatment increased liver weight as compared to vehicle treatment. It has been shown that clofibrate induces hepatomegaly associated with peroxisomal proliferation, which, to some extent, can contribute to the hypolipidaemic effect of clofibrate through increased fatty acid oxidation (Vainio *et al*., 1983; Alegret *et al*., 1994). On the other hand, KRN4884 did not affect liver to body weight ratio. Thus, the mechanism of action of KRN4884 may differ from that of clofibrate.

Serum triglyceride levels are thought to be regulated by triglyceride secretion from the liver and intestine, and triglyceride removal in peripheral tissues. To test the possibility that the fall in serum triglyceride seen after drug treatment may be due to a decrease in triglyceride secretion, we measured TGSR in rats where clearance of triglyceride-rich lipoproteins was blocked by intravenous injection of Triton WR-1339 (Steiner *et al*., 1984). The results showed that clofibrate significantly decreased TGSR, but KRN4884 did not. Our finding of a decrease in TGSR by clofibrate is in agreement with those of Odonkor & Rogers (1984) and Sato *et al*. (1991). In the present study, clofibrate reduced serum free fatty acid levels. Because a reduction in circulating levels of free fatty acid should decrease the availability of free fatty acid to the liver to serve as a substrate for the synthesis of triglyceride (Grundy & Vega, 1987), clofibrate-induced reduction in TGSR is likely to be related to the inhibition of hepatic triglyceride formation by clofibrate (Adams *et al*., 1971). The reduction in serum free fatty acid levels by clofibrate is thought to result from: decrease in free fatty acid synthesis (Maragoudakis *et al*., 1972; Strandberg *et al*., 1983); reduced mobilization of free fatty acid (Arnold *et al*., 1979) and increased β-oxidation of fatty acid (Vainio *et al*., 1983; Alegret *et al*., 1994). On the other hand, while KRN4884 resulted in a TGSR reduction, it was not significant and was less extensive as compared to that induced by clofibrate treatment. Since KRN4884 and clofibrate decreased serum triglyceride to a similar extent, KRN4884 may reduce serum triglyceride levels by a mode of action different from that of decreasing triglyceride secretion.

In this study, administration of clofibrate did not affect tissue LPL activity, which is in an agreement with earlier findings (Vainio *et al*., 1983; Sato *et al*., 1991). Therefore, the serum triglyceride lowering effect of clofibrate could be explained mainly by the decrease in triglyceride secretion (Odonkor & Rogers, 1984). In contrast with clofibrate, KRN4884 signicantly increased LPL activities both in adipose tissue and in skeletal muscle. A similar increase in LPL activity was observed in rats treated with the cyanoguanidine type K_{ATP} channel opener, AL0671 (Matzno *et al*., 1994). Furthermore, we found a significant inverse correlation between tissue LPL activity and serum triglyceride levels in vehicle- and KRN4884-treated rats, strongly suggesting that an elevation in LPL activity contributes to the triglyceride lowering effect of KRN4884. LPL is thought to play a crucial role in hydrolysing triglyceride in triglyceride-rich lipoproteins, such as chylomicron and VLDL (Nilsson-Ehle *et al*., 1980). Accordingly, KRN4884 reduced triglyceride levels in chylomicron and VLDL, but not in LDL and HDL. Increased free fatty acid levels observed in KRN4884-treated rats might be explained by increased triglyceride metabolism by LPL in peripheral tissues. The mechanism by which KRN4884 induces an increase in LPL activity is not clear. Nevertheless, *in vitro* addition of KRN4884 to tissue LPL samples showed no enhancement in LPL activity, therefore LPL up-regulation by KRN4884 seems to result from an increase in LPL expression (Tatsumi *et al*., 1993). The finding in the present study indicating that KRN4884 does not affect HTGL activity contrasts with that of Matzno *et al*. (1994), who obtained an increase in HTGL activity in AL0671-treated rats.

Several data indicate that a multitude of risk factors leading to atherosclerosis, such as hypertension and hypertriglyceridaemia, may interact with each other and accelerate the incidence of cardiovascular disease in a cooperative manner (Fujioka *et al*., 1987; Reaven, 1988; Kaplan, 1989). Kashiwabara *et al*. (1994) found that KRN4884 lowered blood pressure in spontaneously hypertensive rats. Furthermore, preliminary experiments in our laboratory showed that KRN4884 reduced serum triglyceride levels in hyperlipidaemic rats (unpublished observation), suggesting that this compound may be useful in preventing atherosclerosis.

In summary, the present study has demonstrated that administration of a novel pyridinecarboxamidine type K_{ATP} channel opener, KRN4884, to Sprague-Dawley rats produces a serum triglyceride reduction which is associated with reductions in triglyceride levels in triglyceride-rich lipoproteins. This effect may be due to an increase in LPL activities both in adipose tissue and in skeletal muscle.

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