EFFECTS OF N-(4-METHYLBENZYLTHIOCARBONYL)-L-PHENYLALANINE (KF 1492), A NEW HYPOLIPIDEMIC DRUG, AND CLOFIBRATE ON LIPIDS METABOLISM

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The effects of N-(4-methylbenzylthiocarbonyl)-L-phenylalanine (KF 1492) on the intestinal lipid absorption, the biliary lipid composition and α-glycerophosphate dehydrogenase (GPD) activity have been investigated in rats in comparison with clofibrate. KF 1492 did not have inhibitory activity on intestinal absorption of cholesterol and triglyceride. In the KF 1492-treated group (100 mg/kg, 8 d), an increase of bile flow (25.9%) per g liver was observed. The increase of excretion of bile acids (29.9%), phospholipids (45.2%) and cholesterol (33.4%) due to the increase of bile flow was clearly observed but no significant change in the concentration of each lipid was observed. In clofibrate-treated group, the concentration of bile acids and cholesterol in bile was decreased and output of biliary phospholipids was increased. Approximately 5 to 10 times increase of GPD activity was observed in mitochondrial fraction of the KF 1492- or clofibrate-treated rats (0.25% w/w) in rat chow, 3 weeks). Thus, the increased degradation and excretion of cholesterol to bile may explain the hypcholesterolemic activity of KF 1492.

Keywords — KF 1492; clofibrate; hypolipidemic drug; cholesterol; triglyceride; bile acid; phospholipid; α-glycerophosphate dehydrogenase; rat

INTRODUCTION

N-(4-Methylbenzylthiocarbonyl)-L-phenylalanine (KF 1492) was shown to inhibit hepatic 3-hydroxy-3-methylglutaryl-Co A (HMG-Co A) reductase correlated with reduction of incorporation of 14C-acetate into digitorin-precipitable sterols.1) Stereoisomers, metabolites, and analogs of KF 1492 inhibited HMG-Co A reductase activity, and this effect was correlated with their cholesterol-lowering activity.1) Recently, Yamada has shown that KF 1492 had different hypcholesterolemic activity from clofibrate in thiouacil-fed rats and decreasing phase of Triton-induced hyperlipemia of rats.2) In these model, since hepatic cholesterol synthesis is fairly suppressed, it is suggested that some other mechanisms may play important roles.2) Generally, lowering of lipids is affected by inhibition of intestinal lipids absorption, increased cholesterol degradation and excretion, or increased mobilization of cholesterol from blood to tissues in addition to inhibition of cholesterol biosynthesis. Therefore, we have examined the effects of KF 1492 on intestinal lipid absorption, catabolism and/or excretion of cholesterol, and α-glycerophosphate dehydrogenase (EC 1.1.99.5, abbreviated as GPD) activity in comparison with clofibrate.

MATERIALS AND METHODS

Materials — KF 1492 was synthesized in Kyowa Hakko Kogyo Co., Ltd. Clofibrate from Sumitomo Kagaku Kogyo was used as reference drug. Cholesterol and KCN were purchased from Wako Chemical Industries, triolein from Tokyo Kasei, 5α-cholestan from Nakarai Chemicals, DL-α-glycerophosphate and phenazine methosulfate from Kanto Chemical Co., and hydroxysteroid dehydrogenase (grade II, from Pseudomonas testosteroni) from Sigma.
Effects of KF 1492 on Intestinal Absorption of Lipids — Male Wistar rats weighing 230—250 g (7—8 animals per group) were used. Each animal was fasted overnight, a polyethylene tubing was inserted into the thoracic duct of the rats under light anesthesia with ether by the modified method of Bollman et al. After the operation, the rats were restrained in Bollman cages and given 10 ml of saline by subcutaneous injection. At the time when the lymph flow became constant, cholesterol and triolein sufficiently emulsified in 1 ml of 1% carboxymethyl cellulose (CMC) by ultrasonication were administered by gastric intubation at a dose of 200 mg/kg each. KF 1492 suspended in 1 ml of 1% CMC was orally given simultaneously with the above emulsion at a dose of 100 mg/kg. The lymph flow was measured every hour up to 8 h to determine amounts of cholesterol and triglyceride in the lymph. The same emulsion without the drug was administered to the control group. Throughout the experiment, saline was available, but food was not given. The collected lymph was frozen until the lipid levels were measured. The total cholesterol and triglyceride contents in the lymph were measured utilizing Determiner TC and TG (Kyowa Medex Co.), respectively. The absorbed lipids were calculated as follows; the lipids before the experiment were subtracted from those in the corresponding hour zone during the experiment, and these calculated values for each hour were accumulated.

Effects of KF 1492 and Clofibrate on Bile Composition — Male Wistar rats (190—200 g) received KF 1492 or clofibrate by gavage 100 mg/kg/d for 8 d. Four hours after the last dose, pentobarbital was injected in the peritoneal cavity at the dose of 50 mg/kg, immediately thereafter a polyethylene tubing was cannulated in the bile duct, and bile was collected for 1 h. After the liver weight and the bile volume were measured, the phospholipid and bile acids contents in the bile were determined by the use of Determiner PL (Kyowa Medex Co.) and hydroxysteroid dehydrogenase, respectively. The cholesterol level was determined by means of gas chromatography on SE-30 (1.5%) Chromosorb W (AW-DMCS) column (5α-cholestan as an internal standard, column temperature 270°C).

Effects of KF 1492 and Clofibrate on α-Glycerophosphate Dehydrogenase — Male Wistar rats weighing 190—210 g were fed on ground chow (manufactured by Funabashi Nojo) for one week before the experiment. The rats were divided into three groups (5 animals per group) and given 0.25% (w/w) KF 1492 or 0.25% (w/w) clofibrate mixed with diet as described by Krishnakantha et al. and Kurup et al. for 3, 7, and 21 d. The control animals received ground chow without the drug. At the end of the period the livers of non-fasting rats were excised and homogenized for 1 min with 5 volumes of ice-cold 1.15% KCl solution. An aliquot of the homogenate (0.5 ml) was utilized for the measurement of GPD activity.

Preparation of Liver Mitochondria — Two grams of rat liver were homogenized for 1 min with 20 ml of 0.25 M ice-cold sucrose solution in Potter-Elvehjem homogenizer. After centrifugation (700 × g, 10 min), the supernatant was collected. The pellet was suspended in 10 ml of 0.25 M sucrose and centrifuged again. The supernatant together with the former one was recentrifuged (8250 × g, 10 min). Liver mitochondrial fraction was isolated by the method of Johnson et al. The pellet obtained from 2 g of rat liver was suspended in 10 ml of 1.15% KCl solution, and 0.5 ml of the suspension (calculated as 100 mg of fresh liver) was utilized for the measurement.

Measurement of GPD Activity — The GPD assay was performed by the method of Krishnakantha. One ml of 0.1 M KCl, 0.1 ml of 0.1 M MgCl₂, 0.3 ml of 0.01 M KCN, 0.5 ml of 0.5 M potassium phosphate buffer (pH 7.4), 0.1 ml (1.8 mg) of bovine serum albumin solution and 0.5 ml of liver homogenate or mitochondrial suspension were injected in the body of Warburg flask. A filter paper immersed in 0.2 ml of 20% KOH solution was set in the center well. Point
two ml of 0.6 M DL-α-glycerophosphate (disodium salt) and 0.2 ml (1.8 mg) of phenazine methosulfate were injected in the side arm. After pre-incubation for 10 min at 30°C, the side arm solution was injected in the body, and reaction was started. The oxygen consumption was measured for 30 min at 5 min intervals (Yanagimoto MFG, Type G-1-7). GPD activity was expressed as µl/10 min/100 mg of fresh tissue. Protein concentrations were assayed by the method of Lowry et al. Plasma cholesterol, triglyceride, and phospholipids were determined by Determiner TC, TG, and PL (Kyowa Medex Co.), respectively. The Student’s t-test was utilized to establish significant differences in the mean values between the control group and the treated group.

RESULTS

Effects of KF 1492 on Absorption of Cholesterol and Triglyceride

Fig. 1 and 2 show the lymph flow, the concentration of each lipid and the cumulative absorption of lipid when cholesterol, triolein (200 mg/kg each) and KF 1492 (100 mg/kg) were orally administered to rats. The average lymph flow of the control group was approximately constant at 0.40 ml/h during the test period. In the KF 1492-treated group, the lymph flow was 0.41 ml/h for 5 h after administration and increased thereafter. In the control group, the absorption of exogeneous cholesterol began immediately, and the maximum concentration was attained 3 h after administration. Thereafter, a slow decrease was observed until 8 h. At the eight hour after administration, the concentration decreased to the initial level. In the KF 1492-treated group, the concentration was maximum 4 h after administration, but the pattern of the concentration-time curve was the same as that in the control group. Regarding the

FIG. 1. Effects of KF 1492 on Lymphatic Absorption of Cholesterol

Broken lines represent control group. Solid lines represent KF 1492-treated group. Each point represents the mean±S.D.

FIG. 2. Effects of KF 1492 on Lymphatic Absorption of Triglyceride

Broken lines represent control group. Solid lines represent KF 1492-treated group. Each point represents the mean±S.D.
amount of cholesterol, a lag time of approximately 1 h appeared in the drug-treated group. Although the lymph flow increased after 6 h, cumulative absorption of cholesterol did not differ from the control group throughout the experiment. The percentage of cholesterol absorption was 3.12% in the control group and 3.50% in the KF 1492-treated group (not significant). The concentration of triglyceride was maximum 3 h after administration in the control group, and the concentration approached the initial level after 8 h. In the KF 1492-treated group, a lag time was observed with absorption of triglyceride. However, at the end of the test the cumulative absorption of triglyceride in the treated group was almost the same as that in the control group (control group, 73%; KF 1492-treated group, 69.8%).

Effects of KF 1492 and Clofibrate on Bile Composition

It has been pointed out that the biliary excretion rate of bile acids, cholesterol and phospholipids were significantly influenced by the time after the bile duct cannulation.\textsuperscript{10) In the present investigation, bile was collected for 1 h after the cannulation.\textsuperscript{11,12) The bile flow, biliary phospholipids, bile acids and cholesterol in the KF 1492- and clofibrate-treated groups are shown in Table I. In the KF 1492-treated group, an increase of bile flow (25.9%) per g of liver was observed in comparison to the control group. Regarding the concentration of each lipids, no significant change was observed, and the increase of excretion of cholesterol, bile acids and phospholipids due to the increase of bile flow was clearly observed. On the other hand, in the clofibrate-treated group, the bile acids and cholesterol concentration were decreased. The increase of phospholipids excretion due to the increase of bile flow (54.3%) was recognized.

\textbf{Effects of KF 1492 and Clofibrate on }\alpha\textsuperscript{-}Glycerophosphate Dehydrogenase (GPD)

The effects of KF 1492 and clofibrate on the GPD activity in liver homogenate and liver mitochondria are shown in Table II. The uptake of oxygen due to GPD activity linearly increased up to 30 min in liver homogenate and mitochondrial fraction. In either one of the drug-

\begin{table}[h]
\centering
\caption{Effects of KF 1492 and Clofibrate on Rat Bile Composition and Output\textsuperscript{a)}}
\begin{tabular}{lccc}
\hline
Treatment & Control & KF 1492 & Clofibrate \\
\hline
\textit{n} & 9 & 10 & 10 \\
Liver size body wt. % & 3.45 ± 0.16 & 3.61 ± 0.14\textsuperscript{b)} & 5.60 ± 0.51\textsuperscript{**} \\
Bile flow \textmu{l}/h & 588 ± 80 & 800 ± 104\textsuperscript{**} & 907 ± 87\textsuperscript{**} \\
\textmu{l}/g liver/h & 74.2 ± 7.9 & 93.4 ± 11.2\textsuperscript{**} & 69.3 ± 7.2 \\
Cholesterol \mu{g}/ml & 89.9 ± 18.1 & 89.3 ± 15.9 & 67.5 ± 10.7\textsuperscript{**} \\
\mu{g}/h & 53.6 ± 16.9 & 71.5 ± 16.8\textsuperscript{*} & 61.0 ± 10.7 \\
Bile acids \mu{mol}/ml & 18.3 ± 2.5 & 17.5 ± 2.6 & 12.6 ± 2.0\textsuperscript{**} \\
\mu{mol}/h & 10.7 ± 1.7 & 13.9 ± 2.1\textsuperscript{**} & 11.4 ± 2.0 \\
Phospholipids mg/dl & 248 ± 41 & 264 ± 31 & 236 ± 32 \\
mg/h & 1.46 ± 0.38 & 2.12 ± 0.31\textsuperscript{**} & 2.13 ± 0.31\textsuperscript{**} \\
\hline
\end{tabular}
\textsuperscript{a)} Results are expressed as mean± S.D.
\textsuperscript{b)} Significantly different from control (\(*p<0.05, \,**p<0.01\)).
\end{table}
treated group, the liver weight and the amount of protein increased, and especially in mitochondria the latter increased by 31–59%. The significant increase of GPD activity was recognized from the third day, and the increasing tendency was observed up to the 21st day. On the 21st day, approximately 5 to 10 times increase of GPD activity was observed in mitochondria of the KF 1492- and clofibrate-treated rats, compared to the control level. The enhanced GPD activity in the KF 1492-treated group was approximately half as large as that in the clofibrate-treated group. Cholesterol and phospholipids in plasma were significantly decreased in both of the drug-treated groups.

**DISCUSSION**

Cholesterol is absorbed through intestinal mucosa, mostly esterified and transported as lipoprotein to the lymphatics. Triglyceride is hydrolyzed into 2-monoacylglycerol and fatty acids with lipase and then absorbed. It was reported that some hypolipidemic drugs; e.g. β-sitosterol, colestipol, sucrose polyester, and neomycin inhibited intestinal cholesterol absorption. Clofibrate, used as the reference drug in the present experiment, was reported to reduce cholesterol absorption in man. In the KF 1492-treated group, a significant difference from the control group could not be found in cumulative absorption of cholesterol and triglyceride. This result supported that KF 1492 produced no reduction of serum cholesterol in cholesterol-fed rats soon after taking off the diet. Clofibrate caused the reduction of biliary cho-

**TABLE II.** Effects of KF 1492 and Clofibrate on Protein Content, Liver α-Glycerophosphate Dehydrogenase (GPD) and Plasma Lipids

<table>
<thead>
<tr>
<th></th>
<th>Day</th>
<th>Control</th>
<th>KF 1492</th>
<th>Clofibrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver size (body wt. %)</td>
<td>3</td>
<td>4.11</td>
<td>4.27 (104) a)</td>
<td>5.36 (130)*</td>
</tr>
<tr>
<td>Protein (mg/g liver)</td>
<td>7</td>
<td>3.78</td>
<td>4.78 (126)** b)</td>
<td>6.39 (169)**</td>
</tr>
<tr>
<td>Mitochondrial protein (mg/g liver)</td>
<td>21</td>
<td>3.86</td>
<td>5.12 (133)**</td>
<td>6.67 (173)**</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Protein</td>
<td>7</td>
<td>188±21 c)</td>
<td>215±2 (114)*</td>
<td>210±13 (112)</td>
</tr>
<tr>
<td>(mg/g liver)</td>
<td>21</td>
<td>193±11</td>
<td>215±9 (111)*</td>
<td>239±7 (124)**</td>
</tr>
<tr>
<td>Mitochondrial protein (mg/g liver)</td>
<td>7</td>
<td>35.4±2.3</td>
<td>46.8±4.0 (132)**</td>
<td>46.2±3.1 (131)**</td>
</tr>
<tr>
<td>GPD activity d)</td>
<td>21</td>
<td>36.7±2.0</td>
<td>48.8±5.9 (133)**</td>
<td>56.6±2.9 (154)**</td>
</tr>
<tr>
<td>Liver homogenate</td>
<td>3</td>
<td>19.5±1.3</td>
<td>37.0±1.8 (190)**</td>
<td>53.9±3.3 (276)**</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>20.0±2.7</td>
<td>55.2±2.5 (276)**</td>
<td>92.9±6.3 (465)**</td>
</tr>
<tr>
<td>Mitochondrial fraction</td>
<td>21</td>
<td>11.4±2.9</td>
<td>75.6±6.7 (663)**</td>
<td>121±11.4 (1063)**</td>
</tr>
<tr>
<td>Plasma cholesterol (mg/dl)</td>
<td>7</td>
<td>13.5±0.9</td>
<td>39.2±3.8 (290)**</td>
<td>87.8±7.9 (650)**</td>
</tr>
<tr>
<td>(mg/dl)</td>
<td>21</td>
<td>9.4±3.8</td>
<td>53.0±7.6 (564)**</td>
<td>96.4±11.1 (1026)**</td>
</tr>
<tr>
<td>Plasma phospholipids (mg/dl)</td>
<td>7</td>
<td>63.4±3.9</td>
<td>43.4±4.4**</td>
<td>36.7±2.8**</td>
</tr>
<tr>
<td>(mg/dl)</td>
<td>21</td>
<td>60.9±5.2</td>
<td>48.4±5.1**</td>
<td>39.2±2.4**</td>
</tr>
<tr>
<td>Plasma triglyceride (mg/dl)</td>
<td>7</td>
<td>94.1±17.9</td>
<td>57.4±7.8**</td>
<td>54.3±3.7**</td>
</tr>
<tr>
<td>(mg/dl)</td>
<td>21</td>
<td>145±18.2</td>
<td>115±14.5**</td>
<td>98.3±3.1**</td>
</tr>
</tbody>
</table>

a) Values in parenthesis represent % of control.
b) Significantly different from the control group (*p<0.05, **p<0.01).
c) Values represent mean± S.D.
d) The activity is expressed as μl O₂/ 10 min/ 100 mg tissue.
lesterol concentration. While KF 1492 inhibited the hepatic cholesterogenesis, no change was found in biliary cholesterol concentration, and the increase of cholesterol excretion appeared to some degree. It has been shown that clofibrate increase the bile flow in proportion to the liver size.11) The bile flow per g of liver in the clofibrate-treated group did not differ from that in the control group, but the significant increase in the KF 1492-treated group was observed as compared to the control group. Additionally, the bile flow is related to hepatic microcirculation, and also to the blood flow of the portal vein and the secretion of bile acids.18) The secretion of bile acids is affected by enterohepatic circulation, synthesis of bile acids, hormones, vitamins or dietary factors.19) The question as to which one of these factors plays a role in the elevation of bile flow is unknown at this time.

As shown in the above description, the hypocholesterolemic effect of KF 1492 can be attributed to the increased degradation and excretion of cholesterol to bile, and the inhibition of hepatic cholesterol synthesis. Recently, Watanabe et al.20) reported that KF 1492 (300 mg/kg, 2 weeks) has little influence on the hepatic peroxisomal enzymes, indicating that the action mechanism of this drug may be different from that of clofibrate. In the present study, after administration of KF 1492 or clofibrate at a dose of 100 mg/kg p.o. for 2 weeks in rats, the liver catalase activity was not changed in the KF 1492-treated group (96% of control) but markedly increased in the clofibrate-treated group (148% of control). The formation of micromolecules seen in electron micrograph after KF 1492-treatment was less than that seen after clofibrate treatment (data not shown). These results may suggest that the participation of hepatic peroxisomes in hypolipidemic activities of KF 1492 may be little if any.20)

It has been reported that KF 1492 reduced plasma triglyceride levels in cholesterol-fed, Triton-injected, thioaurcil-fed or glycerol-induced hyperlipemia as well as in normal rats.2) Although there are many obscure points regarding the mechanism of triglyceride reduction by clofibrate, it is generally considered that the serum triglyceride level is reduced by the inhibition of hepatic triglyceride formation21) or by the indirect mechanism which increases the GPD activity of the mitochondrial fraction.22) Therefore, the effect of KF 1492 on intestinal absorption of triglyceride and the GPD activity of hepatic homogenate and mitochondrial fractions have been studied. It was confirmed that KF 1492 did not have any inhibitory activity on intestinal absorption of triglyceride.

On the other hand, the GPD activity after KF 1492- or clofibrate-treatment increased linearly during the test period. As shown in Table II, 70% or more of the GPD activity in the liver existed in the mitochondrial fraction, and mitochondrial localization of this enzyme was assumed. KF 1492 had no influence on plasma triglyceride level in this experiment, and clofibrate significantly reduced triglyceride level in normolipidemic rats. The reduction in percentage of plasma triglyceride did not change at the time of the 7th day and the 21st day in both drugs, while the GPD activity was increased.

REFERENCES
6) T.P.Krishnakantha and C.K.R.Kurup: Increase in


