EFFECTS OF MEPANIPYRIM ON LIPID METABOLISM IN RATS

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ABSTRACT — Our preceding paper reported that mepanipyrim, a new fungicide, induced fatty liver in the rat. This study was undertaken to examine this phenomenon further on hepatic triglyceride (TG) synthesis, on liver and serum lipid concentrations, and on concentration of serum very-low-density lipoprotein (VLDL) in rats fed for 3 weeks on the drug at 4,000 ppm. Mepanipyrim decreased the incorporation of ¹⁴C-acetate into hepatic TG, total cholesterol (TC) and total lipids. In addition, mepanipyrim treatment induced a drastic increase in hepatic TG accompanying a decrease in serum TG. Esterified cholesterol (CE), phospholipid (PL) and non-esterified fatty acid (NEFA) also increased in the liver with a concomitant decrease in the serum. The decrease of serum VLDL by mepanipyrim was comparable to the decrease in serum TG. Because hepatic TG is secreted into the blood by forming VLDL, which consists of TG, TC, PL, and apoprotein, the decrease in serum TG would be mainly ascribable to that in serum VLDL. Mepanipyrim also decreased serum concentrations of low-density lipoprotein (LDL) and high-density lipoprotein (HDL), and the relative weights of the epididymal adipose tissue, indicating that a reduction in serum VLDL does not reflect acceleration of serum VLDL dissimulation. These results suggest that the fatty liver induced by mepanipyrim would be due to the inhibition of hepatic VLDL synthesis or its secretion into the blood.

KEY WORDS: Mepanipyrim, Fatty liver, Rat, Lipids, Triglyceride, VLDL

INTRODUCTION

Mepanipyrim, N-(4-methyl-6-prop-1-ynylpyrimidin-2-yl) aniline, is a new fungicide which has a controlling activity against gray mold disease caused by *Botrytis cinerea*. In our previous study (Terada *et al.*, 1998), hepatic swelling accompanied by yellowish coloring was observed in rats fed a diet containing 4,000 ppm of mepanipyrim for 13 weeks, but these changes were not observed in mice and dogs fed mepanipyrim up to the maximum tolerated dose in each species. Histopathological examination revealed that there was severe vacuolation with lipids in the peripheral region of the hepatic lobules in the mepanipyrim treated rats. This indicated that mepanipyrim induced fatty liver in rats.

Fatty liver is a pathological increase in fat content,

commonly restricted to TG in hepatocytes. TG accumulation results from an imbalance between the synthesis of hepatic TG and its discharge into the blood. It is known that fatty livers can be induced in experimental animals by administration of many agents such as barbiturate (Tuma *et al.*, 1974), ethanol (Di Luzio and Hartman, 1967), isopropanol (Nordmann *et al.*, 1973) and orotic acid (Standerfer and Handler, 1955). These drugs also induce the imbalance between the synthesis of hepatic TG and its discharge into the blood. This imbalance can also be caused by the following four mechanisms singly or by their combinations : a) increased lipid synthesis, b) decreased fatty acid oxidation, c) increased uptake of blood lipids, or d) decreased secretion of lipids into the blood (Lambardi, 1966 and Hoyumpa Jr. *et al.*, 1975).

In order to gain an insight into the mechanisms of mepanipyrin-induced fatty liver, we have examined the effects on hepatic lipid synthesis, the liver and serum lipid concentrations, and serum VLDL components in rats fed mepanipyrin.

MATERIALS AND METHODS

Chemicals

Mepanipyrin, N-(4-methyl-6-prop-1-ynylpyrimidin-2-yl) aniline, was synthesized by Ihara Chemical Industry Co., Ltd., (Shizuoka, Japan) and was 98.2% pure according to HPLC.

Sodium [$1\text{-}^{14}\text{C}$] acetate (^{14}C -Ac, 58 mCi/mmol) was purchased by Amersham Japan Co., Ltd., (Tokyo, Japan).

Animals and housing

Male Fischer 344 rats (F344/DuCrj) supplied by Charles River Japan, Inc., (Kanagawa, Japan), 4-6 weeks old on arrival, were acclimatized to laboratory conditions with the temperature at $22 \pm 2^\circ\text{C}$, relative humidity at $55 \pm 5\%$, a 12-hr lighting cycle (7:00-19:00), and frequency of ventilation 13/hr. The rats were thus 6 to 8 weeks old and their body weights were 95-169 g at the start of the treatment. Food (basal diet, M-powder, Oriental Yeast Co., Ltd., Shizuoka, Japan) and tap water were supplied *ad libitum*.

Administration of mepanipyrin

Rats were fed a diet mixed with 4,000 ppm mepanipyrin for 3 weeks, because the treatment with 4,000 ppm mepanipyrin for 3 weeks induced hypertrophy in the liver and severe fatty vacuolation in hepatocytes without serious reduction in body weight. This condition was used in a subsequent experiment. Control animals received only the basal diet.

Incorporation of [$1\text{-}^{14}\text{C}$] acetate into lipids

In this examination, the animals were sacrificed without previous fasting on completion of treatment. The liver was dissected after perfusion with Krebs-Ringer original phosphate buffer (pH 7.4) and was sliced. A liver slice was incubated in a buffer containing $4\text{ }\mu\text{Ci}$ of ^{14}C -Ac at 37°C for 90 min with aeration ($\text{O}_2\text{:CO}_2$ 95:5). After incubation, the hepatic lipids were extracted with chloroform-methanol (1:2) from the liver homogenate according to the method of Bligh and Dyer (1959). Then TG and TC were separated by the method of Schlierf and Wood (1965) using a one-dimensional TLC system. A glass-backed silica gel plate with the lipid extracts applied was developed with

the solvent, petroleum ether - ether - acetate (82:18:1 v/v/v). The radioactivities of TG, TC and total lipid fractions were measured by liquid scintillation counting.

Lipid concentrations in serum and liver

For this examination, blood samples were obtained from the subabdominal aorta under ether anesthesia following 15 hr of fasting. The livers were obtained after perfusion with saline solution. Lipids in the liver were extracted and dried in a N_2 gas stream. The lipids suspended in propanol-2% Triton X-100 (3:20) were emulsified at 70°C for 20 min. Concentrations of lipids; TC, non-esterified cholesterol (FC), CE, TG, NEFA, and PL, in the serum and liver were measured using Kyowa Medex kits (Tokyo, Japan).

Serum VLDL concentration and composition

Serum VLDL ($d < 1.006\text{ g/ml}$) was isolated by single vertical spin density gradient ultracentrifugation at a serum density of 1.30 g/ml at 50,000 rpm for 2.5 hr at 10°C (Chung *et al.*, 1986). The VLDL fraction was collected and concentrated for further purification by disc electrophoresis or gel filtration using fast protein liquid chromatography (FPLC). In the examination using gel filtration, serums from five rats per group were combined. This examination was repeated in triplicate.

Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis was performed using an ATTO-disc apparatus (Tokyo, Japan) according to the method of Tanimoto and Fujikawa (1972), except that samples were suspended in 40% saccharose before application. Absorbance of the VLDL band was analyzed by densitometry.

Gel filtration

Gel filtration using FPLC was performed as follows: The crude VLDL samples were applied to a $2.2 \times 50\text{ cm}$ column of Sephacryl HR (Pharmacia Fine Chemicals, Uppsala, Sweden) previously equilibrated with the elution buffer (10 mM Tris-HCl, pH 7.4, containing 0.15 M NaCl and 1 mM EDTA) at 4°C using a Pharmacia-FPLC apparatus (Uppsala, Sweden), and the eluate was collected fractionally. The first major peak fractions were pooled. Protein contents in the VLDL samples were measured by the method of Lowry *et al.* (1951) and lipids in them by the same kits described above.

Serum LDL concentration

LDL concentration in the serum was measured

using a Kyowa Medex kit (heparin-Ca method; Tokyo, Japan).

Serum HDL-Triglyceride and HDL-cholesterol concentrations

Serum chylomicron, VLDL, and LDL were separated from serum HDL using a Kyowa Medex kit (dextran sulfate-Mg and phosphotungstic acid-Mg method), and cholesterol (C) and TG in the HDL fraction were measured by commercial kits (Kyowa Medex, Tokyo, Japan).

Adipose tissue weight

On completion of the treatment, the animals were fasted for 15 hr before sacrifice. The weight of the epididymal adipose tissue was measured.

Statistical analysis of data

Statistical analysis of data was performed using the Student's *t*-test for comparison between the treated and control groups (Gad and Weil *et al*).

Fig. 1. Effects of mepanipyrim on the incorporation of sodium [1-¹⁴C] acetate into total cholesterol, triglyceride and total lipids in rat livers.

Rats were fed a diet containing 4,000 ppm mepanipyrim for 3 weeks. The rats were 7 weeks old, and their body weights were 162 ± 6 g in control and 160 ± 3 g in treated groups at the start of the treatment.

Each column and vertical bar represent mean $\pm$ SD for 3 animals, and asterisks indicate a significant difference from the respective control value (** $P < 0.01$).

RESULTS

Incorporation of [1-¹⁴C] acetate into hepatic TG

¹⁴C-Ac incorporation into TC, TG and total lipids in a liver slice was determined to prove the effects of mepanipyrim on the lipid synthesis. As shown in Fig. 1, mepanipyrim decreased ¹⁴C-Ac incorporation into TC, TG and total lipids to 36, 24 and 29% of the control, respectively.

Lipid concentrations in serum and liver

The effect of mepanipyrim on the concentrations of lipids in the serum and liver are summarized in Fig. 2.

The concentrations of all serum lipids, TC, FC, CE, TG, PL and NEFA, decreased due to mepanipyrim treatment. They decreased to 61-82% of the controls except for serum TG, which decreased to 37%. In contrast, the concentrations of all hepatic lipids increased. Hepatic FC and PL increased 1.5-fold of the controls comparable to their decrease rates in the serum; however, TC, CE, TG and NEFA showed a drastic increase at 4-, 24-, 4- and 3-fold, respectively.

Fig. 2. Lipid concentrations of the liver and serum in rats treated with Mepanipyrim.

Rats were fed a diet containing 4,000 ppm mepanipyrim for 3 weeks. The rats were 6 weeks old, and their body weights were 124 ± 4 g in control and 124 ± 4 g in treated groups at the start of the treatment.

TC: total cholesterol, FC: non-esterified cholesterol, CE: esterified cholesterol, TG: triglyceride, PL: phospholipid, NEFA: non-esterified fatty acid.

Each column and vertical bar represent mean $\pm$ SD for 5 animals, and asterisks indicate a significant difference from the respective control value (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

Serum VLDL concentration and composition

Serum VLDL concentrations are summarized in Fig. 3. Patterns of polyacrylamide gel electrophoresis are shown in Photo. 1. In both cases, mepanipyrim decreased the concentration of serum VLDL to about 30% of the controls. The composition of serum VLDL in mepanipyrim-treated rats purified by FPLC was similar to that in the controls, except for a slight increase in TC (Fig. 4).

Serum LDL concentration

The concentration of serum LDL in rats treated with mepanipyrim significantly decreased to 49% that of the controls (Fig. 5).

Fig. 3. Serum VLDL concentration in rats treated with mepanipyrim.

Rats were fed a diet containing 4,000 ppm mepanipyrim for 3 weeks. VLDLs were isolated using two procedures; ultracentrifugation followed by polyacrylamide gel electrophoresis (EL) and gel filtration (GF). In EL examination, the rats were 8 weeks old and their body weights were 183 ± 2 g in control and 183 ± 6 g in treated groups at the start of the treatment. In GF examination, the rats were 6 weeks old and their body weights were 120 ± 5 g in control and 120 ± 5 g in treated groups at the start of the treatment. The serums from 5 rats per group were combined and this examination was repeated in triplicate.

Each column and vertical bar represent mean $\pm$ SD for 3 animals in EL examination and for 3 trials in GF examination, and asterisks indicate a significant difference from the respective control value (** $P < 0.01$).

Serum HDL-TG and HDL-C concentrations

Both HDL-TG and HDL-C concentrations in the serum of treated rats showed a slight decrease to about 80% of the controls (Fig. 6). The statistical difference in HDL-C level, however, was not significant compared to the controls.

Adipose tissue weight

Relative adipose tissue weight of the treated rats decreased gently to 76% of the control group (Fig. 7).

DISCUSSION

The aim of the present study is to clarify the mechanism of induction of fatty liver by mepanipyrim. Similar to mepanipyrim, some chemicals are known to induce fatty livers, and some possible mechanisms causing the imbalance between the synthesis of hepatic TG and its discharge into the blood have been reported. The major causes of induced fatty livers have been claimed to be stimulation of fatty acid (NEFA) synthesis in the liver for barbiturate (Tuma *et al.*, 1974), blocking of NEFA oxidation by mitochondria for

Fig. 4. Composition of serum VLDL from rats treated with mepanipyrim.

Rats were fed a diet containing 4,000 ppm mepanipyrim for 3 weeks. The rats were 6 weeks old and their body weights were 120 ± 5 g in control and 120 ± 5 g in treated groups at the start of the treatment. The serums from 5 rats per group were combined and this examination was repeated in triplicate. VLDL was isolated by ultracentrifugation, and purified by FPLC.

The value represents mean $\pm$ SD for 3 trials.

Effects of mepanipyrim on lipid metabolism.

Photo 1. Polyacrylamide gel disc electrophoresis patterns of VLDL in VLDL layer fractionated from the serum in rats treated with 4,000 ppm mepanipyrim for 3 weeks by ultracentrifugation.

Fig. 5. Serum LDL concentration in rats treated with mepanipyrim.

Rats were fed a diet containing 4,000 ppm mepanipyrim for 3 weeks. The rats were 7 weeks old, and their body weights were 156 ± 7 g in control and 156 ± 5 g in treated groups at the start of the treatment.

Each column and vertical bar represent mean $\pm$ SD for 5 animals, and asterisks indicate a significant difference from the respective control value (***) $P < 0.001$.

Fig. 6. Serum HDL-triglyceride (HDL-TG) and HDL-cholesterol (HDL-C) concentrations in rats treated with mepanipyrim.

Rats were fed a diet containing 4,000 ppm mepanipyrim for 3 weeks. The rats were 7 weeks old, and their body weights were 139 ± 8 g in control and 138 ± 8 g in treated groups at the start of the treatment.

Each column and vertical bar represent mean $\pm$ SD for 5 animals, and asterisks indicate a significant difference from the respective control value (*) $P < 0.05$.

ethanol (Di Luzio and Hartman, 1967), an enhancement of NEFA mobilization into the liver from adipose tissues for isopropanol (Nordmann *et al.*, 1973) and inhibition of lipid secretion into the blood for orotic acid (Stauderfer and Handler, 1955). Our study was conducted as based on these four viewpoints.

At first, we examined whether or not mepanipyrim enhances the synthesis of hepatic TG, TC and total lipids; mepanipyrim, however, inhibited rather than enhanced their syntheses.

In the determination of lipid concentrations in the liver and serum, an increase in concentrations of all hepatic lipids, TG, FC, CE, PL and NEFA, and in contrast, a decrease in those in the serum were observed in mepanipyrim-treated rats. The most distinctive changes among them were an increase in hepatic TG 4-fold and a decrease in serum TG to 37% that of the control level. Based on these facts, it was considered that mepanipyrim inhibited the release of these lipids from the liver to the serum.

VLDL, which is a TG-rich lipoprotein consisting of TC, PL and apoproteins, plays an important role in the

release of lipids, mainly TG. It is known that VLDL is synthesized through a process in which a precursor, nascent VLDL, is formed by attachment of TC, PL and TG to apoproteins in smooth endoplasmic reticulum and transported to the Golgi apparatus where sugar moieties are finally added to it (Janero and Lane, 1983 and Gotto *et al.*, 1986). Thus, hepatic TG is secreted into the blood by VLDL. Every lipid which was a component of VLDL revealed quantitative changes in both the liver and serum. Hepatic NEFA showed a relatively large increase in comparison with the degree of decrease in serum NEFA. In addition, the adipose tissue weight decreased only slightly. As described above, isopropanol-induced fatty liver is known to be caused by enhanced NEFA mobilization from adipose tissue and thus increased hepatic triglyceride synthesis (Nordmann *et al.* 1973). Then a marked increase in the blood NEFA level has been observed after the administration of isopropanol. From these facts, changes in the amount of NEFA seen in mepanipyrim-treated rats appear to be secondary phenomena, caused by regulation of TG synthesis but not by enhanced NEFA mobilization from adipose tissue.

As shown in Fig. 4 and Photo 1, mepanipyrim reduced the serum VLDL volume significantly. The degree of decrease in VLDL corresponded to that of TG in the serum. It has been reported that VLDL is metabolized and converted into LDL to give the PL, cholesterol and apoprotein to HDL (Sigurdsson *et al.*, 1975 and Chajek and Eisenberg, 1978). Triglyceride, which is released from VLDL in the dissimulating process, is stored in adipose tissue in the form of NEFA (Brown and Goldstein, 1986). HDL is also synthesized in the liver and intestines to accept cholesterol from tissues and return it to the liver. Regarding the decrease in serum VLDL, it was suggested that an enhancement of the dissimulation of VLDL would not occur, because the concentrations of LDL and HDL in the serum and the weight of adipose tissue were instead decreased by mepanipyrim treatment.

The results in this study indicate that a major cause of induction of fatty liver by mepanipyrim is the inhibition of the synthesis or secretion of hepatic VLDL.

Miura *et al.* (1994a) have reported in their studies using *B. cinerea* that mepanipyrim inhibited the incorporation of ^{14}C -labeled precursors, such as glucose, acetate, uridine, thymidine, alanine or glucosamine, into proteins, lipids, cell walls, DNA, or RNA in mycelia at $100\ \mu\text{g/ml}$. However, no apparent inhibition of this incorporation was observed at concentrations below $1\ \mu\text{g/ml}$, where mepanipyrim apparently exhibited antifungal

Fig. 7. Weights of the epididymal adipose tissue from rats treated with mepanipyrim.

Rats were fed a diet containing 4,000 ppm mepanipyrim for 3 weeks. The rats were 7 weeks old, and their body weights were 164 ± 5 g in control and 164 ± 5 g in treated groups at the start of the treatment.

Each column and vertical bar represent mean $\pm$ SD for 5 animals, and asterisks indicate a significant difference from the respective control value (** $P < 0.01$).

activity. So they concluded that the mode of action of mepanipyrim might be different from inhibition of nutrient uptake or macromolecular biosyntheses in the fungi. Because mepanipyrim also prevented the secretion of host-cell wall-degrading enzymes of *B. cinerea* without inhibition of their activity resulting in their intracellular accumulation, the inhibition of enzyme secretion is suggested to be an important mechanism in mepanipyrim disease control (Miura *et al.*, 1994b). These findings indicate that mepanipyrim affects the intracellular synthesis and/or transport process of VLDL in rat hepatocytes. For further causes of decreased secretion of lipids performed by formation of VLDL, the following factors could be related: disturbance of apoprotein synthesis, inhibition of glycosylation to apoproteins at the final stage of lipoprotein assembly, and obstruction of transport through the membrane of hepatocytes (Hoyumpa Jr. *et al.*, 1975 and Mori and Ogawa, 1986).

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