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EFFECT OF 4-(4′-CHLOROBENZYLOXY)BENZYL NICOTINATE (KCD-232) ON CHOLESTEROL METABOLISM IN RATS

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Abstract—The effects of 4-(4′-chlorobenzyloxy)benzyl nicotinate (KCD-232), a new hypolipidemic agent, on serum cholesterol level and cholesterol biosynthesis were studied in normolipidemic rats. KCD-232 dose-dependently reduced the serum cholesterol level. The *in vivo* incorporation of [^{14}C]-acetate and ^{3}H from [^{3}H]water into liver digitonin-precipitable sterols was inhibited by oral administration of KCD-232, while that of [^{14}C]mevalonic acid into the sterols was not inhibited. Hepatic 3-hydroxy-3-methylglutaryl (HMG)-CoA reductase activity was suppressed significantly by the oral administration of the drug. A KCD-232 metabolite, 4-(4′-chlorobenzyloxy)benzoic acid (MII), strongly inhibited [^{14}C]acetate incorporation and weakly inhibited [^{14}C]mevalonic acid incorporation into the sterols in liver slices. MII also significantly inhibited the sterol synthetic rate measured with [^{3}H]water and the HMG-CoA reductase activity in dispersed hepatocytes. MII and its CoA thioester (MII-CoA) inhibited the incorporation of [^{14}C]acetate, [^{14}C]acetyl-CoA and [^{14}C]HMG-CoA into nonsaponifiable lipids in a cell-free enzyme system from rat liver. MII-CoA further showed a weak inhibition of [^{14}C]-mevalonic acid incorporation into nonsaponifiable lipids in the system, while MII showed no effect on mevalonic acid incorporation. These results indicate that KCD-232 possesses a major inhibitory site for sterol synthesis on HMG-CoA reductase due to both MII and MII-CoA, and a possible second site of action beyond mevalonic acid due to MII-CoA. The latter inhibitory site, however, is considered to play a minor role in the inhibition of sterol synthesis *in vivo*.

KCD-232 [4-(4′-chlorobenzyloxy)benzyl nicotinate], a new hypolipidemic agent, has been reported to possess hypotriglyceridemic and hypocholesterolemic activities in experimental animals [1–3] without any appreciable toxicity [2]. Its hypotriglyceridemic action is based on the inhibition of hepatic triglyceride synthesis due to both decreased fatty acid synthesis and increased fatty acid oxidation in the liver, the latter being apparently due to increased mitochondrial oxidation activated by a KCD-232 metabolite, 4-(4′-chlorobenzyloxy)benzoic acid (MII) [4]. MII, like other benzoic acids [5, 6], has been found to be esterified to glycerol in the rat liver and to form a thioester with CoA by rat liver microsomes [4]. This thioester, 4-(4′-chlorobenzyloxy)benzoyl-CoA (MII-CoA), inhibits fatty acid synthesis in cell-free enzyme systems and has been considered to be a chemical entity for the inhibition of hepatic fatty acid synthesis by KCD-232 [4].

Of the possible mechanisms for the hypocholesterolemic activity, KCD-232 has been found to inhibit cholesterol (Ch) absorption from the intestine [2]. As the next possibility, the effect of KCD-232 on Ch biosynthesis is described in the present paper.

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MATERIALS AND METHODS

Chemicals. Sodium [1-^{14}C]acetate (60.2 mCi/mmole) was obtained from The Radiochemical Centre, and [1-^{14}C]acetyl-CoA (51.5 mCi/mmole), DL-[3-^{14}C]3-hydroxy-3-methylglutaryl (HMG)-CoA (57.6 mCi/mmole), DL-[2-^{14}C]mevalonic acid dibenzylethylenediamine (DBED) salt (40.8 mCi/mmole) and [^{3}H]water (100 mCi/ml) were from New England Nuclear. Dibenzylethylenediamine was removed from [^{14}C]mevalonic acid DBED salt following the direction of the supplier before mevalonic acid was used. Specific radioactivities of these labeled compounds were diluted with corresponding unlabeled compounds, if necessary. KCD-232, sodium salt of MII and sodium salt of clofibric acid (4-chlorophenoxyisobutyric acid) were synthesized in this laboratory. MII-CoA was enzymatically synthesized [7] and purified [8] as described previously [4]. Crystallized bovine serum albumin (Fr. V), CoA, acetyl-CoA, acetoacetyl-CoA, HMG-CoA, glucose-1-phosphate, reduced glutathione (GSH), ATP, NAD, NADP and NADPH were purchased from the Sigma Chemical Co., and collagenase and hyaluronidase from Boehringer Mannheim GmbH; Eagle's minimum essential medium was from the Nissui Seiyaku Co. Other chemicals were of the best grade commercially available.

Animals and treatment. Male Wistar rats (CLEA Japan Inc.) were used throughout these experiments. The animals were fed a commercial stock pellet (CE-

2, CLEA Japan Inc.) and water *ad lib.* in an air-conditioned room with a 6:00 a.m. to 6:00 p.m. light cycle. Where indicated, animals were kept under a reversed light and dark cycle. KCD-232 was suspended in 0.5% carboxymethyl cellulose solution and given to rats by oral intubation (0.5 ml/100 g body wt). Control rats received 0.5% carboxymethyl cellulose solution alone.

Estimation of cholesterol lowering effect of KCD-232. Rats weighing about 200 g were divided into six groups, and KCD-232 was administered for 10 days at doses of 0, 10, 20, 40, 80 and 160 mg/kg body weight once a day. The final administration of the drug was at 9:00 a.m., at which time rats were deprived of their diet but allowed free access to water. They were killed by decapitation 4 hr later, and blood was collected in a glass tube. From the serum (1 ml), total lipids were extracted and purified according to the method of Folch *et al.* [9]. The serum Ch level was determined by the colorimetric method of Zak [10].

In vivo *incorporation of* [^{14}C]*acetate and* [^{14}C]*-mevalonic acid into hepatic sterols.* Rats were maintained under an 8:00 p.m. to 8:00 a.m. light cycle for 2 weeks, after which time they reached body weights of 230–280 g. Animals were then given 0, 100 and 300 mg/kg/day of KCD-232 for 16 days under the reversed light and dark cycle. The last administration of the drug was 5.5 hr before decapitation at mid-dark around 2:00 p.m. while the animals were in a lipogenic state. They were allowed free access to their diet and water until sacrifice. They received an intraperitoneal injection of [1-^{14}C]acetate (60.2 mCi/mmole, 9.7 μCi/100 g body wt/0.2 ml isotonic NaCl solution) 50 min prior to being killed. In another experiment, DL-[2-^{14}C]mevalonic acid (40.8 mCi/mmole, 1.4 μCi/100 g body wt/0.2 ml isotonic NaCl solution) was intraperitoneally injected into rats 60 min before sacrifice. After decapitation, the liver was quickly removed, washed and weighed. A minced liver sample (*ca.* 1 g) was placed in a screwcap glass tube, and dissolved and saponified in 3 ml of 10% (w/v) KOH in 67% (v/v) ethanol at 85° for 3 hr. Nonsaponifiable lipids were then extracted three times with 4 ml of petroleum ether. The extracts were pooled in a glass tube, dried under nitrogen, and dissolved in 1 ml of acetone–ethanol (1:1, v/v). Subsequently, 2 ml of 0.5% digitonin in 50% ethanol was added to the tube to form digitonin-precipitable sterols [11]. The digitonides were isolated [11] and dissolved in 1 ml of methanol. The methanol solution was quantitatively transferred to a scintillation vial and counted with a liquid scintillation spectrometer (Packard Tri-Carb model C2425) after addition of a toluene-based scintillator. The quenching correction was performed on each vial by the external standard method.

Estimation of rate of sterol synthesis in vivo *using* [^{3}H] *water.* Rats kept under an 8:00 p.m. to 8:00 a.m. light cycle for 2 weeks received KCD-232 (0 and 300 mg/kg/day) for an additional 16 days. The last administration was 5.5 hr before sacrifice and rats were decapitated at mid-dark around 2:00 p.m. [^{3}H] Water was intraperitoneally injected at a dose of 4 mCi/rat/0.2 ml isotonic NaCl solution 1 hr prior to sacrifice. The animals were given no further diet and water after the injection of [^{3}H]water. The liver and small intestine were quickly removed, washed with cold isotonic NaCl solution, and weighed. An aliquot of plasma was taken to determine the plasma Ch as mentioned above. The small intestine was divided into 3 parts of equal length, and the most distal part from the stomach was taken for the ileum. The minced liver (2 g), ileum (2 g) and plasma (2 ml) were placed in screwcap tubes and saponified at 85° for 5–6 hr after adding 2 ml of 15% (w/v) ethanolic KOH. The whole remaining body, including the rest of the small intestine and the clot obtained from the aforementioned blood sample, was placed in a large beaker containing 250 ml of aqueous 30% KOH solution and heated in a boiling-water bath with stirring until totally dissolved. Contents of the beaker were then brought to 500 ml with water in a volumetric flask. To 5 ml of the body lysate placed in a screwcap tube was added 5 ml of ethanol and the mixture was heated at 85° for 2 hr.

After nonsaponifiable lipids were extracted with petroleum ether, the digitonides were precipitated [11], and free sterols cleaved from the digitonides with pyridine were extracted with diethyl ether into a scintillation vial and taken to dryness under air. The dried free sterols were further heated at 80° for an additional 1 hr [12]. Radioactivity was counted after adding toluene scintillator.

Radioactivity present in 20 μl of the plasma was counted by adding 10 ml of Insta Gel (Packard Instrument Co., Inc.). The specific radioactivity of plasma water at the time each rat was killed was calculated by looking upon the water content of plasma as 92%, and the nmoles or μmoles of water incorporation into sterols was calculated using the specific radioactivity [12].

Measurement of cholesterogenic enzyme activities. Rats maintained under an 8:00 p.m. to 8:00 a.m. light cycle for 4 weeks received KCD-232 (0 and 300 mg/kg) at 9:00 a.m. The animals were decapitated at mid-dark around 2:00 p.m., until which time they were allowed free access to water and diet. Blood was collected, and the serum Ch level was determined as mentioned above. Liver enzyme preparations (cytosolic fraction and microsomes) were performed as described by Kuroda and Endo [13]. The cytosolic fraction was precipitated with ammonium sulfate at 40–80% saturation, and the precipitate was dissolved in 100 mM potassium phosphate buffer (pH 7.4) and dialyzed against the same buffer [13]. This fraction was used as enzyme source for the assays of cytosolic acetoacetyl-CoA thiolase and HMG-CoA synthase. Cytosolic acetoacetyl-CoA thiolase and HMG-CoA synthase activities were determined by the spectrophotometric methods described by Clinkenbeard *et al.* [14, 15]. Microsomal HMG-CoA reductase was assayed by the method described by Ide *et al.* [16]. Protein was determined by the method of Lowry *et al.* [17].

Estimation of sterol synthesis in liver slices. Rats kept under a 4:00 p.m. to 4:00 a.m. light cycle for 2 weeks were decapitated at mid-dark around 10:00 a.m. As reference, age-matched rats maintained under a normal light and dark cycle were also used at the same time. After decapitation, the liver was quickly removed, washed with cold isotonic NaCl

solution, and sliced with a tissue slicer (YH-10D, Hotta Rika Co., Ltd., Tokyo) to a thickness of 0.5 mm. Sterol synthesis was measured by a slightly modified version of the procedure described by Endo *et al.* [18]. Liver slices weighing 100–120 mg were placed in a screwcap tube (16 × 100 mm) containing 1 ml of Krebs–Ringer phosphate buffer (pH 7.4) with 0.5% bovine serum albumin and 1.0 μCi of [1-^{14}C]acetate (1 mCi/mmole) or 0.4 μCi of [2-^{14}C]-mevalonic acid (1.6 mCi/mmole) in the absence or presence of MII (sodium salt) and nicotinic acid. Under 100% O_2 phase, the tube was incubated at 37° in a metabolic shaker at 120 strokes/min for 2 hr. The reaction was terminated with 1 ml of 15% ethanolic KOH. After saponification, nonsaponifiable lipids were extracted, and digitonin-precipitable sterols were formed, isolated, dissolved in methanol, and counted as described above.

Hepatocyte preparation and incubation procedure. Hepatocytes were isolated from the liver of rats kept under a 4:00 p.m. to 4:00 a.m. light cycle for at least 4 weeks by *in situ* perfusion with 0.05% collagenase and 0.1% hyaluronidase in calcium-free Hanks' solution as described by Berry and Friend [19]. The perfusion was started from 10:00 a.m. Cell viability was found to be at least 85% by trypan blue exclusion. The final density of 5 × 10^6 cells/ml was attained in Eagle's minimum essential medium containing 1.5% bovine serum albumin. Two milliliters of the cell suspension (1 × 10^7 cells) was placed in a plastic tube (18 × 100 mm) with or without 1 mM MII (sodium salt) and incubated at 37° in a metabolic shaker with gentle shaking under 95% O_2–5% CO_2 phase. In an experiment, hepatocytes were incubated with 4 mCi of [^{3}H]water for 1 hr, and the sterol synthetic rate in the cells was determined. The result was expressed as nmoles of H_2O incorporated into sterols/hr/10^7 cells. In another experiment, hepatocytes were collected after a 1-hr incubation, and microsomes were isolated and used for HMG-CoA reductase assay [16].

Incorporation experiment with cell-free enzyme system. Rats (*ca.* 300 g) maintained under a 4:00 p.m. to 4:00 a.m. light cycle were decapitated at 10:00 a.m., and liver microsomes and cytosolic enzyme fraction were isolated as described above. The reaction mixture (0.2 ml) contained 1 mM ATP, 10 mM glucose-1-phosphate, 6 mM GSH, 6 mM $MgCl_2$, 0.25 mM NADP, 0.25 mM NAD, 100 mM potassium phosphate buffer (pH 7.4), 0.15 to 0.18 mg microsomal protein, 1.5 to 1.8 mg cytosolic enzyme protein fraction [13] and 0–50 μM concentrations of various test compounds. As radiolabeled precursors, 0.5 μCi of [1-^{14}C]acetate (3 mCi/mmole, in this case 40 μM CoA was added to the reaction mixture), 0.1 μCi of [1-^{14}C]acetyl-CoA (2 mCi/mmole), 0.1 μCi of DL-[3-^{14}C]HMG-CoA (9.4 mCi/mmole) and 0.26 μCi of DL-[2-^{14}C]mevalonic acid (2 mCi/mmole) were contained in the reaction mixture. After incubation at 37° for 1 hr under air with gentle shaking, the reaction was terminated by adding 1.5 ml of 15% KOH in 67% ethanol. After saponification nonsaponifiable products were extracted three times with 4 ml of petroleum ether. Pooled extracts in a scintillation vial were evaporated to dryness under an infrared lamp and radioactivity was counted after adding toluene scintillator. Proteins of microsomes and cytosolic fraction were determined [17], and the results were expressed as dpm/hr/mg protein.

Statistical method. Statistical analysis was performed using Student's *t*-test for paired data, and a P value of <0.05 was considered significant.

RESULTS

As illustrated in Fig. 1, KCD-232 reduced the serum Ch level of rats in a dose-dependent manner. Significant decreases were observed at doses above 20 mg/kg/day.

The oral administration of KCD-232 significantly suppressed the *in vivo* incorporation of [^{14}C]acetate into hepatic digitonin-precipitable sterols by 51%

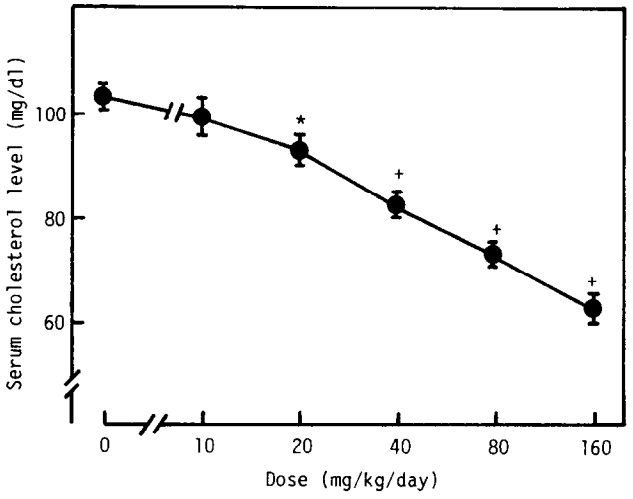

Fig. 1. Dose-dependent reduction of serum cholesterol level in rats orally given KCD-232. Rats orally received KCD-232 for 10 days at the indicated doses. The serum cholesterol level was determined as described in Materials and Methods. Each point represents the mean of seven rats. Vertical bars indicate standard errors. Key: (*) $P < 0.05$ and (†) $P < 0.001$, respectively, compared to the control group.

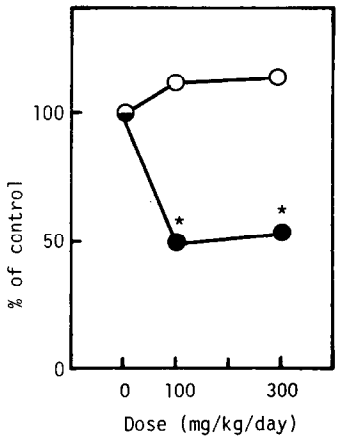

Fig. 2. *In vivo* incorporation of [1-^{14}C]acetate and [2-^{14}C]-mevalonic acid into digitonin-precipitable sterols in the liver of rats orally given KCD-232. Rats kept under an 8:00 p.m. to 8:00 a.m. light cycle for 2 weeks received KCD-232 for another 16 days. The incorporation of each labeled substrate was determined as described in Materials and Methods. Each point represents the mean of five rats. Control values for [^{14}C]acetate (●) and [^{14}C]mevalonic acid (○) incorporation were 10,476 ± 1173 and 44,579 ± 3429 dpm/g liver (mean ± S.E.M.) respectively. Key: (*) $P < 0.01$, compared to the incorporated radioactivity of the control group.

(100 mg/kg/day) and 47% (300 mg/kg/day). By contrast, that of [^{14}C]mevalonic acid into the sterols was enhanced slightly (12–14%), though not significantly, by the drug administration (Fig. 2).

The absolute synthetic rate of sterols was measured *in vivo* using [^{3}H]water (Table 1). KCD-232 significantly suppressed the sterol synthetic rate in the liver by 46% (per unit tissue) and 40% (per whole tissue) at a dose of 300 mg/kg/day. The sterol synthetic rate of ileum (per unit tissue) tended to be lowered ($P < 0.1$) by the drug administration. KCD-232 also decreased those of plasma (in this case, not a synthetic rate but rather an influx from other compartments) and carcass by 27–30% in both units, although the decrease in plasma was not significant. The total sterol synthetic rate of the whole animal was suppressed significantly (by 32%) with the drug administration. KCD-232 significantly decreased the plasma Ch level by 41%. There were no significant differences between control and KCD-232-treated rats in body, liver and carcass weights.

Table 2 shows the activities of liver enzymes related to the Ch synthetic pathway from acetyl-CoA to mevalonic acid production. The single oral dose of KCD-232 at 300 mg/kg significantly decreased the microsomal HMG-CoA reductase activity by 65%, while it enhanced the cytosolic acetoacetyl-CoA thiolase by 12% and HMG-CoA synthase by 20%. KCD-232 significantly reduced the serum Ch level by even a single oral dose at 300 mg/kg.

To examine which metabolites of KCD-232, MII or nicotinic acid, possessed the inhibitory effect on sterol synthesis, liver slices were incubated with [^{14}C]acetate and [^{14}C]mevalonic acid in the absence or presence of the metabolites (Table 3). Liver slices obtained from rats maintained under a reversed light and dark cycle incorporated 12.8-fold and 2.5-fold as much [^{14}C]acetate and [^{14}C]mevalonic acid, respectively, into sterols as did those obtained from rats under a normal light and dark cycle. In these cholesterogenic slices, MII depressed the [^{14}C]acetate incorporation into sterols in a dose-dependent manner and inhibited almost completely the incorporation of [^{14}C]acetate into sterols at a concentration of 2 mM where nicotinic acid showed no inhibitory effect. The concentration of MII required for 50% inhibition was 0.13 mM. On the other hand, MII showed a significant decrease in the [^{14}C]mevalonic acid incorporation into sterols at only a high dose of 2 mM where nicotinic acid had no significant effect. In this case, the MII concentration required

Table 1. *In vivo* sterol synthetic rate, measured with [^{3}H]water, in rats orally given KCD-232*

Group	Incorporation of ^{3}H from [^{3}H]water into DPS				
	Liver	Ileum	Plasma	Carcass	Whole animal
	Per unit tissue (nmoles H_2O/hr/g or ml)				
Control	4562 ± 642	2412 ± 250	400 ± 86	240 ± 8	
KCD-232	2473 ± 305†	1740 ± 155	291 ± 56	175 ± 20†	
	Per whole tissue (μmoles H_2O/hr/whole tissue)				
Control	48.6 ± 5.9		5.0 ± 1.2	73.3 ± 3.9	126.9 ± 7.2
KCD-232	29.4 ± 4.9†		3.5 ± 0.6	52.8 ± 5.9†	85.7 ± 7.4‡

* After rats were kept under an 8:00 p.m. to 8:00 a.m. light cycle for 2 weeks, they were given orally KCD-232 (300 mg/kg/day) or 0.5% carboxymethyl cellulose (control) for an additional 16 days. Six (control) or five (KCD-232) rats were used. The nmoles or μmoles of water incorporation into digitonin-precipitable sterols (DPS) was determined as described in Materials and Methods. Carcass means the whole animal excluding liver and whole plasma, where whole plasma mass was regarded as 4% of body weight. Each value represents the mean ± S.E.M. The body weight (g, mean ± S.E.M.) of rats at starting drug administration was 249 ± 8 (control, C) or 249 ± 6 (KCD-232, K) and that at sacrifice was 305 ± 8 (C) or 302 ± 7 (K). The relative liver weight (g/100 g body wt) was 3.56 ± 0.10 (C) or 3.87 ± 0.16 (K) and the carcass weight (g) was 287 ± 7 (C) or 282 ± 6 (K). KCD-232 significantly ($P < 0.001$) reduced the plasma cholesterol concentration (mg/dl) from 123 ± 6 (C) to 73 ± 4 (K).

† $P < 0.05$, compared to the control group.

‡ $P < 0.005$, compared to the control group.

Table 2. Activities of hepatic enzymes related to cholesterol biosynthesis in rats receiving a single oral dose of KCD-232*

Group	Liver enzyme activity†		
	Thiolase (nmoles/min/mg protein)	Synthase	Reductase (pmoles/min/mg protein)
Control	172 ± 11	2.35 ± 0.11	540 ± 107
KCD-232	192 ± 11	2.81 ± 0.11‡	188 ± 45‡

* Rats kept under an 8:00 p.m. to 8:00 a.m. light cycle received a single oral administration of KCD-232 at 9:00 a.m. and were decapitated at mid-dark around 2:00 p.m. Then, activities of three enzymes were assayed as described in Materials and Methods. Each value represents the mean ± S.E.M. of five rats. The body weight (g, mean ± S.EM.) of rats at sacrifice was 288 ± 4 (control) or 287 ± 4 (KCD-232). The relative liver weight (g/100 g body wt) was 4.01 ± 0.15 (control) or 3.65 ± 0.17 (KCD-232). KCD-232 significantly ($P < 0.025$) reduced the serum cholesterol concentration (mg/dl) from 98 ± 2 (control) to 84 ± 4 (KCD-232).

† Thiolase, acetoacetyl-CoA thiolase (nmoles of acetoacetyl-CoA converted to acetyl-CoA/min/mg protein); Synthase, 3-hydroxy-3-methylglutaryl (HMG)-CoA synthase (nmoles of HMG-CoA synthesized from acetoacetyl-CoA/min/mg protein); Reductase, HMG-CoA reductase (pmoles of mevalonic acid formed from HMG-CoA/min/mg protein).

‡ $P < 0.025$, compared to the control group.

Table 3. Incorporation of [1-^{14}C]acetate and [2-^{14}C]mevalonic acid into digitonin-precipitable sterols in rat liver slices with or without KCD-232 metabolites*

Group	Concn (mM)	[^{14}C]Acetate		[^{14}C]Mevalonic acid	
		(dpm/g liver) × 10^{-5}	% of Control	(dpm/g liver) × 10^{-5}	% of Control
Control		5.65 ± 1.09	100.0	3.15 ± 0.56	100.0
MII	0.1	3.45 ± 0.69	61.1	3.15 ± 0.43	100.0
MII	0.5	0.57 ± 0.13†	10.1	2.04 ± 0.29	64.8
MII	2.0	0.25 ± 0.05†	4.4	1.59 ± 0.22‡	50.5
Nicotinic acid	2.0	6.17 ± 0.91	109.2	2.71 ± 0.33	86.0
Normal§		0.44 ± 0.14†	7.8	1.27 ± 0.20†	40.3

* Rats (230–269 g) maintained under a 4:00 p.m. to 4:00 a.m. light cycle for 2 weeks were decapitated at mid-dark around 10:00 a.m., and the liver was removed. From each liver, five or six (normal) groups of slices weighing 100–120 mg each were prepared and incubated with 1.0 μCi of [1-^{14}C]acetate (1 mCi/mmole) or 0.4 μCi of DL-[2-^{14}C]mevalonic acid (1.6 mCi/mmole) in the absence (control, normal) or presence of drugs. The incorporation of labeled substrates into digitonin-precipitable sterols was determined as described in Materials and Methods. Each value represents the mean ± S.E.M. of five or six (normal) rats.

† $P < 0.01$, compared to the control group.

‡ $P < 0.05$, compared to the control group.

§ Slices of this group were obtained from rats (225–264 g) kept under a 6:00 a.m. to 6:00 p.m. light cycle.

Table 4. Sterol synthetic rate, measured using [^{3}H]water, and 3-hydroxy-3-methylglutaryl-coenzyme A reductase activity, in rat hepatocytes with or without MII*

Group	Sterol synthetic rate (nmoles H_2O/hr/10^7 cells)	HMG-CoA reductase (pmoles/min/mg protein)
Control	4.83 ± 0.30	59.5 ± 6.4
MII	2.29 ± 0.17†	35.2 ± 3.3‡

* Hepatocytes (1×10^7 cells) isolated from rats kept under a 4:00 p.m. to 4:00 a.m. light cycle were incubated for 1 hr in the absence or presence of 1 mM MII. The incorporation of ^{3}H from [^{3}H]water into sterols and the activity of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase were determined as described in Materials and Methods. Each value represents the mean ± S.E.M. of five (sterol synthetic rate) or six (HMG-CoA reductase activity) incubations.

† $P < 0.001$, compared to the control group.

‡ $P < 0.01$, compared to the control group.

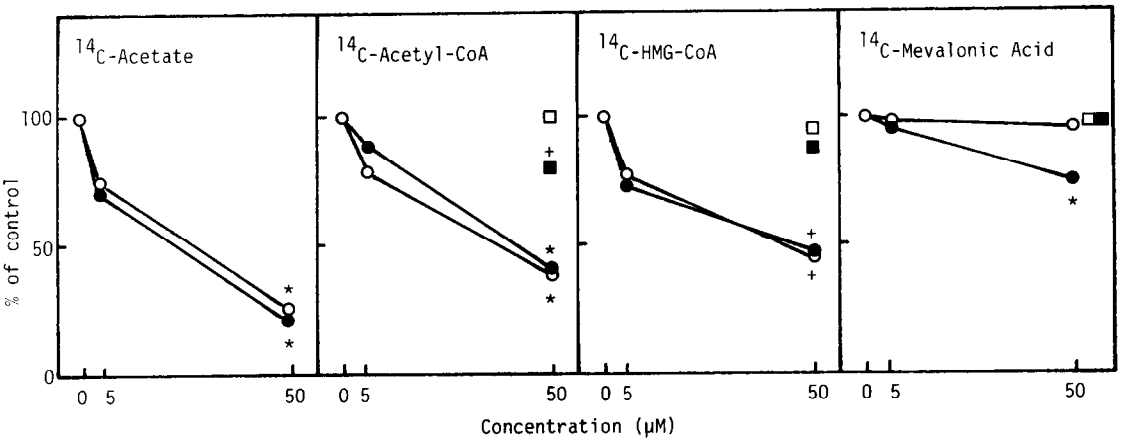

Fig. 3. Incorporation of various radiolabeled substrates into nonsaponifiable lipids in cell-free enzyme system from rat liver in the absence or presence of MII, MII-CoA, clofibric acid or free CoA. Liver microsomes and cytosolic enzyme fraction were isolated from rats kept under a 4:00 p.m. to 4:00 a.m. light cycle. The reaction mixture (0.2 ml) was incubated for 1 hr with MII (○), MII-CoA (●), clofibric acid (□) or CoA (■). The incorporation of [1-^{14}C]acetate, [1-^{14}C]acetyl-CoA, DL-[3-^{14}C]3-hydroxy-3-methylglutaryl (HMG)-CoA or DL-[2-^{14}C]mevalonic acid into nonsaponifiable lipids (NSL) was determined as described in Materials and Methods. Each value represents the mean of triplicate analyses except for the control group. Control values for the incorporation of [^{14}C]acetate, [^{14}C]acetyl-CoA, [^{14}C]HMG-CoA and [^{14}C]mevalonic acid into NSL were 2490 ± 180 (4), 1452 ± 55 (4), 466 ± 51 (4) and 85,597 ± 665 (5) dpm/hr/mg protein (mean ± S.E.M. for analyses indicated in parentheses) respectively. Key: (*) $P < 0.001$ and (†) $P < 0.05$, respectively, compared to the incorporated radioactivity of the control group.

for 50% inhibition was 2.02 mM. In dispersed hepatocytes (Table 4), MII significantly inhibited the absolute synthetic rate of sterols by 53% and the activity of HMG-CoA reductase by 41% at a concentration of 1 mM. From these results, MII was shown to be a KCD-232 metabolite which caused the suppression of hepatic sterol synthesis. In addition, the main site of inhibitory action of MII was suggested to exist in a step(s) before mevalonic acid formation.

To examine the effects of MII and its intracellular metabolite MII-CoA on sterol synthetic activity, the incorporation of various radiolabeled substrates into nonsaponifiable lipids was determined in a cell-free enzyme system from rat liver (Fig. 3). Both MII and MII-CoA equally inhibited the incorporation of [^{14}C]acetate, [^{14}C]acetyl-CoA and [^{14}C]HMG-CoA. The incorporation of [^{14}C]mavalonic acid was not inhibited at all by MII, whereas it was inhibited slightly (24%) by MII-CoA at 50 μM. Clofibric acid which has been reported to inhibit competitively HMG-CoA reductase [20] did not affect at all the incorporation of [^{14}C]acetyl-CoA, [^{14}C]HMG-CoA and [^{14}C]mevalonic acid into nonsaponifiable lipids at a concentration of 50 μM. CoA reduced the incorporation of [^{14}C]acetyl-CoA by 20% and that of [^{14}C]HMG-CoA by 14% at 50 μM, while it had no effect on that of [^{14}C]mevalonic acid at this concentration.

DISCUSSION

KCD-232 exhibits hypocholesterolemic activity [2, 3] by decreasing the serum very low- and low-density lipoprotein levels and, slightly, the high-density lipoprotein level in normal rats [2]. In the present study, KCD-232 was confirmed to reduce the serum total Ch level in a dose-dependent manner (Fig. 1). KCD-232 inhibited *in vivo* sterol syntheses from [^{14}C]acetate and [^{3}H]water, while it failed to inhibit that from [^{14}C]mevalonic acid (Fig. 2 and Table 1). These results indicate that KCD-232 actually inhibited sterol synthesis most likely before the step(s) of mevalonic acid formation. This assumption is supported by the results shown in Table 2 where HMG-CoA reductase activity was decreased by a single oral administration of KCD-232. The inhibitory effect of KCD-232 on sterol synthesis was attributable not to nicotinic acid but to MII (Table 3). MII was thought to be formed by oxidation of 4-(4'-chlorobenzyloxy)benzyl alcohol which was derived from hydrolysis of KCD-232 and could not be detected in the serum. The concentration of MII required for 50% inhibition of sterol synthesis from [^{14}C]acetate in liver slices was 130 μM, which was found to be less than half of the MII level in the liver 3 (0.30 μmole/g ≈300 μM) or 7 (0.35 μmole/g ≈ 350 μM) hr after a single oral dose of KCD-232 at 100 mg/kg body weight in normal rats. On the other hand, the MII concentration required for 50% inhibition of sterol synthesis from [^{14}C]mevalonic acid in liver slices was 2020 μM, which was much higher than the MII level in the liver, suggesting a minor role *in vivo* of MII in the inhibition of sterol synthesis at a step(s) after mevalonic acid formation. The inhibitory effects of MII on sterol synthetic activity and HMG-CoA reductase were further verified in hepatocytes (Table 4).

Usual fatty acyl-CoA thioesters have been reported to inhibit not only the activities of acetyl-CoA carboxylase [21] and fatty acid synthetase [22], enzymes related to fatty acid biosynthesis, but also

that of HMG-CoA reductase [23]. Therefore, to examine the possible inhibitory effect of MII-CoA on HMG-CoA reductase and confirm the site(s) of MII-derived inhibitory action on sterol synthesis, the incorporation experiments were performed in a cell-free enzyme system from rat liver (Fig. 3). MII itself, as well as MII-CoA, equally inhibited sterol synthetic activity at the step catalyzed by HMG-CoA reductase. The inhibitory effects of MII and CoA were not synergestic for that of MII-CoA. Hence, MII as such or MII moiety of MII-CoA appears to play a significant role in the inhibition of the reductase. MII-CoA, however, showed a weak inhibition on sterol synthetic activity at a step(s) following mevalonic acid formation, being consistent with a possible second site of action beyond mevalonic acid. The latter inhibition, however, appears to play only a minor role in the inhibition of sterol synthesis *in vivo*, since the *in vivo* incorporation of [^{14}C]mevalonic acid into sterols was not suppressed by the oral administration of KCD-232 (Fig. 2).

In rodents such as mice and rats, compactin, a specific inhibitor of HMG-CoA reductase, has been reported to lack in hypocholesterolemic activity in normolipidemic animals of these species that had received multiple doses of the drug and these animals showed a marked increase in hepatic levels of HMG-CoA reductase [24]. KCD-232, on the other hand, significantly reduced the serum/plasma Ch levels (Fig. 1 and Table 1) and hepatic synthetic rate of sterols (Table 1) when multiple doses of the drug were given. KCD-232 is therefore different from compactin in its mode of action on Ch metabolism. In our studies using microsomal suspensions and assessing changes in microsomal HMG-CoA reductase activity, MII and MII-CoA essentially had no direct inhibitory effects on the reductase (data not shown). Nevertheless, KCD-232 administration inhibited the reductase activity (Table 2). It is therefore concluded that KCD-232, unlike compactin, inhibits the reductase through some indirect mechanism(s), for example, an involvement in phosphorylation (inactive form)-dephosphorylation (active form) of HMG-CoA reductase [25]. In addition to the inhibition of Ch synthesis, KCD-232 has an inhibitory effect on Ch absorption [2]. Accordingly, possible modes of action of KCD-232 on the serum Ch level are considered as follows: the two inhibitory actions of KCD-232 on Ch absorption and synthesis lead to a reduction of body Ch pool size, which results in a reduction of the serum Ch level. Its hypotriglyceridemic action [1, 4] may play a role in the reduction of the serum Ch level.

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