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EFFECT OF ERITADENINE ON CHOLESTEROL METABOLISM IN THE RAT

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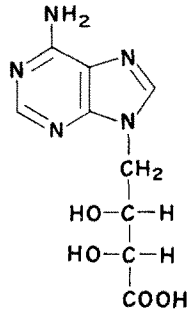
Abstract—Eritadenine, a hypocholesterolemic adenine derivative occurring in the Japanese mushroom *Lentinus edodes*, had no effect on hepatic cholesterol biosynthesis from 1-^{14}C-acetate and 2-^{14}C-mevalonate but accelerated tissue uptake of plasma cholesterol.

ERITADENINE is a hypolipidemic substance[1] isolated from the mushroom "shiitake" (*Lentinus edodes*) and its chemical structure has been identified as 2 (R), 3 (R)-dihydroxy-4-(9-adenyl) butyric acid by two independent research groups.[2,3]



Eritadenine

When eritadenine was administered orally to normal rats, it lowered all the lipid components (cholesterol, triglyceride and phospholipid) of plasma lipoproteins.[4,5] Dietary hypercholesterolemia in the rat was also suppressed by eritadenine.[4,5]

The present investigation was undertaken to study the effect of eritadenine on cholesterol metabolism in the rat in an attempt to elucidate its mechanism of action. In preliminary experiments, eritadenine showed no effect on fecal excretion of radioactive neutral sterols and bile acids after oral or intravenous administration of 4-^{14}C-cholesterol. Therefore, we investigated effects of eritadenine on hepatic cholesterol biosynthesis from 1-^{14}C-acetate and 2-^{14}C-mevalonate and the plasma level of labelled cholesterol after intravenous injection of 2-^{14}C-mevalonate and 4-^{14}C-cholesterol-labelled rat serum.

MATERIALS AND METHODS

Chemicals. Eritadenine was synthesized by Okumura *et al.*[6] of the Chemical Research and Development Laboratory of Tanabe Seiyaku Co., Ltd. Sources and specific

radioactivities of the labelled compounds used were as follows: 4-^{14}C-cholesterol 59·2 mCi/mmole (The Radiochemical Center, Amersham), 1-^{14}C-sodium acetate 50·0 mCi/mmole (Daiichi Pure Chemicals Co. Ltd., Tokyo), and DL-2-^{14}C-mevalonic acid lactone 6·33 mCi/mmole (Daiichi Pure Chemicals Co. Ltd., Tokyo). DL-2-^{14}C-mevalonate was obtained by warming the lactone in 0·001 M $NaHCO_3$ at 37° for 30 min immediately before use.

Animal experiments. Male Sprague–Dawley rats (Nihon CLEA Co. Ltd., Tokyo) were maintained on commercial laboratory chow (Nihon CLEA CE-2 pellets). When test compounds were administered in the diet, they were mixed with the powder chow (Nihon CLEA CE-2 powder) and fed *ad lib.* from powder-feeding dishes (Natsume Seisakusho Co. Ltd., Tokyo), while control rats were fed the powder chow only.

When small amounts (less than 0·4 ml) of blood were needed for determination of serum cholesterol or blood radioactivity before and during an experiment, blood samples were obtained from the tail tip where a small cut was made with a razor blade under light ether anesthesia.

4-^{14}C-Cholesterol-labeled rat serum was obtained by intravenously injecting a 140 g rat with 4-^{14}C-cholesterol (33·5 μCi, 74·3 × 10^6 dis/min, suspended in 0·5 ml saline plus one drop of Tween 80) and withdrawing blood from the abdominal aorta 5 hr after the injection of the isotope. The serum contained 1·463 × 10^6 dis/min per ml.

Isolation and chemical determination of cholesterol. Liver tissue (500 mg wet wt) was digested by refluxing in 4 ml of 10% KOH in 90% ethanol for 90 min and then extracted twice with 8 ml of petroleum ether. The combined extracts were evaporated to dryness and the residue was dissolved in 2–3 ml of ethanol–acetone (1:1). Cholesterol was then precipitated with 1% digitonin in 50% ethanol by the method of Sperry and Webb.[7] The digitonide was dissolved in 0·5 ml of methanol and used for radioactivity measurement and chemical assay of cholesterol. Total cholesterol in rat serum (0·05 ml) and cholesterol of the digitonide were determined by the method of Zak.[8]

When the appearance of ^{14}C-cholesterol in plasma after injection of 2-^{14}C-mevalonate was pursued, radioactivity of the combined petroleum ether extracts of the alkaline digest of 0·05–0·15 ml serum (the volumes of 10% KOH in 90% ethanol and petroleum ether were 1 and 2 × 2 ml, respectively) was counted without isolation of cholesterol with digitonin.

Preparation of samples for measurement of radioactivity. Radioactive samples were counted in a liquid scintillation spectrometer equipped with an automatic monitoring system for quenching. Samples for counting were prepared in the following ways: 20 μl of blood was warmed in 0·2 ml of 60% NHO_3–30% H_2O_2 (8:1) at 50° in a counting vial until the reddish color disappeared and then to this was added 15 ml of toluene–ethanol (1:1) scintillation fluid containing 0·7% DPO and 0·005% POPOP; 20–50 μl of serum was dissolved in 1 ml of Hyamine (hydroxide, 10-X, Packard) at 40° and mixed with 15 ml of the scintillation fluid; the combined petroleum ether extracts of the alkaline digest of serum were washed once with water (pH 8–9), transferred quantitatively into a vial, evaporated to dryness and dissolved in 15 ml of the scintillation fluid; 500 mg of liver tissue was homogenized in 8 ml chloroform–methanol (2:1), the homogenate filtered, 2 ml of the

filtrate (10 ml) evaporated to dryness, and the residue dissolved in 15 ml of the scintillation fluid; 0·5 ml of cholesterol digitonide solution in methanol was mixed with 15 ml of toluene containing 0·4% DPO and 0·01% POPOP.

RESULTS

The effect of eritadenine on hepatic cholesterol biosynthesis was studied by estimating the incorporation of the labeled precursors, 1-^{14}C-acetate and 2-^{14}C-mevalonate, into hepatic cholesterol *in vivo* in eritadenine-fed and control rats. There was no significant difference between the two groups either in the level of radioactivity incorporated into cholesterol per gram of liver or in the specific radioactivity of hepatic cholesterol with either of the two precursors used, while clofibrate markedly depressed the incorporation of labeled acetate into cholesterol (Table 1).

Tissue uptake of plasma cholesterol was examined by intravenously injecting 4-^{14}C-cholesterol-labeled rat serum into rats and then following the disappearance of radioactivity from the circulation for 1 hr. This disappearance of blood radioactivity can be taken as a measure of the tissue uptake of plasma cholesterol, since a major fraction of the isotope of the *in vivo* labeled serum is in esterified cholesterol,[9] which, unlike free cholesterol, does not readily exchange with the cholesterol of red blood cells.[10] The experimental group was given eritadenine orally by intubation 5 hr prior to the injection of labeled serum. The isotope in blood decreased at a slightly, but significantly, faster rate in the experimental group than in the control group (Fig. 1). When the animals were sacrificed two hours after the injection, the radioactivities remaining in plasma were calculated to be 24·3 ± 2·2% of the dose for the experimental group and 33·6 ± 2·8% for the control

TABLE 1. EFFECTS OF ERITADENINE AND CLOFIBRATE ON HEPATIC CHOLESTEROL SYNTHESIS IN RATS

Precursors	Addition in diet	Incorporation of ^{14}C into liver cholesterol (Means ± S.E.M.)	
		dis/min/g liver	dis/min/mg cholesterol
1-^{14}C-Acetate	None	6492 ± 1948	3300 ± 1010
	Eritadenine 0·02%	6372 ± 1796	3410 ± 660
	Clofibrate 0·2%	910 ± 132	550 ± 90
2-^{14}C-Mevalonate	None	12,728 ± 2556	7070 ± 1380
	Eritadenine 0·02%	12,644 ± 2664	6200 ± 1300
	Clofibrate 0·2%	6232 ± 1680	3850 ± 1280

1-^{14}C-Acetate (10 μCi/0·2 mole/100 g body wt) was injected intraperitoneally into three groups of 5 rats (*ca.* 200 g) on the last day of the 7-day dietary period. Fifteen min after injection the rats were anesthetized with ether and bled from the femoral artery, and the livers were quickly excised. 2-^{14}C-Mevalolactone (1·5 μCi/rat) was injected intravenously into three groups of 5 rats (*ca.* 120 g) on the last day of the 7-day dietary period. Four min after injection the animals were sacrificed as described above. Liver cholesterol was isolated and its quantity and radioactivity were determined as described in the text.

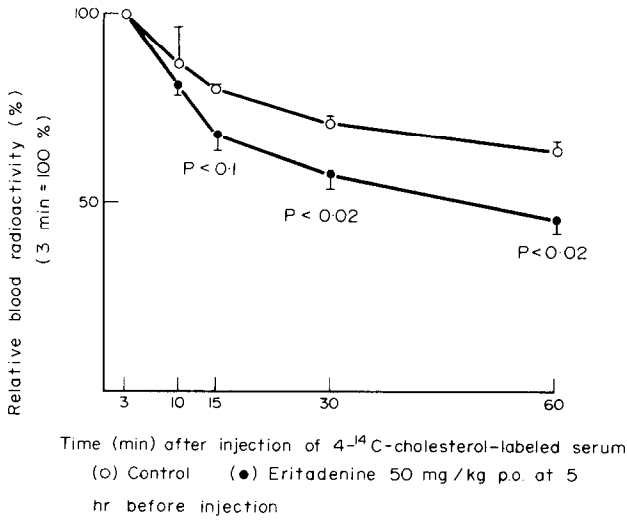


FIG. 1. Effect of eritadenine on the disappearance of 4-^{14}C-cholesterol from the circulation after injection of 4-^{14}C-cholesterol-labeled rat serum into rats. Two groups of four rats (*ca.* 120 g) were fasted for 24 hr, and the experimental rats were orally intubated with an eritadenine solution (50 mg/10 ml/kg) and the control rats with saline (10 ml/kg). Five hr after intubation of eritadenine or saline all the rats were injected in the tail vein with 4-^{14}C-cholesterol-labeled rat serum (146,400 dis/min/0·1 ml/rat; see the text for its preparation). Blood samples were taken from the tail tip at indicated times and their radioactivities were measured as described in the text.

group (the calculation was based on an assumed plasma volume of 3·42% of body wt[11]), while those in liver were 23·3 ± 1·9% and 23·6 ± 2·5% of the dose, respectively.

An attempt to estimate the release of plasma cholesterol from the liver was made by following the appearance of radioactivity in plasma cholesterol after injecting 2-^{14}C-mevalonate into rats. The radioactivity of serum cholesterol rose

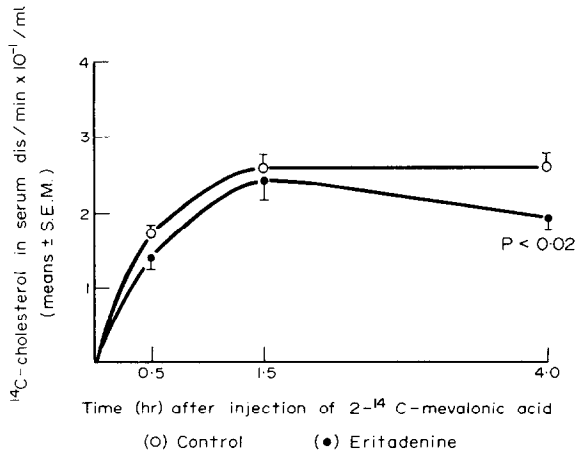


FIG. 2. Effect of eritadenine on the level of serum 4-^{14}C-cholesterol after injection of 2-^{14}C-mevalonate into rats. Thirteen rats (*ca.* 150 g) were fasted for 22 hr. Five experimental rats were orally intubated with an eritadenine solution (200 mg/kg) and eight control rats were intubated with water (10 ml/kg). Two hr after receiving eritadenine or water, all the rats were injected in the tail vein with DL-2-^{14}C-mevalonate (1·48 μCi/rat). Blood samples were taken from the tail tip at indicated times and radioactivity of the serum cholesterol fraction was determined as described in the text.

rapidly up to 40 min after the injection in both eritadenine-treated and control groups, and thereafter it leveled off (Fig. 2). There was no significant difference between the two groups in the rate of the increase in plasma ^{14}C-cholesterol. Four hr after the injection, however, the isotope level in the experimental group was past its peak value and significantly lower than the level in the control group, which was still at its peak level. At this time the amounts of hepatic radioactive cholesterol (purified as digitonide), i.e. the cholesterol synthesized from 2-^{14}C-mevalonate and remaining in this main production site of cholesterol, were nearly equal in the control ($110{\cdot}0 \pm 10{\cdot}2 \times 10^2$ dis/min per g liver) and eritadenine ($114{\cdot}7 \pm 7{\cdot}3 \times 10^2$ dis/min per g liver) groups.

DISCUSSION

The plasma cholesterol level will decrease if the flow of cholesterol into the plasma compartment decreases and/or the transfer of cholesterol out of the plasma compartment increases. This could arise by one of the following mechanisms; (1) inhibition of intestinal absorption of cholesterol, (2) inhibition of cholesterol biosynthesis, (3) acceleration of fecal steroid excretion and, (4) shifting of the equilibrium of cholesterol between plasma and tissues toward the latter without a net change in the total cholesterol content in the body.

Eritadenine did not lower the hepatic cholesterol level in rats maintained on the normal cholesterol-low diet, and its hypocholesterolemic effect was apparent even when rats were fed the hypercholesterolemic diet which should have suppressed cholesterol biosynthesis to a minimum.[4,5] Evidence presented in Table 1 indicates that the mechanism of the hypocholesterolemic action of eritadenine is not an inhibition of cholesterol biosynthesis.

The bile acid sequestering agent cholestyramine is known to be ineffective in reducing the serum cholesterol level in the rat, since this specis can easily maintain the normal cholesterol level by a compensatory increase in cholesterol biosynthesis.[12,13] Therefore, the lack of a stimulatory effect on cholesterol biosynthesis in the rat treated with eritadenine could be taken as indirect evidence that the hypocholesterolemic effect of eritadenine is not due to an increase in cholesterol catabolism or fecal steroid excretion. Similarly, inhibition of intestinal absorption of dietary cholesterol is not the likely mechanism. Eritadenine is effective even in fasted rats.[4]

It is possible therefore, that eritadenine may shift the equilibrium of cholesterol between plasma and tissues towards the latter by influencing the processes which are more or less directly involved in the transfer of cholesterol into and/or out of the plasma compartment (mechanism (4)). Since the total quantity of plasma cholesterol is much less than the total amount of cholesterol present in the liver only, a substantial alteration in the plasma level by the equilibrium shift may not significantly influence the tissue levels of cholesterol. Such an equilibrium shift by eritadenine is suggested by somewhat faster disappearance of radioactive cholesterol from the circulation in eritadenine-treated rats than in control rats whether the isotope injected was in the form of ^{14}C-cholesterol-labeled rat serum or 2-^{14}C-mevalonate (Figs. 1 and 2). It cannot be decided from those data whether the faster decline of serum ^{14}C-cholesterol is due to a stimulated tissue uptake or an inhibition of ^{14}C-cholesterol release (recycling) from the tissues. At least one can deduce from Fig. 2 that

the release of newly synthesized cholesterol into plasma is not significantly inhibited by eritadenine. From the therapeutic view-point, eritadenine would be useful only if blood vessel walls are not involved in the equilibrium shift.

The precise mechanism of the action of eritadenine in shifting the equilibrium of plasma cholesterol towards tissues has yet to be determined. In view of its chemical structure it may interfere with the metabolic functions of adenine nucleotides. The effects of eritadenine on the metabolic processes influenced by cyclic AMP are being investigated in this laboratory.

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