Effect of policosanol on the hepatic cholesterol biosynthesis of normocholesterolemic rats

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We have suggested previously, measuring ^{14}C-acetate incorporation into free cholesterol, that oral administration of policosanol inhibits hepatic cholesterol biosynthesis in rats. Nevertheless, since acetate has limitations to study cholesterol synthesis ***in vivo****, we now investigate rates of incorporation of labeled water into hepatic sterol after policosanol treatment. Absolute rates of incorporation of ^{3}H-water in sterols were depressed by policosanol by about 20%, giving a more accurate degree of cholesterol biosynthesis inhibition in this species. Since policosanol did not inhibit labeled mevalonate incorporation into cholesterol in rat liver, we also studied the effect of policosanol on hydroxymethylglutaryl-coenzyme A (HMG-CoA) reductase. Reductase activity assayed in microsomes treated with policosanol remained unchanged, suggesting that cholesterol synthesis is not inhibited by a direct action of policosanol on this enzyme.*

Key terms: *cholesterol biosynthesis, hepatic sterol, policosanol, rat liver.*

INTRODUCTION

Policosanol is a defined mixture of high molecular weight aliphatic alcohols isolated from sugar cane (*Saccharum officinarum*, L.) wax, wherein octacosanol is its main component.

Previous studies have demonstrated cholesterol-lowering effects induced by policosanol in experimental models (4, 5, 8, 17), human healthy volunteers (2, 10) and type II hypercholesterolemic patients (3, 7, 15, 16, 18).

Recent studies have shown that policosanol depressed ^{14}C-acetate incorporation into total cholesterol of cultured fibroblasts, meanwhile the radioactivity incorporated from ^{14}C-mevalonate was not inhibited. These facts suggest an inhibition of cholesterol biosynthesis *in vitro* at some step prior to mevalonate generation (13).

Oral administration of policosanol (0.15-1 g/kg) to normocholesterolemic rats for one month inhibited radioactivity incorporation from ^{14}C-acetate into hepatic free cholesterol, whereas the incorporation from labeled mevalonate was not depressed. These results suggest that policosanol also inhibits cholesterol biosynthesis *in vivo* prior to mevalonate formation (14). However, it is known that ^{14}C-acetate is not a useful substrate for estimating absolute rates of cholesterol biosynthesis *in vivo*. Limitations of acetate are either related to the rate of entry of this substrate into the cell and its partially rate-limited step in its metabolism to acetyl-CoA or to errors associated with the intracellular pool of acetyl-CoA (1, 9).

The rates of cholesterol biosynthesis can be quantitated by incorporation of ^{3}H-water. This substrate rapidly penetrates cell membranes and attains a uniform and constant

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specific activity (SA) in total body water (11, 19).

The present study was undertaken using ^{3}H-water to quantitate the effect of oral administration of policosanol (500 mg/kg) for one month on hepatic cholesterol biosynthesis in normocholesterolemic rats. Also, considering that the inhibition on the cholesterol biosynthetic pathway occurs prior to mevalonate generation, the effect of policosanol on hydroxymethylglutaryl-coenzyme A (HMG-CoA) reductase was also investigated.

MATERIALS AND METHODS

Animals

Male Wistar rats weighing 150 to 180 g at the beginning of the experience were used. Animals were conventionally kept under laboratory conditions a week before starting policosanol treatment. In this period standard rat chow from CENPALAB (*Centro Nacional para la Producción de Animales de Laboratorio, La Habana, Cuba*) and water were allowed *ad libitum*. After this period, rats were allocated randomly to the two experimental groups (10 animals per group).

Policosanol was orally administered as a suspension in Acacia gum/water (10 mg/ml) by gavage (1 ml/100 g body weight). Control animals received only vehicle, while treated animals received policosanol (500 mg/kg) for 4 weeks.

Effect of policosanol on cholesterol biosynthesis ***in vivo***.

Five animals each from the control and the treated group were used. Animals were administered ^{3}H-water intravenously as a bolus (200 mCi/kg) using the penile vein. Following the injection, the animals were returned to individual cages and killed after 1 h. During this period they received neither food nor water. Blood (5 ml) from the abdominal aorta was taken and specimens of the liver were removed and rinsed in cold saline. Three slices (2-3 mm thick) were cut, which were again rinsed in cold isotonic saline. Blood samples were centrifuged to obtain plasma for determining SA of plasma water. The slices were saponified in alcoholic KOH and radiolabeled sterols were determined essentially as described by Jeske and Dietschy (11); non-saponifiable lipids were extracted into petroleum ether-acetone from which digitonin precipitable sterols (DPS) were obtained. The radioactivity of DPS was determined in a Rackbeta (LKB) scintillation spectrometer by adding 15 ml of Cocktail T (BDH). The value of SA of body water (cpm of ^{3}H/nmol of water) and the rate of hepatic sterol synthesis (μmol of ^{3}H-water incorporated into DPS/h/g of tissue) were calculated according to Spady and Dietschy (19) and Turley *et al* (20).

Effect of policosanol on HMG-CoA reductase activity

Preparation of liver microsomes. Two aliquots of the liver (approximately 1.5 g) of a control rat were weighed and placed 9:1 (v:w) into cold isotonic 50 mM Tris-HCl buffer, pH 7.4, containing 0.3 M sucrose, 10 mM EDTA and 10 mM dithiothreitol. Microsomes were prepared in presence of fluoride (50 mM NaF) to capture the expressed activity of reductase and in fluoride free buffer (50 mM NaCl) to obtain total reductase activity (6) by differential centrifugation. The washed microsomal fraction was suspended in the homogenizing buffer.

Assay of HMG-CoA reductase activity. Microsomal reductase activity was assayed according to Brown et al (6). The microsomal fraction (100-200 μg of protein) was preincubated for 15 min at 37° C in a total volume of 100 μl containing phosphate buffer 100 mM, pH 7.4, 100 mM imidazole, 5 mM dithiothreitol, 10 mM EDTA, 0.5 units of glucose-6-phosphate dehydrogenase and 3 mM NADP. Microsomes were preincubated with or without policosanol (5 and 50 μg/ml in a vehicle Tween-20 0.4%; controls were added similar volumes of detergent solution). The assay was initiated by adding 10 μl of [3-^{14}C] HMG-CoA (30 μM, 0.01 μCi). After 60 min the incubation was stopped by the addition of 25 μl 2 M HCl. Tritium labeled mevalonic acid (0.01 μCi) was added together with unlabeled mevalonic acid lactone (10 mM) and the incubation mixture was allowed

to stand 30 min at 37°C for complete lactonization. Then, the incubation mixture was subjected to thin-layer chromatography with benzene-acetone (1:1, v:v) as developing solvent. The mevalonic acid lactone zone was scrapped off into counting vials and 15 ml of Cocktail T (BDH) was added. A RACKBETA liquid scintillation spectrometer was used to determine radioactivity. Corrections for losses were made by the internal standard. Protein was determined by Lowry's method (12). In all the experiments, enzyme assays were carried out in triplicate.

Statistical analysis

Comparisons between treated and control groups were performed using non parametric Mann Whitney *U* tests.

RESULTS AND DISCUSSION

Table I shows the incorporation of tritiated water into DPS of control and treated animals. As can be seen, the incorporation of radioactivity into sterols is significantly lower after oral administration of policosanol, demonstrating that policosanol orally administered for one month inhibits hepatic cholesterol biosynthesis from tritiated water in normocholesterolemic rats. These results agree with previous studies in this species, where an inhibition of cholesterol biosynthesis from ^{14}C-acetate was observed after policosanol administration at the same doses (14). However, differences between the results with regard to the magnitude of the absolute inhibition occur, since inhibition of cholesterol biosynthesis from labeled acetate reached near 40%, meanwhile in the present study an inhibition of about 20% was observed.

The above difference could be due to methodological conditions. Thus, cholesterol biosynthesis from acetate was estimated from the incorporation of radioactivity into hepatic free cholesterol and was expressed as a percentage of the whole lipid extract content of radioactivity. In contrast, the rate of cholesterol biosynthesis from tritium labeled water was assessed by measuring the velocity at which [^{3}H]-water was incorporated into DPS in the intact animal and was expressed as the absolute rate of radioactivity incorporated into total sterols by estimating the SA of body water.

Taking into consideration the limitation of ^{14}C labeled acetate to determine the absolute biosynthesis of cholesterol, present results may provide a more accurate determination of the extent of inhibition of hepatic cholesterol biosynthesis after policosanol treatment.

Previous results in different experimental models have shown that policosanol inhibited cholesterol biosynthetic pathway prior to mevalonate generation, suggesting an effect on HMG-CoA reductase, the key enzyme of cholesterol synthesis (13, 14).

However, the experiments performed to examine whether policosanol suppressed preformed reductase directly in microsomes showed that, when policosanol was added to the reductase assay at concentrations ranging from 5 to 50 µg/ml, the enzyme activity

TABLE I

Hepatic cholesterol biosynthesis from tritiated water in controls and animals treated with policosanol

Treatment	^{3}H-water incorporated into DPS (µmol/h/g tissue)					
	Animal					
	1	2	3	4	5	$\bar{x} \pm$ SD
Controls	1.26	2.05	2.45	1.94	1.58	1.85 ± 0.45
Policosanol	1.46	1.40	1.38	1.15	1.45	1.36 ± 0.12*

Rats orally administered with policosanol (500 mg/kg) or vehicle (controls) for one month.
Cholesterol biosynthesis measured through ^{3}H-water incorporation into digitonin-precipitable sterols.
* Statistically significant differences ($p < 0.05$).

TABLE II

Effect of policosanol on HMG-CoA reductase activity of hepatic microsomes

Treatment	n	HMG-CoA reductase activity (pmol/mg/min)		
		Total	Expressed	Ratio E/T
Control	3	18.94 ± 4.14	6.40 ± 2.09	0.33
Policosanol 5 µg/ml	3	19.94 ± 3.86	6.80 ± 2.02	0.34
Policosanol 50 µg/ml	3	21.00 ± 4.91	8.62 ± 1.44	0.41

Microsomes prepared from livers of non treated rats in presence of fluoride to capture expressed activity of reductase and in fluoride free buffer to obtain total reductase activity. Reductase assay performed as described in Text. Results, means ± SEM's of triplicates.

remained unchanged (Table II). Thus, there was no evidence of enzyme inhibition induced by policosanol. Also, policosanol addition does not interfere in the state of activation of the enzyme, since no major differences were observed in the fraction of expressed activity in control and policosanol-treated hepatic microsomes (Table II). These results suggest that policosanol does not act on the reversible phosphorylation of the enzyme.

In conclusion, our results are consistent with the inhibitory effect of policosanol on hepatic cholesterol biosynthesis from [^{14}C]-acetate. They also suggest that this effect is not elicited by a direct action of policosanol on HMG-CoA reductase. Further research on the effect of policosanol on the cholesterol biosynthetic pathway is required to determine the primary site of action of policosanol.

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