

Cholesterol-lowering effect of policosanol on rabbits with hypercholesterolaemia induced by a wheat starch–casein diet

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The effect of policosanol, a mixture of high-molecular-weight aliphatic alcohols isolated from sugar-cane wax, on casein-induced hypercholesterolaemia in rabbits was studied. When policosanol was administered by the oral route once daily for 30 d (50 mg/kg) the increases in plasma total cholesterol and LDL-cholesterol (LDC-C) were significantly reduced when compared with the control group. The incorporation of $^3\text{H}_2\text{O}$ into sterols in the liver was significantly depressed, suggesting inhibition of hepatic cholesterol biosynthesis. The oral administration of policosanol raised the rate of removal of ^{125}I -labelled LDL from serum. Kinetic parameters calculated following injection of [^{125}I]LDL showed that in casein-fed rabbits, the terminal half-life ($t_{1/2}$) was significantly decreased after policosanol treatment. The hepatic LDL-binding activity was increased after policosanol administration which suggested that the enhanced clearance was due, at least in part, to increased receptor-mediated uptake of LDL by the liver. Considered together, these results suggest that policosanol can significantly reduce the increase of plasma LDL-C in rabbits fed on a wheat starch–casein diet by reducing cholesterol biosynthesis in the liver. Such an effect could account for the enhancement of LDL catabolism through the receptor-mediated pathway.

Policosanol: Hypercholesterolaemia: Cholesterol biosynthesis: LDL

Policosanol is a mixture of high-molecular-mass aliphatic alcohols isolated and purified from sugar cane (*Saccharum officinarum* L.) wax. Octacosanol (M_r 410.7) is its major component, followed by triacontanol (M_r 438.5) and hexacosanol (M_r 382.4) while other alcohols (tetracosanol, heptacosanol, nonacosanol, dotriacontanol and tetratriacontanol) are minor components.

Previous studies have demonstrated that serum cholesterol levels of pigs are significantly decreased by supplementation of the diet with policosanol (Cruz-Bustillo *et al.* 1991). Also, oral administration of policosanol decreases the serum cholesterol level in normocholesterolaemic rabbits (Arruzazabala *et al.* 1994), monkeys (Rodríguez-Echenique *et al.* 1994), healthy human volunteers (Hernández *et al.* 1993) and patients with hypercholesterolaemia (Pons *et al.* 1992, 1994; Aneiros *et al.* 1993, 1995; Soltero *et al.* 1993; Canetti *et al.* 1995; Castaño *et al.* 1995a,b; Torres *et al.* 1995).

Evidence has accumulated to support the hypothesis that policosanol inhibits cholesterol biosynthesis and enhances LDL processing. When cultured human fibroblasts were incubated with policosanol, [^{14}C]acetate incorporation into total cellular cholesterol was inhibited. This inhibition was accompanied by increases in LDL binding, internalization and metabolism (Menéndez *et al.* 1994). In rats it was also found that

policosanol inhibited cholesterol biosynthesis, because oral administration of policosanol decreased the incorporation of [^{14}C]acetate into hepatic cholesterol (Menéndez *et al.* 1993).

It is reported that normal rabbits fed on a low-fat, cholesterol-free, semipurified diet containing casein become hypercholesterolaemic (Kroon *et al.* 1982; Khosla *et al.* 1991). Although the exact mechanism for this hypercholesterolaemia remains to be resolved, it is known that the hepatic LDL-receptor activity is reduced in rabbits fed on a wheat starch-casein diet (Chao *et al.* 1983; Samman *et al.* 1989; Khosla *et al.* 1991). Treating these rabbits with mevinolin, a highly potent inhibitor of cholesterol biosynthesis, prevents the increase in plasma and LDL-cholesterol. Such an effect is mediated through increased receptor-dependent catabolism of LDL by the liver (Chao *et al.* 1983).

In the current research we studied the effect of policosanol on hepatic cholesterol biosynthesis in New Zealand rabbits fed on a fat-free, casein-rich diet. We also studied the effect of policosanol on hepatic LDL-binding and the rate of removal of [^{125}I]LDL from serum.

EXPERIMENTAL METHODS

Animals

Young male New Zealand and White rabbits (2.26 (SD 0.28) kg) were obtained from the National Center for Laboratory Animal Production (CENPALAB, Havana, Cuba). The rabbits were maintained on rabbit chow supplied by CENPALAB and feeding was restricted to 100 g/d per animal (Canadian Council of Animal Care, 1980). They were housed in individual cages and adapted to laboratory conditions for 15 d before being transferred to the semipurified diet containing casein, the consumption of which was similar to those described by Kroon *et al.* (1982) (g/kg): wheat flour 333, cellulose 300, casein 270, water 20, maize oil 10, mineral mixture 7. Semipurified diet consumption was restricted to 100 g/d per animal (Kroon *et al.* 1982).

Animals were randomly distributed into two experimental groups (fifteen animals per group) according to their cholesterol levels and were fed on the diet for 30 d. Policosanol (50 mg/kg body weight (BW)) was orally administered as a suspension in a Tween 20-water vehicle (1 ml/kg BW) for 30 d. Control rabbits were given the same volumes of Tween 20-water vehicle. Body weights were recorded on a weekly basis. Fresh food and water were provided daily. All the animals were observed at least once daily to evaluate their health status.

Lipid profile determinations

Blood was drawn from the marginal ear vein on days 0, 15 and 30 of treatment. Total cholesterol and triacylglycerol concentrations in serum and lipoproteins were assayed enzymically using diagnostic kits (Boehringer, Mannheim, Germany). Plasma lipoprotein fractions were obtained by a one-step density-gradient ultracentrifugation technique as described by Terpstra *et al.* (1981). The samples were centrifuged at 20° for 24 h at 40 000 rev./min in a Beckman L-7 ultracentrifuge (Beckman Instruments Inc., Spinco Division, Palo Alto, CA, USA) using a SW 41Ti rotor.

Effect of policosanol on in vivo cholesterol biosynthesis

Experiments were carried out according to Spady & Dietschy (1983). In the present experiment five animals from the control and treated groups were used. Newly synthesized cholesterol was measured in liver after injection of $^3\text{H}_2\text{O}$ (7.4 MBq). At 60 min after injection rabbits were killed by exsanguination and livers were collected. Samples were cut into at least three slices (2–3 mm thick) and the lipids were saponified by incubating in alcoholic KOH for 2 h at 100°. Non-saponifiable lipids were extracted into hexane, and digitonin-precipitable sterols (DPS) were obtained. The specific activity (SA) of body water and the rate of hepatic sterol synthesis were calculated according to the method of Jeske & Dietschy (1980). Cholesterol biosynthesis was expressed as nmol $^3\text{H}_2\text{O}$ incorporated into DPS per h per g tissue (nmol/h per g).

Preparation and labelling of lipoprotein

Before the metabolic and binding studies, blood was obtained from the marginal ear vein of unanaesthetized animals in the fasted state. Plasma was separated and LDL ($1.019 < \rho < 1.063$ g/ml) from wheat starch–casein-fed rabbits was isolated by sequential flotation at 4° using a Ti60 rotor in a preparative Beckman ultracentrifuge as described by Havel *et al.* (1955). Purified LDL was iodinated to a specific activity of 150–200 disintegrations/min (dpm)/ng using a modification of the ICl method of McFarlane (1958). The tracer contained less than 2% as free I as determined by precipitation with TCA.

Effect of policosanol on the removal of [^{125}I]LDL by intact rabbits

Animals were fasted for 12–16 h before the kinetic study. ^{125}I -labelled-LDL (SA 100–200 counts/min (cpm)/ng protein) was injected into control and policosanol-treated rabbits (four animals per group, approximately 148 KBq per animal) through the marginal ear vein. The amount of protein injected per animal was about 0.05% of the circulating pool of LDL protein. Blood samples were drawn from each rabbit at different time intervals for 42 h. The [^{125}I]LDL contained in 500 μl samples of serum was precipitated with TCA (50 ml/l). Radioactivity of the precipitated LDL in the samples was measured with a gamma counter (Miniganma, Pharmacia LKB Biotechnology, Uppsala, Sweden) and was expressed as a percentage of that precipitated from the sample taken 5 min after injection of the labelled LDL. The plasma radioactivity decay curves fitted a two-compartment model and the terminal elimination rate constant (β) was estimated by linear regression of the log-plasma concentration *v.* time curve (see Fig. 2). Plasma elimination half-life ($t_{1/2}$) was determined from β , by the relationship $t_{1/2} = 0.693/\beta$ (Notari, 1980).

Effects of policosanol on [^{125}I]LDL binding in liver homogenates

The heparin-sensitive binding of LDL to liver-tissue homogenates was determined with a filter assay as described by Rudling & Peterson (1985*a,b*). The results, expressed as ng [^{125}I]LDL bound/mg protein, represent heparin sensitive binding calculated by subtracting the radioactivity of the filters incubated with heparin (heparin-resistant binding) from the radioactivity of the filters incubated in the absence of heparin (total binding). The non-specific (heparin-resistant) binding was about 50% of the total binding and the CV of the assay was 12%. Protein determinations were done according to Lowry *et al.* (1951).

Statistical analysis

Comparisons between control and treated animals were performed using the non-parametric Mann–Whitney *U* test.

RESULTS

As can be seen in Table 1 there was a similar weight gain in all the groups. Table 2 lists the lipid profile values during the study. At day 0 there were no significant differences between groups. Control animals maintained on the casein diet had increases in serum total and LDL-cholesterol (LDL-C) over the 30 d period. Although animals treated with policosanol also showed increases in these variables, the values attained were significantly lower than those of the control rabbits. The hypercholesterolaemia exhibited by the control rabbits fed on the casein–wheat starch diet was mainly related to increases in the LDL-C fraction.

The effect of policosanol on hepatic cholesterol synthesis is illustrated in Fig. 1, which shows the content of newly synthesized sterol in the liver of control and treated animals. There was a significant decrease in the content of [³H]DPS in liver of the treated animals compared with the controls.

Table 1. *Body weights (kg) of rabbits fed on a casein-containing diet, at different times during the experiment*

(Mean values and standard deviations for fifteen animals per experimental group)

Day of expt...	0		15		30	
	Mean	SD	Mean	SD	Mean	SD
Control	2.30	0.24	2.55	0.25	2.76	0.24
Policosanol	2.27	0.30	2.49	0.27	2.72	0.30

Table 2. *Effects of policosanol on the serum lipid profile in rabbits fed on a wheat starch–casein diet†*

(Mean values and standard deviations for ten animals per group)

Day of expt...	0		15		30	
	Mean	SD	Mean	SD	Mean	SD
Total cholesterol (mmol/l)						
Control	1.54	0.35	3.63	0.70	8.00	0.80
Policosanol	1.53	0.40	2.46*	0.70	5.21*	0.79
LDL-cholesterol (mmol/l)						
Control	0.43	0.10	3.02	0.32	6.52	0.45
Policosanol	0.40	0.11	1.77	0.23	4.03*	0.33
HDL-cholesterol (mmol/l)						
Control	0.52	0.10	0.46	0.14	0.74	0.34
Policosanol	0.54	0.22	0.45	0.24	0.82	0.16
Triacylglycerols (mmol/l)						
Control	1.19	0.31	1.25	0.90	1.13	0.22
Policosanol	1.43	0.98	1.09	0.50	1.24	0.42

* Mean values were significantly different from those for controls, $P < 0.05$ (Mann–Whitney *U* test).

† For details of diet and procedures, see pp. 924–925.

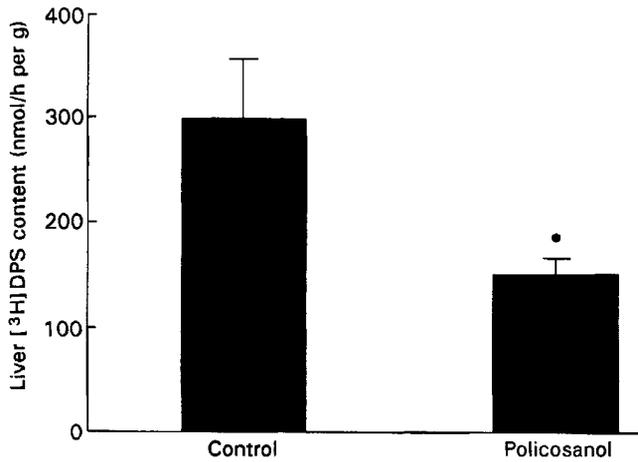


Fig. 1. Content of newly synthesized sterol (measured as digitonin-precipitable sterols, DPS) in liver of control and policosanol-treated rabbits fed on a wheat starch-casein diet. Values are means and standard deviations of five animals. * Mean value was significantly different from that of the control, $P < 0.05$ (Mann-Whitney U test).

Fig. 2 shows that the clearance of [125 I]LDL from the plasma of control hypercholesterolaemic rabbits was slower than that observed in the treated animals. The LDL kinetic parameters calculated from the radioactivity decay curves showed that the clearance of LDL for the rabbits treated with policosanol was considerably faster than the decline of radioactivity observed in the control group. Thus, $t_{1/2}$ was significantly reduced in the treated rabbits when compared with controls (12.6 (SD 1.03) h v. 59.5 (SD 12.42) h, $P < 0.05$).

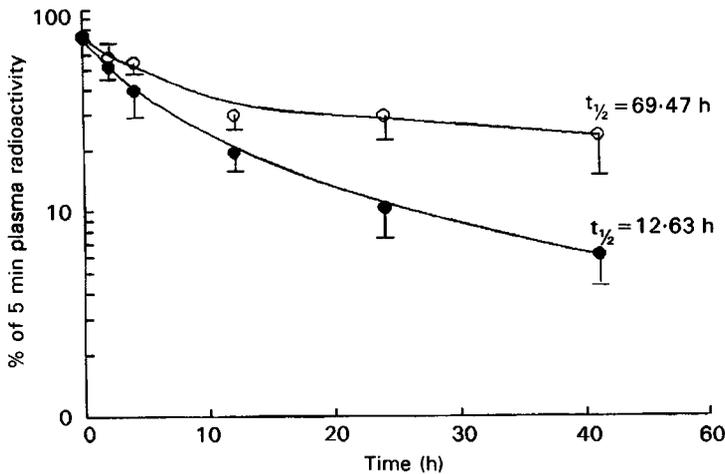


Fig. 2. The decline of plasma radioactivity following the injection of 125 I-native-LDL into controls (○) and policosanol-treated (●) casein-fed rabbits. Each point represents the precipitable-associated radioactivity expressed as percentage of the level 5 min after injection of radio-iodinated LDL. Values are means and standard deviations for four animals per experimental group.

Table 3. *Effect of policosanol on the heparin-sensitive binding of [¹²⁵I]LDL in rabbit liver homogenates†*

(Mean values and standard deviations for homogenates from five rabbits measured in triplicate)

Source of homogenates	[¹²⁵ I]LDL added (µg/ml)	[¹²⁵ I]LDL binding (ng/mg protein)	
		Mean	SD
Control rabbits	12.5	4.00	0.77
Policosanol treated	12.5	22.08*	3.40
Control rabbits	25.0	5.00	0.77
Policosanol treated	25.0	31.85*	6.46

* Mean values were significantly different from those for controls, $P < 0.05$ (Mann-Whitney U test).

† For details of diet and procedures, see pp. 924–925.

Since most plasma cholesterol is internalized into cells by LDL through a well-defined LDL-receptor pathway (Brown & Goldstein, 1986), we investigated the hypothesis that enhancement of LDL clearance is related to increased LDL-receptor activity in hepatic tissue, by determining the binding of labelled LDL to liver homogenates. In these experiments the heparin-releasable binding sites of liver homogenates from hypercholesterolaemic control rabbits were almost abolished, while LDL-binding sites were present in liver homogenates from rabbits treated with policosanol (Table 3).

DISCUSSION

Our results show that oral treatment with policosanol (50 mg/kg) for 30 d significantly reduced hypercholesterolaemia induced in rabbits by a wheat starch-casein diet. Policosanol diminished the increases in serum total and LDL-cholesterol induced by the diet, but levels of triacylglycerols and HDL-cholesterol (HDL-C) were not significantly affected.

Normal rabbits became hypercholesterolaemic when fed on a cholesterol-free wheat starch-casein diet. Since this diet is free of cholesterol, the hypercholesterolaemia is believed to be related to enhanced cholesterol biosynthesis (Kroon *et al.* 1982; Scholz *et al.* 1982), although controversial data on this subject exist (Carroll, 1971; Beynen *et al.* 1986; Khosla *et al.* 1991). It has been proposed that casein induces a rise in LDL concentration by stimulating cholesterol and/or bile acid absorption at the level of the gut. This increases the content of cholesterol in liver leading to a down-regulation of LDL-receptors and later to an increase in the output of cholesterol associated with this lipoprotein (Samman *et al.* 1989; Khosla *et al.* 1991).

Effects of cholesterol-lowering compounds that act through an inhibition of cholesterol biosynthesis have been examined using this rabbit model. An effective inhibitor of hydroxymethylglutaryl (HMG) CoA reductase (*EC* 1.1.1.88) such as mevinolin, prevented hypercholesterolaemia in rabbits fed on a casein-rich diet by raising the catabolic removal rate of plasma LDL in response to an increase in LDL-receptor activity (Chao *et al.* 1983).

The present study also showed that policosanol inhibited hepatic cholesterol biosynthesis, since ³H₂O incorporation into hepatic DPS was significantly depressed, by about 50%, after policosanol administration. Also, after policosanol administration, a higher removal of [¹²⁵I]LDL from plasma was observed. We therefore considered the possibility that the observed enhancement of the LDL clearance could be due to increased

hepatic LDL-receptor activity. In support of this hypothesis, we observed that in rabbits given policosanol, there was an increase in the heparin-sensitive binding of [125 I]LDL in comparison with the specific receptor activity seen in casein-fed rabbits not treated with policosanol. Furthermore, the increased LDL removal achieved by policosanol is consistent with the proportional increase in the number of receptors for LDL in liver homogenates. Taken together, these findings suggest that the enhancement of the LDL clearance could be due, at least in part, to increased LDL uptake by the liver.

However, the fact that 125 I-radiolabelled native LDL can be metabolized via either the receptor mediated process or the receptor-independent pathway, requires the measurement of the effect of policosanol on the LDL catabolism via each of these processes. In this regard, future experiments will need to investigate the metabolism of radiolabelled native and modified LDL after policosanol treatment, to show more clearly whether the LDL-catabolism induced by policosanol is exclusively due to an effect on the receptor-dependent route.

Rabbits fed on casein protein show a tendency to increase LDL apo B production via the VLDL-independent pathway (Khosla *et al.* 1989; Samman *et al.* 1989). Although it is clear that the primary response to a casein diet is a decrease in the number of hepatic LDL receptors and not an increase in LDL apo B synthetic rate, we cannot rule out an effect of policosanol on the LDL apo B production rate. If this exists, policosanol might also decrease the LDL-C level in plasma by inhibiting the secretion of lipoprotein by the liver.

It has been proposed that casein-induced hypercholesterolaemia involves stimulation of cholesterol and/or bile acid absorption from the gut (Samman *et al.* 1989; Khosla *et al.* 1991). A corollary of this would be that inhibition of cholesterol and/or bile acid absorption from the gut by policosanol also could decrease this hypercholesterolaemia. The possibility remains open, since the effect of policosanol on intestinal absorption of cholesterol and excretion of faecal bile acids was not investigated. However, several findings argue against this effect. First, reduction of cholesterol and bile acid absorption can block diet-induced rises in serum cholesterol as reported previously (Topping, 1991; de Deckere *et al.* 1993; McCarthy, 1993; Nielsen *et al.* 1993). However, previous experiments performed in our laboratory have demonstrated that policosanol orally administered does not effectively prevent hypercholesterolaemia in cholesterol-fed rabbits (Noa *et al.* 1997). Second, Kato *et al.* (1995) found that the dietary incorporation of octacosanol into a high-fat diet did not affect lipid absorption from the gut in rats. All these findings suggest that factors other than an inhibition of cholesterol and/or bile acid absorption from the gut regulate LDL-receptor activity in the liver after policosanol administration.

To our knowledge, there are few studies on the absorption and metabolism of very-long-chain alcohols. Studies carried out after oral administration of the 14 C-labelled compound to rats show that octacosanol absorption is limited, but that which is absorbed is distributed between several tissues and may be partially degraded to fatty acids (Kabir & Kamura, 1993). We have obtained similar findings in kinetic studies using 3 H-labelled octacosanol. In addition, we have demonstrated that the radioactivity is mainly present in liver within the first 24 h after oral administration and we also identified octacosanoic acid in liver early after oral administration of octacosanol (R. Menéndez, R. Más, V. Fraga, A. M. Amor, R. M. González, L. González, J. Magraner, D. Marrero, S. Jiménez and R. Gámez, unpublished results). Experiments performed by Rizzo *et al.* (1988, 1990) and Kosuke *et al.* (1986) have demonstrated that fatty alcohol oxidation can be produced in cultured cells and intestinal mucosa via a reversible fatty alcohol cycle. Recent experiments by Kawamura *et al.* (1981), Singh & Poulos (1986), Singh *et al.* (1987) and Wanders *et al.* (1987) have shown that β -oxidative chain shortening of very-long-chain

fatty acids (VLCFA) occurs in rat liver peroxisomes. Therefore, although this present study cannot demonstrate the exact mechanism for the policosanol-induced inhibition of hepatic cholesterol biosynthesis and increase of LDL receptor activity in casein-fed rabbits, the available data are consistent with the hypothesis that changes in hepatic cholesterol metabolism are mainly caused by the presence not only of aliphatic alcohols but also of VLCFA and chain-shortened secondary metabolites. Further research on the metabolism of policosanol may help determine the primary causes of its effects on casein-induced hypercholesterolaemia.

In conclusion, this work demonstrates that policosanol reduces the hypercholesterolaemia induced by a wheat starch–casein diet in rabbits, and that one of the actions of policosanol is that it prevents the loss of hepatic LDL receptors. According to our results, oral administration of policosanol also reduces the synthesis of cholesterol in the liver. However, since the hypercholesterolaemia induced by casein-feeding of rabbits also involves an increase in LDL synthesis rate, additional experiments must be performed to elucidate the effect of policosanol on LDL synthesis by the liver.

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