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STUDIES ON THE REGULATION OF CHOLESTEROL METABOLISM BY THE USE OF THE STRUCTURAL ANALOGUE, DIOSGENIN

JUAN C. DÍAZ ZAGOYA, JOSÉ LAGUNA and JESÚS GUZMÁN-GARCÍA

Departamento de Bioquímica, Facultad de Medicina, Universidad Nacional Autónoma de México, Ciudad Universitaria, México, D. F.

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Abstract—In white male Wistar rats, some aspects of the regulation of cholesterol metabolism were studied using a structural analogue, diosgenin (25α-spirost-5-en,3-β-ol). In everted gut sacs and in the whole animal, the analogue impairs the intestinal absorption of cholesterol without altering the intestinal absorption of the bile acids. The conversion of acetate to cholesterol, both *in vitro* and *in vivo*, was larger in the diosgenin-fed animals than in the respective controls. On the other hand, the incorporation of mevalonate was not altered by diosgenin. The catabolism of the sterol, as judged by production of acidic steroids and radioactive CO_2 from labeled cholesterol, was slightly diminished in the diosgenin-fed animals. The results are discussed on the basis of both biosynthesis and regulation of catabolism of cholesterol.

PREVIOUS experiments made in this laboratory[1] suggested that diosgenin lowers blood and liver cholesterol levels by inhibiting the absorption of cholesterol in the intestine. Accordingly, an effort has been made to test this hypothesis and to quantitate the extent of the inhibition of cholesterol absorption by diosgenin. The data obtained indicate that diosgenin indeed inhibits cholesterol absorption without affecting the intestinal absorption of bile acids.

The regulation of cholesterol metabolism in diosgenin-treated rats has also been studied by utilizing labeled precursors of cholesterol and by analyzing some of the steps involved in the transformation of cholesterol into bile acids. Since diosgenin does not affect the absorption of bile acids, the diosgenin-treated animals may be a better experimental system for the study of cholesterol metabolism than the bile fistula technique,[2] since in the latter both cholesterol and bile acids are lost. Also, our experimental approach has certain advantages over that in which cholesterol biosynthesis is inhibited by dietary cholesterol.[3–5]

EXPERIMENTAL

All the experiments were carried out with male Wistar albino rats weighing 150–250 g and maintained in wire-floored cages with food and water available *ad lib*. The basal diet was a commercial Chow; cholesterol (1%) or diosgenin (1%) was dry mixed with the powdered Chow.

The animals were sacrificed by a blow on the head, avoiding bleeding. The liver

was excised, blotted and homogenized immediately. Dissection of intestine, when necessary, was also carried out immediately.

Cholesterol was obtained from E. Merck (Darmstadt), and crystallized diosgenin, obtained from Diosynth (México, D.F.), was recrystallized as indicated elsewhere.[1] Cholic acid-24-[^{14}C] and cholesterol-26-[^{14}C] were purchased from New England Nuclear Corp. (Boston); acetate-1-[^{14}C], DL-mevalonic acid-2-[^{3}H] lactone and cholesterol-4-[^{14}C] were obtained from the Radiochemical Centre (Amersham, England). All other chemicals were analytical reagents.

Except for acidic steroids, the radioactivity was measured in a Packard TriCarb model 4322 liquid scintillation spectrometer. The scintillation fluids were a solution of 0·4% Omnifluor (New England Nuclear Corp.), 6% naphthalene in dioxane or a solution of 0·05% 1,4-bis-2-(4-methyl-5-phenyloxazolyl)-benzene (dimethyl POPOP), 0·5% 2,5-diphenyloxazole (PPO) in toluene. All values were corrected for quenching by the use of an external standard; nitromethane was employed as quenching material for the calibration curve.

Experiments with everted gut sacs were carried out with jejunal loops, following the procedure of Lack and Weiner[6] with cholesterol-4-[^{14}C] "solubilized" in acetone as indicated by Day.[7] Diosgenin and cholesterol were "solubilized" together, and the suspension was immediately incubated with the intestinal sacs. At the end of the incubation period, the sac was withdrawn from the incubation medium and thoroughly washed with fresh Ringer's solution; the washings were combined with the incubation medium. The fluid in the loops was collected by squeezing the sac and washing the serosal side; then the washings and the squeezed fluid were combined. The washed loop was homogenized with 25 vol. of Folch's solvent[8] ($CHCl_3$–MeOH; 2:1, v/v). Aliquots of each solution were assayed for radioactivity in the Omnifluor–naphthalene–dioxane mixture. When labeled cholic acid was used instead of cholesterol, it was dissolved in 0·308 M NaOH and the resulting solution was used to prepare the Krebs–Ringer phosphate fluid. The incubation technique and radioactivity measurement were as described for cholesterol experiments.

To study fecal radioactivity, control rats and rats which had been fed diosgenin for 10 days were injected intraperitoneally with the labeled cholesterol; immediately after the injection, the rats were placed individually in metabolic cages with a wire floor and feces were collected daily for a period of 8 days; the control or the diosgenin diet and water were offered *ad lib.* Daily fecal outputs were homogenized with 20 vol. of Folch solvent and, after standing overnight, the homogenates were filtered; an aliquot of each sample was evaporated in a scintillation vial and radioactivity was measured with the dimethyl POPOP–PPO–toluene mixture. At the end of the experimental period the rats were sacrificed, liver and intestines were excised, and the skin was removed by dissection. Livers and carcasses were homogenized in Folch solvent. The isolation of cholesterol and the measurement of its radioactivity were carried out as mentioned below for the extracts of liver slices.

A similar experimental design was used in order to measure radioactivity in feces after the intraperitoneal injection of cholic acid-24-[^{14}C] except that the experimental period was 9 days. In this case, carcasses were not analyzed for radioactivity.

The incorporation of acetate-1-[^{14}C] into cholesterol was measured in liver slices from control rats and from rats fed diosgenin or cholesterol diets for 48 hr. Slices were incubated in a Dubnoff shaking bath under an oxygen atmosphere with 2·5 ml

of Krebs–Ringer phosphate medium, pH 7·4, per 0·5 g of liver slice. To this mixture, 1·0 μc acetate-1-[^{14}C] was added to give a final concentration of 2·4 mM. At the end of the 2 hr of incubation, the slices were thoroughly washed with fresh Ringer's solution, and 2·0 ml aqueous KOH and 5 ml ethanol were added. The mixture was wet-digested at 65° until complete saponification occurred and monitored with thin-layer chromatography.[9] The saponified mixture was extracted with petroleum ether (b.p. 40–60°) until no radioactivity was detected in the extracts. From an aliquot of the mixed extracts, cholesterol was precipitated with digitonine and recovered from the digitonide with pyridine in a scintillation vial according to Gould and Swyryd.[3] The radioactivity was measured in the dimethyl POPOP–PPO–toluene mixture.

Incorporation *in vivo* of labeled acetate or mevalonate into cholesterol was also studied in control rats and in those fed diosgenin or cholesterol diets for 48 hr. Ten μc of either acetate-1-[^{14}C] or DL-mevalonate-2-[^{3}H] was injected intraperitoneally into each rat; the animals were sacrificed 1 hr later. The livers were excised, wet-digested and saponified. The isolation of cholesterol and the measurement of its radioactivity were carried out as indicated in the experiments with the liver slices.

Control rats and rats fed diosgenin for 10 days were used for the study of the conversion *in vitro* of cholesterol-4-[^{14}C] to acidic steroids. A 20,000 *g* liver supernatant, prepared as described by Mendelsohn *et al.*[10] with minor modifications, was employed: the final concentration of ATP and AMP was 8 mM and of NADPH, 8·7 mM; the 3·0 ml final volume included also 0·2 ml (175,000 dis./min) of a "solution" of cholesterol-4-[^{14}C] (sp. act., 58·5 mc/m-mole) in 1% albumin, prepared as described by Lee and Whitehouse.[11] The incubation time was 6 hr. The acidic steroids were extracted as indicated by Siperstein and Chaikoff,[12] and radioactivity was measured in an aliquot of the final alcoholic extract with a D_{47} Q-gas flow Geiger tube (Nuclear Chicago) in a zone in which autoabsorption was not detected.

The production of CO_2-[^{14}C] from cholesterol-26-[^{14}C] (86,000 dis./min; sp. act., 55·5 mc/m-mole) was studied by adding mitochondria from 2 g liver to 3 ml of the preparation used for the cholesterol-4-[^{14}C] experiments; this concentration of mitochondria corresponds to that used by Lee and Whitehouse[11] in their experiments on the oxidation of the lateral chain of cholesterol. The solubilization of the sterol in bovine serum albumin and the trapping of CO_2 were also done according to Lee and Whitehouse.[11] Radioactivity was measured with the dimethyl POPOP–PPO–toluene mixture.

Expired CO_2-[^{14}C] from control rats and rats fed diosgenin for 7 days was measured according to the method of Lerner *et al.*,[13] with a $Ba(OH)_2$ flask introduced before the glass cage and another after the NaOH flasks to assure that the input air was free of CO_2 and that all the expired CO_2 was trapped by the NaOH flasks. The trapped CO_2 was converted to $BaCO_3$ and recovered from an aliquot, employing the device of Towe *et al.*,[14] with 2 ml of a 1:1 (v/v) mixture of ethanolamine and methylcellosolve as trapping mixture. The [^{14}C] radioactivity was measured with dimethyl POPOP and PPO as scintillators.

RESULTS AND DISCUSSION

After the administration of labeled cholesterol by stomach tube to rats fed several combinations of cholesterol and diosgenin in the diet, no direct evidence of impairment of cholesterol absorption by diosgenin was found when total carcass radioactive

cholesterol was measured. This was due to the wide dispersion of the results and a high individual variability. Accordingly, the inverted intestinal loop technique[6] was used to test any possible action of diosgenin on intestinal cholesterol absorption. The highest diosgenin concentration in which no gross morphological alterations of the intestinal mucosa occurred and in which normal absorption of glucose took place was 0·05 mg/ml of incubation fluid; therefore, this was the final concentration used throughout these experiments. This concentration of diosgenin reduced the trapping of cholesterol by the intestinal wall to 30 per cent or less of the residue detected in loops incubated without diosgenin (Table 1). The results were statistically significant.

On the other hand, when the absorption of cholic acid was measured, there was no difference between the values obtained in the presence or in the absence of diosgenin (Table 1).

TABLE 1. CHOLESTEROL-4-[^{14}C] OR CHOLIC ACID-24-[^{14}C] TRAPPING BY EVERTED JEJUNAL SACS, WITH OR WITHOUT DIOSGENIN ADDED TO THE INCUBATION MEDIUM*

Diosgenin (mg/ml of incubation medium)	Serosal fluid	Intestinal wall	Mucosal fluid
Cholesterol trapping			
None (6)	0·12 ± 0·02	92·3 ± 1·4†	4·4 ± 1·4‡
0·05 (4)	0·41 ± 0·06	58·3 ± 3·1†	38·1 ± 3·2‡
Cholic acid trapping			
None (4)	12·4 ± 2·3	22·5 ± 4·0	59·2 ± 6·1
0·05 (4)	13·7 ± 3·5	26·7 ± 4·7	51·1 ± 6·7

* The results represent the per cent of added radioactivity plus or minus the standard error of the mean. Figures in parentheses indicate the number of experiments. Where indicated, the following were added: cholesterol, 2·4 nmoles ($3·0 \times 10^5$ dis./min) and cholic acid 12 nmoles ($4·8 \times 10^5$ dis./min) per 10 ml of Krebs–Ringer bicarbonate medium. The incubation time was 1 hr at 37°.

†,‡ The differences between these averages are statistically significant; the P value for the average differences is < 0·001 (*t*-test).

As both cholesterol and bile acids undergo an enterohepatic circulation which causes considerable reabsorption of both compounds, the effect *in vivo* of diosgenin on the intestinal absorption of cholesterol and bile acids may be evaluated by measuring the amount of the compound lost in feces after its parenteral administration. Thus cholesterol-26-[^{14}C] radioactivity in feces from intraperitoneal labeled cholesterol becomes a function of the amount of cholesterol or other neutral 27-C sterols which has not been reabsorbed. When cholic acid-24-[^{14}C] is injected, radioactivity in feces is a measure of unreabsorbed bile acids, since all the transformations of cholic acid into other cholanic acids preserve the C-24 label.

Figure 1 shows a typical experiment of the cumulative daily excretion of radioactivity after the parenteral administration of cholesterol-26-[^{14}C] or cholic acid-24-[^{14}C]. When the former substance was injected, the fecal radioactivity was almost 15-fold higher in diosgenin-fed animals than in the controls during the first 24 hr (35,108 dis./min vs. 2397 dis./min) and 2·5-fold higher in the second 24-hr period. From the third to the eighth day (the length of the experiment), the radioactivity was always greater in the diosgenin-fed rats, usually by a factor of 1·5 to 2·0. At the end of the experimental period, the radioactivity in feces was almost three times larger

in the diosgenin-fed animals (87,276 dis./min) than in the controls (31,276 dis./min), the difference being statistically significant at a level of $P < 0.001$. These results indicate that in diosgenin-fed rats the intestinal absorption of cholesterol is approximately one-third of that found in normal rats.

On the other hand, the same amount of radioactivity was recovered in the feces of control and diosgenin-treated animals after the intraperitoneal administration of cholic acid-24-[^{14}C]; moreover, although the difference was not statistically significant, the fecal radioactivity was slightly lower in the diosgenin-fed rats throughout the length of the experiment (Fig. 1).

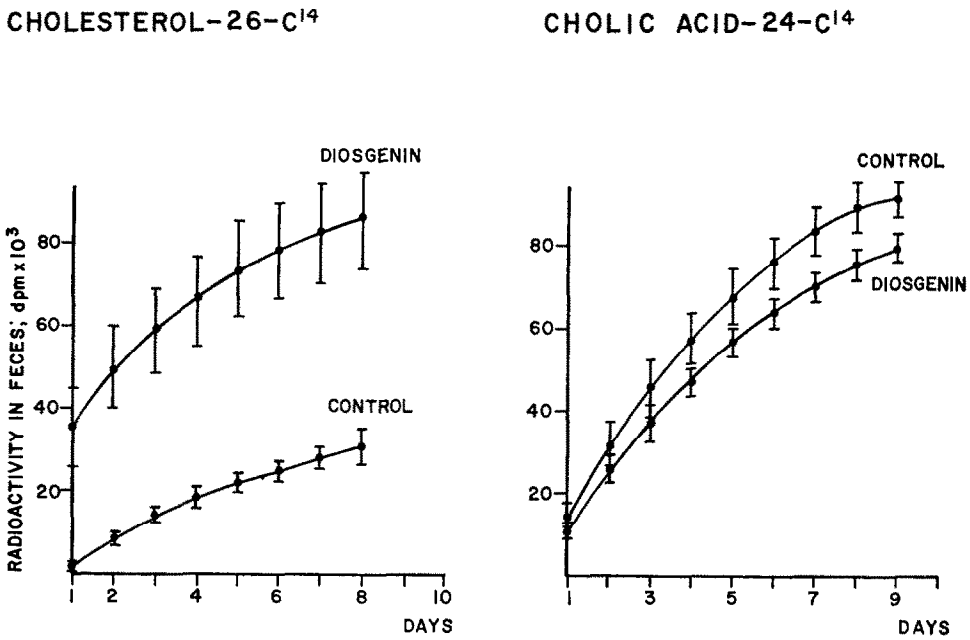

FIG. 1. Cumulative daily radioactivity in feces after intraperitoneal injection of cholesterol-26-[^{14}C] or cholic acid-24-[^{14}C] to control or diosgenin-fed rats. Each animal received, per 100 g of body weight, 0·5 ml (4×10^5 dis./min) of a "solution" of cholesterol prepared according to the procedure of Lee and Whitehouse[11] or 0·5 ml ($7{\cdot}5 \times 10^4$ dis./min) of a solution of labeled cholic acid in Krebs–Ringer phosphate, pH 7·4.

The results obtained in the isolated intestinal loop and in the whole animal substantiate the hypothesis that diosgenin inhibits the absorption of cholesterol without affecting the enterohepatic circulation of bile acids. This action of diosgenin could be used advantageously in the study of the regulation of cholesterol biosynthesis.

Excess dietary cholesterol depresses liver cholesterol biosynthesis, as can be shown by the experiments in which an inhibition of the incorporation of labeled acetate into the sterol molecule has been measured.[4,5,15,16] On the other hand, the incorporation of mevalonate into cholesterol is much less affected by excess dietary cholesterol and thus it is generally accepted that the enzymatic site for this feedback regulation is on the reduction of β-hydroxy-β-methyl-glutaryl coenzyme A to mevalonate.[4,15,17,18]

The incorporation of acetate-1-[^{14}C] into cholesterol in liver slices from rats fed diosgenin for 48 hr was much greater than that found in control animals (sp. act.,

1066 vs. 311 counts/min), the difference being statistically significant at a level of $P < 0.01$.

Besides the approach *in vitro*, an effort was made to duplicate the observations in whole animals. Rats fed diosgenin or cholesterol for 48 hr were injected with acetate-1-[^{14}C] or mevalonate-2-[^{3}H]. In the former animals, the incorporation of acetate into cholesterol was twice that observed in the control animals (Table 2). On the other hand, the incorporation of labeled mevalonate into cholesterol was slightly higher in the diosgenin-treated animals (Table 2). This difference was not statistically significant. In animals on high dietary cholesterol, the results obtained are in agreement with those reported in the literature.[3,4,15,19]

TABLE 2. INCORPORATION *in vivo* OF LABELED PRECURSORS INTO CHOLESTEROL BY THE LIVER OF CONTROL, CHOLESTEROL-FED OR DIOSGENIN-FED RATS*

Additions to diet	Labeled precursor	
	Acetate-1-[^{14}C]	Mevalonate-2-^{3}H
None	1772 ± 276	40,183 ± 2968
1% Diosgenin	4376 ± 878 ($P < 0.02$)†	52,329 ± 5016 ($P < 0.1$)
1% Cholesterol	267 ± 146 ($P < 0.001$)	22,162 ± 4055 ($P < 0.005$)

* The results express the disintegrations per minute per milligram of cholesterol plus or minus the standard error of the mean. Six animals were used for each group. Ten μc acetate-1-[^{14}C] (30 μmoles) or DL-mevalonate-2-[^{3}H] (0·1 μmole) was injected per rat. Immediately before the injection, DL-mevalonate-2-[^{3}H] lactone was converted into the salt by dissolving it in 0·068 M phosphate buffer, pH 7·8.

† P values (*t*-test) are for the difference between the means of the indicated test group and the corresponding control animals (no additions to the diet).

We believe that the diosgenin-induced loss of cholesterol through the feces results in an increase in the biosynthesis of cholesterol by the liver. Also, since diosgenin affects acetate incorporation rather than mevalonate incorporation, the possibility exists that the critical step influenced by diosgenin through the impairment of cholesterol absorption is before the formation of mevalonate, most probably at the β-hydroxy-β-methylglutaryl coenzyme A level. This finding is of special significance, since no previous results have been reported in relation to cholesterol biosynthesis when cholesterol is excluded from the intestine; most of the data in the literature are derived from studies on cholesterol biosynthesis in animals fed cholestyramine[16,20] or in bile fistula rats.[2,21] These two conditions induce the loss of bile acids or cholesterol and bile acids. An indirect approach to avoid this problem has been employed by Grundy *et al.*,[22] who applied balance methods in human beings and obtained an increase in cholesterol synthesis when the intestinal cholesterol absorption was lowered by administering vegetable sterols in the diet.

Since the effect on cholesterol biosynthesis caused by the feeding of diosgenin could also alter the transformation of the sterol in bile acids, a subcellular preparation designed after that of Mendelsohn *et al.*,[10] which can convert cholesterol into cholic acid, was employed to test this possibility. In the diosgenin-treated animals, a trend

(not statistically significant) toward a lower transformation rate of cholesterol into cholic acid was found (Table 3).

TABLE 3. OXIDATION *in vitro* AND *in vivo* OF LABELED CHOLESTEROL BY NORMAL OR DIOSGENIN-FED RATS*

Additions to diet	Labeled cholesterol		
	(*In vitro*)		(*In vivo*)
	4-^{14}C	26-^{14}C	26-^{14}C
None	1481 ± 293† (6)	4711 ± 105 (4)	1851 ± 220 (6)
1% Diosgenin	1030 ± 149 (6)	4253 ± 87 (5)	1155 ± 72 (5)
	P < 0·10	P < 0·02	P < 0·025

* The results of the experiment *in vitro* express the disintegrations per minute of acidic steroids produced from cholesterol-4-[^{14}C] by the 20,000 *g* supernatant from 0·5 g liver and the disintegrations per minute of the CO_2 liberated from cholesterol-26-[^{14}C] by the 20,000 *g* supernatant from 0·5 g liver plus mitochondria from 2 g liver. Incubation time was 6 hr at 37°. The rats employed received their respective diets for 10 days prior to the experiment. The results of the experiments *in vivo* are expressed as disintegrations per minute of the total CO_2 expired per gram of rat, collected for 48 hr after the parenteral administration of cholesterol-26-[^{14}C] (0·5 μc/100 g of rat). The respective diets were offered 7 days before the experimental period as well as during the experimental period. Figures in parentheses indicate the number of rats.

† Standard error of the mean.

Similar results were obtained when the production of CO_2-[^{14}C] from cholesterol-26-[^{14}C] was measured in the Mendelsohn type preparation with added mitochondria. This study of the oxidation of the last three carbons of the chain of cholesterol in the formation of bile acids again showed a tendency toward a lower CO_2-[^{14}C] formation in the preparation from diosgenin-treated rats (Table 3).

Our results indicate that the diosgenin-induced fecal loss of cholesterol results in an increase in the incorporation of acetate into cholesterol, possibly through a lowering of the body cholesterol pool; at the same time no increase in the production of bile acids could be demonstrated. A likely explanation for these results may reside in the existence of two regulatory pathways, one for the biosynthetic process from acetate to cholesterol and the other for the catabolic pathway, from cholesterol to cholanic acids. Danielsson *et al.*[2] have suggested that the 7 α-hydroxylation of cholesterol might be the rate-determining step in the conversion of cholesterol to bile acids. With the data presented in our paper, however, no speculations can be made on the nature of the metabolites responsible for the regulation of each of the two processes. If cholic acid or another cholanic acid were involved in the regulation of the biosynthetic pathway, as Back *et al.*[19] have proposed, a diminished conversion of cholesterol to cholanic acids could be expected in situations leading to increased cholesterol biosynthesis. In this respect, the data in Table 3 indicate a discrete statistically significant lowering of the catabolism of cholesterol in diosgenin-fed animals. It is important to note that Fimognari and Rodwell[23] have shown that, in a partially purified preparation from yeast, some bile acids inhibited the activity of mevalonate reductase (mevalonate-NADP-oxidoreductase, EC 1.1.1.34). Also, when radioactive CO_2 was

measured in the expired air from animals that received cholesterol-26-[^{14}C], a statistically significant diminution (about 40 per cent) in labeled CO_2 was found in diosgenin-fed animals (Table 3). The sample was obtained during a period of 48 hr after 7 days on either the diosgenin diet or a control diet, a period sufficient to allow the oxidation of the lateral chain of cholesterol. Since the intestinal absorption of cholesterol is seriously impaired (Fig. 1), the lowering of the expired CO_2 in diosgenin-treated animals does not necessarily imply a blocking of the oxidation of the sterol lateral chain, but rather, it may be the result of an impairment of cholesterol reabsorption through the intestinal wall.

It may be argued also that diosgenin causes a redistribution of cholesterol to the different organs of the animal so that the metabolism of cholesterol by the liver would be affected, as Merola *et al.*[24] have shown in the case of estrogen administration.* In our experiments this possibility is untenable, since the analysis of labeled cholesterol in different parts of the carcass did not show a significant difference between diosgenin-fed and control animals.

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* The authors have unpublished data which suggest that estrogens also cause mobilization of cholesterol from peripheral tissues to the liver.