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ENHANCEMENT OF CHOLESTEROL TURNOVER IN RATS BY A CATATOXIC STEROID (PCN) AND A BILE ACID SEQUESTRANT (COLESTIPOL-HCl)*

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Abstract—Catatoxic steroids induce hepatic microsomal enzymes. To determine if cholesterol catabolism to bile acids by microsomal enzymes is stimulated by a catatoxic steroid, effects of pregnenolone-16α-carbonitrile (PCN) alone or with colestipol-HCl on cholesterol 7α-hydroxylase, cholesterol synthesis and cholesterol turnover were studied. Male rats fed diets containing colestipol (1%), PCN (0·085%), colestipol plus PCN, or basic diet were injected with [1,2-^{3}H]-cholesterol complexed with serum lipoproteins. Serum cholesterol specific radioactivity was measured for 49 days. Hepatic cholesterol and bile acid synthesis were estimated by [1-^{14}C]-acetate incorporation and cholesterol 7α-hydroxylase activity. Turnover curves were analyzed using a three-pool model. PCN significantly increased all rate constants and cholesterol production rate (10·75 to 12·87 mg/day), which in this model is a measure of total body cholesterol turnover. Colestipol significantly increased total body cholesterol turnover (10·75 to 19·91 mg/day) and the excretion rate constant (0·44 to 0·88 day^{-1}) and increased acetate incorporation 6-fold and cholesterol 7α-hydroxylase activity 2-fold. PCN only slightly inhibited the latter. Effects of colestipol plus PCN were not different from those of colestipol alone. No treatment significantly changed cholesterol serum levels overall. Colestipol results are consistent with reported data for bile acid sequestrants. PCN markedly increased cholesterol flux between pools; it does not appear to induce and may, in fact, inhibit bile acid synthesis, perhaps by decreasing availability of necessary cofactors or cholesterol substrate.

Catatoxic steroids are known to induce hepatic microsomal mixed-function oxygenases [1] and phenobarbital has been shown to produce similar effects [2, 3]. Phenobarbital has been reported both effective [4, 5] and ineffective [6, 7] as an inducer of the microsomal enzyme, cholesterol 7α-hydroxylase, the probable rate-limiting enzyme of bile acid synthesis [8–10]. To see if cholesterol catabolism to bile acids might be stimulated by a catatoxic steroid, effects of pregnenolone-16α-carbonitrile (PCN) alone or with a bile acid sequestrant (colestipol-HCl) on bile acid and cholesterol synthesis and cholesterol turnover were studied. If PCN enhanced bile acid synthesis, an increase in the amount of bile acids entering the gut or a change in their composition might result in a potentiation of effects of the bile acid sequestrant.

MATERIALS AND METHODS

Four groups of 13 male Upjohn:TUC(SD)spf rats (initially 300 g each) were used in this study. All animals were caged individually and had free access to a synthetic diet‡ and water. The control group received diet alone while the remaining groups received diet containing PCN (0·085 per cent), colestipol (1·0 per cent) or PCN plus colestipol (0·085 and 1·0 per cent, respectively). Food consumption and change in body weight were measured for each animal over the 10 weeks of the study.

Methods used in acquisition and analysis of the turnover data closely followed those of Phillips and Elfring [11]. On day 18 of the feeding regimen, ten rats from each group received an intravenous dose of 4·5 μCi [1,2-^{3}H]-cholesterol (New England Nuclear; sp. act., 52·6 Ci/m-mole) complexed with rat serum lipoproteins [12], while the remaining three rats per group received unlabeled serum.

Blood samples, 0·4 or 0·8 ml, were taken without anesthesia from the external jugular vein of each rat [13] at 2 hr, 1, 2, 3, 6, 10 and 15 days, and thereafter at weekly intervals up to 49 days after injection of cholesterol. Serum cholesterol concentrations were determined by gas–liquid chromatography of the hexane-extractable material from saponified samples [14] using 5α-cholestane as an internal standard. Samples were chromatographed on a glass column (18 × 1/8 inch, i.d.) packed with 3·8% UC-W98 on 80–100 mesh Diaport-S® (Hewlett–Packard) in a Hewlett–Packard model 5700A gas chromatograph with an oven temperature of 220°.

Either 0·1 or 0·2 ml of the serum samples (0·2 ml at periods after 28 days) was counted for radioactivity in 15 ml of scintillation fluid prepared with 4 g PPO§ and 50 mg POPOP§ per liter of toluene and containing 12% Bio-Solv Solubilizer Formula BBS-3® (Beckman Instruments, Inc.) and three drops of 8% $SnCl_2$ in 0·1 N HCl. A quench curve prepared with hemolyzed blood using an automatic external standard allowed calculation of the absolute radioactivity in each sample

* Colestid®, The Upjohn Co.; hereafter referred to as colestipol.

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‡ Diet of Phillips and Berg [15] except coconut oil at 10 per cent was substituted for corn oil, and 18 per cent casein and 0·2 per cent methionine served as the protein source.

§ PPO = 2,5-diphenyloxazole; POPOP = 1,4-*bis*-2-(4-methyl-5-phenyloxazolyl)benzene.

Table 1. Weight, cholesterol concentration and residual [^{3}H] cholesterol of livers upon termination of the turnover study.*

	Control	PCN	Colestipol	PCN + Colestipol	S.D. (%)
Liver weight (g)	14·04	17·04†	14·90	17·50†	9·60
Hepatic cholesterol concentration (mg/g)	2·28	2·57†	2·08	2·43	13·74
Hepatic [^{3}H] cholesterol specific activity (dis./min/mg)	3,710	2,591‡	414§	507§	5·36
Total hepatic [^{3}H] cholesterol dis./min	117,733	110,912	12,699§	21,414§	3·25

* Group mean, ten animals per group.
† Significantly different from control, $P < 0{\cdot}01$.
‡ Significantly different from control, $P < 0{\cdot}05$ (antilog of log means).
§ Significantly different from control, $P < 0{\cdot}01$ (antilog of log means).

after counting in a TriCarb® model 3375 liquid scintillation spectrometer (Packard Instrument Co., Inc.). Counting efficiencies ranged from 35 to 48 per cent. Semilogarithmic plots of serum cholesterol specific activity versus time for each animal were analyzed by a nonlinear estimation program [16] employing a three-pool model. The use of a three-pool model for analysis of long-term cholesterol turnover data has been shown to be appropriate in the rat [12], monkey [17] and man [18]. Data were transformed by $1/Y^2$ in order to achieve the most precise estimates of turnover parameters.

Rats which initially received unlabeled serum were sacrificed by cervical disolation 51 days after injection. Their livers were perfused with ice-cold saline *in situ*, excised and weighed. Liver homogenates were prepared (1:4, w/v) in Bucher buffer [19] with the addition of EDTA (2·5 mM). Cholesterol 7α-hydroxylase activity was assayed by the method of Berséus *et al.* [19] using the 20,000 *g* supernatant of the above homogenate with [4-^{14}C]-cholesterol (Amersham/Searle Corp.; sp.act., 55·6 mCi/m-mole) as the substrate. The incubation medium was made 1 mM with respect to NADPH. Protein concentrations of the incubation mixtures were determined using the biuret method of Yonetani [20]. Assay results were expressed as nmoles 7α-hydroxycholesterol produced per mg of protein per hr.

The remaining ten rats from each of the groups received intraperitoneal injections of an aqueous solution (from 0·45 to 0·65 ml) containing 25 μCi [1-^{14}C]-acetic acid, sodium salt/100 g of body weight (Amersham/Searle Corp.; sp.act., 58 mCi/m-mole) 53 days after the injection of [1,2-^{3}H]-cholesterol. One hr after the [1-^{14}C]-acetate injections, the rats were sacrificed by cervical dislocation and their livers were perfused with ice-cold saline, excised, weighed and frozen. Later, incorporation of [1-^{14}C]-acetate into hepatic cholesterol was estimated by the method of Duncan and Best [21]. Liver samples (3 g) were digested in 9 ml of 25% KOH in 95% ethanol at 37° overnight. Three 20-ml hexane extracts of the liver saponifications were combined and aliquots were analyzed for carbon-14, tritium and cholesterol, while the remaining portions of the samples were taken through the digitonin precipitation. Digitonin-precipitable material was assayed for carbon-14, tritium and cholesterol after the cleavage of the digitonide. Cholesterol assays were performed as described above. Tritium and carbon-14 were measured simultaneously [22] employing dual channel counting. The counting efficiencies for each isotope in each sample were estimated by the use of the automatic external standard and appropriate quench curves. The average counting efficiencies for carbon-14 were 6·5 per cent for the low energy channel and 68 per cent for the high energy channel, while tritium was counted with efficiencies of 32 and 0·002 per cent respectively. The gross counts and counting efficiencies were used to solve simultaneous equations yielding the absolute radioactivities for the individual isotopes.

Statistical differences from control means were determined by Student's *t*-test using pooled error variance. Where indicated, data were transformed to logs in order to achieve more homogeneous within-group variance; data are reported as the anti-log of the log means [23]. An indication of the amount of variability for data in each table is expressed as overall per cent standard deviation (S.D., %).

RESULTS

Over the 10 weeks of the study, food consumption and change in body weight for treatment groups did not differ significantly from those of control groups. The average daily dose was 37·43 mg/kg for PCN and 440 mg/kg for colestipol. PCN treatment increased liver weight by about 20 per cent over control values,

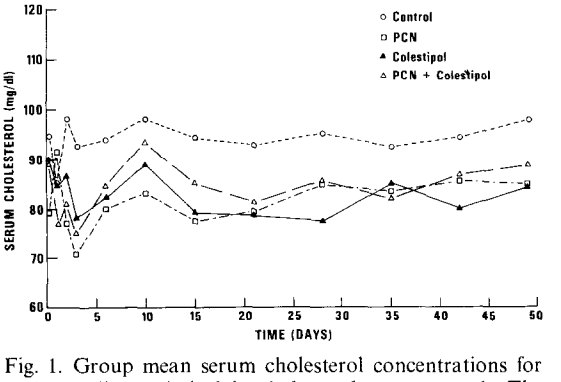

Fig. 1. Group mean serum cholesterol concentrations for each sampling period of the cholesterol turnover study. The PCN group was significantly different from control at 2 days ($P < 0{\cdot}05$) while at 3 days both the PCN and colestipol groups were significantly different from control ($P < 0{\cdot}01$), although overall changes for the entire study period were not significantly different.

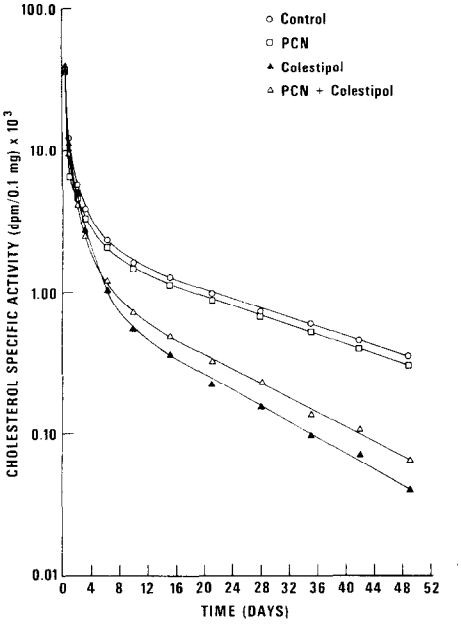

Fig. 2. Semilogarithmic plots of serum cholesterol specific activity versus time following injection of 4·5 μCi [1,2-^{3}H]-cholesterol complexed with rat serum lipoproteins. Each point represents the group mean for a specific sampling time.

while colestipol treatment had little or no effect (Table 1). Concentration of heptatic cholesterol was increased by PCN treatment alone; the increase was not significant when PCN was given in combination with colestipol (Table 1). Serum cholesterol concentrations were not significantly changed for the overall study (Fig. 1), although cholesterol levels were significantly different at a few individual sampling periods.

Semilogarithmic plots of serum cholesterol specific activity versus time for the four groups are presented in Fig. 2. Group mean parameters calculated from the turnover data for each individual animal are presented in Table 2. These parameters are defined by the three-pool model described in Fig. 3. Although the specific activity curve for the PCN group does not appear to differ from that of control (Fig. 2), differences actually exist at the early sampling intervals which have marked effects on the calculated turnover parameters. PCN treatment significantly increased above control all the rate constants (k_{12}, k_{21}, k_{10}, k_{13} and k_{31}), production (PR_1), and the specific activity of cholesterol in pool 1 (M_1) at time zero (D/V) and reduced the size of pool 1 and the areas under the 0 to ∞ and 0 to 49 day curves. This model assumes that excretion from the system occurs only from pool 1 so that PR_1, which is equal to $k_{10} \times M_1$, represents total body turnover of cholesterol. PCN significantly increased the rate constant k_1, which represents rate of total cholesterol removal from pool 1, but did not decrease significantly the calculated amount of tritium remaining in the animals at the termination of the study (calculated tritium remaining). However, the actual amount of tritiated cholesterol remaining in the liver at sacrifice (Table 1) was slightly, but significantly, decreased, indicating relatively greater retention in extrahepatic tissues.

In the colestipol group, a greatly increased excretion rate (k_{10}) was reflected in a significant decrease in area under the 0 to ∞ and 0 to 49 day curves and the calculated and actual tritium remaining. Rate constant k_{13} was decreased significantly, while changes in other rate constants were not significant. Due to the reduction of both k_{13} and k_{12}, the k_1 for colestipol did not differ from that of control despite the dramatic increase in k_{10}.

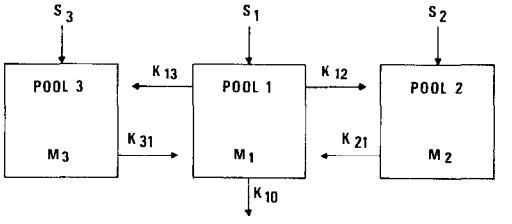

Fig. 3. Three-pool cholesterol turnover model with k values representing rate constants and M values representing pool sizes; S values denote addition of exogenous or endogenous cholesterol to the various pools. It is assumed that excretion from the system occurs only via pool 1; therefore, $k_{10} \times M_1$ (PR_1) represents total body cholesterol turnover. The units of the various parameters are indicated in Table 2.

Table 2. Calculated cholesterol turnover parameters*

Parameter	Control	PCN	Colestipol	PCN + Colestipol	S.D. (%)
D/V (dis./min/mg) × 10^4	40·948	47·605†	44·953	40·230	15·01
k_{12} (day^{-1})	0·499	1·356‡	0·372	0·458	43·50
k_{21} (day^{-1})	0·524	0·833§	0·715	0·533	36·77
k_{10} (day^{-1})	0·439	0·609§	0·883§	0·801§	10·91
k_{13} (day^{-1})	0·474	0·842‡	0·260‡	0·348‡	35·87
k_{31} (day^{-1})	0·079	0·106‖	0·083	0·085	20·28
M_1 (mg)	24·679	21·408†	21·764†	25·323	13·18
PR_1 (mg/day)	10·746	12·872§	19·913§	20·097§	10·80
Area (0–49 days) × 10^4	82·990	70·720§	50·100§	48·922§	9·70
Area (0–∞) × 10^4	93·890	78·300§	50·820§	50·433§	10·78
$k_1 \cdot [-(k_{10} + k_{12} + k_{13})]$	−1·412	−2·807	−1·544	−1·607	24·87
Calculated tritium remaining (dis./min) × 10^3	1031	957	137‡	213‡	48·86

* Group mean, ten animals per group.
† Significantly different from control, $P < 0.05$.
‡ Significantly different from control, $P < 0.01$ (antilog of log means).
§ Significantly different from control, $P < 0.01$.
‖ Significantly different from control, $P < 0.05$ (antilog of log means).

Table 3. Hepatic 7α-hydroxycholesterol synthetic rate, an estimate of bile acid synthesis, and [1-^{14}C]-acetate incorporation, an estimate of cholesterol synthesis*

	Control	PCN	Colestipol	PCN + colestipol	S.D. (%)
7α-Hydroxycholesterol (nmoles/mg protein/hr)	0·029	0·017†	0·071‡	0·056‡	5·81
7α-Hydroxycholesterol (nmoles/liver/hr)	7·48	6·07	17·68‡	15·68‡	6·39
[^{14}C]-cholesterol specific activity (dis./min/mg)	1,740	1,360	11,311‡	9,640‡	9·57
Total hepatic [^{14}C]-cholesterol (dis./min)	55,204	73,290	347,216‡	406,824‡	6·89

* Group mean, three animals per group for the former and ten animals per group for the latter.
† Significantly different from control, $P < 0·05$ (antilog of log means).
‡ Significantly different from control, $P < 0·01$ (antilog of log means).

PCN–colestipol combination treatment resulted in turnover parameters closely resembling those seen for colestipol alone. Rate constant k_{10} decreased slightly for the combination, but the larger size of pool 1 resulted in a production rate equal to that seen for colestipol.

Rate of formation of 7α-hydroxycholesterol in the liver, an estimate of the rate of bile acid synthesis, was significantly increased in colestipol treatment groups and decreased with PCN treatment (Table 3). Hepatic cholesterol synthesis, as estimated by [1-^{14}C]-acetate incorporation into non-saponifiable digitonin-precipitable materials, was increased more than 6-fold by colestipol, while PCN had no significant effect (Table 3). The specific activities of the carbon-14-labeled hepatic sterols were not significantly different before and after digitonin precipitation. This indicates no accumulation of non-sterol cholesterol precursors in any treatment group.

DISCUSSION

Einarsson and Gustafsson [24] have reported a slight, but insignificant, reduction in hepatic cholesterol 7α-hydroxylase activity in rats treated with a daily dose of 67 mg PCN per kg for 5 days. In man [25] and monkey [26] the increase in bile acid synthesis upon phenobarbital treatment appears to be secondary to a rise in cholesterol synthesis. In the present study hepatic cholesterol 7α-hydroxylase is inhibited and there is an insignificant increase in hepatic cholesterol synthesis. Therefore PCN does not appear to induce bile acid synthesis in the present rat model. Although the microsomal mixed-function oxygenases induced by PCN are not identical to cholesterol 7α-hydroxylase [27, 28], the enzyme systems may be similar enough to be in competition for common cofactors, resulting in reduced cholesterol 7α-hydroxylase activity. Turnover parameters indicate enhanced cholesterol excretion from the organism upon treatment with PCN. However, direct estimates of cholesterol and bile acid synthesis suggest that this is not taking place via bile acids. A possible alternative route is via fecal neutral sterols. On the other hand, calculated changes in cholesterol flux rate with PCN may be entirely nonspecific.

Alone or with PCN, colestipol significantly increased hepatic cholesterol and bile acid synthesis and total cholesterol turnover (PR_1) while reducing the calculated tritium remaining. In the rat, cholesterol synthesis is increased to compensate for loss due to excretion [29]; therefore, enhanced excretion indicated by the turnover parameters is supported by the direct measurements of hepatic cholesterol and bile acid synthesis. These data are consistent with similar colestipol data reported previously from this laboratory [11, 30]. These data represented several separate experiments; present data were obtained in one experiment employing the same animals for the various determinations. Turnover and cholesterol 7α-hydroxylase data correspond closely for both studies, but acute dosing (8 days) in the earlier study resulted in a 2-fold increase in estimates of hepatic cholesterol synthesis, while the present chronic dosing (53 days) resulted in a 6-fold increase. The present data are also consistent with those reported previously for cholestyramine [29, 31–33], another bile acid sequestrant.

PCN and phenobarbital seem to induce microsomal mixed-function oxygenases in rats by a similar mechanism [24]. However, in contrast to our results with PCN, phenobarbital has been shown, in some laboratories, to induce bile acid synthesis [4, 5]. Because microsomal enzyme induction by drugs may be genetically controlled [5, 25, 34, 35], PCN may have different effects on bile acid synthesis in species other than the rat.

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