Effect of *p*-chlorophenoxyisobutyrate (CPIB) fed to rats on hepatic biosynthesis and catabolism of ubiquinone

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ABSTRACT The effect of *p*-chlorophenoxyisobutyrate (CPIB) feeding on cholesterol and ubiquinone metabolism in rats was investigated. The results obtained from acetate-1-^{14}C and mevalonate-2-^{14}C incorporation studies both in vivo and in vitro confirm the results of other workers that CPIB feeding caused a metabolic block in the conversion of acetate to mevalonate, thereby inhibiting over-all steroidogenesis. Liver ubiquinone synthesis was inhibited in CPIB-fed rats, but a block in the catabolism of the ubiquinone resulted in accumulation of ubiquinone in CPIB-fed animals.

KEY WORDS CPIB · cholesterol · ubiquinone · hydrocarbon · biosynthesis · turnover · rat · liver · acetate · mevalonate

Chlorophenoxyisobutyrate (CPIB) is a potent hypolipidemic drug that depresses the concentrations of serum cholesterol and other lipids in blood and liver (1, 2). Investigations of the possible mechanism of action of this drug by Gould and his coworkers (3, 4) revealed that the biosynthesis of hepatic cholesterol was markedly inhibited in CPIB-fed rats. Studies with liver slices demonstrated that the site of inhibitory action of CPIB was on the biosynthetic pathway between acetate and mevalonate, since incorporation of acetate-1-^{14}C into liver sterols was strongly inhibited, but that of mevalonate-2-^{14}C unaffected. These workers also observed that CPIB did not inhibit hepatic sterol synthesis when it was added to homogenates of normal liver, and concluded that it is only an indirect inhibitor.

The isoprenoid side chain of ubiquinone is also synthesized by the pathway from acetate via mevalonate, at least up to isoprenyl pyrophosphate (5). Consequently, it is logical to expect that CPIB feeding will also inhibit biosynthesis of ubiquinone from acetate. We have, however, demonstrated that feeding CPIB to rats caused a dramatic *increase* in liver content of ubiquinone as well as a reduction in serum cholesterol levels (6). This finding implies that other regulatory mechanisms exist in the rat to account for increased ubiquinone levels after CPIB feeding. Recently Ramasarma and his associates (7) reported that steroidogenesis in rats can be regulated by the feeding of ubiquinone, and proposed the novel hypothesis that inhibition of sterol biosynthesis by CPIB feeding may also be mediated through the increased levels of ubiquinone which simultaneously result. No attempt has been made, however, to explain the effect of CPIB feeding on liver ubiquinone.

The present investigations were undertaken to provide further insight into the mechanism of CPIB action not only on the metabolism of sterols but also on that of ubiquinone. Acetate-1-^{14}C and mevalonate-2-^{14}C incorporation was studied in normal and CPIB-fed rats in vivo as well as in liver homogenates.

METHODS

Radioactive Tracers

Acetate-1-^{14}C (specific activity 2.85 mc/mmole), obtained from New England Nuclear Corp., Boston, Mass., and mevalonic acid-2-^{14}C lactone (specific activity 3.35 mc/mmole), obtained from Merck, Sharpe & Dohme

Abbreviations: CPIB (Atromid-S, clofibrate), *p*-chlorophenoxyisobutyrate; PPO, 2,5-diphenyloxazole; POPOP, 1,4-bis[2-(5-phenyloxazolyl)]benzene; Hyamine hydroxide, *p*-(diisobutylcresoxy-ethoxyethyl) dimethyl benzyl ammonium hydroxide.

Ltd., Montreal, Canada, were dissolved in water in suitable concentrations. The pH of the solutions were adjusted to 7.2.

Animals and Drug Administration

Wistar male rats were weaned at 21 days of age and divided into two groups, one (control group) maintained on a basal diet of ground stock cubes mixed with 3.0% of corn oil and the other (CPIB-fed group) maintained on the same diet thoroughly mixed with 0.2% (w/w) of CPIB (Atromid-S, clofibrate, generously supplied by Ayerst, McKenna, and Harrison Ltd., Montreal, Quebec). Feed and water were allowed ad libitum.

In Vivo Studies

In studies of the incorporation of acetate-1-^{14}C, rats were maintained on the respective diets for 15 days, while in those with mevalonate-2-^{14}C the animals were fed for 35 days. Each rat then received either 10 μc of acetate-1-^{14}C or 2 μc of mevalonate-2-^{14}C by stomach tube. 4, 24, and 72 hr after the tracer dose rats in each group were killed by a blow on the head. The livers were excised immediately and stored at -10°C. After saponification and extraction, the nonsaponifiable constituents were chromatographed on deactivated alumina as described previously (8).

Hydrocarbons were eluted with petroleum ether. Ubiquinone, eluted with 6% diethyl ether in petroleum ether, was determined spectrophotometrically at 275 mμ after reduction with potassium borohydride ($\Delta E^{1\%}_{1\ cm}$ = 156). Sterol was determined gravimetrically from dried Liebermann–Burchard-positive material eluted with 20% diethyl ether in petroleum ether. Aliquots of each fraction were then evaporated to dryness in a counting vial under nitrogen and finally taken up in 10 ml of the scintillation fluid (0.5% PPO plus 0.03% POPOP in toluene) to be assayed for radioactivity.

In Vitro Studies

After 21 days on the test diets, the rats were killed by a blow on the head. For long-term studies the animals were fed the respective diets for 150 days. The liver was perfused with ice-cold 0.25 M sucrose, passed through a tissue mincer to remove any connective tissue, and subsequently homogenized in a loose-fitting Potter–Elvehjem homogenizer with 2.5 volumes of a buffer solution (10 strokes at a rotor speed of 1000 rpm). The composition of the buffer was KH_2PO_4 5.98 g, nicotinamide 3.42 g, sucrose 43.1 g, and 0.1 N KOH 25–35 ml in 1000 ml of water, adjusted to pH 7.2. The homogenate was centrifuged at 400 *g* in a Beckman model L2 ultracentrifuge. The supernatant solution, which will henceforth be referred to as "homogenate", was used for in vitro incubations.

Acetate-1-^{14}C or mevalonate-2-^{14}C incorporation in vitro was determined in triplicate for each rat and the mean values are reported. Each incubation mixture consisted of either 5 μc of acetate-1-^{14}C (approximately 1.75 μmole, giving a concentration of 0.5 mM) or 0.2 μc of mevalonate-2-^{14}C (approximately 0.5 μmole, 0.15 mM), 1.3 mg of ATP, and 3.8 mg of NAD in 0.2 ml of water plus 2.5 ml of the homogenate (equivalent to 1.0 g of liver); the final volume was always made up to 3.0 ml. After incubation of the mixture for 90 min at 37°C in a Dubnoff water bath in an atmosphere of oxygen with slow automatic shaking (40–50 excursions per minute), 5 ml of ethanol containing 5 mg of carrier cholesterol was added to stop the reaction. The incubation mixture was saponified for 45 min after which nonsaponifiable constituents were thoroughly extracted with light petroleum (bp 40–60°C). The extract was washed free of alkali with water and finally with ethanol–water 1:1. The solvent was evaporated to dryness under nitrogen and the residue redissolved in 10 ml of ethanol. Cholesterol was precipitated as the digitonide by the addition of a 2 ml solution of 1% digitonin in 50% ethanol. After centrifugation, the precipitate was washed twice with acetone–ether 1:2 and once with acetone, the supernate being discarded after each washing. The precipitate was then filtered and obtained as a pad. The digitonide pad was finally transferred to a counting tube and dissolved in 1 ml of Hyamine hydroxide, (Rohm & Haas Co.), after which 9 ml of scintillation fluid was added and the mixture shaken well before being assayed for its ^{14}C content.

Assay of Radioactivity

We measured the ^{14}C content of samples in a Packard liquid Scintillation spectrometer and used the channel ratio method to correct for quenching. The specific activity represents the total radioactivity (dpm) in the various nonsaponifiable fractions per g of liver in the in vivo studies and per mg of cholesterol in the in vitro studies.

RESULTS

In Vivo Experiments

The incorporation at 4 hr was taken as a measure of synthesis, while the values at 24 and 72 hr showed the changes in the amount of the labeled compound remaining in the liver at these longer time intervals.

Acetate-1-^{14}C Incorporation

The results on the incorporation of acetate-1-^{14}C into liver sterols in normal and CPIB-fed rats are presented in Fig. 1. It is apparent that specific activity in the sterol fraction is significantly lower in CPIB-fed rats at all

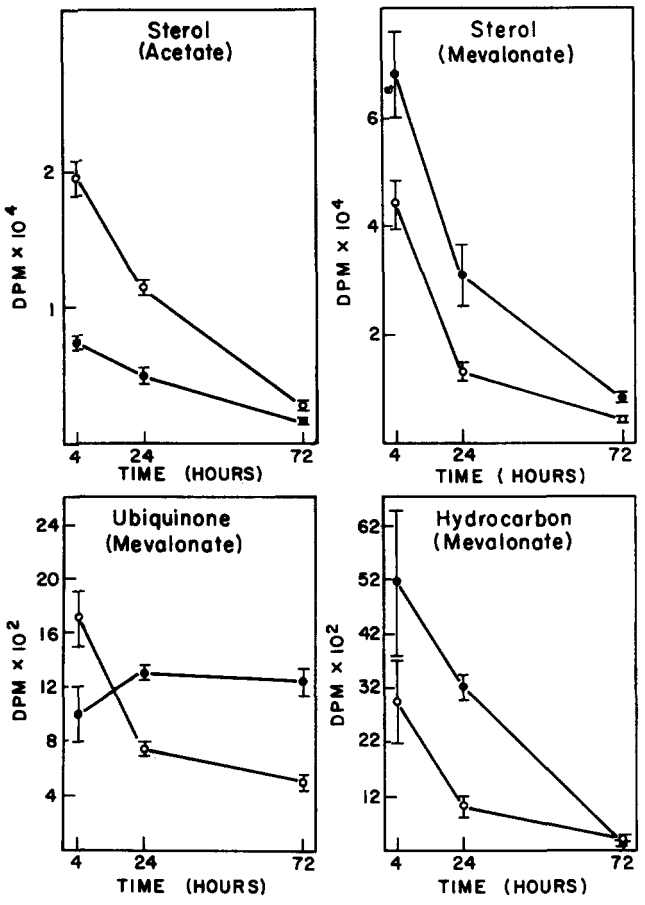

FIG. 1. Total radioactivity per g of liver in specific nonsaponifiable fractions from normal (○) and CPIB-fed (●) rats with time after oral administration of acetate-1-^{14}C (10 μc) or mevalonate-2-^{14}C (2 μc). Isolation of nonsaponifiable fractions and assay of radioactivity are described in the Methods section. Each value represents the mean of four observations with SEM for acetate incorporation, and three observations with SEM for mevalonate incorporation.

three time intervals. The data from the 4 hr time interval showed a 62% inhibition in CPIB-fed rats. The rate of decrease in the specific activity of the liver sterols at the two later time intervals was lower for the CPIB-fed rats than the controls. This reflects the inhibition of liver cholesterol synthesis relative to other tissues. The incorporation into the liver ubiquinone and hydrocarbon fractions at this level of acetate-1-^{14}C administration was low so that no attempt could be made to study specific activity of these compounds.

Mevalonate-2-^{14}C Incorporation

The results obtained for the mevalonate-2-^{14}C incorporation into various liver nonsaponifiables—sterol, ubiquinone, and hydrocarbon—from normal and CPIB-fed rats are also presented in Fig. 1. The specific activity in the sterol fraction was consistently and significantly higher in CPIB-fed animals at all the time intervals. However, the rate of fall of radioactivity in both groups was similar up to 72 hr. The total amount of ubiquinone isolated per liver was 746 ± 65 μg from animals of the control group and 1205 ± 151 μg from animals of the CPIB-fed group. The specific activity in the ubiquinone fraction in the CPIB-fed group was considerably lower than in the normal group at 4 hr, but significantly higher at 24 and 72 hr. Consequently, the specific activity for the CPIB-fed group showed an initial increase from 4 to 24 hr and thereafter stayed constant until 72 hr, whereas the control group showed a progressive fall until 72 hr. The specific activity in the hydrocarbon fraction in CPIB-fed animals, although higher at 4 and 24 hr, dropped almost to the same level as in the controls after 72 hr.

In Vitro Experiments

Incorporation of acetate-1-^{14}C and mevalonate-2-^{14}C into cholesterol of liver homogenates from control and CPIB-fed rats is presented in Table 1. In confirmation of in vivo results, the incorporation of acetate-1-^{14}C was significantly (P = 0.01) inhibited by 50–86%. The inhibition was slightly higher in rats fed CPIB for 150 days than in those fed for 21 days, but not significantly so. Older animals showed lower incorporation rates than younger ones when acetate-1-^{14}C was administered. With mevalonate-2-^{14}C, there was little difference in the specific activities between control and CPIB-fed animals. In three of five experiments, mevalonate incorporation into cholesterol of homogenates from CPIB-fed animals was slightly higher, but only at a level of significance of P = 0.05.

TABLE 1 EFFECT OF CPIB FEEDING ON THE IN VITRO INCORPORATION OF ACETATE AND MEVALONATE INTO LIVER CHOLESTEROL

Experiment	Treatment	No. of Days on Diet	Acetate-1-^{14}C Specific Activity ($\times 10^{-3}$)	Acetate-1-^{14}C % of Control	Mevalonate-2-^{14}C Specific Activity ($\times 10^{-4}$)	Mevalonate-2-^{14}C % of Control
			dpm/mg cholesterol		*dpm/mg cholesterol*	
1	Control	21	5.39 ± 0.62		0.96 ± 0.03	
	CPIB	"	2.72 ± 0.47	50*	1.17 ± 0.06	122†
2	Control	"	10.22 ± 0.91		1.27 ± 0.21	
	CPIB	"	2.70 ± 0.44	26*	1.29 ± 0.04	102
3	Control	"	13.83 ± 0.11		1.35 ± 0.01	
	CPIB	"	4.81 ± 0.15	36*	1.56 ± 0.05	119†
4	Control	150	1.94 ± 0.17		1.46 ± 0.05	
	CPIB	"	0.72 ± 0.08	33*	1.71 ± 0.07	113†
5	Control	"	3.83 ± 0.17		1.37 ± 0.08	
	CPIB	"	0.52 ± 0.01	13*	1.26 ± 0.04	93

Acetate-1-^{14}C (5 μc) or mevalonate-2-^{14}C (0.2 μc) was incubated with liver homogenates from normal and CPIB-fed rats in the presence of NAD and ATP. Results for individual experiments are presented with the SEM.

* Significantly different ($P < 0.01$) from control group by Student's t test.

† Significantly different ($P < 0.05$) from control group by Student's t test.

DISCUSSION

Our previous studies (6) demonstrated that CPIB fed to rats increases the hepatic concentration of ubiquinone. Reports from other laboratories have indicated that the possible site of inhibition of steroidogenesis caused by this drug is between acetate and mevalonate (3, 4) or between mevalonate and cholesterol (9). The results obtained in the present investigations have confirmed that biosynthesis of liver sterol is, in fact, strongly inhibited at an early step between acetate and mevalonate, as evidenced by marked inhibition of incorporation of acetate-1-^{14}C but not of mevalonate-2-^{14}C into liver sterols in CPIB-fed rats (Fig. 1). These in vivo observations have been confirmed by in vitro experiments with cell-free liver homogenates, where again it has been shown that the incorporation of mevalonate-2-^{14}C into sterols is unaffected, whereas that of acetate-1-^{14}C is strongly inhibited (Table 1).

The in vivo studies also revealed that the rate of disappearance of radioactive sterols from the liver was slightly lower in CPIB-fed animals than in control rats. It is obvious, therefore, that CPIB has no activating effect whatsoever on the catabolism of liver sterols. That CPIB fed animals had liver sterols with markedly higher specific activity than in those of the controls might be partially explained by a decreased pool size of mevalonate resulting from the block in the conversion of acetate to mevalonate. The inhibition of ubiquinone synthesis from mevalonate, in contrast to the lack of inhibition of mevalonate incorporation into sterols, indicates an inhibitory site specific for the biosynthesis of the ubiquinones rather than one on the common pathway of isoprenoid metabolism to sterols. However, since the specific activities of ubiquinone at 24 and 72 hr were significantly higher than in controls, the catabolism of ubiquinone seems to be blocked. This would explain our finding increased levels of liver ubiquinone in CPIB-fed animals (6).

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