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The Effects of Phenobarbital, Spironolactone and Diazepam on Hepatic 3-Hydroxy-3-methylglutaryl Coenzyme A Reductase Activity in Male and Female Rats

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The specific activity of hepatic microsomal 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA reductase) was examined at noon and at midnight in rats of both sexes pretreated with phenobarbital, spironolactone, or diazepam. In female rats, phenobarbital pretreatment significantly increased the activity of the enzyme at midnight, but the activity at noon remained unaltered. In male rats, the activity was unchanged by phenobarbital pretreatment both at noon and at midnight, while in castrated rats, the activity was increased significantly at midnight by pretreatment with phenobarbital. Spironolactone pretreatment decreased the activity at midnight but increased the activity at noon in both sexes. In adrenalectomized male rats, the activity in rats pretreated with spironolactone was unchanged both at noon and at midnight, compared with untreated adrenalectomized rats. Diazepam pretreatment increased the activity at noon and kept it unchanged at midnight in both sexes. It is suggested that the effect of these drugs on HMG-CoA reductase should be carefully examined in view of the variability of the effect between day and night as well as the sex-dependent differences in rats.

Keywords——HMG-CoA reductase; phenobarbital; spironolactone; diazepam; castration; adrenalectomy

Cholesterol is an essential component of various biomembranes and also serves as the precursor of bile acids and steroid hormones in the body. Furthermore, various pathological conditions, such as atherosclerosis and cholesterol gall stone disease, are believed to be closely related to abnormal cholesterol metabolism. Thus, it is important to monitor the effects of drugs frequently used in clinical wards on cholesterol biosynthesis.

3-Hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase (EC 1.1.1.34) is believed to be the rate-limiting enzyme and the most important regulatory site of cholesterol biosynthesis.[1] In this paper, we report the effects of three drugs, phenobarbital, spironolactone and diazepam, on the activity of HMG-CoA reductase in the liver microsomes of rats of both sexes. All of these drugs are frequently prescribed in clinical wards, and known to influence the activity of microsomal enzymes. Phenobarbital has been shown to increase the incorporation of acetate into cholesterol, though this finding was not always supported by other investigators.[2] Repeated administration of spironolactone increased not only the bile flow rate, but also the biliary excretion and the biliary concentration of cholesterol.[3] However, there is no information concerning the effect of this drug on cholesterol biosynthesis. Diazepam was reported to increase the incorporation of acetate into cholesterol in a human liver specimen.[4] The dearth of information with regard to the effects of these drugs on cholesterol biosynthesis is apparent. In the present study, we examined the activity of HMG-CoA reductase two times a day, at noon and at midnight, because the activity of this enzyme is known to vary 5 to 10 fold between the peak at midnight and the nadir at noon.[5]

Experimental

Animals——Five-week-old Sprague–Dawley rats of both sexes were used in all experiments. The rats were housed under daily lighting schedules of light on from 6:00 a.m. to 6:00 p.m. for 1 week prior to the experiment. Some male rats were castrated 2 weeks before administration of phenobarbital. Bilateral adrenalectomy was performed in another group of male rats 2 d prior to the start of the administration of spironolactone.

Administration of Drugs——Phenobarbital sodium salt was injected intraperitoneally, 8 mg/100 g body weight, daily for 4 d. Spironolactone, 10 mg/100 g b.w. (Aldactone-A, G. D. Searle and Co., Chicago, Ill.), or diazepam, 5 mg/100 g b.w. (Cersine powder, Takeda Chemical Industries Ltd., Osaka), was administered orally, once daily for 4 d. Control rats received 1 ml water/100 g body weight orally for 4 d.

Preparation of Microsomes——The rats were sacrificed at midnight of the day of the last treatment or at noon on the following day. Microsomes were prepared by the method of Shapiro *et al.*[6]

Assay of HMG-CoA Reductase Activity——The assay method was based on the thin layer chromatography (TLC) separation of mevalonolactone described by Shapiro *et al.*[6] Microsomes (15—30 μg protein) and nicotineamide adenine dinucleotide phosphate (NADPH) (7.5 μmol) were preincubated in 125 μl of the buffer at 37 °C for 5 min. The reaction was then started by the addition of [3-^{14}C]HMG-CoA (0.3 μmol/25 μl, 5000 dpm/nmol). The percent recovery of mevalonate from TLC was 92.6±1.1% (mean±S.D., $n=4$). The specific activity of the reductase was expressed as pmol of mevalonate production per min per mg microsomal protein.

Statistical Analysis——Significance of differences between experimental groups and the respective control groups was assessed using Student's unpaired t-test. A p value lower than 0.05 was considered to be significant.

Results and Discussion

In Table I, the specific activities of HMG-CoA reductase in these rat groups are summarized. In control rats of both sexes, the activities were 4 to 6 times higher at midnight compared with the values at noon. Furthermore, male values were lower than female values both at noon and at midnight, but the difference was significant only at midnight ($p<0.005$). Higher activities in female rats in our study agree well with previous reports by Shefer *et al.*[7] and Carlson *et al.*[8]

Phenobarbital pretreatment caused a significant increase in the specific activity of HMG-CoA reductase in female rats at midnight, but the increase in the activity at noon was not significant. In male rats, the activity showed no significant difference either at noon or at midnight between control and phenobarbital-pretreated rats.

TABLE I. Effects of Drugs on Hepatic HMG-CoA Reductase Activity

Treatment	HMG-CoA reductase activity							
	Male				Female			
	Noon (pmol/min per mg protein)[a]	(%)[c]	Midnight (pmol/min per mg protein)	(%)	Noon (pmol/min per mg protein)	(%)	Midnight (pmol/min per mg protein)	(%)
Water (*p.o.*)	208±36 (16)[b]	100	967±306 (14)	100	236±30 (8)	100	1412±552 (17)	100
Phenobarbital (*i.p.*)	205±49 (12)	99	991±265 (11)	103	271±53 (9)	115	2654±699[d] (6)	188
Spironolactone (*p.o.*)	267±62[d] (13)	128	615±195[d] (18)	64	320±57[d] (9)	136	838±324[d] (12)	59
Diazepam (*p.o.*)	297±77[d] (19)	143	776±251 (12)	80	334±62[d] (8)	142	1533±623 (6)	109

a) Values are given as the mean±S.D.
b) Values in parentheses indicate the number of rats examined.
c) Values are expressed as percent of the respective control values.
d) Significantly different from the respective control values by Student's t-test, $p<0.005$.

TABLE II. Effect of Phenobarbital on the Activity of Hepatic HMG-CoA Reductase in Testectomized Rats at Midnight

Treatment		HMG-CoA reductase activity[a]	
			(%)[c]
Saline	(*i.p.*)	1112±322 (11)[b]	115
Phenobarbital	(*i.p.*)	1501±435[d] (12)	155

a) Values are expressed in pmol·min^{-1}·mg microsomal protein^{-1} and given as the mean±S.D.
b) Values in parentheses indicate the number of rats examined.
c) Values are expressed as percent of the control value in non-castrated male rats shown in Table I.
d) Significantly different from the control value by Student's *t*-test, $p<0.025$.

Schoenfield *et al.*[9] reported that HMG-CoA reductase activity in the liver was increased in man and hamsters by repeated administration of phenobarbital. Our observations suggest, however, that cholesterogenesis may not be enhanced, at least in male rats. This agrees with the previous study on male rats reporting an unchanged conversion rate from acetate to cholesterol.[2c] Wada *et al.* also reported that cholesterogenesis (examined by using acetate as the substrate) was only marginally increased, although they demonstrated an enhancement of cholesterol production from mevalonate.[2b]

In castrated male rats, phenobarbital pretreatment increased the activity, as in intact female rats (Table II). These findings suggest that testosterone may interfere with the enhancement of activity by phenobarbital in intact male rats. Thus, it seems important to take sex differences as well as species differences into account with regard to the effect of phenobarbital on hepatic cholesterogenesis.

In rats pretreated with spironolactone, the activity of the enzyme was significantly decreased compared with control values at midnight in both sexes. On the other hand, at noon, the activity was increased significantly in both sexes. In adrenalectomized male rats, the activity in rats pretreated with spironolactone was not significantly different from the respective control values without spironolactone pretreatment, either at noon or at midnight (Table III). Table III also shows that HMG-CoA reductase activity at noon in adrenalectomized male rats was two times higher than in control rats without adrenalectomy, while values at night did not differ significantly between the two groups. However, a diurnal rhythm was still distinctly observed in adrenalectomized rats.

Several investigators[10] reported that spironolactone destroyed adrenal or testicular microsomal cytochrome P-450 and lowered the production of corticosteroids and androgen. Our observations appear to suggest that the action of adrenal hormone may be involved in the effect of spironolactone on cholesterogenesis.

Bergmann *et al.* reported that spironolactone pretreatment increased cholesterol excretion into the bile more than two fold.[3] Mellon *et al.* reported that spironolactone did not alter the activity of cholesterol 7α-hydroxylase, the rate-limiting enzyme of cholesterol catabolism.[11] From these observations, it is speculated that the increased biliary excretion of cholesterol may be due to the possible increase in cholesterol synthesis induced by this drug. Since the specific activities of HMG-CoA reductase were increased at noon and decreased at night in intact rats by spironolactone in our study, we cannot decide in a rigorous sense whether total cholesterogenesis per day was increased or decreased by this drug. However, since the rate of cholesterol synthesis was 5 times higher at midnight compared to noon, the two fold increase in the activity ony at noon does not appear to suggest a significant increase

TABLE III. Effect of Spironolactone on the Activity of Hepatic HMG-CoA Reductase in Adrenalectomized Male Rats

Treatment		HMG-CoA reductase activity[a]			
		Noon		Midnight	
			(%)[c]		(%)
Saline	(*p.o.*)	537±156 (5)[b]	258	1101±269 (6)	114
Spironolactone	(*p.o.*)	482±201 (6)	232	1133±219 (5)	117

a) Values are expressed in pmol·min^{-1}·mg microsomal protein^{-1} and given as the mean±S.D.
b) Values in parentheses indicate the number of rats examined.
c) Values are expressed as percent of the respective control values in nonadrenalectomized rats shown in Table I.

in net cholesterol synthesis rate in the rat. Thus, our observations suggest that the enhanced biliary excretion of cholesterol induced by spironolactone pretreatment[3] is unlikely to be due to increased cholesterol synthesis in the liver.

Pretreatment with diazepam increased the activities of the enzyme at noon in both sexes compared with the respective control values. At midnight, the activity was not significantly different between control rats and diazepam-pretreated rats in either sex (Table I).

Orlandi *et al.*[4] reported that pretreatment with diazepam in patients without liver disease increased the rate of cholesterol synthesis. They also stated that cholesterol synthesis in rats was not increased by this drug. In our study, the activity of HMG-CoA reductase was increased by diazepam only at noon, and then for both sexes.

In summary, phenobarbital pretreatment did not change the activity of HMG-CoA reductase expressed as per mg microsomal protein, except in the case of female rats examined at midnight, in which the activity was increased. In castrated male rats, phenobarbital pretreatment increased the activity at midnight. Spironolactone and diazepam had different effects on the activity of HMG-CoA reductase depending on the time of examination. These findings suggest that it is important to study carefully the effect of drugs on the activities of enzymes such as HMG-CoA reductase, which have pronounced diurnal rhythms, by examinations at least twice a day.

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