DIFFERENCES IN EFFECTS OF SPIRONOLACTONE AND PHENOBARBITAL ON THE HEPATIC-THYROID AXIS IN MALE RATS

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Abstract: Treatment with 150 mg/kg of spironolactone (SPL) and 100 mg/kg of phenobarbital (PB) for 13 weeks increased thyroid weight, numbers of apical vesicles and colloid droplets in thyroid follicular cells, and thyroid follicular hypertrophy. The patterns of elevated serum thyrotropin (TSH) and transiently decreased serum thyroxine (T_4) and triiodothyronine (T_3) suggest that thyroid follicular hypertrophy is a compensatory reaction to lowered thyroid hormone levels. Treatment with PB or SPL induced increased liver weight, hepatocellular hypertrophy, and smooth endoplasmic reticulum proliferation in hepatocytes. PB-treatment increased Uridine diphosphate glucuronosyl transferase (UDP-GT) activity toward *p*-nitrophenol and P-450 content but SPL-treatment had no such effect on hepatic microsomal enzymes. PB and SPL have been shown not to directly affect thyroid hormone synthesis. It is concluded that the effect of PB on thyroid function in male rats is secondary to increased hepatic UDP-GT and P-450 and that in SPL-treated male rats a distinct spectrum of isozymes of cytochrome P-450 and UDP-GT and/or other enzymes may play an important role for induction of thyroid hypertrophy. (*J Toxicol Pathol* 7: 211~222, 1994)

Key words: Spironolactone, Phenobarbital, Thyroid, Liver, Uridine diphosphate glucuronosyl transferase

Introduction

Hepatic microsomal enzymes alter both the metabolism of xenobiotic substances and that of various endogenous substances such as steroids and endocrine hormones including cortisol¹, testosterone², estrogens³, and thyroxine^{4,5}. Hormones cannot be regulated by synthesis and secretion alone; they must also be metabolized and eliminated. Thus, the hepatic-endocrine axis plays an important role in hormone homeostasis.

Phenobarbital (PB) has been extensively studied as a prototype for a class of microsomal enzyme inducers that induce a similar spectrum of cytochrome P-450 isozymes⁶. The goitrogenic effect of PB in rats is characterized by increased

thyroid gland weight and hypertrophy and hyperplasia of thyroid follicular cells⁷. Goldstein and Taurog concluded that the increased biliary excretion of thyroxine (T_4) as caused such thyroid gland change in PB-treated rats⁸. Such changes in thyroid and liver are likely due to activated thyrotropin (TSH) response that occurred in compensation for decreased serum T_4 concentration caused by an increase in its metabolic clearance⁹⁻¹¹.

Spironolactone (SPL), an aldosterone antagonist, is effective against primary aldosteronism^{12,13}, low ascites, renin essential hypertension¹⁴⁻¹⁶, and disorders of spontaneous or iatrogenic hypokalemia^{17,18}. Thyroid follicular hypertrophy and hyperplasia and a dose-related increase in liver weight have been described in 78- and 104-week study on the effects of SPL in rats¹⁹. By increasing hepatic clearance of T_4 , SPL caused a decrease in the serum concentration of this hormone and followed by a release of feed back

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inhibition and an increase in TSH resulting in the increased in thyroid gland size and activity²⁰. Thus, the effects of thyroid and liver in SPL-treated rats resemble those in PB-treated rats. Long-term administration of PB in rats has induced no thyroid follicular tumors without the initiation by nitrosamine or nitrosourea²¹. On the other hand, long-term administration of SPL in rats has induced the thyroid follicular lesions categorized as "adenomas". We have been interested in the difficult results of two long-term administration tests. The objectives of this investigations were to characterize and determine the relationship between thyroid and hepatic effects of SPL compared with that of PB and to discover the difference in these two drugs.

Materials and Methods

Chemicals

Spironolactone was obtained from Wako Pure Chem. Co. (Osaka, Japan). Phenobarbital was purchased from Mitubishikasei Co. (Tokyo, Japan).

Animals and treatment

Male Sprague-Dawley-derived CD rats (6 weeks of age), obtained from Charles River Laboratories (Kanagawa, Japan) were assigned to three groups of 18 animals each by random selection and

were orally administrated with 100 mg/kg/day of PB, 150 mg/kg/day of SPL or 0.5% (w/v) carboxymethylcellulose sodium, used as vehicle. Six animals from each group were sacrificed after 3 days, 3 weeks, and 13 weeks of the treatment (Table 1). Two animals each were housed in a polycarbonate cage with hardwood chip bedding under an air-conditioned environment at 20–24°C, 40–70% humidity and a 12 h light/dark cycle. All the animals were given rodent chow (MF, Oriental Yeast Co., Ltd., Tokyo, Japan) and tap water *ad libitum*. At the end of dosing, the animals were anesthetized by intraperitoneal administration of pentobarbital and were exsanguinated *via* the abdominal vein after blood collection was sampled. The serum samples were stored at –80°C. TSH, triiodothyronine (T₃) and T₄ were determined by radioimmunoassay. Anti-TSH antibody was rat-specific and assayed by Dr. Wa-

Table 1. Experimental Design

Group	Dose (mg/kg)	Number ^a	Sacrifice schedule ^b		
			3 day	3 week	13 week
Control	0	18	6	6	6
SPL	150	18	6	6	6
PB	100	18	6	6	6

^a Number of rats/group.

^b Days or weeks are times following start of the test.

Values are number of rats killed.

SPL, spironolactone; PB, phenobarbital.

Table 2. Thyroid Weight and Thyroid-to-body Weight Ratios^a

Group	thyroid weight (mg)			thyroid/body weight ratio (×10 ⁻³)		
	3 day	3 week	13 week	3 day	3 week	13 week
Control	12.9±1.48	18.7±2.23	23.3±4.44	6.8±0.93	5.5±0.95	4.0±0.67
SPL	14.9±3.49	25.2±4.13**	27.7±2.18	8.3±2.15	7.4±1.18*	4.9±0.49*
PB	12.7±3.75	22.1±4.87	30.0±7.76 ^b	6.7±1.84	7.0±1.33*	6.0±0.97 ^{b*}

Data represent the mean±SD for 6 rats per group.

^a Days or weeks are times following start of the test.

^b Means with standard deviation of 3 animals.

*, p<0.05; **, p<0.01 for comparison with control values (Student's *t*-test)

SPL, spironolactone; PB, phenobarbital.

Fig. 1. Thyroids from control (A) and from rats receiving 150 mg/kg of SPL (B) and 100 mg/kg of PB (C) for 13 weeks. The colloids have diminished and are nonhomogeneous. Follicular epithelial cells in the rats receiving SPL or PB are taller and wider than in control rats and their cytoplasm is finely vacuolated. HE×180.

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After the animals were sacrificed, the thyroids and livers were removed, weighed, and fixed in 10% buffered formalin. The tissues were embedded in paraffin, sectioned at 4 μ m thick and stained with hematoxylin and eosin (HE). For electron microscopy, thyroid and liver tissues were sampled from two animals per group at 13 weeks. Small blocks of these organs were fixed in 2.5% glutaraldehyde for 2 hours, postfixed in 2% osmium tetroxide for 2 hours, dehydrated in ethanol, passed through propylene oxide, and embedded in Quetol 812.

Ultrathin sections were made with LKB ultramicrotome, stained with uranyl acetate and lead nitrate, and examined with a Hitachi H-7000 electron microscope (Hitachi, Ltd., Tokyo, Japan) at 75 kV.

P-450 content and Uridine diphosphate glucuronosyl transferase (UDP-GT) activity

Microsomal fractions of liver samples from 5 control, 6 SPL-treated and 3 PB-treated animals sacrificed at week 13 were collected and measured for P-450 content and UDP-GT activity. P-450 content was determined by as described by Omura and Sato²². UDP-GT activity was determined

based on the disappearance of *p*-nitrophenol using methods first described by Bock *et al.*²³.

Statistical analysis

Statistical significant differences in means with standard deviations against the non-treated group were assessed by the Student's *t*-test, with $p < 0.05$ as minimum significance.

Results

Pathologic examination

Thyroid: Thyroid weights and thyroid morphological lesions in SPL-treated animals resembled closely those in PB-treated animals. Increased thyroid weight was noted in SPL and PB groups at week 3 (Table 2). Thyroid-to-body weight ration significantly increased in the same groups at weeks 3 and 13. These changes were slight at day 3 but quite prominent at weeks 3 and 13. In histologic examination, follicles in PB or SPL treated animals were smaller in size and lined by taller and wider epithelial cells with finely vacuolated cytoplasm when compared with the controls (Figs. 1A-1C). Some animals showed microfollicular hyperplasia featuring closely packed small follicles with little or no colloid

Table 3. Liver Weight and Liver-to-body Weight Ratios^a

Group	liver weight(mg)			liver/body weight ratio ($\times 10^{-3}$)		
	3 day	3 week	13 week	3 day	3 week	13 week
Control	8.35 $\pm$ 0.58	14.9 $\pm$ 1.97	20.2 $\pm$ 3.94	4.4 $\pm$ 0.17	4.3 $\pm$ 0.31	3.5 $\pm$ 0.38
SPL	9.37 $\pm$ 0.86*	16.9 $\pm$ 1.36**	25.0 $\pm$ 1.85*	5.2 $\pm$ 0.47**	5.0 $\pm$ 0.24**	4.4 $\pm$ 0.29**
PB	10.5 $\pm$ 0.76***	18.1 $\pm$ 2.44*	22.7 $\pm$ 5.60 ^b	5.6 $\pm$ 0.37***	5.5 $\pm$ 0.22***	4.5 $\pm$ 0.49 ^b *

Data represent the mean $\pm$ SD for 6 rats per group.

^a Days or weeks are times following start of the test.

^b Means with standard deviation of 3 animals.

*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ for comparison with control values (Student's *t*-test)

SPL, spironolactone; PB, phenobarbital.

Fig. 2. Electron micrographs of thyroids from control and rats treated with 150 mg/kg of SPL and 100 mg/kg of PB for 13 weeks.

A. Control rat, $\times 10,500$.

B. Rat treated with SPL, $\times 10,500$. The follicular cells are enlarged with delayed rough endoplasmic reticulum and there are increased numbers of reabsorbed colloids. Apical vesicles are prominent.

C. Rat treated with PB, $\times 7,700$. The follicular cells are enlarged and dilated with rough endoplasmic reticulum and there are increased numbers of resorbed colloids and lysosomal bodies. Apical vesicles are prominent.

Fig. 3. Livers from control (A) and rats treated with 150 mg/kg of SPL (B) or 100 mg/kg of PB (C, D) for 13 weeks. HE

A. A control rat, $\times 180$.

B and C. Rats treated with SPL (B) or PB (C), $\times 180$.

Centrilobular hepatocytes are swollen and round.

D. A rat treated with PB, $\times 360$. Hepatocytes have intracellular eosinophilic inclusion bodies.

content. Electron microscopy revealed some changes in follicular cells in PB or SPL treated animals killed at 13 weeks (Figs. 2A–2C). The changes were characterized mainly by increased numbers of apical vesicles beneath the apical plasma membrane of follicular epithelial cells, representing secretion into the follicular lumen and increased numbers of colloid droplets located basally, representing reabsorbed intracellular colloid. Also, follicular cells showed dilated cisternae of rough endoplasmic reticulum and increased numbers of lysosomal bodies.

Liver: Animals given 150 mg/kg of SPL or 100 mg/kg of PB showed increased liver weight and liver-to-body weight ratio during the period of treatment (Table 3). Histologically, centrilobular hepatocellular hypertrophy was seen in animals from SPL and PB groups (Figs. 3A–3D). This change was slight at day 3 but well-defined at weeks 3 and 13. Intracellular eosinophilic inclusion bodies were seen in all animals from PB group at week 13 (Figs. 3D). No SPL-treated animals showed intracellular eosinophilic inclusion bodies. Electron microscopy demonstrated increased smooth endoplasmic reticulum in enlarged centrilobular hepatocytes in SPL or PB treated animals (Figs. 4A and 4B). Concentric

membranous bodies of the endoplasmic reticulum were seen only in PB-treated animals (Figs. 4C). These membranous bodies appeared to correspond to intracellular eosinophilic inclusion bodies observed by light microscopy.

Serum TSH, T_4 , and T_3 concentrations

Serum TSH concentrations significantly increased in SPL group at weeks 3 and 13 and in PB group at week 3 when compared with control group (Fig. 5A). An upward trend of serum TSH concentrations was noted in PB group at week 13. Serum T_4 concentrations significantly decreased in PB group at 3 days and 3 weeks of treatment (Fig. 5B). Serum T_4 concentration was slightly lower in SPL group than in control group at 3 and 13 weeks. But there was no statistical difference between these two groups. The serum T_3 concentrations were significantly lower in PB group and in SPL group than in control at day 3 (Fig. 5C).

Hepatic microsomal enzyme levels

UDP-GT activity, as measured using *p*-nitrophenol as the substrate on the basis of one milligram of protein and one gram of liver significantly increased in the liver from animals treated with 100 mg/kg of PB for 13 weeks (Table 4). Moreover,

Table 4. Liver Microsomal Enzyme Levels^a

Group	Number of animals	P-450 ^b	UDP-GT ^c
Control	5	0.534±0.120	11.3±1.45
SPL	6	0.563±0.049	11.2±2.38
PB	3	1.069±0.134***	32.9±2.04***

^a All rats were treated for 13 weeks with SPL or PB.

^b Cytochrome P-450 contents are expressed as mol/mg protein.

^c One unit of activity is equal to 1 nmol *p*-nitrophenol glucuronide formed per mg protein per minute.

Data represent the mean±SD.

***, $p < 0.001$ for comparison with control values (Student's *t*-test).

SPL, spironolactone; PB, phenobarbital

significant increase in cytochrome P-450 content could be detected in the same group at week 13. On the other hand, male rats treated with SPL had neither changes in UDP-GT activity nor in P-450 content.

Discussion

Thirteen weeks of treatment with 150 mg/kg of SPL and 100 mg/kg of PB induced increased thyroid weights and follicular cell hypertrophy in male rats. The patterns of elevated serum TSH and transiently decreased serum T_4 and T_3 suggest that TSH stimulation occurs secondary to declined T_4 and T_3 levels, giving rise to morphologic changes in the thyroid gland²⁴. Serum thyroid hormone level are regulated by a feed back mechanism in the pituitary gland; decreased levels of thyroid hormone resulted in increased TSH release from the pituitary. In thyroids of animals treated with PB or SPL, ultrastructurally the follicular epithelial cells possessed abundant apical vesicles beneath the apical plasma membrane. These changes appear not to imply degenerative process, but to represent colloid biosynthesis²⁵. Decreases in T_4 and T_3 observed in animals treated with PB

or SPL were probably not due to lowered iodide uptake and organification by thyroid. Because, McClain *et al.* and Semler *et al.* reported that each drug treatment results in increased iodide uptake and organification by thyroid^{20,26}. Their data also indicate that PB or SPL do not directly affect thyroid hormone synthesis.

In animals treated with PB or SPL, enlarged hepatocytes accompanied by smooth endoplasmic reticulum proliferation were observed, and hepatic microsomal enzyme activity was enhanced. The induction of hepatic microsomal enzymes enhances the metabolism of the thyroid hormone^{4,5,11}. Comer *et al.* demonstrated that thyroid change induced by potassium propenoate (analogue of SPL) administration, follicular epithelial hypertrophy took place secondary to increased hepatic microsomal enzyme activity²⁷.

Thyroid hormone can be metabolized *via* glucuronidation (UDP-GT), oxidation (P-450), and deiodination (monodeiodinase) *etc.*¹⁰. In rodent, it is well known that UDP-GT is the most common pathway of T_4 clearance²⁸. By studies with benzpyrene, the induction of UDP-GT activity toward *p*-nitrophenol has been shown to be a good marker for induction of UDP-GT activity toward T_4 ^{8,29}. In this study, treatment with PB increased both UDP-GT activity toward *p*-nitrophenol and induction of P-450. These data indicate that the induction of hepatic microsomal enzymes (UDP-GT and P-450) by PB treatment becomes an important factor for metabolism of the thyroid hormone. On the other hand, although SPL-treated animals showed hepatic smooth endoplasmic reticulum proliferation, induction of UDP-GT and P-450 in their livers was not observed. Two hundred mg/kg of SPL administered for two weeks has been shown to increase the glucuronidation of *p*-nitrophenol in rats²⁰. In our another study, however, SPL administration at the same dose for two weeks increased thyroid weight but not glucuronidation. Increased thy-

Fig. 4. Electron micrographs of livers from rats treated with 150 mg/kg of SPL (A) and 100 mg/kg of PB (B, C) for 13 weeks.

A. A rat treated with SPL. ×10,500. Smooth endoplasmic reticulum proliferation can be seen in a centrilobular hepatocyte.

B. and C. A rat treated with PB. ×10,500. The membranes of the endoplasmic reticulum formed a whorl in the cytoplasm, enclosing lipid droplets (concentric membranous body) that are present in a centrilobular hepatocyte.

Fig. 5. Serum TSH (ng (RP-2)/ml) (A), T_4 (µg/dl) (B), and T_3 (pg/ml) (C) concentrations after 3 days, 3 weeks, and 13 weeks of treatment with spironolactone at 0, 150 mg/kg and phenobarbital at 100 mg/kg. Values are means $\pm$ SD of three to six rats per group. The asterisk indicates values significantly different from the control at $p < 0.05$ (Student's t -test).

roid weight in rats fed at 6 mg/kg of SPL, while no effect on T_4 level could be detected²⁴. Ten mg/kg administration of SPL failed to increase UDP-GT activity toward *p*-nitrophenol in rats³⁰. Thus, it appears unlikely that decreases in serum T_4 and T_3 seen in the SPL-treated group, resulted from hepatic clearance by UDP-GT and P-450.

Inducers of the enzymes involved in foreign-compound metabolism have been divided into the least two different categories on the basis of their characteristic effects on cytochrome P-450 and monooxygenase activity³¹⁻³³. One of these, PB, led to a significant increase in liver size and weight and caused the substantial proliferation of hepatic endoplasmic reticulum. This induction was associated with increases in cytochrome P-450, in rats, is referred to as cytochrome P-450b³³. The other one, 3-methyl cholanthrene (3MC) did not cause large increases in liver size or in the proliferation of endoplasmic reticulum; neither did it result in large increases in cytochrome P-450. 3MC resulted in the induction of qualitatively differed from cytochrome P-450, in rats, is referred to as cytochrome P-450c^{31,33}. In this study, SPL led to a significant increase in liver weight and substantial proliferation of hepatic endoplasmic reticulum, but did not result in large increases in cytochrome P-450. Therefore, our study suggests the possibility that increased levels of a distinct spectrum of isozymes of cytochrome P-450 and other enzymes may cause decreases in T_4 and T_3 in SPL-treated rats. Many of the same comments apply to the UDP-GT assay.

In conclusion, the thyroidal effect of PB in rats is mainly due to TSH stimulation, a compensatory response to decreased thyroid hormone level by induction of the hepatic microsomal enzymes (UDP-GP and P-450). In contrast, in SPL-treated rats, a distinct spectrum of isozymes of P-450 and UDP-GT and other enzymes may play an important role for thyroid hormone homeostasis.

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