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EFFECT OF SPIRONOLACTONE AND PREGNENOLONE-16α CARBONITRILE ON BILIRUBIN METABOLISM AND PLASMA LEVELS IN MALE AND FEMALE RATS

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Abstract—The effect of 5 days of treatment with spironolactone (100 mg/kg/day) or pregnenolone-16α carbonitrile (40 mg/kg/day) on the activity of hepatic bilirubin glucuronyltransferase (EC 2.4.1.17) in 10,000 *g* supernatant fraction from male and female rats was studied. Bilirubin plasma levels were measured after 5 and 12 days of treatment with spironolactone or pregnenolone-16α carbonitrile. Both spironolactone and pregnenolone-16α carbonitrile significantly increased the rate of bilirubin conjugation in male and female rats after 5 days of treatment. Plasma bilirubin levels were significantly reduced after 5 days of treatment with spironolactone in male rats. Both drugs caused significant decreases in plasma bilirubin levels in male and female rats after 12 days of treatment.

STUDIES in humans have shown that treatment with phenobarbital, a known stimulator of hepatic drug-metabolizing enzyme activity,[1] can lower total serum bilirubin levels in normal and hyperbilirubinemic individuals.[2–5] Recent studies in rats have shown that phenobarbital treatment increases the activity of hepatic bilirubin glucuronyltransferase (UDP glucuronate glucuronyltransferase, EC 2.4.1.17) and suggest that at least part of the clinical effects of phenobarbital on serum bilirubin levels are due to an increase in the rate of bilirubin metabolism.[6]

Since spironolactone and pregnenolone-16α carbonitrile have been shown to be effective stimulators of hepatic drug-metabolizing enzyme activity,[7,8] it was of interest to examine their effects on bilirubin metabolism. While the present work was being completed. Solymoss and Zsigmond[9] presented results of their studies showing that spironolactone and pregnenolone-16α cargonitrile treatment caused an increase in the rate of bilirubin metabolism in female rats. The studies reported here confirm their findings and expand the investigations to effects in male rats and on plasma bilirubin levels in male and female rats.

MATERIALS AND METHODS

Animals and drug treatments. Male and female rats of the Charles River strain (100–120 g) were used throughout the study. The animals were allowed to acclimate to animal facilities for 5 days before use and were allowed free access to food and water. Animals were divided into three treatment groups (8 or 12 animals per group).

One group was treated with 0·9% (w/v) sodium chloride solution (saline; 1 ml/kg/day), a second with an aqueous suspension of spironolactone (100 mg/kg/day), and the third group with an aqueous suspension of pregnenolone-16α carbonitrile (40 mg/kg/day). The drugs were administered by intraperitoneal injection for either 5 or 12 days as indicated in the tables. Methylcellulose (4000 cP) was used as the suspending agent for spironolactone and pregnenolone-16α carbonitrile, and preliminary experiments showed it had no effect on bilirubin conjugation.

Tissue preparation and blood sampling. For the metabolism studies *in vitro*, animals were sacrificed by cervical dislocation 24 hr after the last drug treatment. Livers were removed, chilled on ice, minced through a tissue press and homogenized in 0·1 M sodium phosphate buffer (pH 7·4) using a Teflon–glass homogenizer. The liver homogenates (330 mg liver/ml) were centrifuged at 10,000 *g* for 15 min, and the supernatant fraction was used as the enzyme source. For the determination of plasma bilirubin, blood was obtained by heart puncture under ether anesthesia 24 hr after the last drug treatment. The blood was stored on ice in the dark during collection. Plasma was obtained rapidly and stored at −20° in the dark until analyzed to prevent photodecomposition of the bilirubin. Preliminary experiments showed that ether anesthesia had no effect on plasma bilirubin levels.

Enzyme assays and analysis methods. Incubation mixtures consisted of 1 ml of 10,000 *g* liver supernatant fraction, 1 ml of 0·2 M Tris buffer (pH 8·0), 50 μmoles $MgCl_2$, 5 μmoles uridine-5′-diphosphoglucuronic acid (UDPGA, triammonium salt; Sigma Chemical Co.) and 400 μg bilirubin (Calbiochem) in a final volume of 3 ml. The bilirubin solution was prepared by rapidly dissolving 8 mg in 2·5 ml of 0·2 N NaOH and diluting the solution to 10 ml with a 2% (w/v) aqueous solution of bovine serum albumin (Pentex).

Mixtures were incubated for 30 min at 37° under an atmosphere of 95% oxygen and 5% carbon dioxide in a Dubnoff metabolic shaking incubator with blanks. The blanks used were identical to the incubation mixtures, except that the bilirubin was not included. Duplicate samples were employed. Preliminary experiments established that reaction rate was linear over the time period used, over the range of tissue concentrations used, and that the concentrations of substrate and cofactors were saturating. The reaction was stopped by addition of 1 ml of 1·0 N HCl to the incubation mixtures. The amount of conjugated and unconjugated bilirubin was measured by the method of Weber and Schalm,[10] except that the volumes of all reagents were quadrupled. Protein concentration of the 10,000 *g* supernatant fraction was determined by the method of Lowry *et al.*[11]

Plasma samples were analyzed for total bilirubin concentration by a Beckman Discrete Sample Analyzer (Beckman Instruments, Inc.) using the method of Jendrassik and Grof.[12]

Data from these studies were statistically analyzed by analysis of variance using the *t* statistic.

RESULTS AND DISCUSSION

Treatment of animals for 5 days with either spironolactone or pregnenolone-16α carbonitrile caused a significant increase in the rate of conjugation of bilirubin by the 10,000 *g* supernatant fraction from male and female rat livers (Table 1). Male rats

TABLE 1. EFFECT OF SPIRONOLACTONE OR PREGNENOLONE-16α CARBONITRILE TREATMENT ON THE METABOLISM OF BILIRUBIN *in vitro* BY 10,000 × *g* SUPERNATANT FRACTION FROM MALE AND FEMALE RAT LIVER*

Drug pretreatment	Sex	No. of animals	Bilirubin glucuronyltransferase activity†
Saline	Male	8	3·37 ± 0·14
Spironolactone		8	4·56 ± 0·24‡
Pregnenolone-16α carbonitrile		8	4·35 ± 0·16‡
Saline	Female	12	1·49 ± 0·18
Spironolactone		12	2·46 ± 0·17‡
Pregnenolone-16α carbonitrile		8	2·32 ± 0·07‡

* Rats were pretreated for 5 days with saline (1 ml/kg/day) or spironolactone (100 mg/kg /day) or pregnenolone-16α carbonitrile (40 mg/kg/day). Drugs were given by the intraperitoneal route and the animals were used 24 hr after the last treatment.

† Expressed as the mean micrograms of bilirubin conjugated/milligram protein/30 min ± S.E.

‡ Significantly different from the respective saline group; $P < 0·01$.

in the saline-treated group had a higher rate of bilirubin metabolism than the female rats. However, treatment with spironolactone and pregnenolone-16α carbonitrile caused a larger per cent increase in the rate of bilirubin metabolism in female rats (65 and 56 per cent respectively) than in male rats (35 and 29 per cent respectively). Sex differences in the metabolism of various compounds in rats have been reported.[1] Further, in accord with the data presented here, Inscoe and Axelrod[13] showed that the rate of glucuronidation of *O*-aminophenol was greater in liver microsomes from male rats than those from female rats, and that treatment with 3,4-benzypyrene, an enzyme stimulator, caused a larger per cent increase in the glucuronyltransferase activity in female rats.

TABLE 2. EFFECT OF SPIRONOLACTONE OR PREGNENOLONE-16α CARBONITRILE PRETREATMENT ON PLASMA BILIRUBIN LEVELS IN MALE RATS*

Drug pretreatment	Days of pretreatment	No. of animals	Total plasma bilirubin (mg/100 ml) ± S.E.)
Saline	5	7	0·14 ± 0·01
Spironolactone	5	8	0·12 ± 0·01†
Pregnenolone-16α carbonitrile	5	8	0·13 ± 0·01
Saline	12	8	0·13 ± 0·01
Spironolactone	12	8	0·08 ± 0·01‡
Pregnenolone-16α carbonitrile	12	8	0·07 ± 0·01‡

* Rats were pretreated with saline (1 ml/kg/day) or spironolactone (100 mg/kg/day) or pregnenolone-16α carbonitrile (40 mg/kg/day). Drugs were given by the intraperitoneal route and the animals were used 24 hr after the last treatment.

† Significantly different from saline group; $P < 0·05$.

‡ Significantly different from saline group; $P < 0·01$.

Treatment of male rats with spironolactone for 5 days caused a significant decrease in plasma bilirubin (Table 2). Pregnenolone-16α carbonitrile treatment for 5 days did not significantly alter the plasma bilirubin in male rats (Table 2). Treatment of male rats for 12 days with either spironolactone or pregnenolone-16α carbonitrile caused a significant decline in the level of plasma bilirubin (Table 2). Treatment of female rats for 5 days with spironolactone or pregnenolone-16α carbonitrile did not significantly alter plasma bilirubin levels but, when the treatment was extended for 12 days, both compounds showed the ability to decrease significantly bilirubin levels in plasma (Table 3). It might be expected that bilirubin plasma levels should show a

TABLE 3. EFFECT OF SPIRONOLACTONE OR PREGNENOLONE-16α CARBONITRILE PRETREATMENT ON PLASMA BILIRUBIN LEVELS IN FEMALE RATS*

Drug pretreatment	Days of pre-treatment	No. of animals	Total plasma bilirubin (mg/100 ml ± S.E.)
Saline	5	12	0·14 ± 0·03
Spironolactone	5	12	0·14 ± 0·02
Pregnenolone-16α carbonitrile	5	8	0·11 ± 0·01
Saline	12	13	0·13 ± 0·01
Spironolactone	12	14	0·11 ± 0·01†
Pregnenolone-16α carbonitrile	12	8	0·10 ± 0·01†

* Rats were pretreated with saline (1 ml/kg/day) or spironolactone (100 mg/kg/day) or pregnenolone-16α carbonitrile (40 mg/kg/day). Drugs were given by the intraperitoneal route and the animals were used 24 hr after the last treatment.

† Significantly different from saline group; $P < 0·05$.

decrease after 5 days of treatment with spironolactone or pregnenolone-16α carbonitrile, since the activity of the glucuronyltransferase is increased at that time. This discrepancy may be due to the fact that at normal plasma concentrations of bilirubin the liver does not metabolize bilirubin at a maximal rate.[14] If the animals were given a load of exogenous bilirubin to saturate the glucuronyltransferase, the effects of these compounds on plasma bilirubin might be observed earlier in the treatment schedule. In agreement with this postulate, Solymoss and Zsigmond[9] have presented data which show that, in female rats, 3 days of pretreatment with either spironolactone of pregnenolone-16α carbonitrile caused a significant increase in the rate of decline of bilirubin in the plasma after an intravenous injection of bilirubin. While the data presented here support the hypothesis that the decrease in plasma bilirubin may be due to increases in the activity of glucuronyltransferase, alteration in other factors which have been shown to affect plasma bilirubin levels, such as rate of bile flow,[15] rate of heme oxidation[16] or changes in hepatic uptake of bilirubin,[17] may be involved. Data presented by other investigators suggest that treatment of female rats with either spironolactone or pregnenolone-16α carbonitrile can cause an increase both in bile flow and hepatic uptake of bilirubin.[9] The role that each of these factors plays in the decrease in plasma bilirubin after treatment with spironolactone or pregnenolone-16α carbonitrile will await further study.

The data presented in this study show that treatment of male and female rats with spironolactone or pregnenolone-16α carbonitrile causes an increase in the hepatic bilirubin metabolism by stimulating the activity of the bilirubin glucuronyltransferase. Plasma bilirubin levels are also decreased by treatments with these drugs. These results suggest that spironolactone or pregnenolone-16α carbonitrile might be useful in treating clinical hyperbilirubinemia where the excess bilirubin in the plasma is in the unconjugated form.

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