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SHORT COMMUNICATIONS

Effect of spironolactone and phenobarbital administration on bilirubin glucuronidation in hepatic and extrahepatic rat microsomes

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It is known that conjugation with glucuronic acid catalyzed by microsomal UDP-glucuronyltransferase (EC 2.4.1.17) (GT) is quantitatively the most important phase 2 reaction of drug metabolism [1]. GT activity is at least partially latent and the degree of latency depends upon the tissue, mode of preparation of the microsomal suspension, and the activating agent used [2]. Bilirubin IXα, the hydrophobic end product of heme catabolism, is one of the endogenous substrates of GT. The enzyme transfers the glucuronyl group of UDP-glucuronic acid (UDPGA) to the carboxyl group of one or both propionic acid side chains of the bilirubin molecule. These reactions have an absolute requirement for the cosubstrate UDPGA and may result in the formation of two distinct bilirubin monoglucuronides (BMG) (C-8 and C-12 isomers) as well as bilirubin diglucuronide (BDG) [3].

Although the liver is a major site of bilirubin glucuronidation, other tissues show, *in vitro* GT activity towards bilirubin [4]. Thus, the renal cortex [5] and the intestinal mucosa [6, 7] have been studied particularly, but the proportions of BMG isomers and BDG formed in these extrahepatic tissues are unknown.

On the other hand, phenobarbital (PB) [8–10] and spironolactone (SP) [11–14] induce GT activity in rats, increasing plasma bilirubin clearance and bile bilirubin secretion. In addition, SP is a more effective and specific inducer of hepatic bilirubin-GT activity in rats than PB, and joint treatment with both inducers suggested an additive effect on liver enzyme activity [15].

In this study, we compared bilirubin glucuronidation in liver, renal cortex and intestinal mucosa rat microsomes in order to investigate the effect of individual and combined administration of PB and SP on the formation of BMG isomers and BDG. To determine whether the induction of GT by PB and SP was influenced by the activation status of the enzyme, inducer effects were measured in native microsomes and in preparations activated with the membrane perturbant digitonin.

Materials and Methods

Chemicals. Bilirubin, UDPGA (ammonium salt), bovine serum albumin (fraction V), and SP were from the Sigma Chemical Co. (U.S.A.). All other chemicals were of analytical grade purity and used as supplied.

Animals. Male Wistar rats (250–300 g) were used. All rats were housed in individual metabolic cages in a 22° temperature-controlled room with alternating 12-hr light/dark cycles for at least 1 week prior to the study. The animals were maintained *ad lib.* on a standard laboratory pellet diet and were allowed free access to water and saline solution during treatment.

Experimental groups. A group of rats was injected i.p. with PB (sodium salt), given as a daily dose of 100 or 200 μmol/kg body wt (25.4 or 50.8 mg/kg body wt, respectively), dissolved in 0.9% (w/v) NaCl solution, for 3 consecutive days prior to the experiment. Another group received equal molar doses of SP (41.7 or 83.4 mg/kg body wt) in the same way, but dissolved in propylene glycol. A third group of animals received simultaneously both inducers in the same way, in the relationship 100 to 100 and 200 to 200 μmol/kg body wt. The doses of PB and SP used were below those capable of producing a maximum induction of the liver enzyme [15]. A fourth group of rats was injected with 0.9% (w/v) NaCl solution or propylene glycol, and was used as the control.

Isolation of microsomes. The animals were deprived of food for 12 hr before the experiments. After sacrifice, liver and mucosa specimens were obtained as described previously [7]. For intestinal mucosa, the mucus was separated carefully [16] prior to scraping from the inner wall. The kidneys were promptly removed and placed in cold saline solution. Then, renal cortical tissue was separated for isolation of microsomes. Hepatic, renal and intestinal microsomal suspensions were prepared in 250 mM sucrose–1 mM EDTA (pH 7.4), as described [17]. The total protein concentration determined [18] in the three tissue preparations varied from 18.7 to 27.5 mg/mL. The suspensions thus obtained will be referred to hereafter as latent microsomes.

Enzyme activation. Aliquots of latent preparations were diluted to about 18.5 mg protein/mL with the isolation medium and then mixed with an equal volume of 1.2% (w/v) digitonin in sucrose—EDTA solution to yield a final detergent concentration of about 0.6 mg/mg microsomal protein. The mixtures thus obtained were left at 0° for 30 min.

Assay of bilirubin glucuronidation. Total bilirubin glucuronidation capacity was measured according to Black *et al.* [19]. The optimal concentrations of the reagents in the mixtures were: 65 mM triethanolamine–HCl buffer (pH 7.4), 9.6 mM $MgCl_2$, 137 μM bilirubin, 75 μM bovine serum albumin, and 12 mM UDPGA. The final volume was 1.35 mL. The reaction was initiated by adding 0.4 mL of latent microsomes (previous to dilution of 1 vol. of the original suspension with 1 vol. of sucrose–EDTA solution) or digitonin-activated preparation to the reaction mixtures. These mixtures were then incubated at 37° in a shaking water bath for 15 min. During this period, GT activity was linear with time for all tissue preparations. Protein concentrations in all the mixtures varied from 2.8 to 4.0 mg/mL.

At the end of the incubation, a volume of 0.7 mL was used to determine GT activity with diazotized ethyl anthranilate [19]. The remainder was used to estimate the relative amounts of BMG isomers and BDG formed as described previously [20].

Statistical analysis. The results are expressed as mean values ±SD. Student's *t*-test was used for comparison of the data: $P < 0.05$ was considered to be statistically significant.

Results and Discussion

Bilirubin glucuronidation in preparations from control rats. When latent preparations were used, the highest GT activity was seen in liver while intestinal preparations showed the lowest activity (Fig. 1A).

Digitonin activation (Fig. 1B) produced important increases of enzyme activity in the three tissue preparations but the degree of activation was much greater in the intestinal ones. Thus, percent increases over basal values were 95, 102 and 231% for liver, renal cortex and intestinal mucosa respectively. This suggested a greater latency of

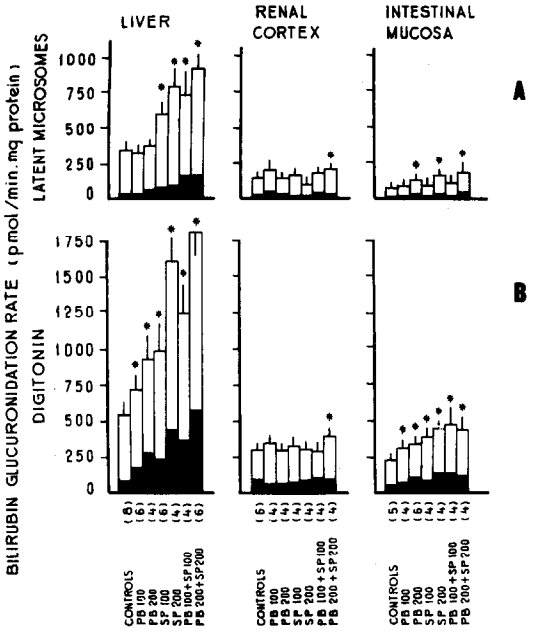

Fig. 1. Effects of inducer treatment and activation status on bilirubin glucuronidation in liver, renal cortex, and intestinal mucosa microsomes. Abbreviations: PB, phenobarbital; and SP, spironolactone. Rats were given daily doses of PB, SP or PB + SP (100 or 200 μmol/kg body wt), for 3 consecutive days prior to the experiment. See Materials and Methods for experimental details. Open bars indicate BMG and solid bars, BDG formation. Values of total conjugates are means ± SD. The asterisks indicate a significant difference in comparison with controls ($P < 0.05$). The number of experiments is given in parentheses.

the intestinal enzyme *in vivo*, and that the use of detergent-activated microsomes may lead to overestimating GT activity as was observed for 1-naphthol [21], which is probably glucuronidated by a different GT isoenzyme [22].

Most of the bilirubin glucuronide formed in the three tissues was BMG; BDG represented only about 12%. Digitonin activation increased the proportions of BDG (liver: 18 ± 2%; renal cortex: 22 ± 9%; intestinal mucosa: 21 ± 3%), which may be attributed to a modification of the physical state of the hydrophobic core of the microsomal membrane [23]. The isomeric composition of BMG was similar in hepatic and extrahepatic tissues, irrespective of the activation status of the enzyme. Thus, the ratio C-8 BMG/C-8 BMG + C-12 BMG in latent and activated preparations was 0.62 ± 0.08 (N = 16) for liver, 0.58 ± 0.12 (N = 12) for renal cortex, and 0.68 ± 0.04 (N = 10) for intestinal mucosa. These results indicated that no differences existed between tissues in the mode of interaction of unconjugated bilirubin with the postulated unique catalytic center at which BMG is formed [24].

Bilirubin glucuronidation in preparations from inducer-treated rats. As shown in Fig. 1A, the enzyme activity of latent hepatic microsomes seemed to be preferentially induced by SP pretreatment while the intestinal enzyme appeared to be similarly affected by the higher doses of both inducers. In contrast, the renal enzyme activity was practically unaffected by inducer treatment. This lack of effect of enzyme inducers on renal cortex preparations has been described for Phase 1 enzymes and cytochrome P450 which are unaffected by PB [25]. Such behavior may be indicative of differences in structure–activity relationships of the renal enzyme [26] or in the inducer accessibility to the sites of receptor proteins in renal tissue [27].

Digitonin activation modified membrane constraint of the hepatic and intestinal enzyme in such a way that all the inducer treatments enhanced GT activity significantly (Fig. 1B). However, a different pattern of induction was observed for both tissues. While SP seemed to be the most effective inducer of hepatic bilirubin glucuronidation, intestinal GT was similarly induced by all the treatments irrespective of inducer, dose or combination used. This suggested that factors regulating enzyme stimulation in the intestinal mucosa could be different from those in liver.

On the other hand, the lack of effect of digitonin activation in modifying the pattern of induction observed for latent renal microsomes reinforces the assumption discussed above.

As shown in Fig. 1, BDG proportion was only modified by inducer treatment when digitonin-activated microsomes were examined. In this regard, the higher capacity to form BDG was mainly observed in liver SP preparations which is in agreement with the results obtained in living rats [14]. The isomeric composition of BMG was not modified by inducer pretreatment irrespective of the degree of enzyme activation, suggesting that the interaction unconjugated bilirubin-GT was unaffected.

It may be concluded that (i) liver GT seemed to be the more active and that BMG and BDG formations were biochemically similar in the three tissues examined, (ii) digitonin activation revealed a high degree of latency of intestinal GT which probably depended on a particular microenvironment of the enzyme, (iii) hepatic and intestinal GT activity increased significantly in response to inducer treatment while the renal enzyme was practically unaffected, and (iv) SP was more effective than PB as an inducer of hepatic GT irrespective of its activation status.

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REFERENCES

1. Dutton GJ, The biosynthesis of glucuronides. In: *Glucuronic Acid, Free and Combined* (Ed. Dutton GJ), pp. 185–299. Academic Press, New York, 1966.
2. Dutton GJ, *Glucuronidation of Drugs and Other Compounds*. CRC Press, Boca Raton, FL, 1980.
3. Gollan JL and Schmid R, Bilirubin metabolism and hyperbilirubinaemic disorders. In: *Liver and Biliary Disease. Pathophysiology, Diagnosis, Management* (Eds. Wright R, Alberti KGMM, Karran S and Millward-Sadler GH), 2nd Edn, pp. 301–357. Baillière Tindall, London, 1985.
4. Stevenson IH and Dutton GTJ, Glucuronide synthesis in kidney and gastrointestinal tract. *Biochem J* **82**: 330–340, 1962.

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5. Fevery J, Van de Vijver M, Michiels R and Heirwegh KPM, Comparison in different species of biliary bilirubin-IXα conjugates with the activities of hepatic and renal bilirubin-IXα uridine diphosphate glycosyltransferases. *Biochem J* **164**: 737–746, 1977.
6. Hartmann F and Bissell DM, Metabolism of heme and bilirubin in rat and human small intestinal mucosa. *J Clin Invest* **70**: 23–29, 1982.
7. Morisoli LS, Mottino AD, Pellegrino JM, Guibert EE and Rodríguez Garay EA, Effect of spironolactone on bilirubin metabolism in rat liver and small intestinal mucosa. *Biochem Pharmacol* **31**: 1469–1474, 1982.
8. Robinson SH, Yannoni C and Nagasawa S, Bilirubin excretion in rats with normal and impaired bilirubin conjugation. Effect of phenobarbital. *J Clin Invest* **50**: 2606–2613, 1971.
9. Jansen PLM and Henderson PTh, Influence of phenobarbital treatment on *p*-nitrophenol and bilirubin glucuronidation in Wistar rat, Gunn rat and cat. *Biochem Pharmacol* **21**: 2457–2462, 1972.
10. Berk PD, Tavoloni N, Okuda H, Jones MJT and Kiang CL, Dose related effects of phenobarbital on rat liver microsomal UDP-glucuronyltransferase. In: *Advances in Glucuronide Conjugation* (Eds. Matern S, Bock KW and Gerok W), pp. 145–152. MTP Press, Lancaster, England, 1985.
11. Solymoss B and Zsigmond G, Effect of various steroids on the hepatic glucuronidation and biliary excretion of bilirubin. *Can J Physiol Pharmacol* **51**: 319–323, 1972.
12. Radzialowski FM, Effect of spironolactone and pregnenolone 16α carbonitrile on bilirubin metabolism and plasma levels in male and female rats. *Biochem Pharmacol* **22**: 1607–1611, 1973.
13. Klaassen CD, Effect of microsomal enzyme inducers on the biliary excretion of an exogenous load of bilirubin in newborn rats. *Proc Soc Exp Biol Med* **153**: 370–373, 1976.
14. Mottino AD, Rassero JA and Rodríguez Garay EA, Effect of spironolactone on bilirubin conjugation by the rat liver. *Biochem Pharmacol* **28**: 2215–2217, 1979.
15. Mottino AD, Guibert EE and Rodríguez Garay EA, Additive effect of combined spironolactone and phenobarbital treatment on hepatic bilirubin UDP-glucuronyltransferase. *Biochem Pharmacol* **38**: 851–853, 1989.
16. Koster ASj and Noordhoek J, Similarity of rat intestinal and hepatic microsomal 7-hydroxycoumarin-UDP-glucuronyltransferase: *In vitro* activation by Triton-X100, UDP-*N*-acetylglucosamine and $MgCl_2$. *Biochem Pharmacol* **31**: 2701–2704, 1982.
17. Siekevitz P, Preparation of microsomes and submicrosomal fractions. *Methods Enzymol* **5**: 61–68, 1962.
18. Suranyi EM and Avi-Dor Y, Swelling—contraction of mitochondria in hypotonic medium. *Biochim Biophys Acta* **118**: 445–452, 1966.
19. Black M, Billing BH and Heirwegh KPM, Determination of bilirubin UDP-glucuronyltransferase activity in needle–biopsy specimens of human liver. *Clin Chim Acta* **29**: 27–35, 1970.
20. Blanckaert N, Analysis of bilirubin mono- and di-conjugates. Determination of their relative amounts in biological samples. *Biochem J* **185**: 115–128, 1980.
21. Koster ASj and Noordhoek J, Glucuronidation in the rat intestinal wall. Comparison of isolated mucosal cells, latent microsomes and activated microsomes. *Biochem Pharmacol* **32**: 895–900, 1983.
22. Bock KW, von Clausbruch UC, Kaufmann R, Lilienblum W, Oesch F, Pfeil H and Platt KL, Functional heterogeneity of UDP-glucuronyltransferase in rat tissues. *Biochem Pharmacol* **29**: 495–500, 1980.
23. Smith DJ and Gordon ER, Role of the physical state of the hepatic microsomal membrane in the formation of bilirubin diglucuronide. *J Hepatol* **4**: 1–7, 1987.
24. Vanstapel F and Blanckaert N, On the binding of bilirubin and its structural analogues to hepatic microsomal bilirubin UDP-glucuronyltransferase. *Biochemistry* **26**: 6074–6082, 1987.
25. Kluwe WM and Hook JB, Comparative induction of xenobiotic metabolism in rodent kidney, testis and liver by commercial mixtures of polybrominated biphenyls and polychlorinated biphenyls, phenobarbital and 3-methylcholanthrene: Absolute and temporal effects. *Toxicology* **20**: 259–273, 1981.
26. Fournel S, Magdalou J, Villoutreix J, Siest G, Caldroell J and André JC, Structure-dependent induction of bilirubin glucuronidation and lauric acid 12-hydroxylation by arylcarboxylic acids chemically related to clofibrate. *Biochim Biophys Acta* **842**: 202–213, 1985.
27. Bock KW and Lilienblum W, Immunochemical studies on a 3-methylcholanthrene-inducible UDP-glucuronyltransferase of rat liver. In: *Advances in Glucuronide Conjugation* (Eds. Matern S, Bock KW and Gerok W), pp. 51–59. MTP Press, Lancaster, U.K., 1985.

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Effects of different glycosaminoglycans on myosin ATPase activity in platelets

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Heparin is well known for increasing microvascular bleeding and capillary permeability [1]. Heparin also affects many functional characteristics of platelets, such as agonist-induced aggregation, platelet adhesion [2], release of serotonin, thromboglobulin and PF4 [3]. Furthermore, heparin induces thrombocytopenia [4], prolongs the bleeding time in patients and has antithrombotic activity [5].

Myosin is present in platelets [6], and its importance in the release reaction, clot retraction and interaction with actin is widely known [7]. Myosin–actin interaction produces kinetic energy, cytoplasmatic consistency and cellular protrusions as a consequence of G-actin quick polymerization [8]. Here we report studies performed on the ATPase activity of washed platelets in the presence of heparins having different molecular weights, native and desulfated dermatans and heparans. ATPase activity is determined in the presence of endogenous ATP.