Biochemical Pharmacology, Vol. 28, pp. 2215 - 2217.

0006-2952/79/0715-2215 $02.00/0

PRELIMINARY COMMUNICATIONS

EFFECT OF SPIRONOLACTONE ON BILIRUBIN CONJUGATION BY THE RAT LIVER

Aldo D. Mottino, Jorge A. Rassero and Emilio A. Rodriguez Garay

Departamento de Fisiología, Facultad de Ciencias Bioquímicas,

Universidad Nacional de Rosario, 2000 Rosario, Argentina
(Received 12 March 1979; accepted 16 April 1979)

Glucuronidation of bilirubin is a prominent pathway by which lipid soluble pigment is converted into a water soluble form that may be excreted into the bile (1,2). UDP-glucuronyltransferase is the microsomal enzyme which catalyzes the transfer of glucuronic acid from UDP-glucuronic acid to bilirubin (3). It was suggested that conjugation of bilirubin may follow two different steps: a priming formation of bilirubin monoglucuronide which requires UDP-glucuronyltransferase and further transglucuronidation of bilirubin monoglucuronide, with formation of bilirubin diglucuronide (preferentially excreted into the bile) and unconjugated bilirubin (4,5). However, recent findings provide no evidence for the transglucuronidation mechanism in normal rats in vivo (6). Another enzyme, UDP-glycosyltransferase, catalyzes the transfer of glucose from uridine diphosphate glucose to several substrates like bilirubin (7), although it represents a mechanism of less relevance (8). It was demonstrated in rats that the level of UDP-glucuronyltransferase correlated with the apparent maximal biliary secretion of bilirubin and that phenobarbitone produces an increase of both parameters, as well as of bilirubin diglucuronide in bile (9).

The present studies were designed to examine inducer properties of spironolactone on bilirubin conjugation in comparison to the well known effects described for phenobarbital.

All the experiments employed Wistar rats weighing between 280 and 320 g. The animals were used for in vitro and in vivo experiments. (1) In vitro experiments: five rats were treated with phenobarbital sodium, i.p., given as a daily dose of 395 μmoles/kg body wt, dissolved in 1 ml of 0.9% NaCl for 3 consecutive days prior to the experiment. This dose has been demonstrated effective as inducer of bilirubin conjugation in adult rats (10). Six rats received the steroid spironolactone (Sigma Chemical Co., U.S.A.) in the same way but as a daily dose of 240 μmoles/kg body wt (11), dissolved in 1 ml propylene glycol. Another six rats were injected with 0.9% NaCl or propylene glycol and were used as controls. Treated and control rats were killed by cervical dislocation 24 hr after the last injection. The livers were perfused with 0.9% NaCl through the portal vein, then removed, blotted on filter paper, and weighed. The ratio liver weight/body weight was calculated. Liver homogenates were prepared for the assay of glucuronyltransferase and glycosyltransferase activities as described (12,13). Total proteins in liver homogenates were determined by the method of Lowry et al.(14). Enzyme activities were expressed as nmoles of bilirubin conjugated/10 min/mg of protein. (2) In vivo experiments: these studies were conducted to examine the response of treated animals during bilirubin overload. Rats from the three groups (three rats for each group) were used. The animals were allowed free access to water and to saline solution during treatment. A polyethylene catheter was inserted into the bile duct (PE-50, Intramedic, U.S.A.) and another into the femoral vein (PE-10, Intramedic, U.S.A.), under light ether anaesthesia. Rats were then placed in restraining cages and remained conscious throughout the experiment. After complete recovery from anaesthesia (3 hr after surgery), collection of bile was started (15). Bile was collected in the dark for 30 min. Body temperature during bile collection was maintained by a warming lamp. Volume of bile was estimated gravimetrically and expressed as μl/min/100 g body wt. Bile flow was significantly greater in phenobarbital-treated rats (8.1 ± 0.4) and in spironolactone-treated rats (6.1 ± 0.4), as compared to controls (4.1 ± 0.1). The difference was also statistically significant between both treated groups ($P < 0.05$). Then, a priming dose of unconjugated bilirubin (Sigma Chemical Co., U.S.A.) (2 mg/100 g body wt) was injected, i.v., followed by the i.v. administration of pigment for 40 min (100 μg/100 g body wt/min) (16). Bile was collected every 10 min during the infusion and total bilirubin was determined in bile samples by the diazoreaction (17). Bile samples

collected during the infusion were diluted (1:50) and treated with diazotized ethyl anthranilate (ethyl anthranilate was from Eastman Organic Chemicals, U.S.A.) (18) at pH 6.0 (19). The azoderivatives were extracted and submitted to thin-layer chromatography (20) and the relative amounts of monoglucuronide and diglucuronide were determined by densitometry (7,8) (Densicord 542 A, Photovolt, U.S.A.). Student's t-test was used in the comparison of data.

Results of in vitro experiments are shown in Table 1.

Table 1. Liver weight, hepatic protein concentration and enzyme activities in liver homogenates of normal rats and rats treated with spironolactone or phenobarbital *

Rats	(Liver wt/body wt)100	P	Hepatic proteins (g/dl)	P	Glucuronyl-transferase activity	P	Glycosyl-transferase activity	P
Control	3.2 ± 0.1		4.2 ± 0.2		1.0 ± 0.1		0.6 ± 0.1	
Phenobarbital	4.2 ± 0.2	†	3.9 ± 0.1	‡	2.0 ± 0.5	< 0.05	1.2 ± 0.2	< 0.01
Spironolactone	4.1 ± 0.2	†	3.9 ± 0.1	‡	3.3 ± 0.4	< 0.05	1.7 ± 0.1	< 0.01

* Data are means ± S.E. Enzyme activities were expressed as nmoles of conjugated bilirubin/10 min/mg of protein.
† $P < 0.001$, as compared to controls.
‡ not significant differences, as compared to controls.

The ratio liver weight/body weight was significantly greater in both treated groups as compared to controls. However, glucuronyltransferase and glycosyltransferase activities were induced significantly more by spironolactone.

In vivo experiments showed that the apparent maximal rate of bilirubin excretion in controls and in phenobarbital-treated rats was reached after 30 min of unconjugated bilirubin infusion. However, rats treated with spironolactone exhibited the highest rate of bilirubin excretion that was reached, even earlier than in the other two groups. Furthermore, the rate of biliary excretion of bilirubin in the three groups was due mainly to the excretion of bilirubin diglucuronide (Fig. 1).

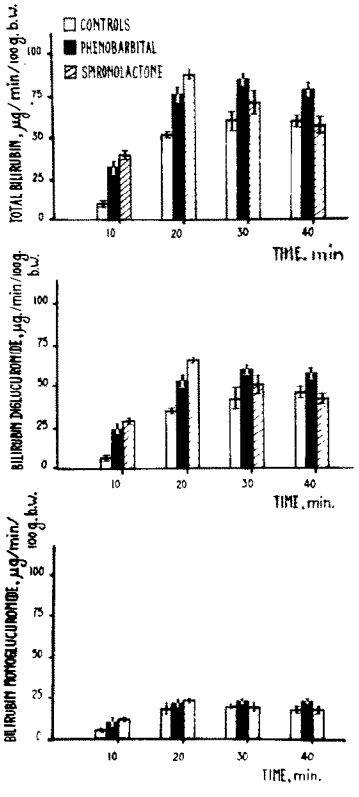

Fig. 1. Time course of biliary excretion rate of total bilirubin, bilirubin diglucuronide and bilirubin monoglucuronide in rats infused, i.v., with unconjugated bilirubin. A priming dose was injected followed by continuous infusion (see text). The results are mean values ± S.E.M. The rates of excretion of total bilirubin and bilirubin diglucuronide observed at 20 min were significantly greater in spironolactone-treated rats as compared to phenobarbital-treated animals ($P < 0.05$).

The present studies support the concept that the rate-limiting step for bilirubin hepatic transport is the mechanism of transfer of conjugated bilirubin from liver to bile (21) and that formation of bilirubin diglucuronide at the surface membrane of the liver cell (5) may be a determinant factor in the mechanism. On this basis, spironolactone, recognized as an inducer of hepatic microsomal drug-metabolizing enzymes (11), was found more effective than phenobarbital in the induction of bilirubin conjugation by the rat liver, at the respective specific doses used in the present experiments. This is in agreement with the observation that spironolactone is more effective than phenobarbital on the clearance of bilirubin in the newborn rat (22). As shown in Fig. 1, biliary excretion of bilirubin in spironolactone-treated rats decreased with time after the peak was reached, despite the fact that bile flow was well maintained. This might be due to a deficiency in the rate of bilirubin infusion for these rats or to an increased uptake of pigment by extrahepatic tissues. No explanation is possible from the present studies.

We conclude that spironolactone treatment to rats results in an important induction of the enzymes involved in bilirubin conjugation and in a more rapid excretion rate of bilirubin diglucuronide into the bile. The latter effect may afford a deeper insight into the mechanism of bilirubin transfer from liver cell to bile and of the mode of action of inducers on bilirubin conjugation.

Acknowledgements - This work was supported by research grants from CONICET, República Argentina, and from Consejo de Investigaciones de la Universidad Nacional de Rosario, República Argentina. The authors are grateful to Dr. K.P.M. Heirwegh and to Dr. J. Fevery for stimulating discussion and valuable suggestions.

REFERENCES

1. R.Schmid, J. biol. Chem. 229,881 (1957).
2. B.H.Billing, P.G.Cole and G.H.Lathe, Biochem. J. 90,261 (1964).
3. G.J.Dutton, Biochem. Pharmac. 24,1835 (1975).
4. A.W.Wolkoff, J.N.Ketley, J.G.Waggoner, P.D.Berk and W.B.Jakobi, J. clin. Invest. 61,142 (1978).
5. P.L.M.Jansen, J.R.Chowdhury, E.B.Fischberg and I.M.Arias, J. biol. Chem. 252,2710 (1977).
6. N.Blanckaert, J.L.Gollan and R.Schmid, Abstracts of IASL-meeting, p.9. Fuenguirola, Spain. October 20-21, 1978.
7. J.Fevery, M.Van de Vijver, R.Michiels and K.P.M.Heirwegh, Biochem. J. 164, 737 (1977).
8. J.Fevery,B.Van Damme, R.Michiels, J.De Groote and K.P.M.Heirwegh, J. clin. Invest. 51, 2482 (1972).
9. W.Van Steenbergen and J.Fevery, Gastroenterology, in press.
10. S.L.Vaisman, K.-S.Lee and L.M.Gartner, Pediat. Res. 10, 967 (1976).
11. D.R.Feller and M.C.Gerald, Biochem. Pharmac. 20, 1991 (1971).
12. M.Black, B.H.Billing and K.P.M.Heirwegh, Clinica chim. Acta 29, 27 (1970).
13. J.Fevery, P.Leroy and K.P.M.Heirwegh, Biochem. J. 129, 619 (1972).
14. O.H.Lowry, N.J.Rosebrough, A.L.Farr and R.J.Randall, J. biol. Chem. 193, 265 (1951).
15. N.Blanckaert, F.Compernolle, P.Leroy, R.Van Houtte, J.Fevery and K.P.M. Heirwegh, Biochem. J. 171, 203 (1978).
16. K.Weinbren and B.H.Billing, Br. J. exp. Path. 37, 199 (1956).
17. H.T.Malloy and K.A.Evelyn, J. biol. Chem. 119, 481 (1937).
18. F.P.Van Roy and K.P.M.Heirwegh, Biochem. J. 107, 507 (1968).
19. N.Blanckaert, J.Fevery, K.P.M.Heirwegh and F.Compernolle, Biochem. J. 164, 237 (1977).
20. K.P.M.Heirwegh, G.P.Van Hess, P.Leroy, F.P.Van Roy and F.H.Jansen, Biochem. J. 120, 877 (1970).
21. L.M.Gartner, D.L.Lane and C.E.Cornelius, Am. J. Physiol. 220, 1528 (1971).
22. C.D.Klaassen, Proc. Soc. exp. Biol. Med. 153,370 (1976).