Effect of spironolactone on p-nitrophenol glucuronidation in isolated rat hepatocytes[1]

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Summary. The effect of spironolactone (SP) on p-nitrophenol (PNP) glucuronidation was studied in isolated rat hepatocytes with appropriate viability conditions. A significant increase of protein concentration and PNP glucuronidation was found in the hepatocytes from SP-treated rats. Increased enzyme activity apparently was related to the SP dose. The results favor the conclusion that SP may induce PNP glucuronidation in the hepatocyte.

The influence of the aldosterone antagonist spironolactone (SP) on hepatic microsomal enzyme activity has been extensively investigated[3]. SP has been shown to increase bile flow[4], hepatic cytochrome P-450[5], and biliary excretion rate of some organic anions[6-8]. Conjugation with glucuronic acid, a reaction catalyzed by microsomal UDP-glucuronyltransferase (EC.2.4.1.17), has been recognized as a prominent pathway by which the body inactivates and eliminates a wide variety of xenobiotics and endogenous compounds[9]. In previous investigations we demonstrated that SP treatment to rats increased the activity of hepatic enzymes involved in bilirubin conjugation and the rate of bilirubin diglucuronide excretion into the bile[10,11]. As far as we know inducer properties of SP treatment on the enzyme system involved in glucuronidation has not been examined in isolated liver cells, a model capable of performing metabolic reactions similar to those observed in the whole animal[12]. Therefore, in this study we assayed the glucuronidation of para-nitrophenol (PNP) as substrate by using isolated hepatocytes from normal and SP-treated rats.

Materials and methods. All the experiments employed adult male Wistar rats, weighing between 230 and 280 g. One group of rats was injected i.p. for 3 consecutive days with SP dissolved in propylene glycol (240 µmoles/kg b.wt (100 mg/kg)). Another group received a daily dose of SP in the amount of 120 µmoles/kg b.wt (50 mg/kg). Control rats were injected with propylene glycol. Hepatocytes were isolated according to the procedures of Berry and Friend[13] and Seglen[14], with some modifications. Animals were anesthetized with sodium pentobarbital (50 mg/kg b.wt, i.p.). After a midline abdominal incision, the liver was exposed, the animal heparinized, and the vena porta cannulated. The liver was perfused 'in situ' with a calcium free buffer (pH 7.4)[15] at a rate of 18-23 ml/min, for 6-8 min, and at 37 °C. The perfusion medium was continuously bubbled with a gas mixture (95% O_2-5% CO_2). The perfusion was continued with 250 ml of the same buffer but with collagenase (0.05% w/v)[16,17] (nominally calcium free buffer[18]) at a rate of 10-12 ml/min. After the perfusion the remanent tissue was transferred to a plastic reservoir containing collagenase-calcium buffer (pH 7.4)[15], and then it was incubated in a Dubnoff metabolic shaker, at 50 strokes/min for 10 min, and at 37 °C in a 95% O_2-5% CO_2 atmosphere. Purification of hepatocytes was carried out by filtration through a nylonlayer and centrifugation (50 g, 90 sec), and the viability of liver cells as judged by the exclusion of Trypan blue dye was estimated by light microscopy[14]. The percentage of stained cells by Trypan blue averaged 16%. The number of cells stained increased slightly after 30 min of incubation at 37 °C. Cell diameter was measured with an ocular micrometer of a Leitz Wetzlar microscope (FRG)[19], and intracellular potassium concentration by flame photometry[20]. Release of lactate dehydrogenase was expressed as percent of total enzyme determined after the lysis of the cells with 0.1% Triton X-100[21]. Total water content of cells[14] and ultrastructural characteristics (Emiskope - 1A - Siemens, FRG) of the hepatocytes fixed in a glutaraldehyde mixture[13] were also tested. Table 1 shows the results of viability tests used. Glucuronidation of PNP was determined following the method of Isselbacher[22] with slight modifications. Isolated cells were resuspended in a solution containing 137 mM NaCl, 5.4 mM KCl, 0.4 mM KH_2PO_4, 0.4 mM Na_2HPO_4, 4 mM $NaHCO_3$, 1.26 mM $CaCl_2$, 0.8 mM $MgSO_4$, and 5.5 mM glucose. Appropriate amounts of the cell suspension ($5-9\times10^6$ cells equivalent to 16-25 mg of protein) were incubated at 37 °C in a medium containing 50 mM Tris-HCl buffer (pH 7.4), 3.3 mM $MgCl_2$, 3 mM UDPGA, 1 mM PNP, 1 mM saccharic acid-1,4-lactone[22,23] in a final volume of 1.5 ml which was given by the addition of a suitable amount of 150 mM NaCl solution. Appropriate blanks without UDPGA were similarly processed. The reaction was stopped by adding 10% trichloroacetic acid[24] after 30 min of incubation since rates are linear up to this time, at which point 60-70% of the substrate has reacted and substrate concentration becomes rate-limiting[12]. The formation of glucuronide was determined by measuring the disappearance of PNP. For this purpose aliquots of the deproteinized medium were diluted with 0.1 N NaOH and assayed spectrophotometrically at 400 nm (Varian 634 S,

Table 1. Parameters tested for estimation of the viability of isolated liver cells

Parameters	Mean ± SE	
Exclusion of Trypan blue (%)	83.5 ± 1.4	(n = 8)
Intracellular K^+ (mM)	96.3 ± 12.1	(n = 5)
LDH release (% of total LDH)	14.8 ± 2.9	(n = 6)
Wet weight (mg/10^6 cells)	11.7 ± 0.8	(n = 8)
Dry weight (mg/10^6 cells)	2.1 ± 0.4	(n = 8)
(Dry weight/wet weight) 100	17.4 ± 3.1	(n = 8)
Dry weight (mg/g liver weight)	256.8 ± 48.2**	(n = 8)
Cell diameter (µm)	10.23 ± 0.11	(n = 160)*
Diameter of nucleus (µm)	3.46 ± 0.03	(n = 160)*

n, No. of experiments; *No. of cells; **calculated according to P.O. Seglen from the ratio 128×10^6 cells/g liver weight. The data correspond to hepatocytes obtained from control rats. There were no differences in the parameters tested between hepatocytes from normal and SP-treated group.

Table 2. Effect of spironolactone on protein concentration and PNP glucuronidation in isolated liver cells

	Total protein concentration mg/10^6 cells	UDP-Glucuronyltransferase activity nmoles of PNP conjugated/30 min	
		Per 10^6 cells	Per mg protein
Controls	1.95 ± 0.10 (n = 8)	20.94 ± 2.98 (n = 8)	10.72 ± 1.55 (n = 8)
+ SP (daily dose 120 µmoles/kg)	2.86 ± 0.24** (n = 5)	34.95 ± 4.47** (n = 5)	12.64 ± 2.20* (n = 5)
+ SP (daily dose 240 µmoles/kg)	2.83 ± 0.24** (n = 6)	49.44 ± 8.35** (n = 5)	17.12 ± 2.02** (n = 5)

Results are mean values ± SE. n, No. of experiments. *Not significant difference; **$p < 0.05$ (refer to the statistical significance of the differences between treated and control groups).

Australia). Total protein was determined by digestion of the membrane fraction with 10% sodium deoxycholate before the addition of biuret reagent[25]. Collagenase type IV, SP, Trypan blue, PNP, UDPGA, D-saccharic acid-1,4-lactone, Triton X-100 were purchased from Sigma Chem. Co., USA, and monotest LDH opt., test UV from Boehringer GmbH, Mannheim, FRG. Sodium deoxycholate came from Fluka AG, Switzerland.

Results and discussion. The results presented in table 2 indicated that protein concentration in the hepatocytes increased significantly after SP pretreatment irrespective of the dose, and that the increase was about 46%. On the other hand UDP-glucuronyltransferase activity estimated by the rate of PNP glucuronidation, showed an increase that seemed to be proportional to the SP dose when data were expressed per 10^6 cells. The increase observed was 67% when the daily SP dose was 120 µmoles/kg, and 136% when the daily SP dose was 240 µmoles/kg, respectively. When data were expressed per mg of protein, enzyme activity was only significantly increased (60%) after the administration of the higher dose of SP.

Glucuronidation is probably the most important truly detoxicatory process, and UDPG-glucuronyltransferase is the enzyme, or enzyme system responsible for glucuronylating a wide variety of endogenous and exogenous compounds[26]. The glucuronidation of PNP has been extensively used as an estimation of enzyme activity in liver by using microsomes, homogenates or isolated hepatocytes[23,26,27]. Data obtained from single cell experiments can, in a sense, prepare us for the results of the in vivo experiments[12].

The viability of isolated cells and electron microscopy studies show that the preparation used in this investigation was suited to our purpose. However, artificially high levels of UDPGA[28] had to be added since preliminary experiments showed no glucuronidation of PNP in the absence of UDPGA. Furthermore the addition of the beta-glucuronidase inhibitor, 1,4-saccharolactone, prevented the hydrolysis of some of the conjugated product[12]. The results described in this paper demonstrate that SP treatment increase protein concentration and enzyme activity in the isolated cells, and that the increase in enzyme activity apparently is related to SP dose. In previous as yet unpublished studies we observed that increased enzyme activity in - presumably fully - digitonin-activated homogenates from SP-treated rats was unrelated to the SP dose. Thus we conclude that SP may induce the enzyme system involved in PNP glucuronidation in the hepatocyte, and that isolated cells are suitable for detecting the effect of potential inducers of such a process.

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Paracelsin, a peptide antibiotic containing α-aminoisobutyric acid, isolated from *Trichoderma reesei* Simmons. Part A*

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Summary. A peptide antibiotic has been isolated from *Trichoderma reesei* QM 9414. Although crystalline and uniform in TLC, this antibiotic could be resolved by HPLC into 3 sequence analogues. The close relationship to alamethicin was proved by chemical and spectroscopic methods, and the formation of ion-conducting pores in lipid bilayers.

In recent years the structure of several peptides with the following properties has been clarified[2]: a) molecular masses between 1600 and 2000, b) a high content (up to 50%) of α-aminoisobutyric acid (Aib, 2-methylalanine), c) some contain isovaline (Iva, 2-ethylalanine), d) N-termini acetylated, and C-termini linked with phenylalaninol (Phol) or, as in the so far unique case of trichotoxin[3,4], with valinol (Vol). With the exception of Iva, which is D[5,6] both