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STUDIES ON THE MECHANISM OF TOXICITY OF REDUCED LANTADENE A IN RATS

MICHAEL A. PASS, MIRIAM W. PUGH and LEIGH FINDLAY
Department of Physiology and Pharmacology, University of Queensland, St. Lucia, Queensland, 4067, Australia

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Abstract—Experiments were performed to test the effects of some hepatic enzyme inducers and inhibitors on the hepatotoxicity of the triterpene acid reduced lantadene A in rats, and to determine the relationship of injury of bile canaliculi to the onset of cholestasis. Pretreatment of female Wistar rats with phenobarbitone, SKF525A or carbon disulphide failed to alter the toxicity of reduced lantadene A but when female rats were pretreated with spironolactone there was a marked increase in the severity of toxicity. Male rats which are normally resistant to the toxicity of reduced lantadene A were still resistant after pretreatment with spironolactone. In other experiments rats were sacrificed at various times after dosing with reduced lantadene A and liver plasma membrane fractions enriched in bile canaliculi were prepared for estimation of the activities of 5′-nucleotidase and Mg^{2+}-ATPase. The activity of 5′-nucleotidase was reduced at 4 hr after administration of reduced lantadene A and by 6 hr the activities of both enzymes were significantly reduced. At 24 hr after dosing with the triterpene the enzyme activities were further decreased. The reduction in the activities of the bile canalicular enzymes preceded retention of bilirubin as estimated by serum bilirubin levels. It is concluded that reduced lantadene A may cause hepatotoxicity through a toxic metabolite and that injury to bile canaliculi is an early lesion in the development of cholestasis.

Reduced lantadene A (22β-angeloyloxyoleanolic acid) is a triterpene acid which has been isolated from the plant *Lantana camara* L. [1]. Together with other triterpene acids it is responsible for the cholestatic syndrome in ruminant animals which eat toxic lantana [2]. Reduced lantadene A is also toxic to female rats and it causes cholestatis characterised by jaundice, inappetance, reduced faecal output, polyuria, polydipsia and accumulation of predominantly conjugated bilirubin in blood [3].

Although female rats are readily intoxicated by reduced lantadene A male animals are not [3]. This difference in susceptibility could be due to differences in metabolism between male and female rats as it is known that male rats metabolise many foreign compounds at different rates than females [4, 5]. If the susceptibility to reduced lantadene A is due to metabolism of the triterpene, then induction or inhibition of drug-metabolising enzymes in the liver may be expected to alter the toxicity of the triterpene.

The initial site of injury in the hepatocytes is not known but injury to bile canaliculi is a consistent feature of triterpene-induced cholestasis [6–8]. Although it has been shown that incubation of isolated plasma membranes with reduced lantadene A decreases the activity of enzymes associated with bile canaliculi [9], the relationship between the changes observed in bile canaliculi and the initiation of cholestasis is still not clear.

Experiments were done to investigate the effects of some hepatic enzyme inducers and inhibitors on reduced lantadene A toxicity and to relate the time of injury to bile canaliculi to the onset of cholestasis.

MATERIALS AND METHODS

Effect of hepatic enzyme inducers and inhibitors on the toxicity of reduced lantadene A

Female Wistar rats which weighed 150–200 g were used. Food was withheld for 24 hr before and 6 hr after dosing with reduced lantadene A. The triterpene was prepared by Dr. J. Lamberton, C.S.I.R.O., Australia, by sodium borohydride reduction of lantadene A which had been extracted from *Lantana camara* L. [1, 10]. It was dissolved in olive oil and given to the rats by stomach tube.

Five groups of female rats, each containing 20 animals were used. One group was pretreated with phenobarbitone, one with spironolactone, one with β-diethylaminoethyl phenylpropyl acetate (SKF525A) and one with carbon disulphide. The fifth group was the control group. Phenobarbitone was given intraperitoneally at 80 mg/kg 48 hr and again 24 hr before dosing with reduced lantadene A. Rats were treated orally with spironolactone at 100 mg/kg twice daily for three days before receiving reduced lantadene A. SKF525A was given intraperitoneally at a dose of 40 mg/kg 1 hr before dosing with reduced lantadene A and carbon disulphide was given at 1 mg/kg orally in a 1:1 mixture with arachis oil 1 day before reduced lantadene A was given. Following pretreatment with the respective drugs each group was dosed with reduced lantadene A at 6.3 mg/kg (4 rats), 12.5 mg/kg (4 rats), 25 mg/kg (4 rats), 50 mg/kg (4 rats) and 100 mg/kg (4 rats). Two days later the rats were anaesthetised and blood was collected by heart puncture for estimation of total serum bilirubin (11). Urine bilirubin was esti-

mated on a scale from negative to +++ by a clinical screening test (Multistix-Ames Company, Australia). The rats were then killed and autopsied. Rats were considered to have been intoxicated if they were jaundiced, had a pale or yellow liver and if there was elevation of the serum and urine bilirubin levels [3].

The $ED_{50} \pm$ S.D. dose was calculated for each experiment [12] and the ED_{50} dose of treated groups compared with the control group using a *t*-test [13]. These calculations were based on the number of animals intoxicated at each dosage level.

In another trial three male Wistar rats were pretreated with spironolactone and then dosed with reduced lantadene A at 200 mg/kg. Three untreated male rats were also dosed with reduced lantadene A. They were killed two days later and post-mortem examinations were conducted.

Effect of reduced lantadene A on bile canalicular enzymes

Food was withheld from rats for one day before dosing and thereafter until they were killed. Reduced lantadene A was given orally to rats at a dose of 50 mg/kg body weight [3]. Control rats were dosed with equivalent volumes of olive oil. At 4, 6 or 24 hr after dosing, the rats were anaesthetised with ether and blood was collected by heart puncture for estimation of the total serum bilirubin [11]. The animals were then decapitated and the liver removed into ice cold 1 mM sodium borate buffer containing 0.5 mM calcium chloride, pH 7.5 for preparation of plasma membrane fractions and estimation of 5′-nucleotidase and Mg^{2+}-ATPase activities.

Liver plasma membrane fractions rich in bile canaliculi were prepared by a similar method to that of Dorling and Le Page [14]. All procedures were carried out at 4°. The liver from a rat was cut into small pieces and gently homogenized by 10 strokes of a loose-fitting Dounce homogenizer using 10 ml borate buffer per gram of liver. The homogenate was centrifuged at 150 *g* for 10 min and the supernatant retained. The pellet was resuspended, centrifuged as before and the supernatant retained. The supernatants from the 150 *g* centrifugations were centrifuged at 2000 *g* for 10 min. The pellets were combined, resuspended in borate buffer and centrifuged at 2000 *g* twice more. The final pellet was suspended in 15 ml of 66% sucrose (w/v) in a Beckman number 326825 polyallomer tube. A discontinuous sucrose gradient was formed above this base by layering 2 ml 54%, 2 ml 49%, 5 ml 45%, 5 ml 41% and 3 ml 37% sucrose solutions. All sucrose solutions were prepared with the borate buffer and were at pH 7.5. The sucrose gradient was centrifuged at 90,000 *g* for 100 min in a Beckman SW25.1 rotor. The plasma membrane fraction was removed from the 37–41% sucrose interface, washed twice in 7 mM Tris–HCl buffer, pH 7.5 and then resuspended in the same buffer.

The degree of purity of the plasma membrane fractions was estimated by the relative specific activities of marker enzymes in homogenates and membrane fractions, and by electron microscopy. 5′-Nucleotidase was used as the plasma membrane marker, acid phosphatase for lysosomes, succinate cytochrome *c* reductase for mitochondria and NADPH cytochrome *c* reductase for endoplasmic reticulum. Samples of plasma membrane fractions were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4 for 2 hr, transferred to 0.1 M cacodylate buffer and then prepared for electron microscopy [15].

5′-Nucleotidase activity was estimated in liver homogenates and plasma membrane fractions using 5 mM Na 5′-AMP (Sigma) as substrate in 50 mM Tris–HCl buffer containing 100 mM KCl and 5 mM $MgCl_2$ at pH 7.4 [16]. The activities of non-specific phosphatases in the reaction mixture were estimated using 5 mM phenyldisodium orthophosphate (BDH) as substrate instead of 5′-AMP. Mg^{2+}-ATPase activity was estimated in the same buffer solution as 5′-nucleotidase using 5 mM Tris–ATP (Sigma) as substrate [16]. Acid phosphatase was estimated in 50 mM sodium acetate buffer, pH 5.0 using 50 mM sodium β-glycerophosphate (Hopkin and Williams) as substrate [17]. Inorganic phosphate liberated in the above assays was estimated by the method of Hurst [18]. NADPH and succinate cytochrome *c* reductases were estimated in 33 mM potassium phosphate buffer, pH 7.4 containing 27 mM nicotinamide (Sigma), 0.2 mM sodium cyanide, 0.04 mM oxidised cytochrome *c* (Sigma) and either 0.075 mM NADPH (Sigma) or 33 mM succinic acid (Sigma) [19]. The concentration of protein in liver homogenates and plasma membrane fractions was estimated by the method of Lowry using crystalline bovine serum albumin (Sigma) as the standard [20].

Results are exrpessed as mean ± S.E. Comparisons of treated with control groups were made by Student's *t*-tests.

RESULTS

Effect of hepatic enzyme inducers and inhibitors on the toxicity of reduced lantadene A

The ED_{50} dose for reduced lantadene A in the control group was 14.3 ± 3.1 mg/kg. In the groups pretreated with phenobarbitone, SKF525A and carbon disulphide the ED_{50} doses of reduced lantadene A were 21.5 ± 5.4, 25.0 ± 4.8 and 21.5 ± 5.4 mg/kg respectively and were not statistically different from the control group. The clinical and postmortem findings in affected animals in the control group and the phenobarbitone, SKF525A and carbon disulphide treated groups were typical of cholestasis caused by reduced lantadene A [3].

The ED_{50} dose for the spironolactone pretreated group was 9.4 ± 3.2 mg/kg and this also was not statistically different from the control group. However, there was evidence that spironolactone significantly increased the severity of the disease. None of the rats in the control, phenobarbitone, SKF525A or carbon disulphide groups died during the experiment but 14 of the 20 rats in the group treated with spironolactone died within two days of being dosed with reduced lantadene A. Examination of affected livers revealed an increase in the severity of the lesion in many animals. Massive hepatic necrosis was widespread in the livers from animals receiving doses of reduced lantadene A at 25, 50 and 100 mg/kg and

Table 1. Enzyme activities in rat liver homogenates and plasma membrane fractions*

Enzyme	Enzyme activity Homogenate	Enzyme activity Plasma membrane fraction	Relative specific activity†	% Yield recovered in plasma membrane fraction
5′-Nucleotidase (μmoles P_i released/mg protein/hr)	1.7 ± 0.1	32.7 ± 2.1	19.5 ± 1.3	22.9 ± 0.6
Mg^{2+}-ATPase (μmoles P_i released/mg protein/hr)	4.6 ± 0.2	49.3 ± 2.0	10.8 ± 0.7	12.9 ± 1.1
Acid phosphatase (μmoles P_i released/mg protein/hr)	0.7 ± 0.04	1.0 ± 0.2	1.5 ± 0.3	1.7 ± 0.3
Succinate cytochrome *c* reductase (nmoles/mg protein/min)	37 ± 0.2	23 ± 2	0.6 ± 0.04	0.8 ± 0.05
NADPH cytochrome *c* reductase (nmoles/mg protein/min)	12 ± 1	10 ± 1	0.9 ± 0.04	1.0 ± 0.07

* Mean ± S.E. of 6 livers.
† Ratio of enzyme activity in the membrane fraction to that in the homogenate.

in two rats receiving 12.5 mg/kg. In livers affected by lower doses there was cholestatic injury [3].

The three male rats pretreated with spironolactone and the three normal male rats were not affected by reduced lantadene A.

Effect of reduced lantadene A on bile canalicular enzymes

Marker enzyme activities in liver homogenates and membrane fractions demonstrated concentration of 5′-nucleotidase and Mg^{2+}-ATPase in the membrane fractions but not of acid phosphatase, succinate cytochrome *c* reductase or NADPH cytochrome *c* reductase (Table 1). Electron microscopic examination revealed numerous bile canaliculi in the plasma membrane fractions.

In rats dosed 4 hr previously with reduced lantadene A the activity of 5′-nucleotidase in the plasma membrane fractions was significantly lower than in the control animals but there was no difference in the activity of Mg^{2+}-ATPase (Fig. 1). By 6 hr the activities of both enzymes in the treated animals were significantly below those in the control animals and by 24 hr the differences were even greater (Fig. 1). There were no significant differences between the total serum bilirubin levels in the rats dosed 4 and 6 hr previously with reduced lantadene A, and their respective control groups. In the rats dosed 24 hr previously with reduced lantadene A the serum bilirubin levels were elevated from the normal of 4 ± 0.8 μmoles/l to 45 ± 5.1 μmoles/l.

DISCUSSION

An increase in the hepatotoxicity of a compound following induction of hepatic drug-metabolising enzymes suggests that the compound may be metabolised to a toxic product. Spironolactone is a catatoxic steroid which induces hepatic drug-metabolising enzymes in rats and this results in increased

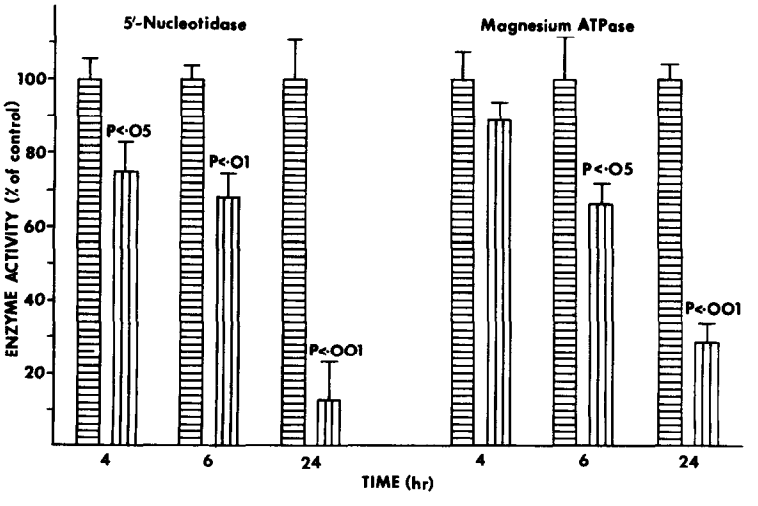

Fig. 1. Effect of reduced lantadene A (50 mg/kg orally) on the activities of 5′-nucleotidase and magnesium-ATPase in liver plasma membrane fractions of normal rats (horizontal stripes) and of rats 4, 6 and 24 hr after dosing with reduced lantadene A (vertical stripes). Each value represents the mean ± S.E. of 6 rats. Significant differences of treated animals from control animals are indicated.

metabolism of a number of compounds [21–25]. The increased toxicity of reduced lantadene A following pretreatment of rats with spironolactone indicates that reduced lantadene A may be converted to a toxic metabolite.

Although spironolactone increased the toxicity of reduced lantadene A, phenobarbitone which induces drug metabolism, and the inhibitors of drug metabolism, SKF525A and carbon disulphide, had no effect. A possible explanation for the different effects is that phenobarbitone, SKF525A and carbon disulphide do not affect the pathway for metabolism of reduced lantadene A or that their effects are qualitatively or quantitatively different to those of spironolactone. There is evidence that differences in the effects of inducers of drug metabolism do occur [25–30].

The failure of spironolactone to alter the toxicity of reduced lantadene A in male rats is not surprising as sex-differences in the effects of spironolactone on the metabolism of some drugs have been noted [25]. In male rats the hepatic effects of spironolactone are believed to be negated by the effects on the liver of reduced androgen production caused by the steroid [31].

If reduced lantadene A is toxic through a metabolite then an apparent contradiction exists between the effect of spironolactone pretreatment in female rats and the lack of toxicity in normal male rats. The rate of metabolism of many compounds is greater in male rats than female rats [4, 5]. Therefore, if reduced lantadene A acts through a toxic metabolite it would be reasonable to expect that male rats would be more susceptible than females. The reverse is in fact true [3]. A possible explanation is that male and female rats may metabolise reduced lantadene A to different products, males to a non-toxic compound and females to a toxic one. Evidence that male and female rats metabolise some compounds though different pathways was provided by Jacobson and Kuntzman [32].

The results also confirm previous observations that injury to bile canaliculi occurs in triterpene-induced cholestasis and provide evidence that this injury occurs *in vivo* and precedes the onset of cholestasis. There were significant decreases in the activities of 5′-nucleotidase and Mg^{2+}-ATPase at 4 and 6 hr respectively after dosing but the serum bilirubin did not increase until later. This indicates that injury to bile canaliculi could in part be responsible for initiating cholestasis. A similar situation has been reported in sheep with lantana poisoning in that morphological changes in bile canaliculi were detected 6 hr after lantana was given to sheep but increases in serum bilirubin did not occur until 10 hr after dosing [7, 8, 33].

It is still unclear whether injury to bile canaliculi is the primary lesion in triterpene-induced cholestasis because, at least in sheep, there are also morphological changes in the agranular endoplasmic reticulum before there are increases in serum bilirubin [7, 8, 33]. In some other cholestatic conditions it seems unlikely that injury to the endoplasmic reticulum is the cause of cholestasis [34, 35] but the relationship between changes in the functions of the endoplasmic reticulum and the onset of cholestasis caused by triterpene compounds is not known.

It is concluded that in reduced lantadene A-induced cholestasis there is injury to bile canaliculi before retention of bilirubin and that the toxin may act through formation of a toxic metabolite.

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