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Fasting increases the sensitivity of hepatic harmol glucuronidation to hypoxia

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Abstract—Livers from fasted (N = 16) and fed (N = 22) rats were perfused with harmol (50 μM) for an initial 30 min with normal oxygen delivery (6–10 μmol/min/g liver), then for 45 min with perfusate equilibrated with O_2/N_2 mixtures, which reduced hepatic oxygen delivery to 0.9–6 μmol/min/g liver, and finally for a further 30 min period of normal oxygenation. Seventy per cent of the harmol eliminated was accounted for as the glucuronide conjugate and approximately 5% as the sulphate conjugate. During the hypoxia phase with *fed* preparations, decreasing oxygenation did not reduce harmol clearance or harmol glucuronide formation clearance until oxygen delivery was less than 2.5 μmol/min/g liver, whereas with *fasted* preparations this hypoxic threshold was much higher (5 μmol/min/g liver). Below the hypoxic threshold, harmol clearance was linearly related to oxygen delivery in both groups. Hepatic tissue concentrations of unchanged harmol at the end of the hypoxia phase were double those after the same period of normal oxygenation, whereas tissue harmol glucuronide concentrations were similar. By establishing a hypoxic threshold for reduced oxygen availability this study shows that harmol glucuronidation is relatively insensitive to hypoxia, but sensitivity increases markedly in fasted animals.

Studies on drug metabolizing pathways in isolated hepatocytes [1, 2] and in isolated perfused liver preparations (e.g. Angus *et al.* [3, 4]) have shown that several of these pathways are oxygen dependent and that the metabolism of some drugs can be impaired under hypoxic conditions. In particular, oxidative drug metabolism has been shown, in both of these systems, to be sensitive to even mild reductions in oxygen delivery [1, 4, 5].

We have shown previously, using the recirculating isolated perfused rat liver preparation, that the hepatic elimination of harmol, which is metabolized almost entirely by glucuronidation, is impaired by acute, severe hypoxia [6]. Studies in isolated hepatocytes have indicated that the availability of glucose-1-phosphate and thus of glucuronic acid is a major determinant of the rate of glucuronidation, and that a decrease in this availability is important in the impairment seen in glucuronidation during acute hypoxia [2, 7]. Using the same recirculating isolated perfused rat liver preparation, we also showed that the impairment of harmol elimination by severe hypoxia was potentiated by fasting [8], which is known to deplete hepatic intracellular glycogen stores [9, 10].

By using a range of rates of hepatic oxygen deliveries in single-pass perfused livers from fed rats, we found that the hepatic clearance of salbutamol, also metabolized almost entirely by glucuronidation, was not impaired until oxygen delivery was reduced below a relatively low threshold of about 2 μmol/min/g liver [3]. The aim of the present study was to determine in the single-pass perfused liver preparation the relationship between the rate of hepatic oxygen delivery and the rate of glucuronidation of harmol in both fed and acutely fasted liver preparations, and to establish the threshold levels of hypoxia which are required to produce impairment of this drug elimination pathway under both conditions.

Materials and Methods

Chemicals and enzymes. Harmol hydrochloride, D-saccharic-1,4-lactone and β-glucuronidase–arylsulphatase enzyme (partially purified powder from *Helix pomatia*, Type H-1) were obtained from the Sigma Chemical Co. (St Louis, MO, U.S.A.). Bovine serum albumin was obtained from the Commonwealth Serum Laboratories (Melbourne, Australia) and sodium taurocholate was purchased from Calbiochem (San Diego, CA, U.S.A.). Other chemicals used were of analytical reagent grade quality.

Experimental preparation. Livers of fed (N = 22) and 24 hour fasted (N = 16) male Sprague–Dawley rats (weight 130–155 g) were perfused via the portal vein in a constant flow (10 mL/min) single-pass system [3]. The perfusate consisted of 20% (v/v) washed human red blood cells, 1% (w/v) bovine serum albumin, 0.1% (w/v) glucose (8 mM), sodium taurocholate (30 μM) and harmol 50 μM in a Krebs–Henseleit electrolyte solution at 37°. Viability of the liver preparation was determined by macroscopic appearance, oxygen consumption of greater than 3 μmol/g liver/min during normal oxygenation, stable perfusion pressure of 4–8 cm of H_2O and initial bile flow of greater than 0.3 mL/hr [3]. Perfusate pO_2, pCO_2, pH, HCO_3^- and per cent saturation were monitored throughout. For normal oxygenation the perfusate was oxygenated by equilibrium with 100% oxygen in a Silastic membrane oxygenator and for hypoxia the perfusate was equilibrated with mixtures of oxygen and nitrogen, producing a range of oxygen deliveries from 0.8 to 10 μmol/g liver/min [3].

Experimental design. Steady state studies of graded hypoxia: The experiments consisted of three consecutive phases: an initial 30 min normoxia phase, a 45 min hypoxia phase and a 30 min normoxia (recovery) phase. A different rate of oxygen delivery was used in the hypoxia phase in each liver preparation. During the recovery phase, oxygen consumption and oxygen extraction returned to within 10% of the initial control values. Inflow and outflow perfusate samples were collected at 0, 15, 20, 25 and 30 min (phase 1), at 60, 65, 70 and 75 min (phase 2), and at 90, 95, 100 and 105 min (phase 3) for measurement of steady-state harmol concentration and oxygen content.

In 11 of the 22 non-fasting liver preparations and nine of the 16 fasting liver preparations, additional samples of outflow perfusate were taken at steady state for measurement of harmol metabolites (harmol glucuronide and harmol sulphate). In these preparations, bile from each of the three phases was also assayed for these metabolites and for unchanged harmol.

Hepatic uptake studies: In two further experiments, perfusion was stopped after the 75 min sample, and the liver was immersed in 10 mL of ice-cold Sorensen's solution and homogenized with a Potter–Elvehjeim homogenizer. Two control liver perfusions were performed in which the oxygen delivery was not reduced in the second experimental phase. These perfusions were also stopped at 75 min and the liver homogenized as for the "hypoxia" group. The homogenates were then assayed for harmol and its conjugated metabolites.

Assays. Harmol concentrations in perfusate (following

Table 1. Effect of hypoxia and fasting on harmol metabolism

Experimental phase	Harmol glucuronide formation clearance (mL/min) Perfusate	Bile	Harmol sulphate formation clearance (mL/min) Perfusate	Bile	Harmol clearance (mL/min) Bile	Total
Fed preparations (N = 11)						
Control	1.86 ± 0.52	3.74 ± 1.90	0.20 ± 0.28	0.23 ± 0.19	0.17 ± 0.10	7.98 ± 0.61
Hypoxia	0.77† ± 0.52	2.51 ± 1.35	0.10 ± 0.17	0.10 ± 0.15	0.07 ± 0.06	6.21 ± 2.02
Recovery	3.29 ± 0.63	2.97 ± 1.13	0.02 ± 0.16	0.22 ± 0.30	0.06† ± 0.03	7.53 ± 0.59
Fasted preparations (N = 9)						
Control	1.73 ± 0.68	2.77 ± 1.10	0.15 ± 0.11	0.08 ± 0.06	0.09 ± 0.09	7.35* ± 0.60
Hypoxia	1.08† ± 1.37	1.90 ± 1.37	0.25 ± 0.28	0.02 ± 0.02	0.08 ± 0.08	3.56 ± 2.48
Recovery	2.27 ± 1.22	2.38 ± 1.63	0.16 ± 0.12	0.02 ± 0.03	0.15 ± 0.20	6.73* ± 0.78

* Significantly different from fed preparations, $P < 0.005$.
† Significantly different from normoxia, $P < 0.01$.

removal of red blood cells), bile and liver were quantified using a sensitive and specific HPLC method [11]. The conjugated metabolites of harmol (i.e. glucuronide and sulphate) were determined after enzymatic incubation with β-glucuronidase and arylsulphatase [11].

Calculations and statistics. The results are expressed in text and tables as the mean and the standard deviation (mean ± SD). Statistical comparisons of data made using the Student's *t*-test for paired observations, accepting $P < 0.05$ as significant. Correlations between variables were analysed using linear regression analysis [Statworks™ (version 1.2), Cricket Software Inc., Philadelphia, PA, U.S.A.] performed on a Macintosh SE computer (Apple Computer Inc., Cupertino, CA, U.S.A.).

Results and Discussion

After the 24 hour fast the mean body weight of the sixteen rats (132 ± 10 g) was significantly less than that of the fed rats (142 ± 8 g) ($P = 0.001$). Similarly, the mean weight of the livers isolated from the fasted rats (4.40 ± 0.66 g) tended to be less than that of the livers from fed animals (4.93 ± 0.88 g) ($P = 0.05$). The viability parameters of the liver preparations were comparable in the two groups during the control phase. For example, there were no differences in mean bile flow (0.66 ± 0.09 mL/min fasted rats, 0.59 ± 0.16 mL/min fed rats; $P = 0.11$) or perfusion pressure in the two groups. Using a haematocrit of 20%, hepatic oxygen delivery and consumption during normal oxygenation were 7.82 ± 1.43 and 4.20 ± 1.02 μmol/min/g liver, respectively, which are comparable to *in vivo* values in the rat (5–7 and 2–3 μmol/min/g liver, respectively [12–15]).

Consistent with previous studies [16, 17], we found that harmol was efficiently cleared by the livers of fed animals under conditions of normal oxygenation and the major metabolic pathway was glucuronidation (approximately 70%, Table 1). Another 5% was eliminated as harmol sulphate and less than 3% as unchanged drug in bile. The relative importance of these metabolic pathways was maintained during hypoxia (Table 1). Total clearance for the fed group was slightly but significantly greater than that for the fasted group during the control period, as indicated previously [8].

A *harmol clearance ratio* was calculated for each preparation as the ratio of clearance during hypoxia to clearance during the control, normal oxygen, phase. In this way each liver preparation acted as its own control. In the preparations from *fed* animals, decreasing oxygen delivery had no effect on harmol clearance (i.e. the clearance ratio was 1.0) until the rate of oxygen delivery was less than about 2.5 μmol/min/g liver (Fig. 1). Below this "hypoxic threshold", the harmol clearance ratio progressively declined in an approximately linear manner ($r = 0.96$, $P < 0.001$). A similar linear decline in the harmol clearance ratio was seen below an oxygen consumption of approximately 1.8 μmol/min/g liver (data not shown). In the livers from *fasted* rats the threshold oxygen delivery below which harmol clearance began to fall was much higher [5 μmol/min/g liver (Fig. 1)]. Below this threshold there was more variability in the effect of hypoxia than was seen for the fed preparations. However, there was a correlation between the clearance ratio and oxygen delivery below this threshold ($r = 0.55$, $P = 0.05$). Similarly, the level of oxygen consumption at which the harmol clearance ratio began to fall was much higher in the fasted liver preparations than in the fed preparations (approximately 4.2 μmol/min/g liver). In all preparations there was recovery of harmol clearance to prehypoxia values during the final recovery phase.

There were similar differences in the effect of decreasing oxygen delivery on the formation clearance ratio of harmol glucuronide in the two groups of livers (Fig. 2). The rate of oxygen delivery at which the formation clearance ratio of harmol glucuronide began to fall was identical to that of the parent harmol clearance in both the fed and fasted preparations (Figs 1 and 2). Thus, as demonstrated previously in isolated hepatocytes, there is a greater sensitivity of hepatic glucuronidation to hypoxia in the fasted state [7]. The amounts of harmol sulphate formed in this study were too small to allow analysis of the effect of hypoxia or fasting on sulphation. The hepatic tissue concentration of harmol after normal oxygenation perfusion was approximately half that of the livers which had 45 min of hypoxic perfusion. The ratio of tissue harmol glucuronide/harmol in the normoxia group was approximately twice

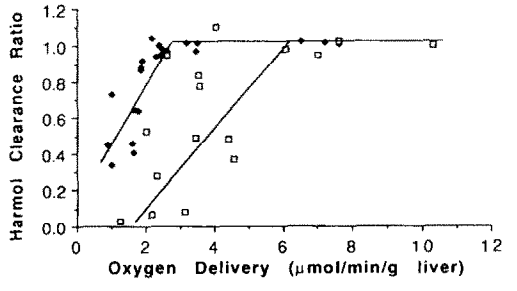

Fig. 1. Relationship between the ratio of harmol clearance during the hypoxia phase/control phase for perfused livers from fasted (□) and fed (◆) rats.

Table 2. Liver concentrations of harmol and its glucuronide metabolite at 75 min in livers isolated from fed rats

Experiment No.	Harmol (μM)	Harmol glucuronide (μM)	Harmol glucuronide/ harmol concentration ratio
Control*			
1	23.8	16.3	0.68
2	25.2	16.1	0.72
Hypoxia†			
1	52.7	18.8	0.36
2	57.2	21.1	0.37

* 75 min normal oxygenation.
† 30 min normal oxygenation followed by 45 min hypoxia.

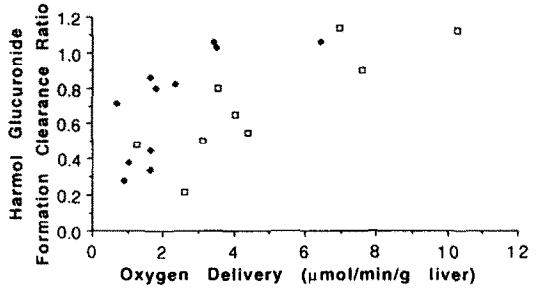

Fig. 2. Relationship between the ratio of harmol glucuronide formation clearance during the hypoxia phase/control phase for perfused livers from fasted (□) and fed (◆) rats.

that of the hypoxia group (Table 2). The tissue harmol sulphate concentration was in each case below the sensitivity of the assay. These findings indicate that the decrease in harmol clearance during hypoxia is due predominantly to impairment of glucuronidation rather than a decrease in hepatic uptake or impairment along some other elimination route.

The relationships between harmol clearance and oxygen delivery and consumption in the fed group are similar to those obtained previously from similar experiments in a group fed with salbutamol [3], a drug almost exclusively glucuronidated in the rat [18]. This was despite the differences in drug concentrations studied (salbutamol 0.2 μM, harmol 50 μM). The significance of the heterogeneity of UDP-glucuronosyl transferases [19] in the effect of hypoxia on the glucuronidation of different drugs remains to be determined.

An acute 24 hr fast markedly depletes hepatic glycogen and UDP-glucose in rats [9, 10, 20]. The combined effect of fasting and hypoxia (Figs 1 and 2) is probably via more profound depletion of ATP (and UTP) than hypoxia alone [21]. These findings imply the need for reduced drug dosage *in vivo* when hypoxia and poor nutrition co-exist, e.g. alchoholism [22]. They also suggest the possibility of increased toxicity of drugs, such as paracetamol, under such circumstances, where the impairment of glucuronidation could result in increased formation of its hepatotoxic metabolite [23].

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REFERENCES

1. Jones DP, Hypoxia and drug metabolism. *Biochem Pharmacol* **30**: 1019–1023, 1981.
2. Jones DP, Aw TY and Shan X, Drug metabolism and toxicity during hypoxia. *Drug Metab Rev* **20**: 247–260, 1989.
3. Angus PW, Mihaly GW, Morgan DJ and Smallwood RA, Oxygen dependence of salbutamol elimination in the isolated perfused rat liver. *Biochem Pharmacol* **38**: 1443–1449, 1989.
4. Angus PW, Mihaly GW, Morgan DJ and Smallwood RA, Oxygen dependence of omeprazole clearance and the production of its sulphone and sulphide metabolites in the isolated perfused rat liver. *J Pharmacol Exp Ther* **250**: 1043–1047, 1989.
5. Roth RA and Rubin RJ, Role of blood flow in carbon monoxide- and hypoxic hypoxia-induced alterations in hexobarbital metabolism in rats. *Drug Metab Dispos* **4**: 460–467, 1976.
6. Angus PW, Mihaly GW, Morgan DJ and Smallwood RA, Hypoxia impairs conjugation and elimination of harmol in the isolated perfused rat liver. *J Pharmacol Exp Ther* **240**: 931–936, 1987.
7. Aw TY and Jones DP, Control of glucuronidation during hypoxia. Limitation by UDP-glucose pyrophosphorylase. *Biochem J* **291**: 707–712, 1984.
8. Angus PW, Mihaly GW, Morgan DJ and Smallwood RA, Synergistic effects of hypoxia and fasting on harmol elimination in the isolated perfused rat liver. *Biochem Pharmacol* **37**: 1207–1212, 1988.
9. Reinke LA, Belinsky SA, Evans RK, Kauffman FC and Thurman RG, Conjugation of *p*-nitrophenol in the perfused rat liver: the effect of substrate concentration and carbohydrate reserves. *J Pharmacol Exp Ther* **217**: 863–870, 1981.
10. Jennische E, Effects of ischaemia on the hepatic cell membrane potential in the rat. Differences between

fed and fasted animals. *Acta Physiol Scand* **118**: 69–73, 1983.
11. Ching MS, Mihaly GW, Angus PW and Smallwood RA, High performance liquid chromatographic analysis of harmol and its conjugated metabolites. *J Chromatogr* **380**: 190–195, 1986.
12. Preisig RJ, Bircher J and Bauereisen E, Physiological and pathophysiological aspects of the hepatic haemodynamics. *Prog Liver Dis* **4**: 201–216, 1972.
13. Mitzkat HJ and Meyer U, Metabolic state of isolated perfused rat liver and model-induced metabolism modifications. In: *Isolated Liver Perfusion and its Applications* (Eds. Bartosek I, Guaitani A and Miller LL), pp. 79–86. Raven Press, New York, 1973.
14. Lutz J, Henrich H and Bauereisen E, Oxygen supply and uptake in the liver and intestine. *Pflugers Arch* **360**: 7–15, 1975.
15. Lautt WW, Method for measuring hepatic uptake of oxygen or other blood born substances in situ. *J Appl Physiol* **40**: 269–274, 1976.
16. Pang KS, Koster H, Halsema ICM, Scholtens E and Mulder GJ, Aberrant pharmacokinetics of harmol in the perfused rat liver preparation: sulfate and glucuronide conjugations. *J Pharmacol Exp Ther* **219**: 134–140, 1981.
17. Pang KS, Koster H, Halsema ICM, Scholtens E, Mulder GJ and Stillwell RN, Normal and retrograde perfusion to probe the zonal distribution of sulphation and glucuronidation activities of harmol in the perfused rat liver preparation. *J Pharmacol Exp Ther* **224**: 647–653, 1983.
18. Martin LE, Hobson JC, Page JA and Harrison C, Metabolic studies of salbutamol-^{3}H: a new bronchodilator, in rat, rabbit, dog and man. *Eur J Pharmacol* **14**: 183–199, 1971.
19. Burchell B and Coughtrie MWH, UDP-glucuronosyltransferases. *Pharmacol Ther* **43**: 261–289, 1989.
20. Price VF and Jollow DJ, Mechanism of decreased acetaminophen glucuronidation in the fasted rat. *Biochem Pharmacol* **37**: 1067–1075, 1988.
21. Bradford BV, Marotto M, Le Masters JJ and Thurman RG, New simple models to evaluate zone-specific damage due to hypoxia in the perfused rat liver: time course and effects of nutritional state. *J Pharmacol Exp Ther* **236**: 263–268, 1985.
22. French SW, Biochemical basis for alcohol-induced liver injury. *Clin Biochem* **22**: 41–49, 1989.
23. Aw TY, Shan X, Sillau AH and Jones DP, Effect of chronic hypoxia on acetaminophen metabolism in the rat. *Biochem Pharmacol* **42**: 1029–1038, 1991.