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SYNERGISTIC EFFECTS OF HYPOXIA AND FASTING ON HARMOL ELIMINATION IN THE ISOLATED PERFUSED RAT LIVER

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Abstract—In isolated hepatocytes the availability of intracellular glucose appears to be a key factor controlling the rate of xenobiotic glucuronidation during hypoxia. This study in the isolated perfused rat liver examines the effect of both a 24-hr fast and removal of glucose (8 mM) from liver perfusate on the elimination of bolus doses of harmol (20 μmol) under normoxic and hypoxic conditions. In the preparations used in these experiments, harmol glucuronide is the major metabolite (> 80%) with the remainder being sulphate. During *normal oxygenation*, in the livers from fed rats, harmol was rapidly eliminated ($t_{\frac{1}{2}} = 4.2 \pm 0.4$ min; mean ± SD, N = 4). Fasting led to a small reduction in harmol elimination rate ($t_{\frac{1}{2}} = 5.6 \pm 0.4$ min; $P < 0.025$) while removal of glucose from perfusate made no difference in either fed or fasted preparations. In the same livers, a second bolus dose of harmol was given during *hypoxia*. This produced a modest decline in harmol elimination in fed rats ($t_{\frac{1}{2}} = 7.1 \pm 2.0$ min; $P < 0.05$). However, in fasted rats there was a striking reduction in harmol elimination ($t_{\frac{1}{2}} = 109.8 \pm 54.0$ min; $P < 0.025$). The removal of glucose from perfusate made no significant difference to these results ($t_{\frac{1}{2}} = 253 \pm 209$ min in fasted preparations, $P > 0.1$). In all preparations, reoxygenation resulted in a rapid recovery of drug elimination. We conclude that nutritional state is important in determining the impact of hypoxia on harmol elimination by the liver. This study suggests that clinically significant reductions in xenobiotic glucuronidation are most likely to occur in poorly nourished or fasted subjects who became hypoxaemic.

The hepatic clearance of a number of drugs has been shown to be sensitive to changes in oxygen supply [1–4]. This would be expected with drugs primarily metabolized by the mixed function oxidase system, since cytochrome P-450 uses oxygen directly as a substrate. However, recent studies in isolated hepatocytes and in the intact liver indicate that glucuronidation, a reaction which does not require oxygen directly as substrate, is also sensitive to hypoxia [5, 6].

The supply of the co-factor UDP glucuronic acid is a major determinant of glucuronidation rate in the intact liver and isolated hepatocytes [7, 8], and during hypoxia inhibition of glucuronidation can be related to falling levels of UDP glucuronic acid [5]. In isolated hepatocytes this fall is significantly greater when glucose availability is limited by excluding glucose from the incubation medium or by using cells from fasted animals [5]. The applicability of these findings to drug glucuronidation in the intact liver is not known.

We have recently shown that the elimination of harmol by the isolated perfused rat liver (IPRL)‡ is considerably reduced during acute hypoxia, and that this is due to a reduction in its glucuronidation [6]. The present study in the IPRL examines the effect of fasting and of reduction in exogenous glucose supply on the elimination of harmol during normal oxygenation and during episodes of hypoxia.

EXPERIMENTAL PREPARATION

Livers of fed and 24-hr fasted male Sprague–Dawley rats (weight 200–250 g) were isolated by standard surgical procedures [9] and perfused via the portal vein in a constant flow recycling system [10]. The perfusate (100 ml total volume) consisted of 10% (v/v) washed human red cells in a standard electrolyte solution, which normally contains glucose (8 mM). The perfusate was oxygenated by equilibration with 100% oxygen in a silastic membrane oxygenator. Hypoxia was achieved by replacing the oxygen with 100% nitrogen [1]. A constant infusion of sodium taurocholate (30 μmole/hr) into the perfusate reservoir maintained perfusate bile salt concentration and bile was collected in pre-weighed vials. The principal indices of liver viability were steady O_2 consumption during normal oxygenation (2.0–2.5 μmole/g liver/min), bile flow rates of 0.4–0.6 ml/hr, constant perfusion pressure and stable transaminase levels [1, 2].

Study design. The elimination of consecutive bolus doses of harmol during normal oxygenation and then under hypoxic conditions was studied in four experimental groups using livers from (i) fed rats with

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‡ Abbreviations used: IPRL, isolated perfused rat liver.

glucose (8 mM) in perfusate, (ii) fed rats without exogenous glucose, (iii) fasted rats with glucose (8 mM), (iv) fasted rats without added glucose (N = 4 in each group).

The study period in each experiment was divided into three consecutive 1-hr segments. In the first hour the perfusate was equilibrated with 100% oxygen and at time zero a 20 μmole bolus of harmol was administered into the perfusate reservoir. Perfusate was sampled (1 ml) from the reservoir predose and then at 5, 10, 15, 20, 25, 30, 40 and 60 min for measurement of harmol concentrations. An equal volume of fresh perfusate was added to replace that lost by sampling.

During the second hour, fresh perfusate was used to ensure minimal carry-over of drug from the previous phase. Hypoxia was achieved by equilibrating the perfusate with 100% nitrogen and at the commencement of the hour, a second bolus dose of harmol (20 μmole) was given into the perfusate. Perfusate was again sampled for 60 min in the same way as in the initial hour.

At the beginning of the final hour normal oxygenation with 100% O_2 was restored. No further drug was given nor was the perfusate changed. Perfusate samples were again taken over 60 min, as in the first hour, to examine the elimination of drug remaining from the second harmol dose.

Control experiments (N = 3 in each of the four groups) were also performed, their design being identical to the hypoxia experiments except that there was normal oxygenation throughout all three segments of the experiment.

In all experiments the pO_2 was measured at 0, 60, 90, 120 and 150 min in both portal (inflow) and hepatic (outflow) perfusate and bile was collected half hourly. Additional samples of venous effluent (2 ml) were taken at 30, 60, 75, 90, 105, 120, 135 and 150 min for estimation of lactate:pyruvate ratios to assess the redox state of the liver. The lactate:pyruvate ratio in perfusate provides an estimate of the NADH:NAD^+ ratio in cytosol [11].

Assays. Harmol concentrations in perfusate were quantified by a selective and sensitive HPLC technique which has previously been described [12]. Lactate:pyruvate ratios in hepatic venous effluent were estimated using a commercially available kit (Sigma Chemical Co., St. Louis, MO) to assess the redox state of the liver.

Calculations and statistics. The elimination half-life ($t_{\frac{1}{2}}$) was calculated by least squares regression analysis of the terminal log-linear phase of the perfusate concentration–time data.

Data in tables and text are expressed as mean ± SD and graphically as mean ± SE of mean. Statistical comparisons were made using the Mann Whitney rank sum test for non-parametric data, and the Student's paired *t*-test for paired parametric observations accepting $P < 0.05$ as significant [13].

RESULTS

Indices of hypoxia

Oxygen delivery and consumption

Mean hepatic inflow and outflow pO_2 values for the four groups in each experimental phase are shown in Table 1, with the corresponding oxygen delivery and oxygen consumption data summarized in Tables 2 and 3. As has previously been described [14], oxygen consumption was consistently lower in livers from fasted rats. The hypoxic episode produced a similar reduction in O_2 delivery and consumption in all groups. The recovery in O_2 consumption in all groups indicates that the hypoxic episode did not lead to irreversible impairment of cellular respiration.

Lactate:pyruvate ratio

Mean lactate:pyruvate ratios in all groups are shown in Table 4. Consistent with previous studies, during normal oxygenation lactate:pyruvate ratios

Table 1. pO_2 in hepatic inflow (pO_2 in) and outflow (pO_2 out) in all experiments (mmHg) expressed as mean ± SD

		Control 0–60 min		Hypoxia 60–120 min		Reoxygenation 120–180 min	
Experimental group	N	pO_2 in	pO_2 out	pO_2 in	pO_2 out	pO_2 in	pO_2 out
Fed + glucose	4	400 ± 33	28 ± 7	18 ± 3	15 ± 4	390 ± 29	33 ± 3
Fed + no glucose	4	406 ± 32	30 ± 4	23 ± 3	16 ± 2	407 ± 15	32 ± 9
Fasted + glucose	4	428 ± 25	31 ± 3	23 ± 2	18 ± 4	403 ± 26	34 ± 6
Fasted + no glucose	4	439 ± 40	34 ± 2	22 ± 2	20 ± 2	429 ± 39	36 ± 11

Table 2. Hepatic oxygen delivery in all hypoxia experiments (μmol/g liver/min) expressed as mean ± SD

Experimental group	N	Control 0–60 min	Hypoxia 60–120 min	Reoxygenation 120–180 min
Fed + glucose	4	3.30 ± 0.13	0.73 ± 0.25	3.25 ± 0.13
Fed + no glucose	4	3.31 ± 0.79	0.82 ± 0.13	3.30 ± 0.13
Fasted + glucose	4	3.74 ± 0.06	0.84 ± 0.12	3.75 ± 0.07
Fasted + no glucose	4	3.68 ± 0.12	0.67 ± 0.10	3.71 ± 0.19

Table 3. Hepatic oxygen consumption (μmole/g liver/min) in all hypoxia experiments expressed as mean ±SD

Experimental group	N	Control 0–60 min	Hypoxia 60–120 min	Reoxygenation 120–180 min
Fed + glucose	4	2.19 ± 0.37	0.42 ± 0.22	2.22 ± 0.24
Fed + no glucose	4	2.13 ± 0.20	0.48 ± 0.20	2.08 ± 0.41
Fasted + glucose	4	1.82 ± 0.14	0.42 ± 0.14	1.88 ± 0.08
Fasted + no glucose	4	1.80 ± 0.27	0.33 ± 0.14	1.63 ± 0.22

Table 4. Lactate:pyruvate in all hypoxia experiments expressed as mean ± SD

Experimental group	N	Control	Hypoxia	Reoxygenation
Fed + glucose	4	10.4 ± 3.9	123.2 ± 43.1	21.4 ± 8.5
Fed + no glucose	4	14.3 ± 4.8	130.4 ± 43.2	18.1 ± 4.0
Fasted + glucose	4	33.7 ± 11.8	90.7 ± 27.1	23.3 ± 4.7
Fasted + no glucose	4	36.9 ± 8.1	76.1 ± 29.8	26.8 ± 9.6

Table 5. Elimination half-lives for harmol in all experiments*

Treatment groups	$t_{\frac{1}{2}}$ values (min) First hour	Second hour	Third hour	P value+
Control experiments, N = 12				
Fed + glucose	4.2 ± 0.6	4.1 ± 0.8	—	N.S.
Fed + no glcose	4.3 ± 0.9	4.2 ± 0.8	—	N.S.
Fasted + glucose	5.5 ± 1.1	5.3 ± 0.9	—	N.S.
Fasted + no glucose	5.6 ± 1.0	5.0 ± 0.6	—	N.S.
Hypoxia experiments, N = 16				
Fed + glucose	4.2 ± 0.4	7.1 ± 2.0	—	<0.05
Fed + no glucose	4.2 ± 0.3	9.1 ± 1.8	—	<0.01
Fasted + glucose	5.5 ± 0.4	109.8 ± 54.0	5.3 ± 0.8	<0.025
Fasted + no glucose	5.5 ± 0.4	253.0 ± 209	6.9 ± 1.3	<0.025

* Values expressed as mean ± standard deviation in control studies (N = 3 in each group) and hypoxia studies (N = 4 in each group)

+P value comparing elimination half-life in second hour versus first hour for each group. N.S. no significant difference.

were higher in the preparations from fasted rats [15, 16]. In the fed groups, there was an approximately 10-fold rise during hypoxia ($P < 0.05$), while in fasted preparations there was a 2–3 fold rise ($P < 0.05$). Following reoxygenation, in all groups the lactate:pyruvate ratio returned to near control values, providing further evidence of the ability of the liver to regain normal function after a hypoxic episode.

Harmol elimination

Control studies

In the control studies, for all four groups, there was no change in the elimination rate when the second bolus of harmol was given with normal oxygenation (Table 5).

Hypoxia studies

Normal oxygenation. During normal oxygenation harmol was eliminated rapidly in livers from fed animals, and the rate of elimination was not influenced by the absence of glucose in perfusate ($t_{\frac{1}{2}}$ = 4.2 ± 0.4 min in fed with glucose; $t_{\frac{1}{2}}$ = 4.2 ± 0.3 min in fed without glucose) (Fig. 1, a and b).

In preparations from fasted rats, harmol disposition once again did not appear to be influenced by the absence of exogenous glucose in perfusate ($t_{\frac{1}{2}}$ = 5.5 ± 0.4 min in fasted with glucose in perfusate; $t_{\frac{1}{2}}$ = 5.5 ± 0.4 min in fasted without glucose) (Fig. 1, c and d). However, there was a small but statistically significant reduction in harmol elimination compared with the rate of elimination in preparations from fed animals ($t_{\frac{1}{2}}$ = 4.2 ± 0.4 min in fed, $t_{\frac{1}{2}}$ = 5.5 ± 0.4 min in fasted, $P < 0.025$) (Table 5).

Hypoxia phase. When a second bolus dose of harmol was given under hypoxic conditions in preparations from fed animals with the standard concentration of glucose in perfusate, there was a gradual slowing in the rate of fall of drug concentrations in the first 15 min. This was followed by a monoexponential fall in drug levels with a half-life of 7.1 ± 2.0 min, which was significantly greater than that seen in the same group in the normoxic period ($P < 0.05$) (Fig. 1, a). The use of a glucose free medium in fed rats produced a similar pattern of

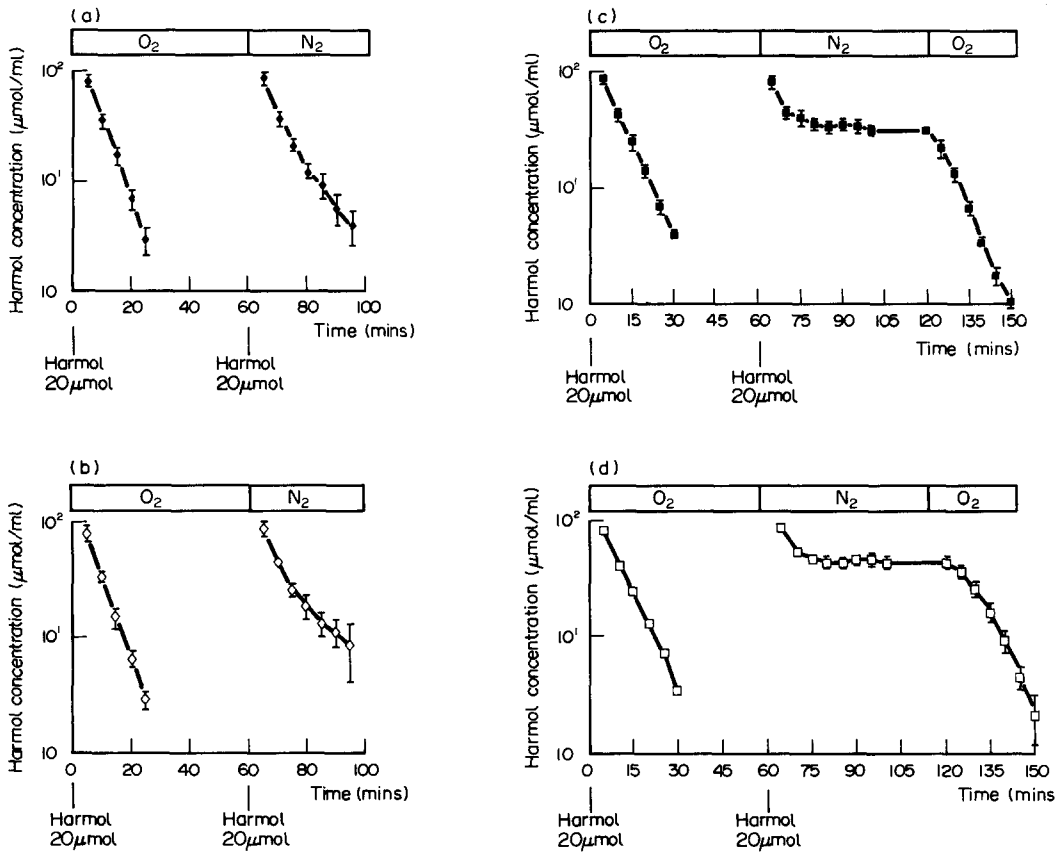

Fig. 1. Perfusate elimination of two consecutive 20 μmole bolus doses of harmol under normoxic (dose given at 0 min) and hypoxic conditions (dose given at 60 min) in preparations from: (a) fed rats with added glucose (8 mM) in perfusate (although experiments ran for 180 min, harmol levels fell below detectable limits by 100 min); (b) fed animals without exogenous glucose in perfusate (harmol levels were undetectable by 100 min); (c) fasted animals with added glucose in perfusate; (±) fasted animals without added glucose in perfusate.

decline in harmol elimination during hypoxia, although the half-life during the log-linear phase appeared slightly greater again ($t_{\frac{1}{2}} = 9.1 \pm 1.8$ min (Fig. 1, b)). This difference was not significant ($P < 0.1$).

In both groups harmol concentrations fell below the detectable range before the end of the hypoxia phase. It was therefore not possible to follow the elimination of harmol following reoxygenation of the system in the final hour (Fig. 1, a and b).

Harmol elimination in livers from fasted rats was substantially more sensitive to hypoxia than in the preparations from fed animals. As in the fed livers, elimination gradually slowed during the first 10–15 min of hypoxia (Fig. 1, c). After this initial phase, the mono-exponential decline of harmol levels was much slower ($t_{\frac{1}{2}} = 109.8 \pm 54.0$ min, $P < 0.025$), indicating a considerable reduction in elimination rate of the drug. The absence of glucose from the perfusion medium did not significantly augment this effect ($t_{\frac{1}{2}} = 253.0 \pm 209.0$ min, $P > 0.10$) (Fig. 1, d). As a result of this striking reduction in drug elimination rate, harmol concentrations were still easily measurable at the end of the hypoxic period (Fig. 1, c and d). In these preparations from fasted animals it was therefore possible to follow the time course of drug levels in perfusate upon restoration of normal oxygenation. As can be seen (Fig. 1, c and d; Table 5) there was recovery of harmol elimination to pre-hypoxic levels [$t_{\frac{1}{2}} = 5.5 \pm 0.3$ min (normoxia) vs $t_{\frac{1}{2}} = 5.3 \pm 0.8$ min (recovery) with glucose in the perfusion medium and $t_{\frac{1}{2}} = 5.5 \pm 55$ min (normoxia) vs $t_{\frac{1}{2}} = 6.9 \pm 1.3$ min (recovery) without glucose present].

DISCUSSION

The oxygen dependence of a number of pathways of drug metabolism has been demonstrated in isolated hepatocytes and in subcellular systems [4]. However, until recently there had been few studies of the effects of hypoxia on hepatic drug elimination in the intact liver. The isolated perfused rat liver has proved to be a useful model to assess the effect of hypoxia on hepatic drug disposition [1–3]. Oxygen delivery and consumption can be monitored, the lactate : pyruvate ratio in hepatic venous effluent provides an index of the redox state of the liver, hepatic perfusion rates can be precisely controlled, the vol-

ume of the system is constant and other routes of drug clearance are not present.

Earlier work by ourselves and others has indicated that the effect of hypoxia on hepatic drug elimination is largely related to the oxygen dependence of the principal pathways of drug metabolism, rather than to the oxygen dependence of uptake or excretory mechanisms [1–4]. This conclusion was further supported by our recent study of harmol elimination by the IPRL [6], in which we showed that impaired elimination was due primarily to inhibition of glucuronidation rather than inhibition of harmol uptake or of biliary excretion of harmol glucuronide.

Studies *in vivo* and *in vitro* have demonstrated that agents which reduce intracellular UDP glucuronic acid levels such as ethanol, diethyl ether and galactosamine also decrease the rate of xenobiotic glucuronidation [17, 8, 18]. Similarly under hypoxic conditions the fall in rate of drug glucuronidation which occurs both in the intact liver and in isolated hepatocytes [6, 5] is likely to result from depletion of UDP glucuronic acid [5]; UDP glucuronic acid is synthesized in an NAD^+-dependent reaction from UDP glucose, which is in turn derived from glucose-1-phosphate and UTP [19]. Aw and Jones concluded that in isolated hepatocytes, limitation of UDP glucuronic acid supply and a subsequent fall in glucuronidation rates during hypoxia was directly related to reduced availability of glucose-1-phosphate and UTP for the synthesis of UDP glucose [5]. Although the $NADH/NAD^+$ ratio rises during hypoxia [1, 2, 20] limitation of the conversion of UDP glucose to UDP glucuronic acid by reduced availability of NAD^+ did not appear to be the rate controlling factor, since under all conditions, the rate of UDP glucuronic acid synthesis was directly proportional to the concentration of UDP glucose [5].

In the current study, during normal oxygenation, fasting produced a minor reduction in harmol elimination compared to the fed state. Similarly Mulder *et al.* [21] observed that following *in vivo* infusion of harmol to fasted rats, the rate of harmol glucuronidation was mildly reduced compared with that in fed animals. In contrast, fasting dramatically increased the effect of hypoxia on harmol elimination, leading to a greater than 20-fold increase in elimination half-life (Fig. 1, c and d; Table 5).

In 24-hr fasted rats hepatic glycogen levels are reduced to less than 10% of levels in the fed state [22, 23]. Hypoxia leads to a more rapid fall in ATP (and thus UTP) production in the livers from fasted rats than in those from fed animals where glycogen reserves are large and ATP levels can be maintained by anaerobic glycolysis [24]. Moreover, stimulation of glycolysis by hypoxia in livers with pre-existing low carbohydrate reserves might be expected to produce a greater reduction in the availability of carbohydrate for the synthesis of UDP glucuronic acid than occurs in glycogen replete livers. Thus, as previously demonstrated in isolated hepatocytes [5] one might expect hypoxia to lead to lower levels of intracellular glucose-1-phosphate and UTP in fasted compared to fed animals, resulting in a greater sensitivity of hepatic glucuronidation to hypoxia in the fasted state (Fig. 1, a and b). In support of this hypothesis, the differences between the experimental groups did not appear to be related to variations in the sensitivity of the $NADH/NAD^+$ ratio to hypoxia, since the rise in lactate : pyruvate ratios during hypoxia appeared to be greater in the preparations from fed animals than in the fasted group, with the presence or absence of added glucose making no difference to the pattern. Thus, the combined affect of fasting and hypoxia is probably not due to the inhibition of conversion of UDP glucose to UDP glucuronic acid.

In isolated rat livers under *normoxic* conditions Reinke demonstrated that 10 mM glucose infusion produced a 3-fold rise in hepatic glucuronidation rates in the livers from fasted rats during high dose *p*-nitrophenol infusion [22]. In our experiments, under *hypoxic* conditions, addition of glucose (8 mM) to perfusate resulted in no significant stimulation of harmol elimination in either fed or fasted groups. This suggests that during acute hypoxia, at these concentrations, glucose cannot be taken up and used by the liver in sufficient quantities to overcome the combined effects of fasting and hypoxia on intracellular carbohydrate supplies.

A number of recent studies in the intact liver have shown that the hepatic clearance of drugs which primarily undergo microsomal oxidation reactions is significantly impaired by reductions in oxygen supply. In our previous study we demonstrated that in fed animals the glucuronidation and elimination of harmol is also impaired during acute hypoxia. However, the effect does not appear to develop as rapidly, nor is it as severe as has been seen with some oxidatively metabolized drugs [1, 2, 6]. The current study demonstrates that fasting dramatically increases the impact of hypoxia on harmol elimination by the liver, suggesting that nutritional status may be critical in determining the effect of hypoxia on xenobiotic glucuronidation and elimination. We might therefore speculate that clinically significant changes in the elimination of substrates undergoing extensive hepatic glucuronidation is most likely to be seen in fasted or poorly nourished patients who are subjected to hypoxia.

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