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THE TOXICOLOGICAL RELEVANCE OF PARACETAMOL-INDUCED INHIBITION OF HEPATIC RESPIRATION AND ATP DEPLETION

O. STRUBELT* and M. YOUNES†

Institut für Toxikologie der Medizinischen Universität zu Lübeck, Katzeburger Allee 160, D-2400 Lübeck 1; and †Max von Pettenkofer-Institut, BGA, Postfach 330013, D-1000 Berlin 33, Germany

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Abstract—In order to elucidate the role of mitochondrial dysfunction in paracetamol-induced hepatotoxicity, the effects of paracetamol on the oxygen consumption and ATP content of the isolated perfused rat liver were correlated with parameters of hepatic viability and hepatotoxicity. Paracetamol at 5 g/L reduced the oxygen consumption of the livers by about 80% and hepatic ATP content by 96%. Hepatotoxicity was evident from the nearly complete interruption of bile secretion, a marked release of enzymes [glutamate–pyruvate transaminase (GPT), lactate dehydrogenase (LDH)] in the perfusate, a depletion of hepatic glutathione and an accumulation of calcium in the liver. Paracetamol-induced hepatotoxicity could be prevented completely by using livers from non-fasted rats as well as by addition of fructose to the perfusate of livers from fasted animals. Both treatments resulted in an increased energy supply from anaerobic glycolysis as evidenced by a large release of lactate and pyruvate into the perfusate, but did not inhibit paracetamol-induced decline of oxygen consumption. The decrease in hepatic oxygen consumption depended on the dose of paracetamol and occurred first at a concentration of 0.2 g/L (−10%). LDH and GPT release, on the other hand, was elevated at 2 and 5 g/L and calcium accumulation occurred at 5 g/L paracetamol only. Inhibition of mixed-function oxidases by dithiocarb did not prevent the decrease in oxygen consumption and the resulting hepatic injury induced by paracetamol. The oral administration of the high dose of 5 g/kg paracetamol *in vivo* to rats exerted strong hepatotoxicity but produced maximal serum levels of 800 mg/L paracetamol only and did not decrease hepatic oxygen consumption as measured *in vitro*. Our results show that in the isolated perfused rat liver *in vitro*, only high concentrations of paracetamol can produce "chemical hypoxia" by attacking mitochondria so as to cause hepatic injury. Such high concentrations of paracetamol are not attained *in vivo*, however. "Chemical hypoxia", thus, seems not to be relevant to the well-known hepatotoxic action of paracetamol.

The well-known hepatotoxic activity of paracetamol is due to the fact that a small proportion of the drug undergoes cytochrome P450-mediated metabolism [1, 2] to form *N*-acetyl-*p*-benzoquinone imine (NAPQI‡), a highly reactive intermediate [3]. Following a therapeutic dose, this metabolite is detoxified by conjugation with glutathione. At higher doses, however, liver glutathione is depleted and can initiate a number of primary effects including covalent binding to hepatic macromolecules. The amount of binding correlates with the severity of the hepatotoxicity of paracetamol [4]. The molecular mechanisms of the events that follow this binding and produce cellular injury, however, have not been established until now.

Meyers *et al.* [5] recently raised the hypothesis that alteration in mitochondrial function resulting in an inhibition of mitochondrial respiration might be involved in the initiation and/or progression of paracetamol-induced hepatotoxicity. They founded this hypothesis on the fact that oral treatment of mice with a hepatotoxic dose of 600 mg/kg paracetamol inhibited state 3 hepatic mitochondrial respiration (ADP-stimulated) by 19 and 30% at 2 and 4 hr after treatment, respectively, when glutamate was used as a substrate and, consequently, decreased the respiratory control rate. No such inhibition was observed after a non-hepatotoxic dose of paracetamol (300 mg/kg p.o.). Toxic doses of paracetamol in rats caused also a 25–47% decrease in state 3 mitochondrial respiration rate after 24 hr [6]. Furthermore, a reduction in hepatic ATP content by 40–60% was seen in mice 60–90 min after oral treatment with 250–500 mg/kg paracetamol [7, 8]. *In vitro*, an inhibition of oxygen consumption by 15–30% was observed in the isolated perfused rat liver in the presence of paracetamol at a concentration of 5 mM [9, 10], whereas the same concentration of paracetamol inhibited the respiration of mouse liver mitochondria by 80% [11] and that of isolated mouse hepatocytes by about 75% [12]. Furthermore, *N*-acetyl-*p*-benzoquinone imine, the reactive metabolite of paracetamol, was shown to inhibit oxygen consumption of rat hepatocytes and to deplete mitochondrial ATP [13, 14].

In order to elucidate the role of mitochondrial dysfunction in paracetamol-induced hepatotoxicity, the effects of paracetamol on the oxygen consumption and ATP content of the isolated perfused rat liver were determined and correlated with some parameters of hepatic viability and hepatotoxicity.

* Corresponding author.

‡ Abbreviations: NAPQI, *N*-acetyl-*p*-benzoquinone imine; LDH, lactate dehydrogenase; GPT, glutamate–pyruvate transaminase; DTC, dithiocarb, diethyldithiocarbamate; GSH, reduced glutathione; GSSG, oxidized glutathione.

MATERIALS AND METHODS

Animals and treatments. Male Wistar rats (conventional animals, 320–380 g; breeder: Winkelmann, Borchen) were used throughout. They had free access to a standard diet (Altromin pellets) and tap water until use. Fasting was achieved by deprivation of feed but not of drinking water for 16 hr. To deplete hepatic glutathione, the animals were treated with phorone (diisopropylidene acetone, 250 mg/kg in 10 mL/kg olive oil i.p.) 2 hr prior to the start of the relevant experiment. This dose was shown to decrease hepatic glutathione concentrations by 90% without affecting major xenobiotic-metabolizing enzymes [15]. In order to inhibit mixed-function oxidase we treated the rats with dithiocarb (diethyldithiocarbamate, DTC, 200 mg/kg in 5 mL/kg saline i.p.) 1 hr before the experiments.

In the case of *in vivo* experiments, rats were treated orally with 2 or 5 g/kg paracetamol in 10 mL/kg of a 1% tylose (carboxymethylcellulose) solution. In experiments designed to study the hepatotoxic potential of paracetamol at these doses, the animals were anesthetized after 24 hr with 60 mg/kg pentobarbital i.p. and killed by exsanguination from a carotid artery. Plasma was used to measure the activities of liver-specific enzymes.

In experiments designed to study the oxygen consumption rates, rats were treated with paracetamol as described above. After 1, 2 or 3 hr, the animals underwent surgery and their livers were perfused *in vitro* for 2 hr. Blood samples were collected from these animals at the time of death for determination of plasma paracetamol concentrations.

Liver perfusion. Removal of the liver and its connection to a recirculating perfusion system was performed as described previously [16]. The perfusion medium consisted of 250 mL Krebs–Henseleit buffer, pH 7.4 (118 mmol/L NaCl, 6 mmol/L KCl, 1.1 mmol/L $MgSO_4$, 1.2 mmol/L KH_2PO_4, 24 mmol/L $NaHCO_3$). $CaCl_2$ (1.25 mmol/L) was added to the prewarmed medium (37°) immediately before starting the perfusion experiments. The perfusion medium was gassed continuously with carbogen (95% O_2, 5% CO_2) yielding an oxygen partial pressure of about 600 mm Hg. Sodium taurocholate (36.7 g/L) was infused into the perfusate at a rate of 12 mL/hr to stimulate bile secretion. Bile was sampled every 30 min and weighed. The rate of bile secretion was calculated per gram liver and minute. Oxygen consumption of the isolated livers was calculated from the difference in the oxygen concentrations of the influent and the effluent perfusate using a Micro pH/Blood Gas Analyzer 413 (Instrumentation Laboratory). Paracetamol was added to the perfusate after a 30-min equilibration period at time 0 to yield a final concentration of 0.2, 0.5, 1.2 or 5 g/L. Fructose (20 mmol/L) or dithiocarb (100 mg/L) was added to the perfusate in the relevant experiments at the start of the perfusion period.

Biochemical determinations. The activities of glutamate–pyruvate transaminase (GPT) and lactate dehydrogenase (LDH) in the perfusate and in the plasma, as well as the concentrations of glucose, lactate and pyruvate in the perfusate were assayed using commercial kits of Boehringer (Mannheim, Germany). Calcium concentrations in the liver were measured colorimetrically following acid extraction also with reagent kits of Boehringer. Total glutathione was determined in liver and perfusate samples according to Brehe and Burch [17]. Oxidized glutathione (GSSG) was estimated by the same procedure after blocking reduced glutathione (GSH) with 2-vinylpyridine [18]. For ATP determination, hepatic tissue was frozen immediately in liquid nitrogen and extracts were prepared according to Williamson and Corkey [19]. ATP was assayed enzymatically using a reagent kit of Sigma (Munich). The concentrations of paracetamol in plasma were determined by means of high performance liquid chromatography according to Howie *et al.* [20].

Statistics. Means ± SEM were calculated in the usual manner. The difference between two means was calculated with Dunnett's *t*-test [21] taking $P = 0.05$ as the limit of significance.

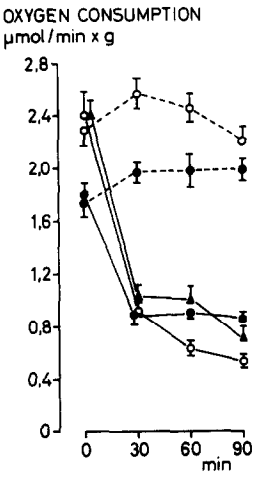

Fig. 1. Effect of paracetamol (5 g/L) added at time 0 on the oxygen consumption of the isolated perfused rat liver. Values are means ± SEM. Solid lines, experiments with paracetamol; dotted lines, control experiments without paracetamol. (○) Fasted animals, (●) fed animals, (▲) fasted animals + fructose (3.6 g/L = 20 mmol/L).

RESULTS

Oxygen consumption and ATP concentration

In control experiments without paracetamol, the oxygen consumption of the isolated perfused livers of fasted and fed rats lay between 1.7 and 2.4 μmol/min × g and did not decline during the 2 hr-perfusion period (Fig. 1). The administration of 5 g/L paracetamol resulted in an immediate decrease in oxygen consumption by about 60% in the livers from both fasted and fed animals (Figs 1 and 2). The oxygen consumption in the livers of fasted but not in those of fed animals declined further to a final value of 0.54 ± 0.05 μmol/min × g at the end of the experiments (fed livers, 0.8 ± 0.05 μmol/min × g). The addition of 20 mmol/L fructose did not influence

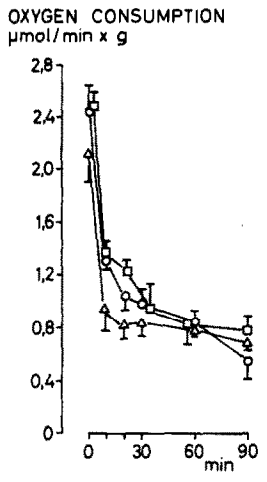

Fig. 2. Effect of paracetamol (5 g/L) added at time 0 on the oxygen consumption of isolated perfused livers from: (○) fasted rats; (□) fasted rats, treated with 200 mg/kg DTC 1 hr prior to surgery; (△) fasted rats with addition of 100 mg/kg DTC to the perfusate 30 min before paracetamol. Values are means ± SEM.

considerably the paracetamol-induced decline in oxygen consumption (Fig. 1).

Hepatic ATP concentrations in the control experiments at the end of the perfusion period (Table 1) were slightly higher in the livers from fed than in those from fasted rats (statistically not significant). Paracetamol-induced reduction in oxygen consumption led to a nearly complete depletion of hepatic ATP by 96% of the control value in the livers from fasted rats. Paracetamol-induced ATP decrease in the livers of fed rats, on the other hand, amounted to only 55% of the respective control value.

In some further experiments, the exposure of isolated livers from fasted rats to 5 g/L paracetamol was limited to a period of 30 min only. The perfusion was then continued with a paracetamol-free Krebs–Henseleit buffer for 2 hr. Hepatic respiration impaired by paracetamol did not recover upon cessation of the exposition: oxygen consumption was 2.46 ± 0.20 µmol/min × g before paracetamol, 0.84 ± 0.16 µmol/min × g after 30 min exposure to 5 g/L paracetamol and 0.90 ± 0.24 µmol/min × g after 2 hr of paracetamol-free perfusion (N = 3). Hepatic concentrations of ATP, calcium and glutathione were also the same after 30 and 90 min exposure to paracetamol (Table 1).

Bile flow

As a further measure of viability, hepatic bile flow was determined during the experiments (Fig. 3). In the control experiments without paracetamol, bile flow did not decline during the 2-hr perfusion period. The administration of 5 g/L paracetamol to the livers of fasted rats produced a strong and continuous decline in hepatic bile secretion to zero levels at the end of the experiments. This decline did not occur in the livers of fed rats and was also prevented by addition of fructose to livers obtained from fasted animals (Fig. 3).

Hepatotoxic effects

As a measure of hepatotoxicity, the release of GPT and LDH into the perfusate was measured. In control experiments without paracetamol, enzyme release remained low over the whole perfusion period (Fig. 4). The addition of 5 g/L paracetamol to the livers of fasted rats led to a clear enhancement of enzyme release: at the end of the experiments, perfusate activities of LDH were elevated 17-fold and those of GPT 29-fold over control values. Feeding the animals prior to surgery or the addition of fructose to the perfusate prevented paracetamol-induced enzyme leakage completely (Fig. 4).

As a further index of cytotoxicity, calcium accumulation in the livers at the end of the experiments was studied. The addition of 5 g/L paracetamol elevated the calcium content of the livers of fasted rats by 85% over control values (Table 1). This increase in hepatic calcium

Table 1. Concentrations of ATP, glutathione and calcium in the isolated perfused rat livers at the end of the experiments, i.e. after an exposure period of 90 min

Group	Paracetamol (g/L)	Additional treatment	N	ATP (µmol/g)	Calcium (µmol/g)	GSH (µmol/g)	GSSG (µmol/g)
1	—	Fasted	5	2.05 ± 0.17	1.42 ± 0.14	3.60 ± 0.88	0.07 ± 0.01
2	—	Fed	5	2.60 ± 0.09	1.30 ± 0.07	5.60 ± 0.48*	0.13 ± 0.05
3	5	Fasted	5	0.08 ± 0.02*	2.62 ± 0.19*	0.28 ± 0.09*	0.02 ± 0.02
4	5	Fed	8	1.17 ± 0.27†	1.50 ± 0.09†	2.10 ± 0.22†	0.11 ± 0.02
5	5	Fasted, fructose	5	ND	1.28 ± 0.04†	0.87 ± 0.12†	0.03 ± 0.01
6	5	Fasted, 30 min exposure	3	0.16 ± 0.01*	2.75 ± 0.15*	0.15 ± 0.05*	0.02 ± 0.01

Values are means ± SEM.
ND, not determined.
* Statistically significant difference to group 1 ($P < 0.05$).
† Statistically significant difference to group 3 ($P < 0.05$).

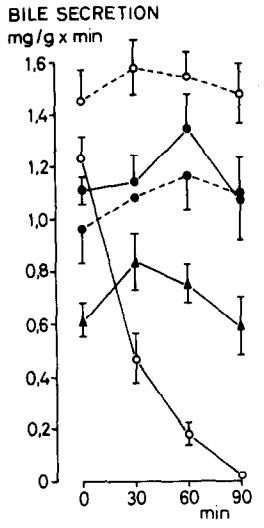

Fig. 3. Effect of paracetamol (5 g/L) added at time 0 on bile secretion of the isolated perfused rat liver; values refer to the 30 min before the time indicated. Values are means ± SEM. Solid lines, experiments with paracetamol; dotted lines, control experiments without paracetamol. (○) Fasted animals, (●) fed animals, (▲) fasted animals + fructose (3.6 g/L = 20 mmol/L).

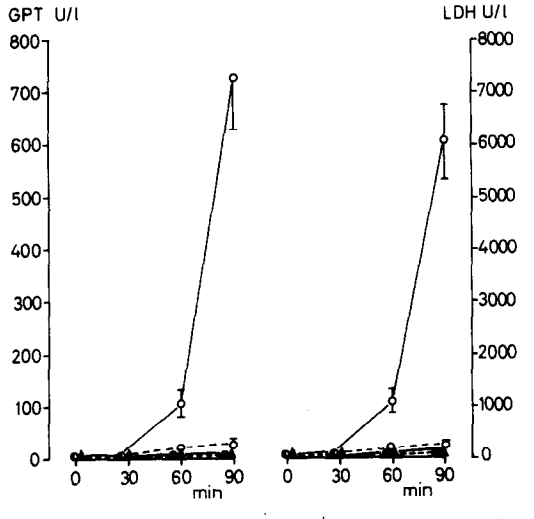

Fig. 4. Effect of paracetamol (5 g/L) added at time 0 on the activities of GPT and LDH released by perfused rat livers into the perfusate. Values are means ± SEM. Solid lines, experiments with paracetamol; dotted lines, control experiments without paracetamol. (○) Fasted animals, (●) fed animals, (▲) fasted animals + fructose (3.6 g/L = 20 mmol/L).

concentration was prevented completely by feeding the animals or by the addition of fructose (Table 1).

Treatment with paracetamol resulted in a strong depletion of hepatic GSH to 8% and of GSSG to 28% of the control values (Table 1). This loss of glutathione could be partially prevented by feeding the animals as well as by addition of fructose.

Glycogenolysis and glycolysis

The concentrations of glucose, lactate and pyruvate in the perfusate were determined as a measure of glycogenolysis and glycolysis (Table 2). In the control experiments without paracetamol, livers from fasted rats released only about 1/10 the amount of glucose, lactate and pyruvate as compared with livers from fed animals. Paracetamol did not influence the release of glucose, lactate and pyruvate from the livers of fasted rats. However, it stimulated glycolysis and glycogenolysis in the livers from fed animals as indicated by increased levels of glucose (+33%), lactate (+434%) and pyruvate (+38%) in the perfusate (Table 2). A 3-fold increase in the glucose concentration, a 16-fold increase in the lactate concentration and a 12-fold increase in the pyruvate concentration were observed upon addition of fructose to paracetamol-treated livers obtained from fasted animals.

The lactate/pyruvate ratio was higher in the livers from fasted rats as compared with those of fed animals. Paracetamol did not influence the ratio in the livers of fasted rats but increased it in those of rats fed 4-fold. Addition of fructose to the livers of fasted rats treated with paracetamol increased the lactate/pyruvate ratio by about 50% (Table 2).

Role of glutathione depletion

In the control experiments without paracetamol, glutathione concentration in the livers of fasted rats were 36% lower than in the livers from fed animals (Table 1). In order to elucidate the role of these low glutathione levels in paracetamol-induced hepatotoxicity, we performed some experiments with livers from fed rats treated with phorone 2 hr prior to the start of the experiments. Phorone treatment without paracetamol resulted in a decline of hepatic glutathione to 0.50 ± 0.08 μmol/g liver (N = 3), i.e. 91% depletion as compared to the control values in fed rats (cf. Table 1). Administration of paracetamol to the livers of phorone-pretreated, fed rats led to a further decrease to 0.28 ± 0.07 μmol glutathione/liver (N = 5). However, no hepatotoxic response was evident after addition of paracetamol to the glutathione-depleted livers although the paracetamol-induced decrease in oxygen consumption was the same as in the livers from fasted rats (not shown). Presumably this is the consequence of the energy yield from glycogen depletion as evidenced by an increased release of glucose and lactate from the livers of these phorone-pretreated, fed rats upon addition of paracetamol (not shown).

Dose dependence

Experiments with the livers from fasted rats were also performed with 0.2, 0.5, 1 and 2 g/L paracetamol. The values of some parameters measured at the end of these experiments are demonstrated in Fig. 5. Depression of hepatic oxygen consumption was already evident at a concentration of 0.2 g/L paracetamol whereas a decrease in hepatic bile secretion first occurred at 0.5 g/L. LDH and GPT release, on the other hand, was elevated at the

Table 2. Glucose, lactate and pyruvate concentrations in the perfusate at the end of the experiments

Group	Paracetamol (g/L)	Additional treatment	N	Glucose (mmol/L)	Lactate (mmol/L)	Pyruvate (μmol/L)	Lactate/ pyruvate*
1	—	Fasted	10	0.55 ± 0.05	0.26 ± 0.03	14.3 ± 0.9	18.5
2	—	Fed	8	4.30 ± 0.36†	1.68 ± 0.23†	224 ± 29.1†	7.7
3	5	Fasted	9	0.42 ± 0.05	0.27 ± 0.05	13.3 ± 1.1	22.0
4	5	Fed	8	5.72 ± 0.57‡	8.97 ± 0.61‡	310 ± 45.9‡	31.3
5	5	Fasted, fructose	5	1.60 ± 0.08‡	4.40 ± 0.66‡	166 ± 50.1‡	27.5

Values are means ± SEM.
* Means of the ratios calculated for individual experiments.
† Statistically significant difference to group 1 ($P < 0.05$).
‡ Statistically significant difference to group 3 ($P < 0.05$).

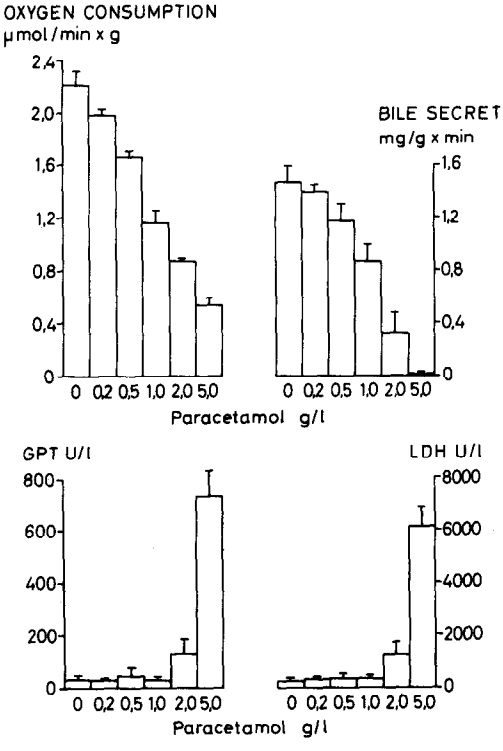

Fig. 5. Oxygen consumption, bile secretion and the perfusate activities of GPT and LDH at the end of the experiments with different concentrations of paracetamol added to the livers from fasted rats. Values are means ± SEM from 5–10 experiments each.

concentrations of 2 and 5 g/L paracetamol only (Fig. 5), and Ca^{2+} accumulation at 5 g/L only (Table 1, other parameters are not shown).

Influence of dithiocarb

DTC is a potent inhibitor of mixed-function oxidases [22–24] and has a strong protective effect against paracetamol-induced liver injury *in vivo* [25, 26]. In order to elucidate the inhibitory role of cytochrome P450-mediated metabolism of paracetamol to NADPH in hepatic respiration, we performed experiments: (1) with livers from fasted rats treated with a high dose of DTC (200 mg/kg i.p.) 1 hr prior to surgery and (2) with livers obtained from fasted animals to the perfusate of which DTC (100 mg/L) was directly added 30 min prior to paracetamol exposure. The administration of 5 g/L paracetamol to these livers resulted in a similar decrease in oxygen consumption to that in the control livers not pretreated with DTC (Fig. 2). Treatment with DTC *in vivo* or *in vitro* also did not attenuate the depletion of hepatic ATP and GSH, and the accumulation of hepatic calcium and exhaustion of bile flow induced by paracetamol (not shown). However, addition of DTC *in vitro* (but not pretreatment with DTC *in vivo*) prevented paracetamol-induced release of LDH and GPT into the perfusate (not shown). This effect may be due to the radical-scavenging activity of DTC and was also seen with livers exposed to hypoxia or ischemia (unpublished results).

Table 3. Plasma concentrations of LDH and GPT in fasted rats 24 hr after oral treatment with paracetamol

Paracetamol (g/kg)	N	LDH (U/L)	GPT (U/L)
—	6	19 ± 5.7	287 ± 57
2	8	4197 ± 2812	792 ± 467
5	3	15,650 ± 9321	2266 ± 1072

Values are means ± SEM.

Experiments in vivo

We questioned whether the concentrations of paracetamol which inhibit hepatic oxygen consumption *in vitro* can also be obtained in the rat *in vivo*. Therefore, fasted rats were treated orally with 2 or 5 g/kg paracetamol. These doses of paracetamol exerted a strong hepatotoxic activity as evidenced by the increase in plasma LDH and GPT levels 24 hr after treatment (Table 3). Plasma concentrations of paracetamol were determined 1, 2 and 3 hr after the treatment and amounted maximally to 433 mg/L

Table 4. Plasma concentrations of paracetamol and oxygen consumption of the isolated perfused livers in fasted rats 1, 2 and 3 hr after oral treatment with paracetamol

Paracetamol (g/kg)	Plasma concentration of free paracetamol (mg/L)			Oxygen consumption* of the isolated livers (μmol/min × g)		
	1 hr	2 hr	3 hr	1 hr	2 hr	3 hr
—	—	—	—	ND	2.20 ± 0.12	ND
2	433 ± 55	360 ± 50	136 ± 8	2.32 ± 0.15	2.36 ± 0.13	2.30 ± 0.15
5	626 ± 109	800 ± 295	530 ± 93	2.09 ± 0.03	2.12 ± 0.09	2.21 ± 0.11

Values are means ± SEM.
N = 4 in each case.
ND, not determined.
* Values measured at the end of the experiments after 2 hr of perfusion.

after 2 g/kg paracetamol and to 800 mg/L after 5 g/kg paracetamol (Table 4).

Perfusion experiments were also performed with the livers of rats treated with paracetamol *in vivo*. The oxygen consumption of the livers was quite normal (Table 4) and did not change consistently during the experiments. The livers also did not exhibit other signs of hepatotoxicity (enhanced enzyme release, decrease in bile secretion, calcium accumulation).

DISCUSSION

In the isolated livers from fasted rats, paracetamol (at a concentration of 5 g/L) produced a strong decline in oxygen consumption and a nearly total depletion of hepatic ATP. This disruption of energy homeostatis was followed by an exhaustion of hepatic bile flow and by a release of enzymes into the perfusate indicating cellular membrane damage. Hepatotoxicity was further evidenced by an accumulation of calcium in the liver and by a depletion of cellular glutathione.

Two lines of evidence suggest that a decreased energy supply from mitochondrial respiration is the cause of paracetamol-induced injury in the isolated liver. The first line of evidence is that hepatic injury was observed only in the livers from fasted rats. Fasting is known to result in a substantial loss of hepatic glycogen. In a previous experiment of ours, food deprivation for 16 hr was shown to decrease rat hepatic glycogen concentration from 47.1 mg/g tissue (fed rats) down to 2.2 mg/g tissue [27]. Thus, if aerobic energy conservation reactions are impaired, anaerobic glycolysis cannot take place and hepatic injury may occur. This proved true in the case of hypoxic liver injury [27–29]. The second line of evidence is the observation that feeding the animals prior to surgery or addition of fructose to the perfusion medium strongly attenuated paracetamol-induced toxicity towards isolated perfused livers. Both treatments allow for anaerobic synthesis of ATP to take place: with livers from fed animals, glycogenolysis may proceed yielding glucose-1-phosphate while fructose is phosphorylated to fructose-6-phosphate in a hexokinase-catalysed reaction. Both are substrates in the glycolytic pathway. In fact, glycogenolysis and glycolysis were evident in our experiments by an increase in the formation and release of glucose, lactate and pyruvate caused by both treatments. The liver, thus, proved resistant to cellular damage resulting from paracetamol-induced inhibition of oxidative metabolism as long as anaerobic energy conservation was available.

Similar results as those with paracetamol were obtained with cyanide in the isolated perfused rat liver [30]. In these experiments, too, energy supply from anaerobic glycolysis protected the liver from the damage induced by the inhibition of oxygen utilization. "Chemical hypoxia" [31] due to inhibition of mitochondrial respiration, thus, can be produced in the isolated perfused rat liver by paracetamol as well as by cyanide.

Besides depleting hepatic glycogen stores, fasting is also known to result in a substantial loss of liver glutathione [32, 33]. As glutathione is involved in the detoxification of reactive metabolites of paracetamol, fasting-induced glutathione depletion might have contributed to our observation that only livers from fasted rats were susceptible to paracetamol-induced damage *in vitro*. Near total depletion of glutathione in livers from fed animals induced by phorone, however, did not render the isolated perfused livers susceptible to paracetamol-induced injury although oxygen consumption was decreased considerably. As glycogen was still present in these livers and glycogenolysis evident upon administration of paracetamol, loss of glycogen but not of glutathione must render the isolated liver susceptible to the cytotoxicity ensuing from paracetamol-induced inhibition of oxygen consumption.

In contrast to our results with high concentrations of paracetamol *in vitro*, depletion of hepatic glutathione markedly increases the hepatotoxicity of paracetamol *in vivo* [34]. Furthermore, inhibition of mixed-function oxidases by DTC did not attenuate the toxic effects of paracetamol in the present study, although DTC exerts a strong protective effect against paracetamol-induced liver injury *in vivo* [25, 26]. These discrepancies between *in vitro* and *in vivo* experiments may result from the fact that the parent compound paracetamol is mainly

responsible for paracetamol-induced inhibition of hepatic respiration and hepatotoxicity *in vitro* whereas its metabolite NAPQI induces mainly the hepatotoxicity of paracetamol *in vivo*.

The question remains as to whether "chemical hypoxia" resulting from mitochondrial dysfunction also accounts for the well-known hepatotoxic activity of paracetamol *in vivo*. To our knowledge, the influence of paracetamol on hepatic oxygen consumption *in vivo* has not been investigated until now. Nonetheless, treatment with hepatotoxic doses of paracetamol *in vivo* has been shown to decrease hepatic mitochondrial respiration *in vitro* [5, 6] and to reduce hepatic ATP content [7, 8]. However, these effects were much smaller (cf. Introduction) than those of hepatotoxic concentrations of paracetamol in the present study. Furthermore, these *in vivo* effects have not been proven to be an initial event in paracetamol-induced liver injury because the earliest determination was performed 60 min after administration of paracetamol (cf. Introduction).

It remains doubtful, therefore, as to whether paracetamol can produce an inhibition of hepatic respiration of toxicological relevance *in vivo*. The doubts are supported by comparing the paracetamol concentrations producing "chemical hypoxia" and ensuing hepatotoxicity *in vitro* with those inducing hepatic damage *in vivo*. For instance, peak plasma concentrations of about 200 mg/mL paracetamol are connected with severe hepatotoxic injury after administration of 1 g/kg paracetamol orally to rats [35]. According to our present results, this level of paracetamol is not sufficient to produce chemical hypoxia of toxicological relevance (Fig. 5). In the present study also, we administered 2 or 5 g/kg paracetamol orally to rats and attained peak plasma concentrations of around 400 or 800 mg/L, respectively (Table 4). According to our experiments *in vitro*, these concentrations might decrease hepatic oxygen consumption by 20–40% (cf. Fig. 5). However, the livers from these rats exhibited quite normal oxygen consumption when perfused *in vitro* 1, 2 or 3 hr after the treatment *in vivo*. The exposure of the livers to paracetamol was long enough in these experiments to allow for the irreversible decrease in oxygen consumption observed after 30 min of exposure to paracetamol *in vitro*. It might be concluded that different mechanisms critically limit cell survival at different concentrations of paracetamol. At the very high concentration used in the present model (5 g/L), impairment of energy production becomes the limiting factor, resulting in early necrosis of livers from fasted rats. In contrast, at the much lower concentrations present *in vivo* the energy production is not impaired critically. Hepatotoxicity develops at a slower pace as a consequence of other alterations such as alkylation and/or oxidation of biological macromolecules and oxidative stress.

In summary, high concentrations of paracetamol produce "chemical hypoxia" of the isolated rat liver resulting in toxic injury. Such high concentrations of paracetamol are not attained *in vivo*, however. The lower concentrations of paracetamol present *in vivo* do not seem to impair energy production critically but rather induce hepatotoxicity as a consequence of other alterations resulting from the metabolic activation of paracetamol to NAPQI. It cannot be ruled out, however, that "chemical hypoxia" might aggravate liver damage at least in severe cases of paracetamol intoxication.

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