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EFFECT OF CHRONIC HYPOXIA ON ACETAMINOPHEN METABOLISM IN THE RAT

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Abstract—The effect of chronic hypoxia (10.5% O_2 for 8–9 days) on acetaminophen metabolism was studied *in vivo* or in isolated cell or microsomal systems. Results from *in vivo* studies with oral administration of acetaminophen showed that in hypoxic rats, the plasma appearance of the drug was delayed and the plasma half-life was increased. Analyses of the area under the curve (AUC_{oral}) showed that this value was higher in hypoxic rats, whereas the rate constants for elimination (k_{elim}) and absorption (k_{abs}) were lower in these animals. Formation of the glucuronide and sulfate conjugates was decreased significantly ($P < 0.05$) in hypoxic animals. The calculated volume of distribution (V_d) after an intravenous dose was not different in either group but total clearance (CL) was 35% lower in hypoxic rats. Studies with isolated hepatocytes from both groups revealed that glucuronidation and sulfation were inhibited markedly at low O_2 concentrations. The O_2 concentrations required for half-maximal production (P_{50} values) of glucuronide (2.3 μM O_2) and sulfate (1.8 μM O_2) conjugates in cells from hypoxic animals were lower than for control cells (5.3 μM and 3.9 μM O_2 for glucuronide and sulfate conjugates, respectively). Maximal rates of conjugation in cells from hypoxic rats were 60–70% of control rates. Similar decreases in microsomal UDP-glucuronosyltransferase and cytosolic sulfotransferase activities were found in livers of animals exposed to chronic hypoxia. These lower P_{50} values are consistent with a lower P_{50} for oxidation of mitochondrial cytochromes in hypoxic cells. In comparison, the P_{50} for glutathione conjugation (4.1 μM O_2) was not statistically different from control (4.6 μM O_2), but the maximal rate was 65% higher. The results show that chronic hypoxia causes a change of absorptive processes and decreased glucuronidation and sulfation reactions which affects the disposition of acetaminophen and potentially the disposition of a variety of other exogenous and endogenous compounds.

Hypoxia is a common clinical occurrence and, at the cellular level, causes altered biochemical and physiological functions [1]. Studies on drug-metabolizing pathways in cellular systems [1–3] or in isolated perfused organs [4, 5] indicate that metabolism of a variety of drugs is O_2 dependent and can be impaired under hypoxic conditions. However, little information is available on drug absorption, metabolism and elimination *in vivo* as a function of O_2 supply. One of the limitations has been the difficulty of performing these *in vivo* studies under controlled hypoxic conditions. In the current paper, we describe a system for induction of chronic hypoxia in rats and for maintenance of controlled hypoxic conditions throughout the period of blood sampling following drug administration. This approach provides a means to directly examine the effects of O_2 deficiency on drug metabolism *in vivo*.

In earlier studies [6] we found that the metabolism of acetaminophen in freshly isolated hepatocytes is markedly dependent upon O_2; the formation of the glucuronide, sulfate and glutathione conjugates was decreased at low O_2 concentrations. To examine the effect of chronic hypoxia on acetaminophen disposition *in vivo*, we administered an oral dose of the drug to rats that had been exposed to hypoxia (10.5% O_2) for 8–9 days and measured the time dependence of the appearance of plasma acetaminophen and its metabolites. The results showed that in hypoxic animals, attainment of maximal acetaminophen concentrations in plasma was delayed, and formation of the glucuronide and sulfate conjugates was decreased. Further studies on the O_2 dependence of acetaminophen metabolism in isolated hepatocytes showed that the sulfation and glucuronidation pathways were selectively inhibited in cells isolated from hypoxic animals while conjugation with glutathione was increased. These results show that an *in vivo* change in O_2 supply affects glucuronidation and sulfation processes such that the disposition of acetaminophen and potentially a variety of other exogenous and endogenous compounds metabolized by these pathways will be altered.

METHODS

Materials. Collagenase (Type IV) and acetaminophen were purchased from the Sigma Chemical Co., St. Louis, MO. Surgical tools and supplies were obtained from the office of the Emory University veterinarian. All other chemicals used were of reagent grade and purchased from local sources.

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Animal protocols were reviewed and approved by the Emory University IACUC Committee (File No. 138-89).

Induction of chronic hypoxia in rats. Male rats (Kng: (SD) Br, King Animal Laboratories, Oregon, WI) weighing between 200 and 250 g were exposed to either normoxia (140 torr) or hypoxia (100 torr for 24 hr followed by 70–80 torr for 8–9 days) in specially constructed plastic cages. This protocol induces a severe but not life-threatening hypoxia. Major acclimatization occurs within several days (by 8–9 days for the current studies); however, additional changes can occur for several weeks especially under more severe hypoxic conditions. The 22-L cages were sealed at the top by plastic covers secured by lug bolts. Small openings were made in the top covers to allow inflow and outflow gases and to accommodate water bottles. The desired pO_2 was achieved by using air (normoxia) or by mixing air and nitrogen (hypoxia) in a Matheson gas mixer (Matheson Gas Products, NJ). The pO_2 in the chambers was monitored using an O_2 electrode (Clark-type, Yellow Springs Instruments, Yellow Springs, OH) inserted through an opening in the top cover. Total gas flow was set at approximately 1.8 L/min to prevent excessive accumulation of moisture and ammonia and to keep pCO_2 below the limit of detection of the pCO_2 electrode. Cages were opened daily for 5 min to change bedding and food. Normoxic rats housed in standard open rat cages showed no difference in the kinetic parameters studied when compared to those kept in closed cages.

Catheterization of the jugular vein. Following hypoxic or normoxic treatment, surgery was performed in rats to provide an indwelling catheter in the jugular vein. Rats were lightly anesthetized with a mixture (1:1, v/v) of rompum (20 mg/mL) and ketamine (100 mg/mL) at 0.1 mL/kg body weight, and catheterization of the external jugular vein was performed by the procedure of Juarbe and Sillau [7]. After surgery, rats were injected with 0.2 mL penicillin (300,000 units/mL) to prevent infection and were allowed to recover for 1 hr in open cages before being returned to normoxic or hypoxic chambers. The recovery following surgery was similar for normoxic and hypoxic rats. In experiments where fasted animals were used, food was removed 24 hr prior to drug administration.

In vivo *studies.* All experiments were conducted in rats 24 hr after surgery. An initial aliquot of blood was removed via the indwelling catheter prior to drug administration to determine the baseline values. Rats were then given an oral dose of acetaminophen (70 mg/kg) in approximately 0.5 mL of 0.9% NaCl by gavage. During these procedures, the animals were exposed to room air for approximately 2 min and were returned to hypoxic chambers. Brief exposure to normoxia did not apparently affect the results because when hypoxic animals were returned to normoxia, kinetic characteristics did not return to normoxic values for at least 48 hr. In some experiments, acetaminophen was given intravenously (20 mg/kg) via the catheter. This was followed by an equivalent volume of normal saline to wash the catheter and minimize contamination of the catheter for subsequent sampling. At various times after drug administration, blood (0.2 mL) was drawn into heparinized syringes, and the catheter was washed with 0.2 mL heparin (100 units/mL) in saline. The blood was centrifuged immediately to remove erythrocytes and an aliquot of plasma was added to 3 M perchloric acid. The acid supernatants were analyzed for acetaminophen and its conjugates. Between each sampling (2 min), the rats were returned to hypoxic chambers to minimize exposure to air.

Liver cell preparations and incubations. Hepatocytes from normoxic and hypoxic rats were prepared by the method of Moldeus *et al.* [8]. The cells were typically >90% viable immediately after isolation as ascertained by exclusion of 0.2% trypan blue. Cells were maintained at 20° in a gyratory shaker water bath under air for 4 hr without loss of viability. Incubations (10^6 cells/mL) were performed at 37° in rotating round-bottom flasks in Krebs–Henseleit buffer, containing 25 mM HEPES, pH 7.4, and 5 mM acetaminophen. The O_2 dependence of acetaminophen metabolism was studied under different steady-state O_2 concentrations as previously described [6]. Cell incubations were terminated by the addition of perchloric acid (3 M, 0.1 mL/0.2 mL incubation volume).

Subcellular preparations and incubations. Subcellular fractions of livers from normoxic and hypoxic rats were prepared by differential centrifugation as previously described [9]. Incubations with microsomes and cytosol were carried out in buffer containing the following (in mM): 125 KCl, 2 K_2HPO_4, 4 $MgCl_2$ and 25 HEPES, pH 7.0, in the presence of 10 mM acetaminophen. Formation of the glucuronide and sulfate conjugates was carried out in the presence of 2 mM UDP-glucuronic acid and 250 μM 3′-phosphoadenosine 5′-phosphosulfate, respectively. At various times, 1-mL samples were taken and added to 0.5 mL of 3 M perchloric acid. Acetaminophen glucuronide and sulfate were analyzed in the acid supernatants.

Quantification of acetaminophen metabolites. Acetaminophen metabolites were quantified by high performance liquid chromatography according to the method of Howie *et al.* [10] as modified by Moldeus [11]. Conjugates were measured in the total incubation mixture following centrifugation of the acid extracts to remove insoluble protein and were quantified by integration. Total plasma proteins were determined as the trichloroacetic acid-insoluble fractions by the method of Smith *et al.* [12].

Data analyses. The area under the curve (AUC) was calculated using the trapezoid method [13]. The apparent volume of distribution (V_d) and plasma half-life ($T_{1/2}$) were calculated from the zero intercept and the slope of line of the log plasma concentration–time data. Total clearance was calculated as the product of V_d and $0.693/T_{1/2}$, the rate constant for elimination (k_{elim}) [13]. Statistical analyses were performed using Student's *t*-test.

RESULTS

In vivo *disposition of acetaminophen.* Chronic exposure of rats to hypoxia for 8 or 9 days resulted in a smaller weight gain in hypoxic rats (20 g vs 40 g

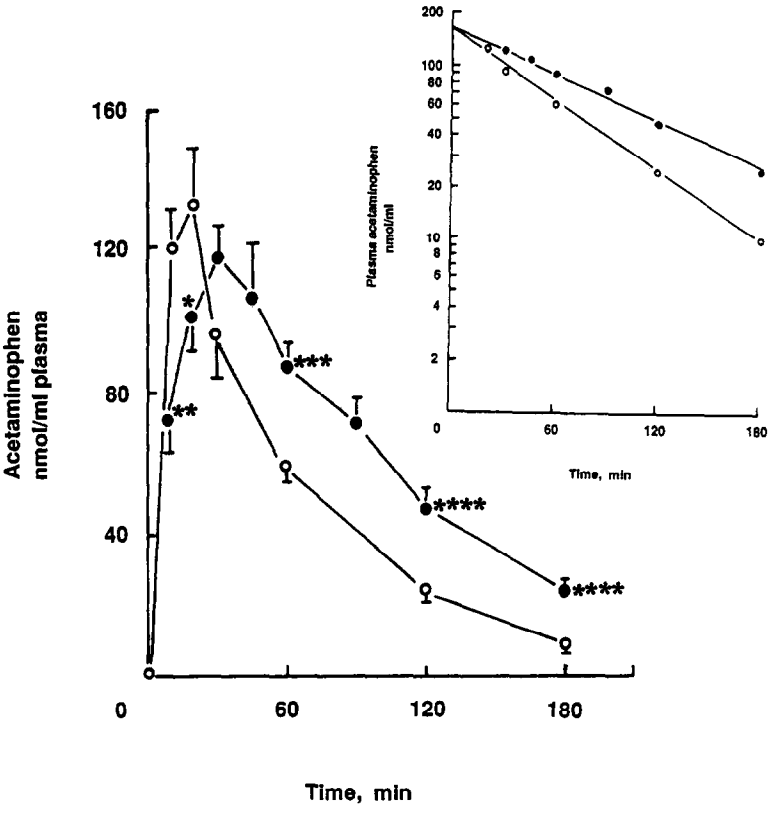

Fig. 1. Effect of normoxia or hypoxia *in vivo* on plasma acetaminophen concentration in rats under fed conditions. Rats were given an oral dose of acetaminophen (70 mg/kg) in 0.9% NaCl by gavage and at various times after drug administration, 0.2 mL blood was obtained from the external jugular vein via an indwelling catheter. Erythrocytes were removed by centrifugation and the plasma were acidified with 3 M perchloric acid. Acetaminophen and conjugates were analyzed in the acid supernatants by HPLC [10, 11]. Between sampling, animals were returned to their respective chambers to maintain steady-state normoxic or hypoxic conditions. The plasma half-lives of acetaminophen elimination under the two O_2 conditions were estimated from the semi-log plot of the data (inset). Key: (○) normoxia; and (●) hypoxia. Values are means ± SEM of five animals. The 90-min value in the normoxic group was not collected. Values from hypoxic rats were significantly different from those of normoxic animals at (*) $P < 0.05$, (**) $P < 0.025$, (***) $P < 0.01$, and (****) $P < 0.005$.

for normoxic rats) but was without other visible adverse effects on the animals. To examine the effect of hypoxia *in vivo* on the disposition of acetaminophen, the kinetics of absorption and metabolism were determined for 3 hr in normoxic and hypoxic rats following an oral dose of the drug. Measurements of plasma concentrations of acetaminophen showed that appearance of the drug in normoxic, fed animals was rapid. The peak concentration occurred at 20 min and decreased to about 7% by 3 hr post gavage (Fig. 1). In contrast, plasma appearance of acetaminophen in hypoxic rats was noticeably delayed; peak concentration occurred at 30 min (Fig. 1). Moreover, the plasma concentrations in these animals were higher than those of normoxic rats at 30 min and all subsequent time points. Comparison of the plasma half-lives ($T_{1/2}$) of acetaminophen (Fig. 1, inset) showed that the half-life was nearly twice as high in hypoxic ($T_{1/2}$ = 72 min) as in normoxic rats $T_{1/2}$ = 43 min). The respective rate constants for elimination (k_{elim}) were 0.00963 min^{-1} and 0.0161 min^{-1}. These results show that chronic hypoxic exposure of rats caused a delay in attainment of the maximal plasma concentration and in elimination of orally administered acetaminophen.

To determine whether the difference in kinetics of acetaminophen appearance in the plasma was due to the difference in weights between the normoxic and hypoxic animals, rats were pair fed, and the food intake was monitored. Under these controlled conditions, similar weight gains were found for normoxic and hypoxic rats (23 g vs 21 g, respectively). The peak concentrations of plasma acetaminophen occurred at 15 and 30 min, respectively, for normoxic and hypoxic rats. These values were similar to those found in rats fed *ad lib.* (see Fig. 1), indicating that the delay in acetaminophen absorption in hypoxic animals was a consequence of O_2 limitation rather than a consequence of weight differences of the two groups. The $T_{1/2}$ value for the normoxic, pair-fed rats was comparable to that for the normoxic rats

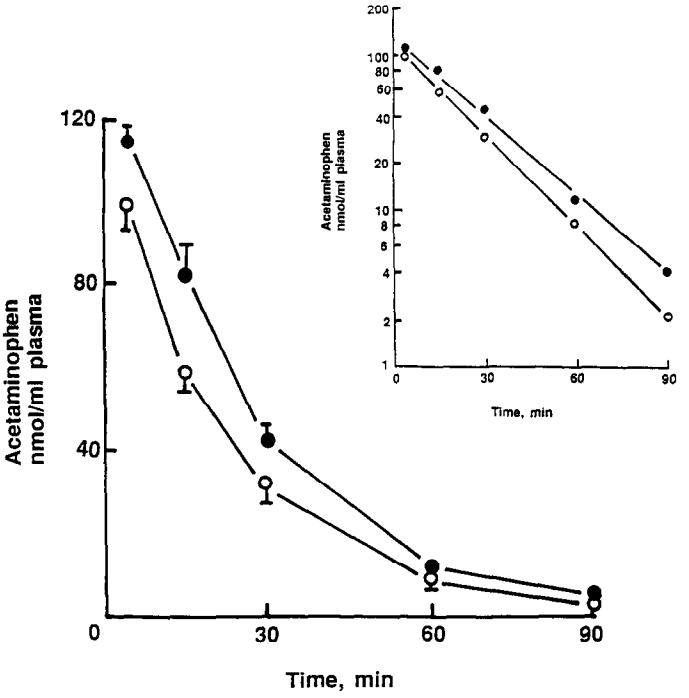

Fig. 2. Plasma acetaminophen concentrations in hypoxic and normoxic rats under fed conditions following intravenous administration. A 20 mg/kg dose of acetaminophen in 0.9% saline was given to hypoxic and normoxic rats intravenously via the indwelling catheter. At various times following drug administration, blood samples were removed and analyzed for plasma acetaminophen concentrations. The plasma half-lives of acetaminophen elimination under the two O_2 conditions were estimated from the semi-log plot of the data (inset). Key: (○) normoxia, and (●) hypoxia. Values are means ± SEM of five animals. Hypoxic data were significantly different from normoxic data at all time points ($P \leq 0.05$).

fed *ad lib*. Thus, the changes appeared to be independent of direct changes due to differences in energy consumption; however, indirect effects of hypoxia on glycogen metabolism or UDP-glucuronic acid concentrations none-the-less could be involved in the overall kinetics of elimination.

To confirm that the altered pharmacokinetics involved a decrease in elimination rate, direct measurement of the effect of chronic hypoxia on plasma half-life, volume of distribution (V_d) and total clearance (CL) of acetaminophen in normoxic and hypoxic rats was obtained following an intravenous dose of the drug (Fig. 2). The solubility of acetaminophen prevented use of the same total dose as used for the oral administration studies described above. The results with a lower dose (Fig. 2) showed that the plasma $T_{1/2}$ in normoxic rats was 17.4 ± 0.7 min, a value similar to that reported previously for a comparable dose for the rat [14], while the plasma half-life was increased in hypoxic rats ($T_{1/2}$ = 23.1 ± 0.8 min). The apparent V_d of acetaminophen in control rats (1.08 L/kg), obtained by extrapolating back to zero time on the acetaminophen concentration versus time plot, was comparable to previously published values in rats (0.97 L/kg, [14]). The estimated V_d in hypoxic rats was similar to that in normoxic rats (1.01 ± 0.08 L/kg), indicating that the total aqueous space available for acetaminophen distribution was not affected by exposure of animals to chronic hypoxia. The calculated total clearance of acetaminophen was decreased from 41.6 ± 3.7 $mL \cdot min^{-1} \cdot kg^{-1}$ in normoxic rats to 29.9 ± 1.2 $mL \cdot min^{-1} \cdot kg^{-1}$ in hypoxic rats. Taken together with the above data, these results indicate that the exposure of rats to chronic hypoxia slows the elimination of acetaminophen.

Because k_{elim} was decreased by chronic hypoxia, a delayed attainment of maximal plasma acetaminophen was expected. However, a decreased absorption rate could also contribute to the delay in attaining maximal concentration. To address this possibility, mean absorption time (MAT) for hypoxic and normoxic rats was calculated as the difference between mean residence time (MRT) of orally administered and intravenously administered acetaminophen. Mean residence time was calculated as the area under the first moment curve (AUMC) divided by the area under the curve (AUC) [13]. The partial AUC_{oral} for the first 3 hr was 8.80 $\mu mol \cdot mL^{-1} \cdot min^{-1}$ for normoxic rats and 11.5 $\mu mol \cdot mL^{-1} \cdot min^{-1}$ for hypoxic rats. Extrapolation to obtain an estimate of the total AUC was obtained using the plasma acetaminophen concentration at 3 hr divided by the rate constant for elimination [13]. The resulting total AUC_{oral} values were 9.29 and 13.9 $\mu mol \cdot mL^{-1} \cdot min^{-1}$. The

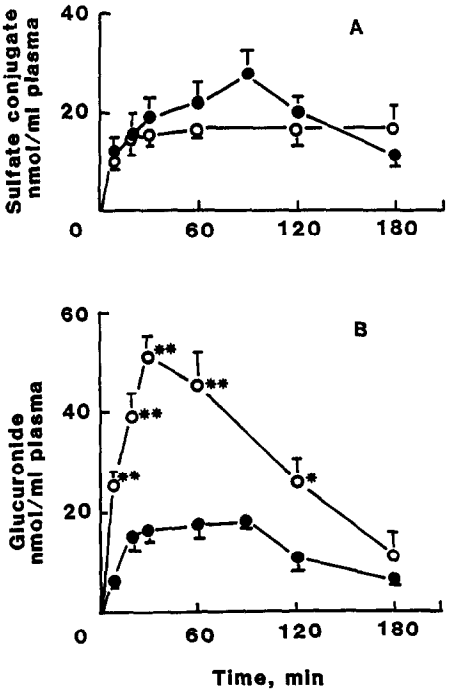

Fig. 3. Effect of normoxia or hypoxia *in vivo* on plasma concentrations of acetaminophen conjugates in rats under fed conditions. Oral administration of acetaminophen and determination of plasma concentrations of the sulfate (A) and glucuronide (B) conjugates in normoxic and hypoxic rats were as described in the legend of Fig. 1. Key: (○) normoxia, and (●) hypoxia. Values are means ± SEM of five animals. The glucuronide conjugate in normoxic rats was significantly higher than that in hypoxic animals at (*) $P < 0.025$, and (**) $P < 0.005$, but the sulfate conjugates were not different.

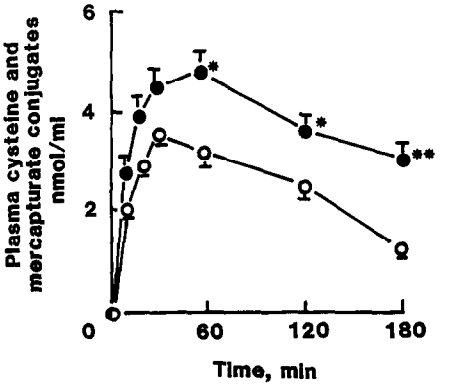

Fig. 4. Effect of normoxia or hypoxia *in vivo* on plasma cysteine and mercapturate concentrations. Plasma concentrations of the total cysteine and mercapturate conjugates of acetaminophen were determined in normoxic (○) or hypoxic (●) rats as described in the legend of Fig. 1. Values are means ± SEM of five animals. As indicated by asterisks, values from hypoxic rats were significantly higher than the corresponding values from normoxic animals at (*) $P < 0.025$ and (**) $P < 0.01$.

total $AUMC_{oral}$ was calculated similarly using the trapezoidal rule for the first 3 hr and the concentration at 3 hr and k_{elim} to estimate the area for 3 hr to infinity [13]. The respective hypoxic and normoxic values were 147 and 702 $\mu mol \cdot mL^{-1}$. MRT_{oral} values were 15.8 and 50.4 min.

For normoxic rats, AUC_{iv} was 2.46 $\mu mol \cdot mL^{-1} \cdot min^{-1}$ and $AUMC_{iv}$ was 9.32 $\mu mol \cdot mL^{-1}$. For hypoxic rats AUC_{iv} was 3.32 and $AUMC_{iv}$ was 21.0 $\mu mol \cdot mL^{-1}$. MRT_{iv} values were 3.79 and 6.32 min. Thus, $MAT_{normoxia}$ was 12.0 min and $MAT_{hypoxia}$ was 43.7 min. Taking the absorption rate constant (k_{abs}) as 1/MAT, this indicates that the rate constant for absorption is 0.083 min^{-1} in normoxic rats and 0.023 min^{-1} in hypoxic rats. Thus, the results indicate that chronic exposure to hypoxia decreases both absorption and elimination of acetaminophen. The direction of this change further suggests that the hypoxia-induced decrease in gut wall metabolism is not responsible for the altered k_{abs}, but decreased metabolism in the gut could contribute to the overall change in k_{elim}.

To determine whether the delayed elimination of acetaminophen in hypoxic animals was due to impaired metabolism, plasma levels of metabolites were measured. The results (Fig. 3) showed that in fed, normoxic rats, the major metabolite was the glucuronide conjugate (75% of total conjugates), consistent with glucuronidation being the predominant pathway for detoxication of acetaminophen [15, 16]. In contrast, formation of the glucuronide conjugate in hypoxic rats was decreased substantially (Fig. 3B), indicating that chronic hypoxic exposure caused a reduction in the glucuronidation capacity. This may occur due to reduced uptake of acetaminophen by the liver or to decreased metabolic activity. In normoxic fed rats, the sulfate conjugate was maximal at about 20 μM and was not different for hypoxic, fed animals (Fig. 3A).

The glutathione conjugate was not detectable in the plasma extracts under these conditions (limit of detection was 3 nmol), but was present predominantly as the cysteine and the mercapturate conjugates (Fig. 4). This indicates that the former is cleared very rapidly from the plasma. Analysis of the sum of the cysteine and mercapturate conjugates revealed that the plasma concentrations were higher in the hypoxic rats (Fig. 4), a result that is consistent with an enhancement in metabolism by cytochrome P-450-catalyzed reactions. Despite this increased activity, a substantial decrease in acetaminophen elimination occurred in hypoxic rats due to reduced sulfation and glucuronidation reactions.

Because glucuronidation during hypoxia can be limited by glucose availability [6, 17], we examined the effect of a 24-hr fast on the disposition of acetaminophen in hypoxic and normoxic rats. The results showed that a pronounced delay occurred in plasma appearance and clearance of acetaminophen (Fig. 5) in hypoxic fasted rats compared to normoxic fasted animals, a result that was similar to that found for hypoxic rats under fed conditions (Fig. 1). Plasma half-lives were 72 and 40 min respectively (Fig. 5, inset). Analyses of the profiles for the glucuronide and sulfate conjugates revealed that, in contrast to the fed condition, the sulfate conjugate was the

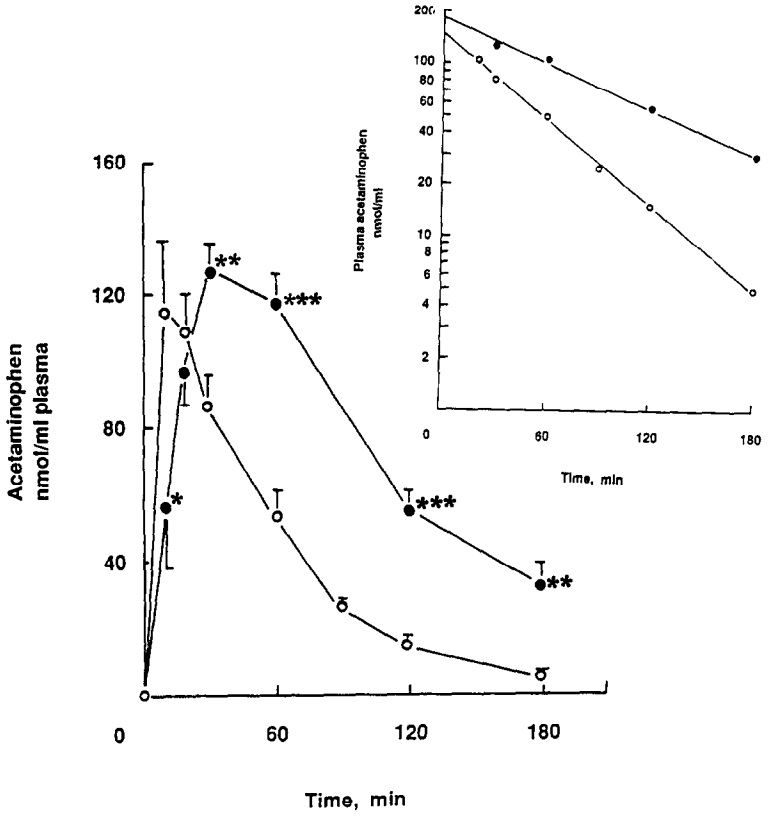

Fig. 5. Plasma acetaminophen concentrations in normoxic or hypoxic rats following 24 hr of fasting. Measurement of plasma acetaminophen concentrations was performed as described in the legend of Fig. 1 in normoxic or hypoxic rats after a 24-hr fast. Key: (○) normoxia, and (●) hypoxia. Values are means ± SEM of four animals for normoxia and three animals for hypoxia. Data from hypoxic rats were significantly different from the data from normoxic animals at (*) $P < 0.05$, (**) $P < 0.025$, and (***) $P < 0.005$.

predominant metabolite in fasted animals under normoxic conditions, accounting for 75% of the total conjugates (Fig. 6A). Formation of the glucuronide conjugate was relatively less during hypoxia (25% of the total, Fig. 6B) which is consistent with a compensatory shift to sulfation as the major route of detoxication due to a limited supply of substrate for glucuronidation during fasting [18]. In comparison, plasma concentrations of the glucuronide and sulfate conjugates in fasted hypoxic animals were low (Fig. 6). Significantly, the decrease in formation of the sulfate conjugate in hypoxic compared to normoxic rats shows that the sulfation pathway is sensitive to O_2 deficiency *in vivo*. Thus, hypoxia selectively alters the kinetics of acetaminophen absorption and metabolism.

Studies with isolated hepatocytes. To further characterize the effect of chronic hypoxia on glucuronidation, sulfation and conjugation with glutathione, we examined the response of these reactions to O_2 changes in freshly isolated cells prepared from normoxic and hypoxic rats. Hepatocytes that were prepared from hypoxic rats were comparable to those from normoxic animals in gross morphological characteristics (shape, size, appearance) as observed by light microscopy. Cell viability as assessed by exclusion of 0.2% trypan blue was between 89 and 95% which compared well with cells from normoxic rats (typically >90%). Other parameters, including cell yield ($2–4 \times 10^8$ cells/liver), O_2 consumption rate (22 nmol $O_2 \cdot (10^{-6}$ cells$)^{-1} \cdot$ min^{-1}) and protein concentration (1.4 mg/10^6 cells) were comparable to normoxic cells in this study and those previously reported [6, 19].

The O_2 dependence of acetaminophen metabolism was studied under various steady-state O_2 concentrations [6]. The results showed that sulfation and glucuronidation reactions were lower in hypoxic cells than in normoxic cells (Fig. 7, A and B); maximal rates were 60–70% of that of control (Table 1). The half-maximal changes for conjugate formation also occurred at lower half-maximal production (P_{50}) values than controls (Fig. 7, A and B; Table 1). These lower P_{50} values for glucuronide formation (2.3 μM O_2) and for sulfate conjugation (1.8 μM O_2, Table 1) in hypoxic cells parallel the lower P_{50} for oxidation of mitochondrial cytochromes in these cells [20].

Examination of the effect of chronic hypoxia on O_2 dependence of conjugation with glutathione revealed important differences from those on sulfation and glucuronidation (Fig. 7C). The P_{50}

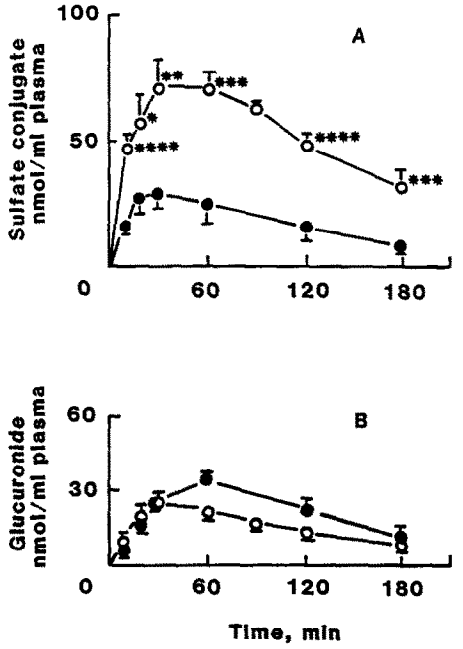

Fig. 6. Plasma concentrations of sulfate and glucuronide conjugates in normoxic or hypoxic rats after 24 hr of fasting. Determination of plasma concentrations of sulfate (A) and glucuronide (B) conjugates of acetaminophen were performed in rats after a 24-hr fast. Key: (○) normoxia, and (●) hypoxia. Values are means ± SEM of four animals for normoxia and three animals for hypoxia. The sulfate conjugate in normoxic animals was significantly higher than that in hypoxic animals at (*) $P < 0.05$, (**) $P < 0.025$, (***) $P < 0.01$, and (****) $P < 0.005$. The glucuronide conjugates were not different.

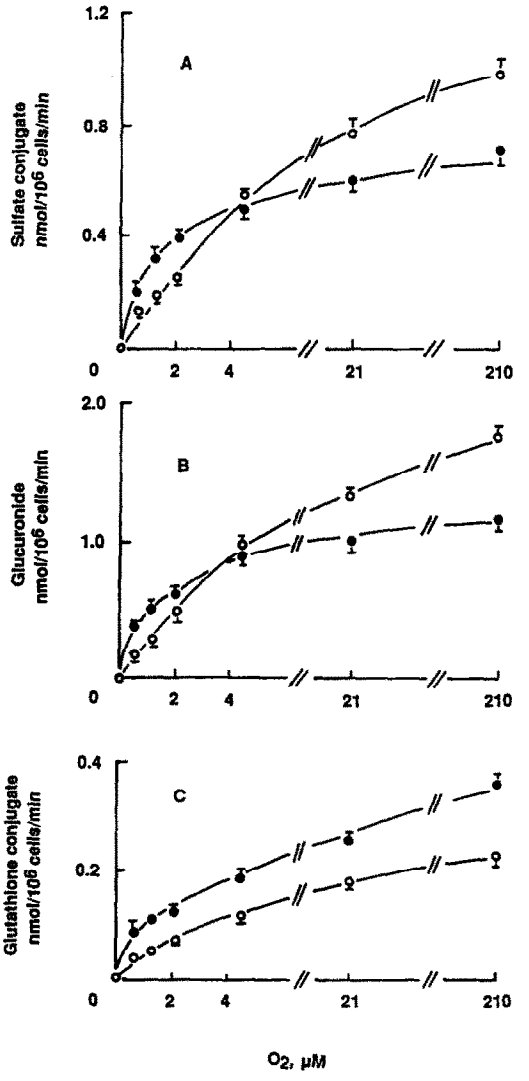

Fig. 7. O_2 dependence of acetaminophen metabolism in isolated hepatocytes from normoxic or hypoxic rats. Incubations (10^6 cells/mL) were performed at 37° in rotating round-bottom flasks in Krebs–Henseleit buffer, containing 25 mM HEPES, pH 7.4, in the presence of 5 mM acetaminophen. The O_2 dependence of acetaminophen metabolism was studied under different steady-state O_2 concentrations as previously described [6]. Sulfate (A), glucuronide (B), and glutathione (C) conjugates were determined by HPLC [10, 11]. Key: (○) normoxia, and (●) hypoxia. Values are means ± SEM of six animals for normoxia and nine animals for hypoxia.

value in cells from hypoxic rats (4.1 μM O_2) was not significantly different from that in normoxic animals (4.6 μM O_2, Table 1), but the maximal rate of conjugation was 0.38 nmol·(10^6 cells)$^{-1}$·min^{-1}, a value that is 65% higher than in normoxic cells (0.23 nmol·(10^6 cells)$^{-1}$·min^{-1}, Table 1). These results suggest that, of the three major pathways for acetaminophen detoxication, glucuronidation and sulfation are vulnerable to *in vivo* O_2 deficiency, while conjugation with glutathione is enhanced.

Studies with subcellular fractions. The decreased maximal rates of production of glucuronide and sulfate conjugates in cells from hypoxic rats even at normal O_2 concentrations suggest that hypoxia *in vivo* causes a decrease in levels of UDP-glucuronosyltransferases and sulfotransferases. To examine this, the respective enzyme activities were measured in microsomal and cytosolic fractions of livers from normoxic and hypoxic rats. The results show that under optimal substrate concentrations, UDP-glucuronosyltransferase activities in isolated microsomes from normoxic rats were 0.20 ± 0.02 nmol·(mg protein)$^{-1}$·min^{-1} compared to 0.13 ± 0.01 nmol·(mg protein)$^{-1}$·min^{-1} in microsomes from hypoxic rats, a 35% decrease in activity. This difference was not due to differences in latencies of the enzyme consequent to the two oxygen conditions since activation of microsomes with either 0.2% Triton X-100 or 0.1% digitonin did not eliminate the differences (data not shown). Thus, the decrease in glucuronidating capacity in rats exposed chronically to hypoxia appeared to be, at least in part, a direct result of decreased microsomal UDP-glucuronosyltransferase activity. This effect occurs in addition to the previously reported decrease in UDP-glucuronic acid during hypoxia [17], and, therefore, both factors could contribute to rate limitation during chronic hypoxia.

Table 1. P_{50} values and maximal rates for conjugation of acetaminophen in hepatocytes from normoxic and hypoxic rats

Conditions	Conjugates	P_{50} (μM O_2)	Maximal rate (nmol·(10^6 cells)$^{-1}$·min^{-1})
Normoxia	Sulfate	3.9 ± 0.5	1.04 ± 0.08
(N = 6)	Glucuronide	5.3 ± 0.9	2.06 ± 0.05
	Glutathione	4.6 ± 1.0	0.23 ± 0.03
Hypoxia	Sulfate	1.8 ± 0.1*	0.73 ± 0.05*
(N = 9)	Glucuronide	2.3 ± 0.2*	1.33 ± 0.05*
	Glutathione	4.1 ± 0.5†	0.38 ± 0.02*

Cells (10^6/mL) were incubated with 5 mM acetaminophen and reactions were terminated by the addition of 3 M perchloric acid. Values are means ± SEM.

* Significantly different from value for normoxic animals, $P \leq 0.005$.

† Not significantly different from value for normoxic animals.

Measurements of cytosolic sulfotransferase activities in normoxic and hypoxic rats showed that the function of this soluble enzyme was also decreased (0.31 ± 0.02 nmol·(mg protein)$^{-1}$·min^{-1} for normoxia vs 0.24 ± 0.02 nmol·(mg protein)$^{-1}$·min^{-1} for hypoxia). These results, together with those from isolated cells, suggest that the hypoxia-induced inhibition of sulfation *in vivo* is also, in part, a direct result of decreased sulfotransferase activity.

DISCUSSION

The combination of techniques for induction and maintenance of controlled chronically hypoxic conditions and for rapid sampling of blood provided a useful approach to the study of the effect of chronic hypoxia on *in vivo* drug absorption, metabolism and elimination in rats. In addition, use of isolated hepatocytes and subcellular fractions prepared from livers of normoxic and hypoxic animals allowed a direct examination at the cellular level of glucuronidation and sulfation pathways in response to hypoxia *in vivo*.

The results showed that there was a significant reduction in absorptive activity *in vivo* in response to limited O_2 supply as reflected in delayed acetaminophen absorption. In initial experiments with isolated perfused intestine [21], we found no difference in the rate of appearance of radiolabeled acetaminophen into the perfusate in normoxic versus hypoxic rats, indicating that the delay in absorption observed *in vivo* is not due to enhanced metabolism of acetaminophen by the intestinal cells. At present, we are not able to distinguish whether the delay in *in vivo* absorption is due to decreased gastric emptying or decreased intestinal perfusion. Hypoxia is known to affect both functions [22].

A decrease in absorption due to hypoxia is an important consideration for oral administration of drugs in therapy. More generally, a malfunction of absorptive processes due to *in vivo* O_2 deficiency means that compromised uptake of a variety of other nutrient substrates during hypoxia could severely limit cell function and energy metabolism. Clinically, impairment of absorptive capacities by chronic hypoxia can be critical, especially in certain disease conditions, such as pulmonary cachexia and anemia, in which severe hypoxia can limit essential nutrient supply and cause tissue wasting.

Decreased metabolism by glucuronidation and sulfation in hypoxic individuals could result in increased conversion of acetaminophen to the toxic product, *p*-benzoquinonimine. In addition, the plasma concentration of unbound acetaminophen could be increased in hypoxic rats because a substantial amount of acetaminophen is normally bound to plasma proteins [23] and the measured total plasma proteins in hypoxic rats were lower (5.3 ± 0.1 mg/mL) than normoxic rats (6.3 ± 0.2 mg/mL). Thus, a decrease in binding of the free drug by plasma proteins under hypoxic conditions could decrease the therapeutic index for actaminophen.

Mechanistically, the decreases in activities of glucuronidation and sulfation by chronic hypoxia appeared to be different from the inhibitory effects of acute hypoxia. In hypoxic animals, such decreases in activities are consistent with inhibition of *in vivo* protein synthesis by hypoxia [24], and studies using immunochemical techniques are needed to determine the forms of UDP-glucuronosyltranferases and sulfotransferases that are affected. In earlier studies, we found that the decrease in glucuronidation at low O_2 concentrations in isolated hepatocytes from normoxic rats was due primarily to the decrease in UTP and glucose for synthesis of UDP-glucose and UDP-glucuronic acid [6, 17]. Similarly, the inhibition of sulfation was the result of decreased ATP availability for synthesis of the activated sulfate, 3′-phosphoadenosine 5′-phosphosulfate [6]. Thus, the effects of acute O_2 deficiency on drug glucuronidation and sulfation are related primarily to decreased energetics of the cell as a result of impaired cytochrome oxidase function, while decreased enzyme activities are important contributing factors to the reduced conjugation capacities during chronic hypoxia.

Because functions of glucuronidation and sulfation pathways are dependent on functions of the mitochondria, chronic hypoxic effects on mitochondrial oxygenation characteristics could have further impact on these detoxication systems. In recent studies on the O_2 dependence of mitochondrial function in hepatocytes from hypoxic rats, we found that hypoxia *in vivo* caused a significant decrease in

the O_2 concentration required for oxidation of mitochondrial cytochromes [20]. This shift to a lower P_{50} value for mitochondrial function appeared to be the result of a cellular redistribution of mitochondria from a clustered configuration in normoxic cells [25] to a more uniform pattern in hypoxic cells [24, 26]. Thus, functions of a variety of O_2-dependent metabolic processes are expected to be altered by the change in mitochondrial and cellular oxygenation. The measured O_2 concentration for half-maximal activities for acetaminophen sulfation and glucuronidation (Table 2) reflect that for oxidation of mitochondrial cytochromes [20].

The enhancement of acetaminophen conjugation with glutathione in cells from hypoxic rats is consistent with an increase in metabolism by the cytochrome P450-catalyzed reactions because of impairment of sulfation and glucuronidation. An alternate explanation could be that chronic hypoxia caused an induction of P450. Measurements of total hepatic cytochrome P450 contents from CO-induced difference spectra of dithionite-reduced forms in hypoxic and normoxic rats showed that these were not different. However, this does not address whether changes in various P450 forms occur during chronic hypoxia.

The decrease in overall glucuronidation and sulfation capacities during hypoxia has important clinical implications in the disposition of acetaminophen and potentially a variety of other exogenous and endogenous compounds. Of importance is a limitation of acetaminophen glucuronidation by hypoxia *in vivo* in a population of patients with chronic bronchitis and emphysema [27]. Administration of O_2 partially restores normal metabolism [27], and consideration of proper dietary uptake may also promote drug elimination because glucose supply can enhance glucuronidation under hypoxic conditions [6, 17].

Another significant aspect is the effect of chronic hypoxia on zonal toxicities of acetaminophen in the liver. Studies on distribution of drug-conjugating systems showed that the glucuronidation pathway is localized to the centrilobular regions of the liver [16, 28]. Thus, one would expect that this zone would be protected from acetaminophen toxicity. However, during normoxia, hepatocytes in centrilobular regions are exposed to lower O_2 concentrations than cells in the periportal regions due to lobular differences in O_2 supply [16]. Thus, even under normoxic conditions, the increase in P450-catalyzed reactions consequent to a decrease in glucuronidation could result in enhanced vulnerability of centrilobular cells to acetaminophen toxicity [29]. During chronic hypoxia, the additive effects of decreased O_2 supply and further reduction of glucuronidating activities could exacerbate centrilobular necrosis.

In summary, we have developed an approach to induce and maintain controlled hypoxic conditions for the study of absorption, metabolism and elimination of drugs as a function of *in vivo* O_2 supply. The results show that chronic hypoxia causes selective impairment of specific metabolic systems involved in absorption, glucuronidation and sulfation processes. Such knowledge provides a basis for the study of the disposition of a variety of other therapeutic drugs and for improved interventions of drug therapies in hypoxic patients.

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