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Influence of hypoxia on the metabolism and biliary excretion of trimetrexate by the isolated perfused rat liver

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The effect of hypoxia on hepatic drug metabolism is not entirely predictable. The elimination of drugs which primarily undergo microsomal oxidation will be impaired [1–6], but if biotransformation has been suppressed due to the *presence* of oxygen, a compound may well exhibit increased clearance during hypoxia. Such a situation occurs with misonidazole [7]. Therefore, potential consequences of drug administration to a hypoxic patient could include either a decrease in metabolism of the parent drug, resulting in higher plasma concentrations, or an enhancement in drug elimination, causing lower plasma levels. Oxygen deprivation might also influence the formation and elimination of metabolites.

The oxygen dependence of biliary excretion of parent drugs and metabolites has not been widely studied. Although bile formation is complex, at least certain aspects require energy, including the $(Na^+ + K^+)$-ATPase-dependent uptake of bile salts into hepatocytes, and the active transport of solutes into bile canaliculi [8]. The potential exists, therefore, that hypoxia could lead to decreased bile flow and impaired biliary excretion. This could have important implications in cases where bile is a major route of elimination of active compounds.

Trimetrexate [2,4-diamino-5-methyl-6-(3,4,5-trimethoxyanilino) methylquinazoline], a lipophilic inhibitor of dihydrofolate reductase [9], is currently in clinical trials as an anticancer agent. Previous studies have shown that trimetrexate undergoes rapid biphasic clearance by the isolated perfused rat liver [10], and is excreted primarily in the bile as two metabolites (M_1 and M_2). In this study, we have examined the effect of hypoxia on the elimination of trimetrexate by the isolated perfused rat liver. Formation and elimination of its metabolites were also followed, and particular attention was given to changes in bile flow and biliary excretion of parent drug and metabolites.

Materials and methods

Non-fasting male Sprague–Dawley rats (190–260 g) were anesthetized with sodium pentobarbital (65 mg/kg) and the livers were surgically isolated and perfused as previously described [4]. The 100-ml recirculating perfusate was gassed during its passage through 4 m of coiled silastic tubing (i.d. 1.47 mm, o.d. 1.96 mm, Dow Corning) in a closed jar into which either 100% O_2 (for normal oxygenation) or 100% N_2 (to create a hypoxic condition) was delivered at 1.5 l/min. Using an identical experimental design, Jones *et al.* [2] and Webster *et al.* [4] obtained control oxygen consumption values of 1.5 and 2.2 μmoles O_2/min per g liver respectively. This decreased during hypoxia by 70–90%, and recovered to near original values upon reoxygenation. A similar degree of hypoxia was confirmed for the present study by a preliminary experiment, which showed a drop in oxygen consumption from 1.16 (control) to 0.042 (hypoxia) μmoles/min per g liver. A constant infusion of sodium taurocholate (30 μmoles/hr; Sigma Chemical Co., St. Louis, MO) into the perfusate reservoir maintained bile salt concentration. Trimetrexate (as the glucuronate, PD-74, 013–714, Warner-Lambert Co., Ann Arbor, MI; prepared in water) was delivered into, and samples were removed from, the perfusate reservoir. Less than 4% of the total dose was lost in sampling.

Bolus dose studies. In four experiments, trimetrexate and metabolite concentrations were measured in the same liver perfusate during control (normoxic), hypoxic, and reoxygenated (recovery) conditions in sequence. Therefore, 1 mg (1.77 μmoles) of trimetrexate was delivered at the start of a 90-min normoxia phase, and repeated at the beginning of the subsequent 60-min hypoxic period. The perfusate was then reoxygenated, but no further trimetrexate was given.

Steady-state studies. A combination of a 200-μg bolus dose plus a constant infusion (730 μg/hr) of trimetrexate into the perfusate reservoir rapidly achieved a steady-state concentration of 1–2 μg/ml. This was maintained for 3 hr in the control studies, where 100% O_2 was used. The hypoxia studies consisted of three consecutive 1-hr periods (normoxia, hypoxia, and recovery), where O_2 was replaced by 100% N_2 during the hypoxic hour. Bile was collected serially at 30-min intervals.

Perfusate and bile samples were assayed for trimetrexate, M_1 and M_2 by a specific and sensitive high-performance liquid chromatographic method [10, 11]. The limit of detection for all compounds was 50 ng/ml. Extinction coefficients were unavailable for the metabolites; however, since neither metabolite has any alteration to the chromophore (unpublished observation), one can assume no significant change in the extinction coefficient. Therefore, the standard curve for trimetrexate was used to estimate metabolite concentrations, which are thus expressed as "trimetrexate equivalents". Statistical comparisons were made using two-tailed Student's *t*-tests. All results are expressed as mean ± SEM.

Results and discussion

From the steady-state studies, it was possible to calculate the total recovery of trimetrexate and the metabolites in perfusate and bile. Under control conditions, over 85% of the trimetrexate dose was metabolized. Of the total dose, 53 and 19% were found in bile as M_1 and M_2 respectively.

Biliary excretion data for the three compounds during the steady-state studies are shown in Table 1. Less than 6% of the total dose of trimetrexate was recovered as parent drug in bile during the 3-hr experiments, and hypoxia had little effect on this. However, lack of oxygen provoked approximately an 80% decrease in biliary excretion of both M_1 and M_2 by the last 30 min of hypoxia (period 2b, control vs hypoxia, $P < 0.001$). After an hour of reoxygenation (hypoxia studies, period 3b), biliary excretion of the metabolites recovered and was equivalent to both the pre-hypoxic period (period 1b) and the control experiments (control studies, period 3b).

Mean bile flow rates are also given in Table 1, and it can be seen that hypoxia caused a marked cholestasis, reducing bile flow by 60% during the last half-hour of hypoxia (period 2b). Recovery of flow rate was evident but not complete during reoxygenation (period 3). These results suggest that an oxygen-dependent process is a necessary part of bile formation.

The elimination of trimetrexate from the perfusate during normoxia in the bolus dose studies was rapid and biphasic, with a $T_{\frac{1}{2}}\alpha$ of 2.9 ± 0.5 min and $T_{\frac{1}{2}}\beta$ of 14.3 ± 2.2 min (Fig. 1). When the dose was repeated under hypoxic conditions, $T_{\frac{1}{2}}\alpha$ was unchanged (3.8 ± 1.3 min). This phase is often equated with drug uptake and distribution, and was not expected to be impaired since most drugs are believed to enter the hepatocyte by passive diffusion [12]. However, the terminal elimination phase was five to twelve times greater than during normoxia ($T_{\frac{1}{2}}\beta$ =

Table 1. Bile flow and biliary excretion during the steady-state tremetrexate studies*

Period†	Bile flow (ml/hr/10 g liver) Control	Hypoxia	Biliary excretion (μg/30 min) Trimetrexate‡ Control	Hypoxia	M_1§ Control	Hypoxia	M_2‖ Control	Hypoxia
1a	0.84 ± 0.06	0.85 ± 0.06	19 ± 4	17 ± 4	151 ± 28	149 ± 8	28 ± 10	23 ± 4
1b	0.84 ± 0.08	0.80 ± 0.06	19 ± 3	16 ± 1	221 ± 21	246 ± 19	70 ± 16	89 ± 11
2a	0.71 ± 0.08	0.53 ± 0.07	22 ± 1	16 ± 2	240 ± 11	128 ± 16	89 ± 12	61 ± 12
2b	0.68 ± 0.09	0.27 ± 0.04	18 ± 2	11 ± 2	222 ± 15	47 ± 8	83 ± 8	19 ± 5
3a	0.65 ± 0.05	0.49 ± 0.09	29 ± 3	38 ± 4	217 ± 16	146 ± 30	97 ± 9	66 ± 18
3b	0.63 ± 0.08	0.55 ± 0.08	32 ± 3	28 ± 3	209 ± 24	236 ± 40	98 ± 11	88 ± 15

* Results are mean ± SEM.

† Periods 1, 2 and 3 correspond to the normoxia, hypoxia, and reoxygenation hours, respectively, for the hypoxia studies (N = 4), or to the three consecutive control oxygenation hours during the control studies (N = 4). During each hour, two serial (30 min each) bile samples were collected (a and b). The perfusate was equilibrated with 100% O_2 in all periods except 2a and 2b in the hypoxia studies, where 100% N_2 was used.

‡ Total dose over 3 hr was 2390 μg.

§ M_1 = metabolite number one.

‖ M_2 = metabolite number two.

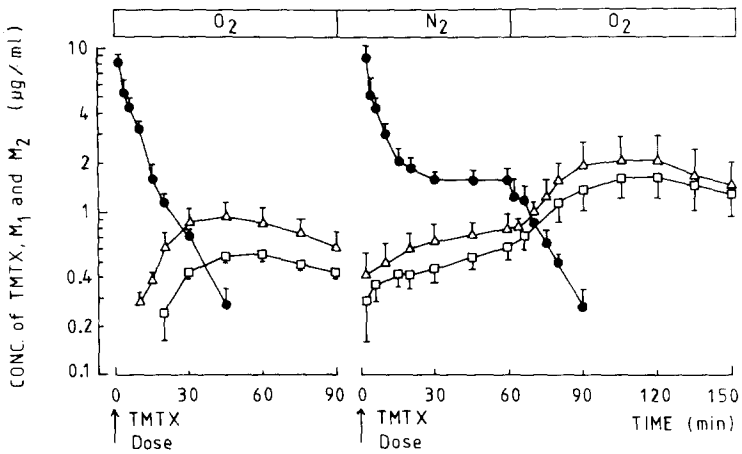

Fig. 1. Perfusate concentration–time profiles of trimetrexate (TMTX; ●) and its metabolites, M_1 (△) and M_2 (□), during the bolus dose studies. A 1-mg bolus of TMTX was given into the perfusate at the start of the normoxia phase (100% O_2). After 90 min, the perfusate was made hypoxic (100% N_2) and allowed to equilibrate for 10 min prior to the second 1-mg dose of TMTX. After 60 min of hypoxia, the perfusate was reoxygenated, but no further TMTX was given. Values are expressed as mean ± SEM, N = 4.

148 ± 32 min; $P < 0.025$), indicating that the overall hepatic elimination of trimetrexate requires oxygen. This is supported by data from the steady-state experiments (Fig. 2), where trimetrexate concentrations increased from 1.41 ± 0.01 to 3.09 ± 0.45 μg/ml after 1 hr of hypoxia ($P < 0.02$). This hypoxia-induced inhibition of drug elimination has been reported for propranolol [2], theophylline [3], omeprazole [4], and antipyrine [5], which also undergo oxidative metabolism. The reoxygenation phase was important to assess the recoverability of the liver after each episode of hypoxia. Perfusate levels of trimetrexate dropped rapidly in all experiments, indicating a lack of permanent damage to the elimination mechanisms of this drug.

Since the perfusate concentration–time profiles for the metabolites reflect their continuous formation and elimination processes, true half-lives could not be calculated. However, it was still possible to elicit qualitative information from their apparent values. In the bolus dose experiments (Fig. 1), the apparent elimination half-lives after the first dose of trimetrexate were 54 ± 7 and 68 ± 2 min for M_1 and M_2 respectively. However, during hypoxia the concentrations gradually increased, and no elimination was evident for either metabolite. Upon reoxygenation, the elimination mechanisms of both metabolites recovered to a certain extent, yielding apparent half-lives of 70 ± 16 and 105 ± 29 min. The slopes of the metabolite (semilogarithmic) profiles from the first 5 to 30 min in each of the three phases give an indication of their apparent rates of formation. For M_1, these were 0.025, 0.007 and 0.015 for normoxia, hypoxia and recovery, respectively, and the numbers were similar for M_2. This suggests that metabolite production diminished during hypoxia, but that it did tend to recover after reoxygenation.

The metabolite perfusate concentrations for the steady-state studies are included in Fig. 2. During both the normoxic and hypoxic hours of the hypoxia studies, M_1 and M_2 concentrations were similar to their respective control profiles; however, after reoxygenation, M_1 and M_2 levels

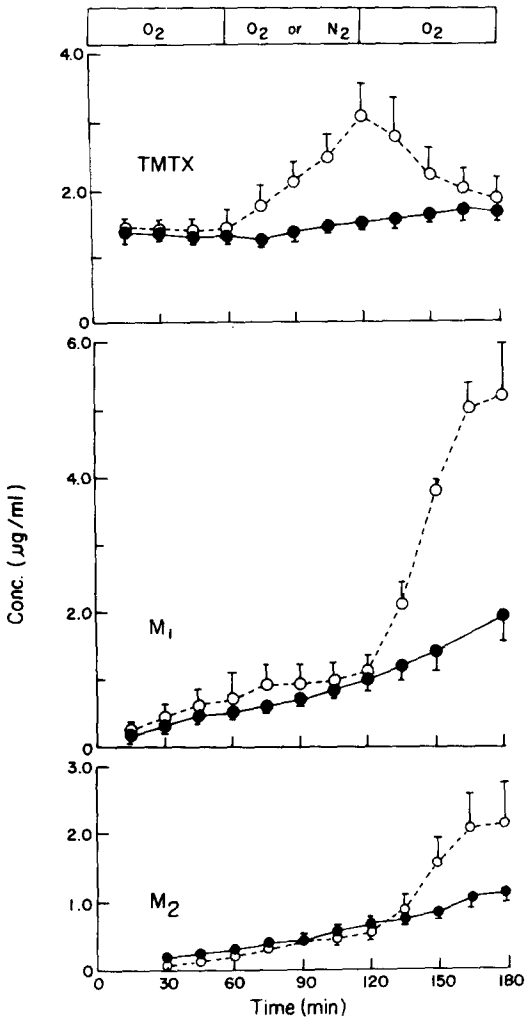

Fig. 2. Perfusate concentration–time profiles of trimetrexate (TMTX, top panel), M_1 (center panel) and M_2 (lower panel) during control (●) and hypoxia (○) steady-state trimetrexate studies. A bolus plus constant infusion of TMTX maintained TMTX levels between 1.4 and 1.6 μg/ml in control studies. The perfusate was equilibrated with N_2 from 60 to 120 min in the hypoxia studies; O_2 was used at all other times. Values are expressed as mean ± SEM, N = 4.

dramatically increased, and were 2- to 3-fold higher than in the control studies at 3 hr. Because most of the trimetrexate is eliminated by metabolism to M_1 or M_2, the inhibition of parent drug elimination during hypoxia must be primarily due to a decrease in metabolite formation. This supports unpublished data from this laboratory which showed that both M_1 and M_2 were O-demethylated products of trimetrexate, resulting therefore from an oxygen-dependent microsomal reaction. The apparent lack of effect of hypoxia on the perfusate metabolite concentrations in the steady-state experiments must be due to a similar degree of inhibition of both the metabolite production and elimination mechanisms. (If hypoxia did not influence metabolite elimination, one would expect a decrease in their concentrations.) This same phenomenon occurred during the steady-state studies with omeprazole, whereby the concentrations of an oxidative metabolite were apparently unchanged during hypoxia [4]. The rapid recovery of trimetrexate metabolism upon reoxygenation probably caused the dramatic increase in perfusate metabolite levels during this phase (Fig. 2): since metabolite elimination occurs primarily via the bile, it is possible that the biliary excretion mechanism is initially unable to compensate for the sudden increase in metabolite production. This slow recovery of metabolite biliary excretion, as well as bile flow, is evident in data from the hypoxia studies (Table 1, period 2b compared with 3a and b).

In summary, using the isolated perfused rat liver, we have shown that the elimination of trimetrexate, and the production and elimination of two metabolites, can be inhibited by hypoxia. Bile flow and biliary excretion of the metabolites were diminished by hypoxia. It is also evident that, for oxidatively produced metabolites which are primarily excreted in the bile, a return to normal oxygenation after a hypoxic episode can lead to sharp increases in perfusate concentrations. Further work using different degrees of hypoxia is necessary to establish the exact oxygen requirements of individual mechanisms and to relate the effects more quantitatively to the clinical situation.

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