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Restriction of hexobarbital metabolism by *t*-butyl hydroperoxide in perfused rat liver

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Hepatic monooxygenation reactions, catalysed by cytochrome P-450, can be supported by organic hydroperoxides as was found with isolated microsomal fractions [1–4]. Further, redox effects of organic hydroperoxides on NADH, NADPH and cytochrome b_5 have been described with such fractions [5–9]. The physiological or toxicological relevance of these effects in more complex experimental systems has so far not been studied extensively.

Experiments with isolated hepatocytes [8] and with isolated perfused liver [10] revealed no effect of added *t*-butyl hydroperoxide on the steady state level of the cytochrome P-450–CO or cytochrome P-450–substrate complexes. In view of the substantial oxidation of NADPH to $NADP^+$ during the reduction of organic hydroperoxides by intact liver [11] the question arises whether added organic hydroperoxide might be capable of supporting cytochrome P-450–dependent drug oxidations in intact cells or organs.

In the present work, the effect of *t*-butyl hydroperoxide on the metabolism of hexobarbital was studied in perfused livers from phenobarbital-pretreated rats.

Materials and methods

Hemoglobin-free liver perfusion. Livers from male Wistar rats, 130–190 g body weight, fed on stock diet (Altromin) and pretreated for at least 7 days with sodium phenobarbital (1 mg per ml of drinking water) were perfused as described previously [12]. The perfusion medium consisted of 115 mM NaCl, 5.9 mM KCl, 1.2 mM $MgCl_2$, 1.2 mM NaH_2PO_4, 1.2 mM Na_2SO_4, 2.5 mM $CaCl_2$, 25 mM $NaHCO_3$ and 1.2 mM L-lactate and 0.3 mM pyruvate (as sodium salts), equilibrated with O_2/CO_2(19/1, v/v). The temperature was maintained at 33°. The perfusion was non-recirculating (open system). Additions were made from stock solutions by means of precision micropumps into the perfusate entering the portal vein.

Assays. The concentration in influent and in effluent perfusate was determined for *t*-butyl hydroperoxide [13], oxygen [12, 13] and hexobarbital ([14]; slightly modified as described in [15]).

Chemicals. t-Butyl hydroperoxide was a gift from Peroxid Chemie, Höllriegelskreuth, München, and sodium hexobarbital was a gift from Bayer, Leverkusen. All other chemicals were from Merck, Darmstadt, or Boehringer, Mannheim.

Results and discussion

Previous experiments had shown that the half-maximal effect for extra O_2 uptake upon infusion of hexobarbital was 59 μM [16]. Therefore, in the present work hexobarbital concentrations of approx. 0.2 mM were employed. As shown in Fig. 1, the uptake of hexobarbital under such condition is about 0.2 μmoles/min per gram of liver; it was 0.21 ± 0.02 μmoles/min per gram (mean ± S.E.M.) in 10 different perfusions. The extra O_2 uptake was 2.01 ± 0.18 μmoles O_2 per μmole of hexobarbital taken up, as determined in 7 different perfusions, similar to our previous results [16]. Since the extra O_2 uptake occurs also in the presence of antimycin A and is suppressed upon addition of 0.12 mM metyrapone [16], it is attributable to the enhanced flux through the monooxygenase system. Thus, it is calculated that 4 μmoles extra NADPH is utilized by the monooxygenase system per μmole of hexobarbital metabolized.

The infusion of *t*-butyl hydroperoxide leads to a substantial decrease of the rate of hexobarbital metabolized (Fig. 1), concomitant with a decrease of O_2 uptake by the liver (not shown). The effect is reversible. The extent of the restriction of hexobarbital metabolism increases with the rate of infusion of the hydroperoxide (Fig. 2). Since the addition of *t*-butyl hydroperoxide in the absence of hexobarbital also leads to a slight decrease in O_2 uptake [11], the inhibitory effect on O_2 uptake in the present experiments may be of composite nature and, therefore, was not analysed further for the present purposes. The addition of *t*-butanol, the product of *t*-butyl hydroperoxide reduction, had no effect on O_2 uptake or on the NAD(P)H-dependent fluorescence even at a high concentration of 2 mM [11].

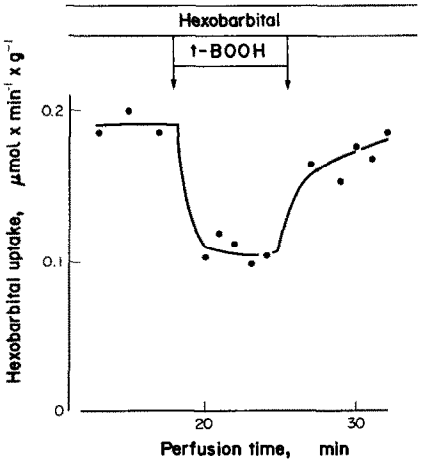

Fig. 1. Reversible inhibition of hexobarbital metabolism by *t*-butyl hydroperoxide in perfused liver. Influent concentrations were: sodium hexobarbital, 0.19 mM; *t*-butyl hydroperoxide (*t*-BOOH), 0.56 mM.

The data shown in Figs. 1 and 2 clearly demonstrate that the hydroperoxide has an inhibitory effect on the cytochrome P-450-linked drug oxidation system in the intact organ. This is interpreted as being due to the substantial oxidation of NADPH to $NADP^+$ during hydroperoxide reduction, an effect of the coupled reactions of GSH peroxidase and GSSG reductase [10, 11, 13]. Under these conditions, the liver is capable of completely reducing the hydroperoxide in a single-pass as shown by the absence of detectable hydroperoxide in the effluent perfusate [13]. Therefore, in a first approximation the rate of infusion of the hydroperoxide shown in Fig. 2 may be equated to the rate of NADPH oxidation by GSSG reductase. Consequently, it can be deduced from Fig. 2 that a 25 per cent inhibition of hexobarbital metabolism is observed at a rate of NADPH utilization by GSSG reductase 1.7-fold the rate elicited by the metabolism of hexobarbital, assuming that the extra O_2 uptake during hexobarbital metabolism is fully accounted for by NADPH utilization (see above). Similarly, the number for 50 per cent inhibition, observed at 3 μmoles hydroperoxide/min per gram, corresponds to 6.3-fold the rate for the monooxygenase reaction (Fig. 2).

Thus, it can be concluded from these experiments that externally added organic hydroperoxide has only a negligible role in supporting drug oxidation at the endoplasmic reticulum in the intact cell and, rather, leads to a net inhibition. The reason is twofold: (i) The steady state concentration of hydroperoxide is kept extremely low by the active GSH peroxidases (Se and non-Se dependent) in the cytosol, and (ii) interference with NADPH supply and concomitant rise in $NADP^+$ by virtue of GSSG reductase activity.

However, that organic hydroperoxide is capable of reaching the membranes of the endoplasmic reticulum to some extent can be deduced from the observation in intact perfused liver and isolated hepatocytes of a substantial oxidation of cytochrome b_5 [8]. A possible role of diversion of electron flow from NADH appears unlikely as the cytosolic free $NADH/NAD^+$ was even slightly increased upon hydroperoxide addition, indicated by an increased lactate/pyruvate ratio [11].

The present work was carried out with externally added hydroperoxide, so that little can be said about endogenously generated hydroperoxides, e.g. lipid hydroperoxides. However, the possibility of a considerable share of endogenous organic hydroperoxides in maintaining drug oxidations in liver can probably be discounted for their extremely low steady state production [17]. In specialized tissues with high rates of lipid hydroperoxide production, cooxidation of xenobiotics can occur. An example is the peroxidatic oxidation of benzo[a]pyrene to a mixture of quinones in sheep seminal vesicles, utilizing prostaglandin G_2 as the hydroperoxide substrate [18], a reaction independent of electron flow through the cytochrome P-450 system.

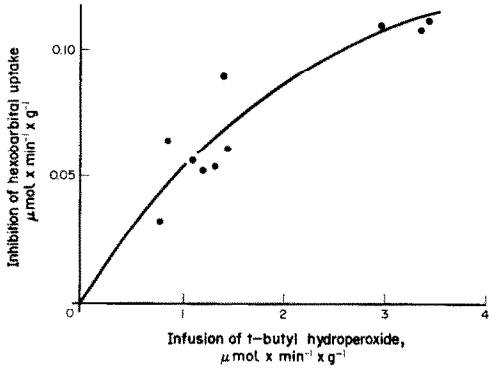

Fig. 2. Dependence of the inhibition of hexobarbital uptake on the rate of infusion of *t*-butyl hydroperoxide. Each point represents the steady state value obtained in a separate perfusion.

In summary, in isolated hemoglobin-free perfused rat liver, the metabolism of hexobarbital was reversibly decreased upon infusion of *t*-butyl hydroperoxide. Thus, although hydroperoxide-supported drug metabolism can be observed with microsomal fractions *in vitro*, there is a restriction of cytochrome P-450-dependent drug oxidation by added hydroperoxide in the intact organ. The effect is attributed to the maintenance of extremely low intracellular hydroperoxide concentrations and to the marked oxidation of NADPH to $NADP^+$ associated with hydroperoxide reduction, catalysed by GSH peroxidases and GSSG reductase, respectively.

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