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DRUG-INDUCED LIPID PEROXIDATION IN MICE—V

ETHANE PRODUCTION AND GLUTATHIONE RELEASE IN THE ISOLATED LIVER UPON PERFUSION WITH ACETAMINOPHEN

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Abstract—Isolated liver from phenobarbital-induced male mice was perfused using infusions of cytochrome *c* pulses as quality control of the system. Livers spontaneously evolved 1.1 pmoles ethane g^{-1} liver min^{-1}, exogenous pentane disappeared with 0.6 pmoles g^{-1} min^{-1}. Infusion of 0.26 mmoles/l. $FeCl_2$ led to immediate ethane production followed later on by lactate dehydrogenase release from the liver. Infusion of acetaminophen resulted in hepatic ethane production at drug concentrations greater than 0.1 mmoles/l. A maximum effect was observed at 2 mmoles/l. of acetaminophen infused while higher concentrations, up to 10 mmoles/l. delayed ethane release although they enhanced the rate of glutathione depletion. This glutathione efflux decreased from 12 nmoles/g^{-1} min^{-1} observed after perfusion of medium alone to 2.9 nmoles/g^{-1} min^{-1} when AAP was infused. The slope of the pseudo first order depletion kinetics depended on the acetaminophen concentrations. This glutathione release represents the perisinusoidal portion of the total efflux measured here independently from the biliary secretion. The results show that in agreement with the *in vivo* findings acute intoxication of liver with high doses of this drug lead to lipid peroxidation, while *in vitro* an apparent antioxidative effect was measured. The implications for drug screening are probably important.

In the previous communications of this series, we showed that high doses of the mild analgesic acetaminophen (AAP, paracetamol, 4-hydroxyacetanilide) led *in vivo* in the mouse to a dose-dependent ethane exhalation which is considered to be an index of lipid peroxidation [1]. The deleterious effects of the drug overdosage were dependent on the turnover of the microsomal mono-oxygenase system as well as on the hepatic glutathione content and were drastically exacerbated in selenium-deficient mice [2]. Liposomally entrapped GSH following intravenous administration protected the animals completely against drug-induced liver necrosis and prevented ethane exhalation [3] while free GSH did not do so even though it led to an apparently similar increase in hepatic GSH content [4]. In liver cell homogenates or microsomal suspensions, however, opposite effects of acetaminophen were observed, i.e. the drug quenched NADPH- as well as Fe/ADP-induced microsomal LPO in a concentration-dependent manner [5]. It is not clear at which level of hepatic cellular organization the LPO-inducing ability of AAP becomes an apparently antioxidative effect. The perfused mouse liver was employed here to resolve the apparent paradox resulting from the above-mentioned studies, particularly as important considerations arise from these concerning drug pre-screening and the legalty of experiments using live animals. Using this sytem we confirm the oxidative potential of the drug, which is misleadingly undetectable *in vitro*.

Abbreviations: LPO, lipid peroxidation; AAP, acetaminophen.

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MATERIALS AND METHODS

Male albino mice (weight 30–35 g) were raised on a stock diet (C 1018, Altromin, Lage, F.R.G.). They received 0.1% phenobarbital for 7 days in the drinking water and were fed a 10% liquid sucrose containing diet prior to the experiments as described [2–4]. Excision and preparation of the livers was carried out after opening the abdomen under mild ether anesthesia as follows: the bile duct was opened, the mesenteric vein was canulated by inserting a 0.5 mm i.d. Teflon catheter (Abbocath T-22 G) and perfused at a flow rate of 4–5 ml/min by Krebs–Henseleit medium at 37° (118 mmoles/l. NaCl, 4.8 mmoles/l. KCl, 1.2 mmoles/l. KH_2PO_4, 1.2 mmoles/l. $MgSO_4$, 2.5 mmoles/l. $CaCl_2$, 25 mmoles/l. $NaHCO_3$ equilibrated with O_2/CO_2, 19:1, v/v; pH 7.4). It is important to note that the gall bladder remained intact. The perfusate ran off the lower part of the inferior vena cava via an inserted 1.5 mm i.d. polyvinyl tube. The liver was removed and hooked at the diaphragm into a 3 × 3 × 5 cm Plexiglass chamber which had a vol. of 43 ml. This collecting chamber for evolved alkanes was equipped with gas-tight in- and outlets for the perfusate; it also had two ports for flushing with gas and for gas sampling, and a funnel-formed bottom which was immersed into a beaker filled with medium at all times which allowed an isobaric gas sampling by reflux of fluid during the aspiration phase. The oxygen concentration was monitored in the effluent perfusage by using a Clarke-type electrode and maintained at about 0.2 mmoles/l. Samples of 5 ml of gas were taken from the chamber surrounding the isolated liver via a three-way port and analysed gas-

chromatographically on an isothermally run Porasil C column at 70° using a Carlo Erba Fractovap 2151 AC instrument [7]. The system was calibrated by flushing the gas chamber which contained a liver with a mixture of 0.83 vpm ethane and 0.5 vpm *n*-pentane in N_2 supplied by Messer-Griesheim, F.R.G. The quality of the flow conditions within the livers was checked according to ref. [8] by injecting a pulse of cytochrome *c* (5 μl, 0.2 mg) into the perfusate as close as possible to the portal inlet and by measuring the elution profile by passing the effluent perfusate through a flow photometer (Gilson Spectrochrome M, 405 nm).

Lactate dehydrogenase was determined according to ref. [9] and the sum of reduced plus oxidized glutathione (GSH + 2 GSSG) by the cyclic method using Ellman's reagent and glutathione reductase [10].

Chemicals. Triethanolamine and bovine serum albumine were from Sigma, acetaminophen from Ega-Chemicals; NADH, NADPH, beef heart cytochrome *c*, glutathione reductase, lactate dehydrogenase and GSH were obtained from Boehringer, Mannheim. All other chemicals were analytical grade substances from Merck, Darmstadt.

RESULTS

A cytochrome *c* pulse was washed out of the perfused mouse livers within 20 sec of injection at the beginning of the perfusion with a symmetrical elution profile. After a perfusion for more than 150 min without any additions to the perfusate, however, the elution lasted up to 35 sec and its profile became asymmetric. These results show that this mouse liver perfusion system meets the quality requirements of the isolated perfused rat liver [8, 11].

The liver was then perfused in a premixed atmosphere of 28.1 pmoles/ml pentane and 37.0 pmoles/ml ethane and the change in hydrocarbon concentration with time was measured against a control without liver (Fig. 1). The pentane concentration decreased biphasically compared to the control. After an initial fall within the first 50 min, which we attribute to a distribution phase, an apparently linear decrease with a rate of 0.6 pmoles/g·min was observed. In contrast, the ethane concentration showed an apparently linear net increase from the beginning of the perfusion with a rate of 1.1 pmoles/g·min^{-1}.

If the liver was subjected to full oxidative stress by infusion of 0.26 mmoles/l. $FeCl_2$, maximum ethane production was reached 8 min after initiating the infusion (Fig. 2). During further infusion the rate of ethane release decreased while massive amounts of lactate dehydrogenase maintained leaking out. The total amount of ethane production was 340 pmoles/g.

Fig. 3 shows a perfusion experiment with a high concentration of AAP and Fig. 4 the corresponding control perfusion with medium alone. While the control exhibited a constant glutathione release of about 12 GSH nano-equivalents/g·min and a basal ethane production of 0.4–0.6 pmoles/g·min, the infusion of APP at a steady-state concentration of 9.3 mmoles/l. led to an immediate decrease of the

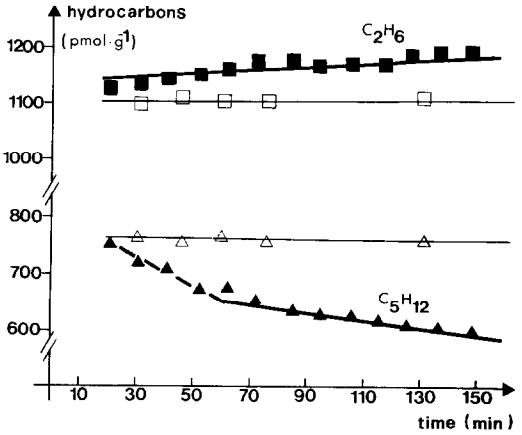

Fig. 1. Perfusion of phenobarbital-induced mouse liver with medium alone in a premixed gas atmosphere of 28.1 pmoles/ml pentane (triangles) and 37 pmoles/ml ethane (squares). Control without liver: open symbols. Data in pmoles hydrocarbon content of the perfusion chamber per gram liver wet weight.

glutathione release to about 20% of the initial rate. The glutathione content of the whole liver, which was determined to be 16 μmoles at the beginning of the perfusion, was found to drop in analogous experiments to 3.5 μmoles after perfusion with 9.3 μmoles/l. AAP for 60 min and to 3.0 μmoles after 110 min.

At a period of 30 min after the beginning of the AAP infusion (Fig. 3), the ethane production rate increased and continued doing so until the end of the experiment where it reached about four times the basal rate. In contrast to the control experiment, the oxygen consumption became less after 90 min of perfusion, this being followed by a drastic increase in lactate dehydrogease release after 100 min. (Basal release was less than 10 mU/g·min, Fig. 2.) The experiment demonstrates that the infusion of AAP induces lipid peroxidation in mouse liver before loss of cellular integrity is observed in the form of lactate dehydrogenase release. The concentration dependence of this AAP-induced lipid peroxidation was then investigated. Below a concentration of 0.01 mmoles/l. of AAP in the perfusion medium only a low, spontaneous ethane release occurred, while concentrations of AAP from 0.07 to 10 mmoles/l. led throughout to a greater hydrocarbon evolution than in the controls. The shape of the curves showing GSH + 2 GSSG release with time, following AAP infusion under conditions which gave a minimum steady state glutathione efflux of 2.9 ± 1.0 nmoles/l·g·min (n = 13) were different at the various AAP concentrations used.

Figure 5(A) demonstrates that the pseudo first order rate constant of this AAP-induced decrease in glutathione efflux becomes greater with increasing AAP concentrations. At low AAP concentrations GSH depletion was slow. The inset in Fig. 5(A) illustrates how these rate constants were determined graphically from the slopes of semilogarithmic plots. It further shows that at the highest AAP concentration used, GSH depletion is faster in livers of

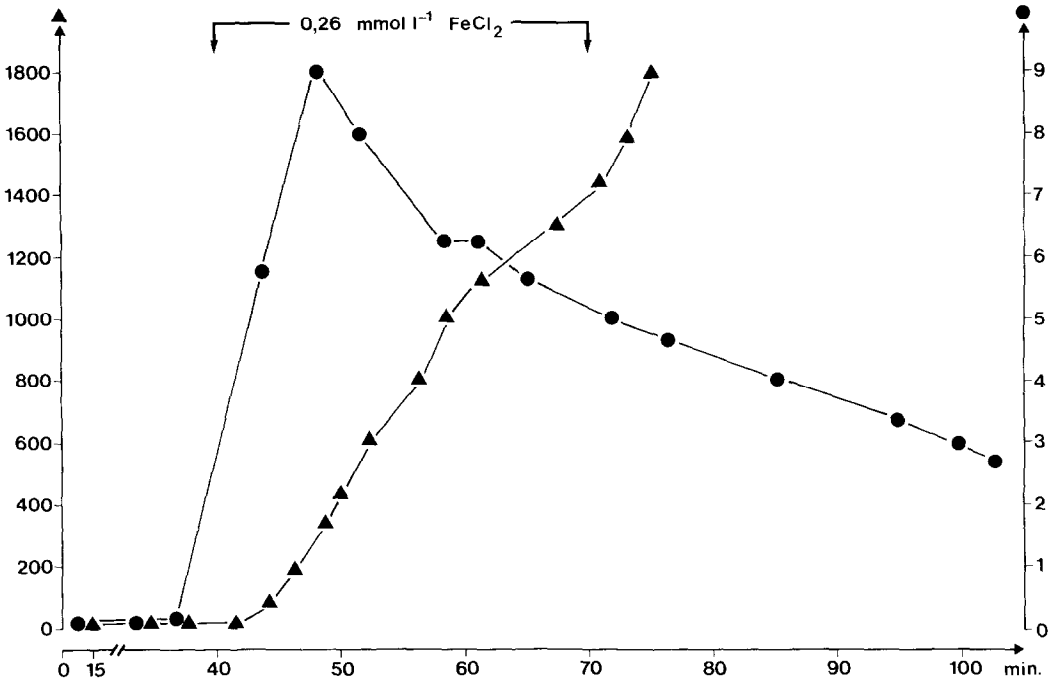

Fig. 2. Ethane (●, pmoles/g·min) and lactate dehydrogenase (▲, mU/g·min) release from isolated perfused phenobarbital-induced mouse liver upon infusion of 0.26 mmoles/l. $FeCl_2$. A freshly anaerobically prepared solution of 26 mmoles/l. $FeCl_2$ was infused immediately prior to the liver at 1% of the flow of the medium by a separate pump.

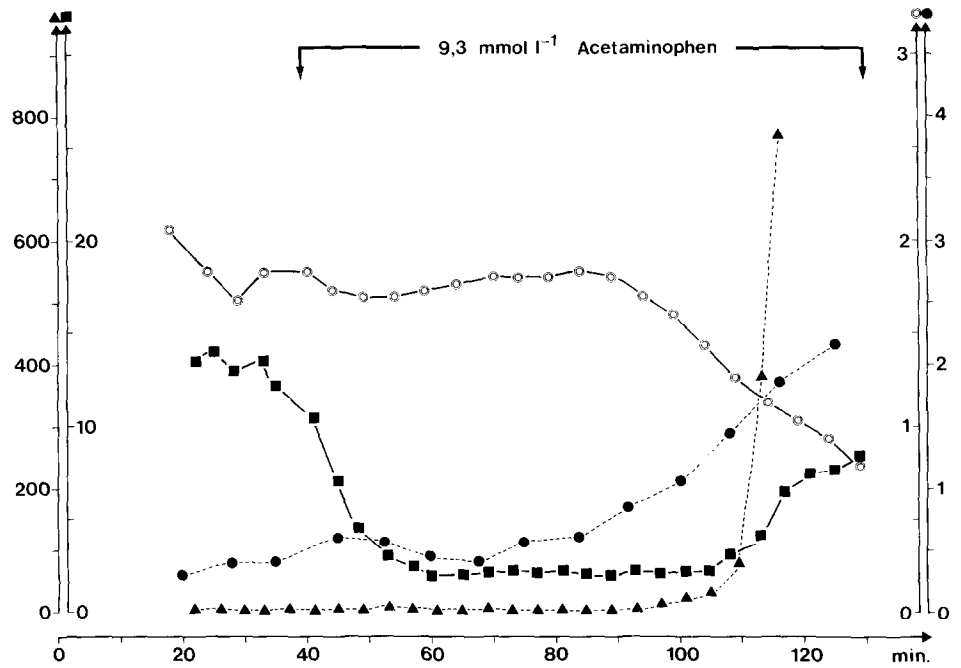

Fig. 3. Ethane evolution (●, pmoles/g·min), glutathione efflux (■, nano-equivalents/g·min), lactate dehydrogenase (LDH) release (▲, mU/g·min) and oxygen utilization (○, μmoles/g·min) of isolated perfused phenobarbital-induced mouse liver following infusion of a steady state concentration of 9.3 mmoles/l. acetaminophen.

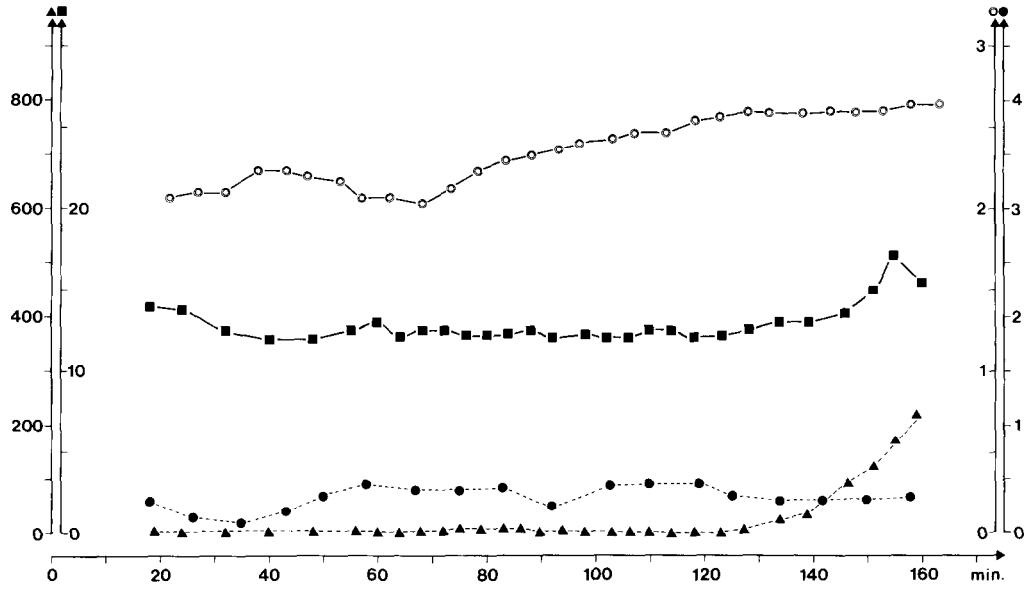

Fig. 4. Spontaneous ethane production (●, pmoles/g·min), glutathione efflux (■, nano-equivalents/g·min), lactate dehydrogenase release (▲, mU/g·min) and oxygen consumption (○, μmoles/g·min) of isolated mouse liver perfused with 4.1 ml/g·min Krebs–Henseleit medium, pH 7.4 at 37°.

phenobarbital-induced animals. One consequence of the dependence of the GSH + 2 GSSH efflux rate on AAP was that, at low AAP levels, the time periods preceding the onset of release of ethane and lactate dehydrogenase became closer to those observed in the absence of the drug. After the minimum glutathione efflux had been reached, however, similar maximum ethane evolution rates were measured at all AAP concentrations. Thus, in order to compare differences in the concentration dependence of AAP-induced LPO the ethane release rate after 50 min (i.e. four times the longest apparent $t_{1/2}$ of the various depletion phases) was chosen as an appropriate parameter (see Fig. 5B). The graph in Fig. 5(B) shows that infusion of about 2 mmoles/l. AAP results in the earliest ethane release while higher concentrations delayed the start of hydrocarbon production despite their ability to still accelerate the glutathione depletion phase. Interestingly, at 1–2 nmoles/l. AAP, where the highest rates of ethane evolution were reached after 50 min of infusion, a total of 300–350 pmoles/g ethane was measured. Comparing these data with those of Fig. 2, we conclude that this total amount of ethane might represent the maximum released from a single liver.

DISCUSSION

Methodology

This work shows that modified conventional techniques allow mouse liver to be perfused for up to 2 hr with maintenance of cell integrity. Similar oxygen consumption and glutathione release rates to those in isolated perfused rat liver were measured [11]. As observed for the rat [6] a linear net production of ethane was measured from the beginning of the perfusion. For pentane, a distribution phase was observed followed by a pseudo first order removal of the gas by the liver. This is interpreted as a diffusion-limited metabolism in this organ, as rat *in vivo* data [14] and experiments with perfused rat liver [17] and with rat liver microsomes [13] suggest. The physical distribution of ethane between gas phase and perfusate was such that at the flow rate used here 25% was found consistently in the perfusion chamber gas and 75% was recovered in the perfusate.

It should be noted that, in contrast to the rat, the mouse's gall bladder, which had an open cystic duct, remained intact in our preparation. Thus, a basic difference between the systems for those two species is that in our perfusion experiments bile did not reach the perfusate. This means that changes in intrahepatic bile flow would not affect metabolite concentrations measured in the perfusate.

Metabolism

The application of the liver perfusion technique to AAP-induced ethane release shows that, in mouse liver, infusion of the drug leads to lipid peroxidation. In quantitative terms, a maximal total ethane production is evoked by the drug which is not even exceeded by the infusion of ferrous chloride. When physical distribution is included in the calculation, a maximum ethane evolution rate of 1.1 nmoles/kg body weight·min is obtained. This matches remarkably well the ethane exhalation rates of phenobarbital-induced mice treated by i.p. injection of 300 mg AAP/kg, i.e. 1.25 nmoles/kg·min [2]. The data indicate that, under conditions of acute intoxication, the liver actually accounts fully for the ethane exhaled and that in contrast to pentane the *in vivo* catabolism of this hydrocarbon is negligible.

The infusion of AAP into mouse liver led to a glutathione release into the perfusate (not including biliary release) which had the following characteristics: (a) first order kinetics; (b) slope dependence of the concentration on AAP infused; (c) less release

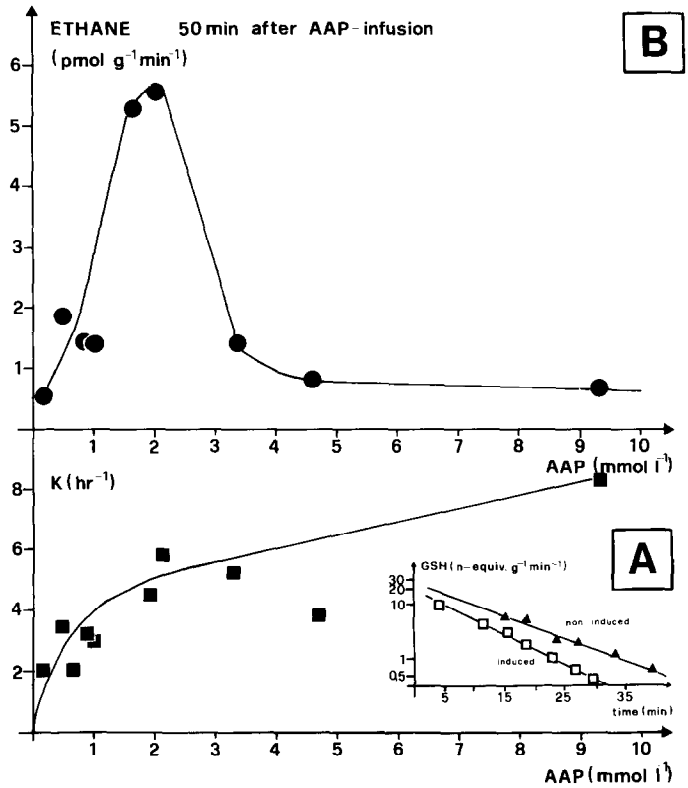

Fig. 5.(A) Relation of the apparent first order rate constant of GSH + 2 GSSG efflux into the perfusate from isolated perfused phenobarbital-induced mouse liver to the concentration of acetaminophen in the infusate. The inset shows the data for 9.3 mmoles/l. acetaminophen in phenobarbital-induced (□) and non-induced control animals (▲). (B) Ethane production rates of isolated perfused phenobarbital-induced mouse liver upon infusion of different concentrations of acetaminophen. The rates were measured 50 min after the beginning of drug infusion (cf. text and Figs. 3 and 4).

in non-induced control livers; and (d) within the range tolerated by the perfused liver it was independent of the flow. Thus, in analogy to the *in vivo* situation, there is good reason to conclude that the actual glutathione efflux rate reflects the intracellular available glutathione concentration over a gradient of three orders of magnitude (8 mmoles/l. inside, e.g. ref. 2–4 and 3.3–4.8 μmoles/l. in the perfusate in the absence of drug). In other words, the observed decreased efflux upon AAP infusion has to be regarded as a consequence of the phase II metabolism of the drug consuming GSH. Actually, at the end of the perfusions 4.1 ± 1.3 nmoles GSH equivalents per mg liver protein were found ($n = 5$) compared to 24 ± 2 ($n = 3$) nmoles/mg in non-perfused livers. In all of the experiments, ethane release preceded lactate dehydrogenase release by 40 ± 9 min ($n = 13$) suggesting that lipid peroxidation, and the subsequent hydrocarbon production, are not a consequence of cellular destruction but rather an early causal event in cell death induced by chemical lesions. We believe, however, that LPO is not sufficient to account for the complexity of cell death unless other feasible mechanisms coincide lethally, e.g. chemical modification of macromolecules or lipids [15], and collapse of ion gradients or cofactor redox states [16]. Thus, it appears that metabolism of AAP induces massive lipid peroxidation in mouse liver. Since the drug caused the opposite effect to this LPO-inducing action in isolated mouse hepatotcytes [12], liver homogenates or microsomal suspensions [5], it must be concluded that the structural organization of the organ decisively contributes to drug metabolism in a manner which is not clear at present.

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