Biochemical Pharmacology, Vol. 36, No. 18, pp. 2973–2977, 1987.
Printed in Great Britain.

0006–2952/87 $3.00 + 0.00

ENHANCEMENT OF HYPOXIC LIVER DAMAGE BY ETHANOL

INVOLVEMENT OF XANTHINE OXIDASE AND THE ROLE OF GLYCOLYSIS

M. YOUNES and O. STRUBELT

Institut für Toxikologie, Medizinische Universität zu Lübeck, Ratzeburger Allee 160, D-2400 Lübeck, Federal Republic of Germany

(*Received* 16 *December* 1986; *accepted* 16 *March* 1987)

Abstract—Using isolated hemoglobin-free perfused rat livers we investigated the hepatotoxic effects of hypoxia, ethanol or the combination of both. Hypoxia only (90 min) led to a weak toxicity as evidenced by the efflux of the enzymes glutamate-pyruvate-transaminase (GPT) and sorbitol dehydrogenase (SDH). This toxic effect was slightly higher in livers treated with ethanol (3 g/l) under normoxic conditions. Ethanol added under hypoxic conditions, however, showed a strong hepatotoxic effect. Under hypoxic conditions, lactate + pyruvate production was increased fivefold over control, indicating that glycolysis was more effectively undergone as main source of energy. Addition of ethanol suppressed this effect, indicating that ethanol inhibited glycolysis. These results indicate that ethanol potentiates hypoxic liver damage by inhibiting the main metabolic pathway yielding ATP under low oxygen tension resulting in a severe energy deficit. Allopurinol (100 mg/l) inhibited the toxic effects seen with ethanol + hypoxia. Also, the inhibitory action of ethanol on glycolysis was antagonized. Our results are consistent with the following model: hypoxia converts NAD-dependent xanthine dehydrogenase (XD) into the oxygen-dependent xanthine oxidase (XO). Due to hypoxia and ethanol, purine metabolites *and* acetaldehyde accumulate and are metabolized via XO. This process leads to the production of oxygen radicals which most probably mediate both the inhibition of glycolysis and the direct toxic effects towards liver cells.

Ethanol treatment was shown to increase the rate of hepatic oxygen consumption in liver slices [1, 2], as well as in perfused livers [3–5]. This observation led to the hypothesis that a relative hypoxia might be involved in the pathogenesis of alcoholic liver injury [2]. Indeed, ethanol treatment selectively increases the oxygen uptake of those parts of the liver which are prone to ethanol-induced lesions, namely the centrilobular zone [5]. Furthermore, centrilobular necrosis was shown to occur in ethanol-fed rats after exposure to a brief period of hypoxia or after hematocrit reduction by bleeding [6–8]. However, the whole organism was exposed to hypoxia in these experiments *in vivo*, and extrahepatic effects (e.g. catecholamine liberation), thus, might have been implicated in the hepatotoxic damage. In order to exclude these confounding factors, we investigated the interrelations between ethanol and hypoxia in the isolated perfused rat liver. In this model, ethanol-induced acute toxicity is evident by an enhanced release of enzymes into the perfusate [9–11].

MATERIALS AND METHODS

Animals. Male Wistar rats (conventional animals, 320–380 g; breeder: Winkelmann, Borchen) were used throughout. They had free access to feed (Altromin® pellets) and tap water until use.

Liver perfusion. Removal of the liver and its connection to a recirculating perfusion system was performed as described previously [12]. The perfusion medium consisted of 250 ml Krebs–Henseleit buffer, pH 7.4 (per l: 118 mmol NaCl, 6 mmol KCl, 1.1 mmol $MgSO_4$, 1.2 mmol KH_2PO_4, 25 mmol $NaHCO_3$). $CaCl_2$ (1.25 mmol/l) was added to the prewarmed perfusion medium (37°) immediately before starting the perfusion. The perfusion medium was continuously gassed with carbogen (95% O_2, 5% CO_2), the partial pressure of O_2 amounting to 600 mm Hg. In order to produce hypoxic conditions in the appropriate experiments, carbogen was replaced by a mixture of 95% N_2 and 5% CO_2 after a 30-min equilibration period. Sodium taurocholate (26.7 g/l) was infused into the perfusate at a rate of 12 ml/hr to stimulate bile secretion. Oxygen consumption of the isolated liver 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). For further details see [12]. Ethanol was added to the perfusion medium at a concentration of 3 g/l 30 min after the start of the respective experiment. Perfusion was continued for another 90 min.

Biochemical determinations. The activities of glutamic-pyruvic-transaminase (GPT) and sorbitol dehydrogenase (SDH) as well as the concentrations of glucose, lactate and pyruvate in the perfusates were assayed using commercial kits of Boehringer Mannheim. Ca^{2+} concentrations in the liver were measured colorimetrically following acid extraction also with reagent kits of Boehringer Mannheim. Total glutathione was determined according to Brehe

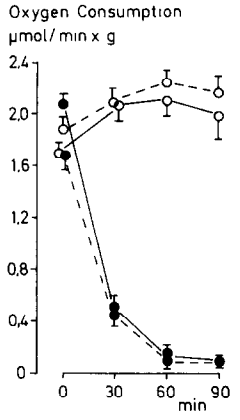

Fig. 1. Oxygen consumption by the isolated perfused rat liver: O---O, Normoxic conditions, no ethanol; O——O, normoxic conditions +3 g/l ethanol; ●---●, hypoxic conditions, no ethanol; ●——●, hypoxic conditions +3 g/l ethanol.

and Burch [13]; oxidized glutathione was estimated by the same procedure after blocking reduced glutathione (GSH) with 2-vinylpyridine [14].

The concentrations of ethanol and acetaldehyde in the perfusate were determined by means of gas chromatography as described previously [11].

Statistics. Means ± SEM were calculated in the usual way. The difference between two means was checked with Dunnett's *t*-test [15] taking P = 0.05 as the limit of significance.

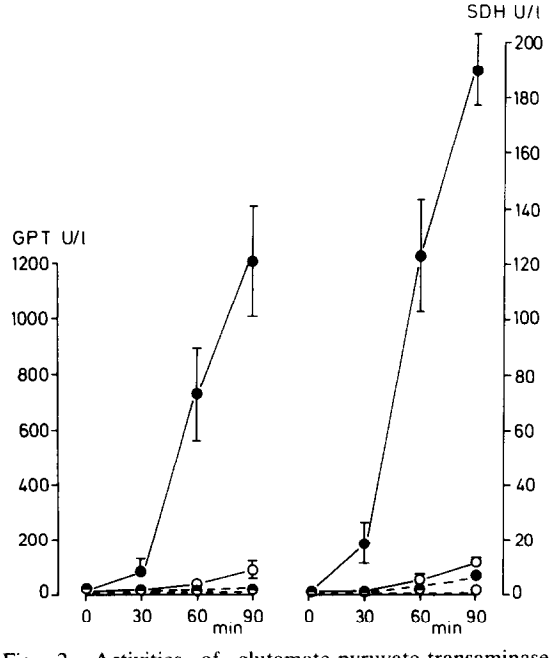

Fig. 2. Activities of glutamate-pyruvate-transaminase (GPT) and sorbitol dehydrogenase (SDH) released from the isolated perfused rat liver: O---O, Normoxic conditions, no ethanol; O——O, normoxic conditions +3 g/l ethanol; ●---●, hypoxic conditions, no ethanol; ●——●, hypoxic conditions + 3 g/l ethanol.

RESULTS

Oxygen consumption following hypoxia and/or ethanol treatment

In control experiments run under normoxic conditions, the partial pressure of oxygen varied from 523 to 626 mm Hg. Under these conditions, oxygen consumption over the whole perfusion period valued around 2 μmol/min g liver (Fig. 1). Addition of ethanol had no effect on this parameter. Replacement of carbogen by a mixture of N_2 (95%) and CO_2 (5%) lowered the oxygen partial pressure of the influent perfusate to 27–66 mm Hg. As a consequence, oxygen consumption by the perfused liver decreased drastically with time, reaching a value of 0.1 μmol/min g liver, i.e. only 5% of the amount of oxygen utilized under normoxic conditions (Fig. 1). Under these conditions, also, addition of ethanol had no effect on this parameter (Fig. 1).

Hepatotoxic effects of hypoxia and ethanol

Damage to the isolated perfused livers was assayed by measuring the efflux of the enzymes GPT and SDH into the perfusate (Fig. 2). Hypoxia alone led to a weak increase in the enzyme concentrations of the perfusate. At the end of the experiment (90 min after the onset of hypoxia), GPT content was 1.9-fold and SDH-content 6.7-fold higher than in control normoxic livers (Fig. 2). Application of ethanol under normoxic conditions resulted in a slightly higher increase of the amounts of GPT and SDH released (8.7- and 10.4-fold, respectively). Combination of ethanol treatment and hypoxia showed a strong hepatotoxic effect: the release of GPT was elevated 111-fold, that of SDH 174-fold over control values at the end of the observation period (Fig. 2).

Neither hypoxia nor ethanol (or the combination of both) had any effect on the glucose concentrations of the perfusate (Table 1). As expected, hypoxia alone resulted in a clear increase in lactate content; as a consequence, lactate + pyruvate production was also increased (Table 1) indicating an elevated level of glycolysis [16]. Addition of ethanol to livers perfused under hypoxic conditions suppressed this effect in accordance with the inhibitory effect of ethanol on glycolysis described by Yuki and Thurman [17]. With ethanol given under normoxic conditions no such inhibition of glycolysis was evident (Table 1). The lactate–pyruvate ratio was elevated by either hypoxia or ethanol, and, more so, by both treatments (Table 1).

While ethanol only tended to reduce the hepatic content of GSH after 90 min (non-significant reduction, Table 2), hypoxia alone resulted in a 33.3% decrease of GSH concentration. Application of ethanol to livers perfused under hypoxic conditions led to a more pronounced GSH depletion by 55.9% of the value seen in controls (Table 2). No significant differences in the content of oxidized glutathione (GSSG) was seen by either treatment (Table 2). At the end of the experiment, the Ca^{2+}-content of control normoxic livers valued 1.46 μmol/g wet weight. Ethanol only had no effect on this parameter. By way of contrast, hypoxia alone led to a 2-fold and hypoxia + ethanol to a 2.3-fold elevated hepatic Ca^{2+}-concentrations (Table 2).

Table 1. Concentrations of glucose, lactate and pyruvate ($\bar{x} \pm$ SEM) in the perfusate at the end of the experiments (i.e. 90 min after administration of 3 g/l ethanol and/or the onset of hypoxia) and the effect of allopurinol (100 mg/l at zero time point, i.e. 30 min prior to ethanol addition)

Group	Hypoxia	Ethanol	N	Glucose (mmol/l)	Lactate (mmol/l)	Pyruvate (μmol/l)	Lactate + pyruvate (mmol/l)	Lactate/pyruvate
1	−	−	8	4.47 ± 0.48	0.94 ± 0.13	160 ± 18	1.10 ± 0.14	5.8 ± 0.4
2	+	−	5	5.49 ± 0.74	5.47 ± 0.95*	40 ± 7*	5.51 ± 0.86*	140.0 ± 20.3*
3	−	+	4	5.14 ± 0.61	1.02 ± 0.14	23 ± 4*	1.05 ± 0.14	47.9 ± 5.6*†
4	+	+	5	5.73 ± 0.89	1.43 ± 0.34	10 ± 3	1.44 ± 0.30	334.0 ± 226.2*
+allopurinol:								
5	+	+	6	6.98 ± 1.15	3.75 ± 0.72*‡	11 ± 2*	3.78 ± 0.66*‡	445 ± 125*

* Statistically significant difference ($P < 0.05$) as compared to group 1.
† Statistically significant difference ($P < 0.05$) as compared to group 3.
‡ Statistically significant difference ($P < 0.05$) as compared to group 4.

Effect of hypoxia on alcohol metabolism

Alcohol metabolism was inhibited under hypoxic conditions. Ninety min after addition of 3 g/l ethanol, 0.94 g/l were metabolized under normoxic conditions, whereas under hypoxic conditions only 0.35 g/l disappeared (Fig. 3). However, acetaldehyde production was not significantly different between normoxic and hypoxic livers (Fig. 3).

Effect of allopurinol on alcohol-induced hepatotoxicity

The xanthine oxidase inhibitor allopurinol was applied at zero time-point in a concentration of 100 mg/l in the experimental set-up leading to maximal liver damage, i.e. in ethanol-treated hypoxic livers. As a consequence, ethanol-induced release of GPT and SDH was clearly inhibited (Table 3). In addition, the production of lactate and pyruvate was elevated, indicating that allopurinol reversed the inhibitory action of ethanol on glycolysis in hypoxic livers (Table 1). Also, allopurinol antagonized the depletion of hepatic glutathione and the elevation of liver Ca^{2+}-content seen with hypoxia and ethanol (Table 2).

DISCUSSION

In our model, ethanol was shown to induce a moderate toxicity towards the isolated perfused liver as evidenced by an increased release of hepatic enzymes, in accordance with previous findings [9–11]. While hypoxia by itself led only to a minor increase in the amounts of enzymes released, the combination of both, hypoxia and ethanol, resulted in a marked hepatotoxicity. Such an effect of ethanol on livers subjected directly to hypoxia has not—to our knowledge—been described previously. This enhanced hepatotoxicity occurred despite an even lower metabolic degradation rate of ethanol itself.

Under hypoxic conditions, the main source of energy is anaerobic glycolysis. This is evident in our experiments by a 5-fold increase in the concentrations of lactate and pyruvate in the perfusate of hypoxic livers. Though the energy yield due to glycolysis is low, it seems to still allow for the basic functions of liver cells to take place. Consequently, only a weak toxicity was evident due to hypoxia only: a slight increase in the released amounts of enzymes, an increased Ca^{2+}- and a decreased glutathione-content.

Table 2. Glutathione (GSH and GSSG) and Ca^{2+}-content ($\bar{x} \pm$ SEM) of the isolated perfused rat livers at the end of the experiments (i.e. 90 min after administration of 3 g/l ethanol and/or after the onset of hypoxia) and the effect of allopurinol (100 mg/l at zero time-point, i.e. 30 min prior to ethanol addition)

Group	Hypoxia	Ethanol	N	GSH (μmol/g)	GSSG (μmol/g)	Ca^{2+} (μmol/g)
1	−	−	8	6.28 ± 0.24	0.11 ± 0.01	1.46 ± 0.12
2	+	−	5	4.19 ± 0.90*	0.13 ± 0.04	2.95 ± 1.09
3	−	+	4	5.72 ± 0.65	0.23 ± 0.12	1.36 ± 0.09
4	+	+	5	2.77 ± 0.72*†	0.10 ± 0.02	3.37 ± 1.48*†
+allopurinol:						
5	+	+	6	5.03 ± 0.07*‡	0.08 ± 0.01	1.58 ± 0.28‡

* Statistically significant difference ($P < 0.05$) as compared to group 1.
† Statistically significant difference ($P < 0.05$) as compared to group 3.
‡ Statistically significant difference ($P < 0.05$) as compared to group 4.

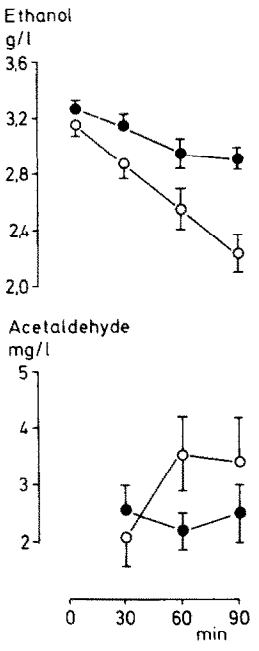

Fig. 3. Concentrations of ethanol and acetaldehyde in the perfusate upon application of 3 g/l ethanol: ○——○, Under normoxic conditions; ●——●, under hypoxic conditions.

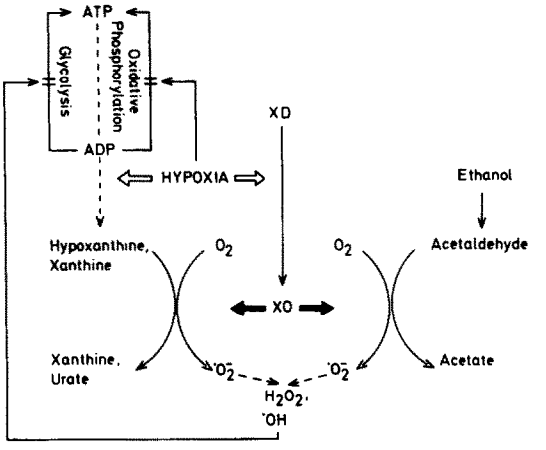

Fig. 4. Schematic representation of the suggested mechanism for alcohol-potentiation of hypoxic liver injury. XD = Xanthine dehydrogenase; XO = xanthine oxidase; ◊ = activation; ◗ = catalysis; →= = Inhibition.

In the presence of ethanol, glycolysis was reported to be inhibited [16]. In our experiments, lactate + pyruvate levels were not elevated after ethanol treatment under normoxic conditions. This lack of inhibitory effect on glycolysis is most probably due to the fact that in the presence of oxygen the main source of energy is oxidative phosphorylation and not anaerobic glycolysis. Addition of ethanol to the liver perfusion system under hypoxia, however, resulted in a clear inhibition of glycolysis: the hypoxia-induced elevation of lactate + pyruvate levels was almost totally abolished. Consequently, marked hepatotoxicity was evident, as ethanol blocked the only source of energy present under hypoxic conditions. Thus, the presence of ethanol under hypoxia results in an energy deficit and in an aggravation of the acute toxic effects of hypoxia towards the liver. This is of practical relevance, as acute ethanol toxicity might be more pronounced under hypoxic conditions due to changes in some hemodynamic parameters.

From another point of view, low oxygen tension is known to stimulate the conversion of the NAD-dependent xanthine dehydrogenase, which converts purine metabolites into uric acid, into the oxygen-dependent xanthine oxidase [18]. Xanthine oxidase is capable of metabolizing both, the purine metabolites which accumulate under hypoxic conditions and acetaldehyde which is formed from ethanol [19]. As oxygen—which is still present in sufficient trace amounts under hypoxic conditions (pO_2 of 27–66 mm Hg in our experiments)—is the electron acceptor in this reaction, the consequence will be the generation of oxygen radicals (superoxide, H_2O_2, hydroxyl radicals) leading to oxidative stress. The combination of hypoxia and ethanol creates a situation under which oxygen radical production is enhanced: firstly, xanthine oxidase is activated, secondly, purine metabolites are formed at higher rates as glycolysis is also inhibited, and thirdly, acetaldehyde is formed from ethanol. Consequently, more substrate for the xanthine oxidase reaction is present and the amount of reactive species produced

Table 3. Effect of inhibition of xanthine oxidase by allopurinol (100 mg/l given at the start of the experiment) on the release of enzymes (GPT, SDH) from the isolated perfused liver within 90 min from the onset of hypoxia and ethanol administration (3 g/l, 30 min after allopurinol). Values are means and their standard errors ($\bar{x} \pm$ SEM)

Hypoxia	Ethanol	Allopurinol	N	GPT (U/l)	SDH (U/l)
+	−	−	5	20.3 ± 6.3	7.3 ± 2.0
+	+	−	5	1211.0 ± 200.0*	190.0 ± 13.1*
+	+	+	6	326.0 ± 211.0*†	68.2 ± 61.0*†

* Statistically significant difference ($P < 0.05$) as compared to the group subjected to hypoxia only.

† Statistically significant difference ($P < 0.05$) as compared to the ethanol-treated group subjected to hypoxia.

is elevated. In fact, the decrease in GSH content in our study after ethanol-treatment of hypoxic livers is indicative of such an oxidative stress situation.

The role played by xanthine oxidase in this process is substantiated by our findings that inhibition of this enzyme by allopurinol clearly reversed ethanol hepatotoxicity and oxidative stress as evidenced by the lower rate of enzyme release and of GSH depletion, respectively. Furthermore, the increase in lactate + pyruvate concentrations after allopurinol shows that the inhibition of glycolysis is not a direct effect of ethanol, but most probably stems from acetaldehyde metabolism via the xanthine oxidase reaction. Oxygen free radicals, thus, might be involved in this process (Fig. 4).

The implication of oxygen free radicals in ethanol-induced liver damage is in accordance with our previous findings under normoxic conditions showing that oxygen radical scavenge systems reversed ethanol-induced enzyme leakage [10, 11].

In conclusion, our results indicate that ethanol-induced hypoxia by itself is not the cause of hepatotoxicity. The presence of ethanol under hypoxic conditions, however, will lead to a marked potentiation of hypoxia-induced toxicity towards the liver. This effect is most probably due to an inhibition of glycolysis mediated by oxygen free radicals.

Acknowledgements—The skillful technical assistance of Cornelia Magnussen and Astrid Röbke is greatly appreciated. Thanks go to Dr. R. Pentz for determination of acetaldehyde.

REFERENCES

1. J. Bernstein, L. Videla and Y. Israel, *Biochem. J.* **134**, 515 (1973).
2. Y. Israel, H. Kalant, H. Orrego, J. M. Khanna, M. J. Phillips and D. J. Stewart, in *Biochemistry and Pharmacology of Ethanol* (Eds. E. Majchrowicz and E. P. Noble), Vol. 1, p. 433. Plenum Press, New York (1979).
3. R. G. Thurman, W. R. McKenna and T. B. McCaffrey, *Molec. Pharmac.* **12**, 156 (1976).
4. T. Yuki, B. U. Bradford and R. G. Thurman, *Pharmac. Biochem. Behav.* **13**, 67 (1980).
5. S. Ji, J. J. Lemasters, V. Christenson and R. G. Thurman, *Pharmac. Biochem. Behav.* **18**, 439 (1983).
6. Y. Israel, H. Kalant, H. Orrego, J. M. Khanna, L. Videla and J. M. Phillips, *Proc. natn. Acad. Sci. U.S.A.* **72**, 1137 (1975).
7. Y. Israel, H. Orrego, J. M. Khanna, D. J. Stewart, M. J. Phillips and H. Kalant, in *Alcohol and the Liver* (Eds. M. M. Fisher and J. G. Rankin), p. 323. Plenum Press, New York (1977).
8. S. W. French, N. C. Benson and P. S. Sun, *Hepatology* **4**, 912 (1984).
9. H. Bassan, J. Kendler and H. J. Zimmerman, *Proc. Soc. exp. Biol. Med.* **137**, 852 (1971).
10. O. Strubelt, M. Younes and R. Pentz, *Naunyn-Schmiedeberg's Arch. Pharmac.* **334** (Suppl.), R 23 (1986).
11. M. Younes and O. Strubelt, *Free Rad. Res. Commun.* **3**, 19 (1987).
12. O. Strubelt, M. Younes and R. Pentz, *Acta pharmac. tox.* **59**, 47 (1986).
13. J. E. Brehe and H. B. Burch, *Analyt. Biochem.* **74**, 189 (1976).
14. O. W. Griffith, *Analyt. Biochem.* **106**, 207 (1980).
15. C. W. Dunnett, *Biometrics* **20**, 482 (1964).
16. R. G. Thurman and R. Scholz, *Eur. J. Biochem.* **75**, 13 (1977).
17. T. Yuki and R. G. Thurman, *Biochem. J.* **186**, 119 (1980).
18. D. H. Granger, G. Rutili and J. M. McCord, *Gastroenterology* **81**, 22 (1981).
19. H. H. H. Oei, H. C. Zoganas, J. M. McCord and S. W. Schaffer, *Res. Commun. Chem. Path. Pharmac.* **51**, 195 (1986).