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CHRONIC ETHANOL, OXYGEN TENSION AND HEPATOCYTE ENERGY METABOLISM

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1. ABSTRACT

Hepatocytes from ethanol-fed animals, isolated from either whole liver or the periportal or perivenous regions of the lobule, exhibited an ethanol-related decrease in energy state only when they were oxygen deficient. This was accompanied by an ethanol-related decrease in hepatocyte viability. Both periportal and perivenous hepatocytes from ethanol-fed rats demonstrated increased respiration. The observations reported here are consistent with an ethanol-induced increase in oxygen utilization which could render the perivenous region of the lobule relatively oxygen deficient in the intact liver. This oxygen deficit may cause the decreases in energy state and cell viability associated with chronic ethanol consumption. Ethanol-associated loss in hepatocyte viability appeared to correlate better with a decrease in energy state than with an increase in the products of oxidative stress. An investigation of the association between viability and cellular malondialdehyde levels revealed no effects of chronic ethanol consumption on MDA levels in hepatocytes that demonstrated ethanol-related decreases in cell viability.

2. INTRODUCTION

It is now well established that chronic ethanol consumption alters those systems involved in oxygen utilization in the liver. With the microsomal system there is an increase in the activity of the mono-oxygenase system which utilizes oxygen to metabolize a variety of organic compounds, including ethanol (1). In contrast, the mitochondrial system, which accounts for about 80% of the hepatocyte's respiratory activity (2), is damaged by chronic ethanol consumption (3,4). Ethanol elicits a decrease in the capacity of the mitochondrion to carry out mitochondrial protein synthesis due to alterations in mitochondrial ribosomes which make them less functional (5). This results in a depression in the translation of the 13 polypeptides encoded by the mitochondrial genome. Since all these proteins are integral components of the oxidative phosphorylation system (6) mitochondrial respiration, measured with isolated mitochondria, is decreased. The net result is a decrease in the rate of ATP synthesis, as has been measured with tightly coupled, isolated mitochondria (3).

It has been assumed that the ethanol-elicited depression in the rate of ATP synthesis translates to a decrease in the energy state of the liver, but this relationship has not been rigorously established to date. References to hepatic energy state indicate either the levels of ATP in the tissue, or comparisons of ATP concentrations to those of its breakdown products, ADP and P_i, as expressed by the phosphorylation potential (7). The studies described in this paper are investigations of the effects of chronic ethanol consumption on the energy state of hepatic tissue. They were implemented as an initial step in evaluating the relationship between changes in mitochondrial function and liver energetics. It is also important to determine the effects of ethanol on hepatic energy state because the viability of the tissue will depend on the availability of ATP to provide the energy to drive anabolic processes in the cell. The adequacy of this energy source is even more critical in tissues being exposed to ethanol, acetaldehyde and reactive oxygen species generated as a result of ethanol metabolism. Under such circumstances the demands for ATP will increase in order to repair damage elicited by these toxic agents.

3. INTERACTION BETWEEN ETHANOL EFFECTS AND OXYGEN TENSION IN INFLUENCING HEPATIC ENERGY STATE OBSERVED IN HEPATOCYTES, WHOLE LIVER AND INTACT ANIMALS

In all the studies presented from our laboratory rats were fed for 31 days on the Lieber-DeCarli diet (8) with ethanol and lipid comprising 36 and 35% of total calories, respectively. Control animals were fed isocalorically with carbohydrate substituted for ethanol. Our initial observations demonstrated that effects of

Table 1. Energy state of hepatocytes incubated in presence of 95% O_2***

ENERGY-RELATED PARAMETER	SOURCE OF HEPATOCYTES	UNINCUBATED	INCUBATED
ATP concentration**	Liquid diet control	7.9±0.9	11.1±0.8†
	Ethanol-fed	6.1±0.8	12.2±1.2†
P_i concentration	Liquid diet control	74±5	17±2†
	Ethanol-fed	104±12*	17±2†
Phosphorylation potential (7)	Liquid diet control	340±63	1894±276†
	Ethanol-fed	165±36*	1817±328†

Hepatocytes (1 x 10^6/ml) were incubated for 20 min at 37^o in Krebs-Ringer bicarbonate solution containing 2 mM glutamate and 2.5% bovine serum albumin while being maintained in a 95% O_2 - 5% CO_2 atmosphere. **nmol per 10^6 viable cells; *$p < 0.05$ or lower for a difference from control samples; n = 9 pairs of animals; †$p < 0.05$ or lower for a difference due to the incubation procedure ***Data obtained from the study reported in Spach *et al.* (9) (reproduced with permission).

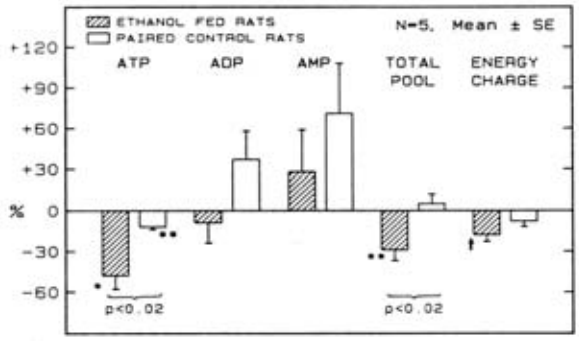


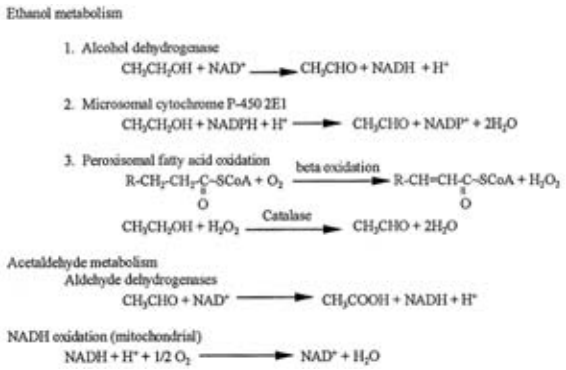
Figure 1. The effect of acute hypoxia on the hepatic adenine nucleotides and energy charge in rats fed ethanol. An outline of the experiment is provided in the text and details are provided in the original article published by Miyamoto, K. and French, S. (1988) Hepatology 8, 53-60 (reproduced with permission).

chronic ethanol consumption on hepatic energy stores were most pronounced when oxygen tension was very low. As shown in table 1, the energy state of ethanol hepatocytes was lower than that of controls when analyses were carried out on suspensions of freshly prepared cells. The ATP concentrations were lower in ethanol than in control hepatocytes and the phosphorylation potentials in the ethanol hepatocytes were half that of the controls. Analyses of oxygen content of these unincubated suspensions revealed that the preparations were anoxic (9). The ethanol-related difference in hepatocyte energy state was not retained when the cells were incubated in a highly aerobic environment. Both ATP concentrations and phosphorylation potential increased as a result of aeration and there were no differences in these parameters between ethanol and control hepatocytes. The phosphorylation potential increased primarily due to a decrease in the P_i concentrations since ADP concentrations stayed relatively constant (9). When the same experiment was repeated in the presence of breathing air, it was confirmed that the ethanol-related decrease in hepatocyte energy state occurred only when the cells were anaerobic, with the difference disappearing when hepatocytes were incubated in an oxygen-containing environment.

The above mentioned observations made with isolated hepatocytes are consistent with those carried out with live, intact animals which had been administered ethanol using the intragastric intubation procedure developed by Tsukamoto and French (10). Miyamoto and French (11) demonstrated more dramatic decreases in hepatic ATP in ethanol-fed rats than in controls when the animals were subjected to a hypoxic episode (figure 1). There was approximately a 50% decrease in ATP in ethanol animals, as compared to a 10% decrease in hepatic ATP in control rats when the animals were maintained in a gaseous environment containing only 10% rather than the normoxic 21% oxygen atmosphere.

The studies above emphasize that if hepatic oxygen tension is normal it is unlikely there will be ethanol-related effects on liver energy state. Our observations with livers freeze-clamped *in situ* while being perfused continuously by the animal's blood supply verify that a normal energy state can be maintained in livers from ethanol-fed animals if oxygenation is adequate (9). However, if any region of the alcoholic liver were to become anoxic, or hypoxic, that region might experience a localized decrease in energy state. Earlier studies suggest that in livers of ethanol consumers there may be a decrease in oxygen tension in the perivenous region of the liver lobule (12, 13). Furthermore, it is in the perivenous region of the liver lobule that irreversible damage to the liver from ethanol consumption seems to originate (14). Cell necrosis is more prevalent in this region of the liver lobule (15) and centrilobular fibrosis (16) is often observed at the alcoholic hepatitis stage of the disease.

One of the theories derived to explain the pronounced damage in the perivenous region of the liver lobule accompanying alcoholic hepatitis is termed the ethanol-induced hypermetabolic state which gives rise to perivenous hypoxia (12, 17). The evidence for this hypermetabolic effect were the observations that chronic ethanol treatment resulted in an increase in oxygen utilization in both liver slices (18, 19) and the perfused liver (20). An increase in hepatic respiratory activity has also been observed when ethanol is administered acutely (21) which demonstrates that ethanol metabolism is associated with increased oxygen utilization. The following scheme demonstrates the participation of molecular oxygen in the oxidation of ethanol to acetate in liver.



Scheme 1. Oxygen utilization associated with ethanol metabolism.

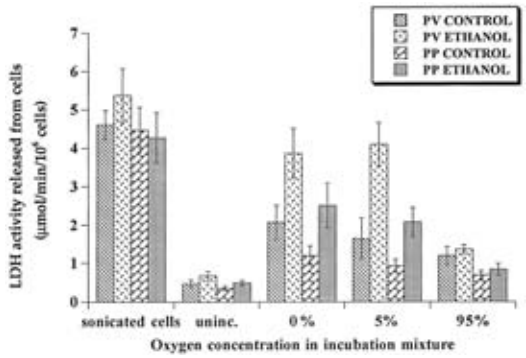


Figure 2. Lactate dehydrogenase released from periportal (PP) and perivenous (PV) hepatocytes. The LDH activity released into the medium during a 20-min incubation period at the oxygen concentrations designated was compared with that released from cells disrupted by sonication. The inset designates the cell type and diet. The details of the procedures and statistical analyses for these data are provided in Ivester et al. (1995) Arch. Biochem. Biophys. 322, 14-21 (reproduced with permission).

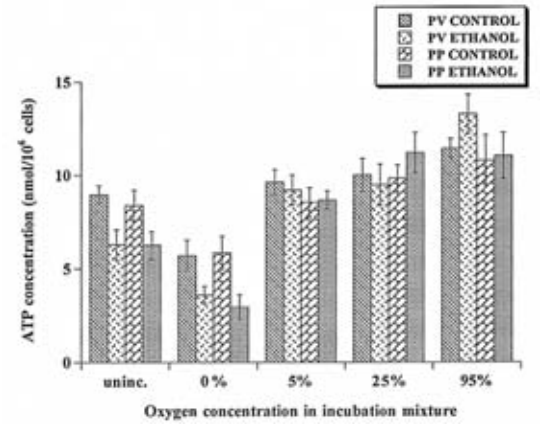


Figure 3. Influence of oxygen tension on the ATP concentrations in periportal (PP) and perivenous (PV) hepatocytes. The ATP concentrations were measured after the hepatocytes had been incubated for 20 min under the oxygen concentrations designated. The details of the procedures and statistical analyses are provided in Ivester et al. (1995) Arch. Biochem. Biophys. 322, 14-21 (reproduced with permission).

Oxygen is utilized when ethanol is oxidized by the cytochrome P-450 and peroxisomal fatty acid oxidation-catalase mechanisms. Molecular oxygen is also utilized to reoxidize the $NADH + H^+$ that is generated via the alcohol dehydrogenase and aldehyde dehydrogenase catalyzed oxidations of ethanol and acetaldehyde, respectively. Thus the increased oxygen utilization is likely associated with ethanol metabolism itself. The increase in hepatic respiratory activity observed in chronically fed animals suggests that ethanol consumption may initiate adaptations in liver tissue, making them "hypermetabolic", such that they utilize more oxygen even in the absence of ethanol (12, 17). When ethanol is oxidized a greater portion of the oxygen in the lobule is utilized in the periportal region, resulting in less oxygen available for the perivenous hepatocytes (12, 13). This is likely to affect aerobic energy metabolism such that the energy state of the perivenous hepatocytes is lowered, rendering them less viable. Thus, an oxygen deficit in this region of the lobule may contribute to the development of perivenous inflammation and necrosis.

The following studies were designed to evaluate the role of chronic ethanol consumption and oxygen tension on the energy state, viability and oxygen utilization of periportal and perivenous hepatocytes. The particular questions addressed were: 1) Do ethanol-perivenous cells exhibit a lower energy state and lower viability than do ethanol-periportal hepatocytes? 2) Do ethanol-perivenous cells respond differently to low oxygen tension than do ethanol-periportal hepatocytes? 3) Are ethanol hepatocytes "hypermetabolic"?

Animals were administered the Lieber-DeCarli diet and either periportal or perivenous hepatocytes were isolated by the digitonin-collagenase procedure (22). These cells were incubated at various oxygen tensions until the adenine nucleotide levels reached steady state and were then analyzed for viability and for their content of ATP and its metabolites, ADP, AMP and P_i. There was an ethanol-related difference in the viability of hepatocytes, both perivenous and periportal, that was noted only at low oxygen tensions (figure 2). In this study there were significant ethanol-related increases in lactate dehydrogenase (LDH) activity released from both periportal and perivenous cells that occurred under anoxic conditions or at low oxygen tensions. The release of LDH was greatest in perivenous-ethanol hepatocytes, but was significantly different from control hepatocytes in both cell types.

The losses in cell integrity were accompanied by ethanol-related decreases in the ATP content of both the periportal and perivenous hepatocytes when they were incubated under anoxic conditions (figure 3). This is also illustrated in table 2 which demonstrates that freshly prepared ethanol hepatocytes from both periportal and perivenous regions exhibited lowered ATP concentrations, high P_i concentrations and decreased phosphorylation

Table 2. Effect of incubation on energy state of periportal and perivenous hepatocytes*

ENERGY RELATED PARAMETER	SOURCE OF HEPATOCYTES	UNINCUBATED	INCUBATED*
ATP Concentration**	Periportal ethanol	8.4± 0.8	9.8±0.7†
	Periportal ethanol	6.3±0.7***	11.2 ±1.1†
	Perivenous control	9.0±0.5	10.0±0.9
	Perivenous ethanol	6.3±0.8***	9.5±1.1†
P_i concentration**	Periportal control	44.2±8.6	20.6±1.3†
	Periportal ethanol	86.5 ± 1.8***	23.6±2.4†
	Perivenous control	38.5 ± 6.4	21.7±1.7
	Perivenous ethanol	64.4±9.5***	18.3±2.0†
Phosphorylation Potential (M^{-1})	Periportal ethanol	441±79	1695±219†
	Periportal control	184±33***	1313±162†
	Perivenous control	556±114	1316±143†
	Perivenous ethanol	247±47	1669±353

*Incubated in an atmosphere of 25% O_2; **nmol per 10^6 viable cells; ***$p < 0.05$ or lower for a difference from control cells (ethanol effect); †$p < 0.05$ or lower for a difference from unincubated cells. The data were derived from studies reported in Ivester *etal*.(22)(reproduced with permission).

potential compared to control cells. This ethanol-related depression in ATP concentrations in both periportal and perivenous hepatocytes under anoxic conditions parallels the decrease we observed earlier in whole liver hepatocytes (table 1). Furthermore, at physiologically relevant concentrations of oxygen the ethanol effect disappeared in both periportal and perivenous hepatocytes (table 2). These results emphasize that the ethanol-related decrease in energy state which occurs under anoxic conditions is experienced by both periportal and perivenous cells. Moreover, there is no apparent permanent effect of ethanol, as administered in these studies, on ATP concentrations in either cell type that can't be reversed by an adequacy of oxygen.

These results still provide for the possibility that, if by ethanol consumption and its subsequent metabolism perivenous hepatocytes are made more hypoxic, there could be an ethanol-related decrease in energy state in this region of the liver lobule. It should be noted that oxygen tension in the perivenous portion of the lobule is normally below that which would exist in an incubation medium equilibrated with a gaseous phase consisting of 5% oxygen. The average oxygen concentration reported for the surface of the liver is 32 micromolar (13), which is equivalent in oxygen tension to an incubation medium equilibrated with a gas mixture containing approximately 3.5% oxygen. Clearly, energy state and cell viability need to be evaluated under conditions where oxygen is varied over a much more narrow range than we have utilized in the past. Nevertheless, the studies included suggest that the energy state of the hepatocyte is a factor which will affect its viability. Recent studies from our laboratory (23) demonstrate that ATP synthesis via glycolysis is depressed in hepatocytes from ethanol-fed animals, thus providing an explanation for depressed ATP concentrations observed in ethanol hepatocytes under hypoxic conditions.

As mentioned above, it has been suggested that ethanol consumption initiates adaptations in hepatocytes, making them "hypermetabolic" such that they utilize more oxygen even in the absence of ethanol. This possibility was evaluated using both periportal and perivenous hepatocytes that were assayed for oxygen utilization over a range of media oxygen tensions varying from 16 -140 micromolar oxygen. At every oxygen concentration utilized, respiratory activity of both periportal and perivenous hepatocytes from ethanol-fed animals was significantly higher than that of cells from control animals. For example, at a media oxygen concentration of 70 micromolar (approximately 50 torr), there were ethanol-related increases in cellular respiration of 50% and 70% in perivenous and periportal hepatocytes, respectively (23). There were no significant differences in the respiratory activities between periportal and perivenous hepatocytes isolated either from control or ethanol-fed animals. It has to be recognized, however, that in preparation of these hepatocytes Kupffer cells from ethanol-fed animals may have released higher levels of prostaglandin E_2 when they were lysed by digitonin. The increased respiration in ethanol hepatocytes may reflect unavoidable exposure to higher concentrations of this compound known to increase cellular respiration (24). Indeed, it has been demonstrated that Kupffer cell extracts from ethanol-fed rats cause increases in respiration in hepatocytes isolated from whole liver (24).

4. OXYGEN TENSION, CELL VIABILITY AND LIPID PEROXIDATION IN ETHANOL HEPATOCYTES

The energy state of the cell may assume even more importance in maintaining viability of the hepatocyte under conditions of oxidative stress. The following experiment was designed to evaluate the effect of energy state on cell viability under conditions where oxidative stress was increased, as indicated by an increase in lipid peroxidation. In this experiment perivenous hepatocytes were exposed to conditions which gave rise to a small, but significant, loss in viability when they were incubated in the absence of oxygen. This is demonstrated in figure 4 by an ethanol-related decrease in trypan blue exclusion and also by increased LDH release from ethanol hepatocytes.

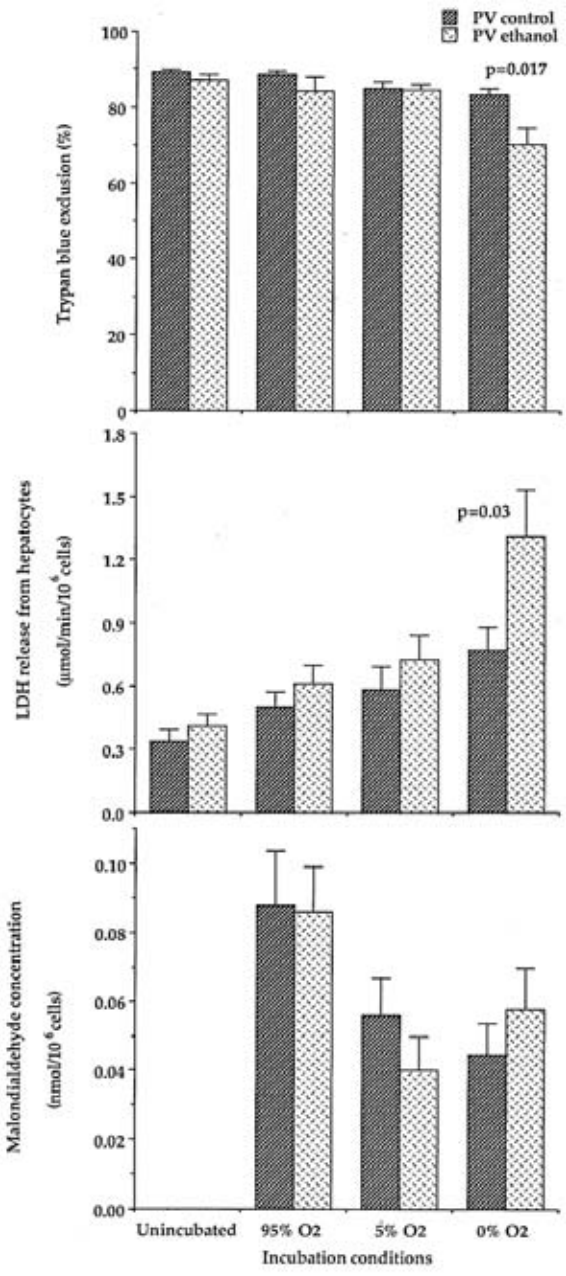


Figure 4. Influence of oxygen tension on viability and malondialdehyde production in perivenous (PV) hepatocytes. Cells were subjected to an incubation protocol similar to that published previously (22). At the end of the incubation period trypan blue exclusion and LDH release were measured as described previously (22) and thiobarbituric acid positive production was measured by a modification of the method of Recknagel et al. (26) in which product was detected fluorimetrically (excitation and emission wavelengths were 515 and 553 nm, respectively). Thiobarbituric positive material was not measured in the unincubated hepatocytes.

This loss in cell viability was accompanied by the production of some thiobarbituric acid positive material which was not significantly different between ethanol and control hepatocytes. The concentration of this material, which has been expressed as malondialdehyde, was equivalent under anoxic conditions and in a 5% oxygen environment. When the cells were incubated at high oxygen tension (95% oxygen) there is increased malondialdehyde production, but no loss in viability in either ethanol and control hepatocytes. This experiment suggests that if ATP concentrations are adequate the cell may be able to repair damage caused by oxidative stress and thus maintain cell integrity. However, when ATP concentrations decrease as a result of ethanol consumption and oxygen depletion (figure 3, table 2), cell viability is compromised by a decrease in intracellular energy state.

In a more recent study from our laboratory (25) loss of cell viability in ethanol hepatocytes was measured as a function of both intracelluar ATP content and levels of reactive oxygen species. In experiments where hepatocytes from ethanol-fed and control rats were incubated for one hour, it was demonstrated that loss in hepatocyte viability was correlated with decreases in ATP concentrations(correlation coefficient = 0.74), but not increases in the levels of reactive oxygen species (correlation coefficient = -0.34). These studies and the measurements of lipid peroxidation, discussed above, provide examples where ethanol-related loss in hepatocyte viability is dissociated from oxidative stress. It also suggests that the mechanism for alcohol-induced liver damage should be considered a multi-factorial process which is affected by the energy state of the cell as well as those agents with the potential to alter cell structure such as the active oxygen species.

5. GENERAL CONCLUSIONS

Ethanol-related alterations in hepatocyte energy state are noted only when the cells are maintained in an oxygen deficient state. The ethanol-related decrease in cell viability appears to correlate with a depression in intracellular energy state. This may be important in the intact organism since there is evidence for an ethanol-related depression in oxygen tension in the perivenous portion of the liver lobule after either acute or chronic ethanol consumption. This presumably results from both increased oxygen utilization associated with ethanol metabolism and perhaps the induction of a "hypermetabolic" state in the hepatocytes after chronic ethanol consumption related to increased Kupffer cell activity. This in turn could result in perivenous hypoxia *in situ* with the potential to decrease the hepatic energy state in that region of the lobule. The decreased energy state predisposes the region to ethanol-related damage since there may not be a sufficient source of energy for the endergonic process of repair of cell components damaged by the reactive agents generated as a result of ethanol metabolism.

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