

³¹P NMR Studies on Perfused Liver from Mouse with Chronic Ethanol Ingestion

Haripalsingh M. Sonawat*, Shunji Yamamoto**, and Dieter Leibfritz

Fachbereich Chemie/Biologie, Universität Bremen, Postfach 33 04 40,
D-28334 Bremen, Bundesrepublik Deutschland

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Changes in the levels of phosphate metabolites as affected by acute ethanol administration and chronic ethanol ingestion were investigated in perfused mouse liver by ³¹P NMR spectroscopy. Acute ethanol administration decreases intracellular P_i and the P_i/ATP ratio, and increases phosphomonoester levels in normal-fed animals. No such change was observed in the liver from ethanol-fed mice. Chronic ethanol ingestion renders the liver more prone to ischemia-induced changes in ATP, intracellular P_i and phosphomonoesters. The P_i/ATP ratio increases fivefold in control mice and fourfold in alcohol-fed mice when ischemia is induced in the presence of ethanol. Intracellular pH of 7.45 ± 0.05 is not affected by ethanol perfusion. Cellular acidosis resulting from ischemia in the presence or absence of alcohol was similar. However, longer period of ischemia leads to an additional 0.11 unit drop in pH in the presence of ethanol.

Introduction

Acute ethanol ingestion causes cellular and sub-cellular changes in several organs. Ethanol intake decreases ATP [1–4], intracellular P_i and ATP/ADP ratio and increases phosphomonoesters [4] in rat liver. A difference in response, however, exists between fed animals – where ethanol administration increased the P_i/ATP, and fasted animals – which exhibited a decrease in this ratio [1]. Ethanol also causes a lowering of intracellular cytosolic pH. This has been demonstrated by subjecting perfused liver to hypoxia in presence of ethanol leading to a pH drop 0.20 pH unit more than hypoxia in the absence of ethanol [4]. Enhanced synthesis of phosphomonoesters (mainly glyc-3-P) has also been reported [4–6].

Chronic ethanol intake on the other hand leads to adaptive changes. Its metabolic degradation to acetaldehyde in liver cytosol, by NAD-linked alcohol dehydrogenase, is followed by conversion of acetaldehyde to acetyl-CoA in mitochondria. In addition, ethanol is also degraded by a microsomal oxidizing system and peroxidase [7]. Furthermore, phosphatidyl ethanol [8] and acyl esters of ethanol [9] are also formed. Adaptive responses to high circulating levels of ethanol include processes ranging from specific enzyme activities like Na⁺/K⁺-ATPase, monoamine oxidase [10] to functions such as oxidative phosphorylation, transmembrane signal transduction [11, 12] and alteration in mitochondrial coupled respiration through electron transport chain [13]. Since these processes are associated with membrane, and membranes of organs, including liver, and since after an initial period of alcohol ingestion ethanol fed animals show resistance to further disordering by ethanol *in vitro* [14–17], the adaptive responses are proposed to be the result of interaction of ethanol with biological membranes.

In this paper, we have used ³¹P NMR spectroscopy to elucidate the effect of chronic alcohol intake on phosphate metabolites in perfused mouse liver. Since ischemia and hypoxia induce major changes in liver metabolic activity, intracellular pH and membrane structure [18–21], in our experiments ischemia was applied in combination with ethanol perfusion to elicit this information.

Reprint requests to Prof. Dr. D. Leibfritz.

Present addresses:

* Chemical Physics Group, Tata Institute of Fundamental Research, Homi Bhabha Road, Bombay – 400 005, India.

** Kitakaneyascho, Ogawa, Imadegawadori, Kami-gyoku, Kyoto 602, Japan.

Abbreviations: HPV, hepatic portal vein; KH, Krebs-Henseleit buffer, pH 7.4; NAD(H), nicotinamide adenine dinucleotide (reduced); P_i, cellular inorganic phosphate; PME, phosphomonoester.

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Materials and Methods

Liver perfusion

Male mice (NMRI) were fed *ad libitum* on standard chow and water for one week. One group of mice were continued on this regimen for 5 weeks and served as normal-fed. In another group ethanol was introduced into the water bottles and its concentration was increased every week *i.e.* 10, 20, 30, 40, and 50% to induce chronic ethanol consumption. After one week on 50% alcohol the mice were assumed to be adapted to chronic alcohol consumption. NMR experiments were conducted on liver of mice so adapted and of normal-fed mice. The animals weighed 36–46 g before the experiment.

Prior to surgery the animals were injected with 0.2 ml nembutal (6 mg/ml) intraperitoneally. The liver was perfused through the hepatic portal vein at the rate of 13.5 ml/min per 100 g body weight (2.6 ml/min per g wet weight of liver). The perfusate (Krebs-Henseleit buffer, pH 7.4, with NaCl 118 mM, NaHCO_3 25 mM, KCl 4.7 mM, KH_2PO_4 1.2 mM, CaCl_2 1.9 mM, MgSO_4 1.2 mM, glucose 5.0 mM and heparin 5 units/ml) in the reservoir was gassed with 95% O_2 and 5% CO_2 and temperature maintained constant at 45 °C so as to have the temperature in the NMR tube at 25 °C. The liver was bathed with the perfusate in the NMR tube (20 mm o.d.), the level of the perfusate being maintained above the liver by an outflow tubing connected to another channel of the same peristaltic pump running at about 20 ml/min. Initially, perfusate coming out of the liver was discarded. After about 10 min the outlet was connected to the reservoir and perfusion was continued in recirculation mode (total volume 450 ml). Effort was made to minimize the length of time between cannulation of HPV by the catheter and initiation of perfusion in NMR tube, and a period of less than 10 min was usually sufficient, after which time data acquisition for NMR experiments was started.

NMR experiments

^{31}P NMR spectra were obtained at 145.78 MHz on a Bruker AM360 spectrometer interfaced with an Aspect 3000 computer. Acquisition parameters were 12,000 Hz spectral width, 2 k data points, 0.4 s relaxation delay between pulses, 15 μs (90°) obser-

vation pulse resulting in an acquisition time of 0.16 s and repetition time of 0.56 s. The field was shimmed on the FID of the proton signal of water and linewidths of less than 20 Hz were routinely obtained. No proton decoupling was used. Zero filling to 4k data points and exponential multiplication, equivalent to a further line broadening of 20 Hz, was employed before Fourier transformation of 20 averaged transients. Time domain contour plot of series of 1D spectra were obtained by processing the FIDs by the method of Offermann *et al.* [22]. Vertical cross sections from the contour plots are presented in this paper as concentration change with time. NMR detectable energy-rich phosphates in living tissue are in the range of 1–10 mmol. Concentrations are given in arbitrary units as high energy phosphates are not visible totally and only relative concentration changes matter in this context. Resonances in the individual 1D spectra were integrated and these integrals were used to calculate P_i/ATP and ADP/ATP ratios. Integral for ADP was obtained from the difference of the integrals of $\gamma\text{-ATP}$ + $\beta\text{-ADP}$ and $\beta\text{-ATP}$ signals. Chemical shifts are given in ppm with respect to phosphocreatine which was in a capillary in the NMR sample tube.

Results

Fig. 1 shows the concentration of phosphate metabolites obtained from a perfused mouse (fed normal chow) liver. With recirculating mode of perfusion the $\beta\text{-ATP}$ signal remains constant. A slight gradual decrease in $\alpha\text{-ATP}$ + $\alpha\text{-ADP}$ and $\gamma\text{-ATP}$ + $\beta\text{-ADP}$ resonances is observed. As a result, the ADP/ATP ratio also declines. The phosphomonoester signal increases gradually in the initial phases of the perfusion but stabilizes at later stages. Level and chemical shift of P_i , as also the P_i/ATP ratio, remains steady for the duration of the perfusion and hence it is clear that no acidosis occurs. Only after 5 h of perfusion some deterioration is observed in terms of decreasing $\beta\text{-ATP}$ signal. Since most experiments were of much shorter duration, the changes observed in subsequent experiments were due to the various effects under investigation. From the chemical shift of P_i resonance, a value of 7.45 ± 0.05 is established for the intracellular pH.

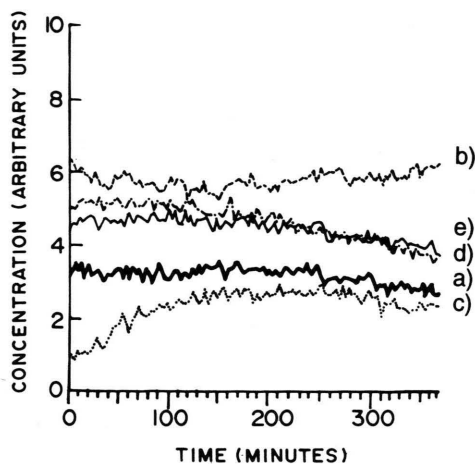


Fig. 1. Metabolite concentrations as a function of time from ^{31}P NMR spectra of mouse liver perfused with Krebs-Henseleit buffer, pH 7.4. Perfusion details as in Materials and Methods section. The curves are β -ATP (a), intracellular P_i (b), phosphomonoester (c), α -ATP + α -ADP + NAD(H) (d), and γ -ATP + β -ADP (e).

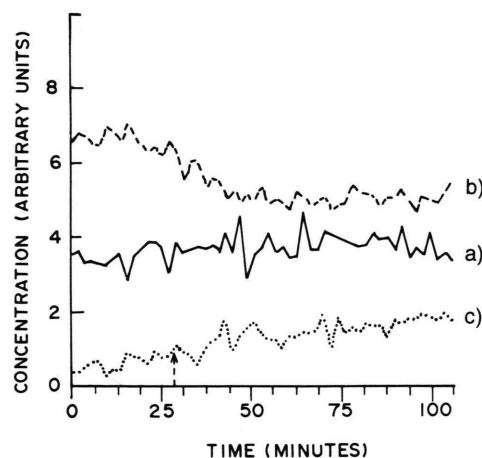


Fig. 2. Effect of alcohol perfusion on β -ATP (a), intracellular P_i (b) and phosphomonoester (c) levels obtained from ^{31}P NMR spectra of normal-fed mouse liver. At the point indicated by dashed arrow, alcohol was added to the perfusate to a final concentration of 200 mM.

Fig. 2 shows the levels of phosphate metabolites derived from ^{31}P NMR spectra obtained on perfusion of the liver with alcohol. For this purpose the liver was first perfused with Krebs-Henseleit buffer for a period of ~ 30 min following which 200 mM ethanol was included in the perfusate. The cellular P_i decreases by 30% and the PME signal correspondingly increases. While P_i level

stabilizes after ~ 15 min at a lower level, the PME concentration gradually increases. No change in the ATP level is observed. P_i/ATP ratio decreases by 20% (Table I). The ADP/ATP ratio, however, drops by as much as 60–90% thereby indicating a drastic reduction in ADP level. In addition, intracellular pH remains unchanged.

Table I. Effect of alcohol perfusion and ischemia on P_i/ATP , pH and ADP/ATP ratio in perfused mouse liver. P_i/ATP is the ratio of integrals of P_i and β -ATP resonances. The difference of the integrals of γ -ATP + β -ADP and β -ATP signals provided a measure of ADP and this is used for determination of the ADP/ATP ratio. The figures are mean \pm SD, $n = 5$. Values corresponding to ischemia are from spectra at the end of 7 min ischemic period. pH was determined from chemical shifts of P_i relative to external phosphocreatine. Values are mean \pm SD, $n = 5$. Figures in the ischemia column are pH values from spectra at the end of a 7 min ischemia. Those in double parentheses are pH from spectra at the end of 18 min ischemia.

Perfusate ^a		Normal-fed		Alcohol-fed	
		Krebs-Henseleit buffer	Krebs-Henseleit buffer + alcohol	Krebs-Henseleit buffer	Krebs-Henseleit buffer + alcohol
P_i/ATP (pH)	perfusion	1.93 \pm 0.39	1.39 \pm 0.50	1.60 \pm 0.15	1.67 \pm 0.09
		(7.45 \pm 0.05)	(7.40 \pm 0.03)	(7.45 \pm 0.05)	(7.42 \pm 0.02)
	ischemia	2.43 \pm 0.49	7.47 \pm 1.01 ^b	5.00 \pm 0.23 ^b	7.07 \pm 0.35 ^{b,c}
		(6.94 \pm 0.02)	(6.94 \pm 0.02)	(6.92 \pm 0.02)	(6.90 \pm 0.03)
		((6.89 \pm 0.02))	((6.78 \pm 0.02))		
ADP/ATP	perfusion	0.35 \pm 0.23	0.24 \pm 0.15	0.18 \pm 0.12	0.18 \pm 0.04
	ischemia	0.30 \pm 0.12	1.08 \pm 0.75	0.74 \pm 0.26	1.40 \pm 0.53

^a Before induction of ischemia and on reperfusion.

^b Significantly different from KH perfused normal-fed mouse at 0.05.

^c Significantly different from KH perfused alcohol-fed mouse at 0.05.

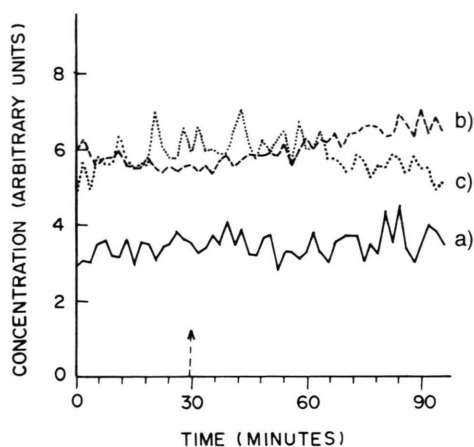


Fig. 3. Effect of alcohol perfusion on β -ATP (a), intracellular P_i (b) and phosphomonoester (c) levels derived from ^{31}P NMR spectra of alcohol-fed mouse liver. Dashed arrow indicates time of addition of alcohol (final concentration 200 mM) to the perfusate.

In liver from alcohol-fed mice, perfusion with alcohol initially results in almost no change in β -ATP level (Fig. 3). In contrast to the normal-fed mouse liver, intracellular P_i gradually increases. P_i/ATP ratio follows the pattern of P_i with progressive increment during alcohol perfusion. The PME level remains constant.

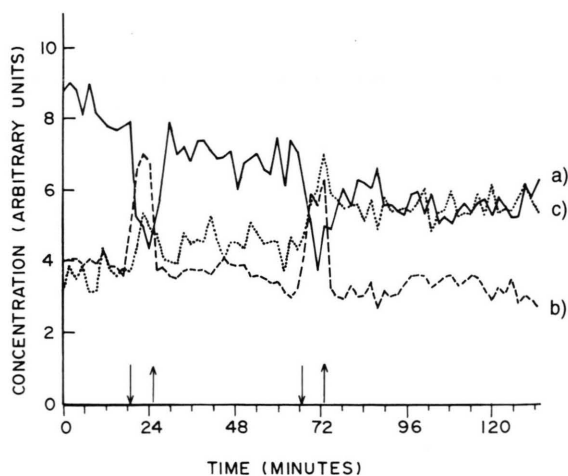


Fig. 4. Concentrations of β -ATP (a), intracellular P_i (b) and phosphomonoester (c) obtained from ^{31}P NMR spectra of normal-fed mouse liver subjected to ischemia. Arrows pointing down indicate start of ischemia. Arrows pointing up indicate reperfusion.

Ischemia was induced for a period of 7 min by stopping the perfusion. The levels of phosphate metabolites obtained from ^{31}P NMR spectra of liver from normal-fed mouse recorded under these conditions are presented in Fig. 4. The ATP level decreases with a concomitant increase in intracellular P_i . Moreover the P_i signal shifts upfield corresponding to a more acidic intracellular pH of 6.9. Also observed is an increase in the phosphomonoester signal. The drop in the level of ATP and increase of intracellular P_i (reflected in the P_i/ATP ratio (Table I)) and PME during ischemia was more pronounced in liver from alcohol-fed mice. The upfield shift of intracellular P_i at the end of the ischemic period was the same as that observed for liver from normal mice. On restarting the perfusion, ATP recovery to 70–80% of its pre-ischemic level takes place within 10 min. The intracellular P_i concentration, on reperfusion, drops back to its pre-ischemic level. Recovery from acidosis after reperfusion is almost immediate and the intracellular pH is again re-established at 7.45. Through the period of reperfusion PME decrease at a slower rate to their levels before introduction of ischemia. Upon reperfusion, intracellular P_i in liver from alcohol-fed mice initially drops below pre-ischemic level, rises again above this level before decreasing once again to a lower value (Fig. 6). Also, the recovery of ATP is complete in this case. About 40 min after the first a second ischemia of 7 min duration was induced. The concentration profiles of various phosphorylated metabolites during the progress of ischemia and recovery during reperfusion were similar to those for the first (Fig. 4). However, the effect was much more pronounced in terms of ATP decrease and intracellular P_i increase. The extent of acidosis and PME increase, and recovery thereof, were similar to that of the first ischemic period.

Two successive ischemic periods were induced on perfused liver in another set of experiments. The first one when the liver was being perfused with normal Krebs-Henseleit buffer and another after ethanol was added to the perfusate to a concentration of 200 mM. The levels of various phosphate metabolites from these experiments are presented in Fig. 5. Accordingly, the expected pattern of decrease in ATP with corresponding increase in P_i (along with its upfield shift) and PME levels, and

recovery from ischemia is observed. On perfusion with ethanol, P_i continues to exhibit a decreasing trend. Ischemia in the presence of ethanol causes a more pronounced decrease in ATP with increase in P_i , the peak concentration being much lower than during the first ischemia, and extensive production of PME. Although the ADP/ATP ratio was not significantly affected, the P_i/ATP was fivefold above the value prior to induction of ischemia. The corresponding value for liver from alcohol-fed mice was fourfold (Table I). Also the ADP/ATP ratio in this case was increased by about sevenfold. Reperfusion with ethanol leads to recovery of ATP to 85% of its pre-ischemic level and PME and P_i decrease to the pre-ischemic values. In case of alcohol-fed mice, the intracellular P_i first drops below pre-ischemic level and then increases and stabilizes above this level (Fig. 6). Reperfusion establishes ATP to pre-ischemic concentration. The recovery is, however, slow.

Alcohol perfusion by itself does not affect the intracellular pH as has been reported by Desmoulin *et al.* [4] and unlike Cunningham *et al.* [1] who reported a 0.1 pH unit acidification in presence of alcohol. Chronic ethanol ingestion does not change this observation in our experiments. A longer duration (18 min) of ischemia (Fig. 7) does bring about 0.2 ppm (0.11 pH unit) additional

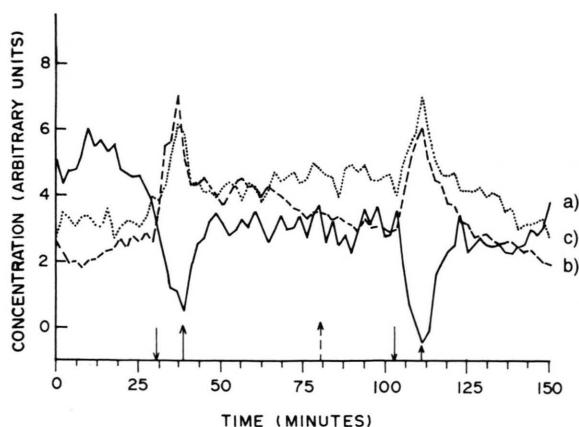


Fig. 5. Concentration of $\beta\text{-ATP}$ (a), intracellular P_i (b) and phosphomonoester (c) obtained from ^{31}P NMR spectra of perfused mouse liver subjected to ischemia in absence and presence of alcohol in the perfusate. Arrows pointing down and up indicate start of ischemia and reperfusion, respectively. Dashed arrow indicates inclusion of alcohol in perfusate from this time onwards.

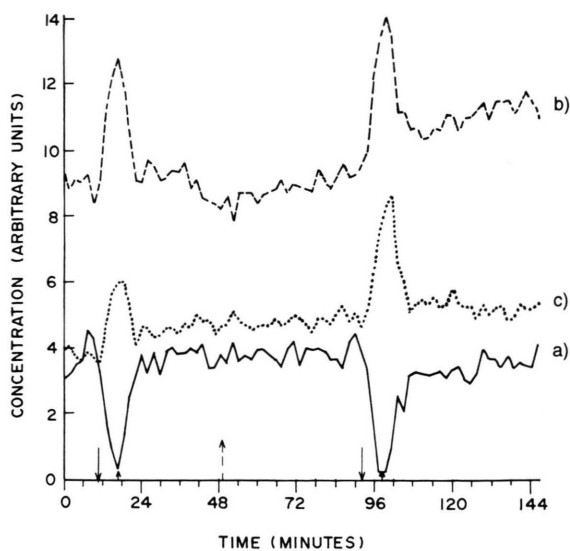


Fig. 6. Same as in Fig. 5 but with alcohol-fed mouse liver.

shift of the intracellular P_i resonance in the presence of alcohol (Table I).

Discussion

Under the perfusion conditions used, *i.e.* 25 °C, 13.5 ml/min per 100 g body weight (2.6 ml/min per g liver wet weight) circulation rate of Krebs-Henseleit buffer, pH 7.4, saturated with 95% O_2 and 5% CO_2 , homeostasis was maintained as regards the phosphate metabolites in mouse liver for at least 5 h. In most of the experiments a single phosphate resonance at 5.41–5.53 ppm, representing intracellular P_i , with ~3% contribution from P_i in the circulating buffer, corresponding to a pH of 7.45 ± 0.05 was observed. Since the entire liver was positioned in the NMR active region of the sample tube, the pH observed is an average of all the regions of the liver. This is in agreement with the cellular pH of 7.4 reported by McLaughlin *et al.* [23].

Alcohol administration causes decrease in cellular P_i . Similar observations on alcohol perfusion have been reported by Desmoulin *et al.* [4] and during fructose metabolism [24]. Cunningham *et al.* [1], however, have not observed this effect on ethanol administered rat liver, and on the contrary, an increase in

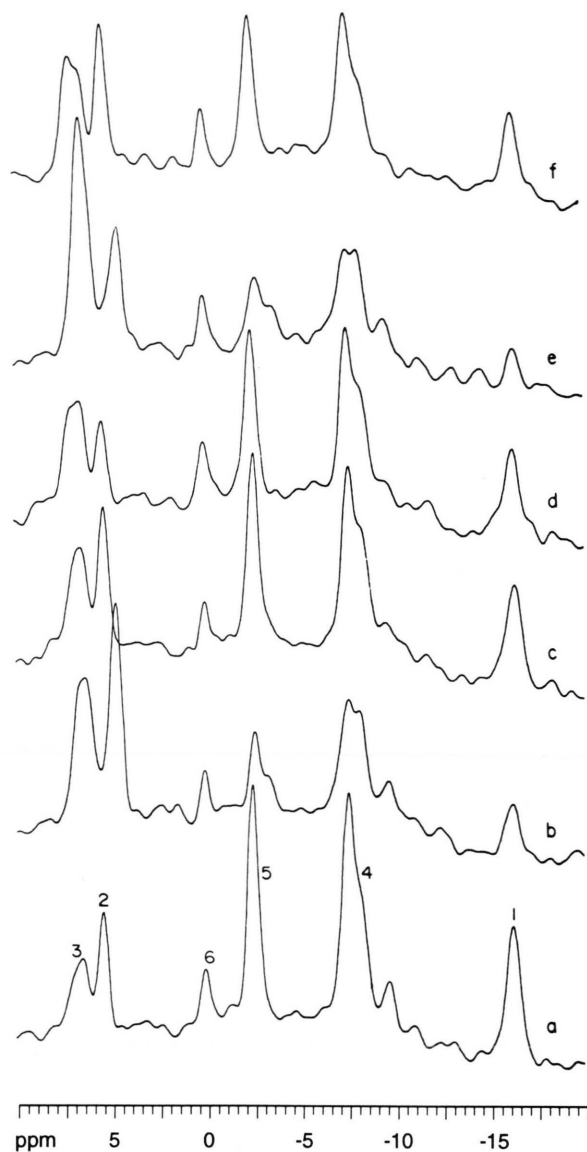


Fig. 7. ^{31}P NMR spectra of perfused liver from normal-fed mouse (a) 20 min after perfusion with Krebs-Henseleit buffer, (b) 16–18 min into ischemia, (c) 40 min after reperfusion with Krebs-Henseleit, (d) 48 min after introducing 200 mM alcohol into the perfusate, (e) 16–18 min into second ischemia in presence of alcohol and (f) 40 min after reperfusion with alcohol containing Krebs-Henseleit. Resonance assignments are (1) β -ATP, (2) intracellular P_i , (3) phosphomonoester, (4) α -ATP + α -ADP + NAD(H), (5) γ -ATP + β -ADP and (6) external phosphocreatine as ref. Note the additional upfield shift of P_i in spectrum (e) compared with that in spectrum (b). ppm is the unit of the chemical shift.

P_i , although not significant, has been reported in fed rats. In our study, the ATP level is not affected by alcohol perfusion. A drop in the P_i/ATP ratio therefore reflects merely the P_i decrease. This decrease during ethanol perfusion is caused by utilization of P_i for substrate level phosphorylation leading to the synthesis of phosphomonoesters [4]. In our study, we do not observe any change in cellular P_i in liver from alcohol-fed mice when subjected to alcohol perfusion. In addition, the ATP level also remains unaffected. Thus it is clear that the energy state of liver exposed to chronic alcohol, low to begin with, is maintained during further short periods of exposure to alcohol. The PME synthesis increases in normal-fed mice liver, and remains constant in alcohol-fed, on perfusion with alcohol. Ischemia-induced changes, that is, fall in ATP level and increase in PME and P_i and the accompanying acidosis are recovered even after 7 min of ischemia. Liver from alcohol-fed mice were more prone to changes in concentration of phosphate metabolites. The extent of acidosis was the same as with liver from normal-fed mice. Similar results in response to hypoxia and ischemia [23] and hypoxia [4, 20] have been reported.

The presence of alcohol during ischemia enhances the drop in ATP and surge in PME. The P_i/ATP ratio is higher suggesting a low energy state. This is less so in alcohol-fed mice where the increase in the ratio was 90% as compared to normal-fed mice and more than 4 times the value before ischemia. Recovery of ATP from ischemia was immediate in normal-fed as well as alcohol-fed mice. The behaviour of intracellular P_i during ischemia in the presence of alcohol observed in our experiments is in agreement with that reported for hypoxia in presence of alcohol [4]. We observe similar variation in cellular P_i in alcohol-fed mice during recovery from ischemia in absence of alcohol.

PME increase due to the influence of alcohol, and also during ischemia, has been observed in liver from both normal and alcohol-fed mice. This enhancement, also observed in rat liver [4], has been ascribed mainly to increase in glycerol-3-phosphate and thus the glycerol-3-phosphate/dihydroxyacetone phosphate shuttle has been suggested to play a dominant role in the transfer of reducing equivalents under conditions of acidosis.

Finally, our data suggest that chronic alcohol consumption leads to a decrease in the P_i/ATP ratio. Further exposure to alcohol, as during perfusion,

results in no additional variation. Mitochondrial membrane phospholipids are perturbed during chronic ethanol ingestion [17]. Our observations on the P_i/ATP ratio could be explained on the basis of the activities of Na^+/K^+ -ATPase [25] and cytochrome oxidase [26], which are affected due to the altered phospholipids composition.

Acknowledgements

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