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Toxicity of ethanol and acetaldehyde in hepatocytes treated with ursodeoxycholic or tauroursodeoxycholic acid

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Abstract

In hepatocytes ethanol (EtOH) is metabolized to acetaldehyde and to acetate. Ursodeoxycholic acid (UDCA) and tauroursodeoxycholic acid (TUDCA) are said to protect the liver against alcohol. We investigated the influence of ethanol and acetaldehyde on alcohol dehydrogenase (ADH)-containing human hepatoma cells (SK-Hep-1) and the protective effects of UDCA and TUDCA (0.01 and 0.1 mM). Cells were incubated with 100 and 200 mM ethanol, concentrations in a heavy drinker, or acetaldehyde. Treatment with acetaldehyde or ethanol resulted in a decrease of metabolic activity and viability of hepatocytes and an increase of cell membrane permeability. During simultaneous incubation with bile acids, the metabolic activity was better preserved by UDCA than by TUDCA. Due to its more polar character, acetaldehyde mostly damaged the superficial, more polar domain of the membrane. TUDCA reduced this effect, UDCA was less effective. Damage caused by ethanol was smaller and predominantly at the more apolar site of the cell membrane. In contrast, preincubation with TUDCA or UDCA strongly decreased metabolic activity and cell viability and led to an appreciable increase of membrane permeability. TUDCA and UDCA only in rather high concentrations reduce ethanol and acetaldehyde-induced toxicity in a different way, when incubated simultaneously with hepatocytes. In contrast, preincubation with bile acids intensified cell damage. Therefore, the protective effect of UDCA or TUDCA in alcohol- or acetaldehyde-treated SK-Hep-1 cells remains dubious.

Keywords: Cytotoxicity; Cell viability; Liver enzyme; Membrane structure; EPR

1. Introduction

The liver is the main site of ethanol metabolism and offers three metabolic pathways: the cytosolic enzymes alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (AlDH), the microsomal ethanol oxidizing system (MEOS: CYP2E1) and catalase in both peroxisomes and mitochondria.

Bile acids like chenodeoxycholic acid (CDCA), ursodeoxycholic acid (UDCA), glycoursodeoxycholic acid (GUDCA) or tauroursodeoxycholic acid (TUDCA) influence hepatocyte membrane structure and function [1,2]. Investigations with electron paramagnetic resonance spectroscopy (EPR) and experiments on enthalpy and entropy using differential scanning calorimetry revealed that the localization of the steroid nucleus of UDCA in the apolar core and of TUDCA and GUDCA in the interface region of erythrocyte and hepatocyte membranes near to the phospholipid head groups, like cholesterol stabilizes and protects membranes against toxic and more apolar bile acids [1,3]. Recent investigations with the isolated perfused rat liver model revealed that TUDCA also incorporates the bile salt export pump (Bsep) and the multidrug-resistant protein 2 (Mrp2) into intact hepatocyte membranes [4]. Others have shown UDCA, TUDCA and GUDCA to protect hepatocytes against apoptosis induced by ethanol, transforming growth

Abbreviations: ADH, alcohol dehydrogenase; AlDH, acetaldehyde dehydrogenase; ASH, alcoholic steatohepatitis; CDCA, cheno 3α,7β-dihydroxy-5β-cholan-24-oic acid; 5-DSA, 5-doxylstearic acid; 16-DSA, 16-doxylstearic acid; EPR, electron paramagnetic resonance spectroscopy; EtOH, ethanol; G, Gauss; GSH, glutathione; GUDCA, glyco-urso 3α,7β-dihydroxy-5β-cholan-24-oic acid; LDH, lactate dehydrogenase; MEOS, microsomal ethanol oxidizing system; α-MEM, α-minimal essential medium; NASH, nonalcoholic steatohepatitis; TUDCA, tauro-urso 3α,7β-dihydroxy-5β-cholan-24-oic acid; UDCA, urso 3α,7β-dihydroxy-5β-cholan-24-oic acid; WST-1, (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate

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factor β, Fas ligand, and hydrophobic bile acids [5–8]. These findings suggest that UDCA and TUDCA not only prevail membrane structure but also function and cell survival. Since ethanol and acetaldehyde influence both phospholipid composition and fluidity of the bilayer membrane [9] and cell metabolism, UDCA and TUDCA could also have protective effects on ethanol-induced hepatocyte damage. Consequently, UDCA has been recommended for the treatment of alcohol steatohepatitis (ASH).

The aim of this study was to investigate the effects of the more hydrophilic bile acids UDCA and TUDCA on acute ethanol and acetaldehyde alterations in hepatocytes. HepG2 cells have served as a good model for studying hepatotoxicity of chemicals and drugs, but have the disadvantage that they have lost most of their ability to express ethanol-metabolizing enzymes as ADH [10] and cytochrome P4502E1 [11]. The hepatic carcinoma cell line SK-Hep-1 is of human origin and expresses a manifold higher ADH activity than previously used HepG2 cells but no AlDH. Therefore, SK-Hep-1 cells are a suitable model system to elucidate alcohol hepatotoxicity.

2. Materials and methods

Culture media, antibiotics, phosphate-buffered saline (PBS), trypsin and fetal bovine serum were purchased from Gibco/BRL (Karlsruhe, Germany). Ethanol (EtOH) was from Merck (Darmstadt, Germany) and acetaldehyde was obtained from Sigma (Munich, Germany) and maintained as 1 M stock solution. The sodium salts of UDCA (purity >99%) and TUDCA (purity >97%) were obtained from Calbiochem (La Jolla, CA) and maintained as 100 mM stock solutions. The spin labels 5-doxylstearic acid (5-DSA) and 16-doxylstearic acid (16-DSA) as well as the ADH-inhibitor 4-methylpyrazole were purchased from Sigma.

2.1. Experiments

Cell cultures were incubated with EtOH (100 and 200 mM; physiological blood concentration of a heavy drinker) or acetaldehyde (1.5 and 2.5 mM) together with UDCA or TUDCA. Since preceding investigations have shown that low physiological concentrations had no effect on the hepatocytes and high doses are toxic, concentrations of 0.01 and 0.1 mM were used. Incubation time was 24 h. Dose dependency of ethanol and acetaldehyde toxicity were investigated with the WST-1 test in the range of 20–200 mM and 0.5–10 mM, respectively. Hepatocyte membrane structure was assessed measuring membrane order, polarity and leakage of lactate dehydrogenase (LDH) as well as glutathione (GSH) depletion of cells. GSH also informs on the metabolic state of mitochondria. The integrity of the inner mitochondrial membrane was investigated with the WST-1 test to assess metabolic function. Trypan blue exclusion was measured to ascertain cell viability, cell morphology was investigated microscopically. To find out whether EtOH, when not being metabolized to acetaldehyde, is toxic and whether toxicity can be prevented by bile acids oxidation of EtOH by ADH and liver microsomal (MEOS) CYP-2E1 was inhibited by means of 2 μM 4-methylpyrazole. In a second series of experiments, cells were preincubated for 1 and 4 h with UDCA or TUDCA (0.01 and/or 0.1 mM each) followed by EtOH or acetaldehyde.

2.2. Cell culture

SK-Hep-1 cells were obtained from "Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH" (DMSZ, Braunschweig, Germany). Stock cultures in 175 cm^2 Falcon culture flasks were maintained in a humidified CO_2-incubator at 37 °C. The cell line was fed with α-minimal essential medium (α-MEM medium), supplemented with heat-inactivated fetal bovine serum (10%, v/v), 100 U/ml penicillin and 10 μg/ml streptomycin. Cells were seeded in Petri dishes and microplates at $5 \times 10^4/cm^2$ and examined microscopically (Leica DM-IL; Zeiss, Wetzlar, Germany). The cell count was monitored with a "Neubauer" haemacytometer (Fisher Scientific, Nidderau, Germany).

The media were replaced by serum-free α-MEM medium containing ethanol (100 and 200 mM) and acetaldehyde (1.5 and 2.5 mM), respectively. Media were supplemented with 0.01 and/or 0.1 mM UDCA or TUDCA. Thereafter, cells were grown for 24 h and harvested by scraping or trypsinization. Control cells were grown for 24 h in the plain α-MEM. For preincubation experiments, cells were grown for 1 and 4 h in bile acid (0.01 and/or 0.1 mM) containing α-MEM medium followed by ethanol or acetaldehyde for additional 24 h.

2.3. Biochemical studies

2.3.1. Metabolic activity

The WST-1 (Boehringer, Mannheim, Germany) reduction assay is commonly used as an index of reductive capacity of electron carriers to evaluate integrity of the inner mitochondrial membrane. The amount of the highly water-soluble yellow/red formazan dye generated by dehydrogenases in cells is directly proportional to the number of viable cells. Cells were seeded in 96-well microplates and grown for 24 h in plain α-MEM and for an additional 24 h in modified media which contained UDCA or TUDCA (0.01 and 0.1 mM each) and EtOH (100 and 200 mM) or acetaldehyde (1.5 and 2.5 mM). Afterwards, 20 μl of WST-1 solution was added to each well and the cells were allowed to metabolize for 2 h at 37 °C in the incubator. Optical density was measured at two wavelength modes (540 and 690 nm) using a microtiter plate spectrophotometer (Rainbow). All values are calculated as the percentage of the nontoxic control (100%).

2.3.2. Glutathione release

Total GSH levels were measured with a GSH-test-kit (Calbiochem). For this colorimetric assay, 300 μl cell culture supernatant was diluted with 600 μl phosphate buffer (200 mM potassium phosphate, pH 7.8 (25 °C), containing 0.2 mM diethylene-triamine-pentaacetic acid (DTPA) and 0.025% LUBROL). After adding 50 μl of a chromogenic reagent R1, the samples were incubated for 10 min at 25 °C in the dark. Absorbance was measured at 400 nm and GSH concentration was calculated from a GSH standard curve.

2.3.3. Cell viability

Cell viability was measured as the ability of living cells to exclude trypan blue vital dye. Cells were trypsinized from the culture dishes, combined with any floating cells present in the media, and pelleted by centrifugation at $300 \times g$ for 10 min at 4 °C. Cells were washed twice with PBS, pH 7.2–7.4, and trypan blue was added at 0.2% final concentration. Living cells were counted in a haemacytometer and calculated as the percentage of the total count.

2.3.4. Lactate dehydrogenase leakage

Plasma membrane integrity was assessed using the cytotoxicity-detection-kit-LDH from Boehringer. Cells were grown for 24 h in plain α-MEM. The medium was exchanged by a modified medium containing bile acids (UDCA or TUDCA, 0.1 mM each) with or without EtOH or acetaldehyde and incubated for another 24 h. Supernatant (100 μl) was transferred into a 96-well microplate, 100 μl substrate was added and after 15 min, incubation reaction was stopped by adding 50 μl 1 M hydrochloric acid. The optical density was measured at two wavelength modes (492 and 690 nm) using a microtiter plate spectrophotometer (Rainbow). LDH release was calculated as the percentage cytotoxicity according to the instructions.

For determination of protein concentrations, a modification of Lowry's method was used (Sigma).

2.4. EPR spectroscopy

Changes in membrane order (fluidity) and polarity were monitored by EPR. As paramagnetic reporter groups, we used the spin label 5-DSA and 16-DSA. These fatty acid spin labels become incorporated into the plasma membrane lipid bilayer. 16-DSA reports on the deeper apolar and 5-DSA on the superficial polar (interface) membrane regions. Order parameter s and the correction factor c were calculated according to Gaffney and Lin [12]. S informs on membrane fluidity and rigidity. Order parameter $s = 0$ results from unhindered motion, whereas $s = 1$ shows "rigid glass spectra" or membrane inflexibility [13]. The distances A_{II} and $A_{\perp}$ were measured in the spectra and the order parameter s was calculated. Polarity can be estimated by measuring a_N values [1]. In addition to a_N, the ratio h_{-1P}/h_{-1H} is a sensitive parameter of the relative amount of spin label molecules in polar and apolar environments [13] and is calculated from the spectra.

2.4.1. EPR investigations with intact hepatocytes

Cells were seeded in Petri dishes and grown for 24 h in plain α-MEM. The medium was exchanged by media containing UDCA or TUDCA (each 0.1 mM) with or without EtOH or acetaldehyde and incubated for another 24 h. After harvesting, cells were adjusted to 2×10^7cells/ml PBS. For each experiment, 50 μl cell suspension was labeled with 5 mM 5-DSA or 5 mM 16-DSA to a final concentration of 0.1 mM 5-DSA or 0.1 mM 16-DSA and incubated for 120 s at room temperature. Samples were inserted into a Bruker B-R 70 X-band spectrometer with a B-E 25 magnet and spectra of 5-DSA and 16-DSA were performed at room temperature (25 °C). For computer evaluation of EPR spectra, the Bruker WIN-EPR program was used.

2.5. Statistics

At least three tests were performed in each experiment. All data are expressed as means ± S.D. The statistical significance of the data was assessed by two-tailed Student's t-test. A difference at $P \leq 0.05$ was considered statistically significant.

3. Results

3.1. ADH-concentrations in SK-Hep-1 cells

ADH activity in the SK-Hep-1 cells was found to be 48 mU/mg protein and was 17.8 times higher than in HepG2-cells, where it was 2.7 mU/mg protein. SK-Hep-cells did not express AlDH.

3.2. Biochemical studies

All biochemical methods used revealed no change when cells were incubated with UDCA or TUDCA alone compared to cells grown in plain α-MEM medium. The same results were obtained when oxidation of the added EtOH was inhibited by 4-methylpyrazole. Even the high dose of 100 μM UDCA or TUDCA did not inhibit ADH activity.

3.2.1. Metabolic activity (WST-1 test)

Metabolic activity of SK-Hep-1 cells after 24 h incubation with α-MEM medium was set to 100% and was used as control (Table 1). Investigation of the dose dependency of the toxic effect of acetaldehyde showed at concentrations of 0.5, 1.5, 2.5, 5 and 10 mM significantly decreasing metabolic activities (versus control, all $P < 0.002$) from 88.5 ± 2.1%, 61.4 ± 2.7%, 50.1 ± 4.5%, 31.2 ± 7.1% to 13.3 ± 6.3%. Concentrations below 60 mM EtOH did not modify cell viability, whereas 60, 80, 100, 150 and 200 mM EtOH decreased metabolic activity to 93.7 ± 3.1%, 92.1 ± 1.2%

Table 1
Metabolic activity (%) of SK-Hep-1 cells after 24 h simultaneous incubation with bile acids and ethanol or acetaldehyde and after 1 and 4 h preincubation with bile acids followed by 24 h simultaneous incubation with ethanol or acetaldehyde

	Acetaldehyde (1.5 mM)	Acetaldehyde (2.5 mM)	EtOH (100 mM)
α-MEM			
Simultaneous incubation	61.4 ± 2.7	50.1 ± 4.5	89.2 ± 2.1
0.01 mM UDCA			
Simultaneous incubation	71.7 ± 3.5**	57.3 ± 5.1*	89.6 ± 2.2
1 h preincubation	39.4 ± 2.6***	38.3 ± 3.4***	89.1 ± 2.0
4 h preincubation	33.5 ± 3.3***	32.3 ± 6.1***	65.2 ± 5.6***
0.1 mM UDCA			
Simultaneous incubation	75.8 ± 9.3*	62.0 ± 8.1	94.9 ± 2.4*
1 h preincubation	34.4 ± 4.9***	35.9 ± 4.2***	68.6 ± 7.1***
4 h preincubation	34.1 ± 6.2***	31.2 ± 4.5***	60.3 ± 6.8***
0.01 mM TUDCA			
Simultaneous incubation	68.3 ± 3.8	66.1 ± 4.1*	91.3 ± 2.0
1 h preincubation	40.0 ± 3.9***	31.5 ± 2.8***	82.0 ± 11.0
4 h preincubation	35.3 ± 3.5***	36.5 ± 3.5***	62.7 ± 4.6**
0.1 mM TUDCA			
Simultaneous incubation	72.5 ± 4.3*	70.9 ± 4.2**	91.5 ± 4.6
1 h preincubation	42.8 ± 7.1***	35.1 ± 7.4***	79.8 ± 7.8*
4 h preincubation	37.9 ± 6.1***	37.0 ± 3.4***	63.9 ± 9.4***

Statistical significance was calculated versus acetaldehyde and ethanol, respectively. *$P<0.05$; **$P<0.01$; ***$P<0.002$. Metabolic activity in α-MEM medium was set to 100%. Values are means of four to six independent measurements ± S.D.

($P<0.05$), 89.2 ± 2.1% ($P<0.05$), 71.7 ± 2.8% ($P<0.01$) and 64.8 ± 5.8% ($P<0.01$). Addition of 1.5 mM acetaldehyde decreased metabolic activity to 61.4% (versus control $P<0.002$), whereas simultaneous incubation of 1.5 mM acetaldehyde with 0.1 mM UDCA yielded a decrease to only 75.8%. UDCA improved the situation by 14.4% ($P<0.05$). When 0.1 mM TUDCA was used metabolic activity decreased to 72.5%, which was a significant ($P<0.05$) but only 11.1% improvement compared to acetaldehyde. UDCA or TUDCA (0.05 mM) reduced toxicity of 1.5 and 2.5 mM acetaldehyde to 71.4 ± 3.7% and 61.0 ± 8.4% or to 71.7 ± 3.3% and 70.0 ± 3.0% (all $P<0.05$). Also, 0.01 mM UDCA or TUDCA reduced toxicity of 1.5 mM acetaldehyde: 0.01 mM UDCA by 10.3% ($P<0.01$) and TUDCA by 6.9% (n.s.). Even the toxic effect of 2.5 mM acetaldehyde was reduced by bile acids. TUDCA had a better effect than UDCA. 100 mM ethanol did not reveal significant damage on mitochondrial reductive capacity, however, 0.1 mM UDCA improved the metabolic activity by 5.7% ($P<0.05$), while 0.1 mM TUDCA in the ethanol experiments had no effect. EtOH did not alter metabolic activity (98.7 ± 2.8% at 100 mM) when ADH and CYP-2E1 was inhibited by 4-methylpyrazole. Preincubation for 1 and 4 h with 0.01 or 0.1 mM bile acids drastically decreased the metabolic activity of 1.5 and 2.5 mM acetaldehyde by about more than 20% ($P<0.002$; Table 1).

3.2.2. Glutathione release

GSH release of cells after 24 h incubation with α-MEM medium was 0.83 ± 0.02 μmol/mg protein and was used as control. At 1.5 mM acetaldehyde, there was a slight but not significant increase of GSH in the supernatant (Table 2), 2.5 mM caused a GSH increase to 1.14 μmol/mg protein (versus control $P<0.01$). UDCA and TUDCA (0.1 mM) reduced this effect ($P<0.05$). EtOH only slightly increased GSH content in the supernatant. Preincubation of hepatocytes for 1 h with 0.1 mM bile acids increased the GSH release and intensified the toxicity of acetaldehyde and only slightly changed the effects of ethanol. The inhibition of the alcohol-metabolizing enzymes by 4-MPA had no influence.

3.2.3. Cell viability (trypan blue exclusion test)

Another parameter of plasma membrane integrity, but commonly used as an index of cell viability is the ability of living cells to exclude trypan blue. Living cells were counted and calculated as a percentage of the total count. In the presence of 1.5 mM acetaldehyde viability decreased to 68.8%, with 2.5 mM acetaldehyde to 42.1%. The high concentration of 200 mM EtOH induced a decrease to only

Table 2
GSH release (μmol/mg protein) after 24 h simultaneous incubation of SK-Hep-1 cells with bile acids and acetaldehyde and 1 and 4 h preincubation with bile acids followed by 24 h simultaneous incubation with ethanol or acetaldehyde

	Acetaldehyde (1.5 mM)	Acetaldehyde (2.5 mM)	EtOH (100 mM)	EtOH (200 mM)
α-MEM				
Simultaneous incubation	1.00 ± 0.12	1.14 ± 0.02$^{\#\#}$	0.85 ± 0.6	0.85 ± 0.04
0.1 mM UDCA				
Simultaneous incubation	0.86 ± 0.05	1.05 ± 0.07*	0.77 ± 0.02$^{\#}$	0.88 ± 0.08
1 h pre-incubation	1.04 ± 0.16	1.65 ± 0.15**,$^{\#\#}$	0.83 ± 0.10	0.86 ± 0.02
4 h pre-incubation	1.00 ± 0.15	1.27 ± 0.38	0.80 ± 0.04	0.90 ± 0.01
0.1 mM TUDCA				
Simultaneous incubation	1.06 ± 0.11*	0.93 ± 0.05*	0.84 ± 0.07	0.87 ± 0.07
1 h pre-incubation	1.19 ± 0.02*,$^{\#\#\#}$	1.42 ± 0.14*,$^{\#\#}$	0.84 ± 0.08	0.89 ± 0.08
4 h pre-incubation	1.02 ± 0.13	1.48 ± 0.10**,$^{\#\#}$	0.80 ± 0.08	0.92 ± 0.07

Cells, grown in plain α-MEM medium were used as control and showed a GSH release of 0.83 ± 0.02 μmol/mg protein (means of three independent measurements ± S.D.). Statistical significance was calculated versus control ($^{\#}P<0.05$; $^{\#\#}P<0.01$; $^{\#\#\#}P<0.002$) as well as versus ethanol and acetaldehyde.(*$P<0.05$; **$P<0.01$).

76.6% (Table 3). UDCA significantly reduced 1.5 mM acetaldehyde toxicity from 68.8% to 72.0% ($P<0.05$) and from 42.1% to 51.1% ($P<0.01$) at 2.5 mM acetaldehyde. The effect of TUDCA was significantly ($P<0.002$) stronger. Also EtOH-induced toxicity was significantly prevented by UDCA and TUDCA. Better protective effects for TUDCA than for UDCA where seen with acetaldehyde. EtOH did not alter viability (90.4 ± 0.8% at 100 mM and 90.8 ± 2.0% at 200 mM) when ADH and CYP-2E1 was inhibited by 4-methylpyrazole. Preincubation with bile acids for 1 h did not much change the effects of acetaldehyde and ethanol, whereas 4 h preincubation significantly increased cytotoxicity (Table 3).

3.2.4. Lactate dehydrogenase leakage

LDH activity was expressed as the percentage of LDH released into the medium of the total activity present in hepatocytes (Table 4). Acetaldehyde induced an increase of cytotoxicity to 4.66% at 1.5 mM and to 10.02% at 2.5 mM. Simultaneous incubation of 1.5 mM acetaldehyde and 0.1 mM UDCA or TUDCA completely decreased LDH activity to the control level. UDCA (0.1 mM) when simultaneously incubated with 2.5 mM acetaldehyde reduced enzyme activity from 10.02% to 4.15% and 0.1 mM TUDCA completely abolished cytotoxicity. Ethanol and simultaneous incubation with bile acids likewise reduced cytotoxicity. Inhibition of ethanol-oxidation abolished cytotoxicity at 100 and 200 mM EtOH. Investigation of the efficacy of 1 and 4 h preincubation with UDCA or TUDCA followed by 24 h simultaneous incubation with 1.5 or 2.5 mM acetaldehyde, 100 or 200 mM ethanol in all experiments revealed an increase of plasma membrane leakage compared to controls (Table 4).

Table 3
Viability (%) of SK-Hep-1 cells after 24 h simultaneous incubation with bile acids and ethanol or acetaldehyde and after 1 and 4 h preincubation with bile acids followed by 24 h simultaneous incubation with ethanol or acetaldehyde

	Acetaldehyde (1.5 mM)	Acetaldehyde (2.5 mM)	EtOH (100 mM)	EtOH (200 mM)
α-MEM				
Simultaneous incubation	68.8 ± 4.0	42.1 ± 1.2	85.5 ± 1.6	76.6 ± 4.9
0.1 mM UDCA				
Simultaneous incubation	72.0 ± 1.9*	51.1 ± 2.0**	91.7 ± 0.6*	86.8 ± 1.1*
1 h pre-incubation	70.8 ± 2.1	44.2 ± 2.0	81.9 ± 4.1	77.7 ± 1.9
4 h pre-incubation	63.8 ± 1.3*	42.1 ± 2.5	77.0 ± 3.6**	71.1 ± 3.3*
0.1 mM TUDCA				
Simultaneous incubation	76.2 ± 1.3***	59.6 ± 1.9***	93.7 ± 2.1*	85.7 ± 2.3*
1 h pre-incubation	71.1 ± 3.1	44.0 ± 3.1	83.8 ± 1.8	75.9 ± 3.1
4 h pre-incubation	62.7 ± 3.1*	42.3 ± 2.1	68.9 ± 2.5***	66.2 ± 3.6**

Cells, grown in plain α-MEM medium were used as control and showed a viability of 90.9 ± 1.9% (means of three independent measurements ± S.D.). Statistical significance was calculated versus acetaldehyde and ethanol, respectively. *$P<0.05$; **$P<0.01$; ***$P<0.002$.

Table 4
LDH release (% cytotoxicity) after 24 h simultaneous incubation of SK-Hep-1 cells with bile acids and ethanol or acetaldehyde and after preincubation with bile acids for 1 and 4 h followed by 24 h simultaneous incubation with ethanol or acetaldehyde

	Acetaldehyde (1.5 mM)	Acetaldehyde (2.5 mM)	EtOH (100 mM)	EtOH (200 mM)
α-MEM				
Simultaneous incubation	4.66 ± 0.93	10.02 ± 4.01	1.38 ± 0.07	9.84 ± 2.46
0.1 mM UDCA				
Simultaneous incubation	0 ± 0.20	4.15 ± 1.04	0 ± 0.01	3.8 ± 0.68
1 h pre-incubation	4.32 ± 0.94	16.93 ± 15.74	3.45 ± 0.55	14.85 ± 10.99
4 h pre-incubation	6.22 ± 3.10	32.64 ± 21.87	6.22 ± 1.43	13.99 ± 3.63
0.1 mM TUDCA				
Simultaneous incubation	0 ± 1.08	0 ± 0.02	0.52 ± 0.14	2.76 ± 0.75
1 h pre-incubation	8.64 ± 1.64	12.26 ± 10.66	4.84 ± 0.48	18.31 ± 12.26
4 h pre-incubation	11.92 ± 6.08	25.91 ± 8.03	12.44 ± 5.72	17.44 ± 5.93*

Statistical significance was calculated versus acetaldehyde and ethanol, respectively (*$P<0.05$). Values are means of three independent measurements ± S.D.

3.3. EPR investigations with intact hepatocytes

Fig. 1 shows the effect of acetaldehyde on the order parameter *s* revealed with the 5-DSA spin label. Incubation with 1.5 mM acetaldehyde reveals a drastic and significant decrease of *s* to 0.64 (versus control $P<0.002$), with 2.5

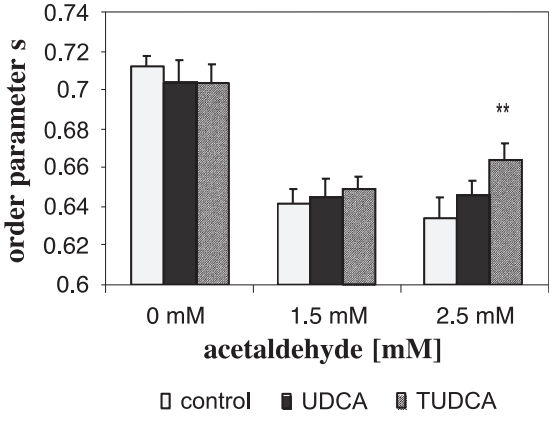

Fig. 1. Order parameter *s* after 24 h simultaneous incubation of SK-Hep-1 cells with 0.1 mM bile acids and 1.5 or 2.5 mM acetaldehyde. EPR measurements were carried out with the 5-DSA spin label (means of five measurements ± S.D.). Statistical significance was calculated versus 2.5 mM acetaldehyde (**$P<0.01$).

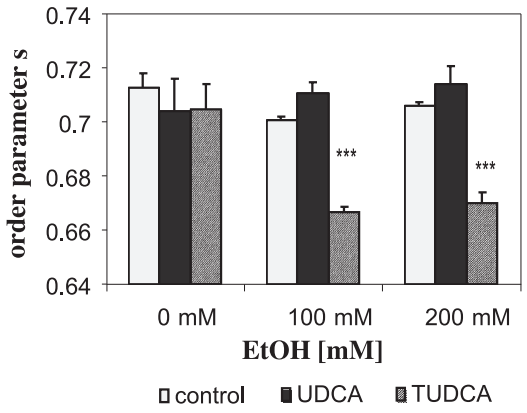

Fig. 2. Order parameter *s* after 24 h simultaneous incubation of SK-Hep-1 cells with 0.1 mM bile acids and 100 or 200 mM EtOH. EPR measurements were carried with the 5-DSA spin label (means of five measurements ± S.D.). Statistical significance was calculated versus EtOH (***$P<0.002$).

mM it further decreased to 0.63 (versus control $P<0.002$). Under these conditions both bile acids distinctively increase the order parameter to 0.64 for UDCA and 0.665 (versus acetaldehyde $P<0.01$) for TUDCA.

Fig. 2 shows the influence of 100 and 200 mM ethanol on the order parameter *s* revealed by the 5-DSA spin label. EtOH in both concentrations exerts no significant effect on order parameter. In contrast to UDCA, which also had no effect, surprisingly 0.1 mM TUDCA lowers the oder parameter from the control level (0.712) to 0.667 ($P<0.002$) at 100 mM ethanol and 0.670 ($P<0.002$) at 200 mM ethanol. Preincubation of hepatocytes for 1 and 4 h with bile acids yielded similar effects on membrane order as obtained during simultaneous incubation (data not shown).

Table 5 shows the influence of UDCA and TUDCA on acetaldehyde- and ethanol-induced toxicity in the deeper, more apolar region of the membrane (16-DSA). Both, acetaldehyde and EtOH did not significantly alter these domains (Table 5). UDCA or TUDCA did not significantly change a_N as well as the ratios during incubation with 2.5 mM acetaldehyde. Incubation with 200 mM EtOH and 0.1 mM UDCA or 0.1 mM TUDCA increased the ratio h_{-1P}/h_{-1H} significantly indicating an increase in polarity. Preincubation with bile acids yielded similar or even stronger alterations.

Table 5
Interactions of bile acids with hepatocyte membranes (16-DSA) after simultaneous and after 1 and 4 h preincubation of SK-Hep-1 cells with UDCA or TUDCA followed by 24 h simultaneous incubation with bile acids and ethanol or acetaldehyde

	a_N (G)	h_{-1P}/h_{-1H}
Control	14.05 ± 0.05	0.46 ± 0.06
0.1 mM UDCA	14.07 ± 0.06	0.45 ± 0.07
0.1 mM TUDCA	14.13 ± 0.04	0.46 ± 0.05
2.5 mM acetaldehyde	14.22 ± 0.03	0.60 ± 0.12
200 mM EtOH	14.22 ± 0.15	0.61 ± 0.06
0.1 mM UDCA and 2.5 mM acetaldehyde		
Simultaneous incubation	14.14 ± 0.05	0.49 ± 0.07
1 h preincubation	14.11 ± 0.05	0.53 ± 0.03
4 h preincubation	14.19 ± 0.09	0.67 ± 0.12$^{\#}$
0.1 mM TUDCA and 2.5 mM acetaldehyde		
Simultaneous incubation	14.19 ± 0.03	0.55 ± 0.03
1 h preincubation	14.10 ± 0.05	0.49 ± 0.05
4 h preincubation	14.17 ± 0.09	0.65 ± 0.10$^{\#}$
0.1 mM UDCA and 200 mM EtOH		
Simultaneous incubation	14.26 ± 0.03	0.87 ± 0.19$^{*,\#}$
1 h preincubation	14.29 ± 0.05	0.87 ± 0.21$^{*,\#}$
4 h preincubation	14.24 ± 0.07	0.97 ± 0.10$^{*,\#\#}$
0.1 mM TUDCA and 200 mM EtOH		
Simultaneous incubation	14.18 ± 0.05	0.85 ± 0.04$^{**,\#}$
1 h preincubation	14.22 ± 0.10	0.93 ± 0.07$^{**,\#}$
4 h preincubation	14.21 ± 0.03	0.93 ± 0.10$^{**,\#}$

Statistical significance was calculated versus control ($^{\#}P<0.05$; $^{\#\#}P<0.01$) as well as versus ethanol and acetaldehyde, ($^{*}P<0.05$; $^{**}P<0.01$). Values are means of five measurements ± S.D.

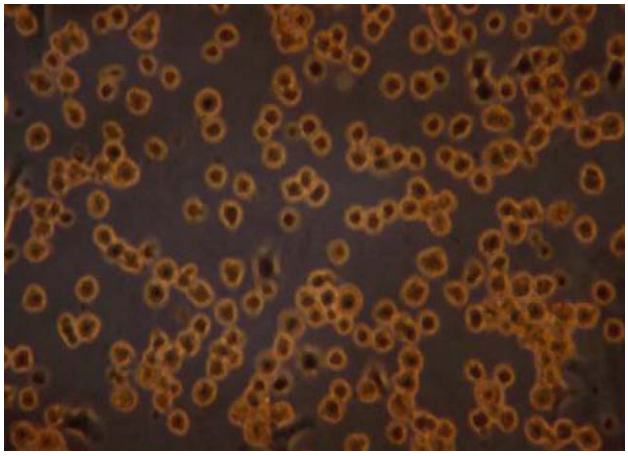

Fig. 3. The morphology of SK-Hep-1 cells exposed to 2.5 mM acetaldehyde for 24 h was determined under the light microscope (magnification 20 × 0.5).

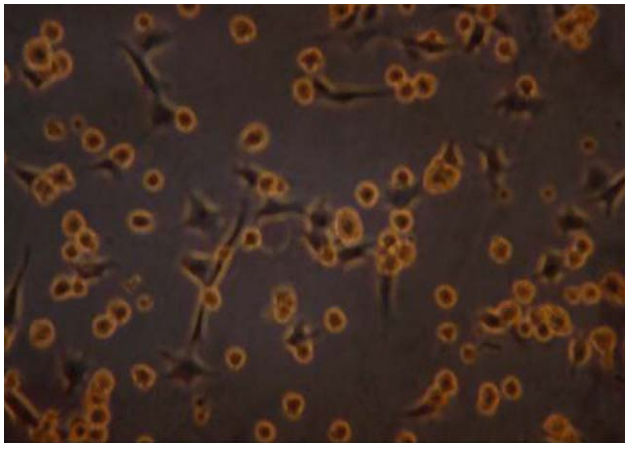

Fig. 4. SK-Hep-1 cells were exposed to 2.5 mM acetaldehyde for 24 h in the presence of 0.1 mM UDCA (magnification 20 × 0.5).

3.4. Morphology

Cells incubated with 1.5–2.5 mM acetaldehyde appeared morphologically rounded off (Fig. 3), whereas simultaneous incubation with acetaldehyde and 0.1 mM UDCA or TUDCA showed spreading and attachment to the surface (normal finding) of the microplates (Fig. 4).

4. Discussion

There is no unequivocal medicamentous treatment of alcoholic liver disease. In previous studies, steroids [14] and UDCA or its conjugate TUDCA, have been recommended [15]. Treatment studies in patients did not reveal convincing effects. The toxicity of ethanol probably represents a summation of a series of adverse effects [16]. This makes it difficult or practically impossible to study this drug in the intact animal; in particular, the investigation of the effect of ethanol per se without superimposed effects of other substances caused by alcohol metabolism, like acetaldehyde and its metabolites [17]. We used a tissue culture system which has the advantage that specific phenomena can be studied more easily. In our in vitro study we investigated in ADH-containing SK-Hep-1 cells whether alcohol or acetaldehyde in concentrations found in heavy drinkers influenced mitochondrial metabolic activity and external hepatocyte membranes and whether UDCA or TUDCA were able to prevent cell damage. Whereas we could not find any AlDH in SK-Hep-1 cells, the ADH activity was 48 mU/mg protein. The bile acid concentrations used in our experiments were rather high (0.01 and 0.1 mM), but lower concentrations had no detectable effects and higher concentrations induced cell damage (not shown). As observed by Neuman et al. [18], the physiological dose of 0.05 mM bile acids showed similar but in our experiments weaker effects than 0.1 mM. In contrast, they determined the lowest hepatoprotective concentration of bile acids at 0.025 mM, whereas in our experiments even 0.01 mM UDCA or TUDCA showed positive effects. Because of the discussions about dose administration in hepatobiliary diseases and the promise that high doses of UDCA may be required for therapeutic success [19], we decided to perform the cytotoxicity experiments at low (0.01 mM) and high (0.1 mM) bile acid concentrations. For acetaldehyde and EtOH, we also used high concentrations, since low concentrations had no detectable effects, as shown by others. These investigators also used concentrations as 80–200 mM EtOH or more to study toxicity in hepatocytes [20–23]. Neuman et al. [18,21] observed that culturing of HepG2 cells for 24 h with 60–80 mM EtOH lead to 30–40% loss of viability, whereas others [22] could not see any toxicity in their HepG2 cell strain after incubating them for up to 3 days with 100 mM EtOH. It seems that the cytotoxic dose of EtOH to hepatocytes differs between the cell strains and can probably be accounted for by a variety of mechanisms which are due to the metabolism of ethanol by ADH and cytochrome P450 [22]. Our findings, that the addition of the ADH- and MEOS-inhibitor 4-methylpyrazole to the ethanol-containing medium completely abolished the impairment caused by ethanol-oxidation correlate with investigations of others and demonstrate that ethanol oxidation is required for this cell dysfunction [24]. This is also explained by Wu and Cederbaum [22] who made the observation that ethanol toxicity is observed in cells which express cytochrome P4502E1 but not in cells which do not express this cytochrome and are therefore not able to metabolize ethanol.

Our and other data indicate that acetaldehyde provides much stronger toxic effects than ethanol [17,24,25] and that both UDCA and TUDCA are able to diminish some cell damage when added to SK-Hep-1 cells simultaneously. Since 100 μM UDCA or TUDCA did not affect ADH activity, the beneficial effects of both bile acids must be due to other mechanisms. It is assumed that the strong effect of acetaldehyde is attributable to its reaction with biomolecules producing free radicals [26,27]. EtOH is oxidized to 1-hydroxyethyl radical. This radical produces a state of oxidative stress, but seems to be less toxic than the acetaldehyde-derived radicals [28,29]. From these data, we suppose that the beneficial effects of TUDCA and UDCA at least in part depend on the interaction with the acetaldehyde and EtOH adducts, and not only on interactions with biomolecules. Further investigations should elucidate this hypothesis. It has been shown that hydrophilic bile acids such as TUDCA counteract the inhibitory effect of ethanol on bile secretion and vesicular exocytosis as well as the ethanol-induced cytolytic effect in the isolated perfused rat liver [30] and that TUDCA prevents hepatotoxicity because of its hydrophilic properties and the ability to neutralize toxic compounds as hydrophobic bile acids by competition [31]. This may also be an explanation for the beneficial effects of UDCA and TUDCA.

Interestingly, preincubation with bile acids increased EtOH and acetaldehyde toxicity. Others have shown that preincubation with alcohol and consecutively with bile acids reduced toxicity in HepG2 cells [18]. But in these experiments the cell strain was different from ours and incubation time was 5-fold. During this long period of time, cells could have recovered and become resistant to toxins.

UDCA protects against ethanol-induced mitochondrial injury by improving ATP synthesis and preserving liver mitochondrial morphology, which may contribute to the decrease in fat accumulation [32]. Our biochemical investigations revealed that UDCA obviously better protects mitochondrial function, while TUDC better prevents membrane damage. This was shown by the WST-1 test with both reagents, EtOH and acetaldehyde. In investigations not only testing for cell function but also for membrane structure and integrity, TUDCA was superior to UDCA. This, e.g., was the case investigating the GSH release and cell viability. The antioxidant GSH, besides being distributed in the cytoplasm, is also located in liver cell mitochondria. As shown

previously, UDCA and TUDCA protect hepatocyte mitochondria through modulation of mitochondrial membrane fluidity and subsequent normalization of GSH levels [33,34]. TUDCA selectively increased GSH levels in the mitochondria of ethanol-fed rats but not the cytosolic GSH and preserved membrane fluidity of mitochondria and mitoplasts, whereas without TUDCA, the order parameter increased [33]. Others found that HepG2 cells exposed for 24 h to 80 mM EtOH in the presence of 0.05 mM UDCA or TUDCA increased GSH levels in mitochondria as well as in the cytosol [5]. This is supported by recent investigations [35] that UDCA specifically enhanced methionine adenosyltransferase activity and hepatic GSH levels.

Since in our experiments GSH was measured in the supernatant, we assessed both the cytoplasmic and the mitochondrial GSH fraction which had passed through the outer cell membrane. Therefore, GSH also informs about cell viability on which we focused as an universal parameter. The cell viability test secondarily depends on membrane integrity. Trypan blue only enters the hepatocyte through a leaky membrane of an injured cell. But since there is a strong correlation between dye uptake and cell death, the trypan blue test not only informs us on hepatocyte membranes but also on viability. Even early stages of cell death can be recorded. The LDH test does not report on cell function but predominantly on the degree of membrane damage. Accordingly, in the above-mentioned tests, TUDCA protected cell membranes better than UDCA, which may be due to the different cellular uptake mechanisms for UDCA and TUDCA and the different concentrations in biomembranes and cell organelles [36,37]. Therefore, the higher intracellular UDCA concentration could react with the deeper membrane domains and intracellular structures.

Another explanation for the different actions of UDCA and TUDCA is provided by our EPR investigations which are in accordance with the biochemical investigations. Both bile acids were found not to interfere with the low polarity in the membrane's hydrophobic, deeper domain (spin label 16-DSA). Both, ethanol and acetaldehyde, however, increased polarity. Simultaneous incubation of acetaldehyde and bile acids did not significantly increase polarity in this deeper domain, but simultaneous incubation of ethanol and bile acids did. These findings suggest that EtOH interacts with the more apolar domain of the membrane and is influenced by bile acids, while the effect of acetaldehyde is not.

In the polar, more superficial part of the membrane (spin label 5-DSA), there was a maximal decrease of order observed in the presence of acetaldehyde. Evidently, polar acetaldehyde severely and instantly damages the superficial polar domain of the cell membrane surface. Consequently, the more polarly anchored TUDCA is most effective in reducing the damage, shown by the increase of the order parameter. Ethanol only in the presence of the more polar TUDCA decreased the order parameter of 5-DSA, whereas the combination of EtOH with the less polar UDCA is without effect. This result fits very well with the observation that in most experiments UDCA proved superior in restitution of ethanol-induced damage. Like in the biochemical tests, also in the EPR investigations preincubation with bile acids for 1 and 4 h before ethanol increased polarity or membrane damage.

To summarize, ethanol appears to induce much more damage at the apolar membrane site than at the polar membrane surface. Acetaldehyde, in contrast, mostly damages the membrane surface due to its more polar character. The acetaldehyde-induced superficial membrane damage is counteracted by the polarly anchored TUDCA, while UDCA better preserves mitochondrial function. The alterations in cell integrity during alcohol metabolism seem dependent on acetaldehyde rather than ethanol [23].

Taking into account our in vitro investigations and the experience of others, we believe bile acid therapy in patients with alcoholic liver disease appears rather dubious: beneficial effects of bile acids were only seen with rather high and unphysiological concentrations; preincubation of hepatocytes with bile acids significantly deteriorated cell damage caused by ethanol and acetaldehyde while simultaneous incubation reduced cell damage, and in contrast to our results, others had positive as well as negative effects [35]. Our preincubation studies with bile acids suggest that in sober alcoholics, it is likely that the bile acids may in fact be toxic to the liver.

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