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Effect of Chronic Ethanol Feeding on Oxysterols in Rat Liver

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It was our hypothesis that, as a consequence of increased oxidative stress, cholesterol-derived hydroperoxides and oxysterols are increased in livers of rats exposed to ethanol. To test this we dosed Wistar rats (approximately 0.1 kg initial body weight) with ethanol chronically (rats fed a nutritionally complete liquid diet containing ethanol as 35% of total calories; sampled liver at approximately 6–7 weeks). We measured concentrations of 7α- and 7β-hydroperoxycholest-5-en-3β-ol (7α-OOH and 7β-OOH) as well as 7α- and 7β-hydroxycholesterol (7α-OH and 7β-OH), and 3β-hydroxycholest-5-en-7-one (also termed 7-ketocholesterol; 7-keto).

In response to chronic alcohol feeding, there were significant elevations in the concentrations of 7α-OOH (+169%, $P = 0.005$) and 7β-OOH (+199%, $P = 0.011$). Increases in the concentrations of hepatic 7-keto (+74%, $P = 0.01$) and decreases in cholesterol (−43%; $P = 0.03$) also occurred. In contrast, the concentrations of both 7α-OH and 7β-OH were not significant (NS). However, when oxysterols in chronic ethanol-fed rats were expressed relative to cholesterol there were significant increases in 7-keto/cholesterol ($P = 0.0006$), 7α-OH/cholesterol ($P = 0.0018$) and 7β-OH/cholesterol ($P = 0.0047$).

In conclusion, this is the first report of increased 7α-OOH, 7β-OOH, and 7-keto in liver of rats and their elevation in chronic experimental alcoholism represent evidence of increased oxidative stress.

Keywords: Liver; 7-Hydroperoxycholesterol; Oxysterol; Alcohol; Rat

INTRODUCTION

Liver disease is a debilitating consequences of chronic and excessive alcohol ingestion and has effects on morbidity and mortality via a number of different processes, including toxic interactions, changes in gross or subcellular architecture and nutrient inactivation.[1] Contributing factors include inflammation,[2] induction of apoptosis,[3] DNA damage,[4,5] changes in protein turnover,[6,7] and increased lipid peroxidation via the excessive generation of reactive oxygen species (ROS).[8] Chronic ethanol administration can generate hydroxyl radicals through P4502E1(CYP2E1) induction in liver, which ethanol-induced CYP2E1 was associated with the pathogenesis of alcoholic liver disease. In addition, xanthine oxidase has a role in ethanol-induced lipid peroxidation.

ROS-induced products are cholesterol hydroperoxides (derivatives with a OOH group on the cholesterol ring) and oxysterols (derivatives with a OH group on the cholesterol ring). Some recent attention has focused on the possibility that increased hydroperoxides and oxysterol levels may occur in alcohol-induced tissue injury as a consequence of ROS. For example, the hepatic concentrations of the cholesterol hydroperoxides 7α-hydroperoxycholest-5-en-3β-ol (7α-OOH) and 7β-hydroperoxycholest-5-en-3β-ol (7β-OOH) are increased in alcoholics with fatty liver though not in samples from alcoholics with cirrhosis.[9] The oxysterols cholesta-3,5-dien-7-one, and cholesta-4,6-dien-3-one are also increased in liver of alcoholics though 7-ketocholesterol is reduced.[10]

However, studies in alcohol misusers are often prone to artefactual observations which are occasionally due to different diets or nutritional status.

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Perturbations in nutritional status has the potential to alter ROS generation and/or antioxidant status in both man and animals.[11,12] In some clinical studies, adequate and fresh human tissue for biochemical analysis is also limiting, which necessitates the use of autopsy material.[9]

Animal models have been developed in an attempt to unravel some of the pathogenic processes involved in alcohol-induced liver injury. Alcohol feeding studies in rats circumvents potential criticism that the resulting data is due to malnutrition by utilising *pair-feeding* protocols.[13] With *pair-feeding*, both control and alcohol-treated rats receive identical amounts of the same diet albeit with differences due to the contribution of either ethanol or isoenergetic glucose.[13] Animal studies also have the advantage of facilitating analysis of freshly harvested tissue. Thus, to address some of these issues above, we assayed cholesterol hydroperoxides and oxysterols in liver tissue from rats pair-fed either glucose- or ethanol-containing liquid diets.

MATERIAL AND METHODS

Materials

3,5-Di-tert-butyl-4-hydroxytoluene, luminol (3-aminophthaloylhydrazine) and cytochrome c (from horse heart, type VI) were purchased from Wako Pure Chemical (Osaka, Japan). Cholesterol hydroperoxides, 7α-OOH, 7β-OOH and β-sitosterol 5α-hydroperoxide (as an internal standard, IS) for HPLC with postcolumn chemiluminescence (HPLC–CL) were prepared as described previously.[14] 7-Keto, 7α-OH, 7β-OH and β-sitosterol as the IS for HPLC–UV were purchased from Steraloids (Wilton, NH, USA). Fresubin, a nutritionally complete diet with added vitamins and minerals was obtained from the Department of Dietetics, King's College Hospital, and "Orovite 7", a vitamin supplement (Beecham Group, Brentford, UK) was purchased from the high street retailers Boots Chemists (UK).

Treatment of Animals in Chronic Studies

Male Wistar rats were obtained from accredited commercial suppliers at about 60 g body weight. They were maintained in a temperature and humidity controlled animal house for approximately 1 week until they weighed approximately 0.1 kg. They were then ranked and divided into two groups of equal mean body weight and subjected to a pair-feeding alcohol-dosing regimen in which treated rats were given a nutritionally complete liquid diet containing 35% of total calories as ethanol (see below).[13] Controls were pair-fed the same diet in which ethanol was replaced by isocaloric glucose. After 6–7 weeks, animals were sacrificed by decapitation and livers dissected. The work has been carried out there under institutional supervision that ensured humane treatment of the animals.

Fresh liquid diets used for the 6 week chronic ethanol feeding experiment were prepared on a daily basis according to the recipe. A food blender was used to thoroughly mix the ingredients. To prevent the possibility of ethanol precipitating the protein in the alcohol diet, absolute ethanol was the last ingredient to be added carefully, and contents were then thoroughly stirred during the addition. The diets were freshly prepared each day and presented to the animals between 09:00 and 12:00 h. Control and alcohol-containing diets were isolipidic, isonitrogenous and isoenergetic.

Tissue Extraction Procedures for Lipids

Total lipid was extracted by adding 4 ml of ice-cold chloroform/methanol (3:1, v/v), containing 0.005% (v/v) butylated hydroxytoluene (as antioxidant) and 500 pmol β-sitosterol 5α-hydroperoxide as the IS for HPLC–CL, and 60 nmol β-sitosterol as the IS for HPLC–UV, to approximately 0.1 g of tissue, and homogenised under ice-cold conditions. The homogenate was mixed with 4 ml of chloroform/methanol (3:1, v/v) and 1 ml of distilled water, vortexed vigorously and centrifuged ($\times 800g$) for 20 min. The chloroform layer was aspirated, concentrated in a rotary evaporator and dried under nitrogen. A cholesterol-rich fraction was isolated from the total lipid by solid phase extraction. A silica column (Sep-Pak, Waters, Milford, MA, USA) packed with aminopropyl-derivatised silica ($-NH_2$) was initially conditioned by washing with 5 ml of acetone and 10 ml of *n*-hexane. The total lipid sample, dissolved in a small amount of chloroform, was added to the column, which was flushed with a mixture of 2 ml chloroform and 1 ml iso-propanol, giving an eluate that mainly consisted of cholesterol. This was concentrated in a rotary evaporator and dried under a nitrogen stream. The residue was dissolved in methanol and stored until analysis.

HPLC–CL Analysis of Cholesterol Hydroperoxides

Cholesterol hydroperoxides were determined by reverse phase HPLC–CL as described previously.[15] A TSK gel Octyl-80Ts column (Tosoh, Tokyo, Japan) was used (150 × 4.6 mm internal diameter) with methanol/water/acetonitrile (89:9:2, v/v/v) as the mobile phase.

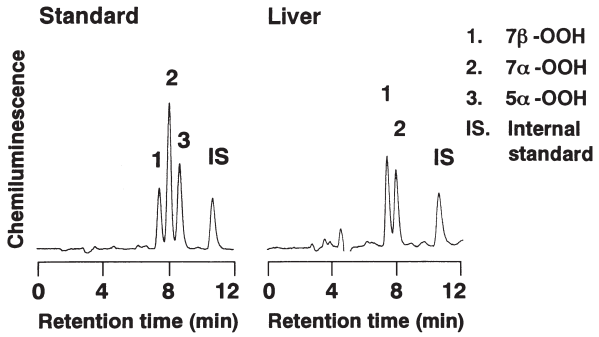

FIGURE 1 Chromatograms of cholesterol hydroperoxide in standard compounds and rat liver by HPLC with chemiluminescent detection: 7β-OOH, 7β-hydroperoxycholest-5-en-3β-ol; 7α-OOH, 7α-hydroperoxycholest-5-en-3β-ol; 5α-OOH, 5α-hydroperoxycholest-6-en-3β-ol.

HPLC–UV Analysis of Oxysterols

Oxysterols were determined by HPLC comprised L-7100 pump (Hitachi, Tokyo, Japan), SPD-10Avp UV detector (Shimadzu) set at 210 and 245 nm, and a Chromatopac C-R8A integrator (Shimadzu). An Inertsil ODS-2 column (GL Sciences) was used (5 μm, 150 × 4.0 mm internal diameter) and acetonitrile/methanol/water (46:45:9) was used as the mobile phase at the flow rate of 0.7 ml/min. All oxysterols were detected at 210 nm, while 7-keto was detected at 245 as well as 210 nm. The area of absorbance at 245 nm was 2.6 times as large as at 210 nm (hence the determination of 7-keto at 245 nm). Standard curves were prepared by the analyses of 25–200 ng of 7α-OH, 50–200 ng of 7β-OH, and 7-keto using 250 ng of IS (β-sitosterol).

Statistical Analysis

All data are expressed as mean ± SEM of seven observations in each group. Differences between groups were assessed by Students *t*-test.

RESULTS

Liver and Body Weights

Liver weights of the control rats at the end of the study were 9008 ± 364 mg compared to 8772 ± 405 mg in ethanol-fed rats ($P > 0.05$). Body weights were 0.215 ± 0.004 kg and 0.199 ± 0.004 kg, respectively ($P = 0.012$).

Separation of Cholesterol Hydroperoxides and Oxysterols

HPLC chromatograms of standard 7β-OOH and 7α-OOH, 5α-OOH and the internal standard β-sitosterol 5α-hydroperoxide show successful separation of

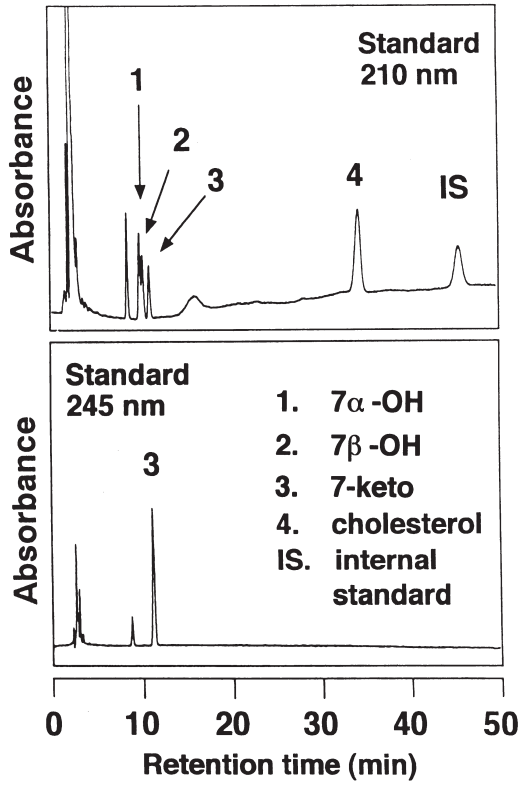

FIGURE 2 Chromatograms of standard oxysterols by HPLC–UV: 1, 7α-hydroxycholesterol; 2, 7β-hydroxycholesterol; 3, 7-ketocholesterol; 4, cholesterol; IS internal standard.

compounds, with respective retention times of approximately 7.2, 7.7, 8.2 and 9.9 min (Fig. 1). Similar successful separation of cholesterol hydroperoxides in a liver sample was also achieved (Fig. 1). Retention times of 7α-OOH and 7β-OOH were virtually identical to standard solutions.

HPLC chromatograms of standard 7α-OH, 7β-OH, 7-keto, cholesterol, and the internal standard β-sitosterol show successful separation of compounds, with respective retention times of approximately 9.7, 10.1, 10.8, 34.0, and 45.6 min (Fig. 2). Similar

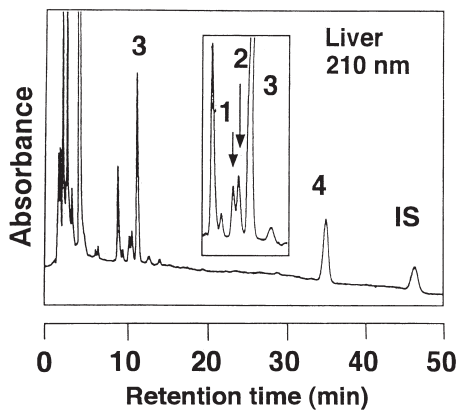

FIGURE 3 Chromatograms of oxysterols in rat liver by HPLC–UV at 210 nm: 1, 7α-hydroxycholesterol; 2, 7β-hydroxycholesterol; 3, 7-ketocholesterol; 4, cholesterol; IS internal standard.

TABLE I Cholesterol hydroperoxides in liver of chronically (6 weeks) dosed rats

	n	Hydroperoxides (nmol/g)	
		7α-OOH	7β-OOH
Control	7	1.80 ± 0.19	3.23 ± 0.51
Ethanol	7	4.84 ± 0.72	9.66 ± 1.78
P		0.005	0.011

Data as mean ± SEM. 7α-OOH, 7α-hydroperoxycholest-5-en-3β-ol; 7β-OOH, 7β-hydroperoxycholest-5-en-3β-ol.

successful separation of oxysterols in a liver sample was also achieved (Fig. 3). Retention times of 7α-OH, 7β-OH, and 7-keto (Fig. 3) were virtually identical to standard solutions (Fig. 2).

Cholesterol Hydroperoxides and Oxysterols in Liver of Control Rats

The liver of control rats in the chronic ethanol feeding studies contained 7α-OOH (1.80 ± 0.19 nmol/g), 7β-OOH (3.23 ± 0.51 nmol/g) (Table I), 7α-OH (276 ± 47 nmol/g), 7β-OH (331 ± 61 nmol/g), 7-keto (730 ± 138 nmol/g), and cholesterol (4306 ± 661 nmol/g) (Table II). The concentration of 7-keto was two times greater than both 7α-OH and 7β-OH. 7-Keto concentrations was about 150 times higher than the sum of 7α-OOH and 7β-OOH (Table II). The mean ratio of 7-keto to cholesterol (17.0 ± 1.6%) was higher than that of 7α-OH (6.7 ± 0.6%) and that of 7β-OH (7.7 ± 0.5%; Table II).

The concentration of 7-hydroperoxycholesterol (7-OOH) was less than 2% of 7-hydroxycholesterol (7-OH). The area of 7-OH with HPLC–UV was about 30,000 arbitrary units, which was well above the lower area of detection of 600 arbitrary units. To separate 7-OH from 7-OOH[16] a normal phase column was used and thus the sensitivity of 7-OH was low because 7-OH appeared later.

Response of Hydroperoxides and Oxysterols to Alcohol Feeding

In response to chronic alcohol feeding there were increases in 7α-OOH ($P = 0.005$) and 7β-OOH ($P = 0.011$) by 169 and 199%, respectively (Table I). Chronic alcohol feeding also significantly increased 7-keto ($P = 0.01$) (Table II). The increase of 7-keto, was 74% (Table II). As the cholesterol concentration was decreased by 43% following chronic alcohol (Table II), the ratios of 7α-OH and 7β-OH, to cholesterol were increased significantly (Table II). The relative increase in 7-keto was also markedly increased when expressed relative to cholesterol (Table II).

DISCUSSION

In the present study we showed increased cholesterol hydroperoxides and oxysterols in livers of rats chronically exposed to ethanol. These data are not due to malnutrition since both control and ethanol-treated rats were fed identical amounts of the same diet, albeit with isoenergetic contributions from ethanol or carbohydrate.[13] Otherwise the ingestion of lipids, proteins, micro- and macro-nutrients were identical in both groups of rats.[13]

Lipid peroxidation was monitored by thiobarbituric acid assay as malondialdehyde (MDA) in the liver,[17] by conjugated diene formation,[18] and by 4-hydroxynonenal (HNE) concentration in microsomes of rats fed ethanol intragastrically.[19] These methods lack specificity, because in that lipid peroxide itself was not measured, or from which fatty acid hydroperoxide of phospholipid the MDA or HNE was generated.

Oxysterols and Liver Disease

In considering the raised levels of oxysterol observed in the present study it is important to introduce the concept that it may reflect decreased clearance as well as modification of regulatory enzymes in cholesterol biosynthesis such as 3-hydroxy-3-methylglutaryl CoA (HMG-CoA) reductase which catalyses HMG-CoA to mevalonate + CoASH.[20] In fact, 7-keto was a potent inhibitor of sterol synthesis in cultured mammalian cells and lowered the levels of HMG-CoA reductase activity in these cells.[21] Thus, possible increase in 7-keto occurring in liver of alcoholics would be expected to reduce cholesterol in liver. In our studies, an increase in 7-keto as well as a decrease of cholesterol in rats after chronic ethanol

TABLE II Changes in oxysterols and oxysterols relative to cholesterol in rat liver after chronic alcohol

	n	Oxysterols (nmol/g)				Oxysterols/cholesterol ratio (%)		
		7-keto	7α-OH	7β-OH	Cholesterol	7-keto/cholesterol	7α-OH/cholesterol	7β-OH/cholesterol
Control	7	730 ± 138	276# ± 47	331 ± 61	4306 ± 661	17.0 ± 1.6	6.7 ± 0.6	7.7 ± 0.5
Ethanol	7	1270 ± 117	367 ± 42	303 ± 39	2449 ± 196	55.4 ± 6.2	15.8 ± 1.8	15.8 ± 1.2
P		0.01	NS	NS	0.03	0.0006	0.0018	0.0047

Data as mean ± SEM. 7-keto, 7-ketocholesterol; 7α-OH, 7α-hydroxycholesterol; 7β-OH, 7β-hydroxycholesterol.

feeding was observed, being consistent with the *in vitro* studies.[21] Accordingly, the increase of 7-keto must not result from the increased cholesterol synthesis but from the reduced clearance of oxysterol.

Why 7-keto was significantly higher in chronic ethanol group than control but 7-OH was not, the oxidative reaction (from 7-OOH to 7-keto) was elevated but not reductive reaction to 7-OH was not elevated. Another reason is chronic ethanol reduced the activity of glutathione peroxidase.[22,23]

We believe that the increased hepatic oxysterols have important implications for alcohol-induced liver injury due to their putative cytotoxic effects. Oxysterols increase apoptosis in rat and human smooth muscle cells *in vitro*.[24,25] Increased cytokine production (i.e. interleukin-8) by monocytes and monocyte-derived macrophages exposed to oxysterols also occur.[26] In cultured rat hepatocytes, 7β-OH but not 7-keto inhibit gap junction communications without compromise of cell viability.[27] However, when 7-keto has been shown to reduce hepatocyte cell viability, the release of nitric oxide and superoxide radicals are initiated.[28] In rat hepatocytes *in vitro*, the 7-keto *per se* alters also cytosolic enzymes responsible for maintaining the oxidative equilibrium by increasing the activities of catalase and superoxide dismutase without affecting lipid peroxidation.[29] *In vitro*, 7α-OH is toxic towards hepatic epithelial and fibroblast lines.[30]

The 7-keto (and 25-hydroxycholesterol) also inhibit albumin synthesis in hepatocytes *in vitro*.[31] This is of importance in alcoholic subjects since reductions in both plasma albumin concentrations and liver protein synthesis are common feature of alcoholism.[32] Moreover, recent evidence has implicated the possibility that, in the presence of alcohol, the apoptosis in human arterial endothelial cells induced by the oxysterols 7β-OH, 7-keto, and cholesterol-5,6-epoxides is enhanced.[33] This may be related to calcium influx.[33]

Cholesterol Hydroperoxides

Cholesterol hydroperoxides have also been shown to increase in red blood cells and liver of alcoholics[9,34] which has been ascribed to increased ROS. Our present results (Table I) are compatible with the aforementioned study in liver. Cholesterol hydroperoxides are also important in tissue pathology as they can be converted to free radical species via iron-catalysed reactions.[35] It has been suggested that such radicals can initiate peroxidative damage via a chain reaction.[35] Cholesterol hydroperoxides also inhibit calmodulin *in vitro* although inhibitory activity appears to depend upon the nature of the modified cholesterol molecule.[36,37] However, compared to oxysterols, little is known about their possible cytotoxic effects. Nevertheless, their formation, as a consequence of ROS-mediated conformational changes of the parent cholesterol molecule, suggests that they are particularly suitable as an indices of tissue damage. For example, protein carbonyl, a well characterised marker of ROS activity and/or reduced anti-oxidant defences, is unaltered in this model of 6 week alcohol-feeding (Preedy, V.R., Mantle, D., unpublished data). Thus, carbonyl can be considered insensitive compared to oxysterols and cholesterol hydroperoxides which increase in the present studies.

With respect of oxysterols and cholesterol hydroperoxides, it is imprudent to ascribe these changes to membrane changes *per se*. This is because oxidised LDL, which is localised in the liver, also contain both cholesterol hydroperoxides[38,39] and oxysterols.[40] Thus, further studies are clearly warranted to ascertain if the increased cholesterol hydroperoxides and oxysterols are located within the hepatic cell membrane or cytosolic milieu. However, regardless of their location, the altered cholesterol hydroperoxides and oxysterols composition in chronic ethanol feeding can be ascribed to enhanced oxidative stress.

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