Chronic Ethanol Feeding Increases 7-Hydroperoxycholesterol and Oxysterols in Rat Skeletal Muscle

Tetsuo Fujita, Junko Adachi, Yasuhiro Ueno, Timothy J. Peters, and Victor R. Preedy

It was our hypothesis that, as a consequence of increased oxidative stress, cholesterol-derived hydroperoxides and oxysterols are increased in skeletal muscles chronically exposed to ethanol. To test this we fed male Wistar rats (0.1 kg initial body weight) a nutritionally complete liquid diet containing ethanol as 35% of total calories: controls were pair-fed identical amounts of the same diet in which ethanol was replaced by isocaloric glucose. At the end of 1½ months, soleus (type I fiber–predominant) and plantaris (type II fiber–predominant) skeletal muscles were dissected out. We measured 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). We identified and confirmed by LC-MS the presence of 7α-OOH, 7β-OOH and 7α-OH, 7β-OH and 7-keto in skeletal muscle of rats. We also showed that in response to chronic alcohol feeding, there were significant increases in soleus 7α-OH ($P = .0005$), 7β-OH ($P = .0005$), and 7-keto ($P = .0007$). In the plantaris, mean 7α-OH and 7-keto were not significantly altered ($P > .05$), but 7β-OH increased ($P = .0418$). This is the first report of 7α-OH, 7β-OH, and 7-keto oxysterols being identified in skeletal muscle of rats. Their elevation in chronic experimental alcoholism, together with increases in cholesterol hydroperoxides, may possibly represent evidence of increased oxidative stress.

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ALCOHOLIC MYOPATHY is one of the most prevalent skeletal muscle diseases in the Western hemisphere and is characterized by atrophy of type II (anaerobic, glycolytic, fast switch) fibers.[1,2] In contrast, type I fibers are relatively resistant, though they may atrophy in severe alcoholism.[1-3] Concomitant pathologies include disordered gait, myalgia, loss of muscle mass, and impaired quality-of-life measures. Although the causal agent is known, the pathogenic mechanisms between ethanol ingestion and symptomatic developments are poorly understood.[1-3] Proposed etiological factors have included impaired protein synthesis, loss of ribosomal RNA, increased RNAase activities, and protein adduct formation.[1-4]

Animal models provide an excellent way of studying alcohol toxicity, as anatomically distinct skeletal muscles can be used to represent either type I or II fibers. Thus, the soleus contains mainly type I fibers and the plantaris mainly type II fibers.[5] The suitability of the models is supported by the observation that all of the myopathic lesions in human alcoholic myopathy can be reproduced in the rat.[2,6-8] In our pathogenic investigations in this model we have used 2 models defined as either "acute" or "chronic."[2,7,8] In the acute model, rats are injected with a single bolus of ethanol, at a dose of 75 mmol/kg body weight, and killed after 1 to 24 hours. In the chronic ethanol dosing models, rats are fed an ethanol-containing diet for approximately 2 to 6 weeks.[2,7,8] In the acute model, there is no overt loss of muscle until 24 hours, whereas in the chronic model, a reduction in muscle protein is demonstrable after 2 weeks.[2,7,8]

Rats fed ethanol as 35% of their total dietary energy has reduced muscle mass almost identical to the changes seen clinically, ie, approximately 20%.[8] Other similarities include lack of malnutrition, neuropathy, and liver disease as causative factors, reduced muscle RNA, unchanged apoptotic index, reduced muscle protein, and impaired protein synthesis.[8]

More recently, reactive oxygen species–mediated damage has also been described within skeletal muscle-exposed to ethanol, particularly affecting the lipid, ie, the membrane-located domain.[9,10] Thus, we have shown that 7α- and 7β-hydroperoxycholest-5-en-3β-ol (7α-OOH and 7β-OOH) increase in rats acutely exposed to ethanol with corresponding alterations in membrane phospholipids such as increased C18:2.[9,10] However, these studies are limited in their scope for understanding the pathophysiology of alcoholic myopathy for 2 reasons.

First, there are presently no data on the chronic effects of alcohol on the hydroperoxide composition. Long-term studies may be considered to be more relevant to the clinical counterpart of prolonged and excessive ethanol misuse. The second point relates to the possibility that other cholesterol oxidation products may alter in alcohol toxicity. The oxysterols constitute biologically important pathways for the nonenzymatic modification of cholesterol such as the generation of 7α- and 7β-hydroxycholesterol (7α-OH and 7β-OH), and 3β-hydroxycholest-5-en-7-one (also termed 7-ketocholesterol; 7-keto) (Fig 1). With few exceptions, the pathways for their formation include oxidation by microsomes (P450), mitochondria, or auto-oxidation.[11] Subsequently, oxysterols may be converted to bile acids, esters, or other sterols.[11] However, some oxysterols may be derived by enzymatic reaction.[12] 7-Hydroxycholesterols are produced enzymatically by glutathione S-transferase (Ya-Ya or Ya-Yc) and selenium-containing glutathione peroxidase from rat liver cytosol.[13] The generation of oxysterols has been reviewed previously.[12]

Recently, oxysterols have received considerable attention, as these cholesterol-derived products are themselves cytotoxic.[14]

From the Department of Legal Medicine, Kobe University Graduate School of Medicine, Kobe, Japan; Department of Clinical Biochemistry, Kings College Hospital, London; and the Nutrition and Dietetics Department, King's College, London, UK.

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Address reprint requests to Junko Adachi, PhD, Department of Legal Medicine, Kobe University Graduate School of Medicine, Kusunoki-cho 7 Chuo-ku, Kobe 650-0017, Japan.

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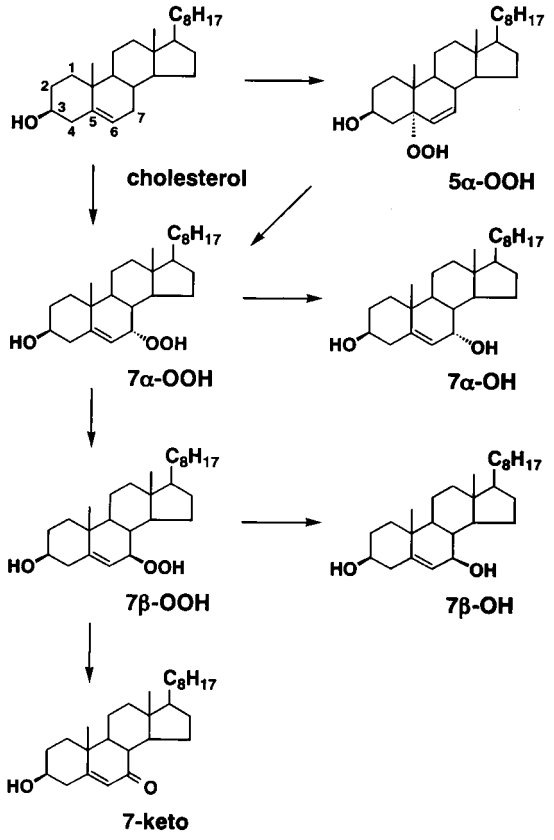

Fig 1. Schematic representation of pathways for cholesterol hydroperoxide and oxysterol production.

However, they have never previously been assayed in skeletal muscle of laboratory animals with myopathy.

To address these issues, we measured cholesterol hydroperoxides and the oxysterols 7α-OH, 7β-OH, and 7-keto in muscles from rats chronically fed ethanol-containing diets.

MATERIALS 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 Co (Osaka, Japan). Cholesterol hydroperoxides, 7α-OOH, 7β-OOH, and β-sitosterol 5α-hydroperoxide (as an internal standard, IS) for high-performance liquid chromatography with postcolumn chemiluminescence (HPLC-CL) were prepared as described previously.[15] 7-Keto, 7α-OH, 7β-OH, and β-sitosterol as the IS for HPLC-UV were purchased from Steraloids (Wilton, NH). 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).

Animal Treatments

Twenty 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 2 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[16] (see below). Controls were pair-fed the same diet in which ethanol was replaced by isocaloric glucose.

After 6 to 7 weeks, the animals were killed by decapitation and muscles dissected out. Muscle were dissected out by first removing the hind limb. The skin was removed and the hind limb muscle was exposed. The soleus and plantaris muscles were then located and dissected out. The entire muscle was ground up, but lipids were extracted from a representative portion ($<$100 mg of the muscle) to facilitate optimum assay conditions. This does not invalidate our results in any way as all tissues from both treatment groups were treated identically. The work has been carried out there under institutional supervision that ensured humane treatment of the animals.

Liquid Diets

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 9 AM and noon. 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, vol/vol), containing 0.005% (vol/vol) 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 homogenized under ice-cold conditions. The homogenate was mixed with 4 mL of chloroform/methanol (3:1, vol/vol) and 1 mL of distilled water, vortexed vigorously, and centrifuged ($\sim$800 *g*) for 20 minutes. 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 Co, Milford, MA) packed with aminopropyl-derivatized 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 quantified by HPLC-CL as previously described.[17]

HPLC-UV Analysis of Oxysterols

Oxysterols were determined by HPLC comprised of an L-7100 pump (Hitachi, Tokyo, Japan), SPD-10Avp UV detector (Shimadzu, Kyoto, Japan) set at 210 nm and 245 nm, and a Chromatopac C-R8A integrator (Shimadzu). An Inertsil ODS-2 column (GL Sciences, Tokyo, Japan) was used (5 μm, 150 $\times$ 4.6 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 to 200 ng of 7α-OH, 50 to 200 ng of 7β-OH, and 7-keto using 250 ng of IS (β-sitosterol).

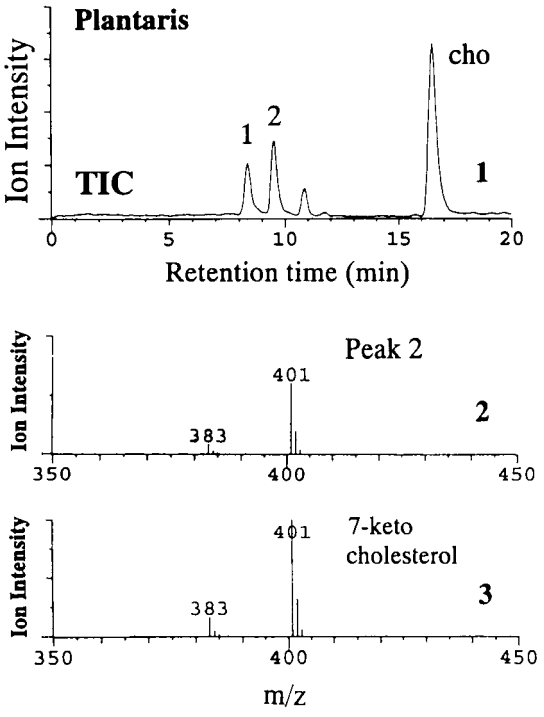

Fig 2. Total ion chromatograms of lipid extracts from rat plantaris by LC-MS. Mass spectra of standard 7-keto and peak 2.

Liquid Chromatography–Mass Spectrometry

A Hitachi L-7000 series liquid chromatography system fitted with TSKgel ODS-80Ts column (Tosoh, Tokyo, Japan) (250 × 4.6 mm internal diameter) and a model M-1200AP liquid chromatography–mass spectrometry (LC-MS) system that incorporated an atmospheric chemical ionization system (Hitachi) were used to identify 7-OOH, 7-OH, and 7-keto. The mobile phase, methanol containing 10 mmol/L ammonium acetate, was delivered at a flow rate of 0.7 mL/min.

Statistical Analysis

All data are expressed as the mean ± SEM of 4 to 10 observations in each group. Due to limitations in the availability of assay space, we were unable to analyze all analytes in every single muscle. However, the number of observations or analytes measured in these studies are displayed in the appropriate text or tables. Differences between groups were assessed by Student's *t* test.

RESULTS

In the ensuing synopsis of the data, we first report on the verification and quantification of the oxysterols in skeletal muscle, which is a novel feature of these studies. This is followed by a description of the effects of alcohol on muscle oxysterols and cholesterol hydroperoxides in chronic ethanol-fed rats.

Skeletal Muscle Weights

The weights of soleus and plantaris in control rats at the end of the study were 214 ± 5 (n = 10) and 451 ± 11 (n = 10) mg, respectively. Corresponding weights of the soleus and plantaris muscles in alcohol-fed were (all *P* values are compared to controls) 174 ± 2 mg (n = 10; *P* = .0001) and 377 ± 14 mg (n = 10; *P* = .0100), respectively.

The mean ratios of body weight, soleus, plantaris after chronic ethanol to control group were 93%, 81%, and 84%, respectively. Accordingly, the drop in skeletal muscle weights were greater than that in body weight after chronic ethanol. However, as we have described in detail previously, in the chronic alcohol feeding model, body weights alone are difficult to interpret.[18] This is because rats fed liquid diet engorge themselves causing marked changes in body weight.[18]

The mechanisms responsible for the decrease in muscle weights pertain to changes in protein turnover.[1-5] Specifically, reduction in protein synthesis may be contributory factors although as mentioned earlier the connection between changes in protein turnover and altered protein synthesis are uncharacterized.[1-5]

Oxysterols in Skeletal Muscle

Oxysterols were identified by LC-MS. Figure 2 shows total ion chromatograms of lipid extract from rat plantaris by LC-MS. Peaks 1 and 2 appeared at retention times (RTs) of 8.4 and 9.6 minutes, respectively. The mass spectra of standard 7-OOH (column 2), 7-OH (column 4), and peak 1 (columns 1 and 3) are shown in Fig 3. Standard 7-OOH had an ion $[M+H\text{-}H_2O]^+$ at m/z 401 and fragment ions at m/z 385 and m/z 367, while standard 7-OH had an ion $[M+H\text{-}H_2O]^+$ at m/z 385 and a fragment ion at m/z 367. The RTs of standard 7-OOH and 7-OH were about 8.4 minutes. Accordingly, peak 1 at RT of 8.4 minutes (column 3), which had a larger ion at m/z 385 than at

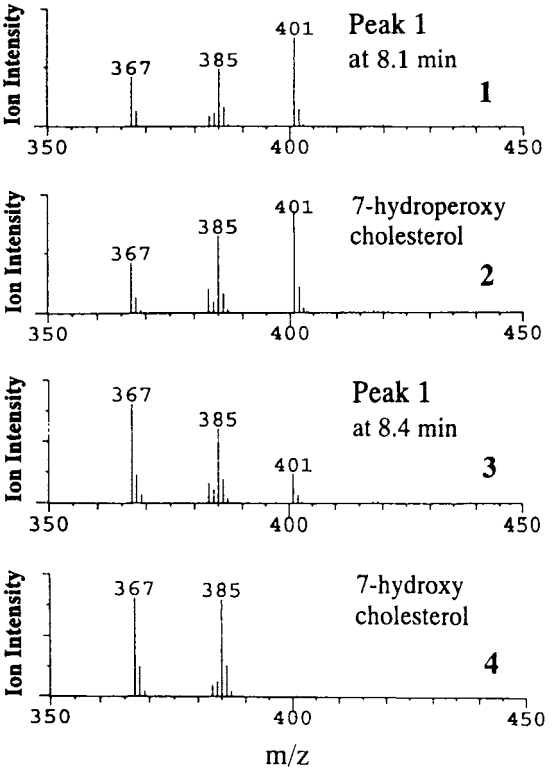

Fig 3. Mass spectra of standard 7-OOH and 7-OH and peak 1 by LC-MS.

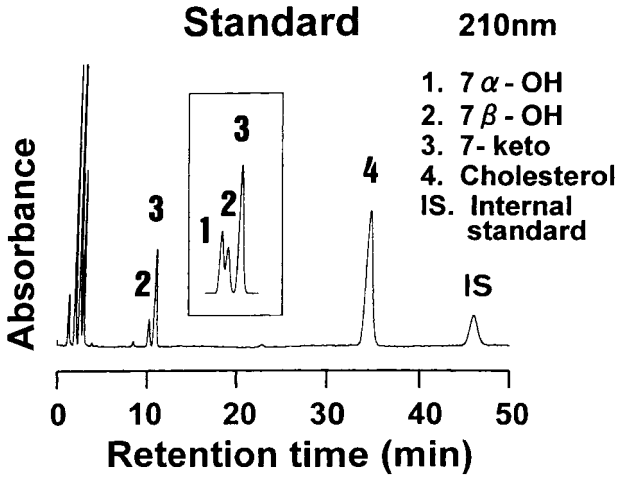

Fig 4. HPLC analysis of standard cholesterol oxidation products with UV detection at 210 nm.

m/z 401, was assumed to be a mixture of 7-OOH and 7-OH, whereas peak 1 subjected to MS at 8.1 minutes (column 1) was assumed to be 7-OOH. The peak of 7-OOH appeared faster than that of 7-OH. The mass spectra of standard 7-keto and peak 2 from plantaris are shown in Fig 2. Standard 7-keto had an ion $[M+H]^+$ at m/z 401 and an ion $[M+H\text{-}H_2O]^+$ at m/z 383. The RT and mass spectrum of peak 2 were almost identical with 7-keto. Accordingly, peak 2 was identified as 7-keto.

HPLC of standard 7α-OH, 7β-OH, and 7-keto and IS showed successful separation of compounds, with respective retention times of approximately 9.7, 10.1, 10.8, and 45.6 minutes (Fig 4). Similar successful separation of oxysterols in skeletal muscle samples was also achieved (Fig 5). Retention times of 7α-OH, 7β-OH, and 7-keto were virtually identical to standard solutions (Fig 4).

In soleus muscle of control rats, 7α-OH, 7β-OH, and 7-keto concentrations were 24.7, 29.1, and 80.1 nmol/g, respectively. 7-Keto concentration was 3-fold higher than 7α-OH or 7β-OH (Table 1). Similar data were obtained in plantaris muscle of control rats, although 7β-OH level was much lower than 7α-OH. 7-Keto concentration in plantaris was lower compared to the soleus muscle (Table 1).

Cholesterol Hydroperoxides

As with the hydroperoxides, there was complete separation of these compounds. The hydroperoxides 7α-OOH and 7β-OOH were similar to those reported previously in skeletal muscle (Table 2).[9,10]

Response of Oxysterols to Alcohol Feeding

In response to chronic alcohol feeding there were increases in soleus 7α-OH (P = .0005), 7β-OH (P = .005), and 7-keto (P = .0007) (Table 1). Similar results were obtained in the plantaris, but changes in 7α-OH and 7-keto failed to achieve significance, presumably due to the larger coefficients of variation (Table 1).

Response of Cholesterol Hydroperoxides to Alcohol Feeding

Alcohol feeding had significant effect on 7α-OOH and 7β-OOH (Table 2) in soleus and plantaris. In both muscles, the

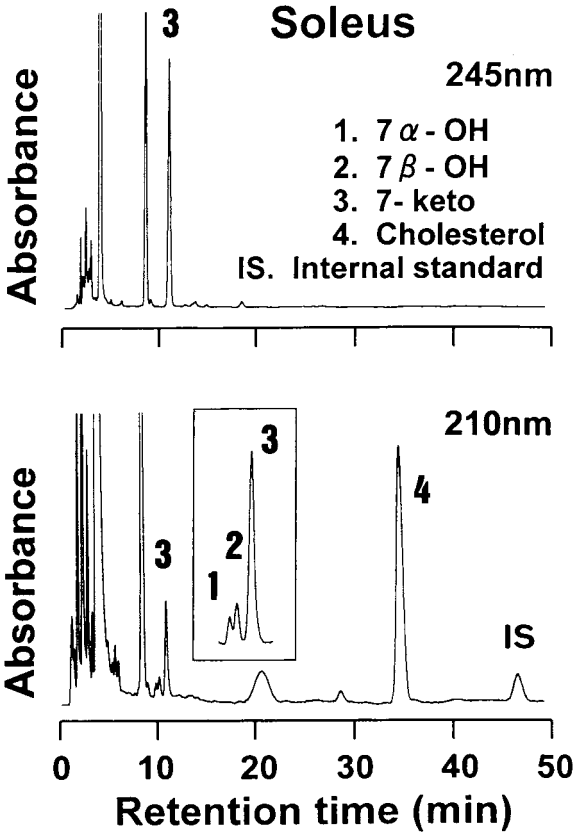

Fig 5. HPLC analysis with UV detection at either 245 or 210 nm of cholesterol oxidation products in soleus skeletal muscle. The wavelength at 245 nm is employed to show enhanced detection of 7-keto.

increase of 7α-OOH, was about 50%, while that of 7β-OOH was about 70%.

DISCUSSION

In this experimental model, blood ethanol concentrations are similar to those seen clinically in alcohol abusers, ie, 50 to 60 mmol/L.[19] Pathophysiological changes within skeletal muscle are also similar to those seen clinically.[20] These perturbations

Table 1. Oxysterols in Skeletal Muscles of Control and Ethanol-Fed Rats

	nmol/g Wet Weight			
	Control	Ethanol-Fed	% Change	*P*
Soleus				
7α-OH	24.74 ± 1.19 (5)	33.79 ± 1.07 (5)	+37	.0005
7β-OH	29.07 ± 1.36 (5)	39.80 ± 1.35 (5)	+37	.0005
7-keto	80.13 ± 3.37 (5)	108.31 ± 4.05 (5)	+35	.0007
Plantaris				
7α-OH	29.33 ± 3.18 (5)	36.32 ± 5.55 (5)	+24	>.05
7β-OH	14.98 ± 2.16 (5)	21.41 ± 1.55 (5)	+43	.0418
7-keto	37.28 ± 4.45 (5)	53.46 ± 6.74 (5)	+43	>.05

NOTE. Oxysterols were measured in control rats and ethanol-fed rats by HPLC-UV. Data are means ± SEM with the number of observations in parenthesis; n values pertain to the number of individual muscles from individual rats.

Table 2. Cholesterol Hydroperoxides in Skeletal Muscle of Control and Ethanol-Fed Rats

	nmol/g Wet Weight			
	Control	Ethanol-Fed	% Change	*P*
Soleus				
7α-OOH	0.96 ± 0.09 (6)	1.48 ± 0.19 (4)	+54	.0247
7β-OOH	2.35 ± 0.19 (6)	4.11 ± 0.75 (4)	+75	.0262
Plantaris				
7α-OOH	3.39 ± 0.29 (6)	4.80 ± 0.53 (5)	+42	.0384
7β-OOH	10.49 ± 1.12 (6)	17.89 ± 2.73 (5)	+71	.0250

NOTE. Cholesterol hydroperoxides were measured in control rats and ethanol-fed Wistar rats by HPLC-CL. Data are means ± SEM with the number of observations in parenthesis; n values pertain to the number of individual muscles from individual rats.

within ethanol exposed muscle include reduced protein synthesis, loss of ribosomal RNA, reduced fiber diameters, and induction of protein-acetaldehyde adduct levels. These changes have been reviewed by us previously.[2,6-8]

In these studies, we investigated the response of skeletal muscle to an increased oxidative load induced by chronic alcohol feeding. Increased oxidative stress has been shown to occur in a variety of tissues exposed to ethanol such as the liver, CNS, and heart.[21] In contrast, the responses of skeletal muscle antioxidant defences and/or markers of increased oxidative stress are poorly characterized, even though such changes have been postulated to occur.[22]

We have recently described changes in skeletal muscle in response to acute ethanol dosage.[9,10] At 24 hours after ethanol dosage, both 7α-OOH and 7β-OOH are increased equally in soleus and plantaris, reflecting enhanced oxidative stress in these muscles. Subsequent studies at earlier time points demonstrated significant increases in 7α-OOH and 7β-OOH in plantaris at 2.5 hours following acute alcohol injection, but not in the soleus.[10] In the present study, chronic alcohol feeding increased both 7α-OOH and 7β-OOH in soleus and plantaris muscles, which is further evidence that alcohol increased oxidative stress in skeletal muscle. The increase in soleus by chronic alcohol was greater than those by acute alcohol reported previously.[9]

As far as we are aware, this is the first report of oxysterols in skeletal muscle of rats with concomitant confirmation of molecular identity by LC-MS. The oxysterols 7α-OH and 7β-OH and 7-keto have been reported in chicken meat and these were shown to be increased on cooking.[23] 7-Keto, 7α-OH, and 7β-OH have also been identified in pig and mouse skeletal muscle.[24] In vitro, 25-hydroxycholesterol has also been shown to be toxic to chick myoblasts as defined by their transformation to myotubes.[25] However, this is the first time that oxysterols have been identified in skeletal muscle in a pathophysiological situation characterized by reduced muscle weights.

We found that 7α-OH, 7β-OH, and 7-keto increased significantly in soleus after chronic alcohol feeding, while in the plantaris only 7β-OH increased significantly, which supports the supposition that oxysterols as good markers of oxidative stress. However, an unusual feature of the present studies relates to the phenomena of type I atrophy as reflected by the reduction in soleus weight. In contrast, the change in type II plantaris is similar to those recorded previously. We are unable to explain why this occurred in this particular series of animals and as far as we are aware this is an exceptional finding although consistent with other studies showing that type I fibers atrophy in severe alcoholism.[26] For example, in one study, needle biopsy of quadriceps from alcoholic patients showed concomitant atrophy of both type I ($P < .05$) and type II ($P < .001$) fibers.[26] Alcohol-induced type I fiber atrophy has also been reported in rats studies.[27] We can only assume that the present study represents one end of the spectrum of muscle changes (ie, a statistical phenomena whereby over the 13-year period since our studies commenced, a significant type I atrophy is to be expected by chance in 1 study).

Oxysterols may be used as a general indicator of lipid peroxidation and can be reduced with vitamin E.[28] However, they also have defined and well-characterized cytotoxic properties. Thus, in smooth muscle and endothelial cells, oxysterol are responsible for the pathogenicity of oxidized low-density lipoprotein and may target cells directly.[29] In a comparison of 7β-OH and 7-keto, both agents displayed cytotoxicity as reflected by increased cell permeability and cell-impaired adhesion.[30] Additionally, increased apoptosis also occurs with DNA fragmentation and/or condensation in rat and human smooth muscle cells exposed to oxysterols.[30] However, the pathogenic role of oxysterols in fibroblasts is less certain.[30] In contrast, cholesterol alone has no pathogenicity compared to the oxysterols per se, which induces cell death in aortic smooth muscle in vitro.[31] Indirect effects have been supported by studies showing increased cytokine production (ie, interleukin-8) by monocytes and monocyte-derived macrophages exposed to oxysterols.[32]

Most of the skeletal muscle cholesterol is located within the tissue membrane (ie, sarcolemma[33]) and increased hydroperoxides and oxysterols suggest defective functional sarcolemma. Indeed, early studies have shown that ethanol exposure causes dilatation of sarcoplasmic reticulum and increases Na^+-K^+ adenosine triphosphatase (ATPase) activity.[34] Increased activity of Na^+-K^+ ATPase may possibly occur via upregulation of [^{3}H]-ouabain binding sites,[35] although this has been refuted.[34] Sarcoplasmic reticulum from skeletal muscles of ethanol-fed rats is also defective with respect to calcium transport and storage (ie, ascribed as being "leakier" and "less ordered"[36,37]). However, other studies suggest that skeletal muscle Ca^{2+} ATPase activity is unaffected by chronic ethanol feeding.[38]

In conclusion, this is the first report of 7α-OH, 7β-OH, and 7-keto oxysterols being identified in skeletal muscle of rats. Their elevation in chronic experimental alcoholism, together with increases in cholesterol hydroperoxides, may possibly represent evidence of increased oxidative stress.

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