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Comparative Effects of Acute Ethanol Dosage on Liver and Muscle Protein Metabolism

Matthew E. Reilly, David Mantle,† Jonathan Salisbury,‡ Timothy J. Peters* and Victor R. Preedy§‖*

Departments of *Clinical Biochemistry and ‡Histopathology, King's College School of Medicine, London SE5 9PJ; †Department of Neurochemistry, Regional Neurosciences Centre, Newcastle General Hospital, Newcastle-Upon-Tyne NE4 6BE; and §Departments of Nutrition and Dietetics, School of Life Sciences, King's College London, London SE1 9NN, U.K.

ABSTRACT. Experiments were performed to address some outstanding issues and investigate possible mechanisms relating to the acute comparative effects of ethanol on liver and skeletal muscle protein metabolism. Ethanol (EtOH)-treated rats were injected (i.p.) with a bolus of EtOH (75 mmol/kg body weight) and sacrificed at 20 min, 1-, 2.5-, 6-, and 24-hr time points. Control rats were injected with saline (Con-Sal; 0.15 mmol/L NaCl). All 24-hr ethanol-treated animals were compared with saline-injected rats subjected to controlled feeding (i.e. pair-fed controls for 24 hr EtOH). At 24 hr, there was no measurable alcohol in the plasma, whereas high levels were seen from 20 min to 6 hr (up to 448 mg/dL). Plasma levels of albumin were reduced at initial time points, and activities of aspartate aminotransferase increased, but there was no histological evidence of overt tissue damage either in muscle or liver. Hepatic protein and RNA contents and indices of tissue (C_s and k_s) and whole-body (V_s) protein synthesis were significantly increased in ethanol-dosed rats relative to saline-injected pair-fed controls at 24 hr. In the liver, four of the seven cytoplasmic proteases investigated (alanyl-, arginyl-, and pyroglutamyl-aminopeptidases and proline-endopeptidase) showed significant increases in activity at 24 hr relative to pair-fed controls; four of the six lysosomal proteases showed significant decreases in activity (dipeptidyl-aminopeptidase II and cathepsins B, L, and H). In skeletal muscle, k_s fell progressively between 1 and 24 hr (−25 to −69%; $P < 0.001$), but no significant changes in skeletal muscle protease activities were seen at 24 hr. At 24 hr after ethanol dosage *in vivo*, there were no significant increases in protein carbonyl content in liver or skeletal muscle compared to pair-fed controls (muscle levels actually decreased slightly). However, using either rat or human tissue, both liver and muscle carbonyl increased *in vitro* in response to superoxide and hydroxyl radicals: muscle was more susceptible to carbonyl formation than liver and both tissues were more sensitive to hydroxyl compared to superoxide radicals. These results show divergent effects of acute ethanol treatment on liver and skeletal muscle protein metabolism, which may not be linked to *in vivo* free radical-mediated protein damage (as indicated by carbonyl formation), at least in the short term. BIOCHEM PHARMACOL **60**;12:1773–1785, 2000. © 2000 Elsevier Science Inc.

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Skeletal muscle accounts for between one-fifth and one-quarter of whole-body protein metabolism. A reduction in protein synthesis is initially seen that is thought to be central to altered muscle composition and function over longer treatment periods [1]. This has implications for whole-body nitrogen economy [2], which must also take into account protein degradative pathways. However, skeletal muscle proteolysis is thought to be relatively insensitive to ethanol, although there is some inconsistency in reported data, for example in studies showing increased or decreased muscle protein breakdown [3]. This inconsistency may relate to either the method used to measure protein breakdown or the failure to examine multiple time points in the experimental design (reviewed in [3]). Furthermore, measurements in virtually all alcohol-dosing studies have been carried out in the presence of circulating ethanol, whereas the effects during the immediate post-ethanol period have been comparatively ignored. In addition, the examination of muscle protein metabolism has very often been carried out in isolation, without reference to the other major contributors to whole-body protein metabolism such as the liver, which also accounts for 20–25% of whole-body protein synthesis.

‖ Corresponding author: Dr. Victor R. Preedy, Dept. of Nutrition and Dept. of Dietetics, King's College London, 150 Stamford Street, London SE1 9NN, UK. Tel. +44-0207-848.4255; FAX +44-0207-848.4185; E-mail: victor.preedy@kcl.ac.uk

¶ *Abbreviations:* C_s, synthetic capacity; H&E, hematoxylin and eosin; EtOH, ethanol; k_s, fractional rate of protein synthesis; k_{RNA}, RNA activity; $O_2^{-\cdot}$, superoxide radicals; $OH^{\cdot}$, hydroxyl radicals; S_i, specific radioactivity of free amino acid in intracellular pools; S_b, specific radioactivity of protein-bound amino acid; S_p, specific radioactivity of free amino acid in the plasma pool; ROS, reactive oxygen species; V_s, absolute rate of protein synthesis.

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Alcoholic liver disease is characterised by diverse but well-defined morphological changes including increased fatty deposits, alcoholic hepatitis, and fibrosis [4]. In advanced cases, hepatitis and cirrhosis may develop [4]. Some studies have suggested that there is a relationship between alcoholic liver disease and alcohol-induced muscle disease (AIMD) [5], though this has been refuted [3]. Ethanol itself, rather than malnutrition, has been confirmed as a major contributor to changes in liver acting mainly through metabolic and biochemical mechanisms [3, 6]. However, there are inconsistent data on ethanol-induced defects in liver protein metabolism *in vivo*, which appear to show decreased [7] or maintained [8] rates of protein synthesis. Similarly to muscle, hepatic protein degradation, measured directly or indirectly, has been observed to be either maintained [9], reduced [10], or increased [9]. Furthermore, there is also controversy over the cause of the perturbations in hepatic protein metabolism following ethanol administration.

There is increasing evidence that free radicals contribute to the pathogenesis of alcoholic liver and muscle disease, although not all experimental studies support this hypothesis (see [11–14]). There are numerous indices of oxidative stress of varying specificity and/or sensitivity [15–17] and recent studies have suggested that, in terms of muscle protein metabolism, the measurement of protein carbonyl is a suitably sensitive and specific indicator of oxidative stress, particularly as these proteins are readily formed under conditions of atrophy or wasting [18]. However, as there is no information on carbonyl formation in muscle in response to ethanol, we concur that the role of free radicals in this disorder remains a matter for debate.

The present study was undertaken to resolve some of the issues raised above. Acute changes in liver protein synthesis and indices of protein degradation due to ethanol administration were measured. These were compared with acute changes in skeletal muscle. We chose the gastrocnemius as its fibre-type composition is representative of the musculature as a whole, and its response to alcohol toxicity is identical to that occurring in the entire body muscle [19]. The possibility that alcohol exposure increases carbonyl formation was also investigated, as this is generally recognised as a specific and sensitive marker of oxidative damage to proteins. It is important to note that many studies on free radical-related damage during alcohol misuse have focused on lipid changes, such as the use of thiobarbituric acid reactive substance (TBARS). However, our study overall was mainly concerned with protein rather than lipid metabolism. Controlled feeding was used to discern alterations due to ethanol *per se* rather than those due to anorexia, a common feature of alcohol dosage regimens [20].

MATERIALS AND METHODS

Animals and Chemicals

L-[4-^{3}H]Phenylalanine was obtained from Amersham International and all other reagents were obtained from Merck/BDH. Male Wistar rats (approx. 90 g) were obtained from Charles River and fed *ad lib.* on a commercial pelleted diet (ERD diet, Labsure, UK) until they reached a body weight of approximately 150 g. They were housed in cages in an air-conditioned (20–25°), humidity-controlled (40–60%) animal house with a 12-hour light–dark cycle starting at 8 a.m.

Ethanol Dosage Time–Course Experiment

Animals (approximately 150 g) were ranked and divided into groups of equal mean body weight. Control rats were injected with saline (0.15 mol/L of NaCl in the proportion of 1 mL per 100 g body weight, i.p.) and killed 2.5 hr later. Ethanol-dosed rats were injected with a bolus of 43% ethanol (75 mmol/kg body weight, prepared in 0.15 mol/L of NaCl; 1 mL per 100 g body weight, i.p.) and killed either 20 min, 1, 2.5, 6, or 24 hr later. Preliminary experiments showed that ethanol caused marked reductions in food intake (i.e. anorexia) over 24-hr treatments. This has the potential to alter protein synthesis [20]. While the *ad lib.* fed, saline-treated control rats consumed an average of 23 ± 1 g/day, the 24-hr ethanol-treated animals consumed 1.1 ± 0.3 g. In the alcohol-treated group, some rats ate nothing at all, whilst the others consumed only a few grams. To control for this, we introduced a weight-matched group of animals, subsequently termed *pair-fed* for comparison with the 24-hr ethanol-treated group, to account for intoxication-induced undernutrition [1]. The pair-feeding regime was designed on a treatment group basis with the amount of diet given to the pair-fed groups based on that consumed by the respective treatment groups [1]. In some 24-hr studies, we did not present these pair-fed rats with any diet, which essentially resolved problems of some rats eating only a fraction of a gram of diet. There was no discernible difference in metabolic parameters between rats consuming only a few grams and nothing at all. We do not believe that there were overt differences in tissue hydration as DNA concentrations in liver of control 24-hr pair-fed saline-injected and 24-hr ethanol-injected rats were similar, i.e. 6.80 ± 0.40 and 6.96 ± 0.08 mg/g wet weight, respectively ($P > 0.05$). In muscle, corresponding values were 1.59 ± 0.06 and 1.68 ± 0.05 mg/g wet weight, respectively ($P > 0.05$).

Determination of Fractional Protein Synthesis Rates In Vivo

The fractional rate of protein synthesis (k_s,¶ defined as the percentage of tissue protein renewed each day, i.e. %/day) was measured as described previously [21] using a flooding dose of L-[4-^{3}H]phenylalanine. Briefly, L-[4-^{3}H]phenylalanine was injected into rats via a lateral tail vein (150 mmol/L, 1 mL/100 g body weight). Exactly 10 min after injection of isotope, rats were killed by decapitation and tissues were quickly dissected out, blotted dry, weighed, and frozen in liquid nitrogen. All tissue samples were stored at

−70° until processing for specific radioactivities of phenylalanine in free (S_i), protein-bound (S_b), and plasma-free (S_p) amino acid pools for calculation of k_s [21] using the formula:

$$k_s = \frac{S_b \times 100\ (\%/\text{day})}{S_i \times t}$$

where 't' was the period between injection of isotope and freezing of the tissue in liquid nitrogen during which the label was incorporated, S_i was the specific radioactivity of the free amino acid in intracellular pools, and S_b the specific radioactivity of the amino acid in protein-bound pools.

Calculations of protein synthesis rates are usually based on the assumption that amino acids are incorporated into proteins via intracellular amino acid pools as represented by acid supernatants of tissue homogenates (i.e. S_i). For skeletal muscle, values of S_i were taken from tissues dissected from rats at the end of 10-min labelling, as these change little over the labelling period. However, it is important to note that methodologically, it is necessary to define the change in liver S_i accurately, since liver S_i falls rapidly over short time periods. To accommodate for this potential source of error, k_s values were calculated using time-corrected values of the free specific radioactivity of phenylalanine in the intracellular amino acid pools (i.e. mean S_i was used; for a fuller explanation of the rationale for this calculation, see [22]). For comparative purposes, synthesis rates were also calculated on the assumption that amino acids were incorporated into proteins via extracellular pools as represented by acid-soluble fractions of the plasma (S_p), i.e. by substituting S_i with S_p in the formula used to calculate k_s. Values of k_s obtained using either S_p or S_i showed good agreement and conclusions were identical. The RNA activity (k_{RNA}, defined as the amount of protein synthesis per unit of RNA; mg protein/mg RNA/day) is derived by division of k_s by protein synthetic capacity (C_s, mg RNA/g protein).

Measurement of Cellular Protease Activities

Cytoplasmic and lysosomal protease activities were measured in gastrocnemius muscle and liver from 24-hr ethanol- and pair-fed, saline-dosed control rats as described previously [23].

In Vitro *Generation of Reactive Oxygen Species, Hydroxyl ($OH^{\cdot}$), or Superoxide ($O_2^{-\cdot}$) Radicals*

Reactive oxygen species were generated *in vitro* using a ^{60}Co gamma radiation source, as described previously [24]. Tissue homogenates (1:10, tissue:buffer, weight: volume ratio) were placed in 10-mL glass tubes, sealed, and then exposed to gamma radiation (100 krad per hour) to produce the equivalent of 600 nmol/mL/hr of either $OH^{\cdot}$ or $O_2^{-\cdot}$ radical species. For generation of $OH^{\cdot}$ radicals, samples in 100 mmol/L of KH_2PO_4/K_2HPO_4 buffer pH 7.5 were gassed to saturation with N_2O. For the generation of $O_2^{-\cdot}$ radicals, the tissue homogenates in 100 mmol/L of $KH_2PO_4/2HPO_4$:20 mmol/L of sodium formate buffer pH 7.5 were gassed to saturation with O_2; in the latter, formate was used as an active $OH^{\cdot}$ scavenger [24].

Other Assays

Total RNA, DNA, and protein were measured as previously described [1]. Protein carbonyl content was quantified via a method based on absorption spectrophotometry of dinitrophenylhydrazine–carbonyl adducts as described previously [25]. Plasma analytes were measured in blood collected into ice-cold, heparinised tubes. Plasma was assayed by standard laboratory procedures.

Histology

Conventional light microscopic analysis of H&E-stained sections of liver and whole gastrocnemius muscle was carried out according to previously described methods [26].

Statistics

All data are expressed as means ± SEM of 4 to 8 observations in each group unless stated otherwise. Differences between groups in the time–course experiment were assessed with ANOVA and Student's unpaired *t*-test using the pooled estimate of variance. Analysis between just the 24-hr ethanol-treated and pair-fed controls was performed using Student's unpaired *t*-test. All tests were performed at the 5% ($P < 0.05$), 1% ($P < 0.01$), and 0.1% ($P < 0.001$) levels.

RESULTS

In the following section, attention is primarily focused on the liver. For comparison, the effects of ethanol on gastrocnemius muscle (taken to represent the skeletal musculature as a whole) are also described.

Histological Findings

Liver and gastrocnemius sections taken at 20 min, 1, 2.5, 6, and 24 hr showed that very little visible tissue damage occurred due to the ethanol dosing (only the histology for the 24-hr time points are displayed in Fig. 1). H&E-stained liver sections from ethanol-treated rats showed a sparse degree of scattered hepatocyte necrosis, fat droplets, and mild lymphocyte infiltration which was also seen in control livers. Gastrocnemius muscle sections from 24-hr ethanol-treated rats showed normal histology (Fig. 1). No overt evidence of fibre necrosis or lymphocyte infiltration due to ethanol was visible in any of the sections, though there were varying numbers of eosinophilic fibres in muscle sections from both ethanol- and saline-treated rats (Fig. 1).

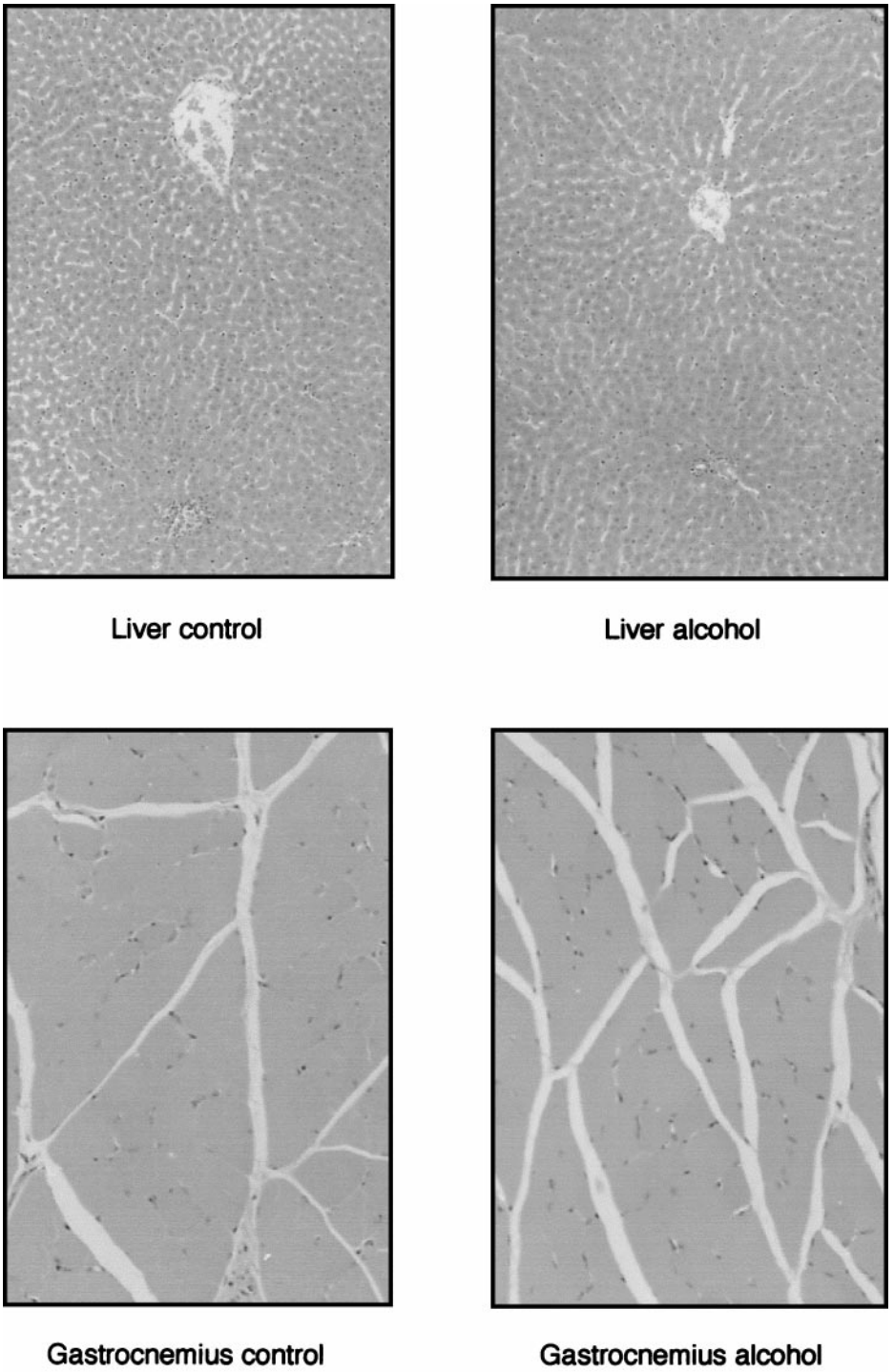

FIG. 1. Light microscopic sections of livers and muscle from 24-hr saline or ethanol-treated, pair-fed rats (see Methods for details of 24-hr pair-feeding regime). Liver sections from both 24-hr saline and ethanol-treated animals were similar, with both showing either normal liver pathology or, in a few examples, minimal degrees of fatty changes, lymphocyte infiltration or hepatocyte necrosis. Light microscopic sections of gastrocnemius muscle from 24-hr saline and ethanol-treated rats showed no discernible difference between samples from control and ethanol-treated rats.

Changes in Plasma Analytes due to Acute Ethanol Toxicity

Initially, there were rapid and significant reductions in total plasma protein and albumin. The concentrations of both total plasma protein and albumin fell by between 7% and 14%, respectively, until 6 hr after injection, at which point levels rose back toward normal values (Fig. 2). At the 24-hr time point, control plasma albumin values were reached, while in the case of total plasma protein they were surpassed, with a rise of 13% relative to pair-fed controls ($P < 0.001$, Fig. 2).

The activities of the marker enzyme, aspartate amino-

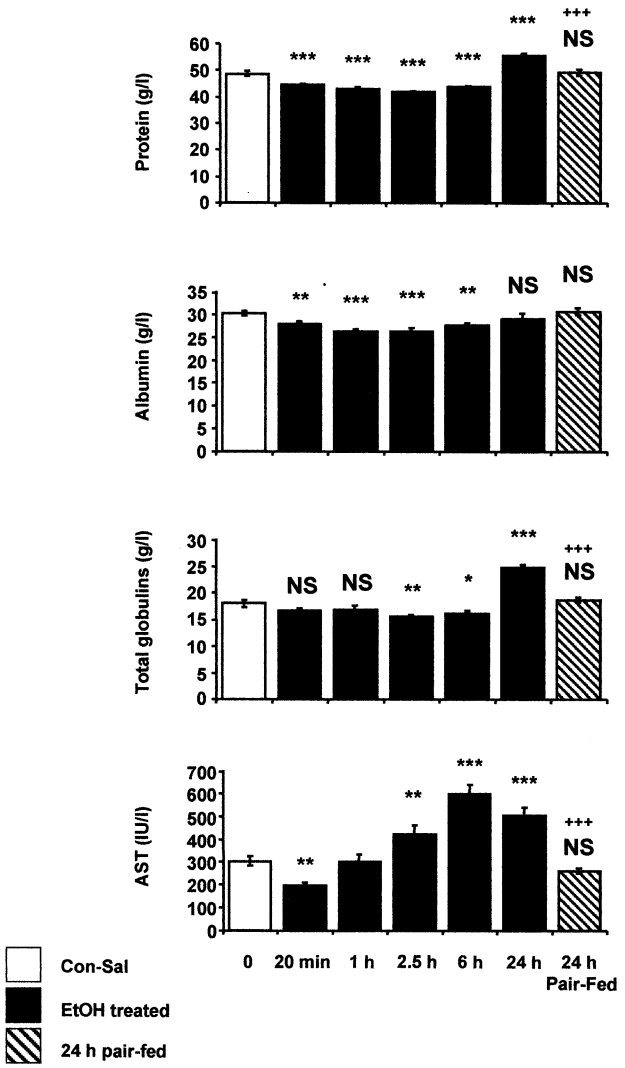

FIG. 2. Plasma analyte levels in male Wistar rats during the 24-hr period following acute (i.p.) saline or ethanol bolus injection (see Methods for additional details). Groups of ethanol-treated rats were killed at 20 min, 1, 2.5, 6, and 24 hr. Control (i.e. saline-injected; Con-Sal) rats were treated identically with isovolumetric saline (0.15 mol/L of NaCl) and fed *ad lib.* on standard laboratory chow. In a second control group, rats were injected with saline as in the Con-Sal group, but were fed identical amounts of food as consumed by 24-hr ethanol-treated animals. Following killing, blood was sampled into heparinised tubes. All data points represent means ± SEM (N = 4–8). Significance was assessed by Student's unpaired *t*-test using the pooled estimate of variance after one-way ANOVA (N = 4–6). *P* values for differences between the control saline (Con-Sal: open bars) group and the ethanol-treated groups (filled bars) at different time points are denoted by asterisks: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; NS, not significant. $P > 0.05$. Comparisons between 24-hr EtOH-treated rats and the 24-hr pair-fed control group (hatched bars) are denoted by $^{+++}$, $P < 0.001$ or otherwise differences did not attain significance. AST, aspartate aminotransferase. For a detailed discussion of the need for pair-fed controls, see the Methods section and [1].

transferase (AST), showed a similar, although more acute, rebound effect. Following initial reductions in the first hour, AST activities surpassed control values after 2.5 hr ($P < 0.01$) and after 24 hr, reached levels 65% and 91% above those of the saline-injected and pair-fed controls ($P < 0.001$; Fig. 2). Activities of alkaline phosphatase (data not shown) did not show any significant alteration due to ethanol, which is probably related to the fact that in the rat, but not in man, this enzyme is derived largely from the gastrointestinal tract [27].

Changes in Tissue Weights and Composition

Liver weights fell 12% at 6 hr ($P < 0.01$) and by 21% after 24 hr ($P < 0.001$) relative to saline-injected controls (Fig. 3). Changes in liver weight were similar in 24-hr ethanol-treated and pair-fed rats. Similarly, hepatic protein contents were reduced by 14% in the 24-hr ethanol-treated animals ($P < 0.05$) and by 27% in the pair-fed for 24-hr group relative to saline-injected controls ($P < 0.001$; Fig. 3). Total liver RNA content was significantly reduced only in the pair-fed for 24-hr group ($P < 0.05$). Both total RNA and protein contents were significantly lower in the pair-fed group relative to the 24-hr ethanol-treated animals (*P* values 0.05 and 0.001, respectively; Fig. 3).

Changes in muscle protein and nucleic acid composition were refractory at initial time points. Significant nutrition-independent reductions in total gastrocnemius muscle protein and RNA contents were observed only at the 24-hr time point. Protein contents fell by 12% ($P < 0.001$) and RNA by 26% ($P < 0.01$) relative to saline-injected controls (Fig. 3). Levels of both protein and RNA were significantly lower in 24-hr ethanol-treated rats relative to pair-fed controls (*P* values between 0.01 and 0.001; Fig. 3). No significant alterations in cellular DNA content were seen in either liver or skeletal muscle at any time point after ethanol administration (Fig. 3).

Changes in Rates of Liver and Muscle Protein Synthesis in response to Ethanol Dosage

Fractional rates of liver protein synthesis (k_s) were largely maintained up to 2.5 hr post alcohol dosage. After 6 hr, however, increase up to 14% were seen ($P < 0.05$; Table 1). This effect progressed up until the 24-hr time point, where k_s was up to 18% higher than values from control saline-injected rats ($P < 0.025$; Table 1). Similar changes occurred in the hepatic protein synthetic capacity (C_s; μg RNA/mg protein—an approximate measure of the amount of protein synthetic machinery) and as a consequence RNA activity (k_{RNA}; mg protein/day/mg RNA—a parameter which describes in dynamic terms the synthesis of protein per unit of RNA) was not significantly affected (Table 2).

Gastrocnemius muscle k_s fell by up to 31% over the initial 6 hr following ethanol administration and by 69% at the 24-hr time point (*P* values between 0.01 and 0.001; Table 1). These changes were again reflected by similar alterations in k_{RNA} and C_s and, as in the liver, alterations in k_{RNA} were similar in direction and magnitude as those

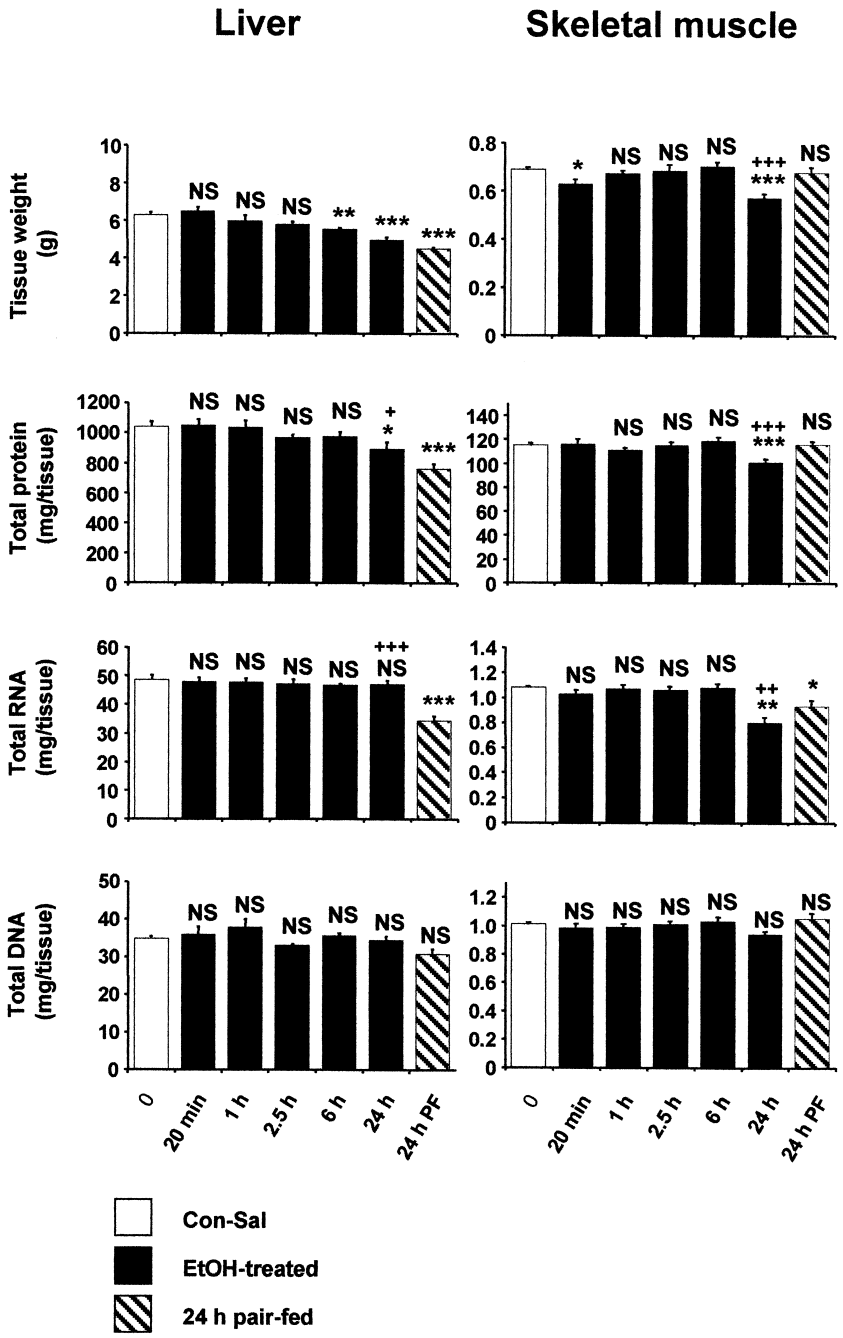

FIG. 3. Liver and skeletal muscle composition in male Wistar rats during the 24-hr period following acute (i.p.) saline or ethanol bolus injection (see legend to Fig. 1 for further details). All data are presented as means ± SEM (N = 4–8). Significance was assessed using Student's *t*-test after ANOVA using the pooled estimate of variance (N = 4–8). *P* values for differences between the control saline (Con-Sal: open bars) group and the ethanol-treated groups (filled bars) at different time points are denoted by asterisks: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; NS, not significant. $P > 0.05$. Comparisons between 24-hr EtOH-treated rats and the pair-fed control group (hatched bars) are denoted by $^{+++}$, $P < 0.001$ or otherwise differences did not attain significance.

for k_s (Table 2). Muscle C_s changes were later in onset, decreasing by 19% at 24 hr ($P < 0.001$; Table 2).

In both liver and muscle, similar qualitative effects of ethanol were obtained when S_i was replaced by S_p in the formula used to calculate k_s (see Table 1). In addition, it should be noted that none of the changes in liver or skeletal muscle k_s were due to alterations in the specific radioactivity of the precursor, aminoacyl tRNA, as no significant alterations were seen in either mean S_i (liver) or S_i (muscle) during the 24 hr following ethanol injection in any group. Values of mean S_i in the liver ranged from 265 to 290 dpm/nmol and in skeletal muscle, values of S_i ranged from 231 to 243 dpm/nmol. Group mean values were ≤ 5% different (NS) from *ad-lib.* fed, saline-injected control (Con-Sal) rats in all ethanol treatment groups and in the 24-hr pair-fed group (not shown).

TABLE 1. Protein synthesis in rat liver and muscle

	Precursor used to calculate k_s	k_s (%/day)	% change from control	Significance (P)
Liver				
Con-Sal	S_i*	72 ± 2	—	—
	S_p†	66 ± 2	—	—
20 min EtOH	S_i	67 ± 4	−7	NS
	S_p	64 ± 3	−3	NS
1 hr EtOH	S_i	65 ± 3	−10	NS
	S_p	58 ± 1	−12	NS
2.5 hr EtOH	S_i	72 ± 2	0	NS
	S_p	70 ± 3	+6	NS
6 hr EtOH	S_i	82 ± 3	+14	$P < 0.05$
	S_p	79 ± 4	+19	$P < 0.025$
24 hr EtOH	S_i	85 ± 4	+18	$P < 0.025$
	S_p	85 ± 4	+29	$P < 0.01$
Pair-fed for 24 hr	S_i	72 ± 4	0	NS
	S_p	70 ± 6	+6	NS
Skeletal muscle				
Con-Sal	S_i‡	13.1 ± 0.4	—	—
	S_p§	10.5 ± 0.2	—	—
20 min EtOH	S_i	12.2 ± 1.0	−7	NS
	S_p	9.8 ± 0.7	−7	NS
1 hr EtOH	S_i	9.9 ± 0.2	−25	$P < 0.01$
	S_p	8.1 ± 0.2	−23	$P < 0.001$
2.5 hr EtOH	S_i	9.1 ± 0.6	−31	$P < 0.001$
	S_p	7.6 ± 0.6	−28	$P < 0.001$
6 hr EtOH	S_i	9.3 ± 0.6	−29	$P < 0.001$
	S_p	7.3 ± 0.3	−30	$P < 0.001$
24 hr EtOH	S_i	4.1 ± 0.3	−69	$P < 0.001$
	S_p	3.3 ± 0.4	−69	$P < 0.001$
Pair-fed for 24 hr	S_i	9.2 ± 0.4	−30	$P < 0.001$
	S_p	7.6 ± 0.5	−28	$P < 0.001$

*S_i, Pair-fed for 24 hr versus 24 hr EtOH: +18%, $P < 0.05$.
†S_p, Pair-fed for 24 hr versus 24 hr EtOH: +21%, $P < 0.05$.
‡S_i, Pair-fed for 24 hr versus 24 hr EtOH: −55%, $P < 0.001$.
§S_p, Pair-fed for 24 hr versus 24 hr EtOH: +57%, $P < 0.001$.

Acute time–course of changes in rates of protein synthesis (k_s, %/day) in liver and skeletal muscle (gastrocnemius) from rats dosed acutely (i.p.) with ethanol (see legend to Fig. 1 for details). One hundred and fifty minutes following acute i.p. bolus injections, rats were injected with a *flooding dose* of L-[4-^{3}H]phenylalanine and processed for specific radioactivities of phenylalanine in plasma (S_p) and in tissue-free (S_i) and protein-bound (S_b) amino acid pools. Data are means ± SEM (N = 4–8). Significance was tested using Student's unpaired *t*-test by ANOVA with pooled estimates of variance. Values of muscle k_s were calculated using S_i and S_p, and liver k_s was calculated using mean values of both tissue- and plasma-free phenylalanine specific radioactivities (S_i and S_p, respectively) as denoted in the second column. Values of mean S_i (i.e. S_i) were calculated as described previously (see Ref. 22) and Methods for further details). For a detailed discussion on the need for pair-fed controls, see the Methods section and [1].

Absolute values of the amounts of protein synthesised by liver and muscle (i.e. V_s, mg protein/day/rat: Table 3) were determined according to the percentage of total body weight represented by each tissue and hence, their total contribution to the whole-body protein pool. Each tissue contributed approximately 20–25% of the whole-body value [28]. Absolute protein synthesis in the liver was significantly elevated after 24 hr by 199 mg protein/day, whereas in skeletal muscle it was reduced by 580 mg protein/day (Table 3).

Changes in Protease Activities in Saline- and Ethanol-Treated Rats

Liver cytoplasmic protease activities were generally raised 24 hr after ethanol treatment (Table 4). Alanyl-, arginyl-, and pyroglutamyl-aminopeptidase as well as proline-endopeptidase activities were all significantly higher in ethanol-treated rats (increases ranged between +19 and +108%, *P* values from 0.05 to 0.001; Table 4). In contrast, hepatic lysosomal protease activities were generally reduced. Significant reductions in the activities of dipeptidyl-aminopeptidase II and cathepsins B, L, and H were observed (−14 to −27%, *P* values ranged from 0.05 to 0.01; Table 4). The values of cytoplasmic and lysosomal protease activities in skeletal (gastrocnemius) muscle showed no significant changes 24 hr after ethanol administration (% changes ranged between −28% and +10%, $P > 0.05$ in all instances).

Effects of Ethanol on Tissue Protein Carbonyl Levels

Protein carbonyl levels were assayed in both liver and skeletal muscle 24 hr after ethanol treatment. Significant

TABLE 2. Changes in RNA activities in alcohol-dosed rats

	Parameter	Mean ± SEM	% change from control	Significance (*P*)
Liver				
Con-Sal	C_s*†	43.1 ± 0.9	—	—
	k_{RNA}‡§	16.8 ± 0.8	—	—
20 min EtOH	C_s	42.2 ± 0.9	−2	NS
	k_{RNA}	15.8 ± 1.2	−6	NS
1 hr EtOH	C_s	41.4 ± 1.0	−4	NS
	k_{RNA}	15.7 ± 0.9	−7	NS
2.5 hr EtOH	C_s	43.0 ± 0.9	0	NS
	k_{RNA}	16.8 ± 0.4	0	NS
6 hr EtOH	C_s	43.2 ± 1.4	0	NS
	k_{RNA}	18.9 ± 0.6	+13	NS
24 hr EtOH	C_s	48.3 ± 1.0	+12	$P < 0.001$
	k_{RNA}	17.3 ± 1.0	+3	NS
Pair-fed for 24 hr	C_s	40.1 ± 0.6	−7	NS
	k_{RNA}	18.1 ± 1.1	+8	NS
Skeletal muscle				
Con-Sal	C_s‖	9.7 ± 0.6	—	—
	k_{RNA}¶	13.7 ± 0.4	—	—
20 min EtOH	C_s	9.5 ± 0.3	−2	NS
	k_{RNA}	12.8 ± 1.1	−6	NS
1 hr EtOH	C_s	9.5 ± 0.1	−2	NS
	k_{RNA}	10.3 ± 0.2	−25	$P < 0.01$
2.5 hr EtOH	C_s	9.4 ± 0.1	−3	NS
	k_{RNA}	9.5 ± 0.8	−31	$P < 0.01$
6 hr EtOH	C_s	9.1 ± 0.1	−6	NS
	k_{RNA}	10.2 ± 0.7	−25	$P < 0.01$
24 hr EtOH	C_s	7.9 ± 0.1	−19	$P < 0.001$
	k_{RNA}	5.3 ± 0.4	−62	$P < 0.001$
Pair-fed for 24 hr	C_s	8.0 ± 0.1	−18	$P < 0.001$
	k_{RNA}	11.6 ± 0.3	−15	NS

*C_s, pair-fed for 24 hr versus 24 hr EtOH: +20%, $P < 0.001$.

‡k_{RNA}, pair-fed for 24 hr versus 24 hr EtOH: −4%, NS.

‖C_s, pair-fed for 24 hr versus 24 hr EtOH: +1%, NS.

¶k_{RNA}, pair-fed for 24 hr versus 24 hr EtOH: −54%, $P < 0.001$.

Acute time–course of changes in protein synthetic capacity and RNA activities were measured in liver and skeletal muscle (gastrocnemius) from rats dosed acutely with ethanol. RNA activity is derived by division of k_s by protein synthetic capacity. See legend to Table 1 for further details.

†C_s, mg RNA/g protein.

§mg protein/day/mg RNA.

effects were only noted in the gastrocnemius muscle, where the protein carbonyl level fell by 28% ($P < 0.05$; Table 5). As these results are inconsistent with the concept of elevated tissue protein carbonyl formation following increased free radical generation, a study was carried out to investigate the generation of protein carbonyl in these tissues *in vitro* following exposure to $O_2^{-\cdot}$ and $OH^{\cdot}$ radicals generated radiolytically. These data showed that, following 2.5- and 24-hr exposure to $O_2^{-\cdot}$ radicals, hepatic protein carbonyl concentrations increased by 16% (NS) and 71% ($P < 0.05$) at 2.5 and 24 hr, respectively (Table 6). Greater increases in this parameter were seen following exposure to $OH^{\cdot}$ radicals, with hepatic protein carbonyl concentrations increased at 2.5 and 24 hr by 43% (NS) and 121% ($P < 0.05$), respectively. Similar effects were seen in gastrocnemius muscle. Thus, $O_2^{-\cdot}$ exposure for 2.5 and 24 hr increased muscle protein carbonyl levels by 32% (NS) and 117% ($P < 0.01$), respectively. With $OH^{\cdot}$ exposure, muscle protein carbonyl was raised by 26% (NS) and 253% ($P < 0.01$) at 2.5 and 24 hr, respectively (Table 6). Similar results were obtained for control human tissue subjected to similar treatments (for 2 hr and 20 hr). Thus, liver and muscle carbonyl contents at 0 min were (data as means ± SEM nmol/mg protein, N = 3) 0.82 ± 0.07 and 1.51 ± 0.12, respectively. After a 2-hr treatment with $OH^{\cdot}$, mean values in liver and muscle were 1.05 ± 0.10 and 2.49 ± 0.22, respectively ($P > 0.05$ in both instances), whereas after 20 hr corresponding values were 4.18 ± 0.37 ($P < 0.001$) and 18.10 ± 1.09 ($P < 0.001$). With superoxide generation studies, values were as follows: time 0 min, 0.87 ± 0.07 and 1.72 ± 0.12 in liver and muscle, respectively. After 2 hr of $O_2^{-\cdot}$, corresponding values were 1.13 ± 0.13 and 2.01 ± 0.17, respectively ($P > 0.05$ in both instances). Twenty hours of $O_2^{-\cdot}$ increased carbonyl to 1.79 ± 0.15 in liver ($P > 0.05$, NS) and 4.54 ± 0.48 nmol/mg protein in muscle ($P < 0.001$). These data also

TABLE 3. Changes in absolute rates of protein synthesis in rat liver and skeletal muscle following ethanol injection

	V_s (mg protein/ day/150 g rat)	% change from control	Significance (P)
Liver			
Con-Sal	815 ± 40	—	—
20 min EtOH	791 ± 69	−3	NS
1 hr EtOH	723 ± 28	−11	NS
2.5 hr EtOH	779 ± 4	−4	NS
6 hr EtOH	877 ± 30	+8	NS
24 hr EtOH	814 ± 71	−<1	NS
Pair-fed for 24 hr	615 ± 55	−25	$P < 0.001$
Pair-fed for 24 hr versus 24 hr EtOH: +24%, $P < 0.001$			
Skeletal muscle			
Con-Sal	1327 ± 57	—	—
20 min EtOH	1053 ± 55	−20	NS
1 hr EtOH	940 ± 14	−29	$P < 0.001$
2.5 hr EtOH	952 ± 78	−28	$P < 0.001$
6 hr EtOH	975 ± 61	−27	$P < 0.001$
24 hr EtOH	229 ± 14	−83	$P < 0.001$
Pair-fed for 24 hr	809 ± 51	−39	$P < 0.001$
Pair-fed for 24 hr versus 24 hr EtOH: −72%, $P < 0.001$			

Absolute rates in whole liver compared with skeletal muscle. Gastrocnemius was taken as representative of the entire skeletal musculature. V_s, the total amount of protein synthesised per day was calculated from the formula [$V_s = (M \times k_s)/100$]. M is the protein content and was calculated from the product of tissue protein concentration and the proportion of body weight made up by each tissue (approx. 40% for skeletal muscle). See legend to Table 1 for further details.

show that failure to identify increased carbonyl levels in tissues following ethanol exposure *in vivo* did not result from methodological limitations of the assay technique employed.

DISCUSSION

Light microscopy analysis on liver showed no overt ethanol-induced morphological changes in this tissue compared to controls. Parallel studies on sections of gastrocnemius muscle also showed no overt signs of tissue injury at the light microscopic level. However, consideration needs to be given to the fact that discrete alterations in membrane function may have occurred. The increase in plasma aspartate aminotransferase activities attributable to ethanol dosage confirms that there was a direct toxic effect on the liver. This may have been due to the membrane-altering properties of the high levels of circulating ethanol. In a previous study incorporating an identical systematic time–course analysis and ethanol-dosing method, plasma levels of ethanol were 448 ± 44, 376 ± 26, 291 ± 24, and 184 ± 16 mg/dL at 20 min, 1, 2.5, 6, and 24 hr following bolus injection, respectively, as reported previously [1]. However, it is arguable whether there are reliable markers for skeletal muscle sarcolemmal changes in the rat. For example, in the absence of anaesthesia, circulating creatine kinase activities increase acutely in both control and alcohol-treated rats in response to the process of killing *per se* ([29] and *).

Ethanol-Induced Changes in Protein Metabolism

The reason for the apparent rise in hepatic protein synthesis rates 6 hr after ethanol administration is unclear. One possibility is that the raised liver k_s is associated with increased plasma protein production, representing an acute-phase response. Temporary loss of gut barrier function and prolonged translocation of bacterial endotoxin from the intestine could produce acute endotoxaemia [30], resulting in immunological activation and cytokine release. This in turn would stimulate hepatic protein synthesis and increase production of acute-phase proteins such as C-reactive protein and haptoglobin. Another mechanism relates to increased cytokine production, which has been reported to increase in acute alcohol toxicity [31]. Certainly, cytokine production concomitantly increases liver and reduces muscle protein synthesis, a pattern seen in this study [32].

Regarding the observed changes in hepatic protease activities, our results are in agreement with studies which have demonstrated reduced hepatic lysosomal protease activities following acute *in vitro* [10] and chronic *in vivo* ethanol treatments [33]. However, it has been reported that the total activities of cathepsins B, D, H, and L do not alter 12 hr after ethanol administration via gastric intubation, while the free (non-latent) activities of cathepsins D and H increase [9]. In the present study, total activities of hepatic cathepsins were reduced. This difference could be due to methodological variations between the two studies. In the former, isocaloric glucose was administered to the control animals [9], whereas a controlled feeding regime was employed in the present study (see Methods). An alternative explanation for the reduced cathepsin activities could be that lysosomal fragility is increased during acute ethanol treatments, leading to leakage of lysosomal proteases from this compartment into the cytosol [33]. The neutral cytosolic pH would render lysosomal proteases (with acidic pH optima) inactive, and there would thus be an overall reduction in lysosomal proteolytic capacity. This has been demonstrated previously, albeit over longer periods of ethanol treatment [33]. However, consideration also needs to be given to the non-lysosomal proteases, the activities of which increased. Since the precise function of any of the protease types remains to be elucidated, the effect of ethanol on the mechanisms of protein degradation must remain a matter for speculation.

The observed changes in skeletal muscle protein metabolism and composition due to acute ethanol administration are also in agreement with previous studies [34]. Gastrocnemius protease activities were generally unaffected by acute ethanol administration. Previous studies have demonstrated that proteolysis may decrease [35] in response to

* Reilly ME and Preedy VR, unpublished observations.

TABLE 4. Skeletal muscle and liver protease activities from 24-hr saline- or ethanol-dosed rats

	Mean activity* ± SEM (nmol/hr/mg protein)		% Change from control	Significance (P)
	Control	Ethanol		
Liver				
Cytoplasmic proteases				
Alanyl-aminopeptidase	227 ± 8	279 ± 13	+23	$P < 0.01$
Arginyl-aminopeptidase	489 ± 22	580 ± 33	+19	$P < 0.05$
Leucyl-aminopeptidase	196 ± 8	212 ± 7	+8	NS
Pyroglutamyl-aminopeptidase	19 ± 1	39 ± 2	+108	$P < 0.001$
Dipeptidyl-aminopeptidase IV	817 ± 47	801 ± 45	−2	NS
Tripeptidyl-aminopeptidase	133 ± 8	131 ± 7	−1	NS
Proline-endopeptidase	161 ± 4	204 ± 8	+28	$P < 0.001$
Lysosomal proteases				
Dipeptidyl-aminopeptidase I	13467 ± 710	13673 ± 501	+2	NS
Dipeptidyl-aminopeptidase II	68 ± 3	50 ± 4	−27	$P < 0.01$
Cathepsin B	310 ± 20	263 ± 11	−15	$P = 0.05$
Cathepsin L	2552 ± 99	2138 ± 86	−16	$P < 0.01$
Cathepsin H	370 ± 22	317 ± 12	−14	$P = 0.05$
Cathepsin D	37 ± 2	42 ± 2	+12	NS
Skeletal muscle				
Cytoplasmic proteases				
Alanyl-aminopeptidase	918 ± 156	980 ± 71	+6	NS
Arginyl-aminopeptidase	670 ± 113	690 ± 50	+3	NS
Leucyl-aminopeptidase	49 ± 6	46 ± 5	−7	NS
Pyroglutamyl-aminopeptidase	ND	ND	—	—
Dipeptidyl-aminopeptidase IV	83 ± 15	74 ± 6	−13	NS
Tripeptidyl-aminopeptidase	196 ± 31	153 ± 16	−28	NS
Proline-endopeptidase	148 ± 26	149 ± 15	<+1	NS
Lysosomal proteases				
Dipeptidyl-aminopeptidase I	656 ± 93	650 ± 72	−1	NS
Dipeptidyl-aminopeptidase II	66 ± 6	61 ± 4	−9	NS
Cathepsin B	66 ± 6	61 ± 4	−9	NS
Cathepsin L	256 ± 33	260 ± 26	+2	NS
Cathepsin H	157 ± 27	174 ± 14	+10	NS
Cathepsin D	ND	ND	—	—

Tissue protease activities in liver and skeletal muscle (gastrocnemius) after acute (24-hr) i.p. bolus injection of ethanol. Control, saline-dosed rats were pair-fed for 24 hr with identical amounts of diet as those consumed by ethanol-treated animals (see Methods for details). All data are means ± SEM (N = 4–8). Significance between 24-hr ethanol and pair-fed for 24 hr control groups was assessed using Student's unpaired *t*-test. NS, non-significant. ND, not determined.

*Protease activity units are nmol/hr/mg muscle protein, except cathepsin D as enzyme units per mg soluble protein, where 1 unit of activity represents an increase in absorbance of 0.01/hr at 280 mm, 37°, 1-cm pathlength.

ethanol. However, it could be that, as already discussed, ethanol may give rise to enhanced lysosomal fragility, which would mask any visible effect in total activities measured here. Secondly, it is possible that some proteases not included in the present study may have been altered. For instance, the ATP–ubiquitin-dependent proteolytic pathway has recently received attention as a major system for enhanced proteolysis in various catabolic states (reviewed in [36]); macropain was not measured in this study, since its activity on synthetic fluorogenic substrates does not designate this enzyme as a major component of overall tissue proteolytic activity. Nevertheless, the assessment of

TABLE 5. Liver and skeletal muscle protein carbonyl content in acutely ethanol-treated rats

	Mean content ± SEM		% Change from control	Significance (P)
	Control	Ethanol		
Concentration (nmol/mg protein)				
Liver	3.19 ± 0.69	2.37 ± 0.36	−26	NS
Skeletal muscle	2.44 ± 0.30	1.76 ± 0.26	−28	$P < 0.05$
Total tissue content (nmol/tissue)				
Liver	2412 ± 146	2106 ± 197	−13	NS
Skeletal muscle	246 ± 73	204 ± 82	−17	NS

Protein carbonyl content in young male Wistar rats (approximately 150 g) treated with ethanol or saline for 24 hr (see Methods section for details). Data are presented as means ± SEM (N = 4–8). Significance for acute analysis was assessed using Student's *t*-test for unpaired samples. NS, not significant.

TABLE 6. Rat liver and skeletal muscle protein carbonyl content following superoxide ($O_2^{-\cdot}$) and hydroxyl ($OH^{\cdot}$) radical exposure *in vitro*

Exposure period (hr)	Tissue	Carbonyl concentration (nmol/mg protein) after generation of $O_2^{-\cdot}$	% Change from control	Significance (P)
0 hr	Liver	5.50 ± 1.17	—	—
	Gastrocnemius	6.17 ± 0.72	—	—
2.5 hr	Liver	6.40 ± 0.90	+16	NS
	Gastrocnemius	8.17 ± 1.59	+32	NS
24 hr	Liver	11.62 ± 2.27	+71	$P < 0.05$
	Gastrocnemius	13.41 ± 1.55	+117	$P < 0.01$
Exposure period (hr)	**Tissue**	**Carbonyl concentration (nmol/mg protein) after generation of $OH^{\cdot}$**	**% Change from control**	**Significance (P)**
0 hr	Liver	5.50 ± 1.17	—	—
	Gastrocnemius	6.17 ± 0.72	—	—
2.5 hr	Liver	7.85 ± 1.31	+43	NS
	Gastrocnemius	7.80 ± 1.22	+26	NS
24 hr	Liver	12.13 ± 2.75	+121	$P < 0.05$
	Gastrocnemius	21.78 ± 3.43	+253	$P < 0.01$

Protein carbonyl content in liver and muscle were measured before (0 hr) and after *in vitro* generation of either $OH^{\cdot}$ or $O_2^{-\cdot}$ radicals. Data are presented as means ± SEM. Significance was assessed using Student's *t*-test for paired samples. NS, not significant.

protease activities is considered to be a satisfactory approach in assessing the contribution of the protein degradative pathway to muscle pathologies [37].

Relation between Liver and Muscle

It is possible that acute liver dysfunction was indirectly responsible for the reduction in muscle protein synthesis at 24 hr. Subtotal hepatectomy leads to a 25% increase in liver k_s, concomitant with a reduction in gastrocnemius muscle k_s of between 10 and 20% [38]. However, studies have demonstrated that alcohol-induced muscle pathology occurs independently of the severity of liver disease in alcohol misusers (reviewed in [3]). It is difficult to speculate as to the source of these divergent changes in liver and muscle from 6 hr onwards. In this respect, there appears to be a large nutritional influence on hepatic V_s, since it was reduced by 28% in the pair-fed group. Changes in skeletal muscle V_s occurred irrespective of reduced nutritional intake, since V_s was 54% lower in ethanol-treated rats relative to pair-fed controls. V_s in the liver was significantly elevated after 24 hr by approx. 200 mg protein/day, whereas in skeletal muscle it was approx. 600 mg protein/day lower than in pair-fed rats. Overall, this represents a shift towards a net reduction in protein synthesis, which is consistent with studies showing that acute doses of ethanol increase nitrogen excretion [39].

Changes in Tissue Protein Carbonyl Content

Ethanol dosage leads to an enhanced risk of tissue damage due to raised levels of tissue ROS (reviewed in [12–14, 16, 17]). In the liver, increased production of ROS following ethanol administration may induce peroxidation of membrane lipids. In muscle, ROS may arise from ethanol and acetaldehyde oxidation by oxygen-dependent intramuscular xanthine oxidase activity [40]. In the present study, increased protein carbonyl levels were predicted following ethanol administration *in vivo*, particularly in muscle, as this tissue has an inferior antioxidant capacity compared to the liver [16, 17]. However, contrary to the expected finding, no increase in protein carbonyl levels was found in liver or muscle (carbonyl levels in the latter were reduced). This is surprising, given that several studies have reported raised lipid peroxidation and reduced levels of free radical scavenging (antioxidant) moieties [41] following ethanol treatment. The mechanism responsible for the changes reported here is therefore unclear. One possible explanation could involve the accelerated removal of carbonyl-containing, oxidatively damaged cellular proteins that are preferentially targeted for degradation within the cytoplasm [18]. However, although significant increases in activity were noted for a number of cytoplasmic proteases in liver tissue following ethanol treatment, there was no increase in the corresponding protease activities in muscle tissue. In addition, it is difficult to assign changes in the activity of these proteases to such a specific role in the intracellular maintenance of oxidised protein levels, since the role of these enzymes (other than in more general terms) has still to be determined.

An alternative explanation for the above relates to the relative susceptibility of muscle carbonyl formation. Specific antioxidants within muscle may have prevented the formation of protein carbonyl and this was evaluated in a controlled experiment. For example, muscle contains high concentrations of imidazole dipeptides, which are putative

antioxidants. However, after exposure of tissue preparations to hydroxyl and superoxide radicals generated radiolytically *in vitro* (an established model for examining free radical-mediated protein damage [24]), protein carbonyl levels were increased in both liver and muscle, although only to a significant degree after 24 hr. Muscle was more susceptible to carbonyl formation, particularly in response to $OH^{\cdot}$ radicals. It is therefore possible to conclude that carbonyl formation readily occurs in muscle. This does not preclude the possibility that other macromolecules, such as lipids, were affected by ROS.

In conclusion, the acute model of ethanol toxicity used in the present study produces overt biochemical alterations in liver and skeletal muscle in the absence of observable cellular damage, necrosis, or inflammation. The divergent alterations in protein metabolism may be due to variations in the demand placed on whole-body pools of amino acids, although the factors driving the different tissue responses remain unclear. There seems no clear connection between ethanol-induced perturbations in tissue protein metabolism and ROS-mediated protein damage.

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