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The effect of chronic ethanol ingestion on protein metabolism in Type-I- and Type-II-fibre-rich skeletal muscles of the rat

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1. The effects of chronic ethanol feeding on muscles containing a predominance of either Type I (aerobic, slow-twitch) or Type II (anaerobic, fast-twitch) fibres were studied. Male Wistar rats, weighing approx. 90 g or 280 g, were pair-fed on a nutritionally complete liquid diet containing 36% of total energy as ethanol, or isovolumetric amounts of the same diet in which ethanol was replaced by isoenergetic glucose. After 6 weeks feeding, fractional rates of protein synthesis were measured with a flooding dose of L-[4-^{3}H]-phenylalanine and muscles were analysed for protein, RNA and DNA. 2. Ethanol feeding decreased muscle weight, protein, RNA and DNA contents in both small and large rats. Type-II-fibre-rich muscles showed greater changes than did Type-I-fibre-rich muscles. Changes in protein paralleled decreases in DNA. 3. The capacity for protein synthesis (RNA/protein), fractional rates of protein synthesis and absolute rates of protein synthesis were decreased by ethanol feeding in both small and large rats. The amounts of protein synthesized relative to RNA and DNA were also decreased. Changes were less marked in Type-I than in Type-II-fibre-rich muscles. Loss of protein, RNA and DNA was greater in small rats, but protein synthesis was more markedly affected in large rats. 4. It was concluded that chronic ethanol feeding adversely affects protein metabolism in skeletal muscle. Fibre composition and animal size are also important factors in determining the pattern of response.

INTRODUCTION

Chronic ethanol ingestion in man causes skeletal-muscle dysfunction. Pathological changes include muscle weakness and electrophysiological disturbances (Ekbom *et al.*, 1967; Martin *et al.*, 1985; Mills *et al.*, 1986). Histochemical examinations of myosin-ATPase-stained proximal-muscle biopsies reveal a selective decrease in the diameter of Type II (also defined as anaerobic, glycolytic, fast-twitch) fibres. The diameters of Type I (also defined as aerobic, oxidative, slow-twitch) fibres are relatively unaffected (Martin *et al.*, 1985; Hanid *et al.*, 1981).

To study the biochemical basis of these lesions a suitable animal model is required. Ward *et al.* (1987) established histochemically that in the laboratory rat Type II fibre diameter was decreased after feeding them on a liquid diet containing ethanol as 36% of total energy. As protein mass is dependent on the rate of protein turnover, defects in either one or both of its constituent processes (i.e. synthesis or degradation) could be responsible for the lesion. To test this hypothesis, rates of protein synthesis in a variety of skeletal muscles (selected to represent different fibre types) from ethanol-fed rats were measured. In this study, the soleus and diaphragm were taken to represent Type I fibres, and plantaris and quadratus lumborum were considered to be Type-II-fibre-rich (Hopkins *et al.*, 1983; Eddinger *et al.*, 1985; Ariano *et al.*, 1973).

MATERIALS AND METHODS

Materials

L-[4-^{3}H]Phenylalanine was purchased from Amersham International (Amersham, Bucks., U.K.). All other reagents were from Sigma (Poole, Dorset, U.K.).

Experimental feeding

Male Wistar rats were obtained from the National Institute for Medical Research (Mill Hill, London N.W.7, U.K.) and weighed either 50–70 g or 230–270 g. They were maintained in a temperature-controlled animal house on a 12 h-light/12 h-dark cycle, with light commencing at 07:00 h. Rats were fed *ad lib.* on a standard laboratory chow for 3–5 days, at which time they were pair-matched on the basis of weight. Control rats were then fed on a nutritionally complete liquid diet prepared from a commercial food drink, supplemented with glucose, casein and a vitamin mixture (Preedy *et al.*, 1988; Venkatesan *et al.*, 1987). The proportions of fat, protein and carbohydrate in the control diet comprised 19, 15 and 66% of total energy respectively. Treated rats were given the same diet *ad libitum* in which glucose was replaced with ethanol of equal energy content. For the first 3 days ethanol comprised 18%, and thereafter 36%, of total energy (Lieber *et al.*, 1963; DeCarli & Lieber, 1967). Blood ethanol concentrations attained by ethanol-fed rats are 50–250 mg/100 ml. Control and ethanol-containing diets were isolipidic, isonitrogenous and isoenergetic.

Treated rats had an aversion to the ethanol-containing liquid diet (Preedy *et al.*, 1988). To compensate for this, control rats were also given identical volumes of diet. The preparation of the liquid diets and pair-feeding were carried out on a daily basis between 08:00 and 12:00 h. In the week preceding measurement of synthesis, the diet was prepared and administered between 08:00 and

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10:00 h. On the day of synthesis measurements, diet was administered between 08:00 and 08:30 h.

Measurement of protein synthesis

After 6 weeks, rates of muscle protein synthesis were measured by the flooding-dose technique of McNurlan *et al.* (1979) as described by Garlick *et al.* (1980) for L-[4-^{3}H]phenylalanine. The radiochemical (150 mM, 0.1 Ci/mol, measured sp. radioactivity 208 d.p.m./nmol; 2 ml/100 g body wt.) was injected intraperitoneally into conscious unrestrained rats between 11:00 and 15:00 h. After 15 min rats were decapitated, and blood was collected into cold (4 °C) heparinized tubes for 15 s for subsequent separation of plasma. Muscles were quickly dissected and immersed into a mixture of ice/water. The period between injection of radioisotope and immersion of muscle was accurately timed. Only a small portion of the diaphragm was taken for analysis. Tissues were blotted, weighed and stored at −70 °C until analysis.

Muscles were processed as described previously (Garlick *et al.*, 1980; Preedy *et al.*, 1984). All subsequent steps were carried out at 0–4 °C. Briefly, muscles were homogenized, protein was precipitated with 0.2 M-$HClO_4$ and centrifuged (2000 *g*, 15 min). The acid supernatant was neutralized with saturated tripotassium citrate and the specific radioactivity of free phenylalanine determined. Protein precipitates were then washed twice with 0.2 M-$HClO_4$ and digested in 0.3 M-NaOH. After incubation at 37 °C for 1 h, 2 ml of digest was removed for measurement of protein and DNA by modifications of the methods of Gornall *et al.* (1949) and Downs & Wilfinger (1983) respectively. The alkaline digest was re-precipitated in 2 M-$HClO_4$ for measurement of RNA in the acid supernatant (Munro & Fleck, 1969). Protein pellets were then washed repeatedly (eight times) in 0.2 M-$HClO_4$, and heated in 6 M-HCl for 36 h at 105 °C. Hydrolysates were dried *in vacuo* (over P_2O_5 and solid NaOH) and suspended in citrate buffer (1.5 M, pH 6.3) for measurement of the specific radioactivity of phenylalanine in tissue protein. Specific radioactivities of free phenylalanine in plasma were obtained after precipitation in 0.2 M-$HClO_4$ and subsequent neutralization. Phenylalanine specific radioactivities were assayed after conversion into 2-phenethylamine (Garlick *et al.*, 1980).

It was essential that data between different muscles were strictly comparable. Samples with known concentrations of RNA, DNA, protein or 2-phenethylamine specific radioactivities were therefore interdispersed throughout subsequent assays to ensure comparability.

Calculation of data

Fractional rates of protein synthesis (defined as percentage of tissue protein renewed each day by synthesis, i.e. k_s) were calculated from the formula

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

where S_B was the specific radioactivity of L-[4-^{3}H]phenylalanine in tissue protein, S_i was the specific radioactivity of L-[4-^{3}H]phenylalanine in acid-soluble supernatants of muscle homogenates, and t was the incorporation time in days (Garlick *et al.*, 1980). Integration of the time course of S_i was also used. Jepson *et al.* (1986) showed that administration of the radioisotope via the intraperitoneal route in rats of various nutritional states was followed by rapid rises to a plateau, and the value of the time integral for S_i was 0.9 of that which would have occurred if plateau was achieved instantaneously.

Rates of protein synthesis were also expressed relative to RNA and DNA. The 'RNA efficiency' was defined as the amount of protein synthesized per unit of RNA, i.e. k_{RNA} (mg of protein/day per mg of RNA), and calculated from:

$$k_{RNA} = \frac{k_s \times 10}{(\text{RNA/protein})}$$

The 'cellular efficiency' was defined as the amount of protein synthesized per day per nucleus, i.e. k_{DNA} (mg of protein/day per mg of DNA):

$$k_{DNA} = \frac{k_s \times 10}{(\text{DNA/protein})}$$

Statistics

All data are presented as means ± S.E.M. for six to nine pairs of observations. As a 'pair-feeding' regime for individual animals was employed, differences between means were assessed by paired analysis (two-tailed Student's t test). Significance was indicated at $P < 0.05$. To ascertain if the response to treatment in Type-I-fibre-rich muscles was different from that of Type II, the ratio Type I/Type II for various parameters in individual rats was measured.

RESULTS

The weights of individual muscles are displayed in Table 1. In small rats, weights of plantaris and quadratus lumborum muscles were lower after ethanol treatment, when expressed both as absolute values (i.e. mg) or relative to body weight (i.e. mg/kg body wt.), by 12–39%. There was no statistically significant difference in soleus wet weight. In contrast, changes in the weight of soleus, plantaris and quadratus lumborum muscles from large rats were not statistically significant.

Table 2 shows that in small and large rats decreases in muscle weight were accompanied by decreases in protein content, either as an absolute value or relative to body weight. Significant increases in the ratio of soleus/plantaris protein content, in young rats, also indicated greater sensitivity of plantaris muscles. There were also small, but statistically significant, increases in protein concentrations in plantaris and quadratus lumborum from small rats and quadratus lumborum from large rats.

In small and large rats the concentration (mg/g wet wt.), and absolute (mg) and relative amounts (mg/kg body wt.) of RNA in soleus were unchanged (Table 3). RNA concentrations in plantaris and quadratus lumborum muscles from small rats were not altered by ethanol feeding. The increase in diaphragm RNA concentration was not statistically significant. However, decreases in RNA concentrations were observed in corresponding muscles of large rats. The total amount of RNA in plantaris and quadratus lumborum muscles from small and large rats was decreased by 15–40%. Smaller changes in RNA content relative to body weight were also obtained. Significant increases in the ratio of soleus/plantaris RNA concentrations in small and large rats confirmed that the plantaris muscles were more

Table 1. Effect of chronic ethanol feeding on muscle weights

Male Wistar rats were pair-fed with a nutritionally complete liquid diet (controls) or isovolumetric amounts of the same diet in which glucose was replaced by isoenergetic ethanol, as described in the Materials and methods section. Data for small and large groups pertain to rats which were sexually immature and mature, respectively, at the onset of treatment. At the end of 6 weeks, rats were killed and analysed for body composition. Data for quadratus lumborum are for left muscle only, whereas soleus and plantaris represent analysis of combined left and right muscles. No data are presented for diaphragm weights, as this tissue was not dissected out quantitatively. All data are presented as means ± S.E.M. for six to nine pairs of observations. Differences between means were assessed by Student's *t* test for paired samples: †$P > 0.05$ (not significant); **$P < 0.025$; ***$P < 0.01$; ****$P < 0.001$.

Rats	Parameter	Muscle	Control	Ethanol	Difference (%)
Small	Body wt. (g)				
	Initial		85±2	85±2	0†
	Final		225±3	197±4	−12***
	Tissue wt. (mg)	Soleus	223±8	201±4	−10†
		Plantaris	457±10	355±15	−22****
		Soleus/plantaris ratio	0.49±0.02	0.57±0.02	+17**
		Quadratus lumborum	1420±87	870±48	−39****
	Tissue wt./body wt. (mg/kg)	Soleus	990±30	1020±20	+3†
		Plantaris	2040±40	1800±70	−12***
		Quadratus lumborum	6340±410	4420±220	−30****
Large	Body wt. (g)				
	Initial		278±7	278±80	0†
	Final		390±8	361±9	−7****
	Tissue wt. (mg)	Soleus	367±9	357±12	−2†
		Plantaris	743±14	699±21	−6†
		Soleus/plantaris ratio	0.49±0.01	0.51±0.01	+4†
		Quadratus lumborum	2650±210	2110±130	−20†
	Tissue wt./body (mg/kg)	Soleus	940±24	988±19	+6†
		Plantaris	1910±35	1930±40	+2†
		Quadratus lumborum	6810±570	5830±330	−14†

Table 2. Effect of chronic ethanol feeding on muscle protein

Protein in various muscles was measured as described in the Materials and methods section and calculated as a concentration (mg/g wet wt.) or absolute value (mg) or relative to body wt. (mg/kg). Other details are contained in the legend to Table 1. All data are presented as means ± S.E.M. for six to nine pairs of observations. Differences between control and ethanol-fed rats were assessed by Student's *t* test for paired samples: †$P > 0.05$ (not significant); *$P < 0.05$; **$P < 0.025$; ***$P < 0.01$; ****$P < 0.001$.

Rats	Parameter	Muscle	Control	Ethanol	Difference (%)
Small	Protein concn. (mg/g wet wt.)	Soleus	184±3	183±3	0†
		Plantaris	183±3	190±3	+4*
		Soleus/plantaris ratio	1.01±0.02	0.97±0.02	−4†
		Quadratus lumborum	176±3	187±2	+7***
		Diaphragm	129±6	131±3	+2†
	Total protein (mg)	Soleus	40.9±1.5	36.8±0.8	−10**
		Plantaris	83.8±2.6	67.3±3.0	−20****
		Soleus/plantaris ratio	0.49±0.02	0.55±0.02	+12***
		Quadratus lumborum	248±13	163±9	−34****
	Total protein/body wt. (mg/kg)	Soleus	182±5	187±5	+2†
		Plantaris	373±8	341±12	−9**
		Quadratus lumborum	1106±57	828±41	−25****
Large	Protein concn. (mg/g wet wt.)	Soleus	187±3	185±2	−1†
		Plantaris	185±2	183±4	−1†
		Soleus/plantaris ratio	1.01±0.02	1.01±0.02	0†
		Quadratus lumborum	182±2	188±2	+3****
	Total protein (mg)	Soleus	68.3±1.3	66.0±1.9	−3†
		Plantaris	137±3	128±2	−7**
		Soleus/plantaris ratio	0.50±0.01	0.52±0.01	+4†
		Quadratus lumborum	482±37	397±24	−18†
	Total protein/body wt. (mg/kg)	Soleus	175±3	183±2	+4†
		Plantaris	351±7	354±5	+1†
		Quadratus lumborum	1240±100	1100±60	−11†

Table 3. Effect of chronic ethanol feeding on muscle RNA

RNA was measured in various skeletal muscles as described in the Materials and methods section and expressed as a concentration (mg/g wet wt.), absolute value (mg) or relative to body wt. (mg/kg). Other details are contained in the legend to Table 1 and the Materials and methods section. All data are presented as means ± S.E.M. for six to nine pairs of observations. Differences between means were assessed by Student's *t* test for paired samples: †$P > 0.05$ (not significant); *$P < 0.05$; **$P < 0.025$; ***$P < 0.01$; ****$P < 0.001$.

Rats	Parameter	Muscle	Control	Ethanol	Difference (%)
Small	RNA concn. (mg/g wet wt.)	Soleus	1.19±0.03	1.26±0.03	+5†
		Plantaris	0.757±0.011	0.748±0.017	−1†
		Soleus/plantaris ratio	1.58±0.04	1.69±0.04	+7*
		Quadratus lumborum	0.731±0.012	0.712±0.024	−3†
		Diaphragm	0.845±0.032	0.991±0.069	+17†
	Total RNA (mg)	Soleus	0.267±0.014	0.253±0.009	−5†
		Plantaris	0.346±0.009	0.265±0.011	−23***
		Soleus/plantaris ratio	0.77±0.04	0.96±0.04	+24***
		Quadratus lumborum	1.037±0.063	0.626±0.051	−40****
	Total RNA/body wt. (mg/kg)	Soleus	1.19±0.05	1.28±0.05	+8†
		Plantaris	1.54±0.03	1.34±0.05	−13**
		Quadratus lumborum	4.63±0.28	3.17±0.25	−31****
Large	RNA concn. (mg/g wet. wt.)	Soleus	1.027±0.032	1.036±0.020	+1†
		Plantaris	0.601±0.012	0.542±0.012	−10***
		Soleus/plantaris ratio	1.71±0.05	1.92±0.06	+12*
		Quadratus lumborum	0.572±0.008	0.511±0.013	−11**
	Total RNA (mg)	Soleus	0.377±0.019	0.370±0.016	−2†
		Plantaris	0.446±0.010	0.378±0.009	−15****
		Soleus/plantaris ratio	0.84±0.03	0.98±0.03	+16***
		Quadratus lumborum	1.515±0.124	1.084±0.088	−28*
	Total RNA/body wt. (mg/kg)	Soleus	0.97±0.04	1.02±0.03	+6†
		Plantaris	1.14±0.02	1.05±0.02	−9***
		Quadratus lumborum	3.89±0.33	2.99±0.24	−23†

Table 4. Effect of chronic ethanol feeding on muscle DNA

DNA was measured as described in Materials and methods section and expressed as concentration (mg/g wet wt.), absolute value (mg) or relative to body wt. (mg/kg). Other details are contained in the legend to Table 1 and the Materials and methods section. All data are presented as means ± S.E.M. for six to nine pairs of observations. Differences between means were assessed by Student's *t* test for paired samples: †$P > 0.05$ (not significant); *$P < 0.05$; **$P < 0.025$; ***$P < 0.01$; ****$P < 0.001$.

Rats	Parameter	Muscle	Control	Ethanol	Difference (%)
Small	DNA concn. (mg/g wet wt.)	Soleus	1.123±0.027	1.110±0.043	−1†
		Plantaris	0.638±0.013	0.649±0.018	+2†
		Soleus/plantaris ratio	1.76±0.04	1.72±0.07	−3†
		Quadratus lumborum	0.530±0.012	0.562±0.016	+6†
		Diaphragm	1.069±0.060	1.164±0.071	+10†
	Total DNA (mg)	Soleus	0.251±0.012	0.223±0.012	−11*
		Plantaris	0.292±0.009	0.230±0.011	−21****
		Soleus/plantaris ratio	0.86±0.03	0.98±0.04	+13***
		Quadratus lumborum	0.754±0.051	0.493±0.035	−35****
	Total DNA/body wt. (mg/kg)	Soleus	1.12±0.04	1.13±0.05	+1†
		Plantaris	1.30±0.03	1.17±0.05	−10***
		Quadratus lumborum	3.36±0.22	2.49±0.16	−26****
Large	DNA concn. (mg/g wet wt.)	Soleus	1.108±0.015	1.090±0.022	−2†
		Plantaris	0.614±0.009	0.601 +0.012	−2†
		Soleus/plantaris ratio	1.81±0.04	1.81±0.04	0†
		Quadratus lumborum	0.453±0.006	0.486±0.013	+7*
	Total DNA (mg)	Soleus	0.406±0.011	0.388±0.012	−4†
		Plantaris	0.456±0.011	0.419±0.007	−8**
		Soleus/plantaris ratio	0.89±0.02	0.93±0.02	+4†
		Quadratus lumborum	1.197±0.093	1.018±0.056	−5†
	Total DNA/body wt. (mg/kg)	Soleus	1.04±0.02	1.08±0.03	+3†
		Plantaris	1.17±0.02	1.16±0.01	−1†
		Quadratus lumborum	3.08±0.26	2.82±0.16	−8†

Table 5. Effect of chronic ethanol feeding on derived parameters

Derived parameters were calculated from data contained in Tables 2–4. RNA per nucleus was calculated from RNA/DNA (mg/mg), cell size from protein/DNA (mg/mg), and synthetic capacity from RNA/protein (mg/g). Other details are contained in the legend to Table 1 and the Materials and methods section. All data are presented as means ± S.E.M. for six to nine observations. Differences between means were assessed by Student's t test for paired samples: †$P > 0.05$; *$P < 0.05$; **$P < 0.025$; ***$P < 0.01$.

Rats	Parameter	Muscle	Control	Ethanol	Difference (%)
Small	RNA/DNA (mg/mg)	Soleus	1.07±0.04	1.15±0.04	+7†
		Plantaris	1.19±0.03	1.16±0.04	−3†
		Soleus/plantaris ratio	0.90±0.04	1.00±0.05	+10*
		Quadratus lumborum	1.383±0.032	1.278±0.064	−8†
		Diaphragm	0.796±0.021	0.857±0.043	+8†
	Protein/DNA (mg/mg)	Soleus	164±3	167±5	+1†
		Plantaris	287±4	293±7	+2†
		Soleus/plantaris ratio	0.57±0.02	0.57±0.02	0†
		Quadratus lumborum	332±8	335±8	+1†
		Diaphragm	121±3	115±5	−5†
	RNA/protein (mg/g)	Soleus	6.50±0.16	6.88±0.17	+6†
		Plantaris	4.14±0.07	3.96±0.12	−4†
		Soleus/plantaris ratio	1.57±0.05	1.75±0.05	+11*
		Quadratus lumborum	4.17±0.09	3.81±0.14	−9***
		Diaphragm	6.60±0.20	7.53±0.42	+14†
Large	RNA/DNA (mg/mg)	Soleus	0.926±0.023	0.952±0.019	+3†
		Plantaris	0.981±0.024	0.902±0.018	−8*
		Soleus/plantaris ratio	0.95±0.03	1.06±0.03	+12**
		Quadratus lumborum	1.265±0.030	1.056±0.040	−16***
	Protein/DNA (mg/mg)	Soleus	168±2	170±4	+1†
		Plantaris	301±3	304±1	+1†
		Soleus/plantaris ratio	0.56±0.01	0.56±0.01	0†
		Quadratus lumborum	403±7	390±11	−3†
	RNA/protein (mg/g)	Soleus	5.51±0.18	5.60±0.13	+2†
		Plantaris	3.26±0.06	2.96±0.06	−9***
		Soleus/plantaris ratio	1.691±0.06	1.89±0.05	+12**
		Quadratus lumborum	3.14±0.07	2.71±0.07	−14***

sensitive. Similarly, there were significant increases in the ratio of soleus/plantaris total RNA, in small and large rats.

There was little change in DNA concentrations in soleus and plantaris muscles from small and large rats (Table 4). The quadratus lumborum showed increases in concentrations which were only significant for larger animals. The total amounts of DNA decreased in soleus, plantaris and quadratus lumborum of small rats, by 11–35%. In small rats the increase in the soleus/plantaris ratio for total DNA signified a greater response in the plantaris. The decline in soleus DNA content was not apparent when expressed relative to body weight. Muscle DNA changed very little in large rats, though a significant decrease in total plantaris DNA content was observed.

Data for protein, RNA and DNA were also expressed in terms of various ratios (Table 5). Similar expressions of data for skeletal muscle have been used in a variety of nutritional studies (for a review, see Waterlow *et al.*, 1978). The amount of RNA per DNA or nucleus was increased in soleus and diaphragm of small rats, but was not statistically significant. In the plantaris of small rats the RNA/DNA ratio was relatively unchanged. However, significant increases in the soleus/plantaris ratio in small and large rats signified differential susceptibility of the plantaris. The quadratus lumborum from small and large rats showed 8–16% decreases in RNA/DNA. The DNA unit (indicated by the amount of protein per DNA, or cell size) was unaffected by ethanol feeding in all muscles of small and large rats. The capacity or potential for protein synthesis (indicated by the amount of RNA per unit of protein) in plantaris and quadratus lumborum muscles from small and large rats decreased by 4–14%. There was no significant change in soleus. Confirmation of differential sensitivity was obtained by significant increases in the soleus/plantaris ratio for synthesis capacity in small and large rats. In contrast, there was an increase in protein-synthetic capacity in diaphragm, though $P = 0.06$.

The data for phenylalanine specific radioactivity are displayed in Table 6. Ethanol feeding had no significant effect on the value of S_i in the various muscles from small or large rats. The data also showed that k_s in soleus and plantaris muscles of the control group of small rats were slightly higher (6–12%) than the corresponding values in ethanol-fed rats, but these differences were not statistically significant. The decline in k_s in soleus and plantaris of large rats was also small (19–21% decline) and was significant for plantaris only. We could not measure the k_s in quadratus lumborum, as this muscle was difficult and time-consuming to dissect out quantitatively. A

Table 6. Effect of chronic ethanol feeding on L-[4-^{3}H]phenylalanine specific radioactivities and fractional synthesis rates

Specific radioactivities and fractional rates of protein synthesis were measured after intraperitoneal injection of a large flooding dose of L-[4-^{3}H]phenylalanine; k_s was calculated from S_I. Other details are contained in the Materials and methods section. All data are presented as means ± S.E.M. for six or seven pairs of observations. Differences between means were assessed by Student's t test for paired samples: †$P > 0.05$ (not significant); **$P < 0.025$.

Rats	Parameter	Muscle	Control	Ethanol	Difference (%)
Small	S_I (d.p.m./nmol)	Soleus	173±3	169±5	−2†
		Plantaris	179±2	173±3	−4†
		Soleus/plantaris ratio	0.96±0.02	0.98±0.01	+1†
		Diaphragm	201±3	199±3	−1†
	S_B (d.p.m./nmol)	Soleus	0.192±0.009	0.164±0.007	−15†
		Plantaris	0.125±0.004	0.112±0.004	−10†
		Soleus/plantaris ratio	1.51±0.05	1.46±0.04	−3†
		Diaphragm	0.205±0.008	0.215±0.022	+5†
	k_s, calculated from S_I (%/day)	Soleus	11.16±0.53	9.85±0.43	−12†
		Plantaris	6.95±0.21	6.56±0.17	−6†
		Soleus/plantaris ratio	1.58±0.06	1.49±0.05	−5†
		Diaphragm	8.96±0.45	9.45±0.99	+6†
Large	S_I (d.p.m./nmol)	Soleus	153±12	167±3	+10†
		Plantaris	163±9	175±3	+8†
		Soleus/plantaris ratio	0.93±0.03	0.96±0.02	+3†
	S_B (d.p.m./nmol)	Soleus	0.138±0.006	0.126±0.006	−8†
		Plantaris	0.083±0.003	0.071±0.005	−14†
		Soleus/plantaris ratio	1.66±0.06	1.80±0.13	+9†
	k_s, calculated from S_I (%/day)	Soleus	9.30±0.84	7.49±0.31	−19†
		Plantaris	5.15±0.24	4.07±0.28	−21**
		Soleus/plantaris ratio	1.80±0.10	1.89±0.13	+5†

Table 7. Effect of ethanol feeding on protein synthesis relative to RNA and DNA

k_{RNA} and k_{DNA} were calculated as described in the Materials and methods section for six or seven pairs of observations; values are means ± S.E.M. Differences between means were assessed by Student's t test for paired samples: †$P > 0.05$ (not significant); *$P < 0.05$; **$P < 0.025$.

Rats	Parameter	Muscle	Control	Ethanol	Difference (%)
Small	k_{RNA} (g of protein/day per g of RNA)	Soleus	17.1±0.9	14.4±0.6	−16†
		Plantaris	16.8±0.5	16.4±0.6	−2†
		Soleus/plantaris ratio	1.01±0.04	0.89±0.04	−12**
		Diaphragm	14.2±0.7	12.9±0.5	−9†
	k_{DNA} (g of protein/day per g of DNA)	Soleus	18.3±1.1	16.4±0.7	−10†
		Plantaris	19.9±0.8	19.3±0.6	−3†
		Soleus/plantaris ratio	0.90±0.05	0.83±0.04	−7†
		Diaphragm	11.2±0.3	10.9±0.9	−3†
Large	k_{RNA} (g of protein/day per g of RNA)	Soleus	16.8±1.0	13.4±0.5	−20**
		Plantaris	15.8±0.6	13.9±1.2	−12†
		Soleus/plantaris ratio	1.06±0.04	1.00±0.08	−5†
	k_{DNA} (g of protein/day per g of DNA)	Soleus	15.6±1.3	12.7±0.4	−18*
		Plantaris	15.5±0.8	12.4±0.9	−20*
		Soleus/plantaris ratio	1.00±0.05	1.05±0.07	+5†

considerable period of time elapsed between decapitation and dissection (approx. 10 min), so no confidence could be attached to any synthesis data pertaining to these muscles.

Table 7 shows that ethanol feeding was accompanied by an apparent decrease in k_{RNA} in soleus, but not in plantaris or diaphragm, of small rats. A significant decrease in the ratio of soleus/plantaris k_{RNA} demonstrated a greater response in the soleus. Similar results were obtained in large rats.

The amount of protein synthesized per unit of DNA, or cellular efficiency (k_{DNA}), in soleus, plantaris and

Table 8. Effect of chronic ethanol feeding on absolute synthesis rates

Absolute rates of protein synthesis were measured from protein contents (Table 2) and k_s (Table 6). Data are presented as means ± S.E.M. for six or seven pairs of observations. Differences between means were assessed by Student's t test for paired samples: †$P > 0.05$ (not significant); *$P < 0.05$; **$P < 0.25$; ***$P < 0.01$.

Rats	Parameter	Muscle	Control	Ethanol	Difference (%)
Small	Absolute synthesis rate (mg of protein/day per rat)	Soleus	4.56±0.26	3.61±0.22	−21**
		Plantaris	5.76±0.29	4.52±0.19	−22***
		Soleus/plantaris ratio	0.79±0.05	0.81±0.04	+3†
	Absolute synthesis rate/ body wt. (mg of protein/ day per kg)	Soleus	20.4±1.0	18.2±1.2	−11†
		Plantaris	25.8±1.1	22.7±1.0	−12*
		Soleus/plantaris ratio	0.79±0.05	0.81±0.04	+3†
Large	Absolute synthesis rate (mg of protein/ day per rat)	Soleus	6.41±0.70	4.95±0.27	−23†
		Plantaris	7.05±0.37	5.21±0.40	−26***
		Soleus/plantaris ratio	0.90±0.06	0.98±0.07	+9†
	Absolute synthesis rate/ body wt. (mg of protein/ day per kg)	Soleus	16.3±1.5	13.7±0.6	−16†
		Plantaris	18.0±0.7	14.4±1.0	−20*
		Soleus/plantaris ratio	0.90±0.06	0.98±0.07	+9†

diaphragm in small rats was unaltered by ethanol treatment. However, in large rats k_{DNA} in soleus and plantaris fell by 18% and 20% respectively.

Table 8 shows that absolute rates of protein synthesis in soleus and plantaris muscles from small and large rats were decreased by ethanol treatment. Smaller decreases were also obtained for absolute rates as a proportion of body weight.

Similar differences between control and treated groups for k_s, k_{RNA}, k_{DNA} and absolute rates of synthesis were obtained when data were calculated from the assumption that the precursor amino acid was derived from extracellular pools as represented by S_p, i.e. by substituting S_p for S_i in the formula used to calculate k_s (results not shown).

DISCUSSION

Use of sexually immature and mature rats

The rationale for using young (85 g) rats was based on the observations that muscle protein metabolism in younger animals is particularly responsive to nutritional disturbances (Goodman & Ruderman, 1980; V. R. Preedy, unpublished work). For the present study, the sexually immature rats (approx. 5 weeks *post partum*) had a growth rate of 10%/day and an estimated rate of mixed-muscle protein synthesis of 15%/day at the onset of treatment. However, a potential flaw is that sexually immature rats do not have equivalent alcohol-abuse problems to those in man. To circumvent this criticism, the same feeding regime and analytical procedures were applied to rats which were sexually mature at the onset of treatment. These (approx. 9–10 weeks *post partum*) had an initial growth rate of 2%/day and an estimated fractional rate of mixed-muscle protein synthesis of 5%/day, at the onset of treatment. As immature rats were sexually mature at the end of treatment, we have distinguished between both sets of animals by the terms 'small' and 'large'.

Effect of chronic ethanol feeding on muscle composition

Data from small and large rats clearly demonstrated that ethanol feeding decreased the weights of individual muscles. Type I-fibre-rich muscles and large rats showed the least change. The results also showed that chronic ethanol feeding caused a decrease in protein mass. This represented a diminished rate of accretion rather than a loss of protein, as both sets of rats were in an active stage of growth. It was difficult to explain why protein concentration was increased in some skeletal muscles. This was similar to that seen in starvation (Li & Goldberg, 1976), and may reflect alterations in the amount of muscle water, glycogen and/or fat.

Control and treated rats were given isovolumetric, isolipidic, isonitrogenous and isoenergetic amounts of the same diet. The decrease in total muscle protein was therefore due to defects in nutrient absorption, retention or excretion. For example, studies with chronically treated ethanol-fed rats have shown impaired intestinal absorption of glucose, calcium and zinc (Lieber, 1982). Evidence for altered urinary excretion of nutrients, e.g. calcium, has also been obtained (Baran *et al.*, 1980). Data to support the hypothesis that ethanol causes defects by affecting the metabolism and retention of nutrients by the tissue itself have also been obtained in both man and laboratory animals. Ethanol ingestion increases urinary nitrogen excretion without overt evidence of increased faecal nitrogen loss (Rodrigo *et al.*, 1971; Klatskin, 1961; Bunout *et al.*, 1987). This would be compatible with the present study, in which diminished protein accretion occurred despite giving isonitrogenous diets to control and ethanol-fed rats.

Mechanisms for decreased muscle protein

Data were presented to suggest possible mechanisms for diminished protein mass in Type-II-fibre-rich muscles. In both sets of rats, muscle RNA was decreased. With the assumption that muscle RNA is predominantly ribosomal, the data implied a decrease in the capacity or potential for the Type-II-fibre-rich muscles to synthesize protein, i.e. a decrease in synthetic machinery. In contrast, in the Type-I-fibre-rich soleus, the rate at which RNA was translated was significantly decreased in both small and large rats without evidence of protein loss. It

is difficult to explain why k_{RNA} in Type-I-fibre-rich muscles was more sensitive than that for Type-II-fibre-rich muscles.

In large and small rats k_s and k_{RNA} were decreased by only a small percentage by chronic ethanol treatment. These changes were smaller than those in acute ethanol studies (Preedy & Peters, 1988), which showed decreases of approx. 30% in k_s and k_{RNA}. In chronic studies on mixed-muscle protein synthesis in rats, treatments such as dexamethasone administration for 5 days, or diabetes mellitus for 56 days, caused 35% and 50% decreases in k_s respectively (Kelly & Goldspink, 1982; Pain *et al.*, 1983). Thus it appeared that the changes in k_s and k_{RNA} in our experiment were relatively small in comparison with the response in other studies.

Two explanations are possible for the small changes in k_s. The first relates to feeding patterns. Control rats consumed most of their food during the first 6–9 h of feeding, whereas treated rats gradually ate their food during the night and day. It is possible that the timing of the synthesis measurements may have influenced k_s. This is a problem that occurs in many protein-synthesis studies. We have attempted to resolve this by ensuring that synthesis was measured in both control and ethanol-treated rats when in the fed state. The adverse reaction to ethanol withdrawal precluded any measurement of k_s in the starved state. Also, withdrawal of liquid diets would have resulted in diminished circulating concentrations of ethanol in the treated group. Furthermore, the measurement of k_s itself uses a validated technique which overcomes methodological problems associated with immobilization or stress and precursor pools (Garlick *et al.*, 1980). Thus the conclusion seems that these small changes in k_s and k_{RNA} were real and may be a characteristic of chronic ethanol feeding. However, consideration should be given to the concept that even small perturbations in synthesis will have an accumulative effect on muscle protein content. Thus it would seem that after 6 weeks the rats (particularly from the small group) attained a transitional phase, adapting to their diet by producing small changes in k_s. Evidence for such adaptation in prolonged ethanol feeding has been obtained for phosphorus metabolism for example (Ferguson *et al.*, 1981).

The second explanation for the small changes in k_s was that after 6 weeks rats may have attained a stage in which the regulation of protein may also have been determined by changes in the rate of protein breakdown (k_d). Theoretically this can be determined from the difference between the fractional rate of growth of the whole body and synthesis (Waterlow *et al.*, 1978). For example, k_d in plantaris of small control and ethanol-fed rats would be 4.6 and 4.5%/day respectively, whereas corresponding values for the large rats would be 4.6 and 3.7%/day respectively. This indicates an apparent decrease in the rate of protein degradation in response to ethanol feeding. Similar decreases in k_d were observed for the soleus. However, k_d measurements are complicated by the fact that the fractional rates of growth of individual muscles are different, and accumulation of body mass may include increasing amounts of fat. A more appropriate study, to measure k_d, would be the sequential killing of alcohol-fed and control rats and measurement of muscle protein content. However, it is very difficult to measure accurately small changes in protein content. More experimental work is required to determine the quantitative role of degradative pathways in alcohol-induced myopathy.

Differences between small and large rats

Small rats showed greater decrease in protein content of Type II muscles than did large rats. A possible explanation for this was that between 50 and 200 g body wt. DNA deposition in rat skeletal muscle occurs quite rapidly (Lewis *et al.*, 1984). As the amount of protein per nucleus is relatively constant during growth (Waterlow *et al.*, 1978), decreased protein mass would be a predicted outcome if decreases in the amount of DNA occurred. The data showed that in small rats DNA content was decreased whereas in large rats it was unchanged. This gives support to the above hypothesis. However, the term k_{DNA} has been defined in a functional way as the amount of protein synthesis controlled by each nucleus, or cellular efficiency. Although the multinucleated nature of skeletal muscle complicates this description, the data showed that k_{DNA} in large rats was decreased to a greater extent than in small rats. This therefore suggests that ethanol exerts regulatory control by two other mechanisms, i.e. by altering the number of nuclei or the amount of protein synthesized per unit of DNA.

Differences between Type-I- and Type-II-fibre-rich muscles

The data showed that Type-I- and Type-II-fibre-rich muscles displayed differential sensitivity for a number of parameters. In small rats the soleus/plantaris ratios were statistically significant for tissue weight, protein concentration and content, DNA content, RNA per cell, synthetic capacity and k_{RNA}. In large rats, significant differences were also obtained for some of these parameters. This was very similar to the occurrence in human skeletal muscle, which showed that the cross-sectional area of Type II fibres was decreased, though the Type I fibres were relatively unaffected (Martin *et al.*, 1985). Factors for these contrasting responses may be sensitivity to various hormones which are known to affect Type-I- and Type-II-fibre-rich muscles differently, i.e. insulin and glucagon (Preedy *et al.*, 1980; Preedy & Garlick, 1985). Measurements of insulin (V. R. Preedy & T. J. Peters, unpublished work) precluded that this hormone decreased k_s, as no changes were observed in large animals. As we have argued previously (Preedy & Peters, 1988), ethanol causes a variety of changes in the biochemistry of tissue and blood, which may be responsible for its toxic effects (Lieber, 1982). Alternatively, the degree of fibre stretch may have afforded a protective mechanism against ethanol-induced damage. For example, various studies have demonstrated that activity or stretch can ameliorate the skeletal-muscle myopathy observed in denervation and dystrophy (Frankeny *et al.*, 1983; Kane *et al.*, 1984).

Conclusion

In conclusion, skeletal-muscle protein synthesis was inhibited by ethanol, and muscles containing a predominance of anaerobic (Type II) fibres were relatively more susceptible. Significant decreases were also obtained for absolute synthesis rates, in individual muscles from both small and large rats. Ethanol-induced disturbances in nitrogen excretion may therefore be due to detrimental changes in skeletal-muscle protein turnover, as this tissue comprises 40% of whole-body weight.

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