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Role of thyroid hormones in early postnatal development of skeletal muscle and its implications for undernutrition

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Energy intake profoundly influences many endocrine axes which in turn play a central role in development. The specific influence of a short period of mild hypothyroidism, similar to that induced by undernutrition, in regulating muscle development has been assessed in a large mammal during early postnatal life. Hypothyroidism was induced by providing methimazole and iopanoic acid in the feed of piglets between 4 and 14 d of age, and controls were pair-fed to the energy intake of their hypothyroid littermates. Thyroid status was evaluated, and myofibre differentiation and cation pump concentrations were then assessed in the following functionally distinct muscles: *longissimus dorsi* (*l. dorsi*), *soleus* and *rhomboideus*. Reductions in plasma concentrations of thyroxine (T_4 ; 32%, $P < 0.01$), triiodothyronine (T_3 ; 48%, $P < 0.001$), free T_3 (58%, $P < 0.001$) and hepatic 5'-monodeiodinase (EC 1.11.1.8) activity (74%, $P < 0.001$) occurred with treatment. Small, although significant, increases in the proportion of type I slow-twitch oxidative fibres occurred with mild hypothyroidism, in *l. dorsi* (2%, $P < 0.01$) and *soleus* (7%, $P < 0.01$). Nuclear T_3 -receptor concentration in *l. dorsi* of hypothyroid animals compared with controls increased by 46% ($P < 0.001$), a response that may represent a homeostatic mechanism making muscle more sensitive to low levels of circulating thyroid hormones. Nevertheless, Na^+, K^+ -ATPase (EC 3.6.1.37) concentration was reduced by 15–16% in all muscles (*l. dorsi* $P < 0.05$, *soleus* $P < 0.001$, *rhomboideus* $P < 0.05$), and Ca^{2+} -ATPase (EC 3.6.1.38) concentration was significantly reduced in the two slow-twitch muscles: by 22% in *rhomboideus* ($P < 0.001$) and 23% in *soleus* ($P < 0.05$). It is concluded that during early postnatal development of large mammals a period of mild hypothyroidism, comparable with that found during undernutrition, induces changes in myofibre differentiation and a down-regulation of cation pumps in skeletal muscle. Such changes would result in slowness of movement and muscle weakness, and also reduce ATP hydrolysis with a concomitant improvement in energetic efficiency.

Ca²⁺-ATPase: Muscle: Na⁺,K⁺-ATPase: Thyroid: Undernutrition

Normal muscle development is essential for many functions including maintenance of posture, movement and thermogenesis. The acquisition of contractile ability is achieved in part by both the differentiation of muscle fibres and the maturation of two cation pumps: the sarcolemmal (SL) Na^+, K^+ -ATPase (EC 3.6.1.37) and the sarcoplasmic reticulum (SR) Ca^{2+} -ATPase (EC 3.6.1.38) (Clausen *et al.* 1991). Both these ATPases undergo marked developmental up-regulation in porcine muscle during the first 2 weeks postnatally (Dauncey *et al.* 1994; Harrison *et al.* 1994a), indicating that this period of life may be particularly susceptible to modifications by environmental factors such as nutrition, ambient temperature, activity, or hormonal status which could in turn impair the normal development of muscle.

Both the quantity and composition of food eaten influence thyroid hormone (TH) status

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(Dauncey, 1990) which in turn plays a major role in regulating myofibre differentiation (d'Albis & Butler-Browne, 1993; Dauncey & Gilmour, 1996) and cation pump concentrations (Clausen *et al.* 1991). In adult rats severe hypothyroidism over prolonged periods results in conversion of fast- to slow-twitch fibres (McAllister *et al.* 1991) and reduced rates of muscle contraction and relaxation (Gold *et al.* 1970), which explains in part the associated slowness of movement and muscle weakness. Moreover, muscle function would also be affected by the down-regulation of Na⁺-K⁺- and Ca²⁺-ATPase concentrations which occurs in severe hypothyroidism (Kjeldsen *et al.* 1986a; Simonides & van Hardeveld, 1989).

Much less well understood is the extent to which the mild hypothyroidism associated with undernutrition (Dauncey, 1990) affects muscle development and whether changes can be exerted within a short period of time, especially in young growing animals. The aim of the present study, therefore, was to determine the effect of a short period of pharmacologically-induced mild hypothyroidism, comparable with that due to a 50% reduction in energy intake, on myofibre proportions and cation pump concentrations in three morphologically and functionally distinct skeletal muscles during the first 2 weeks of postnatal development in a large mammalian species. To avoid any influence of changes in feed intake with induced hypothyroidism, care was taken to ensure that controls were pair-fed to the feed intake of hypothyroid animals. Furthermore, animals were kept at thermal neutrality to ensure that environmentally-induced changes in energy expenditure were avoided. Part of this work has been the subject of preliminary communications (Harrison *et al.* 1993, 1994c).

METHODS

Subjects and experimental design

Piglets (*n* 18) of the Large White breed were left with the sow until 2 d of age to enable the feeding of colostrum. Animals within the same litter were paired for similar birth weight, of approximately 1.4 kg. Piglets were housed in pairs in temperature-controlled rooms at thermal neutrality, which is approximately 34° at 2 d of age. The temperature was reduced by 1° every 3 d to allow for the known decrease in critical temperature with age (Mount, 1979). Each pair of animals was bottle-fed to repletion four times daily (at 12.00, 18.00, 00.00, 06.00 hours) for the first 2–4 d with sow's milk-replacement formula (Lifeline; British Denkavit, Poole, Dorset). This formula is available as a complete milk replacer for piglets and as such it can be used to rear piglets in the absence of the sow. Lifeline contains (g/kg): sugar (as lactose) 482, protein 173, fat 159, ash 101, moisture 26, starch 2, and added vitamins, and provides 17.8 kJ/g. It is reconstituted with water at a concentration of 150 g powder/l mixed milk.

At the start of day 4 (12.00 hours) animals were weighed and within each pair were assigned randomly to either the control or hypothyroid group. Mild hypothyroidism was induced by providing the following compounds in the feed: methimazole, which blocks thyroidal production of TH, and iopanoic acid, which blocks peripheral conversion of T₄ to T₃. Initial starter doses of 30 mg methimazole/kg and 4.1 mg iopanoic acid/kg were provided. At subsequent six-hourly feeds hypothyroid piglets were given 7.5 mg methimazole/kg and 1.0 mg iopanoic acid/kg. The daily dose of methimazole was estimated on the basis of previous studies in the young pig (Morovat & Dauncey, 1995) and that for iopanoic acid was based on the amount used for human subjects (Acheson & Burger, 1980). This treatment induced mild hypothyroidism (see p. 846) but otherwise the animals were in good health and had a good appetite at each meal. Control piglets were pair-fed to the feed intake of their hypothyroid littermates on the basis of metabolic body size (g/kg^{0.67}). Animals were maintained on this treatment until aged 14 d.

Plasma and tissue samples

After 3 d of treatment, at the age of 7 d, 0.5 ml blood from the vein corresponding to the median vein of the elbow in human subjects was obtained from both control and hypothyroid animals, for measurement of plasma TH concentrations. After 10 d of treatment piglets were sedated at least 9 h after their last meal by an intramuscular injection of ketamine hydrochloride (0.5 ml Vetalar (100 mg/ml); Parke-Davis Veterinary, Pontypool, Gwent). Blood samples (5 ml) were then taken by cardiac puncture into heparinized tubes and plasma was stored at -40° . Animals were killed by injection of 0.7 ml pentobarbitone sodium (Lethobarb, 200 g/l; Duphar Veterinary Ltd, Southampton, Hants)/kg. All procedures were carried out with full authorization from the UK Home Office. The liver and three functionally and morphologically distinct skeletal muscles were dissected and small pieces were frozen rapidly and stored at -40° . Tissue samples for histochemistry were frozen in 2-methylbutane (isopentane) cooled by liquid N_2 while all other tissue samples were frozen directly in liquid N_2 . The muscles investigated were *l. dorsi*, a predominantly type II fast-twitch muscle of the thoracic-lumbar region, used for rapid movement; *soleus*, with a high proportion of type I slow-twitch fibres, associated with maintenance of posture; and *rhomboideus*, also a postural muscle but of interscapular origin with a postulated key role in thermoregulation.

Plasma thyroid hormone concentrations

Total 3,5,3',5'-tetraiodo-L-thyronine (thyroxine; T_4) and total 3,5,3'-triiodothyronine (T_3) concentrations were measured using commercially available kits (Coat-a-Count; Diagnostic Products Corporation, Los Angeles, CA, USA), which incorporate a solid-phase radioimmunoassay (RIA) system. Free T_3 was measured using a Liso-phase Sclavo kit (Metachem Diagnostics Ltd, Northampton). The method incorporated separation of free T_3 by chromatography and RIA of the adsorbed hormone using solid-phase separation of the bound antibody from the free antigen.

Hepatic 5'-monodeiodinase (EC 1.11.1.8) activity

Hepatic 5'-monodeiodinase activity was assessed because peripheral conversion of T_4 to T_3 represents a major source of circulating T_3 in the young pig (Slebodziński *et al.* 1985). The method of Kühn *et al.* (1987) was used, with some modification. Liver (approximately 4 g) was homogenized in 4 vol. phosphate buffer (150 mmol/l), pH 7.4, and spun at 700 g for 10 min. Portions (100 μ l) of the microsomal fraction (approximately 2 mg protein) were incubated for 30 min at 37° with 100 μ l T_4 (1.0 μ mol/l) in the presence of 100 μ l dithiothreitol (7.5 mmol/l). The reaction was stopped by cooling the tubes on ice and addition of 1 ml ethanol (950 ml/l). Blank tubes contained no T_4 during the incubation but had T_4 added after the addition of ethanol. An appropriate dilution of each sample was then assayed for T_3 content using the method described previously.

Isolation of muscle nuclei and assay of thyroid hormone receptors

The methods were based on those described previously (Dauncey *et al.* 1988; Morovat & Dauncey, 1995). Briefly, muscle samples (approximately 10 g) were homogenized in buffer A containing (mmol/l): K_2HPO_4 0.2, KH_2PO_4 0.6, sucrose 250, $MgCl_2$ (pH 6.8) 1, with the addition of Triton X-100 (1 ml/l). The homogenates were then filtered through mesh of 35 μ m pore size. The filtrate was centrifuged at 750 g for 10 min and the pellet was washed twice in buffer A. The final pellet was layered over buffer B containing (mmol/l): K_2HPO_4 3.5, sucrose 230, $MgCl_2$ (pH 6.8) 1, and centrifuged at 70000 g for 40 min. Muscle nuclei were further purified with a centrifugation at 30000 g for 60 min in buffer B. The pelleted

nuclei were washed in buffer A and finally suspended in buffer C containing (mmol/l): tris(hydroxymethyl)aminomethane 20, sucrose 320, MgCl_2 1, dithiothreitol (pH 7.4) 5. All procedures were carried out at 4°.

The DNA content of the nuclear extract was determined by fluorimetry. Portions (200 μl) of the nuclear suspension containing 20 μg DNA were incubated for 30 min at 37° with 100 μl ^{125}I -labelled T_3 and increasing concentrations of unlabelled T_3 in buffer C. Tubes used to measure non-specific binding contained a 100-fold excess of unlabelled T_3 . The nuclei were then centrifuged at 3500 g for 10 min. The pellet was washed in buffer A containing Triton X-100 (10 ml/l) and its radioactivity measured on a gamma counter (Autogamma; Canberra Packard, Berks.). Values for specific maximal T_3 -binding capacity (B_{max}) were obtained from Scatchard plots after correction for non-specific binding.

Histochemical analysis and identification of myofibre types

Serial sections of each muscle were cut on a cryostat (Bright Instruments, Huntingdon, Cambs.) at -22° and a thickness of 10 μm . The plane of the sections was at right angles to the longitudinal axis of the fibres. Muscle sections were air-dried for 30 min and stained for myosin ATPase (mATPase) (EC 3.6.1.32) after acid (pH 4.35) or alkaline (pH 9.4) pre-incubation using the method of Brooke & Kaiser (1970). To determine the metabolic characteristics of individual fibres (oxidative *v.* non-oxidative) serial sections were treated to reveal succinate dehydrogenase (SDH) (EC 1.3.99.1) activity using the cytochemical method described previously (Dauncey & Ingram, 1988). Type I slow-twitch fibres were characterized by their acid-stable-alkali-labile mATPase activity, while type II fast-twitch fibres were characterized by their alkali-stable-acid-labile mATPase activity. SDH staining used in conjunction with mATPase enabled overall classification of fibres into one of the following three categories: type I slow oxidative (SO), type II fast oxidative and glycolytic (FOG), and type II fast glycolytic (FG) fibres. A fourth fibre type (IIC) was also found to occur in skeletal muscles, although the proportion of these fibres was always very small and less than 5%. These IIC fibres were both acid- and alkali-stable and it is generally thought that such fibres probably contain a mixture of fast and slow myosins and are in transition between the fast and slow types.

Measurement of myofibre proportion and size

The relative proportion of each fibre type was assessed in a standard field area of 109 700 μm^2 . Fibre typing was carried out on 250–700 fibres counted over at least three fields for each muscle. Thus, proportions of fibre type were based on counts of the number of fibres; these can be converted to fibre volume by correcting for differences in fibre cross-sectional area. Mean fibre-type cross-sectional area was determined for a minimum of fifty fibres for each fibre type over three to five fields. All measurements were made using a Seescan A010 research grade image-analysis system (Seescan plc, Cambridge).

^3H -labelled ouabain binding

The concentration of Na^+ , K^+ -ATPase in muscle samples was determined from the maximal ^3H -labelled ouabain-binding capacity in the presence of vanadate (Nørgaard *et al.* 1983). This method enables quantification of the total concentration of Na^+ , K^+ -ATPase in small samples of muscle and the values obtained correspond to the total population of functional Na^+ , K^+ ATPases (Clausen *et al.* 1987). In brief, frozen muscles samples were cut into segments weighing 2–8 mg, washed and incubated in buffer of the following composition (mmol/l): tris(hydroxymethyl)aminomethane 10, MgSO_4 3, tris-vanadate 1, and sucrose 250, pH 7.2–7.4. The specimens were equilibrated for 2 h at 37° in buffer containing ^3H -labelled ouabain (0.6 $\mu\text{Ci/ml}$) and unlabelled ouabain at a final concentration of 10^{-6} mol/l.

This has been shown previously to allow saturation of all ^3H -labelled ouabain-binding sites in porcine skeletal muscle (Dauncey & Burton, 1989). This was followed by four 30 min washes in ice-cold unlabelled buffer to remove ^3H -labelled ouabain not bound to the receptors. The specimens were then blotted, weighed on a microbalance and soaked overnight in mini-vials containing 0.5 ml trichloroacetic acid (TCA; 0.3 M) with 0.1 mmol ouabain carrier/l before addition of 2.5 ml optifluor (Pico-fluor; Packard Instrument Co., Groningen, The Netherlands) and liquid-scintillation counting of ^3H activity. The amount of ^3H -labelled ouabain taken up and retained in the specimens was calculated and corrected for non-specific uptake. From each muscle sample, four specimens were taken for measurement, allowing quadruplicate determinations of maximum binding capacity (B_{max}).

Ca²⁺-dependent ³²P-labelled ATP incorporation

The concentration of Ca^{2+} -ATPase was determined by measurement of the Ca^{2+} -dependent steady-state phosphorylation from ^{32}P -labelled ATP in crude muscle homogenates (Everts *et al.* 1989). Ca^{2+} catalyses the dephosphorylation of ^{32}P -labelled ATP leading to incorporation of ^{32}P into the Ca^{2+} -ATPase. Briefly, muscle samples of approximately 40 mg were homogenized at 0° in buffer of the following composition (mmol/l): hepes 5, sucrose 300, pH 7.4, and diluted to a final concentration of 10 mg wet wt/ml. A portion (0.2 ml) of homogenate was incubated for 30 s at 0° with 2.5 ml of a solution of the following composition (mmol/l): imidazole 100, KCl 100, MgCl_2 5, ethyleneglycol-bis-(β -aminoethyl ether) N,N,N',N'-tetraacetic acid (EGTA) 0.5, in the presence of 0.05 mM-ATP, γ - ^{32}P -labelled ATP (0.3 $\mu\text{Ci/ml}$), pH 7.4. The reaction was stopped with 3 ml of a solution of the following composition (mmol/l): $\text{Na}_4\text{P}_2\text{O}_7$ 10, KH_2PO_4 10, TCA 600, and the solution was centrifuged at 0° and 1200 *g* for 40 min. The resulting pellet was washed twice with a solution of the following composition (mmol/l): $\text{Na}_4\text{P}_2\text{O}_7$ 10, KH_2PO_4 10, TCA 60. The pellet was dissolved in 0.5 ml NaOH (1 mM) and ^{32}P activity was measured by liquid-scintillation counting. Ca^{2+} -ATPase concentration was calculated as the difference between the ^{32}P incorporation in the absence and presence of CaCl_2 , added to obtain a concentration of free Ca^{2+} of 50 $\mu\text{mol/l}$.

This technique involving crude muscle homogenates determines the concentration of both SR Ca^{2+} -ATPase and SL Ca^{2+} -ATPase. The contribution made to total Ca^{2+} -ATPase concentration by the SL Ca^{2+} -ATPase is, however, minimal since the contribution of SL to SR membrane area is approximately 1% and, moreover, SL Ca^{2+} -ATPase activity in comparison with the SR Ca^{2+} -ATPase is approximately 3% (Mickleton *et al.* 1985). Therefore, most of the total Ca^{2+} -ATPase concentration in skeletal muscle can be attributed to the SR Ca^{2+} pump.

Plasma cortisol and aldosterone concentrations

Plasma concentrations of cortisol and aldosterone were measured using commercially available ^{125}I -labelled cortisol and ^{125}I -labelled aldosterone RIA kits (ICN Biomedicals Inc., Thame, Oxon). These two hormones were measured in addition to plasma TH concentrations because they have both been implicated in regulation of Na^+ , K^+ -pump concentration (Hegyvary, 1977; Dørup, 1996).

Statistical analysis

The results were analysed using Genstat (Lawes Agricultural Trust, 1987). A paired *t* test was considered to be the most appropriate statistical test because comparisons were made between pair-fed control and hypothyroid littermate animals of identical parentage. Data are presented as means with their standard errors. Results were considered statistically significant at the $P < 0.05$, 0.01 or 0.001 levels.

RESULTS

Growth rates and feed intakes

The mean body weight of animals at birth was 1.4 (SE 0.07) kg. Piglets gained approximately 0.4 kg between birth and 2 d of age, and then maintained weight for the next 2 d, after they were removed from the sow and the start of bottle-feeding. The pair-feeding regimen ensured that feed intakes were very similar in the two groups of animals. Thus, at the start of treatment on day 4 average feed intakes were 425 and 428 g for control and hypothyroid groups respectively, and these increased to 739 and 762 g at 14 d of age. Growth rates for the two groups were also similar during the 10 d of treatment. Mean body weights (kg) at 6, 10 and 14 d were 1.9 (SE 0.09), 2.2 (SE 0.08) and 2.5 (SE 0.08) for the controls, and 2.0 (SE 0.10), 2.3 (SE 0.09) and 2.7 (SE 0.09) for the hypothyroid group. The very small difference in growth rate was probably due to a lower resting metabolic rate in the hypothyroid animals.

Thyroid hormone status

Plasma TH levels. After 10 d of treatment the mean plasma total T_4 concentration was reduced by 32% ($P < 0.01$) in the hypothyroid group compared with the control group (Table 1). Plasma total T_3 concentration was affected similarly, with a mean reduction of 48% ($P < 0.001$) in the hypothyroid animals compared with the control animals. Analysis of plasma samples taken during treatment showed that at 7 d of age (i.e. 3 d of treatment), total T_3 concentration (nmol/l) in the hypothyroid animals was 2.4 (SE 0.07) compared with 2.9 (SE 0.09) in the controls ($P < 0.05$), indicating that hypothyroidism was induced rapidly after the start of treatment. Plasma total T_4 and T_3 concentrations were very similar between animals within a treatment group. By contrast, the concentrations of free T_3 were more variable within each group (Fig. 1), ranging from 5.5 to 10.8 pmol/l for controls and from 1.3 to 7.2 pmol/l for the hypothyroid group; the average reduction induced by hypothyroidism was 58% ($P < 0.001$).

Hepatic deiodinase activity. The hepatic 5'-monodeiodinase activities of the control and hypothyroid animals are given in Table 1, which shows a marked difference in the rate of peripheral conversion of T_4 to T_3 between the two groups. Thus, after only 10 d of administration of methimazole and iopanoic acid there was a 74% reduction in deiodinase activity ($P < 0.001$). Deiodinase activity (pmol T_3 /min per mg protein) ranged from 434 to 666 in the controls and from 20 to 172 in the hypothyroid animals. Fig. 1 shows the relationship between hepatic deiodinase activity and plasma free T_3 concentration. In general, within each group free T_3 concentration was highest in animals with a high hepatic deiodinase activity, although in some animals with similar deiodinase activity different free T_3 concentrations were observed, a finding that may be due to temporal fluctuations in the concentration of free T_3 (Dauncey & Morovat, 1993).

Myonuclear T_3 receptors. A short period of mild hypothyroidism was found to induce a striking up-regulation of nuclear T_3 receptors in skeletal muscle (Fig. 2): the maximal T_3 -binding capacity of *l. dorsi* muscle was increased by 46% in hypothyroid animals compared with control animals ($P < 0.001$). Because of the large amount of muscle needed for determination of nuclear T_3 -receptor binding, this analysis was performed only on *l. dorsi* and not on *soleus* and *rhomboideus* muscles.

Muscle morphology

Fibre proportions. Table 2 shows that in *l. dorsi* muscle a short period of mild hypothyroidism resulted in a very small, although statistically significant, increase in the relative amount of type I SO fibres, with mean values of 5.4 (SE 0.5) and 7.4 (SE 0.7)% in

Table 1. Plasma total thyroxine (T_4) and total and free 3,5,3'-triiodothyronine (T_3) concentrations, and hepatic 5'-monodeiodinase activity†

(Mean values with their standard errors for nine pairs of control and hypothyroid animals)

Treatment group	Plasma thyroid hormone concentrations						Hepatic 5'-monodeiodinase activity	
	Total T_4 (nmol/l)		Total T_3 (nmol/l)		Free T_3 (pmol/l)		(pmol T_3 /min per mg)	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Control	54.8	3.4	1.8	0.1	7.7	0.7	500	27
Hypothyroid	37.0**	8.6	0.9***	0.1	3.2***	0.6	132***	48

Mean values were significantly different from those for the controls (paired t test): ** $P < 0.01$, *** $P < 0.001$.

† For details of animals and procedures, see pp. 842-843.

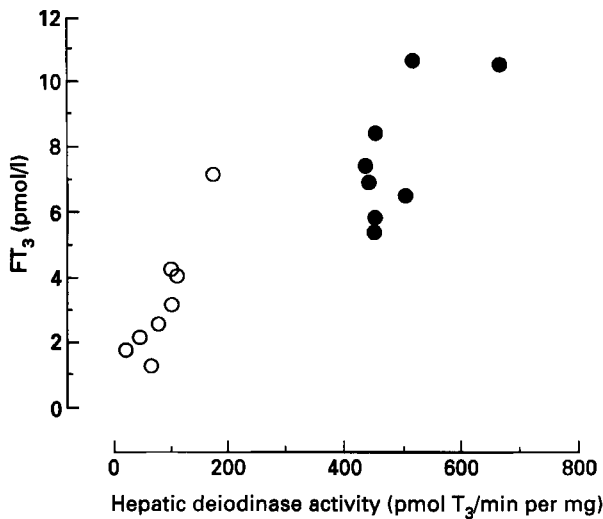


Fig. 1. Relationship between hepatic 5'-monodeiodinase (*EC* 1.11.1.8) activity and plasma free 3,5,3'-triiodothyronine (FT_3) concentration in control (●), and hypothyroid (○) animals at 14 d of age. For details of animals and procedures, see pp. 842-843.

control and hypothyroid groups respectively ($P < 0.01$). A more substantial increase in the amount of type I SO fibres was also found in *soleus* muscle, with mean values of 37.1 (SE 1.0) and 43.4 (SE 1.8)% in control and hypothyroid animals respectively ($P < 0.01$). However, in *rhomboideus* muscle no significant difference in fibre-type amounts was observed after the 10 d period of treatment; mean values for type I SO fibre proportions were 35.2 (SE 2.3) and 32.4 (SE 3.6)% for control and hypothyroid groups respectively ($P = 0.65$).

Fibre cross-sectional areas. Table 3 shows that, in general, no significant difference in fibre-type area was observed between the control and hypothyroid groups. In *l. dorsi* muscle, however, where very little variation between litters occurred in the mean cross-sectional area of type II FOG fibres, a statistically significant reduction in area occurred with hypothyroidism, mean values were 297 (SE 20) and 245 (SE 16) μm^2 in control and hypothyroid animals respectively ($P < 0.05$). Moreover, there was also a tendency for type

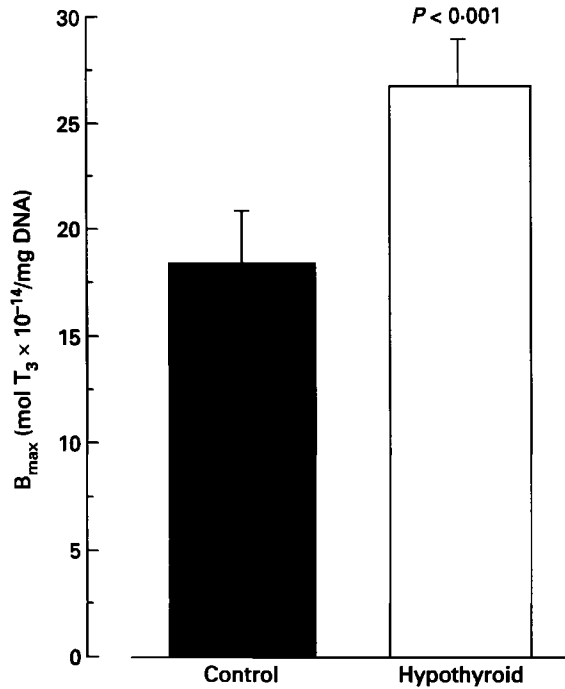


Fig. 2. Maximal T_3 -receptor-binding capacity (B_{max}) of nuclei from *longissimus dorsi* muscle of control (■) and hypothyroid (□) animals at 14 d of age. Values are means with their standard errors from nine pairs of littermate animals. For details of animals and procedures, see pp. 842–844.

Table 2. *Relative amounts (%) of type I slow oxidative (I SO), type II fast oxidative and glycolytic (II FOG), type II fast glycolytic (II FG), and type IIC oxidative and glycolytic fibres in longissimus dorsi (l. dorsi), soleus and rhomboideus muscles†*

(Mean values with their standard errors for nine pairs of control and hypothyroid animals)

Muscle	Treatment group	I SO		II FOG		II FG		IIC	
		Mean	SE	Mean	SE	Mean	SE	Mean	SE
<i>L. dorsi</i>	Control	5.4	0.5	48.1	0.9	45.5	1.0	1.0	0.2
	Hypothyroid	7.4**	0.7	45.4	1.2	46.3	1.7	0.9	0.2
<i>Soleus</i>	Control	37.1	1.0	58.2	1.3	0	0	4.7	0.6
	Hypothyroid	43.4**	1.8	52.4**	2.2	0	0	4.2	0.7
<i>Rhomboideus</i>	Control	35.2	2.3	59.0	2.4	3.1	0.7	2.7	1.0
	Hypothyroid	32.4	3.6	61.4	4.0	2.8	1.1	3.4	0.6

Mean values were significantly different from those for the controls (paired t test): ** $P < 0.01$.

† For details of animals and procedures, see pp. 842–844.

I SO fibres to be smaller in *l. dorsi* but larger in *soleus* muscles of hypothyroid animals compared with controls.

Skeletal muscle Na^+, K^+ -ATPase concentration

It was found that a short period of mild hypothyroidism during early postnatal development leads to a reduction in Na^+, K^+ -ATPase concentration in skeletal muscle, irrespective of muscle type (Fig. 3). Reductions of 15 ($P < 0.05$), 16 ($P < 0.001$) and 16%

Table 3. Cross-sectional areas (μm^2) of type I slow oxidative (I SO), type II fast oxidative and glycolytic (II FOG), type II fast glycolytic (II FG), and type IIC oxidative and glycolytic fibres in longissimus dorsi (l. dorsi), soleus and rhomboideus muscles†

(Mean values with their standard errors for nine pairs of control and hypothyroid animals)

Muscle	Treatment group	I SO		II FOG		II FG		IIC	
		Mean	SE	Mean	SE	Mean	SE	Mean	SE
<i>L. dorsi</i>	Control	426	20	297	20	291	28	243	21
	Hypothyroid	401	26	245*	16	256	20	216	11
<i>Soleus</i>	Control	213	13	162	9	—	—	125	10
	Hypothyroid	253	14	171	13	—	—	141	10
<i>Rhomboideus</i>	Control	393	42	415	33	364	22	263	15
	Hypothyroid	447	37	375	38	388	54	321	49

Mean values were significantly different from those for the controls (paired *t* test): * $P < 0.05$.

† For details of animals and procedures, see pp. 842–844.

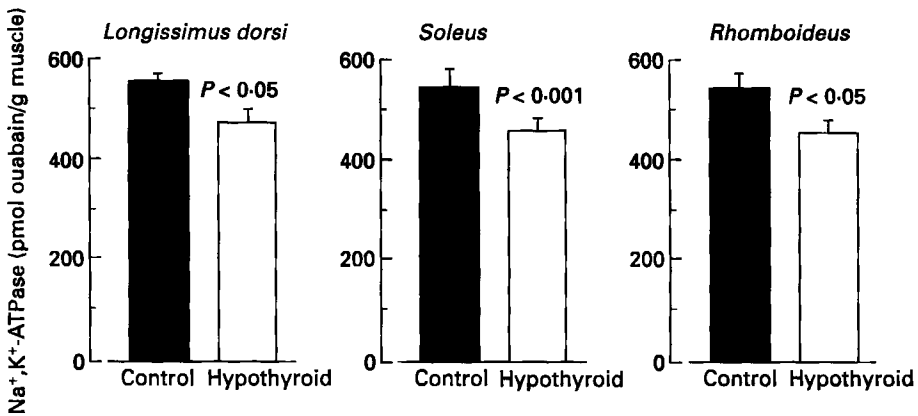


Fig. 3. Total Na^+, K^+ -ATPase (EC 3.6.1.37) concentration in skeletal muscles from control (■) and hypothyroid (□) animals at 14 d of age; estimated using the vanadate-facilitated ^3H -labelled ouabain-binding technique. Values are means with their standard errors from nine pairs of littermate animals. For details of animals and procedures, see pp. 842–845.

($P < 0.05$) occurred in the ^3H -labelled ouabain-binding site concentration of *l. dorsi*, *soleus* and *rhomboideus* muscles respectively, of hypothyroid compared with control animals.

In treatment groups with very large differences in muscle fibre size it can be more meaningful to present results for Na^+, K^+ -ATPase concentration in relation to SL area rather than to muscle weight (Harrison *et al.* 1994b). However, differences in fibre size in the present study were very small (Table 3) and, therefore, results have been presented only in relation to muscle weight. It can, nevertheless, be estimated that the difference in Na^+, K^+ -ATPase concentration between control and hypothyroid animals would have been even greater if results had been expressed in relation to SL area.

Skeletal muscle Ca^{2+} -ATPase concentration

A short period of mild hypothyroidism was found to reduce the concentration of Ca^{2+} -ATPase in skeletal muscle (Fig. 4). However, in the fast-twitch muscle *l. dorsi* the 11% reduction was not statistically significant ($P = 0.20$) because of the wide variation between

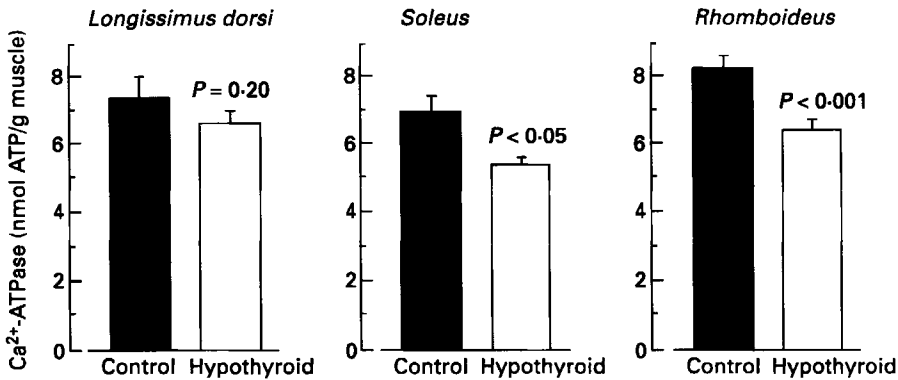


Fig. 4. Total Ca^{2+} -ATPase (EC 3.6.1.38) concentration in skeletal muscles from control (■) and hypothyroid (□) animals at 14 d of age; estimated from Ca^{2+} -dependent steady-state phosphorylation from ^{32}P -labelled ATP. Values are means with their standard errors from nine pairs of littermate animals. For details of animals and procedures, see pp. 842–845.

litters. By contrast, in the slow-twitch muscles *soleus* and *rhomboideus* a more consistent decrease in Ca^{2+} -ATPase concentration occurred, with a period of mild hypothyroidism resulting in mean reductions of 23 ($P < 0.05$) and 22% ($P < 0.001$) respectively.

Plasma cortisol and aldosterone concentrations

Plasma cortisol concentration was 18% lower in hypothyroid compared with control animals; mean values were 42.8 (SE 11.7) and 52.4 (SE 8.1) nmol/l respectively. However, there was wide variation between litters and no significant difference between the two treatment groups ($P = 0.50$). No difference in aldosterone levels were observed between the control and hypothyroid groups; mean values were 47.8 (SE 6.1) and 47.7 (SE 9.2) pmol/l respectively.

DISCUSSION

The present results have shown that in early postnatal life a short period of mild hypothyroidism, comparable with that occurring during undernutrition, induced down-regulation of two cation pumps essential for normal muscle function, the SL Na^+, K^+ -ATPase and the SR Ca^{2+} -ATPase. This response was rapid, occurring within 10 d of treatment, and was not associated with other effects of chronic severe hypothyroidism such as reduced feed intake or marked changes in fibre type proportions. Up-regulation of nuclear T_3 receptors with pharmacologically-induced hypothyroidism in animals receiving an adequate energy intake may have buffered the effects of low plasma TH levels, suggesting a homeostatic mechanism at the level of the myocyte which increases the sensitivity to low levels of TH. Comparisons between three functionally different muscles suggest that in early postnatal development Na^+, K^+ -ATPase concentration is affected to the same extent in all muscles irrespective of fibre type, whereas Ca^{2+} -ATPase concentration may be regulated by TH in a muscle-specific fashion. The significance of these findings is considered in the following sections.

Down-regulation of Na^+, K^+ -ATPase by hypothyroidism

The present study has shown that a pharmacologically-induced 50% reduction in plasma TH concentrations results in a 15% down-regulation in Na^+, K^+ -ATPase concentration in both slow- and fast-twitch muscles, in 2-week-old piglets compared with their pair-fed control littermates (Fig. 3). These results extend previous findings in which down-

regulation of this enzyme has been shown to occur after long periods of chronic severe hypothyroidism (Kjeldsen *et al.* 1986a), by showing that down-regulation of pump concentration can be induced by a short period of mild hypothyroidism, and that the effects of hypothyroidism on pump concentration are not related to a reduction in feed intake. Moreover, even within the control group there was a direct correlation between plasma T_3 levels and Na^+,K^+ -ATPase concentration of the slow-twitch muscles *soleus* ($r\ 0.85$, $P < 0.01$) and *rhomboideus* ($r\ 0.76$, $P < 0.05$), and this correlation was reinforced when the hypothyroid piglets were taken into account. In the fast-twitch *l. dorsi* there was, however, no such correlation within the control group ($r\ 0.46$, $P < 0.20$).

The probability is that this down-regulation in Na^+,K^+ -ATPase was directly due to the reduction in TH levels rather than to indirect changes in other endocrine factors. Thus, aldosterone, which modulates the concentration of Na^+,K^+ -ATPase in cardiac SL (Hegyvary, 1977), was not significantly affected by the short period of hypothyroidism. Similarly, cortisol, which can up-regulate Na^+,K^+ -ATPase concentration in muscle (Dørup, 1996), was not significantly altered in hypothyroid piglets compared with control piglets. The tendency for plasma cortisol to be lower in the hypothyroid group may, however, have made a small contribution to the lower Na^+,K^+ -ATPase values in these animals. It is unlikely that the changes in concentration of Na^+,K^+ -ATPase induced by hypothyroidism resulted from changes in fibre type because these changes were small (Table 2) and not consistent with any previously suggested relationship between Na^+,K^+ -ATPase concentration and fibre type (Dauncey *et al.* 1994; Harrison *et al.* 1994b).

Despite the clear relationship between thyroid status and Na^+,K^+ -ATPase concentration reported in the present and previous studies (Clausen *et al.* 1991), it is relevant to note that these two variables are not invariably related. Thus, reduced TH levels in young pigs living in a warm environment and receiving 50% of the energy intake of controls for 4 weeks did not alter Na^+,K^+ -pump concentrations (Dauncey & Burton, 1989; Harrison *et al.* 1994b). By contrast, in 12-week-old rats a similar level of undernutrition for only 2 weeks was related to a reduction in both plasma T_3 and Na^+,K^+ -ATPase concentration (Kjeldsen *et al.* 1986b). In keeping with these results in the rat is the finding that T_3 administration to fasting rats restores normal Na^+,K^+ -ATPase concentration (Matsumura *et al.* 1992) suggesting that the fasting-induced decrease in pump concentration was due at least in part to the decrease in plasma T_3 levels. These contrasting results are not easy to interpret but may be related to differences in age, species or relative severity of the undernutrition. A potentially important factor is that the young pig has a high *ad libitum* feed intake and, therefore, even those on a 50% intake continue to grow, whereas the 50% restricted rat shows a severe reduction in growth rate.

An additional factor which may help to explain differences between studies is the number of nuclear T_3 receptors, which is likely to affect the tissue response to the hormone. The 46% up-regulation of T_3 receptors in *l. dorsi* muscle in the present study has been reported previously after removal of the thyroid gland or pharmacologically-induced hypothyroidism (Duchamp *et al.* 1994; Morovat & Dauncey, 1995). By contrast, however, a nutritionally-induced reduction in plasma TH levels results in down-regulation of T_3 receptors in *l. dorsi* muscle of young pigs (Dauncey *et al.* 1988). Reasons for this difference are not known but may be related to differences in energy balance (Morovat & Dauncey, 1995). Future investigations, therefore, need to clarify the complex interactions between energy balance and TH status in the acute and long-term regulation of Na^+,K^+ -ATPase concentration.

Down-regulation of Ca^{2+} -ATPase by hypothyroidism

The present study has shown that mild hypothyroidism in pair-fed piglets during the first 2 weeks of postnatal life results in a 22–23% reduction in Ca^{2+} -ATPase concentration in

skeletal muscle (Fig. 4). These results extend the findings of earlier studies in which the Ca^{2+} -accumulating ability of SR from rat muscles was decreased after thyroidectomy (Nwoye *et al.* 1982) as a result of down-regulation of Ca^{2+} -ATPase concentration (Simonides & van Hardeveld, 1989), by showing that a short period of mild hypothyroidism also reduces Ca^{2+} -ATPase concentration in skeletal muscle. It has been observed also that the mRNA levels of SR Ca^{2+} -ATPase are affected; they decrease in hypothyroidism and increase in hyperthyroidism (Rohrer & Dillmann, 1988; Arai *et al.* 1991), confirming that in chronic hypothyroidism, at least, regulation of Ca^{2+} uptake is accomplished primarily by decreasing the number of functional pumps. The present results further increase understanding of the hormonal regulation of Ca^{2+} -ATPase by showing that down-regulation of pump numbers by hypothyroidism is not related to alterations in feed intake, and that regulation of Ca^{2+} -ATPase concentration may be muscle-specific because it was significantly altered only in *soleus* and *rhomboideus* muscles.

Thus, not only do TH exert different effects on myosin genes in different types of muscles (Izumo *et al.* 1986; d'Albis & Butler-Browne, 1993; Dauncey & Gilmour, 1996) but they may also regulate the expression of Ca^{2+} -ATPase in a muscle-specific fashion. Indeed, it has been reported previously that TH can have dual and opposite effects on the expression of different genes in skeletal muscle fibres (Izumo *et al.* 1986; Muller *et al.* 1994). The present results suggest that the muscle-specific regulation of Ca^{2+} -ATPase cannot be related simply to the proportions of fibre type in muscles because there were no marked differences in Ca^{2+} -ATPase concentration in the three muscles studied from control piglets despite differences in their proportions of fibre type (Fig. 4 and Table 2). Thus, muscle-specific differences in nuclear T_3 receptors, local TH metabolism, and neuronal and hormonal signals altered by TH may also play a role. Indeed, different isoforms of nuclear T_3 receptors have been described (Lazar, 1993) and it has been shown that their expression as well as their response to changes in thyroid status may differ according to muscle phenotype (Hoffman *et al.* 1994).

Changes in muscle function induced by hypothyroidism

Despite the short period of administration of methimazole and iopanoic acid, small, but significant, increases in type I SO fibre proportions occurred in *l. dorsi* and *soleus* muscles (Table 2). This suggests that the effects of hypothyroidism on the expression of myosin ATPase isoforms are rapid and can occur within 10 d, especially in young animals. In support of this finding, rapid effects of T_3 on proportions of fibre type have also been reported in 20-week-old hypothyroid rats 14 d after administration of T_3 (Montgomery, 1992). By contrast with the present findings for *l. dorsi* and *soleus* muscles, changes in fibre type were not observed in *rhomboideus* muscle. Although it is difficult to interpret this finding, it may be associated with muscle-specific effects of TH on the myosin gene family (Izumo *et al.* 1986). Moreover, the specific role of *rhomboideus* muscle in thermoregulation (Harrison *et al.* 1996) may also contribute to such a difference.

Although there was only a slight change in the proportions of fibre type in *l. dorsi*, the observation that it changes over a relatively short period suggests that more prolonged hypothyroidism will be of importance for energy conservation, especially as this muscle is one of the largest in the body. Moreover, a consequence of an increase in proportion of type I SO fibres combined with down-regulation of Na^+ , K^+ - and Ca^{2+} -ATPases is probably slowness of movement, muscle weakness and improved energetic efficiency due to a reduction in the quantity of ATP hydrolysed to maintain Na^+ , K^+ homeostasis and sequester Ca^{2+} . Alterations in contractile properties of the muscles may even have preceded changes in their fibre types because changes in isometric force and velocity

properties of muscles have been detected as early as 2 d after T_3 administration to hypothyroid rats (Montgomery, 1992). Indeed, the rapidity with which these changes in contractile properties occurred may have been related to rapid changes in the number of cation pumps because these can occur within a 24 h period (Dauncey *et al.* 1991). Moreover, the responsiveness of muscle to TH may be particularly marked during early development, when there are marked changes in cation-pump concentrations (Harrison *et al.* 1994a; Dauncey *et al.* 1994; Dauncey & Harrison, 1996). Not only is hypothyroidism associated with a reduction in Ca^{2+} -ATPase gene expression but there is also over-expression of its inhibitor, phospholamban (Kiss *et al.* 1994), and these changes would contribute to a depression of both Ca^{2+} uptake and muscle relaxation rate.

Whether the present effects on myofibre cation pumps would impair subsequent development after a euthyroid state had been achieved remains to be investigated. However, studies on intact rat skeletal muscle (Kjeldsen *et al.* 1986a) and cultured rat skeletal myotubes (Brodie & Sampson, 1989) indicated that although TH increased the concentrations of Na^+ - K^+ pumps and Na^+ channels, the newly synthesized pumps had a reduced affinity for their respective ligands. Moreover, preliminary data from small-for-gestational age piglets, which have been undernourished before birth, suggest that not only are Na^+ , K^+ - and Ca^{2+} -ATPase concentrations lower at birth than those of control littermates but they remain lower at 14 d postnatally (Dauncey & Harrison, 1996).

Conclusions

A short period of mild hypothyroidism during early postnatal development was found to induce down-regulation of two cation pumps essential for normal muscle function, the SL Na^+ , K^+ -ATPase and the SR Ca^{2+} -ATPase. This effect on cation-pump concentrations was rapid and could not be explained by any indirect effects of the hypothyroid state such as reduced feed intake, changes in myofibre type or alterations in the circulating levels of other hormones. The possibility that proportions of myofibre type and Ca^{2+} -ATPase concentration may be regulated by TH in a muscle-specific fashion via the differential expression of the various isoforms of nuclear T_3 receptors should be the focus of future investigation. The consequence of these changes in muscle development at the cellular level would be slowness of movement, muscle weakness and improved energetic efficiency. Results hold important implications for muscle function of neonates with low thyroid status resulting from a reduction in energy intake due to illness or restricted food supply.

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