Effects of Growth Hormone on the Expression of Carbonic Anhydrase III and Phosphoglucoisomerase in Rat Slow and Fast-Twitch Skeletal Muscles

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Abstract

Changes in carbonic anhydrase III (CAIII) and phosphoglucoisomerase (PGI) at both the protein and mRNA levels were studied in rat soleus and extensor digitorum longus (EDL) muscles following hypophysectomy and subsequent growth hormone (GH) treatment. Levels of CAIII and PGI proteins were detected in tissue sections using antibodies and the corresponding mRNAs were assessed by Northern and slot-blot analyses. Following hypophysectomy there were increases in CAIII protein and mRNA and decreases in PGI protein and mRNA in both muscles suggesting a shift towards a more oxidative phenotype. In soleus muscles GH-treatment of hypophysectomised rats restored CAIII protein and mRNA levels to those found in control animals but had little effect on PGI gene products over a time course of treatment of 11 days. In EDL muscle PGI protein was restored to control levels and there was a partial recovery of mRNA levels following GH-treatment but CAIII protein and mRNA remained elevated suggesting that the different muscles had individual patterns of response to GH-treatment. Results of histochemical assessment of myosin ATPase generally supported these conclusions.

Key words: skeletal muscle, growth hormone, carbonic anhydrase III, phosphoglucoisomerase.

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Growth hormone (GH) is necessary for normal somatic growth and is used clinically for the treatment of human GH-deficient dwarfism [23]. Its most potent effects include stimulation of skeletal muscle growth promoting both DNA synthesis [14] and protein synthesis [5, 13]. The influence of GH is not, however, confined to young animals as implantation of GH producing tumours induces muscle hypertrophy in adult rats [21, 26, 27] and depletion of GH by hypophysectomy has been shown to result in a decrease in weight of gastrocnemius muscles [6].

GH has also been shown to influence the fibre type composition of fully differentiated adult skeletal muscles [2]. These effects are most clearly observable when the tissue has first been deprived of GH for a prolonged period such as by hypophysectomy. Adult rats subjected to such treatment have been reported to show a loss of type 2 fibres in soleus muscles [28] and an increase in "transitional" fibres with myosin ATPase activity between type 1 and type 2B fibres in extensor digitorum longus (EDL) muscle as assessed by histochemical analysis [3]. The latter authors also reported that after human GH treatment the number of these "transitional fibres" in EDL were reduced and the number of type 1 fibres increased. A decrease in mitochondrial succinate dehydrogenase (SDH) activity has also been reported in soleus and EDL muscles of hypophysectomised animals and this was partially reversed by GH treatment [1, 3].

These changes in muscle fibre type following hypophysectomy may result either from a general loss of characteristics specific for fast and slow twitch muscles or a directional shift in phenotype, which is reversed by GH-treatment. Previous studies, which point to a directional shift in muscle phenotype in response to hypophysectomy which is counteracted by GH-treatment, have largely been confined to fibre type analysis using myosin ATPase in predominantly fast EDL muscles. To extend this work on

contractile proteins we analysed changes in levels of two metabolic enzymes associated with specific fibre types, carbonic anhydrase III (CAIII) and phosphoglucoisomerase (PGI). These enzymes and their corresponding mRNAs were analysed following hypophysectomy and GH-treatment over a period of up to 11 days by immunohistochemical and slot-blotting techniques. These studies were carried out using two rat hind limb muscles, EDL and soleus, which differed substantially in their ratio of type 1 to type 2 fibres. CAIII is an enzyme normally found in muscles composed largely of type 1 fibres and levels of this enzyme have been shown to be responsive to electrical stimulation [8], denervation [10] and limb immobilisation [19]. PGI is a glycolytic enzyme predominant in anaerobic type 2B fibres [4]. Changes in fibre profile of the two muscles in response to GH-treatment was further assessed by histochemical analysis of SDH and acid myosin ATPase activity.

Materials and Methods

Animals

Male Sprague-Dawley rats (200g), purchased from Interfauna (Huntingdon, Cambs, UK) were hypophysectomised by the parapharyngeal method and maintained on minimum diet RH1 (Special Diet Services, Witham, Essex, UK) for 14 days. During this time animals were weighed daily and only those that did not exhibit a weight increase were used for further experiments. After 14 days, groups of rats received daily s.c. injections of recombinant human GH (National Institute of Biological Standards and Control; Code No. 88/624, 60 mIU/injection) in 0.5 cm^3 of diluent (phosphate buffered saline with 0.1% bovine serum albumin) or 0.5 cm^3 of diluent alone for 2,4,7,9 and 11 days. The animals were killed 16 hours after the last injection, since this is within the time-scales reported for maximum response of muscle IGF-1 mRNA to GH-treatment and other stimuli [15, 24]. The animals were killed by asphyxiation with CO_2, the soleus and EDL muscles were rapidly dissected out and immediately frozen in liquid N_2 and stored at -70°C until used. For immunohistochemical studies composite blocks of EDL and soleus muscles were mounted on cork with Tissue-tek and immediately frozen in an isopentane bath immersed in liquid N_2 for 10 seconds. Tissue blocks were stored in an airtight container at -70°C.

Acrylamide gel electrophoresis and Western blotting

Aqueous protein extracts (10% w/v) were prepared by homogenisation of EDL and soleus muscles from treated and untreated rats in extraction buffer (0.25 M sucrose, 10 mM Tris HCl, pH 8.0 containing 1 mM phenylmethyl-sulphonyl fluoride) and subsequent centrifugation at 10000 g for 10 min. at 4°C. Cytoplasmic proteins (10 µg) in the supernatant fraction were separated by SDS-PAGE at pH 8.6 using 10% gels and subsequently transferred to nitrocellulose filters using an LKB dry-blot continuous buffer system according to the manufacturer's description. The filters containing the cytoplasmic proteins were blocked with 0.5 x phosphate buffered saline (PBS) containing 0.5% Tween 80, 3% albumin and then incubated with polyclonal antibodies to either CAIII [16] or PGI for 2 hours.

A new preparation of anti-PGI was raised by injecting a commercial source of rabbit muscle PGI (Sigma Chemical, P9544) intra-dermally (10 sites) into guinea pigs. All anti-PGI samples were checked against the original PGI sample as well as against extracts from soleus and EDL muscles. The greater intensity of the peroxidase-conjugated second antibody obtained with EDL extracts when compared with those from soleus confirmed the association of the PGI antibody with glycolytic fibres. CAIII protein was located using a peroxidase-conjugated goat anti-rabbit IgG, and PGI protein was located with a peroxidase-conjugated goat anti-guinea pig IgG.

Histochemical analysis of actomyosin ATPase

Tissue sections (10 µ) were cut at -20°C in a cryostat (Slee, London) and collected onto poly-L-lysine treated slides. Muscle sections from a number of animals in the hypophysectomised group and in the GH-treatment groups were mounted on the same slide to ensure comparability for both histochemical and immunohistochemical staining. The percentage of different fibre types in each section determined by histochemical and immunohistochemical staining were counted manually in a representative population of approximately 100 fibres.

For analysis of myosin ATPase the procedure of Brook and Kaiser [7] was used. Muscle sections were incubated at 20°C for exactly 5 minutes in 0.2 M sodium acetate, pH 4.3 and then rinsed 3 times in buffer at pH 9.4 (142 mM sodium acetate, 143 mM sodium barbital and 1.5% calcium chloride, pH 9.4). The ATPase incubation was then carried out for 1 hour in pH 9.4 buffer containing 1 mg/cm^3 ATP. The sections were then immersed 3 times in 3% calcium chloride for 30 seconds each, in 1% cobalt chloride for 3 minutes and then rinsed 4 times in distilled water before immersion in 1% ammonium polysulphide for 2 minutes. Slides were then rinsed 3 times with distilled water and dehydrated through a graded series of ethanol solutions. The stained sections were mounted with DPX and viewed and photographed using a Zeiss microscope and Kodak Gold film. For these histochemical analyses a minimum of four animals was used for each treatment.

Immunohistochemical analysis of CAIII and PGI

Tissue sections were cut and collected onto slides as described in the previous section. Slides containing sections were prefixed for 2 minutes with cold absolute ethanol at -20°C and then equilibrated for 5 mins at 20°C with 0.1% albumin in PBS before incubating with the appropriate antibody (anti-CAIII or anti-PGI diluted in 1:100 and 1:200 respectively in 1% albumin in PBS) in a humid chamber for 1-2 hours. Slides were washed 3 times with PBS prior to incubation with the FITC-conjugated second antibody (anti-rabbit IgG for CAIII detection and anti-gui-

nea pig IgG for PGI detection) in a humid chamber for 45-60 mins. After washing 4 times with PBS, slides were mounted using UNIVERT mountant (BDH). Sections were examined using a Zeiss standard epifluorescence microscope equipped with suitable filters for FITC detection and photographs were taken using Kodak T_{Max} 400 film.

Slot-blot analysis of total RNA

RNA was prepared from soleus and EDL muscles of control and treated animals. Tissue, frozen in liquid N_2, was pulverized and added to 8 M urea/4M LiCl, stored at 4°C for 40 hours and then phenol/chloroform extracted as previously described [8]. Total RNA was isolated by ethanol precipitation. The resultant pellets were washed and dissolved in TE buffer (DEPC treated 10 mM Tris, 1 mM EDTA pH 7.5) and stored at -70°C for subsequent analysis. Total RNA isolated from muscles was analysed by slot-blotting techniques using cDNA probes to rat CAIII and PGI. The concentration of poly A^+ RNA in each slot-blot was checked using oligo-dT probes. The cDNA corresponding to the rat CAIII mRNA is well characterised [18]. The rat PGI cDNA has been characterised in our laboratory by studies using Northern analysis and sequencing (in collaboration with L Gibbs, Royal Veterinary College: unpublished results), in comparison with the documented mouse PGI cDNA [22]. The densities of the bands on the slot-blot autoradiographs were measured with a Joyce Loebl Scanning Densitometer and related to µg of total RNA. Integrated peak areas/µg of RNA from muscle extracts from hypophysectomised and GH treated hypophysectomised animals were expressed as a percentage of the value obtained for normal animals.

The integrity of total RNA was checked by reference to the 18S and 28S rRNA bands using agarose urea gels and the integrity of the mRNA by Northern analysis using the specific cDNA probes for each sample.

Results

Protein analysis

The specificity of the polyclonal antibodies to CAIII [16] and PGI was first checked by immunoblotting following SDS PAGE of muscle extracts (Fig. 1). CAIII was detected as a single band in soleus muscle extracts but was below a detectable level in extracts of EDL muscle. Analysis of the PGI antibody raised in guinea pigs against rabbit PGI showed that the sample bound strongly to the original commercially purified enzyme sample and with a protein of similar size in extracts of both soleus and EDL muscles (Fig. 1). The intensity of staining was greater with EDL muscle extracts than with soleus. Tissue sections from control and treated soleus and EDL muscles were then analysed by immunohistochemistry using the above antibodies.

Soleus muscles

Figure 2a shows soleus tissue sections after immunohistochemical analysis with antibody to CAIII detected with FITC-labelled anti-rabbit IgG. All fibres in sections from normal animals show positive staining. It has been observed previously that sections analysed using anti-CAIII show strong positive reactions in the interfibre space [29] and this is apparent with all muscle sections used in our studies. This heavy labelling around the periphery of fibres was also observed by Kelly *et al.* [17] using *in situ* hybridisation to detect CAIII mRNA. The pattern of localisation of the staining, particularly the more intense regions at multi-fibre junctions, suggests that it is due to red cell CAI and/or CAII in the blood vessels in the inter-fibre space.

From Figure 2a it can be seen that CAIII is present in the majority of fibres in the control soleus muscle at uniformly high levels. Following hypophysectomy the CAIII staining exhibits a mosaic effect of very intensely stained and moderately stained fibres. This may be due to enhanced levels of CAIII in a select population of fibres (approximately 30%) or a decrease of CAIII in the remainder. This mosaic effect disappears with GH-treatment and the sections at 2, 4 and 11 days of such treatment appear uniformly stained as those from normal animals.

Figure 2b shows the results of the immunohistochemical analysis of muscle sections for PGI which was detected with FITC-labelled anti-guinea pig IgG. In normal soleus muscle sections there are two fibre types evident; those which stain intensely (40%) for PGI and those which give a very weak signal (60%). On hypophysectomy the number of strongly staining fibres appears to decrease (30%) and the intensity of these positive fibres is less than in normal animals. There is no evidence that GH has an effect on this loss of PGI protein even after 11 days of treatment.

This analysis suggests that soleus muscle from hypophysectomised rats has a more oxidative phenotype than that from normal animals. For CAIII this change is reversed by GH-treatment. Further investigation of the oxidative profile of the fibres was carried out using histochemical analysis of myosin ATPase. Fibre types were determined by myosin ATPase activity following preincubation at pH 4.3. In this regime type 1 fibres are dark staining, type 2A are pale staining while the type 2B fibres stain with an intermediate intensity. The results for soleus muscle are shown in Figure 3. In control muscles two fibre types are apparent corresponding to type 2A (pale) and type 1 (dark staining) fibre populations. On hypophysectomy there is a decrease in the number of type 2A (pale) fibres. After GH-treatment pale type 2A fibres reappear along with some very dark staining fibres. This pattern of changes in myosin ATPase after hypophysectomy and subsequent GH-treatment is consistent with that observed for CAIII and PGI.

EDL muscles

The immunohistochemical assessment of CAIII in EDL muscles is shown in Figure 4a. The sections from normal animals stain weakly since CAIII levels are normally low in EDL muscles, being confined to type 2A fibres where it is expressed to a small extent. As seen for the soleus

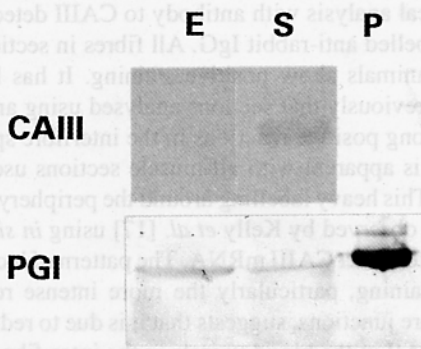

Figure 1. Immunodetection on Western blots of carbonic anhydrase III (CAIII) and phosphoglucoisomerase (PGI) in soleus (S) and EDL (E) muscle extracts from normal rats. P = Rabbit muscle PGI from Sigma (cat. no. P9544).

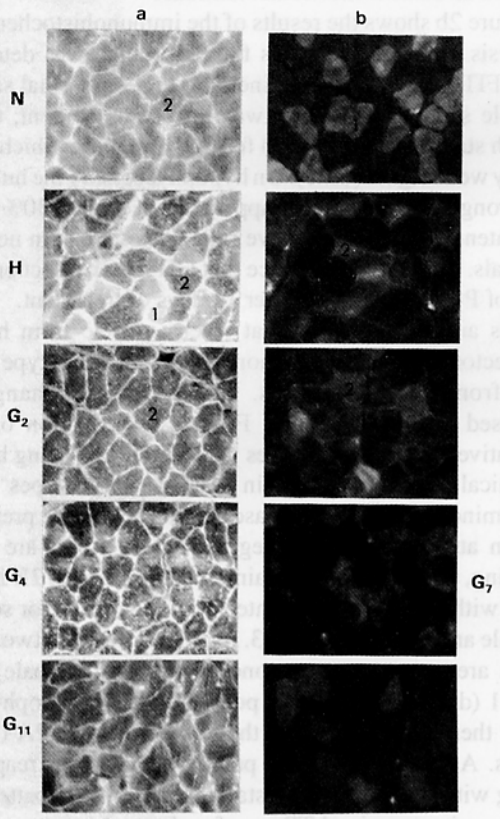

Figure 2. FITC-labelled antibody detection of carbonic anhydrase III (a) and phosphoglucoisomerase (b) in soleus muscle sections from normal (N), hypophysectomised (H) and 2 day (G_2), 4 day (G_4), 7 day (G_7) and 11 day (G_{11}) growth hormone treated hypophysectomised rats. CAIII: 1. Intensely staining, 2. Moderately staining. PGI: 1. Intensely staining, 2. Intermediate staining,3. Weak staining.

muscles there is extensive staining in the inter-fibre space in all sections which is probably due to red cell CA. Following hypophysectomy there is moderate staining of CAIII in certain fibres (30-40%) giving rise to a mosaic pattern and in this muscle GH-treatment maintains this elevation.

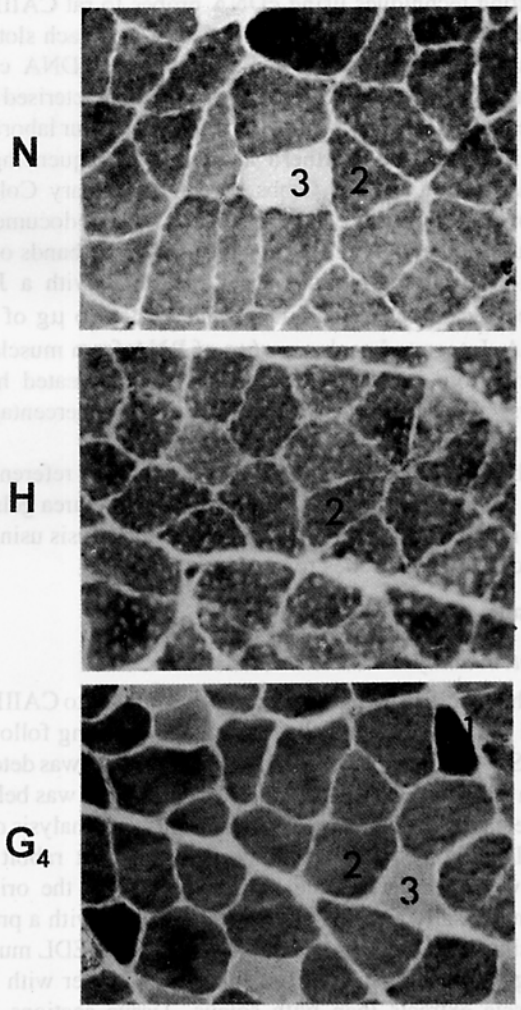

Figure 3. Histochemical analysis of myosin ATPase in soleus muscle sections from normal (N), hypophysectomised (H) and 4 day growth hormone treated hypophysectomised (G_4) rats. 1. Very dark staining 2. Dark staining 3. Pale staining.

Figure 4b shows that the enzyme PGI is present at high levels (intense staining) in a distinct population of fibres in EDL muscle from control animals. Following hypophysectomy there appear to be fewer positive fibres present in this muscle than in those from normal animals. There is some evidence of reversal of this situation after 2 days of GH treatment and after 11 days muscle sections from these animals appear similar to those from control animals (Fig. 4b). The above results suggest that some fibres in EDL muscle acquire a more oxidative capacity on hypophysectomy and for PGI this is reversed by GH-treatment.

Possible changes in the fibre profile of EDL muscle were also investigated using myosin ATPase staining (Fig. 5). Sections from normal animals show three fibre types, dark (type 1, 2%), intermediate (type 2B, 53%) and pale (type 2A, 45%). The appearance of an increased number of dark staining fibres following hypophysectomy again suggests an increase in fibres with slow properties. The number of dark staining fibres in EDL muscle of GH-treated animals is similar to that of the hypophysectomised group but a number of them stain more intensely.

mRNA analysis

Alterations in protein levels in muscle in response to stimuli such as electrical stimulation [9] and mechanical stretch [20] are associated with changes in transcriptional patterns and it was of interest to see if the changes which occurred in protein levels reported above were associated with similar changes in mRNA levels. In our study we used cDNA probes to detect CAIII and PGI mRNAs from muscles of normal, hypophysectomised and 9 day GH-treated animals on Northern blots (Fig. 6). The results are largely consistent with those from studies of the corresponding protein described above and were quantified by slot-blot analysis. Table 1 shows the results of these slot-blot analyses of the mRNAs relative to total RNA in soleus and EDL muscles from normal, hypophysectomised and hypophysectomised GH-treated rats. Following hypophysectomy CAIII levels in soleus muscle are approximately 1.5-fold higher than those found in this muscle from normal animals. This elevation is largely reversed on GH-treatment after 9 days ($P < 0.05$). Levels of PGI mRNA are decreased in this muscle following hypophysectomy and this did not appear to be reversed following GH-treatment.

Levels of CAIII mRNA in EDL muscles from normal animals are extremely low reflecting the non-expression of this enzyme in type 2B fibres and the limited expression in type 2A fibres. From such low backgrounds it is difficult to carry out quantitative estimations of the extent of the increase in levels of CAIII mRNA exhibited in muscles from hypophysectomised animals. However, analysis of results obtained from at least 13 animals indicated that the increase was of the order of 5-fold. These elevated levels of CAIII mRNA in EDL muscles from hypophysectomised animals were maintained after GH-treatment. Levels of PGI mRNA were decreased in this muscle following hypophysectomy and were not statistically different after GH-treatment but an upwards trend was observed.

Table 1 Relative levels of carbonic anhydrase (CAIII) and phosphoglucoisomerase (PGI) mRNAs in soleus (SOL) and EDL muscles from normal (N), hypophysectomised (HYP) and 4 and 9 day growth hormone (GH) treated rats.

muscle	TREATMENT		CAIII	PGI
SOL	N		100	100
	HYP		152 ± 10	72 ± 12
	HYP/GH 4 days		129 ± 8	70 ± 13
	HYP/GH 9 days	*	105 ± 23	73 ± 18
EDL	N		100	100
	HYP		465 ± 78	37 ± 8
	HYP/GH 4 days		556 ± 82	65 ± 11
	HYP/GH 9 days		428 ± 66	45 ± 16

The figures given are derived from integrated peak areas/µg of total RNA determined by slot blot analysis expressed as a percentage of the value obtained for normal animals.
CAIII n = 6-9; PGI n = 3-9
* $P < 0.05$ for soleus muscles of 9 day GH-treated relative to hypophysectomised animals

Discussion

In the present studies CAIII, PGI and myosin ATPase were analysed in sections of soleus and EDL muscles from rats which had been subjected to hypophysectomy and subsequent GH-treatment. Our results demonstrate that there are changes in fibre phenotype in both muscles of treated animals. They suggest that hypophysectomy induces an oxidative directional shift in phenotype in both soleus and EDL muscles but the effects of growth hormone are more complex and differ in the two muscle types.

Hypophysectomised animals

Sections of soleus muscles of hypophysectomised animals incubated with anti-CAIII exhibit a mosaic effect compared with the uniform staining pattern of fibres in normal muscles. The increase observed in CAIII mRNA in whole muscle extracts following hypophysectomy suggests that this pattern of staining for the corresponding protein in tissue sections is a consequence of increased levels of CAIII in a select population of fibres. The level of the enzyme PGI, which is present at higher concentrations in glycolytic type 2B fibres, decreased in select populations of fibres in soleus muscles of hypophysectomised animals and a parallel decrease occurred in total PGI mRNA.

The response to hypophysectomy was similar in EDL muscles ie. there is an increase in CAIII and a decrease in PGI proteins in a distinct population of fibres. It seems

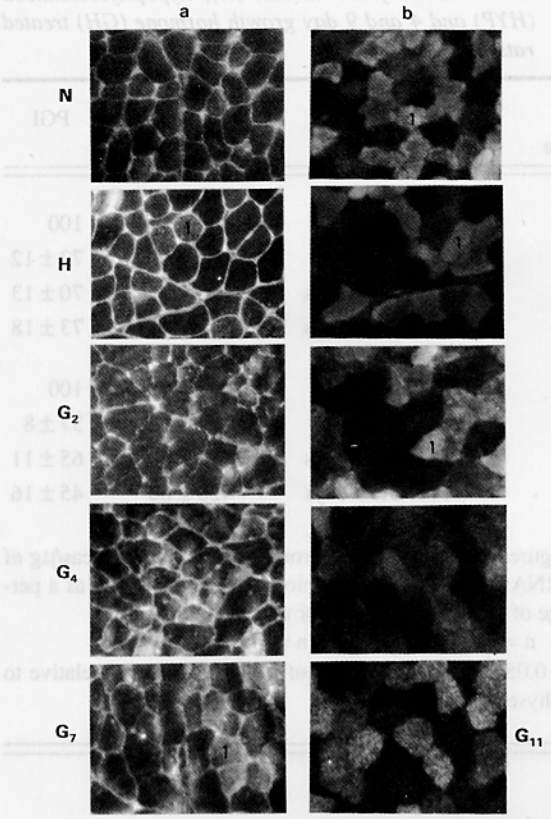

Figure 4. FITC-labelled antibody detection of carbonic anhydrase III (a) and phosphoglucoisomerase (b) in EDL muscle sections from normal (N), hypophysectomised (H) and 2 day (G_2), 4 day (G_4), 7 day (G_7) and 11 day (G_{11}) growth hormone treated hypophysectomised rats. CAIII: 1. Moderately staining, 2. Weak staining. PGI: 1. Intensely staining, 2. Weak staining.

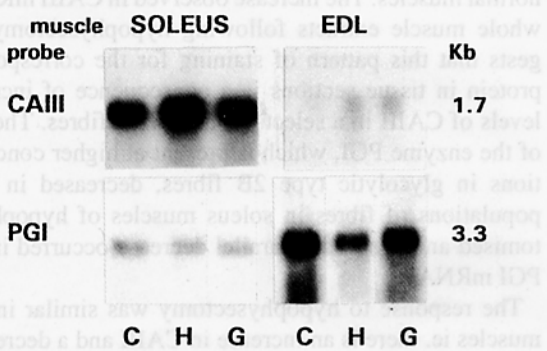

Figure 6. Northern analysis of carbonic anhydrase III (CAIII) and phosphoglucoisomerase (PGI) mRNAs in soleus and EDL muscles from control (C), hypophysectomised (H) and 9 day growth hormone (G) treated rats.

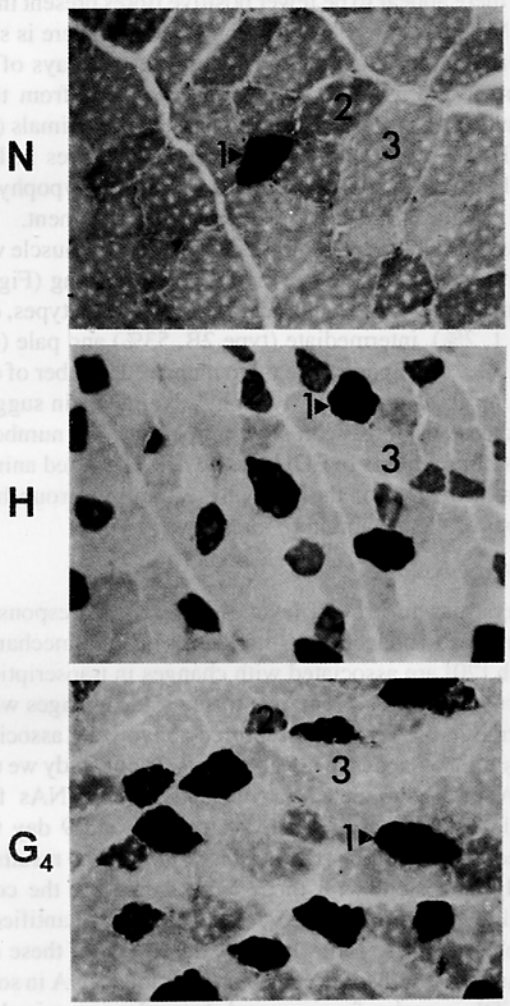

Figure 5. Histochemical analysis of myosin ATPase in EDL muscle sections from normal (N), hypophysectomised (H) and 4 day growth hormone treated hypophysectomised (G_4) rats. 1. Dark staining 2. Intermediate staining 3. Pale staining.

likely that the CAIII staining fibres are the more oxidative fibres which were increased in number in hypophysectomised animals as ascertained by myosin ATPase staining. The corresponding CAIII and PGI mRNAs followed a similar pattern of change in whole muscle extracts of EDL muscles.

Dark staining fibres identified by myosin ATPase analysis (pH 4.3 pre-incubation) which are normally associated with oxidative metabolism are increased in both soleus and EDL muscles after hypophysectomy suggesting a shift towards a more oxidative state in both muscles. These results for myosin ATPase activity are in agreement with those of other workers who demonstrated a loss of type 2 fibres in soleus muscles [28] and an increase of "transitional" oxidative fibres in EDL muscles [3]. In general, our results indicate a directional shift towards a more oxidative phenotype in both soleus and EDL muscles. CAIII would appear to be more responsive than the other proteins studied to hypophysectomy.

GH-treated animals

The effects of GH treatment are different in the two muscle types. In soleus muscles GH-treatment of hypophysectomised animals for 11 days caused a decrease in CAIII protein and mRNA levels and a return of the pattern of myosin ATPase fibre staining to that found in normal animals. However, in this muscle levels of PGI protein and mRNA were unaffected by such treatment compared with hypophysectomised animals.

In contrast, in EDL muscles levels of CAIII protein and mRNA, already increased on hypophysectomy, were maintained by GH administration and the pattern of myosin ATPase staining does not return to that of normal animals. The number of fibres staining strongly for PGI in EDL muscles of 4 day GH-treated animals has increased and by 11 days are similar to those found in control animals. This implies that GH influences the level of PGI protein. PGI mRNA does not appear to return to control levels in most animals studied over 9 days which could imply post-translational regulation of PGI by GH. As the changes in the PGI protein are small and confined to sub-populations of fibres within each muscle any corresponding mRNA changes may be masked when using whole muscle extracts. Further studies using *in situ* hybridisation are necessary before such conclusions can be drawn.

Hypophysectomy causes depletion of all pituitary hormones. In these studies only GH was administered following such treatment and is, therefore, the most likely cause of any changes observed. It is clear that administration of GH over a period of 11 days does not reverse all the changes induced in enzyme levels following hypophysectomy of rats in either of the two muscles studied but for some proteins, such as CAIII in soleus and PGI in EDL, there appears to be a direct responsiveness between GH treatment and protein expression. There may be several explanations for the observation that GH-treatment did not reverse the effects of hypophysectomy on all proteins analysed in this study. It is possible that different GH thresholds are required to influence the expression of individual proteins in each muscle type. Other studies have shown that changes in skeletal muscle fibre profiles were only observed after much longer periods and in some cases after higher doses of GH administration [3, 12]. A second explanation may involve the effects of hypophysectomy on other endocrine influences on muscle such as loss of thyroid stimulating hormone (TSH), prolactin and adrenocorticotrophic hormone. Evidence has shown that administration of thyroid hormone influences muscle phenotype in dwarf mice [25] and thus a reduction in thyroid hormone levels, due to decreased levels of TSH, may be the major influence on regulation of muscle proteins not influenced by GH. In these studies GH appears to exert most influence on the metabolic enzymes normally dominant in the particular fibre type associated with each muscle, that is, CAIII in soleus and PGI in EDL. Other fibre specific enzymes would need to be studied before it was ascertained whether this was a general phenomenon.

In conclusion, analysis of four different muscle proteins, in two rat muscles composed of different fibre types, indicate that there is a directional shift towards a more oxidative phenotype in both soleus (largely type 1 fibres) and EDL (type 2A and type 2B fibres) muscles of rat following hypophysectomy. These effects occur predominantly in discrete populations of fibres. In the short term, GH-treatment causes partial reversion back towards the differentiated state of the muscles by restoration of the levels of the oxidative proteins in soleus and the glycolytic proteins in EDL muscles. The parallel changes in the corresponding mRNAs suggest that these effects operate at the level of transcription. Whether regimes of longer term administration or more frequent injections of GH would re-establish the lower levels of glycolytic proteins in soleus and oxidative proteins in EDL found in normal animals is a subject for further study. These current studies provide further evidence of the complex interplay of stimuli in the differential regulation of gene expression in adult skeletal muscles.

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