Role of denervation in modulating IIb MHC gene expression in response to T₃ plus unloading state

NICK A. DI MASO, FADIA HADDAD, MING ZENG, SAMUEL A. McCUE, AND KENNETH M. BALDWIN
Department of Physiology and Biophysics, University of California, Irvine, California 92697

Di Maso, Nick A., Fadia Haddad, Ming Zeng, Samuel A. McCue, and Kenneth M. Baldwin. Role of denervation in modulating IIb MHC gene expression in response to T₃ plus unloading state. J. Appl. Physiol. 88: 682–689, 2000.—Previously, we have reported that the combination of hind-limb suspension (HS) and thyroid hormone [triiodothyronine (T₃)] treatment induces the de novo expression of the fast IIb myosin heavy chain (MHC) gene in the soleus. Thus we tested the hypotheses that the induction of IIb MHC expression with HS + T₃ is prevented with denervation and that this IIb induction is regulated by transcriptional processes. Adult female rats were subjected to 2 wk of combined HS + T₃ in which one side of the lower leg was simultaneously denervated. HS + T₃ caused decreased expression of the slow type I MHC and concomitant increases in both the fast type IIx and IIb MHC isoforms in the intact soleus muscle. Denervation prevented the endogenous expression of the IIb MHC gene at both the protein and mRNA levels. Although HS + T₃ intervention was able to markedly increase the expression of the 2.6-kb IIb MHC promoter-reporter construct using direct gene transfer, this induction, however, was not inhibited by denervation. These findings collectively suggest that normal innervation is essential for inducing the unique expression of the IIb MHC in a slow muscle in response to HS + T₃; however, in the denervated muscle, there is a discordance between the regulation of the endogenous IIb MHC gene relative to the exogenous IIb MHC promoter-reporter construct.

METHODS

Animal Care and Experimental Manipulations

Sixteen adult female Sprague-Dawley rats (weight ~250 g; Taconic Farms, Germantown, NY) were randomly assigned to either a normal control (NC, n = 8) or a HS plus thyroid hormone-treated (HS + T₃, n = 8) group. The animals were housed individually in temperature- and light-controlled living quarters and were provided food and water ad libitum.

Animals designated for suspension were treated with a noninvasive tail-casting technique as described previously (9). The same group was treated with daily intraperitoneal injections of T₃ (150 µg·kg⁻¹·day⁻¹) throughout the 14-day experimental period. This protocol has been shown to 1) elevate plasma T₃ levels, 2) induce cardiac hypertrophy and MHC isoform shifts in striated muscle favoring a fast phenotype, and 3) increase resting metabolic rate; together, these three effects are the hallmarks of a hyperthyroid state (8, 9, 13).
To accomplish denervation, animals in the HS + T₃ group were anesthetized with ketamine-acepromazine (80:2 mg/kg). After an incision was made in the popliteal region of the left leg, the sciatic nerve was isolated, ligated in two places, and severed between the ligations so that 1 cm of nerve was removed to prevent regeneration. Denervation at this level eliminates innervation of both the anterior and posterior compartments of the lower leg while avoiding disruption of the structural integrity of the targeted muscles and their blood supply. After suturing, each animal was allowed to recover to full ambulation, at which time the combined suspension and T₃-treatment procedures were initiated. A unilateral denervation approach was used to expose both the denervated and the innervated contralateral muscles to the same environmental conditions, i.e., T₃ treatment and hind-limb unloading. The separate interventions of hindlimb unloading and of T₃ treatment were not performed in the context of the present experiment because neither intervention by itself has been shown to cause a significant upregulation of the Iib MHC at the protein level in the soleus muscle (2, 9).

Finally, a 2-wk period was selected because previous findings on this model suggest that upregulation of the Iib MHC, at both the mRNA and protein levels of expression, occurs in this time frame (2, 9). All the procedures reported above were approved by our institutional animal care and use committee.

In Vivo Gene Transfer

For promoter assays, plasmid DNA mixtures were directly injected into the soleus muscle of young rodents according to methods published previously (Refs. 4, 6, 15). Separate groups of NC (n = 4) and HS + T₃-treated (n = 8) animals were used for the gene injection study; a left-leg denervation procedure was performed on the latter group. Each injection consisted of 10 µg of the test plasmid combined with an equimolar amount of a reference plasmid in 25 µl of sterile PBS. The test plasmid consisted of a mouse Iib MHC promoter fragment extending from −2554 bp to +13 bp relative to the transcription start site and linked to a firefly luciferase reporter gene (pGL3 basic, Promega) and was designated as 2.6-kb Iib MHC-FLuc. The reference plasmid consisted of the promoter sequence of another sarcomeric protein, the human skeletal α-actin (4), which was linked to a Renilla luciferase reporter (pRL null, Promega) and designated as HSA2000RLuc. Both promoter fragments were the kind gift of Dr. Steve Swoap, Williams College, Williamstown, MA (15). The reference plasmid was used to control for transfection efficiency within a group and as a general marker for sarcomeric gene transcription. After plasmid injection in the soleus muscle, the sciatic nerve of the left leg of the HS + T₃ group was cut, and the rats were immediately subjected to 7 days of HS + T₃ as described above, whereas the NC rats were allowed to bear weight in standard vivarium cages. After 7 days of treatment, the animals were euthanized (see below), and the soleus muscles were removed and quickly frozen and stored at −80°C. Each frozen muscle was homogenized in 1 ml of ice-cold passive lysis buffer (Promega) supplemented with protease inhibitors (0.2 mM AEBSF, 5 µg/ml aprotinin, 5 µg/ml leupeptin), and the homogenate was centrifuged at 10,000 g for 10 min in the cold. Five microliters of the supernatant were immediately assayed for luciferase activity with use of the Promega dual luciferase assay kit, which was designed for sensitive detection of both firefly and Renilla luciferase activities in a single extract aliquot. Light output from each specific luciferase activity was measured for 10 s with an analytic luminometer (Monolight 2010-C, Analytical Luminescence Laboratory, Ann Arbor, MI). Background activity levels, based on measurements in noninjected tissue for both luciferases, were established and subtracted from the activities measured in the DNA-injected tissue. Activities were reported as relative light units per muscle per second. Firefly luciferase activity represents the Iib MHC promoter activity, whereas the Renilla luciferase activity represents that of the α-actin promoter.

Tissue Processing and Biochemical Analyses

At the termination of the 2-wk HS + T₃ experiment, the rats were killed by a lethal injection of Nembutal (50 mg/kg), immediately after which the heart, soleus, and plantaris muscles (both sides) were rapidly removed, trimmed of connective tissue, weighed, and stored at −80°C until they were analyzed for MHC mRNA and protein content. Each soleus muscle was divided into two pieces. One piece was homogenized in a solution that contained (in mmol/l) 250 sucrose, 100 KCl, 5 EDTA, and 10 Tris-HCl, pH 7.0. Total protein content was determined by the Biuret method (7), and the homogenate protein was adjusted to 1 mg/ml with a storage buffer containing 50% glycerol, 50 mmol/l Na₃PO₄, 2.5 mmol/l EGTA, and 0.5 mmol/l β-mercaptoethanol (pH 8.8) and stored at −20°C until subsequent analyses for MHC protein determination.

The other piece of muscle was processed for total cellular RNA extraction by homogenization in the TRI reagent (Molecular Research Center, Cincinnati, OH) according to the company's protocol, which is based on the method described by Chomczynski (3). Total RNA was precipitated from the aqueous phase with isopropanol; after it was washed with ethanol, it was dried and suspended in a small volume of nuclease-free water. The RNA concentration was determined by using an optical density at 260 nm equivalent to 40 µg/ml. The RNA samples were stored frozen at −80°C until they were subsequently analyzed by RT-PCR technology (see below).

Reverse Transcription

One microgram of total RNA was reverse transcribed for each muscle sample by using the SuperScript II RT from Gibco BRL and a mix of oligo dT and random decamer primers (100 ng/reaction) according to the provided protocol. At the end of the RT reaction, the tubes were heated at 85°C for 5 min to stop the reaction and were stored frozen at −80°C until they were used in the PCR reactions for MHC isoforms as well as for MyoD mRNA analyses.

MHC Protein Analysis

Skeletal MHC protein isoforms were separated with an SDS-PAGE technique that is capable of separating both neonatal and embryonic MHCs in addition to the four adult MHCs typically expressed in rodent leg muscles. The separation technique for adult and neonatal/embryonic MHC isoforms was described in detail previously (1). Included in these analyses were Western blotting analytic techniques to identify the appearance of any detectable embryonic MHC isofrom in the denervated muscle by using procedures described previously (1). The MyoC protein isoform profile for each muscle was determined by laser scanning densitometry of the stained gel as described previously (1, 8, 9).

MHC mRNA Analysis

Analyses of MHC mRNA isoforms used a modification of a RT-PCR technique designed to quantitate relative amounts of
The PCR reaction was carried out in the presence of 1.5 mM MgCl₂ by using standard PCR buffer (GIBCO), 0.2 mM dNTP, 1 µM MyoD primers, 0.5 µM 18S primers mix, and 0.75 unit of DNA Taq polymerase (GIBCO) in 25 µl total volume. Amplifications were carried out in a Stratagene Robocycler with an initial denaturing step of 3 min at 95°C, followed by 25 cycles of 1 min at 96°C, 1 min at 59°C, 1 min at 72°C, and a final step of 3 min at 72°C. PCR products were separated on a 2% agarose gel by electrophoresis, and signal quantification was done as reported previously (17). For the MyoD amplification, we used the following primers: 5'-CTACAGGCGGACTCAGACG-3' (5' sense primer) and 5'-TTGGGGCCGATGTAAG-3' (3' antisense primer). The MyoD primers yielded a 563-bp product. For the 18S, we used the alternate 18S internal standards (Ambion), which yields a 324-bp product. The 18S competimers and primers were mixed at a 10-1 to 1 ratio, which allowed linear amplification of the 18S to the same range as the MyoD target mRNA (Quantum RNA, Ambion). One microliter of a 10-fold dilution of each RT reaction was used for the PCR amplification. The PCR reaction was carried out in the presence of 1.5 mM MgCl₂ by using standard PCR buffer (GIBCO), 0.2 mM dNTP, 1 µM MyoD primers, 0.5 µM 18S primers mix, and 0.75 unit of DNA Taq polymerase (GIBCO) in 25 µl total volume. Amplifications were carried out in a Stratagene Robocycler with an initial denaturing step of 3 min at 96°C, followed by 25 cycles of 1 min at 96°C, 1 min at 59°C, 1 min at 72°C, and a final step of 3 min at 72°C. PCR products were separated on a 2% agarose gel by electrophoresis and stained with ethidium bromide; signal quantification was performed by laser scanning densitometry as reported previously (17). By this method, each MyoD signal was normalized to its corresponding 18S.

Under these PCR conditions, both the MyoD and 18S product yield are still in the linear range of the semilog plot when the yield is expressed as a function of the number of cycles.

Statistical Analyses

All data are reported as means ± SE. Statistical differences between the NC, HS + T3, and HS + T3 + denervation groups were tested by using a one-way ANOVA procedure; when differences were detected for a given variable, a Newman-Keuls post hoc test was used. All statistical analyses were performed using a computer software package (Prism, Graphpad Software). Statistical significance was set at P < 0.05.

RESULTS AND DISCUSSION

Body and Muscle Weights

The combination HS + T3 induced a 12% reduction in body weight (P < 0.05; Table 2), which is a typical adaptive response to the combined interventions (9). Heart weight and the heart weight-to-body weight ratio were significantly elevated by 35% in the treated group (Table 2), a finding that is also consistent with the action of T3 on this muscle type (13). Both the innervated soleus and plantaris muscles of the HS + T3 group underwent the typical atrophy response that has been associated with the action of T3.
been routinely reported with this model (9). Denervation induced additional atrophy in the plantaris muscle but not in the soleus muscle (Table 2). These findings on the denervated muscle, as well as other findings in which denervation was interacted with thyroid deficiency (8), clearly suggest that fast muscle appears to be more sensitive to the denervation-induced atrophy response than slow muscle, especially when either the

Fig. 1. Myosin heavy chain (MHC) protein isoform expression in soleus muscle in three groups: normal control (NC), hindlimb suspended (HS) and thyroid hormone (T3) treated (HS + T3), and HS, T3 treated, and denervated (HS + T3 + Den). Each isoform is presented as % of total MHC pool and expressed as mean ± SE. Note that embryonic and neonatal MHC protein isoforms were not detected; therefore, they are not represented. *P < 0.05, vs. NC; #P < 0.05, HS + T3 vs. HS + T3 + Den.

Fig. 2. MHC mRNA isoform expression in the soleus muscle in the 3 groups as analyzed with the RT-PCR technique. Each isoform is presented as % of total MHC mRNA pool and is expressed as mean ± SE. *P < 0.05 vs. NC; #P < 0.05, HS + T3 vs. HS + T3 + Den; ns, not significant, i.e., P > 0.05 vs. NC. Emb, embryonic; Neo, neonatal.
loading status or the thyroid state is altered in combination with denervation (8). Clearly, more information is needed to unravel this differential response between fast and slow skeletal muscle.

Endogenous MHC Isoform Responses

The NC soleus expresses mainly two MHC isoforms, i.e., type I and IIa, which combine to account for more than 95% of the MHC protein pool. This is true at both the protein and mRNA levels (Figs. 1 and 2). Trace amounts of IIx (≤3%) also were detected. In addition to types I, IIa, and IIx MHC, some IIb and embryonic MHC isoforms could be detected only at the mRNA level, accounting for ~1% and ~8% of the pool, respectively (Fig. 2). The expression of the embryonic mRNA is somewhat surprising given that its encoded MHC protein is seldom detected in normal adult slow muscle even with the use of more sensitive detection methods such as immunoblotting techniques (Fig. 3). This unique observation of a low level of embryonic MHC mRNA expression in a NC soleus was validated by using the more conventional Northern blot hybridization (Fig. 4B) and was also noted by others using RT-PCR methods (12).

The combination of HS + T3 induced a significant remodeling of MHC expression by causing a decrease in the relative content of the type I MHC. This response was offset by significant increases in the relative content of both the fast type IIx and IIb MHCs (P < 0.05), whereas essentially no change in the relative content of the fast IIa MHC was produced. This pattern of response across the MHC pool is similar at both the protein and mRNA levels (Figs. 1 and 2), although the magnitude of shifts is much more significant at the mRNA level because the mRNA has a faster turnover rate compared with the MHC proteins. Also, the MHC isoform response to HS + T3 is similar to what was previously reported (2, 9).

When the variable of denervation was introduced in combination with the HS + T3 intervention, the de novo expression of the IIb MHC protein and induction of IIb MHC mRNA were repressed, whereas significant amounts of the IIx MHC continued to be expressed along with the type I and IIa MHCs (Figs. 1 and 2). In addition to blunting the induction of the IIb MHC mRNA expression, denervation was also associated with an induction of both embryonic (22%) and neonatal (3%) MHC mRNAs, which were not detected at the protein levels (Figs. 1–4). This observation was somewhat surprising especially for the embryonic MHC, which comprised as much as 22% of the total MHC mRNA pool even though it apparently was not translated (Fig. 1).

Interestingly, other reports (14) have shown detectable amounts of embryonic and neonatal MHC protein in denervated muscles using the same antibody (BF-45) used in this study. One explanation for this discrepancy is that the immunoblotting method we used (Fig. 3) is...
not as sensitive as the immunofluorescence method used by Schiaffino et al. (14). Alternatively, one cannot compare the denervated muscles in the two studies (this study and Ref. 14) because in this present study denervation was coupled to a hyperthyroid state, whereas in the study by Schiaffino et al. it was carried out in the euthyroid state, and this difference could possibly account for the difference in results. In fact, in a previous study (8), we were able to detect the embryonic MHC protein isoform expression in denervated soleus muscle under both hypothyroid and euthyroid states, but not under a hyperthyroid state, by using Coomassie blue-stained protein gels. Thus, based on our results, it is clear that there is a disproportionality between the amount of embryonic MHC mRNA vs. protein that is expressed. If embryonic MHC protein were expressed, it had to be at a very low level, i.e., below the limits of our detection method. Thus denervation, although essentially blunting the de novo expression of the IIb MHC in terms of both protein and mRNA, induced a unique profile at the mRNA level but not at the protein level. That is, denervation essentially mimics a pattern typically seen as a cross between undifferentiated fast and slow muscle during the early stages of neonatal development (1).

IIb MHC Promoter Activity

The 2.6-kb IIb MHC promoter fragment had a very low activity in the NC soleus based on total firefly luciferase reporter activity expressed and compared with the actin promoter activity (Fig. 5). This was not surprising because the former is normally expressed chiefly in fast-twitch muscles, whereas the latter is expressed in both fast- and slow-twitch skeletal muscles (4). HS + T₃ caused an approximately four- to fivefold increase in the absolute activity of the 2.6-kb IIb MHC-Fluc promoter. Interestingly, this response to HS + T₃ occurred even though the response of the HSA2000-Rluc promoter was markedly inhibited, thereby producing a marked increase (~40-fold) in the IIb-to-actin ratio (Fig. 5). When denervation was imposed in the context of the HS + T₃ stimulus, the response of the 2.6-kb IIb MHC-Fluc remained elevated and was not different from that seen with HS + T₃ (Fig. 5). Similarly, denervation in the context of HS + T₃ did not alter the HSA2000-Rluc promoter activity any further than the inhibition due to HS + T₃ alone. Thus these data suggest a discord between the response of the endogenous IIb MHC gene and the exogenous 2.6-kb IIb MHC promoter fragment under the unique conditions of denervation, and this response occurs when the 2.6-kb IIb MHC-Fluc promoter-reporter data are assessed independently of or in conjunction with the actin response.

MyoD Expression

Analysis of the 2.6-kb IIb MHC promoter nucleotide sequence reveals that at least 14 E-box elements are located throughout the 2.6-kb promoter upstream of the transcription start site (16). The E-box element...
served that the denervated HS in the denervated side. Surprisingly, however, we have expected a marked reduction in the MyoD signal of the IIb MHC isoform (11), this factor could play a role in the adaptive process by impacting transcriptional activity of the IIb MHC in HS + T₃ soleus muscle. If MyoD expression is a key factor in regulating IIb MHC transcriptional activity in the HS + T₃ soleus, we would have expected a marked reduction in the MyoD signal in the denervated side. Surprisingly, however, we observed that the denervated HS + T₃ soleus also expressed significant levels of MyoD mRNA. In fact, the levels were higher in the denervated side compared with HS + T₃ alone (Fig. 6). These data suggest that the MyoD factor, in and of itself, is unlikely to be the primary factor in the regulation of endogenous IIb MHC expression in response to HS + T₃, particularly in the context of the denervation response. Clearly, some other factor(s) must be involved in both the upregulation and repression of IIb MHC gene expression in our model system.

Integrated Response

The collective data presented in this paper provide convincing evidence that normal muscle innervation is an essential factor in the de novo expression of the endogenous IIb MHC gene that occurs in the soleus muscle in response to the unique combination of hind-limb unloading and T₃ treatment. This conclusion is based on the MHC protein and mRNA isoform expression data and confirms the first hypothesis. Using the in vivo gene transfer approach, we demonstrated that the activity of the exogenous 2.6-kb IIb MHC promoter fragment increases severalfold relative to control in response to the combined treatment (HS + T₃). This increase correlated well with the endogenous IIb MHC expression in HS + T₃ soleus and suggests that denervation synthesis of IIb is due to transcriptional regulation. Also, this finding implies that the −2554/+13 bp IIb MHC promoter fragment contains cis regulatory elements that are responsive to HS + T₃ treatment, which, in turn, act to enhance the promoter activity. Surprisingly, judging by reporter gene activity level, which, in turn, act to enhance the promoter activity. Thus we have observed that the denervated HS + T₃ soleus also expressed significant levels of MyoD mRNA. In fact, the levels were higher in the denervated side compared with HS + T₃ alone (Fig. 6). These data suggest that the MyoD factor, in and of itself, is unlikely to be the primary factor in the regulation of endogenous IIb MHC expression in response to HS + T₃, particularly in the context of the denervation response. Clearly, some other factor(s) must be involved in both the upregulation and repression of IIb MHC gene expression in our model system.

On the other hand, one should not ignore the alternative possibility that the chromosomal context in which the endogenous IIb MHC gene exists may contain the required information for the full regulatory mechanism. It is known that the exogenous plasmid constructs are taken up and remain episomal. Although these plasmid constructs are exposed to the same transcription factors and nuclear milieu, some critical information linked to chromatin structure and interaction may be lacking. In conclusion, the exact mechanism by which denervation inhibits IIb MHC isoform expression in HS + T₃ soleus remains to be elucidated.

REFERENCES