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MINI REVIEW

Mechanism of Thyroid-Hormone Regulated Expression of the SERCA Genes in Skeletal Muscle: Implications for Thermogenesis

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Thyroid hormone increases the Ca^{2+}-ATPase activity of the sarcoplasmic reticulum (SR) in skeletal muscle, thereby increasing the energy-turnover associated with Ca^{2+}-cycling during contraction and rest. The fast-muscle isoform of the Ca^{2+}-ATPase (SERCA1) and the slow-muscle isoform (SERCA2a), are encoded by two genes that are transcriptionally regulated by T_3. The SERCA1 isoform can be expressed to considerably higher levels than the SERCA2a isoform. The stimulation of transcription of the SERCA1 gene by T_3 is mediated by two thyroid hormone response elements, located in the promoter of this gene. The intracellular $[Ca^{2+}]$ can modulate the effect of T_3. The increase in SR Ca^{2+}-ATPase activity seen when T_3-levels rise above normal, results from the induction of SERCA1 expression in slow muscle fibers. Concomitant high levels of Ca^{2+}-ATPase activity are associated with down-regulation of SERCA2a expression in these fibers. The observed T_3-dependent increase in SERCA1 expression and associated Ca^{2+}-ATPase activity will increase the overall metabolic rate of the organism significantly under normal conditions, because of the high average level of contractile activity of slow fibers. Given the rise in serum T_3-levels during prolonged cold exposure, these data suggest that fiber-specific stimulation of SERCA1 expression contributes to the thermogenic response in non-shivering thermogenesis. This mechanism may be particularly relevant in larger mammals, which have a relatively high percentage of slow fibers in skeletal muscle, and which need to rely on tissues other than brown fat for the generation of extra heat.

KEY WORDS: SERCA1; SERCA2a; ATPase; calcium; sarcoplasmic reticulum; skeletal muscle; thyroid hormone.

INTRODUCTION

The resting metabolic rate (RMR) is a function of the thyroid status and the general stimulatory effect of thyroid hormone (T_3) on metabolism of practically all tissues is perhaps the best known action of this hormone. Equally important, however, is that T_3 enhances energy turnover during cellular activity. This is particularly true during contraction of skeletal muscle, which is a principle target tissue of T_3. The contribution of resting muscle metabolism to the RMR of mammals is estimated to

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be around 20% [1], but this percentage rises sharply with increasing activity of the organism. This heat-generating capacity of contraction is used as a first line of defense against hypothermia in the shivering response. During prolonged exposure to the cold, shivering subsides and non-shivering thermogenesis (NST) takes over with increased circulating levels of norepinephrine (NE) and T_3, resulting in a rise in RMR. Small mammals, like the mouse and rat, have a relatively high rate of heat loss, and at 4°C, the RMR needs to double to maintain body temperature. This NST resides almost exclusively in brown adipose tissue (BAT) and is mediated by the mitochondrial uncoupling protein UCP1 [2, 3]. T_3 is merely permissive, yet essential, for the thermoregulatory action of NE on UCP1 activity. In fact, the rise in serum T_3 seen during cold exposure is secondary to the increase in NE and not required for the effect on BAT [4, 5]. Although a rise in T_3-levels will stimulate the RMR in most tissues, this thermogenic effect is not important for successful cold adaptation in mice or rats. However, it could come into play in larger animals, which need to rely on tissues other than BAT for extra heat generation. Skeletal muscle is thought to play a significant role in NST in larger animals, because of the metabolic capacity and total mass of this tissue. Various mechanisms may contribute to enhanced muscle metabolism, but several lines of evidence suggest increased expression of the sarcoplasmic reticulum (SR) Ca^{2+}-ATPase—and a concomitant increase in Ca^{2+}-cycling—as a principle source of extra heat generation [6–9]. This points to a possible direct action of T_3 in NST, rather than a permissive one, because the expression of SR Ca^{2+}-ATPase is stimulated by T_3. The focus of this report is therefore on the regulation of the multiple Ca^{2+}-ATPase genes by T_3 in different muscle fiber types. We will begin with a summary of the data which identified the SR Ca^{2+}-ATPase as a target in T_3-dependent muscle metabolism, and we will then focus on the mechanism of regulation of SR Ca^{2+}-ATPase expression and the implications of this regulation for thermogenesis.

THYROID STATUS, Ca^{2+}-HANDLING AND SKELETAL MUSCLE FIBER TYPES

In general, the RMR in hypothyroid mammals is 70% of euthyroid (control) values, whereas in hyperthyroidism this is 150% [10]. This is also found in rats and the 2-fold stimulation of RMR by T_3 mostly reflects effects on tissues other than BAT, since this tissue is not activated in hyperthyroidism. Actually, UCP1 expression is suppressed in hyperthyroidism, possibly as a compensatory mechanism for the increased heat production in other tissues (5). When studying the rat at thermoneutrality the T_3-dependent metabolism in the range between hypo- and euthyroidism can be viewed as the contribution to obligatory thermogenesis, whereas the range between eu- and hyperthyroidism indicates the capacity for energy turnover in tissues other than BAT, which in principle could be mobilized by T_3 in NST. Serum levels of T_3 in cold-adapted rats are in the range of mild to overt hyperthyroidism, resulting from increased hormone production by the thyroid, but primarily from a NE-dependent increase in peripheral conversion of T_4 to T_3 by type II 5′-deiodinase [4, 5].

A series of experiments using perfused hind limb of the rat provided the basis for our work on T_3-regulation of SR Ca^{2+}-ATPase (see Refs. 1, 10–12 for a more detailed review of these data). The essence of these results was that the characteristic 2-fold difference between hypothyroid and hyperthyroid rats in resting O_2-consumption of the muscle preparation could be ascribed primarily to increased Ca^{2+}-cycling by the SR. The T_3-dependent increase in SR Ca^{2+}-ATPase activity suggested by these data was subsequently confirmed by determination of SR Ca^{2+}-uptake activity, coupled ATPase activity, and level of phosphorylated intermediate of the enzyme in purified SR and muscle homogenates [13–18]. A 2-fold increase in muscle SR Ca^{2+}-ATPase activity from hypo- to euthyroidism was found for slow muscle and again a doubling in the transition to hyperthyroidism, whereas in fast muscle T_3 induced a maximal 50% increase, with little effect in hyperthyroidism. Measurement of the actual heat production associated with SR Ca^{2+}-cycling during contraction correlated quantitatively with the stimulation of Ca^{2+}-ATPase activity by T_3 in slow and fast muscle [19]. These measurements also showed that SR Ca^{2+}-cycling accounts for 20 to 50% of the heat produced during contraction. The effect on the SR activity in both muscle types also provided a basis for the well known stimulation of the relaxation rate by T_3, which together with an enhanced contraction rate are classic symptoms in increased thyroid activity.

Since the effect of T_3 is an increase in the speed of the contractile process it is not surprising that the effects are more pronounced in slow muscle. The expression of myosin heavy chain (MHC) isoforms with higher catalytic turnover rates was already well established as the basis for the stimulation of the rate of contraction [20, 21]. These isoforms are part of the phenotype that make up the different fiber types present in skeletal muscle, which are furthermore characterized by their principle pathway for generating ATP, i.e., oxidative for sustained activity and glycolytic for short bursts of activity with high ATP turnover. The major isoforms of MHC are MHC I, IIa and IIb, ranked in order of increasing catalytic turnover rate. Therefore, MHC I is found in slow oxidative fibers (type I), MHC IIa in fast oxidative/glycolytic fibers (type IIA), which have intermediate contraction rates, and MHC IIb in the fastest, glycolytic fibers (type IIB). Type IIC fibers contain a mixture of MHC I and IIa, and MHC IIx is an intermediate isoform between IIa and IIb. Under normal conditions the different fiber types are optimally geared to their task. Type I fibers are the principle fiber in slow twitch, fatigue resistant muscles, used for maintaining posture and relatively slow movement. These slow fibers are typically active 20–30% of the time at a low frequency (20 Hz) [22]. Fast fibers, on the other hand, are active for only about 0.2% of the time, but at frequencies of up to 200 Hz [22], and fast-twitch muscles are composed of roughly equal amounts of type IIA and IIB fibers, with small amounts of type I fibers mixed in [23]. The phenotype which is expressed in a fiber is mainly determined by T_3 and the motor nerve innervating a group of fibers (motor unit) (reviewed in [24–26]). Development and maintenance of slow characteristics requires low frequency, almost continuous stimulation, typically imposed by a slow motor nerve. In contrast, development of fast characteristics is independent of innervation, but dependent on T_3 [20, 26–28]. Moreover, T_3 counteracts the effect of slow innervation by stimulating the fast phenotype. Consequently, the degree of motor nerve activity and the level of T_3

determine to which extent the slow or fast genetic programs are expressed within a motor unit, although intrinsic properties of individual fibers may limit the range of phenotypic changes induced by these factors [20].

The identification of the family of myogenic regulatory factors (MRF) provided a possible mechanism for the coordinated expression of phenotype [29]. The MRF's initiate the muscle gene program and low levels of these transcription factors are most likely involved in maintenance of the differentiated state. Even though considerable redundancy exits between the various MRF's, phenotype specificity was observed with MyoD predominantly expressed in fast muscles and myogenin in slow muscles [30, 31]. Conversion of the phenotype by denervation, cross-innervation, and to some extent by T_3, was usually accompanied by an appropriate change in the relative expression of MyoD and myogenin [30, 32, 33]. However, a strict correlation between fiber type and expression of a particular MRF was not supported by further analysis, and additional factors are probably involved [34–36]. Notably, the fast-to-slow phenotypic changes induced by electrical stimulation preceded the change in MRF expression by several days [37], supporting the suggested role of MRF's in consolidating a specific phenotype, rather than being a primary regulator.

Irrespective of the underlying mechanism, the dynamic balance between innervation and T_3 illustrates the characteristic plasticity of skeletal muscle which allows (adaptive) changes in response to altered workload or thyroid status. So, depending on the type of muscle, increasing levels of T_3 induce a consecutive shift from MHC I to IIa, to IIx, to IIb, inducing one gene, while turning off the other [21]. The most prominent shift in contractile properties seen in slow muscle from hypo- to hyperthyroidism (MHC I to IIa), is in keeping with the large increase in SR Ca^{2+}-ATPase activity in slow muscle described above. Because T_3 does not increase the amount of force generated, the effects on Ca^{2+}-cycling and cross-bridge cycling result in an energetically less efficient force production and a greater dissipation of heat during contraction [19, 38–40].

Summarizing, these data showed that T_3 enhances the Ca^{2+}-cycling capacity of skeletal muscle through increased expression of the SR Ca^{2+}-ATPase, thereby speeding-up the contraction-relaxation cycle at an increased energy cost. This applies to fast and slow muscle in the transition from hypo- to euthyroidism, but mainly to slow skeletal muscle in the transition to hyperthyroidism.

T_3 AND EXPRESSION OF Ca^{2+}-ATPase ISOFORMS

Three genes, SERCA1, 2 and 3 encode a total of five isoforms of the sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase (reviewed in Ref. 41). SERCA1 and SERCA2a are the SR Ca^{2+}-ATPases expressed in adult muscle. SERCA1 is predominantly expressed in fast muscle and SERCA2a in slow muscle. The heart expresses exclusively SERCA2a, and SERCA1 cannot be induced in this tissue under any condition. Unlike the MHC isoforms there are few, if any, intrinsic differences between both SERCA isoforms [42]. The difference lies in the maximal expression levels that can be attained for both genes. Under normal conditions fast muscles express 5 to 7-times higher levels of SERCA1 compared to the level of SERCA2a in slow muscles [16, 17, 41, 43–46]. This muscle-specific expression suggested that

SERCA1 and SERCA2a are reciprocally regulated by T_3, similar to the fast and slow MHC isoforms, as part of a slow or fast genetic program.

Analysis of SERCA mRNA, -protein and Ca^{2+}-ATPase activity in slow and fast muscle indicated that the effect of T_3 is more complex and highly fiber-specific [44, 45, 47]. Figure 1 shows the effect of the thyroid status on the total SR Ca^{2+}-ATPase activity in homogenates of the slow soleus muscle (SOL) and the fast extensor digitorum longus (EDL) (note that the absolute activity level in euthyroid EDL is 6-times higher than that in SOL, see legend). The EDL expressed almost exclusively SERCA1 and the T_3-independent increase in Ca^{2+}-ATPase activity from hypothyroidism to euthyroidism correlated with a 50% rise in SERCA1 mRNA and protein [44, 45, 47]. Analysis of SERCA isoform expression in the SOL indicated that T_3 strongly stimulates the expression of SERCA1, with only marginal effects on SERCA2a expression (Fig. 2) [44, 45, 47]. Figure 3 shows the stimulation by T_3 of SERCA1 expression in individual fibers of the SOL, and illustrates the large increase in SR Ca^{2+}-ATPase activity in the transition to hyperthyroidism. Although overall SERCA2a expression seemed unaffected by T_3, differential effects became evident when expression was analyzed at the level of individual fibers [47]. As shown in Fig. 4, SERCA2a was expressed in all fibers in the hypothyroid SOL and co-expressed with MHC I in 85% of them, those being the slow type I fibers. In the transition to hyperthyroidism, expression of MHC I and SERCA2a was shut off in half of the type I fibers, implying that the level of SERCA2a expression in the remaining fibers increased, since total SERCA2 mRNA and protein levels were only marginally lower [44, 45, 47]. SERCA1 and MHC II (fast) isoforms were cordinately induced in SOL

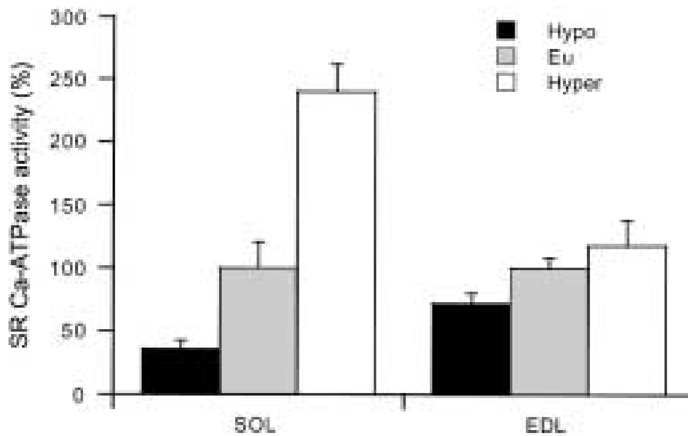

Fig. 1. SR Ca^{2+}-ATPase activities in homogenates of SOL and EDL muscles of hypothyroid (filled columns) and hyperthyroid rats (2 weeks of T_3 treatment) (open columns), expressed relative to euthyroid activity levels (100%, shaded columns) (mean ± SD, $n = 5$). Absolute activities in euthyroid preparations: SOL: 13.5 ± 2.9 [5]; EDL: 79 ± 7 [5] (μmol/g wet weight · min at 25°C). Effects of hyper- and hypothyroidism relative to euthyroid control were significant ($p < 0.05$), except for hyperthyroidism in EDL. Data from Ref. 45.

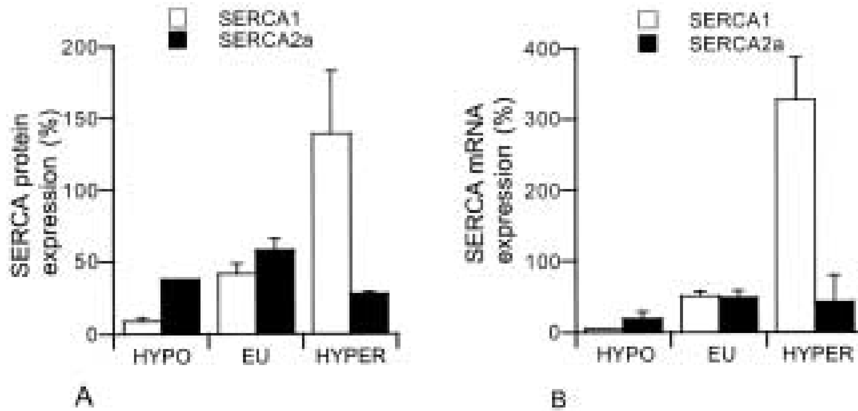

Fig. 2. Total SERCA1 and SERCA2a protein (panel A) and mRNA (panel B) content of SOL muscle from hypo- (HYPO), euthyroid (EU), and euthyroid rats treated with T_3 for 2 weeks (HYPER) ($n = 2$, mean ± SD). Total SERCA protein or mRNA content of the euthyroid SOL was set to 100%, and HYPO and HYPER values were expressed relative to the EU expression levels (from Ref. (7). These data confirmed earlier extensive analyses of mRNA [45] and SERCA protein [44]. Reproduced with permission from The American Physiological Society: *Am. J. Physiol.* **271** (*Cell Physiol.* **40**) C1908–C1919, (1996).

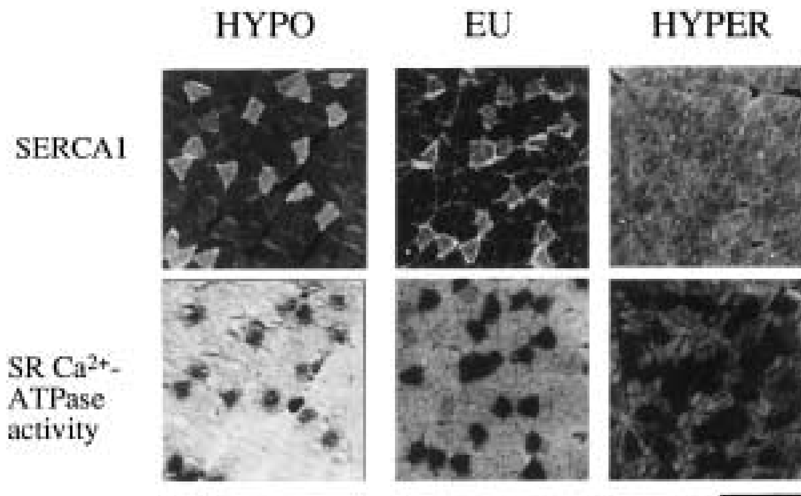

Fig. 3. Immunofluorescent and histochemical stainings of serial cross-sections of SOL muscle from hypo- (HYPO), euthyroid (EU), and euthyroid rats treated with T_3 for 2 weeks (HYPER). SERCA1 was detected with an isoform-specific antibody, and these fibers stain light. SR CA^{2+}-ATPase activity was visualized as described in Ref. 89, and fibers with high SR Ca^{2+}-ATPase activity stain dark (from Ref. 47). Bar: 0.25 mm. Reproduced with permission from The American Physiological Society: *Am. J. Physiol* **271** (*Cell Physiol.* **40**) C1908–C1919 (1996).

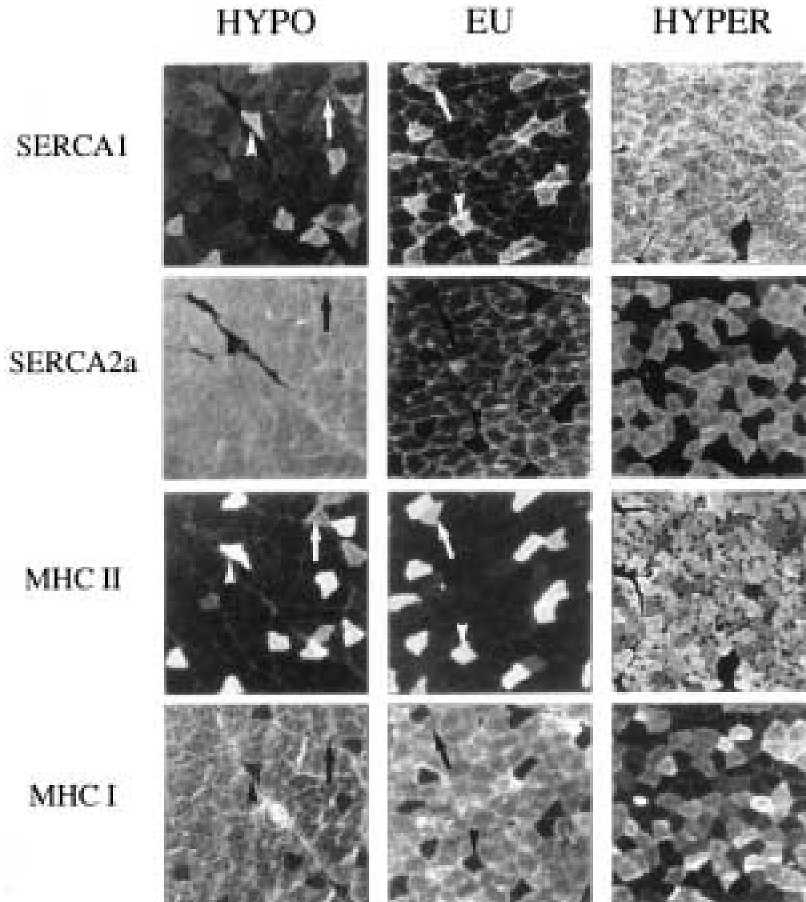

Fig. 4. Immunofluorescent stainings of serial cross-sections of SOL muscle from hypo- (HYPO), euthyroid (EU) and euthyroid rats treated with T_3 for 2 weeks (HYPER). Monoclonal antibodies for SERCA1, MHC I and MHC II, and a polyclonal serum for SERCA2a were used. Fibers binding the antibodies stain light. Type I fibers are positive with the MHC I, and negative with the MHC II antibody (85% of the fibers in HYPO). Type IIA fibers stain with MHC II antibody and are negative with the MHC I antibody (examples indicated by arrow heads), and type IIC fibers stain with both MHC antibodies (examples indicated by arrows) (from Ref. 47). Reproduced with permission from The American Physiological Society: *Am. J. Physiol.* **271** (*Cell Physiol.* **40**) C1908–C1919 (1996).

from 13% of the fiber population in hypothyroidism to 20% in euthyroidism and 100% in hyperthyroid muscle. The levels of SERCA1 mRNA and protein were further increased by T_3 in fibers that already expressed SERCA1. In the hyperthyroid SOL the Ca^{2+}-ATPase activity was highest in the fibers expressing solely SERCA1 and MHC II isoforms [47]. In other words, T_3 induces a coordinated switch of MHC and SERCA isoforms in practically all fibers of the SOL; approximately half of them are converted from a slow to a fast phenotype (IIA), the other half being converted to a mixed slow/fast phenotype (type IIC).

The full conversion of only half of the SOL fibers probably reflects intrinsic differences between fiber populations, since the range of activities of the slow motor units in SOL is small (see Ref. 47 for a detailed discussion). The mechanism for the down-regulation of SERCA2a in fibers that do convert completely to the fast phenotype remains unclear. However, we noted that inhibition of SERCA2a expression occurred at a pre-translational level, and only after fibers had attained high relative SERCA1 expression levels and concomitant SR Ca^{2+}-ATPase activities [47]. This led us to suggest that changes in intracellular [Ca^{2+}], as a consequence of T_3-induced changes in SR Ca^{2+}-uptake activity, may differentially affect expression of SERCA isoforms (see section on Ca^{2+}-dependency below).

In the fast EDL muscle SERCA1 was the major isoform detected, with only low levels of SERCA2a mRNA and protein corresponding to a small population of type I fibers (<3%) [47]. The T_3-response of these fibers was the same as seen for type I fibers in SOL. The expression level of SERCA1 mRNA in the type II fibers was only modestly affected by the thyroid status, which correlated with a 1.5-fold change in SERCA1 protein content and SR Ca^{2+}-ATPase activity in the transition from hypothyroidism to hyperthyroidism (see also Fig. 1). The limited responsiveness of SERCA1 in adult fast muscle contrasts with the effect of T_3 in neonatal muscle development. Earlier we had shown that the large increase in SR Ca^{2+}-ATPase activity in neonatal fast muscle is absolutely dependent on the rise in serum T_3, which starts in the first week of life [28]. Subsequent analysis showed that T_3 induced a 6-fold increase in SERCA1 mRNA [48]. This is a direct T_3 effect and not secondary to stimulation of growth hormone or insulin like growth factor I (IGF-I), which are both involved in muscle growth and differentiation [28].

Summarizing, these results indicated that the regulation of both isoforms of SR Ca^{2+}-ATPase by T_3 is established primarily at a pre-translational level and generally coordinated with expression of MHC isoforms. Furthermore, the SERCA1 gene is a principle target of T_3 and high levels of SR Ca^{2+}-ATPase activity, as in fast muscle and the hyperthyroid SOL, can only be attained by expression of SERCA1. Taken together with studies showing the repression of the fast gene program by slow type innervation [24], the data illustrate the dynamic equilibrium between T_3 and contractile activity in determining the phenotype and SR Ca^{2+}-ATPase activity, with SERCA1 being the pivotal player.

MECHANISM OF T_3-REGULATED EXPRESSION OF SERCA1

Skeletal muscle cell lines were used to analyze the regulation of SERCA1 expression by T_3 in more detail. IGF-I was initially included in these analyses because it is regulated by T_3 and involved in skeletal muscle differentiation (cf. [49]). T_3 stimulated the expression of SERCA1 mRNA and functional protein in L6 myotubes, and the effect was potentiated in the presence of IGF-I [49]. IGF-I alone had little effect, but in the presence of T_3 it doubled the half-life of both SERCA1 mRNA and protein. The principle action of T_3 was a 3-fold stimulation of the transcription rate of the SERCA1 gene as determined by nuclear run-on analysis. This mode of action of T_3 is in agreement with the generally accepted view that

nuclear T_3-receptors (TR) mediate the effect of the hormone on gene expression [50, 51].

The three known TR isoforms are encoded by two genes, c-erbAα and c-erbAβ. They belong to a family of ligand-dependent transcription factors which bind to *cis*-regulatory DNA sequences in the promoters of target genes, allowing hormone-dependent modulation of transcription rates [50, 51]. In the case of T_3 these *cis*-elements are termed thyroid hormone response elements (TRE) [52]. The optimal binding site for a single TR has the consensus sequence AGGTCA. A TRE typically consist of two to four TR binding sites, oriented as direct repeats (head to tail) spaced by 4 nucleotides, or as everted repeats (tail to tail) spaced by 6 nucleotides, or as inverted repeats with no spacing (palindrome). This organization allows for cooperative receptor binding and dimerization or multimerization, which positively correlates with transactivation. TR binding and transactivation is increased by heterodimerization with the ubiquitous retinoid X receptor (RXR), a member of the TR-receptor family. It recognizes the same DNA binding motif, and is considered the natural partner for TRs *in vivo*. Deviations from the optimal binding-site sequence and, to a lesser extent, from the optimal spacing between them, are common in naturally occurring TRE's and responsible for large differences in TR-binding affinity and transactivation potency. A promoter may contain several TRE's contributing to the overall T_3-respectiveness. In contrast to other members of the nuclear receptor family, TRs bind to their response elements in the unliganded state, resulting in a substantial repression of promoter activity. Binding of T_3 to the TR relieves this repression and induces an interaction with the transcription initiation complex, stimulating transcriptional activity. T_3-induced transactivation is therefore the result of two separate phenomena, relief of repression and true activation. Both processes are now known to involve numerous proteins forming large coactivator or corepressor complexes, that act as adapters between receptors and the transcription initiation complex (reviewed in Ref. 53). Recent analyses of TR knock-out mice indicate considerable redundancy in isoform function, although some aspects of T_3 action are isoform specific [54–56]. For instance, TRβ is required for the development of hearing and the feed-back regulation of thyroid hormone production, whereas the absence of TRα results in a reduced heart rate and slightly lower body temperature. Mice lacking both TR genes are viable and, surprisingly, they show a much less pronounced reduction of body temperature than observed in hypothyroid neonatal rats, which die within 6 weeks [28, 54, 55, 57]. This is most likely explained by the active repression of T_3-responsive genes in hypothyroidism by the unliganded receptor, which does not occur in TR-deficient animals.

Skeletal muscle expresses the two principle receptor isoforms TRα1 and TRβ1, as well at TRα2. The latter isoform is not a true T_3-receptor since it cannot bind T_3, but because the domains for DNA-binding and dimerization are conserved, it can act as a dominant negative regulator of T_3-responsive genes. Its proper physiological function is not known. Although data on the expression of TR-isoform mRNAs indicate no differences between fast and slow muscle, species differences may exist in the relative levels of TRα1 and TRβ1 [58, 59]. Immunohistochemical analyses indicated similar levels of TRα1 and TRβ1 protein in slow and fast muscle in the rat [60]. These data do not suggest a muscle-type specific role of TR isoforms,

which is supported by a recent analysis of MHC-isoform expression in SOL and EDL in TR knock-out mice [61]. This study did, however, indicate that both TRα and TRβ isoforms are required for the full effect of T_3 on slow muscle.

To analyze the T_3-regulated expression of SERCA1 in detail we cloned the 5′-flanking sequence of the SERCA1 gene of the rat. Analysis of the first 962 base pairs (bp) of this promoter in COS (kidney) cells confirmed the T_3-responsiveness of the gene [62]. T_3-induced a TR-dependent, 4-fold increase of the transcription rate which was doubled in the presence of RXR. TRα1 and TRβ1 were equally potent in repressing transcription and mediating stimulation by T_3. Three sequences were identified that met the criteria for a TRE, i.e., they bound TR with high affinity (dimers and oligomers), and conferred T_3-responsiveness when inserted in a heterologous promoter (thymidine kinase, TK). The TRE at position −545 (R3) bound TR with the highest affinity and also conferred the highest T_3-induction ratio to the TK promoter (6-fold). Detailed analysis showed that it consisted of an unusual combination of 4 half sites. The SERCA1 sequence also contained several binding sites for MRFs so called "E-boxes" (CANNTG) [29]. Comparison of the SERCA1 promoter with the published sequence of the SERCA2 promoter showed no similarities. However, multiple TREs were also found to be responsible for T_3-responsiveness of this gene [63, 64].

Analyses of the 962 bp SERCA1 promoter in L6 muscle cells showed only a 2-fold T_3-induction, but this increased to 5-fold when an extended promoter fragment (2658 bp) was used [65]. This long promoter generated a 20-fold higher transcriptional activity in L6 than in COS cells, indicating the effect of tissue specific factors (Simonides and Thelen, unpublished results). Recently we identified a strong TRE at position −1710 (R) responsible for the extra T_3-induction in L6 cells. In conferred an 8-fold T_3-induction to the TK-promoter and site-directed mutagenesis indicated a half-site organization similar to other potent TREs. Figure 5 gives the sequences and TR-binding sites of TRE R3 and R5, and the deletion analysis of the SERCA1 promoter in L6 myoblasts which indicated the position of these elements. We now conclude that TRE R5, together with TRE R3, confer T_3-responsiveness to the SERCA1 gene in muscle cells (Simonides and Thelen, unpublished results).

Ca^{2+}-DEPENDENCY OF T_3-REGULATED SERCA1 EXPRESSION

The average intracellular free calcium concentration ($[Ca^{2+}]_i$) is approximately 50–100 nM in fast fibers, and 200–300 nM in slow fibers, as a result of the difference in contractile activity (*see above*). Several lines of evidence suggested that these differences in $[Ca^{2+}]_i$ may be involved in regulating gene expression [66–69]. In line with this we found that contractions suppressed the basal expression of SERCA1 in cultured primary myotubes at a pretranslational level. This correlated with a reduced MyoD/myogenin mRNA ratio, but a coordinate fast-to-slow switch was not indicated, since SERCA2a expression was not affected [70]. However, the effect of contractions on SERCA1 expression was in line with the $[Ca^{2+}]_i$-dependency of SERCA1 expression which we found earlier in non-contracting L6 myotubes [71]. In that study basal expression was optimal around 120 nM $[Ca^{2+}]_i$ and decreased to

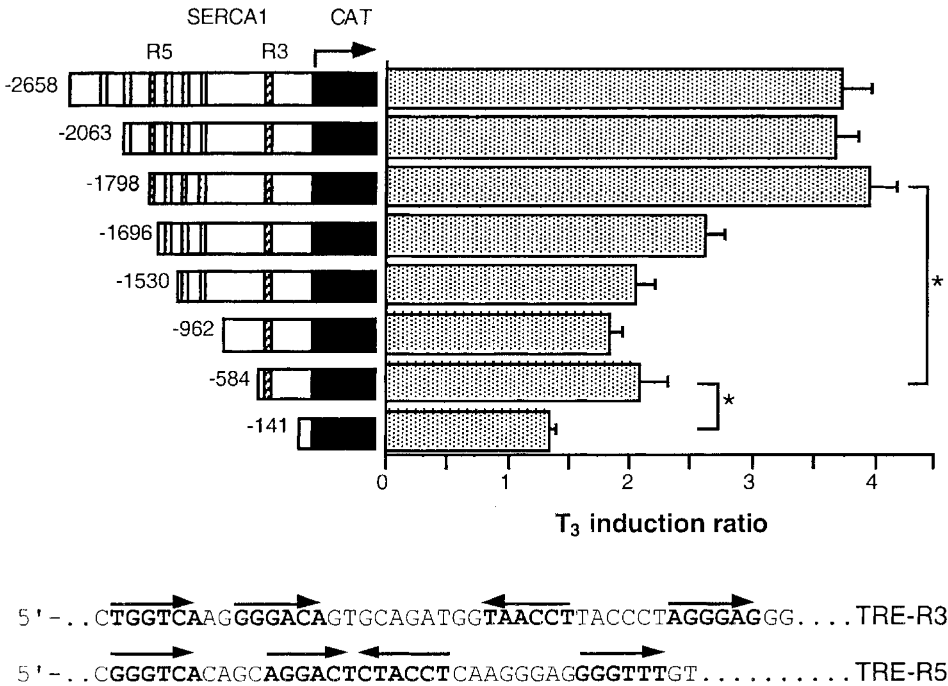

Fig. 5. Progressive 5′-deletion fragments of the 2658 bp promoter of the rat SERCA1 gene were inserted in the promoterless pOCAT2 vector and analyzed for T$_3$-responsiveness in transient transfection assays using L6 myoblasts. Suspected TRE sequences are indicated in the promoter. Only deletion of regions containing R3 and R5 resulted in a significant loss of T$_3$-stimulation of transcription. Mobility-shift analyses and site-directed mutagenesis identified R3 and R5 as functional TREs. TR binding-sites essential for transactivation are indicated in the DNA sequences (bold face) and their orientation is indicated by arrows (from Ref. 62 and unpublished results (Simonides and Thelen)).

about 50% at 185 nM, but, surprisingly, the 5-fold T$_3$-stimulation of SERCA1 protein expression at 120 nM was practically abolished at 185 nM. The same effect was found in C2C12 myotubes, when contractions were used to increase $[Ca^{2+}]_i$ and this was shown to result from a specific block of the 7-fold T$_3$-stimulation of the 2658 bp SERCA1 promoter [72]. These results suggest that the switch of fast muscles to the slow phenotype, induced by continuous electrical stimulation [24], is at least in part due to suppression of the stimulatory effect of T$_3$ on fast isoforms.

Analysis in L6 cells confirmed that the effect of increasing $[Ca^{2+}]_i$ on the T$_3$-dependent expression of SERCA1 protein occurs at a pre-transional level. Furthermore, it showed that this is correlated with induction of myogenin expression [65]. Figure 6 shows the Ca^{2+}-dependent inhibition of the effect of T$_3$ on SERCA1 mRNA expression, as well as the strong induction of myogenin mRNA expression with increasing $[Ca^{2+}]_i$. Subsequent co-transfection experiments with SERCA1 promoter constructs and myogenin expression vetors showed that myogenin specifically blocks the effect of T$_3$ on SERCA1 promoter activity [65]. With respect to SERCA1 expression, these data suggest a mechanism for the opposing effects of contractile

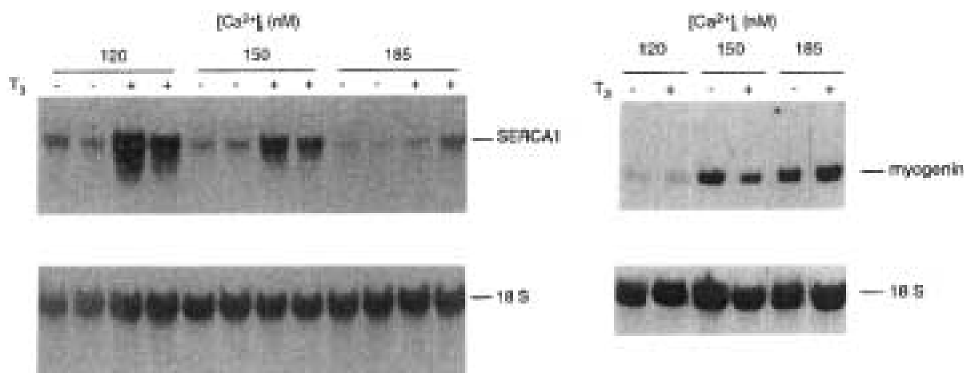

Fig. 6. L6 myotubes were cultured in the presence or absence of T_3, and different concentrations of ionomycin were used to obtain the intra-cellular $[Ca^{2+}]$ indicated. Total RNA was isolated at day 3 and Northern blots were probed for SERCA1 (left panel) or myogenin mRNA (right panel). Hybridization with an 18S rRNA oligonucleotide provided normalization. T_3 had no effect on myogenin mRNA levels (from Ref. 65). Reproduced with permission from Thelen, M. H., Simonides, W. S., Muller, A., and van Hardeveld, C. (1998) *Biochem. J.* **329:**(1)**:**131–136. © The Biochemical Society.

activity and T_3 on the fast phenotype, with $[Ca^{2+}]_i$ being a driving force. It should be noted that for any given level of motor-nerve activity, enhancement of SERCA activity by T_3 will affect the average $[Ca^{2+}]_i$. It was shown recently that the $[Ca^{2+}]_i$-dependent calcineurin pathway of gene regulation may play a role in phenotypic regulation in skeletal muscle [73]. This signaling route could provide a link between the changes in $[Ca^{2+}]_i$—either resulting from altered contractile activity or altered SR Ca^{2+}-ATPase activity—and the expression of MRFs and other factors regulating muscle gene transcription.

ROLE OF T_3-REGULATED SERCA EXPRESSION IN THERMOGENESIS

The data discussed thus far show that expression of SR Ca^{2+}-ATPase is to a large extent dependent on direct regulation by T_3. Apart from the absolute dependency during neonatal development, 30–50% of the expression in skeletal muscle of the adult, euthyroid animal is T_3-dependent. Furthermore, the net stimulation of Ca^{2+}-ATPase activity by higher levels of T_3, is solely the result of increased expression of the SERCA1 isoform. The possible role of this regulation in a thermogenic effect of T_3 during prolonged cold exposure is discussed in the following sections.

CONTRIBUTION OF SR CA^{2+}-CYCLING TO THE METABOLIC RATE DURING REST

Maintenance of Ca^{2+}-homeostasis by the SR Ca^{2+}-ATPase in resting muscle is estimated to account for 10–25% of the RMR of this tissue, based on *in vitro* analyses of Ca^{2+}-ATPase function in purified SR [74–76]. Maintenence of the steep Ca^{2+}-gradient over the SR membrane is a balance between active uptake and efflux of Ca^{2+} back into the cytosol. Passive efflux through the SR membrane is low, but the

Ca^{2+}-ATPase can "slip", i.e., the optimal Ca^{2+}/ATP coupling ratio of 2 decreases to much less than 1 as the gradient builds. This accounts for a substantial energy turnover at steady state [74–76]. De Meis recently showed that the SERCA1 isoform of the Ca^{2+}-ATPase can mediate so-called uncoupled Ca^{2+}-efflux in which osmotic energy is converted to heat [6, 77]. It was suggested that a variable proportion of the SR Ca^{2+}-ATPases may operate in this mode, determining the overall ATP turnover and dissipation of heat during maintenance of the gradient. It is tempting to speculate that such variation underlies the earlier reported higher coupling ratio and 30% lower ATP turnover associated with maintenance of a Ca^{2+}-gradient, in hypothyroid vs. euthyroid fast muscle SR, where all other parameters, such as Ca^{2+}-ATPase/mg SR and passive efflux rates, were equal [74]. T_3-induced changes in SR membrane lipid composition, which correlated with slightly altered catalytic properties of the Ca^{2+}-ATPase [13, 78], could provide a basis for such variation. Most studies on Ca^{2+}-ATPase kinetics and energetics have been done with fast muscle SR and it is not known whether SERCA2a can operate in the uncoupled efflux mode. Recently it was shown that the SERCA2b isoform of endoplasmic reticulum in blood platelets does not show uncoupled efflux, unless platelet activating factor is present [79]. SERCA2b is a splice variant of the SERCA2 gene in which the 4 c-terminal amino acids present in SERCA2a are replaced by an extended 49 or 50 amino acid sequence [80, 81].

The estimate of the energy cost of Ca^{2+}-homeostasis at rest derived from the *in vitro* data mentioned above, is generally supported by indirect calorimetric and O_2-consumption measurements of different types of isolated or perfused, denervated muscles, which yield an estimate between 5 and 25% of the RMR of muscle [1, 82, 83]. Given the fact that skeletal muscle accounts for approximately 20% of the RMR of the animal, the contribution of SR Ca^{2+}-ATPase activity at rest to the overall RMR is not more than 5% [1]. As a result, the T_3-dependent stimulation of SR Ca^{2+}-ATPase activity may account for at most 10% of the increase in RMR in the transition from hypothyroidism to hyperthyroidism. The possible contribution of T_3-dependent SR Ca^{2+}-cycling to NST is therefore small. However, the T_3-induced increase in SERCA1 expression will potentiate the effect of any mechanism that selectively stimulates Ca^{2+}-cycling. Several such mechanisms have been proposed in which Ca^{2+}-leakiness of the SR is increased (cf. Ref. 7), but their contribution to NST has not yet been firmly established. An alternative hypothesis for skeletal-muscle dependent NST, which does not involve Ca^{2+}-cycling, suggests induction of the mitochondrial uncoupling protein UCP3, a homologue of UCP1. Such a mechanism has not been confirmed yet and is in fact debated [2, 84].

CONTRIBUTION OF SR Ca^{2+}-CYCLING TO THE METABOLIC RATE DURING ACTIVITY

As mentioned earlier, SR Ca^{2+}-cycling accounts for up to 50% of the energy turnover in muscle during contraction [1, 10, 11]. The overall metabolic rate during exercise is therefore primarily determined by SR Ca^{2+}-ATPase activity and the ATPase activity of the contractile filaments. It should be noted that the maximal energy consumption by SR Ca^{2+}-ATPase alone, is 5 to 10 times higher than the RMR of the muscle. The enormous capacity to generate heat by Ca^{2+}-cycling in muscle is further illustrated in malignant hyperthermia, where a defective SR

Ca^{2+}-release channel can be triggered to leak, leading to a lethal rise in body temperature (reviewed in Ref. 7). Consequently, SR Ca^{2+}-ATPase activity accounts for a substantial part of the extra heat generation during shivering, with 30–50% of the contribution being dependent on T_3 in the euthyroid animal.

Primarily slow, fatigue-resistant motor units are recruited in the shivering response during acute cold exposure [85, 86]. These motor units are already active 20–30% of the time, and even though they have a relatively low maximal capacity for energy turnover, their increased activity apparently generates sufficient heat. It is intriguing that these slow fibers are also the ones most responsive to increased T_3-levels. The presented data indicate that as shivering subsides and serum T_3-levels rise and NST takes over, expression of high levels of SERCA1 is induced in these fibers, as well as faster MHC isoforms, and the phenotype gradually shifts from slow oxidative type I, to fast oxidative type IIC or IIA. As a result, the energy turn-over of these motor units almost doubles—at the same level of force production—but the motor-nerve activity will not have changed, i.e., they will still be active 20–30% of the time. Consequently, these effects on fiber phenotype, and the associated increase in heat production at normal levels of physical activity, may provide a mechanism for a T_3-dependent thermogenic response during prolonged cold exposure. The higher percentage of slow motor units in muscles of larger mammals is a relevant factor for the possible importance of the mechanism we propose here. In man the average percentage of type I fibers in all skeletal muscles is about 50%, whereas this is 10% in, for instance, rat, guinea pig and cat, with the exception of some pure slow muscles like the SOL [23, 87]. The capacity to generate heat through this route is therefore substantially greater in those larger species that cannot rely on BAT for NST. A role in facultative thermogenesis of phenotype modulation by T_3, particularly with respect to SERCA1 expression, is supported by observations in cold-acclimated ducks. Like larger mammals, ducks also need to rely on tissues other than BAT to survive in the cold, and up to 70% of NST in these animals was found to originate in skeletal muscle [88]. This was correlated with increased expression of SERCA1, resulting in a 30–50% increase in SR Ca^{2+}-ATPase activity [8, 9]. Perhaps the ultimate adaptive use of the high levels of Ca^{2+}-ATPase activity that can be attained with the SERCA1 gene, is found in the modified extraocular muscles that harness the heat generated by Ca^{2+}-cycling to warm the brain and eyes in several species of fish [7]. This heater-tissue has no contractile function, but instead is filled with mitochondria and SR packed with SERCA1.

In conclusion, the T_3-induced up-regulation of SERCA1 in slow muscle fibers appears to provide a robust way to generate extra heat, at the same level of physical activity, and we suggest that this route contributes to the thermogenic response in those animals that need to rely on skeletal muscle for adaptation to the cold.

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