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Differential regulation of the expression of fast-type sarcoplasmic-reticulum Ca^{2+}-ATPase by thyroid hormone and insulin-like growth factor-I in the L6 muscle cell line

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The aim of this study was to investigate the mechanism(s) underlying the thyroid-hormone (L-tri-iodothyronine, T_3)-induced elevation of fast-type sarcoplasmic-reticulum Ca^{2+}-ATPase (SERCA1) levels in L6 myotubes and the potentiating effect of insulin-like growth factor-I (IGF-I) [Muller, van Hardeveld, Simonides and van Rijn (1991) Biochem. J. **275**, 35–40]. T_3 increased the SERCA1 protein level (per μg of DNA) by 160%. The concomitant increase in the SERCA1 mRNA level was somewhat higher (240%). IGF-I also increased SERCA1 protein (110%) and mRNA levels (50%), whereas IGF-I + T_3 increased SERCA1 protein and mRNA levels by 410% and 380% respectively. These SERCA1 mRNA analyses show that the more-than-additive action of T_3 and IGF-I on SERCA1 expression is, at least in part, pre-translational in nature. Further studies showed that the half-life of SERCA1 protein in L6 cells (17.5 h) was not altered by T_3. In contrast, IGF-I prolonged the half-life of SERCA1 protein 1.5–1.9-fold, which may contribute to the disproportional increase in SERCA1 protein content compared with mRNA by IGF-I. Measurements of SERCA1 mRNA half-life (as determined by actinomycin D chase) showed no difference from the control values (15.5 h) in the presence of T_3 or IGF-I alone. When T_3 and IGF-I were both present, the SERCA1 mRNA half-life was prolonged 2-fold. No significant effects of T_3 and IGF-I were observed on the half-life of total protein (37.4 h) and total RNA (37.0 h). The absence of an effect of T_3 on SERCA1 protein and mRNA stability, when it was present alone, suggested transcriptional regulation, which was confirmed by nuclear run-on experiments, showing a 3-fold increase in transcription frequency of the SERCA1 gene by T_3. We conclude that the synergistic stimulating effects of T_3 and IGF-I on SERCA1 expression are the result of both transcriptional and post-transcriptional regulation. T_3 acts primarily at the transcriptional level by increasing the transcription frequency of the SERCA1 gene, whereas IGF-I seems to act predominantly at post-transcriptional levels by enhancing SERCA1 protein and mRNA stability, the latter, however, only in the presence of T_3.

INTRODUCTION

Thyroid hormone (T_3) is a major determinant of the fast-type sarcoplasmic-reticulum Ca^{2+}-ATPase (SERCA1) level in skeletal muscle [1–4]. This Ca^{2+}-transporting protein is responsible for the removal of Ca^{2+} from the cytosol during a contraction–relaxation cycle. The T_3-induced increase in SERCA1 expression is mainly responsible for the enhanced muscle relaxation rate which is characteristic of the hyperthyroid status [5–7]. Evidence that T_3 regulates SERCA1 expression *in vivo* at least partly at a pre-translational level was provided in one of our previous studies, in which it was shown that T_3 increases SERCA1 mRNA levels in rat soleus and extensor digitorum longus muscle [8]. Other factors, such as growth hormone and insulin-like growth factor-I (IGF-I), which are involved in growth and differentiation of skeletal muscle, are directly or indirectly controlled by T_3 [9–11]. In a previous study with the L6 rat muscle cell line we investigated the possible involvement of IGF-I in T_3-stimulated sarcoplasmic-reticulum development [12]. It was shown that not only T_3, but also IGF-I, elevated SERCA1 levels (2.3- and 1.6-fold respectively), and that T_3 and IGF-I acted synergistically (4.3-fold increase). The goal of the present study was to gain further insight into the regulatory mechanism(s) by which T_3 and IGF-I enhance SERCA1 expression in L6 myotubes.

The primary mechanism of T_3 action in diverse cellular processes involves an interaction of the hormone–receptor complex with specific DNA sequences, leading to altered transcription rates of target genes (reviewed in [13]). In addition, T_3 may also act at post-transcriptional levels. Evidence has been presented that T_3 increased the stability of growth hormone and malic enzyme mRNA [14–16], whereas a negative influence of T_3 was demonstrated on the stability of thyrotropin β-subunit mRNA [17]. Additionally, it has been known for a long time that T_3 enhances protein turnover in skeletal muscle by stimulating both protein synthesis [18–20] and protein degradation [18,21].

IGF-I has a broad range of effects, comprising a general stimulation of protein, RNA and DNA synthesis, as well as induction of specific proteins involved in cell proliferation or differentiation (reviewed in [22]). These effects are accomplished after binding of IGF-I to the type 1 IGF plasma-membrane receptor, followed by the activation of an intracellular signalling system, which is not yet completely elucidated [23,24]. This eventually results in transcriptional [25–27] or post-transcriptional regulation, for example by increasing mRNA stability

Abbreviations used: AraC, 1-(β-D-arabinofuranosyl)cytosine; DMEM, Dulbecco's modified Eagle's medium; IGF-I, insulin-like growth factor-I; PMSF, phenylmethanesulphonyl fluoride; SERCA1, fast-type sarcoplasmic-reticulum Ca^{2+}-ATPase; SERCA2a, cardiac/slow-type sarcoplasmic-reticulum Ca^{2+}-ATPase; T_3, L-tri-iodothyronine (thyroid hormone); T_xS, thyroid-hormone-depleted fetal-calf serum.

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[28,29]. The latter was also demonstrated in L6 muscle cells, in which insulin (acting as an IGF-I analogue) increased the stability of creatine kinase mRNA [30]. Furthermore, the increase in total protein content that was induced by IGF-I in cultured muscle cells was shown to be mediated partly through inhibition of protein breakdown [31–33]. Taken together, these data illustrate that T_3 and IGF-I may regulate gene expression at various levels.

In this study we present evidence that T_3 and IGF-I control SERCA1 expression at the transcriptional and post-transcriptional level respectively, although not exclusively. Part of this study has been presented in a preliminary form [34].

MATERIALS AND METHODS

Materials

Eagle's modified essential medium without methionine, and Dulbecco's modified Eagle's medium (DMEM), were purchased from Flow Laboratories (Irvine, Scotland, U.K.). Fetal-calf serum was from GIBCO–BRL (Gaitersburg, MD, U.S.A.) and culture plastics were from NUNC (Roskilde, Denmark). IGF-I (human recombinant) was obtained from Boehringer (Mannheim, Germany). Insulin (bovine) was from Serva (Heidelberg, Germany). 1-(β-D-Arabinofuranosyl)cytosine (AraC), actinomycin D and T_3 were obtained from Sigma (St. Louis, MO, U.S.A.). AG-1 X8 anion-exchange resin and anti-(mouse immunoglobulin) immunobeads were obtained from Bio-Rad (Richmond, CA, U.S.A.). Hybond-N membrane was from Amersham International (Amersham, Bucks., U.K.). [^{35}S]Methionine, [α-^{32}P]dATP and [α-^{32}P]GTP (triethylammonium salt) were from Du Pont–New England Nuclear (Wilmington, DE, U.S.A.). RNAase inhibitor (recombinant) was obtained from Promega Corp. (Madison, WI, U.S.A.). The monoclonal antibody A52 and the *Bam*HI(+1874)–*Pvu*II-(+2373) rabbit SERCA1 cDNA fragment were kindly provided by Dr. D. H. MacLennan, Charles H. Best Institute, Toronto, Canada. Autoradiography was performed at −80 °C using RX medical film (Fuji, Tokyo, Japan) and Cronex Lightning Plus intensifying screens (Dupont). Autoradiograms were analysed by laser-densitometric scanning (LKB 220 Ultroscan; LKB, Uppsala, Sweden).

Cell culture

The subclone $L6_{AM}$ of the rat myogenic cell line L6, originally isolated by Yaffe [35], was grown to confluence on DMEM + 10% (v/v) fetal-calf serum depleted of thyroid hormones (T_xS) by AG-1 X8 resin treatment [36]. Next, differentiation was initiated by replacing the medium by DMEM + 2.5% (v/v) T_xS and 3.3 nM IGF-I (differentiation medium), and 2 days later, AraC (4 μg/ml) was added to the medium. When fusion was complete, the cultures were switched to DMEM containing 2.5% (v/v) T_xS. The next day (day 0) the following additions were made: T_3 (5 nM), IGF-I (3.3 nM) or T_3 plus IGF-I. Controls received no additions. For the analysis of SERCA1 mRNA and protein levels, cells were harvested on day 3, and SERCA1 mRNA and protein levels were determined by Northern-blot analysis and competitive e.l.i.s.a. respectively.

E.l.i.s.a.

For SERCA1 protein analysis, cell cultures were washed twice with PBS, covered with 300 μl of PBS and frozen at −20 °C. After thawing, the cells were harvested and stored at −20 °C until analysis. A specific monoclonal antibody, A52 [37], raised against rabbit SERCA1, but also specific for the rat SERCA1, was used to quantify SERCA1 protein in muscle cell homogenates by competitive e.l.i.s.a. essentially as described previously [12].

Determination of SERCA1 protein half-life

Cells were grown in 6-well plates essentially as described in the 'Cell culture' section, except that IGF-I in differentiation medium was replaced by insulin (175 nM). We have shown previously that effects of insulin on SERCA1 expression are similar to those of IGF-I [38]. T_3 (5 nM) was added to guarantee high SERCA1 levels. When fusion was complete, the degradation rate of SERCA1 protein was measured, by using the protocol followed by Zubrzycka-Gaarn et al. [39] to determine the degradation rate of sarcoplasmic-reticulum Ca^{2+}-ATPase in L6 cells. Cells were labelled for 24 h in Eagle's modified essential medium without methionine supplemented with 2.5% (v/v) T_xS, T_3 (5 nM), insulin (175 nM), [^{35}S]methionine (10 μCi/ml; sp. radioactivity 1000 Ci/mmol) and 20 μM unlabelled methionine. After a 24 h labelling period, the cells were washed with PBS and then covered with 2 ml of DMEM + 2.5% T_xS, with or without T_3 (5 nM) and/or IGF-I (3.3 nM). The chase medium contained 10-fold excess of unlabelled methionine compared with the labelling medium, to minimize isotopic re-incorporation. The cells were collected at the time of removal of the labelling medium ($t = 0$) and 4, 8, 24 and 48 h later. Cells were washed twice with ice-cold PBS, covered with 200 μl of PBS, pH 7.35, containing 1% (v/v) Triton X-100, 0.5% sodium deoxycholate, 1% (w/v) SDS and 1 mM phenylmethanesulphonyl fluoride (PMSF), and were then placed on ice for 30 min. The cell lysate was resuspended and centrifuged for 10 min at 10000 *g*. The supernatant was removed and stored at −80 °C until analysis.

Isolation of SERCA1 protein

SERCA1 was isolated by immunobeads-mediated precipitation using monoclonal antibody A52. A52 was bound to anti-(mouse immunoglobulin) immunobeads by incubation of beads with hybridoma tissue-culture supernatant (50 μl/mg of immunobeads) in PBS containing 1% (w/v) BSA during 2 h at 37 °C. The A52-coupled immunobeads were washed three times with PBS, pH 7.35, containing 1% Triton X-100, 0.5% deoxycholate, 1% SDS and 1 mM PMSF. Cell lysates were supplemented with BSA to a final concentration of 1% before immunoprecipitation. Then 100 μl of lysate (containing approx. 200 μg of cellular protein) was incubated with 0.5–1.0 mg of A52-coupled immunobeads for 1 h at room temperature. Next, the immunobeads were washed three times with ice-cold PBS, pH 7.35, containing 1% Triton X-100, 0.5% deoxycholate, 1% SDS, 1 mM PMSF and 1% BSA. Precipitated proteins were extracted from the immunobeads by incubation at 85 °C for 10 min in 25 μl of 60 mM Tris, containing 10% (v/v) glycerol, 100 mM dithiothreitol, 2% (w/v) SDS and 0.05% Bromophenol Blue, pH 6.8. Proteins were electrophoresed by SDS/PAGE in 7% mini-slab gels [40]. After fixation in 40% (v/v) methanol/10% (v/v) acetic acid, the gels were incubated in dimethyl sulphoxide (1 h), next in dimethyl sulphoxide + 15% (w/v) 2,5-diphenyloxazole (3 h), and finally in water (1 h), and were subsequently dried and exposed for autoradiography at −20 °C. The intensity of the 100 kDa ^{35}S-labelled protein band was determined by laser densitometry. It was verified by immunoblot analysis [12] that the 100 kDa ^{35}S-labelled protein band corresponded to that of SERCA1 protein (Figure 1). The slope of the degradation curve

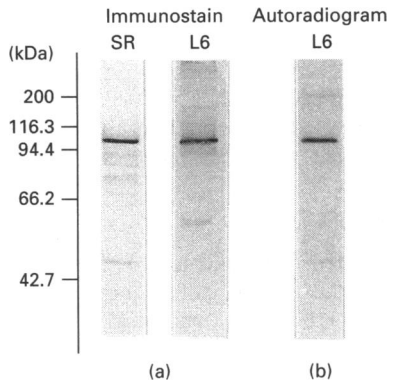

Figure 1 SDS/PAGE profile of immunoprecipitated protein isolated from L6 myotubes by using the monoclonal antibody A52 against SERCA1

^{35}S-labelled extract of $L6_{AM}$ myotubes obtained by immunoprecipitation was separated by Laemmli SDS/PAGE (6% gels) and then transferred to a nitrocellulose sheet. The blot was immunostained with A52 (**a**) and exposed for autoradiography (**b**). Purified sarcoplasmic reticulum from rat gastrocnemius muscle (containing 4.25 nmol of sarcoplasmic-reticulum Ca^{2+}-ATPase/mg of protein), immunostained with A52, served as a standard (SR).

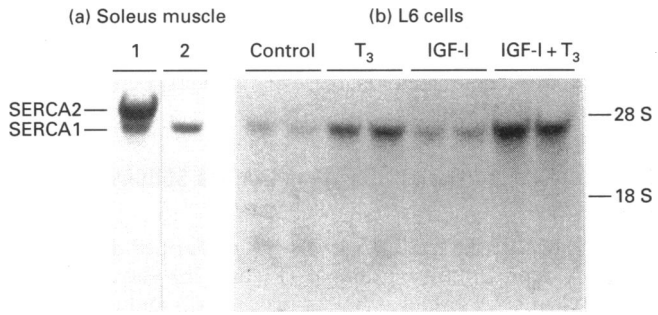

Figure 2 Northern-blot analysis (a) of rat soleus RNA demonstrating the specificity of the 395 bp *Bgl*II(+2914)–[poly(A)$^+$ tail] probe for SERCA1 mRNA, and (b) of SERCA1 mRNA in L6 myotubes (cultured as described in the Materials and methods section) subjected to T_3 (5 nM), IGF-I (3.3 nM) or T_3 plus IGF-I for 3 days

(**a**) A 30 μg portion of total RNA was electrophoresed, subsequently blotted and hybridized as described in the Materials and methods section. The following probes were used: (1) 520 bp *Bam*HI(+1854)–*Pvu*II(+2373) rabbit SERCA1 cDNA, which does not discriminate between SERCA1 and SERCA2a mRNA; (2) 395 bp *Bgl*II(+2914)–[poly(A)$^+$ tail] specific for SERCA1 mRNA. In (**b**) controls received no additions. Portions (40 μg) of total RNA from duplicate samples of the different experimental groups were electrophoresed, subsequently blotted and hybridized with the probe specific for SERCA1 mRNA and exposed for autoradiography. In the right margin the positions of 28 S and 18 S rRNA are indicated.

was determined by least-squares analysis; the half-life is defined as the time point at which the radioactivity in SERCA1 protein was decreased to 50% of the value at $t = 0$.

Determination of total protein half-life

A 10 μl portion of lysate (containing approx. 20 μg of cellular protein) was mixed with 20 μl of PBS+1% (w/v) BSA. Then 0.5 ml of ice-cold 10% (v/v) trichloroacetic acid was added, and after incubation for 30 min on ice, the mixture was centrifuged at 10000 *g* for 10 min at 4 °C. The pellet was washed once more with 0.5 ml of trichloroacetic acid, and then dissolved in 0.15 ml of 1 M NaOH; 10 ml of scintillation fluid was added and the samples were counted for radioactivity.

Isolation of RNA and Northern-blot analysis

Northern-blot analysis was performed essentially as described by Simonides et al. [8]. Total RNA was extracted from the combined cell material of three 25 cm^2 culture flasks. RNA isolation was performed in duplicate for each variable. The concentration of total RNA was determined spectrophotometrically at 260 nm, and equal amounts of total RNA (up to 40 μg per lane) were electrophoresed. The probe consisted of a 395 bp *Bgl*II(+2914)–[poly(A)$^+$ tail] cDNA fragment (translation initiation site at +1) of SERCA1 of the rat. Specificity of this probe for the SERCA1 transcript was demonstrated with RNA samples from rat soleus muscle {a slow-twitch muscle containing mRNA for both SERCA1 and cardiac/slow-type sarcoplasmic-reticulum Ca^{2+}-ATPase (SERCA2a) at a ratio of 1:10 [8]} by using a previously described probe consisting of the *Bam*HI(+1854)–*Pvu*II(+2373) cDNA fragment from the coding region of rabbit SERCA1 that does not discriminate between SERCA1 and SERCA2a transcripts [8] (Figure 2a). For determination of the degradation rate of SERCA1 mRNA by actinomycin D chase, the SERCA1 transcript was quantified by using a 586 bp (−65)–*Sal*I(+521) cDNA fragment of rat SERCA1. The specificity of this probe for SERCA1 mRNA was confirmed as described above. When using the (−65)–*Sal*I(+521) probe, the hybridization protocol was slightly adapted, i.e. 1% SDS was included in the prehybridization and hybridization buffer. Blots were subjected to autoradiography and the signals were quantified by scanning. The integrated absorbance values were multiplied by the RNA/DNA ratio to yield the mRNA abundance/unit of DNA.

Determination of SERCA1 mRNA half-life

SERCA1 mRNA stability was measured by actinomycin D chase. Cells were grown as described in the section on 'Cell culture'. Actinomycin D (5 μg/ml) was added to the cultures at day 2. To correct for possible loss of IGF-I during 2 days of culture, the IGF-I-treated cultures were supplemented with an additional amount of IGF-I, 12.5 ng/ml. No significant decrease in the T_3 concentration was observed during the experiment. Total RNA was isolated at the time of actinomycin D addition ($t = 0$) and 3, 6, 10 and 24 h later. SERCA1 mRNA levels were determined by Northern-blot analysis. The slope of the degradation curve of total RNA and SERCA1 mRNA was determined by least-squares analysis.

Determination of the SERCA1 transcription rate

For nuclear run-on analysis, L6 cells were grown to confluence on DMEM+10% T_xS. Next, the cultures were switched to differentiation medium, containing 2.5% T_xS and 1 μg/ml insulin. At this point of time (designated 'day 0') treatment with 5 nM T_3 started (controls received no T_3). On day 3 AraC (4 μg/ml) was added to the medium. On day 4 cells were harvested for determination of SERCA1 mRNA levels by Northern-blot analysis and the relative SERCA1 transcription rate by nuclear run-on analysis.

Isolation of nuclei from L6 cells

Nuclei of L6 cells were isolated by adaptations of the protocols described by Groudine et al. [41] and Marzluff and Huang [42].

From ten 180 cm^2 culture flasks, cells were harvested by scraping in 5 ml of homogenization buffer (10 mM Hepes buffer, pH 7.9, 10 mM $MgCl_2$, 0.3 M sucrose, 1 mM dithiothreitol, 0.2 mM PMSF, 0.1% Triton X-100, 10 units/ml RNAase inhibitor). Cells were ruptured with a glass/Teflon Potter homogenizer, and subsequently filtered through a 45 μm nylon filter and left on ice for 30 min to allow lysis. The sucrose concentration was raised to 1.8 M, and the homogenates were layered on a 2.1 M sucrose cushion (in 10 mM Hepes, pH 7.9, 10 mM $MgCl_2$, 1 mM dithiothreitol, 0.2 mM PMSF). After centrifugation at 77000 *g* for 100 min at 4 °C, the pellet, containing the nuclei, was resuspended in storage buffer [20 mM Tris, pH 7.9, 5 mM $MgCl_2$, 0.5 mM EDTA, 1 mM dithiothreitol, 0.2 mM PMSF, 50% (v/v) glycerol], frozen in liquid nitrogen and stored at −80 °C.

Nuclear run-on transcription assay

The protocol used for nuclear run-on assays was an adaptation of that described by Linial et al. [43] and Marzluff and Huang [42]. Transcription reactions were started by addition of 200 μl of freshly thawed nuclei [$(2–4) \times 10^7$ nuclei] to 100 μl of reaction buffer {0.25 M KCl, 5 mM magnesium acetate, 2.25 mM each of ATP, UTP and CTP, 1.5 μM [α-^{32}P]GTP (3000 Ci/mmol), 0.75 μM unlabelled GTP, 350 units/ml RNAase inhibitor, 1 mM dithiothreitol} at 30 °C. Reactions were performed with equal numbers of L6-myotube nuclei isolated from T_3-treated or control cells, and were terminated after 30 min by addition of 10 vol. of stopping buffer (1% SDS, 10 mM EDTA, pH 7.0) and put on ice. Transcriptional activity was determined by monitoring total incorporation of radioactive GTP into the nuclei. The relative activity of RNA polymerase II was estimated from the α-amanitin (0.5 μg/ml)-inhibitable and -insensitive components of the total incorporation [44]. Nuclear RNA was isolated by hot-phenol/chloroform extraction [45] followed by propan-2-ol precipitation. The dry pellet was resuspended in 200 μl of RNA dissolvent (0.3 M NaCl, 0.1% SDS, 1 mM EDTA, 10 mM Tris, pH 7.5), and the RNA was purified on a Sephadex G50 column.

Hybridization of run-on RNA

To quantify the relative rate of SERCA1 gene transcription, RNA labelled *in vitro* was hybridized to 1400 nt sense and antisense single-stranded (ss) SERCA1 DNA probes, which were obtained by subcloning a rat SERCA1 cDNA fragment [*Bam*HI(+1852)–(poly(A)$^+$ tail] in M13mp18/19. This probe proved specific for SERCA1, with no cross-hybridization with SERCA2a mRNA. Non-recombinant M13mp18 served as a control for background hybridization, and 5 μg portions of this vector and the probes were immobilized on Bio-Rad Zetaprobe GT nylon membranes by using a Bio-Rad Biodot SF slot-blot apparatus according to the manufacturer's protocol. To facilitate hybridization, the RNA synthesized *in vitro* was mildly digested by supplementing the solution with NaOH (final concn. 0.2 M) and incubation on ice for 10 min. Reactions were quenched by addition of Hepes (final concn. 0.24 M). After precipitation, the RNA was resuspended in hybridization fluid [50% (v/v) formamide, 5 × SSC, 10 mM Tris, pH 7.5, 1 mM EDTA, 0.1% SDS, 10 ng/ml poly(A), 4 × Denhardt's, 100 μg/ml denatured herring sperm DNA]. [SSC: 0.15 M NaCl/15 mM sodium citrate, pH 7.0; 100 × Denhardt's: 2% (w/v) Ficoll, 2% (w/v) polyvinylpyrrolidone, 2% (w/v) BSA.] Hybridizations with several dilutions of RNA were carried out in small volumes (< 4 ml) at 42 °C for 72 h in hybridization buffer. Subsequently the blots were rinsed in 5 × SSC and washed for 30 min in, successively: 2 × SSC/0.1% SDS, 52 °C; 2 × SSC, 37 °C; 2 × SSC containing 50 μg/ml RNAase A, 37 °C; 0.5 × SSC/0.1% SDS, 52 °C; 0.1 × SSC/0.1% SDS, 58 °C. Finally the blots were rinsed in 5 × SSC and subjected to autoradiography. Signals were quantified by laser-densitometric scanning.

Other determinations

DNA was extracted from cells and assayed by the method of Burton [46], with DNA from calf thymus as a standard. Protein was determined by the Lowry method [47], with crystalline BSA as standard.

Statistics

Data were evaluated by Student's paired *t* test. Differences were considered significant at $P < 0.05$.

RESULTS

DNA, RNA and protein content

Effects of T_3 and IGF-I on DNA, RNA and protein content of the cell cultures are presented in Table 1. The only effect of T_3 was a moderate increase in the total RNA content. IGF-I, on the contrary, increased the contents of all parameters under study, i.e. DNA (10%), RNA (40%) and protein (25%). The effects of IGF-I and T_3 combined on these parameters were similar to those of IGF-I alone.

Effects of T_3 and IGF-I on SERCA1 mRNA and SERCA1 protein levels

Analysis of SERCA1 mRNA levels was performed on Northern blots. A representative example (Figure 2b) shows that the SERCA1 mRNA level was low in the controls and only slightly higher in IGF-I-treated cultures. Addition of T_3 resulted in a considerable increase in the SERCA1 mRNA content, which was further enhanced when T_3 and IGF-I were added simultaneously. The effects of T_3 and IGF-I on SERCA1 protein and mRNA levels are compared and summarized in Figure 3. The synergistic action of T_3 and IGF-I is evidenced by a 410% increase in the SERCA1 protein level, since, when given separately, the stimulation amounted to 160% for T_3 and 110% for IGF-I. T_3 induced a larger increase in the SERCA1 mRNA level (240%) than in the SERCA1 protein level, whereas for IGF-I the SERCA1 mRNA level was increased much less (50%) than the SERCA1 protein level. In the presence of both compounds

Table 1 Effects of T_3 and IGF-I on DNA, RNA and protein content

L6 myotubes (cultured as described in the Materials and methods section) were subjected to T_3 (5 nM), IGF-I (3.3 nM) or T_3 plus IGF-I (5 nM and 3.3 nM respectively) for 3 days. Controls received no additions. DNA, RNA and protein contents of the different experimental groups were measured as described in the Materials and methods section. Values represent the means ± S.E.M. of 7 experiments: * $P < 0.05$ versus control.

	DNA (μg/flask)	RNA (μg/flask)	Protein (mg/flask)	RNA/DNA (mg/mg)	Protein/DNA (mg/mg)
Control	14.7 ± 0.2	22.0 ± 0.9	0.75 ± 0.03	1.53 ± 0.10	50.3 ± 1.1
T_3	14.2 ± 0.2	24.0 ± 1.1*	0.70 ± 0.02	1.70 ± 0.07*	49.8 ± 1.0
IGF-I	15.3 ± 0.2*	32.3 ± 1.6*	0.94 ± 0.03*	2.12 ± 0.12*	61.1 ± 2.0*
T_3 + IGF-I	15.8 ± 0.2*	30.8 ± 2.2*	0.91 ± 0.04*	1.95 ± 0.15*	57.4 ± 1.8*

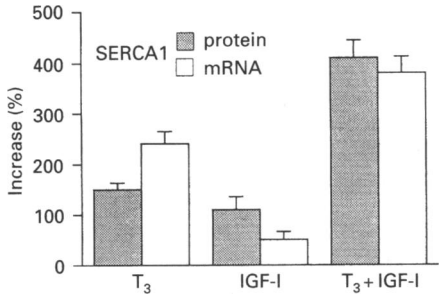

Figure 3 Effects of T_3 and IGF-I on SERCA1 protein and SERCA1 mRNA levels

L6 myotubes (cultured as described in the Materials and methods section) were subjected to T_3 (5 nM), IGF-I (3.3 nM) or T_3 plus IGF-I for 3 days. Controls received no additions. The SERCA1 protein content was determined by e.l.i.s.a.; the SERCA1 mRNA content was determined by Northern-blot analysis, followed by laser-scanning densitometry of the autoradiograms (both as described in the Materials and methods section). SERCA1 protein and mRNA levels are expressed per unit of DNA. Data are expressed as percentage increase versus the control value (no additions) and represent the means of four experiments ± S.E.M. All increases shown were significant ($P < 0.05$).

Table 2 Effects of T_3 and IGF-I on the half-life of SERCA1 protein (A) and total protein (B)

Cells were grown and labelled with [^{35}S]methionine as described in the Materials and methods section. After 24 h, the labelling medium was replaced with DMEM containing T_3 (5 nM), IGF-I (3.3 nM) or T_3 + IGF-I (5 nM and 3.3 nM respectively). Controls received no additions. Cells were collected at the time of replacement of medium and 4, 8, 24 and 48 h thereafter. The radioactivity remaining in SERCA1 protein was determined by immunoprecipitation, and the radioactivity remaining in total protein was analysed by the trichloroacetic acid precipitation method (both as described in the Materials and methods section). Data represent means ± S.E.M. of four experiments: * $P < 0.05$ versus T_3.

	(A) SERCA1 protein half-life (h)	(B) Total protein half-life (h)
Control	17.5 ± 1.7	30.9 ± 5.7
T_3	15.2 ± 2.3	32.3 ± 6.3
IGF-I	26.8 ± 6.1	40.8 ± 7.5
T_3 + IGF-I	29.0 ± 6.6*	43.2 ± 5.3

these effects appear to average out, resulting in a proportional increase in SERCA1 mRNA and protein content (380 and 410 % respectively).

SERCA1 protein half-life studies

The relatively larger increase in the levels of SERCA1 protein compared with SERCA1 mRNA that was induced by IGF-I could imply regulation at a post-translational level, for example by increasing SERCA1 protein stability. The degradation rate of SERCA1 protein was measured by determining the total radioactivity in SERCA1 at different points of time after labelling the cells with [^{35}S]methionine. The results, summarized in Table 2(A), show that the half-life of SERCA1 protein was 1.5–1.9 times longer in the presence of IGF-I than in its absence, although the effect was only significant in the case of IGF-I + T_3. In the presence of T_3 alone the degradation rate of SERCA1 protein was slightly increased (T_3 versus control, $P = 0.052$). Figure 4 shows representative examples of degradation curves of SERCA1 protein in the presence of T_3 and of T_3 + IGF-I, which

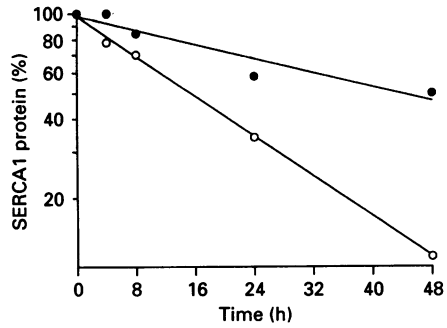

Figure 4 SERCA1 protein degradation in the presence of T_3 (○) or T_3 + IGF-I (●)

Cells were cultured and labelled with [^{35}S]methionine as described in the Materials and methods section. After 24 h ($t = 0$) the labelling medium was replaced with DMEM containing T_3 (5 nM) with or without IGF-I (3.3 nM). The radioactivity remaining in SERCA1 protein was determined by immunoprecipitation. Data points represent means of duplicate determinations and are expressed as percentage of the value at $t = 0$.

Table 3 Effects of T_3, IGF-I and T_3 + IGF-I on the half-life of SERCA1 mRNA (A) and total RNA (B)

Cells were grown as described in the Materials and methods section. At 2 days after addition of T_3 (5 nM), IGF-I (3.3 nM) or T_3 + IGF-I (5 nM and 3.3 nM respectively), the medium was supplemented with actinomycin D (5 μg/ml) ($t = 0$). Total RNA was isolated at $t = 0$ and 3, 6, 10 and 24 h thereafter. The decrease in SERCA1 mRNA was determined by Northern-blot analysis; the decrease in total RNA was determined spectrophotometrically, both as described in the Materials and methods section. Data represent means ± S.E.M. of five (control, T_3) or four (IGF-I, T_3 + IGF-I) experiments. Significance was tested by analysis of paired values: * $P < 0.05$ versus T_3; † $P < 0.05$ versus control.

	(A) SERCA1 mRNA half-life (h)	(B) Total RNA half-life (h)
Control	15.5 ± 2.5	37.0 ± 3.8
T_3	13.3 ± 2.0	41.7 ± 6.9
IGF-I	16.4 ± 4.7	40.7 ± 8.5
T_3 + IGF-I	32.5 ± 7.9*†	49.2 ± 11.4

demonstrate that IGF-I prolonged the half-life of SERCA1 protein considerably. In the presence of IGF-I, a 30 % decrease in the degradation rate of total protein was observed, which was, however, not significant (control versus IGF-I, $P = 0.054$; T_3 versus T_3 + IGF-I, $P = 0.093$). The degradation rate of total protein was not influenced by T_3 (Table 2B).

SERCA1 mRNA half-life studies

To examine the possibility that regulation at a post-transcriptional level contributes to the elevated SERCA1 mRNA levels in the presence of T_3 and IGF-I, we studied the effects of both compounds on mRNA stability. The degradation rate of SERCA1 mRNA was determined by actinomycin D chase experiments. The results of these experiments are summarized in Table 3(A), which shows that there is no evidence that SERCA1 mRNA stability was influenced by T_3 alone, since the half-life of the mRNA in T_3-treated cultures was not significantly different from the SERCA1 mRNA half-life in control cultures. Also, in the presence of IGF-I alone, no significant effect on the SERCA1 mRNA half-life was observed. However, the simultaneous presence of IGF-I and T_3 prolonged the half-life of SERCA1 mRNA 2-fold. Representative SERCA1 mRNA degradation curves in

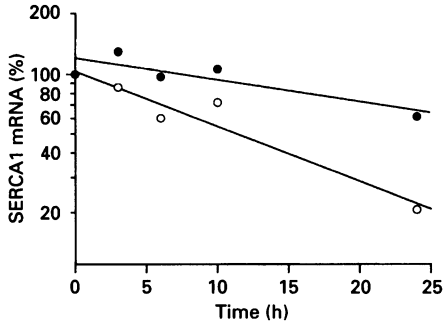

Figure 5 SERCA1 mRNA degradation in the presence of T_3 (○) or T_3+IGF-I (●)

Cells were grown as described in the Materials and methods section. At $t = 0$, actinomycin D (5 μg/ml) was added. Total RNA was isolated at the indicated time points. SERCA1 mRNA half-life was determined by measuring the decrease in SERCA1 mRNA, which was determined by Northern-blot analysis as described in the Materials and methods section. Data points represent means of duplicate determinations and are expressed as percentage of the value at $t = 0$.

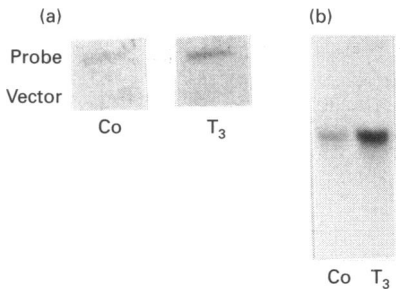

Figure 6 (a) Autoradiograms of a nuclear run-on assay performed with L6 myotubes; (b) Northern-blot analysis

(**a**) Cell culture and run-on analysis were performed as described in the Materials and methods section. Equal amounts (c.p.m.) of the isolated radiolabelled nuclear RNA were hybridized to nylon blots containing a SERCA1 probe (5 μg of M13mp18 ssDNA harbouring a 1400 nt SERCA1 probe [*Bam*HI(+1852)–[poly(A)$^+$ tail]] and an equal amount of vector DNA (native M13mp18 ssDNA) to be able to correct for background hybridization. Hybridization signals of RNA elongated *in vitro* from control cultures (Co) and T_3-treated cultures (T_3) are shown. (**b**) In parallel with the nuclear run-on assay, Northern-blot analysis was performed on material from cultures identical with the cultures described for (**a**), to verify the increase in the SERCA1 mRNA level by T_3. Northern-blot analysis was performed as described in the Materials and methods section.

T_3- and T_3+IGF-I-treated cultures are shown in Figure 5. Similarly to the SERCA1 protein half-life results, it shows that in the presence of T_3+IGF-I the half-life of SERCA1 mRNA was prolonged. No significant differences in total RNA stability were observed between controls and T_3-, IGF-I- or T_3+IGF-I-treated cultures (Table 3B).

Transcription rate of the SERCA1 gene

The mRNA half-life studies showed that the elevation of SERCA1 mRNA levels by T_3 or IGF-I alone was not mediated by an increase in the SERCA1 mRNA stability. These results are strongly in favour of transcriptional regulation of SERCA1 expression by both agents. Since there was only a moderate stimulating effect on SERCA1 mRNA content by IGF-I (50%), in contrast with the much larger effect by T_3 (240%), transcriptional regulation was tested by nuclear run-on analysis only in the latter case. Total RNA polymerase activity in run-on reactions was found to be independent of T_3 treatment of the cells. In two independent experiments α-amanitin-inhibitable RNA polymerase II activity was 65 and 66% of total RNA polymerase activity in nuclei from T_3-treated cells, compared with 67 and 75% in nuclei isolated from control cells. This indicates that there was no general effect of T_3 on mRNA synthesis. Hybridization experiments with RNA elongated *in vitro* revealed that the relative transcription frequency of the SERCA1 gene was increased 3-fold by T_3 (3.1 ± 0.4, mean ± S.E.M. of 3 experiments). Figure 6 shows a representative example of a run-on experiment. The antisense product of the SERCA1 gene was also detectable (in parallel hybridizations with M13mp19 ssDNA containing the 1400 nt SERCA1 probe), but was not stimulated by T_3 (results not shown). We confirmed that excess probe was immobilized on the membrane by rehybridization of the RNA-containing hybridization fluid after 72 h of hybridization with a new blot, which yielded no SERCA1 signal (results not shown), indicating that all SERCA1 mRNA was bound by the filter in the prior hybridization. In addition, when hybridizing different amounts of ^{32}P-labelled RNA of the same sample, the intensity of the signals detected on autoradiograms corresponded to the amounts of ^{32}P-labelled RNA in hybridizations, which also evidenced probe excess.

DISCUSSION

Our previous work with myotubes of the $L6_{AM}$ muscle cell line demonstrated that IGF-I potentiates the T_3-induced increase in net SERCA1 protein synthesis and Ca^{2+}-uptake activity [12]. The present study was initiated to gain insight into the regulation of SERCA1 expression by T_3 and IGF-I.

The T_3-induced increase in SERCA1 mRNA content (240%) in L6 cells is in accordance with the previously observed T_3-induced increase in SERCA1 mRNA in skeletal muscle of the rat [8], and demonstrates at least pre-translational control of SERCA1 expression by T_3. The less than proportional increase of SERCA1 protein, however, suggests some post-translational action of T_3 as well. Such regulation, but now resulting in enhanced expression, is also clearly suggested for the IGF-I-mediated increase in SERCA1 protein content, which was twice as high as that of the SERCA1 mRNA content. These post-translational effects appear to balance each other out when both T_3 and IGF-I are present, resulting in a proportional increase in SERCA1 mRNA and protein.

In a search for the level(s) of SERCA1 regulation by T_3 and IGF-I, half-life studies were performed which showed that the half-life of SERCA1 protein ranged from 15.2 to 29 h in the different experimental groups. This agrees well with the earlier reported half-life of sarcoplasmic-reticulum Ca^{2+}-ATPase in L6 cells (22 h) [39] and in primary rat myoblasts (20 h) [48]. The half-life of the protein *in vitro* is apparently much shorter than the half-life of the protein *in vivo*, which was reported to be 9–11 days [49]. The 1.5–1.9-fold increase in the half-life of SERCA1 protein which was observed in the presence of IGF-I probably contributes to the 2-fold greater increase in SERCA1 protein content relative to its mRNA. This positive regulatory effect of IGF-I on protein stability is in accordance with the previously reported prolongation of total protein half-life by IGF-I in muscle cell cultures [31–33]. In the present study, IGF-I increased the half-life of total protein as well, although this effect did not reach significance. These data indicate, nevertheless, that the effect of IGF-I on SERCA1 protein half-life may be part of a

general effect of IGF-I on protein stability. The mechanism(s) underlying the SERCA1 protein-stabilizing effect of IGF-I are as yet unclear, but a suggestive observation was made in a previous study of ours, in which an equal increase in SERCA1 protein induced by either IGF-I or T_3 resulted in a larger increase in sarcoplasmic-reticulum Ca^{2+}-uptake activity in the case of IGF-I [12]. One might speculate that such increased formation of functional sarcoplasmic-reticulum membrane is involved in the prolongation of SERCA1 protein half-life. As shown in Figure 3, however, this effect of IGF-I is offset by T_3. This is in accordance with the less than proportional increase in SERCA1 protein relative to its mRNA in cultures treated with T_3 alone. This suggests that T_3 increases the degradation rate of SERCA1 protein, which would be in line with studies reporting acceleration of protein degradation by T_3 [18,21]. However, our study does not provide clear evidence that the half-life of the SERCA1 protein is decreased by T_3, since the slight decrease in SERCA1 protein stability by T_3 observed in this study did not reach significance. Another possibility is that T_3 diminishes the translation efficiency of SERCA1 mRNA.

The half-life of SERCA1 protein was similar to the half-life of SERCA1 mRNA (ranging from 13.3 to 32.5 h). Although proteins are usually more stable than the corresponding mRNA, it is not uncommon that mRNA and protein turn over with an equal rate [50]. Our study also presents some evidence that the increased SERCA1 mRNA levels observed in the presence of T_3 and IGF-I were for a significant part due to post-transcriptional regulation, i.e. stabilization of SERCA1 mRNA. The SERCA1 mRNA half-life was twice as long in IGF-I + T_3-treated cultures compared with T_3-treated cultures, which is in reasonable agreement with the 1.6-fold greater increase in the SERCA1 mRNA levels by IGF-I + T_3 compared with T_3. T_3 or IGF-I alone did not affect the SERCA1 mRNA half-life, which indicates that both T_3 and IGF-I are involved in the process leading to the T_3 + IGF-I induced increase in SERCA1 mRNA stability. The mechanism underlying this interaction between T_3 and IGF-I is at present unknown. Regarding the mechanisms operating in selective stabilization of mRNA, a number of possibilities have been proposed (reviewed in [51,52]), such as mRNA modification at the 5-end or the 3-terminus, or protection of mRNAs against RNAases by interaction with specific proteins. For instance, evidence was obtained in a study with L6 muscle cells that the increased creatine kinase mRNA stability that was induced by insulin, here acting as an IGF-I analogue, was mediated by a short-lived protein [30].

In the presence of T_3 alone, a 3.4-fold increase in SERCA1 mRNA content was observed, which could not be attributed to effects on SERCA1 mRNA stability. This pointed to transcriptional regulation, which was confirmed in the nuclear run-on analyses. The 3-fold increase in the transcription initiation frequency of the SERCA1 gene induced by T_3 strongly suggests that enhanced transcription is the primary mechanism leading to elevated SERCA1 mRNA levels in the presence of T_3. These results confirm what a preliminary analysis *in vitro* of the SERCA1 promoter had already suggested. In that study, using transient transfection assays, it was shown that the transcriptional activity of the initial 2400 bp of the SERCA1 promoter is stimulated 3.5-fold by T_3 [53]. Similar results have also been reported for the T_3 regulation of SERCA2a promoter activity in transient transfection assays using cardiomyocytes, although these results were not substantiated by run-on analyses [54]. With respect to IGF-I regulation of SERCA1 expression, our results do not exclude the possibility of transcriptional regulation. However, since the IGF-I induced increase in SERCA1 mRNA content is only small compared with the increase induced by T_3, there is no need to postulate a major role of IGF-I in transcriptional regulation of SERCA1 expression.

In summary: this study suggests that T_3 controls SERCA1 expression primarily by increasing the transcription frequency of the SERCA1 gene, whereas IGF-I seems to act predominantly at post-transcriptional levels by enhancing SERCA1 protein and mRNA stability, the latter, however, only in the presence of T_3.

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