Thyroid states regulate subcellular glucose phosphorylation activity in male mice

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Abstract

The thyroid hormones (THs), triiodothyronine (T3) and thyroxine (T4), are very important in organism metabolism and regulate glucose utilization. Hexokinase (HK) is responsible for the first step of glycolysis, catalyzing the conversion of glucose to glucose 6-phosphate. HK has been found in different cellular compartments, and new functions have been attributed to this enzyme. The effects of hyperthyroidism on subcellular glucose phosphorylation in mouse tissues were examined. Tissues were removed, subcellular fractions were isolated from eu- and hyperthyroid (T3, 0.25 µg/g, i.p. during 21 days) mice and HK activity was assayed. Glucose phosphorylation was increased in the particulate fraction in soleus (312.4% ± 67.1, n = 10), gastrocnemius (369.2% ± 112.4, n = 10) and heart (142.2% ± 13.6, n = 10) muscle in the hyperthyroid group compared to the control group. Hexokinase activity was not affected in brain or liver. No relevant changes were observed in HK activity in the soluble fraction for all tissues investigated. Acute T3 administration (single dose of T3, 1.25 µg/g, i.p.) did not modulate HK activity. Interestingly, HK mRNA levels remained unchanged and HK bound to mitochondria was increased by T3 treatment, suggesting a posttranscriptional mechanism. Analysis of the AKT pathway showed a 2.5-fold increase in AKT and GSK3B phosphorylation in the gastrocnemius muscle in the hyperthyroid group compared to the euthyroid group. Taken together, we show for the first time that THs modulate HK activity specifically in particulate fractions and that this action seems to be under the control of the AKT and GSK3B pathways.

Introduction

Thyroid hormones exert important effects on basal and adaptive metabolism (1). Although several studies have shown that triiodothyronine (T3) may regulate glucose uptake and oxygen consumption (2, 3, 4, 5, 6, 7, 8), the effects of T3 on glucose metabolism remain unclear.

Glycemic control can be achieved through different factors such as hormone action and cell metabolism. Insulin and glucagon are hormones that stimulate glucose uptake and synthesis, respectively. Insulin stimulates glucose uptake in muscle and adipose tissues through glucose transporter (GLUT4) translocation to cell surface. Besides that, other crucial step to glucose uptake is its phosphorylation in a reaction catalyzed by the enzyme hexokinase (HK) (9, 10). The increase in glucose uptake observed in models of hyperthyroidism has been studied by many research groups, with a particular focus on...
the effects of THs on GLUT expression (11, 12, 13, 14). However, little is known about the roles of these hormones on glucose phosphorylation by hexokinase.

There are four important mammalian hexokinase isozymes, namely HK I–IV, which differ with respect to kinetic parameters, subcellular localization and physiological roles. Different levels of HK isoforms are co-expressed. The prevalence of each isofrom varies in a species-specific manner. For instance, in rat skeletal muscle, HK II predominates over HK I, whereas the total quantity of HK I is similar to HK II in human skeletal muscle (15). In addition, HK activities may also differ depending on the organ and subcellular fraction in which they are located (16). An important characteristic of HK I and II is the ability to interact with the outer-mitochondrial membrane specifically through a mitochondria binding motif (10). This mitochondrial interaction occurs through the voltage-dependent anion channel (VDAC), also known as the mitochondrial porin, which is an integral protein of the mitochondrial outer membrane (17, 18). Its main function is to transport anions, cations, adenine nucleotides and other metabolites into and out of the mitochondria (19). The HK-VDAC complex has been described as an important mechanism coupling oxidative phosphorylation to glycolysis as the ATP synthesized by the Fo–F1 ATPase can be used promptly by the enzyme to fix glucose inside the cell (20). Additionally, many other functions have been proposed for this interaction with mitochondria, such as higher cell resistance to chemotherapy (21) and modulation of reactive oxygen species generation (22).

The amount of HK bound to the mitochondria is under the regulation of different factors, such as PKCe activity and glucose 6-phosphate concentration. While the accumulation of the HK reaction product glucose 6-phosphate leads to conformational changes and the consequent detachment of HK from mitochondrial VDAC, PKCe promotes HK binding through phosphorylation of this channel (23, 24, 25, 26, 27). In the last decade, many other mediators have been directly or indirectly identified as regulators of hexokinase binding to the mitochondria, including phosphorylation by some protein kinases. Curiously, some of these pathways are also related to increase in glucose uptake. Gottlob (28) and Majewski (29) found that AKT, also known as protein kinase B, increases the basal levels of HK units bound to mitochondria. A few years later, it was demonstrated that this interaction was a consequence of direct phosphorylation of HK by AKT (30). However, glycogen synthase kinase 3β (GSK3B), a downstream target of AKT, can negatively affect the mitochondria-HK interaction by phosphorylating VDAC. Thus, although part of the same pathway, AKT and GSK3B have opposing effects on the regulation of HK binding to mitochondria.

Because thyroid hormones modulate glucose metabolism and HK compartmentalization is a dynamic process, our hypothesis is that THs alter the distribution of HK activity in mouse tissues. Using a hyperthyroidism mouse model, which was compared to a euthyroid group, we provide novel evidence that T3 increases mitochondria-bound hexokinase activity and that this phenomenon seems to occur as result of the AKT–GSK3B pathway activation.

Materials and methods

Reagents and materials

Unless otherwise specified, all reagents were purchased from Sigma Chemical, Vetec Química Fina Ltda. (Duque de Caxias, RJ, Brazil), Invitrogen Corporation, Applied Biosystems, Millipore and Fermentas/Thermo Fisher Scientific.

Hyperthyroidism induction

Male BALB/c mice (3 weeks old) were treated with vehicle or T3 to induce eu- and hyperthyroidism, respectively. T3 was first solubilized in 0.04 mol/L NaOH, diluted in PBS and then administered i.p. daily (supraphysiological dose: 0.25 µg/g body mass) for 21 days. The same volume of PBS plus 0.04 mol/L NaOH was injected into the euthyroid group as vehicle. Animals were killed after 24 h of last T3 administration. Acute treatment was achieved by a single i.p. injection of T3 (1.25 µg/g body mass), and animals were killed after 2, 4, 8 or 24 h of T3 administration. Animal handling and killed were approved by the Institutional Animal Care and Use Committee (Comissão de Ética no Uso de Animais do Centro de Ciências da Saúde, CEUA/CCS) of the Federal University of Rio de Janeiro (protocol number IBQ40).
sucrose, 1 mmol/L EDTA, 1 mmol/L EGTA and 10 mmol/L Tris–HCl, pH 7.4. The total homogenate was centrifuged at 2500g for 10 min. The supernatant was collected and then centrifuged at 20,000g for 40 min. The resulting supernatant was saved (soluble fraction), and the pellet (particulate fraction) was resuspended in the same buffer. All steps were performed at 4°C. Both of the fractions were stored at −80°C. Protein concentrations were determined by the Folin-Lowry method using bovine serum albumin as a standard (31).

**Hormone measurements**

Serum total T<sub>4</sub>, total T<sub>3</sub> and free T<sub>3</sub> were measured using, respectively, a total thyroxine (total T<sub>4</sub>) antibody coated tube (125I RIA kit); a total triiodothyronine (total T<sub>3</sub>) antibody coated tube (125I RIA kit); and a free triiodothyronine (free T<sub>3</sub>) antibody coated tube (125I RIA kit) (MP Biomedicals, Santa Ana, CA, USA), following the manufacturer’s instructions.

**Cell culture**

The C2C12 myoblast cell line was obtained from BCRJ (Banco de Células do Rio de Janeiro) and certified to be free from mycoplasma contamination. The cells were grown in DMEM supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 mmol/L sodium selenite and 2 mmol/L glutamine and maintained at 37°C in a 5% CO<sub>2</sub> atmosphere. To induce differentiation into myotubes, the cells were seeded in petri dishes with DMEM plus 10% FBS until 90% confluence was achieved. Afterward, the culture medium was changed to DMEM supplemented with 2% horse serum for 7 days, and the medium was changed on days 2, 4 and 7 of differentiation. At the end of differentiation, the culture medium was changed to DMEM plus 0.1% BSA for 24 h, and the cells were subsequently treated with vehicle or 1.85 nmol/L T<sub>3</sub> for an additional 24 h.

**Cell culture subcellular fractionation**

At the end of treatment, cells were washed with PBS and trypsinized. To stop trypsin action, cells were resuspended in DMEM plus 0.1% BSA with vehicle or T<sub>3</sub>, collected in conical tubes and centrifuged at 200g for 5 min. The cell pellet was washed with PBS and centrifuged at 200g for 5 min. The new pellet was resuspended in extraction buffer containing 10 mmol/L Tris–HCl, pH 7.4, 0.25 mol/L sucrose, 1 mmol/L EDTA, 1 mmol/L sodium vanadate, 1 mmol/L NaF, 0.5 mmol/L PMSF, 1 mmol/L EGTA and 1 mmol/L β-mercaptoethanol, homogenized in a glass potter and centrifuged at 100,000g for 15 min. The supernatant (soluble fraction) was collected, and the pellet (particulate fraction) was resuspended in extraction buffer. All steps were performed at 4°C. Both fractions were stored at −80°C. The protein concentration was determined using the bicinchoninic acid method (Thermo Scientific Pierce BCA Protein Assay Kit), using bovine serum albumin as a standard.

**Hexokinase activity**

HK and glucokinase (GK) specific activities were determined by NADH formation, following the absorbance at 340 nm, using a coupled assay. Tissue extract HK assay medium contained 50 mmol/L Tris–HCl, pH 7.4, 6 mmol/L MgCl<sub>2</sub>, 0.5 mmol/L β-NAD<sup>+</sup>, 0.5 U/mL Glucose 6-Phosphate Dehydrogenase (G6PDH) from *Leuconostoc mesenteroides*, 2 mmol/L ATP, 0.1% Triton (v/v) and 5 mmol/L glucose. GK activity was determined by the difference between the activities measured in the media containing 100 mmol/L glucose or 0.5 mmol/L glucose. Besides this difference in glucose concentrations, both media contained 50 mmol/L Tris–HCl, pH 7.4, 6 mmol/L MgCl<sub>2</sub>, 0.5 mmol/L β-NAD<sup>+</sup>, 0.5 U/mL G6PDH, 2 mmol/L ATP, 0.1% Triton (v/v) and 5 mmol/L dithiothreitol. The cell extract HK assay medium contained 50 mmol/L Tris–HCl, pH 7.4, 7.7 mmol/L MgCl<sub>2</sub>, 0.5 mmol/L β-NAD<sup>+</sup>, 6.7 mmol/L ATP, 0.05% Triton, 4.2 mmol/L glucose, 0.1 mmol/L rotenone, 45 mmol/L KCl, 0.5 mmol/L EDTA, 5 mmol/L NaN<sub>3</sub> and 1 unit/mL G6PDH. All enzymatic assays were performed at 35°C, and the final protein concentration was 0.05 mg/mL (tissue extract) or 0.02 mg/mL (cell extract).

The effects of T<sub>3</sub> on enzyme activity were reproducible in all of the experiments. To better compare and decrease inter-experimental variability, enzyme activities were normalized to the euthyroid experimental group (considered 100% in each experiment).

**RNA isolation and quantification**

Liver and gastrocnemius muscle RNA were isolated from eu- and hyperthyroid animals using TRIzol reagent (Invitrogen) according to the manufacturer’s instructions. cDNA synthesis and quantitative real-time PCR were performed using a High-Capacity...
cDNA Reverse Transcription Kit protocol (Applied Biosystems) and a Power SYBR Green Master Mix (Thermo Fischer Scientific), according to manufacturer's instructions. The following primers were used: hexokinase (Forward 3′- CAACGCTGGACTGCTCTTCC 5′; Reverse 3′- TGGTGCAAGATGGTCCGGAC 5′), Dio1 (Forward 3′-CCACCTCTCCAGCATCC-5′; Reverse 3′-AGTCTCATCGAGTTCCTTG-5′) and 36β (Forward 3′-GTGTGCACACGCGACATTT-5′; Reverse 3′-CCGAGGCAACAGTTGGTA 5′). The 36β gene was used as a housekeeping gene.

Western blot analysis

Immunoblot analyses were performed with the antibodies listed in Supplementary Table 1 (see section on Supplementary data given at the end of this article). The proteins from the soluble fraction were separated in 10% polyacrylamide gels and then transferred to PVDF membranes. The membranes were blocked using 3% BSA (w/v) or 3% low fat milk (w/v) in Tris-buffered saline with 0.1% Tween 20 (v/v) (TBS-T) for 1 h. Then, they were washed with TBS-T and probed overnight with the following primary antibodies. After primary antibody incubation, the membranes were incubated with secondary antibodies conjugated to horseradish peroxidase for 1 h. The membranes were developed using Luminata Forte Western HRP Substrate (Millipore), and images were quantified using NIH ImageJ software (32).

Statistical analysis

All data were analyzed using PRISM software (GraphPad Software) and are expressed as mean ± S.E.M. One-way ANOVA was used to compare more than two groups, followed by Student–Newman–Keuls test to detect differences between groups. Student’s t-test was used to compare the differences between two groups. P < 0.05 was used to reject the null hypothesis.

Results

Thyroid states in mice

To examine the effects of T₃ on glucose phosphorylation in different subcellular fractions, we induced hyperthyroidism in mice by injecting T₃ (0.25 µg/g) over 3 weeks. There is no consensus about T₃ doses to induce hyperthyroidism in mouse model. In the literature, we found a broad range of doses and decided to use a dose that is considered as supraphysiological and frequently used (33, 34). To confirm whether this pathological condition was achieved, we measured physiological parameters of hyperthyroidism, such as total T₃ and T₄ levels (Fig. 1). As expected, total T₃ levels were 10 times higher in hyperthyroid animals (Fig. 1A), while total T₄ levels were decreased by T₃ treatment (Fig. 1B), in agreement with the negative feedback exerted by thyroid hormones at the pituitary and hypothalamic levels (35). Hyperthyroid mice showed increased heart weight to body weight ratio (Fig. 1C), suggestive of heart hypertrophy, which is commonly observed in hyperthyroidism. Moreover, Dio1 mRNA expression, a marker of thyroid hormone action, was 50-fold higher in the livers of the hyperthyroid groups (Fig. 1D). It is important to note that there was no difference in housekeeping gene expression (Fig. 1D, inset). Collectively, these results confirm the induction of a hyperthyroid state in our experimental model and tissue responsiveness to increased serum T₃ levels.

Figure 1

Assessment of hormonal status and markers of hyperthyroidism in mice. Hyperthyroidism was induced by treating animals with T₃, as described in the ‘Materials and methods’ section. (A) Total T₃ and (B) total T₄ levels were measured using RIA. (C) HW/BW (heart weight/body weight) was obtained by dividing heart weight (mg) by body weight (g). (D) Liver D1 mRNA expression was assayed by real-time PCR and normalized to the housekeeping gene 36β. (Inset) Ct values from housekeeping gene 36β. Values are means ± S.E.M. of 3–7 animals per experimental group. Open bars represent control, and black bars, T₃-administered groups. *P < 0.05, **P < 0.0001 compared to the euthyroid group (Eu).
Thyroid hormones increase hexokinase activity in particulate but not in cytosolic fractions in mouse muscles

As little is known about the effects of THs on HK compartmentalization in muscle tissue, we evaluated whether T₃ treatment could affect the distribution of HK activity in cell compartments in different tissues. Accordingly, once our hyperthyroidism model was established, glucose phosphorylation by HK was investigated in two enriched subcellular fractions, namely, the soluble fraction, corresponding to cytosolic proteins, and the particulate fraction, which is enriched in mitochondria (15, 36). We observed an increase in HK activity in the particulate fraction from the heart and soleus and gastrocnemius muscles (Fig. 2A, C and E). The magnitude of the effect was variable and tissue specific. The fold change observed in these tissues varied between 1.5 (heart) and 3–4 (soleus and gastrocnemius muscles) compared to the euthyroid group. In the soluble fraction, no alterations were observed, except for the gastrocnemius muscle, which also presented a slightly higher HK activity (Fig. 2B, D and F). We could also confirm incremental hexokinase activity in the particulate fraction of the heart, soleus and gastrocnemius using a lower dose of T₃ (0.1 µg/g body weight) to induce hyperthyroidism in mouse (Supplementary Fig. 1). We also investigated the effect of T₃ treatment in vitro using C2C12 myoblast cell line differentiated into myotubes during 7 days. After 24 h of T₃ treatment, we did not observe any difference in particulate HK activity (Fig. 3A) and only a slight decrease in HK soluble activity (Fig. 3B) was observed.

Glucose phosphorylation was also measured in the brain and liver, where T₃ administration showed no effect on HK and GK activity (Table 1). Despite the trend observed in the liver-soluble fraction from the hyperthyroid group, this difference did not reach statistical significance. These results suggest that the T₃ effects may be tissue specific and act preferentially in the muscle tissues tested. A possibility could be that T₃ was acting directly by interacting and regulating the enzyme activity. However, this hypothesis was discarded once we did not observe an effect on HK activity when tissue extracts from control mice were incubated with different T₃ concentration (Supplementary Fig. 2).

To identify whether the increase in HK activity was related to the acute action of thyroid hormone, we also evaluated glucose phosphorylation from 2 to 24 h after the administration of a single dose of T₃ (1.25 µg/g). We found no differences in hexokinase activity in the particulate fraction at any time point investigated (Fig. 4A), whereas a slight increase in HK activity in the soluble fraction was observed 2 h after T₃ injection (Fig. 4B). This effect, however, was lost over exposure time. Therefore, it seems that only chronic T₃ treatment is able to increase HK activity in the particulate fraction.

T₃ treatment does not modulate Hk2 mRNA, but increases HK2 protein

It is well known that thyroid hormones exert their functions mainly through transcriptional regulation of target genes (37). As HK subcellular activity was modified...
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Thyroid hormones do not affect subcellular glucose phosphorylation in mouse brain and liver.

Table 1

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Subcellular fraction</th>
<th>Thyroid state</th>
<th>Euthyroid</th>
<th>Hyperthyroid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brain</td>
<td>Soluble</td>
<td>100.0 ± 3.9 (10)</td>
<td>95.9 ± 3.4 (10)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Particulate</td>
<td>100.0 ± 2.3 (10)</td>
<td>99.9 ± 2.6 (10)</td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td>Soluble</td>
<td>100.0 ± 21.0 (10)</td>
<td>49.8 ± 12.6 (10)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Particulate</td>
<td>N.D.</td>
<td>N.D.</td>
<td></td>
</tr>
</tbody>
</table>

Reactions were started by adding protein preparations at a final concentration of 0.05 mg/mL. Values are expressed as percentages of activity in the euthyroid group and correspond to means ± S.E.M. Specific activities (μmol G6P mg⁻¹ min⁻¹) in the euthyroid group were 0.114 ± 0.065 (soluble) and 0.227 ± 0.034 (particulate) in brain and 0.013 ± 0.001 (soluble) in liver. The numbers shown in parenthesis represent the numbers of animals per group. N.D.: not detected.

Acute T₃ treatment on subcellular HK activity in gastrocnemius muscle.

Figure 4

Mice were treated with a single T₃ dose (1.25 µg/g) and killed at the indicated time points after T₃ injection. HK activity was assessed as described in the 'Materials and methods' section in the particulate (A) and soluble (B) fractions. Values are means ± S.E.M. of 4 animals for each time point and group. Open bars represent control, and black bars, T₃-administered groups. *P < 0.05 compared to the euthyroid group.

HK2 mRNA and protein levels in mouse gastrocnemius muscle.

Figure 5

HK2 mRNA expression. T₃-induced hyperthyroidism did not modify HK2 mRNA levels (Fig. 5A), but induced an increase in mitochondrial-bound hexokinase (Fig. 5B). These results suggest that T₃ may regulate HK2 activity and subcellular localization involving a posttranscriptional mechanism.

Figure 3

Effect of TH on HK activity from C2C12 myotubes. C2C12 cells were differentiated as described in the 'Materials and methods' section for 7 days. The cells were treated with vehicle (open bars) or 1.85 nM T₃ (black bars). HK activity was assessed as described in the 'Materials and methods' section in the soluble (A) and particulate (B) fractions. Values are means ± S.E.M. of 3 independent experiments. *P < 0.05 compared to the vehicle (control).

only after chronic treatment, we sought to investigate whether the effects observed on HK activity in the particulate fraction could be explained by an increase in Hk2 mRNA expression. T₃-induced hyperthyroidism did not modify HK2 mRNA levels (Fig. 5A), but induced an increase in mitochondrial-bound hexokinase (Fig. 5B). These results suggest that mitochondrial-bound hexokinase activity in the euthyroid group and correspond to means ± S.E.M. of 3 independent experiments. *P < 0.05 compared to the vehicle (control).

T₃ increases AKT and GSK3B phosphorylation in gastrocnemius muscle

Previous studies have shown that HK isoforms may be post-translationally regulated by different mechanisms, such as SUMOylation and phosphorylation (28, 38). One of these mechanisms involves the protein AKT, whose activation is correlated with higher mitochondrial-bound HK2 activity (29, 39).

As we observed similar effects on HK activity in different muscle types, we focused our subsequent experiments on the gastrocnemius, which has a higher mass and presented higher fold change increases after T₃ treatment. As it has been shown that TH induces AKT phosphorylation in gastrocnemius (40) and cardiac muscles of rat (41), we decided to examine whether AKT could similarly be regulated by T₃ in mouse gastrocnemius muscle, which would explain the observed increase in glucose phosphorylation in the particulate fraction.

In the gastrocnemius muscle, there was a substantial increase in both total and phosphorylated AKT protein levels (Fig. 6A and B), confirming our assumption that this pathway is activated by T₃-induced hyperthyroidism.
Miyamoto and coworkers (30) showed that AKT activation by phosphorylation regulates the amount of hexokinase bound to mitochondria. This occurs through direct phosphorylation of HK2, promoting its translocation to the mitochondria (30). Additionally, as previously mentioned, the interaction of HK to mitochondria can be affected by phosphorylation of VDAC in a process mediated by GSK3B. Both kinase pathways can converge once AKT phosphorylates and inhibits GSK3B. In this way, when GSK3B is inhibited (phosphorylated form), less VDAC is phosphorylated, and more HK can bind to mitochondria (42, 43). We observed that both total and phosphorylated GSK3B levels were increased in hyperthyroid animals (Fig. 6C and D). As GSK3B phosphorylation may also be mediated by ERK (44), we measured ERK levels in the euthyroid and hyperthyroid groups (data not shown). These results suggest that, in our model, the AKT–GSK3B pathway is regulated by T₃ in the gastrocnemius muscle and is responsible to the increase in particulate HK activity observed.

Discussion

The maintenance of glucose homeostasis has been considered for many years as result of glucagon and insulin actions. More recently, several other hormones have been shown to directly or indirectly be associated with glucose utilization and uptake (45, 46). A considerable consensus exists in the literature regarding the involvement of thyroid hormones as regulators of glycemia, but the mechanisms underlying these effects are still unclear. Therefore, countless efforts have been devoted to understand these mechanisms. For this reason, we decided to investigate thyroid hormones actions on glucose phosphorylation by hexokinase in mouse tissues. To address this point, we used mouse models of eu- and hyperthyroidism.

Our results indicate that T₃ can be a potent stimulus to induce changes in subcellular hexokinase activity in mouse muscles. Here we observed an increased HK activity in the particulate fraction from mouse cardiac, soleus and gastrocnemius muscles (Fig. 2 and Supplementary Fig. 1) after T₃-induced hyperthyroidism.

Curiously, the effect observed seems to be very specific for muscle tissues, as we did not observe any change in glucose phosphorylation activity in liver and brain. The absence of effect in the liver is probably related to the fact that GK, the predominant isoform, does not have the motif responsible for mitochondria interaction. On the other hand, HK1, that has the ability to interact to mitochondria, also did not have its activity regulated in brain. In this case, our hypothesis is that AKT–GSK3B pathway may be not modulated in this tissue, but additional experiments should be performed to address this point.

Our next step was to check whether we could reproduce our animal findings in vitro using a myoblast cell line. C2C12 cells were differentiated for 7 days and then exposed to T₃ (1.85 nmol/L) for 24 h. However, T₃-treated differentiated C2C12 myotubes did not show changes in particulate HK activity (Fig. 3A). Although a possible interpretation would be the absence of thyroid hormone receptors expression, this seems not to be the case as it has been shown that C2C12 myotubes are responsive to T₃ and express TRα1, TRα2 and TRβ1 (47, 48). Other possibility is that T₃ effects on HK activity are mediated by a systemic effect. For example, T₃ affects the secretion of some factor that could act on muscles and alter HK activity. As we are studying glucose metabolism, insulin might be a candidate. However, it has been described that insulin has no effect on subcellular HK activity (49). Other possibility is leptin, a hormone secreted by adipocytes.
Although the effect of THs on leptin levels is controversial, there are evidence showing that T₃ treatment increases leptin expression and secretion in adipocytes (50, 51, 52). In addition, it has been observed that high and low leptin levels are able to, respectively, stimulate glucose uptake (53) and decrease HK activity (54) in muscle. Another possibility is that prolonged T₃ treatment in cells is needed to achieve results similar to those observed in animal models. Of note, studies in which T₃ acute effect is not the aim usually perform treatments for no longer than 24 h (55, 56).

The absence of effect in cell line limited our investigation to comprehend T₃ actions. For example, we could not address the participation of translational mechanism by using inhibitors such as cycloheximide. Next, we investigated the direct effect of T₃ by testing it during the measurement of enzyme activity using the particulate fraction from the gastrocnemius. Currently, the molecular nature of the posttranslational effects of THs on certain enzymes remains unclear. It has been shown that short-term exposure of L6 myoblasts to either T₃ or T₄ activates the Na⁺/H⁺ antiporter (57). In addition, thyroid hormone analogs stimulated the Ca²⁺-ATPase in the sarcoplasmic reticulum-enriched membranes of rabbit skeletal muscle (58). However, we did not find such activation of HK at the range of concentrations and time of exposure used in this study (Supplementary Fig. 2).

We also checked whether T₃ could increase the transcription of the Hk gene, but no changes were observed using the gastrocnemius muscle (Fig. 5A). These results suggest that T₃ does not interact directly with HK nor is able to regulate its gene expression, but this does not exclude a genomic action in other pathway targets.

The subcellular localization of hexokinase has been described as a dynamic process that can be regulated by kinase activity. Using an AKT activator, Miyamoto and coworkers showed that cardiomyocytes treated with an AKT activator had higher levels of phosphorylated hexokinase associated with mitochondria. This association protected mitochondria against a loss of membrane potential (30). Moreover, it has been shown that the hyperthyroid rats present increased levels of phosphorylated AKT in the heart (59). Thus, an excessive amount of TH would promote AKT activation, which, in turn, increases phosphorylation and the amount of HK bound to mitochondria, a fact that may explain our results. Our data are in line with these observations, as we observed a dramatic increase of total AKT, phosphorylated AKT and mitochondrial-bound HK2 in gastrocnemius from hyperthyroid experimental group (Fig. 4B, 5A and 5B).

Glycogen synthase kinase 3β (GSK3β) is a protein kinase involved in several biological processes, such as glucose metabolism, regulation of cell differentiation and cell survival. Its regulation can be achieved by multiple pathways, such as PKA, AKT and ERK (via p90RSK, a downstream target), which inhibit GSK3β by phosphorylation. Once activated, Akt phosphorylates HK, increasing its association with mitochondria. In parallel to this effect, Akt also phosphorylates and inactivates GSK3β. This inactivation may decrease VDAC phosphorylation, allowing more HK binding to mitochondria. Taken together, these effects result in increased mitochondrial hexokinase activity. It seems that the ERK pathway does not have a relevant effect on HK activity.
As it has been shown that TH treatment increases Akt and ERK phosphorylation in several cell models (59, 62, 63, 64), we expected to observe in our hyperthyroidism model a situation where more GSK3B is phosphorylated and, therefore, inhibited. Thus, phosphorylated VDAC levels would probably be lower, favoring a greater HK association with mitochondria. In fact, we found elevated levels of both total and phosphorylated GSK3B (Fig. 6C and D), whereas no differences were observed in total and phosphorylated ERK (data not shown), suggesting that ERK1/2 are not involved in this process. However, these results do not exclude the possibility of a genomic action of T₃ as Akt can be activated by a transcriptional pathway (Fig. 7).

With this in mind, we are able to highlight some possibilities regarding the physiological functions related to increased HK activity in hyperthyroid animals. Excessive TH signaling is known to induce cell apoptosis in several cell lines through the activation of different mechanisms (65, 66, 67, 68). On the other hand, several studies have demonstrated that the presence of HK bound to mitochondria prevents apoptosis (26, 28, 69). Thus, increased HK activity in the mitochondrial fraction of hyperthyroid mice may be an attempt to minimize cell death.

Common characteristics of the hyperthyroid state are increased oxygen consumption and reactive oxygen species (ROS) production (70) that can result in cellular damage and may even cause cell death. Thus, it is important to the cell to develop mechanisms to regulate excessive ROS production and their effects. In line with this, it has been demonstrated that mitochondrially bound HK can decrease ROS productions in neurons (22). Perhaps, the increased mitochondrial enzyme activity in hyperthyroidism is an effort to reduce ROS production in hyperthyroidism.

It has also been shown that thyroid hormones are able to increase glucose uptake in several tissues (8). Frequently, increases in glucose uptake are related to an increase in the exposure of the glucose transporter (GLUT4) in the cell membrane, as in the EDL and soleus muscle of rats (14). However, mitochondrial-bound HK is able to modulate glucose uptake without changing GLUT at the plasma membrane (71). Both steps, uptake and phosphorylation, are crucial to determine if glucose will be oxidized by the tissue or not. Here, we did not exclude the participation of GLUT, but we pointed out that, in hyperthyroidism, mitochondrial-bound HK could be relevant to glucose uptake process.

In addition, hyperthyroidism has been associated to muscle impairment function, such as weakness, atrophy and wasting (72, 73, 74). However, most of these studies did not relate the symptoms to glucose metabolism. The HK association to mitochondria has been reported as a manner to couple oxidative phosphorylation to glycolysis and supply the high demand of ATP in some physiological, but also pathological, conditions (41, 75). We could speculate that to solve the damages observed in thyrotoxic patients some adaptations are necessary. The well functioning of ATPases, ions gradient and muscle contraction needs to be maintained by higher oxidative phosphorylation. In this way, mitochondria need to oxidize more substrates that could be provided, among others, by glycolysis. Our data suggest that the increment of HK activity in mitochondria could be an adaptive mechanism to keep cell metabolism and function. However, further studies need to be performed.

Taken together, our results show an increase in mitochondrial-bound hexokinase activity in different muscles from hyperthyroid mice, which could be implicated in many physiological processes. Moreover, these changes seem to be a result of Akt–GSK3B pathway modulation.

Supplementary data
This is linked to the online version of the paper at http://dx.doi.org/10.1530/EC-17-0059.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement
F L M P, R S S and W S S conceived and designed the experiments, analyzed the data and wrote the paper. F L M P and R S S performed the experiments.

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References


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69 Pastorino JG & Hoek JB. Regulation of hexokinase binding to VDAC. *Journal of Bioenergetics and Biomembranes* 2008 40 171–182. (doi:10.1007/s10863-008-9148-8)


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