The Effect of TSH-Suppressive Dose of Levothyroxine On Skeletal Muscle In Ovariectomized Rats

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Abstract

Hyperthyroidism is often observed in postmenopausal women due to conditions such as thyroiditis and toxic nodular goiter, Grave's disease or thyroid stimulating hormone suppressive therapy for treating differentiated thyroid carcinoma (DTC). However, the effect of such hormonal changes on skeletal muscles in females remain unclear. Therefore, this study aimed to observe the effects of hyperthyroidism on the skeletal muscle of ovariectomized rats. We randomly divided female Sprague-Dawley rats into sham-operated (Sham), ovariectomized (OVX), and levothyroxine-treated ovariectomized groups (OVX+LT4). Levothyroxine was administered intraperitoneally at 0.3 mg/kg, daily for six weeks. Protein synthesis was increased after ovariectomy whereas protein synthesis was suppressed and protein degradation was increased in response to levothyroxine treatment. However, there was no difference in lean mass between the two groups. Collagen I levels were similar between the Sham and OVX groups, but were significantly decreased in the OVX+LT4 group. The mRNA levels of matrix metalloproteinase (MMP) -2 and -9 were similar between the Sham and OVX groups but were upregulated in the OVX+LT4 group. After ovariectomy, mitochondrial biogenesis and dynamics were changed; these changes were exacerbated in hyperthyroidism. Our findings indicate that in postmenopausal rats with hyperthyroidism, the progression of muscle weakness occurs through impaired regulation of signaling pathways related to extracellular matrix homeostasis, protein turnover, and mitochondrial quality.

Introduction

Thyroid hormone signals have metabolic functions in regulating skeletal muscle growth, contractile function, and muscle regeneration ¹. Skeletal muscles comprise a combination of fiber types, classified according to the distribution of the myosin heavy chain, and can be affected by thyroid hormone ². Moreover, thyroid hormone stimulation induces autophagy, which is essential for mitochondrial biogenesis and activity in skeletal muscles ³. However, excessive thyroid hormone affects muscle regeneration negatively by reducing proliferation ⁴.

Administration of intentional thyrotropin suppressive therapy to prevent recurrence of differentiated thyroid carcinoma (DTC) is one of the most common causes of subclinical hyperthyroidism ^{5,6}. Hyperthyroidism is prevalent among women ⁷. Postmenopausal women often have hyperthyroidism due to conditions such as Grave's disease or TSH suppressive therapy for differentiated thyroid cancer (DTC). In a previous meta-analysis, subclinical hyperthyroidism has been associated with an increased risk of fractures ⁸. TSH suppressive levothyroxine (LT4) administration has been reported to adversely affect several target organs, particularly the bones and myocardium.⁵ LT4 treatment in thyroidectomized subjects due to DTC is also reported to regulate numerous genes in skeletal muscles ⁹. Yet, studies on the histological and molecular biological changes of TSH-suppressive administration of LT4 in skeletal muscle are limited.

Skeletal muscle weakness is the reduction of muscle mass, strength, and function, leading to decreased performance. Some clinical studies have examined the effects of thyroid hormone on skeletal muscles; however, the results of these studies are controversial. For instance, patients with hyperthyroidism were reported to complain of muscle weakness¹⁰, and showed reduced muscle mass and muscle strength¹¹. Treatment of hyperthyroidism was found to increase muscle area¹². Conversely, long-term, minimally thyrotropin-suppressive doses of LT4 did not have major salutary or adverse effects on energy expenditure or body composition¹³; further, LT4 therapy did not impair the grip strength of patients compared to the healthy controls¹⁴.

Women have lower peak muscle mass and weaker muscle strength compared to men over the course of life^{15,16}. Estrogen plays an important role in comprehensively maintaining the number of satellite cells and muscle function, and its insufficiency affects muscle strength and regeneration^{17–19}. Menopause induces a remarkable reduction in bone function as well as a decline in muscle mass²⁰. Similar to a high fat diet, estrogen deficiency due to ovariectomy can lead to skeletal muscle dysfunction due to increased oxidative stress in muscles and mitochondrial dysfunction²¹. Some previous preclinical studies have investigated the effects of thyroid hormone on skeletal muscles^{3,22}, but studies investigating hormonal changes in a female model remain insufficient. Therefore, the present study was performed to investigate changes in the histology and molecular mechanisms of skeletal muscles in levothyroxine-induced hyperthyroidism in ovariectomy animal model.

Materials And Methods

Animals

Twenty-four 9-week old female Sprague-Dawley rats were used in this study (Koatech, Gyeonggi, Korea). Each group was weight-matched at the beginning of the study. After a week of acclimatization, the rats were randomly classified into three groups as follows: Group I (n = 8) with sham-operated rats as the Sham group, group II (n = 8) with surgically ovariectomized rats as the OVX group, and group III (n = 8) including surgically ovariectomized rats treated with levothyroxine as the OVX+LT4 group. The rats were euthanized via CO₂ inhalation at six weeks after ovariectomy. All animals were maintained in a controlled condition on a 12 h/12 h light/dark cycle and were provided rat chow with water *ad libitum*. The animal care and research protocols were based on the principles and guidelines of the Guide for the Care and Use of Laboratory Animals (National Institutes of Health publication) and all experiment procedures were conducted in accordance with the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines. This study was approved by the institutional Animal Care and Ethics Committee of Pusan National University Hospital (IACUC Approval No. PNUH-2017-117).

Establishment of the ovariectomized rat model

The rats were anesthetized by isoflurane inhalation (3% dissolved in oxygen) (Matrix VIP 3000™, Midmark Co., USA). Ovariectomy was performed as follows. An 1cm incision was made at the midline of the

abdomen to expose both ovaries. In the OVX group, the ovaries were ligated, cut off bilaterally. Then, the abdominal cavity was closed. Sham operations (for the Sham group) were performed by exposing the ovaries without excision of the ovaries. After ovariectomy, the rats in the OVX+LT4 group were intraperitoneally injected with LT4 at 0.3 mg/kg, daily for 6 weeks.

Body composition

Body composition was measured using dual-energy X-ray absorptiometry (DXA). The animals were scanned using an animal DXA system (InSight, Osteosys R&D Center, Seoul, Korea). The rats were anesthetized using an isoflurane inhalation vaporizer (Matrix VIP 3000™, Midmark Co., USA), ventrally positioned, and then scanned. The device was subjected to daily quality assurance and was calibrated at each study performance. For the whole-body composition analysis, the software provided body weight (g), fat proportion (%) and lean mass (g).

Histology and morphometric analysis

The soleus muscle tissue was isolated from each rat and prepared for fixation overnight in 10% neutral buffered formalin. We used an automatic tissue processor for paraffin embedding (Leica, TP1020, Semi-enclosed benchtop tissue processor) and dispensing (Leica EG1150H, Heated paraffin embedding module). The cross-sections were placed on glass slides, and the sections were subjected to H&E staining and Sirius red staining (Abcam, Cambridge, MA, USA, #ab150681). For staining analyses, the slides were de-paraffinized with xylene, and then hydrated through a series of sequential washes in 100% ethanol, 85% ethanol, 75% ethanol, 50% ethanol, and finally water. We selected an appropriate middle part of the muscle tissue as a representative figures using images taken at 10X using a light microscope (Leica DM4000/600M, Versatile upright microscope for materials analysis). For quantitative analyses, each slide was evaluated at 6 randomly selected non-overlapping areas and the positively stained areas were measured by image analysis. The results were denoted as the ratio of the stained area to the total area.

Electron microscopy

The skeletal muscle tissue was pre-fixed with 2.5% glutaraldehyde phosphate buffer solution (0.1M, pH 7.4) at 4°C and post-fixed with 1% osmium tetroxide in the same buffer. The fixated samples were dehydrated with a series of graded ethyl alcohol solutions, substituted in propylene oxide and embedded in epoxy resin mixture (Epon 812). Thick sections (1 µm) were stained with 1% toluidine blue and examined under a light microscope to select the observed areas. Then, thin sections (50–60 nm) were prepared using an ultramicrotome (EM UC7, Leica) and stained with uranyl acetate and lead citrate on copper mesh grids. These prepared sections were examined using a transmission electron microscope (JEM-1200EXII, JEOL, Tokyo, Japan).

Quantitative PCR

Total RNA from the soleus muscle tissue was isolated using TRIzol reagent (Life Technologies, Rockville, MD, USA). cDNA was synthesized from 1µg of total RNA using a reverse transcription kit (Elpis Bio, Daejeon, Korea) according to the manufacturer's protocol. Real-time PCR was performed on an ABI 7500

instruments (Applied Biosystems, Foster City, CA, USA) including HiPi Real-Time PCR 2x Master Mix SYBR Green mixture (Elpis Bio, Daejeon, Korea). In the reaction mixture, 25ng cDNA was used for amplification, the final concentration of each primer was 10pmole/μl, and the final volume was adjusted with PCR grade water. The reaction conditions were set as follows: initial denaturation for 2 min at 50°C and 10 min at 95°C (1 cycle) followed by 15 s at 95°C and 30 s at 58°C (1 cycle) for 40 cycles. The primers sequences (Macrogen CO., Ltd., Seoul, Korea) are listed in Table 1. Each reaction was performed as a technical duplicate and the average of 5 or 6 independent biological replicates was calculated. Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) gene expression levels were used to normalize gene expression as the endogenous control. Relative expression (fold change) was calculated as $2^{-\Delta\Delta C_t}$. Raw data were analyzed using ExpressionSuite v. 1.0.3 (Thermo Fisher Scientific, Darmstadt, Germany).

Table 1
Sequence of primers.

	Sequence (5'→3')	
Col1a1 (Collagen, type I, alpha 1)	Forward	TTGCCAGGAGAACCAGCAGAG
	Reverse	AAGGGTGAGACAGGCGAACAA
Col3a1 (Collagen, type III, alpha 1)	Forward	AATGGTGACAGAGGAGAAACG
	Reverse	CCTCGATGTCCTTTGATGCC
MMP1 (Matrix metalloproteinase 1)	Forward	ATGAGACGTGGACCGACAAC
	Reverse	TGAGTGAGTCCAAGGGAGTG
MMP2 (Matrix metalloproteinase 2)	Forward	GTAACCTCACTACGCTTTTCTCG
	Reverse	GACACATGGGGCACCTTCTGA
MMP8 (Matrix metalloproteinase 8)	Forward	CAGACAACCCTGTCCAACCT
	Reverse	GGATGCCGTCTCCAGAAGTA
MMP9 (Matrix metalloproteinase 9)	Forward	CGGAGCACGGGGACGGGTATC
	Reverse	AAGACGAAGGGGAAGACGCACATC
GAPDH (Glyceraldehyde 3-phosphate dehydrogenase)	Forward	ACCCCAATGTATCCGTTGT
	Reverse	TACTCCTTGGAGGCCATGTA

Western blot analysis

Total soleus muscle tissue protein lysates were extracted using lysis buffer containing protease and phosphatase inhibitors (Translab, Daejeon, Korea). Protein contents quantification carried out using a BCA assay (Thermo Fisher Scientific, Rockford, IL, USA). Each sample at the same concentration was denatured in Laemmli’s sample buffer at 100°C for 5 min. Equal amount (30μg) of prepared samples

were subjected to 10% SDS-PAGE (Gradi-Gel II Gradient PAGE Analysis Kit, Elpis Bio, Daejeon, Korea) to separate proteins by size, and transferred onto a polyvinylidene fluoride (PVDF) membrane. Then, the membranes were incubated in 5% non-fat dry milk at room temperature for 1 hr, treated overnight with primary antibodies at 4°C and followed by incubation with the horseradish peroxidase-conjugated secondary antibodies at room temperature for 1 hr. To visualize the band signal, immunoblot signals were developed using Amersham ECL Select (GE healthcare) with the ATTO Chemiluminescence Imaging System (AE-9150 Ez-capture II, Tokyo, Japan). Band signal intensity was quantified using Image analyzer CS4 analysis tool. GAPDH protein expression intensity was normalized as the loading control. The average of 5 or 6 independent biological replicates was calculated. The list of the antibodies used is included in Table 2. Cropped images are presented for clarity of bands, full-length blots are shown in the supplementary figures.

Table 2
List of antibodies

	Host	Source (Cat No.)	Dilution
mTOR (Mammalian target of rapamycin)	Rabbit	Cell signaling (#2983)	1:1000
phospho-mTOR (Phosphorylated mammalian target of rapamycin)	Rabbit	Cell signaling (#5536)	1:1000
phospho-70S6 kinase (Phosphorylated ribosomal protein 70 kDa S6 kinase 1)	Rabbit	Cell signaling (#9234)	1:1000
phospho-4EBP1 (Phosphorylated eukaryotic translation initiation factor 4E-binding protein 1)	Rabbit	Cell signaling (#2855)	1:1000
MuRF-1 (Muscle RING-finger protein-1)	Rabbit	ECM bioscience (MP3401)	1:1000
PGC-1 α (Peroxisome proliferator-activated receptor gamma coactivator 1-alpha)	Rabbit	Abcam (ab54481)	1:2000
TFAM (Mitochondrial transcription factor A)	Rabbit	Abclonal (A13552)	1:1000
NRF2 (Nuclear factor erythroid 2-related factor 2)	Rabbit	Abclonal (A1244)	1:1000
OPA1 (Optic atrophy 1)	Rabbit	Abclonal (A9833)	1:1000
MFN2 (Mitofusin 2)	Rabbit	Abclonal (A12771)	1:1000
DRP1(Dynamin-related protein 1)	Rabbit	Abclonal (A2586)	1:1000
MTFP1 (Mitochondrial fission process 1)	Rabbit	Abclonal (A7110)	1:1000
Cytochrome C	Rabbit	Abclonal (A0225)	1:1000
COX IV (Mitochondrial cytochrome c oxidase subunit IV)	Rabbit	Abclonal (A6564)	1:1000
GAPDH (Glyceraldehyde 3-phosphate dehydrogenase)	Mouse	Santa cruz (sc-365062)	1:2000
Anti-mouse IgG HRP	Rabbit	Santa cruz (sc-516102)	1:4000
Anti-rabbit IgG HRP	Goat	Cell signaling (#7074)	1:2000

Statistical analysis

Statistical analyses were performed using GraphPad Prism 7.0. All quantitative data are presented as the mean $\pm$ standard error. The normality and homogeneity of variance of data distribution were verified using the Shapiro–Wilk normality test and Levene test. One-way analysis of variance (one-way ANOVA) was used to determine significant differences among the groups followed by Bonferroni's post-hoc analysis. $P < 0.05$ was considered statistically significant.

Results

Effect of hyperthyroidism on body composition

To investigate the effect of ovariectomy and hyperthyroidism on body composition, we examined the body composition of the rats using DXA as shown in Fig. 1. Food intake was increased in the OVX group; further, LT4 treatment significantly increased food intake compared with the other groups (Fig. 1A). Based on the DXA analysis, body weight was significantly increased in the OVX group compared to the Sham group and was similar between the OVX and OVX+LT4 groups (Fig. 1B). The fat proportion was significantly increased after OVX, but maintained to the Sham levels in the OVX+LT4 group (Fig. 1C). However, we did not observe a difference in lean mass between OVX and OVX+LT4 groups (Fig. 1D).

Effect Of Hyperthyroidism On Protein Turnover

We then used immunoblotting to evaluate whether LT4 treatment affects protein turnover (Fig. 2). Protein turnover is regulated by signaling through mammalian target of rapamycin (mTOR) and the ubiquitin-proteasome pathway, which is responsible for degradation^{23,24}.

The protein expression of mTOR and phospho-mTOR was similar among the groups. The expression of downstream mTOR targets, initiation of protein synthesis, phosphorylated ribosomal protein 70 kDa S6 kinase 1 (phospho-p70s6 kinase) and phosphorylated eukaryotic translation initiation factor 4E-binding protein 1 (phospho-4EBP1) were higher in the OVX group compared with the Sham group, but were lower in the LT4 treatment group ($P < 0.01$). The protein expression of MuRF1, which indicates ubiquitin-proteasome mediated protein degradation, was not significantly different in the OVX group compared to the Sham group, but tended to increase and was significantly upregulated in the OVX group treated with LT4 ($P < 0.05$) (Fig. 2F).

Effect Of Hyperthyroidism On Soleus Muscle Histology

We confirmed the morphology of skeletal muscle by H&E staining (Fig. 3A). Consistent with our previous report²⁵, the interstitial space was significantly increased in the LT4 treatment group compared to that in the other groups ($P < 0.05$) (Fig. 3B). The skeletal muscle fiber was surrounded by extracellular matrix (ECM).²⁶ Thus, we performed Sirius red staining to identify collagen type I and III, which account for most of the ECM components. We found that stained area was similar in the OVX group compared with Sham

group and the LT4 treatment group was significantly reduced compared with other groups ($P < 0.05$) (Fig. 3C).

Effect Of Hyperthyroidism On Extracellular Matrix Remodeling

We next performed quantitative PCR to investigate the expression level of genes involved in ECM remodeling (Fig. 4). The mRNA expression level of Col1a1 was similar in the OVX group compared with that in the Sham group ($P > 0.05$), and the OVX+LT4 group showed significantly downregulated Col1a1 mRNA ($P < 0.01$) relative to the OVX group (Fig. 4A). No significant difference was observed in the expression levels of Col3a1 among the groups ($P > 0.05$) (Fig. 4B). The expression levels of matrix metalloproteinase (MMP) 1 and MMP8 were similar among the groups ($P > 0.05$) (Fig. 4C-D). Moreover, the expression levels of MMP2 and MMP9 were similar in the OVX group compared with the Sham group, whereas that in the OVX+LT4 group was the highest among the groups ($P < 0.05$) (Fig. 4E-F).

Effect Of Hyperthyroidism On Ultrastructure

We next confirmed the skeletal muscle ultrastructure by transmission electron microscopy (Fig. 5). There was no difference in the myofibril structure among the groups. We observed disintegrated cristae in the OVX group. Further, an increased number of mitochondria and aberrant enlarged mitochondria were observed in the OVX+LT4 group. Therefore, we confirmed the mitochondrial quality control process (mitochondrial biogenesis and dynamics), which plays an important role in mitochondrial function.

Effect Of Hyperthyroidism On Mitochondrial Biogenesis And Dynamics

To evaluate the effect of LT4 on mitochondrial quality control, we performed immunoblotting analysis (Fig. 6). Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1 α) levels were significantly decreased in the OVX group ($P < 0.05$), which were increased after LT4 treatment ($P < 0.001$). Mitochondrial transcription factor A (TFAM) levels were similar between the Sham and OVX groups, but were significantly reduced in the OVX+LT4 group ($P < 0.001$). Nuclear factor erythroid 2-related factor 2 (NRF2) levels were similar among the groups. The expression of optic atrophy 1 (OPA1) in the OVX group was significantly lower than that in the Sham group ($P < 0.05$), whereas LT4 treatment maintained these downregulated levels ($P < 0.001$). The expression of mitofusin 2 (MFN2) was similar among the groups. Mitochondrial fission process 1 (MFP1) levels were similar between the Sham and OVX groups, but were significantly reduced in the OVX+LT4 group ($P < 0.001$); dynamin-related protein 1 (DRP1) levels were similar among the groups. Moreover, cytochrome c and mitochondrial cytochrome c oxidase subunit IV

(COX IV) were similar between the Sham and OVX groups, but were significantly increased in the OVX+LT4 group ($P < 0.01$).

Discussion

DTC has a high survival rate and good prognosis, but long-term administration of levothyroxine has both benefits and adverse effects including cardiovascular and skeletal risks⁵. The association between menopause and sarcopenia onset is controversial, but menopause is reported to be associated with muscle mass²⁰. Sarcopenia plays an important role as a factor affecting the life of women after menopause as life expectancy increases. Our previous study evaluated body composition using DXA in a sex hormone deficiency model²⁵, and demonstrated that ovariectomy resulted in increased fat mass, muscle mass, and fat proportion in the whole body, but the lean body mass proportion of the whole body was not changed, leading to sarcopenic obesity. Further, we reported that an estradiol deficiency state with levothyroxine treatment may result in worsened muscle quality without any change in muscle mass. In this study, we explored the molecular mechanisms underlying the effect of hyperthyroidism on skeletal muscles in a postmenopausal rat model.

We initially evaluated body composition using animal DXA. In the group treated with levothyroxine, although food intake was increased, lean body mass was not different from that in the ovariectomized group, but the fat content was decreased. The balance of protein synthesis and degradation regulates the determinants of muscle mass^{23,24,27}. We found that mTOR/p-mTOR did not differ among the groups, whereas phospho-p70S6k and phospho-4EBP1 expression were upregulated in the ovariectomized group. We observed that MuRF1 tended to increase after ovariectomy, but were not significantly different, whereas they were increased significantly in the hyperthyroidism group. Activation of the mTOR downstream pathway after ovariectomy also appears to occur due to an increase in body weight and lean mass following early estrogen insufficiency as an adaptation to weight gain due to ovariectomy. Although we observed that lean mass increased through activation of the mTOR downstream pathway after ovariectomy, the proportion of lean mass in the body composition did not change, but rather increased the proportion of fat mass²⁵. Recent evidence suggests that estrogen deficiency causes dysregulation in muscle protein turnover with the balance tipping from protein synthesis to protein degradation, thus contributing to the loss of muscle mass¹⁹. Prolonged estrogen insufficiency results in muscle atrophy, reduced muscle force generation, and a shift in muscle fiber-type distribution¹⁷. It is thus speculated that the changes due to decreased estradiol may vary with the induction period. We observed that the mTOR pathway was inhibited by thyroid hormones, and the catabolic action of thyroid hormone excess enhanced the expression of MuRF1, but lean mass did not decrease compared to the ovariectomy group. The demand for energy in the skeletal muscles is provided for by the increased hepatic glucose production rate and metabolic activity induced by hyperthyroidism²². As in previous studies^{25,28}, creatinine kinase (CK), a marker of muscle damage, was not elevated in hyperthyroidism. However, in hyperthyroidism, a sharp decrease in thyroid hormone levels results in increased muscle wasting, which is manifested by elevated CK²⁸. In this manner, these results indicate that despite increased metabolic

demands, a lack of supply due to drastic changes in thyroid hormone is expected to contribute directly to muscle loss.

Next, we observed that ovariectomized rats with hyperthyroidism displayed muscle tissues with increased interstitial space and decreased intramuscular connective tissue upon histomorphological observation. Moreover, we showed that collagen type I mRNA expression was decreased and that MMP (MMP2 and MMP9) mRNA expression was increased by levothyroxine, leading to a decline in intramuscular connective tissue. Perimysium is a connective tissue composed of ECM that binds muscle fiber bundles, and is composed primarily of collagen types I and III. Endomysium attaches to the basement membrane, which is attached to the sarcolemma, and is mostly composed of collagen type IV ^{26,29}. MMPs are produced by both damaged muscle fibers and infiltrating cells and can break down the ECM components ²⁹. Muscle stiffness was lower in patients with hyperthyroidism and was sensitive to severity depending on the level of elevated free thyroid hormone in the serum ³⁰. Clinical studies do not consistently report changes in muscle mass and strength, but there are frequent complaints of muscle fatigue symptoms ^{10,14,30}. ECM homeostasis plays a role in increasing the efficiency of muscle contraction by acting as a mediator of muscle contraction as well as in protecting muscle fibers from excessive stress and promoting micro-trauma healing ²⁶. Thus, our data support that hyperthyroidism-related changes in ECM homeostasis lead to collagen degradation by increasing MMP levels with reduced synthesis of collagen structures. These changes in ECM homeostasis can be considered one of the factors that cause symptoms of increased muscle fatigue and weakened muscle strength, even if there is no change in muscle mass.

We showed that ovariectomy results in changes of mitochondrial cristae structures in agreement with a previous report ²¹. Further, levothyroxine treatment resulted in an increased number of mitochondria and aberrant enlarged mitochondria. Excessive thyroid hormone affects mitochondrial morphology rather than sarcomere structures. Strong evidence indicates that estrogen protects skeletal muscles from apoptosis through its effects on mitochondria ¹⁹. The mitochondrial shape is essential for mitochondrial activity ³¹. Morphological abnormalities can remarkably manifest as a result of altered mitochondrial quality control processes by impairing biogenesis, dynamics, and mitophagy, leading to sarcopenia ^{31,32}.

In the present study, reduced expression of PGC-1 α , which plays a major role in mitochondrial biogenesis, was observed in female animals without gonads, but this was upregulated by levothyroxine treatment. We also observed downregulated expression of TFAM and unchanged expression of NRF2 upon levothyroxine treatment. Although PGC-1 α overexpression in skeletal muscles is reported to protect from sarcopenia in male animals but not in female ³³, it is well documented that increased expression of PGC-1 α prevents muscle atrophy ³⁴. TFAM participates in mitochondrial DNA (mtDNA) replication and binds mtDNA to increase mitochondrial function while protecting against ROS ³⁵. The ROS generated in oxidative phosphorylation via activation of the AMPK pathway by thyroid hormone, increases the expression of PGC-1 α ³. Although ROS production by continuous thyroid hormone stimulation increases

mitochondrial biogenesis via PGC-1 α , a decrease in TFAM is thought to be associated with muscle loss due to excessive oxidative stress.

Mitochondrial dynamics include the fusion/fission process of the outer and inner membranes, and influence metabolism as well as complex cellular signaling events³¹. In the fusion process, outer membrane fusion is followed by inner membrane fusion. MFN2, located on the mitochondrial outer membrane, is essential for fusion of the outer membranes and OPA1 is important for cristae junction formation and maintenance³¹. We found downregulated OPA1 expression in the ovariectomized rats. Further, ovariectomized rats with hyperthyroidism showed upregulation of OPA1 and downregulation of MTFP1. Ablation of OPA1 results in a disorganized mitochondrial inner membrane structure³¹, loss of MFN2 function, induction of endoplasmic reticulum stress, ROS production, and impaired metabolic homeostasis³⁶. Reduced MFN2 and OPA1 expression in the skeletal muscle exacerbates the progression of sarcopenia in an accelerated aging SAMP8 model, as a result of impaired mitochondrial quality and autophagy²⁷. Next, we observed upregulated expression of cytochrome c and COX IV in ovariectomized rats with hyperthyroidism. Cytochrome c, which is trapped in the cristae junction compartment, is released upon damage to the mitochondrial ultrastructure and activates the caspase pathway that executes apoptosis³¹. Excessive or prolonged energy demands and oxidative stress can lead to ROS-mediated mitochondrial damage and dysfunction²². Short duration exogenous levothyroxine administration (for 3 days) increased the expression of uncoupling proteins and decreased the mitochondrial efficiency of skeletal muscles in male subjects³⁷. Our results suggest that estrogen is involved in mitochondrial biogenesis and dynamics in the skeletal muscle. Further, excessive thyroid hormone with an estrogen deficient state aggravates disruption of mitochondrial biogenesis and dynamics in the skeletal muscle. Our limitations are that we did not elucidate the sole effect of hyperthyroidism in a female model.

Overall, our data indicate that the skeletal muscle is a target organ affected by both thyroid and ovarian hormones. We found that early estrogen deprivation affects mitochondrial quality control signaling pathways in the skeletal muscle. We highlighted that estrogen deficiency with an excess of thyroid hormone can result in the accumulation of harmful effects in the progression of muscle weakness via dysregulation of the signaling pathways involved in ECM homeostasis, protein turnover, and mitochondrial quality.

Declarations

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Conflicts of Interest

None

Author contributions

J.H.K. and J.M.K performed experiments. J.H.K. and K.K. analyzed experiments. K.K. designed the study. J.H.K. and K.K. wrote the manuscript with the contribution of all coauthors. B.J.L, I.J.K., K.P. and K.K. supervised the study.

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Figures

Figure 1.

Figure 1

Body composition and food intake. (A) Food intake (B) Body weight (C) Fat proportion (D) Lean mass *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001

Figure 2.

Figure 2

Protein turnover. (A) Representative images of immunoblotting for soleus muscle (B) mTOR protein expression (C) phospho-mTOR protein expression (D) phospho-p70s6k protein expression (E) phospho-4EBP1 protein expression (F) MuRF1 protein expression. Protein expression data were normalized to the GAPDH expression levels. *P < 0.05, **P < 0.01, and ***P < 0.001

Figure 3.
(A)

Figure 3

Histological findings. (A) H&E and Sirius red staining (B) Morphometric analysis of interstitial space after H&E staining (C) Morphometric analysis of Sirius red staining in the skeletal muscles of the groups. Scale bars = 250 µm; Magnification, 10x. *P < 0.05, **P < 0.01

Figure 4.

Figure 4

Extracellular matrix homeostasis. mRNA expression of (A) Col1a1 (B) Col3a1 (C) MMP1 (D) MMP8 (E) MMP2 and (F) MMP9 Gene expression data are normalized to the expression levels of GAPDH. *P < 0.05, **P < 0.01, and ***P < 0.001

Figure 5.

Figure 5

Electron microscopy. Representative electron micrograph of the subsarcolemmal and intermyofibrillar area of the soleus muscle. Magnification, 20000x.

Figure 6.

Figure 6

Mitochondrial biogenesis and dynamics. Representative immunoblotting image of mitochondrial biogenesis and dynamics in the soleus muscle. Protein expression data are normalized to the GAPDH expression levels. *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001

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