Impacts of isoflavones and physical activity on skeletal muscle anabolic adaptation and fatty acid metabolism

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ABSTRACT

Introduction: Menopause transition often leads to an imbalance homeostasis of skeletal muscle and energy in females. Consequently, elderly women are at higher risks in developing sarcropenia, osteoporosis and metabolic syndrome related diseases such as obesity and type 2 diabetes. The loss of skeletal muscle mass is associated with a loss of strength but also with the development of metabolic syndrome. Isoflavones (ISOs) and physical activity have shown some beneficial impacts on alleviating the changes that happen in postmenopausal women. Therefore, the major objectives of this study were to examine effects and underlying mechanisms of ISOs, training and the combined intervention on modulating muscle anabolic adaptation and fatty acid metabolism which are interfered by estrogen decline in females through both in vivo and in vitro experiments.

Methods: In the animal experiment, female Wistar rats were assigned to six groups: (1) ovary intact (Sham); (2) Sham-operated rats with exercise (Sham+T); (3) ovariectomized (OVX) rats; (4) OVX+T; (5) OVX with an ISO enriched diet (OVX+ISO); (6) OVX+ISO+T. Rats with training were trained 10 min/time, twice/day, a rest day every four days on a treadmill with an incline of 25° for 61 days and a gradually increasing velocity from 12 to 20 m/min. In addition, two different diets, an ISO depleted diet (4 mg ISO aglycone equivalent/kg diet) and an ISO enriched diet (479 mg ISO aglycone equivalent/kg diet), were used to feed the rats. Sizes of cross-sectional areas (CSAs) of soleus muscle, gastrocnemius muscle as well as adipose tissue were determined by histochemical methods. Serum leptin and IGF-1 levels were measured by ELISA kits and serum lipids were analyzed by colorimetry. In addition, trabecular bone mineral content (Tb.BMC) and trabecular bone mineral density (Tb.BMD) were measured by peripheral quantitative computed tomography (pQCT). Moreover, gene and protein expression related to skeletal muscle anabolic adaptation or fatty acid metabolism was investigated by real-time PCR and western blot. In the cell culture experiment, C2C12 myoblastoma cells were proliferated and differentiated into myotubes. Afterwards, a soy extract (Soy), genistein (Gen), daidzein (Dai), glycitein (Gly), and a mixture of Gen-Dai-Gly (Mix) were used to treat the myotubes. The number and diameters of myotubes were measured under a microscope. In addition, the mRNA and protein expressions related to muscle anabolic activity were analyzed.

Results: In the animal experiment, OVX in rats resulted in decreases of relative gastrocnemius muscle weight and bone mass. Meanwhile, body weight, fat mass, leptin and lipid levels were increased by OVX. The training effectively antagonized the effects by decreasing visceral fat mass, adipocyte size and leptin. However, ISOs only showed effects on reducing serum leptin. The combination of training and ISOs showed additive effects, reflecting in an increase of relative gastrocnemius muscle weight and a decrease of serum triglyceride level. In the part of this study regarding muscle adaptation, in soleus muscle insulin-like growth factor (IGF)-1R, MyoD and Myogenin expressions were only up-regulated by training in Sham groups. However, a stimulation of IGF-1R and MyoD expression could be observed when ISOs and training were combined. In gastrocnemius muscle MyoD and Myogenin expressions were stimulated by either training or ISOs. Additive effects were detected when combining the two interventions. In the part of this study in regard to lipid metabolism, training stimulated the expressions of genes associated with fatty acid synthesis (SREBP-1c and FAS) in adipose tissue, soleus muscle, liver and genes associated with fatty acid oxidation (PPARδ and PGC-1α) in adipose tissue. ISOs stimulated the expression of SREBP-1c and FAS in soleus muscle and PGC-1α in adipose tissue, whereas suppressed hepatic SREBP-1c and FAS expression. Strong additive effects of ISOs combined with training were observed for PPARδ and PGC-1α expressions in soleus muscle. In the C2C12 cell culture experiment, treatment with Soy, Gen and Mix led to a significant increase of myotube diameter and an increase of the number of myotubes per area compared to control group. The increased diameter by Soy was antagonized by antiestrogen ZK 191703 (ZK), whereas not by antiandrogen flutamide (Flut). Furthermore, gene expressions of IGF-1, IGF-1R as well as protein expression of myosin heavy chain (MHC) were significantly increased by Soy, Gen, Mix. The effects induced by Gen and Mix were comparable to Soy.

Conclusion: The training combined with ISOs could be an effective strategy to promote muscle growth and reduce a risk of developing metabolic syndrome such as obesity for postmenopausal women. The combination of ISOs and exercise is more efficient in increasing relative skeletal muscle mass and the expression of molecular markers related to anabolic adaptation in the skeletal muscle of female rats. Gen might be the most efficient compound in the soy extract exerting anabolic activity on skeletal muscle growth. Furthermore, the training seems to have a higher impact on visceral fat prevention than dietary ISO intake. Nevertheless, the strongest effects for several of the addressed

parameters related to fatty acid metabolism could be observed in the combined group of ISOs and exercise especially in the soleus muscle.

ZUSAMMENFASSUNG

Einleitung: Der Übergang in die Menopause führt für Frauen häufig zu einer gestörten Homöostase in der Skelettmuskulatur und dem Energiehaushalt. Folglich haben ältere Frauen ein höheres Risiko an Sarkopenie, Osteoporose und dem metabolischen Syndrom zugehörigen Krankheiten, wie zum Beispiel Adipositas und *Diabetes mellitus Typ 2*, zu erkranken. Der Verlust der Skelettmuskelmasse steht im Zusammenhang mit einem Rückgang der Muskelkraft, aber auch mit der Entwicklung des metabolischen Syndroms. Isoflavone (ISO) und körperliche Aktivität zeigten einige positive Auswirkungen in dem sie zur Linderung dieser Effekt führten. Die Hauptziele dieser Studie waren daher die Untersuchung der Effekte und des zugrungeliegenden Mechanismus der ISO, des Trainings und der kombinierten Intervention auf die durch einen Östrogenrückgang ausgelösten Veränderungen der anabolen Muskeladaption und des Fettsäurestoffwechsels. Dafür wurden verschiedene in-vitro und in-vivo Experimente durchgeführt.

Methoden: Im Tierversuch wurden weibliche Wistar-Ratten in sechs Gruppen eingeteilt: (1) scheinoperierte Ratten (Sham); (2) scheinoperierte Ratten mit Training (Sham + T); (3) ovariektomierte (OVX) Ratten; (4) OVX + T; (5) OVX mit einer ISO-angereicherten Diät (OVX + ISO); (6) OVX + ISO + T. Ratten mit Training wurden für 10 min, zweimal täglich auf einem Laufband mit einer Neigung von 25° und einer allmählich zunehmender Geschwindigkeit von 12 bis 20 m/min für 61 Tage trainiert. Alle vier Tage bekamen die Ratten einen Ruhetag.

Zusätzlich wurden zwei verschiedene Tierfutter, eine ISO-armes Futter (4 mg ISO-Aglycon-Äquivalent/ kg Futter) und eine ISO-angereichertes Futter (479 mg ISO-Aglycon-Äquivalent/ kg Futter) verwendet. Die Größen der Querschnittsflächen (CSAs) des Soleus-Muskels, des Gastrocnemius-Muskels sowie des Fettgewebes wurden mit Hlfe histochemischer Methoden analysiert. Die Bestimmung der Serumleptin- und IGF-1-Spiegel wurden mittels ELISA-Kits und die Serumlipide durch Kolorimetrie bestimmt. Zusätzlich wurden der trabekuläre Knochenmineralgehalt (Tb.BMC) und die trabekuläre Knochenmineraldichte (Tb.BMD) durch periphere quantitative Computertomographie (pQCT) gemessen. Darüber hinaus wurde die mit der anabolen Anpassung der Skelettmuskulatur oder die mit dem Fettsäuremetabolismus im Zusammenhang stehende Gen- und Proteinexpression mittels Real-Time PCR und Western Blot untersucht. Im Zellkulturversuch wurden C2C12-Myoblastomzellen vermehrt und zu

Myotuben differenziert. Danach erfolgte die Behandlung der Myotuben mit Sojaextrakt (Soy), Genistein (Gen), Daidzein (Dai), Glycitein (Gly) oder einer Mischung aus Gen-Dai-Gly (Mix). Anzahl und Durchmesser der Myotuben wurden unter einem Mikroskop bestimmt. Zusätzlich erfolgte die Anaylse der mRNA- und Proteinexpression, der Gene die mit der muskelanabolen Aktivität in Zusammenhang standen.

Ergebnisse: Im Tierversuch führte die OVX bei Ratten zu einer Abnahme des relativen Gewichts des Gastrocnemius-Muskels und des Knochens. Das Körpergewicht, die Fettmasse, sowie die Leptin- und Lipidspiegel erhöhten sich durch die OVX. Das Training wirkte diesem Effekt entgegen, indem es die viszerale Fettmasse, die Adipozytengröße und den Leptinspiegel verringerte. Die Wirkung der ISO zeigte sich nur in der Verringerung des Serum-Leptins. Die Kombination aus Training und ISO führte zu additiven Effekten, welche sich in einem Anstieg des relativen Muskelgewichtes des Gastrocnemius und einer Abnahme des Serumtriglyceridspiegels zeigten. In dem Teil der Studie zur Muskeladaption wurde durch Training die Expression des Insulin-like growth factor (IGF) -1R, des MyoD und des Myogenins im Soleus-Muskel nur durch Training in den Sham-Gruppen hochreguliert. Jedoch konnte eine Stimulation der IGF-1R- und MyoD-Expression beobachtet werden, wenn ISOs und Training kombiniert wurden. Im Gastrocnemius-Muskel wurden die MyoD- und Myogenin-Expression entweder durch Training oder ISOs stimuliert. Bei der Kombination der beiden Interventionen konnten additive Effekte festgestellt werden. In dem Teil dieser Studie, der den Lipidstoffwechsel betrifft, stimulierte das Training die Expression von Genen, die im Fettgewebe, dem Soleus-Muskel und der Leber mit der Fettsäuresynthese (SREBP-1c und FAS) assoziiert sind, sowie, sowie Genen, die im Fettgewebe im Zusammenhang mit Fettsäureoxidation stehen (PPARδ & PGC-1α). ISOs stimulierten die Expression von SREBP-1c und FAS im Soleus-Muskel und PGC-1α im Fettgewebe, während die hepatische SREBP-1c- und FAS-Expression unterdrückt wurde. Bei der PPARδ- und PGC-1α-Expression im Soleus-Muskel wurden starke additive Effekte der ISOs in Kombination mit dem Training beobachtet. Im C2C12-Zellkulturexperiment führte die Behandlung mit Soja, Gen und Mix zu einer signifikanten Zunahme des Myotubendurchmessers und einer Zunahme der Anzahl der Myotuben pro Fläche im Vergleich zur Kontrollgruppe. Der erhöhte Durchmesser der mit Soja behandelten Myotuben wurde durch das Antiöstrogen ZK 191703 (ZK) antagonisiert, aber nicht durch das Antiandrogen Flutamid (Flut). Darüber hinaus wurden die Genexpression von IGF-1, IGF-1R, sowie die Proteinexpression der

schweren Myosinkette (MHC) durch Soy, Gen, Mix signifikant erhöht. Die durch Gen und Mix induzierten Effekte waren vergleichbar mit Soja.

Schlussfolgerung: Das Training in Kombination mit ISOs könnte eine effektive Strategie sein, um das Muskelwachstum zu fördern und das Risiko eines metabolischen Syndroms wie Adipositas bei postmenopausalen Frauen zu reduzieren. Die Kombination von ISOs und Training führt zu einer effizienteren Steigerung der Erhöhung der relativen Skelettmuskelmasse und der Expression von molekularen Markern im Zusammenhang mit der anabolen Anpassung in der Skelettmuskulatur der weiblichen Ratten. Gen könnte die wirksamste Verbindung im Sojaextrakt sein, die eine anabolische Aktivität auf das Skelettmuskelwachstum ausübt. Darüber hinaus scheint das Training einen höheren Einfluss auf die viszerale Fettprävention zu haben als die ISO-Aufnahme. Dennoch konnten die stärksten Effekte für einige der angesprochenen Parameter im Zusammenhang mit dem Fettsäurestoffwechsel in der kombinierten Gruppe von ISOs und Training insbesondere im Soleus-Muskel beobachtet werden.

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LIST OF ABBREVIATIONS

AR	Androgen receptor
bHLH	Basic helix-loop-helix
BMC	Bone mineral content
BMD	Bone mineral density
CSA	Cross-sectional area
Dai	Daidzein
DHEA	Dehydroepiandrosterone
DHT	Dihydrotestosterone
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethylsulfoxide
E1	Estrone
E2	17β-estradiol
E3	Estriol
ER	Estrogen receptor
ERE	Estrogen response element
ERK	Extracellular response kinase
FAS	Fatty acid synthase
FBS	Fetal bovine serum
Flut	Flutamide
Gen	Genistein
GH	Growth hormone
Glut	Glucose transporter
Gly	Glycitein
HDL	High density lipoprotein
HRT	Hormone replacement therapy
IGF-1	Insulin-like growth factor-1
IGF-1R	Insulin-like growth factor-1 receptor
IRS	Insulin receptor substrate protein
ISO	Isoflavone
LDL	Low density lipoprotein
LPL	Lipoprotein lipase

LXR	Liver X receptor
MAPK	Mitogen activated protein kinase
MGF	Mechano growth factor
MHC	Myosin heavy chain
Mix	A mixture of genistein-daidzein-glycitein
MRF	Myogenic regulatory factor
mTOR	Mechanistic target of rapamycin
MuRF1	Muscle RING finger-containing protein 1
O-DMA	O-desmethylangolensin
OVX	Ovariectomy
Pax	Paired-box
PGC	Peroxisome proliferator-activated receptor-γ coactivator
PKB	Protein kinase B
PI3K	Phosphatidylinositol 3-kinase
PND	Postnatal day
PPAR	Peroxisome proliferator-activated receptor
pQCT	Peripheral quantitative computed tomography
RXR	Retinoid X receptor
SERM	Selective estrogen receptor modulator
SIRT1	Sirtuin 1
SREBP	Sterol regulatory element-binding protein
Soy	A soy extract
Tb	Trabecular area
TNF	Tumor necrosis factor
ZK	ZK 191703

CHAPTER 1: INTRODUCTION

1.1 Background

1.1.1 Negative impacts by menopause

Menopause is often defined as the time when women no longer have menstrual periods and not able to bear children. It typically occurs in a woman's late 40s to early 50s. Menopause means a transition from a reproductive to non-reproductive phase, which is characterized by a decrease of hormone production from ovaries. As a result, the concentration of circulating estradiol (E2) is strongly reduced to a lowered basal value, which is maintained by secondary sources such as adipose tissue and adrenal glands [1]. Postmenopause is the period after the menopause and is often associated with multiple menopause symptoms such as hot flashes, sleep disturbances or mood swings due to a huge change of endogenous estrogen in females. In addition, it is well known that postmenopause is associated with increased risks to develop metabolic diseases like obesity, type 2 diabetes, atherosclerosis, muscle loss and osteoporosis [2-4].

1.1.2 Isoflavone intake as an alternative for hormone replacement therapy

There are several ways to alleviate symptoms induced by menopause in females. Hormone replacement therapy (HRT) and selective estrogen receptor modulators (SERMs) are the two main medical therapies. HRT is considered as a potential treatment for metabolic syndrome [5, 6], muscle loss [7, 8] and bone loss [9]. Our previous in vivo studies also demonstrated the beneficial effects of estrogen on preventing obesity in ovarian dysfunction rats [6, 10]. However, the risk of taking HRT is still controversially discussed. HRT may be associated with increased risks of breast and endometrial cancer. Several findings did not support the use of HRT for chronic disease prevention, although it might appropriate for symptom management in some women [11]. Therefore, people are seeking for an alternative approach to HRT. Phytoestrogens arouse public attentions because of their more natural source and similar structure/properties to E2. Soy isoflavones (ISOs) are the most important dietary source of phytoestrogens for humans. They have a non-steroidal structure but possess a phenolic ring which enables them to bind the estrogen receptors (ERs) and act as estrogen agonists or antagonists [12, 13]. SERMs are defined as a group of chemically diverse non-steroidal compounds that bind to and interact with the ERs, therefore ISOs are characterized as naturally occurring SERMs [14, 15]. Similar to synthetic SERMs, soy ISOs have been proposed to exert beneficial effects like estrogen without its side effects [15]. For example, several health declarations about Asian populations consuming high dietary ISOs reported that in these people the frequencies of

hormone-related cancer and menopausal disorders are lower than in Western countries where soy is not commonly consumed. In addition, it has been shown that long-term dietary ISO intake exerts beneficial effects on increasing muscle mass, bone mass and reducing fat or lipid accumulation [16]. However, no significant effects were also reported in some studies regarding those aspects due to different experimental designs such as ISO exposure time, ISO concentration, food composition and analytical methods [16]. Further, the molecular mechanisms involved in these effects remain not very clear.

1.1.3 Effects of physical activity on muscle adaptation

Modern lifestyle is often characterized by a lack of physical activity and a consumption of high calorie, unhealthy diets. Exercise exerts a variety of benefits on human health, therefore it is regarded as a nonpharmacological intervention to overcome health issues associated with menopause. Physical activity is often recommended for increasing muscle mass and strength, especially for elder people [17]. Skeletal muscle adapts to the functional demands imposed by an increased loading of an individual, leading to changes in both fiber size and the structural/functional properties of myofibers in mammals. The adaptation process of skeletal muscle in response to exercise depends on the speed, force, duration and characteristics of contraction patterns, which is associated with a network of signal transduction pathways and a specific modulation of gene expression. Resistance training mainly results in an increased rate of protein synthesis which leads to myofibrillar adaptation and eventually hypertrophy [18]. Endurance training has been shown to result in metabolic and mitochondrial adaptations [18]. Heavy training is characterized as an “all-out” effort exercise. It is typically associated with resistance training from a cell-signaling perspective. However, some studies pointed out that high intensity training induces the phenotypic changes that resemble traditional endurance training [19]. In addition, muscle adapts to exercise to become a more effective energy provider. The energy provision is primarily supplied by aerobic metabolism including the consumption of oxygen to drive the oxidation of carbohydrates and fatty acids. Mitochondria are increased for a greater rate of fatty acid oxidation and a change of reaction to biochemical signals controlling energy metabolism [20]. Except exerting effects on muscle, exercise can delay the expansion of obesity and in consequence prevent insulin resistance, atherosclerosis or type 2 diabetes by modulating the chronic energy balance [6, 21].

1.1.4 Combinatory effects of isoflavones and exercise on regulating fuel molecules

Some studies have pointed out that a combination of ISOs and exercise is more efficient in preventing bone loss and body fat elevation in postmenopausal women [22, 23], indicating additive positive effects might be induced by combining the two interventions. The combinatory effects of ISOs and exercise on muscle mass are rarely reported. A study used soy protein (also contains ISOs) combined with a resistance training of 12-16 weeks in postmenopausal women did not result in greater increases in strength or muscle mass compared with placebo group [24]. Although a study showed a combination of ISOs and a 6-month walking training increases lean body mass and reduces fat mass in overweight postmenopausal women [23], more studies need to be done to make clear of energy homeostasis including glucose and fatty acid metabolism induced by the combination of ISOs and exercise. Adipose tissue, skeletal muscle and liver are three of the most important tissues participated in fatty acid metabolism. Adipose tissue is not only a passive storage for energy but also an active metabolic and endocrine organ. It regulates its own size and is actively communicating with other organs such as hypothalamus, pancreas, skeletal muscle, liver or immune system [25]. Moreover, in white adipose tissue, hormones, growth factors or adipocytokines are expressed. Some adipocytokines, such as leptin, resistin or adiponectin, have a huge impact on insulin sensitivity [26]. Therefore, adipose tissue is very relevant for the development of metabolic diseases. A liver plays an essential role in several stages of lipid synthesis and transportation. It is a major site for converting excess carbohydrates and proteins into fatty acids and triglycerides which are then mainly exported and stored in adipose tissue. In addition, the liver synthesizes large quantities of cholesterol, phospholipids and lipoproteins. Therefore, liver dysfunction is often diagnosed through observing an abnormal lipid profile. Moreover, circulating IGF-1 is mostly secreted from the liver [27], indicating an important role of a liver in regulating insulin sensitivity. The healthy human contains ~3 times of triglycerides in whole body skeletal muscle than in a liver [28]. The triglycerides make muscle available for lipolysis and providing energy. Therefore, some genes and proteins related to lipid synthesis, lipolysis as well as muscle growth are investigated in adipose tissue, liver and skeletal muscle in our animal study.

1.2 Objectives

As noted above, menopause in females is associated with negative metabolic changes. ISO intake and physical activity have shown some beneficial impacts on alleviating the changes. Therefore, the major objectives of this study were to examine effects and some underlying

mechanisms of ISOs, training and the combined intervention on modulating muscle anabolic adaptation and fatty acid metabolism which are interfered by estrogen decline in females through both in vivo and in vitro experiments.

In general, the following questions should be answered:

1. What are negative impacts caused by estrogen deficiency in females?
2. How do ISOs, training and a combined treatment of ISOs and training affect skeletal muscle mass in postmenopausal women?
3. How do ISOs, training and combined treatment of ISOs and training affect fat accumulation and lipid metabolism in postmenopausal women?
4. What are distinguishing effects of a soy extract and the soy ISO aglycones on muscle growth?

The above mentioned objectives should be achieved by the conduction of one in vivo animal study and one in vitro cell culture study.

In the animal study, female Wistar rats were Sham or ovariectomized (OVX) operated. OVX is normally used as a model to mimic a hormone decline in postmenopausal women. In addition, two different diets, an ISO depleted diet and an ISO enriched diet, were used to feed the rats. The ISO enriched diet could result in a plasma concentration in rats which is comparable to those in Asians consuming moderate to high amounts of soy products according to our previous study [29]. Meanwhile, a heavy training protocol based on and modified from previous studies [30, 31] was conducted on a motor-driven treadmill with an incline of 25° for 61 days and the rats ran uphill.

In the cell culture experiment, C2C12 myoblastoma cells were differentiated into myotubes, followed by treatments of a soy extract (Soy), genistein (Gen), daidzein (Dai), glycitein (Gly), and a mixture of Gen-Dai-Gly (Mix).

Fig. 1 drafts an overview of the main issues addressed in this Ph.D. project.

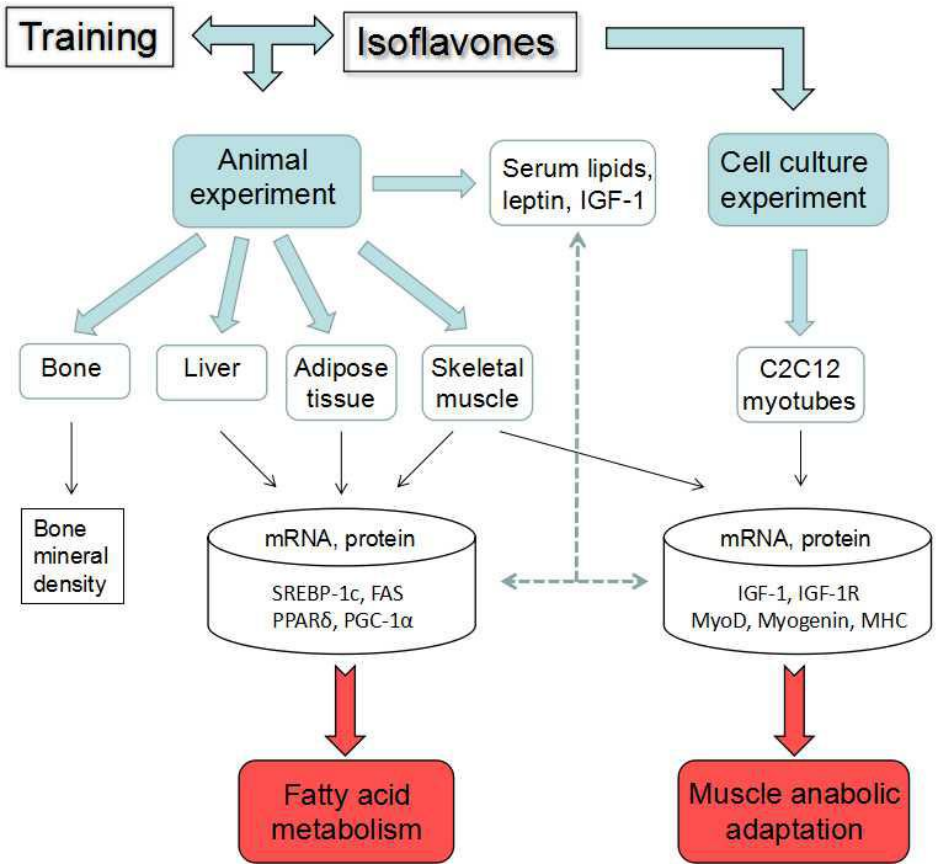

Fig.1. 1 Project overview

1.3 Outline of this thesis

A short outline of the other chapters of this work is given:

- **Chapter 2** reviews the literature concerning health issues of muscle loss, bone loss and metabolic syndrome related to estrogen deficiency, ISO supplementation and physical activity. A detailed description of molecular mechanisms is also included. Moreover, a brief overview of analyzed genes in this study is given in the last section of this part.

- **Chapter 3** is about a part of this study showing that an ISO enriched diet increased skeletal muscle adaptation in response to physical activity in OVX rats. In this part, relative muscle weight and muscle fiber size were measured. In addition, gene expression related to muscle growth factors and myogenesis (IGF-1, IGF-1R, MyoD and Myogenin) was investigated in soleus muscle and gastrocnemius muscle.

- **Chapter 4** is about a part of this study showing combinatory effects of ISOs and exercise on regulating fatty acid metabolism in OVX female rats. In this part, adipose tissue weight, adipocyte size, serum leptin and lipid profile were measured. In addition, bone mineral density (BMD) was analyzed. Moreover, expression of genes associated with fatty acid synthesis (SREBP-1c and FAS) and fatty acid oxidation (PPARδ and PGC-1α) was investigated in adipose tissue, soleus muscle and liver.

- **Chapter 5** is about a part of this study showing that ISOs enhanced anabolic activity in C2C12 myotubes through a modulation of both IGF-1 and myosin heavy chain (MHC) expression. The number and diameters of myotubes were measured. In addition, expression of IGF-1, IGF-1R, MHC, MyoD was investigated in C2C12 myotubes. Moreover, ERβ was demonstrated that involved in skeletal muscle hypertrophic effects.

- **Chapter 6** concludes main results of this study and gives an outlook for future research regarding ISOs and exercise.

- Finally, contributions to the experiments are given. Since the results in this thesis involve contributions of my own and those from colleagues, a master student and a bachelor student, my specific contributions to publications and publications in preparation are outlined.

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CHAPTER 2: LITERATURE REVIEW

2.1 Estrogens and health

2.1.1 Estrogens

Estrogen is the primary female sex hormone which responsible for the development and regulation of the female reproductive system and secondary sex characteristics. 17β-estradiol (E2), estrone (E1) and estriol (E3) are three main naturally occurring estrogens in women. E2 is a main and the most potent estrogen found in premenopausal and non-pregnant women. E1 is a predominant estrogen in postmenopausal women. E3 is the main hormone in pregnant women.

2.1.2 Synthesis of estrogens

The ovary is the primary site of producing E2 in premenopausal women. The placenta is mainly responsible for producing estrogen during pregnancy. A small but significant amount of estrogen can also be produced by other organs, such as livers, adrenal glands, and breasts. The primary source of estrogen in postmenopausal women is from the conversion of androstenedione to E1 in adipose tissue. The biosynthesis of estrogens starts in theca interna cells in the ovary, followed by series of reactions delivered cholesterol to androstenedione. Afterwards, androstenedione was converted either immediately into E1, or into testosterone and then E2 with an additional step in the granulosa cells.

2.1.3 Mechanisms of estrogen actions

Estrogen receptor (ER) α and ERβ are nuclear receptors and two distinct subtypes mediating biological effects by estrogens. They are expressed in a lot of different organs in both males and females, including ovaries, uteri, prostates, testes, epididymides, bones, skeletal muscles, breasts, livers, kidneys, white adipose tissue and various regions of the brain. Most of the effects of estrogens in target organs are exerted through the action of ERs on gene regulation (genomic actions), but also some other effects are so rapid (within seconds or minutes) that they cannot depend on DNA transcription or protein synthesis (non-genomic actions).

The genomic actions include a ligand-dependent mechanism that estrogens bind to ERs forming dimmers in the nucleus and then bind to specific elements estrogen response elements (EREs) or other transcription factor complexes located in the promoters of target genes. However, ERs can also regulate gene expression without directly binding to DNA, which triggers a ligand-independent mechanism. Additionally, membrane-associated ERs mediate non-genomic actions, which lead to both altered functions of proteins in cytoplasm

and a regulation of gene expression [1].

2.1.4 Health risks in postmenopausal women

2.1.4.1 Postmenopause and muscle loss

Increasing evidence showed that aging is associated with a loss of muscle mass and muscle strength, which known as sarcopenia [2, 3]. Sarcopenia is increasingly present in postmenopausal women according to a New Mexico elderly population study [3]. Although some studies pointed out that muscle mass is negatively correlated with age [4], increasing cross-sectional studies supported that there is a correlation specifically between muscle mass and estrogen metabolism [5].

There are changes in muscle composition after menopause. One study showed that women after menopause have much more non-contractile muscle tissue per unit of cross-sectional area (CSA) compared to younger women [6], which indicates more intramuscular fat is stored in postmenopausal women. Women utilize more fat than glycogen as fuel in muscle compared to men. However, women after menopause maintain their ability to store fat in muscles under the form of triglycerides but lose their ability to oxidize it since a decrease of lipoprotein lipase (LPL) activity was found [7]. LPL is an enzyme that plays an important role in lipid metabolism and lipid transport.

Several factors contribute to sarcopenia in postmenopausal women. The major factor is an imbalance between muscle protein synthesis and muscle protein breakdown [8]. Physical inactivity, protein insufficiently intake and oxidative stress all lead to the sarcopenia in postmenopausal women [9-11]. Additionally, growth hormone (GH), insulin-like growth factor-1 (IGF-1), insulin and dehydroepiandrosterone (DHEA) appear to be correlated positively with muscle mass [3, 11, 12]. Estrogens exert effects through ER isoforms expressed in human skeletal muscle. Recent studies in our group showed that ERβ is the major ER isoform mediating estrogenic effects on skeletal muscle growth and repair [13, 14].

The loss of muscle strength may also be related to menopausal status. A study showed that muscle strength is correlated with circulating estrogen levels [15]. Estrogens exhibit anabolic effects on muscle by stimulating IGF-1 receptors (IGF-1Rs) [16]. Additionally, ERs play their roles on muscle strength through the action of both estrogens and IGF-1 [17, 18]. However, there is a study which compared muscle strength of four groups of women from 45 to 64 years old pointing out that aging rather than menopause status makes a contribution to the loss of muscle strength [19]. Regarding the muscle composition, a study

by Widrick et al showed type II fibers are reduced in number and size in postmenopausal women [20]. Furthermore, contractile properties, power output and isometric force are affected by estrogens [21-24].

2.1.4.2 Postmenopause and metabolic syndrome

Metabolic syndrome is a combination of various medical disorders caused by dysregulation of energy homeostasis and represents a cluster of obesity related diseases, such as cardiovascular disease and type 2 diabetes, which are characterized by hypertension, dyslipidemia and insulin resistance [25-27]. Postmenopausal women tend to have a higher risk of developing metabolic syndrome because of changes in metabolic and hormonal parameters. Approximately 20–30% of the middle-aged population is affected by metabolic syndrome, and it is associated with an increased risk of 60% in postmenopausal women [28].

Central obesity is one obvious feature of metabolic syndrome. Obesity is characterized by an enlargement of adipose tissue either in terms of adipocyte size increase or a combination of increases in adipocyte size and adipocyte number [29]. It is well known that estrogens affect energy homeostasis in females by changing body fat distribution, fatty acid mobilization and glucose absorbing capacity [30-32]. Estrogen deficiency leads to significant changes in body composition including an increase of adipose tissue and a loss of muscle mass [33]. It is reported that women after menopause are 20-28% more body fat compared to premenopausal women [34], meanwhile, 0.6% muscle mass of postmenopausal women are lost every year [35]. The underlying molecular mechanisms of effects on obesity by menopause are normally recognized to be associated with decreased activity of fatty acid oxidation [36].

Some studies have shown that the decline of estrogens with menopause is associated with a preferential increase in intra-abdominal fat depots, independent of the whole body gain or the total amount of adipose tissue [37]. Also, ovariectomy (OVX) in rats is associated with visceral fat gain, whereas subcutaneous fat mass does not change [38]. The accumulation of visceral fat is considered as a major determinant of metabolic syndrome. Women with high amounts of visceral fat suffer insulin resistance, cardiovascular mortality and associated metabolic abnormalities [39]. Meanwhile, studies showed that body visceral fat is correlated with serum leptin levels [40, 41]. Leptin is a hormone produced by adipose cells, which helps to regulate energy homeostasis by inhibiting hunger. People with obesity show resistance to leptin, similar as a resistance of insulin in type 2 diabetes, leading to a

failing of controlling hunger and modulating weight. There are a lot of explanations regarding this. One is correlated with a leptin receptor signaling pathway [42]. Some studies also pointed out that OVX rats develop a leptin resistance because leptin is very sensitive and therefore the weight gain of 10% in the OVX animals results in an increase of 50% serum leptin level compared to Sham rats [43, 44].

Besides, the effects of estrogens on serum lipid profile were well documented. Postmenopausal women or OVX animals tend to have higher levels of total cholesterol, low density lipoprotein (LDL) and triglycerides [45, 46] compared to premenopausal women or Sham animals. The abdominal obesity induced by estrogen deficiency is associated with increased insulin resistance, increased free fatty acid and decreased adiponectin which all contribute to increased secretion of apolipoprotein B-containing particles, leading to a hypertriglyceridemia and an increased hepatic lipase activity [47].

In general, the fatty acid metabolism induced by estrogen deficiency is rather complex and interacted with glucose metabolism. OVX in mice results in glucose intolerance and lowered plasma insulin level, whereas these effects are ameliorated by E2 substitution [48].

2.1.4.3 Postmenopause and osteoporosis

Osteoporosis is a disease with weaken bones and increased risks of unexpected fractures. There is a direct correlation between the lack of estrogen and the development of osteoporosis. After menopause, bone breakdown overtakes the building of new bone. Many similar characteristics are shared with OVX induced bone loss in rats and postmenopausal bone loss [49]. Studies in our lab have demonstrated that movement drive and bone mineral density (BMD) are positively influenced by E2 [50]. The steroid estrogens affect trabecular area (Tb) of the tibia in a bone-protective manner by increasing movement drive [50].

2.1.4.4 Treatments of postmenopausal symptoms

There are several ways to alleviate symptoms induced by menopause in females. Hormone replacement therapy (HRT) and selective estrogen receptor modulators (SERMs) are the two main medical therapies. HRT is considered as a potential strategy to play a protective role on muscle strength and power [51, 52]. Besides, it was reported that HRT reduces abdominal obesity, lipids, insulin resistance, blood pressure, new-onset diabetes, adhesion molecules and procoagulant factors in women, therefore HRT is an option of treating

postmenopausal metabolic syndrome [33, 53]. Moreover, HRT appears effective for preventing bone loss and osteoporotic fractures [54]. However, the risk of taking HRT is still controversially discussed. HRT may be associated with increased risks of breast and endometrial cancer. The Women's Health Initiative reported that HRT has a complex pattern of risks and benefits. Several findings do not support the use of HRT for chronic disease prevention, although it may be appropriate for symptom management in some women [55]. Therefore, people are seeking for an alternative approach to HRT. SERMs are defined as a group of chemically diverse non-steroidal compounds that bind to and interact with the ERs. Phytoestrogens arouse public attentions because of their more natural source and similar structure/properties to E2. Isoflavones (ISOs) are important phytoestrogens and have shown various biological actions including interacting with ERs, which leads them being characterized as naturally occurring SERMs.

2.2 Isoflavones

2.2.1 Phytoestrogens

Phytoestrogens are natural plant-derived compounds that are structure/properties resemble mammalian estrogens [56]. They are categorized into 4 groups based on their chemical structures: ISOs, coumestans, lignans and stil-benes. ISOs are the most well-known phytoestrogens and will be focused discussed in this review. Genistein (Gen), daidzein (Dai), glycitein (Gly), biochanin A and formonetin have received great interests among all ISOs. ISOs are mainly found in soybeans and other legumes but also exist in berries, wine, grains and nuts [57]. Coumestans are another group of plant phenols that show estrogenic activities, but less common in the human diet than ISOs. Coumestrol is the main compound in coumestans. They are mainly found in legumes, particularly food plants such as mung bean and sprouts of alfalfa [58]. The most well-known lignans are secoisolariciresinol and matairesinol. Lignans are first identified in plants and later in biological fluids of mammals. They are mainly found in seeds (particularly flaxseeds) and many fiber-rich foods such as berries, grains, nuts and fruits [59]. In addition, resveratrol is one of stil-benes arousing great interests. It is found in a variety of plants and functions as a phytoalexin to protect against fungal infections.

2.2.2 Source and chemical structure of isoflavones

ISOs are natural occurring diphenolic compounds and mainly present in the Favaceae family which has food legumes such as soy, peanut and clover [60]. Soybeans, red clover

and kudzu root contain the highest ISO concentrations, although the ISO compositions are very diverse in those foods. Several naturally occurring ISOs exhibit estrogenic activities. They are mainly ISO aglycones (Gen, Dai, Gly), ISO glycosides (genistin, daidzin, glycitin), biochanin A and formononetin [61, 62]. Gen, Dai and Gly are principal ISOs found in soy foods. In contrast, red clover contains much formononetin and biochanin A, whereas puerarin and Dai are the major ISOs in kudzu root. The chemical structure of the main soy ISO aglycons and E2 are depicted in Fig. 1.

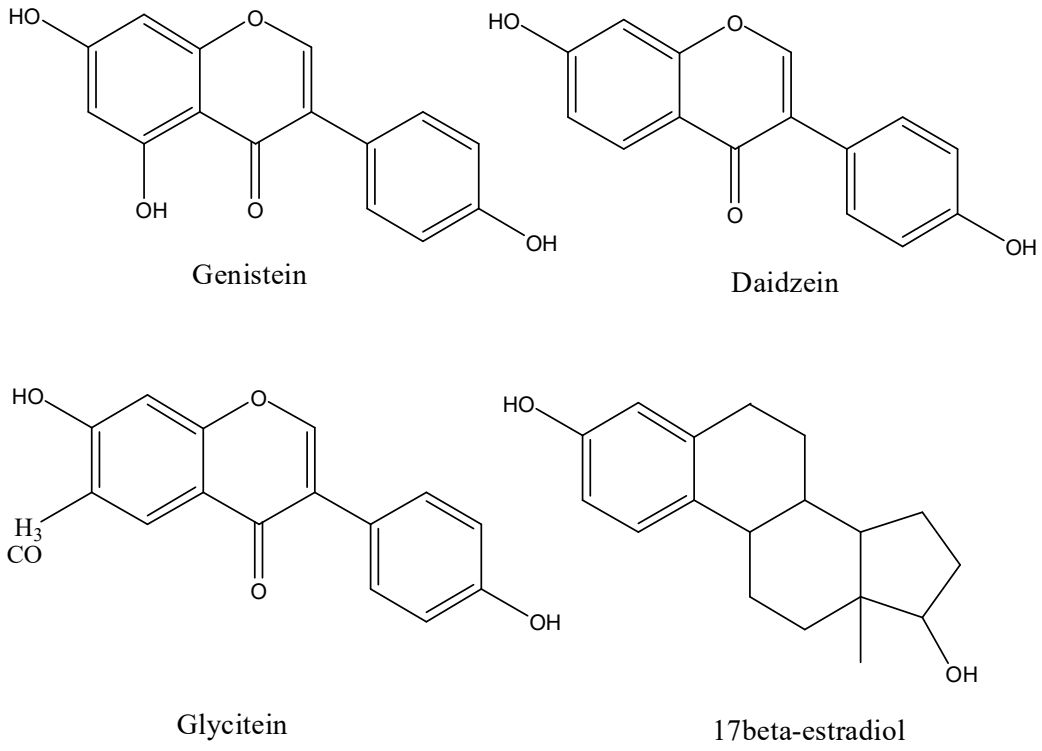

Fig.2. 1 Chemical structure of genistein, daidzein, glycitein and 17β-estradiol

The main consumed food source of ISOs is from a variety of soy products in Asian countries. According to the studies of Nagata et al. [63] and Messina et al.[64], the mean ISO intake in Asian countries is ranged from 30 mg/day to 50 mg/day. The ISO concentrations in different soy products are diverse (Table 1). There are between 5.1 and 64 mg ISO per 100 g tofu; 1.3 to 21 mg ISO per 100 g soy milk; and 0.1 to 2.3 mg ISO per 100 g soy sauce (relatively low). However, soy is not commonly consumed in Western countries. The main consumed food sources are soy flour or soy protein isolated products. Soy flour of 100 g contains in total 60 to 265 mg ISO aglycones (sum of Gen, Dai and Gly in equivalents) in different types (U.S. Department of Agriculture, 2002). Soy infant

formula of 100 g contains up to 31 mg ISO aglycones (U.S. Department of Agriculture, 2002). Nevertheless, the highest ISO concentration is found in nutritional supplements (up to 40%) which are generally manufactured from extracts of soybeans or red clover [65]. Investigations of ISO intake in several countries such as Ireland (0.73±1.77 mg/day), Italy (0.55±1.51 mg/day), the United Kingdom (0.70±1.04 mg/day), and Netherlands (0.91±1.90 mg/day) indicate low dietary intake of ISOs in Western diets [66].

Table 2. 1 The average isoflavone content of several dietary soy products

Food item	Isoflavone content (mg/100g)	
	Genistein	Daidzein
Soybean	69.66	56.22
Soybean curd	5.77	3.93
Soybean curd residue	13.93	11.24
Bean sprouts	8.42	3.81
Soybean paste	42.54	39.43
Seasoned soybean paste	6.14	5.47
Miso	24.56	16.13
Soy milk	7.84	4.51

2.2.3 Isoflavone absorption and metabolism

The metabolism of ISOs in animals and humans is a complex process with a combination of in mammals and in gut microbes. ISOs are considered to be metabolized in the gastrointestinal tract after being consumed in mammals. During the process, ISOs are firstly hydrolyzed to the aglycones Gen, Dai, Gly by β-glucosidases from both intestinal mucosa and gut microbiota [67, 68]. Glucocerebrosidase, lactase phlorizin hydrolase and a broad-specificity cytosolic enzyme are three native β-glucosidases which have been identified in humans [69]. Secondly, the compounds may be absorbed or further metabolized in the distal intestine with forming specific metabolites [70]. Dai may be further metabolized to dihydrodaidzein and then to O-desmethylangolensin (O-DMA) and equol [57]. Equol is not metabolized equally in all humans [71]. It was reported that 50-60% of the adult in Asian countries but only 30% of the Western population excrete equol after consumption of soy foods [72]. However, equol is representing up to 70-90% of all circulating ISOs in rodents [73]. The ability of transforming soy ISOs into equol may

explain the reason why the results of present phytoestrogen studies are varied [74]. Gen, similar as Dai, is firstly metabolized to dihydrogenistein, followed by a formation of metabolite 6′-hydroxy-O-DMA [57]. p-ethylphenol is the putative end product in humans [62]. Until now, there is limited information about metabolites of Gly. In vitro and in vivo studies identified several metabolites, including dihydroglycitein, 6-OH-Dai, 6-OH-dihydrodaidzein, 6-methoxy-equol, 6-OH-equol, 5'-OH-O-DMA and 5-methoxy-O-DMA [75, 76]. Considering the steps in ISO metabolism, ISO aglycones are thought to be absorbed faster and in greater amounts than their glucosides in humans [77]. Maximal ISO concentrations in blood are generally observed after 4-8 h of dietary intake [70, 78]. The rate of Dai in urinary excretion is greater than that of Gen throughout the postmeal period [79]. The overview of absorption and metabolism of ISOs is shown in Fig.2.

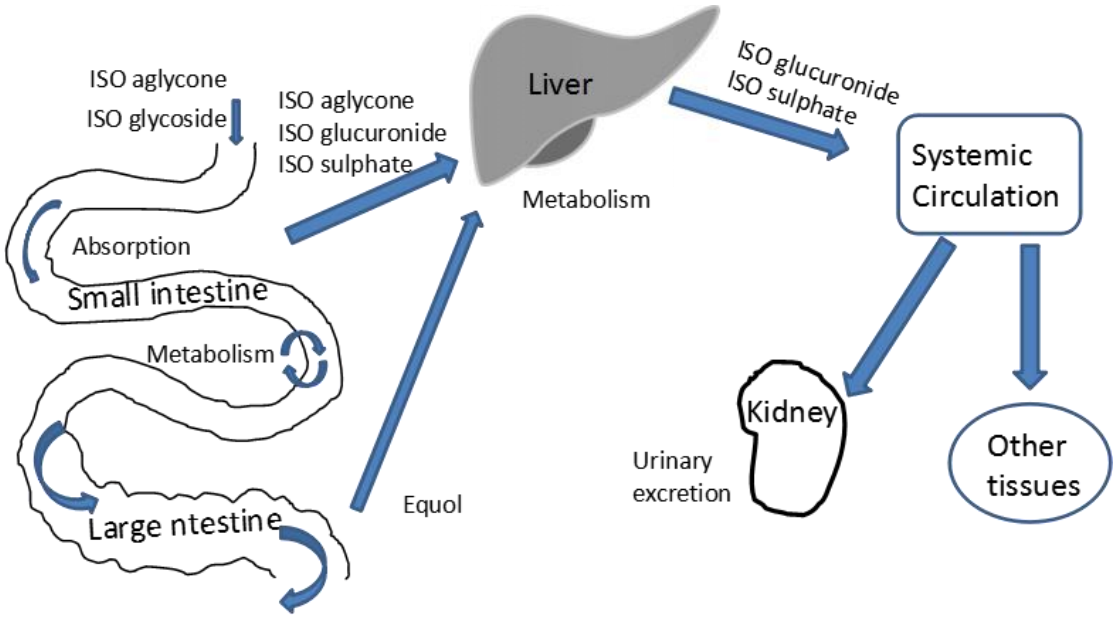

Fig.2. 2 Overview of absorption and metabolism of isoflavones (ISOs)

ISOs can be detected in many tissues in animals and humans. Yueh et al. using rats injected of Dai found that the concentrations are high in plasma, livers, lungs and kidneys at around 30 μg/g wet weight; moderate in skeletal muscles, spleens, and hearts at around 15-20 μg/g wet weight; and low in brains and testis at around 2-5 μg/g wet weight [80]. Plasma ISO concentrations (sum of Gen, Dai and equol) were about 0.2-3.2 μmol/L in adults consuming modest quantities of ~50 mg ISOs per day from soy products [81, 82]. Additionally, mean plasma ISO concentrations in Japanese women and men on traditional

diets are about 874 and 806 nmol/L [83].

2.2.4 Health and isoflavones

2.2.4.1 Isoflavones and muscle mass

Aubertin-Leheudre et al [84] showed that lean body mass and muscle mass of obese-sarcopenic postmenopausal women are significantly increased after supplementation of 70 mg/d ISOs for 24 weeks. One potential mechanism is that ISO supplementation may attenuate high concentrations of pro-inflammatory markers which contribute to muscle protein breakdown [85]. The promising results lead to the assumption that ISO supplementation may be beneficial for postmenopausal women as an alternative strategy for HRT. A previous study in our group demonstrated that E2 and Gen antagonize the effects induced by OVX on skeletal muscle myosin heavy chain (MHC) expression via ERβ mediated pathways [13], indicating an important role of ERβ pathway involved in muscle growth and regeneration which is activated by ISOs. An in vitro study with C2C12 cells showed that ISOs are able to prevent muscle atrophy by reducing proinflammatory cytokine tumor necrosis factor (TNF)-α through an activation of Sirtuin 1 (SIRT1) [86]. However, the molecular mechanisms involved in the anabolic effects of ISOs on muscle mass are still poorly understood.

2.2.4.2 Isoflavones and fatty acid metabolism

Adipocytes play an essential role in lipid homeostasis and energy regulation in mammals. E2 is a major regulator of adipogenesis in both females and males by affecting adipocyte size and adipocyte number [87]. E2 was shown to bind to ERs and decrease the activity of LPL, leading to an inhibition of lipogenesis. LPL regulates lipid uptake in adipocytes [88]. ISOs have a similar structure to E2 and have also been found to decrease LPL mRNA in adipose tissue [89]. This action is mediated by an activation of ERs. ERα and ERβ are both expressed in various cell types. E2 has equal binding affinities for ERα and ERβ, whereas Gen in ISOs was found to have a greater binding affinity for ERβ than ERα [90]. The previous in vivo study in our group demonstrated that the activation of either ER subtype reduces fat enrichment and improves insulin sensitivity [90]. Additionally, an activation of ERβ ameliorates lipid and glucose utilization in muscle [90]. In addition, peroxisome proliferator-activated receptor (PPAR) families are the primary adipogenic transcription factors. They interact with the members of CCAAT/enhancer binding proteins to regulate

the adipogenesis process [91]. Unlike the highly specific ERs, PPARs bind a wide number of ligands and directly affect lipid metabolism by enhancing transcription of PPAR-regulated genes [92]. An in vitro study with bone marrow cells and KS483 cells has shown that Gen inhibits adipogenesis at 0.1-10 μM, whereas stimulates adipogenesis at 10-50 μM [93]. A direct activation of PPARγ2 was supposed to explain the anti-estrogenic effect of Gen exposing at high concentrations [93]. In addition to the ability of interacting with ERs and PPARs, Gen at high concentrations acts as a potent inhibitor of protein tyrosine kinase, DNA topoisomerases I and II, and ribosomal S6 kinase. An induction of apoptosis induced by ISOs may also contribute to the inhibition of adipogenesis.

A liver is also a major site for cholesterol synthesis. LDL and cholesterol are associated with cardiovascular diseases. It has been reported that both Gen and Dai at 50-100 μM inhibit cholesterol synthesis through multiple mechanisms [94]. A study investigated the changes in hepatic transcriptional profiles showed that the hypolipidemic effect by Gen can be ascribed partly to the up-regulation of genes involved in fatty acid catabolism in the liver [95]. Others have reported that Gen supplementation decreases triglyceride, total cholesterol, and LDL levels in both serum and liver in mice [96]. Clinical experiments with postmenopausal women also showed high density lipoprotein (HDL) is significantly increased and apolipoprotein B, a primary apolipoprotein in LDL particles, is decreased by taking isolated ISO extracts [97]. In contrast to those findings reporting positive effects of ISOs on liver metabolism, some adverse effects were reported as well [98]. ISOs are able to reduce serum lipid levels through PPARα-dependent and PPARα-independent pathways, however, sustained activation of PPARα leads to a development of liver tumors in mice and rats [98].

Until now, there are a number of studies focusing on ISOs. However, results from different studies are often difficult to compare because of many variables, such as food composition, the duration time of experiments and differences in analytical methods. One important factor contributing to the confounding results in in vivo experiments might be the age of experimental animals. Another factor that seems to cause different results might due to gender [99]. ISOs influence metabolism in males and females distinguishingly because males have a much smaller number and different distribution of ERs compared to females [100]. The previous study in our group also showed distinct results by ISOs on males and females [101].

2.2.4.3 Isoflavones and bone health

The osteoporosis is a major public health concern. It is reported that about 1 million Americans suffer fragility fractures every year [102]. The beneficial effects of HRT on prevention of postmenopausal osteoporosis are well known. However, due to the concerns about the risks of taking HRT, more researchers have begun turning to ISOs as an alternative therapy. The in vitro study by Dang et al. investigated adipogenesis with mouse bone marrow cells and KS483 cells also demonstrated that Gen has a biphasic effect, showing a stimulation of osteogenesis at low doses (0.1-10 μM) and an inhibition of osteogenesis at high doses (25-50 μM) [93]. Vincent and Fitzpatrick [103] reviewed three animal studies and got the similar conclusions with OVX rats as in the in vitro study by Dang et al. Data from a human study showed a dose of ISOs at 50-90 mg/d seems to exert a benefit on skeleton in postmenopausal women [104]. However, some studies are still failed to find bone-repairing effects of ISOs in postmenopausal women [105, 106].

The possible mechanisms of ISOs on bone protection are multiple proposed, which include prevention of urinary calcium loss and suppressing the secretion of calcitonin. Therefore ISOs show beneficial effects on osteoblasts and bone resorption [57]. ERs have been found in osteoblasts, which may bind to EREs, inhibit or suppress specific gene expression, and therefore cause alterations of some protein productions [107].

2.3 Physical activity

2.3.1 Effects of exercise on skeletal muscle adaptation

2.3.1.1 Skeletal muscle biology and metabolism

Skeletal Muscle is one organ that transforms chemical energy into kinetic energy and responsible for the actual movement. Mammalian skeletal muscle is made up of a number of muscle fascicles which composed of single muscle fiber. Skeletal muscle fibers are the cellular level of the muscle. Their abilities and compositions strongly depend on muscle type. There are type I (slow-twitch) or type II (fast-twitch) in mammalian skeletal muscle according to their ATPase activity and contractile properties. Type I muscle fibers are classically red in appearance, which reflects an abundance of the oxygen transport protein myoglobin, high level of mitochondrial density and the relative high oxidative metabolic capacity. Type IIa and type IIx are sub-classifications of type II existing in humans, while type IIb is primarily found in rodents. Type IIx and IIb fibers are white in appearance and type IIa fibers have an intermediate color. Therefore, type I muscle fibers mainly use

oxidation as an energy source and require a better oxygen supply. They are slow in reaction time but have a high endurance and are also capable of fat oxidation. In contrast, type II muscle fibers tend to use anaerobic glycolytic pathway as energy supply and are much better in utilizing very quick energy sources like phosphocreatine. They can produce much more force but are less resistant to fatigue as their mitochondrial density is lower. A study with normal human subjects showed that the soleus muscle contains predominantly type I fibers (~80%), while the gastrocnemius muscle only contains ~57% slow twitch fibers [108]. In rodents, type IIb muscle fiber is the main muscle fiber type in gastrocnemius muscle [109, 110].

2.3.1.2 Modalities of exercise

Exercise can be classified into 3 subtypes: endurance, resistance and patterned movements. Resistance and endurance exercise have profound influences on muscle phenotype, and therefore impact muscle function and metabolism. Generally, resistance exercise imposes a low frequency, high resistance demand on muscular contraction and maintains muscle mass, basal metabolic rate, physical function in the elderly. The exercise of a short duration and high intensity is normally considered to produce skeletal muscle hypertrophy [111]. Endurance exercise imposes a repetition, low loading demand, which results in increases in muscle capillary density, mitochondrial proteins, fatty acid oxidation enzymes, and more metabolically efficient forms of contractile and regulatory proteins [112]. An early review by Hickson [113] showed that doing strength and endurance training at the same time has less adaptation compared to either one alone. But some recent studies reported that a combination of endurance and resistance training is more effective for reducing insulin resistance, obesity and metabolic syndrome [114].

2.3.1.3 Activation of satellite cells

Satellite cells are mononucleated stem cells of skeletal muscle and locate between the basal lamina layer and sarcolemma in adult muscle. The satellite cells are normally quiescent and arrested at an early stage of the myogenic process. Following injury or in response to increased muscle loading, satellite cells become activated, migrate to the site, proliferate, differentiate into myoblasts and fuse to form multinucleated myofibers with undamaged ones (hypertrophy) or to repair damaged myofibers by forming new myofibers (hyperplasia) [115]. With increasing cytoplasmic volume and development of organelles, the nuclei in satellite cells become myonuclei. Since myonuclei in adults are unable to

divide, repair and growth of muscle depend on the availability of satellite cells to provide new myonuclei in muscle fibers. Estrogens seem to affect muscle regeneration by influencing satellite cell number and activity. Treating male rats with E2 activates more satellite cells (an increase in paired-box (Pax) 7 expression) after a bout of muscle damaging downhill running compared with control group [116, 117]. Pax7 is a transcription protein expressed in cells that closely associated the activation of satellite cells. A study showed Pax7 knock out muscle have no satellite cells [118]. Myf5 or MyoD is also expressed in satellite cells after being activated and before going into the differentiation process [119].

2.3.1.4 Skeletal muscle growth and myogenesis

Skeletal muscle is a highly adaptive tissue which grows and develops in response to growth factors, nutrition, and exercise [120]. The regulation of muscle mass and fiber size is essentially the balance between protein synthesis and degradation within the muscle fibers. Addition or loss of myonuclei could also affect protein turnover [121]. During the process of protein synthesis or muscle growth in adult, myoblast cells are differentiated into myotubes with further developing to different cell types by producing myogenic regulatory factors (MRFs)-MyoD, Myf5, Myogenin and Mrf4 [122]. The MRFs belong to a superfamily of basic helix-loop-helix (bHLH) transcription factors and contain basic regions that facilitate DNA binding and the HLH domain mediating dimerizations [123]. MyoD and Myf5 are expressed in proliferating myoblasts, as primary MRFs, promoting satellite cells entry into the cell cycle [124]. Myogenin and Mrf4 are secondary MRFs, presenting in myocytes and promoting differentiation and cell cycle exit [125]. Since MyoD and Myogenin in myoblasts and myotubes have a high number of binding sites, they exhibit direct and great influences on other transcription genes correlated with myoblast proliferation [126].

Upstream of MRFs, the Pax family of transcription factors is also responsible for muscle growth. It is associated with the activation of satellite cells, particularly Pax3 and Pax7, expressed uniquely in satellite cells [127]. The Pax transcription factor levels are reduced with concurrent increases of Myf5 and MyoD, resulting in differentiation of myoblasts [128, 129]. The terminal differentiated, multinucleated myotubes are distinguished by expression of MHC and troponin T. During the process, p53 controls nuclear apoptosis without degrading the whole myofibers [130]. The process of myogenesis is depicted in Fig. 3

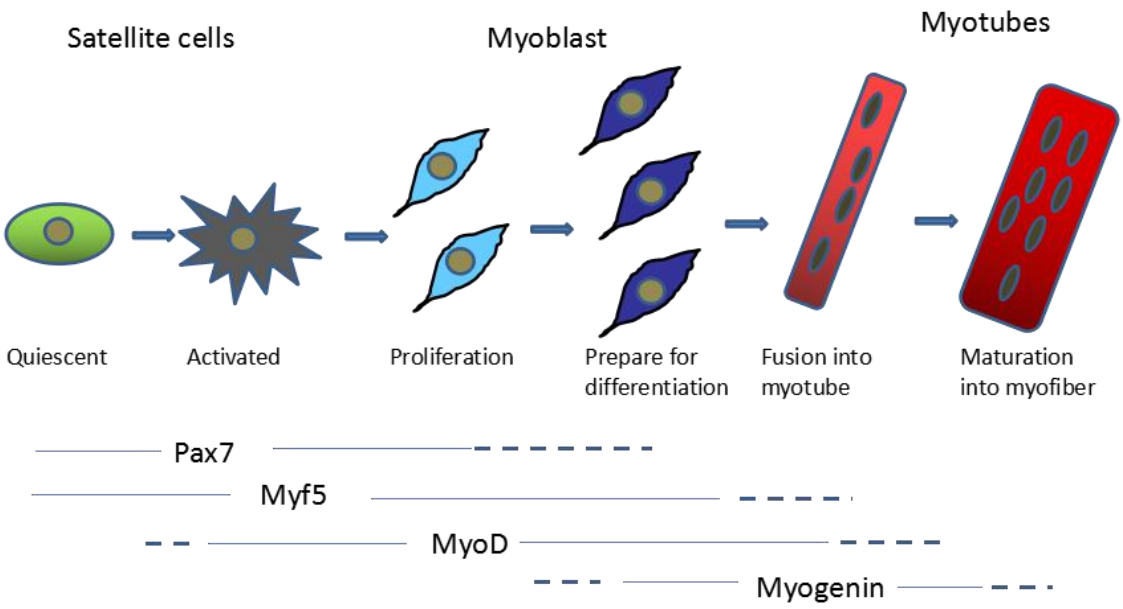

Fig.2. 3 The process of myogenesis

Several studies pointed out that exercise is effective for preserving muscle mass and strength [131]. The combination of exercise and HRT may be more beneficial than either intervention alone for muscle mass and muscular performance. Sipila et al [132] studied CSA of quadriceps and lower-leg muscle of randomly assigned 80 postmenopausal women showed that groups receiving a combination of exercise and HRT have more significant increases in muscle mass than exercise or control group. However, studies investigated changes induced by a combination of exercise and phytoestrogens on muscle mass in postmenopausal women are still limited.

2.3.1.5 Associated bone health

Several studies demonstrated that increases in muscular strength have been associated with the prevention of BMD loss. High intensity resistance training is normally considered to prevent the loss of BMD and has shown osteogenic effects [133, 134]. A study compared resistance training of high intensity to low intensity found that BMD of the hip is only increased after high loading, low repetition resistance training in postmenopausal women [135].

2.3.2 Effects of exercise on energy homeostasis

A chronic imbalance of energy homeostasis is a major cause of obesity and its co-morbidities, such as insulin resistance and type 2 diabetes. It is well shown that exercise prevents negative modifications on body composition induced by menopause. Marked changes in body composition consist of increases in total body fat mass, abdominal fat, and a progressive reduction in muscle mass (sarcopenia) [31, 136]. Skeletal muscle accounts for the most mass of an individual and daily energy consumption [137], indicating that increased muscle mass is associated with a decrease in total and abdominal fat [138]. Several studies showed resistance training decreases body fat and reverses the sarcopenia process associated with menopause [139, 140]. A study by Teixeira et al. [140] demonstrated with or without HRT, 1 year of resistance training in postmenopausal women induces an increase in lean body mass and a decrease of fat tissue size. Also, it is well described that endurance training increases skeletal muscle insulin sensitivity by a modulation of glucose transporter type (Glut)-4 protein expression. A study with female OVX rats has shown that training reduces fat deposition and enhances insulin sensitivity [141]. Another study showed that insulin sensitivity is stimulated with improved glucose metabolism in response to exercise in OVX rats [142], even though the weight gain was unaffected. Nevertheless, there is evidence that physical activity catabolizes adipose tissue [131]. Adipose tissue is mainly responsible for glucose and lipid metabolism. According to the study of Horowitz et al. [36], body fat gain up to a manifested obesity level is associated with decreased metabolic rates of lipolysis and fat oxidation. Exercise training, in contrast, stimulates lipolysis due to an increased activity of fat utilization [143].

Lipid profile is an essential diagnosis for metabolic syndrome. The effects of exercise on lipid profile are mixed. Prabhakaran et al. [144] examined the effects of resistance training on the blood lipid profile showed a decrease in total cholesterol and LDL, without affecting triglycerides and HDL. Similar results were found in another study with endurance exercise in OVX rats [33]. Within a Heritage family study, 48 postmenopausal women, with and without HRT did not show any significant changes in serum lipid levels after an endurance-training program [145], which was proved by another study with 173 obese postmenopausal women who underwent a one-year lasting aerobic exercise training program [146]. Recently, Zois et al. [147] studied the effects of a combined resistance and endurance exercise of 16 weeks in postmenopausal women with type 2 diabetes found that HDL is increased and plasma triglycerides are decreased by training.

2.4 Brief overview of analyzed genes and proteins

2.4.1 Skeletal muscle growth

2.4.1.1 MyoD

MyoD, also known as Myf3, is one of the master regulatory genes in muscle myogenesis. In the early development of an embryo, MyoD plays a key role in facilitating the progression of somitic cells to myoblasts and therefore determining myogenic cell fate with suppressing other cell fates simultaneously. Failure to express MyoD early in mammal development leads to reduced differentiation and growth of muscles [117]. Cultured MyoD-null myoblasts grow fast, but abnormally express target muscle promoter genes (i.e., MHC) and differentiate poorly [148].

MyoD acts as myogenic determinator together with Myf5 [149]. When MyoD gene was deleted in mouse models, Myf5 overexpressed and compensated for MyoD, however, the muscle developed abnormally and showed defects in regeneration [150]. Another study investigated mice deficient in both MyoD and Myf5 found that the mice were failed to form skeletal muscle and dead at birth [150]. Except functioning together with Myf5, MyoD is capable to target a greater number of genes involved in differentiation (i.e., Mrf4 and Myogenin) [149, 151]. Furthermore, mice lacking functional MyoD gene shifts muscle fiber type distribution and MHC isoforms from metabolizing glycogen toward more to oxygen [152].

2.4.1.2 Myogenin

Myogenin, also known as Myf4, is mainly expressed at the beginning of terminal differentiation progression, but before fusing to myotubes [153]. In classical myogenic development, Myogenin is functionally downstream of MyoD. They are functionally and structurally closely related to each other. Just as the other MRFs, Myogenin has its distinct spatial and temporal role in myogenic fate [149]. In mice models with Myogenin-knockout, myoblasts are unable to fuse and form differentiated myofibers and therefore leads to death at birth [154, 155]. Muscle progenitors in these Myogenin-null mice still express MyoD and Myf5, but muscle differentiation is defective [150]. Another animal study showed that myotubes can be formed without Myogenin but the muscle fibers are disorganized, becoming shorter and lacking z-lines [156]. Unlike MyoD, Myogenin transcript mRNA has been found to be expressed greater in slow MHC isoforms [157]. Additionally, over-expression of Myogenin results in a decrease in glycolytic enzyme concentration, indicating an improvement of oxidative metabolism [158].

2.4.1.3 Myosin heavy chain

Several isoforms of MHC proteins are expressed in the sarcomere according to different contractile speed based on ATPase properties. It is normally considered that three distinct MHC isoforms (MHC I, MHC IIa and MHC IIb) exist in human skeletal muscle. In small mammals such as rats or mice, a fourth MHC isoform (MHC IId/x) is identified. This isoform has an intermediate contractile speed between MHC IIa and MHC IIb and has been shown to be homologous to the human type IIb isoform [159]. Fibers mainly composed of MHC IIa, IId/x and IIb display faster contracting speeds, possess higher ATPase activities and stronger muscle forces compared with fibers mainly composed of MHC I [160].

Since the types of muscle fibers can be shifted by exercise or growth factors, the activity of MHC is altered as well. A study showed that the protein expression of MHC IIb was reduced and MHC IIa was increased followed by a 19-week heavy resistance training program in humans [161]. Similar results of MHC isoforms shifting were observed in two respective studies using electrostimulation resistance training in humans [162] and rats [163]. Staron et al [164] reported that weight lifters have exceptionally larger type IIa fibers and a lower percentage of type IIb fibers compared to sedentary control subjects. Together with findings showing that MHC IId/x is down-regulated and MHC IIa is up-regulated in the biceps muscle of male bodybuilders after training [165], it suggests that the type IIa fibers are used, with MHC IIa predominantly expressed, after accustomed resistance training performing. Moreover, hormonal status affects MHC expression and muscle fiber type. MHC I expression in soleus muscle is increased due to hypothyroidism [166]. In addition, muscle fiber type is shifted toward type I after menopause in females, leading to a reduction in skeletal muscle force [167]. The same results were also observed in several different muscle tissues (soleus, extensor digtitorum longus, plantaris) in OVX animals [168, 169].

2.4.1.4 Insulin-like growth factor-1

IGF-1 is one of the major growth factors showing influences on hormone system and regulating physiological processes in cell survival and differentiation of not only prenatal growth but also overall adult growth. IGF-1 was reported to coordinate the process of myogenesis by affecting downstream MyoD, Myf5 and Myogenin expression [170]. The encoded gene of IGF-1 is well characterized and extensively studied. Some of the domains

of the resulting peptide are homologous to chains of insulin. However, IGF-1 is post-processed differently and comes with additional domains which have no analog in insulin. The isoforms of IGF-1 are various due to different ways of splicing IGF-1 transcripts and different exons, leading to different C-terminal domains and N-terminal domains.

The isoforms differ not only in structure but also in their localization and physiological effects. The systemic circulating IGF-1 is thought to be connected to overall postnatal growth, which is mostly secreted from a liver. A study showed that non-hepatic IGF-1 production from skeletal muscle and adipose tissue also contributes to circulating IGF-1 levels [171]. Recently, some studies also pointed out locally expressed IGF-1 splice variants in muscle, called as Mechano Growth Factor (MGF), react to physical stress and muscle damage [172]. In addition to anabolic effects of IGF-1, hepatic IGF-1 was reported to mainly regulate carbohydrate and lipid metabolism rather than growth promotion because IGF-1 in liver is not required for postnatal body growth [173, 174]. Sjogren et al. [173] also showed that mice lacking hepatic IGF-1 develop into decreased fat mass and increased serum cholesterol.

2.4.1.5 Insulin-like growth factor-1 receptor

It is well documented that the endocrine, autocrine and paracrine functions of IGF-1 are mediated through binding to IGF-1R, which is a ligand-activated tyrosine kinase [175]. IGF-1R consists of two extracellular α-subunits and two transmembrane β-subunits which contain IGF-1 binding site and a cluster of tyrosine residues respectively [176]. When IGF-1 binds to IGF-1R, it induces autophosphorylation which recruits specific cytoplasmic molecules like insulin receptor substrate proteins (IRS) [177]. Two main signaling pathways have been proposed to be associated with the activation of the IGF-1R [178]. One pathway is activation of phosphatidylinositol 3-kinase (PI3K) through phosphorylation of IRS-1. PI3K is involved in cellular processes such as protecting from apoptosis by activating protein kinase B (PKB/AKT) [179]. Another pathway is Ras-Raf to extracellular response kinases (ERKs), which in turn activates phosphorylation of several other protein kinases and transcription factors. The Ras-Raf-ERK signaling pathway has also been shown to promote cell proliferation in in vitro studies with muscle cells [175]. Moreover, the binding of IGF-1 to IGF-1R is a key to initiate many other mitogenic and myogenic signaling cascades, including mitogen-activated protein kinase (MAPK) and mechanistic target of rapamycin (mTOR) pathways [180].

2.4.1.6 Muscle RING finger-containing protein 1

Muscle RING finger-containing protein 1 (MuRF1) is normally known as a marker for muscle atrophy that represents protein degradation. It encodes a protein that contains four domains and is localized to the sarcomere. MuRF1 has been shown to bind the giant sarcomeric protein titin [181]. In addition, MuRF1 was reported to interact with and control the half-life of many important muscle structural proteins, including troponin I, actin, MHCs, myosin light chains and myosin binding protein C [121]. Mice lacking MuRF1 are resistant to muscle atrophy induced by denervation [182] and dexamethasone [183].

2.4.2 Lipogenesis and lipolysis

2.4.2.1 Sterol regulatory element-binding protein-1c

Sterol regulatory element-binding proteins (SREBPs) belong to the bHLH-zipper family of transcription factors and are synthesized as inactive precursors binding to the endoplasmic reticulum [184]. Two separate genes encode for different SREBPs, SREBP1 and SREBP2. The SREBP1 gene is further able to produce two proteins, SREBP-1c and SREBP-1a, which results from the use of two different transcription start points [185]. SREBP1 is mainly expressed in livers and adrenal glands, whereas SREBP2 is expressed in many tissues. SREBP-1a applies wide uses in gene transcription which potentially activate all genes mediating the synthesis of cholesterol, fatty acids, and triglycerides which are responsive to SREBP. However, SREBP-1c preferentially activates transcription of genes required for fatty acid synthesis and SREBP2 is mainly for cholesterol synthesis [185]. Genes responsive to SREBP-1c contain those for ATP citrate lyase, acetyl-CoA carboxylase and fatty acid synthase.

Regulation of SREBPs occurs at transcriptional and post-transcriptional levels. The transcriptional regulation of the SREBPs is rather complex. Liver X receptors (LXRs) are nuclear receptors forming heterodimers with retinoid X receptors (RXRs), which are well reported to regulate the gene expression of SREBP1c in the liver and adipose tissue through an insulin-mediated activation [186]. Exposure to insulin increases hepatic SREBP1c mRNA expression in mice and rats [187]. LXR and RXR are well studied in liver and adipose tissue. The agonists of LXR/RXR are able to markedly induce SREBP1c gene expression, even if cholesterol levels are high [188]. The polyunsaturated fatty acids inhibit LXRα/RXRα binding to the LXR-response elements in the SREBP1c promoter

region, which reduces SREBP1c gene expression and suppressed lipogenesis [189]. Furthermore, LXR and RXR activate the SREBP1c promoter in C2C12 cell line and an over-expression of RXR increases SREBP1c gene expression with an increase of triglyceride content in skeletal muscle [190]

2.4.2.2 Fatty acid synthase

Fatty acid synthase (FAS) is encoded by the FASN gene. The main function of FAS is to catalyze the reaction of acetyl-CoA and malonyl-CoA to the major product palmitate (C 16:0). Stearate (C 18:0) and shorter acids may also be produced. In this process, the FAS protein functions as a homodimer, assembled by 273 kDa subunits, which is only enzymatically active in a dimeric form [191]. A study using immunofluorescent showed that the FAS protein seems to be localized in the cytoplasm [192]. Livers, adipose tissue and lungs were found to have high protein expression of FAS [193]. The FASN promoter region contains binding sites which control regulatory elements and transcription factor activating FAS transcription [194]. The sterol regulatory elements in the binding sites of promoter regions of FASN contribute to the regulatory role of SREBP1c on activating FAS expression [195]. In addition, the FAS expression was reported that can be inhibited through inverting CCAAT box binding site [196].

2.4.2.3 Peroxisome proliferator-activated receptor δ

PPARs are members of the nuclear hormone receptor superfamily. PPARα, PPARβ/δ and PPARγ are three isoforms that have been identified. PPARs, together with 9-cis-retinoic receptors, bind to specific PPAR-response DNA elements as heterodimers, therefore regulate various metabolic and developmental pathways. PPARs are activated by dietary fatty acids, particularly polyunsaturated fatty acids. PPARδ, also called PPARβ, is activated by long-chain fatty acids, prostacyclin and some synthetic molecules [197]. It showed that PPARδ is abundantly expressed throughout the body but at low levels in liver. Through the help of synthetic PPARδ agonists, genetic studies have revealed the role of PPARδ in regulating fatty acid catabolism and energy homeostasis [198]. The PPARδ agonist GW501516 was shown to exert a great impact on lowering plasma triglyceride levels whilst raising HDL levels in obese monkeys, suggesting that PPARδ agonists might be effective drugs to treat hyperlipidemic patients [199].

Furthermore, studies in muscle and adipose tissue started to uncover the biological functions of PPARδ. PPARδ-deficient mice were shown susceptible to obesity with

high-fat diet, while activation of PPARδ in adipocytes and skeletal muscle cells promoted fatty acid oxidation and utilization [200]. PPARδ is also associated with improvement of insulin resistance induced by diet [201], probably as a result of increased fat-burning by muscle and overall improvement in systemic lipid metabolism.

2.4.2.4 Peroxisome proliferator-activated receptor-γ coactivator-1α

Peroxisome proliferator-activated receptor-γ coactivator (PGC)-1α is a member of a family of transcription coactivators that interacts with many transcription factors involved in mitochondrial biogenesis, adaptive thermogenesis, skeletal muscle fiber type shifting and glucose/fatty acid metabolism. The gene of PGC-1α is located on chromosome 5 (mice) or chromosome 4 (human) and encodes a protein containing 797 (mouse) or 798 amino acids (human). PGC-1α is abundantly expressed in the brown adipose tissue, heart and skeletal muscle where tissues have a number of mitochondria and are active in oxidative metabolism. So it is expressed relatively low in liver and very low in white adipose tissue [202].

The transcription of PPARs is subjected to PGC-1α. PGC-1α was originally discovered as a cold-inducible transcription coactivator of exhibiting effects on adaptive thermogenesis in brown adipose tissue, which is also found in the functions of PPARδ [202, 203]. Additionally, the PGC-1α expression is strongly associated with PPARδ in both cultured cells and tissues [200], suggesting that many metabolic effects of PGC-1α might be mediated through PPARδ. In skeletal muscle, it is now well established that PGC-1α stimulates mitochondrial biogenesis, resulting in a remodeling of skeletal muscle fiber composition to a fiber-type metabolically more oxidative and less glycolytic. PGC-1α-deficient mice showed reduced ability on adaptation to muscle performance, including decreases in mitochondrial number and respiratory capacity of slow-twitch muscle [204]. Furthermore, PGC-1α was reported to suppress fat accumulation in muscle, therefore increase insulin sensitivity and activate insulin sensitive Glut-4 expression [205]. In type 2 diabetic subjects, PGC-1α expression was observed to decrease in skeletal muscle [206]. In contrast, the hepatic PGC-1α expression was reported to increase in a type 2 diabetic mice model [207].

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CHAPTER 3: AN ISOFLAVONE ENRICHED DIET INCREASES SKELETAL MUSCLE ADAPTATION IN RESPONSE TO PHYSICAL ACTIVITY IN OVARIECTOMIZED RATS

This study has been accepted as:
Wenya Zheng, Jonas Hengevoss, Sebastian T. Soukup, Sabine E. Kulling, Mingyong Xie, Patrick Diel*. *Molecular nutrition & food research* DOI:10.1002/mnfr.201600843

Abstract

Scope: This study was to investigate anabolic adaptation of skeletal muscle in response to an isoflavone (ISO) enriched diet, training and their combinations in ovariectomized (OVX) rats.

Methods and results: Female Wistar rats were sedentary, performed treadmill uphill running, received ISOs, or a combination of ISOs and running after ovariectomy. Body weight was increased by OVX. Both ISO and training treatment antagonized this increase. The weights of soleus and gastrocnemius muscles were increased only when training and ISOs were combined. In soleus muscle insulin-like growth factor (IGF)-1R, MyoD and Myogenin expressions were only up-regulated by training in Sham groups. However, a stimulation of IGF-1R and MyoD expression could be observed when ISOs and training were combined. In gastrocnemius muscle MyoD and Myogenin expressions were stimulated by either training or ISOs. Additive effects were detected when combining the two interventions.

Conclusion: Our results indicate that the combination of ISOs and exercise is more efficient in increasing relative skeletal muscle mass and the expression of molecular markers related to anabolic adaptation in the skeletal muscle of female rats.

Key words: Female, Ovariectomy, Soy isoflavones, Skeletal muscle, Training

Abbreviations: CSAs, cross-sectional areas; Dai, daidzein; E_2, 17β-estradiol; Gen, genistein; HRT, hormone replacement therapy; IGF-1, insulin-like growth factor 1; IGF-1R, insulin-like growth factor 1 receptors; ISOs, isoflavones; MRFs, myogenic regulatory factors; OVX, ovariectomized; PND, postnatal day.

3.1 Introduction

Due to the increase in life expectancy, the time women spend in postmenopause throughout their lifetime has expanded. The decline in ovarian sex hormone production after menopause is associated with an increased risk to develop metabolic diseases like obesity, type 2 diabetes, coronary heart disease, metabolic syndrome and bone loss [1-3]. The decrease in serum estrogen levels also alters the skeletal muscles' metabolic, structural and functional characteristics [4]. Losing skeletal muscle mass is associated with a loss of strength but also with the development of metabolic diseases especially type 2 diabetes [5]. Nutrition, hormone replacement therapy (HRT), Vitamin D consumption and exercise have been shown to attenuate loss of muscle mass and improve the quality of life [6].

Administration of phytoestrogens is discussed to be an alternative for HRT. Isoflavones (ISOs) are the most well known phytoestrogens. They are structurally similar to 17β-estradiol (E_2) and display estrogenic activities in mammals [7]. Lean body mass and muscle mass of obese-sarcopenic postmenopausal women were significantly increased after supplementation of ISOs [8]. Furthermore, exercise has been reported to overcome negative health effects associated with menopause and is often recommended for increasing muscle mass and strength, especially for elder people [9].

The knowledge of adaptation of skeletal muscle on muscle growth regarding a combination of an ISO-enriched diet and exercise is still limited. Therefore, it was the major aim of this study to investigate combinatory effects of an ISO-enriched diet and exercise on skeletal muscle anabolic adaptation in an animal model representing the situation of postmenopausal women. Female OVX Wistar rats were fed with an ISO-depleted diet or ISO-enriched diet. The rats either remained sedentary or performed a specific uphill running. Body weight and several organs (muscle soleus, muscle gastrocnemius, heart, uterus, ovarian and bone tibia) were weighed after the dissection. IGF-1, IGF-1R, MyoD and Myogenin expressions were investigated in skeletal muscle. IGF-1 levels were additionally measured in serum.

3.2 Materials and methods

3.2.1 Animal treatment

Forty-four female Wistar rats (~250g, Janvier, Le Genest St Isle, France) were randomly grouped and got either Sham or OVX operated at postnatal day (PND) 84. Rats either received standard ISO-depleted diet or an ISO-rich diet. The study design is shown in Fig. 1. Treatment groups are assigned as follows: (1) Sham; (2) Sham operated with training

(Sham+T); (3) OVX; (4) OVX+T; (5) OVX with an ISO-enriched diet (OVX+ISO); (6) OVX+ISO+T. The training was conducted on an uphill treadmill for 61 days. We provided more explanation about animal treatment in Supporting Information.

Group	Isoflavone-free diet	Isoflavone-rich diet	Training
Sham (n=7)	×		
Sham+T (n=6)	×		×
OVX (n=12)	×		
OVX+T (n=6)	×		×
OVX+ISO (n=7)		×	
OVX+ISO+T (n=6)		×	×

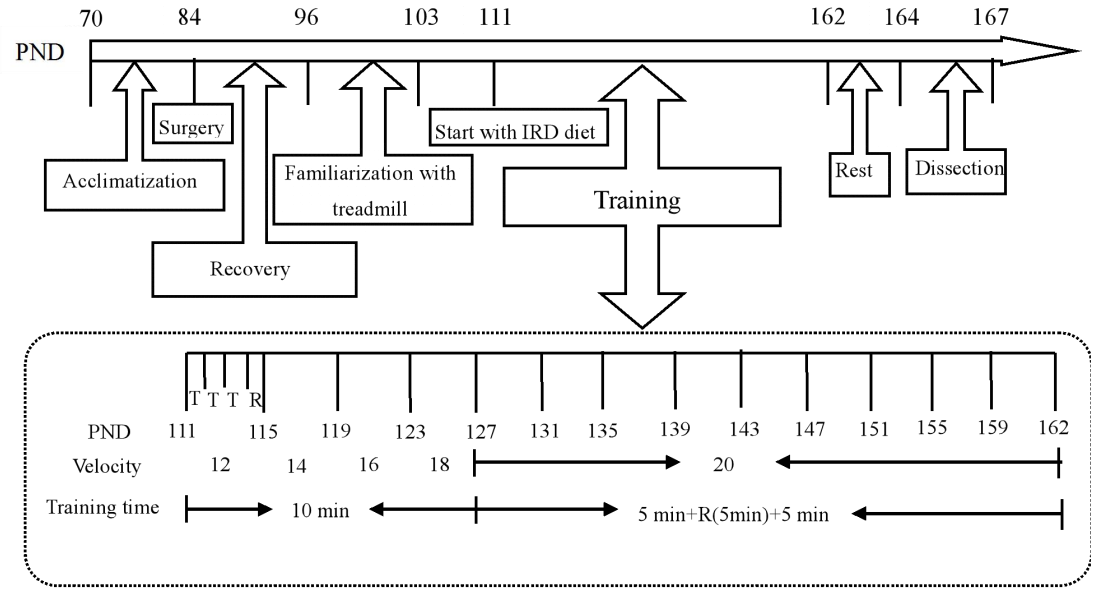

Fig.3. 1 Experimental design

T=Training, R=Rest, Sham = sham operated, OVX =ovariectomized. The rats were OVX or Sham operated and allocated into six different treatment groups. Two different diets, an isoflavone-depleted diet and an isoflavone-enriched diet (ISO), were used. The uphill-training was conducted on a motor-driven treadmill with an incline of 25°. The animals of the training groups were trained for 10 min, twice a day. Every fourth day was a rest day.

3.2.2 Quantification of ISOs in animal diet

Quantification of ISO levels in animal diets were performed as described previously [10].

3.2.3 Quantification of ISOs in plasma

Quantification of the ISOs genistein (Gen), daidzein (Dai) and their corresponding phase II metabolites in plasma were determined by ultra-performance liquid chromatography tandem mass-spectrometry (UHPLC-MS/MS) as described previously [11, 12] and summarized as Gen+Dai aglycone equivalents. The measuring conditions for the analytes are given in Supporting Information Table 1.

3.2.4 Immunohistochemical staining and determination of skeletal muscle fiber size

Immunohistochemical staining and determination of muscle fiber size of soleus and gastrocnemius muscles were performed as previously described [13].

3.2.5 Determination of IGF-1 in serum

The serum concentrations of IGF-1 were measured in triplicates using ELISA kits for rats according to the manufacturer's instruction (IGF-1 mouse/rat ELISA DEE025, Demeditec Diagnostics, Kiel, Germany).

3.2.6 Real-time PCR experiments

mRNA analysis was performed like previously described [14, 15]. More detailed explanation is provided in Supporting Information. The following rat-specific primers were synthesized by Invitrogen™ (Germany): cyclophillin (housekeeping gene), fwd: 5'-GGATTCATGTGCCAGGGTGG-3', rev: 5'-CACATGCTTGCCATCCAGCC-3'; IGF-1, fwd: 5'-CCGCTGAAGCCTACAAAGTC-3', rev: 5'-TGTTTTGCAGGTTGCTCAAG-3'; IGF-1R, fwd: 5'-TTCTTCGTTTCGTCATGGAG-3', rev: 5'-AAACTGGGCTCCATCTCATC-3'; MyoD, fwd: 5'-GGAGACATCCTCAAGCGATGC-3', rev: 5'-AGCACCTGGTAAATCGGATTG-3'; Myogenin, fwd: 5'-CCAGTGAATGCAACTCCCAC-3', rev: 5'-GCAGACAATCTCAGTTGGGC-3'.

3.2.7 Western blotting

Western blotting was performed as previously described with pooled samples [14, 16]. Proteins were detected using primary anti-MyoD antibodies (45-47 kDa, mouse anti-MyoD, D7F2, Developmental Studies Hybridoma Bank, University of Iowa, United States) and

primary anti-alpha-Tubulin antibodies (50 KDa, mouse anti-alpha-Tubulin antibody, 12G10, Developmental Studies Hybridoma Bank, University of Iowa, United States) over night at 4°C. Protein expression of MyoD was related to the reference protein alpha-Tubulin.

3.2.8 Statistical analysis

All data were expressed as means ± standard deviation (SD). Statistical significance of differences was calculated using Kruskal–Wallis test with a subsequent Mann–Whitney U-test (GraphPad Prism, version 5). Statistical significance was established at $p \leq 0.05$.

3.3 Results

3.3.1 ISO content in plasma

Measurement of the plasma ISO concentrations of three groups (OVX, OVX+ISO and OVX+ISO+T) (Fig.2A) showed that mean Gen+Dai aglycone equivalents in OVX+ISO and OVX+ISO+T were 1947 and 1556 nM, whereas small amounts of Gen+Dai aglycone (mean of 10 nM) were detected in OVX animals with ISO-depleted diet. There was no significant difference between OVX+ISO and OVX+ISO+T group.

3.3.2 Parameters related to skeletal muscle mass

Body weight gain from PND 103 to PND 164 was shown in Fig.2B. OVX in rats was associated with an increase in body weight ($p<0.05$). Both training and ISO diet antagonized body weight gain in OVX rats ($p<0.05$). Mean weight gain of OVX+ISO+T group was lower (0.82 and 0.75-fold, respectively) compared to OVX+T and OVX+ISO groups, although these differences were not statistically significant. The relative soleus muscle weight (Fig.2C) was not significantly affected by OVX, whereas the relative gastrocnemius muscle weight (Fig.2D) was decreased by OVX ($p<0.05$). OVX induced skeletal muscle weight loss couldn't be prevented by ISOs. An increase in relative muscle weight of 10.1% of soleus and 4.3% of gastrocnemius was detected after the training, although these differences were not statistically significant. However, ISO+T antagonized OVX induced muscle loss significantly ($p<0.05$). Moreover, the organ weights of uterus, heart, bone tibia were shown in Supporting information Fig.1. OVX resulted in a strong atrophy of the uterus and decreased relative tibia weight which was antagonized by ISO+T. Additionally, training restored the relative heart weight in OVX rats.

The fiber sizes of cross-sectional areas (CSAs) in the soleus and gastrocnemius muscles

were not significantly affected by OVX, training or ISOs (Fig.2E and 2F). However, the mean fiber size of soleus muscle in OVX+T group was 14.7% higher than in OVX group. Also, mean fiber size of OVX+T and OVX+ISO groups in gastrocnemius muscle were 16.0% and 20.9% higher than OVX group.

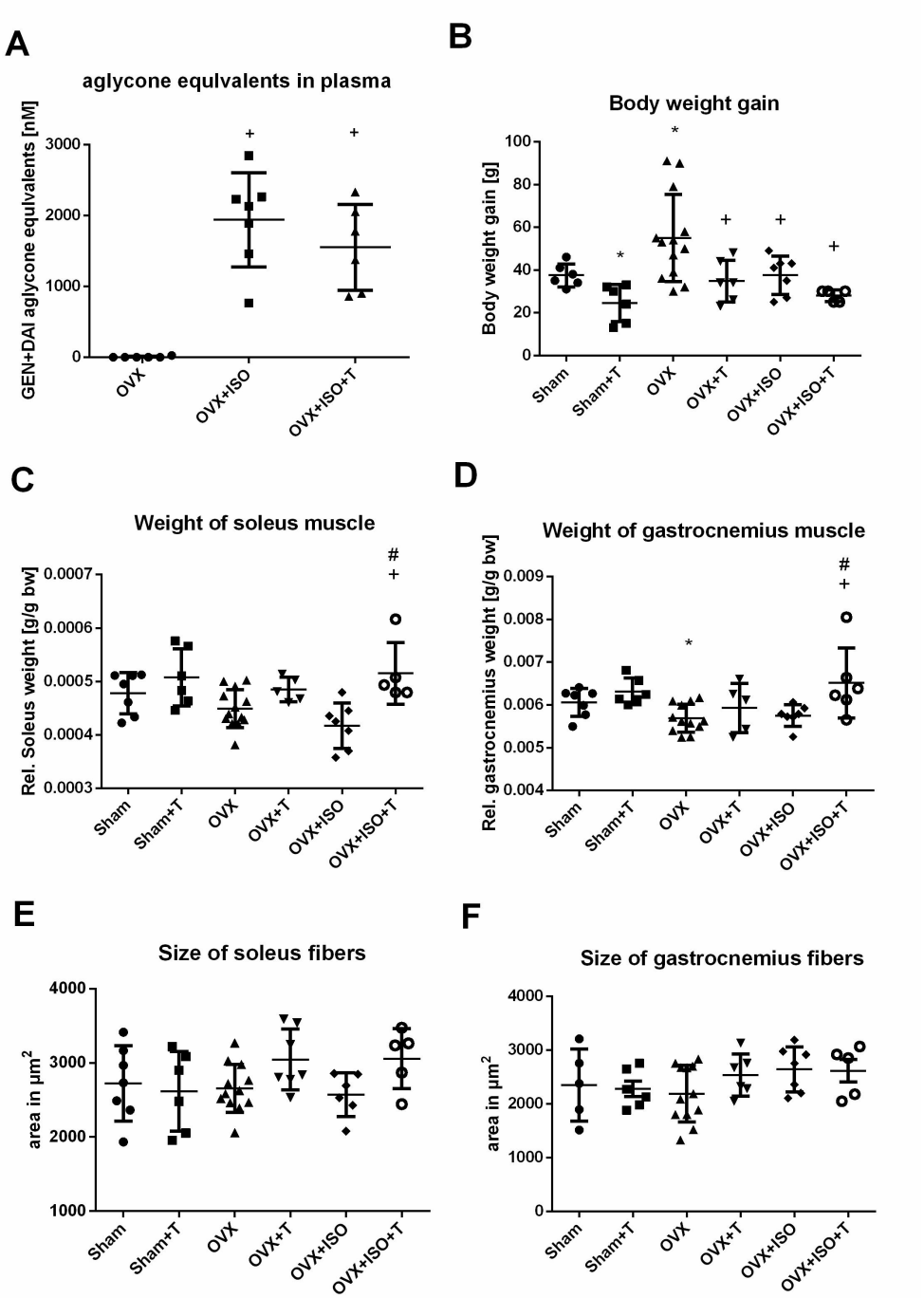

Fig.3. 2 Plasma ISO concentrations (A) as sum of daidzein (Dai), genistein (Gen) and their corresponding phase II metabolites (Gen+Dai aglycone equivalents) after dietary ingestion in OVX rats

and effects of training, ISOs, ISO+T on body weight gain (B), relative soleus muscle weight (C), relative gastrocnemius muscle weight (D), soleus muscle fiber size (E) and gastrocnemius muscle fiber size (F)

Mean values were significantly different for the following comparisons: * marks when significant differences between Sham and Sham+T, Sham and OVX ($p \leq 0.05$); +vs. OVX ($p \leq 0.05$) when compared within OVX groups; # marks when significant differences between OVX+ISO and OVX+ISO+T ($p \leq 0.05$).

3.3.3 IGF-1 and IGF-1R expressions

Circulating levels of IGF-1 in serum were higher in OVX than in Sham rats ($p<0.05$). Training or ISO showed no effects (Fig.3A).

In soleus muscle IGF-1 mRNA expression was increased by training in both Sham and OVX rats ($p<0.05$, 1.20 and 1.27-fold). The strongest increase of IGF-1 in OVX animals was observed in the OVX+ISO+T group ($p<0.05$, 1.42-fold compared to OVX group) (Fig.3B). Regarding IGF-1R, training increased its gene expression in Sham rats ($p<0.05$, 1.48-fold). ISO+T resulted in a significant increase of IGF-1R in OVX animals ($p<0.05$, 1.24-fold) (Fig.3C).

In gastrocnemius muscle no significant regulation of IGF-1 and IRF-1R mRNA expression was observed (Fig. 3D and 3E).

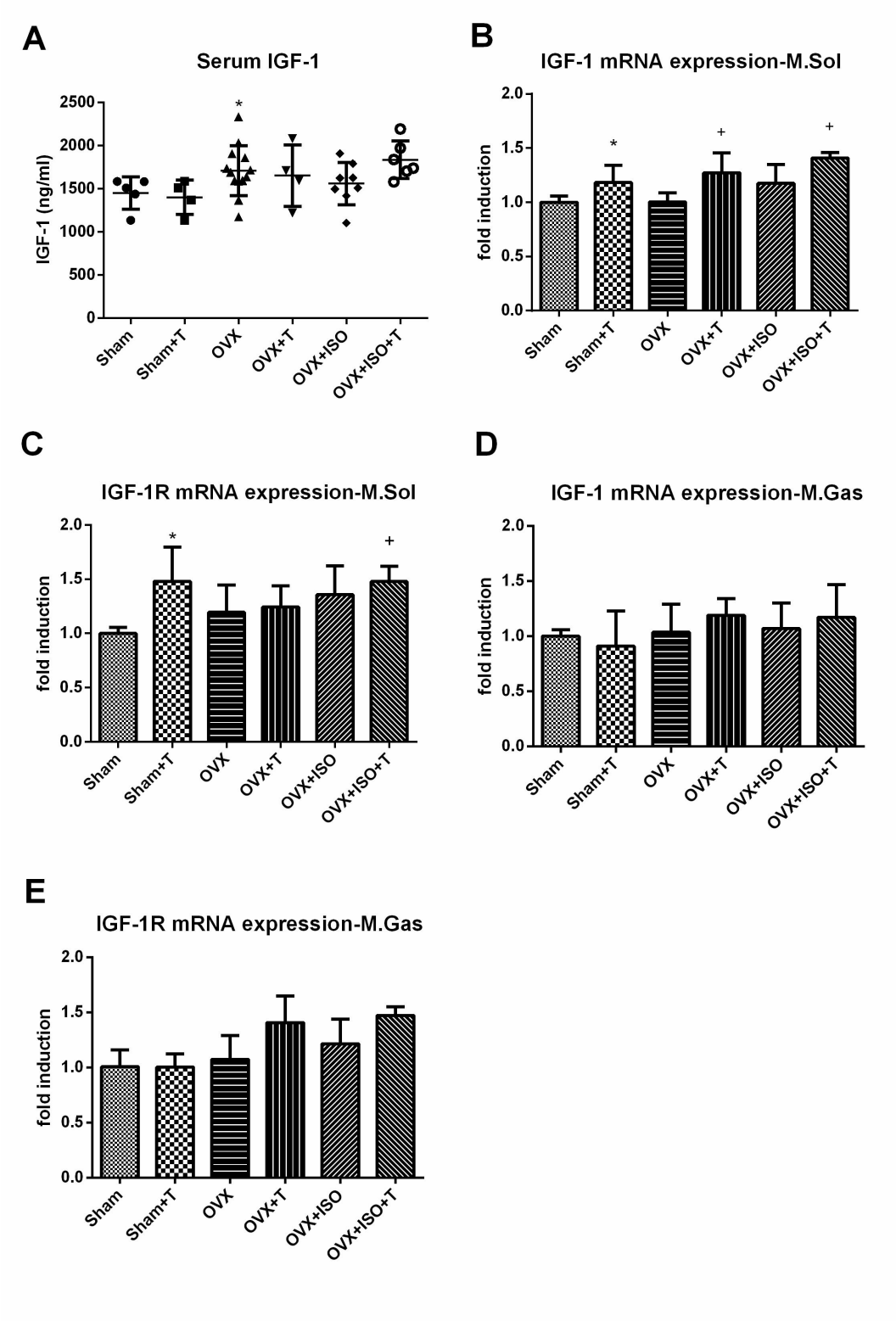

Fig.3. 3 Effects of training, ISOs and ISO+T on serum IGF-1 levels (A), the relative mRNA expressions of IGF-1 and IGF-1R in soleus muscle (B and C), and gastrocnemius muscle (D and E)

Mean values were significantly different for the following comparisons: * marks when significant differences between Sham and Sham+T, Sham and OVX ($p \leq 0.05$); +vs. OVX ($p \leq 0.05$) when compared within OVX groups.

3.3.4 MyoD and Myogenin expressions

MyoD and Myogenin, indicating proliferation and differentiation of satellite cells, were further investigated in soleus and gastrocnemius muscle. MyoD expressions in soleus muscle were up-regulated by training in Sham and ISO+T in OVX rats ($p<0.05$, 1.71 and 1.58-fold) (Fig.4A). Myogenin expression was only stimulated by training in Sham groups ($p<0.05$, 1.69-fold). No effects were observed by training, ISOs or ISO+T in OVX rats (Fig.4B).

Gene expressions of MyoD (Fig.4C) and Myogenin (Fig.4E) in gastrocnemius muscle were reduced by OVX ($p<0.05$, 0.56 and 0.63-fold). The effects were antagonized by either training ($p<0.05$, 1.39 and 2.30-fold compared to OVX group) or ISOs ($p<0.05$, 1.91 and 1.79-fold compared to OVX group). Moreover, MyoD expression of OVX+ISO+T showed an additive character compared to OVX+T ($p<0.05$, 1.86-fold), and OVX+ISO+T induced an additive effect on Myogenin expression compared to OVX+ISO ($p<0.05$, 1.38-fold). The strongest antagonizing effect was observed in the combined group (2.59 and 2.49-fold compared to OVX group). Because the effect on MyoD mRNA expression was most striking, its expression was also investigated on protein levels. As shown in Fig.4D the protein data is completely in line with the observed effects of the MyoD mRNA expression.

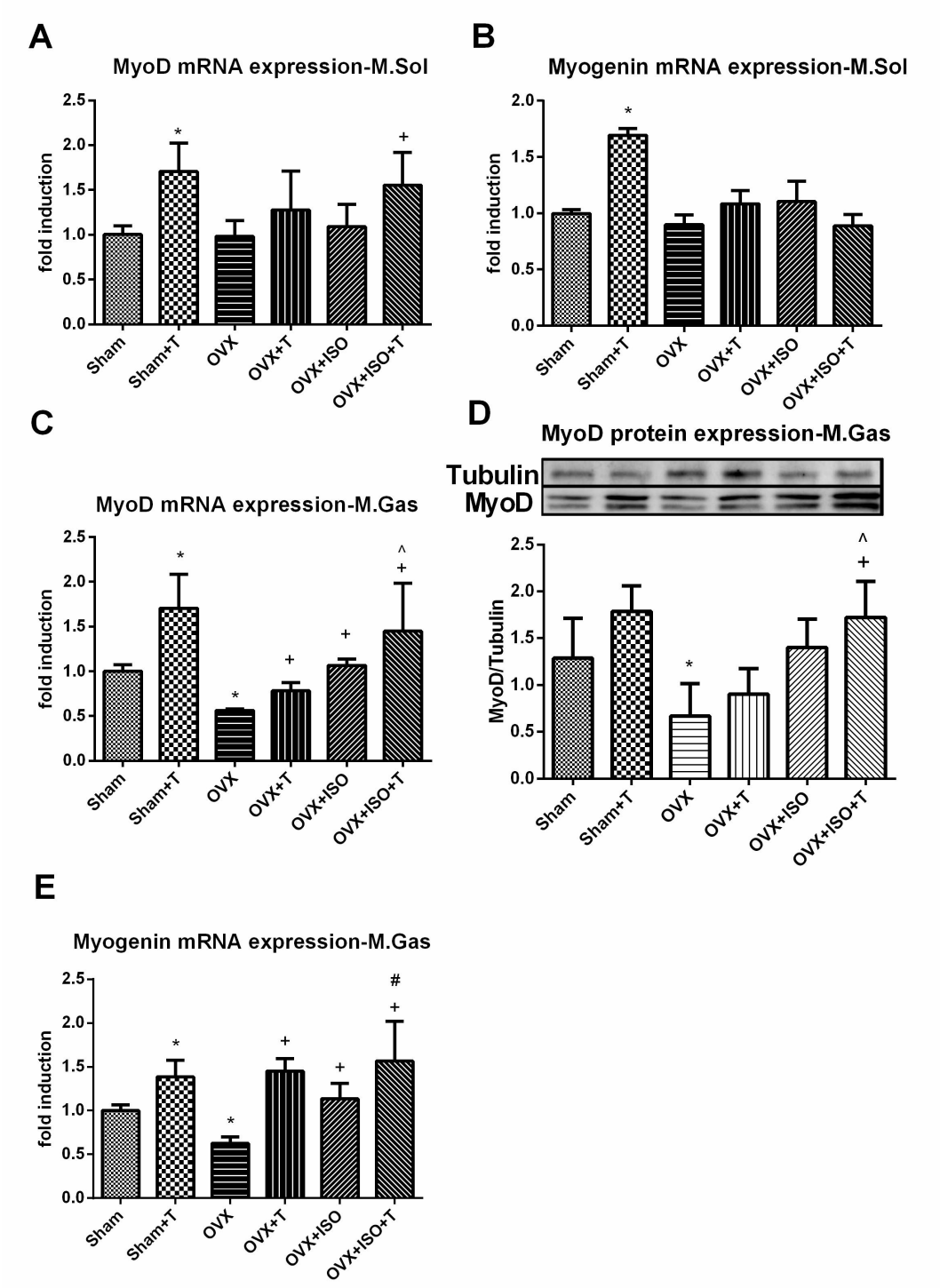

Fig.3. 4 Effects of training, ISOs and ISO+T on the relative mRNA expressions of MyoD and Myogenin in soleus muscle (A and B), and gastrocnemius muscle (C and E). (D) shows effects on MyoD protein expression in gastrocnemius muscle

The bands are representative of four individual runs of western blot results for detecting MyoD and Tubulin. Mean values were significantly different for the following comparisons: * marks when significant differences between Sham and Sham+T, Sham and OVX ($p \leq 0.05$); +vs. OVX ($p \leq 0.05$) when compared groups within OVX groups; # marks when significant differences between OVX+ISO and

OVX+ISO+T ($p \leq 0.05$); ^ marks when significant differences between OVX+T and OVX+ISO+T ($p \leq 0.05$).

3.4 Discussion

The main findings of this study are 1) the combination of training and ISO diet increased the relative weights of soleus and gastrocnemius muscle and thereby antagonized the loss of muscle mass caused by OVX.; 2) in soleus muscle an increase of IGF-1, IGF-1R and MyoD gene expression was observed in the OVX+T+ISO group compared to OVX control; 3) in gastrocnemius muscle of the OVX+ISO+T group mRNA expression of MyoD and Myogenin was increased compared to OVX control.

Summarizing our experimental design we can conclude that a) OVX Wistar rats were chosen as an animal model to mimic the situation of postmenopausal women. b) The used training protocol was designed based on our previous studies [4, 17] and modified according to the reaction of the rats. The speed was finally kept at 20 m/min at which point the training reached as heavy exercise [18]. c) Animals exposed to the ISO enriched diet reached ISO plasma concentrations which are comparable to those in Asians consuming moderate to high amounts of soy products [12].

Training and ISO diet influenced a variety of physiological parameters in this study. In our study estrogen deficiency leads to increased body weight. This is in line with literature data and described to be caused by changes in the body composition including a reduction of skeletal muscle mass and an increase of fat mass [1, 19]. We also observed an antagonizing effect of training on body weight gain, which is line with in our previous study [20]. Additionally, the antagonizing effect of ISOs on body weight gain was demonstrated that comparable to effects observed for E_2 in our previous study [20]. A combination of ISOs and training was most effective in antagonization of weight gain which is in line with published data [21]. Additionally, the ISO+T resulted in an increase of the relative weights of the musculus gastrocnemius and soleus (Fig.2C&2D). However, the increase in muscle weight is not associated with an increase in muscle fiber diameter (Fig.2E&2F). It has already been demonstrated that muscle fiber size of CSAs does not account for all the observed increases in muscle mass, and other mechanisms, such as muscle fiber hyperplasia, may play a role in contributing to muscle mass increases [22].

The combinatory effects of training and ISOs could be also observed in this study analyzing molecular markers related to skeletal muscle adaptation. IGF-1 is one of the main effectors involved in the regulation of cell growth, cell survival and differentiation in

many tissues [23], Our results showed that circulating IGF-1 levels in OVX rats were higher than in Sham rats (Fig.3A), which was in line with a study in OVX rats where E_2 supplementation was shown to down-regulate plasma IGF-1 levels in OVX rats [24]. In this study ISO intake did not result in similar effects. However, the result was in line with one of our previous studies reporting that no effects were observed on serum IGF-1 by ISOs [12]. IGF-1 has been described as a potent anabolic marker for muscle tissue [23]. Studies investigating influences on circulating IGF-1 levels by training are conflicting which might be due to different training protocols. The gene expression of IGF-1 in gastrocnemius muscle (Fig.3D) was also not affected by training, which partially was in line with results from studies that were done with 12-week long-term resistance training [25]. In contrast, IGF-1 expression in soleus muscle (Fig.3B) was up-regulated by training. The reason could be that overloading stimulated early increases in IGF-1 in slow twitch oxidative fibers than in fast twitch glycolytic fibers in rodents.

It is well known that the endocrine, autocrine and paracrine functions of IGF-1 are all mediated through binding to IGF-1R [26]. IGF-1R mRNA and protein levels increased in response to various types of exercise [27, 28], which could be observed in our study of soleus muscle (Fig.3C). In OVX rats, ISOs alone did not show a significant effect on regulating IGF-1R, whereas an up-regulating effect could be seen by ISO+T in soleus muscle. The effect on IGF-1R expression was in consistent with the IGF-1 expression, and showed that ISOs partly act as estrogen in OVX rats to increase the IGF-1R expression when combined with training because IGF-1R mRNA expression could also be increased by training in Sham rats with endogenous estrogen.

Proliferation and differentiation of satellite cells play an essential role in the adaptation process of skeletal muscle. MyoD and Myogenin are two important transcription factors belonging to the family of MRFs that function as main activators of skeletal muscle differentiation and also interact with certain growth factors [29]. The training effect of increasing MyoD and Myogenin expressions in gastrocnemius muscle (Fig.4C&4E) in this study was in line with many previous studies [25, 30]. ISO diet is also supposed to influence postnatal muscle growth [12]. However, the combinatory effects have not been deeply investigated so far. Our present study demonstrated for the first time that ISO+T results in strong additive effects on the regulation of gene expression of MyoD and Myogenin in gastrocnemius muscle. In addition, western blotting with a specific antibody against MyoD confirmed the mRNA result of MyoD in gastrocnemius muscle (Fig.4D). Time course studies of myogenic gene expressions in response to loading in human

skeletal muscle [30] indicated time dependent effects on the expression of these genes which might be a reason for missing significant changes of Myogenin expression in soleus muscle (Fig.4B).

In summary, our data showed that estrogen deficiency led to an increase in body weight. The combination of designed exercise and ISO diet antagonized this most efficiently resulting in an increase of relative skeletal muscle mass. Molecular markers related to muscle anabolic activity were significantly modulated by combining ISO diet and exercise. Therefore, the results of our present study provide evidence that a combination of ISO-enriched diet and exercise could be a promising intervention concept for increasing skeletal muscle anabolic activity and therefore preventing muscle loss particularly in postmenopausal females.

Author contributions

W.Z planned and conducted the animal experiment, collected and analyzed data, and wrote the manuscript. J.H was involved in conducting animal experiment. S.T.S and S.E.K. performed ISO analytics. M.X was involved in designing the study. P.D. mainly designed the study and gave conceptual advices for the manuscript.

Acknowledgments

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Conflicts of interest

The authors have declared no conflicts of interest.

3.5 Supporting Information

Materials and Methods

Animal treatment

Forty-six female Wistar rats (~250g, Janvier, Le Genest St Isle, France) were kept under controlled conditions of illumination (12/12 h day/night cycle) and constant room temperature (20 ± 1°C, relative humidity 50–70%). The rats had free access to food (SSniff GmbH, Soest, Germany) and water. Body weight and food intake were monitored twice/week. All animal handling and experiments were approved by the Committee of Animal Care (permission number 84.02.04.2013.A234).

Before experimental procedures, all rats were randomly grouped and got either Sham or OVX operated via the dorsal route at postnatal day (PND) 84. The rats were further divided into sedentary or training groups. One week before the training, rats either remained on the standard ISO-depleted diet (4 mg ISO aglycone equivalent (sum of genistein, daidzein and glycitein)/kg diet; Ssniff Sm R/M-H, 10 mm, phytoestrogen free, Ssniff, Soest, Germany) or received an ISO-rich diet (479 mg ISO aglycone equivalent (sum of genistein, daidzein and glycitein)/kg diet). The ISO-rich diet is based on the ISO-depleted diet with an added ISO extract (NovaSoy650, ADM, Decatur, Illinois, USA). Treatment groups are assigned as follows: (1) Sham; (2) Sham operated with training (Sham+T); (3) OVX; (4) OVX+T; (5) OVX with an ISO-enriched diet (OVX+ISO); (6) OVX+ISO+T.

Animals were sacrificed two days after the last training, whereby Sham rats were sacrificed in the proestrous or estrous phase and blood was collected. Uterus, heart, tibia, soleus muscle and gastrocnemius muscle were dissected and weighed. The relative weights were calculated as the organ weight divided by body weight.

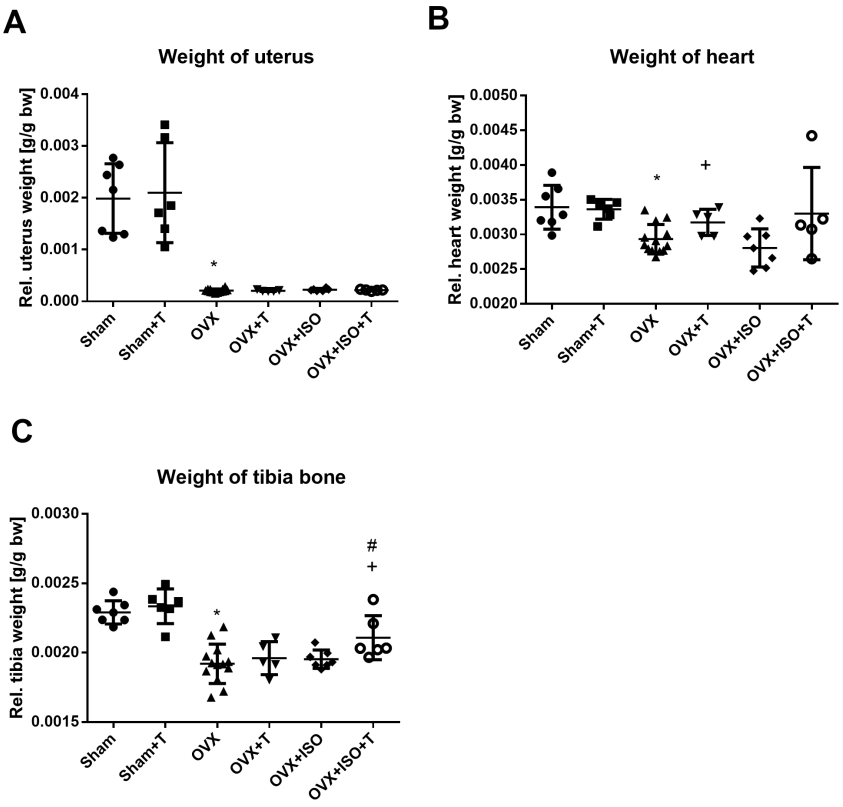
An uphill high intensity training protocol was designed for the exercise groups. The training was conducted on a motor-driven treadmill with an incline of 25°. After a period of familiarization with the treadmill lasting several days, rats were trained for 10 min, twice a day (at 08:00 am and 08:00 pm) for 61 days. Every fourth day was a rest day. The velocity was gradually increased from 12 to 20 m/min and maintained at 20 m/min until the end of the training protocol. The 10 min training was divided into 2x5 min followed by a 5 min break after the velocity reached 20 m/min. The break was necessary, as a continuous running performance of 10 min was too exhausting for the rats.

Real-time PCR experiments

Total RNA was extracted from frozen soleus and gastrocnemius muscle of individual rats using the standard TRIzol method (Invitrogen). The quality of RNA was checked by agarose gel electrophoresis and the RNA was quantified by spectrophotometry (NanoDrop™ 1000, Thermo Scientific, Wilmington, DE 19810, USA). cDNA synthesis of each group was performed with the equal amount of pooling mRNA sample which was a mixture of the same amount of mRNA from each rat. Real-time PCR was performed with Taq DNA polymerase (Invitrogen, Germany) and a fluorescent dye (SYBR Green, BioRad) on an Mx3005P™ qPCR System (Stratagene). All reactions were run in triplicate in a 50 µl total volume. The final results of real-time PCR consisted of a minimal of three different

cDNA synthesis. The PCR program was as follows: 95°C for 3 min for 1 cycle, followed by 40 cycles of 95°C for 30 s, 59°C for 30 s, 72°C for 30 s, and 1 cycle of 95°C for 1 min, 58°C for 30 s, 95°C for 30 s. Fluorescence was quantified during the 59°C annealing step and product formation was confirmed by melting curve analysis (59-95°C). mRNA levels were normalized to cyclophillin.

Fig.3. 5 Supporting information Figure



Supporting Information Fig. 1. Effects of training, ISOs and ISO+T on the relative weights of uterus (A), heart (B), tibia bone (C). Mean values were significantly different for the following comparisons: * marks when significant differences between Sham and Sham+T, Sham and OVX ($p \leq 0.05$); +vs. OVX ($p \leq 0.05$) when compared within OVX groups; # marks when significant differences between OVX+ISO and OVX+ISO+T ($p \leq 0.05$).

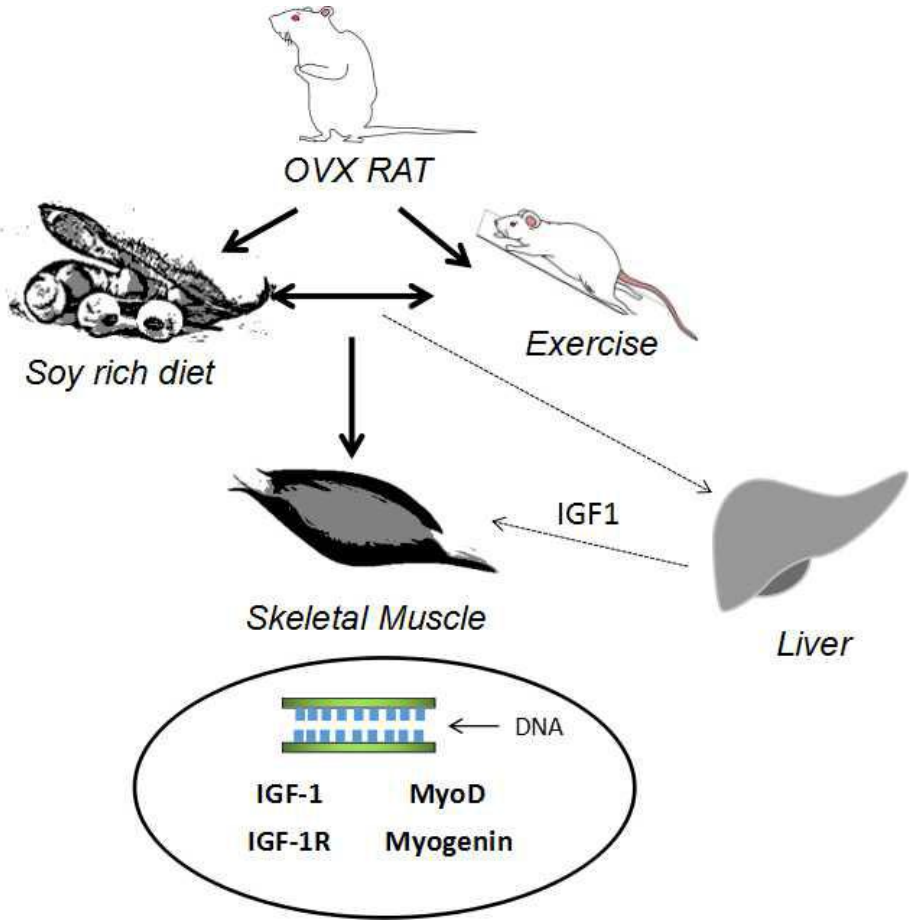
Table 3. 1 Supporting Information Table

Supporting Information Table 1. Measuring conditions (sMRM) for daidzein-7,4'-β-D-diglucuronide and gensitein-7,4'-β-D-diglucuronide

Compound	Transition	Mass 1	Mass 2	RT [min]	DP [V]	CE [V]	CXP [V]
Dai-7,4'-DiGlcA	1	605.0	429.1	7.22	-145	-24	-9
	2	605.0	253.0	7.22	-145	-52	-12
Gen-7,4'-DiGlcA	1	621.0	445.1	8.17	-140	-27	-14
	2	621.0	269.0	8.17	-140	-54	-10

Compound-dependent parameters and scheduled MRM settings (AB Sciex QTrap 5500) of daidzein-7,4'-β-D-diglucuronide (Dai-7,4'-DiGlcA) and gensitein-7,4'-β-D-diglucuronide (Gen-7,4'-DiGlcA) in the UHPLC-MS/MS method. MRM detection window: 60 sec; target scan time: 0.4 sec; transition 1: quantifier; transition 2: qualifier; RT: retention time; DP: declustering potential; CE: collision energy voltage; CXP: collision cell exit potential.

Graphical abstract



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CHAPTER 4: COMBINATORY EFFECTS OF PHYTOESTROGENS AND EXERCISE ON BODY FAT MASS AND FATTY ACID METABOLISM IN OVARIECTOMIZED FEMALE RATS

This study has been submitted to *The journal of steroid biochemistry and molecular biology* as:
Wenya Zheng*, Jana Rogoschin, Anja Niehoff, Kristina Oden, Sabine E. Kulling, Mingyong Xie, Patrick Diel

Abstract

Purpose: Investigate the combinatory effects of an isoflavone (ISO)-rich diet and exercise on fat mass and fatty acid metabolism in ovariectomized (OVX) rats.

Methods and results: Female Wistar rats were sedentary, performed treadmill uphill running, received ISOs, or a combination of ISOs and running after ovariectomy. Exercise reduced visceral fat mass, adipocyte size and serum leptin in Sham animals and antagonized the increases of these parameters induced by OVX. ISOs reduced OVX induced increase of serum leptin. The combination of training and ISOs was most effective in reducing serum triglycerides. In OVX rats training stimulated the expression of genes associated with fatty acid synthesis (SREBP-1c and FAS) in adipose tissue, soleus muscle, liver and genes associated with fatty acid oxidation (PPARδ and PGC-1α) in adipose tissue. ISOs stimulated the expression of SREBP-1c and FAS in soleus muscle and PGC-1α in adipose tissue, whereas suppressed hepatic SREBP-1c and FAS expression. Strong additive effects of ISOs combined with training were observed for PPARδ and PGC-1α expressions in soleus muscle.

Conclusion: Our results demonstrate that both training and ISOs affect fat mass and fatty acid metabolism in OVX rats. Training seems to have a higher impact than ISO exposure in regulating gene expression in adipose tissue. However, the strongest effects for several of the addressed parameters could be observed in the combination group especially in the soleus muscle. Therefore a combination of training and an ISO-rich diet may have beneficial effects on fatty acid metabolism and could be a concept for the prevention of metabolic disease in postmenopausal females.

Keywords: Exercise; Female; Ovariectomy; Soy isoflavones; Fatty acid metabolism.

Abbreviations: E_2, 17β-estradiol; FAS, fatty acid synthase; HDL, high-density lipoproteins; HRT, hormone replacement therapy; ISOs, isoflavones; LDL, low-density lipoproteins; OVX, ovariectomized; PGC, peroxisome proliferator-activated receptor-γ coactivator; PND, postnatal day; PPAR, peroxisome proliferator-activated receptor; SREBP, sterol regulatory element-binding protein; Tb.BMC, trabecular bone mineral content; Tb.BMD, trabecular bone mineral density.

4.1 Introduction

Metabolic syndrome is a combination of medical disorders caused by dysregulation of energy homeostasis and represents a cluster of obesity related diseases, such as cardiovascular disease, type 2 diabetes, which are characterized by hypertension, dyslipidemia and insulin resistance [1-3]. Postmenopausal women tend to have a higher risk of developing metabolic syndrome because of changes in metabolic and hormonal parameters. Estrogen deficiency exerts a huge impact on mobilization of fatty acids, body fat distribution and bone mass [4-6]. Several exogenous factors such as diet and lifestyle could affect body composition and thereby also be associated with the risk of developing metabolic syndrome [4].

As estrogen directly influences energy homeostasis through a modulation of lipid metabolism [7], hormone replacement therapy (HRT) was regarded as an effective treatment for preventing postmenopausal women from metabolic disorders for several decades [8]. However, some reports showed that HRT increases the risk of developing breast cancer, endometrial cancer and irregular bleeding [9, 10]. Therefore, there is a need for alternative treatment strategies such as administration of isoflavones (ISOs) which is controversially debated [11]. ISOs are the most well known phytoestrogens. They are structurally similar to 17β-estradiol (E_2) and display weak estrogenic activities in mammals [12]. ISOs derived from soybeans and their products are widely discussed regarding disease prevention [12]. Epidemiologic data showing that a lower rate of obesity and related metabolic diseases in Asia might be linked to a higher soy ISO consumption in their diet [13].

Apart from nutrition exercise has also been used as a non-pharmacological intervention to overcome negative health impacts caused by menopause. It is well known that training has a potency to prevent the development of many diseases, such as obesity, type 2 diabetes, hypertension and osteoporosis [14, 15]. Training experiments in animals have shown to decrease fat deposition and increase insulin sensitivity [16].

Previously studies of our group have used ovariectomized (OVX) rat models demonstrating that a combination of exercise and E_2 administration is an effective strategy to increase insulin sensitivity and prevent the development of metabolic syndrome [17, 18]. However, the combinatory effects of dietary ISO intake and training to treat the metabolic syndrome yet remain to be solved. Therefore, the major aim of this study was to investigate combinatory effects of dietary ISO intake and exercise on parameters related with lipid metabolism in an OVX Wistar rat animal model. Visceral fat mass, adipocyte size, serum

leptin levels and lipid profile were determined. Bone mineral density was also measured. Furthermore, the expressions of genes involved in the regulation of lipid metabolism (sterol regulatory element-binding protein (SREBP)-1c, fatty acid synthase (FAS), peroxisome proliferator-activated receptor (PPAR) δ and peroxisome proliferator-activated receptor-γ coactivator (PGC)-1α) were investigated in adipose tissue, liver and soleus muscle.

4.2 Materials and methods

4.2.1 Animals

Forty-four female Wistar rats (~250g, Janvier, Le Genest St Isle, France) were kept under controlled conditions of illumination (12/12 h day/night cycle) and constant room temperature (20 ± 1°C, relative humidity 50–70%). The rats had free access to food (SSniff GmbH, Soest, Germany) and water. Body weight and food intake were monitored twice/week. All animal handling and experiments were approved by the Committee of Animal Care.

4.2.2 Animal treatment and diets

The study design is shown in Fig.1. Before experimental procedures, all rats were randomly grouped and got either Sham or OVX operated via the dorsal route at postnatal day (PND) 84. The rats were further divided into sedentary or training groups. One week before the training started, rats either remained on the standard ISO-depleted diet (4 mg ISO aglycone equivalent (sum of genistein, daidzein and glycitein)/kg diet; Ssniff Sm R/M-H, 10 mm, phytoestrogen-free, Ssniff, Soest, Germany) or received an ISO-rich diet (479 mg ISO aglycone equivalent (sum of genistein, daidzein and glycitein)/kg diet). The ISO-rich diet is based on the ISO-depleted diet with an added ISO extract (NovaSoy650, ADM, Decatur, Illinois, USA). Treatment groups are assigned as follows: (1) Sham; (2) Sham-operated with training (Sham+T); (3) OVX; (4) OVX+T; (5) OVX with an ISO-rich diet (OVX+ISO); (6) OVX+ISO+T.

Animals were sacrificed two days after the last training session, whereby Sham rats were sacrificed in the proestrous or estrous phase and blood was collected. Visceral fat (periovarian, perirenal, omental) was removed, pooled and weighed. Perirenal fat pads were immediately fixed in 4% formalin solution for morphological analysis. Adipose tissue, liver and soleus muscle for gene analysis were snap frozen immediately after removal.

4.2.3 Training protocol

An uphill training protocol (Fig.1) was designed for the exercise groups [19]. The training was conducted on a motor-driven treadmill with an incline of 25°. Rats were trained for 10 min, twice a day for 61 days. Every fourth day was a rest day. The velocity was gradually increased from 12 to 20 m/min and maintained at 20 m/min until the end of the training protocol. The 10 min training was divided into 2x5 min followed by a 5 min break after the velocity reached 20 m/min. The break was necessary, as a continuous running performance of 10 min was too exhausting for the rats.

Group	Isoflavone-free diet	Isoflavone-rich diet	Training
Sham (n=7)	×		
Sham+T (n=6)	×		×
OVX (n=12)	×		
OVX+T (n=6)	×		×
OVX+ISO (n=7)		×	
OVX+ISO+T (n=6)		×	×

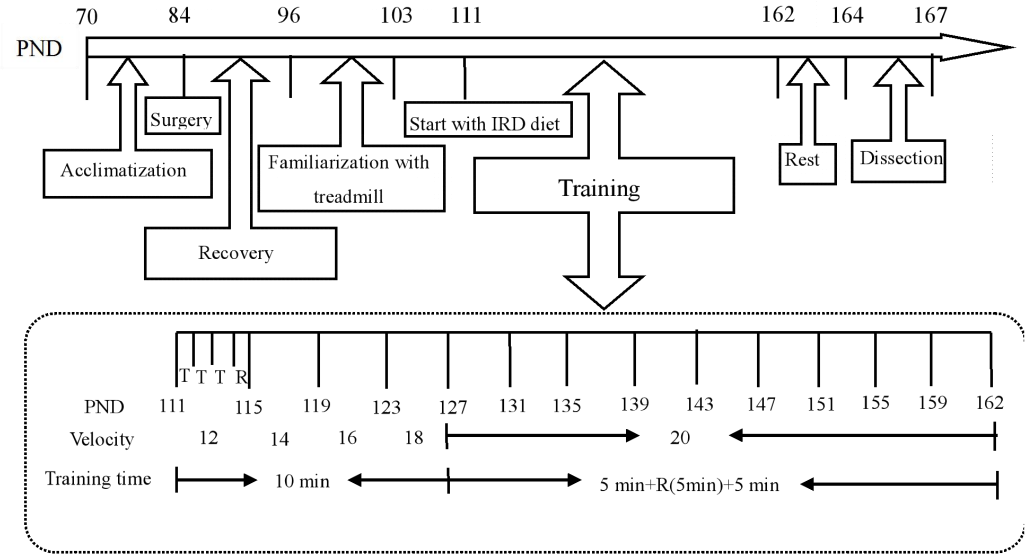


Fig.4. 1 Experimental design

T=Training, R=Rest, Sham = sham operated, OVX =ovariectomized. The rats were OVX or Sham operated and allocated into six different treatment groups. Two different diets, an isoflavone-depleted diet and an isoflavone-enriched diet (ISO), were used. The uphill-training was conducted on a

motor-driven treadmill with an incline of 25°. The animals of the training groups were trained for 10 min, twice a day. Every fourth day was a rest day.

4.2.4 HE staining and determination of adipocyte size

HE staining and determination of adipocyte size were performed as previously described [20].

4.2.5 Determination of leptin in serum

The serum concentration of leptin was measured in triplicates using ELISA kits for rats according to the manufacturer's instruction (mouse/rat leptin ELISA E06, Mediagnost, Reutlingen, Germany).

4.2.6 Determination of serum lipids

Serum triglyceride levels were analyzed by colorimetry using ABX Pentra reagent (ABX Diagnostics Montpellier, France). Total cholesterol, high-density lipoproteins (HDL) and low-density lipoproteins (LDL) were determined by photometry using reagents from DIALAB (Wiener, Neudorf, Austria). To measure serum lipids, a chemistry analyzer (Roche Hitachi Cobas Mitra Plus) was used.

4.2.7 Peripheral quantitative computed tomography measurements of bone

Trabecular bone mineral content (Tb.BMC) and trabecular bone mineral density (Tb.BMD) were measured by peripheral quantitative computed tomography (pQCT, XCT Research SA+, StraTec Medizintechnik, Pforzheim, Germany) as previously described [21].

4.2.8 Real-time PCR experiments

mRNA analysis and PCRs were performed as previously described [18, 22]. Real-time PCR was performed with Taq DNA polymerase (Invitrogen, Germany) and a fluorescent dye (SYBR Green, BioRad) on an Mx3005P™ qPCR System (Stratagene). Relative mRNA amounts of target genes were calculated after normalization to an endogenous housekeeping gene (Hypoxanthine-guanine phosphoribosyltransferase-HPRT was used with adipose tissue, cytochrom c oxidase subunit-1A was used with hepatic tissue, cyclophillin was used with soleus muscle). Specific primer pairs were designed as previously described [22] and depicted in the Supplementary Table 1.

4.2.9 Western blotting

Protein extraction and concentration measurement were performed as previously described [21, 22]. After electrophoretic protein separation by size, proteins were transferred onto a nitrocellulose membrane (PROTRANR Nitrocellulose Transfer Membrane, WhatmanR, Little Chalfont, England) and blocked with 5% non-fat milk in a Tris buffered saline solution with 0.1% Tween 20 (TBST, 100 mM; pH 7.4) at room temperature for 1 h, proteins were detected using specific antibodies against FAS (fatty acid synthase, rabbit anti-FAS, ab22759, Abcam, Cambridge, United Kingdom) with 1:600 dilution and actin (mouse anti-actin antibody, sc-27578, Santa Cruz, Heidelberg, Germany) with 1:20000 dilution overnight at 4°C. Afterwards membranes were incubated for 1 h with peroxidase-conjugated secondary antibodies (Swine anti-rabbit antibody; Rabbit anti-mouse antibody, HRP, Dako, Hamburg, Germany). The protein expression of FAS was related to the reference protein actin.

4.2.10 Statistical analysis

All data were expressed as means ± standard deviation (SD). Statistical significance of differences was calculated using Kruskal–Wallis test with a subsequent Mann–Whitney U-test (GraphPad Prism, version 5). Statistical significance was established at $p \leq 0.05$.

4.3 Results

4.3.1 Visceral body fat mass, adipocyte size and serum leptin levels

As shown in Fig. 2, the largest visceral fat mass (Fig. 2A), adipocyte size (Fig. 2B) and serum leptin level (Fig. 2C) were observed in OVX rats. In trained rats visceral fat mass, adipocyte size and leptin levels were significantly reduced in Sham and OVX rats. In OVX rats an ISO-rich diet reduced leptin serum levels but showed no significant effects on fat mass and adipocyte size. A combination of ISO+T did not result in additive effects.

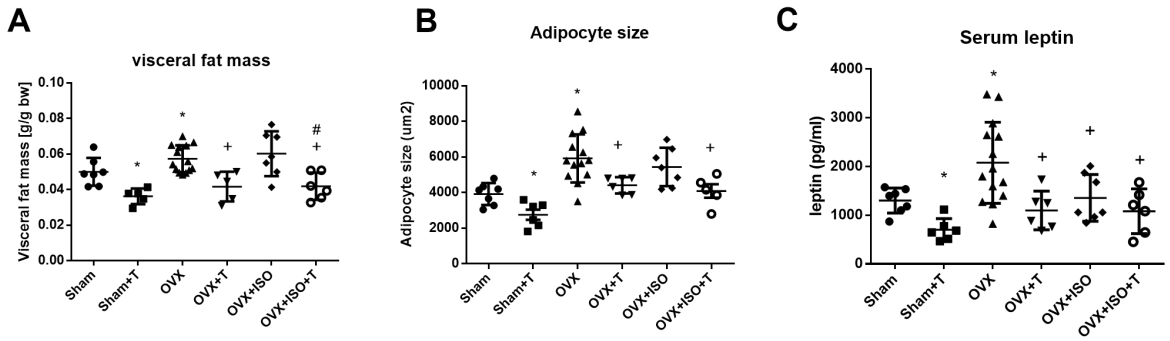


Fig.4. 2 Effects of ISO and training treatment on the visceral body fat mass (A), adipocyte size (B) and serum leptin (C)

Mean values were significantly different for the following comparisons: * marks when significant differences between Sham and Sham+T, Sham and OVX ($p \leq 0.05$); +vs. OVX ($p \leq 0.05$) when compared within OVX groups; # marks when significant differences between OVX+ISO and OVX+ISO+T ($p \leq 0.05$).

4.3.2 Serum lipid levels

As shown in Fig.3, OVX resulted in significant increases of cholesterol (Fig. 3A), HDL (Fig. 3B) and LDL (Fig. 3D) which was neither affected by training or an ISO-rich diet. In contrast serum triglycerides (Fig. 3C) were reduced by training in Sham rats and a combination of ISO+T in OVX rats.

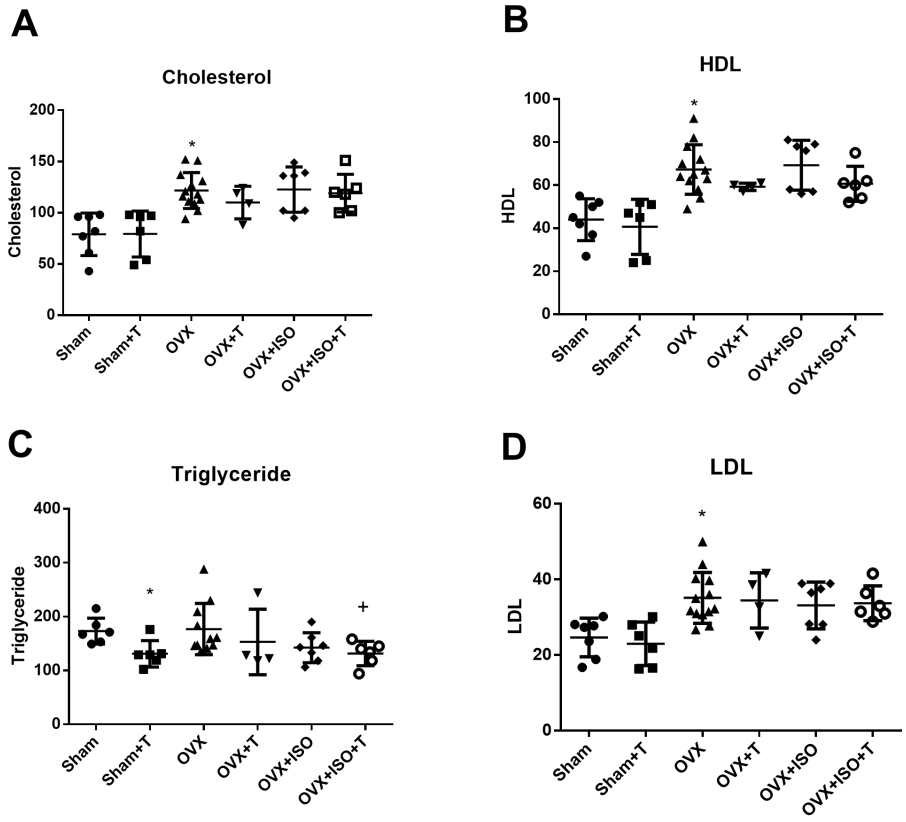


Fig.4. 3 Effects of ISO and training treatment on the serum levels of cholesterol (A), HDL (B), triglycerides (C) and LDL (D)

Mean values were significantly different for the following comparisons: * marks when significant differences between Sham and Sham+T, Sham and OVX ($p \leq 0.05$); +vs. OVX ($p \leq 0.05$) when compared within OVX groups.

4.3.3 Bone analysis

In Fig.4, it is obvious that Tb.BMD (Fig. 4A) and Tb.BMC (Fig. 4B) were significantly decreased by OVX. However, training, ISOs or ISO+T showed no significant changes in the parameters.

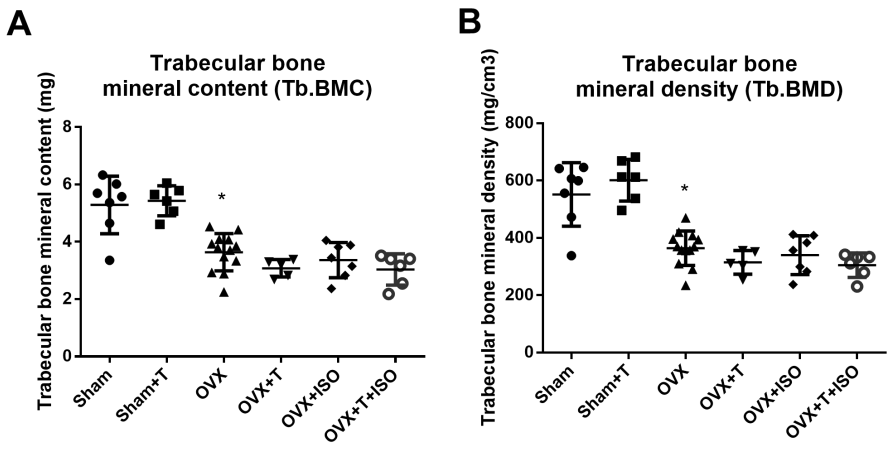


Fig.4. 4 Effects of ISO and training treatment on the Tb. BMC (A) and Tb. BMC (B)
Mean values were significantly different for the following comparisons: * marks when significant differences between Sham and Sham+T, Sham and OVX ($p \leq 0.05$).

4.3.4 SREBP-1c and FAS expression in adipose tissue, soleus muscle, liver

As shown in Fig.5A, the SREBP-1c gene expression in adipose tissue was reduced by OVX. The effect was able to be antagonized by training, but not by ISOs. Training resulted in a strong induction of SREBP-1c gene expression in adipose tissue. In soleus muscle (Fig. 5B) SREBP-1c gene expression was also reduced by OVX. Interestingly here both training and ISOs resulted in increased SREBP-1c gene expressions in OVX animals. However, ISOs decreased the hepatic SREBP-1c expression (Fig. 5C). As shown in Fig. 5C, the hepatic SREBP-1c expression was increased by OVX. Training resulted in a further increase in OVX rats. The gene expression induction of ISO+T was similar as training in adipose tissue and liver.

FAS gene expression was not significantly modulated by OVX in all the analyzed tissues (Fig. 5D&5E&5F). However, the effects on FAS regulation induced by training, ISOs or ISO+T were completely in line with on SREBP-1c regulation (Fig. 5D&5E&5F). Further, FAS protein expression (Fig. 5G) in adipose tissue was consistent with the FAS gene expression.

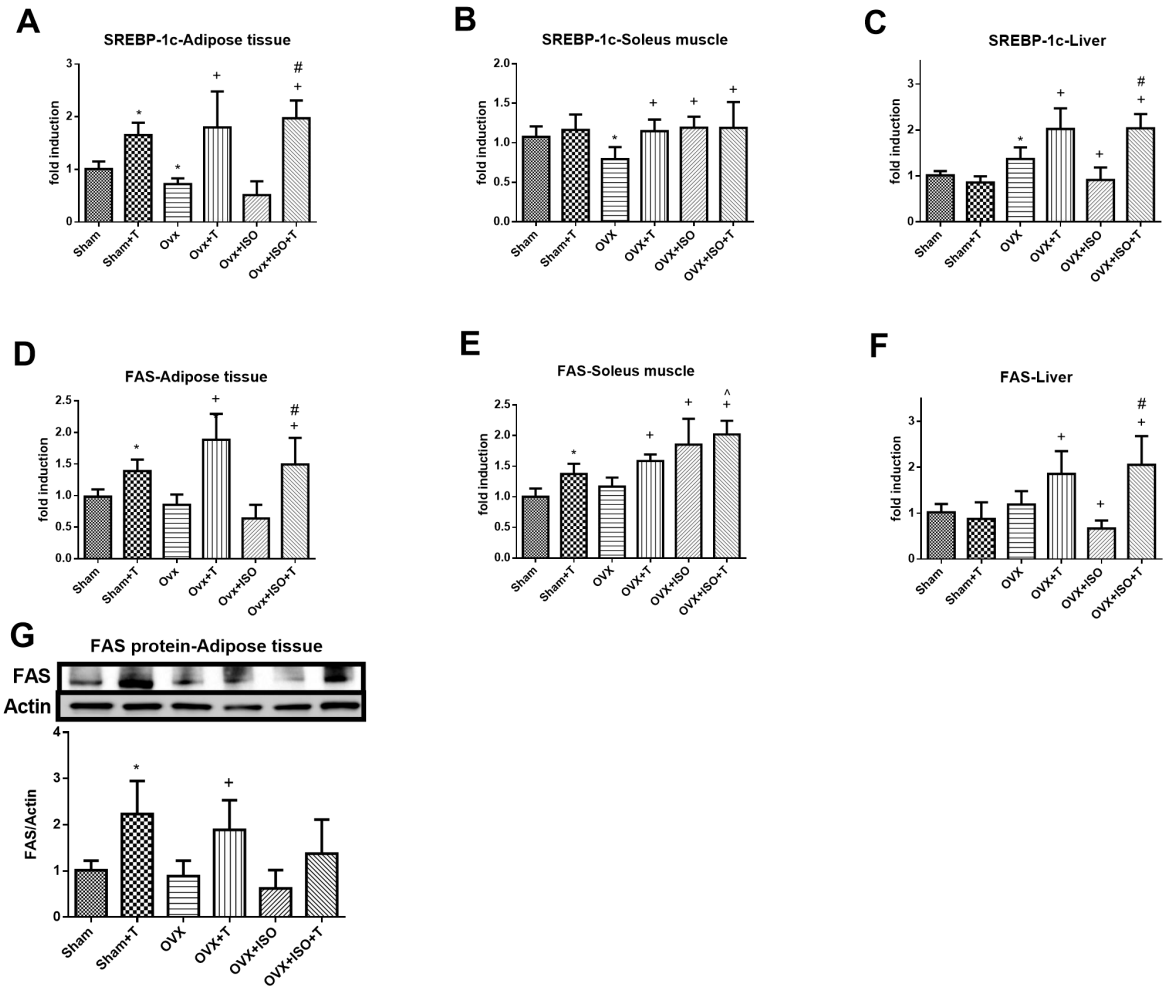


Fig.4. 5 Effects of ISO and training treatment on gene expressions of SREBP-1c and FAS in adipose tissue (A&D), soleus muscle (B&E), liver (C&F) and protein expressions of FAS in adipose tissue (G) Western blot bands are representative for detection of FAS (273 kDa) and the control Actin (42 kDa). Mean values were significantly different for the following comparisons: * marks when significant differences between Sham and Sham+T, Sham and OVX ($p \leq 0.05$); +vs. OVX ($p \leq 0.05$) when compared groups within OVX groups; # marks when significant differences between OVX+ISO and OVX+ISO+T ($p \leq 0.05$); ^ marks when significant differences between OVX+T and OVX+ISO+T ($p \leq 0.05$).

4.3.5 PPARδ, PGC-1α mRNA expression in adipose tissue, liver and soleus muscle

As shown in Fig. 6A&6B&6D&6E, PPARδ and PGC-1α gene expressions were significantly reduced by OVX in either adipose tissue or soleus muscle. The effect of OVX was antagonized by training in adipose tissue (Fig 6A&6D). Also, ISOs antagonized the OVX effect in adipose tissue by up-regulating PGC-1α expression (Fig. 6D). In the soleus muscle (Fig. 6 B&6E) PPARδ or PGC-1α gene expression was induced by training in intact animals. In OVX animals neither training nor ISO affected PPARδ or PGC-1α gene

expression. However, ISO+T showed additive effects, thereby led to increased expression of PPARδ and PGC-1α. Hepatic PPARδ or PGC-1α gene expression was not regulated by OVX, training, ISOs or ISO+T (Fig. 6C&6F).

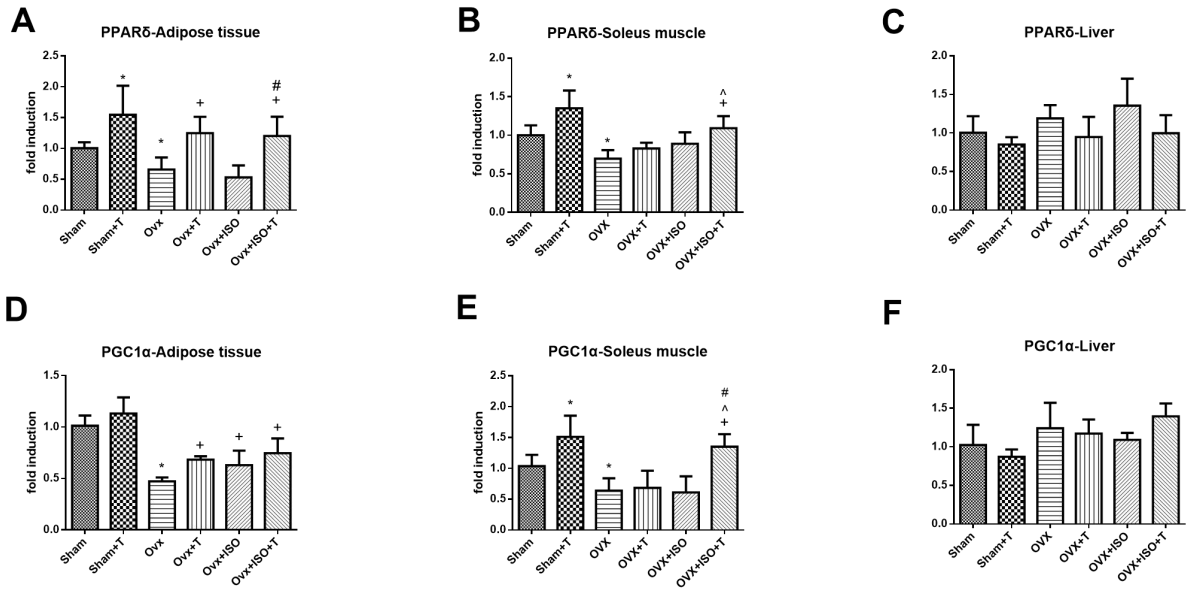


Fig.4. 6 Effects of ISO and training treatment on gene expressions of PPARδ and PGC-1α in adipose tissue (A&D), soleus muscle (B&E), liver (C&F)

Mean values were significantly different for the following comparisons: * marks when significant differences between Sham and Sham+T, Sham and OVX ($p \leq 0.05$); +vs. OVX ($p \leq 0.05$) when compared groups within OVX groups; # marks when significant differences between OVX+ISO and OVX+ISO+T ($p \leq 0.05$); ^ marks when significant differences between OVX+T and OVX+ISO+T ($p \leq 0.05$).

4.4 Discussion

Estrogens are important regulators of body composition and fatty acid metabolism in females [23]. Onset of menopause, females suffer from estrogen removal in their body. Exercise and ISOs have been reported to exert beneficial effects on preventing metabolic syndrome [17, 24]. However, there is still a limited research on their combinatory effects and the underlying molecular mechanism. The key findings of these investigations are:

1) Training and ISOs affect body fat mass and fatty acid metabolism.
2) Training seems to have a higher impact than dietary ISO intake in regulating gene expression related with lipid metabolism especially in adipose tissue.
3) Nevertheless for several of the addressed parameters such as triglycerides and gene expression in soleus muscle, the strongest effects could be observed in the

combination group of ISO+T.

Suitability of the chosen interventions

In our investigations OVX Wistar rats were chosen as an animal model to mimic the situation of postmenopausal women. In line with earlier studies in our group [17, 20], OVX rats showed a significantly higher visceral fat mass than Sham rats. Meanwhile, OVX rats resulted in obesity related risk factors, such as an increased adipocyte size and serum leptin levels.

As nutritive intervention animals were fed with two different kinds of diets, an ISO-depleted diet and an ISO-enriched diet. The average ISO intake was 9.8 mg/bw/d in the OVX+ISO group and 10.6 mg/bw/d in the OVX+ISO+T group. This resulted in mean plasma concentrations of Genistein (Gen)+Daidzein (Dai) aglycone equivalents in these two groups were 1947 and 1556 nM (data published) [19]. Very small amounts of Gen+Dai aglycone were detected in OVX animals with an ISO-depleted diet [19]. A previous study in our group showed similar plasma concentrations of Gen+Dai aglycone equivalents in ISO-treated rats and reported that the concentrations are comparable to those in Asians consuming moderate to high amounts of soy products [24, 25].

The training protocol used in this study was an uphill running on a treadmill. The speed was gradually increased from 12 to 20 m/min progressing at a rate of 2 m/min per session, which leads to an effective exercise stimulus. Sufficient regeneration periods between every training session were also scheduled within the protocol. This heavy training model was developed and modified according to seven basic principles of physical training [26] and our previous studies [27, 28]. High intensity training was reported to induce mitochondrial biogenesis and an increased capacity for fatty acid metabolism [29]. As expected, training in this study reduced the risk of being obesity by decreasing visceral fat mass, adipocyte size and serum leptin levels. The results were also in line with our previous study [17].

Effects on fat mass, adipocyte size and leptin

In our investigations OVX rats showed a significantly higher visceral fat mass, fat cell size and serum leptin than Sham rats (Fig. 2), which was in line with earlier studies in our group [17, 24, 25]. The increased parameters could be all antagonized by training. In contrast, ISOs significantly reduced the leptin level, whereas the effects on visceral fat mass and adipocyte size were only faint and not statistically significant. A possible explanation for these differences is the time of exposure. In previous studies lifelong but

not short-term ISO intake decreased visceral fat mass and adipocyte size in OVX rats [24]. Moreover, leptin is a very sensitive indicator for total body fat mass [30]. Therefore it is possible that this parameter displays a reduction of fat mass which was not detectable by just analyzing specific fat pads.

Regarding the combinatory effects of training and ISOs, it has to be stated that in this study no additive effects in decreasing visceral fat mass, adipocyte size and serum leptin could be observed. Effects of a combination of ISO+T are controversially debated due to the different exposure time of ISOs and protocols of training. A study in OVX mice showed that a combination of training and ISOs exerted an additive effect on reducing fat mass compared to the single intervention [31], while no additive effect was observed in a clinical study [32]. Here a possible explanation for our results is that the training used in this study was so effective that we already reached the maximum of possible effects in the relatively short period of intervention time.

Effects on blood lipids

Blood lipid profile is an essential component for determining metabolic syndrome. Postmenopausal women tend to have negative changes in lipid profile and higher incidences of atherosclerotic damage [16]. The increases of cholesterol and LDL by OVX shown in this study were in line with our previous studies [17, 20, 24]. The effects of training on lipid profile are controversially discussed. Some studies reported positive effects [33, 34], while others showed no effects [35, 36]. ISO intake also showed no antagonizing effect on lowering lipids, which was consistent with the results from a study focusing on Gen (a main component in ISOs) [20]. However, triglycerides were significantly reduced by ISO+T in OVX rats (Fig.3C), which was in line with another study that also reported high lipid levels induced by OVX were normalized by combining exercise and ISOs [31].

Effects on bone mass

Associations between metabolic syndrome and bone health in postmenopausal women have been described in several investigations [37, 38]. In agreement to data from the literature [31, 39], OVX induced bone loss also in our animal model (Fig. 4). Tb.BMC and Tb.BMD is well described as markers for bone diseases such as osteoporosis [40], which were strongly reduced in OVX rats. In our investigation neither training nor ISOs showed protective effects regarding bone mass and density. Many studies reported beneficial

effects of training on increasing bone mass [31, 39]. However, one study reports that high intensity training could have no effects on BMD in OVX rats although the bone strength was improved [41]. It is very likely that parameters like duration and training intensity have a huge impact on training effects on the bone. This is also the case for ISO intake. One previous study showed that ISO-rich diet exhibited bone protective effects in OVX rats [42]. The differences in the results could be most probably caused by the period of time for observation because the amount of ISO intake per day and the data of BMD of ISO group were similar in this study to the previous one, while the data of OVX group was not. Altogether the amount of ISOs and the training intensity must be adapted very carefully to result in bone protective effects.

Effects on molecular markers related to fatty acid metabolism

In our study we analyzed several genes related to fatty acid metabolism (SBEBP-1c, FAS, PPARδ and PGC-1α) in adipose tissue, soleus muscle and liver. SBEBP-1c and FAS are key players in de novo lipogenesis. SREBP-1c is a regulator of FAS transcription [43], which partly proved in our results. FAS is expressed mostly in tissues with high rates of fatty acid synthesis, namely, adipose tissue and liver [43]. Skeletal muscle is the main tissue for fatty acid oxidation. It reported that SREBP is regulated in skeletal muscle in response to exercise and ISOs [44, 45].

In our study OVX decreased the expression of SREBP-1c in adipose tissue and soleus muscle, which was in line with data from the literature [46, 47]. Leptin was pointed out to be a regulator of the gene expression [46]. In contrast, OVX increased the SREBP-1c expression in liver which may be an indication for increased lipogenesis [48].

Exercise up-regulated expressions of SREBP-1c and FAS in adipose tissue, soleus muscle and liver in OVX rats. Again this up-regulation effect may be mediated by leptin. As we have observed, training reduced fat mass, adipocyte size and leptin levels (Fig. 2). Because high leptin level was reported to be directly associated with down-regulation of SREBP-1c, FAS mRNA expression [46], a reduction of serum leptin may result in an increase of the expressions of the mentioned genes. Moreover, a number of studies demonstrated that training up-regulated expressions of SREBP-1c and FAS in skeletal muscle [44], which was consistent with our results in this study.

In this study we found that ISOs inhibited hepatic SREBP-1c and FAS expressions, which was in line with previous results of our group [24]. The effects might be modulated through the estrogen receptor β (ERβ) [49]. However, ISO intake up-regulated SREBP-1c

and FAS expressions in soleus muscle of OVX rats. The mechanism was still not very clear. As OVX decreased SREBP-1c and FAS gene expressions in soleus muscle, one hypothesis is that ISOs have estrogenic potency and compensate for the loss of endogenous estrogen induced by OVX.

Regarding combinatory effects, it is interesting that regulation of SREBP-1c is similar in the OVX+ISO+T group as in the OVX+T group in adipose tissue and liver. In these tissues training plays a dominant role in modulating SREBP-1c when combined with ISOs. The treatment of ISOs did not exert effects strong enough to antagonize the effect induced by training. Interestingly in the soleus muscle there have additive effects of ISOs and training on FAS expression (Fig.5E). The additive effects are more clearly visible for the expression of PPARδ and PGC-1α in this muscle (Fig.6B&6E). The combinatory effects of training and ISOs on the regulation of gene expression associated with lipid metabolism are still limited. One study combining ISOs and exercise reported an additive prevention effect on body fat accumulation [31]. In contrast, a clinical study showed that a combination of training and ISOs had no favorable changes in body composition [32]. Unfortunately, the molecular response was not systematically investigated in the studies.

Additionally, PPARδ is a key regulator of fatty acid catabolism and energy homeostasis [50]. It is abundantly expressed throughout the body but at a very low level in liver, which might be a reason for no regulation found with hepatic PPARδ expression. OVX significantly down-regulated PPARδ expressions in both adipose tissue and soleus muscle, which was in line with a number of studies [47, 51]. The activation of PPARδ in adipose tissue produces lean mice resistant to obesity [50]. An overexpression of PPARδ increases the number of mitochondria and results in an induction of genes responsible for oxidative metabolism and fatty acid catabolism [52, 53]. Our data showed that training clearly induced the PPARδ expression in adipose tissue, whereas ISOs have no effects. However, ISOs might act as a compensation for estrogen in soleus muscle. Therefore, an additive effect and a significant increase of the PPARδ expression could be observed in the ISO+T group of soleus muscle. The PGC-1α expression strictly correlates with PPARδ expression in cultured cells and adipose tissue [50]. In our study OVX significantly reduced the PGC-1α gene expression in adipose tissue, which was in line with another study [47]. Both the training and ISOs were observed to enhance the PGC-1α expression in adipose tissue in OVX rats. In skeletal muscle it is known that PGC-1α acts as a co-activator of PPARδ to control mitochondrial biogenesis [50, 52]. Interestingly especially in the soleus muscle in our study we observed a strong combinatory effect of ISOs and training on PGC-1α

expression. This could be an indication that training in OVX animals only in the presence of an activation of the ER, in our case by binding of ISOs, are able to stimulate mitochondrial biogenesis.

In conclusion, estrogen deficiency in OVX rats triggered metabolic syndrome related risks such as increases of visceral fat mass, adipocyte size, serum leptin, cholesterol, LDL and a decrease of BMC, BMD. In our study a combination of exercise and dietary ISO intake was effectively reducing the visceral fat mass, adipocyte size, serum leptin, and serum triglycerides. Although training has a dominant role regarding visceral fat prevention, several parameters were most efficiently influenced in the combination group of ISO+T. These effects occurred at serum concentration comparable to such observed in Asian population who has a soy-rich nutrition. Therefore we believe that our data provide evidence that combining training with a nutrition resulting in such ISO serum concentration may be a promising intervention concept of preventing metabolic syndrome in postmenopausal women.

Acknowledgments

W.Z planned and conducted the animal experiment, collected and analyzed data, and wrote the manuscript. J.R and K.O helped in analyzing hepatic gene expression. A.N did bone mineral density analysis. S.E.K. performed ISO analytics. M.X was involved in designing the study. P.D. mainly designed the study and gave conceptual advice for the manuscript. This project is funded by the German Research Foundation (DFG) grant Di 716/12-1. And the authors thank the China Scholarship Council for grant support to W.Z.

Conflicts of interest

The authors have declared no conflicts of interest.

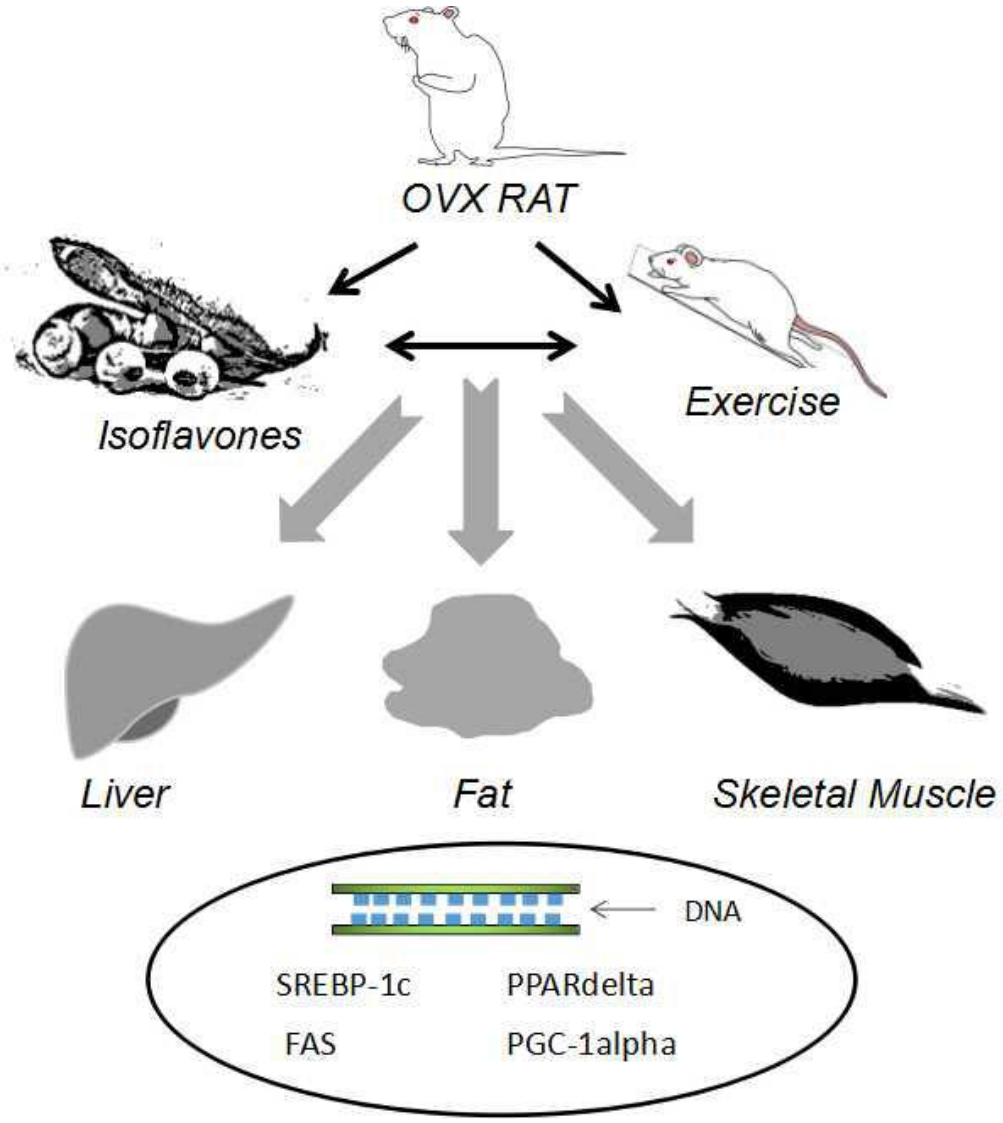
4.5 Supplementary Information

Table 4. 1 Supplementary Table 1

Primers used in real-time PCR experiments

Gene	Direction	Primer sequence (5'-3')
HPRT	fwd	AGGACCTCTCGAAGTGTTGG
	rev	TGGCCACATCAACAGGACTC
cyclophillin	fwd	GGATTCATGTGCCAGGGTGG
	rev	CACATGCTTGCCATCCAGCC
1A	fwd	CGTCACAGCCCATGCATTCG
	rev	CTGTTCATCCTGTTCCAGCTC
SREBP-1c	fwd	GTACCTGCGGGACAGCTTAG
	rev	CAGGTCATGTTGGAAACCAC
FAS	fwd	GGCTAGAGACCTTGGCACTG
	rev	TAGCCCTCTGCTCTGGTCAC
PPARδ	fwd	TCATTGAGCCCAAGTTCGAG
	rev	GGAAGAGGTACTGGCTGTCG
PGC-1α	fwd	TTACACCTGTGACGCTTTCG
	rev	GTGGAAGCAGGGTCAAAATC

Graphical abstract



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CHAPTER 5: ANABOLIC ACTIVITY OF A SOY EXTRACT IN C2C12 MYOTUBES

This study has been prepared to submit as:
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Abstract

Isoflavones (ISOs) have been reported to stimulate muscle growth. The aim of this in vitro study was to examine anabolic activity and associated molecular mechanisms of a soy extract (Soy), ISO aglycones and a mixture simulating the composition of Soy in C2C12 myotubes. C2C12 cells were differentiated into myotubes. Effects of Soy, genistein (Gen), daidzein (Dai), glycitein (Gly) and a mixture of Gen, Dai and Gly (Mix) on myotube diameter and number were determined. In addition, the expression of genes and proteins associated with anabolic activity was analyzed. Treatment with Soy, Gen and Mix led to a significant increase of myotube diameter and an increase of the number of myotubes per area compared to control cell. The increase of diameter by Soy was antagonized by antiestrogen ZK 191703, whereas not by antiandrogen flutamide. Furthermore, gene expressions of insulin growth factor (IGF)-1 and its receptor (IGF-1R), as well as protein expression of myosin heavy chain (MHC) were significantly increased by Soy, Gen, Mix. The effects induced by Gen and Mix were comparable to Soy. In conclusion, Soy enhances the anabolic activity of C2C12 myotubes by binding to ER and modulating IGF-1 and MHC expression. Our studies with ISO aglycones and Mix indicate that the ISO aglycone with the highest anabolic bioactivity in Soy is Gen.

Keywords: Isoflavones, C2C12 myotube, IGF-1, MHC

Abbreviations: AR, androgen receptor; Dai, daidzein; DHT, dihydrotestosterone; DMEM, dulbecco's modified eagle medium; DMSO, dimethylsulfoxide; E2, 17β-estradiol; ER, estrogen receptor; FBS, fetal bovine serum; Flut, flutamide; Gen, genistein; Gly, glycitein; HRT, hormone replacement therapy; IGF, insulin-like growth factor; ISO, isoflavone; MHC, myosin heavy chain; Mix, a mixture of genistein-daidzein-glycitein; mTOR, mechanistic target of rapamycin; MuRF1, muscle RING finger-containing protein 1; PKB, protein kinase B; PI3K, phosphatidylinositol 3-kinase; Soy, a soy extract; ZK, ZK 191703.

5.1 Introduction

Skeletal muscle accounts for the most mass of an individual and daily energy consumption. It is able to respond and adapt to changing environmental stimuli. Endocrine factors influence muscle growth and development throughout life. Increasing evidence shows that menopause is associated with a decline in muscle mass and muscle strength [1], which indicates a correlation specifically between muscle mass and estrogen status in females [2]. Hormone replacement therapy (HRT) is regarded as a treatment for women who are suffering the loss of muscle mass and muscle function because of the anabolic effect of 17β-estradiol (E2). However, HRT has a complex pattern of risks and benefits. Several findings did not support a use of HRT for chronic disease prevention [3]. Isoflavones (ISOs) are natural occurring diphenolic compounds and discussed to be an alternative for HRT because of their similar chemical structure to E2. Soy is the main source for ISOs [4]. Aubertin-Leheudre et al [5] showed that lean body mass and muscle mass of obese-sarcopenic postmenopausal women were significantly increased after supplementation of 70 mg ISOs per day for 24 weeks. Genistein (Gen), daidzein (Dai) and glycitein (Gly) are principal ISOs found in soy foods. There are other chemicals identified in soy products such as genistin, daidzin, glycitin. However, Gen has been shown to exhibit greater biological activity than genistin [6, 7].

Skeletal muscle is composed of multinucleated fibers which formed after fusion of myoblast and followed by differentiated to myotubes. C2C12 cells, a myoblast cell line derived from murine satellite cells, have been used extensively as an in vitro model to study both muscle differentiation and hypertrophy [8]. Differentiated myotubes of C2C12 cells exhibit a hypertrophic response to growth factors like IGF-1 and E2 [8], characterized by changes of diameters in myotubes and an activation of protein synthesis. Previously, our group demonstrated that estrogen receptor (ER) β is involved in the hypertrophy of C2C12 myotubes induced by a phytoecdysteroid ecdysterone [9]. Another in vivo study in our group showed that Gen is able to affect muscle growth through ERβ signaling pathway, showing an increase expression of myosin heavy chain (MHC) [10]. MHC determines muscle fiber type and contractile speed based on ATPase properties in skeletal muscle. In mammals several isoforms of MHC proteins are expressed in the sarcomere, of which fibers composed display distinct contractile properties in muscles. An in vivo study showed that MHC composition was shifted in soleus and extensor digitorum longus by ovariectomy in rats [11]. In C2C12 neo-formed myotubes, MHC is a major structural protein and act as a differentiation marker [12]. Moreover, insulin growth factor-1 (IGF-1)

is among the best-characterized factors involved in muscle hypertrophy. IGF-1 acts through direct interaction with its own receptor IGF-1R, followed by activating PI3K/Akt pathway [13].

Therefore, the major aim of this study was to identify the effects of a soy extract (Soy), its three major ISO components (Gen, Dai, Gly) and a mixture simulating the composition of the extract on hypertrophy of C2C12 myotubes and underlying molecular mechanisms.

5.2 Methods

5.2.1 Substance and chemicals

Soy was obtained from Novasoy® 650 (ADM, Decatur, Illinois, USA). Gen, Dai, and Gly were purchased from LC Laboratories (Woburn, MA, USA) and exhibit purities>98%. E2, dihydrotestosterone (DHT) and a selective antagonist of androgen receptor (AR) flutamide (Flut) were purchased from Sigma-Aldrich (Steinheim, Germany). The antiestrogen ZK 191703 (ZK) was provided by Bayer Pharma AG (Berlin, Germany). Dimethylsulfoxide (DMSO), ethanol, methanol and glutaraldehyde were purchased from Merck (Darmstadt, Germany). Dulbecco's modified eagle medium (DMEM) and medium components were all purchased from Gibco®-Life technologies (Darmstadt, Germany).

5.2.2 Quantification of ISO derivates

Quantification of ISO derivates in Soy extract was measured by LC/DAD analysis and performed as described previously [14].

5.2.3 C2C12 hypertrophy cell culture model

C2C12 cells (American Type Culture Collection, Manassas, VA, USA) were grown in DMEM supplemented with 10% fetal bovine serum (FBS), 4 nM glutamine, 1.5 g/L sodium bicarbonate, 100 mM sodium pyruvate, and 100 units/mL penicillin/streptomycin. Differentiation toward myotubes was induced at confluence by shifting the proliferation medium to differentiation medium (DMEM with 2% horse serum). During the whole experiment, the cells were stored at atmospheric conditions of 5% CO_2 and 37°C.

Differentiated myotubes were incubated for 2 d in the presence of either Soy (0.0005-50 ug/mL), E_2 (1 or 10 nM), ZK (100 nM), DHT (1 mM), Flut (1 mM), Gen (1 nM), Dai (2.1 nM), Gly (0.6 nM), or control (0.1% DMSO only). For the antagonization study, cells were treated with combinations of E2-ZK (10 nM/100 nM), Soy-ZK (0.05 ug/mL-100 nM), DHT-Flut (1 mM each), Soy-Flut (0.05 ug/mL-1 mM). Moreover, the myotubes were also

treated with a mixture of Gen-Dai-Gly (1 nM-2.1 nM-0.6 nM).

5.2.4 Measurement of myotube diameters

Cells were fixed and photographed by glutaraldehyde-induced autofluorescence after the treatment with different chemicals. Myotube diameters of minimal 50 myotubes per group were measured every 10-20 μm along the length of the myotube using a fluorescence microscope (Axiovert 200M, Zeiss) and the Axiovision LE software.

5.2.5 Measurement of myotube number in defined areas

Before and after treatment with the respective substances pictures of alive cells were taken with a microscope (Axiovert 200 M, Zeiss). In order to avoid that irregular myotube growth would affect the results; pictures were taken of marked spots before chemical treatment which could be compared to the pictures of the same spot after the treatment. At the bottom of each well, two dots were drawn with a colored marker. For each group ≥ 7 pictures were taken. The dots were surrounded by circles in order to find them more easily under the microscope. In the field of view of the microscope, the shadow of the circle and the dot could be detected.

5.2.6 Real-time PCR experiments

Total RNA was extracted from C2C12 myotubes using the standard TRIzol method (Life Technologies, Germany). The quality of RNA was checked by agarose gel electrophoresis and the RNA was quantified by spectrophotometry (NanoDrop™ 1000, Thermo Scientific, Wilmington, DE 19810, USA). cDNA synthesis and Real-time PCR (RT-PCR) was performed as previously described [15]. The PCR program was as follows: 95°C for 3 min for 1 cycle, followed by 40 cycles of 95°C for 30 s, 59°C for 30 s, 72°C for 30 s, and 1 cycle of 95°C for 1 min, 58°C for 30 s, 95°C for 30 s. Fluorescence was quantified during the 59°C annealing step and product formation was confirmed by melting curve analysis (59-95°C). Relative mRNA amounts of target genes were calculated after normalization to a housekeeping gene (GAPDH). The following oligonucleotide primers (Life Technologies, Germany) were used for amplification: GAPDH [16], fwd: 5'-ACCCAGAAGACTGTGGATGG-3', rev: 5'-TTCAGCTCTGGGATGACCTT-3'; IGF-1, fwd: 5'-AGCTGCAAAGGAGAAGGAAAGGAAG-3', rev: 5'-GGTGATGTGGCATTTCCTGCT-3'; IGF-1R, fwd: 5'-GGACAACTGCCCTGATATGC-3', rev: 5'-CTCCATC TCCAGCTCCTCTG-3';

muscle Ring finger-containing protein 1 (MuRF1) [17], fwd: 5'-AGGACAACCTCGTGCCTACAAG-3', rev: 5'-ACAACCTGTGCCGCAAGT G-3'.

5.2.7 Western blotting

Total protein was extracted from C2C12 myotubes using lysis buffer (50 mM Tris pH 8.0, 2 mM $CaCl_2$, 80 mM NaCl, 1% Triton-X 100) containing protease inhibitor PMSF (10 mM, phenylmethanesulfonylfluoride). Measurement of protein concentration, gel electrophoresis and protein transfer were performed as previously described [15]. Proteins were detected using primary antibodies of mouse anti-MHC (MF 20, Developmental Studies Hybridoma Bank, University of Iowa, United States), mouse anti-MyoD (D7F2, Developmental Studies Hybridoma Bank, University of Iowa, United States), mouse anti-actin (MAB1501, Millipore GmbH, Schwalbach, Germany) and mouse anti-alpha-Tubulin (12G10, Developmental Studies Hybridoma Bank, University of Iowa, United States) over night at 4°C. Afterwards membranes were incubated for 1 h with peroxidase-conjugated secondary antibodies (Rabbit anti-mouse antibody, HRP, Dako, Hamburg, Germany). Visualization of the blot signals was obtained by chemiluminescent reaction of the substrate Luminol (Lumi-LightPlus Western Blotting Substrate, Roche, Basel, Switzerland) and the following detection by a fluorescent detection system (FluorChem, Alpha Innotech, Santa Clara, USA). ImageJ software (NIH, Bethesda, Maryland) was used for densitometric determination of protein expression. Protein expression of MHC, MyoD was respectively related to the reference protein actin, alpha-Tubulin.

5.2.8 Statistical analysis

All data were expressed as means ± standard deviation (SD). Statistical significance of differences was calculated using Kruskal–Wallis test with a subsequent Mann–Whitney U-test (GraphPad Prism, version 5). Statistical significance was established at $p \leq 0.05$.

5.3 Results

5.3.1 Content of ISO derivatives in the soy extract

As shown in Table 1, the soy extract contains 19.62 µmol ± 5.0 % Gen/g, 40.91 µmol ± 4.3 % Dai/g, and 11.27 µmol ± 3.6 % Gly/g. Other ISO derivatives Genistin, 6"-O-Ac-Genistin, Daidzin, 6"-O-Ac-Daidzin, Glycitin, 6"-O-Ac-Glycitin were also detected (data shown in our previous paper) [18].

Table 5. 1 Concentration of ISOs in Soy extract

Analytes	Content (umol/g soy extract)	RSD (%)
Genistein	19.62	5.0
Daidzein	40.91	4.3
Glycitein	11.27	3.6

Mean of ISO aglycones in soy extract; RSD, relative standard deviation

5.3.2 Effect of soy extract on myotube diameters

As shown in Fig.1A, different dilutions of Soy (0.0005-50 ug/mL) resulted in a bell-shaped dose–response curve, inducing a significant increase in myotube diameter at a concentration of 0.05 ug/mL. The increase was similar as induced by E2 at a concentration of 10 nM (Fig.1A&1C). Further, the diameters of C2C12 myotubes treated with Gen, Dai, Gly at concentrations of the same as determined in 0.05 ug/mL Soy and a mixture of the Gen, the Dai and the Gly (Mix) were measured (Fig.1B). Treatment of Gen and Mix led to significant increases of the diameters, which was comparable to the effect induced by Soy.

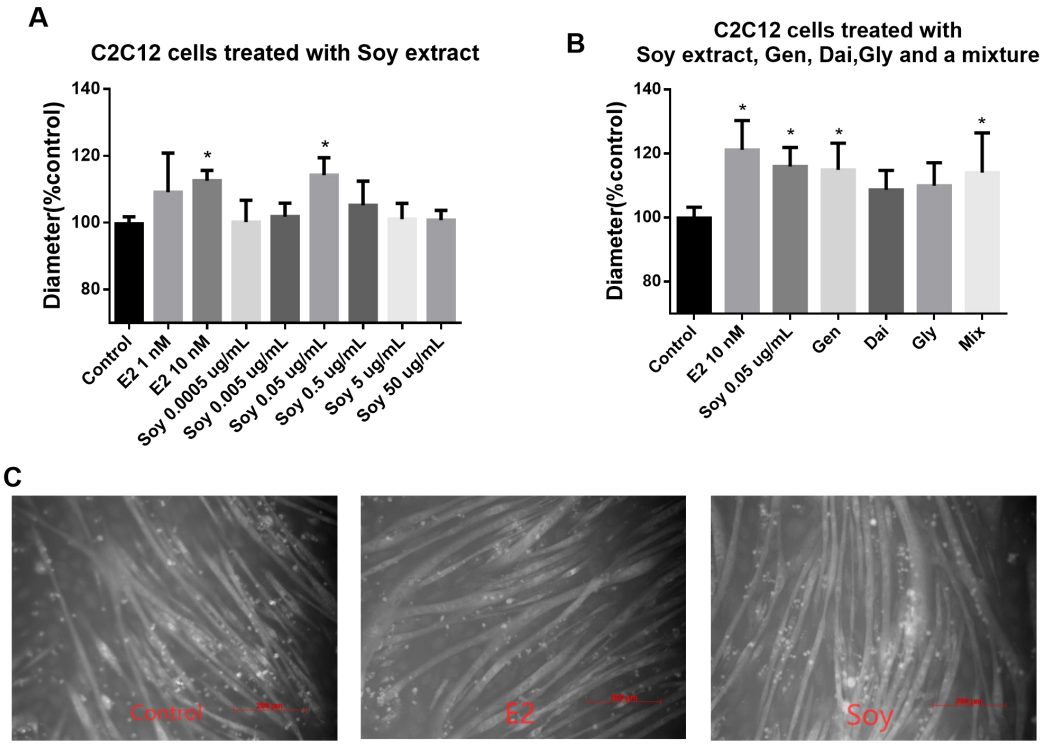

Fig.5. 1 Diameter measurement of C2C12 myotubes treated with increasing doses of Soy (A), and Gen, Dai, Gly at concentrations of the same as determined in 0.05 ug/mL Soy, as well as a mixture of the

Gen, the Dai and the Gly (Mix) (B). (C) shows differences of the diameters in control, E2 (10 nM) and Soy (0.05 ug/mL) groups.

The diameter of control group was set as 1,*$p \leq 0.05$ vs. control

5.3.3 Effect of soy extract on myotube number

The effect of the respective substances on the number of myotubes in a defined area was shown in Fig.2. Fig.2A&2B showed the same area of a 6-well plate before and 48 h after treatment with Soy of 0.05 ug/mL. It is clearly visible the treatment with Soy results in an increase of the myotube diameter and that the number of cells in this area has not changed. Comparing the number of myotubes per area between the treatment groups (Fig.2C) revealed treatment with all substances resulted in a significantly higher number of myotubes in the treated groups compared to the control group.

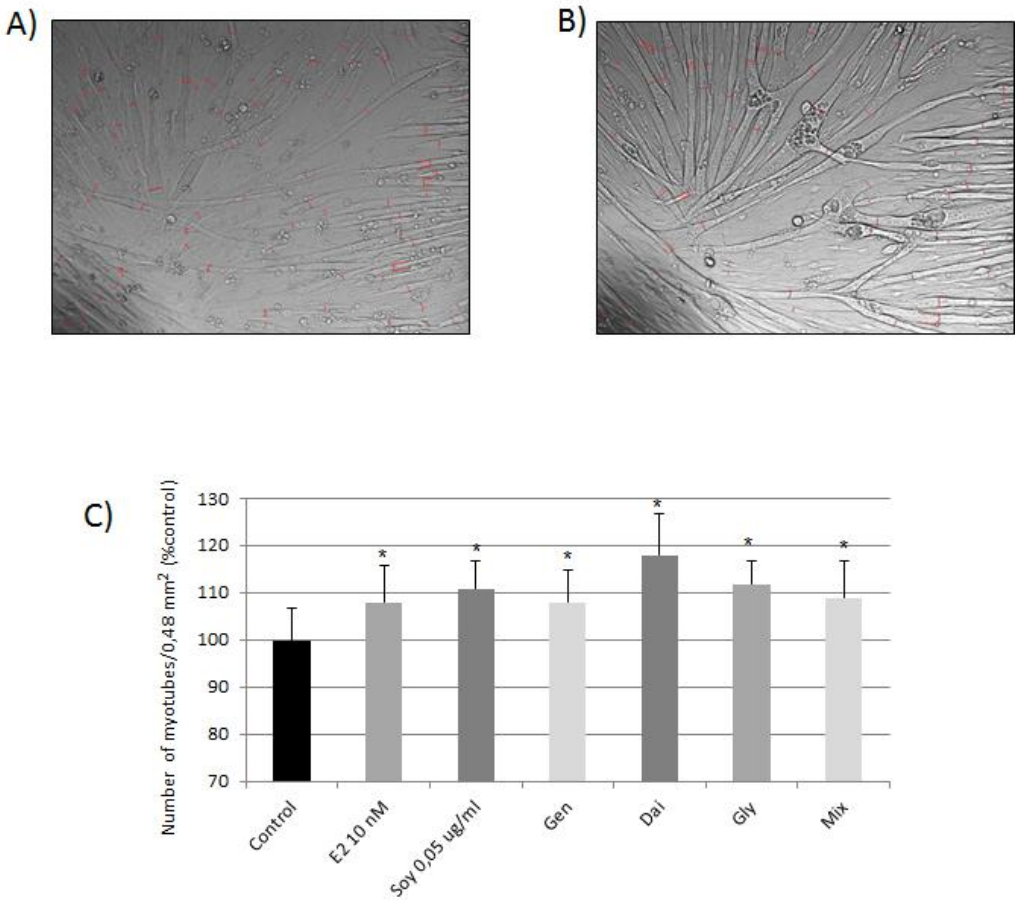

Fig.5. 2 Measurement of C2C12 myotube number in specific areas. (A) and (B) showed the same area on a 6-well plate before and 48 h after the treatment with Soy. (C) showed the number of myotubes in a 0.48 mm^2 area after 48 h incubation with E2, Soy, Gen, Dai, Gly and Mix.

The number of control group was set as 1, *p≤0.05 vs. control

5.3.4 Role of ER, AR in the anabolic effect of soy extract

As shown in Fig.3A, the increased diameter induced by either E2 or Soy was antagonized by ZK. In contrast, antiandrogen Flut significantly antagonized the myotube hypertrophy induced by DHT, whereas was not able to decrease the diameter when co-treatment with Soy (Fig.3B).

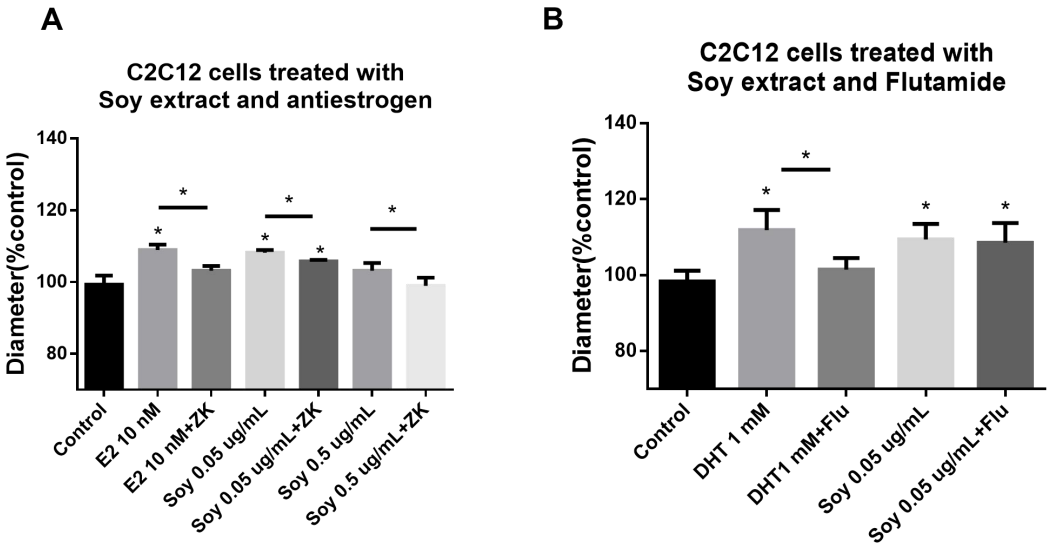

Fig.5. 3 Investigation of the role of AR (A) and ER (B) in affecting the increases of diameters of C2C12 myotubes that treated with Soy.

*$p \leq 0.05$ vs. control

5.3.5 Gene expression of IGF-1, IGF-1R, MuRF1 in C2C12 myotubes

The mRNA expressions of IGF-1, IGF-1R, MuRF1 in C2C12 myotubes are shown in Fig.4. The IGF-1 expression (Fig.4A) was significantly increased by the E2, Soy, Gen or Mix. There were no significant differences found between either E2 and Soy, or Soy and Gen, or Gen and Mix. Also, the IGF-1R expression (Fig.4B) was increased by E2, Soy, Gen or Mix in C2C12 myotubes, which was similar as regulated with IGF-1. However, the expression of MuRF1 (Fig.4C) was not affected by any of the treatments.

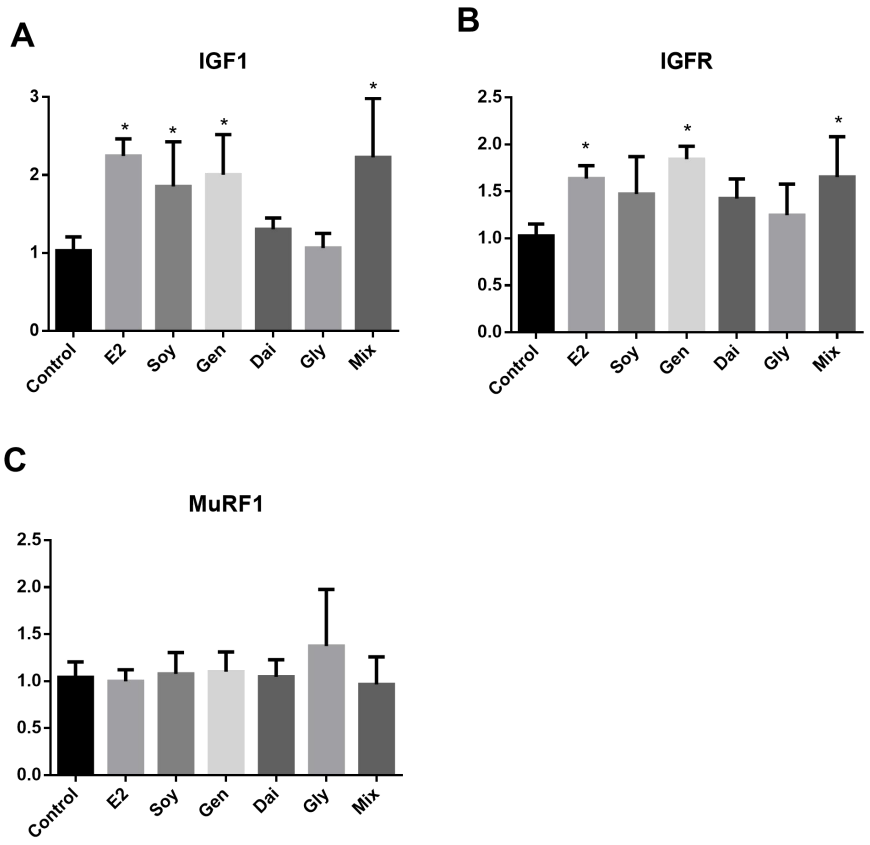

Fig.5. 4 Effects of Soy, Gen, Dai, Gly, Mix on relative mRNA expressions of IGF-1 (A), IGF-1R (B), and MuRF-1 (C) in C2C12 myotubes.

*$p \leq 0.05$ vs. control

5.3.6 Protein expression of MHC, MyoD in C2C12 myotubes

As shown in Fig.5A, E2, Soy, Gen, Mix significantly increased MHC protein expression and the highest expression was detected in E2 group. The effect of Gen was more striking compared to Dai or Gly. Moreover, the MyoD protein expression (Fig.5B) was not affected by any of the treatments.

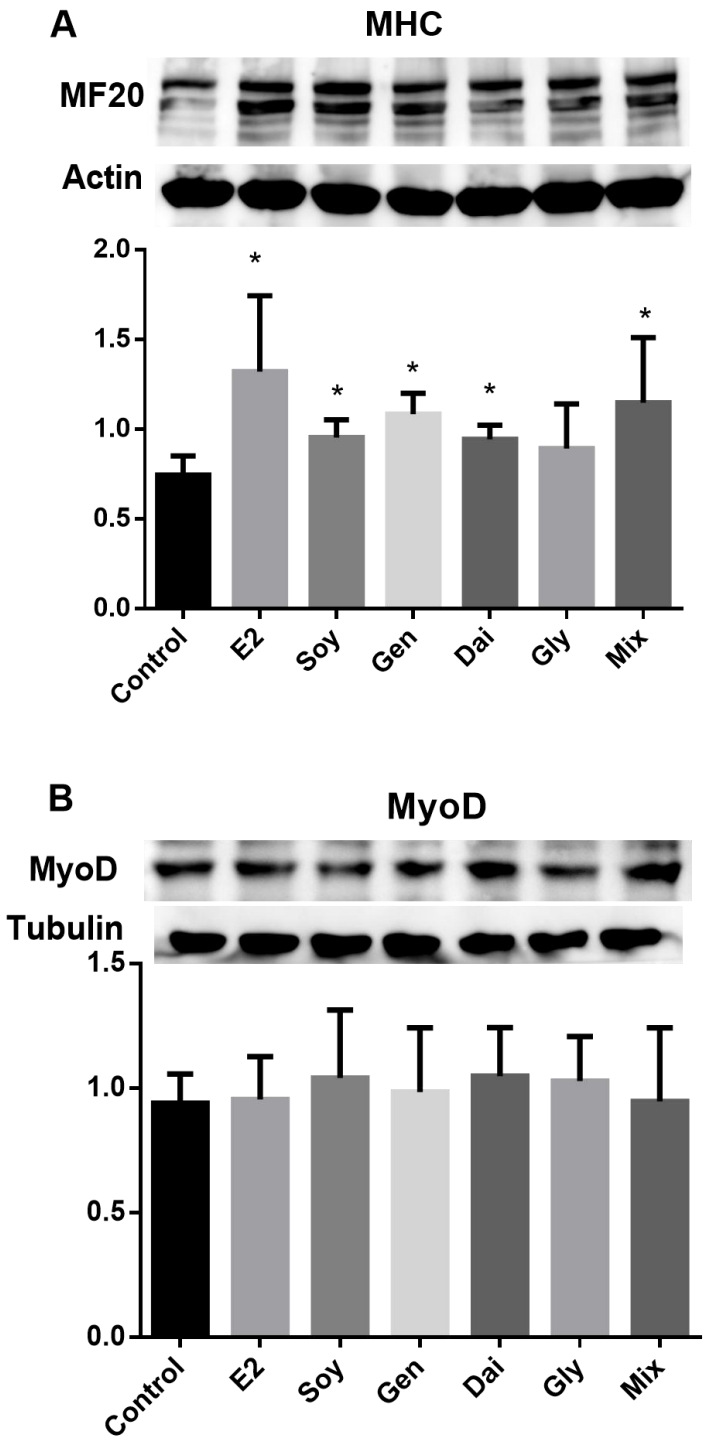

Fig.5. 5 Effects of Soy, Gen, Dai, Gly, Mix on protein expressions of MHC (A) and MyoD (B) in C2C12 myotubes.

*$p \leq 0.05$ vs. control

5.4 Discussion

Skeletal muscle growth, also known as hypertrophy, is characterized as an increase in muscle mass and myofiber size, which can be induced by anabolic hormonal substances (i.e. DHT and E2) [9]. C2C12 myoblastoma cells differentiated into myotubes is an accepted cell culture model to study anabolic activity [8]. Previously, studies of our group using a model of C2C12 myotubes demonstrated that ecdysterone affects muscle growth via ER mediated mechanisms [9]. In this study we could demonstrate dose dependent anabolic activity of Soy on C2C12 myotubes, which is quantitatively comparable to the effects observed for E2 (Fig.1A). There are a few in vitro studies investigating anabolic effects of Soy on muscle cell line. A study using rat L6 skeletal muscle cells showed Gen promoted cells proliferation at ≤ 1 μM and a trend of stimulation of protein synthesis could be observed at 1 μM Gen or Gly [19]. Previously, our group using in vivo studies demonstrated that ISOs increased soleus muscle weight in ovariectomized female rats [20, 21].

Interestingly our investigations also demonstrate that treatment with E2, Soy, ISOs and Mix resulted in a higher number of myotubes per area compared to the control cells (Fig. 2C). To our knowledge, comparable investigations have not been performed so far. Because in all groups the same number of cells was seeded and a comparable amount of myotubes were differentiated, we speculate that in the absence of the used substances in the control group some myotubes may die by mechanisms related to autophagy. But it needs to be investigated in future studies in detail.

To further investigate the molecular mechanisms of Soy ISO activity and to identify steroid hormone receptors serving as a target for Soy action, a co-incubation experiment with Soy and the antiestrogen ZK, or Soy and the antiandrogen Flut was performed. It is well known that C2C12 cells express both AR and ER [22, 23]. In Fig. 2, it is shown that the hypertrophic effect of Soy can be antagonized by the antiestrogen, but not the antiandrogen, indicating the anabolic effect of Soy is mediated via an interaction with ER signaling pathway rather than AR pathway. Some studies pointed out that Gen inhibited cell growth by suppressing AR transcription in prostate cancer cell line [24]. However, based on our results as well as many other studies which have already shown that Gen binds to ER [25], we conclude that AR is not involved in promoting myotube growth induced by Gen or Soy.

Gen, Dai, Gly are major ISO aglycones found in our soy extract. Apart from them, ISO glycosides (genistin, daidzin, glycitin) were also detected in the Soy. In intestines, ISO

glycosides are firstly hydrolyzed and converted to corresponding bioactive aglycones [26]. It was found that the aglycones are absorbed faster and in higher amounts than their glycosides after dietary ISO intake in humans [27], therefore the anabolic effects of ISO aglycone compositions on C2C12 myotubes were examined. The ISO aglycone compositions were the same amounts as in the determined analytically corresponding Soy (Fig.1B). Previously, a study in our group showed a mixture of Gen-Dai-Gly did not reach the same stimulation as the Soy on the proliferation of MCF-7 breast cancer cells [18]. However, in this study we found that ISO aglycones determined in the soy extract accounted for its anabolic potential on C2C12 myotubes in vitro. Moreover, the results showed that Gen acted as the most functional compound in the Soy on regulating the anabolic activity of C2C12 myotubes.

The regulation of skeletal muscle mass depends on the balance between protein synthesis and degradation. IGF-1 - phosphoinositide-3-kinase (PI3K)-Akt/protein kinase B (PKB) - mammalian target of rapamycin (mTOR) pathway acts as a positive regulator of muscle growth [28]. Many studies have reported an important role of IGF-1 in muscle hypertrophy [29]. In addition, muscle-specific inactivation of the IGF-1 receptor (IGF-1R) impairs muscle growth due to reduced muscle fiber number and size [30]. In this study both IGF-1 and IGF-1R mRNA expressions were detected being up-regulated by Soy. Meanwhile, Gen increased IGF-1 and IGF-1R expressions and the effects can be comparable to the Soy. The mRNA results of IGF-1 and IGF-1R were consistent with our data of diameters. Sometimes the protein synthesis and degradation are intimately connected. One study reported that IGF-1 stimulates muscle growth by decreasing MuRF1 [17]. MuRF1 is a muscle-specific ubiquitin ligase and regarded as an important regulator for muscle atrophy. Here in this study the regulation of MuRF1 gene expression was not involved in muscle hypertrophy induced by the Soy or Gen. The explanation could be that muscle hypertrophy is independent of MuRF1 expression, which was demonstrated in another study [31].

MyoD and MHC play important roles during the process of myogenesis in C2C12 cells [32] and they are associated with myostatin expression and localization which known as a major negative regulator of muscle growth [33]. The analysis of these two protein expressions in C2C12 myotubes showed MHC was affected by Soy, whereas MyoD was not. One main reason could be that MHC is characterized positively after formation of myotubes, but MyoD is mainly expressed before cells entering into the process of differentiation [32]. Several isoforms of MHC proteins are expressed in C2C12 myotubes. Among them, MHC-II is strongly expressed, whereas a relatively small amount of MHC-I

is expressed [33]. Therefore, the MHC-IIb might be the isoform modulated in this study. However, this needs to be further confirmed. Our previous study showed that MHC protein expressions of both soleus and gastrocnemius muscles were increased by E2 or Gen in an ovariectomized rat model [34]. In this study the distinct effects of the compositions in ISO aglycones on MHC expression in C2C12 myotubes were also investigated. The MHC expression pattern was similarly regulated as IGF-1 gene expression, which further proved the main role of Gen in Soy on stimulation of muscle growth. The effect was mediated by increasing MHC protein expression. Dai and Gly also increased MHC protein expressions, however, the effects were weaker than Gen. This result was consistent with the data of IGF-1 mRNA expression and diameter measurement.

Altogether, Soy ISOs enhance an anabolic activity of C2C12 myotubes by binding to ER and increasing IGF-1 and MHC expression. Among the compositions in ISO aglycones, Gen exhibits the strongest bioactivity on stimulating muscle growth.

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Conflicts of interest

The authors have declared no conflicts of interest.

Related work and a publication

In addition to ISOs, the effect of another phytoestrogen ecdysterone on skeletal muscle hypertrophy has been studied by measuring diameters of C2C12 myotubes. Further, ERβ has been demonstrated that plays an important role in the hypertrophic effect. This part of our study has been published as:

Estrogen receptor beta is involved in skeletal muscle hypertrophy induced by the phytoecdysteroid ecdysterone

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Abstract:

Scope: The phytoectysteroid ecdysterone (Ecdy) was reported to stimulate protein synthesis and enhance physical performance. The aim of this study was to investigate underlying molecular mechanisms particularly the role of ER beta (ERβ).

Results: In male rats, Ecdy treatment increased muscle fiber size, serum IGF-1 increased, and corticosteron and 17-estradiol (E2) decreased. In differentiated C2C12 myoblastoma cells, treatment with Ecdy, dihydrotestosterone, IGF-1 but also E2 results in hypertrophy. Hypertrophy induced by E2 and Ecdy could be antagonized with an antiestrogen but not by an antiandrogen. In HEK293 cells transfected with ER alpha (ERα) or ERβ, Ecdy treatment transactivated a reporter gene. To elucidate the role of ERβ in Ecdy-mediated muscle hypertrophy, C2C12 myotubes were treated with ERα (ALPHA) and ERβ (BETA) selective ligands. Ecdy and BETA treatment but not ALPHA induced hypertrophy. The effect of Ecdy, E2, and BETA could be antagonized by an ERβ-selective antagonist (ANTIBETA). In summary, our results indicate that ERβ is involved in the mediation of the anabolic activity of the Ecdy.

Conclusion: These findings provide new therapeutic perspectives for the treatment of muscle injuries, sarcopenia, and cachectic disease, but also imply that such a substance could be abused for doping purposes.

(This paper is attached in the appendix)

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CHAPTER 6: GENERAL DISCUSSION

In this final chapter, the key findings of the thesis are represented and subsequently will be discussed and compared with the current literature. Against this background, a conclusion and outlook are given. The aims of the present thesis were to evaluate the effects of dietary isoflavone (ISO) intake, physical activity and the combined intervention on alleviating menopausal symptoms such as muscle loss, osteoporosis, obesity, hyperlipemia and to elucidate molecular mechanisms involved in skeletal muscle adaptation and fatty acid metabolism which are influenced by the interventions.

Key findings of the presented work

1. Ovariectomy (OVX) in rats results in a decrease of relative gastrocnemius muscle weight. Meanwhile, body weight, fat mass, leptin and lipid levels are increased by OVX. All these effects induced by OVX can be antagonized by the combination of ISOs and training.
2. Both ISOs and training are able to increase relative skeletal muscle mass and the expression of molecular markers related to anabolic adaptation in skeletal muscle. The combined intervention shows additive effects.
3. Genistein (Gen) might be the most efficient compound in soy extract exerting anabolic activity on skeletal muscle growth.
4. Training seems to have a higher impact than ISO exposure on visceral fat prevention. However, the strongest effects for several of the addressed parameters could be observed in the combination group especially in the soleus muscle. Therefore a combination of training and an ISO-rich diet may have beneficial effects on fatty acid metabolism and could be a concept for the prevention of metabolic diseases in postmenopausal females.

6.1 Effects of training and isoflavones on preventing negative impacts caused by estrogen deficiency

OVX Wistar rats were chosen as an animal model to mimic the situation of estrogen deficiency in postmenopausal women. As expected, OVX resulted in lower uterus wet weights, indicating that the OVX rats were estrogen deficient. Our previous studies reported that estrogen substitution antagonized OVX induced uterus weight reduction [1], whereas dietary ISO intake in this study did not show such effects. The uterus growth is a physiological end point which is mainly mediated by ERα [2]. This inability of ISOs to stimulate uterine weight can be discussed due to the lower binding affinities to ERα than to

ERβ [3, 4].

Regarding health risks, it is important to figure out that important changes occur in body composition of females including fat mass, muscle mass and bone mass after menopause. OVX led to an increase in body weight in this study, which was mainly caused by the increase of visceral fat mass. The accumulation of visceral fat is considered as a major determinant of the metabolic syndrome. Women with high amounts of visceral fat are normally associated with insulin resistance, high cardiovascular mortality [5]. Meanwhile, adipocyte size and serum leptin level were increased by OVX in this study. An enlargement of adipose tissue leading to obesity was characterized by an increase of adipocyte size in this study. Leptin is the most important protein that regulates energy intake and expenditure by acting on several neurons in the hypothalamus and people with obesity show resistance to leptin. Body weight gain and leptin level were antagonized by either training or ISOs. The effect of training on body weight gain has been reported in our previous study [1]. Additionally, the effect of ISOs on body weight gain was comparable to effects observed for E_2 which was demonstrated in our previous study [1]. However, only training showed effects on reducing fat mass and adipocyte size in this study. Our previous study demonstrated that lifelong but not short-term ISO intake decreases visceral fat mass and adipocyte size in OVX rats [6], which could be an explanation for the results in this study. Leptin is a very sensitive indicator for total body fat mass [7]. However, one study reported that leptin plasma concentration is dependent on body fat distribution in obese patients, whilst independent of the amount of fat mass [8]. These all might be reasons to explain the different results between visceral fat mass and leptin level induced by ISOs.

Reductions of skeletal muscle mass and bone mass, and increments of lipid levels were observed in OVX rats in this study. The combination of ISOs and training led to significant effects of increasing the relative weights of the musculus gastrocnemius and soleus, and decreasing serum triglyceride level. It is still controversial discussed whether a combination of ISOs and exercise exerts additive effects. No synergistic effects were observed between ISOs and exercise on body composition in several studies [9, 10]. In contrast, there are studies showing a combination of ISOs and exercise improves lean body mass and bone mass more effectively [11, 12].

6.2 IGF-1, IGF-1R, MyoD and Myogenin expressions are regulated by training and isoflavones and involved in muscle anabolic adaptation

The activation of IGF-1 ligand binding to IGF-1R triggers a signaling pathway which is

important for muscle growth. The release of IGF-1 is considered to be a compensatory process of muscle hypertrophy [13]. The IGF-1 and IGF-1R gene expressions were both up-regulated by the combination of training and ISOs in soleus muscle, whereas no effects were observed in gastrocnemius muscle. In rodents, overloading stimulates early increases in IGF-1 in slow twitch oxidative fibers (type I) than in fast twitch glycolytic fibers (type II). Therefore, the explanation for increasing IGF-1 gene expression only in soleus muscle could be a feedback of promoting type I muscle fiber to type IIA muscle fiber. Training alone did not show effects on increasing IGF-1R in OVX rats, whereas the gene expression could be enhanced by either training in Sham rats or the combined intervention. Therefore, ISOs functioned as estrogen substitution, regulating IGF-1R expressions in soleus muscle. Moreover, the MyoD gene expression was similarly modulated as IGF-1R in soleus muscle. The supporting theory could be that the expression of local IGF-1 is able to increase levels of myogenic regulatory factors (MRFs) and promotes myofiber regeneration and hypertrophy [14]. Both MyoD and Myogenin belong to the family of MRFs and are involved in myogenesis processes of regulating satellite cells from quiescent status to myotubes [15]. In this study MyoD and Myogenin expressions were reduced by OVX in gastrocnemius muscle, however, the expression could be increased by either ISOs or training. The results demonstrated that the muscle anabolic effects are affected by training and ISOs. The combination of the two interventions showed additive effects on regulating MyoD expression in both soleus muscle and gastrocnemius muscle, which was consistent with our data of relative muscle weights.

6.3 Genistein is an active form in isoflavones on stimulating C2C12 myotube growth

Skeletal muscle hypertrophy is a dynamic process including both an increase of the number of nuclei (due to the stimulation of the proliferation, fusion and differentiation of quiescent satellite cells) and an increase of cytoplasmic volume (due to an increase in protein synthesis or remodeling). As demonstrated in our animal experiment, ISO intake in vivo showed potential ability to promote skeletal muscle growth by up-regulating gene expressions involved in myogenesis, which is a process of increasing number of nuclei in muscles. However, we were also interested in the effects of ISOs on the increase of cytoplasmic volume. Therefore, a model of C2C12 myotubes that fusion and differentiation from myoblasts was established [16]. The myotubes were used to mimic the existing myofibers and were treated with ISOs in this study. E2 has been shown to induce C2C12 myotube hypertrophy as a positive control previously [16]. This in vitro experiment

showed the soy extract induced muscle hypertrophy and the effect was mediated through ER rather than AR. Further, ERβ was demonstrated that involved in skeletal muscle hypertrophy [16]. ISOs in vivo after consuming is firstly hydrolyzed to the ISO aglycones Gen, daidzein (Dai) and glycitein (Gly) [17, 18]. Therefore the anabolic effects of the three aglycones were investigated. Gen was found to be more active than Dai and Gly on increasing myotube diameter as well as enhancing IGF-1, IGF-1R and MHC expressions. In addition, the effects of Gen could be comparable to the soy extract.

6.4 Effects of training and isoflavones on regulating SREBP-1c, FAS, PPARδ, PGC-1α expressions in adipose tissue, soleus muscle and liver

The expression of genes associated with fatty acid synthesis (SREBP-1c and FAS) and fatty acid oxidation (PPARδ and PGC-1α) were distinguishingly modulated by ISOs or training in different tissues. Adipose tissue and liver play important roles in energy and fatty acid metabolism. Skeletal muscle accounts for the most mass of an individual and daily energy consumption. Regarding the genes, SREBP-1c belongs to a family of transcription factors originally involved in regulating cholesterol from a cellular availability [19]. SREBP-1c induces the expression of a family of genes involved in glucose utilization and fatty acid synthesis. In this study OVX led to decreases of SREBP-1c and FAS gene expressions in adipose tissue and skeletal muscle, which was in line with other studies [20, 21]. Leptin regulation could be a main reason for the effect. High leptin level was reported to be directly associated with down-regulation of SREBP-1c and FAS mRNA expressions [21]. Therefore, the training increased SREBP-1c and FAS expressions in adipose tissue and soleus muscle due to a down-regulation of leptin level. Similarly, ISOs increased the two gene expressions in soleus muscle. The effects of ISOs on modulating SREBP-1c and FAS expressions in adipose tissue were not significant. However, it is generally consistent with our results of fat mass and adipocyte size. The training played a dominant role in regulating SREBP-1c and FAS when combined with ISOs in adipose tissue, as we also observed in the data of fat mass and adipocyte size.

OVX increased hepatic SREBP-1c expression in this study, which could be antagonized by ISO supplementation. Several studies have shown soy composition or soy protein reduced hepatic SREBP-1c or FAS expression, therefore reducing fatty acid synthesis [22]. Insulin induces SREBP-1c expression in insulin sensitive tissues, particularly in the liver. Through the observation in lipid profile, it is assumable that OVX induced insulin resistance from increased cholesterol, HDL, LDL and triglyceride content, thereby leading to a high

SREBP-1c level. However, similar as in adipose tissue and soleus muscle, the training also increased SREBP-1c and FAS expression in liver. Therefore, probably there are two different signaling pathways in liver mainly mediating the expression of SREBP-1c and FAS induced by estrogen and exercise respectively.

In regard to PPARδ and PGC-1α expression level in this study, OVX resulted in decreases of their expressions in adipose tissue and soleus muscle, which could be antagonized by the combination of training and ISOs. The additive combinatory effect was obviously observed in soleus muscle. PPARδ is abundantly expressed throughout the body but at low levels in liver, which could be an explanation for that no significant regulation of hepatic PPARδ and PGC-1α was observed by ISOs or training. In adipose tissue the activation of PPARδ results in lean mice resistant to obesity [23]. An over-expression of PPARδ and PGC-1α increases the number of mitochondria and induces expression of genes responsible for oxidative metabolism and fatty acid catabolism [24, 25]. Therefore, in this study we demonstrated that a combination of training and ISOs is an effective strategy to promote oxidation of fatty acids and therefore reduce fat accumulation at least in adipose tissue and soleus muscle.

Conclusion and outlook

With menopause transition, the decline of endogenous estrogens, as well as physical inactivity, results in a variety of negative physiological changes. New strategies particularly focus on prevention and therapy of muscle loss and metabolic syndrome in postmenopausal women have to be found.

The present study made a contribution to a sophisticated comprehension of the effects of dietary ISO intake, physical activity and the combined intervention on preventing muscle loss and metabolic syndrome which induced by menopause transition. Our data provide evidence that ISOs combined with training could be an effective strategy to promote muscle growth and reduce a risk of developing metabolic diseases in postmenopausal women. However, the combination of ISOs and training seems to exert no additive effects on reducing body weight and fat mass compared to training. The reason might be that ISO intake was not long enough and the training was very heavy, therefore the effects of ISOs were hidden. Potentially, in the future the effects of a longer term of ISO intake combined with a low to moderate density of training are worth to be investigated.

As an animal experiment, it permits a detailed analysis of each essential tissue and gives the first insight into the underlying molecular mechanisms. IGF-1, IGF-1R, MyoD,

Myogenin and MHC are demonstrated that involved in the skeletal muscle anabolic adaptation. However, other molecular responses involved in IGF-1-induced signaling pathway are not examined yet. In addition, the muscle physiology changes induced by training are definitely worth to investigate in the future.

To clarify the molecular mechanism involved in lipogenesis and lipid oxidation, SREBP-1c, FAS, PPARδ and PGC-1α expressions are analyzed in three different tissues-adipose tissue, liver and soleus muscle. Our results clearly showed the four gene expressions were regulated by training or ISOs. Leptin seems to be the main factor to regulate SREBP-1c and FAS gene expression by training. Moreover, insulin seems to be a very important regulator in determining SREBP-1c and FAS gene expression affected by ISOs. Further research is needed concerning the interaction between ISOs, physical activity and leptin, insulin secretion. In addition to leptin, other adipocytokines such as resistin, adiponectin and ghrelin might be also interesting to be measured as they have a close connection with the aetiology of obesity and its consequences. Additionally, serum adipocytokines and hyperinsulinemia are closely correlated with inflammatory markers and chronic inflammation which are associated with the development of many diseases. Therefore, in the future the research could establish a connection between lipid metabolism and inflammation.

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CONTRIBUTIONS TO CHAPTER 3, 4 AND 5 (EXPERIMENTS)

The work in this thesis involves contributions of my own and my colleagues and a master student of Prof. Dr. Diel. In the following, my specific contributions to each publication (and publication in preparation) are outlined.

Chapter 3

- Performance of animal training together with Jonas Hengevoss
- Animal caring together with Ute Laudenbach-Leschowski
- Tissue collection and preparation in collaboration with Ute Laudenbach-Leschowski, Jonas Hengevoss, Anne Kurrat and Dennis Mueller
- Immunohistochemical staining and determination of skeletal muscle fiber size
- Determination of IGF-1 in serum by ELISA
- Real-time PCR of several gene analysis in soleus muscle and gatrocnemius muscle
- Western blot of MyoD protein analysis in gastrocnemius muscle
- Preparation of manuscript together with Prof. Dr. Patrick Diel

Chapter 4

- Same animals used as in the study of Chapter 3
- HE staining and determination of adipocyte size
- Determination of leptin in serum by ELISA
- Real-time PCR of several gene analysis in adipose tissue, soleus muscle and liver. Part of the work with hepatic gene expression analysis was helped by Jana Rogoschin and Kristina Oden
- Western blot of FAS protein analysis in adipose tissue
- Preparation of manuscript together with Prof. Dr. Patrick Diel

Chapter 5

- All the experiments with C2C12 myotubes treated with soy extract and isoflavones only except the measurement of the number of myotubes
- Measurement of the diameters of C2C12 myotubes treated with β agonist for the study of investigating effects of ecdysterone
- Preparation of manuscript with Prof. Dr. Patrick Diel

PUBLICATIONS AND PRESENTATIONS

Published paper

[1] **Wenya Zheng**, Jonas Hengevoss, Sebastian T. Soukup, Sabine E. Kulling, Mingyong Xie, Patrick Diel*. An isoflavone enriched diet increases skeletal muscle adaptation in response to physical activity in ovariectomized rats [J]. *Molecular nutrition & food research* DOI:10.1002/mnfr.201600843
[2] Maria Kristina Parr, Piwen Zhao, Oliver Haupt, Sandrine Tchoukouegno Ngueu, Jonas Hengevoss, Karl Heinrich Fritzemeier, Marion Piechotta, Nils Schloerer, Peter Muhn, **Wenya Zheng**, Mingyong Xie, Patrick Diel*. Estrogen receptor beta is involved in skeletal muscle hypertrophy induced by the phytoecdysteroid ecdysterone[J]. *Molecular nutrition & food research*, 2014, 58, 1861–1872

Submitted manuscript

[1] **Wenya Zheng***, Jana Rogoschin, Anja Niehoff, Kristina Oden, Sabine E. Kulling, Mingyong Xie, Patrick Diel. Combinatory effects of phytoestrogens and exercise on body fat mass and fatty acid metabolism in ovariectomized female rats. *The journal of steroid biochemistry and molecular biology*

Prepared manuscript

[1] **Wenya Zheng**, Patrick Diel. Anabolic activity of a soy extract in C2C12 myotubes

Presentations

[1] **Wenya Zheng**, Jonas Hengevoss, Patrick Diel. Effects of estrogen deficiency and isoflavone on skeletal muscle adaptation in response to physical activity. *American college of sport medicine annual meeting (ACSM conference)*, 2016, poster presentation (Highlighted in the conference and a student award was given)
[2] **Wenya Zheng**, Valentina Lionello, Matthieu Raess, Narjoussa Batti (as a group). Functional exploration of skeletal muscle in rodents and human. *Myograd summer school Paris, 2015*, slide presentation
[3] **Wenya Zheng**, Jonas Hengevoss, Patrick Diel. Effects of estrogen deficiency and isoflavone on body composition in response to physical activity. *ESE Bregenz summer school on endocrinology, 2014*, poster presentation

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APPENDIX

Chapter 5 → A published paper related to the anabolic effects of a phytoestrogen on C2C12 myotubes

RESEARCH ARTICLE

Estrogen receptor beta is involved in skeletal muscle hypertrophy induced by the phytoecdysteroid ecdysterone

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Scope: The phytoectysteroid ecdysterone (Ecdy) was reported to stimulate protein synthesis and enhance physical performance. The aim of this study was to investigate underlying molecular mechanisms particularly the role of ER beta (ERβ).

Results: In male rats, Ecdy treatment increased muscle fiber size, serum IGF-1 increased, and corticosteron and 17β-estradiol (E2) decreased. In differentiated C2C12 myoblastoma cells, treatment with Ecdy, dihydrotestosterone, IGF-1 but also E2 results in hypertrophy. Hypertrophy induced by E2 and Ecdy could be antagonized with an antiestrogen but not by an antiandrogen. In HEK293 cells transfected with ER alpha (ERα) or ERβ, Ecdy treatment transactivated a reporter gene. To elucidate the role of ERβ in Ecdy-mediated muscle hypertrophy, C2C12 myotubes were treated with ERα (ALPHA) and ERβ (BETA) selective ligands. Ecdy and BETA treatment but not ALPHA induced hypertrophy. The effect of Ecdy, E2, and BETA could be antagonized by an ERβ-selective antagonist (ANTIBETA). In summary, our results indicate that ERβ is involved in the mediation of the anabolic activity of the Ecdy.

Conclusion: These findings provide new therapeutic perspectives for the treatment of muscle injuries, sarcopenia, and cachectic disease, but also imply that such a substance could be abused for doping purposes.

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1 Introduction

Ecdysteroids are polyhydroxylated steroids playing an important role during the molting (ecdysis) of insects. They are also found in a variety of plants such as spinach and are main bioactive components of herbs such as *Cyanotis vaga*, *Leuzea carthamoides*, or *Rhaponticum carthamoides* used in traditional Chinese medicine.

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Abbreviations: **β-gal**, β-galactosidase; **AR**, androgen receptor; **Dexa**, dexamethasone; **DHT**, dihydrotestosterone; **E2**, 17β-estradiol; **Ecdy**, ecdysterone; **ER**, estrogen receptor; **ERα**, ER alpha; **ERβ**, ER beta; **FBS**, fetal bovine serum; **Flut**, antiandrogen flutamide; **IGF-1**, insulin-like growth factor 1; **m. lev. ani**, musculus lev ani; **s.c.**, subcutaneously; **TBS-T**, Tris-buffered saline supplemented with 0.1% Tween 20

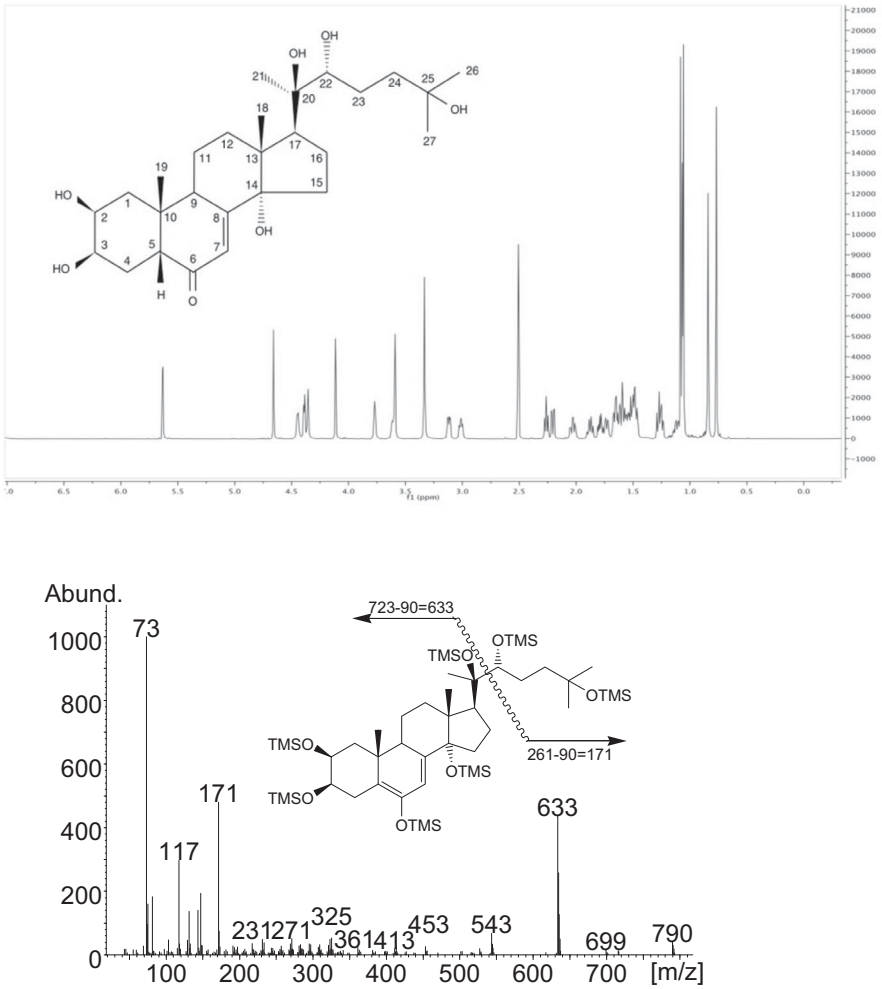

Figure 1. Confirmation of the chemical identity of ecdysterone. Chemical structure and ^{1}H NMR spectrum [δ ppm] of ecdysterone in DMSO–d6 and mass spectrum of ecdysterone heptakis-TMS, M^{+} = 984.

The first characterized ecdysteroid was α-ecdyson (ecdyson), which was isolated from the silkworm *Bombyx mori* [1]. In spinach (*Spinacia oleracea* L.), the major ecdysteroid is ecdysterone (Ecdy; chemical structure in Fig. 1) which, depending of the growth rate of the plant, can occur in concentrations between 50 and 800 μg/g [2].

Numerous studies describe a variety of pharmacological effects of Ecdy-like increase in carbohydrate and fatty acid metabolism, stimulation of immune response, and an increase in protein synthesis and physical power [3]. For these reasons, Ecdy is marketed as dietary supplement for athletes. It is advertised to induce anabolic effects but to have none of the classical side effects of anabolic steroids such as virilization.

The molecular mechanisms involved in the Ecdy-induced effects are only barely characterized. In arthropods, the effects of Ecdy are mediated via a specific nuclear receptor complex consisting of two proteins, the Ecdy receptor (EcR) and ultraspiracle protein (USP). Mammalians do not express this receptor complex, but it has been speculated that Ecdy could bind to other nuclear receptors such as the human steroid hormone receptors [4]. In receptor-binding assays, no significant binding affinity of Ecdy to the androgen receptor (AR) could be detected [5, 6]. Alternatively, it was discussed that Ecdy might interact with receptors located in the cell membrane of mammalian cells. Antioxidative effects of Ecdy on cell membrane lipids were described [7]. Moreover, it has been demonstrated that treatment with Ecdy activates the PI3K/AKT signal transduction pathway, which is also activated by insulin-like growth factor 1 (IGF-1) [6]. A calcium-dependent G-protein-coupled mechanism is also debated. However, the associated G-protein has not been identified so far [8].

There is increasing evidence that, besides androgens and IGF-1, estrogens are also involved in the regulation of skeletal muscle homeostasis. Estrogen receptors (ERs) are ligand-activated transcription factors belonging to the family of nuclear receptors [9]. Upon ligand binding, ERs bind to estrogen response elements on target genes and modulate gene expression by interacting with transcription factors and other coregulatory factors. There are two ER subtypes, ER alpha (ERα) and ER beta (ERβ), with homology of amino acid

sequences in the DNA-binding domain and similar binding affinity to the ligand 17β-estradiol (E2) but different tissue distribution and expression levels. While ERα is predominantly expressed in reproductive tissues such as the uterus and mammary gland, as well as in the heart, liver, and kidney, ERβ is mainly expressed in the gastrointestinal tract, vascular endothelial cells, and the prostate [10]. However, there are a number of tissues and cell types where both receptors are coexpressed, such as epididymis, thyroid, bone, and regions of the brain [10]. Both ERα and ERβ are expressed in skeletal muscle of mice [11, 12] and humans [13, 14].

With respect to molecular mechanisms mediating the anabolic effects of Ecdy, it seems of high interest to investigate whether Ecdy may be able to act through ERs. Recently, it has been demonstrated that selective activation of ERβ strongly induces regeneration and probably also de novo fiber formation in toxin-damaged muscles of female rats [15]. Moreover, a selective ERβ agonist was shown to be anabolic in male and female rats [15, 16]. These observations indicate a major role of ERβ in skeletal muscle homeostasis in both genders. The question whether Ecdy can interact with ER is controversially discussed. In receptor-binding assays, no significant binding affinity of Ecdy to ERα has been described [5]. Data describing the binding affinity of Ecdy to ERβ have not been published so far. However, Gao et al. [17] have demonstrated that Ecdy is able to transactivate ERβ.

The aim of this study was to further elucidate the anabolic mechanism of Ecdy and to get a deeper insight into the molecular mechanisms involved. Hereby, a specific focus was put on the potential role of the ERs. First, an in vivo study was conducted in male rats. Male Wistar rats were treated for 21 days subcutaneously (s.c.) either with Ecdy or placebo. Effects on the weight, fiber composition, and fiber size of the soleus and gastrocnemius muscles of the rats were studied. Serum concentrations of IGF-1, thyroxin (T4), testosterone, estradiol, inhibin, prolactin, and corticosterone were determined.

To elucidate whether the ER may be involved in the anabolic activity of Ecdy, differentiated C2C12 cells were treated with Ecdy, ERα (ALPHA) and ERβ (BETA) selective ligands, IGF-1, dexamethasone (Dexa), or dihydrotestosterone (DHT), and effects on the myotube diameter were investigated. In addition, myotubes were cotreated with Ecdy and the unselective ER-antagonist ZK or ANTIBETA. Myotubes cotreated with E2 and ZK or ANTIBETA served as control.

2 Materials and methods

2.1 Substances and chemicals

Ecdy was obtained from Steraloids (Wilton, NH, USA). Purity (Ecdy, purity labeled ≥93%) and structure were confirmed by NMR and MS. DHT, Dexa, IGF-1, E2, and the antiandrogen flutamide (Flut) were purchased from Sigma-Aldrich (Steinheim, Germany). The unselective antiestrogen ZK 191703 (ZK), selective ERβ antagonist ZK 283361 (ANTIBETA, European patent EP 1 365 768 B1), ERα-selective agonist 16α-LE2 (ALPHA), and selective ERβ agonist 8β-VE2 (BETA) were provided by Bayer Pharma AG (Berlin, Germany). Corn oil was purchased from Sigma-Aldrich. Glutaraldehyde and ethanol were from Merck (Darmstadt, Germany). Cell culture medium and medium components were all purchased from Gibco-Invitrogen (Darmstadt, Germany).

2.2 NMR spectroscopy

The NMR data were obtained using a Bruker DRX 500 instrument, equipped with a z-gradient coil. The spectra were recorded at 600 MHz (^{1}H) and 150 MHz (^{13}C) in DMSO-d6. For confirmation of the structure, ^{1}H, ^{13}C, and distortionless enhancement by polarization transfer (DEPT) spectra were measured together with 2D HH-correlated spectroscopy (HH-COSY), heteronuclear single quantum coherence (HSQC), and heteronuclear multiple bond correlation (HMBC) spectra. Chemical shifts were determined in δ values (ppm) relative to tetramethylsilane.

2.3 MS

Mass spectra of the trimethylsilyl (TMS)-derivatized analyte were recorded on an Agilent 5973 Network mass-selective detector with electron ionization (70 eV) after separation on an Agilent 6890 Series gas chromatographic (GC) system (Agilent, Waldbronn, Germany). The column used was a 17.0 m HP5MS (0.25 mm inner diameter, 0.25 m film thickness). Oven temperature was increased from 140 to 320°C by 20°C/min, and held for 3 min. Injection volume was 2 μL in split mode (1:10) for the reference material. Helium was used as carrier gas (1.5 mL/min).

2.4 Animal study

Male Wistar rats were obtained from Janvier Laboratories (Le Genest St. Isle, France) and kept under controlled conditions of temperature (20 ± 1°C, relative humidity: 50–80%) and illumination (12-h light/12-h dark). The rats had free access to standard diet (SSniff GmbH, Soest, Germany) and water. All animal procedures were approved by the Committee on Animal Care (Approval no. 9.93.2.10.35.07.240) and complied with accepted veterinary medical practice.

Male Wistar rats were randomly allocated to treatment and vehicle groups ($n = 6$) and treated for 21 days either with 5 mg/kg Ecdy or vehicle. Ecdy was dissolved in ethanol and diluted in corn oil (ethanol 20%, corn oil 80%) and was administrated s.c. once daily. The treatment dose of Ecdy was based on previous studies showing an increase in muscle mass [18]. After completion of treatment, the animals were sacrificed by decapitation and blood was collected. The weight

of prostate, seminal vesicles, and levator ani muscle was measured immediately. For histological analysis, soleus and gastrocnemius muscles were removed, embedded in TissueTek (Sakura, Staufen, Germany), cooled in isopentane close to freezing, and frozen in liquid nitrogen.

2.5 Histological analysis

Cryosections from the soleus muscle were cut at 7 μm thickness and stained with hematoxylin and eosin. Areas of the muscle fibers were determined using a light microscope (Axiovert 200M, Zeiss, Jena, Germany) and the Axiovision LE software.

Gastrocnemius cryosections (7 μm) were stained by the myofibrillar adenosine triphosphatase (mATPase) method. The areas of the muscle fibers were measured in images captured with a light microscope (KS 300, Zeiss) and the ImageJ 3.0 software.

2.6 C2C12 hypertrophy cell culture model

For cell culture experiments, C2C12 cells (American Type Culture Collection, Manassas, VA, USA) were grown in DMEM supplemented with 10% fetal bovine serum (FBS), 4 nM glutamine, 1.5 g/L sodium bicarbonate, 100 mM sodium pyruvate, and 100 units/mL penicillin/streptomycin. Differentiation toward myotubes was induced at confluence by shifting the proliferation medium to differentiation medium (DMEM with 2% horse serum). During the whole experiment, the cells were stored at atmospheric conditions of 5% CO_2 and 37°C.

Differentiated myotubes were incubated for 48 h in the presence of either Ecdy (1 μM), DHT (100 nM), Dexa (1 μM and 10 μM), IGF-1 (10 ng/mL), E2 (10 nM), ALPHA (0.01 nM-100 nM), BETA (0.01 nM-100 nM), or vehicle (medium) only. For the antagonization study, cells were treated with combinations of DHT–Flut (100 nM/1 μM), Ecdy–Flut (1 μM each), E2–ZK (10 nM/1 μM), E2–ANTIBETA (10 nM/1 μM), BETA–ANTIBETA (0.01 nM/1 μM), Ecdy–ZK (1 μM each), and Ecdy–ANTIBETA (1 μM each).

To determine myotube diameter, cells were fixed and photographed by glutaraldehyde-induced autofluorescence. Myotube diameters of 50 myotubes per group were measured every 10–20 μm along the length of the myotube using a fluorescence microscope (Axiovert 200M, Zeiss) and the Axiovision LE software.

2.7 Plasmid transfection and luciferase reporter gene assay in HEK293 cells

HEK293 cells (American Type Culture Collection, Manassas, VA, USA) were grown in phenol red free DMEM containing CDT-FBS for 4 days. One day before transfection, HEK293 cells were plotted in 24-well plates at a concentration of 2×10^4 cells per well. For transfection, maintenance media were aspirated and 100 μL transfection mix was added. Cells were cotransfected with 0.1 μg ERα or 0.1 μg ERβ, 0.8 μg ERE-luc, and 0.1 μg β-galactosidase (β-gal) plasmid (Clontech Laboratories, Inc., Mountain View, CA, USA) as control using lipofectamine 2000 (2 μL/well) (Invitrogen, Carlsbad, CA, USA). After an incubation of 4 h for transfection according to the manufacturer's instructions, the experimental medium prepared with phenol red free DMEM, 10% charcoal-stripped FBS (Hyclone, Logan, UT, USA), and Ecdy with the final concentrations from 10^{-6} to 10^{-11} M was added without removal of the transfection media. Each experiment also included an estradiol standard curve with tenfold dilutions from 10^{-8} to 10^{-13} M. A vehicle control containing 0.1% ethanol, which was the maximal level of ethanol used in the treatment media, was also included. After incubated for 24 h at 37°C, media was aspirated and cells were lysed with 100 μL of Glo Lysis Buffer (Promega, Madison, WI, USA). Lysates were then transferred to microcentrifuge tubes and stored at –70°C. For luciferase assay, 50 μL lysate was added to 50 μL steady Glo Luciferase Assay reagent (Promega). Luminescence was measured by multifunctional microplate reader (Tecan). A β-gal assay kit (Promega) was used for enzyme activity assay, and the final result of luciferase activity was standardized by β-gal value so as to correct well-to-well variation. The maximal achievable response to 10^{-8} M estradiol was arbitrarily set at 100%, and the luciferase activity in response to Ecdy was measured by relative light units. Effects of ER-antagonist ZK on luciferase activities induced by 10^{-8} M estradiol or 10^{-6} M Ecdy in transfected HEK293 cells were also observed in the test.

2.8 Protein preparation and Western blot

The cells were seeded and incubated as previously described. At the end of the incubation period, cell culture dishes were placed on ice and the cells were washed twice with ice-cold PBS. Then, ice-cold lysis buffer (50 mM Tris, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100) supplemented with protease enzyme inhibitor PMSF (dissolved in isopropanol) at a final concentration of 1 mM was added directly to the dishes and the cells were scraped off using a cold plastic scraper. The cell suspensions were transferred into precooled microcentrifuge tubes and incubated for 30 min on ice. After 20-min centrifugation at 12 000 rpm, the supernatants were transferred in new tubes and the protein concentration was determined using the DC Protein Assay (Bio-Rad, Munich, Germany). Protein samples (20 μg) were mixed with equal amounts of SDS loading buffer loaded on a NuPAGE Novex 4–12% Bis–Tris Midi Gel (Invitrogen, Karlsruhe, Germany), and electrophoresed at 90 mA for 60 min. Proteins were blotted for 30 min at 25 V onto nitrocellulose membranes that had been blocked for 1 h at room temperature with 5% dry milk powder in Tris-buffered saline

supplemented with 0.1% Tween 20 (TBS-T). Then, the membranes were incubated overnight at 4°C with primary antibodies against β-actin, 1:5000 (Sigma-Aldrich, Deisenhofen, Germany), or ERβ, 1:200 (Santa Cruz, CA, USA), diluted in 1% dry milk powder in TBS-T. After four wash cycles with TBS-T for 10 min each, the membranes were incubated for 1 h at RT with the corresponding secondary antibody diluted in TBS-T, namely polyclonal rabbit anti-mouse HRP (P0260) and swine anti-rabbit HRP (P0217) both from Dako (Hamburg, Germany). After another four washes (10 min each) with TBS-T, blot signals were visualized by the chemoluminescent POD substrate (LumiLight Plus, Roche Diagnostics, Mannheim, Germany). The images were analyzed using the ImageJ 1.40 g software.

2.9 RNA preparation and real-time RT-PCR

This method was applied to detect the expression of ERα in differentiated C2C12 cells. The total cytoplasmic RNA was extracted from the cells using the standard TRIzol® method (Invitrogen Life Technologies). cDNA synthesis including genomic DNA removal was performed using the QuantiTect® Rev. transcription kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. RT-PCR was performed in an MX3005P thermal cycler (Stratagene). The protocol consisted of a denaturing cycle at 95°C for 4 min, followed by 40 cycles of 20 s at 95°C, 20 s at 58°C, and 30 s at 72°C. Fluorescence was quantified during the 58°C annealing step. Product formation was confirmed by a melting curve analysis and agarose gel electrophoresis/ethidium bromide staining. The following mouse-specific primers were used: cyclophillin as housekeeping, *fwd*: 5′-GGATTCATGTGCCAGGGTGG-3′, *rev*: 5′-CACATGCTTGCCATCCAGCC-3′; ERα, *fwd*: 5′-TGTTTGCTCCTAACTTGCTCCT-3′, *rev*: 5′-GGTGGATGTGGTCCTTCTCTT-3′.

2.10 Statistical analysis

Statistical analyses were performed using the SPSS 20.0 statistical analysis program. Statistical differences in tissue weight, soleus muscle size, and serum concentrations were calculated using the Mann–Whitney *U*-test. Data on gastrocnemius muscle size were analyzed using the Kruskal–Wallis one-way analysis of variance followed by pair-wise comparisons with the Mann–Whitney *U*-test. All data shown are means ± SD. Significance level was established at $p \leq 0.05$.

For the cell culture experiments, differences in myotube diameters were assessed by Kruskal–Wallis one-way analysis of variance with a following Mann–Whitney *U*-test. Data shown are means ± SD. Significance level was set at $p \leq 0.05$.

Table 1. Body and tissue weights

	Control	Ecdy
Body weight (g)	365 ± 18	366 ± 21
Heart weight (g)	1.20 ± 0.10	1.28 ± 0.13
Liver (g)	14.35 ± 1.06	13.80 ± 1.57
Seminal vesicle (g)	0.39 ± 0.06	0.30 ± 0.04
Prostate (g)	1.27 ± 0.,26	1.25 ± 0.24
M. lev. ani (g)	0.23 ± 0.03	0.26 ± 0.04
M. gastrocnemius (g)	1.85 ± 0.4	1.99 ± 0.15
M. soleus (g)	0.16 ± 0.02	0.15 ± 0.01

Table 2. Fiber-type composition

Fiber type	I	IIa	IIb
Control	27	12	60
Ecdysterone	28	9	63

Data show the number of fibers in percent of all analyzed fibers.

3 Results

3.1 Confirmation of the chemical identity of Ecdy

Ecdy (Fig. 1) was obtained from Steraloids. Purity and structure were confirmed by NMR and GC-MS as described in Section 2. The spectra are displayed in Fig. 1. The generation of the characteristic fragments in MS is dominated by a cleavage of the side chain between C20 and C22 yielding either m/z 261 or m/z 723 as indicated in Fig. 1. Subsequent losses of trimethylsilanol (TMSOH) from these fragments result in m/z 171 (from m/z 271) and m/z 633, 543, and 453 (from m/z 723). The base peak m/z 73 can be assigned to the TMS cation.

3.2 Animal study

To investigate the effects of Ecdy in vivo, male Wistar rats were treated s.c. with a daily dose of 5 mg/kg BW for 21 days. The dose was chosen based on published data [18]. In Table 1, body weight and the tissue weights of the liver, heart, musculus lev ani (m. lev. ani), prostate, seminal vesicles, and the m. soleus and m. gastrocnemius are listed. No significant differences were found between control and Ecdy-treated animals. However, for m. lev. ani as well as m. gastrocnemius, a clear tendency for an increased weight was observed. To further investigate whether this tendency is reflected at the structural level, m. gastrocnemius and m. soleus histology was investigated. Ecdy-treated animals had a significantly increased soleus muscle fiber size (Fig. 2A). Fiber composition of the m. gastrocnemius was determined (Fig. 2). As shown in Table 2, Ecdy treatment did not affect fiber composition. However, the diameter of type IIb fibers showed a significant increase (Fig. 2B), and for type IIa fibers a clear tendency was detectable.

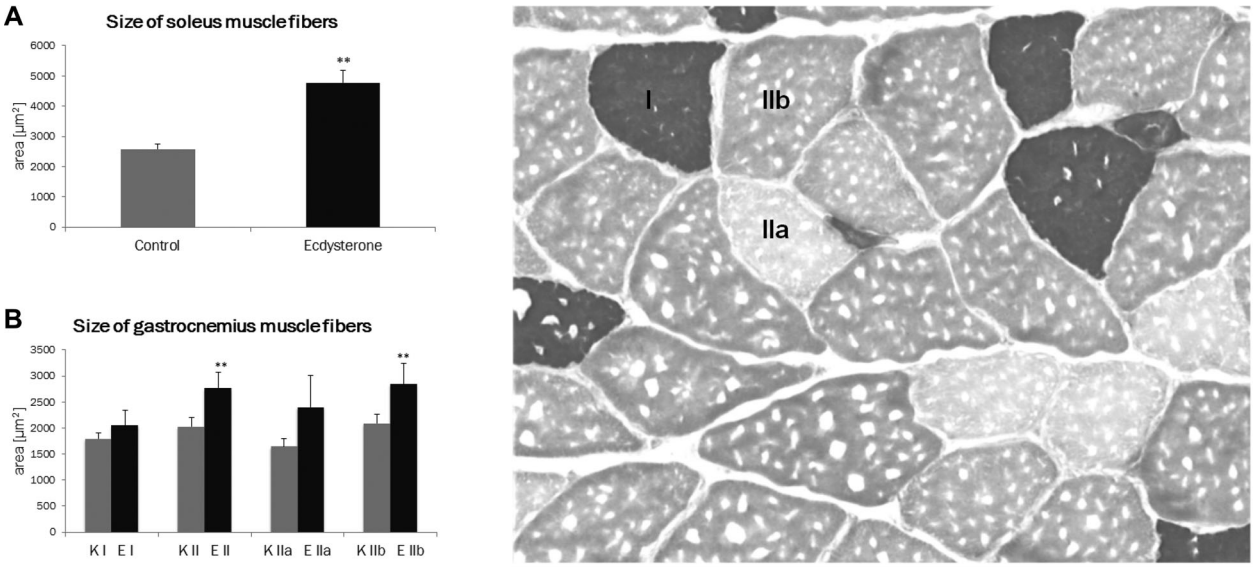

Figure 2. Fiber size of the soleus muscle (A) and gastrocnemius muscle (B) after treatment with ecdysterone. K, placebo group; E, ecdysterone group; I, fast muscle fibers; II, slow muscle fibers; IIa/IIb, subtypes of fast muscle fibers (rat soleus muscle presented a predominance of type I fibers). Representative picture of mATPase staining of gastrocnemius muscle. Data shown are means ± SD. Statistical differences were calculated using Kruskal–Wallis one-way analysis of variance with a following Mann–Whitney *U*-test for gastrocnemius fiber size and only Mann–Whitney *U*-test for soleus muscle fiber size. $^{**}p \leq 0.01$ versus placebo group.

To investigate the effects of Ecdy on pituitary–gonad feedback axis and some other endogenous hormones or growth factors, the serum concentration of IGF-1, thyroxin (T4), testosterone, E2, inhibin, prolactin, and corticosterone was determined (Fig. 3). An increase in serum IGF-1 and a decrease in serum corticosterone were observed. Moreover, Ecdy-treated animals showed reduced E2 serum concentrations.

Table 3. The EC50 values of estradiol and ecdysterone for ERα and ERβ[a)]

Compound	EC50 for ERα (M)	EC50 for ERβ (M)
Estradiol	5.66×10^{-11}	8.66×10^{-11}
Ecdysterone	2.57×10^{-8}	1.30×10^{-8}

a) A dose-dependent curve was fit to the data shown in Figure 6 to determine EC50.

3.3 Cell culture experiments

To investigate the molecular mechanisms involved in the anabolic activity of Ecdy, the effects of different anabolic and catabolic hormones in an in vitro cell culture model for hypertrophy were analyzed. C2C12 cells were differentiated as described in Section 2 and incubated for 48 h with the test compounds. In a first experiment aimed to verify the anabolic activity of Ecdy, C2C12 myotubes were incubated with Dexa (1 and 10 μM) known to be catabolic, IGF-1 (10 ng/mL) and DHT (100 nM), both known to be anabolic, and Ecdy (1 μM). Drug concentrations were chosen on the basis of published data and previous experiments [6]. As shown in Fig. 4, treatment with Dexa resulted in a dose-dependent decrease in myotube diameter, whereas treatment with IGF-1, DHT but also Ecdy resulted in a significant increase in myotube diameter.

To investigate the role of the glucocorticoid receptor, ER, or AR in the effects of Ecdy, co-incubation experiments with either Ecdy or Dexa (Fig. 5A), or Ecdy and the Flut (Fig. 5B), or Ecdy and the antiestrogen ZK (Fig. 5C) were performed. As shown in Fig. 5A, the anabolic effect of Ecdy could be antagonized by Dexa but not by flutamide (Fig. 5B). Interestingly, treatment with E2 also resulted in myotube hypertrophy (Fig. 5C). Cotreatment with an unselective antiestrogen antagonized E2-mediated but also Ecdy-mediated hypertrophy (Fig. 5C).

To investigate whether Ecdy has direct binding affinity to ERα and ERβ, a luciferase reporter gene assay in ERα- and ERβ-transfected HEK293 cells was performed as described in Section 2. Luciferase activities induced by Ecdy at various concentrations are shown in Fig. 6A as percentage of the maximal response to estradiol treatment. The data indicate that Ecdy could induce dose-dependent response in estrogen response element mediated gene expression at a range of 10^{-11}–10^{-6} M, especially through ERβ. The EC50 for ERα or ERβ was determined with a full dose–response curve. All these data are summarized in Table 3. The induction of luciferase in HEK293 cells by Ecdy could be specifically blocked by cotreatment with the antiestrogen ZK (Fig. 6B). This provides further evidence for the role of ER in mediating the action of Ecdy in the test.

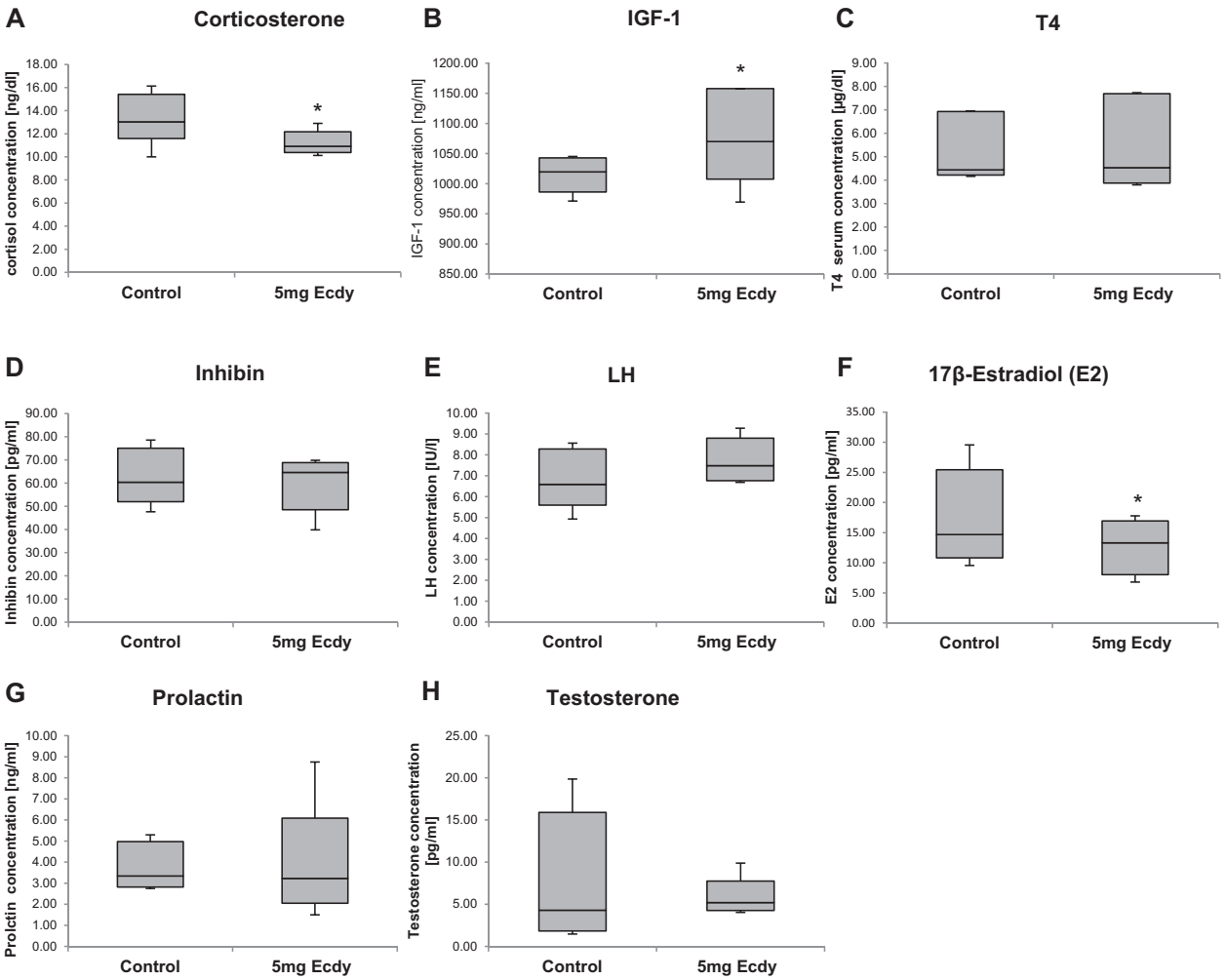

Figure 3. Serum concentration of corticosterone in intact male rats (A), IGF-1 (B), T4 (C), inhibin (D), LH (E), E2 (F), prolactin (G), and testosterone (H). Statistical analysis was performed with the Mann–Whitney *U*-test. $*p \leq 0.05$ compared to control.

To investigate whether the hypertrophic effects of E2 in C2C12 cells are mediated via ERα or ERβ, C2C12 cells were incubated with various concentrations of ERα- and ERβ-selective ligands (ALPHA and BETA; Fig. 7B). Incubation with increasing concentrations of ALPHA did not result in a significant increase in myotube diameter. BETA treatment resulted in a bell-shaped dose–response curve, inducing a significant increase in myotube diameter at a concentration of 0.01 nM. The presence of both ERα and ERβ in C2C12 cells was confirmed at the mRNA and protein level, respectively (Fig. 7A).

To further verify the specificity of the observed effects, coincubation experiments with the ERβ-selective antagonist ZK 283361 were performed. C2C12 cells were also incubated for 48 h in the presence of either E2 or Ecdy together with ANTIBETA as described in Section 2. As shown in Fig. 6C, the anabolic effect of E2, Ecdy, and BETA on myotube hypertrophy could be antagonized by the ERβ-selective antagonist ANTIBETA.

4 Discussion

The aim of our study was to investigate the anabolic activity of Ecdy in vivo and to further characterize underlying molecular mechanisms. The activity of Ecdy was analyzed in intact male rats. Our results demonstrate that Ecdy did not show any androgenic activity on prostate and seminal vesicles (Table 1). This is in agreement to published data [19]. In contrast to many anabolic steroids, the activity of Ecdy is believed to be AR-independent. Receptor-binding assays document only a weak binding affinity of Ecdy to the AR [6]. M. lev. ani is a muscle highly sensitive to androgens [20], which may explain why Ecdy had no effect on this tissue. We also investigated the anabolic activity of Ecdy on the m. soleus and m. gastrocnemius of the same animals. Histological analysis revealed a statistically significant difference in muscle fiber size (Fig. 2): An increase in fiber diameter by Ecdy has already been described in the literature [18]. Anabolic steroids such as testosterone affect mainly type I fibers [21, 22]. In agreement with these

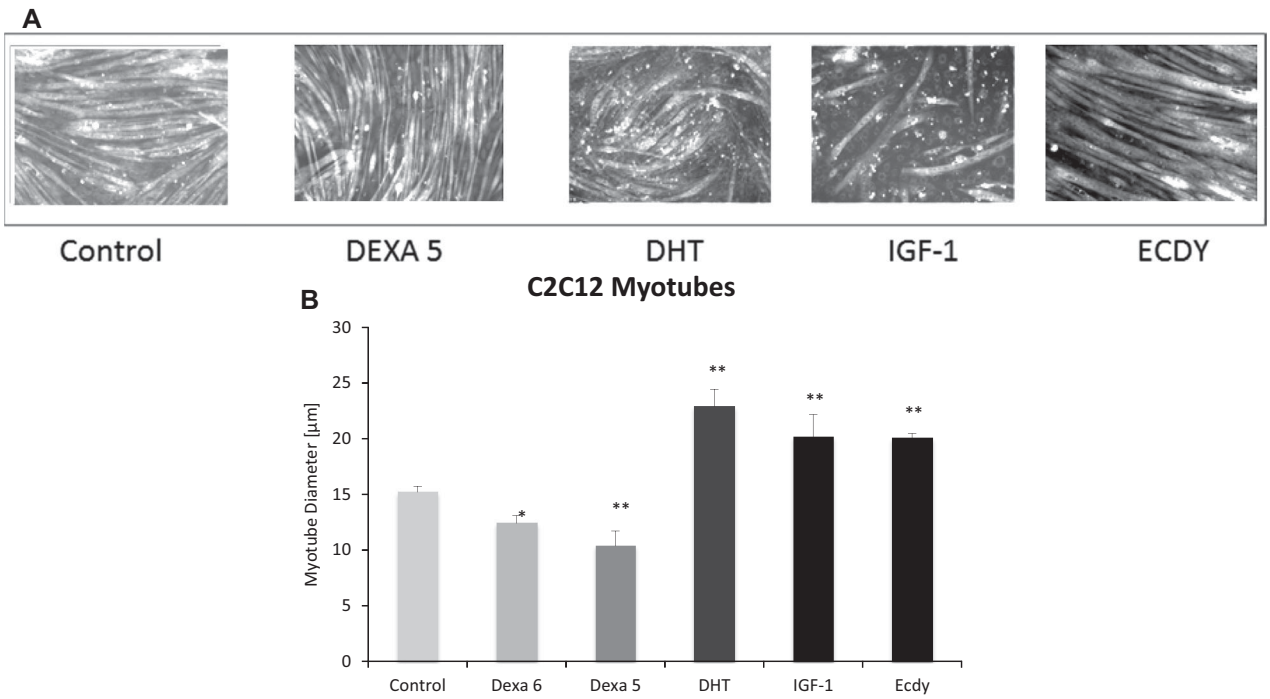

Figure 4. Effects of dexamethasone (Dexa 6 = 10^{-6} M, Dexa 5 = 10^{-5} M), DHT (10^{-6} M), IGF-1 (10 ng/mL), and Ecdy (10^{-6} M) on the diameter of C2C12 myotubes. Four- to six-day-old myotubes were incubated for 48 h with test chemicals, and were fixed and photographed by glutaraldehyde-induced autofluorescence. (A) Representative pictures of $n = 3$ independent experiments, magnification is 100-fold. (B) Myotube diameter. Shown is the mean of $n = 3$ independent experiments. $^*p \leq 0.05$ versus control by Kruskal–Wallis *H*-test and Mann–Whitney *U*-test.

data, we found Ecdy stimulation of m. soleus type I fibers. In m. gastrocnemius, mainly fibers of the subtype IIa und IIb were stimulated, whereas type I fibers remained unaffected. Thus, in the m. gastrocnemius, mainly the fast type II fibers are target of the anabolic activity of Ecdy. The observed increase of type II fiber diameter is believed to increase muscle force, which is in agreement with animal studies showing that Ecdy treatment results in an increase in muscle force [6].

As mentioned before, experiments were performed in intact male rats. Therefore, we also analyzed the effects of Ecdy treatment on the serum concentrations of distinct endogenous hormones. As shown in Fig. 3, treatment of the animals with Ecdy resulted in an increase in IGF-1 concentrations and a decrease in E2 and corticosterone levels. Corticosterone is described as an inducer of catabolic processes in the skeletal muscle [23], whereas an increase in IGF-1 serum levels is always related to an anabolic activation [24]. The increased IGF-1 and decreased corticosterone levels as a result of Ecdy treatment explain the observation that Ecdy induces muscle growth. Surprisingly, Ecdy reduced E2 but not testosterone levels in the treated rats. This observation may indicate that Ecdy may influence gonadotropin secretion and activate negative feedback mechanisms on the hypothalamic–pituitary axis. A decrease in serum E2 levels is observed after gonadotropin administration [25]. Recently, anabolic effects of Ecdy have also been reported in ovariectomized female rats [26]. The authors argue that these effects are not mediated via the ER because no signs for classical estrogenic activity such as uterotrophy were visible in these animals. However, recently we have demonstrated the anabolic effects of estrogens including an activation of ERβ [15]. Selective activation of ERβ does not result in any of the classical E2-mediated effects such as uterotrophy. Therefore, it is possible that the anabolic activity of Ecdy may be mediated by a selective activation of ERβ. To further investigate the molecular mechanisms involved in the activity of Ecdy, we performed cell culture experiments in differentiated C2C12 cells. This cell line is derived from murine skeletal myoblasts [27]. C2C12 cells can be cultured and differentiated toward myotubes [28]. Recently, we could demonstrate that anabolic compounds affect the differentiation of C2C12 cells toward myotubes [29]. C2C12 cells express a variety of steroid hormone receptors, including AR and ER [12, 29].

In our first experiment (Fig. 4), C2C12 myotubes were treated with Ecdy, Dexa, IGF-1, and DHT. The Ecdy dose used in this experiment was chosen based on the dose–response results published by Gorelick-Feldmann et al. [6]. This group showed an increase in protein synthesis in C2C12 cells following Ecdy treatment. In our experiments, treatment with Dexa resulted in a dose-dependent decrease in myotube diameter, which is in line with the described catabolic activity of

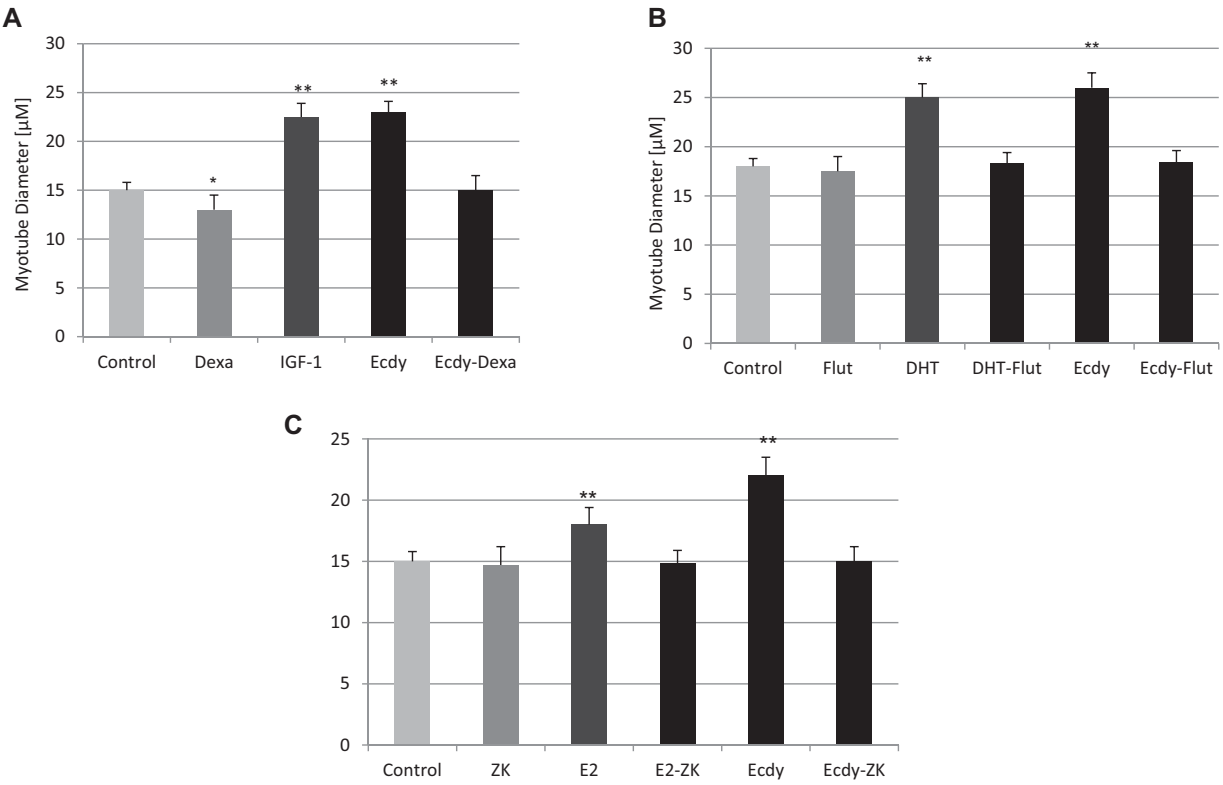

Figure 5. Investigation of the role of AR and ER in the anabolic activity of Ecdy. Four- to six-day-old myotubes were incubated for 48 h with test chemicals, were fixed, and their diameter was measured. Shown is the mean of $n = 3$ independent experiments. Significant versus control by Kruskal–Wallis *H*-test and Mann–Whitney *U*-test; $*p \leq 0.05$, $**p \leq 0.01$. (A) Antagonization of the effects of Ecdy (10^{-6} M) by dexamethasone (Dexa 10^{-6} M). (B) Antagonization of the effects of Ecdy (10^{-6} M) by the antiandrogen flutamide (Flut 10^{-6} M, DHT 10^{-7} M). (C) Antagonization of the effects of Ecdy by an unselective antiestrogen (ZK 10^{-6} M, E2 10^{-9} M, Ecdy 10^{-6} M).

Dexa [30]. In contrast, DHT and IGF-1 treatment resulted in an increase in myotube diameter, demonstrating an anabolic activity. In agreement with our in vivo findings, treatment of myotubes with Ecdy also resulted in an increase in the diameter, indicating an anabolic activity. The effect of Ecdy is quantitatively comparable to the effects observed for IGF1 and DHT.

To further investigate the molecular mechanisms of Ecdy activity and to find out which steroid hormone receptors may be the target of Ecdy action, we performed coincubation experiments, in which C2C12 myotubes were treated with Ecdy, Dexa, DHT, Flut, antiestrogen ZK, and combinations thereof. In Fig. 5, it is shown that the hypertrophic effect of Ecdy can be antagonized by Dexa and the antiestrogen ZK, but not by an antiandrogen. The antagonistic effect of Dexa may be mediated by a binding of Ecdy to the glucocorticoid receptor. However, referring to the study of Bathori et al. [5], which showed that Ecdy does not bind to the GR, this effect may probably be due to direct interaction on protein synthesis cascade, for example the PI3K/Akt pathway. In general, these data demonstrate that the hypertrophic effect of Ecdy is not mediated via an interaction of Ecdy with the AR. The data support the hypothesis that Ecdy activity is mediated via the ER. As shown in Fig. 6, Ecdy is indeed able to stimulate dose dependently reporter gene expression ER in ERα- or ERβ-transfected HEK293 cells. These data confirm observations of Gao et al. [17] and indicate that Ecdy directly binds to both ER subtypes. Moreover, this is in line with our observation that Ecdy affects E2 serum concentrations in the animal experiment. Recently, we could demonstrate that activation of ER by E2 results in skeletal muscle hypertrophy [15, 16]. In agreement to this, the anabolic activity of E2 was also observed in our experiments on C2C12 cells. In our previous work, we could demonstrate that the anabolic effect of E2 on skeletal muscle is mainly mediated via ERβ. To validate this result in the present study, C2C12 myotubes were treated dose dependently with both, ALPHA and BETA. According to Hillisch et al. [31], both agonists are highly selective to their respective receptor at low concentrations and lose their selectivity at higher concentrations may be through interaction with other steroid receptors, that is ERα or phosphorylation cascade. As shown in Fig. 7B, at a concentration of 10^{-11} M, were selectivity to the respective receptors is guaranteed, BETA but not ALPHA induced myotube hypertrophy. Remarkably, BETA has a clear bell-shaped dose–response curve. We only can speculate why higher doses of BETA do not stimulate

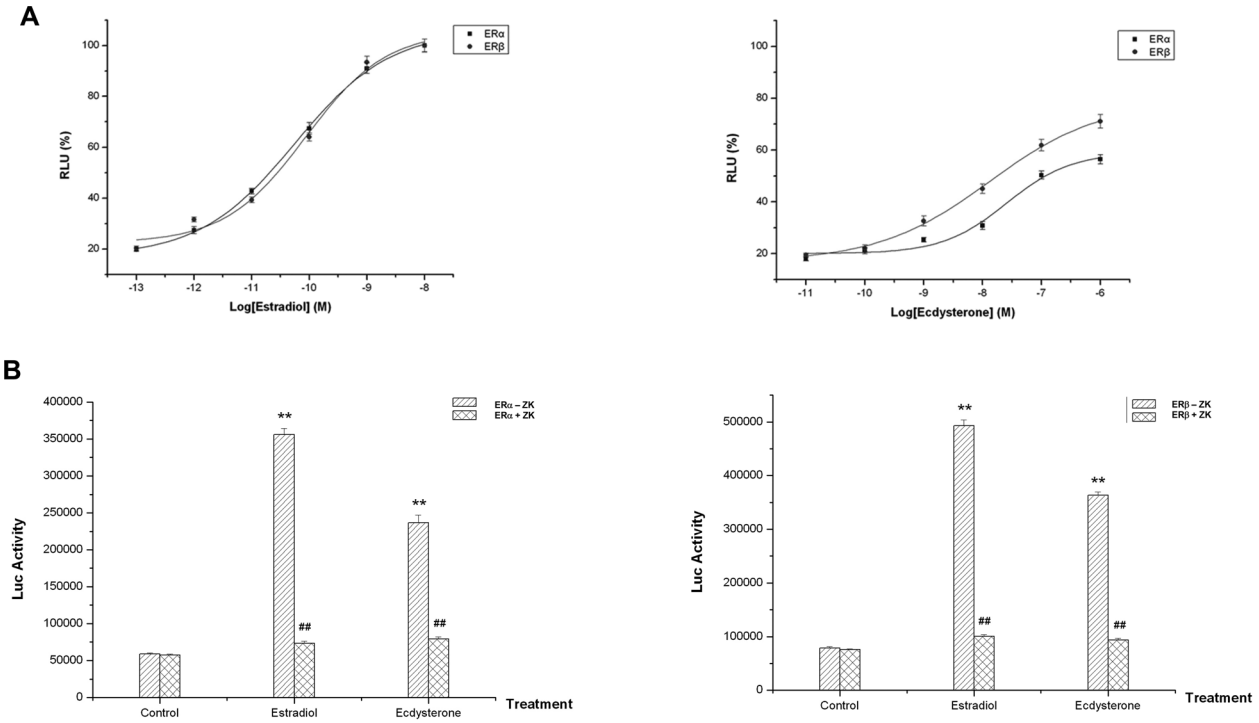

Figure 6. (A) Dose–response curve for estradiol and ecdysterone. HEK293 cells were transfected with plasmids containing either ERα or ERβ and a recombinant luciferase reporter plasmid pTAL-ERE-luc containing 5× ERE. After incubation with estradiol between 10^{-8} and 10^{-13} M or ecdysterone between 10^{-6} and 10^{-11} M for 24 h, the cells were lysed and reacted with substrate luciferin. Transcriptional activity is represented as relative light units (RLUs) calculated as percentage of the maximal induction by 10^{-8} M estradiol and presented normalized to β-galactosidase activity. (B) ER-antagonist ZK specifically suppressed the stimulation of luciferase activities by estradiol or ecdysterone in transfected HEK293 cells. The cells were treated with 10^{-8} M estradiol or 10^{-6} M ecdysterone with or without 10^{-8} M ZK. When a combination of ecdysterone and ZK was used together in the pretreatment, the luciferase activity induced by ecdysterone alone was significantly inhibited. Results were obtained from three different experiments with each dose assayed in triplicate wells. $^{**}p < 0.01$ represents significant difference compared with control. $^{\#\#}p < 0.01$ represents significant difference compared with same dose of estradiol- or ecdysterone-treated groups without ZK. All data are expressed as mean ± SD from three experiments.

hypertrophy. Very likely such doses may have an inhibitory effect on mechanisms involved in the mediation of the anabolic effects. These mechanisms, for example effects on phosphorylation, need to be studied in future investigations. Nevertheless, it is remarkably that such a low concentration of a pharmacological active substance can induce such a specific physiological effect. This is also relevant with respect to Ecdy, because it may indicate that even low tissue concentrations of ERβ-activating substances may have physiological effects. In summary, this result provides evidence that ERβ is the ER isoform driving the hypertrophic effect of E2 on skeletal muscle.

To investigate whether the effects of Ecdy are mediated via ERβ, we performed an additional antagonization experiment with the selective ERβ antagonist ZK 283361 (ANTIBETA). As shown in Fig. 6C, ANTIBETA is able to antagonize the effects of E2, BETA but also Ecdy. This result may be interpreted as an indication for ERβ-mediated anabolic activity of Ecdy in C2C12 cells.

In conclusion, Ecdy has an anabolic activity in vitro and in vivo. Analysis of the regulatory feedback mechanisms as well the results of cell culture experiments in C2C12 cells indicate that the anabolic effects of Ecdy are mediated via the ER. Moreover, experiments with ER-selective agonists and antagonists indicate that mainly ERβ may be involved in the anabolic activity of Ecdy. A more specific molecular mechanism involved in the interaction of ERβ and Ecdy may be the modulation of phosphorylation effects. In C2C12 cells, it has been demonstrated that PI3K/Akt phosphorylation cascade is involved in the hypertrophic activity of Ecdy [8]. Because it has been also demonstrated that ERβ can modulate Akt phosphorylation [32], this could be a mechanistically linkage between ERβ and Ecdy-induced hypertrophy in C2C12 cells.

Our data clearly confirm that Ecdy is a compound with strong anabolic activity in vivo and in vitro, and that ERβ seems to be involved at least in vitro. This opens therapeutic perspectives for the treatment of muscle injuries, sarcopenia, and cachectic disease, especially in woman, but also needs to be considered with respect to classification of Ecdy as a substance that could be misused for doping purposes.

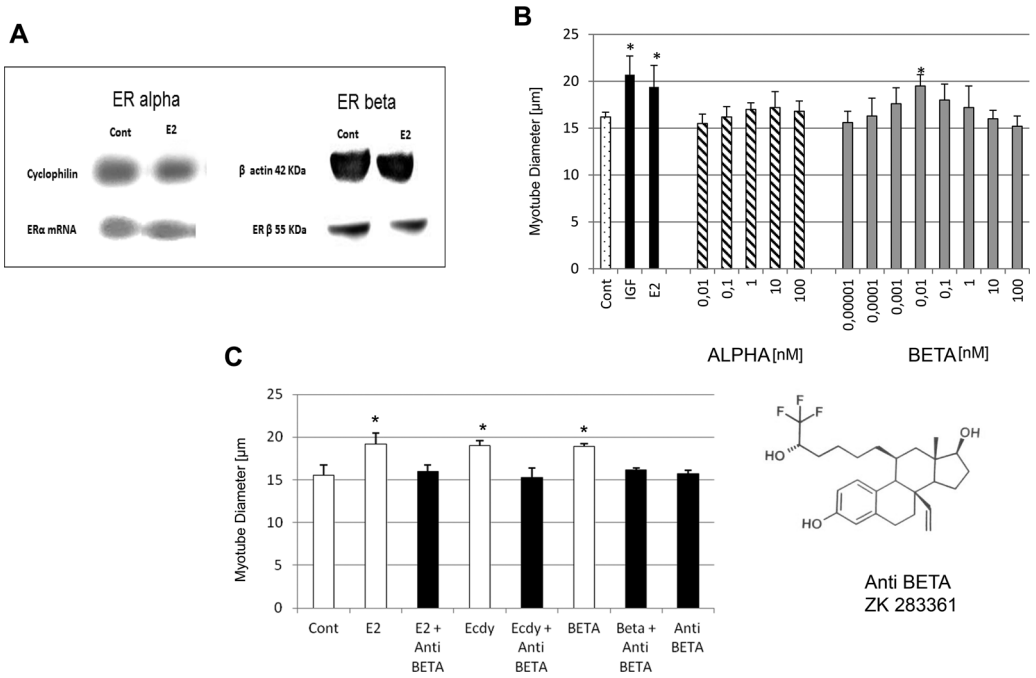

Figure 7. Role of ER alpha and ER beta in the ER-mediated hypertrophy of C2C12 cells. Four- to six-day-old myotubes were incubated for 48 h with test chemicals, were fixed, and their diameter was measured. Shown is the mean of $n = 3$ independent experiments. *Significant versus control. $^{*,+}p \leq 0.05$ by Kruskal–Wallis *H*-test and Mann–Whitney *U*-test. (A) Expression of ER alpha and ER beta in C2C12 cells. Western blot after RT-PCR. (B) Dose-dependent effects of ER alpha (ALPHA) and ER beta (BETA) selective ligands on C2C12 myotube diameter. (C) Antagonization of the effects of Ecdy (10^{-11} M) by an ER beta selective antagonist (ANTIBETA, 10^{-6} M).

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The authors have declared no conflict of interest.

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