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Estradiol and genistein antagonize the ovariectomy effects on skeletal muscle myosin heavy chain expression via ER-β mediated pathways

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ABSTRACT

The age-related decline in ovarian sex hormone production following the onset of menopause alters skeletal muscle metabolic, structural and functional characteristics. The myosin heavy chain (MHC) expression pattern defines skeletal muscle contraction velocity and is therefore an important factor in skeletal muscle function. The present study was designed to examine the effects of 17β estradiol (E2), estrogen receptor (ER) subtype selective agonists (ERα, ERβ) or genistein (Gen) following ovary removal (OVX) in female Wistar rats in combination with a high intensity treadmill-based exercise protocol (Ex) or normal cage-based activity (NoEx) on MHC protein expression patterns in the slow fiber type m.Soleus (Sol) and the fast fiber type m.Gastrocnemius (Gas). Gen and E2 in the Sol significantly stimulated MHC-I expression relative to OVX only in the absence of exercise (NoEx). MHC-IIb expression in the Gas was significantly increased relative to OVX in Gen Ex and E2 Ex and NoEx groups. The estrogenic effects in the Sol and Gas were both predominantly mediated via ERβ pathways, since the ERβ agonist induced greater MHC increases than OVX or ERα. We therefore propose that high intensity exercise in combination with exposure to E2, Gen, ERα or ERβ agonists in OVX rats exerts differential effects on MHC expression in skeletal muscles composed of mainly slow type I MHC (Sol) or fast type II MHC (Gas). In summary, the data shows that MHC composition is affected by estrogens and exercise in a fiber type specific manner and that these effects are mainly mediated by ER-β. This is of great importance with respect to skeletal muscle health and potential treatment with ER selective agonists.

1. Introduction

Women become postmenopausal usually by the sixth decade of life and consequently spend approximately one third of their lifetime in an estrogen-deficient state [1]. Loss of ovarian hormone production following the onset of menopause is associated with a decline of approximately 15% in skeletal muscle mass (sarcopenia) and muscle strength in addition to the age-related loss [2]. This association strongly implies that a relationship exists between decreasing estrogen levels and reduced muscle mass and strength.

Studies on the effects of hormone replacement therapy on skeletal muscle mass and strength in humans have reported mixed and sometimes contradictory findings. Sorensen et al. found that a 12-week hormone replacement program in postmenopausal women was sufficient to significantly increase lean body mass [3]. Results from the 'estrogen plus progestin trial of the women's health initiative (WHI)' also show that a 3-year estrogen plus progestin therapy significantly reduced the loss of lean tissue mass compared to placebo [4]. In contrast, others found no significant effect of 64 weeks of hormone replacement on skeletal muscle mass [5] and no difference in the prevalence of sarcopenia in older women receiving hormone-replacement therapy (HRT) and those not receiving HRT [6]. As for the effects of HRT on skeletal muscle mass, similar mixed results exist for the preservation of muscle strength with almost as many studies reporting no effects as there are studies that have found a significant effect of HRT on maintaining skeletal muscle strength. For example, 6-month of HRT did not influence knee flexion, extension or handgrip strength in postmenopausal women [7,8] while results from a cross-sectional study show that handgrip strength loss can be partly prevented by HRT [9]. Discrepancies between studies are likely due to the varying doses of estrogen given, age of the study participants, diet and physical activity patterns.

The molecular mechanisms responsible for reducing muscle strength and mass after the onset of menopause are thus far largely undefined. Moran and colleagues have shown that myosin binding dynamics are altered in ovariectomized mice leading to a reduction in tetanic force that is restored with estrogen replacement [10]. Skeletal muscle fiber type and contractile properties that determine force output are mainly determined by the myosin heavy chain

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(MHC) molecule, of which there are a number of isoforms. Fibers composed of MHC-I display a slow contracting velocity and produce less force than the faster contracting MHC-IIa, IIx, IIb [11,12]. MHC-Isoform expression patterns change with age in humans at the gene transcription level towards increased MHC-I mRNA expression and reduced levels of MHC-IIx [13]. Shifts in MHC composition towards increased expression of the slower MHC-I in the Soleus (Sol) and MHC-IIa in the extensor digitorum longus (EDL) and reduced expression levels of the fast MHC-IIb in the EDL have also been reported following ovariectomy (OVX) in rats [14]. Exercise in combination with estrogen treatment prevented the OVX-induced fiber type shift in the Sol and EDL [14]. OVX also induced a decrease in MHC-IIx expression in the plantaris (PLA), which was reversed by estrogen treatment [15]. In addition to the observed fiber type shift, estrogen replacement also stunts body weight gain and decreases Sol fiber size and in vitro contractile properties when it is the only ovarian hormone present following OVX in young, growing animals [16]. Functionally, estrogen replacement restored maximal isometric force in adult ovariectomized mice that failed to fully recover skeletal muscle mass in an estrogen-deficient state following disuse [10]. Four weeks [17] and ten days [18,19] of skeletal muscle disuse (hindlimb unloading) induced significant skeletal muscle atrophy in OVX rats, which was only completely reversed following estrogen replacement combined with reloading. This shows the importance of the presence of estrogens for the regeneration of skeletal muscle mass after a period of disuse. Overall, the majority of long-term human studies report favorable effects of estrogen replacement on maintaining lean body mass and stimulating muscle mass recovery after disuse or injury following the onset of menopause.

Based on these findings and the fact that a HRT has been linked to the development of cancer in the breast and in the reproductive organs [20–22], major research efforts are currently made to investigate tissue-specific effects of ER-subtype selective ligands (ERα, ERβ). Estrogen receptors (ER) belong to the nuclear receptor superfamily of ligand-regulated transcription factors [23]. Upon ligand binding, ERs dimerize and bind with their DNA binding domain to estrogen response elements within estrogen-sensitive target genes [24,25] or influence gene expression via protein–protein tethering with transcription factors [26]. Co-regulator proteins are able to bind to the ER complex, thereby influencing the magnitude of the transcriptional response [23,27]. Both ER subtypes are expressed in a variety of tissues. While the ERα is primarily expressed in the uterus, liver, kidney and heart, the ERβ is mainly expressed in the lung, ovaries, gastrointestinal tract, bladder and central nervous system. A number of tissues show overlapping expression of both subtypes. Both the ERα and ERβ are expressed in the mammary gland, skeletal muscle, bone, thyroid- and adrenal glands [24].

In this context, the aim of the present study was to assess the effects of estrogens on skeletal muscle structural composition (MHC expression) in a predominantly slow fiber type muscle (Soleus, Sol) and a fast fiber type muscle (Gastrocnemius, Gas). In particular, we analyzed the effects of 17β estradiol (E2), the isoflavone genistein (Gen) and ER subtype selective agonists (ERα, ERβ) in combination or without a high intensity exercise program (Ex) on MHC protein expression.

2. Methods

2.1. Animals and study design

8-Week-old female Wistar rats (125–150 g) were obtained from Janvier (Janvier, Le Genest St Isle, France) and were maintained under controlled conditions of temperature (20 ± 1 °C, relative humidity 50–80%) and illumination (12 h light, 12 h dark). All animal experiments were approved by the Committee of Animal Care and complied with accepted veterinary medical practice. Rats were ovariectomized (OVX) or sham-operated (Sham) following 1 week of acclimatization (weighing 200–220 g) after anesthesia with Ketanest (40 kg^{-1} b.wt,) and Rompun (4 mg kg^{-1} b.wt) followed by 14 days of endogenous hormonal decline. The study was divided into three parts: in the first part OVX rats were randomly allocated to treatment with either E2 Genistein, or vehicle (OVX) for 3 weeks. In the second part, rats were allocated to treatment with vehicle (OVX), ERα or ERβ agonists and in the third part of the study, MHC expression patterns of animals with normal ovarian hormone production (Sham surgery) were compared to OVX. Moreover, all treatment groups were further divided into exercise (Ex) or normal cage-based activity (NoEx) groups (all $n = 6$). Rats in the exercise groups (Ex) were trained twice per day on a motorized rat treadmill at 10% incline for 15 min per running session. Running velocity increased incrementally during the 3-week long training from 20 m/min in the first week to 24 m/min during the last week of training. We have previously shown that the intensity and duration of the training regime was sufficient to alter skeletal muscle sarcomeric protein expression [28]. E2 (17β-Estradiol, Estra-1,3,5(10)-trien-3,16α,17 β-diol), was obtained from Sigma–Aldrich (Deisenhofen, Germany). The specific estrogen receptor agonists for ERα (16α-LE2, 3,17-dihydroxy-19-nor-17α-pregna-1,3,5 (10)-triene-21,16α-lactone) and ERβ (8β-VE2, 8-vinylestra-1,3,5 (10)-triene-3,17β-diol) were provided by the Bayer Schering Pharma AG (Berlin, Germany). E2 (4 μg kg^{-1} b.wt d^{-1}), 16α-LE2 (10 μg kg^{-1} b.wt d^{-1}) and 8β-VE2 (100 μg kg^{-1} b.wt d^{-1}) were dissolved in dimethylsulfoxide (DMSO) (200 μl kg^{-1} b.wt d^{-1}) and corn oil (800 μl kg^{-1} b.wt d^{-1}) for subcutaneous (s.c.) administration. Because the ERα and ERβ agonists activate both receptors at higher concentrations [29,30], doses of 10 μg kg^{-1} b.wt d^{-1} (16α-LE2) and 100 μg kg^{-1} b.wt d^{-1} (8β-VE2) were chosen, respectively. For these doses activation and subsequent signaling through either ERα or ERβ, respectively can be anticipated [29,30].

2.2. Diet

Animals had free access to a standard diet (Genistein, Gen: <10 μg/g, Daidzein, Dai: <10 μg/g) and water. The Gen groups received a special diet high in Gen content (Gen: 700 μg/g, Dai: <10 μg/g) (SSniff GmbH, Soest, Germany). Daily food intake did not differ between the different diets [31].

2.3. Tissue collection and processing

Animals were sacrificed immediately after the last training session by decapitation after light anesthesia with CO_2 inhalation and Uteri were prepared free of fat and the wet weights were determined. Skeletal muscles (Soleus and superficial white region of the lateral head of the Gastrocnemius) from the right hindlimb were removed and weighed. 84% of the Sol MHC distribution consists of MHC type I, while 92% of the white region of the Gas muscle is composed of MHC-IIb [32].

2.4. Protein analysis

Frozen Soleus and Gastrocnemius muscles from the right hindlimb of each animal were separately pooled, powdered and homogenized in buffer (623,5 mM Tris/EDTA, pH 8) containing protease inhibitors (5 mg/ml aprotonin, 5 mg/ml leupeptin, 1 mg/ml pepstatin-A, 5 mg/ml antipain, 100 mM pefac in 0.5 M EDTA, pH 8). Protein concentration was determined following the method of Lowry (DC Protein Assay, Bio-Rad, Munich, Germany). Equal amounts of sample were loaded onto 4–12% Bis-Tris Criterion™ XT gels (Bio-Rad, Munich, Germany). Following electrophoresis (200 V, 90 mA), one gel was used for silver staining (Silver

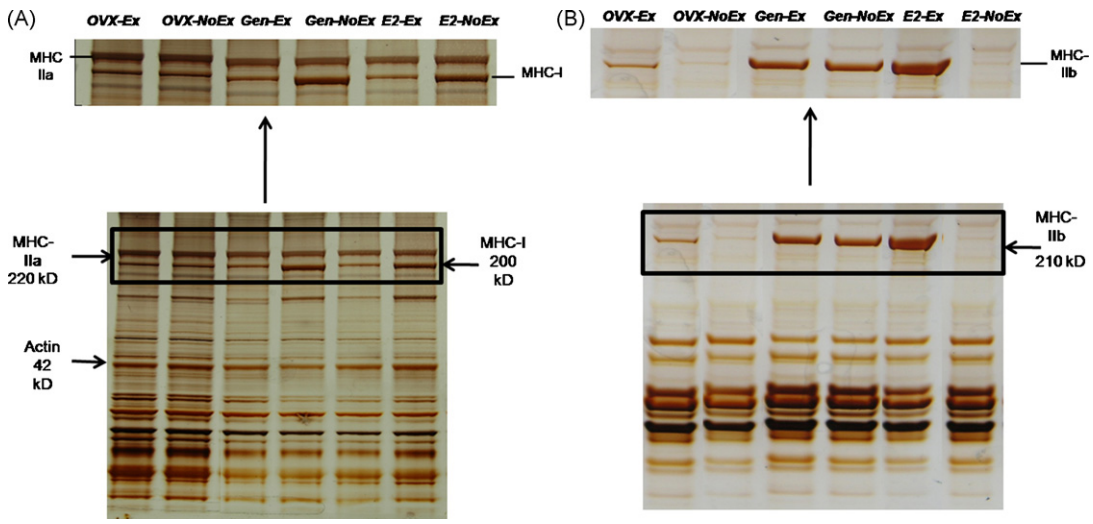

Fig. 1. Genistein and E2 stimulate MHC-I expression without exercise in the Soleus and in combination with exercise in the Gastrocnemius muscle. Silverstain showing total protein expression in the (A) Sol and (B) Gas of OVX, Gen and E2 groups. The inserts show a magnification of the silver-stained bands of MHC-IIa and MHC-I in the Sol (A) and MHC-IIb in the Gas (B). Gen and E2 stimulate MHC-I expression in the Sol in the absence of Ex and MHC-IIb in the Gas in combination with Ex.

Stain Plus Kit, Bio-Rad, Munich, Germany) while a second gel was used for specific protein detection by western blot analysis. Proteins were transferred onto nitrocellulose membranes (Schleicher u. Schuell, Dassel, Germany) and blocked with 5% BSA in phosphate buffered saline Solution (100 mM; pH 7.4) at room temperature for 1 h. The immobilized proteins were detected and quantified using a myosin heavy chain specific antibody (MF-20, Developmental Studies Hybridoma Bank, University of Iowa) and an anti-actin antibody (A5060, Sigma–Aldrich, Schnelldorf, Germany) as a reference for equal protein loading. A polyclonal rabbit anti-mouse HRP-conjugated secondary antibody (Dako, Glostrup, Denmark) was used. Blot signals were visualized by the chemoluminscent POD substrate (Lumi-Light Plus, Roche Diagnostics, Mannheim, Germany) and a Fluorchem Luminescent imager (Alpha Innotech, CA, USA). The correct protein bands were identified with molecular weight markers. Protein bands were quantified by densitometric analysis using a Nikon D70 camera (Nikon, Japan) and ImageJ 1.38 sofware (National Institute of Health, USA, http://rsb.info.nih.gov/ij/). Expression levels of the reference protein actin were calculated to account for non-homogenous protein loading. The OVX NoEx groups were set to one throughout the data anaylsis process.

2.5. Statistical analysis

Statistical analyses were performed using the SPSS Statistical Analysis System, Version 15.0. All data are expressed as arithmetic means with their standard errors. Statistical significance of differences was calculated using a 2×2 ANOVA followed by Tukey's HSD post hoc test. Statistical significance was established at $P < 0.05$.

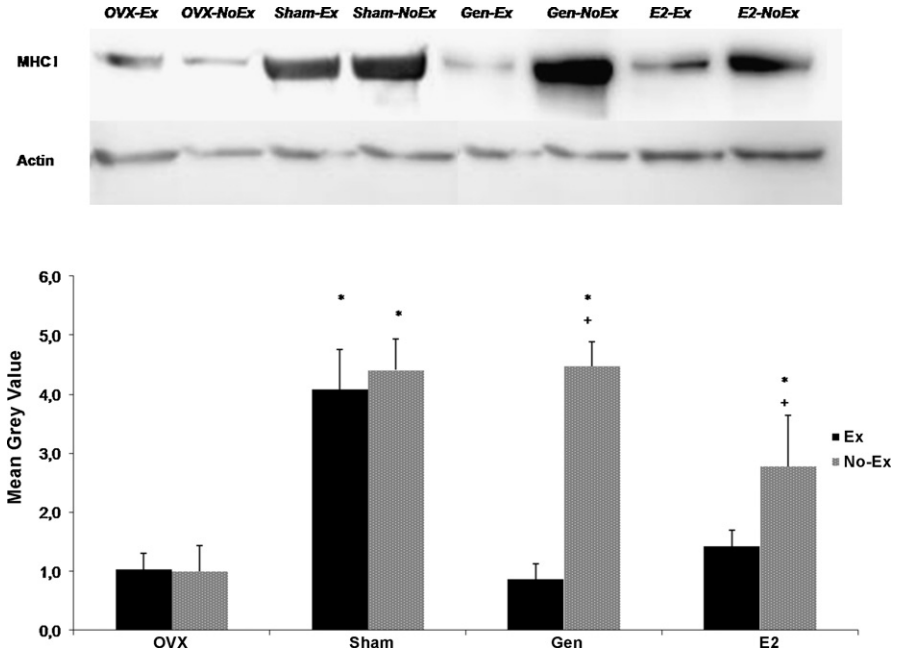

Fig. 2. Treatment with Gen and E2 without exercise normalizes MHC-I expression in ovariectomized rats relative to Sham levels. Western blot and densitometric analysis of MHC-I expression in the Sol of OVX, Sham, Gen and E2 groups. *Significant difference relative to OVX, +significant difference between Ex and NoEx.

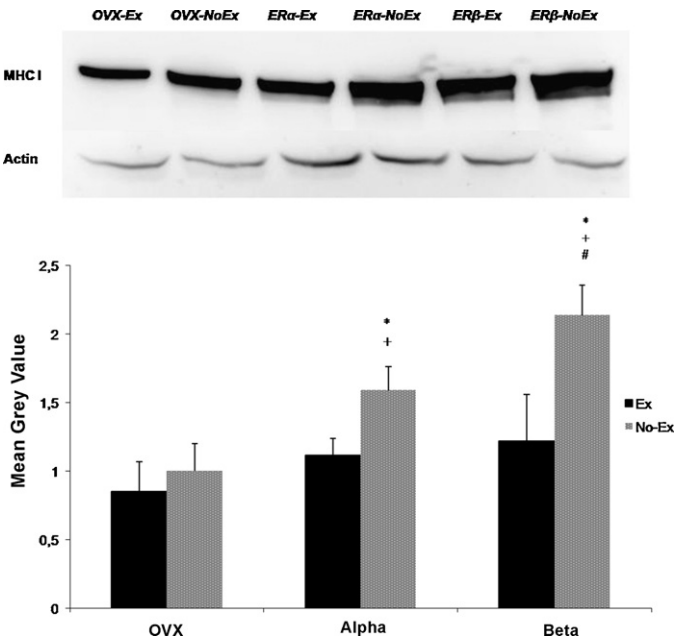

Fig. 3. Estrogenic effects on MHC-I expression are predominantly mediated via ER-β. Western blot and densitometric analysis of MHC-I protein expression in the Sol of OVX, ER-α agonist (Alpha) and ER-β agonist (Beta) groups. *Significant difference relative to OVX, +significant difference between Ex and NoEx, #significant difference relative to Alpha.

3. Results

3.1. E2 and Gen increase Sol MHC-I expression only in the absence of high intensity exercise

Silverstain (Fig. 1A) and western blot analysis (Fig. 2) in the slow fiber type Sol showed that MHC-I expression was significantly higher in the Gen and E2 NoEx groups compared to OVX or the exercised and hormone-exposed groups. Gen and E2 treatment normalized MHC-I expression only in NoEx groups relative to Sham levels. There were no differences between groups for MHC-IIa expression (Fig. 1A). The highest MHC-I expression was detected in the Gen NoEx group (Fig. 2).

3.2. E2 and Gen increase Sol MHC-I expression via ERβ mediated pathways

Exposure to the selective ERβ agonist in the NoEx group significantly increased MHC-I expression above levels seen in OVX and ERα agonist Ex and NoEx groups (Fig. 3). There was also a significant difference between Ex and NoEx groups for ERα and ERβ groups, with higher MHC-I expression levels detected in the NoEx groups. There were no significant differences in Sol MHC-I expression between OVX NoEx and OVX Ex.

3.3. E2 increases Gas MHC-IIb expression only in combination with high intensity exercise

E2 in combination with Ex significantly increased the expression of MHC-IIb in the Gas relative to OVX and Gen Ex and NoEx groups and normalized levels relative to Sham Ex groups (Fig. 4). E2 also significantly increased MHC-IIb expression in the NoEx groups relative to OVX Ex and NoEx (Fig. 4). MHC-I was not detected in the Gas (Fig. 1B). Gen had a smaller albeit significant effect on MHC-IIb expression in the Gas Ex groups compared to OVX. There were no significant differences in Gas MHC-IIb expression between OVX NoEx and OVX Ex.

3.4. E2 and Gen increase Gas MHC-IIb expression in combination with exercise via ERβ mediated pathways

MHC-IIb expression was significantly higher in ERβ treated Ex groups compared to OVX or ERα treated groups (Fig. 5). There were no significant differences in Gas MHC-IIb expression between OVX NoEx and OVX Ex. A significant difference was detected between

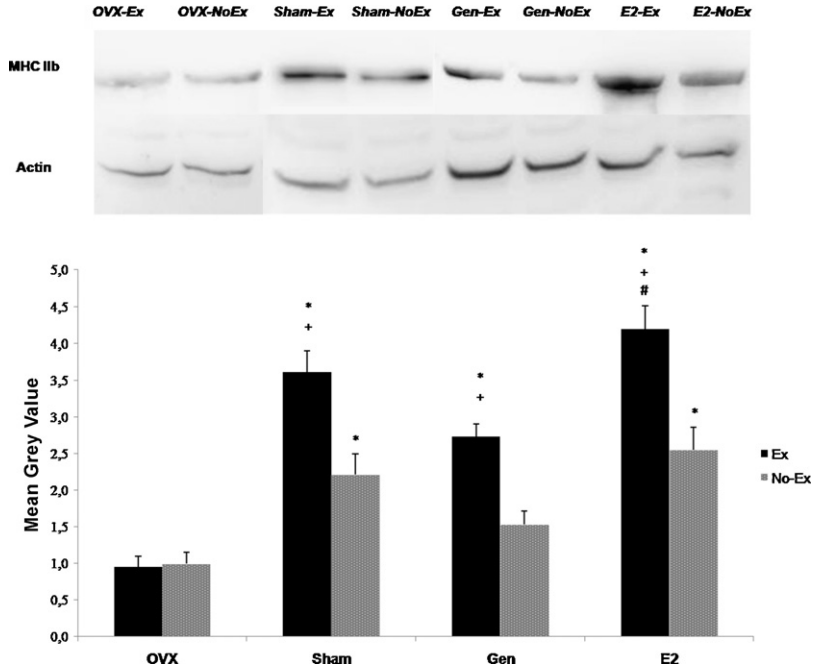

Fig. 4. E2 normalizes MHC-IIb expression with or without exercise and Gen only in combination with exercise relative to Sham levels. Western blot and densitometric analysis of MHC-IIb protein expression in the Gas of OVX, Gen and E2 groups. *Significant difference relative to OVX, +significant difference between Ex and NoEx, #significant difference relative to Gen.

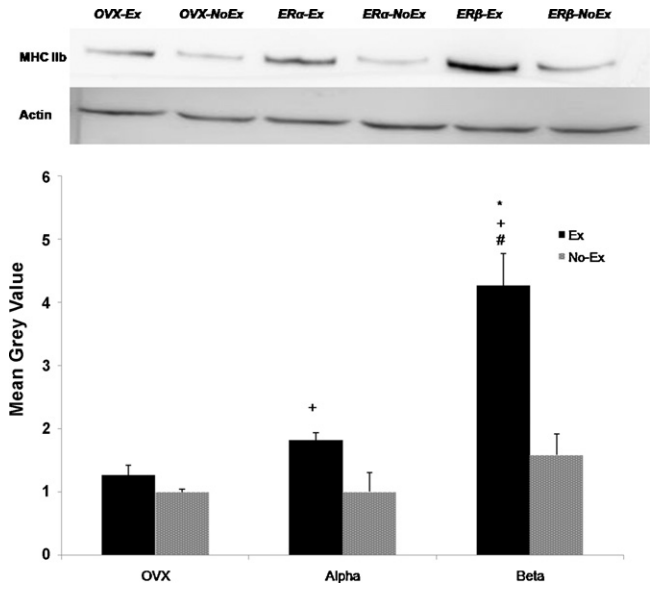

Fig. 5. Estrogenic effects on MHC-IIb expression are predominantly mediated via ER-β. Western blot and densitometric analysis of MHC-IIb protein expression in the Gas of OVX, ER-α agonist (Alpha) and ER-β agonist (Beta) groups. *Significant difference relative to OVX, +significant difference between Ex and NoEx, #significant difference relative to Alpha.

the Ex and No Ex groups of ERβ and ERα groups, with higher levels of MHC-IIb expressed in the Ex groups.

4. Discussion

The presence of both estrogen receptor (ER) subtypes (ERα, ERβ) in skeletal muscle cells at the mRNA and protein level of expression strongly indicates that estrogens exert direct ER mediated effects in skeletal muscle [33,34]. Besides the known positive effects of estrogens on skeletal muscle metabolism [35,36], reducing adipocyte size and down-regulating lipogenic gene expression [37], skeletal muscle structural parameters and contractility patterns are also affected by the presence of estrogens.

Mixed results are reported regarding the effects of removal of ovarian hormone production (OVX) and estrogen replacement on MHC expression patterns, which determine contractile speed. Estrogen replacement in combination with voluntary wheel running exercise for 5 weeks normalized the OVX-induced fast to slow fiber type shifts in the slow fiber type Soleus and the fast fiber type extensor digitorum longus of female rats back to control levels [14]. In contrast to Kadi et al. [14] we have shown that OVX reduces MHC-I expression in the Soleus relative to Sham and that exercise was unable to bring MHC-I expression levels in OVX groups back to Sham levels (Fig. 2). It is possible that the overall MHC expression per gram of muscle was lower in OVX animals. Similar reports have been made by Sanchez-Ortiz et al. [38] who investigated the effects of estrogen on MHC expression in smooth muscle and showed that total MHC mRNA and protein expression was reduced in OVX animals. We show here that MHC-I expression levels remained unaltered after a high intensity training in the Soleus of rats with intact ovaries (Sham) and rats with removed ovaries (OVX) (Fig. 2). In contrast to Kadi et al. [14] who used voluntary wheel running as an endurance exercise model and showed that exercise normalized OVX MHC-I expression back to Sham levels, rats in our study were trained on a treadmill at high intensity twice per day (15 min, 10% incline, 20–24 m/min) to have a sufficiently strong stimulus to alter MHC expression at the molecular level [39]. The MHC-I isoform is predominantly expressed in slow, oxidative skeletal muscle fibers that are important for endurance type activities. E2 and Gen treatment normalized OVX MHC-I expression levels only in the absence of high intensity exercise (Fig. 2). This finding suggests that high intensity exercise resulted in a remodeling process in the Soleus muscle and interfered with the regulation of MHC-I by Gen and E2, and that other ovarian hormones in the Sham exercise group had an effect on MHC-I expression. The specific signaling mechanisms behind the hormone-dependent alteration of MHC expression remain to be elucidated.

The results of our study confirm the result of Kadi et al. [14] showing that MHC-IIb expression was depressed after OVX in a fast fiber type muscle and returned to control levels with exercise and E2 (Fig. 4). However, in contrast to Kadi et al. we show that MHC-IIb expression increased in the exercised Gastrocnemius muscles of Sham, Gen and E2 animals compared to non-exercised groups (Fig. 4). Again, the discrepancies between studies regarding the exercise effect can be attributed to the different training regimes. The high intensity training in our study most likely resulted in enhanced recruitment of fast muscle fibers, activating gene expression pathways leading to altered MHC-IIb expression. According to Hennemann's size principle, faster motor units are recruited as the activation stimulus increases [40].

The results of this study suggest that E2 exposure mimics the physiological state by increasing MHC-IIb expression with exercise in OVX groups to MHC-IIb expression levels detected in the Sham exercise group (Fig. 4). In addition to Kadi et al. who did not study the effects of E2 alone, we also showed that E2 without high intensity training was also able to increase MHC-IIb in the Gastrocnemius above OVX levels. Regulation of MHC-II expression in the Gastrocnemius appears to be dependent on ovarian hormones and training can only up regulate MHC-IIb expression levels in animals with normal ovarian hormone status or OVX animals treated with Gen or E2.

We therefore propose that the MHC-IIb adaptation to intense training is hormone-dependent since the exercise effect evident in the Sham groups, were ovarian hormones are present, was not seen in the OVX groups (Fig. 4). Hormonal replacement with Gen and E2 enabled the Gastrocnemius muscle to respond to high intensity exercise with increased MHC-IIb expression. This finding has relevance with respect to skeletal muscle function and adaptation in older people participating in exercise programs.

The effects of ER-subtype selective agonists on skeletal muscle MHC expression are thus far unknown. Using the ER selective agonists 16alpha-LE2 and 8beta-VE2 [29], we were able to show that the E2 and Gen effects on Gastrocnemius MHC-IIb and Soleus MHC-I are both predominantly mediated via the ERβ (Figs. 3 and 5). It has been postulated that the effects and actions of E2 depend on the ER subtype that is activated and that the ER subtypes are often antagonistic against one another [41]. For example, the uterine proliferative response to E2 is unaltered or increased in ERβ knockout animals [42]. This is due to the fact that ERα is the predominant ER subtype expressed in the uterus and therefore loss of ERβ has no functional relevance. In breast cancer cell lines, the presence of the ERα stimulates proliferation, whereas ERβ inhibits proliferation [43]. This has led to the belief that the two receptor subtypes have distinctly different biological functions. While both ER subtypes have been found in skeletal muscle cells at the gene expression and also protein level of expression [34,44], our results show that the effects of E2 and Gen on skeletal muscle MHC expression appear to be predominantly mediated through the ERβ. This result agrees with findings showing that Gen binds with higher affinity to the ERβ than ERα [45]. The biological implications of ER-subtype specific signaling in skeletal muscle tissue remain to be determined. In future experiments we plan to further validate this finding by conducting experiments using ER-subtype selective antagonists.

To date there is very limited data on the effects of soy isoflavones that exert estrogenic effects (phytoestrogens) in combination with physical activity on skeletal muscle MHC expression levels. Qin et al. showed that a phytoestrogen rich formula resulted in enhanced mRNA expression levels of MHC-I, IIa and IIb compared to OVX in the abductor muscles of rats [46]. Therefore, one aim of the current study was to evaluate the effects of Gen in combination with exercise on MHC expression. Gen, similarly to E2, increased MHC-I expression in the Soleus in the absence of Ex (Fig. 2). In the Gastrocnemius, MHC-IIb expression was significantly higher in Gen Ex groups compared to OVX (Fig. 4). Some of the effects of Gen on skeletal muscle are presumably non-ER mediated. Little is known about non-genomic pathways activated by phytoestrogens. Signaling pathways such as ERK1/2, PI3-Kinase/Akt cascades have been described to play a role in non-genomic actions of phytoestrogens [47]. The precise mechanisms behind isoflavone induced signaling in skeletal muscle remain to be fully elucidated.

In summary, we show that the combinatory effects of exercise and hormonal exposure to E2, ERα, ERβ selective agonists or soy isoflavones with estrogenic activity (Gen) on MHC expression in skeletal muscle depend on exercise intensity and skeletal muscle fiber type composition. A resting, slow fiber type muscle such as the Soleus responds to E2 or Gen exposure with increased MHC-I expression. The fast fiber type Gastrocnemius responds to Gen exposure only in combination with exercise and to E2 with or without exercise with increased MHC-IIb expression. The exposure of OVX rats to ER selective agonists revealed that the estrogenic effects on MHC expression patterns in Soleus and Gastrocnemius are predominantly ERβ mediated. To understand the differential effects of estrogenic action in skeletal muscle during rest or exercise, research needs to focus on the distribution of ER expression in different fiber types and the signaling mechanisms downstream of receptor activation.

The combinatory effects of exercise and exposure to E2, ER selective agonists or Gen on skeletal muscle structural composition and muscle health should be considered in a fiber type specific context.

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