Beneficial Effects of Estradiol on Murine Skeletal Muscle Function

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effect size (ES)

17-β estradiol (E_2)

estrogen receptors (ER)

estrogen receptor α (ER-α)

estrogen receptor β (ER-β)

extensor digitorum longus (EDL)

G-protein coupled receptor 30 (Gper)

hormone therapy (HT)

ovariectomized (OVX)

Physiotherapy Evidence-Based Database Scale (PEDro)

randomized controlled trial (RCT)

tibialis anterior (TA)

Chapter 1

Introduction

Aging is a multidimensional physiological process that affects all people. Biological systems age at various rates with declines in structure and function of up to 3% per year due to a number of different mechanism (90). Many systems have well defined pathologies related to aging; cardiovascular disease, diabetes, osteoporosis, and dementia. The age-related pathology of skeletal muscle is sarcopenia. Sarcopenia is primarily characterized by muscle atrophy and weakness and is highly associated with functional impairment and immobility of the elderly (45). With age, both absolute force production and that normalized to the size of the muscle, or specific force, have noted decrements compared to younger individuals. It is likely that numerous mechanisms with various contributions of each account for the pathology of sarcopenia; such as physical (in)activity, hormonal changes, nutrition, oxidative stress, inflammation, and mitochondrial dysfunction. The main focus in this dissertation is on hormonal changes in females and secondary changes in physical (in)activity.

The timing of skeletal muscle force loss with age has been well documented in cross-sectional studies of both men and women (59, 86). However, detailed longitudinal studies including men and women have indicated various rates of force loss between the sexes, men have a noted gradual decline beginning about the 6^{th} decade of life and women having a sharp decline around the time of menopause (81). It has been hypothesized that this decline in women is due to the loss of ovarian hormones secondary to the menopause transition (58). During the menopause transition there is an increase in follicle-stimulating hormone in combination with decreases in inhibin A and B,

progesterone, and estrogen (16). However the direct effect of ovarian hormones on skeletal muscle is not known.

While the characterization of age related strength loss had been conducted in both men and women, there is a lack of consensus in animal studies (14). It is common to only use male rodents in aging studies, likely because researchers have been trying to control the confounding effects of ovarian hormones. Further studies need to be conducted to thoroughly characterize both this disconnect between the males and females and the influence of ovarian hormones of age-related force loss.

The primary goal of this dissertation is to further comprehend the connection between strength loss and ovarian hormones, specifically estrogens, in females. The first aim was to statistically synthesize the literature on strength loss in postmenopausal women to come to a consensus on how estrogens are related to the muscle strength. Also to synthesize data on the loss of force generating capacity in ovarian hormone-deficient rodents. The second aim was to contribute to our understanding of the relationships between physical activity, estrogen, and force generating capacity, and to discern if aged ovarian-senescent mice are affected in parallel to what has been determined in younger ovariectomized mice.

Sex difference in the decline in muscle function

There is evidence to suggest a sex difference exists for declining muscle strength with age. A study out of the Baltimore Longitudinal Study on Aging found that there was a decrease in muscle quality with age and a difference between the males and females (59). Specifically, the muscle quality of arm and leg muscles from both men and women

declined with age, although the women had a different decline than men. Women over the ~50 year age range of study participants, had varying rates of decline between the muscle groups studied with a greater decline in muscle quality of the leg muscle, while the men had similar rates of decline in the two muscle groups tested.

A cross-sectional study by Samson et al. (86) examined both men and women between 20 to 90 years of age. While the men in the study had a gradual decline in strength of both the knee extensors and hand grip strength over increasing ages, this was not found in the women. There was a gradual decline in strength found in the women only up until ~55 years of age, or around the menopause transition. At this point there was a sharp decline in strength, with the hypothesis that this strength loss was due to loss of estrogen production.

Few studies have specifically examined age-related strength differences between men, pre-menopausal and post-menopausal women. Phillips and colleagues investigated this in a cross-sectional study (81). Examining specific force, they again confirmed the gradual decline of strength in men and women up to the point of menopause. However, they also examined a group of women who were replaced with estrogen-based hormone therapy (HT). Interestingly, it appeared that HT prevented the drastic strength loss around the time of menopause (Figure 1). However, this finding was novel and has since been heavily debated in the literate. Phillips' data has been by far the most supportive evidence of a positive effect of HT on muscle strength, with many other studies finding no or minimal effect of HT on muscle strength. Traditional literature reviews have investigated this and concluded that although it is possible that HT may be beneficial, the results are still inconclusive (66, 93). To systematically determine the effect of HT on muscle

strength, I conducted a statistical analysis of all relevant literature regarding muscle strength and HT treatment. The results of that study compromise the second chapter of this dissertation.

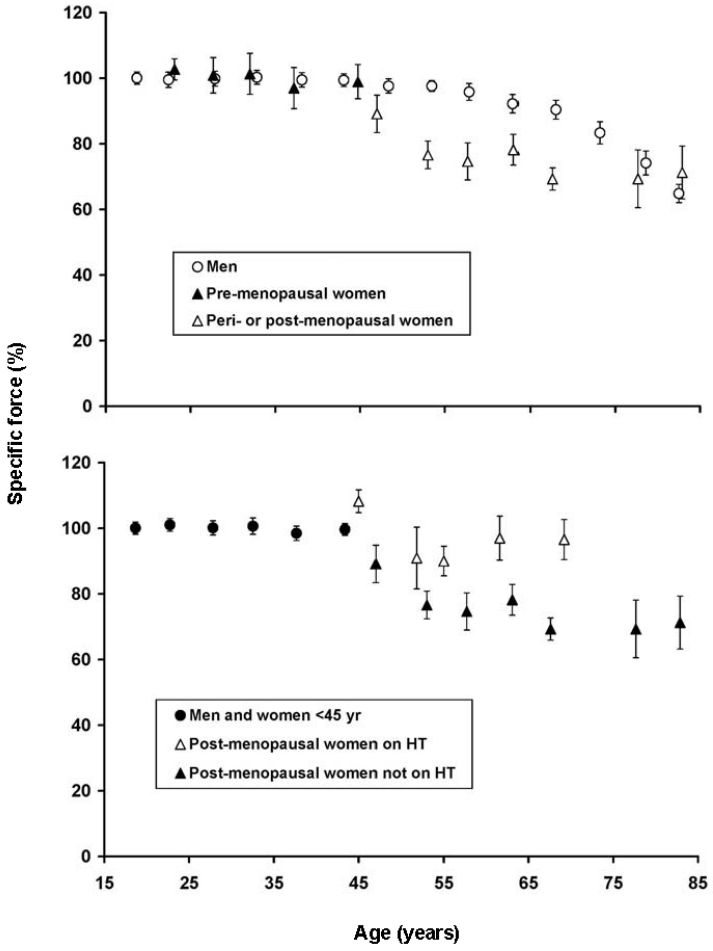

Figure 1. Cross-sectional data indicating the age related decline in specific force of the adductor pollicis muscle. Notably there is a drastic decline in strength around the time of menopause in women (top), however when women were replaced with estrogen-based hormone therapy (HT) there is a preservation of strength (bottom). Data from Phillips et al. (81), graph is used with permission from Lowe et al. (58) (see Appendix 1).

The strength loss reported in men and women with age is also apparent in male and female rodents. For example, soleus muscle of female mice had decreased force-generating capacity with age (70). Interestingly, there was a sharp decline of ~25% in soleus muscle force generating capacity around the age of 16 months. This dramatic decline was hypothesized to be due to the a decline in normal estrus cycling because C57BL/6 mice have normal estrous cycling between 3- and 14-months of age (76). However, there was not conclusive evidence that the loss of estrogens was the mechanism for the loss of soleus muscle force because ovarian function and hormones were not assessed in that study.

Estrogen and skeletal muscle.

Estrogen and muscle contractility. The basic science literature on the effects of estrogen on skeletal muscle force generating capacity has been limited, with mixed results. The use of rodent models can be advantageous because there is control of many environmental factors within a shorten lifespan. There are 10 papers that specifically address the question of force generating capacity and ovarian hormones in rodents. Within these papers there have been proposed mechanisms for estrogenic effects on skeletal muscle contractility. I will summarize the mechanistic aspects of these 10 studies.

1) Early and novel work by Suzuki and colleagues examined the effects of estrogen on skeletal muscle in growing rats (100). Briefly, very young rats were randomized at 21 days of age to an ovariectomized (OVX) placebo treated group, an OVX+17-β estradiol (E_2) treated group, and a sham operated and placebo treated group.

Rats were than aged to 10 weeks in these groups. Physiological *in vitro* contractile and histological parameters were measured in the extensor digitorum longus (EDL) and soleus muscles. There was no difference in the maximal twitch for the soleus muscle, although the maximal isometric force was lower in E_2-replaced rats. For the EDL muscle both maximal twitch and tetanus were lowest in the E_2-replaced rats. There was no difference in the fiber type distribution or total muscle fiber number within the experimental groups. These data suggested that during development E_2 may have an inhibitory role in skeletal muscle fiber development. However, the authors hypothesized that due to the neural component of the development of skeletal muscle fibers, there may be an indirect effect of E_2 on skeletal muscle through this pathway. Although in my perspective this hypothesis can not account for the total differences seen in these experiments.

2) Warren and colleagues designed a study with the aim of determining the effects of E_2 in bone and skeletal muscle, both tissues with known changes due to estrogens (110). Young immature mice, 6 weeks of age, underwent OVX surgery then were randomized to E_2- or placebo-treatment for 21 days. Following the treatment duration mice underwent *in vivo* and *in vitro* skeletal muscle analysis of the anterior hindlimb (tibialis anterior (TA), extensor hallucis longus, and EDL muscles) and EDL muscles, respectively. The isometric torque of the anterior muscles was 18% greater in the mice treated with E_2. In parallel, the absolute force of the EDL of the mice treated with E_2 was 14% greater than the E_2-deficient mice. The tibial bone ultimate load and stiffness of these mice were 16% greater in the E_2-treated mice. Warren's data show that there was

not a mismatch between anterior crural muscles and the tibial bone function within this relatively short time frame and that estrogens were beneficial to both tissues.

3) Progressing this line of research, Fisher and colleagues conducted a study on mature rats, 6.5 months of age (26). The purpose of their study was to examine skeletal muscle maintenance and function in respect to ovarian hormones. Rats were initially randomized to either sham or OVX surgical conditions. In addition to control states of normal cage ambulation, some rats were randomized to hindlimb unloading groups. The rats were given 1 month in these conditions. Following this the EDL, plantaris, peroneous longus, and soleus muscles were surgically exposed and contractility was measured *in situ*. Examining the data from the control (i.e., normal loaded) rats, maximal force showed mixed results with no differences in the soleus and plantaris muscles between sham and OVX rats, but the EDL muscle had increased strength in the OVX rats, and the peroneous longus muscle had about 40% greater force in the sham, ovary-intact rats. Force normalized to muscle mass followed the same trends, with the only significant finding being the EDL muscle in favor of greater normalized force in OVX rats. Additional findings on the twitch contraction kinetics (time to peak twitch and ½ relaxation time) were that OVX rats had greater times indicating that loss of ovarian hormones slowed muscle contraction. It was hypothesized that this change in contraction relaxation may be due to the effects of estrogens at the sarcoplasmic reticulum, with an inhabitation of skeletal muscle uptake of Ca^{+2}. In addition to studying control rats, there was also an unloaded group. They found that in the OVX rats there was a modification of the endocrine response that was deemed necessary for recovery from atrophy, with a

hypothesized protective effect of ovarian hormones on skeletal muscle size following disuse.

4) Work by Wattanapermpool and colleagues tried to match the fields of cardiac and skeletal muscle (112). Their aim was to determine if ovarian hormone-deficiency causes changes in both the mechanical properties of soleus muscle fibers and in cardiac myofilaments. Young (~9 weeks of age) rats were randomized to sham or OVX surgical conditions and rats remained in these states for either 10 or 14 weeks. At these time points, single permeabilized soleus muscle fibers underwent contractile measurements. The normalized force of soleus muscle fibers from the E_2-deficient rats were 19 and 20% lower at both 10 and 14 week time points, respectively. Measurements of Ca^{+2}-sensitivity of these fibers indicated no change in the presence of this force change. These data lead to the hypothesis that there may be an involvement of the actin and myosin cross-bridge, with more data needed to definitely discern this.

5) McCormick and colleagues examined estrogens effects on skeletal muscle in growing rats (7 weeks of age) (64). The rats were randomized to sham or OVX surgical conditions, then the E_2-deficient rats were randomized to be treated with placebo or E_2. Treatment continued for 28 days. Following this, soleus muscles were tested for both the E_2-deficient and –circulating rats. Twitch contraction kinetics, specifically time to peak twitch and ½ relaxation time, were decreased in the OVX rats by 16 and 17%, respectively. The investigators hypothesized that the changes in twitch kinetics may be due to changes in Ca^{2+} release, likely dependent upon E_2.

6) Work by Schneider and colleagues continued the field by studying young (6 weeks of age) mice (87). Specifically, they sought to determine if there was an interaction

of estrogen and progesterone on isometric torque of mice. Following OVX surgery, mice were randomized to receive placebo, E_2, progesterone, or a combination of E_2 and progesterone, in addition there was an intact control group. Following 16 days of hormone treatment mice underwent *in vivo* muscle testing of the anterior muscle compartment. The maximal isometric torque of the anterior compartment of the intact control mice was ~27% greater than the OVX-placebo treated group.

7) Hubal and colleagues took on a broader question to include exercise and bone, in addition to muscle (41). The primary aim of their study was to investigate eccentric muscle training in E_2-deficient mice; here I will specifically report their findings on the contractile data in the unexercised groups. Young (9 weeks of age) mice were randomized to sham, OVX, or control conditions. The OVX mice were randomized further to include an exercise training group. Anterior compartment muscle contractility was tested *in vivo* at baseline, 4, and 8 weeks. In the sham and OVX mice there was no change over time or difference between groups in peak isometric torque. However, there was a trend for the peak torque in the E_2-deficient mice to be ~5-10% lower than that of the sham group. It was noted that this study may have been underpowered to detect this small difference.

8) More recent studies by Moran and colleagues further probed the question on ovarian hormones and skeletal muscle contractile function (69). The purpose of their study was to determine the effects of ovarian hormones on muscle contractility of the EDL and soleus muscles. Six-month old, skeletally mature mice were randomized to OVX or sham-operated conditions for 60 days. Following this, the EDL and soleus muscles were analyzed *in vitro* for contractile parameters and a force-frequency

relationship and the contralateral EDL muscle was used to study actin and myosin interaction by electron paramagnetic resonance. EDL and soleus muscles from OVX mice had ~19% less normalized force than those from sham mice. Those results paralleled by the electron paramagnetic resonance spectroscopy findings, in which the EDL muscles of the E_2-deficient mice had a fraction of strongly-bound myosin during contraction ~15% less than the sham operated mice. These data indicated that ovarian hormones are affecting skeletal muscle and more importantly the contractile apparatus of the muscle, actin and myosin. It was found that this reduction of strength with ovarian hormone loss may be due to a reduction of strong-binding myosin in muscle contraction.

9) To follow this study, Moran and colleagues designed an additional study to specifically study estrogens effects on skeletal muscle (68). The purpose of this study was to determine if E_2 was the key hormone affecting muscle strength in skeletally mature mice following removal of ovarian hormones. Mature 4 month old mice underwent sham or OVX surgery, then a subset of mice were immediately treated with E_2 or placebo and the other subset was treated following 30 days of hormone-deficiency. Either 30 or 60 days following surgery the soleus muscle was test for contractility *in vitro*. Maximal soleus muscle force was ~10% less in E_2-deficient mice (i.e. OVX placebo treated), additionally this loss was recovered with E_2 treatment to the level of force in the sham operated mice. Plasma E_2 levels were positively correlated with maximal soleus muscle force and active stiffness. Overall, E_2 was shown to be the key hormone affecting skeletal muscles and myosin function.

10) More recently Wholers et al., investigated the effects of circulating ovarian hormones on contraction-induced activation of enzymes related to the gene expression

and metabolic function of skeletal muscle (specifically AMPK and MAPK) (116). Using 8 week old mice, half underwent OVX surgery with the remaining sham operated. The mice were allowed 8 weeks until both the TA and EDL muscles underwent contractile testing. Although there was no difference in muscle force between E_2-deficient and sham-operated mice, there were noted contraction- included changes in phosphorylation of AMPK and ERK2. Specifically, there were increases in both signaling cascades following contractions in OVX mice. They concluded that ovarian hormones impact cellular signaling in skeletal muscle although the downstream effects of this increased activation are still unknown.

While the most conclusive data indicate a decrease of skeletal muscle function of ~10-15% following the loss of ovarian hormones (Figure 2) (69, 110, 112), other data have not (64, 116). Some mechanisms of the effects of estrogen on skeletal muscles emerged from these 10 studies summarized above, however there was a number of conflicting ideas and the specific mechanism is not currently understood. Based on knowledge of estrogenic mechanisms in non-muscle tissues, it is likely that estrogens work in muscle through estrogen receptors (ER) via genomic mechanisms. Estrogen functions by binding to the ligand (i.e. the nuclear ER) and then working as a transcription factor by dimerizing to bind to estrogen response elements in the promoter region of genes. This action is followed by an increase in the transcription of target genes.

In muscle E_2 could also be working through non-genomic mechanisms as E_2 interacts with a number of factors within signaling cascades. Theoretically, those

mechanisms could affect skeletal muscle by improving the oxidation state of fibers. First, it was hypothesized that there is a decrease of strong-binding of myosin during contraction in E_2-deficient states. How the improvement in myosin function is occurring is not known, although it is possible that estrogen is working through an antioxidant mechanism (58). Second, it has been suggested that the change in contraction speed may be due to Ca^{2+} release, with an inhibition of Ca^{2+} uptake at the sarcoplasmic reticulum in E_2-deficient states.

With the conflicting nature of this literature, a conclusive analysis of this data may clear up the effects of estrogens on skeletal muscle as well as if E_2 is the key ovarian hormone inducing these changes. The first step that is needed is to comprehensively conclude that the loss of E_2 is detrimental to skeletal muscle function, which I have done and report in chapter two. It is also necessary to determine if E_2 is acting directly on the skeletal muscle and not through secondary mechanisms, such as physical activity, which I have completed and report in chapter three. The importance of considering physical activity in light of hormonal affects on skeletal muscle is summarized in the next section of this chapter.

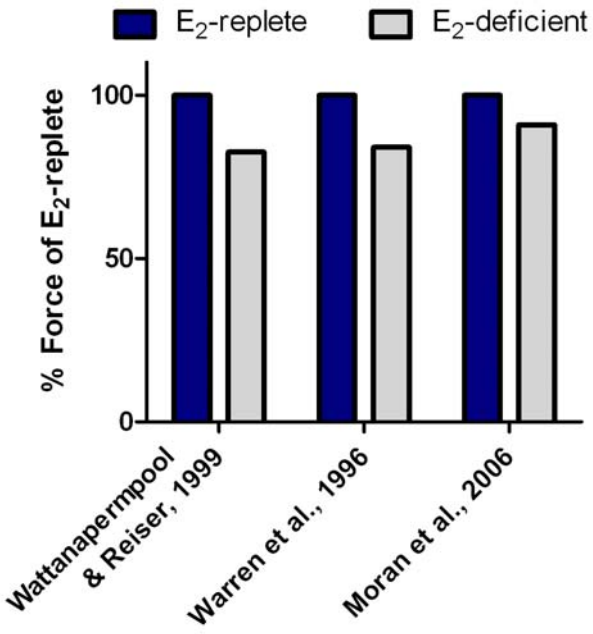

Figure 2. Collective strength loss with the loss of ovarian-hormones due to ovariectomy in young rodents. Data is soleus muscle normalized force (112), EDL and anterior cruel muscle force and torque (110), and EDL and soleus muscle force (69).

Estrogen and physical activity.

Combined effects of estrogens and exercise. Exercise as an intervention in estrogen-dependent models has also been examined. Returning to a previously summarized paper by Hubal, a model of eccentric exercise in addition to control unexercised mice were investigated (41). It was found that eccentric training of the anterior crural muscle group in OVX mice, trained three times over an 8-week period, resulted in trends with 7-10% greater strength in the E_2-deficient mice that were trained over the untrained E_2-deficient mice. Again, it was noted that the study was underpowered. Another approach was to use an acute bout of exercise to induce muscle injury. Sotiriadou and colleagues used force downhill running to determine if E_2 in an

OVX model could protect skeletal muscle following injury (97). Through *in situ* testing of the soleus muscle, they determined that rats treated with E_2 had greater force than non-treated E_2-deficient rats following the injury indicating the E_2 can be protective against muscle injury.

Influences of estrogens on wheel running. Spontaneous exercise in estrogen-dependent models has been studied. The loss of estrogens has been shown to severely affect spontaneous physical activity in mice. Physical activity in the form of wheel running is decreased in E_2-deficinet mice (30, 48, 71, 111). However, it can be recovered following replacement of E_2 (Figure 3). Mice treated with the selective estrogen receptor modulator tamoxifen, ran distances comparable to those of mice replaced with E_2 (30). These data indicate that the wheel running is responsive to circulating ovarian hormones, specifically estrogen levels. Using estrogen receptor knock out mice it was determined that the increase in physical activity is mediated by the alpha estrogen receptor (ER-α) in the hypothalamus (77). It has been hypothesized that the alterations in activity as mediated through the ER-α pathway may be due to enhanced release of dopamine as well as changes in dopamine transport and receptors (56).

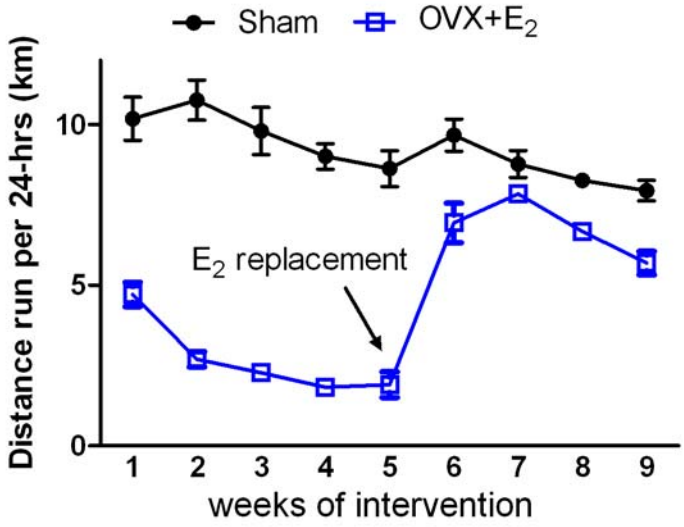

Figure 3. Weekly running averages for mice with circulating E_2 levels (sham) or those deficient in E_2 due to ovariectomy (OVX). Mice were replaced with E_2 following 5 weeks, running distances rebound to those of sham mice within 3 days of E_2-replacement.

Influences of estrogens on acute physical activity. There has been a minimal amount of research conducted on short term monitoring of fear behaviors in relationship to ovarian hormones, with effects of E_2 noted on fear (71, 77). Data indicate estrogens can increase fear behaviors in rodents, which in turn decreases activity acutely due to the stressful stimuli (72). There have also been noted decreases in ambulation during the dark photo-period in mice deficient in ovarian hormones, relative to mice with normal levels of these hormones (85, 91). However, the impact that estrogens have on normal daily cage activites was not clearly established. This is an important consideration because it is possible that decreases in cage activites may affect skeletal muscle function negatively (Figure 4).

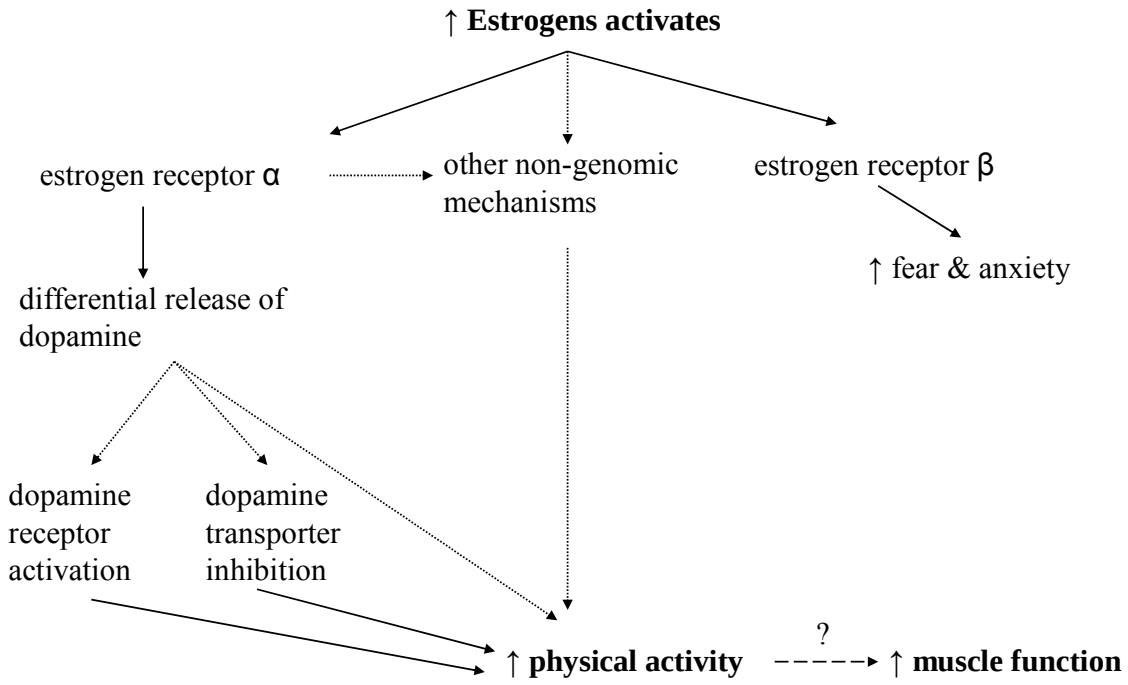

Figure 4. Schematic of the proposed pathway of estrogens effect on physical activity. It is possible that the variation in long term physical activity may be influencing skeletal muscle function. Dashed arrows are hypothesized mechanisms, adapted and modified from Ligthfoot, 2008 (56).

To address the possibility that estrogenic effects on muscle are secondary to changes in physical activity (as opposed to direct estrogenic effects on the muscle), I both characterized the normal cage activities of mice with and without estrogen. Furthermore, I designed and carried out studies described in chapter three to control physical activity, in efforts to determine the direct effect of E_2 on skeletal muscle.

Estrogens

Estrogens are the primary steroidal sex hormones in females. Estrogens are comprised of estrone, estradiol, and estriol. The most biologically active estrogen is 17-β estradiol (E_2).The function of E_2 is either through genomic or non-genomic mechanisms. Estrogens' genomic actions are functioning through estrogen receptors (ER), distinctively the isoforms of ER-α and ER-β, additional effects may be through a non-genomic effects via a G-protein coupled receptor 30 (Gper). Notably all three isoforms have been found in skeletal muscle of mice (6).

Estrogen deficiency as a model of hormonal aging. The most common approach for studying estrogen-deficiency in rodents is ovariectomy. Specifically, the surgical removal of both ovaries results in the loss of the primary endocrine gland producing estrogens as well as the other female sex steroids, follicle stimulating hormone, progesterone and inhibin. There are secondary sites of estrogen production in mammals but those sites produce limited amounts of estrogens and the circulating concentration is considered negligible. Even though the ovariectomy approach is not a direct reflection of menopause since the ovary is removed and the hormone decline is not gradual, it does allow for the study of estrogens without the complex, interactive effects of aging. To address this limitation, the investigation of aged mice that are naturally ovarian-senescent is needed. In chapter four, I designed studies to investigate muscle function and physical activity in aged, ovarian-senescent mice.

There is a newly available model of progressive ovarian-senescent that I used as well (see Figure 5). Mice treated with the chemical 4-vinylcyclohexene diepoxide (VCD) have accelerated apoptosis and atresia of ovarian follicles resulting in state of ovarian

senescence and low circulating E_2 levels similar to what occurs with natural aging (40, 63). It is possible to treat young mice with VCD, again eliminating the complex effects of aging, but this time being able to keep the ovaries intact. Thus, to further investigate the effects of natural ovarian aging on skeletal muscle, I also examined VCD-treated mice and report those results in the forth chapter of this dissertation.

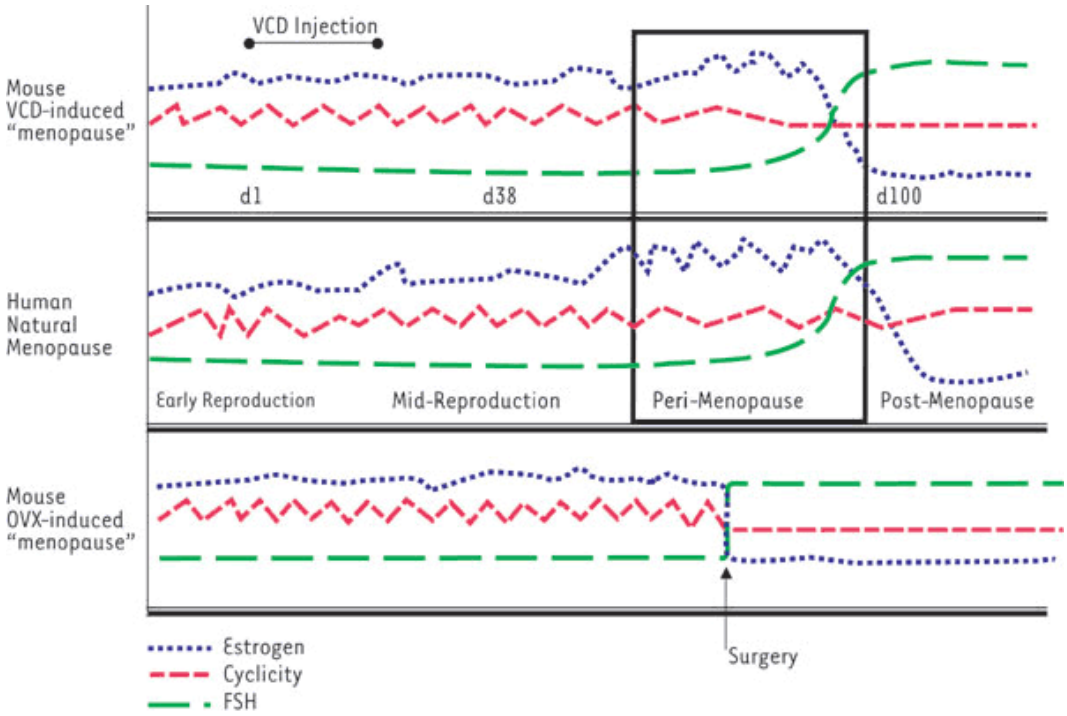

Figure 5. Cyclicity and hormone profiles of VCD treatment, natural age-induced ovarian senescence, and surgical removal of the ovaries. Notably, natural menopause and VCD treatment have a similar rate of decline of estrogen while the ovariectomy (OVX) model has an immediate drop off following the surgical procedure.

Aims & Hypotheses

Aim one (Chapter 2) of this dissertation was to perform a rigorous systematic review and statistical analysis (meta-analysis) of the few studies that had compared muscle strength in post-menopausal women who were and were not on HT. Due to variation, a secondary objective was to analyze several experimental factors that varied among the studies. Finally, to specifically assess the effect of E_2 on strength, a systematic search and meta-analysis were also conducted on studies of muscle strength in rodents that were and were not E_2-deficient.

Aim two (Chapter 3) of this dissertation was to determine if E_2 was beneficial to muscle function independent of an increase in physical activity. I hypothesized that under reduced total body physical activity (hindlimb suspension), E_2 replacement would cause muscle function to be better than in E_2-deficient mice. Additionally, under the condition of reduced muscle activity (tibial nerve transection), E_2 replacement would cause muscle function to be greater than that of E_2-deficient mice. The significance of this project was to determine if there is an indirect effect of estrogen on muscle function through physical activity.

Aim three (Chapter 4) of this dissertation was to the first determine the effects of E_2 treatment on physical activity of female mice that had experienced natural, age-induced ovarian senescence. The second purpose was to determine the extent to which muscle of aged, ovarian-senescent mice adapt to physical activity and to E_2 treatment. The final aim of this study was to examine the effects of E_2 on young mice that have been treated with VCD to induce progressive ovarian senescence, in comparison to both non-treated and VCD treated and replaced with E_2. I hypothesized that mice deficient of

ovarian hormones either due to aging or chemical treatment would have decreased soleus muscle function. The significance of this project was to decipher the effects of ovarian hormones on skeletal muscle contractile function following natural ovarian senescence.

Chapter 2

Hormone Therapy and Skeletal Muscle: A Meta-Analysis

The specific literature review on the loss of muscle strength in the rodent E_2-deficient model, as previously detailed, has mixed results. The literature on muscle strength in post-menopausal women is also varied. Thus the aim of this study and chapter is to clarify these issues by performing a systematic review and meta-analysis.

The content of this chapter has been previously published (33). Copyright permission has been granted by Oxford University Press on behalf of the Journals of Gerontology Series A, Biological and Medical Sciences (see Appendix 2).

Background. Our objective was to perform a systematic review and meta-analysis of the research literature that compared muscle strength in post-menopausal women who were and were not on estrogen-based hormone therapy (HT). *Methods.* Twenty-three relevant studies were found. Effect sizes (ES) were calculated as the standardized mean difference and meta-analyses were completed using a random effects model. *Results.* HT was found to result in a small, beneficial effect on muscle strength in post-menopausal women (overall ES=0.23; P=0.003) that equated to an ~5% greater strength for women on HT. Among the 23 studies, various muscle groups were assessed for strength and those that benefitted the most were the thumb adductors (ES=1.14; P<0.001). Ten studies that compared muscle strength in rodents that were and were not estradiol deficient were also analyzed. The ES for absolute strength was moderate but not statistically significant (ES=0.44; P=0.12), while estradiol had a large effect on strength normalized to muscle size (ES=0.66; P=0.03). *Conclusions.* Overall, estrogen-based treatments were found to beneficially affect strength.

Introduction

There is a loss of skeletal muscle mass and strength, i.e., sarcopenia, with age and this loss contributes to increased risks for co-morbidities related to falls, diminished ability to carry out activities of daily living, and an overall decreased quality of life (51). In addition to the age-induced loss of muscle strength, in women there are reports of accelerated rates of strength loss associated with menopause (66, 81, 86). Therefore, it is probable that changes in ovarian hormones due to menopause contribute to muscle weakness. To counter the loss of ovarian hormones, women have the option of taking hormone therapy (HT). In this manuscript we will use the term HT because it was recently recommended that this terminology be used for menopause-related therapies that encompass estrogen therapy and combined estrogen-progestogen therapy (1, 2). The primary active ingredients in HT are estrogenic compounds such as estra-1,3,5,(10)-triene-3, 17-β estradiol, estrone, and dihydroequilin. Progestogen and testosterones are secondary components of some HTs. The decision to take HT is largely based on known and perceived benefits versus risks on menopausal symptoms, bone loss, cancers of the female reproductive organs, and cardiovascular events.

In contrast to HT's known effects on bone, reproductive tissues, and the cardiovascular system, the effects of HT on skeletal muscle are not as well understood nor considered clinically. Skeletal muscle is the largest tissue containing estrogen receptors so there is a logical connection between ovarian hormones and skeletal muscle. There have been a number of studies that have reported the effects of HT on the primary function of skeletal muscle, that is, to generate force, which we evaluate as muscle strength. However, the results of these studies have been equivocal and even when

analyzed collectively in traditional narrative reviews, there is no clear answer as to whether or not HT for menopausal women is advantageous for improving muscle strength (66, 93). In this study, our objective was to clarify this issue by performing a rigorous systematic review and statistical analysis (meta-analysis) of the studies that have compared muscle strength in post-menopausal women who were and were not on HT. We found a substantial amount of variability in the effects of HT on strength among the studies. Thus, a secondary objective was to analyze several experimental factors whose levels varied among the studies and thus could have affected study outcomes. Finally, to specifically assess the effect of estradiol on strength, a systematic search and meta-analysis were also conducted for studies of muscle strength in rodents that were and were not estradiol-deficient.

Methods

Meta-analyses of studies on post-menopausal women

Systematic review. We searched for studies in the research literature in which muscle strength was assessed in post-menopausal women who were and were not administered HT. Strength was operationally defined as a maximal force, torque or power generated by a group of muscles. Our search for studies began October, 2007 and continued through April, 2009. PubMed, Cochrane Library, Biological Abstracts, Web of Science, Digital Dissertation Database, and ClinicalTrials.gov databases were searched. The search terms and strategy were as follows: 1) hormone replacement therapy, 2) estrogen, 3) human, 4) muscle, 5) skeletal muscle, 6) strength, 7) muscle function 8) muscle force, 9) muscle performance, 10) muscle strength, and 11) (1 or 2) and 3 and (4 or 5 or 6 or 7 or 8 or 9 or 10). The reference lists from the 56 fully-evaluated publications (described below) and those of relevant review articles (8, 44, 66, 92, 93, 95) were also examined for studies not found with the online database searches. Searches were completed independently by two of the authors (SG, KB), searching through the same databases with the same search strategies.

Study inclusion and exclusion criteria. Studies meeting the following criteria were considered for review: 1) women were post-menopausal at the start of the study, 2) the study contained both HT and control (non-HT) groups, 3) the HT was estrogen based, 4) an objective measurement of muscular strength was performed, 5) there was an explanation of how muscle strength was tested, 6) details on subject age were included, 7) explanations of subject inclusion and exclusion criteria were given, and 8) the results were published in English. All studies determined to be relevant were checked for

inclusion criteria by two authors (SG, KB) and the data selected for analysis were based on a consensus of the two. Studies were excluded for the following reasons: 1) there was a concurrent exercise intervention with no group that did not perform exercise either with or without HT, 2) strength data were presented as adjusted means (e.g., data adjusted for age, BMI, or smoking status), or 3) strength data could not be extracted from the study (i.e. raw or summary data were not provided).

Selection. A total of 3,824 relevant publications were originally identified through the database searches. Of those, 3,768 were initially excluded based on the title and/or a brief review of the abstract. Fifty-six publications were identified at this point and fully evaluated via careful review of the full text. Based on the inclusion and exclusion criteria, 32 articles were excluded, leaving a total of 24 articles to be included in the primary meta-analysis. Of the 24 articles, two reported findings from the same subjects though the strength analyses differed (94, 103). Thus, data from these two publications were combined, giving a total of 23 studies that were included in the analysis.

Data extraction and study quality assessment. In most instances, mean muscle strength was extracted for the HT and control groups from the 23 studies along with standard deviations and sample sizes. Data for strength normalized to muscle cross-sectional area were also extracted when available. If mean values were not available, percent changes and p-values were extracted. If data were presented only in graphical form, means and standard deviations were extracted from the graphs. All data extractions were completed by one author (SG) and cross-checked by a second (KB). From the 23 studies, three corresponding authors were contacted via e-mail in attempts to retrieve data

on strength normalized to muscle cross-sectional area. Two sets of these data were obtained and included in the meta-analyses of normalized strength.

The 23 studies were assessed for quality based on the Physiotherapy Evidence-Based Database Scale (PEDro) independently by two authors (SG, KB). This scale yields a total possible score of 11 points with more points corresponding to higher quality (73). We deemed this quality assessment to be useful as the results yielded a range of scores.

Meta-analysis. For studies that were cross-sectional in design, the extracted strength data were converted to a standard format by calculating a standardized mean difference using the equation: (HT mean – control mean) / pooled standard deviation (SD) (12). Alternatively, standardized mean difference was determined from percent differences in strength and p-values when only those data were available. For studies that reported paired data from pre and post trials (i.e., studies that were longitudinal in design), the SD of change from pre to post trials was calculated for each group: $((SDpre^2 + SDpost^2) – (2r \times SDpre \times SDpost))^{½}$, where r is the correlation of pre and post trial measurements (12). The within-group SD was calculated by dividing the SD of change by $(2 \times (1 - r))^{1/2}$ (12). Because correlations of pre and post trial measurements were not reported in any study, a range of correlation coefficients (i.e., 0.7-0.9) was tried in calculating the SDs of change from pre to post trials. This range was based on test-retest correlations (i.e., 0.8-1.0) reported for strength measurements in the literature (98). However, these correlations were mostly derived from studies with short intervals between trials, as opposed to the studies in our meta-analysis that averaged about a year between trials. We rationalized that test-retest correlations would decrease as the time

between trials increased and therefore we tested a slightly lower range of correlation coefficients. The correlation coefficient used in all final calculations was 0.8.

When a longitudinal study had more than one time point, the standardized mean differences and variances for the midpoint, endpoint, and any points between the mid- and end-points were averaged across time points. Likewise, when a study measured strength in more than one muscle group, the standardized mean differences and standard errors for those muscle groups were averaged. Meta-analyses were run using a random-effects model that accounts for true variation in effects that vary from study to study and also for random error within each study. Examples of true variation among the 23 studies that could potentially affect study effect sizes are experimental factors including characteristics of the subjects, details of HT administration, and parameters related to muscle strength testing.

We next sought to determine the role of experimental factors in explaining the large inter-study variation observed in effect sizes. These experimental factors can be treated as moderator variables in a meta-analysis. Therefore, meta-regressions (conducted using the method of moments model) or meta-analyses comparing subsets of studies were used to probe the roles of the following factors: 1) the subjects' time since menopause (by meta-regression), 2) the subjects' time on HT (by meta-regression), 3) previous use of HT (no previous use vs. previous use), 4) study design (randomized controlled trial (RCT) experimental design vs. non-RCT design), 5) muscle group type (thumb adductors vs. forearm flexors vs. hip abductors vs. knee extensors vs. knee flexors), and 6) type of muscle contraction used in testing (isometric vs. isokinetic). Some studies measured strength of multiple muscle groups or tested strength both isometrically and

isokinetically. In those cases, data subsets from a study were treated as separate studies in the meta-analysis.

Meta-analyses and meta-regressions were conducted using the software Comprehensive Meta-Analysis Version 2.2 (Biostat, Englewood, NJ). An α-level of 0.05 was used in all analyses except when a moderator variable with more than two levels was being probed in a meta-analysis. In this situation, a Bonferroni correction was applied to the α-level to correct for multiple post-hoc comparisons. Effect sizes of 0.2, 0.5, and 0.8, were considered small, moderate, and large, respectively (21).

Meta-analyses of studies on rodents

Systematic review. It was difficult to explore some of the potential moderator variables among the studies on women, particularly those associated with the treatment of HT. For example, the type and amount of HT that women took was quite variable among studies and not controlled within many of the studies. To address this shortcoming, meta-analyses were performed using studies in which strength was measured in estrogen-replete versus estrogen-deficient rodents. In these studies, the type of HT, namely 17β-estradiol, was consistent and dosages were similar among studies. Also, although only a few of the studies on women measured size of the muscles that were tested for strength, the results of those studies suggest that the effect of HT on strength normalized to muscle cross-sectional area is important. We were able to further explore the effect of HT on normalized strength in the rodent studies because muscle size was measured in many of those.

A search for studies on the effects of estrogen on muscle strength in rodents was conducted using the following terms and strategy: 1) estradiol, 2) estrogen, 3) ovariectomy, 4) rodent, 5) mouse, 6) rat, 7) muscle, 8) skeletal muscle, 9) strength, 10) muscle function, 11) muscle force, 12) muscle strength, 13) muscle performance, and 14) (1 or 2 or 3) and (4 or 5 or 6) and (7 or 8 or 9 or 10 or 11 or 12 or 13). Searches were completed on PubMed, Cochrane Library, Biological Abstracts, Web of Science, and Digital Dissertation Database by two of the authors (SG, KB).

Inclusion and exclusion criteria of rodent studies. Studies meeting the following criteria were considered for review: 1) the rodents were young (i.e., no age-induced ovarian failure), 2) there was an estrogen-replete group (i.e., sham ovariectomy or ovariectomized followed by estradiol treatment) and an estrogen-deficient group (i.e., ovariectomized) and 3) skeletal muscle strength was determined *in situ*, *in vitro,* and/or *in vivo*. Studies on rodents were excluded for the following reasons: 1) all groups received voluntary or forced exercise, 2) all groups were hindlimb unweighted, 3) no strength data were presented (e.g., papers only provide data on fatigue), 4) estradiol replacement was mixed with another hormonal intervention such as androgen plus estrogen, with no pure estradiol group.

Selection of rodent studies. The search strategy utilized for the rodent studies found a total of 3,674 studies. Examination of the title and/or abstract led to the exclusion of 3,654 studies. The remaining 20 studies were thoroughly evaluated based on the full text. After careful examination, 10 studies were excluded leaving 10 studies that were used to complete the meta-analysis. Two authors (SG, KB) independently checked

studies for inclusion and exclusion criteria and came to a consensus regarding the final selection of studies to be included in the rodent analyses.

Data extraction from rodent studies. Extraction of absolute strength (force or torque) and strength normalized to muscle size were done as described for the studies on women. All data extraction was completed by one author (SG) and cross-checked by a second (KB). From the 10 selected studies, five corresponding authors were contacted for missing information. Of those, one was for an explanation of group size, one was for absolute muscle strength data because only normalized strength data were reported, one was for absolute and normalized data of a second muscle that was analyzed but not reported, and two were contacted to request data on normalized muscle strength. The rodent studies were not analyzed for quality because of the very similar experimental designs of the studies.

Meta-analysis of rodent studies. An effect size for each of the ten studies was calculated from extracted strength data as described for the studies on women. Meta-analyses were completed on effect sizes determined from absolute and normalized strength measurements of hindlimb muscles from rodents with circulating estradiol and those without. Meta-analyses probing the moderator variables of species type (rat vs. mice) and estradiol source (intact ovaries vs. estradiol supplementation) were also conducted.

Results

Meta-analyses of studies on post-menopausal women

Description of included studies. In total, 23 human studies published between 1987 and 2007 were included in the primary meta-analysis and characteristics of those studies are summarized in Table 1. The mean subject age for the individual studies ranged from 51 to 77 years. The mean time since menopause for subjects in the individual studies ranged from 0.5 to 30 years, with an average of ~12 years. Overall, there were 9,956 post-menopausal women included in the analysis, 7,288 in control groups and 2,668 in HT groups. The type of HT utilized in the 23 studies was not consistent. Women in 15 of the studies were permitted to take various preparations of HT. For the eight studies that stated a specific HT, seven different preparations were used. The most frequent dosage of HT was equivalent to ~0.6 mg of estrogen per day with the lowest being ~0.4 mg and the highest 4.3 mg per day. Nine studies did not provide any dosage information. There were eight studies that were longitudinal in design and the average duration of those studies was 12.2 months (Table 1). Six of the eight longitudinal studies stipulated no use of HT prior to the study while all cross-sectional studies permitted previous use. Typically, studies that permitted previous use of HT placed those women in the HT group (although six studies allowed previous users to be allocated to the control group if the individual had been off HT for a given period of time). The mean previous HT use for the 12 studies that allowed and reported a length of time for this use, was 110 months (~9 years; Table 1).

Table 1. Characteristics of the 23 studies on post-menopausal women.

Author, publication year[a]	Mean subject age[b] (years)	Mean time since menopause[d] (years)	Number of subjects (Control /HT)	Type of estrogen- based HT	HT dosage of estrogen component (mg/day)	Study design and duration (months)	Use of HT prior to strength measurement[e] (months)	PEDro scale[f]
Widrick et al., 2003 (114)	53	2	9/8	Various	~0.625	CS	24	5
Seeley et al., 1995 (89)	72	-	5616/1331	Various	~0.625	CS	178	5
Armstrong et al., 1996 (4)	61	13	54/54	Prempak-C ® or Premarin (Wyeth)	0.625	Long (11)	0	9
Bemben & Langdon, 2002 (11)	59	14	20/20	Various	~0.65	CS	136	5
Taaffe et al., 1995 (101)	69	21	48/37	Various	~0.625	CS	214	5
Bassey et al., 1996 (9)	51	-	91/14	Various	-	CS	-	5
Maddalozzo et al., 2004 (61)	51	1	59/67	Premarin ® (Wyeth)	0.625	Long (12)	16	4
Krintz-Silverstein et al., 1994 (50)	77	30	176/465	Various	-	CS	-	4
Maddalozzo et al., 2007 (62)	52	2	29/34	Premarin ® (Wyeth)	0.625	Long (12)	26	5
Baumgartner et al., 1999 (10)	76	-	132/48	Various	-	CS	-	5
Taaffe et al., 2005 (102)	74	-	581/259	Various	-	CS	214	5
Uusi-Rasi et al., 2003 (107)	62	11	35/42	Various	-	CS	127	5
Ribom et al., 2002 (83)	67	-	17/17	Menorest ® (Novartis)	4.3	Long (6)	0	9
Greeves et al., 1999 (31)	51	1-3[c]	10/11	Various	-	Long (9)	0	5
Preisinger et al., 1995 (82)	60	10	22/21	Various	-	CS	47	5
Cauley et al., 1987 (19)	57	9	255/55	Various	~0.37	CS	91	5
Heikkinen et al., 1997 (36)	53	0.5-3[c]	26/52	Divina ® or Divitren ® (Orion Pharma, Finland)	2.0	Long (24)	0	7
Sipila & Taaffe, 2001 & 2003* (94, 103)	50-57[c]	0.5-5[c]	17/17	Kilogest ® (Novo Nordisk, Denmark)	2.0	Long (12)	0	10
Carville et al., 2006 (18)	69	-	14/15	Various	-	CS	158	5
Onambele et al., 2006 (78)	63	16	8/14	Various	~0.3	CS	-	5
Brooke-Wavell et al., 2001 (13)	65	-	14/22	Estraderm TTS 50 ® (Ciba-Geigy, Switzerland)	2.0	CS	-	4
Skelton et al., 1999 (96)	61	10	50/44	Premak-C ® (Wyeth-Lederle)	0.625	Long (12)	0	7
Phillips et al., 1993 (81)	42-72[c]	5-17[c]	35/21	Various	-	CS	94	4

[a] Studies arranged from low to high effect size to correspond with Figure 1. [b] Mean age for all subjects in a study regardless of treatment group. [c] Range. [d] Mean time post-menopausal for all subjects regardless of treatment group. [e] Initial strength measurement for the longitudinal studies. [f] Quality assessment by the PEDro Scale (highest quality=11, lowest=1). * Combined study data for Sipila et al., 2001 and Taaffe et al., 2005. HT, hormone therapy; CS, cross-sectional; Long, longitudinal; -, information not provided

The quality of the studies ranged from 4 to 10 based on the PEDro scale (Table 1). Studies with the higher quality rankings, between 7 and 10, were those that encompassed a RCT design. Studies that had a cross-sectional design scored 4 or 5.

Meta-analysis results. A large variation was observed among studies in the effect of HT on strength, with effect sizes ranging from -0.56 to 1.25 (Figure 6). Only six of the 23 studies showed negative effects of HT on strength (i.e., the first 6 studies in Figure 1). Conversely, 17 of the 23 studies showed positive effects of HT on strength. Overall, the meta-analysis on the 23 human studies indicates a small, beneficial effect of HT on skeletal muscle strength that was statistically significant [overall effect size (ES)=0.23; P=0.003; Figure 6]. This effect size equates approximately to a 5% greater strength for the post-menopausal women on HT compared to those not on HT.

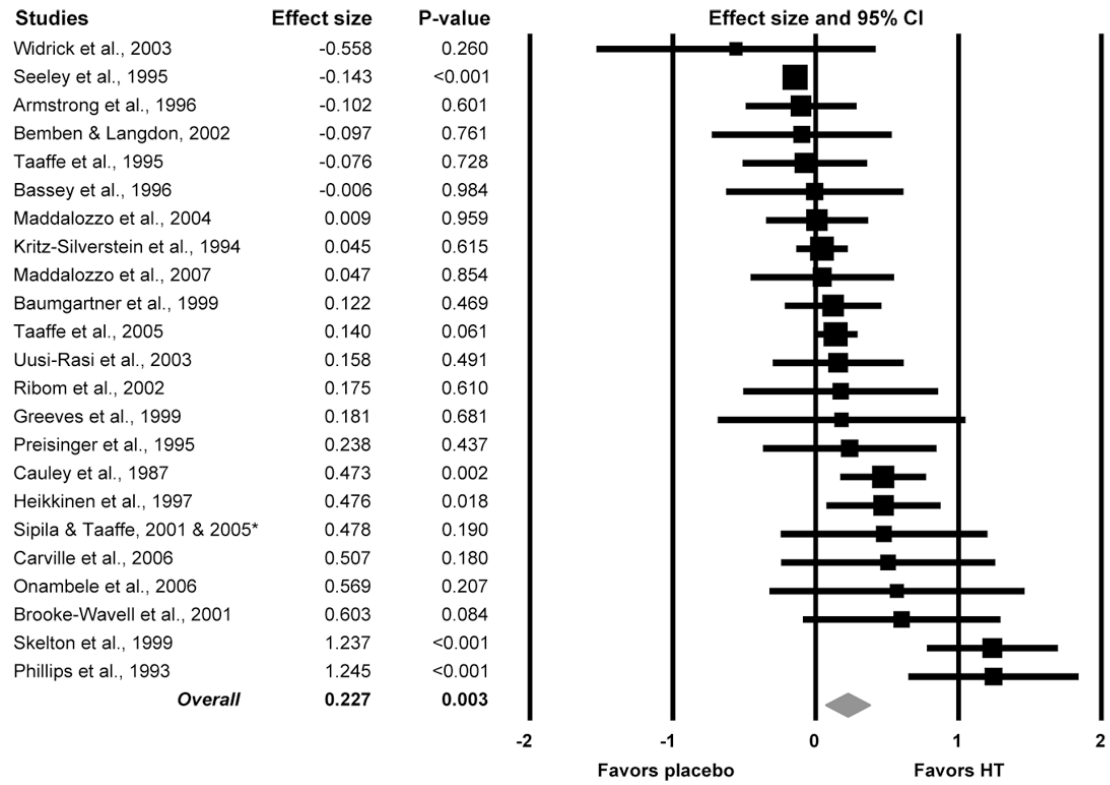

Figure 6. Forest plot of effect sizes from the 23 studies that assessed muscle strength in post-menopausal women who were and were not on HT. A square represents the effect size for a given study with the size of the square being proportional to the weighting of that study in the meta-analysis. A horizontal line indicates the effect size's 95% confidence interval (CI). Studies are arranged from lowest to highest effect sizes. The diamond at the bottom represents the overall effect size for HT on muscle strength. The width of the diamond represents the 95% CI for the overall effect size. * Combined study data for Sipila et al., 2001 and Taaffe et al., 2005.

Five of the 23 studies reported strength normalized to muscle size, which provides a measure of muscle quality. In those studies, muscle size was measured by computed tomography or an equivalent method. Imaging was conducted at the times of strength testing such that strength could be normalized to the cross-sectional area of the tested muscles. Results from those studies indicate that HT may have a moderate effect on normalized strength, but the effect size (0.45) was not statistically significant due to the low number of studies (Figure 7).

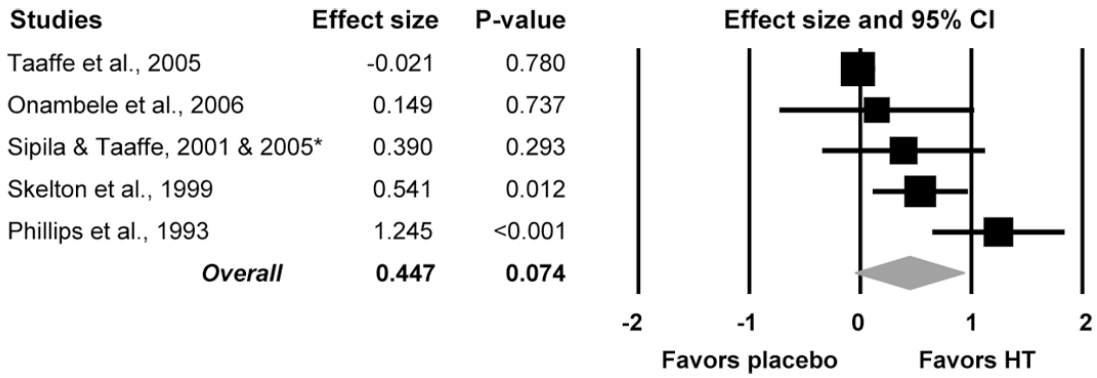

Figure 7. Forest plot of effect sizes from the meta-analysis conducted on the five studies that reported muscle strength normalized to muscle size in post-menopausal women who were and were not on HT. * Combined study data for Sipila et al., 2001 and Taaffe et al., 2005.

Analyses of potential moderator variables. In an attempt to explain the large variability observed in HT's effect on absolute strength among the 23 studies, experimental factors that varied among studies were explored as potential contributors to the variability. The factors that might affect muscle strength were grouped into three general categories, i.e., subject characteristics, HT administration, and muscle testing.

1. Subject characteristics. Twelve studies reported an average for the subject's mean time since menopause (see Table 1) and effect sizes calculated from strength data in those studies were used in a meta-regression. The relationship between mean time since menopause and a study's strength effect size was not significant (P=0.49). There was also a large range across the 23 studies for the length of time that subjects were on HT. This can be seen in Table 1 within the columns "Use of HT prior to strength measurement" and "Study duration" for the longitudinal studies. To determine if the subject's length of time on HT influenced the study's effect size, we conducted a meta-regression but did not find a significant relationship between these variables (P=0.23).

2. HT administration. Only nine of the 23 studies required women to take a specific preparation of HT, and even among those nine studies the type and dosage of HT varied. Therefore, it was not possible to assess many potential moderator variables related to the type of hormone treatment. There was sufficient information available however, to probe whether or not two aspects of HT treatment contributed to variability in effect sizes. First, an analysis of prior HT use was undertaken. Women in six of the studies were not permitted to have had prior HT, while the other 17 studies accepted women with prior or current use of HT. There was no statistical difference in effect sizes between studies that did and did not allow previous HT use, but a trend existed toward HT being more favorable when there was no prior use (P=0.12; Table 2).

Five of the 24 studies rigorously controlled the HT treatment. Characteristics of these studies were that: 1) HT and placebo treatments were randomly assigned to the women, 2) women who were in the HT group received a specific type and dosage of HT, 3) the length of time each subject received HT was controlled, and 4) women were not permitted to have had prior HT use. As such, each of these studies were a RCT which is considered the strongest type of clinical study design. The combined effect size of those five tightly-controlled studies was nearly triple that of the 18 studies that were less controlled (ES 0.46 vs. 0.16) though this difference was not quite statistically significant (P=0.10; Table 2).

Table 2. Summary of analyses of potential moderator variables that theoretically could influence the effect sizes of hormone therapy on strength in post-menopausal women.

Moderator variable	Comparison	Between-group P-value
Previous HT use	Yes (n=17; ES=0.161) No (n=6; ES=0.430)	0.124
Study design	Non-RCT studies (n=18; ES=0.161) RCT studies (n=5; ES=0.455)	0.104
Type of muscle contraction	Isokinetic (n=8; ES=0.077) Isometric (n=20; ES=0.265)	0.245

HT, estrogen-based hormone therapy; ES, effect size; RCT, randomized controlled trials

3. Muscle testing. There was a variety of muscle groups tested in the 23 studies. If a muscle group was tested in three or more studies, it was included in assessment of how the moderator variable, muscle group type, influenced variability of the effect sizes among studies. We found that studies measuring strength of the thumb adductor muscles reported a markedly higher effect size compared to studies assessing other muscle groups ($P<0.001$; Figure 8). The overall effect size in studies testing the thumb adductor muscles equates to a ~17% greater strength for women on HT versus those not on HT. There was a negative overall effect size for studies testing hip abductors and this effect size was significantly less than those for the forearm flexors and knee extensors ($P<0.001$; Figure 8).

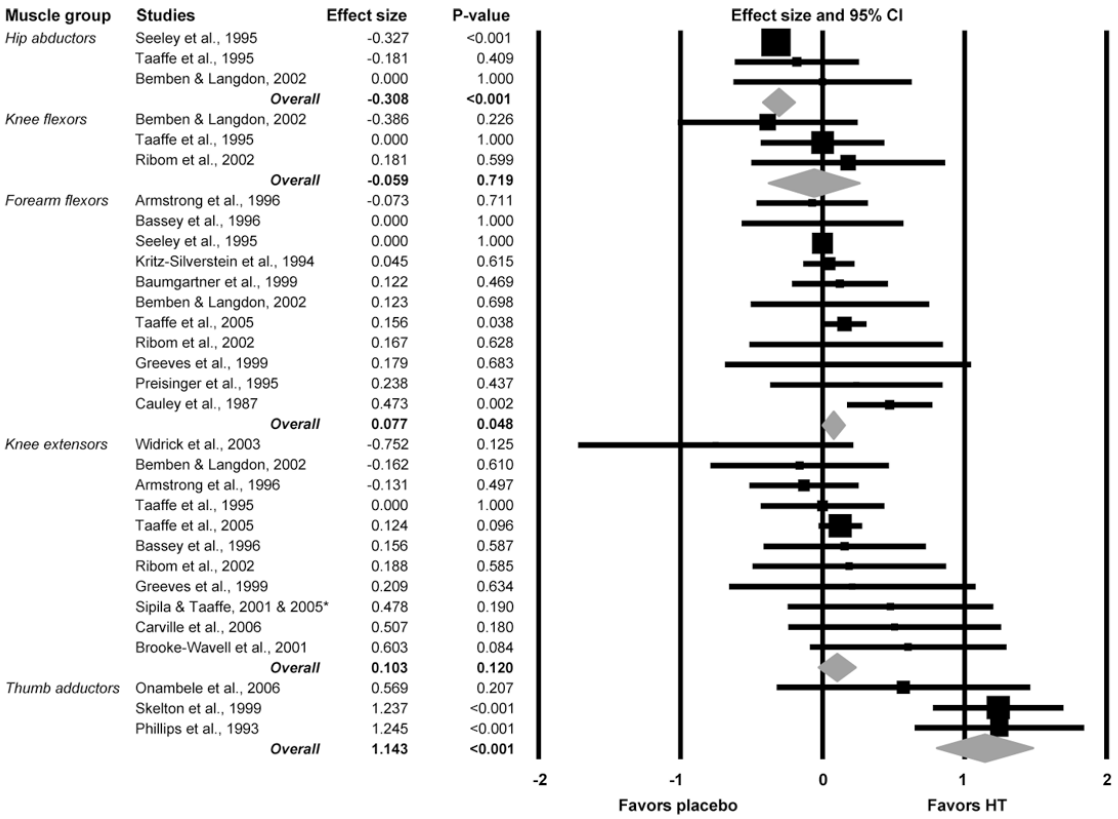

Figure 8 Forest plot depicting analysis of the moderator variable, muscle group type. Effect sizes were compared between studies that measured strength of five different muscle groups. A diamond represents the combined effect size for that level of the moderator variable. * Combined study data for Sipila et al., 2001 and Taaffe et al., 2005.

To determine if the type of muscle contraction used during strength assessments contributed to the variability in effect sizes observed among studies, studies that evaluated strength via isometric contractions were compared to those that used isokinetic contractions. There was no difference in effect size between these two groups of studies (P=0.25; Table 2).

Meta-analyses of studies on rodents

Description of included studies. Our literature search yielded 10 studies on rodents that investigated the effects of estradiol on muscle strength. Characteristics of those studies are presented in **Table 3**. All studies were of cross-sectional design and included Wistar or Sprague Dawley rats, or ICR or C57BL/6 mice. Surgical removal of the ovaries (ovariectomy, OVX) occurred between the ages of 3 wk and 7 mo. Thus, hormone manipulation occurred in animals that are considered to be young or young adult and as such, all rodents had similar hormone histories (e.g., they were virgin rodents and age-induced ovarian failure had not occurred). Data from a total of 250 rodents were included in the meta-analysis. Of that number, 91 were ovary-intact or sham-operated rodents (i.e., controls), 109 were estrogen deficient due to OVX, and 50 OVX rodents were replaced specifically with 17β-estradiol. Estradiol treatment was accomplished by either subcutaneous injections or time-release devices. Duration of estrogen deficiency or replacement ranged from 2.5 to 14 wk.

Table 3. Characteristics of the 10 studies on rodents.

Author, Publication year[a]	Species	Age at OVX	Number of animals (Intact/OVX/ OVX+estradiol)	Estradiol treatment	Study duration (wk)
Suzuki & Yamamuro, 1985 (100)	Wistar rats	3 wk	6/6/6	20 µg, 2x/wk, sc injection	7
McCormick et al., 2004 (64)	Sprague Dawley rats	7 wk	8/8/9	sc implant of silastic tube	4
Fisher et al., 1998 (26)	Sprague Dawley rats	6-7 mo	8/10/-	-	4
Wohlers et al., 2009 (116)	C57BL/6 mice	8 wk	5/5/-	-	8
Hubal et al., 2005 (41)	ICR mice	13 wk	12/9/-	-	8
Moran et al., 2007 (68)	C57BL/6 mice	4 mo	20/31/18	0.18 mg, 60-day release pellets	8
Schneider et al., 2004 (87)	C57BL/6 mice	< 8 wk	13/13/9	0.05 mg, 21-day release pellets	2.5
Warren et al., 1996 (110)	ICR mice	6 wk	-/8/8	40 µg/kg/day, sc injection	3
Moran et al., 2006 (69)	C57BL/6 mice	6 mo	10/13/-	-	8
Wattanapermpool & Reiser, 1999 (112)	Sprague Dawley rats	8-10 wk	3/3/-	-	14

[a] Studies are arranged corresponding to Figure 9. OVX, ovariectomy; sc, subcutaneous

Meta-analysis results. Absolute muscle strength was reported in nine of the 10 studies on rodents. It appears that circulating estradiol may have a moderate effect on absolute strength, but the effect did not quite reach statistical significance (ES= 0.44; P=0.12). These findings were inconclusive because of a significant difference in effect sizes between studies that used mice versus those that used rats (P<0.01). There was a

large effect of estradiol on absolute muscle strength in mice (ES=0.88), whereas there was no beneficial effect of estradiol on absolute strength in rats (Figure 9).

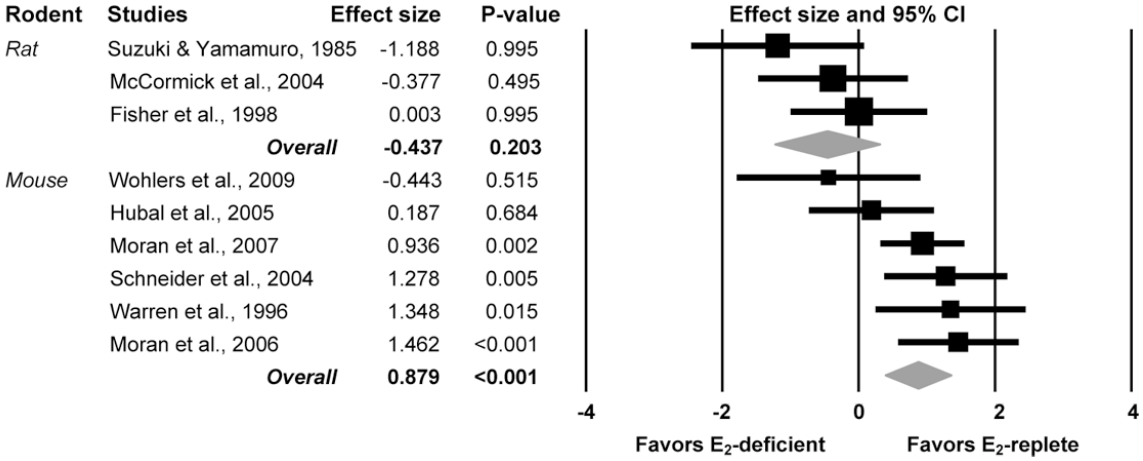

Figure 9. Forest plot depicting analysis of the moderator variable, species type, in the meta-analysis of estradiol's effect on absolute strength in rodents. E_2-deficient represents rodents that were ovariectomized. E_2-replete represents control rodents (e.g., sham-operated rodents) or rodents that were ovariectomized and treated with estradiol.

We also explored whether or not estradiol was the specific ovarian hormone imparting strength benefits. All nine studies that reported absolute strength data included a group of estrogen-deficient rodents via ovariectomy; within those studies the deficient rodents were compared to those with circulating estrogen either due to intact ovaries (n=8; ES= 0.38) or specifically from estradiol replacement following ovariectomy (n=5; ES=0.39). These resulting effect sizes were not different (P=0.99) indicating that estradiol is the key ovarian hormone.

Seven of the 10 studies reported muscle strength normalized to an indicator of muscle size (muscle mass, muscle cross-sectional, fiber cross-sectional area, or contractile protein content). Those data show that estradiol has a large, significant effect on normalized strength (ES=0.66; Figure 10). This effect equates to rodents with

circulating estradiol having 7% greater normalized strength than those that were depleted of estradiol. There was no difference in effect sizes between studies that assessed normalized strength in mice versus those in rats (P=0.60).

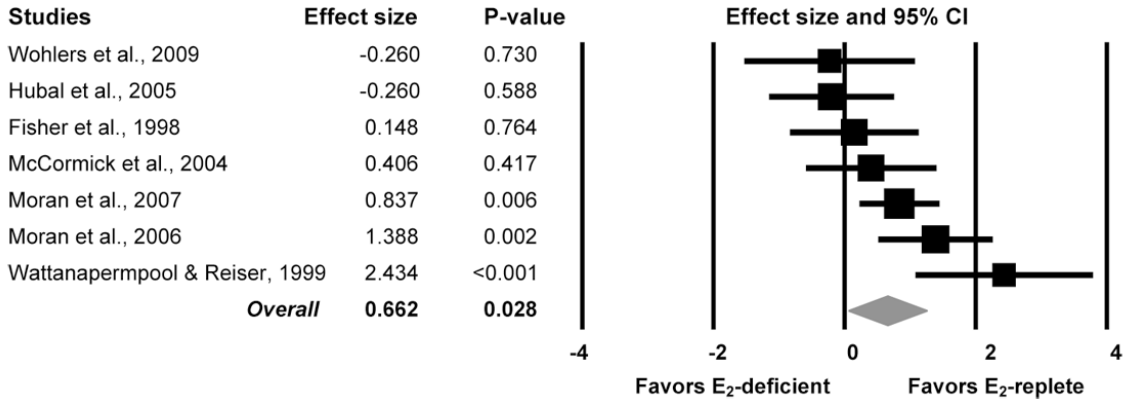

Figure 10. Forest plot of effect sizes from the meta-analysis conducted on the seven studies that reported muscle strength normalized to muscle size in rodents that were estradiol deficient and estradiol replete.

Discussion

The results of our meta-analyses show that estrogen-based HT is beneficial to skeletal muscle strength in post-menopausal women. Overall, we found 23 studies in which strength was compared under conditions of HT and non-HT in post-menopausal women. Statistical synthesis of the data from these studies revealed that women on HT were ~5% stronger than those not on HT, which is a small but statistically significant finding. This relatively small effect could be quite clinically meaningful given that women lose strength at the rate of ~1% per year after menopause and at a higher rate after the age of 70, and that these losses lead to frailty (15). Thus, for post-menopausal women who choose to be on HT it is possible that they may reduce the effects of sarcopenia and the loss of independence.

A potential limitation of a systematic review and meta-analysis is the possibility of publication bias (i.e., unpublished studies with nonsignificant findings and/or small or negative effect sizes). To address this we qualitatively examined a funnel plot of the 23 studies on post-menopausal women and found it to be symmetric. Quantitative confirmation was done by calculating Duval and Tweedie's trim and fill correction (22) which showed that the adjusted and unadjusted effect sizes were equal. Thus, it is very unlikely that publication bias influenced our findings. Another limitation of our meta-analysis is that the correlation between pre- and post-measurements was not reported in any study and that we were forced to estimate a value in order to calculate a standardized mean difference for each longitudinal study. We tested a range of correlations in order to determine the most appropriate one to use and found that effect sizes were altered minimally. For example, using a correlation of 0.7 resulted in an overall ES of 0.24

(P=0.003) while using a correlation of 0.9 resulted in an overall ES of 0.21 (P=0.005). Thus, in all our final analyses, a correlation of 0.8 was used.

Despite the overall result that HT was beneficial to strength of post-menopausal women, there was a large variability in effect sizes among the 23 individual studies. Study-to-study variability has also been noted in traditional narrative reviews on the effects of HT on muscle strength (66, 93). In an attempt to determine what might be contributing to this variability, we considered three categories of possible moderator variables. The first set of possible moderator variables that we examined was subject characteristics. This is important to consider because there is currently an effort to "individualize" HT prescription focusing on early treatment, preferably beginning in peri- or early-menopause (1, 23). HT treatment beyond five years is not considered appropriate due to increased risks of cardiovascular events and breast cancer (1). In many of the studies included in the main meta-analysis, women were beyond this ideal time point to start HT treatment and many had been treated well past the recommended five-year duration. If time since menopause and treatment duration are important for HT's effects on strength, we hypothesized that studies in which women started HT soon after menopause would have greater effect sizes. Likewise, we thought we would find greater effect sizes in studies where the duration of HT was not too long. These type of correlations have been speculated previously (53, 94), but our meta-regression analyses revealed no significant relationships that would support the contention.

A second set of possible moderator variables considered was related to administration of hormone treatment. Variables that we would have liked to have explored included type of estrogen in the HT, delivery mode of the HT, and dosage of the

estrogen component in the HT, but we were not able to explore these variables due to insufficient information. We were able to investigate whether or not previous use of HT influenced a study's effect size, but only found a trend for HT to have a more beneficial effect on strength when the women had no previous use. There was also a trend for larger effect sizes in studies that were randomized controlled trials, and thus ranked higher on the PEDro quality scale. In the five RCT studies, women tended to be younger, had no previous use of HT, received a given type and dosage of HT (or placebo), and took that specific HT for a given duration, typically one year.

The third set of possible moderator variables that we examined included aspects of the muscle strength testing. We found that the type of muscle contraction used, isometric or isokinetic, did not explain any of the variability of effect sizes among studies. However, the muscle group that was tested was important. HT had a small negative effect in studies testing hip abductor strength and a large positive effect on strength in studies testing the thumb adductors. The primary adductor of the thumb is the adductor pollicis muscle and it functions to adduct and extend the thumb, allowing for movements such as grasping and gripping. A distinguishing feature of this muscle compared to other muscle groups that were tested is its fiber type composition, which is ~80% type I fibers. The forearm flexors, hip abductors, knee extensors, and knee extensors are very mixed muscles, containing a more equal distribution of type I and II fibers (46). This suggests the possibility that HT might affect force production of type I fibers more than type II fibers. This is intriguing because muscle composed of type I fibers has been reported to have more α-estrogen receptor mRNA relative to muscle composed of type II fibers (54) and thus may be more responsive to estrogen. However, it

appears that this is not the only explanation. Rat soleus muscle is comprised predominately of type I muscle fibers (5), and the three studies assessing that muscle reported no beneficial effect sizes of estradiol on absolute strength. Additionally, the beneficial effects of estradiol on strength were equivalent in mouse EDL and soleus muscle (69), which are predominated by type II and type I fibers, respectively (17). Another distinguishing feature related to the studies of the adductor pollicis is that all three were conducted in the same laboratory using an apparatus specifically designed for precise measurements of this small muscle group. Therefore, in addition to possible physiological explanations for the differing effect sizes observed among studies testing different muscles, there exists the possibility that non-physiological factors (e.g., experimental procedures and instrumentation) may have also contributed to the effect size differences.

An interesting observation was the effect of HT on strength normalized to muscle size. Among the studies on women, HT tended to have a greater effect on strength when normalized to muscle size (ES=0.45), but the result was not significant (P=0.074). While only a small subset of the studies on post-menopausal women reported those data, several of the studies on rodents did. Those collective results show that rodents with circulating estradiol had an ~7% greater normalized strength than estradiol-deficient rodents (ES=0.66). Normalized muscle strength is an indication of muscle quality. Thus these results suggest that estrogens may influence muscle strength due to a qualitative effect, as opposed to a quantitative or muscle size effect.

A second important finding from the analyses of rodent studies was that it appears that mice mimic women in their response to estrogen more so than do rats. This result

should influence one's choice of a rodent model in future studies of estrogen's effect on skeletal muscle. Finally, the combined results of the rodent studies confirm that the specific ovarian hormone important for strength is estradiol.

A limitation in translating the results from the rodent studies to the studies on post-menopausal women is that in all rodent studies, the animals were young adults. The ovariectomy model is advantageous for specifically investigating ovarian hormones independent of age, but falls short in that hormonal affects on muscle in young rodents and women may be different than those that occur in aged rodents and women who have traversed a natural failure of the ovaries and estrogen production. We are not aware of any published research reporting the effects of HT or estradiol on muscle strength in aged rodents.

In conclusion, the results of our meta-analysis which pooled data from 23 studies show that when estrogen-based HT is given to post-menopausal women there is a small, beneficial effect on muscle strength. This finding is supported by analyses of rodent studies in which estradiol was found to beneficially affect normalized muscle strength. It should be noted that the increases in strength with HT are modest (~5%), especially when compared to other means for improving strength, e.g., resistance exercise training, where increases in strength can be at least 8-14% in one year (62, 103). Our intent is not to suggest that HT be prescribed for age-related sarcopenia, but that skeletal muscle should be recognized clinically as a target tissue when HT is prescribed for other reasons. For example, if HT can benefit muscle in addition to bone then this argues more for considering the use of HT despite its potential adverse effects of cancer and cardiovascular events. Additionally, future research should further investigate the

mechanisms of estrogen's actions on muscle as this may lead to new strategies for combating sarcopenia, particularly in the menopausal woman.

Chapter 3

Estradiol's beneficial effect on murine muscle function is independent of muscle activity

Knowing that estrogen-based HT has a beneficial affect on muscle strength in post-menopausal women and confirming this in rodent models I went on to examine the relationship of E_2, muscle, and physical activity. I found E_2 beneficially affects normalized muscle strength in rodents, however estrogens are also affecting physical activity. My next question was to address physical activity in E_2-deficinet mice, and then to control activity to examine this relationship. The aim was to determine if E_2 is beneficial to muscle contractility and myosin function independent of an changes in physical activity.

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Estradiol (E_2) deficiency decreases both muscle strength and wheel running in female mice. It is not known if the muscle weakness results directly from the loss of E_2 or indirectly from mice becoming relatively inactive with presumably diminished muscle activity. The first aim of this study was to determine if cage activities of ovariectomized mice with and without E_2 treatment differ. Ovariectomized mice were 19-46% less active than E_2–replaced mice in terms of ambulation, jumping, and time spent being active ($P \leq 0.033$). After finding that E_2-deficient mice have low cage activities, the second aim was to determine if E_2 is beneficial to muscle contractility, independent of physical activities by the mouse or its hindlimb muscles. Adult, female mice were ovariectomized or sham operated and randomized to receive E_2 or placebo and then placed in conditions that should maintain physical and muscle activity at a constant low level. Following two weeks of hindlimb suspension or unilateral tibial nerve transection, muscle contractile function was assessed. Soleus muscles of hindlimb-suspended mice that were ovariectomized generated 31% lower normalized (relative to muscle contractile protein content) maximal isometric force than suspended mice with intact ovaries ($P \leq 0.049$). Irrespective of whether the soleus muscle was innervated or not, muscles from ovariectomized mice generated ~20% lower absolute and normalized maximal isometric forces, as well as power, relative to E_2-replaced mice ($P \leq 0.004$). In conclusion, E_2 affects muscle force generation even when muscle activity is equalized.

Introduction

17 β-estradiol (E_2) is implicated as a factor influencing muscle strength. With the loss of E_2, via removal of ovarian tissue, there is a noted decrease in the force-generating capacity of hindlimb muscles in mice (69). Following replacement of E_2, this decrement in strength fully recovers showing that E_2 is a key hormone affecting skeletal muscle function and quality (68). Those findings are substantiated by the results of a systematic review and meta-analysis reporting an overall ~7% greater normalized muscle strength, or muscle quality, in E_2-replaced than ovariectomized rodents (33). Similarly, a meta-analysis on data from nearly 10,000 postmenopausal women showed that those taking an estrogen-based hormone therapy were stronger than those not taking the treatment (33). Collectively, these results provide evidence that E_2 is beneficial in enhancing muscle force production. There are several hypothesized mechanisms by which the loss of E_2 detrimentally affects skeletal muscle, including a decrease in the fraction of strongly-bound myosin during contraction (69), inhibition of Ca^{2+}-uptake at the sarcoplasmic reticulum (112), an increase in muscle damage due to a destabilized muscle membrane (7, 105), and oxidative damage (80). It is possible that any or all of these proposed mechanisms may contribute to the force-generating decrements in muscle when E_2 is diminished.

It is also conceivable that any or all of the effects of E_2 on muscle are not direct effects, but rather are secondary to an estrogenic effect on some non-skeletal muscle tissue or cell. A notable systemic effect of E_2 in rodents is its strong influence on physical activity, particularly voluntary wheel running (30, 48, 71, 77, 111). For example, ovariectomized mice ran ~80% less compared with ovarian-intact mice, but within three

days of E_2 replacement the mice rebounded to a running level equivalent to that of ovarian-intact mice (30). Similarly, ovariectomized mice treated with tamoxifen, a selective estrogen receptor modulator, ran distances comparable to those of ovariectomized mice replaced with E_2 (30). To what extent circulating E_2 levels influence physical activities other than voluntary wheel running, such as typical activities in a cage, is not completely known. There is limited evidence showing reduced ambulatory activity in the dark by ovariectomized compared to ovarian-intact mice, with no difference during the photoperiod (85). Cage activities are important to consider because it is possible that E_2-deficient mice become less active in their cages and that reduced rearing, jumping, ambulating, etc. causes all or part of the ovariectomy-induced muscle weakness. Thus, the first aim of the present work was to determine if E_2 deficiency in female mice adversely affects normal cage activity.

Upon finding that cage activities were reduced in ovariectomized mice, we sought to address the second aim of this work, which was to determine if E_2 is beneficial to muscle force generation, fatigue resistance, and power independent of physical and muscle activities. We used hindlimb suspension and unilateral tibial nerve transection to control muscle loading and activity, respectively, at constant low levels in both ovariectomized mice with and without E_2 replacement. We hypothesized that under the condition of equalized activity and loading, E_2 deficiency would still cause detrimental effects on muscle contractile function.

Methods

Animals. Female C57BL/6J mice, 3-5 months of age, were purchased from Jackson Laboratories (Bar Harbor, ME). This age range was selected because regular estrous cycling is known to occur between 3 and 14 months of age and we have reported muscle functional capacity to be at a constant level between 4 and 8 months of age for female mice of this strain (70, 76). Upon arrival, mice were given 1-3 weeks to acclimate to the facility with free access to water and a phytoestrogen-free diet (2019 Teklad Global 19% Protein Rodent Diet, Harland Teklad, Madison, WI). Mice were group housed for Study I and housed individually for Studies II and III. At the end of each study, mice were weighed and anesthetized by an intraperitoneal injection of sodium pentobarbital (100 mg/kg body weight) with supplemental doses given as required. While under anesthesia, mice were euthanized by exsanguination. All protocols were approved by the Institutional Animal Care and Use Committee at the University of Minnesota and complied with the American Physiological Society guidelines.

Design of Study I: Daily cage activity of ovariectomized mice with and without E_2 treatment. At ~14 weeks of age, mice underwent ovariectomy and then were randomized into one of two groups, immediate treatment with a slow-release E_2-containing pellet (OVX+E_2; n=8) or placebo pellet (OVX; n=8). Cage activities of each mouse were measured 50-59 days after the ovariectomy procedure.

Design of Study II: Control of soleus muscle loading by hindlimb suspension. At 21 weeks of age, mice were randomized into one of three groups; surgical removal of

ovarian tissue and immediate treatment with E_2 (OVX+E_2; n=5) or placebo pellet (OVX; n=6), or a sham operation (sham; n=6). Following 3-5 days of recovery from surgery, all mice were hindlimb suspended as described previously (109). Briefly, mice were restrained and harnesses were attached to their tails. The harness was then attached to the cage top so that the hindlimbs could not touch the cage bedding. Mice remained suspended for two weeks such that the hindlimb muscles were unloaded for all mice.

At the end of the two-week suspension period, the soleus muscles from both limbs and the extensor digitorum longus (EDL) muscle from one limb were analyzed *in vitro* for contractility. The soleus muscle is affected by hindlimb suspension as indicated by amass loss of ~40% compared to weigh-bearing mice (109).The EDL muscle is less affected by hindlimb suspension but was studied because previous work showed contractile functions of both of these muscles are affected by E_2. In addition to contractile measurements (described in detail in a following section), a fatiguing contraction protocol was conducted on the soleus muscles. This was done because there are noted effects of sex on fatigue (38), with most hypotheses linking this difference to sex hormones. Following the *in vitro* contractility analyses, muscles were weighed, and then snap frozen in liquid nitrogen, and stored at -80 ˚C.

Design of Study III: Control of soleus muscle activity by denervation. At 14 weeks of age, all mice in this study underwent ovariectomy, tibial nerve transection, and simultaneously received a pellet, either E_2 (OVX+E_2; n=10)or placebo (OVX; n=10). Following the two weeks of soleus muscle denervation and E_2 manipulation, each mouse was anesthetized. Soleus muscles from both limbs were analyzed *in vitro* for contractile

function. In addition, a protocol assessing the force-velocity relationship was conducted. This protocol was selected because of previous reports on E_2 affecting V_{max} (69) and myofibrillar ATPase (113). EDL muscles were not assessed in this study because Study II showed that two weeks of hormone manipulation was not long enough to significantly affect contractility of that muscle. Soleus muscles were weighed, snap frozen in liquid nitrogen, and stored at -80 ˚C immediately following the *in vitro* contractile analyses.

E_2 manipulation and related assessments common to all studies. In all three studies, ovariectomy and sham operations were conducted as previously described (69). For the OVX procedure, mice were initially anesthetized in an induction chamber using isoflurane and then maintained by mask using inhalation of 1.75% isoflurane mixed with oxygen at a flow rate of 200 ml/min. Sham-operated mice underwent the same protocol with no removal of ovarian tissue. While still under anesthesia, ovariectomized mice were implanted with 0.18-mg, 60-day slow-release E_2 or placebo pellets (Innovative Research of America, Sarasota, FL) at the base of the neck. All mice were administered 0.15 μg of buprenorphine subcutaneously ~5 minutes after end of the surgery.

Blood was collected by facial vein bleed prior to any surgical procedures and/or during exsanguination at the end of a study. Plasma was separated and stored at 4 ^{o}C for analysis of circulating plasma E_2. Circulating plasma E_2 levels for mice in Study I were measured by ELISA (RE50241, Immuno Biological Laboratories (IBL); Minneapolis, MN). A diethylether extraction procedure as directed by IBL preceded the ELISA to concentrate the mouse plasma. The assay was then performed following manufacturer specifications. For Studies II and III, an estradiol ELISA kit (KA0234, Abnova

Corporation, Taiwan) was used following manufacturer specifications. Data for the standards were fit using a 4-parameter logistic curve fit. It was necessary to change to the Abnova estradiol assay because the IBL kit used in Study I had been discontinued. Circulating E_2 levels were measured as a marker of successful ovarian-hormone depletion and replacement. The uteri were dissected and weighed from each mouse in Studies II and III as a secondary marker of E_2 status.

Physical activity assessment. Cage activity of individual mice in Study I was monitored for 24 hours using open-field activity chambers (Med Associates Inc., St. Albans, VT) (52). Photo beam arrays in the chamber register an activity count and duration each time one or more of the beams is disrupted. It is important to recognize that an "activity count" represents any single photo beam break. Because multiple photo beams may break with one jump, the total jump count does not represent the actual number of times a mouse jumps. Activity data were acquired using Activity Monitor version 5 software (Med Associates Inc.) with a box size set to "3" (4.8 cm^2); this box size was established in order to calculate movement, delay, and to filter the data such that stereotypic movements are not counted as ambulation. Stereotypic movement is considered movement contained within the 4.8 cm^2 box, for example while the mouse is grooming or eating. Immediately prior to measuring activity, each mouse was familiarized to the activity-monitoring cage by spending 24 hours in a mock chamber.

Tibial nerve transection. Immediately following the pellet implantation, while still under isoflurane anesthesia, all mice in Study III underwent transection of the left

tibial nerve. The mouse was positioned in right lateral recumbency and the left foot was stabilized for the duration of the surgery. Under aseptic conditions, an ~15-mm incision was made on the skin parallel and slightly posterior to the femur. Then, an incision was made in the biceps femoris muscle, in line with the muscle fiber orientation, directly under the skin incision. The flaps of biceps femoris muscle were retracted, exposing the common peroneal and tibial nerves. Care was taken to dissect the tibial nerve from surrounding musculature, connective tissue, and fat. A 7-0 sterile silk suture was used to make a knot on the most distal portion of the tibial nerve. The nerve was cut just distal to the knot and then retracted. The nerve was cut as proximal as possible such that a ≥3-mm segment of nerve was removed. The biceps femoris muscle flaps were sutured together and the skin incision closed, both using 6-0 silk suture. The left tibial nerve of all mice was cut and removed, while the right limb remained intact and served as the intra-animal control.

In vitro assessment of skeletal muscle contractility. Procedures used for isolation and testing of soleus and EDL muscles have been described previously (70, 109). Contractile characteristics that were measured in all studies included maximal isometric tetanic force (P_o) and active stiffness. Active stiffness is an indirect estimate of myosin strongly bound to actin during contraction (29, 88). Muscle length was measured from proximal to distal myotendinous junction using digital calipers after being set to their anatomic resting muscle length (L_o). Fiber lengths were calculated as 71 and 44% of the measured muscle length for the soleus and EDL muscles, respectively (14, 65). Normalized P_o was calculated as P_o divided by the ratio of contractile protein content

(sum of myosin heavy chain and actin contents) to fiber length. P_o was adjusted for muscle size using contractile protein content rather than physiological cross-sectional area because OVX causes an increase in non-protein mass that inflates cross-sectional area (69).

In Study II, soleus muscles underwent a protocol employing a bout of fatiguing contractions that was initiated two minutes following the force and stiffness measurements (35). Muscles were subjected to 1-second tetanic contractions, at 150 V and 40 Hz, at a rate of 12 tetani per minute for 5 minutes, for a total of 60 contractions. For the 20 min following this test protocol, maximal tetanic contractions were done at 5-minute intervals to assess the extent of recovery. Because soleus muscles were atrophied from the two-week suspension and required delicate dissection, muscles from both limbs of each mouse were tested. The soleus muscle with the higher P_o was used in all statistical analyses (the overall results were not different if both muscles were averaged together).

In Study III, soleus muscles completed a protocol assessing the force-velocity relationship by measuring 12 shortening velocities using quick releases from P_o to given afterloads that corresponded to 5-50% of P_o (69). The muscle's maximal shortening velocity (V_{max}) and maximal power were determined by fitting the data as a hyperbolic-linear curve using Table Curve 2D (version 5.0), Systat Software, Richmond, CA) (14, 20, 47). Maximal power was normalized by the ratio of contractile protein content to fiber length, in parallel to normalized P_o.

Determination of contractile protein content. Soleus and EDL muscles that were tested *in vitro* for contractility were subsequently homogenized and electrophoresed on SDS-PAGE gels to determine contents of actin and myosin heavy chain (69).

Statistical analysis. Independent Student t-tests were used to determine if differences in: 1) cage activities existed between ovariectomized mice with and without E_2 treatment in Study I and 2) plasma E_2 and uterine masses between mice with and without E_2 in Studies II and III. Body masses were analyzed using two-way repeated measures ANOVA (treatment x time) for all studies. Muscle contractile parameters were evaluated using independent Student t-tests in Study II and by two-way repeated measures ANOVAs (limb x treatment) in Study III. Pearson correlations were used to determine if cage activities were associated with plasma E_2 levels. Effect sizes for correlations of cage activity data were calculated by converting the correlation to the Fisher's *z* scale (12). All statistical analyses were done using SigmaStat version 3.5 (Systat Software Inc; Point Richmond, CA). Significance was accepted with an α level of 0.05. Values are reported as means ± SD.

Results

Effects of E_2 manipulation common to all studies

Body mass of rodents may be affected by ovarian hormone status. At the end of the ~60-day hormone manipulation in Study I, OVX mice had 13% greater body mass than OVX+E_2 mice (Table 4). In Study II, OVX mice gained more body mass during the two-week intervention than did the sham mice (19.4% ± 3.7 vs. 11.2% ± 3.1 gain; Table 1, P<0.001). The OVX+E_2 mice in Study II lost body mass during the first 2-5 days of treatment. In Study III, mice gained ~14% body mass over the two-week study (Table 4).

Table 4. Body masses before and following 60 (Study I) or 14 (Studies II and III) days of hormone manipulation.

	Pre (g)	**Post (g)**	**Statistical findings**
Study I			
OVX+E_2 (n=8)	20.2 (1.9)	21.9 (1.3) *	Interaction between estradiol and time; P=0.004
OVX (n=8)	19.3 (1.0)	24.7 (1.1) *†	
Study II			
Sham (n=6)	19.7 (0.8)	21.6 (1.3) †	
OVX (n=6)	19.8 (0.9)	24.5 (0.9) *†	Interaction between estradiol and time; P=0.007
OVX+E_2 (n=5)	19.3 (1.1)	17.4 (0.6) $^{*\#}$	
Study III			
OVX+E_2 (n=10)	19.7 (1.3)	22.5 (0.9)	Main effect of time; P<0.001
OVX (n=10)	19.6 (0.9)	22.4 (1.1)	

Mean (SD). Data within each study were analyzed by a two-way repeated measures ANOVA (estradiol by time, with time being the repeated factor). *Significantly different than pre within group. †Significantly different than OVX+E_2 group at same time point. $^{\#}$This post measurement was made 2-5 days following E_2 treatment.

The effectiveness of ovariectomy and E_2 replacement was evaluated by measuring plasma E_2 levels and uterine mass. The mean plasma E_2 levels of OVX and OVX+E_2 mice in Study I were 13.8 ± 3.1 pg/ml and 38.9 ± 7.4 pg/ml at the end of the 60-day study ($P \leq 0.001$). Prior to randomization into surgical procedures in Study II, circulating plasma E_2 of all mice averaged 25.1 ± 12.8 pg/ml. At sacrifice, sham mice had circulating levels of 30.7 ± 13.1 pg/ml while 4 out of 6 of the OVX mice had plasma levels below the detection level of the assay, i.e., < 10 pg/ml. The average of the other 2 OVX mice was 18.5 ± 5.4 pg/ml. The uterus atrophies with estrogen-deprivation (67) and indeed there was a substantial difference between OVX and sham mice in Study II (24.7 ± 8.3 vs. 71.5 ± 25.9 mg, respectively; P=0.002), further confirming successful reduction of estrogens. Following two weeks of denervation and E_2 manipulation in Study III, OVX+E_2 mice had circulating E_2 levels averaging 220.0 ± 116.8 pg/mL, while 7 out of 10 of the OVX mice were below the detection level of the assay. The average of the other 3 OVX mice was 23.0 ± 10.2 pg/ml. Uterine mass, averaged 25.5 ± 3.4 and 148.1 ± 21.7 mg for the OVX and OVX+E_2 mice, respectively ($P<0.001$).

Study I: Daily cage activity of ovariectomized mice with and without E_2 treatment

To test the hypothesis that mouse physical activity was diminished with E_2-deficiency, several parameters of daily cage activities were measured in OVX mice treated with placebo or E_2 (Figure 11). During a 24-hr period, the OVX mice spent 19% less time being active than did the OVX+E_2 mice (P=0.020). This percentage equates to a difference of 60 minutes of physical activity per day, 263 ± 45 vs. 323 ± 46 min for OVX and OVX+E_2 mice, respectively. Ambulation, or distance traveled over 24 hrs, was

~30% less in OVX compared to OVX+E_2 mice (426 ± 65 vs. 580 ± 171 m, respectively; P=0.033). OVX mice did about 20% fewer stereotypic movements(41964 ± 3054 vs. 52019 ± 11096 counts), i.e., these mice had fewer beam breaks while grooming or eating (P=0.027), furthermore OVX mice jumped 46% less than OVX+E_2 mice (1757 ± 469 vs. 3226 ± 1624 counts, respectively; P=0.028). Additionally, OVX mice tended to rear, or go onto their hind limbs, less than OVX+E_2 mice (3634 ± 538 vs. 5677 ± 933 counts, respectively; P=0.072).

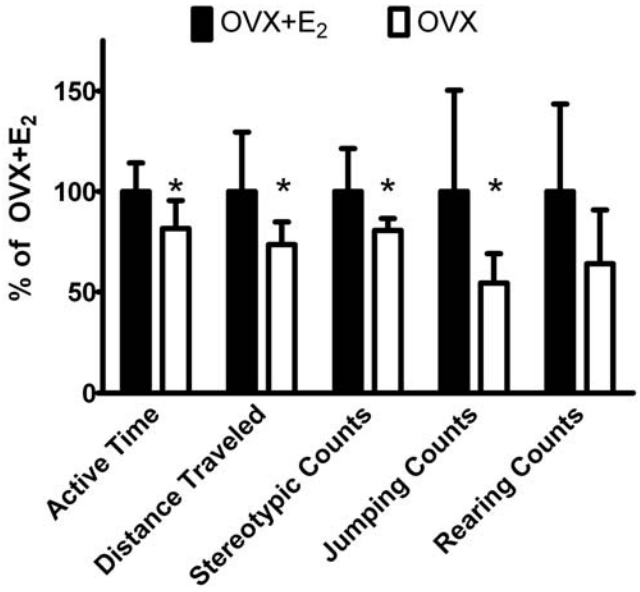

Figure 11. Daily cage activities of ovariectomized mice that received a placebo treatment (OVX) relative to ovariectomized mice that received 17β-estradiol treatment (OVX+E_2). All data were collected ~55 days post-ovariectomy surgery (means, SD). "Count" is a measure of one single photo beam break and stereotypic counts are movements while the mouse is contained within the computer generated box. N=8 per group. *Significantly different than OVX+E_2

Correlational analyses were performed to determine if cage activities were associated with plasma E_2 levels. Active time was correlated with plasma E_2 levels (r=0.503; P=0.047). Ambulation, jumping, rearing and stereotypic activity were not

significantly correlated with plasma E_2 (P=0.072 to 0.188), although trends existed for mice with lower E_2 levels to be less active. Effect sizes for correlations were calculated and found to be "large" (21). For example, effects sizes for correlations between plasma E_2 levels and ambulation distance, rearing, and jumping were 1.04 to 1.16.

Study II: Control of soleus muscle loading by hindlimb suspension

In attempt to equalize the use and loading of the soleus and EDL muscles in mice with and without normal levels of circulating E_2, all mice in this study were hindlimb suspended. The 5 OVX+E_2 mice had to be euthanized 2-5 days following hindlimb suspension. Once suspended, these mice ate ~70% less chow than those that received a placebo pellet or were sham operated; these mice lost ~13% of their body mass during those 2-5 days (Table 4). Since all mice underwent comparable anesthesia, surgeries, and hindlimb suspension, stresses caused by these individual interventions were likely not responsible; the E_2 treatment was the differentiating intervention and when combined with hindlimb suspension may have placed excessive stress on the animals. Nonetheless, to move forward with the question of ovarian hormones' direct versus indirect effects on skeletal muscle, the sham and OVX groups were compared. The caveat of this study design is that any difference found between sham and OVX mice could not be attributed solely to E_2 because ovariectomy results in more than the loss of this single ovarian hormone.

Two weeks of ovarian hormone deficiency in hindlimb suspension mice resulted in a modest decrement in soleus muscle contractility relative to suspended mice with normal levels of ovarian hormones (Figure 12). Absolute P_o for soleus muscle was not

affected by ovarian hormone status (83.6 ± 13.5 vs. 84.8 ± 18.6 mN for sham and OVX mice, respectively; P=0.907; Figure 12). Normalized P_o of soleus muscle in OVX mice was ~30% lower than that in sham mice (0.178 ± 0.05 vs. 0.268 ± 0.08 N·cm/mg, respectively; P=0.049; Figure 12). Two weeks of ovarian hormone manipulation did not result in significant effects on EDL muscle contractility (Figure 12).

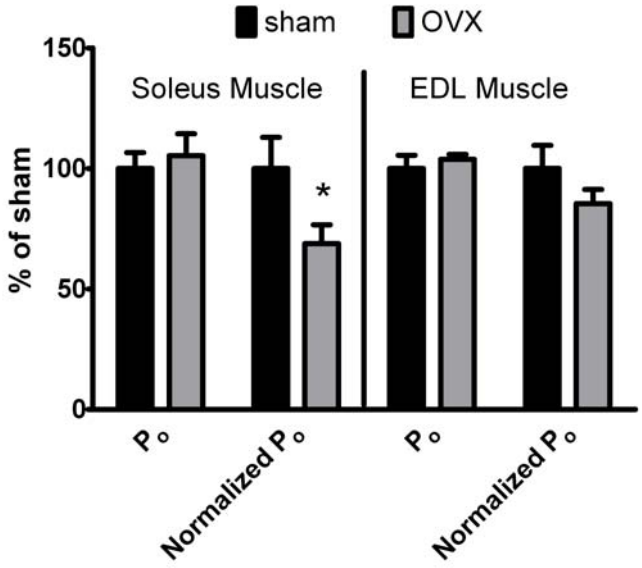

Figure 12. Force-generating capacities of soleus and EDL muscles of sham and OVX, i.e., control and ovarian hormone-deficient mice, respectively, following two weeks of hindlimb unloading (means, SD). Normalized P_o is P_o divided by the ratio of contractile protein content to fiber length. N=6 per group. * Significantly different than sham.

Absolute and normalized P_o of EDL muscles were not different between sham and OVX mice (294.5 ± 39.5 vs. 301.8 ± 16.3 mN and 0.210 ± 0.05 vs. 0.175 ± 0.02 N·cm/mg, respectively; P≥0.168). Ovarian hormone status did not affect active stiffness, a marker of myosin-actin strong-binding, of either soleus or EDL muscles (P≥0.197). Active stiffness was 229.8 ± 24.6 vs. 194.8 ± 51.3 N/m for the soleus muscle and 393.2 ± 53.2 vs. 371.1 ± 33.2 N/m for the EDL muscle, respectively, for the sham and OVX mice.

There was no indication that fatigue of the soleus muscle was affected by ovarian hormone status either. Soleus muscles from both groups lost ~30% of their force-generating capacity from the first to the sixtieth contraction of the protocol (P=0.947; Figure 13). Fatigue as opposed to injury was confirmed because 20 min following the sixtieth fatiguing contraction, P_o was not different than that measured prior to the fatigue protocol (P=0.183; Figure 13).

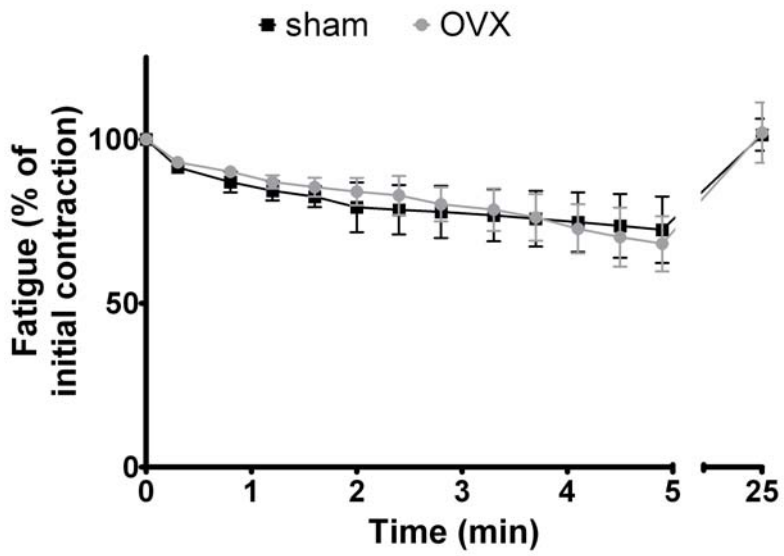

Figure 13. Force decrements and recovery from a fatiguing bout of contractions performed by soleus muscles from sham and OVX mice following two weeks of ovarian hormone manipulation and hindlimb unloading (means, SD). Fatigue is calculated as the percentage of force relative to that of the 1st contraction of the protocol. Plotted are the relative forces of every fifth contraction during the 60-contraction fatiguing protocol and a recovery index 20 min following the protocol. N=5 per group.

The mass of soleus muscles was ~17% greater in OVX mice than those of sham mice (Table 5). There were no differences in soleus muscle length or total or contractile protein contents between sham and OVX mice (Table 5), indicating that the OVX-induced increase in mass was due to fluid accumulation. The size and composition of EDL muscles were not significantly different between OVX and sham mice (Table 5).

Table 5. Composition of hindlimb suspended mice that were either sham operated or ovariectomized for 14 days.

	sham	***OVX***	**P-value**
Soleus muscle (n)	6	6	
Muscle mass (mg)	4.0 (0.5)	4.7 (0.5)	0.046
Muscle length (mm)	10.0 (0.6)	9.6 (0.9)	0.388
Total protein (mg/mg)	0.106 (0.02)	0.109 (0.01)	0.625
Contractile protein (mg)	0.24 (0.09)	0.33 (0.08)	0.111
EDL muscle (n)	6	6	
Muscle mass (mg)	8.1 (0.8)	8.7 (0.2)	0.112
Fiber Length (mm)	12.0 (0.5)	12.16 (0.6)	0.665
Total protein (mg/mg)	0.130 (0.01)	0.136 (0.01)	0.422
Contractile protein (mg)	0.77 (0.15)	0.94 (0.21)	0.130

Mean (SD). Data analyzed by Student's t-test.

Study III: Control of soleus muscle activity by denervation

All mice in this study underwent unilateral tibial nerve transection to equalize the use of the soleus muscle, irrespective of the physical activity level of the mouse. All mice also were ovariectomized with half of the mice receiving E_2 replacement. This allowed for the direct investigation of E_2 on skeletal muscle.

Soleus muscles from ovariectomized mice had low capacities to generate force relative to those from mice that were replaced with E_2, regardless of whether or not the muscle was innervated (i.e., no interaction between E_2 status and innervation for all variables with the exception of normalized power; Table 6). Specifically, soleus muscles of OVX mice generated less absolute force than those of OVX+E_2 mice (Figure 14A, left and Table 6). More importantly, P_o normalized by contractile protein content was 23% less for OVX

than OVX+E_2 mice (Figure 14A, right and Table 6), substantiating that E_2 status affects force generation independent of muscle activity. There was a main effect of innervation status on absolute P_o of soleus muscle , which is explained by the low contractile protein content because there was no effect of denervation on normalized P_o (Table 6). Active stiffness, indicative of strong-binding myosin to actin, was also 21% lower in OVX than OVX+E_2 mice, irrespective of innervation (Table 6).

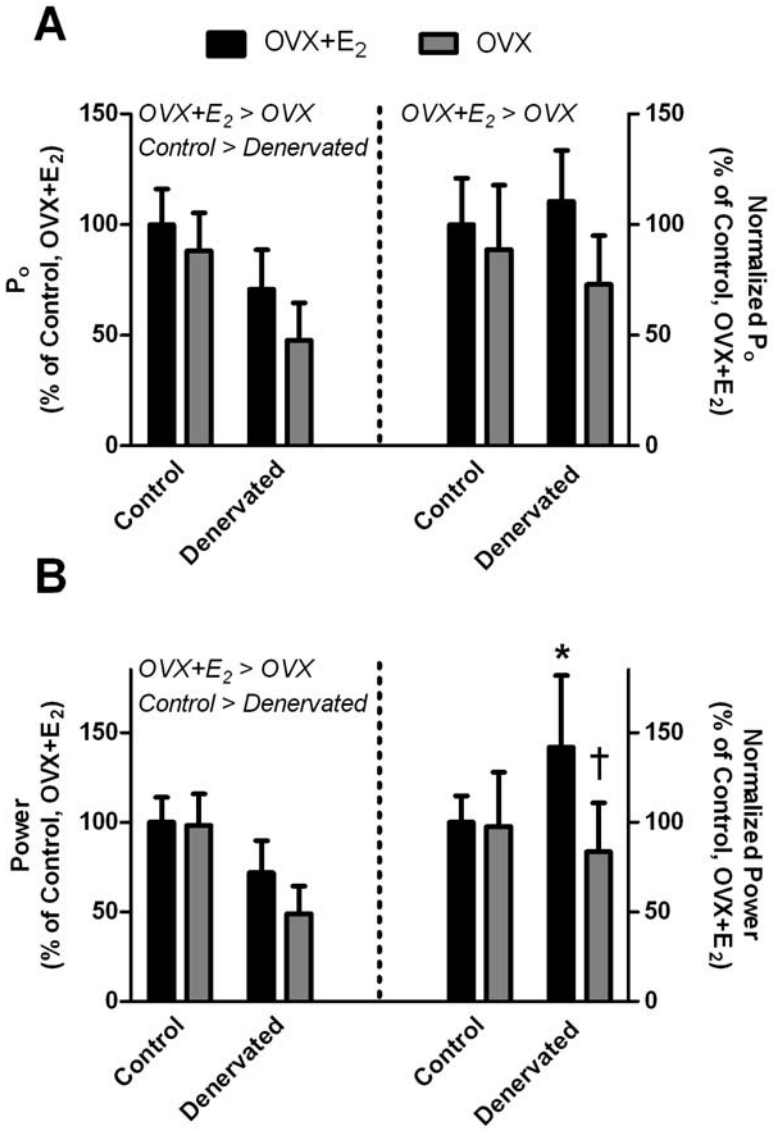

Figure 14. Contractility of soleus muscles from ovariectomized mice following two weeks of 17β-estradiol (OVX+E_2) or placebo (OVX) treatment combined with unilateral soleus muscle denervation. Data are shown relative to the control, nerve-intact limb of the OVX+E_2 mice (means, SD). Normalized P_o and power are those adjusted by the ratio of contractile protein content to fiber length. Data were analyzed by two-way repeated measures ANOVA (treatment x limb), N=10 per group. Main effects of estradiol or denervation are indicated above the bars in text. Interaction post-hoc results are indicated by: * Significantly different than Control+E_2; †Significantly different than Denervated+E_2.

Table 6. Composition and *in vitro* contractile properties of denervated and contralateral, nerve-intact soleus muscles of mice that were either sham operated or ovariectomized for 14 days.

	Control Limb		**Denervated Limb**		**2-Way repeated measure ANOVA P-value**		
	OVX+*E*$_2$	**OVX**	**OVX+*E*$_2$**	**OVX**	**Estradiol effect**	**Denervation effect**	**Interaction**
(n)	10	10	10	10			
Muscle mass (mg)	8.8 (0.8)	9.1 (0.8)	5.7 (0.8)	5.5 (0.6)	0.955	<0.001	0.298
Muscle length (mm)	11.0 (0.6)	10.9 (0.5)	10.0 (0.5)	9.9 (0.5)	0.636	<0.001	0.831
Total protein (mg)	1.024 (0.23)	0.907 (0.08)	0.596 (0.13)	0.570 (0.08)	0.173	<0.001	0.282
Contractile protein (mg)	0.76 (0.15)	0.73 (0.13)	0.42 (0.12)	0.42 (0.08)	0.661	<0.001	0.767
P_o (mN)	144.0 (23.1)	126.8 (24.9)	102.0 (25.5)	68.7 (24.5)	0.004	<0.001	0.363
Normalized P_o (N·cm/mg)	0.158 (0.03)	0.140 (0.05)	0.175 (0.04)	0.115 (0.03)	0.002	0.682	0.165
Active stiffness (N/m)	334.5 (40.5)	283.6 (37.9)	270.6 (43.4)	213.6 (62.2)	0.043	<0.001	0.590
V_{max} (fl/s)	4.5 (0.8)	4.2 (1.0)	4.5 (0.3)	4.6 (1.0)	0.578	0.659	0.597
Maximal power (mW)	0.36 (0.05)	0.35 (0.06)	0.26 (0.06)	0.18 (0.06)	0.008	<0.001	0.165
Normalized power (mW/mg)	0.50 (0.07)	0.48 (0.15)	0.70 (0.20) *	0.41 (0.14) †	--	--	0.048

Mean (SD). P_o, maximal isometric tetanic force; V_{max}, maximal shortening velocity per muscle fiber length (fl); P_o and power normalized by contractile protein content. Data within each study were analyzed by a two-way repeated measures ANOVA (estradiol by intervention with intervention being the repeated factor). * Significantly different than Control+E_2; †Significantly different than Denervated+E_2.

Maximal shortening velocity and maximal power of soleus muscles were also investigated as additional contractility parameters potentially affected by E_2 status. There was no effect of either E_2 or denervation on soleus muscle V_{max} with the average for all muscles equaling 4.5 ± 0.8 fiber lengths/sec (Table 6). Despite this, there were main effects of both E_2 and denervation on maximal power (Figure 14B, left and Table 6).

Innervated soleus muscles generated ~33% more power than denervated soleus muscles and soleus muscles from OVX+E_2 mice generated ~25% more power than did those from OVX mice. When maximal power was normalized to contractile protein content, an interaction between E_2 status and innervation was detected (Figure 14B, left and Table 6), These *in vitro* parameters of soleus muscle indicate that, with the exception or normalized power, E_2 has positive effects on muscle contractility that are independent of skeletal muscle activity.

Discussion

We know that skeletal muscle function is detrimentally and beneficially affected by low and high levels of physical activity, respectively, and that E_2 status influences physical activity, specifically wheel running, in rodents. This brings forth the possibility that effects of E_2 on skeletal muscle could result indirectly through altered physical activity levels. The effects of E_2 on muscle that we are most interested in are the decrements in force generation and myosin function that occur as a result of E_2 deficiency (58, 68, 69, 110). To help elucidate if those functional decrements are due directly to ovariectomy-induced E_2 deficiency or if declines in the mouse's physical activity contribute to the decrements, we first investigated the influence of ovariectomy on cage activities of mice. The chief finding was that ovariectomized mice which received only a placebo treatment were less active in their standard mouse cages than ovariectomized mice which received E_2 replacement. This equated to the E_2-deficient mice spending one hour less time being physically active every day, which theoretically over the duration of 30-60 days could detrimentally impact the contractile function of those lesser-recruited muscles.

Our finding that cage activities were reduced in ovariectomized mice fits with previous observations in female rodents. The most commonly-used measure of rodent physical activity has been wheel running, with distances run by E_2-deficient females being only 20-40% of that run by E_2-replete females (30, 48, 71, 77, 111). Acute monitoring of ovariectomized and ovarian-intact mice has also shown effects of E_2 on physical activity behaviors associated with fear and arousal (71, 77). Finally, previous studies in both mice and rats have shown that ambulatory activity is reduced in ovarian

hormone-deficient females, particularly during the dark phase (43, 85, 91). The decreases in 24-hr cage activity we measured by active time, distance traveled, and rearing, jumping, and stereotypic counts were 20-55% less in ovariectomized mice, broadening our knowledge of systemic treatment effects of E_2 on rodents. We concluded that the effects of E_2 on physical activity need to be considered as a potentially confounding factor in efforts to determine the mechanism(s) of E_2's effects on skeletal muscle and myosin functions. Thus, in the next two studies the direct influence of E_2 was examined by attempting to equalize physical and muscle activities of mice with low and normal levels of E_2 by hindlimb suspension and tibial nerve transection. Our hypothesis was that E_2-mediated effects on skeletal muscle are independent of physical activity. This hypothesis was supported by our main findings from Studies II and III.

The first main result supporting our hypothesis was that when the degree of load bearing by the mouse's hindlimbs was clamped at a low level by hindlimb suspending the mice, there were still significant effects of ovarian hormones on the capacity of soleus muscle to generate force. In particular, normalized P_o was ~30% lower in soleus muscles from ovariectomized mice, relative to those from ovary-intact mice. These findings were further substantiated when soleus muscle activity was controlled by tibial nerve transection. This second major result was that soleus muscles from ovariectomized mice generated 20-25% less absolute and normalized force than those from E_2-replaced mice, regardless of innervation state. The results of the hindlimb suspension study implicated ovarian hormones whereas data from the denervation study directly showed that E_2 is the key ovarian hormone affecting contractility, because treatment with that specific ovarian hormone reversed the detrimental contractile effects of ovariectomy. Overall, our

hypothesis that the effects of E_2 on soleus muscle function is independent of physical and muscle activity was supported.

While 14 days of hormone manipulation was long enough to cause functional changes in soleus muscle, significant changes in the ability of EDL muscle to generate force were not elicited. Two previous studies showed that relatively short durations of E_2 manipulation, i.e., 2.5-3 weeks, were long enough to affect dorsiflexor muscle function in mice (87, 110) and we have shown that manipulating ovarian hormones for 30 or 60 days affects contractility of EDL and soleus muscles similarly (68). We can only surmise that soleus muscle is slightly quicker to respond to E_2 manipulation, compared with EDL muscle, though the reason for this is not known.

Beyond E_2's influence on force generation there are indications that the hormone may affect muscle fatigue and power. There are conflicting results as to E_2's effect on muscle fatigue with one study indicating a beneficial effect (39), one study indicating a negative effect *in situ* and no effect *in vitro* (116), and a third study indicating no effect (106). Tiidus and colleagues went on to suggest that progesterone, not E_2, may improve fatigue resistance (87). In the present Study II, we found no effect of ovarian hormones on the fatigability of isolated soleus muscle. Muscle power was also investigated because E_2 has been shown to influence parameters related to velocity of contraction. We previously showed that soleus muscle V_{max} increased by ~10% following the loss of ovarian hormones for 60 days (69). In addition, others have determined faster muscle twitch parameters of ovariectomized rats (64). In Study III reported here, V_{max} was not affected by E_2, but maximal power was affected, being ~30% lower in soleus muscles from ovariectomized mice than those from E_2-treated mice. This effect of E_2 on power

was due to the hormone's impact on force generation, not velocity, and again was elicited irrespective of chronic muscle activity.

One mechanism underlying E_2's influence on skeletal muscle force generation is its effect on myosin function and we hypothesize that this mechanism is the result of chronic exposure of E_2 on skeletal muscle (58). This hypothesis is based on our previous work showing that the fraction of myosin in its strong-binding, force-generating structural state during contraction is greater in skeletal muscle from sham-operated or ovariectomized, E_2-replaced mice than that from untreated, ovariectomized mice (69). This has been measured directly in fibers by site-specific spin labeling of myosin combined with electron paramagnetic resonance. In those studies, the same result was obtained by analyzing muscle's active stiffness, which is an indirect measure of the degree of actin strongly bound to myosin during contraction (57). In both Studies II and III reported here, active stiffness was 15% lower in soleus muscles from OVX mice relative to sham or E_2-replaced mice, with this being statistically significant in Study III. These data suggest a positive effect of E_2 on myosin's function to generate force at the molecular level. This result combined with E_2's beneficial effect on normalized force generation indicate an E_2 effect on skeletal muscle quality, as opposed to some effect on muscle growth or size (58).

In summary, by minimizing the effects of physical and muscle activity on muscle function, the results of the present work clearly show that the effects of E_2 are directly on the muscle and are not an indirect result of E_2 changing the behavior and thus physical and muscular activities of the mice. Based on this work and our previous work, we believe that E_2 affects the intrinsic ability of skeletal muscle to generate force (58).

Future studies to determine the underlying mechanisms by which E_2 influences skeletal muscle and myosin functions are warranted.

Chapter 4

Estradiol treatment, physical activity, and muscle function in ovarian-senescent mice

With all of the previous studies being conducted on a young mouse model, including all studies analyzed in the meta-analysis, the next step I took was to investigate the relationships between skeletal muscle, physical activity, and E_2 in aged naturally ovarian-senescent mice. The second portion of this study was to determine this relationship in a young model of ovarian senescence due to 4-vinylcyclohexene diepoxide (VCD) treatment.

The contents of this chapter are under final preparation to be submitted Experimental Gerontology.

Estradiol (E_2) treatment in young adult ovariectomized mice reverses deleterious effects on skeletal muscle and also increases physical activity. Here we test the hypothesis that E_2 treatment also improves muscle function and physical activity in aged, ovarian-senescent mice. Plasma E_2 levels and vaginal cytology confirmed ovarian senescence in 20-month-old C57BL/6 mice. Mice were then randomly divided into activity groups, wheel runners or non-runners, and further into those receiving E_2 or placebo. Placebo-treated mice wheel ran more than E_2-treated mice ($p=0.027$), with no difference between treatment groups in cage activities such as time spent being active and ambulation distance ($p\geq0.551$) or soleus muscle maximal isometric tetanic force ($p\geq0.786$). Because E_2 treatment did not improve muscle function or physical activity in the aged, ovarian-senescent mice as predicted, a second study was initiated to examine E_2 treatment of young adult mice prematurely ovarian-senescent due to chemical treatment with 4-vinylcyclohexene diepoxide (VCD). 4-month-old C57BL/6 mice were treated with oil (control) or VCD and then a subset of VCD mice received E_2. VCD-treated mice wheel ran the same distance as control and VCD+E_2 mice ($P\geq0.338$), indicating that the presence of the ovaries is important for running, whether or not ovarian hormones are being produced. Concentric, isometric, and eccentric force was greater in VCD+E_2 mice than controls ($P<0.039$), however there was no decrement in force production by VCD-treated mice compared to controls. These results bring forth the possibility that residual ovarian tissue in senescent mice may be contributing the inconsistent responsiveness to E_2-treatment.

Introduction

Aging results in many structural, functional, and chemical changes that can impact the ability or the willingness to do physical activity (42). This scenario is complicated in females because ovarian hormonal changes occur simultaneously with aging and some of the tissues important for doing or regulating physical activity are affected by both age and ovarian hormones. For example, skeletal muscle weakness occurs with age and there is evidence for a greater decline in strength at the time of menopause in women (81) and ovarian failure in female mice (70). Hypothalamic areas that regulate physical activity are also affected by both aging and alterations in estrogen status (24, 49, 99). One approach for dissecting out the influences of aging versus ovarian hormones on physical activity is to ovariectomize young female rodents. Although this is closer to a model of surgical menopause in young women, it is advantageous because the effects of ovarian hormones can be assessed independently. Using this approach, physical activity in terms of wheel running decreases by as much as 10-fold and cage activities are about 50% reduced in female rodents post-surgery (30, 32, 71). Furthermore, it appears that estrogens are a primary hormone responsible for the decreases, as 17β-estradiol (E_2) treatment reverses ovariectomy-induced physical inactivity in young mice within a few days (30). Similarly, female rodents become less active with age (42), but whether or not low circulating estrogen levels contribute to this age-reported physical inactivity is not known. Thus, the first purpose of this study was to determine the effects of E_2 treatment on physical activity of female mice that had experienced natural, age-induced ovarian senescence. Physical activity was monitored by both voluntary wheel running and several

parameters of daily cage activities. We hypothesized that physical activities would be greater in aged, ovarian-senescent mice treated with E_2 compared with those not treated.

In post-menopausal women it is not known if positive skeletal muscle adaptations can occur due to additive effects of estrogen-based hormone therapy and exercise. There is evidence that strength can be gained in post-menopausal women, although no differences were determined between interventions of exercise alone or exercise in combination with estrogen-based hormone therapy (27, 62, 104), while others have shown positive additive effects (94, 103). Beneficial skeletal muscle adaptations do occur in response to both physical activity and estradiol treatment in young, ovariectomized mice (32, 68, 111). As such, a second purpose of this study was to determine the extent to which muscle of aged, ovarian-senescent mice adapt to physical activity and to E_2 treatment. We hypothesized soleus muscle contractile function would be greater in aged, ovarian-senescent mice that were treated with E_2 versus those that remained untreated, and that mice that engaged in physical activity would have greater soleus muscle contractility compared with those that were relatively sedentary. Specifically, we hypothesized that maximal isometric force and active stiffness would be greater in soleus muscles of aged, ovarian-senescent mice that were treated with E_2 compared with those not treated. Furthermore, because there have been sex implications in resistance to muscle fatigue (38), we hypothesized a protective effect of E_2 on soleus muscle fatigability.

In general our hypotheses were not supported as the aged, ovarian-senescent mice treated with E_2 did not have improved physical activities or soleus muscle function over the placebo-treated mice. Thus, the E_2 benefits on physical activity and muscle function

observed in young ovariectomized mice appear to be influenced by the absence of the ovaries. Alternatively, those tissues important for doing or regulating physical activity and muscle contractility are responsive to E_2 treatment in a young but not aged environment. We addressed these possibilities using a model of premature ovarian senescence in young adult mice allowing us to investigate senescent ovaries in otherwise young, healthy, ovary-intact mice. Chemical treatment with 4-vinylcyclohexene diepoxide (VCD) accelerates the normal process of apoptosis and atresia of ovarian follicles resulting in state of persistent diestrus and low circulating E_2 levels similar to what occurs with age (40, 63). The advantages of this approach is that young adult mice can be utilized to study the influences of ovarian hormones without the confounding effects of advanced age, similar to ovariectomy, but while having the ovarian tissue remain intact. Furthermore, the hormone profile of the follicle-depleted, ovary intact young mouse after VCD treatment is similar to the aged ovarian-senescent mouse as apposed to the abrupt change in hormones that occur with ovariectomy (3, 84). Specifically, with aging or VCD treatment there are increases in follicle stimulating hormone and luteinizing hormone, decreases in progesterone and androstenedione, and a decrease in E_2 to levels found with age-induced ovarian senescence (63, 84).

Using young, VCD-treated mice with and without subsequent E_2-treatment, we tested the hypothesis that physical activity and soleus muscle function would be reduced when circulating E_2 was diminished and preserved when E_2 was restored. Support of this hypothesis would lead us to believe that indeed E_2 is a key ovarian hormone for physical activity and skeletal muscle function and that perhaps in our previous study using aged mice the dosing regimen was not or that the aged mouse was not able to respond to the E_2

treatment. Alternatively, if the hypothesis was not supported that is, if VCD-induced ovarian senescence in the young mouse was not affective in changing physical activity or muscle function, then there might be some component of ovarian tissue removal that impacts physical activity and muscle function.

Methods

Animals. Female C57BL/6 mice (n = 60) were purchased through the NIA at 17 months of age and given 1 week to acclimate to our facility upon arrival for Study 1. Twelve of the initial 60 mice were not used in the study. Six, of the mice were excluded before being designated to a group; three had skin irritations in the neck area that would have compromised pellet implantation, one had an abdominal tumor, and two died of unknown causes at 19 and 21 months of age. The other six mice were eliminated during the study due to a bacterial infection (n=1), inner ear infection (n=1), distended urinary bladder (n=1), and death of undetermined cause (n=3).

A second set of female C57BL/6 mice (n=24) were purchased from Jackson Laboratories (Bar Harbor, ME) at 3 months of age for Study 2. These mice were housed at the Jackson In Vivo Laboratory Facility and were treated daily for 15 days with either sesame oil (n=8) or VCD (n=16) via intraperitoneal injections at a dose of 160 mg/kg body mass/day (63). Five of the VCD-treated mice died during treatment at Jackson Laboratories; technical reports indicated the mice appeared fine except for abnormal gait patterns following their last injection prior to death. Thus, a total of 19, 4-month-old mice arrived in our facilities.

Aged mice in Study I were housed five per cage until persistent diestrus was confirmed and at that time were placed in individual cages. Mice in Study II were group housed for the duration of the study. All mice were maintained on a 12-hr light cycle to simulate natural activity patterns. Mice had free access to water and phytoestrogen-free chow (2019 Teklad Global 19% Protein Rodent Diet, Harland Teklad, Madison, WI), which was used to eliminate potential complications from exogenous forms of estrogens.

At the end of each study, when mice were ~22- and 8-months of age for Study I and II respectively, mice were weighed and anesthetized by intraperitoneal injection of sodium pentobarbital (100 mg/kg body weight), with supplemental doses given as needed prior to muscle contractility testing. After muscles were harvested, mice were euthanized by exsanguination and blood collected was used for determination of plasma hormone levels. All protocols were approved by the Institutional Animal Care and Use Committee at the University of Minnesota.

Experimental design of Study I. Ovarian senescence was determined by vaginal cytology. Cytology was assessed 2-3 times per week and a mouse was deemed to be in diestrus when two consecutive samples showed complete acellular leukocytes. Of the 60 mice initially received from NIA, 7 were in diestrus upon arrival. By 18- and 19-months of age, 10 and 19 mice entered diestrus, respectively. In all, mice had failed ovaries by an average of 19.7 ± 0.2 (mean ± SE) months of age. This was confirmed by the low plasma levels of E_2; 38 of the 40 tested had values below the assay detection level (10 pg/ml) the remaining 2 mice averaged 15.2 ± 0.4 pg/ml. Within 2-4 weeks of when mice entered diestrus, the following study design was initiated.

Approximately half of the mice were given running wheels for 1 week in their home cages for acclimation purposes. Distance run during the last 3 days was used to rank mice according to running performance. These mice were divided into two groups based on a rank-order scheme designed to minimize baseline differences in wheel running activity. The mice then received pellet implants, either E_2 (run-E_2; n=12) or placebo (run-placebo; n=9). Wheels were taken out of cages for 3 days to allow recovery

from the minor pellet-implantation surgery. Wheels were then reintroduced and voluntary running continued for approximately 8 weeks. Mice that did not wheel run, i.e., remained sedentary, were also subdivided into two groups and received pellets; either E_2 (sed-E_2; n=10) or placebo (sed-placebo; n=9) and remained in their individual cages for ~8 week. 24-hr cage activity measurements were conducted on all mice during week 7 of the hormone manipulation period. Body mass of each mouse was measured weekly and food intake was monitored throughout the study. At ~22 months of age each mouse was anesthetized and underwent soleus muscle testing.

A subset of 8 mice not included in the previous study design was used to determine effect of ovarian tissue on wheel running. Once diestrus was confirmed all mice were given running wheels for 1 week. The mice were then randomized to either a sham (n=3) or ovariectomy (n=5) procedure. Following ~1 week of recovery mice were re-introduced to running wheels for 2 weeks.

Experimental design of Study II. VCD-treated mice were assessed daily for estrous cycle stage until persistent diestrus and thus ovarian senescence was confirmed. VCD-treated mice were considered acyclic after 10 days of diestrus, which occurred ~63 days following the onset of VCD-treatment (range 47-79 days). Once diestrus was confirmed, each mouse underwent physical activity testing including wheel running and cage activities. At ~6 months of age, hormone manipulation began and lasted for 8 weeks, with experimental groups of VCD (n=5), VCD+E_2 (n=6), and control (n=8). The control group was the ovary-intact mice that had received sesame oil injections at Jackson Labs. During the study, mice underwent assessment of running motivation (at weeks 2 and 5 of the 8 week study), cage activities and food intake (at weeks 1 and 7 of

the 8 week study), and weekly body mass. At ~8 months of age mice were anesthetized and underwent soleus muscle testing.

Hormone manipulation and related procedures. Mice were anesthetized via inhalation of 1.75% isoflurane mixed with oxygen at a flow rate of 200 ml/min. Once anesthetized, mice were implanted with a 60-day time-release E_2 or placebo pellet (Innovative Research of America, Sarasota, FL) on the dorsal aspect of the neck using a trochar. In study I, E_2 pellets contained 0.09 mg of 17β-estradiol. The dosage was intended to mimic the normal circulating levels of E_2 in adult female mice, i.e., ~20 pg/ml serum (75). Placebo pellets consisted of the same matrix as the E_2 pellets. In Study II, a subset of the VCD-treated mice (n=6) were implanted with 0.18 mg, 60-day slow release E_2 pellets. We used the lower dose of E_2 when treating the aged mice because in preliminary studies we found that the higher does caused bladed retention (reported by others as well (28, 37, 55)). This was minimized with the lower does via 0.09 mg pellets, only 1 mouse was excluded due to a distended urinary bladder. We used the higher does pellet for the younger mice in Study II because we desired to mimic our previous experiments in young ovariectomized mice (30, 32, 68).

Ovariectomy of mice was conduced as previously described (32, 68, 69). While under isoflurane anesthesia the ovaries were removed bilaterally. Mice undergoing the ovariectomy procedure were administered 0.15 μg of Buprenorphine subcutaneously ~5 minutes post procedure.

Blood was collected by facial vein bleed prior to the hormone manipulation and/or during exsanguination at the study completion. Plasma was separated from whole

blood, frozen in liquid nitrogen, and stored at -80^{o} until analysis. Plasma E_2 was measured by ELISA (KA0234, Abnova Corporation, Taiwan). The uteri were dissected and weighed at the study completion as an additional marker of ovarian-hormone depletion and treatment.

Physical activity assessments. Voluntary wheel running was measured using an exercise wheel, 11 cm in diameter with a 2 inch running surface, that was mounted to the top of a standard mouse cage (47 x 26 x 15 cm) (30). A digital magnetic counter was fastened to the wheel and connected to a microprocessor which stored the number of revolutions per 24 hr. In Study II, mice were allowed access to the wheels on three separate ~3 day periods to assess physical activity without any anticipated training effects (6).

Cage activities were evaluated by first placing individual mice in a mock activity chamber to acclimate to the new environment for 24 hr immediately prior to the test. Mice were then placed individually into an activity chamber (Med Associates Inc., St. Albans, Vermont) for 24 hr (32, 52). Data presented are total active time, jumping counts, rearing counts, stereotypic counts, and ambulatory distance and were collected using Activity Monitor version 6.01 software (Med Associates) with a box size set to "3" (4.8 cm^2). While in the activity chamber, food intake was also measured for each mouse in Study II.

In vitro assessment of soleus muscle contractility. The isolation and testing protocol for the soleus muscle has been described previously (70, 109). Measurements included passive muscle stiffness determined by stretching inactive muscle sinusoidally from 97.5% L_o to 102.5% L_o at 0.5 Hz and measuring the force response (300B-LR;

Aurora Scientific Inc., Aurora, ON, Canada), peak twitch force (P_t) elicited by stimulating the muscle with a 0.5-ms pulse at 150 V (Grass S48 stimulator delivered through a SIU5D stimulus isolation unit; Grass Telefactor, Warwick, RI), maximal isometric tetanic force (P_o) determined by stimulating muscles for 400 ms at 150 V and 120 Hz, and active stiffness determined during a second maximal tetanic contraction by eliciting a sinusoidal length oscillation of 0.01% L_o at 500 Hz. Additional parameters measured from twitch and tetanic contractions include; time to peak twitch (TPT), one-half relaxation time ($RT_{½}$), maximal rate of tetanic force development (+d*P*/d*t*), and maximal rate of relaxation (-d*P*/d*t*).

Soleus muscles from mice in Study I then underwent a protocol employing a bout of fatiguing contractions that began 2 min following the final force and stiffness measurements (32). Soleus muscles from Study II were measured for maximal concentric force that was elicited by passively lengthening the muscle to 105% of L_o over 3 s, and then stimulating tetanically for 133 ms as the muscle was shortened to 95% of L_o at 1.5 L_o/s, and eccentric force, as the muscle was passively shortened to 95% of L_o and then stimulated tetanically as it lengthened to 105% of L_o.

Following contractility testing, each soleus muscle was removed from the bath, trimmed at the myotendinous junctions, blotted, weighed, and stored at -80^o until further analysis. Fiber length was calculated as 71% of the soleus muscle length (65). Specific P_o was calculated by correcting for physiological cross-sectional area of the muscle (14).

Statistical analysis. In Study I, two-way repeated measure ANOVAs with Tukey's post-hoc tests were used to determine if differences existed for wheel-running distances between mice treated and not treated with E_2 over time. Students t-test were

used to examine possible differences in cage activities between mice treated and not treated with E_2. Two-way ANOVAs (E_2 treatment x activity (wheel runners or sedentary)) were used to determine if there were differences between the three groups for body mass, organ masses, muscle composition, and soleus muscle contractility parameters. Three-way repeated measure ANOVAs (E_2 treatment x activity x time) were used to determine differences in food intake.

For Study II, two-way repeated measures ANOVAs (group x time) were conducted to assess body mass, activity and running. One-way ANOVAs were used to determine differences between groups for food intake, muscle contractile parameters, uterine masses, muscle masses, and terminal hormone analyses.

Statistical analyses were done using SigmaStat version 3.5 (Systat Software Inc; Point Richmond, CA), with the exception for the three-way repeated measures ANOVA, which was conducted using JMP version 7.0 (SAS Institute, Inc; Cary NC). Significance was accepted with an α level of 0.05. Values are reported as means ± SE.

Results

Study I: Ovarian senescence in aged mice

Body mass was not different among groups at the beginning or the end of the 8-wk study, although there was a trend for E_2-treated, aged, ovarian-senescent mice to have lower body mass than those treated with placebo (Table 7). On average, mice ate 4.1 ± 0.1 g of food per 24-hr with no effect of hormone status or physical activity (P>0.457). Mice treated with E_2 had uterine masses nearly twice that of placebo mice indicating effective E_2 treatment (Table 7).

Table 7. Body and uterus mass from naturally aged, ovarian-senescent mice treated with placebo or E_2 for 8 weeks.

	Sedentary		**Runner**		**2-Way ANOVA P-value**		
	placebo	***E_2***	***placebo***	***E_2***	**Activity effect**	**Estradiol effect**	**Interaction**
(n)	9	10	9	12			
Body mass at ~20-mo (g)	27.8 ± 1.2	25.6 ± 0.6	27.7 ± 1.6	26.7 ± 0.6	0.602	0.137	0.543
Body mass at ~22-mo (g)	28.8 ± 1.3	26.7 ± 0.8	28.7 ± 1.9	26.5 ± 0.5	0.909	0.067	0.917
Uterus mass (mg)	94.4 ± 7.4	199.0 ± 10.3	98.9 ± 10.8	182.5 ± 25.7	0.069	<0.001	0.593

Mean ± SE. Body masses were measured when all mice were ovarian-failed but had not yet been randomized to receive E_2- or placebo-treatment ~20-mo of age and at the termination of the study ~22-mo of age when soleus muscles were analyzed for contractile properties. Data were analyzed by a two-way ANOVA (activity by estradiol).

Physical activities. Of the non-wheel running, sedentary mice, between those treated with E_2 and placebo there was no difference in any parameter of daily cage

activities. Mice were active for about 4 hr per day, 234 ± 31 vs. 248 ± 12 min for placebo and E_2 mice, respectively (P=0.555). Distance ambulated per 24 hr was equivalent between groups; 414.8 ± 50.1 vs. 365.2 ± 71.7 m for placebo and E_2 mice, respectively (P=0.608). During the active periods, placebo- and E_2-treated mice had equal activity counts for stereotypic, jumping, and rearing as well (P≥0.551).

Mice that had access to running wheels were affected by E_2 as far as distances run. Prior to receiving placebo or E_2 treatments, mice ran an average 2.8 ± 0.3 km per 24 hr (Figure 15). There was an effect of both time and treatment on running distances (P≤0.027; Figure 15) as running gradually declined over time and the E_2–treated mice ran significantly less than placebo-treated mice for the duration of the 8-wk study.

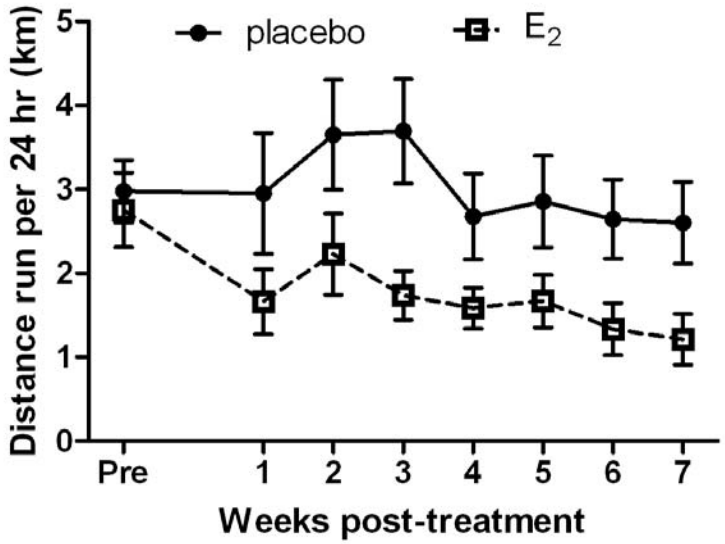

Figure 15. Distances run on wheels per 24-hr as weekly averages for aged ovarian-senescent mice treated with placebo (control) or E_2 (means ± SE). Pre time point was prior to treatment. Data analyzed by 2-way repeated measures ANOVA (treatment by time). Main effects of E_2-treatment and time. N=9-12 per group.

To begin to investigate discrepancies of E_2 effects on physical activities between previously-reposted young, ovariectomized mice and aged, ovarian-senescent mice in these studies, an additional set of 8 ovarian-senescent female mice were investigated. Once diestrus was confirmed, these mice wheel ran for 1 week, running an average of 4.5 ± 0.4 km per 24 hr, and were then randomized to a surgical group, either ovariectomy or sham. During the next 2 wks following surgeries, sham ovary-intact mice ran 3.5 ± 0.6 km per 24 hr while ovariectomized mice ran only 1.9 ± 0.4 km (P=0.012; Figure 16). These data suggest that the presence of residual, non-E_2 producing ovarian tissue in aged mice may affect wheel running.

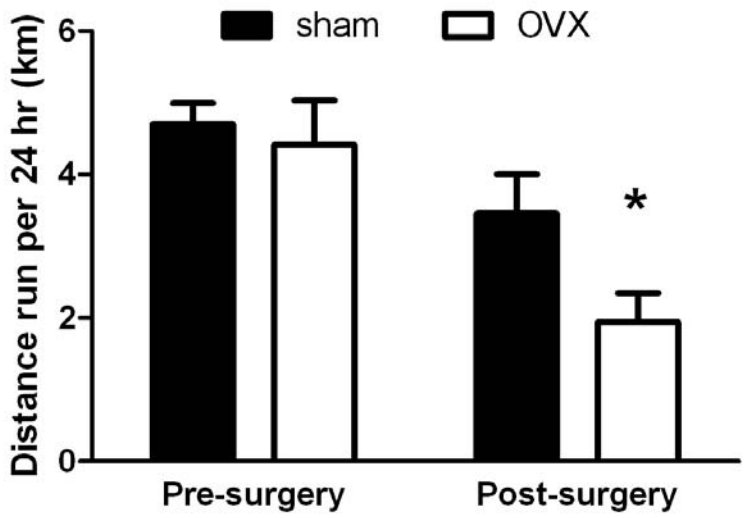

Figure 16. Wheel running distances for aged ovarian-senescent mice, before and after a surgical procedure and following randomization to ovariectomy (OVX) or sham procedure (means ± SE). Data analyzed by 2-way repeated measures ANOVA (surgical group by time). *Significantly less than sham at given time point. N=3-5 per group.

Muscle contractility. Soleus muscles from aged, ovarian-senescent mice that ran on wheels for ~8 wk had greater capacity to generate force than those without running wheels, irrespective of E_2 treatment. Specifically, soleus muscles of running mice had 21

to 23% greater mass, P_o, and active stiffness (Table 8). Examination of force-time tracing of the twitch and tetanic contractions revealed no effect of either wheel running or E_2, as TPT, $RT_{½}$, and rates of contraction (+d*P*/d*t*) and relaxation (-d*P*/d*t*) were not affected (Table 8). E_2 treatment of the aged, ovarian-senescent mice affected the fatigability of the soleus muscle, but no other contractile parameter (Table 8). With the 60-contraction fatiguing bout, there was a main effect of E_2 on fatigability from the 10^{th} contraction onward ($P \leq 0.032$; Figure 17). Specifically, E_2 was protective against soleus muscle fatigue with ~10% greater fatigue resistance in E_2-treated mice, irrespective of activity. The force decrement was confirmed to be fatigue as opposed to muscle injury as P_o 5 min following the fatigue protocol was not different than P_o pre-fatigue ($P>0.229$; Figure 17 insert).

Table 8. *In vitro* contractile properties of soleus muscles from naturally aged, ovarian-senescent mice treated with placebo or E_2 for 8 weeks.

	Sedentary		**Runner**		**2-Way ANOVA P-value**		
	placebo	***E_2***	***placebo***	***E_2***	**Activity effect**	**Estradiol effect**	**Interaction**
(n)	9	10	9	12			
Soleus mass (mg)	9.4 ± 0.7	8.7 ± 0.5	10.8 ± 0.4	9.6 ± 0.5	0.024	0.076	0.612
Muscle length (mm)	11.3 ± 0.1	11.1 ± 0.2	11.2 ± 0.1	11.2 ± 0.1	0.937	0.292	0.552
P_t (mN)	23.6 ± 2.2	24.3 ± 1.7	25.9 ± 1.2	27.9 ± 1.3	0.380	0.899	0.186
TPT (ms)	40.4 ± 1.5	42.3 ± 1.6	41.9 ± 1.0	44.2 ± 1.5	0.249	0.155	0.890
$RT_{½}$ (ms)	57.6 ± 7.2	52.4 ± 7.7	52.3 ± 4.0	58.1 ± 2.9	0.973	0.949	0.324
+d*P*/d*t* (N/s)	1.5 ± 0.1	1.3 ± 0.1	1.3 ± 0.1	1.6 ± 0.1	0.060	0.695	0.547
-d*P*/d*t* (N/s)	1.9 ± 0.2	1.7 ± 0.3	2.2 ± 0.2	2.2 ± 0.1	0.635	0.519	0.073
P_o (mN)	139.5 ± 14.9	133.3 ± 10.5	161.5 ± 9.0	173.3 ± 6.8	0.005	0.788	0.389
Specific P_o (N/cm^2)	12.9 ± 1.4	13.0 ± 1.1	12.7 ± 0.7	15.3 ± 0.7	0.314	0.186	0.195
Active stiffness (N/m)	272.4 ± 15.2	278.6 ± 23.4	323.9 ± 10.0	341.1 ± 8.0	<0.001	0.716	0.437
Passive stiffness (N/m)	20.9 ± 3.3	17.3 ± 2.8	15.8 ± 1.9	16.3 ± 2.4	0.254	0.567	0.439
Fatigue (% of initial contraction)	49.1 ± 5.7	55.1 ± 6.4	43.3 ± 5.3	63.1 ± 4.0	0.837	0.022	0.206

Mean ± SE. P_t, peak twitch force; TPT, time to peak twitch force; $RT_{1/2}$, one-half relaxation time; +d*P*/d*t*, maximal rate of tetanic force development; -d*P*/d*t*, maximal rate of relaxation; P_o, maximal isometric tetanic force; Specific P_o is normalized by physiological cross sectional area; Fatigue is the percentage of maximal isometric tetanic force on the 60^{th} contraction relative to that of the 1^{st} contraction of the protocol. Data were analyzed by a two-way ANOVA (activity by estradiol).

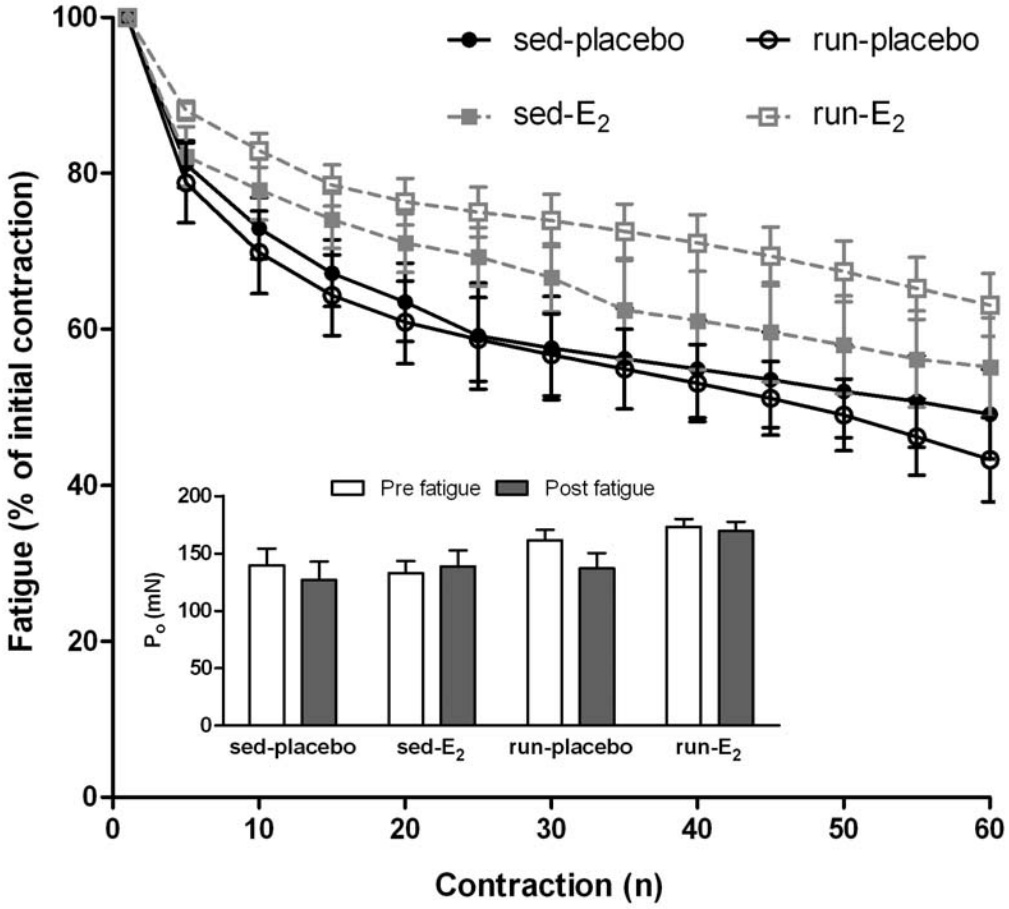

Figure 17. Force decrements and recovery from a fatiguing bout of contractions performed by soleus muscles from mice following 60 days of ovarian hormone manipulation and being sedentary in cage or wheel running (means ± SE). Fatigue is calculated as the percentage of force relative to that of the 1st contraction of the protocol. Plotted are the relative forces of every fifth contraction during the 60-contraction fatiguing protocol. Main effect of E_2 from 10^{th} – 60^{th} contractions. Insert is pre and post P_o, showing that the fatiguing bout did not induce injury to the soleus muscle because post P_o recovered equivalent to pre P_o in all groups. N=7-12 per group.

Study II: Premature ovarian senescence in adult mice

Ovarian senescence of VCD mice was confirmed 63 ± 3 days from the first VCD treatment by 10 consecutive days of persistent diestrus. Periodically throughout the 8-wk

study cytology was conducted to confirm that control mice were cycling normally and that VCD and E_2 interventions were affective.

Body mass was only affected by the duration of the study with no affect of treatment (P=0.251). Initially mice weighed 23.0 ± 0.3 g and at the end of the 8-wk study mice weighed 25.1 ± 0.4 g (main effect of time, P<0.001). Mice ate an average of 3.4 ± 0.1 g of food per 24 hr with no effect of treatment group (P=0.485). Control mice had uterine weights different than both VCD and E_2-treated groups, indicating that both the VCD- and E_2-treatments were affective in atrophying and hypertrophying the uterus, respectively (118.2 ± 13.0, 50.4 ± 7.6, and 131.9 ± 27.5 g for control, VCD, and VCD+E_2, respectively; P=0.021).

Physical activities. Acute wheel running and 24-hr cage activity were measured three times during the study as indicators of physical activity. There was no difference in running among groups (Table 9). Cage activities were not conclusively different among groups (Table 9). Total active time in the cage increased over time for the VCD+E_2 mice, and these mice were more active in the final week of testing with a difference of ~1.5 hrs between the VCD+E_2 and control groups. Comprised within the total time active only some parameters were different between groups, there was no difference in jumping counts and ambulatory distance (P≥0.064; Table 9). However, both stereotypic and rearing counts differed among groups, with rearing counts increasing over time (main effect P=0.044) and stereotypic counts greater in the VCD+E_2 between the pre and wk 7 testing, and within the wk 7 testing the VCD+E_2 had greater counts than the control group (interaction P=0.032).

Table 9. Physical activities of control mice, mice prematurely ovarian-senescent due to treatment with VCD, and VCD-treated mice with E_2-treatment.

Wheel running (km/24 hr)						
	Control	**VCD**	**VCD+E_2**	**2-Way ANOVA P-value**		
				Time effect	**Hormone effect**	**Interaction**
Pre	7.2 ± 0.5	8.0 ± 0.5	8.5 ± 0.3	0.338	0.710	0.434
Week 2	8.0 ± 0.5	8.3 ± 0.6	8.0 ± 0.9			
Week 5	8.5 ± 0.5	8.1 ± 0.5	8.8 ± 0.5			
Distance ambulated in cage (m/24 hr)						
	Control	**VCD**	**VCD+E_2**	**2-Way ANOVA P-value**		
				Time effect	**Hormone effect**	**Interaction**
Pre	756 ± 114	905 ± 170	890 ± 150	0.958	0.064	0.748
Week 1	1389 ± 125	833 ± 169	553 ± 109			
Week 7	1084 ± 379	966 ± 49	1210 ± 115			
Total time active (min/24 hr)						
	Control	**VCD**	**VCD+E_2**	**2-Way ANOVA P-value**		
				Time effect	**Hormone effect**	**Interaction**
Pre	267 ± 23	288 ± 22	291 ± 21			0.018
Week 1	270 ± 21	292 ± 33	242 ± 29			
Week 7	270 ± 20	309 ± 5	366 ± 16 *†‡			

Pre time point was prior to hormone treatment, mean ± SE; analyzed by 2-way repeated measures ANOVA (hormone by time). N=5-8 per group.*Significant difference between pre time point within the group, †Significant difference between week 1 time point within group. ‡ Significant difference between control at individual time point.

Muscle contractility. Mice that were prematurely ovarian-senescent due to VCD-treatment and then replaced with E_2 for 8-wks had greater soleus muscle function than VCD mice not treated (Figure 18). There was no difference among groups in soleus muscle size (Table 10). Soleus muscles from VCD+E_2 mice generated 16-19% more concentric, isometric, and eccentric force than VCD mice ($P \leq 0.039$). This was also confirmed in concentric and isometric specific forces which were 10-12% greater in VCD

treated with E_2 ($P \leq 0.009$), but eccentric specific force did not differ among groups ($P=0.218$). There were no differences in passive stiffness or peak twitch force (Table 10). Active stiffness, indicative of strong-binding myosin to actin, had a trend for the VCD+E_2 to be relatively greater (Table 10). The force-time tracing of twitch and tetanic contractions revealed no effect of VCD- or E_2-treatment on the rates of muscle contraction or relaxation (TPT, $RT_{½}$, and $-dP/dt$) with the $+dP/dt$ which was greater in VCD+E_2 than VCD mice (Table 10).

Table 10. *In vitro* contractile properties of soleus muscles from prematurely ovarian-senescent mice.

	Control	**VCD**	**VCD+E_2**	**ANOVA P-value**
(n)	*8*	*5*	*6*	
Soleus mass (mg)	9.9 ± 0.4	9.4 ± 0.5	9.9 ± 0.2	0.616
Muscle length (mm)	11.6 ± 0.1	11.7 ± 0.1	11.6 ± 0.2	0.828
P_t (mN)	36.8 ± 1.9	37.5 ± 2.7	38.0 ± 1.4	0.905
TPT (ms)	38.5 ± 1.0	39.4 ± 1.6	40.6 ± 1.4	0.794
$RT_{½}$ (ms)	43.8 ± 1.3	43.8 ± 1.2	45.3 ± 2.3	0.771
$+dP/dt$ (N/s)	2.9 ± 0.1	2.6 ± 0.2	3.4 ± 0.2 *	0.026
$-dP/dt$ (N/s)	2.3 ± 0.1	2.3 ± 0.1	2.7 ± 0.1	0.115
Active stiffness (N/m)	291.9 ± 7.4	311.9 ± 9.4	328.6 ± 14.6	0.056
Passive stiffness (N/m)	12.0 ± 0.6	11.4 ± 0.6	11.0 ± 0.2	0.381

Mean ± SE. P_t, peak twitch force; Specific P_o is normalized by physiological cross sectional area; Data were analyzed by one-way ANOVA, * Significantly different than VCD

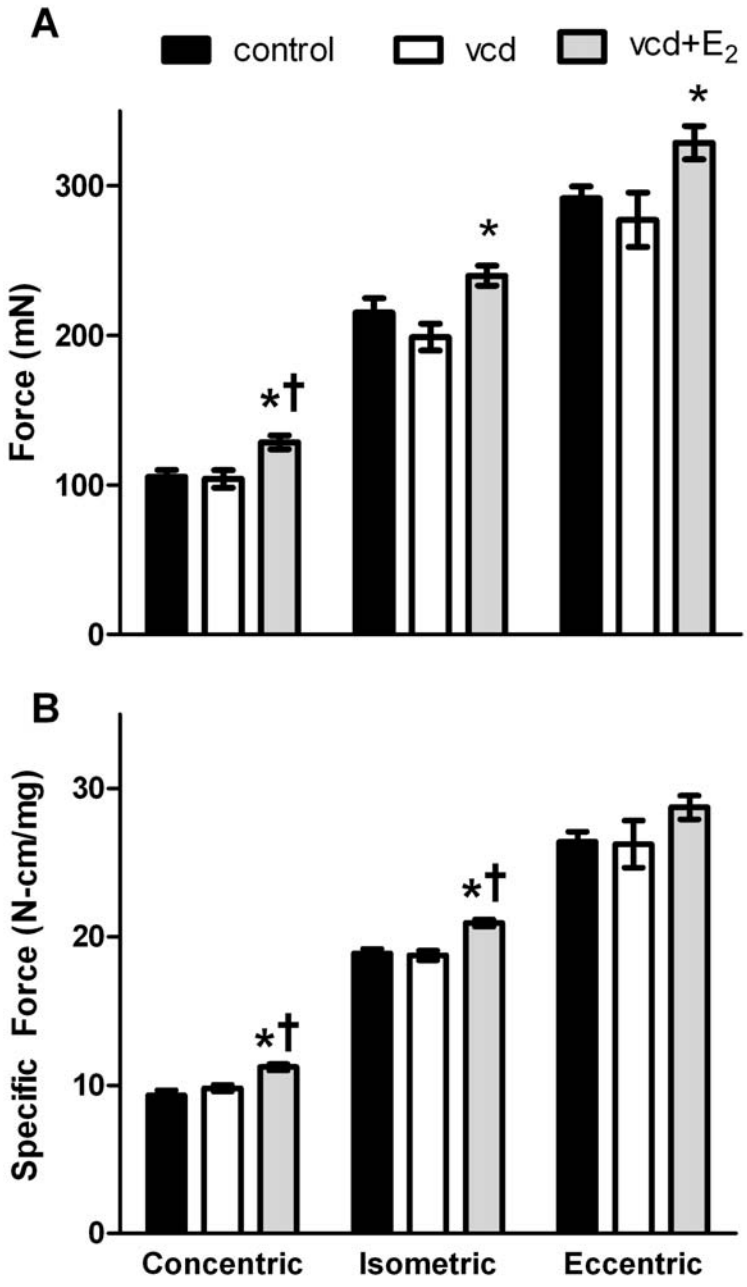

Figure 18. Contractility of soleus muscles from mice treated with sesame oil (control), VCD, or VCD+E_2 for eight weeks (means ± SE). Specific forces are corrected for physiological cross-sectional area of soleus muscle. Data analyzed by one-way ANOVA, N=5-8 per group. *Significantly different than VCD, †Significantly different than control.

Discussion

The results of these studies indicate that physical activity and skeletal muscle function following E_2 treatment in mice that are ovarian-senescent, either due to age or chemical treatment, differ from what has previously been shown following the removal of ovarian tissue in young mice. When young ovariectomized mice are replaced with E_2 there is a preservation of voluntary wheel running and physical activites (30, 32, 48), and reversal of the ovarian hormone-induced strength loss (32, 58, 68, 111). In the first study reported here, we investigated physical activity and soleus muscle force in 22-mo-old, ovarian-senescent mice and found that 8 wks of E_2 treatment in these mice did not affect cage activites, decreased wheel running, and had no affect on soleus muscle force generation. In contrast, we and others have previously shown that 4-8 wks of E_2 treatment in young, ovariectomized mice resulted in a maintenance of physical activity and wheel running and preserved muscle force (30, 68, 111). These conflicting results of E_2 treatment in estrogen-deficient aged and young mice prompted us to design a second study to examine the effects of failed, non-hormone producing ovaries in a young ~8-mo-old mouse model of premature ovarian senescence. In the young ovarian-senescent mice, our primary findings were that physical activities were minimally altered with or without E_2 treatment and soleus muscles produced greater forces with E_2 treatment. These findings indicate that there may be a component of the non-estrogen producing ovary that impacts physical activity and skeletal muscle function.

Physical activity decreases with aging in many species (42). In the studies reported here, ~22-mo-old female mice were less active than young ~8-mo-old mice. Young mice were active for ~5 hrs per day and ambulated about the cage ~1000 m while

aged mice were active for ~4 hrs and ambulated only ~400 m per day. Similarly, in young estrogen-deficit mice, cage activities are low by 20-55% relative to those replaced with E_2 (32). This is the first study to show that the effects of aging may outweigh the effects of ovarian hormones on physical activity because we found no effect of E_2 treatment on daily cage activites in aged, ovarian-senescent mice. In young mice prematurely ovarian-senescent due to VCD, we found minor differences between those replaced with E_2, those not replaced, and control mice. VCD mice with E_2 replacement spent more total time being active in the cage and ambulatory distance tended to be greatest in those mice. Previous research indicated decreased stereotypic activity as female mice aged from 4.5 to 15-mo, while other parameters of cage activities were not significantly changed, likely due to the nature of these highly variable cage activity measurements (28).

Similar to cage activities, wheel running is detrimentally affected by the loss of ovarian hormones in young mice, and can be preserved with treatment of E_2 (30, 48, 71, 77, 111). In aged ovarian-senescence mice, E_2 treatment evoked a decrease in running distances, with mice running about half the distances of those that were treated with placebo. To further examine this discrepancy, we compared naturally ovarian-senescent mice with intact ovaries to those that had their non-functioning ovaries surgically removed. Interestingly, following the removal of the non-functioning ovaries, running decreased more than that in age-matched mice that underwent sham surgeries. This result was confirmed in Study II as VCD mice that retained their non-functioning ovaries did not have a decrement in wheel running performance. Similarly, treatment of young mice with Faslodex, an estrogen receptor antagonist , had no effect on wheel running (6). From

these three observations, it appears that some component of the non-functioning ovary may influence wheel running in mice.

It has been shown that the ovaries of VCD mice still produce low levels of androgen, such that androstenedione levels are decreased by a third while E_2 levels decrease to a level of non-detection (63). Therefore, the ratio of androstenedione to E_2 is significantly altered in these ovarian senescent mice and possibly influence wheel running. Androgen has been implicated as a behavioral modifier as exemplified in androgen receptor knock out mice which show a drastic decrease in running distances and cage activites compared to control mice (25, 79). In humans, androgen is primarily produced in the adrenal gland, however in mice that is not the case, instead it is exclusively produced in the ovary or testis in female and male mice, respectively (108). To our knowledge androgen levels of age-induced ovarian senescent mice has not been reported, as it has for VCD mice (63, 84). Thus, the link between androgens' and estrogens' interaction in running motivation in mice is unclear.

We previously reported that in young mice, wheel running as little as 1.45 km per day can induce positive skeletal muscle adaptations (111). In this study, the aged mice only ran 2.3 km per day and this was enough to produce favorable adaptations in soleus muscle. Irrespective of ovarian hormone status, soleus muscles from mice that wheel ran for 8 wks had more mass and generated more force compared to mice that did not wheel run. Absolute force and active stiffness were both ~22% greater in aged mice that ran on wheels. This is an important finding in that muscle adaptations can occur in response to exercise independent of ovarian hormone status.

While treatment of E_2 in aged mice did not affect soleus muscle force generation, E_2 did have a protective effect on soleus muscle fatigue. There was ~10% greater fatigue resistance in aged mice replaced with E_2, than those that remained E_2 deficient. There has been a noted difference in fatigue resistance between sexes (38). Recently this sex difference has been hypothesized to be independent of androgen because androgen receptor knockout mice had equivalent fatigue resistance of the soleus muscle to that of wild type female mice both of which were more resistant than muscles from wild type males (60). It is possible that the direct effects of E_2 on soleus muscle fatigue are central to the commonly found sex difference. We have previously determined that E_2-mediated effects on skeletal muscle are independent of physical activity, thus we believe that the fatigue results presented in this study are due to direct effects of E_2 on skeletal muscle (32). In Study II, there was no loss of muscle function in VCD mice compared to controls. However, these premature ovarian-senescent young mice replaced with E_2 had greater soleus muscle force generation. Concentric, isometric, and eccentric absolute forces were 16-19% higher in E_2-replaced mice over those only treated with VCD indicating that in young mice, E_2 treatment can be beneficial to muscle similar to what has been shown with the OVX model (33).

Collectively, it appears that skeletal muscle of ovarian senescent mice is not as responsive to E_2 treatment as are young mice with the ovaries removed. One possible explanation for this is that estrogen receptor dynamics could be altered with aging, that is, aging of the mouse or specific aging of the ovaries. Early work indicated that there was decreased binding of nuclear estrogen receptors in the hypothalamus, pituitary, and uterus of 23-month aged female mice following E_2 treatment (74). In support of this, decreases

in estrogen receptor alpha and beta positively-stained nuclei in quadriceps muscle of postmenopausal women compared to young women have been reported (115). Skeletal muscle of young female mice has been shown to contain both estrogen receptors alpha and beta (6). While young ovariectomized mice respond to the loss and treatment of E_2 by increasing and decreasing estrogen receptor alpha mRNA levels, respectively (6), a preliminary study showed that skeletal muscle of aged mice did not respond to E_2 treatment in terms of estrogen receptor alpha mRNA levels (34). Thus there is a possibility that due to aging there are altered estrogen receptors in skeletal muscle. Further studies are needed to determine both content and function of skeletal muscle estrogen receptors in aged compared to young females.

In summary, this study is the first to report a number of key findings on skeletal muscle in ovarian-senescent mouse models. The loss of ovarian hormones due to age-induced, ovarian senescence has dissimilar effects on physical activity and skeletal muscle function than does the removal of ovarian tissue in young mice. In aged ovarian-senescent mice there was no effect of E_2-treatment on soleus muscle force, but a protective effect on fatigue resistance. In young mice exposed to chemical VCD, producing a senescence only of the ovarian tissue, greater soleus muscle force generation occurred following E_2 replacement.

Chapter 5

Conclusions

The contents of this dissertation add significant findings to the fields of skeletal muscle physiology and ovarian hormone actions. The loss of ovarian hormones is a consequence of normal aging, likewise, so is age-induced strength loss. Prior to my studies, the effects of the loss of ovarian hormones on muscle weakness in women had been debated with no clear conclusion. I conducted a meta-analysis of the published literature and concluded that estrogen-based HT when given to postmenopausal women is beneficial to muscle strength. Specifically, women who were on HT had 5%-10% greater strength than those not on HT. This finding was supported by an analogous meta-analysis of rodents in which I determine there were beneficial effects of E_2 on normalized muscle strength and that E_2 is the key ovarian hormone affecting muscle contractility.

With the loss of ovarian hormones there are detrimental effects not only on muscle function, but also on the physical activity of rodents. Therefore, it was possible that there were combined estrogenic effects on muscle function in previous studies using rodent models. While the relationship between any two of the variables, physical activity, muscle strength, and estrogens are fairly-well understood, the combined effects of all three were not. Through controlling physical and muscle activity of mice by hindlimb suspension and tibial nerve transection, I determined that the effects of E_2 are directly on skeletal muscle function and not through the indirect results of changes in physical and muscle activity of the mice.

Finally, while the loss of ovarian hormones due to surgical removal of ovaries has been determined to be detrimental to muscle function in mice, the effects of ovarian

hormone loss due to natural aging had not been investigated. In aged 22-month old, ovarian-senescent mice I found minimal effects of E_2 replacement on skeletal muscle strength and physical activity. Despite finding no benefits E_2, there were positive adaptations of the soleus muscle in response to wheel running. The second component of this study on young mice chemically treated with VCD, producing a premature senescence only of the ovarian tissue, concluded no loss of soleus muscle function but improvement in function when mice were treated with E_2.

Overall, the results of these studies show that the loss of estrogens are detrimental to skeletal muscle and conversely that E_2 treatment is beneficial. As such, more research should be conducted to determine the mechanisms by which E_2 affects skeletal muscle and how this can be translated to women as they age.

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