Expression of sex steroid hormone receptors in human skeletal muscle during the menstrual cycle

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Short title: Hormone receptors in skeletal muscle

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/apha.12757

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Abstract

**Aim:** Variations in sex hormone levels during the menstrual cycle may affect neuromuscular performance and the risk of sustaining musculoskeletal injury in women. The aim of this study was to investigate mRNA and protein levels for sex steroid hormone receptors in skeletal muscle in three distinct phases of the menstrual cycle.

**Methods:** Fifteen, healthy women with regular menstrual cycles participated in the study. Muscle biopsies from the vastus lateralis were obtained in three hormonally verified phases of the menstrual cycle for each individual, i.e. the follicular phase, the ovulatory phase and the luteal phase. mRNA and protein levels of oestrogen (ER\(\alpha\) and ER\(\beta\)), progesterone (PR) and androgen (AR) receptors were analysed.

**Results:** There was an overall significant variation in mRNA and protein levels of ER\(\alpha\) and PR across the menstrual cycle. mRNA and protein levels of ER\(\alpha\) were highest in the follicular phase when estradiol levels were low, whereas protein levels of PR were highest in the luteal phase when progesterone levels were high. mRNA levels of PR were highest in the ovulatory phase. No significant variation in AR levels was detected across the menstrual cycle. ER\(\beta\) levels were very low in all three phases of the menstrual cycle.

**Conclusion:** Significant variations in mRNA and protein levels of ER\(\alpha\) and PR were detected in skeletal muscle during three confirmed phases of the menstrual cycle. These results may have an impact on effects of muscular training and sports injuries in women.
Key words: Androgen receptor, Oestrogen receptor alpha, Oestrogen receptor beta, Progesterone receptor, Skeletal muscle

Introduction

Several studies indicate that variation in sex hormone levels during the menstrual cycle may affect neuromuscular performance and the risk of sustaining musculoskeletal injury. An association between musculoskeletal knee injuries and the menstrual cycle has been reported, although the association to a specific cycle phase has varied between different studies (Wojtys et al., 1998, Wojtys et al., 2002, Möller-Nielsen and Hammar, 1989, Myklebust et al., 1998). Data on the influence of the menstrual cycle on muscle strength is not conclusive. Some studies have reported a significant variation in muscle strength over the cycle (Davies et al., 1991, Phillips et al., 1996, Sarwar et al., 1996), whilst this was not observed in other studies (Fridén et al., 2003a, Gür, 1997, Gür et al., 1999, Lebrun, 1994, Janse de Jonge, 2003, Montgomery and Shultz, 2010). However, significant variations in knee joint laxity, neuromuscular coordination, muscle stretch reflex, and postural control during the menstrual cycle have been suggested.

A systematic review found a significant increase in knee joint laxity during the ovulatory or post-ovulatory phase of the menstrual cycle (Zazulak et al., 2006). Furthermore, neuromuscular coordination appears to be enhanced during ovulation (Fridén et al., 2005), while the muscle stretch reflex response seems to be lowest at this phase (Casey et al., 2014). Postural control has been reported to be impaired during the premenstrual phase (Darlington et al., 2001, Fridén et al., 2003b, Fridén et al., 2005). These variation might at least partly explain the higher injury rate in women than in men (Casey et al., 2014, Darlington et al., 2001, Fridén et al., 2003b, Fridén et al., 2005, Fridén et al., 2006, Zazulak et al., 2006).

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The effects of different sex hormones on skeletal muscle are not fully elucidated. Animal studies support that oestrogen exerts anabolic effects including muscle contractile function, post-exercise muscle repair and regeneration (Skelton et al., 1999, Enns and Tiidus, 2010). The mechanisms seem to involve activation and proliferation of satellite cells (Skelton et al., 1999, Enns and Tiidus, 2010). However, results in humans have been less clear. Some studies in women after menopause support beneficial effects of oestrogen replacement therapy on muscle mass and function, while other studies do not support such effects (Skelton et al., 1999, Enns and Tiidus, 2010). Whether oestrogen variation during the menstrual cycle can influence muscle function is not known.

Little is known about the physiological effect of progesterone on skeletal muscle but recent findings suggest that high circulating levels of progesterone increase protein catabolism (Oosthuyse and Bosch, 2010). Exogenous testosterone is known for its anabolic effect on skeletal muscle (Crewther et al., 2011). However, the effect of endogenous testosterone on muscle tissue is less studied, particularly in women.

Sex steroid hormones promote their effects via classical genomic activation by binding to intracellular nuclear receptors or more rapid non-genomic mechanisms via membrane-bound receptors (Sakamoto et al., 2012). In human skeletal muscle, the nuclear steroid receptors oestrogen receptor (ER) α and β, progesterone receptor (PR) and the androgen receptor (AR) have been detected (Wiik et al., 2003, Wiik et al., 2005, Sinha-Hikim et al., 2004, Copas et al., 2001). Steroid hormone receptors have also been detected in human anterior cruciate ligaments (Liu et al., 1996, Liu et al., 1997, Yu et al., 1999). Oestrogen has been shown to decrease the proliferation of fibroblasts, the synthesis of pro-collagen (Yu et al., 1999) and to lead to decreased musculotendinous stiffness (Eiling et al., 2007). However, the role of sex steroid hormone receptors for variation in neuromuscular performance during the menstrual cycle is not known.
To our knowledge, there are no previous studies on sex steroid hormone receptors in human skeletal muscle during the menstrual cycle. The aim of this study was to investigate mRNA and protein levels of sex hormone receptors in skeletal muscle tissue in three specific phases of the menstrual cycle: the early follicular phase with low levels of estradiol, progesterone and testosterone, the ovulation phase with increased levels of estradiol and testosterone, and in the mid luteal phase with high levels of estradiol and progesterone in healthy women.

Materials and methods

Participants

Thirty-six women volunteered to participate in the study. Thirty of them fulfilled the inclusion criteria for the study: healthy women without any neurological or movement disorder, 20 to 35 years of age, non-smokers, regular menstruation, no oral contraceptives or other hormonal treatment for at least three months. Of the 30 women, 14 did not complete the study and the reasons were missed ovulation in four cases, unpleasant biopsy in nine cases and one woman moved. Furthermore, one individual was excluded after hormonal determination, which did not confirm the specific menstrual cycle phases. Finally, 15 women completed the study with three biopsies. All women gave their written informed consent prior to entering the study which was approved by the Stockholm Regional Ethical Review Board (2006/198-31/1).

Experimental design

Muscle biopsies from the vastus lateralis of the quadriceps femoris muscle were obtained in three different phases of the menstrual cycle; the early follicular phase (cycle day 3-5 of the menstrual cycle), the ovulatory phase detected by daily identification of the luteinizing hormone (LH) surge in urine (Ovustix, Clearplan, Unipath Limited, Bedford, England), and the mid luteal phase (seven days after ovulation). Each subject was randomly examined due to three different schedules in one phase per cycle and in different phases during three consecutive menstrual cycles (Fig 1.). The biopsies
were obtained at rest using the percutaneous needle biopsy technique. All biopsy samples were immediately frozen in isopentane, precoolied in liquid nitrogen, and stored at -80°C until analysed. Blood samples for hormonal assays were collected in the morning the same day as the muscle biopsies were obtained.

**RNA purification and quantification of mRNA levels**

For the extraction of RNA from the muscle biopsies, a combination of Trizol reagent and RNeasy Clean Up kit (Qiagen) was used. Specifically, 50-100 mg of tissue was homogenized with 1 ml Trizol reagent using the Tissuelyser (2 x 5min- 25 Hz; Qiagen). The supernatant, after a centrifuge (10000 rpm, 4°C, and 10 min), was collected and 200 µl chloroform was added at RT for 3 min. Centrifuge at 10000 rpm, 4°C was performed for 15 min and the aqueous phase (approximately 400 µl) was transferred to the RNeasy Clean Up column. Total RNA was purified using the RNeasy Clean Up kit (Qiagen), according to the manufactures’ instructions. 100 ng of total RNA from each sample was reverse transcribed into cDNA using TaqMan Reverse Transcription Reagents (Applied Biosystems) with random hexamer primers. Real-time (RT) PCR assays were conducted using the Applied Biosystems 7500 fast RT PCR system with SYBR green master mix RT-PCR reagent (Applied Biosystems, Stockholm, Sweden). All RT-PCRs were performed in duplicate. Acidic ribosomal phosphoprotein PO (36B4) was used as internal control gene (Akamine et al., 2007). The sequences of the primers are listed in Table 1.

**ELISA analysis of ERα, AR and PR protein levels**

The tissue homogenization procedure was carried out as follows; approximately 200 mg tissue was homogenized with 1 ml of ice-cold lysis buffer (20m M Hepes, 400m M NaCl, 0.1 mM EDTA, 10 mM NaF, 10µM NaMoO₄, 1mM NaVO₃, 20% glycerol, 10 mM PNPP, 10 mM b-glycerophosphate pH 7.5, in the presence of protease inhibitors cocktail 10 mM and DTT 1 mM). Tissue extract was prepared by centrifugation at 10000 g for 10 min at 4°C. The protein concentration of the tissue extract was
calculated, using the Bradford assay method (BioRad). Then, 20 µg protein was used for the
determination of ERα protein levels, while 30 µg protein was used for the determination of PR, and
AR protein levels. In all cases NR Sandwich ELISA by Active Motif was used, according to the
manufactures’ instructions.

**Serum hormone levels**

Blood samples for hormone determination were collected in the morning after 15 minutes of bed
rest. After centrifugation, serum was separated and stored at -20°C until analysed. Enzyme
immunoassays were used for determination of serum levels of follicular stimulating hormone (FSH)
and luteinizing hormone (LH) (AutoDelfia, Perkin Elmer), estradiol and progesterone (Dxl, Beckman
Coulter AB) and sex hormone-binding globulin (SHBG) (IMMULITE Automated Analyser, DPC). Serum
levels of testosterone were determined by a sensitive radioimmunoassay (RIA) (Spectria
testosterone RIA, Orion Diagnostica, Espoo, Finland). Apparent concentrations of free testosterone
were calculated from values of total testosterone, SHBG and fixed albumin concentration of 40 g L-1
by successive approximation using a computer program based on an equation derived from the law
of mass action (Södergård et al., 1982). Detection limits and coefficients of variation were for FSH
0.05 IU L-1, 3.5%; for LH 0.05 IU L-1, 4%; for estradiol 73 pmol L-1, 9%; for progesterone 0.25 nmol L-
1, 14%; for testosterone 0.03 nmol L-1, 7%; and for SHBG 0.2 nmol L-1, 6.5%, respectively.

**Statistics**

Data are presented by mean and standard deviation (SD). A Mixed Model was used with phase as
the within-group factor and three levels (follicular phase, ovulatory phase and luteal phase).
Correlations were performed with Pearson correlation coefficient as well as Spearman rank
correlation. All tests were two sided and the level of significance was set to \( p \leq 0.05 \).
Results

The mean (SD) age, height, weight and body mass index (BMI) of the 15 participating women were 29 (1.2) years, 168 (4) cm, 62 (2) kg and 22 (2) kg/m², respectively. All women were sedentary and reported recreational physical activity only.

Hormone levels

Mean FSH (SD) in the three phases of the menstrual cycle (follicular phase, ovulation phase and luteal phase) were 5.9 (1.2), 7.7 (3.3) and 2.1 (0.8) IU L⁻¹ and mean LH (SD) were 4.8 (1.6), 16.2 (13.2) and 3.0 (2.0) IU L⁻¹, respectively. Hormone levels of estradiol, progesterone and free testosterone in the three phases of the menstrual cycle are presented in Fig. 2. Estradiol levels were low in the early follicular phase and increased as expected in the ovulation phase and midluteal phase. Serum levels of progesterone were also low in the early follicular phase and increased in the midluteal phase compared to the other phases. Levels of free testosterone were significantly increased in the ovulation phase as expected.

mRNA expression levels of steroid hormone receptors

Data on mRNA expression in muscle tissue is presented as relative mRNA levels compared with acidic ribosomal phosphoprotein PO (36B4). There was a significant overall effect (p<0.0001) in ERα levels between the three phases of the menstrual cycle (Fig. 3). ERα mRNA levels were highest in the follicular phase of the menstrual cycle, significantly higher than in the ovulatory phase (p<0.05) and the luteal phase (p<0.001). Furthermore, ERα levels were significantly higher in the ovulatory phase compared to the luteal phase (p<0.001). The mRNA levels of ERβ were in general undetectable in all three phases (data not presented). There was also a significant overall difference in PR mRNA levels between the phases (p<0.05) (Fig. 3). PR mRNA levels were lowest in the luteal phase, significantly lower than in the ovulatory phase (p<0.01) with the same tendency for the follicular phase (p=0.06). There was no difference in AR mRNA levels between the phases (Fig. 3).
Protein levels of steroid hormone receptors

Protein levels of ERα displayed a similar pattern across the menstrual cycle as the mRNA levels (p<0.01). Thus, the levels of ERα protein were highest in the follicular phase, and significantly higher than in the luteal phase (p<0.01) with a trend to be higher compared to the ovulatory phase (p=0.07) (Fig. 4). Since ERβ mRNA was not detected, analysis of protein levels was not performed. There was also a significant overall effect across the menstrual cycle for protein levels of PR (p<0.01). However, in contrast to PR mRNA levels, PR protein levels were highest in the luteal phase of the menstrual cycle and significantly higher than both the follicular phase (p<0.01) and the ovulatory phase (p<0.01) (Fig. 4). There was no significant difference for protein levels of AR over the menstrual cycle.

There were no significant correlations between steroid hormone levels and steroid receptor levels for the three different phases of the menstrual cycle (data not shown).

Discussion

To our knowledge, this is the first study reporting mRNA and protein levels of steroid hormone receptors in human skeletal muscle during the three different phases of the menstrual cycle. The main findings were significant variations in mRNA and protein levels of ERα and PR across the menstrual cycle with the highest mRNA and protein levels of ERα in the follicular phase when estradiol levels were low. In contrast, mRNA and protein levels of PR were not consistent. The lowest PR mRNA levels were detected in the luteal phase when progesterone levels are high, whereas PR protein levels were highest in this phase. No significant variation was detected in AR mRNA or protein levels over the menstrual cycles. ERβ mRNA was close to undetectable in all three phases and therefore we did not proceed to determine ERβ protein levels.
Expression of ERα and ERβ in human skeletal muscle at the mRNA and protein levels have been demonstrated in small cross-sectional studies of both men and women (Wiik et al., 2003). The mRNA expression of ERα was 180 fold higher than that of ERβ, whereas immunohistochemistry demonstrated positive staining only for ERβ and not for ERα (Wiik et al., 2003). Our study is consistent with the data by Wiik et al (Wiik et al., 2003) showing that ERα mRNA levels are much higher than ERβ mRNA levels. As opposed to the studies by Wiik et al, (Wiik et al., 2003, Wiik et al., 2005) we observed consistently high levels of both ERα mRNA and of ERα protein. It is possible that the use of different methods explain the different results for the two studies. In the studies by Wiik et al. (Wiik et al., 2003, Wiik et al., 2005) an immunohistochemistry protocol was used to detect ERα and ERβ protein, whilst in the present protocol an ELISA assay was explored for detection of ERα protein. Additionally, the studies by Wiik et al (Wiik et al., 2003, Wiik et al., 2005) include only four and six samples, respectively.

To our knowledge, there are no studies of oestrogen receptors in skeletal muscle during different phases of the menstrual cycle. We found the highest mRNA and protein levels of ERα in the early follicular phase, the levels then decreased to be lowest in the mid-luteal phase. In comparison, steroid hormone receptors have been studied in the endometrium during the menstrual cycle. In this tissue, the expression of both ERα and ERβ increases during the follicular phase to the highest levels at ovulation and then gradually decreases during the luteal phase (Giudice, 2006). Animal studies have also shown a significant variation of ERα immunoreactivity in bovine cervix and vagina during the sexual cycle with greatest intensity during the follicular phase (Sagsöz et al., 2010), as well as, fluctuating expression of ERβ in the rhesus monkey corpus luteum during the menstrual cycle (Duffy et al 2000).
Animal studies suggest an anabolic effect of oestrogen on skeletal muscle (Skelton et al., 1999, Enns and Tiidus, 2010), and some studies in women have indicated a better trainability of muscle strength in the follicular phase compared to the luteal phase of the menstrual cycle (Reis et al., 1995, Sung et al., 2014). Potential mechanisms to explain the anabolic effects of oestrogen on skeletal muscle have been described, but some of these effects have only been studied in animals and have not been fully delineated in humans (Enns and Tiidus, 2010). Furthermore, oestrogen seems to have a direct effect on connective tissue and to decrease musculotendinous stiffness (Eiling et al., 2007). However, if short-term changes in oestradiol levels during the menstrual cycle can explain variation in knee joint laxity and/or affect muscle growth and strength is still not clear.

Progesterone receptors have also been detected in skeletal muscle (Copas et al., 2001), but the physiological effects of progesterone on this tissue is still not fully understood. Serum levels of progesterone are high in the luteal phase, and recent studies have suggested an increased amino acid catabolism during this phase of the menstrual cycle (Oosthuyse and Bosch, 2010, Reis et al., 1995, Sung et al., 2014). Furthermore, better trainability of muscle strength in the follicular phase than the luteal phase as mentioned above could also be explained by the possible catabolic effect on muscle tissue of progesterone in the luteal phase (Reis et al., 1995). In agreement, we found the highest levels of protein expression of PR in this phase of the menstrual cycle.

The muscle stretch reflex as well as postural control seem to change over the menstrual cycle (Casey et al., 2014). Impaired postural control has been detected in the luteal phase when progesterone is high, but only in women with premenstrual symptoms (Fridén et al., 2005). The neurosteroid allopregnanolone increases during the luteal phase and has been shown to induce disturbances in motor function (Söderpalm et al., 2004). Furthermore, impaired concentration, which is one of the typical premenstrual symptoms, might also have an impact (Bolmont et al., 2002). Thus, a central

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effect by the fluctuating sex hormone levels during the menstrual cycle on neuromuscular control could not be excluded.

Testosterone is a well known anabolic steroid (Crewther et al., 2011). However, the effect of endogenous testosterone in women is less studied. Testosterone is under LH control and therefore the serum levels are low during the early follicular phase and highest just prior to or during ovulation (Jabbour et al., 2006). AR has been detected in skeletal muscle (Copas et al., 2001) but has not, to our knowledge, been studied in different phases of the menstrual cycle. In the endometrium, AR is gradually decreasing from the early follicular phase until the mid-luteal phase (Mertens et al., 1996). However, in the present study there was no significant difference in AR between menstrual cycle phases.

There was a high dropout rate of subjects mainly because of discomfort when collecting biopsies. Still, we succeeded to collect biopsies in three hormonally verified phases in 15 subjects, which to our knowledge makes this study the largest in the literature. Furthermore, it is difficult to perform studies in different phases of the menstrual cycle when subjects are supposed to detect ovulation with LH surge in urine. In some cases the women failed to detect ovulation exactly, which resulted in delayed sampling during this phase of the menstrual cycle.

We used NR Sandwich ELISA methods to analyse the steroid receptor content of tissue samples. This method uses two antibodies that recognize distinct epitopes on steroid receptors providing high sensitivity and specificity (Schuurs and van Weemen, 1980). However, one limitation is that the subcellular distribution of the protein is not revealed using this method.

In conclusion, this well controlled study shows significant variation in mRNA and protein levels of ERα and PR in skeletal muscle during three different phases of the menstrual cycle. The importance of these results needs further investigation. However, variation in levels of sex steroid hormone
receptors in muscle tissue during the menstrual cycle might influence neuromuscular performance, effect of training and the risk of sustaining sports injuries.

Conflict of interest

All authors declare no conflicts of interest.

References


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Tables

Table 1. Primer pairs employed to quantify mRNA levels of receptors (ERα, ERβ, AR, PR) and the internal control gene, acidic ribosomal phosphoprotein PO (36B4).

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<td>GTG TTC GAC AAT GGC AGC AT GAC ACC CTC CAG GAA GCG A</td>
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Figure legends

Figure 1. Three different schedules for muscle biopsies.

Figure 2. Hormone levels for estradiol, progesterone and free testosterone in the three phases of the menstrual cycle, follicular phase (FP), ovulatory phase (OP) and luteal phase (LP). Box plots showing median and 25-75% and bars showing non-outlier range.

Figure 3. Relative mRNA levels of ERα, PR and AR in the three phases of the menstrual cycle, follicular phase (FP), ovulatory phase (OP) and luteal phase (LP). Box plots showing median and 25-75% and bars showing non-outlier range.
Figure 4. Protein levels of ERα, PR and AR. For ERα a recombinant protein was used to make a standard curve, for PR and AR cell extract was used to make a standard curve. Measured levels of ERα, PR and AR are normalized to ug of total protein. Box plots showing median and 25-75% and bars showing non-outlier range.

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