Author(s): Pöllänen, Eija; Ronkainen, Paula; Horttanainen, Mia; Takala, Timo; Puolakka, Jukka; Suominen, Harri; Sipilä, Sarianna; Kovanen, Vuokko

Title: Effects of combined hormone replacement therapy or its effective agents on the IGF-1 pathway in skeletal muscle

Year: 2010

Version:

Please cite the original version:

Pöllänen, E., Ronkainen, P., Horttanainen, M., Takala, T., Puolakka, J., Suominen, H., Sipilä, S., & Kovanen, V. (2010). Effects of combined hormone replacement therapy or its effective agents on the IGF-1 pathway in skeletal muscle. Growth Hormone and IGF Research, 20(5), 372-379. https://doi.org/10.1016/j.ghir.2010.07.003

Effects of Combined Hormone Replacement Therapy or its Effective Agents on the IGF-1 pathway in Skeletal Muscle

Eija Pöllänen*[,a,b], Paula H.A. Ronkainen*[,a,b], Mia Horttanainen[a], Timo Takala[c], Jukka Puolakka[d], Harri Suominen[a], Sarianna Sipilä[a,b], Vuokko Kovanen[a,b] .

*contributed equally to the work

[a]Gerontology Research Centre, University of Jyväskylä, P.O. Box 35, FIN-40014 University of Jyväskylä, Jyväskylä, Finland
[b]Department of Health Sciences, University of Jyväskylä, P.O. Box 35, FIN-40014 University of Jyväskylä, Jyväskylä, Finland
[c]Oulu Deaconess Institute & Department of Health Sciences, University of Oulu, Oulu, Finland
[d]Central Finland Central Hospital, Jyväskylä, Finland

Corresponding author:
Vuokko Kovanen
Gerontology Research Centre
Department of Health Sciences
University of Jyväskylä
P.O. Box 35
FIN-40014 University of Jyväskylä
Tel. +358 14 260 2163
Fax. +358 14 260 4600
Email: vuokko.kovanen@jyu.fi

Running title: Effects of HRT on IGF-1 pathway

ABSTRACT

Objectives: To investigate the effects of combined hormone replacement therapy (HRT) and its effective agents on the IGF-1 signaling pathway.

Design and methods: To examine the effects of HRT on skeletal muscle *in vivo,* we utilized pre- and post-intervention samples from a randomized double blinded trial with 50-57-year-old women. The intervention included the year-long use of either HRT preparation (2 mg 17β-estradiol, E_2; 1 mg norethisterone acetate, NETA, n=10) or placebo (CO, n=9). Microarray technology and quantitative PCR (qPCR) were used to study the expression of *insulin-like growth factor I* (*IGF-1*) and its splice variants as well as *IGF-1 receptor, Akt1, mTOR, FOXO1, FOXO3, atrogin, estrogen receptors* and *androgen receptor* in muscle samples. Serum concentrations of IGF-1, E_2 and testosterone were measured. C2C12 myotubes were fed with E_2 or NETA followed by analyzing the expression of essentially the same gene transcripts as in human samples by qPCR and phosphorylation of Akt and mTOR by Western blotting.

Results: The gene expression of *IGF-1* and its splice variant, *IGF-1Ec* (also known as the mechano growth factor or MGF), *mTOR*, *FOXO3*, and *AR* was up-regulated among the HRT users compared to the CO ($P<0.05$), while *Akt1* was down-regulated ($P<0.05$). The change in the level of *IGF-1Ec* transcript correlated positively with muscle size at post-intervention ($r=0.5$, $P<0.05$). In C2C12 myotubes, no statistically significant effects of either E_2 or NETA at the level of gene transcripts studied were identified. The amount of phosphorylated Akt appeared to respond to NETA, albeit the response was not statistically significant. Phosphorylation of mTOR did not respond to either of the treatments.

Conclusion: Year-long postmenopausal HRT was found to affect the expression of the genes along the IGF-1 signaling cascade reflecting the higher muscle mass compared to the CO women. By using cell culture model we were, however, unable to confirm the possible differential role of E_2 and NETA. It appears that synchronous presence of both effective agents of the HRT or the presence of yet unidentified microenvironmental factors providing proper paracrine signals naturally existing in the intact muscle tissue are critical for appropriate signaling via sex steroid-IGF-1 axis to occur.

Keywords: hormone replacement therapy, IGF-1 signaling, muscle, estradiol, norethisterone acetate, postmenopausal women

INTRODUCTION

Insulin-like growth factor I (IGF-1) signaling pathway is one of the key regulatory pathways of muscle mass [1]. Together with insulin and IGF-II, IGF-1 is the only extracellular growth factor known to promote the terminal differentiation of myoblasts thus enhancing muscle growth [2, 3]. The respective pathway is activated as IGF-1 binds to its receptor, which initiates a phosphorylation cascade further leading to activation of the renowned Akt (also referred to as protein kinase B and Rac). Akt is a multifunctional protein kinase, which has been shown to exhibit an essential role in the control of cell growth, cell survival and apoptosis [4-6] and contribution to the regulation of glycogen synthesis is evident as well [7, 8]. A significant proportion of the growth promotion by IGF-1 and Akt results from the induction of protein synthesis. At least a part of the positive effect on muscle mass comes through Akt-activated mammalian target of rapamycin (mTOR), which further activates a pathway leading to increase of protein synthesis [9, 10]. In addition, Akt is reported to attenuate the activity of forkhead box O (FOXO) transcription factors [11, 12]. FOXOs induce the transcription of atrophy genes [4], muscle ring finger protein 1 (MuRF1, also known as RNF28) and atrogin (also known as FBXO32 and MAFbx), and reduces the expression of the transcript for androgen receptor (AR) [13]. The phosphorylation of FOXO by Akt results in its sequestration in the cytoplasm away from the target genes [14].

In our previous study, the Exercise and Hormone Replacement Therapy-trial (Ex/HRT), we examined the effects of a year-long power training and HRT on bone and muscle structure and function in early postmenopausal women [15-17]. We found for instance that HRT induced an average 6 % increase in knee extensor muscle cross-sectional area and 7 % increase in jumping height [17]. Also other studies have documented that HRT increases muscle mass, improves body and muscle composition or promotes muscle function [18-23]. A direct involvement of HRT as a

modulator of the effects of IGF-1 signaling in skeletal muscle has been poorly, if at all studied. However, a clue concerning the involvement of the respective pathway in this context comes from the study of Vasconsuelo and colleagues [24]. They reported that 17β-estradiol (E_2) activated the signaling cascade including PI3K and Akt in an estrogen receptor-dependent manner in C2C12 myoblasts.

The purpose of the study was to investigate the effects of estrogen- and progestogen-containing HRT and its effective agents, E_2 and norethisterone acetate (NETA), on the expression of genes along the IGF-1 signaling pathway. To build up a general view concerning the *in vivo* effects of HRT on the pathway as a whole, we assessed the expression of genes encoding IGF-1 and its splice variants, IGF-1 receptor, Akt1, mTOR, FOXO1, FOXO3 and atrogin and further analyzed, whether the expression of these genes changed differently among women using HRT in comparison with the non-users during the one year intervention. We also measured the expression of genes encoding estrogen receptors (ESR1 and ESR2) and androgen receptor (AR), since these receptors may deliver the signal induced by HRT or its components. Furthermore, we utilized an *in vitro* model to examine, if the possible effects of combined HRT are due to E_2 or its progestogenic counterpart, NETA, used in the respective preparation. According to our hypothesis the progestogenic agent may not represent just a passive component, but rather change the molecular milieu in muscle tissue together with estradiol.

MATERIALS AND METHODS

Study design and intervention

Details of the study design and interventions of the year-long Ex/HRT trial (ISCTN number ISRCTN49902272) are described in detail elsewhere [17]. Briefly, 1333 women at age 50 to 57

years were enrolled from which 118 went through an extensive medical and physical examination including determination of their menopausal status, as well as eligibility to the study (no serious medical conditions; no current or previous use of medication including estrogen, fluoride, calcitonin, biophosphonates or steroids; last menstruation at least six months but no more than 5 years ago; FSH level above 30 IU/L; no contraindications for exercise and HRT). Finally, 80 women were randomly assigned to one of the four study groups: power training (PT, n=20), HRT (n=20), PT+HRT (n=20) and control (CO, n=20). Since the current study focuses on the effects of HRT, only the HRT and CO groups were included in further analysis. From these groups ten women discontinued the intervention and both baseline and post-intervention muscle samples from eleven women were not available. Final group sizes were ten for the HRT and nine for the CO groups.

HRT was conducted double blinded. All study participants used either continuous, combined HRT preparation (2 mg of E_2, 1 mg of NETA, Kliogest, Novo Nordisk, Copenhagen, Denmark) or placebo one tablet every day. Pure effective agents of the HRT, namely E_2 and NETA (both from Novo Nordisk), were used in cell culture experiments. The study was carried out in accordance with the Declaration of Helsinki (1989) of the World Medical Association and was approved by the ethics committee of the Central Finland Health Care District. Informed consent was given by all participants.

Anthropometrics

Body mass index (BMI) was calculated from height and weight measured with standard procedures. Lean body mass (LBM) and body fat mass were assessed using bioelectrical impedance (Spectrum II, RJL Systems, Detroit, MI). Thigh muscle mass was assessed with computed tomography (Siemens AG, Erlangen, Germany) and analyzed using BonAlyse software (version 1.0, Commit Ltd, Espoo, Finland) as previously described [17].

Serum hormone measurements

Blood samples were taken from the antecubital vein in a supine position between 07.00 and 10.00 AM. Serum samples were collected at baseline and post-intervention during the same day as muscle sampling was performed. The aliquoted sera were stored in -70 °C until analyses. Serum concentration of IGF-1 was measured using solid-phase, chemiluminescent immunometric assay (Immulite 1000, Diagnostic Products Corporation, Los Angeles, CA). Serum concentration of E_2 and testosterone were measured using time-resolved fluoroimmunoassay (DELFIA, Wallac, Turku, Finland). The intra-assay coefficient of variation for detection of IGF-1, E_2 and testosterone was 4.3%, 3.8% and 8.2%, respectively.

Muscle biopsy sampling

Muscle biopsies were obtained from the mid-part of *m. vastus lateralis* defined as a midpoint between the greater trochanter and the lateral joint line of the knee both at baseline and after completion of the intervention as previously described [25]. The biopsy protocol was standardized within the study in order to avoid variation due to sampling. Both baseline and post-intervention muscle samples for the current analysis were available from ten participants in the HRT and nine in the CO group.

Microarray experimentation: RNA preparation, cRNA generation and array hybridization

RNA preparation, cRNA generation and array hybridization procedures have been previously described [25]. Briefly, total RNA was extracted (Trizol, Invitrogen, Carlsbad, CA) from the muscle samples homogenized with FastPrep FP120 apparatus (MP Biomedicals, Illkrich, France). The Experion equipment (Bio-Rad Laboratories, Hercules, CA, USA) was used to inspect RNA concentration and quality. Biotin-labeled cRNA from 500 ng of total RNA was produced (Illumina

RNA amplification kit, Ambion, Austin, TX) and quality controlled (Experion) before hybridizations. The samples were hybridized to the HumanRef-8 v1.0 or HumanWG-6 v1.0 BeadChips (Illumina Inc., San Diego, CA) beadchips. Both baseline and post-intervention samples from each participant were always hybridized onto the same chip. The chips were scanned by confocal laser scanning system (Illumina BeadReader Rev. C, Illumina Inc.). The data were acquired by the BeadStudio Direct Hybridization V.1.5.0.34. The MIAME guidelines were followed during array data generation, preprocessing and analysis. The complete microarray data is accessible through GEO Series accession number GSE16907 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE16907).

Microarray data preprocessing and validation

Gene expression data from ten users and five non-users was produced by HumanRef-8 BeadChips [26] and from four non-users by HumanWG-6 BeadChips [27]. Data from these two separate array types were combined and validated as presented in [27]. Briefly, hybridization of both samples from three CO subjects onto both Illumina platforms followed by a correlation analysis of the performance between the array types indicated that the resemblance of the two platforms was high enough (Pearson correlation coefficient, r=0.88-0.94) to allow the combination of the data. However, using two different platforms caused detectable batch effect, which was corrected by using array type as a covariate in statistical testing. Before analysis, the microarray data was quantile normalized utilizing the affy package [28] of the R/Bioconductor analysis software (www.r-project.org, www.bioconductor.org).

Cell culture, differentiation and hormonal treatments

C2C12 murine (*Mus Musculus*) skeletal muscle cells (ATCC, LGC Standards AB, American Type Culture Collection, Manassas, VA) were maintained in Dulbecco's Modified Eagle Medium

(DMEM) with GlutaMAX^{TM} (Gibco, InVitrogen) supplemented with 10 % inactivated fetal bovine serum (FBS) and 1 % penicillin-streptomycin (both from InVitrogen). Cells were grown in monolayer cultures in a humidified 5% CO_2 atmosphere at +37ºC and subcultured as 70-80 % confluence was reached. Myoblasts were differentiated into myotubes for six days with DMEM+GlutaMAX^{TM} supplemented with 2 % horse serum (HS, Invitrogen) and 1 % penicillin-streptomycin. The E_2 and NETA treatments were launched as the cells exhibited high degree of differentiation and only a minute amount of myoblasts were detected.

Myotubes were incubated for one hour in serum-free DMEM without phenol red supplemented and with 1 mM sodium pyruvate, 2 mM L-glutamine and 1 % penicillin-streptomycin (all from InVitrogen). After this pre-incubation, the cells were treated with 1 nM and 10 nM of either E_2 or NETA. The dilutions were made into the above-mentioned medium, which was applied to the control cells as well. The cells for the time point of 0 h served as a basal control. In addition to this baseline control, control cells with no hormones in the treatment medium were collected at each time point. Cells were collected at 0 h, 2 h, 6 h, and 24 h for RNA analysis and at 0 h, 5 min, 20 min, 40 min, and 2 h for the analysis of protein phosphorylation. At least three independent experiments were carried out. Total RNA was extracted with RNeasy Mini kit (QIAGEN Corp., Gaithersburg, MD). RNA concentration and purity were measured with NanoDrop equipment (NanoDrop products, Thermo Fisher Scientific Inc., Wilmington, DE). Cells for Western blotting were homogenized with a 20G needle in fresh, ice-cold homogenization buffer (20 mM Hepes, pH 7.4, Sigma-Aldrich, St. Louis, MO; 1mM EDTA, IDRANL®III, Sigma; 5 mM EGTA, Sigma; 10 mM magnesium chloride, Merck & Co. Inc., Whitehouse Station, NJ; 100 mM β-glyserofosfaatti, Sigma; 1 mM sodium orthovanadate, Sigma; 2 mM DTT, Sigma; 1 % Triton X-100, Fluka Chemie GmbH, Buchs, Switzerland; 40 µg/ml leupeptin, Fluka; 40 µg/ml aprotinin, Sigma, 80 µg/ml PMSF, Sigma; 1 µl/100 µl phosphatase inhibitor cocktail, Sigma) during 15 min incubation on ice followed by

centrifugation (12 000 g, 10 min, +4ºC) and collection of the supernatant. Total protein concentration was assessed using BCA Protein Assay Kit (Pierce, Rockford, IL).

Real Time RT-PCR

One microgram of RNA from both the muscle samples (TaqMan Reverse Transcription Reagents, N808-0234, Applied Biosystems, Foster City, CA) and cell cultures (High Capacity cDNA Reverse Transkription Kit, Applied Biosystems) was reverse transcribed into cDNA for Real Time RT-PCR analysis. Before cDNA synthesis the RNA from the muscle biopsies was subjected to DNase treatment (Turbo DNA-free kit, AM1907, Ambion). All the gene expression kits were ordered from Applied Biosystems. For human samples, unique TaqMan gene expression assays were designed for each of the IGF-1 splice variants, *IGF-1Ea* (forward primer: AGCGCCACACCGACATG, reverse primer: TCCCTCTACTTGCGTTCTTCAAA, probe: CAAGACCCAGAAGGAAGTA), *IGF-1Eb* (forward primer: GAGGAGCAGACAGCAAGAATGA, reverse primer: CCAGCAGGCCTACTTTTCTTCA, probe: AAGCAGAAAATACAATAGAGG) and *IGF-1Ec* (forward primer: CACGAAGTCTCAGAGAAGGAAAGG, reverse primer: CTTGTTTCCTGCACTCCCTCTAC, probe: AAGTACATTTGAAGAACGCA), since the probe for *IGF-1* used in the microarray platforms do not separate three different isoforms. Taqman Gene Expression Assays were utilized for cell samples to investigate the expression of *IGF-1* (Mm01233960_m1), *ESR1* (Mm 00433149_m1), *ESR2* (Mm 01281854_m1,) *AR* (Mm 01238475_m1), *Akt1* (Mm 01331624_m1), *mTOR* (Mm00444968_m1), *atrogin-1* (Mm01207879_m1), *Foxo1(a)* (Mm00490672_m1), and *Foxo3a* (Mm00490673_m1). All RT-PCR assays were run with an Applied Biosystems' ABI 7300 unit using standard PCR conditions recommended by the manufacturer. Each sample was run in triplicate and the reference sample was included in all plates in order to control for inter-assay variation. *GAPDH* (Hs99999905_m1) and *18S* (Hs99999901_s1) were tested with human samples as candidates for endogenous control and

GAPDH chosen due to its better reliability. The corresponding gene was used as control in the cell samples as well (*GAPDH*: Mm 99999915_g1). The dilution series of the reference sample was used as a standard curve. The mean intra-assay coefficient of variation was 8.2 % for our Real Time RT-PCR assays.

Western blotting

The homogenized samples (35 μg protein/sample) were solubilized in 6x Laemmli buffer, heat-denatured (5 min, 95°C), separated by 4-15 % gradient gels (Bio-Rad Laboratories) for 90 min (p-AKT) or 120 min (p-mTOR), and transferred to nitrocellulose membrane (Amersham^{TM}Hybond^{TM}-ECL, GE Healthcare UK Ltd, Little Chalfont, England) at 300 mA (p-AKT) or 400 mA (p-mTOR) constant current for 180 min on ice at +4ºC. The uniformity of blotting was checked by staining the membrane with Ponceau S. Samples from a single treatment were run on the same gel. The blots were analyzed using phosphospecific rabbit polyclonal primary antibodies recognizing Akt on Ser^{473} and mTOR on Ser^{2448} (both 1:1000 and from Cell Signaling Technology, Beverly, MA). After incubation with the secondary antibody (horse radish peroxidase-conjugated anti-rabbit IgG, A9169, Sigma, 1:40 000) the phosphorylated proteins were visualized by enhanced chemiluminescence utilizing Amersham^{TM} ECL Advance^{TM} Westernblotting Detection Kit (GE Healthcare) according to manufacturer's protocol. Quantification of specific protein bands was performed using a ChemiDoc XRS together with Quantity One software (version 4.6.3, Bio-Rad Laboratories).

Data analysis

Univariate analysis of variance with the baseline measurement, and when appropriate the array type as covariates was used to compare the changes in the gene expressions between the HRT and CO groups. When a significant F-score was found the Bonferroni *post hoc* procedure was employed to locate the source of significant differences in means. The same analysis strategy was applied for

serum concentrations as well. Analysis of variance for repeated measures was applied for cell culture data to investigate whether different treatments affected the amount of given gene transcripts or phosphorylated proteins.

RESULTS

The basic characteristics and serum hormone concentrations of the study participants are presented in Table 1. The mean age (±SD) of the participants was 53.3 ± 1.8 yr. The HRT users and non-users had very similar mean BMI at the baseline and post-intervention measurements (change in the HRT users vs. non-users: 0.6 % vs. -0.1 %, P=0.15). The HRT users significantly increased their LBM compared to the non-users (change in the HRT users vs. non-users: 2.1 % vs. -0.7 %, P=0.03). The opposite, albeit not significant, trend was seen for percent body fat. The cross-sectional area of thigh muscle increased 8.6 % among the HRT users and 1.5 % among the non-users (P for trend 0.003). As shown in Table 1, HRT had no effect on the serum IGF-1 or the serum testosterone concentrations between the users and the non-users. An expected increase in the concentration of E_2 was observed among the HRT users (214 %, P<0.001).

The intramuscular expression of *IGF-1* gene was up-regulated among the HRT users (13 %) compared to the CO (-16 %, P=0.014, Fig 1). The level of *IGF-1 receptor* was too low to be determined accurately in the microarray data. All three splice variants of *IGF-1*, i.e., *IGF-1Ea*, *IGF-1Eb* and *IGF-1Ec*, also known as the *mechano growth factor* or *MGF*, were up-regulated in the HRT group compared to the CO group, in which their expression was down-regulated (change in the HRT users vs. controls: *IGF-1Ea*: 62 % vs. -30 %, P=0.10; *IGF-1Eb*: 10 % vs. -61 %, P=0.31; *IGF-1Ec*: 58 % vs. -31 %, P=0.003, Figure 2). However, the only significant change between the groups was observed in the expression of *IGF-1Ec*. One participant had extremely high fold change

(>40). However, excluding this individual from the analysis, did not change the significance of the analysis (P=0.005). Intriguingly, the change in the muscle expression of *IGF-1Ec* was associated with muscle mass measured at post-intervention (r=0.50, P=0.035, Fig 3), but no such significant correlation with muscle mass was seen for any other splice variant of IGF-1 or other genes investigated.

Figure 1 shows that the gene expression of *Akt1* was down-regulated in the HRT group (-8 %) compared to the CO (0.7 %, P=0.036). The expression of *mTOR* was slightly, but significantly up-regulated in the HRT group (2 %) compared to the CO, in which it was down-regulated (-13 %, P=0.043). On the other hand, the expression of *FOXO3*, representing the key molecule for atrophy signaling, was also up-regulated in the HRT group (2 %) compared to the CO (-9 %, P=0.021). The expression of *FOXO1*, exhibited a similar trend (change in the HRT group vs. controls: 2 % vs. 9 %, P=0.06), but was not statistically significant even though the absolute expression level of *FOXO1* was much higher than that of *FOXO3*. The downstream target of FOXO proteins, *atrogin*, was not expressed significantly differently between the HRT and CO groups (change in the HRT users vs. non-users: 23% vs. 15%, P=0.36). The expression of *AR* was clearly up-regulated among the HRT users (12 %) compared to the CO (-8 %, P=0.001). The level of *ESR1* did not differ significantly between the HRT and CO groups (change in the HRT users vs. controls: 6 % vs. 11 %, P=0.36). The transcript level of *ESR2* was too low to be reliably detected from the array data.

To dissect the possible role of the effective agents of HRT on the modulation of the expression of genes or on the activation of proteins along the IGF-1 signaling pathway, we used an *in vitro* cell model, in which either E_2 or NETA were fed to C2C12 derived myotubes. In our hands neither of these compound were able to induce statistically significant changes on the levels of transcripts investigated (P>0.05, data not shown). Even though a clear indication of the effect of 10 nM NETA

on p-Akt was seen, it did not reach statistical significance (Fig. 4). Moreover, no significant effects of E_2 or NETA on the phosphorylation status of Akt or mTOR were observed (Fig 4).

DISCUSSION

This is the first study to investigate the effects of a combined HRT and the effective agents of the same HRT preparation (E_2 and NETA) on the IGF-1 signaling pathway. Our results indicate that one-year use of HRT may change the expression of several genes along the IGF-1 signaling cascade in comparison with the non-users. This notion may perhaps hold relevance, since the observed effects on the gene expression along the IGF-1 signaling route in skeletal muscle coincides with improved muscle mass. Several RCTs have previously shown that the use of postmenopausal HRT has positive effect on either the whole body composition or directly on muscle mass [15, 17, 21, 22, 29, 30], while in others the effects were not found [31-35]. Our study goes deeper into the detailed molecular mechanisms through which HRT may exert at least a part of its effects leading to preservation of muscle mass after menopause.

The change in the expression of one splice variant of *IGF-1* gene, the *IGF-1Ec*, which is better known as the *MGF*, was associated with post-intervention muscle mass. MGF, which was first identified as a factor responding to muscle contraction, is nowadays regarded as a major activator of muscle satellite cells and as a direct growth factor [36]. Even though the synthetic MGF E-peptide has been shown to promote cellular proliferation and survival the actual role or even existence of the endogenous MGF E-peptide has recently confronted criticism [37]. However, there is no doubt that the *IGF-1Ec* mRNA exists and coincides with improvements in muscle mass. Hereby, greater muscle mass observed among the HRT users may hypothetically be a result from improved muscle repair systems, as well as, activation of growth promoting signaling cascades even though we do

not know whether the effect is due to related (cleaved or not cleaved) E-peptide or the whole pro-IGF-1Ec. In our recent transcriptome-wide study, the expression of several myogenesis-related genes was found to be up-regulated in muscles of both the HRT users and the non-users suggesting an increased demand for muscle regeneration in the early stage of the postmenopause [27]. Studies investigating the influence of HRT or estrogen on exercise induced myogenesis or on satellite cell proliferation have also concluded that the preservative function of HRT on muscle mass may emerge from improved muscle regeneration due to activation of satellite or other adult stem cells [38-42]. These studies support our results suggesting that HRT may modulate its positive effects on body composition and muscle mass possibly through the activation of IGF-1-related signaling.

We found no difference between the HRT and the CO groups in the serum levels of testosterone and IGF-1. This indicates that the observed increment in muscle mass is not reflected by endocrine effects of IGF-1 or testosterone, but rather is due to the local activation of the IGF-1 pathway through autocrine or paracrine manner in response to HRT. To our knowledge, only two previous studies have tried to dissect the role of separate HRT regimens [18, 22] and none the role of different effective agents of the combined HRT preparation on body composition or muscle mass. Thereby, the question of how and which components of the HRT is able to activate the IGF-1/IGF-1Ec pathway or induce the expression of its components is open to dispute.

According to our hypothesis the effects of HRT on IGF-1 signaling are delivered through downstream components of this pathway, namely Akt, mTOR, FOXO and atrogin or through steroid hormone receptors such as ESRs or AR. Our results indicated that the gene expression of *mTOR*, *FOXO3* and *AR* is significantly up-regulated in the muscle tissue of the HRT users compared to the non-users while *Akt1* was down-regulated. Akt, mTOR and AR are considered as anabolic factors whereas FOXO regulates atrophy signaling. The discrepancy between anabolic and

catabolic signals observed at the level of gene expression may be due to combined effects of E_2 and NETA. Therefore, a cell culture setting with myotubes fed with the respective agents was utilized. However, we were unable to identify differential effects on transcription in this model.

Since the level of gene expression does not always reflect the true activation of signaling pathways, we analyzed the effects of E_2 and NETA on the phosphorylation of Akt and mTOR. Akt1 is known to be activated by phosphorylation at two key residues: at Thr^{308} by PDK1 and at Ser^{473} by mTOR [43-45]. An indication of the possible effect of NETA on Akt at Ser^{473} was observable, but statistical significance was blurred by high variation between the triplicates (Fig. 4C). More specifically, the 10 nM treatment was observed to induce the phosphorylation of Akt even up to about five fold suggesting that NETA may exert an androgenic effect on the pathway under investigation, although the findings were not significant. A recent study by Vasconsuelo and colleagues reported that E_2 induces the phosphorylation of Akt at Ser^{473} in single-nucleated, undifferentiated C2C12 myoblasts. A striking difference in comparison with our multinucleated myotubes was that their myoblasts had no p-Akt in control cells, whereas p-Akt was expressed in our differentiated, control myotubes during the entire time frame studied. Importantly, the activation of Akt is known to govern the early and late steps of myogenic differentiation [4]. Our notion is supported by this fact and a recent study documenting that p-Akt is present between 24 and 120 hours during the differentiation of myoblasts into myotubes [46]. Thus, we may have been unable to catch the possible effects of the hormonal treatments due to high phosphorylation of Akt already present owing to myogenesis, albeit several different concentrations of the hormones and time points of treatments were tested during optimization of the experimental procedure. Therefore, it is possible that we were unable to observe the changes in phosphorylation status of Akt due to too narrow time window.

Activation of mTOR, a key regulator of muscle protein synthesis [47], was studied using phosphospecific (Ser^{2448}) antibody. Since Akt is a direct activator of mTOR [9], but also mTOR can activate Akt [44] these two kinases form a complex activation loop involved in the regulation of muscle hypertrophy and atrophy. However, neither E_2 nor NETA was found to affect the amount of p-mTOR. There are only a limited number of studies investigating the effects of estrogen or progesterone on the activation of mTOR and, to the best of our knowledge, none using murine muyotubes as a model. For instance, Jaffer *et al.* were able to demonstrate estradiol-mediated up-regulation of the amount of p-mTOR in rat uterine myocytes [48], while, by using bovine mammary epithelial cells, Sobolewska and colleagues showed that both estradiol and progesterone suppressed the phosphorylation of mTOR thereby supporting our results [49]. As in the case of p-Akt, however, we cannot dissect out the possibility that high initial phosphorylation status of mTOR may have restrained us from detecting true induction of phosphorylation. These contradictory results from different cell types indicate the presence of some cell type specific effects of estrogen and progesterone.

Aside from the traditional anabolic signaling via activation of Akt/mTOR, IGF-1 or its splice variants may induce its effects on skeletal muscle through other pathways as well [50, 51, 52]. Our results concerning the induction of IGF-1 gene expression by HRT is supported by a previous study reporting that E_2 treatment increases the level of IGF-1 in the rat uterus [53] and natural progesterone in glial cells [54]. Also testosterone has been shown to induce IGF-1 expression in human muscle samples [55]. Hewitt and colleagues have proposed a model along which E_2 has the capacity to interact with AR in order to modulate transcription [56]. In our data, the gene expression of *AR, IGF-1 and MGF* were significantly up-regulated suggesting possible cross-talk between the given sex steroids and growth factors. Furthermore, AR-mediated induction of *IGF-1* and *MGF* has been suggested to promote muscle growth by activating the β-catenin pathway [52]. Despite the

somewhat confusing effects of HRT on muscle gene expression in this study the net sum appears to be positive since concomitant increase of muscle mass was observed.

The relatively small sample size in our RCT may have affected the results. On the other hand, the long duration of our study enables us to examine true adaptation to the HRT both at the level of phenotype and gene expression. The RCT design with repeated measures and using a single HRT preparation strengthens our study by eliminating possible physiological and genetic differences in responses to HRT and, therefore, enables the detection of true intra-individual responses. A clear methodological observation was made in the cell culture part of this study. Traditionally these kinds of experiments investigating the induction of gene expression or the amount of total or activated protein in response to a given treatment utilize a single sample as a control sample, to which the treated samples are compared. The control sample usually represents a basal level control. In our experimentation, we pursued accurate and reliable findings and chose to track cells with supplement free medium along the entire time frame of the experiment. We found that the expression of several transcripts or the level of protein phosphorylation changed as the time passed regardless of the samples; untreated or treated. If we were to compare treated samples of each time point to the control sample of 0 hours, we would perhaps have had some statistically significant effects due to hormonal treatments.

In conclusion, the combined HRT used in the current study appeared to affect the expression of several genes along the IGF-1 signaling cascade when compared to the non-users. The observed muscle growth may perhaps result from cross-talk within sex steroid-IGF-1/MGF axis. Since we were unable to clearly identify, which of the components (E_2 or NETA) in the combined HRT might be responsible for the observed effects it appears that either both effective agents are simultaneously needed, or that yet unidentified microenvironmental factors providing proper

paracrine signals are critical for appropriate signaling to occur. Nevertheless, further research is needed to clarify the role of sex steroids and their interaction with other signaling pathways regulating muscle properties.

ACKNOWLEDGEMENTS

We are grateful to the women participating in the present study. Satu Koskinen is acknowledged for her precious contribution to the original Ex/HRT trial. Erkki Helkala is thanked for assistance in biopsy sampling and Juha Hulmi for advice related to experiments on phosphorylation and critical reading of manuscript. Paavo Rahkila is acknowledged for valuable advice in cell culture-related experiments and Kaisa-Leena Tulla for the serum analyses. Markku Kauppinen and Timo Törmäkangas are thanked for all the comments in statistical issues. We thank personnel at the Turku Centre for Biotechnology (Turku, Finland) for carrying out the cRNA generation, array hybridizations and quality control of the raw data. This work was supported by the grants from the Finnish Ministry of Education, the Academy of Finland, the Finnish Cultural Foundation, “Aging, well-being and technology” graduate school, National Graduate School of Musculoskeletal Disorders and Biomaterials, the Jenny and Antti Wihuri Foundation and the EC FP7 Collaborative Project MYOAGE (GA-223576).

REFERENCES

[1] M.E. Coleman, F. DeMayo, K.C. Yin, H.M. Lee, R. Geske, C. Montgomery, R.J. Schwartz, Myogenic vector expression of insulin-like growth factor I stimulates muscle cell differentiation and myofiber hypertrophy in transgenic mice, J. Biol. Chem. 270 (1995) 12109-12116.

[2] M. Sheffield-Moore, R.J. Urban, An overview of the endocrinology of skeletal muscle, Trends Endocrinol. Metab. 15 (2004) 110-115.

[3] F. Lluis, E. Perdiguero, A.R. Nebreda, P. Munoz-Canoves, Regulation of skeletal muscle gene expression by p38 MAP kinases, Trends Cell Biol. 16 (2006) 36-44.

[4] S.C. Bodine, T.N. Stitt, M. Gonzalez, W.O. Kline, G.L. Stover, R. Bauerlein, E. Zlotchenko, A. Scrimgeour, J.C. Lawrence, D.J. Glass, G.D. Yancopoulos, Akt/mTOR pathway is a crucial regulator of skeletal muscle hypertrophy and can prevent muscle atrophy in vivo, Nat. Cell Biol. 3 (2001) 1014-1019.

[5] T.F. Franke, D.R. Kaplan, L.C. Cantley, PI3K: downstream AKTion blocks apoptosis, Cell 88 (1997) 435-437.

[6] B.M. Burgering, P.J. Coffer, Protein kinase B (c-Akt) in phosphatidylinositol-3-OH kinase signal transduction, Nature 376 (1995) 599-602.

[7] E. Hajduch, G.J. Litherland, H.S. Hundal, Protein kinase B (PKB/Akt)--a key regulator of glucose transport? FEBS Lett. 492 (2001) 199-203.

[8] D.A. Cross, D.R. Alessi, P. Cohen, M. Andjelkovich, B.A. Hemmings, Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B, Nature 378 (1995) 785-789.

[9] B.T. Nave, M. Ouwens, D.J. Withers, D.R. Alessi, P.R. Shepherd, Mammalian target of rapamycin is a direct target for protein kinase B: identification of a convergence point for opposing effects of insulin and amino-acid deficiency on protein translation, Biochem. J. 344 Pt 2 (1999) 427-431.

[10] C. Rommel, S.C. Bodine, B.A. Clarke, R. Rossman, L. Nunez, T.N. Stitt, G.D. Yancopoulos, D.J. Glass, Mediation of IGF-1-induced skeletal myotube hypertrophy by PI(3)K/Akt/mTOR and PI(3)K/Akt/GSK3 pathways, Nat. Cell Biol. 3 (2001) 1009-1013.

[11] T.N. Stitt, D. Drujan, B.A. Clarke, F. Panaro, Y. Timofeyva, W.O. Kline, M. Gonzalez, G.D. Yancopoulos, D.J. Glass, The IGF-1/PI3K/Akt pathway prevents expression of muscle atrophy-induced ubiquitin ligases by inhibiting FOXO transcription factors, Mol. Cell 14 (2004) 395-403.

[12] M. Sandri, C. Sandri, A. Gilbert, C. Skurk, E. Calabria, A. Picard, K. Walsh, S. Schiaffino, S.H. Lecker, A.L. Goldberg, Foxo transcription factors induce the atrophy-related ubiquitin ligase atrogin-1 and cause skeletal muscle atrophy, Cell 117 (2004) 399-412.

[13] W. Fan, T. Yanase, H. Morinaga, T. Okabe, M. Nomura, H. Daitoku, A. Fukamizu, S. Kato, R. Takayanagi, H. Nawata, Insulin-like growth factor 1/insulin signaling activates androgen signaling through direct interactions of Foxo1 with androgen receptor, J. Biol. Chem. 282 (2007) 7329-7338.

[14] A. Brunet, A. Bonni, M.J. Zigmond, M.Z. Lin, P. Juo, L.S. Hu, M.J. Anderson, K.C. Arden, J. Blenis, M.E. Greenberg, Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor, Cell 96 (1999) 857-868.

[15] D.R. Taaffe, S. Sipila, S. Cheng, J. Puolakka, J. Toivanen, H. Suominen, The effect of hormone replacement therapy and/or exercise on skeletal muscle attenuation in postmenopausal women: a yearlong intervention, Clin. Physiol. Funct. Imaging 25 (2005) 297-304.

[16] S. Cheng, S. Sipila, D.R. Taaffe, J. Puolakka, H. Suominen, Change in bone mass distribution induced by hormone replacement therapy and high-impact physical exercise in post-menopausal women, Bone 31 (2002) 126-135.

[17] S. Sipila, D.R. Taaffe, S. Cheng, J. Puolakka, J. Toivanen, H. Suominen, Effects of hormone replacement therapy and high-impact physical exercise on skeletal muscle in post-menopausal women: a randomized placebo-controlled study, Clin. Sci. (Lond) 101 (2001) 147-157.

[18] P.H. Ronkainen, V. Kovanen, M. Alen, E. Pollanen, E.M. Palonen, C. Ankarberg-Lindgren, E. Hamalainen, U. Turpeinen, U.M. Kujala, J. Puolakka, J. Kaprio, S. Sipila, Postmenopausal hormone replacement therapy modifies skeletal muscle composition and function: a study with monozygotic twin pairs, J. Appl. Physiol. 107 (2009) 25-33.

[19] S.K. Phillips, K.M. Rook, N.C. Siddle, S.A. Bruce, R.C. Woledge, Muscle weakness in women occurs at an earlier age than in men, but strength is preserved by hormone replacement therapy, Clin. Sci. (Lond) 84 (1993) 95-98.

[20] J.P. Greeves, N.T. Cable, T. Reilly, C. Kingsland, Changes in muscle strength in women following the menopause: a longitudinal assessment of the efficacy of hormone replacement therapy, Clin. Sci. (Lond) 97 (1999) 79-84.

[21] M.B. Sorensen, A.M. Rosenfalck, L. Hojgaard, B. Ottesen, Obesity and sarcopenia after menopause are reversed by sex hormone replacement therapy, Obes. Res. 9 (2001) 622-626.

[22] A.S. Dobs, T. Nguyen, C. Pace, C.P. Roberts, Differential effects of oral estrogen versus oral estrogen-androgen replacement therapy on body composition in postmenopausal women, J. Clin. Endocrinol. Metab. 87 (2002) 1509-1516.

[23] D.A. Skelton, S.K. Phillips, S.A. Bruce, C.H. Naylor, R.C. Woledge, Hormone replacement therapy increases isometric muscle strength of adductor pollicis in post-menopausal women, Clin. Sci. (Lond) 96 (1999) 357-364.

[24] A. Vasconsuelo, L. Milanesi, R. Boland, 17Beta-estradiol abrogates apoptosis in murine skeletal muscle cells through estrogen receptors: role of the phosphatidylinositol 3-kinase/Akt pathway, J. Endocrinol. 196 (2008) 385-397.

[25] E. Pollanen, P.H. Ronkainen, H. Suominen, T. Takala, S. Koskinen, J. Puolakka, S. Sipila, V. Kovanen, Muscular transcriptome in postmenopausal women with or without hormone replacement, Rejuvenation Res. 10 (2007) 485-500.

[26] G. Kopernik, Z. Shoham, Tools for making correct decisions regarding hormone therapy. Part II. Organ response and clinical applications, Fertil. Steril. 81 (2004) 1458-1477.

[27] E. Pöllänen, V. Fey, T. Törmäkangas, P.H.A. Ronkainen, D. Taaffe, T. Takala, S. Koskinen, S. Cheng, U.M. Kujala, J. Puolakka, H. Suominen, V. Kovanen, S. Sipilä, Power training and postmenopausal hormone therapy affect transcriptional control of specific co-regulated gene clusters in skeletal muscle, (2010) DOI 10.1007/s11357-010-9140-1.

[28] L. Gautier, L. Cope, B.M. Bolstad, R.A. Irizarry, affy--analysis of Affymetrix GeneChip data at the probe level, Bioinformatics 20 (2004) 307-315.

[29] C. Hassager, C. Christiansen, Estrogen/gestagen therapy changes soft tissue body composition in postmenopausal women, Metabolism 38 (1989) 662-665.

[30] J. Jensen, C. Christiansen, P. Rodbro, Oestrogen-progestogen replacement therapy changes body composition in early post-menopausal women, Maturitas 8 (1986) 209-216.

[31] R.J. Walker, N.J. Lewis-Barned, W.H. Sutherland, A. Goulding, E.A. Edwards, S.A. de Jong, E. Gold, H.L. Walker, The effects of sequential combined oral 17beta-estradiol norethisterone acetate on insulin sensitivity and body composition in healthy postmenopausal women: a randomized single blind placebo-controlled study, Menopause 8 (2001) 27-32.

[32] S.R. Davis, K.Z. Walker, B.J. Strauss, Effects of estradiol with and without testosterone on body composition and relationships with lipids in postmenopausal women, Menopause 7 (2000) 395-401.

[33] L.B. Tanko, L. Movsesyan, O.L. Svendsen, C. Christiansen, The effect of hormone replacement therapy on appendicular lean tissue mass in early postmenopausal women, Menopause 9 (2002) 117-121.

[34] A.M. Kenny, A. Kleppinger, Y. Wang, K.M. Prestwood, Effects of ultra-low-dose estrogen therapy on muscle and physical function in older women, J. Am. Geriatr. Soc. 53 (2005) 1973-1977.

[35] I.H. Thorneycroft, R. Lindsay, J.H. Pickar, Body composition during treatment with conjugated estrogens with and without medroxyprogesterone acetate: analysis of the women's Health, Osteoporosis, Progestin, Estrogen (HOPE) trial, Am. J. Obstet. Gynecol. 197 (2007) 137.e1-137.e7.

[36] G. Goldspink, S.D. Harridge, Growth factors and muscle ageing, Exp. Gerontol. 39 (2004) 1433-1438.

[37] R.W. Matheny, Jr., B.C. Nindl, M.L. Adamo, Minireview: Mechano-growth factor: a putative product of IGF-1 gene expression involved in tissue repair and regeneration, Endocrinol. 151 (2010) 865-875.

[38] D.L. Enns, P.M. Tiidus, Estrogen influences satellite cell activation and proliferation following downhill running in rats, J. Appl. Physiol. 104 (2008) 347-353.

[39] E. Kamanga-Sollo, M.S. Pampusch, G. Xi, M.E. White, M.R. Hathaway, W.R. Dayton, IGF-I mRNA levels in bovine satellite cell cultures: effects of fusion and anabolic steroid treatment, J. Cell. Physiol. 201 (2004) 181-189.

[40] E. Kamanga-Sollo, M.E. White, M.R. Hathaway, K.Y. Chung, B.J. Johnson, W.R. Dayton, Roles of IGF-I and the estrogen, androgen and IGF-I receptors in estradiol-17beta- and trenbolone acetate-stimulated proliferation of cultured bovine satellite cells, Domest. Anim. Endocrinol. 35 (2008) 88-97.

[41] C.M. Dieli-Conwright, T.M. Spektor, J.C. Rice, F.R. Sattler, E.T. Schroeder, Influence of hormone replacement therapy on eccentric exercise induced myogenic gene expression in postmenopausal women, J. Appl. Physiol. 107 (2009) 1381-1388.

[42] D.L. Enns, S. Iqbal, P.M. Tiidus, Oestrogen receptors mediate oestrogen-induced increases in post-exercise rat skeletal muscle satellite cells, Acta Physiol. (Oxf) 194 (2008) 81-93.

[43] D.R. Alessi, M. Andjelkovic, B. Caudwell, P. Cron, N. Morrice, P. Cohen, B.A. Hemmings, Mechanism of activation of protein kinase B by insulin and IGF-1, EMBO J. 15 (1996) 6541-6551.

[44] D.D. Sarbassov, D.A. Guertin, S.M. Ali, D.M. Sabatini, Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex, Science 307 (2005) 1098-1101.

[45] E. Jacinto, V. Facchinetti, D. Liu, N. Soto, S. Wei, S.Y. Jung, Q. Huang, J. Qin, B. Su, SIN1/MIP1 maintains rictor-mTOR complex integrity and regulates Akt phosphorylation and substrate specificity, Cell 127 (2006) 125-137.

[46] A. Alisi, A. Spaziani, S. Anticoli, M. Ghidinelli, C. Balsano, PKR is a novel functional direct player that coordinates skeletal muscle differentiation via p38MAPK/AKT pathways, Cell. Signal. 20 (2008) 534-542.

[47] D.J. Glass, Skeletal muscle hypertrophy and atrophy signaling pathways, Int. J. Biochem. Cell Biol. 37 (2005) 1974-1984.

[48] S. Jaffer, O. Shynlova, S. Lye, Mammalian target of rapamycin is activated in association with myometrial proliferation during pregnancy, Endocrinology 150 (2009) 4672-4680.

[49] A. Sobolewska, M. Gajewska, J. Zarzynska, B. Gajkowska, T. Motyl, IGF-I, EGF, and sex steroids regulate autophagy in bovine mammary epithelial cells via the mTOR pathway, Eur. J. Cell Biol. 88 (2009) 117-130.

[50] S.D. Harridge, Plasticity of human skeletal muscle: gene expression to in vivo function, Exp. Physiol. 92 (2007) 783-797.

[51] A. Philippou, E. Papageorgiou, G. Bogdanis, A. Halapas, A. Sourla, M. Maridaki, N. Pissimissis, M. Koutsilieris, Expression of IGF-1 isoforms after exercise-induced muscle

damage in humans: characterization of the MGF E peptide actions in vitro, In Vivo. 23 (2009) 567-575.

[52] M.A. Gentile, P.V. Nantermet, R.L. Vogel, R. Phillips, D. Holder, P. Hodor, C. Cheng, H. Dai, L.P. Freedman, W.J. Ray, Androgen-mediated improvement of body composition and muscle function involves a novel early transcriptional program including IGF1, mechano growth factor, and induction of {beta}-catenin, J. Mol. Endocrinol. 44 (2010) 55-73.

[53] L.J. Murphy, L.C. Murphy, H.G. Friesen, Estrogen induces insulin-like growth factor-I expression in the rat uterus, Mol. Endocrinol. 1 (1987) 445-450.

[54] D. Chesik, J. De Keyser, Progesterone and dexamethasone differentially regulate the IGF-system in glial cells, Neurosci. Lett. 468 (2010) 178-182.

[55] A.A. Ferrando, M. Sheffield-Moore, C.W. Yeckel, C. Gilkison, J. Jiang, A. Achacosa, S.A. Lieberman, K. Tipton, R.R. Wolfe, R.J. Urban, Testosterone administration to older men improves muscle function: molecular and physiological mechanisms, Am. J. Physiol. Endocrinol. Metab. 282 (2002) E601-7.

[56] S.C. Hewitt, J. Collins, S. Grissom, B. Deroo, K.S. Korach, Global uterine genomics in vivo: microarray evaluation of the estrogen receptor alpha-growth factor cross-talk mechanism, Mol. Endocrinol. 19 (2005) 657-668.

TABLES

Table 1. Clinical data of the study participants. The values are mean ± standard deviation.

	HRT group (n=10)		**CO group (n=9)**		**P value**
	Baseline	Post-intervention	Baseline	Post-intervention	
Age (years)	53.45±1.46		53.22±2.12		0.79[§]
BMI(kg/m^2)	28.05±2.92	28.23±2.94	25.92±2.13	25.9±2.12	0.15
LBM (kg)	47.50±3.95	48.50±3.95	47.00±4.56	46.67±3.39	0.03
Body fat (%)	34.30±7.78	32.50±6.36	30.56±3.64	31.33±4.80	0.32
Thigh muscle size (cm^2)	47.49±6.14	51.58±7.73	46.32±6.77	47.03±6.57	0.003
Estrogen (nmol/l)	0.07±0.13	0.22±0.05	0.03±0.08	0.01±0.01	<0.001
Testosterone (nmol/l)	1.07±0.82	1.02±0.92	0.67±0.52	0.74±0.55	0.38
IGF-1 (nmol/l)	15.00±3.65	16.67±4.41	15.00±4.50	16.48±7.23	0.77

[§]independent samples t-test.

FIGURE LEGENDS

Figure 1. Fold change (FC) values of gene expression. Black dots are FC values for each individual, ** represents P value less than 0.005, * represents P value less than 0.05 when the HRT group was compared to the CO group by univariate analysis of variance.

Figure 2. Fold change (FC) values for change in gene expression of *IGF-1* splice variants. Black dots are FC values for each individual, ** represents P value less than 0.005 when the HRT group was compared the CO group by univariate analysis of variance.

Figure 3. Correlation between the change in the gene expression of *IGF-1Ec* (from the baseline to post-intervention) and post-intervention lean tissue area. The two outcomes correlated significantly (r=0.50, P=0.035).

Figure 4. The amount of phosphorylated Akt and mTOR in C2C12 myotubes. The figure shows the amount of p-Akt (A) and p-mTOR (B) after treatment with E_2, as well as p-Akt (C) and p-mTOR (D) after treatment with NETA. No statistically significant effects due to the treatments were observed. Black line with diamonds represent the control samples, dark blue line with squares the 1 nM treatment and light blue with triangles the 10 nM treatment. Each value represents the mean value of three independent experiments and error bars standard errors.