Age and estrogen-based hormone therapy affect systemic and local IL-6 and IGF-1 pathways in women

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Abstract A thorough understanding of the role of estrogens on aging-related muscle weakness is lacking. To clarify the molecular mechanisms underlying the effects of hormone replacement therapy (HRT) on skeletal muscle, we analyzed systemic protein and local

mRNA levels of factors related to interleukin 6 (IL-6) and insulin-like growth factor 1 (IGF-1) pathways in 30- to 35-year-old ($n=14$) women (without hormonal contraceptives) and in 54- to 62-year-old monozygotic female twin pairs discordant for HRT ($n=11$ pairs, mean duration of HRT 7.3 ± 3.7 years). Biopsies were taken from vastus lateralis muscle and from abdominal adipose tissue. We found, first, that the systemic levels of IL-6 receptors sIL-6R and sgp130 are sensitive to both age and HRT concomitant with the changes in body composition. The serum levels of sgp130 and sIL-6R were 16% and 52% ($p\leq 0.001$ for both variables) higher in postmenopausal women than in premenopausal women, and 10% and 9% lower ($p=0.033$ and $p<0.001$, respectively) in the HRT using than in their non using co-twins. After adjustment for body fat amount, the differences were no more significant. Second, the transcript analyses emphasize the impact of adipose tissue on systemic levels of IL-6, sgp130 and sIL6R, both at pre- and postmenopausal age. In muscle, the most notable changes were 28% lower gene expression of IGF-1 splice variant Ea (IGF-1Ea) and 40% lower expression of splice variant Ec (IGF-1Ec) in the postmenopausal non-users than in premenopausal women ($p=0.016$ and 0.019 , respectively), and 28% higher expression of IGF1-receptor in HRT users than in non-users ($p=0.060$). The results tend to demonstrate that HRT has positive anti-catabolic effect on aging skeletal muscle.

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Introduction

The menopause transition in women includes ovarian involution and estrogen deficiency. The resultant hypogonadism affects the metabolism of organs as diverse as bone, blood vessels and adipose tissue, and consequently affects several age-related diseases, such as atherosclerosis, cancer, diabetes, osteoporosis and dementia (Chung et al. 2009). Muscle tissue and the immune system can also be included in the list, owing to the existence of estrogen receptors (ESRs) (Lemoine et al. 2003; Wiik et al. 2003; Scariano et al. 2008). So far, no unifying mechanism has been identified to explain these effects (Farhat et al. 1996; Mendelsohn and Karas 1999). To date, the 2- to 4-fold increase in proinflammatory cytokines, i.e., the so-called low-grade inflammation, which is associated with aging and the decline in ovarian function as well, has been thought to best explain the various above-mentioned age-related health problems (Ferrucci et al. 2002; Roubenoff 2004; Franceschi et al. 2000). Among the cytokines, signaling through interleukin 6 (IL-6) is regarded as one of the main signaling pathways affecting age-related adverse outcomes (Ershler and Keller. 2000; Maggio et al. 2006). The activation of the IL-6 pathway is a complex interaction between IL-6 and its both membrane-bound and soluble receptors (Maggio et al. 2006), and has not been fully clarified in different tissues and cell types. Ex vivo studies have shown the association of estrogen deficiency with the increased expression and secretion of the proinflammatory cytokines interleukin 1 (IL-1), IL-6 and tumor necrosis factor- α (TNF- α), as well as the association of administration of estrogen with decreases in cytokine levels (Pfeilschifter et al. 2002). However, trials to demonstrate the same association in specific tissues or in circulation have been less successful. This has led to the hypothesis that the effects of estrogen on cytokine gene expression may be dependent on the target tissue or cellular context as well as on the pharmacological differences between the hormones administered.

In addition to above-mentioned age-related diseases, the decline in estrogen production during

the perimenopausal phase is also associated with decreased muscle mass and strength (Phillips et al. 1993). A central player in the regulation of muscle mass is the phosphatidylinositol 3 kinase (PI3K)/AKT pathway, as it activates protein synthesis and inhibits protein degradation (Velloso 2008). The (PI3K)/AKT pathway is activated by anabolic factors such as exercise, growth hormone, insulin-like growth factor 1 (IGF-1) and sex hormones. IGF-1 is nowadays regarded as one of the most important factors in muscle growth and repair (Goldspink 2007), and the modulation of the IGF system is suggested as a potential strategy to improve the condition of skeletal muscle in older adults (Giovannini et al. 2008; Kandalla et al. 2011). Recent studies have underlined the balance between the catabolic effects of cytokines and the anabolic effects of IGF-1 (Ferrucci et al. 2002; Roubenoff 2004; Payette et al. 2003), and these factors have been suggested to antagonize each other's effects on skeletal muscle protein synthesis (Frost and Lang 1999; Jurasinski and Vary 1995).

Despite the many studies that have been conducted on the effects of estrogen-based hormone therapy (HRT) on skeletal muscle, discrepancy on whether HRT is associated with or has an effect on skeletal muscle has been existing (Brown 2008). Recently, a few studies have found evidence for the positive influence of HRT on skeletal muscle, including one of our own, showing a strong relationship between muscle performance and long-term HRT (Ronkainen et al. 2009), and another, demonstrating for the first time that short-term HRT promotes a proanabolic muscle environment by enhancing the gene expression of myogenic regulatory factors (Dieli-Conwright et al. 2009). Also, a recent meta-analysis concludes that HRT is beneficial for muscle strength, as HRT users have approximately 5% greater strength than non-users (Greising et al. 2009). The objective of the current study was to clarify the association of IL-6 and IGF-1 pathways with estrogen-based hormone therapy in skeletal muscle of postmenopausal women. For this purpose, we determined IL-6 and its receptors, both systemically and locally in muscle and adipose tissue as well as in leukocytes. Furthermore, we analyzed the transcriptional activity of the muscular IGF-1 pathway to evaluate the crosstalk between the IL-6 and IGF-1 pathways. We used a unique study design with postmenopausal identical

twin pairs discordant for the use of HRT which enabled us to study the HRT-exposed subjects with their non-exposed co-twins with identical genetic background at sequence level. Additionally, we had a group of 30- to 35-year-old women not using any hormonal contraceptives for age control.

Methods

Study design and participants

This study is a part of a larger research project, "Sarcopenia and Skeletal Muscle Adaptation to Postmenopausal Hypogonadism: Effects of Physical Activity and Hormone Replacement Therapy in Older Women — a Genetic and Molecular Biology Study on Physical Activity and Estrogen-related Pathways" (SAWEs), investigating the molecular events involved in maintaining proper muscle mass and function after menopause (Ronkainen et al. 2009). The participants for this study were recruited from the Finnish Twin Cohort ($n=13,888$ pairs) (Kaprio et al. 1978). An invitation was sent to all monozygotic (MZ) female twin pairs born in 1943–1952 ($n=537$ pairs). Only twin pairs in which one co-twin was a current HRT user and the other co-twin was not currently using HRT were asked to respond to the invitation. From the total of responders ($n=114$ pairs), twin pairs where one sister had never used HRT, while the other sister was a current user, and both sisters reported willingness to participate in the laboratory measurements, were contacted ($n=21$ pairs). Finally, a total of 16 MZ pairs aged 54–62 years discordant for HRT use and without any contraindications participated in the study. One twin pair turned out to be dizygotic (DZ) and was excluded from the further analyses. The HRT users consisted of five women using estradiol-only preparations (1–2 mg), six women were on combined treatment including estrogenic (1–2 mg) and progestogenic compounds, and four women were using tibolone (2.5 mg). In the present study, our focus was on the effects of estrogen-based hormone therapy including estrogen therapy (ET) and combined estrogen – progestogen therapy (EPT). In this pooled HRT group ($n=11$ pairs), nine women used an oral preparation and two used a transdermal preparation. The number of participants is rather small, but it is comparable to previous reports of MZ co-twin

control studies (Laustiola et al. 1988; Bouchard et al. 1990; Mustelin et al. 2008), and has sufficient statistical power for clinically relevant results. The mean duration of HRT usage was 6.9 ± 4.1 years (range, 2–16 years). For the recruitment of premenopausal women, an invitation was sent to two thousand premenopausal women (39.1% of the entire cohort) randomly selected from the entire 30–40 years age cohort (born in 1967–1977) living in the City of Jyväskylä. According to inclusion criteria (Pollanen et al. 2011), a group of 59 premenopausal women aged 30–40 years, not being treated with hormonal contraceptives or progesterone preparations within the past 5 years, participated to the study. For the present study, a subgroup of 14 women aged 30–35 years was randomly selected from the original study population for the analysis described below. All randomization steps were performed by the random sampling feature of the PASW Statistic Software (SPSS Inc., IBM, Illinois, USA).

Body and thigh muscle composition

Body mass index (BMI) was calculated from height and weight measured with standard procedures. Percentage body fat was measured with bioelectrical impedance (InBody [720], Biospace Co. Ltd., Seoul, South Korea). Computed tomography (CT) scans (Siemens Somatom Emotion scanner, Siemens AG, Erlangen, Germany) were obtained from the midpoint between the greater trochanter and the lateral joint line of the knee. The scans were analyzed using software developed at the University of Jyväskylä (Jyväskylä, Finland) for cross-sectional CT image analysis (Geanie 2.1, Commit Ltd., Espoo, Finland). The software separates fat and lean tissue based on given radiological density limits. Total thigh muscle and fat cross-sectional areas (CSA) were analyzed and the relative proportion of muscle in the CSA of the whole thigh was calculated.

Blood and tissue sampling

Muscle (m. vastus lateralis) needle biopsies, abdominal subcutaneous adipose tissue biopsies (needle aspiration) and a set of blood samples (whole blood, serum, plasma, leucocytes) were taken under standard fasted conditions with supine participants. Tissue

samples were snap frozen and stored at -80°C for biochemical analyses. Sera samples were stored at -70°C until analyzed.

Serum cytokine and hormone analyses

Circulating concentrations of IL-6 was measured by Immulite® 1,000 (DPC, Los Angeles, CA) and the IL-6 receptors sIL6R and sgp130 were measured by Quantikine® immunoassays (R&D Systems, Minneapolis, MN, USA). Serum 17β -estradiol (E_2) levels were assessed in duplicate by an extraction RIA method optimized to measure especially low estradiol levels in serum, as described previously (Ankarberg-Lindgren and Norjavaara 2008). The levels of serum testosterone were measured as described previously (Turpeinen et al. 2008).

Gene expression analyses from muscle, adipose tissue and leukocytes

Total RNA from muscle and leukocytes was extracted using Trizol reagent (Invitrogen, Carlsbad, CA) and from fat tissue using RNAqueous Micro-kit (Applied Biosystems). For muscular microarray, RNA quality was determined using Experion electrophoresis station (Bio-Rad Laboratories, Hercules, CA), and Sentrix Human-WG6 V3 Expression BeadChip microarrays (Illumina, San Diego, CA) were used for the transcriptome-wide expression analysis (Turku Center for Biotechnology, BTK, University of Turku). Data mining according to a list of selected genes encoding proteins related to the IGF-1 pathway was performed utilizing R software (<http://www.R-project.org>) together with BioConductor development software (<http://www.bioconductor.org>). The gene expression levels were analyzed as relative expression levels compared to GAPDH. For the qPCR analysis, one microgram of RNA was reverse transcribed into cDNA (TaqMan Reverse Transcription Reagents, N808-0234; Applied Biosystems). Taqman Gene Expression Assays (Applied Biosystems) were used to investigate the expression of IL-6 (Hs00174131_m1), IL-6R (Hs01075664_m1), gp130 (Hs00174360_m1), IGF-1R (Hs99999020_m1), IGFBP3 (Hs00181211_m1) and IGFBP5 (Hs01052296_m1). The previously published unique *TaqMan* gene expression assays were used for IGF-1 splice variants, IGF-1Ea and IGF-1Ec (Pollanen et al.

2010). Assays were performed with an Applied Biosystems ABI 7,300 unit using standard PCR conditions as recommended by the manufacturer. Samples were run in triplicate and the reference sample was included in all plates to control for inter-assay variation. GAPDH (Hs99999905_m1) was used for endogenous control.

Statistics

Data are reported as means and standard deviations unless otherwise stated. The analyses are based on three groups: (1) premenopausal women aged 30–35 years and not using any hormonal contraceptives ($n=14$), (2) postmenopausal, 54- to 62-year-old women who had never used HRT (“non-users”, $n=11$) and (3) their identical co-twins who were currently on long-term estrogen-based therapy (“HRT users”, $n=11$). The inter-group comparisons were performed between the premenopausal women and postmenopausal non-users, while paired comparisons were conducted between the postmenopausal MZ twin sisters, i.e., the non-users and the HRT users. The statistical analyses for group comparisons included either parametric independent samples *t*-test and paired-sample *t*-test, or non-parametric Mann–Whitney and Wilcoxon signed ranks tests, depending on the normal distribution of the means tested by Shapiro–Wilk test. The adjustment for body composition was done for the values of serum inflammatory factors by dividing the values with fat percentage, and thereafter differences between the groups and twin pairs were analyzed. The strength of the associations between the variables was assessed by Pearson’s correlation coefficient (*r*). Data analyses were carried out using SPSS (version 15.0, SPSS, Chicago, IL).

Ethics

This study follows the guidelines of good clinical and scientific practice as well as the Helsinki declaration. The SAWEs study was approved by the Ethics Committee of the Central Finland Health Care District on 6.6.2006. An informed consent explaining the possible risks and personal benefits associated with the examinations and permission to use the data for research purposes and in publications was signed by the subjects before the measurements.

Results

Anthropometric characteristics and systemic sex steroids of study subjects

Anthropometric characteristics of study subjects are presented in Table 1. Compared to the premenopausal women, the postmenopausal women tended to have higher percentage body fat ($p=0.068$). No significant differences were observed in BMI or in relative thigh muscle area between the pre- and postmenopausal women. In the HRT users, the percentage of fat was lower and the relative thigh muscle area was larger ($p=0.026$ and 0.013 , respectively) compared to the non-using co-twins. Also, BMI tended to be lower ($p=0.091$) in the HRT users compared to non-users. The observed concentrations of serum sex steroids were as expected (Table 1). Both estradiol and testosterone levels were clearly lower in postmenopausal compared to premenopausal women ($p\leq 0.001$ and 0.003 , respectively) and higher in HRT users compared to their non-using co-twins ($p=0.003$ and 0.044 , respectively). There were no significant differences in serum sex steroid levels between ET users and EPT users (data not shown).

Systemic status of IL-6 and its receptors

Due to the high inter individual variation, no significant differences were seen in the serum levels of IL-6 between the study groups. However, the levels

of both soluble IL-6 receptors, sgp130 and sIL-6R, were 16% and 52% higher in postmenopausal women compared to premenopausal women ($p\leq 0.001$ for both variables), and 10% and 9% lower ($p=0.033$ and $p<0.001$, respectively) in the HRT using than in their non using co-twins (Fig. 1). After adjusting for body fat amount, the differences were no longer significant.

Local transcript levels of IL-6, gp130 and IL-6R in muscle, adipose tissue and leukocytes

As the serum levels of IL-6 receptors proved to be sensitive to postmenopausal status regardless of HRT, we analyzed their local mRNA levels by qPCR, to evaluate the impact of each tissue on systemic inflammation status and to obtain a general view of IL-6 and its receptors at different ages and in relation to HRT use. The results show that the contribution of adipose tissue to serum levels of IL-6 and gp130 is overwhelming compared to that of muscle or leukocytes (Fig. 1). Muscle tissue in the dormant state appears to be rather inactive. In the case of the *IL-6R* gene, the mRNA level in leukocytes was found to be the same as that in the adipose tissue. The mRNA levels of *IL-6R* and *gp130* were 55% and 26% higher in the adipose tissue of postmenopausal women compared to premenopausal women ($p=0.043$ and 0.154 , respectively). In leukocytes, the mRNA level of *IL-6R* was 32% higher in postmenopausal women than in premenopausal women ($p=0.019$). The observed differences in serum IL-6R and gp130 levels

Table 1 Anthropometric characteristics and sex steroid levels of premenopausal women and postmenopausal MZ twin pairs discordant for long-term HRT

	Premenopausal women ($n=14$)	Postmenopausal MZ twin sisters ($n=11$ pairs)			
		Non-users	p^*	HRT users	$p^{\#}$
Age (years)	32.3 $\pm$ 1.9	57.2 $\pm$ 1.8		57.2 $\pm$ 1.8	
BMI (kg/m ²)	25.0 $\pm$ 5.5	28.1 $\pm$ 6.5	0.204 ^a	25.6 $\pm$ 3.8	0.091 ^c
Percentage of fat (%)	27.9 $\pm$ 9.9	35.2 $\pm$ 8.9	0.068 ^b	30.7 $\pm$ 7.1	0.026 ^c
Relative thigh muscle area (%)	54.4 $\pm$ 11.1	49.8 $\pm$ 12.7	0.354 ^b	54.2 $\pm$ 10.0	0.013 ^c
Estradiol (pmol/l)	281.2 $\pm$ 158.3	33.3 $\pm$ 27.4	< 0.001 ^a	172.9 $\pm$ 203.2	0.003 ^b
Testosterone (nmol/l)	1.3 $\pm$ 0.5	0.6 $\pm$ 0.3	< 0.001 ^a	0.7 $\pm$ 0.3	0.061 ^b

Values are mean $\pm$ standard deviation. The p values are obtained from Mann–Whitney U -test,^a independent samples t -test^b and ^c Wilcoxon signed ranks test

*Compared with premenopausal women

[#] Compared with non-using co-twin

Fig. 1 Systemic protein levels and local transcript levels of IL-6 (a), IL-6R (b) and gp130 (c) in adipose tissue, skeletal muscle and leukocytes emphasizing the contribution of adipose tissue on systemic levels. Data are mean $\pm$ standard deviation, $n=14$ premenopausal women (white bar), 11 non-users (grey bar) and 11 HRT users (black bar). The transcript levels are shown as the relative expression of each gene compared to the expression level of GAPDH. The p values are obtained from Mann–Whitney U -test and paired-sample t -test (compared between pre- and postmenopausal women and between twins, respectively; * $p\leq 0.05$; *** $p\leq 0.001$)

were not significant on transcript level between HRT users and their non-using co-twins.

Systemic levels of IGF-1 and IGFBP3

The serum concentration of IGF-1 in the postmenopausal women not on HRT was only 63% of that of the premenopausal women ($p<0.001$). There were no significant differences in serum IGF-1 and IGFBP3 levels between the HRT users and their non-using co-twins. Serum IGF-1 was positively correlated with serum IGFBP3 in the premenopausal women ($r=0.869$, $p<0.001$), but not in either the postmenopausal non-users or HRT users. When the connections between serum IGF-1 and IL-6 and its receptors were analyzed, a strong negative correlation between IGF-1 and sIL-6R

was found in HRT-using postmenopausal women (Fig. 2b), but not in their non-using co-twins (Fig. 2a).

Local transcript levels of IGF-1Ea, IGF-1Ec, IGF-1R, IGFBPs and IRSs in muscle

The local mRNA levels from the genes related to IGF-1 signaling were either selected from the transcriptome-wide expression analysis of muscle and/or analyzed by qPCR (Table 2). The mean transcript levels of *IGF-1Ea* and *IGF-1Ec* in the non-users were 72% and 60% of the values in the premenopausal group ($p=0.016$ and 0.019), and in the HRT users 112% and 131% of the mean values in their non-using co-twins ($p>0.05$). The expression level of *IGF1R* in the HRT users was 128% of that of

Fig. 2 Association of systemic IGF-1 with sIL6R levels in non-users ($r=-0.269, p=0.423$; **a** and in their HRT using MZ twin sisters ($r=-0.710, p=0.014$; **b**)

the non-using co-twins ($p=0.060$). In HRT users, the transcript level of *IGFBP3* was 88% and the expression level of *IGFBP5* was 113% of the mean values in non-users ($p=0.025$ and 0.026 , respectively). The changes in qPCR results were parallel to those obtained from the arrays, but did not reach statistical significance. The expression levels of *IRS1* and *IRS2*

Table 2 Relative muscular mRNA levels in MZ twin pairs discordant for long-term HRT as determined by quantitative PCR (qPCR) and/or microarray

Gene	Non-users (% of mean value in premenopausal women)	HRT users (% of mean value in non-using co-twin)	
		Array	qPCR
<i>IGF1Ea</i>	72 ($p=0.016$)	—	112 ($p=0.424$)
<i>IGF1Ec</i>	60 ($p=0.019$)	—	131 ($p=0.477$)
<i>IGF-1R</i>	90 ($p=0.458$)	—	128 ($p=0.060$)
<i>IGFBP3</i>	84 ($p=0.646$)	88 ($p=0.025$)	92 ($p=0.213$)
<i>IGFBP5</i>	90 ($p=0.702$)	113 ($p=0.026$)	122 ($p=0.657$)
<i>IRS1</i>	—	128 ($p=0.002$)	—
<i>IRS2</i>	—	114 ($p=0.088$)	—

Data are expressed as% of mean value in respective control group. The p values are determined by independent samples t -test (compared with premenopausal women) in non-users and by paired-sample t -test (compared with non-users) in HRT users. *IGF1Ea* insulin-like growth factor 1Ea, *IGF1Ec* insulin-like growth factor 1Ec, *IGF-1R* insulin-like growth factor 1 receptor, *IGFBP3* insulin-like growth factor 1 binding protein 3, *IGFBP5* insulin-like growth factor 1 binding protein 5, *IRS1* insulin receptor substrate 1, *IRS2* insulin receptor substrate 2.

in the HRT users were 128% and 114% of that of the non-using co-twins ($p=0.002$ and 0.088 , respectively).

The muscular transcript level of *IGF-1Ec* was positively correlated with the levels of *IGF-1Ea*, *IGF-1R* and *IGFBP5* in premenopausal women but only with the level of *IGF-1Ea* in non-users (Table 3). However, the same positive correlations as in premenopausal women were found in HRT users (Table 3). The muscular transcript level of *IGF-1R* was positively correlated with the levels of *IGF-1Ea*, *IGF-1R*, *IGFBP3* and *IGFBP5* in premenopausal women. No significant correlation was found in postmenopausal women not using HRT (Table 4), while the level of *IGF-1R* was positively correlated with the levels of *IGF-1Ea* and *IGFBP5* in their HRT-using co-twins (Table 4).

Discussion

The purpose of our study was to clarify the molecular mechanisms underlying the estrogen-based hormone therapy (HRT) effects on skeletal muscle. Therefore, we determined, in premenopausal women without external hormonal therapies and in postmenopausal identical twin sisters with and without current HRT, the IL-6 and its receptors, both systemically and locally in muscle and adipose tissue as well as in leukocytes. Furthermore, we analyzed the transcriptional status of the muscular IGF-1 pathway in these women to evaluate the crosstalk between the IL-6 and IGF-1 pathways. We found, first, that the systemic

Table 3 Association between muscular transcript levels of IGF1Ec and IGF-1-related genes in premenopausal women and postmenopausal MZ twin pairs discordant for long-term HRT

Gene	Premenopausal women (n=14) <i>IGF1Ec</i>		Postmenopausal MZ twin sisters (n=11 pairs)			
			Non-users <i>IGF1Ec</i>		HRT users <i>IGF1Ec</i>	
	<i>r</i>	<i>p</i>	<i>r</i>	<i>p</i>	<i>r</i>	<i>p</i>
<i>IGF1Ea</i>	0.741	0.002	0.731	0.002	0.703	0.016
<i>IGF-1R</i>	0.568	0.034	−0.252	0.364	0.666	0.025
<i>IGFBP3</i>	0.362	0.203	−0.113	0.689	0.614	0.045
<i>IGFBP5</i>	0.648	0.012	0.415	0.124	0.535	0.090

IGF1Ea insulin-like growth factor 1Ea, *IGF1Ec* insulin-like growth factor 1Ec, *IGF-1R* insulin-like growth factor 1 receptor, *IGFBP3* insulin-like growth factor 1 binding protein 3, *IGFBP5* insulin-like growth factor 1 binding protein 5

levels of IL-6 receptors; sIL-6R and sgp130, are sensitive to both age and HRT concomitantly with the changes in body composition. Secondly, the transcript analyses emphasized the contribution of adipose tissue on systemic levels of IL-6, sgp130 and sIL6R, both at pre- and postmenopausal age. In muscle, the most notable changes were the lower gene expression of *IGF-1Ea* and *IGF-1Ec* in the postmenopausal women not on HRT compared to the premenopausal women, and higher expression of *IGF1R* in HRT users than non-users.

To deepen understanding of the cross-talk between the tissues contributing to inflammatory response, we analyzed the local gene expression levels of IL-6 and its receptors in adipose tissue, muscle and leukocytes. IL-6 signaling is coordinated by membrane-bound and soluble receptors (Maggio et al. 2006). Soluble IL-6R works as an agonist; it forms a complex with IL-6 and associates

with the ubiquitously expressed signal-transducing membrane protein gp130 initiating the intracellular signaling. This process, called trans-signaling, enables IL-6 also to influence those tissues and cells not expressing the membrane-bound IL-6R. Furthermore, the trans-signaling is regulated by a soluble form of gp130 (sgp130), which can bind and inactivate the IL-6/sIL-6R complex (Jostock et al. 2001). Altogether, the biological effect of IL-6 is a sensitive interplay between all these factors both on the systemic level and locally in target tissues. Our results emphasize the role of adipose tissue on systemic levels of IL-6, sgp130 and sIL6R, both in pre- and postmenopausal women as well as in women with and without HRT. Only the transcription of the *IL-6R* gene in leukocytes was found to be equal to that in adipose tissue. The serum levels of IL-6, sIL-6R and sgp130 were lower in the HRT users compared to non-users, but the differences

Table 4 Association between muscular transcript levels of IGF-1R and IGF-related genes in premenopausal women and postmenopausal MZ twin pairs discordant for long-term HRT

Gene	Premenopausal women (n=14) <i>IGF-1R</i>		Postmenopausal MZ twin sisters (n=11 pairs)			
			Non-users <i>IGF-1R</i>		HRT users <i>IGF-1R</i>	
	<i>r</i>	<i>p</i>	<i>r</i>	<i>p</i>	<i>r</i>	<i>p</i>
<i>IGF1Ea</i>	0.778	0.001	−0.186	0.507	0.489	0.127
<i>IGF1Ec</i>	0.568	0.034	−0.252	0.364	0.666	0.025
<i>IGFBP3</i>	0.466	0.093	0.075	0.790	0.459	0.155
<i>IGFBP5</i>	0.619	0.018	0.310	0.260	0.733	0.010

IGF1Ea insulin-like growth factor 1Ea, *IGF1Ec* insulin-like growth factor 1Ec, *IGF-1R* insulin-like growth factor 1 receptor, *IGFBP3* insulin-like growth factor 1 binding protein 3, *IGFBP5* insulin-like growth factor 1 binding protein 5

were abolished after adjustment for body fat. Visceral adipose tissue has been shown to release 2–3 times more IL-6 than subcutaneous adipose tissue (Fried et al. 1998). However, we suggest that the transcript analysis of IL-6 and its receptors from subcutaneous adipose tissue provides a good estimation of the levels released into circulation. Estrogen deficiency has been found to be associated with increases in IL-6 activity in several experiments (Pfeilschifter et al. 2002). Girasole et al. (1999) showed in fertile women undergoing surgery for benign uterine diseases that estrogen treatment prevents the alterations of the IL-6 system induced by ovariectomy, and suggested that sIL-6R is under direct inhibitory control of estrogen both in vivo and in vitro. Our previous study showed that body and thigh fat content was lower in the users of estrogen-containing HRT compared to non-users (Ronkainen et al. 2009). Consequently, the lower levels of IL-6 and its receptors observed in the HRT users might be a direct consequence of decreased amount of adipose tissue.

As the evidence of the importance of local over systemic regulation of the IGF-1 system in skeletal muscle continues to accumulate (Phillips et al. 1993; Philippou et al. 2007), we analyzed the muscular mRNA levels of the IGF-1 signaling factors with respect to age and use of HRT. IGFs are regulators of skeletal muscle mass and have a positive effect on the protein balance by increasing protein synthesis and decreasing protein degradation (Frost and Lang 1999; Clemmons 2009). In muscle, IGF-1 exists in two isoforms resulting from alternative splicing of the *IGF-1* gene. IGF-1Ea is equivalent to the systemic isoform and is produced in both the liver and muscle. IGF-1Ec, also referred to as mechano-growth factor (MGF), is produced mainly in the overloaded muscle (Goldspink et al. 2002). It has been suggested that age-related changes in body composition and physical function are related to decreased IGF-1 signaling (Giovannini et al. 2008). Studies investigating the effect of resistance training on the expression of the IGF-1 splice variants have shown that the IGF-1Ea and IGF-1Ec isoforms are differentially regulated in young and older human skeletal muscle, i.e., elderly subjects do not respond to resistance exercise by increased IGF-1Ec mRNA production (Hameed et al. 2003). At the protein level, a synthetic MGF E-peptide has been shown to promote cell proliferation and survival, but no analogous peptide has yet been

detected in vivo (Kandalla et al. 2011; Matheny et al. 2010). Our results showed that the gene expression of these two IGF-1 isoforms was lower in postmenopausal non HRT women compared to premenopausal women. Also, a correlation between the expression of IGF-1Ea and IGF-1Ec was observed in all three groups. Contrary to the suggested role of IGF-1Ec as a transient and load-sensitive growth factor, our study showed differential expression of the IGF-1Ec gene between age groups and with respect to postmenopausal HRT. We suggest that the production of IGF-1Ec is, in addition to overload and stretch, also dependent on the overall endocrine status of the body and needs to be studied further.

Importantly, our study showed that the gene expression level of muscular IGF1R was higher in postmenopausal HRT users compared to their non-using co-twins. Although the importance of IGF-1 in preventing muscle atrophy has been widely recognized in experimental studies and reinforced in human studies (Clemmons 2009), information on the local and long-term anabolic effects of IGF-1 in humans is lacking. It has been shown in mice that aging is associated with lower IGF1R number in skeletal muscle (Li et al. 2003; Willis et al. 1997). The binding of IGF-1 to IGF1R activates the insulin receptor substrate proteins (IRS-1-IRS-6), which have variable roles in further signal transduction. IRS-1 is regarded as a mediator for other downstream signaling molecules and pathways in skeletal muscle, including PI-3 K, the predominant pathway in protein synthesis, reviewed by (Philippou et al. 2007). Our results show that the gene expression of IRS-1 and IRS-2 in skeletal muscle was clearly higher in the HRT users than in their non-using co-twins, which we consider as a further evidence for the increased activity of the IGF-1 pathway in skeletal muscle in women under HRT. Furthermore, our recent work on a different study group showed a similarly increased level of activity of the IGF-1 pathway in HRT users compared to non-users (Pollanen et al. 2010).

In IGF-1 signaling, IGF binding proteins (IGFBPs) have an essential role. IGFBPs, by functioning as carrier proteins for systemic IGFs, determine the physiological concentrations of IGFs, as they regulate IGF turnover, transport and tissue distribution (Jones and Clemmons 1995). In addition to their endocrine role, IGFBPs act locally as paracrine/autocrine factors, inhibiting and/or promoting IGF activities (Duan and Xu 2005). Rodent

skeletal muscle expresses IGFBP3, -4, -5 and -6 (Jennische and Hall 2000), of which, and in line with our human results, IGFBP5 is predominant (James et al. 1993). According to the previous studies, the expression of IGFBP5 has been localized into regenerating muscle cells, while IGFBP3 is expressed mainly in macrophages (Jennische and Hall 2000). IGFBP5 has both stimulatory and inhibitory effects on IGF action, depending on the cell type and context (Beattie et al. 2006; Schneider et al. 2002), while IGF-independent actions of IGFBP5 have also been described (Mohan and Baylink 2002). In our study, the levels of the muscular transcripts of IGFBP3 and IGFBP5 responded differentially to the long-term use of HRT, i.e., the expression level of IGFBP3 was lower and that of IGFBP5 higher in the HRT users than in their non-using co-twins. The expressions of IGFBP3 and IGFBP5 were also found to correlate with the levels of muscular IGF-1Ec and IGF-1R, but only in the premenopausal and in the postmenopausal HRT using women. Enns and Tiidus (2010) recently extensively reviewed the effects of estrogen on skeletal muscle, and found increasing evidence for the advantageous influence of estrogen on macrophage infiltration and muscle regenerative processes such as satellite cell activation. Whether the differential gene expression of IGFBP3 and IGFBP5 reflects these phenomena remains to be clarified. Altogether, our results show that the activity of the IGF-1 pathway in HRT users resembles the activity in premenopausal women, although no significant differences were seen in the systemic levels of IGF-1 or IGFBP3.

The interaction between the immune and endocrine systems with respect to IL-6 and IGF-1 is nowadays considered to be a bi-directional, well-balanced process, highly essential both in several chronic inflammatory diseases and in normal aging (O'Connor et al. 2008). Proinflammatory cytokines are known to act as negative regulators that attenuate the action of IGF-1 in several ways: they can control the secretion of IGF-1, they can decrease IGF-1 sensitivity by increasing the production of IGFBPs, and they can cause IGF-1 resistance, i.e., decrease the response to IGF-1. If we consider our results from this point of view, we can speculate that the postmenopausal inflammatory change causes IGF-1 resistance in skeletal muscle, which HRT then is able to resist by increasing the amount of IGF-1R and by stimulating the post-receptor signaling. Based on our results, however, we cannot distinguish the direct

effects of HRT on the IGF-1 pathway from the possible indirect effects via IL-6 signaling, as the results are compatible with either of these possibilities. Despite the strong negative correlation between serum IGF-1 and sIL-6R in HRT users, the present study is not able to clarify the causality and mechanisms behind the observed effects of age and HRT on the IL-6 and IGF-1 pathways.

In conclusion, our study showed that the systemic levels of IL-6 and its soluble receptors, mainly produced in adipose tissue, are lower in premenopausal women than in postmenopausal women, as well as in long-term HRT users compared to their non-using co-twins. Equally, the muscular expression of the genes related to IGF-1 signaling is higher in premenopausal women than in postmenopausal women, as well as in HRT users compared to their non-using co-twins. In sum, we suggest that the changes observed in the IL-6 and IGF-1 pathways account at least partly for the positive effects of HRT on muscle composition and mass as well.

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