Thesis for doctoral degree (Ph.D.) 2008

ESTROGEN RECEPTORS IN SKELETAL MUSCLE

Anna Wiik

Karolinska Institutet

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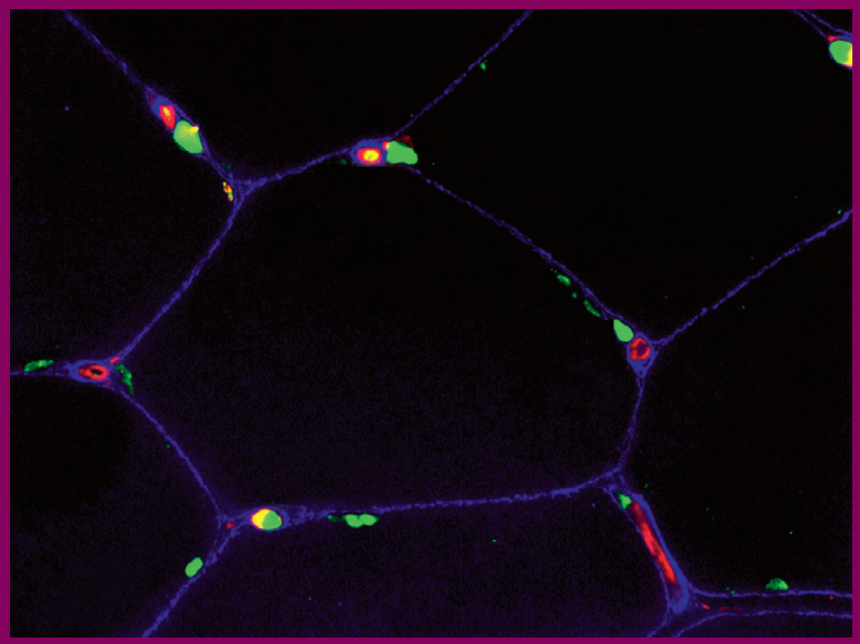
EXPRESSION AND ACTIVATION



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Den som inte åker vilse får inte se mycket.

JOLO

ABSTRACT

There are two estrogen receptors (ERs), ERα and ERβ, which are ligand activated transcription factors. Estrogen, which exerts its effect via ERs, is not only a female reproductive hormone, but acts almost ubiquitously in the human body and is involved in physiological and pathological states in both males and females. Estrogen has effects in bone maintenance and the cardiovascular and central nervous systems as well as the reproductive system. Estrogen reduces blood lipid levels and blood pressure as well as increases insulin sensitivity and endothelial function. The effects of estrogen on skeletal muscle tissue have not been studied extensively, although a few reports indicate a role in muscle strength development and involvement in carbohydrate and lipid metabolism. Just like estrogen physical activity reduces blood lipid levels and blood pressure as well as insulin sensitivity and endothelial function. Interestingly, physical activity transcriptionally activates similar genes as estrogen does, for example vascular endothelial growth factor (VEGF). Thus, considering that physical activity and estrogen have actions in common, the question whether estrogen signalling is induced by physical activity and thus could be involved in down-stream exercise-induced gene expression arises.

The overall aim of this thesis was to study the expression of ERs and their activation in skeletal muscle tissue. The specific aims were to investigate if ERα and ERβ are present in human skeletal muscle. Thereafter, the ER expression was studied in subjects with low endogenous estrogen levels such as men, children and postmenopausal women. Furthermore, the localisation and possible co-expression of the both receptors were investigated. The expression levels of ERs were compared in highly endurance-trained men and moderately active men together with the target gene VEGF. Finally, the activation of ERs by estrogen as well as by muscle contractions was investigated. It was hypothesised that ERs in skeletal muscle are functional and activated by estrogen and by muscle contractions and are involved in the adaptation of skeletal muscle to physical training.

For the first time ERα and ERβ were shown to be expressed in human skeletal muscle representing both sexes and various ages. Approximately 65 % of all nuclei expressed ERα and 70 % expressed ERβ. The ERα and ERβ were located not only to the nuclei of muscle fibres themselves but also to capillaries. Of all ERα- or ERβ-positive nuclei about 25 % were located to capillaries. The two receptors were to a major extent co-expressed in the same nuclei. Endurance-trained men had a higher steady-state mRNA level of both ERα and ERβ compared to normally active men, together with higher expression of VEGF. Muscle contractions of myotubes from rat also increased ERβ mRNA levels without any effect on ERα mRNA. An increase in ERβ mRNA was also seen with estrogen stimulation of the myotubes. Muscle contractions had a similar functional effect as estrogen in myotubes causing activation of estrogen response elements (ERE). In contrast to estrogen, the effects of muscle contractions were most likely independent of ERs.

That ERs are present in the skeletal muscle fibres suggests that this tissue is a target for estrogen action, which was confirmed in myotubes by ERE activation when stimulated with estrogen. In the muscle tissue, estrogen might also have direct effects on the capillaries, since ERs are located to capillaries too. The finding that contraction of myotubes activates ERE-sequences and increases ERβ mRNA levels as well as the higher mRNA levels of ERs in endurance trained men suggest an involvement of ERs and ER target genes in the adaptation of skeletal muscle to physical exercise.

LIST OF PUBLICATIONS

This thesis is based on the following articles, which are referred to in the text by their Roman numerals:

I. **Wiik A**, Glenmark B, Ekman M, Esbjörnsson-Liljedahl M, Johansson O, Bodin K, Enmark E, Jansson E.
Oestrogen receptor beta is expressed in adult human skeletal muscle both at the mRNA and protein level.
Acta Physiol Scand, 2003, 179, 381-387.

II. **Wiik A**, Ekman M, Morgan G, Johansson O, Jansson E, Esbjörnsson M.
Oestrogen receptor beta is present in both mucle fibres and endothelial cells within human skeletal muscle tissue.
Histochem Cell Biol, 2005, 124, 161-165.

III. **Wiik A**, Ekman M, Johansson O, Jansson E, Esbjörnsson M.
Expression of both oestrogen receptor alpha and beta protein in human skeletal muscle.
Submitted

IV. **Wiik A**, Gustafsson T, Esbjörnsson M, Johansson O, Ekman M, Sundberg CJ, Jansson E.
Expression of oestrogen receptor alpha and beta is higher in skeletal muscle of highly endurance-trained than of moderately active men.
Acta Physiol Scand, 2005, 184, 105-112.

V. **Wiik A**, Hellsten Y, Berthelson P, Lundholm L, Fischer H, Jansson E.
Activation of estrogen response elements is mediated both via estrogen and muscle contractions in rat skeletal muscle myotubes.
Submitted

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LIST OF ABBREVIATIONS

ab	Antibody
AEC	Aminoethyl-carbazol
AF-1	Activation function-1
AF-2	Activation function-2
AMCA	Aminomethylcoumarin
ANOVA	Analysis of variance
AP-1	Activator protein-1
cAMP	Cyclic adenosine monophosphate
Cy3	Cyanine 3
DAB	3,3´-diaminobenzidine
DAPI	4´,6 diamidino-2-phenylindole
DHEA	Dehydroepiandrosterone
DMEM	Dulbecco's modified Eagle's medium
E2	17β-estradiol
eNOS	Endothelial nitric oxide synthase
ER	Estrogen receptor
ERE	Estrogen response element
ERK	Extra-cellular regulated kinase
ERKO	Estrogen receptor knock out
ERR	Estrogen related receptor
FITC	Fluorescein-isothiocyanate
GLUT-4	Glucose transporter-4
HDL	High density lipoprotein
IGF-1	Insulin like growth factor-1
LDL	Low density lipoprotein
MAPK	Mitogen-activated protein kinase
MPP	Methyl-piperidon- pyrazole
n	Number of subjects
NO	Nitric oxide
NOS	Nitric oxide synthase
PGC-1	PPAR gamma coactivator-1
SP-1	Specificity protein-1
VEGF	Vascular endothelial growth factor
VLDL	Very low density lipoprotein

INTRODUCTION

SKELETAL MUSCLE HISTOLOGY

The muscular system represents one of the largest systems in the body and comprises over 40 % of the body's mass. Skeletal muscle is composed of elongated cells called muscle fibres and contains several nuclei in contrast to other cell types that have only one nucleus. The nuclei are located just beneath the plasma membrane (sarcolemma). Basal lamina (basement membrane) is located just outside of the sarcolemma and contains collagen IV. Satellite cells are located between the plasma membrane and the basal lamina (fig. 1). They are reserve cells that when activated can proliferate, differentiate and fuse together to form myotubes, which in turn undergo further differentiation to become mature muscle fibres. Mature and functional muscle fibres are parallel to their neighbour and are arranged in bundles called fascicles, which are surrounded by connective tissue. Contracting muscle requires tremendous quantities of energy. Each muscle fibre is also surrounded by an extensive network of capillaries that supply the cell with the necessary oxygen and nutrient required by the activated skeletal muscle (Peachy et al. 1983).

Resting skeletal muscle fibres rely mostly on the aerobic metabolism of fatty acids, which are absorbed from the circulation. When skeletal muscle contractions occur, mitochondria break down glucose either from the surrounding or from glycogen reserves. During physical activity the milieu surrounding the skeletal muscle changes, the temperature increases, pH and oxygen levels decrease. These and other factors affect a lot of proteins in the muscle cell. For example the degree of phosphorylation of protein kinases are affected which in turn can activate genes that lead to an adaptation to training (Saltin and Gollnick 1983).

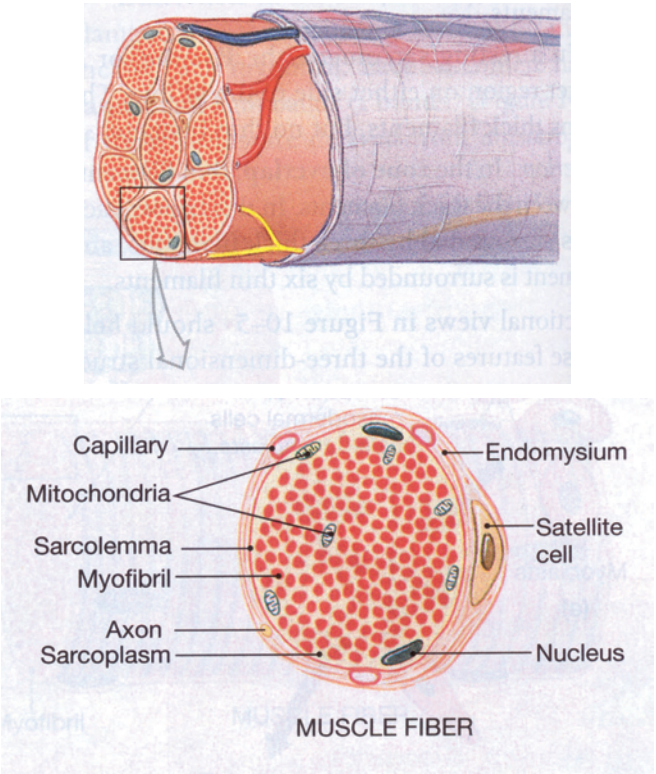

Figure 1. Schematic figure of a muscle and a cross-cut of a muscle fibre indicating the location of the muscle nuclei, capillaries and satellite cells.

ESTROGENS

Estrogens are steroid hormones that are synthesized from cholesterol. The most potent and dominant estrogen in humans is 17β-estradiol (E2), but lower levels of the estrogens estrone and estriol are also present. Estrone is the most abundant estrogen in postmenopausal women. In premenopausal women, most of the estrogens are produced in the ovary, while in men and postmenopausal women it is produced by aromatization of androgens in peripheral tissue (fig. 2). Tissues that have been reported to synthesise estrogen includes muscle, fat, liver and brain (Brodie and Inkster 1993; Matsumine et al. 1986; Miller 1991; Naftolin et al. 1975). The great mass of muscle and fat could thereby be expected to be the main contributor to total peripheral estrogen formation. Although aromatase activity and level of expression are low in skeletal muscle, such

small activity can be compensated for by the bulk of the tissue in the body (Larionov et al. 2003).

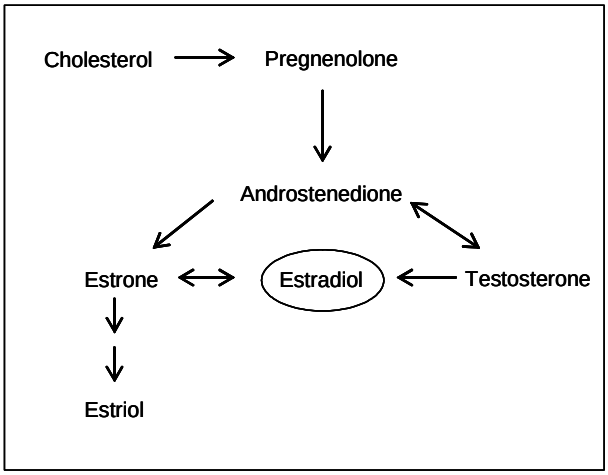

Figure 2. Steroid synthesis.

When estrogens are released into the circulation most of it is bound to plasma proteins and transported to target tissues. The steroid hormones are lipophilic and have a low molecular weight that enables them to enter the target cells by passive diffusion. Estrogens have a broad range of target tissues in the human body (Nilsson et al. 2001). For example, estrogens are required for female sexual maturation and affects growth, differentiation and function of the female reproductive system. In addition, estrogens have an important role in for instants the liver (Fisher et al. 1984), in bone metabolism (Manolagas 2000), the nervous system (Joels 1997), the cardiovascular system (Mendelsohn and Karas 1999) as well as the development and function of the immune system (Carlsten et al. 1989) in both sexes. In skeleton, estrogens prevent bone resorption and estrogen replacement therapy are known to reduce osteoporosis in postmenopausal women (Rossouw et al. 2002). In the nervous system estrogen has a numerous of different effects such as beneficial for learning and memory as well as controlling the hypothalamic-pituitary-gonadal axis (Birge 1996; Lamberts et al. 1997). In the cardiovascular system estrogen exerts protective effects by influencing the vascular function with effects on vascular tone and blood flow and subsequently arterial blood pressure (Mendelsohn and Karas 1999).

SIMILARITIES OF PHYSICAL ACTIVITY AND ESTROGEN

Estrogen and skeletal muscle

Very little is known about the effect of estrogen in skeletal muscle. Some animal studies indicate that estrogen can influence skeletal muscle growth. Estrogen administrated to ovariectomized immature female rats increased the weights of gastrocnemius and soleus muscles (Sillence et al. 1995). In humans high estrogen levels during the normal menstrual cycle have been suggested to influence muscle strength with increased maximum voluntary muscle force when the level of estrogen is highest in healthy regularly menstruating women (Phillips et al. 1996; Sarwar et al. 1996). In addition, some published prospective randomized studies, strongly suggest that estrogen has an anabolic effect on skeletal muscle, as shown by a positive or preserving effect on muscle strength in postmenopausal women (Heikkinen et al. 1997; Skelton et al. 1999). However, the effects of estrogen on muscle strength are conflicting since other studies have not been able to detect any effect of hormone replacement therapy (Greeves et al. 1997; Ribom et al. 2002; Taaffe et al. 1995). Furthermore, estrogen has been shown to be an important factor in protecting muscle from exercise-induced muscle damage (Tiidus 1995).

Proliferation and/or activation of muscle satellite cells may be involved in steroid-induced muscle growth. Satellite cells from mice stimulated with estrogen shows an increased proliferation (Deasy et al. 2007). Estrogenic influence on satellite cell proliferation may be mediated by insulin like growth factor-1 (IGF-1), which is known to simulate satellite cell proliferation. Treatment of bovine satellite cell cultures with estrogen increase IGF-1 mRNA level and proliferation (Kamanga-Sollo et al. 2004). This is consistent with in vivo studies showing that treatment of steers with estrogen increases muscle IGF-1 mRNA levels and muscle growth (Pampusch et al. 2003). RALGRO®, an estrogen-active compound, also exerts strong anabolic effects in farm animals (Pfaffl et al. 2001). In a study by Tiidus et al. (2005) downhill running in male rats was shown to significantly elevate the number of satellite cells in both soleus and white vastus muscle samples (Tiidus et al. 2005). Interestingly, estrogen supplementation resulted in greater post-exercise increase in satellite cells

detected compared to trained muscle without estrogen and this was mediated by ERs (Enns et al. 2008).

Estrogen and the cardiovascular system

The female sex and physical activity are both well-known factors that reduce the risk to die in cardiovascular disease. The incidence of cardiovascular disease demonstrates significant gender-based differences and is low in premenopausal women, rises in postmenopausal women and is reduced in postmenopausal women who receive estrogen therapy (Mendelsohn and Karas 1999), although available data on the benefits of estrogen therapy are inconsistent (Rossouw et al. 2002). The biological function of estrogen has been studied in a man with no functional estrogen receptors (ERs), which demonstrates, among others, evidence for early endothelial dysfunction, atherosclerosis and low levels of the good cholesterol (Sudhir et al. 1997). All these are risk factors for cardiovascular diseases and indicate that estrogen has a protective role in the development of cardiovascular disease. If the positive effects of estrogen involve the skeletal muscle system is unknown.

Endothelium dependent vasodilatation varies during the menstrual cycle along with the estrogen level and vasodilatation through endothelial production of nitric oxide (NO) can be enhanced in females by estrogen through both short-term and long-term effects on the vasculature. Flow mediated dilatation is enhanced by estrogen in vessels (Kublickiene et al. 2008). Thus, the rapid vasodilatation caused by estrogen may be mediated by estrogen receptors located to the cell membrane and activates endothelial nitric oxide synthase (eNOS) to release NO (Chen et al. 1999). Estrogen have also long-term effects on eNOS gene expression (Mendelsohn 2002). Furthermore, estrogen regulates the expression of a number of other vasodilators and vasoconstrictor proteins as well, including multiple components of the renin-angiotensin system (Mendelsohn 2002).

Estrogen has also indirect beneficial effects on the vasculature by affecting plasma lipoproteins by decreasing low density lipoprotein (LDL) cholesterol and increasing high density lipoprotein (HDL) cholesterol, which has been shown in postmenopausal women receiving estrogen.

There are now a number of studies, both in humans and rodents, that link estrogen to the maintenance of glucose metabolism (Louet et al. 2004). Knockout mice with a deletion of the aromatase gene cannot synthesise estrogen. These animals

develop insulin resistance. The same is true for the estrogen receptor knock out (ERKO) mice where the ERα gene has been deleted. In addition, postmenopausal women have a higher risk for type 2 diabetes than younger women and treatment of healthy postmenopausal women with estrogen has been shown to improve insulin sensitivity and lowers blood glucose levels (Crespo et al. 2002; Espeland et al. 1998; Saglam et al. 2002).

Several studies on hormone replacement therapy after the menopause reveal a substantial reduction in the incidence of cardiovascular disease. However, estrogen may not prevent further cardiovascular events in women who already have established disease at the time of instituting hormone replacement therapy. Also negative effects have been shown with hormone therapy such as genital bleeding and increased risk of breast cancer and thrombosis (Mijatovic et al. 1999). A clinical trial including 17.000 postmenopausal women on a combined hormonal replacement therapy (estrogen and progesterone) was interrupted because of an increased incidence of myocardial infarction in the hormonal treated women (Rossouw et al. 2002). Several authors have heavily criticized this study, claiming that the conclusions drawn were not warranted due to flawed design of the study.

Physical activity and skeletal muscle

Skeletal muscle is a tissue that can easily adapt to changes. Physical activity is known to have a great impact on the structure and function of the muscle depending on subjects, training mode etc (Fluck 2006; Saltin and Gollnick 1983). A typical adaptation to for instance endurance training is an increase in the number of mitochondria and capillaries. An obvious effect of increasing the number of capillaries would be to reduce the diffusion distances within the muscle, which may be crucial for gas and substrate transport from the blood to the muscle cell. The number and volume of mitochondria increase, which increases the surface area for the exchange of metabolites and end products between the cytosol and the mitochondrial matrix. These changes give rise to a reduced anaerobic metabolism at a certain work load. Adaptation to resistance training on the other hand results in increases in muscle bulk with increased fibre areas (Saltin and Gollnick 1983).

Physical activity and the cardiovascular system

Regular physical activity is an important factor in the prevention of cardiovascular diseases and the skeletal muscle system of course plays an important role in this

prevention. During the last decades a great number of studies have shown that regular physical activity, in a dose-dependent manner, is associated to reduced morbidity and risk to die in cardiovascular disease (Booth et al. 2000; Kushi et al. 1997; Manson et al. 1999). The positive effects of physical activity are possibly related to reduced blood lipid levels, increased insulin sensitivity and fibrinolytic activity and an adaptation of the circulatory system with increased endothelial function and decreased blood pressure (Libonati 1999; Linke et al. 2006; Shephard and Balady 1999).

A suggested beneficial effect of physical activity is the endothelium-dependent vasodilatation in the vasculature caused by NO. Release of NO is stimulated by a rise in shear stress associated with increases in blood flow in activated muscle during physical exercise (Miller and Vanhoutte 1988). Exercise-mediated increase in NO levels are largely due to an up-regulation of eNOS mRNA and protein expression (Sessa et al. 1994; Woodman et al. 1997). Another positive example is that physical exercise has been shown to increase both the number and the diameter of arterial blood vessels in skeletal muscle and the myocardium. These changes of architecture of the vascular tree are likely associated with functional changes and improved organ blood flow (Linke et al. 2006; Shephard and Balady 1999).

Contracting skeletal muscles increase their glucose uptake. The insulin sensitivity and glucose tolerance are both increased with physical activity due to an increase in glucose transporters (GLUT-4) in the membrane of the skeletal muscle, which facilitates the uptake of glucose (Yu et al. 2001). Body composition and fat distribution are linked to cardiovascular mortality and are improved by exercise which increases muscle mass that utilizes more glucose than does adipose tissue.

Furthermore, exercise leads to a reduction in total cholesterol, in LDL and an increase in the good cholesterol HDL. After just one bout of exercise, changes in blood lipid levels are detected with an increase in HDL and a decrease in the levels of triglycerides and very low lipoprotein (VLDL) (Borsheim et al. 1999). These rapid changes in blood lipid composition are one of the factors behind the reduced risk of cardiovascular disease found in well-trained subjects. HDL has an important function in the reverse transport of LDL from the periphery tissues back to the liver, thereby reducing LDL. Furthermore, muscle cells take up LDL and degrade it (Gurusinghe et al. 1988). High LDL levels are a leading factor in the formation of atherosclerosis.

To observe is the great similarities between the possible effects of estrogen and regular physical activity (fig. 3). During the menstrual cycle systemic vascular resistance, blood pressure and cardiac output changes. These fluctuations trigger the cardiovascular system comparable to the in the present study described effects of physical activity. Since the estrogen level fluctuates during the menstrual cycle and all of these changes can be induced by estrogen, it seems to be responsible for the cardiovascular changes occurring in menstruating women which are comparable to the circulatory efforts of athletes (Eskes and Haanen 2007). This continuous biological challenge during the reproductive years creates optimal cardiovascular compliance in women comparable to the effects of exercise.

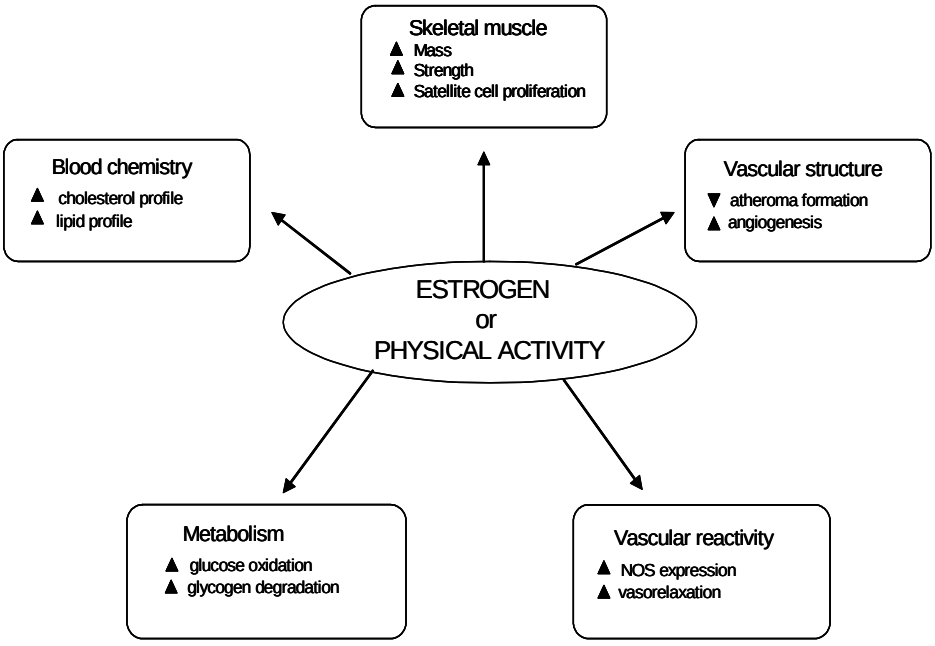

Figure 3. Effects of estrogen and physical activity.

ESTROGEN RECEPTORS

In the early sixties the presence of an estrogen binding receptor was first reported by Jensen and Jacobsen (Jensen 1962). This was isolated and cloned in the middle of the eighties by Green et al. (1986) and was for a long time believed to be the only existing

ER (Green et al. 1986). Then in 1996 an additional ER was discovered (Kuiper et al. 1996). This new ER was named ERβ and consequently the first ER was renamed ERα.

The ERs belong to the nuclear receptor super family and share a common structure including five distinguishable domains. They are named A/B, C, D, E and F domains (fig. 4). The N-terminal A/B domain contains a transactivation function that activates transcription of target genes. This domain varies the most between ERα and ERβ. The C domain, the DNA-binding domain, is involved in specific DNA binding and receptor dimerization. This domain is highly similar between ERα and ERβ, which indicates that the target genes are the same for the two receptors. The D domain works as a flexible hinge between the DNA-binding domain and the E domain. The E domain is referred to as the ligand-binding domain. It is important for ligand binding, receptor dimerization and transcriptional activation. The function of the F domain is still poorly understood. There are two activation function sites in the ER, AF-1 and AF-2.

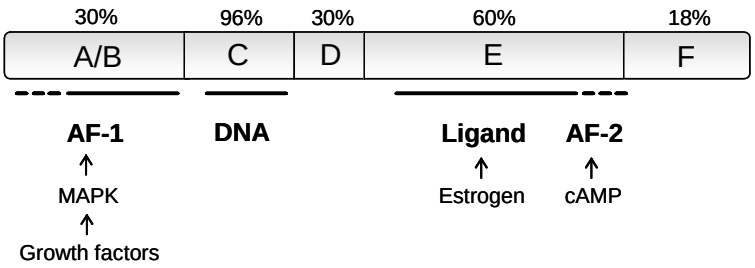

Figure 4. Schematic figure of ERα protein with its different domains. The percentage number indicates the sequence similarity with ERβ. The locations of the two activation functions and their possible activation are also indicated.

The AF-1 is located in the N-terminal and AF-2 within the ligand binding domain of the receptor and induces ligand-dependent activation of transcription. They are believed to function by binding co-activators and bringing them to the promoter of the target gene. It can function autonomously and in the absence of estrogen. AF-1 is not well understood but seems to be weaker in ERβ than the AF-1 of ERα. To give full transcriptional response of an ER agonist, a synergism between the weaker AF-1 and the stronger hormone inducible AF-2 is required (Kraus et al. 1995).

Several different spliced forms of ER have been reported, whether all are translated to protein and have any biological function is not established. Even though ERα and ERβ are highly homologous their alternative splicing pattern differs. Two splice variants of ERα have been shown to inhibit the wild-type receptor and might act as regulators of gene transcription (Bollig and Miksicek 2000). Also for ERβ different splice variants have been identified. ERβcx has a deletion which makes it unable to bind ligand. However it can heterodimers with preferentially ERα and inhibit ERα induced gene transcription. Another ERβ splice variant is called ERβ2 and shows impaired E2 binding ability. ERβ2 may function as a dominant negative partner of both ERα and ERβ with reduced transcriptional activity. The expression of splice variants appears to be tissue specific (Poola et al. 2002).

Tissue distribution of ERs

The tissue distribution of ERα and ERβ is in part different. Classical oestrogen targets are the uterus, mammary gland, placenta, central nervous system, cardiovascular system and bone. These tissues have a high ERα content. Non-classical target tissues include prostate, testis, ovary, adrenals, pancreas, skin and urinary tract (Ciocca and Roig 1995). The expression of ERα is either low or not measurable in these tissues. Besides the classic estrogen tissues, ERβ is also highly expressed in many non-classical estrogen target tissues (Taylor and Al-Azzawi 2000). ERβ has a broader tissue distribution than ERα suggesting that the two receptors have distinct biological functions. This is evident when studying the different phenotypes of ERα and ERβ knock-out mice (αERKO and βERKO, respectively). Both single and double knock-out mice can survive to adulthood, albeit with retarded growth. The most striking phenotypes in αERKO mice are complete infertility in both sexes. In contrast, male βERKO mice are fertile whereas the females are sub fertile; they have fewer litters with reduced number of pups (Couse and Korach 1999). The basal release of endothelium-derived NO is decreased in male αERKO (Rubanyi et al. 1997) and the estrogen mediated production of NO is abolished (Pendaries et al. 2002). βERKO mice develop hypertension in both sexes as they age (Zhu et al. 2002), which confirms their role in the cardiovascular system.

ERs in skeletal muscle

At the start of the work with this thesis there were only a few reports on ER in the non-classical estrogen target tissue skeletal muscle. ERα mRNA had been detected in mouse (Couse et al. 1997) and binding studies had indicated the protein expression of ERs in skeletal muscle of various animals (Dahlberg 1982; Saartok 1984). *Direct evidence for ER protein in skeletal muscle had not been found in humans or in any other species.* In parallel with the work of the present thesis studies on different species showed an expression of ERα in skeletal muscle, among them human skeletal muscle (Couse et al. 1997; Lemoine et al. 2002; Lemoine et al. 2003; Pfaffl et al. 2001). Pfaffl et al. (2001) showed for the first time the expression of ERβ mRNA in skeletal muscle from heifer. Immunohistochemical studies of sex steroid receptors in human skeletal muscle (m. levator ani) demonstrated nuclear expression of progesterone and androgen receptors but not of ERs (Copas et al. 2001; Oettling and Franz 1998). Recently, however both ERα and ERβ protein has been found to be present in mouse and pig skeletal muscle as well as in myoblasts from rat and mouse (Barros et al. 2006; Kalbe et al. 2007) which confirms the findings of this thesis.

ESTROGEN RECEPTOR SIGNALLING

It was for many years believed that the only mechanism by which estrogen affected expression of estrogen-responsive genes was by direct binding of the activated ER to specific estrogen response elements (EREs) on DNA. However, evidence for signalling pathways that deviate from this classical model has emerged. Today, it is accepted that ER may regulate transcription from target genes by a number of distinct mechanisms, both in the presence and absence of estrogen (fig. 5). Activation of ER appears to be a multi-step process relying on a number of molecular events, including dimerization, the actual binding of ligand, phosphorylation, interaction with cofactors and DNA binding. *If ERs are functional and how they are activated in skeletal muscle is largely unknown.* In myoblasts, Kahlert et al. (1997) demonstrated functional ERs, which were activated by estrogen.

Classical ligand-dependent activation of ER

In the absence of ligand, ERs are located to the cell nucleus in a multiprotein complex containing heat shock proteins (Baulieu et al. 1990). When estrogens, which can diffuse across the plasma and nuclear membranes of cells, bind to the ER a conformational change occurs that promotes receptor dimerization. The activated ERs bind as homodimers or heterodimers to EREs located in the regulatory regions of target genes. The ERE sequence is a 13 base par palindromic inverted repeat with the consensus sequence: 5' –GGTCAnnnTGACC-3'. The binding of ERs to EREs facilitates the assembly of basal transcription factors into a stable pre-initiation complex and increases transcription rate for target mRNA synthesis (Nilsson and Gustafsson 2002). The conformational change of activated ERs also lead to that an interaction surface for co-activators is provided. Ligand-dependent activation of transcription by ERs is mediated by the interactions of a number of different nuclear receptor co-activators.

Ligand-independent activation of ER

The ERs can also be activated without any estrogen present. Within the AF-1 site of ER there are well-conserved serine residues, which are target for phosphorylation. Binding of growth factors, such as IGF-1 and epidermal growth factor, to its cognate receptor results in the intracellular activation of mitogen-activated protein kinase (MAPK) signal transduction cascade that influences the transcriptional activity of the ERα by phosphorylation of serine residues (Bunone et al. 1996; Kato et al. 1995). Trembley et al. (1999) showed a similar ligand-independent activation of the ERβ (Tremblay et al. 1999). Phosphorylation events have been demonstrated to be the foremost mechanism in the ligand-independent activation of ER. Estrogen also induces phosphorylation of serine residues, but this appears to be independent of MAPK (Joel et al. 1998). It is also described that a combined stimulation with growth factors and estrogen gives potentiated effect (Smith 1998). In bone cells, mechanical strain has a similar effect on increasing ERE activity as more prolonged exposure to estrogen (Zaman et al. 2000). It is suggested that strain has its effects on increased ERE activity by phosphorylation of the ER using kinase-dependent signalling pathways (Lee and Lanyon 2004). Strain-induced ER phosphorylation does not require the presence of estrogen, but is dependent on extra-cellular regulated kinase (ERK), a member of the MAPK family (Jessop et al. 2002).

ER may also be activated by cAMP induced signalling (El-Tanani and Green 1997). Activation via cAMP signalling pathways requires the AF-2 site, in contrast to the MAPK which requires the AF-1 site, and appears to be dependent on protein kinase A that is activated by cAMP. This represents a pathway distinct from activation via peptide growth factors.

Non-ERE-dependent actions of ER

In addition to binding to the ERE, the activated ERs can interact with other DNA-bound transcription factors to regulate the transcription of certain sets of genes. In this mechanism, ERs do not themselves bind DNA; instead it is tethered by protein-protein interactions to a transcription factor complex that contacts the DNA. AP-1 sites and SP-1 sites are well characterized motifs that could mediate estrogen signalling via other bound transcription factors, such as FOS/JUN (Bjornstrom and Sjoberg 2005; Porter et al. 1997; Webb et al. 1995). The discovery of this mechanism explains how estrogen regulates genes in which no consensus ERE has been found.

Non-genomic signalling

There is evidence suggesting that estrogen has non-genomic effects too, since very rapid effects of estrogen have been observed (Mendelsohn 2002). These effects are too rapid to be accounted for by transcriptional activation or repression of target genes, which occurs with a time lag of several hours. These effects occur within seconds to minutes after estrogen treatment and cannot be blocked by transcription or translational inhibitors. Studies have suggested that these effects may be the result of estrogen activation of MAPK and ERK signalling (Pedram et al. 2006) or release of intracellular calcium (Mermelstein et al. 1996). The MAPK pathway is rapidly activated by estrogen in various cell types, for example endothelial cells (Chen et al. 1999). Some of the protective effects of estrogen in the cardiovascular system are mediated by a non-genomic mechanism involving rapid activation of eNOS by estrogen through the MAPK pathway (Simoncini and Genazzani 2000). The activated eNOS releases NO which promotes vasodilatation. eNOS is also regulated on the genomic level by estrogen by activating an ERE-sequence in its promoter region (Mendelsohn 2002).

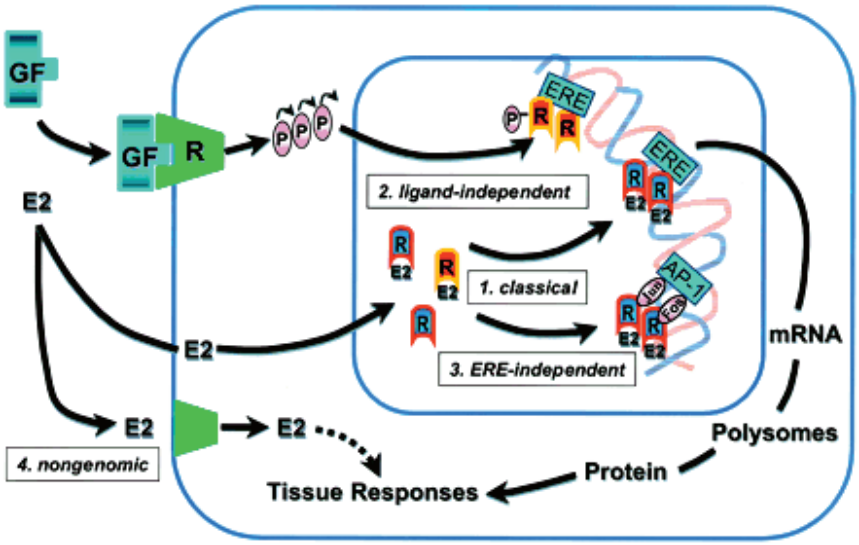

Figure 5. Proposed mechanisms of ER signalling pathways.

ESTROGEN RELATED RECEPTORS

There are three estrogen related receptors (ERRs) ERRα, ERRβ and ERRγ. These receptors as well as ERs belong to the nuclear receptor super family and have high similarity to ERs, especially in the DNA-binding domain. The ERRs can bind to the same EREs as ERs. Thus, the ERs and ERRs can regulate common target genes and in tissues where they are both expressed, collaborate with each other to dictate the overall response. The ERRs cannot bind estrogen; they are orphan receptors with no known ligand. ERRα is ubiquitously expressed in adult tissues (Giguere et al. 1988; Sladek et al. 1997) and is highly expressed in skeletal muscle (Bonnelye et al. 1997; Sladek et al. 1997), while ERRβ expression in adult tissues appears to be low and is found only in a few organs (Giguere et al. 1988). ERRγ appears to be expressed in several tissues, in particular brain and kidney (Heard et al. 2000). In adults, ERRα and ERRγ expression

is enriched in tissues that rely primarily on mitochondrial oxidative metabolism for energy generation, such as heart, brown adipose and slow-twitch skeletal muscle (Huss et al. 2004).

ERRα is one of the major regulators of mitochondrial function in response to exercise and is also involved in a novel angiogenic pathway of oxygen and substrate delivery (Arany et al. 2008). ERRα activates genes involved in multiple key energy production pathways and is a critical regulator of energy metabolism in heart and skeletal muscle. A potent co-activator for ERR are the PPAR gamma co-activtor-1 (PGC-1) family. PGC-1 is a transcriptional co-activator and a key regulator of an array of cellular energy metabolic pathways, but its primary effect in target tissues is to enhance mitochondrial oxidative metabolism by increasing cellular mitochondrial number, fatty acid oxidation and respiration via co-activation of nuclear receptor transcription factors (Vega et al. 2000). PGC-1 distinguishes itself from other co-activators by its tissue specificity and regulated expression (Kamei et al. 2003). ERRα interacts physically with PGC-1 and enables activation of transcription and suggests that ERRα plays a role in some of the known PGC-1α-regulated pathways (Huss et al. 2004).

PGC-1β, which was first called ERR ligand 1, can function as a protein ligand of ERRs and activate ERR-mediated transcription, at least in cell cultures. PGC-1β mRNA is highly expressed in skeletal muscle and generally the expression pattern of PGC-1β closely resembles that of ERRα (Kamei et al. 2003).

AIMS OF THE STUDY

The overall aim of this thesis was to study the expression of estrogen receptors and their activation in skeletal muscle tissue. It was hypothesised that ERs in skeletal muscle are functional and that estrogen as well as muscle contractions induce activation of ERE-sequenes in skeletal muscle and that this activation is ER dependent.

The specific aims of the thesis were:

- To investigate if ERα and ERβ are present in human skeletal muscle.

- To study the protein expression of ERα and ERβ in skeletal muscle in relation to sex and age.

- To study localisation of ERs in human skeletal muscle with regard to:
    1. distribution between muscle fibres and capillaries
    2. co-localisation to the same nuclei.

- To study the skeletal muscle expression of ERα and ERβ in trained muscle and to compare the expression level of ERs and its target gene VEGF.

- To investigate the activation of EREs by estrogen and muscle contractions in skeletal muscle and to assess whether the activation is ER dependent.

MATERIAL AND METHODS

SUBJECTS AND SKELETAL MUSCLE BIOPSIES

All subjects participating were healthy volunteers. The subject characteristics are presented in table 1. In paper I and II the presence and location of ERs were studied in men and women. To characterize the ERs in different age groups children and postmenopausal women not on hormone replacement treatment were included in paper III. In paper IV highly endurance-trained men (elite cyclists and triathletes) were compared to normally active men. The level of physical activity in all studies was assessed by the subjects' evaluated leisure-time physical activity. Prior to the respective study, the procedure was explained in both oral and written forms. All subjects gave their informed consent before inclusion in the study, as did the parents of the children. The Ethics Committee of the Karolinska Institutet approved all studies. Muscle biopsies were performed at rest from the vastus lateralis of the quadriceps femoris muscle using percutaneous needle biopsy (Bergström 1962). The muscle tissue samples were frozen in isopentane precooled in liquid nitrogen and stored at –80° C until analysed.

Table 1. Subject characteristics

Study	Subject	n	Age (years)	Weight (kg)	Height (cm)
I	Women	3	25 (24-26)	59 (56-62)	168 (166-171)
	Men	3	22 (19-24)		
II	Women	2	25, 26	58, 58	167, 171
	Men	2	19, 21	85, 74	179, 182
III	Women	4	24 (21-28)	65 (50-75)	170 (160-176)
	Postmenopausal women	2	54, 59	54, 55	155, 166
	Girl	1	10	36	152
	Men	4	24 (21-29)	74 (58-92)	176 (169-185)
	Boy	1	10	50	155
IV	Trained men	10	22 (18-27)	72 (65-81)	182 (174-195)
	Control men	10	24 (19-28)	74 (65-92)	180 (174-190)

PRIMARY RAT SKELETAL MUSCLE CELL CULTURES

Wistar male rats (M&B, Denmark) weighing 100 g were anaesthetized with 0.1 ml sodium pentobarbital (50 mg/ml). All treatment of animals complied with the European Convention for the Protection of Vertebrate Animals Used for Experimental or other Scientific Purposes (Council of Europe No. 123, Strasbourg, France, 1985).

Carefully the muscle fascia was removed and soleus, gastrocnemius and quadriceps femoris were removed and minced into small pieces with scissors and then digested to extract the satellite cells. The cells were suspended in primary growth medium counted and seeded out onto 35 mm dishes (Nunc, Denmark) coated with 1% matrigel and incubated at 8% CO_2 and 37º C. Rat myoblasts were grown to a density of 90-95% confluency in phenol red-free Dulbecco's modified Eagles' medium (DMEM) supplemented with dextran-coated charcoal-treated serum. Phenol red-free medium was used throughout all experiments, as phenol red is known to act as a weak estrogen. Cells were transfected with ERE-LUC, a reporter containing three copies of the vitellogenin estrogen responsive element driving expression of the firefly luciferase cDNA. Transfection was performed using Lipofectamine 2000 (Invitrogene) in Opti-Mem (Invitrogene) according to standard protocol. A plasmid expressing β-galactosidase was included to allow for normalization of the transfection efficiency and to exclude a general effect of estrogen in the transfected cells. After 5 h the transfection medium was changed to phenol red-free DMEM. The cells were than differentiated into myotubes by changing to primary fusion medium. After 5-6 additional days the primary skeletal muscle cells were ready for experiments.

Before stimulation, the cell medium was changed to serum free medium for 12 h. Muscle cells transfected with ERE-luc and non-transfected cells were either stimulated with estrogen (10 nM) for 6 h or were electrically stimulated to contract in an incubator for 1.5 h or 3 h at 10 V and a frequency of 50 Hz. The stimuli consisted of 0.5 s trains with 0.5 s pauses between the trains and 1 ms pulse width. The pure ER-antagonist ICI 182 780 (100 nM) and the ERα specific antagonist methyl-piperidon-pyrazole (MPP) (1 μM) were added 30 min prior to the stimulation to study the ER-dependent activation. Directly after estrogen stimulation or at 3 h after the end of stimulation, the transfected cells were lysed and collected for determination of ERE activation. Luciferase activity was determined by a luciferase reporter assay (Biothema) and β-galactosidase by a Galacto-Star assay (Applied Biosystems) according to the

manufacturers' instructions on a luminometer Tecan Infinite M200. The non-transfected cells were lysed with Trizol reagent and collected for mRNA analysis.

mRNA MEASUREMENTS

Total RNA was prepared by the acid phenol method (Chomczynski and Sacchi 1987) and quantified spectrophotometrically by absorbance at 260 nm. Integrity of total RNA was determined by 1% agarose-gel electrophoresis. Two micrograms of RNA were reverse transcribed by Superscript reverse transcriptase (Invitrogene) using random hexamer primers (Roche Diagnostics GmbH) in a total volume of 20 µl. Real-time PCR analysis was performed with the ABI-PRISM® 7700 Sequence Detector (Applied Biosystems). In paper I and IV oligonucleotide primers and TaqMan probes were designed by using Primer Express version 1.0 (Perkin-Elmer Applied Biosystems). The primers and probes designed for ERα, ERβ and VEGF are shown in Table 2. The probes were designed to cover exon–exon boundaries to avoid amplification of genomic DNA. The specificity of primers and probes for ERα and ERβ was verified by sequencing the amplified products from ERα and ERβ plasmids, respectively. In paper V primers and probes were achieved as pre-designed assays (Applied Biosystems, table 2). mRNA levels were calculated by the Standard Curve Method according to instructions in User Bulletin no.2 (Applied Biosystems). The mRNA expression levels were normalized to 18S rRNA or β-actin (Applied Biosystems) to correct for potential variations in RNA loading.

In paper I β-actin and 18S were compared. The data expressed relative to 18S rRNA showed the same pattern as those related to β-actin mRNA, when comparing the expressions of ERα and ERβ mRNA. However, when making intra-individual comparisons for either ERα or ERβ, the agreement between 18S rRNA and β-actin mRNA-related values was low. In repeated analyses on different days, the intra-individual variability in 18S rRNA-related value was much greater than in β-actin mRNA-related values. Therefore in paper I, we chose to present only the β-actin mRNA-related values. In paper IV and V we normalized to 18S because in paper IV the β-actin values differed between the two subject groups. The highly endurance trained men had higher β-actin expression than the moderately active men. In paper V we thought that 18S might be more stable to muscle contractions than β-actin. The

methodological error for repeated mRNA analysis from the same cDNA was calculated with the formula:
SD differences/mean value x 100 x 1/√2 and ranged between 0.3% - 1% (Ct values) for the different genes.

Table 2. Primers and probes. The 5' end of the probes was labelled with either FAM for ERα, ERβ and VEGF and with TAMRA for 18S and β-actin.

Study	Gene	Species	GenEMBL Acc. Nr	Primers and probes	Applied Biosystems nr
I, IV	ERα	Human	M12674	F: GGCCCAGCTCCTCCTCAT R: GGCACCACGTTCTTGCACTT P: CCACATCAGGCACATGAGTAACAAAGGCA	
I, IV	ERβ	Human	AF051427	F: CGACAAGGAGTTGGTACACATGA R: CGAACAGGCTGAGCTCCAC P: AAGCCGGGAATCTTCTTGGCCCA	
I, IV	VEGF	Human	M11167	F: ACTGCCATCCAATCGAGACC R: GATGGCTTGAAGATGTACTCGATCT P: TGGTGGACATCTTCCAGGAGTACCCTGAT	
V	ERα	Rat			RN 562610m1
V	ERβ	Rat			Rn 664737m1
I, IV, V	18S	Human, rat			4310893E
I	β-actin	Human			4310881E

IMMUNOHISTOCHEMISTRY

Cross-cut sections from frozen skeletal muscle tissue were fixed in either cold methanol followed by cold acetone or in formalin. In study II, paraffin sections were also used and were deparafinised, rehydrated and processed in citrate buffer pH 6.0 for antigen retrieval by microwave treatment. Endogenous peroxidase activity was quenched with hydrogen peroxide and to reduce non-specific binding of the secondary antibody, the sections were treated with 5% normal rabbit or swine serum depending on the secondary antibody before incubation with primary antibody (see table 3 for details). The sections were incubated with primary antibody over night at 4° C and thereafter

extensively rinsed followed by secondary antibody. All dilutions were made in phosphate-buffered saline or tris-buffered saline containing 1% bovine serum albumin and 0.3% Triton X-100. For visualisation with light microscopy the colour reaction was developed either by aminoethyl-carbazol (AEC) or by 3,3´-diaminobenzidine (DAB). The biotin labelled antibodies had to be incubated with an avidin-biotin complex kit (Dako) before visualisation with DAB. To study the nuclear localization, sections were counterstained with haematoxylin (for light microscopy) and 4′,6 diamidino-2-phenylindole (DAPI) (for fluorescent microscopy), which binds to chromatin and stains the nuclei blue. As negative controls, the primary antibody was replaced with buffer or for ERβ a pre-absorbed ERβ 503 antibody (Saji et al. 2000). The sections were mounted in Vectashield (Vector Laboratories, Burlingame, CA, USA) or Prolong Gold (Invitrogene, Eugene, OR, USA) and evaluated with a light microscope or fluorescence microscope equipped with the appropriate filters.

There have been a lot of difficulties in finding the ERα protein in human skeletal muscle. In paper I we tried three different antibodies without any success in locating the ERα even though the uterine muscle tissue used as a positive control stained positive with all three antibodies. Although we tried several different antibodies and staining protocols with different fixations and amplification steps and used both fluorescence and light microscope we could not find ERα protein until recently. The antibody in paper III was the first to succeed in demonstrating ERα protein in human skeletal muscle.

Table 3. Overview of the immunohistochemistry protocols.

Study	Thickness	Fixation	Primary ab	Dilution	Secondary ab labelling	Dilution	Chromogen/ fluorochrome
I	14 µm	methanol/ acetone	ERβ 503 ERα ID5 HC20 6F11	1:100	peroxidase biotin	1:100 1:200	DAB DAB
II	3 µm	paraffin	Coll IV ERβ 503 CD34 CD45	1:450 1:45 1:75 1:30		1:100	AMCA FITC Rhodamine Red-x
	5 µm	methanol/ acetone	ERβ 503 Coll IV CD3 CD11b	1:50 1:800 1:40 1:40	peroxidase biotin	1:1000 1:20 1:200 1:200	AEC DAB
III	5 µm	formalin	ERα C311 ERβ 503 Coll IV	1:50 1:400 1:450	donkey	1:500 1:75 1:100	Cy3 FITC AMCA
IV	5 µm	methanol/ acetone	ERβ 503	1:100	peroxidase rabbit	1:1000	DAB

Quantitative evaluation of immunohistochemistry

For quantification of the immunostaining an image analysis system QWin 500IW was used (Leica Microsystems). An average of five different areas of each muscle section (approximately 200 fibres) were analysed and measured for the number of nuclei expressing ERα, ERβ and total numbers of nuclei. The area of each section was also measured. The proportions of ERα- and ERβ-positive nuclei were calculated, respectively. The number of ERα- and ERβ-positive nuclei was expressed per muscle fibre or per square millimetre.

Staining the basement membrane collagen IV made it possible to identify the capillaries and differentiate between ER-positive nuclei within myofibres and positive nuclei outside the myofibres in the interstitium or the capillaries. In paper II and III the proportion of ER-positive nuclei in muscle fibres and capillaries, respectively, were calculated by dividing the number of ERα- or ERβ-positive nuclei within the

capillaries with the total number of ERα- or ERβ-positive nuclei in an area of a transverse cryostat section. Sections were viewed at a magnification of 500 times and readings taken until an area containing a total of approximately 50 muscle fibres was included for the following variables: number of muscle fibres, total number of ERα- or ERβ-positive nuclei, and the number of ERα- or ERβ-positive nuclei within capillaries. Three calculations were conducted from these measurements as follows: 1) the proportion of ERα- or ERβ-positive nuclei located in capillaries, 2) the proportion of ERα- or ERβ-positive nuclei located in muscle fibres and 3) the proportion of ERα- or ERβ-positive nuclei in capillaries per muscle fibre. Less than 1% of the ER-positive nuclei were located outside muscle fibres or capillaries so they were not recorded separately and are included in the number of ERα or ERβ-positive nuclei, respectively, located to the capillaries.

Of all stained nuclei the proportion of ERα-positive nuclei that co-expressed ERβ was also calculated.

The methodological error for two different measurements of the number of ER-positive staining from one section including approximately 50 fibres was 4%.

STATISTICS

Values are expressed as mean ± standard deviation in study III and IV and ± standard error in study V. Unpaired Student's *t*-tests were used to test for any significant differences between groups in study III and IV. In study V, ANOVA was used to test for effects of stimulation and estrogen receptor antagonists. Single regression analysis was performed to study the relationships between variables in study IV. Significance was accepted at the statistical level of $P < 0.05$.

RESULTS AND DISCUSSION

This study is the first one to demonstrate that human skeletal muscle express ERα and ERβ. The expression of ERs was demonstrated both at the mRNA and protein level.

mRNA EXPRESSION

In paper I we found that both ERα and ERβ mRNA were expressed in human skeletal muscle from both men and women. The mRNA level of ERα was higher than ERβ. Whether there were any differences in ER expression between the sexes could not be determined because of lack of statistical power for that purpose. Both ERα and ERβ mRNAs were also expressed in both myoblasts and myotubes from rat.

PROTEIN EXPRESSION

In paper I-IV both ERα and ERβ proteins were for the first time found and studied in skeletal muscle (fig. 6). Both types of ERs were present in all subjects tested, women and men as well as children and postmenopausal women. The ERs were located to the nuclei in muscle tissue, approximately 65% of all nuclei expressed ERα and about 70% expressed ERβ. The localisation of ERs to the nuclei of the skeletal muscle fibres suggests that this tissue is a target for estrogen action.

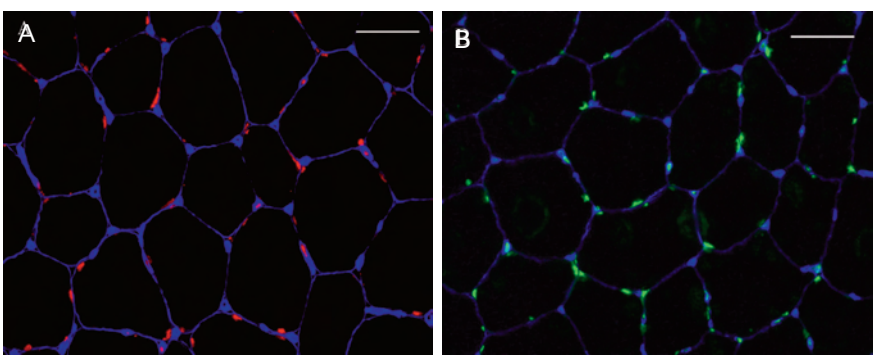

Figure 6. Immunohistochemical double-staining for A) ERα (red) and B) ERβ (green) together with collagen IV (blue) in human skeletal muscle from an adult woman.

Sex difference

For ERβ the immunohistochemistry results from paper I, III and IV were pooled together to look at sex differences. This includes 7 premenopausal women and 17 moderately active men. Women have significantly higher number of ERβ per square millimetre than men do (fig. 7). Also when looking at the number of stained muscle nuclei compared to all nuclei, there was a similar sex difference. In contrast, no significant difference was seen when relating the number of ERβ positive nuclei per muscle fibre, which could be explained by the larger cross-sectional area of the fibres from men.

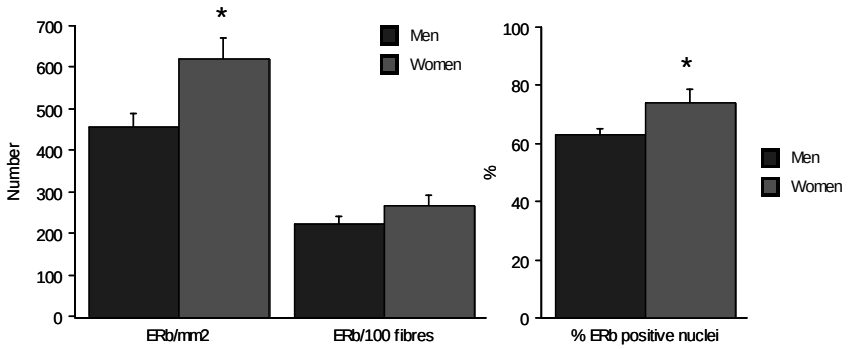

Figure 7. Number of ERβ-positive nuclei in relation to sex.

The statistical power was too low to study any sex differences in ERα.

The number of ERs in different fibre types was not studied, although all muscle fibres expressed ERs with no obvious difference between various fibres. In animal studies a fibre type difference between ERs has been suggested. In a binding study of rabbit muscle, Gustafsson *et al*. (1984) demonstrated that the total amount of ERs was higher in slow twitch oxidative muscle (soleus) than in the fast twitch oxidative-glycolytic muscle (gastrocnemius/plantaris muscle complex) (Gustafsson et al. 1984). In rats, similar results were found with a higher mRNA expression of ERα in slow twitch muscle than in fast twitch oxidative-glycolytic or glycolytic muscle (Lemoine et al. 2002).

ERs in subjects with low estrogen levels

An interesting question is why subjects with low estrogen level express ERs in muscle and relates to the mechanism of ER activation. There are several different possibilities. Firstly, substances such as androstenediol and estrone are beside estrogen also activators of ER (Maggiolini et al. 1999). These are breakdown products from dehydroepiandrosterone (DHEA), which is a hormone released from the adrenal glands (Kraemer et al. 2001). Physiologically it is important in its role in the peripheral synthesis of testosterone and estrogen. The level of DHEA determines the concentration of estrogen in peripheral tissue. Furthermore, DHEA can also function directly via ER as an agonist, although the activity at physiologically relevant concentration of DHEA is low (Chen et al. 2005). During exercise circulating levels of DHEA in blood are increased (Tremblay et al. 2004) to a level that might be able to activate the ERs (see fig. 14).

Secondly, some natural dietary components, like genistein, may be another candidate for ER activation in skeletal muscle and has shown a preference for binding ERβ (Kuiper et al. 1998). A third possibility is that aromatase may locally convert testosterone to oestrogen, which in turn activates the ERs. ER activation at low estrogen levels, such as in men, children and postmenopausal women, could also be due to ligand-independent pathways of activation. Recently, the activation of ERs in mice was studied by in vivo imaging (Ciana et al. 2003). In reproductive organs, it was shown that the peak transcriptional activity of ERs coincided with the highest level of circulating estrogen. This was in contrast to the findings in non-reproductive organs such as bone and brain, where the transcriptional activity of the ERs was inversely related to the circulating estrogen levels. Instead the ERs activity co-varied with circulating IGF-1 levels and IGF-1 was suggested for the activation of ERs in the non-reproductive organs. This was supported by administration of IGF-1 to the mice in the absence of estrogen, which also increased the transcriptional activity of ERs (Ciana et al. 2003).

ERs in skeletal muscle capillaries

Of all ERα positive nuclei, 25% were localized to the capillaries and 75% to muscle fibres. The same was true for ERβ (fig. 8).

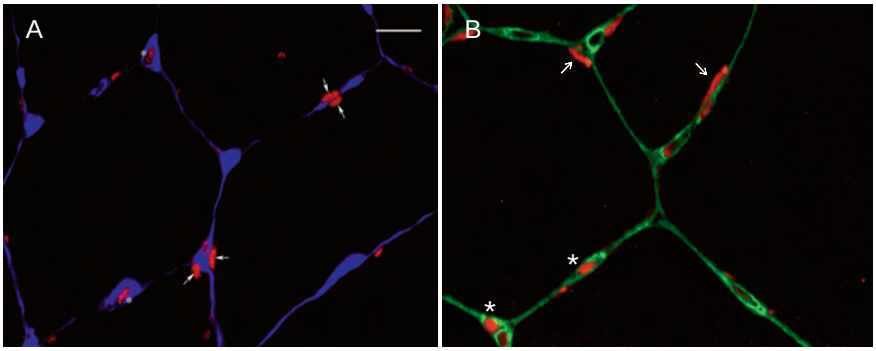

Figure 8. Immunohistochemical double-staining forA) ERα (red) and collagen IV (blue) and B) ERβ (red) and collagen IV (green), which stains the basement membranes of the muscle fibres and capillaries. Arrows indicate ERα-positive or ERβ-positive nuclei located to muscle fibres and ERα- or ERβ-positive nuclei located to capillaries are indicated with asterisks.

The ER-positive nuclei located to capillaries were interpreted as being those of endothelial cells, since triple-staining with ERα or ERβ together with a marker for endothelial cells and collagen IV indicates that ER is present in endothelial cells (fig. 9).

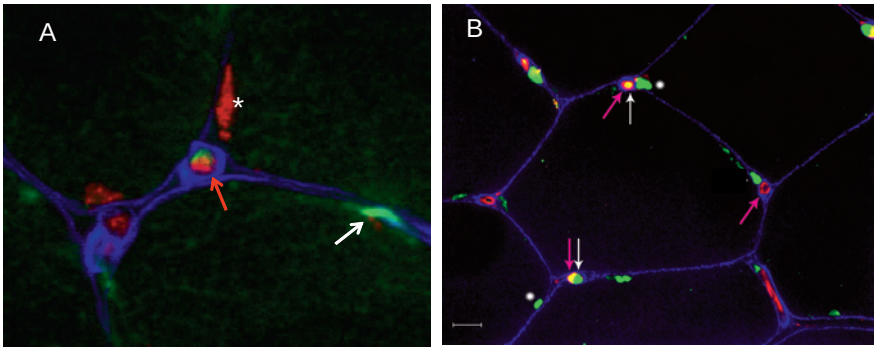

Figure 9. A) Immunohistochemical triple-staining for ERα (red), endothelial cells (green) and collagen IV (blue). The star indicates an ERα-positive nucleus located within a muscle fibre, while the white arrow points at an endothelial cell. ERα present in an endothelial cell is indicated by a red arrow. In B) the green colour marks ERβ. The star indicates a localisation within a muscle fibre, while the white arrows point at endothelial nuclei. The blue colour marks the collagen IV basement membrane of the muscle fibres and capillaries. Endothelial cells are demonstrated by pink colour where the pink arrows point at capillary endothelial cells. ERβ located in endothelial cells appear in yellow.

The relative distribution of ERs between muscle fibres and capillaries may be important in the physiological response to ER-mediated transcriptional activity and may thereby modulate the biological effects of estrogen. However, at this stage, we can only speculate upon the physiological role of ERs in skeletal muscle tissue. For instance, VEGF and NOS are both expressed in skeletal muscle (Frandsen et al. 1996; Gustafsson et al. 1999) and have been shown to be regulated by estrogen among other stimuli (Mueller et al. 2000; Weiner et al. 1994). The gene for VEGF has a functional estrogen response element in its promoter region (Hyder et al. 2000) and is known to enhance both myogenesis and angiogenesis in skeletal muscle (Arsic et al. 2004; Gustafsson and Kraus 2001). Nitric oxide can function as a signal transducer in muscle and as a vessel dilator (Steensberg et al. 2007), and the production of nitric oxide might be regulated by ERs. Such ER-mediated effects may favour muscle tissue repair and also muscle adaptation to physical training.

Co-localisation

ERα and ERβ was to a large extent co-expressed in the same nuclei (fig. 10). Of all stained nuclei 72% co-expressed ERα and ERβ. The number of nuclei that only expressed ERβ exceeded the number of nuclei expressing only ERα. Men had more nuclei expressing only ERα than did women ($P < 0.05$).

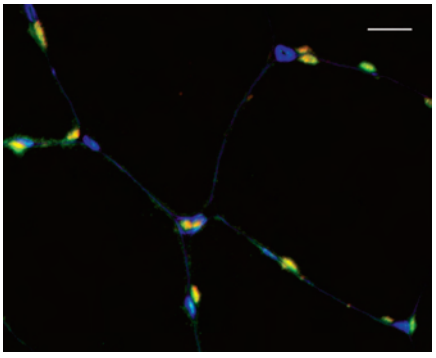

Figure 10. Immunohistochemical triple-staining for ERα (red), ERβ (green) and collagen IV (blue). Co-localizations of ERα and ERβ appears as yellow.

The co-localization of ERα and ERβ in nuclei has implications for the action of estrogen in muscle. If both receptors are present in the same cell, they can form heterodimers and interact in the regulation of transcriptional activity (Pettersson et al. 1997). ERβ has the capacity to repress the transcriptional activity of ERα. On the other hand, it can partially replace ERα if it is absent. In cells and tissues where ERα

and ERβ are co-expressed the ratio of ERα to ERβ determines what type of action estrogen will have. In the case of muscle, manipulations that decrease the levels of ERβ have been shown to improve glucose tolerance in mice (Barros et al. 2006).

Methodological considerations

Some of the ERs located to the capillaries might be nuclei of immune cells because such cells also express ERs as seen in paper II. However, in previous studies, immune cells were found to be rare and present only in the range of 0.02-0.05 cells per muscle fibre (Malm et al. 2000). We found approximately 0.7 ERα-positive and 0.5 ERβ-positive capillaries per muscle fibre which is more than 10 times the frequency of immune cells. Therefore, it is most likely that only a small fraction of the ER-positive nuclei located to capillaries could have been nuclei of immune cells. In our view, most of the ER-positive capillary nuclei do represent endothelial cell nuclei.

Some of the ER-positive nuclei in the muscle fibres may have been nuclei of satellite cells. These can not be differentiated from nuclei of the mature muscle fibre with the methods used in the present study, so this possibility was not investigated. However, the proportion of satellite cells in human skeletal muscle is known to be at most 5% of the total number of nuclei (Kadi et al. 2005), which is apparently much fewer than the number of ER-positive nuclei found in the present study. Therefore, our results of the ER-positive nuclei located to the muscle cells mainly represent those of the mature fibres.

It is important to be careful when interpreting morphometric data on the density of ER. The numbers of ER-positive nuclei per unit area and nuclei may be distorted due to possible section-thickness-dependent and volume-dependent redundancy in counting the nuclei twice as well as underestimating information about smaller nuclear fragments. At best immunohistochemistry provide only a semi-quantitative measure of the amount of protein, therefore group differences of ER at the protein level might be missed.

ER EXPRESSION IN TRAINED VERSUS NORMAL SUBJECTS

The mRNA levels for ERα and ERβ were higher in skeletal muscle samples from highly endurance-trained athletes than from moderately active control subjects (fig. 11).

VEGF mRNA, which can be a target gene for ER, was also higher in the highly trained group. Muscle contractions of myotubes from rat increased the mRNA level of ERβ. ERα mRNA on the other hand was not increased by muscle contractions in contrast to the higher amount of both receptors among the highly endurance trained men. In an endurance training study in rats Lemoine et al. (2002) found increased expression of ERα mRNA in gastrocnemius muscle after training while ERβ expression was not studied.

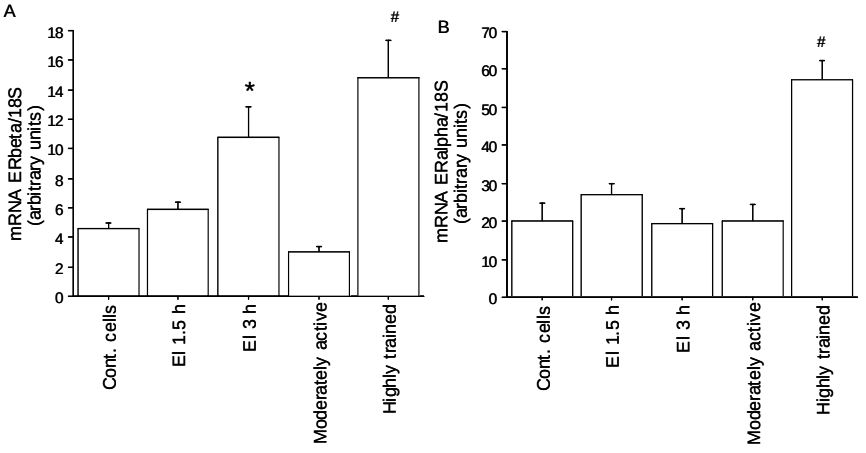

Figure 11. ERβ (A) and ERα (B) mRNA levels relative to 18S in myotubes from rat and in moderately and endurance trained men. Myotubes were electrically stimulated for 1.5 h or 3 h before harvesting 3h after end of stimulation. Bars represent the mean mRNA level of ERβ respectively ERα relative to 18S with standard error. * indicates a significant difference from control cells and # indicates a significant difference between the moderately active and endurance trained men. $P < 0.05$.

As for ER mRNA the angiogenic factor VEGF mRNA was higher in skeletal muscle from highly endurance-trained athletes than from moderately active men. Estrogen has been shown to regulated VEGF mRNA levels in vivo (Charnock-Jones et al. 1993) and most likely through ER-mediated processes. In rat uterus VEGF has been shown to be up regulated by very low estrogen levels. Functional EREs are present in the VEGF gene (Hyder et al. 2000; Mueller et al. 2000). ERs might therefore be involved in the increase of VEGF mRNA and in the regulation of human skeletal muscle adaptation to physical training. However, other mechanisms behind exercise induced VEGF expression has been suggested such as activation by the hypoxia

inducible factor (Ameln et al. 2005; Tang et al. 2004). The higher VEGF mRNA levels in the trained athletes are probably related to the large differences compared to the controls regarding the amount of weekly training dose, which is supported by the differences in skeletal muscle oxidative characterization.

Positive correlations between ERs and maximal oxygen uptake and the number of capillaries were observed as well as for muscle citrate synthase activity. This could indicate that ERs are involved in the regulation of mitochondrial biogenesis. Furthermore, ERβ has been shown to be located to the mitochondria in human heart (Yang et al. 2004) and EREs have been found in the regulatory part of the mitochondrial genome (Demonacos et al. 1996). Thus, ERs have been proposed to be involved in the regulation of the mitochondrial transcription (Chen et al. 2004). The transcriptional activity of ERα has also been reported to associate with the important regulator of mitochondrial biogenesis PGC-1 (Tcherepanova et al. 2000).

For the protein level, no differences in the proportion of ERβ-positive nuclei or in the number of ERβ-positive nuclei per unit area or per fibre were observed between the trained and the control group. ERα was not studied at the protein level in these subjects. Why there is an increase in ER mRNA levels without any effect on the protein level in this study is unknown. A possible explanation is a shorter half-life for the protein than the mRNA. In different cell systems the half-life of ER protein has been shown to be quite short, 4-5 h. Ligand binding can influence the stability of the receptor and accelerates degradation, reducing its half-life to 3-4 h (Eckert et al. 1984). Without ligand, ER elimination is compensated by synthesis which maintains receptor homeostasis. Ligand-binding enhances the degradation by ubiquitination of the receptor via the proteasomal pathway (Nawaz et al. 1999) and essentially targets newly synthesized receptor molecules (Laios et al. 2005). In a study by Alarid et al. (1999) estrogen stimulation decreased ER protein levels without any decline in mRNA levels (Alarid et al. 1999). The increase in ER mRNA level in the present study could be due to an increased expression of ER or an increase in the stability of the mRNA.

As mentioned earlier, group differences of ER at the protein level might also be missed because of difficulties in evaluating the immunohistochemistry staining because of the semi quantitative nature of such methods.

ACTIVATION OF ERs BY ESTROGEN

Estrogen induces an activation of the ERs in skeletal muscle myotubes (fig. 12). The estrogen induced activation of the ERE-sequence is in line with the study by Kahlert et al. (1997), which in myoblasts showed an increase in ERE activation. The effect of estrogen was dependent of the ERs since the ERE activity was totally blocked by the estrogen receptor antagonist ICI 182 780. MPP, which is an ERα specific antagonist, showed a partially blocked ERE activity. The activity seen when co-incubating MPP with estrogen can be caused by ERβ, which is not affected by MPP. This suggests that both ERα and ERβ are involved in the estrogen induced activation of the ERE sequence.

ERβ mRNA levels were also increased after stimulation with estrogen, whereas ERα mRNA levels were unaffected.

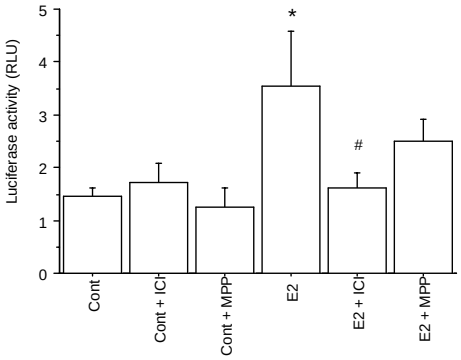

Figure 12. Activation of an estrogen responsive reporter plasmid in myotubes from rat. Myoblasts were transfected, differentiated into myotubes and then exposed to estrogen for 6 h in the absence or presence of the estrogen receptor antagonists ICI 182 780 or MPP before harvesting. Bars represent the mean luciferase activity with standard error. * indicates a significant difference from control cells. # indicates a difference between estrogen stimulation and estrogen stimulation together with antagonist. $P < 0.05$.

ACTIVATION OF ERs BY MUSCLE CONTRACTIONS

Muscle contractions induced by electrical stimulation also activated the ERE-sequence (fig. 13). The activation by muscle contractions though, seemed to be independent on

ERs since ER antagonists did not affect the activation. An explanation to the increased activation of the ERE-sequence by muscle contractions could instead be due to activation of ERRs, which have been shown to be increased in human skeletal muscle by acute exercise (Cartoni et al. 2005).

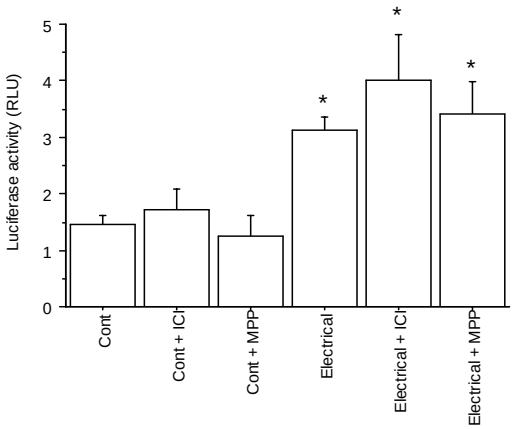

Figure 13. Activation of an estrogen responsive reporter plasmid by muscle contractions in myotubes from rat. Myoblasts were transfected, differentiated into myotubes and than electrically stimulated in the absence or presence of the estrogen receptor antagonists ICI 182 780 or MPP for 3 h before harvesting. Bars represent the mean luciferase activity with standard error. * indicates a significant difference from control cells. $P < 0.05$.

However, an activation of ERs, which was indicated in the present thesis by the finding of increased levels of ERα and ERβ mRNA, cannot be excluded. Physical exercise in vivo, in contrast to the in vitro isolated muscle cell contractions in paper V, involves a number of systemic factors that could affect ERs. For example, immediately after strenuous anaerobic exercise plasma levels of estradiol, testosterone and androstenedione are increased (Kuoppasalmi et al. 1976; Vincent et al. 2004), although with moderate endurance training or acute endurance exercise the circulation concentration of estradiol is not altered (Bullen et al. 1984). DHEA is produced from the adrenal glands especially during stress such as physical activity and serves as a precursor for testosterone and estrogen. The biosynthesis of active androgens and estrogens from DHEA is achieved by metabolism of DHEA first to testosterone by hydroxyl steroid dehydrogenases and than to estrogen by aromatase. All of the enzymes in this process are expressed and functional in skeletal muscle and are increased by

exercise (Aizawa et al. 2007). The aromatase gene can be regulated by different mechanisms, for example cAMP (Larionov et al. 2003) that is also increased during exercise (Goldfarb et al. 1989). Basal muscle estrogen levels are elevated by exercise in males (Aizawa et al. 2008) which shows a local and active metabolic pathway for synthesis of estrogen in skeletal muscle and provides further evidence for the role of steroid hormones in skeletal muscle function (fig. 14).

None of these possible effects on ER can be detected in the in vitro situation in paper V where the effect of muscle contraction alone is studied. Therefore, ER could still be activated by physical activity and involved in the process of adaptation, even though isolated muscle contractions do not seem to activate them. Furthermore, as described in the introduction, ERs can interact with other DNA-bound transcription factors at for example AP-1 sites to regulate the transcription of certain set of genes in an ERE-independent way. The MAPK-system is activated by muscle contractions and exercise (Widegren et al. 1998; Wretman et al. 2000) and IGF-1 levels have also been shown to increase by muscle contraction (Berg et al. 2007). MAPK and growth factors such as IGF-1 can activate ER to bind to ERE sequences and probably also to AP-1 sites.

ERRα is another possible activator of the ERE-sequence. ERRα is increased in human skeletal muscle after acute exercise and its activity is increased by the MAPKs, which could explain the increase in ERE activation by muscle contractions. The co-activator PGC-1, which interacts with ERRα and further increases its activity, is also increased by exercise (Norrbom et al. 2004; Schreiber et al. 2003).

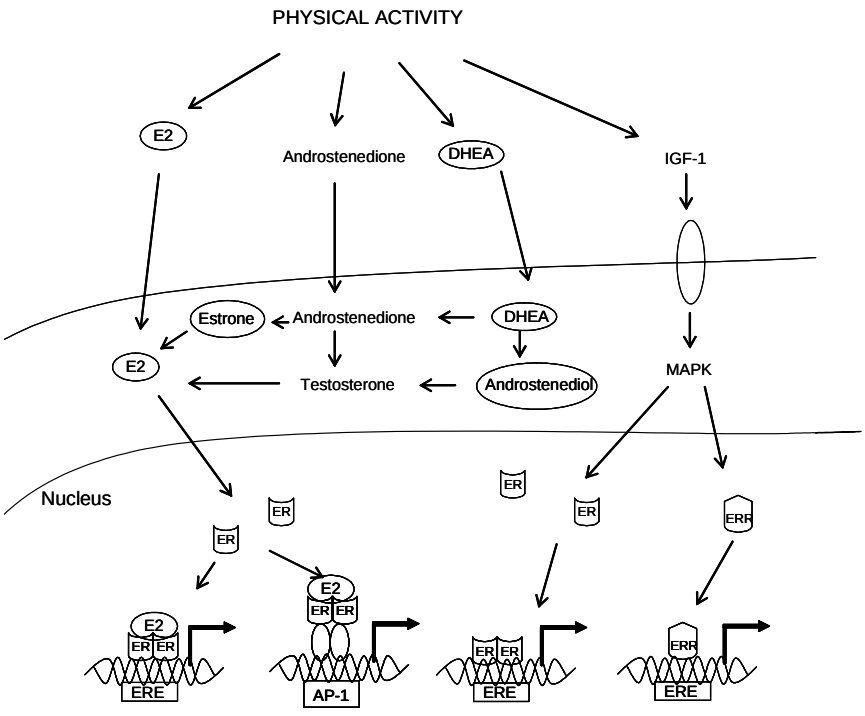

Figure 14. Possible mechanisms by which physical activity can influence ER signalling. Encircled substances can directly bind to ERs.

CONCLUSIONS

From the work of this thesis it was concluded that:

- Skeletal muscle expresses both ERα and ERβ mRNA and protein in adult men and women as well as in children of both sexes and postmenopausal women.

- The ERα and ERβ are localized not only to the nuclei of muscle fibres themselves but also to the nuclei of capillary endothelial cells. The two receptors are to a major extent co-expressed in the same nuclei.

- Endurance trained men have a higher steady state mRNA level of both ERα and ERβ than normally active men. This is concurrent with higher expression of the angiogenic factor VEGF. The expression of ERs is correlated with muscle oxidative capacity.

- Both estrogen and muscle contractions of skeletal muscle myotubes increases the mRNA of the ER target gene ERβ.

- Estrogen activates ERs in primary skeletal muscle cells.

- Muscle contractions have a similar functional effect as estrogen causing ERE-sequence activation in skeletal muscle myotubes. In contrast to estrogen the effects of muscle contractions are most likely independent of ERs.

These results suggest an involvement of ERs and ER target genes in the adaptation of skeletal muscle to physical training.

FUTURE PERSPECTIVE

The findings of this thesis arises new questions and ideas for further studies. There seems to be a sex difference in the level of ERβ protein in human skeletal muscle. If there is a sex difference for ERα protein as well is unknown and need further studies. In paper III we found that ERα and ERβ are present in skeletal muscle in children and postmenopausal women as well as in women and men. Although the number of subjects included was too low for any conclusions to be drawn, children seemed to have higher concentrations of ERα- and ERβ-positive nuclei per unit area than did adults, whereas the postmenopausal women showed fewer ERα- and ERβ-positive nuclei. This needs further investigation. In addition, animal studies indicate a fibre type specific expression of ERs. This has never been studied in humans and should be done for the basic characterisation of ERs in human skeletal muscle.

In this thesis we suggest an involvement of ERs and ER target genes in the adaptation process of physical activity. This is based on the findings that ERs are present and functional in skeletal muscle, contractions of myotubes activate ERE-sequences and endurance-trained men have a higher expression of ER mRNA than normally active men. The increased activation of the ERE-sequence by muscle contractions seemed to be independent of ERs and could instead be due to activation of ERRs. The activation of ERRs by muscle contraction needs further investigations. The effect of muscle contractions was studied in rat myotubes, which needs to be confirmed in human myotubes and than of course in a whole body system. If ERs and ER target genes are activated in exercising human skeletal muscle is still unknown and needs further investigations. For a better understanding of ER signalling and the different genes activated by ERs during physical exercise for example chromatin immunoprecipitation (ChIP) and the chip-on-ChIP techniques might be used.

SVENSK SAMMANFATTNING

Kvinnors risk för hjärt-kärlsjukdomar ökar dramatiskt när östrogennivåerna i kroppen sjunker efter menopaus och är den vanligaste orsaken till sjukdom och död hos äldre kvinnor. För att utföra sin effekt binder östrogen till specifika transkriptionsfaktorer, östrogenreceptorer (ER), ERα och ERβ. Den klassiska aktiveringen av ER är via östrogen. När östrogen bundit till receptorn aktiveras den och kan då binda in till specifika sekvenser i målgener och öka deras uttryck. Ytterst intressanta alternativa singaleringsvägar har beskrivits där ER aktiveras utan närvaro av östrogen. Kanske fysisk aktivitet kan leda till en aktivering av ER och därmed ge liknande effekter på hjärta och kärl som östrogen.

För att karaktärisera uttrycket av ER i skelettmuskel inklusive tillhörande kapillärnät togs vilobiopsier från kvinnor i olika åldersgrupper och från män (både normalaktiva och uthållighetstränade). Uttryck och lokalisering av ERα och ERβ studerades med hjälp av realtids-PCR (TaqMan) och immunohistokemi. Aktiviteten av ER undersöktes i elektriskt stimulerade celler från råtta.

ERα och ERβ uttrycks på både mRNA – och proteinnivå i human skelettmuskel hos både kvinnor och män oberoende av ålder och träningsstatus. Immunohistokemi visar att ER uttrycks både i muskelcellernas och i endotelcellernas kärnor. Östrogen skulle alltså kunna verka direkt på muskelcellerna och på kärlen i muskelvävnaden. mRNA-uttrycket av ERα och ERβ i skelettmuskulaturen är uppreglerat hos uthållighetstränade jämfört med normalaktiva. Detsamma gäller för kapillärtillväxtfaktorn VEGF, som även är en målgen till ER. Myotuber uttrycker ER och muskelkontraktioner ökar den transkriptionella aktiviteten av målsekvenser till ER, detta verkar dock ske oberoende av ER.

Sammanfattningsvis uttrycks ERα och ERβ i human skelettmuskel oavsett ålder och kön. Uthållighetsträning uppreglerar nivån av ERα och ERβ, vilket kan tyda på att ER är kopplad till adaptationsprocessen vid fysisk träning, dock visar el-stimulering på en ER-oberoende aktivering av målsekvenser. Våra fortsatta studier förväntas ge ökad kunskap om betydelsen av muskelarbete och östrogen för kardiovaskulär hälsa och bör kunna utnyttjas för en förbättrad rådgivning vad gäller fysisk träning i hälsofrämjande syfte.

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