Activation of estrogen response elements is mediated both via estrogen and muscle contractions in rat skeletal muscle myotubes

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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. Am J Physiol Cell Physiol 296: C215–C220, 2009. First published November 19, 2008; doi:10.1152/ajpcell.00148.2008.—The aim of the present study was to investigate the activation of estrogen response elements (EREs) by estrogen and muscle contractions in rat myotubes in culture and to assess whether the activation is dependent on the estrogen receptors (ERs). In addition, the effect of estrogen and contraction on the mRNA levels of ERα and ERβ was studied to determine the functional consequence of the transactivation. Myoblasts were isolated from rat skeletal muscle and transfected with a vector consisting of sequences of EREs coupled to the gene for luciferase. The transfected myoblasts were then differentiated into myotubes and subjected to either estrogen or electrical stimulation. Activation of the ERE sequence was determined by measurement of luciferase activity. The results show that both ERα and ERβ are expressed in myotubes from rats. Both estrogen stimulation and muscle contraction increased (P < 0.05) transactivation of the ERE sequence and enhanced ERβ mRNA, whereas ERα was unaffected by estrogen and attenuated (P < 0.05) by muscle contraction. Use of ER antagonists showed that, whereas the estrogen-induced transactivation is mediated via ERα, the effect of muscle contraction is ERβ independent. The muscle contraction-induced transactivation of ERβ and increase in ERβ mRNA were instead found to be MAP kinase (MAPK) dependent. This study demonstrates for the first time that muscle contractions have a similar functional effect as estrogen in skeletal muscle myotubes, causing ER activation and an enhancement of ERβ mRNA. However, in contrast to estrogen, the effect is independent of ERs and dependent on MAPK, suggesting activation via the estrogen related receptor (ERR).

electrostimulation; estrogen-related receptor; ligand-independent activity; luciferase; mRNA

ESTROGEN RECEPTORS (ERs) are ligand-activated transcription factors that belong to the nuclear hormone receptor super family. 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 many important effects on the cardiovascular, reproductive, and central nervous system as well as for bone maintenance (14). In skeletal muscle, it has been reported that estrogen for example is involved in regulating carbohydrate and lipid metabolism (22). During exercise, estrogen modifies the energetic substrate mobilization improving fat oxidation while sparing muscle glycogen (22). Previous reports indicate a role of estrogen in muscle growth and strength development (31) but available data are not consistent (13).

The two estrogen receptors ERα and ERβ are expressed at the mRNA level in human skeletal muscle in both females and males (25, 37). ERα and ERβ have been identified at the protein level by immunohistochemistry and was found to be localized to the nuclei of both muscle fibers and endothelial cells (35–37). ERs in myoblasts appear to be functional when stimulated with estrogen (20), and estrogen stimulation increases expression of insulin-like growth factor (IGF-1) in both myotubes and proliferating myoblasts (21). The mRNA levels of ERs in skeletal muscle has been shown to be increased with endurance training in rats (24), and in a cross-sectional study it was reported that the muscle contents of both ERα and ERβ mRNA were higher in well-trained endurance athletes than those in moderately active men. In accordance with these observations the mRNA expression of ERs have also been found to be correlated with muscle oxidative capacity (citrate synthase) (38). These findings may suggest that ERs, in being transcription factors, may be involved in the process of muscle adaptation to physical training.

The transcriptional activation of ERs by estrogen is initiated when the receptor binds to specific DNA sequences called response elements (EREs) in the promoter region of target genes. Interestingly, besides estrogen, other signaling pathways and extracellular signals can stimulate the transcriptional activity of ERs such as growth factors and mitogen-activated protein kinases (MAPKs) (ligand-independent activation). Recently, the activation of ERs in mice was studied by in vivo imaging (10). 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 nonreproductive organs such as bone and brain, where the transcriptional activity of the ERs was inversely related to the circulating estrogen levels (10). Instead the activity of ER covaried with circulating IGF-1 levels and IGF-1 was suggested for the activation of ERs in the nonreproductive 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 (10). The physiological implications of such interplay between alternative ways of enhancing the transcriptional activity of the ER are not known, although IGF-1 levels have been shown to be increased by muscle contractions (4) and there may be a prerequisite for an ER activation during physical exercise.

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EREs can, beside ERs, also be activated by estrogen-related receptors. The estrogen-related receptors α, β, and γ (ERRα, ERRβ, and ERRγ, respectively) are nuclear receptors with high similarities to the ERs, especially in the DNA-binding domain (12), but they do not bind estrogen. They are instead activated by MAPKs (2). The action of ERR is not affected by ER antagonists (33), so ERRs are able to activate ERE-regulated genes even in the presence of such antagonists. 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. Especially ERRs is ubiquitously expressed in adult tissues (12, 32) and is highly expressed in skeletal muscle (6, 32) and may therefore be of special interest in relation to the idea of ERE activation during physical exercise.

The aim of the present study was to investigate the activation of EREs in cultured rat skeletal muscle cells by estrogen and muscle contractions and to assess whether the activation is ER dependent. In addition, the effects of estrogen and contraction on the mRNA levels of ERα and ERβ were studied to determine the functional consequence of the transactivation. The hypothesis was that estrogen as well as muscle contractions induce activation of EREs in skeletal muscle and that ER target genes are involved in the adaptation of skeletal muscle to physical training.

**METHODS**

*Materials.* Dulbecco’s modified Eagle’s medium (DMEM), fetal calf serum (FCS), horse serum (HS), Dulbecco’s phosphate-buffered saline (DBS), matrigel, Penstrep (10,000 U/ml penicillin and 10,000 U/ml streptomycin), trypsin, culture dishes, Lipofectamine 2000, Opti-Mem, Trizol reagent, and Superscript reverse transcriptase were all from Invitrogen. Collagenase (type II) and DNase were from Sigma. Luciferase reporter assay was from BioThema. Galacto-Star assay, primers, and probes were from Applied Biosystems. Random hexamer primers were from Roche Diagnostics. ER inhibitors ICI-182780 and methyl-piperidon-pyrazole (MPP) were from Tocris Bioscience, and the MAPK inhibitor PD-98059 (50 μM) was added 30 min before the stimulation to study the ER-dependent activation. Furthermore, the MAPK inhibitor PD-98059 (50 μM) was added 30 min before electrical stimulation to investigate the influence of MAPKs on ERE activation. Directly after estrogen stimulation or at 3 h after the end of electrostimulation, the transfected cells were lysed and collected for analysis. All samples were stored at −80°C.

*Reporter gene assays.* For determination of ERE activation, luciferase activity was analyzed by a luciferase reporter assay and β-galactosidase by a Galacto-Star assay according to the manufacturers’ instructions on a luminometer Tecan infinite M200 (Tecan trading AG, Männedorf, Switzerland).

*RNA extraction and reverse transcription.* Total RNA was prepared from the nontransfected myotubes using TRIZol Reagent as previously described (9). The RNA was quantified spectrophotometrically by absorbance at 260 nm, and the integrity of total RNA was determined by 1% agarose gel electrophoresis. Two microgram of RNA was reverse transcribed by Superscript reverse transcriptase using random hexamer primers in a total volume of 20 μl.

*Real-time PCR analysis.* Analysis was performed with the ABI-PRISM 7700 Sequence Detector (Applied Biosystems, Foster City, CA). A TaqMan probe-based protocol was used with ERα (Rn00604673m_1), ERβ (Rn00526261_m1), and ERRα (Rn01479215) primers, and probes were achieved as predesigned assays. 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 (4310893E Applied Biosystems) to correct for potential variations in RNA loading.

*Statistics.* Values are expressed as means ± SE. ANOVA was used to test for effects of stimulation and antagonists. The effects of antagonists were tested against controls with added antagonists. Significance was accepted at the statistical level of P < 0.05.

**RESULTS**

To characterize the primary rat skeletal muscle cells (myotubes) used for the experiments ERα and ERβ mRNA levels were analyzed. Both ERα and ERβ mRNA were present in all cultures, both myoblasts and myotubes (data not shown).

To study the activation of EREs in skeletal muscle, primary myoblasts were transfected with ERE-LUC and then differentiated into myotubes. Estrogen stimulation for 6 h showed transactivation of the reporter construct (P < 0.05) (Fig. 1). To
investigate whether the activation was dependent on ER, the ER antagonist ICI-182780 was used. The activation of the ERE sequence was blocked by ICI-182780 ($P < 0.05$) (Fig. 1). The ERα-specific antagonist MPP was also used to differentiate between activation by ERα or ERβ. MPP tended ($P = 0.17$) to attenuate the activation of ERs by estrogen (Fig. 1). ERβ mRNA levels were increased after 6 h of estrogen stimulation ($P < 0.01$) (Fig. 2), whereas ERα mRNA was not affected by estrogen stimulation (Fig. 3).

Muscle contractions induced by electrostimulation for 3 h increased the transactivation of the reporter construct ($P < 0.05$), whereas electrostimulation for 1.5 h did not alter the transactivation (Fig. 4A). Addition of ICI-182780 or MPP did not block the electrostimulated activation of the ERE sequence. The MAPK inhibitor PD-98059 abolished ($P < 0.05$) the ERE activity (Fig. 4B).

**DISCUSSION**

This is the first study demonstrating that estrogen and muscle contraction induce an enhanced ERE activity in skeletal muscle myotubes and an elevated level of ERβ mRNA. The data also show that, whereas the estrogen-induced transactivation is mediated via ER, the muscle contraction induced by electrical stimulation is dependent on MAPK, suggesting that the transactivation occurs via ERR activation.

The estrogen-induced activation of the ERE sequence and increase in ERβ mRNA level is in line with the study by Kahlert et al. (1997), in which estrogen was found to increase ERE activation and ERβ protein in myoblasts. In the present study the effect of estrogen was dependent of ERs since the increase in ERE activity was abolished by the estrogen receptor antagonist ICI-182780. MPP, which is an ERα-specific antagonist, showed a partially blocked ERE activity (Fig. 1). The activity seen after cotreatment with MPP and 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.

An ERE sequence is located to the promoter region of the ERβ gene (26), therefore ERβ itself can act as a target gene for ERs. ERα promoter regions on the other hand do not contain...
physiological role of ERβ seems to be to modulate ERα-mediated gene transcription, supporting a “Ying Yang” relationship between ERα and ERβ (27).

Muscle contractions induced by electrical stimulation also activated the ERE sequence (Fig. 4) and increased ERβ mRNA level while ERα mRNA was decreased. The electrostimulation showed a dose-response effect as indicated by a higher ERβ mRNA level after 3 h of stimulation compared with after 1.5 h (Fig. 5A). In the present study, the activation by muscle contractions seemed to be independent on ERs since ER antagonists did not affect the activation (Fig. 4B). The increased activation of the ERE- sequence by muscle contractions could instead be due to activation of ERRs. ERRα is one of the major regulators of mitochondrial function in response to exercise and is also involved in a novel angiogenic pathway (1). ERRα activation is dependent on MAPK where phosphorylation of ERRα by MAPK can increase the transcriptional activity of ERRα by affecting its response to coactivators (40). The MAPK system has been shown to be activated in rat skeletal muscle in vivo by electrostimulation (39) as well as in

an ERE sequence and is therefore not a target gene for ERs. The ERα mRNA levels were not increased by estrogen in contrast to ERβ (Figs. 2 and 3). The increase in ERβ mRNA level could also be explained by an increase in mRNA stability. Estrogen can upregulate ER mRNA levels 400% after 24 h of treatment in endometrial cells by stabilizing the ER mRNA without affecting the rate of ER gene transcription (17, 18). However, in the breast cancer cell line MCF7 estrogen treatment downregulates ER mRNA and protein, and the effect appears to be due to reduced stability of ER mRNA (5, 29). An increase in the mRNA stability or activation by some other factor than ER is a probable cause to the increased ERβ mRNA level in myotubes since ICI-182780 did not affect the levels (Fig. 2). Why the ERβ mRNA is affected by MPP and not ICI might be due to a complicated relation between ERα and ERβ gene regulation where ERβ can have different effects when ERα is present than when it is by itself (15).
human skeletal muscle after one bout of exercise (34). MAPK inhibitors attenuated the activation of ERE by muscle contractions (Fig. 4), which demonstrates that the transactivation of ERE induced by muscle contraction is MAPK dependent and suggests that the effect is mediated by activation of ERRs. As ERRα mRNA has been shown to be increased in human skeletal muscle by acute exercise (7), the ERRα mRNA level was examined after electrostimulation in the current study. Muscle contractions were not found to enhance the ERRα mRNA levels (Fig. 7). However, there was an increased ERRα mRNA level when using MAPK inhibitors together with electrical stimulation. The explanation for this observation could be that when MAPK inhibitors were used the phosphorylation of ERRα was blocked, which resulted in decreased ERE activation and might function as a feedback mechanism to produce more ERRα mRNA. ERRα interacts physically with the transcriptional coactivator PGC-1 and enables activation of transcription (30). The expression of PGC-1 has been shown to be increased by exercise (3, 28). Thus in the present study muscle contractions might both activate the MAPK system and increase PGC-1 interaction, which could lead to activation of ERRα and increased binding to the ERE sequence.

Previous studies in bone cells show that a short period of mechanical strain has a similar effect on increasing ERE activity as more prolonged exposure to estrogen (41). In contrast to the myotubes in the present study, activation of ERE in bone cells is dependent on ERs, although ER antagonists could not completely block the strain-induced activity (41). It is likely that strain has its effects on increased ERE activity by phosphorylation of the ER using kinase-dependent signaling pathways (23). Strain-induced ER phosphorylation does not require the presence of estrogen but is dependent on extracellular-regulated kinase (ERK), a member of the MAPK family (19). Although both strain and estrogen activate ER in bone cells, they do not compete for the same domain of the receptor because the maximum effects of strain and estrogen are additive (8, 11). The question of whether estrogen and contractions have an additive effect on myotubes was not investigated in the present study.

In conclusion, this study demonstrates for the first time that muscle contractions have a similar functional effect as estrogen in skeletal muscle myotubes, causing ERE-sequence activation and an enhancement in ERα mRNA. In contrast to estrogen, the effects of muscle contractions are independent of ERs but dependent on MAPK, indicating involvement of ERR activation. These findings may suggest an involvement of ER target genes in the adaptation of skeletal muscle to physical training.

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Fig. 6. ERα mRNA levels relative to 18S in myotubes from rat. Myotubes were electrically stimulated for 1.5 h or 3 (A) h or electrically stimulated for 3 h (B) in the absence or presence of the estrogen receptor antagonists ICI or MPP or MAPK inhibitor PD-98059 before harvesting 3 h after end of stimulation. Bars represent the mean mRNA level of ERα relative to 18S with SE. *Significant difference from control cells. P < 0.05.
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