Menopause: The Journal of The North American Menopause Society
Vol. 24, No. 7, pp. 815-823
DOI: 10.1097/GME.0000000000000829

OPEN

Time since menopause and skeletal muscle estrogen receptors, PGC-1α, and AMPK

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Abstract

Objective: Short-term administration of estradiol (E_2) improves insulin-stimulated glucose disposal rate in early postmenopausal (EPM) women compared with a reduction in late postmenopausal (LPM) women. The underlying mechanisms by which E_2 action on glucose disposal rate reversed from beneficial early to harmful late in menopause is unknown, but might include adverse changes in estrogen receptors (ERs) or other biomarkers of cellular energy metabolism with age or duration of estrogen deficiency.

Methods: We retrospectively analyzed skeletal muscle samples from 27 postmenopausal women who were 6 years or less past menopause (EPM; n = 13) or at least 10 years past menopause (LPM; n = 14). Fasted skeletal muscle (vastus lateralis) samples were collected after 1 week administration of transdermal E_2 or placebo, in random cross-over design.

Results: Compared with EPM, LPM had reduced skeletal muscle ERα and ERβ nuclear protein. Short-term E_2 treatment did not change nuclear ERα or ERβ, but decreased cytosolic ERα, so the proportion of ERα in the nucleus compared with the cytosol tended to increase. There was a group-by-treatment interaction ($P < 0.05$) for nuclear proliferator-activated receptor γ co-activator 1-α and phosphorylated adenosine monophosphate-activated protein kinase, such that E_2 increased these proteins in EPM, but decreased these proteins in LPM.

Conclusions: These preliminary studies of skeletal muscle from early and late postmenopausal women treated with E_2 suggest there may be declines in skeletal muscle ER and changes in the E_2-mediated regulation of cellular energy homeostasis with increasing time since menopause.

Key Words: AMPK – Estradiol – Estrogen receptors – Menopause – PGC-1α – Skeletal muscle.

Women today live up to four decades without production of ovarian hormones, predisposing them to metabolic dysregulation and development of type 2 diabetes.[1] Estrogen-based hormone therapy (HT) not only relieves vasomotor symptoms (eg, hot flashes, sleep disturbances),[2] but reduces incidence of diabetes in postmenopausal women.[3] Our studies suggest estrogen-mediated improvement in insulin sensitivity may contribute to the reduced incidence of diabetes in HT-treated postmenopausal women.[4] However, the timing of treatment relative to menopause seems to be important. We recently demonstrated that estradiol (E_2) action on insulin-stimulated glucose disposal rate (GDR) was improved in early postmenopausal (EPM) women, but worsened in late postmenopausal (LPM) women.[5] The mechanism for this reversal in E_2 action with increasing time since menopause is not known, but may be through changes in estrogen receptor (ER) expression or other downstream estrogen-signaling pathways with aging or estrogen deficiency.

Skeletal muscle is the major site of whole body glucose disposal.[6] Preclinical studies demonstrate glucose tolerance and insulin-mediated glucose disposal are impaired after ovariectomy and restored with estrogen treatment.[7,8] Estrogens exert their classical genomic actions via two known ER subtypes, ERα and ERβ,[9,10] the balance of which varies across tissues and appears to be important for glucoregulation.[11-13] Globally knocking out ERα, but not ERβ, in rodents leads to impairments in glucose tolerance, insulin-mediated glucose disposal, and insulin signaling.[14,15] Moreover, knocking out ERα specifically from muscle leads to impairments in skeletal muscle mitochondrial function

Received August 8, 2016; revised and accepted December 6, 2016.

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Funding/support: The following awards from the National Institutes of Health supported this research: R01 DK088105, P50 HD073063, T32 DK007446, P30 DK048520, UL1 TR001082.

Financial disclosure/conflicts of interest: None reported.

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(eg, diminished oxidative metabolism, increased reactive oxygen species) and reduced insulin-mediated glucose uptake.[16] Preliminary evidence suggests that the relative expression of ERα to ERβ in various tissues might be shifted after ovariectomy in animals or menopause in humans.[13,17] However, it is not yet known whether ERα or ERβ (content or function) in human tissues changes with increasing time since menopause (ie, duration of estrogen deficiency). If so, this would be expected to impact the metabolic actions of E_2 that are mediated through its receptor.

In addition to the classical genomic effects of estrogens acting through nuclear ER, estrogens may further impact bioenergetic pathways associated with insulin action through rapid nongenomic mechanisms (ie, extranuclear ER). For example, E_2 has been shown to rapidly (within minutes) increase adenosine monophosphate-activated protein kinase (AMPK) in rat soleus.[18] Moreover ER-mediated transcription can occur through ligand-independent mechanisms (eg, phosphorylation of ER by Akt).[19] Thus, the relative abundance of extranuclear ER, irrespective of available ligand (eg. E_2), is important for tissue metabolic function.[20] However, to our knowledge, no studies have reported age or menopause-related changes in nuclear/extranuclear ER protein in human skeletal muscle.

As a follow-up to our initial studies, the aim of these secondary studies was to determine whether late, compared with early, postmenopausal women had changes in skeletal muscle ER (nuclear and cytosolic fractions) that might explain the adverse effect of E_2 on glucose disposal that we previously observed.[5] We further assessed the impact of E_2 on master regulators of cellular bioenergetic pathways (AMPK and peroxisome proliferator-activated receptor γ co-activator 1-α [PGC-1α]) that might be expected to contribute to changes in E_2 action on glucose disposal with time since menopause.

METHODS

Participants

The present study retrospectively analyzed muscle samples from a subset of women enrolled in a previous study[5]: 13 EPM women (≤6 years of menopause) and 14 LPM women (≥10 years past menopause). Detailed inclusion and exclusion criteria were previously reported.[5] In brief, women were healthy, sedentary to moderately active, nonobese (body mass index [BMI] $<30\,kg/m^2$), postmenopausal women (age 45-70 years) who had never used any formulation of estrogen-based HT. All participants provided informed consent before enrollment. The protocol was approved by the Colorado Multiple Institutional Review Board.

Study design

At the screening visit, body composition, oral glucose tolerance, and physical activity were assessed. All participants completed two treatment conditions (1 week of transdermal placebo [PL] or E_2) in random order with a washout period of 6 ± 2 weeks between study visits. Under both conditions, fasted skeletal muscle was collected before measurement of insulin-mediated GDR by hyperinsulinemic-euglycemic clamp.

Body composition assessment

Total fat mass (FM) and fat-free mass (FFM) were measured by a dual x-ray absorptiometry (DXA; Hologic Discovery W, software version 11.2) as previously described.[21]

Glucose tolerance and insulin sensitivity

At the screening visit, a 2-hour 75 g oral glucose tolerance test (OGTT) was administered in the morning after an overnight fast as previously described.[5] Glucose tolerance was assessed by area under the curve (AUC) over the 2-hour period using the trapezoidal method. On two separate occasions, women underwent a hyperinsulinemic-euglycemic clamp to assess whole insulin sensitivity as previously described.[5] Briefly, insulin-mediated GDR was determined from the steady-state glucose infusion rate needed to maintain euglycemia (90 mg/dL) during an insulin infusion rate of $40\,\mu U/m^2/min$. Glucose and insulin samples collected during the OGTT and clamp visits were stored at $-80^\circ C$ and analyzed in batch by the University of Colorado Anschutz Medical Campus (UC-AMC) Clinical Translational Research Center (CTRC) Laboratory. Plasma glucose concentration was determined enzymatically (Beckman Coulter, Inc.); insulin concentration was assessed using radioimmunoassay (EMD Millipore).

Three-day dietary lead-in period

All participants were required to maintain their current body weight within ±2 kg before enrollment and throughout testing. To make the macronutrient intake among the participants in the days before the testing day consistent, a standardized diet was given by the UC-AMC CTRC metabolic kitchen 3 days before each testing day as previously described.[5]

Estradiol treatment

The clamp visit was repeated on two occasions after 1 week of transdermal E_2 (0.15 mg) and 1 week of matching transdermal PL treatment in a randomized, blinded, cross-over design. Patches were changed out mid-week and the second set removed after the biopsy and clamp procedures.

Human muscle biopsy

On the morning before each clamp, skeletal muscle samples were percutaneously obtained from the vastus lateralis. After sanitizing and draping the skin in a sterile manner, 1% lidocaine (with no epinephrine) was injected under the skin. An approximately 0.75 cm incision was made in the skin and fascia over the belly of the vastus lateralis. Approximately 100 mg of muscle tissue was removed using a 5-mm Bergstrom side-cut biopsy needle with suction applied. Muscle tissues were immediately flash frozen with liquid N_2.

Cellular protein fractionation

The method for cellular protein fractionation was modified from previous studies.[9,22] Approximately 30 mg skeletal muscle was pulverized and homogenized using a tissue homogenizer (Bullet blender, Next Advance, Averill, NY) in cold Buffer A containing 20 mM 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid, N-(2-Hydroxyethyl)piperazine-N′-(2-ethanesulfonic acid), 50 mM sodium fluoride, 100 mM NaCl, 1 mM ethylenediamine tetraacetic acid (EDTA), 1 mM ethyleneglycol tetraacetic acid (EGTA), 1 mM dithiothreitol (DTT), 1 mM sodium orthovanadate, 2 mM 4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF), 20 μg/mL leupeptin, 20 μg/mL aprotinin, 20 μg/mL antipain, 10 mM iodoacetamide, 5 mM *p*-chloromercuri phenylsulfonate (pCMBS), and 6 μL/mL phosphatase inhibitor cocktail 2 and 3; and centrifuged at 500 *g* at 4°C for 5 minutes. Supernatant (cytosol/membrane fraction) was stored at −80°C. Crude nuclei (pellet) were resuspended and sonicated in cold buffer B (buffer A + 25% glycerol, 0.1% sodium dodecyl sulfate [SDS], and 400 mM NaCl), and incubated at room temperature for 15 minutes. After a centrifugation at 3,000 *g* at 4°C for 5 minutes, supernatant (nuclear fraction) was obtained and stored at −80°C for further analyses.

SDS-PAGE western blot

Protein concentrations in nuclear and cytosol/membrane fraction in skeletal muscle were determined using the Pierce BCA protein assay kit (Thermo Scientific, Rockford, IL). Samples containing the protein homogenates (30 μg of protein) and laemmli buffer were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred onto polyvinylidene difluoride (PVDF) membranes by electroblotting. After blocking in 5% non-fat milk, membranes were probed with primary antibodies (1:200-1,000 concentrations in 5% bovine serum albumin). ERα, ERβ, and PGC-1α antibodies were obtained from Cell Signaling (cs8644, Beverly, MA), R&D Systems (mab7106, Minneapolis, MN), and EMD Millipore (st1202, Temecula, CA), respectively. Oxidative phosphorylation (OxPhos) complexes antibody containing complex II (30 kDa), complex IV subunit I, and complex V alpha subunit was purchased from Abcam (ab110411, Cambridge, MA), and total AMPK-α, pAMPK Thr^{172} (activation site), and pAMPK $Ser^{485/491}$ (inhibition site) antibodies from Cell Signaling (#2532, #2531, and #4185, respectively; Beverly, MA). Individual protein bands were quantified using a densitometer (Bio-Rad), and normalized to β-actin antibody (loading control, #4967, Abcam, Cambridge, MA). We used ER-positive MCF7 (human breast adenocarcinoma cell line, ab3871, Abcam) and ER-negative MDA-MB-231(sc2232, Santa Cruz Biotechnology, Inc, Dallas, TX) as positive and negative controls of ER protein expression, respectively (Fig. 1A). Hela cells (sc2200, Santa Cruz Biotechnology, Inc, Dallas, TX), PGC-1α-positive control (kp9804, EMD Millipore, Billerica, MA), and human heart mitochondria Western blot control (ms801, Abcam) were used as positive controls for AMPK, PGC-1α, and mitochondrial oxidative phosphorylation proteins, respectively. Empty well (loading buffer only) was used as a negative control for those proteins (Fig. 1A). α-Tubulin and lamin were used to test the purity of each fraction (Fig. 1B).

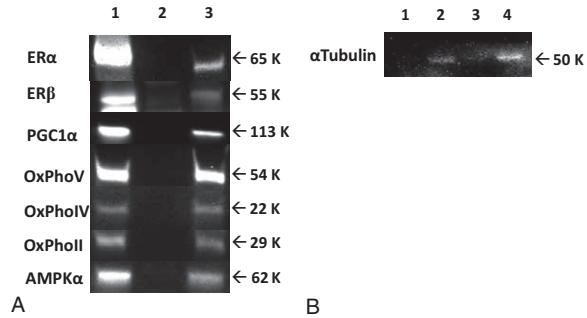

FIG. 1. Evidence of correct band detection on the Western blot and the purity of nuclear fraction. (**A**) Lane 1: positive control, lane 2: negative control, lane 3: 30 μg protein from skeletal muscle; MCF7 (positive) and MDA-MB-231 (negative) were used for ERα and ERβ; PGC-1α positive control, human heart mitochondria Western blot control, and Hela cells were used as positive controls for PGC-1α, mitochondrial oxidative phosphorylation (OxPho) proteins, and AMPKα, respectively. Empty well (loading buffer only) was used as negative controls for those proteins. (**B**) Lanes 1 and 3: nuclear fraction, lanes 2 and 4: cytosolic fraction. α-Tubulin was used as a cytosolic marker; no bands were detected in nuclear fraction. AMPK, adenosine monophosphate-activated protein kinase; ER, estrogen receptor; PGC-1α, proliferator-activated receptor γ co-activator 1-α.

Statistical analysis

The present study utilized a two-group (EPM vs LPM) repeated-measure (PL vs E_2) general linear model to test for main effects of menopausal group or E_2 treatment and group-by-treatment interactions. When a significant group-by-treatment interaction existed, least significant difference post-hoc test was used for pair-wise comparisons. Bivariate Pearson's correlations were performed to determine the association between protein expressions. Baseline group differences (LPM vs EPM) were assessed using *t* tests. All data were analyzed using IBM SPSS Statistics version 22.0. $P < 0.05$ was considered statistically significant and data are reported as mean ± SEM unless otherwise specified.

RESULTS

Participant characteristics

Compared with EPM women, LPM women were on average 7 years older and 9 more years past menopause (Table 1). The EPM and LPM groups included: non-Hispanic White (n = 11 and 11, respectively), non-Hispanic Black (n = 1 and 1), Hispanic (n = 1 and 1), and Asian (n = 0 and 1). LPM women were leaner (less total FM and FFM) compared with EPM women, but percent fat did not differ between groups. All women had normal fasting and postchallenge glucose and insulin concentrations. Circulating E_2 concentrations were not different between EPM and LPM women at baseline (16 ± 17 vs 10 ± 6 pg/mL) or in response to 1 week of 0.15 mg transdermal E_2 (180 ± 102 vs 149 ± 56 pg/mL). Although our

TABLE 1. *Participant characteristics*

	EPM (n = 13)	LPM (n = 14)
Age, y	55 ± 3	62 ± 3[a]
Time since menopause, y	3 ± 1	12 ± 2[a]
Weight, kg	69.4 ± 7.8	60.8 ± 6.1[a]
BMI, kg/m^2	26.0 ± 2.5	23.6 ± 3.0[a]
Total fat mass, kg	25.2 ± 5.4	20.9 ± 5.3[a]
Fat-free mass, kg	44.2 ± 4.4	39.8 ± 2.8[a]
%Fat, kg/kg	36.1 ± 4.7	34.1 ± 5.8
Fasting glucose, mg/dL	92.1 ± 5.7	89.1 ± 12.6
Fasting insulin, μU/mL	13.2 ± 4.0	11.4 ± 4.0
OGTT glucose AUC (×10^4)	1.3 ± 0.2	1.4 ± 0.4
OGTT insulin AUC (×10^3)	7.5 ± 3.9	6.4 ± 2.5
ΔGDR (mg/kg FFM/min)	0.2 ± 0.8	−0.5 ± 1.5[b]

Mean ± SD.
ΔGDR, estradiol-mediated change in glucose disposal rate during clamp; AUC, area under the curve; BMI, body mass index; EPM, early (≤6 years) postmenopausal; FFM, fat-free mass; LPM, late (≥10 years) postmenopausal; OGTT, 2-hour oral glucose tolerance test.
[a] $P < 0.05$ group difference.
[b] $P = 0.14$.

previous study[5] reported a significant group–by-treatment interaction ($P < 0.05$) for GDR, such that E_2 increased GDR in EPM women, but decreased GDR in LPM women, in this smaller subset, the interaction (interaction, $P = 0.14$) did not reach statistical significance (Table 1).

Estrogen receptors

Nuclear protein contents of ERα and ERβ were lower in LPM compared with EPM women (group main effect, $P < 0.05$ in both ER α and β; Fig. 2A and B). E_2 treatment decreased cytosolic ERα protein in both groups (treatment main effect, $P < 0.05$; Fig. 2C). Cytosolic ERβ protein was lower in LPM than EPM women (group main effect, $P < 0.01$; Fig. 2D). There was a nonsignificant trend for the ratio of ERα nuclear/cytosolic protein to increase after E_2 treatment in both groups (treatment main effect, $P = 0.072$), and this ratio tended to be lower in LPM compared with EPM women (group main effect, $P = 0.069$; Fig. 2E). There were no group differences or treatment effects in the ratio of ERβ nuclear/cytosolic protein (Fig. 2F) or in the ratio of ERα/ERβ protein within the nuclear and cytosolic fractions (data not shown).

Markers of cellular bioenergetics

A significant group–by-treatment interaction was found in PGC-1α nuclear protein, such that E_2 treatment increased nuclear PGC-1α by 22% in EPM women, but decreased it by 23% in LPM women (interaction, $P < 0.05$; Fig. 3A). There was a nonsignificant trend for E_2 to reduce cytosolic PGC-1α protein in both groups (treatment main effect, $P = 0.10$; Fig. 3C), so the ratio of nuclear/cytosolic PGC-1α protein was increased (treatment main effect, $P < 0.05$; Fig. 3E). OxPhos complex V protein expression was lower in LPM compared with EPM women (group main effect, $P < 0.01$; Fig. 3B), whereas no significant differences were found in OxPhos complex IV and II protein expression between groups (Fig. 3D and F). The nuclear/cytosolic ratio of PGC-1α protein expression was correlated with the nuclear/cytosolic ratio of ERα protein expression ($r = 0.718$, $P < 0.001$; Fig. 4A), but not with the nuclear/cytosolic ratio of ERβ protein expression ($r = 0.265$, $P = 0.10$; Fig. 4B). No associations were found between ERα/ERβ, PGC-1α, or OxPhos complex proteins and GDR (data not shown).

In EPM women, transdermal E_2 treatment increased phosphorylation of AMPK by 14% at the activation site Thr^{172} (pAMPK Thr^{172} protein), but decreased it 10% in LPM women (group–by-treatment interaction, $P < 0.05$; Fig. 5A). No significant differences were found between groups in phosphorylation of AMPK at the inhibition site Ser$^{485/491}$ (pAMPK Ser$^{485/491}$; Fig. 5B). pAMPK Thr^{172} protein content was significantly associated with GDR ($r = 0.216$, $P < 0.05$; Fig. 5C), but not with the nuclear/cytosolic ratio of ERα protein ($r = -0.005$, $P = 0.95$; Fig. 5D).

DISCUSSION

These studies are the first to demonstrate that skeletal muscle ERα and ERβ proteins are lower in LPM compared with EPM women. These data suggest that the beneficial effect of E_2 on GDR early in menopause compared with the harmful effect late in menopause that we previously observed[5] were not explained by changes in the balance of ERα to ERβ in skeletal muscle. Although declines in ER content with time since menopause could contribute to a reduced E_2 action late in menopause, it does not account for the reversal in E_2 action from early to late menopause. Instead, nuclear PGC-1α and AMPK activation (pAMPK Thr^{172}) were increased in EPM women, but decreased in LPM women. These new data suggest there may be adverse changes in E_2-mediated effects on master regulators of cellular energy homeostasis (nuclear PGC-1α and AMPK activation) with increasing time since menopause.

Muscle estrogen receptors and time since menopause

Estrogens exert many of their biologic actions through ERα and ERβ. ERα appears to be particularly important to metabolic health given that global knockout of this receptor subtype, but not ERβ, leads to a metabolic syndrome phenotype in rodents.[13] If aging or prolonged duration of estrogen deficiency leads to loss of ERα or possibly the balance of ERα to ERβ, this would have important implications to the metabolic action of E_2 in women with increasing time since menopause. However, to our knowledge, the effects of age or duration of estrogen deficiency on ER in human skeletal muscle have not been studied previously. Cross-sectional comparisons of adipose tissue ER mRNA expression in premenopausal versus postmenopausal women have been inconsistent, reporting either no group differences[23] or greater ERβ, but not ERα, in postmenopausal compared with premenopausal women.[17] These data are in contrast to the present study, in which we found reduced skeletal muscle nuclear ERα and ERβ protein in late compared with early postmenopausal women. One week of transdermal E_2 did not alter muscle nuclear ERα or ERβ protein. Our studies are in contrast to previous studies due to differences in the tissues

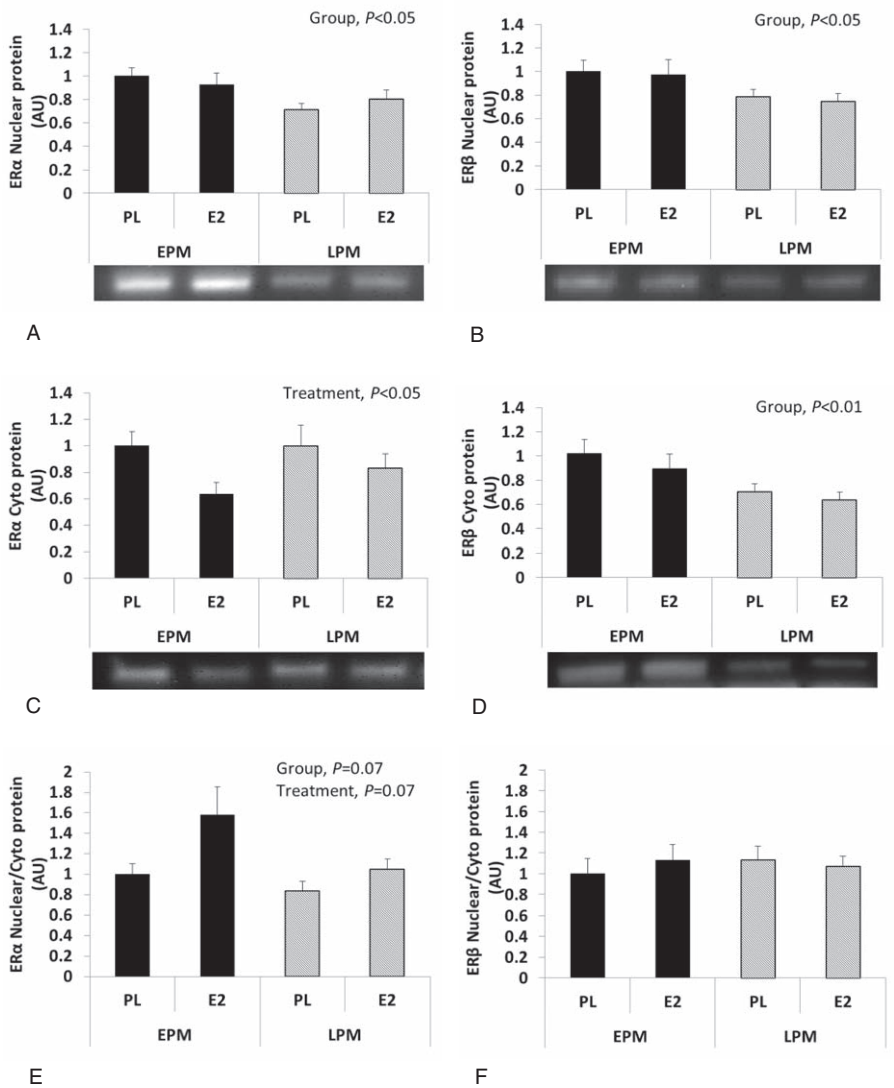

FIG. 2. Skeletal muscle estrogen receptor (ER) protein. (**A**) ERα protein in nuclear fraction; (**B**) ERβ protein in nuclear fraction; (**C**) ERα protein in cytosolic fraction; (**D**) ERβ protein in cytosolic fraction; (**E**) ratio of ERα nucleus/cytosol protein; and (**F**) ratio of ERβ nucleus/cytosol protein. Values are means ± SE (n = 12-13 per group). Group = group main effect, early postmenopausal women (EPM; ≤6 years) versus late postmenopausal women (LPM; ≥10 years); treatment = treatment main effect, estradiol (E_2) versus placebo (PL). AU, arbitrary unit.

studied (muscle vs adipose); method of measure (protein vs mRNA); and menopausal groups studied (early/late vs pre/post). Our data are the first to suggest there may be declines in skeletal muscle ER protein (both α and β subunits) with increasing time since menopause.

Estrogens, estrogen receptors, and insulin sensitivity

In rodent and nonhuman primates, glucose tolerance and insulin-mediated glucose disposal are impaired after ovariectomy and restored with estrogen,[7,8,24,25] demonstrating exogenous estrogens have favorable effects on systemic insulin action shortly after loss of ovarian function. Consistent with this, our well-controlled human physiology studies demonstrated improved systemic insulin-stimulated glucose disposal after both short-term conjugated estrogens and E_2 administration in early postmenopausal women.[4,26] Evidence suggests E_2-mediated changes in glucose uptake are through ERα signaling.[13,27] Expression of ERα is high in insulin-sensitive tissues and ERα (but not ERβ) knockout animals have impaired glucose tolerance, insulin-mediated glucose disposal, hepatic glucose production, and insulin signaling.[14,15] However, in the present study, the amount of ERα and ERβ in skeletal muscle was not related to E_2-mediated changes in whole body glucose disposal. Thus, loss of ER (or the balance of ERα/ERβ) with time since menopause did not appear to account for the reversal in E_2 action from early to late menopause that we previously observed.[5]

Localization of estrogen receptors and PGC-1α

While most ERα is found in the nucleus, a small proportion resides in the cytoplasm and translocates to the nucleus after binding to ligand (eg, E_2).[28] In the present study, the ratio of

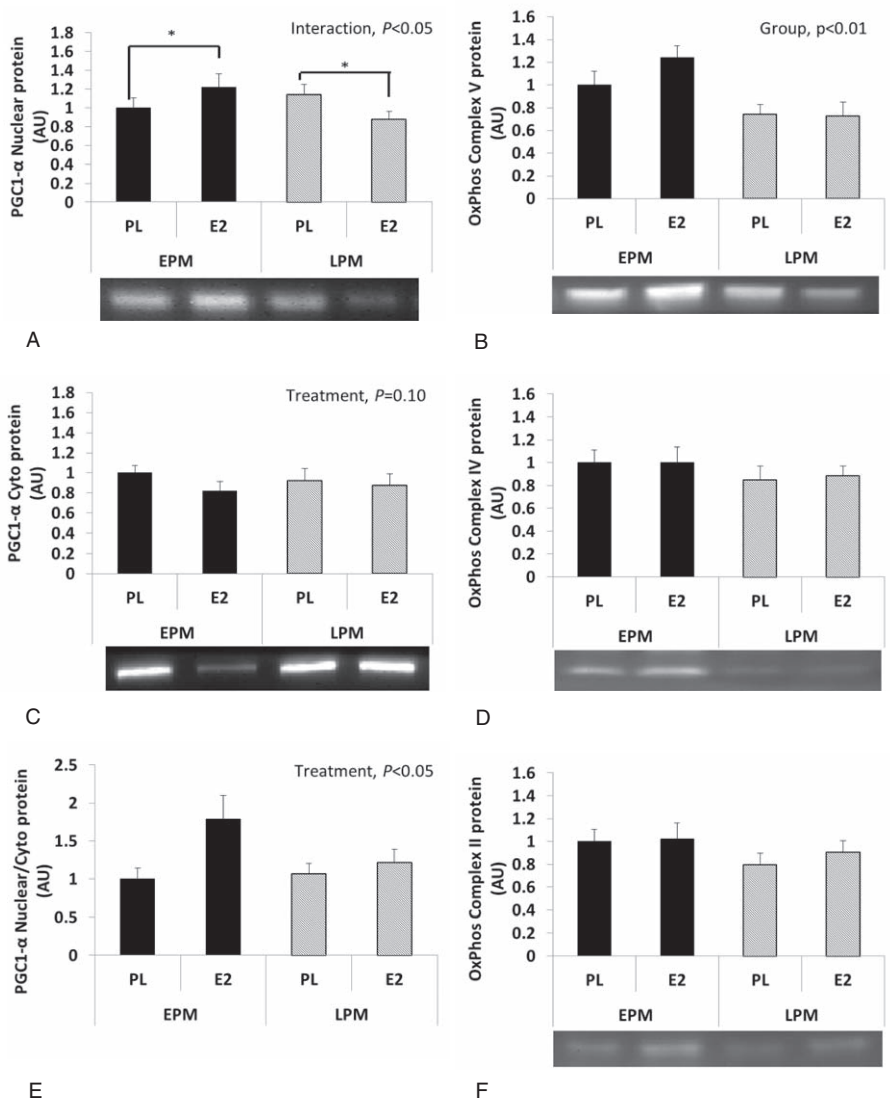

FIG. 3. Skeletal muscle peroxisome proliferator-activated receptor γ co-activator 1α (PGC-1α) and mitochondrial oxidative phosphorylation (OxPhos) complex proteins. (**A**) PGC-1α protein in nuclear fraction; (**B**) OxPhos complex V protein; (**C**) PGC-1α protein in cytosolic fraction; (**D**) OxPhos complex IV protein; (**E**) ratio of PGC-1α protein in nucleus/cytosol; and (**F**) OxPhos complex II protein. Values are means ± SE (n = 12-13 per group). Group = group main effect, early postmenopausal women (EPM; ≤6 years) versus late postmenopausal women (LPM; ≥10 years); treatment = treatment main effect, estradiol (E_2) versus placebo (PL); interaction = group × treatment interaction. (*) Denotes significant ($P < 0.05$) within-group change in response to E_2 treatment. AU, arbitrary unit.

ERα in the nucleus relative to the cytosol tended to increase in response to E_2 in both early and late postmenopausal women. This ratio of nuclear/cytosolic ERα was highly related to the ratio of nuclear/cytosolic PGC-1α, suggesting colocalization. PGC-1α is a well-known inducible transcription coactivator that acts as a key regulator of energy metabolism.[29-31] Thus, as a coactivator for ERα, PGC-1α may be an important convergence point for ERα signaling and cellular energy homeostasis.[32]

Muscle estrogen receptors and mitochondrial markers

A growing body of evidence suggests that reduced mitochondrial content and function in skeletal muscle contributes to insulin resistance and increased risk for diabetes.[33,34] Impairment in skeletal muscle mitochondrial content and oxidation after ovariectomy in rodents further suggest a role for estrogens.[35] Importantly, recent studies of muscle-specific ERα knockout (αMERKO) mice demonstrated a role for skeletal muscle ERα on mitochondrial function and metabolic homeostasis.[16] The impaired glucose homeostasis observed in the αMERKO mice was paralleled by abnormal mitochondrial morphology, impaired mitochondrial fission dynamics, and overexpression of reactive oxygen species in skeletal muscle. To our knowledge, no studies have evaluated the relation between skeletal muscle ERα and mitochondrial function in humans. In the present study, E_2-mediated localization of ERα and PGC-1α from the cytosol to the nucleus was strongly correlated. However, the different E_2-mediated

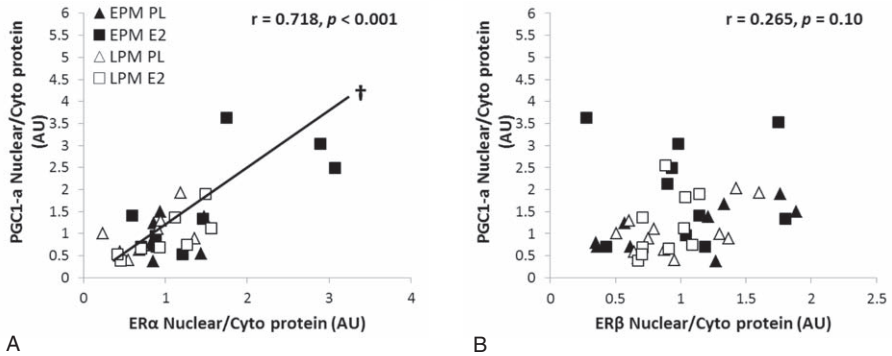

FIG. 4. Relation between nuclear/cytosol PGC-1α and estrogen receptor (ER). (**A**) Association between nuclear/cytosolic PGC-1α and nuclear/cytosolic ERα; and (**B**) association between nuclear/cytosolic PGC-1α and nuclear/cytosolic ERβ. (†) Denotes a significant ($P < 0.05$) correlation. AU, arbitrary unit; EPM, early postmenopausal women; E_2, estradiol; PGC-1α, proliferator-activated receptor γ co-activator 1-α; PL, placebo; LPM, late postmenopausal women.

changes in PGC-1α and AMPK across menopausal groups (increases early, decreases late) were not accompanied by corresponding E_2-mediated changes in ER across groups. There were also no E_2-mediated changes in mitochondrial oxidative phosphorylation complex proteins. Thus, the E_2-mediated effects on PGC-1α may not result in changes in mitochondrial content, though future studies directly assessing mitochondrial respiration are needed to confirm this.

Estrogen and muscle AMPK

Like PGC-1α, evidence implicates AMPK as a master regulator of many cellular pathways including insulin action and oxidative metabolism.[36,37] In rodents, ovariectomy is accompanied by reduced skeletal muscle AMPK activity, and restored by E_2 treatment.[38] Moreover, acute (10 minutes) E_2 stimulation of rat soleus muscle in vitro increased AMPK.[18] This latter observation is consistent with a rapid nongenomic action of E_2 on AMPK-mediated pathways. In

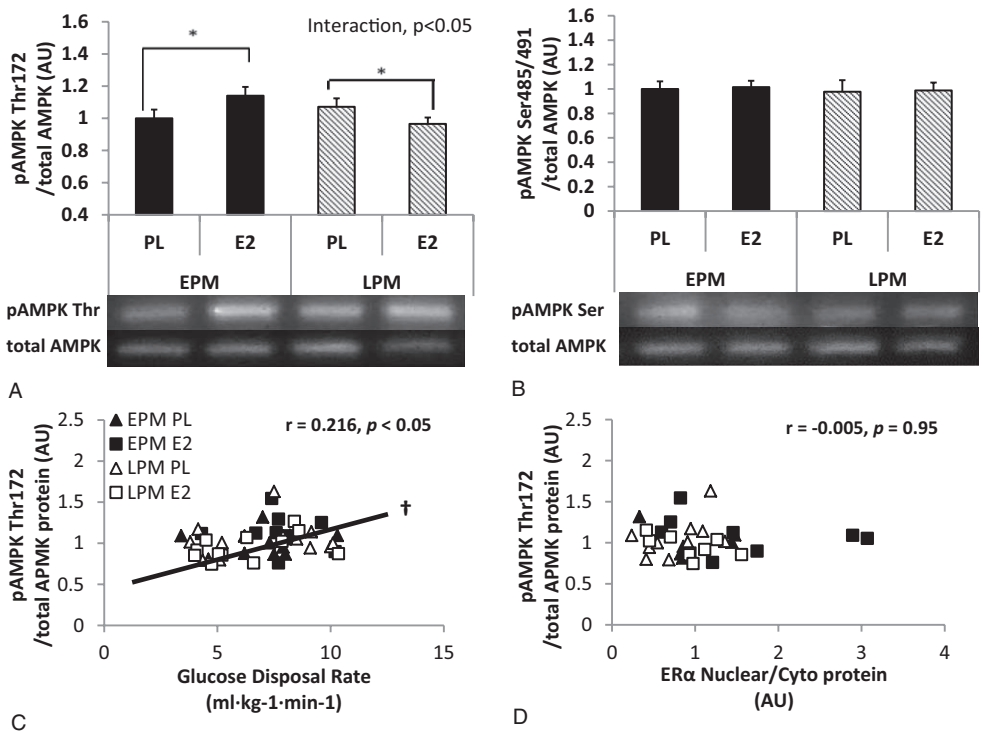

FIG. 5. Skeletal muscle adenosine monophosphate-activated protein kinase (AMPK) protein. (**A**) pAMPK Thr^{172} (activation site) and (**B**) pAMPK $Ser^{485/491}$ (inhibition site); normalized for total AMPK; (**C**) association between pAMPK Thr^{172} protein and insulin-stimulated glucose disposal rate (ie, insulin sensitivity); and (**D**) association between pAMPK Thr^{172} and nuclear/cytosolic ERα protein. Values are means ± SE (n = 12-13 per group). Group = group main effect, early postmenopausal women (EPM; ≤6 years) versus late postmenopausal women (LPM; ≥10 years); treatment = treatment main effect, estradiol (E_2) versus placebo (PL); interaction = group × treatment interaction. (*) Denotes significant ($P < 0.05$) within-group change in response to E_2 treatment. (†) Denotes a significant ($P < 0.05$) correlation. AU, arbitrary unit.

the present study, a marker of AMPK activation (phosphorylated AMPK Thr^{172}) was increased in early, but decreased in LPM women after short-term (1 week) E_2 treatment. This reversal in E_2 action on AMPK activation from early to late menopause was consistent with our previously observed reversal in E_2 action on insulin-mediated glucose disposal. Moreover, in the present study, skeletal muscle AMPK activation was correlated with whole body glucose disposal. On the contrary, AMPK activation was not correlated with ERα protein in skeletal muscle, consistent with the possibility that the effect of E_2 on AMPK was through a nongenomic (ER-independent) mechanism. These data are in contrast to the observations that phosphorylation of AMPK in murine skeletal muscle is increased in response to ERα-selective agonists[39] and decreased in ERα knockouts.[15] Additional studies in human tissue are needed to resolve these discrepant findings.

Potential limitations

There are limitations to the current studies that should be considered. First, these were secondary analyses of tissues collected as part of a larger trial which was powered to detect group differences in E_2-mediated change in GDR. This ancillary study was not statistically powered a priori for any of the outcomes reported herein and as such results should be interpreted with caution and reproduced when possible. A second important limitation of the current study was lack of additional biopsies collected during the clamp (ie, in the insulin-stimulated condition). This would have allowed for further assessment of changes in insulin-signaling pathways with time since menopause and in response to E_2 treatment. There were no group differences in basal expression of insulin signaling intermediates (IRS1, Akt, AS160) or glucose transport (GLUT4) protein under control or E_2-treated conditions (data not shown), but future studies are needed to determine whether E_2 impacts these signaling molecules during insulin stimulation. Third, we cannot tease out the respective effects of age and duration of estrogen deficiency on group differences in ER. In addition to being 9 years further past menopause, LPM women were on average 7 years older than EPM women. None of the women had used any type of HT in the past, so their years since menopause reflected duration of estrogen deficiency. Future studies of late postmenopausal women with and without past use of HT will be needed to determine the independent effect of estrogen deficiency from aging per se on ERs. Lastly, we found lamin, a marker of nuclear proteins, in the cytosolic fraction from many samples, suggesting there was nuclear contamination in our cytosolic fractions (possibly due to the freezing process). On the contrary, α-tubulin, a cytosolic marker, did not appear in the nuclear fraction of our samples, suggesting these fractions were pure (Fig. 1B). Thus, we have the most confidence in our nuclear fraction results.

CONCLUSIONS

These studies are the first to show that skeletal muscle ERα and ERβ protein are reduced in LPM women compared with EPM women; 1 week of transdermal E_2 administration did not alter ER protein. Short-term E_2 administration increased nuclear PGC-1α and AMPK activation in early postmenopausal women compared with a decrease in LPM women. Taken together, these novel preliminary data in skeletal muscle collected from EPM and LPM women treated with E_2 suggest there may be declines in skeletal muscle ER and a reversal in the E_2-mediated regulation of cellular energy homeostasis with increasing time since menopause. Additional well-controlled human physiology studies are needed to further elucidate the mechanism of E_2 action on cellular bioenergetics in EPM women.

Acknowledgments: The authors wish to thank the staffs of the University of Colorado Anschutz Medical Campus (UC-AMC) Clinical Translational Research Center (CTRC), and Energy Balance Core of the Colorado Nutrition and Obesity Research Unit (NORC) for their assistance in conducting this study. The authors would also like to thank the members of their research group for carrying out the day-to-day activities of the project and the study volunteers for their time and efforts.

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