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Menopause is associated with decreased whole body fat oxidation during exercise

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Abildgaard J, Pedersen AT, Green CJ, Harder-Lauridsen NM, Solomon TP, Thomsen C, Juul A, Pedersen M, Pedersen JT, Mortensen OH, Pilegaard H, Pedersen BK, Lindegaard B. Menopause is associated with decreased whole body fat oxidation during exercise. *Am J Physiol Endocrinol Metab* 304: E1227–E1236, 2013. First published April 2, 2013; doi:10.1152/ajpendo.00492.2012.—The purpose of this study was to examine if fat oxidation was affected by menopausal status and to investigate if this could be related to the oxidative capacity of skeletal muscle. Forty-one healthy women were enrolled in this cross-sectional study [premenopausal (n = 19), perimenopausal (n = 8), and postmenopausal (n = 14)]. Estimated insulin sensitivity was obtained from an oral glucose tolerance test. Body composition was measured by dual-energy X-ray absorptiometry and magnetic resonance imaging. Fat oxidation and energy expenditure were measured during an acute exercise bout of 45 min of ergometer biking at 50% of maximal oxygen consumption ($\dot{V}O_{2\,max}$). Muscle biopsies from the vastus lateralis of the quadriceps muscle were obtained before and immediately after the exercise bout. Postmenopausal women had 33% [confidence interval (CI) 95%: 12–55] lower whole body fat oxidation (P = 0.005) and 19% (CI 95%: 9–22) lower energy expenditure (P = 0.02) during exercise, as well as 4.28 kg lower lean body mass (LBM) than premenopausal women. Correction for LBM reduced differences in fat oxidation to 23% (P = 0.05), whereas differences in energy expenditure disappeared (P = 0.22). No differences between groups were found in mRNA [carnitine palmitoyltransferase I, β-hydroxyacyl-CoA dehydrogenase (β-HAD), peroxisome proliferator-activated receptor-α, citrate synthase (CS), pyruvate dehydrogenase kinase 4, peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α)], protein [phosphorylated AMP-activated protein kinase (AMPK), vascular endothelial growth factor, pyruvate dehydrogenase-1Eα, cytochrome oxidase I], or enzyme activities (β-HAD, CS) in resting skeletal muscle, except for an increased protein level of cytochrome *c* in the post- and perimenopausal women relative to premenopausal women. Postmenopausal women demonstrated a trend to a blunted exercise-induced increase in phosphorylation of AMPK compared with premenopausal women (P = 0.06). We conclude that reduced whole body fat oxidation after menopause is associated with reduced LBM.

menopause; skeletal muscle; fat oxidation; biopsy

MENOPAUSE SIGNALS THE END of the fertile phase of a woman's life and is defined after 12 mo of amenorrhea, as the last menstrual bleeding. At this time point, a marked increase in the incidence of the metabolic syndrome (19, 37), cardiovascular diseases (1, 27, 35), and type 2 diabetes (48) is observed. The increased occurrence of metabolic diseases is likely to be related to changes in body composition, such as an increased amount of body fat (22, 29, 30, 49), changed body fat distribution (22, 30, 51), and loss of lean body mass (LBM) (12, 49, 53).

Menopause leads to a significant decrease in circulating levels of estrogen as well as increases in gonadotropins. Estrogen seems to influence the substrate choice in humans toward fat oxidation. Hence, fat oxidation is lower in males than in females during endurance exercise (8, 17, 26, 50) and increases in males taking estrogen supplementation (11, 16). Furthermore, these gender differences in substrate utilization are only seen when comparing males with females at fertile ages (8, 17, 26, 50). Therefore, it has been hypothesized that reduced estrogen levels with menopause may influence substrate utilization.

To our knowledge, only a single previous study has investigated whole body fat oxidation in relation to menopause showing that menopause is associated with lower whole body fat oxidation at rest (30). However, it is still unknown whether fat oxidation during exercise is lower in postmenopausal than premenopausal women. Because decreased fat oxidation and mitochondrial dysfunction have been shown to be important factors preceding obesity (24, 44, 56) and type 2 diabetes (4, 33), it is possible that a change in substrate utilization with menopause, at rest and during exercise, could, in part, explain the changed metabolic profile leading to an increased incidence of metabolic diseases after menopause.

Furthermore, menopause has, in large cross-sectional cohort studies, been shown to be associated with changes in skeletal muscle mass (12, 49, 53). The age span in the studies has, however, been wide, and information about when in the menopausal transition the loss of LBM is happening is still sparse. Loss of skeletal muscle mass is associated with serious and expensive health effects, including functional impairments and physical disability (14, 20). Furthermore, muscle mass has proven to play a critical role in whole body metabolism. It is the far most abundant insulin-sensitive tissue (2), and it is

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responsible for up to one-third of the oxygen consumption at rest (55). We therefore hypothesized that changes in skeletal muscle mass could be related to the changes in metabolism seen following menopause.

Animal studies point to changes in molecular mechanisms in skeletal muscle as important contributors to the changes in the metabolic profile seen when removing the female sex hormone stimulus. Ovariectomized rodents have been shown to have decreased protein levels and activity of proteins involved in fat oxidation [carnitine palmitoyltransferase I (CPTI) and β-hydroxyacyl-CoA dehydrogenase (β-HAD)] and regulation of oxidative capacity [peroxisome proliferator-activated receptor-α (PPARα) and pyruvate dehydrogenase kinase 4 (PDK4)] (3, 6, 7). In addition, estrogen supplementation could restore both the oxidative capacity and the ability to oxidize fat by increasing the activity of CPTI and β-HAD, as well as protein levels of PPARα and PDK4 (3, 6, 7, 10). This indicates that female sex hormones may influence oxidative capacity, fatty acid availability, and fatty acid oxidation in skeletal muscle. In humans, it is still unknown if menopause affects whole body fat oxidation during exercise and whether the content of enzymes/proteins involved in fat oxidation in human skeletal muscle at rest and during exercise are changed.

We hypothesized that menopause is associated with a lower whole body fat oxidation during exercise, and changes in whole body fat oxidation are related to changes in mRNA, protein levels, or enzyme activity in skeletal muscle. Furthermore, we aimed at investigating if these potential changes in fat oxidation were related to changes in whole body metabolic functioning.

METHODS

Subjects. Using a cross-sectional study design, 41 women between 47 and 54 yr of age were enrolled in the study. Subjects were recruited by advertising in a local newspaper, and information about overall health and menopausal status was collected from each subject.

Exclusion criteria were: *1*) infections during the last 4 wk, *2*) chronic disease such as diabetes and other metabolic disorders, *3*) use of medication, including hormone therapy, *4*) smoking, *5*) hysterectomy and/or oophorectomy, *6*) premature ovarian failure, and *7*) body mass index >30 kg/m^2. Women with a menstrual period within the last 3 mo were enrolled in the follicular phase of the first coming menstrual period (on *days 3–7* of their menstrual cycle). The remaining women were enrolled on a random day. Participants were given both oral and written information about the experimental procedures before giving their written informed consent. The study was approved by the Ethical Committee of Copenhagen (H-3–2010-073), Denmark, and performed according to the Declaration of Helsinki.

The women were divided into premenopausal [menstrual bleeding within the last 12 mo and follicle-stimulating hormone (FSH) <20 IU/l] and postmenopausal (amenorrhea for >12 mo and FSH >20 IU/l). Eight women did not meet the subdivision criteria because of irregular menstrual bleeding within the last 12 mo despite of an FSH >20 IU/l. These were classified as perimenopausal, and, because of their higher FSH levels, they were considered hypoestrogenic compared with the premenopausal women. Because the perimenopausal women were characterized by high circulating levels of gonadotropins above the limit of 20 IU/l, equivalent to the postmenopausal women, the two groups were pooled in the statistical comparisons with the premenopausal women with regard to the molecular markers of oxidative capacity in the skeletal muscle. Hence, results are given for two groups of women with low respectively high circulating gonadotropins. Of note, the perimenopausal women did not differ from the postmenopausal women with regard to levels of specific mRNAs, proteins, or enzymes involved in fat oxidation.

Study design. The subjects were instructed not to perform any vigorous exercise 24 h before the experiments and reported to the laboratory after an overnight fast between 7:00 and 9:00 A.M. A general health examination was performed, and blood samples were drawn. Muscle biopsies were taken, and a 2-h oral glucose tolerance test (OGTT) was performed followed by a dual-energy X-ray absorptiometry (DXA) scan and a maximal oxygen consumption ($\dot{V}O_{2\,max}$) test. Participants completed a Minnesota Leisure Time Physical Activity Questionnaire (MLTPAQ) (40) to determine their physical activity levels over the last 4 mo, and the physical activity was divided into activity with a light, medium, or heavy activity metabolic index. The daily energy intake was estimated from a 3-day food diary before the visit. Magnetic resonance imaging (MRI) was performed to assess abdominal adiposity on one of the following days. Three premenopausal and four postmenopausal women were not scanned because of coinciding working hours.

All participants were asked to participate in one additional experimental day. Eighteen (11 premenopausal and 7 postmenopausal) of the 41 women accepted to participate. On this second experimental day, subjects underwent 45 min of indirect calorimetry measurements during cycle ergometry exercise performed at 50% of $\dot{V}O_{2\,max}$. A muscle biopsy was taken before and immediately after the exercise bout. If the duration between *experiment day 1* and *2* exceeded 2 mo, a new DXA scan was performed, and new blood samples were drawn to confirm the participants' hormonal status. Subjects completed the MLTPAQ and the 3-day food diary once again and were asked to report major changes in life style, including physical activity, menstrual bleeding, and food intake.

Body composition. Fat and fat-free masses were measured using DXA scanning (Lunar Prodigy Advance; GE Medical Systems, Lunar, Milwaukee, WI). Software (Prodigy enCORE 2004 version 8.8; GE Lunar, Madison, WI) was used to estimate fat and LBM. The amount of intra-abdominal fat was measured using an MRI (Siemens Magnetom Total imaging matrix magnetic resonance scanner, Erlangen, Germany) of the abdomen. Software (Multi-image Analysis GUI; Research Imaging Institute) was used to calculate the amount of visceral fat. Any adipose tissue located from diaphragm to pelvic floor except subcutaneous tissue was characterized as intra-abdominal fat. A single reader, who was blinded to the menopausal status of the subjects, performed all image analyses.

$\dot{V}O_{2\,max}$ test. $\dot{V}O_{2\,max}$ was determined using a standard progressive exercise test on a bicycle ergometer (Monark 839E; Monark, Varberg, Sweden). The subjects started with a 3-min warm up at 30 watts followed by a 20-watt increase in workload every other minute until subjects were unable to maintain a cadence on 60 rpm, heart rate had reached a plateau, and respiratory exchange ratios were higher than 1.1.

$\dot{V}O_2$ was continuously measured by indirect calorimetry (Quark b^2; Cosmed, Rome, Italy).

Submaximal exercise bout. Subjects performed a 45-min exercise bout on a bicycle ergometer (Monark 839E; Monark). In- and expired volumes of oxygen and carbon dioxide were measured using an indirect calorimetry system (Quark b^2; Cosmed). Rates of fat oxidation and energy expenditure were calculated from the following formulas (21):

$$\text{fat oxidation (g/min)} = 1.695\dot{V}O_2 - 1.701\dot{V}CO_2$$

$$\text{energy expenditure (kcal/min)} = 0.550\dot{V}O_2 + 4.471\dot{V}CO_2$$

Hormone analyses. FSH and estradiol were measured by sandwich electrochemiluminescens-immunoassay (Modular E-module; Roche) with detection limits of 0.2 IU/l and 0.04 nmol/l, respectively. Inhibin A and B were determined by specific two-site enzyme immunoanaly-

ses (Beckman Coulter), with detection limits of 12 and 3 pg/ml, respectively (43).

OGTT and blood lipids. Blood samples were drawn at time points −10, −5, 0, 30, 60, 90, and 120 min after drinking 75 g of anhydrous glucose dissolved in 300 ml of water. An estimate of insulin sensitivity was calculated using the Matsuda index (32), and area under curve (AUC) was calculated from serum insulin and plasma glucose. Blood lipids were measured from plasma samples obtained from the fasting subjects and analyzed using standard laboratory procedures.

Biopsies. Muscle biopsies from the vastus lateralis were obtained with a modified Bergström needle (including suction) under local anesthesia with 2% lidocaine. Muscle tissue was immediately frozen in liquid nitrogen and stored at −80°C.

Two postmenopausal and three perimenopausal women did not have muscle biopsies taken on the 1st day of experiments.

Skeletal muscle mRNA. Total RNA was extracted from 20–30 mg of wet weight muscle tissue, from biopsies taken at rest, with triZol (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. RNA was resuspended in 30 μl of elution buffer. The concentration of the isolated RNA and the ratio of absorbance at 260 to 280 nm were measured using a spectrophotometer (BMG Labtechnologies, Offenburg, Germany). RNA was reverse transcribed using random hexamers employing a high-capacity reverse transcription kit (Applied Biosystems, Foster City, CA) according to the manufacturer's protocol. The mRNA levels of analyzed genes and the endogenous control 18S were determined by real-time PCR using a ViiA 7 sequence detector (Applied Biosystems). Primers and MGB probes [CPTI, β-HAD, PPARα, citrate synthase (CS), 18S] or TAMRA probes (PDK4, PGC-1α) were designed using Primer Express software (Applied Biosystems) or obtained using the Universal Probe Library (Roche Applied Science). Primers and probes were premixed with Master Mix (Applied Biosystems) and distributed into 384-well MicroAmp optical plates (Applied Biosystems). A twofold dilution series was made from a pooled sample. This was run on each plate together with the samples and used to construct a standard curve from which the mRNA content of the target genes was calculated in triplicates, using the standard curve method.

Muscle lysate. Twenty micrograms of the vastus lateralis muscle biopsies were homogenized in an ice-cold lysis buffer [50 mM Tris, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 50 mM NaF, 5 mM NaP, 2 mM NaV, 1 mM dithiothreitol (DTT), 0.2% Ipegal-CA-630, protease inhibitor (Roche Applied Science), and 1% phosphatase inhibitor cocktail, pH 7.4] for 2 min using a tissue lyser with 30 oscillations/s (TissueLyser II; QIAGEN). Lysates were generated by centrifugation (16,000 *g*) for 15 min at 4°C. Protein concentration in lysates was measured by the Bradford reagent (Bio-Rad) (5).

Immunoblotting. Twenty micrograms of whole tissue lysates were subjected to SDS-PAGE using Bio-Rad 4–15% precast gels and an I-blot wet transfer machine according to the manufacturer's instructions. Lysates from exercise biopsies were run on Invitrogen 4–12% precast gels and the I-blot dry transfer machine according to the manufacturer's instructions. Samples from all groups were loaded in even numbers on the same gel. Samples from the exercise trials were run with both before and after exercise samples for each subject on the same gel, and both pre- and postmenopausal women were represented on each gel.

Polyvinylidene difluoride membranes were probed with primary antibodies raised against the protein of interest as follows: phosphorylated (p) AMP-activated protein kinase (AMPK) (no. 2535; Cell Signaling Technology, Danvers, MA), vascular endothelial growth factor (VEGF, A-20; Santa Cruz, Santa Cruz, CA), cytochrome *c* (CytC) (cytC, no. 556433; BD Pharmigen, San Diego, CA), PDH-1Eα (38), and cytochrome oxidase I (COX-I, no. 459600; Invitrogen). Detection of primary antibodies was performed using appropriate peroxidase-conjugated IgG, and protein signals were visualized using FEMTO enhanced chemiluminescence and a Biorad Chemidoc XRS imager. Total protein content was quantified using reactive brown 10 (Sigma-Aldrich, St. Louis, MO). Quantification of immunoblots was done using ImageJ (NIH, Bethesda, MD, http://rsb.info.nih.gov/ij).

Activity assays. Thirty milligrams of vastus lateralis skeletal muscle biopsy (wet weight) were homogenized in an ice-cold buffer containing 25 mM glycyl-glycin, 150 mM KCl, 5 mM $MgSO_4$, 5 mM EDTA, pH 7.5 with 1 mM DTT, 0.02% BSA, 0.1% Triton X-100, and complete protease inhibitor cocktail (Roche) using a tissue lyser at 30 Hz for two times 1 min (Quiagen). To ensure complete cell disruption, homogenates were then quick-frozen in liquid N_2 and thawed on ice, followed by centrifugation at 22,000 *g* for 2 min at 4°C. The supernatant was frozen at −80°C.

The maximal activities of CS (EC 4.1.3.7) and β-HAD (EC 1.1.1.35) were determined spectrophotometrically according to Passonneau and Lowry (37a). Briefly, CS activity (CS, EC 4.1.3.7) was determined spectrophotometrically at 409.5 nm at 30°C by measuring the reduction rate of β-dystrobrevin (DTNB) in a reaction mixture with 100 mM glycyl-glycin, 0.5 mM EGTA and 2 mM $MgCl_2$, 100 μM DTNB, and 50 μM acetyl-CoA, pH 8.2. The reaction was initiated by adding oxaloacetate to a final concentration of 50 μM. Likewise, β-HAD was determined spectrophotometrically at 340 nM at 37°C by measuring the oxidation of NADH to NAD^+ in a reaction mixture containing 150 mM imidazole, 1 mM EDTA, 0.15 mM NADH, and 0.05% BSA, pH 6.1. The reaction was initiated by adding acetoacetyl-CoA to a final concentration of 0.24 mM.

Because an earlier study (36) showed that 3 h of ergometer biking did not lead to increases in skeletal muscle maximum activity of β-HAD measured by these assays, we did not run the assays on muscle biopsies taken after exercise.

Statistics. Data are presented as means ± SE, unless otherwise stated.

All parameters were tested for normal distribution and log-transformed if not normally distributed (age, estradiol, FSH, inhibin A, inhibin B, high-density lipoprotein, CPTI, CS, PGC-1α, PDK4, CytC, PDH). Comparison of data with repeated sampling was performed using two-way ANOVA. On the second day of experiments, pre- and postmenopausal groups were compared using Student's *t*-test. Pearson correlation analyses were used to test for linear relationships between normally distributed variables. Statistical analyses were performed using SAS 9.2 (SAS Institute, Cary, NC). A P value <0.05 was considered statistically significant.

RESULTS

Study participant characteristics. Study participant characteristics are shown in Table 1.

The pre- and postmenopausal women differed in age by 2.37 yr [confidence interval (CI) 95%: 0.78–3.96] ($P = 0.003$), and postmenopausal women had on average been postmenopausal for 2.50 yr (CI 95%: 1.16–3.84). The postmenopausal women had a 4.28 kg (CI 95%: 0.72–7.84) lower LBM ($P = 0.02$) and a 91% (CI 95%: 28–172) higher visceral fat mass ($P = 0.003$) than the premenopausal women. After adjusting for age, the differences in LBM ($P = 0.03$) and visceral fat ($P = 0.003$) between the groups remained. There were no differences in total body weight.

Postmenopausal women showed significantly higher AUC for plasma insulin ($P = 0.01$) and C-peptide ($P = 0.008$) as well as significantly lower insulin sensitivity measured by the Matsuda Index ($P = 0.02$). Insulin sensitivity was closely correlated with LBM ($r = 0.61$, $P = 0.0001$). The women showed no significant differences in daily physical activity measured by MLTPAQ or in $\dot{V}O_{2\,max}$, since premenopausal women had a $\dot{V}O_{2\,max}$ of 34 ± 5 ml·min^{-1}·kg^{-1} and postmenopausal had a $\dot{V}O_{2\,max}$ of 31 ± 5 ml·min^{-1}·kg^{-1} (Table 1).

Table 1. *Characterization of subjects*

				Day 2	
	Pre	Peri	Post	Pre	Post
n	19	8	14	11	7
Age, yr	49.6 ± 1.8	51.1 ± 1.5	52.0 ± 2.0*	50.1 ± 2.1	52.0 ± 2.0
Body composition					
Height, cm	167 ± 6	168 ± 7	166 ± 5	168 ± 5	165 ± 5
Weight, kg	64.6 ± 5.3	63.0 ± 4.1	62.0 ± 7.6	63.1 ± 6.2	61.8 ± 3.5
LBM, kg	43.5 ± 4.5	41.4 ± 4.9	39.2 ± 3.1*	43.8 ± 4.6	38.2 ± 3.5†
Fat mass, kg	18.5 ± 4.8	19.0 ± 5.7	20.4 ± 3.6	16.8 ± 3.9	21.2 ± 3.4†
Trunk fat mass, kg	7.1 ± 3.1	6.7 ± 3.2	8.3 ± 2.3	5.8 ± 2.1	8.6 ± 2.4†
Limb fat mass, kg	10.7 ± 2.2	11.5 ± 3.3	11.4 ± 1.9	10.3 ± 2.3	11.8 ± 1.8
Visceral fat mass, ml	721 ± 397	626 ± 288	1,379 ± 632*	693 ± 395	1,289 ± 560†
Female sex hormones					
Estradiol, nmol/l	0.50 ± 0.47	0.18 ± 0.11*	0.05 ± 0.02*	0.51 ± 0.12	0.05 ± 0.01†
FSH, IU/l	10 ± 6	45 ± 13*	82 ± 40*	11 ± 2	93 ± 20†
Inhibin A, pg/ml	22 ± 11	14 ± 3	11 ± 5*	23 ± 3	12 ± 2†
Inhibin B, pg/ml	62 ± 36	9 ± 16*	3 ± 2*	57 ± 12	4 ± 1†
Metabolic parameters					
Total cholesterol, mmol/l	4.71 ± 0.16	5.33 ± 0.25	5.29 ± 0.18	4.47 ± 0.16	5.51 ± 0.28†
LDL cholesterol, mmol/l	2.61 ± 0.15	3.09 ± 0.20	3.13 ± 0.16	1.97 ± 0.10	2.03 ± 0.14
HDL cholesterol, mmol/l	1.84 ± 0.08	1.97 ± 0.11	1.83 ± 0.11	2.32 ± 0.12	3.20 ± 0.21†
Triglycerides, mmol/l	0.78 ± 0.06	0.74 ± 0.06	0.82 ± 0.09	0.76 ± 0.04	0.74 ± 0.13
Systolic BP, mmHg	125 ± 2	126 ± 3	129 ± 3	123 ± 2	131 ± 3†
Diastolic BP, mmHg	77 ± 2	78 ± 2	79 ± 2	77 ± 3	81 ± 4
Heart rate, beats/min	57 ± 2	60 ± 2	64 ± 3	58 ± 2	72 ± 4†
Maximal oxygen uptake					
$\dot{V}O_{2max}$, ml/min	2,221 ± 353	2,091 ± 454	1,914 ± 304	2,271 ± 357	1,917 ± 332
$\dot{V}O_{2max}$/body wt, ml·min^{-1}·kg^{-1}	34 ± 5	33 ± 6	31 ± 5	36 ± 5	31 ± 6
$\dot{V}O_{2max}$/FFM, ml·min^{-1}·kg^{-1}	51 ± 5	50 ± 6	49 ± 7	52 ± 6	50 ± 7
Insulin sensitivity					
Glucose AUC	776 ± 142	754 ± 189	862 ± 126	780 ± 156	816 ± 109
Insulin AUC	30,624 ± 12,224	32,790 ± 8,494	44,241 ± 12,573*	29,666 ± 14,002	45,318 ± 10,838†
C-peptide AUC	230,000 ± 54,000	232,000 ± 52,000	286,000 ± 44,000*	222,760 ± 63,163	282,720 ± 24,630†
Insulin sensitivity	9.63 ± 2.73	8.43 ± 1.83	6.75 ± 2.25*	9.92 ± 3.03	5.80 ± 1.50†
Caloric intake					
Total caloric intake, kJ	7,818 ± 909	8,279 ± 1,036	8,140 ± 1,704	7,818 ± 1,572	8,962 ± 1,572
Protein intake, %	18 ± 4	16 ± 3	17 ± 3	18 ± 2	17 ± 2
Carbohydrate intake, %	56 ± 6	53 ± 7	52 ± 7	56 ± 8	52 ± 8
Fat intake, %	26 ± 5	31 ± 6	31 ± 7	26 ± 8	31 ± 8
Physical activity					
Total METs	481 ± 229	589 ± 463	382 ± 204	497 ± 227	338 ± 211

Data are presented as means ± SD; *n*, no. of subjects. Pre, premenopausal; Peri, perimenopausal; Post, postmenopausal; LBM, lean body mass; FSH, follicle-stimulating hormone; LDL, low-density lipoprotein; HDL, high-density lipoprotein; BP, blood pressure; $\dot{V}O_{2max}$, maximal oxygen consumption; AUC, area under the curve; MET, metabolic equivalent. *Significantly different from premenopausal, $P \leq 0.05$ on *day 1*. †Significant difference from premenopausal, $P \leq 0.05$ on *day 2*. *Day 2* refers to the second experimental day, where fat oxidation measurements and biopsies for molecular analyses were completed.

Energy intake estimated from a 3-day food diary did not differ between the three groups of women (Table 1).

As expected, the postmenopausal women showed lower circulating levels of the female sex hormone estradiol ($P < 0.0001$) and higher levels of FSH ($P < 0.0001$) than the premenopausal women.

Whole body fat oxidation during exercise. Fat oxidation data are presented in Table 2. During submaximal exercise, the postmenopausal women had a 33% (CI 95%: 12–55) lower whole body fat oxidation ($P = 0.005$) and a 19% (CI 95%: 9–22) lower energy expenditure than the premenopausal women. LBM correlated closely with fat oxidation ($r = 0.52$,

Table 2. *Oxygen uptake, fat oxidation, and energy expenditure*

	Pre	Post
n	11	7
Fat oxidation, exercise, g/min	0.31 ± 0.03	0.21 ± 0.07*
Fat oxidation, exercise/FFM, g·min^{-1}·kg^{-1}	7.18 ± 1.03	5.51 ± 1.69*
Energy expenditure, exercise, kcal/min	5.40 ± 0.79	4.35 ± 0.60*
Energy expenditure, exercise/FFM, kcal·min^{-1}·kg^{-1}	0.12 ± 0.01	0.11 ± 0.01
Fat oxidation (kcal/min)/energy expenditure (kcal/min)	0.52 ± 0.06	0.43 ± 0.12
RER exercise	0.83 ± 0.02	0.86 ± 0.04

Data are presented as means ± SD; *n*, no. of subjects. *Significantly different from premenopausal, $P \leq 0.05$. Indirect calorimetry data during a 45-min acute bout of exercise performed on a bicycle ergometer.

$P = 0.04$) and energy expenditure ($r = 0.68$, $P = 0.003$). After adjusting for LBM, the differences in whole body fat oxidation between pre- and postmenopausal women were reduced to 23% (CI 95%: 3–47) ($P = 0.05$), whereas differences in energy expenditure between groups were 7% ($P = 0.22$). Furthermore, high levels of circulating FSH levels correlated with low fat oxidation ($r = -0.58$, $P = 0.02$) and low energy expenditure ($r = -0.51$, $P = 0.04$).

The fraction of energy expenditure derived from fat oxidation did not differ significantly between the groups and was 52% (CI 95%: 46–58) in the premenopausal women and 43% (CI 95%: 31–56) in the postmenopausal women.

High whole body fat oxidation correlated with low visceral fat mass ($r = 0.74$, $P = 0.004$), and insulin sensitivity ($r = 0.56$, $P = 0.05$). The same applied for energy expenditure, since high energy expenditure correlated with low visceral fat mass ($r = -50$, $P = 0.04$) and high insulin sensitivity ($r = 0.88$, $P < 0.0001$).

Markers of oxidative capacity. To evaluate the molecular markers of oxidative capacity in the skeletal muscle in a group of women with high vs. low circulating gonadotropins, respectively, peri- and postmenopausal women were pooled for further analyses and compared with premenopausal women.

Primer sequences are shown in Table 3.

At rest the pre-, peri-, and postmenopausal women did not differ in the skeletal muscle mRNA content of the fat oxidation-related genes CPTI, CS, PPARα, β-HAD, PGC-1α, and PDK4 (Fig. 1).

The activity of both β-HAD and CS reflected mRNA expression and showed no differences between the groups (Table 4), and none of them were correlated to either whole body fat oxidation or energy expenditure in this group of women.

The post- and perimenopausal women had lower protein levels of the mitochondrial protein CytC than the premenopausal women ($P = 0.03$) (Fig. 2). COX-I (another component of the respiratory chain) protein levels did, however, not differ significantly between premenopausal and postmenopausal women ($P = 0.15$). PDH-E1α, the catalyst of the irreversible conversion of pyruvate to acetyl-CoA, protein level was also unaffected by menopausal status.

To investigate if the differences in fat oxidation were because of differences in regulation of capillarization in the skeletal muscle, we measured the protein levels of VEGF and found no differences between groups.

For the subset of postmenopausal women, a single 45-min exercise bout did not elicit increased AMPK phosphorylation in skeletal muscle as seen in the premenopausal women, resulting in a trend to blunt the exercise-induced increase in the phosphorylation of AMPK (24%, $P = 0.06$) (Fig. 3). No differences in AMPK phosphorylation at rest were evident (Fig. 2).

Table 3. *Primer sequences used for real-time PCR*

Gene	Primer Sequence
18S	
Forward	5′-GCA ATT ATT CC CAT GAA CG-3′
Reverse	5′-GGC CTC ACT AAA CCA TCC AA-3′
β-HAD	
Forward	5′-GGC TTA GTG GCT GCG TGT-3′
Reverse	5′-ATA AGC TTC CAC TAT CAT AGC ATG-3′
CPTI	
Forward	5′-GAG TGA CTG GTG GGA AGA GTA CA −3′
Reverse	5′-CTT GAT GAG CAC AAG GTC CA-3′
CS	
Forward	5′-GCA TCT TGT CTT GTT CTT GCA-3′
Reverse	5′-TGG CCT GCT CCT TAG GTA TC-3′
PPARα	
Forward	5′-GCA CTG GAA CTG GAT GAC AG-3′
Reverse	5′-TTT AGA AGG CCA GGA CGA TCT-3′
PDK4	
Forward	5′-TCC ACT GCA CCA ACG CCT-3′
Reverse	5′-TGG CAA GCC GTA ACC AAA A-3′
Probe	5′-ATA ATT CCC GGA ATG CTC CTT TGG CTG-3′
PGC-1α	
Forward	5′-CAA GCC AAA CCA ACA ACT TTA TCT CT-3′
Reverse	5′-CAC ACT TAA GGT GCG TTC AAT AGT C-3′
Probe	5′-AGT CAC CAA ATG ACC CCA AGG GTT CC-3′

β-HAD, β-hydroxyacyl-CoA dehydrogenase; CPTI, carnitine palmitoyltransferase I; CS, citrate synthase; PPARα, peroxisome proliferator-activated receptor-α; PDK4, pyruvate dehydrogenase kinase 4; PGC-1α, peroxisome proliferator-activated receptor-γ coactivator-1α.

DISCUSSION

The major findings of this study were that postmenopausal women had a lower whole body fat oxidation and energy expenditure during exercise. Lower LBM seems to be a critical factor for the low fat oxidation and energy expenditure, since these factors were closely correlated. Lower energy expenditure correlated closely with lower insulin sensitivity and increased visceral fat mass. Fat oxidation showed a close correlation to visceral fat and a borderline correlation to insulin sensitivity. This indicates that the low whole body oxidative capacity together with low LBM in the postmenopausal women could be important contributors to the aggravated metabolic profile seen after menopause. Interestingly, none of the differences in whole body fat oxidation were reflected in differences in mRNA levels of proteins involved in fat oxidation and energy expenditure (CPTI, CS, PPARα, β-HAD, PGC-1α, and PDK4) during resting conditions. Also, the activity of important oxidative enzymes (β-HAD or CS) was unchanged by menopausal status. However, we found that postmenopausal women did not elicit an exercise-induced AMPK phosphorylation in skeletal muscle as premenopausal women ($P = 0.06$). Although only a trend, this finding is compatible with earlier results showing that AMPK is activated by estrogen (9, 10).

The finding that postmenopausal women have lower whole body fat oxidation during exercise adds new knowledge to the question of how menopause affects substrate utilization and is in accordance with a previous finding by Lovejoy et al. (30) during resting conditions. Hence, the present results indicate that physiologically higher levels of FSH, seen with menopause, are associated with the lower fat oxidation. The lower fat oxidation and energy expenditure may partially be the reason why postmenopausal women experience an increased total and visceral fat mass (39, 44, 56), since these correlated negatively with the amount of visceral fat seen in this group of pre- and postmenopausal women. Increased abdominal fat and visceral fat mass is known to be associated with several metabolic diseases, such as cardiovascular disease (28) and diabetes (52), which are already known to increase in incidence with menopause (1, 19, 27, 35). Furthermore, low fat oxidation was correlated to decreased insulin sensitivity, which is suggested to precede type 2 diabetes and metabolic syndrome (31). It is well known that early stages of diabetes and prediabetic

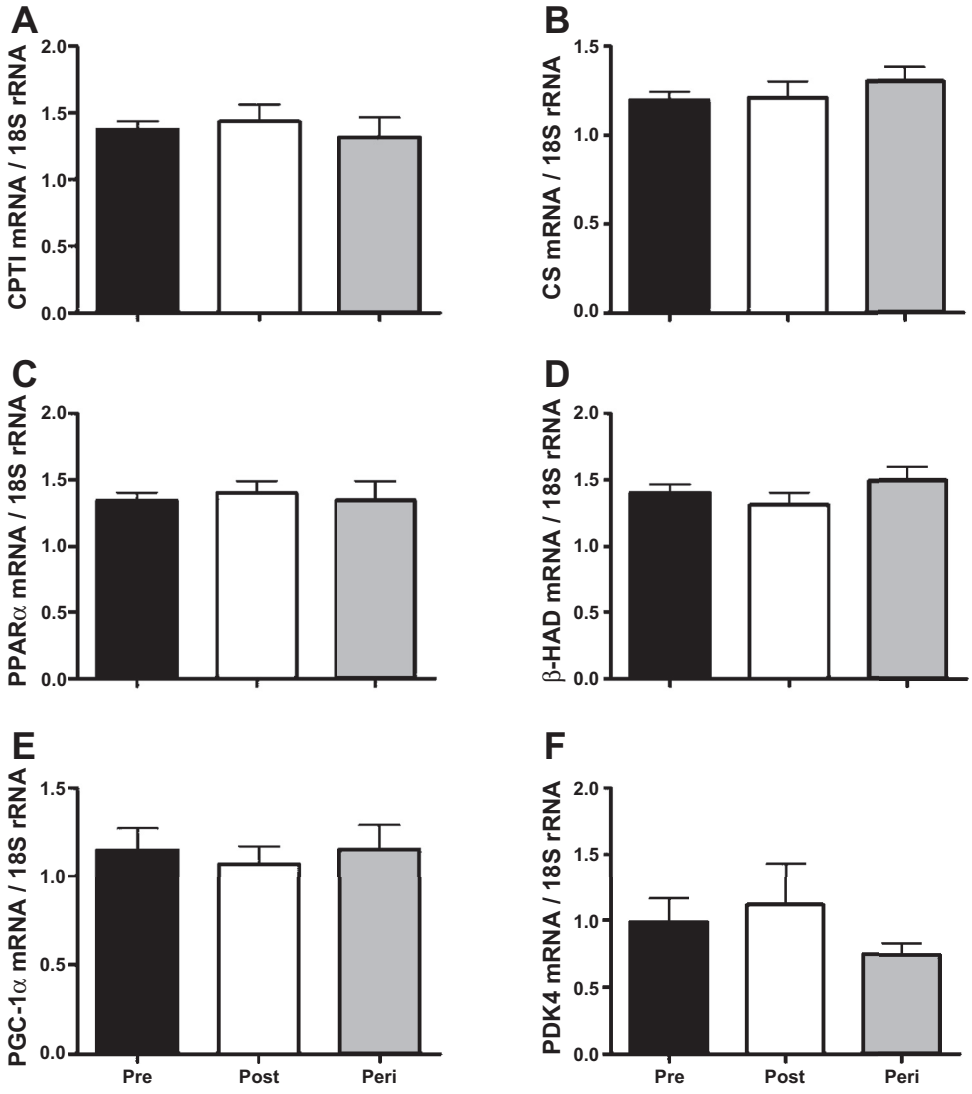

Fig. 1. mRNA expression of carnitine palmitoyltransferase I (CPTI, *A*), citrate synthase (CS, *B*), peroxisome proliferator-activated receptor-α (PPARα, *C*), β-hydroxyacyl-CoA dehydrogenase (β-HAD, *D*), peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α, *E*), and pyruvate dehydrogenase kinase 4 (PDK4, *F*) in the vastus lateralis of the quadriceps muscle of premenopausal (Pre, $n = 19$), postmenopausal (Post, $n = 12$), and perimenopausal (Peri, $n = 5$) women. 18S was used as a housekeeping gene and did not differ between groups.

stages are related to impairments in fatty acid oxidation (4, 25, 33), and it has been hypothesized that the impaired fat oxidation plays a crucial role in the development of diabetes; however, this causality is still theoretical. During postprandial conditions, obese and insulin-resistant individuals have been shown to have a higher fat oxidation (23). However, this is because of an inability to switch easily between fat and glucose oxidation in response to homeostatic changes, also referred to as metabolic inflexibility; whether postmenopausal women show the same metabolic inflexibility remains to be seen.

All in all, this study confirms the fact that changes in fat oxidation might be crucial in the pathogenesis of metabolic diseases and proposes that changes in fat oxidation could play an important role in the development of metabolic diseases after menopause.

Lower LBM in the postmenopausal women seemed by far to be the most important reason for the lower oxidative capacity in this group of women. Furthermore, loss of LBM is a strong predictor of mortality in the elderly (28). In relation to metabolism, LBM has been found to be important for both oxidative capacity (36) and insulin sensitivity (45), which was also applicable in this study. It therefore seems crucial for this group of women to maintain skeletal muscle mass through, for example, resistance exercise (41). Differences in LBM did not seem to be the result of differences in activity level, since there were no differences in $\dot{V}O_{2\,max}$ or daily physical activity

Table 4. *Activity assays of oxidative proteins*

	Premenopausal	Perimenopausal	Postmenopausal
n	19	8	14
β-HAD, μmol·min^{-1}·g wet muscle^{-1}	123.63 ± 29.82	109.20 ± 38.83	120.53 ± 24.68
CS, μmol·min^{-1}·g wet muscle^{-1}	26.20 ± 8.23	25.19 ± 10.71	23.01 ± 5.27

Data are presented as means ± SD; *n*, no. of subjects.

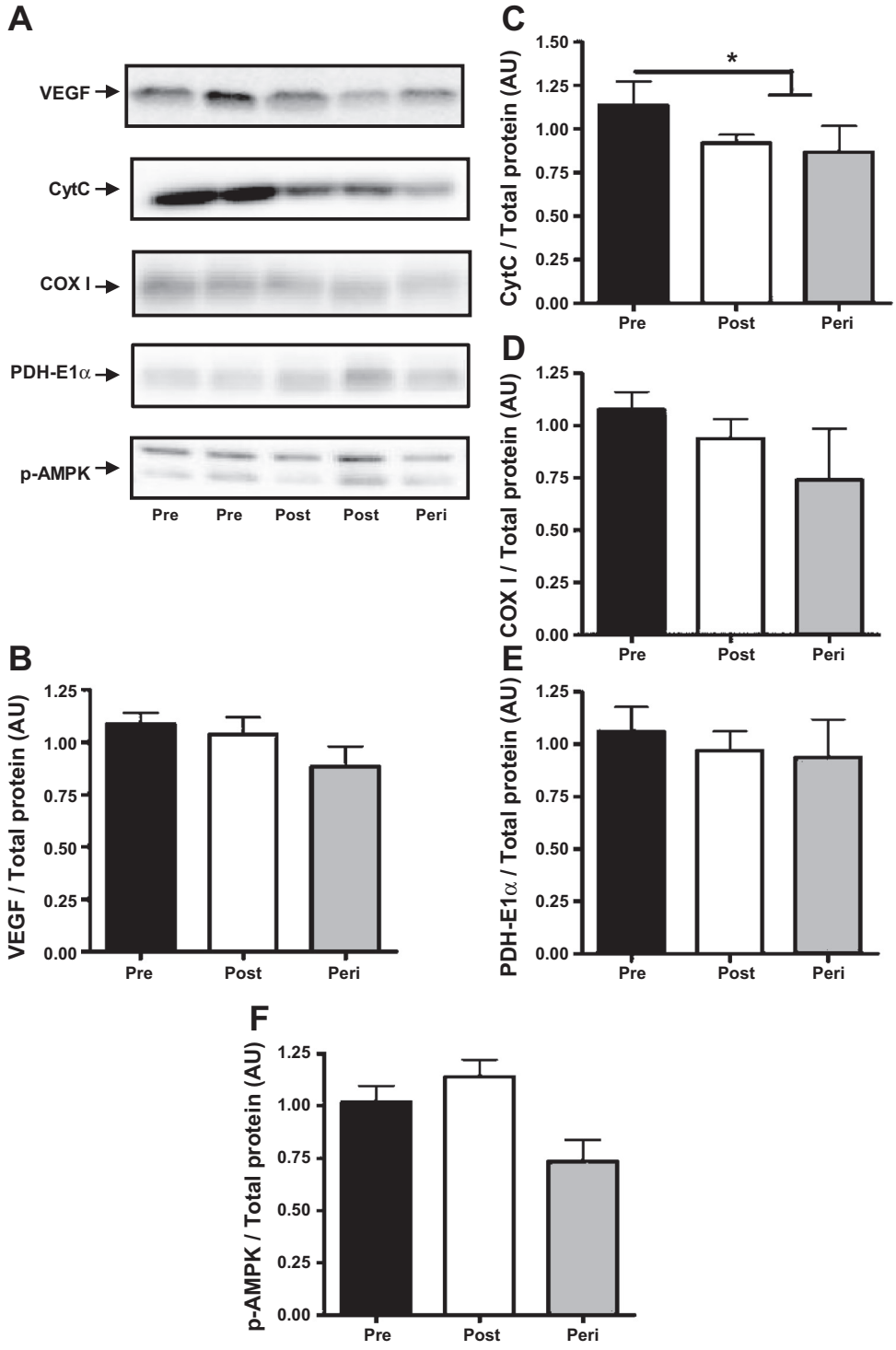

Fig. 2. Western blot analysis of protein levels in the vastus lateralis of the quadriceps muscle in premenopausal (n = 19), postmenopausal (n = 12), and perimenopausal (n = 5) women. *A*: representative immunoblot of all investigated proteins. *B*: quantification of vascular endothelial growth factor (VEGF) Western blot (21 kDa). *C*: quantification of cytochrome *c* (cytC) Western blot (15 kDa). *$P < 0.05$. *D*: quantification of cytochrome oxidase I (COX-I) Western blot (37 kDa). *E*: quantification of pyruvate dehydrogenase-E1α (PDH-E1α) Western blot (40 kDa). *F*: quantification of phosphorylated AMP-activated protein kinase (p-AMPK^{Thr172}) Western blot (62 kDa).

assessed by questionnaire between pre- and postmenopausal women nor did the women differ in dietary intake and composition. This minimizes the likelihood that the difference in LBM is a result of random sampling differences in physical activity level.

Even though LBM seemed crucial for whole body oxidative metabolism, a borderline significant (P = 0.05) difference in whole body fat oxidation during exercise remained, after adjustment for skeletal muscle mass. We therefore hypothesized that menopause status also affects oxidative enzymes in skeletal muscle. This idea is supported by previous animal studies (6, 7, 9, 10) that showed that ovariectomy decreased activity of central oxidative pathways in skeletal muscle and that estrogen supplementation could restore these changes. However, we found no molecular differences in skeletal muscle that could explain the differences in whole body metabolism between pre- and postmenopausal women. Thus, the changed pattern of fat oxidative capacity did not seem to be because of changes in

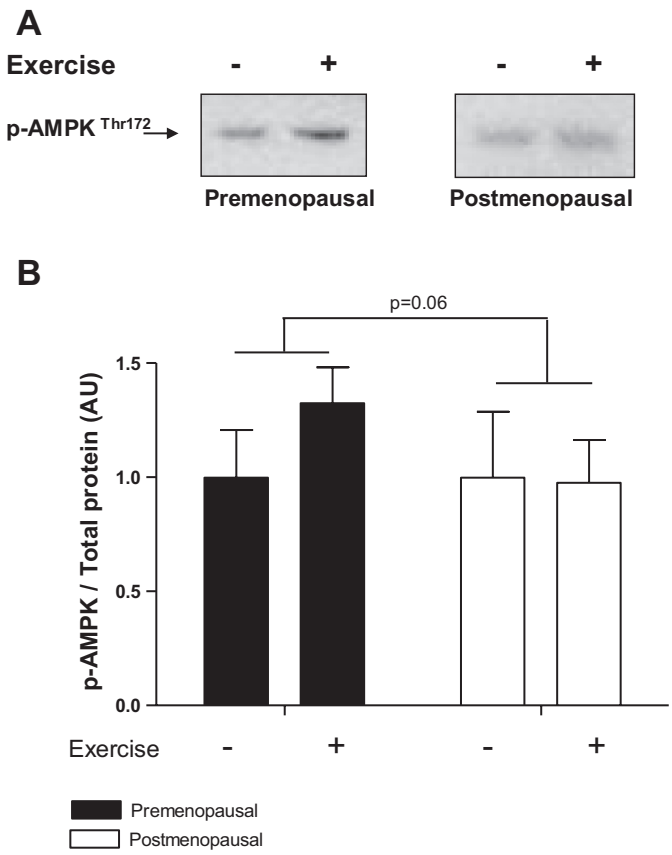

Fig. 3. *A*: Western blot of p-AMPK^{Thr172} (62 kDa) in the vastus lateralis of the quadriceps muscle in premenopausal and postmenopausal women before and after exercise. *B*: quantification of p-AMPK^{Thr172} before and after exercise in pre- and postmenopausal women, $P = 0.06$. Exercise effect in premenopausal women, $P = 0.07$. All before and after exercise samples for each subject were run on the same gel, and on each gel both pre- and postmenopausal women were represented. No perimenopausal women participated in the 2nd day of experiments.

skeletal muscle mRNA expression of fat oxidative genes. This is in accordance with a previous human study (15) investigating changes in mRNA content of metabolically related genes with menstrual cycle phase. However, we did find a CytC protein level that was lower in skeletal muscle of the peri- and postmenopausal women than of the premenopausal women. This indicates that the oxidative capacity of skeletal muscle could be reduced in women after menopause, since the CytC protein levels have been shown to correlate to oxidative capacity (42). However, the COX-I protein levels in the skeletal muscle were unaffected by menopausal status, potentially indicating that CytC is the limiting step of the respiratory capacity in the postmenopausal women. In addition, there were no differences in CS activity, a marker of mitochondrial density, which is in accordance with a previous finding in mice (6) that, however, in contrast to our findings, found that ovariectomy led to a decreased activity of β-HAD in skeletal muscle.

The molecular markers of oxidative capacity were assessed in vastus lateralis of the quadriceps muscle, which is a muscle with a mixed fiber-type composition containing approximately one-half glycolytic fibers and one-half oxidative fibers (47). Because the fat oxidative capacity of this specific muscle has been shown to correlate to both whole body fat oxidation and oxidative capacity (34), we found it appropriate to use this muscle.

The present observation that exercise increased AMPK phosphorylation in skeletal muscle in premenopausal but not postmenopausal women may at least in part account for the differences seen in whole body fat oxidation, with a higher fat oxidation during exercise in the premenopausal women than in the postmenopausal women. In support of this, animal studies in vivo (10) and in vitro (9, 10) have shown that estrogen is a strong activator of AMPK. The overall increase in p-AMPK in the premenopausal group was 35% ($P = 0.07$) and none ($P = 0.73$) in the postmenopausal group. The two main reasons for the only relatively small increase in the premenopausal group is probably the relatively low intensity (50%) and time (45 min) of the exercise, since exercise-induced AMPK phosphorylation in human skeletal muscle has previously been shown to be intensity-dependent (54).

In contrast to the aforementioned animal studies (9, 10), we did not find any differences in resting phosphorylation of AMPK in skeletal muscle of pre- and postmenopausal women, which could suggest that a more severe difference in estrogen may be necessary to observe resting differences in the phosphorylation of AMPK. However, it has previously been described (13) that differences in phosphorylation of AMPK only appear after a stimulus such as exercise, since activation of AMPK is connected to energy depletion in the cell. Furthermore, the physiological effect of circulating female sex hormone on phosphorylation of AMPK might only appear modest. Another reason for the blunted increase in p-AMPK in the postmenopausal group could be the increased visceral fat mass or lower insulin sensitivity, since both obesity and type 2 diabetes is associated with attenuated exercise-induced AMPK phosphorylation (46).

The discrepancy in molecular changes between our findings and earlier animal studies could be a result of species differences. However, it could also indicate that a greater, or a more sudden, change in female sex hormones is necessary to regulate fat oxidation-related molecular mechanisms. Furthermore, the postmenopausal women in this study were in a very early state of menopause. Estrogen depletion with menopause is gradual over the years. This could be adequate to keep up the stimulation of fat oxidative pathways of skeletal muscle. Therefore, to detect differences in molecular mechanisms of oxidative pathways in the muscle, it might be necessary to investigate menopause in a later stage after the menopausal transition.

Because this study was cross-sectional, it is not possible to interpret on causality, which is a limitation. However, the study indicates that the lower fat oxidation in the postmenopausal women is related to important factors of the development of metabolic disease, e.g., low LBM, insulin sensitivity, and high visceral fat mass.

In conclusion, our findings suggest that menopause and higher circulating levels of FSH are associated with lower whole body fat oxidation and energy expenditure during exercise. Decreased LBM seems to be the most important contributor to the observed changes in metabolism in women in early stages after menopause, since lower LBM in the postmenopausal women correlates with both low whole body fat oxidation and energy expenditure. The low fat oxidation and energy

expenditure were associated with an aggravated metabolic profile with both high visceral fat mass and low insulin sensitivity and could play a role in the development of metabolic diseases. Surprisingly, we found no changes in key molecular markers of metabolic function in skeletal muscle that could explain the large differences in whole body metabolism in this early stage after menopause.

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DISCLOSURES

The authors have nothing to disclose.

AUTHOR CONTRIBUTIONS

Author contributions: J.A., A.T.P., B.K.P., and B.L. conception and design of research; J.A., C.J.G., N.M.H.-L., C.T., A.J., J.T.P., and O.H.M. performed experiments; J.A., M.P., and O.H.M. analyzed data; J.A., A.T.P., C.J.G., T.P.S., H.P., B.K.P., and B.L. interpreted results of experiments; J.A. and B.L. prepared figures; J.A. and B.L. drafted manuscript; J.A., A.T.P., C.J.G., N.M.H.-L., T.P.S., C.T., A.J., M.P., J.T.P., O.H.M., H.P., B.K.P., and B.L. edited and revised manuscript; J.A., A.T.P., C.J.G., N.M.H.-L., T.P.S., C.T., A.J., M.P., J.T.P., O.H.M., H.P., B.K.P., and B.L. approved final version of manuscript.

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