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ORIGINAL ARTICLE

Regulation of LKB1 expression by sex hormones in adipocytes

KJ McInnes[1,5], KA Brown[2,3,5], NI Hunger[2] and ER Simpson[2,4]

OBJECTIVE: In the adipose tissue, activation of AMP-activated protein kinase (AMPK) by phosphorylation favours local fatty acid oxidation and inhibition of lipogenesis. We have previously shown that the potent androgen dihydrotestosterone (DHT) can inhibit phosphorylation of AMPK in adipose tissue and 3T3-L1 adipocytes in a dose-dependent manner. This negative effect of DHT was reversed by oestrogen treatment. The purpose of this current study was to determine the underlying mechanisms whereby androgens and oestrogens can regulate AMPK phosphorylation in adipocytes, and whether this mechanism is receptor dependent.

RESULTS: Phosphorylation of AMPK was assessed by western blot in cells treated for 24 h with testosterone or DHT (1–1000 nM). Testosterone and DHT significantly inhibited basal phosphorylation of AMPK. Addition of the androgen receptor antagonist Flutamide (1 μM) to the media reversed the negative effect of testosterone and DHT by returning AMPK phosphorylation levels to those of basal. To further dissect the mechanism underlying AMPK inhibition by testosterone or DHT, we examined the mRNA expression of the upstream activator of AMPK, namely LKB1. Testosterone and DHT treatment of murine 3T3-L1 or human SGBS adipocytes for 24 h significantly decreased the mRNA expression of LKB1. In contrast, 17β-estradiol treatment increased LKB1 mRNA, an effect mediated by oestrogen receptor alpha.

CONCLUSION: We conclude that regulation of AMPK phosphorylation by androgens and oestrogens is receptor-dependent, and demonstrate for the first time that LKB1 is regulated by sex hormones in adipocytes.

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INTRODUCTION

Clinical observations have suggested a role for sex steroid hormones in both men and women in the regulation of adiposity, through either direct or indirect physiological mechanisms. Changes in the hormonal milieu at menopause are associated with an increase in total adiposity.[1] Circulating oestrogen levels decline rapidly around menopause, while simultaneously there is a more gradual decline of androgen levels.[2] Recently, we have shown that the androgen to oestrogen ratio may have an important role in adipose homeostasis.[3] Some years ago, we generated an aromatase knockout (ArKO) mouse by homologous recombination.[4] As aromatase is the enzyme responsible for the conversion of androgens to oestrogens, this mouse has no endogenous oestrogens, but has testosterone levels, which are 3–5 times the normal range. The ArKO mouse develops a number of phenotypes, among which is a full-blown metabolic syndrome.[5] A similar phenotype has been observed in men with natural inactivating mutations of the aromatase gene.[6] In both cases, replacement with 17β-estradiol (E2) resulted in reversal of the phenotype. This metabolic phenotype can be recapitulated in wild-type ovariectomised mice that are given dihydrotestosterone (DHT), but not by ovariectomy alone, indicating that it is the ratio of androgen to oestrogen that is important.[3] In these mice, activation of the AMP-activated protein kinase (AMPK) pathway in the adipose tissue was decreased, suggesting that AMPK activity can be regulated by sex hormones. Subsequent *in vitro* experiments in murine 3T3-L1 adipocytes revealed that DHT can directly decrease phosphorylation of AMPK, whereas E2 increases AMPK phosphorylation.[3] This opposing regulation of AMPK activity by androgens and oestrogens provides at least in part an explanation for the obesity phenotype of the ArKO mouse and suggests that the increase in the androgen to oestrogen ratio following the menopausal transition in humans may contribute to the shift in fat distribution from subcutaneous to visceral depots because of decreased AMPK phosphorylation, and subsequent increased fatty-acid synthesis in visceral adipose tissue.[3] However, the mechanism whereby androgens and oestrogens can differentially regulate the phosphorylation of AMPK remains to be elucidated. AMPK activity is regulated covalently through phosphorylation of the α-catalytic subunit at Thr-172 by the upstream serine/threonine kinase LKB1.[7] In this study, we used mouse and human adipocytes to establish whether LKB1 is regulated by sex hormones, and to determine the involvement of androgen (AR) and oestrogen receptors (ER) in the regulation of AMPK activity.

MATERIALS AND METHODS

Tissue culture

3T3-L1 cells or SGBS cells were seeded at 3×10^5 cells per ml in six-well plates and maintained at no higher than 70% confluence in Dulbecco's modified Eagle's medium (DMEM) or DMEM-F12, respectively (Trace Scientific Ltd, Melbourne, Victoria, Australia), supplemented with 10% (v/v) fetal calf serum (FCS; Trace Scientific Ltd) 100 IU ml^{-1} penicillin, 100 μg ml^{-1} streptomycin and 200 mM L-glutamine (Gibco, Auckland,

[1]Endocrinology Unit, BHF/University Centre for Cardiovascular Science, Queen's Medical Research Institute, University of Edinburgh, Edinburgh, Scotland, UK; [2]Prince Henry's Institute of Medical Research, Clayton, Victoria, Australia; [3]Department of Physiology, Monash University, Clayton, Victoria, Australia and [4]Department of Biochemistry, Monash University, Clayton, Victoria, Australia. Correspondence: Dr KJ McInnes, Endocrinology Unit, BHF/University Centre for Cardiovascular Science, Queen's Medical Research Institute, University of Edinburgh, 47 Little France Crescent, Edinburgh EH16 4TJ, Scotland, UK.
E-mail: kerry.mcinnes@ed.ac.uk
[5]These authors contributed equally to this work.
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New Zealand). For 3T3-L1 cells, differentiation was induced as previously described.[3] Briefly, cells were grown 2 days post confluence in DMEM/FCS and then for 2 days in DMEM/FCS containing 10 nM insulin (Novo Nordisk, Bagsvaerd, Denmark), 1 μM dexamethasone (Sigma-Aldrich, Sydney, New South Wales, Australia) and 0.5 mM isobutylmethylxanthine (Sigma-Aldrich). The media was then changed to DMEM/FCS supplemented only with 10 nM insulin for 2 days and then to DMEM/FCS alone for 3–5 days. For SGBS cells, differentiation was induced as previously described.[3] Briefly, cells were grown to confluence and subsequently cultured in serum-free DMEM-F12 medium, containing penicillin (100 IU ml^{-1}), streptomycin (100 μg ml^{-1}), biotin (33 μM), pantothenic acid (17 μM), transferrin (0.01 mg ml^{-1}), cortisol (0.1 μM), triiodotyronine (200 pM), human insulin (20 nM), Dexamethasone (0.25 μM), isobutylmethylxanthine (500 μM) and rosiglitazone (2 μM) for 3 days. On day 4, the medium was changed to differentiation medium lacking rosiglitazone, and from day 8 until the completion of differentiation (12 days), cells were cultured in the differentiation medium lacking rosiglitazone, isobutylmethylxanthine and Dexamethasone.

For both cell lines, media was replaced 24 h before treatment with steroid hormones in phenol red-free and serum-free DMEM or DMEM-F12.

Western blot analysis

After treatment with hormones, cells were washed with ice-cold phosphate-buffered saline and lysed in lysis buffer as previously described.[8] In all, 50 μg of protein was diluted in sample buffer containing dithiothreitol and denatured. The denatured protein was then run on 8% polyacrylamide gels and transferred to nitrocellulose for western blotting. Phosphorylation of AMPK in 3T3-L1 cells was assayed by western blotting with antibodies to phosphopeptides based on the amino-acid sequence surrounding Thr-172 of the α-subunit of human AMPK (Cell Signaling, Beverley, MA, USA), and normalised to the level of total AMPK protein using antibodies against the catalytic α1 and α2 subunits of AMPK (Cell Signaling). LKB1 protein was quantified by probing the membrane with an anti-LKB1 antibody (Cell Signaling). Proteins were visualised with an Alexa Fluor-680 goat anti-rabbit secondary antibody (Molecular Probes, Inc., Eugene, OR, USA) and band intensities were quantified using the Odyssey infrared imaging system (Licor Biosciences, Lincoln, NE, USA).

Reverse transcription and real-time PCR

Total RNA was extracted using the RNeasy Mini kit (Qiagen, Doncaster, Victoria, Australia) and reverse transcription was performed using AMV reverse transcriptase and random primers (Promega, Alexandria, New South Wales, Australia) as directed by the manufacturer. Briefly, 1.0 μg RNA was incubated with 0.5 μg random primers at 70 °C for 5 min, and reverse transcriptase reaction was incubated at 37 °C for 1 h. Real-time PCR amplifications were performed on the Lightcycler (Roche, Castle Hill, New South Wales, Australia) or the Rotor-Gene (Corbett Life Sciences, Sydney, New South Wales, Australia). Quantification of mouse LKB1 transcript and 18S was performed on the Lightcycler using Fast Start Master SYBR Green 1 (Roche) and specific primer pairs: mLKB1-F: 5′-ACGGCCTGGAATACCTACA C-3′and mLKB1-R: 5′-CCATTGGTGGTGAGTAGCAG-3′, 18S-F: 5′-CGGCTACCA CATCCAAGGAA-3′ and 18S-R: 5′-GCTGGAATTACCGCGGCT-3′. Cycling conditions were one cycle at 95 °C for 10 min, followed by a variable number of cycles of 95 °C for 10 s, 58 °C for 5 s and 72 °C for 10 s. Quantification of human LKB1 transcript was performed on the RotorGene as previously described.[8] Experimental samples were quantified by comparison with standards of known concentration. All samples were normalised to 18S or L32 transcript levels.

Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) was performed to examine ERα binding to the LKB1 promoter. Sample preparation was performed as previously described.[8] Briefly, serum-starved cells were grown to 50% confluency and treated for 45 min at 37 °C for the study of binding of transcriptional regulators to the LKB1 promoter. Cells were then cross linked for 5 min at room temperature and collected in phosphate-buffered saline containing protease inhibitors. Cells were lysed and sonicated at 20% max power 8 times for 30-s pulses using a Sonics sonifier. After sonication, one-tenth of the total sample was removed for input. ChIP was performed using the ChIP-IT express kit (Active Motif, Carlsbad, CA, USA) as directed by the manufacturer. Briefly, 5 μg of DNA was immunoprecipitated overnight at 4 °C with 5 μg antibody (ERα, Santa Cruz Biotechnology, Santa Cruz, CA, USA). Protein–DNA complexes were eluted from the beads and treated with proteinase K solution at 37 °C for 1 h. A number of putative EREs were identified in the region 2.5 kb upstream of the LKB1 promoter transcription start site using several online tools, including *PROMO* (http://alggen.lsi.upc.es/cgi-bin/promo_v3/promo/promoinit.cgi?dirDB = TF_8.3), *AliBaba2.1* (http://www.gene-regulation.com/pub/programs/alibaba2/index.html) and *Prediction of Nuclear Hormone Receptor Response Elements* (http://asp.ii.uib.no:8090/cgi-bin/NHR-scan/nhr_scan.cgi). Real-time PCR was performed on the purified DNA as described above using primers designed −2287 to −2020 (LKB1-ChIP-F: 5′-CTGCCTTCTTCCTGTTTTGC-3′; LKB1-ChIP-R: 5′-TTCTCCTCCTCCTCCTCCTC-3′) for ERα binding to the LKB1 promoter. Images presented are representative of three separate experiments.

Statistical analyses

The experiments were performed at least three times and data are reported as mean ± s.e. m. Statistical analyses were performed by two-tailed Student's *t* test. Single, double and triple asterisks/number signs indicate statistically significant differences: $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.005$. GraphPad Prism Version 3.00 (GraphPad Software Inc., San Diego, CA, USA) was used for the statistical analyses.

RESULTS

The inhibitory effect of androgens on pAMPK in adipocytes is mediated by androgen receptor (AR)

To determine whether the inhibition of phosphorylation of AMPK in adipocytes is mediated by AR, the effect of pre-incubation with the AR antagonist Flutamide was investigated. Twenty-four-hour incubation with testosterone or DHT inhibited basal phosphorylation of AMPK in these cells in a dose-dependent manner (Figures 1a and b). Addition of Flutamide to the media, 1 h before incubation with either androgen reversed the negative effect of testosterone or DHT on AMPK activity and returned phosphorylated levels of AMPK to those of basal (Figures 1a and b).

LKB1 expression is regulated by sex hormones in mouse and human adipocytes

To further investigate the mechanism underlying the regulation of phosphorylation of AMPK by androgens and oestrogens, expression of the upstream regulator of AMPK, LKB1, was investigated in adipocytes. The treatment of human SGBS and mouse 3T3-L1 adipocytes with either DHT or testosterone at nanomolar concentrations resulted in a significant decrease in LKB1 mRNA levels (Figures 2a and b, respectively).

Importantly, E2 had the opposite effect on LKB1 transcript expression. Treatment of differentiated human SGBS cells or mouse 3T3-L1 cells with E2 resulted in a significant and dose-dependent increase in LKB1 transcript levels (Figures 2c and d, respectively).

The regulation of LKB1 expression by oestrogens and androgens is mediated by ER and AR, respectively

ER α and β, as well as the AR, have been reported in the white adipose tissue of mice.[9,10] To gain insight into the involvement of ERα and β in the regulation of LKB1 expression, adipocytes were incubated with the ERα-specific agonist PPT or the ERβ-specific agonist DPN. Incubation of adipocytes with the ERα-specific agonist PPT at concentrations from 10 to 1000 nM increased LKB1 mRNA expression up to twofold (Figure 3a). The ERβ-specific agonist DPN had no effect on LKB1 expression (data not shown).

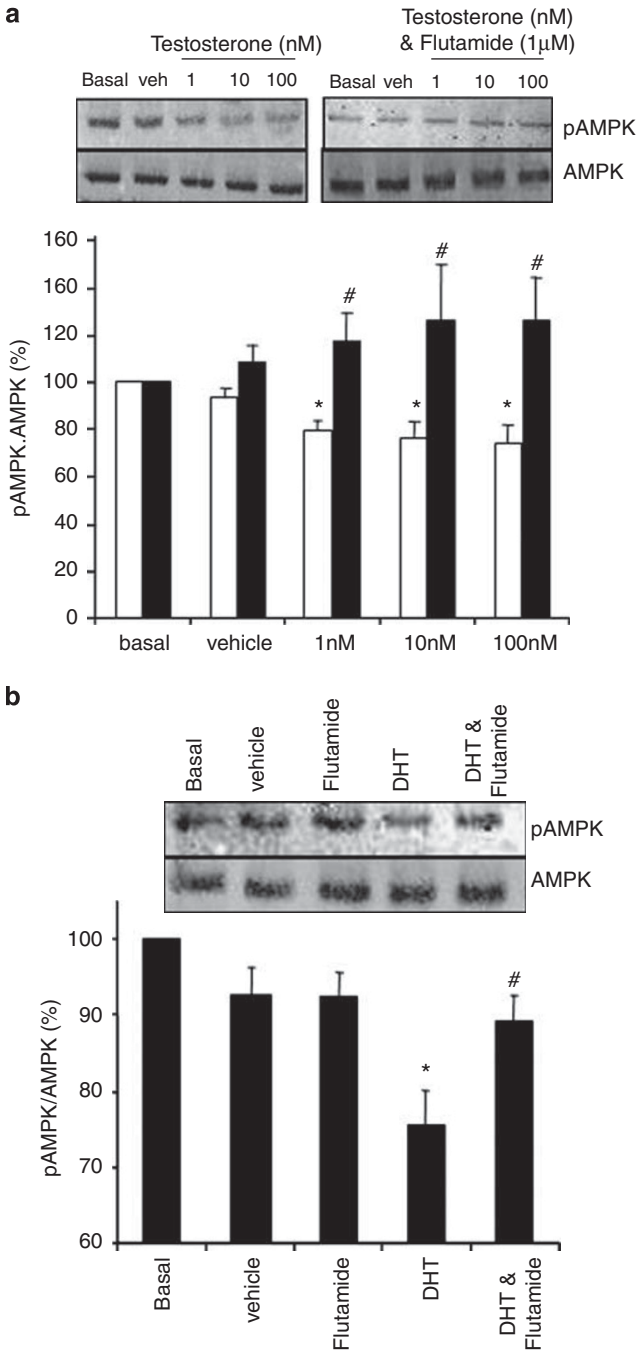

Figure 1. The inhibitory effect of androgens on pAMPK in adipocytes is mediated by the AR. (**a**) Testosterone (T) (24-h treatment) and (**b**) dihydrotestosterone (DHT) (24-h treatment) inhibits phosphorylation (p) of AMPK in differentiated 3T3-L1 cells in a dose-dependent manner. (**a**, **b**) Pre-incubation with Flutamide (1 μM; 1 h before androgen treatment) reverses the negative effect of T or DHT on AMPK phosphorylation. Western blots of phosphorylated (Thr-172) AMPK (upper blot) and total AMPK (lower blot) are shown. $^{*}P<0.05$ vs vehicle (veh); $^{\#}P<0.05$ vs T or DHT alone.

Human LKB1 promoter sequence up to 2.5 kb upstream of the transcriptional start site has been characterised (KA Brown, unpublished observations). Putative EREs were identified within this region of the LKB1 promoter. ChIP assays showed that ERα binds to a region from −2287 to −2020 bp of the LKB1 promoter and that this interaction is increased in the presence of E2 (Figure 3b).

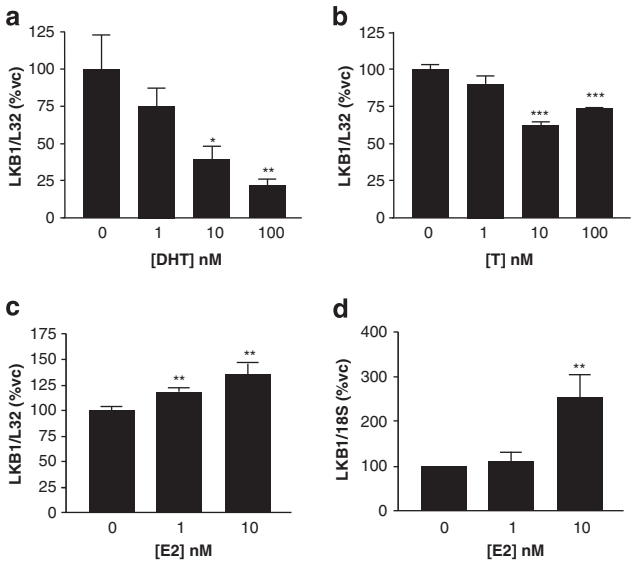

Figure 2. LKB1 expression is regulated by sex hormones in mouse and human adipocytes. (**a**, **b**) DHT and T inhibit LKB1 mRNA expression in differentiated (**a**) SGBS cells and (**b**) 3T3-L1 cells as measured by quantitative reverse transcriptase (qRT-PCR). (**c**, **d**) E2 increases LKB1 mRNA expression in differentiated (**c**) SGBS and (**d**) 3T3-L1 cells as measured by qRT-PCR. $^{*}P<0.05$ vs 0 nM treatment; $^{**}P<0.01$ vs 0 nM treatment; $^{***}P<0.005$ vs 0 nM treatment.

To determine whether the the inhibition of LKB1 expression by androgens is mediated by the AR, Flutamide, an AR antagonist, was employed. As can been seen in Figure 3c, pre-incubation of adipocytes with Flutamide at a concentration of 1 μM for 1 h before testosterone treatment blocked the ability of testosterone to inhibit LKB1 mRNA expression. However, no putative ARE sequences were detected in this 2.5-kb region of the LKB1 promoter.

DISCUSSION

Ageing-associated obesity is associated with multiple alterations of the endocrine system, including changed circulating sex hormone concentrations due to alterations in their pattern of secretion and metabolism, as well as altered hormone transport or action at the level of target tissues.[11] Here, we have shown that the opposing effects of androgens and oestrogens on AMPK activity are mediated, at least in part, by differential regulation of the upstream AMPK kinase, LKB1. The regulation of LKB1 in various tissues has previously been examined.[12,13] The majority of these studies have focussed on the regulation of LKB1 phosphorylation by PKCζ and its resultant action on AMPK, leaving few indices regarding the transcriptional regulation of the *STK11/LKB1* gene. Results presented herein are therefore the first to describe the transcriptional regulation of LKB1 in adipose tissue by androgens and oestrogens. The reciprocal regulation observed, whereby androgens inhibit and oestrogens stimulate expression of LKB1 transcripts, is striking and provides an explanation for the previously reported reciprocal phosphorylation of AMPK on Thr-172.[3] Because AMPK inhibits fatty acid biosynthesis and stimulates fatty acid β-oxidation, these data provide, in turn, an explanation for the obesity phenotype of ovariectomised mice treated with DHT, and of the ArKO mouse, which are both characterised by a high plasma testosterone:oestrogen ratio. Following the menopausal transition, the ratio of plasma testosterone to E2 increases sharply because of the failure of the ovaries to continue to synthesise oestrogens. Extrapolation of our previous work and that presented in this report to the human

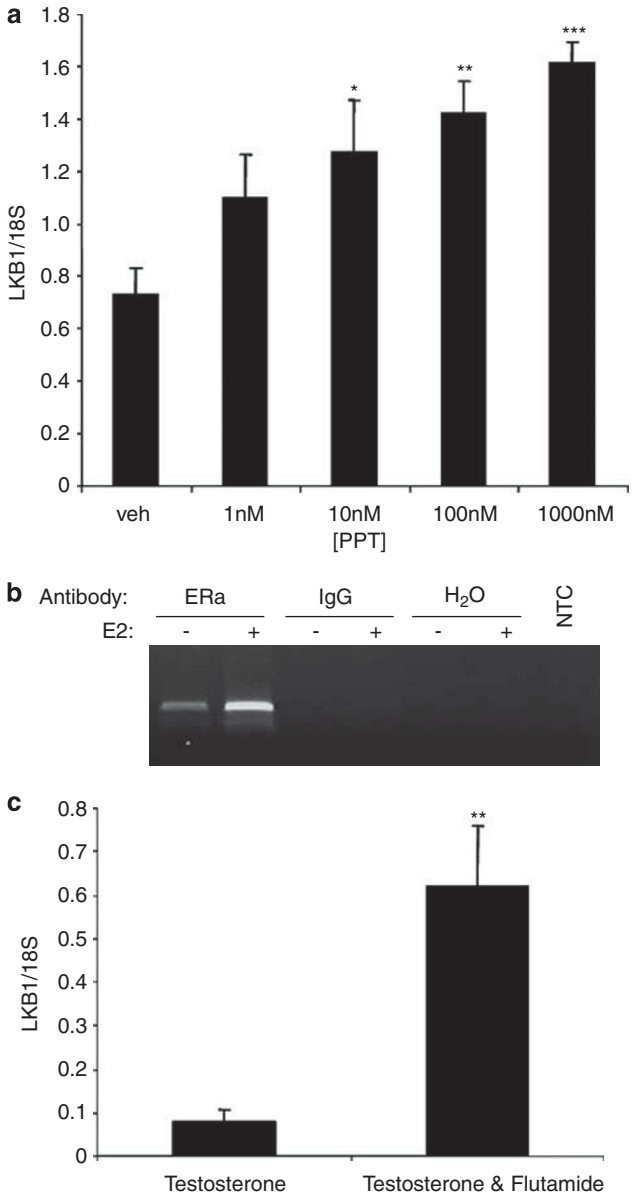

Figure 3. The regulation of LKB1 expression by androgens and oestrogens is mediated by the AR and ER, respectively. (**a**) The ERα-specific agonist PPT significantly increased LKB1 mRNA expression in SGBS adipocytes in a dose-dependent manner as measured by quantitative reverse transcriptase (qRT-PCR). $*P<0.05$ vs vehicle (v) treatment; $**P<0.01$ vs vehicle treatment; $***P<0.005$ vs vehicle treatment. (**b**) Occupancy of the LKB1 promoter by ERα in the presence of E2 as measured by ChIP assay in differentiated SGBS cells. Fixed cell extracts were incubated with anti-ERα antibody, IgG or H_2O (negative controls). ChIP DNA was analysed by real-time PCR using primers to the −2287 to −2020 region of the LKB1 promoter as described in Materials and methods. The result is representative of three separate experiments. (**c**) Pre-incubation of SGBS adipocytes with Flutamide (1 μM; 1 h before testosterone treatment) increases LKB1 mRNA expression as measured by qRT-PCR. $**P<0.01$ vs testosterone treatment.

situation suggests that downregulation of the LKB1/AMPK pathway in adipocytes as a result of a change in the ratio of androgens to oestrogens may provide an explanation for the increase in adiposity frequently observed in postmenopausal women, and for the prevention/reversal of this phenomenon by hormone replacement therapy. Preliminary characterisation of the mechanisms underlying these actions would indicate that they are mediated by the AR and ERα. Moreover, consistent with the stimulation of LKB1 expression by E2, sequencing of 2.5 kb of the LKB1 promoter region revealed putative EREs. Furthermore, ChIP analysis showed direct binding of the ERα to this region, which was stimulated upon addition of E2 at a concentration of 10 nM, thereby identifying ERα as a direct modulator of LKB1 promoter activity. The situation with regard to androgen regulation of LKB1 expression is less clear, and as it is an inhibitory action, the mechanism is likely to be indirect. Studies are presently in progress to investigate this further.

These observations emphasise once more that sex steroids play an important underlying role in energy homeostasis, and moreover suggest a potent mechanism whereby this occurs, namely mediation by the LKB1/AMPK pathway.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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REFERENCES

1 Poehlman ET. Transversing the menopause: change in energy expenditure and body composition. *Coron Artery Dis* 2005; **16**: 511.
2 Liu YM, Ding JZ, Trudy TL, Longenecker JC, Nieto FJ, Golden SH *et al.* Relative androgen excess and increased cardiovascular risk after menopause: a hypothesized relation. *Am J Epidemiol* 2001; **154**: 489–494.
3 McInnes KJ, Corbould A, Simpson ER, Jones ME. Regulation of adenosine 5′,monophosphate-activated protein kinase and lipogenesis by androgens contributes to visceral obesity in an estrogen-deficient state. *Endocrinol* 2006; **147**: 5907–5913.
4 Fisher CR, Graves KH, Parlow AF, Simpson ER. Characterization of mice deficient in aromatase (ArKO) because of targeted disruption of the cyp19 gene. *Proc Natl Acad Sci USA* 1998; **95**: 6965–6970.
5 Simpson ER, Misso M, Hewitt KN, Hill RA, Boon WC, Jones ME *et al.* Estrogen—the good, the bad, and the unexpected. *Endocr Rev* 2005; **26**: 322–330.
6 Jones MEE, Boon WC, McInnes K, Maffei L, Carani C, Simpson ER. Recognizing rare disorders: aromatase deficiency. *Nat Clin Pract Endocrinol Metab* 2007; **3**: 414–421.
7 Woods A, Johnstone SR, Dickerson K, Leiper FC, Fryer LGD, Neumann D *et al.* LKB1 is the upstream kinase in the AMP-activated protein kinase cascade. *Curr Biol* 2003; **13**: 2004–2008.
8 Brown KA, McInnes KJ, Hunger NI, Oakhill JS, Steinberg GR, Simpson ER. Subcellular localization of cyclic AMP-responsive element binding protein-regulated transcription coactivator 2 provides a link between obesity and breast cancer in postmenopausal women. *Cancer Res* 2009; **69**: 5392–5399.
9 Barros RPA, Gabbi C, Morani A, Warner M, Gustafsson JA. Participation of ER alpha and ER beta in glucose homeostasis in skeletal muscle and white adipose tissue. *Am J Physiol-Endocrinol Metabol* 2009; **297**: E124–E133.
10 Yu IC, Lin HY, Liu NC, Wang RS, Sparks JD, Yeh S *et al.* Hyperleptinemia without obesity in male mice lacking androgen receptor in adipose tissue. *Endocrinol* 2008; **149**: 2361–2368.
11 Pasquali R. Obesity and androgens: facts and perspectives. *Fertil Steril* 2006; **85**: 1319–1340.
12 Alessi DR, Sakamoto K, Bayascas JR. LKB1-dependent signaling pathways. *Annu Rev Biochem* 2006; **75**: 137–163.
13 Carling D. LKB1: a sweet side to Peutz-Jeghers syndrome? *Trends Mol Med* 2006; **12**: 144–147.