

Prevention of Obesity and Insulin Resistance by Estrogens Requires ER α Activation Function-2 (ER α AF-2), Whereas ER α AF-1 Is Dispensable

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The beneficial metabolic actions of estrogen-based therapies are mainly mediated by estrogen receptor α (ER α), a nuclear receptor that regulates gene transcription through two activation functions (AFs): AF-1 and AF-2. Using mouse models deleted electively for ER α AF-1 (*ER α AF-1^o*) or ER α AF-2 (*ER α AF-2^o*), we determined their respective roles in the actions of estrogens on body composition and glucose homeostasis in response to either a normal diet or a high-fat diet (HFD). *ER α AF-2^o* males and females developed accelerated weight gain, massive adiposity, severe insulin resistance, and glucose intolerance—quite reminiscent of the phenotype observed in mice deleted for the entire ER α protein (*ER α ^{-/-}*). In striking contrast, *ER α AF-1^o* and wild-type (wt) mice shared a similar metabolic phenotype. Accordingly, 17 β -estradiol administration regulated key metabolic genes in insulin-sensitive tissues and conferred a strong protection against HFD-induced metabolic disturbances in wt and *ER α AF-1^o* ovariectomized mice, whereas these actions were totally abrogated in *ER α AF-2^o* and *ER α ^{-/-}* mice. Thus, whereas both AFs have been previously shown to contribute to endometrial and breast cancer cell proliferation, the protective effect of estrogens against obesity and insulin resistance depends on ER α AF-2 but not ER α AF-1, thereby delineating new options for selective modulation of ER α . *Diabetes* 62:4098–4108, 2013

Estrogens are now recognized as key regulators of energy balance and glucose homeostasis since estrogen deficiency promotes visceral adiposity and insulin resistance in menopausal women, resulting in an increased risk of type 2 diabetes (1). Accordingly, in experimental animal models, bilateral ovariectomy induces metabolic disturbances, including significant weight gain, adipose tissue accumulation, and glucose intolerance. Noteworthy, 17 β -estradiol (E2) prevents or even reverses this phenotype (2,3). The beneficial role of estrogens on energy metabolism is also observed in humans. Indeed large randomized clinical trials evidenced that estrogen-based replacement therapies improve insulin

sensitivity and strongly reduce type 2 diabetes incidence in postmenopausal women (4–6). Although all data concur to demonstrate that estrogens elicit beneficial actions on body composition and glucose metabolism, their proliferative effects on reproductive tissues, uterus and breast, majorly contribute to limit the use of hormone replacement therapy in menopausal women.

The actions of estrogens are mainly mediated by two nuclear receptors, estrogen receptor α (ER α) and β (ER β), respectively encoded by *Esr1* and *Esr2* genes (7,8). The major influence of ER α in the control of body composition and glucose homeostasis was first suggested by the unique clinical observation of a man bearing a mutation in the *Esr1* gene, who developed premature visceral adiposity and insulin resistance associated with vascular dysfunction (9). Accordingly, both male and female ER α -null (*ER α ^{-/-}*) mutant mice demonstrated dysmetabolic characteristics with massive adiposity, insulin resistance, and impaired glucose tolerance (10,11). Finally, whereas chronic E2 administration prevents fat mass accumulation and insulin resistance in wild-type (wt) ovariectomized mice submitted to a high-fat diet (HFD), this hormonal treatment appears totally ineffective in *ER α ^{-/-}* mice (12). Although the major role of ER α in the control of energy metabolism has now been evidenced, the corresponding molecular mechanisms are still unknown but could depend on the molecular structure of the receptor.

As a member of the nuclear receptor family, ER α shares the six-domain (A–F) structure, which includes two distinct activation functions (AF-1 and AF-2) located in the A/B and E domains, respectively (13). The regulation of gene transcription by ER α can be promoted through a functional cooperation between both AFs or through each AF independently (14). Upon estrogen binding, ER α undergoes a conformational change that facilitates the recruitment of coregulators to the promoter regions of target genes, either directly through interaction with cognate DNA sequences (estrogen-responsive element [ERE]) or through protein/protein interaction with transcriptional binding sites such as AP1 and SP1 (8).

Although the pivotal metabolic role of ER α is well established, the involvement of its two AFs has never been reported to date. We here explored in vivo their respective contribution to energy and glucose homeostasis, thanks to recent mouse models deficient in either ER α AF-1 (*ER α AF-1^o* mice) or ER α AF-2 (*ER α AF-2^o* mice) (15,16). We first investigated the metabolic phenotype of *ER α AF-1^o* and *ER α AF-2^o* mice fed with a chow diet or submitted to an HFD. In ovariectomized mice, we then determined the contribution of each ER α AF to E2-mediated regulation of key metabolic genes in peripheral insulin-sensitive tissues as well as protection against HFD-induced obesity and insulin resistance.

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See accompanying commentary, p. 3996.

RESEARCH DESIGN AND METHODS

Animals. ER α -deficient (*ER α ^{-/-}*), ER α AF-1-deficient (*ER α AF-1^o*), and ER α AF-2-deficient (*ER α AF-2^o*) mice (all on a C57BL6/J background) have been previously described (15–18). Wt littermates were systematically used as control. Animals were housed in groups of six and kept in a specific pathogen-free and temperature-controlled facility on a 12-h light/dark cycle. All experimental procedures were performed in accordance with the principles and guidelines established by the National Institute of Medical Research and approved by the local Animal Care and Use Committee.

According to the experimental settings, mice were fed with either a normal chow diet (NCD; 2.9 kcal/g; SAFE, Augy, France) or HFD (45% fat, 3.7 kcal/g; Research Diets, New Brunswick, NJ). To standardize the chronic exposure to estrogens, 4-week-old female mice were ovariectomized, and then a placebo or E2-releasing (0.1 mg for 60 days, i.e., 80 μ g/kg/day; Innovative Research of America, Sarasota, FL) pellet was subcutaneously implanted. Food intake and body weight were recorded weekly, and glucose tolerance tests were performed before sacrifice. Body composition determination by EchoMRI, assessment of basal metabolism in metabolic cages, insulin tolerance tests, and hyperinsulinemic-euglycemic clamps were performed as previously described (12).

Primary hepatocyte and adipocyte cultures. Cells were isolated from 10-week-old, NCD-fed, ovariectomized female mice. Hepatocytes were then 12-h cultured in 60-mm petri dishes (2.5×10^6 cells per well) in Dulbecco's modified Eagle's medium (BE12-614F; LONZA) supplemented with 10% steroid-free FCS, 1% penicillin/streptomycin, and 0.2 nmol/L L-glutamine. Mature adipocytes were placed in fibrin gels (1.5 mg fibrinogen/mL; Sigma-Aldrich) supplemented with thrombin (25 units/mL, 1/3 vol/vol; Sigma-Aldrich) and cultured in Dulbecco's modified Eagle's medium supplemented with 1% steroid-free FCS, 1% penicillin/streptomycin, and 0.2 nmol/L L-glutamine. Then, cells were cultured for 24 h with 17 β -estradiol (10^{-8} mol/L; Sigma-Aldrich) or vehicle alone (DMSO), lysed with TriPure reagent (Roche), and stored at -20°C for RNA extraction.

Real-time quantitative PCR. Total mRNAs were extracted from tissues or primary cell cultures using TriPure reagent (Roche) and reverse transcribed (High-Capacity cDNA Reverse Transcription Kits; Applied Biosystems). The relative gene expression was determined by RT-quantitative PCR performed on Biomark (Fluidigm) using the specific primers listed in Supplementary Table 1.

Adipose tissue histology. Paraffin-embedded 95% ethanol-fixed adipose tissue sections were stained with hematoxylin-eosin. The number and mean size of adipocytes were estimated by using the ImageJ quantification software.

Biochemical analysis. Plasma levels of insulin and adipokines were determined using the Multiplex Immunoassay Technology Xmap (MILLIPEX, 4 Plex; Millipore, Saint-Quentin-en-Yveline, France). Homeostasis model assessment of insulin resistance (HOMA-IR) was calculated as follows: (insulin [mU/L]/glycemia [mg/dL])/405. Testosterone and 17 β -estradiol were measured by gas chromatography-mass spectrometry according to the protocol described by Liere et al. (19), with minor modifications.

Statistical analysis. Results are presented as mean \pm SEM. Statistical analyses were performed using GraphPad Prism 4.00 (GraphPad Software, San Diego, CA). Since no significant differences between groups were observed for all the studied parameters, data from wt littermates (ER α ^{+/+}, AF1^{+/+}, and AF2^{+/+}) were pooled for statistical analyses. One-way or one-way repeated ANOVA tests were performed to address the influence of genotype in intact mice. Two-way ANOVA test was used to explore the respective effect of E2 treatment and day/night period on activity and metabolic parameters. The effect of E2 administration was considered in each genotype using Student *t* test.

RESULTS

ER α AF-2^o mice spontaneously develop adiposity and glucose intolerance. To address the respective role of each ER α AF in body composition and glucose homeostasis regulation, ER α ^{-/-}, ER α AF-1^o, and ER α AF-2^o mutant mice and their respective wt littermates were first maintained on an NCD from weaning to 7 months of age. These animal models have been previously characterized (15–17). Nevertheless, prior to our analyses, we confirmed the profile of ER α protein expression in each genotype (Supplementary Fig. 1).

As expected, female ER α ^{-/-} mice exhibited a significant rise in weight gain, compared to wt mice (Fig. 1A). No difference was observed between ER α AF-1^o and wt mice, whereas weight gain significantly increased in ER α AF-2^o mice, although to a lesser extent than in ER α ^{-/-} mice (Fig. 1A). Importantly, daily food consumption was not

influenced by the different genotypes (Supplementary Fig. 2). At sacrifice, quantification of adipose tissues revealed the significant expansion of subcutaneous (dorsolombar, inguinal, and gluteal fat pads) and deep (perigonadic and mesenteric) adiposity in ER α ^{-/-} and ER α AF-2^o mice (Fig. 1B), with an important increase in mean adipocyte size (Fig. 1C), and larger heterogeneity in adipocyte size distribution within perigonadic samples (Supplementary Fig. 2). In striking contrast, adipose tissue accumulation and histological characteristics were similar in ER α AF-1^o and wt control mice (Fig. 1B and C). A significant increase in HOMA-IR was observed in ER α ^{-/-} and, to a lesser extent, in ER α AF-2^o female mice, contrasting with the identical values in wt and ER α AF-1^o (Fig. 1D). Finally, as ER α ^{-/-} mice, female ER α AF-2^o mice spontaneously developed pronounced glucose intolerance, whereas an intermediate but significant hyperglycemic value was only observed 30 min after glucose challenge in ER α AF-1^o females, as compared with wt controls (Fig. 1E). Males displayed characteristics similar to females since both ER α ^{-/-} and ER α AF-2^o exhibited an increased body weight gain, adiposity, and glucose intolerance, as compared with ER α AF-1^o and wt mice (Fig. 1A–E and Supplementary Fig. 2).

ER α AF-2^o females are prone to HFD-induced obesity and insulin resistance in contrast to ER α AF-1^o mice.

To further investigate the involvement of the two ER α AFs in the preventive action of estrogens against obesity and type 2 diabetes, female mutant mice and their respective wt littermates were then submitted to a 3-month HFD period. Both ER α ^{-/-} and ER α AF-2^o mice gained more weight than wt controls and developed a severe obese phenotype (Fig. 2A and B), although monitoring of daily food intakes revealed no change between wt and mutant mice (Fig. 2C). They were also characterized by a greater increase in adiposity, with identical histological characteristics demonstrating adipose hypertrophy (Fig. 2D–F and Supplementary Fig. 3). On the contrary, weight gain and adipose tissue deposits were similar in ER α AF-1^o and wt mice (Fig. 2A–F and Supplementary Fig. 3).

Moreover, fasting plasma insulin concentrations and insulin tolerance tests demonstrated that ER α ^{-/-} and ER α AF-2^o female mice developed a severe insulin-resistant state under HFD (Fig. 2G and K). Plasma concentrations of leptin and resistin were also considerably increased in ER α ^{-/-} and ER α AF-2^o mice as compared with wt and ER α AF-1^o females, whereas no differences were observed between the different strains in plasma adiponectin levels (Fig. 2H–J). Plasma lipid profiles only showed a significant increase in total and HDL cholesterol levels in ER α ^{-/-} mice (Supplementary Table 2). Although fasting plasma glucose levels were higher in ER α ^{-/-} than in ER α AF-2^o mice, these mouse models showed a similar profile of glucose intolerance when submitted to a glucose challenge (Fig. 2L). Noteworthy, no signs of insulin resistance were observed in ER α AF-1^o mice since insulin and adipokine plasma concentrations, as well as their responses to insulin and to glucose challenge, were not different from those observed in wt mice (Fig. 2G–L).

Both ER α AF-1 and ER α AF-2 deficiencies affect plasma levels of sexual steroids. Targeting ER α signaling affects the hypothalamic-pituitary-gonadal axis and the reproductive tract, in agreement with previous reports (7,15–17). Indeed, ER α ^{-/-} and ER α AF-2^o female mice displayed a severe uterine hypoplasia, not related to the type of diet (Fig. 3A and B). Suggesting a different level of involvement of each activation function in the

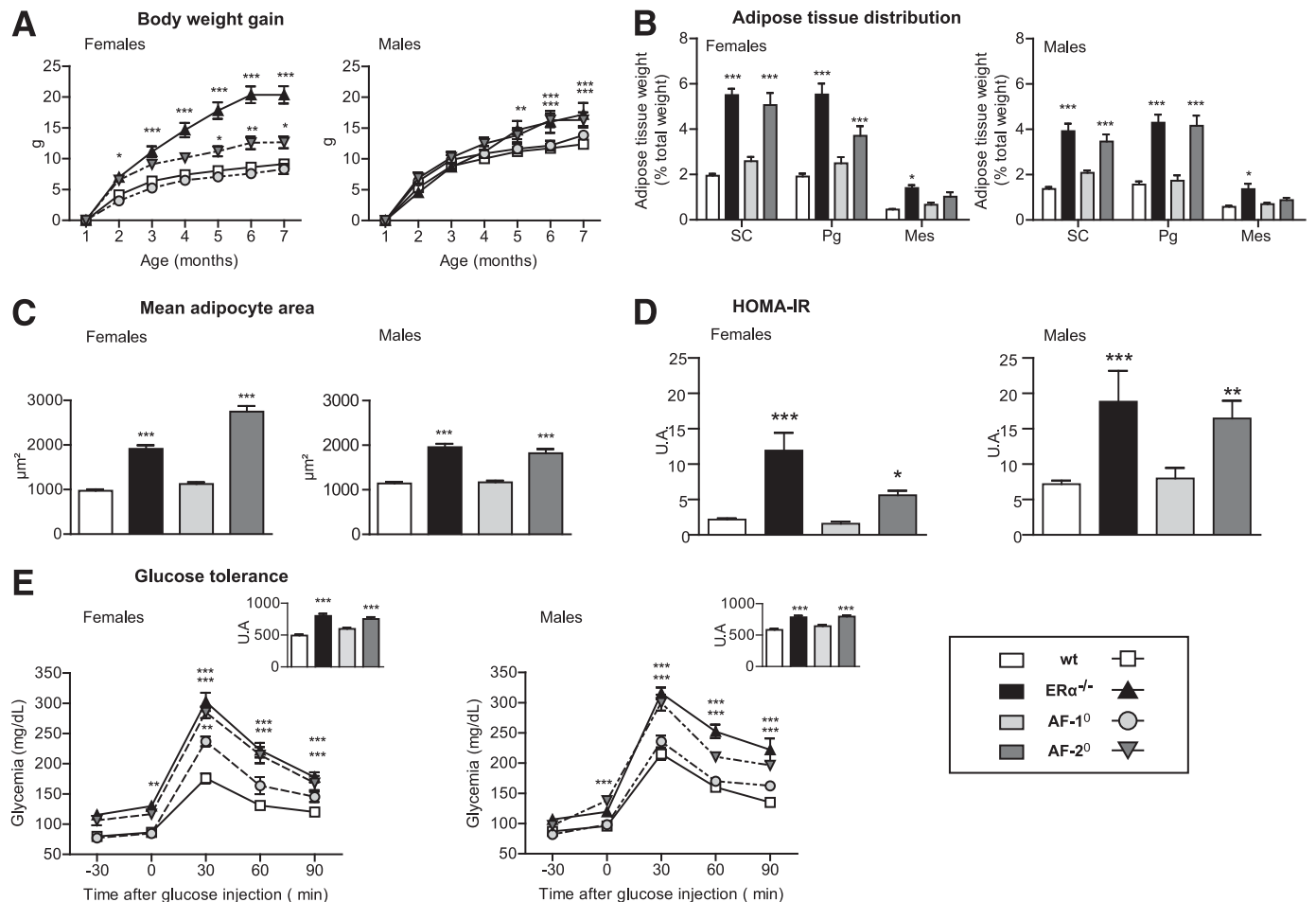


FIG. 1. *ERαAF-2°* mice develop obesity and glucose intolerance under NCD. *ERα^{-/-}*, *ERαAF-1°*, and *ERαAF-2°* female and male mice and their wt littermates were maintained on an NCD until they were killed at 7 months of age. **A:** Body weight gain during the follow-up period. **B:** Subcutaneous (SC), perigonadic (Pg), and mesenteric (Mes) adipose tissue distribution at sacrifice. **C:** Adipocyte mean area in perigonadic deposits (μm^2). **D:** HOMA-IR. **E:** Plasma glucose (mg/dL) during intraperitoneal glucose tolerance test. The inset represents the area under the curve for each group. Data are means \pm SEM ($n = 7\text{--}14$ animals per group). One-way repeated ANOVA (**A** and **E**) or one-way ANOVA (**B**, **C**, and **D**) statistical analyses: * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ for *ERα^{-/-}*, *ERαAF-1°*, or *ERαAF-2°* vs. wt mice.

postpubertal development of the uterus, uterine hypoplasia was significant, but less pronounced, in *ERαAF-1°* female mice (Fig. 3A and B). Furthermore, we noticed that under HFD conditions, circulating levels of endogenous sex steroid hormones, E2 and testosterone, were significantly altered. As compared with wt mice, the greater increase in E2 and testosterone plasma concentrations was observed in *ERα^{-/-}* female mice, followed by *ERαAF-2°* and then *ERαAF-1°* mice (Fig. 3C and D). Similar trends were observed in NCD-fed mice, as previously reported (data not shown). Thus, these data confirm that the present mouse models display alterations in endogenous sexual steroid levels, with various severities according to the type of mutation.

Prevention of HFD-induced adiposity by exogenous E2 is abolished in *ERαAF-2°* mice, but fully maintained in *ERαAF-1°* mice. To standardize the exposure to estrogens and avoid interferences with endogenous sexual steroids, experiments were then carried out with HFD-fed ovariectomized female mice receiving a chronic subcutaneous administration of E2, which induces high physiological hormonal concentrations averaging 100 pg/mL (data not shown), as previously described (12,15,16). The treatment prevented uterine involution in

wt mice, an effect that was totally abrogated in *ERα^{-/-}* and *ERαAF-2°* mice and highly attenuated in *ERαAF-1°* females (Supplementary Fig. 4). E2 treatment exerted a strong preventative effect against HFD-induced body weight gain in wt mice. This protective effect was fully maintained in *ERαAF-1°* female mice but totally abolished in both *ERα^{-/-}* and *ERαAF-2°* mice (Fig. 4A). Body composition analyses revealed that E2 administration was associated with considerable reduction in fat mass accumulation (both at subcutaneous and deep sites) in wt and *ERαAF-1°* mice, with a significant increase in lean mass and water content (Fig. 4B and C). In contrast, E2 administration did not influence body composition, as well as adipose tissue distribution and histology in *ERα^{-/-}* and *ERαAF-2°* mice (Fig. 4B–E).

Daily food intakes were not affected by E2 along the follow-up period, regardless of the genotype (Supplementary Fig. 4). However, E2 raised the respiratory quotient (VCO_2/VO_2 ratio) in wt and *ERαAF-1°*, but not in *ERα^{-/-}* or *ERαAF-2°*, ovariectomized mice (Fig. 5A and B). Accordingly, separate values of VCO_2 and VO_2 , as well as energy expenditure measurements, were significantly influenced by E2 in wt and *ERαAF-1°*, but not in *ERα^{-/-}* or *ERαAF-2°*, mice (Supplementary Fig. 5). Finally, as

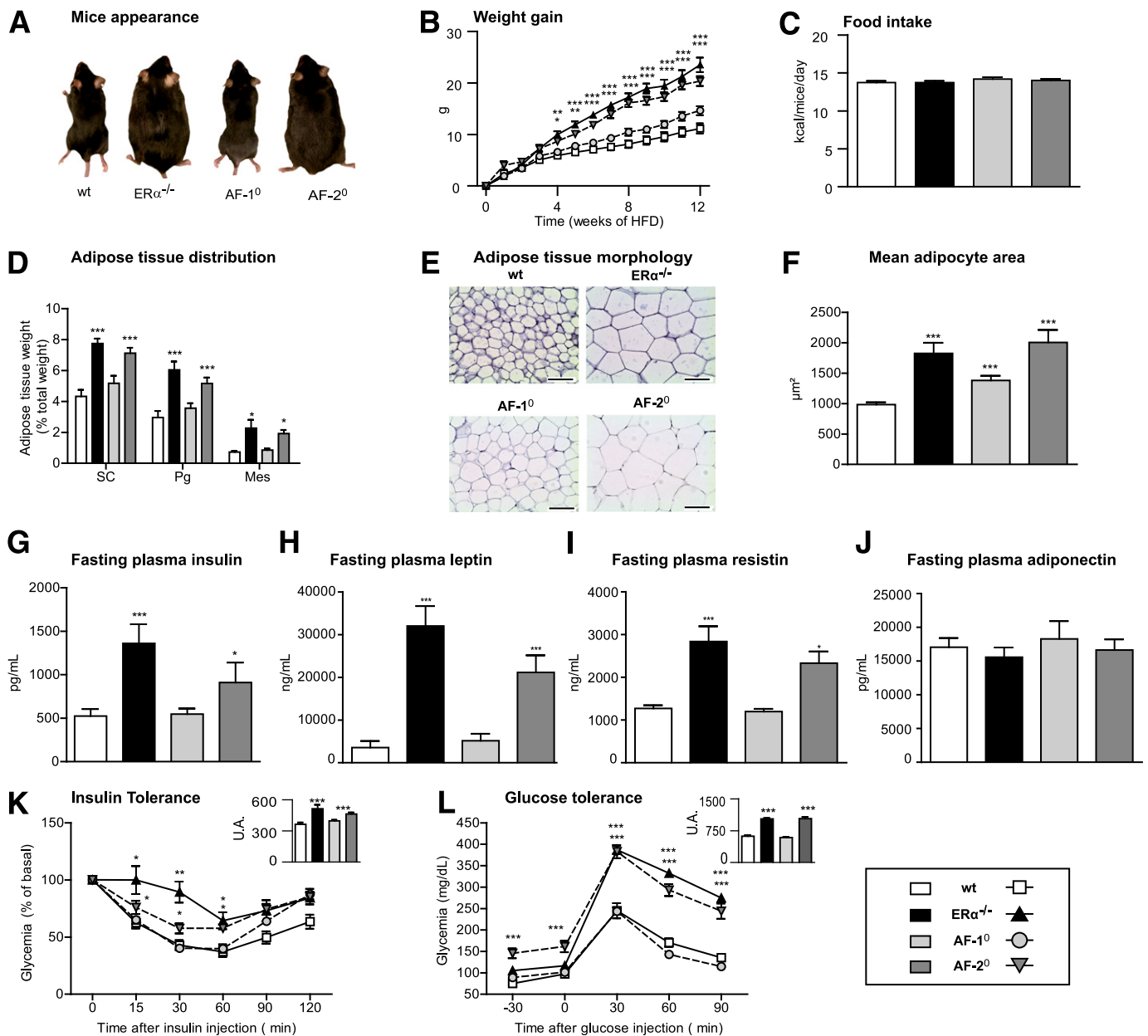


FIG. 2. $ER\alpha AF-2$ deficiency worsens the obese and insulin-resistant phenotype induced by HFD. Four-week-old $ER\alpha^{-/-}$, $ER\alpha AF-1^{\circ}$, and $ER\alpha AF-2^{\circ}$ female mice and their wt littermates were fed with HFD for 3 months. **A:** Mice appearance at time they were killed. **B:** HFD-induced body weight gain. **C:** Daily food intake. **D:** Subcutaneous (SC), perigonadic (Pg), and mesenteric (Mes) adipose tissue distribution. **E:** Representative photomicrographs of perigonadic adipose tissue sections (hematoxylin-eosin staining; original magnification $\times 250$). **F:** Adipocyte mean area in perigonadic deposits (μm^2). **G:** Fasting plasma insulin levels (pg/mL). **H:** Fasting plasma leptin levels (ng/mL). **I:** Fasting plasma resistin levels (ng/mL). **J:** Fasting plasma adiponectin levels (pg/mL). **K:** Intraperitoneal insulin tolerance test (0.6 mU/kg). The inset represents the area under the curve (AUC) for each group. **L:** Intraperitoneal glucose tolerance test (1 g/kg). The inset represents the AUC for each group. Data are means \pm SEM ($n = 6-15$ animals per group). One-way repeated ANOVA (**B, K, and L**) or one-way ANOVA (**C, D, F-J**) statistical analyses: * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ for $ER\alpha^{-/-}$, $ER\alpha AF-1^{\circ}$, or $ER\alpha AF-2^{\circ}$ vs. wt mice.

previously reported (20,21), E2 administration elicited a marked increase in the locomotor activity in wt and $ER\alpha AF-1^{\circ}$ mice, mainly during the dark/active phase (night), but not in $ER\alpha^{-/-}$ or $ER\alpha AF-2^{\circ}$ mice (Fig. 5C).

Preservation of insulin sensitivity and glucose homeostasis by E2 depends on $ER\alpha AF-2$, but not on $ER\alpha AF-1$. Besides the crucial role of $ER\alpha AF-2$ to mediate the prevention of HFD-induced adiposity, we next determined the respective contribution of the two $ER\alpha AF$ s in the beneficial actions of E2 on insulin sensitivity and glucose tolerance. The demonstration that E2 treatment exerted a protective effect against HFD-induced insulin

resistance was first evidenced by the decrease in fasting plasma insulin concentration in wt mice (Fig. 6A). This metabolic parameter was still favorably influenced by E2 in $ER\alpha AF-1^{\circ}$ mice, but not in $ER\alpha^{-/-}$ or $ER\alpha AF-2^{\circ}$ mice (Fig. 6A). In contrast, we did not find any significant influence of E2 administration on fasting lipid profile and free fatty acid concentrations (Supplementary Table 3).

The effect of E2 on insulin sensitivity was then assessed during hyperinsulinemic-euglycemic clamps, which clearly demonstrated that E2 administration similarly improved whole insulin sensitivity in HFD-fed wt and $ER\alpha AF-1^{\circ}$ mice, whereas this beneficial action was totally abolished

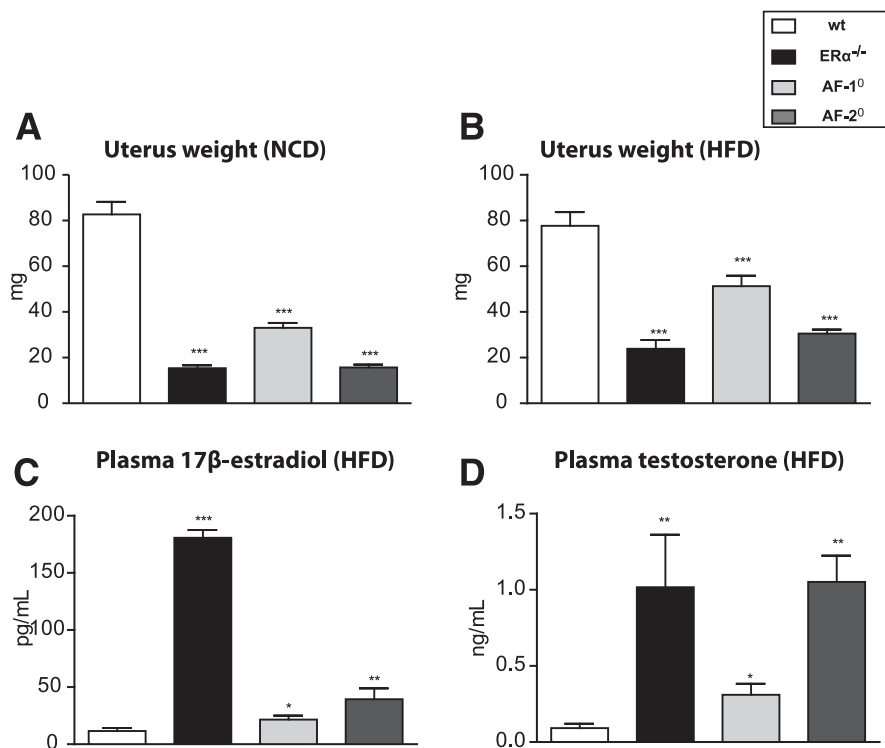


FIG. 3. Either ER α AF-1 or ER α AF-2 deficiency alters uterine weight and sex steroid plasma levels. Wet uterus weight at sacrifice in wt and mutant mice fed with NCD (A) and HFD (B). C: Plasma 17 β -estradiol concentration in HFD-fed wt and mutant mice. D: Plasma testosterone concentration in HFD-fed wt and mutant mice. Data are means \pm SEM ($n = 5-6$ per group). One-way ANOVA statistical analysis: * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ for ER $\alpha^{-/-}$, ER α AF-1 $^{\circ}$, or ER α AF-2 $^{\circ}$ vs. wt mice.

in ER $\alpha^{-/-}$ and ER α AF-2 $^{\circ}$ mice (Fig. 6B). Finally, chronic E2 administration similarly preserved wt and ER α AF-1 $^{\circ}$ mice from HFD-induced glucose intolerance, contrasting the severe hyperglycemia observed after glucose challenge in both placebo- and E2-treated ER $\alpha^{-/-}$ and ER α AF-2 $^{\circ}$ mice (Fig. 6C).

ER α -mediated gene regulation in insulin-sensitive tissues depends on ER α AF-2. As the ER α AFs contribute to the effects of estrogens through the modulation of target gene transcription, we finally explored the expression profiles of genes that have been previously shown to be regulated by chronic exposure to estrogens in insulin-sensitive tissues (liver, adipose tissues, and skeletal muscles) (22–24). First, we considered several genes involved in the lipogenic pathway in the liver (*Stat3*, *Srebp1C*, *Scd1*, and *Fas*) and found that E2 significantly influenced their expression in wt and ER α AF-1 $^{\circ}$ mice, but not in ER $\alpha^{-/-}$ or ER α AF-2 $^{\circ}$ mice (Fig. 7A). Next, genes (*Fas*, *Acc1*, *Acc2*, and *Perilipin2*) that contribute to lipogenesis and lipid storage in white adipose tissues were studied. In wt mice, E2 downregulates the abundance of these gene mRNAs. This E2-induced downregulation only reached a significant level for *Acc1* and *Perilipin2* in ER α AF-1 $^{\circ}$ mice, but was totally abrogated in ER $\alpha^{-/-}$ and ER α AF-2 $^{\circ}$ mice (Fig. 7B). *LPL* gene expression was not influenced by E2, irrespective of the genotype (data not shown). Then, we examined several mRNAs encoding for proteins involved in skeletal muscle metabolism (*Pgc1 α* , *Ppara α* , *Ppar γ 1*, *Ppar γ 2*, *Pdk4*, and *Ucp3*) (Supplementary Fig. 6A). All genes were regulated by E2 in wt and, except *Ppar γ 2*, in ER α AF-1 $^{\circ}$ mice, but this regulation was abolished in ER $\alpha^{-/-}$ and ER α AF-2 $^{\circ}$ mice (Supplementary Fig. 6A). E2 also significantly increases the expression of genes

involved in brown adipose tissue metabolism (*Pgc1 α* , *Ppar γ 1*, *Ppar γ 2*, and *Ucp1*) in wt mice. This enhancing effect was totally abrogated in ER $\alpha^{-/-}$ and ER α AF-2 $^{\circ}$ mice, whereas *Pgc1 α* and *Ucp1* expression remained increased by E2 in ER α AF-1 $^{\circ}$ mice (Supplementary Fig. 6B).

Finally, we aimed to confirm the respective role of ER α AF-1 and ER α AF-2 in gene regulation at the cellular level, in response to short-term exposure to E2. Thus, hepatocytes and mature adipocytes were isolated from ovariectomized female mice and were cultured for 24 h in the presence of 10 $^{-8}$ mol/L E2 or vehicle. E2 significantly enhanced the Stat3 mRNA level in hepatocytes from wt and ER α AF-1 $^{\circ}$ mice, but not in cells from ER $\alpha^{-/-}$ or ER α AF-2 $^{\circ}$ mice (Fig. 8A). The expression of two key genes of the lipogenic pathway in the liver (*Srebp-1c* and *Scd1*) was also only repressed in wt and ER α AF-1 $^{\circ}$ hepatocytes (Fig. 8A). In contrast to in vivo treatment, the *Fas* mRNA level was significantly decreased by E2, but only in freshly isolated wt and ER α AF-1 $^{\circ}$ hepatocytes (Fig. 8A). E2 exerted no influence on *Acc-2* and *Perilipin2* gene expression in primary adipocyte cultures (Fig. 8B). However, according to our in vivo data, E2 significantly downregulated *Fas* and *Acc-1* mRNA levels in adipocytes from both wt and ER α AF-1 $^{\circ}$ mice, but not in cells from ER $\alpha^{-/-}$ or ER α AF-2 $^{\circ}$ mice (Fig. 8B). These data provide further evidence that the regulation of crucial metabolic genes by E2 absolutely requires ER α AF-2 activation, whereas ER α AF-1 appears to be dispensable.

DISCUSSION

Although strong clinical and experimental evidence supports a pivotal role for ER α in the regulation of metabolism

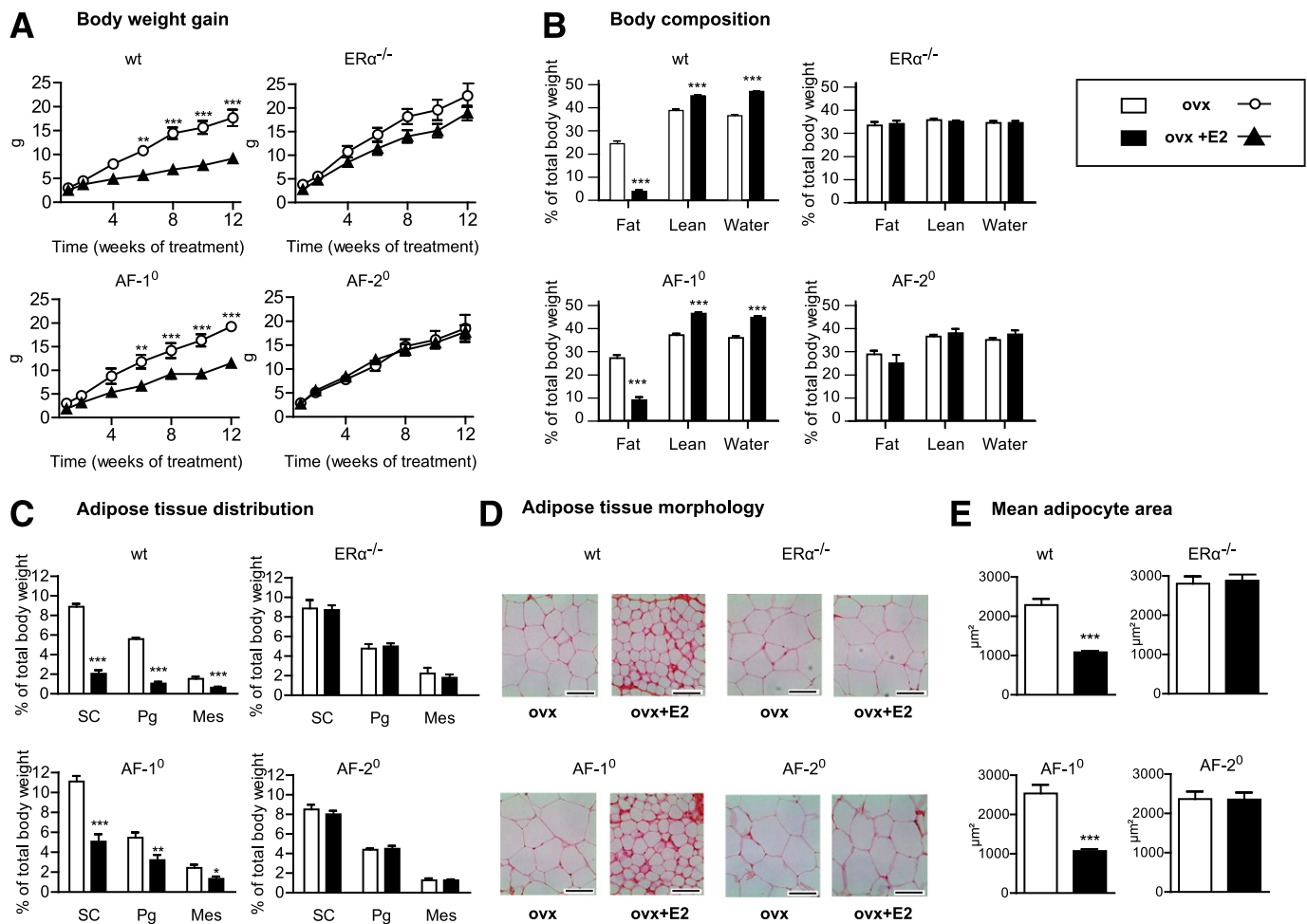


FIG. 4. Exogenous estradiol prevents HFD-induced adiposity in $ER\alpha AF-1^{\circ}$, but not in $ER\alpha AF-2^{\circ}$, ovariectomized female mice. Four-week-old wt, $ER\alpha^{-/-}$, $ER\alpha AF-1^{\circ}$, and $ER\alpha AF-2^{\circ}$ female mice were ovariectomized and received either 17 β -estradiol (ovx+E2) or placebo (ovx) subcutaneous administration for a 3-month period and were concomitantly fed with HFD. **A:** Body weight gain during the treatment period. **B:** Body composition analysis (magnetic resonance technology). **C:** Subcutaneous (SC), perigonadic (Pg), and mesenteric (Mes) adipose tissue distribution. **D:** Representative photomicrograph of perigonadic adipose tissue sections (hematoxylin-eosin staining; original magnification $\times 250$). **E:** Mean adipocyte area in perigonadic deposits (μm^2). Data are means \pm SEM ($n = 5-13$ animals per group). Student t test statistical analysis: * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ for E2-treated vs. placebo-treated mice.

(i.e., energy balance, adipose tissue distribution, insulin sensitivity, and glucose homeostasis), the molecular mechanisms involved in these beneficial actions remain largely unknown (25,26). The current study demonstrates that the activation of $ER\alpha AF-2$ is absolutely required to mediate the protective actions of estrogens against adiposity, insulin resistance, and hyperglycemia.

Indeed, we first confirmed that both female and male $ER\alpha^{-/-}$ mice spontaneously develop global adiposity, insulin resistance, and glucose intolerance, as previously reported (10,11), and demonstrated that $ER\alpha AF-2$ deficiency results in a very close obese and dysmetabolic phenotype. In contrast, either on NCD or HFD, the body composition and glucose homeostasis showed no significant differences between $ER\alpha AF-1^{\circ}$ and wt mice, except for the glycemic peak at 30 min in response to glucose challenge in NCD-fed intact $ER\alpha AF-1^{\circ}$ female mice. We report that $ER\alpha$ deficiency, but also $ER\alpha AF-1$ - or $ER\alpha AF-2$ -specific targeting, alters plasma concentrations of endogenous sexual steroids under HFD, as previously reported under chow diet (17). This confirms that both $ER\alpha AF$ s are involved in the negative feedback of estrogens. Since increased levels of E2 and testosterone are

more pronounced in $ER\alpha^{-/-}$ and $ER\alpha AF-2^{\circ}$ than in $ER\alpha AF-1^{\circ}$ females, we considered the hypothesis that this complex endocrine profile could have contributed to the dysmetabolic phenotype. However, after suppression of endogenous hormones by ovariectomy, the administration of exogenous E2 highly prevented adiposity and insulin resistance in wt and $ER\alpha AF-1^{\circ}$ mice, whereas this protective effect was totally abrogated in $ER\alpha^{-/-}$, as in $ER\alpha AF-2^{\circ}$ mice, definitely demonstrating the crucial role of this latter AF in the metabolic actions of estrogens.

It is now recognized that the beneficial actions of estrogens on energy balance, insulin sensitivity, and glucose homeostasis result from their combined effects on numerous ER-expressing tissues (25,26). For instance, new important insights on the action of estrogens on the central nervous system have been provided in the last years, demonstrating that hypothalamic $ER\alpha$ plays an essential role in the control of energy balance and glucose homeostasis (21,27-29). Altogether, the data indicate that the obese and dysmetabolic phenotype induced by the disruption of $ER\alpha$ signaling results from decreased energy expenditure rather than from increased food intake (20,21,27,28). Although most of these previous studies

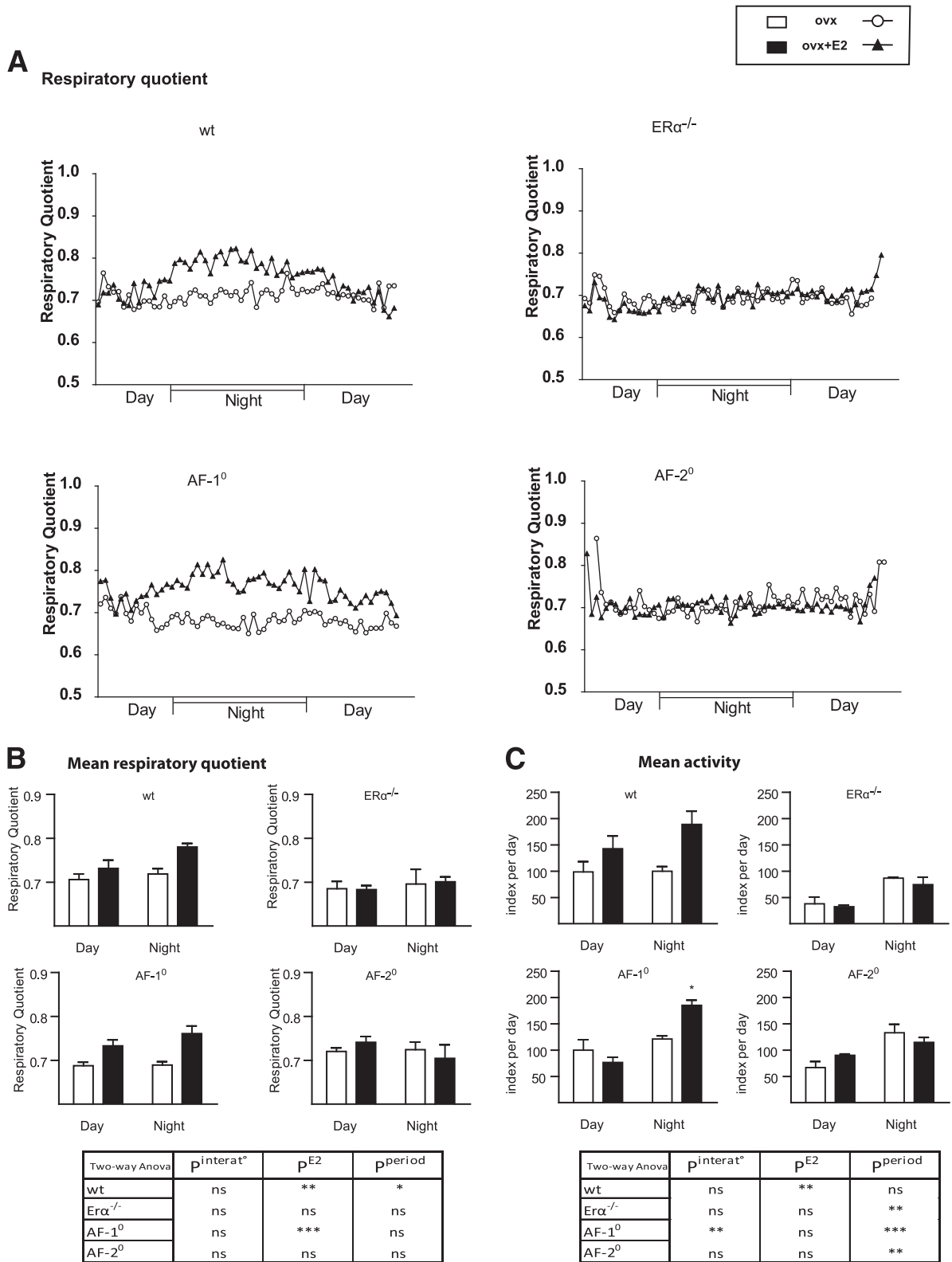


FIG. 5. Exogenous E2 enhances energy expenditure in $ER\alpha AF-1^0$, but not $ER\alpha AF-2^0$, ovariectomized female mice. Four-week-old wt, $ER\alpha^{-/-}$, $ER\alpha AF-1^0$, and $ER\alpha AF-2^0$ female mice were ovariectomized, received either 17 β -estradiol (ovx+E2) or placebo (ovx) subcutaneous administration for a 3-month period, and were concomitantly fed with HFD. **A:** Daily profile of respiratory quotient measured in metabolic chambers. **B:** Mean values of respiratory quotient in each group according to the day (light) and night (dark) periods. **C:** Total activity measured in metabolic chambers during the day (light) and night (dark) periods. Data are means \pm SEM ($n = 5-6$ per group). Two-way ANOVA statistical analyses: * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

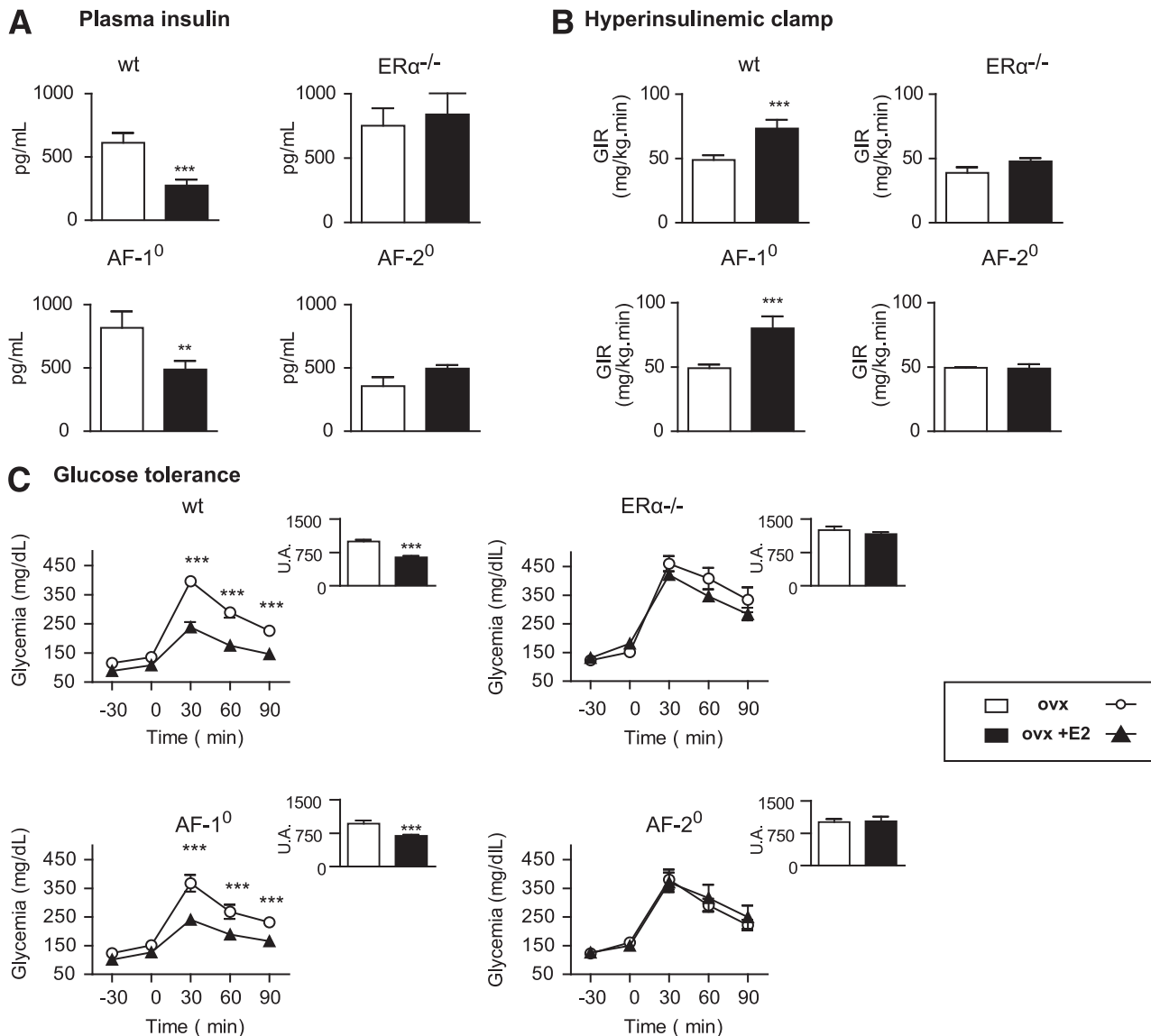


FIG. 6. Prevention of HFD-induced insulin resistance and hyperglycemia by estradiol is abolished in $ER\alpha AF-2^\circ$, but fully maintained in $ER\alpha AF-1^\circ$, ovariectomized mice. Four-week-old wt, $ER\alpha^{-/-}$, $ER\alpha AF-1^\circ$, and $ER\alpha AF-2^\circ$ female mice were ovariectomized, received either 17 β -estradiol (ovx+E2) or placebo (ovx) subcutaneous administration for a 3-month period, and were concomitantly fed with HFD. **A:** Fasting plasma insulin levels (pg/mL). **B:** Glucose infusion rate (GIR) during hyperinsulinemic-euglycemic clamp procedure. **C:** Intraperitoneal glucose tolerance test (1 g/kg). The inset represents the area under the curve for each group. U.A., arbitrary unit. Data are means \pm SEM ($n = 6-16$ animals per group). Student t test statistical analyses: ** $P < 0.01$ and *** $P < 0.001$ for E2-treated vs. placebo-treated mice.

reported an anorexic action of estrogens, in our experiments neither endogenous nor exogenous estrogens influenced food intake, irrespective of both genotype and type of diet. In contrast and in perfect agreement with previous reports, E2 administration significantly promotes physical activity and thereby enhanced energy expenditure. Interestingly, this effect was totally abolished in $ER\alpha AF-2^\circ$ mice but fully maintained in $ER\alpha AF-1^\circ$ mice, suggesting that $ER\alpha AF-1$ activation is dispensable for recruiting the central action of estrogens involved in the control of energy expenditure.

Estrogens are also recognized to exert specific influences on peripheral tissues involved in the maintenance of insulin sensitivity (22,23,29-31). Particularly, estrogens regulate energy balance in adipose tissue, acting on both lipogenesis and lipolysis processes to prevent adipocyte hypertrophy (22,32). Like $ER\alpha^{-/-}$ mice, $ER\alpha AF-2^\circ$ mice

are characterized by severe adipocyte hypertrophy, whereas either endogenous or exogenous estrogens protect $ER\alpha AF-1^\circ$ mice, in accordance with the E2-mediated downregulation of lipogenesis genes in this latter mutant strain. We previously demonstrated that E2 administration significantly enhances insulin-mediated glucose utilization by both adipose tissues and skeletal muscles in HFD-fed ovariectomized mice (12). In addition, specific deletion of hepatic $ER\alpha$ was recently shown to induce severe steatosis and insulin resistance in the liver, resulting in a significant alteration of whole-body insulin sensitivity, without any influence on body weight and adiposity (33). The total abolition of estrogen-protective effects on adiposity, whole-body insulin sensitivity, and glucose tolerance in $ER\alpha AF-2^\circ$ mice suggests that the crucial role of $ER\alpha AF-2$ also applies to these peripheral tissues, contrasting with the dispensable role of $ER\alpha AF-1$ on the same targets. Interestingly, since

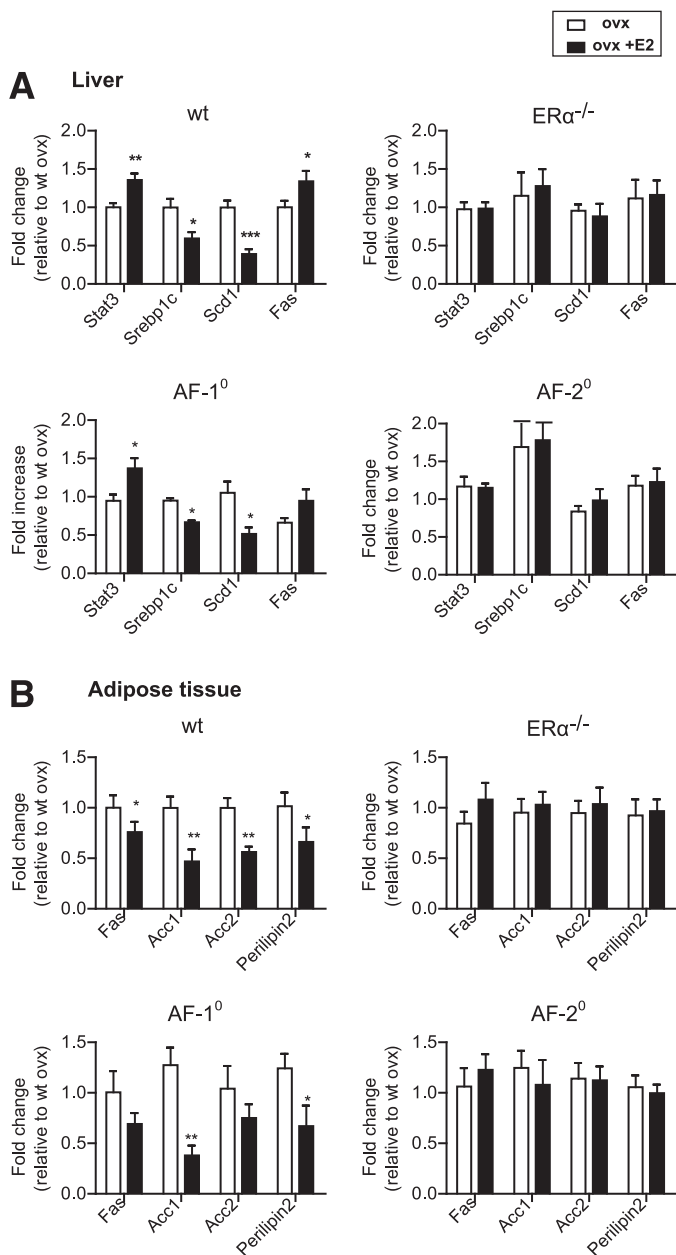


FIG. 7. Regulation of metabolic gene expression by estradiol is abrogated in peripheral tissues from $ER\alpha AF-2^{-0}$ mice. Four-week-old wt, $ER\alpha^{-/-}$, $ER\alpha AF-1^{-0}$, and $ER\alpha AF-2^{-0}$ female mice were ovariectomized, received either 17 β -estradiol (ovx+E2) or placebo (ovx) subcutaneous administration for a 3-month period, and were concomitantly fed with HFD. Quantification of mRNA levels (relative to Hprt), expressed in terms of fold change relative to wt ovx mice, from selected metabolic genes in the liver (A) and subcutaneous adipose tissue (B). Data are means \pm SEM ($n = 4-6$ animals per group). Student t test statistical analyses: * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ for E2-treated vs. placebo-treated mice.

ER α activation by estrogens has been recognized to enhance glucose-stimulated insulin biosynthesis and to promote β -cell survival (34), future studies should also investigate the role of the respective ER α AFs on the endocrine pancreas.

Our data indicate that the influence of estrogens on the expression of some crucial metabolic genes in vivo relies on ER α AF-2 activation. Indeed, we considered metabolic genes previously shown to be regulated by estrogens in adipose tissues, skeletal muscle, and the liver (22-24) and

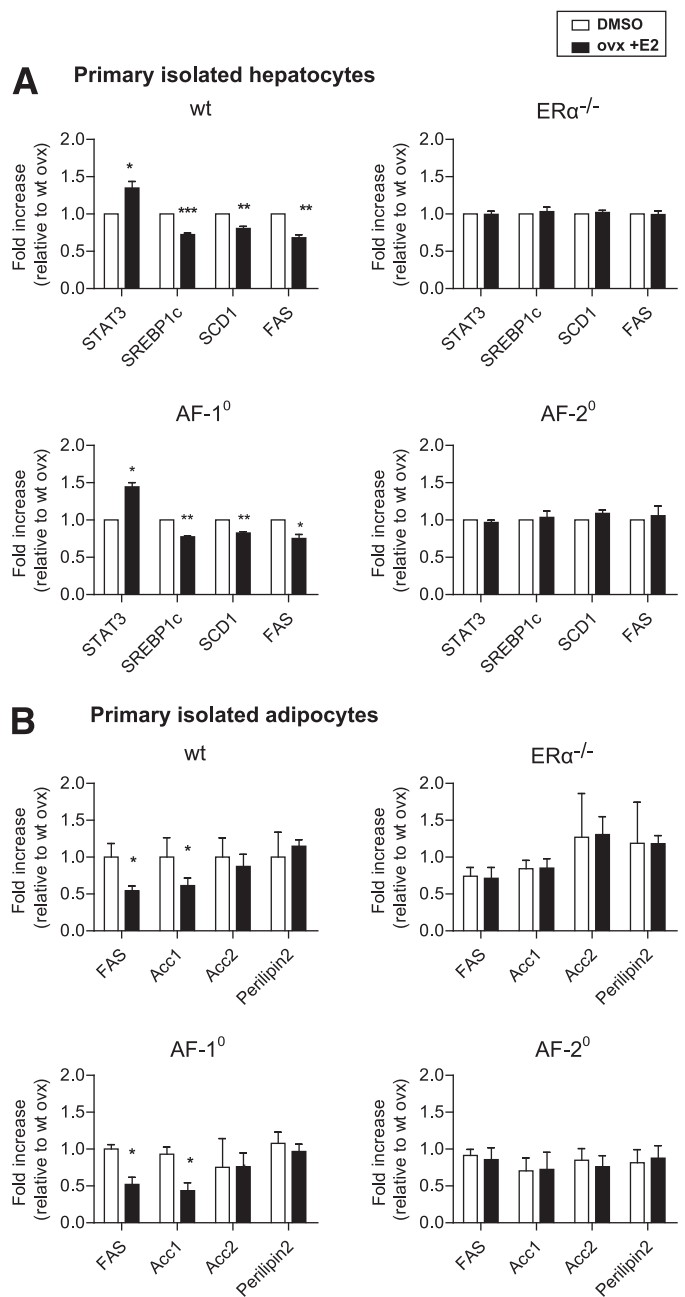


FIG. 8. Regulation of gene expression in isolated hepatocytes and mature adipocytes requires ER α AF-2, but not ER α AF-1, activation. Cells were isolated from 10-week-old wt, $ER\alpha^{-/-}$, $ER\alpha AF-1^{-0}$, and $ER\alpha AF-2^{-0}$ mice, previously ovariectomized at 4 weeks of age. These cells were cultured for 24 h with 17 β -estradiol (10^{-8} mol/L) or vehicle (DMSO). A: Relative gene expression of Stat3, Srebp-1c, Scd1, and Fas in primary isolated hepatocytes. B: Relative gene expression of Fas, Acc-1, Acc-2, and perilipin in primary isolated mature adipocytes. Data are means \pm SEM ($n = 3-4$ individual animals per group from three separate experiments). Student t test statistical analyses: * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ for cells stimulated with E2 vs. vehicle.

found that the regulation of most of them by E2 absolutely required ER α AF-2, but not ER α AF-1. Only Ppar γ 2 expression in skeletal muscle, Fas expression in the liver and adipose tissue, and Ppar γ 1 and Ppar γ 2 in brown adipose tissue were not significantly influenced by the hormone in $ER\alpha AF-1^{-0}$ mice. Importantly, in vitro gene regulation by E2 in hepatocytes and mature adipocytes freshly isolated from ovariectomized mice led to similar conclusions,

demonstrating at the cellular level the dispensable role of ER α AF-1. Experimental settings, including the fasting state of animals at sacrifice, timing of E2 administration (short-term versus chronic), and potential cellular interactions in vivo, could have contributed to the opposite regulation of Fas gene expression in hepatocytes during in vivo and in vitro experiments, and to the lack of E2 effect on *Acc2* and *Perilipin2* mRNAs in adipocyte cultures.

So far, the respective roles of each ER α AF have been explored mainly in cultured cell lines, and these in vitro studies revealed that the impact of ER α AF-1 and ER α AF-2 on ligand-induced transcriptional activity of ER α varied in a cellular type- and cell differentiation stage-dependent manner (35–38). These cell-specific variations mainly depend on cofactor expression and posttranslational modifications. Besides, several studies suggested ER α AF-1 and ER α AF-2 synergism for the recruitment of different cofactors (39–43). However, although minor contribution of ER α AF-1 cannot be excluded in the beneficial effects of E2 on body composition, insulin sensitivity, and glucose homeostasis, the full preventative effect observed in *ER α AF-1*⁰ mice clearly demonstrates that ER α AF-1 activation is dispensable for mediating these protective effects of estrogens.

Our results suggest that estrogen metabolic actions mainly result from their direct regulatory effects on gene expression, but the contribution of nongenomic actions, also called membrane-initiated steroid signaling (MISS), remains to be addressed (21). These effects are mediated by a pool of intracellular receptors localized at the plasma membrane in caveolae rafts and have been mainly described in cultured cells in vitro (44,45). In vivo, a significant increase in body weight and visceral fat was reported in a transgenic mouse model that only expresses a functional E domain of ER α at the plasma membrane (MOER), leading to the conclusion that the control of body composition by estrogens requires nuclear ER α signaling (46). Furthermore, the lack of E2 effect in our *ER α AF-2*⁰ mice probably indicates that MISS effects are not sufficient to mediate E2 metabolic protective actions. Indeed, these MISS effects appear to be, at least in part, preserved in these mutant mice since we recently showed that E2-induced reendothelialization, the unique MISS effect of estrogens described in vivo to date (47), is totally preserved in *ER α AF-2*⁰ mice (16). It is also tempting to speculate that the persistence of MISS effects in *ER α AF-2*⁰ mice could contribute to a partial negative feedback of estrogens on the hypothalamus/pituitary structures. However, the development of new appropriate mouse models is now required to definitely address the role of MISS effects in ER α -mediated metabolic protection.

We, and others, previously showed that the protective effects of estrogens on arteries (15,16) and bone (17) are essentially ER α AF1 independent and ER α AF-2 dependent. From our present results, it also appears to hold true with the beneficial actions of estrogens on energy balance, body composition, insulin resistance, and glucose homeostasis. Of major importance, this is in striking contrast with their influence on the reproductive tract, where both ER α AF-1 and ER α AF-2 are clearly involved in uterine hypertrophy (15–17). Furthermore, in a newly generated ER α AF2-mutated ER α knock-in (AF2ERKI) mouse model, Arao et al. (48) demonstrated that ER α AF-2 activation is absolutely required for endometrial proliferation. We recently confirmed this observation in *ER α AF-2*⁰ mice and found that ER α AF-1 deficiency also abolished E2-induced endometrial

proliferation (49). Interestingly, in vitro studies also demonstrated that a full ER α AF1 activity is absolutely required for the E2-dependent proliferation of cultured MCF-7 (38,50). Altogether, these data thus provide a molecular proof of concept of the ability to uncouple ER α -mediated protective actions of estrogens from their potentially deleterious effects on reproductive target organs.

In conclusion, the current study reinforces the hypothesis that a selective modulation of ER α AF-2, with minimal activation of ER α AF-1, would retain many protective effects of E2 not only on arteries and bone, but also on glucose metabolism, and would not elicit the deleterious proliferative effect on uterus and breast. The identification of the respective role of ER α subfunctions in vivo would therefore represent a crucial step toward the development of an optimized therapeutic approach to overcome the rising risk of abdominal obesity, metabolic syndrome, and type 2 diabetes in postmenopausal women.

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S.H. and E.R. researched and analyzed the data and wrote the manuscript. A.F. contributed to the experiments and discussed the data. A.W. and P.L. performed clamp studies and hormone measurements, respectively. R.B., A.K., P.C., and J.-F.A. contributed to discussion and reviewed and edited the manuscript. P.G. designed the experiments, supervised experiments and data analysis, and reviewed and edited the manuscript. S.H., E.R., and P.G. are the guarantors of this work and, as such, had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

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