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Participation of ER α and ER β in glucose homeostasis in skeletal muscle and white adipose tissue

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Barros RP, Gabbi C, Morani A, Warner M, Gustafsson JA. Participation of ER α and ER β in glucose homeostasis in skeletal muscle and white adipose tissue. *Am J Physiol Endocrinol Metab* 297: E124–E133, 2009. First published April 14, 2009; doi:10.1152/ajpendo.00189.2009.—Glucose uptake and homeostasis are regulated mainly by skeletal muscle (SM), white adipose tissue (WAT), pancreas, and the liver. Participation of estradiol in this regulation is still under intense investigation. We have demonstrated that, in SM of male mice, expression of the insulin-regulated glucose transporter (GLUT)4 is reduced by estrogen receptor (ER) β agonists. In the present study, to investigate the relative contributions of ER α and ER β in glucose homeostasis, we examined the effects of tamoxifen (Tam) on GLUT4 expression in SM and WAT in wild-type (WT) and ER $-/-$ mice. ER β $-/-$ mice were characterized by fasting hypoglycemia, increased levels of SM GLUT4, pancreatic islet hypertrophy, and a belated rise in plasma insulin in response to a glucose challenge. ER α $-/-$ mice, on the contrary, were hyperglycemic and glucose intolerant, and expression of SM GLUT4 was markedly lower than in WT mice. Tam had no effect on glucose tolerance or insulin sensitivity in WT mice. In ER α $-/-$ mice, Tam increased GLUT4 and improved insulin sensitivity. i.e., it behaved as an ER β antagonist in SM but had no effect on WAT. In ER β $-/-$ mice, Tam did not affect GLUT4 in SM but acted as an ER α antagonist in WAT, decreasing GLUT4. Thus, in the interplay between ER α and ER β , ER β -mediated repression of GLUT4 predominates in SM but ER α -mediated induction of GLUT4 predominates in WAT. This tissue-specific difference in dominance of one ER over the other is reflected in the ratio of the expression of the two receptors. ER α predominates in WAT and ER β in SM.

estrogen receptor- α ; estrogen receptor- β ; glucose transporter 4; tamoxifen

FOR SEVERAL DECADES the multiple and sometimes contradictory effects of estrogens on human physiology and disease have puzzled endocrinologists. Even today, the modulatory effects of estrogen on glucose homeostasis are not completely understood. The effects of estrogen on gene regulation are mediated by two sometimes opposing forces, estrogen receptor (ER) α and ER β (20, 28), in both females and males (19, 25). Recent experimental evidence has revealed that estradiol (E₂) is an important modulator in tissues previously not considered to be classical estrogen targets.

In mice, insulin resistance develops when there is no estrogen (in aromatase-knockout mice) and when ER α is inactivated (ER α $-/-$ mice) (5, 17, 19). In women, a clear relationship between E₂ and glycemia has been observed

during the menstrual cycle (32), in gestation (7), in gestational diabetes mellitus, and in polycystic ovarian syndrome (11). In normal males, because of the absence of estrogen cyclicity, the effect of estrogen on glycemia is less obvious, but men with hypogonadism or with mutations in the aromatase (13) or ER α (31) genes do develop insulin resistance.

Insulin is the most important hormone for the maintenance of euglycemia. It regulates carbohydrate metabolism in the liver and glucose uptake in insulin-sensitive tissues, i.e., skeletal muscle (SM) and white adipose tissue (WAT) (12). On binding to its receptors on the cell membrane of SM and WAT cells, insulin initiates a signaling cascade of phosphorylation leading to the translocation of vesicles containing glucose transporter (GLUT)4 to the plasma membrane. Once GLUT4 is anchored to the membrane, glucose can enter the cell by facilitated diffusion (33). GLUT4 is expressed mainly in SM and WAT, and its inappropriate expression, translocation, or anchorage to the membrane can lead to insulin resistance and consequently impaired glucose homeostasis (16).

In an effort to investigate the role of E₂ on glucose homeostasis, we previously studied the expression of the two ERs in muscle. We found that ER α and ER β are coexpressed in SM, where they have opposing effects on modulation of GLUT4 expression. In SM, ER α is a positive regulator of GLUT4 expression while ER β has a suppressive role (4), and the net effect of estrogen depends on the balance between the two receptor isoforms (3). It is therefore pertinent to ask whether the use of ER agonists influences glucose homeostasis.

Tamoxifen (Tam) is a nonsteroidal selective ER modulator (SERM) that binds to both ERs with high affinity, causing agonist or antagonist actions in a tissue-specific way (18). Because of its ability to specifically inhibit the proliferative actions of E₂, it is the antiestrogen most widely used in breast cancer therapy and it is used as a research tool to evaluate specific actions of ERs. We have selected Tam because it is of paramount importance to rule out potential side effects of such a widely used drug on glucose homeostasis. The use of more specific SERMs could be more helpful to identify the role of each ER; however, the question of whether Tam could interfere with glycemia would remain open.

In the present study, we have treated wild-type (WT), ER α $-/-$, and ER β $-/-$ mice with Tam for 21 days and evaluated whether Tam affects 1) the expression of GLUT4 in WAT and SM and 2) glucose homeostasis, as measured by tolerance tests for insulin (ITT) and glucose (GTT).

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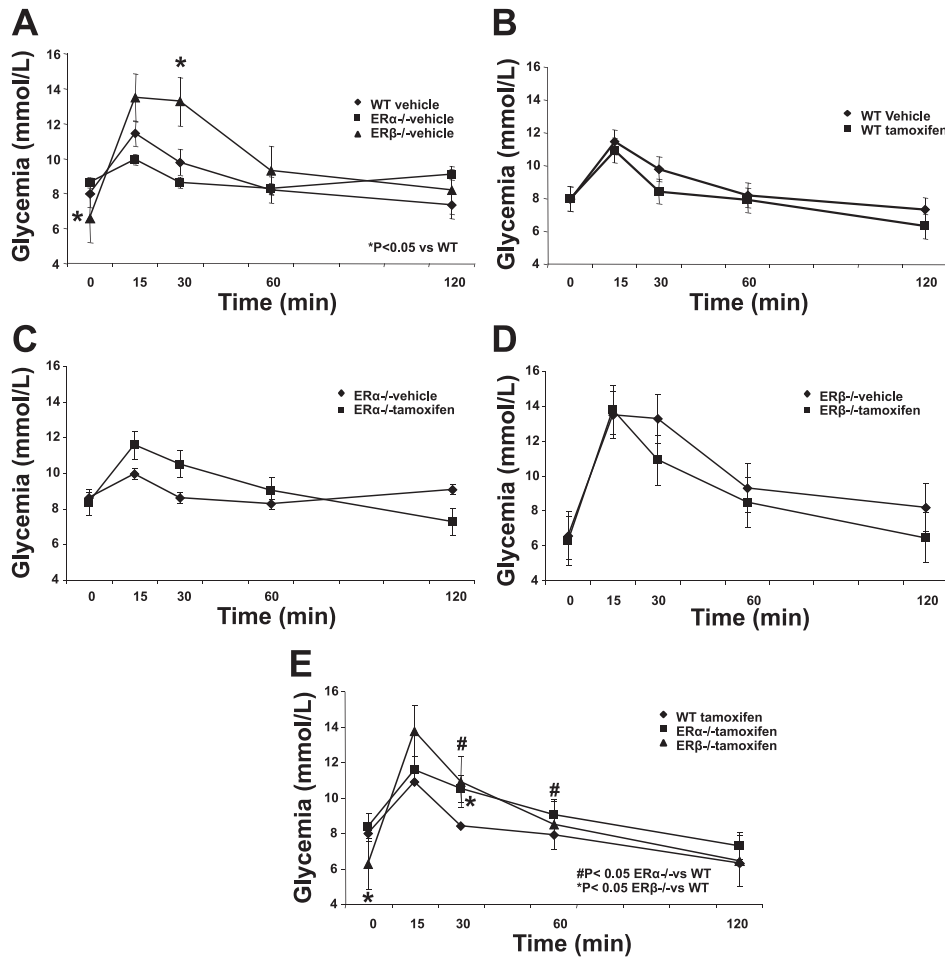


Fig. 1. Comparison of glucose tolerance test (GTT) in wild-type (WT), estrogen receptor (ER) α -/-, and ER β -/- mice treated with vehicle and tamoxifen (Tam). A: WT, ER α -/-, and ER β -/- mice treated with vehicle. B: WT mice treated with vehicle (same as in A) and Tam. C: ER α -/- mice treated with vehicle (same as in A) and Tam. D: ER β -/- mice treated with vehicle (same as in A) and Tam. E: WT, ER α -/-, and ER β -/- mice treated with Tam. Each data point represents mean value of measurements from 5 or 6 mice. Values are means \pm SE (*P* values by Student's *t*-test).

MATERIALS AND METHODS

Animals. ER β -/- mice were from our colony (21), and ER α -/- mice were purchased from Taconic Europe. Mice were housed at the Huddinge University Hospital Animal Facility in a controlled environment on a 12:12-h light-dark illumination schedule and fed a standard pellet diet with water provided ad libitum. Mice were asphyxiated by CO₂ after overnight fasting. Gastrocnemius muscle, periepididymal WAT, and pancreas were excised and frozen immediately for Western blotting or fixed in 4% paraformaldehyde overnight and routinely embedded in paraffin wax for immunohistochemistry or immunofluorescence. All experiments were approved by the local Animal Experimentation Ethics Committee (Stockholm's Södra Djurförsöksetiska Nämnd; protocol no. S-127-08).

Chemicals and antibodies. We purchased Tam (Sigma E-361) and vehicle pellets from Innovative Research of America. Insulin was from Novo Nordisk (Actrapid Penfill). The following antibodies were used: rabbit polyclonal anti-ER α (MC-20) from Santa Cruz Biotechnology; goat anti-rabbit antibody from Zymed Laboratories; FITC anti-chicken and Cy3 anti-rabbit from Jackson ImmunoResearch; and rabbit affinity-purified anti-GLUT4 from FabGennix. The chicken polyclonal anti-ER β 503 was produced in our laboratory (30). 4',6-Diamidino-2-phenylindole (DAPI) was from Sigma-Aldrich.

Treatment of mice with tamoxifen. To observe the effect of Tam on GLUT4 expression and glucose homeostasis, 8-mo-old male mice were treated with subcutaneous pellets (1.5 mg/21-day release; 0.07 mg/day) for 21 days. Thirty-two mice were divided into three groups as follows: *group 1*, 10 WT mice, 5 treated with vehicle and 5 with Tam; *group 2*, 10 ER α -/- mice, 5 treated with vehicle and 5 with

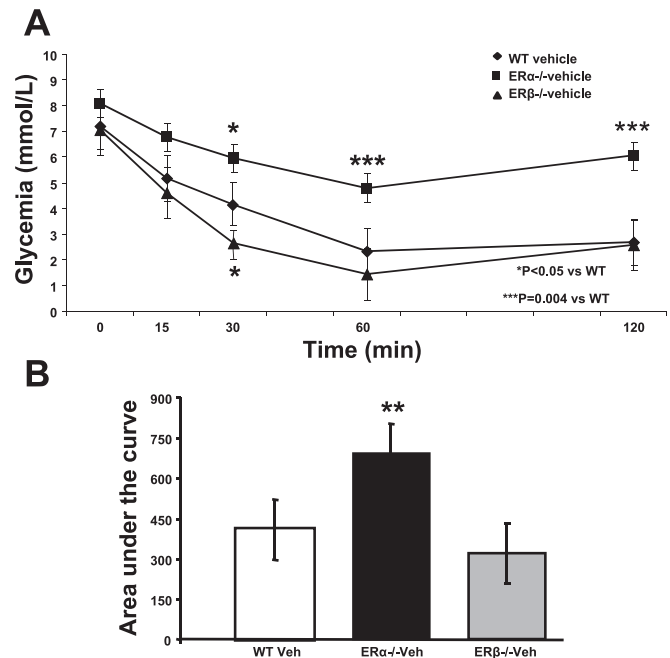
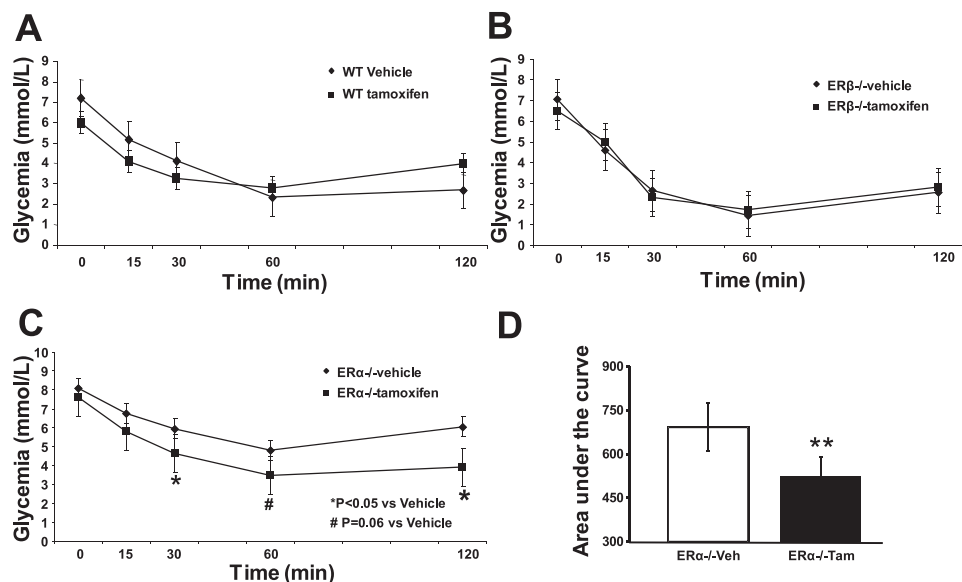


Fig. 2. Comparison of insulin tolerance test (ITT) in WT, ER α -/-, and ER β -/- mice treated with vehicle only. A: WT, ER α -/-, and ER β -/- mice treated with vehicle. B: measurement of the area under the curve of ITT in WT, ER α -/-, and ER β -/- mice treated with vehicle. Each data point represents mean value of measurements from 5 or 6 mice. Values are means \pm SE. ***P* = 0.01 vs. WT vehicle (Student's *t*-test).

Fig. 3. Comparison of ITT in WT (A), ER β ^{-/-} (B), and ER α ^{-/-} (C) mice treated with vehicle and Tam. D: measurement of the area under the curve of ITT in ER α ^{-/-} mice treated with vehicle and Tam. Each data point represents mean value of measurements from 5 to 6 mice. Values are presented as means \pm SE. ** $P = 0.01$ vs. ER α ^{-/-} treated with vehicle (Student's t -test).



Tam; and group 3, 12 ER β ^{-/-} mice, 6 treated with vehicle and 6 with Tam. GTT was performed after 14 days and ITT after 21 days of Tam treatment.

GTT and ITT. After an overnight fast, mice were administered a bolus of 1.0 g/kg glucose or 1 U/kg insulin intraperitoneally. Blood samples were obtained via a tail nick at 0, 15, 30, 60, and 120 min after glucose or insulin administration, and plasma glucose was immediately determined with the Accu-Chek Aviva Blood Glucose Meter System (Roche Diagnostics). GTT and ITT were performed 1 wk apart in all mice.

Immunohistochemical staining. Representative blocks of paraffin-embedded tissues were cut at 4- μ m thickness, dewaxed, and rehydrated. For ER staining, antigens were retrieved by boiling in 10 mM citrate buffer (pH 7.0) for 20 min. The sections were incubated in 0.5% Triton X-100 in PBS for 30 min. To block nonspecific binding, sections were incubated in 3% BSA for 1 h at 4°C. Sections were incubated with anti-ER α , anti-ER β , or anti-GLUT4 antibodies at a dilution of 1:200, in 1% BSA and 0.1% P40 in PBS overnight at 4°C. For colocalization evaluation, sections were incubated with both anti-ER α and anti-ER β antibodies. BSA replaced primary antibodies in negative controls. After washing, sections were incubated with the corresponding secondary antibodies (1:200 dilution) for 1 h at room temperature. The sections were lightly counterstained with hematoxylin or DAPI, dehydrated through an ethanol series to xylene, and mounted. For immunofluorescence, slides were directly mounted in Vectashield antifading medium (Vector Laboratories). The sections were examined under a Zeiss fluorescence microscope with filters suitable for selectively detecting the fluorescence of FITC (green) and Cy3 (red) or a light microscope. For colocalization, images from the same section but showing different antigen signals were overlaid. Cells in which both FITC- and Cy3-conjugated secondary antibodies are present have an orange or yellow color.

Western blotting. Total membrane fractions were analyzed by Western blotting. Briefly, frozen tissues were homogenized for 1 min each with a Polytron in 25 ml of PBS buffer containing protease inhibitor mixture according to manufacturer's instructions (Roche Diagnostics). Homogenates of WAT were centrifuged at 1,000 g for 15 min. The fat cake was discarded, and the supernatant, a fat-free extract, was recovered as a total cellular membrane fraction. SM was homogenized in the same buffer. Fat-free extracts from WAT and SM homogenate were centrifuged for 10 min at 800 g (rotor JA-17; Beckman). The pellet contained nuclear protein, and the supernatant contained mitochondrial, cytosolic, and membrane proteins. Mito-

chondrial proteins were precipitated and removed by centrifugation of the supernatant at 8,000 g for 20 min (rotor JA-17). Membrane proteins were precipitated by ultracentrifugation at 146,000 g for 60 min (rotor Ti50; Beckman) and resuspended in PBS. The protein content was measured by Bio-Rad protein assay with BSA as standard. Thirty micrograms of protein were dissolved in SDS sample buffer and resolved on 4–20% gradient SDS-polyacrylamide gels (Invitrogen) in Tris-glycine buffer. Proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Amersham Biosciences) by electroblotting in Tris-glycine buffer. Molecular mass markers

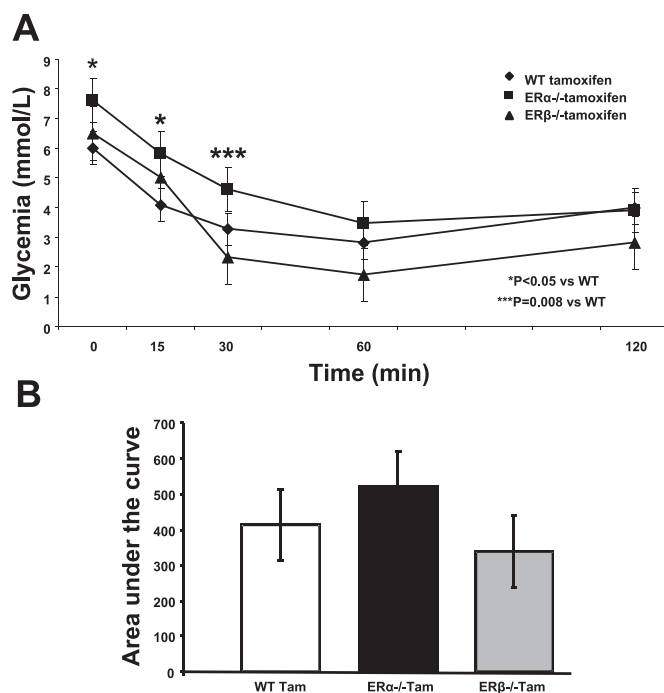


Fig. 4. Comparison of ITT in WT, ER α ^{-/-}, and ER β ^{-/-} mice treated with Tam only. A: WT, ER α ^{-/-}, and ER β ^{-/-} mice treated with Tam. B: measurement of the area under the curve of ITT in WT, ER α ^{-/-}, and ER β ^{-/-} mice treated with Tam. Each data point represents mean value of measurements from 5 or 6 mice. Values are presented as means \pm SE (P values by Student's t -test).

were Precision Plus protein standards (Bio-Rad). Colon tissue was used as a negative control. The PVDF membranes were incubated in blocking solution containing 10% fat-free milk and 0.1% Nonidet P-40 in PBS for 2 h at room temperature. This was followed by incubation with rabbit anti-GLUT4 (FabGennex; 1:1,000) in blocking solution overnight at 4°C. After washing, membrane was incubated with secondary peroxidase-conjugated goat anti-rabbit antibody (Sigma; 1:5,000) in blocking solution for 1 h at room temperature. After washing, detection with an enhanced chemiluminescence (ECL) kit (Amersham) was performed. For evaluation of expression of ER α and ER β in SM, nuclear extracts were prepared as follows: muscle homogenates were centrifuged at 1,000 g for 10 min to obtain a nuclear crude nuclear pellet. Nuclear pellets were resuspended in 30% sucrose-PBS and centrifuged at 8,000 g for 20 min. Resulting pellets were resuspended in 0.5 M NaCl PBS and centrifuged at 8,000 g for 10 min. Twenty milliliters of ethanol was added to the supernatant, and the samples were kept in dry ice for 1 h. After a centrifugation of 7,000 g for 10 min, pellets were resuspended in PBS and analyzed by

Western blotting as described above, with specific antibodies for ER α and ER β . ER β commercial standard (purchased from Panvera) and MCF7 cells were used as positive controls for ER β and ER α , respectively.

First-phase insulin secretion in WT and ER β ^{-/-} mice. After an overnight fast, mice were divided into four groups: 0, 2, 5, and 10 min. At *time 0*, mice were anesthetized with Avertin (240 mg/kg) and blood was collected from the periorbital sinus to evaluate fasting insulin. To evaluate glucose-stimulated insulin secretion, the other three groups were anesthetized and administered a bolus of 1.0 g/kg glucose intraperitoneally, and blood was collected as mentioned above after 2, 5, and 10 min. Plasma insulin was measured by radioimmunoassay (Mercodia Ultrasensitive Mouse Insulin ELISA; Mercodia, Uppsala, Sweden).

Evaluation of islet area and percent distribution. One block of paraffin-embedded pancreas from four different WT mice and two blocks from eight different ER β ^{-/-} mice were cut at 4- μ m thickness, dewaxed, rehydrated, and stained with hematoxylin and eosin. Two sections from each block, at least 25 μ m apart, were evaluated

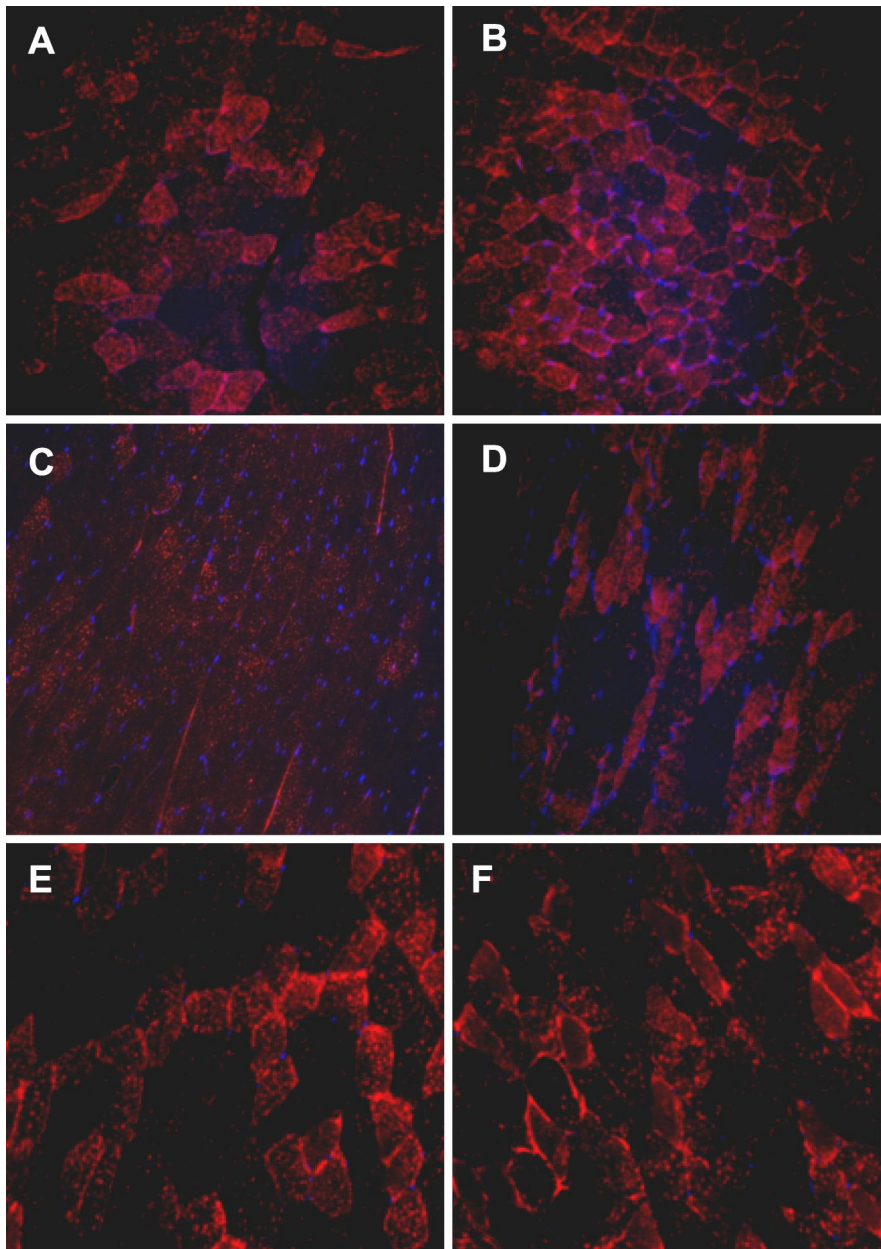


Fig. 5. Detection of GLUT4 in SM of WT, ER β ^{-/-}, and ER α ^{-/-} mice by immunofluorescence. GLUT4 was visualized with Cy3 (red). There was clear staining of the cell membrane and cytosolic vesicles of WT (A) and ER β ^{-/-} (E) mice treated with vehicle. In ER α ^{-/-} mice treated with vehicle (C), the presence of GLUT4 was substantially reduced in the plasma membrane and vesicles. Treatment with Tam increased GLUT4 expression in the plasma membrane and vesicles of WT (B) and ER α ^{-/-} (D) mice. No changes were observed in ER β ^{-/-} mice treated with Tam (F). Nuclei were stained with DAPI (blue).

under light microscopy. All visible islets were photographed with a Carl Zeiss Axioplan2 microscope, and the areas were measured with the Carl Zeiss AxioVision Rel. 4.6 program. The area average was then statistically evaluated. For the evaluation of islet percent distribution by size, islets were classified into three groups: small (smaller than $5,000 \mu\text{m}^2$), medium (between $5,000$ and $10,000 \mu\text{m}^2$), and large (larger than $10,000 \mu\text{m}^2$). The distribution of islets according to these groups was determined in each mouse separately, and results were used for statistical evaluation.

Data analysis. All values are reported as means \pm SE. The contents of GLUT4 protein were quantified in at least three different experiments using different mice from all animal groups. Each group was compared with the corresponding WT or vehicle-treated mice. For comparison of the mean values, we used Student's *t*-test, SPSS version 13.0 (SPSS, Chicago, IL).

RESULTS

GTT. At *time 0*, after an overnight fast, vehicle-treated $\text{ER}\beta^{-/-}$ mice had lower glucose levels than WT mice (Fig. 1A). Thirty minutes after a bolus of 1.0 g/kg glucose was given intraperitoneally to vehicle-treated WT, $\text{ER}\alpha^{-/-}$, and $\text{ER}\beta^{-/-}$ mice, the glucose peak levels in $\text{ER}\beta^{-/-}$ mice were higher than in the other two groups, but the clearance was similar to that of WT mice (Fig. 1A), and there was no difference in the area under the plasma concentration/time curve (AUC). Treatment with Tam did not cause significant changes in the GTT in mice of any genotype (Fig. 1, B–D): After Tam treatment, $\text{ER}\beta^{-/-}$ mice still maintained lower fasting glucose levels and $\text{ER}\alpha^{-/-}$ mice remained glucose intolerant (vs. WT mice) (Fig. 1E).

ITT. Comparison of vehicle-treated WT, $\text{ER}\alpha^{-/-}$, and $\text{ER}\beta^{-/-}$ mice showed that after 1 U/kg insulin intraperitoneally, $\text{ER}\alpha^{-/-}$ mice were more resistant to insulin at 30, 60, and 120 min (Fig. 2A) than WT mice and had an increased AUC (Fig. 2B). $\text{ER}\beta^{-/-}$ mice were more sensitive to insulin at 30 min than WT mice (Fig. 2A). Treatment with Tam did not change

insulin sensitivity in WT (Fig. 3A) or $\text{ER}\beta^{-/-}$ (Fig. 3B) mice but increased insulin sensitivity at 30 and 120 min in $\text{ER}\alpha^{-/-}$ mice and reduced the AUC (Fig. 3, C and D). Despite the improvement elicited by Tam, $\text{ER}\alpha^{-/-}$ mice still had higher glucose levels at *time 0* and remained more insulin resistant than WT mice at 15 and 30 min (Fig. 4A). However, as judged by the AUC, there was a clear improvement in glucose disposition after Tam treatment, with values in $\text{ER}\alpha^{-/-}$ mice similar to those in WT mice (Fig. 4B).

Expression and distribution of GLUT4 by immunofluorescence. SM from 8-mo-old WT, $\text{ER}\alpha^{-/-}$, and $\text{ER}\beta^{-/-}$ male mice, treated with vehicle or Tam, was evaluated for expression and distribution of GLUT4 by immunofluorescence. In vehicle-treated WT mice, GLUT4 was present on the cell membrane and on vesicles localized in the cytoplasm (Fig. 5A). Treatment with Tam increased the expression of GLUT4 both on the cell membrane and in cytoplasmic vesicles (Fig. 5B). In $\text{ER}\alpha^{-/-}$ mice, the presence of GLUT4 on the cell membrane and cytoplasmic vesicles was markedly lower than in WT mice (Fig. 5C) and treatment with Tam increased GLUT4 expression mainly in cytoplasmic vesicles (Fig. 5D). These results observed in WT and $\text{ER}\alpha^{-/-}$ mice indicate that Tam acts as an $\text{ER}\beta$ antagonist in SM. In $\text{ER}\beta^{-/-}$ mice, there was a robust expression of GLUT4 in the plasma membrane and cytoplasmic vesicles (Fig. 5E). Treatment with Tam (which should block $\text{ER}\alpha$) had no detectable effect on GLUT4 expression (Fig. 5F). Thus, as discussed below, $\text{ER}\alpha$ seems to have minor effects on GLUT4 expression in SM.

Expression of GLUT4 in SM and WAT by Western blotting. On Western blots the specific GLUT4 antibody recognized two bands of molecular mass 48 kDa and 47 kDa in extracts from SM and WAT. This doublet pattern is commonly observed in many studies (14, 26) and is thought to reflect glycosylation states of the transporter. In SM of 8-mo-old WT mice, Tam

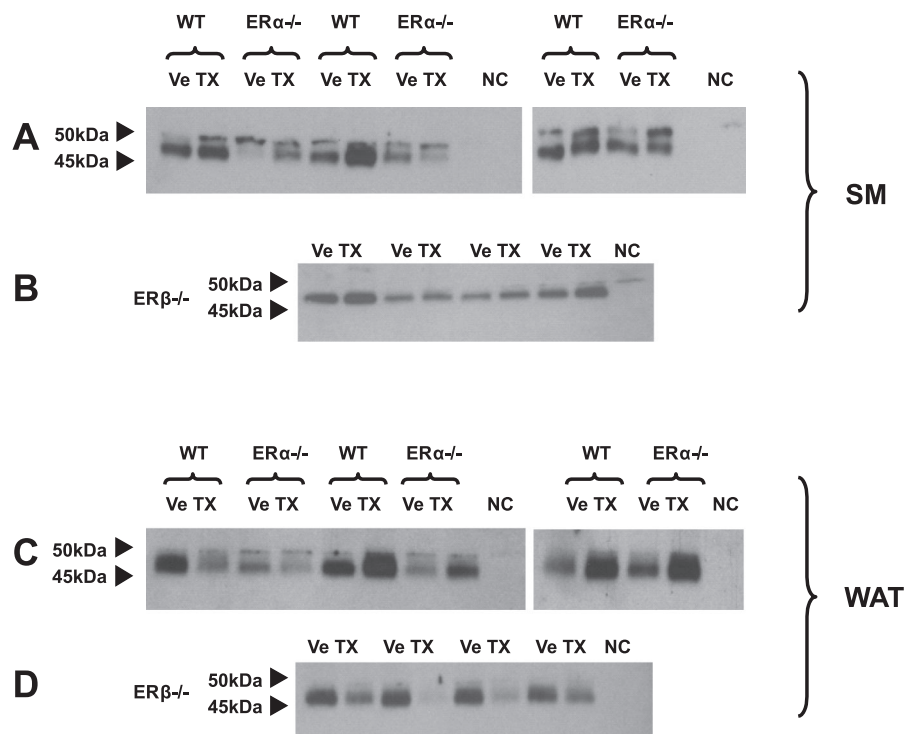


Fig. 6. Detection of GLUT4 expression in SM and WAT by Western blotting. In SM of WT and $\text{ER}\alpha^{-/-}$ mice, treatment with Tam increased GLUT4 expression (A) and had little effect in $\text{ER}\beta^{-/-}$ mice (B). In WAT of WT and $\text{ER}\alpha^{-/-}$ mice, Tam treatment did not elicit a consistent response on GLUT4 expression (C). However, in $\text{ER}\beta^{-/-}$ mice, Tam reduced GLUT4 expression (D). Each blot of GLUT4 represents tissue extracted from 1 mouse. Both bands were considered in the evaluation. Ve, vehicle; TX, tamoxifen; NC, negative control.

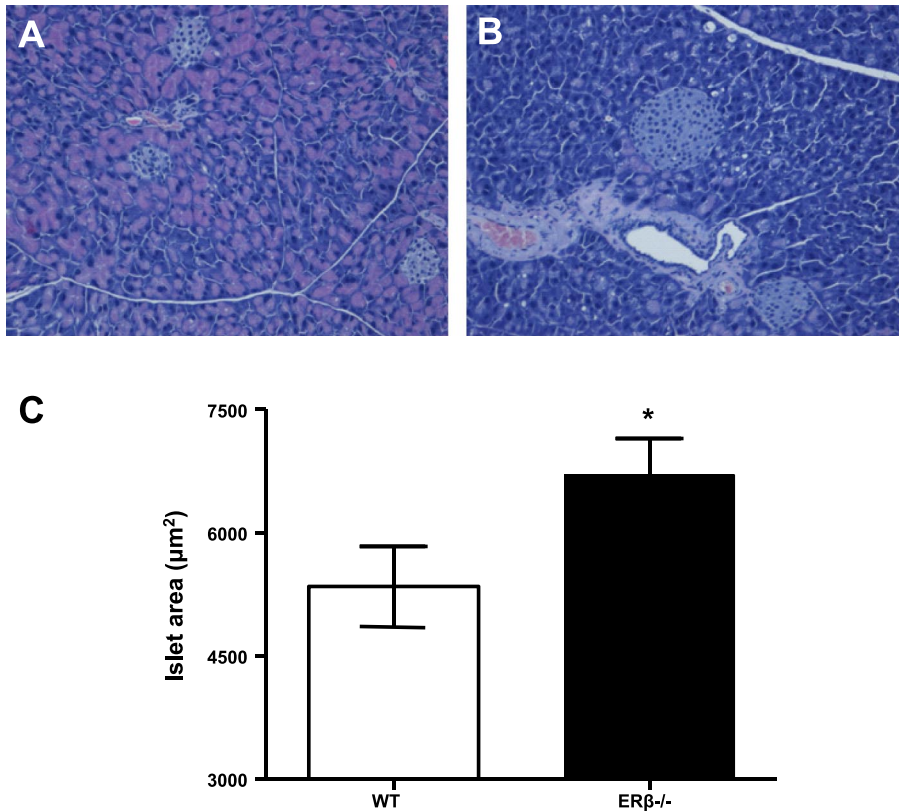


Fig. 7. Analysis of the size of pancreatic islets of WT and ER β ^{-/-} mice. Pancreata of 5 WT and 8 ER β ^{-/-} mice were sectioned and stained with hematoxylin and eosin, and islet areas were evaluated. A: pancreas of WT mice showing small size islets (<math><5,000 \mu\text{m}^2</math>). B: pancreas of ER β ^{-/-} mice showing medium-size islets (5,000–10,000 μm^2). C: comparison of the average islet area showed islet hypertrophy in ER β ^{-/-} mice. * $P < 0.05$ vs. WT (Student's t -test).

treatment increased the expression of both upper and lower bands of GLUT4 (Fig. 6A). In ER α ^{-/-} mice Tam increased predominantly the expression of the upper band in SM (Fig. 6A), while in ER β ^{-/-} mice only the lower band of GLUT4 was detected in SM (Fig. 6B) and treatment with Tam had only a small effect (Fig. 6B). Thus as with immunofluorescence, Western blot data indicate a repressive role of ER β on GLUT4 expression.

In WAT of WT and ER α ^{-/-} mice Tam treatment did not elicit a consistent response on GLUT4 expression (Fig. 6C), but in ER β ^{-/-} mice (Fig. 6D) it caused a substantial reduction in the lower GLUT4 band (Fig. 6D). As in the SM, no upper GLUT4 band was detected in ER β ^{-/-} WAT. This result reveals the importance of ER α in WAT and shows that antagonism of ER α by Tam reduces total GLUT4 expression in this tissue.

Islet area in WT and ER β ^{-/-} mice. The presence of decreased glycemia in some ER β ^{-/-} mice after overnight fasting was associated with symptoms (tremulousness, dis-

turbed gait and balance, and spinning movements when mice were lifted by the tail). Plasma glucose levels in ER β ^{-/-} mice after overnight fast ranged between 5.5 and 8.0 mmol/l, while in fasted WT mice glucose levels were maintained between 7.0 and 9.4 mmol/l. These observations led us to evaluate the size and percent distribution of pancreatic islets. Hematoxylin and eosin-stained pancreas sections from 8-mo-old WT and ER β ^{-/-} mice (Fig. 7, A and B) revealed that the average islet area in ER β ^{-/-} mice was larger than that in WT mice (Fig. 7C). In ER β ^{-/-} mice, the percentage of small islets (<math><5,000 \mu\text{m}^2</math>) was decreased while the percentage of larger islets (between 5,000 and 10,000 μm^2) was increased (Table 1).

Table 1. Percent distribution of pancreatic islets in WT and ER β ^{-/-} mice

	<math><5,000 \mu\text{m}^2</math>	5,000–10,000 μm^2	>10,000 μm^2
WT	72% \pm 6 (4)	10% \pm 2 (4)	18% \pm 3 (4)
ER β ^{-/-}	53% \pm 4 (8)*	22% \pm 3 (7)*	25% \pm 6 (8)

Data are means \pm SE for number of mice in parentheses. Islets were arbitrarily classified as small (<math><5,000 \mu\text{m}^2</math>), medium (5,000–10,000 μm^2), or large (>10,000 μm^2), and the percent distribution was evaluated in each pancreas. WT, wild type; ER β , estrogen receptor- β . * $P < 0.05$ vs. WT (Student's t -test).

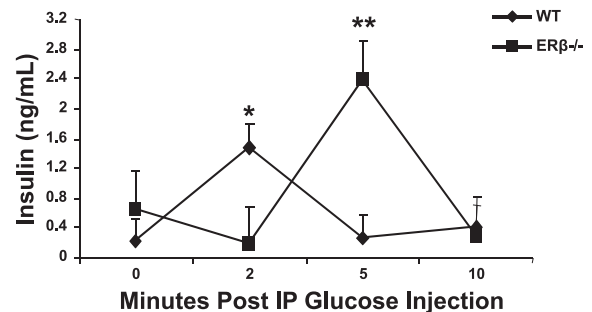


Fig. 8. Comparison of acute phase glucose-stimulated insulin secretion in WT and ER β ^{-/-} mice. Eight-month-old male mice were fasted overnight. Glucose (1 g/kg) was injected intraperitoneally (IP), and blood was collected from the periorbital sinus at 0, 2, 5, and 10 min. In WT mice, insulin peaked at 2 min and returned to values similar to baseline at 5 min. In ER β ^{-/-} mice there was a delayed response, and insulin peaked at 5 min. Values returned to basal levels at 10 min. Each data point represents mean value of measurements from 5 mice. Values are presented as means \pm SE. * $P < 0.05$, ** $P = 0.003$ vs. WT (Student's t -test).

Fig. 9. Colocalization of ER α and ER β in adipocyte cell nuclei of WT mice by immunofluorescence. WAT was stained for ER α with Cy3 (red; A) and ER β with FITC (green; B). Overlap of A and B shows the colocalization of both ERs (yellow), indicated by arrows (C). Nuclei were stained with DAPI (blue; D).

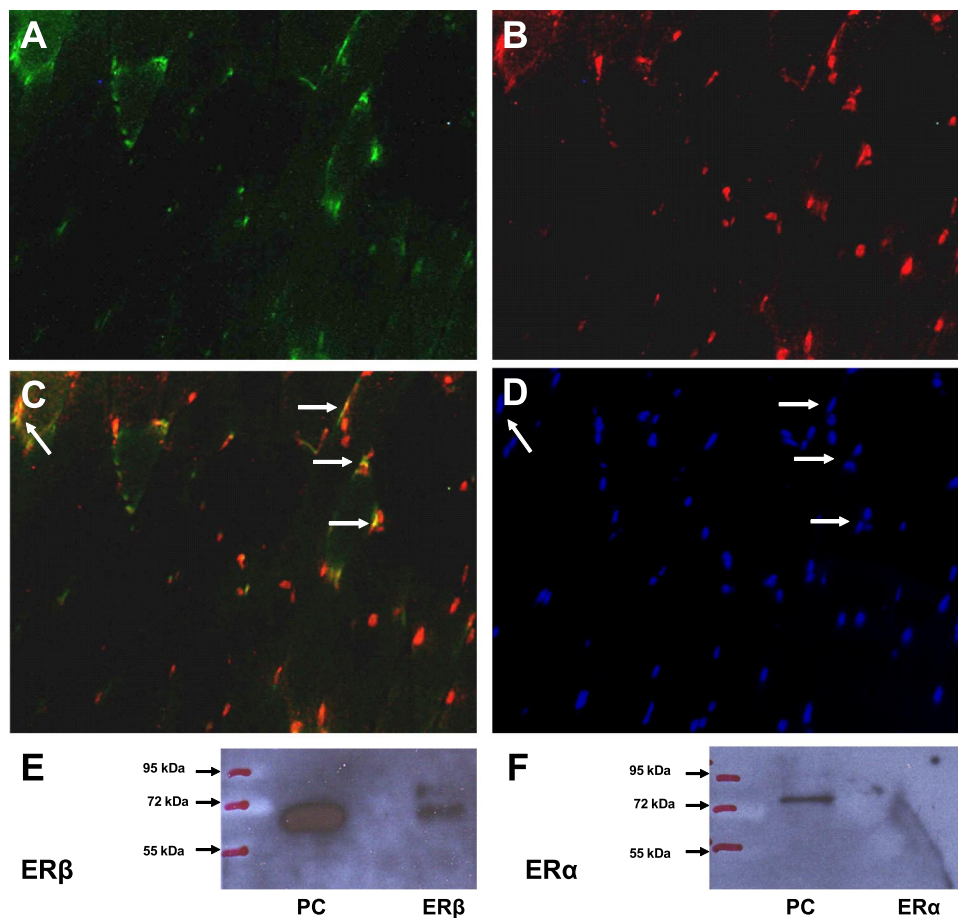
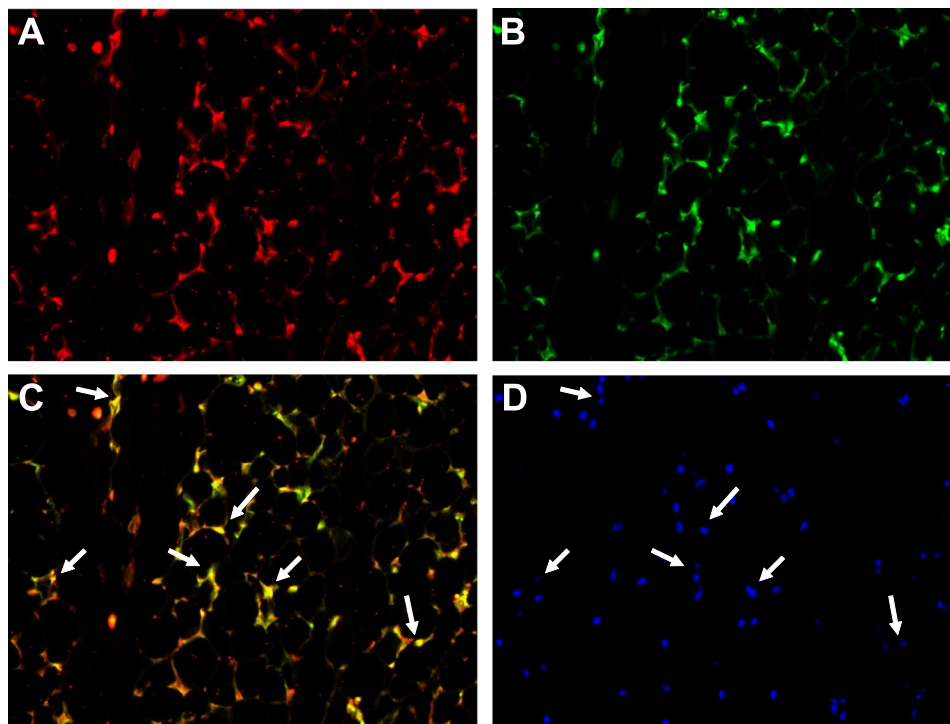


Fig. 10. Colocalization of ER α and ER β in SM nuclei of WT mice by immunofluorescence and expression of ER α and ER β in SM by Western blot. SM was stained for ER α with FITC (green; A) and ER β with Cy3 (red; B). Overlap of A and B shows the colocalization of both ERs (yellow), indicated by arrows, and the predominance of ER β (red) in the SM (C). Nuclei were stained with DAPI (blue; D). Presence of ER β was confirmed by Western blotting showing a band at the expected molecular mass of 63 kDa (E). The presence of ER α could not be detected by Western blotting (F). ER β 503 prepared in this lab and MCF7 cells were used as positive controls (PC) for ER β and ER α , respectively.

Table 2. Consequences of predominance of ER α in WAT and ER β in SM

	SM	WAT	Plasma Glucose	BW
ER dominance	ER β > ER α	ER α > ER β		
Loss of ER α	GLUT4 ↓	GLUT4 ↓	↓ Glucose uptake	↑ (NS)
Loss of ER β	GLUT4 ↑		↓ Fasting glycemia; delayed insulin release	↓ (NS)
Tam in WT mice	GLUT4 ↑			
Tam in ER α ^{-/-} mice	GLUT4 ↑		↑ Glucose uptake	
Tam in ER β ^{-/-} mice		GLUT4 ↓		

In skeletal muscle (SM), ER β is predominant, and the loss of ER β caused an increase in glucose transporter (GLUT)4 expression and reduced glucose uptake. Block of ER β by tamoxifen (Tam) in ER α ^{-/-} mice increased GLUT4 expression in SM and improved glucose uptake. In white adipose tissue (WAT), ER α is predominant, the loss of ER α decreased GLUT4 expression, and diminished glucose uptake was observed. Block of ER α by Tam in ER β ^{-/-} mice decreased GLUT4 expression in WAT, with no consequences to overall glycemia. Body weight (BW) was increased in ER α ^{-/-} and decreased in ER β ^{-/-} mice [nonsignificant (NS) ($P = 0.06$)]. Treatment with Tam did not interfere with body weight of any of the groups.

Glucose-stimulated insulin secretion in WT and ER β ^{-/-} mice. To confirm the difference between WT and ER β ^{-/-} mice in the first 30 min of the GTT (Fig. 1, A and E), we evaluated the first phase of insulin secretion after an intraperitoneal bolus of glucose (1.0 g/kg) (Fig. 8). In WT mice, insulin secretion peaked 2 min after stimulus and returned to values similar to fasting values after 5 min. In ER β ^{-/-} mice, the time to peak insulin secretion was delayed and occurred only after 5 min.

Colocalization of ER α and ER β in WAT and SM by immunofluorescence. WAT and SM from 8-mo-old WT mice were examined by immunofluorescence for the colocalization of ER α and ER β in nuclei. Double-staining with anti-ER α and anti-ER β antibodies revealed the presence of ER α and ER β in nuclei and colocalization of these receptors in the same nuclei of some adipose cells. Additionally, there were nuclei that expressed only ER α (Fig. 9). In SM, as described previously (4), colocalization was seen in some nuclei and there were, in addition, nuclei that expressed only ER β (Fig. 10). These results confirm the predominance of ER α in WAT and ER β in SM.

Expression of ER α and ER β in SM by Western blotting. Nuclear extracts of SM from 8-mo-old WT mice were examined by Western blotting for the expression of ER α and ER β . ER β was easily detected when 100 μ g of nuclear protein was loaded on the lane (Fig. 10E), but ER α could not be detected under these conditions (Fig. 10F). The positive controls for the respective antibodies were ER β purchased from Panvera and MCF7 cell extracts.

DISCUSSION

In a previous study, we showed that in SM of male mice ER α is required for appropriate GLUT4 expression and that ER β has a suppressive role (4). These results led us to investigate the physiological importance of ERs in glucose homeostasis. In the present study, with the use of the nonsteroidal SERM Tam, we confirmed our previous finding of a diabetogenic effect of ER β and demonstrated a novel distribution of labor between the two receptors, with ER α dominating in WAT and ER β in SM. The results of the present study are summarized in Table 2.

We found that in SM of WT mice there was colocalization of both ERs in some muscle cell nuclei, but there was a clear predominance of ER β expression and many nuclei expressed ER β only. Treatment of ER α ^{-/-} mice with Tam increased GLUT4 expression in SM. Thus the blocking of ER β had a

positive effect on GLUT4 expression. In contrast, Tam had very small effects on GLUT4 expression in SM in ER β ^{-/-} mice.

In WAT of WT mice, changes in GLUT4 expression in response to Tam treatment were erratic. Although some studies have shown the expression of ER α and ER β in human adipose tissue with predominance of ER α (9), no colocalization studies have been done. In the present study we found that both ERs are expressed in WAT but that ER α is the predominant ER. There was colocalization of the receptors in many nuclei, but, in addition, there were nuclei that expressed only ER α . Recent studies have demonstrated a role for ER α in the physiology of adipose tissue and development of obesity (27). Regulation of GLUT4 expression by estrogen in WAT has not been described, but, on the basis of our present results, regulation of GLUT4 by ERs in WAT is different from that in SM.

Glucose homeostasis is mainly dependent on the combined effects of glucose uptake by SM and WAT (16). The opposite

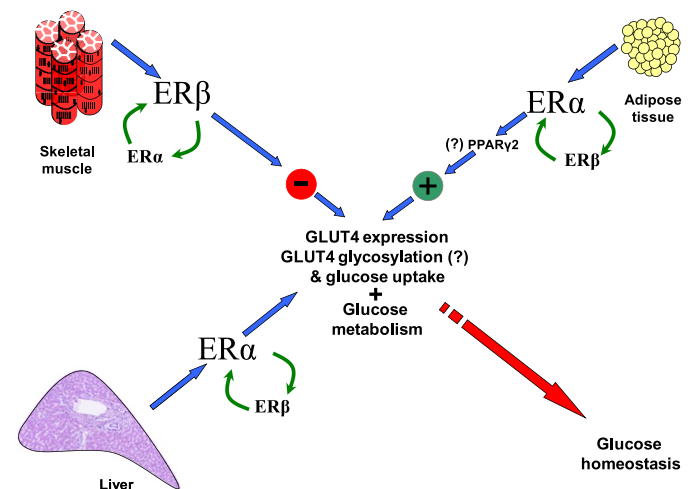


Fig. 11. Model summarizing the interaction of SM, WAT, and liver with glucose uptake and metabolism. ER α and ER β are expressed in all tissues involved in glucose homeostasis but have different roles in its regulation. In SM, ER β is predominant and regulates GLUT4 expression. In WAT, ER α is predominant and may regulate GLUT4 expression through peroxisome proliferator-activated receptor- γ (PPAR γ). GLUT4 glycosylation seems to be another step in GLUT4 function regulated by ER α /ER β . In the liver, ER α has an important role in the regulation of genes involved in glucose metabolism. Additionally, interplay between ER α and ER β may also be important in the regulation of GLUT4 expression and glucose uptake in SM and WAT and glucose metabolism in the liver.

effects of estrogen on GLUT4 expression in SM and WAT (presented in the present study) may help to explain why it is so difficult to understand estrogen effects on glucose homeostasis. Indeed, in WT mice Tam treatment did not affect overall glucose metabolism, insulin sensitivity, or body weight. ER α -/- mice had increased body weight ($P < 0.05$ vs. WT) and insulin resistance as expected (17). In addition, we found that loss of ER α expression was associated with very low expression of GLUT4 on the cell membrane and vesicles of SM. ITT confirmed the insulin resistance in these mice. The overall effect of Tam treatment of ER α -/- mice was an improvement in glucose uptake, observed at 30 and 120 min of the ITT, and a significantly reduced AUC.

No Tam-induced changes were detectable by immunofluorescence in GLUT4 expression in SM of ER β -/- mice. In contrast to its effects in SM of ER β -/- mice, in WAT Tam caused a clear decrease in GLUT4 expression without affecting body weight. Regulation of GLUT4 in WAT is known to be different from that in muscle (8); however, the mechanisms responsible for the discordant regulation are still unclear. Several factors involved in glucose metabolism and GLUT4 regulation are known to have different expression patterns in insulin-sensitive tissues, and among them is the peroxisome proliferator-activated receptor- γ (PPAR γ) (6). One of its isoforms, PPAR γ 2, is adipose specific and involved in GLUT4 regulation (2).

In contrast to a previous study in young ER β -/- mice, in which there was normal fasting glycemia and normal glucose tolerance (5), in the present study there was decreased fasting glycemia accompanied by signs of glucose intolerance at the initial 30 min of the GTT. The maintenance of euglycemia is mainly dependent on the balance between glucose uptake by peripheral tissues and glucose production by glycogenolysis and gluconeogenesis by the liver, and alteration in this balance can lead to hyper- or hypoglycemia. E₂ acts on pancreatic β cells and interferes with insulin release (1); however, the role of ER α and ER β in the regulation of islet function and insulin release remains to be investigated. In ER β -/- mice we observed islet hypertrophy but no detectable increase in basal plasma insulin after an overnight fast. Another plausible explanation that needs further investigation is the aging process. As ER β -/- mice age, adaptations of the pancreas could well interfere with glucose uptake in SM and WAT, causing abnormal fasting glycemia in older mice.

In normal C57BL/6 mice, the first phase of glucose-induced insulin secretion is characterized by a peak of insulin 2 min after an intraperitoneal glucose load, with plasma insulin returning to basal levels at 5 min (15). In ER β -/- mice we observed a delayed insulin secretion, with the peak 5 min after the initial load and return to basal levels at ~10 min (Fig. 8). This delay could partially explain the sustained plasma glucose levels observed during the first 30 min of the GTT in ER β -/- mice.

As they age, male ER β -/- mice develop hypertension due to abnormal adrenergic regulation of small artery tone (24). One of the consequences of hypertension is reduction in insulin-stimulated blood flow in SM (22). Such a reduction may be the cause of the delayed uptake of glucose in the initial 30 min of the GTT in ER β -/- mice. After 30 min a pronounced decrease in glucose levels was observed, and the final

result was a normal AUC comparable with that seen in WT mice.

Together with the results from the ITT of ER β -/- mice, we speculate that the abnormal glucose tolerance during the initial 30 min of the GTT is not related to reduced insulin sensitivity in peripheral tissues but to delayed insulin secretion. Additionally, one of the most important paracrine actions of insulin is the suppression of glucagon release by pancreatic α -cells (29). If insulin release is delayed, glucagon secretion is not suppressed and plasma glucose is maintained by glycogenolysis and gluconeogenesis.

In ER α -/- mice Tam treatment resulted in an abnormal response to glucose load, the cause of which is multifactorial. As discussed above, hepatic glucose metabolism is impaired (5) and GLUT4 expression in SM is reduced in ER α -/- mice. In addition, pancreatic β -cells of these mice are less protected from apoptosis induced by oxidative injury (23).

On Western blots of SM and WAT extracts of WT and ER α -/- mice, we detected two immunoreactive bands with GLUT4 antibodies. These bands are thought to possibly result from posttranslational modifications of GLUT4, such as glycosylation (10, 26). Glycosylation has an important role in the maturation of glucose transporters (26). Treatment of WT mice with Tam increased both protein bands, while in ER α -/- mice Tam increased the upper band. This change in pattern of expression indicates that Tam can both increase protein expression and perhaps also change the glycosylation state of GLUT4. Strikingly, in ER β -/- mice, in both SM and WAT only the lower band was detected, suggesting the predominance of the nonglycosylated form of GLUT4. Treatment with Tam slightly increased this band in SM but clearly decreased it in WAT. In light of the present results, we suggest that ERs may have a role in the posttranslational modification of GLUT4 protein.

Regulation of glucose homeostasis is a complex phenomenon, involving several tissues, hormones, cytokines, and coregulators. Evidence presented in the present study indicates that ERs and cofactors associated with expression of ERs play a crucial role in the maintenance of euglycemia. ER α and ER β have distinct functions in SM and WAT and opposite actions on GLUT4 expression, and continued dissection of this network could lead to new options to improve glucose metabolism and insulin resistance in prediabetes and diabetes (Fig. 11).

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