Estrogen Can Prevent or Reverse Obesity and Diabetes in Mice Expressing Human Islet Amyloid Polypeptide

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Type 2 diabetes is characterized by loss of β-cell mass and concomitant deposition of amyloid derived from islet amyloid polypeptide (IAPP). Previously we have shown that expression of human IAPP (huIAPP) in islets of transgenic mice results in either a rapid onset of hyperglycemia in mice homozygous for the huIAPP transgene on a lean background (FVB/N) or a gradual hyperglycemia in mice hemizygous for the huIAPP transgene on an obese background (A^{vy}/A). In both strains, only the males routinely develop diabetes. To investigate this sexual dimorphism, we treated young prediabetic A^{vy}/A mice transgenic for huIAPP (huIAPP-A^{vy}) with 17β-estradiol (E2). The treatment completely blocked the progression to hyperglycemia but also prevented the associated weight gain in these mice. Immunohistochemistry of pancreatic sections demonstrated normal islet morphology with no apparent deposition of islet amyloid. E2 treatment of 1-year-old huIAPP-A^{vy} diabetic males rapidly reverses obesity and hyperglycemia. To determine the effects of E2 in a nonobese model, we also treated prediabetic, ad libitum–fed and pair-fed Lean-huIAPP transgenic males. E2 completely blocked the progression to hyperglycemia with no significant effect on body weight. Pancreatic insulin content and plasma insulin concentration of Lean-huIAPP transgenic mice increased in a dose-dependent manner. We demonstrated the presence of estrogen receptor (ER)-α mRNA in mouse and human islets. By also confirming the presence of ER-α protein in islets, we discovered a novel 58-kDa ER-α isoform in mice and a 52-kDa isoform in humans, in the absence of the classic 67-kDa protein found in most tissues of both species. The demonstrated presence of ER-α in mouse and human islets is consistent with a direct effect on islet function. We conclude that exogenous E2 administered to male mice may block human IAPP-mediated β-cell loss both by direct action on β-cells and by decreasing insulin demand through inhibition of weight gain or increasing insulin action.
***Diabetes* 51:2158–2169, 2002**

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Received for publication 5 November 2001 and accepted in revised form 15 April 2002.

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J.G.G. has received research support from Pfizer Inc. A.J.M. holds stock in Pfizer Inc.

AR, androgen receptor; E2, 17β-estradiol; ER, estrogen receptor; EST, estrogen sulfotransferase; FBG, fasting blood glucose; huIAPP, human IAPP; IAPP, islet amyloid polypeptide; PPAR, peroxisome proliferator–activated receptor; TBST, Tris-buffered saline plus Tween.

Type 2 diabetes is characterized by both impaired insulin action (1) and failure of β-cells to compensate with increased insulin secretion (2). Some studies have found an association of this β-cell failure with the deposition of islet amyloid in the pancreas (3). We and others have previously reported the generation of transgenic animal models that exhibit human islet amyloid polypeptide (huIAPP)-dependent hyperglycemia associated with loss of β-cell mass and/or amyloid deposition (3–5). One model is described herein as the Lean-huIAPP transgenic line [FVB/N-*Tg(IAPP)6Jdm/Tg(IAPP)6Jdm*] (6). It was generated with a transgene that directs expression of huIAPP to pancreatic β-cells under the regulation of rat insulin II promoter fragment, in mice bred to homozygosity for the transgene. This model exhibits a rapid onset of severe hyperglycemia associated with the progressive loss of islet area and insulin immunoreactivity in the pancreas. This β-cell loss is specific for huIAPP since the expression of murine IAPP to comparable levels in transgenic mice has the normal complement of β-cells (7). With the aim of obtaining a phenotype more akin to human type 2 diabetes, we crossed Lean-huIAPP transgenics to the Agouti viable yellow (A^{vy}/a) mouse on the C57BL/6 background to generate an obese hemizygous transgenic mouse [FVBB6F1-*Tg(IAPP)6Jdm/-A^{vy}/A*] (8). These mice, herein described as huIAPP-A^{vy}, develop a much more gradual onset of hyperglycemia that is associated with the appearance of copious deposits of islet amyloid in the pancreas. Interestingly, in both these models, either the females are much less severely affected (huIAPP-A^{vy}) (8) or diabetes appears at a much lower frequency (~5% of Lean-huIAPP males) (6).

Such sexual dimorphism has been described in a number of rodent models of diabetes or insulin resistance: *Cpe^{fat}* (9), *Lepr^{db}/Lepr^{db}* mice on a C3HeB/FeJ genetic background (10), obese Agouti viable yellow (A^{vy}/a) mice overexpressing Agouti (11,12). Sexual dimorphism may be caused by several factors stemming from the different levels of circulating estrogen between sexes. First, a cohort of genes may be transcriptionally modulated by estrogen if they possess an estrogen response element within their promoters (13). Second, estrogen sulfotransferases (ESTs) inactivate estrogen and may play a role in diminishing available reservoirs, which thereby androgenizes or estrogenizes the liver (10,14). High levels of EST

mRNA were detected in male rat livers, whereas no EST mRNA was found in the livers of females (15), which would maintain higher levels of 17β-estradiol (E2) for females within the local hepatic environment. A null mutation of the mouse EST did not alter circulating levels of estrogen or testosterone, and mice were phenotypically normal for both sexes (16). Finally, one report has shown that estrogen can increase the number of insulin receptors in the liver (17). The sexual dimorphic phenotype suggests either that E2 is having some protective effect against hyperglycemia or that androgens have a detrimental effect. A role for E2 in the regulation of lipid and glucose metabolism has recently emerged in the characterization of aromatase-deficient mice (ArKO) (18,19) and estrogen receptor (ER)-α–deficient mice (20). ArKO mice have increased adiposity, hepatic steatosis, and hyperinsulinemia. ER-α null mutant mice display increased adiposity, insulin resistance, and glucose intolerance. In humans, the E2 antagonist tamoxifen can induce hepatic steatosis (21). In addition, an individual with a genetic ER-α deficiency exhibited glucose intolerance and hyperinsulinemia (22). Given these reports, we sought to determine the effect of administering E2 on the diabetic phenotype of male Lean-huIAPP and huIAPP-A^{vy} transgenic mice.

RESEARCH DESIGN AND METHODS

Animal maintenance and techniques. All animal maintenance protocols and procedures on animals performed in these studies were carried out in accordance with the Pfizer Inc. Institutional Animal Care and Use Committee guidelines. E2 was administered as subcutaneously implanted pellets containing a continuous 90-day release of either 0.18 mg or 0.72 mg E2 or vehicle carrier (Innovative Research of America, Sarasota, FL). A small incision was made in the skin on the back between the scapulae of mice under anesthesia using 0.3 cm^3 10 mg/ml Avertin. The pellet was inserted toward the left side of the neck and a surgical staple was used to close the incision. Fasting blood glucose (FBG) concentrations were determined as described (8) from animals subjected to an overnight fast. Mice were fed standard chow (Purina 5001). Pair feeding of Lean-huIAPP transgenic mice was conducted by providing in the morning 20 g of chow per cage of the E2-treated mice and determining consumption by the following morning. The average consumption of all E2-treated mice was determined, and that amount was provided to vehicle-treated mice.

Transgenic animals. Transgenic mice used in this study have been described previously (6,8). To conform with established guidelines of the Mouse Genome Informatics (MGI) Nomenclature Committee, the mice homozygous for the Rip2-huIAPP transgene are now referred to as FVB/N-*Tg(IAPP)6Jdm/Tg(IAPP)6Jdm*. For brevity we confer the alias of Lean-huIAPP on this line. The MGI designation for the huIAPP-A^{vy} mice with slow-onset diabetes is FVBB6F1-*Tg(IAPP)6Jdm/-A^{vy}/A*. They are the F1 generation of Lean-huIAPP transgenic females (FVB/N) crossed with obese Agouti viable yellow (A^{vy}/a) males (C57BL/6 background strain).

Histology. During excision of the pancreas, mice were kept alive, under anesthesia, to maintain blood flow to the pancreas. Mice were given ~0.3 cm^3 16 mg/ml sodium pentobarbital as a general anesthetic. The pancreas was excised such that the main portal vein was not severed until the majority of the pancreas was cut away from the intestines and stomach. The spleen was kept attached to the pancreas for orientation purposes. Pancreas/spleen was rinsed briefly in Hanks' buffered saline solution (Hanks) (Gibco BRL, Rockville, MD), orientated anatomically correct in a small petri dish, and overlaid with 2 ml of 4% paraformaldehyde fixative (Electron Microscopy Sciences, Port Washington, PA) in 0.1 mol/l sodium cacodylate buffer (pH 7.4, room temperature, 2–4 h). Following a 4-h fixation, the tissue was quickly rinsed with 0.1 mol/l sodium cacodylate buffer and then infiltrated with 30% sucrose (4°C, overnight) in PBS. In the morning, the tissues were transferred to a freezing block containing OCT compound (Tissue-Tek) and snap frozen using the Gentle Jane System (Instrumedics). Frozen sections (8 μm) were cut on a Leica CM3000 microtome and picked up using the Cryo Jane tape system (Instrumedics). Sections were stored at −20°C until they were stained. Tissues were stained with 1:500 dilution in PBS of primary guinea pig anti-insulin antibody stock (Peninsula Laboratories, San Carlos, CA), at room temperature for 1 h, followed by several 5-min washes with 1 ml PBS, and continued with a 1:500 in PBS Alexa 594 goat anti-guinea pig (Molecular Probes, Eugene, OR) conjugate stain for 1 h at room temperature in PBS. Pancreatic sections were then stained with a 1:500 dilution of 0.05 mg/ml thioflavine S (Sigma, St. Louis, MO) in PBS. Sections were washed again three times with 1 ml PBS and overlaid with 80% glycerol in PBS, and a coverslip was secured with enamel. Amyloid was detected by fluorescence at 488 nm and insulin by fluorescence at 594 nm. Fluorescent images were captured digitally on a Nikon FXA microscope with a Magnafire camera. Morphometric analysis of islet size was conducted on these images using Optimus 6.5 software (Media Cybernetics).

Estrogen enzyme immunosorbent assay. Plasma samples obtained from retro-orbital sinus bleeds were stored at −20°C until analysis. Plasma E2 levels were measured using Estradiol ELISA (American Laboratory Products Co., Windham, NH), following the manufacturer's instructions.

Insulin content. Pancreata of Lean-huIAPP transgenic mice and nontransgenic A^{vy}/A mice were removed while the mice were anesthetized with ~0.3 cm^3 16 mg/ml sodium pentobarbital and snap frozen in liquid nitrogen. Insulin was extracted from pancreas by incubating the pancreas diced into small fragments in 5 ml acid alcohol at 4°C for several days. A 1:400 or 1:100 dilution of this in Hanks provided a working solution for the assay. The assay for insulin has previously been described (23).

RT-PCR. Hand-picked islets of Langerhans, liver tissue, and brain tissue from mouse strain FVB/N were homogenized and dissolved in 1,000 μl Trizol (Gibco BRL). Total RNA was extracted according to manufacturer's recommendation. RNA was resuspended in 200 μl DEPC-treated water and treated with 10 μl DNase (Promega) for 1 h at 37°C. DNase treatment was stopped by incubating reaction for 10 min at 70°C, followed by EtOH precipitation. RNA was resuspended and quantified, and 5 μg was used for RT using Superscript 2 (Gibco) according to the manufacturer's recommendation. For each PCR reaction, 1 μg of reverse-transcribed total RNA was used. As a control for DNA contamination, 1 μg of non-RT [(−)RT] total RNA was also assayed for each oligonucleotide set. Mouse ER-α oligonucleotide set was 5′-ACCAATG CACCATTGACAAGAACC-3′ and 5′-TATCTGCTAGGTTGGTCAATAAGC-3′, which generates a 381-bp fragment. ER-β oligonucleotide set was 5′-CTATG CAGAACCTCAAAAGAGTCC-3′ and 5′-TCTCTCCTGGATCCACACTTGA CC-3′, generating a 372-bp mouse fragment. The mouse androgen receptor (AR) mRNA was detected using oligonucleotide set 5′-GATGACTGTATC ACACATTGAAGG-3′ and 5′-GGTCAAAAGTGAACTGATGCAGC-3′, which generates a 665-bp fragment. Human ER-α primers were 5′-ATTTGACCCTCCAT GATCAGGTCCACCTTCTAGAAT-3′ and 5′-ACTGTGGCAGGGAAACCCTCT GCCTCCCCCGTGATG-3′, which generates a 679-bp fragment. Expand High Fidelity PCR System (Roche Diagnostics, Indianapolis, IN) was used under the following conditions: denature at 95°C for 30 s, anneal at 68°C for 30 s, and extend at 72°C for 1 min, repeated for 40 cycles.

Islet isolation. One-year-old male huIAPP-A^{vy} transgenic mice or A^{vy}/A mice were anesthetized using ~0.3 cm^3 16 mg/ml sodium pentobarbital. An incision into the abdominal cavity provided access to the pancreas. The ampulla of Vater was clamped off. A small nick in the common bile duct was made from the origin (near the liver). A 22-gauge needle was inserted into the bile duct, and 5 ml cold Hanks was infused to distend the pancreas. Pancreas was excised away from other tissues and diced into fine pieces using a scissors. Tissue was transferred to a 15-ml test tube and washed several times with cold Hanks, and light centrifugation was performed using a clinical centrifuge (Dynac). Collagenase P (5 mg; Boehringer Mannheim, Indianapolis, IN) was added, and the tissue was digested by vigorously shaking the tube within a 37°C water bath for 2–3 min. Ice-cold Hanks was used to stop digestion by washing several times. Islets were hand picked in cold Hanks under a light microscope and transferred into fresh Krebs-Ringer bicarbonate buffer with 1% BSA. Islets were collected and transferred to a 1.5-ml Eppendorf tube and centrifuged at low speed, the supernatant was discarded, and 1 ml Trizol (Gibco BRL) was used to directly dissolve islet tissue with vortexing. Human islets were provided as a gift from the JDF Human Islet Distribution Program at the University of Alberta. Islets provided in tissue culture media were centrifuged to collect islets, washed in PBS, and centrifuged again, and the resulting pellet was resuspended in 1 ml Trizol (Gibco BRL).

Statistical analysis. A standard two-tailed Student's t test was used with a 5% significance level or a P value <0.05.

Plasma leptin and insulin levels. Plasma samples were obtained from retro-orbital sinus bleeds of E2- or vehicle-treated mice and stored at −20°C. Leptin concentrations were determined using a mouse Leptin-RIA kit (Linco, St. Charles, MO), and insulin concentrations were determined using a Mouse Ultrasensitive Insulin ELISA (Alpco Diagnostics, Windham, NH) according to the manufacturer's instructions.

Western blot. FVB/N male mouse brain, liver, and islet protein or human islets, liver, brain, skeletal muscle, and pancreas were extracted from isolated

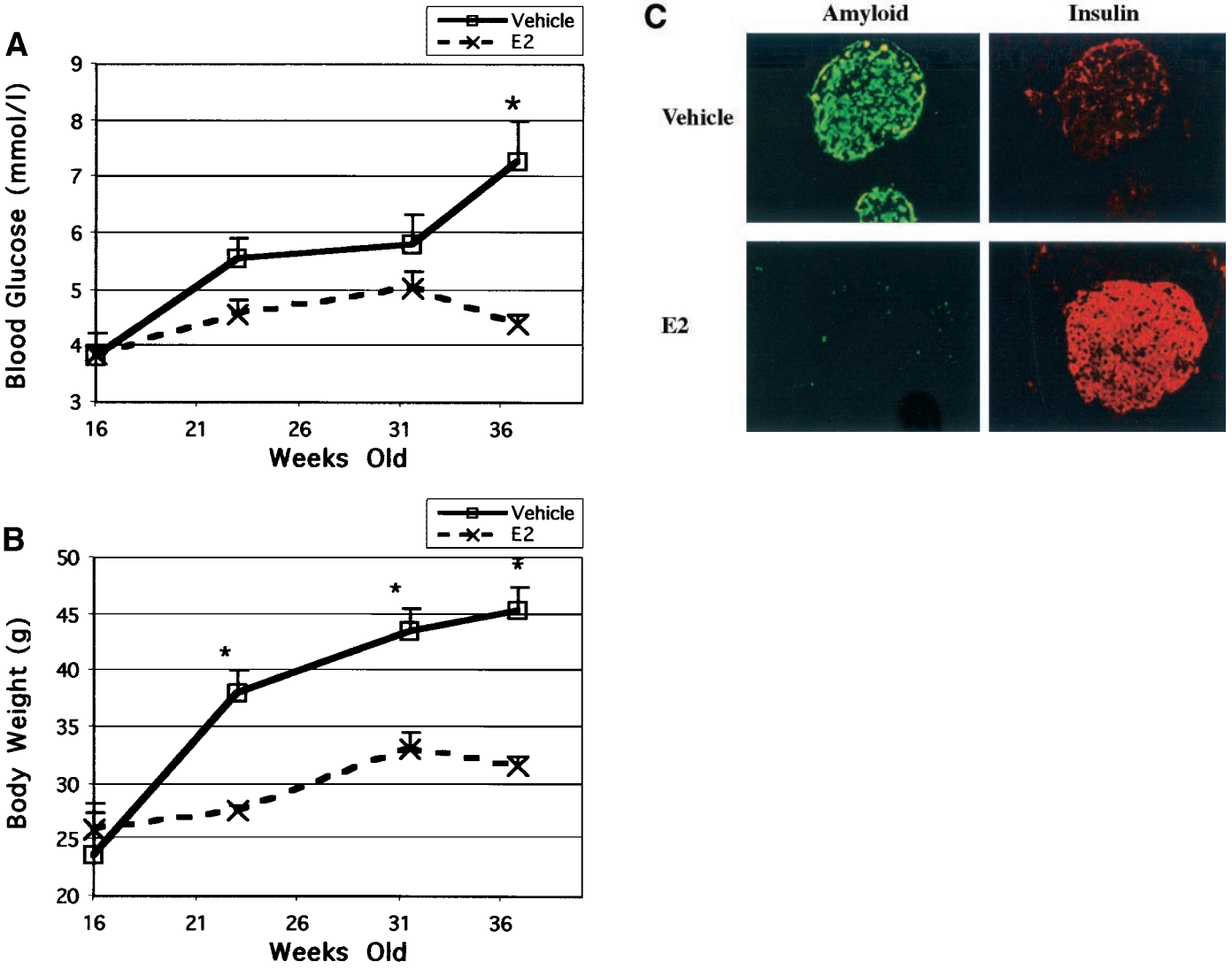

FIG. 1. Prediabetic huIAPP transgenic males treated with E2. E2 (0.72 mg/90-day release) or vehicle pellets were implanted in seven huIAPP-A^{vy} males beginning at 16 weeks of age (4 months). E2 and vehicle pellets were replenished at 32 weeks of age in each of the corresponding groups. *A*: Fasting blood glucose concentrations (mmol/l glucose) monitored over the 21-week study period. *B*: Fasted body weight (g) versus time of mice monitored in *A*. *C*: Double staining for amyloid (thioflavine S) and insulin (anti-insulin conjugated with Alexa 594) in the E2- and vehicle-treated mice shows that islets are devoid of amyloid and have intact β-cells. Vehicle-treated animals develop large amyloid plaques, rendering the greater plaque area of the islet absent of insulin or β-cells. *$P < 0.05$.

tissue using 500 μl M-PER Mammalian Protein Extraction Reagent (Pierce, Rockford, IL) with 10 μl of 0.1 mol/l dithiothreitol (DTT), 2.5 μl of 200 mmol/l phenylmethylsulfonyl fluoride (PMSF), and a 1× Protease Inhibitor Cocktail Tablet (Roche Diagnostics, Mannheim, Germany). Tissues were briefly sonicated on ice, followed by 20 min of incubation on ice. Protein concentrations were determined using Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's recommendation. Protein samples (10, 20, 40, or 80 μg) were heated to 100°C in 1× NuPAGE LDS Sample Buffer (Invitrogen, Carlsbad, CA) 5 min or 2 μl/lane of Cruz Markers (Santa Cruz Biotechnology, Santa Cruz, CA) without heat and run on a NuPAGE 10% Bis-Tris Gel in 1× NuPAGE MOPS SDS Running Buffer (Invitrogen) 100 V for ~2 h, and protein gel transfer was conducted using a PVDF Membrane Filter and 1× NuPAGE Transfer Buffer (Invitrogen) 30 V for 1 h at room temperature. Blots were preincubated in 10 ml of 1× TBST (Tris-buffered saline, pH 8.0, with 0.5% Tween-20) with 5% Carnation Non-Fat Dry Milk (Nestle, Solon, OH) (1 h at room temperature), followed by replacing the solution with 10 ml 1× TBST with 5% nonfat dried milk and 1 μl monoclonal anti–ER-α antibody MAB463 (Chemicon, Temecula, CA) for 1 h at room temperature with gentle shaking. Nylon filter was washed several times with 10 ml 1× TBST 5 min with shaking, followed by adding 10 ml 1× TBST, 5% nonfat dried milk, and 12.5 μl anti-mouse IgG-HRP conjugate (Santa Cruz Biotechnology) for 1 h at room temperature. Western blot was washed several times for 5 min with 1× TBST, then developed using ECL Western Blotting Detection Reagents (Amersham Pharmacia Biotech, Arlington Heights, IL) solutions for 5 min and analyzed on a Kodak Image Station 440 CF (Eastman Kodak Company, Rochester, NY) for a 3-min exposure. Band size was determined by Kodak DS 1D version 3.0.2 software (Kodak Imaging Systems, New Haven, CT).

RESULTS

Our initial intent was to determine if estrogen could prevent the onset of hyperglycemia in the huIAPP-A^{vy} gradual-onset obese model. HuIAPP-A^{vy} males that were still euglycemic at 16 weeks of age were subjected to vehicle or E2 slow-release pellet (0.72 mg/90-day release) implantation. Fasting blood glucose concentrations and body weight were monitored for the next 22 weeks (Fig. 1*A*). Implanted pellets were replenished at week 32. The fasting blood glucose concentrations of the vehicle-treated males steadily climbed to a plateau of 7.3 mmol/l by week 37. In contrast, the E2-treated mice remained euglycemic (4.4 mmol/l) throughout this time period. The mean body weight of the vehicle group climbed from 25 g at the time

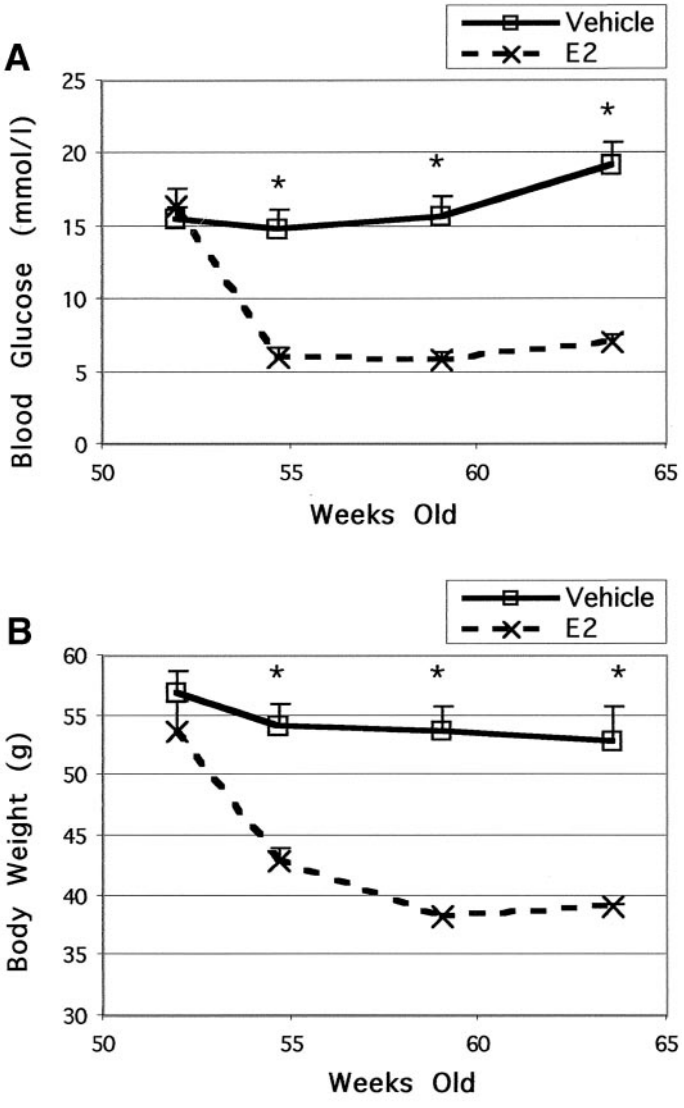

FIG. 2. Diabetic huIAPP-A^{vy} transgenic males treated with E2. E2 0.72/90-day ($n = 4$) and vehicle ($n = 8$) slow-release pellets were implanted in huIAPP-A^{vy} 52-week-old males with frank diabetes. *A*: Fasting blood glucose concentrations (mmol/l glucose) monitored over a 12-week period. In E2-treated animals, blood glucose levels rapidly dropped from an average of 16.3 to 5.9 mmol/l in 19 days. *B*: Fasting body weight (g) of E2- and vehicle-treated mice in *A*. On average, E2-treated mice started at 54 g and lost 11 g in weight by 19 days. *$P < 0.01$.

of the first implantation to 45.3 g at 37 weeks (Fig. 1*B*). The mean body weight of the E2-treated group climbed to only 31.5 g, 31% less than the control animals. Plasma insulin concentrations were determined to be significantly elevated ($P = 0.01$) in vehicle-treated males (4.9 ± 1.01 ng/ml, $n = 7$) compared with E2-treated males (1.31 ± 0.47 ng/ml, $n = 6$). We previously had demonstrated the accumulation of copious amounts of islet amyloid in huIAPP-A^{vy} mice at 10 months of age by means of thioflavine S staining (8). To confirm the islet morphology in this cohort, a subset ($n = 3$) were killed at the end of this study, and their pancreata were fixed and stained with thioflavine S and anti-insulin antiserum (Fig. 1*C*). Islets of vehicle-treated mice stained intensely with thioflavine S, indicating the presence of large amyloid deposits, while the insulin staining was very weak. In contrast, islets of E2-treated mice stained intensely for insulin with no apparent thioflavine S staining. Thus, it appeared that E2 could block both the progression to hyperglycemia and amyloid deposition, presumably relieving insulin resistance through suppression of body weight gain.

The next study utilized the same treatment but in aged, obese huIAPP-A^{vy} mice to determine if E2 could reverse the state of frank diabetes. HuIAPP-A^{vy} males aged 52 weeks on average weighed 55.2 g and exhibited a mean fasting blood glucose concentration of 15.9 mmol/l (Fig. 2). E2 treatment resulted in a rapid and dramatic drop in both fasting blood glucose concentrations and body weight. At 64 weeks, after 84 days of E2 or vehicle treatment, mice were killed and examined for gross morphology. E2-treated mice had no apparent white adipose tissue in any of the common fat stores, whereas the vehicle-treated mice had copious amounts (data not shown). The absence of engorged white adipose tissue suggests that E2 treatment resulted in depletion of major fat depots. The plasma insulin concentrations were determined not to be significantly different between vehicle-treated males (6.22 ± 0.92 ng/ml, $n = 8$) and E2-treated males (6.04 ± 1.86 ng/ml, $n = 4$); however, both groups were clearly elevated relative to the younger cohort in Fig. 1 ($P < 0.01$).

To determine if such effects would also occur in a euglycemic mouse prone to insulin resistance and obesity but not diabetes, we treated 16-week-old nontransgenic A^{vy}/A males with two successive implants of E2 (0.72 mg/90-day release) (Fig. 3*A* and *B*).

No significant differences in fasting blood glucose concentrations were observed. However, the body weight steadily increased in the vehicle population from 30.7 to 49.0 g by week 37, whereas the E2-treated animals maintained the same weight of 31.0 g. Thus, the resistance to body weight gain was just as dramatic as that seen in the huIAPP-A^{vy} males (Fig. 1*B*). The mean pancreatic insulin content of the E2-treated males was 2.4-fold lower than the vehicle-treated group (221 vs. 520 μg), consistent with an E2-mediated increase in insulin sensitivity due to lower body weight ($P < 0.05$) (Fig. 3*C*). Plasma insulin levels were also significantly lower in E2-treated mice (0.42 ± 0.11 ng/ml, $n = 4$) compared with the vehicle-treated group (5.97 ± 0.61 ng/ml, $n = 7$) measured at the end of the study.

We next shifted our attention to the Lean-huIAPP transgenic mouse. This strain also exhibits a strong sexual dimorphism with respect to the diabetic phenotype. However, unlike the huIAPP-A^{vy} strain, this animal is a model of insulin deficiency that lacks the obese and insulin resistance phenotype of the A^{vy}/A mouse. Vehicle- and E2-treated males were monitored for blood glucose concentrations and body weight from 7 to 13 weeks of age. Vehicle-treated mice exhibited a very rapid onset of fasting hyperglycemia (18.8 mmol/l blood glucose by 13 weeks of age) (Fig. 4*A*) as reported previously (6). E2-treated mice remained euglycemic (6.2 mmol/l). A slight body weight lowering was noted between 10 and 11 weeks of age ($P > 0.2$) (Fig. 4*B*).

To determine if this slight drop in body weight could be due to an E2-mediated effect on food intake, we measured the 5-day average ad libitum food consumption of a new

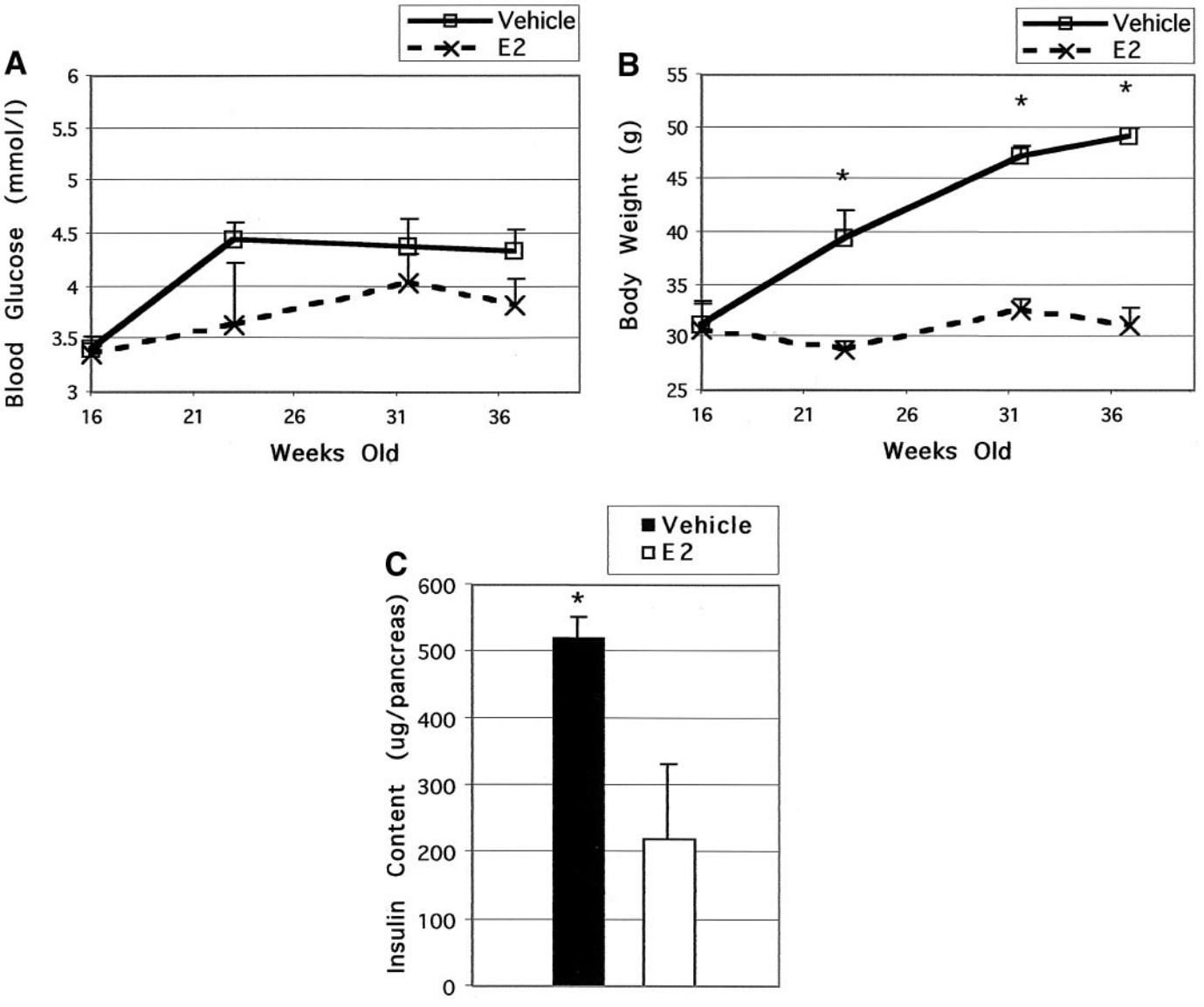

FIG. 3. Preobese nontransgenic male A^{vy}/A mice treated with E2. To determine the effect of estrogen in a nontransgenic obese/insulin-resistant model, 0.72 mg/90-day ($n = 4$) and vehicle ($n = 7$) pellets were implanted in 16-week-old A^{vy}/A males. At 31 weeks of age, a second E2 or vehicle pellet was inserted for each corresponding group. *A*: Fasting blood glucose concentrations (mmol/l glucose) were monitored over a 21-week period. *B*: Fasting body weight (g) of mice in *A*. *C*: At the end of the study, the pancreas was removed, and total insulin content per pancreas was determined. The insulin content rose 2.35-fold in the vehicle population relative to the E2-treated mice. *$P < 0.01$.

cohort of the Lean-huIAPP transgenic mice. These mice had been implanted with vehicle, 0.18 mg/90-day E2 slow-release pellets, or 0.72 mg/90-day E2 slow-release pellets (Fig. 5*A*). E2 at either dose suppressed food consumption by 33%, consistent with a recent report (24). At the end of the 5-day food intake study, the vehicle-treated mice were food restricted to match the average intake of the E2-treated mice, and all three groups were monitored for fasting blood glucose concentrations and body weight for the next 5 weeks (Fig. 5*B* and *C*). Again, the vehicle-treated Lean-huIAPP transgenic males became extremely hyperglycemic (>20 mmol/l) by 8 weeks of age, while both the low- and high-dose E2-treated mice remained euglycemic. The mean body weight of the vehicle-treated mice did not differ significantly from either estradiol-treated group ($P > 0.2$ for all postpellet implantation time points). Plasma insulin concentrations were determined for these three groups and compared with euglycemic, vehicle-treated Lean-huIAPP females and both sexes of nontransgenic FVB/N mice (Fig. 5*D*). Plasma insulin concentration rose in a dose-dependent manner greater than twofold relative to the vehicle-treated Lean-huIAPP males ($P < 0.01$). Both sexes of the FVB/N mice and the female Lean-huIAPP mice had plasma insulin levels threefold greater than vehicle-treated Lean-huIAPP males ($P < 0.001$). Only the low-dose E2-treated cohort was significantly lower ($P < 0.05$) than male and female wild-type mice, but not Lean-huIAPP females ($P > 0.1$).

Plasma E2 concentrations were measured for Lean-huIAPP transgenic males treated with 0.18 mg/90-day pellets, 0.72 mg/90-day pellets, or vehicle after 4 weeks of treatment (Fig. 6*A*). The mean E2 concentration in plasma of vehicle-treated mice was 13.8 pg/ml. The low- and high-dose group E2 concentrations were 323 and 838 pg/ml, respectively. The low-dose value represents an ~10-fold increase of what is typically observed in a

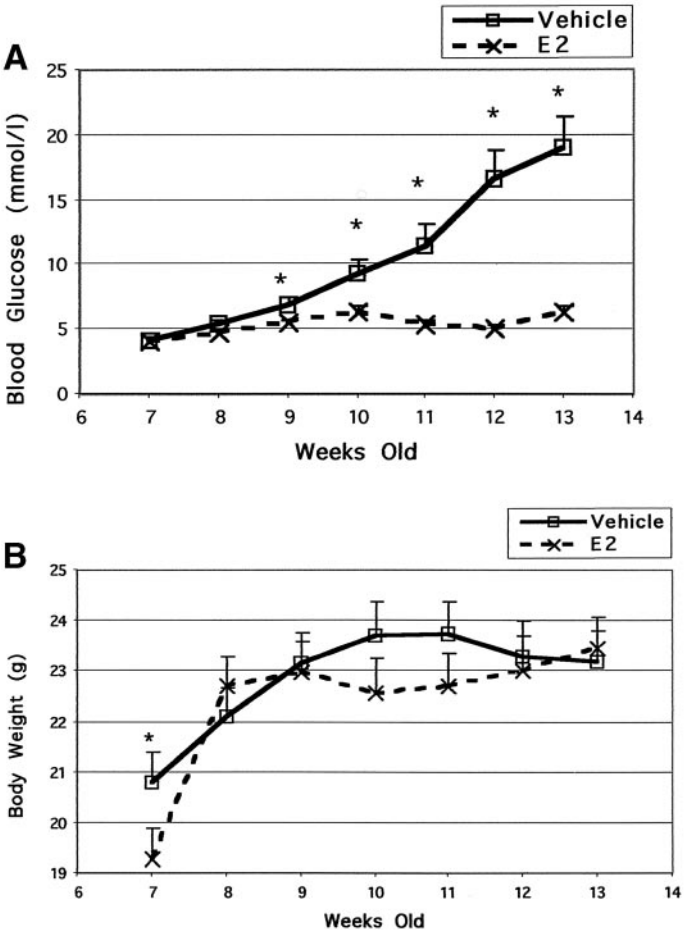

FIG. 4. Ad libitum–fed E2-treated prediabetic Lean-huIAPP male mice. Seven-week-old Lean huIAPP male mice were implanted with 0.72 mg/90-day E2 (n = 10) or vehicle (n = 10) pellets. *A*: Fasting blood glucose concentrations (mmol/l glucose) were measured each week. *B*: Fasting body weight (g). At 10 weeks, the vehicle-treated group had a 1-g body weight gain over the E2-treated group; however, the weight normalized by 12 weeks. *P < 0.05.

nonpregnant female mouse (~30 pg/ml) (25) or an ~5-fold increase if pregnant (~60 pg/ml) (26). The pancreas was removed and weighed from each of these animals, and the insulin content was determined (Fig. 6*B*). Pancreatic insulin content increased dramatically and dose-dependently with E2 treatment (Fig. 6*C*). To determine the morphology of the Lean-huIAPP transgenic mice with or without E2 treatment, pancreatic sections from the first Lean-huIAPP transgenic cohort (Fig. 4) were treated with anti-insulin antisera to visualize insulin and thioflavine S to detect amyloid fibrils (Fig. 7*A*). A stained section from a huIAPP-A^{vy} male is shown for comparison. No thioflavine S staining was detected for either vehicle-treated or E2-treated mice. This confirms previous reports that failed to observe islet amyloid deposits under light microscopy but noted the detection of amyloid fibrils in these mice only by electron microscopy (6,27). Insulin staining in the 13-week-old vehicle-treated pancreata was weak. In contrast, E2-treated mice stained intensely for insulin in a level comparable to that seen in wild-type nontransgenic FVB/N (data not shown). The average islet size of vehicle-treated mice was dramatically smaller than that seen for E2-treated mice. The tissue sections from two mice of each group were then subjected to morphometric analysis. Islet area was measured for every islet detected on three whole pancreatic cross-sections stained for insulin. The mean islet areas for two of the vehicle-treated mice (17 and 22 mmol/l fasting blood glucose) were 150 and 650 μm^2, respectively, while the values for two of the E2-treated mice (5 and 6 mmol/l fasting blood glucose) were 2,650 and 1,600 μm^2, respectively (Fig. 7*B*). To visualize the distribution of islet sizes within each group, the islet number was plotted against the islet area in 1,000 μm^2 increments. Islet size was very heterogeneous in every section. However, it is clear that the vast majority of vehicle-treated islets were smaller than 500 μm^2 in size, and none were larger than 5,000 μm^2. Islets in the E2-treated mice are skewed to the larger increments, with some areas as large as 45,000 μm^2 (data not shown).

To determine if the effects of estrogen could be due to the estrogen receptor inducing transcription of leptin, we analyzed plasma samples of both E2- and vehicle-treated mice. The plasma leptin concentration of the ER-treated Lean-huIAPP transgenic males (3.00 ± 0.33 ng/ml, n = 10 mice) was not significantly different from either the vehicle-treated Lean-huIAPP transgenic males (2.60 ± 0.32 ng/ml, n = 10 mice) (P = 0.43) or the vehicle-treated Lean-huIAPP transgenic females (2.50 ± 0.35 ng/ml, n = 10 mice) (P = 0.29). The glucose-lowering effects of E2 observed in a model of insulin deficiency (Lean-huIAPP transgenic) suggests that some of its effects could be mediated directly on the islet itself. We took the first step in exploring this possibility by determining if the two ER isoforms and AR are expressed in mouse islets. RNA was extracted from purified mouse islets and subjected to RT-PCR utilizing primers specific for murine ER-α, ER-β, and AR. Mouse liver and brain RNAs, as well as RNA in the absence of reverse transcriptase [(−)RT], were run as controls. The results are shown in Fig. 8*A*. ER-α primers generated a 381-bp PCR product from brain and liver RNA, as expected. These primers also demonstrated the presence of ER-α RNA in islets. ER-β–specific primers generated a 372-bp product in brain but not liver, as expected. No ER-β–specific product was detected in islet RNA. AR (665-bp product) was detected in all three tissues. No PCR products were formed in the absence of reverse transcriptase, (−)RT. Human islet RNA was also extracted, reverse transcribed, and used to PCR a human ER-α gene fragment (Fig. 8*B*). The expected 679-bp product was detected, whereas no product was found in the (−)RT. Thus murine and human islets specifically express ER-α, consistent with a direct role for E2 in maintaining islet function and/or viability. To confirm translation of ER-α message in islets, we analyzed mouse islet protein for ER-α product by Western blot (Fig. 8*C*). Surprisingly, Western blot analysis revealed an ER-α isoform in an islet tissue of novel size (i.e., 58 kDa, ~9 kDa smaller than the known 67-kDa product in brain or liver). The abundance of this novel isoform in islets is about half of that seen of the 67-kDa product in liver or brain, and the classic 67-kDa ER-α is not detected at all. To verify if humans also have a truncated isoform specifically expressed in islets, Western blot analysis was conducted using human islets, whole pancreas, liver, brain, and skeletal muscle (Fig. 8*D*). Again, a novel 52-kDa ER-α isoform was detected in human islets

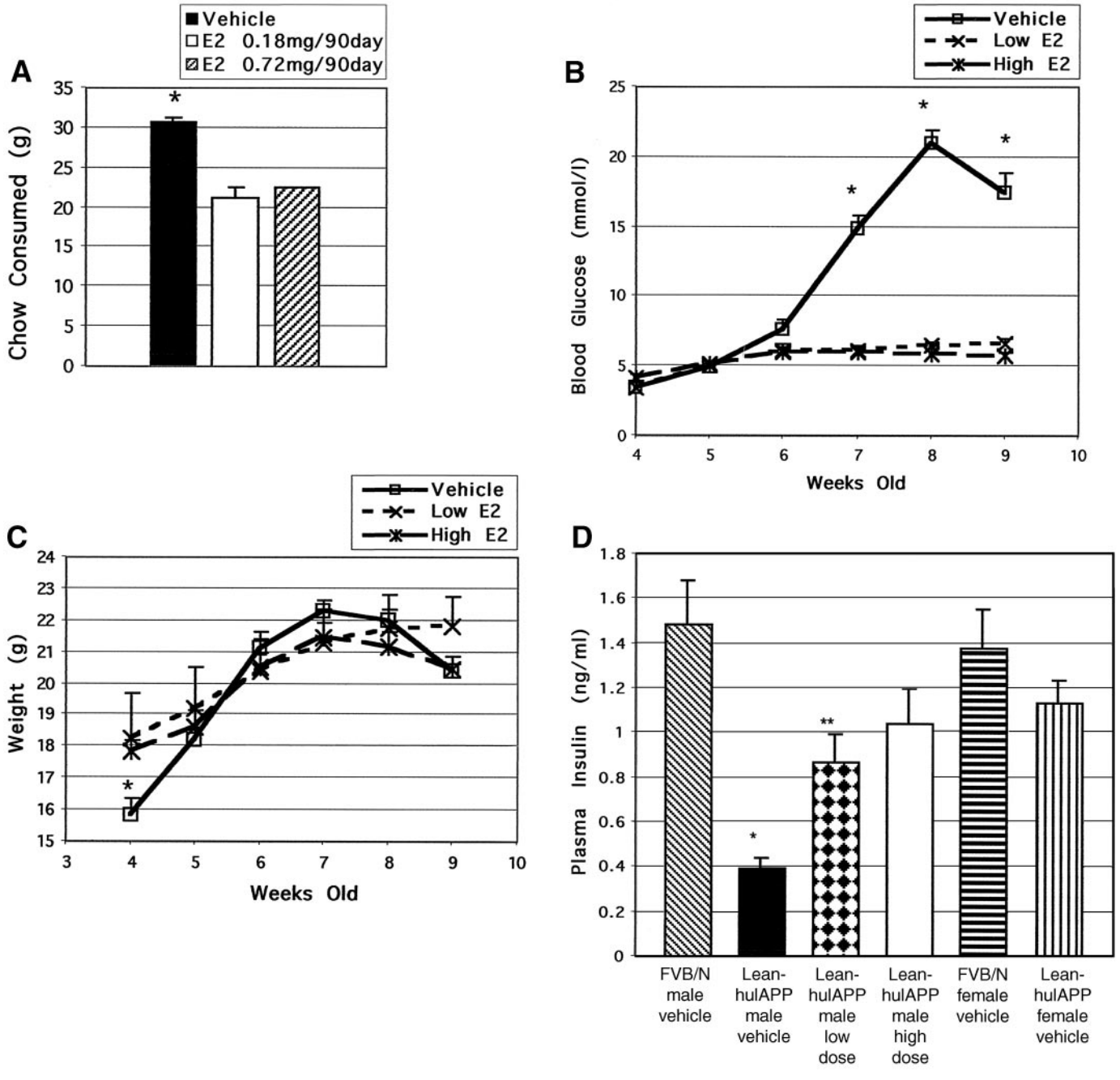

FIG. 5. Pair feeding of vehicle- versus E2-treated prediabetic Lean-huIAPP male mice. In a second study, 4-week-old Lean-huIAPP males were treated with 0.72 mg/90-day E2 (n = 8) or vehicle (n = 9) implants and fourfold lower dose of E2 (0.18 mg/90-day) (n = 9). *A*: Ad libitum food consumption was monitored for 5 days. *B*: Fasting blood glucose concentrations (mmol/l glucose) were measured each week in pair-fed animals. *C*: Fasting body weights (g) of mice monitored in *B*. *D*: Fasting plasma insulin concentration (ng/ml) at the end of study with the addition of wild-type males (n = 8) and females (n = 12) and Lean-huIAPP females (n = 12). *P < 0.01; **P < 0.05.

in the absence of the classic 67-kDa protein detected in most tissues. Since whole pancreas detects the 67-kDa protein and not the 52-kDa isoform and islets detect only the 52-kDa isoform, these Western blot results suggest that the exocrine portion of the pancreas expresses the classic ER-α and the endocrine portion of the pancreas expresses the novel isoform.

DISCUSSION

In one mouse model of islet amyloid, Kahn et al. (28) reported that ovariectomy promoted islet amyloid formation in females. However, no effects on blood glucose concentration were observed. Sexual dimorphism in the diabetic phenotype of rodents has been extensively documented in the literature (9–12). Our primary aim in the present study was to determine if E2 can stop the progression to hyperglycemia in two distinct huIAPP-dependent murine models of diabetes. In the huIAPP-A^{vy} transgenic mouse, E2 inhibited the weight gain and the associated onset of hyperglycemia normally observed in this mouse model. Examination of the islet morphology determined that E2 also prevented the degeneration of β-cells and

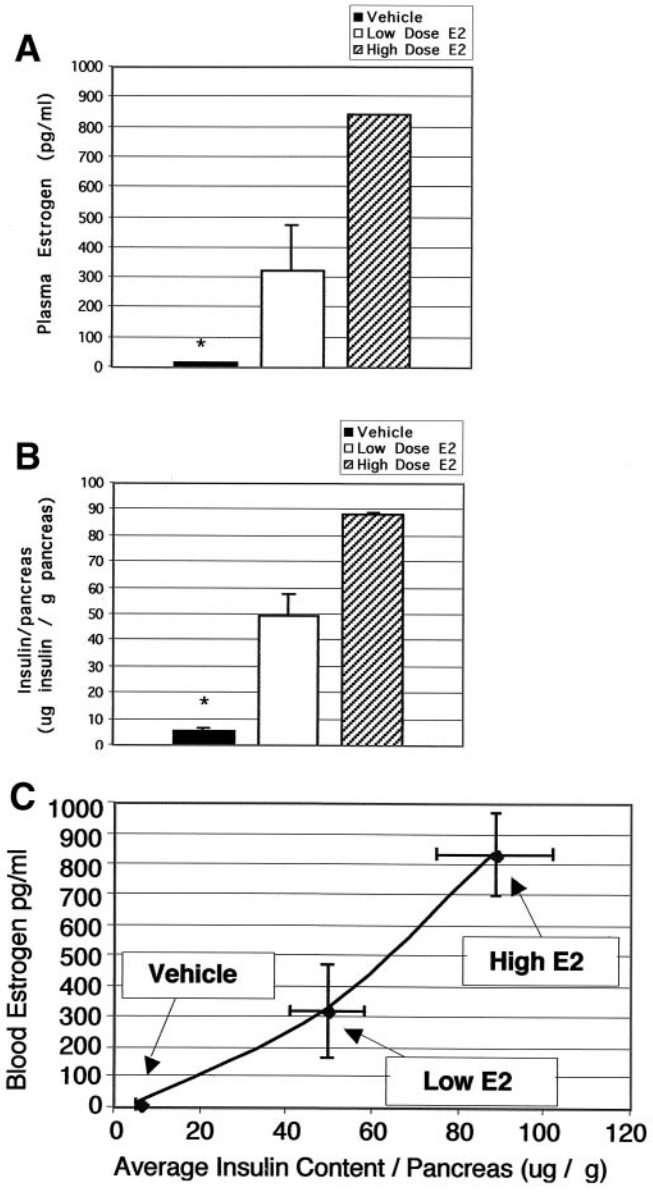

FIG. 6. Plasma estrogen and pancreatic insulin content. Plasma E2 levels were measured for Lean-huIAPP males provided 0.18 mg/90-day pellets (n = 6), 0.72 mg/90-day pellets (n = 7), or the vehicle (n = 9). In addition, the insulin content of the pancreas was measured upon termination of the study. *A*: 17β-Estradiol plasma levels were measured to determine the blood E2 levels achieved. The average E2 levels achieved in the vehicle-treated males was 13.8 pg/ml; low dose (0.18 mg/90-day) of E2 achieved 323 pg/ml, and the high dose (0.72 mg/90-day) of E2 was 838 pg/ml. *B*: Insulin content per pancreas was measured. Results are expressed as microgram of insulin per gram of wet weight pancreas. A substantial conservation of insulin content was found in mice with either the low E2 dose (49 μg insulin) or the high dose (88 μg insulin) relative to the vehicle mice (5.7 μg/ml insulin). *C*: A plot of the plasma E2 levels relative to the pancreatic insulin content. *P < 0.01.

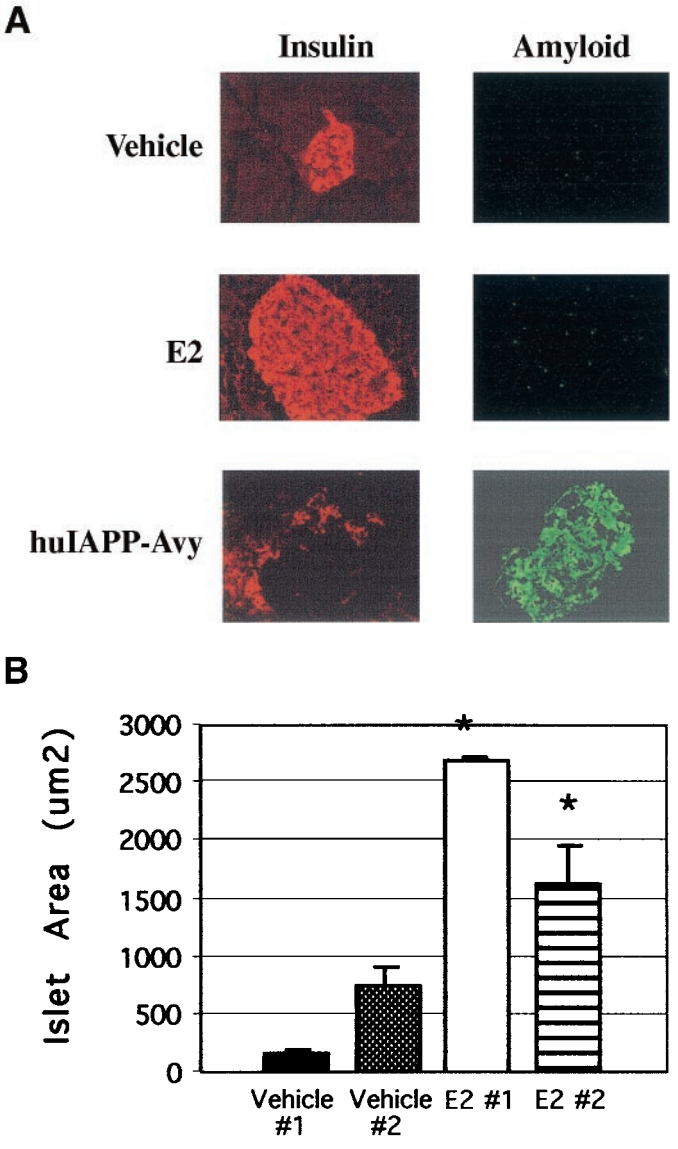

FIG. 7. Morphometric analysis. The islet areas were measured for every islet found in three pancreatic cross-sections of two vehicle-treated and two E2-treated Lean-huIAPP male mice. *A*: Costained pancreatic sections with anti-insulin conjugated with Alexa 594 (left column) and thioflavine S, a marker for amyloid (right column). Neither the vehicle-treated mice (top row) nor the E2-treated mice (middle row) developed amyloid. A 1-year-old male huIAPP-A^{vy} was used as a positive control for islet amyloid (bottom row). *B*: Average islet area for each vehicle- and E2-treated animal. *P < 0.05 relative to either vehicle.

deposition of islet amyloid. The effect of E2 on weight gain was also manifested in nontransgenic A^{vy}/A mice but with no significant effect on fasting blood glucose concentrations. More surprisingly, E2 was able to restore euglycemia in older huIAPP-A^{vy} males with established frank diabetes. This restoration was also associated with a dramatic and rapid weight loss (23 g in 2.5 weeks) primarily due to reduction of adipose tissue mass (data not shown). Because weight gain primarily in the form of amassed adipose tissue is well known to induce insulin resistance, a likely explanation for the prevention and reversal of hyperglycemia in the huIAPP-A^{vy} model would be a restoration of insulin sensitivity mediated by the reduction in adipose mass. Unlike the significant E2-mediated lowering of plasma insulin concentrations seen in the younger huIAPP-A^{vy} and nontransgenic A^{vy}/A mice, the hyperinsulinemia in 1-year-old huIAPP-A^{vy} males was not reduced despite the restoration of euglycemia. This suggests that some insulin resistance independent of adipose mass persists in these E2-treated mice.

That E2 plays a role in body fat deposition has long been known from studies of ovariectomized rats (29). Several recent studies of null mutation animal models have further elucidated a role for E2 in energy homeostasis and regu-

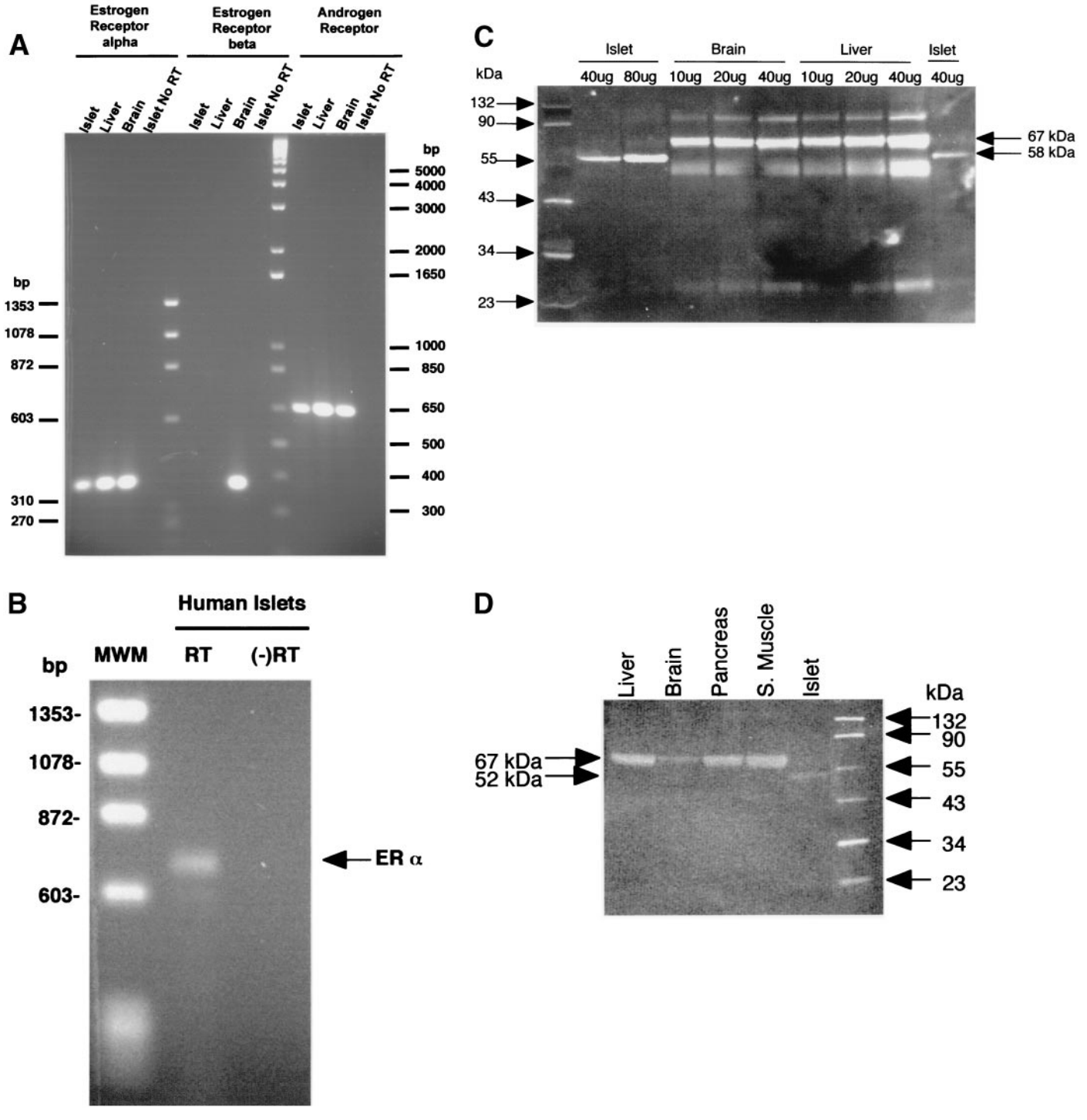

FIG. 8. Expression of ER-α and ER-β and the AR in islets. *A*: RT-PCR of 1 μg total islet RNA was conducted using oligonucleotide primers corresponding to regions of the mouse ER-α/β and the AR cDNAs. Liver and brain tissues were used as a positive control and non-RT islet RNA was used as a negative control. No product was detected in the non-RT lanes for all three oligonucleotide sets. *B*: RT-PCR of human islet RNA using ER-α primers detected a 679-bp fragment. No products were detected in the (−)RT reaction. *C*: Western blot of mouse islet protein at 40 μg (first and last lanes) and 80 μg probed with monoclonal ER-α antibody. Liver and brain protein was used as a positive control at 10, 20, and 40 μg. An ER-α isoform of novel size is detected in mouse islet protein revealing a 58-kDa protein, ~9 kDa smaller than the known 67-kDa ER-α found in liver or brain. Extra bands are nonspecific in brain and liver tissues. *D*: Western blot of 40 μg of human islet, pancreas, brain, liver, and skeletal muscle protein probed with monoclonal ER-α. The classic 67-kDa ER-α protein is detectable in all tissues except islets, which show a novel 52-kDa isoform.

lation of adipose mass in rodents. Jones et al. (18) examined the adipose depots of male and female mice null for aromatase and thus incapable of synthesizing endogenous estrogen. These mice were found to possess fatty livers and accumulate more adipose tissue, especially in the abdominal area, relative to their wild-type counterparts. Surprisingly, the increase in weight gain was not due to increased food intake or decreased resting energy expenditure. Rather, these mice displayed reduced physical activity and reduced glucose oxidation. Such null muta-

tions were also independently generated by Nemoto et al. (19). Although this group did not comment on adipose stores they did observe impaired hepatic expression of enzymes involved in fatty acid oxidation in their model. This resulted in hepatic steatosis that could be reversed by estradiol treatment. These findings are consistent with reports of hepatic steatosis in humans induced by the E2 antagonist tamoxifen (21,30). Reports of sex-related defects in lipid and glucose metabolism in peroxisome proliferator–activated receptor (PPAR)-α null mice point to cross-talk between signaling pathways involving ERs and PPARs in the maintenance of energy metabolism (31).

Another key finding supporting a role for E2 in energy homeostasis is increased white adipose tissue in ER-α null mice of both sexes (20). These mice exhibit not only adipocyte hypertrophy and hyperplasia but also insulin resistance and glucose intolerance. This occurs in the absence of any change in food intake. These findings indicate that E2/ER-α signaling in both sexes plays an important role in development and function of white adipose tissue, which in turn affects overall energy metabolism. It also has recently been reported that ER-α null mice have lower metabolic rates (32).

In searching for possible mechanisms whereby E2 could be mediating this effect, leptin is one obvious target, given its crucial role in regulating whole-body energy homeostasis. Plasma leptin concentrations are higher in females independent of body composition (33) and ovariectomy in rats leads to decreases in adipose leptin mRNA levels and serum leptin concentrations that can be reversed by E2 replacement (34). Furthermore, E2 can regulate leptin synthesis and secretion directly through ER-mediated processes (35). It also has recently been discovered that the leptin promoter contains an ER response element (ERE) and in vitro functionality studies demonstrate that a combination of E2 and ER-α can induce expression from the leptin promoter (36). In light of this data, we measured plasma leptin levels in E2- and vehicle-treated Lean-huIAPP transgenic males and found no significant changes. This is consistent with the finding that E2 administered to $Lepr^{db}/Lepr^{db}$ mice also had an antihyperglycemic and weight-reducing effect (37). Since increasing expression of leptin in $Lepr^{db}/Lepr^{db}$ mice should be ineffective, we expected that the role of E2 would be independent of leptin signaling. It should be noted, however, that treatment of 1-year-old huIAPP-A^{vy} (~55 g) with E2 is closely reminiscent of the effects of Lep^{ob}/Lep^{ob} mice provided recombinant leptin (38). Leptin-treated Lep^{ob}/Lep^{ob} mice lost 30% body fat in 2 weeks or 40% in 33 days. Leptin also had a satiety effect in that these animals consumed less food but the means of losing this much fat in such a short time frame was primarily due to increases in energy expenditure, metabolic rate, and physical activity.

In addition to the effects of E2 seen in the huIAPP-A^{vy} animal, we also report dramatic glucose-lowering effects in lean mice that are transgenic for huIAPP, even after controlling for E2-mediated energy intake effects. Although hyperglycemia per se can induce insulin resistance, the sheer magnitude of this effect suggests that E2 could also be having a direct effect on islet function and viability. Our finding that ER-α is expressed in both mouse and human islets is consistent with this notion. The discovery of a novel ER-α 58-kDa isoform in islet tissue, ~9 kDa smaller than the normal 67-kDa product in the absence of the 67-kDa isoform, is intriguing. The sequence of the coding region and the arrangement of the exons required to generate this 58-kDa product are presently unknown. Although several alternative ER-α splice variants encoding gene products 46–52 kDa in size have been described in human tissues and cell lines, only the native 67-kDa ER-α isoform has been observed in the mouse (39–41). However, islet tissue was not included in previous analyses of ER-α tissue distribution (40,42,43). The discovery of a 52-kDa protein in human islets in the absence of the classic 67 kDa expressed in whole pancreas (as well as other tissues) suggests that the endocrine pancreas exclusively expresses the novel isoform and the exocrine portion expresses the 67-kDa isoform. The exclusive presence of this alternate 58-kDa product in mice or 52-kDa product in human islets suggested that the ER-α gene undergoes alternative splicing in this tissue and that this novel isoform may play a special regulatory role there. In fact, preliminary sequence analysis of human ER-α cDNAs from islets suggests that the 52-kDa product may be a result of alternative splicing in the ligand binding domain (J.G.G. and W.C.S., data unpublished). If confirmed, then the 46-kDa product found in primary osteoblasts generated by skipping the first exon and starting at exon 2 (41) is not the same product found in islets. It will be of interest to determine the functional differences between the novel isoform and the classic 67-kDa estrogen α receptors.

The salient observations in the treatment of mice with estrogen are that islet mass and insulin content are protected in E2-treated male Lean-huIAPP and huIAPP-A^{vy} transgenic mice, recapitulating the phenotype of female littermates and resolving the diabetic sexual dimorphism. Such protection may stem from a lower demand by peripheral tissues for insulin and thereby feedback to decrease expression of the huIAPP transgene under the Rip2 promoter or from a local effect within the environment of the β-cell. Although E2 has a pleiotrophic effect in lowering food intake, decreasing body weight in the obese animals and lowering blood glucose, the effect of preventing amyloid formation may be a result of the novel ER-α isoform specifically expressed in islets. The mechanism for E2 effect in peripheral tissues or within the islets is unknown. It should be noted that even with the lower dose formulation, the plasma concentrations of E2 achieved in these mice are 11-fold elevated compared to the endogenous concentrations found in nonpregnant female mice. Although the ER-α null and aromatase null mice argue for physiological rather than pharmacological effects of E2 on glucose metabolism, the studies described here should be extended to male mice subjected to physiological doses of E2.

Relevance of these findings to obesity and diabetes in humans is suggested by the phenotype of people possessing point mutations or variations in ER-α or aromatase. The patient with an ER-α deficiency was glucose intolerant and hyperinsulinemic (22). Hyperinsulinemia was also reported in male individuals harboring single amino acid

substitutions in the aromatase gene (44). Although treatment of disorders of glucose and lipid metabolism with E2 itself would not be suitable, elucidation of the signaling pathway in the various target organs/tissues would be useful in identifying other targets for therapeutic intervention.

Interestingly, humans also appear to display sexual dimorphism related to glucose metabolism and diabetes. Normal women in the general population have twice the adipose mass with only two-thirds the skeletal muscle/bone mass (45), yet the incidence of type 2 diabetes remains roughly equal between sexes up until the time of menopause. It was shown in Western society in the early 20th century (46) and confirmed in the later half of the century (47) that there is a significant spike in the incidence of diabetes for women aged 55–60 years. The time frame and data suggest that metabolic protection is attenuated when E2 levels fall during menopause, perhaps through decline in lipid partitioning (48).

Nearly 100% of type 2 diabetic subjects who have been tested for islet amyloid deposition have some deposits in contrast to nondiabetic subjects (49). The relationship between islet amyloid and hyperglycemia has been known since 1901 (50); however, the mechanism or factor which causes the monomeric IAPP to form into fibrils in persons with diabetes is still unknown (51). There is a strong association between obesity and diabetes (52), and it is known that fat metabolites have a pleiotrophic effect (53–55). Although a relationship between lipids and amyloid formation is still unclear, it is possible that fat metabolites may also play a role in shifting the balance from IAPP to amyloid, perhaps by acting as a locus for seeding or altering expression of chaperone proteins.

ACKNOWLEDGMENTS

We thank the JDF Human Islet Distribution Program at the University of Alberta for providing human islets and the Program in Critical Technologies in Molecular Medicine of the Department of Pathology at Yale University for providing all other human tissues. J.G.G. has received grant support from Pfizer Inc. W.Z. and K.Z. contributions were supported by grants from the American Diabetes Association and by the National Institute of Diabetes and Digestive and Kidney Diseases (National Institutes of Health Grant 41230).

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