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17-β Estradiol regulates proglucagon-derived peptide secretion in mouse and human α- and L cells

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Clinical and experimental data indicate a beneficial effect of estrogens on energy and glucose homeostasis associated with improved insulin sensitivity and positive effects on insulin secretion. The aim of the study was to investigate the impact of estrogens on proglucagon-producing cells, pancreatic α cells, and enteroendocrine L cells. The consequences of sexual hormone deprivation were evaluated in ovariectomized mice (ovx). Ovx mice exhibited impaired glucose tolerance during oral glucose tolerance tests (OGTT), which was associated with decreased GLP-1 intestinal and pancreatic secretion and content, an effect that was reversed by estradiol (E_2) treatment. Indeed, E_2 increased oral glucose–induced GLP-1 secretion in vivo and GLP-1 secretion from primary culture of mouse and human α cells through the activation of all 3 estrogen receptors (ERs), whereas E_2-induced GLP-1 secretion from mouse and human intestinal explants occurred only by ERβ activation. Underlying the implication of ERβ, its selective agonist WAY20070 was able to restore glucose tolerance in ovx mice at least partly through plasma GLP-1 increase. We conclude that E_2 directly controls both α- and L cells to increase GLP-1 secretion, in addition to its effects on insulin and glucagon secretion, highlighting the potential beneficial role of the estrogenic pathway and, more particularly, of ERβ agonists to prevent type 2 diabetes.

Introduction

The control of glucose homeostasis predominantly depends on the coordinated secretion of insulin by pancreatic β cells and proglucagon-derived peptides, glucagon, and glucagon-like peptide-1 (GLP-1) produced by pancreatic α- and gut L cells. Type 2 diabetes (T2D) has been attributed to insulin resistance and alterations of insulin secretion, but it is also characterized by a disrupted coordination between glucagon, insulin, and GLP-1 secretions. Glucagon regulates glycemia mainly through the stimulation of glucose production by the liver (1), whereas GLP-1 increases insulin and decreases glucagon levels (2). A newly developed model shows that GLP-1 production by pancreatic α cells plays a key role in the regulation of glucose homeostasis by acting on GLP-1 receptors of adjacent β cells, whereas GLP-1 produced by the enteroendocrine L cells acts on GLP-1 receptors located on vagal afferent nerves, thereby controlling hepatic glucose production (3, 4). Thus, the understanding of proglucagon-derived peptide biosynthesis and secretion appears to be highly relevant for a better design of therapeutic approaches for diabetes care.

Beside their pivotal role in sexual development and reproduction, estrogens prevent the occurrence of visceral adiposity, insulin resistance, and glucose intolerance in both humans and animal models (5, 6). Supporting the beneficial effects of estrogens on glucose homeostasis, women of reproductive age present higher sensitivity to insulin than age-matched men (7). Furthermore, estrogen-based replacement therapies reduce T2D incidence in postmenopausal women (8, 9). Importantly, women also exhibit about 20% higher GLP-1 responses to glucose than age-matched men, suggesting that sexual hormones may control GLP-1 release (10).

In rodent models, the beneficial effect of sexual hormones on glucose homeostasis is mediated by estradiol (E_2), the main endogenous reproductive hormone; its administration to ovariectomized (ovx) mice prevents diet-induced obesity and stimulates insulin secretion (11, 12). The beneficial effects of estrogens on energy balance, insulin sensitivity, and glucose homeostasis result from their combined actions both centrally and peripherally (13). Estrogens exert specific actions in insulin-sensitive tissues, notably by modulating the expression of key genes of glucose and lipid metabolism (14). It has been well established that

E_2 improves β cell function and viability (11); however, E_2 effects in proglucagon cells are unclear. In rodent models, ovariectomy increases plasma glucagon concentrations, and estrogen administration decreases the hepatic portal vein insulin/glucagon ratio (15). Furthermore, in vitro, E_2-treatment decreases glucagon secretion from male and female mice islets (16). Thus, better characterization of E_2 effects on proglucagon-producing cells is highly relevant to more fully understand the beneficial effects of hormonal treatment on metabolism, and it could lead to new therapeutic approaches to treat, or prevent, glucose homeostasis dysregulation occurring during and after menopause.

We have explored the regulation of glucose homeostasis by estrogens through their action on proglucagon-producing cells. We first investigated the metabolic phenotype of 1-week-old ovx adult female mice, compared with their littermate intact controls (sham), and showed that hormonal deprivation led to an alteration of glucose tolerance, along with a decrease in plasma GLP-1 levels. Short-term E_2 treatment reversed these effects. Then, we showed that E_2 directly controls pancreatic α- and intestinal L cells to stimulate GLP-1 production. Interestingly, whereas all estrogen receptors (ERs) appear to mediate the effect in α cells, only the ERβ pathway is involved in L cells. Finally, we demonstrated that E_2 effects were also observed on human α cells and intestinal explants from nondiabetic female donors.

Results

Impact of sexual hormonal deprivation on glucose homeostasis in female mice. To address the impact of sexual hormones on glucose homeostasis, we first used ovx mice and evaluated glycemic status 1 week after surgery, a duration allowing discrimination between acute and chronic effects of estrogens on glucose homeostasis (17, 18). Ovx mice and their control sham-operated littermates were maintained under normal chow diet (NCD); 1 week after ovariectomy, plasma E_2 in the ovx mice was undetectable, consistent with the 59.2% lower uterus weight observed in this group compared with their sham littermate controls (Supplemental Figure 1, A–C; supplemental material available online with this article; https://doi.org/10.1172/jci.insight.98569DS1). Importantly, we observed no difference in total body weight before and after surgery in the 2 groups (Supplemental Figure 1D). We then evaluated glycemia, insulinemia, and glucagonemia during oral glucose tolerance tests (OGTT) and observed that ovx mice exhibited higher fasting plasma glucose values (8.4 ± 0.3 mmol/l) compared with sham (7.5 ± 0.2 mmol/l) (Figure 1A); this was associated with lower insulinemia and higher glucagonemia measured at 0 minutes in the ovx group (Figure 1, C and D). Ovx mice had also glucose intolerance assessed by an 18.9 % increase in the AUC during OGTT (Figure 1B), with lower insulinemia at 5 and 120 minutes and higher glucagonemia at 30 and 120 minutes compared with sham mice (Figure 1, C and D). Circulating levels of GLP-1 during OGTT were lower in ovx than in sham 5 minutes after oral glucose load (Figure 1E). We also assessed pancreatic insulin, glucagon, and GLP-1 content. Insulin content was decreased, while glucagon content was increased in ovx mice. Interestingly, we found that pancreatic GLP-1 content was lower in ovx mice compared with control (0.0028 ± 0.0001 ng/μg of protein and 0.0047 ± 0.0008 ng/μg of protein for ovx and sham mice, respectively) (Supplemental Figure 2, A–C). In addition, we found higher glucagon and lower GLP-1 content in FACS-purified α cells and lower content of insulin in FACS-purified β cells from ovx compared with sham mice (Supplemental Figure 2, D–F). Of note, there was no difference in α-Venus[+] and β-Cherry[+] cell sorted numbers between sham and ovx mice, indicating that changes in pancreatic hormone content are due to changes in cell content (Supplemental Figure 2G). Overall, we demonstrated that short-term sexual hormone deprivation significantly altered glucose homeostasis in female mice, with decreases of plasma insulin and GLP-1 levels and an increase of glucagonemia.

E_2 administration improves glucose tolerance in ovx mice. We then investigated whether E_2 administration was able to restore glucose tolerance in ovx female mice. Ovx mice were treated during 48 hours with vehicle or E_2 (80 μg/kg/day). Treatment was effective, since ovx+E_2 female mice presented circulating E_2 levels close to those measured in sham mice and a significant 2.84-fold increase in uterus weight compared with the vehicle-treated group (Supplemental Figure 1, A–C). During OGTT, ovx+E_2 mice exhibited improved glucose tolerance, as assessed by lower glycemia at 15, 30, and 60 minutes, along with lower AUCs (Figure 2, A and B). This was correlated with higher plasma insulin at 5 minutes and lower glucagon at 120 minutes, as well as higher GLP-1 levels after glucose load at 5 minutes (Figure 2, C–E). We also measured insulin and glucagon contents from pancreases of both groups. We observed that E_2 treatment led to increased insulin but decreased glucagon contents compared with sham mice (Figure 3, A and B). Moreover, E_2 administration also induced increases of pancreatic GLP-1 content (Figure 3C), an effect also observed in
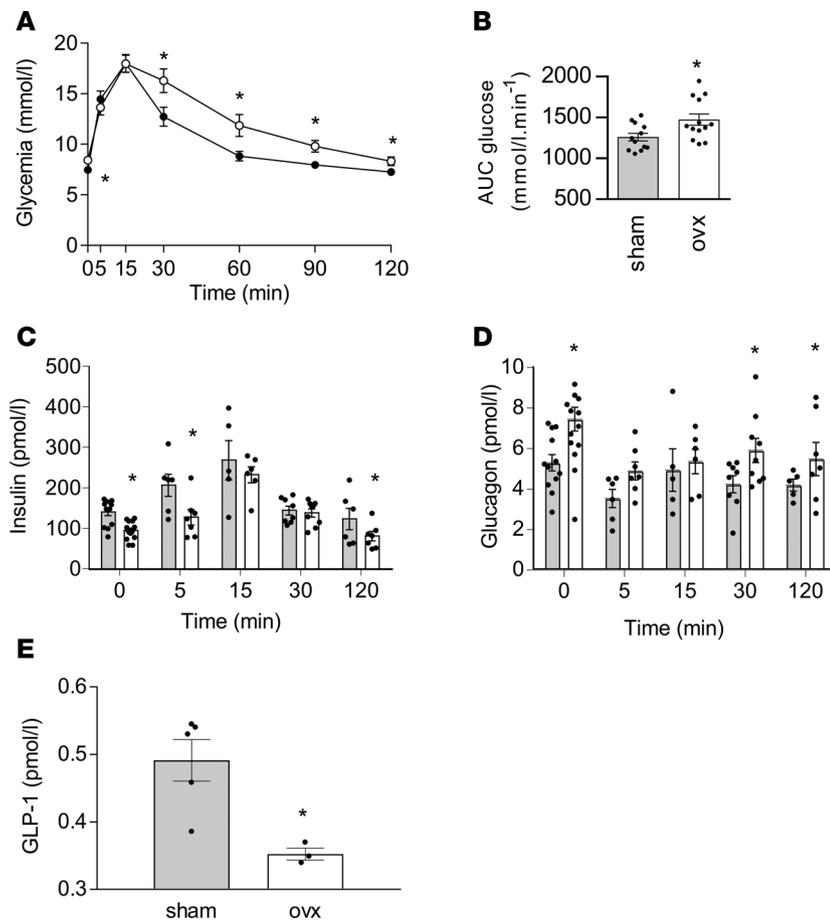
Figure 1. Ovariectomized female mice develop glucose intolerance. Plasma glucose levels during OGTT (2 g/kg) (**A**) and the related AUCs (**B**) in sham (gray bars, n = 12) and ovx (white bars, n = 13) mice, 1 week after the surgery. Plasma insulin (**C**), glucagon (n = 6 at least per group) (**D**), and GLP-1 levels 5 minutes after gavage (sham, n = 5; ovx, n = 3) (**E**) during the OGTT. One-way repeated ANOVA with Bonferonni post hoc test analyses were performed for glycemia (**A**), and 2-tailed Student's t test statistical analyses were performed for the other results. *P ≤ 0.05 for sham vs. ovx mice.

isolated islets (data not shown). To better characterize E_2 effects, we measured cellular contents of insulin from sorted β cells, as well as glucagon and GLP-1 cellular contents of sorted α cells. We observed that insulin contents from β cells of ovx mice were increased after E_2 administration (+47.3%), whereas glucagon content was decreased (–44.9%) and GLP-1 increased (+71.2%) in α cells (Figure 3, D–F). No difference in α-Venus+ and β-Cherry+ cell numbers was observed between the groups (Figure 3G). We conclude that E_2 administration for 48 hours to ovx mice partially reverses the consequences of sexual hormonal deprivation on glucose metabolism and regulates pancreatic insulin, glucagon, and GLP-1 synthesis and secretion in response to glucose.

E_2 directly acts on α- and β cells to regulate their function. Since E_2 is known to modulate the transcription of target genes, we assessed the expression of proglucagon mRNA and of genes coding for enzymes responsible for its maturation, prohormone convertase 1/3 and -2 (PC1/3 and PC2). To this end, we isolated purified fractions of α cells (Venus+cells) and β cells (Cherry+cells) from ovx mice and treated them in vitro with 1 × 10−8 mol/l E_2 for 48 hours. E_2 administration had no effect on proglucagon mRNA (*Gcg*) levels but was able to modulate *Pcsk1/3* and *Pcsk2* mRNA levels. Indeed, E_2 led to a 38.1% decrease of *Pcsk2* mRNA levels and an 80.1% increase of *Pcsk1/3* mRNA levels (Figure 4A). These observations were coupled with decreased glucagon and increased GLP-1 contents in E_2-treated α cells (Figure 4, B and C), leading to an increase of the GLP-1/glucagon ratio (Figure 4D). Then, we assessed the glucagon and GLP-1 release of sorted α cells after E_2 administration and found decreased glucagon (–34.8%) and increased GLP-1 releases (+71.6%) (Figure 4, E and F). Similarly, we investigated E_2 effects on insulin content and release from sorted β cells. As expected, E_2 was able to directly increase *Ins1* and *Ins2* mRNA levels, along with insulin content in β cells (Figure 4, G and H). This effect was associated with an increased capacity of these cells to release insulin (Figure 4I).

We next assessed if the increase in GLP-1 secretion induced by E_2 in α cells could modulate insulin secretion and if glucose was able to modulate GLP-1 secretion by α cells. To this end, we decided to perform α/β cell cocultures; α cells were cultured separately in the presence of E_2 during 48 hours to avoid
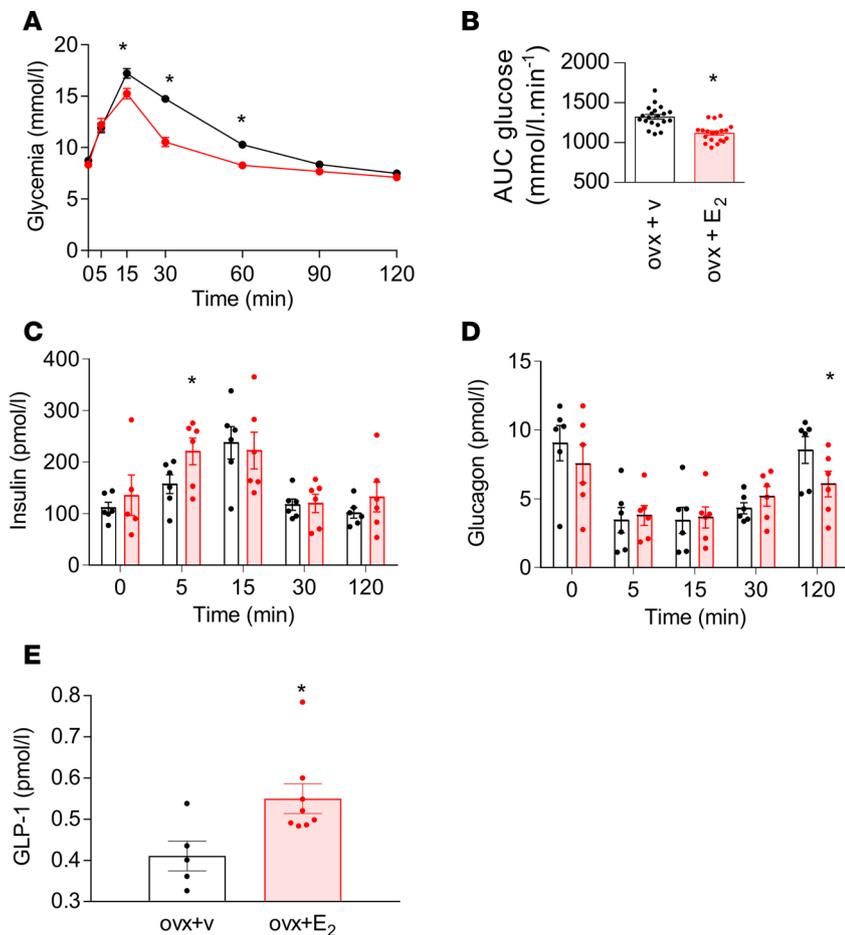
Figure 2. Exogenous estradiol improves glucose tolerance during OGTT in ovx mice. Plasma glucose levels during OGTT (2 g/kg) (**A**) and the related AUCs (**B**) in ovx+vehicle (black circles, white bars; n = 20) and ovx+E$_2$ (80 μg/kg/48 hours; red circles, red bars; n = 19) mice. Plasma insulin (ovx, n = 6; ovx+E$_2$, n = 6) (**C**), glucagon (ovx, n = 6; ovx+E$_2$, n = 6) (**D**), and GLP-1 levels 5 minutes after gavage (ovx, n = 5; ovx+E$_2$, n = 8) (**E**) during an OGTT. One-way repeated ANOVA with Bonferonni post hoc test analyses were performed for glycemia (**A**), and 2-tailed Student's t test statistical analyses were performed for the other results. *$P \le$ 0.05 for ovx+vehicle vs. ovx+E$_2$ mice.

direct effects of E$_2$ on β cells. We next assessed the response to glucose and to the GLP-1 receptor antagonist Exendin 9-39 (Ex9-39) after mixing E$_2$-treated α cells with untreated β cells. E$_2$-treated cocultures exhibited higher insulin secretion in basal conditions (5.6 mmol/l glucose) and at high glucose concentration (16.7 mmol/l) (Figure 4J); we also observed that glucose was able to increase GLP-1 secretion by α cells, as recently described (19) (Figure 4K). Interestingly, insulin responses to high glucose from E$_2$-treated α cells mixed with β cells were lowered by treatment with Ex9-39, compared with vehicle-treated cells (Figure 4J). These data indicate that increased GLP-1 secretion from α cells induced by E$_2$ leads to improved glucose-stimulated insulin secretion (GSIS) from adjacent β cells.

Functional and molecular characterization of intestinal L cells from ovx mice. We showed that E$_2$ administration to ovx mice increased plasma GLP-1 levels to values similar to those observed in intact female mice (Figure 1E and Figure 2E). We thus analyzed the effects of E$_2$ on enteroendocrine L cells. We first investigated expression of *Gcg* and *Pcsk1/3* genes and GLP-1 contents of sorted L cells from ovx+v and ovx+E$_2$ mice (Figure 5, A and B). *Pcsk1/3* mRNA levels and GLP-1 contents were higher in intestinal L cells isolated from E$_2$-treated ovx female mice (+61.8% and +59.1 %, respectively), while *Gcg* mRNA levels were unaffected (Figure 5, A and B). We found no difference in L cell number between the groups (Figure 5C). We next evaluated GLP-1 release in response to E$_2$ treatment using intestinal explants from ovx mice. E$_2$-treated explants exhibited 75.5% higher GLP-1 release than explants treated with vehicle (Figure 5D). Interestingly E$_2$ did not affect gastric inhibitory polypeptide (GIP) release in the same conditions (Supplemental Figure 3). These data indicate that E$_2$ increases release and content of GLP-1 but not GIP from intestinal cell explants.

E$_2$ increases GLP-1 secretion and decreases glucagon in α cells through the activation of all 3 ERs, whereas only ERβ activation increased GLP-1 secretion from L cells. To investigate by which receptors E$_2$ affects pancreatic α- and intestinal L cells, we first measured mRNA levels of ER from sorted α cells, L cells, and enteroendocrine GLUTag cells. All 3 ERs — ERα, ERβ, and G protein-coupled estrogen receptor (GPER) — were expressed at relatively similar levels in α cells, whereas ERβ was largely dominant in L cells. In GLUTag cells, only
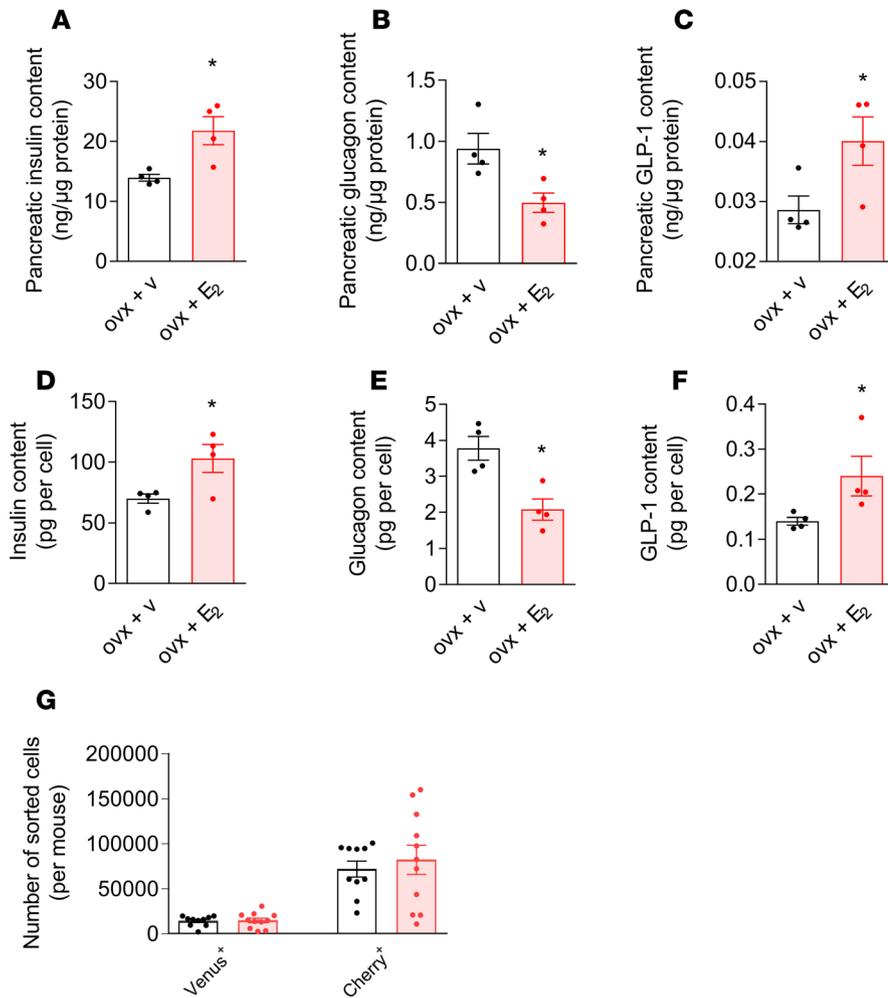
Figure 3. In vivo effects of exogenous estradiol administration on the pancreas of ovx mice. Insulin (**A**), glucagon (**B**), and GLP-1 (**C**) contents were evaluated from pancreases of ovx+vehicle (white bars, $n = 4$) and ovx+E_2 (80 μg/kg/48 hours; red bars, $n = 4$) mice. Insulin was measured in FACS-purified β cells (ovx, $n = 4$; ovx+E_2, $n = 4$) (**D**), glucagon (**E**), and GLP-1 (**F**) measured in FACS-purified α cells (ovx, $n = 4$; ovx+E_2, $n = 4$). Both α- (Venus$^+$ cells) and β cell (Cherry$^+$ cells) numbers were evaluated by FACS from ovx ($n = 10$) and ovx+E_2 ($n = 11$) dissociated pancreatic islets (**G**). Two-tailed Student's t test statistical analyses. *$P \leq 0.05$ for ovx+vehicle vs. ovx+E_2 mice.

ERβ was expressed (Figure 6A). To assess the role of ERs, we performed in vitro assays using selective ER modulators (SERMs). Treatment of α cells with E_2, the ERα selective agonist PPT (20), the GPER agonist G1 (21), and the ERβ selective agonist WAY (22) separately resulted in increases of GLP-1 release (Figure 6B). We also measured the expression of *Gcg* and *Pcsk1/3*, as well as *Pcsk2*, and showed that, as for E_2, all estrogen receptor agonists increase *Pcsk1/3* and decrease *Pcsk2* mRNA levels, whereas *Gcg* mRNA levels are unchanged (Figure 7). We performed the same experiments on intestinal explants of ovx mice; only E_2 and the ERβ selective agonist WAY were able to significantly increase GLP-1 release (Figure 6C). We next analyzed the effects of E_2 and SERMs on GLUTag cells, as we could not obtain viable pure primary L cells in culture. We measured expression of *Gcg* and *Pcsk1/3*, as well as GLP-1 content and release after 48 hours of incubation with E_2 (1×10^{-8} mol/l). As observed in sorted L cells from ovx+E_2 mice, E_2 treatment increased *Pcsk1/3* (+22.7%) but not *Gcg* mRNA levels (Supplemental Figure 4A). This was correlated with an increase of GLP-1 content and release (Supplemental Figure 4, B and C). As observed in intestinal explants, only ERβ activation was able to increase GLP-1 release from intestinal cells (Figure 6, C and D). We conclude that E_2 regulates GLP-1 release from L cells through ERβ, whereas all 3 receptors mediate E_2 effects on GLP-1 release from α cells.

In vivo administration of WAY200070 increases GLP-1 circulating levels in ovx mice. We then determined if chronic WAY administration was able to restore glucose tolerance in ovx female mice. Ovx mice were treated 48 hours with vehicle or WAY (10 mg/kg/day, a dose that has previously been described to be effective; ref. 22). During OGTT, ovx+WAY mice exhibited a better tolerance to glucose than vehicle-treated mice, as assessed by a lower glycemia at 15 and 30 minutes, along with a lower AUC (Figure 8, A and B), whereas the activation of ERα or GPER had weaker or no significant effects on glucose tolerance at the same dose (Supplemental Figure 5 and 6). Indeed, only WAY administration increased plasma insulin at 5 minutes and decreased plasma glucagon at 5 and 120 minutes after glucose load (Figure 8, C and D). The improvement of glucose tolerance under WAY administration was associated with higher plasma GLP-1
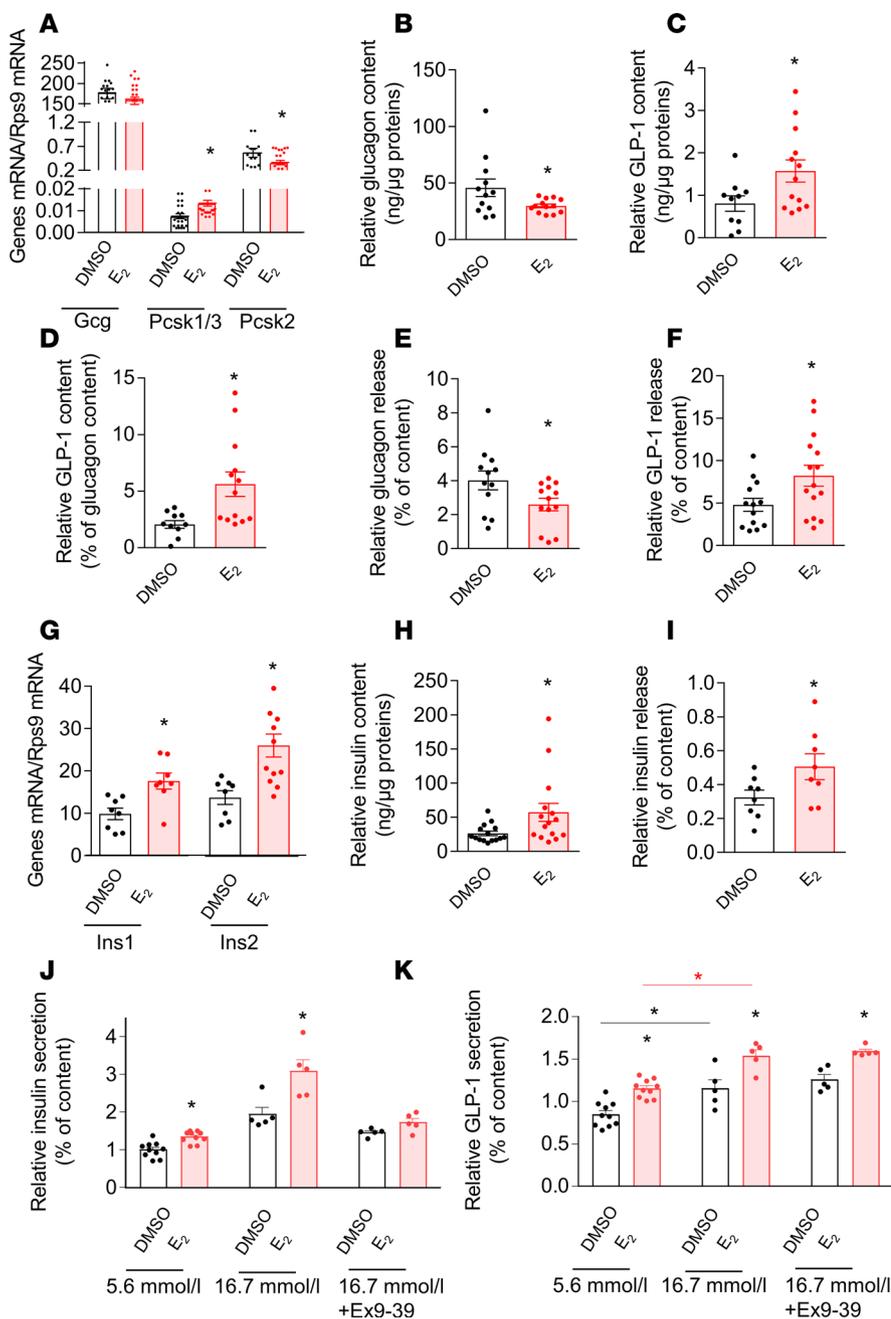
Figure 4. Estradiol directly affects α- and β cells from ovx mice in vitro. Both α- and β cells isolated from ovx mice were cultured with vehicle (white bars), or 17β-estradiol (E_2, 1 × 10^{-8} mol/l) (red bars). Relative mRNA levels were quantified in sorted α cells (n = 11 in each group) (**A**). Glucagon (**B**) and GLP-1 (**C**) contents were assessed in sorted α cells treated for 48 hours with vehicle (white, n = 11) or with E_2, 1 × 10^{-8} mol/l (red, n = 13). Contents are normalized to total protein. Ratios of GLP-1 to glucagon content of sorted α cells are represented in **D**. Glucagon (**E**) and GLP-1 (**F**) levels were quantified in supernatants relative to the contents of glucagon and GLP-1, respectively, after 6 hours of continuous release in complete medium of purified α cells (vehicle, n = 11; E_2, n = 13). Relative mRNA level quantification was assessed in sorted β cells (n = 12 in each group) (**G**), as well as insulin contents relative to total protein amount (**H**) and release relative to insulin content (**I**) (vehicle, n = 15; E_2, n = 15). Purified α- and β cells isolated from ovx mice were cultured in 2 separate drops, and only α cells were treated either with vehicle (white bars, n = 5) or 17β-estradiol (E_2, 1 × 10^{-8} mol/l) (red bars, n = 5); both drops were then joined during the secretion tests. Insulin (**J**) and GLP-1 (**K**) levels were assessed in response to glucose and a GLP-1 receptor antagonist (Ex9-39). Two-tailed Student's t test statistical analyses. *$P ≤$ 0.05 for vehicle- vs. E_2-treated cells.

levels 5 minutes after glucose load (Figure 8E). Our data suggest that the activation of the ERβ pathway in vivo is critical to glucose homeostasis, not only by regulating pancreatic α- and β cells, but also by regulating intestinal L cell function to increase stimulated plasma GLP-1 levels.

Primary human α- and intestinal mixed cells are responsive to E_2 and SERMs. We then investigated the effects of SERMs on primary isolated human α cells. These cells were isolated from pancreases from nondiabetic, nonobese woman (Supplemental Figure 7). After 48 hours in culture in hormonal-deprived medium, cells were treated for 48 hours with vehicle, E_2, or the different SERMs (1 × 10^{-8} mol/l). As observed in mouse α cells, E_2, and all 3 ERs, agonists were able to significantly increase GLP-1 release from human α cells (Figure 9A). We also observed a trend toward a decrease of glucagon by all 3 agonists (Figure 9B). In isolated β cells, E_2 treatment also increased GSIS (Figure 9C). We also investigated GLP-1 secretion from intestinal explants coming from the same donors and showed that, similar to mice, activation of the ERβ pathway increased GLP-1 release (Figure 9D). We conclude that E_2 has similar effects on both mice and human pancreatic α- and intestinal L cell to increase GLP-1 and decrease glucagon release.
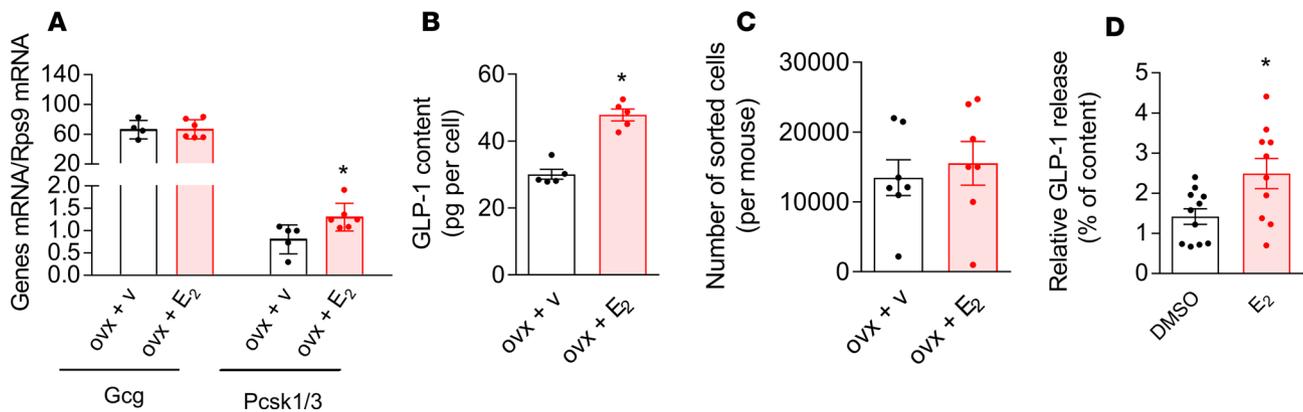
Figure 5. Effects of estradiol on intestinal L cells of ovx mice in vivo and in vitro. Relative *Gcg* and *Pcsk1/3* mRNA levels ($n = 6$ animals in each group) (**A**) and GLP-1 cellular contents were measured from sorted intestinal L cells of ovx+vehicle (white bars, $n = 5$) and ovx+E$_2$ (red bars, $n = 5$) mice (**B**). L cell numbers (Venus$^+$ cells) from the small intestine were also evaluated by FACS ($n = 7$ animals in each group) (**C**). GLP-1 levels were quantified in supernatants and contents from primary cultures of mixed intestinal cells (**D**) of ovx mice, cultured for 48 hours with vehicle DMSO (white bars, $n = 11$) or with 17β-estradiol (E$_2$, 1×10^{-8} mol/l, red bars, $n = 10$) after 2 hours of continuous release in complete fresh medium. Two-tailed Student's *t* test statistical analyses. *$P \leq 0.05$ for ovx+vehicle vs. ovx+E$_2$ mice, and for DMSO- vs. E$_2$-treated mixed cells.

Discussion

Besides their pivotal role in sexual development and reproduction, estrogens prevent the occurrence of visceral obesity, insulin resistance, and glucose intolerance in women. This is supported by reduction of T2D incidence in postmenopausal women who received hormonal replacement therapy (8, 9). Furthermore, experimental data indicate that disruption of estrogen signaling results in an obese and dysmetabolic phenotype, induced by decreased energy expenditure and increased food intake. Estrogens also exert specific actions on the pancreas and, notably, on insulin biosynthesis and secretion (11).

We investigated further E$_2$ effects on endocrine pancreas and intestine and, notably, its impact on glucagon and GLP-1 synthesis and secretion by proglucagon-producing cells, pancreatic α cells, and enteroendocrine L cells. We show that 1 week of sexual hormonal washout was able to impair glucose tolerance, without any change in weight under NCD, consistent with unchanged fat and lean body mass, as previously shown (18). We chose 1 week as the period, allowing us to study the impact of hormonal deprivation before the occurrence of significant insulin resistance or weight gain in these mice, as longer periods of hormonal deprivation induce several metabolic changes with increased body weight and food intake, as well as a reduction of locomotor activity (18, 23, 24). Conversely, glucose intolerance in ovx mice was improved by 48 hours of E$_2$ administration. We observed a higher capacity of β cells to release insulin, both basally and in response to glucose, and reduced glucagon secretion by α cells during fasting and after oral glucose load. Whether a longer duration of treatment would have led to more pronounced effects on glucose homeostasis remains to be evaluated. Several previous studies, using whole mice islets, have suggested that E$_2$ has a direct insulinotropic effect by blocking the ATP-sensitive potassium channels (25). This is in agreement with GLP-1/E$_2$–based studies, where the administration of this estrogenic component, targeting the GLP-1–responsive cells like β cells, has been shown to have beneficial effects on glucose homeostasis (26).

Previous studies have indicated that all 3 ERs — ERα, ERβ, and GPER — are expressed in both rodent and human α- and β cells (27, 28). E$_2$ has been reported to have rapid insulinotropic effects on mouse isolated β cells (29, 30), attributed to ERα activation, since mice presenting a β cell–specific inactivation of ERα exhibit insulin secretion defects (31, 32). However, the other receptors, ERβ and GPER, also play a role in β cells, since they both have been demonstrated to regulate insulin secretion in mouse isolated islets (33, 34). E$_2$ also increases insulin gene expression through the stimulation of NeuroD1 binding to the insulin gene promoter under ERα activation (32, 35). The use of FACS-purified β-Cherry$^+$ and α-Venus$^+$ cells from ovx mice allowed us to confirm the direct functional E$_2$ effects on insulin secretion and to demonstrate effects on α cells — particularly on glucagon secretion and content. Previously, E$_2$ had been shown to acutely decrease glucagon secretion in streptozotocin-treated male mice implanted with human or mouse islets and to reduce glucagon secretion through the activation of GPER in human isolated islets (27, 28). Here, we show that E$_2$ directly acted on isolated α cells through
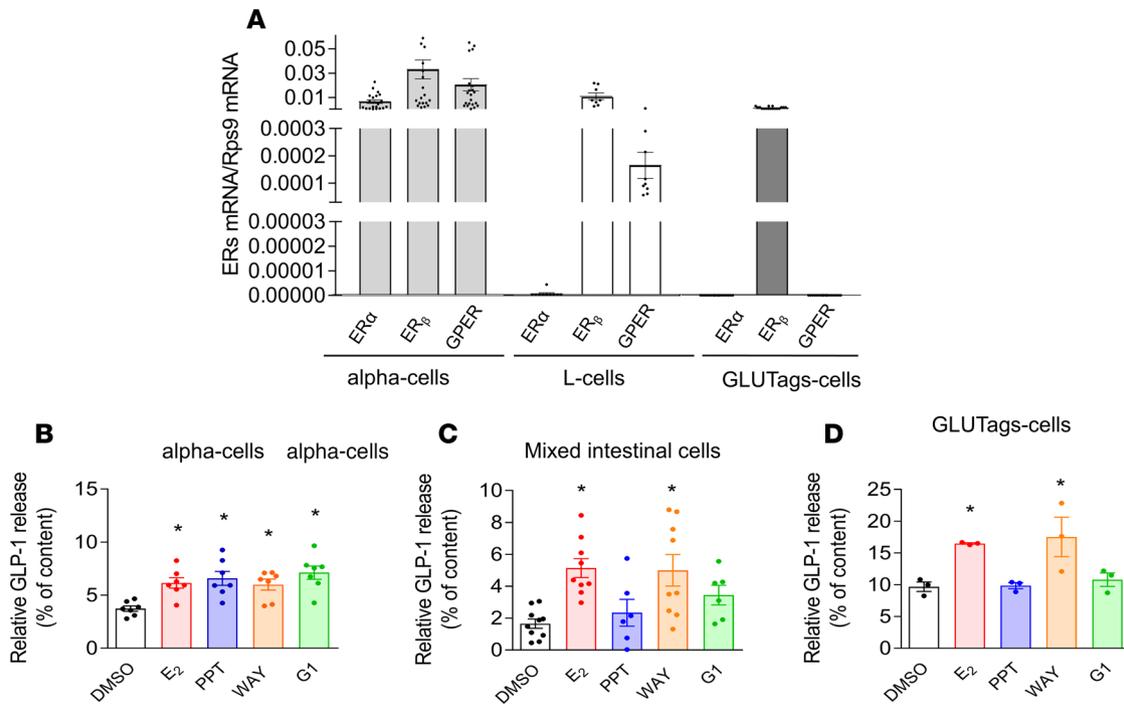
Figure 6. Estrogen receptors mediate estradiol effects in α- and L cells. Sorted α- and L cells isolated from 13-week-old female mice ovariectomized for a week and GLUTag cells were studied for the expression of estrogen receptors (ERs) (**A**). GLP-1 releases during 2 hours in fresh medium were quantified in supernatants relative to contents of purified α cells (n = 7 experiments in each group) (**B**), of dissociated mixed cells from small intestine (jejunum/ileum) (n = 6 experiments in each group) (**C**), and of GLUTag cell cultures (n = 5 experiments in each groups) (**D**), cultured during 48h hours with vehicle (DMSO) or the several SERMs (E_2, PPT, WAY, and G1 at 1×10^{-8} mol/l). One-way ANOVA with the Dunnett's multiple comparison post test. *$P \le 0.05$ for vehicle- vs. E_2 agonist–treated cells.

all 3 ERs; however, by contrast to its effects on insulin gene expression, E_2 did not modulate glucagon gene expression. Rather, E_2 altered the *Pcsk1/3*-to-*Pcsk2* mRNA ratio and changed the maturation of proglucagon-derived peptides to increase GLP-1 and decrease glucagon synthesis and secretion. PC2 levels have previously been shown to be crucial for the maturation of proglucagon into glucagon (36). The modulation of the expression of the PC1/3 and PC2 has been previously observed in α cells. In rats treated by streptozotocin, an increase of PC1/3 and PC2 that colocalize with glucagon in the same granules of α cells was reported (37). Hansen et al. also demonstrated that PC1/3 expression was increased in the *P. obesus* α cells in response to hyperglycemia (38), leading to an increase in GLP-1/GLP-2 levels and improved glucose tolerance (39). Sexual hormones have also been suggested to increase PC1/3 expression in α cells; indeed, Kilimnik et al. demonstrated an increased number of PC1/3+/glucagon+ cells at 9 days after coitus, associated with higher plasma E_2 levels in mice during pregnancy, consistent with the observed increased GLP-1 plasma levels in female rats prior to estrus (40–42).
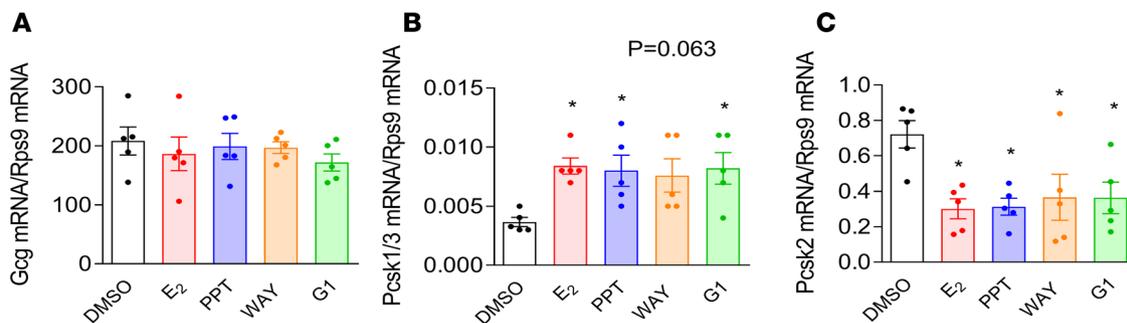

Figure 7. Estrogen receptors mediate the PC2-to-PC1/3 switch in mouse α cells. Sorted α cells isolated from 13-week-old female mice ovariectomized for a week were cultured during 48 hours with vehicle (DMSO), E_2, or SERMs (PPT, WAY, and G1 at 1×10^{-8} mol/l). Expression of *Gcg* (**A**), *Pcsk1/3* (**B**), and *Pcsk2* (**C**) mRNA (n = 5 per group) was assessed. One-way ANOVA with the Dunnett's multiple comparison post test was performed. *$P \le 0.05$ for vehicle- vs. E_2 agonist–treated cells.
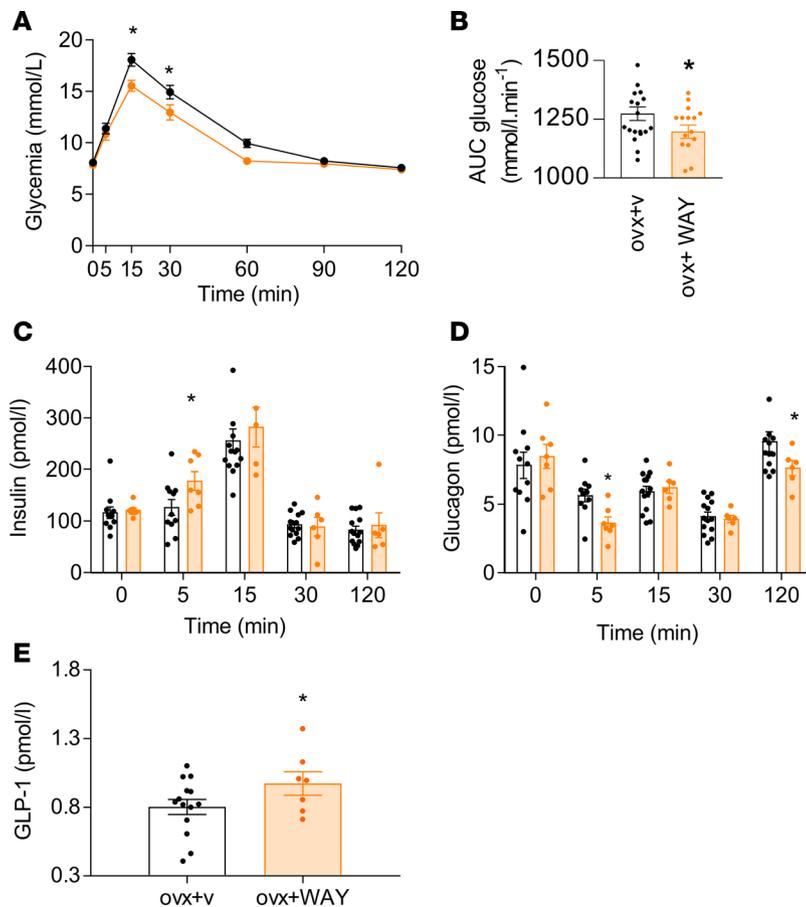
Figure 8. ERβ activation improves glucose tolerance during OGTT in ovx mice. Plasma glucose levels during OGTT (2 g/kg) (**A**) and the related AUC (**B**) in ovx+vehicle (black circles, white bars; $n = 20$) and ovx+WAY (100 mg/kg/48 hours; orange circles, orange bars; $n = 13$) mice are represented. Plasma insulin (ovx, $n = 11$; ovx+WAY, $n = 6$) (**C**), glucagon (ovx, $n = 11$; ovx+WAY, $n = 6$) (**D**), and GLP-1 levels 5 minutes after gavage (ovx, $n = 9$; ovx+WAY, $n = 8$) (**E**) during an OGTT. One-way repeated ANOVA with Bonferroni post hoc test analyses were performed for glycemia (**A**), and 2-tailed Student's t test statistical analyses were performed for the other figures. *$P \leq 0.05$ for ovx+vehicle vs. ovx+WAY mice.

Marchetti et al. demonstrated that only 21% of nondiabetic human α cells coexpressed glucagon and PC1/3 (43). We propose that the number of α cells, which express PC1/3 and thus produce GLP-1, may be increased under E_2 treatment or, alternatively, that PC1/3 expression or activity are increased in these subpopulation of glucagon$^+$/PC1$^+$ cells.

We demonstrated that all 3 ERs are implicated in the increase of GLP-1 secretion by α cells and, most importantly, that this increase is important for GSIS and probably for glucose tolerance in vivo, in agreement with Chambers et al., who demonstrated that pancreatic production of GLP-1 is necessary for the regulation of glycemia (3). In addition to their effects on the endocrine pancreas, we show that E_2 also directly acted on enteroendocrine L cells to increase GLP-1 production and secretion, improving glucose tolerance. Indeed, during OGTT, ovx mice had decreased GLP-1 secretion in response to glucose compared with their sham-operated littermates, supporting the importance of GLP-1 secretion in intact mice for the control of glucose homeostasis and its effects on the inhibition of glucagon secretion. This is consistent with what is observed in humans, where it was shown that women had a better GLP-1 response to glucose than aged-matched men (10). Progesterone (P_4) might have been a candidate for this effect, as P_4 increases GLP-1 biosynthesis and secretion when administered orally (44). We show, however, that E_2 restored GLP-1 secretion in ovx mice and directly stimulated L cells; E_2 also increased the binding of P_4 to its receptor in islets, resulting in an increased response of insulin to glucose (45–47). It is then possible that in vivo estrogens maximize their effects through the action of P_4 on both pancreatic α- and intestinal L cells. Our observations are not limited to mouse α- and L cells, but also extend to humans, indicating that E_2 effects on GLP-1 and glucagon are conserved throughout species. Interestingly, whereas E_2 affects L cells, it does not regulate GIP secretion, indicating that E_2 does not regulate all incretins.

Thus, our results show that E_2 induces GLP-1 secretion in ovx mice by increasing GLP-1 release from pancreatic α cells through the activation of any of the 3 ERs, both in mouse and human cells, and from human and mouse intestinal L cells in a ERβ-dependent pathway. Indeed, the ERβ agonist WAY200070 was able to restore glucose tolerance in ovx mice by stimulating GLP-1 secretion from L cells and probably
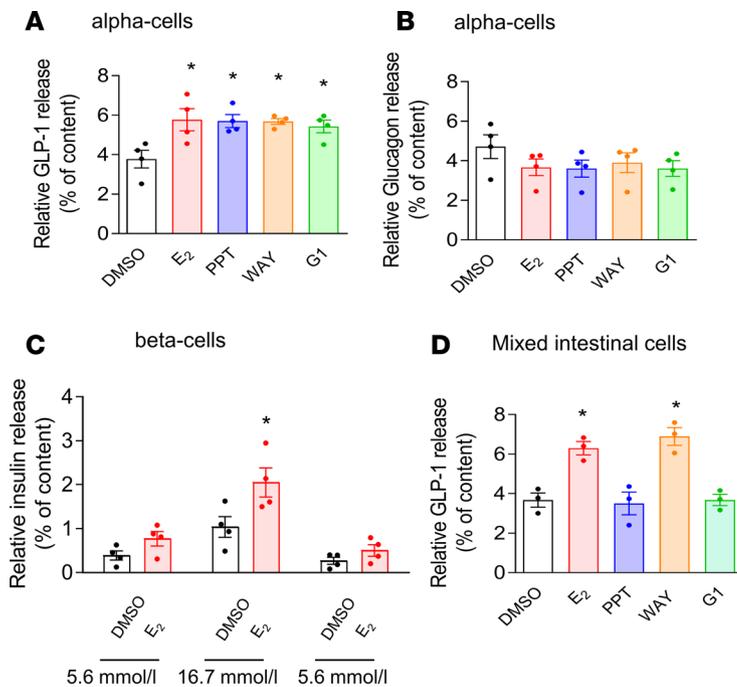
Figure 9. Estradiol increases GLP-1 release from human α- and L cells in vitro. GLP-1 (**A**) and glucagon (**B**) releases were quantified in supernatants relative to content of sorted human α cells (n = 4 experiments in each group). (**C**) Glucose-stimulated insulin secretion in Human isolated β cells (n = 4 in each group). (**D**) GLP-1 secretion of dissociated mixed cells from human small intestine (duodenum) (n = 3 experiments in each group). One-way ANOVA with the Dunnett's multiple comparison post test. *$P \leq 0.05$ for vehicle- vs. E_2 agonist–treated cells.

from α cells, as indicated in vitro. Thus, WAY200070 has a strong antidiabetic action, supporting the therapeutic potential of the ERβ pathway (22), contrasting with the previously proposed diabetogenic role of ERβ, since KO mice exposed to a high-fat diet had improved insulin sensitivity and glucose tolerance (48).

Overall, the present study highlights that estrogens, and more particularly E_2, improve glucose homeostasis by direct effects on β cells and on pancreatic α- and intestinal L cells to stimulate GLP-1 secretion in both mice and humans. The activation of the ERβ pathway was sufficient to mediate this effect, making the targeting of this specific ER subtype a promising drug target, as is already the case for treatment of cancer and multiple sclerosis (49).

Methods

Animals. Transgenic mice C57Bl/6J-Tg (GLU-Venus x INS-Cherry) were generated by Pedro L. Herrera (Geneva, Switzerland). The GLU-Venus x INS-Cherry mice express, specifically, the Venus fluorochrome in proglucagon-producing cells and the Cherry fluorochrome in insulin-producing cells (50, 51). Mice were bred in conventional housing (University of Geneva Medical School) according to ethical approbation by the Swiss federal committee. For the ovx and sham groups, 12-week-old female littermate mice were fed NCD and were ovx or sham operated (23). Experiments were performed 1 week after the surgery. For the ovx+vehicle and ovx+E_2/WAY groups, 12-week-old female littermate mice were ovx and were s.c. daily injected with castor oil containing vehicle (EtOH/DMSO), 17β-E_2 (80 μg/kg/day, MilliporeSigma), or WAY200070 (WAY, 10 mg/kg/day, MilliporeSigma) 1 week after the surgery, 48 hours before the final test. It is to note that ovx and ovx+vehicle mice are different animals.

SERM injections were performed 1 week after ovariectomy at 13 weeks of age, as described in Supplemental Figure1A. The 13-week-old littermate ovx mice were s.c. daily injected with castor oil containing vehicle (DMSO), or 4,4',4''-(4-Propyl-[1*H*]-pyrazole-1,3,5-triyl)trisphenol (PPT, 10 mg/kg/day, Tocris, half-maximal effective concentration [EC_{50}], 0.2 nmol/l), WAY200070 (WAY, 10 mg/kg/day, MilliporeSigma, EC_{50}, 2.3 nmol/l) or G1 (G1, 10 mg/kg/day, Tocris, EC_{50}, 2.0 nmol/l) during 48 hours.

In vivo experiments and blood sample collections. OGTT, ex vivo experiments and sample collections were performed 1 week after hormonal deprivation (for sham/ovx groups) or 48 hours after vehicle and E_2/WAY/PPT/G1 injections (for ovx+v, ovx+E_2, ovx+WAY, ovx+PPT, ovx+G1 groups). Glycemia, plasma insulin, glucagon, and GLP-1 levels were measured before and after D-glucose gavages at 2 g/kg on 6 hours–fasted mice. Blood glucose levels were measured by sampling from the tail vein of gently held conscious mice from 0–120 minutes after glucose administration. At the different time points (only 2 points per animal per experiment), a blood sample (60 μl) was collected and immediately mixed with 6 μl of a chilled

solution containing 1.5 mmol/l aprotinin (Applichem) and 0.01 mmol/l Diprotin A (MilliporeSigma) for measurement of plasma insulin, glucagon, and total GLP-1 levels.

Glucagon, insulin, GLP-1, and GIP measurements. Plasma, tissues (whole pancreas), cell extracts, and supernatants from sham, ovx, ovx+vehicle, and ovx+E$_2$/WAY mice were evaluated with specific ELISA kits for mature glucagon, insulin (Mercodia AB), total GLP-1 (Meso Scale Discovery), and total GIP (MilliporeSigma) peptide detections.

Murine primary cell preparation and cellular cultures. At the end of the protocol, primary α-Venus[+] and L cells and primary β-Cherry^{+} cells from sham, ovx, ovx+v, and ovx+E$_2$ mice were separated from non-Venus and non-Cherry cells by FACS using Bio-Rad S3 and Moflo Astrios, after standard isolation procedures on the pancreas and small intestine, as described (50–52). FACS-purified α-, β- , and L cells were collected in acid/ethanol mixture (1.5% HCl/75% ethanol) for glucagon, insulin, and GLP-1 cellular content measurements or in RNAse-free lysis buffer (Qiagen RLT+ Buffer) for gene expression analyses. We confirmed that Venus^{+} cells correspond to highly enriched glucagon-expressing cells and that Cherry^{+} cells correspond to highly enriched insulin gene–expressing cells (Supplemental Figure 1, A and B).

Primary cultures of sorted α- and β cells of ovx mice were seeded on 804G matrix (51) in DMEM 5.6 mmol/l glucose phenol red free medium, supplemented with 10% Charcoal Stripped FBS (MilliporeSigma), 10 mmol/l Na-pyruvate, and antibiotics (100 μg penicillin-streptomycin [Thermo Fisher Scienetific] and gentamycin [Thermo Fisher Scientific]). After an overnight recovery, these cells were treated with vehicle (DMSO), E$_2$ (1 × 10^{-8} mol/l, MilliporeSigma), PPT (1 × 10^{-8} mol/l, Tocris), WAY (1 × 10^{-8} mol/l, MilliporeSigma), or G1 (1 × 10^{-8} mol/l, Tocris) during 48 hours.

For α/β cell cocultures, after FACS purification, these cells were seeded in 2 different drops, and only the α cells were treated with vehicle (DMSO) or E$_2$ (1 × 10^{-8} mol/l, MilliporeSigma), as described above for the α- and β cells.

For intestinal explants, mixed intestinal cells from small intestine (jejunum/ileum) were isolated and seeded in Matrigel (Corning) as previously described (53). After overnight recovery in 5.6 mmol/l glucose phenol red free medium, supplemented with 10% Charcoal Stripped FBS (MilliporeSigma), 10 mmol/l Na-pyruvate, and antibiotics, intestinal cells were treated with vehicle (DMSO), E$_2$ (1 × 10^{-8} mol/l, MilliporeSigma), PPT (1 × 10^{-8} mol/l, Tocris), WAY (1 × 10^{-8} mol/l, MilliporeSigma), or G1 (1 × 10^{-8} mol/l, Tocris) during 48 hours.

GLUTag cells were grown to 80% confluence in DMEM 5.6 mmol/l glucose phenol red medium, supplemented with 10% FBS (MilliporeSigma), 10 mmol/l Na-pyruvate, and antibiotics and next cultured for 48 hours more in deprivating conditions (DMEM 5.6 mmol/l glucose phenol red free medium, supplemented with 10% Charcoal Stripped FBS (MilliporeSigma), 10 mmol/l Na-pyruvate, and antibiotics). These cells were then treated with DMSO (vehicle), E$_2$, PPT, WAY, G1 (1 × 10^{-8} mol/l for each) during 48 hours.

Human primary cell preparations and cellular cultures. Human islets were obtained from Prodo Laboratories and shipped in PIM(T) media (Prodo Laboratories). Islet cultures were performed according to the manufacturer's instructions using the complete PIM(S) medium (supplemented by PIM[G] and PIM[ABS], Prodo Laboratories). Twenty-four hours after transfer, 2,000 islets (islet equivalents [IEQ]) were dissociated using Accutase treatment (StemPro Accutase, Invitrogen; 10 minutes at 37°C) with pipetting every 2 minutes. Dissociated islets cells were then labeled with anti–human α cell antibody HPa1 (DHIC2-2C12, Thermo Fisher Scientific; 1/50, 30 minutes at 4°C) and Alexa Fluor 488 goat anti–mouse IgM (Invitrogen; A21042, 1/200, 20 minutes at 4°C) as previously described (54). Dead cells were marked with DRAQ7 (Far-Red Fluorescent Live cell Impermeant DNA Dye, Biostatus). Human α- and β cell fractions were collected using a Bio-Rad S3 cell sorter, excluding cell doublets and dead cells. Cells were finally cultured using standard procedures (51) in complete PIM(S) medium during 48 hours. For the next 48 hours, the cells were cultured in the supplemented DMEM medium with 10% charcoal stripped serum, with DMSO, E$_2$ (1 × 10^{-8} mol/l, MilliporeSigma), PPT (1 × 10^{-8} mol/l, Tocris), WAY (1 × 10^{-8} mol/l, MilliporeSigma), or G1(1 × 10^{-8} mol/l, Tocris).

For human intestinal explants, the protocol was similar to the one used for mouse samples, and it is described above.

Ex vivo secretion assays. For secretion assays, primary cultures of sorted α- and β cells, as well as mixed intestinal cells from the small intestine and GLUTag cells of ovx mice, were seeded in DMEM 5.6 mmol/l glucose phenol red free medium, supplemented with 10% Charcoal Stripped FBS (MilliporeSigma), 10

mmol/l Na-pyruvate, and antibiotics. After an overnight recovery, these cells were treated with vehicle (DMSO), E_2 (1×10^{-8} mol/l, MilliporeSigma), PPT (1×10^{-8} mol/l, Tocris), WAY (1×10^{-8} mol/l, MilliporeSigma), or G1 (1×10^{-8} mol/l, Tocris) during 48 hours.

The pancreatic cells and GLUTag cells were respectively incubated for 2 or 6 hours with fresh medium. Supernatants and cell lysates (acid/ethanol mixture) were collected for insulin, glucagon, and GLP-1 measurements.

For α/β cell cocultures, cells were incubated in 2 separated drops in Krebs 5.6 mmol/l glucose medium during 2 hours. Then, both drops were joined in 1 single drop, thus containing α- and β cells. They were assessed for insulin and GLP-1 release during 1 hour in Krebs buffer containing 5.6 mmol/l glucose and were finally incubated in 16.7 mmol/l glucose or 16.7 mmol/l glucose with Ex9–39 (MilliporeSigma, 10 nmol/l) for 1 hour. Supernatants and cell lysates (acid/ethanol mixture) were collected for insulin and GLP-1 measurements.

The mixed intestinal cells from small intestine (jejunum/ileum) explants were seeded in Matrigel (Corning), as previously described (53), in DMEM 5.6 mmol/l glucose phenol red free medium, supplemented with 10% Charcoal Stripped FBS (MilliporeSigma), 10 mmol/l Na-pyruvate, and antibiotics. After overnight recovery and 48-hour treatment with the different SERMs, intestinal cells were incubated for 2 hours with fresh medium as described (55). Supernatants and cell lysates (cell lysis buffer; ref. 50) were collected for GLP-1 measurements.

Glucagon, insulin, and GLP-1 releases were expressed relative to glucagon and GLP-1 cellular contents (percent of content).

Target gene analysis. Total mRNA was isolated from mouse sorted Venus⁺ pancreatic α cells, Cherry⁺ pancreatic β cells, and Venus⁺ intestinal (jejunum/ileum) L cells, as well as GLUTag cells with RNeasy plus micro kit (Qiagen). After reverse transcription (Prime-script RT Reagent, Takara Bio Inc.) and preamplification (cDNA Pre-Amp Master, Roche Diagnostics) following manufacturer recommendations, specific cDNA levels were analyzed by quantitative PCR (qPCR). qPCR reactions are performed using Light-Cycler 480 SYBR Green technology (Roche Diagnostics). Each target gene amplification was previously validated by evaluation of the melting temperature of the products and of the slope obtained with the standard curve, as well as by sequencing the PCR product. Mean values of gene expression were calculated from technical duplicates of each qPCR analysis and normalized to the housekeeping gene *Rps9*, exhibiting no significant variability of its expression levels throughout each experiment. It therefore served as internal control. Data are expressed as follow: $2^{(Ct_{Rps9} - Ct_{gene})}$, where Ct corresponds to the number of cycles needed to generate a fluorescent signal above a predefined threshold.

Statistics. Data are presented as mean ± SEM and analyzed using GraphPad Prism software (v.7.0). Statistical analyses were performed using 1-way repeated ANOVA with Bonferroni post hoc tests for glycemia during OGTT. Unpaired 2-tailed Student's *t* tests were used for comparison between 2 groups for sham vs. ovx comparison, or for ovx vs. ovx+E_2 or ovx+WAY comparisons. Finally, 1-way ANOVA with the Dunnett's multiple comparison post hoc tests were performed for experiments using the several SERM. Data are statistically significant at $P \leq 0.05$.

Author contributions

All authors took part in the conception and design of the study, acquisition, and analysis and interpretation of data, as well as revision and approval. SH, RD, and FV researched data (design, acquisition, and analysis of data) and contributed to revision; SH, JP, and YG researched data design and contributed to revision of manuscript, project management, and writing of the manuscript. YG and JP are the guarantors of this

work and, as such, had full access to all data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

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