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# The Islet Estrogen Receptor- $\alpha$ Is Induced by Hyperglycemia and Protects Against Oxidative Stress-Induced Insulin-Deficient Diabetes

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## Abstract

The female steroid, 17 $\beta$ -estradiol (E2), is important for pancreatic  $\beta$ -cell function and acts via at least three estrogen receptors (ER), ER $\alpha$ , ER $\beta$ , and the G-protein coupled ER (GPER). Using a pancreas-specific ER $\alpha$  knockout mouse generated using the Cre-lox-P system and a Pdx1-Cre transgenic line (PER $\alpha$ KO<sup>-/-</sup>), we previously reported that islet ER $\alpha$  suppresses islet glucolipotoxicity and prevents  $\beta$ -cell dysfunction induced by high fat feeding. We also showed that E2 acts via ER $\alpha$  to prevent  $\beta$ -cell apoptosis *in vivo*. However, the contribution of the islet ER $\alpha$  to  $\beta$ -cell survival *in vivo*, without the contribution of ER $\alpha$  in other tissues is still unclear. Using the PER $\alpha$ KO<sup>-/-</sup> mouse, we show that ER $\alpha$  mRNA expression is only decreased by 20% in the arcuate nucleus of the hypothalamus, without a parallel decrease in the VMH, making it a reliable model of pancreas-specific ER $\alpha$  elimination. Following exposure to alloxan-induced oxidative stress *in vivo*, female and male PER $\alpha$ KO<sup>-/-</sup> mice exhibited a predisposition to  $\beta$ -cell destruction and insulin deficient diabetes. In male PER $\alpha$ KO<sup>-/-</sup> mice, exposure to E2 partially prevented alloxan-induced  $\beta$ -cell destruction and diabetes. ER $\alpha$  mRNA expression was induced by hyperglycemia *in vivo* in islets from young mice as well as in cultured rat islets. The induction of ER $\alpha$  mRNA by hyperglycemia was retained in insulin receptor-deficient  $\beta$ -cells, demonstrating independence from direct insulin regulation. These findings suggest that induction of ER $\alpha$  expression acts to naturally protect  $\beta$ -cells against oxidative injury.

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## Introduction

The female steroid, 17 $\beta$ -estradiol (E2), is important for pancreatic  $\beta$ -cell function in mammals [1–4]. E2 acts through at least three estrogen receptor (ER)s in  $\beta$ -cells, ER $\alpha$ , ER $\beta$  and the G-protein coupled ER (GPER). These ERs are expressed in rodent and human  $\beta$ -cells in both sexes, where they exhibit a predominant extranuclear localization [3,5]. The islet ER $\alpha$  is important for enhancing insulin biosynthesis *in vivo* via an extranuclear ER $\alpha$ -dependent mechanism that amplifies the effect of glucose in stimulating the insulin gene promoter [6,7]. The islet ER $\alpha$  also suppresses excess *de novo* lipogenesis, which prevents glucolipotoxic  $\beta$ -cell failure in rodent models of type 2 diabetes (T2D) [8]. E2 also acts as a survival hormone that prevents  $\beta$ -cell apoptosis *in vivo* in both sexes at physiological concentrations. This

protection is lost in mice globally deficient in ER $\alpha$  [9]. In cultured mouse and human islets, E2 protection is mediated mainly via ER $\alpha$  and GPER, and it protects from diabetes-associated injury resulting from oxidative stress and pro-inflammatory cytokines [5,9–11]. Further, during pancreatic islet transplantation, use of an ER $\alpha$ -selective agonist enhances human islet graft survival, thus protecting islet functional mass [12]. Overall, global expression of ER $\alpha$  is necessary for islet survival in mice, and pharmacological activation of ER $\alpha$  protects islet survival in culture and following *in vivo* treatment. Nonetheless, the direct and singular impact of ER $\alpha$  in islet  $\beta$ -cells on islet survival *in vivo*—without contribution from the effects of ER $\alpha$  action in other tissues—has not been addressed. In this study we used the PER $\alpha$ KO<sup>-/-</sup> mouse to examine the role of islet ER $\alpha$  in islet survival from alloxan induced-oxidative stress *in vivo*.

## Materials and Methods

### Generation of mutant mice and animal care

Pancreas specific ER $\alpha$  knockout mice were generated using the Cre-lox-P system and a Pdx1-Cre transgenic line (PER $\alpha$ KO<sup>-/-</sup>) as previously described [7]. Pdx1-Cre mice were bred onto the cre-inducible Rosa26-LacZ line at the University of Michigan. Animal had free access to food and water. They were kept on a 12-h light/dark cycle. All animal experiments were approved by Northwestern University or University of Michigan Institutional Animal Care and Use Committee.

### Induction of experimental diabetes and tissue collection

Diabetes was induced in 10–12 week-old female and male mice by a single intraperitoneal (IP) injection of 150 mg/kg of alloxan (ALX) (2,4,5,6-Tetraoxypyrimidine) (Sigma-Aldrich) freshly prepared in sterile cold saline (0.9%). Mice ER $\alpha$ lox<sup>+/+</sup> were used as control for PER $\alpha$ KO<sup>-/-</sup> mice. Blood glucose was measured every 48 h after ALX injection using One Touch Ultra Glucose Monitor (Lifescan). At day 11 after ALX injection, mice were killed and blood and pancreata were collected.

### In vivo drug administration

17 $\beta$ -Estradiol (4  $\mu$ g/25 g); Tocris Biosciences) and vehicle (10% ethanol and 90% sesame oil) were administered subcutaneously (s.c.) twice daily for two days.

### Pancreas insulin concentration

Tails of the pancreata were collected, weighed, and homogenized in acid/ethanol. Then, pancreas homogenates were centrifuged, and supernatants were used to measure pancreas insulin concentration by radioimmunoassay (Linco) as described [7].

### Plasma insulin concentrations

Plasma insulin concentrations were measured by ELISA (Millipore).

### Pancreas immunohistochemistry

Deparaffinized pancreatic sections (5  $\mu$ m) were blocked for 30 min with blocking solution (20% Fetal Bovine Serum + 2% Roche Blocking Reagent). Sections were incubated overnight with primary antibodies and 1–2 h with secondary antibodies at room temperature with the following primary antibodies: guinea pig anti-human insulin (1:1000; Linco Research), rabbit anti-glucagon (1:1000, Linco Research), rat anti-mouse CD31 (1:400; BD Biosciences). Secondary antibodies FITC-conjugated donkey anti-guinea pig, CY3-conjugated donkey anti-rabbit, AMCA-conjugated donkey anti-guinea pig, and CY3-conjugated goat anti-rat (Jackson ImmunoResearch Laboratories) were used at concentrations recommended by the manufacturer. The nuclei were stained with DAPI (Invitrogen, Molecular Probes). Images were obtained with either Nikon Eclipse E400 microscope or Tissue Genostics Tissue/Cell High Throughput Imaging and Analysis System at Northwestern University Cell Imaging Facility.

### Brain immunohistochemistry

Perfusion and immunohistochemistry were performed as previously described [13]. Briefly, mice were anesthetized with a lethal dose of intraperitoneal pentobarbital (150 mg/kg) and transcardially perfused with sterile PBS and then either 4% paraformaldehyde or 10% formalin. Brains were removed, post-fixed overnight and dehydrated in a 30% sucrose solution.

Following cryoprotection, brains were sectioned into 30  $\mu$ m coronal slices, collected in four consecutive series and stored at  $-20^{\circ}\text{C}$  until further use. For immunohistochemistry, brain sections were pretreated with ice-cold methanol, 0.3% glycine and 0.3% SDS before blocking. Sections were then incubated with primary antibodies either rabbit anti-ER $\alpha$  (1:1000, Sigma) or goat anti- $\beta$ Gal (1:1000, Biogenesis Ltd) overnight at  $4^{\circ}\text{C}$ . Detection of primary antibodies was done by either immunofluorescence using secondary antibodies anti-rabbit Alexa 488, anti-goat Alexa 568, both 1:200 dilution (Invitrogen) or using the avidin-biotin/diaminobenzidine method (secondary antibody anti-rabbit-biotin, 1:200 dilution).

### Cell counts and statistic

Stained sections were imaged using Leica microscope using 10X and 20X air objectives and processed using Adobe Photoshop CSII (Adobe Systems, San Jose, CA). Photoshop was only used to overlay matched images in different RGB channels such that dual-labeled cells would become apparent and could be quantified as described [13].

### Calculation of pancreatic $\beta$ -cell mass

$\beta$ -cell area was measured in insulin-stained 5  $\mu$ m thick pancreatic sections. Three to four sections per tissue were randomly chosen for morphometric analysis. Insulin positive area was determined by using ImageJ 1.37v program. To calculate  $\beta$ -cell mass (mg), insulin positive area was divided by pancreas area and then multiplied by pancreas weight.

### Calculation of vessel density in islets

Blood vessel density was calculated by dividing the mouse-CD31-positive area by the insulin-positive islet area by using ImageJ 1.37v program.

### Rat model of glucolipotoxicity

Two-month and 6-month old male Wistar rats (Charles River, St.-Constant, QC), were maintained hyperglycemic and hyperlipidemic for 72 hours, following a co-infusion of 70% dextrose plus 20% Intralipid, compared to their 0.9% saline infused controls as described [14].

### Islets isolation

At the end of infusion, islets were isolated by pancreas collagenase digestion as described [14].

### Rat islets culture

Wistar rat islets were pre-cultured for a week in serum-free RPMI medium supplemented with 5 g/L BSA ( $37^{\circ}\text{C}$ , humidified atmosphere containing 5% CO<sub>2</sub>). Islets were further cultured for 18 h or 1 week in the same medium containing 5, 10 or 30 mM glucose (medium was renewed every other day) [15].

### Mouse model of moderate hyperglycemia

To study ER $\alpha$  expression under mild hyperglycemia conditions, a 4 days glucose infusion in mice was performed as is described in [16]. Briefly, C57bl/6J male mice of 8–12 weeks-old and 20–25 g received a 4 days infusion of saline or 50% glucose. After this, mice were anesthetized and islets isolated by digestion with 1.7 ml/cc Collagenase P (Sigma) [9].

### $\beta$ IRKO cell culture

We used insulin-secreting cell lines established from groups of  $\beta$ IRKO, and Lox control mice as was described previously

[17,18]. Cells were maintained at 37°C and 5% CO<sub>2</sub> in Dulbecco's modified Eagle's medium (DMEM) containing 25 mM glucose, 10% fetal bovine serum, and penicillin and streptomycin. Experiments were performed using 80–90% confluent cells. Lox and  $\beta$ IRKO cells were seeded in 6-well plates and incubated for 24 h to recover. Cells were first washed with PBS before incubating in 16.7 or 33 mM glucose in DMEM containing 10% serum and penicillin and streptomycin for 3 days.

#### Q-PCR

Total RNA was extracted using RNeasy<sup>®</sup> Micro kit (Qiagen, Valencia, CA) for islets or RNeasy<sup>®</sup> kit (Qiagen, Valencia, CA) for cells according to the manufacturer's protocols. cDNA was prepared from 1  $\mu$ g of total RNA using the High Capacity cDNA Reverse Transcription Kit (Invitrogen) with random hexamer primers, according to the manufacturer's instructions. Real-time PCR amplification of ER $\alpha$  and Tbp (TATA-box binding protein) cDNAs was carried out in a CFX96 using iQ-SYBR green supermix (Bio-Rad, Hercules, CA). Results were normalized to TBP expression and expressed as arbitrary units. Primer sequences are the following: Rat islets: 5'-ACCCTTCACCAATGACTCC-TATG-3' and 5'-TCAGCATTCTGGCAGCAAGT-3' for TBP and -5'AATTCTGACAATCGACGCCAG3' and 5'-GTGCT-TCAACATTCTCCCTCCTC-3' for ER $\alpha$ . Mouse islets and cells: 5'-ACCCTTCACCAATGACTCC-TATG-3' and 5'-ATGATG-ACTGCAGCAAATCGC-3' for TBP and 5'-GCTTCTCTT-GGCCTGTA-CTT-3' and 5'-CTCTCCCAGTTTCCACATC-TT-3' for ER $\alpha$ .

#### Statistical analysis

Data are presented as mean  $\pm$  SEM unless otherwise stated. Data were analyzed by Student's *t* test. A value of  $p < 0.05$  was considered statistically significant.

## Results

### Recombination of ER $\alpha$ in hypothalamic neurons of PER $\alpha$ KO<sup>-/-</sup> mice

To investigate the role of pancreatic ER $\alpha$  on  $\beta$ -cell biology *in vivo*, we used PER $\alpha$ KO<sup>-/-</sup> mice in which ER $\alpha$  was inactivated in all pancreatic lineages using a Pdx1-Cre transgenic mouse [7,8]. Because Pdx1-Cre transgenic mice were reported to promote recombination in nutrient sensing hypothalamic neurons [19], we first sought to determine whether recombination of ER $\alpha$  occurs in the hypothalamus of PER $\alpha$ KO<sup>-/-</sup> mice. Accordingly, using a transgenic Pdx1-Cre/LacZ mouse [19], we observed that Pdx1 is co-expressed with ER $\alpha$  in ~26% of neurons of the ventromedial hypothalamus (VMH), ~17% of neurons in the preoptic area (POA), and ~15% of neurons of the arcuate nucleus (ARC) (Fig. 1A–B). Female PER $\alpha$ KO<sup>-/-</sup> mice exhibited a 20% decrease in the number of ER $\alpha$  positive cells in the ARC, without a parallel decrease in the VMH (Fig. 1D–F). They also exhibited decreased fertility (data not shown), suggesting that ER $\alpha$  expression was also decreased in the POA.

### No alteration in islet vascularization in absence of ER $\alpha$

E2 stimulates angiogenesis and promotes endothelial cell recovery after injury [13–17]. We previously observed that estrogens improve islet revascularization during islet transplantation [12]. Thus, prior to exploring islet predisposition to oxidative stress, we sought to determine whether islet vascularization was altered in female PER $\alpha$ KO<sup>-/-</sup> mice. Because loss of ER $\alpha$  in  $\beta$ -cells or in endothelial cells can alter endothelial cell function via paracrine or endocrine mechanisms, respectively, we studied

vascular density in PER $\alpha$ KO<sup>-/-</sup> and mice globally deficient in ER $\alpha$  (ER $\alpha$ KO<sup>-/-</sup>). When we quantified the endothelial cell area in pancreas section using the mouse endothelial cell marker CD31, we observed no difference in islet vascularization among ER $\alpha$ KO<sup>-/-</sup>, PER $\alpha$ KO<sup>-/-</sup> and female control mice (Fig. 2). The absence of islet vascular defects demonstrated that ER $\alpha$  was not essential for islet angiogenesis in mice.

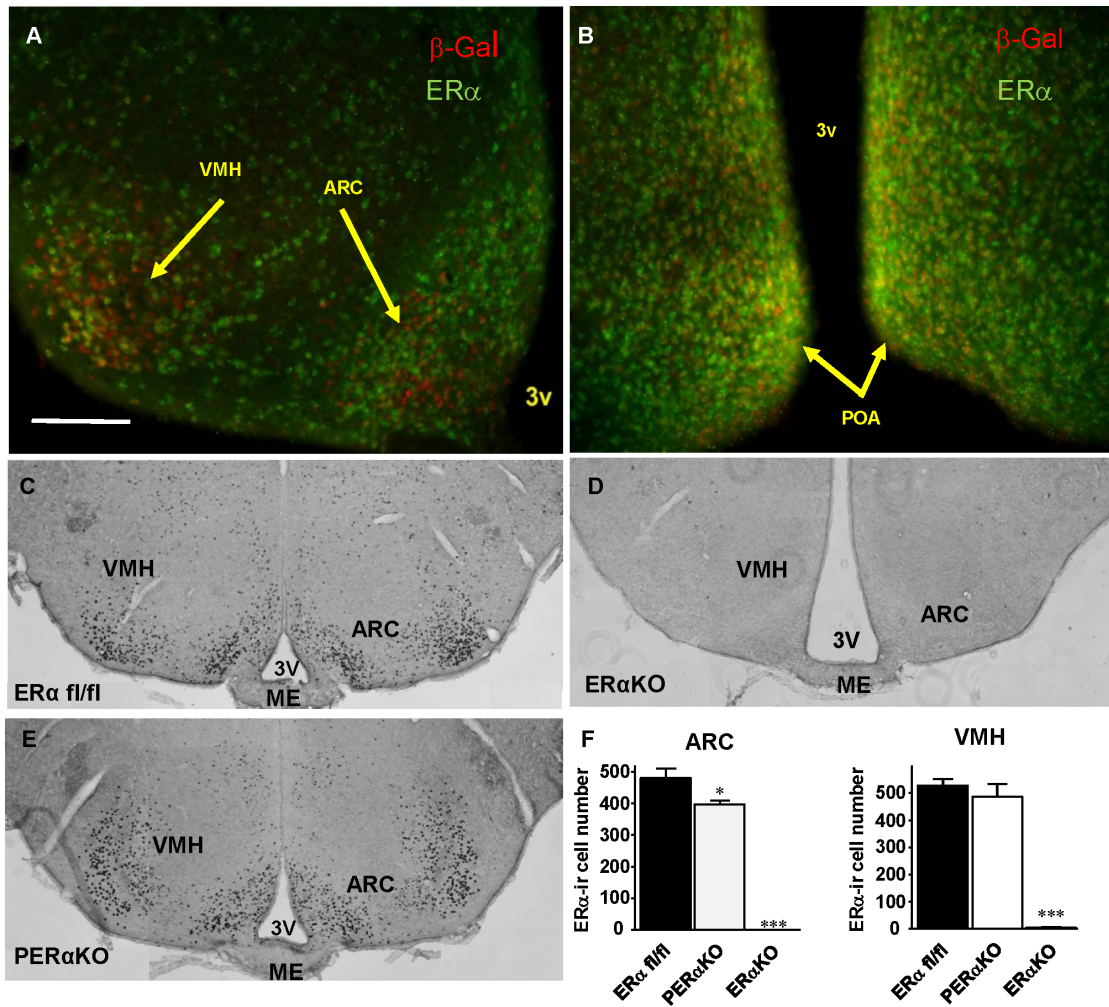
### The absence of islet ER $\alpha$ predisposes to oxidative stress-induced diabetes in mice

We induced oxidative stress in  $\beta$ -cells *in vivo* using a single high-dose injection of alloxan (ALX; 150 mg/kg of body weight), which augments the generation of reactive oxygen species (ROS) in pancreatic islets [20]. We initially observed that female C57BL/6 mice were protected from ALX-induced diabetes (Fig. 3). Next, we induced oxidative stress in  $\beta$ -cells of PER $\alpha$ KO<sup>-/-</sup> female mice. In basal conditions (time = 0, prior to ALX injection), control and PER $\alpha$ KO<sup>-/-</sup> female mice displayed similar blood glucose (Fig. 4A–B) and insulin concentrations (data not shown). They also exhibited normal islet architecture, with insulin-producing  $\beta$ -cells in a central location and glucagon-producing  $\alpha$ -cells at the periphery (Fig. 4D). PER $\alpha$ KO<sup>-/-</sup> female mice showed a trend toward decreased pancreatic insulin concentration, an observation that was consistent with the known effect of ER $\alpha$  in stimulating insulin synthesis [6,7]. Following exposure to ALX, control female mice showed relative protection compared to PER $\alpha$ KO<sup>-/-</sup> female mice. Control female mice displayed only a minor increase in blood glucose despite hypoinsulinemia and an 87% decrease in  $\beta$ -cell mass and pancreatic insulin concentration (Fig. 4C–F). This finding was consistent with the fact that only 20% of  $\beta$ -cells are needed to maintain euglycemia [12]. In contrast, relative to controls, exposure of PER $\alpha$ KO<sup>-/-</sup> female mice to ALX, induced marked hyperglycemia and insulin deficiency that resulted from a more severe  $\beta$ -cell destruction (97%) and decrease in pancreatic insulin concentrations (Fig. 4A–F). Thus, PER $\alpha$ KO<sup>-/-</sup> female mice exhibited a predisposition to alloxan-induced  $\beta$ -cell destruction. Note that we did not observe differences in  $\alpha$ -cell density between alloxan-injected control and PER $\alpha$ KO<sup>-/-</sup> female mice.

Regarding males, control and PER $\alpha$ KO<sup>-/-</sup> mice were normoglycemic and normoinsulinemic in basal conditions (Fig. 5A–C), and displayed normal islet architecture (Fig. 5D). After ALX exposure, both control and PER $\alpha$ KO<sup>-/-</sup> male mice developed hyperglycemia and insulin deficiency and exhibited decreased  $\beta$ -cell mass and pancreatic insulin concentrations. However, the reduction in all of these parameters was more dramatic in PER $\alpha$ KO<sup>-/-</sup> than in control mice (Fig. 5A–F). In addition, after E2 administration, we observed partial protection from alloxan-induced  $\beta$ -cell destruction and insulin deficiency in both controls and PER $\alpha$ KO<sup>-/-</sup> male mice (Fig. 5A–F). Thus, as observed in females, male PER $\alpha$ KO<sup>-/-</sup> mice exhibited a predisposition to alloxan-induced  $\beta$ -cell destruction (although to a lesser extent), but estrogen still provided some protection from alloxan in the absence of islet ER $\alpha$ . Note that unlike in the case of Fig. 3, experiments of ALX injections described in Fig. 4 and 5 were performed independently in male and female mice. Therefore, males and female mice described in Fig. 4 and 5 are not comparable with regard to the female protection from diabetes observed in Fig. 2.

### Altered islet ER $\alpha$ expression during hyperglycemia and hyperinsulinemia

Having determined that islet ER $\alpha$  is important to oxidative stress protection *in vivo*, we next sought to determine whether ER $\alpha$



**Figure 1. ER $\alpha$  expression in PER $\alpha$ KO $^{-/-}$  hypothalamus.** Pdx1-Cre/LacZ mouse and immunofluorescence in frontal brain sections from PER $\alpha$ KO $^{-/-}$  mice. Pdx-1 expression (red) marked by beta galactosidase ( $\beta$ -gal) and ER $\alpha$  (green) show PDX-1 and ER $\alpha$  co-expression in the ventromedial hypothalamus (VMH), the preoptic area and arcuate nucleus (ARC) (A–B), and quantification (C) Bar represents 250  $\mu$ m. Representative images of immunohistochemical analysis showing ER $\alpha$  expression in frontal brain sections from ER $\alpha$  fl/fl, (D) ER $\alpha$ KO $^{-/-}$  (E) and PER $\alpha$ KO $^{-/-}$  (F) mice. Pictures taken at 10X magnification and quantification (G). doi:10.1371/journal.pone.0087941.g001

mRNA expression was altered in islets during hyperglycemia-induced oxidative stress *in vivo*. We used two established rodent models of glucotoxicity and glucolipotoxicity. We first studied ER $\alpha$  mRNA expression in islets from non-diabetic Wistar rats that received a 72 h glucose and intralipid co-infusion to mimic glucolipotoxicity (mean glucose 15 mM) [14]. Under these conditions, hyperglycemia was associated with increased ER $\alpha$  mRNA expression in 2 month-old rat islets (Fig. 6A). However, hyperglycemia did not increase ER $\alpha$  mRNA in islets from 6 month-old rats. We next studied ER $\alpha$  mRNA expression in a mouse model of mild hyperglycemia that was achieved by a 4-day glucose infusion (mean glucose 7 mM) [16]. In this model, we observed no increase in islet ER $\alpha$  mRNA (Fig. 6B). To ascertain whether ER $\alpha$  mRNA induction under severe hyperglycemic conditions resulted from a direct glucose effect on islets, we further studied ER $\alpha$  mRNA expression in Wistar rat islets cultured one week in hyperglycemic conditions [15]. ER $\alpha$  expression was increased when glucose was raised from 5 mM to 10 mM, but there was no further increase at 30 mM (Fig. 6C). Therefore,

moderate to severe hyperglycemia [15] is associated with increased ER $\alpha$  mRNA expression *in vitro* and *in vivo* in rats.

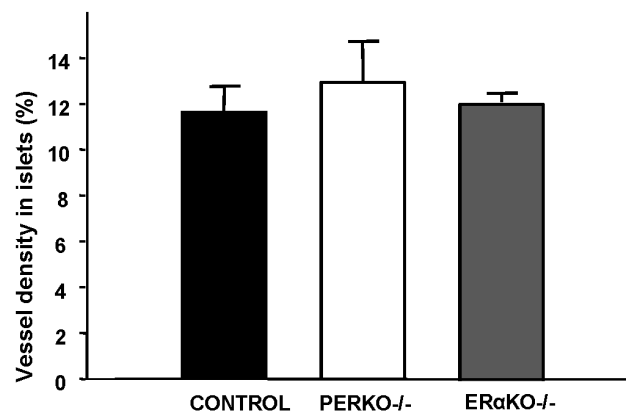
#### ER $\alpha$ expression in insulin resistant $\beta$ -cells

We hypothesized that the increased ER $\alpha$  mRNA expression in islets exposed to hyperglycemia could be due to the stimulatory effect of high glucose or to the impact of elevated insulin on the IR in the islets. To address this question, we quantified ER $\alpha$  expression in  $\beta$ -cells isolated from normal and  $\beta$ -cell IR knockout ( $\beta$ IRKO) mice [18]. These islets were cultured in hyperglycemic conditions to increase insulin secretion. Consistent with the effect of glucose described above (Fig. 5A), ER $\alpha$  mRNA expression was increased in both control (lox/lox) and  $\beta$ IRKO  $\beta$ -cells when glucose was increased from 16.7 mM to 33 mM (Fig. 6D). However, at both glucose concentrations, ER $\alpha$  mRNA expression was higher in  $\beta$ IRKO compared to lox/lox  $\beta$ -cells, demonstrating that insulin action in  $\beta$ -cells inhibits ER $\alpha$  mRNA expression.

A



B

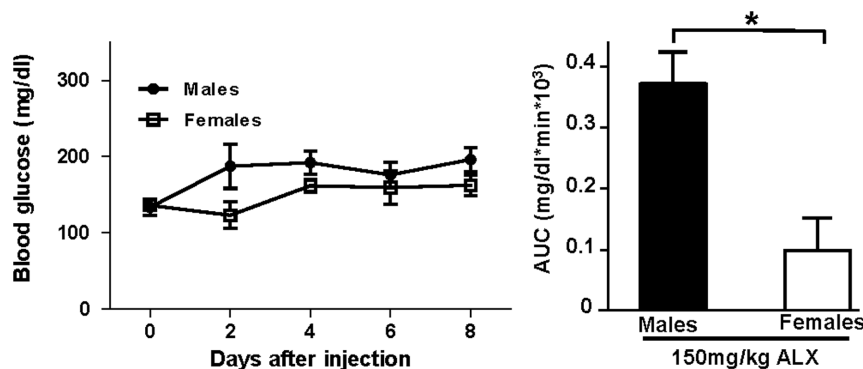


**Figure 2.** No difference in islet vascularization between ER $\alpha$ KO $^{-/-}$ , PER $\alpha$ KO $^{-/-}$  and control female mice. **(A)** Representative sections showing immunofluorescence staining for insulin (blue) and mouse CD31 (red) positive cells in control, PER $\alpha$ KO $^{-/-}$ , and ER $\alpha$ KO $^{-/-}$  pancreata. **(B)** Quantification of islets vessel density. Values represent the mean  $\pm$  SEM,  $n = 3-4$ /group. Bar represents 100  $\mu$ m. doi:10.1371/journal.pone.0087941.g002

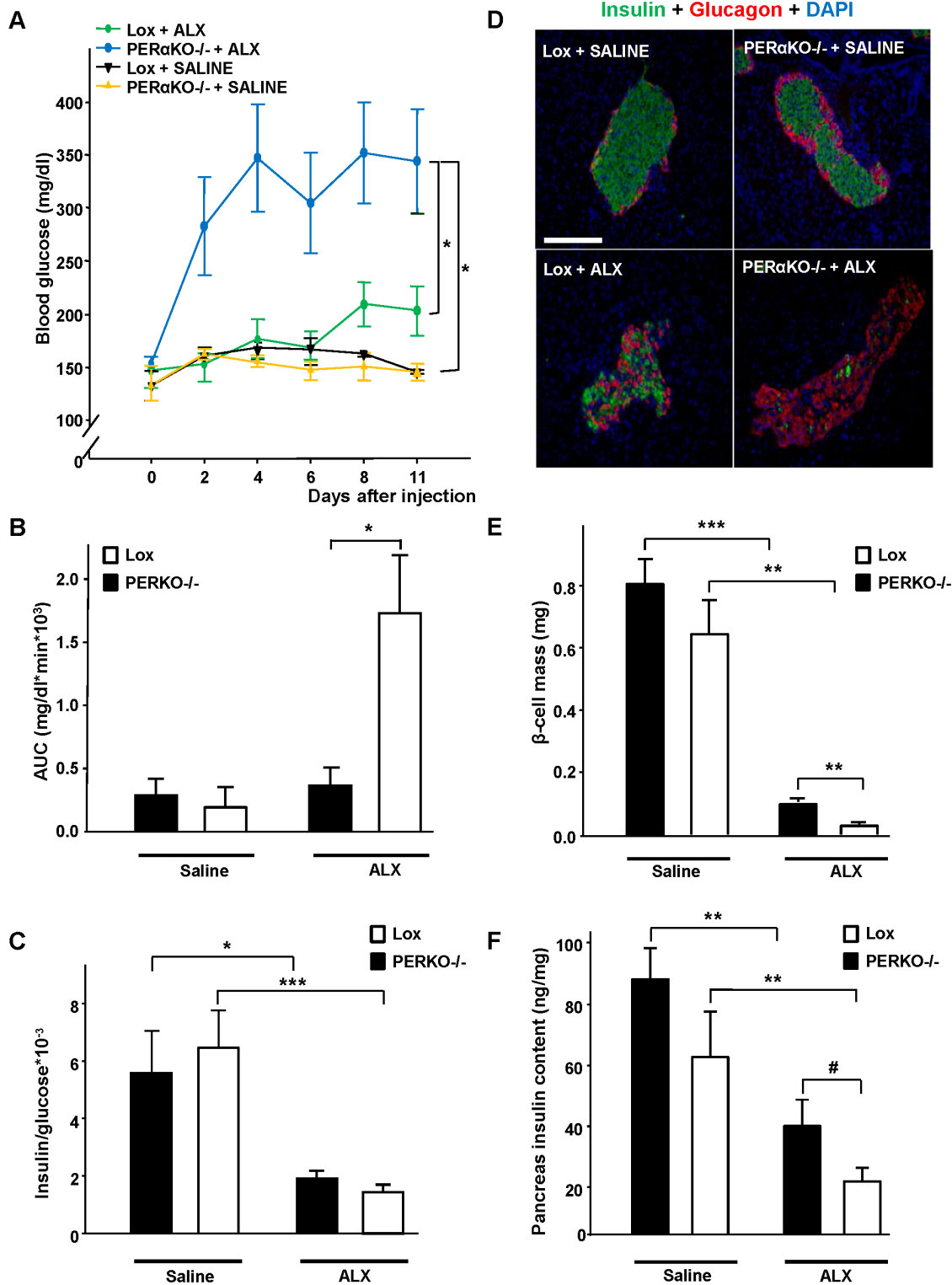
## Discussion

Having established that ER $\alpha$  is not essential to islet angiogenesis in mice, we focused on the role of ER $\alpha$  in protecting islets from glucotoxicity and oxidative stress *in vivo* and report that both male and female mice lacking ER $\alpha$  selectively in the pancreas are more susceptible to alloxan-induced  $\beta$ -cell destruction, insulin

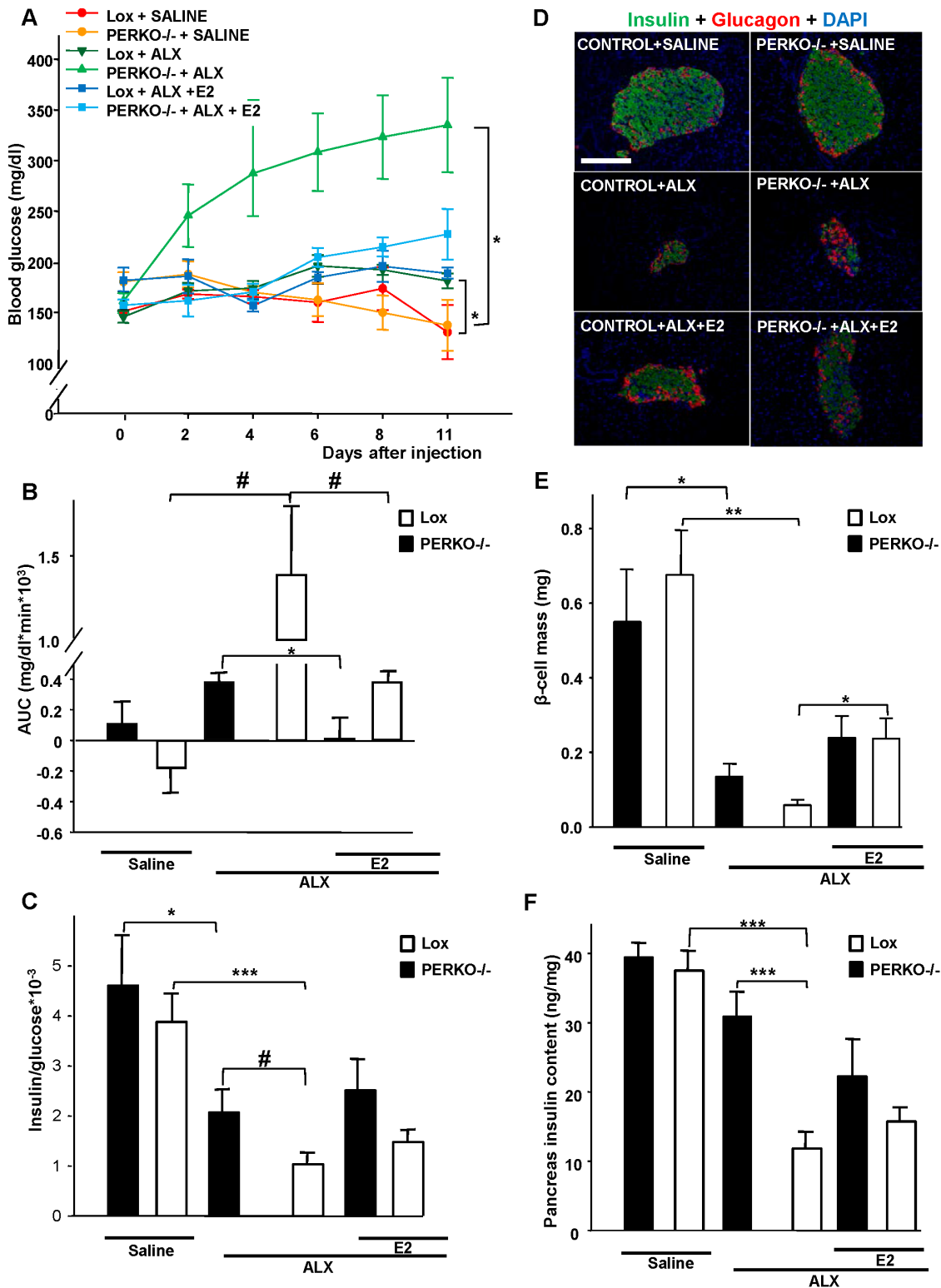
deficiency, and hyperglycemia. Although these experiments demonstrated a mild decrease in ER $\alpha$  mRNA expression in hypothalamus of PER $\alpha$ KO $^{-/-}$  mice, the absence of alteration in energy homeostasis [7,8] and the pancreas-specific phenotype observed in this model both suggest that the PER $\alpha$ KO $^{-/-}$  phenotype results exclusively from pancreatic elimination of ER $\alpha$ .



**Figure 3. Gender dimorphism in alloxan sensitivity.** Comparison of blood glucose values and area under the curve for glucose (AUC) above basal (T0) between male and female C57bl/6J wild type mice. Blood glucose was measured every 48 h for 8 days after injection of 150 mg/kg of alloxan (ALX). Values represent the mean  $\pm$  SEM,  $n = 4$ /group. \* $P < 0.05$ . doi:10.1371/journal.pone.0087941.g003



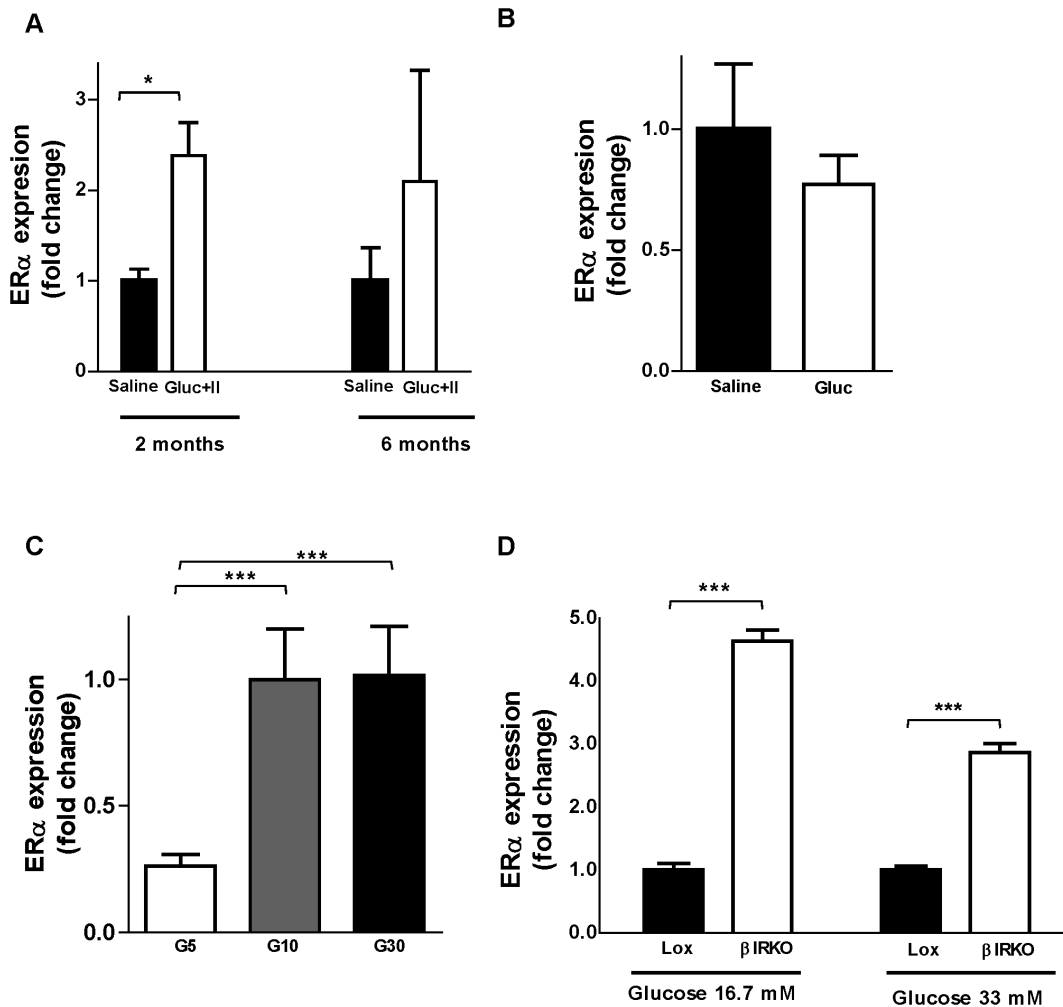
**Figure 4. Female PER $\alpha$ KO $^{-/-}$  mice are susceptible to ALX-induced diabetes.** (A) Random-fed blood glucose from day 0 to day 11 after injection of either 150 mg/Kg of ALX or saline. (B) Corresponding area under the curve (AUC) for glucose. (C) Ratio of random-fed of insulin (ng/ml) and glucose (mg/dl) at day 11 was used as an index of insulin deficiency. (D) Representative sections showing immunofluorescent histochemical analysis of pancreas sections stained for insulin (green) and glucagon (red) in control ER $\alpha$ lox $^{+/+}$  and PER $\alpha$ KO $^{-/-}$  mice (E)  $\beta$ -cell mass quantification. (F) Pancreas insulin concentration 11 day after ALX injection. Values represent the mean  $\pm$  SEM, n = 4–13/group. \*P < 0.05, \*\*P < 0.001 \*\*\*P < 0.01, Bar represents 100  $\mu$ m. doi:10.1371/journal.pone.0087941.g004



**Figure 5. Male PER $\alpha$ KO<sup>-/-</sup> mice are susceptible to ALX-induced diabetes.** (A) Random-fed blood glucose from day 0 to day 11 after injection of either 150 mg/Kg of ALX or saline. (B) Corresponding area under the curve (AUC) for glucose. (C) Ratio of random-fed of insulin (ng/ml) and glucose (mg/dl) at day 11 was used as an index of insulin deficiency. (D) Representative sections showing immunofluorescent histochemical analysis of pancreas sections stained for insulin (green) and glucagon (red) in control ER $\alpha$ lox<sup>+/+</sup> and PER $\alpha$ KO<sup>-/-</sup> mice (E)  $\beta$ -cell mass quantification (F) Pancreas insulin concentration 11 day after ALX injection. Values represent the mean  $\pm$  SEM, n = 4–19/group. \*P < 0.05, \*\*\*P < 0.001, # = 0.06. Bar represents 100  $\mu$ m.

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**Figure 6.** Messenger RNA levels of ER $\alpha$  were measured by QT-PCR from (A) 2 and 6 months-old male Wistar rats after 72 h glucose and intralipid co-infusion. (B) C57bl/6J male mice of 8–12 weeks-old after a 4 day glucose infusion. (C) Wistar rat islets cultured for one week in glucose 5, 10 or 30 mM. (D)  $\beta$ -cells cell line from control Lox/Lox and  $\beta$ IRKO mice cultured in 16.7 or 33 mM glucose. Values represent the mean  $\pm$  SEM, n = 4–5/group. \*P < 0.05, \*\*P < 0.01, \*\*\*P < 0.001. doi:10.1371/journal.pone.0087941.g006

Since alloxan induces oxidative stress, these findings demonstrate that normal islet ER $\alpha$  expression is required to protect  $\beta$ -cells from oxidative stress-induced apoptosis *in vivo* in both sexes. The harmful effect of ER $\alpha$  deletion is more pronounced in female mice, presumably as a result of higher E2 serum concentrations that are required to activate the islet ER $\alpha$  in this gender. Nonetheless, the negative effect of ER $\alpha$  deletion on islet cells is also observed in males, demonstrating that islet protection by ER $\alpha$  is sex independent. We previously reported that mice of both sexes globally lacking ER $\alpha$  ( $\alpha$ ERKO $^{-/-}$ ) were predisposed to streptozotocin-induced  $\beta$ -cell apoptosis and insulin-deficient diabetes [9]. However, the beneficial actions of estrogen on glucose homeostasis results from the combined actions of ER $\alpha$  in different tissues [2]. Thus  $\alpha$ ERKO $^{-/-}$  mice globally lacking estrogen action in skeletal muscle, adipose tissue, and the brain become obese and insulin resistant as well as mildly hyperglycemic. This could produce additional  $\beta$ -cell stress that would synergize streptozotocin toxicity to alter  $\beta$ -cell survival. The current study demonstrates that loss of ER $\alpha$  selectively in islets – while ER $\alpha$  is normally expressed in other tissues – is sufficient to induce  $\beta$ -cell destruction in the presence of another  $\beta$ -cell stress and without any influence of altered body weight [2,7]. Given the mild decrease in ER $\alpha$

expression observed in PER $\alpha$ KO $^{-/-}$  hypothalamic ARC, this abnormality is unlikely to play a role in the PER $\alpha$ KO $^{-/-}$  phenotype.

We previously reported that ER $\alpha$  gene dosage plays a role in the islet protection from streptozotocin injury because heterozygous  $\alpha$ ERKO $^{-/-}$  mice of both sexes were predisposed to streptozotocin-induced diabetes [9]. Thus, increased ER $\alpha$  expression could function to protect  $\beta$ -cells against oxidative stress. To evaluate this hypothesis, we used established models of glucolipotoxicity and moderate hyperglycemia. We observed that in both cultured rat islets and in mouse islets *in vivo*, moderate to severe hyperglycemia increased ER $\alpha$  mRNA. In cultured mouse islets and MIN6 cells, short term exposure to high glucose is also associated with an increase in ER $\alpha$  mRNA expression [21]. Overexpression of ER $\alpha$  prevents apoptosis in PC12 neuronal cells, [22] and in the SK-N-MC human neuroblastoma cell line [23]. In contrast, in a model of moderate hyperglycemia, ER $\alpha$  mRNA is not upregulated in islets.

During hyperglycemia, is ER $\alpha$  induced by glucose itself or by insulin? In IR-deficient  $\beta$ -cells cultured in high glucose, ER $\alpha$  mRNA was increased compared to normal cells. This demonstrates that compared to hyperglycemia, insulin action in  $\beta$ -cells is unlikely to play a direct role in inducing ER $\alpha$  mRNA in  $\beta$ -cells.

Thus, the induction of ER $\alpha$  expression by hyperglycemia could function as a  $\beta$ -cell protection against oxidative injury when hyperglycemia reaches a threshold beyond which oxidative injury occurs. Further, glucolipotoxicity, upregulates ER $\alpha$  mRNA in young rats, but this feature is lost in older animals. Since ER $\alpha$  improves  $\beta$ -cell survival [3,5,9], the loss of ER $\alpha$  induction in old islets may alter their resistance to diabetic injuries, as we observe in the PER $\alpha$ KO<sup>-/-</sup> mouse. This weakness may further increase  $\beta$ -cell susceptibility to oxidative injuries such as glucotoxicity, setting the stage for  $\beta$ -cell failure in old age.

ER $\alpha$  protection from oxidative stress could involve a combination of rapid antiapoptotic actions that are independent of nuclear events and that potentially lead to alteration in protein phosphorylation [3,24]. Alternatively, it could involve a more classical

genomic mechanism that induces an anti-inflammatory cascade via expression of the liver receptor homolog [25].

In conclusion, ER $\alpha$  mRNA expression is induced in islets from young mice by exposure to hyperglycemia and oxidative stress, and mice of both sexes that selectively lack ER $\alpha$  in the islets are susceptible to both oxidative stress in  $\beta$ -cells and insulin-deficient diabetes.

### Author Contributions

Conceived and designed the experiments: GK FMJ. Performed the experiments: GK BZ CWL DO. Analyzed the data: GK AAM MGM JCJ RNK LCA FMJ. Contributed reagents/materials/analysis tools: LCA JCJ MGM VP RNK FMJ. Wrote the paper: GK AAM FMJ.

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