Sex-steroid milieu determines diabetes rescue in \textit{pttg}-null mice

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Abstract

Male mice that are \textit{pttg}-null develop sexually dimorphic diabetes with hypoinsulinemia secondary to reduced postnatal \(\beta\)-cell proliferation and an inability to expand islet cell mass with aging. We therefore examined the effects of sex-steroid manipulation on diabetes development in \textit{pttg}\(^{-/-}\) male mice. Surgical gonadectomy was followed by implantation of 90-day slow-release pellets releasing 17\(\beta\)-estradiol (0\(::\)36 mg/pellet), placebo or dihydrotestosterone (DHT; 12\(::\)5 mg/pellet). Mean fasting blood sugars at the end of the study were 414 \pm 54 mg/dl for \textit{pttg}\(^{-/-}\) controls and 371 \pm 14 mg/dl for \textit{pttg}\(^{-/-}\) mice gonadectomized and treated with DHT compared with 124 \pm 40 and 85 \pm 12 mg/dl in gonadectomized \textit{pttg}\(^{-/-}\) males treated with placebo or estradiol, respectively (\(P<0\cdot01\) compared with control \textit{pttg}\(^{-/-}\)). Gonadectomy with and without estradiol treatment did not increase the very low circulating insulin levels in \textit{pttg}-null males (fasting insulin 0\(::\)44 \pm 0\(::\)04 ng/ml in \textit{pttg}\(^{-/-}\) controls, 0\(::\)47 \pm 0\(::\)07 and 0\(::\)4 ng/ml in \textit{pttg}\(^{-/-}\) gonadectomized males treated with placebo or estradiol, respectively). Gonadectomy increased serum adiponectin levels (4\(::\)9 \pm 0\(::\)08 \(\mu\)g/ml in \textit{pttg}\(^{-/-}\) controls versus 13 \pm 0\(::\)08 and 7\(::\)5 \pm 0\(::\)6 \(\mu\)g/ml in \textit{pttg}\(^{-/-}\) gonadectomized males treated with placebo or estradiol, respectively; \(P<0\cdot001\) and \(P<0\cdot05\)), accompanied by increased insulin sensitivity. The results show that gonadectomy delayed, and gonadectomy with additional estradiol treatment prevented, diabetes development in \textit{pttg}\(^{-/-}\) males, possibly through increased insulin sensitivity mediated by elevated serum adiponectin levels. Male-selective effects of disrupted \(\beta\)-cell proliferation in the absence of \textit{pttg} are restored by sex-steroid effects on peripheral insulin sensitivity.


Introduction

\textit{pttg} is the functional mammalian homolog of yeast securin (Pei & Melmed 1997, Zou et al. 1999), which facilitates sister-chromatid separation during the mitotic transition from metaphase to anaphase (Pei & Melmed 1997). \textit{pttg}-null male mice develop hyperglycemia secondary to hypoinsulinemia starting at 6 months of age (Wang et al. 2003). By 1 year, >80\% of male \textit{pttg}\(^{-/-}\) mice are diabetic with hypoinsulinemia secondary to reduced postnatal \(\beta\)-cell proliferation and an inability to expand islet cell mass with aging. Diabetes onset is accompanied by loss of fat tissue. In contrast, \textit{pttg}\(^{-/-}\) female mice rarely develop diabetes before 1 year of age and the incidence of diabetes in older \textit{pttg}-null females (more than 1 year) is increased. Moreover, ovariectomy causes earlier onset of diabetes in both sexes. Male mice were diabetic at 6 months of age in female \textit{pttg}\(^{-/-}\) mice (Wang et al. 2003).


To elucidate the role of sex steroids in the pathogenesis of diabetes in \textit{pttg}-null animals, males were surgically gonadectomized at the age of 4 weeks and implanted with 90-day slow-release pellets releasing 17\(\beta\)-estradiol, placebo or dihydrotestosterone (DHT). Fasting blood glucose levels were monitored, and glucose- and free fatty acid (FFA) levels were measured. Insulin sensitivity was assessed by the glucose clamp technique, and FFA and glucose were infused to maintain euglycemia and euglycemia, respectively. The results show that gonadectomy delayed, and gonadectomy with additional estradiol treatment prevented, diabetes development in \textit{pttg}\(^{-/-}\) males, possibly through increased insulin sensitivity mediated by elevated serum adiponectin levels. Male-selective effects of disrupted \(\beta\)-cell proliferation in the absence of \textit{pttg} are restored by sex-steroid effects on peripheral insulin sensitivity.
insulin–tolerance tests were preformed to assess the effects of these manipulations on diabetes development in this model.

**Materials and Methods**

**Animals**

pttg$^{-/-}$ mice (Wang et al. 2001) were kept in a hybrid background derived from C57/BL6 and 129 SvJ mouse strains. Animals were housed with a 12-h light:12-h darkness cycle and fed standard chow ad libitum. Experiments were approved by the Institutional Animal Care and Use Committee of Cedars-Sinai Research Institute, Los Angeles, CA, USA.

Four-week-old male pttg$^{+/+}$ and pttg$^{-/-}$ mice were surgically gonadectomized (or sham operated) under isoflurane anesthesia and implanted every 90 days with estradiol (0.36 mg/pellet; Innovative Research of America, Sarasota, FL, USA) or placebo (Saba et al. 2002, van Eickels et al. 2003). Controls included pttg$^{+/+}$ and pttg$^{-/-}$ males with no intervention, or gonadectomized and treated with DHT (12.5 mg/pellet; same time points as other pellets).

**Blood assays**

Fasting blood glucose was measured twice a month starting at 4 weeks of age from tail blood samples after an overnight fast, using OneTouch Ultra glucometer (Lifescan; Johnson and Johnson, Milpitas, CA, USA). Animals with blood-glucose measurements above 150 mg/dl were considered as having diabetes.

Blood collected from the tail vein after an overnight fast was allowed to clot and then separated by ultracentrifugation. The following serum analytes were measured: insulin concentrations were measured in samples collected during glucose loading using an ultra-sensitive rat ELISA kit (CrystalChem, Downers Grove, IL, USA); fasting concentrations of adiponectin were measured using a mouse adiponectin RIA kit (Linco Research, St Charles, MO, USA); and C-peptide levels were measured by RIA using a rat C-peptide RIA kit (Linco Research).

Leptin and insulin were measured using the Lincoplex Adipokine panel (Linco Diagnostics, St Charles, MO, USA). Estradiol levels were measured by RIA using a third Generation Estradiol RIA kit (Diagnostic Systems Laboratories, Webster, TX, USA).

Intraperitoneal insulin and glucose-tolerance test

For glucose-tolerance testing (GTT), mice were fasted 16 h before i.p. glucose injection (1 g/kg body weight) and tail-vein blood collected at the indicated times. For insulin–tolerance testing (ITT), mice were fasted 6 h before i.p. insulin injection (1 unit/kg body weight), and glucose measured at specific time points.

**Histological, immunohistological and morphometric analysis**

Pancreata were isolated immediately after CO$_2$ euthanasia and fixed overnight at 4 °C in 10% buffered formalin, followed by processing and paraffin embedding. Blocks were sectioned (3–5 μM) and stained with hematoxylin and eosin. For immunostaining following antigen retrieval with citrate buffer, guinea-pig anti-insulin and rabbit anti-glucagon (both from Dako–cytometry, Carpinteria, CA, USA) were used. Primary antibodies were visualized using rhodamine-conjugated goat anti-guinea-pig antibody (Jackson ImmunoResearch, West Grove, PA, USA) and fluorescein-conjugated goat anti-rabbit antibody (Molecular Probes, Eugene, OR, USA) using an Olympus fluorescence microscope and digital camera. For morphometric analysis, 1 × images of the hematoxylin and eosin sections were used to calculate pancreatic surface area using ImageJ software (National Institutes of Health, Bethesda, MD, USA). Consecutive sections from the same block were immunostained for insulin and glucagon. Using ImageJ software the numbers of islets (defined as a minimum of five insulin-positive cells) in the same section were counted and the surface area of insulin-positive and glucagon-positive cells measured and averaged for all islets in the section. β-Cell area in the section was calculated by summing the individual insulin-positive cells area expressed as a percentage of the total pancreatic area observed.

**Statistical analysis**

Insulin resistance was assessed by the homeostasis model assessment-insulin resistance (HOMA-IR) index (Matthews et al. 1985), which was calculated as [fasting blood glucose (mg/dl) × fasting insulin (mg/ml)]/405 (Umeda et al. 2003).

Statistical comparisons were performed using the one tailed unpaired t-test with Welch’s correction, unless otherwise stated. One-way ANOVA was used when appropriate and is stated in the text. Data were analyzed using Prism software (Prism 4; Graphpad Software Inc, San Diego, CA, USA). Data in graphs are depicted as means ± S.E.M. Circulating leptin concentrations were log-transformed to normalize the distribution.

**Results**

Diabetes is prevented in gonadectomized pttg$^{-/-}$ males treated with estradiol, and delayed in gonadectomized pttg$^{-/-}$ males

Eighty percent of sham-operated males and all males with no intervention (control) pttg$^{-/-}$ mice developed
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hypoglycemia by 10 months (mean fasting blood glucose; sham pttg−/− 276 ± 57 mg/dl versus pttg+/+ 115 ± 28 mg/dl, and control pttg−/− 414 ± 54 mg/dl versus pttg+/+ 104 ± 7 mg/dl; P<0.05 and P<0.01 respectively, n=4–5 animals per group; Fig. 1).

Gonadectomized pttg−/− males treated with estradiol (n=4) had normal fasting blood glucose levels for up to 13 months (mean fasting blood glucose: 85 ± 12 mg/dl compared with control pttg−/− males at 10 months of age; P<0.01; Fig. 1B). Mean serum estradiol levels in treated pttg−/− and pttg+/+ mice were higher than in control pttg−/− mice (253 ± 97, 230 ± 56 and 13 ± 1 pg/ml respectively; P<0.01 by one-way ANOVA with Kruskal–Wallis test). GTT (1 g glucose/kg body weight) was performed serially on mice gonadectomized and treated with estradiol (n=4) at 3, 6, 9 and 12 months, and these values remained normal (data not shown).

Gonadectomized pttg−/− male mice treated with placebo (n=5) were protected from developing diabetes mellitus (mean fasting blood glucose at 13 months: 124 ± 40 mg/dl versus pttg−/− controls 414 ± 54 mg/dl; P<0.01; Fig. 1B), while a single gonadectomized pttg−/− male developed overt fasting hyperglycemia (260–370 mg/dl). By 12 months, gonadectomized pttg−/− males treated with placebo were glucose-intolerant compared with gonadectomized males with additional estradiol treatment. The mean area under the curve of GTT values was lower in gonadectomized pttg−/− animals treated with estradiol when compared with gonadectomy alone (18.5 ± 2.3 versus 35.2 ± 7.4 arbitrary units; P<0.05; Fig. 2).

Gonadectomized pttg−/− male mice treated with DHT (n=4) developed diabetes as expected (mean fasting blood glucose at 9 months: 371 ± 14 versus 108 ± 16 mg/dl in pttg+/+ similarly treated animals; n=5 P<0.001; Fig. 1C), and were also glucose intolerant, similar to control pttg−/− males.

Diabetes onset was accompanied by weight loss in pttg−/− male controls and in sham-operated and gonadectomized animals with supplemental DHT. pttg−/− gonadectomized males with and without estradiol treatment had lower body weights at an early age, irrespective of diabetes onset, when compared with pttg−/− and pttg+/+ control mice (data not shown).

Gonadectomy with or without additional estradiol therapy fails to elicit fasting and post-challenge insulin responses in pttg−/− males

Insulin levels were measured to determine whether the observed protection from diabetes conferred by sex-steroid manipulation was due to increased circulating insulin concentrations. Fasting serum insulin levels in control pttg+/+ males rose gradually with age with no similar increase noted in pttg−/− males (1.95 ± 0.6 versus 0.44 ± 0.04 ng/ml at 10 months; P<0.05; Fig. 3A). Interestingly, although gonadectomized pttg−/− males treated with estradiol were protected from developing hyperglycemia, they did not exhibit increased fasting insulin levels (Fig. 3A). Measurement of C-peptide levels in the same animals confirmed this observation (data not shown). Eight-month-old gonadectomized pttg−/− males had

Figure 1 Fasting blood glucose in pttg−/− (KO) and pttg+/+ (WT) male mice after sex-steroid manipulation. (A) Fasting blood glucose in 10-month-old sham-operated pttg−/− (mean 276 ± 57 mg/dl) and pttg+/+ (123 ± 7 mg/dl) males; *P<0.05. (B) Fasting blood glucose in 10-month-old male pttg−/− controls (414 ± 54 mg/dl) and male pttg+/+ controls (104 ± 7 mg/dl), 13-month-old pttg−/− gonadectomized males treated with estradiol (KO G+E2; 85 ± 12 mg/dl) and gonadectomized pttg−/− males treated with placebo (KO G+placebo; 124 ± 40 mg/dl); **P<0.01 compared with pttg−/− control males. (C) Fasting blood glucose in 9-month-old gonadectomized pttg−/− (KO G+DHT; 371 ± 14 mg/dl) and pttg+/+ (WT G+DHT; 108 ± 16 mg/dl) males treated with DHT; ***P<0.001.

Figure 2 GTT in 12-month-old pttg−/− (KO) and pttg+/+ (WT) male mice after sex-steroid manipulation. Glucose (1 g/kg body weight) was injected intraperitoneally and blood glucose measured after 15, 30, 45, 60, 90 and 120 min. Glucose values are from GTT of gonadectomized pttg−/− males treated with estradiol (KO G+estradiol) and placebo (KO G+placebo); n=4–5 in each group.
modestly increased fasting insulin levels compared with control pttg−/− animals (0.734 ± 0.06 versus 0.43 ± 0.03 ng/ml; P<0.001 by one-way ANOVA; Fig. 3B), an increase that was not evident at other experimental time points and was not confirmed by C-peptide levels. Most circulating fasting insulin measurements in pttg−/− control males were below the detection sensitivity of the Lincoplex kit. It is therefore possible that insulin levels in gonadectomized pttg−/− males exceeded those of controls, but the assay was not sufficiently sensitive to detect differences. Thus it remains unclear as to whether gonadectomy alone resulted in unambiguously elevated insulin levels in pttg−/− males. Serial insulin levels in gonadectomized pttg−/− and pttg+/− males treated with DHT were in accord with those of control animals (data not shown).

The appropriate wild-type insulin response to a glucose challenge (GTT) was markedly attenuated in pttg−/− males (for the 0, 30, 60 min time points (P<0.05) and for the 120 min time point (P<0.02); Fig. 4A). Moreover, gonadectomy of pttg−/− males with or without estradiol treatment did not normalize insulin levels following a glucose challenge, compared with age-matched control pttg−/− males (0, 30, 60 and 120 min time points; P>0.05; Fig. 4). Gonadectomized pttg−/− males treated with DHT also failed to raise insulin levels following a glucose load when compared with pttg+/− mice that exhibited a normal response to glucose (data not shown).

These results indicate that gonadectomy, followed by additional estradiol or no added treatment, protected male pttg−/− mice from developing diabetes in both the fasted and the post-glucose-challenged state without appreciably increasing insulin levels.

Increased insulin sensitivity accompanied by elevated serum adiponectin and leptin levels in gonadectomized pttg−/− males

To assess whether diabetes rescue occurred as a result of increased insulin sensitivity, ITTs were performed on mice aged 8–11 months (n=3 animals per group). Control pttg+/+ and pttg−/− males were assessed at 8 months.
(mean fasting glucose: 107 ± 21 and 204 ± 81 mg/dl respectively), gonadectomized \( pttg^{+/+} \) and \( pttg^{-/-} \) males treated with DHT at 9 months (mean fasting glucose: 108 ± 16 and 371 ± 14 mg/dl respectively), and gonadectomized \( pttg^{+/+} \) and \( pttg^{-/-} \) males treated with placebo or estradiol were assessed at 10 and 11 months respectively (all were normoglycemic).

A modest increase in insulin sensitivity was observed in gonadectomized \( pttg^{+/+} \) and \( pttg^{-/-} \) males both with and without estradiol treatment. The time required to attenuate basal glucose levels by 50% was ~60 min in control \( pttg^{-/-} \) males versus 30 and 40 min for gonadectomized \( pttg^{-/-} \) males receiving placebo or estradiol respectively. Gonadectomized \( pttg^{-/-} \) males treated with DHT did not improve insulin sensitivity.

The HOMA-IR (Homeostasis Model Assessment) index (Matthews et al. 1985, Umeda et al. 2003), a physiologic marker for insulin resistance, was calculated for each group of animals at 2 month intervals starting at the age of 2 months. A higher HOMA index score is an estimated reflection of increased insulin resistance. Gonadectomized \( pttg^{-/-} \) males with and without added estradiol treatment had a lower HOMA index at 10 months when compared with control \( pttg^{-/-} \) and \( pttg^{+/+} \) males, indicating that gonadectomy with or without estradiol treatment likely enhanced insulin sensitivity (0·096 ± 0·02 in gonadectomized \( pttg^{-/-} \) males treated with placebo, 0·108 ± 0·18 in gonadectomized \( pttg^{-/-} \) males treated with estradiol and 0·368 ± 0·08 in control \( pttg^{-/-} \); \( P<0·025 \) comparing \( pttg^{-/-} \) gonadectomized with and without additional estradiol with control \( pttg^{-/-} \)). These observations were confirmed by correlating fasting glucose and insulin levels (data not shown). \( pttg^{+/+} \) animals have low glucose levels in the face of high insulin levels, while \( pttg^{-/-} \) animals have high glucose levels in the face of low insulin levels. Gonadectomy with and without additional estradiol treatment resulted in changing \( pttg^{-/-} \) mice to the area on the correlation plot associated with low insulin and low glucose levels, thus implying increased insulin sensitivity.
To determine potential mechanisms for the observed enhanced insulin sensitivity, fasted serum adipokine levels were measured \((n=3–5\) in each group; Fig. 5). \(pttg^{−/−}\) males exhibit a gradual decrease in adiponectin and leptin concentrations with age, as compared with \(pttg^{+/+}\) males (for adiponectin at 8 months: \(4.9 \pm 0.8 \text{µg/ml}\) versus \(9.4 \pm 0.9 \text{µg/ml}\) \((P<0.05)\); and at 10 months: \(3.9 \pm 1\) versus \(9.6 \pm 0.9 \text{µg/ml}\) \((P<0.01)\); for leptin at 8 months: \(1.9 \pm 1.4\) versus \(8.6 \pm 1.5 \text{ng/ml}\) \((P<0.05)\); and at 10 months: \(1 \pm 0.5\) versus \(9.2 \pm 1.8 \text{ng/ml}\) \((P<0.01)\) in control \(pttg^{−/−}\) and \(pttg^{+/+}\) respectively; all by one-way ANOVA). Similar observations were made in gonadectomized \(pttg^{−/−}\) males treated with DHT (data not shown). In contrast, gonadectomized \(pttg^{−/−}\) males have elevated levels of both adipokines as compared with age-matched control \(pttg^{−/−}\) males (black bars): \(*P<0.05\); \(**P<0.01\); \(***P<0.001\). Gonadectomized \(pttg^{−/−}\) males treated with estradiol (diagonally striped bar) have elevated adiponectin (A) but not leptin (B) levels at 8 months compared with age-matched control \(pttg^{−/−}\) males (black bars); \(*P<0.05\) \((n=3–5\) in each group). G, gonadectomy; P, placebo; E2, estradiol.

Figure 5 Serum adipokine levels in male \(pttg^{−/−}\) (KO) and \(pttg^{+/+}\) (WT) mice after sex-steroid manipulation. (A) Serum adiponectin levels. (B) Serum leptin levels. Control \(pttg^{−/−}\) males (black bars) had lower adiponectin and leptin levels when compared with age-matched \(pttg^{+/+}\) control males (white bars); \(*P<0.05\); \(**P<0.01\). Gonadectomized \(pttg^{−/−}\) males (gray bars) had elevated adiponectin (A) and leptin levels (B) at 8 and 10 months when compared with age-matched control \(pttg^{−/−}\) males (black bars); \(*P<0.05\); \(**P<0.01\); \(***P<0.001\). Gonadectomized \(pttg^{−/−}\) males treated with estradiol (diagonally striped bar) have elevated adiponectin (A) but not leptin (B) levels at 8 months compared with age-matched control \(pttg^{−/−}\) males (black bars); \(*P<0.05\).
control pttg−/+ (P<0.01; both by one-way ANOVA). Leptin levels at 8 months (Fig. 5B) were 14.3±5.7 ng/ml in gonadectomized pttg−/+ mice versus 1.9±1.3 ng/ml in control pttg−/+ (P<0.05), and at 10 months they were 17.3±8.4 ng/ml in gonadectomized pttg−/+ versus 1±0.5 ng/ml in control pttg−/+ mice (P<0.01). Serum adiponectin but not leptin levels were also elevated in 8-month-old gonadectomized pttg−/+ mice treated with estradiol (Fig 5A; 7.5±0.6 µg/ml in gonadectomized pttg−/+ mice treated with estradiol versus 3.9±1 µg/ml in control pttg−/+ mice; P<0.05).

Pancreatic β-cell area is not increased in gonadectomized pttg−/− males with or without estradiol therapy

The percentage β-cell area occupying total pancreatic sections was markedly reduced in pttg−/− control males when compared with pttg+/+ controls (0.08±0.07 versus 7.9±2.5%; P<0.001 by ANOVA and Bonferroni group analysis). Although gonadectomy followed by estradiol or no added therapy increased the percentage β-cell area in pttg−/− males (0.27±0.24 and 0.18±0.05 versus 0.08±0.07% respectively) this difference was not significant when assessed by ANOVA and Bonferroni group analysis. The average islet size and the number of islets per µm² was also unaffected by gonadectomy in pttg−/− male mice with and without added estradiol therapy, compared with pttg−/− control male mice (data not shown).

Discussion

In pttg-null mice, sexually dimorphic diabetes is associated with β-cell hypoplasia, likely a consequence of disrupted securin function. Failure of differentiated islets to proliferate to maturation results in hypoinsulinemia, hyperglycemia and severe lipodystrophy at 6 months of age (Wang et al. 2003). It is unclear why β-cells are apparently selectively affected by securin deficiency. β-Cells undergo a proliferation phase before the end of the first postnatal month, when the pancreas is subject to dynamic changes in response to variations in insulin demand. Securin loss and resultant abnormal cell-cycle progression directly affect β-cell mass expansion, resulting in diabetes. It is likely that loss of fat tissue and reduced body weight are secondary to hypoinsulinemia. pttg−/− animals also exhibit selective pituitary, splenic and testicular hypoplasia, with no apparent functional deficits (Wang et al. 2001, 2003).

The results shown here demonstrate that gonadectomy alters the course of diabetes in pttg−/− males, with four of five males being free of diabetes for up to 13.5 months, and one male animal developing diabetes at 12 months. As a group these gonadactomized males showed glucose intolerance for the first time at 12 months of age. Sexually dimorphic hyperglycemia has been described in several rodent diabetes models in which female sex conferred complete or partial protection from diabetes development (Rossini et al. 1978, Paik et al. 1982, Kava et al. 1989, 1992, Leiter 1989, Efrat 1991, Shi et al. 1994, Kim et al. 2001, Thomas et al. 2001, Weksler-Zangen et al. 2001, Geisler et al. 2002). Several of the models cited demonstrated that male gonadectomy is protective for diabetes development. These include streptozotocin-induced diabetes, in which gonadectomy ameliorated (Rossini et al. 1978) or attenuated (Maclaren et al. 1980) hyperglycemia, the obese Zucker rat (Kava et al. 1992), the Cohen diabetic rat (Weksler-Zangen et al. 2001) and the OLET fatty rat (Shi et al. 1994). In most of these studies, removal of testosterone from the sex-steroid milieu of the male animal improved insulin sensitivity, while in others, like the streptozotocin model, the mechanism for the negative effect of testosterone on blood sugar levels is unclear (Maclaren et al. 1980, Kromann et al. 1982, Paik et al. 1982). The negative effects of testosterone have been shown in a study where neonatal therapy of female rats with testosterone caused fetal imprinting followed by subsequent development of insulin resistance at an older age (Nilsson et al. 1998). In humans male hypogonadism is associated with glucose intolerance, decreased lean body mass and increased fat mass, mostly through alterations in insulin-sensitivity markers (Fukui et al. 2000, Stellato et al. 2000, Oh et al. 2002, Bhasin 2003). Gonadectomy of pttg−/− male mice further lowered body weight which might account for protective effects of gonadectomy on diabetes development in pttg-null mice.

In the work shown here, we demonstrate that the delay and attenuation of diabetes development in gonadactomized pttg−/− males was not caused by a consistent rise in insulin levels but rather through a decrease in insulin resistance, as reflected in the ITT and the HOMA-IR index. Insulin sensitivity as assessed by the ITT measures mainly muscle glucose clearance, while insulin sensitivity of fat and liver are not appreciably reflected by this test (Goren et al. 2004). This might explain the lack of a more robust increase in insulin sensitivity in gonadectomized pttg−/− male mice with or without estradiol therapy, as shown by the ITT. Tests directly assessing fat or liver insulin sensitivity may point to greater differences between these groups. For technical reasons, we performed ITT on control mice at 8 months of age and compared results with those of older gonadectomized mice treated with estradiol or placebo. Control pttg−/− and pttg+/+ male mice would have been expected to show more insulin resistance if assayed at an older age, so the finding that gonadectomy increases insulin sensitivity would have likely been magnified, rather than diminished, if the ITT was performed at similar ages.

Increased insulin sensitivity was associated with a dramatic rise in serum adiponectin levels following gonadectomy of both pttg−/− and pttg+/+ males. Adiponectin has been identified as an insulin-sensitizing peptide. Low
adiponectin levels are a feature of insulin resistance in humans and rodents with insulin resistance, whether accompanied by either lipoatrophy or obesity (Yamauchi et al. 2001, Berg et al. 2002, Haque et al. 2002), and exogenous adiponectin administration reverses insulin resistance in rodents (Haque et al. 2002). In insulinopenic rodent models for diabetes like the non-obese diabetic (NOD) mouse and streptozotocin-induced diabetes, treatment with adiponectin normalized glucose levels, without a rise in insulin (Berg et al. 2001). Given these results, adiponectin is a strong candidate for mediating the observed improvement in diabetes onset in gonadectomized pttg<sup>−/−</sup> male mice. Decreased adiponectin levels seen in age-matched pttg<sup>−/−</sup> control male mice when compared with pttg<sup>+/+</sup> control animals is likely secondary to the pttg-null lipodystrophy, which is caused primarily by the low insulin levels. Several studies in humans and rodents have shown sexual dimorphism in plasma levels of adiponectin, with males having lower levels than females (Berg et al. 2002, Nishizawa et al. 2002). Furthermore, hypogonadal males with low testosterone levels have higher levels of adiponectin when compared with eugonadal males, and testosterone treatment attenuates adiponectin levels (Lanfranco et al. 2004). In rodents, male but not female gonadectomy was followed by increased adiponectin levels and improved insulin sensitivity (Nishizawa et al. 2002). Increased adiponectin levels in females is likely related to the low-testosterone state (Nishizawa et al. 2002).

Serum leptin levels also increased in gonadectomized pttg<sup>−/−</sup> male mice when compared with control pttg<sup>−/−</sup> males. Leptin, secreted from fat tissue, is a body-weight regulator through its control of feeding and energy expenditure. The association between leptin levels and insulin resistance are not yet fully delineated (Ceddia et al. 2002). The observed increase in serum leptin levels in gonadectomized pttg<sup>−/−</sup> males was not as marked as the increased adiponectin levels, and therefore leptin is not a major candidate for diabetes protection in our model.

Compared with gonadectomy alone, estradiol treatment of gonadectomized pttg<sup>−/−</sup> male mice conferred additional protection from diabetes development. These animals had normal fasting blood glucose levels for up to 13-5 months and a normal response to IPGTT for up to 12 months. These changes were accompanied by increased indices of insulin sensitivity and higher levels of adiponectin, but not leptin.

Several studies have shown that estrogen exerts protective effects on male animals with a genetic predisposition to develop diabetes. In db/db mice on the C57BL/KsJ background chronic low-dose estradiol therapy effectively ameliorated the severity of diabetes and obesity typical of this model, reduced body mass and restored functional pancreatic islet cytoarchitecture (hypercortisolipidemia, cytohypertrophy and atrophy). These changes were accompanied by increased pancreatic and serum insulin levels (Garris & Garris 2005). In obese hIAPP (human islet amyloid polypeptide) males, diabetes was prevented when estradiol therapy was started at a young age, and diabetes controlled when estradiol therapy was initiated at an older age. These protective effects were mediated by increased insulin sensitivity secondary to estrogen-induced weight loss. Estradiol therapy in these mice also prevented β-cell degeneration and amyloid deposition (Geisler et al. 2002).

Estradiol has been shown both in vivo and in vitro to have positive effects on the structure, size, number and function of pancreatic islet β-cells (Bailey & Ahmed-Sorour 1980, Puah & Bailey 1985, Zhu et al. 1998, Choi et al. 2005). Recent evidence supports the presence of a novel non-nuclear estrogen receptor which exerts rapid actions in the endocrine pancreas, enabling calcium fluxes that favor insulin secretion (Ropero et al. 2002, Sutter-Dub 2002). In our experiments serum insulin levels were not increased following gonadectomy and estradiol therapy; moreover, we did not observe beneficial trophic effects of estradiol on islet morphology. The results favor increased insulin sensitivity induced by estradiol in pttg-null mice. Mechanisms by which estradiol conferred almost total protection against diabetes development in pttg<sup>−/−</sup> males are still undetermined.

Estrogen has a key role in body fat composition. Lack of estrogen, as seen in the postmenopausal state, is characterized by increased body fat and changes in body fat distribution shifting from peripheral to central adiposity concomitant with increased insulin resistance (Gambacciani et al. 2001, Wu et al. 2001, Liu et al. 2004). Male mice lacking estrogen receptors have increased adipose tissue, increased insulin resistance and impaired glucose tolerance (Heine et al. 2000).

The additional protection conferred by estradiol in our model could be due to decreased adipose tissue or its mobilization from central (android) to peripheral (gynoid) depots, which indirectly increases insulin sensitivity. Estradiol treatment in gonadectomized pttg<sup>−/−</sup> and pttg<sup>+/+</sup> male mice was accompanied with weight loss (data not shown), most probably through decreased fat mass, but we did not perform quantitative assessment of fat tissue and/or its distribution.

Taken together, the results suggest that gonadectomy with and without estradiol therapy confers diabetes protection in pttg<sup>−/−</sup> male mice by increased insulin sensitivity associated with increased serum adiponectin levels and elevated leptin levels. Delayed diabetes onset in pttg<sup>−/−</sup> females may also in part occur secondarily to higher levels of adiponectin and leptin when compared with age-matched pttg<sup>−/−</sup> control males. Supporting these conclusions is the fact that the percent β-cell area, average islet size and the number of pancreatic islets per mm<sup>2</sup> were not increased in pttg<sup>−/−</sup> males following gonadectomy, either with or without estradiol therapy. This conclusion is also supported by the unaltered fasted or challenged insulin and C-peptide levels following these
sex-steroid interventions. These results show that, in the absence of pttg, effects of male-selective β-cell hypoplasia are restored by sex-steroid environmental changes and their effect on peripheral insulin sensitivity.

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