Original Article

PED/PEA-15 Regulates Glucose-Induced Insulin Secretion by Restraining Potassium Channel Expression in Pancreatic β-Cells

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The phosphoprotein enriched in diabetes/phosphoprotein enriched in astrocytes (*ped/pea-15*) gene is overexpressed in human diabetes and causes this abnormality in mice. Transgenic mice with β-cell–specific overexpression of *ped/pea-15* (β-tg) exhibited decreased glucose tolerance but were not insulin resistant. However, they showed impaired insulin response to hyperglycemia. Islets from the β-tg also exhibited little response to glucose. mRNAs encoding the *Sur1* and *Kir6.2* potassium channel subunits and their upstream regulator *Foxa2* were specifically reduced in these islets. Overexpression of PED/PEA-15 inhibited the induction of the atypical protein kinase C (PKC)-ζ by glucose in mouse islets and in β-cells of the MIN-6 and INS-1 lines. Rescue of PKC-ζ activity elicited recovery of the expression of the *Sur1, Kir6.2*, and *Foxa2* genes and of glucose-induced insulin secretion in PED/PEA-15–overexpressing β-cells. Islets from *ped/pea-15*–null mice exhibited a twofold increased activation of PKC-ζ by glucose; increased abundance of the *Sur1*, *Kir6.2*, and *Foxa2* mRNAs; and enhanced glucose effect on insulin secretion. In conclusion, PED/PEA-15 is an endogenous regulator of glucose-induced insulin secretion, which restrains potassium channel expression in pancreatic β-cells. Overexpression of PED/PEA-15 dysregulates β-cell function and is sufficient to impair glucose tolerance in mice. *Diabetes* 56: 622–633, 2007

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2-DG, 2-[1-^{3}H]deoxy-D-glucose; PED/PEA-15, phosphoprotein enriched in diabetes/phosphoprotein enriched in astrocytes; PKC, protein kinase C; RIA, radioimmunoassay.

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Type 2 diabetes is a genetically determined disorder for which the pathogenesis is characterized by development of impaired secretogogue-regulated insulin secretion and insulin resistance (1,2). Metabolic abnormalities caused by insulin resistance contribute to β-cell dysfunction in type 2 diabetes (3–5). Deranged β-cell function is also determined by genetic factors, but their identity remains elusive (3,5).

Phosphoprotein enriched in diabetes/phosphoprotein enriched in astrocytes (PED/PEA-15) was originally identified as a major astrocyte phosphoprotein (6,7) and found to be widely expressed in different tissues (8) and highly conserved among mammals (7). It is a highly regulated protein (6,9,10) whose gene maps on human chromosome 1q21-22 (8). Several studies in cultured cells and rodent tissues have revealed that PED/PEA-15 is a cytosolic scaffold protein, as it regulates multiple cellular functions by binding to distinct components of major intracellular transduction pathways (10–14). These include extracellular signal–related kinase-1/2, Akt, and ribosomal protein S6kinase2. PED/PEA-15 is a death effector domain–containing protein (15). In part through this interaction domain, it inhibits a number of molecules conveying apoptotic signals to the nucleus (10–12). PED/PEA-15 also binds to and increases the stability of phospholipase D, enhancing its activity and controlling important mechanisms in cell metabolism (14).

Gene profile studies evidenced that *ped/pea-15* is commonly overexpressed in individuals with type 2 diabetes (8,16). In cultured muscle and adipose cells and in peripheral tissues from transgenic mice, high levels of PED/PEA-15 impair insulin-stimulated GLUT4 translocation and glucose transport, suggesting that PED/PEA-15 overexpression may contribute to insulin resistance in type 2 diabetes (13,17). Indeed, more recent studies revealed that in offspring of type 2 diabetic subjects, PED/PEA-15 levels negatively correlate to insulin sensitivity (16). Other studies have demonstrated that PED/PEA-15–induced resistance to insulin action on glucose disposal is accompanied by phospholipase D–dependent activation of the classical protein kinase C (PKC) isoform PKC-α (13). In turn, the induction of PKC-α by PED/PEA-15 prevents subsequent activation of the atypical PKC-ζ by insulin (13,17). Rescue of PKC-ζ activity in cells overexpressing PED/PEA-15 restores normal sensitivity to insulin of the glucose transport machinery (17). Thus, in muscle and adipose cells,

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PED/PEA-15 generates resistance to insulin action on glucose disposal by impairing normal regulation of PKC-ζ (17). Characterization of transgenic mice overexpressing PED/PEA-15 ubiquitously evidenced that, in addition to generating insulin resistance and altered glucose tolerance, high levels of PED/PEA-15 impair glucose-regulated insulin secretion (13). Whether this effect is directly caused by the action of PED/PEA-15 in the β-cell and through which mechanism was not conclusively established in these studies. Whether PED/PEA-15–determined β-cell abnormalities are sufficient to impair glucose tolerance in these mice is also unknown.

RESEARCH DESIGN AND METHODS

Generation of transgenic mice. The *rip-I/ped/pea-15* chimeric gene was obtained by introducing a 2.3-Kb *Eco*RI fragment containing the entire human *ped/pea-15* cDNA at the *Eco*RI site in a pB5-RIP-β-globin expression vector (kindly provided by Prof. D. Accili, Columbia University, NY). This chimeric gene was microinjected into fertilized C57BL6/SJL mouse eggs. The general procedures used for microinjection and animal selection have been previously described (18). Six F_0 founders were identified by PCR as in reference 13. Mice were fed ad libitum with a standard diet (Research Diets formulas D12328; Research Diets, New Brunswick, NJ) and kept under a 12-h light/12-h dark cycle. Tissue samples were collected rapidly after mice were killed by pentobarbitone overdose. Tissues were snap frozen in liquid nitrogen and stored at −80°C. All procedures described below were approved by the institutional animal care and utilization committee. Blood glucose levels were measured with glucometers (A. Menarini Diagnostics, Florence, Italy); insulin was measured by radioimmunoassay (RIA) with rat insulin as standard (Insulin Rat RIA kit; Linco Research, St. Louis, MO). Fasting plasma free fatty acids were measured with the Wako NEFA C kit (Wako Chemicals, Richmond, VA), and triglycerides were measured with the Infinity triglyceride reagent (Sigma-Aldrich, St. Louis, MO).

Determination of glucose and insulin tolerance and assessment of insulin secretion and glucose utilization. Glucose tolerance tests, insulin tolerance tests, and insulin secretion were measured as previously described (19,20). For analyzing glucose utilization by skeletal muscle, an intravenous injection of 1 μCi of the nonmetabolizable glucose analog 2-[1-^{3}H]deoxy-D-glucose (2-DG) (Amersham Pharmacia Biotech, Piscataway, NJ) and an intraperitoneal injection of insulin (0.75 mU/g body wt) were administered to random fed mice. The specific blood 2-DG clearance was determined with 25-μl blood samples (tail vein) obtained 1, 15, and 30 min after injection as previously reported (21). Skeletal muscles were removed 30 min after the injections. Glucose utilization index was determined by measuring the accumulation of radiolabeled compound (22). The amount of 2-DG-6 phosphate per milligram of protein was then divided by the integral of the concentration ratio of 2-DG to the measured unlabeled glucose. Glucose utilization indexes were expressed as picomoles per milligram of protein per minute.

Immunoistochemistry and morphometric analysis. Generation of PED/PEA-15 antibody has been previously reported (8). Immunoistochemical detection of PED/PEA-15, insulin, and glucagon in pancreases from control and transgenic mice were analyzed as described (13). Analysis of serial consecutive islet sections stained with either insulin or PED/PEA-15 antibodies was used to confirm PED/PEA-15 expression in insulin-immunopositive β-cells. Sections were also stained with hematoxylin and eosin. For morphometry, pancreases were obtained from 6-month-old control and transgenic mice, and immunohistochemical detection of insulin was performed in three sections (2–3 μm) separated by 200 μm as in reference 18.

Islet isolation, ex vivo insulin secretion assessment, and estimation of pancreatic insulin content. Islets were isolated from 6-month-old mice by collagenase digestion and subsequent centrifugation on a Histopaque (Sigma-Aldrich) gradient as in reference 23. A total of 20 islets were manually selected and preincubated in Dulbecco's modified Eagle's medium (Life Technologies) at 37°C in a 5% CO_2 atmosphere for 24 h. Islets were then further incubated in Krebs-Ringer buffer (120 mmol/l NaCl, 1.2 mmol/l $MgSO_4$, 5 mmol/l KCl, 10 mmol/l $NaHCO_3$, 1.3 mmol/l $CaCl_2$, and 1.2 mmol/l KH_2PO_4) for 30 min and then stimulated at 37°C with various concentrations of either glucose for 1 h, KCl for 30 min, or glyburide (Sigma-Aldrich) for 1 h. Islets were subsequently collected by centrifugation and supernatants assayed for insulin content by RIA. For measuring total pancreatic insulin content, pancreases were solubilized in acid-ethanol solution (75% ethanol, 1.5% HCl) overnight at 4°C and centrifuged for 15 min at 800*g*. Insulin was measured in the supernatants by RIA.

Cell culture procedures and transfection. MIN-6 cells were cultured in Dulbecco's modified Eagle's medium containing 25 mmol/l glucose, 50 μmol/l 2-mercaptoethanol, and 10% FCS (Biochrom) at 37°C in a 5% CO_2 atmosphere (24). INS-1 cells were cultured in RPMI-1640 medium (Life Technologies) containing 11 mmol/l glucose, 50 μmol/l 2-mercaptoethanol, and 10% fetal bovine serum (Biochrom) at 37°C in a 5% CO_2 atmosphere (25). Stable transfection of the *ped/pea-15* cDNA was performed with the lipofectamine method (13) according to the manufacturer's instructions. Transient transfection of the PKC-ζ cDNA was also performed with the lipofectamine method. By using PCAGGS-β-Gal as a reporter, transfection efficiency was between 65 and 85%, staining with the chromogenic substrate 5-bromo-4-chloro-3-indolyl b-D-galactopyranoside.

Northern blot and real-time RT-PCR analysis. Total cellular RNA was isolated from pancreatic islets and tissue samples by using the RNeasy kit (Qiagen Sciences) according to the manufacturer's instructions. Northern blot analysis was performed as in reference 18. For real-time RT-PCR analysis, 1 μg islet or cell RNA was reverse transcribed using Superscript II Reverse Transcriptase (Invitrogen, Carlsbad, CA). PCR were analyzed using SYBR Green mix (Invitrogen). Reactions were performed using Platinum SYBR Green qPCR Super-UDG using an iCycler IQ multicolor Real Time PCR Detection System (Biorad, Hercules, CA). All reactions were performed in triplicate, and β-actin was used as an internal standard. Primer sequences used were as follows: PED/PEA-15 forward 5′-TTCCCGCTGTTCCCTTAGG-3′, PED/PEA-15 reverse 5′-TCTGGCTCATCCGCATCC-3′; Kir6.2 forward 5′-TC CACCAGGTAGACATCCC-3′, inwardly rectifying K^+ channel 6.2 (Kir6.2) reverse 5′-TAGGAGCCAGGTCGTAGAG-3′; sulfonylurea receptor 1 (SUR1) forward 5′-GCATCAACTTGTCTGGTG-3′, SUR1 reverse 5′-ACTGTCCTCTT GTCATCC-3′; forkhead box A2 (Foxa2) forward 5′-CACCTGAGTCCGAGTCTG 3′, Foxa2 reverse 5′-CGAGTTCATGTTGGCGTAG-3′; glucose transporter 2 (Glut2) forward 5′-ACAGTCACACCAGCATAC-3′, Glut2 reverse 5′-ACCCA CCAAAGAATGAGG3′; glucokinase (GK) forward 5′-GCTGTACGAAAAGAT CATTGG-3′, GK reverse 5′-CTCCGTGGAACAGAAGATTC-3′; hexokinase (HK)1 forward 5′-ACGACACCCCAGAGAACATC-3′, HK1 reverse 5′-AGTCTC CGAGGCATTCAGC-3′; HK2 forward 5′-CTCAGGACACCACAGGCTAC-3′, HK2 reverse 5′-TTCACTCACTGGGTCACTAAGG-3′; and β-actin forward 5′-GCGTGACATCAAAGAGAAG-3′, β-actin reverse 5′-ACTGTGTTGGCATA GAGG-3′.

Western blot analysis and PKC assay. Tissues homogenates and cell lysates were separated by SDS-PAGE and analyzed by Western blot as previously described (13,17,26). Membranes were probed with antibodies to PED/PEA-15 (8), PKC-ζ (Santa Cruz Biotechnology, Santa Cruz, CA), or tubulin (Santa Cruz Biotechnology). For analyzing atypical PKC activity, cells were incubated with Krebs-Ringer buffer for 30 min and then incubated with either 2.8 or 16.7 mmol/l glucose containing medium for 1 h at 37°C in a 5% CO_2 atmosphere. Cells were solubilized in lysis buffer (25 mmol/l Tris-HCl, pH 7.4, 0.5 mmol/l EDTA, 0.5 mmol/l EGTA, 0.05% Triton X-100, 10 mmol/l β-mercaptoethanol, 1 μg/ml leupeptin, and 1 μg/ml aprotinin) for 1 h at 4°C. Lysates were precipitated with a PKC-ζ antibody (17), and PKC activity was assayed using the SignaTECT PKC assay system (Promega, Madison, WI) according to the manufacturer's instructions. Determination of PKC activity using the Ac-MBP (4–14) or the pseudosubstrate region of PKC-ϵ (specific for atypical PKC) provided consistent results.

Statistical procedures. Data were analyzed with Statview software (Abacus Concepts) by one-factor ANOVA. P values <0.05 were considered statistically significant. The total area under the curve for glucose response during the insulin tolerance test was calculated by the trapezoidal method (13).

RESULTS

To investigate the relevance of PED/PEA-15–induced β-cell dysfunction to glucose tolerance, we used the insulin promoter to generate transgenic mice featuring selective overexpression of PED/PEA-15 in the β-cells (β-tg; Fig. 1*A*). Three lines of β-tg mice (L5, L8, and L9) were established in which *ped/pea-15* mRNA as well as PED/PEA-15 protein showed a 10- to 20-fold increased expression in the β-cells but unchanged expression in other tissues (Fig. 1*B* and *C*). Transgenic mice were fertile and generated viable offsprings showing no significant growth alterations compared with their nontransgenic littermates (controls) or other apparent abnormalities. L8 *ped/pea-15* transgenic mice, both male and female, exhibited slightly reduced fasting blood glucose levels compared with control mice (males 84 ± 6 vs. 91 ± 7 mg/dl; females 82 ± 8 vs. 92 ± 6 mg/dl; significant at $P < 0.05$). Similarly reduced fasting blood glucose levels were mea-

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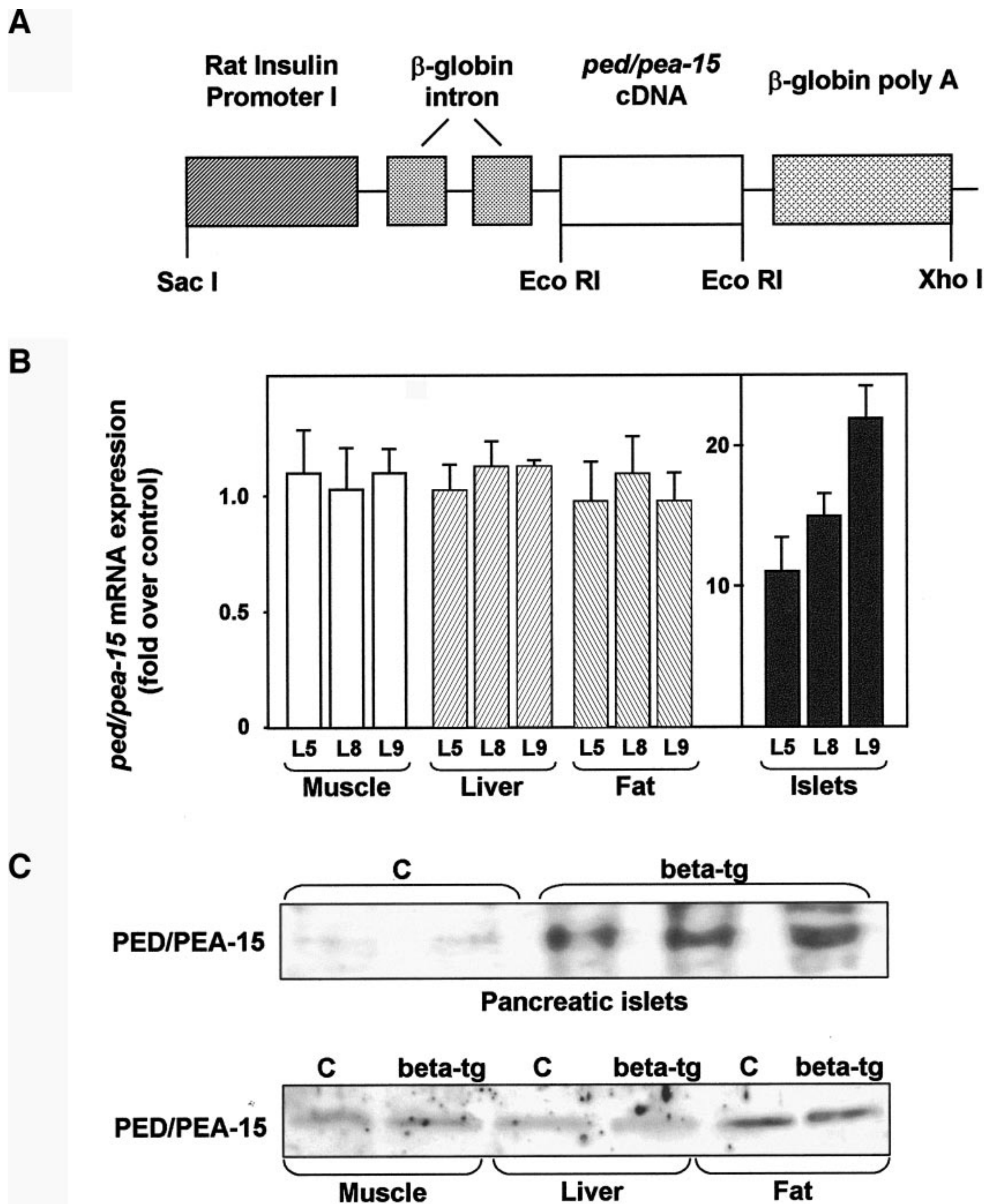

FIG. 1. Generation of β-tg (beta-tg) mice. ***A*****: Subcloning of the** ***ped/pea-15*** **cDNA in the pB5-RIP-β-globin expression vector. Tissues are from three lines of β-tg mice (L5, L8, and L9) and from their nontransgenic littermates (C) and were subjected to Northern (*****B*****) or Western (*****C*****) blotting. Northern blots (30 μg RNA/lane) were probed with** ***ped/pea-15*** **cDNA. Loading of the same amount of RNA in each lane was ensured by further blotting the filters for β-actin. Quantitation was performed by densitometric analysis. Data are plotted as increase of** ***ped/pea-15*** **mRNA expression in transgenic versus control mice. Each bar represents the means ± SD of determinations in five transgenic and five nontransgenic animals. For Western blotting, tissue lysates (100 μg proteins/lane) were blotted with the PED/PEA-15 antiserum. The autoradiographs shown are representative of five (*****top*****) and six (*****bottom*****) independent experiments.**

sured in the other transgenic lines (data not shown). However, glucose loading (2 g/kg) rendered the β-tg mice significantly more hyperglycemic during the following 120 min compared with control mice (Fig. 2*A* and *B*). Very similar abnormalities were observed in the L5 and L9 lines (data not shown), indicating that β-cell overexpression of PED/PEA-15 is sufficient to impair glucose tolerance in mice. As shown in Fig. 2, sex had no effect on the impairment in glucose tolerance caused by β-cell overexpression of PED/PEA-15. Aging (3–14 months) also determined no significant changes in PED/PEA-15–induced decreases in glucose tolerance (data not shown).

L8 β-tg mice featured no significant differences in fasting nonesterified free fatty acid and triglyceride blood concentrations compared with their controls (free fatty acids 0.82 ± 0.03 vs. 0.78 ± 0.05 mmol/l; triglycerides 115 ± 7 vs. 111 ± 8 mg/dl). However, fasting insulin levels were 80% increased in the transgenics (0.31 ± 0.07 vs. 0.17 ± 0.03; $P < 0.001$). The same results were obtained in the L5 and the L9 lines, with no sex- or age-related variation (data not

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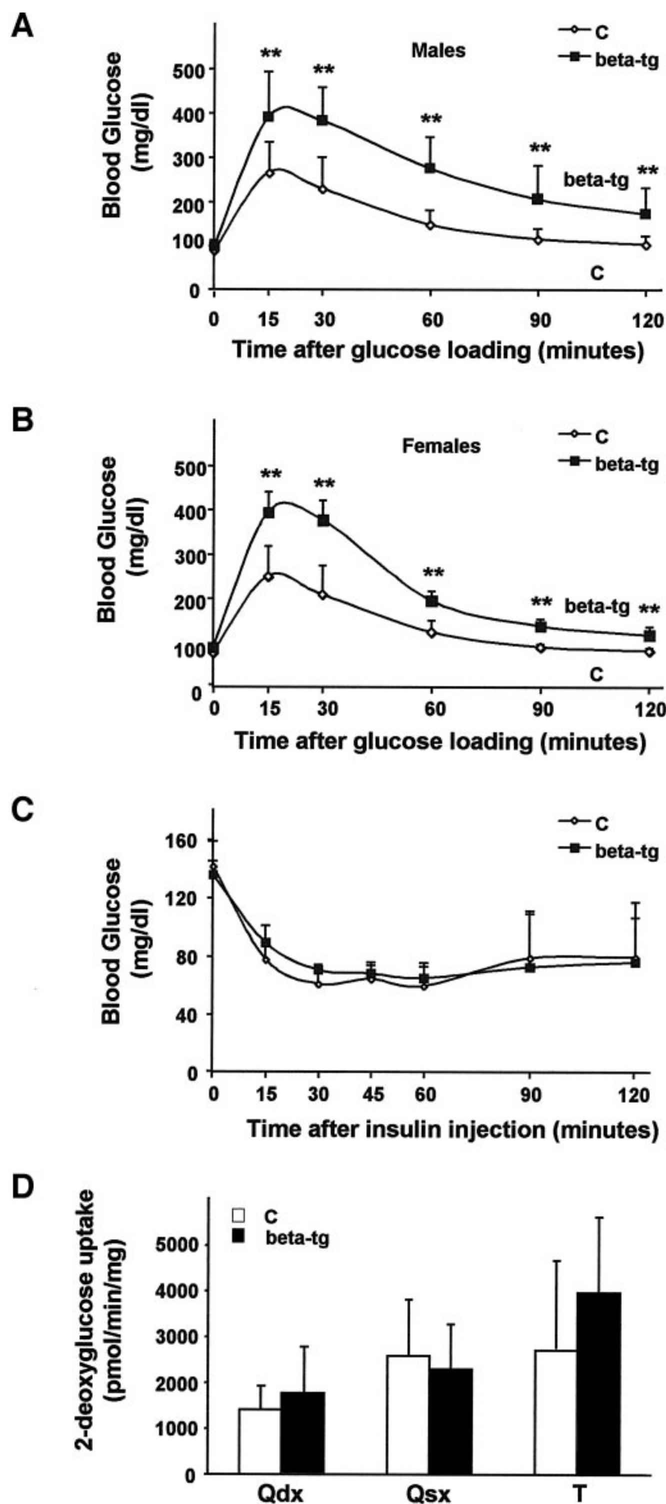

FIG. 2. Glucose tolerance, insulin sensitivity, and glucose transport in β-tg mice. Three-month-old β-tg male (*A*) and female (*B*) mice and their nontransgenic littermates (C) were fasted for 16 h and subjected to intraperitoneal glucose loading (2 g/kg body wt). Blood glucose levels were determined before and at the indicated times following the load. Values are expressed as means ± SD of determinations in at least 12 (*A*) and 15 (*B*) mice per group. *Statistically significant differences (*$P < 0.05$; **$P < 0.01$). *C*: Three-month-old random, fed β-tg mice and their nontransgenic littermates (*C*) ($n = 12$/group; 6 males and 6 females) were injected intraperitoneally with insulin (0.75 mU/g body wt), followed by determinations of blood glucose levels at the indicated times. Values are expressed as means ± SD. *D*: Weight-matched, random, fed β-tg mice and their nontransgenic littermates were subjected to intravenous injection of 1 μCi of 2-DG and intraperitoneal injection of insulin. Tibialis (T) and right (dx) and left (sx) quadriceps (Q) muscles were removed 30 min after and snap frozen in liquid nitrogen. 2-DG accumulated in muscle tissues was quantitated as described in RESEARCH DESIGN AND METHODS. Bars represent mean values ± SD of determinations in at least seven mice/group.

shown). To verify whether β-cell overexpression of PED/PEA-15 is accompanied by reduced insulin sensitivity, we performed insulin tolerance tests. Intraperitoneal injection of insulin (0.75 mU/g) evoked comparable hypoglicemic responses in both β-tg and control mice (Fig. 2*C*) with no significant difference in glucose areas under the curve in all of the transgenic lines (C 9,180 ± 1,900 mg/dl per 120 min; L5 8,898 ± 1,800 mg/dl per 120 min; L8 8,805 ± 1,600 mg/dl per 120 min; L9 9,121 ± 1,600 mg/dl per 120 min). Furthermore, insulin-dependent glucose uptake in quadriceps and tibialis muscles from β-tg and control mice exhibited no difference (Fig. 2*D*).

We then investigated how glucose tolerance is reduced in the β-tg and analyzed their islets by immunohistochemistry and morphometry. Islets from β-tg mice immunostained with PED/PEA-15 antibody significantly more intensely than those from control mice (Fig. 3*A*). Importantly, islets from the β-tg mice were larger and showed a more elongated and irregular shape but feature normal distribution of α- and β-cells compared with control islets (Fig. 3*B*). Also, quantitative morphometry revealed twofold increased islet and β-cell mass per unit of total pancreatic area in the β-tg mice ($P < 0.01$) with no significant difference in their pancreas weight (Table 1).

In control mice, a sevenfold increase in insulin secretion was observed 3 min after intraperitoneal glucose injection, and the levels remained higher than baseline values for up to 30 min, indicating second-phase response (Fig. 3*C*). Based on the area under the curve, the acute first-phase insulin secretory response to glucose was reduced by more than threefold in β-tg mice ($P < 0.001$). The second-phase response was also significantly impaired in the β-tg compared with control mice ($P < 0.001$). Again, almost identical decreases in early and late insulin responses were observed in both male and female β-tg mice and in all of the lines produced (data not shown). Thus, in vivo, isolated β-cell overexpression of PED/PEA-15 increases fasting insulinemia but impairs further insulin secretion response to hyperglycemia.

Similar as in intact mice, β-tg mouse islets also exhibited a twofold increased insulin release when incubated in medium containing 2.8 mmol/l glucose (Fig. 4*A*; $P < 0.001$). Raising glucose concentration did not further increase the insulin release by these islets, while enhancing that from control mice by 3.9-fold ($P < 0.001$). Similarly, islets from transgenic mice ubiquitously expressing PED/PEA-15 (13) showed 2.4-fold increased insulin release when exposed to 2.8 mmol/l glucose ($P < 0.001$) and no further release upon exposure to 16.7 mmol/l glucose (Fig. 4*B*). At variance with glucose, exposure to the membrane depolarising agent potassium chloride (33 mmol/l) caused a comparable release of insulin by the β-tg and the control islets, suggesting an effect of PED/PEA-15 on earlier events of β-cell response to glucose. Total insulin content in pancreases was 25% increased in β-tg mice (2.9 ± 0.4 ng/mg in β-tg vs. 2.3 ± 0.2 ng/mg in control mice; $P < 0.05$).

To gain further insight into the mechanisms leading to dysfunction of β-cells overexpressing PED/PEA-15, we have profiled the expression of several genes relevant to the glucose sensory apparatus by real-time RT-PCR analysis of total RNA isolated from islets of β-tg mice (Fig. 4*C*).

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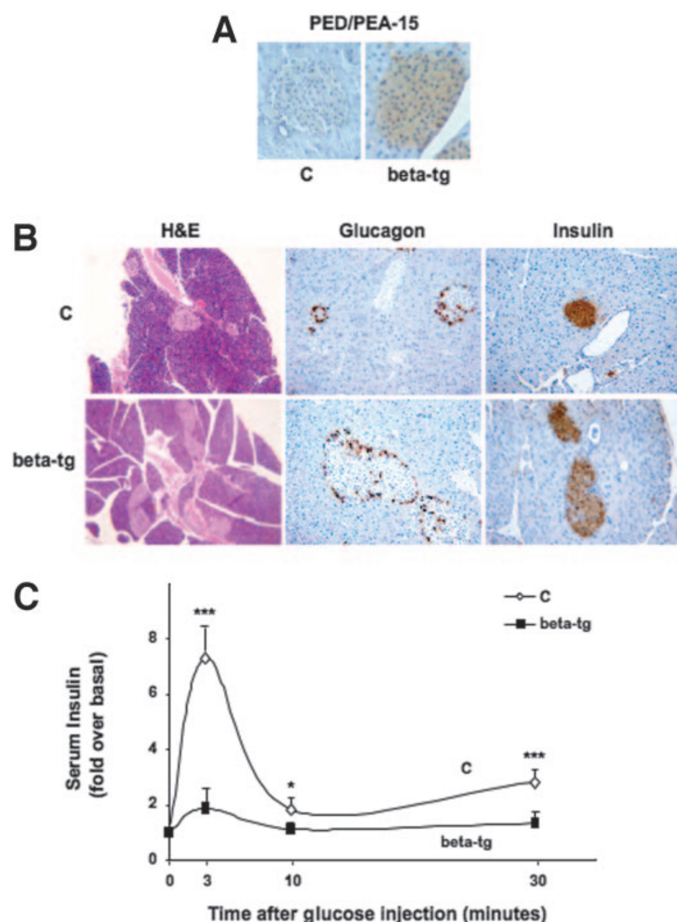

FIG. 3. Immunohistochemical analysis and insulin secretion in β-tg mice. *A* and *B*: Pancreases from β-tg and control (C) 6-month-old mice were fixed and embedded in Tissue-Tek OTC, and sections were prepared as described in RESEARCH DESIGN AND METHODS. Sections were stained with hematoxylin and eosin (H&E). Immunoistochemical analysis of the islets was carried out using PED/PEA-15 (*A*) or insulin or glucagon (*B*) antibodies, as indicated. Anti-goat or anti-rabbit immunoglobulin G was used as the second antibody. Immunoreactivity was revealed by peroxidase-labeled streptavidin. The microphotographs shown (H&E 200×; glucagon and insulin 400×) are representative of images obtained from eight transgenic (male/female = 1) and eight nontransgenic (male/female = 1) mice. Note that β-tg mice show an increased number of enlarged islets. *C*: β-Tg and control mice (C) were fasted overnight and then injected with glucose (3 g/kg body wt) intraperitoneally. Serum insulin concentrations were measured at the indicated times by RIA. Data points represent the means ± SD of determinations in 10 β-tg and 12 control mice. *Statistically significant differences (*$P < 0.05$; ***$P < 0.001$).

The amounts of mRNAs for the *Sur1* subunit of the ATP-sensitive K^+ channel was significantly reduced by 30% in these islets compared with those in control mice ($P < 0.001$). Consistently, islets from both the β-tg and the transgenic mice featuring ubiquitous overexpression of PED/PEA-15 exhibited no secretory response to the K^+ channel locking agent glyburide (Fig. 4*D*). RNAs encoding the *Kir6.2* potassium channel subunit and the *Sur1/Kir6.2* upstream regulator *Foxa2* were also reduced, respectively, by 30 and 35% in the islets from the *ped/pea-15* β-cell–specific transgenics ($P < 0.01$). Decreased expression of *Sur1*, *Kir6.2*, and *Foxa2* mRNAs was confirmed by Northern blot analysis (Fig. 4*E*). At variance, the abundance of *glucokinase*, *HK1*, *HK2*, and *GLUT2* mRNAs did not differ between transgenic and control mice (Fig. 4*C* and *E*).

A ~35% decrease in the abundance of the *Sur1*, *Kir6.2*, and *Foxa2* mRNAs has also been observed in the glucose-responsive MIN-6 and INS-1 β-cell lines upon transfection with *ped/pea-15* cDNA (Min-$6_{ped/pea-15}$, INS-$1_{ped/pea-15}$; Fig. 5*A*; $P < 0.001$). As in the mouse islets, these changes were accompanied by a significantly greater release of insulin when in low-glucose medium (2.8 mmol/l glucose) with no further insulin secretion upon exposure to 16.7 mmol/l glucose (Fig. 5*B*; $P < 0.001$). This raise in glucose concentration caused a 2.5- and 4-fold enhancement in insulin secretion, respectively, by the untransfected INS-1 and MIN-6 cells, however. In both the mouse islets and the cultured β-cell lines, the effect of glucose on insulin release was paralleled by a twofold increase in immunoprecipitated PKC-ζ activity, with no measurable change in its expression (Fig. 5*C*). Interestingly, however, the effect of glucose was almost completely abolished in cells overexpressing PED/PEA-15 whether the MIN-6 or the β-tg mouse islets. Similar data were obtained in the INS-1 cells (data not shown), further suggesting an important role of atypical PKCs in regulating genes of the insulin secretion pathway. To further analyze this issue, we forced the expression of PKC-ζ in the MIN-$6_{ped/pea-15}$ and the INS-$1_{ped/pea-15}$ cells. The overexpression largely rescued PKC-ζ activity in the precipitates from glucose-exposed MIN-$6_{ped/pea-15}$ cells (Fig. 6*A*). Importantly, this rescue was accompanied by recovery of the *Sur1*, *Kir6.2*, and *Foxa2* gene expression (Fig. 6*B*). In part, the changes in insulin release caused by PED/PEA-15 were also reverted, determining recovery of glucose-induced insulin secretion (Fig. 6*C*). The same results were obtained using the INS-1 cells (data not shown), indicating that the changes in atypical PKC function caused by PED/PEA-15 overexpression represent upstream abnormalities impairing the function of multiple genes involved in regulating insulin secretion by the β-cell.

To further address this issue, we used islets from *ped/pea-15*–null mice. These animals have been previously characterized and reported (27) and feature no PED/PEA-15 expression in β-cells (Fig. 7*A*). Importantly, islets from these animals evidenced a twofold increased PKC-ζ activation following glucose exposure, as compared with islets from control mice ($P < 0.01$; Fig. 7*B*). In parallel with this enhanced response, islets from the *ped/pea-15*–null mice exhibited a significant twofold increase in sensitivity to glucose and glyburide effect on insulin secretion compared with control islets, with unchanged response to potassium chloride ($P < 0.001$; Fig. 7*C*). Abundance of the *Sur1*, *Kir6.2*, and *Foxa2* mRNAs was also increased by 45% in these islets ($P < 0.001$; Fig. 7*D*), indicating that these genes represent major effectors through which PED/PEA-15 physiologically controls insulin secretion.

TABLE 1
Morphometric analysis of β-tg pancreas

	Control	β-Tg
% islet area/pancreatic area	0.52 ± 0.06	1.04 ± 0.11**
% β-cells/islet	76.87 ± 4.66	81.33 ± 2.08*
% α-cells/islet	17.23 ± 1.92	14.54 ± 1.85 NS
% other cells/islet	5.90 ± 0.71	4.13 ± 0.41 NS
Pancreas weight (mg)	182 ± 16	188 ± 12 NS
Islet cell mass (mg)	0.95 ± 0.08	1.96 ± 0.06**
β-Cell mass (mg)	0.73 ± 0.05	1.59 ± 0.06**

Data are means ± SD in six β-tg and six control (nontransgenic littermates) mice. Mice were analyzed as described in RESEARCH DESIGN AND METHODS. * and ** denote statistically significant differences, respectively, at the $P < 0.05$ and 0.01 levels.

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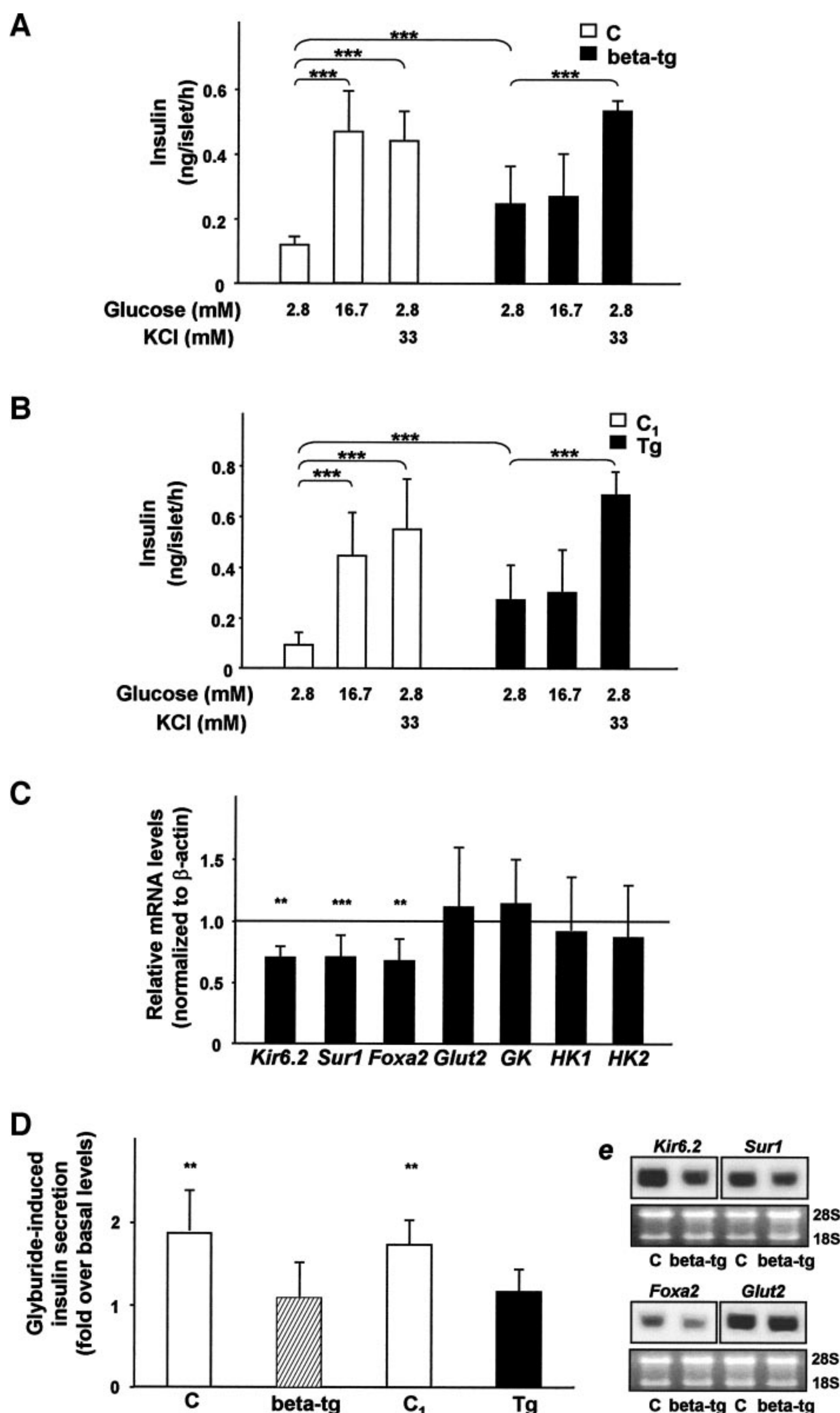

FIG. 4. Insulin secretion and gene expression profile in isolated islets from PED/PEA-15–overexpressing mice. Islets were isolated from 6-month-old mice, either from the β-tg and their nontransgenic littermates (C, open bars) (*A*) or from transgenic mice ubiquitously overexpressing PED/PEA-15 (33) and their nontransgenic littermates (C1, open bars) (*B*). Insulin release was determined upon exposure to the indicated concentrations of glucose or potassium chloride as described in RESEARCH DESIGN AND METHODS. Bars represent the means ± SD of determinations in 12 (*A*) and 11 (*B*) independent experiments in duplicate. *C*: The abundance of mRNAs for the indicated proteins was determined by real-time RT-PCR analysis of total RNA isolated from islets of β-tg mice and their nontransgenic littermates using β-actin as internal standard. The mRNA levels in β-tg islets are relative to those in control animals (C). Each bar represents the mean ± SD of four independent experiments in each of whom reactions were performed in triplicate using the pooled total RNAs from six mice/genotype. *D*: Islets were isolated from 6-month-old β-tg and ubiquitously PED/PEA-15–overexpressing transgenic mice and their nontransgenic littermates (respectively, C and C1). Insulin release was determined after exposure to 10 μmol/l glyburide for 60 min, as described in RESEARCH DESIGN AND METHODS. Bars represent the means ± SD of data from four independent experiments, each with at least three mice/group. *Statistically significant differences ($P < 0.01$; ***$P < 0.001$). *E*: Total RNA isolated from β-tg and control (C) islets was analyzed by agarose-formaldehyde electrophoresis and hybridized to the indicated cDNA probes. 28S and 18S ribosomal RNA is shown as a loading control.**

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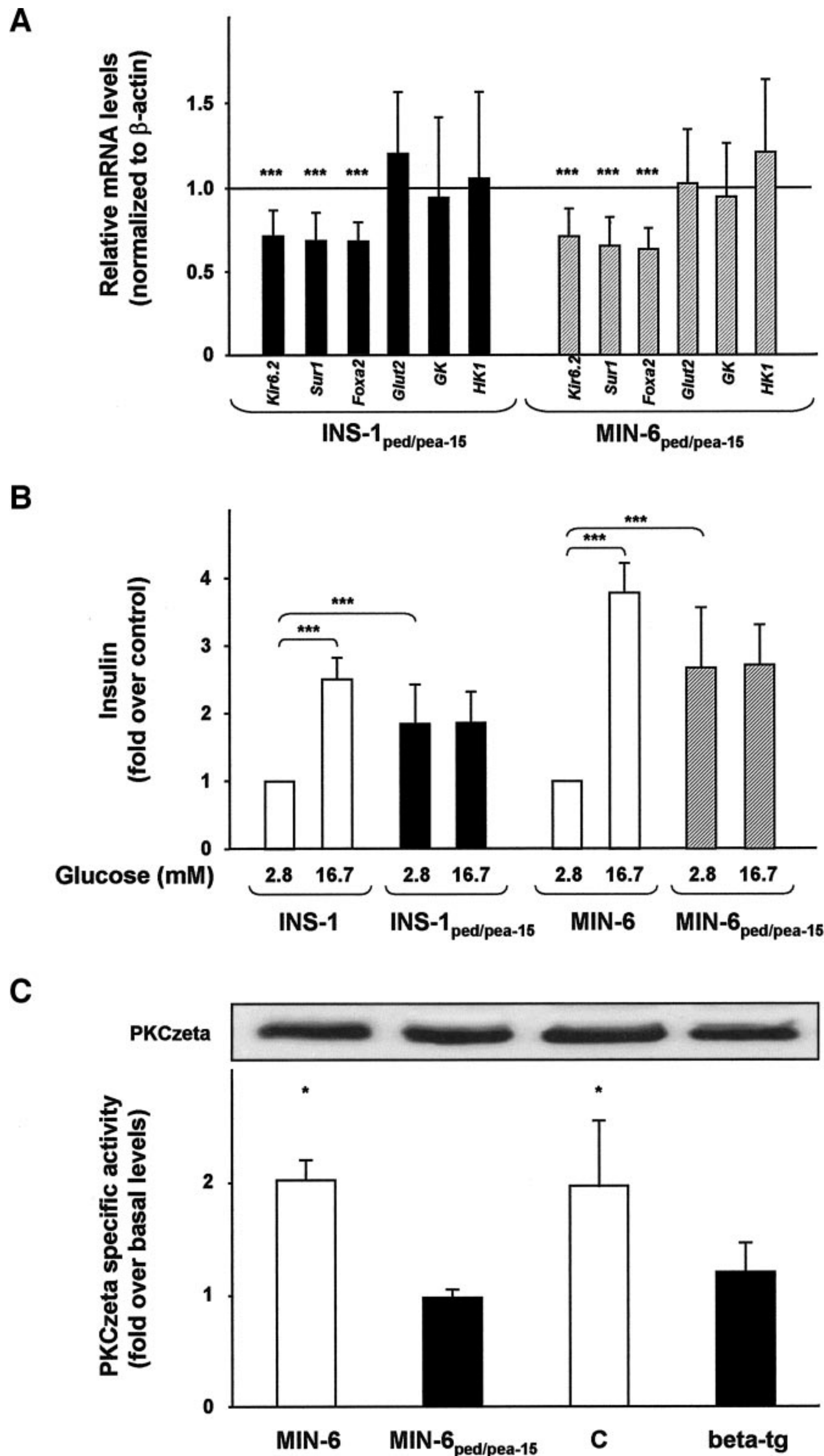

FIG. 5. Gene expression profile, glucose-stimulated insulin secretion and PKC-ζ activity in β-cell lines overexpressing PED/PEA-15. *A*: The abundance of mRNAs for the indicated proteins was determined by real-time RT-PCR analysis of total RNA isolated from INS-1 and MIN-6 wild-type cells and from the same cells stably transfected with a *ped/pea-15* cDNA (INS-$1_{ped/pea-15}$ and MIN-$6_{ped/pea-15}$). Bars represent the mRNA levels in the transfected cells and are relative to those in wild-type (control) cells. Data are expressed as means ± SD of triplicate reactions for total RNAs from each cell type in five independent experiments. *B*: Insulin release was assayed by RIA in the culture medium of the wild-type and the *ped/pea-15*–transfected β-cells upon 60 min incubation with the indicated glucose concentrations. Bars represent mean values ± SD from six independent experiments each in duplicate. *C*: Islet from β-tg and control mice (*right*) and wild-type MIN-6 cells and those stably expressing the *ped/pea-15* cDNA (MIN-$6_{ped/pea-15}$; *left*) were exposed to 16.7 mmol/l glucose for 60 min and then solubilized. A total of 100 μg proteins were then precipitated with PKC-ζ antibody, and PKC activity was assayed as described in RESEARCH DESIGN AND METHODS. PKC activity is expressed as fold increase over basal activity (measured in the presence of 2.8 mmol/l glucose). Bars represent the mean ± SD of data from four independent experiments. For control, aliquots of the cell lysates were normalized for protein and directly blotted with PKC-ζ antibodies. Blots were revealed by enhanced chemiluminescence and autoradiography. The blot shown in the inset is representative of those in the other three experiments. *Statistically significant differences (*$P < 0.05$; ***$P < 0.001$).

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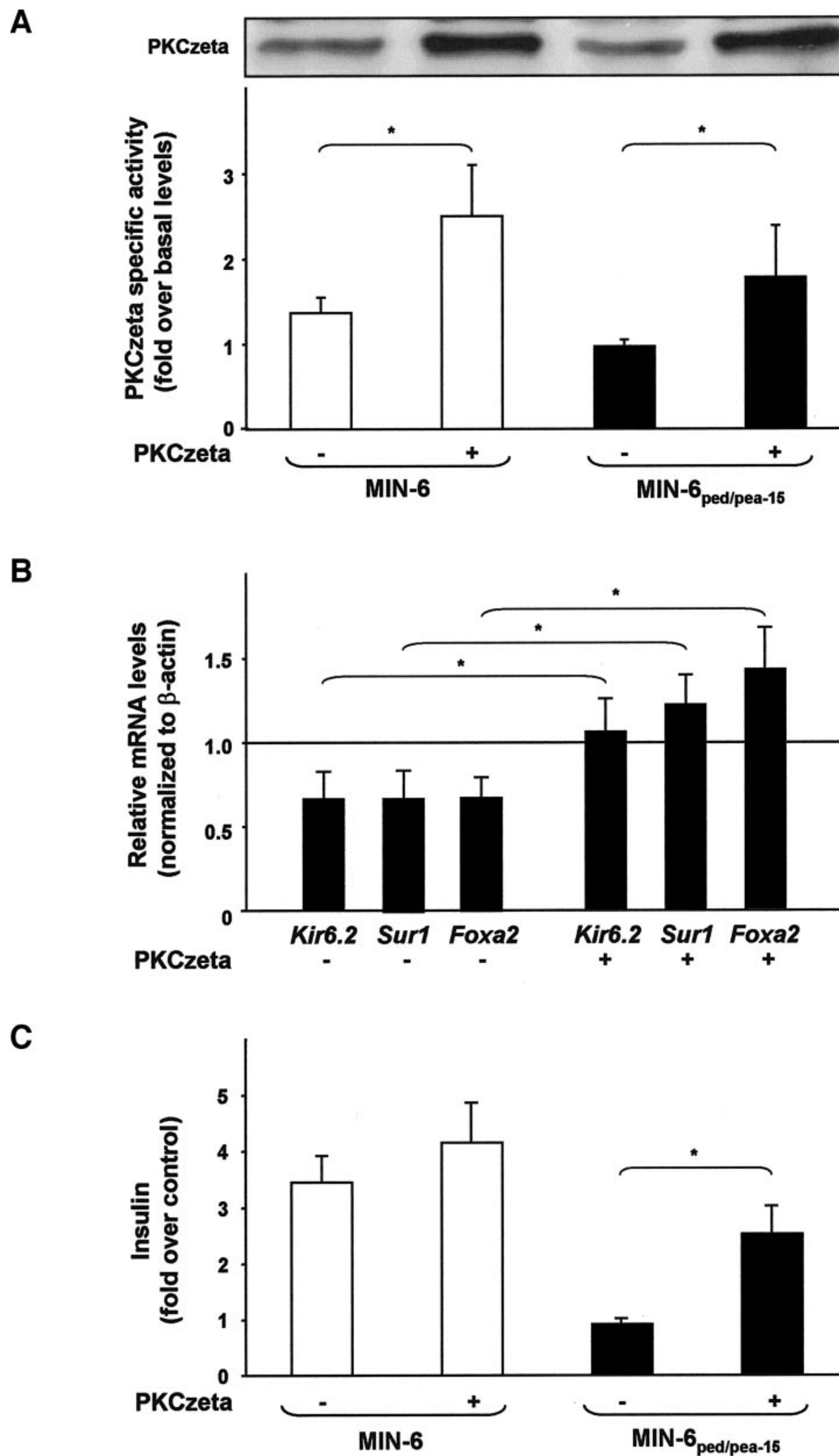

FIG. 6. Rescue of PKC-ζ activity, gene expression profile, and glucose-stimulated insulin secretion in $MIN_{ped/pea-15}$ cells. *A*: MIN-6 and MIN-$6_{ped/pea-15}$ cells were transiently transfected with a PKC-ζ cDNA, solubilized, and lysates immunoprecipitated with a PKC-ζ antibody. PKC activity was assayed in the immunoprecipitates. For control, aliquots of the lysates were blotted with the PKC-ζ antibody (*inset*). Bars represent the means ± SD of five independent experiments in duplicate. The autoradiograph shown in the inset is representative of blots from the five experiments. *B*: The abundance of mRNAs for the indicated proteins was determined by real-time RT-PCR analysis of total RNA isolated from MIN-6 and MIN-$6_{ped/pea-15}$ cells. Bars represent the mRNA levels in the MIN-$6_{ped/pea-15}$ cells and are relative to those in the untransfected cells (MIN-6). Data are expressed as means ± SD of triplicate reactions for total RNAs from each cell type in four independent experiments. *C*: The MIN-6 cells, either those transiently transfected with the PKC-ζ cDNA and the untransfected cells, were exposed to 16.7 mmol/l glucose for 60 min, and insulin release in the culture medium was assayed by RIA as described in RESEARCH DESIGN AND METHODS. Bars represent the means ± SD of duplicate determinations in three independent experiments. *Statistically significant differences (*$P < 0.05$).

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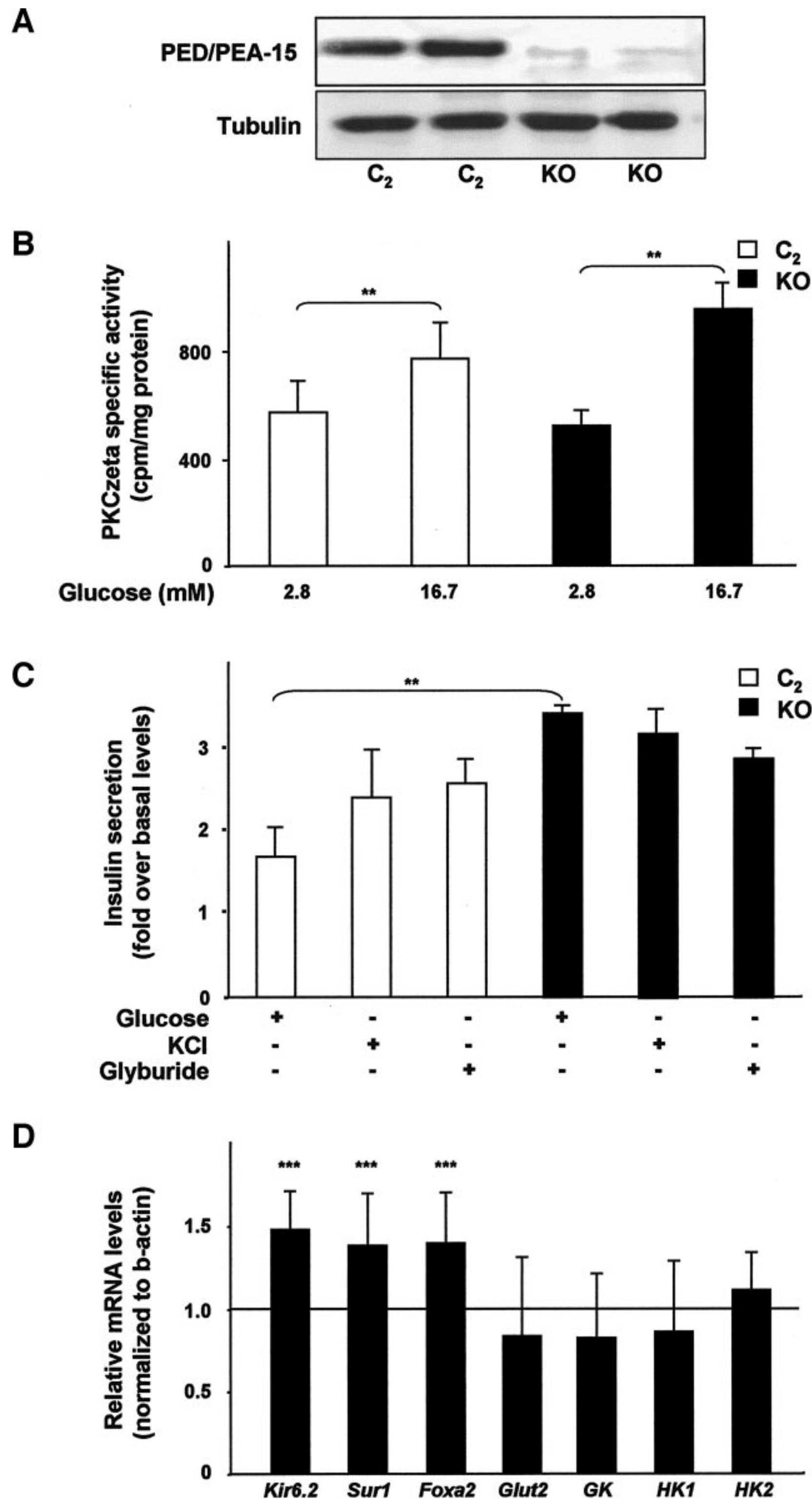

FIG. 7. PKC-ζ activity, insulin secretion, and gene expression profile in isolated islets from ped/pea-15–null mice. *B*: Islets were isolated from 6-month-old *ped/pea-15*–null mice (KO) and, for control, from their unmodified littermates (C2). The islets were exposed to glucose as indicated, solubilized, and precipitated with PKC-ζ antibodies. PKC activity was assayed in the immunoprecipitates as described in RESEARCH DESIGN AND METHODS. PKC activity is expressed as fold increase over basal activity (measured in the presence of 2.8 mmol/l glucose). Bars represent the means ± SD of four independent experiments, each in which two mice/group were used. Aliquots of the lysates were blotted with PED/PEA-15 antibodies (*A*). For control, filters were reprobed with tubulin antibodies. The autoradiographs shown are representative of the blots performed in each of the four experiments. *C*: Islets from knockout and control mice were exposed to glucose, potassium chloride, or glyburide as indicated, and insulin was assayed in the culture medium by RIA. Insulin release is expressed as fold increase in medium insulin concentration over the basal (measured with cells exposed to 2.8 mmol/l glucose alone). Bars represent mean values ± SD of data with islets from at least six mice in each group. *D*: The abundance of mRNAs for the indicated proteins was determined by real-time RT-PCR analysis of total RNA isolated from islets of *ped/pea-15*–null mice and their unmodified littermates, using β-actin as internal standard. The mRNA levels in knockout islets are relative to those in islets from the unmodified littermates. Each bar represents the mean ± SD of four independent experiments, each in which reactions were performed in triplicate using the pooled total RNAs from six mice/genotype. *Statistically significant differences ($P < 0.01$; ***$P < 0.001$).**

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DISCUSSION

In the present study, we generated transgenic mice overexpressing PED/PEA-15 selectively in β-cells (β-tg mice). We found that PED/PEA-15 overexpression determines islet hyperplasia with a specific increase in β-cell mass. This may result from a direct effect on β-cell survival as PED/PEA-15 exerts a broad antiapoptotic action in a number of different cell types (10,15,27–30), including β-cells (C.M., unpublished observation). Increased β-cell mass is consistent with the increased fasting serum insulin levels detected in the β-tg mice. Indeed, these mice do not feature resistance to insulin action. They do show grossly impaired glucose tolerance and reduced insulin secretion in response to hyperglycemia, however. This latter defect is comparable with that occurring in mice with ubiquitous overexpression (13), leading us to conclude that β-cell overexpression of PED/PEA-15 is sufficient to impair glucose tolerance in mice. Previous studies in isolated cells and in vivo evidenced that chronic insulin resistance may impair β-cell function, ultimately reducing glucose response (2). Secondary impairment of β-cell function may also occur in the transgenic mice overexpressing PED/PEA-15 ubiquitously. However, the findings in the present study evidence that impaired response to glucose is a direct consequence of PED/PEA-15 overexpression in the β-cells. Consistently, islets from β-tg mice persistently show similarly impaired glucose response in culture as well as in the intact animal. Also, the expression of *ped/pea-15* cDNA in different β-cell lines inhibits insulin release following increased glucose concentration in the culture medium.

The defective insulin secretion induced by β-cell overexpression of PED/PEA-15 affected both the early and the late phases of insulin response to glucose, raising the possibility that the overexpression of PED/PEA-15 impairs the glucose-sensing machinery. Earlier reports evidenced that the KIR6.2 and SUR1 potassium channel subunits couple glucose metabolism to the electrical activity of β-cell membrane and insulin secretion by setting the resting membrane potential below the activation threshold for voltage-gated Ca^{2+} channels (31). We found that the membrane depolarizing agent potassium chloride causes a similar release of insulin by both transgenic and control mouse islets, indicating that the site of PED/PEA-15 action on glucose-induced secretion is upstream the membrane depolarization step. Indeed, β-cell expression profiling revealed that mRNA abundance of both the *Kir6.2* and the *Sur1* transcripts are significantly depressed by the overexpression of *ped/pea-15*. PED/PEA-15 action on the *Kir6.2* and *Sur1* genes represents a specific abnormality, as other genes encoding key components of the glucose-sensitive insulin secretion machinery were unaffected by PED/PEA-15. The latter include *GLUT2*, *HK1*, and *HK2*. Consistent with the functional consequence of this defect, glyburide showed impaired action on insulin secretion in PED/PEA-15–overexpressing islets.

Previous studies in mice evidenced that ablation of either the *Sur1* or the *Kir6.2* genes results in a phenotype reminiscent of that characterizing the β-tg mice (32–34). Indeed, both the $Sur1^{-/-}$ and the $Kir6.2^{-/-}$ mice feature blunted insulin secretion response to sulfanylureas. The defective insulin response to the sulfanylurea glyburide seems even more pronounced in the β-tg mice as, in these animals, <30% decrease in *Sur1* and *Kir6.2* gene expression blocks glyburide action. The simultaneous impairment in *Sur1* and *Kir6.2* gene function may result in an additive effect on glyburide action in the β-tg mice. Similar to the β-tg, fasted $Sur1^{-/-}$ mice are more hyperinsulinemic than control animals due to persistent activation of voltage-gated calcium channels (33,34). These mice are also significantly more hypoglycemic than controls when fasted. Furthermore, both the $Sur1^{-/-}$ and the $Kir6.2^{-/-}$ mice feature impaired glucose tolerance and glucose-induced first- and second-phase insulin secretion. Thus, at least in part, the loss of potassium channels caused by β-cell overexpression of PED/PEA-15 may account for the abnormalities in basal and glucose-stimulated insulin secretion and in glucose tolerance observed in the β-tg mice.

Recent evidence in skeletal muscle and fat indicated that high cellular levels of PED/PEA-15 impair the function of the atypical PKC isoform PKC-ζ through a phospholipase D–mediated mechanism (13,17). Interestingly, the activity of PKC-ζ is also decreased in islets from the β-tg transgenics compared with those from control mice. PKC-ζ overexpression in β-cells transfected with a *ped/pea-15* cDNA simultaneously rescues glucose-induced insulin secretion and the expression of the *Sur1* and *Kir6.2* genes. These data evidence that PKC-ζ activation is sufficient to upregulate these genes, while not proving that the loss of PKC-ζ accompanying PED/PEA-15 overexpression causes *Sur1* and *Kir6.2* downregulation. Another atypical PKC isoform, PKC-λ, has recently been shown to play a major role in regulating glucose-induced insulin secretion by modulating the expression of a number of pancreatic β-cell genes (35). However, the latter report did not exclude that, as we now show, the activation of PKC-ζ may also upregulate *Sur1* and *Kir6.2* gene function. Indeed, while clearly distinct and involved in a number of specialized mechanisms, PKC-ζ and -λ also feature structural similarities and many common functions (36). Downregulation of PKC-ζ and/or -λ may cause impaired glucose-induced insulin response in β-tg mouse islets by reducing *Sur1* and *Kir6.2* gene expression.

Atypical PKCs have been reported to control the function of a number of transcription factors (37,38). Very recent data by Hashimoto et al. (35) evidenced that in *PKC-λ*–null mice, the abundance of pancreatic β-cells, the expression of the *Foxa2* mRNA in addition to that of *Sur1* and *Kir6.2*, is significantly reduced. In addition, in pancreatic β-cells, the expression of the *Sur1* and *Kir 6.2* genes is dependent on *Foxa2* activity (25,39,40). Similar to the $PKC\lambda^{-/-}$ mice, we now report that *Foxa2* mRNA abundance is also reduced in islets from β-tg transgenics. Thus, the impaired activity of PKC-ζ in β-cells overexpressing PED/PEA-15 may also prevent normal potassium channel generation and glucose-regulated secretion of insulin by depressing *Foxa2* expression. Supporting this conclusion, we evidenced that forcing the expression of PKC-ζ in PED/PEA-15–overexpressing β-cells rescued F*oxa2* levels, in addition to those of *Kir6.2* and *SUR1*, and glucose-induced insulin secretion. Importantly, islets from *ped/pea-15*–null mice feature upregulated PKC-ζ function. The enhanced PKC-ζ activity is accompanied by specific increases in the abundance of *Kir6.2*, *Sur1*, and *Foxa2* mRNAs and augmented insulin secretion in response to glucose. Thus, F*oxa2*-mediated control of glucose sensitivity represents a physiological function of PED/PEA-15 in the β-cell. Dysregulation of this mechanism may be relevant for type 2 diabetes in humans as well.

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