

Age-Related Impairment in Insulin Release

The Essential Role of β_2 -Adrenergic Receptor

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In this study, we investigated the significance of β_2 -adrenergic receptor (β_2 AR) in age-related impaired insulin secretion and glucose homeostasis. We characterized the metabolic phenotype of β_2 AR-null C57Bl/6N mice (β_2 AR^{-/-}) by performing in vivo and ex vivo experiments. In vitro assays in cultured INS-1E β -cells were carried out in order to clarify the mechanism by which β_2 AR deficiency affects glucose metabolism. Adult β_2 AR^{-/-} mice featured glucose intolerance, and pancreatic islets isolated from these animals displayed impaired glucose-induced insulin release, accompanied by reduced expression of peroxisome proliferator-activated receptor (PPAR) γ , pancreatic duodenal homeobox-1 (PDX-1), and GLUT2. Adenovirus-mediated gene transfer of human β_2 AR rescued these defects. Consistent effects were evoked in vitro both upon β_2 AR knockdown and pharmacologic treatment. Interestingly, with aging, wild-type (β_2 AR^{+/+}) littermates developed impaired insulin secretion and glucose tolerance. Moreover, islets from 20-month-old β_2 AR^{+/+} mice exhibited reduced density of β_2 AR compared with those from younger animals, paralleled by decreased levels of PPAR γ , PDX-1, and GLUT2. Overexpression of β_2 AR in aged mice rescued glucose intolerance and insulin release both in vivo and ex vivo, restoring PPAR γ /PDX-1/GLUT2 levels. Our data indicate that reduced β_2 AR expression contributes to the age-related decline of glucose tolerance in mice. *Diabetes* 61:692–701, 2012

Impairment of glucose metabolism with age represents a major determinant of type 2 diabetes epidemics within the elderly population. The molecular mechanisms underlying these changes have not been fully elucidated and are likely attributable to multiple causes (1,2). Aging per se is associated with a continuous decrease in basal insulin release (3). The size of this effect is sufficient to increase the likelihood of developing abnormalities in glucose tolerance and even overt diabetes (2,4). The consequence of aging on glucose tolerance occurs in different species, having been identified in rats

(5,6) as well as in humans (4,7,8). However, why insulin secretion deteriorates with aging remains a moot point.

The noradrenergic system provides fine-tuning to the endocrine pancreas activity through the function of α - and β -adrenergic receptors (ARs) (9,10). The reciprocal regulation exerted by insulin and the adrenergic system has been well documented through a large number of studies (11–13). More recent evidence shows that mice with simultaneous deletion of the three known genes encoding the β ARs (β_1 , β_2 , and β_3) present a phenotype characterized by impaired glucose tolerance (14). Studies with β_2 AR agonists further suggest that the β_2 AR may play an important role in regulating insulin secretion (15). In addition, different human polymorphisms in the β_2 AR gene have been associated with higher fasting insulin levels (16). Nevertheless, the impact of the β_2 AR subtype on glucose tolerance and insulin secretion is still unclear.

Similar to glucose tolerance, β AR function and responsiveness deteriorate with aging (17–20), but the precise mechanisms involved are unknown. However, current evidence indicates that aging may downregulate β AR signaling, β_2 AR in particular, by decreasing the expression of molecular components of the adrenergic signaling machinery (21–24). We have therefore hypothesized that age-dependent alterations in β AR function impair glucose-regulated insulin release by the pancreatic β -cells and may contribute to deterioration of glucose tolerance. To test this hypothesis, we explored the consequences of β_2 AR knockout on insulin secretion in mice and investigated the significance of the age-related changes in β_2 AR function with regard to glucose tolerance.

RESEARCH DESIGN AND METHODS

In vivo studies. We studied male mice with a homozygous deletion of the β_2 AR gene (β_2 AR^{-/-}) and backcrossed >12 generations onto C57Bl/6N background. Founders were provided by Brian Kobilka (Stanford University, Stanford, CA) (25). Wild-type littermates (β_2 AR^{+/+}) were used as controls. The animals were housed in a temperature-controlled (22°C) room with a 12-h light/dark cycle in accordance with the *Guide for the Care and Use of Laboratory Animals* published by the National Institutes of Health (NIH publication no. 85-23, revised 1996), and experiments were approved by the ethics committee of the Federico II University. Mice were killed by cervical dislocation. Pancreata were excised and collected rapidly after mice were killed. Samples were weighted, fixed by immersion in 4% paraformaldehyde for histology, homogenized for determination of total insulin content, or snap-frozen in liquid nitrogen and stored at -80°C for subsequent analyses. For determination of insulin or glucagon content, pancreatic tissue was homogenized in acid ethanol and extracted at 4°C overnight. The acidic extracts were dried by vacuum, reconstituted, and subjected to insulin and glucagon measurements.

Glucose tolerance test and assessment of insulin secretion. Glucose tolerance test (GTT) was performed as previously described (9,26). Briefly, mice were fasted overnight and then injected with glucose (2 g/kg i.p.). Blood glucose was measured by tail bleeding (Glucose Analyzer II; Beckman Coulter, Brea, CA) at indicated time points. The assessment of insulin secretion before

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and during glucose challenge was performed as previously described (9,27). Blood from the mandibular vein of overnight-fasted mice was collected at the indicated time for serum insulin assessment. The evaluation of glucagon secretion was performed by collecting blood from the mandibular vein of randomized mice before and after injection of insulin (0.75 IU/kg i.p.). Serum insulin and plasma glucagon were assayed by radioimmunoassay (Millipore, Billerica, MA). **Histological analysis.** Immunohistochemistry was carried out on paraffin sections using the H38 rabbit insulin antibody or the N-17 goat glucagon antibody (1:200 dilution; both from Santa Cruz Biotechnology, Santa Cruz, CA). Goat anti-rabbit serum coupled with peroxidase (Immunotech, Marseille, France) was used as secondary antibody. After primary antibody exposure, slides were incubated with biotinylated anti-rabbit or anti-goat IgG and peroxidase-labeled streptavidin (Dako Corporation). All reactions were revealed with diaminobenzidine. Sections were counterstained with hematoxylin and mounted (28,29).

Ex vivo studies

Isolation of mouse pancreatic islets. Islets of Langerhans were isolated by collagenase digestion (26,30). In brief, mice were killed as described above, the fur was soaked with ethanol, and the abdomen was opened to locate and excise the pancreas. Digestion was completed with collagenase P (Roche Applied Sciences, Penzberg, Germany) in a shaking water bath (37°C) for 5–8 min. The digested pancreas was treated with DNase I (New England Biolabs, Ipswich, MA). The islets were hand-picked under a stereomicroscope using a syringe with a 25-gauge needle and cultured at 37°C with 95% air and 5% CO₂ in complete RPMI-1640 supplemented with 5% heat-inactivated FBS, 1 mmol/L sodium pyruvate, 50 μmol/L 2-mercaptoethanol, 2 mmol/L glutamine, 10 mmol/L HEPES, 100 units/mL penicillin, and 100 μg/mL streptomycin (all from Sigma-Aldrich, Saint Louis, MO) as previously described (31,32).

Evaluation of insulin secretion. Isolated islets were preincubated at 37°C for 30 min in Krebs-Ringer bicarbonate buffer (120 mmol/L NaCl, 4.7 mmol/L KCl, 1.2 mmol/L MgSO₄, 1.2 mmol/L KH₂PO₄, 2.4 mmol/L CaCl₂, and 20 mmol/L NaHCO₃) supplemented with 10 mmol/L HEPES and 0.2% BSA and gassed with a mixture of 95% O₂ and 5% CO₂ containing 2 mmol/L glucose. Twenty size-matched islets collected in each tube were incubated for 1 h in a 37°C water bath with 500 μL Krebs-Ringer bicarbonate buffer medium containing 2.8 mmol/L glucose, 16.7 mmol/L glucose, or 2.8 mmol/L glucose plus 33 mmol/L KCl. Islets were then pelleted by centrifugation (9,000g, 2 min, 4°C), and supernatants were collected for insulin secretion. Insulin concentrations were determined using radioimmunoassay (31).

Adenovirus-mediated reinstallation of β₂AR in vivo and ex vivo.

Twenty-month-old β₂AR^{+/+} mice were anesthetized by isoflurane (4%) inhalation and maintained by mask ventilation (isoflurane 1.8%) (28). After laparotomy, we identified and mobilized the distal pancreas and we performed two injections (50 μL each) of adenovirus (7 × 10¹⁰ plaque-forming units [pfu]/mouse) using a 30-gauge needle. Given the high homology between the human and mouse β₂AR (33) and the validated use of the adenoviruses expressing the human β₂AR (Adβ₂AR) for in vivo gene transfer (34–36), we used the human Adβ₂AR or, as control, an empty expression cassette derived from pcDNA3.2/V5/GW/D-TOPO (AdEmpty), kindly provided by Walter J. Koch, Jefferson University (Philadelphia, PA). Finally, abdominal incision was quickly closed in layers using 3–0 silk suture, and animals were observed and monitored until recovery. Isolated islets were infected (12 × 10⁴ pfu/islet) with Adβ₂AR or AdEmpty. After 3 h incubation with the adenoviruses, pancreatic islets were incubated with fresh medium and under normoxic conditions (95% air, 5% CO₂) at 37°C for 24 h.

In vitro studies. The INS-1E β-cells (provided by P. Maechler, University of Geneva, Geneva, Switzerland), derived and selected from the parental rat insulinoma INS-1 β-cell line, were maintained in monolayer culture in RPMI-1640 medium as described above for the ex vivo experiments. For the insulin secretion assays, cells were seeded at a density of 5 × 10⁵ cells/cm² for at least 96 h before use (31). Transient transfection of the PPARγ cDNA and control empty plasmid was performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) (37). We also tested the effects of the specific pharmacological β₂AR antagonist ICI 118,551 (0.1 μmol/L) or agonist (1 μmol/L fenoterol) (both from Sigma-Aldrich) (36).

Short hairpin RNA design, generation, and transfection and radioligand-binding assay. Stealth short hairpin (sh)RNA oligoribonucleotides against β₂AR and scramble were designed (sequences and efficiency shown in Supplementary Table 1 and Supplementary Fig. 2, respectively) and then synthesized by Invitrogen. The shRNA hairpin configuration is cleaved by the cellular machinery into small interfering RNA, which is then bound to the RNA-induced silencing complex. This complex binds to and cleaves mRNAs that match the small interfering RNA that is bound to it. We transfected INS-1E β-cells with 100 nmol/L sh-β₂AR RNA or sh-scramble RNA using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Assays were performed at least 3 days after shRNA transfection. Membrane fractions from pancreatic tissue, isolated islets, and INS-1E β-cells were used for

radioligand-binding studies to assess the density of ARs as previously described and validated (35,36,38,39).

Real-time RT-PCR analysis. Total cellular RNA was isolated from INS-1E β-cells, isolated islets, and pancreatic tissue samples with the RNeasy kit (Qiagen, Germantown, MD), according to the manufacturer's instructions, as previously described (31,40). PCRs were analyzed using SYBR Green mix (Invitrogen). Reactions were performed in triplicate using Platinum SYBR Green qPCR Super-UDG by means of an iCycler IQ multicolor Real Time PCR Detection system (Bio-Rad, Hercules, CA). Cyclophilin was used as an internal standard. Primer sequences are reported in Supplementary Table 2.

Immunoblotting. Immunoblot analysis was performed as previously described (31,37). Blots were probed with mouse monoclonal antibodies against adenylate cyclase type VI (AC-VI) (Abcam, Cambridge, MA), pancreatic and duodenal homeobox (PDX)-1, GLUT2, peroxisome proliferator-activated receptor (PPAR)γ, G-protein-coupled receptor (GRK)2, G protein α_s (Gα_s), clathrin heavy chain, and actin (Santa Cruz Biotechnology). Experiments were performed in triplicate to ensure reproducibility. Membrane extracts were obtained as previously described (29,38). Data are presented as arbitrary units using actin as internal control (clathrin heavy chain for membrane extracts) as indicated.

Measurement of cAMP production in vitro and ex vivo. Intracellular content of cAMP was determined using a cAMP^{125I}-scintillation proximity assay (GE Healthcare, Piscataway, NJ) according to the manufacturer's instructions. Briefly, we used 20 size-matched islets (for the ex vivo assays) and 4,000 cells/well INS-1E (for the in vitro assays). Islets and β-cells were washed once and preincubated at 37°C in HEPES-buffered Krebs-Ringer solution containing 1 mmol/L glucose and 0.5 mmol/L isobutylmethylxanthine (a phosphodiesterase inhibitor) for 1 h and incubated for another 15 min in the same buffer with or without 100 μmol/L forskolin (MP Biomedicals, Solon, OH), 3 mmol/L NaF (Thermo Fisher Scientific, Pittsburgh, PA), or 1 μmol/L isoproterenol (Tocris Bioscience, Ellisville, MO). The reaction was stopped by addition of 50 mmol/L HCl and neutralized with NaOH. The cAMP levels were normalized to the protein concentration.

Statistical analysis. All data are presented as means ± SE. Statistical differences were determined by one-way or two-way ANOVA as appropriate, and Bonferroni post hoc testing was performed when applicable. A *P* value <0.05 was considered significant. Statistical analysis was performed using GraphPad Prism (version 5.01; GraphPad Software Inc., San Diego, CA).

RESULTS

Metabolic phenotype of β₂AR^{-/-} mouse. To investigate in vivo the relevance of the β₂AR gene in the regulation of insulin secretion, we compared the metabolic phenotype of adult (6 months old) β₂AR^{-/-} and β₂AR^{+/+} mice. Blood glucose was significantly higher in the null mice compared with that in their wild-type littermates both upon fasting and under random feeding conditions (Table 1). In addition, their fasting serum insulin levels were significantly reduced (Table 1). Upon glucose loading (GTT), the β₂AR^{-/-} mice displayed a marked reduction in glucose tolerance (Fig. 1A and B). In β₂AR^{+/+} mice, we observed a threefold increase in insulin secretion 3 min after intraperitoneal glucose injection, presumably corresponding with the peak of first-phase insulin release. This was followed by a decrease at 10

TABLE 1
Metabolic characteristics of adult (6 months old) wild-type and knockout mice

	β ₂ AR ^{+/+}	β ₂ AR ^{-/-}
<i>n</i>	15	14
Body weight (g)	29.6 ± 1.1	28.2 ± 0.7
Food intake (g/day)	3.1 ± 0.6	3.1 ± 0.8
Water intake (mL/day)	5.8 ± 0.6	6.3 ± 0.9*
Random-fed blood glucose (mg/dL)	170.1 ± 12.3	198.4 ± 11.1*
Fasting blood glucose (mg/dL)	75.7 ± 8.2	135.3 ± 11.5*
Fasting serum insulin (ng/mL)	0.41 ± 0.03	0.30 ± 0.07*

Data are means ± SE unless otherwise indicated. **P* < 0.05 vs. β₂AR^{+/+}.

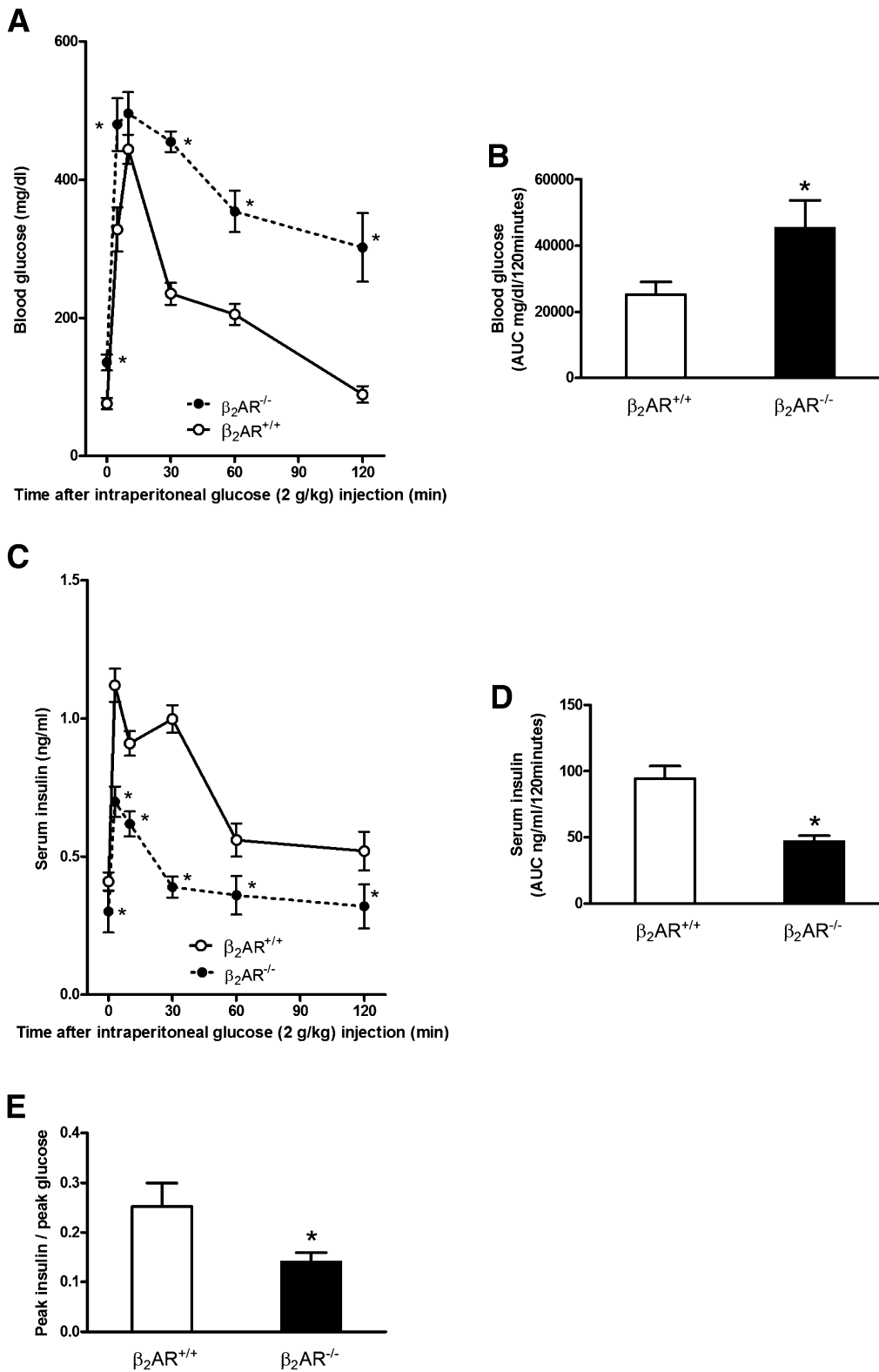


FIG. 1. Metabolic profile of $\beta_2AR^{-/-}$ mice. Six-month-old $\beta_2AR^{-/-}$ mice and their wild-type littermates ($\beta_2AR^{+/+}$) were fasted for 16 h and subjected to intraperitoneal glucose loading (2 g/kg body weight). Blood glucose (A and B) and serum insulin (C and D) were monitored for 120 min after glucose administration ($n = 14-18$ animals per group). $\beta_2AR^{-/-}$ mice displayed glucose intolerance (A) and impaired insulin secretion (C). We calculated the AUC from glucose (B) and insulin excursion (D) curves. Peak insulin-to-peak glucose ratio (E) represents β -cell function, as better described in RESEARCH DESIGN AND METHODS. Bars represent means \pm SE. * $P < 0.05$ vs. $\beta_2AR^{+/+}$, Bonferroni post hoc test. AUC, area under the curve.

min and then a gradual increase over 30 min that may indicate a second-phase response (27,41).

In $\beta_2AR^{-/-}$ mice, the early phase of insulin secretory response to glucose was reduced by more than twofold. The late response was also significantly impaired in the $\beta_2AR^{-/-}$ compared with $\beta_2AR^{+/+}$ mice (Fig. 1C and D). The peak insulin-to-peak glucose ratio was also decreased (Fig. 1E), further indicating impaired insulin response to hyperglycemia in the null mice.

To investigate whether the alterations in glucose tolerance identified in the $\beta_2AR^{-/-}$ mice were contributed by deranged glucagon release, we further measured blood glucose and plasma glucagon levels 30 min after insulin administration. Indeed, insulin administration determines a fall in blood glucose and a counterregulatory rise in plasma glucagon (2,30). However, $\beta_2AR^{-/-}$ and $\beta_2AR^{+/+}$ mice exhibited comparable glucose and glucagon responses to insulin administration (Supplementary Fig. 1A and B). Pancreatic islet histology also did not show any significant difference in these mice (Fig. 2A), similar to total insulin and glucagon pancreatic content (Fig. 2B and C).

We then posed the further question of whether the reduced glucose insulin secretion observed in the $\beta_2AR^{-/-}$ mice in vivo may represent the direct consequence of the $\beta_2AR^{-/-}$ lack in the β -cells or whether it is indirectly mediated by other regulatory factors. To answer this question, we analyzed glucose effect on islets isolated from the null mice. As shown in Fig. 2D, these islets responded poorly to increased glucose concentration in the culture medium compared with the islets from their wild-type littermates but were fully responsive to KCl depolarization.

Islets and β -cell profiling after β_2AR deletion. To gain further insight into the mechanism leading to impaired insulin secretion in mice lacking β_2AR , we profiled the expression of different genes relevant to β -cell regulation by real-time RT-PCR of islet mRNA. As shown in Fig. 3A and B, mRNA levels of both *PDX-1* and *GLUT2*, two major genes involved in β -cell function, were decreased in islets from $\beta_2AR^{-/-}$ mice by 75 and 60%, respectively. Also, mRNA levels of the *PDX-1/GLUT2* upstream regulator *PPAR γ* were decreased by 54% compared with islets from wild-type mice (Fig. 3C). Reliable results were obtained in

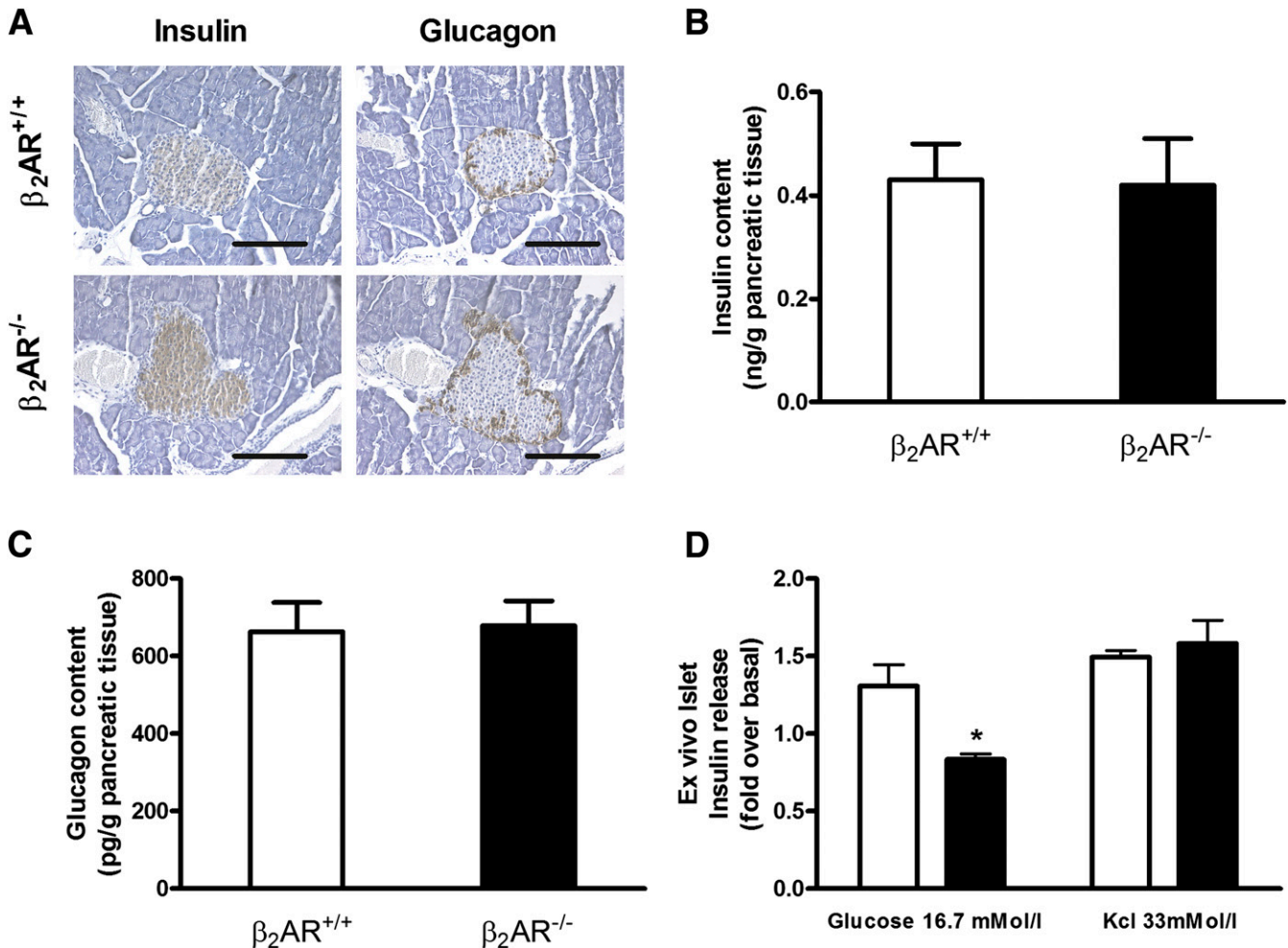


FIG. 2. Comparison of $\beta_2AR^{+/+}$ and $\beta_2AR^{-/-}$ pancreatic islets. Immunohistochemical analysis (A) of the islets was carried out on paraffin sections using insulin (left panel) or glucagon (right panel) antibodies. Microphotographs are representative of images obtained from pancreas sections of five 6-month-old $\beta_2AR^{+/+}$ (upper panel) or $\beta_2AR^{-/-}$ (lower panel) mice. Insulin (B) and glucagon (C) content in isolated islets from $\beta_2AR^{+/+}$ ($n = 10$) or $\beta_2AR^{-/-}$ ($n = 13$) mice. Insulin secretion in response to basal (2.8 mmol/L) or high (16.7 mmol/L) glucose concentration and to KCl (33 mmol/L) was measured in isolated islets from $\beta_2AR^{+/+}$ (□) and $\beta_2AR^{-/-}$ (■) mice (D). Bars represent means \pm SE of data from 10 mice per group. * $P < 0.05$ vs. $\beta_2AR^{+/+}$, Bonferroni post hoc test. (See also Supplementary Fig. 1.) (A high-quality digital representation of this figure is available in the online issue.)

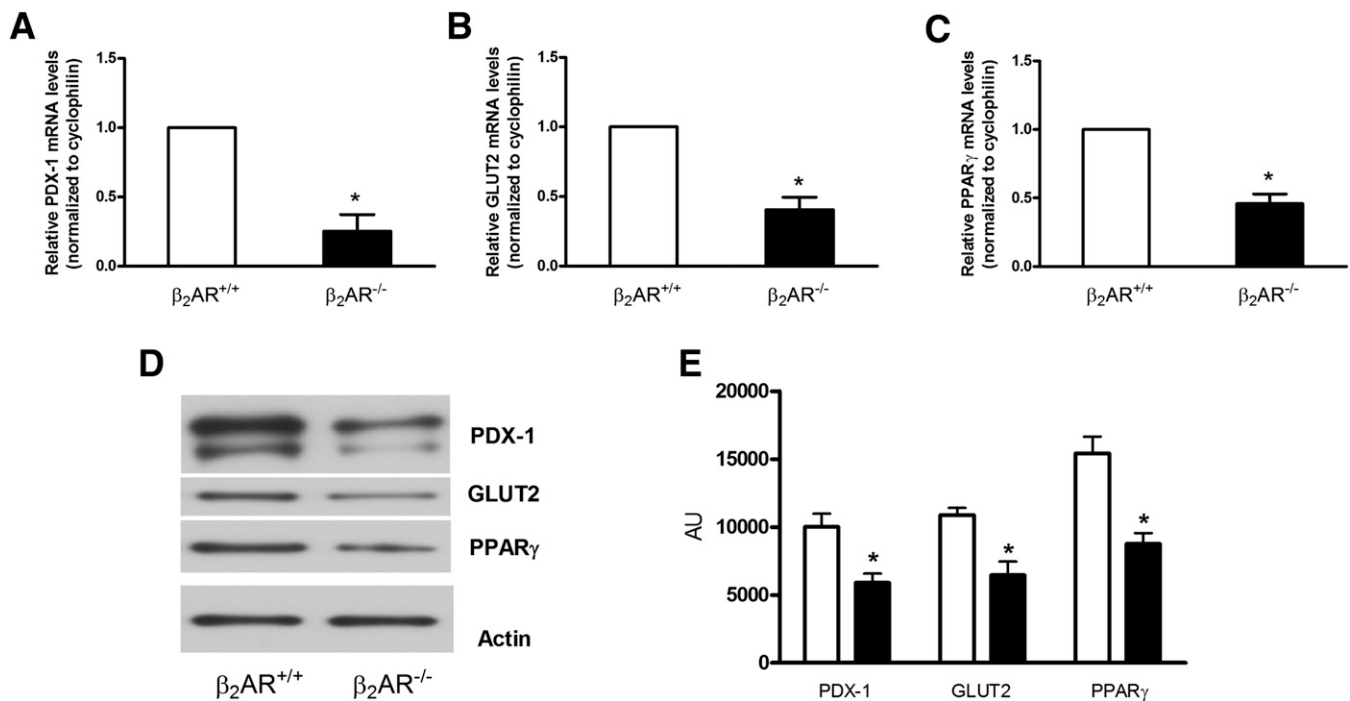


FIG. 3. Gene expression profile in isolated Langerhans islets from $\beta_2AR^{+/+}$ and $\beta_2AR^{-/-}$ mice. The abundance of mRNAs for PDX-1 (A), GLUT2 (B), and PPAR γ (C) was determined by real-time RT-PCR analysis of total RNA, using cyclophilin as internal standard. The mRNA levels in $\beta_2AR^{-/-}$ mice are relative to those in control animals. Each bar represents means \pm SE of four independent experiments in each of which reactions were performed in triplicate using the pooled total RNAs from five mice/genotype. Proteins from a Western blot representative of three independent experiments were quantified by densitometry (D and E). * $P < 0.05$ vs. $\beta_2AR^{+/+}$, Bonferroni post hoc test. AU, arbitrary units.

immunoblotting experiments (Fig. 3D and E). PDX-1 and GLUT2 mRNAs were also reduced to a similar extent in total pancreatic tissue from the $\beta_2AR^{-/-}$ mice (data not shown).

We then sought to demonstrate whether these abnormalities in gene expression were directly caused by β_2AR silencing. To pursue this objective, we silenced with a specific shRNA (Supplementary Fig. 2) the β_2AR gene in the glucose-responsive INS-1E β -cell line (INS-1E $_{sh\beta_2AR}$) (Fig. 4A and B). As shown in Fig. 4C, this specific knock-down impaired glucose-induced insulin secretion by 58% in these cells. A similarly sized effect was achieved by treatment with the specific β_2AR antagonist ICI, while the β_2AR agonist fenoterol showed an opposite action (Supplementary Fig. 3A). Consistent with our ex vivo results, the INS-1E $_{sh\beta_2AR}$ displayed a reduction in PDX-1, GLUT2, and PPAR γ mRNA (Fig. 4D–F) and protein levels (Fig. 4G and H). Interestingly, transient transfection of a PPAR γ cDNA in INS-1E $_{sh\beta_2AR}$ β -cells increased glucose-induced insulin secretion compared with the wild-type INS-1E control β -cells (Fig. 4C). In addition, overexpression of PPAR γ prevented the downregulation of both PDX-1 and GLUT2 occurring in the INS-1E $_{sh\beta_2AR}$ β -cells (Fig. 4D–H). Consistently, treatment of INS-1E β -cells with ICI decreased PDX-1 and GLUT2 mRNA and protein levels, while PPAR γ overexpression completely prevented the effect of ICI (Supplementary Fig. 3B–D), suggesting that β_2AR controls insulin secretion through a PPAR γ /PDX-1-mediated mechanism.

To better define the β_2AR downstream mechanism leading to PPAR γ activation, we assessed the cAMP levels in these cells, observing an impaired production of cAMP in INS-1E $_{sh\beta_2AR}$ β -cells both in basal condition and after stimulation with the βAR agonist isoproterenol (Supplementary

Fig. 4A). Accordingly, to rule out possible involvement of other components of β_2AR signaling machinery, we assessed the protein level of AC-VI, GRK2, and $G\alpha_s$, and we found no significant difference (Supplementary Fig. 4B and C). Parallel results were obtained in ex vivo experiments, performed to investigate the possible age-related alterations in the β_2AR transduction pathway, comparing pancreatic islets isolated from adult (6 months old) and old (20 months old) $\beta_2AR^{+/+}$ mice (Supplementary Fig. 4D and E).

β_2AR overexpression rescued the age-related impairment in insulin release. Based on radioligand binding and real-time RT-PCR analysis, the expression of both β_2AR protein and mRNA was significantly decreased in islets from aged (20 months old) $\beta_2AR^{+/+}$ mice compared with those isolated from adult (6 months old) mice (Fig. 5A and B). PDX-1, GLUT2, and PPAR γ expression (both in terms of mRNA and protein level) was also reduced, and insulin release in response to glucose, though not that evoked by KCl depolarization, was impaired in islets from the aged mice (Fig. 5C–H), suggesting that the reduced β_2AR density constrains islet glucose response in these animals. To prove this hypothesis, we used an adenoviral construct driving overexpression of human β_2AR in mouse islets. Interestingly, infection of islets isolated from wild-type old mice with this construct induced a twofold increase in β_2AR expression (Fig. 5A and B) and returned glucose-induced insulin secretion to levels comparable with those of islets from 6-month-old mice (Fig. 5C) accompanied by restored expression of PDX-1, GLUT2, and PPAR γ (Fig. 5D–H).

In the in vivo setup, 20-month-old $\beta_2AR^{+/+}$ mice exhibited a significant reduction in fasting serum insulin levels (Table 2) accompanied by impaired glucose tolerance and

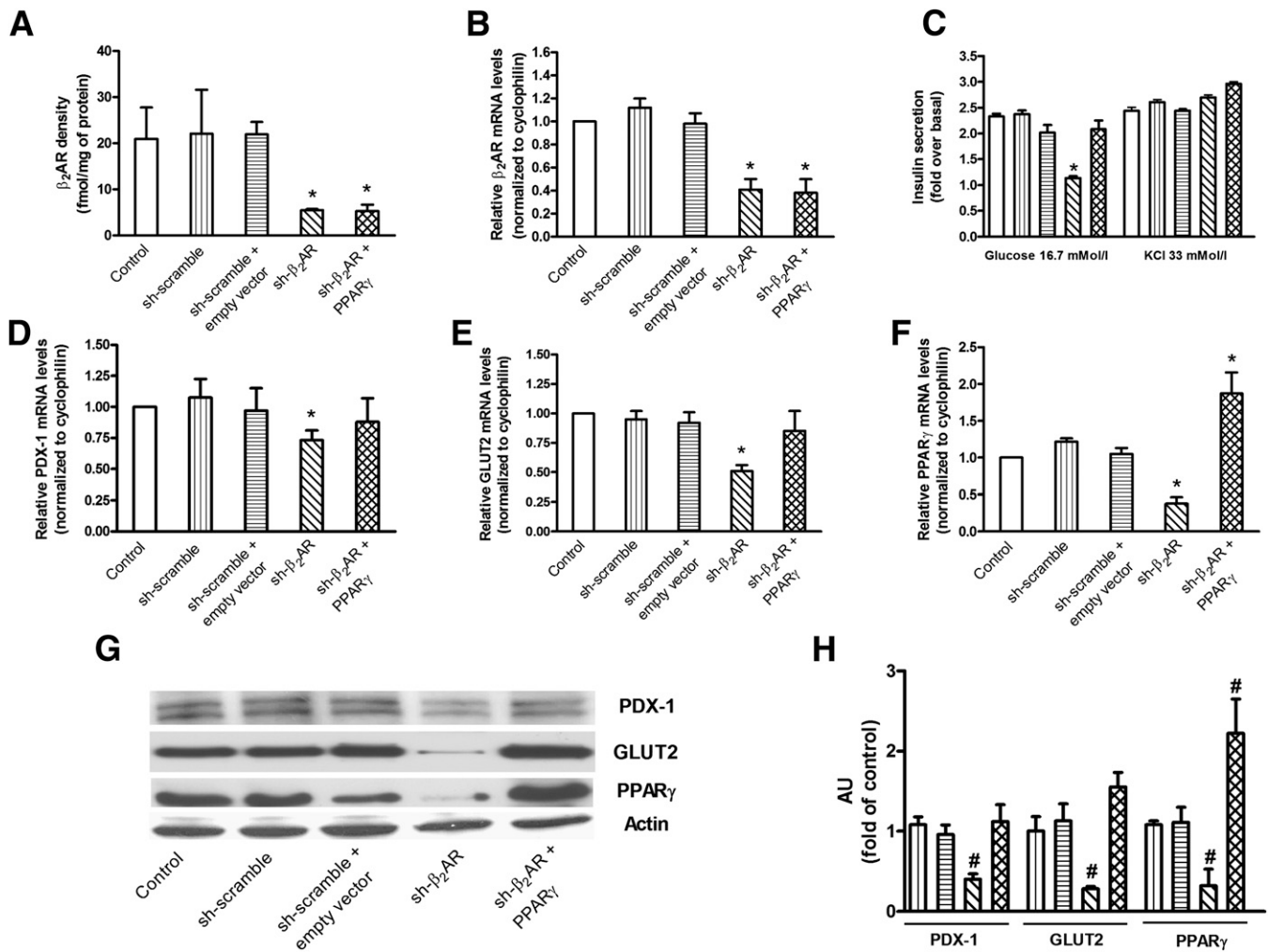


FIG. 4. β_2 AR levels, glucose-stimulated insulin secretion, and gene expression profile in silenced INS-1E β -cells. Treatment with a specific β_2 AR-shRNA significantly decreased the density (by 73.7% [A]) and mRNA levels (by 59.1% [B]) of β_2 AR in INS-1E β -cells. β_2 AR-shRNA inhibited the insulin secretory response to 16.7 mmol/L glucose, which was rescued by the overexpression of PPAR γ (C). KCl-induced insulin release (C) was not significantly different among the studied groups. β_2 AR-shRNA also determined a significant reduction in mRNA level of PDX-1 (D), GLUT2 (E), and PPAR γ (F) that was prevented by the overexpression of PPAR γ . Bars represent means \pm SE from four to five independent experiments in each of which reactions were performed in triplicate (□, control, i.e. untreated INS-1E β -cells; ▨, sh-scramble; ▩, sh-scramble+empty vector; ▪, sh- β_2 AR; ▫, sh- β_2 AR+PPAR γ ; * P < 0.05 vs. control, Bonferroni post hoc test; basal is glucose 2.8 mmol/L. Equal amount of proteins from three independent experiments was analyzed by Western blotting and quantified by densitometry (G and H). * P < 0.05 vs. sh-scramble. AU, arbitrary units. (See also Supplementary Figs. 2–4.)

insulin response upon GTT (Fig. 6A–E). We have therefore designed a gene therapy protocol aimed to prove that these abnormalities can be corrected by restoring β_2 AR density. Accordingly, we infected the pancreas of aged mice by Ad β_2 AR injection. This injection effectively rescued β_2 AR expression in the pancreatic tissue, returning it to levels comparable with those of 6-month-old mice (Supplementary Fig. 5A and B), and restored the expression of PDX-1, GLUT2, and PPAR γ (Supplementary Fig. 5C–E). Injections in the distal pancreas did not induce β_2 AR expression in other tissues, such as the liver (Supplementary Fig. 6A and B) or the skeletal muscle (Supplementary Fig. 6C and D).

These effects were paralleled by significant improvement in glucose tolerance and insulin secretion during GTT (Fig. 6A–E). Fasting insulin levels also increased, reaching values similar to those measured in 6-month-old mice (Table 2), further underlining the relevance of β_2 AR function in enabling adequate pancreatic β -cell response to hyperglycemia.

DISCUSSION

In the present work, we provide evidence that β_2 AR gene deletion in mice causes reduction of glucose-stimulated insulin release by pancreatic β -cells. This phenotype is reminiscent of that observed in mice with targeted β -cell disruption of the $G\alpha_s$ gene (30). In these mice, however, the impairment of $G\alpha_s$ prevented response to multiple $G\alpha_s$ -related receptors, causing a severe phenotype, with gross abnormalities in pancreatic islets. Interestingly, in islets from β_2 AR $^{-/-}$ mice, PPAR γ expression was reduced by 50%, leading to repression of the PPAR γ downstream molecules PDX-1 and GLUT2, two key effectors of β -cell function (26,42,43). This downregulation resulted in a clear impairment in insulin release, though islet architecture and insulin content were not affected by the β_2 AR gene deletion.

Rosen et al. (44) showed that islets from mice with targeted elimination of PPAR γ in β -cells were approximately twice as large as those from control mice. Thus, we can speculate that in our model the 50% reduction in

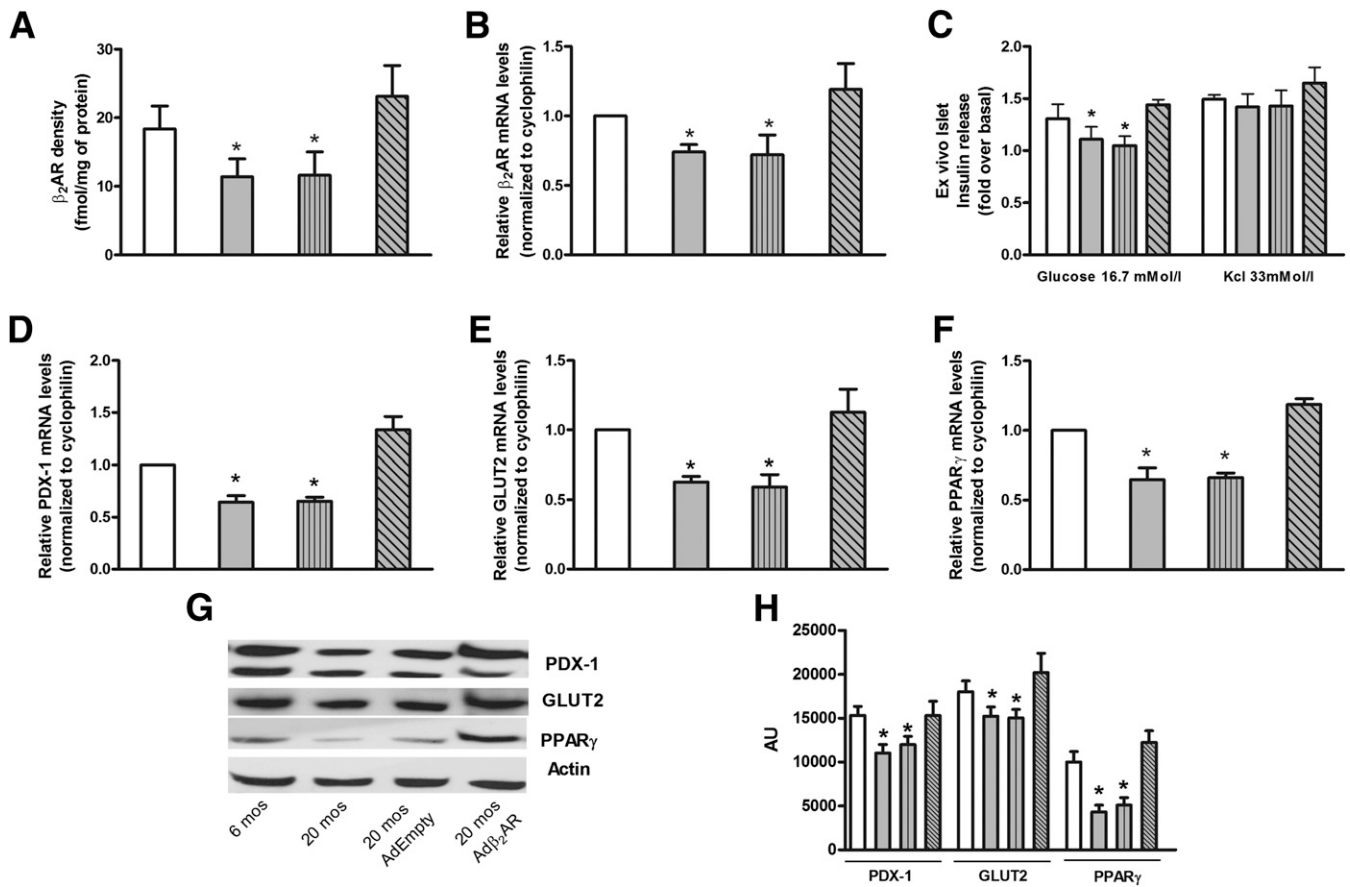


FIG. 5. β_2AR ex vivo infection rescued age-dependent impairment of β -cell function. Density (A) and mRNA levels (B) of β_2AR were evaluated on cell membranes of islets isolated from $\beta_2AR^{+/+}$ mice. Insulin release (C) was determined upon exposure to the indicated concentration of glucose or KCl as described in RESEARCH DESIGN AND METHODS. mRNA levels of PDX-1 (D), GLUT2 (E), and PPAR γ (F) were determined by real-time RT-PCR using the pooled total RNAs from five mice/group with cyclophilin as internal standard. Each bar represents means \pm SE of five independent experiments in each of which reactions were performed in triplicate. Islets isolated from $\beta_2AR^{+/+}$ mice were solubilized and aliquots of the lysates were blotted with PDX-1, GLUT2, and PPAR γ antibodies. Actin was used as loading control. The autoradiographs shown (G) are representative of three independent experiments, which are quantified in H. \square , age 6 months (mos); \blacksquare , 20 months; ▨ , 20 months AdEmpty; ▩ , 20 months Ad β_2AR . * $P < 0.05$ vs. $\beta_2AR^{+/+}$ 6 months, Bonferroni post hoc test.

PPAR γ levels is sufficient to restrain β -cell function without altering islet mass.

The mechanistic significance of β_2AR gene knockout was further sustained by in vitro studies in the INS-1E pancreatic β -cells, showing that the silencing of the β_2AR as well as the pharmacological treatment with a specific β_2AR antagonist impaired glucose response and down-regulated PPAR γ expression, reducing both PDX-1 and GLUT2 levels. No alteration of αARs was observed instead

(data not shown). In addition, treatment with the β_2AR agonist fenoterol activated PPAR γ /PDX-1/GLUT2 signaling, indicating that, at least in part, β_2AR controls insulin secretion through this pathway. Indeed, in this study we show that exogenous PPAR γ expression in INS-1E β -cells silenced for β_2AR led to recovery of PDX-1/GLUT2 levels and glucose-stimulated insulin secretion. This finding is supported by recent evidence that directly relates β_2AR to PPAR γ (42,45–47), a key element in the process of insulin

TABLE 2
Metabolic effects of β_2AR overexpression in aged (20 months old) $\beta_2AR^{+/+}$ mice

	20 months old			6 months old
	Untreated	AdEmpty	Ad β_2AR	Untreated
<i>n</i>	10	6	8	15
Body weight (g)	38.4 \pm 1.7*	38.1 \pm 2.4*	38.6 \pm 2.1*	29.6 \pm 1.1
Food intake (g/day)	4.0 \pm 1.1*	4.2 \pm 1.8*	3.9 \pm 1.5*	3.1 \pm 0.6
Water intake (mL/day)	6.8 \pm 1.2*	6.9 \pm 1.7*	6.6 \pm 1.8*	5.8 \pm 0.6
Random-fed blood glucose (mg/dL)	176.5 \pm 8.6	178.4 \pm 12.7	173.2 \pm 10.4	170.1 \pm 12.3
Fasting blood glucose (mg/dL)	84.2 \pm 10.4	83.6 \pm 11.9	77.7 \pm 11.7	75.7 \pm 8.2
Fasting serum insulin (ng/mL)	0.32 \pm 0.04*	0.33 \pm 0.05*	0.42 \pm 0.1	0.41 \pm 0.03

Data are means \pm SE unless otherwise indicated. * $P < 0.05$ vs. adult (6 months old) $\beta_2AR^{+/+}$ mice.

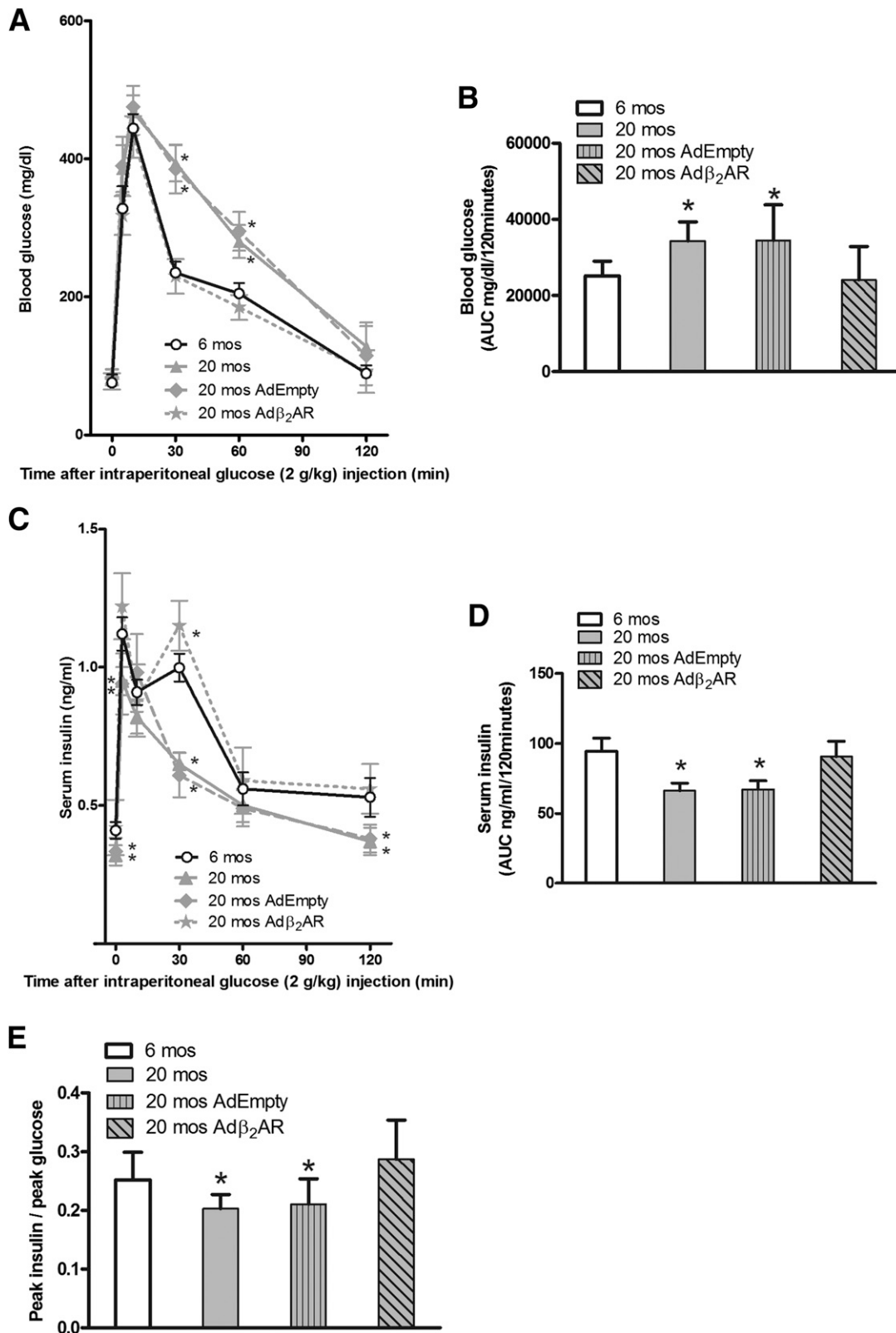


FIG. 6. Adenoviral vector-mediated β_2 AR gene transfer in the mouse pancreas rescued age-related reduction in glucose tolerance. Blood glucose levels (A) and serum insulin (C) after 120 min of glucose administration ($n = 14$ – 18 animals per group). We calculated the AUC from glucose (B) and insulin excursion (D) curves. Twenty-month-old β_2 AR $^{+/+}$ mice showed glucose intolerance (A and B), impaired insulin secretion (C and D), and also an impairment in β -cell function, evaluated measuring the peak insulin-to-peak glucose ratio (E). All of these parameters were restored after Ad β_2 AR in vivo infection. * $P < 0.05$ vs. β_2 AR $^{+/+}$ at 6 months (mos) of age, Bonferroni post hoc test. (See also Supplementary Figs. 5 and 6.)

secretion that has also recently been investigated in aging (43,48). Our results are consistent with these observations, sustaining also the hypothesis that cAMP levels could act as a connecting link through which β_2 AR signaling leads to activation of PPAR γ (49,50). Moreover, the cAMP assays, performed both in INS-1E_{sh β_2 AR} pancreatic β -cells and in islets isolated from aged mice, showed an impairment in basal conditions and after stimulation with isoproterenol, while the responses to NaF and forskolin were not affected. Also, G α_s and AC-VI protein levels were not significantly different among the explored settings. This combination of events is usually observed in models of β_2 AR gene deletion or impaired β_2 AR signaling (18,34).

Whether and to what extent β_2 AR gene knockout in liver and peripheral tissues affects glucose homeostasis in the β_2 AR^{-/-} mice remain to be conclusively addressed. Indeed, variations in the β_2 AR locus have also been reported to associate with insulin resistance in type 2 diabetic patients (16). However, as shown in this work, the impaired glucose tolerance of β_2 AR^{-/-} mice is likely contributed by the defective β -cell function, as indicated by the major effect of β_2 AR lack on glucose-evoked insulin secretion.

In humans, glucose tolerance declines with age, resulting in a high prevalence of type 2 diabetes and impaired glucose tolerance in the elderly population (2,8). How, at the individual level, glucose tolerance declines remains unclear, but it is likely determined by multiple factors including diminished insulin secretion (3,7). In rat models and in humans, a progressive decline in β -cell activity with age has been documented (4,6). In the present work, we show that the same occurs in the C57Bl/6N mouse and is paralleled by the development of abnormal glucose tolerance. Similar to previous findings in several human tissues (18–21,24), our results show that these changes are accompanied by reduced β_2 AR levels in mouse pancreatic islets. The decreased β_2 AR density in islets from aged mice recapitulates the mechanisms leading to the insulin secretory defect occurring in β_2 AR-null mice, indicating that it may contribute to the age-related impairment in glucose tolerance. Indeed, both in vivo and ex vivo experiments of β_2 AR gene transfer revealed that recovery of normal β_2 AR levels rescued insulin release and glucose tolerance in aged mice. Thus, in the mouse model progressive decline of islet β_2 AR density appears to contribute to the reduction in glucose tolerance that accompanies aging. Whether the same also occurs in humans needs to be clarified and is currently under investigation in our laboratory.

In conclusion, we have shown that β_2 AR physiologically regulates pancreatic β -cell insulin secretion by modulating PPAR γ /PDX-1/GLUT2 function. Reduced β_2 AR expression contributes to the age-dependent deterioration of glucose tolerance.

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No potential conflicts of interest relevant to this article were reported.

G.S. conceived the project, performed experiments, analyzed data, and wrote the manuscript. A.L. performed experiments, analyzed data, and wrote the manuscript. D.S. performed experiments and contributed to discussion. A.A. performed experiments. C.D.G. performed experiments. P.F. analyzed data and contributed to discussion. F.B. analyzed data and wrote the manuscript. B.T. designed

research and supervised the project. C.M. analyzed data and wrote the manuscript. G.I. designed research, analyzed data, and wrote the manuscript. G.S. and G.I. are the guarantors of this work and, as such, had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

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SUPPLEMENTARY DATA

Supplementary Table 1. shRNA sequences

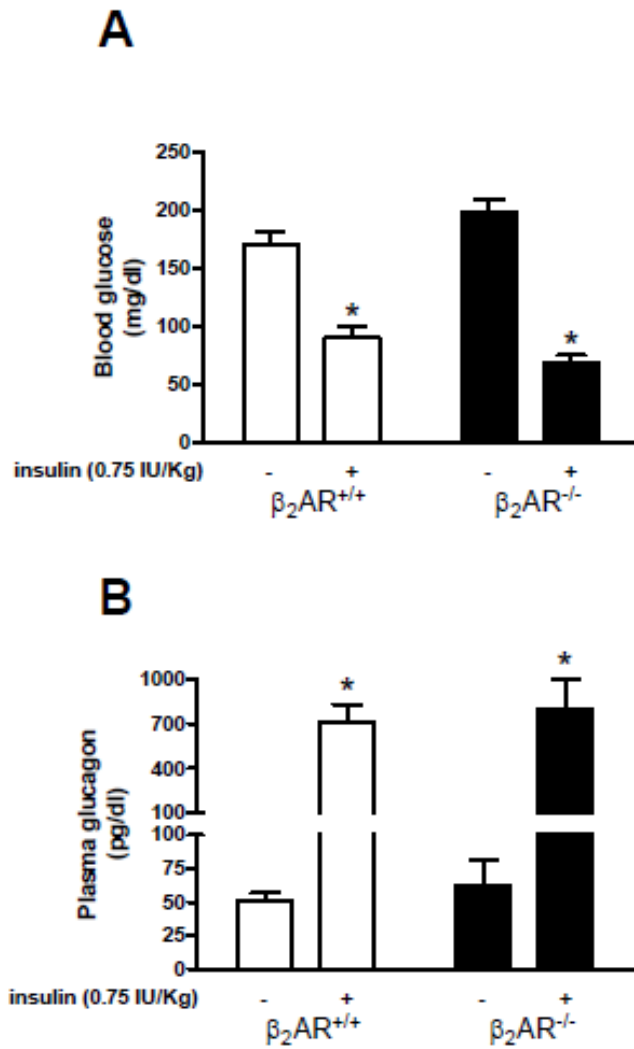
	5'- 3'
shRNA scramble	GCUGACGUAUCCUAAAUGUGUAAUA
shRNA β_2AR	CCACAAGCAAGCCAUCGACUGUUAU
shRNA β_2AR (x)	GGGAGGAAACUGUAAACACAAACGACU
shRNA β_2AR (y)	GAAGGUCCUCCAAAGUUCUGCUUGAA

Supplementary Table 2. Real Time RT-PCR primer sequences (5'- 3')

	Forward	Reverse
β_2AR (rat)	ACGAGGATGGGAGGAGCGGG	GGTTGGCCCGGATGACGTGG
β_2AR (mouse)	GAGTGTGCAGGACGCACCCC	CTGTCGTTCCCGTGTGGCCC
PDX-1 (rat and mouse)	AAAACCGTCGCATGAAGTGG	CCCGCTACTACGTTTCTTATCT
GLUT2 (rat and mouse)	ACAGTCACACCAGCATAAC	ACCCACCAAAGAATGAGG
PPARγ (mouse)	ACGGGGTCTCGGTTGAGGGG	AGTTGGTGGGCCAGAATGGCA
PPARγ (rat)	AAGGTGCTCCAGAAGATGACA	TCAGCGACTGGGACTTTTCT
Cyclophilin (rat and mouse)	GCAGACAAAGTTCCAAAGACAG	CACCCTGGCACATGAATCC

SUPPLEMENTARY DATA

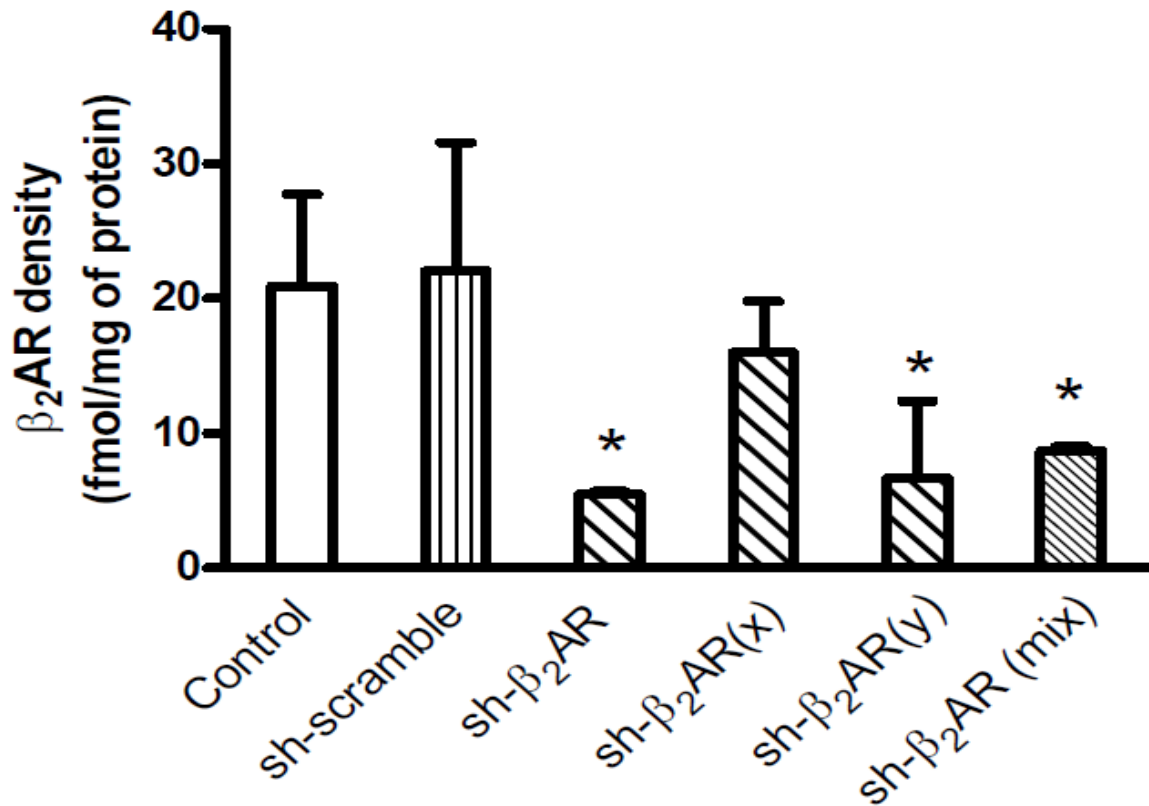
Supplementary Figure 1. Blood glucose and plasma glucagon assessed after intraperitoneal insulin administration. Blood glucose (A) and plasma glucagon (B), evaluated in basal (random fed) conditions and 30 minutes after intraperitoneal administration of insulin (0.75 IU/Kg), were not significantly different among the two studied groups of adult (6-month-old) mice. Each bar represents the mean±SE of eight independent experiments. *:p<0.05 vs basal condition; Bonferroni *post hoc* test.







SUPPLEMENTARY DATA

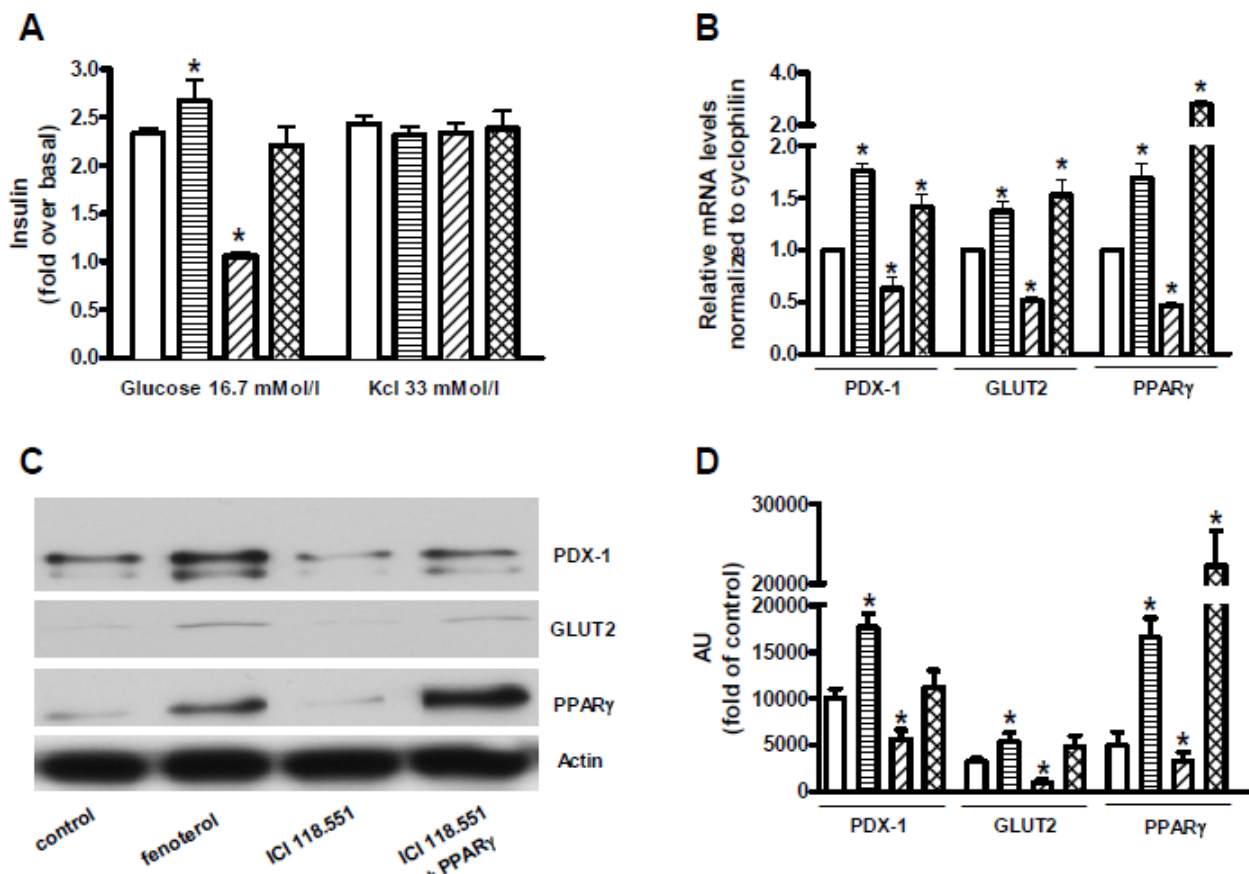
Supplementary Figure 2. β_2 AR silencing in pancreatic INS-1E β -cells.

To silence the expression of β_2 AR in INS-1E β -cells, we designed (sequences are shown in Supplementary Table 1) three shRNA, namely sh- β_2 AR, sh- β_2 AR(x), sh- β_2 AR(y). We decide to use the first one, which showed the best effectiveness, inducing a 73.7% decrease in the membrane density of β_2 AR, also better than the pooled mix of the three sh- β_2 AR. Each bar represents mean \pm SE from five independent experiments in each of whom reactions were performed in triplicate. *:p<0.05 vs control; Bonferroni *post hoc* test).







Supplementary Figure 3. Pharmacological modulation of β_2 AR.

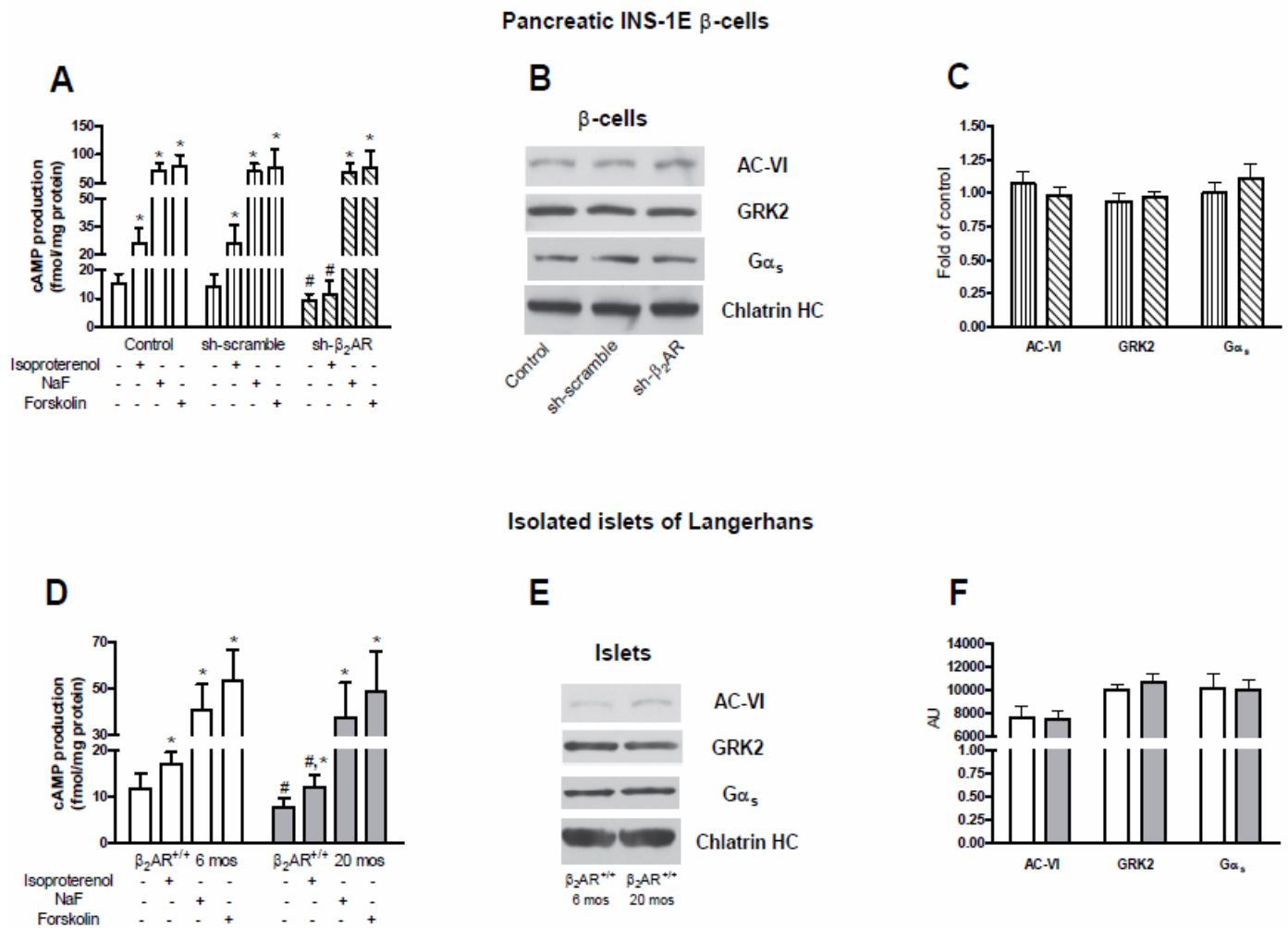
INS-1E pancreatic β -cells were incubated (1 hour) with the β_2 AR selective agonist fenoterol (1 μ Mol/l) or antagonist ICI 118.551 (ICI, 0.1 μ Mol/l). This latter inhibited the insulin secretory response to 16.7 mMol/l glucose, that was completely rescued by overexpression of PPAR γ (A). Neither fenoterol nor ICI determined significant changes in KCl-induced insulin release. Each bar represents the mean \pm SE from five independent experiments in each of whom reactions were performed in triplicate. (*:p<0.05 vs control, *i.e.* untreated INS-1E β -cells; Bonferroni *post hoc* test; basal is glucose 2.8 mMol/l). Fenoterol treatment caused a marked rise in mRNA level of PDX-1 (by 76%), GLUT2 (by 53%) and PPAR γ (by 80.5%). On the contrary, ICI determined a significant decrease in mRNA level of the same genes: PDX-1 (by 37%), GLUT2 (by 48%) and PPAR γ (by 53%), compared to untreated INS-1E β -cells (B). Bars represent the mRNA levels in treated cells and are relative to those in control cells; data are expressed as mean \pm SE of triplicate reactions for total RNA from each group in four independent experiments. Reliable results were obtained from western blot analysis (C-D); representative images (C) of triplicate experiments are shown; actin was used as loading control; bar graph in panel D show densitometry. *:p<0.05 vs control, *i.e.* untreated INS-1E cells; Bonferroni *post hoc* test; : control, *i.e.* untreated INS-1E cells; : fenoterol; : ICI 118.551; : ICI 118.551 + PPAR γ .



SUPPLEMENTARY DATA





Supplementary Figure 4. Assessment of β_2AR downstream.

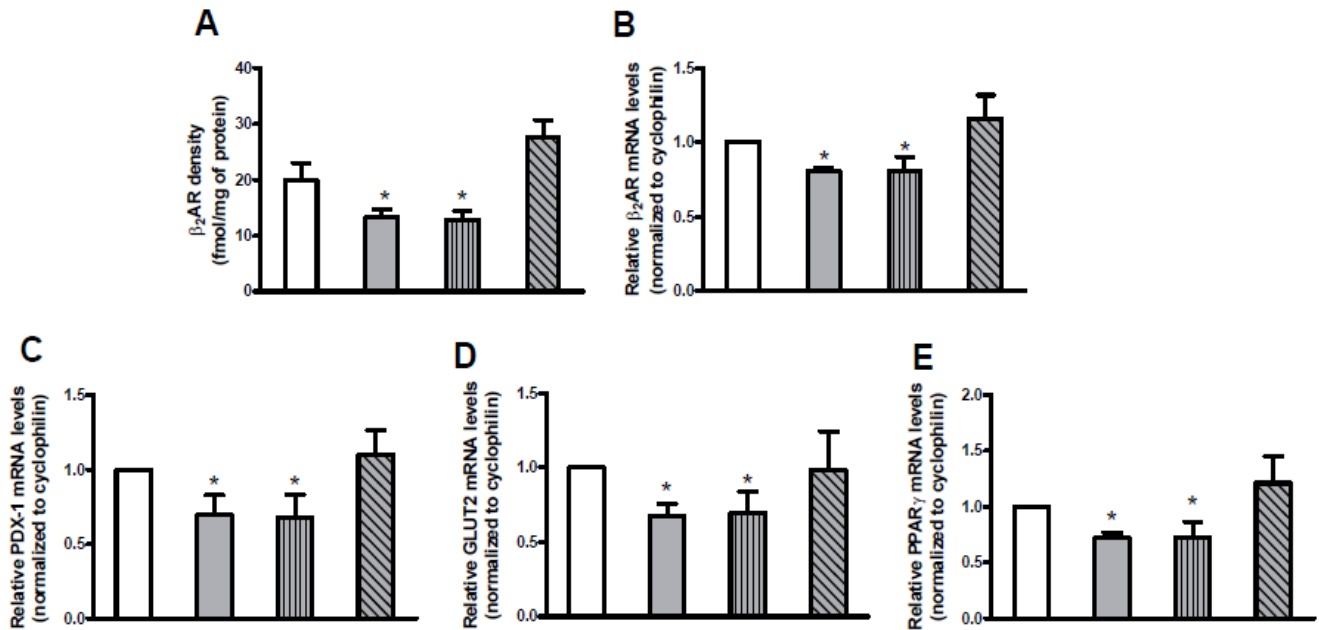
In vitro (A-C) and *ex vivo* (D-F) experiments were performed to investigate cAMP production and β_2AR downstream. cAMP levels (A, D) were measured in basal condition and after stimulation with isoproterenol (1 $\mu\text{Mol/l}$), NaF (3 mMol/l) and forskolin 100 $\mu\text{Mol/l}$, as described in RESEARCH DESIGN AND METHODS. Each bar represents the mean \pm SE of four independent experiments in each of whom reactions were performed in triplicate. We found an impaired cAMP production in basal conditions and after stimulation with isoproterenol both in INS-1E_{sh β_2AR} β -cells (A, *:p<0.05 vs unstimulated INS-1E β -cells; #:p<0.05 vs control) and in islets of Langerhans isolated from 20-month-old $\beta_2AR^{+/+}$ mice (D, *:p<0.05 vs unstimulated islets; #:p<0.05 vs $\beta_2AR^{+/+}$ 6 mos). We assessed by Western blot (B,E) protein levels of adenylate cyclase type VI (AC-VI), GRK2 and $G\alpha_s$ on membrane extracts, using chlatrin heavy chain (chlatrin HC) as loading control. Representative images of three independent experiments are shown. We quantified blots by densitometry in INS-1E pancreatic β -cells (B,  : sh-scramble;  : sh- β_2AR ; *:p<0.05 vs sh-scramble) and in isolated islets (E,  : 6 mos,  : 20 mos; *:p<0.05 vs $\beta_2AR^{+/+}$ 6 mos). Control indicates INS-1E β -cells not treated with shRNA; AU indicates arbitrary units; mos is months of age.



SUPPLEMENTARY DATA




Supplementary Figure 5. Efficacy of β_2AR *in vivo* infection.

Assessment of β_2AR expression on cell membranes of pancreatic tissue from $\beta_2AR^{+/+}$ mice after β_2AR overexpression *in vivo*. β_2AR density (A) was evaluated by radioligand assay. mRNA levels of β_2AR (B), PDX-1 (C), GLUT2 (D) and PPAR γ (E) were determined by Real-time RT-PCR using the pooled total RNAs from four mice/group, using cyclophilin as internal standard. AdEmpty: Pancreata infected with a control adenovirus; Ad β_2AR : Pancreata infected with an adenovirus encoding for the human β_2AR gene. Each bar represents the mean \pm SE of four independent experiments in each of whom reactions were performed in triplicate; : 6 mos, : 20 mos, : 20 mos Adempty, : 20 mos Ad β_2AR ; *:p<0.05 vs $\beta_2AR^{+/+}$ 6 mos; Bonferroni *post hoc* test; mos is months of age.



SUPPLEMENTARY DATA

Supplementary Figure 6. Effect of Ad β_2 AR pancreatic injection on liver and muscle.

The localized injections in the distal pancreas do not induce β_2 AR expression in other tissues. Density (A, C) and mRNA levels (B, D) of β_2 AR were evaluated on cell membranes of liver and quadriceps muscle (C, D) from β_2 AR^{+/+} mice. mRNA levels were determined by Real-time RT-PCR using the pooled total RNAs from four mice/group with cyclophilin as internal standard. Each bar represents the mean \pm SE of five independent experiments in each of whom reactions were performed in triplicate. : 20 mos, : 20 mos Adempty, : 20 mos Ad β_2 AR; AdEmpty: Mice infected with a control adenovirus; Ad β_2 AR: Mice infected with an adenovirus encoding for the human β_2 AR gene; mos is months of age.

