Age-Related Impairment in Insulin Release

The Essential Role of β2-Adrenergic Receptor

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In this study, we investigated the significance of β2-adrenergic receptor (β2AR) in age-related impaired insulin secretion and glucose homeostasis. We characterized the metabolic phenotype of β2AR-null C57Bl/6N mice (β2AR−/−) 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 β2AR deficiency affects glucose metabolism. Adult β2AR−/− 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 β2AR rescued these defects. Consistent effects were evoked in vitro both upon β2AR knockdown and pharmacologic treatment. Interestingly, with aging, wild-type (β2AR+/+) littermates developed impaired insulin secretion and glucose tolerance. Moreover, islets from 20-month-old β2AR−/− mice exhibited reduced density of β2AR compared with those from younger animals, paralleled by decreased levels of PPARγ, PDX-1, and GLUT2. Overexpression of β2AR in aged mice rescued glucose intolerance and insulin release both in vivo and ex vivo, restoring PPARγ/PDX-1/GLUT2 levels. Our data indicate that reduced β2AR 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 βARs (β1, β2, and β3) present a phenotype characterized by impaired glucose tolerance (14). Studies with β2AR agonists further suggest that the β2AR may play an important role in regulating insulin secretion (15). In addition, different human polymorphisms in the β2AR gene have been associated with higher fasting insulin levels (16). Nevertheless, the impact of the β2AR subtype on glucose tolerance and insulin secretion is still unclear.

Similar to glucose tolerance, βAR function and responsiveness deteriorate with aging (17–20), but the precise mechanisms involved are unknown. However, current evidence indicates that aging may downregulate βAR signaling, β2AR 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 βAR 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 β2AR knockout on insulin secretion in mice and investigated the significance of the age-related changes in β2AR function with regard to glucose tolerance.

RESEARCH DESIGN AND METHODS

In vivo studies. We studied male mice with a homozygous deletion of the β2AR gene (β2AR−/−) and backcrossed >12 generations onto C57Bl/6N background. Founders were provided by Brian Koblika (Stanford University, Stanford, CA) (25). Wild-type littermates (β2AR+/+) 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
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 random-fed 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). The evaluation of glucagon secretion was performed 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.** An immunoblot analysis was performed as previously described (31,37). Blots were probed with mouse monoclonal antibodies against adenylyl 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 (GRK2), G protein αs (Gαs), 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 Biomedical, 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. LA 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

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**Table 1**

| Metabolic characteristics of adult (6 months old) wild-type and knockout mice | β2AR+/+ | β2AR−/− |
|---|---|---|
| n | 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+/+. 

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FIG. 1. Metabolic profile of β2AR−/− mice. Six-month-old β2AR−/− mice and their wild-type littermates (β2AR+/+) 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). β2AR−/− 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 ± SE. *P < 0.05 vs. β2AR+/+, 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 β2AR−/− 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 β2AR−/− compared with β2AR+/+ 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 β2AR−/− mice were contributed by deranged glucagon release, we further measured blood glucose and plasma glucagon levels 30 min after insulin administration. Indeed, insulin administration determined a fall in blood glucose and a counterregulatory rise in plasma glucagon (2,30). However, β2AR−/− and β2AR+/+ 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 β2AR−/− mice in vivo may represent the direct consequence of the β2AR−/− 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 β2AR deletion.** To gain further insight into the mechanism leading to impaired insulin secretion in mice lacking β2AR, 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 β2AR−/− mice by 75 and 60%, respectively. Also, mRNA levels of the PDX-1/GLUT2 upstream regulator PPARγ were decreased by 54% compared with islets from wild-type mice (Fig. 3C). Reliable results were obtained in
immunoblotting experiments (Fig. 3D and E). PDX-1 and GLUT2 mRNAs were also reduced to a similar extent in total pancreatic tissue from the β2AR−/− mice (data not shown).

We then sought to demonstrate whether these abnormalities in gene expression were directly caused by β2AR silencing. To pursue this objective, we silenced with a specific shRNA (Supplementary Fig. 2) the β2AR gene in the glucose-responsive INS-1Esh β-cell line (INS-1Eshβ2AR) (Fig. 4A and B). As shown in Fig. 4C, this specific knockdown impaired glucose-induced insulin secretion by 58% in these cells. A similarly sized effect was achieved by treatment with the specific β2AR antagonist ICI, while the β2AR agonist fenoterol showed an opposite action (Supplementary Fig. 3A). Consistent with our ex vivo results, the INS-1Eshβ2AR displayed a reduction in PDX-1, GLUT2, and PPARγ mRNA (Fig. 4D–F) and protein levels (Fig. 4G and H). Interestingly, transient transfection of a PPARγ cDNA in INS-1Eshβ2AR β-cells increased glucose-induced insulin secretion compared with the wild-type INS1-E control (Fig. 4C). In addition, overexpression of PPARγ prevented the downregulation of both PDX-1 and GLUT2 occurring in the INS-1Eshβ2AR β-cells (Fig. 4D–H). Consistently, treatment of INS-1E β-cells with ICI decreased PDX-1 and GLUT2 mRNA and protein levels, while PPARγ overexpression completely prevented the effect of ICI (Supplementary Fig. 3B–D), suggesting that β2AR controls insulin secretion through a PPARγ/PDX-1–mediated mechanism.

To better define the β2AR downstream mechanism leading to PPARγ activation, we assessed the cAMP levels in these cells, observing an impaired production of cAMP in INS-1Eshβ2AR β-cells both in basal condition and after stimulation with the β2AR agonist isoproterenol (Supplementary Fig. 4A). Accordingly, to rule out possible involvement of other components of β2AR signaling machinery, we assessed the protein level of AC-VI, GRK2, and Gαs, 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 β2AR transduction pathway, comparing pancreatic islets isolated from adult (6 months old) and old (20 months old) β2AR+/+ mice (Supplementary Fig. 4D and E).

**β2AR overexpression rescued the age-related impairment in insulin release.** Based on radioligand binding and real-time RT-PCR analysis, the expression of both β2AR protein and mRNA was significantly decreased in islets from aged (20 months old) β2AR−/− mice compared with those isolated from adult (6 months old) mice (Fig. 5A and B). PDX-1, GLUT2, and PPARγ 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 β2AR density constrains islet glucose response in these animals. To prove this hypothesis, we used an adenoviral construct driving overexpression of human β2AR in mouse islets. Interestingly, injection of islets isolated from wild-type old mice with this construct induced a twofold increase in β2AR 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 PPARγ (Fig. 5D–H). In the in vivo setup, 20-month-old β2AR+/+ mice exhibited a significant reduction in fasting serum insulin levels (Table 2) accompanied by impaired glucose tolerance and
We have therefore designed a gene therapy protocol aimed to prove that these abnormalities can be corrected by restoring $\beta_2$AR density. Accordingly, we infected the pancreas of aged mice by Ad$\beta_2$AR injection. This injection effectively rescued $\beta_2$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$\gamma$ (Supplementary Fig. 5C–E). Injections in the distal pancreas did not induce $\beta_2$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 $\beta_2$AR function in enabling adequate pancreatic $\beta$-cell response to hyperglycemia.

**DISCUSSION**

In the present work, we provide evidence that $\beta_2$AR gene deletion in mice causes reduction of glucose-stimulated insulin release by pancreatic $\beta$-cells. This phenotype is reminiscent of that observed in mice with targeted $\beta$-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 $\beta_2$AR$^{-/-}$ mice, PPAR$\gamma$ expression was reduced by 50%, leading to repression of the PPAR$\gamma$ downstream molecules PDX-1 and GLUT2, two key effectors of $\beta$-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 $\beta_2$AR gene deletion.

Rosen et al. (44) showed that islets from mice with targeted elimination of PPAR$\gamma$ in $\beta$-cells were approximately twice as large as those from control mice. Thus, we can speculate that in our model the 50% reduction in
PPARγ levels is sufficient to restrain β-cell function without altering islet mass.

The mechanistic significance of β2AR gene knockout was further sustained by in vitro studies in the INS-1E pancreatic β-cells, showing that the silencing of the β2AR as well as the pharmacological treatment with a specific β2AR antagonist impaired glucose response and down-regulated PPARγ expression, reducing both PDX-1 and GLUT2 levels. No alteration of αARs was observed instead (data not shown). In addition, treatment with the β2AR agonist fenoterol activated PPARγ/PDX-1/GLUT2 signaling, indicating that, at least in part, β2AR controls insulin secretion through this pathway. Indeed, in this study we show that exogenous PPARγ expression in INS-1E β-cells silenced for β2AR led to recovery of PDX-1/GLUT2 levels and glucose-stimulated insulin secretion. This finding is supported by recent evidence that directly relates β2AR to PPARγ (42,45–47), a key element in the process of insulin

**TABLE 2**

Metabolic effects of β2AR overexpression in aged (20 months old) β2AR+/+ mice

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

Data are means ± SE unless otherwise indicated. *P < 0.05 vs. adult (6 months old) β2AR+/+ mice.
FIG. 6. Adenoviral vector-mediated β2AR 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 β2AR−/− 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 Adβ2AR in vivo infection. *P < 0.05 vs. β2AR+/+ 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 \( \beta_2 \) AR signaling leads to activation of PPAR\( \gamma \) (49,50). Moreover, the cAMP assays, performed both in INS-1E\(_{ab2AR}\) pancreatic \( \beta \)-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, Go\(_s\) and AC-VI protein levels were not significantly different among the explored settings. This combination of events is usually observed in models of \( \beta_2 \) AR gene deletion or impaired \( \beta_2 \) AR signaling (18,34).

Whether and to what extent \( \beta_2 \) AR gene knockout in liver and peripheral tissues affects glucose homeostasis in the \( \beta_2 \) AR\(^{−/−}\) mice remain to be conclusively addressed. Indeed, variations at the \( \beta_2 \) 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 \( \beta_2 \) AR\(^{−/−}\) mice is likely contributed by the defective \( \beta \)-cell function, as indicated by the major effect of \( \beta_2 \) 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 \( \beta \)-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 \( \beta_2 \) AR levels in mouse pancreatic islets. The decreased \( \beta_2 \) AR density in islets from aged mice recapitulates the mechanisms leading to the insulin secretory defect occurring in \( \beta_2 \) 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 \( \beta_2 \) AR gene transfer revealed that recovery of normal \( \beta_2 \) AR levels rescued insulin release and glucose tolerance in aged mice. Thus, in the mouse model progressive decline of \( \beta_2 \) 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 \( \beta_2 \) AR physiologically regulates pancreatic \( \beta \)-cell insulin secretion by modulating PPAR\( \gamma \)/PDX-1/GLUT2 function. Reduced \( \beta_2 \) AR expression contributes to the age-dependent deterioration of glucose tolerance.

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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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