Deletion of β-Arrestin2 in Mice Limited Pancreatic β-Cell Expansion under Metabolic Stress through Activation of the JNK Pathway

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β-Arrestin2 (βarr2) is an adaptor protein that interacts with numerous signaling molecules and regulates insulin sensitivity. We reported previously that βarr2 was abundantly expressed in mouse pancreatic β-cells, and loss of βarr2 leads to impairment of acute- and late-phase insulin secretion. In the present study, we examined the dynamic changes of β-cell mass in βarr2-deficient (βarr2−/−) mice in vivo and explored the underlying mechanisms involved. βarr2−/− mice with exclusively luciferase overexpression in β-cells were generated and fed a high-fat diet (HFD). β-Cell mass was determined by in vivo noninvasive bioluminescence imaging from 4 to 20 wks of age. Proliferation was measured by 5-bromo-2-deoxyuridine (BrdU) incorporation and fluorescence-activated cell sorter analysis. Quantitative real-time polymerase chain reaction (qRT-PCR) and immunoblotting were conducted for gene and protein expression. We found that β-cell mass was reduced dramatically in βarr2−/− mice at 12 wks old compared with that of their respective HFD-fed controls. The percentage of BrdU- and Ki67-positive cells reduced in islets from βarr2−/− mice. Exposure of βarr2−/− islets to high levels of glucose and free fatty acids (FFAs) exacerbated cell death, which was associated with upregulation of the JNK pathway in these islets. Conversely, overexpression of βarr2 amplified β-cell proliferation with a concomitant increase in cyclinD2 expression and a decrease in p21 expression and protected β-cells from glucose- and FFA-induced cell death through JNK-activation inhibition. In conclusion, βarr2 plays roles in regulation of pancreatic β-cell mass through the modulation of cell cycle regulatory genes and the inhibition of JNK activation induced by glucolipotoxicity, which implicates a role for βarr2 in the development of type 2 diabetes.

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INTRODUCTION

Type 2 diabetes (T2D) is caused by relative insulin deficiency due in part to the reduction of pancreatic β-cell mass (1–3). The relative β-cell deficits at the onset of diabetes and impaired glucose tolerance found at autopsy were 64% and 21%, respectively, indicating that loss of β-cell mass could exist within the normal glucose tolerance stage (4). However, the initiation of β-cell loss in humans is difficult to determine, due to the unavailability of current in vivo tracing techniques for β-cell mass measurement. In addition, the exact mechanisms underlying the loss of β-cell mass are not fully understood. Identification of key signal molecules involved in regulating β-cell mass is, thus, essential to reveal potential therapeutic targets in diabetes.

β-Arrestin2 (βarr2), an adaptor protein ubiquitously expressed in cells, modulates G-protein–coupled receptor desensitization and internalization (5–7). It also functions as a scaffold and mediates the strength and duration of a series of cellular signaling pathways, including modulation of peripheral insulin sensitivity (8–10). We reported previously that βarr2 was expressed abundantly in mouse pancreatic β-cells, and its expression was significantly decreased in obese and diabetic mouse models. Loss of βarr2 led to impairment of acute- and late-phase insulin secretion with β-cell mass tending to decrease in βarr2-null mice (11).
The roles of βarr2 in the modulation of cell mass have been reported in smooth muscle and hepatic stellate cells (12,13). βarr2 aggravated atherosclerosis after carotid injury through mechanisms involving smooth muscle cell proliferation (12) and exacerbated hepatic fibrosis induced by porcine serum through its effect on hepatic stellate cell proliferation (13), suggesting a role of βarr2 regulation of cell mass expansion under environmental stress. Interestingly, Ravier et al. reported recently that knocking out βarr2 in female mice decreased the β-cell mass (14). However, how β-cell mass changes upon βarr2 deletion and its mechanism in regulating β-cell mass still remain largely unknown.

In the current study, using noninvasive bioluminescence imaging (BLI), we traced the dynamics of β-cell loss in mice with βarr2 deletion during the progression of impaired glucose tolerance and explored the underlying mechanisms. As overnutrition is becoming the leading risk factor of T2D, discovering molecules that protect β-cells against glucolipotoxicity is of great importance to the prevention and treatment of T2D.

**MATERIALS AND METHODS**

**Animals and Glucose Tolerance Test**

βarr2 knockout mice (βarr2−/−) were generated and identified as described previously (15,16). Transgenic mice expressing luciferase under the control of the mouse ins2 promoter (MIP-TF) were purchased from The Jackson Laboratory (C57BL/6-Tg[Ins2-luc/EGFP/Tk]300Kauf/J, stock number 012943). MIP-TF-βarr2−/− mice were generated by interbreeding MIP-TF and βarr2−/− mice. Mice were genotyped by polymerase chain reaction (PCR) using primers as described previously (15,17; Supplementary Figure S1). Mice were fed either a normal chow diet (20% kcal protein, 10% kcal fat and 70% kcal carbohydrate; Slacca Co.) or a high-fat diet (HFD) (20% kcal protein, 45% kcal fat and 35% kcal carbohydrates; Research Diets) from 6 wks of age. They were housed at 23°C ± 1°C under an artificial 12-h light-dark cycle with free access to food and water. All the procedures involving the care and use of animals were in accordance with Shanghai Jiao Tong University Guidelines for the Care and Use of Laboratory Animals (Permit Number SYXK 20110128). The MIP-TF-βarr2+/+ and MIP-TF-βarr2−/− mice were mainly used in the in vivo imaging study; otherwise βarr2−/− and βarr2+/+ mice were used. All experiments were performed with male mice, and littermate controls were used throughout this study. Glucose tolerance tests (GT Ts) and insulin secretion tests were performed after 12 h of fasting. Glucose (1.5 g/kg for GT Ts and 3.0 g/kg for insulin secretion tests) was injected intraperitoneally. Blood samples were taken from the tail vein. Glucose levels were measured using an ACCU-CHEK Performa glucose monitor (Roche). Insulin levels were measured using enzyme-linked immunosorbent assay (ELISA) kits (Mercodia).

**Islet Isolation, INS-1 (832/13) Cell Culture and Gene Silencing or Overexpression**

Pancreatic islets were isolated from 16-wk-old βarr2+/+ and βarr2−/− male mice as described previously (18) and cultured in Ham F10 (Gibco, Invitrogen Corp.) supplemented with 6.1 mmol/L glucose, 0.5% BSA (charcoal treated) and penicillin-streptomycin. INS-1 (832/13) cells (gift from Yong Liu) were maintained in RPMI 1640 (Gibco) supplemented with 6.1 mmol/L glucose, 10% fetal bovine serum (FBS) and 10 mmol/L HEPES, as described previously (19). Overexpression or knockdown of βarr2 in INS-1 (832/13) cells were conducted by infecting the cells with 10 multiplicity of infection adenovirus expressing βarr2 (Ad-βarr2) or its shRNA (Ad-shβarr2) (gift from Gang Pei; 20). For overexpression of the plasmid expressing c-Jun N-terminal kinase-1 (JNK1) (gift from Yan Lu), INS-1 (832/13) cells were transfected with 0.05 or 0.2 μg of plasmid expression of JNK1 in 200 μL culture medium.

**Histology Study and Morphometric Analyses**

Pancreases were removed from 16-wk-old βarr2+/+ and βarr2−/− mice, immediately weighed, fixed and embedded. To determine the count and area of islets, 8–10 randomly chosen sections per mouse that were separated by at least 100 μm were stained with hematoxylin and eosin (H&E). The entire pancreatic sections were scanned using a Nikon Eclipse Ni-E Microscope (Nikon), and a tile image of the tissue section was generated using the NIS-Elements AR 4.20 (Nikon). The fractional area of the islet in the pancreas, islet count per unit pancreatic area (islet density) and islet size were manually quantified using Image-Pro Plus 6.0 (Media Cybernetics), as described previously (21). The average of all the sections was taken as a measure for the entire organ. The total islet mass was calculated as pancreatic weight × mean fractional pancreatic islet area.

For immunofluorescence analysis, pancreatic sections were incubated overnight at 4°C with guinea pig anti-insulin antibody (Abcam), followed by staining with secondary goat anti-guinea pig antibody conjugated with Alexa Fluor 488 (Invitrogen). Sections were imaged using a Zeiss AxiosImager Standard Microscope (Carl Zeiss).

To analyze cell replication, βarr2+/+ and βarr2−/− mice were administered 50 mg/kg 5-bromo-2-deoxyuridine (BrdU) (Sigma-Aldrich) intraperitoneally, twice daily for 7 d. Immunohistochemistry of BrdU or Ki67, a cellular marker for proliferation (22), was performed using an anti-BrdU antibody (Boster) and an anti-Ki67 antibody (Abcam) with overnight incubation at 4°C, followed by incubation with corresponding horseradish peroxide (HRP)-conjugated secondary antibodies. The proliferation rate was represented as the percentage of BrdU- or Ki67-positive nuclei per total islet nuclei.

**Cell Viability and Cell Cycle Analysis**

Cell viability was assayed either by Hoechst 33342 (HO; Sigma) and
propidium iodide (PI; Sigma) staining, as described previously (18), or by flow cytometer analysis. Mice islets from βarr2+/+ and βarr2–/– mice or βarr2-overexpressing INS-1 (832/13) cells were exposed to indicated concentrations of glucose or palmitate for 60 or 48 h, respectively. For flow cytometer analysis, the single cell suspension was incubated with Annexin V (Invitrogen) and examined using the Navios flow cytometer (Beckman Coulter).

For the cell cycle analysis, INS-1 (832/13) cells were cultured in 3 mmol/L glucose without FBS for 6 h for synchronization and then incubated in 11 mmol/L glucose with 10% FBS. After 16 h, cells were harvested for mRNA expression or for cell cycle distribution. The cell cycle phase distribution was determined by the above-mentioned flow cytometer with PI staining.

**Microarray Expression Analysis and Quantitative Real-Time PCR**

Gene expression profiles of isolated islets from βarr2+/+ and βarr2–/– mice were analyzed with the Affymatrix GeneChip Mouse Genome 430 2.0 Array (Affymetrix). Sample preparation and hybridization were done at the Shanghai Biotechnology Corporation (SBC). Microarray data were analyzed using SBC Analysis System (including significant analysis of microarray, fold change, pathway enrichment analyses and hierarchical cluster). Fold changes ≥1.4 and P values <0.05 are considered significant difference. Quantitative real-time PCR was performed using the ABI 7500 Fast Real-Time PCR System (Applied Biosystems) as described previously (18). Primers for each target gene are shown in Supplementary Table S1. The relative expression level of each sample was calibrated by the comparative Ct method, using β-actin as an endogenous control.

**Immunoblotting Analysis**

Protein was extracted from cells with radioimmunoprecipitation assay buffer containing protease inhibitors, separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes. The membranes were incubated overnight at 4°C with anti-phosphorylated JNK (p-JNK) antibody, anti-total JNK antibody (Cell Signaling Technology), anti-Cyclin D2 antibody (Cell Signaling Technology) and anti-p21 antibody (Millipore), followed by incubation with corresponding HRP-conjugated secondary antibodies. Signals were detected using Fujifilm Luminescent Image Analyzer LAS4000 System (Fujifilm).

**BLI**

BLI was performed using a Lumazone Imaging System (Roper Scientific Photometrics) as described previously (23). MIP-TF–β cells were anesthetized with isoflurane (1.5% in 95% O2) and injected with 126 mg/kg of D-luciferin (15 mg/mL in phosphate-buffered saline) intraperitoneally. Then, mice were placed in the supine orientation in a light-tight chamber, and a gray-scale reference image was obtained under low-level illumination. Bioluminescent images were captured 12 min after luciferin injection with an integration time of 3 min. Isometric regions of interest were drawn over the location corresponding to the pancreas for quantification using MAG Biosystems Software 7.6 (MAG Biosystems). Luciferase activity is presented in photon intensity per second. Bioluminescence attenuation was adjusted as described previously by Yong et al. (17).

**Statistical Analysis**

Data are expressed as mean ± standard error of the mean. Statistical analysis was performed with GraphPad Prism 5 for Windows (GraphPad software), and significance was calculated by Student t test or analysis of variance (ANOVA) and followed with Bonferroni correction as appropriate. For all analyses, P values were two-tailed, and P < 0.05 was considered statistically significant.

All supplementary materials are available online at www.molmed.org.
Loss of αarr2 Exacerbated Pancreatic β-Cell Death Induced by High Levels of Glucose and FFA

We next examined the effect of αarr2 on β-cell viability in glucolipotoxicity, a condition that occurred during HFD feeding in mice. As expected, exposure of islets or INS-1 (832/13) cells to high levels of glucose or palmitate led to cell death compared with that of their respective controls (Figures 5A, B). Deletion of αarr2 in islets increased the cell death compared with wild-type islets (Figure 5A), whereas overexpression of αarr2 in INS-1 (832/13) cells greatly alleviated the cell death (Figures 5B, C and Supplementary Figure S4).

Taken together, our results suggested a role of αarr2 in protection of β-cells from high glucose- and FFA-induced cell death.

Overexpression of αarr2-Protected Cells through Inhibition of JNK Pathway

To understand the biological processes related to β-cell survival in αarr2−/− mice,
a microarray analysis was performed to compare the gene expression between islets isolated from βarr2+/− and βarr2+/+ mice. In total, 819 genes were found to be changed significantly in the βarr2-null islets. Among them, 558 genes were upregulated and 261 genes were downregulated. As shown in Figure 6A, components in the JNK/mitogen-activated protein kinase (MAPK) signaling pathway were significantly altered in βarr2−/− islets (35 genes involved, 13% of the total genes in the pathway, enrichment test P < 0.01) with upregulation of gene expression in MAPK-upstream protein kinase (MUK)/MAP kinase kinase 7 (MKK7)/JNK1/2/c-Jun cascade. The real-time PCR validated the expression pattern of MKK7 observed in the array analysis (Figure 6B). Moreover, exposure of INS-1 (832/13) to high glucose or FFA led to the phosphorylation of JNK, whereas overexpression of βarr2 in the cells diminished the JNK activation (Figure 6C), suggesting a role of βarr2 in the regulation of the JNK signaling pathway. To confirm findings in islets, the gain- and loss-of-function experiments were performed in the β-cell line. As shown in Figure 6D, knocking down of βarr2 in INS-1 (832/13) cells with shRNA significantly aggravated the high glucose- and FFA-induced cell death to 1.8-fold (glucose) and 1.6-fold (FFA). The addition of SP600125, a JNK inhibitor, dose-dependently abolished the above effect. In contrast, the cytoprotective effects of βarr2 on high glucose- and FFA-induced death were diminished dose-dependently in the presence of JNK1 (Figure 6E). Taken together, these observations suggested that βarr2 modulates β-cell survival through the regulation of proapoptosis JNK pathway.

**DISCUSSION**

In the present study, using a real-time tracing imaging technique, we characterized the dynamic changes of β-cell mass in βarr2-null mice under HFD conditions from 4 to 20 wks and provided in vivo evidence demonstrating that
deficiency of βarr2 in mice led to much earlier and more severe decompensation of β-cell mass under HFD (Figure 2). Moreover, we provide evidence supporting the novel roles of βarr2 in protection of β-cells from glucotoxicity- and lipotoxicity-induced cell death (Figure 5) through inhibition of proapoptosis JNK pathway activation (Figure 6) and in regulation of cell proliferation through cyclin D2 and p21 expression modulation (Figure 4). Revealing novel roles and mechanisms of βarr2 in regulating dynamic β-cell mass changes is essential to provide information necessary for the development of new therapy strategies for T2D.

It is well known that β-cell mass changes during the development and progression of T2D (1–4). Mass quantification with noninvasive approaches is important for basic science and clinical studies. However, quantification of β-cell mass in vivo is relatively difficult due to the lack of reliable and noninvasive biomarkers for cells (24). Recently, the use of in vivo imaging techniques including positron-emission tomography (PET) and single-photon emission computed tomography (SPECT) with isotope-labeled ligands [such as 18F-fluoropropyl-(+)-DTBZ and 111In-labeled exendin] have been utilized in the field of human β-cell image studies (25–27). Likewise, BLI has been widely used with luciferase transgenic mice to evaluate β-cell mass under both normal and pathological conditions (17,28). BLI is advantageous because it saves the animals from being killed and allows sequential studies in the same animal. In contrast to other imaging methods, BLI additionally provides quantifiable data with high throughput and inherently low background. However, a drawback of limited tissue penetration in BLI makes it impossible to apply to humans. Nevertheless, the lower cost of BLI compared with other imaging methods and good correlation with pancreatic β-cell mass makes BLI highly useful for studies in small animals.

In the present study, to trace the effect of βarr2 loss on the changes of β-cell mass during mouse growth in real

Figure 3. Quantification of islet mass in pancreas sections. (A) Pancreas sections (8–10 sections per mouse) of 16-wk-old βarr2+/+ and βarr2−/− mice fed a chow diet or a HFD were stained with H&E. Representative micrographs of H&E staining of pancreatic sections from βarr2+/+ and βarr2−/− mice. Scale bar: 500 μm. (B) Quantification of islet mass, (C) percentage islet area relative to pancreas, (D) islet density, (E) mean islet size and (F) islet size distribution of βarr2+/+ and βarr2−/− mice fed with a HFD (n = 3–5). *P < 0.05, **P < 0.01, versus βarr2+/+ mice.
Figure 4. The effect of βarr2 on β-cell proliferation. (A) Representative micrographs of BrdU and Ki67 staining in islets of βarr2+/+ and βarr2−/− mice. Scale bar: 50 μm. Arrows: BrdU- or Ki67-positive cells. (B) Quantification of BrdU- or Ki67-positive cells in the total islet cells (n = 3–5). (C) Cell cycle distribution analyzed by flow cytometry in INS-1 (832/13) cells infected with adenovirus expressing GFP or βarr2 and exposed to 3 mmol/L (G3) or 11 mmol/L (G11) glucose. (D) Relative mRNA transcripational levels of cell cycle genes from INS-1 (832/13) cells with βarr2 overexpression (n = 6–8). Expression of mRNA was normalized with β-actin. (E) Cyclin D2 and p21 protein levels in INS-1 (832/13) cells infected with adenovirus expressing GFP or βarr2. Pictures are representative of four independent experiments. *P < 0.05, **P < 0.01, versus control.
of βarr2 deletion on glucose-stimulated insulin secretion are controversial (11,14). This discrepancy may result from the islets isolated from different mice, as while we used islets from 12-wk-old male mice, Ravier et al. used islets from 6- to 7-month-old female mice. Further studies may be needed to explore the possibility.

The failure of β-cells to compensate for the lost mass under metabolic stress observed in βarr2–/– mice indicates the role of βarr2 in regulation of β-cell mass. One mechanism underlying the loss of β-cell mass in HFD-fed βarr2–/– mice is likely the consequence of reduced β-cell proliferation via modulation of cell cycle regulation. We showed in the study that both BrdU- and Ki67-positive cells were decreased significantly in islets with βarr2 deletion. Overexpression of βarr2 increased the proportion of cells in the S and G2/M phases of the cell cycle with concomitant upregulation of cyclin D2 and attenuation of p21 expression in INS-1 (832/13) cells (Figure 4). Both cyclin D2 and p21 are cell cycle–related proteins involved in regulating release of the G1 brake and entry of cell cycle (31,32). Cdk inhibitor p21 functions as a molecular brake during β-cell expansion and is upregulated during pregnancy and mitogen stimulation to avoid excessive proliferation (33–36). Cyclin D2 is the major D-type cyclin in β-cells and plays a vital role in the regulation of adult β-cell proliferation (31,37). Knocking out cyclin D2 in mice resulted in a four-fold reduction in β-cell mass and led to development of impaired glucose tolerance or even diabetes in these mice (38,39). In contrast, overexpression of cyclin D2 in human islets increased BrdU-positive cells (40). While cyclin D2 might be a regulator driving adaptive β-cell proliferation, it is currently unclear how βarr2 manipulates cyclin D2 transcription in β-cells. It is known that activation of the insulin receptor/Akt signaling pathway was associated with β-cell proliferation (41). βarr2 has been reported to enhance insulin-stimulated Akt activity in mouse liver, skeletal muscle and
Moreover, we showed in the present study that βarr2 protected β-cells from glucolipotoxicity-induced cell apoptosis. The antiapoptosis effects for βarr2 are in agreement with several previous studies.}

adipose tissue (10). In INS-1E cells, activation of Akt is associated with phosphorylation of FoxO1a and prevents FoxO1a from translocation into the nucleus, thus releasing transcription repression of cyclin D2 (42). It is, thus, speculated that the effect of βarr2 on cyclin D2 transcription might be through the Akt/FoxO1a pathway. Further studies are needed to explore that possibility.

Moreover, we showed in the present study that βarr2 protected β-cells from glucolipotoxicity-induced cell apoptosis. The antiapoptosis effects for βarr2 are in agreement with several previous studies.

**Figure 6.** βarr2 inhibited FFAs or glucose-induced activation of the JNK pathway in β-cells. (A) Total RNA was obtained from islets of βarr2+/+ and βarr2−/− mice and subjected to whole-genome expression profiling using Affymetrix arrays. Heat diagram depicting genes in the JNK/MAPK pathway for which expression was significantly upregulated (28 genes) and downregulated (7 genes) in the islets of βarr2−/− as compared with βarr2+/+ animals (n = 3). (B) Quantification of islet mRNA expression in the JNK pathway normalized with β-actin (n = 4–6). (C) INS-1 (832/13) cells were infected with adenovirus encoding GFP or βarr2 and assayed for p-JNK and total JNK expression in the presence or absence of 30 mmol/L glucose (G30) and 0.4 mmol/L palmate (PA0.4) for 24 h. Pictures are representative of four independent experiments. (D, E) INS-1 (832/13) cells were infected with either adenovirus-encoding shRNA of βarr2 (shβarr2) (D) or βarr2 (E) and incubated with G30 or PA0.4 in the presence of SP600125 (D) or plasmid-expressing JNK (E). Data were presented as fold-change over cells infected with scrambled shRNA (D, white bar) or GFP (E, white bar) (n = 4). *P < 0.05, **P < 0.01, versus indicated group. Muk, mitogen-activated protein kinase upstream protein kinase; Mkk7, mitogen-activated protein kinase kinase 7. Jnk, c-Jun N-terminal kinase.
in other cell lines (43,44). Sun et al. reported that overexpression of βarr2 decreased the percentage of apoptosis and inhibited caspase-3 activation in endometrial cancer (44). Furthermore, we proved, for first time, that the cytoprotective effect of βarr2 was mediated through its inhibition of the JNK pathway activated by high levels of glucose and FFA. The following lines of evidence support this conclusion: a) deletion of βarr2 in islets enhanced the gene transcription in the JNK pathway shown in microarray, and aggravated cell death; b) overexpression of βarr2 inhibited the phosphorylation of JNK induced by glucose and FFA and protected β-cell from high levels of glucose- and FFA-induced apoptosis; and c) the cytoprotective effect of βarr2 on cells could be abolished dose-dependently in JNK1-overexpression cells. JNK1 and JNK2 are stress-activated proapoptotic proteins in pancreatic β-cells against glucolipotoxicity-induced cell death through inhibition of the JNK pathway and in regulation of β-cell proliferation associated with modulation of cell cycle regulatory genes. Loss of βarr2 leads to limited β-cell mass expansion under metabolic stress. Taken together with our previous discoveries (10,11), we conclude that βarr2 plays an important role in the development of T2D by modulating peripheral insulin sensitivity, insulin secretion and β-cell mass.

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DISCLOSURE

The authors declare that they have no competing interests as defined by Molecular Medicine, or other interests that might be perceived to influence the results and discussion reported in this paper.

REFERENCES


