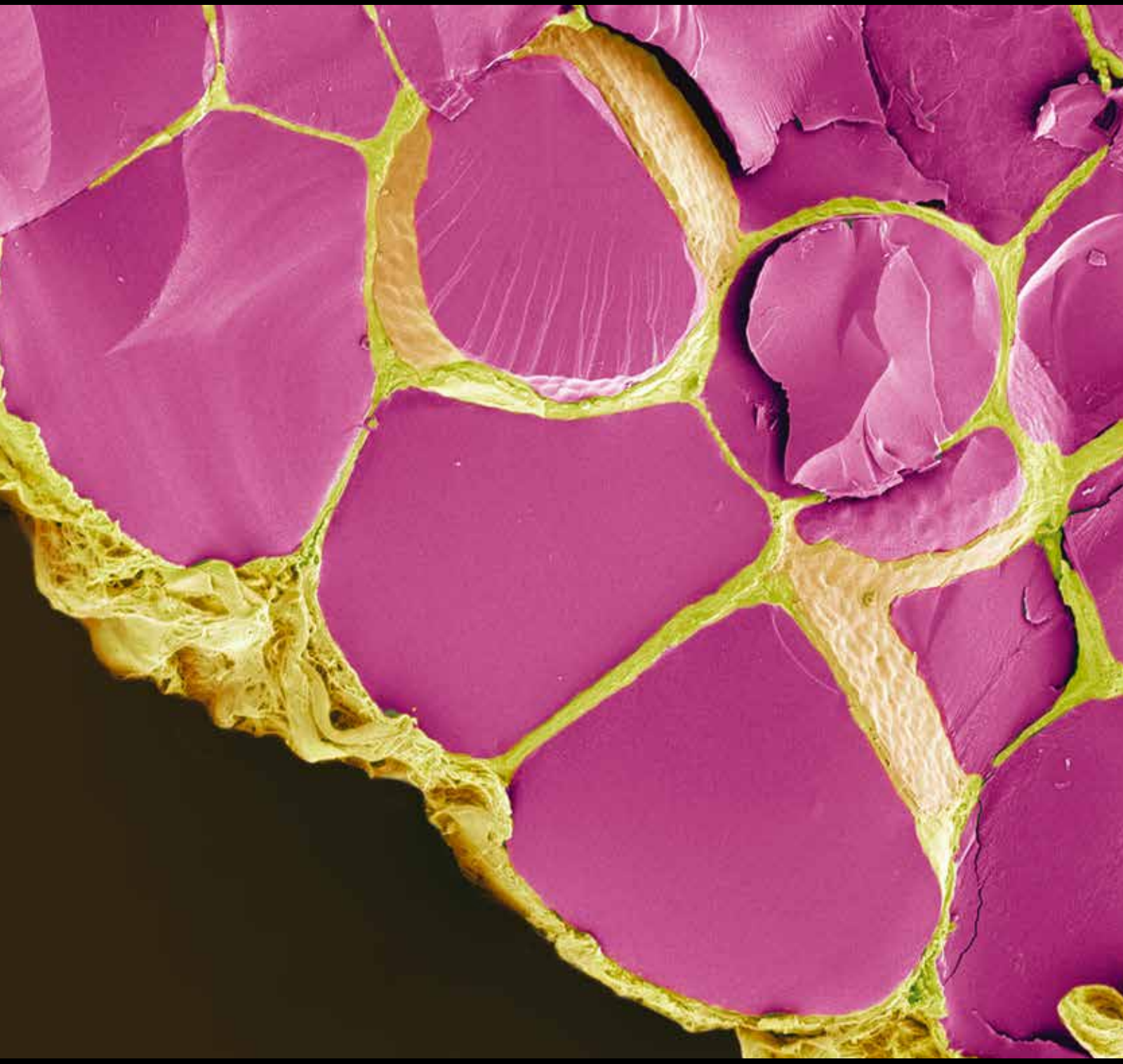
International Journal of Endocrinology

Islets and Glucose Homeostasis

Guest Editors: Tien-Jyun Chang, Gang Xu, and Jyuhn-Huarng Juang



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Editorial Board

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Editorial

Islets and Glucose Homeostasis

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Diabetes mellitus is a chronic progressive metabolic disease, resulting from both insulin resistance and the dysfunction of beta-cells. Beta-cell apoptosis is a crucial pathophysiology leading to diabetes [1]. Aberrant immune system leads to destroying of beta-cells occurring in type 1 diabetes. Infiltration of immune cells around beta-cells and attack of beta-cells by cytokines or chemokines through upregulating the proapoptotic molecule Bid and subsequently the release of cytochrome c from mitochondria contributed to apoptosis. Fas/FasL and TNF pathways also elicit the same downstream molecules as the above-mentioned apoptotic pathway [2]. Unlike type 1 diabetes, metabolic disorders mainly cause type 2 diabetes, such as chronic glucotoxicity, lipotoxicity, and endoplasmic reticulum (ER) stress [3, 4]. Apoptosis in pancreatic beta-cells is observed in response to various stimuli such as glucose, cytokines, free fatty acids, leptin, islet amyloid polypeptide, ER stress, and sulfonylureas [4, 5]. Regardless of the underlying cause of the disease, insufficient beta-cell mass leads to dependence on exogenous insulin for blood glucose regulation. The morbidities associated with diabetes are significant. The knowledge about pancreas or islets transplantation and factors attributing to changing the secretory function and/or mass of islet beta-cells might help to develop a novel treatment to diabetes.

The past 15–20 years has seen a dramatic increase in the prevalence of T2D in children and adolescents [6–8]. Type 2 diabetes is generally believed to be a polygenic disorder, with disease development being influenced by both hereditary and environmental factors [9]. Support for the role of genetic factors comes from epidemiological evidence that type 2 diabetes in youth is most common in individuals from racial groups with a high prevalence of diabetes and in individuals with a strong family history [10]. A search for the contribution of certain candidate genes in the early onset type 2 diabetes is mandatory for further understanding of pathogenesis of type 2 diabetes in childhood. In this issue, Y.-D. Jiang et al. reported that E23K polymorphism of the *KCNJ11* gene contributed to an increased risk for type 2 diabetes in school-aged child and adolescence. K23-allele-containing genotypes conferring increased plasma insulin level during OGTT in normal subjects. However, the diabetic subjects with the K23-allele-containing genotypes had lower fasting plasma insulin levels after adjustment of age and BMI percentiles.

T2DM is a multifactorial metabolic disease mainly characterized by hyperglycemia [11], but before the occurrence of overt hyperglycemia, peripheral insulin resistance leads to compensatory insulin hypersecretion by pancreatic islets [12]. A. P. O. Protzek et al. reported that augmented β-cell function and increased β-cell mass developed in response to the glucocorticoid-induced insulin resistance involve inhibition of the islet AS160 protein activity.

Recently, human islet transplantation has achieved insulin independence in type 1 diabetes and the success rates have been markedly improved [13]. However, most successful cases need 2 or more implants and the long-term follow-up shows their insulin independence declines with time [14, 15]. Therefore, the critical issue in clinical islet transplantation is to further improve and maintain its successful rate. J.-H. Juang et al. reported that posttransplant DPP-4 inhibition with MK-0431 in the diabetic recipient with a marginal number of islets

is not beneficial to transplantation outcome or islet grafts. We cannot exclude the possibility that higher dose of MK-0431 or more islets graft may have beneficial effects on the outcome of islet transplantation. M. A. Kanak et al. reviewed recent advances in understanding the role of inflammation in islet transplantation and development of strategies to prevent damage to islets from inflammation. Details on cell signaling pathways in islets triggered by cytokines and harmful inflammatory events during pancreas procurement, pancreas preservation, islet isolation, and islet infusion are presented. The authors also discussed several potent anti-inflammatory strategies that show promise for improving islet engraftment.

Type 1 diabetes is characterized by the progressive loss of pancreatic beta-cells caused by autoimmune attack [16]. Although beta-cell mass is markedly diminished in long-standing type 1 diabetics, residual beta-cells can be detected and new beta-cell formation may occur in these patients several decades after the disease onset [17]. This observation has led to researches to induce remission of diabetes by targeting beta-cell autoimmunity and regeneration. J.-H. Juang et al. reported that before the onset of autoimmune diabetes, all-*trans*-retinoid acid (ATRA) and exendin-4 treatment alone preserves pancreatic beta-cells; ATRA and ATRA plus exendin-4 treatment delays the onset of autoimmune diabetes. However, after the onset of autoimmune diabetes, ATRA and/or exendin-4 treatment is unable to reverse hyperglycemia or improve survival.

Tien-Jyun Chang
Gang Xu
Jyuhn-Huarng Juang

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Research Article

The Estimation of First-Phase Insulin Secretion by Using Components of the Metabolic Syndrome in a Chinese Population

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Aims. There are two phases of insulin secretion, the first (FPIS) and second phase (SPIS). In this study, we built equations to predict FPIS with metabolic syndrome (MetS) components and fasting plasma insulin (FPI). *Methods.* Totally, 186 participants were enrolled. 75% of participants were randomly selected as the study group to build equations. The remaining 25% of participants were selected as the external validation group. All participants received a frequently sampled intravenous glucose tolerance test, and acute insulin response after the glucose load (AIRg) was obtained. The AIRg was considered as FPIS. *Results.* When MetS components were only used, the following equation was built: log (FPIS) = 1.477 − 0.119 × fasting plasma glucose (FPG) + 0.079 × body mass index (BMI) − 0.523 × high-density lipoprotein cholesterol (HDL-C). After FPI was added, the second equation was formulated: log (FPIS) = 1.532 − 0.127 × FPG + 0.059 × BMI - 0.511 × HDL-C + 0.375 × log (FPI), which provided a better accuracy than the first one. *Conclusions.* Using MetS components, the FPIS could be estimated accurately. After adding FPI into the equation, the predictive power increased further. We hope that these equations could be widely used in daily practice.

1. Introduction

Both deteriorated insulin sensitivity (S_I) and impaired insulin secretion are recognized as 2 of the foremost forms of pathophysiology for type 2 diabetes (T2DM) [1, 2]. In the natural course of developing diabetes, the plasma glucose does not rise until the decompensation of the β-cell function to insulin resistance (IR) occurs. Moreover, the impaired β-cell function is a more critical factor than decreased S_I to determine the development of T2DM, especially in Asian people [3, 4].

Two phases of insulin secretion are widely known: the first-phase insulin secretion (FPIS) and the second-phase insulin secretion (SPIS). The FPIS is normally secreted by the β-cells within 10 min after being exposed to an acute rise in plasma glucose levels to reduce their emission [5], whereas the SPIS is the newly secreted insulin from β-cells after the FPIS. A better FPIS results in longer maintenance of normal

glucose homeostasis before the occurrence of diabetes [5]. The FPIS is a sensitive indicator for the deterioration of insulin sensitivity [6, 7] because it decreases rapidly even in prediabetes stage (PreDM) [8]. Normally, it becomes completely disappeared when diabetes is diagnosed.

Numerous studies have documented the link between metabolic syndrome (MetS), the clustering of hyperglycemia, hypertension, obesity, and dyslipidemia, and an elevated risk of developing cardiovascular disease and diabetes [9, 10]. A consensus exists that IR is the core of MetS [11–14]. Simultaneously, each abnormal MetS component adversely affected insulin secretion independently [15]. Components such as the body mass index (BMI), triglyceride (TG), high-density lipoprotein cholesterol (HDL-C), and fasting plasma glucose (FPG) levels were all shown to be related to insulin secretion [11, 16]. Although, as mentioned, the FPIS is important, no readily available accurate method exists for measuring the first ISEC. In this study, we built an equation to estimate the FPIS by using routine clinical variables and MetS components. We hope that the estimated FPIS can be widely used in clinical settings.

2. Participants and Methods

2.1. Participants. In total, 186 participants were enrolled in this study. Either they were self-referred or health professionals had referred them for diabetes screening. They had no history of diabetes and, therefore, they took no diabetes medications at the time of the study. They were defined as having a normal glucose tolerance (NGT), PreDM, and T2DM according to the criteria published by the American Diabetes Association in 2012 [17]. Otherwise, none of the participants had a remarkable medical or surgical history. In total, 51 participants had NGT, 40 had PreDM, and 95 had T2DM. Before participating in the study, they were instructed by physicians and dietitians not to receive any medication known to affect glucose or lipid metabolism and to remain on a stable diet for at least 1 wk before the study. On the day of the first study, an entire routine workout was completed by participants to exclude those with significant cardiovascular, endocrine, renal, hepatic, and respiratory disorders. The study protocol had been approved by the hospital's institutional review board and ethics committee; all participants provided written informed consent prior to participating. To validate our equation, 75% of the participants were randomly selected. Based on these participants, an optimal equation was built for estimating the FPIS. This equation was subsequently used to calculate the FPIS among the remaining 25%, who constituted the external validation group.

2.2. Study Protocol. Frequently sampled intravenous glucose tolerance test (FSIGT): all tests were performed at the clinical research center. On the day of the study visit, after a 12 h overnight fast, one catheter was placed on both arms of each participant. A bolus of 10% glucose water (0.3 g/kg) was given. Another bolus of regular human insulin (Novo Nordisk Pharmaceutical, Princeton) 0.05 units/kg was injected 20 min after the glucose load. Blood samples for plasma glucose and insulin levels were collected at 0 min, 2 min, 4 min, 8 min, 19 min, 22 min, 30 min, 40 min, 50 min, 70 min, 100 min, and 180 min. The data were inputted into a Bergman minimal model [1], and then the S_I, glucose effectiveness (GE), and acute insulin response after the glucose load (AIRg) were obtained. The AIRg was considered the FPIS, and the product of the S_I and the AIRg was the disposition index (DI).

The calculations of HOMA-IR and HOMA-β (homeostasis model assessment of insulin resistance and the β-cell function) were performed according to Matthew's equation [18].

Plasma was separated within 1 h of blood withdrawal and stored at −30°C until the analysis. Plasma glucose was measured using a glucose analyzer by employing an oxidase method (YSI Model 203, Scientific Division, Yellow Spring Instrument Company, Inc., Yellow Spring, OH, USA). Plasma insulin was assayed using a commercial solid phase radioimmunoassay technique (Coat-A-Count insulin kit, Diagnostic Products Corporation, Los Angeles, CA, USA) with intra- and interassay coefficients of variance of 3.3% and 2.5%, respectively. Serum TG was measured using the Fuji Dri-Chem 3000 analyzer (Fuji Photo Film Corporation, Minato-Ku, Tokyo, Japan) by employing the dry multilayer analytical slide method. The serum HDL-C concentration was determined using the enzymatic cholesterol assay method after dextran sulfate precipitation.

2.3. Statistical Analysis. The data were tested for normal distribution by using the Kolmogorov-Smirnov test and for the homogeneity of variances by using the Levene test. Continuous variables were expressed as mean ± standard deviation. Among the data, FPIS, FPI, S_I, and DI were not normally distributed and were logarithmically transformed. An independent t-test was used to evaluate the demographic data, the clinical characteristics, and the parameters derived from the FSIGT between the 2 groups (a study and external validation group). To build the equation to estimate the FPIS, we used the stepwise method in multiple regression analysis. We adopted sex, age, and the MetS components as independent variables and the FPIS as the dependent variable. Although FPI is not a component of MetS, it was found to be strongly related to FPIS; another equation with the FPI as the independent variable was also built.

These equations were subsequently used to calculate the FPIS among the remaining 25% of participants. The correlation between the calculated FPIS and measured FPIS was measured using Pearson's r correlation coefficient. Higher correlation coefficients (r) indicate a superior prediction accuracy. Hierarchical multiple regression method was also used to examine predicting power between these equations.

All statistical analyses were performed using the SPSS software system, version 13.0 (SPSS Inc., Chicago, IL, USA). All P values less than 0.05 were considered statistically significant.

3. Results

In the study, 140 and 46 participants were classified into the study group and the external validation group, respectively.

Table 1: Demographic data of the study and external validation groups.

	Study group	Ext. val. group	P value
n	140	46	
Sex (male/female)	69/71	25/21	0.552
Age (y)	50.7 ± 13.5	50.8 ± 14.7	0.910
Body mass index (kg/m^2)	25.2 ± 3.9	25.8 ± 5.1	0.366
Systolic blood pressure (mmHg)	121.5 ± 13.0	118.3 ± 16.2	0.186
Diastolic blood pressure (mmHg)	76.3 ± 8.0	73.4 ± 7.7	0.076
Triglyceride (mmol/L)	1.3 ± 0.6	1.5 ± 0.6	0.112
HDL-C (mmol/L)	1.1 ± 0.3	1.1 ± 0.4	0.350
Fasting plasma glucose (mmol/L)	7.8 ± 2.8	7.5 ± 3.0	0.540
Fasting plasma insulin (pmol/L)	30.5 (12.2–61.3)	27.6 (14.4–62.0)	0.353
First-phase insulin secretion (μU/min)	115.0 (23.5–426.4)	114.9 (24.4–430.4)	0.822
Insulin sensitivity ($10^{-4}\cdot$min$^{-1}\cdot$pmol$^{-1}\cdot$L^{-1})	1.274 (0.5–3.4)	1.6 (0.3–3.3)	0.501
Disposition index	87.8 9 (20.3–900.8)	95.3 (17.0–408.7)	0.830
Glucose effectiveness ($10^{-2}\cdot$dL$\cdot$min$^{-1}\cdot$kg^{-1})	0.016 ± 0.010	0.015 ± 0.010	0.314
HOMA-IR	1.7 (0.6–3.1)	1.7 (0.7–3.1)	0.615
HOMA-β	22.1 (6.9–83.0)	20.0 (8.5–92.0)	0.334

Data are expressed as mean ± SD or median (interquartile range). Ext. val. group: external validation group.
HDL-C: high-density lipoprotein cholesterol; HOMA-IR and HOMA-β: homeostasis model assessment of insulin resistance and β-cell function.

Table 2: Demographic data of normal glucose tolerance, prediabetes, and diabetes groups.

	Normal glucose tolerance	Prediabetes	Diabetes
n	51	40	95
Age (y)	42.5 ± 17.2[2,3]	54.4 ± 11.9[1]	53.7 ± 10.3[1]
Body mass index (kg/m^2)	26.1 ± 5.9	24.9 ± 3.1	25.1 ± 3.5
Systolic blood pressure (mmHg)	118.1 ± 10.9	121.0 ± 14.7	121.9 ± 14.9
Diastolic blood pressure (mmHg)	74.0 ± 6.8	76.1 ± 8.5	76.4 ± 8.5
Triglyceride (mmol/L)	1.2 ± 0.6	1.4 ± 0.6	1.4 ± 0.6
HDL-C (mmol/L)	1.1 ± 0.3	1.1 ± 0.3	1.1 ± 0.3
Fasting plasma glucose (mmol/L)	4.6 ± 0.5[2,3]	6.4 ± 0.4[1,3]	9.9 ± 2.2[1,2]
Fasting plasma insulin (pmol/L)	49.5 (9.3–81.1)	25.5 (7.5–61.7)	23.0 (14.4–44.3)
First-phase insulin secretion (μU/min)	517.5 (183.0–5144.7)[2,3]	123.6 (35.5–390.7)[1]	37.8 (11.6–158.3)[1]
Insulin sensitivity ($10^{-4}\cdot$min$^{-1}\cdot$pmol$^{-1}\cdot$L^{-1})	0.8 (0.2–3.2)	1.9 (0.6–4.4)	1.4 (0.6–2.9)
Disposition index	893.9 (240.0–2447.1)[2,3]	54.8 (21.7–894.6)[1]	40.7 (8.3–182.5)[1]
Glucose effectiveness ($10^{-2}\cdot$dL$\cdot$min$^{-1}\cdot$kg^{-1})	0.020 ± 0.010[2,3]	0.014 ± 0.008[1]	0.014 ± 0.010[1]
HOMA-IR	1.7 (0.4–2.7)	1.3 (0.3–3.1)	1.7 (0.8–3.3)
HOMA-β	134.0 (27.9–352.4)[2,3]	29.0 (10.4–72.6)[1,3]	13.0 (6.2–26.4)[1,2]

Data are expressed as mean ± SD or median (interquartile range). HDL-C: high-density lipoprotein cholesterol; HOMA-IR and HOMA-β: homeostasis model assessment of insulin resistance and β-cell function.
[1] P value < 0.05 when compared with "Normal glucose tolerance" group; [2] P value < 0.05 when compared with "Pre-diabetes" group; [3] P value < 0.05 when compared with "diabetes" group.

Table 1 shows the demographic data, FPG and FPI, plasma lipids, and variables derived from the FSIGT of these two groups. There was no significant difference in these measurements between the two groups. The demographic data of NGT, PreDM, and T2DM are shown in Table 2. The participants in the T2DM group were older and had a higher FPG compared to the NGT group. Log (FPIS), log (DI), and log (GE) were significantly lower in the DM group.

To identify the parameters that contribute most to the FPIS, the correlations between the FPIS and different parameters were evaluated; the results are shown in Table 3. The FPIS was significantly correlated to age ($r = -0.398$, $P = 0.000$), BMI ($r = 0.264$, $P = 0.002$), FPG ($r = -0.475$, $P = 0.000$), HDL-C ($r = -0.190$, $P = 0.034$), and log (FPI) ($r = 0.382$, $P = 0.000$).

Only MetS components were used in multiple linear regression analysis. Three of them were selected from regression analysis, and the equation was built and is shown as log (FPIS) = 1.477 − 0.119 × FPG + 0.079 × BMI − 0.523 × HDL-C (standard coefficients are shown in Table 4). Subsequently, as mentioned, this equation was used to calculate the FPIS of the external validation group. The correlation

Table 3: Pearson correlation between the clinical parameters and log (first-phase insulin secretion) in the study group.

Variables	r	P value
Age	−0.398	0.000
Body mass index	0.264	0.002
Systolic blood pressure	−0.044	0.623
Diastolic blood pressure	0.030	0.740
Triglyceride	−0.064	0.463
HDL-C	−0.190	0.034
Fasting plasma glucose	−0.475	0.000
Log (FPI)	0.382	0.000
Log (insulin sensitivity)	−0.184	0.035
Log (HOMA-IR)	0.231	0.006
Log (HOMA-β)	0.551	0.000

HDL-C: high-density lipoprotein cholesterol; FPI: fasting plasma insulin; HOMA-IR and HOMA-β: homeostasis model assessment of insulin resistance and β-cell function.

Table 4: Multiple linear regression of the associated factors with log (first-phase insulin secretion) in the 2 equations.

Variables	MetS components Beta (P value)	MetS components + FPI Beta (P value)
Fasting plasma glucose	−0.386 (0.000)	−0.415 (0.000)
Body mass index	0.361 (0.000)	0.269 (0.001)
HDL-C	−0.181 (0.028)	−0.177 (0.017)
Log (FPI)	—	0.288 (0.005)

Beta: standardized coefficients; MetS: metabolic syndrome; HDL-C: high-density lipoprotein cholesterol; FPI: fasting plasma insulin.

between calculated log (FPIS) and the measured log (FPIS) was assessed, and the results are shown in Figure 1. The r value was 0.671, and P was 0.000.

Because the FPI is also considered a surrogate for the FPIS, it was also added to multiple linear regression analysis to build a second equation and, unlike the first, 4 factors were selected, and the following equation was formulated: log (FPIS) = 1.532 − 0.127 × FPG + 0.059 × BMI − 0.511 × HDL-C + 0.375 × log (FPI). The difference of predicting power of FPIS between the first and the second equation was determined using hierarchical multiple regression method. The r^2 increased significantly in both of the study ($P = 0.000$) and external validation groups ($P = 0.049$) after adding log (FPI) into FPG, BMI, and HDL-C.

The correlation between the calculated FPIS and the measured FPIS in the external group was also evaluated, and the results are shown in Figure 2. The calculated log (FPIS) determined the measured log (FPIS) with good accuracy in the external validation group ($r = 0.722$, $P = 0.000$). Compared to HOMA-β, both equations showed a better predictive accuracy for the FPIS ($r = 0.551$, $P = 0.000$). The standardized coefficient between the associated factors and the FPIS is shown in Table 4.

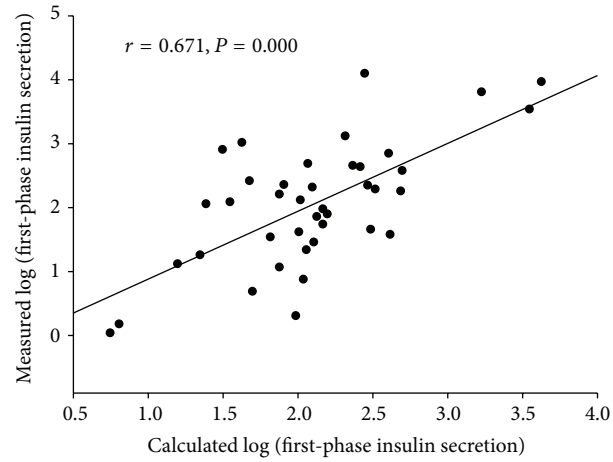

Figure 1: The correlation between the calculated first-phase insulin secretion and measured first-phase insulin secretion by using metabolic syndrome components in the external validation group.

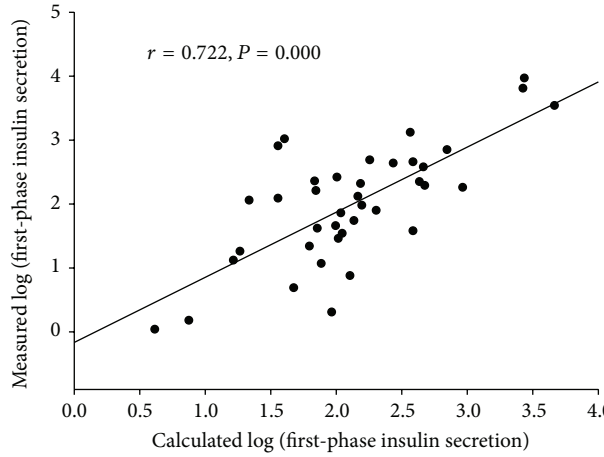

Figure 2: The correlation between the calculated first-phase insulin secretion and measured first-phase insulin secretion by using metabolic syndrome components and fasting plasma insulin in the external validation group.

4. Discussion

In this study, we built an equation by using routine clinical measurements and MetS components to predict the FPIS in participants with different levels of glucose tolerance. Because of the tight correlation between FPI and FPIS, FPI was also added into analysis to build a second equation to improve predictive accuracy. To verify our results, external validation was also performed. Although previous studies have been done to predict the FPIS, most of them enrolled only nondiabetic participants [3, 11, 19–21]. Because we believe that our study results are informative and reliable, they could be applied widely to the public health domain and in clinical settings.

The FPIS is the immediately releasable stored insulin in β-cell granules [22]. The most widely used standardized methods for measuring the FPIS are the hyperglycemic clamp and the FSIGT. By using the hyperglycemic clamp, Chiu et al.

reported that the combination of ethnicity and BMI could be used to predict 16.6% of the variance of the FPIS in NGT participants [21]. Similarly to this study, van Haeften et al. found that a combination of sex, BMI, and the family history of T2DM (FH) could predict the FPIS in the NGT and PreDM participants with similar accuracy ($r^2 = 0.152$, $P < 0.0001$ and 0.250, $P < 0.0001$, resp.) [19]. By using the FSIGT, Alford et al. showed the best prediction accuracy among these 3 studies ($r^2 = 0.27$). In our study, by using only the MetS component, r^2 was as elevated as 0.45, which is a substantially superior result compared to those of the mentioned studies. After adding the FPI into the model, r^2 could have been improved to 0.521 in the external validation group.

In our study, the FPG, BMI, HDL-C, and FPI were selected among all other factors and inputted into multiple linear regression analysis. Because the predominant function of β-cells is to maintain glucose in homeostasis, the FPG provides the most substantial contribution in the regression model [11]. Our results are in line with earlier studies which showed that the FPIS deteriorated as the FPG levels increased from NGT to diabetic range, which suggested that the FPG level is the most critical determinant for assessing the deterioration of β-cell function [7].

Following the FPG, the BMI was the second most critical factor inputted in the model. The results were not surprising because the evidence has shown that people with a higher BMI would have a better β-cell function because of the larger amount of β-cell mass [11, 19, 21, 23]. van Haeften et al. reported that the BMI is a critical contributor to the FPIS in the NGT and the PreDM participants ($r^2 = 0.096$ and 0.090, resp.). In agreement with their findings, our study also demonstrated that the BMI explained a similar level of variance for the FPIS in participants across the spectrum of glucose tolerance ($r^2 = 0.107$, data not shown). It could be questioned that waist circumference was not added into analysis in the study as it is the key component of MetS. However, waist circumference was not measured in the study, so we could not estimate FPIS using waist circumference. Evidence showed that BMI was highly correlated with waist circumference ($r = 0.900$ in men and $r = 0.889$ in women) in Chinese [24]. Moreover, Chiu et al. demonstrated that BMI is better marker than waist-hip ratio to predict first insulin secretion [21]. Therefore, BMI could replace the waist circumference to predict first insulin secretion.

Because the lower HDL-C is associated with IR [11, 16], we postulate that a negative correlation between HDL-C and the FPIS should exist. Both Hanley et al. and Gower et al. have supported this hypothesis [11, 16]. This study also produced similar findings ($r = -0.190$, $P = 0.034$). However, the Bardini study results demonstrated that there was no significant correlation between HDL-C and early-phase insulin secretion in NGT subjects but there was positive correlation between HDL-C and early-phase insulin secretion in impaired glucose tolerance subjects, which do not match ours [25]. Several explanations are available that could be used to resolve this dispute. First, ethnic differences might be the reason for the discrepancies between studies [16, 21]. Second, the FPIS was measured in the current study by using the FSIGT, which was identical with the Hanley and Gower studies. In addition, early-phase insulin secretion in the Bardini study was assessed using only a surrogate marker derived from the oral glucose tolerance test (OGTT), which is a less accurate method compared to the FSIGT. Third, in the Bardini study, the participants with T2DM were not enrolled. These differences could have a profound effect on the relationship between the FPIS and the HDL-C.

The FPI, which is not a routinely used measurement, is associated with β-cell function [7, 11, 18, 26]. Our results, which are compatible with the observations by Hanley et al., showed a significant association between the FPI level and the first ISEC ($r = 0.361$, $P = 0.000$). After combining the FPI and the MetS components into the equation, the predictive accuracy improved further (r^2 increase from 0.450 to 0.521 in the external validation group). This finding might also imply that the FPI and MetS could have affected the FPIS via different pathways because the improvement of r^2 is substantial. Further well-designed study is needed to address the issue.

The β-cell function declines as age increases, even in participants with NGT [21, 27]. Earlier study has shown that this negative influence of aging on the β-cell function might be attributed to the gradual loss of the abilities of both proinsulin converted to insulin and the decreased baseline proliferative activity of β-cell compared with younger adults [28, 29]. Moreover, evidence also showed that aging positively correlated with enhanced glucose-induced β-cell apoptosis in vitro [29]. In our study, we demonstrated that age was negatively correlated with the FPIS ($r = -0.398$, $P = 0.000$). However, age was not selected using multiple linear regression analysis. The finding might be attributed to the strong correlation between age and either FPG, BMI, or FPI which could reduce the impact of age on FPIS. In other words, the effect of age was masked by other stronger relationships between the FPIS and the MetS components.

To the best of our knowledge, the current study is the first to formulate an equation for estimating the FPIS by using the MetS components and the FPI level. However, our study has limitations. First, the body fat content and its distribution, which were known to be associated with IR and the β-cell function [30], were not measured in the study. Measuring these factors in the study might help further improve predictive power of the equation. Second, FH was not assessed in the study. It has been established that participants with an FH of T2DM have a reduced β-cell function and a decreased β-cell response to IR compared to those without [31]. Third, this study investigated only one ethnic group: the Han people. Thus, the application of our results to other ethnic groups should be exercised with caution. Finally, this study is only a cross-sectional study. In future studies, using a baseline that incorporates MetS components to estimate the FPIS would be more valuable. However, even with these limitations, we still believe that our finding could be easily and widely used in clinical settings.

In conclusion, by using the MetS components, the FPIS could be predicted with reliable accuracy ($r = 0.671$). After adding the FPI to the equation, the predictive power increases further ($r = 0.722$). These equations could be widely used in daily practice.

Abbreviations

S_I:	Insulin sensitivity
T2DM:	Type 2 diabetes
IR:	Insulin resistance
FPIS:	First-phase insulin secretion
SPIS:	Second-phase insulin secretion
PreDM:	Prediabetes
MetS:	Metabolic syndrome
BMI:	Body mass index
TG:	Triglyceride
HDL-C:	High-density lipoprotein cholesterol
FPG:	Fasting plasma glucose
FPI:	Fasting plasma insulin
NGT:	Normal glucose tolerance
FSIGT:	Frequently sampled intravenous glucose tolerance test
AIRg:	Acute insulin response after glucose load
GE:	Glucose effectiveness
DI:	Disposition index
HOMA-IR:	Homeostasis model assessment of insulin resistance
HOMA-β:	Homeostasis model assessment of the β-cell function.

Conflict of Interests

The authors have no conflict of interests.

Authors' Contribution

Chang-Hsun Hsieh analyzed the data. Jiunn-diann Lin wrote the paper. Chung-Ze Wu and Yen-Lin Chen reviewed and edited the paper. Dee Pei contributed to the discussion and edited the paper. Wei-Cheng Lian, Chun-Hsien Hsu, An-Tsz Hseih, and Chuan Chieh Liu analyzed the data and contributed to the discussion.

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Research Article

Genetic Variations in the Kir6.2 Subunit *(KCNJ11)* of Pancreatic ATP-Sensitive Potassium Channel Gene Are Associated with Insulin Response to Glucose Loading and Early Onset of Type 2 Diabetes in Childhood and Adolescence in Taiwan

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To investigate the role of E23K polymorphism of the *KCNJ11* gene on early onset of type 2 diabetes in school-aged children/adolescents in Taiwan, we recruited 38 subjects with type 2 diabetes (ages 18.6 ± 6.6 years; body mass index percentiles 83.3 ± 15.4) and 69 normal controls (ages 17.3 ± 3.8 years; body mass index percentiles 56.7 ± 29.0) from a national surveillance for childhood/adolescent diabetes in Taiwan. We searched for the E23K polymorphism of the *KCNJ11* gene. We found that type 2 diabetic subjects had higher carrier rate of E23K polymorphism of *KCNJ11* gene than control subjects ($P = 0.044$). After adjusting for age, gender, body mass index percentiles, and fasting plasma insulin, the E23K polymorphism contributed to an increased risk for type 2 diabetes ($P = 0.047$). K23-allele-containing genotypes conferring increased plasma insulin level during OGTT in normal subjects. However, the diabetic subjects with the K23-allele-containing genotypes had lower fasting plasma insulin levels after adjustment of age and BMI percentiles. In conclusion, the E23K variant of the *KCNJ11* gene conferred higher susceptibility to type 2 diabetes in children/adolescents. Furthermore, in normal glucose-tolerant children/adolescents, K23 allele carriers had a higher insulin response to oral glucose loading.

1. Introduction

Diabetes mellitus in children and adolescents has long been considered primarily type 1 diabetes. Although type 2 diabetes (T2D) is generally considered to be a disease of adults, the past 15–20 years have seen a dramatic increase in the prevalence of T2D in children and adolescents [1–9]. This increased prevalence of pediatric T2D suggests impending future morbidity from diabetic complications in a large number of relatively young adults.

In a nationwide surveillance program with mass urine screening in Taiwan [10, 11], the incidence of T2D is 6 times that of type 1 diabetes in recent years. The identified risk factors for T2D in youth are similar to those for adult type 2 diabetes with the most prominent risk of childhood obesity for T2D [11]. T2D is generally believed to be a polygenic disorder, with disease development being influenced by both hereditary and environmental factors [12]. Genetic factors are important in determining the children who become obese and also the obese children who develop T2D [13]. Support for the role of genetic factors comes from epidemiological evidence that T2D in youth is most common in individuals from racial groups with a high prevalence of diabetes and in individuals with a strong family history [14]. A search for

the contribution of certain candidate genes in the early onset T2D is mandatory for further understanding of pathogenesis of T2D in childhood.

The pancreatic islet ATP-sensitive potassium channel complex (K_{ATP}) plays a major role in glucose-stimulated insulin secretion, thus serving as a strong candidate for T2D. This channel is a heterooctameric complex composed of four sulfonylurea receptor (SUR1) subunits and four Kir6.2 subunits [15, 16]. Mutations in the SUR1 (*ABCC8*) and the Kir6.2 (*KCNJ11*) cause familial hyperinsulinemia in infancy [17], while some polymorphisms in these genes (exon 16-3t/c and exon 18 C/T of *ABCC8* and E23K of *KCNJ11*) have been reported to be associated with T2D in several populations at different degrees [18–23]. The K23 allele is associated with higher risk of T2D, providing an overall odds ratio (OR) of 1.23 [23] and 1.26 [21] in Caucasians and Asians, respectively. According to a recent systemic meta-analysis, the E23K polymorphism was significantly associated with increased T2D risk with per-allele odds ratio (OR) of 1.12. When stratified by ethnicity, significantly increased risks were found for the polymorphism in Caucasians and East Asians. However, no such associations were detected among Indian and other ethnic populations [24]. Normoglycemic lysine carriers are shown to consistently display a defect in insulin secretion [23, 25, 26]. Furthermore, the codon 23 *KCNJ11* polymorphism is shown to be related to glucose intolerance in Caucasians and progression from glucose intolerance to T2D [27, 28].

Recent studies have provided evidence that the E23K variant alters channel function by inducing spontaneous overactivity of pancreatic β-cells, thus increasing the threshold of ATP concentration for insulin release [29, 30]. Therefore, in this study, we analyzed the E23K polymorphism of *KCNJ11* gene in a group of subjects with T2D and a group of controls identified in a nationwide surveillance program for diabetes in schoolchildren aged 6~18 in Taiwan. We demonstrated that the E23K polymorphism of *KCNJ11* gene increased susceptibility to T2D in childhood and adolescence.

2. Materials and Methods

2.1. Subjects. With a nationwide surveillance program for diabetes in Taiwanese school-aged children, 137 subjects were newly diagnosed with T2D [10, 11]. Only 38 newly diagnosed T2D subjects and 69 nondiabetic subjects from the northern part of Taiwan were recruited for genetic analysis. Body mass index (BMI) is a measure of body fat based on body height (BH) and body weight (BW) (BMI = BW (kg)/BH^2 (m^2)). After BMI is calculated for children and teens, the BMI number is plotted on the Centers for Disease Control and Prevention (CDC) BMI-for-age growth charts (for either girls or boys) to obtain a percentile ranking (http://www.cdc.gov/healthyweight/assessing/bmi/childrens_bmi/about_childrens_bmi.html, searched on 8.14.2014). A standard oral glucose tolerance test with 1.75 g glucose/kg of body weight or maximally with 75 g glucose was performed to classify the state of glucose tolerance, except for subjects diagnosed with a fasting plasma glucose level equal to or over 126 mg/dL. Informed consent was obtained from each participant and their parents of those under 18. This study was approved by the Institutional Review Boards.

2.2. Measurements of Metabolic Parameters. The fasting plasma glucose, serum insulin, cholesterol, and TG were measured according to the previous reports [31]. Insulin resistance index was calculated with homeostasis model assessment (HOMA-IR) as described previously [32]. The estimated β-cell function based on the HOMA-B was calculated based on the following formula: %B = 20 × fasting plasma insulin (FPI, μU/mL)/(fasting plasma glucose (FPG, mM) − 3.5) [33]. Another set of estimates of β-cell function proposed by Stumvoll et al. was also calculated using the two formulae: 1st PH_s = 1283 + (1.829 × plasma insulin concentration at 30 min) − (138.7 × plasma glucose concentration at 30 min) + (3.772 × FPI) and 2nd PH_s = 287 + (0.4164 × plasma insulin concentration at 30 min) − (26.07 × plasma glucose concentration at 30 min) + (0.9226 × FPI). These estimations were based on plasma glucose concentrations in mmol/L and plasma insulin concentrations in pmol/L [34]. Insulinogenic index (30 minutes) was estimated as follows: (Ins 30 − Ins 0)/(Glu 30 − Glu 0) [35]. Area under curve (AUC) of glucose and insulin during OGTT was also calculated.

2.3. Genotyping for the Polymorphism of KCNJ11. The E23K polymorphism of *KCNJ11* was genotyped by PCR-restriction fragment length polymorphism (PCR-RFLP). PCR was performed with forward primer 5′-GACTCTGCAGTGAGGCCCTA-3′ and reverse primer 5′-ACGTTGCAGTTGCCTTTCTT-3′ starting with a denaturing step at 95°C for 3 min followed by 35 cycles of 95°C for 30 s, annealing at 60°C for 30 s, and elongation at 72°C for 30 s with a final elongation step at 72°C for 9 min. The PCR product was 209 bps, and it was digested with *Ban*II (New England Biolabs, Beverly, MA) and separated on 3% agarose gels. The substitution of G with A eliminated the *Ban*II site.

2.4. Statistical Analysis. Data were represented as mean ± SD. Due to relatively small sample size, EK/KK were grouped together for regression analyses. Fisher's exact test was used to detect the distribution difference between diabetic and nondiabetic groups. Logistic regression model was further performed to adjust demographic difference. Student's t-test was applied to compare the difference of various parameters between different genotypes or between normal control and diabetic subjects. MANOVA was applied to compare the difference of glucose and insulin levels during OGTT test between different genotypes. SAS program version 8.1 (SAS institute Inc., Cary, NC) was applied for statistical analyses. A value of $P < 0.05$ was considered statistically significant.

3. Results

3.1. Demographic and Metabolic Characteristic. The demographic and metabolic data of the study subjects are shown in Table 1. Obesity, dyslipidemia, higher fasting plasma insulin,

Table 1: Clinical and metabolic features between normal controls and type 2 diabetic subjects in the present study.

	Non-DM ($n = 69$)	DM ($n = 38$)	P value*
Age (years)	17.3 ± 3.8	18.6 ± 6.6	0.289
BMI percentiles	56.7 ± 29.0	83.8 ± 15.4	<0.001
Glucose (mmol/L)	5.19 ± 0.40	10.65 ± 4.05	<0.001
TCH (mmol/L)	4.05 ± 0.77	4.22 ± 1.02	0.4159
TG (mmol/L)	0.74 ± 0.26	1.17 ± 0.45	0.0005
HDL (mmol/L)	1.43 ± 0.39	1.20 ± 0.50	0.0320
Fasting insulin (pmol/L)	7.66 ± 4.47	17.99 ± 20.43	0.0043
HOMA-IR	1.78 ± 1.06	7.48 ± 8.09	<0.001
Log (HOMA-IR)	0.402 ± 0.624	1.449 ± 1.143	<0.001
HOMA-B	94.62 ± 58.17	94.13 ± 168.52	0.987
Log (HOMA-B)	4.372 ± 0.625	3.490 ± 1.458	0.001
Sex (M : F)	26 : 43	20 : 18	0.157$

*P value with Student's t-test.
$By chi-squared test.
BMI percentiles: body mass index age- and sex-specific percentiles; TCH: total cholesterol; TG: triglyceride; HDL: high density lipoprotein-cholesterol; HOMA-IR: homeostasis model assessment-insulin resistance; HOMA-B: homeostasis model assessment-β cell.

Table 2: Genotypic distribution of E23K polymorphism of the Kir6.2 between normal control and type 2 diabetes subjects.

	Non-DM ($n = 69$)	DM ($n = 38$)	P value*	Odds ratio	95% confidence interval
Genotype*					
EE, n (%)	24 (34.8%)	6 (15.8%)			
EK/KK, n (%)	45 (65.2%)	32 (84.2%)	0.044	2.84	1.04–7.75
Allele					
E-allele, n (%)	81 (58.7%)	38 (50.0%)			
K-allele, n (%)	57 (41.3%)	38 (50.0%)	0.251	1.42	0.81–2.50

*Chi-squared test.

higher insulin resistance, and worsened β-cell function were found in subjects with T2D in childhood and adolescence (Table 1).

3.2. Genotypes of KCNJ11 Genes. As shown in Table 2, K-allele-containing genotypes were significantly higher in subjects with T2D as compared to those of control (84.2% versus 65.2%, $P = 0.044$) (Table 2). To further adjust for potential confounding variables, logistic regression analysis was performed (Table 3). After adjustment of age, sex, and BMI age- and sex-specific percentiles (model 1), we found that higher BMI percentiles is an independent risk factor of type 2 diabetes (odds ratio = 1.060, 95% CI: 1.027–1.094, and $P < 0.001$). If we adjust for age, sex, BMI age- and sex-specific percentiles, and fasting plasma insulin levels (model 2), the K-allele-containing genotype is an independent risk factor of type 2 diabetes (odds ratio = 4.105, 95% CI: 1.0008–16.831, and $P = 0.047$). The fasting plasma insulin levels and BMI age- and sex-specific percentiles are also independent risk factors for T2D (odds ratio = 1.066, 95% CI: 1.001–1.135, and $P = 0.045$ for fasting insulin; odds ratio = 1.047, 95% CI: 1.014–1.080 for BMI age- and sex-specific percentiles, and $P = 0.004$, resp.).

3.3. Effect of E23K Polymorphism of the KCNJ11 in Normal Glucose-Tolerant Subjects. To study the effect of genetic polymorphism of the E23K on insulin and glucose homeostasis during oral glucose tolerance test, we firstly compared those with EE genotype and the K23-allele-containing genotypes (EK or KK) in the normal glucose-tolerant subjects. There was no difference in the glucose levels during OGTT between subjects with different genotypes (Figure 1(a)). In contrast, subjects with EK/KK genotypes did have a significantly higher level of plasma insulin level at 60 min after glucose loading and higher AUC of insulin during OGTT (Figure 1(b)). However, this association of genotype of *KCNJ11* with plasma insulin levels at 60 min after glucose loading became insignificant after adjustment of age, sex, and BMI percentiles.

3.4. Effect of E23K Polymorphism of the KCNJ11 on Clinical Phenotypes in Diabetic and Nondiabetic Subjects. To further search for the features of E23K variants on development of type 2 diabetes in children and adolescents, we then compared various metabolic parameters between those with EE genotype and the K23-allele-containing genotypes (EK or KK) in both diabetic and nondiabetic subjects (Table 4). In general, the fasting insulin levels were higher in diabetes group. However, the diabetic subjects with the K23-allele-containing genotypes had a borderline significantly lower level of fasting plasma insulin than the diabetic subjects without K23 allele. With adjustment for age and BMI age- and sex-specific percentiles, the diabetic subjects with K-allele-containing genotypes had significantly lower fasting plasma

Table 3: Logistic regression analysis with type 2 diabetic status as dependent variables, age, sex, BMI age- and sex-specific percentiles, and genotype of E23K polymorphism in Kir6.2 as independent variables.

Independent variables	Odds ratio	95% CI	*P* value
Model 1			
EK/KK versus EE	2.941	0.764–11.323	0.117
Age (every 1 year increment)	1.156	0.920–1.454	0.214
Sex (male = 1, female = 2)	0.672	0.224–2.019	0.479
BMI age- and sex-specific percentiles (every 1 percentile increment)	1.060	1.027–1.094	<**0.001**
Model 2			
EK/KK versus EE	4.105	1.0008–16.831	**0.047**
Age (every 1 year increment)	1.240	0.960–1.601	0.099
Sex (male = 1, female = 2)	0.826	0.252–2.711	0.752
BMI age- and sex-specific percentiles (every 1 percentile increment)	1.047	1.014–1.080	**0.004**
Fasting plasma insulin levels (every 1 pmol/L increment)	1.066	1.001–1.135	**0.045**

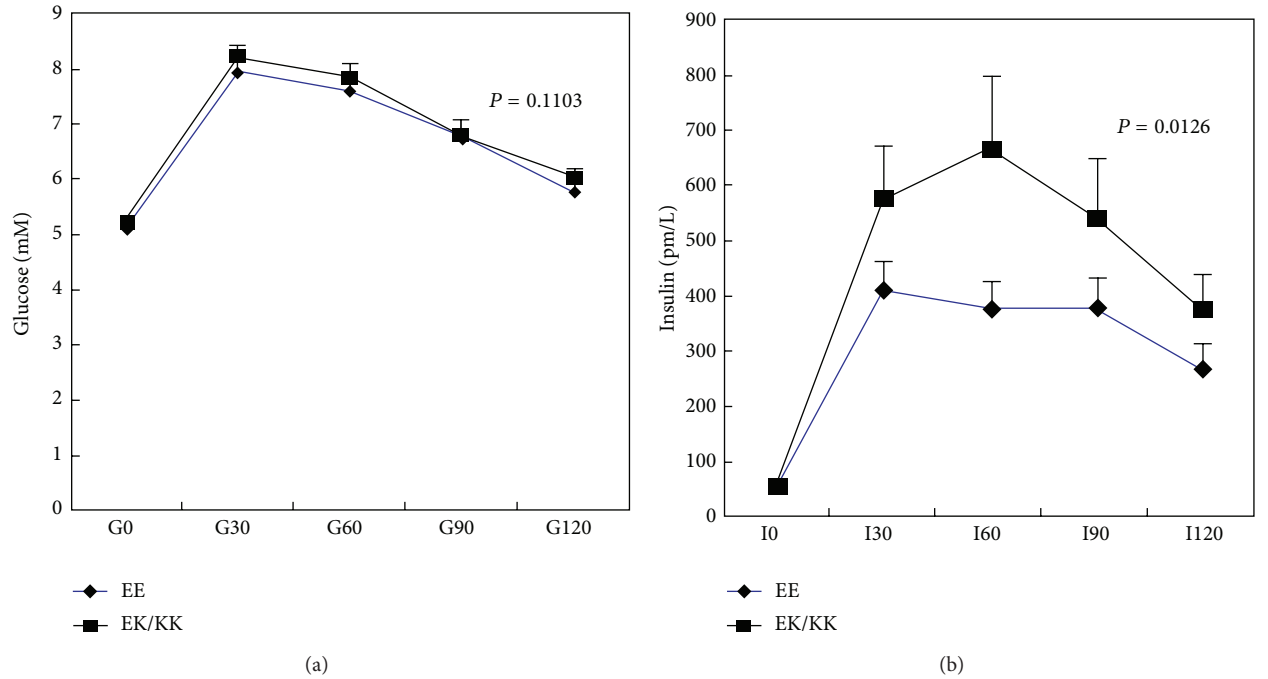

Figure 1: Glucose and insulin homeostasis during OGTT in the normal glucose-tolerant subjects according to genotypes of the *KCNJ11* gene. There was no significant difference in plasma glucose levels in subjects with EE genotype compared with those with K23-allele-containing genotypes (EK or KK) (a). Subjects with EK/KK genotypes tended to have a higher level of plasma insulin level during OGTT (b). ∗ indicates significant difference between the two groups.

insulin levels (fasting plasma insulin as dependent variable: β for age: -2.958 ± 1.385, $P = 0.043$; β for BMI age- and sex-specific percentiles: 0.548 ± 0.238, $P = 0.030$; β for E/E or E/K + K/K genotype: -21.451 ± 10.312, $P = 0.046$). In contrast, there were no significant differences of these metabolic parameters between the K23-allele-containing and the EE genotype subjects in the nondiabetic group.

4. Discussion

In our present study, we found that a common polymorphism of E23K of the *KCNJ11* confers higher susceptibility to T2D in childhood and adolescence of the Han-Chinese in Taiwan.

Childhood obesity is the single most important risk factor for type 2 diabetes in our schoolchildren [10, 11] and the present study. In the present study, we found that even with adjustment for age, sex, BMI age- and sex-specific percentiles, and fasting plasma insulin level, the K-allele-containing genotypes (EK and KK) confer an independent risk, with a relative high odds ratio of 4.105, for T2D in youth. Until recently, the Pro12Ala polymorphism in the peroxisome proliferator-activated receptor gamma (*PPARG*) was one of the other few polymorphisms that demonstrated an alteration in type 2 diabetes susceptibility across different populations [36]. More recently, several genome-wide association studies (GWAS) independently confirmed the strong

Table 4: Clinical and metabolic features between those with E/E genotype and K-containing allele among type 2 diabetic and nondiabetic subjects, respectively.

	Type 2 diabetic subjects			Nondiabetic subjects		
Genotypes	E/E ($n = 6$)	E/K + K/K ($n = 32$)	P value*	E/E ($n = 24$)	E/K + K/K ($n = 45$)	P value*
Sex (M : F)	4 : 2	16 : 16	0.663$	11 : 13	15 : 30	0.434$
Age (years)	21.5 ± 9.7	18.0 ± 5.8	0.242	17.2 ± 3.3	17.4 ± 4.1	0.861
BMI age- sex-specific percentiles	84.02 ± 22.07	83.73 ± 14.31	0.970	56.65 ± 25.63	56.79 ± 30.87	0.987
Glucose (mmol/L)	10.0 ± 3.8	10.8 ± 4.1	0.715	5.1 ± 0.4	5.2 ± 0.4	0.133
Fasting plasma insulin levels (pmol/L)	237.21 ± 230.48	112.18 ± 126.24	0.076	52.2 ± 31.1	56.5 ± 32.8	0.602
Ins-30′	—	—		414.67 ± 53.23	582.09 ± 94.71	0.222
Ins-60′	—	—		381.81 ± 51.51	671.57 ± 132.39	**0.046**
Ins-90′	—	—		379.09 ± 55.41	545.79 ± 107.51	0.280
Ins-120′	—	—		268.32 ± 48.95	375.18 ± 67.10	0.283
AUC-glucose	—	—		832.92 ± 21.83	855.72 ± 17.02	0.422
AUC-insulin				40074.46 ± 4153.74	60457.87 ± 10721.20	0.180
HOMA-B	127.0 ± 194.8	89.89 ± 168	0.685	97.69 ± 70.03	92.98 ± 51.55	0.751

* P value with Student's t-test.
$ P value with Fisher's exact test.

associations of SNP rs7903146 in the *TCF7L2* locus with type 2 diabetes [37–40]. Evidence accumulated so far suggests that the E23K polymorphism of the *KCNJ11* gene, which encodes the Kir6.2 subunit of the K_{ATP} channel, is a candidate gene for type 2 diabetes reported mostly from adults [24, 41]. In children, one recent study indicated that six single nucleotide polymorphisms, including an activating R201H mutation on *KCNJ11* gene, contribute to permanent neonatal diabetes [42]. Besides, several mutations on *KCNJ11* gene have been reported to cause permanent hyperinsulinemic hypoglycemia of infancy [43–45]. A recent study reported that E23K variant did not affect metabolic disorders in prepubertal children who is small for gestational age at birth [46]. On the other hand, the association of the E23K polymorphism with type 1 diabetes was not statistically significant in the evaluated Korean population [47]. To our knowledge, no study has been reported for the impact of E23K polymorphism of the *KCNJ11* gene on the early onset type 2 diabetes in children/adolescents. In consistence with previous studies in adult populations, we showed that school-aged children/adolescents with T2D in this study had higher E23K carrier rate of *KCNJ11* gene than normal subjects. According to Genetic Power Calculator (S. Purcell et al., 2003; http://pngu.mgh.harvard.edu/~purcell/gpc/), the estimated number of diabetic cases for 80% power will be 181 with P value less than 0.05. Though only 38 diabetic subjects were recruited in our study group, the E23K polymorphism still contributed to a significantly increased risk for type 2 diabetes independent of age, gender, BMI age- and sex-specific percentiles, and fasting plasma insulin level. Furthermore, from the meta-analysis of candidate-gene studies and GWAS for T2D in adults, the average odds ratio of each genetic variant to increased T2D risk is in the range from 1.10 to 1.37 [48]. According to a recent systemic meta-analysis, the E23K polymorphism was significantly associated with increased T2D risk with per-allele odds ratio (OR) of 1.12. However, in this study, the odds ratio of K-allele of *KCNJ11* to increased T2D risk in childhood and adolescence reached 4.105 after adjusting age, gender, BMI age- and sex-specific percentiles, and fasting plasma insulin levels. It inferred that the E23K polymorphism of *KCNJ11* contributed a much higher risk to T2D in children and adolescence than in adults.

How E23K variation leads to diabetes is not completely understood. In previous studies, it has been shown that insulin secretion is significantly reduced in both heterozygous (E/K) and homozygous (K/K) variants among the normal glucose-tolerant adults [49, 50]. In contrast, we found that those carrying K-allele exhibited a higher insulin response after oral glucose loading in the normal glucose-tolerant children (Figure 1(b)). In support of our findings, studies in the glucose-tolerant offspring of T2D patients carrying the E23K variants demonstrated significantly higher 2-hour insulin concentrations compared with those with control subjects [51]. Moreover, the E23K variant has been linked to an increase in BMI in the Danish population [50]. Taken together, these data including ours suggest that the higher response in insulin secretion to oral glucose loading might be due to the compensatory hypersecretion of insulin to maintain normal glucose homeostasis in the presence of insulin resistance. In spite of the small case number in this study, we found that there is a decline in fasting plasma insulin levels in diabetes subjects carrying K-alleles compared to those with homozygous EE genotype when adjusted for age, sex, and BMI age- and sex-specific percentiles. Whether the reduced fasting insulin levels observed in the diabetic children/adolescents with K23-allele-containing genotypes are due to inadequate compensation of β-cell failure is not known. Future longitudinal study will be required to establish the effect of E23K polymorphism in the *KCNJ11* gene on changes of body build, insulin resistance, and β-cell dysfunction during disease progression.

5. Conclusions

In conclusion, a common E23K variant of the *KCNJ11* gene conferred higher susceptibility to T2D in children/adolescents in Taiwan. Furthermore, in the normal glucose-tolerant children and adolescents, K23 allele carriers had a significantly higher insulin response to oral glucose loading, suggesting a compensatory insulin secretion in the presence of insulin resistance. However, the functional impact of the E23K polymorphism on progression of glucose intolerance and diabetes needs further investigation.

Conflict of Interests

The authors report no conflict of interests.

Authors' Contribution

Yi-Der Jiang, Lee-Ming Chuang, Tien-Jyun Chang, Jun-Nan Wei, and Fung-Chang Sung participated in concept/design. Lee-Ming Chuang, Dee Pei, and Yann-Jinn Lee participated in the collection of clinical and laboratory data. Yi-Der Jiang, Lee-Ming Chuang, and Tien-Jyun Chang participated in data analysis/interpretation and drafting of the paper. Tien-Jyun Chang participated in critical revision of the paper and approval of the paper. Yi-Der Jiang and Lee-Ming Chuang contributed equally to this work.

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Research Article

Augmented β-Cell Function and Mass in Glucocorticoid-Treated Rodents Are Associated with Increased Islet Ir-β/AKT/mTOR and Decreased AMPK/ACC and AS160 Signaling

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Glucocorticoid (GC) therapies may adversely cause insulin resistance (IR) that lead to a compensatory hyperinsulinemia due to insulin hypersecretion. The increased β-cell function is associated with increased insulin signaling that has the protein kinase B (AKT) substrate with 160 kDa (AS160) as an important downstream AKT effector. In muscle, both insulin and AMP-activated protein kinase (AMPK) signaling phosphorylate and inactivate AS160, which favors the glucose transporter (GLUT)-4 translocation to plasma membrane. Whether AS160 phosphorylation is modulated in islets from GC-treated subjects is unknown. For this, two animal models, Swiss mice and Wistar rats, were treated with dexamethasone (DEX) (1 mg/kg body weight) for 5 consecutive days. DEX treatment induced IR, hyperinsulinemia, and dyslipidemia in both species, but glucose intolerance and hyperglycemia only in rats. DEX treatment caused increased insulin secretion in response to glucose and augmented β-cell mass in both species that were associated with increased islet content and increased phosphorylation of the AS160 protein. Protein AKT phosphorylation, but not AMPK phosphorylation, was found significantly enhanced in islets from DEX-treated animals. We conclude that the augmented β-cell function developed in response to the GC-induced IR involves inhibition of the islet AS160 protein activity.

1. Introduction

Glucocorticoids (GCs), such as dexamethasone (DEX), are widely prescribed in clinical practice due to their anti-inflammatory, antiallergic, and immunosuppressive properties. GCs are the standard treatment for asthma, rheumatoid arthritis, systemic lupus erythematosus, and inflammatory bowel diseases [1, 2], as well as the protection against rejection of transplanted organ [1]. However, supraphysiological levels of GCs (either exogenous or endogenous) induce adverse effects related to glucose homeostasis, such as decreased peripheral insulin sensitivity, glucose intolerance, and dyslipidemia [3–6]. Depending on the genetic background, age, and time and dose of the exposure, it can also lead to type 2 diabetes mellitus (T2DM) [4, 6–11].

T2DM is a multifactorial metabolic disease mainly characterized by hyperglycemia [12], but before the occurrence of overt hyperglycemia, peripheral insulin resistance (IR) leads to compensatory insulin hypersecretion by pancreatic islets [13]. This adaptive islet compensation leads to a state of

hyperinsulinemia together with normoglycemia or a modest increase in glycemic values (prediabetic state) that persist until the β-cells can handle the required demand for insulin [14]. The major mechanisms by which β-cells generate hyperinsulinemia during adaptive compensation consist of functional (e.g., increased insulin biosynthesis and/or secretion) and structural adaptations (e.g., increased β-cell hyperplasia and hypertrophy that may result in increased β-cell mass) [14–16]. Thus, when β-cells can no longer compensate, a glucolipotoxicity process progressively develops that induces β-cell death accompanied by hypoinsulinemia, hyperglycemia, and hyperlipidemia [15].

The β-cell compensations [17] can be rapidly obtained experimentally by 5-day treatment with DEX (5 days) [18, 19] that induces peripheral IR [20, 21], which is associated with increased hepatic gluconeogenesis and lipolysis [8].

Rats and mice are widely used as laboratory models to elucidate the mechanisms (at the functional, structural, and molecular levels) involved in the pancreatic islets compensations during the development of T2DM, such as observed in GC-induced IR [9, 10, 18–27]. These compensations are strongly associated with increased insulin signaling in islets (insulin receptor [Ir] and the protein kinase B [AKT]) [9, 19, 25, 28]. The AKT1 overexpression in β-cells lead to increased islet mass [29], while the β-cell-specific Ir knockout (βIrKO) lead to reduced glucose-stimulated insulin secretion (GSIS), lower islet insulin content, and glucose intolerance, supporting the consensus that the loss of insulin action on β-cells leads to β-cell failure and T2DM [30].

It has been demonstrated that a major downstream effector of AKT, the AKT substrate with 160 kDa (AS160), previously recognized as an important protein in insulin signaling in skeletal muscles and adipose tissue, is found to be expressed in β-cells and is also a major effector of AKT in the β-cell [31, 32].

In skeletal muscles, both insulin and AMP-activated protein kinase (AMPK) pathways phosphorylate and inhibit AS160 [32], inducing the trafficking of vesicles containing glucose transporter-4 (GLUT4) to the plasma membrane [33, 34]. In β-cells, AS160 plays an important role in the GSIS [31] and evidences suggest that AS160 is also involved in the trafficking of vesicles containing insulin to the plasma membrane [35]. AS160 is expressed in human islets and its phosphorylation is increased when stimulated with glucose (16.7 mmol/L) [31]. Also, T2DM humans display reduced AS160 mRNA and the AS160 knockdown in primary mouse islets lead to increased basal insulin secretion (2.8 mmol/L glucose), whereas GSIS (16.7 mmol/L glucose) is impaired [31].

Considering the importance that has been given to the involvement of the AS160 in islet function, we sought to investigate whether the AS160 content and phosphorylation could be modulated by the GC treatment. By using two experimental models, Swiss mice and Wistar rats, we demonstrated that the augmented β-cell function (insulin hypersecretion and increased β-cell proliferation) caused by 5-day DEX administration is associated with significant reduction of the AS160 protein content and marked increase of the AS160 protein phosphorylation in islets of both insulin-resistant rodents. Furthermore, this AS160 inhibition is islets from GC-treated animals which were accompanied by high AKT, but not AMPK phosphorylation.

2. Materials and Methods

2.1. Reagents. Dexamethasone phosphate (Decadron) was purchased from Aché (Campinas, SP, Brazil). Human recombinant insulin (Humalin R) was obtained from Lilly (Indianapolis, IN, USA). Trizol was purchased from Gibco-BRL (Gaithersburg, MD, USA) and Triton X-100 was purchased from Cromato Products (Diadema, SP, BR). The ^{125}I-labeled insulin used in the radioimmunoassay (RIA) was purchased from Perkin Elmer (Boston, MA, USA). SDS-PAGE and immunoblotting were performed using Bio-Rad systems (Hercules, CA, USA), and all chemicals were from Bio-Rad and Sigma Aldrich (St. Louis, MO, USA). Western blot detection of specific proteins used the following primary antibodies from Santa Cruz (Santa Cruz, CA, USA): anti-phospho-Ir-$\beta^{\text{Tyr1162/1163}}$, anti-Ir-$\beta$, anti-phospho-$\text{AKT}_{1/2/3}{}^{\text{Thr308}}$, anti-$\text{AKT}_{1/2/3}$, anti-phospho-$\text{ERK}^{\text{Tyr204}}$, anti-$\text{ERK}_1$, anti-PKC, and anti-GAPDH. Anti-phospho-$\text{AMPK}^{\text{Thr172}}$, anti-AMPK, anti-phospho-$\text{ACC}^{\text{Ser79}}$, anti-ACC, anti-phospho-$\text{AS160}^{\text{Thr642}}$, anti-phospho-PKC substrates, and anti-PCNA were from cell signaling (Temecula, CA, USA). Anti-phospho-$\text{mTOR}^{\text{Ser2448}}$ and anti-mTOR were from Abcam (Cambridge, UK), and anti-CX36 was from Invitrogen (Camarillo, CA, USA). The secondary antibodies used were anti-rabbit IgG and anti-mouse IgG from cell signaling. Urea antiprotease/antiphosphatase buffer was composed of 7 M urea, 2 M thiourea, 5 mmol/L EDTA, 1 mmol/L sodium fluoride, 1 mmol/L sodium orthovanadate, 1 mmol/L sodium pyrophosphate, 2 mM PMSF, 1% Triton X-100, and 1 μg/mL aprotinin (Trasylol from Bayer Health Care Pharmaceuticals, Berkley, CA). Immunohistochemical detection of insulin was performed using an anti-insulin primary antibody (guinea pig polyclonal) (Dako, Carpinteria, CA, USA) and detection of KI-67 was performed using an anti-KI-67 antibody (Spring Bioscience, Pleasanton, CA, USA). The secondary antibodies used to detect the anti-insulin and anti-KI-67 antibodies were anti-guinea pig IgG (Invitrogen, Carlsbad, CA, USA) and HRP-conjugated anti-rabbit IgG (Nichirei Bioscience, Tokyo, JP), respectively.

2.2. Animals and Experimental Design. Experiments were performed on groups of male Swiss mice and male Wistar rats (80 to 100 days) obtained from the State University of Campinas Animal Breeding Center. They were maintained in appropriate animal cages and kept at 24°C on a 12 : 12-hour light-dark cycle. Both mice and rats had access to food and water ad libitum. The experiments with animals were approved by the Institutional State University of Campinas Committee for Ethics in Animal Experimentation under protocol number 2285-1. Mice and rats were divided into the following two groups: DEX-treated rodents (DEX) that received a daily injection of dexamethasone phosphate (intraperitoneally (i.p.), 1.0 mg/kg body weight (b.w.) in 0.9%

NaCl for 5 consecutive days) and control rodents (CTL) that received a daily injection of saline (i.p., 1.0 mL/kg b.w., for 5 consecutive days) between 08:00 and 09:00. All experiments were performed 24 h after the last DEX injection (at the sixth day) to avoid the overlapping of acute and chronic effects of GCs.

2.3. Metabolic, Hormonal, and Biochemical Measurements. Body weight was measured 2 days before the start of treatment and each day thereafter until the day of euthanasia. On the day after the last DEX administration, blood was collected from the tails of a group of fasted (10–12 h) animals and blood glucose levels were measured with a glucometer (Accu-Chek Advantage, Roche Diagnostic, Switzerland). Immediately after blood glucose determination, the animals were sacrificed (by exposure to CO_2 followed by decapitation) and the trunk blood was collected. The serum was obtained by centrifugation and was used to measure the following parameters: insulin by RIA, nonesterified-free-fatty-acids (NEFA) (Wako Chemicals; Richmont, USA), triacylglycerol (TG), and total cholesterol (CHOL) (Roche/Hitachi; Indianapolis, IN, USA) by spectrophotometer according to the manufacturers' instructions.

2.4. Intraperitoneal Insulin Tolerance Test (ipITT). A separate group of fed animals received an intraperitoneal injection of insulin (1 U/kg b.w. in 0.9% NaCl). Blood glucose was measured at baseline (before insulin administration; 0 min) and at 5, 10, 15, 30, 45, and 60 min after insulin administration. Blood glucose measurements were then converted into the natural logarithm (Ln); the slope was calculated using linear regression (time × Ln[glucose]) and multiplied by 100 to obtain the constant rate of glucose decay per minute (%/minute) during the ipITT (KITT) [12].

2.5. Intraperitoneal Pyruvate Tolerance Test (ipPTT). A separate group of fasted (14 h) animals received an i.p. injection of pyruvate (1 g/kg b.w.). Blood glucose was measured at baseline (before pyruvate administration; 0 min) and at 5, 15, 30, and 60 min after pyruvate administration. The area-under-curve (A.U.C.) for blood glucose values was obtained from the 30 min of the ipPTT after normalization of the data [10]. The constant rate of glucose appearance per minute (%/min) during the first 15 min of the ipPTT (KPTT) was calculated as described above.

2.6. Intraperitoneal Glucose Tolerance Test (ipGTT). A separate group of fasted (10 h) animals received an i.p. injection of 25% glucose solution (1 g/kg b.w.). Blood glucose was measured at baseline (before glucose administration; 0 min) and at 15, 30, 60, 90, and 120 min after glucose administration. The A.U.C. was calculated as described above.

2.7. Islet Isolation and Static Insulin Secretion. Islets were isolated by collagenase digestion of the pancreas as described [27]. Insulin secretion and quantification by RIA were performed using a similar method as described previously [10].

2.8. Immunohistochemistry and Morphometry in the Endocrine Pancreas. For morphometric analysis, at least 6 pancreases from each group of mice and rats were removed, weighed, and fixed for 24 hours in 4% paraformaldehyde solution, as previously described [25]. For morphometry analysis, all islets present in the sections were obtained systematically by capturing images with a digital camera (Olympus DP52, Tokyo, JP) coupled to a microscope (Olympus BX51TF, Tokyo, JP). The islet, β-cell and section areas were analyzed using the free software ImageJ (http://rsbweb.nih.gov/ij/download.html). The relative β-cell area was calculated by dividing the β-cell area per section by the total pancreas area per section and the absolute β-cell mass was calculated by multiplying the pancreas weight by the relative β-cell area per pancreas. The relative number of islets was obtained by dividing the number of islets per section by the total area of the section [25].

2.9. β-Cell Proliferation. Average β-cell proliferation was obtained by counting the total islet cell nuclei stained for insulin and KI-67 using the same software cited above. β-cell proliferation was estimated by dividing the number of KI-67-positive nuclei by the total number of insulin-positive cells [25].

2.10. β-Cell Death. Fragmented DNA was isolated using buffer *A* containing 50 nM Tris-Hcl pH 8.1, 10 nM EDTA, and 1% Triton X100. Total DNA was isolated using buffer *B* containing 50 nM Tris-Hcl pH 8.1, 10 nM EDTA, and 1% SDS. After isolation, total DNA and fragmented DNA were precipitated with phenol, chloroform and isoamyl alcohol (25 : 24 : 1) and quantified by SybrGreen fluorescence using a standard curve (0.5 ng/mL to 50 ng/mL). The data are expressed as the ratio of fragmented to total DNA.

2.11. Protein Extraction and Immunoblotting. Protein extraction and immunoblotting were performed as previously reported [36] with minor modifications. Images were captured by the luminescent image analyzer LAS-3000 (Fujifilm, Tokyo, JP) and the specific band intensity was quantified by optical densitometry using ImageJ.

2.12. RNA Isolation and Quantitative RT-PCR Analysis. Groups of 600 islets were homogenized in Trizol following phenol chloroform RNA extraction, as previously described [36]. Relative quantities of target transcripts were calculated from duplicate samples after normalization of the data against the endogenous control, GAPDH. The primers used were as follows: PDX1 (S: aaccggaggagaataagagg and AS: gttgtcccgctactactgtt), insulin (S: ttgcagtagttctccagtt and AS: attgttccaacatggccctgt), and GAPDH (S: cctgcaccaccaactgctta and AS: gccccacggccatcacgcca).

2.13. Statistical Analysis. The results are expressed as the mean ± s.e.m. of the indicated number (n) of animals. A paired or unpaired Student's t-test was used for intragroup (before and after) or intergroup (CTL versus DEX group) comparisons. All analyses were performed using GraphPad

Table 1: Body and adrenal weight in DEX-treated mice and rats.

	Mice			Rats		
	Body weight (g)		Adrenal weight (mg/100 g b.w.)	Body weight (g)		Adrenal weight (mg/100 g b.w.)
	Before	After	After	Before	After	After
CTL	38.7 ± 0.6	40.2 ± 0.6	18.21 ± 1.74	340.1 ± 6.5	351.2 ± 11.5	17.5 ± 1.12
DEX	39.4 ± 0.8	37.9 ± 0.7†	12.68 ± 0.72*	346.7 ± 11.2	309.6 ± 10.8†	11.38 ± 0.78*

†Significantly different using unpaired t-test versus DEX before treatment; *significantly different using unpaired t-test versus CTL after treatment $P < 0.05$; $n = 6$–8; values are mean ± s.e.m.

Table 2: Metabolic variables in 12 hour fasting DEX-treated mice and rats.

	Mice		Rats	
	CTL	DEX	CTL	DEX
Cholesterol (mg/dL)	169.7 ± 14.2	302.0 ± 18.3*	23.8 ± 1.8	30.6 ± 1.7*
Triacylglycerol (mg/dL)	106.9 ± 12.4	201.0 ± 13.8*	111.2 ± 14.2	210.1 ± 26.6*
NEFA (mMol/L)	1.2 ± 0.09	1.1 ± 0.08	0.6 ± 0.04	0.9 ± 0.09*
Glycemia (mMol/L)	5.5 ± 0.3	5.0 ± 0.4	5.5 ± 0.08	8.8 ± 0.8*
Insulinemia (pMol/L)	31.9 ± 3.09	59.9 ± 9.6*	268.6 ± 64.3	2697.4 ± 439.0*

*Significantly different using unpaired t-test versus CTL $P < 0.05$; $n = 8$–10; values are mean ± s.e.m.

Prism version 5.0 (GraphPad Software, San Diego, CA, USA). A P value less than or equal to 0.05 was considered significant.

3. Results

3.1. DEX Treatment Reduced Body and Adrenal Gland Weights in Mice and Rats. It is known that 5-day DEX treatment in rats produces a dose-dependent reduction in adrenal gland mass in a reciprocal reduction of endogenous corticosterone concentration [10]. As expected, DEX treatment induced a significant decrease in the mass of the adrenal glands in mice (30%) and rats (35%) compared with their respective controls (Table 1), which demonstrates the effectiveness of exogenous GC treatment on adrenal hypotrophy. In addition, the mice and rats showed reduced body weight (4% and 11%, resp.) (Table 1), which is a feature commonly observed in rats made insulin-resistant by DEX treatment [10, 37].

3.2. DEX Treatment Induced a Reduction in Insulin Sensitivity in Mice and in Rats but Increased Hepatic Gluconeogenesis and Glucose Intolerance Only in Rats. We first confirmed the reduction in insulin sensitivity in both mice and rats. The ipITT revealed a significant reduction in insulin sensitivity in both DEX-treated groups (Figures 1(a) and 1(f), resp.) as indicated by the reduction in the KITT (inset in Figures 1(a) and 1(f)), although this effect occurred to a lesser extent in mice. We also analyzed whether GC treatment increased hepatic gluconeogenesis. DEX treatment did not alter gluconeogenesis in mice; however, DEX-treated rats showed increased glucose production in response to pyruvate administration, as indicated by the increased A.U.C. and the KPTT (Figures 1(b)–1(d) and 1(g)–1(i), resp.), which indicates hepatic insulin resistance. Despite a reduction in insulin sensitivity, DEX-treated mice remain glucose tolerant (Figure 1(e)). Compared to their controls, DEX-treated rats showed the well-known negative impact of GC excess on glucose tolerance (Figure 1(j)), which reflects the association of increased hepatic glucose production with a possible reduction of peripheral glucose disposal.

3.3. DEX Treatment Induced Dyslipidemia and Hyperinsulinemia in Both Mice and Rats. DEX treatment increased fasting serum cholesterol (CHOL) and triacylglycerol (TG) concentrations in both mice and rats compared to their respective controls, which indicates a negative impact of GCs on lipid metabolism in both species. In addition, DEX treatment increased the NEFA levels only in rats (Table 2), which may indicate an increased rate of lipolysis of adipose tissue. DEX-treated rodents also showed a marked increase in serum insulin levels that were 1- and 9-fold higher in mice and rats, respectively. Blood glucose was not altered in DEX-treated mice, while it was 60% higher in DEX rats compared to CTL (Table 2). Thus, the hyperinsulinemia corroborates the IR state and seems to protect against the disruption of glucose homeostasis, though GC-treated rats were glucose intolerant.

3.4. DEX Treatment Increased the Responsiveness to Glucose in Islets from Both Mice and Rats. Due to the increased insulinemia that was observed in both DEX-treated mice and rats, we assessed the GSIS. Compared to the control groups, isolated islets from DEX-treated rats were more responsive to all glucose concentrations used (from 2.8 mmol/L to 22.2 mmol/L), (Figures 2(c) and 2(d)), whereas islets from DEX-treated mice were more responsive to glucose up to a concentration of 11.1 mmol/L (Figures 2(a) and 2(b)). These data point to species differences in the increase of β-cell function that contributes to the different degrees of hyperinsulinemia found in each species.

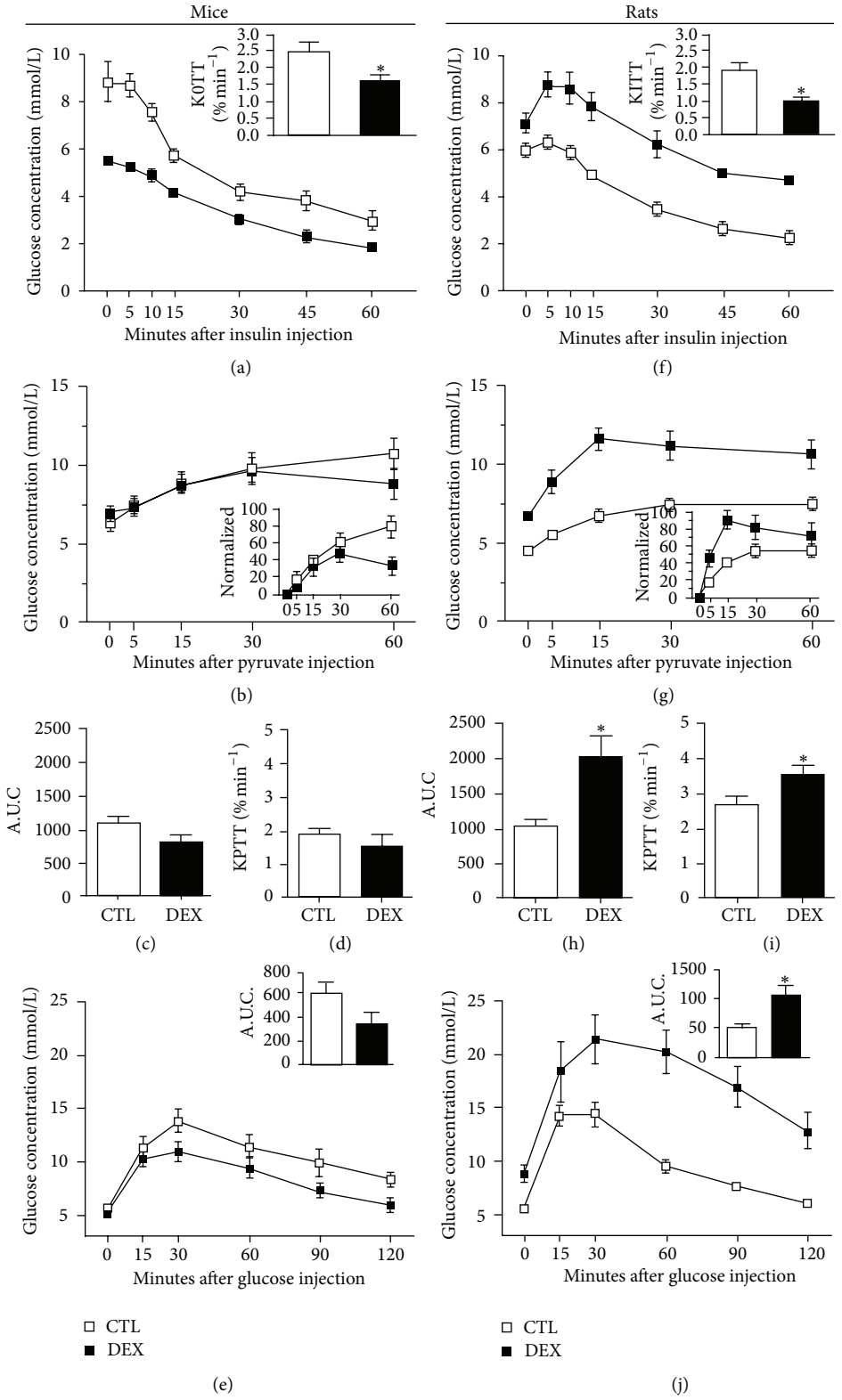

Figure 1: DEX treatment induces a reduction in insulin sensitivity in mice and in rats but increases hepatic gluconeogenesis and glucose intolerance only in rats. ((a), (f)) Blood glucose during intraperitoneal insulin tolerance test (ipITT; 1 U/Kg b.w.) in DEX-treated mice and rats, respectively; the inset in (a) and (f) depicts the constant rate of glucose disappearance (KITT). ((b), (g)) Intraperitoneal pyruvate tolerance test (ipPTT; 1 g/Kg b.w.) in DEX-treated mice and rats, respectively; the inset in (b) and (g) depicts the ipPTT data normalized by minute 0 ((c), (h)) A.U.C and ((d), (i)) the constant rate of glucose appearance (KPTT) during ipPTT in DEX-treated mice and rats, respectively. ((e), (j)) intraperitoneal glucose tolerance test (ipGTT; 1 g/Kg b.w.) in DEX-treated mice and rats, respectively; the inset in (e) and (j) depicts the A.U.C. from ipGTT; values are mean ± S.E.M.; n = 8–10 animals per group. *Significantly different compared to CTL. Unpaired Student's t-test, $P \leq 0.05$.

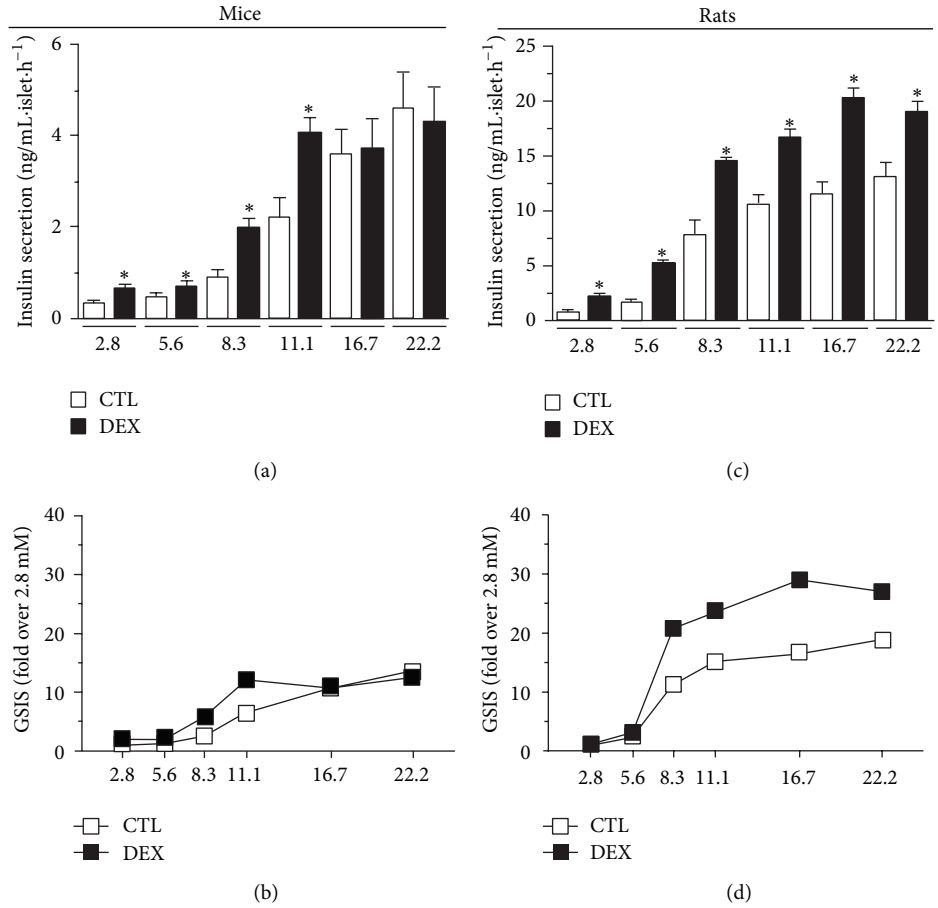

Figure 2: Islets from DEX-treated rats are more responsive to glucose than islets from DEX-treated mice. ((a), (c)) Static cumulative insulin secretion in isolated islets from DEX-treated mice and rats in response to different glucose concentrations, respectively. ((b), (d)) Normalized glucose-stimulated insulin secretion (GSIS) (fold increase in relation to 2.8 mmol/L glucose) in mice and in rats, respectively. Values are mean ± S.E.M.; $n = 4$–6 wells from 5 different animals. *Significantly different compared to CTL. Unpaired Student's t-test, $P \leq 0.05$.

3.5. DEX Treatment Leads to Increased β-Cell Mass in the Pancreas of Mice and Rats. Because an increase in β-cell mass may also favor compensatory hyperinsulinemia, we investigated this parameter in DEX-treated mice and rats. Pancreatic sections stained for insulin revealed a significant increase in the number of islets per pancreatic area in both DEX-treated rats and mice compared to their respective controls (Figures 3(a) and 3(c)). Additionally, DEX treatment significantly increased the absolute β-cell mass in the pancreas from both mice and rats (Figure 3(e)). These data indicate a compensatory structural islet adaptation in response to DEX-induced IR in both species.

3.6. DEX Treatment Increases β-Cell Proliferation without Affecting Apoptosis in Mouse and in Rat Islet Cells. The β-cell mass is the result of a dynamic balance between cell death and proliferation. We found that DEX treatment significantly increased β-cell proliferation in islets from rats (420%) and mice (200%) compared with their respective controls, as indicated by the higher number of KI-67-positive β-cell nuclei (Figures 4(a) and 4(b)). In addition, DEX treatment increased the protein content of the proliferating cell nuclear antigen (PCNA) in islets to a greater extent in rats than in mice (Figure 4(c)). DEX treatment did not affect apoptosis in the islets of rats or mice as judged by DNA fragmentation (Figure 4(d)) and caspase-3 cleavage data (Figure 4(e)).

3.7. Increased β-Cell Function and Mass Is Associated with Increased Ir-β/AKT and Reduced AMPK/ACC Pathway Activities in Pancreatic Islets from DEX-Treated Mice and Rats. Because insulin plays an important role in insulin secretion and in β-cell proliferation and can act directly upon the islet cells, we investigated whether insulin signaling was modulated by DEX treatment. We analyzed the canonical insulin pathway through the insulin receptor β-subunit (Ir-β) and its downstream protein, AKT. In islets from DEX-treated mice, we observed an increase in p-Ir-β (Figure 5(a)) without alterations in the total Ir-β protein content (Figure 5(b)). In islets from DEX-treated rats, the levels of p-Ir-β and total Ir-β protein increased significantly (Figures 5(a) and 5(b)). DEX-treated mice had increased islet p-AKT (Figure 5(c)) without alteration of the total AKT content (Figure 5(d)). In rat islets, DEX treatment resulted in higher amounts of p-AKT and total AKT (Figures 5(c) and 5(d)). We also assessed whether the extracellular signal-regulated kinase

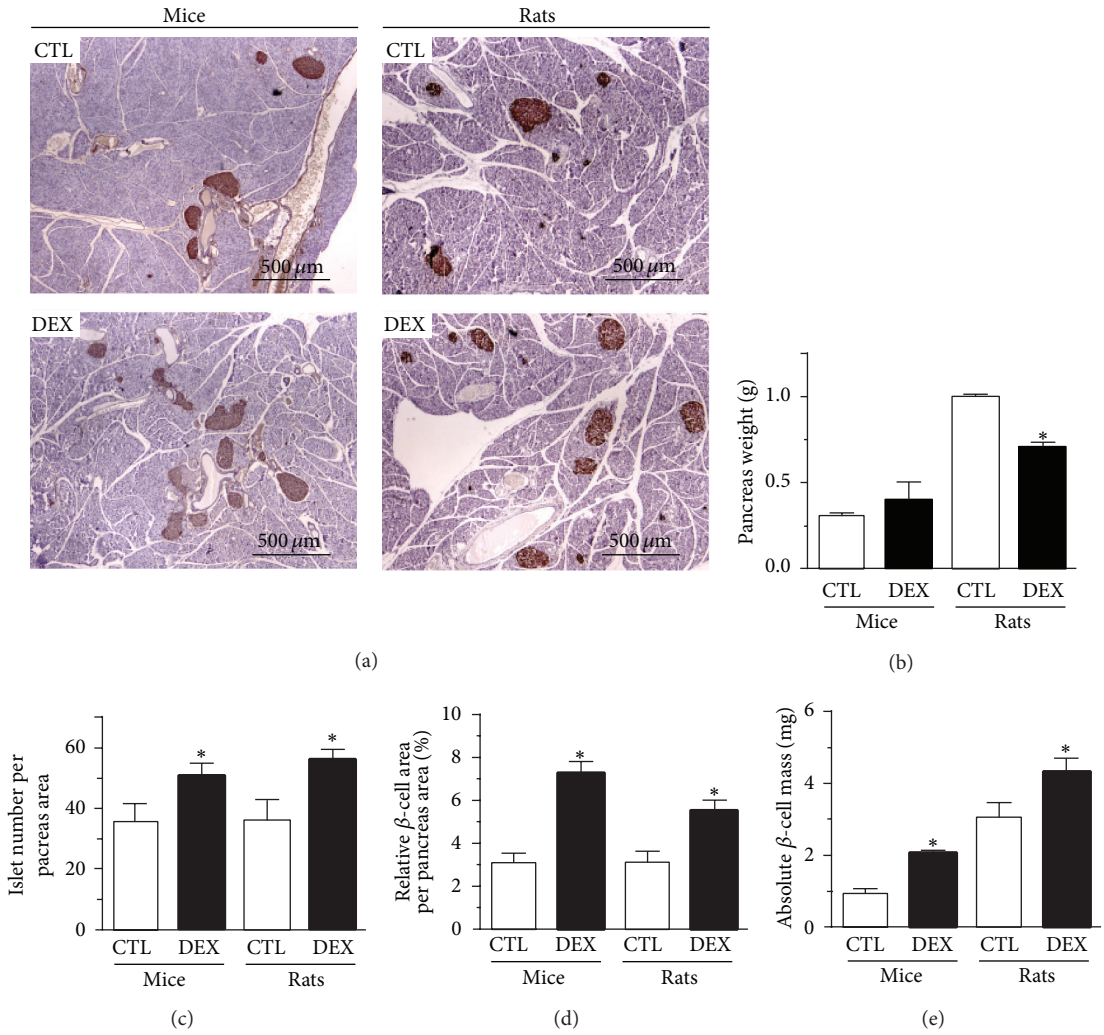

Figure 3: DEX treatment increases β-cell mass in the pancreas of mice and rats. (a) Representative pancreas sections stained for insulin with Hematoxylin counterstaining. (b) Pancreas weight, (c) relative islet number per pancreas area, (d) relative β-cell area per pancreas area, and (e) absolute β-cell mass in DEX-treated mice and rats. Values are mean ± S.E.M.; n = 5-6 animals per group (≈150 islets from mice and ≈300 islets from rats). *Significantly different compared to CTL. Unpaired Student's t-test, $P \leq 0.05$.

(ERK) pathway, which can be activated by insulin and participates in cell proliferation and differentiation [38], is modulated by DEX treatment. In mice islets, only p-ERK was augmented (Figures 5(e) and 5(f)), while DEX-treated rat islets showed a significant increase in p-ERK and total ERK content (Figures 5(e) and 5(f)). The AMPK pathway can also modulate the insulin secretion [39]. Islets from DEX-treated mice and rats had lower p-AMPK without alterations in the total AMPK content (Figures 5(g) and 5(h)). ACC, a downstream AMPK target protein, has diminished activity when phosphorylated. In both species, DEX treatment was associated with decreased levels of phosphorylated ACC (Figure 5(i)) without altering the total ACC levels in islets (Figure 5(j)), which indicates increased lipid synthesis in islets. Thus, DEX treatment results in increased insulin and decreased AMPK signaling pathways in islets from mice and rats.

3.8. DEX Treatment Modulates Proteins Related to Vesicle Trafficking, Protein Synthesis, Cell Growth, and Insulin Secretion in Pancreatic Islets from Both Mice and Rats. AKT and AMPK pathways were modulated in islets from GC-treated rodents. Since, in skeletal muscle, these pathways interact and inhibit AS160 through phosphorylation, allowing the GLUT4 vesicle trafficking to the plasma membrane [34], we investigated whether AS160 could be modulated in islets from GC-treated rodents. In mice and rats, DEX treatment resulted in higher p-AS160 (Figure 6(a)) and lower total AS160 contents (Figure 6(b)) compared to the control groups, which is in accordance with increased GSIS and hyperinsulinemia. AKT and AMPK pathway can also modulate the mammalian target of rapamycin (mTOR), a key protein that induces protein synthesis. Islets from DEX-treated mice and rats showed increased p-mTOR and total mTOR protein (Figures 6(c) and 6(d)).

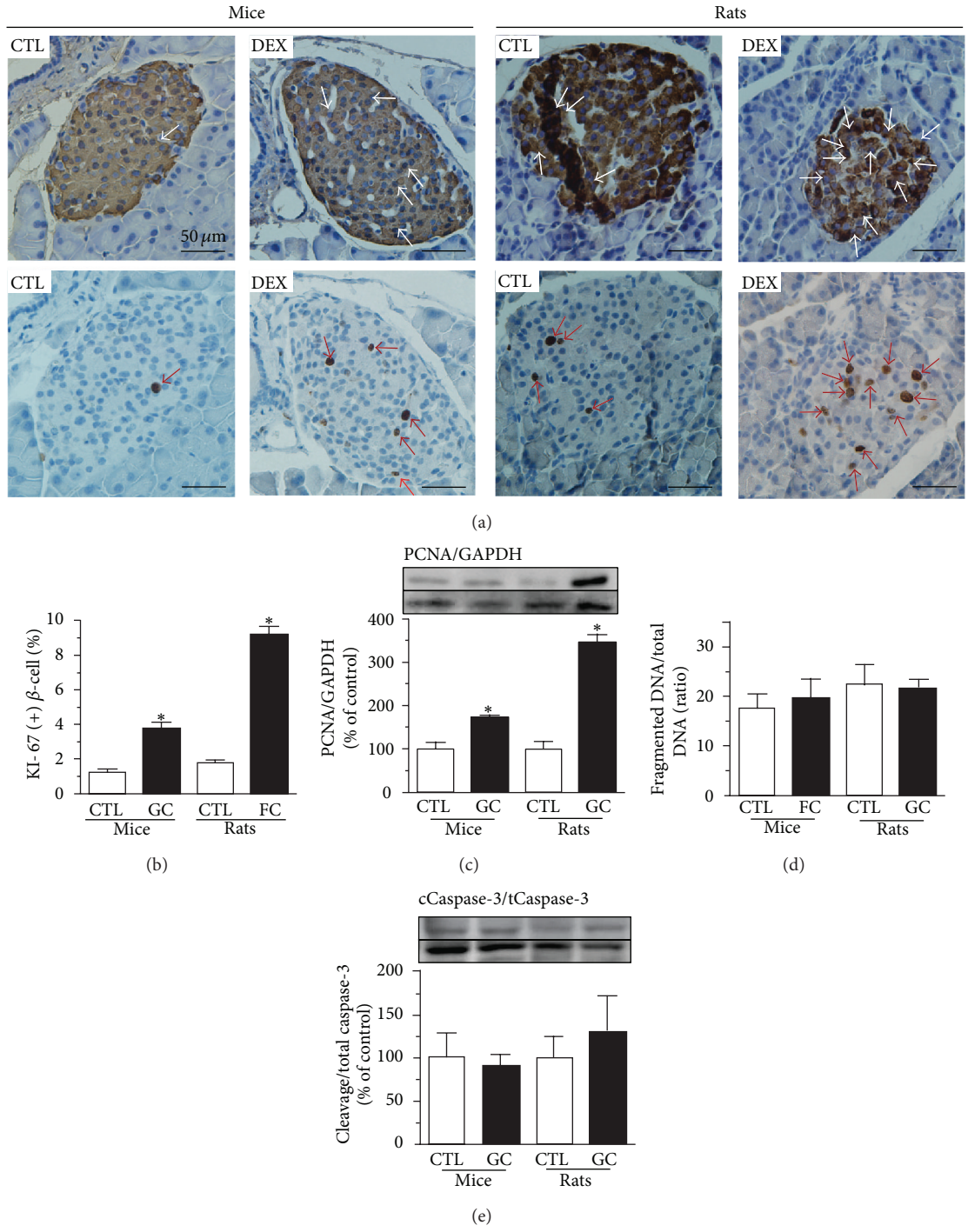

FIGURE 4: DEX treatment increases β-cell proliferation without affecting apoptosis in the islets of mice and rats. (a) Representative pancreas sections stained for insulin (on the top) and KI-67 (at bottom), (b) percentage of KI-67-positive nuclei (+) β-cell, (c) PCNA content, (d) DNA fragmentation/total DNA (ratio), and (e) caspase-3 cleavage/total caspase-3 ratio in islets from DEX-treated mice and rats. Values are mean ± S.E.M.; n = 5-6 rodents per group; ≈100 islets per species (≈8500 nuclei per group). *Significantly different compared to CTL. Unpaired Student's t-test, $P \leq 0.05$.

Due to its importance in the insulin secretion process, we also investigated proteins related to calcium (Ca^{2+}) influx, such as protein kinase C (PKC) and connexin 36 (CX36). The levels of phosphorylated PKC-target proteins (Figure 6(e)), total PKC (Figure 6(f)), and CX36 (Figure 6(g)) were increased in islets from DEX-treated mice and rats compared to their control groups. These data support an increased insulin secretion and β-cell mass in DEX-treated rodents.

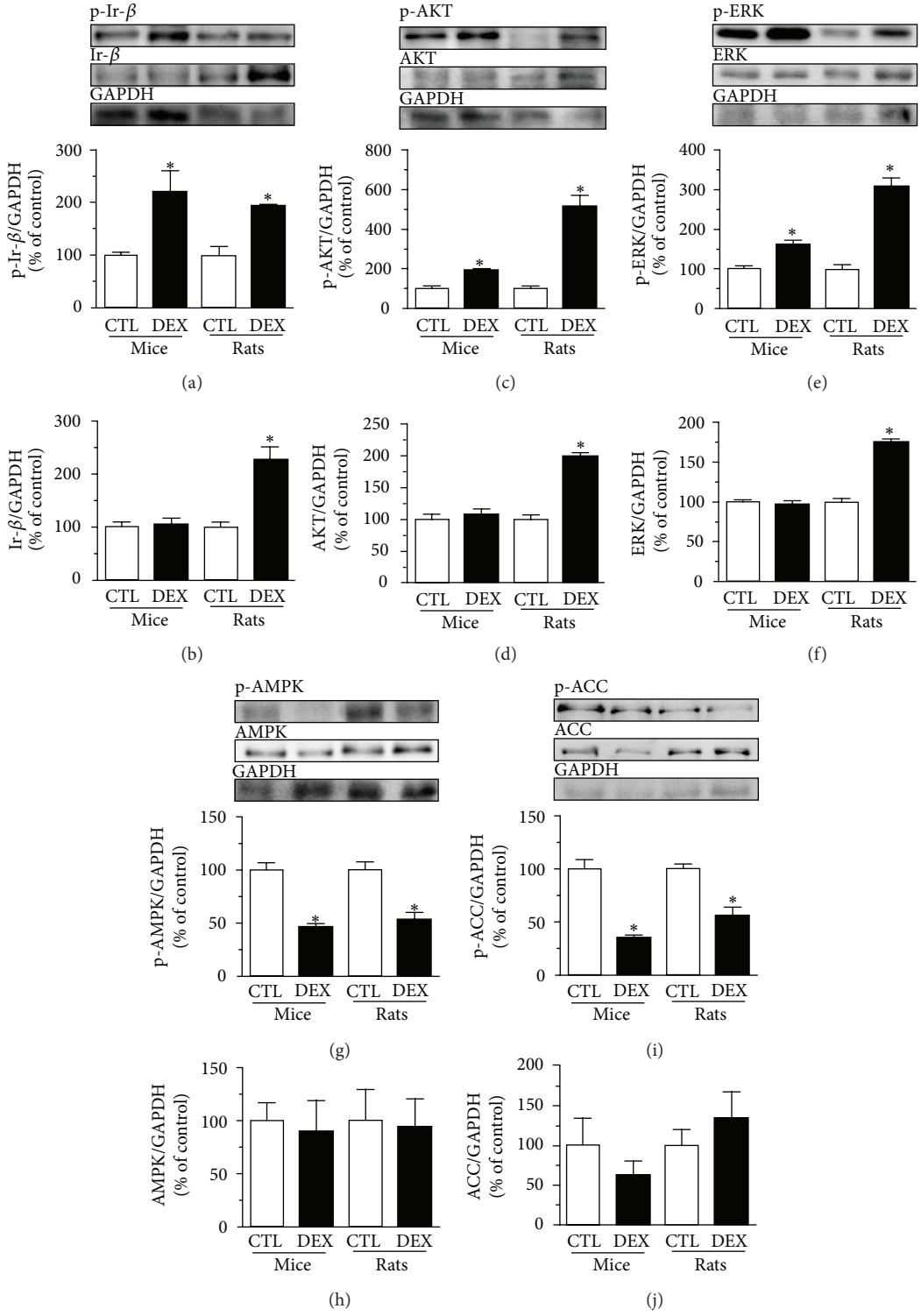

Figure 5: DEX treatment stimulates the canonical insulin pathway and inhibits the noncanonical insulin pathway in pancreatic islets from mice and rats. (a) Representative immunoblotting of phosphorylated and (b) total Ir-β content. (c) Phosphorylated and (d) total AKT content. (e) Phosphorylated and (f) total ERK content. (g) Phosphorylated and (h) total AMPK content. (i) Phosphorylated and (j) total ACC content in islets from DEX-treated mice and rats. Values are mean ± S.E.M.; $n = 4$ rodents per group. *Significantly different compared to CTL. Unpaired Student's t-test, $P \leq 0.05$.

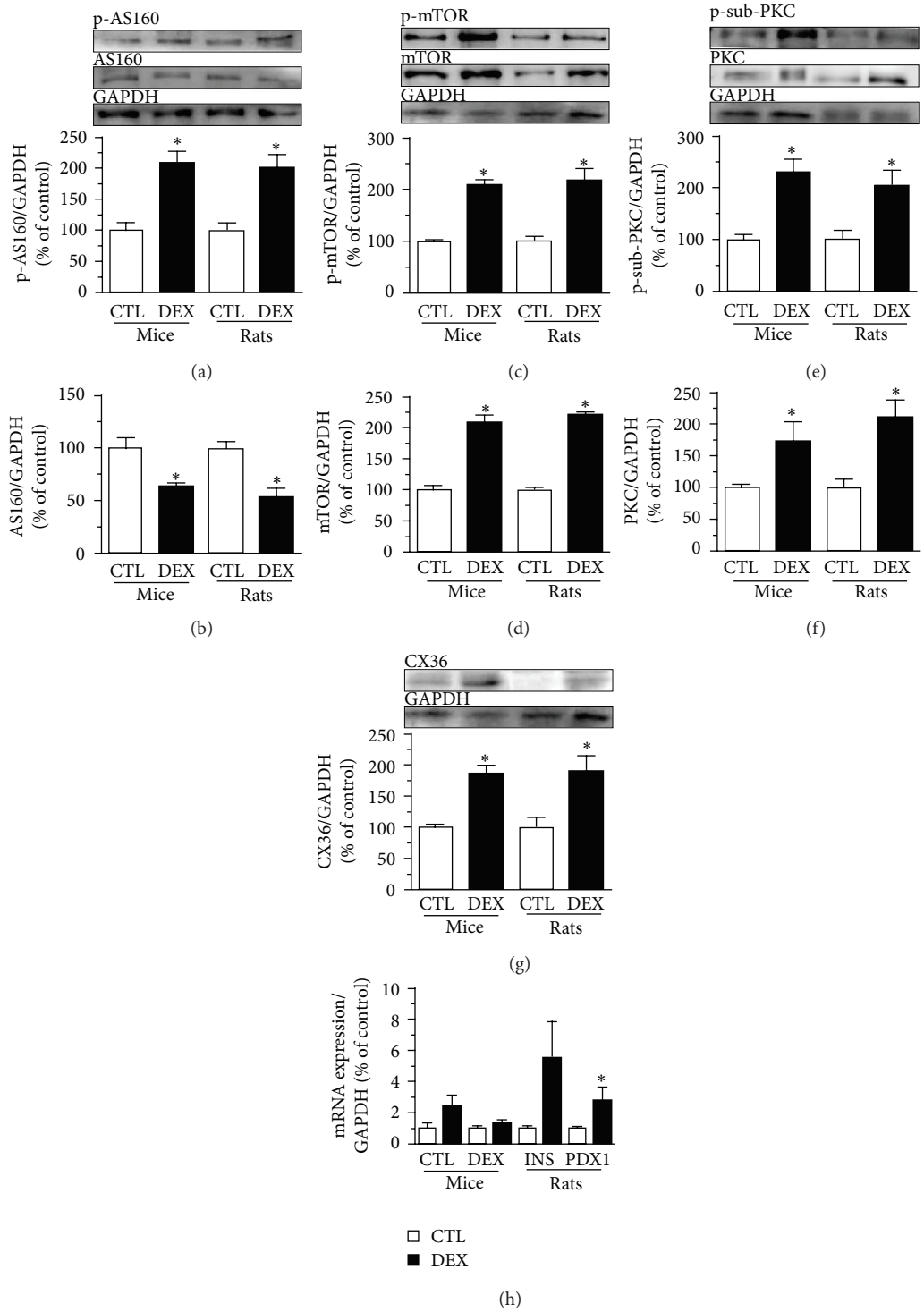

Figure 6: DEX treatment modulates proteins related to vesicle trafficking, protein synthesis, cell growth, and insulin secretion in pancreatic islets from both mice and rats. (a) Representative immunoblotting of phosphorylated and (b) total AS160 content. (c) Phosphorylated and (d) total mTOR content. (e) Phosphorylated substrates of PKC (range from 70 to 110 kDa) and (f) total PKC content. (g) CX36 expression and (h) mRNA expression of INS gene and PDX1 in islets from DEX-mice and rats. Values are mean ± S.E.M.; $n = 4$ rodents per group. *Significantly different compared to CTL. Unpaired Student's t-test, $P \leq 0.05$.

3.9. DEX Treatment Is Associated with Increased mRNA Levels of PDX1 in Rat Islets, but Not in Mice Islets. We also evaluated whether DEX treatment could alter the mRNA levels of the insulin and pancreatic duodenal homeobox-1 (PDX1) genes. DEX treatment tended to increase insulin mRNA levels in pancreatic islets from both mice and rats (Figure 6(h); $P = 0.056$ and 0.055, resp.). In addition, DEX treatment increased PDX1 mRNA levels in rat islets, but not in mouse islets (Figure 6(h)).

4. Discussion

In this study, we showed that *in vivo* GC treatment induced IR, hyperinsulinemia, and dyslipidemia in both species, but glucose intolerance and hyperglycemia only in rats. Both species displayed increased β-cell function (insulin hypersecretion) and mass (β-cell hyperplasia) as compensatory mechanisms. These compensatory responses were associated with reduced AS160 protein content and increased AS160 phosphorylation in islets as well as augmented AKT, but not AMPK phosphorylation (Figure 7).

The alterations in insulin action as well as in plasma insulin concentrations induced by the GC excess are compensated by enhanced insulin secretion in response to glucose, as can be frequently observed in GC-treated subjects [40–43]. Despite the pronounced hyperinsulinemia that results from a marked enhancement of the GSIS and increased β-cell mass, DEX-treated rats were not able to properly counteract the peripheral IR and both glucose intolerance and hyperglycemia developed being in accordance with previous studies [9, 19, 22, 37].

Although DEX-treated mice presented an increase in peripheral IR, they remained normoglycemic and glucose tolerant, indicating that the islet compensations (e.g., increased GSIS and β-cell mass) were sufficient to prevent any significant disruption of glucose homeostasis. Thus, at similar conditions of treatment (time and doses of DEX), mice seem to be less vulnerable than rats to the deleterious effects of GCs upon glucose homeostasis. This difference between species may be ascribed to difference in the GC receptor expression or activity in GC responsive tissues and/or in the GC metabolism, which does not exclude that for a more prolonged period and/or higher GC concentrations mice could develop an imbalance of the glucose homeostasis. These species-specific responses to the GCs regarding glucose tolerance highlight the importance to perform individualized analysis in patients receiving GC-based therapies.

Basal hyperinsulinemia may be explained by several factors, including the reduction of hepatic insulin clearance [44] and/or an increase of basal insulin secretion [10, 18]. The data obtained regarding insulin secretion demonstrated that both DEX-treated rats and mice had higher insulin responses to glucose, including at subthreshold glucose levels (e.g., 2.8 mmol/L and 5.6 mmol/L). It is well known that hydrocortisone administration acutely suppresses insulin release in mice by a mechanism that most likely involves the central activation of sympathetic nerves [45]. This was not the case here, as our experiments were performed 24 h after a 5-day course of DEX, thus eliminating the possible overlapping of acute and chronic effects.

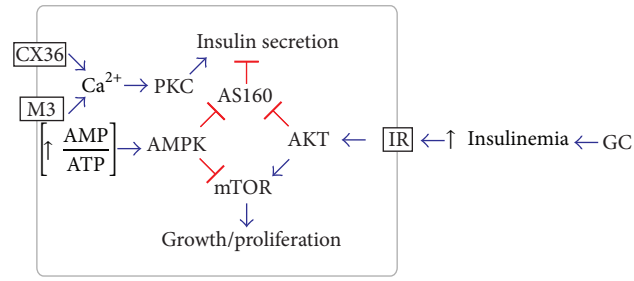

Figure 7: Hypothetical scheme of the main signaling pathways modulated in pancreatic β-cells by the *in vivo* glucocorticoid treatment. Decreased insulin sensitivity in peripheral tissues leads to hyperinsulinemia, which stimulates the insulin signaling in the pancreatic β-cell. Activated AKT inactivates the AS160, releasing its inhibitory effect on insulin secretion (functional compensation). Also, pAKT activates mTOR, favoring β-cell growth (structural compensation). In parallel, dephosphorylated AMPK, which may reflect a low AMP : ATP ratio, favors the activation of mTOR by AKT and suggest that AS160 inhibition is modulated by the insulin signaling, rather than AMPK signaling. An increased parasympathetic drive through muscarinic receptor (M3) and/or increased β-cells communication, through CX36 channels, may increase cytosolic Ca^{2+} and activates PKC, which stimulates insulin secretion. $\rightarrow$ represents stimulations and $\dashv$ represents inhibition.

Basal hyperinsulinemia in DEX-treated rodents can also be explained by the increased β-cell mass which is usually observed in insulin-resistant rodents fed a high-fat [17] or high sucrose diet [46]. Herein, we showed that increased β-cell mass resulted from, at least in part, increased β-cell proliferation as judged by the increased PCNA and KI-67 expression.

β-cell function that includes an increase of insulin response to glucose and increased β-cell proliferation are modulated by several stimuli through various intracellular pathways, and one of these major stimuli is insulin itself. Previous studies have documented the important role of insulin signaling in the β-cell function, survival, and growth [29, 30]. In our study, the compensatory insulin hypersecretion and β-cell proliferation in both DEX-treated rodents were associated with increased p-Ir-β and p-AKT in the islets, corroborating the hypothesis that enhanced insulin pathway (Ir-β/AKT) signaling is a common mechanism involved in the endocrine pancreas compensation, for example, after exposition to GCs (Figure 7). A previous study elegantly demonstrated that AKT overexpression sustains an elevation of the β-cell function that includes an enhancement of both GSIS and β-cell mass [29]. Our data with islet AKT protein content and phosphorylation are in accordance with other GC-related studies [19, 28]. Increased p-ERK also reinforces the hypothesis that insulin is involved with the pancreatic islet compensations during GC exposures. In DEX-treated rats, the increased Ir-β, AKT, and ERK total contents indicate an additive pancreatic compensatory mechanism compared to mice, in which the mechanism is limited to increased phosphorylation of the aforementioned proteins.

We also investigated the potential of AMPK signaling for mediation of islet adaptation in this context of GC excess. In muscle, AMPK is an energy-sensitizing enzyme that is active at low cellular energy (increased AMP/ATP ratio) [47]. In the β-cell lineage (MIN6), AMPK inhibition by glucose is essential for the activation of the insulin secretion process [39]. The decreased AMPK phosphorylation in islets from both groups of DEX-treated rodents supports the increased GSIS and may reflect the abundance of energy substrates available in the plasma. AMPK downregulation also underlies the increase in ACC activity and also suggests higher energy availability. Thus, we hypothesized that reduction in the AMPK/ACC pathway is one of the important mechanisms involved in the modulation of the altered insulin secretory process found under GC treatment.

In muscle and adipose cells, the AS160 is recognized as an inhibitor of GLUT4 vesicle trafficking to the plasma membrane [34]. In muscle cells, both insulin and AMPK signaling inhibit AS160 through phosphorylation, favoring GLUT4 vesicle trafficking [34]. β-cells also express AS160 that seems to exert several actions on the GSIS, survival and growth; and is under the control of insulin signaling [31, 35]. The crosstalk between AMPK signaling with the AS160 in islets is not yet well established. The increased AS160 phosphorylation in islets from GC-treated rodents indicates that insulin pathway, through the AKT, rather than the AMPK pathway, mediates AS160 inhibition, thus favoring the exocytosis of the insulin vesicles. In addition, the reduction in the AS160 expression is another mechanism that may contribute to increased insulin secretion (Figure 7).

The AKT and AMPK signaling also modulate mTOR function, a kinase that integrates multiple cell signals [13, 48] and regulates β-cell function and growth [48]. Our results agree with those from previous studies [49, 50] and indicate that the inhibition of AMPK and the activation of AKT synergistically activate mTOR in islets from GC-treated mice and rats, which may corroborate the increased GSIS and β-cell mass.

Another mechanism that contributes to the increased islet function after exposition to GCs may remain at the improvement of Ca^{2+} handling in β-cells. A Ca^{2+} influx contributes to the first (triggering) and second phase (amplifying) of insulin secretion [51]. An additional increase in intracellular Ca^{2+}, under stimulatory glucose concentrations, is associated with a higher GSIS in islets from GC-treated rats [18]. The higher GSIS is also associated with increased CX36 expression in islets [27], which synchronizes Ca^{2+} transit between β-cells across the islets [52]. Activation of PKC, which is stimulated by Ca^{2+} and participates in the amplification of insulin secretion, is another mechanism associated with increased GSIS in rats treated with GCs [18]. Here, we also observed an increase in CX36 expression and an indirect increase in PKC activity in islets from both rats and mice treated with GCs, indicating the participation of Ca^{2+} in increased β-cell function. Finally, increased expression of β-cell markers (PDX1 mRNA) only in DEX-treated rats islets indicates that pancreatic compensations in this species also involve modifications at transcriptional levels.

5. Conclusions

We conclude that *in vivo* GC treatment induced in rodents, rats and mice, the deleterious effect on glucose homeostasis as observed in humans (IR, hyperinsulinemia, and dyslipidemia), which were compensated by the increased GSIS and β-cell mass. These compensations were associated with islet upregulation of AKT, but not AMPK signals that parallel with inhibited AS160 activation (Figure 7). We suggest that basal hyperinsulinemia in GC treated subjects may also involve the inhibition of AS160 in β-cells.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Effects of Dipeptidyl Peptidase-4 Inhibition with MK-0431 on Syngeneic Mouse Islet Transplantation

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Dipeptidyl peptidase (DPP)-4 inhibitors increase circulating levels of glucagon-like peptide-1 and glucose-dependent insulinotropic polypeptide which may promote β-cell proliferation and survival. This study tested if DPP-4 inhibition with MK-0431 is beneficial for diabetic mice syngeneically transplanted with a marginal number of islets. We syngeneically transplanted 150 C57BL/6 mouse islets under the kidney capsule of each streptozotocin-diabetic mouse and then treated recipients with ($n = 21$) or without ($n = 17$) MK-0431 (30 mg/kg/day, po) for 6 weeks. After islet transplantation, blood glucose levels decreased in both MK-0431-treated and control groups. However, the blood glucose and area under the curve of the intraperitoneal glucose tolerance test at 2, 4, and 6 weeks were not significantly different between MK-0431-treated mice and controls. During 6 weeks, both groups exhibited increased body weights over time. However, the weight between two groups did not differ throughout the study period. At 6 weeks after transplantation, the graft beta-cell mass (0.024 ± 0.005 versus 0.023 ± 0.007 mg, $P = 0.8793$) and insulin content (140 ± 48 versus 231 ± 63 ng, $P = 0.2939$) were comparable in the MK-0431-treated group and controls. Our results indicate posttransplant DPP-4 inhibition with MK-0431 in the diabetic recipient with a marginal number of islets is not beneficial to transplantation outcome or islet grafts.

1. Introduction

Recently, human islet transplantation has achieved insulin independence in type 1 diabetes and the success rates have been markedly improved [1]. However, most successful cases need 2 or more implants and the long-term follow-up shows their insulin independence declines with time [2, 3]. Therefore, the critical issue in clinical islet transplantation is to further improve and maintain its successful rate. Allograft failure may be due to nonimmunological (e.g., insufficient beta-cell mass and islet engraftment problems) as well as immunological (e.g., immune rejection, toxicity of immunosuppressants, and autoimmune recurrence) factors. To improve the outcome of islet transplantation, these problems have been intensively investigated [4]. The shortage of human donor pancreata has prompted efforts to expand the human donor pool and modify islet processing and preservation methods as well as identifying alternative islet sources. Another important approach is the generation of new beta-cells either from preexisting beta-cells or from progenitor/stem cells.

The glucagon-like peptide (GLP)-1 improves glycemic control in type 2 diabetic patients by stimulating glucose-dependent insulin secretion and biosynthesis and by suppressing glucagon secretion, gastric emptying, and appetite [5, 6]. Additionally, GLP-1 is also known to expand

beta-cell mass by stimulating β-cell proliferation and inhibiting β-cell apoptosis [7–10]. However, clinical application of native GLP-1 is limited due to its very short plasma half-life [11]. Exenatide (exendin-4) is a GLP-1 receptor agonist resistant to dipeptidyl peptidase (DPP)-4-mediated inactivation and thus exhibits more sustainable effects [12]. It also had the ability to expand beta-cell mass via stimulation of beta-cell replication and neogenesis as well as prevention of beta-cell death in rodents [4]. We and others have shown exendin-4 not only improved transplantation outcome [13–15] but also expanded the graft beta-cell mass [15]. In contrast, DPP-4 inhibitors increase circulating active incretin hormones, GLP-1, and glucose-dependent insulinotropic polypeptide (GIP), by blocking their degradation [11]; thus, they can be beneficial for beta-cells. Previously, many studies have shown DPP-4 inhibitors improved glucose tolerance, insulin secretion, beta-cell glucose responsiveness [16], and insulin sensitivity [16, 17]; promoted beta-cell survival [18–20], islet neogenesis [18, 21], and proliferation [22]; reduced beta-cell death [23]; and preserved beta-cell mass and function [24–26] in diabetic rodents. In contrast, there is limited information regarding the effects of DPP-4 inhibitors on islet transplantation. One report used positron emission tomography (PET) imaging and demonstrated DPP-4 inhibitor, MK-0431, protected against the loss of islet grafts in streptozotocin (STZ)-diabetic mice [20]. In that study, 300 islets transfected with rAD-TK were transplanted and PET imaging instead of histology was used to assess the graft islet mass. The aim of this study is to test if DPP-4 inhibition with MK-0431 could be beneficial to transplantation outcome and islet grafts in diabetic recipients with a marginal number of islets (150 islets). Here, we used freshly isolated islets for transplantation and directly measured graft beta-cell mass by immunohistochemistry with point counting morphometry and insulin content.

2. Materials and Methods

2.1. Animals. Male inbred C57BL/6 mice (National Laboratory Animal Center, Taipei, Taiwan), aged 8–12 weeks, were used as transplantation donors and recipients. The recipients were made diabetic by a single intraperitoneal injection of STZ (Sigma Immunochemicals, St. Louis, MO, USA, 200 mg/kg body weight, freshly dissolved in citrate buffer, pH 4.5). Before transplantation, diabetes was confirmed by the presence of hyperglycemia, weight loss, and polyuria. Only those mice with blood glucose above 350 mg/dL at 2 weeks after STZ injection underwent transplant. Blood glucose values were determined on blood obtained from the snipped tail, with measurements performed with a portable glucose analyzer (One Touch II, Lifescan Inc., Milpitas, CA, USA). The animal experiments were approved by the Ethics Committee of Chang Gung Memorial Hospital [27–29].

2.2. Islet Isolation. Under anesthesia with sodium amobarbital, pancreases were distended with 2.5 mL of RPMI-1640 medium (GIBCO BRL, Grand Island, NY, USA) containing 1.5 mg/mL of collagenase (collagenase from *Clostridium histolyticum*, type XI, Sigma Immunochemicals), excised, and incubated in a water bath at 37°C. Islets were separated by a density gradient (Histopaque-1077; Sigma Immunochemicals), and purified islets were then handpicked under a dissecting microscope. Islets >75 and <250 μm in diameter were collected and carefully counted into groups of 150 islets [27–29].

2.3. Islet Transplantation. One hundred and fifty C57BL/6 mouse islets were syngeneically transplanted under left kidney capsule of each inbred STZ-diabetic mouse on the same day as the isolation. Blood glucose and body weight were measured periodically after transplantation and normoglycemia was defined as nonfasting blood glucose levels <200 mg/dL [27–29].

2.4. MK-0431 Treatment. After islet transplantation, twenty-one recipients were treated with MK-0431, 30 mg/kg/day po, for 6 weeks. Seventeen recipients who had not received MK-0431 served as controls.

2.5. Intraperitoneal Glucose Tolerance Test (IPGTT). After an overnight fast, a 5% glucose solution (1.5 g/kg) was injected intraperitoneally, and blood glucose was measured at 0, 30, 60, 90, and 120 min by tail snipping. The IPGTT was performed at 2, 4, and 6 weeks after transplantation [27–29].

2.6. Removal of the Islet Graft. Six weeks after transplantation, animals intended for graft removal were anesthetized with amobarbital. An abdominal incision was made and the kidney was exposed. Under dissecting microscope, the kidney capsule surrounding the graft was excised and removed with the adherent graft. The weight of each graft was determined on a Mettler balance type AE200 (Mettler Instruments Corp., NJ, USA) [29].

2.7. Immunohistochemistry and β-Cell Mass of the Islet Graft. The removed grafts were fixed in formalin solution and processed for paraffin embedding and sectioning. Sections of grafts were stained for the endocrine β-cells with immunoperoxidase by a guinea pig anti-swine insulin antibody (Dako Co., Glostrup, Denmark). Graft β-cell mass was measured by point counting morphometry on immunoperoxidase stained sections. Each section was covered systematically using a 48-point grid to obtain the number of intercepts over β-cells, endocrine non-β-cells, and other tissues. The β-cell relative volume was calculated by dividing the intercepts over β-cells by intercepts over total tissue; β-cell mass was then estimated by multiplying β-cell relative volume by graft weight [28, 29].

2.8. Insulin Content of the Islet Graft. At 6 weeks after transplantation, the graft-bearing kidneys were removed and homogenized in acid ethanol. After homogenization, the samples were extracted overnight at 4°C. On the following day, they were centrifuged at 2,400 rpm for 30 min and the supernatant was stored at −20°C. The pellet was again

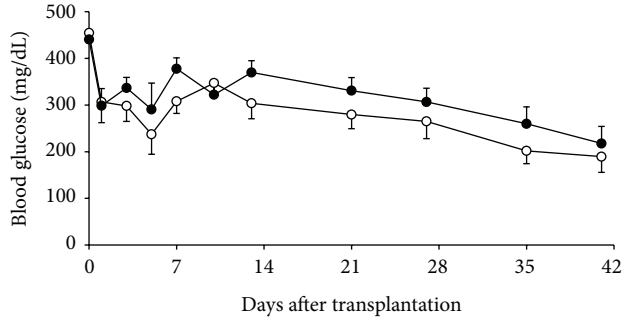

Figure 1: Blood glucose changes in islet recipients with (solid circle) and without (open circle) MK-0431 treatment.

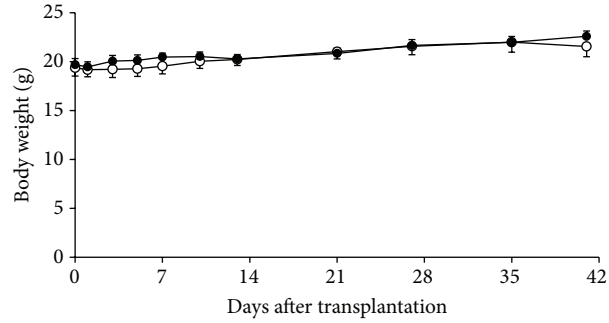

Figure 2: Body weight changes in islet recipients with (solid circle) and without (open circle) MK-0431 treatment.

homogenized in acid ethanol and insulin was extracted overnight. After centrifugation, this second supernatant was added to the first extraction sample. Insulin was measured by radioimmunoassay with rat insulin RIA kit (Millipore Corporation, Billerica, MA, USA) [27–29].

2.9. Statistical Analysis. Results were expressed as mean and standard error of the mean (M ± SEM). Paired and unpaired Student's t-test were employed to compare values in a group and values between two groups, respectively. A value of $P < .05$ was considered significant.

3. Results

3.1. Effects of MK-0431 on Recipients' Blood Glucose after Islet Transplantation. After islet transplantation, recipients' blood glucose levels decreased progressively in both MK-0431-treated and control groups (Figure 1). However, the blood glucose levels were not significantly different between MK-0431-treated mice and controls throughout the study period. At 6 weeks, the blood glucose was 218 ± 37 and 189 ± 34 mg/dL in the MK-0431-treated group and controls, respectively ($P = 0.5776$).

3.2. Effects of MK-0431 on Recipients' Body Weight after Islet Transplantation. During 6 weeks after islet transplantation, both groups exhibited increased body weights over time (MK-0431-treated group: 19.6 ± 0.7 to 22.6 ± 0.7 g, $P = 0.0001$; controls: 19.7 ± 0.9 to 21.8 ± 1.2 g, $P = 0.0454$) (Figure 2). However, the weight between two groups did not differ throughout the study period.

3.3. Effects of MK-0431 on Recipients' Glucose Tolerance after Islet Transplantation. After islet transplantation, the area under the curve (AUC) of the IPGTT at 2 weeks (38038 ± 2847 versus 35806 ± 3433 mg/dL, $P = 0.6204$), 4 weeks (31187 ± 2835 versus 26848 ± 3159 mg/dL, $P = 0.3144$), and 6 weeks (30634 ± 2954 mg/dL versus 22549 ± 2949, $P = 0.0614$) was not significantly different between MK-0431-treated mice and controls (Figure 3).

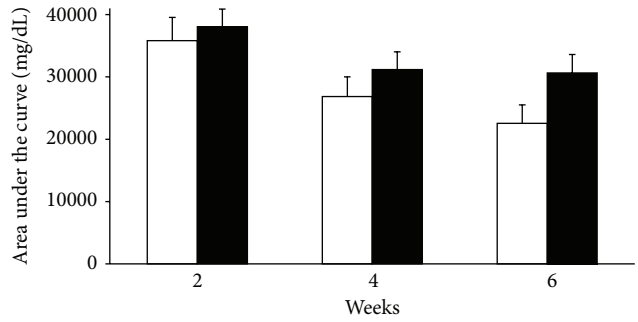

Figure 3: The area under the curve (AUC) of the intraperitoneal glucose tolerance test (IPGTT) at 2, 4, and 6 weeks in islet recipients with (black column) and without (white column) MK-0431 treatment.

3.4. Effects of MK-0431 on Recipients' Graft Insulin Content and β-Cell Mass. At 6 weeks after transplantation, the graft beta-cell mass (MK-0431: 0.024 ± 0.005 mg, $n = 14$ versus controls: 0.023 ± 0.007 mg, $n = 10$, $P = 0.8793$) (Figure 4(A)) and insulin content (MK-0431: 140 ± 48 ng, $n = 4$ versus controls: 231 ± 63 ng, $n = 5$, $P = 0.2939$) (Figure 4(B)) were comparable in both groups.

4. Discussion

Although we and others previously showed exendin-4 improved transplantation outcome and expanded the graft beta-cell mass in diabetic mice transplanted with a marginal number of islets (150 islets) [15], this study demonstrated DPP-4 inhibition with MK-0431 did not. This observation is consistent with the fact that exendin-4 rather than DPP-4 inhibitors reduced blood glucose and increased pancreatic beta-cell mass in STZ-diabetic mice [19]. The above different effects on beta-cells are possibly due to the pharmacologic (exendin-4) and physiologic (GLP-1) bindings to GLP-1 receptors on beta-cells. In contrast, Lamont and Drucker treated high fat-fed mice with a remarkably higher dose (2.75 times) of MK-0431 which produced ~90% inhibition of plasma DPP-4 activity and found a significant reduction in glycated hemoglobin observed with DPP-4 inhibition but not with exendin-4 therapy. In addition, neither of the therapies

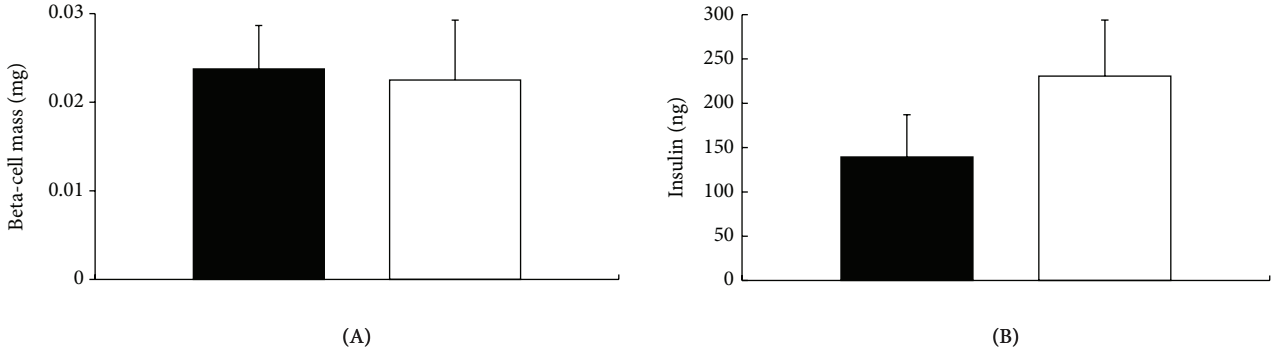

Figure 4: The graft β-cell mass (A) and insulin content (B) at 6 weeks in islet recipients with (black column) and without (white column) MK-0431 treatment.

increased beta-cell mass [30]. Although the effects of MK-0431 depend on its dose, we were unable to obtain sufficient plasma from mice to measure DPP-4 enzyme activity or active GLP-1 concentration after treatment with MK-0431. Hence, we cannot exclude the possibility that higher dose of MK-0431 may have beneficial effects on islet transplantation.

Our study design is different from that of Kim et al. who used PET imaging and showed MK-0431 protected against the loss of islet grafts in STZ-diabetic mice [20]. In contrast to our transplant with 150 freshly isolated islets, they transplanted 300 islets transfected with rAD-TK to each STZ-diabetic mouse. Our results are similar to their pilot studies in which mice receiving transplants of 100 islets remained hyperglycemic and did not benefit from treatment with MK-0431. Moreover, they assessed the graft islet mass by PET imaging which is not specific for beta-cells because [^{18}F]FHBG was taken up by islets instead of beta-cells. In contrast, we directly measured graft beta-cell mass by point counting morphometry and insulin content which are more accurate than PET imaging for quantifying beta-cell mass. Even though we found MK-0431 is not beneficial to the beta-cell mass of 150-islet grafts, whether it did so to 300-islet grafts needs to be further confirmed.

5. Conclusions

Our results indicate posttransplant DPP-4 inhibition with MK-0431 in the diabetic recipient with a marginal number of islets is not beneficial to transplantation outcome or islet grafts.

Conflict of Interests

The authors declared no conflict of interests.

Acknowledgments

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Research Article

Prevention and Reversal of Diabetes by All-Trans Retinoid Acid and Exendin-4 in NOD Mice

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It has been shown that all-trans retinoid acid (ATRA) hinders the development of autoimmune diabetes by inducing immune tolerance status. Meanwhile, exendin-4 increases beta-cell function and mass. Thus, we hypothesized that ATRA and exendin-4 combination therapy would prevent and reverse autoimmune diabetes. NOD/scid mice were intravenously transferred with splenocytes isolated from 12-week-old female NOD mice. After adoptive transfer, mice were treated with vehicle, ATRA (0.5 mg/mouse intraperitoneally every other day), exendin-4 (3 μg/kg subcutaneously twice daily), or combination for 6 weeks. Compared with vehicle, ATRA ($P = 0.022$) and ATRA plus exendin-4 ($P = 0.013$) treatment delayed the onset of diabetes. The pancreatic insulin content in mice treated with ATRA ($P = 0.013$) and exendin-4 ($P < 0.02$) was significantly higher than that of control mice. All but one spontaneous diabetic NOD mouse treated with ATRA and/or exendin-4 remained persistent hyperglycemic. ATRA and/or exendin-4 treatment did not alter their blood glucose levels and survival. Our results indicate that, before the onset of autoimmune diabetes, ATRA and exendin-4 treatment alone preserves pancreatic beta cells; ATRA and ATRA plus exendin-4 treatment delays the onset of autoimmune diabetes. However, after the onset of autoimmune diabetes, ATRA and/or exendin-4 treatment is unable to reverse hyperglycemia or improve survival.

1. Introduction

Type 1 diabetes is characterized by the progressive loss of pancreatic beta cells caused by autoimmune attack [1]. Although beta-cell mass is markedly diminished in long-standing type 1 diabetics, residual beta cells can be detected and new beta-cell formation may occur in these patients several decades after the disease onset [2]. This observation has led to researches to induce remission of diabetes by targeting beta-cell autoimmunity and regeneration.

All-trans retinoid acid (ATRA) is a potent vitamin A derivative that has been clinically used for the treatment of acute promyelocytic leukemia [3] and skin disease [4]. Recently, Van et al. demonstrated ATRA treatment inhibited diabetes in NOD mice by inducing Treg cell-dependent immune tolerance [5]. Exendin-4 (exenatide) is a glucagon-like peptide-1 (GLP-1) receptor agonist resistant to dipeptidyl peptidase-IV-mediated inactivation [6] which exhibits sustainable GLP-1 effects including stimulation of glucose-dependent insulin secretion and biosynthesis and suppression of glucagon secretion, gastric emptying, and appetite [7]. In addition, exendin-4 stimulates beta-cell growth and differentiation [8], inhibits beta-cell apoptosis [9], and delays the onset of diabetes [10]. Thus, we hypothesized that the combination therapy of ATRA and exendin-4 would inhibit autoimmune destruction and enhance the growth and function of pancreatic beta cells, therefore, prevent and reverse autoimmune diabetes.

2. Materials and Methods

2.1. Animals. NOD/scid (National Laboratory Animal Center and National Taiwan University, Taiwan) and NOD (National Defense Medical Center, Taiwan) mice were bred and housed in a specific pathogen-free environment in the animal facility at the Chang Gung Memorial Hospital. Blood was obtained from the snipped tail, and glucose was measured with a portable glucose meter (One Touch II, Lifescan Inc., Milpitas, CA, USA). Normoglycemia was defined as nonfasting blood glucose levels <200 mg/dL. All animal protocols were approved by the Ethics Committee of Chang Gung Memorial Hospital.

2.2. Adoptive Transfer. Splenocytes were isolated from 12-week-old female NOD mice. Nondepleted splenocytes (1×10^7/mouse) were intravenously transferred into NOD/scid mice [5].

2.3. ATRA and Exendin-4 Treatment. NOD/scid mice with adoptive transfer and spontaneous diabetic NOD mice were treated with vehicle (as control), ATRA (Sigma, St. Louis, MO, 0.5 mg/mouse intraperitoneally every other day) [5], exendin-4 (Sigma, St. Louis, MO, 3 μg/kg subcutaneously twice daily) [11], or combination for 6 weeks.

2.4. Immunohistochemistry of the Pancreas. The pancreases of NOD/scid mice were removed before death, fixed in formalin solution, and processed for paraffin embedding and sectioning. Sections of the pancreases were stained for the endocrine beta cells with a guinea pig antiswine insulin antibody (Dako Co., Glostrup, Denmark) [11], Pdx1, and Ki67 (Abcam, California, CA). The degree of insulitis was evaluated by the mononuclear cell infiltration around or in the islet.

2.5. Insulin Content of the Pancreas. The pancreases of NOD/scid mice were removed before death and homogenized in acid ethanol. After homogenization, the samples were extracted overnight at 4°C. On the following day, they were centrifuged at 2,400 rpm for 30 min, and the supernatant was stored at −20°C. The pellet was again homogenized in acid ethanol and left 2 h at 4°C. Above procedure was repeated and insulin was extracted overnight. After centrifugation, the supernatant was added to the previous extraction sample and kept in −20°C freezer till assay. Insulin was measured by radioimmunoassay with INSI-PR kit (CIS US Inc., USA) [11].

2.6. Statistical Analysis. Results were expressed as mean and standard deviation. Kaplan-Meier survival analysis was used to compare cumulative diabetes incidence. ANOVA were employed for comparisons among multiple groups. A value of $P < 0.05$ was considered significant.

3. Results

3.1. Prevention Study. After adoptive transfer, the mean diabetes-free time in NOD/scid mice treated with vehicle ($n = 8$), ATRA ($n = 7$), exendin-4 ($n = 9$), and ATRA plus exendin-4 ($n = 6$) was 6.5, 8.0, 7.6, and 11.2 weeks, respectively. Compared with vehicle, ATRA ($P = 0.022$) and ATRA plus exendin-4 ($P = 0.013$) treatment delayed the onset of diabetes (Figure 1). Although mice with different treatments had varied degrees of insulitis and a variable number of beta cells per islet (Figure 2), the pancreatic insulin content in mice treated with ATRA (932 ± 182 ng/mL, $n = 3$, $P = 0.013$) and exendin-4 (705 ± 479 ng/mL, $n = 5$, $P < 0.02$) was significantly higher than that of control mice (41 ± 9 ng/mL, $n = 4$) (Figure 3). Moreover, ATRA treated group had more

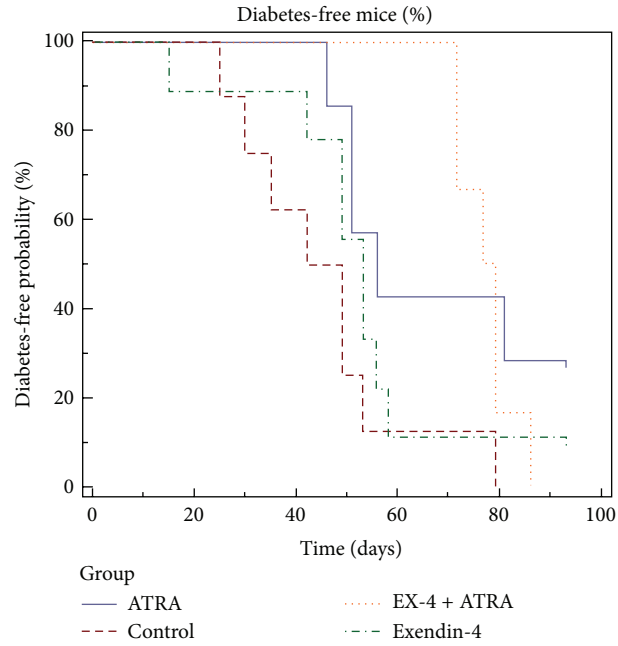

Figure 1: Kaplan-Meier curve of the percentage of diabetes-free mice over time in vehicle (brown line), ATRA- (blue line), exendin-4- (EX-4, green line), and ATRA plus exendin-4- (orange line) treated groups. Adoptive-transfer was performed and treatment was started at day 0. Compared with vehicle, ATRA ($P = 0.022$) and ATRA plus exendin-4 ($P = 0.013$) treatment delayed the onset of diabetes.

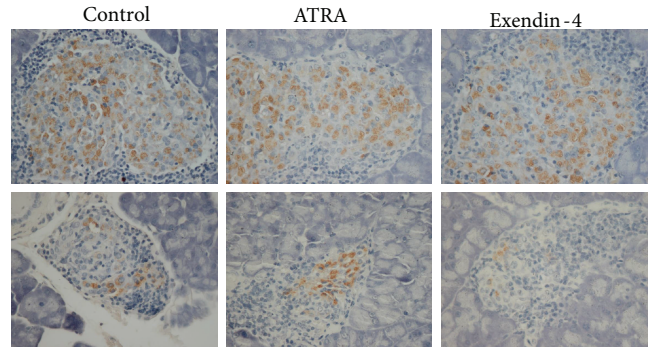

Figure 2: Varied degrees of insulitis with abundant (upper panel) to scanty (lower panel) beta cells were observed in the pancreases of adoptive transferred NOD/scid mice treated with vehicle, ATRA, and exendin-4.

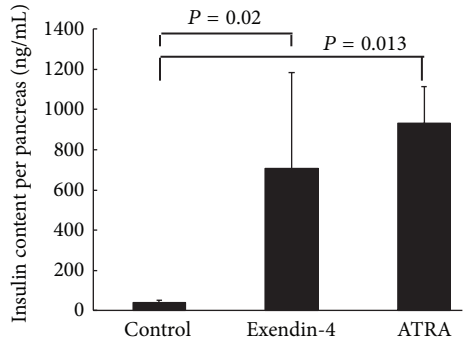

Figure 3: The pancreatic insulin content in adoptive transferred NOD/scid mice treated with vehicle, ATRA, and exendin-4.

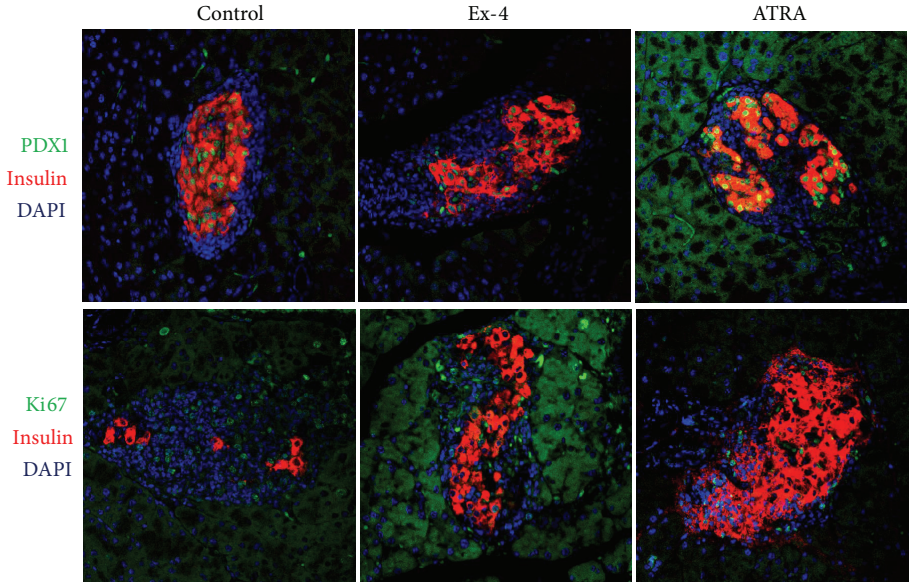

Figure 4: Insulin, Pdx-1, and Ki67 staining for islets in the pancreases of adoptive transferred NOD/scid mice treated with vehicle, ATRA, and exendin-4 (Ex-4).

Pdx-1- and Ki67-positive beta cells compared with control and Ex-4-treated animals (Figure 4).

3.2. Reversal Study. All but one spontaneous diabetic NOD mouse remained persistent hyperglycemic. The lowest blood glucose level in control ($n = 35$) and mice treated with ATRA ($n = 5$), exendin-4 ($n = 15$), and ATRA plus exendin-4 ($n = 27$) was 325 ± 83, 410 ± 22, 286 ± 136, and 308 ± 125 mg/dL, respectively ($P = 0.157$, Figure 5(a)). Meanwhile, ATRA and/or exendin-4 treatment did not alter their survival time [56 ± 39, 45 ± 19, 59 ± 33, and 46 ± 36 days in control ($n = 40$) and mice treated with ATRA ($n = 16$), exendin-4 ($n = 24$), and ATRA plus exendin-4 ($n = 29$), respectively; $P = 0.369$, Figure 5(b)]. One mouse achieved normoglycemia 10 days after ATRA treatment and then had occasional hyperglycemia between 88 and 366 days (Figure 6).

4. Discussion

In the present study, we tested if ATRA and exendin-4 treatment could prevent and reverse autoimmune diabetes. In the prevention experiment, due to limited space in our animal facility, NOD/scid mice were adoptive transferred with splenocytes isolated from 12-week-old female instead of new-onset diabetic NOD mice presented by Van et al. [5]. Even though 100% of our control NOD/scid mice developed diabetes with the mean onset of 6.5 weeks after adoptive transfer [12], in contrast, those treated with ATRA and ATRA plus exendin-4 developed diabetes with the mean onset of 8.0 and 11.2 weeks, respectively. Clearly, ATRA and ATRA plus exendin-4, but not exendin-4, treatment delayed the onset of autoimmune diabetes. This finding indicates that before the onset of diabetes, the inhibition of autoimmunity (exerted by ATRA) is more important than the promotion of beta-cell regeneration (exerted by exendin-4). Since there were varied degrees of insulitis and a variable number of beta cells per islet in the pancreas of mice before death with different treatment, we compared their pancreatic insulin content, an indicator of

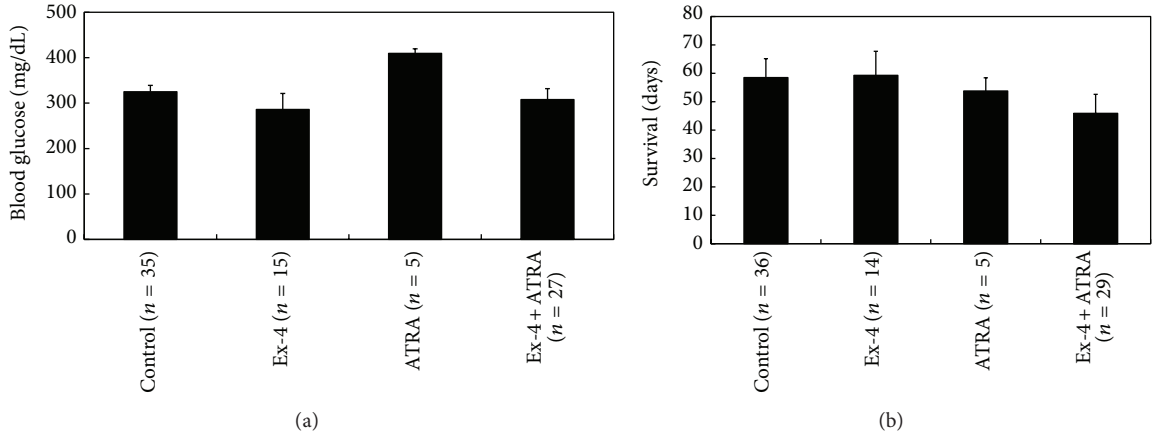

Figure 5: The lowest blood glucose levels (a) and survival time (b) in spontaneous diabetic NOD mice treated with and without ATRA and exendin-4 (Ex-4).

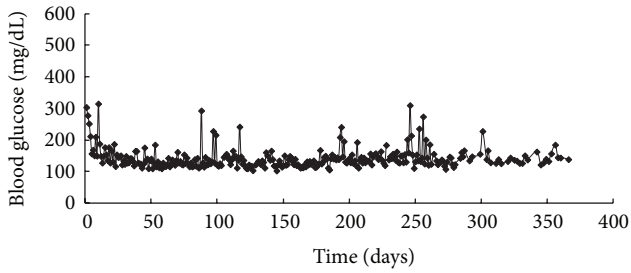

Figure 6: One spontaneous diabetic NOD mouse achieved normoglycemia 10 days after ATRA treatment but then had occasional hyperglycemia between 88 and 366 days.

pancreatic beta-cell mass. Higher pancreatic insulin content was observed in mice treated with ATRA and exendin-4 than in control mice after adoptive transfer. It supports the beneficial effect of ATRA and exendin-4 treatment on beta-cell preservation before the onset of autoimmune diabetes. Our finding of more Pdx-1- and Ki67-positive beta cells in ATRA-treated group compared with control and Ex-4-treated animals indicating that ATRA has replicating and differentiating effects on pancreatic beta cells.

In the reversal experiment, we used spontaneous diabetic NOD mice instead of NOD/scid mice with adoptive transfer. In this slowly progressive diabetic model [13], residual beta cells in the pancreas could be rescued. Unfortunately, nearly all diabetic NOD mice remained persistent hyperglycemic regardless of ATRA and/or exendin-4 treatment. Meanwhile, ATRA and/or exendin-4 treatment did not improve their survival time. Therefore, after the onset of autoimmune diabetes, ATRA cannot halt autoimmune beta-cell destruction. There was only one mouse that achieved normoglycemia after ATRA treatment. It needs to be further studied.

5. Conclusions

Before the onset of autoimmune diabetes, ATRA and exendin-4 treatment alone preserves pancreatic beta cells; ATRA and ATRA plus exendin-4 treatment delays the onset of autoimmune diabetes. After the onset of autoimmune diabetes, ATRA and/or exendin-4 treatment is unable to reverse hyperglycemia or improve survival.

Conflict of Interests

The authors declared no conflict of interests.

Authors' Contribution

Jyuhn-Huarng Juang and Yang-Hau Van contributed equally to this paper.

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Review Article

Inflammatory Response in Islet Transplantation

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Islet cell transplantation is a promising beta cell replacement therapy for patients with brittle type 1 diabetes as well as refractory chronic pancreatitis. Despite the vast advancements made in this field, challenges still remain in achieving high frequency and long-term successful transplant outcomes. Here we review recent advances in understanding the role of inflammation in islet transplantation and development of strategies to prevent damage to islets from inflammation. The inflammatory response associated with islets has been recognized as the primary cause of early damage to islets and graft loss after transplantation. Details on cell signaling pathways in islets triggered by cytokines and harmful inflammatory events during pancreas procurement, pancreas preservation, islet isolation, and islet infusion are presented. Robust control of pre- and peritransplant islet inflammation could improve posttransplant islet survival and in turn enhance the benefits of islet cell transplantation for patients who are insulin dependent. We discuss several potent anti-inflammatory strategies that show promise for improving islet engraftment. Further understanding of molecular mechanisms involved in the inflammatory response will provide the basis for developing potent therapeutic strategies for enhancing the quality and success of islet transplantation.

1. Background

Transplantation of pancreatic islets is a minimally invasive procedure involving infusion of islet cells into the portal vein of the liver, and it was first demonstrated in an experimental diabetic model by Kemp et al. in 1973 [1]. Results from the initial clinical trials for type 1 diabetes were only partially successful, with only 10% of the patients achieving insulin independence at 1 year after transplantation [2]. A quantum leap in islet transplantation occurred when the Edmonton group introduced a steroid-free immunosuppression and showed insulin independence in all 7 of their patients [3]. With this advancement in immunosuppression and the continuous improvement in islet isolation techniques, islet transplantation has entered into a new era of heightened success. Currently, two types of clinical islet transplantation are performed: allogenic and autologous islet transplantation. Allogenic islet transplantation is typically performed on patients with severe type 1 diabetes, while autologous islet transplantation is performed on patients suffering from severe chronic pancreatitis (CP) and undergoing partial/total pancreatectomy.

CP is a progressive inflammatory disease that leads to irreversible damage of the pancreatic parenchyma [4]. In its early stages, the disease affects pancreatic exocrine function, which could be followed by impaired endocrine function that results in the onset of diabetes mellitus [5, 6]. The mechanisms underlying the development of CP are not clearly defined. Inflammation, heavy alcohol consumption, pancreatic ductal obstructions, calcification, sphincter of Oddi dysfunction, certain genetic mutations, and periampullary tumors are considered the major causes of CP. In addition to gradual loss of exocrine and endocrine functions, complications of CP include biliary or duodenal stenosis and intractable pain. Total or partial pancreatectomy followed by intrahepatic transplantation of autologous islets has emerged

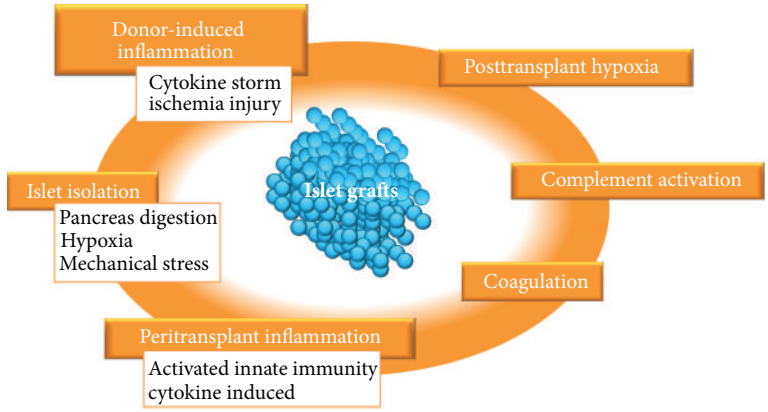

Figure 1: Factors that induce an inflammatory reaction to islet grafts.

as a promising approach to treat CP, due to its ability to reduce or eliminate pain while retaining endocrine function. The first pancreatectomy followed by autologous islet transplantation was performed at the University of Minnesota in 1977 [7]. Since then, more than 500 CP patients have successfully undergone this procedure at several centers in North America and worldwide [8]. Posttransplant results have shown that at 3 years, 30% of patients were insulin independent, 33% had partial islet function, and 94% showed improved pain control [9].

Comparison of patients receiving similar doses of autologous versus allogeneic islets has shown that the survival of autologous islets is far better over time [9]. In contrast to allogeneic islet transplants, autologous islet transplants are devoid of autoimmune response and alloimmune rejection and recipients do not require beta-cell-toxic immunosuppressive drugs. Similar to intrahepatic allogeneic islet transplants, however, autologous islet transplants are subjected to ischemic, hypoxic, and innate inflammatory damage.

Despite recent progress in clinical outcomes, several obstacles for allogenic and autologous islet transplantation remain to be addressed. These include improvement in the technical aspects of the isolation procedure, improvement in the quality of isolated islets, inflammation during the peritransplant period triggered by incompatibility between islets and the blood interface, known as instant blood-mediated inflammatory reaction (IBMIR), and long-term survival of transplanted islets. The common underlying cause that largely contributes to each of the obstacles to clinical success is the production and secretion of inflammatory mediators, which induce apoptosis and impair function of transplanted islets (Figure 1). In this paper, we discuss the causes of inflammation in islets, including inflammation related to donor factors, islet isolation, pretransplant islet culture, the transplant itself, and posttransplant factors.

2. Pancreas Donors

Damage to the islet cells begins with the donor. Immune cell infiltrates and inflammatory mediators are produced in the donor pancreas upon brain death. Most islet transplants use organs from heart-beating, brain-dead (BD) cadaveric donors. Organs from BD donors have much better outcomes than organs from non-heart-beating donors. However, acute physiological changes after brain death in BD donors may still result in significant damage to the islets from inflammatory events. Indeed, several reports have documented poor transplantation outcomes due to organ damage caused by extremely high levels of inflammatory cytokines in the circulation [10–13]. This systemic elevation of inflammatory cytokines, the so-called "cytokine storm" in BD donors, is due in part to hemodynamic and metabolic changes occurring after brain death [14]. Inflammatory cytokines are effective mediators of beta-cell dysfunction and death [15].

Recent studies have demonstrated methods to improve islet graft function by inhibiting BD inflammatory mediators upon procurement. One study showed that maintaining normoglycemia after brain death by insulin reduces inflammatory shock [16]. Transfusing sivelestat sodium, a selective neutrophil elastase inhibitor, was shown to increase islet yield, protect islet function and quality, and inhibit hypercytokinemia-mediated beta-cell death in a brain-dead rat model [17].

3. Ischemic Time

Islets are subjected to hypoxic conditions from the onset of ischemia up until graft revascularization. Hence, ischemic time is a major concern to be addressed to improve islet quality for transplantation. Since islets are physiologically adapted to receiving an ample supply of oxygen, prolonged ischemic times increase damage to islets. Previous study had shown that isolations performed with pancreas with cold ischemic time of >8 hours produces significantly lower islet yields with impaired islet function [18]. The quality of the islets can be improved by reducing organ ischemic time. Another strategy has been used to reduce islet ischemic damage by maintaining the procured pancreas in a high oxygen content solution. A mixture of University of Wisconsin solution along with perfluorocarbon, which has high affinity for oxygen, has been used to reduce islet damage by hypoxia [19].

The viability of islets is significantly reduced due to depletion of ATP and apoptosis induced by hypoxia [20]. Hypoxia in islets activates NF-κB signaling and induces transcription

of inducible nitric oxide synthase (iNOS) and increases expression of MCP-1, leading to infiltration of macrophages and destruction of islets [21]. During hypoxia, islets try to compensate for the low oxygen availability by activating the transcription factor hypoxia-induced factor (HIF) [22]. When activated, the HIF-α and HIF-β subunits translocate to the nucleus and bind to the hypoxia response element. This induces transcription of several genes, including toll-like receptors. Under normal conditions, HIF activity is rendered inactive by prolyl hydroxylases (PHD) and factor inhibiting HIF (FIH). Both PHD and FIH prevent the activation of IκB kinase B and hence inhibit NF-κB activation [23–27].

4. Islet Isolation Process

Isolation of high-quality islets in sufficient quantity remains a major impediment for the further success of this procedure, due to chronic inflammatory conditions affecting the pancreas. The enzymes used for islet isolation could be a factor in the lack of satisfactory results in islet transplantation. It is well documented that enzymatic and mechanical stress can induce inflammatory mediators in islets. Gene array studies have shown upregulation of genes related to inflammation, apoptosis, cell growth, and angiogenesis immediately after isolation, with further increase after 72-hour culture [28]. The most highly upregulated genes have been the ones for inflammation and apoptosis. Isolation and culture of islets have also been shown to downregulate expression of IL-10, which is a negative regulator of cytokine expression and also induces development of regulatory T cells. Conversely, isolation and culture of islets have upregulated the expression of IL-8, which is a potent inflammatory and angiogenic factor, and the expression increased several folds after 3 days in culture [28]. IL-8 has been shown to play a negative role in the longevity of the graft in kidney, liver, and lung transplants; overexpression of IL-8 has been associated with poor graft life [29–31]. Gene array analysis of cultured islets has also shown that the top 15 upregulated genes were all induced by the NF-κB pathway [28]. Therefore, inhibiting the NF-κB pathway during isolation and/or culture might improve islet quality for transplantation.

5. Pretransplant Islet Culture

There has been a continuous debate as to which islets are superior: freshly isolated or cultured islets. Several reports have shown that freshly isolated islets are better than islets cultured for at least 24 hours [32–34]. A recent report from our center confirmed that freshly isolated islets outperform in transplantation when compared to cultured islets to address concerns over the rapid deterioration of islet counts after culture. The study showed a decrease in the islet counts by 13%, 24%, and 35% on days 1, 2, and 3, respectively. When transplanted into diabetic nude mice, the achievement of normoglycemia was significantly improved with fresh islets compared to cultured islets [35, 36]. It has been reported that culturing islets can lead to overexpression of inflammatory mediators caused directly by islets, by stimuli in the culture medium, or by contamination of exocrine tissue in the culture [37]. During culture, islets have to depend on oxygen through diffusion, but the core of the islet begins to undergo hypoxia, causing upregulation of genes induced by oxidative stress and apoptosis [38]. Islets in the culture have been shown to induce expression of proteins such as tissue factor and monocyte chemoattractant protein-1 (MCP-1); this induction could be abrogated by the use of nicotinamide in the culture media [39]. Still, there remains the argument that culturing islets before transplantation will improve the purity of the islets, as acinar cells and damaged islets would die off in the culture. It has also been suggested that providing a culturing period would allow islets to recover from the stress of the isolation process, although there is no compelling data to support this hypothesis. Nonetheless, the Consortium of Islet Transplantation (CIT) has adopted pretransplant culture as a standard procedure for clinical transplants [40].

6. Peritransplant Inflammation

One of the major causes of islet destruction during transplantation is the instant blood-mediated inflammatory reaction (IBMIR) (Figure 2). Bennet et al. first proposed that when freshly isolated "naked" islets come into direct contact with the ABO compatible allogenic blood, activation of innate immune reaction ensues [41]. Tissue factor (TF) expressed on islets could be a major trigger for such reaction. The islets along with resident antigen-presenting cells (APCs) secrete cytokines and chemokines, which play major role in the inflammatory process. Infiltration of leukocytes and macrophages initiate destruction of the islet cells before engraftment [41, 42]. Blocking TF using antibodies significantly reduced the clotting reaction [42]. IBMIR is also characterized by coagulation, complement activation, secretion of chemokines that attract innate immune cells, and release of proinflammatory cytokines leading to islet damage by apoptosis [43].

Although previous studies have shown induction of IBMIR under allogenic and xenogeneic conditions [41, 42], the existence of IBMIR following autologous islet infusion had not been demonstrated in either experimental or clinical settings. Our group recently demonstrated a role of IBMIR in autologous settings [44]. IBMIR was observed in patients undergoing total pancreatectomy followed by autologous islet transplantation. During the initial 0 to 3 hours after infusion of islets, there was a significant increase in markers for coagulation such as thrombin-antithrombin and in proinflammatory cytokines such as interleukin (IL)-6, IL-8, and IP-10 in conjunction with C-peptide, indicating damage to the transplanted islets by the inflammatory response. Further analysis using a miniaturized *in vitro* tube model corroborated the *in vivo* observations and also showed expression of tissue factor (TF) on islets mixed with autologous blood [44]. Therefore, it was concluded that IBMIR is a problem not only in allogenic and xenogeneic islet transplantation but also in autologous islet transplantation.

The IBMIR effect has been characterized by an immediate increase in the thrombin-antithrombin levels after islet

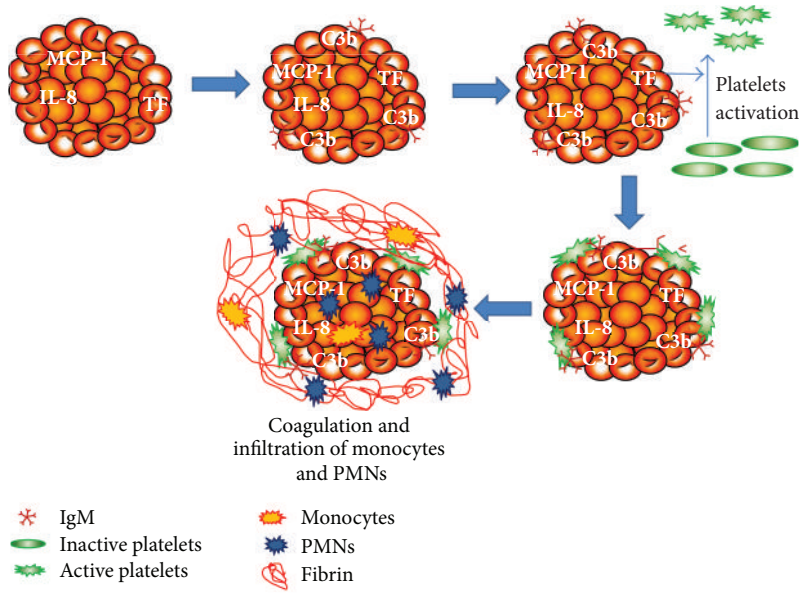

Figure 2: Mechanisms of the instant blood-mediated inflammatory reaction. PMNs: polymorphonuclear cells.

infusion and in the levels of C-peptide in the blood. A recent clinical study utilized labeled islets combined with positron emission tomography and computed tomography, and it has been demonstrated that about 25% of the islets are lost immediately after transplantation [45]. The mechanisms underlying IBMIR and methodologies to block this phenomenon are currently being investigated as described below.

6.1. Coagulation. Coagulation is activated immediately when the islets come in direct contact with the portal blood during transplantation. The coagulation occurs through the extrinsic pathway, which requires TF for activation. TF expressed on the surface of isolated islets interacts with factor VIIa and initiates the coagulation cascade by formation of thrombin and generation of fibrin clots [42, 46, 47]. The production of thrombin at high concentrations can exacerbate the destruction process because at high concentrations thrombin is proinflammatory and an inducer of apoptosis [48, 49]. Özmen et al. showed that the use of anticoagulants such as melagatran could significantly reduce IBMIR *in vitro* in a blood loop model [50]. Johansson et al. previously showed that low-molecular-weight dextran sulfate (LMWDS), a potent inhibitor of coagulation and complement activation, could efficiently suppress IBMIR. They used an *in vitro* tubing loop model to investigate the effect of LMWDS on islets when exposed to human blood, showing effects on activated partial thromboplastin time, platelet function, and complement activation [51]. Other strategies to block IBMIR that have been intensely investigated have included encapsulating islets or coating the islet surface with inhibitory molecules to promote successful islet engraftment. The islet surface has been coated with heparin complex by exploiting biotin-avidin chemistry to inhibit coagulation [52]. Endothelial cells have also been used to coat the islets; transplantation of these coated islets has reduced coagulation, complement activation, and leukocyte infiltration [53, 54]. Alternatively, the use of mesenchymal stem cell-coated islets has been shown to promote endothelial cell proliferation, revascularization, islet neogenesis, and immune modulation [55]. Another study showed that IBMIR could be targeted by overexpressing CD39, which is an ectonucleotidase that degrades ATP required for platelet activation in the islets. *In vivo* and *in vitro* experiments showed less coagulation activity after transplantation using islets from CD39 overexpressing transgenic mice [56].

6.2. Complement Activation. Another major component of IBMIR is the activation of complement cascade. Complement activation is shown by the increase in the concentration of complement proteins in the serum of islet transplant recipients [43, 57]. Activation of complement proteins C3a and C5a leads to recruitment and accumulation of leukocytes, upregulation of adhesion molecules on the endothelium and platelets, and production of reactive oxygen species (ROS) and cytokines [57]. Also, the accumulation of IgG, IgM, and complement proteins C3, C4, and C9 on the surface of human islets immediately after treatment with ABO-compatible blood indicates that immunoglobulin is involved in the activation of the classical complement pathway [57]. Complement protein C5a has been proposed as a critical molecule responsible for activation of coagulation and inflammation by mediating TF expression in neutrophils [58, 59]. A recent short report from Tokodai et al. showed the combined effect of anticoagulant gabexate mesilate and complement protein C5a inhibitory peptide to successfully improve islet transplantation compared to individual use of the drugs in a syngeneic rat transplant model [60]. Thrombin-antithrombin levels were also reduced with the combined treatment. TF was downregulated by complement protein

C5a inhibitory peptide treatment, but only on the liver granulocytes and not on the islet grafts [61]. The use of a complement inhibitor such as compstatin has been shown to significantly improve graft survival in *in vivo* models [62]. In a recent study, it has been reported that immobilization of soluble complement receptor on the islet surface using polyethylene glycol inhibited complement activation significantly and protected islets from damage due to xenoreactive antibodies [63]. In clinical autologous islet transplantation, there is no influence of complement proteins during IBMIR [44].

6.3. Cytokine Secretion, Leukocyte Infiltration, and Beta-Cell Damage. Syngeneic transplant models have shown that the damage to islet grafts is primarily due to a nonspecific inflammatory response [64, 65]. Similarly, clinical data on autologous islet transplantation on patients with CP showed elevated levels of proinflammatory cytokines immediately after transplantation [44]. The islet resident macrophages, Kuppfer cells, and neutrophils secrete IL-1β, which impairs insulin secretion and induces islet cell apoptosis [66–68]. IL-1β activates NF-κB signaling and induces expression of several other inflammatory mediators, including IL-1β, IL-8, IP-10, IL-6, TNF-α, MCP-1, intercellular adhesion molecule-1, vascular cell adhesion molecule-1, endothelial leukocyte adhesion molecule-1, inducible nitric oxide synthase, prostaglandin E2, and prostaglandin EP3 [69–72]. These mediators further induce a cascade of inflammatory events that have deleterious effects on islet graft survival.

In addition to several proinflammatory cytokines, islets also are known to express chemokines after isolation and during culture, of which CXCL10 (IP-10), CXCL8 (IL-8), and CCL2 (MCP-1) are the major ones [28, 43]. IP-10 is widely recognized as a chemokine that attracts leukocytes to the site of infection or inflammation. On the other hand, IL-8 and MCP-1 are known to recruit neutrophils and macrophages, respectively. The role of chemokines receptors and ligands in various transplant rejections has been reviewed elsewhere [73]. In the context of islet transplantation, it has been shown that allograft function is prolonged under anti-CXCL10 antibody therapy and this was due to the lack of infiltration of leukocytes to the graft site [74]. Using $CCL2^{-/-}$ knockout mice, it was demonstrated that reducing CCL2 expression in recipients is more important than knocking out CCL2 in donors. Knocking out CCL2 in the recipient also resulted in abrogation of other chemokines (CXCL1, CCL4, CCL6, and CXCL9) that were upregulated in the liver after transplantation [75]. A recent approach using reparixin, an inhibitor of CXCR1/2, has shown improved islet transplant outcome in allogeneic mouse models. Furthermore, a pilot study on human islet allotransplant patients also showed promising results. Comparison of reparixin-treated group with nontreated controls showed reduced insulin requirement, less HbA1c levels after transplantation, and also improved fasting C-peptide levels. The proposed mechanism is the lack of recruitment of PMN (polymorphonuclear) leukocytes and NKT cells to the liver [76]. Based on these reports, it is conceivable that reducing secretion of proinflammatory cytokines and chemokines during the initial stages of transplantation could improve islet transplant outcomes.

Several reports have shown that allogeneic islet transplant recipients develop antibodies to both human leukocyte antigen (HLA) classes I and II antigens of donor origin [77–79]. While it is evident that islets constitutively express HLA class I antigens, the expression of HLA class II on isolated human islets has not been clearly demonstrated. Upon stimulation with inflammatory cytokines of TNF-α and IFN-γ, islet-enriched pancreatic cultures and MIN6 cell lines have been shown to express HLA class II antigens [80, 81]. Recently, our group has demonstrated that under inflammatory conditions, there is induction of HLA class II expression on the islet cell surface leading to the development of antidonor class II antibodies in the recipient [82]. Islets isolated from deceased donor pancreases were used to demonstrate induction of HLA class II molecules through real-time polymerase chain reaction analysis, immunofluorescent staining, and flow cytometry. One of the transplant recipients developed antibodies to a mismatched donor HLA class II allele (DR7). Posttransplant serum from this patient showed significant binding to cytokine-stimulated islet cells from DR7-positive donors only. These results clearly demonstrate that human islets express HLA class II under inflammatory conditions. This could lead to an anticlass II response, which in turn may play a critical role in rejection of islet allografts.

7. Posttransplant Inflammation

7.1. Hypoxia. Like other transplantable organs, islets are prone to ischemic reperfusion injury. When isolated islets are infused into the blood circulation, several cytotoxic products are produced due to oxygen metabolism, which is catalyzed by xanthine oxidase [83]. Activation of this enzyme results in the formation of superoxide and hydrogen peroxide, which can interact with free iron and copper radicals to produce cytotoxic reactive oxygen species (ROS) [84]. ROS can induce cell death by damaging and degrading proteins, lipids, and nucleic acids and activating NF-κB inflammatory pathways [85]. Beta cells of the islets are more vulnerable to attack by ROS because of their low expression of antioxidants, including glutathione peroxidase, catalase, and superoxide dismutase [86–88]. Indeed, increasing the expression of cellular antioxidants and treatment with exogenous antioxidants have both been shown to protect islets in a diabetes models [89, 90].

Following intraportal infusion, there is a low oxygen supply at the graft site in the liver, creating a hypoxic environment for the islets [91, 92]. Islet cells have countermechanisms to restore the oxygen supply [22]. Eventually, islet grafts begin to vascularize, but the amount of oxygen supply even after hepatic vascularization is significantly lower than in the pancreas (5 mm Hg as opposed to 40 mm Hg) [93]. Although liver is currently the preferential site for islet transplantation, it is still not considered the optimal host site because of the lack of oxygen; therefore other sites that might improve the oxygen availability to the islets are still under consideration.

Cantarelli et al., for example, have provided evidence that bone marrow may be a more suitable graft site for islet transplantation because of the increased vasculature and abundant supply of oxygen [94]. Furthermore, pilot studies on clinical autologous islet transplants have been performed in bone marrow which outperformed islet grafts in the liver [95].

7.2. Innate Immunity. Pancreatic islet inflammation after transplantation is induced by innate immune cells such as neutrophils, islet resident macrophages, and Kupffer cells. Kupffer cells can induce damage to other cells by releasing free radicals and secreting proinflammatory cytokines [96]. The amount of proinflammatory cytokines IL-1β and TNF-α secreted by Kupffer cells is increased after islet transplantation [97]. This inflammatory cytokine elevation is blocked in the case of a macrophage-depleted recipient. Several factors have been postulated to explain the activation of Kupffer cells in the liver after islet transplantation. One report suggested that the collagenase used for islet isolation contains endotoxin, which could activate production of proinflammatory cytokines by islet-resident macrophages and in turn activate Kupffer cells [98]. The presence of acinar tissue in the islet transplant preparation has also been investigated as the cause for activation of Kupffer cells. It has been demonstrated that the amount of exocrine contamination is directly correlated to posttransplant necrosis and lower viability of pancreatic islet cells. It has been postulated that when these acinar cells die, they release enzymes that trigger an inflammatory response leading to islet cell death [99]. Another hypothesis is that the professional APCs residing inside the islets are responsible for inducing the inflammatory response. A recent study on the intraislet immune cell population showed that 67% of the immune cells in the islets are APCs, and 50% of these APCs are B cells [100]. Use of anti-inflammatory agents that block the production and secretion of these proinflammatory cytokines and chemokine would be a useful strategy to protect islets from innate immune cell-mediated damage. Recently we have shown that use of Withaferin A, which is an NFκB inhibitor, protected islet viability, reduced secretion of proinflammatory chemokines and cytokines, and reduced infiltration of neutrophils in a tube model [101].

8. Inflammatory Response from Islets

Islets can also produce inflammatory mediators in response to metabolic, inflammatory, physical, and chemical stress. For example, IL-1β induces production of cytokines, chemokines, and iNOS in pancreatic beta cells [102–104]. In the context of islet transplantation, multiple islet stress events may contribute to cascading cytokine-induced cytokine production in islets. Thus, cytokines produced and released by beta cells throughout the islet transplant process may exacerbate islet inflammation.

MAP kinases ERK1/2, p38, and JNK have roles in islet dysfunction and apoptosis in response to stress-specific signaling pathways [105, 106]. Calcineurin-dependent ERK1/2 activation has short-term stimulatory effects on insulin production and islet function in response to glucose and secretagogues; however, sustained activation due to metabolic and inflammatory stress may have deleterious effects [107–110]. Upon exposure to cytokines, JNK and p38 are activated by MAP kinase kinase (MKK) 4 and MKK3/6, respectively [106]. Additionally, exposure of islets to stress from the islet isolation process activates JNK and p38 via MKK7 signaling. One study showed that an increased p38/JNK activation relative to ERK1/2 activation after islet isolation correlates with decreased islet survival [111]. Hence, identifying methods to selectively block p38 and JNK pathways, while preserving acute ERK1/2 signaling, may enhance islet graft function and survival in islet transplantation [112–116].

Acute exposure of islets to low dose of IL-1β has been shown to stimulate proliferation and enhance beta-cell function, whereas exposure to high dose of IL-1β impairs secretion and induces apoptosis [117–119]. Induction of TNF-α expression in beta cells by IL-1β requires activation of calcineurin and downstream transcription factor nuclear factor of activated T cells (NFAT) [102]. Extended exposure of beta cells to IL-1β results in sustained MAP kinase signaling, which activates nuclear factor-kappa B (NF-κB-)dependent genes that are proapoptotic [102, 120, 121]. Although a number of reports suggest that NF-κB is responsible for apoptosis of transplanted islet cells [122], other reports have presented conflicting evidence suggesting that NF-κB prevents islet cell death [123]. This is also likely attributed to the strength and duration of exposure as well as the type of stimulus, as both NFAT and NF-κB have overlapping roles in cytokine gene expression and beta-cell proliferation and function [102, 123–126]. Thus, minimizing sustained activation of MAP kinase and NFAT/NF-κB signaling during the transplantation process is likely key to reducing islet inflammation and improving islet transplant outcomes.

9. Anti-inflammatory Strategies to Improve Islet Transplantation

Anti-inflammatory agents have been incorporated in immunosuppressive regimens in recent clinical allogeneic islet transplant protocols [127, 128]. A recent review has summarized progress related to this approach [14]. In the era of 1999 to 2002, TNF-α inhibitors were utilized in a small proportion (11.8%) of islet transplants, while in the 2007 to 2010 period, they were used in 33.8% of transplants [129]. The first report of islet transplantation involving an anti-inflammatory agent came from the University of Minnesota, where etanercept was incorporated in peritransplant immunosuppression therapy [130]. All the islet recipients achieved insulin independence after a single islet infusion. The University of Miami group implemented an immunosuppression protocol containing infliximab, which resulted in a high success rate of insulin independence (87.5%) [131]. Recently, a meta-analysis showed significant improvement in the insulin independence rate when a TNF-α inhibitor was coupled with a T-cell depletion regimen, compared with T-cell depletion only [132]. The success rate

with T-cell depletion immunosuppression plus a TNF-α inhibitor is now likely comparable to that of whole-organ pancreas transplantation alone, offering approximately 50% success at 5 years after transplant [129, 132, 133].

No significant difference in the success rate, however, was seen between a T-cell-depleting islet protocol with and without infliximab in a clinical study [131]. In an immunodeficient rodent model transplanted with a marginal mass of human islets, no significant benefits in islet engraftment were observed when only etanercept was given, whereas significant improvement in diabetes reversal was demonstrated when both etanercept and anakinra, an IL-1β receptor antagonist, were administered [134]. Thus, administration of a TNF-α inhibitor alone might be insufficient to fully control peritransplant inflammation. Interestingly, our group has implemented combined therapy of etanercept and anakinra in clinical allogenic islet transplantation and has shown that all patients achieved insulin independence after a single infusion, although the number of islet recipients was limited [135]. A large, well-designed clinical study is required to evaluate the impact of anti-inflammatory agents in islet engraftment. The use of anti-inflammatory agents in clinical allogeneic islet cell transplantation is summarized in Table 1.

In our recent study, we have shown that a marginal dose of withaferin A, an anti-inflammatory compound and a potent NF-κB inhibitor, can significantly improve graft survival in a syngeneic mouse islet transplant model. The islet damage due to proinflammatory cytokines and apoptosis was abolished [116]. The use of an NF-κB inhibitor to reduce islet inflammation and protect islets from apoptosis is helpful in improving graft function and survival. Since NF-κB is a critical transcription factor that can induce the expression of various proinflammatory cytokines, its activation needs to be prevented during transplantation until the graft revascularization is complete.

Identification of islet-specific biomarker(s) is necessary to evaluate islet damage by inflammation. Recently, high-mobility group box 1 (HMGB1) protein was shown to be released specifically from islet grafts immediately after transplant in a syngeneic rodent model [136]. The HMGB1 release was also observed following cytokine stimulation, and anti-HMGB1 treatment was able to restore glycemic control in diabetic mice with a marginal mass of syngeneic islet grafts. Furthermore, similar findings were seen in a study using human islets, which released a higher amount of HMGB1 under hypoxic conditions *in vitro*, corresponding to the degree of apoptosis [137]. In human autologous islet transplantation for patients with CP, significant elevation of serum HMGB1 levels was also observed during islet infusion, although no elevation of HMGB1 levels was found in patients who underwent total pancreatectomy alone [138]. Interestingly, there was a significant difference in changes in HMGB1 levels during the peritransplant period between patients achieving insulin independence and those who continued to require insulin therapy after transplant. These findings suggest that serum HMGB1 levels can be a useful clinical biomarker to evaluate islet damage.

Several anti-inflammatory compounds are currently being investigated and more emerging to improve the outcome of islet transplantation. A very exhaustive review on the strategies and approaches to improve clinical islet transplantation has been mentioned elsewhere [14]. Pileggi et al. have shown that expression of heme oxygenase-1 (HO-1) in islet cells protected the islets from apoptotic death. To induce HO-1 in islets, the cells were cultured in ferriprotoporphyrin IX chloride and cobaltic protoporphyrin IX chloride before transplantation [139]. The HO-1 gene plays an important role in iron homeostasis but also behaves as a potent anti-inflammatory and antiapoptotic gene [140, 141]. A similar study has shown the antiapoptotic effect when islets are treated with carbon monoxide. This treatment causes islets to produce cytoprotective cyclic guanosine monophosphate and its downstream kinases [142]. Another strategy that has shown promise is the expression of the A20 gene in islets by *ex vivo* gene transfer, which protects these islets from cytokine-induced damage. A20 is an inhibitor of NF-κB, which is a transcriptional regulator of several proinflammatory cytokines [143]. A different study involving the use of diannexin, which is a homodimer of annexin 5, has demonstrated a significant decrease in β-cell apoptosis and improved graft function after *in vivo* transplantation [144]. The use of JNK inhibitor SP600125 in combination with nicotinamide and simvastatin protected porcine islets from peritransplant inflammation and apoptosis. Intraductal administration of JNK inhibitor protected islets by inhibiting the production of proinflammatory cytokines IL-1β, TNF-α, IFN-γ, IL-6, IL-8, and MCP-1 *in vivo* [145].

10. Future Direction

Inflammation during islet transplantation is multifaceted and the underlying mechanisms are quite diverse. Several factors could play critical roles in the induction or exacerbation of inflammation. Therefore, it is challenging to identify one particular approach or strategy to eliminate all inflammatory events in islets. A combinatorial approach based on the current understanding of inflammatory mechanisms will likely be required to improve islet transplant outcomes. Overall, isolation of islets from pancreas with less ischemic time is an important aspect. Furthermore, culturing islets in conditions that are suitable for islet survival and use of antioxidants and anti-inflammatory compounds including potent inhibitors of NFκB, which are known to regulate several proinflammatory genes, is important. Since a significant mass of islets is immediately lost to IBMIR, major focus should be to reduce this response. Although the use of anticoagulants or complement inhibitors shows benefits in *in vitro* and *in vivo* settings, they fail to perform effectively in the clinical settings. Moreover, in allogenic transplants, the inflammation is severe and therefore a combined anti-inflammatory regimen to reduce ensuing immune response would be required. For instance, LMWDS has shown potential benefits to prevent clotting. This could be combined with anti-inflammatory compounds such as anakinra, etanercept, and with complement inhibitors to form a complete regimen to block IBMIR. Some of the

Table 1: Anti-inflammatory agents in clinical allogeneic islet cell transplantation.

Publication year	Pt number	Anti-inflammatory agents	Dose of anti-inflammatory agents	Induction therapy	Maintenance therapy	Major outcomes	References
2005	8	Etanercept	50 mg i.v. pretransplant and 25 mg s.c. on days 3, 7, and 10	rATG Daclizumab	Sirolimus Tacrolimus MMF	100% II after single infusion	[130]
2005	8	Infliximab	5 mg/kg i.v. pretransplant	Daclizumab	Sirolimus Tacrolimus	7/8 recipients achieved II	[131]
2008	7	Infliximab or Etanercept	5 mg/kg i.v. pretransplant or 50 mg i.v. pretransplant and 25 mg s.c. twice weekly for 2 weeks	Daclizumab	Sirolimus Tacrolimus (Low-dose steroids for 3 pts)	6/7 recipients achieved II	[146]
2008	3	Etanercept	50 mg i.v. pretransplant and 25 mg s.c. twice weekly for 2 weeks	Alemtuzumab	Sirolimus Tacrolimus MMF	2/3 recipients achieved II	[147]
2008	6	Etanercept	50 mg i.v. pretransplant and 24 mg s.c. on days 3, 7, and 10	rATG (for first transplant) Daclizumab (for 2nd transplant)	Cyclosporine Everolimus	5/6 recipients achieved II	[148]
2008	6	Etanercept	50 mg i.v. pretransplant and 24 mg s.c. on days 3, 7 and 10	Daclizumab	Sirolimus Tacrolimus	6/6 recipients achieved II	[149]
2011	3	Etanercept and anakinra	50 mg i.v. pretransplant and 25 mg s.c. on days 3, 6, and 10 and 100 mg i.v. pretransplant and 100 mg s.c. for 7 days after transplant	rATG	Tacrolimus MMF	3/3 recipients achieved II	[135]
2012*	22	Anti-TNF-α	NA	T-cell depletion protocol	NA	50% of recipients kept II for 5 years	[132]

i.v. indicates intravenous injection; s.c., subcutaneous injection; rATG, rabbit antithymocyte globulin; MMF, mycophenolate mofetil; II, insulin independence; TNF, tumor necrosis factor.
*Based on collaborative islet transplant registry, collecting data from multiple centers.

recent clinical studies, such as use of reparixin to block CXCR1/2, have shown promising outcomes. Several inflammatory mechanisms that cause dysfunction of islets that have been discussed here should be addressed at the clinical level. Future studies should focus on these mechanisms to develop strategies for successful islet transplantation.

11. Conclusion

The inflammatory response has been shown to be a major cause of posttransplant islet graft failure in both an experimental animal model and in clinical studies. Inflammation can be triggered by multiple factors: brain death in the pancreas donor, ischemia during pancreas preservation, physiologic and enzymatic stress during islet isolation, islet infusion, and recipient's innate immunity. TNF-α inhibition has been demonstrated as a potent anti-inflammatory strategy in clinical allogeneic islet transplantation, although other agents, including NF-κB inhibitors, have also been studied for the prevention of an inflammatory reaction in experimental models. More precise and deeper understanding of the inflammatory response to transplanted islets is warranted. Control of inflammation could improve the efficacy of islet cell transplantation, which in turn could enhance the long-term function of transplanted islets.

Conflict of Interests

The authors of this paper have no conflict of interests to disclose.

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