Figure 2

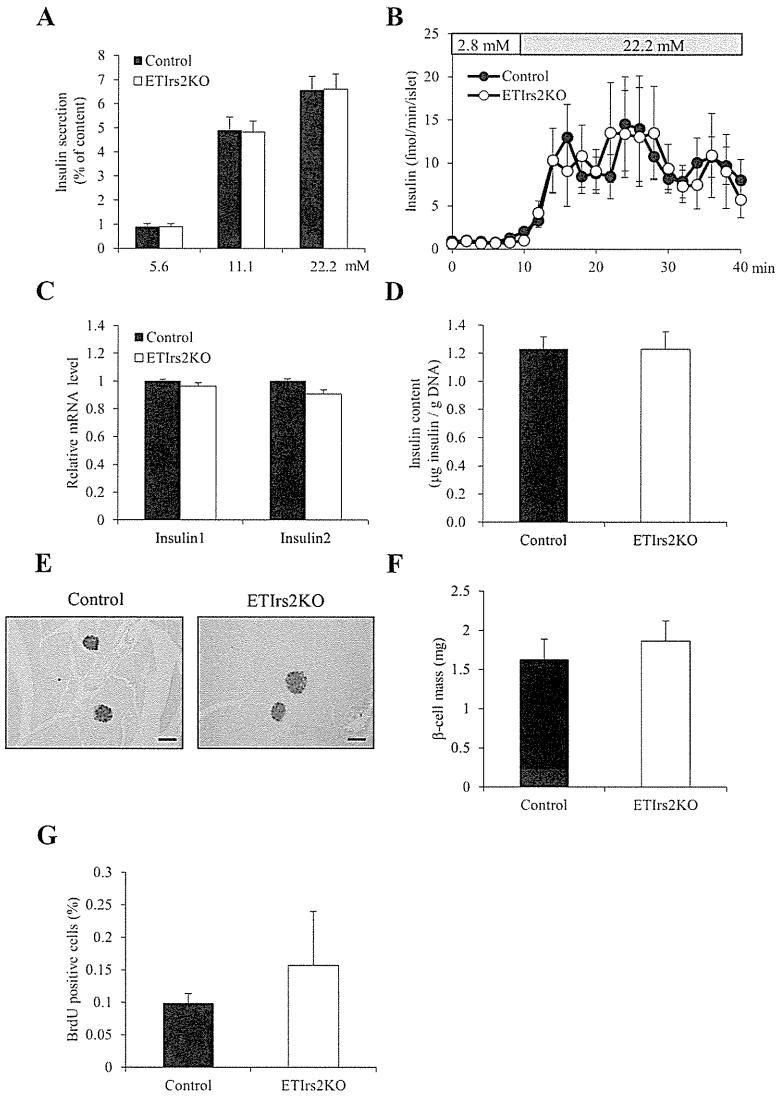

Figure 2- Insulin secretion in the isolated islets was not impaired in the ETIrs2KO mice. (A) Glucose-induced insulin secretion using batch-incubated islets at 12 weeks (n = 9). (B) Glucose-induced insulin secretion as assessed in an islet perifusion experiment at 12 weeks (n = 8). (C) mRNA expression levels expressed as the values in the islets of the ETIrs2KO mice relative to those of the control mice at 12 weeks (n = 5). (D) Insulin content per DNA concentration in islets isolated from the control and the ETIrs2KO mice at 12 weeks (n = 8). (E) Insulin staining of pancreatic sections from the control and the ETIrs2KO mice at 24 weeks (scale bar = 100 μm). (F) The β-cell mass was calculated as estimated islet weight (n = 5). (G) Replication rate of β-cells assayed on the basis of BrdU incorporation in the control and the ETIrs2KO mice at 24 weeks (n = 7). Results are shown as the percentage of BrdU-positive cells relative to the total number of β-cells. Values are the mean ± SE.
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Figure 3

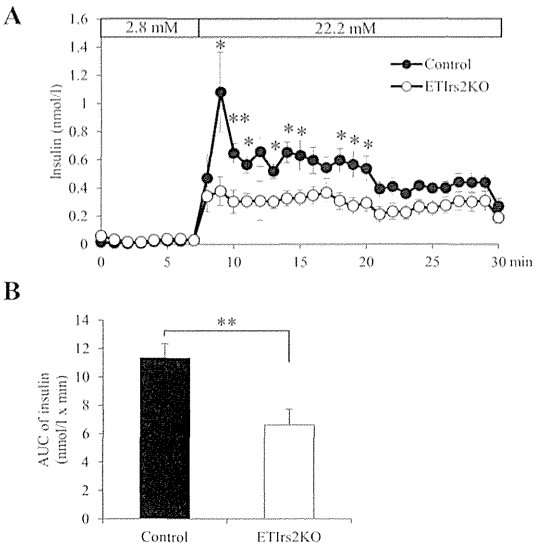

Figure 3- Insulin secretion was significantly decreased in the ETIrs2KO mice during pancreatic perfusion. (A) Insulin secretion from perfused pancreata of the control and the ETIrs2KO mice at 12 weeks (n = 8). (B) Amounts of insulin secreted in the control and the ETIrs2KO mice after glucose stimulation expressed as the AUCinsulin values from 5 to 30 min in A. Values are the mean ± SE. Statistical significance is depicted as * (P < 0.05) and ** (P < 0.01).
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Figure 4

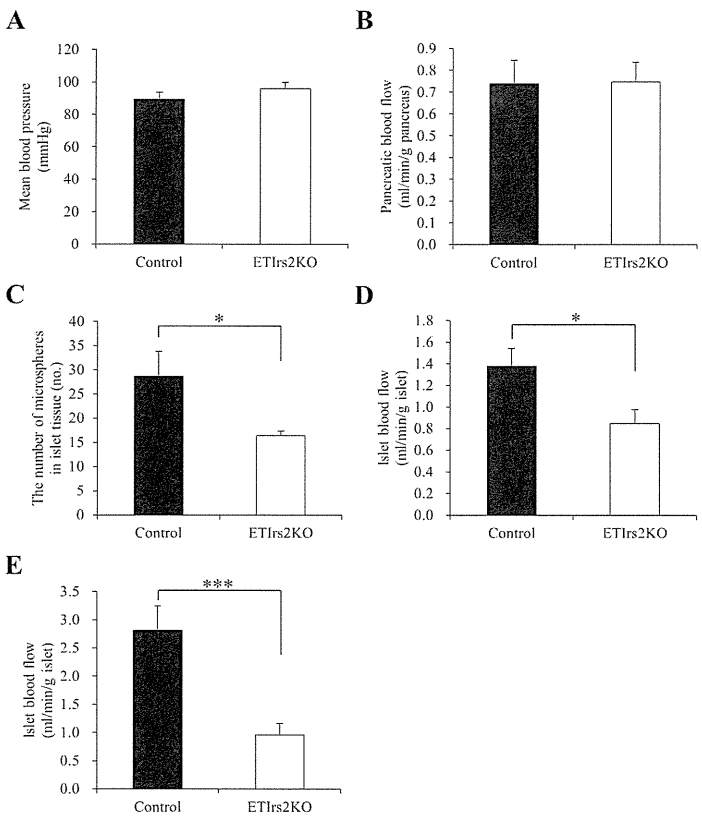

Figure 4- The islet blood flow was significantly decreased in the ETIrs2KO mice. The mean blood pressure (A), pancreatic blood flow (B), the number of microspheres in islet tissue (C) and islet blood flow (D) in anaesthetized 9-12-week-old control and ETIrs2KO mice in the basal state (n = 10). The islet blood flow (E) in anaesthetized 9-week-old control and ETIrs2KO mice in insulin-treated state (n = 10) Values are the mean ± SE. Statistical significance is depicted as * ($P < 0.05$) and *** ($P < 0.001$).
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Figure 5

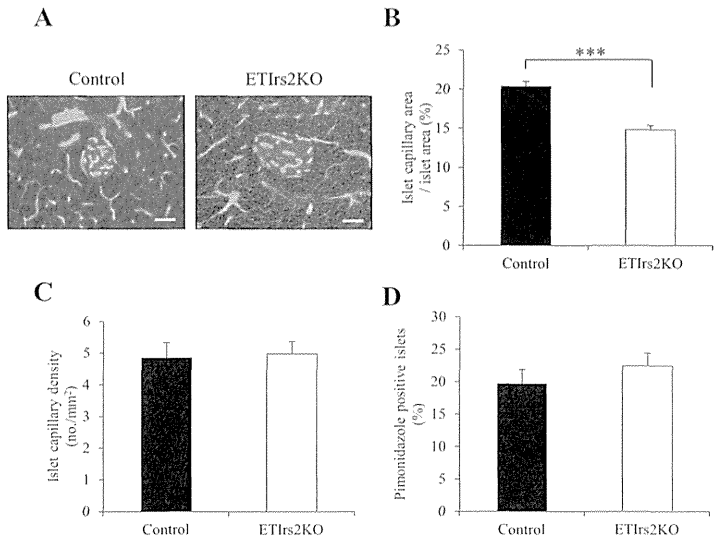

Figure 5- The capillary area stained by lectin was significantly decreased in the islets of the ETIrs2KO mice. Lectin staining of pancreatic section from the control and the ETIrs2KO mice at 12 weeks (scale bar = 50 μm). The approximate islet boundary is marked by a dotted line (A). Vessel area expressed as a relative value to the total islet volume % (B) and the number of capillaries per square millimeters of islet (C). Pimonidazole-positive islets were quantitatively assessed as a percentage of the total number of islets (D). Values are the mean ± SE for 5 animals. Statistical significance is depicted as *** ($P < 0.001$).
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Figure 6

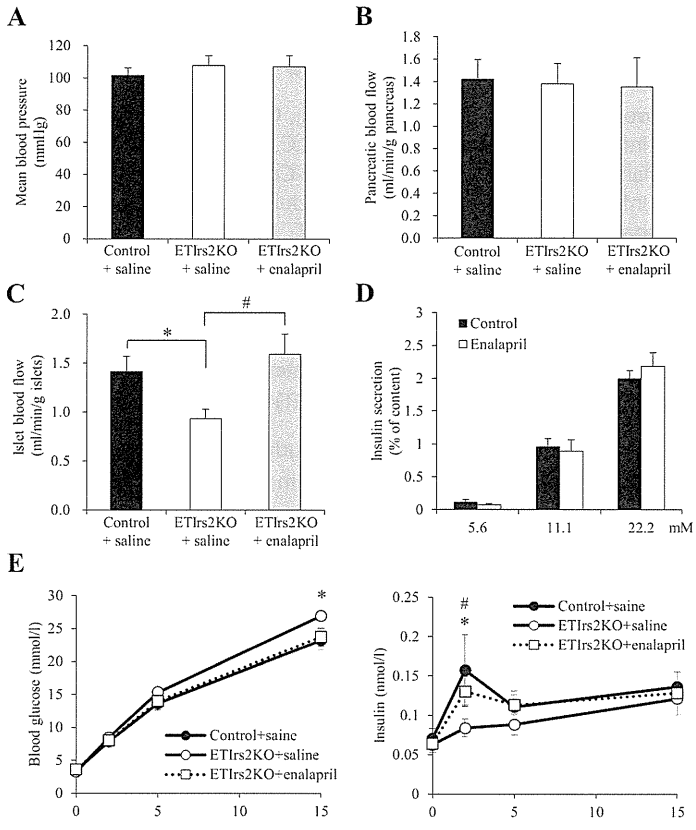

Figure 6- The islet blood flow was improved by treatment with an ACE-inhibitor, enalapril maleate, resulting in the amelioration of insulin secretion in the ETIrs2KO mice. The mean blood pressure (A), the pancreatic blood flow (B), and the islet blood flow (C) in anaesthetized 9-12-week-old control and ETIrs2KO mice at 10 min after the intravenous injection of saline or enalapril maleate (n = 10). (D) Effect of enalapril maleate on glucose-induced insulin secretion using batch-incubated islets of C57BL/6J mice at 12 weeks (n = 5). (E) Blood glucose levels (left) and plasma insulin levels (right) during an intraperitoneal glucose tolerance test in 12-week-old control and ETIrs2KO mice at 10 min after the intravenous injection of saline or enalapril maleate (n = 11-13). Values are the mean ± SE. Statistical significance is depicted as * ($P < 0.05$, control + saline vs. ETIrs2KO mice + saline) and # ($P < 0.05$, ETIrs2KO mice + saline vs. ETIrs2KO mice + enalapril).
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Ethnic Differences in Insulin Sensitivity, β-Cell Function, and Hepatic Extraction Between Japanese and Caucasians: A Minimal Model Analysis

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Context: Ethnic differences have previously been reported for type 2 diabetes.

Objective: We aimed at assessing the potential differences between Caucasian and Japanese subjects ranging from normal glucose tolerance (NGT) to impaired glucose tolerance (IGT) and to type 2 diabetes.

Design: This was a cross-sectional study with oral glucose tolerance tests to assess β-cell function, hepatic insulin extraction, and insulin sensitivity.

Participants: Participants included 120 Japanese and 150 Caucasian subjects.

Main Outcomes: Measures of β-cell function, hepatic extraction, and insulin sensitivity were assessed using C-peptide, glucose, and insulin minimal models.

Results: Basal β-cell function (Φ_b) was lower in Japanese compared with Caucasians ($P < .01$). In subjects with IGT, estimates of the dynamic (Φ_d) and static (Φ_s) β-cell responsiveness were significantly lower in the Japanese compared with Caucasians ($P < .05$). In contrast, values of insulin action showed higher sensitivity in the Japanese IGT subjects. Hepatic extraction was similar in NGT and IGT groups but higher in Japanese type 2 diabetic subjects ($P < .01$). Despite differences in insulin sensitivity, β-cell function, and hepatic extraction, the disposition indices were similar between the 2 ethnic groups at all glucose tolerance states. Furthermore, the overall insulin sensitivity and β-cell responsiveness for all glucose tolerance states were similar in Japanese and Caucasians after accounting for differences in body mass index.

Conclusion: Our study provides evidence for a similar ability of Japanese and Caucasians to compensate for increased insulin resistance. **(*J Clin Endocrinol Metab* 99: 4273–4280, 2014)**

The existence of ethnic difference in the pathogenesis of type 2 diabetes has been reported by several groups (1–16). Many of these studies investigated the hypothesis that Japanese cannot compensate with increased insulin secretion when progressing from normal glucose tolerance (NGT) to impaired glucose tolerance (IGT) to the same degree as Caucasians. Some of these studies refer specifically to the difference in first-phase insulin response (2, 7), although conflicting results have been reported (6).

ISSN Print 0021-972X ISSN Online 1945-7197
Printed in U.S.A.
Copyright © 2014 by the Endocrine Society
Received March 13, 2014. Accepted August 5, 2014.
First Published Online August 13, 2014

Abbreviations: BMI, body mass index; DI, disposition index; HE, hepatic extraction ratio; IGT, impaired glucose tolerance; NGT, normal glucose tolerance; OGTT, oral glucose tolerance test; OMM, oral minimal model; S_I, insulin sensitivity index.

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Comparisons between Japanese and Caucasians have generally been based on data from studies with different inclusion criteria, which potentially could lead to biased and misleading conclusions. To our knowledge, there are no reports from studies where enrolled subjects have been matched for the purpose of studying potential ethnic differences in type 2 diabetes development. We therefore designed a cross-sectional study with Japanese and Caucasians and included subjects with NGT, IGT, and type 2 diabetes. To obtain indices for pathophysiological characterization of disease state, an oral glucose tolerance test (OGTT) was performed for all subjects. Based on the OGTT data, we estimated classic measures for β-cell function and insulin sensitivity such as the homeostatic indices (17) and the insulinogenic index (18).

These measures for β-cell function and insulin sensitivity have been widely applied in previous analyses of ethnic differences (1, 19). One drawback with these methods is that they take into account only a fraction of the available data obtained from the OGTT and that they are typically estimated using glucose and insulin samples, instead of C-peptide. Because approximately 50% of newly secreted insulin is degraded in the liver (20) and the hepatic extraction ratio (HE) can be different from one individual to another, such measures can potentially lead to inaccurate conclusions.

Alternatively, β-cell function may be estimated by mathematical models based on C-peptide concentrations to obtain measures of the insulin secretion rate. For such an assessment, the oral minimal model of C-peptide secretion (21) has been developed to provide indices for both first- and second-phase insulin secretion estimated as the dynamic and static β-cell responsiveness indices, respectively (22–24). When combined with a model for insulin kinetics, information of hepatic extraction during an OGTT can be obtained, thus providing a more complete metabolic description as compared with classic methods (25).

It is well established that type 2 diabetes is characterized by insulin resistance accompanied by β-cell defects (26, 27). Initially, insulin resistance may be compensated by increased β-cell response, and progression to type 2 diabetes is thought to occur when β-cells fail to compensate to a sufficient degree (28). The disposition index (DI) reflects β-cell function relative to the prevailing insulin sensitivity and can be used as a surrogate marker for the diabetes state from a pathophysiological point of view (29).

The present study was undertaken to further investigate our previously reported results (30) obtained in matched cohorts of Caucasian and Japanese subjects ranging from NGT to IGT and type 2 diabetes, by means of minimal models for β-cell function, insulin sensitivity, and hepatic extraction. The aim was to obtain further insights into the pathophysiology that characterizes each state of glucose tolerance in Japanese and Caucasians, beyond the results obtained using simple indices. We applied the oral minimal models (21, 25, 31) on data from frequently sampled OGTTs, which allowed for determination of measures of basal, static, and dynamic β-cell function, insulin sensitivity, hepatic extraction, and DIs in each glucose tolerance state.

Subjects and Methods

Study design and participants

This study included 150 subjects enrolled at Rigshospitalet, Copenhagen, Denmark (of Northern European background for at least 3 generations), and 120 subjects enrolled at Tokyo University Hospital in Japan (of Japanese background for at least 3 generations). The 270 subjects were stratified according to body mass index (BMI) and glucose tolerance state (NGT, IGT, or type 2 diabetes, as defined in the World Health Organization criteria) (32). The aim was to recruit cohorts with similar distribution of subjects, with high and low BMI within each glucose tolerance group. Cutoffs for high/low BMI were 25 kg/m^2 in Japanese and 30 kg/m^2 in Caucasians in accordance with regional definitions of obesity. Subjects were aged 40 to 65 years, and each cohort had a similar distribution of males and females. Baseline characteristics and distribution of subjects are presented in Table 1. The study protocol was approved by the Regional Committee on Biomedical Research Ethics in Denmark (Journal no. H-C-2008–101) and by the Research Ethics Committee of the Graduate School of Medicine, University of Tokyo, Japan. Informed consent was obtained from all participants, and the handling of data was approved by the Danish Data Protection Agency. The present study is part of a clinical trial having the registration number NCT 00897169.

Procedures

Procedures were carried out as previously described (30). Briefly, all subjects received an oral bolus corresponding to 75 g glucose. Plasma samples were collected at times −30, 0, 10, 20, 30, 60, 90, 120, 150, 180, 240, and 300 minutes for determination of plasma glucose and serum insulin and C-peptide and were assayed at the same laboratory (Uni-Labs, Copenhagen, Denmark) using validated methods. To obtain measures for β-cell responsiveness and insulin sensitivity, we applied the oral minimal models (OMMs) for C-peptide and glucose, respectively (29). The OMM for C-peptide provides measures of basal, dynamic, and static (Φ_b, Φ_d, and Φ_s) β-cell responsiveness, whereas the OMM for glucose provides measures of insulin sensitivity (insulin sensitivity index [S_I]). The combination of the C-peptide model with a model for posthepatic insulin delivery rate proposed by Campioni et al (25) was applied for estimation of hepatic extraction in the basal condition and after glucose challenge. Disposition indices (DI_d and DI_s) were calculated as the product of indices for β-cell responsiveness and insulin sensitivity, respectively. All estimation procedures for the minimal models were carried out using a single-subject approach and commercial software package MATLAB version 14 (MathWorks, Inc).

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Table 1. Baseline Demographics of Study Participants[a]

	Caucasians			Japanese		
	NGT	IGT	Type 2 diabetes	NGT	IGT	Type 2 diabetes
Total	63	39	48	46	26	48
Low BMI[b]	32 (51%)	14 (36%)	24 (50%)	25 (54%)	12 (46%)	27 (56%)
Age, y	53 (7)	54 (8)	57 (7)	49 (7)	54 (8)	57 (7)
Sex (male)	29 (46%)	15 (38%)	29 (60%)	21 (46%)	12 (46%)	33 (69%)
Height, m	1.7 (0.1)	1.7 (0.1)	1.8 (0.1)	1.6 (0.1)	1.6 (0.1)	1.6 (0.1)
BMI, kg/m^2	29.8 (5.9)	33.0 (6.3)	30.4 (5.7)	24.0 (3.2)	26.3 (5.0)	25.3 (4.4)
Waist-to-hip ratio	0.91 (0.09)	0.94 (0.08)	0.97 (0.08)	0.93 (0.05)	0.94 (0.05)	0.95 (0.05)
FPG, mmol/L	5.5 (0.5)	5.9 (0.4)	8.3 (2.0)	5.5 (0.5)	6.0 (0.6)	7.7 (1.3)
FSI, pmol/L	46 (36)	56 (34)	77 (47)	30 (19)	36 (24)	36 (23)

Abbreviations: FPG, fasting plasma glucose; FSI, fasting serum insulin.

[a] Data are presented as number of participants (percentage) or mean (SD). To convert the glucose values to milligrams per deciliter, divide by 0.05551. To convert the values for insulin to milliunits per liter, divide by 6.

[b] Cutoffs for the low-BMI group were <25 and <30 kg/m^2 for Japanese and Caucasians, respectively.

Statistical analysis

Data are presented as means ± SE. For each index (Y) of insulin sensitivity, β-cell function, and hepatic extraction, an ANOVA was used to test for overall effects and interaction of glucose tolerance state and ethnicity: logY ~ state + ethnicity + ethnicity:state, where state and ethnicity indicate effects of glucose tolerance state (NGT, IGT, type 2 diabetes) and ethnicity (Japanese, Caucasian), and ethnicity: state indicates interaction between effects of ethnicity and glucose tolerance state. *P* values for effects of ethnicity and interactions between glucose tolerance state and ethnicity were obtained from this ANOVA model with and without BMI included in the model as covariate.

In addition, comparisons between Japanese and Caucasians were performed using the unpaired *t* test in each subgroup to get further insight into possible differences in each glucose tolerance state. A *P* value $< .05$ was considered significant.

Results

Plasma glucose, insulin, and C-peptide profiles

Mean concentration vs time profiles of glucose, insulin, and C-peptide are shown for NGT, IGT, and type 2 diabetes groups in Figure 1. For glucose profiles, both baseline and maximal concentrations appeared similar between Caucasians and Japanese, but slightly different in their return to baseline for the NGT and IGT groups.

In contrast, basal and maximum concentrations of insulin were significantly higher in Caucasians in all glucose tolerance groups ($P < .05$). For all groups, it was further observed that the insulin response in Caucasians appeared higher than in Japanese at time points after 30 minutes. A similar pattern was observed for C-peptide concentrations, with higher concentrations in Caucasians than in Japanese both at baseline and at maximum concentrations in all glucose tolerance groups.

Insulin action

Mean values and SEM for indices of insulin sensitivity (S_I), β-cell responsiveness (Φ_b, Φ_d, and Φ_s), and hepatic extraction ratios (HE_b and HE_{post}) in each glucose tolerance state are presented in Figure 2. A decrease in insulin sensitivity from NGT to type 2 diabetes was observed for both Japanese and Caucasians. The insulin sensitivity was found to be significantly lower in Caucasian IGT subjects compared with Japanese IGT subjects ($P < .05$), but no significant difference was found for NGT or type 2 diabetes subjects. Results from an ANOVA of S_I which included all glucose tolerance states showed that the overall effect of ethnicity was borderline significant ($P = .06$, Table 2).

β-Cell function

No clear trend for β-cell responsiveness during fasting was observed between NGT, IGT, and type 2 diabetes, but basal β-cell responsiveness was significantly lower in Japanese compared with Caucasians in all glucose tolerance groups ($P < .01$, Table 2). In contrast, the dynamic and static β-cell function indices for both cohorts decreased markedly from NGT to type 2 diabetes. For the dynamic response, a significantly lower mean value was found for Japanese IGT subjects compared with Caucasian IGT subjects ($P < .05$), and a similar difference between the 2 IGT groups was found for the static index ($P < .01$). The ANOVA (Table 2) revealed no overall ethnic difference for the dynamic index, whereas the static index was borderline significantly lower in Japanese compared with Caucasians ($P = .05$).

Hepatic extraction

Caucasians and Japanese had similar hepatic insulin extraction in both the NGT and the IGT group, although

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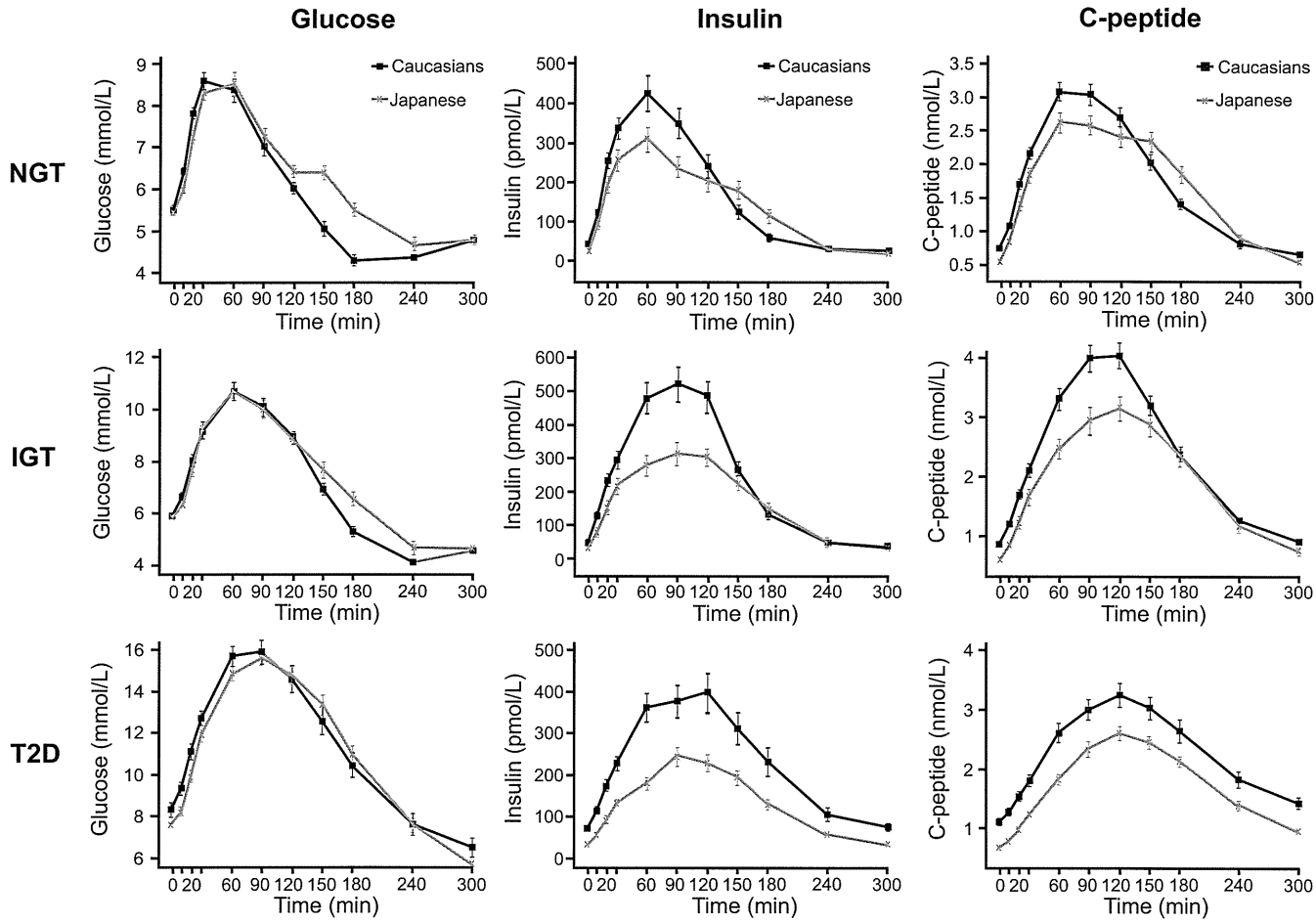

Figure 1. Glucose, insulin, and C-peptide profiles for Caucasian (black) and Japanese (gray) in the 3 different glucose tolerance states (NGT, IGT, and type 2 diabetes). Data are mean ± SEM.

Japanese had numerically slightly higher mean values (Figure 2). For the type 2 diabetes group, the difference was significant ($P < .01$), indicating higher hepatic extraction indices (HEs), both basal (HE_b) and postglucose load (HE_{post}), in Japanese compared with the Caucasians. Testing the overall effect on the HEs revealed that ethnicity in itself was significant both for basal and postglucose HEs ($P < .01$ and $P < .01$, respectively, Table 2).

Disposition indices

Values of DIs, calculated to adjust insulin secretion with the degree of insulin resistance, are presented in Figure 3. No statistically significant difference for any of the glucose tolerance states were observed for DI_d or DI_s between Caucasians and Japanese, which was supported by an ANOVA indicating no significant effect of ethnicity.

Effect of BMI on indices of insulin sensitivity, β-cell function, and hepatic extraction

To evaluate to what extent the ethnic differences in the above mentioned indices could be explained by differences in BMI, P values for ethnicity, and interaction between ethnicity and glucose tolerance state were obtained with and without BMI included as a covariate in the ANOVA. As seen in Table 2, the ethnic differences of S_I and of the dynamic and static indices of β-cell function (Φ_d and Φ_S) were no longer significant after accounting for differences in BMI. However, ethnic differences were still present for the basal insulin secretion index (Φ_b, $P < .01$) and for the postglucose load HE (HE_{post}, $P < .05$) when BMI was included as a covariate in the ANOVA.

Discussion

The present study evaluates and provides new insights into the pathogenesis of type 2 diabetes in Japanese relative to Caucasians. β-Cell responsiveness, insulin sensitivity, HEs, and DIs were estimated using mathematical methods applied to data from a frequently sampled OGTT. Indices were calculated for 3 glucose tolerance subgroups (NGT, IGT, and type 2 diabetes) in Caucasian and Japanese cohorts.

A lower insulin sensitivity in IGT compared with NGT subjects was observed in both Japanese and Caucasians,

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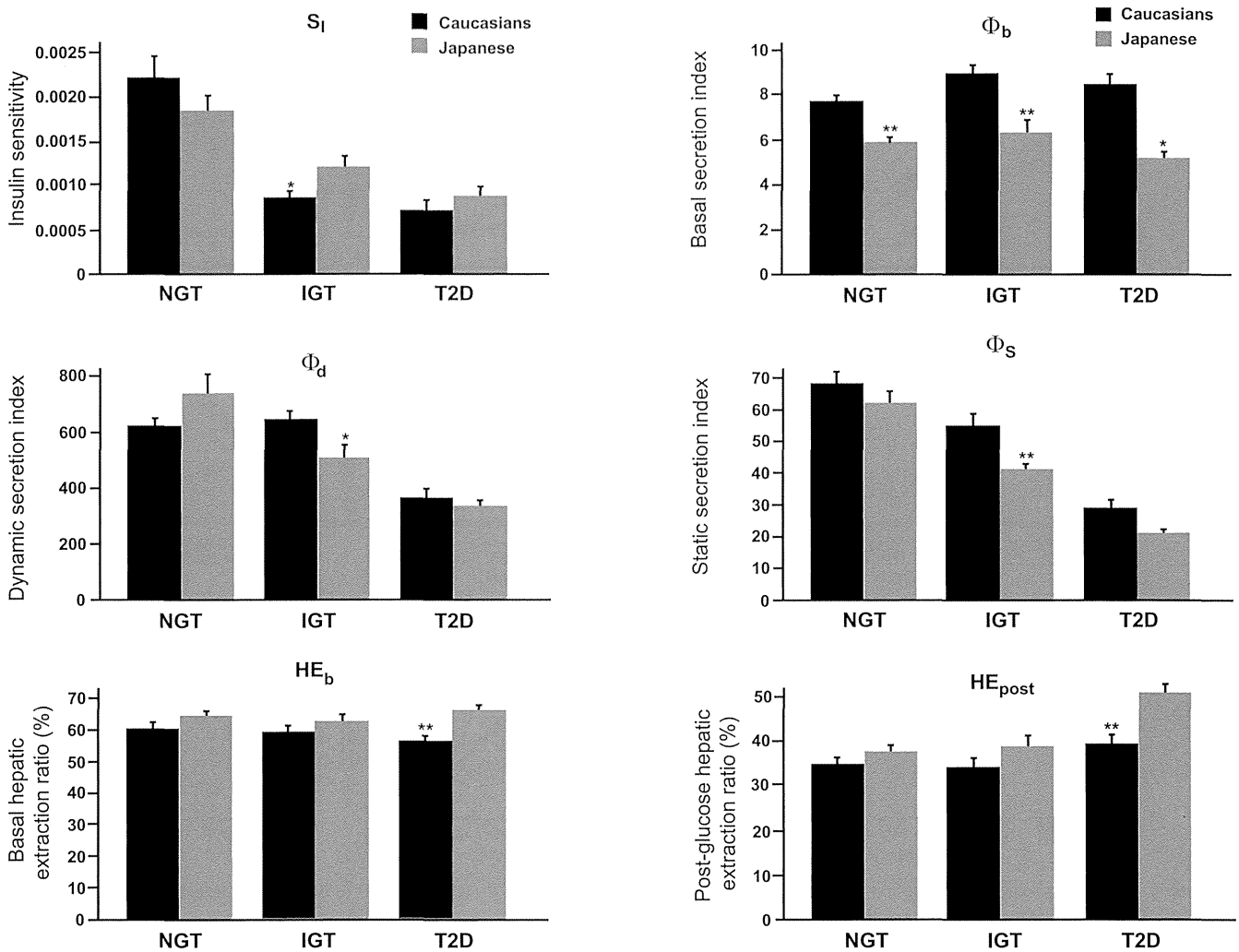

Figure 2. Measures of insulin sensitivity (S_I); basal, dynamic, and static β-cell function (ϕ_b, ϕ_d, and ϕ_s); and basal HE and HE during an OGTT (HE_b and HE_{post}) in NGT, IGT, and type 2 diabetes Caucasian (black) and Japanese (gray) subjects. *, $P < .05$; **, $P < .01$ obtained from unpaired t tests for differences between Caucasians and Japanese within each tolerance group.

although the decrease in insulin sensitivity was more pronounced in Caucasians, resulting in higher insulin sensitivity in Japanese than in the Caucasians in the IGT state. A decrease of insulin sensitivity from NGT to IGT and to type 2 diabetes has been reported both for Caucasians and Japanese (1, 5, 33). In line with the present study, Nishi et al (34) observed decreased insulin sensitivity from NGT to IGT to type 2 diabetes in Japanese.

Table 2. *P* Values From ANOVA for Effects of Ethnicity and Interaction of Ethnicity and Glucose Tolerance State for Indices of Insulin Sensitivity, β-Cell Function, and Hepatic Extraction

	Without BMI in Model		With BMI in Model	
Index	***P* Value for Ethnicity**	***P* Value for Interaction Between Ethnicity and Glucose Tolerance State**	***P* Value for Ethnicity**	***P* Value for Interaction Between Ethnicity and Glucose Tolerance State**
Insulin sensitivity (S_I)	.06	.13	.76	.11
β-Cell index				
Basal (Φ_b)	<.01	.28	<.01	.15
Dynamic (Φ_d)	.90	.29	.98	.29
Static (Φ_s)	.05	.68	.16	.70
Hepatic Extraction Ratio				
Basal (HE_b)	<.01	.13	.55	.17
Post glucose load (HE_{post})	<.01	.20	<.05	.16

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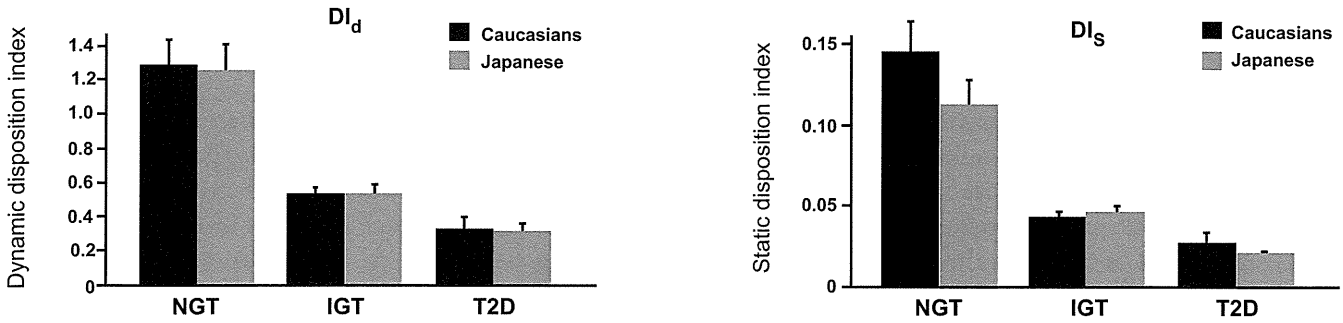

Figure 3. Dynamic and static DIs (DI_d and DI_s), calculated as the product of insulin sensitivity (S_I) dynamic and static β-cell function indices (ϕ_d and ϕ_s), respectively, in Caucasians (black) and Japanese (gray).

Higher maximum insulin and C-peptide concentrations were observed in Caucasian subjects compared with Japanese in all glucose tolerance states, and the difference seemed more pronounced for insulin than for C-peptide. We hypothesized that this could be due to a higher HE in Japanese and therefore applied the newly developed model by Campioni et al (25) for assessment of hepatic extraction based on an OGTT. The HE was actually found to be higher in Japanese both before and during the OGTT, and the difference was significant for the type 2 diabetes groups.

The β-cell function relative to the degree of insulin resistance was assessed using dynamic as well as static DIs. Both indices were found to be similar for the 2 ethnicities in all glucose tolerance states, indicating that Japanese and Caucasians have similar β-cell responsiveness for a similar degree of insulin resistance.

In our previous publication, we applied simple indices for the insulin sensitivity (homeostatic model assessment-insulin resistance [17] and the Matsuda index [34]) and β-cell function (homeostatic model assessment-beta cell function, insulinogenic index, and insulin secretion ratio [35]). In the present study, the indices were supplemented by a minimal model-based approach for several reasons. First, the model-based approach enabled us to accurately assess the HEs before and after the OGTT. Second, the model-based approach takes all samples from the OGTT into account and can handle uncertainty in measurements, and third, it also accounts for intersubject variation in the length and timing of the first- and second-phase insulin secretion.

In the present study, we also assessed ethnic differences between Caucasians and Japanese within each tolerance group. We applied a series of separate analyses (*t* tests) of the insulin sensitivity, β-cell function, and hepatic extraction. The increased risk for raising the probability of type 1 error could have been counteracted by means of the Bonferroni correction. However, this would have caused the probability of making a type 2 error to increase and would most likely have led to too conservative conclusions (36). In addition, the inherent relation between the different indices causes the tests not to be completely independent. Thus, we found it reasonable not to perform Bonferroni adjustments for the *t* tests.

The findings presented here are in line with our earlier findings based on classic indices of insulin sensitivity and β-cell responsiveness, although some differences became apparent. These originated mainly from the above-mentioned difference in the amount of samples included in the estimation of insulin sensitivity and β-cell function. One such difference is the insulin sensitivity in NGT subjects measured by the Matsuda index vs the glucose minimal model estimate (S_I). The Matsuda index uses data up to 120 minutes after glucose ingestion, whereas the minimal model uses the full profiles, which in our case extended up to 300 minutes. The part of the glucose curve that undershoots, ie, proceeds below baseline values for Caucasians with NGT, does not contribute to the estimation of the Matsuda index, which was numerically higher in Japanese subjects than in Caucasians (30). This is in contrast to the findings presented here for the model-based calculation of insulin sensitivity, which suggested higher sensitivity in the Caucasian NGT subjects, albeit not significant.

In our previous publication (30), we reported first-phase insulin secretion using the insulinogenic index, and we found overall significantly higher values in Caucasian than in Japanese subjects. This was in line with the present analysis that showed a statistical difference between the IGT groups for the dynamic index (Φ_d), which, like the insulinogenic index, is a surrogate measure of first-phase insulin secretion.

The static insulin responsiveness (Φ_s) was numerically higher in Caucasians than in Japanese for all glucose tolerance groups and reached statistical significance for the 2 IGT groups. This compared well with the previously reported results for the insulin secretion ratios (based on insulin as well as on C-peptide), which were overall higher in Caucasian than in Japanese subjects (30).

In our study, compared with Caucasians, we found lower β-cell function in Japanese IGTs, when using the dynamic and static β-cell index. This is in line with findings presented previously using the insulinogenic index (12, 16).

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To obtain a measure of β-cell responsiveness corrected for the degree of insulin resistance, we calculated the dynamic and static DIs, which can be used as measures for the ability of the β-cell to compensate for prevailing insulin resistance. As expected, both indices declined from NGT to IGT and type 2 diabetes. Furthermore, for the dynamic as well as the static indices, similar values were obtained for Japanese and Caucasians in all glucose tolerance states. This provides further evidence for a similar ability to compensate for insulin resistance in the 2 cohorts, which supports our previous findings based on classic indices for β-cell function and insulin sensitivity (30). This finding contrasts with previous suggestions that Japanese cannot compensate insulin resistance with increased β-cell function to the same extent as Caucasians (2).

In the present analysis, we assessed the hepatic extraction of insulin, which was not estimated in our previous publication. We found higher hepatic extraction in Japanese than Caucasian type 2 diabetes patients, but similar values in the 2 ethnic groups for NGT and IGT subjects. One assumption for the model-based assessment of hepatic extraction is that the clearance of C-peptide is similar in Caucasians and Japanese. This assumption cannot be verified using our data, and hence, a similar hepatic extraction of insulin but different clearance of C-peptide in Japanese and Caucasian type 2 diabetes patients cannot be ruled out.

For the 2 cohorts in our study to be representative for their corresponding populations, each of the cohorts was stratified into high and low BMI groups according to regional obesity definitions: 25 kg/m^2 for Japanese and 30 kg/m^2 for Caucasians. Because we strived to obtain a well-balanced design with similar numbers of subjects in the high and low BMI groups, Caucasians had on average higher mean BMI compared with the Japanese. In our previous publication, we showed that the BMI difference could explain the major part of the difference in insulin sensitivity and β-cell function in each glucose tolerance state. In the present study, we also evaluated the role of BMI for explaining ethnic differences of the model-based indices of insulin sensitivity, β-cell function, and hepatic insulin extraction. In accordance with our previous study based on classic indices (30), this study showed similar insulin sensitivity and β-cell responsiveness in the 2 ethnic cohorts after accounting for difference in BMI. However, the basal β-cell function and the hepatic insulin extraction following the glucose challenge were still higher and lower, respectively, in Caucasians compared with Japanese after accounting for BMI differences.

The 2 cohorts studied here included subjects who lived in their respective countries of ethnic origin. Thus, our results may not be applicable to second- or third-generation Japanese or Caucasian migrants in other regions of the world. This is underlined by previous findings of altered phenotypes of Japanese migrants who have adapted a Westernized lifestyle (12).

In the present study, we applied a cross-sectional rather than a longitudinal study approach. The results can therefore not provide evidence for whether Japanese and Caucasians tolerate the same period of insulin resistance before progression to type 2 diabetes, or in other words, whether Japanese can cope with β-cell stress during the same period of time as Caucasians. A longitudinal study is a suggestion for future investigation into this.

In summary, our study showed that Caucasian subjects with IGT had lower insulin sensitivity and higher dynamic and static β-cell responsiveness than their Japanese counterparts after an OGTT. Basal insulin secretion was higher in Caucasians than in Japanese in all 3 glucose tolerance groups. Hepatic insulin extraction was higher in Japanese than Caucasian subjects with type 2 diabetes but was similar for the 2 ethnic groups of NGT and IGT subjects. The overall insulin sensitivity and β-cell responsiveness across the range of glucose tolerance was similar in Japanese and Caucasians after accounting for differences in BMI. Finally, the combined effect of insulin sensitivity and β-cell responsiveness as estimated by the DI showed that Japanese and Caucasian NGT, IGT, and type 2 diabetes subjects have similar β-cell function relative to insulin resistance. This provides evidence that the ability to compensate for increasing insulin resistance is similar in Caucasians and Japanese.

Acknowledgments

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The study was funded by the Japan Science and Technology Agency, the Danish Agency for Science Technology and Innovation, and Novo Nordisk A/S. The Centre of Inflammation and Metabolism is supported by a grant from the Danish National Research Foundation (DNRF55). The Centre for Physical Activity Research (CFAS) is supported by a grant from Trygfonden.

These results were partially presented at the IDF-Western Pacific Region Congress in Kyoto, November 2012.

M.O., R.V.O., K.A., C.K., K.U., S.H.I., B.K.P., and T.K. contributed to study design. J.L. and S.H.I. coordinated the study. J.B.M., M.P., H.T., and M.O. contributed to data collection. J.B.M., C.D. R.V.O. and C.C. contributed to data analysis. J.B.M., M.P., H.T., M.O., R.V.O., J.L., K.A., N.-M.V, S.H.I., and B.K.P., contributed to data interpretation. M.P. and H.T. contributed to patient care. All authors contributed to writing or review of the manuscript and gave their approval of the final manuscript.

Disclosure Summary: J.B.M., R.V.O., J.L., K.A., N.-M.V., C.K. and S.H.I. are employees and shareholders in Novo Nordisk A/S. H.T. has received financial support from Novo Nordisk A/S. M.O. has been a speaker for Novo Nordisk, Takeda, MSD,

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Sanofi-Aventis, Eli-Lilly, Novartis, and Kissei. C.D.M., C.C., M.P., and B.K.P. have no conflicts of interest to declare.

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Deregulation of Pancreas-Specific Oxidoreductin ERO1β in the Pathogenesis of Diabetes Mellitus

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A growing body of evidence has underlined the significance of endoplasmic reticulum (ER) stress in the pathogenesis of diabetes mellitus. ER oxidoreductin 1β (ERO1β) is a pancreas-specific disulfide oxidase that is known to be upregulated in response to ER stress and to promote protein folding in pancreatic β cells. It has recently been demonstrated that ERO1β promotes insulin biogenesis in β cells and thus contributes to physiological glucose homeostasis, though it is unknown if ERO1β is involved in the pathogenesis of diabetes mellitus. Here we show that in diabetic model mice, ERO1β expression is paradoxically decreased in β cells despite the indications of increased ER stress. However, overexpression of ERO1β in β cells led to the upregulation of unfolded protein response genes and markedly enlarged ER lumens, indicating that ERO1β overexpression caused ER stress in the β cells. Insulin contents were decreased in the β cells that overexpressed ERO1β, leading to impaired insulin secretion in response to glucose stimulation. These data indicate the importance of the fine-tuning of the ER redox state, the disturbance of which would compromise the function of β cells in insulin synthesis and thus contribute to the pathogenesis of diabetes mellitus.

Diabetes mellitus has long been a worldwide threat. One of the essential aspects of diabetic pathogenesis is the progressive dysfunction of pancreatic β cells. It is widely believed that during the course of diabetes progression, insulin secretion from β cells gradually declines, eventually leading to hyperglycemia with an insufficient insulin supply to compensate for the increased insulin demand imposed by peripheral insulin resistance (1, 2). This state is called pancreatic β cell failure, the pathophysiology of which has, however, still not been fully elucidated. Endoplasmic reticulum (ER) stress is one of the strong candidates for the mechanisms underlying β cell failure (3, 4), and thus, the molecules and signaling pathways involved in the ER stress response have been intensively investigated as possible therapeutic targets for diabetes mellitus (5–7).

ER stress is known to be induced in response to multiple stimuli, all of which essentially interfere with proper protein folding in the ER. These mechanisms include impairing protein glycosylation, causing malfunctions of chaperones, or compromising oxidized protein folding, and they eventually lead to an accumulation of unfolded proteins (8, 9). Oxidized protein folding, or disulfide bond formation within a nascent polypeptide, is a facilitated process aided by protein disulfide isomerases (PDIs) (10) that is dependent on the highly oxidizing condition of the ER (11). Recently it has been reported that several ER resident proteins play essential roles in maintaining the ER oxidizing condition (12, 13), among which are a family of conserved genes termed ER oxidoreductin 1 (ERO1). ERO1p, the protein encoded by ERO1, couples the oxidizing power of molecular oxygen to generate disulfide bonds, which are eventually transferred from PDIs to client secretory proteins (11). Thus, ERO1 loss-of-function mutants of *Saccharomyces cerevisiae* accumulate reduced misfolded proteins in the ER (14, 15). Previous reports have shown that in *S. cerevisiae*, ERO transcripts are induced upon ER stress in the course of the unfolded-protein response (UPR), establishing that EROs are members of the UPR gene family (14, 15). In contrast, mammals have two isoforms of ERO, ERO1α and ERO1β, which have distinct functions with different tissue distributions (16). Importantly, only ERO1β transcripts are induced upon ER stress (16), whereas the regulation of ERO1α expression seems to be associated with hypoxia (17, 18). Furthermore, ERO1β transcripts are abundant in the pancreas (16), with preferentially higher expression in the islets than in the exocrine cells (19). Together with the facts that β cells are highly professionalized cells for insulin synthesis, with proinsulin accounting for up to 50% of the total protein (20, 21), and that the folding of proinsulin requires three intrachain disulfide bond formations (4, 22), it has been speculated that ERO1β would play significant roles in the physiological function of pancreatic β cells and not any less in the pathogenesis of diabetes mellitus.

Recently, Zito et al. have reported that whole-body deletion of ERO1β specifically affects pancreatic β cells, compromising the oxidative folding of insulin and thus leading to glucose intolerance in mice (23). Another report has demonstrated that suppressed ERO1β expression in pancreatic β cells leads to an increased susceptibility to ER stress and a reduction of insulin content (24). While these data clearly indicate that ERO1β plays an important role in insulin biogenesis in β cells and contributes to physiological glucose homeostasis, it is as yet unclear how the

Received 16 December 2013 Returned for modification 5 January 2014
Accepted 16 January 2014
Published ahead of print 27 January 2014
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doi:10.1128/MCB.01647-13

expression or function of ERO1β is changed during pancreatic β cell failure and what its precise roles in the pathogenesis of diabetes are. Here we report that, unlike the expression of other UPR genes, that of ERO1β transcripts in the islets paradoxically declines during the course of diabetes progression despite increased ER stress. However, mice overexpressing human ERO1β specifically in pancreatic β cells showed impaired glucose tolerance due to reduced insulin secretion. In β cells overexpressing ERO1β, the expression of UPR genes was upregulated and the ER lumens were markedly enlarged, indicating that ERO1β overexpression caused ER stress in the β cells.

MATERIALS AND METHODS

Animals. BKS.Cg-$m^{+/+}$ $Lepr^{db}$/J (*db*/*db*) mice and control *misty*/*misty* mice were purchased from Japan CLEA. Akita mice were purchased from Japan SLC. For the generation of hERO1βTg mice, a fusion gene was designed that comprised the rat insulin promoter and human ERO1LB cDNA coding sequences with a Flag tag at its C terminus so that its expression was targeted to β cells. The linearized construct was microinjected into the pronuclei of fertilized C57BL/6 mouse (Japan CLEA) eggs. Transgenic founder mice were identified by PCR analysis by using a primer for the Flag sequence, which was also used to determine the tissue distribution of the transgene by PCR after reverse transcription (RT). All experiments were conducted with heterozygote male mice. High-fat diet (HFD) feeding was started at 7 weeks of age where required. The Animal Care Committee of the University of Tokyo approved the animal care conditions and experimental procedures used.

Quantitative real-time PCR. Total RNA was prepared with the RNeasy kit (Qiagen). RT reagents (Applied Biosystems) were used to prepare cDNA. Quantitative real-time PCR was performed with ABI Prism and PCR Master Mix reagent (Applied Biosystems). The sequences of the primers and probe used for the simultaneous detection of human *ERO1B* and mouse *Ero1b* were as follows: forward primer, TGGAGTTCTGGAT GATTGCTT; reverse primer, TCTTCTGCCCAGAAAGGACA; probe, CGTTATTACAAGGTTAATCTGAA. All of the other primers and probes used were purchased from Applied Biosystems. The levels of mRNAs were normalized to that of cyclophilin (25).

Immunoblotting. Immunoblotting was conducted as previously described (25). The antibodies used for immunoblotting were anti-phospho-PERK antibody (Thr980; Cell Signaling Technology); anti-phospho-eukaryotic transcription initiation factor 2 alpha subunit (anti-phospho-eIF2α) antibody (Ser51; Cell Signaling Technology); anti-4E-BP1 antibody (Cell Signaling), and anti-β-actin antibody (Sigma-Aldrich).

Metabolic assays. A glucose tolerance test (GTT) was performed as described previously (26). The mice were fasted for 16 h, and blood samples were obtained at the indicated time points after the intraperitoneal injection of 1 g/kg body weight of D-glucose (WAKO). Blood glucose levels were checked at indicated time points (Glutest Pro; Sanwa Kagaku Kenkyusho).

Immunohistochemical and morphometric analyses of the pancreas. Immunohistochemical and morphometric analyses of pancreas sections were performed as described earlier (27) with a slight modification. Six mice under each condition at 11, 22, and 36 weeks of age were subjected to morphometric analysis. Sections were stained with antibodies as indicated. For morphometric analysis, the images of islets were traced manually and analyzed by ImageJ software (NIH). The mean of four different sections of each pancreas was used for the analysis.

Islet isolation. Islets were isolated by Liberase RI (Roche) with pancreatic perfusion and subsequent digestion for 24 min at 37°C (28). Islets were picked manually in Hanks' balanced salt solution (Sigma) buffer supplemented with 10% fetal calf serum and 25 mM HEPES buffer and then immediately used for further experiments, except for the pulse-chase analysis, where the islets were subjected to the experiments after overnight incubation in RPMI 1640 medium (GIBCO) supplemented with 10% (vol/vol) fetal bovine serum (GIBCO).

Glucose-stimulated insulin secretion (GSIS) assay. Freshly isolated islets were maintained in Krebs-Ringer bicarbonate (KRB) buffer (129 mM NaCl, 4.8 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 2.5 mM $CaCl_2$, 5 mM $NaHCO_3$, 10 mM HEPES [adjusted to pH 7.4]) containing 0.2% bovine serum albumin supplemented with 2.8 mM glucose for 30 min at 37°C. The islets were then incubated for 30 min in the same buffer containing 22.4 mM glucose or 50 mM KCl as indicated. For the MIN6 β cell experiment, cells were cultured in Dulbecco's modified Eagle's medium (GIBCO) supplemented with 10% (vol/vol) fetal bovine serum (GIBCO). The cells were incubated with KRB buffer with 2.8 mM glucose for 60 min at 37°C, and then the medium was changed to KRB buffer with 22.4 mM glucose for further incubation for 30 min. For measurement of insulin content, insulin was extracted from islets or cultured cells by overnight incubation with acid ethanol at −20°C. Insulin concentrations were measured with an insulin radioimmunoassay kit (Institute of Isotopes) according to the manufacturer's instructions.

Electron microscopy. Two mice of each genotype were anesthetized and subjected to cardiac perfusion with 0.1 M sodium phosphate buffer (pH 7.2) containing 2% glutaraldehyde and 2% paraformaldehyde. The pancreas was excised from each mouse, cut into small pieces, and immersed overnight in the same fixative. The tissue was then exposed to 2% osmium tetroxide, stained with 2% uranyl acetate, dehydrated with ethanol, and embedded in Epon812 (TAAB). Thin sections were stained with uranyl acetate and lead citrate before examination with a Hitachi 7100 electron microscope (Hitachi). The quantification of ER luminal areas was done by a previously described method (29) in which 22 to 34 pictures were taken per animal and then by using a double-lattice test system with a spacing of 1 cm, the points that fell on the ER lumen were counted. The ratio of the points falling on the ER lumen to the points falling in the entire 20-by-20 double lattice was recorded as the ER luminal area percentage.

Detection of superoxide. Superoxide was detected in frozen pancreas sections with dihydroethidium (DHE; 10 μmol/liter) in phosphate-buffered saline (PBS) for 30 min at 37°C in a humidified chamber protected from light. DNA-bound ethidium bromide, which was formed from DHE on reaction with superoxide, was detected as red fluorescence (30).

Generation and infection of adenoviruses. Adenovirus encoding human ERO1β was generated according to the manufacturer's protocol (TaKaRa Biotechnology) by using the same construct as that used to generate hERO1βTg mice. An adenovirus encoding LacZ was purchased from TaKaRa Biotechnology and used as the negative control. Prior to use, all adenoviruses were purified on a cesium chloride gradient and dialyzed into PBS plus 10% glycerol. MIN6 β cells were infected with the adenoviruses at a multiplicity of infection (MOI or number of viral particles per cell) of 3,000 PFU/cell. The cells were subjected to experiments 48 h after adenovirus infection.

Pulse-chase analysis. A total of 65 islets were preincubated in 500 μl of methionine- and cysteine-free RPMI 1640 medium (GIBCO) for 1 h and then labeled in the same medium containing [^{35}S]methionine-cysteine (EXPRE^{35}S^{35}S protein labeling mix; PerkinElmer) at a concentration of 10 μCi/ml for 30 min. When necessary, a subsequent radiolabel-free chase was performed with complete RPMI medium supplemented with 10% (vol/vol) fetal bovine serum (GIBCO) after the islets were washed twice with the same medium, and islets were frozen in liquid nitrogen at the indicated times. The lysates were immunoprecipitated with anti-insulin antibody (ab8304 insulin plus proinsulin antibody; Abcam). Immunoprecipitated proteins were resolved by Tricine-SDS-PAGE with 15% polyacrylamide gel and detected by autoradiography with a phosphorimager (Typhoon FLA 7000; GE Healthcare).

Statistical analysis. Statistical analysis was performed by using the paired two-sample *t* test for means. Analysis of variance (ANOVA) and Tukey's *post hoc* analyses were used when more than two groups were compared. Repeated-measures ANOVA was used for analyzing the results of metabolic assays. Statistical significance was accepted at P values <0.05.

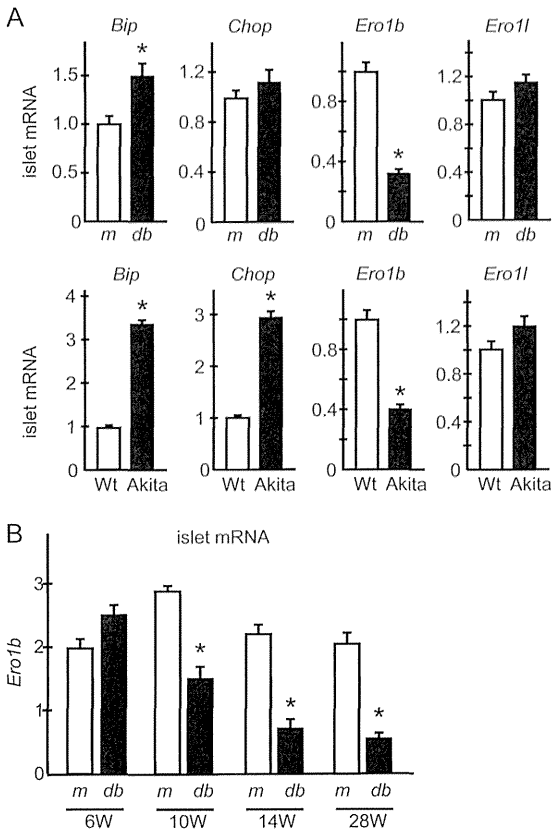

FIG 1 *Ero1b* expression in the islets of diabetic model mice. (A) *Ero1b* expression in the islets of *db*/*db* and Akita mice. Pancreatic islets were isolated from *db*/*db* (*db*) and *misty*/*misty* (*m*) mice at 14 weeks of age or from Akita and control C57BL/6 mice at 7 weeks of age. Total mRNA was extracted and subjected to RT-PCR analysis of the genes indicated. $n = 4$ to 6; *, $P < 0.05$. (B) Pancreatic islets were isolated from *db*/*db* (*db*) or *misty*/*misty* (*m*) mice at the indicted weeks of age. Total mRNA was extracted and subjected to RT-PCR analysis of *Ero1b* expression. $n = 4$ to 6; *, $P < 0.05$. The data shown are means ± the standard errors of the means.

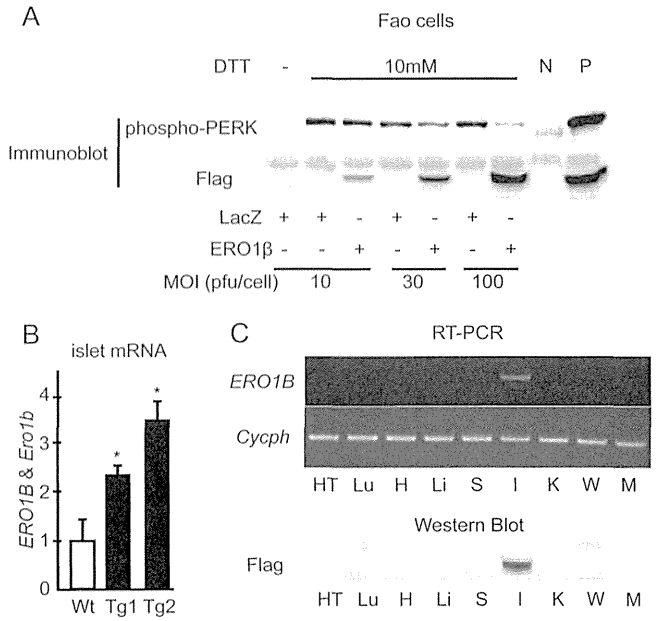

FIG 2 Human *ERO1B* overexpression in Fao cells and hERO1βTg mouse islets. (A) Adenoviral overexpression of human *ERO1B* in Fao cells. Fao cells were infected with adenovirus encoding human ERO1β or the control LacZ at the indicated MOIs. The cells were incubated with 10 mM DTT for 0.5 h. Total cell lysates were prepared and subjected to immunoblotting with anti-phospho-PERK or anti-Flag antibody. N, negative control; P, positive input. (B) *Ero1b* and *ERO1B* expression in the islets of hERO1βTg mice. Islets were isolated from hERO1βTg (Tg1 and Tg2) or control Wt mice at 14 weeks of age. Total mRNA was extracted from the islets and subjected to RT-PCR analysis, which detects mouse *Ero1b* and human *ERO1B*, as described in Materials and Methods. $n = 6$ to 9; *, $P < 0.05$. The data shown are means ± the standard errors of the means. (C) Distribution of the transgene *ERO1B* in the tissues of hERO1βTg mice. Each tissue type was removed from hERO1βTg mice at 9 weeks of age. Total mRNA was extracted from the tissues, 0.2 μg of which was subjected to RT and subsequent PCR analysis amplifying Flag-tagged human *ERO1B* cDNA or cyclophilin (upper panel). Lysate of protein from each tissue type was prepared and subjected to immunoblotting with Flag antibody at 10 μg/lane (lower panel). Tissue types: HT, hypothalamus; Lu, lung; H, heart; Li, liver; S, spleen; I, islet; K, kidney; W, epididymal white adipose tissue; M, skeletal muscle.

RESULTS

ERO1β expression was decreased in the islets of diabetic model mice despite evidence of ER stress. To investigate the roles of ERO1β in the pathogenesis of diabetes, we first examined ERO1β mRNA expression in the islets of diabetic *db*/*db* mice. As widely accepted, the expression of UPR genes such as *Bip* and *Chop* tended to be upregulated in *db*/*db* islets, most likely reflecting the increased ER stress in the β cells. In contrast, the expression of *Ero1b* was paradoxically lower than that in control *misty*/*misty* mouse islets (Fig. 1A, upper panels). The expression levels of *Ero1l*, which encodes the other isoform of ERO1 protein, ERO1α, were relatively similar in *db*/*db* and control *misty*/*misty* mouse islets (Fig. 1A, upper panels). We next investigated the islets of Akita mice as another diabetic model mouse that harbors a C96Y mutation in the insulin-2 gene resulting in misfolded proinsulin accumulation and progressive β cell loss due to ER stress-induced apoptosis (7, 31). Again, the expression of *Ero1b* was paradoxically decreased despite the robust upregulation of other typical UPR genes (Fig. 1A, lower panels). Moreover, the reduction of *Ero1b* in *db*/*db* islets occurred in an age-dependent manner, which was consistent with the time course of diabetes progression, with its expression being maintained, or tending to be higher, at early ages (Fig. 1B). These results highlight the special nature of ERO1β among UPR genes, namely, its lack of any upregulation under ER-stressed conditions. These data prompted us to hypothesize that ERO1β overexpression in β cells would benefit the β cells and rescue the glucose intolerance seen under pathological conditions such as those experienced during HFD feeding.

First we overexpressed Flag-tagged human ERO1β with adenovirus in Fao rat hepatoma cells. The Flag tag was added at the C terminus of the construct so that the tag would not interfere with the signal sequence at the N terminus of ERO1β (32). Overexpression of ERO1β in Fao cells ameliorated the dithiothreitol (DTT)-induced UPR response in a dose-dependent manner, as revealed by reduced pancreatic ER kinase (PERK) phosphorylation during DTT treatment (Fig. 2A), suggesting not only that human ERO1β was functionally valid as a redox regulator in rodent cells but also that ERO1β overexpression could counteract the reducing effects of DTT in Fao cells. Thus, we created a mouse line overexpressing Flag-tagged human ERO1β specifically in β cells under the control of a rat insulin promoter (hERO1βTg mice). We obtained two

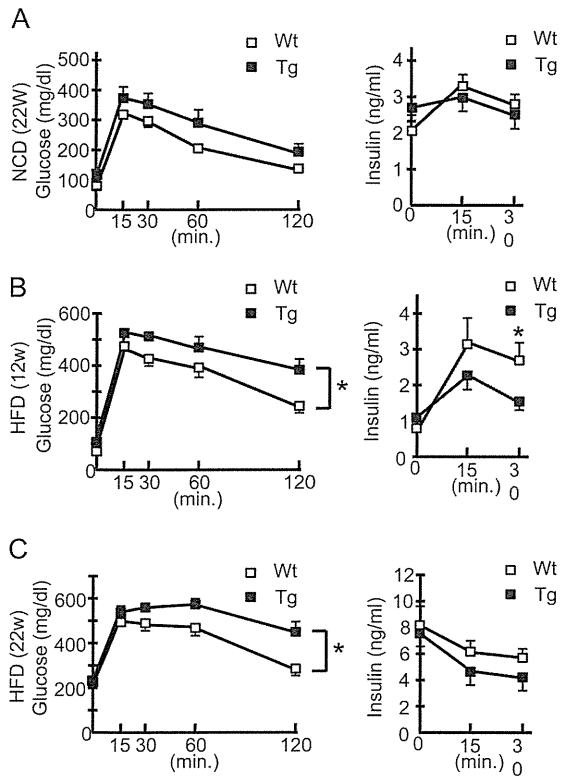

FIG 3 Metabolic phenotypes of hERO1βTg mice. Shown are blood glucose levels (right panels) and serum insulin concentrations (left panels) after the intraperitoneal injection of glucose. hERO1βTg (Tg) or Wt control mice were intraperitoneally injected with glucose at 1 g/kg body weight during NCD feeding at 22 weeks of age (A), during HFD feeding at 12 weeks of age (B), or during HFD feeding at 22 weeks of age (C). Blood samples were collected at the indicated time points and subjected to glucose and insulin measurements. $n = 10$; *, $P < 0.05$. The data shown are means ± the standard errors of the means.

lines (Tg1 and Tg2) with different but similar levels of overexpression of the *ERO1B* gene, the mRNA expression levels of which were quantified by an RT-PCR analysis designed to amplify the mRNA region common to human *ERO1B* and mouse *Ero1b* (Fig. 2B). In the following experiments we essentially used the Tg1 line (referred to as Tg in this report) to characterize our overexpression model, while key experiments were also repeated with the Tg2 line. The expression of human ERO1β in these Tg mice, which was determined by measuring Flag expression, was detected specifically in pancreatic islets (Fig. 2C). These mice were born normally and showed no obvious abnormalities in their appearance.

hERO1βTg mice showed impaired glucose tolerance with reduced insulin secretion. To explore the effects of ERO1β overexpression in β cells under physiological as well as pathological conditions, we fed hERO1βTg mice with a normal chow diet (NCD) or an HFD and examined their metabolic phenotypes. hERO1βTg mice showed a body weight gain similar to that of control wild-type (Wt) mice during both NCD and HFD feeding (data not shown). Unexpectedly, hERO1βTg mice showed impaired glucose tolerance in the GTT compared to findings for the control Wt mice, with a statistically significant difference only under the HFD feeding condition (Fig. 3A to C, left panels). The exacerbated glucose intolerance seen in the HFD-fed hERO1βTg mice was due to their lower insulin secretion than that of the Wt control mice (Fig. 3B and C, right panels). No difference in insulin sensitivity between hERO1βTg and Wt control mice was detectable in an insulin tolerance test (data not shown). To confirm that ERO1β overexpression does not benefit β cells, we also created hERO1βTg mice with a *db/db* background (Wt/*Lepr*^{db} or Tg/*Lepr*^{db} mice) by crossing Tg2 line mice with C57BLKS-*Lepr*^{db} heterozygotes. We then tested their glucose tolerance with a GTT, in which we observed no improvement or worsening of the glucose levels in Tg/*Lepr*^{db} mice compared with those of Wt/*Lepr*^{db} mice, where the blood glucose level had already reached >500 mg/dl after a half-dose glucose challenge (data not shown).

hERO1βTg islets showed an impaired GSIS response with reduced insulin content. To explore the mechanisms whereby ERO1β overexpression led to reduced insulin secretion in glucose challenge tests during HFD feeding, we first examined the morphology of hERO1βTg islets by light microscopy. Microscopic analyses of hERO1βTg mouse islets showed no morphological changes detectable by insulin and glucagon staining (Fig. 4A, upper left panels). Insulin staining showed that the insulin-positive areas of hERO1βTg and control mouse islets were similar under both of the feeding conditions at 12 and 22 weeks, the time points when the glucose intolerance phenotype was already observed in hERO1βTg mice, whereas there was a nonsignificant reduction of the insulin-positive areas of HFD-fed hERO1βTg mouse islets only at 36 weeks of age (Fig. 4A, upper right and lower panels). Single-stranded DNA (ssDNA) staining and proliferating cell nuclear antigen (PCNA) staining showed no evidence of accelerated apoptosis or cell proliferation in the islets under any of the conditions (Fig. 4B). These data indicated that the exacerbated glucose intolerance in hERO1βTg mice was not associated with β cell mass reduction.

We next investigated the GSIS response of islets isolated from HFD-fed hERO1βTg mice. Islets isolated from HFD-fed hERO1βTg mice showed a weaker GSIS response than those from HFD-fed control mice, and the difference was more pronounced and reached statistical significance after longer HFD feeding (Fig. 5A and B, left panels). The weaker GSIS responses in Tg islets were due to reduced insulin contents in the islets (Fig. 5A and B, right panels), as insulin secretion did not differ between hERO1βTg and control Wt islets when normalized to their insulin contents (Fig. 5A and B, middle panels). The analyses of mRNA expression in Tg islets revealed a marginal reduction in *Ins1* and *Ins2* expression by about 15%, the degree of which was, however, relatively small compared to the reduction in the insulin contents of Tg islets (Fig. 5C). Collectively, these data suggested that HFD-fed hERO1βTg mice showed exacerbated glucose intolerance, which was attributed to the reduced islet insulin contents with the possible involvement of posttranscriptional mechanisms.

ERO1β overexpression caused ER stress in pancreatic β cells. To further characterize the phenotypes of ERO1β-overexpressing β cells, we investigated the morphology of hERO1βTg β cells in detail with an electron microscope. Electron microscopic analyses revealed severely enlarged ER lumens in the β cells of hERO1βTg mice (Fig. 6A and B), showing a sharp contrast to the scarce changes observed in the light microscopic analyses. The ER dilation of hERO1βTg β cells was already observed under NCD-fed conditions, the magnitude of which did not change further under HFD-fed conditions (data not shown). No apparent changes were detected in the organelles other than the ER, such as the Golgi apparatus or insulin-containing granules, with regard to

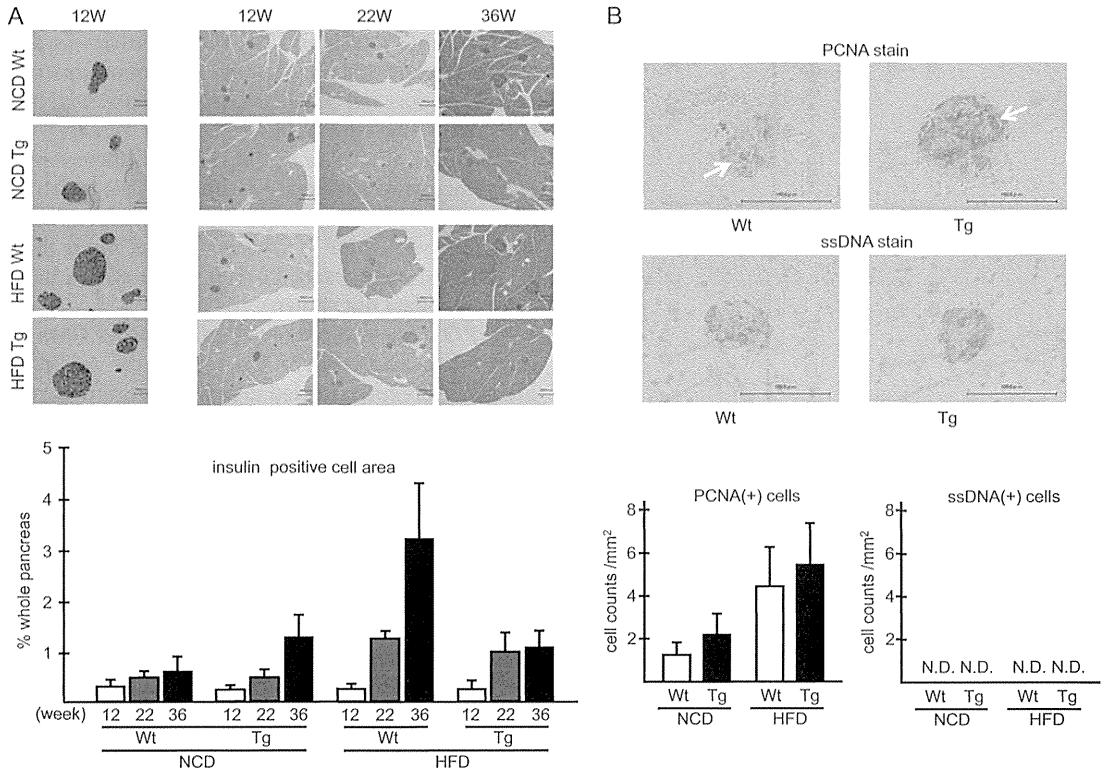

FIG 4 Histological analysis of islets of hERO1βTg mice. (A) Representative images of islets of hERO1βTg mice. Pancreas sections of hERO1βTg (Tg) or Wt control mice under NCD or HFD feeding conditions at 12 weeks of age were stained with insulin (red) or glucagon (dark red) antibody (left panels). Pancreas sections of the mice at the indicated weeks (W) of age were stained with insulin antibody (brown) (right side), and β cell areas determined as insulin-positive areas by staining were quantified (bottom). The occupancy of pancreatic β cells in the whole pancreas was determined as described in Materials and Methods. $n = 6$. Bars, 100.0 μm (left column) and 300.0 μm (right three columns). (B) Cell proliferation and apoptosis markers in hERO1β islets. Pancreas sections of hERO1βTg (Tg) or Wt control mice under NCD or HFD feeding conditions at 12 weeks of age were stained with insulin (brown) and PCNA or ssDNA (dark purple) antibody (representative images are shown at the top). The arrows indicate PCNA-positive cells. The PCNA- or ssDNA-positive cells were counted. Bars, 100.0 μm. Cell counts normalized to the insulin-positive area are shown at the bottom. $n = 6$. The data shown are means ± the standard errors of the means. N.D., not detected.

their numbers or their morphology. As previous reports have indicated, cells under ER stress or with compromised ER homeostasis often show ER enlargement (31, 33, 34), suggesting that the β cells of hERO1βTg mice were also subjected to ER stress. In fact, the analyses of mRNA expression showed upregulation of the expression of multiple UPR genes in Tg islets, including *Bip*, *Chop*, *Derl3*, and *Trb3* (Fig. 7A). Upregulation of UPR genes was again observed in the islets of NCD-fed hERO1βTg mice, the degree of which tended to be higher in the islets of HFD-fed Tg mice. These data collectively indicated that ERO1β overexpression caused ER stress in β cells. Interestingly, however, phosphorylation of PERK and the α subunit of eukaryotic transcription initiation factor 2 (eIF2) or upregulation of 4E binding protein 1 (4E-BP1) was not evident in hERO1βTg islets (Fig. 7B).

Next we investigated whether reactive oxygen species (ROS) could contribute to the β cell dysfunction of hERO1βTg mice. As previously described, in the relay of oxidative equivalents among EROs, PDIs, and client proteins during oxidative protein folding, the final acceptor of the electron is molecular oxygen; thus, ERO-mediated oxidative protein folding could lead to ROS production (35). However, we did not observe any evidence of ROS accumulation in hERO1βTg islets, as revealed by DHE staining of hERO1βTg islets (Fig. 7C). In addition, the mRNA expression of genes involved in the antioxidant pathway, such as *Sod1*, *Sod2*, and *Cat*, was unaltered in hERO1βTg islets compared to that in control Wt islets (Fig. 7D). These results suggested the absence of ROS overproduction in hERO1βTg islets.

ERO1β overexpression caused impaired insulin secretion with ER stress in MIN6 cells. To investigate whether ERO1β overexpression in cultured cells could lead to phenotypes similar to those in islets, we next overexpressed human ERO1β with adenovirus in MIN6 β cells. The amount of insulin secreted under the high-glucose condition was significantly lower in ERO1β-overexpressing MIN6 cells, while the insulin secretion ratio, normalized to the insulin content, did not decrease with ERO1β overexpression (Fig. 8A). These results indicated that the reduced insulin secretion under ERO1β overexpression was due to reduced insulin contents in MIN6 β cells, essentially mimicking the phenotypes of hERO1βTg islets. The mRNA analyses showed that ERO1β overexpression caused UPR gene upregulation (Fig. 8B), while mRNA expression of antioxidant pathway genes such as *Sod1*, *Sod2*, and *Cat* was unaltered (data not shown), suggesting that ERO1β overexpression led to ER stress in MIN6 cells without collateral ROS overproduction, again showing characteristics similar to those in hERO1βTg islets. Importantly, mild DTT treatment paradoxically led to attenuation of UPR gene upregulation (Fig. 8B, gray bars), which was associated with restored insulin contents under ERO1β overexpression (Fig. 8C). These data sug-

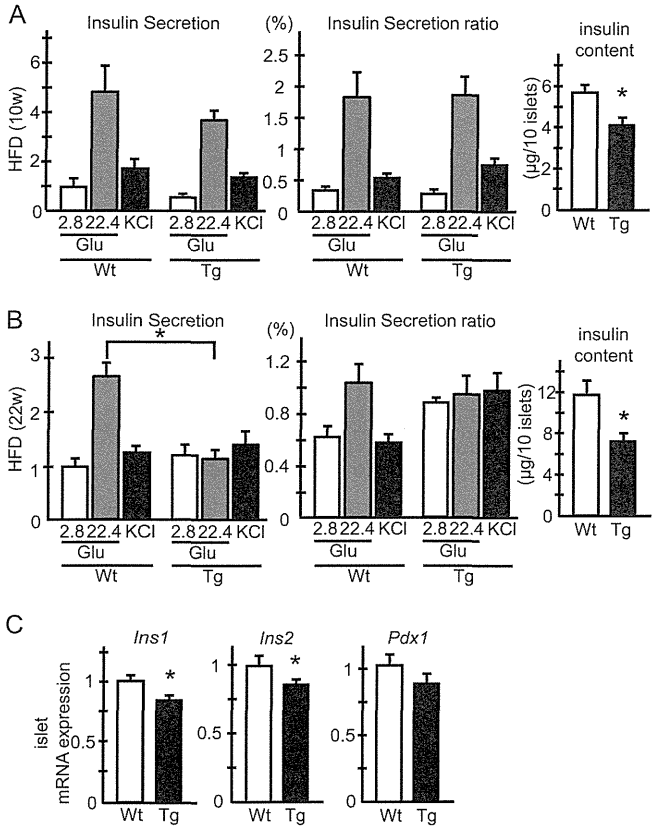

FIG 5 Static-incubation study of islets from hERO1βTg mice. (A and B) GSIS of hERO1βTg islets. Islets were freshly isolated from 10-week-old (A) or 22-week-old (B) hERO1βTg (Tg) or control Wt mice fed an HFD from 7 weeks of age. The islets were incubated for 30 min in KRB buffer containing 2.8 mM glucose (Glu), 22.4 mM glucose, or 50 mM KCl, respectively, and the media were collected. The insulin concentrations in the incubation media were measured with an insulin radioimmunoassay kit. Insulin secretion is displayed as a ratio normalized to the basal secretion of Wt mice (left panels). Insulin secretion was determined as the ratio of secreted insulin to the insulin content of the islets (middle panels). $n = 6$ to 18; 6 islets for each condition. For measurements of the insulin contents of islets, insulin was extracted from the islets by overnight incubation with acid ethanol and measured with an insulin radioimmunoassay kit (right panels). $n = 18$ to 54; 6 islets for each condition; *, $P < 0.05$. (C) mRNA expression of insulin-related genes in hERO1βTg islets. Pancreatic islets were isolated from 10-week-old Tg or control Wt mice fed an HFD from 7 weeks of age. Total mRNA was extracted and subjected to RT-PCR analysis of *Ins1*, *Ins2*, and *Pdx1* mRNA expression. $n = 6$ to 8; *, $P < 0.05$. The data shown are means ± the standard errors of the means.

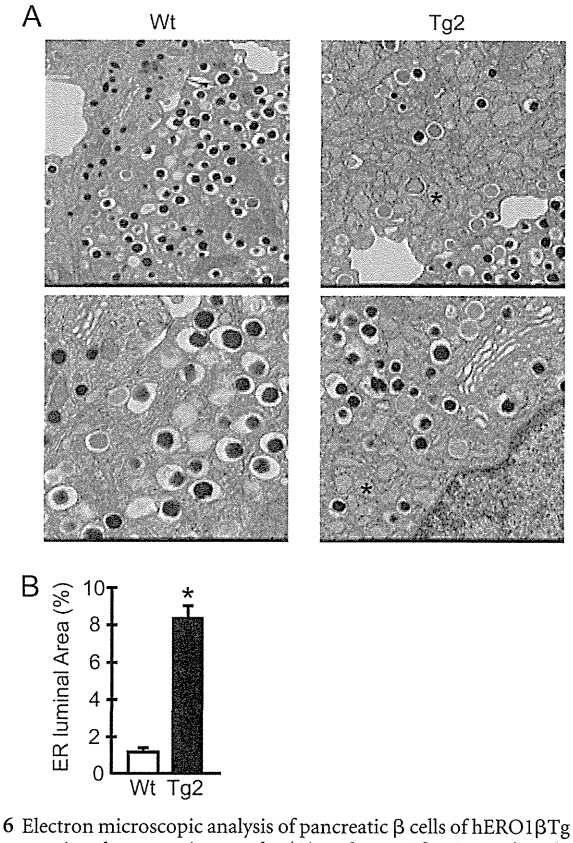

FIG 6 Electron microscopic analysis of pancreatic β cells of hERO1βTg mice. Representative electron micrographs (A) and quantifications of ER luminal areas (B) of pancreatic β cells of hERO1βTg (Tg2) or Wt control mice during NCD feeding at 12 weeks of age are shown. The asterisks indicate the markedly dilated ER lumens. ER lumen areas were quantified as described in Materials and Methods.

gested the possibility that ERO1β overexpression caused ER stress by shifting ER redox states toward overly oxidizing conditions, which was countersuppressed by the reducing effect of the mild DTT treatment.

Insulin maturation was not compromised in hERO1βTg β cells. How did the overexpression of ERO1β cause ER stress in β cells? Generally, ER stress can result from an accumulation of misfolded proteins, which is due to either accelerated misfolding of client proteins or impaired removal of irreparably misfolded proteins from the ER lumens by a mechanism called ER-associated degradation (ERAD). Recent studies have pointed out that the reduction of protein disulfides is required for the dislocation and degradation of misfolded proteins targeted for ERAD (36, 37). To directly address these issues, we conducted a pulse-chase analysis with hERO1βTg islets and investigated proinsulin processing and insulin maturation with an antibody detecting proinsulin and insulin with equal efficiency. The pulse-chase analyses showed that there was no delay in the appearance of processed insulin, which was reflected in the band shift downward (Fig. 9A, upper panel), suggesting that the proinsulin maturation with the C-peptide cleavage occurred in hERO1βTg β cells as smoothly as that in the control Wt β cells. Additionally, no delay in the disappearance of proinsulin was observed, as reflected in the similarly remaining upper bands in Tg and Wt islets until the end of the chase period. The decrease in insulin content in the islets of hERO1βTg mice was confirmed in the proinsulin immunoblot assay, as detected by anti-C-peptide immunoblotting of the same membrane (Fig. 9A, lower panel). In fact, the amount of newly synthesized proinsulin, which was investigated by collecting islets just after 30 min of metabolic "pulse" labeling, was lower in the islets of hERO1βTg mice than in control Wt mouse islets (Fig. 9B). These data collectively suggested the possibility that the decrease in insulin contents in the islets of hERO1βTg mice could be accounted for by reduced protein synthesis, while the conversion of proinsulin to insulin occurred normally in the hERO1βTg β cells, and that the misfolded proinsulin, if it existed, could be removed from the ER with similar efficiency in Tg β cells compared to its clearance from control Wt β cells.

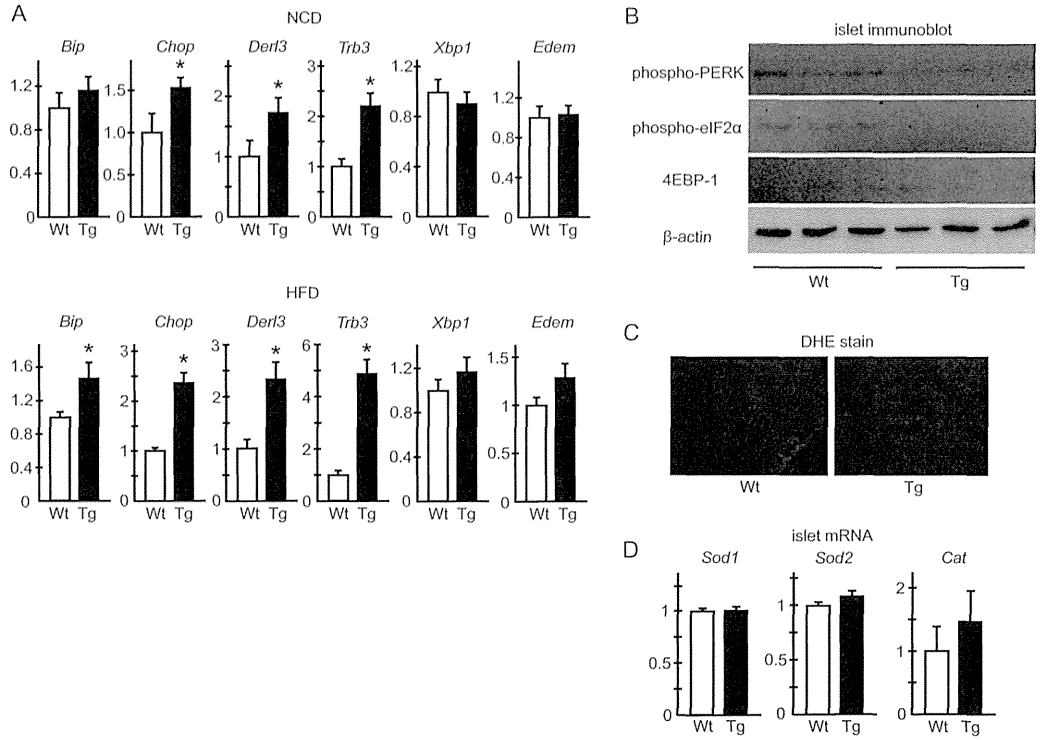

FIG 7 ER stress and oxidative stress markers in hERO1βTg islets. (A) UPR gene mRNA expression in hERO1βTg islets. Pancreatic islets were isolated from 12-week-old hERO1βTg (Tg) or control Wt mice fed either an NCD or an HFD from 7 weeks of age. Total mRNA was extracted and subjected to RT-PCR analysis of the genes as indicated. $n = 7$ or 8; *, $P < 0.05$. (B) UPR signaling pathways in hERO1β islets. Pancreatic islets were freshly isolated from 12-week-old hERO1βTg (Tg) or control Wt mice fed an NCD. Total cell lysates were prepared from the islets, and the same amounts of protein were loaded and subjected to immunoblotting with anti-phospho-PERK, anti-phospho-eIF2α, or anti-4E-BP1 antibody. The same membrane was reblotted with anti-β-actin antibody. Representative images of immunoblotting of hERO1β islets are shown. (C and D) Oxidative stress in hERO1β islets. (C) Representative images of DHE staining of islets of hERO1βTg mice. Pancreas sections of hERO1βTg (Tg) or Wt control mice during NCD feeding at 12 weeks of age were stained with DHE. (D) Antioxidant pathway gene mRNA expression in hERO1βTg islets. Pancreatic islets were isolated from 12-week-old hERO1βTg (Tg) or control Wt mice fed an HFD from 7 weeks of age. Total mRNA was extracted and subjected to RT-PCR analysis of *Sod1*, *Sod2*, and *Cat* mRNA expression ($n = 6$ to 8). The data shown are means ± the standard errors of the means.

DISCUSSION

Here we report for the first time the phenotypes of mice with ERO1β overexpression specifically in pancreatic β cells. While it has been well documented that EROs play critical roles in ER protein folding or in ER homeostasis (12, 14, 15), their roles in the pathogenesis of diseases such as diabetes mellitus have remained obscure, with only one report showing that the disruption of ERO1β expression compromised oxidative folding of insulin and thus led to glucose intolerance in mice (23).

In the first place, we observed a special feature of ERO1β among other UPR genes, which showed a paradoxical decrease in its expression in the islets of *db/db* and Akita mice despite the evidence of increased ER stress. Considering that the β cell mass itself is decreased in these model mice and that ERO1β is specifically expressed in β cells, it would be reasonable to assume that the observed reductions in ERO1β expression could be partly accounted for by the reduction in β cell mass itself. Nevertheless, the reduction of ERO1β showed a striking contrast to findings for other UPR genes like *Bip*, the mRNA upregulation of which in response to ER stress is due to its induction exclusively within β cells (31), indicating that there occurred either a reduction or, more precisely, an inadequate upregulation of ERO1β expression in the stressed β cells in these models.

EROs are essentially double-bladed molecules; they are necessary proteins for the cells to facilitate disulfide protein folding but at the same time could be toxic to the cells by imposing oxidative stress, as EROs produce ROS as by-products when they couple the oxidizing power to molecular oxygen during disulfide bond formation (11). In *Saccharomyces cerevisiae*, cell death under ER stress is attributed partly to ROS production resulting from ERO1 upregulation (35), while $Perk^{-/-}$ cells, in which protein synthesis is not properly attenuated under ER stress, accumulate ROS, leading to apoptosis (33), which is ameliorated by ERO1 abrogation (38).

Interestingly, ERO1β overexpression did not lead to ROS accumulation in the β cells in our model, nor was upregulation of antioxidant pathways observed. In contrast, we observed evidence of severe ER stress induced by ERO1β overexpression. However, despite the upregulation of proapoptotic genes such as *Chop* or *Trb3*, as well as the severe dilation of the ER lumen of hERO1βTg β cells, which is generally regarded as indicative of unfolded protein accumulation and ER stress (31, 33, 34), hERO1βTg islets did not show evidence of ER stress-induced β cell death; thus, the glucose intolerance of hERO1βTg mice was mild and became obvious only after an HFD load. This lack of apoptosis could simply be explained as a consequence of successful compensations achieved through the strongly invoked UPRs, possibly via the downregulation of insulin synthesis, leading to a sort of balanced and maintained status within the ER.