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Drak2 Overexpression Results in Increased β-Cell Apoptosis After Free Fatty Acid Stimulation

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ABSTRACT

Drak2 is a serine threonine kinase in the death-associated protein family. In this study, we investigated its role in free fatty acid (FFA)-induced islet apoptosis. Drak2 mRNA and protein were rapidly induced in islet β-cells after FFA stimulation. Such Drak2 upregulation was accompanied by increased β-cell apoptosis, which was inhibited by Drak2 knockdown using siRNA. Conversely, transgenic (Tg) Drak2 overexpression led to aggravated β-cell apoptosis triggered by FFA. Drak2 overexpression in islets compromised the increase of anti-apoptotic factors, such as Bcl-2, Bcl-xL and Flip, upon FFA assault. Further in vivo experiments demonstrated that Drak2 Tg mice presented compromised glucose tolerance in a diet-induced obesity model. Our data show that Drak2 is detrimental to islet survival in the presence of excessive lipid. J. Cell. Biochem. 105: 1073–1080, 2008.

KEY WORDS: Drak2; TYPE 2 DIABETES; ISLET

Due to calorie-rich diet and sedative life-style, obesity is epidemic in industrialized countries. Taking the US as an example, 30% of its population are obese and 50% are overweight [Wild et al., 2004] Obesity favors the development of the metabolic syndrome, of which type 2 diabetes (T2D) is one manifestation. In T2D, reduced insulin sensitivity is the major problem [Lockwood and Amatruda, 1983] initially, but at a later stage, islet apoptosis is also involved.

Blood free fatty acids (FFA) are generated mainly from lipolysis occurring in adipose tissues [Delarue and Magnan, 2007]. They are the main energy source for non-glucose dependent tissues such as the liver, heart and muscles. In obesity, the blood FFA level is permanently increased [Delarue and Magnan, 2007], and such increase causes insulin resistance in the liver and skeleton muscles. This then sets the condition for the T2D development. The chronic elevation of blood FFA, especially saturated FFA such as palmitate, has been shown to cause β-cell endoplasmic reticulum stress and apoptosis [Lai et al., 2008].

Recent research has also revealed that adipose and other tissues in T2D release harmful inflammatory cytokines, which are detrimental to islet function and survival [Kahn et al., 2006]. In the late stage of T2D, increased blood glucose in combination of elevated FFA contribute to β-cell apoptosis [Rhodes, 2005].

It is, therefore, conceivable that genes controlling β-cell apoptosis and survival are important in determining susceptibility to islet destruction and T2D risks.

Drak2 is a serine/threonine kinase belonging to a family of death-associated protein kinases (DAP kinases), which consists of DAP [Deiss et al., 1995], DRP-1 [Inbal et al., 2000], ZIP kinase [Kawai et al., 1998], DAPK2 [Kawai et al., 1999], Drak1 and Drak2 [Sanjo et al., 1998]. Drak2 shares about 50% identity in the kinase domain with other members of the family [Deiss et al., 1995]. While DAP, DRP-1 and DAPK2 have a calmodulin regulatory domain in their C-terminal, ZIP, Drak1 and Drak2 do not [Deiss et al., 1995; Kawai et al., 1998, 1999; Sanjo et al., 1998; Inbal et al., 2000]. DAP, DAPK2, and DRP-1 are localized in the cytosol [Deiss et al., 1995; Kawai

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et al., 1999; Inbal et al., 2000], ZIP kinase and Drak1 reside mainly in the nuclei [Kawai et al., 1998; Sanjo et al., 1998], whereas Drak2 is found in both the cytosol and nuclei [Sanjo et al., 1998; Matsumoto et al., 2001], suggesting different mechanisms of action. Drak2 autophosphorylates itself, and phosphorylates myosin light chain as an exogenous substrate [Sanjo et al., 1998]. Its endogenous substrates, other than itself, have not been identified. Drak2 interacts with a calcineurin homologous protein [Matsumoto et al., 2001], but the biological significance of this interaction is not clear.

When DAP family kinases are overexpressed in various cells, apoptosis ensues [Deiss et al., 1995; Kawai et al., 1998, 1999; Sanjo et al., 1998; Inbal et al., 2000], indicating their involvement in apoptosis. In Drak2 transgenic (Tg) mice, Tg T cells manifest augmented apoptosis after TCR stimulation followed by culture in the presence of IL-2; as a consequence, the memory T-cell pool is diminished, and the Tg mice incur compromised secondary but not primary in vivo T-cell responses [Mao et al., 2006].

In this study, we discovered that Drak2 expression in islets was rapidly induced by FFA. The induction was accompanied by islet apoptosis. Truncation of such Drak2 upregulation protected β-cells from apoptosis thus induced. Drak2 overexpression in Tg islets resulted in increased β-cell death in vitro upon FFA stimulation, and Drak2 Tg mice developed glucose intolerance after diet-induced obesity. The implications of these findings are discussed.

MATERIALS AND METHODS

ISLET PURIFICATION

Islet purification is performed as we described before [Wu et al., 2003, 2004]. Briefly, 2-ml of digestion solution (Hanks' balanced salt solution [HBSS]) containing 20 mM HEPES and 2 mg/ml collagenase IV (Worthington Biochemical, Lakewood, NJ) were injected into the common bile duct of Tg or wild type (WT) mice (20–24 g) after the distal end of the duct was ligated. The distended pancreas was isolated and put into a 15-ml tube containing an additional 0.5 ml of digestion solution. The pancreas was digested at 37°C for exactly 28 min, and the digestion process was stopped by the addition of 10 ml of cold HBSS containing 20 mM HEPES. The islet suspension was filtered through No. 7880 cheesecloth gauze (Tyco Healthcare, Mansfield, MA) and centrifuged at 500*g* for 1–2 min. The pellet was washed with cold HBSS once at 500*g* for 1–2 min, and the supernatant was removed completely. The pellet was then resuspended in 3 ml of 25% Ficoll, and 2-ml layers of 23%, 20%, and 11% Ficoll were added sequentially. The Ficoll gradient was centrifuged at 700*g* for 5 min. Most of the islets were in the interface between the 20 and 23% Ficoll layers and were handpicked with Pasteur pipettes. They were then washed twice with cold HBSS. The islets were cultured overnight in RPMI 1640 containing 10% FCS, and then used for experimentation.

REAL-TIME RT-PCR

Drak2 mRNA in Tg and WT cells was measured by real-time RT-PCR; the 5′ and 3′ primers were CACAGCTGGCCACAGACTTC and CAGAGGACCTGAGAGTCAG, respectively. A 160-bp product was detected with the following amplification program: 95°C × 15 min, 1 cycle; 94°C × 15 s, 55°C × 30 s, 72°C × 30 s, 40 cycles.

Real-time RT-PCR was also conducted to assess Bcl-2, Bcl-xL and Flip mRNA levels. The 5′ and 3′ primers for Bcl-2 were CTCGTCGCTACCGTCGTGACTTCG and GTGGCCCAGGTATGCACCCAG, respectively, for the detection of a 380-bp band; the 5′ and 3′ primers for Bcl-xL were TGGAGTAAACTGGGGTCGCATC and AGCCACAGTCATGCCCGTCAGG, respectively, for the detection of a 264-bp band; the 5′ and 3′ primers for Flip were GTGGAAGAGTGTCTTGATGAAG and GAGCGAAGCCTGGAGAGTATT, respectively, for the detection of a 480-bp band. The amplification program was the same as that used for Drak2 mRNA.

β-Actin mRNA levels were measured as internal controls; the 5′ and 3′ primers were TGGTACCACAGGCATTGTGAT and TGATGTCACGCACGATTTCCCT, respectively, with the same amplification program as for Drak2 mRNA.

Real-time PCR was performed in triplicate, and the signal ratios of Drak2/β-actin, Bcl-2/β-actin, Bcl-xL/β-actin and Flip/β-actin represented the normalized expression levels of these genes.

FLOW CYTOMETRY

Drak2 Tg and WT islets were digested with 0.05% trypsin-EDTA to obtain single cell suspensions. The cells were fixed with 4% paraformaldehyde and permeabilized with 0.2% Triton X-100. They were stained with rabbit anti-Drak2 Ab (Abgent, San Diego, CA; 1:50 dilution) and anti-insulin mAb (Sigma, St. Louis, MO; 1:500 dilution). Subsequently, they were stained with FITC-conjugated sheep anti-rabbit antibody (Chemicon, Temecula, CA), and PE-conjugated goat anti-mouse antibody (Jackson Immunoresearch, West Grove, PA), and analyzed by two-color flow cytometry. Dispersed islet cells or small interfering RNA (siRNA)-transfected NIT-1 cells were also analyzed for apoptosis by flow cytometry using FITC-annexin V staining [Murakami et al., 2004].

Drak2 KNOCKDOWN BY siRNA IN NIT-1 CELLS

NIT-1 cells, derived from mouse insulinoma, were transfected with siRNA using Lipofectamine 2000 (Invitrogen, Burlington, Ontario) according to the manufacturer's instructions. For Drak2 siRNA, the oligonucleotide RNA sequences were CAUCCCUGAAGAUGGCAGCtt and GCUGCCAUCUUCAGGGAUGtt. The control was the scrambled sequence of said siRNA with following sequences: 5′CCCUAAGUGUAGGACGCACtt and 3′GUGCGUCCUACACUUAGGGtt. Single stranded RNA pairs were annealed by being incubated for 1 min at 90°C, and then cooled down to room temperature over 45 min. The final concentration of double-stranded siRNA was 20 μM for transfection.

INSULIN RELEASE ASSAY

After 48 h culture in complete F-12K medium with 10% FCS in the absence or presence of various stimulants, the islets were transferred to 12-well plates at a density of 10 islets/well. The islets were gently washed twice with 1 ml Kreb's buffer (NaCl, 135 mM; KCl, 3.6 mM; NaH_2PO_4, 5 mM; $MgCl_2$, 0.5 mM; $CaCl_2$, 1.5 mM; $NaHCO_3$, 2 mM; HEPES, pH 7.4, 10 mM; BSA, 0.07%), and then incubated in Kreb's buffer containing 2.8 mM glucose for 5 min at 37°C. Two hundred microliters of supernatant were removed for determination of basal insulin levels. The islets were cultured for additional 40 min, and all the supernatants were harvested for determination of insulin levels

as 2.8 mM glucose-stimulated release. The islets were then cultured in Kreb's buffer containing 16.7 mM glucose for 45 min at 37 °C, and the supernatants were harvested for determination of insulin levels as 16.7 mM glucose-stimulated release. The insulin was assayed by ELISA (Linco Research, St. Charles, MO). The basal insulin levels, which were near zero, were deducted from the 2.8 and 16.7 mM glucose-stimulated levels in final data presentation, which was the fold increase of insulin levels of high glucose versus low glucose stimulation.

GLUCOSE TOLERANCE TESTS

Tg and WT mice were fed a high-fat diet (45% of total calories in the form of fat; Research Diets Inc. New Brunswick, NJ) from age 9 weeks for 6 weeks. They were then fasted for 16 h and injected i.p. with D-glucose (2 mg/g body weight) in PBS. Blood samples from the tail vein were taken at 15, 30, 60, 90, and 120 min after injection for glucose measurements with a glucose meter (Bayer, Toronto, Ontario).

FFA MEASUREMENTS

Serum samples were collected and stored at −20°C until analysis. Their FFA concentrations were determined utilizing the nonesterified fatty acid assay kit (Wako, Richmond, VA) following a modified version of the manufacturer's protocol to accommodate a 96-well microplate. This method utilizes the acylation of coenzyme A by fatty acids in the presence of added acyl-CoA synthetase. Briefly, reagents A and B were prepared as directed by the protocol and then diluted with 13.3 ml of 0.05 M phosphate buffer. Seven standards, ranging from 0 to 1 mEq/L, were prepared from the supplied stock (1 mEq/L). The samples, standards, controls, and blanks were then added to a 96-well microplate in duplicate (10 μl/well). At that time, 100 μl of reagent A was added to each well and mixed prior to a 30-min incubation at room temperature. Finally, reagent B was added (200 μl per well), and mixed followed by an additional 30-min incubation at room temperature, after which the plate was read at 550 nm. Utilizing the standard curve, sample values were expressed as mEq/L.

RESULTS

RAPID INDUCTION OF Drak2 EXPRESSION IN ISLET β-CELLS AND ITS ASSOCIATION WITH ISLET APOPTOSIS

In obesity and T2D, high serum lipid is known to jeopardize islet function and survival [Ahren, 2005]. When isolated islets were exposed to FFA in vitro, Drak2 mRNA was drastically induced within 24 h (Fig. 1A). When FFA was injected into mice i.p., within

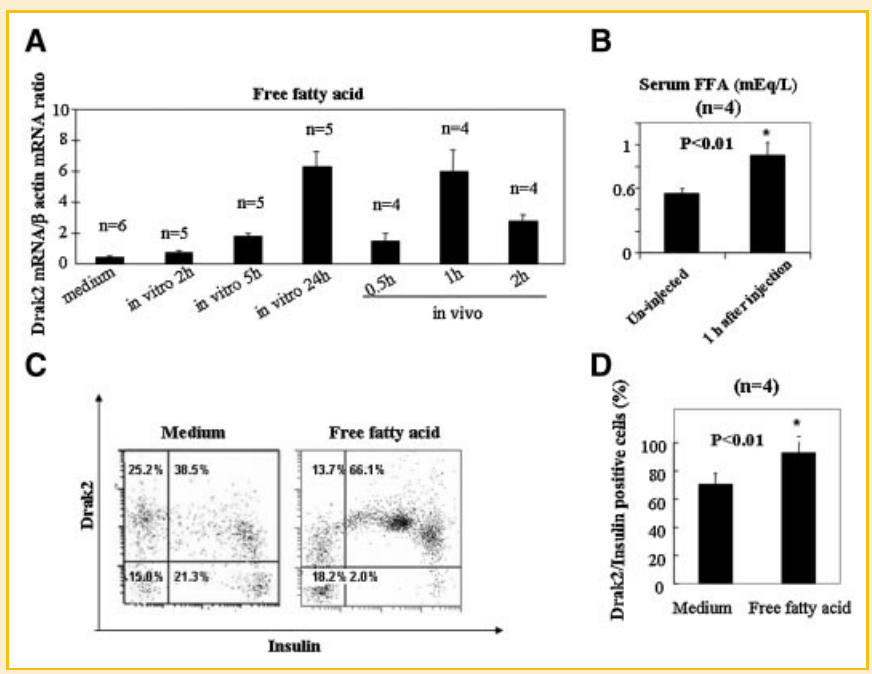

Fig. 1. Drak2 was rapidly augmented in islets treated with FFA. A: Drak2 mRNA expression according to real-time RT-PCR. Islets were stimulated by FFA (0.7 mM oleate and palmitate mixed in a 2:1 ratio) in vitro, or C57BL/6 mice were injected with 15mM FFA 0.5ml (oleate and palmitate mixed in a 2:1 ratio) i.v. in PBS at the indicated time before sacrifice of the mice. For the in vivo experiment, the time indicated was from the time of FFA injection until the sacrifice; the duration of islet isolation (about 1 h) was not calculated in. Drak2 mRNA expression in islet cells was measured by real-time RT-PCR. The ratio of Drak2 mRNA and β-actin mRNA was taken as a measure of Drak2 mRNA levels. The samples were in triplicate, and the means + SD of four to six independent experiments (as indicated) are shown. B: Plasma FFA levels after FFA injection. C57BL/6 mice were injected i.p. with 0.5 ml of 15mM FFA (oleate and palmitate mixed in a 2:1 ratio), and their plasma FFA levels 1 h after the injection were measured by ELISA. Samples were in duplicate. Means + SD of the injected and control groups are shown (n = 4 for each group). The difference is statistically significant ($P < 0.01$, Student's *t*-test). C,D: Drak2 protein expression according to flow cytometry. C57BL/6 islets were cultured for 48 h in the absence or presence of FFA as described in (A). The islets were dispersed after the culture and analyzed by two-color flow cytometry for intracellular insulin and Drak2 staining. The experiment was repeated 4 times. A representative set of histograms is shown in (B) and the summary of all four experiments is illustrated in (C). The asterisk indicates a *P*-value of <0.01 according to Student's *t*-test.

1 h, serum FFA levels were increased (Fig. 1B). Such a FFA concentration increase was accompanied by a significant augmentation of Drak2 expression at 1 h (Fig. 1A; time shown was the duration between FFA injection and mouse sacrifice; the time of islet isolation was not calculated in). The in vivo response was much faster than in vitro response. A likely explanation was that β-cells in vivo in the pancreas were in their natural environment with full viability and function, while isolated islets in vitro had experienced traumatic isolation procedures, and were slow to respond to additional stimuli.

We next assessed Drak2 protein levels in β-cells, employing anti-insulin mAb and anti-Drak2 Ab in two-color flow cytometry. When the islets were stimulated with FFA, Drak2 protein levels in insulin-positive β-cells were significantly augmented at 48 h after FFA assault, as shown in histogram 1C; a summary of 3 independent experiments is illustrated in Figure 1D. The finding on Drak2 protein increase was consistent with the heightened Drak2 mRNA expression at 24h. FFA induced islet cell apoptosis (Fig. 2A, top row, WT islets). Taken together, our data indicate that Drak2 overexpression in islets leads to their apoptosis.

Drak2 KNOCKDOWN BY siRNA PROTECTED NIT-1 INSULINOMA CELLS FROM FFA-TRIGGERED APOPTOSIS

To prove that Drak2 was indeed critical to FFA-induced β-cell apoptosis, we employed siRNA to prevent Drak2 upregulation in NIT-1 insulinoma cells. NIT-1 cells were derived from a transgenic NOD mouse harboring a hybrid rat insulin-promoter/SV40 large T-antigen gene [Hamaguchi et al., 1991]. As shown in Figure 3A, similarly to normal β-cells, Drak2 protein was induced in NIT-1 cells by FFA according to flow cytometry. Two different Drak2 siRNAs (#592 and #1162) significantly truncated Drak2 protein upregulation stimulated by FFA, but a control siRNA had no effect on the Drak2 level. As in normal islet cells, FFA induced NIT-1 cell apoptosis after 24 h (Fig. 3B). However, with protection by the 2 Drak2 siRNAs #592 and #1162, but not the control siRNA, such apoptosis induction was truncated. We noticed that FFA-induced apoptosis in NIT-1 cells occurred at 24 h after the FFA assault, compared with 48 h needed for normal islet cells. The faster apoptosis kinetics in NIT-1 cells was probably due to that they were transformed cells with more active cellular machinery for live and death decisions.

Drak2 OVEREXPRESSION IN Tg ISLETS AGGRAVATED FFA-TRIGGERED APOPTOSIS

To further validate the role of Drak2 in islet survival, actin promoter-driven Drak2 Tg mice, as described in our previous publication [Mao et al., 2006], were studied. These mice are viable, fertile, and have no gross anomalies. They had comparable body weight with WT littermates from 6 to 22 weeks old (Fig. 4A). Their serum inflammatory cytokine IL-6 levels were similarly low as that of

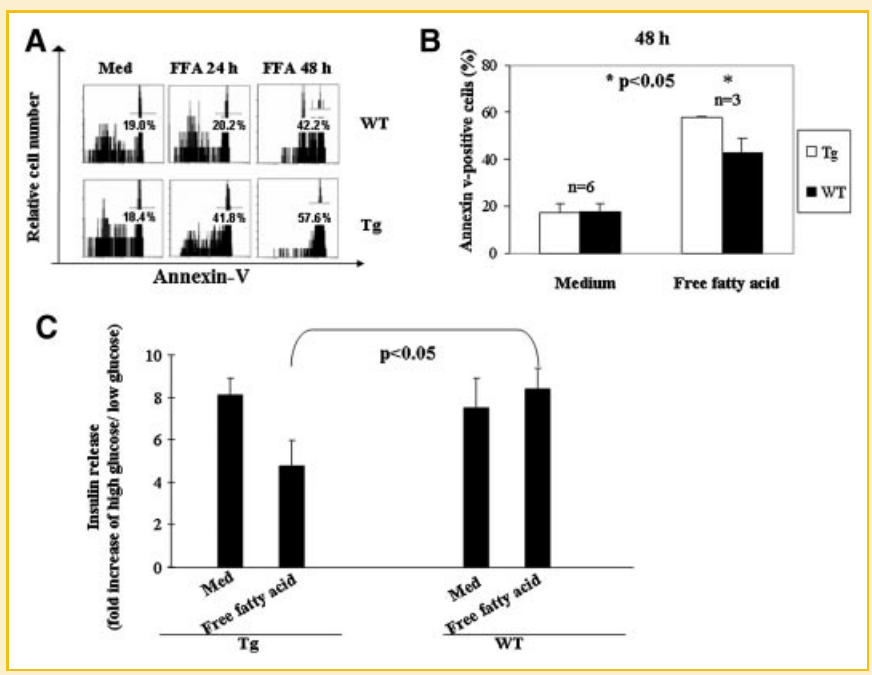

Fig. 2. Drak2 Tg islets were prone to apoptosis upon FFA stimulation. A,B: Flow cytometry analysis of islet cell apoptosis. Drak2 Tg and WT islets were cultured in RPMI 1640 with 10% FCS and stimulated with FFA as described in Figure 1. After 24 or 48 h, as indicated, the islets were dispersed and analyzed by flow cytometry with annexin V staining. The percentage of annexin V-positive cells is shown in the histograms (A). The experiment was repeated 3–6 times and the mean + SD of data at 48 h of all these experiments are illustrated in (B). The asterisk indicates $P < 0.05$, according to Student's *t*-test. C: Islet insulin release after FFA stimulation. Islets from Tg or WT mice were cultured in F-12K medium with 10% FCS in the presence or absence of FFA as described in Figure 1. Insulin release by these cells was conducted after 48 h. For each treatment, the fold increase between low glucose and high glucose stimuli was presented. Insulin release by Tg islets after FFA stimulation was significantly lower than that by WT islets ($P < 0.05$, Student's *t*-test).

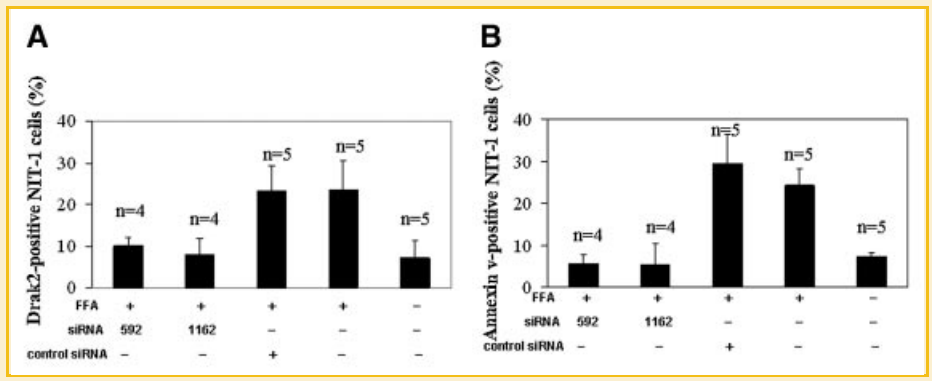

Fig. 3. Drak2 siRNA inhibited Drak2 protein upregulation and reduced apoptosis in NIT-1 cells upon FFA stimulation. A: Drak2 protein levels in NIT-1 cells. NIT-1 cells were transfected with 2 Drak2 siRNAs (#592 and #1162), or a control siRNA, which is with a scrambled sequence of #1162. The cells were cultured for 24 h in the absence or presence of FFA, as indicated, and then analyzed for intracellular Drak2 protein levels by flow cytometry. The experiment was repeated 4–5 times, as indicated, and means + SD of these experiments are shown. B: Drak2 siRNA prevented FFA-induced apoptosis in NIT-1 cells. NIT-1 cells were transfected with 2 different Drak2 siRNAs (#592 and #1162), or a control siRNA, as described in (A). The cells were cultured for 24 h in the absence or presence of FFA, as indicated, and analyzed for apoptosis by flow cytometry with annexin V staining. The experiment was repeated 4–5 times, as indicated, and means + SD of percentage apoptosis of all of these experiments are shown.

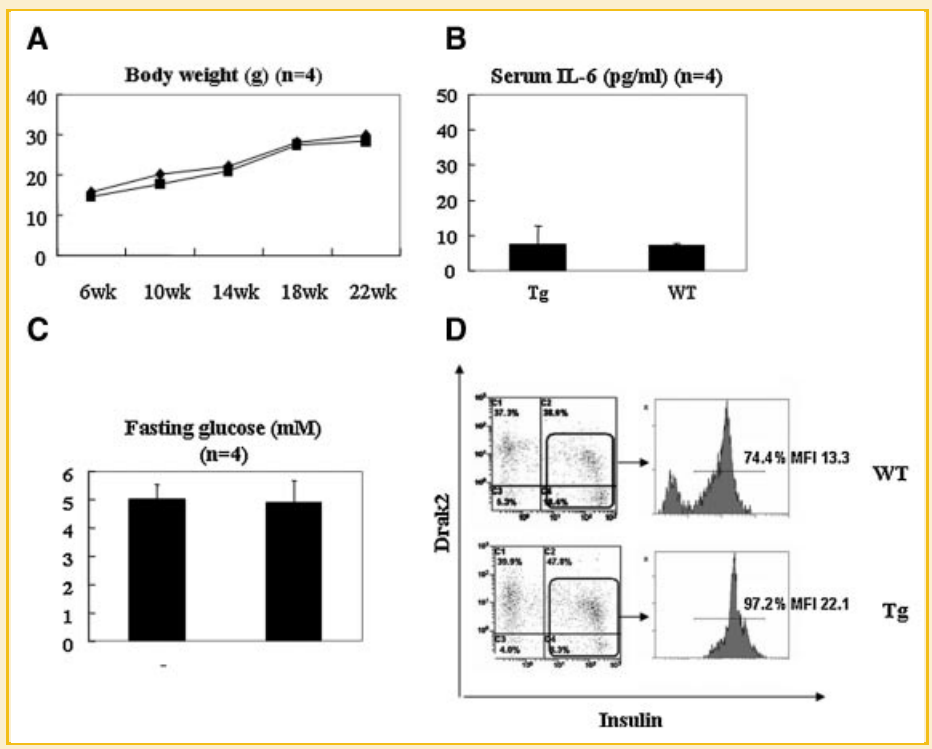

Fig. 4. Features of Drak2 Tg mice. A: Body weight of Tg and WT mice. The body weight (means + SD) of Tg and WT littermates (n = 4 for each group) from 6 to 22 weeks of age are illustrated. No significant difference in any time point is found ($P > 0.05$, Student's *t*-test). B: Serum IL-6 levels of Tg and WT mice. Serum IL6 of Tg and WT mice (n = 4 for each group) were measured by ELISA. The ELISA samples were in duplicate. Means + SD of IL-6 levels are shown. There is no statistical difference between the two groups ($P > 0.05$, Student's *t*-test). C: Fasting serum glucose levels of Tg and WT mice. Blood glucose of Tg and WT mice (n = 4 for each group) were measured after overnight fasting. Means + SD of the glucose levels are shown. There is no statistical difference in the blood glucose levels of the two groups ($P > 0.05$, Student's *t*-test). D: Drak2 overexpression in Tg β-cells. Drak2 Tg or WT islets were analyzed by two-color flow cytometry for Drak2 and insulin expression (right column). The percentage of Drak2 positive cells among insulin-positive cells and their mean fluorescent intensity (MFI) are indicated in the left column. Upper row, WT; bottom row, Drak2 Tg.

WT mice (Fig. 4B). There was no significant difference in fasting blood glucose levels between Tg and WT mice (Fig. 4C). We demonstrated in Figure 4D that Drak2 protein expression in insulin-positive Tg islet cells was augmented both in terms of Drak2 mean fluorescent intensity (MFI) and percentage of Drak2 positive cells, compared with WT islet cells (MFI 22.1 in Tg β-cells versus MFI 13.3 in WT β-cells; 97.2% versus 74.4% Drak2 positive Tg β-cells versus WT β-cells), according to Drak2/insulin two-color flow cytometry.

When Tg islets were stimulated with FFA for 24 h, their apoptosis was significantly increased, compared to WT islets (41.8% vs. 20.2%; Fig. 2A, 2nd column). At 48 h, WT islets also started to suffer from apoptosis, but Tg islets were inflicted with more damage (57.6% vs. 42.2%, Tg vs. WT; Figure 2A, 3rd column; a summary of three independent experiments with data collected at 48 h is illustrated in Fig. 2B).

To assess the function of β-cells under FFA assault, we evaluated islet insulin release after 16.7 mM glucose stimulation. At 48 h after FFA assault, insulin released by the Tg β-cells was significantly lower than by WT β-cells (Fig. 2C). This confirmed that augmented Drak2 expression was harmful to β-cell function.

Drak2 OVEREXPRESSION COMPROMISED ANTI-APOPTOTIC MOLECULE INDUCTION

To understand the molecular mechanisms of β-cell apoptosis associated with Drak2 overexpression, we surveyed the expression levels of a group of anti-apoptotic factors in Tg versus WT β-cells. Anti-apoptotic factors Bcl-2, Bcl-xL and Flip were expressed at low levels in WT and Tg β-cells, but were significantly induced 24 h after FFA stimulation in WT β-cells (Fig. 5); however, such induction was compromised in Tg β-cells. The data indicate that Drak2 overexpression in islets reduce the elevation of anti-apoptotic factors upon detrimental stimulation, and suggests that such compromise might be one of the reasons that renders β-cells prone apoptotic.

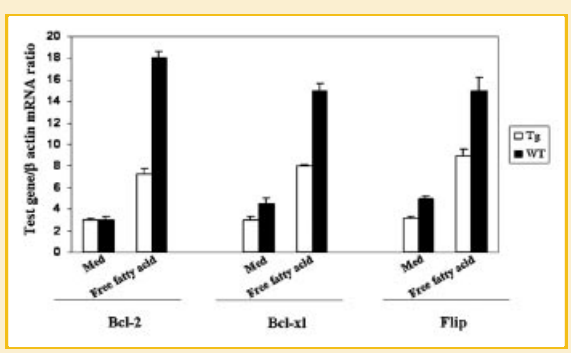

Fig. 5. Compromised anti-apoptotic factor upregulation in Drak2 Tg islets. Drak2 and WT islets were stimulated by FFA as described in Figure 1. The islets were harvested after 24 h, and their Bcl-2, Bcl-xL and Flip mRNA was measured by real-time RT-PCR. The samples were in triplicate. Means + SD of the ratios of signals of these molecules versus those of β-actin from two independent experiments are shown.

Drak2 OVEREXPRESSION LED TO GLUCOSE INTOLERANCE IN MICE UNDER HIGH FAT DIET

With a regular diet, Tg mice in the C57BL/6 background at 9 weeks of age had a moderate elevation of serum FFA, compared with WT mice, although the difference did not reach statistical significance (Fig. 6A). The Tg mouse serum fasting insulin level at this stage was significantly higher than that of WT (Fig. 6B), probably due to insulin resistance caused by the higher serum FFA levels; such FFA-related insulin resistance and the subsequence insulin level increase has been reported previously [Jiao et al., 2008]. When the mice were fed a high fat-diet for 6 weeks to mimic a pre-T2D condition, that is, diet-induced obesity [Winzell and Ahren, 2004], both Tg and WT animals became overweight, on average 10 g heavier than mice on a normal diet (data not shown). After the high-fat diet, both groups maintained normal fasting blood glucose levels (Fig. 6C). However, Tg mouse serum FFA levels were significantly higher than those of WT mice (Fig. 6A); Tg mouse fasting insulin levels were significantly lower than those of WT mice. Moreover, in the glucose tolerance test, Tg mice manifested statistically significantly higher blood glucose levels at 30, 60, and 90 min after glucose injection (Fig. 6C). The interpretation of these results is given in Discussion Section.

DISCUSSION

In this study, we have demonstrated that Drak2 is critical in β-cell apoptosis triggered by FFA. Further in vivo experiments proved that enhanced Drak2 expression reduced glucose tolerance after high-fat diet. It seems that Drak2 is in the pathway downstream of harmful signals received by islets upon FFA stimulation.

We showed that Drak2 was upregulated in islet β-cells upon FFA stimulation, and such upregulation was correlated to decreased islet function and survival. Interestingly, although Tg islets had higher Drak2 expression, such over expression by itself did not manifest harmful effects on the islets, as Tg mice did not develop diabetes, and Tg islets culture in medium did not suffer from increased apoptosis, compared to WT islets, until an exogenous detrimental factor FFA was present. This suggests that Drak2 might act on a two-hit mode, in which signaling event(s) (hit 1) derived from FFA stimulation as well as Drak2 (hit 2) are both required to results in β-cell damage and/or dysfunction. For normal islets, high Drak2 expression (hit 2) could be a consequence of FFA (hit 1). We hypothesize that in individuals with abnormally high basal Drak2 expression in islets, less hit 1 might be sufficient to cause excessive islet damage or dysfunction, and thus these individuals are more prone to T2D development when facing increased serum lipid. In humans, Drak2 gene is located in 2q33.2, and is 14.8 Mb away from a type 2 diabetes risk region at 2q32.1 (http://www.ncbi.nlm.nih.gov/entrez/dispomim.cgi?cmd=entry&id=601724). An epidemiological study on T2D prevalence in individuals having enhanced Drak2 expression will answer the question whether Drak2 is a bona fide diabetes risk gene in humans.

We found Tg mice had a moderately increased serum FFA level compared with WT mice under a normal diet. The mechanism of such an increase is not clear at the present time. It is quite likely that

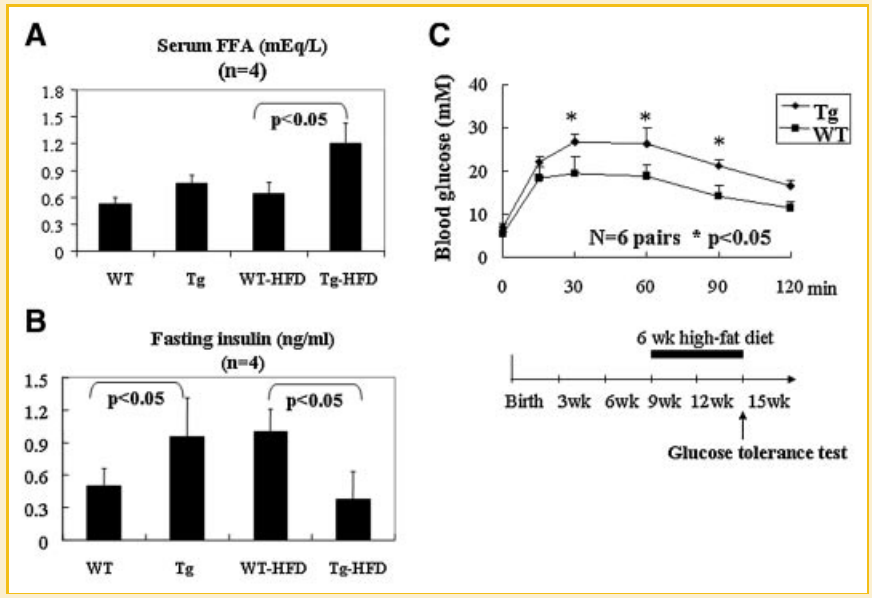

Fig. 6. Features of Drak2Tg mice after diet-induced obesity. A: Serum FFA levels of Tg and WT mice before and after high-fat diet. Tg and WT mouse serum FFA levels were measured by ELISA before and after a 6-week high-fat diet (HFD). The ELISA samples were in duplicate. Means + SD of the FFA levels of the two groups (n = 4 for each group) are shown. The serum FFA levels of the Tg and WT groups were significantly different after HFD ($P < 0.05$, Student's *t*-test). B: Fasting serum insulin levels of Tg and WT mice before and after high-fat diet. Tg and WT mouse fasting serum insulin levels were measured by ELISA before and after 6-week HFD. The ELISA samples were in duplicate. Means + SD of the insulin levels of the two groups (n = 4 for each group) are shown. The insulin levels of the Tg mice were significantly higher than those of WT mice before HFD, and were significantly lower than those of WT mice after HFD ($P < 0.05$ in both cases, Student's *t*-test). C: Reduced glucose tolerance in Drak2 Tg mice after diet-induced obesity. Drak2 Tg and WT mice were fed a high-fat diet for 6 weeks from 9 weeks of age. Both groups became obese at age 15 weeks when the glucose tolerance test was conducted. Tg mice on a high-fat diet presented significantly higher blood glucose at 30, 60, and 90 min after i.p. glucose injection, compared to WT mice (n = 6 pairs, $P < 0.05$, Student's *t*-test).

such a FFA increase caused insulin resistance, which in turn was responsible for the elevated serum insulin levels. It was somewhat surprising that after a high-fat diet, in the presence of prolonged higher serum FFA levels in Tg mice, the fasting insulin levels of Tg mice were significantly lower than those of WT mice. A possible explanation is that the prolonged high FFA levels in Tg mice compromised islet β-cells in terms of insulin release and/or survival. When these mice underwent the glucose tolerance test, they could not cope with the transient surge of blood glucose by providing sufficient insulin, and manifested a prolonged high glucose levels, compared to WT mice.

Currently, our knowledge about the Drak2 activation pathway and Drak2 substrates is limited. We only know that Drak2 is a genuine substrate of itself. Obviously, genes in the Drak2 pathway could all be involved in FFA-induced islet dysfunction and apoptosis, which are critical factors in type 2 diabetes development.

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