Augmented reduction of islet β-cell mass in Goto-Kakizaki rats fed high-fat diet and its suppression by pitavastatin treatment

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ABSTRACT

Aims/Introduction: High fat diet (HFD) is known to be a risk for development of type 2 diabetes. It is unclear, however, how it affects the glucose tolerance or the islet structure in type 2 diabetes. The aim of this study is: (i) to examine the effects of HFD on the islet in GK rats, non-obese type 2 diabetic model; and (ii) to explore if pitavastatin treatment influences the change.

Materials and Methods: To see the effects of HFD on islet changes in type 2 diabetes, 4-week old male GK and Wistar rats were fed HFD for 16 weeks and subjected to glucose tolerance tests and pathological studies of the islet. The effects of pitavastatin (3 mg/kg/day for 16 weeks, oral), one of the lipophilc statins, were also examined in both GK and Wistrar rats fed with or without HFD.

Results: The HFD induced hyperlipidemia and aggravated glucose intolerance in both GK and Wistar rats. Pitavastatin treatment did not influence the glucose tolerance in HFD-fed animals. HFD caused an increase in hepatic lipid contents in all the animals, which was partially suppressed by pitavastatin treatment. GK rats showed reduced β-cell mass, and fibrosis and macrophage migration in the islets. HFD feeding in GK rats augmented these changes which were associated with enhanced expression of 8-hydroxydeoxyguanosine and an increase in apoptotic cells. Pitavastatin treatment improved the HFD-induced islet pathology, and pancreatic insulin contents paralleled the structural changes.

Conclusions: HFD feeding worsened the islet pathology in GK rats which was suppressed by pitavastatin treatment. (J Diabetes Invest, doi: 10.1111/j.2040-1124.2011.00173.x, 2012)

INTRODUCTION

Type 2 diabetes is commonly associated with dyslipidemia and hypertension[1]. Since comorbidity with type 2 diabetes is known to increase the risk of atherosclerotic diseases, comprehensive management of the risk factors is essential to the reduction of mortality caused by lifestyle-related diseases[2]. β-Hydroxy-β-methylglutaryl (HMG)-CoA reductase inhibitors (statins) are now widely used in the treatment of hypercholesterolemia. The efficacy and safety of statins in patients with dyslipidemia complicating diabetes mellitus are still under discussion. Since hyperlipidemia is likely to aggravate diabetes by increasing insulin resistance or by lipotoxic effects on the islets, statin treatment might be expected to reduce such risk factors. Although large-scale clinical studies have revealed that statins had a preventive effect on cardiovascular events in patients with type 2 diabetes on one hand[3], worsening of HbA$_{1c}$ levels and β-cell functions has also been reported[4,5]. It remains unclear whether various statins have different actions on the islet β-cells or the effects are different on lean or obese type 2 diabetic patients.

The spontaneously diabetic Goto-Kakizaki (GK) rat is a model of non-obese type 2 diabetes[6,7]. The pancreas in GK rats is found to be depleted of insulin with reduction of β-cell mass[8,9]. We previously demonstrated that β-cell loss in GK rats was much accelerated when they were fed sucrose[10,11]. Information is sparse, however, as to whether feeding with HFD affects diabetes or influences the pancreatic islet pathology in GK rats. Since most Japanese type 2 diabetic patients are non-obese with poor insulin secretion and are now exposed to a lipid-rich environment, data on the effects of HFD on the islet lesions in GK rats may be useful for the translation to humans. Furthermore, in view of the frequent use of statins to lower the high blood lipids, information on the islet lesions may also be extremely important for the management of type 2 diabetic patients when treated with statins. In this study, we therefore evaluated the effects of HFD and concurrent treatment with pitavastatin, one of the lipophilic statins, on the glucose tolerance and pancreatic islet pathology in GK rats.

MATERIALS AND METHODS

Animals

Four-week-old GK/Jcl rats (GK rats) and Wistar/Jcl rats (Wistar rats) were purchased from CLEA Japan Inc. (Tokyo, Japan). Based on the plasma levels of glucose and insulin, both GK rats and Wistar rats were divided first into two balanced groups; one fed HFD (Quick Fat; CLEA) (protein 25%, lipids 15%) and another fed a standard diet (CE-2, CLEA) (protein 25%, lipids 5%). In either GK or Wistar rats, both HFD-fed and standard-diet-fed groups were further divided into pitavastatin-treated group and untreated group (see footnote of Table 1). Each animal group consisted of six to ten animals. Pitavastatin (3 mg/kg) (Kowa Co. Ltd, Tokyo, Japan) suspended in 0.5% carboxymethylcellulose was given by gavage once daily for 16 weeks and the untreated group received carboxymethylcellulose alone. In a previous study, treatment with pitavastatin 3 mg/kg resulted in an improvement of the glucose tolerance in KKAy mice[12]. Therefore, the dose of pitavastatin in this study was set at 3 mg/kg. Tap water was freely supplied through a water bottle. All the procedures followed the institutional guidelines of Hirosaki University Animal Experimentation for the care and use of laboratory animals (approval #M05032).

Blood Chemistry and Tissue Concentrations of Lipids and Insulin

Body weight and blood glucose concentrations were regularly monitored every 4 weeks on tail blood in a non-fasting state. An oral glucose tolerance test (GTT) (2 g glucose/kg body weight) was performed in all the experimental animals after a 16 week-duration of HFD with or without pitavastatin treatment. Blood glucose and insulin concentrations were examined before and after glucose challenge. Blood glucose and insulin concentrations were measured on tail blood by the Glucose C-II test (Wako Pure Chemical Industries, Tokyo, Japan) and insulin ELISA kit (Shibayagi Co. Ltd., Maebashi, Japan). On the day following the GTT, all the animals at fast were killed by blood withdrawal from cardiac puncture under anesthesia with pentobarbital (50 mg/kg, i.p.) (Abbott Laboratories, Chicago, IL, USA). Then, the liver and pancreas were excised for the measurement of tissue lipid concentrations and insulin contents, respectively. A portion of the body and tail of the pancreas was fixed in Bouin's solution for histological evaluations. Blood concentrations of total cholesterol (TC) and triglyceride (TG) were measured by an enzymatic colorimetric method using cholesterol oxidase and N-ethyl-N-(-2-hydroxy-3-sulfopropyl)-3.5-dimethoxyaniline sodium salt (DAOS) as Cholesterol E-test Wako (Wako Pure Chemical Industries, Ltd., Osaka, Japan) and glycerol -3-phosphate oxidase (GPO) and DAOS as Triglyceride E-test Wako (Wako), respectively. Liver lipid contents were measured as previously described after lipid purification[13]. For the measurement of insulin contents, excised pancreatic tissues were homogenized using 10 times acid-ethanol solution (0.2 N HCl/75% ethanol) (vol/g) in a potter tube and then centrifuged at 15,000 rpm for 15 min, at 4°C. Supernatant was subjected to measurement of insulin by ELISA (Morinaga Institute of Biological Sciences, Yokohama, Japan).

Histological Examinations

All the fixed tissues were embedded in paraffin and stained with hematoxylin and eosin (H&E) for review. For identification of β-cells and α-cells, double immunostaining procedures were applied using streptavidin biotin methods (Nichirei Histofine; Nichirei Co., Tokyo, Japan)[10]. Briefly, deparaffinized sections were first incubated with rabbit anti-glucagon antibody (1:500) (Dako Cytomation, Glostrup, Denmark) followed by incubation with biotinylated anti-rabbit immunoglobulin antibody and alkaline phosphatase-conjugated streptavidin. The alkaline phosphatase reaction was detected by a fresh fuchsin staining kit (Nichirei). The sections were then incubated with polyclonal anti-insulin antibody (1:500) (Dako). They were incubated again with biotinylated immunoglobulin and peroxidase-conjugated streptavidin. The reaction products were visualized with a diaminobenzidine staining kit (Dako). Nuclei were counterstained lightly with hematoxylin.

Table 1 | Laboratory data of experimental animals at the end of experiment

Group	Body weight (g)	Serum triglyceride (mmol/L)	Serum total cholesterol (mmol/L)	Non-fasting blood glucose (mmol/L)
W (*n* = 6)	464 ± 8	1.3 ± 0.2	2.1 ± 0.1	6.8 ± 0.2
W + P (*n* = 6)	434 ± 24	1.2 ± 0.1	2.0 ± 0.1	6.7 ± 0.2
W + HFD (*n* = 8)	510 ± 11*	9.5 ± 0.4*	3.1 ± 0.1*	8.1 ± 0.2
W + HFD + P (*n* = 8)	496 ± .8†	8.0 ± 0.1	2.7 ± 0.2§	8.0 ± 0.2
GK (*n* = 6)	377 ± 6	0.9 ± 0.1	2.8 ± 0.1	12.9 ± 1.3
GK + P (*n* = 6)	370 ± 4	1.2 ± 0.2	2.9 ± 0.1	13.4 ± 1.3
GK + HFD (*n* = 6)	427 ± 6‡	3.7 ± 0.5¶	3.1 ± 0.1‡	16.8 ± 2.2‡
GK + HFD + P (*n* = 6)	397 ± 11	3.9 ± 0.6	2.9 ± 0.1	17.8 ± 1.1

W, Wistar rat; W + P, pitavastatin-treated Wistar rat; W + HFD, Wistar rats fed high fat diet (HFD); W + HFD + P, Piatavastatin-treated Wistar rats fed HFD; GK + P, Pitavastatin-treated GK rats; GK + HFD, GK rats fed HFD; GK + HFD + P, Piatavastatin-treated GK rats fed HFD. Values are mean ± SE, *$P < 0.01$ vs W, †$P < 0.01$ vs W + P, ‡$P < 0.05$ vs GK, §$P < 0.01$ vs W + HFD, ¶$P < 0.01$ vs GK.

To detect the cell death and proliferating cells of islet endocrine cells, immunostaining was conducted using an ApoTag kit (Millipore, Bellerica, MA, USA) and Ki-67 antibody (Abcam, Cambridge, UK). For the evaluation of oxidative stress-induced cell damage, islet fibrosis and inflammatory conditions, immunostains using monoclonal antibodies to 8-hydroxy-deoxyguanosine (8OHdG) (Nikken Seil Co. Ltd., Tokyo, Japan), collagen type III (Abcam), and ED-1 (Serotec Co. Ltd., Sapporo, Japan) were used, respectively. Secondary antibodies of biotinylated anti-mouse immunoglobulin antibody and peroxidase-conjugated streptavidin (Dako) were subsequently incubated and the reaction products were colorized by diaminobenzidine (Dako) or a fresh fuchsin (Nichirei).

Control staining for the specificity was conducted by: (i) omitting the first antibody; (ii) replacing the first antibody by non-immune rabbit or mouse sera; and (iii) use of the first antibody after preincubation of excessive antigens when available, yielding negative staining.

Morphometric Analysis of Islet Cells

Quantitative evaluation of the islet was performed using a computer-assisted point-counting method on an Olympus AX80 microscope connected to a personal computer system using an NIH image (Version 1.56, free software; NIH, Bethesda, MD, USA)[10]. Calculation of the volume density of α- and β-cells with the point-counting method was based on previously described methods[10,14]. During the process of morphometric analysis, the identity of the samples was masked to the examiners.

Quantification of Oxidative Stress-induced Cell Damage, Cell Death, Proliferation, Macrophage Migration and Fibrosis in Islets

Oxidative stress-induced cell damage was reflected by strong immunoreactions against 8OHdG in the islets. Immunoreactions were semiquantified into four grades; 0, negative; 1, weakly positive in the islet relative to completely negative background of exocrine cell nuclei; 2, moderately positive; 3, strongly positive. Frequency of apoptotic cells positive for ApopTag was quantified by counting the cells with positive nuclear reactions among more than 1000 islet endocrine cells. In a similar manner, frequency of proliferating cells positive for Ki67 was quantified and expressed as a percentage. Macrophages infiltrated into the islets were identified by positive staining for ED-1 and their appearance was quantitatively expressed as the number per unit islet area[15]. The levels of fibrosis in each islet were semiquantitatively expressed on the sections stained with antibody to collagen type III, as follows: 0, (no fibrosis); 1, (mild fibrosis; slight fibrosis within islets occupying <1/3 of a single islet); 2, (moderate fibrosis; fibrosis occupying 1/3–1/2 area of a single islet); and 3, (severe fibrosis occupying more than 1/2 area of a single islet). More than 50 islets were subjected to analysis and the average value represented the individual animal. All the evaluations were performed in a blinded manner.

Statistical Analysis

All the values representing the average of each group were expressed as mean ± standard error (SE). The significance of differences when comparing the average values among groups was evaluated first with an axis of untreated Wistar and then GK rats by analysis of variance with *post-hoc* Bonferroni's corrections. Treatment effects were further confirmed by Mann–Whiteney's U test between untreated and pitavastatin-treated group. Differences with *P*-values <0.05 were considered to be statistically significant.

RESULTS

Laboratory Findings and Blood Chemistry

After 16 weeks of HFD, both Wistar and GK rats gained body weight, and there was a trend for slight decrease in body weight in pitavastatin-treated HFD-fed GK rats compared to untreated HFD-fed GK rats, although the differences were not significant (Table 1). During the experimental period, there was no significant difference in the amount of food intake between the pitavastatin-treated group and the untreated group in either Wistar or GK rats (data not shown).

When compared to the HFD-free groups, the blood concentrations of TG were increased by 7.3-fold in HFD-fed Wistar and 4.1-fold in HFD-fed GK rats (both *P* < 0.01) (Table 1). Similarly, blood TC levels were also increased in both groups but to a lesser extent (about 50%) in HFD-fed Wistar rats (*P* < 0.01 vs Wistar) and (about 10%) in HFD-fed GK rats (*P* < 0.05 vs GK). When these HFD-fed animals were administered pitavastatin, the levels of TG and TC in Wistar rats (both *P* < 0.01 vs HFD-fed Wistar), and TC in GK rats were significantly decreased (*P* < 0.05 vs HFD-fed GK). However, there was no significant effect of pitavastatin on TG in HFD-fed GK rats. Non-fasting blood glucose levels were also elevated in GK rats and further increased by 30% in HFD-fed GK rats (*P* < 0.05 vs HFD-free GK). In contrast, HFD-fed Wistar rats showed only a modest, insignificant increase in blood glucose compared to the levels in free Wistar rats. Treatment with pitavastatin did not influence these values.

Glucose Tolerance Measured by GTT

The HFD feeding markedly aggravated glucose intolerance in GK rats, showing elevation of blood glucose 60–120 min after glucose challenge (Figure 1). The HFD-fed Wistar rats also showed modest but significant glucose intolerance with elevation of blood glucose at 60 and 120 min after glucose challenge. The blood glucose levels in Wistar and GK rats with or without HFD feeding were not influenced by pitavastatin treatment.

Histological Findings of the Pancreas

Pancreatic islets in HFD-free Wistar rats were mostly round with smooth contour (Figure 2). In Wistar rats fed the HFD, there was marked hyperplasia of the islet, showing expansion of the central β-cell area, while the peripheral α-cell population appeared to remain intact. Islets in GK rats were mostly
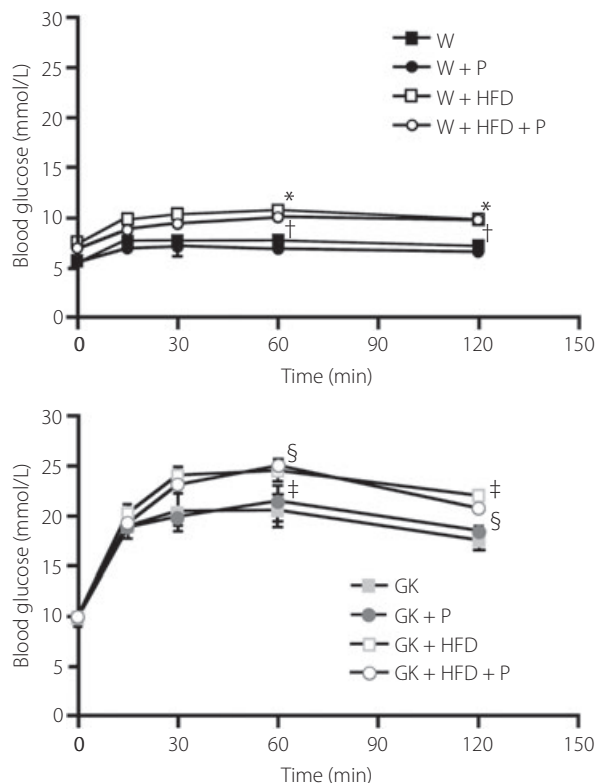
Figure 1 | Results of 2 g/kg oral glucose tolerance test in experimental animals. High fat diet (HFD) caused mild glucose intolerance in the Wistar rat (left). Glucose intolerance in GK rats was markedly augmented by HFD (right). Pitavastatin (P)-treatment had no apparent effects on the glucose tolerance in both groups with or without a HFD. W + P: Pitavastatin treated Wistar rats; W + HFD: HFD-fed Wistar rats; W + HFD + P: Pitavastatin-treated HFD-fed Wistar rats; GK + P: Pitavastatin-treated GK rats; GK + HFD: HFD-fed GK rats; GK + HFD + P: Pitavastatin-treated HFD-fed GK rats. Bar stands for mean ± SE. *$P < 0.01$ vs Wistar, †$P < 0.01$ vs W + P, ‡$P < 0.05$ vs GK, §$P < 0.01$ vs GK + P.

atrophic with depletion of β-cells in the central area. They underwent fibrosis with inflammatory mononuclear cell infiltration. Islets in HFD-fed GK rats demonstrated fibrosis and disorganization of islet contour, showing dispersion of islet cell clusters (Figure 2). While treatment with pitavastatin appeared to slightly suppress the hyperplastic changes of the islets in HFD-fed Wistar rats, the effects on HFD-fed GK rats appeared to show an inhibition of fibrosis and preservation of the β-cell population.

Quantitative Analysis of Islet Endocrine Cells
Islet volume density in GK rats was 30% smaller, occupying 1.2% of pancreatic volume vs 1.7% in normal Wistar rats ($P < 0.01$) (Table 2). The HFD feeding increased the islet volume density by 1.8 times in Wistar rats, but there was no such increase in HFD-fed GK rats. Treatment with pitavastatin did not influence the islet volume density either in GK or Wistar rats with or without HFD feeding.
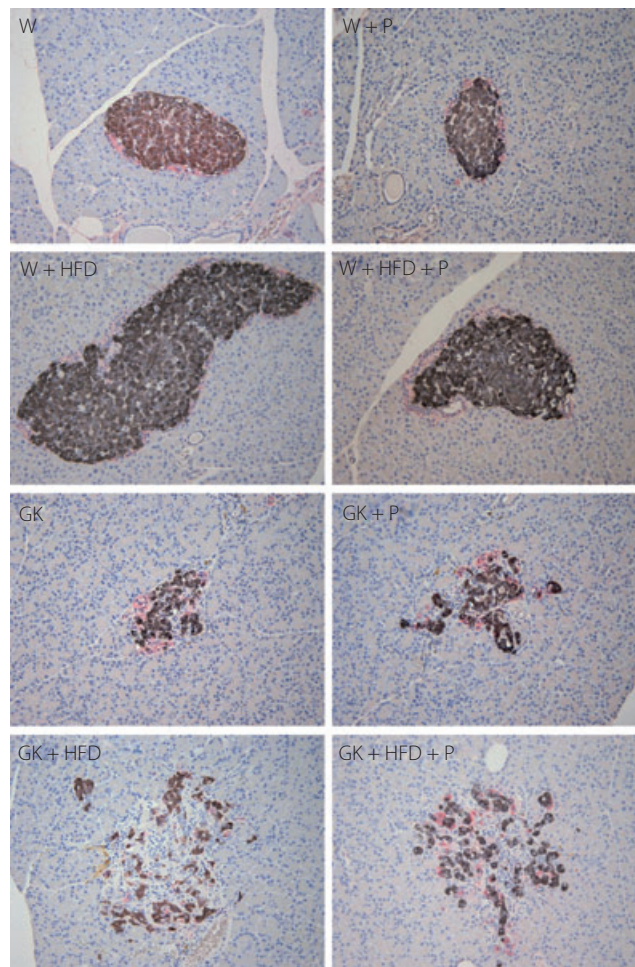

Figure 2 | Immunohistochemistry of the islet in experimental animals. Islets in Wistar rats (W) were mostly round and smooth in contour, consisting mostly of β-cells (brown) in the central area and α-cells (red) in the peripheral area. Pitavastatin (P)-treatment had no apparent effect on the islet architecture (W + P). Islets in the high-fat diet (HFD)-fed Wistar rats (W + HFD) were hyperplastic with expansion of β-cells. Islets in the P-treated HFD-fed Wistar rats (W + HFD + P) were slightly smaller but it was not statistically significant. In contrast to W, islets in GK rats (GK) were atrophic with depletion of β-cells. Irregular contour of the islet with fibrosis and inflammatory cell infiltration were not significantly influenced by P-treatment in GK rats (GK + P). GK rats when fed a HFD showed more severe fibrosis and inflammatory cell infiltration with depletion of β-cells (GK + HFD). P-treatment suppressed inflammatory changes and fibrosis with preservation of β-cells in the islets (GK + HFD + P). (Magnification; all pictures 20×).

On one hand, HFD feeding in Wistar rats produced a twofold increase in β-cell volume density (Table 2). On the other hand, β-cell volume density in GK rats was much decreased, occupying only 39% of normal Wistar rats and further decreased to 30% in HFD-fed GK rats ($P < 0.05$ vs GK). When the animals were treated with pitavastatin, there was no influence on β-cell volume density in HFD-free Wistar rats. In contrast, there

Table 2 | Morphometric data of islets in experimental animals

Group	Pancreas weight (mg)	Islet volume density (%)	β Cell volume density (%)	β Cell mass (mg)	α Cell volume density (%)	α Cell mass (mg)
W	887 ± 32	1.70 ± 0.10	1.20 ± 0.09	11.0 ± 0.6	0.43 ± 0.23	4.0 ± 0.2
W + P	850 ± 48	1.91 ± 0.09	1.37 ± 0.07	12.0 ± 0.6	0.47 ± 0.02	4.0 ± 0.2
W + HFD	888 ± 32	3.12 ± 0.13*	2.21 ± 0.08*	20.0 ± 0.7*	0.78 ± 0.09§	7.0 ± 0.9§
W + HFD + P	896 ± 34	2.88 ± 0.20†	2.07 ± 0.16†	19.0 ± 1.6†	0.69 ± 0.54¶	6.0 ± 0.5¶
GK	906 ± 35	1.17 ± 0.13	0.47 ± 0.05	4.0 ± 0.2	0.45 ± 0.02	4.0 ± 0.3
GK + P	832 ± 30	1.25 ± 0.09	0.56 ± 0.04	5.0 ± 0.3	0.51 ± 0.11	4.0 ± 1.1
GK + HFD	839 ± 41	1.20 ± 0.07	0.33 ± 0.02‡	3.0 ± 0.2‡	0.40 ± 0.04	3.0 ± 0.4
GK + HFD + P	830 ± 22	1.25 ± 0.13	0.42 ± 0.01	4.0 ± 0.2	0.44 ± 0.44	4.0 ± 0.4

Values are mean ± SE. Group names are the same used in Table 1. *$P < 0.01$ vs W, †$P < 0.01$ vs W + P, ‡$P < 0.05$ vs GK, §$P < 0.05$ vs W, ¶$P < 0.05$ vs W + P.

was a trend for preservation of β-cell volume density close to the level of HFD-free GK rats in pitavastatin-treated HFD-fed GK rats ($P = 0.056$, vs HFD-fed GK) (Table 2).

Similar to the increase in β-cell volume density, there was an increase in α-cell volume density in HFD-fed Wistar rats compared to that in HFD-free Wistar rats. Pitavastatin treatment did not influence α-cell volume density in Wistar rats. In contrast, there was no significant effect of HFD on α-cell volume density in GK rats and pitavastatin did not influence the result.

Apoptotic Cells, Proliferating Cells and Oxidative Stress-induced Cell Damage

In normal Wistar rats, cells undergoing apoptosis were rarely detected and HFD did not increase the dead cells. Similarly, apoptotic cells were rarely detected in HFD-free GK rats, but the islets in HFD-fed GK rats contained an increased number of apoptotic cells ($P < 0.01$ vs GK) (Figure 3). The appearance of apoptotic cells in HFD-fed GK rats was inhibited by pitavastatin treatment ($P < 0.05$ vs HFD-fed GK).

Proliferating cells were identified by positive Ki67 reactions in double staining and there was no significant difference in the number of proliferating cells of β cells between Wistar and GK rats at this age. HFD feeding induced a slight but a significant increase in the proliferating β-cells ($P < 0.05$ for both Wistar and GK) which were not affected by pitavastatin treatment (Figure 4).

There was an increased expression of 8OHdG in the islets in GK rats compared to those in normal Wistar rats (Figure 5). While HFD feeding did not significantly affect the expression in Wistar rats, 8OHdG reactions were augmented in HFD-fed GK rats. Pitavastatin treatment did not significantly suppress the expressions of 8OHdG either in Wistar or GK rats.

Infiltration with Inflammatory Cells and Fibrosis of the Islets

In Wistar rats, ED1-positive macrophages were infrequently observed after HFD feeding. In GK rats, on the other hand, infiltration of ED1-positive cells was constantly found, and became more marked after HFD feeding (Figure 6). Pitavastatin
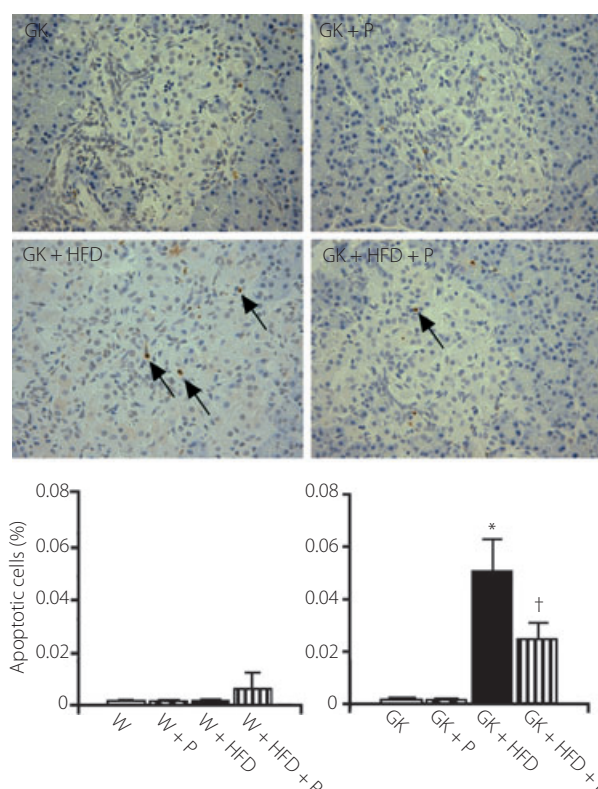

Figure 3 | Immunohistochemical detection of apoptotic cells in the islet stained with ApopTag in experimental animals. In normal Wistar rats (W) and GK rats (GK), apoptotic cells were rarely detected. Absence of apoptotic cells were not altered in pitavastatin (P)-treated Wistar rats (W + P) or GK rats (GK + P). In contrast, there was an emergence of apoptotic cells in high fat diet (HFD)-fed GK rats (GK + HFD) although this effect was not found in HFD-fed Wistar rats (W + HFD). The increase in apoptotic cells was significantly inhibited in P-treated HFD-fed GK rats (GK + HFD + P), while the effect was not found in P-treated HFD-fed Wistar rats (W + HFD + P). Magnification; all pictures 20×. Bar stands for mean ± SE. *$P < 0.01$ vs GK, †$P < 0.05$ vs GK + HFD.

treatment significantly inhibited the infiltration of ED-1 positive cells in HFD-fed GK rats, while there was no influence on Wistar rats.
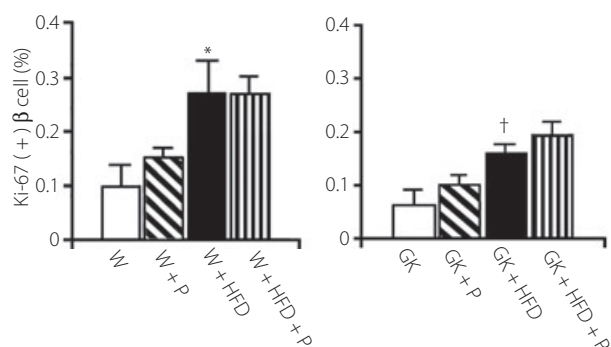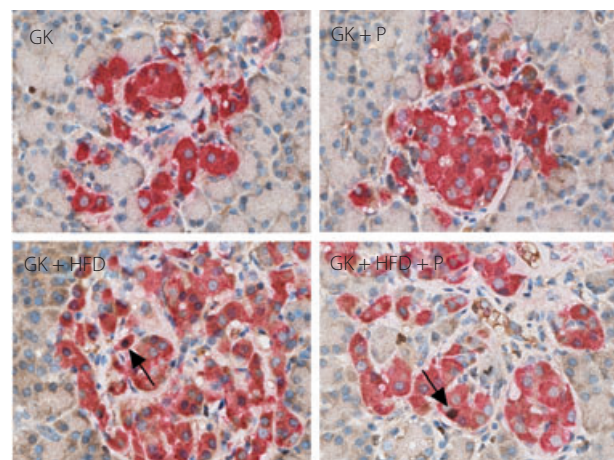
Figure 4 | Immunohistochemical detection of proliferating cells in the islet doubly stained with insulin (red) and Ki67 (brown) in experimental animals. In normal Wistar rats (W) and GK rats (GK) at this age, there were only a few cells positive for Ki67. Pitavastatin (P) treatment did not significantly increase the rate of positive cells either in Wistar rats (W + P) or GK rats (GK + P). In contrast, a high fat diet (HFD) raised the number of proliferating cells either in W (W + HFD) or GK rats (GK + HFD) although the influence was less marked in the latter. P-treatment did not alter the proliferation rate either in HFD-fed Wistar rats (W + HFD + P) or HFD-fed GK rats (GK + HFD + P). (Magnification; all pictures 20×). Bar stands for mean ± SE. *$P < 0.01$ vs Wistar, †$P < 0.05$ vs GK.

Figure 5 | Immunohistochemical detection of oxidative stress-induced cell damage stained with 8-hydroxydeoxyguanosine (8OHdG) in the islet in experimental animals. Compared to the staining of normal Wistar rats (W), the islets in GK rats (GK) showed slightly intensified expressions of 8OHdG. High fat diet (HFD)-fed GK rats (GK + HFD) showed an increased expression, while such reactions were not found in HFD-fed Wistar rats (W + HFD). Pitavastatin (P)-treatment did not significantly influence the reactions in either HFD-free Wistar rats (W + P), HFD-fed Wistar (W + HFD + P), HFD-free GK (GK + P), or HFD-GK rats (GK + HFD + P). (Magnification; all pictures 20×). AU, arbitrary unit. Bar stands for mean ± SE. *$P < 0.01$ vs GK.

The GK rats showed clearly enhanced staining of collagen type III in the islets, regardless of whether the animals were fed normal or HFD, when compared to the staining of the islets of Wistar rats (Figure 7). HFD feeding augmented the fibrotic changes in GK rats and there was a significant decrease in the staining intensities of collagen III in pitavastatin-treated HFD-fed GK rats as compared to the non-treated group.

Hepatic Lipid Concentrations and Pancreatic Insulin Content
The lipid concentrations in the liver in GK rats were comparable to those in normal Wistar rats (Figure 8a). After HFD feeding, nearly twofold increases in both TG and TC were found in Wistar rats. The HFD-fed GK rats also showed significant increases in TG concentrations in the liver but cholesterol levels were not altered. Pitavastatin treatment significantly decreased
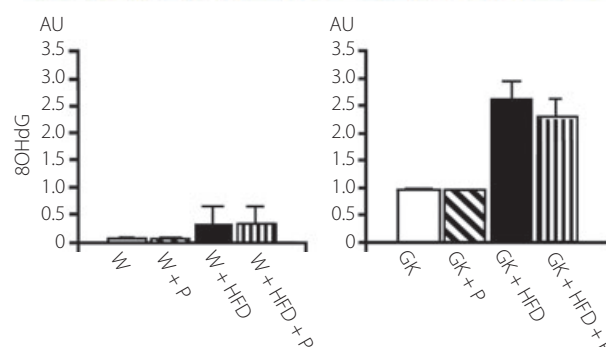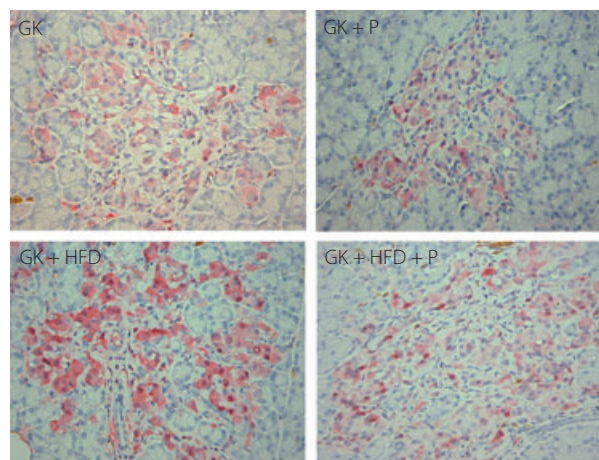
hepatic TG and TC concentrations in HFD-fed Wistar rats and TG concentrations in HFD-fed GK rats.

Feeding the rats HFD for 16 weeks caused a 1.5-fold increase in pancreatic insulin contents in Wistar rats (Figure 8b). Pitavastatin treatment did not influence the values. In contrast to Wistar rats, pancreatic insulin content was reduced in GK rats and further reduced by 67% in HFD-fed GK rats compared to untreated GK rats. While pitavastatin treatment did not influence the values in HFD-free GK rats, there was a trend for preservation of insulin content in pitavastatin-treated HFD-fed GK rats.

DISCUSSION
In this study, we found that islet lesions were differentially influenced by HFD in animals with or without genetic background of type 2 diabetes. Consistent with a previous report, blood lipid
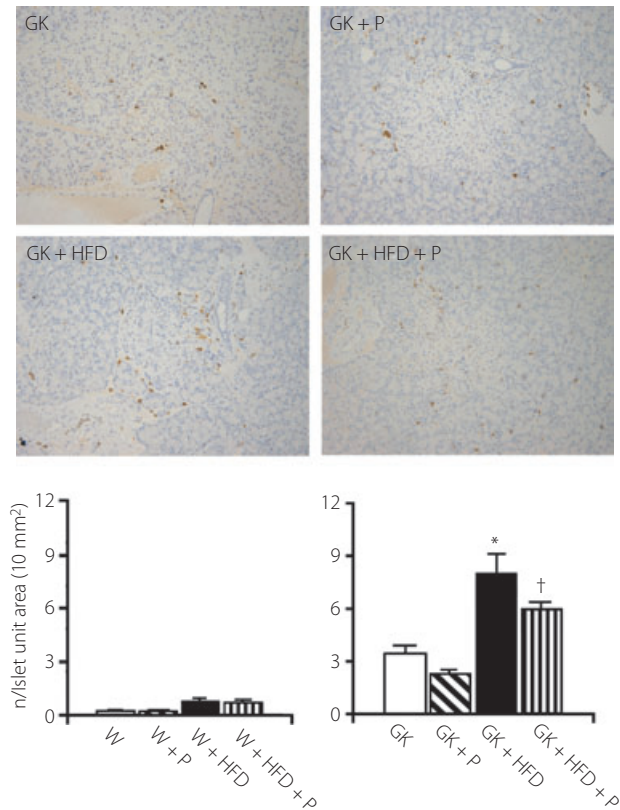
Figure 6 | Immunohistochemical detection of ED-1 positive macrophages in the islet in experimental animals. Sporadic infiltration of ED-1 cells was detected in GK rats (GK) and pitavastatin (P)-treatment did not influence the features (GK + P). In contrast, there was marked infiltration of ED-1 cells in or around the islet in HFD-fed GK rats (GK + HFD). P-treatment appeared to reduce the infiltration of ED-1 cells (GK + HFD + P). In normal Wistar rats (W) and HFD-fed Wistar rats (W + HFD), ED-1 cells were few and P-treatment did not influence the change either in Wistar rats (W + P) or HFD-fed Wistar rats (W + HFD + P). Magnification of pictures 20×, Bar stands for mean ± SE. *$P < 0.01$ vs GK, GK + P, †$P < 0.05$ vs GK + HFD.
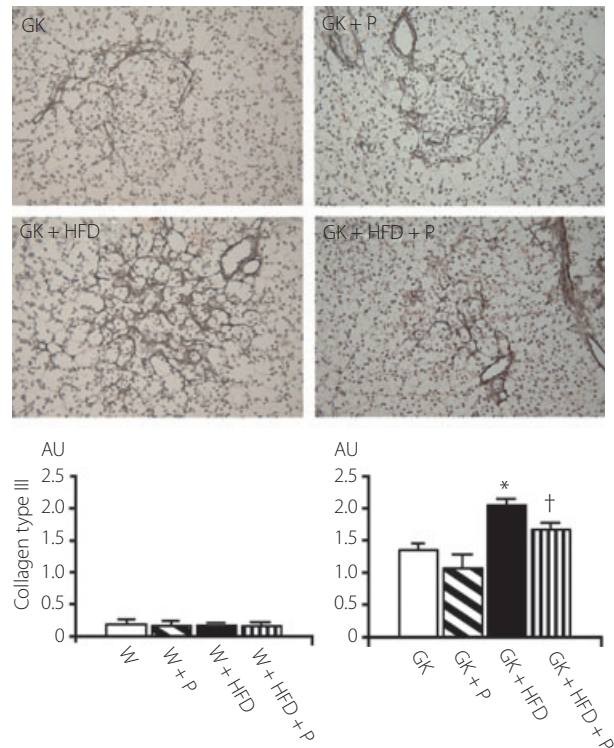

Figure 7 | Immunohistochemistry of type III collagen in the islet in experimental animals. Islets in GK rats (GK) underwent fibrotic changes whereas such changes were not apparent in normal Wistar rats (W). Pitavastatin (P) treatment did not influence the islet in high fat diet (HFD)-free Wistar (W + P) and GK rats (GK + P). HFD feeding accelerated fibrotic changes in GK rats (GK + HFD) and P-treatment improved the fibrosis in HFD-fed GK rats (GK + HFD + P). Such effects were not found in P-treated HFD-fed Wistar rats (W + HFD + P). AU, arbitrary unit. Bar stands for mean ± SE. *$P < 0.01$ vs GK, GK + P, †$P < 0.05$ vs GK + HFD.

concentrations were increased and glucose intolerance was aggravated in both Wistar and GK rats when fed HFD[16]. Glucose intolerance was compensated by islet hyperplasia in Wistar rats. In contrast, β-cell mass was much reduced in HFD-fed GK rats, indicating a vulnerability of GK islets to hyperlipidemic environment. In this setting, treatment with pitavastatin tended to inhibit the decline of islet β-cell mass and preserve pancreatic insulin contents in HFD-fed GK rats. The islet contour was relatively well preserved and inflammatory changes were suppressed in pitavastatin-treated HFD-GK rats, while there was no apparent change in Wistar rats treated with pitavastatin. Concurrently, the effects were associated with suppression of hepatic TG contents and serum TC levels. Since all the measures of endocrine pancreatic function in HFD-free GK rats were not significantly influenced by pitavastatin treatment, as shown in the studies of pravastatin, simvastatin, and

atorvastatin[17], the effects of statin may only be apparent in severely lipotoxic conditions as found in the HFD-fed animals.

Progressive β-cell loss is a hallmark of the islet pathology in type 2 diabetic animal models[18–20]. In obese type 2 animal models such as db/db mice or ZDF rats, early islet hyperplasia is characteristic, followed by β-cell loss[21,22]. Such animals are hyperlipidemic and analogous to human metabolic syndrome. Lipid deposition in the islet is an important attribute to β-cell apoptosis in ZDF rats[23]. In contrast to these animals, GK rats are normolipidemic, non-obese and do not show hyperplasia, but directly develop islet atrophy and β-cell loss[10,15].

In this study, the islet lesions were further aggravated in HFD-fed GK rats, and β-cell mass was only 30% of normal Wistar rats, while HFD caused a marked hyperplasia of β-cells in Wistar rats. The vulnerability of islet β-cells to lipids in GK rats may be ascribed to weak defense mechanisms against oxidative stress in the β-cells[10,11]. In fact, there were an increased number of apoptotic β-cells in HFD-fed GK rats although its rate was low, which were associated with enhanced expression of oxidative
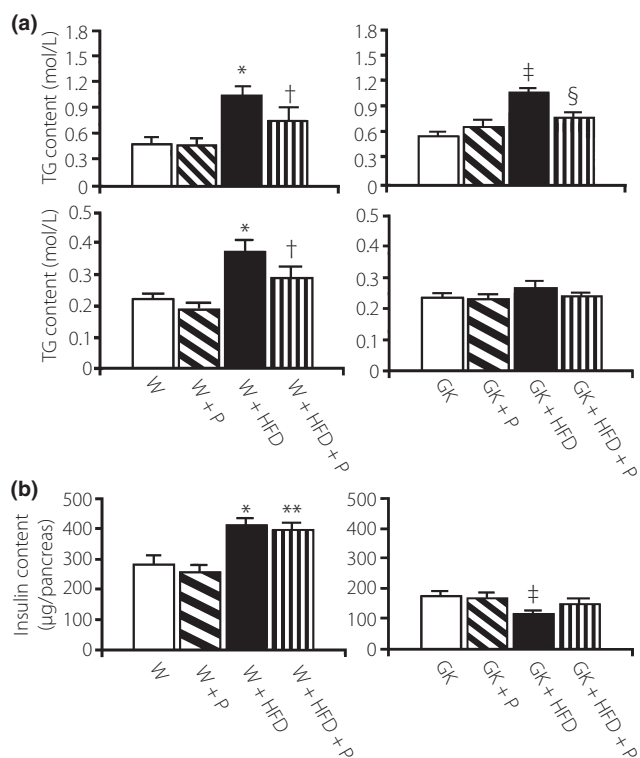
Figure 8 | Hepatic lipid concentrations and pancreatic insulin contents in experimental animals. (a) There were marked increases in concentrations of both triglycerides (TG) and total cholesterol (TC) in Wistar rats (W) fed with high fat diet (HFD). Treatment with pitavastatin (P) significantly suppressed these changes. HFD also caused an increase in TG contents in GK rats (GK) and P-treatment suppressed this rise, while there was no increase in TC in HFD-fed GK rats and P-treatment did not influence the values. (b) There was marked increases in pancreatic insulin contents in HFD-fed Wistar rats compared to those in HFD-free Wistar rats. P-treatment did not influence the values. In contrast, pancreatic insulin contents were much reduced in GK rats compared to normal Wistar rats. The reduction was further augmented in HFD-fed GK rats. Although P-treatment did not influence on HFD-free GK rats, there was a trend to restore the insulin content by P-treatment. W + P: Pitavastatin treated Wistar rats; W + HFD: HFD-fed Wistar rats; W + HFD + P: Pitavastatin-treated HFD-fed Wistar rats; GK + P: Pitavastatin-treated GK rats; GK + HFD: HFD-fed GK rats; GK + HFD + P: Pitavastatin-treated HFD-fed GK rats. Bar stands for mean ± SE. $*P < 0.01$ vs W, $**P < 0.05$ vs W + HFD, $†P < 0.05$ vs W + HFD, $‡P < 0.05$ vs GK, $§P < 0.05$ vs GK + HFD.

stress-induced DNA damage reflected by 8OHdG reactions. As evident from our observations, proinflammatory reactions with lipid oxidation and release of cytokines from macrophages may also have contributed to the augmented β-cell loss in HFD-fed GK rats. When this model was fed sucrose, increased oxidative stress exerted apoptotic processes with mitochondrial dysfunction[11,20]. Our results thus indicate that β-cells in GK rats are likely to be lost under glucotoxic as well as lipotoxic milieu, at least in part mediated by enhanced oxidative stress.

The effects of pitavastatin treatment on the increased concentrations of blood lipid levels were only partial in both Wistar and GK rats. The dose of pitavastatin may have been too small to adequately normalize blood lipid concentrations[24]. Nevertheless, our studies showed a significant suppression of lipid concentrations in the liver of HFD-fed animals, indicating differential effects of pitavastatin on different tissues. In recent large cohort studies, a protective effect of statin on cardiovascular events was demonstrated in type 2 diabetic patients[3,25]. It would therefore be interesting to see the effect of pitavastatin on the vacular wall in GK rats.

Although there are some *in vitro* studies that demonstrated suppressive effects of lipophilic statins such as simvastatin and atorvastatin on insulin secretion while hydrophilic statins exerted β-cell function[5,26], pitavastatin treatment did not aggravate but improved the islet structure in HFD-fed GK rats. The precise molecular mechanisms of how pitavastatin improved the islet pathology are yet to be determined. Pitavastatin is shown to have pleiotropic effects, including anti-inflammatory action and inhibitory effects against the actions of matrix metalloproteinases[27,28]. Immunomodulating actions of statins may also be involved in the prevention of islet changes. By altering cholesterol content in lipid compositions, statins can directly alter T cell and macrophage activation by switching inflammatory cytokines to anti-inflammatory cytokines[29,30]. In fact, augmented islet fibrosis and migration of macrophages in HFD-fed GK rats were suppressed by pitavastatin treatment. Improvement of islet microenvironment by increased islet blood flow or suppressed inflammatory changes in pitavastatin-treated animals may have thus contributed to β-cell preservation, because islet fibrosis enhances ischemic injury and inflammatory cytokines released from macrophages exert death signals in β-cells[31,32]. Statin is also beneficial for β-cells by reducing the intracellular oxidative stress elicited by hyperlipidemia. Alternatively, excessive accumulation of lipid peroxidation products may take place in β-cells by HFD with impairment of autophagic processes[33,34], to which statin is protective.

Clinical effects of statin treatment on the onset and development of diabetes are controversial. In one study, pravastatin treatment inhibited newly onset diabetes in patients with hyperlipidemia[35]. Other studies have shown a significant increase in HbA$_{1c}$ levels in diabetic patients when treated with atorvastatin[4], and rosuvastatin treatment raised the incidence of newly diagnosed diabetes in hyperlipidemic patients[25]. Also, in preclinical studies it has been shown that simvastatin as a lipophilic statin inhibited glucose-induced cytosolic Ca^{2+} signaling and insulin secretion by blocking L-type Ca^{2+} channels in β-cells[5]. Atorvastatin, another lipophilic statin, attenuates adipocyte maturation and glucose transporter 4 (SLC2A4) expression[36]. It is possible that the differences in statin actions may derive from lipophilic or hydrophilic properties. Alternatively, the differential effects on extrapancreatic tissues may operate in the net results on the glucose tolerance among various kinds of statins. Apparently, further studies are warranted to determine whether all

statins, or a specific statin, have an influence on diabetes and islet structure since more than 30% of patients with diabetes are currently treated with statins.

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