Spontaneously diabetic Ins2+/Akita:ApoE-deficient mice exhibit exaggerated hypercholesterolemia and atherosclerosis

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Running head: A Mouse Model of Spontaneous Diabetes and Atherosclerosis

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Type 1 diabetes (T1D) increases the risk of adverse coronary events. Among risk factors, dyslipidemia due to altered hepatic lipoprotein metabolism plays a central role in diabetic atherosclerosis. Nevertheless, the likely alterations in plasma lipid/lipoprotein profile remain unclear especially in the context of spontaneously developed T1D and atherosclerosis. To address this question, we generated Ins2\(^{+/Akita}\):ApoE\(^{-/-}\) mouse by crossbreeding Ins2\(^{+/Akita}\) mouse (that has Ins2 gene mutation causing pancreatic β-cell apoptosis and insulin deficiency) with ApoE\(^{-/-}\) mouse. Ins2\(^{+/Akita}\):ApoE\(^{-/-}\) mice developed T1D spontaneously at 4-5 weeks of age. At 25 weeks of age and while on a standard chow diet, diabetic Ins2\(^{+/Akita}\):ApoE\(^{-/-}\) mice exhibited ~3-fold increase in atherosclerotic plaque in association with ~2-fold increase in plasma non-HDL-cholesterol, predominantly in the LDL fraction, compared with nondiabetic controls. To determine factors contributing to the exaggerated hypercholesterolemia, we assessed hepatic VLDL secretion and triglyceride content, expression of hepatic lipoprotein receptors, and plasma apolipoprotein composition. Diabetic Ins2\(^{+/Akita}\):ApoE\(^{-/-}\) mice exhibited diminished VLDL secretion by ~50%, which was accompanied by blunted Akt phosphorylation in response to insulin infusion and decreased triglyceride content in the liver. While the expression of hepatic LDL receptor was not affected, there was a significant reduction in the expression of lipolysis-stimulated lipoprotein receptor by ~28%. Moreover, there was a marked decrease in plasma ApoB-100 with a significant increase in ApoB-48 and ApoC-III levels. In conclusion, exaggerated hypercholesterolemia and atherosclerosis in spontaneously diabetic Ins2\(^{+/Akita}\):ApoE\(^{-/-}\) mice may be attributable to impaired lipoprotein clearance in the setting of diminished expression of LSR and altered apolipoprotein composition of lipoproteins.

**Keywords:** Type 1 diabetes, atherosclerosis, hypercholesterolemia, lipoprotein metabolism
INTRODUCTION

Despite advances in care, type 1 diabetes (T1D) is associated with an increased risk for coronary heart disease (11, 28, 29, 38, 43). Compared with general population, both the signs of atherosclerosis and coronary heart disease occur much earlier in their life with a more diffuse and accelerated course in patients with T1D (9, 31, 56). The risk factors that may contribute to accelerated atherosclerosis in T1D include hyperglycemia, oxidative stress, vascular cell dysfunction, inflammation, and dyslipidemia (29, 38). Though formerly lipid levels in T1D group were thought to be comparable to those in nondiabetic group, dyslipidemia with elevated cholesterol concentrations in atherogenic lipoproteins has been increasingly recognized even in young adults and adolescents with T1D (1, 17, 42).

The liver normally produces and secretes the circulatory triglyceride-rich very low-density lipoproteins (VLDL) particles that are hydrolyzed by lipoprotein lipase to form VLDL remnants or intermediate-density lipoproteins (IDL). In turn, these particles are rapidly cleared by liver or further processed by hepatic lipase to form cholesterol-rich low-density lipoproteins (LDL) (6). The clearance of VLDL remnants from the circulation depends on the interaction of ApoE with LDL receptors, LDL receptor-related proteins (LRPs) and/or heparan sulfate proteoglycans (HSPGs) in the liver (20, 30). Recently recognized lipolysis-stimulated lipoprotein receptor (LSR) has also been shown to participate in remnant lipoprotein clearance, mainly through its interaction with ApoB (34, 53). On the other hand, LDL particles are cleared by an ApoB-100-mediated mechanism through the LDL receptor (20, 30). In pathologic conditions like diabetes or metabolic syndrome, therefore, either increased secretion of VLDL or decreased clearance of lipoproteins or both are thought to lead to dyslipidemia and consequent atherosclerosis.
To understand the underlying mechanisms for accelerated atherosclerosis in T1D, previous studies have employed different animal models using chemicals like streptozotocin (STZ) to induce diabetes (13, 52). In particular, ApoE-knockout mice and LDL receptor-knockout mice are widely used by several investigators to understand the mechanisms of atherosclerosis. ApoE-deficient mouse models have uniformly shown increased cholesterol in VLDL and LDL fractions, and atherosclerosis by the induction of diabetes while on a standard chow diet. Moreover, they were responsive to various medical interventions. On the other hand, development of atherosclerosis in LDL receptor-deficient mouse has been variable, and often requires dietary intervention that can obscure distinct effects of diabetes. Of note, in human ApoB-expressing transgenic mice, STZ-induced diabetes led to minor or no changes in plasma lipoproteins and atherosclerosis (23). These studies have underscored the critical relationship between enhanced hypercholesterolemia and accelerated atherosclerosis in T1D as a function of mouse strain, normal diet, or high fat-diet. However, these studies have intrinsic limitations because STZ exhibits several nonspecific effects including hepatotoxicity (22, 50). Therefore, the role of spontaneously induced diabetes on accelerated atherosclerosis needs to be further evaluated in a suitable animal model. Previously, Keren and colleagues have made attempts to develop atherosclerosis-prone type 1 diabetic mouse model using non-obese diabetic (NOD) mice (24). Although NOD mice develop spontaneous T1D through an autoimmune process, they do not develop atherosclerosis despite the high fat-diet and the resultant hypercholesterolemia. Clearly, a resistance to the development of atherosclerosis exists in certain mouse strains including NOD mouse.

On the other hand, genetically induced spontaneous T1D on an ApoE-deficient background might provide a realistic alternative approach to study the effects of T1D on
atherosclerosis, avoiding nonspecific effects from chemicals and the need of an artificial diet. Strategies to study the effects of spontaneous T1D on atherogenesis under ApoE-deficient states could provide new insights into: i) altered lipoprotein secretion and clearance by the liver; ii) altered circulatory lipid/lipoprotein profile; and iii) the magnitude of atherosclerosis in the setting of T1D.

The present study is aimed at utilizing Ins2+/Akita mouse (that has Ins2 gene mutation causing pancreatic β-cell apoptosis and insulin deficiency) (49, 55) and ApoE-knockout mouse, the established animal models of spontaneous T1D and spontaneous atherosclerosis, respectively. We have employed a cross-breeding strategy to generate a mouse model of spontaneous T1D and atherosclerosis, that is, the Ins2+/Akita:ApoE−/− mouse. We hypothesize that T1D exaggerates atherogenic phenotype by worsening dyslipidemia. These mice, which develop spontaneous T1D at 4-5 weeks of age, was maintained on a standard chow diet for up to 25 weeks to assess the extent of atherosclerosis. To assess whether the changes in cholesterol or triglyceride concentrations contribute to dyslipidemia, we quantified cholesterol and triglyceride concentrations in plasma and in the individual lipoprotein fraction. We determined hepatic VLDL secretion rate and triglyceride content, and assessed the differences in plasma apolipoprotein composition. To determine whether dyslipidemia results from impaired lipoprotein clearance, we also assessed expression of hepatic lipoprotein receptors. In parallel with all studies using diabetic Ins2+/Akita:ApoE−/− mice, we used littermate non-diabetic Ins2+/+:ApoE−/− mice as controls.
MATERIALS AND METHODS

Experimental animals. All animal experiments were performed in accordance with the Pennsylvania State University College of Medicine Institutional Animal Care and Use Committee guidelines. Ins2+/Akita heterozygous mice, C57BL/6J mice and ApoE\(^{tm1Unc}\) homozygous mutation (ApoE-knockout or ApoE\(^{-/-}\)) mice were originally obtained from Jackson Laboratory (Bar Harbor, ME). All mice were C57BL/6J background.

Generation of Ins2+/Akita:ApoE\(^{-/-}\) mice. Diabetic male Ins2+/Akita:ApoE\(^{t+/+}\) mice (Ins2+/Akita heterozygous mice) were crossed with nondiabetic female Ins2+/+:ApoE\(^{-/-}\) mice (F0). The resulting F1 generation consisted of heterozygous ApoE\(^{t+/+}\) (Ins2+/Akita:ApoE\(^{t+/+}\) and Ins2+/+:ApoE\(^{t+/+}\)) mice. From this F1 generation, diabetic male Ins2+/Akita:ApoE\(^{t+/+}\) mice were crossed with nondiabetic female Ins2+/+:ApoE\(^{-/-}\) mice. The resulting F2 generation consisted of homozygous ApoE\(^{-/-}\) (Ins2+/Akita:ApoE\(^{-/-}\) and Ins2+/+:ApoE\(^{-/-}\)) and heterozygous ApoE\(^{t+/+}\) (Ins2+/Akita:ApoE\(^{t+/+}\) and Ins2+/+:ApoE\(^{t+/+}\)) mice. Subsequently, diabetic male Ins2+/Akita:ApoE\(^{t+/+}\) mice (from F2 generation) and nondiabetic female Ins2+/+:ApoE\(^{-/-}\) were set up as breading pairs to produce an F3 generation of diabetic Ins2+/Akita:ApoE\(^{t+/+}\) mice and nondiabetic control Ins2+/+:ApoE\(^{-/-}\) mice. For the present study, we used the male mice from F3 generation (diabetic and nondiabetic control) because male Ins2+/Akita mice exhibit more severe and homogeneous diabetic phenotypes compared with female mice (55). Male mice were weaned at 3 weeks of age and maintained on a 12h:12h dark-light cycle under controlled temperature (23 °C). The mice had free access to water and standard rodent chow diet (Teklad 2018, Harlan Laboratories, Indianapolis, IN), which contains
cholesterol less than 0.1 % and fat 18% of total calories. Genotypes were determined by PCR amplification of tail DNA using protocols provided by Jackson Laboratory. Diabetic phenotype was confirmed in mice at 4-5 weeks after birth by blood glucose values >250 mg/dL with handheld glucometer (Contour, Bayer Health Care LLC, Tarrytown, NY) measured with a drop of blood from tail puncture. The disease penetrance is 100% in mice with the $Ins2^{Akita}$ mutation (49).

Measurements of body composition. Whole body fat and lean mass were noninvasively measured in awake mice at the age of 10 weeks and 20 weeks using $^1$H-MRS (Magnetic Resonance Spectrometer) (Echo Medical Systems, Houston, TX).

Assessment of atherosclerotic lesion. In the present study, male diabetic $Ins2^{+/Akita}\cdot ApoE^{-/-}$ mice and nondiabetic $Ins2^{+/+}\cdot ApoE^{-/-}$ mice were maintained on a standard chow diet until 25 weeks of age, at which time they were sacrificed to quantify atherosclerotic lesion area. The timeframe of 25 weeks of age was chosen based on earlier studies that show significant atherosclerotic lesion in the aortas of $ApoE^{-/-}$ mice (51). The thoracic and abdominal regions of the aorta, and the aortic arch were dissected out with care to remove adventitial fat, opened longitudinally, and pinned in place on a wax-coated petri dish for en face analysis. The lipid-rich regions on the luminal side of the aorta were stained for fat with oil red-O (0.5% in 60% isopropyl alcohol) for 30 min. Excess stain was removed with 60% isopropyl alcohol, and en face images of each aortic segment were photographed. The atherosclerotic lesion area was expressed as the percentage of the total luminal surface area of the aortic arch using Adobe
Photoshop CS4 (Adobe Systems Inc., San Jose, CA) and ImageJ software (National Institutes of Health, Bethesda, MD).

Biochemical assays. Plasma glucose, total cholesterol, HDL-cholesterol, and triglyceride (TG) concentrations were measured using VITROS DT slides and an enzymatic colorimetric method by VITROS DT60 II Chemistry System (Ortho-Clinical Diagnostics, Inc., Rochester, NY) according to manufacturer’s instructions. Non-HDL cholesterol concentration was calculated by subtracting HDL-cholesterol from total cholesterol. Plasma insulin concentration was measured by ELISA using kits from Alpco Diagnostics (Salem, NH). Whole blood glucose concentration during a hyperinsulinemic-euglycemic clamp study was measured by a handheld glucometer (Contour, Bayer Health Care LLC, Tarrytown, NY).

Measurement of liver triglycerides. Lipids were extracted from liver (0.1 g) in 2 mL of chloroform/methanol (2:1, v/v) using a method adapted from Storlien and colleagues (46). The organic phase was separated by adding 200 μL of 1 M H₂SO₄. 1 mL of organic phase was mixed in 1 mL of 1:1 chloroform/Triton X-100 and evaporated overnight. Dried samples were dissolved in 200 μL of distilled water and assayed for triglycerides using VITROS DT slides and an enzymatic colorimetric method by VITROS DT60 II Chemistry System (Ortho-Clinical Diagnostics, Inc., Rochester, NY).
Lipoprotein fractionation by fast performance liquid chromatography (FPLC). FPLC analysis was provided by the University of Cincinnati Mouse Metabolic Phenotyping Center (MMPC). In brief, the 200 µl of plasma from mice fasted for 5 hours was subjected to FPLC analysis. Plasma sample was chromatographed undiluted through two Superose 6 columns (Pharmacia, Piscataway, NJ) linked in tandem and equilibrated with degassed buffer (50 mM EDTA, 150 mM NaCl, 1 mM EDTA, and 7.7 mM NaN3, pH 7.4). A flow rate of 0.6 mL/min was maintained by a Pharmacia FPLC controller and fifty one 500 µl fractions were collected. VLDL eluted in fractions 1–10, LDL in fractions 11–30, and HDL in fractions 31 –51. Cholesterol and triglyceride concentrations were measured in each of eluted fractions by the Infinity cholesterol assay kit (Thermo Fisher Scientific Inc., Indianapolis, IN) and the Randox triglyceride assay kit (Randox Laboratories Ltd., Antrim, UK), respectively, according to the manufacturer’s instructions.

Hepatic VLDL secretion under hyperinsulinemic-euglycemic conditions. Hepatic VLDL secretion under hyperinsulinemic-euglycemic conditions was assessed as previously described (15) with modifications. Since the majority of TG is found in VLDL fraction according to the FPLC analysis in our animals (Fig. 2B), the secretion rate of TG closely represents that of VLDL. At 4-5 days before clamp experiments, mice underwent surgical placement of right jugular vein catheter (PE-10, Intramedic, BD Biosciences, Sparks, MD) under general anesthesia with intraperitoneal injection of ketamine (100 mg/kg body weight) and xylazine (10 mg/kg body weight). Catheters were externalized to the back of the neck. Following a 5-hour fasting, a 4.5-hour hyperinsulinemic-euglycemic clamp study was conducted in the conscious diabetic Ins2\(^{+/Aki}\).ApoE\(^{−/−}\) mice and nondiabetic Ins2\(^{+/+}\).ApoE\(^{−/−}\) mice at 20 weeks of age (n = 5-7). To
raise plasma insulin within a physiological range (approximately ~300 pM), the mice were
infused with a priming dose (150 mU/kg body weight) followed by a continuous infusion (2.5
mU·kg⁻¹·min⁻¹) of human regular insulin (Humulin; Eli Lilly, Indianapolis, IN) (25). Blood
samples were collected at 15-min intervals for the immediate measurement of glucose
concentration, and 20% glucose was infused at variable rates to maintain euglycemia. All
infusions were performed using the microdialysis pumps (CMA/Microdialysis, North
Chelmsford, MA). Triton WR-1339 (500 mg/kg, Tyloxapol, Sigma-Aldrich, St. Louis, MO)
was injected at 90 min after the infusion of insulin and glucose. Blood samples were collected
from the tail before and at 30, 60, 120, and 180 min after Triton WR-1339 injection for the
measurement of glucose, and TG (Fig. 3A). Insulin concentrations were measured in blood
samples at basal (0 min) and during the clamp study at 210 min (Fig. 3A, B). At the end of the
clamp study, mice were euthanized.

**Immunoblot analysis.** Plasma samples were obtained from mice after a 5-hour fast. The
mouse liver was perfused with ice-cold PBS to remove blood and snap frozen using liquid
nitrogen and stored at -80°C until the further analyses. Hepatic tissue protein extracts were
prepared using T-PER® Tissue Protein Extraction Reagent (Pierce, Rockford, IL). The whole
tissue protein extracts and plasma samples (50 μg protein each) were subjected to electrophoresis
using pre-cast 4-12 % NuPage mini-gels (Invitrogen, Carlsbad, CA), and the resolved proteins
were transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA). The membranes were
blocked and probed with the indicated primary antibodies. The primary antibodies for ApoA-I
(sc-30089), Apo-B (sc-25542), ApoC-III (sc-50378), and LSR (sc-133765) were purchased from
Santa Cruz Biotech, Inc. (Santa Cruz, CA). The primary antibody for LDL receptor (ab3032)
was purchased from Abcam (Cambridge, MA). The primary antibodies for insulin receptor
(#3025), total Akt (#9272), phospho-Ser (473) Akt (#9271), and α-tubulin (#2125) were
purchased from Cell Signaling Tech, Inc. (Danvers, MA). The immunoreactivity was detected
using specific HRP-conjugated secondary antibodies followed by enhanced chemiluminescence
(Pierce, Rockford, IL). The protein bands were quantified using ImageJ software (NIH,
Bethesda, MD).

Statistical analysis. Data are expressed as mean ± SE. Differences between two groups
were assessed using the unpaired two-tailed t test and among more than two groups by analysis
of variance (ANOVA). Values of p < 0.05 were considered statistically significant.
RESULTS

Phenotypic characteristics of the newly generated type 1 diabetic $\text{Ins}^2+/\text{Akita} : \text{ApoE}^{-/}$ mice.

Previous studies have shown that type 1 diabetic $\text{Ins}^2+/\text{Akita}$ mice (that has $\text{Ins}^2$ gene mutation causing pancreatic $\beta$-cell apoptosis and insulin deficiency) exhibit a decrease in body weight by 12-33% as a function of age (4 to 36 weeks) compared with age-matched wild type control littermates (3). In addition, blood glucose concentrations remain significantly elevated to the extent of 390-470 mg/dL in $\text{Ins}^2+/\text{Akita}$ mice (4 to 36 week-old) compared with the control values of 138-187 mg/dL in wild type mice.

In order to study atherogenesis in the context of spontaneous T1D, we generated $\text{Ins}^2+/\text{Akita} : \text{ApoE}^{-/-}$ mouse by crossbreeding $\text{Ins}^2+/\text{Akita}$ mouse with $\text{ApoE}^{-/-}$ mouse as described in ‘Materials and Methods.’ To determine whether the type 1 diabetic phenotype developed on an $\text{Apo-E}^{-/-}$ background exhibit similar characteristics, we compared the body weight, lean and fat mass, glucose and insulin concentrations between the diabetic $\text{Ins}^2+/\text{Akita} : \text{ApoE}^{-/-}$ mice and the nondiabetic control $\text{Ins}^2+/+ : \text{ApoE}^{-/-}$ mice (F3 generation). We also compared them with those of age-matched counterpart $\text{ApoE}$-intact mice (wild-type C57BL/6 and diabetic $\text{Ins}^2+/+ : \text{ApoE}^{-/-}$ mice).

As shown in Table 1, diabetic $\text{Ins}^2+/\text{Akita} : \text{ApoE}^{-/-}$ mice had a decrease in body weight by ~16 % at 20 weeks of age compared with age-matched nondiabetic $\text{Ins}^2+/+ : \text{ApoE}^{-/-}$ mice. The reduced body weight in diabetic $\text{Ins}^2+/\text{Akita} : \text{ApoE}^{-/-}$ mice was the result of a decrease in lean mass by ~12 % and pronounced loss of fat mass by ~41 % compared with control mice. In addition, the diabetic $\text{Ins}^2+/\text{Akita} : \text{ApoE}^{-/-}$ mice showed an increase in plasma glucose concentrations (540 ± 42 mg/dL) by ~3.6-fold compared with the control value of 150 ± 3 mg/dL (n = 6 to 8; p < 0.01).

Hyperglycemia was maintained in the diabetic $\text{Ins}^2+/\text{Akita} : \text{ApoE}^{-/-}$ mice throughout their lifespan in the range of 380 - 580 mg/dL (from 4-5 weeks until 25-30 weeks) compared with nondiabetic
Ins2\(^{+/+}\):ApoE\(^{-/-}\) mice whose blood glucose concentration was in the range of 110 - 200 mg/dL.

Plasma insulin concentrations of diabetic Ins2\(^{+/+}\)::ApoE\(^{-/-}\) mice were significantly lower by ~71% than those of control mice, explaining insulin deficiency as the cause of marked hyperglycemia in these animals. Thus, the newly generated type 1 diabetic Ins2\(^{+/+}\)::ApoE-knockout mice have phenotypic characteristics similar to type 1 diabetic Ins2\(^{+/+}\)::ApoE-knockout mice.

Previous studies demonstrate that ApoE-knockout mice exhibit an increase in plasma total cholesterol level (400-500 mg/dL when fed a standard chow diet) without an increase in plasma triglyceride level (39, 41, 57). To determine whether the ApoE-knockout mice that are subjected to spontaneous induction of type 1 diabetes show similar alterations in plasma lipid profile, we compared the total cholesterol and triglyceride concentrations of the diabetic Ins2\(^{+/+}\)::ApoE\(^{-/-}\) mice with those of the nondiabetic controls under fasting and fed conditions. As shown in Table 1, diabetic Ins2\(^{+/+}\)::ApoE\(^{-/-}\) mice (20 weeks of age) showed increases in plasma total cholesterol concentrations by 1.9-fold and 1.7-fold under fasting and fed conditions, respectively, compared with age-matched nondiabetic Ins2\(^{+/+}\):ApoE\(^{-/-}\) mice. In contrast, in ApoE-intact mice induction of diabetes by Ins2 gene mutation led to decreased cholesterol concentrations. Diabetic Ins2\(^{+/+}\)::ApoE\(^{-/-}\) mice showed a fasting plasma triglyceride value similar to nondiabetic Ins2\(^{+/+}\):ApoE\(^{-/-}\) mice. On a fed state, diabetic mice showed a trend of increase in plasma triglyceride concentration but the difference did not reach statistical significance compared with nondiabetic mice (p = 0.105). In ApoE-intact mice, induction of diabetes by Ins2 gene mutation caused the similar change in triglyceride concentrations as those in ApoE-deficient mice. Thus, diabetic Ins2\(^{+/+}\)::ApoE\(^{-/-}\) mice have pronounced increases in plasma total cholesterol concentrations compared with nondiabetic ApoE-knockout mice.
Enhanced atherosclerotic lesion in diabetic \( \text{Ins}^{+/\text{Akita}} \cdot \text{ApoE}^{-/-} \) mice. Previous studies using ApoE-knockout mice have shown that hypercholesterolemia is associated with the progression of spontaneous atherosclerotic lesion, which is markedly observed at 24-25 weeks of age (51). Since diabetic \( \text{Ins}^{+/\text{Akita}} \cdot \text{ApoE}^{-/-} \) mice showed a pronounced increase in plasma total cholesterol, we examined the likely possibility of enhanced atherosclerosis under these conditions. As shown in Fig. 1A, diabetic \( \text{Ins}^{+/\text{Akita}} \cdot \text{ApoE}^{-/-} \) mice showed an overall increase of atherosclerotic lesion in the entire aortas, as revealed by \textit{en face} analysis using oil red-O-staining. In particular, the atherosclerotic lesion was predominantly clustered in the aortic arch and hence this region was used for lesion quantification (Fig. 1A, B). As shown in Fig. 1C, the atherosclerotic lesion area of the aortic arch in the diabetic \( \text{Ins}^{+/\text{Akita}} \cdot \text{ApoE}^{-/-} \) mice and nondiabetic \( \text{ApoE}^{-/-} \) mice were 14.6 ± 2.1% and 4.7 ± 1.1%, respectively (\( n = 7 \) to 10; \( p < 0.01 \)). These data reveal ~3-fold increase in atherosclerotic lesion area in the aortic arch in the diabetic mice, compared with nondiabetic mice.

Increased plasma concentrations of non-HDL cholesterol in \( \text{Ins}^{+/\text{Akita}} \cdot \text{ApoE}^{-/-} \) mice. The data shown in Fig. 2A compare the plasma levels of triglycerides (TG), total cholesterol (Total-C), and HDL-cholesterol (HDL-C) and non-HDL-cholesterol (non-HDL-C) in diabetic \( \text{Ins}^{+/\text{Akita}} \cdot \text{ApoE}^{-/-} \) mice and nondiabetic \( \text{Ins}^{+/+} \cdot \text{ApoE}^{-/-} \) mice. The TG concentrations in diabetic mice and nondiabetic mice were 147 ± 28 mg/dL and 89 ± 10 mg/dL, respectively (\( n = 4 \); \( p = 0.066 \)). Plasma cholesterol profile showed a significant increase in Total-C and non-HDL-C levels with no change in HDL-C levels in diabetic mice compared with nondiabetic mice. In diabetic \( \text{Ins}^{+/\text{Akita}} \cdot \text{ApoE}^{-/-} \) mice and nondiabetic \( \text{Ins}^{+/+} \cdot \text{ApoE}^{-/-} \) mice, the measured Total-C levels were 936 ± 103 mg/dL and 459 ± 19 mg/dL, respectively (\( n = 4 \); \( p < 0.01 \)) and the
calculated non-HDL-C levels were 881 ± 105 mg/dL and 398 ± 22 mg/dL, respectively (n = 4; p < 0.01). These findings were further confirmed by the fast performance liquid chromatography (FPLC) analysis (Fig. 2B. upper panel). FPLC analysis showed a substantial increase of cholesterol concentrations in VLDL and LDL fractions by 17.6% and 50.1 %, respectively, in diabetic Ins2\(^{+/Akita}\).ApoE\(^{-/-}\) mice compared with those in nondiabetic Ins2\(^{+/+}\).ApoE\(^{-/-}\) control mice. There was no difference in cholesterol concentrations in HDL fraction between two groups. The observed increase in non-HDL-C in Ins2\(^{+/Akita}\).ApoE\(^{-/-}\) diabetic mice may be attributed to: i) increased hepatic VLDL secretion; and/or ii) reduced hepatic clearance of VLDL remnants and LDL.

**Hepatic VLDL secretion is decreased in Ins2\(^{+/Akita}\).ApoE\(^{-/-}\) mice.** To compare hepatic VLDL secretion between type 1 diabetic Ins2\(^{+/Akita}\).ApoE\(^{-/-}\) mice and nondiabetic ApoE\(^{-/-}\) mice, we determined plasma concentration of triglyceride (TG) under hyperinsulinemic-euglycemic clamp conditions. After 90-min of infusion of insulin at a constant rate and glucose at variable rates to achieve euglycemia in mice, Triton WR-1339 was injected through the right jugular vein (Fig. 3A and B). Notably, Triton WR-1339 inhibits lipoprotein lipase thereby preventing the catabolism (removal) of TG-rich lipoproteins from the circulation. Since, as shown in Fig. 2B (lower panel), the majority of TG (more than 90%) is associated with VLDL fraction in fasting plasma of these animals, the consequent accumulation of TG provides a close index of hepatic VLDL secretion rates as previously described (15). TG secretion rates were determined under hyperinsulinemic-euglycemic conditions to control for compounding variables such as glucose, insulin and free fatty acid levels in diabetic Ins2\(^{+/Akita}\).ApoE\(^{-/-}\) mice vs. nondiabetic Ins2\(^{+/+}\).ApoE\(^{-/-}\) mice. The temporal changes in plasma TG concentrations were determined after Triton WR-
1339 injection into mice. As shown in Fig. 3C, the infusion of Triton WR-1339 showed
markedly decreased TG secretion in diabetic mice compared with nondiabetic control mice.
Therefore, VLDL secretion rate in diabetic mice is not higher but lower than that of nondiabetic
mice. These data provide an indirect evidence that the increased accumulation of non-HDL
fraction in diabetic Ins2^{+/Akita}:ApoE^{-/-} mice is likely due to a decrease in clearance of lipoproteins.

Reduced insulin receptor signaling and diminished triglyceride content in the liver of
Ins2^{+/Akita}:ApoE^{-/-} mice. Previous studies have shown that diminished hepatic insulin signaling
leads to a decrease in VLDL secretion (4, 18). Hence, we assessed insulin receptor signaling
pathway in the liver of mice at basal state and after a 4.5-hour hyperinsulinemic-euglycemic
clamp. As shown in Fig. 4A, the expression of hepatic insulin receptor was comparable among
the groups. Insulin infusion during the clamp study resulted in an increase of hepatic Akt
phosphorylation on Ser473 by ~4.8 fold in nondiabetic mice. However, insulin-induced Akt
phosphorylation was markedly blunted in diabetic Ins2^{+/Akita}:ApoE^{-/-} mice. Additionally, in order
to examine whether reduced substrate pool causes a decrease in VLDL formation and secretion
(6), we also measured triglyceride (TG) content in the liver of mice. As shown in Fig. 4B,
hepatic TG content in diabetic Ins2^{+/Akita}:ApoE^{-/-} mice was significantly reduced by ~70%
compared with nondiabetic controls. Together, thses findings suggest that diabetic
Ins2^{+/Akita}:ApoE^{-/-} mice have intrinsic defects in hepatic insulin receptor signaling and diminished
TG content, which may contribute to decreased VLDL secretion.
Altered expression of hepatic lipoprotein receptors in Ins2\(^{+/Akita}\):ApoE\(^{-/-}\) mice. In normal ApoE-intact mice, ApoE-mediated lipoprotein clearance occurs through heparan sulfate proteoglycans (HSPGs), lipoprotein receptor-related protein (LRP), and LDL receptor while ApoB-mediated lipoprotein clearance occurs through LDL receptor (20, 30) and lipolysis-stimulated lipoprotein receptor (LSR) (34). In ApoE-deficient mice, lipoprotein clearance through HSPG and LRP is impaired. Therefore, in these mice, lipoprotein clearance is mainly ApoB-mediated via LDL receptor and/or LSR. To examine whether decreased expression of these receptors contributes to decreased lipoprotein clearance in diabetic Ins2\(^{+/Akita}\):ApoE\(^{-/-}\) mice, we performed immunoblot analysis using the liver tissue extracts. There was no significant change in hepatic LDL receptor expression in the diabetic Ins2\(^{+/Akita}\):ApoE\(^{-/-}\) mice compared with nondiabetic Ins2\(^{+/+}\):ApoE\(^{-/-}\) mice (Fig. 5). On the other hand, the expression of LSR was significantly diminished by 28% in diabetic Ins2\(^{+/Akita}\):ApoE\(^{-/-}\) mice (Fig. 5). These data suggest that factors interfering with ApoB-mediated lipoprotein clearance via LDL receptor and/or decreased LSR expression may be responsible for decreased clearance of lipoproteins and elevated non-HDL-C in diabetic Ins2\(^{+/Akita}\):ApoE\(^{-/-}\) mice.

Reduced ApoB-100 levels with elevated ApoB-48 and ApoC-III levels in Ins2\(^{+/Akita}\):ApoE\(^{-/-}\) mice. Since it is well known that the apolipoprotein composition of lipoproteins affects their clearance in the liver, we performed immunoblot analysis of plasma apolipoproteins such as ApoB-100, ApoB-48, ApoA-I and ApoC-III. In humans, ApoB-100 is synthesized in the liver and ApoB-48 in the small intestine. In mice, however, both ApoB-100 and ApoB-48 are produced in the liver. As shown in Fig. 6, fasting plasma ApoB-100 levels were significantly decreased by approximately 75% (p = 0.004) but ApoB-48 levels were moderately increased by
13% (p = 0.033) in diabetic Ins2+/Akita:Apoe−/− mice compared with nondiabetic Ins2+/+:Apoe−/− mice. There was no significant difference in ApoA-I levels between two groups consistent with no change in plasma HDL-cholesterol concentration (Fig. 2). On the other hand, fasting plasma ApoC-III levels were significantly increased by approximately 50% (p = 0.028) in diabetic mice compared with nondiabetic controls.
DISCUSSION

In dissecting the diverse factors contributing to the pathogenesis of diabetic atherosclerosis, mouse models have been of great value in recent years (21, 22, 52). However, the creation of mouse models that mimic human diabetic cardiovascular disease remains a significant challenge. Here we describe the development of spontaneous diabetes and atherosclerosis in a genetic model of T1D that takes advantage of Ins2\(^{Akita}\) mutation in the background of ApoE deficiency.

Ins2\(^{+/Akita}:ApoE^{-/-}\) mouse is an excellent animal model for spontaneous T1D and atherosclerosis. The Ins2\(^{+/Akita}\) mouse model carries a spontaneous base pair substitution C96Y in the insulin-2 (ins2) gene that leads to misfolding of proinsulin in the endoplasmic reticulum of pancreatic islet cells, and consequent severe β-cell apoptosis and dysfunction (37, 54). As a result, Ins2\(^{+/Akita}\) mouse develops typical features of T1D including persistent hyperglycemia, polydipsia, and weight loss due to significant hypoinsulinemia (49, 55). Moreover, this model replicates several complications of T1D such as retinopathy (3), neuropathy (8) and nephropathy (16, 47). Therefore, Ins2\(^{+/Akita}\) mouse is recognized as a desirable model of T1D free of non-specific effects of STZ, and adopted as an important model for the study of chronic complications of T1D by the NIH-sponsored Animal Models of Diabetic Complications Consortium (AMDCC) (http://www.amdcc.org/). Despite the remarkable phenotype of T1D, however, we did not observe atherosclerotic lesion in the aorta of Ins2\(^{+/Akita}\) mouse even by the age of 40 weeks while on a standard chow diet (data not shown). It is not surprising since it is
well known that mouse is resistant to the development of atherosclerosis since majority of cholesterol exists in the cardioprotective HDL fraction (13).

In our current study, regardless of ApoE gene status Ins2 gene mutation induces typical T1D features of reduced body weight, lean and fat mass, and hyperglycemia, all of which are attributable to hypoinsulinemia. The remarkable difference was seen in cholesterol concentration as predicted by an intermediary role of ApoE in lipoprotein metabolism. While it lowered total cholesterol concentration in ApoE-intact mice, diabetes significantly increased total cholesterol concentration in ApoE-deficient mice. Since HDL is the major cholesterol-carrying lipoprotein in ApoE-intact mice, the impact of T1D on non-HDL metabolism may not be apparent in these animals but instead T1D may decrease total cholesterol concentration possibly through diminished VLDL secretion. On the other hand, in ApoE-deficient mice that already have elevated non-HDL cholesterol concentration due to impaired clearance of remnant lipoproteins, T1D further exaggerated hypercholesterolemia. Most importantly, diabetic Ins2^{+/-Akita}.ApoE^{+-} mice developed significantly increased atherosclerotic lesions by the age of 25 weeks even when maintained on a standard chow diet. In these animals, T1D accelerated atherosclerotic lesions prominently in the aortic arch area as described by previous studies using STZ treatment in ApoE-deficient mice (7, 35, 39, 48). Such site predilection has been thought to result from disturbed blood flow (33) and is possibly associated with increased expression of endothelial cell Toll-like receptors in areas of inflammatory cell accumulation (32).

Ins2^{+/-Akita}.ApoE^{+-} mouse is the first diabetic and atherosclerosis-prone mouse model based on Ins2^{Akita} mutation without the need for both administration of chemicals such as STZ and dietary intervention like an atherogenic or high-fat diet. In addition, maintaining the strain is relatively easy since heterozygous Ins2^{Akita} mutation renders mouse to be diabetic with 100% penetrance,
and their diabetic phenotype is marked and consistent among animals in contrast to STZ 
administration (16, 49). Moreover, since the lifespan of these mice is up to 8-9 months despite 
marked phenotype of T1D probably due to incomplete absence of insulin, this animal model may 
provide the advantage of assessing atherogenesis at various stages, from early stage to advanced 
stage.

The elevated cholesterol in LDL fraction is the major driver for hypercholesterolemia in 
diabetic Ins2+/Akita:ApoE−/− mouse. We next determined lipid profile in mice and found that the 
non-HDL cholesterol concentration was approximately 2-fold higher in diabetic Ins2+/Akita:ApoE−/− mice than in nondiabetic Ins2+/+:ApoE−/− control mice. According to FPLC analysis, diabetic 
mice showed an increase of cholesterol concentration predominantly in LDL fraction and a small 
increase in VLDL fraction with no significant change in HDL fraction. These findings are 
different from previous studies using STZ-induced diabetes in ApoE−/− mice whose elevated 
cholesterol concentration was mainly noted in VLDL fraction (35, 39). However, such changes 
in diabetic Ins2+/Akita:ApoE−/− mice resemble altered lipid profile (elevated total-, LDL- and non-
HDL-cholesterol) observed in human subjects with T1D, particularly, in those with poor 
glycemic control or with increased duration of disease (1, 17, 42).

Diabetic Ins2+/Akita:ApoE−/− mouse has diminished hepatic VLDL secretion and likely 
impaired lipoprotein clearance. Subsequently, we sought to delineate the pathophysiologic 
processes resulting in hypercholesterolemia in spontaneously diabetic Ins2+/Akita:ApoE−/− mice. 
Our present study demonstrates that diabetic Ins2+/Akita:ApoE−/− mice have diminished hepatic
VLDL secretion compared with nondiabetic controls. Additionally, diabetic Ins2+/Akita:ApoE−/− mice have intrinsic defects in hepatic insulin receptor signaling as demonstrated by blunted Akt phosphorylation in response to insulin infusion during a hyperinsulinemic-euglycemic clamp study. This result is consistent with previous findings that reduced hepatic insulin receptor signaling led to diminished VLDL secretion in liver insulin receptor knockout (LIRKO) mice (4) as well as in LDL receptor knockout mice that express low levels of insulin receptor in the liver and lack insulin receptor in peripheral tissues (LiBl6Ldlr−/−), both of which had compensatory systemic hyperinsulinemia (18). It is also possible that since insulin deficiency in T1D decreases lipogenesis and triglyceride content in liver (40) as demonstrated in diabetic Ins2+/Akita:ApoE−/− mice, reduced substrate pool may have caused a decrease in VLDL formation and secretion (6).

Interestingly, while diabetic Ins2+/Akita:ApoE−/− mice showed exaggerated hypercholesterolemia and atherogenesis on a standard chow diet, LIRKO mice developed these findings only when challenged with an atherogenic diet for 12 weeks (4). On the other hand, LiBl6Ldlr−/− mice showed reduced cholesterol concentrations and diminished atherosclerosis despite a Western diet challenge and the absence of LDL receptor (18). These differences may be due to differences in strain used, study design and/or their metabolic properties, such as, systemic insulin concentrations, the degree of hyperglycemia, or systemic versus organ-specific impairment of insulin receptor signaling pathway. Hypercholesterolemia in the setting of diminished VLDL secretion strongly implies that impaired lipoprotein clearance is the major driver for elevated cholesterol concentrations although studies using a direct measurement of lipoprotein clearance are needed to confirm this.
Diabetic Ins2^{+/Akita}:ApoE^{-/-} mouse reveals alteration of hepatic LSR and apolipoprotein composition. Lipoprotein clearance entails complex interaction between apolipoproteins of lipoprotein particles and corresponding receptors in the liver. Since ApoE is an essential ligand for remnant lipoproteins to interact with HSPGs, LRP, and LDL receptor, its absence is expected not only to delay their clearance but also to prompt lipoproteins to be cleared exclusively via ApoB dependent process in the liver via LDL receptor and/or LSR. Diabetic Ins2^{+/Akita}:ApoE^{-/-} mice did not exhibit apparently reduced hepatic LDL receptor expression. Our finding is in agreement with a recent study that revealed STZ-induced diabetes did not affect the expression of the hepatic LDL receptor (36). On the other hand, diabetic Ins2^{+/Akita}:ApoE^{-/-} mice showed significantly reduced expression of LSR. Recent studies showed that liver-specific loss of LSR in mouse models triggered systemic hyperlipidemia (34) and its expression was upregulated by leptin (45). Since uncontrolled T1D is associated with leptin deficiency likely due to fast mass loss (12, 19, 26), it is tempting to speculate that leptin deficiency in T1D may downregulate the expression of hepatic LSR, which in turn exacerbates hyperlipidemia.

While human liver only produces ApoB-100, rodent liver produces both ApoB-48 and ApoB-100. Since ApoB-48 is a truncated form of ApoB-100 and lacks C-terminus LDL receptor binding site of ApoB-100, ApoE deficiency increases ratio of ApoB-48 to ApoB-100 from about 1:1 (in normal animals) to about 20:1 (57). In diabetic Ins2^{+/Akita}:ApoE^{-/-} mice, this ratio was further increased to about 80:1. Our findings are consistent with previous study by Sparks and colleagues showing that STZ-induced diabetes in rats caused pronounced reduction of ApoB-100 levels due to defects at the translational level without change in intracellular degradation (44). A recent study reports that STZ-induced diabetes caused a similar increase in ratio of ApoB-48 to ApoB-100 that was attributable to reduced lipoprotein clearance (14). That study was conducted
in LDL receptor-deficient mice that have ApoB-containing lipoproteins with intact ApoE capable of interacting with LRP as well as HSPGs. They proposed that reduced liver expression of the proteoglycan sulfation enzyme is responsible for this finding. However, Bishop and colleagues found no difference between normal and diabetic littermate mice in liver heparan sulfate content or composition (5). They suggest that dyslipidemia in T1D is likely due to changes in lipoprotein composition that reduce the affinity of the particles for hepatic lipoprotein receptors. Consistently, we found that in addition to the change in ApoB composition, plasma ApoC-III levels were ~50% higher in diabetic Ins2+/Akita:ApoE−/− mice than control Ins2+/+:ApoE−/− mice. Of note, overexpression of ApoC-III caused an accumulation of Apo-B48 containing lipoprotein remnants (10) and is independently associated with increased coronary artery disease in T1D subjects (27). Mechanism for elevated ApoC-III levels is likely due to impaired insulin receptor signaling in the liver either due to insulin deficiency or resistance. Recently, Altomonte and colleagues reported that hepatic ApoC-III expression is upregulated by nuclear transcription factor FoxO1 whose activity is inhibited through its phosphorylation by insulin (2). Further studies examining the exact roles of altered lipoprotein composition will help to define the mechanisms of dyslipidemia and atherosclerosis in T1D.

In summary, the current studies demonstrate the validity of Ins2+/Akita:ApoE−/− mouse as an alternative novel animal model of spontaneous diabetes and atherosclerosis, and offers insight into mechanisms for dyslipidemia and atherogenesis in T1D. This model may be used for future studies to test therapeutic interventions for treatment of dyslipidemia and atherosclerosis in T1D.
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DISCLOURES

No conflicts of interest are declared by the author(s).
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FIGURE LEGENDS

**Fig. 1.** *En face* analysis of atherosclerotic lesion area in the aortas from spontaneously diabetic Ins2+/Akita:ApoE-/- mice. Representative photomicrographs of the entire aorta (magnification x10) (A) and aortic arch (magnification x40) (B) showing enhanced atherosclerotic lesion in the diabetic mice compared with age-matched nondiabetic mice (25 weeks of age, each group). C) Atherosclerotic lesion area is expressed as the percentage of the total luminal surface area of the aortic arch in diabetic mice (n = 10) compared with nondiabetic mice (n = 7). The data shown in the bar graphs are the means ± SE values. * p < 0.01 compared with nondiabetic mice.

**Fig. 2.** Changes in plasma lipid profile in spontaneously diabetic Ins2+/Akita:ApoE-/- mice. A) Plasma samples were obtained by heart puncture from diabetic Ins2+/Akita:ApoE-/- mice and age-matched nondiabetic Ins2+/+ApoE-/- mice after a 5-hour fast (25 weeks of age, n = 4 mice, each group). Total cholesterol (Total-C), HDL-cholesterol (HDL-C), and triglyceride (TG) were measured and non-HDL-cholesterol (non-HDL-C) was calculated by subtracting HDL-cholesterol from total cholesterol. The data shown in the bar graphs are the means ± SE values. * p < 0.01 compared with nondiabetic mice. B) Plasma samples were subjected to fast performance liquid chromatography (FPLC) analysis, and cholesterol (upper) and triglyceride (lower) were measured in each of eluted fractions.
Fig. 3. Hepatic VLDL secretion in diabetic Ins2+/Akita:ApoE−/− mice under hyperinsulinemic-euglycemic conditions. A) Schematic of a hyperinsulinemic-euglycemic clamp study. 20-week-old diabetic Ins2+/Akita:ApoE−/− mice and nondiabetic littermate Ins2+/+:ApoE−/− mice (after a 5-hour fast) were infused with insulin at a constant rate as indicated, and with glucose at variable rates to maintain euglycemia. Triton WR-1339, an inhibitor of lipoprotein lipase, was intravenously injected at 90 min after infusions with insulin and glucose. Tail blood samples were collected at the indicated time points (●) to measure lipids before and after Triton WR-1339 injection. B) Plasma insulin concentrations before (0 min) and during (210 min) the hyperinsulinemic-euglycemic clamp study. C) The linear graphs show the changes in blood glucose concentrations during a 4.5-hour hyperinsulinemic-euglycemic clamp in diabetic mice (closed circle; n = 7) in comparison with nondiabetic control mice (open circle, n = 5). D) The temporal changes in plasma levels of triglyceride (TG) before and after Triton WR-1339 infusion in diabetic Ins2+/Akita:ApoE−/− mice (closed circle; n = 7) compared with nondiabetic Ins2+/+:ApoE−/− mice (open circle, n = 5) under hyperinsulinemic-euglycemic conditions. The data shown in the linear graphs are the means ± SE values. NS denotes not significant. * p < 0.01 compared with nondiabetic mice.

Fig. 4. Hepatic expression of insulin receptor signaling components and triglyceride content in diabetic Ins2+/Akita:ApoE−/− mice. (A) Immunoblot analysis of insulin receptor (IR, β-subunit), pAkt, and Akt from livers of 20-week old nondiabetic (non-DM) Ins2+/+:ApoE−/− mice and diabetic (DM) Ins2+/Akita:ApoE−/− mice at basal state and after a 4.5-hour hyperinsulinemic-euglycemic clamp as described in Fig. 3. Mice were fasted for 5 hours. The relative levels of pAkt/Akt ratio are shown in bar graph. The data are the means ± SE values. n=3, each group. *
p < 0.05 compared with nondiabetic mice. (B) Hepatic triglyceride content of 20-week old nondiabetic Ins2+/+ :ApoE−/− mice and diabetic Ins2+/+ :ApoE−/− mice. Mice were fasted for 5 hours. The data shown in the bar graph are the means ± SE values. n=6-7, each group. * p < 0.01 compared with nondiabetic mice.

**Fig. 5.** The expression of hepatic lipoprotein receptors in diabetic Ins2+/+ :ApoE−/− mice. A) Immunoblot analysis of the LDL receptor (LDLr) and lipolysis-stimulated lipoprotein receptor (LSR) expression in the liver extracts from 5-hour fasted 20-week-old diabetic Ins2+/+ :ApoE−/− mice (n=6) and age-matched nondiabetic control Ins2+/+ :ApoE−/− mice (n = 5). To normalize protein level, superoxide dismutase (SOD)-1 was used an internal control. B) The relative expression of hepatic lipoprotein receptors is shown in the bar graph. The data are the means ± SE values. * p < 0.01 compared with nondiabetic mice.

**Fig. 6.** Diminished ApoB-100 and increased ApoB-48 and ApoC-III in the plasma samples from diabetic Ins2+/+ :ApoE−/− mice. A) Immunoblot analysis of the plasma apolipoproteins such as ApoB-100, ApoB-48, ApoA-I, and ApoC-III from 15-hour fasted 20-week-old diabetic Ins2+/+ :ApoE−/− mice and age-matched nondiabetic control Ins2+/+ :ApoE−/− mice (n = 6, each group). B) The relative levels of plasma apolipoproteins normalized to mean of those in nondiabetic Ins2+/+ :ApoE−/− control. The data shown in the bar graph are the means ± SE values. * p < 0.05 compared with nondiabetic mice.
Table 1. Phenotypic and biochemical characteristics of Ins2+/Akita:ApoE-deficient mice

<table>
<thead>
<tr>
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<th>ApoE-intact mice</th>
<th>ApoE-deficient mice</th>
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</table>
|                      | Nondiabetic C57BL/6 (Ins2+/+;ApoE+/+) | Diabetic Ins2+/Akita (Ins2+/Akita;ApoE+/+)
|                      | Nondiabetic (Ins2+/+;ApoE−/−) | Diabetic (Ins2+/Akita;ApoE−/−) |
| Body weight (g)      |                 |                     |
| 10 wk                | 25.0 ± 0.4      | 22.2 ± 0.7*         | 27.3 ± 2.6 |
|                      | 32.0 ± 0.9      | 25.6 ± 1.1*         | 31.8 ± 0.3 |
| 20 wk                | 23.1 ± 0.5      | 19.4 ± 0.4*         | 22.9 ± 2.3 |
| Lean mass (g)        |                 |                     |
| 10 wk                | 24.6 ± 0.5      | 21.7 ± 1.2*         | 24.9 ± 0.6 |
| 20 wk                | 7.0 ± 0.3       | 3.2 ± 0.1*          | 5.8 ± 0.4  |
| Fat mass (g)         |                 |                     |
| 10 wk                | 2.2 ± 0.1*      | 2.7 ± 0.4           | 2.7 ± 0.1# |
| 20 wk                | 5.8 ± 0.4       | 3.4 ± 0.1$          | 3.4 ± 0.1$ |
| Plasma glucose (mg/dL) | 139 ± 12  | - 487 ± 15* | - 150 ± 3 | - 540 ± 42$ | - |
| Plasma insulin (pM)  | 39.9 ± 6.1      | - 11.4 ± 3.4*       | - 49.1 ± 2.6 | - 14.5 ± 5.0$ | - |
| Plasma total cholesterol (mg/dL) | 107 ± 4 | 107 ± 7 | 77 ± 9* | 82 ± 6* | 462 ± 6# | 534 ± 42# | 865 ± 83$,# | 933 ± 94$,# |
| Plasma triglyceride (mg/dL) | 78 ± 11 | 128 ± 18 | 83 ± 6 | 371 ± 118 | 89 ± 2.0 | 140 ± 25.5 | 83 ± 10.9 | 230 ± 34 |

Body weight was measured on a scale, and whole body fat and lean mass were noninvasively measured in awake mice at 10 and 20 weeks of age using $^1$H-MRS. To determine plasma
glucose, insulin and lipid levels, tail blood samples were collected from diabetic and nondiabetic mice (20 weeks of age) under 15-hour fasting and fed conditions. The data shown are the means ± SE values (n = 6-9 mice, each group). * p < 0.05 compared with littermate nondiabetic C57BL/6 mice. \( \hat{\text{p}} \) p < 0.05 compared with littermate nondiabetic Ins2\(^+/+\):ApoE\(^{-/-}\) mice. \# p < 0.05 compared with age-matched counterpart ApoE-intact mice (Ins2\(^+/+\):ApoE\(^{-/-}\) vs. C57BL/6 (Ins2\(^+/+\):ApoE\(^{+/+}\)), or Ins2\(^+/+\)/Akita:ApoE\(^{-/-}\) vs. Ins2\(^+/+\)/Akita:ApoE\(^{+/+}\)).
Fig. 1

A) Nondiabetic Diabetic

B) Nondiabetic Diabetic

C) Atherosclerotic lesion area (%)

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<thead>
<tr>
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<th>Nondiabetic</th>
<th>Diabetic</th>
</tr>
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<tbody>
<tr>
<td>Atherosclerotic lesion area (%)</td>
<td>5</td>
<td>15</td>
</tr>
</tbody>
</table>

* Indicates statistical significance.
Fig. 2

A) Plasma lipids (mg/dL)

- Total-C
- HDL-C
- Non-HDL-C
- TG

B) Plasma lipids by fraction number

- VLDL
- LDL
- HDL

Cholesterol (µg/Fraction)

Triglyceride (µg/Fraction)
Fig. 3

A)

**Triton WR-1339**

<table>
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<th>Clamp time (min):</th>
<th>-60</th>
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<th>60</th>
<th>120</th>
<th>180</th>
<th>240</th>
<th>300</th>
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Blood sampling for lipids

<table>
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<th>Insulin 2.5 mU/kg/min</th>
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<td>Variable rate of glucose infusion</td>
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B)

![Bar graph showing plasma insulin levels for Nondiabetic and Diabetic groups at Basal and Clamp conditions.](image)

**Nondiabetic**

- Basal (at 0 min): 100 pM
- Clamp (at 210 min): 300 pM

**Diabetic**

- Basal (at 0 min): 10 pM
- Clamp (at 210 min): 300 pM

NS: Not significant

*: Significant difference
Fig. 4

A) Western blot analysis showing the pAkt (Ser473) and Akt levels in both nondiabetic and diabetic groups under basal and clamp conditions. IR, Akt, and α-tubulin bands are shown.

B) Graph illustrating the hepatic triglyceride content in nondiabetic and diabetic groups. The pAkt/Akt ratio is also shown for comparison.

* indicates statistically significant difference.
Fig. 5

A)

Nondiabetic Diabetic

LDLr
LSR
SOD-1

B)

Hepatic receptor expression (arbitrary units)

Nondiabetic
Diabetic

*
Fig. 6

A) Plasma apolipoproteins (arbitrary units)

Nondiabetic  Diabetic

B) Plasma apolipoproteins (arbitrary units)

Nondiabetic  Diabetic

ApoB-100  ApoB-48  ApoA-I  ApoC-III