Spontaneously diabetic Ins2+/Akita:apoE-deficient mice exhibit exaggerated hypercholesterolemia 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 less significant 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 cross-breeding Ins2+/Akita mouse (which 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 wk of age. At 25 wk of age and while on a standard low-fat diet, diabetic Ins2+/Akita:apoE−/− mice exhibited an approximately threefold increase in atherosclerotic plaque in association with an approximately twofold 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. Although the expression of hepatic LDL receptor was not affected, there was a significant reduction in the expression of lipolysis-stimulated lipoprotein receptor (LSR) by 10.220.33.5 on April 26, 2017 http://ajpendo.physiology.org/ Downloaded from http://ajpendo.physiology.org/ by 10.220.33.5 on April 26, 2017
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 for an artificial diet. Strategies to study the effects of spontaneous T1D on atherogenesis under apoE-deficient states could provide new insights into 1) altered lipoprotein secretion and clearance by the liver, 2) altered circulatory lipid/lipoprotein profile, and 3) the magnitude of atherosclerosis in the setting of T1D.

The present study is aimed at utilizing the Ins2\(^{+/+}\)/Akita mouse (which 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 wk of age, were maintained on a standard chow diet until 25 wk of age to assess the extent of atherosclerosis. To assess whether the changes in cholesterol or TG concentrations contribute to dyslipidemia, we quantified cholesterol and TG concentrations in plasma and in the individual lipoprotein fraction. We determined hepatic VLDL secretion rate and the percentage of the total luminal surface area of the aortic arch using 1H-MRS (magnetic resonance spectrometer; Echo Medical Systems, Houston, TX).

**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 and were approved by the committee. Ins2\(^{+/+}\)/Akita heterozygous, C57BL/6, and apoE\(^{-/-}\)/Unc homozygous mutation (apoE-knockout or apoE\(^{-/-}\)) mice were obtained originally from The Jackson Laboratory (Bar Harbor, ME). All mice were C57BL/6 J background.

**Generation of Ins2\(^{+/+}\)/Akita:apoE\(^{-/-}\) mice.** Diabetic male Ins2\(^{+/+}\)/Akita:apoE\(^{-/-}\) mice (Ins2\(^{+/+}\)/Akita heterozygous mice) were crossed with nondiabetic female Ins2\(^{+/+}\)/apoE\(^{-/-}\) mice (F0). The resulting F1 generation consisted of heterozygous apoE\(^{-/-}\) (Ins2\(^{+/+}\)/Akita:apoE\(^{-/-}\) and Ins2\(^{+/+}\)/apoE\(^{-/-}\)) mice. From this F1 generation, diabetic male Ins2\(^{+/+}\)/Akita:apoE\(^{-/-}\) mice were crossed with nondiabetic female Ins2\(^{+/+}\)/apoE\(^{-/-}\) mice. The resulting F2 generation consisted of homozygous apoE\(^{-/-}\) (Ins2\(^{+/+}\)/Akita:apoE\(^{-/-}\) and Ins2\(^{+/+}\)/apoE\(^{-/-}\)) mice, and heterozygous apoE\(^{-/-}\) (Ins2\(^{+/+}\)/Akita:apoE\(^{-/-}\) and Ins2\(^{+/+}\)/apoE\(^{-/-}\)) mice. Subsequently, diabetic male Ins2\(^{+/+}\)/Akita:apoE\(^{-/-}\) mice (from F2 generation) and nondiabetic female Ins2\(^{+/+}\)/apoE\(^{-/-}\) mice were set up as breeding pairs to produce an F3 generation of diabetic Ins2\(^{+/+}\)/Akita:apoE\(^{-/-}\) 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 wk of age and maintained on a 12:12-h dark-light cycle under controlled temperature (23°C). The mice had free access to water and standard rodent chow diet (Teklad, Harlan Laboratories, Indianapolis, IN), which contains cholesterol <0.1% and fat as 18% of total calories. Genotypes were determined by PCR amplification of tail DNA using protocols provided by The Jackson Laboratory. Diabetic phenotype was confirmed in mice at 4–5 wk after birth by blood glucose values >250 mg/dl with a hand-held glucometer (Contour; Bayer Health Care, Tarrytown, NY) measured with a drop of blood from tail puncture. The disease penetrance is 100% in mice with the Ins2\(^{+/+}\)/Akita mutation (49).

**Measurement of body composition.** Whole body fat and lean mass were measured noninvasively in awake mice at the age of 10 and 20 wk using \(^{1}H\)-MRS (magnetic resonance spectrometer; Echo Medical Systems, Houston, TX).

**Assessment of atherosclerotic lesion.** In the present study, male diabetic Ins2\(^{+/+}\)/Akita:apoE\(^{-/-}\) mice and nondiabetic Ins2\(^{+/+}\)/apoE\(^{-/-}\) mice were maintained on a standard chow diet until 25 wk of age, at which time they were euthanized to quantify atherosclerotic lesion area. The time frame of 25 wk of age was chosen on the basis of 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% isopropl alcohol) for 30 min. Excess stain was removed with 60% isopropl 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, San Jose, CA) and ImageJ software (National Institutes of Health, Bethesda, MD).

**Biochemical assays.** Plasma glucose, total cholesterol, HDL cholesterol, and TG concentrations were measured using Vitros DT slides and an enzymatic colorimetric method by Vitros DT60 II Chemistry System (Ortho-Clinical Diagnostics, Rochester, NY) according to the 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 hand-held glucometer (Contour).

**Measurement of liver triglycerides.** Lipids were extracted from liver (0.1 g) in 2 ml of chloroform-methanol (2:1, vol/vol) using a method adapted from Storlien et al. (46). The organic phase was separated by adding 200 μl of 1 M H2SO4. One milliliter of organic phase was mixed in 1 ml of 1:1 chloroform-Trition X-100 and evaporated overnight. Dried samples were dissolved in 200 μl of distilled water and assayed for TGs using Vitros DT slides and an enzymatic colorimetric method by Vitros DT60 II Chemistry System (Ortho-Clinical Diagnostics).

**Lipoprotein fractionation by fast-performance liquid chromatography.** Fast-performance liquid chromatography (FPLC) analysis was provided by the University of Cincinnati Mouse Metabolic Phenotyping Center. In brief, the 200 μl of plasma from mice fasted for 5 h was subjected to FPLC analysis. Plasma sample was chromatographed unidually 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 was eluted in fractions 1–10, LDL in fractions 11–30, and HDL in fractions 31–51. Cholesterol and TG concentrations were measured in each of eluted fractions by the Infinity cholesterol assay kit (Thermo Fisher Scientific, Indianapolis, IN) and the Randox triglyceride assay kit (Randox Laboratories, 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 described previously (15) with modifications. Since the majority of TG is found in VLDL fraction according to the FLPC 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 the right jugular vein catheter (PE-10. Intramedic; BD Biosciences, Sparks, MD) under general anesthesia with intraperitoneal injection of ketamine (100 mg/kg body wt) and xylazine (10 mg/kg body wt). Catheters were externalized to the back of the neck. Following a 5-h fast, a 4.5-h hyperinsulinemic euglycemic clamp study was conducted in the conscious diabetic Ins2+/Akita:apoE−/− mice and nondiabetic Ins2+/+;apoE−/− mice at 20 wk of age (n = 5–7). To raise plasma insulin within a physiological range (~300 pM), the mice were infused with a priming dose (150 mU/kg body wt) 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, Tyloxap; 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. 3, A and B). At the end of the clamp study, mice were euthanized. Immuno blot analysis. Plasma samples were obtained from mice after a 5-h 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 of protein each) were subjected to electrophoresis using precast 4–12% NuPage minigels (Invitrogen, Carlsbad, CA), and the resolved proteins were transferred to nitrocellulose membranes (BioRad, Hercules, CA). The membranes were blocked and probed with the indicated primary antibodies. The primary antibodies for apoA-I (sc-30089), apoB (sc-25542), apoC-III (sc-50378), and LSR (sc-133765) were purchased from Santa Cruz Biotecnology (Santa Cruz, CA). The primary antibody for LDL receptor (ab3032) was purchased from Abcam (Cambridge, MA). The primary antibodies for insulin receptor (no. 3025), total Akt (no. 9272), phospho-Ser473 Akt (no. 9271), and α-tubulin (no. 2125) were purchased from Cell Signaling Technology (Danvers, MA). The immunoreactivity was detected using specific horseradish peroxidase-conjugated secondary antibodies, followed by enhanced chemiluminescence (Pierce). The protein bands were quantified using ImageJ software.

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

RESULTS

Phenotypic characteristics of the newly generated type 1 diabetic Ins2+/Akita:apoE−/− mice. Previous studies have shown that type 1 diabetic Ins2+/Akita mice (which have Ins2 gene mutation, causing pancreatic β-cell apoptosis and insulin deficiency) exhibit a decrease in body weight by 12–33% as a function of age (4–36 wk) 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 Ins2+/Akita mice (4–36 wk old) compared with the control values of 138–187 mg/dl in wild-type mice.

To study atherogenesis in the context of spontaneous T1D, we generated Ins2+/Akita:apoE−/− mouse by cross-breeding Ins2+/+;apoE−/− mouse with apoE−/− mouse, as described in MATERIALS AND METHODS. To determine whether the type 1 diabetic phenotype developed on an apoE−/− background exhibits similar characteristics, we compared the body weight, lean and fat mass, glucose, and insulin concentrations between the diabetic Ins2+/+;apoE−/− mice and the nondiabetic control Ins2+/+;apoE−/− mice (F3 generation). We also compared them with those of age-matched counterpart apoE-intact mice (wild-type C57BL/6 and diabetic Ins2+/+Akita mice). As shown in Table 1, diabetic Ins2+/+;apoE−/− mice had a decrease in body weight by ~16% at 20 wk of age compared with age-matched nondiabetic Ins2+/+;apoE−/− mice. The reduced body weight in diabetic Ins2+/+Akita: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 Ins2+/+Akita: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 = 6–8, P < 0.01). Hyperglycemia was maintained in the diabetic Ins2+/+Akita:apoE−/− mice throughout their lifespan in the range of 380–580 mg/dl (from 4 to 5 wk until 25 to 30 wk) 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+/+Akita: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+/+Akita:apoE-intact knockout mice have phenotypic characteristics similar to type 1 diabetic Ins2+/+Akita 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 TG level (39, 41, 57). To determine whether the apoE-knockout mice that are subjected to spontaneous induction of T1D show similar alterations in plasma lipid profile, we compared the total cholesterol and TG concentrations of the diabetic Ins2+/+Akita:apoE−/− mice with those of the nondiabetic controls under fasting and fed conditions. As shown in Table 1, diabetic Ins2+/+Akita:apoE−/− mice (20 wk of age) showed increases in plasma total cholesterol concentrations by 1.9- 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+/+Akita:apoE−/− mice showed a fasting plasma TG value similar to nondiabetic Ins2+/+;apoE−/− mice. In a fed state, diabetic mice showed a trend of increase in plasma TG 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 a change in TG concentrations similar to those in apoE-deficient mice. Thus, diabetic Ins2+/+Akita:apoE−/− mice have pronounced increases in plasma total cholesterol concentrations compared with nondiabetic apoE-knockout mice.

Enhanced atherosclerotic lesion in diabetic Ins2+/+Akita: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 wk of age (51). Since diabetic Ins2\(^+/-\)/Akita: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 Ins2\(^+/-\)/Akita:apoE\(^-/-\) mice showed an overall increase of atherosclerotic lesion in the entire aorta, as revealed by en face analysis using Oil Red O staining. In particular, the atherosclerotic lesion was clustered predominantly in the aortic arch, and hence, this region was used for lesion quantification (Fig. 1, A and B). As shown in Fig. 1C, the atherosclerotic lesion areas of the aortic arch in the diabetic Ins2\(^+/-\)/Akita:apoE\(^-/-\) and nondiabetic apoE\(^-/-\) mice were 14.6 ± 2.1 and 4.7 ± 1.1%, respectively (n = 7–10, P < 0.01). These data reveal an approximately threefold 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 Ins2\(^+/-\)/Akita:apoE\(^-/-\) mice.** The data shown in Fig. 2A compare the plasma levels of total cholesterol, HDL and non-HDL-cholesterol, and TGs in diabetic Ins2\(^+/-\)/Akita:apoE\(^-/-\) and nondiabetic Ins2\(^+/-\)/apoE\(^-/-\) mice. The TG concentrations in diabetic mice and nondiabetic mice were 147 ± 28 and 89 ± 10 mg/dl, respectively (n = 4, P = 0.066). Plasma cholesterol profile showed a significant increase in total cholesterol and non-HDL-cholesterol levels, with no change in HDL cholesterol levels in diabetic mice compared with nondiabetic mice. In diabetic Ins2\(^+/-\)/Akita:apoE\(^-/-\) and nondiabetic Ins2\(^+/-\)/apoE\(^-/-\) mice, the measured total cholesterol levels were 936 ± 103 and 459 ± 19 mg/dl, respectively (n = 4, P < 0.01), and the calculated non-HDL cholesterol levels were 881 ± 105 and 398 ± 22 mg/dl, respectively (n = 4, P < 0.01). These findings were further confirmed by the FPLC analysis (Fig. 2B, top). FPLC analysis showed a substantial increase in 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 cholesterol in Ins2\(^+/-\)/Akita:apoE\(^-/-\) diabetic mice may be attributed to 1) increased hepatic VLDL secretion and/or 2) 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\(^-/-\) and nondiabetic apoE\(^-/-\) mice, we determined plasma concentration of 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. 3, A and B). Notably, Triton WR-1339 inhibits lipoprotein lipase, thereby preventing the catabolism (removal) of TG-rich lipoproteins from the circulation. Since, as is shown in Fig. 2B, bottom, the majority of TG (>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 and/or reduced hepatic clearance of VLDL remnants and LDL.

#### Table 1. Phenotypic and biochemical characteristics of Ins2\(^+/-\)/Akita:apoE-deficient mice

<table>
<thead>
<tr>
<th></th>
<th>apoE-Intact Mice</th>
<th>apoE-Deficient Mice</th>
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<tbody>
<tr>
<td></td>
<td>Diabetic</td>
<td>Non-diabetic</td>
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<tr>
<td>Body weight, g</td>
<td></td>
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<tr>
<td>10 wk</td>
<td>25.0 ± 0.4</td>
<td>22.2 ± 0.7*</td>
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<tr>
<td>20 wk</td>
<td>32.0 ± 0.9</td>
<td>25.6 ± 1.1*</td>
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<tr>
<td>Lean mass, g</td>
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<tr>
<td>10 wk</td>
<td>23.1 ± 0.5</td>
<td>19.4 ± 0.4*</td>
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<tr>
<td>20 wk</td>
<td>24.6 ± 0.5</td>
<td>19.7 ± 1.2*</td>
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<tr>
<td>Fat mass, g</td>
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<tr>
<td>10 wk</td>
<td>3.0 ± 0.2</td>
<td>2.2 ± 0.1*</td>
</tr>
<tr>
<td>20 wk</td>
<td>7.0 ± 0.3</td>
<td>3.2 ± 0.1*</td>
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<tr>
<td>Plasma glucose, mg/dl</td>
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<tr>
<td>Fasted</td>
<td>39.9 ± 6.1</td>
<td>11.4 ± 3.4*</td>
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<tr>
<td>Fed</td>
<td>107 ± 4</td>
<td>77 ± 9*</td>
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<tr>
<td>Protein</td>
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<tr>
<td>Fasted</td>
<td>107 ± 7</td>
<td>82 ± 6*</td>
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<tr>
<td>Fed</td>
<td>78 ± 11</td>
<td>83 ± 6*</td>
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<td>Plasma TG, mg/dl</td>
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<tr>
<td>Fasted</td>
<td>128 ± 18</td>
<td>371 ± 118</td>
</tr>
<tr>
<td>Fed</td>
<td>128 ± 18</td>
<td>371 ± 118</td>
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Data shown are means ± SE (n = 6–9 mice/group). apoE, apolipoprotein E; TG, triglyceride. Body weight was measured on a scale, and whole body fat and lean mass were noninvasively measured in awake mice at 10 and 20 wk of age using \( ^{1}H \)-magnetic resonance spectroscopy. To determine plasma glucose, insulin, and lipid levels, tail blood samples were collected from diabetic and nondiabetic mice (20 wk of age) under 15-h fasting and fed conditions. \( *P < 0.05 \) compared with littermate nondiabetic C57BL/6 mice; \( \#P < 0.05 \) 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\(^-/-\)].
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, the VLDL secretion rate in diabetic mice is not higher but lower than that of nondiabetic mice. These data provide indirect evidence that the increased accumulation of non-HDL fraction in diabetic Ins2<sup>+/Akita</sup>:apoE<sup>−/−</sup> mice is likely due to a decrease in clearance of lipoproteins.

Reduced insulin receptor signaling and diminished TG content in the liver of Ins2<sup>+/Akita</sup>:apoE<sup>−/−</sup> 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-h 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 in hepatic Akt phosphor-
ylation on Ser473 by ~4.8-fold in nondiabetic mice. However, insulin-induced Akt phosphorylation was markedly blunted in diabetic Ins2+/+Akita−/−apoE−/− mice. Additionally, to examine whether reduced substrate pool causes a decrease in VLDL formation and secretion (6), we also measured 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, these 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 HSPGs, LRP, and LDL receptor, whereas apoB-mediated lipoprotein clearance occurs through LDL receptor (20, 30) and LSR (34). In apoE-deficient mice, lipoprotein clearance through HSPG and LRP is impaired. Therefore, in these mice, lipoprotein clearance is apoB-mediated mainly 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 cholesterol 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. However, in mice, 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 ~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 the 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 ~50% (P = 0.028) in diabetic mice compared with nondiabetic controls.

Fig. 3. Hepatic VLDL secretion in diabetic Ins2+/+Akita−/−apoE−/− mice under hyperinsulinemic euglycemic conditions. A: schematic of a hyperinsulinemic euglycemic clamp study; 20-wk-old diabetic Ins2+/+Akita−/−apoE−/− mice and nondiabetic littermate Ins2+/+apoE−/− mice (after a 5-h 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 injected intravenously 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-h hyperinsulinemic euglycemic clamp in diabetic mice (n = 7; ●) compared with nondiabetic control mice (n = 5; ○) D: the temporal changes in plasma levels of TG before and after Triton WR-1339 infusion in diabetic Ins2+/+Akita−/−apoE−/− (n = 7; ●) compared with nondiabetic Ins2+/+apoE−/− mice (n = 5; ○) under hyperinsulinemic euglycemic conditions. The data shown in the linear graphs are means ± SE. NS, not significant. *P < 0.01 compared with nondiabetic mice.
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 Ins2Akita mutation in the background of apoE deficiency.

Ins2<sup>+/Akita</sup>:apoE<sup>/−</sup> mouse is an excellent animal model for spontaneous T1D and atherosclerosis. The Ins2<sup>+/Akita</sup> 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<sup>+/Akita</sup> 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<sup>+/Akita</sup> mouse is recognized as a desirable model of T1D free of nonspecific effects of STZ and adopted as an important model for the study of chronic complications of T1D by the National Institutes of Health-sponsored Animal Models of Diabetic Complications Consortium (http://www.amdcc.org/). However, despite the remarkable phenotype of T1D, we did not observe atherosclerotic lesion in the aorta of Ins2<sup>+/Akita</sup> mouse even by the age of 40 wk while on a standard chow diet (data not shown). This is not surprising, since it is well known that mouse is resistant to the development of atherosclerosis since the 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. Whereas 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 hypercholester-
different from previous studies using STZ-induced diabetes in apoE−/− mice, whose elevated cholesterol concentration was noted mainly 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 pathophysiological 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 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 (L1B6Ldlr−/−), 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, whereas diabetic Ins2+/Akita::apoE−/− mice showed exaggerated hypercholesterolemia and atherosclerosis on a standard chow diet, liver insulin receptor knockout mice developed these findings only when challenged with an atherogenic diet for 12 wk (4). On the other hand, L1B6Ldlr−/− 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 vs. organ-specific impairment of the 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 the 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 that 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 ex-
pression of LSR. Recent studies showed that liver-specific loss of LSR in mouse models triggered systemic hyperlipidemia (34) and that its expression was upregulated by leptin (45). Since uncontrolled T1D is associated with leptin deficiency likely due to fat 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.

Whereas human liver produces only apoB-100, rodent liver produces both apoB-48 and apoB-100. Since apoB-48 is a truncated form of apoB-100 and lacks COOH-terminal LDL receptor binding site of apoB-100, apoE deficiency increases the ratio of apoB-48 to apoB-100 from ~1:1 (in normal animals) to ~20:1 (57). In diabetic Ins2/Akita:apoE−/− mice, this ratio was further increased to ~80:1. Our findings are consistent with a previous study by Sparks et al. (44) 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. A recent study reported 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 et al. (5) found no difference between normal and diabetic littermate mice in liver heparan sulfate content or composition. They suggested 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 apoB48-containing lipoprotein remnants (10) and is independent of 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, which is due to either insulin deficiency or resistance. Recently, Altomonte et al. (2) reported that hepatic apoC-III expression is upregulated by nuclear transcription factor FoxO1, whose activity is inhibited through its phosphorylation by insulin. 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 study demonstrates 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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