ANIMAL MODELS

Sex-Specific Differences in an ApoE$^{−/−}$:Ins2$^{+/+}$/Akita Mouse Model of Accelerated Atherosclerosis

Daniel E. Venegas-Pino,¹ Pei-Wen Wang,² Heidi K. Stoute,² Nicholas A. Singh-Pickersgill,¹ Brian Y. Hong,¹ Mohammad I. Khan,¹ Yuanyuan Shi,¹ and Geoff H. Werstuck*¹†

From the Departments of Biochemistry and Biomedical Sciences* and Medicine¹ and the Thrombosis and Atherosclerosis Research Institute,¹ McMaster University, Hamilton, Ontario, Canada

Diabetic patients have a twofold to fourfold increased risk of cardiovascular disease. Despite a vast amount of research, the underlying mechanisms that predispose individuals with diabetes to the development of cardiovascular disease are unclear. To further our understanding of how diabetes promotes atherosclerosis, we have established, characterized, and manipulated a new model of hyperglycemia-induced atherosclerosis: the apolipoprotein E−deficient (ApoE$^{−/−}$):Ins2$^{+/+}$/Akita mouse. All mice were fed a standard chow diet. Male ApoE$^{−/−}$:Ins2$^{+/+}$/Akita mice developed chronic hyperglycemia, which significantly accelerated atherosclerosis. Female ApoE$^{−/−}$:Ins2$^{+/+}$/Akita mice presented hyperglycemia that normalized by 15 weeks of age. Despite the transient hyperglycemia, advanced atherosclerosis was observed at 15 weeks of age compared with ApoE$^{−/−}$ females. To better understand these differences, subsets of mice were castrated or ovariectomized at 5 weeks of age. Castrated ApoE$^{−/−}$:Ins2$^{+/+}$/Akita mice showed a reduction in blood glucose levels that correlated with the amelioration of atherosclerosis. Interestingly, castrated normoglycemic ApoE$^{−/−}$ mice developed larger atherosclerotic lesions than sham—operated on controls. Ovariectomized ApoE$^{−/−}$:Ins2$^{+/+}$/Akita mice presented chronic hyperglycemia, and atherosclerosis appeared to be advanced. We have characterized the distinctive sex-specific phenotypes exhibited by the ApoE$^{−/−}$:Ins2$^{+/+}$/Akita mouse model and present evidence for the action of sex hormones on pancreatic β-cell function and the vasculature that affect the regulation of blood glucose levels and the development of atherosclerosis. This model will provide a test bed to further delineate these effects. (Am J Pathol 2016, 186: 67–77; http://dx.doi.org/10.1016/j.ajpath.2015.09.009)

A strong correlation exists between diabetes mellitus (DM) and the prevalence and severity of large-vessel disease. Angiographic and autopsy studies have reported that, compared with nondiabetic patients, individuals with diabetes have a greater degree of both high-grade and subclinical atherosclerosis, the underlying cause of most coronary artery disease.¹,² DM is associated with a cardiovascular disease mortality rate that exceeds 70%, and diabetic patients have a twofold to fourfold increased risk of dying from myocardial infarction or stroke.³

Our understanding of the molecular mechanisms that link diabetes to the accelerated development and progression of atherosclerosis is complicated by the interdependence of circulating glucose and insulin concentrations, as well as the close association of DM with other cardiovascular risk factors, including dyslipidemia, obesity, and hypertension.⁴ To facilitate the investigation and identification of the relevant molecular mechanisms and pathways, several different mouse models of diabetes-induced atherosclerosis have been developed. Most of these models involve the induction of hyperglycemia, in the presence or absence of insulin resistance, in dyslipidemic, atherosclerosis-prone apolipoprotein E−deficient (ApoE$^{−/−}$) or low-density lipoprotein receptor−deficient mice.⁵

The most common, and direct, strategy to induce hyperglycemia in rodents and other experimental animals has been

Supported by the Canadian Institutes of Health Research (CIHR, operating grant number: MOP-142248) and the Canadian Diabetes Association (CDA, operating grant number: OG-3-12-3852-GW), and the Comisión Nacional de Investigación Científica y Tecnológica (Chile) scholarship (D.E.V.-P.). Disclosures: None declared.
through a single, or multiple, i.p. injection of streptozotocin (STZ). STZ is a DNA-alkylating agent that is selectively toxic to pancreatic β cells, resulting in severe insulinopenia. Many studies have shown that STZ-induced hyperglycemia can promote atherosclerosis in adult mice. However, there are several disadvantages associated with the use of STZ, including possible toxicity to other tissues and organs, the severity of the hyperglycemia, and the potential for subsequent β-cell regeneration.

The Ins2+/Akita mouse represents a genetic alternative to chemically induced hyperglycemia. This mouse carries a point mutation (C96Y) in one allele of the insulin 2 gene, which disrupts a disulfide bond between the insulin A and B chains. The resulting proinsulin polypeptide cannot be properly processed and, therefore, accumulates, causing endoplasmic reticulum (ER) stress and β-cell dysfunction. It has been postulated that ER-stress–induced β-cell death is the major cause of insulinopenia in Ins2+/Akita mice. Subsequent studies have suggested that the formation of mutant proinsulin-derived aggregates sequesters the wild-type proinsulin, leading to ER retention and degradation of mutant and wild-type proinsulin, as the underlying cause of the decrease in circulating insulin and the development of hyperglycemia.

We have independently generated and extensively characterized an ApoE−/−:Ins2+/Akita mouse strain in a C57BL/6J genetic background. Herein, we show that glucose regulation and atherosclerotic progression vary significantly between male and female ApoE−/−:Ins2+/Akita mice. We use castration and ovariectomy to expose the roles played by sex hormones in the development of hyperglycemia and atherosclerosis.

### Materials and Methods

#### Mice

Male ApoE−/−:Ins2+/Akita mice (Jackson Laboratory, Bar Harbor, ME) were crossed with female ApoE−/−:Ins2+/+/ mice. The resulting male ApoE−/−:Ins2+/Akita offspring were crossed with female ApoE−/−:Ins2+/+ mice. The male ApoE−/−:Ins2+/Akita offspring produced from this cross and female ApoE−/−:Ins2+/+ were set up as breeding pairs to produce the ApoE−/−:Ins2+/Akita and control ApoE−/−:Ins2+/+ littermates that were used in the following experiments. Genotypes were confirmed by PCR using primers specific for Ins2 and ApoE genes (Supplemental Figure S1). Primers used were as follows: Ins2, 5′-TGCTGGATGCGCCTGCTGTC-3′ (forward) and 5′-GGTTCCCATATGCACTGAGC-3′ (reverse); ApoE, 5′-GGTTCCCTGCTGGACTGAGC-3′ (forward); wild type, 5′-TGCTGGATGCGCCTGCTGTC-3′ (forward); and knockout, 5′-GGTTCCCATATGCACTGAGC-3′ (reverse). The restriction enzyme Fnu4HI was used to identify the presence of the Ins2Akita mutation. All of the mice used in this study were fed a standard chow diet (2018 Teklad Global 18% Protein Rodent Diet; Harlan Teklad, Madison, WI) ad libitum with free access to water. Atherosclerosis was assessed at 5, 15, and 25 weeks of age in male ApoE−/− (n = 25), male ApoE−/−:Ins2+/Akita (n = 25), female ApoE−/− (n = 21), and female ApoE−/−:Ins2+/Akita (n = 25) mice. For castration and ovariectomy experiments, mice were stratified by glucose levels and body weight, and then randomly allocated into each group. All of the animal procedures were approved by the McMaster University Animal Research Ethics Board.

#### Castration

Five-week-old mice were anesthetized with isoflurane and administered a dose of buprenorphine (100 µL of a 0.015 mg/mL solution) for pain control. A 5-mm incision was made through the skin along the midline of the scrotal sac. After crossing the skin, another 5-mm incision was made on the left side of the scrotal sac membrane. The testis was then dissected. The incision made on the scrotal sac membrane was then sutured. This process was repeated to remove the right testis (n = 12 ApoE−/− and 12 ApoE−/−:Ins2+/Akita mice). Identical incisions were made on the sham–operated mice, but testicles were not removed (n = 9 ApoE−/− and 8 ApoE−/−:Ins2+/Akita mice). After surgery, mice recovered on a heating bed, to stabilize corporal temperature, and were returned to sterile cages where they were monitored daily.

#### Ovariectomy

Five-week-old mice were anesthetized with isoflurane and administered a dose of buprenorphine for pain control. Hair was removed from the incision site, and skin was cleaned with 70% ethanol. A 10-mm lateral incision was made through the skin of the left side of the back. After crossing the skin, another 10-mm incision was made to cross the muscle layer. The ovary and oviduct were located, and the ovary was removed. Then, the muscle and skin incisions were sutured. The process was repeated to remove the right ovary (n = 8 ApoE−/− and 9 ApoE−/−:Ins2+/Akita mice). Identical incisions were made on the sham–operated mice, but ovaries were not removed (n = 7 ApoE−/− and 9 ApoE−/−:Ins2+/Akita mice). After surgery, mice recovered on a heating bed, to stabilize corporal temperature, and were returned to sterile cages where they were monitored daily.

#### Harvesting and Storing

Mice were anesthetized with isoflurane, and blood was extracted. Mice were sacrificed by cervical dislocation, and the vasculature was rinsed with 5 mL of 0.9% saline. Liver and fat pad were removed, and the vasculature was perfusion fixed with 10% neutral-buffered formalin. The heart, pancreas, and aorta were extracted. All of the...
organs were stored in 10% neutral-buffered formalin at room temperature.

**Analysis of Atherosclerosis**

Paraffin-embedded hearts were divided into sections with a microtome, and serial sections (4.5 μm thick) from the aortic sinus were collected onto glass slides until atherosclerotic lesions were no longer observed. Sections were stained with hematoxylin and eosin (Sigma, St. Louis, MO), and lesion areas and volumes were determined, as previously described.\(^{20}\) The necrotic core of the atherosclerotic lesions was estimated by Masson’s trichrome staining (Sigma).\(^{21}\) Calcified lesion areas were detected by von Kossa staining.\(^{22}\) Smooth muscle cells and macrophage/foam cells were quantified in the atherosclerotic lesions by immunofluorescence staining for α-actin (Santa Cruz Biotechnology, Santa Cruz, CA) and Mac-3 (BD Pharmingen, San Diego, CA), respectively. All images were captured with an Olympus BX41 microscope (Olympus) mounted onto an Olympus BX41 digital camera (Olympus Imaging, Center Valley, PA) and assessed using ImageJ software version 1.48v (NIH, Bethesda, MD; http://imagej.nih.gov/ij).

**En Face Aortas**

The fixed aortas were cleaned of surrounding muscle and adventitial fat. They were longitudinally opened and stained for lipid content with Sudan IV (Sigma). Images of the whole aorta were captured using a L320 digital camera (Nikon, Mississauga, ON, Canada), and the percentage of the atherosclerotic area was assessed using ImageJ software.

**Analysis of Pancreata**

Paraffin-embedded pancreata were divided into sections (6 μm thick), and consecutive sections were collected onto glass slides. The antibody against GADD153 (growth arrest and DNA damage-inducible protein; Santa Cruz Biotechnology, Santa Cruz, CA) was used to detect the percentage of cells and quantified by von Kossa staining.\(^{22}\) Smooth muscle cells and macrophage/foam cells were quantified in the atherosclerotic lesions by immunofluorescence staining for α-actin (Santa Cruz Biotechnology, Santa Cruz, CA) and Mac-3 (BD Pharmingen, San Diego, CA), respectively. All images were captured with an Olympus DP71 digital camera (Olympus Imaging, Center Valley, PA) mounted on a Leitz Laborlux S bright-field microscope (Leica Microsystems, Concord, ON), and assessed using ImageJ software version 1.48v (NIH, Bethesda, MD; http://imagej.nih.gov/ij).

**Analysis of Plasma**

Nonfasting and fasting (6 hours) blood glucose levels were measured using a glucometer (One Touch Ultra; Life Scan, Burnaby, BC, Canada). Plasma lipid levels were determined in nonfasting and fasting conditions using the colorimetric diagnostic kit for total cholesterol and triglyceride (Thermo Scientific, Middletown, VA). Fasting plasma lipoproteins were separated by fast protein liquid chromatography, and total cholesterol was measured in each fraction. Enzyme-linked immunosorbent assay kits were used to measure insulin (Crystal Chem, Downers Grove, IL), estradiol (Calbiotech, Spring Valley, CA), progesterone, and testosterone (ALPCO Diagnostics, Salem, NH) under fasting conditions.

Surrogate indexes of β-cell function, insulin resistance, and sensitivity were calculated from fasting blood glucose and plasma insulin concentrations as follows:

\[ \text{HOMA} \% \beta = \frac{[(20 \times I_0)/(G_0 - 3.5)]}{(1)} \]

where HOMA is Homeostasis Model Assessment, \( G_0 \) is fasting glucose (mmol/L), and \( I_0 \) is fasting insulin (μIU/mL);\(^{23}\)

\[ \text{HOMA IR} = \frac{[(G_0 \times I_0)/22.5]}{(2)} \]

where HOMA is Homeostasis Model Assessment, IR is Insulin Resistance, \( G_0 \) is fasting glucose (mmol/L), and \( I_0 \) is fasting insulin (μIU/mL); and\(^{24}\)

\[ \text{QUICKI} = \frac{1}{[\log(I_0) + \log(G_0)]} \]

where QUICKI is Quantitative Insulin Sensitivity Check Index, \( I_0 \) is fasting insulin (μIU/mL) and \( G_0 \) is fasting glucose (mg/dL).\(^{24,25}\)

**OGTT and PITT**

Independent groups of mice were prepared for the oral glucose tolerance test (OGTT) and the peritoneal insulin tolerance test (PITT) in male \( ApoE^{-/-} \) (\( n = 11 \)) and \( ApoE^{-/-}:Ins2^{+/+}\) /Akita (\( n = 15 \)) or female \( ApoE^{-/-} \) (\( n = 12 \)) and \( ApoE^{-/-}:Ins2^{+/+}\) /Akita (\( n = 12 \)) mice. Twenty-week-old mice were fasted for 4 hours and then blood samples were collected from the tail vein. After the first collection (time 0), glucose (2 g/kg body weight of a 200 mg/mL solution; Sigma) was administrated by oral gavage, and blood samples were collected at time points of 15, 30, 60, and 120 minutes. Glucose levels were determined using a colorimetric glucose assay reagent (Sigma).

For PITT, 20-week-old mice were fasted for 4 hours and then blood glucose levels were measured with a glucometer (time 0). After the first measurement, bovine insulin (Sigma) was then injected i.p. (0.75 U/kg body weight of a 200 nU/mL solution) and blood glucose was measured at time points of 15, 30, 60, and 120 minutes.

**Data Analysis**

Data were analyzed by \( t \)-test to compare two groups. To compare multiple groups, one- or two-way analysis of variance was used, followed by the Bonferroni multiple comparison test between all groups. Data are expressed as arithmetic means ± SEM. For all experiments, \( P < 0.05 \) was considered statistically significant.
Results

Sex-Specific Effects on Hyperglycemia in ApoE<sup>+</sup>−/−:Ins2<sup>+/−</sup>Akita Mice

Male and female ApoE<sup>+</sup>−/−:Ins2<sup>+/−</sup>Akita mice developed hyperglycemia spontaneously by 5 weeks of age relative to age-matched ApoE<sup>−/−</sup> controls. Nonfasting blood glucose levels for 5-week-old male mice were 20.4 ± 1.1 versus 8.7 ± 0.4 mmol/L (P < 0.001), respectively. For female mice, blood glucose levels were 18.0 ± 1.1 versus 8.8 ± 0.2 mmol/L (P < 0.001). Hyperglycemia was sustained for the entire lifespan of the male mice. The blood glucose levels of female ApoE<sup>+</sup>−/−:Ins2<sup>+/−</sup>Akita mice normalized by 15 weeks of age (Figure 1A). Normalization of blood glucose in female ApoE<sup>+</sup>−/−:Ins2<sup>+/−</sup>Akita mice was at least partially dependent on ApoE deficiency because blood glucose levels in heterozygous ApoE<sup>+</sup>−/−:Ins2<sup>+/−</sup>Akita females remained significantly elevated at 15 weeks of age (Supplemental Figure S2).

OGTT and PITT were performed at 20 weeks of age. Male ApoE<sup>+</sup>−/−:Ins2<sup>+/−</sup>Akita mice were clearly glucose intolerant (Figure 1B) and also exhibited an impaired response to exogenous insulin (Figure 1C). Female ApoE<sup>+</sup>−/−:Ins2<sup>+/−</sup>Akita mice showed marginally impaired glucose tolerance and normal insulin sensitivity (Figure 1, B and C). In addition to chronic hyperglycemia, male ApoE<sup>+</sup>−/−:Ins2<sup>+/−</sup>Akita mice also developed enhanced hypercholesterolemia (Figure 1D), but showed no defined changes in triglycerides (Figure 1E) by 25 weeks of age. There were no differences in plasma lipid levels in female ApoE<sup>+</sup>−/−:Ins2<sup>+/−</sup>Akita mice relative to ApoE<sup>−/−</sup> controls (Figure 1, D and E).

At the end of the study, the average body weight of the hyperglycemic male mice was 5 g less than male
ApoE<sup>−/−</sup> controls (Figure 1F). The hyperglycemic male mice had virtually no epididymal fat pad (Figure 1G). There was no significant difference in liver weight between the male hyperglycemic mice and controls (data not shown). Transiently hyperglycemic ApoE<sup>−/−</sup>:Ins2<sup>+/−</sup>/Akita and normoglycemic ApoE<sup>−/−</sup> female mice presented similar metabolic parameters throughout the study (Figure 1, F and G).
Accelerated Atherosclerosis in ApoE<sup>−/−</sup>:Ins2<sup>+/Akita</sup> Mice

Atherosclerosis was analyzed in 5-, 15-, and 25-week-old male and female ApoE<sup>−/−</sup> and ApoE<sup>−/−</sup>:Ins2<sup>+/Akita</sup> mice. No atherosclerotic lesions were detected in any of the 5-week-old mice (data not shown). Atherosclerotic lesions in the aortic sinus of 15-week-old male ApoE<sup>−/−</sup>:Ins2<sup>+/Akita</sup> mice were relatively small and not significantly different compared with controls (Supplemental Figure S3A). However, the examination of the whole aortas revealed an increase of lipid accumulation, predominantly in the aortic arch of the hyperglycemic male mice (Supplemental Figure S3B). At 25 weeks of age, hyperglycemic male ApoE<sup>−/−</sup>:Ins2<sup>+/Akita</sup> mice presented with advanced atherosclerosis when compared with age- and sex-matched normoglycemic ApoE<sup>−/−</sup> controls. Atherosclerotic lesion volume from male ApoE<sup>−/−</sup>:Ins2<sup>+/Akita</sup> mice was at least 4× larger (P < 0.001) in the aortic sinus (Figure 2A). Similarly, en face aortas from male ApoE<sup>−/−</sup>:Ins2<sup>+/Akita</sup> mice showed 3× (P < 0.05) more atherosclerotic lesion area (Figure 2B).

Transiently hyperglycemic female ApoE<sup>−/−</sup>:Ins2<sup>+/Akita</sup> mice developed accelerated atherosclerosis in the aortic sinus compared with female ApoE<sup>−/−</sup> controls, at 15 weeks of age (P < 0.01) (Figure 2C). A similar trend was observed when the whole aortas were examined for lipid accumulation (Figure 2D). Lesion volume in the aortic sinus (Supplemental Figure S3C) and lipid accumulation in the descending aorta (Supplemental Figure S3D) increased in female ApoE<sup>−/−</sup> and ApoE<sup>−/−</sup>:Ins2<sup>+/Akita</sup> mice by 25 weeks of age; however, there were no longer statistically significant differences in area or volume.

Ovx ApoE<sup>−/−</sup>:Ins2<sup>+/Akita</sup> Mice Develop Chronic Hyperglycemia

To further examine the effects of sex, ApoE<sup>−/−</sup>:Ins2<sup>+/Akita</sup> and ApoE<sup>−/−</sup> mice were ovariec tomized (Ovx) at 5 weeks of age. Sham—operated on female ApoE<sup>−/−</sup>:Ins2<sup>+/Akita</sup> and ApoE<sup>−/−</sup> mice were used as controls. In contrast to the transient hyperglycemia observed in female ApoE<sup>−/−</sup>:Ins2<sup>+/Akita</sup>—sham mice, Ovx ApoE<sup>−/−</sup>:Ins2<sup>+/Akita</sup> mice remained hyperglycemic for the duration of the study (Figure 3A). Examination of the pancreatic islets of 25-week-old mice showed that ovariec tomization resulted in a significant increase of GADD153-stained nuclei (40% stained nuclei per islet) (Figure 3B). Consistent with this finding, β-cell function, evaluated by HOMA %β (Supplemental Figure S4A), and the glucose-induced insulin secretion (Supplemental Figure S5) were impaired in the Ovx group. HOMA IR and QUICKI indexes were also significantly altered in the Ovx ApoE<sup>−/−</sup>:Ins2<sup>+/Akita</sup> mice relative to sham—operated on controls (Figure 3C and Supplemental Figure S4B). Ovariec tomization had no significant effect on fasting blood glucose levels, GADD153 expression, insulin sensitivity, or β-cell function in the ApoE<sup>−/−</sup> control mice (Figure 3, A–C, and Supplemental Figure S4, A and B).

Examination of other metabolic parameters showed no significant differences in body weight, fat pad, liver weight, or plasma lipid levels between 25-week-old ApoE<sup>−/−</sup>:Ins2<sup>+/Akita</sup> and ApoE<sup>−/−</sup>:Ins2<sup>+/Akita</sup>—sham mice (Figure 3, D–G, and Supplemental Figure S6A). Twenty-five-week-old ApoE<sup>−/−</sup>:Ovx mice showed a significant increase of body weight with respect to all of the other groups (Figure 3D). This increase of body weight was accompanied...
by an increase in epididymal fat pad (Figure 3E) but no changes in liver weight (Supplemental Figure S6A). ApoE−/−−/Ovx mice also had increased fasting plasma cholesterol levels (Figure 3F) that correlated with an elevation of the very-low-density lipoprotein (VLDL)-cholesterol and LDL-cholesterol peaks (Supplemental Figure S7A). As expected, Ovx mice had significantly reduced plasma estrogen (estradiol) and progesterone levels compared with sham-operated on controls (Supplemental Figure S8, A and B).

**Ovx ApoE−/−−:Ins2+/Akita Mice Develop Advanced Atherosclerosis**

No differences in atherosclerosis were observed at the aortic sinus among 25-week-old Ovx and sham-operated on mice (Figure 4A, Supplemental Figure S9A, and Supplemental Figure S10A). However, the atherosclerotic lesions found in 25-week-old ApoE−/−−:Ins2+/Akita-Ovx mice showed a trend toward larger necrotic cores (Figure 4B) and increased artery calcification (Figure 4C). Similarly, examination of *en face* aortas showed that 25-week-old ApoE−/−−:Ins2+/Akita-Ovx mice presented with an increased atherosclerotic area in the descending aorta (Figure 4D).

**Castration Reduces Blood Glucose Levels in ApoE−/−−:Ins2+/Akita Mice**

Male ApoE−/−−:Ins2+/Akita and ApoE−/−− mice were castrated (Cx) at 5 weeks of age. After the surgery, ApoE−/−−:Ins2+/Akita-Cx mice presented with a moderate, but significant, decrease in fasting glucose levels when compared with male ApoE−/−−:Ins2+/Akita-sham mice. At 25 weeks of age, this reduction in blood glucose was no longer observed (Figure 5A). In 25-week-old male ApoE−/−−:Ins2+/Akita mice, castration did not alter the percentage of GADD153-positive nuclei in pancreatic islets (Figure 5B). β-Cell function, evaluated by HOMA %β index, was significantly impaired in both the sham—operated on and castrated ApoE−/−−:Ins2+/Akita mice (Supplemental Figure S4C). However, castration did result in improved HOMA IR and

---

*Figure 4* Ovariectomized (Ovx) apolipoprotein E–deficient (ApoE−/−−:Ins2+/Akita) mice have more advanced atherosclerotic lesions than sham-operated on controls. Atherosclerotic lesion area and volume (A), necrotic core (B), calcification in the aortic sinus (C), and lipid accumulation in *en face* aortas (D) were quantified in 25-week-old female mice. *P < 0.05* ApoE−/−−:Ins2+/Akita-sham versus ApoE−/−−:Ins2+/Akita-Ovx. N = 7 to 9 per group (A–D). AUC, area under the curve.
QUICKI indexes (Figure 5C and Supplemental Figure S4D). In ApoE−/− mice, castration had no effect on fasting blood glucose levels, the percentage of GADD153-positive nuclei, insulin resistance or sensitivity, and β-cell function (Figure 5, A–C, and Supplemental Figure S4, C and D).

Castration did not affect body weight of 25-week-old ApoE−/−/Ins2+/Akita-Cx mice (Figure 5D), but they recovered the capability to store fat in the abdominal cavity (Figure 5E). In addition, these mice presented a decrease of liver weight and show indications of advanced atherosclerosis. No changes in plasma lipids were detected (Figure 5, F and G, and Supplemental Figure S7B). Castration effectively reduced plasma testosterone levels. However, testosterone levels were already significantly reduced in the hyperglycemic ApoE−/−/Ins2+/Akita−sham relative to normoglycemic ApoE−/−-sham mice (Supplemental Figure S8C).

Atherosclerosis Is Delayed in Castrated ApoE−/−/Ins2+/Akita−Mice

At 25 weeks of age, the atherosclerotic lesions in the castrated ApoE−/−/Ins2+/Akita− mice were significantly smaller at the aortic sinus (Figure 6A). In addition, these lesions were less advanced, having smaller necrotic cores (Figure 6B), less calcification (Figure 6C), and a reduction in the number of smooth muscle cells but not macrophages (Supplemental Figure S9B and Supplemental Figure S10B), relative to the sham—operated on ApoE−/−/Ins2+/Akita− mice. Descending aortas of ApoE−/−/Ins2+/Akita−Cx mice had significantly less atherosclerosis relative to sham—operated on controls (Figure 6D). Conversely, the castration in the ApoE−/− mice significantly accelerated the development of atherosclerosis (Figure 6, A and B, and Supplemental Figure S9B).

Discussion

Herein, we have characterized the sex-specific differences of the ApoE−/−/Ins2+/Akita− mouse model of hyperglycemia-accelerated atherosclerosis. Specifically, male ApoE−/−/Ins2+/Akita− mice develop chronic hyperglycemia and significantly accelerated atherosclerosis by 25 weeks of age. Castration slows the development of atherosclerosis in ApoE−/−/Ins2+/Akita− mice but enhances disease progression in the normoglycemic controls. Female ApoE−/−/Ins2+/Akita− mice are only transiently hyperglycemic, with glucose levels normalizing by 15 weeks of age. Still, transiently hyperglycemic female mice develop accelerated atherosclerosis. Ovx ApoE−/−/Ins2+/Akita− mice are chronically hyperglycemic and show indications of advanced atherosclerosis.

Differences in the incidence and the prevalence of DM between sexes do exist. In fact, sex differences in prevalence extend to dyslipidemia, obesity, hypertension, atherosclerotic development, and the risk of myocardial infarction. Premenopausal women have a lower incidence of DM, and evidence suggests that estrogen protects against pancreatic β-cell failure in rodent models. Other studies have focused on the multiple benefits of androgens in men. Testosterone levels tend to decrease with age, and this decrease could explain the increased prevalence of age-related disease. In the STZ-hyperglycemic male rats, the lack of circulating insulin leads to the decrease of the circulating pituitary hormones, follicle-stimulating hormone and luteinizing...
hormone, which has a direct impact in the availability of testosterone. Similarly, in human males, an inverse correlation between testosterone levels and type 2 DM has been described, where an increase in the insulin resistance is associated with a deficiency of testosterone.

We observe that castration significantly accelerates the development of atherosclerosis in normoglycemic ApoE−/−:Ins2+/Akita mice, which is consistent with a protective vascular effect of androgens. When the Ins2Akita mutation is incorporated into the male ApoE−/− mice, we and others have observed the early onset of chronic hyperglycemia, enhanced hypercholesterolemia, and accelerated progression of atherosclerosis. We also observed a significant reduction in insulin sensitivity. In these hyperglycemic mice, the testosterone levels are significantly lower than in normoglycemic controls. This is consistent with previous observations in hyperglycemic rodents and humans. Decreased testosterone combined with chronic hyperglycemia and enhanced hypercholesterolemia would exacerbate the development of atherosclerosis. Interestingly, when the castration is performed in the hyperglycemic ApoE−/−:Ins2+/Akita mice, atherosclerosis is attenuated. This effect may be explained by the corresponding reduction in blood glucose and non–high-density lipoprotein-cholesterol levels and the improved insulin sensitivity associated with castration. Together, these results support a model in which testosterone confers vascular protection in the male normoglycemic ApoE−/−:Ins2+/Akita mice, but in diabetic mice, testosterone appears to exacerbate hyperglycemia, hypercholesterolemia, and ultimately atherosclerosis. Further investigations are required to fully delineate the underlying mechanisms of these effects.

Female Ins2+/Akita mice have been reported to be hyperglycemic, but with lower blood glucose levels than male Ins2+/Akita mice. In this study, we show that, by 15 weeks of age, blood glucose levels in female ApoE−/−:Ins2+/Akita mice are similar to ApoE−/− controls. This phenomenon is partially dependent on ApoE deficiency because significant hyperglycemia is sustained at 15 weeks of age in heterozygous ApoE−/−:Ins2+/Akita females. The underlying mechanisms by which female (but not male) mice can produce functional insulin in the presence of the Ins2Akita mutation have not been explained. We show that female ApoE−/−:Ins2+/Akita mice have significantly less β-cell stress (indicated by GADD153 staining) compared with males. We also demonstrate that the transient hyperglycemia...
in female ApoE−/−:Ins2+/Akita mice does not exacerbate the already dyslipidemic profile of ApoE−/− mice, but does promote accelerated atherosclerosis. Ovariectomy of ApoE−/−:Ins2+/Akita mice induces sustained hyperglycemia, effectively abolishing the observed protection from β-cell stress (chronically induced by the Ins2Akita mutation) in the pancreatic β cells and reducing the ability to produce functional insulin. The atherosclerotic lesion volumes of the Ovx ApoE−/−:Ins2+/Akita mice are similar to those observed in the sham-operated on ApoE−/−:Ins2+/Akita and the ApoE−/− mice, but specific characteristics of the lesions appear to be advanced in addition to the lipid accumulation in the whole aortas.

The effects of estrogen on pancreas and the vasculature have been elegantly demonstrated in animal models and in vitro studies. However, the protective effects of estrogen replacement therapy in menopausal women are questioned because of disparate results and the potential cancerogenic effects of estrogens. Finan et al have recently overcome these obstacles and repositioned estrogens as a real alternative to the treatment of the metabolic syndrome.

In conclusion, we have characterized the sex-specific differences of the ApoE−/−:Ins2+/Akita mouse model of hyperglycemia-accelerated atherosclerosis. By using this model, we have shown that the hormonal component from each sex is playing an important role in diabetes and the development of atherosclerosis by affecting the function of the pancreas as well as pathogenesis in the artery wall. More important, in this model, testosterone can have atheroprotective or proatherogenic effects, depending on the glycemic status of the mouse. These findings may have important clinical implications with regard to androgen replacement therapies in the context of chronic disease.

**Supplemental Data**

Supplemental material for this article can be found at http://dx.doi.org/10.1016/j.ajpath.2015.09.009.

**References**


4. Steinberger J, Daniels SR: American Heart Association Atherosclerosis, Hypertension, and Obesity in the Young Committee (Council on Cardiovascular Disease in the Young); American Heart Association Diabetes Committee (Council on Nutrition, Physical Activity, and Metabolism): Obesity, insulin resistance, diabetes, and cardiovascular risk in children: an American Heart Association scientific statement from the Atherosclerosis, Hypertension, and Obesity in the Young Committee (Council on Cardiovascular Disease in the Young) and the Diabetes Committee (Council on Nutrition, Physical Activity, and Metabolism). Circulation 2003, 107:1448–1453.


53. Khoo C, Campos H, Judge H, Sacks FM: Effects of estrogenic oral contraceptives on the lipoprotein B particle system de-