Effects of streptozotocin-induced diabetes in apolipoprotein AI deficient mice

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Abstract

During the past decade a number of investigators have attempted to develop mouse models of diabetic macrovascular disease. Hyperglycemia might increase vascular damage because it increases oxidant stress. For this reason we studied animals that were deficient in HDL; HDL is widely believed to protect against oxidant stress. An inbred line of mice doubly deficient in LDL receptor and apoAI was made diabetic with streptozotocin (STZ); control mice had an average glucose of 7.2 ± 2 mmol/L and STZ-treated mice had an average glucose of 19.4 ± 6.5 mmol/L. The animals were fed a high cholesterol but low fat diet leading to plasma cholesterol levels of 9.4 ± 1.6 mmol/L in control animals and 10.1 ± 1.8 mmol/L in STZ-treated mice. The control and STZ-treated animals had similar plasma lipoprotein profiles. Atherosclerosis assessed at 23 weeks averaged 38154 µm² in control and 32962 µm² in STZ-treated mice. Therefore STZ-induced diabetes does not alter plasma lipoproteins or atherosclerosis in HDL deficient mice.
Introduction

Despite the major advances in development of models for human diseases such as atherosclerosis and diabetes, an acceptable mouse model in which diabetes exacerbates atherosclerotic lesions is not available. Although there are a number of animals in which chemical or genetic induction of diabetes accelerates atherosclerosis, in most cases these models are associated with a more atherogenic lipoprotein profile. One such model is streptozotocin (STZ) treated apoE knockout mice \(^1\). Previously, we had attempted to reproduce the profile of increased VLDL and reduced HDL found in humans with diabetes, especially type 2 diabetes, by reducing lipoprotein lipase (LpL) and introducing a transgene for cholesteryl ester transfer protein\(^2\); most mice have triglyceride and VLDL levels that are much lower than those found in humans. More atherosclerosis was found after STZ treatment of LpL deficient mice, however, those animals had profound hyperlipidemia. Models of type 2 diabetes such as db/db \(^3\) or ob/ob \(^4\) mice that are deficient in the actions of leptin have elevated plasma lipid levels and, as expected, more vascular pathology.

In contrast, a number of investigators have failed to find an increase in atherosclerosis in diabetic mice. STZ-induced diabetes failed to increase lesion size in LDL receptor knockout \(^5\) and human apoB transgenic \(^6\) mice. When LDL receptor knockout mice were fed a high fat diet, the increased body weight, hyperinsulinemia, and hyperglycemia did not alter lesion size in these animals compared to a fructose fed group with similar plasma cholesterol. However, treatment of these animals with a PPAR\(\gamma\) agonist reduced atherosclerosis \(^7,8\). This effect was exclusive of any reduction of plasma glucose. In other mice that were studied after only 6 weeks on a high fat diet, LDL
receptor mice were found to have larger lesions but with a concomitant increase (>two fold) of cholesterol levels. Thus, it appears difficult to directly implicate hyperglycemia, exclusive of hyperlipidemia, as a cause of increased vascular disease in mice.

Hyperglycemia might be vasculotoxic due to a number of mechanisms: alteration of proteins by their irreversible association with glucose leading to advanced glycation endproducts (AGEs); direct effects of hyperglycemia on coagulation; and increased oxidant stress. Although antioxidant compounds may protect against oxidation, a major natural antioxidant is plasma HDL. We hypothesized that mice, especially those placed on a high fat diet, might be protected against the oxidant stress due to levels of plasma HDL that are much higher than those in the average human; over 100 mg/dl or >2mMol in western diet fed mice. We also speculated that since the ingestion of a high fat diet in control mice increases glucose to levels that are diabetic by human standards, studies using this diet only allowed a comparison between mice that were diabetic and those that were very diabetic. To assess the effects of hyperglycemia in low HDL mice with non-diabetic controls, we studied mice doubly deficient in LDL receptor and apoAI (LDLr0/AI0) that were treated with STZ and maintained on a low fat but high cholesterol diet.
Methods

Transgenic Mice and Diet: Mice deficient of both LDL receptor and apo AI were generated by crossing LDL receptor deficient mice $^{14}$ with apo AI deficient mice $^{15}$. LDL receptor mice of the C57BL/B6 (B6) genetic background were purchased from the Jackson Laboratory. Apo AI deficient mice were of mixed genetic background as described previously $^{16}$. LDL receptor and apo AI genes are on mouse chromosome 9 $^{17,18}$, which are approximately 22 cM apart (http://www.ncbi.nlm.nih.gov). A large number of animals were generated to obtain animals homozygous for both LDL receptor and apo AI null alleles.

Mice were maintained in a temperature-controlled (25°C) facility with a 12-h light/dark cycle and given free access to food and water, except when fasting blood specimens were obtained. The mice were fed either standard laboratory rodent chow (Purina P-5001, address) or an enhanced cholesterol diet (TD 99479, Harlan Teklad, Madison, WI). The cholesterol diet was comprised of 995.2 g/Kg of standard chow and 4.8 g/Kg of cholesterol. This yielded a diet containing approximately 0.5% cholesterol. Both diets were free of sodium cholate.

Blood samples were obtained from mice anesthetized with halothane (halocarbon Laboratories, River Edge, NJ) via retroorbital phlebotomy utilizing heparinized capillary tubes (VWR 15401-628). Samples were collected in tubes containing 1mM EDTA as an anticoagulant. Blood was obtained at 8, 12, 16, 20 and 23 weeks of age (shown by the arrows in figure 1).

The initial mice used for this study were screened by polymerase chain reaction to insure that they were homozygous for the deficiency of both LDL receptor and apoAI.
The methods for this are contained in the following references: for LDL receptor knockout \(^{14}\), for apoAI knockout \(^{16}\).

**Mouse genotype analysis:** To determine the proportion of C57BL/6J alleles in our mouse colony we genotyped five randomly selected individual animals using 100 microsatellite markers spaced at the average of \(~15\text{cM}\) across the genome (chromosome Y excluded). Tail tip DNA samples were purified by phenol/chloroform/isoamyl alcohol precipitation and then subjected to PCR using fluorescently labeled primers. The PCR products were analyzed by capillary electrophoresis using the ABI 3700 DNA sequencer. PCR reactions and electrophoresis were robotically automated (Tecan, Genesis RST 100 and Robbins Scientific, Hydra 384 robots) and carried out by the Genotyping Core Facility at the Rockefeller University. Allele scores were analyzed using the ABI Genotyper 3.6 NT software. The genotyping results revealed that, overall, 74.9\% of the alleles in these animals correspond to the C57BL/6J genotype with 50\% of the markers being homozygous for the C57BL/6J allele.

**Diabetes:** Diabetes was induced by streptozotocin (STZ) treatment similar to that described by Kako et al. \(^{6}\) and Kunjathoor et al. \(^{19}\). Male mice were divided into two groups; half were treated with STZ (Sigma Chemical Co., St. Louis, MO). STZ was suspended in a sterile 0.05 M citrate buffer (4 mg/mL) and used within ten minutes of preparation. The solution, injected intraperitoneally at a dosage of 50 mg/Kg, was given for five consecutive days at 8 weeks of age. Control animals received similar injections of citrate buffer only to ensure comparable handling between the two groups. Mice were maintained on standard chow during the injection period and the following three weeks before being switched to the cholesterol diet at 12 weeks of age (figure 1).
**Plasma glucose, lipids, fatty acids and insulin:** Plasma glucose levels were analyzed using an enzymatic kit (#315-100, Sigma Chemical Co., St. Louis, MO) and measured using a SpectraMax 250 Spectrophotometer. Cholesterol levels were determined using enzymatic kits (#402-20, Sigma Chemical Co., St. Louis, MO), as were triglyceride levels (#344-20, Sigma Chemical Co., St. Louis, MO). Insulin determination was done utilizing an ELISA kit (ALPCO, Windham, NH) and measured on a microtiter plate reader (SpectraMax 250, Molecular Devices Inc.).

Lipoproteins were separated via sequential density ultracentrifugation in a TLA-100 rotor (Beckman Instruments, Palo Alto, CA). 60 µl sample volumes were utilized; density ranges were as follows: HDL – d = 1.063-1.21 g/ml; LDL – d = 1.006-1.063 g/ml; and VLDL – d < 1.006 g/ml.

**Gel filtration chromatography:** Pooled plasma (from between 3 and 5 animals, 200 µl total volume) was chromatographed using two Superose 6 columns in series (FPLC; Pharmacia LKB Biotechnology Inc., Piscataway, NJ). Fifty 0.5 ml fractions were collected. Cholesterol and triglyceride levels of FPLC fractions were measured using enzymatic reagents (as in plasma analysis) in colorimetric assays and measured on the SpectraMax 250.

**Quantitative atherosclerosis analysis:** At 23 weeks of age, the mice were sacrificed. The animals were perfused with cold PBS (10 ml) via a catheter (27 gauge needle) inserted into the apex of the heart. The aorta was then removed, the upper third of the heart containing the aortic root was embedded in OCT compound (Sakura Finetek, Torrance, CA) and frozen at -80°C. The heart and aortic outflow tract was sectioned by microtome into 10µm thick sections taken at 80µm intervals. The staining method
utilized Oil Red O to stain for lipids, Harris’ hematoxylin to stain for nuclei and basophilic tissue, and counterstained with light green. Lesions of the proximal aorta were then quantified using ImagePro Plus software and mean lesion area was calculated.

**Statistical analysis:** Statistical analysis for the effects of STZ treatment was done by two tailed student t test.
Results

**STZ induced diabetes in LDLr0/AI0 mice:** For the purposes of this study, diabetes was defined as a plasma glucose concentration of >200 mg/dL (~11mmol/L). In our initial experiments, we treated both male and female animals. However, as noted previously female mice were largely unresponsive to STZ (<31% achieved our definition of diabetic). The female mice were, therefore, excluded from the experiments. Male mice responded to the treatment better; with experience approximately 80% of the mice injected achieved sustained plasma glucose levels greater than 11.1 mmol/L (220 mg/dL). Results of glucose analyses are shown in figure 2A. At 12 weeks (four weeks after injection), on standard rodent chow, the STZ-treated mice had glucose levels of 18.0 ± 4.6 mmol/L compared to control mice, whose glucose averaged 7.7 ± 3.8 mmol/L. Thus, we were able to achieve sustained hyperglycemia and, perhaps most importantly, the use of cholesterol unaccompanied by high fat did not lead to insulin resistant hyperglycemia as evidenced by the insulin levels described below.

**Insulin and fatty acids levels:** Insulin levels, as expected, were reduced in the STZ-treated mice. Plasma levels were too low to accurately assess. By comparison control animals averaged plasma insulin levels of 0.4 ng/mL. Fatty acids were 0.61 ± 0.14 mEq/L in control animals (n=5). Treated mice fatty acid levels were very similar, 0.65 ± 0.23 mEq/L.

**Effects on body weight:** The control and STZ mice had no differences in body weight at any of the time points. These results are summarized in figure 2B. Thus, the diabetes was not severe enough to affect the growth of the mice.
**Plasma lipid levels:** Plasma cholesterol values remained quite consistent between the two groups for the duration of the study. Initial cholesterol averaged $4.2 \pm 1.0 \text{ mmol/L}$ in both groups of mice. As shown in figure 2C, within 4 weeks of institution of the cholesterol-enriched diet cholesterol levels increased to $>9\text{ mM}$. It then remained relatively constant; the STZ mice had cholesterol levels of $10.1 \pm 1.8 \text{ mmol/L}$ at the endpoint, while control mice averaged $9.4 \pm 1.6 \text{ mmol/L}$. The enhanced cholesterol diet, while elevating plasma cholesterol levels by approximately 2.5 fold over animals on chow, did not cause the extreme levels of hyperlipidemia occasionally found in some STZ-treated atherosclerosis-prone mice (table 1). This, we expected, would increase our changes of seeing a glucose-toxic effect over that of the hyperlipidemia.

On chow diets triglyceride levels averaged $1.3 \pm 0.4 \text{ mmol/L}$. After STZ-treatments there were no statistically significant changes in triglyceride (figure 2D).

**Lipoproteins:** Lipoproteins separated by FPLC were not remarkably altered by STZ-treatment. While the mice were consuming chow, figure 3A and B, a LDL cholesterol peak was noted. Cholesterol was found within the usual elution volume of HDL, however, no distinct peak was seen. While on the cholesterol-enriched diet, figures 3C and D, VLDL and LDL peaks increased markedly. Most significantly, STZ did not lead to an alteration in the distribution profile; the increases in peak size in figure 3D versus 3C exaggerate the small differences in cholesterol between these mice.

Lipoproteins were also isolated by ultracentrifugation. In all mice, some cholesterol was recovered within the HDL density; this has previously been shown to be non-apoAI containing particles (Table 1). Cholesterol feeding caused a marked elevation
in VLDL and LDL levels in both groups, but no differences were observed between control and diabetic animals (Table 1).

**Atherosclerosis:** Atherosclerosis in the aortic root was not different between control and STZ-treated mice (figure 4). The average lesion size was $38,154 \pm 21,463 \mu m^2$ in control animals compared to lesions measuring $32,962 \pm 16,730$ in treated mice. The lesions in the aortic root contained lipid-rich areas but did not show any gross differences between the control and diabetic mice.
Discussion

A relationship between diabetes and atherosclerosis is firmly established in humans. Because both insulinopenic diabetics (type 1) and insulin resistant diabetics (type 2) have this complication, it is likely that a common abnormality, e.g. hyperglycemia, is etiologic. Because many of the toxic effects of hyperglycemia might be abrogated by HDL, we assessed this in HDL deficient mice. Our studies showed the following: 1) STZ induced stable hyperglycemia in LDLr0/apoAI0 mice, 2) use of a high cholesterol, but not high fat, diet increased plasma LDL without obesity or hyperglycemia, 3) plasma lipids were not altered by the diabetes, 4) atherosclerosis was equal in control and diabetic mice.

Although a number of animal models have been created to assess the effects of diabetes on atherosclerosis, a clear-cut model that will allow additional investigation of the toxic of effects diabetes on the vasculature is not available. In rabbits, diabetes leads to severe hypertriglyceridemia and the production of lipoproteins that are too large to enter the artery wall 20; atherosclerosis is reduced in diabetic cholesterol fed rabbits. In some models, the diabetes leads to increased plasma cholesterol and it is likely that the dyslipidemia is responsible for greater disease. This occurs in pigs 21, and several genetically modified strains of mice 1,2,4,9. Any conclusion from data on these types of studies is suspect, since the excess hyperlipidemia alone should exacerbate atherosclerosis. In fact the lack of accelerated disease in diabetic models that have no enhanced dyslipidemia 5,6, strongly argues that atherogenic processes purported to be due to hyperglycemic are more likely the result of lipid abnormalities.
One experimental issue in the use of diabetic mouse models has been the need to exacerbate the lipid abnormalities by the use of diets that contain high fat and cholesterol. In some genetically altered mice this has led to cholesterol levels of $>500$ mg/dl (12 mM). Such levels are rarely seen in humans and are certainly not representative of those in most diabetic humans. Moreover, such high cholesterol levels may be so toxic to the artery that they obscure any diabetes-derived toxicity. A second issue is the development of type 2 diabetes in the control, non-STZ-treated, mice placed on these diets. These diets produce control mice that are obese, insulin resistant, and hyperglycemic; therefore the experiments really compare diabetic versus very diabetic animals. By using a cholesterol-rich, but not high fat, diet, we show that atherosclerosis can be studied using a diet that does not produce type 2 diabetes in non-STZ treated mice.

There are limited data suggesting that diabetic patients with high HDL are protected against macrovascular complications. For this reason, we hypothesized the converse, that the toxic effects of diabetes would be evident in HDL deficient mice. Rather we observed no effect of hyperglycemia on the development of atherosclerotic lesions despite HDL deficiency.

These studies are similar to the observations of a number of other investigators. Kunjathoor et al. observed little effect of STZ on vascular lesions in C57BL/6, the major background of the mice used in this study. They did see small differences in BALB/c and their data have suggested that genotypic difference may underlie the lack of glucose-mediated atherosclerosis progression in mice. What genetic alteration is required to produce a diabetic-atherosclerosis mouse model is unknown?
Although microvascular complications are clearly related to glycemic control, why diabetes accelerates diseases of the large vessels is less clear. While past and ongoing human trials provide information related to the best therapeutic approaches to be used, animal models often provide an important way to develop pathophysiological relationships and direct new therapies. Thus, it has been disappointing that despite a large body of in vitro observations connecting hyperglycemia and potentially harmful phenomena, there are not well-developed animal models for diabetic vascular disease. If one assumes from the human clinical data that hyperglycemia is a cause of accelerated atherosclerosis, non-responsive rodent models might either lack an element needed for this response or they may have a protective factor. A likely candidate for the latter is the high HDL in the mouse, however, our studies show that this is not the case. HDL deficient hyperglycemic mice have the same amount of atherosclerosis as their normoglycemia counterparts. In the course of these experiments, however, we have developed dietary and experimental conditions for future investigations.
Acknowledgements

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Table 1: Lipid and lipoprotein levels in LDLr0/AI0 mice with and without streptozotocin treatment (mmol/L)

<table>
<thead>
<tr>
<th>Group</th>
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<th>Cholesterol</th>
<th>Triglyceride</th>
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<tr>
<td></td>
<td></td>
<td>Plasma</td>
<td>VLDL</td>
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<tr>
<td>Baseline</td>
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<td>4.22 ± 0.62</td>
<td>0.91 ± 0.26</td>
</tr>
<tr>
<td>CTR</td>
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<td>9.50 ± 1.67</td>
<td>3.34 ± 1.67</td>
</tr>
<tr>
<td>STZ</td>
<td>10</td>
<td>10.37 ± 1.32</td>
<td>3.47 ± 1.43</td>
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**Figure legends**

**Figure 1:** At 8 weeks of age, the mice were divided and half received streptozotocin (STZ), 50mg/kg body weight for one week via i.p injection. At 12 weeks, animals were switched to a cholesterol-enriched diet (0.5% cholesterol). As indicated by the heavy arrows, mice were bled at 4 week intervals. Aortas were harvested at 23 weeks.

**Figure 2:** Glucose, body weight, cholesterol, and triglyceride levels plotted versus age for control (■) and STZ-treated animals (▲).

**Figure 3:** Lipoprotein profiles by FPLC of control and STZ-treated mice. Plasma was pooled from 3-5 mice in each group and 200 µl was used. The recovered cholesterol in each fraction was assayed and the concentrations shown on the y-axis. Panels A and B are while the mice consumed a chow diet at age 12 weeks; A is control mice and B is from STZ-treated mice. Panels C and D depict control and STZ FPLC cholesterol profiles at 23 weeks, the study endpoint. These mice that consumed a high cholesterol diet, had cholesterol levels that were >2 fold greater and had more cholesterol in VLDL-sized particles.

**Figure 4:** Atherosclerosis assessed by Oil Red O staining of the aortic root. This scattergram shows atherosclerotic lesion areas for control (left side) and STZ-treated mice (right side). The horizontal bar indicates the average lesion size for each group.
FIGURE 1

Age (Weeks)

8 12 16 20 23

S T Z

.5% Cholesterol Diet
FIGURE 2.
FIGURE 3.
FIGURE 4.
References


(22) S. Merat, Casanada, F., Sutphin, M., Palinski, W. and Reaven, P. D. 1999. Western-type diets induce insulin resistance and hyperinsulinemia in LDL receptor-deficient mice but do not increase aortic atherosclerosis compared with normoinsulinemic mice in which similar plasma cholesterol levels are achieved by a fructose-rich diet. *Arterioscler Thromb Vasc Biol.** 19: 1223-1230.

