Dyslipidemia and Vascular Dysfunction in Diabetic Pigs Fed an Atherogenic Diet

J.L. Dixon, J.D. Stoops, J.L. Parker, M.H. Laughlin, G.A. Weisman, M. Sturek

Abstract—Diabetic patients typically have not only hyperglycemia but also dyslipidemia. Study of the pathogenic components of the diabetic milieu and mechanisms of accelerated atherosclerosis is hindered by inadequate animal models. A potentially suitable animal model for human diabetic dyslipidemia is the pig, because it carries a large fraction of total cholesterol in low-density lipoprotein (LDL), similar to humans. In this study, male Sinclair miniature pigs were made diabetic by destroying the insulin-producing cells of the pancreas with alloxan and then were fed a high fat and high cholesterol diet for comparison with pigs fed a nondiabetic high fat and high cholesterol diet and control pigs. Diabetic pigs exhibited hyperglycemia, but plasma urea nitrogen, creatinine, and transaminase levels were in the normal range, indicating no adverse effects on kidney and liver function. The lipoprotein profile in diabetic pigs was similar to that found in human diabetic patients and was characterized by hypertriglyceridemia (2.8-fold increase versus control and high fat–fed pigs) and a profound shift of cholesterol distribution into the LDL fraction (81%) versus the distribution in high fat–fed (64%) and control (57%) pigs. LDL particles were lipid-enriched and more heterogeneous in diabetic pigs. Apolipoprotein B was distributed among a much broader spectrum of LDL particles, and apolipoprotein E was partially redistributed from high-density lipoprotein to apolipoprotein B–containing lipoproteins in diabetic pigs. There was little change in apolipoprotein A-I distribution. Diabetic pigs showed several early signs of excess vascular disease. In diabetic pigs, 75% of the coronary artery segments showed contractile oscillations in response to prostaglandin F2α compared with 25% in high fat–fed pigs and 10% in control pigs. Endothelium-dependent relaxation of brachial arteries was nearly abolished in diabetic pigs but unchanged in high fat–fed versus control pigs. Carotid artery Sudan IV staining for fatty streaks was significantly increased only in diabetic pigs. This porcine model should provide insights into the etiology of human diabetic dyslipidemia and facilitate study of peripheral vascular and coronary artery disease in diabetic patients. (Arterioscler Thromb Vasc Biol. 1999;19:2981-2992.)

Key Words: Sinclair miniature swine ■ animal model ■ lipids ■ VLDL ■ LDL ■ HDL ■ cholesterol ■ triglycerides ■ endothelium ■ vascular smooth muscle ■ atherosclerosis ■ coronary arteries

Several comprehensive reviews emphasize the severity of vascular disease in diabetes.1-4 Diabetes mellitus is the most widespread disease in industrialized nations, afflicting nearly 6% of the population in the United States.5 Large-scale clinical and epidemiological studies indicate that diabetes increases the risk of developing cardiovascular disease 2- to 6-fold1,2 and that 80% of all type 2 diabetics will die of an atherosclerotic event.4 Although the association between excess macrovascular disease and prolonged diabetes has been well documented, pathophysiological mechanisms underlying this relation are not clear.

There is debate about the relative roles of hyperglycemia and dyslipidemia in the excess coronary artery disease (CAD) associated with diabetes.5 Whereas the Diabetes Control and Complications Trial (DCCT) indicated that blood glucose is highly predictive of microvascular disease,6 the contribution of all the commonly measured risk factors can explain no more than 25% of the excess macrovascular CAD associated with diabetes.7 Recent reviews indicate the need for reexamination of traditional hypotheses,8 and increasing attention is being paid to altered plasma lipoprotein profile in the excess atherosclerosis associated with diabetes. Importantly, the plasma lipoprotein profile may be most critical,9-11 because at any total cholesterol level, diabetic individuals have 3- to 5-fold higher CAD mortality rates than do nondiabetic individuals.10

Type 2 diabetic patients typically do not have increased levels of LDL cholesterol.11,12 Instead, a major characteristic of the dyslipidemia of type 2 diabetes is hypertriglyceridemia,11-14 reflecting increased levels of VLDL triglyceride.11,14 The dyslipidemic profile also includes increased VLDL remnants, increased apolipoprotein E in VLDL, increased small dense...
A good animal model for metabolic studies of diabetes. There is virtually uniform agreement that the atherosclerotic lesions in the later, established phases of early functional events that precede gross, structural atherosclerosis.

Because previous studies in our laboratory showed that there are only minor changes in plasma lipids and vascular function30,31 and impaired endothelium-dependent relaxation 30 are assessed altered vascular reactivity (dysfunction) because there is substantial evidence that increased vasoconstriction30,31 and impaired endothelium-dependent relaxation30 are early functional events that precede gross, structural atherosclerotic lesions in the later, established phases of atherosclerosis.

Methods

Experimental Design

The prevalence of insulin resistance in Western societies and the dietary habits that promote hyperlipidemia11 suggested to us that in our porcine model a high fat diet and the resulting hyperlipidemia may interact with impaired insulin-mediated actions to yield hyperglycemia and exacerbation of the lipoprotein profile.15 There are numerous molecular mechanisms for insulin resistance of the major insulin-responsive tissues, ie, skeletal muscle and adipose. In the present study, we impaired insulin action by simply decreasing plasma insulin levels by the destruction of pancreatic beta cells with alloxan.28,32,33 Alloxan-treated diabetic pigs (n=4) were fed a high fat, high cholesterol, atherogenic diet (Minipig chow supplemented with cholesterol, coconut oil, corn oil, and sodium cholate) as shown in Table 1. The term “diabetic” is used in the text for readability, but the figures have been labeled as “diabetic high fat” to indicate more clearly that the alloxan-treated group was fed the atherogenic diet. Because a previous study indicated that there were minimal effects of diabetes alone on plasma lipids,29 an alloxan-treated group on normal pig chow was not included in the present study. A second group of pigs was fed the atherogenic diet (high fat, n=4), and a third group (n=5) was fed only Minipig chow (Purina Mills, Inc). Pigs were fed twice daily and had free access to water. Supplements for the atherogenic diet were obtained from Research Diets.

Animals

All procedures involving animals were approved by the Animal Care and Use Committee of the University of Missouri and complied fully with those approved by the American Veterinary Medical Association Panel on Euthanasia. Male Sinclair miniature swine between 9 and 12 months of age (sexually mature) were obtained from the Sinclair Research Center (Columbia, Mo). Pigs were housed in a temperature-controlled room (20°C to 22°C) with a 12-hour light/dark cycle. Anesthesia was induced with the following drugs given intramuscularly (in mg/kg): atropine 0.05, ketamine 20, and xylazine 2; the level of anesthesia was subsequently maintained with isoflurane gas (up to 4%). A catheter was placed in an ear vein for blood sampling and alloxan (or vehicle) injection. Alloxan monohydrate (175 mg/kg, Aldrich Chemical Co, Inc) was added to 0.9% NaCl, and the pH was adjusted to 7.0 with NaOH to enable solubility and then sterilized filtered. The 40 to 60 mL of alloxan solution was then administered intravenously over a period of ~3 minutes.

The most critical time of care for the alloxan-treated pig was in the first 12 to 16 hours, when blood glucose levels fell to life-threatening

LDL, glycation of LDL, and decreased plasma HDL concentration. There is virtually uniform agreement that the highly abnormal lipid profile (diabetic dyslipidemia) in type 1 and type 2 diabetes should be highly athrogenic,1,2,3,9,14,16,17 but identification of the most critical components of the diabetic milieu that elicit excess vascular disease has been elusive.

It has long been appreciated that identification of pathogenic components of the diabetic milieu and our understanding of the mechanisms of excess vascular disease in diabetes have been limited by the lack of a suitable animal model.1,18,19 We have chosen the porcine model because it is widely accepted that pigs possess a cardiovascular system (particularly a coronary circulation) very similar to that of humans20,21 and that the chronic adaptations of the porcine coronary circulation in experimental CAD models are similar to those of human CAD patients.22,23 Importantly, the pig is a good model in which to study lipoprotein metabolism associated with hyperlipidemic diets,24–26 and alloxan- or streptozocin-treated pigs have been suggested by others to be a be a good animal model for metabolic studies of diabetes.27,28

Because previous studies in our laboratory showed that there were only minor changes in plasma lipids and vascular reactivity in diabetic pigs fed a conventional pig chow diet versus control pigs,29 in the present study we wished to compare diabetic pigs versus non-diabetic pigs when both were fed an experimental high fat, high cholesterol diet. We addressed 2 major criteria to determine the suitability of the porcine model: (1) the plasma lipid and apolipoprotein profile and (2) whether hyperglycemia and the altered lipid profile would result in excess vascular disease/dysfunction. We assessed altered vascular reactivity (dysfunction) because there is substantial evidence that increased vasoconstriction and impaired endothelium-dependent relaxation are early functional events that precede gross, structural atherosclerotic lesions in the later, established phases of atherosclerosis.

<table>
<thead>
<tr>
<th>Composition of Diets</th>
<th>Control Diet</th>
<th>High Fat/Cholesterol Diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein (corn, alfalfa, soy, oats)</td>
<td>16.7</td>
<td>22</td>
</tr>
<tr>
<td>Carbohydrate</td>
<td>53.2</td>
<td>70</td>
</tr>
<tr>
<td>Fiber</td>
<td>14.2</td>
<td>...</td>
</tr>
<tr>
<td>Ash</td>
<td>8</td>
<td>...</td>
</tr>
<tr>
<td>Minerals</td>
<td>3</td>
<td>...</td>
</tr>
<tr>
<td>Other (vitamins, amino acids)</td>
<td>2</td>
<td>...</td>
</tr>
<tr>
<td>Fat (endogenous)</td>
<td>2.6</td>
<td>...</td>
</tr>
<tr>
<td>Coconut oil</td>
<td>0</td>
<td>...</td>
</tr>
<tr>
<td>Corn oil</td>
<td>0</td>
<td>...</td>
</tr>
<tr>
<td>Total fat</td>
<td>(2.6)§</td>
<td>8 (21.4)‖</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>0</td>
<td>...</td>
</tr>
<tr>
<td>Sodium cholate</td>
<td>0</td>
<td>...</td>
</tr>
</tbody>
</table>

*Energy 3.03 kcal/g.
†Energy 4.09 kcal/g.
‡Methionine and l-lysine were added.
§Ratio of polyunsaturated to saturated fatty acids in the diet (P/S) = 1.47.
‖P/S = 1.49.
levels. This initial hypoglycemia is most likely due to massive insulin release triggered by the cytotoxic effects of alloxan on the pancreatic beta cells.28,33 This serious decrease in blood glucose was avoided largely by providing food ad libitum and administering glucose intravenously. The transient hypoglycemic phase of the alloxan response was then followed by sustained hyperglycemia. For blood glucose measurements, a lancet was used to draw blood from an ear vein, and a drop was placed on a Accu-Check ADVANTAGE test strip, and glucose was measured with an Accu-Check monitor (Boehringer Mannheim Corp). Blood samples for glucose measurements were drawn 1 to 2 hours after meals twice per week for the 12 weeks of the study. The glycemic measures fructosamine and percent glycated plasma protein were conducted by the Diabetes Diagnostic Laboratory at the University of Missouri (for review, see References 34 and 35). Plasma indicators for kidney function (urea nitrogen and creatinine) and liver function (alanine transaminase and aspartic transaminase) were assayed by the Veterinary Diagnostic Laboratory at the University of Missouri School of Veterinary Medicine.

Lipid Measures
Plasma was derived from blood samples taken via the anterior vena cava from pigs fasted overnight before alloxan and/or diet treatment and after ≈8 and 12 weeks of treatment. For total cholesterol or triglyceride levels, plasma was assayed directly by standard enzymatic kit (Sigma Chemical Co). For lipoprotein cholesterol and triglyceride levels, fresh plasma samples (1 mL) were chromatographed by fast protein liquid chromatography (FPLC) on a Superose 6 column (HR 16, Pharmacia) and eluted with (in wt/vol) 0.9% NaCl, 0.01% Tris, 0.01% EDTA, and 0.02% sodium azide, pH 7.6. Fractions (2 mL) were collected and assayed for protein (A280) and for cholesterol (standard enzymatic kit). For lipoprotein analysis, the cholesterol and protein profiles for every pig within a treatment group were averaged and plotted versus fraction number (Figure 4). For VLDL, LDL, and HDL lipid content, fractions from each pig corresponding to these lipoproteins were collected and assayed for cholesterol and triglyceride concentration by standard enzymatic assay. These values are shown in Table 3.

Apolipoprotein Electrophoresis
Aliquots of plasma that was frozen and stored at −80°C from each pig in a group were pooled and run from each group on the Superose 6 column. Fractions 11, 12, 16, 18, 20, 22, 24, 26, 28, 29, 30, and 32 from each group were boiled in electrophoresis buffer (4% SDS, 20% glycerol, 10% 2-mercaptoethanol, and 0.125 mol/L Tris, pH 6.8) and analyzed by SDS-PAGE on the same 3% to 15% gradient slab gel.36 Transparency images were taken of the Coomassie-stained gels by use of an Epson Expression 636 scanner. The images were analyzed by using NIH Image (version 1.61). The fraction with the greatest intensity for an apolipoprotein (apoB, apoA-I, or apoE) was set arbitrarily at 1.0, and all other fractions were expressed relative to this fraction. This analysis was for the purpose of determining the relative distribution of an apolipoprotein within a treatment group profile.

Isometric Tension Recordings
After 12 weeks of treatment, the pigs were again anesthetized, and the chest was opened to achieve euthanasia. The left circumflex coronary, brachial, and femoral arteries were rapidly excised for contractile tension recordings as described.37,38 The physiological saline bathing the arteries contained (in mmol/L) CaCl₂ 2.5, NaCl 131.5, MgCl₂ 1.2, KCl 5, NaHCO₃ 25, Na₂HPO₄ 1.2, and glucose 10.1. Prostaglandin F₂α (PGF₂α, 30 μmol/L [Lutalyse, Upjohn]) was used to preconstrict vessels for relaxation studies. Peak relaxation was determined in response to cumulative concentrations of the endothelium-dependent agonist bradykinin (10⁻¹¹ to 10⁻⁶ mol/L).

Sudan IV Staining
Quantification of fatty streaks was performed with Sudan IV stain on the basis of modifications of 2 methods.39,40 Carotid arteries were harvested 5 mm above and below the bifurcation of the main carotid, dissected free of excess connective tissue and fat, rinsed with saline, and then fixed in 10% (vol/vol) buffered formalin. Carotid arteries were opened longitudinally, then exposed to 5 mg/mL Sudan IV in 70% (vol/vol) isopropanol for 15 minutes in a 37°C water bath, and rinsed 3 times with 70% isopropanol. The arteries were then flattened under a glass plate and photographed. The carotid artery stain ratio was derived by analysis of digitally scanned photographs using ImagePro Plus (Media Cybernetics, Inc). Red-stained areas (Figure 8, top) were subjected to the binary process, and a semiautomated edge-tracking function was used to delineate the stained area, which was expressed as the ratio of stained area to total area.

Statistical Analyses
Sigmastat and Sigmaplot (Jandel Scientific) were used for graphics and statistical analyses. Values are expressed as the mean±SE. For blood glucose and plasma lipids, ANOVA was performed and was followed by the Fisher multiple range test for post hoc analysis. The percentage of arteries showing contractile oscillations was analyzed with a χ² test; Sudan IV staining and endothelium-dependent relaxation data were analyzed with a Kruskal Wallis 1-way ANOVA on ranks test and the Dunnett method for post hoc analysis. The significance level chosen was P<0.05.

Results
Glycemia, Kidney Function, and Liver Function
Eight weeks after the administration of alloxan to miniature pigs, a nearly 6-fold increase in blood glucose was evident compared with the value in control pigs (Figure 1). One of the 4 diabetic pigs was given subcutaneous injections of 7 to 8 U porcine insulin for the first 2 weeks of the study. Because these diabetic animals required virtually no exogenous insulin for survival, some beta cell function must have remained. In response to an intravenous glucose load (0.5 g/kg), plasma insulin increased to 25 to 75 μU/mL from the fasting level of 4 μU/mL in control pigs and increased to only 6 to 8 μU/mL from a fasting level of 4 μU/mL in diabetic pigs. Postprandial blood glucose was also increased significantly (27%) in the high fat–fed pigs. In a separate group of diabetic pigs with similar blood glucose values, the glycemic indices fructosamine and glycated plasma protein were increased 59% and 30%, respectively, after only 1 week of diabetes (Figure 2). Although alloxan is a highly specific pancreatic beta cell toxin, renal and liver toxicity is sometimes noted.33 In our studies, if pigs develop renal toxicity, it is apparent within 48 hours of alloxan treatment and is characterized by enormous plasma urea nitrogen levels (>200 mg/dL) and plasma creatinine (>10 mg/dL); because
of the irreversible damage, the pig is euthanized. All pigs given alloxan that completed this study did not show nonspecific alloxan toxicity. As shown in Table 2, levels of plasma urea nitrogen and creatinine in diabetic pigs were low and not different from control levels, thus indicating normal kidney function. Furthermore, plasma concentrations of the liver enzymes alanine transaminase and aspartic transaminase in diabetic pigs were not different from control concentrations, thus indicating normal liver function after alloxan treatment.

### Cholesterol and Triglyceride Concentrations in Plasma and Lipoprotein Fractions

In pigs fed the control diet for 8 weeks (Table 3), the fasting plasma total cholesterol and triglyceride levels were similar to control values reported in numerous studies conducted with swine. Approximately 57% of the cholesterol was in the LDL fraction, whereas 40% was in the HDL fraction (Table 3). When pigs were fed an atherogenic hyperlipidemic diet for 8 weeks, LDL, HDL, and total plasma cholesterol levels increased. When alloxan-treated pigs were concurrently fed the atherogenic diet, the total plasma cholesterol level increased further; this increase was primarily due to an increase in LDL cholesterol. VLDL cholesterol was also increased in diabetic pigs compared with control and high fat–fed pigs. The HDL cholesterol level was slightly but not significantly lower in diabetic pigs compared with pigs fed the high fat diet alone. The large increase in cholesterol in the LDL fraction caused the cholesterol distribution among lipoprotein particles in diabetic pigs to differ greatly from that observed in control and high fat–fed pigs. In diabetic pigs fed the high fat diet, 81% of the cholesterol was in the LDL fraction and only 16% was in the HDL fraction (Table 3). The percent cholesterol found in the VLDL fraction remained small (2.5%) and was not significantly different from that found in the other treatment groups.

Total plasma triglyceride concentration did not change when pigs were fed the hyperlipidemic diet but was significantly greater in diabetic pigs (Table 3). Plasma lipids were also measured at 12 weeks, at the time the pigs were killed for study (Figure 3). Total cholesterol values for high fat–fed and diabetic pigs were no longer significantly different from each other at 12 weeks because of greater variability in each group.

### Lipoprotein Profiles by FPLC

The average cholesterol profile for each group is shown in Figure 4. In control pigs (top, left), plasma cholesterol was distributed primarily between LDL and HDL. When pigs were fed the high fat diet alone, both LDL and HDL cholesterol increased substantially (Figure 4, middle, left). For HDL, peak width increased more than peak height. When pigs were rendered diabetic and concomitantly fed the high fat diet, the cholesterol content of the LDL fraction increased further. The peak for cholesterol in LDL in the diabetic pigs was shifted 2 fractions to the left, indicating that the LDL fraction was enriched in large LDL and cholesterol-rich intermediate-density lipoprotein (IDL) or remnant particles (Figure 4, bottom, left). Cholesterol in individual HDL fractions was either reduced or remained at the same level compared with that seen in pigs fed the high fat diet alone. The cholesterol content of the VLDL fraction either increased greatly or appeared to merge with the LDL fraction, indicating the presence of IDL. The average protein profile for each group (Figure 4, right) indicated that the total protein content of the LDL fractions did not differ between control and high fat–fed pigs but appeared to be slightly elevated in diabetic pigs. Diabetic VLDL protein was increased greatly compared with VLDL in plasma from control or high fat–fed pigs.

### Apolipoprotein Profiles of Lipoprotein Fractions

The relative distributions of apolipoproteins among lipoprotein fractions from Superose 6 chromatography were analyzed by SDS-PAGE. A pooled plasma sample from each group was run on the Superose 6 column, and aliquots from the indicated 12 fractions were electrophoresed on the same gel, stained, and scanned. The relative distribution of an apolipoprotein within a profile was plotted in Figure 5 after setting the fraction with the maximal intensity to 1. Therefore, the data represent relative distribution of an apolipoprotein in the lipoprotein profile within a treatment group and cannot be used to compare the levels of apolipoproteins among treatment groups. apoB (Figure 5, left panels) in control plasma was distributed between fractions 16 and 26, with a peak at fraction 20. In the high fat–fed pigs, the apoB peak was shifted to the left. In the diabetic pigs, the apoB peak was broader. No apoB was detectable in fractions >26. apoA-I (Figure 5, middle panels) in control plasma was distributed in fractions 28 to 32, with much smaller amounts in lower fractions. Although there was a slight relative increase in apoA-I in the lower fractions of plasma from high fat–fed and diabetic/ high fat–fed pigs, a majority of the apoA-I remained above fraction 26. apoE (Figure 5, right

### TABLE 2. Plasma Indicators of Kidney and Liver Function in Control, High Fat–Fed, and Diabetic Swine

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>High Fat</th>
<th>Diabetic High Fat</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kidney</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Urea nitrogen, mg/dL</td>
<td>11.8±2.1</td>
<td>12.5±5.7</td>
<td>15.3±2.8</td>
</tr>
<tr>
<td>Creatinine, mg/dL</td>
<td>1.44±0.1</td>
<td>1.98±0.4</td>
<td>1.43±0.2</td>
</tr>
<tr>
<td>Liver</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ALT, U/L</td>
<td>49.8±4.6</td>
<td>37.5±2.4</td>
<td>57.8±6.4*</td>
</tr>
<tr>
<td>AST, U/L</td>
<td>58.4±16.6</td>
<td>34.5±2.2</td>
<td>54.8±7.3*</td>
</tr>
</tbody>
</table>

Values are mean±SE. ALT indicates alanine transaminase; AST, aspartic transaminase. Plasma was derived from blood samples taken from overnight-fasted pigs after 12 weeks (when they were killed for study).

*Significant difference vs high fat groups.
panels) in control plasma was very low in fractions <26. The peak for apoE was found in the HDL region at fraction 28. In the high fat–fed pigs, a proportion of apoE was redistributed from HDL to fractions 18 to 22; in the diabetic pigs, to fractions 16 to 22. This region probably represents increased IDL found in plasma from both these groups of pigs.

Table 3. Plasma Cholesterol and Triglyceride Levels, Lipoprotein Cholesterol Levels, and Percent Distribution of Cholesterol Among Particles in Control, High Fat–Fed, and Diabetic Swine

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>High Fat</th>
<th>Diabetic High Fat</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total CH, mmol/L</td>
<td>1.94±0.18</td>
<td>7.76±0.58</td>
<td>13.06±1.08</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>VLDL CH, mmol/L</td>
<td>0.06±0.02</td>
<td>0.04±0.03</td>
<td>0.26±0.10</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>LDL CH, mmol/L</td>
<td>0.98±0.07</td>
<td>3.54±0.74</td>
<td>7.96±0.68</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>HDL CH, mmol/L</td>
<td>0.70±0.08</td>
<td>1.91±0.31</td>
<td>1.59±0.23</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>Total TG, mmol/L</td>
<td>0.34±0.03</td>
<td>0.38±0.09</td>
<td>0.09±0.30</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>VLDL CH, %CH</td>
<td>3.5±1.2</td>
<td>0.7±0.4</td>
<td>2.5±1.0</td>
<td>NS</td>
</tr>
<tr>
<td>LDL CH, %CH</td>
<td>56.9±3.9</td>
<td>63.6±3.1</td>
<td>81.4±1.1</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>HDL CH, %CH</td>
<td>39.6±3.8</td>
<td>35.7±3.4</td>
<td>16.1±1.6</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Values are mean±SE. CH indicates cholesterol; %CH, percent CH in each fraction; TG, triglyceride; and NS, not significant. Plasma was derived from blood samples taken after 8 weeks from pigs fasted overnight. Plasma total CH and TG concentrations were determined by direct enzymatic assay of fresh plasma. CH in lipoprotein fractions was determined after FPLC. Plasma samples (1 mL) from each pig were chromatographed on a Superose 6 column (HR 16/50, Pharmacia), and fractions pertaining to VLDL, LDL, and HDL were pooled and assayed for CH and TG by standard enzymatic assay. The recovery of CH in fractions was 89.6% for control plasma and 70.7% and 75.1% for high fat and diabetic high fat plasma, respectively. The percent cholesterol in each fraction was determined on the basis of the total amount of CH recovered in all fractions. Superscripted letters a, b, and c represent significant differences between treatment groups based on ANOVA and Fisher multiple range test.

Taken together, the cholesterol and protein profiles indicate that the cholesterol content of LDL particles, but not the number, was increased in pigs fed the high fat diet alone. In pigs that were also diabetic, LDL cholesterol was further increased, whereas the percentage of cholesterol in the HDL fraction was decreased. The protein content of the LDL fraction was only slightly increased in the diabetic pigs, indicating that the number of LDL particles was not greatly increased in the diabetic state. The major change in diabetic pigs was the development of a more heterogeneous population of both triglyceride- and cholesterol-rich apoB-containing particles with an increased content of apoE.

Excess Vascular Disease

The first apparent difference in vascular function that we noted was the oscillation in contractile tension of coronary arteries from diabetic pigs after exposure to the vasoconstrictor agonist PGF2α (Figure 6A). Coronary arteries from control pigs show a monotonic increase in tension and stable plateau for hours of recordings. Steady-state contractile tension (taken as the average of the maximum oscillatory excursions where applicable) elicited by PGF2α was significantly increased in high fat–fed and diabetic groups compared with the control group (Figure 6B). Isometric contractile responses were studied in 8 segments of left circumflex coronary artery from each pig. In the high fat–fed group, 25% of the segments (2 of 8 total) showed at least one oscillation in contractile tension, whereas 75% of the arterial segments from diabetic pigs (6 of 8 total) showed oscillations (Figure 6C). Nonparametric statistical analysis showed significantly more segments oscillated in the diabetic group compared with control and high fat–fed groups. The effect of diabetes on contractile oscillations is particularly striking because we found contractile oscillations in only 10% (1 of 10 total) control segments in the present study, and we have never found similar oscillations in any of our other studies on nondiseased coronary, brachial, or femoral arteries involving hundreds of
Furthermore, segments with oscillating tension responses were found in every diabetic pig (Figure 6C). Another quantitative assessment of the contractile oscillations was the total number of oscillations occurring in the segments over the 20-minute duration of exposure of each arterial ring segment to PGF$_2$\textsubscript{a} (Figure 6D), which was significantly greater only in arterial segments from diabetic versus control pigs. In contrast, brachial arteries showed no contractile oscillations in any of the groups. The altered contractile responsiveness of coronary arteries was restricted to the receptor-dependent agonist PGF$_2$\textsubscript{a}, in view of the fact that the tension response to depolarization with 80 mmol/L K$^+$ was not different between the groups. Also, the brachial artery contractile response to depolarization by 80 mmol/L K$^+$ was not different between the groups.

We further assessed vascular dysfunction by comparing the endothelium-dependent relaxations of coronary and brachial arteries. As shown in Figure 7A, left circumflex coronary arteries preconstricted with PGF$_{2\alpha}$ and then exposed to cumulative concentrations of the endothelium-dependent dilator bradykinin showed relaxations that were not different between groups. Endothelium-dependent relaxations of brachial artery rings from diabetic pigs were significantly decreased compared with those from high fat–fed and control pigs (Figure 7B). Maximal relaxations to bradykinin in arteries from diabetic pigs were only $\approx50\%$ the maximal relaxation found in arteries from high fat–fed and control pigs.

To assess fatty streaks as a more classical precursor of gross anatomic atherosclerotic lesions,\textsuperscript{43} we used Sudan IV staining.\textsuperscript{39,40} Figure 8 (top) shows the carotid artery bifurcation...
tion and adjacent areas. Staining was virtually absent from control arteries of pigs fed a low fat diet, whereas only the diabetic pigs showed a significant increase in staining. Note also that the staining occurs near the bifurcation at the top of the figure.

Discussion

A previous 12-week study showed that diabetic pigs fed a conventional low fat, low cholesterol diet failed to exhibit altered lipoprotein levels or profiles or vascular dysfunction. Hypertriglyceridemia and altered LDL particle size are currently postulated to play a major role in the atherosclerosis observed in diabetes. For this reason, our goal was to study the effects of diabetes in animals fed a high fat, high cholesterol diet. The major findings of the present study were that the vascular disease/dysfunction and dyslipidemia that mimic human diabetic dyslipidemia were observed concurrently in diabetic pigs fed an atherogenic diet.

Dyslipidemia

Previous studies have indicated that the pig model would provide insight into lipoprotein metabolism associated with the diabetic state for several reasons. The pig has a liprotein distribution similar to that found in humans and carries 50% to 60% of total plasma cholesterol in LDL particles. Numerous studies have shown that high fat, high cholesterol diets cause large changes in the plasma lipoprotein profile of the pig (eg, see References 24 and 26). For example, when diets high in saturated fat (15% to 20%) and cholesterol (1% to 2%) were fed to pigs, plasma total cholesterol increased from 50 to 100 mg/dL on control low fat diets to 500 to 800 mg/dL on the high fat diet. Furthermore, within 3 to 8 months of feeding, these diets stimulate the development of complex atherosclerotic lesions that are similar to those seen in humans. Sex differences have also been noted in lipoprotein profiles of human diabetic patients. Data from

Figure 5. SDS-PAGE analysis of apolipoprotein distribution in FPLC fractions. Aliquots of plasma (frozen and stored at −80°C) from each pig within a group were pooled (1 mL final volume) and chromatographed on the Superose 6 column and analyzed as described in the legend to Figure 4 and in Methods. The elution of lipoprotein fractions occurred one fraction earlier in all 3 groups than observed previously in Figure 4. Fractions 11, 12, 16, 18, 20, 22, 24, 26, 28, 29, 30, and 32 from each group were processed and run on a single 3% to 15% gradient SDS-polyacrylamide slab gel, and the apolipoproteins B, A-I, and E were analyzed as described in Methods. The data are presented as relative intensity, with the fraction containing the greatest intensity set to 1.
pigs fed a high fat diet show that both the LDL and HDL cholesterol fractions increased in this group. Such changes would be in line with observations made in several models of hyperlipoproteinemia. Under the additional stress of diabetes, the LDL cholesterol peak was further increased and became wider, indicating the presence of a more heterogeneous population of IDL/LDL particles (Figure 4). Concurrently, HDL cholesterol was slightly, but not significantly, decreased in diabetic pigs compared with the high fat–fed pigs. Comparison of the Superose 6 profiles of protein and cholesterol indicated that LDL cholesterol increased to a much greater extent than did LDL protein in the diabetic pigs. Therefore, the LDL fraction became cholesterol-enriched,

Figure 6. Excess coronary artery contraction in diabetes. A, Original record shows isometric contractile force in vitro in segments of left circumflex coronary artery. Arteries were exposed to 30 μmol/L PGF\textsubscript{2α} (arrows). B, Steady-state tension obtained after exposure to 30 μmol/L PGF\textsubscript{2α} for >10 minutes was significantly greater in high fat–fed and diabetic groups versus control group (*P<0.05, ANOVA). C, Occurrence of contractile oscillations was significantly greater only in diabetic versus control pigs (*P<0.05, \chi\textsuperscript{2} analysis) whether quantified as the percentage of artery segments (open bars) having oscillations or percentage of pigs from which an artery segment had oscillations (shaded bars). An oscillation was defined as a >10% change in contractile tension from steady-state level; oscillations were quantified in 2 arterial rings from each pig. D, Total number of oscillations occurring over the 20-minute duration of exposure of each ring to PGF\textsubscript{2α} was significantly greater only in rings from diabetic versus control pigs (*P<0.05, Kruskal-Wallis ANOVA on ranks and Dunn method for post hoc analysis).

Figure 7. Impaired endothelium-dependent relaxation in diabetes. A, Left circumflex coronary artery segments preconstricted with 30 μmol/L PGF\textsubscript{2α} showed relaxations that were not different between groups. B, Brachial artery segments preconstricted by PGF\textsubscript{2α} showed relaxations to bradykinin that were significantly less (P<0.05, Kruskal-Wallis test) than those found in control and high fat–fed groups; *At 10\textsuperscript{-8} mol/L bradykinin, the differences were also significant by t test.
larger, and much more heterogeneous in diabetic pigs compared with high fat–fed pigs. Unlike plasma cholesterol, total plasma triglycerides were not elevated when pigs were fed the high fat diet alone (Table 3). This observation reflects the fact that these animals have ample lipoprotein lipase activity and that the hydrolysis of triglycerides in the VLDL fraction was probably not saturated when pigs were fed the high fat diet. However, plasma and VLDL triglycerides (data not shown) were significantly increased in diabetic pigs. The increases in VLDL and IDL/large LDL particles seen in diabetic pigs resembles in profile and distribution those seen in hyperlipidemic humans and may be the result of increases in the production of or decreases in the catabolism of remnant-like lipoprotein particles. The apolipoprotein profiles of the Superose 6 fractions indicated that apoB was shifted to larger particles in high fat–fed pigs and shifted to both larger and smaller particles in diabetic pigs (Figure 5). Although the formation of larger particles (fractions 17 to 20, Figure 5) predominated in diabetic pigs, the amount of smaller LDL particles (fractions 24 and 25, Figure 5) also increased in the diabetic state. More complete fractionation of LDL and subsequent investigation into the relative atherogenicity of each type of particle is required (and is possible in this animal model) in order to ascribe atherogenic blame.

ApoA-I remained largely in the HDL fraction in both high fat–fed and diabetic pigs. Although a small left shoulder appeared in the distribution of apoA-I in the Superose 6 fractions (Figure 5), no new peaks or the appearance of HDL\textsubscript{25} became apparent on Superose 6 chromatography. apoE, found almost exclusively in the HDL fraction in control pigs, was partially redistributed to triglyceride- and cholesterol-enriched particles in both high fat–fed and diabetic pigs. Altogether, the present results suggest that the plasma lipoprotein profile of pigs fed a high fat diet was further modified to become more atherogenic in the diabetic state.

**Excess Vascular Disease**

Our findings clearly indicate that in pigs fed a high fat, high cholesterol diet, diabetes accelerated vascular disease/dysfunction\textsuperscript{3} in that (1) only diabetic pigs showed increased contractile tension oscillations in coronary arteries, (2) only diabetic pigs showed impaired endothelium-dependent relaxation, and (3) only diabetic pigs had significantly increased fatty streaks.

Altered vasomotor tone and increased Sudan staining of fatty streaks in the diabetic pigs strongly argue that these indices of the early stages of atherosclerosis will progress to mature lesions. The significant increase in PGF\textsubscript{2\alpha}-induced contractile oscillations in coronary arteries of the high fat–fed pigs, together with a modest increase in fatty streak formation (not statistically significant), is consistent with the long-held prediction that coronary hyperreactivity precedes formation of gross lesions in epicardial conduit arteries.\textsuperscript{30} Indeed, Golenhofen et al\textsuperscript{21} suggested an association of spontaneous oscillatory activity with proliferation of vascular smooth muscle cells in vasospasm and human pathology. Thollon et al\textsuperscript{15} showed oscillations of membrane potential in smooth muscle of porcine coronary arteries with regenerating endothelium after balloon injury. Furthermore, vascular smooth muscle cells isolated from diabetic rats show an increased incidence of agonist-induced intracellular Ca\textsuperscript{++} oscillations.\textsuperscript{56} Finally, from a longitudinal study of heart transplant recipients, Davis et al\textsuperscript{30} provided intravascular ultrasound evidence that decreased endothelium-dependent vasodilation occurs in arteries defined as structurally normal, ie, probably lacking fatty streaks. Thus, the significant increase (P\textless;0.05) in Sudan staining for fatty streaks in carotid arteries (Figure 8) and the 3-fold greater coronary contractile oscillations in our diabetic pigs compared with the high fat–fed pigs are consistent with the hypothesized progression of vascular dysfunction to complex structural atherosclerotic lesions. Further evidence for profoundly accelerated atherosclerosis in the diabetic pigs was our finding that 9% of the carotid artery was Sudan-stained after only 12 weeks (Figure 8) compared with 34 weeks required for comparable fatty streak development in the carotid artery of nondiabetic miniature pigs fed an atherogenic diet.\textsuperscript{37} Finally, the impaired endothelium-dependent relaxation in brachial arteries of only the diabetic pigs, not the high fat–fed pigs (Figure 7B), confirms a large body of evidence that endothelial dysfunction occurs in diabetes (for review, see Reference 58). The lack of impaired endothelium-dependent relaxation in the high fat–fed pigs in the present study is different from the impairment found by Cohen et al\textsuperscript{19} in hyperlipidemic pigs but could be explained by several differences between the studies, including the artery type, strain of pig, and endothelium-dependent agonists. We were surprised that bradykinin-induced relaxation...
was not impaired in coronary arteries (Figure 7A). However, endothelium-dependent relaxation has not been widely studied in coronary arteries from the animal models (eg, porcine and primate) that most closely mimic humans. Our present study showing that diabetic vasculopathy is vascular bed specific, taken together with the finding that the duration of diabetes greatly influences endothelium-dependent relaxation, points to the importance of studying diabetic vasculopathy under highly controlled experimental conditions.

Future work is needed with the porcine model in which vasoreactivity and morphological lesion formation are monitored in the diabetic high fat–fed pig over longer durations (>12 weeks to 20 or 30 weeks) during which CAD will have progressed further. These measures, combined with intervention studies to attenuate CAD, will provide more definitive evidence for altered vasoreactivity as a predictive tool for structural CAD. Indeed, mature calcified lesions were noted in diabetic high fat–fed pigs but not hyperlipidemic pigs after >20 weeks.

It is too premature to ascribe the excess vascular dysfunction and fatty streaks that were found in the present study (Figures 6 to 8) to altered VLDL, LDL, or triglycerides. We have noted, however, that 12 weeks of diabetes, per se, did not alter lipids or lipoproteins in pigs that were not fed an atherogenic diet and that there was no vascular dysfunction. Thus, taken together, these data argue for components of diabetic dyslipidemia as important factors in accelerated atherosclerosis, whereas blood glucose may either be an “innocent bystander” or necessary, but not sufficient, to induce atherosclerosis.

Need for a Suitable Animal Model: Advantages of the Porcine Model

An American Diabetes Association task force made a recommendation 15 years ago to develop better animal models of accelerated atherosclerosis associated with diabetes. The lack of appropriate animal models is still considered a limitation in the most recent reviews in the field. Although many advances have been made in the fields of insulin signaling and lipid metabolism by using rodent and transgenic mouse models, it will be possible to characterize CAD development in a porcine animal model that enables serial measurements during the course of diabetes.

We destroyed pancreatic beta cells with alloxan to decrease plasma insulin levels. Although this experimentally straightforward maneuver resulted in a diabetic dyslipidemia that is profoundly similar to that in human diabetic patients, this is not identical to early stages of human type 2 diabetes characterized by insulin resistance and concomitant hyperinsulinemia. Instead, this model would best represent the later stages of type 2 diabetes, after the pancreatic beta cells have ceased to function. Importantly, type 1 diabetic patients with poor glycemic control also show dyslipidemia, including hypertriglyceridemia and LDL particle alterations. Thus, the porcine model very likely has relevance to the dyslipidemia in both type 1 and type 2 diabetes.

There are several important similarities between swine and humans that reinforce the advantages of using swine: (1) Swine have omnivorous habits; thus, they will consume a “human-type” diet. (2) Metabolism of foodstuffs, specifically lipoprotein metabolism (References 24, 25, and 51 and the present report), is similar to that in humans. (3) Swine have a propensity toward sedentary habits and obesity; unlike dogs, which will pace in their cages if not adequately exercised, pigs are content to be sedentary. Pigs are intensively used in obesity research with direct relevance to humans. (4) Although the larger size of even miniature swine (~40 to 60 kg) has been considered by others to be an absolute limitation to their use, the ability to sample 30 to 50 mL of blood at weekly intervals is essential for the lipid profiling that we have done. Furthermore, a large blood volume enables the study of coagulation factors (eg, platelets) that is not possible in smaller animals. Very important, the size of the pig is similar to that of humans, thus enabling trials of percutaneous catheter interventions for revascularization with devices identical to those used in humans.

The most important similarity to humans is in the cardiovascular system, specifically, coronary circulation and the susceptibility to CAD. Similar to humans, pigs have few native coronary collateral arteries: the pharmacology of coronary artery reactivity is similar to that in humans; and heart rate and, thus, metabolic demand on the heart and cyclic changes in coronary blood flow are also similar to those found in humans. Finally, atherosclerotic lesions are morphologically similar to those in humans. When fed low fat diets, swine develop modest atherosclerosis, but on high fat diets, they develop the full complement of atherosclerotic lesions. The lipoprotein pattern further suggests a similar etiology of atherosclerosis in swine and humans. The above-mentioned major advantages plus the tight experimental control of blood glucose and lipids in swine will enable the most rigorous assessment of the independent effects of these parameters on atherosclerosis. Studies in humans are limited because both blood glucose and lipids are changing and atherosclerosis is typically quantified by extreme end points, such as mortality or myocardial infarction, rather than more sensitive indices of the early progression of atherosclerosis. We conclude that this porcine model of diabetic dyslipidemia and excess vascular disease/dysfunction will enable further studies to delineate cellular and molecular mechanisms underlying dyslipidemia, CAD, and peripheral atherosclerosis. This animal model should be ideal for preclinical evaluation of pharmacotherapy to prevent the excess CAD associated with diabetes.

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References


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