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Hyperglycemia-Induced Insulin Resistance in Diabetic Dyslipidemic Yucatan Swine

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Hyperglycemia, dyslipidemia, and associated insulin resistance are hallmarks of diabetes mellitus. Purposes of the study reported here were to develop practical methods for assessment of in vivo insulin sensitivity and determine contributions of hyperglycemia and dyslipidemia to insulin resistance in the porcine model of alloxan-induced diabetes mellitus and dyslipidemia. Male Yucatan swine groups were treated for 20 weeks: control (C), high fat-fed (2% cholesterol) hyperlipidemic (H), alloxan-induced diabetic normolipidemic (D), diabetic high fat-fed (diabetic dyslipidemic, DD), and diabetic dyslipidemic treated with the lipid-lowering agent atorvastatin (DDA). Plasma cholesterol concentration increased sixfold in animals of groups H, DD, and DDA, whereas triglyceride concentration increased threefold in animals of group DD only. Diabetics had decreases in glucose tolerance and pancreatic immunostaining for insulin. Use of the gold standard hyperinsulinemic euglycemic clamp procedure indicated that maximal insulin-stimulated glucose uptake was similar to that in humans, but this method was not practical for use in pigs. Instead, a more convenient and valid insulin sensitivity test involving suppression of insulin secretion with somatostatin and a single insulin injection was used. Insulin sensitivity was greatly impaired by anesthesia with isoflurane, but was not affected by use of the anxiolytic agent diazepam. Insulin sensitivity decreased by 75% in diabetics (groups D, DD, DDA), compared with animals of groups C and H, and was inversely related to fasting blood glucose concentration ($r = -0.72$). Insulin treatment to restore blood glucose values of diabetics (> 250 mg/dl) to near control values (< 100 mg/dl) promptly restored insulin sensitivity to control values. We conclude that hyperglycemia is a major cause of insulin resistance in the porcine model of alloxan-induced diabetes mellitus and dyslipidemia.

Results of recent studies have indicated that development of cardiovascular disease is accelerated in porcine models of diabetic dyslipidemia (1-7). The magnitude and histopathologic features of atherosclerotic lesions in swine are virtually indistinguishable from lesions in humans (4). This superb mimicry of atherosclerosis in humans suggests that studies of cellular and molecular mechanisms in swine (2, 5, 6, 8) could have the most relevance to human clinical medicine. One of the many risk factors thought to be involved in the etiopathogenesis of diabetic cardiovascular disease is insulin resistance (9); thus, it is essential to determine the involvement of insulin resistance in these porcine models for comparisons with humans.

Insulin resistance in adipose and skeletal muscle is thought to be the major, primary cause of impaired glucose tolerance, and it initiates a cascade of events (10, 11). Insulin secretion is increased in the earliest stages of glucose intolerance to compensate for insulin resistance, thereby maintaining euglycemia. Ultimately, overt hyperglycemia develops later when insulin secretion decreases, resulting in type 2 diabetes mellitus (hereafter referred to as diabetes). Intrinsic defects in insulin signaling pathways in skeletal muscle and adipose may partly explain decreased glucose uptake (12). Another aspect of insulin resistance is decreased insulin inhibition of adipose tissue lipolysis, leading to hypertriglyceridemia, which may exacerbate impaired uptake of glucose into skeletal muscle (10). Indeed, direct infusion of triglyceride elicits insulin resistance in humans (13) and animal models (14) and acutely alters cardiovascular function (13). Insulin resistance is also involved in the natural course of type 1 (juvenile) diabetes. In this instance, however, destruction of pancreatic beta cells and subsequent insulin deficiency elicit overt hyperglycemia, which causes secondary insulin resistance (15-17).

Several methods have been used for assessment of whole body insulin sensitivity. The common requirement for all methods is measurement of the magnitude of plasma glucose clearance as a function of plasma insulin concentration (18). Glucose tolerance (clearance from the plasma) is adequately measured by use of oral or intravenous glucose tolerance tests (OGTT and IVGTT, respectively), but is influenced by gastrointestinal absorption of glucose, release of insulin from pancreatic beta cells, and finally, uptake of glucose by adipose and skeletal muscle, the major insulin-sensitive tissues (18). Thus, a variation of the IVGTT, the frequently sampled IVGTT, provides a direct measure of in vivo insulin sensitivity by inclusion of insulin, in addition to glucose measurements, and use of minimal model computations (18-23). An insulin tolerance test, using constant infusion of insulin, glucose, and somatostatin to suppress endogenous insulin secretion, yields a steady-state glucose concentration that is inversely related to insulin sensitivity (24-32). The gold standard glucose clamp procedure may be the most widely used method and involves use of constant insulin infusion combined with variable glucose infusion to obtain a steady-

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state blood glucose concentration that is clamped, typically at a euglycemic value (12, 13, 18-20, 33-38). The aforementioned three major methods require that subjects are completely immobilized typically for two to three hours of constant measurements, thus making these methods less practical in conscious animals, because the resultant stress hormones released during physical restraint can significantly alter insulin sensitivity (35, 39-41). An alternate insulin sensitivity test (IST) that requires less than one hour involves monitoring the clearance of glucose from the blood during suppression of insulin secretion with somatostatin and only a single insulin injection (37, 38, 42, 43). Thus, it seems that the IST may be more compatible with low-stress restraint methods devised for swine (44).

Phillips and coworkers used a selective breeding strategy over several years to develop a strain of Yucatan swine with impaired glucose tolerance and modest insulin resistance, thus establishing a natural type 2 model of diabetes (45-47). The involvement of lipids in the pathogenesis of impaired glucose tolerance in the model and the cardiovascular disease were not characterized. In contrast, the current porcine model uses the combination of high fat/high cholesterol feeding and chemically induced beta cell destruction (alloxan, streptozotocin) for eliciting immediate hyperglycemia and dyslipidemia (1-6). This model of diabetic dyslipidemia is more convenient for studies of cardiovascular complications, but to the authors' knowledge, the insulin sensitivity in the model has not been determined. Thus, there were several aims of this study: to develop practical methods for assessment of glycemic status in swine, with principal emphasis on in vivo insulin sensitivity; to determine whether primary insulin resistance can be elicited in swine by dyslipidemia; and to determine the separate contributions of dyslipidemia and hyperglycemia to insulin resistance in the porcine model of alloxan-induced diabetes and dyslipidemia.

Materials and Methods

Swine care, groups, and diets. Young adult male Yucatan swine, weighing 35 to 45 kg, were obtained from the Sinclair Research Center (Columbia, Mo.) and were housed in pens at the Laboratory Animal Center at the University of Missouri. All experimental procedures involving animals were approved by the University of Missouri Animal Care and Use Committee in accordance with the "Principles for the utilization and care of vertebrate animals used in testing, research and training" and complied fully with those approved by the American Veterinary Medical Association Panel on Euthanasia. For all surgical procedures and sacrifice, anesthesia was induced with the following drugs given intramuscularly (mg/kg): atropine 0.05, telazol 6.6, and xylazine 2.2; depth of anesthesia was subsequently maintained with isoflurane gas (up to 4%). During all conscious experimental procedures, the pigs were restrained by use of a minimal stress sling similar to that described by Panepinto and co-workers (44). Tail cuff blood pressure measures were routinely obtained (Fig. 1) in non-invasive manner (Dyna-Map, Critikon, Inc.) to verify minimal stress of the restraint. Animals were principally used for two experiments of the long-term effects of diabetes and dyslipidemia on insulin sensitivity. Baseline experiments (Fig. 2-4 and Table 1) were conducted in non-diabetic clinically normal pigs before they were entered into either of the two long-term experiments. Animals were randomly allotted to groups for the long-term experiments. Because

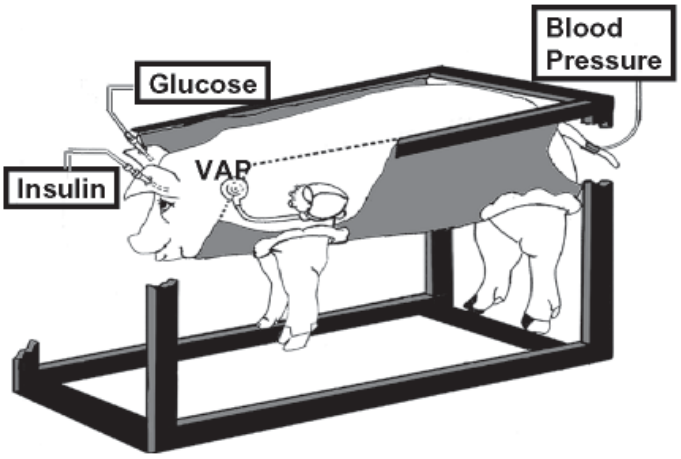

Figure 1. Vascular access port and catheter placement in restrained swine. Vascular access port (VAP) was implanted in the jugular vein and positioned with the opening near the vena cava to obtain central venous blood samples (50). Ear veins were catheterized with a 20-gauge needle for glucose and insulin infusion during glucose clamp procedures. Blood pressure was monitored non-invasively at five- to 10-min intervals, using a tail cuff device. All procedures done on conscious pigs were conducted in the Panepinto sling for low stress restraint (44).

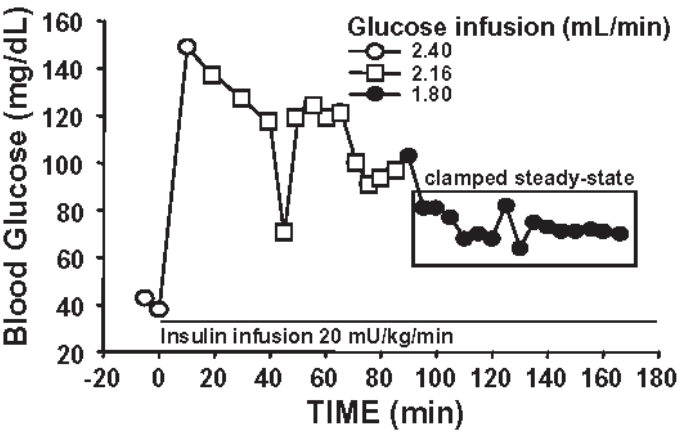

Figure 2. Hyperinsulinemic euglycemic clamp procedure. Insulin (20 mU/kg of body weight/min) and glucose infusion were started simultaneously at time 0. Glucose infusion rate of 2.4 ml/min (open circles) was initiated for the first 10 min to avoid hypoglycemia, then was reduced to 2.16 ml/min (squares), and the final steady-state of 1.80 ml/min (filled circles) was reached at 90 h to achieve a clamped steady-state blood glucose concentration (box) of 65 to 85 mg/dl (n = 8 pigs).

of the low variability in baseline experimental metabolic parameters (i.e., insulin sensitivity [Table 1] and fasting glucose and lipids concentrations [Table 2]) and the random assignment of pigs to experimental groups, there were no differences in baseline metabolic parameters among the groups.

The long-term atorvastatin experiment tested the hypothesis that increased triglyceride concentration would be responsible for decreased insulin sensitivity in animals with diabetic dyslipidemia. Pigs were assigned to four groups and were maintained for 20 weeks: low fat-fed control (C, n = 5); high fat/high cholesterol-fed hyperlipidemic (H, n = 3); high fat/high cholesterol-fed diabetic dyslipidemic (DD, n = 4); and diabetic dyslipidemic treated with atorvastatin ([Lipitor, Parke-Davis,

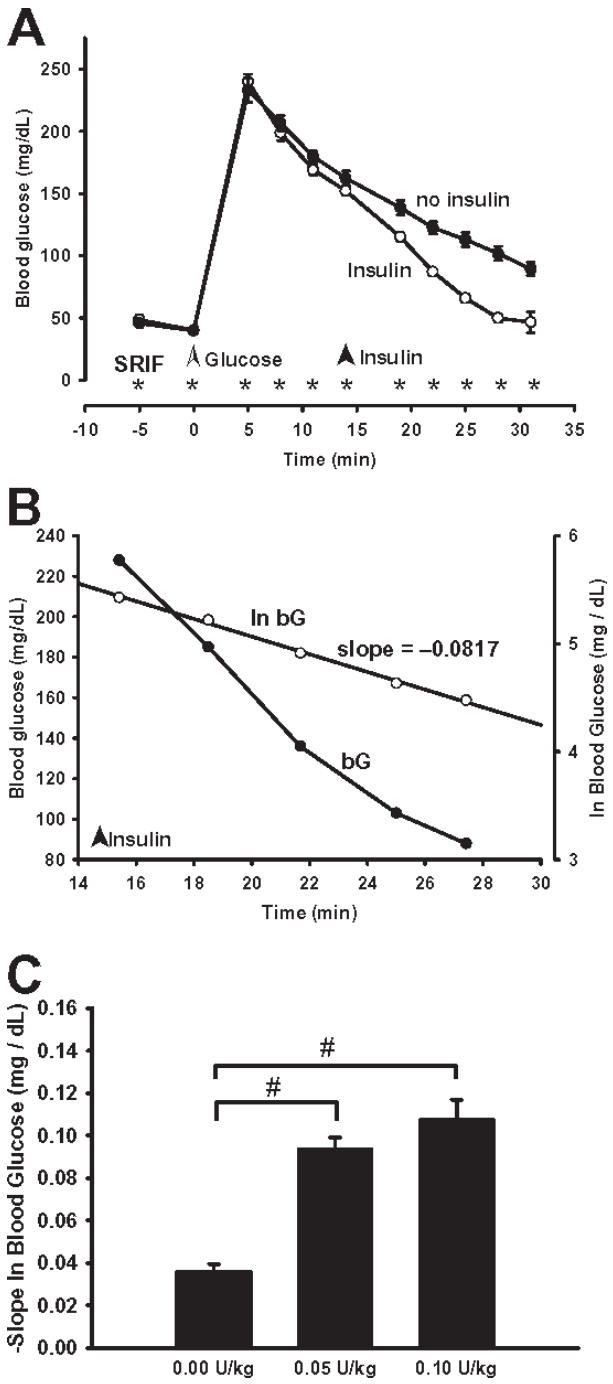

Figure 3. Normal insulin sensitivity test (nIST). (A) A comparison of changes in blood glucose (bG) concentration during a typical nIST in the presence (open circle, n = 24 tests) and absence (filled circle, n = 9 tests) of insulin (0.05 U/kg) in control pigs. A glucose bolus (open arrowhead; 0.5 g/kg) was administered at the 0 h, and insulin (closed arrowhead) was administered at approx. 15 min. *Denotes time when somatostatin (somatotropin release inhibiting factor [SRIF]: initial dose, 4 mg/kg; maintenance dose, 30 µg total) was administered. (B) The natural log (ln) of each bG value was plotted against the respective time point following insulin injection and the slope was obtained. The value of the slope was used as a measurement of insulin sensitivity or rate at which glucose is cleared. Filled circle values represent actual bG value. Open circle values represent ln of each bG value; slope = –0.0817. The graph depicted in (C) represents the average of slopes obtained at insulin doses of 0 (n = 9), 0.05 (n = 24), and 0.10 U/kg (n = 6). #Denotes significant difference between respective doses ($P < 0.05$).

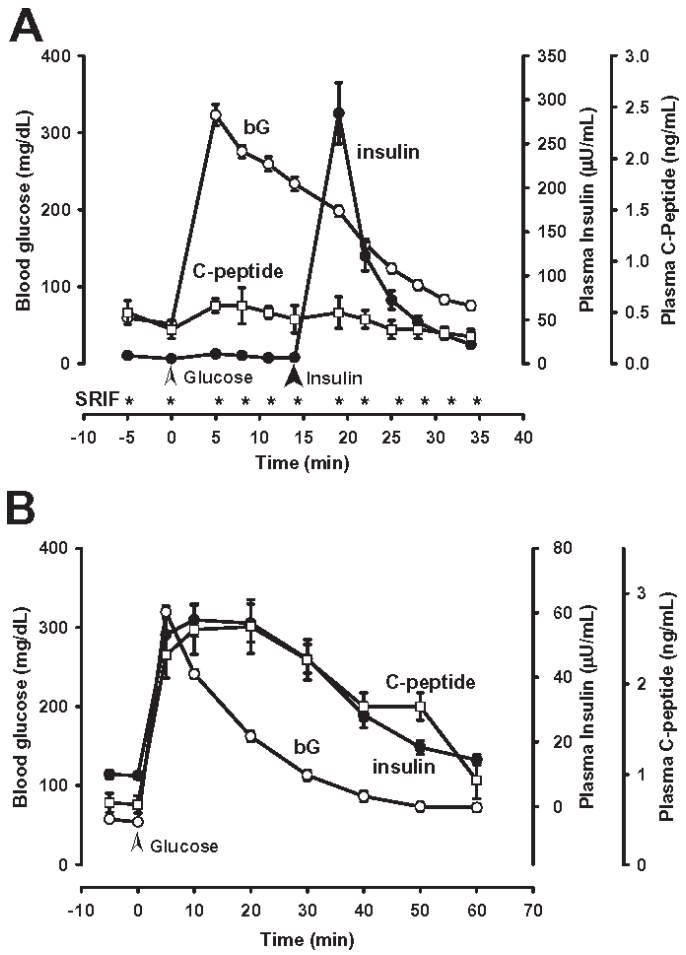

Figure 4. Somatostatin inhibition of endogenous insulin secretion. (A) and (B) represent results of a normal insulin sensitivity test (nIST) and intravenous glucose tolerance test (IVGTT), respectively. The SRIF (*) is administered during the nIST, but not the IVGTT. The absence of an increase in plasma C-peptide (open square) and insulin (filled circle) concentrations during the nIST, but presence of a response during IVGTT confirms that SRIF inhibits endogenous insulin release in response to a glucose challenge (open arrowhead). Insulin (closed arrowhead) was also administered during the nIST at 15 min, resulting in an increase in plasma insulin values. Data points are mean ± SEM; nIST n = 20 tests and IVGTT n = 26 tests.

Table 1. Effects of sedative/anxiolytic and anesthesia on insulin sensitivity in pigs of the study

	Conscious		Unconscious
	Control	Diazepam	Isoflurane anesthesia*
–Slope in bG	0.074 ± 0.003	0.068 ± 0.004	0.032 ± 0.002
N	34	21	34

*Denotes different from control, using paired *t* test.
bG = blood glucose (concentration).

Ann Arbor, Mich.; 40 mg/d given twice daily] DDA, n = 5). The dyslipidemic term was used because of the increase in triglyceride concentration and greater ratio of low to high density lipoproteins that typify lipid abnormalities in human diabetics (48) and this swine model (1-3, 8). Treatment with the lipid-lowering agent atorvastatin prevents only the increase in triglycerides in high fat/high cholesterol-fed swine without altering total cholesterol or glucose concentrations, compared with those in pigs, as reported (2, 3, 8). Lowering of triglyceride values in the absence

Table 2. In vivo blood glucose values and lipid profile

Group	Control	HL	D	DD	DDA
Diabetes					
bG (mg/dl)	49 ± 3[a]	43 ± 2[a]	356 ± 4[b]	316 ± 11[b]	258 ± 47[b]
Lipids (mg/dl)					
TC	79 ± 3[a]	358 ± 57[b]	58 ± 5[a]	420 ± 74[b]	374 ± 74[b]
LDL	35 ± 4[a]	197 ± 42[b]	29 ± 4[a]	256 ± 45[b]	249 ± 57[b]
HDL	35 ± 2[a]	127 ± 12[b]	21 ± 3[a]	103 ± 9[b]	87 ± 14[b]
TG	22 ± 2[a]	22 ± 3[a]	41 ± 17[a]	67 ± 12[b]	35 ± 7[a]
N	5	5	5	5	5

[a,b]Denote difference among treatment groups ($P < 0.05$; one-way analysis of variance, post-hoc least significant difference).
Groups: HL = hyperlipidemic, D = diabetic, DD = diabetic dyslipidemic, and DDA = diabetic dyslipidemic treated with atorvastatin.
TC = total cholesterol, LDL = low-density lipoprotein, HDL = high-density lipoprotein, and TG = total triglyceride (concentration).
See Table 1 for key.
Components of this table were previously published (2, 3, 8).

of lowering of total cholesterol concentration occurs because the high fat/high cholesterol-fed diet basically overrides the cholesterol-lowering action of atorvastatin in this experimental model.

Control pigs were fed Mini-pig breeder diet (Catalog No. 5082, Purina Mills, Inc., Brentwood, Mo.) that consisted of 16% crude protein (minimum), 2.5% crude fat (minimum), 14% crude fiber (maximum), and 8% ash (maximum) by weight. The main components are ground corn, alfalfa, oats, soybeans, and wheat supplemented with vitamins and minerals. This feed composition was consistent for every batch used for the nearly three-year total duration of these experiments. The high fat/high cholesterol-fed pigs consumed Mini-pig chow supplemented with (percentage by weight): cholesterol 2.0, coconut oil 17.1, corn oil 2.3, and sodium cholate 0.7. The high-fat diet increased the %kcal from fat from eight to 46%, decreased protein content from 22 to 13%, and decreased carbohydrate content from 70 to 41%. As in virtually all nutrition studies, other components proportionally decrease when high amounts of fat and cholesterol are added to the diet. Despite this reduction, 32 vitamins and minerals and all essential amino acids remained within acceptable ranges for adequate adult swine nutrition.

The long-term hyperglycemia experiment tested more specifically the hypothesis that hyperglycemia, independent of hyperlipidemia/dyslipidemia, was the cause of the decreased insulin sensitivity associated with diabetic dyslipidemia. Pigs were assigned to four groups and were maintained for 20 weeks in manner similar to that for pigs of the atorvastatin experiment: low fat-fed control (C, n = 5); high fat/high cholesterol-fed hyperlipidemic (H, n = 6); low fat-fed diabetic normolipidemic (D, n = 5); and high fat/high cholesterol-fed diabetic dyslipidemic (DD, n = 7). Control and group-D pigs were fed Mini-pig breeder diet, while group-H and -DD pigs received the high fat/high cholesterol-fed diet similar to that used in the atorvastatin experiment. The initial daily food allocations for control and group-D pigs were 1,050 g of Mini-pig chow and 700 g of the high fat diet to match caloric intake for pigs of groups H, DD, and DDA. Food allocations and insulin therapy were adjusted to maintain body weight as described in the "Glucose concentration and weight maintenance in diabetic pigs" section and in detail elsewhere (49).

Surgical implantation of a vascular access port. Prior to the study, a vascular access port (VAP; Access Technologies, Skokie, Ill.) was surgically implanted into each pig via left external jugular vein catheterization, in manner similar to that described by Bailie and co-workers (50). The VAP provided a non-traumatic and convenient means of serial venous blood sampling and intravenous drug administration. Accessing the venous circulation was accomplished by passing a 20-gauge, 1-in. Huber point needle (Access Technologies) into the access dome of the VAP located subcutaneously on the left side of the neck (VAP, Fig. 1). Blood sampling and drug injection were accomplished by attaching a six- or 12-in. IV tubing with an injection cap. To prevent blood clotting and infection following venous access, a three-milliliter glucose-heparin "lock" solution containing 0.16 mg of vancomycin, 300 U of heparin, and 36% percent glucose was injected to fill the entire lumen of the implanted catheter.

Induction of diabetes. Alloxan, a pancreatic beta cell toxin, was administered to induce diabetes. Prior to alloxan induction of diabetes, animals were fed regular Mini-pig breeder chow for 14 days to alleviate previously documented inhibition of alloxan due to high-fat food components (51). Non-fed pigs were put in a sling designed for minimal stress restraint. Briefly, alloxan (125 mg/kg; Sigma Chemical Co., St. Louis, Mo.) was dissolved in 14 ml of 1*M* NaOH and 11 ml of 0.9% NaCl, for a final volume of 25 ml (pH approx. 7.0). The entire alloxan solution was injected through a 0.22-µm sterile filter into the VAP port, which was then flushed with saline. To protect against possible renal damage, animals were also given 500 ml of 0.9% NaCl through intravenous drip immediately prior to and after the injection of the alloxan solution (52). The pigs were fed ad libitum and received 24 h of critical care following induction of diabetes to prevent hypoglycemic shock that can develop due to massive release of insulin into the blood associated with pancreatic beta cell death.

Glucose concentration and weight maintenance in diabetic pigs. Fasting blood glucose (bG) and blood urea nitrogen (BUN) concentrations were measured once a week, using a lancet to draw blood from an ear vein; measurement of bG values (mg/dl) was done by use of an Accu-Check bG monitor (Boehringer-Ingelheim Corp., North Chicago, Ill.) and of BUN values (mg/dl) by use of Azostix (Bayer, New Haven, Conn.). Blood glucose concentration was maintained between 250 and 400 mg/dl by adjusting daily insulin injections on a weekly basis. Insulin therapy consisted of an insulin mixture containing a third regular (R) insulin (Eli Lilly and Co., Indianapolis, Ind.) and two-thirds NPH (N) insulin (Eli Lilly and Co.) in increments of 0.1 U/kg of body weight (e.g., a 60-kg pig would receive increments/multiples as necessary of two units of R insulin and four units of N insulin).

Insulin therapy, typically 0.1 to 0.3 U/kg, was initiated on the basis of bG concentration and behavior of the pig following induction of diabetes. Injections were administered subcutaneously, and the site of injection was typically a soft area of skin in the flank. Insulin dosage adjustments were additive and dependent on bG concentration, animal behavior, and body weight measurements for that week. For example, if a pig lost 5% body weight in one week and the bG concentration was above the target range of

250 to 400 mg/dl, insulin dosage was increased 0.1 U/kg and food allocation increased 15%. Food ration was increased individually for pigs to elicit comparable increases in body weight. The target percentage increase in body weight for all pigs was 1% (of initial body weight) per week, which is the normal developmental increase in body weight for Yucatan pigs of this age range. All animals (in both long-term experiments) had ad libitum access to water. Each animal's feed portion was weighed out separately in bowls. Feed consumption was confirmed visually, and wastage was rare.

Hyperinsulinemic euglycemic clamp procedure. One day before the hyperinsulinemic euglycemic clamp procedure, two ear vein catheters (20 gauge, 1 in.) were implanted in pigs under general anesthesia. Atropine (0.17 mg/kg), telazol (2.2 mg/kg), and xylazine (0.73 mg/kg), which correspond to a third of a usual dose, were given intramuscularly through a 20-gauge, one-inch butterfly needle. Ear vein catheters were superficially attached with silk sutures, and 1,000 U of heparin/ml of saline was flushed into the catheter to prevent blood clots. Conscious, nonfed pigs were placed in the sling, and the VAP was accessed by use of a Huber needle. Intravenous ear catheters were used for glucose and insulin infusion via infusion pumps, and the VAP was used for drawing blood samples (Fig. 1). Figure 2 shows schematically the steps of the glucose clamp procedure conducted on eight control pigs before random assignment to one of four groups in the hyperglycemia experiment. Blood samples were taken at times –5 and 0 min to obtain the fasting bG value. Both glucose and insulin infusions were started at time 0, using motorized syringe pumps (model 351, Orion Research Inc., St. Louis, Mo.). The rate of insulin infusion was maintained constant at 20 mU/kg/min for the duration of the experiment. Blood glucose concentration was clamped at the euglycemic target range (65 to 85 mg/dl), using a variable infusion rate of 50% glucose. This rate was changed on the basis of bG concentration measured every 10 min until reaching a euglycemic steady-state bG concentration (approx. 100 min), then every five minutes for the next 60 min (Fig. 2).

Plasma was frozen at –80°C for radioimmunoassay (Linco Research Laboratories, St. Louis, Mo.) of insulin and C-peptide. The average rate of glucose infused during the steady-state condition was used for determining whole-body glucose uptake, which is a measure of in vivo tissue insulin sensitivity.

Insulin sensitivity test (nIST). The purpose of an insulin sensitivity test (nIST; where n denotes normal glucose concentration) was to assess glucose uptake and clearance by peripheral tissues, principally skeletal muscle and adipose. Fig. 3A shows schematically the steps of the nIST procedure. Initially, a blood sample was taken at –5 min followed by an intravenous injection of somatostatin (somatotropin release inhibiting factor [SRIF]; 4 μg/kg) to inhibit endogenous release of insulin from the pancreatic beta cells. A 50% glucose solution (0.5 g/kg) was then injected intravenously after the 0-min blood sample was obtained. Five minutes after glucose injection and every three minutes throughout the experiment, bG concentration was monitored by use of an Accu-check Advantage blood glucose meter. Each three milliliters of blood sample collection was followed by a maintenance dose of SRIF (30 μg total). When a stable plateau in bG concentration was reached, insulin (R insulin; 0.05 U/kg) was injected and bG and plasma insulin values were monitored. The rate at which bG values returned to baseline was used to assess the degree of insulin sensitivity. To confirm that SRIF prevented the release of insulin following glucose administration, blood samples were assayed for C-peptide concentration. Effects of the inhalational anesthetic isoflurane (up to 4%; n = 34) and the anxiolytic/sedative diazepam (maximal 0.4 mg/kg given i.v.; n = 21) on insulin sensitivity also were determined in control pigs before random assignment to groups in the hyperglycemia experiment and another long-term experiment reported elsewhere (49).

Cascade insulin sensitivity test (cIST). The cIST was developed to account for the already increased fasting bG concentration associated with diabetes. A blood sample was taken at –5 h, and an initial load of SRIF (4 μg/kg) was injected intravenously to inhibit endogenous secretion of insulin from the beta cells. After the 0-min blood sample was obtained, a bolus of insulin (0.05 U of R insulin/kg) was injected intravenously and bG and plasma insulin values were monitored. When a plateau in bG concentration was reached, a second bolus of insulin was injected intravenously (0.05 U of R insulin/kg) and bG and plasma insulin values were monitored. The rate at which bG values returned to baseline was used to assess the degree of insulin sensitivity. If necessary, a bolus of glucose was given at the end of the test to ensure that hypoglycemia did not ensue.

Intravenous glucose tolerance test. The main purpose of an IVGTT was to assess pancreatic beta cell responsiveness to glucose. Initially, a 50% glucose solution (0.5 g/kg) was injected intravenously via the VAP. To obtain fasting glucose concentration, blood samples (3 ml) were taken at –5 and 0 min before glucose injection, then at 5, 10, 20, 30, 40, 50, and 60 min after glucose injection. Blood glucose values were monitored by use of the Accu-check Advantage glucose meter, and plasma insulin values were obtained by insulin assays done at the University of Missouri Diabetes Clinic or Linco Research Laboratories. C-Peptide assays were conducted on pre-diabetic animals as a positive control for subsequent insulin sensitivity tests in which endogenous insulin secretion was suppressed by administration of SRIF.

In vivo lipid profiling. In vivo blood profile data from animals used in this study have been published (2, 3, 8). Fasting plasma samples were derived from arterial blood samples taken during sacrifice at week 20. As described (1), plasma was directly assayed for total cholesterol and triglyceride concentrations by use of a standard enzymatic kit (Cholesterol EZ, Triglyceride EZ, Sigma Chemical Co.). Cholesterol in lipoprotein fractions were determined after fast protein liquid chromatography (1). Low-density lipoprotein (LDL) and high-density lipoprotein (HDL) fractions were assayed for cholesterol, using the standard enzymatic kit (Cholesterol EZ, Sigma Chemical Co.).

Pancreatic immunohistologic analysis. At the time of necropsy, sections of the tail of the pancreas were fixed in phosphate-buffered 10% formalin and were paraffin embedded. Deparaffinized sections (6-μm thick) were immunostained for the presence of insulin, using a mouse monoclonal anti-insulin antibody (Zymed Laboratories, Inc., South San Francisco, Calif.), and detection of the hormone was completed, using an anti-mouse horse radish peroxidase-conjugated secondary antibody in the Veterinary Medical Diagnostic Laboratory at the University of Missouri. Digital images at 4× magnification were acquired in six individual fields through four cross sections of each pancreatic sample. The percentage of pancreatic area

immunostained for insulin was determined, using Image Pro Plus 3.1 (Media Cybernetics, Silver Springs, Md.).

Statistical analysis. Group data are expressed as mean ± SEM. Comparison of more than two groups involved use of one-way analysis of variance (SigmaStat, Jandel Scientific, Inc., Corte Madera, Calif. and SPSS, Inc., Chicago, Ill.) with least significant difference (LSD) post-hoc analysis. Differences between two groups were compared by use of independent or paired *t* tests where appropriate. Differences were considered significant at a critical value of $P < 0.05$. Pearson correlation and linear regression analyses were used for comparing association of two variables.

Results

Hyperinsulinemic euglycemic clamp procedure. Non-diabetic pigs that were moderately acclimated to the sling were reasonably calm up to three hours (average duration: two hours, 45 min), thus allowing completion of the clamp procedure. As depicted in Fig. 2, the glucose clamp procedure was begun with an infusion rate (2.4 ml/min), higher than the expected steady-state rate to avoid the onset of hypoglycemia due to the sudden hyperinsulinemic state. The glucose infusion rate was adjusted until the bG concentration target of 65 to 85 mg/dl was reached. Blood glucose values varied for approximately 100 min. The glucose infusion rate was changed on the basis of this bG concentration measured every 10 min until reaching euglycemic steady-state bG concentration. The average rate of glucose infused during the steady state was used for determining the whole body glucose uptake, which is a measure of peripheral tissue insulin responsiveness/sensitivity. The amount of glucose required to maintain euglycemia during the steady state ranged between 0.56 and 3 ml/min (mean ± SEM: 2.08 ± 0.26). Mean bG concentration of the clamped steady state was 80 ± 3 mg/dl, with a coefficient of variation of 9%. This reliability is within the range typically accepted for steady-state measures (53). Mean glucose uptake was 16.3 ± 1.4 mg/kg/min (n = 8), which is nearly identical to glucose uptake of control, non-diabetic human subjects (16.2 ± 1.1 mg/kg/min), as reported by Garvey and co-workers (12) using a similar maximal insulin infusion rate. C-Peptide measurement indicated that endogenous insulin secretion was suppressed during the steady-state glucose uptake period. Steady-state insulin values > 2,000 μU/ml were similar to those reported in studies of humans (18: Fig. 18). These supraphysiologic insulin values indicate that the test is a measure of maximal insulin-stimulated glucose uptake.

We noted several drawbacks to the test. In several instances, a hematoma formed by the ear vein catheter, forced us to infuse glucose and insulin in the other ear. For that reason, we suggest having two ear vein catheters for this procedure. Also, even in docile pigs (having normal blood pressure), approximately three hours in the sling resulted in abrasions on the limbs that required topically administered medication. In addition, intramuscularly administered pre-anesthestics (atropine, telazol, xylazine) must be used the day before the clamp procedure for introduction of ear catheters, because the pig requires several hours to recovery fully and insulin sensitivity is impaired during that time. The mask drop technique, using isoflurane anesthesia (54), is the preferred method because it enables ear vein catheters to be introduced on the same day as the clamp procedure, thus minimizing clotting in the ear catheters. Although the pig rapidly recovers consciousness within 15 to 20 min after isoflurane anesthesia, insulin sensitivity may not recover reliably; thus, introduction of catheters requiring anesthesia must be performed several hours before the glucose clamp.

Insulin sensitivity test (IST). Fig. 3A depicts a normal insulin sensitivity test (nIST) result in the presence (0.05 U of insulin/kg, n = 24) and absence (n = 9) of insulin. Initially, nISTs were performed using various doses of insulin (0.00, 0.05, and 0.10 U/kg) to determine an effective dose of insulin to be used in the nIST (Fig. 3C). To compare the results of each nIST, the natural log (ln) of each bG value was plotted against the respective time point following insulin injection and the slope was obtained (Fig. 3B). The value of the slope represents the rate constant for glucose clearance and was used as an index of insulin sensitivity. There was a significant difference in glucose clearance between the 0.00 and 0.05 U/kg doses as well as between the 0.00 and 0.10 U/kg (n = 6; $P < 0.05$) doses; however, there was no significant difference between the 0.05 and 0.10 U/kg doses (Fig. 3C). Therefore, the insulin dose of 0.05 U/kg was used in all insulin sensitivity tests. The ISTs done in humans do not include a glucose load before insulin injection (37, 38, 42, 43). We found, however, in initial experiments on normoglycemic control pigs, that injection of insulin without the glucose load yielded profound hypoglycemia with seizure-like signs that were promptly alleviated by intravenous injection of glucose. The risk of hypoglycemia, combined with our aim to use the IST in hyperglycemic pigs, made necessary the inclusion of the glucose load. The slope of bG clearance before injection of insulin is nearly zero and represents insulin-independent glucose clearance.

Because the glucose bolus was used in the nIST, SRIF was used to inhibit endogenous secretion of insulin during all ISTs. To measure the effectiveness of SRIF, plasma C-peptide values were obtained during an nIST (Fig. 4A; n = 20). Insulin and C-peptide are the products of proinsulin cleavage and are co-secreted in equimolar amounts from pancreatic beta cells. It was evident in the nIST that C-peptide values were low and that insulin values only increased at the point of exogenous insulin infusion. Thus, the initial and maintenance doses of SRIF during the nIST were sufficient to inhibit endogenous insulin secretion and confirm that the rapid decrease in bG concentration following insulin infusion was due to exogenous insulin administration, not endogenous insulin secretion. C-Peptide values were also obtained during an IVGTT in a non-diabetic control pig to confirm that infusion of a bolus of glucose in the absence of SRIF causes an equimolar increase of plasma insulin and plasma C-peptide values (Fig. 4B; n = 26). This positive control provides evidence that C-peptide concentration is increased as predicted in a pig having normal glucose tolerance (i.e., normal pancreatic beta cell function).

Minimal stress of the restraint sling was verified by normal resting values for mean arterial blood pressure. For example, mean arterial pressures obtained while pigs were in the sling during the insulin sensitivity test after 18 weeks of the long-term hyperglycemia experiment were (group, mean ± SEM [mmHg], n): C, 106 ± 9, 5; H, 83 ± 2, 3; D, 97 ± 4, 6; and DD, 104 ± 12, 7. Long-term (three to four weeks) acclimation of pigs to the sling was essential for sling restraint to elicit minimal stress reflected in these normal blood pressures. Sling acclimation could be avoided, and longer durations (three hours) in the sling needed for the glucose clamp procedure could be tolerated by pigs by

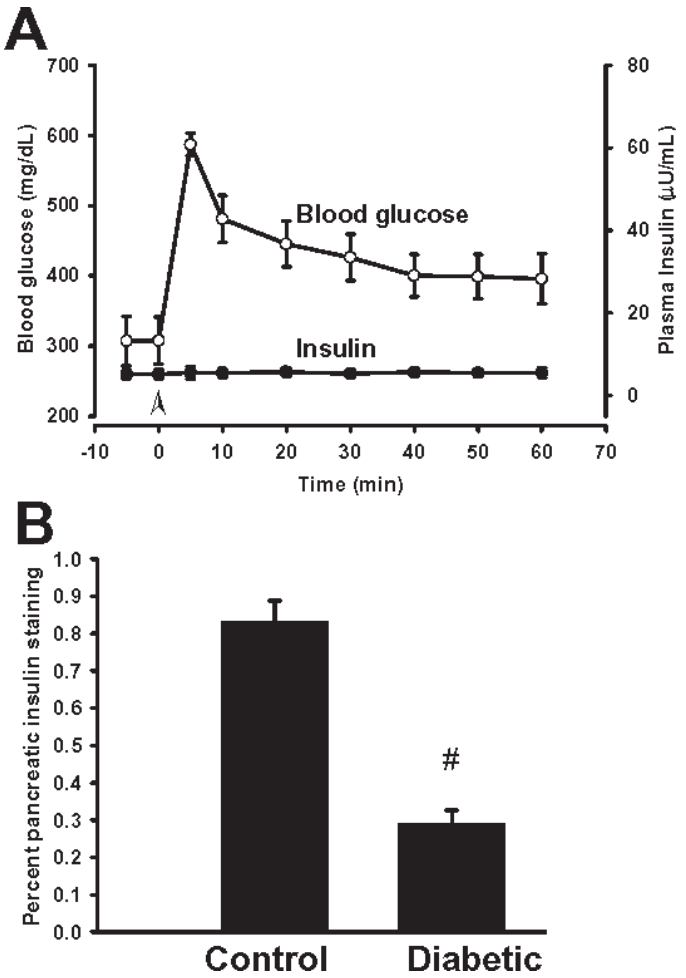

Figure 5. Diabetic IVGTT results and pancreatic histologic examination findings. (A) depicts the average bG (open circle) and insulin (filled circle) responses at various time points during the IVGTT in diabetic pigs (n = 5, 1 test/animal). As depicted in (B), alloxan administration significantly decreased the percentage area of the pancreas that immunostained for insulin in diabetic, compared with control pigs ($P < 0.05$; 24 cross sections analyzed per animal; n = 4 control and n = 3 diabetic dyslipidemic pigs from the atorvastatin experiment).

use of pharmacologic agents that reduce stress. Isoflurane anesthesia was found to impair glucose tolerance (55), but it was not evident whether the effect was due solely to impaired insulin secretion and/or whether target tissue (skeletal muscle) sensitivity also was decreased. Table 1 shows the effects on insulin sensitivity of isoflurane anesthesia and the sedative/anxiolytic agent diazepam. Isoflurane significantly decreased insulin sensitivity, whereas diazepam had no effect, compared with that in conscious control pigs.

Insulin sensitivity in long-term diabetes experiments. During week 0, diabetes was induced in animals of the diabetic groups by infusion of alloxan. The effects of alloxan are evident in IVGTT results of diabetic pigs (Fig. 5A; n = 5), compared with those of non-diabetic pigs (Fig. 4B). Diagnosis of diabetes was indicated on the basis of: high bG values prior to glucose infusion; lack of endogenous insulin response to the glucose bolus; and inability of bG values to return to baseline. We confirmed that alloxan destroyed pancreatic beta cells by examining histologic cross-sections of the pancreas from diabetic dyslipidemic pigs of the long-term atorvastatin study. Positive immunostaining reaction for insulin in control pigs was seen throughout the islets, but only sporadically in diabetic pigs. The relative percentage of pancreatic area immunostained for insulin was 2.5-fold greater in control (n = 4), compared with diabetic (n = 3) pigs ($P < 0.05$; Fig. 5B).

Several important results apply to both long-term diabetes experiments. Pre-experimental metabolic profiles and plasma lipids concentrations were not different among the groups (data not shown). Because of the low variability in baseline experimental metabolic parameters (i.e., insulin sensitivity, [Table 1], fasting glucose concentration, and lipids concentrations [Table 2]) and the random assignment of pigs to experimental groups, there were no differences in baseline metabolic parameters among the groups. Thus, differences among groups to be reported are due solely to the long-term treatments. Also, long-term insulin and food maintenance algorithms elicited appropriate developmental body weight gain in animals of all diabetic groups (D, DD, DDA), compared with that in controls. Hyperlipidemic animals also had the normal developmental percentage increase in body weight per week over the study (e.g., range: 37- to 50-kg beginning, and 47- to 60-kg ending body weight). Thus, the high fat/high cholesterol-fed diet with mildly reduced nutrient amounts provided adequate nutrition for growth of adult pigs fed the same total kilocalories. We cannot state with certainty whether minor reductions in nutrient composition of high- fat/high cholesterol diets fed to rapidly growing, juvenile swine will stunt development. Finally, the alloxan procedure did not elicit renal damage, as evidenced by no difference in BUN or serum creatinine concentration (range, 1.32 to 1.50 mg/dl) among groups. Similarly, alloxan did not elicit liver damage, as evidenced by no difference between groups in activity of liver enzymes, such as aspartate transaminase (range, 29 to 40 U/L).

For the atorvastatin experiment, postprandial bG values were maintained between 250 and 350 mg/dl. The average 20-week bG value was increased almost sevenfold in diabetic versus control pigs (Table 2). Typical fivefold increases in total cholesterol, as well as increases in low density lipoprotein (LDL), and high density lipoprotein (HDL) values were evident in all pigs fed the high-fat/high cholesterol diet (Table 2). Atorvastatin (DDA, Table 2) prevented only the increase in triglyceride concentration, compared with that in pigs of the DD group, as reported (2, 3, 8). Results of the nISTs performed at week 20 indicated that diabetic pigs (DD) had decreased insulin sensitivity, compared with that in control pigs, as indicated by a 75% decrease in the slope of the ln of bG concentration following insulin infusion (Fig. 6A).

Although atorvastatin (group DDA) prevented the increase in triglyceride concentration, it did not improve insulin sensitivity in diabetic pigs (Fig. 6A). Further, non-diabetic high fat/high cholesterol-fed pigs (H) had bG and triglyceride values similar to those of control pigs and high cholesterol values similar to those of pigs of groups DD and DDA, but did not have impaired insulin sensitivity (Table 2, Fig. 6A). The plasma insulin responses to exogenous insulin (measured as in Fig. 4) were similar among all pig groups, thus ruling out the possibility that decreased insulin sensitivity in pigs of groups DD and DDA could be explained by decreased insulin bioavailability. Fig. 6B depicts correlation of the slopes of nISTs versus bG concentration at the time of insulin infusion ($r = 0.72$). To the authors' knowledge, these results are the first evidence that high bG values in diabetic pigs are associated with insulin resistance. Thus, the

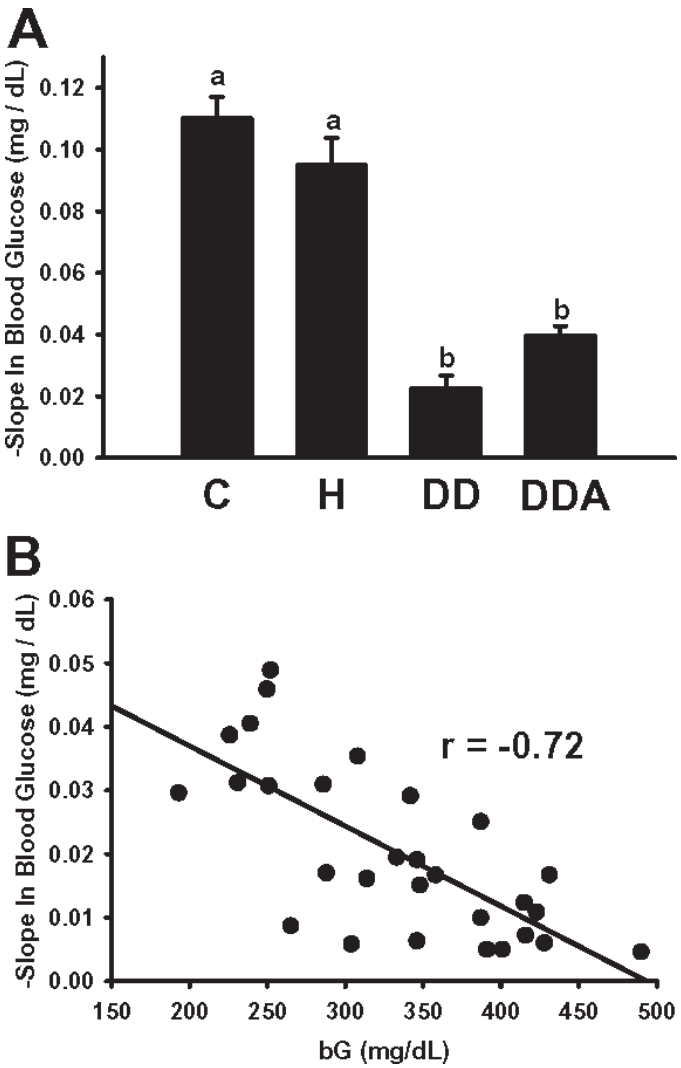

Figure 6. Atorvastatin experiment—no effect of triglyceride lowering with atorvastatin on insulin sensitivity in the diabetic dyslipidemic pig. The graph depicted in (A) represents the average insulin sensitivity values following insulin administration (0.05 U/kg) during an nIST in control (C, n = 5 pigs), high fat/high cholesterol-fed hyperlipidemic (H, n = 3) pigs, diabetic dyslipidemic (DD, n = 4) pigs, and diabetic dyslipidemic pigs fed atorvastatin (DDA, n = 5). Diabetic insulin sensitivity values were decreased by 75%, compared with those in animals of control and hyperlipidemic groups, and were not different from those in diabetic pigs fed atorvastatin (a and b denote significant difference, $P < 0.05$). As depicted in (B), insulin sensitivity values from nISTs in diabetic pigs are inversely proportional to baseline bG values obtained at 0 min of the nIST; $r = -0.72$ (n = 29 nISTs).

lack of effects of triglyceride concentration lowering in animals of the DDA group and hyperlipidemia on insulin sensitivity and the significant inverse correlation of bG concentration with insulin sensitivity lead us to hypothesize that hyperglycemia is the main underlying cause of decreased insulin sensitivity in this model.

To test this hypothesis further, we conducted another long-term, 20-week experiment, using groups C, H, and DD again, but included a diabetic group fed regular mini-pig chow. Animals of the last group were clearly hyperglycemic and normolipidemic (D, Table 2). Figure 7 shows clearly that hyperglycemia alone (D) elicited the same decrease in insulin sensitivity as did the combined effects of hyperglycemia and dyslipidemia (DD).

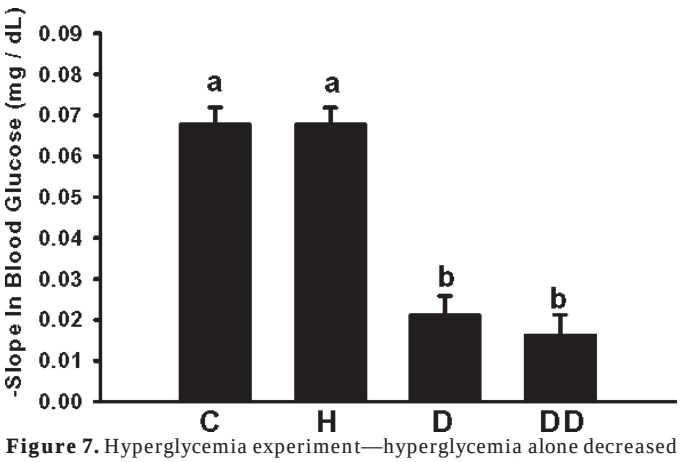

Figure 7. Hyperglycemia experiment—hyperglycemia alone decreased insulin sensitivity. Insulin sensitivity values of hyperglycemic normolipidemic (D, n = 5) and hyperglycemic dyslipidemic (DD, n = 7) pigs were similarly decreased (a and b denote significant difference, $P < 0.05$), compared with those in control (C, n = 5) and hyperlipidemic (H, n = 6) pigs.

The initial evidence of hyperglycemia-induced insulin resistance resulted in the implementation of the cascade IST (cIST) protocol in the diabetic animals. The cIST did not involve initial infusion of glucose since the fasting bG concentration of diabetic animals was already at such a high value. We reasoned that introducing more glucose into the system seemed unnecessary and would only compound hyperglycemia-induced insulin resistance. Consequently, the cIST (Fig. 8A) involved an infusion of insulin after the blood sample was obtained at time 0 min. After bG values plateaued, insulin was infused again and insulin sensitivity was determined as previously described for the nIST. Fig. 8B depicts the average insulin sensitivity values of the first (bG > 250 mg/dl) and second (bG < 250 mg/dl) infusions of insulin in diabetic animals during a cIST, compared with the pre-diabetic nIST where insulin infusion was done at a glucose concentration of approximately 150 to 250 mg/dl. As predicted, insulin sensitivity following the first insulin infusion of the cIST was markedly reduced, compared with insulin sensitivity determined in the pre-diabetic nIST (Fig. 8B). Although bG values in the diabetic pigs during the second insulin infusion of the cIST were similar to those in the control, non-diabetic pigs during the nIST, insulin sensitivity was still markedly reduced in the diabetic pigs. This result provides further evidence of hyperglycemia-induced insulin resistance.

We further demonstrated the concept of chronic hyperglycemia-induced insulin resistance by normalizing bG concentration by use of intensive insulin therapy after establishment of chronic diabetes. Initially, an nIST was performed before induction of diabetes, then diabetes was induced, and a cIST was performed two weeks after alloxan administration when bG concentration had stabilized in the 300 to 400 mg/dl target range. There was a typical > 75% decrease in insulin sensitivity as predicted (Fig. 8C; n = 4). Blood glucose values were then restored to near control values (< 100 mg/dl) by administering subcutaneous injections of insulin. After 24 h of bG normalization, results of an nIST indicated that insulin sensitivity was restored to pre-diabetic values (Fig. 8C). Restoring bG values to the pre-diabetic state did not attenuate the dyslipidemic profile (data not shown). Restoration of normal insulin sensitivity following

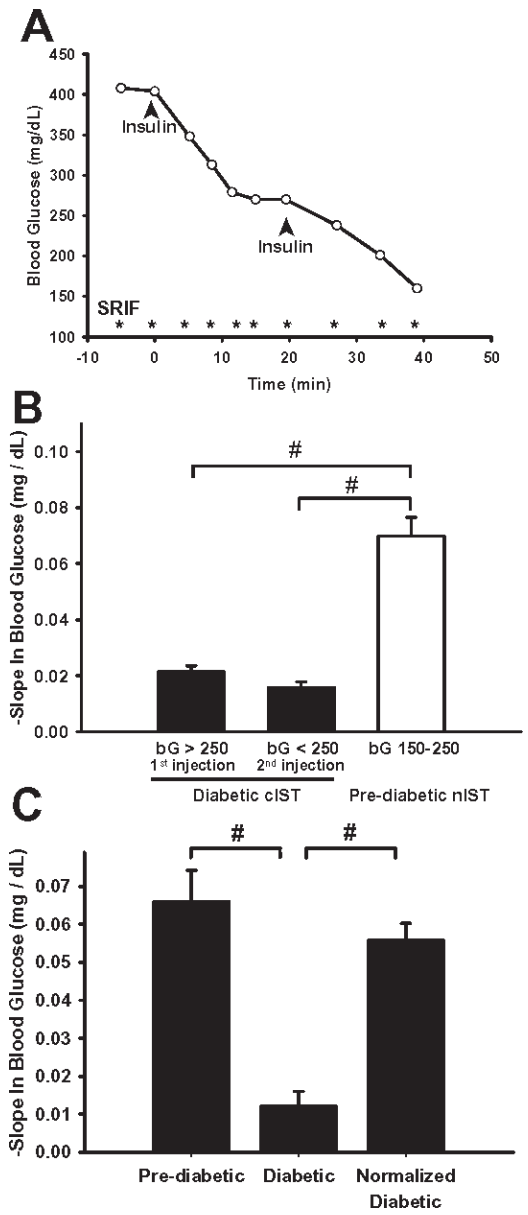

Figure 8. The cascade insulin sensitivity test (cIST) and hyperglycemia-induced insulin resistance. (A) Changes in blood glucose (bG) values during a typical cIST. Insulin (0.05 U/kg, arrowhead) was administered at 0 min and again at 20 min following a plateau in bG values; glucose is not administered. *Denotes SRIF. The closed bars in (B) represent the averaged insulin sensitivity associated with the first and second insulin injections of the cIST in diabetic dyslipidemic pigs of the hyperglycemia experiment. The open bar represents the average insulin sensitivity values for the nIST performed in the same pigs prior to induction of diabetes with alloxan. Insulin sensitivity was significantly reduced after the first insulin injection, compared with that of the pre-diabetic insulin state (n = 13 tests; $^{\#}P < 0.05$). Although the bG values in the diabetic pigs at the time of the second insulin injection were similar to those of the pre-diabetic state at the time of insulin injection, insulin sensitivity was still markedly reduced. (C) Near normalization of bG concentration in diabetic dyslipidemic (DD) pigs from the hyperglycemia experiment returned insulin sensitivity to pre-diabetic values. Insulin sensitivity was determined in four pigs under pre-diabetic, diabetic (diabetic dyslipidemic), and normalized diabetic conditions. Following alloxan-induced diabetes and onset of dyslipidemia and chronic hyperglycemia, fasting bG values were reduced to nearly control/pre-diabetic values (< 100 mg/dl) by daily insulin injection, and an nIST was performed one day (approx. 24 h) after normalization of values.

return to euglycemia in the animals of this study is the third line of evidence that chronic hyperglycemia is a main factor that induces insulin resistance in this porcine model.

Discussion

We achieved all three aims of this study, significantly advancing the body of knowledge on porcine models of diabetes. The major finding is that insulin resistance develops in the porcine model of alloxan-induced diabetes and dyslipidemia and is almost solely attributable to hyperglycemia. Hyperglycemia-induced insulin resistance is well-documented in other animal models (17, 53, 56-59) and humans (15, 16), but to our knowledge, this is the first report in swine, and several lines of evidence strongly support the conclusion that dyslipidemia is not involved. Pigs of the hyperglycemic, normolipidemic (D) group had similarly decreased insulin sensitivity as did diabetic dyslipidemic pigs (DD, Fig. 7). Hyperlipidemia (H) alone did not elicit significant insulin resistance (Fig. 6 and 7). Atorvastatin prevented the increase in triglyceride concentration in diabetic dyslipidemic pigs (DD), but did not restore insulin sensitivity to control values (Fig. 6). Intensive insulin treatment did not restore blood lipid concentrations to control values, but restored bG concentration and insulin sensitivity to control values (Fig. 8C).

An extension of our aim to determine the relative roles of hyperglycemia and dyslipidemia in insulin resistance in the porcine model are novel findings on the effects of the lipid-lowering agent atorvastatin. As we reported previously, atorvastatin prevented the increase in triglycerides, but not plasma cholesterol concentration (Table 2). This was predicted because the high-fat/high cholesterol diet basically overrides the actions of atorvastatin (2, 3, 8), as low-density lipoprotein receptor function would already be greatly decreased due to the large influx of dietary cholesterol into tissues (60). Atorvastatin also did not alter postprandial bG concentration or insulin resistance, but we previously reported that atorvastatin prevented atheroma (2, 3). Results of the current study, thus, provides strong evidence that atorvastatin does not elicit atheroprotection by preventing hyperglycemia-induced insulin resistance.

A second major aim of this study was to develop practical methods for assessment of glycemic status in swine, with a principal emphasis on in vivo insulin sensitivity. We provided several lines of evidence in support of the validity of the IST. Somatostatin fully suppressed insulin secretion, as evidenced by lack of C-peptide response to glucose load. There was a minimal rate of decay of bG values in the absence of insulin injection. The test is not substantially influenced by stress, as non-invasive tail cuff blood pressure recordings were normal. Also, compelling data from human studies indicate that IST measurements correlate with glucose clamp data (37, 38) and insulin receptor binding to isolated fat cells (42). The validity and practicality of completing the IST in less than one hour, compared with three hours for the glucose clamp procedure, make the IST the preferred method. On the other hand, the glucose clamp procedure would be preferred for metabolic studies requiring steady-state conditions. Our finding that maximal insulin-stimulated glucose uptake during the glucose clamp procedure in swine (Fig. 2) was similar to that in humans (12) provides a rational basis for this use of the glucose clamp procedure. We caution that sedation of swine may be required for long duration (> three hours) clamp studies to avoid excess

movement in the restraint sling and resulting skin abrasions. Clearly, general anesthesia with isoflurane is not suitable because of the marked impairment of insulin sensitivity (Table 1). We have extended the important finding of Laber-Laird and coworkers (55) that glucose tolerance is impaired by isoflurane anesthesia by documenting (by use of our insulin sensitivity test) that isoflurane impairs insulin action at the level of the target tissue, skeletal muscle. On the other hand, the anxiolytic agent diazepam induced muscular relaxation and sedation efficaciously, but did not affect insulin sensitivity (Table 1). Thus, if sedation is essential during insulin sensitivity testing in swine, diazepam is an excellent choice.

Despite the advantages of the IST, two main improvements could be made. Inclusion of a lower insulin dose (< 0.05 U/kg) to achieve a lower, submaximal peak insulin concentration may enable differentiation of more subtle changes in insulin sensitivity. Since 0.05 U of insulin/kg elicited only slightly less glucose clearance than did 0.1 U/kg, it is clear that the 0.05 U dose is approaching maximum. However, the peak plasma insulin concentration is transient, thus yielding insulin values approximating postprandial values during most of the IST (Fig. 4A) and establishing the relevance to physiologic in vivo values. The IST would be more practical if it were not necessary to implant a VAP, which is expensive and requires great care to avoid introduction of systemic infection. Use of ear vein venous access for injection and perhaps cephalic or saphenous vein access for repeated blood sampling should be explored.

A major advance of the current study is that the validity of our main conclusions relevant to the hyperglycemia-induced insulin resistance in human diabetes is strengthened by our close attention to the overall metabolic state of the animals. Specifically, we have documented that appropriate feeding and insulin therapy can maintain body weight and hyperglycemia in alloxan-diabetic swine (49), similar to that in diabetic humans (61). Thus, hyperglycemia-induced insulin resistance in diabetic swine is not confounded by the starvation syndrome that is characteristic of many animal studies of diabetes. The widely appreciated usefulness of swine for studies of atherosclerosis (1-6) and cardiac function (62) in diabetes make the swine model similarly useful to the well characterized nonhuman primate model (63).

A final aim of our study was to determine whether primary insulin resistance can be elicited in swine by dyslipidemia. In contrast to the studies of Phillips and co-workers (45-47), our previous study (49) and the one described here (Fig. 6 and 7) did not elicit primary insulin resistance. The most likely explanations are that Phillips and co-workers selected for glucose intolerant pigs by use of a breeding strategy and the duration of their high-fat/high cholesterol feeding study was longer (45) than our 20-week experiments. We conclude that, despite the profound and rapid induction of cardiovascular disease (1-6) elicited by the combination of high fat/high cholesterol feeding and chemically induced beta cell destruction (alloxan, streptozotocin) in swine (1-6), other animal models should be studied as models of type 2 diabetes. Specifically, primary insulin resistance in other models, such as llamas and alpacas (64) and Ossabaw swine (65), should be pursued.

In conclusion, valid and practical measures using the insulin sensitivity test indicated that hyperglycemia is the major cause of insulin resistance in the porcine model of alloxan-induced diabetes and dyslipidemia. This model is excellent for studying the pathogenesis of cardiovascular disease resulting from type 1 diabetes and end-stage type 2 diabetes at which point insulin therapy is required. Further work is needed to establish porcine models that mimic early stages in the pathogenesis of type 1 and type 2 diabetes.

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