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Association of insulin resistance with hyperglycemia in streptozotocin-diabetic pigs
Effects of metformin at isoenergetic feeding in a type 2–like diabetic pig model

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Abstract

Insulin-mediated glucose metabolism was investigated in streptozotocin (STZ)–treated diabetic pigs to explore if the STZ-diabetic pig can be a suitable model for insulin-resistant, type 2 diabetes mellitus. Pigs (~40 kg) were meal-fed with a low-fat (5%) diet. Hyperinsulinemic (1, 2, and 8 mU kg^{-1} min^{-1}) clamps and/or 6,6-^{2}H-glucose infusion studies were performed in 36 pigs. Diabetic (slow, 30-minute infusion of 130 mg STZ/kg) vs normal pigs were nonketotic, showed fasting hyperglycemia (21.7 ± 1.1 vs 5.3 ± 0.2 mmol/L), comparable plasma insulin (9 ± 7 vs 5 ± 1 mU/L), and elevated triglyceride concentrations (1.0 ± 0.3 vs 0.2 ± 0.1 mmol/L). After a standard meal, plasma triglycerides, cholesterol, and nonesterified fatty acid concentrations were significantly higher in diabetic vs normal pigs (1.2 ± 0.3 vs 0.3 ± 0.1, 2.3 ± 0.2 vs 1.7 ± 0.1, and 1.5 ± 0.5 vs 0.2 ± 0.1 mmol/L, respectively, $P < .05$). Fasting whole-body glucose uptake, hepatic glucose production, and urinary glucose excretion were increased ($P < .01$) in diabetic vs normal pigs (9.1 ± 0.6 vs 4.8 ± 0.4, 11.4 ± 0.6 vs 4.8 ± 0.4, and 2.3 ± 0.2 vs 0.0 ± 0.0 mg kg^{-1} min^{-1}). During hyperinsulinemic euglycemia (~6 mmol/L), whole-body glucose uptake was severely reduced ($P < .01$) and hepatic glucose production was moderately increased ($P < .05$) in diabetic vs normal pigs (6.7 ± 1.3 vs 21.1 ± 2.2 and 1.7 ± 0.5 vs 0.8 ± 0.3 mg kg^{-1} min^{-1}) despite plasma insulin concentrations of 45 ± 5 vs 24 ± 5 mU/L, respectively. Metformin vs placebo treatment of diabetic pigs (twice 1.5 g/d) for 2 weeks during isoenergetic feeding (1045 kJ/kg body weight$^{0.75}$) resulted in a reduction in both fasting and postprandial hyperglycemia (14.7 ± 1.5 vs 19.4 ± 0.6 and 24.9 ± 2.2 vs 35.5 ± 4.9 mmol/L), a reduction in daily urinary glucose excretion (~250 vs ~350 g/kg food), and an increase in insulin-stimulated glucose disposal (9.4 ± 2.2 vs 5.8 ± 1.7 mg kg^{-1} min^{-1}; $P < .05$), respectively. In conclusion, a slow infusion of STZ (130 mg/kg) in pigs on a low-fat diet induces the characteristic metabolic abnormalities of type 2 diabetes mellitus and its sensitivity to oral metformin therapy. It is therefore a suitable humanoid animal model for studying different aspects of metabolic changes in type 2 diabetes mellitus. Insulin resistance in STZ-diabetic pigs is most likely secondary to hyperglycemia and/or hyperlipidemia and therefore of metabolic origin.

1. Introduction

The domestic pig is recognized as a valuable model in biomedical research because of its anatomical, physiologic, and metabolic similarities with humans [1-4]. In diabetes and cardiovascular research, the diabetic pig is becoming more and more popular as a humanoid animal model to study endocrinology and metabolism [5-9], and the mechanisms underlying dyslipidemia and atherosclerosis [10,11].

In non–insulin-dependent diabetes mellitus or type 2 diabetes mellitus, the derailed interaction between lipid and glucose metabolism is thought to be responsible for the development of insulin resistance [12-14]. Glucose toxicity, lipotoxicity, and the substrate competition between glucose and nonesterified fatty acids (NEFA) are known to induce

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insulin resistance [15-21]. Because lipoprotein and lipid metabolism in humans and pigs are very alike [3,4], it may be expected that streptozotocin (STZ) treatment for pigs (ie, the chemical induction of diabetes) can create a type 2–like diabetic pig model with regard to insulin resistance, glucose homeostasis, dyslipidemia, and the response to oral anti–type 2 diabetes mellitus drug therapy. Streptozotocin, a chemical agent that damages pancreatic beta cells, has frequently been used to induce insulin-deficient diabetes in many species [22-25], and depending on the dose and strategy of STZ administration, a type 1–like [7,26,27], or a type 2–like [28-31], diabetes mellitus can be generated. Rapid intravenous injection of STZ at doses of around 100 mg/kg or higher produces insulin-dependent diabetes in pigs within days after STZ administration, and diabetes remains present for periods up to 3 years [6,7,25,26]. Lower doses of STZ result, in general, in a transient or absent diabetic reaction in pigs [6,32].

The present study was undertaken to induce diabetes mellitus in pigs by slow (over a period of 30 minutes) intravenous infusion of several doses of STZ (110, 130, or 150 mg/kg). Previously, it has been shown [32] that rapid injection of STZ causes severe beta-cell damage in pigs, whereas a more slow infusion (several minutes) of STZ results in a more moderate damage to beta cells. The latter strategy, using the appropriate dose of STZ, could lead to a diabetic pig model with characteristics of type 2 diabetes mellitus. The type 2–like diabetic pig model should be characterized by hyperglycemia, minimal ketonuria, fasting normo- to hyperinsulinemia, elevated plasma triglyceride concentrations, insulin resistance, and a positive energy balance without exogenous insulin therapy. In addition, the type 2–like diabetic pigs should respond positively to metformin treatment.

To our knowledge, information on insulin-mediated glucose metabolism and the occurrence of insulin resistance in STZ-diabetic pigs is not available yet. Therefore, hyperinsulinemic clamp experiments with 6,6-^{2}H-glucose infusion were performed to quantify insulin-mediated whole-body and hepatic glucose kinetics.

Oral metformin treatment for type 2 diabetic patients leads to improvement of glucose homeostasis and insulin resistance [33-35]. Diabetic pigs were fed daily with 2 isoenergetic meals (1045 kJ/kg body weight [BW]$^{0.75}$ per day) to study the pharmacologic effect of metformin in the absence of changes in food intake. The response to oral metformin therapy was investigated with regard to glucose homeostasis and insulin sensitivity.

2. Materials and methods

2.1. Animals and housing

Experimental protocols describing the management, surgical procedures, and animal care were reviewed and approved by the Animal Sciences Group-Lelystad Animal Care and Use Committee (Lelystad, The Netherlands).

Thirty-six adolescent [1-4], crossbred pigs (Yorkshire × Landrace) of approximately 30 kg BW (12 weeks of age) at surgery were used in this study. Yorkshire × Landrace pigs were used because these pigs are easy to breed at a high level of standardization and at reasonable cost because this type of pig is the most important production animal in the world. Adolescent pigs (30-50 kg) grow continually (3-5 kg/wk) until they reach ~100 kg and growth rate slows down.

Two weeks before surgery the pigs were housed in metabolism cages (1.15 × 1.35 m) and adapted to the light/dark cycle and the feeding regimen. Lights were on and off at 05:00 AM and 10:00 PM, respectively. Ambient room temperature was 20°C.

2.2. Feeding regimen and surgery

A commercial diet (5% crude fat, 16% crude protein, 41% starch and sugars, 20% nonstarch polysaccharides, 6% ash, and 12% water; Startbrok; Agrifirm, Meppel, The Netherlands) was fed twice daily, that is, at 06:00 AM and 03:00 PM with free access to water. Pigs were weighed twice weekly, and meal size was adjusted to the weight of the pig. The nutritive value was equal to 2.5-fold maintenance requirements for metabolizable energy (ME) as established in a normal pig [36]. This corresponded with a feeding level of 1045 kJ ME/kg BW$^{0.75}$ (metabolic weight of the pigs) per day sufficient for moderate growth in a normal pig.

The surgery was preceded with a 24-hour period of fasting, and only water was offered. The day after surgery, the pigs were given 50% of the presurgically consumed food, and afterward their preoperative food intake was established.

Pigs were anesthetized by intramuscular injection of 2 mg azaperone/kg BW (Stressnil; Janssen, Tilburg, The Netherlands) followed by an intravenous injection of 15 mg nesdonal/kg BW (Rhone Merieux, Lyon, France). Pigs were intubated, and general anesthesia was maintained by inhalation of 4% isoflurane in combination with oxygen and nitrous oxide. Afterward, pigs were equipped with 2 polyethylene catheters (Tygon, ID 1.02 mm, OD 1.78 mm, length 1 m; Norton, Akron, OH) in the right carotid artery and the right external jugular vein according to a modified procedure [37]. The catheters were inserted and advanced until the tip of the catheter reached the aorta (carotid artery catheter) or the antrum (jugular vein catheter). The catheters were fixed firmly at the place of insertion and were tunneled subcutaneously to the back of the pig and exteriorized between the shoulder blades. The catheters were filled and sealed with physiologic saline containing 50 IU heparin and 150 000 IU penicillin (Procpen; AUV, Cuijk, The Netherlands) per milliliter and kept in and protected by a backpack, which was glued to the skin of the pig's back. During surgery the pig was given an intramuscular injection of antibiotic (300 000 IU procaine penicillin G, Depocilline, Mycofarm Nederland, De Bilt, The Netherlands) and

anodyne (50 mg flunixine, Finadyne, Schering-Plough Brussels, Belgium).

During the 1-week recovery period after surgery, the pigs were habituated to the blood sampling and infusion procedure. The carotid artery was used for blood sampling, and the jugular vein catheter was used for the infusion of fluids. During the blood sampling procedure, the catheters were flushed and filled with physiologic saline containing 5 IU heparin/mL. After the 1-week postsurgical recovery/habituation period, the pigs were assigned for a hyperinsulinemic clamp experiment or were treated with STZ to induce diabetes.

2.3. Induction of diabetes

Streptozotocin (Pharmacia & Upjohn Company, Kalamazoo, MI) was dissolved in saline (1 g/10 mL) and administrated to pigs via the jugular vein by a continuous infusion over 30 minutes. Streptozotocin treatment (110, 130, or 150 mg/kg) was initiated 4 to 5 hours after the morning meal. During the first 3 days after STZ treatment, food was offered ad libitum to avoid hypoglycemia [6,7]. Over the following 4 days, the food was offered still ad libitum, but now from 06:00 to 7:00 AM and from 03:00 to 04:00 PM, to evaluate the food intake capacity in relation to 24-hour urinary glucose excretion of diabetic pigs. This is (1) to be sure that diabetic pigs consume their meals at the isoenergetic level of 1045 kJ ME/kg $BW^{0.75}$ per day and (2) to underline the importance of isoenergetic feeding when 24-hour urinary glucose excretion is used as one of the parameters to quantify the efficacy of metformin in diabetic pigs. On day 8 after STZ treatment, pigs were fed on a restricted and isoenergetic level of 1045 kJ ME/kg $BW^{0.75}$ per day throughout the study.

2.4. Study protocols

In total, 36 pigs were used in 5 study protocols, comprising 58 experiments in the nondiabetic (normal) and/or diabetic state. Twenty-two pigs were used in 2 study protocols. A 1-week time interval was imposed between the 2 study protocols when a pig was reused.

2.4.1. Protocol 1

Fasting hepatic glucose production and whole-body glucose uptake were studied with 6,6-^{2}H-glucose infusion in 5 normal and 5 STZ (130 mg/kg)–diabetic pigs.

2.4.2. Protocol 2

Insulin-mediated hepatic glucose production and whole-body glucose uptake were studied with the hyperinsulinemic (1 or 2 mU kg^{-1} min^{-1}) euglycemic clamp technique in combination with 6,6-^{2}H-glucose infusion in 6 normal and 7 STZ (130 mg/kg)–diabetic pigs. Insulin clamps of 1 or 2 mU kg^{-1} min^{-1} were chosen because these infusion rates reflect physiologic insulin secretion. In a pig, postprandial plasma insulin concentrations are in the range of 25 to 50 mU/L [1-4].

2.4.3. Protocol 3

Insulin-mediated whole-body glucose disposal was studied with the hyperinsulinemic (2 or 8 mU kg^{-1} min^{-1}) euglycemic clamp technique in 6 normal and 6 STZ (150 mg/kg) diabetic pigs.

2.4.4. Protocol 4

Insulin-mediated whole-body glucose disposal under influence of acute mass action of plasma glucose was studied with the hyperinsulinemic (2 mU kg^{-1} min^{-1}), euglycemic (6 mmol/L) and hyperglycemic (22 mmol/L) clamp technique in 4 STZ (110 mg/kg) diabetic pigs. Pigs were used in a crossover design to eliminate any age or training or habituation effects.

2.4.5. Protocol 5

Insulin-mediated whole-body glucose disposal, after a 2-week placebo or metformin (twice 1.5 g/d) oral treatment period under conditions of isoenergetic feeding, was studied with the hyperinsulinemic (2 mU kg^{-1} min^{-1}) euglycemic clamp technique in 14 STZ (130 mg/kg) diabetic pigs. Metformin was administered to the first 50 g of chow of a meal.

2.5. Infusates

Insulin (Actrapid MC, porcine monocomponent, Novo, Copenhagen, Denmark), 6,6-^{2}H-glucose (Cambridge Isotope Laboratories, Andover, MA), and D-glucose (Merck, Darmstadt, Germany) were prepared as sterile solutions and passed through a 0.22-μm Millipore filter into sterile containers before use. Insulin was diluted in a saline solution containing pig plasma (final plasma concentration was 3%) to avoid sticking of insulin to the plastic containers and tubings. 6,6-^{2}H-Glucose was dissolved in a saline solution and D-glucose was dissolved in Aqua dest.

2.6. Fasting glucose rate of appearance

After overnight fasting, a 6,6-^{2}H-glucose solution was administered as a prime (72 mg)–continuous (1.2 mg/min) infusion for 3 hours in normal pigs. 6,6-^{2}H-Glucose was given as an adjusted prime (216 mg)–continuous (3.6 mg/min) infusion for 3 hours in hyperglycemic diabetic pigs [24] to obtain a similar plasma glucose dilution of 6,6-^{2}H-glucose in normo- and hyperglycemic pigs. Isotopic steady-state conditions were achieved within 90 minutes after initiation of the tracer infusion, and steady-state calculations were carried out during the last hour of the tracer infusion (t = 120, 135, 150, 165, and 180 minutes).

2.7. Hyperinsulinemic euglycemia and glucose rate of appearance

After overnight fasting, at t = 0 minute, insulin was administered as a prime (17 or 34 mU/kg)–continuous (1 or 2 mU kg^{-1} min^{-1}) infusion for 3 hours in normal pigs. Simultaneously, a variable infusion of a 33% D-glucose solution was started and adjusted every 10 minutes to

maintain the plasma glucose concentration at euglycemic levels and a 6,6-^{2}H-glucose solution was given as a prime (216 mg)–continuous (3.6 mg/min) infusion. Steady-state conditions were achieved within 90 minutes after initiation of the hyperinsulinemic clamp, and steady-state calculations were carried out during the last hour of the clamp (t = 120, 135, 150, 165, and 180 minutes). In hyperglycemic diabetic pigs, insulin was given as a prime (34 or 136 mU/kg)–continuous (2 or 8 mU kg^{-1} min^{-1}) infusion for 5 hours. After reaching euglycemic levels, a variable infusion of a 33% D-glucose solution was started and adjusted every 10 minutes to maintain the plasma glucose concentration at euglycemia. At t = 2 hours, 6,6-^{2}H-glucose was administered as a prime (216 mg)–continuous (3.6 mg/min) infusion for the last 3 hours of the clamp in diabetic pigs. Steady-state conditions were achieved during the last hour of the clamp, and steady-state calculations were carried out at t = 240, 255, 270, 285, and 300 minutes.

2.8. Hyperinsulinemic hyperglycemia

Hyperinsulinemic hyperglycemic clamp experiments were carried out in diabetic pigs only. After overnight fasting, insulin was given as a prime (34 mU/kg)–continuous (2 mU kg^{-1} min^{-1}) infusion for 5 hours. Simultaneously, a variable infusion of a 33% D-glucose solution was started and adjusted every 10 minutes to maintain the plasma glucose concentration at hyperglycemia. Steady-state calculations were carried out during the last hour of the clamp (t = 240, 255, 270, 285, and 300 minutes).

2.9. Fasting and postprandial blood samples

Fasting blood samples were taken before the start of hyperinsulinemic clamp experiments, and postprandial blood samples were taken 2 to 3 hours after a morning meal in the week after a clamp study.

2.10. Plasma, urine, and infusate analyses

Blood samples for determination of 6,6-^{2}H-glucose enrichment, glucose, and insulin were collected in heparinized tubes (150 USP U lithium heparin, 10 mL Venoject, Terumo, Leuven, Belgium) and immediately chilled at 0°C on ice, and centrifuged at 4°C for 10 minutes at 3000 rpm. Plasma was stored at −20°C in 4 aliquots of 0.5 mL for further analyses. Glucose was extracted from the plasma with methanol, followed by derivatizing with hydroxylamine and acetic anhydride as described previously [38]. The aldonitrile pentaacetate derivative was extracted in methylene chloride and evaporated to dryness in a stream of nitrogen. The extract was reconstituted with ethyl acetate and injected into a gas chromatograph/mass spectrometer system (HP 6890 series GC system and 5973 Mass Selective Detector, Agilent Technologies, Palo Alto, CA). Separation was achieved on a DB-17 capillary column (30 m × 0.25 mm × 0.25 μm; J&W Scientific, Palo Alto, CA). Selected ion monitoring, data acquisition, and quantitative calculations were performed using the HP Chemstation software. Glucose concentration was determined using the internal standard method (xylose was added as internal standard). The internal standard was monitored at m/z 145. Glucose was monitored at m/z 187 for glucose and m/z 189 for D-2-glucose. Glucose enrichments were calculated by dividing the area of the m/z 189 peak and the m/z 187 peak and correcting for natural enrichments. Isotopic enrichment was calculated as tracer-to-tracee ratio after subtracting the isotopic enrichment of a background plasma sample. An aliquot of the 6,6-^{2}H-glucose infusate was analyzed for the isotope concentration to calculate the actual infusion rate for each infusion experiment.

Plasma insulin concentration was measured using a Delfia assay (test kit no. B080-1101 by PerkinElmer Life Sciences, trust by Wallac Oy, Turku, Finland). This specific pig insulin assay was validated using pig insulin standards. Rapid measurement of plasma glucose concentration was achieved by centrifuging (for 20 seconds) blood samples in a microcentrifuge (Beckman Instruments, Palo Alto, CA). Subsequently, plasma glucose concentrations were analyzed enzymatically on an autoanalyzer of Radiometer (ABL and AML, Copenhagen, Denmark). Urine glucose concentrations were also analyzed on an autoanalyzer of Radiometer (ABL and AML). Urine was collected in buckets containing 0.5 g Halamid-d (sodium-p-toluenesulfonchloramide, Akzo Nobel Chemicals, Amersfoort, The Netherlands) to prevent microbial breakdown of glucose. Urine volume was registered over a given interval to calculate the rate of urinary glucose excretion. Urine samples (0.5 mL) were stored at −20°C for later glucose analyses. Ketones (acetoacetic acid) were determined in fresh urine by a reagent strip test (Ketostix, Bayer Diagnostics, Mijdrecht, The Netherlands).

Blood samples for determination of triglycerides and NEFA were collected in tubes containing EDTA (0.47 mol/L EDTA, 10 mL Venoject, Terumo) and immediately chilled at 0°C on ice, and centrifuged at 4°C for 10 minutes at 3000 rpm. Plasma was stored at −20°C in 4 aliquots of 0.5 mL for further analyses.

Concentrations of triglycerides, cholesterol, and NEFA were measured using commercially available test kits (Boehringer Mannheim, Mannheim Germany; Human, Wiesbaden, Germany; and Wako Chemicals, Neuss, Germany, respectively).

2.11. Calculations

2.11.1. 6,6-^{2}H-Glucose kinetics

Fasting and insulin-mediated rate of appearance (Ra) of glucose was calculated by dividing the 6,6-^{2}H-glucose infusion rate (mg/min) by the plasma 6,6-^{2}H-glucose enrichment (mg/mg%). Insulin-mediated hepatic glucose production was calculated as measured Ra glucose minus exogenous glucose infusion rate. Under hyperglycemic conditions, the rate of disappearance (Rd) of glucose, or

Table 1
Fasting and insulin-stimulated glucose metabolism in normal and diabetic (STZ 130 and 150 mg/kg) pigs[a]

Pigs (n)	Normal (6)	Normal (6)	Diabetic (7)	Diabetic (6)
STZ dose (mg/kg)	0	0	130	150
Insulin clamp (mU kg^{-1} min^{-1})	1	2	2	8
6,6-^{2}H-glucose infusion	Yes	No	Yes	No
Fasting plasma glucose (mmol/L)	5.3 ± 0.2[a]	4.8 ± 0.2[a]	21.7 ± 1.1[b]	22.0 ± 0.8[b]
Fasting plasma insulin (mU/L)	5 ± 1 (range, 2-10)	4 ± 1	9 ± 7 (range, 1-38)	3 ± 1
Time to reach euglycemia in diabetic pigs (min)	NA	NA	145 ± 22	243 ± 21
Steady-state clamp plasma glucose (mmol/L) and CV%	5.1 ± 0.1[a]	4.7 ± 0.2[a]	6.1 ± 0.8[a,b]	7.5 ± 0.9[b]
	8 ± 2	10 ± 2	9 ± 1	9 ± 2
Steady-state clamp plasma insulin (mU/L) and CV%	24 ± 5[a]	51 ± 6[b]	45 ± 5[b]	228 ± 36[c]
	12 ± 2	14 ± 2	14 ± 2	17 ± 3
Steady-state clamp glucose infusion rate (mg kg^{-1} min^{-1})	20.3 ± 2.1[a]	28.1 ± 1.4[b]	5.0 ± 1.4[c]	3.2 ± 1.0[c]
Body weight (kg)	40.5 ± 1.6	39.4 ± 2.0	41.3 ± 2.8	39.7 ± 2.7

Different letters (a, b, c) in a row indicate a significant difference ($P < .05$) between pig groups. Data were generated from protocols 2 and 3. NA indicates not applicable; CV%, coefficient of variation within pigs.

[a] Determined before and during hyperinsulinemic (insulin infusion rates of 1, 2, or 8 mU kg^{-1} min^{-1}) euglycemic clamp studies with or without 6,6-^{2}H-glucose infusion. Tracer-derived results are depicted in Figs. 2 and 3.

whole-body glucose uptake, was corrected for urinary glucose excretion. This implies that whole-body glucose uptake was calculated as measured Ra glucose minus urinary glucose excretion.

2.12. Statistical methods and analyses

Analyses were done with the GenStat software [39] Multiple comparisons were submitted to analyses of variance (ANOVAs). When ANOVA showed a significant difference among pig groups, the unpaired Student *t* test (least significant difference testing) was used as a post hoc analysis to compare individual pig groups (Table 1). Statistical significance between averages from 2 data sets (as within subject comparison) was determined by the paired Student *t* test (Table 2). Statistical significance between averages from 2 data sets (as between subject comparison) were determined by the unpaired Student *t* test (Tables 3-5, Figs. 3 and 4). Correlation between 2 data sets was determined by linear regression analysis (Figs. 1 and 2).

Table 2
Hyperinsulinemic (insulin infusion rate of 2 mU kg^{-1} min^{-1}) clamp studies at euglycemia or hyperglycemia in 4 diabetic (STZ 110 mg/kg) pigs

Clamp	Euglycemia	Hyperglycemia
Fasting plasma glucose (mmol/L)	22.3 ± 2.1	23.0 ± 2.4
Fasting plasma insulin (mU/L)	17 ± 13 (range, 4-51)	12 ± 5 (range, 3-21)
Steady-state clamp plasma glucose (mmol/L) and CV%	6.0 ± 0.1	22.4 ± 2.6
	8 ± 1	3 ± 1
Time to reach euglycemia in diabetic pigs (min)	139 ± 23	NA
Steady-state clamp plasma insulin (mU/L) and CV%	54 ± 16	62 ± 19
	21 ± 2	16 ± 5
Steady-state clamp glucose infusion rate (mg kg^{-1} min^{-1})	5.1 ± 1.0	8.2 ± 0.8[a,*]
Body weight (kg)	46.9 ± 1.7	47.7 ± 3.5

[a] Fasting urinary glucose excretion was estimated to be 2.3 mg kg^{-1} min^{-1}. Urinary glucose excretion was subtracted from the glucose infusion rate to obtain a measure of insulin-mediated whole-body glucose disposal, being 5.9 ± 0.8 mg kg^{-1} min^{-1}.

* $P < .05$.

Daily repeated measurements within pigs (Fig. 5) were submitted to ANOVA for repeated measurements. When ANOVA for repeated measurements showed a significant difference between the 2 pig groups, the unpaired Student *t* test (least significant difference testing) was used as a post hoc analysis to compare daily urinary glucose excretion between the 2 pig groups (Fig. 5). The results are expressed as means ± SEM. The criterion of significance was set at $P < .05$.

3. Results

3.1. Induction of diabetes

One week after STZ treatment, 2 of 6 pigs receiving a dose of 110 mg/kg BW and 3 of 23 pigs receiving a dose of 130 mg/kg BW had overnight fasting plasma glucose concentrations of less than 10 mmol/L and were excluded from the study. All 6 pigs treated with an STZ dose of 150 mg/kg BW showed overnight fasting plasma glucose concentrations of more than 20 mmol/L.

3.2. Food intake and urinary glucose excretion

After inducing diabetes with STZ (4-7 days), food was offered to pigs ad libitum from 06:00 to 07:00 AM and from 03:00 to 04:00 PM to evaluate the rate of food intake in

Table 3
Plasma lipid profiles in the fasting and postprandial state in 6 normal and 6 diabetic (STZ 130 mg/kg) pigs

Pigs	Normal	Diabetic
Fasting plasma triglycerides (mmol/L)	0.2 ± 0.1	1.0 ± 0.3**
Fasting plasma cholesterol (mmol/L)	1.8 ± 0.1	2.1 ± 0.2
Fasting plasma NEFA (mmol/L)	1.0 ± 0.3	0.9 ± 0.3
Postprandial plasma triglycerides (mmol/L)	0.3 ± 0.1	1.2 ± 0.3**
Postprandial plasma cholesterol (mmol/L)	1.7 ± 0.1	2.3 ± 0.2*
Postprandial plasma NEFA (mmol/L)	0.2 ± 0.1	1.5 ± 0.5**

Data were generated from protocol 2.

* $P < .05$.

** $P < .01$.

Table 4
Body weights and postprandial plasma glucose and insulin concentrations of diabetic (STZ 130 mg/kg) pigs, before and after placebo (n = 7) or metformin (n = 7) treatment over a period of 2 weeks

Diabetic pigs	Placebo treated	Metformin treated
Body weight before treatment (kg)	34.5 ± 1.2	36.3 ± 1.0
Postprandial plasma glucose before treatment (mmol/L)	25.9 ± 0.8	31.0 ± 5.8
Postprandial plasma insulin before treatment (mU/L)	1 ± 0	1 ± 0
Body weight at end of treatment (kg)	35.2 ± 2.4	40.7 ± 1.8
Postprandial plasma glucose at end of treatment (mmol/L)	35.5 ± 4.9	24.9 ± 2.2*
Postprandial plasma insulin at end of treatment (mU/L)	2 ± 1	3 ± 1

Data were generated from protocol 5.
* $P < .05$.

relation to 24-hour urinary glucose excretion of diabetic pigs (Fig. 1). Ad libitum food intake ranged from 800 to 2036 g/d and 24-hour urinary glucose excretion ranged from 341 to 886 g/d. From linear regression analysis, the equation was calculated to be $y = 0.33x + 141$ ($R = 0.67$) with a highly significant ($P < .0002$) correlation between both parameters. The slope of the line predicted that each gram of feed intake (ie, 0.41 g starch and sugars) leads to 0.33 g of urinary glucose excretion. The intercept was equal to the fasting 24-hour urinary glucose excretion (141 g/d). Average body weight of all diabetic pigs was 37.3 ± 1.2 kg and, thus, their predicted fasting urinary glucose excretion was equal to 2.6 mg kg^{-1} min^{-1}. Diabetic pigs were fasted overnight to measure a factual rate of fasting urinary glucose excretion and, afterward, urine was collected for 8 hours. It appeared that the factual fasting urinary glucose excretion was similar to the predicted value and amounted 2.3 ± 0.2 mg kg^{-1} min^{-1}.

On day 8 after STZ treatment, pigs were fed on a restricted and isoenergetic level of 1045 kJ ME/kg $BW^{0.75}$ per day throughout the study. The relationship between fasting plasma glucose concentration and 24-hour urinary glucose excretion at isoenergetic feeding (Fig. 2) was

Table 5
Hyperinsulinemic (insulin infusion rate of 2 mU kg^{-1} min^{-1}) euglycemic clamps in diabetic (STZ 130 mg/kg) pigs after placebo (n = 7) or metformin (n = 7) treatment over a period of 2 weeks

Diabetic pigs	Placebo treated	Metformin treated
Fasting plasma glucose (mmol/L)	19.4 ± 0.6	14.7 ± 1.5*
Fasting plasma insulin (mU/L)	14 ± 9 range (1-55)	11 ± 6 range (3-37)
Steady-state clamp plasma glucose (mmol/L) and CV%	6.1 ± 0.9 8 ± 1	5.3 ± 0.3 9 ± 1
Time to reach euglycemia in diabetic pigs (min)	175 ± 36	105 ± 28
Steady-state clamp plasma insulin (mU/L) and CV%	48 ± 6 11 ± 2	42 ± 7 8 ± 1
Steady-state clamp glucose infusion rate (mg kg^{-1} min^{-1})	5.8 ± 1.7	9.4 ± 2.2*

Data were generated from protocol 5.
* $P < .05$.

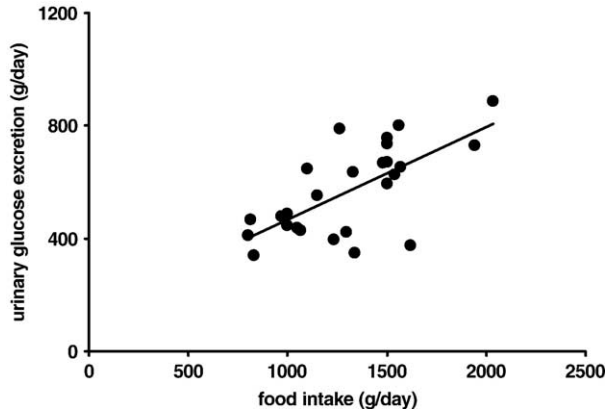

Fig. 1. Relationship between ad libitum food intake and urinary glucose excretion in diabetic (STZ 110, 130, and 150 mg/kg) pigs (n = 26) on day 7 after STZ treatment. Data were generated from protocols 2 to 5.

positive, and from linear regression analysis, the equation was calculated to be $y = 37.6x - 56$ ($R = 0.73$, $P < .001$).

In urine of diabetic pigs (STZ dose of 110 and 130 mg/kg), no ketones or no trace (0.5 mmol/L) amounts of ketones were detected, whereas STZ at a dose of 150 mg/kg induced intermediate to moderately high levels of ketones in urine (1.5-4 mmol/L).

3.3. Glucose metabolism and insulinemia

Glucose metabolism in normal and diabetic (STZ doses of 110, 130, and 150 mg/kg) pigs after overnight fasting and after insulin infusion at the rate of 1, 2, or 8 mU kg^{-1} min^{-1} is presented in Tables 1 and 2 and Figs. 3 and 4. Overnight fasting plasma glucose concentrations were on average 4-fold greater in the 3 diabetic pig groups compared with normal pigs. Overnight fasting plasma insulin concentrations however were comparable and did not differ significantly among normal and diabetic pigs. Diabetic pigs treated with STZ (110 and 130 mg/kg) had a wide range (4-51 and 1-38 mU/L) in fasting plasma insulin concentrations, which on average were 2-fold higher than in normal and diabetic (150 mg STZ/kg) pigs, which showed a narrow range

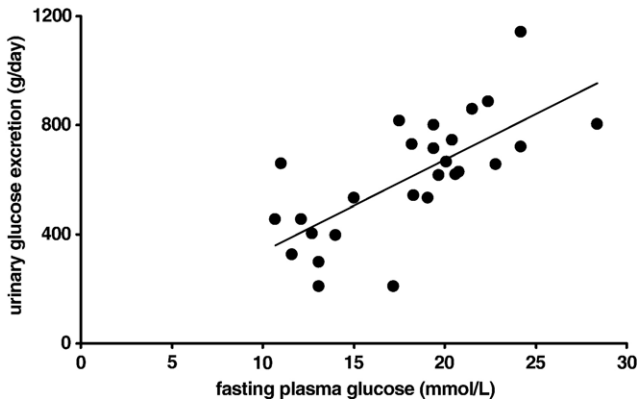

Fig. 2. Relationship between fasting plasma glucose concentration and urinary glucose excretion in diabetic (STZ 110, 130, and 150 mg/kg) pigs (n = 27) at isoenergetic feeding. Data were generated from protocols 2 to 5.

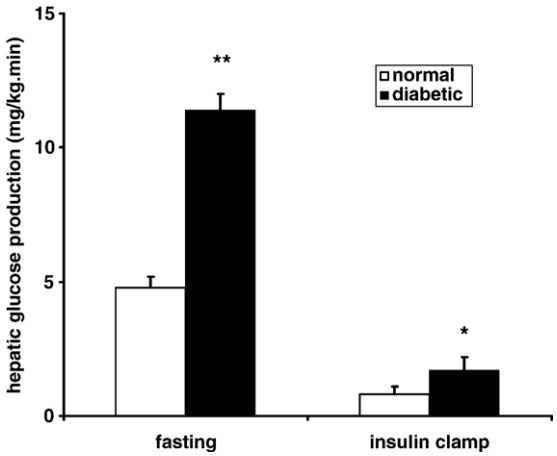

Fig. 3. Hepatic glucose production (HGP) after overnight fasting and during a hyperinsulinemic (insulin infusion rate of 1 and 2 mU kg^{-1} min^{-1} in normal and diabetic [STZ 130 mg/kg] pigs, respectively) euglycemic (~6 mmol/L) clamp with 6,6-^{2}H-glucose infusion. Fasting HGP was measured in 5 normal and 5 diabetic (STZ 130 mg/kg) pigs. Insulin-inhibited HGP was measured in 6 normal and 6 diabetic (STZ 130 mg/kg) pigs. Data were generated from protocols 1 and 2. *P < .05 and **P < .01 compared with normal pigs.

(2-10 and 0-6 mU/L) in fasting plasma insulin concentrations (Tables 1 and 2). Pigs receiving 110 and 130 mg/kg of STZ showed no significant correlation between fasting plasma glucose and fasting insulin concentrations (R = 0.22, P = NS, data not shown). Fasting Ra glucose was approximately 2-fold greater in diabetic (130 mg STZ/kg) pigs compared with normal pigs. Insulin-inhibited hepatic glucose production was 2-fold greater, and insulin-stimulated whole-body glucose uptake was 3-fold lower in diabetic (130 mg STZ/kg) vs normal pigs (Figs. 3 and 4).

Diabetic pigs treated with STZ (150 mg/kg) required an insulin infusion rate of 8 mU kg^{-1} min^{-1} during the hyperinsulinemic euglycemic clamp to reach near-euglycemic levels within the time frame (5 hours) of the clamp study. Nevertheless, at an insulin infusion rate of 8 mU kg^{-1} min^{-1}, 3 of 6 diabetic pigs treated with STZ (150 mg/kg) did not reach euglycemia (5-6 mmol/L) and were clamped at steady-state plasma glucose concentrations of 11.1, 7.8, and 7.8 mmol/L, respectively. The steady-state glucose infusion rate was ~8-fold lower in the diabetic pigs (150 mg/kg STZ) compared with the average glucose infusion rates achieved in normal pigs (Table 1). For diabetic pigs treated with STZ (110-130 mg/kg), an insulin infusion rate of 2 mU kg^{-1} min^{-1} proved to be sufficient to reach near euglycemic levels within the time frame (5 hours) of the clamp study. The steady-state glucose infusion rates were 5- to 6-fold lower compared with the average glucose infusion rates achieved in normal pigs (Table 1). To correct the glucose infusion rate (Table 1) for any differences in steady-state plasma glucose and insulin concentrations between pig groups, we have adjusted the glucose infusion rate per pig group by dividing glucose infusion with steady-state plasma insulin and glucose concentrations. This procedure will give the insulin sensitivity index for glucose clearance per pig group. Any differences in steady-state plasma glucose and insulin concentrations between pig groups will now become a less critical issue. For Table 1, this implies that the glucose infusion rates (mg kg^{-1} min^{-1}) per pig group are transformed to the insulin sensitivity index for glucose clearance (glucose infusion rate divided by insulin concentration times glucose concentration in arbitrary units [au]) as follows: 20.3 ± 2.1^a mg kg^{-1} min^{-1} becomes 16.6 ± 1.8^a au; 28.1 ± 1.4^b mg kg^{-1} min^{-1} becomes 11.7 ± 0.8^b au; 5.0 ± 1.4^c mg kg^{-1} min^{-1} becomes 1.8 ± 0.3^c au; and 3.2 ± 1.0^c mg kg^{-1} min^{-1} becomes 0.2 ± 0.0^d au. The insulin sensitivity index for glucose clearance is significantly lower in both (130 and 150 mg/kg STZ) diabetic pig groups compared with the normal pig groups. When the same procedure is applied to the fasting condition (Fig. 4), whole-body glucose uptake (mg kg^{-1} min^{-1}) is transformed to the insulin sensitivity index for glucose clearance (in arbitrary units) from 4.8 ± 0.4 mg kg^{-1} min^{-1} to 18.1 ± 1.9 au for normal pigs and from 9.1 ± 0.8** mg kg^{-1} min^{-1} to 4.7 ± 1.4** au for (130 mg/kg STZ) diabetic pigs. The insulin sensitivity index for glucose clearance at fasting is significantly lower in (130 mg/kg STZ) diabetic pigs compared with normal pigs.

Diabetic pigs (STZ 110 mg/kg) were clamped both at euglycemic and hyperglycemic levels (Table 2) to study acute mass action of plasma glucose on insulin-stimulated whole-body glucose disposal. Insulin-mediated whole-body glucose disposal was increased by 16% (5.9 vs 5.1 mg kg^{-1} min^{-1}, P < .05) at hyperglycemia compared with euglycemia.

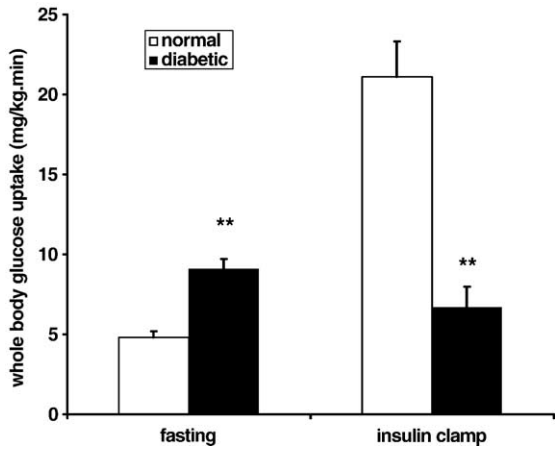

Fig. 4. Whole-body glucose uptake (WBGU) after overnight fasting and during a hyperinsulinemic (insulin infusion rate of 1 and 2 mU kg^{-1} min^{-1} in normal and diabetic [STZ 130 mg/kg] pigs, respectively) euglycemic (~6 mmol/L) clamp with 6,6-^{2}H-glucose infusion. Fasting urinary glucose excretion was estimated to be 2.3 mg kg^{-1} min^{-1}. Urinary glucose excretion was subtracted from fasting hepatic glucose production to provide a measure of fasting whole-body glucose uptake. Fasting WBGU was measured in 5 normal and 5 diabetic (STZ 130 mg/kg) pigs. Insulin-stimulated WBGU was measured in 6 normal and 6 diabetic (STZ 130 mg/kg) pigs. Data were generated from protocols 1 and 2. **P < .01 compared with normal pigs.

3.4. Lipidemia

Plasma concentrations of triglycerides, cholesterol, and NEFA in normal and diabetic (STZ 130 mg/kg) pigs are shown in Table 3. Fasting plasma triglyceride concentrations were found to be 5-fold elevated in diabetic pigs compared with normal pigs, whereas fasting cholesterol and NEFA concentrations did not differ between both groups. In the postprandial phase of diabetic pigs, plasma triglycerides, cholesterol, and NEFA concentrations were elevated (4-, 1.3-, and 7-fold, respectively) compared with normal pigs.

3.5. Metformin treatment

Postprandial plasma glucose and insulin concentrations at the beginning and the end of the 2-week placebo vs metformin treatment of diabetic pigs are shown in Table 4. Initial plasma glucose and insulin concentrations were similar among the pigs, whereas after the treatment period of 14 days with metformin, plasma glucose concentrations declined by 30% compared with placebo-treated pigs at similar plasma insulin concentrations. Metformin-treated diabetic pigs grew faster (+4.4 ± 1.1 kg, $P < .01$) than placebo-treated diabetic pigs (+0.7 ± 1.2 kg, P = NS).

Daily urinary glucose excretion (g/kg food) in diabetic pigs after metformin vs placebo treatment is depicted in Fig. 5. Initial urinary glucose excretion was similar among both groups of pigs, but after 1 week of metformin treatment, urinary glucose excretion became significantly ($P < .05$) less (20%-40%) in the metformin group compared with the placebo group.

Basal plasma glucose concentrations were significantly ($P < .05$) reduced at comparable basal plasma insulin concentrations in metformin-treated pigs compared with placebo-treated pigs (Table 5). Under similar insulin stimulation (3- to 4-fold over fasting insulin concentrations), whole-body glucose disposal was found to be 1.6-fold greater in the metformin group compared with the placebo group with diabetes (9.4 vs 5.8 mg kg^{-1} min^{-1}).

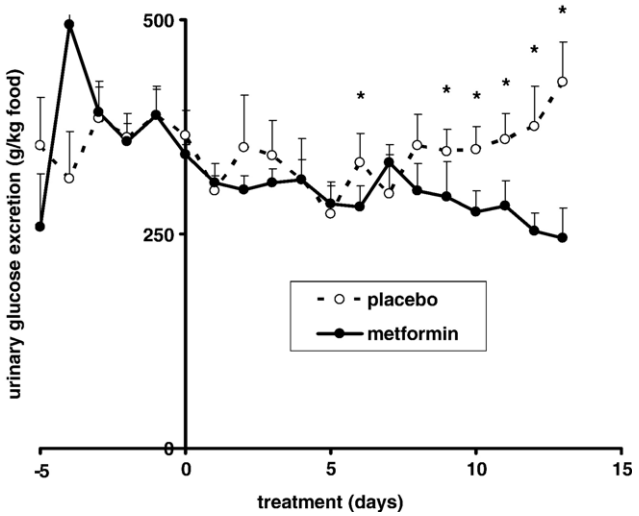

Fig. 5. Urinary glucose excretion in diabetic (STZ 130 mg/kg) pigs treated with metformin or placebo (n = 7 per group). On day −8, pigs were treated with STZ, and on day 0, treatment with metformin or placebo started. On day 14, the experiment was completed with an insulin clamp study (results in Table 5). Data were generated from protocol 5. *$P < .05$ compared with placebo-treated pigs.

4. Discussion

This study demonstrates that the chemical induction of pancreatic beta-cell dysfunction in domestic pigs leads to insulin-resistant diabetes mellitus. Depending on the dose of STZ, infused over a 30-minute period, a type 1–like or a type 2–like diabetes can be created.

We found that an STZ dose of 110 mg/kg did not lead to fasting hyperglycemia (>10 mmol/L) in 2 of 6 pigs, indicating that this dose of STZ was too low to reliably induce diabetes. On the other hand, a dose of STZ (150 mg/kg) resulted in a clear hyperglycemic, hypoinsulinemic, and ketotic diabetic state in all pigs. The latter implies that their diabetic state mimicked type 1 diabetes mellitus. An STZ dose of 130 mg/kg resulted in fasting hyperglycemia (>10 mmol/L) in 20 of 23 pigs with concomitant fasting normoinsulinemia. These diabetic pigs showed minimal ketonuria, were moderately dyslipidemic, developed severe insulin resistance for glucose metabolism, maintained growth without insulin therapy, and therefore resembled type 2 diabetes mellitus.

From a quantitative point of view, the insulin resistance in STZ (130 mg/kg)–diabetic pigs was mainly present at the level of whole-body glucose uptake and to a lesser extent at the level of hepatic glucose production. This is consistent with human type 2 diabetes mellitus where peripheral (extrahepatic) tissues are the primary site of insulin resistance [13,40]. However, human type 2 diabetes mellitus is generally considered to have a significant underlying (genetic) compound of insulin resistance, which predates beta-cell failure and is independent of glucose and/or lipid toxicity exacerbating insulin resistance, whether whole body or hepatic. Insulin resistance in human type 2 diabetes mellitus is therefore both of genetic and acquired origin involving glucose toxicity, lipid toxicity, substrate competition between fat and glucose (the Randle cycle), and an inflammatory response [15-21,41,42]. The insulin resistance in STZ-diabetic pigs is acquired, but not caused by hypoinsulinemia-induced catabolism because STZ-diabetic pigs maintained a positive energy balance. In this respect, the STZ-diabetic pig model seems ideal for studying the mechanisms of "metabolic insulin resistance of human type 2 diabetes mellitus" and the effects of pharma-, nutraceuticals or functional foods on this type of acquired insulin resistance can be investigated. Insulin resistance for glucose metabolism in pigs appeared within 1 week after STZ treatment and remained stable during the following 2 weeks of the study. In addition, the severity of insulin resistance in diabetic pigs was relatively independent on acute changes in

plasma glucose concentrations because we have shown that insulin-mediated glucose metabolism was only 16% increased at hyperglycemia compared with euglycemia. This is in agreement with previous studies in man, dog, and rat [18,22,43], which have shown that insulin resistance at euglycemia was only partially compensated at hyperglycemia. In STZ-diabetic rats, a 30% to 50% reduction in insulin action was reported [24,28,30,43,44], but some rat studies could not demonstrate a defect in insulin action [29,45]. Studies in STZ diabetic dogs reported a 50% reduction [22], or no reduction [46], in insulin action. Compared with rodent and dog studies, insulin resistance in STZ diabetic pigs is reproducible (all 31 STZ diabetic pigs developed insulin resistance) and severe as manifested by a ~75% reduction in insulin action.

In diabetic pigs, whole-body glucose uptake decreased from 9.1 mg kg^{-1} min^{-1} at fasting (no exogenous insulin infusion) to 6.7 mg kg^{-1} min^{-1} during the clamp (during insulin infusion). We have observed this phenomenon before in diabetic rats using a similar experimental protocol [24]. This can be explained by the fact that fasting whole-body glucose uptake in diabetic pigs is mainly driven by mass action of hyperglycemia and amounts 9.1 mg kg^{-1} min^{-1}. During the hyperinsulinemic euglycemic clamp, insulin-stimulated whole-body glucose uptake amounts 6.7 mg kg^{-1} min^{-1} and is mainly driven by insulin and no longer by mass action of hyperglycemia because the clamp is carried out at euglycemic levels. Because of the severity of insulin resistance, insulin-stimulated whole-body glucose uptake at euglycemic levels is lower than glucose-stimulated whole-body glucose at hyperglycemic levels. This phenomenon underlines the severity of insulin resistance for whole-body glucose uptake in STZ diabetic pigs.

Fasting plasma insulin concentrations have invariably been found to be normal or increased in human type 2 diabetes mellitus. Even in studies where normal fasting plasma insulin levels have been reported, they uniformly have been in the high reference range [13]. We documented that mean fasting plasma insulin concentrations were similar in 130 mg/kg STZ-diabetic pigs (mean, 9 mU/L; range, 1-38 mU/L) compared with normal pigs (mean, 5 mU/L; range, 2-10 mU/L). Normal fasting plasma insulin concentrations have been observed before in alloxan-diabetic minipigs [47], but the studies with STZ-diabetic (mini)pigs showed fasting hypoinsulinemia [6-8,25,26,48]. As discussed previously [48], the discrepancy in fasting plasma insulin concentrations may be explained by different strain, age, and sex, but also by different beta-cell toxic compound (alloxan or STZ) and the way of STZ administration. We have infused STZ over a 30-minute period, whereas other studies injected STZ. A slow infusion of STZ induces less severe beta-cell damage [32], which may result in fasting normoinsulinemia. On the other hand, we observed postprandial hypoinsulinemia (close to the detection limit of 1 mU/L) 2 to 3 hours after feeding in STZ diabetic pigs compared with postprandial insulin concentrations of ~25 mU/L in normal pigs (data not shown). The reason for this phenomenon is not clear, but the acute prandial stimulation of insulin secretion superimposed on the chronic stimulation by hyperglycemia may temporarily exhaust the insulin secretory capacity of the remaining beta cells, 2 to 3 hours postprandially. From the latter, it is clear that beta-cell dysfunction exists in STZ-diabetic pigs with regard to postprandial plasma insulin concentrations, which are very low compared with normal pigs. Pigs receiving 110 and 130 mg/kg of STZ showed no significant correlation between fasting plasma glucose and fasting insulin concentrations. This indicates that the variation in fasting (baseline) plasma insulin concentrations in STZ-diabetic pigs is insufficient to affect overnight fasting hyperglycemia. This lack of correlation can be explained by the following: (1) the severity of insulin resistance: a 4- to 5-fold reduction in insulin sensitivity is difficult to overcome with small changes in fasting plasma insulin concentrations; (2) the variation in fasting plasma insulin concentrations may be caused by pulsatility [27] of insulin secretion and, therefore, a high intrapig variation in fasting plasma insulin concentrations could be present. Because we did not collect multiple blood samples per pig in the fasting condition, we cannot verify the latter explanation. However, we did collect fasting blood samples from 8 STZ diabetic pigs on 2 different days, and these insulin concentrations showed an intrapig coefficient of variation of 67%. In future experiments, serial (preferably intraportal) blood sampling will be necessary to investigate any pulsatile insulin secretion in our STZ diabetic pig models.

Dyslipidemia in diabetic pigs was most manifested by elevated plasma triglycerides in the fasting (1.0 mmol/L) and postprandial (1.2 mmol/L) phases compared with normal pigs (0.2 and 0.3 mmol/L, respectively). Plasma cholesterol and NEFA concentrations were elevated in the postprandial phase only. The difference in fasting and postprandial dyslipidemia in diabetic pigs may have been caused by the differences in plasma insulin concentrations. Insulin is known to be a strong regulator of lipid metabolism [27,49-51], and the diabetic pigs in our study had fasting normoinsulinemia but postprandial hypoinsulinemia. Despite dyslipidemic status is theoretically defined when the levels of plasma triglycerides are greater than 1.7 mmol/L [52,53], it is clear that STZ-diabetic pigs, fed with a low-fat diet, reveal an endogenous drive toward elevated plasma triglyceride concentrations compared with normal pigs. Thus, it may be expected that these pigs when fed with a high-fat, high-cholesterol diet will develop a clear-cut dyslipidemia [11].

4.1. Metformin

We found that metformin treatment of diabetic pigs resulted in a 24% reduction in fasting plasma glucose concentrations, a 30% reduction in postprandial plasma

glucose concentrations, a 20% to 40% reduction in 24-hour urinary glucose excretion, and a 60% increase in insulin-stimulated whole-body glucose disposal, as compared with placebo-treated diabetic pigs.

The magnitude of improvement by metformin on glycemic control in diabetic pigs is comparable to that reported in human studies [33,34]. The effect of metformin in pigs progressed over a 2-week period, and the reduction in 24-hour urinary glucose excretion did not reach steady state yet in diabetic pigs (Fig. 5). In human studies, it is a normal phenomenon that the metformin treatment needs to be continued for several weeks before a stable effect of metformin on glucose homeostasis can be observed [33,34]. By contrast, rodent studies [31,54-58] reported an immediate (within days) effect of metformin at high doses (100-500 mg/kg) on glucose homeostasis. These studies were of pharmacologic use, whereas our pig study was designed to serve a more physiologic approach, using a dose of 3 g metformin per day (~75 mg/kg). A metformin treatment of ~75 mg/kg is considered to be the maximum dose, whereas most human dosing is in the order of 10 to 20 mg/kg.

4.2. Isoenergetic feeding

Streptozotocin-diabetic pigs become hyperphagic 1 to 2 weeks after STZ treatment (data not shown), which is in agreement with previous observations in rats [24,43]. Streptozotocin treatment induces postprandial hypoinsulinemia, which may induce, in combination with hypoleptinemia [8], an increase in food intake [59,60]. Diabetic pigs were fed at the isoenergetic level of 1045 kJ/kg $BW^{0.75}$ per day to exclude an effect of food intake on insulin sensitivity and glucose homeostasis. This restricted feeding level proved sufficient for maintaining a constant body weight in diabetic pigs.

We found a significant correlation between daily ad libitum food intake and 24-hour urinary glucose excretion in diabetic pigs (Fig. 1). This indicates that daily changes in food consumption (independent of major changes in body weight and body composition) strongly affect glucose homeostasis in diabetic pigs and underlines the necessity of isoenergetic feeding when studying antidiabetic drug therapy. Indeed, it is well known that energy intake restriction can improve glucose homeostasis and tissue sensitivity to insulin [61]. Part of the antidiabetic effect of metformin may therefore stem from its ability to reduce food intake [62,63], and in most studies, food intake has not strictly been controlled [31,54,56,58]. Our study quantified the antidiabetic effects of metformin at constant food intake. Metformin-treated diabetic pigs grew faster than placebo-treated pigs. This can be explained by the fact that metformin treatment of diabetic pigs reduced urinary glucose excretion compared with placebo treatment and, as a consequence, more energy from the diet remained available for growth in the metformin-treated diabetic pigs.

We found a significant correlation between 24-hour urinary glucose excretion and fasting plasma glucose concentration at isoenergetic feeding in diabetic pigs (Fig. 2). This indicates that 24-hour urinary glucose excretion is a good indicator of hyperglycemia when diabetic pigs are on constant food intake. However, for the true correlation between 24-hour urinary glucose excretion and average 24-hour blood glucose concentrations, blood samples need to be collected over a 24-hour period. We have not done this, but it can be assumed that this will lead to a stronger correlation between 24-hour urinary glucose excretion and hyperglycemia in diabetic pigs.

In summary, domestic pigs fed with a low-fat diet, and infused with STZ (130 mg/kg), revealed a type 2–like diabetes mellitus. We defined *type 2 diabetes mellitus* in the present pig study by the following criteria: hyperglycemia (>10 mmol/L), minimal ketonuria (urinary ketone concentration between 0 and 0.5 mmol/L), fasting normo- to hyperinsulinemia (>9 mU/L), elevated plasma triglyceride concentrations (>1 mmol/L), insulin resistance (several-fold reduction in insulin action), and a positive energy balance without exogenous insulin treatment (no weight loss over a 2-week period). In addition, the type 2–like diabetic pigs responded positively to oral metformin treatment for 2 weeks with a significant improvement in glucose homeostasis and a significant reduction in insulin resistance.

References

[1] Kaneko JJ, Harvey JW, Bruss ML. Clinical biochemistry of domestic animals. 5th ed. San Diego (Calif): Academic Press; 1997.
[2] Swindle MM. Swine as models in biomedical research. Ames (Iowa): Iowa State University Press; 1992.
[3] Tumbleson ME. Swine in biomedical research, vols. 1-3. New York: Plenum Press; 1996.
[4] Tumbleson ME, Schook LB. Advances in swine in biomedical research, vol. 1 & 2. New York: Plenum Press; 1996.
[5] Barb CR, Cox NM, Carlton CA, et al. Growth hormone secretion, serum, and cerebral spinal fluid insulin and insulin-like growth factor–I concentrations in pigs with streptozotocin-induced diabetes mellitus. Proc Soc Exp Biol Med 1992;201:223-8.
[6] Gäbel H, Bitter-Suermann H, Henriksson C, et al. Streptozotocin diabetes in juvenile pigs. Evaluation of an experimental model. Horm Metab Res 1985;17:275-80.
[7] Grussner R, Nakhleh R, Grussner A, et al. Streptozotocin-induced diabetes mellitus in pigs. Horm Metab Res 1993;25:199-203.
[8] Ramsay TG, White ME. Insulin regulation of leptin expression in streptozotocin diabetic pigs. J Anim Sci 2000;78:1497-503.
[9] Romsos DR, Leveille GA, Allee GL. Alloxan diabetes in the pig (*Sus domesticus*). Response to glucose, tolbutamide and insulin administration. Comp Biochem Physiol 1971;40A:557-68.
[10] Fricker J. The pig: a new model of diabetic atherosclerosis. DDT 2001;6:921-2.
[11] Gerrity RG, Natarajan R, Nadler JL, et al. Diabetes-induced accelerated atherosclerosis in swine. Diabetes 2001;50:1654-65.
[12] Bays H, Mandarino L, DeFronzo RA. Role of the adipocyte, free fatty acids, and ectopic fat in pathogenesis of type 2 diabetes mellitus: peroxisomal proliferator-activated receptor agonists provide a rational therapeutic approach. J Clin Endocrinol Metab 2004;89:463-78.

[13] DeFronzo RA, Bonadonna RC, Ferrannini E. Pathogenesis of NIDDM. A balanced overview. Diabetes Care 1992;15:318-68.
[14] Reaven GM. Role of insulin resistance in human disease. Diabetes 1988;37:1595-607.
[15] Bevilacqua S, Buzzigoli G, Bonadonna R, et al. Operation of Randle's cycle in patients with NIDDM. Diabetes 1990;39:383-9.
[16] Boden G. Role of fatty acids in the pathogenesis of insulin resistance and NIDDM. Diabetes 1997;46:3-10.
[17] Felley CP, Felley EM, van Melle GD, et al. Impairment of glucose disposal by infusion of triglycerides in humans: role of glycemia. Am J Physiol 1989;256:E747-52.
[18] Mandarino LJ, Consoli A, Jain A, et al. Interaction of carbohydrate and fat fuels in human skeletal muscle: impact of obesity and NIDDM. Am J Physiol 1996;270:E463-70.
[19] Rossetti L, Giaccari A, DeFronzo RA. Glucose toxicity. Diabetes Care 1990;13:610-30.
[20] Thiebaud D, DeFronzo RA, Jacot E, et al. Effect of long chain triglyceride infusion on glucose metabolism in man. Metab Clin Exp 1982;31:1128-36.
[21] Unger RH. Lipotoxicity in the pathogenesis of obesity-dependent NIDDM: genetic and clinical implications. Diabetes 1995;44:863-70.
[22] Bevilacqua S, Barrett EJ, Smith D, et al. Hepatic and peripheral insulin resistance following streptozotocin-induced insulin deficiency in the dog. Metabolism 1985;34:817-25.
[23] Jones CW, Reynolds WA, Hoganson GE. Streptozotocin diabetes in the monkey. Plasma levels of glucose, insulin, glucagons, and somatostatin, with corresponding morphometric analysis of islet endocrine cells. Diabetes 1980;29:536-46.
[24] Koopmans SJ, de Boer SF, Sips HCM, et al. Whole body and hepatic insulin action in normal, starved, and diabetic rats. Am J Physiol 1991;260:E825-32.
[25] Marshall M, Oberhofer H, Staubesand J. Early micro- and macroangiopathy in the streptozotocin diabetic minipig. Res Exp Med (Berl) 1980;177:145-58.
[26] Canavan JP, Flecknell PA, New JP, et al. The effect of portal and peripheral insulin delivery on carbohydrate and lipid metabolism in a miniature pig model of human IDDM. Diabetologia 1997;40:1125-34.
[27] Koopmans SJ, Sips HCM, Krans HMJ, et al. Pulsatile intravenous insulin replacement in streptozotocin-diabetic rats is more efficient than continuous delivery: effects on glycaemic control, insulin-mediated glucose metabolism and lipolysis. Diabetologia 1996;39:391-400.
[28] Blondel O, Bailbe D, Portha B. Insulin resistance in rats with non–insulin dependent diabetes induced by neonatal (5 days) streptozotocin: evidence for reversal following phlorizin treatment. Metabolism 1990;39:787-93.
[29] Kergoat M, Portha B. In vivo hepatic and peripheral insulin sensitivity in rats with non–insulin-dependent diabetes induced by streptozotocin. Diabetes 1985;34:1120-6.
[30] Kruszynska YT, Home PD. Liver and muscle insulin sensitivity, glycogen concentration and glycogen synthase activity in a rat model of non–insulin dependent diabetes. Diabetologia 1988;31:304-9.
[31] Reed MJ, Meszaros K, Entes LJ, et al. Effect of masoprocol on carbohydrate and lipid metabolism in a rat model of type II diabetes. Diabetologia 1999;42:102-6.
[32] Marshall M. Induction of chronic diabetes by streptozotocin in the miniature pig. Res Exp Med (Berl) 1979;175:187-96.
[33] Bailey CJ, Turner RC. Metformin. N Engl J Med 1996;334:574-9.
[34] Cusi K, DeFronzo RA. Metformin: a review of its metabolic effects. Diabetes Rev 1998;6:89-131.
[35] Hundal RS, Krssak M, Dufour S, et al. Mechanism by which metformin reduces glucose production in type 2 diabetes. Diabetes 2000;49:2063-9.
[36] CVB. Centraal Veevoederbureau. Dutch Norms for Livestock Feeding and Nutritive Values for Feedstuffs. 24th ed Lelystad (The Netherlands): CVB Press; 2000.
[37] Koopmans SJ, Mroz Z, Dekker R, et al. Insulin-stimulated net utilization of plasma glucose and amino acids in growing pigs. In: Souffrant WB, Metges CC, editors. Progress in research on energy and protein metabolism. Wageningen, The Netherlands: Wageningen Publishers, 2003;109. EAAP, 2003. p. 197-200.
[38] Reinauer H, Gries FA, Hubinger A, et al. Determination of glucose-turnover and glucose-oxidation rates in man with stable isotope tracers. J Clin Chem Clin Biochem 1990;28:505-11.
[39] Payne RW, Lane PW, Ainsley AE, et al. Genstat 5. Reference manual. Oxford (UK): Oxford University Press; 1987.
[40] DeFronzo RA. The triumvirate: beta-cell, muscle, liver. A collusion responsible for NIDDM. Diabetes 1987;37:667-87.
[41] Fernandez-Real JM, Ricart W. Insulin resistance and inflammation in an evolutionary perspective. The contribution of cytokine genotype/phenotype to thriftiness. Diabetologia 1999;42:1367-74.
[42] Festa A, D'Agostino R, Howard G, et al. Chronic subclinical inflammation as part of the insulin resistance syndrome: the insulin resistance atherosclerosis study (IRAS). Circulation 2000;102:42-7.
[43] Koopmans SJ, Maassen JA, Radder JK, et al. In vivo insulin responsiveness for glucose uptake at eu- and hyperglycemic levels in normal and diabetic rats. Biochem Biophys Acta 1992;115:230-8.
[44] Nishimura H, Kuzuya H, Okamoto M, et al. Postreceptor defect in insulin action in streptozotocin-induced diabetic rats. Am J Physiol 1989;256:E624-30.
[45] Dallaglio E, Chang H, Hollenbeck CB, et al. In vivo and in vitro resistance to maximal insulin-stimulated glucose disposal in insulin deficiency. Am J Physiol 1985;249:E316-21.
[46] Tobin BL, Finegood DT. Reduced insulin secretion by repeated low doses of STZ impairs glucose effectiveness but does not induce insulin resistance in dogs. Diabetes 1993;42:474-83.
[47] Kjems LL, Kirby BM, Welsh EM, et al. Decrease in β-cell mass leads to impaired pulsatile insulin secretion, reduced postprandial hepatic insulin clearance, and relative hyperglucagonemia in the minipig. Diabetes 2001;50:2001-12.
[48] Larsen MO, Wilken M, Gotfredsen CF, et al. Mild streptozotocin diabetes in the Göttingen minipig. A novel model of moderate insulin deficiency and diabetes. Am J Physiol 2002;282:E1342-51.
[49] Koopmans SJ, Kushwaha RS, DeFronzo RA. Chronic physiologic hyperinsulinemia impairs suppression of plasma free fatty acids and increases de novo lipogenesis but does not cause dyslipidemia in conscious normal rats. Metabolism 1999;48:330-7.
[50] Koopmans SJ, Mandarino L, DeFronzo RA. Time course of insulin action on tissue-specific intracellular glucose metabolism in normal rats. Am J Physiol 1998;274:E642-50.
[51] Newsholm EA, Leech AR. Biochemistry for the medical sciences. Chichester, UK: John Wiley & Sons Ltd.
[52] Howard BV, Howard WJ. Dyslipidemia in non–insulin dependent diabetes mellitus. Endocr Rev 1994;15:263-74.
[53] National Cholesterol Education Program. JAMA 2001;285:2486-97.
[54] Cheng JT, Liu IM, Chi TC, et al. Metformin-like effects of Quei Fu Di Huang Wan, a Chinese herbal mixture, on streptozotocin-induced diabetic rat. Horm Metab Res 2001;33:727-32.
[55] Dutta K, Podolin DA, Davidson MB, et al. Cardiomyocyte dysfunction in sucrose-fed rats is associated with insulin resistance. Diabetes 2001;50:1186-92.
[56] Leng SH, Lu FE, Xu LJ. Therapeutic effects of berberine in impaired glucose tolerance rats and its influence on insulin secretion. Acta Pharmacol Sin 2004;25:496-502.
[57] Pushparaj PN, Tan BKH, Tan CH. The mechanism of hypoglycaemic action of the semi-purified fractions of *Averrhoa bilimbi* in streptozotocin-diabetic rats. Life Sci 2001;70:535-47.
[58] Suzuki M, Odaka H, Suzuki N, et al. Effects of combined pioglitazone and metformin on diabetes and obesity in Wistar fatty rats. Clin Exp Pharmacol Physiol 2002;29:269-74.

[59] Koopmans SJ, Frolich M, Gribnau EH, et al. Effect of hyperinsulinemia on plasma leptin concentrations and food intake in rats. Am J Physiol 1998;274:E998-E1001.
[60] Schwartz MW, Figlewicz DP, Baskin DG, et al. Insulin in the brain: a hormonal regulator of energy balance. Endocr Rev 1992;13:387-414.
[61] DeFronzo RA, Ferrannini E. Insulin resistance. A multifaceted syndrome responsible for NIDDM, obesity, hypertension, dyslipidemia, and atherosclerotic cardiovascular disease. Diabetes Care 1991; 14:173-94.
[62] Lee A, Morley JE. Metformin decreases food consumption and induces weight loss in subjects with obesity with type II non–insulin-dependent diabetes. Obes Res 1998;6:47-53.
[63] Paolisso G, Amato I, Eccellente R, et al. Effect of metformin on food intake in obese subjects. Eur J Clin Invest 1998;28:441-6.