Impaired glucose stimulated insulin secretion is coupled with exocrine pancreatic lesions in the Cohen diabetic rat

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Running title: Impaired GSIS and exocrine lesions

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ABSTRACT

Objective: The Cohen diabetes-sensitive (CDs) rat develops postprandial hyperglycemia when fed a high-sucrose, copper-poor diet (HSD) while the Cohen diabetes-resistant (CDr) rat maintains normoglycemia. The pathophysiological basis of diabetes was studied in the Cohen diabetic rat centering on the interplay between the exocrine and endocrine compartments of the pancreas.

Research Design and Methods: Studies utilized male CDs and CDr rats fed 1-month HSD. Serum insulin and glucose levels were measured during glucose and insulin tolerance tests. The pancreas was evaluated for weight, insulin content, macrophage and fat infiltration. Glucose-stimulated insulin secretion was determined in isolated perfused pancreas and in islets.

Results: Hyperglycemic CDs rats exhibited reduced pancreatic weight with lipid deposits and IL1-β-positive macrophage infiltration in the exocrine pancreas. Islet morphology was preserved and total pancreatic insulin content did not differ from that of CDr rats. Lipids did not accumulate in skeletal muscle, nor was insulin resistance observed in hyperglycemic CDs rats. Intravenous glucose-tolerance test revealed markedly elevated glucose levels associated with diminished insulin output. Insulin release was induced in-vivo by the non-nutrient secretagogues arginine and tolbutamide, suggesting a selective unresponsiveness to glucose. Decreased glucose-stimulated insulin secretion was observed in the isolated perfused pancreas of the hyperglycemic CDs rat while islets isolated from these rats exhibited glucose-dependent insulin secretion and proinsulin biosynthesis.

Conclusions: The association of the in-vivo insulin secretory defect with lipid accumulation and activated macrophage infiltration in the exocrine pancreas suggests that changes in the islet microenvironment are the culprit in the insulin secretory malfunction observed in-vivo.
Type 2 diabetes mellitus (T2DM) is genetically determined, but environmental factors, mainly nutritional, are essential for its manifestation in susceptible individuals (1, 2). Although insulin resistance has long been accepted as the key feature of T2DM, the development of overt hyperglycemia entails a decline in β-cell function (1, 2). The progressive deterioration of insulin secretion could be attributed to the hyperglycemic environment that further promotes β-cell dysfunction (3, 4) and loss of β-cell mass (5).

The Cohen Diabetic (CD) rat is a genetic model of nutritionally induced diabetes. The CD rat consists of two contrasting strains: a sensitive strain (CDs) that develops type 2-like diabetes only when fed a diabetogenic high-sucrose, copper-poor diet (HSD), but maintains normoglycemia when fed regular diet (RD), and a resistant strain (CDr) which remains normoglycemic irrespective of the diet (6, 7). As in the human disease, nutrition-dependent hyperglycemia in the CDs rat is reversible in its early stages by adjusting the diet (6). Furthermore, overt hyperglycemia in this model, apparent following 4-6 months of HSD, results in complications involving several target organs (8). Preliminary histological evaluation of the pancreas of CDs rats fed HSD for 10 weeks showed a significant atrophy of the exocrine acinar tissue while islet morphology was preserved (9).

Exocrine pancreatic lesions were shown to be associated with increased prevalence of diabetes (10-15) and significant impairment of exocrine pancreatic function and morphology are more prevalent in diabetic patients (16, 17). Copper deficiency was shown to induce a highly selective acinar cell degeneration and lipomatosis while islets, ducts, and nerves were not affected (18-20). These observations suggest a link between the function of the endocrine compartment of the pancreas and the surrounding exocrine tissue. The current study describes the pathophysiological basis of diabetes in the CD rat centering on the importance of the interplay between the exocrine and endocrine compartments of the pancreas.

**EXPERIMENTAL DESIGN AND METHODS**

**Animals.** CD rats were bred and maintained in the animal facility, Hebrew University School of Medicine, Jerusalem. Rats were fed RD ad libitum (Koffolk, Petach-Tikva, Israel), composed of 54% carbohydrate (ground whole wheat, ground alfalfa and bran) 21% protein (skimmed milk powder), 6% fat, 5% salts, vitamins and trace elements including an adequate copper content (15 ppm), 7% humidity and 7% ash. Custom-prepared HSD contains 72% sucrose, 18% vitamin-free casein, 5% salt mixture No. II USP (MP Biomedicals, LLC, Ohio, USA), 4.5% butter, 0.5% corn oil, vitamins and low copper (0.9 ppm) (6, 7). Animal studies were approved by the institutional committee for animal use and care.

**Experimental Design.** Seven-week old male rats on RD were switched to HSD for an additional period of one month. Two groups were studied: CDs and CDr rats fed HSD (CDs-HSD and CDr-HSD, respectively). Care was taken to include in each study CDs and CDr rats with similar weights to preclude possible influence of the initial weight on the phenotype. In-vivo studies included: postprandial determination of glucose and insulin, oral or intravenous glucose...
tolerance test (OGTT or IVGTT, respectively), insulin tolerance test (ITT), and insulin secretion in response to non-nutrient secretagogues. Glucose concentration was measured in tail blood using a standard glucometer (Elite®, Bayer, Leverkusen, Germany). Serum insulin was determined using an ultrasensitive rat insulin Elisa assay (Mercodia AB, Uppsala, Sweden). In-vitro studies comprised of insulin secretion in perfused pancreas, and proinsulin biosynthesis and insulin secretion in isolated islets.

**In-vivo studies.** Postprandial test. Rats were fasted overnight. Blood glucose (BG) and serum insulin concentrations were measured after fasting and following 60 minutes of free access to HSD. Additional blood samples were taken at 60 and 120 min after HSD removal.

**OGTT.** BG and serum insulin concentrations were measured after overnight fast and following the oral administration of glucose (3.5 g/kg), as described (6).

**Surgical catheter insertion.** Heparinized catheters were inserted into the right jugular vein and left carotid artery of anesthetized rats (85 mg/kg, Ketalar, Parke-Davis, Gwent U.K. and 3 mg/kg xylazine, XYL-M2 Veterinary, VMD, Belgium) seven days before tests.

**IVGTT.** BG and serum insulin concentrations were measured after overnight fast and during 120 min after a bolus administration of glucose (0.75 g/kg) via the jugular vein.

**Secretagogues tests.** Bolus arginine (350 mg/kg) or saline followed by 45 minutes of 6 mg/min constant infusion were administered intravenously (i.v.) to CDs-HSD rats following an overnight fast or in the postprandial state. Tolbutamide (i.v. bolus of 100 mg/kg) was administered after an overnight fast. Carotid artery BG and serum insulin concentrations were measured before and after arginine, tolbutamide or saline administration (control). Control and tests were performed on the same rats following 4-5 days recovery.

**ITT.** BG concentrations were measured in CDs-HSD and CDr-HSD rats after an overnight fast and following intraperitoneal (i.p.) administration of insulin in 3 doses: 0.25, 0.5 and 1U/kg (Actrapid® HM, Novo Nordisk, Denmark).

**Collection of tissues.** Rats were anesthetized. Liver, spleen, kidney, heart and pancreas were removed, dissected from external fat and weighed. The entire pancreas was flushed-frozen in liquid nitrogen and kept at -80°C for insulin or triglycerides (TG) determination. Alternatively, the pancreas was dissected and immersed in EM fixative solution, or fixed in formalin for further histological analyses.

**Pancreatic insulin determination.** Insulin was extracted from the entire pancreas with acid-ethanol solution (21) and assayed by RIA using a commercial kit (Linco Research Inc., St. Charles, MO, USA).

**Triglycerides extraction and measurement.** TG were extracted from the entire pancreas using the Folch method (22) and determined using the GPO-Trinder kit (Sigma, St Louis, MO).

**Serum free fatty acids (FFA).** were measured in sera of overnight fasted CDs-HSD and CDr-HSD rats using the NEFA kit (Randox Laboratories, Crumlin, County Antrim, UK).

**Immunohistochemistry and histological evaluation.** Paraffin embedded pancreatic sections (5 µm) stained with hematoxylin-eosin were evaluated for islet morphology, macrophage infiltration, and acinar degeneration (at Hadassah Hospital, Jerusalem, and Hannover
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Medical School, Germany). For immunohistochemical analysis, sections were incubated overnight with guinea-pig anti-insulin antibody (1:500) (polyclonal A565, DAKO, Hamburg, Germany), mouse anti-glucagon antibody (1:300) (monoclonal, Sigma), mouse anti-ED1 (1:500) (monoclonal MCA 341) or mouse anti-IL-1β (1:200) (monoclonal AAR15G) (Serotec, Oxford, UK) antibodies, followed by a 30 min incubation with an appropriate biotinylated second antibody, as described (23) and a 30 min incubation with a mixture of streptavidin (1:100) and biotin-peroxidase (1:1000) (Jackson Immuno Research, West Grove, IL) (24). The peroxidase reaction was visualized using 0.7 mM diaminobenzidine and 0.002 % hydrogen peroxide in PBS, pH 7.3. Sections were examined by bright field illumination or under phase contrast using a Zeiss Photomicroscope II (Oberkochen, Germany).

Electron microscopy (EM). Transmission EM (TEM). Thin pancreas sections fixed overnight in cacodylate buffer (pH 7.3), containing 2% glutaraldehyde and para-formaldehyde were stained with 1% osmium tetroxide (lipids stained black), dehydrated, and embedded in Epon. Sections were contrast-stained with saturated solutions of lead citrate and uranyl acetate and evaluated by electron microscopy [EM 9 S2, Zeiss, Oberkochen, Germany (23)].

Scanning EM (SEM) for lipid imaging using the WETSEM™ technology. Sections (400 μm) of formalin fixed pancreas and gastrocnemius muscle were stained with 0.1% osmium tetroxide followed by mild staining (0.05%) with uranyl acetate (lipids appear bright white). Stained samples were kept at 4°C. SEM imaging was performed on samples placed in a capsule (QuantomiX Ltd. Rehovot, Israel) using SEM [FEI XL-30 Eindhoven, The Netherlands, (25)].

Proliferation and apoptosis. Pancreases were removed 4h following 5-bromo-2'-deoxyuridine (BrdU 100 mg/kg, Sigma,) i.p. injection. Cell replication was assessed by scoring BrdU staining (incorporation) of proliferating cell nuclei (Biosciences Pharmingen, Brussels, Belgium). Apoptotic cells were identified by Terminal dUTP 3' Nick End-Labeling (TUNEL) procedure using cell death detection kit (Roche, Mannheim, Germany) (26). Stained nuclei were counted and expressed as percent of the total number of nuclei in the same field (23, 27).

In-vitro studies. Pancreas perfusion. Rats were anaesthetized (pentobarbital, 100 mg/kg). The pancreas was dissected and perfused (3 ml/min) with Krebs-Ringer bicarbonate buffer (KRB) in a 37°C chamber via the abdominal aorta. Subsequently, pancreases were perfused with KRB containing 3.3 mmol/l glucose for 20 min; 16.7 mmol/l glucose for 60 min and 3.3 mmol/l glucose for additional 20 min. Samples were collected from the pancreatic vein and stored at -20°C for insulin RIA (Linco) (28).

Islet isolation and proinsulin biosynthesis (21). Batches (200-300) of similar size islets, washed repeatedly with Hank's balanced salt solution, were incubated at 37°C for 90 min under 5% CO₂ atmosphere in modified KRB buffer containing 20 mmol/l HEPES, 0.25% BSA (KRBH-BSA) and 3.3 mmol/l glucose to allow recovery from the isolation procedure. Groups of 25 islets were washed in KRBH-BSA buffer containing 1.7 mmol/l glucose and incubated for 1-h in the same buffer containing increasing concentrations of glucose (1.7-16.7 mmol/l). Insulin release was determined in supernatants of the incubation buffer.
Islets were then suspended in fresh KRBH-BSA buffer containing 25 µCi L-³H-leucine (150 Ci per mmol/l; Amersham, Aylesbury, UK) (29) and the same glucose concentration and incubated for additional 15 min at 37°C. Leucine incorporation was terminated by addition of 1 ml ice-cold glucose-free KRBH-BSA buffer and rapid centrifugation. Islet insulin content and proinsulin biosynthesis was determined in pellet extracts suspended in glycine buffer and subjected to 4 freeze-thaw cycles in liquid nitrogen, as described (30). Islet insulin secretion and content were determined using rat insulin RIA kit (Linco).

Data analysis. Data shown are means ± SE. Statistical significance of differences between groups was determined by one-way ANOVA followed by Tukey test using the Sigmasstat program (Jandel Corporation, San Rafael, CA, USA). A two-tail paired t-test was used to compare data from tests performed on the same animal or in isolated islets. A P value of less than 0.05 was considered significant.

RESULTS
Postprandial insulin secretion. In CDs-HSD rats, the markedly elevated BG concentrations remained high 120 min after HSD removal (Fig. 1A). The high BG concentrations were coupled with a low flat insulin output (Fig. 1B). The CDr-HSD rats maintained normal BG levels and exhibited a significant postprandial increase in insulin secretion (Figs. 1A and B).

Glucose tolerance test. Glucose-stimulated insulin secretion (GSIS) was assessed in CDs-HSD and CDr-HSD rats in response to oral (Fig. 1C and D) and i.v. (Fig. 1E and F) glucose administration. CDs-HSD rats exhibited an abnormal glucose-tolerance curve, characterized by elevated glucose levels and a low insulin output, while CDr-HSD rats exhibited a normal response (Figs. 1C- F). The calculated glucose area under the curve (AUC) of CDs-HSD rats (Fig. 1G) was higher in both OGTT and IVGTT compared to CDr-HSD rats (Fig. 1H), whereas the insulin AUC was lower. The insulin response was higher in OGTT compared to IVGTT in both CDs-HSD (Fig. 1G) and CDr-HSD rats (Fig. 1H). A comparable normal IVGTT was demonstrated for both CDs and CDr maintained for 30 days on regular diet (RD), as shown in the online appendix (Figs. 1Sa-b [available at http://diabetes.diabetesjournals.org]).

Response to secretagogues. The mechanism underlying the reduced GSIS of the CDs-HSD rats was examined in response to arginine and tolbutamide. The abnormal postprandial (Figs. 2A and B) or fasting (Figs. 2C and D) glucose and insulin response curves observed in CDs-HSD rats were fully normalized by arginine or tolbutamide administration (Figs. 2A-D). The glucose AUC was significantly decreased while the insulin AUC was increased (Figs. 2E and F) compared to control.

Insulin tolerance test. Insulin produced a similar decrease in BG levels in both CDs-HSD and CDr-HSD rats (Fig. 3A). The CDs-HSD rats exhibited a dose-dependent reduction in BG levels (Fig. 3B), indicating a high sensitivity to insulin which may explain the similar basal glucose levels of CDs-HSD and CDr-HSD rats.

Pancreas weight. The hyperglycemic CDs-HSD rats were nearly 20% smaller than the CDr-HSD rats (230g ± 7 vs. 290g ± 6) after 5 weeks on HSD but did not show significant body weight difference after 1-3 weeks of HSD (data not shown). The pancreas weight of the CDs-HSD rats was 40% lower than CDr-
HSD pancreas. No such variance was detected in the weight of the liver, spleen, heart or kidney (Table 1).

**Apoptosis and proliferation.** Considerable apoptotic activity (2.48 ± 0.27%) was detected in the acinar cells of the CDs-HSD rats using the TUNEL assay, whereas only few (< 0.01 %) apoptotic cells were detected in CDr-HSD rats. The acinar cells of the CDs-HSD rats exhibited increased proliferative activity compared to CDr-HSD rats [1.01 ± 0.02 vs. 0.08 ± 0.02 % (BrdU labeling index), respectively, P<0.01]. The very few (<0.01%) TUNEL and BrdU positive cells detected in the islets of the same animals precluded quantitative analysis. Thus, acinar cell apoptosis is likely to be the cause of in the reduced pancreatic weight of the CDs-HSD rats (table 1).

**Insulin and TG content.** Pancreatic insulin content of the CDs-HSD rat was lower but not significantly different compared to CDr-HSD rats (16.8 ± 3.4 vs. 28.8 ± 7.3 nmol/pancreas, n=9, respectively). In line with the loss of exocrine tissue, the relative density of islets increased in CDs-HSD compared with CDr-HSD rats (1.2 ± 0.1 vs. 0.7 ± 0.1 islets/mm² pancreas area; respectively, P<0.05, n = 5). TG content of the pancreas was significantly higher in the CDs-HSD compared to CDr-HSD rats (5.2 ± 0.9 vs. 1.2 ± 0.2 mg/g tissue, P<0.002, n=9). When taking into account the smaller pancreas of the CDs-HSD, the TG content of the CDs-HSD pancreas was approximately 1.5 fold higher than in the CDr-HSD rat.

**Serum FFA concentration.** Levels of serum FFA were significantly higher in fasted CDs-HSD compared to CDr-HSD rats (1.442 ± 0.052 vs. 0.868 ± 0.065 mmol/l respectively, P<0.001).

**Immunohistochemistry of pancreatic islets.** Dense insulin immunostaining was observed in pancreatic β-cell cytoplasm while dense glucagon immunostaining of the α-cells was observed as a rim at the islet periphery (Fig. 4A-D). Macrophages stained positively for IL-1β were found in the transition zone surrounding the islet (Fig. 4E), or as single cells in the intra-islet capillary system of CDs-HSD rats (not shown). These macrophages did not express INFγ and TNFa. Macrophages were only rarely observed in the exocrine tissue of the CDr-HSD rats (Fig. 4F). A significant increase in the number of ED1 positive macrophages was observed in the atrophic regions of the exocrine parenchyma of the CDs-HSD compared to CDr-HSD rats (2.6 ± 0.4 vs. 0.3 ± 0.1 cells/mm², respectively, P<0.01; n = 4).

**Ultrastructural changes and lipid deposits in the pancreas and skeletal muscle.** The ultrastructural analysis of the exocrine parenchyma using TEM (Figs. 5A-D) or the WETSEM™ technology (Figs. 5G and H) confirmed acinar atrophy with lipid deposits in the pancreas of the CDs-HSD rats. Nuclear heterochromatin condensation and margination typical of apoptosis were exhibited by some acinar cells of the CDs-HSD rats (Fig. 5C). No comparable signs of apoptosis or lipid deposits were found in the β-cells (Fig. 5A and B). In the fibrous transition zone between the endocrine and exocrine parenchyma, two layers of macrophages, showing ultrastructural signs of activation including an increased cytoplasmic volume (Fig. 5D), surrounded the islet periphery and were also observed as single macrophages in the islet capillary system (not shown). The β-cells in the islet center exhibited well-preserved cellular organelles (Fig. 5E), while some β-cells in the islet periphery, exhibited signs of cellular damage, dilated cisternae of the rough endoplasmic reticulum and swollen
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mitochondria (Fig. 5F). No lipid deposits were observed in the CDr-HSD rat pancreas (Fig. 5H), in skeletal muscle or in islets of both CDs-HSD and CDr-HSD rats (not shown). No comparable signs of apoptosis, lipid deposits or macrophage infiltration were found in the pancreas of CDr-HSD (Figs. 2Sa-d).

**Isolated perfused pancreas.** To test the relationships between the endocrine and exocrine compartments of the pancreas we evaluated the dynamics of insulin release in the isolated perfused pancreas. When subjected to a square wave of glucose stimulation, the isolated pancreas of the CDr-HSD rats (n = 3) responded with biphasic insulin release (Fig. 6); the transient first phase was followed, 4–5 min later, by a gradual increase of the second phase. In contrast, the CDs-HSD rats pancreas (n = 4) expressed a very low insulin response to glucose, suggestive of a small first phase, followed by a blunted second insulin phase.

**Insulin secretion and biosynthesis in isolated islets.** As opposed to the blunted response of the perfused CDs-HSD rat pancreas to 16.7 mmol/l glucose, islets isolated from these rats responded to a similar glucose challenge with increased secretion of insulin (Fig. 7A and B). However, islets of CDs-HSD rats exhibited a left-shift in insulin secretion, with significant increase already observed at 3.3 mmol/l and no additional increase between 3.3 and 16.7 mmol/l glucose. Conversely, insulin biosynthesis was shifted to the right, with increased insulin biosynthesis only apparent at 16.7 mmol/l, as opposed to 3.3 mmol/l glucose in the CDr-HSD islets. Nonetheless, similar insulin content per islet was observed in CDs-HSD and CDr-HSD rats (14.2 ± 2 and 15.1 ± 3.7 pmol/islet, respectively).

**DISCUSSION**

The inbred CDs-HSD rat demonstrates the importance of the cross-talk between the exocrine and endocrine compartments of the pancreas for proper β-cell function. Glucose intolerance in the CDs rats following high-sucrose, copper-poor diet was associated with lipid deposits and macrophage infiltration in the exocrine pancreas. Yet, lipids did not accumulate in skeletal muscle tissue or in the islets; peripheral insulin resistance or obesity were not observed in hyperglycemic CDs-HSD rats contrasting other animal models of diabetes (31-33). A pancreatic pathophysiological mechanism was therefore considered to explain the CDs-HSD diabetic phenotype. The Cohen diabetic rat provides a good model to study GSIS. It comprises two inbred strains, one sensitive and the other resistant to the same dietary regimen which may enable deciphering the factors responsible for the reduced GSIS in the hyperglycemic CDs-HSD rat. Since a major sex difference in severity of glucose intolerance has been demonstrated, only male rats that exhibit a more severe diabetic phenotype were used in the current study (6, 7). The genetic susceptibility to diet-induced glucose intolerance was observed in the present study already following 1 month of HSD feeding, 1.5 months earlier than in previous studies (6, 7).

The elevated postprandial BG concentrations (>12 mmol/l) and reduced insulin output observed in the hyperglycemic CDs-HSD rat were ascertained also by OGTT and IVGTT. The diminished insulin response suggested that aberrant insulin secretion is the major cause for diabetes in this model. A reduced pancreatic insulin reserve, as reported in other models of nutritional diabetes (32), could not explain
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the diminished insulin output in CDs-HSD rats, since insulin immunostaining, insulin content per animal and islet density were not reduced significantly in the CDs-HSD rats compared to CDr-HSD rat. Furthermore, insulin output was elicited in-vivo in the hyperglycemic CDs-HSD rat by the non-nutrient secretagogues arginine and tolbutamide. This suggests the presence of sufficient insulin reserve and functional distal secretory machinery, confirming selective unresponsiveness to glucose.

Islets isolated from the pancreas of hyperglycemic CDs-HSD rats exhibited significant insulin secretion in response to 16.7 mmol/l glucose. The shifted glucose-insulin concentration-response curve may be related to the prolonged postprandial exposure of the CDs rat derived islets to hyperglycemia prior to their isolation. Similar phenomena occur in isolated islets from other diabetic models (34, 35). Since insulin secretion was observed only in isolated islets and not in-vivo or in the perfused pancreas experiments, we considered interdependency between the exocrine and endocrine compartment in-vivo.

What could be the cause for the major discrepancy between the in vivo and in vitro insulin response to glucose? Although most animal models of T2DM do not share this phenomenon, similar observations were described in a human study performed over 20 years ago (36) and recently also in the Tally-Ho mouse, a transgenic animal model of T2DM (37). In both studies the reduced in-vivo GSIS was not attributed to intrinsic β-cell dysfunction, but was related to extrinsic factors in the surroundings of the islets. A more recent study, supporting a role for exocrine-endocrine interaction in diabetes, linked diabetes to the disrupted function of carboxyl ester lipase; the acinar cell enzyme, responsible for cholesterol esters hydrolysis (38).

In concert with these studies, several such extrinsic factors were considered as contributing to the impaired GSIS in the CDs-HSD rat. Incretin hormones, the activity of which has been shown to be attenuated in T2DM (39) were considered. However, the observation that insulin output in the CDs-HSD rat was higher in OGTT compared to the IVGTT does not support the involvement of the entero-insular hormonal axis in the in-vivo secretory dysfunction.

Increased lipid deposition in the exocrine pancreas and the infiltrating macrophages have been associated with a diabetic-like phenotype in the pancreas of patients with cystic fibrosis (12), long-standing chronic pancreatitis (13), or pancreatic cancer (11) and in several transgenic mouse models (40, 41). Under these conditions, fat infiltration was suggested to be associated with the development of hyperglycemia. In our study, the fasted hyperglycemic CDs-HSD rat exhibited significantly higher levels of serum FFA and increased pancreatic TG content compared to the normoglycemic CDr-HSD rat. The specific pancreatic fat accumulation in the CDs rat was induced by HSD, a diet low in copper and high in sucrose. Diets containing high concentrations of simple sugars were shown to affect enzymes responsible for lipolysis, resulting in fat accumulation in non-adipose tissue (42, 43). Copper deficiency was also reported to be associated with abnormal glucose tolerance, and pancreatic atrophy (18, 19, 42, 43). Simple sugars, such as sucrose and fructose further augment the low copper induced phenomenon possibly by the production of reactive oxygen species that may specifically affect GSIS (42).
Lipid deposits were observed exclusively in the exocrine parenchyma of the CDs-HSD rat. Thus, it is conceivable that the excessive fat storage in the exocrine pancreas of the CDs-HSD rat contributes to the aberrant surrounding of the \( \beta \)-cell and may lead to its dysfunction. On the other hand, the lack of lipids in skeletal muscle explains the high peripheral sensitivity to insulin in hyperglycemic CDs rats.

Previous studies have shown that macrophage infiltration is associated with lipid deposits and that these macrophages are a major source of inflammatory mediators (44-46). The infiltrating activated macrophages in the exocrine tissue of the CDs-HSD rats that expressed exclusively IL-1\( \beta \) could be a crucial factor in their diabetic phenotype. Other proinflammatory cytokines or iNOS were not expressed by the macrophages (data not showed) and T-cells were not observed in the pancreas of the hyperglycemic CDs rats. We believe that this may provide a plausible explanation for the fact that compared to rat models of immune-mediated diabetes (47) the CDs rat exhibits a mild form of T2DM characterized by a selective defect in GSIS rather than a total loss of pancreatic \( \beta \)-cells. This may also explain the relatively fast recovery of the GSIS when islets were isolated from the deleterious environment. Indeed, low levels of IL-1\( \beta \) were shown to inhibit selectively GSIS in vitro (48) and mitochondrial abnormalities similar to those observed by the ultrastructural analysis of \( \beta \)-cells in CDs-HSD rats were induced by IL-1\( \beta \) in an iNOS independent manner (49). Thus, the hyperglycaemic CDs rat may represent a model of a mild cytokine-mediated diabetic syndrome.

In conclusion, glucose intolerance in the diabetic CDs rat is a result of its genetic susceptibility to a copper-poor, sucrose-rich diet. A blunted \( \beta \)-cell response to glucose in-vivo is responsible for the diabetic phenotype. The association of the in-vivo secretory defect with lipid accumulation in the exocrine pancreas and the observation of IL-1\( \beta \) positive activated macrophages in the islet vicinity suggest that changes in the islet microenvironment are the culprit in the in-vivo malfunction of the \( \beta \)-cell.

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**TABLE 1.** Body and organ weights in CDs-HSD and CDr-HSD rats.

<table>
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<th>group</th>
<th>n</th>
<th>body weight</th>
<th>pancreas</th>
<th>spleen</th>
<th>heart</th>
<th>liver</th>
<th>kidney</th>
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<tr>
<td>CDs-HSD</td>
<td>20</td>
<td>230 ± 7*</td>
<td>0.202 ± 0.01*</td>
<td>0.636 ± 0.03</td>
<td>1.05 ± 0.03</td>
<td>10.1 ± 0.26</td>
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<tr>
<td>CDr-HSD</td>
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<td>1.11 ± 0.03</td>
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Data are means ± SE of weights in grams.

*P<0.001 relative to CDr-HSD.
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FIGURE LEGENDS

Figure 1. Postprandial (A and B), OGTT (C and D) and IVGTT (E and F) profiles of CDs (black squares) vs. CDr rats (white circles) fed HSD. A and B: Blood glucose and insulin concentrations were measured after an overnight fast (0), and after a 60 min period of HSD feeding. Additional blood samples were taken at 120 and 180 min (60 and 120 min after HSD removal). C – F: Blood samples glucose and insulin concentrations were measured after an overnight fast (-10 and/or 0) and during the 120 min period after glucose administration. Data are means ± SE for 5 or 6 rats per group. # P< 0.001, CDs-HSD vs. CDr-HSD same parameter, time-point and treatment. G and H- The calculated AUC of glucose (white bars) and insulin (black bars) of CDs-HSD (G) and CDr-HSD (H) rats. # P < 0.002, AUC of OGTT and IVGTT between groups (CDs-HSD vs. CDr-HSD rats, respectively). * P < 0.002 OGTT vs. IVGTT within the same group.

Figure 2. Control (white circles) arginine (black squares) and tolbutamide (white squares) tolerance tests performed on CDs-HSD rats in the postprandial (A and B) and fasting (C and D) states. Glucose and insulin concentrations were measured at fasting (-10, 0 min) and during 120 min of the intravenous administration of arginine as bolus followed by a 45 min constant infusion of 6 mg/min tolbutamide or saline (control). Control and test studies were performed on the same rats after 4 to 5 days recovery. Arginine was administered in both postprandial (A and B) and fasting (C and D) states. Tolbutamide was tested only in the fasting state (C and D). AUC of glucose (white bars) and insulin (black bars) are shown for the postprandial (E) and fasting (F) states. Data are means ± SE for 5-6 rats per group. * P < 0.002 control vs. arginine or tolbutamide same time-point and/or treatment.

Figure 3. Insulin tolerance test. BG concentrations were determined following i.p. administration of insulin to fasted animals. A- BG levels following insulin administration (1U/kg) to CDs-HSD (black squares) and CDr-HSD (white circles) rats. B- BG concentrations in CDs-HSD following i.p. injection of insulin: 1U/kg (white circles), 0.5U/kg (black circles) and 0.25U/kg (black triangles). BG concentrations were measured after an overnight fast (time 0) and at 30-120 after administration of insulin. Data are means ± SE of 5 rats per group. * P< 0.025 vs. 0.25U/kg insulin.

Figure 4. Immunostaining of pancreatic sections of CDs-HSD (A, C, E) and CDr-HSD (B, D, F) for insulin (A, B) glucagon (C, D) and IL-1β (E, F). Dense insulin and glucagon immunostaining of the β- and α-cells was observed with no significant alterations in staining or distribution between the two strains of animals. In the exocrine pancreas of the CDs-HSD rats macrophages expressing IL-1β immunostaining (arrows) were observed (E) in the transition zone between the exocrine (asterisks) and endocrine parenchyma (dashed line depicts the islet boundary). Light microscopy, magnification x 320 (A-D), x 560 (E, F)

Figure 5. Photomicrographs of pancreatic sections of CDs-HSD rats (A-G) and CDr-HSD rats (H). Semithin tissue sections were stained with toluidine blue (A, B). The acinar cells were reduced in size and showed a mild atrophy with increased amounts of adipose tissue inside and between the acini [(arrows, black vacuoles, osmium staining (A,B)]. The islets in (A) and (B) (arrowheads) in close association with the atrophic acini of the exocrine parenchyma show no signs of cell damage such as vacuolisation or

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Ultrastructural analysis of the exocrine (C, G and H) and endocrine (E and F) tissues of the CDs-HSD group. Figure D (arrowheads) shows acinar cells with nuclear heterochromatin condensation and margination of the chromatin (arrows), typical of apoptosis. In the transition zone between the exocrine and endocrine parenchyma two layers of macrophages (arrows) surround the islet periphery of the CDs-HSD rat. The β-cells in the center of the islet (E) exhibit well preserved mitochondria (arrowheads) and normally arranged cisternae of the rough endoplasmic reticulum (arrows), while the β-cell in the islet periphery (F) show swollen mitochondria (arrowheads) and dilated cisternae of the rough endoplasmic reticulum (arrows). Lipid droplets are observed by WETSEM™ technology (bright white, G) in the disorganized exocrine pancreas of CDs-HSD rat, but not in the well organized pancreas of CDr-HSD rat (H). Magnification 320 (A), 480 (B), 6.500 (C, D), 18.000 (E, F), 320 (G, H).

Figure 6. Pancreas perfusion studies. Pancreases of CDs-HSD rats (black squares) and CDr-HSD rats (white circles) were perfused at a rate of 3 ml/min. Following perfusion at 3.3 mmol/l glucose for 20 min the glucose concentration was increased to 16.7 mmol/l for 60 min and reduced again to 3.3 mmol/l glucose for an additional 15 min. One minute effluent fractions were collected every 5-min during the time interval between −10 and 0 min. During the first 10 min of perfusion with 16.7 mmol/l glucose, fractions were collected every 1 min followed by collection of 1 min fractions every 5 min for the remaining 50 min at 16.7 mmol/l glucose and during the additional 15 min at 3.3 mmol/l glucose. Insulin concentration was determined in the effluent fractions by RIA. Values are means ± SE of 3 and 4 perfused pancreases from CDr-HSD and CDs-HSD rats, respectively.

Figure 7. Proinsulin biosynthesis and insulin secretion in isolated islets of CDs-HSD (black bars) and CDr-HSD rats (white bars). Islets were incubated for 1 h at 37°C in KRBH-BSA supplemented with 1.7, 3.3 and 16.7 mmol/l glucose. A- Proinsulin biosynthesis. B- Insulin secretion. Data are means ± SE for 5-6 individual experiments per study. * P <0.03 vs. islets at 1.7 mmol/l glucose.
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Fig 1

- A: Blood glucose (mmol/l) over time (min)
- B: Blood insulin (pmol/l) over time (min)
- C: Glucose (mmol/l)
- D: Insulin (pmol/l)
- E: Glucose (mmol/l)
- F: Insulin (pmol/l)
- G: AUC for glucose (mmol/l/120 min)
- H: AUC for insulin (pmol/l/120 min)

Legend:
- ○ CD(H)-HSD
- ■ CD(H)-HSD

P-values:
- P=0.001
- P=0.004
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Fig 2

A

B

E

C

D

F
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Fig 3

A

B
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Impaired GSIS and exocrine lesions

Fig. 5
Impaired GSIS and exocrine lesions

Fig. 5
Impaired GSIS and exocrine lesions
Impaired GSIS and exocrine lesions

**Fig 7**

![Graph showing impaired GSIS and exocrine lesions with comparisons between CDx-HSD and CDx-HSD treated groups.](image)