

Dietary copper supplementation restores β -cell function of Cohen diabetic rats: a link between mitochondrial function and glucose-stimulated insulin secretion

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¹Diabetes Unit, Department of Internal Medicine and Hadassah Diabetes Center, Hadassah-Hebrew University Medical Center, Jerusalem, Israel; ²Institute of Clinical Biochemistry and ³Centre of Anatomy, Hannover Medical School, Hannover, Germany; ⁴Department of Pathology, Hadassah-Hebrew University Medical Center, Jerusalem, Israel; and ⁵The Monique and Jaques Roboh Department of Genetic Research and Metabolic Diseases, Hadassah-Hebrew University Medical Center, Jerusalem, Israel

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Weksler-Zangen S, Jörns A, Tarsi-Chen L, Vernea F, Aharon-Hananel G, Saada A, Lenzen S, Raz I. Dietary copper supplementation restores β -cell function of Cohen diabetic rats: a link between mitochondrial function and glucose-stimulated insulin secretion. *Am J Physiol Endocrinol Metab* 304: E1023–E1034, 2013. First published March 19, 2013; doi:10.1152/ajpendo.00036.2013.— β -Cell mitochondrial dysfunction as well as proinflammatory cytokines have been suggested to contribute to reduced glucose-stimulated insulin secretion (GSIS) in type 2 diabetes. We recently demonstrated that Cohen diabetic sensitive (CDs) rats fed a high-sucrose, low-copper diet (HSD) developed hyperglycemia and reduced GSIS in association with peri-islet infiltration of fat and interleukin (IL)-1 β -expressing macrophages, whereas CD resistant (CDr) rats remained normoglycemic on HSD. We examined: 1) the correlation between copper concentration in the HSD and progression, prevention, and reversion of hyperglycemia in CDs rats, 2) the relationship between activity of the copper-dependent, respiratory-chain enzyme cytochrome *c* oxidase (COX), infiltration of fat, IL-1 β -expressing macrophages, and defective GSIS in hyperglycemic CDs rats. CDs and CDr rats were fed HSD or copper-supplemented HSD before and during hyperglycemia development. Blood glucose and insulin concentrations were measured during glucose tolerance tests. Macrophage infiltration and IL-1 β expression were evaluated in pancreatic sections by electron microscopy and immunostaining. COX activity was measured in pancreatic sections and isolated islets. In CDs rats fed HSD, GSIS and islet COX activity decreased, while blood glucose and infiltration of fat and IL-1 β -expressing macrophages increased with time on HSD ($P < 0.01$ vs. CDr-HSD rats, all parameters, respectively). CDs rats maintained on copper-supplemented HSD did not develop hyperglycemia, and in hyperglycemic CDs rats, copper supplementation restored GSIS and COX activity, reversed hyperglycemia and infiltration of fat and IL-1 β -expressing macrophages ($P < 0.01$ vs. hyperglycemic CDs-HSD rats, all parameters, respectively). We provide novel evidence for a critical role of low dietary copper in diminished GSIS of susceptible CDs rats involving the combined consequence of reduced islet COX activity and pancreatic low-grade inflammation. diabetes; interleukin-1 β ; pancreatic β -cell; copper; cytochrome *c* oxidase

NUTRIENTS, DIETARY PATTERNS, and chronic inflammation in metabolically responsive organs such as the pancreas play a crucial

role in the development of type 2 diabetes (11, 18). Copper is an essential trace element that serves as a cofactor for metalloenzymes, in particular the mitochondrial respiratory chain enzyme cytochrome *c* oxidase (COX) (17, 30, 36). Proper COX activity crucial for ATP generation couples respiratory chain flux to physiological insulin secretion by pancreatic β -cells (17, 30, 36, 46). Copper deficiency has been shown to be associated with an increased frequency of glucose intolerance, electrocardiographic abnormalities, hypertension, and disturbances in lipid metabolism (33, 39, 46). In rats, copper deficiency has been shown to induce increased lipid storage in liver and pancreas and to cause degeneration and apoptosis of pancreatic acinar cells (14, 24, 49), sparing the islets, ducts, and nerves (48, 49). Restricting dietary copper combined with a high concentration of dietary sucrose induced lipid peroxidation, atrophy of pancreatic acinar tissue, and compromised mitochondrial function (14). Conversely, copper administration in mice prior to diabetes induction by streptozotocin reduced the incidence of diabetes, oxidative stress, lipid peroxidation, and expression of cytokines in mononuclear cells (43).

The Cohen diabetic rat model (5, 50, 51) comprises two genetically derived contrasting strains. The sensitive (CDs) strain develops postprandial hyperglycemia when fed the diabetogenic low-copper high-sucrose diet (HSD) but maintains normoglycemia on regular diet (RD); and the resistant (CDr) strain that does not develop hyperglycemia when exposed to HSD. Hyperglycemic CDs rats have normal fasting glucose levels (50) without insulin resistance, but their pancreatic β -cells exhibit markedly reduced capacity to secrete insulin in response to glucose (GSIS) (50). Furthermore, a pancreatic infiltration with macrophages expressing the proinflammatory cytokine IL-1 β is present in histologic sections and is associated with reduced GSIS. Resistant CDr rats fed HSD remained normoglycemic and did not exhibit these deleterious effects (50).

In this study, we studied the mechanism by which the diabetogenic low-copper diet (HSD) induces hyperglycemia in the genetically derived CDs rats. We examined 1) the correlation between copper concentration in HSD and progression, prevention, and reversal of hyperglycemia in CDs rats and 2) the relationship between the copper-dependent, mitochondrial respiratory enzyme COX activity, infiltration of IL-1 β -expressing macrophages, and defective GSIS in hyperglycemic CDs rats fed HSD.

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MATERIALS AND METHODS

Animals

Studies were performed on male CDs and CDr rats of similar initial weight, aged 8 wk. Breeding and experimentation received approval of the Animal Care Committee of Hebrew University Hadassah Medical School.

Diets

A regular rat diet (RD; Teklad 2918; Harlan Laboratories, Indianapolis, IN) contained 15–22% soy as protein and 16 ppm copper (50). In previous studies (5, 50), CDs rats were fed RD obtained from Koffolk, Petach-Tikva, Israel, that contained as protein 21% skimmed milk powder. Comparing the two different RDs, we found that CDs rats fed either RD exhibited normoglycemia regardless of the protein origin in the diet. The HSD (low-copper high-sucrose diet; MP Biomedicals, Solon, OH) contained a low copper concentration (1.2 ppm), 72% sucrose, 18% vitamin-free casein, 5% salt mixture no. II USP butter (4.5%), corn oil (0.5%), and vitamins (5, 50, 51). The copper-supplemented HSD (HSD+Cu) contained HSD supplemented with 16 ppm copper. This copper concentration is similar to that measured in the regular rat diet.

Study Design

Three study protocols were designed (Fig. 1).

Study protocol 1: diabetes progression. CDs and CDr rats were fed RD for 8 wk and switched to HSD for periods of 10, 20, or 30 days. Of note, this protocol is not a copper-deficient protocol, as the HSD is only copper poor and the body copper stores were not eliminated using a copper chelator. As previously shown, this protocol initiated hyperglycemia within a period of 1 mo only in CDs but not in CDr rats (5, 50, 51).

Study protocol 2: diabetes prevention. CDs- and CDr-rats were fed RD for 8 wk and switched to HSD+Cu for a period of 30 days.

Study protocol 3: diabetes reversal. Hyperglycemic CDs rats were fed HSD for 30 days and switched to HSD+Cu for periods of 10, 20, or 40 days. The hyperglycemia of CDs rats fed HSD for 30 days was

confirmed by a 2-h postprandial blood glucose test of >200 mg/dl (5, 50, 51) before the onset of HSD+Cu feeding. The 40-day HSD+Cu group was designed to confirm the persistence of reversal to normoglycemia.

OGTT and IVGTT

Blood glucose and insulin concentrations were measured during oral (OGTT) and intravenous (IVGTT) glucose tolerance tests. Blood glucose was measured using a glucometer (Elite, Bayer, Leverkusen, Germany) and serum-insulin with an ELISA assay (Mercodia, Uppsala, Sweden). At the different time points in the different study protocols, the rats were killed, and the organs were removed and processed. Pancreases were fixed, and pancreatic morphology, fat, and macrophage infiltration as well as β -cell function were evaluated. COX activity was evaluated during diabetes progression (*study protocol 1*) and diabetes reversal (*study protocol 3*).

In the OGTT, glucose and insulin concentrations were measured in overnight-fasted rats in tail vein blood obtained before and after an oral bolus administration of glucose (3.5 g/kg) (5, 50, 51). In the IVGTT, glucose and insulin concentrations were measured in overnight-fasted rats in blood drawn from the carotid artery before and after intravenous glucose bolus (0.75 g/kg) administration into the jugular vein (50). IVGTT required complicated surgery to insert catheters into the right jugular vein and left carotid artery of anesthetized rats. Therefore, in *study protocol 3*, using a large number of hyperglycemic CDs rats, we performed an OGTT. This was based on our previous study (50) showing that IVGTT and OGTT exhibited comparable phenotypes. Nevertheless, in the current study, the OGTT phenotype was confirmed in 40-day CDs-HSD+Cu by IVGTT.

Collection of Tissues

Liver, spleen, kidney, heart, and pancreas were removed and weighed. The entire pancreas was flash-frozen in liquid nitrogen and kept at –80°C for insulin and TG measurement or for cryostat sections. Alternatively, the pancreas was fixed by immersion either in buffered 4% paraformaldehyde for light microscopy or in a mixture of 2% paraformaldehyde and 2% glutaraldehyde for ultrastructural analysis.

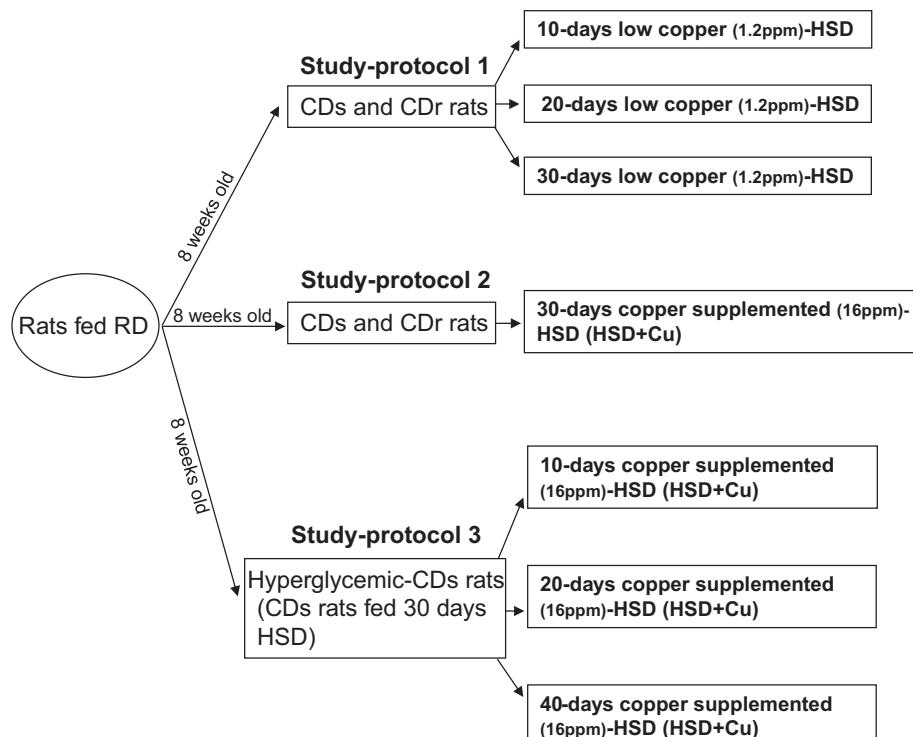


Fig. 1. Study groups. Rats fed regular diet (RD) were switched to one of the following feeding protocols: 1) *study protocol 1* (diabetes progression): Cohen diabetic sensitive (CDs) and resistant (CDr) rats fed 10-, 20-, or 30-day low-copper high-sucrose diet (HSD; copper content 1.2 ppm); 2) *study protocol 2* (diabetes prevention): CDs and CDr rats fed 30-day HSD or HSD+Cu (HSD containing adequate 16 ppm copper concentration); 3) *study protocol 3* (diabetes reversal): CDs rats fed 30-day HSD. Hyperglycemia (>200 mg/dl) was confirmed in these rats by 2-h postprandial glucose test. Hyperglycemic CDs rats were fed HSD+Cu for 10, 20, and 40 days.

Triglyceride and Fatty Acid Measurements

Triglycerides (TGs) were extracted from the whole pancreas (15) and determined using a GPO-Trinder kit (Sigma, St. Louis, MO). Nonesterified fatty acids were measured in serum of overnight fasted rats using a NEFA kit (Randox Laboratories, Crumlin, County Antrim, UK).

Immunohistochemistry and Morphological Evaluation

Immunohistochemistry used the avidin-biotin complex (ABC) or a double immunofluorescence method to identify β -cells, CD68 macrophages, and IL-1 β expression (20, 50) with the following primary antibodies: insulin (A565; DAKO, Hamburg, Germany), CD68 macrophages (clone ED1), and IL-1 β (clone silk6; both from Serotec, Düsseldorf, Germany) (21, 50). Sections were examined using bright-field illumination or fluorescence illumination with a BX61 Olympus microscope. Areas of acinar cells and adipocytes were morphometrically determined, and the proportion of adipose tissue was expressed as a percentage of the total pancreas tissue on the section. Macrophages and their IL-1 β expression were quantified in eight consecutive sections of four different areas in each animal for the different experimental groups. Results are expressed per square millimeter of pancreatic parenchyma.

COX Activity in Freshly Isolated Islets and Pancreas Sections

COX activity was determined spectrophotometrically (40) in homogenates of 200–300 islets freshly isolated from CDs or CDr rats fed for 30 days on HSD (*study protocol 1*) or from hyperglycemic CDs rats fed for 20 days on HSD+Cu (*study protocol 3*). COX activity was expressed per milligram of protein or per activity of the mitochondrial matrix enzyme citrate synthase (CS). Histochemical COX activity staining was performed in cryostat pancreatic sections (11 μ m). COX activity was depicted by oxidative polymerization of 3,3'-diaminobenzidine (DAB) in response to an osmophilic reaction product allowing the localization of COX in the mitochondria of different organs (41). Briefly, pancreatic sections were incubated in fresh incubation solution containing DAB and cytochrome C (type II) in phosphate buffer (41, 42). Sections from the different groups were stained in parallel to avoid variations in the intensity of the brown staining. COX activity in islets was determined densitometrically and calculated as a percentage of COX activity in CDr rats fed 30-day HSD, which was taken as 100%. We analyzed 20–40 islets densitometrically in each experimental group.

Transmission Electron Microscopy

Ultrathin sections were contrast-stained with saturated solutions of lead citrate and uranyl acetate and evaluated by electron microscopy (EM 9 S2; Zeiss, Oberkochen, Germany) (21). Twenty islets were analyzed in each experimental group.

Data Analysis

Data are means \pm SE. Statistical significance of differences between groups was determined by one-way ANOVA followed by the Tukey test using the SigmaStat program (Jandel, San Rafael, CA). A two-tailed paired *t*-test was used to compare data from tests performed on the same animal or in isolated-islets.

RESULTS

Glucose Tolerance Tests

HSD gradually induced hyperglycemia in CDs rats. In *study protocol 1* (Fig. 1), the glucose_{AUC} of CDs rats fed 10 days HSD was not significantly different from the glucose area under IVGTT curve (glucose_{AUC}) of CDr rats fed 10-day HSD (Fig. 2C) or CDs rats fed RD (Fig. 2E), but maximal insulin secretion was already reduced in CDs rats compared with CDr

rats (Fig. 2, *B* and *D*), indicating that the impairment in GSIS in the CDs rats preceded the development of hyperglycemia. The insulin area under the IVGTT curve (insulin_{AUC}) markedly decreased, whereas the glucose_{AUC} markedly increased in relation to the duration on HSD (insulin_{AUC} 19,134 \pm 1,352, 12,669 \pm 2,242, and 4,350 \pm 1,218 pmol·l $^{-1}$ ·120 min; glucose_{AUC} 935 \pm 105, 1,400 \pm 144, and 1,500 \pm 160 mmol·l $^{-1}$ ·120 min for 10, 20, and 30 days on HSD, respectively; Fig. 2, *A* and *B*). CDr rats fed HSD exhibited a small reduction (25%) in insulin_{AUC} only after 30 days on HSD (Fig. 2D), whereas glucose_{AUC} did not significantly change with time on HSD (Fig. 2C).

Copper supplementation to the HSD (HSD+Cu; *study protocol 2*; Fig. 1) prevented the increase in glucose_{AUC} and the decrease in insulin_{AUC} (Fig. 2, *E* and *F*), thus eliminating the difference in glucose and insulin tolerance curves exhibited by CDs-HSD and CDr-HSD rats. In fact, blood glucose of CDs rats fed HSD+Cu rose less than in normoglycemic CDs-RD rats (Fig. 2E), while insulin secretion was comparable (Fig. 2F). Moreover the IVGTT curve of CDs rats fed HSD+Cu was characterized by a large insulin-secretory response to glucose followed by a prompt reduction in blood glucose concentration (Fig. 2, *E* and *F*). HSD+Cu also reversed the diabetic OGTT exhibited by hyperglycemic CDs rats (*study protocol 3*; Fig. 1). After 10 days on HSD+Cu, hyperglycemic CDs rats exhibited a 50% recovery of the glucose_{AUC} and insulin_{AUC} (not shown). Full reversal, i.e. glucose levels and insulin_{AUC} as found in normoglycemic CDs rats (Fig. 2, *G* and *H*), was achieved after 20 days on HSD+Cu and maintained for a total of 40 days as demonstrated by OGTT (Fig. 2, *G* and *H*) and IVGTT (Fig. 2I).

Pancreas Weight

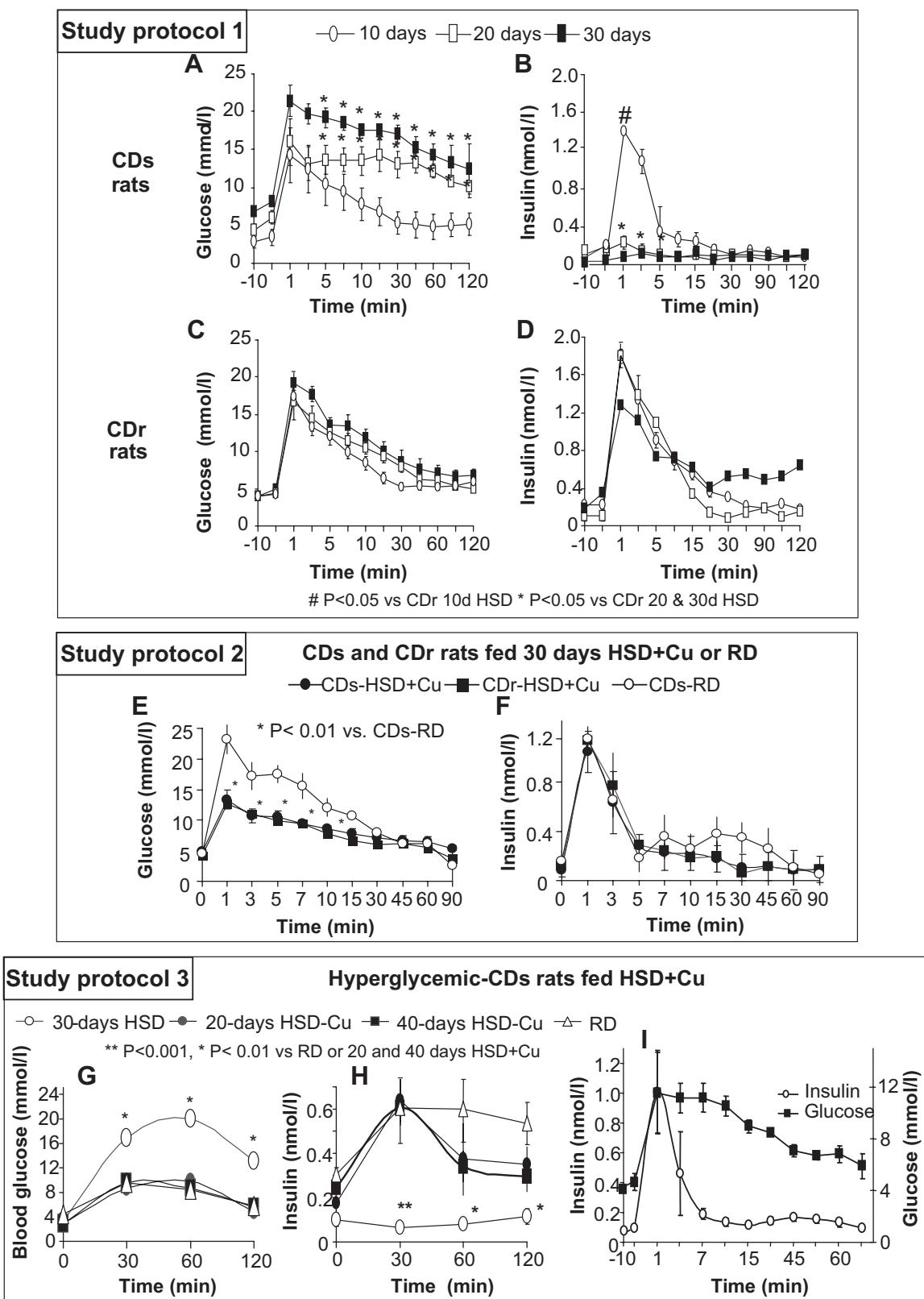
Based on the fact that CDs rats fed 30-day HSD exhibited a marked reduction in pancreatic weight and enhanced pancreatic lesions in our previous studies (50), we studied the relation between the time of exposure to HSD and pancreatic weight (*study protocol 1*; Fig. 1). A significant decrease in pancreatic weight of CDs rats was initiated only after 20 days on HSD. Pancreas weight was further reduced after 30 days on HSD (Fig. 3A, $P < 0.05$ vs. CDr-HSD Fig. 3B). After 10 days of HSD, the pancreas weight of CDs was not significantly different from that of CDr-HSD (Fig. 3B) or CDs-RD (not shown). The reduction in pancreatic weight was prevented (*study protocol 2*; Fig. 1) when the CDs rats were fed HSD+Cu (Fig. 3C). Thus, pancreas weight of CDs rats fed HSD+Cu was comparable to that of CDr rats fed HSD+Cu (Fig. 3D). The pancreas weight of hyperglycemic CDs rats fed 20-day HSD+Cu (*study protocol 3*; Fig. 1) increased by 40% (Fig. 3E). The increased weight was maintained for 20 additional days (hyperglycemic CDs rats fed 40-day HSD+Cu; Fig. 3E). No differences were detected in the weight of liver, spleen, heart, or kidney of rats in the different studies (not shown). Pancreas weight of CDr rats did not change with time on HSD or HSD+Cu (Fig. 3, *B* and *D*).

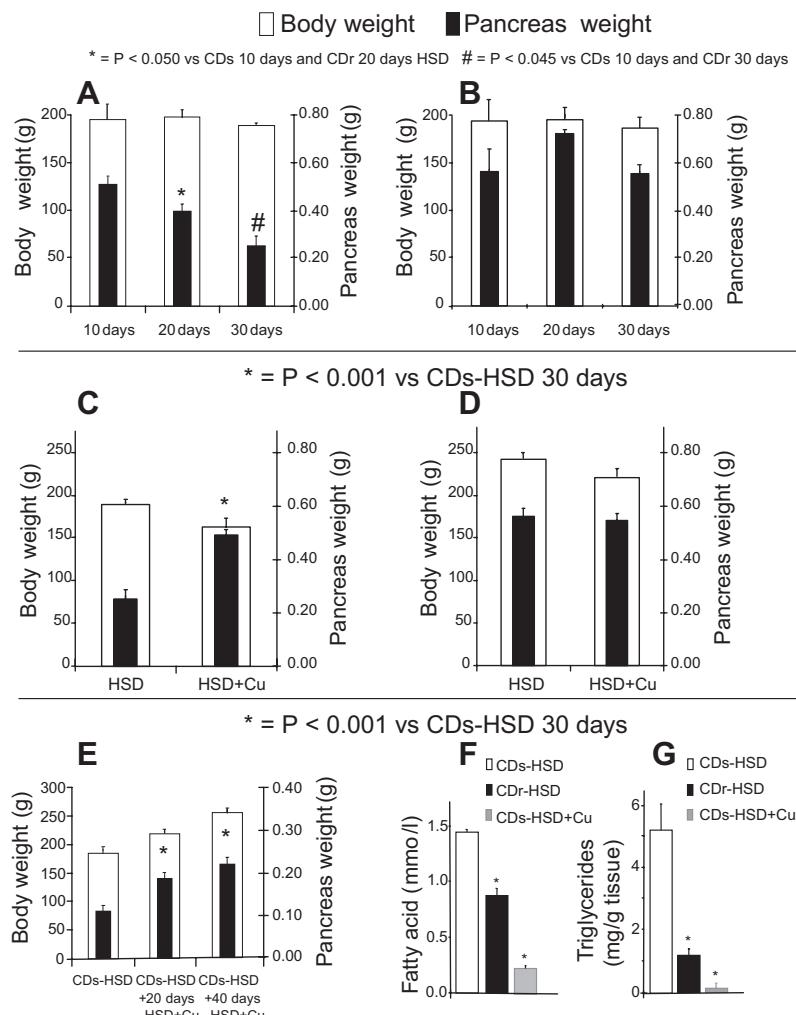
Immunostaining of Insulin in β -Cells and IL-1 β in Macrophages

HSD gradually increased the number of macrophages infiltrating the exocrine pancreas and the intraislet capillary system as well as the extent of their activation (*study protocol 1*; Figs. 1 and 4, *A* and *B*). After 10 days on HSD, the pancreas of CDs rats was infiltrated

with a small number of macrophages (0.6 ± 0.2 macrophages/ mm^2), most of which (0.5 ± 0.0 macrophages/ mm^2) expressed IL-1 β . After 20 and 30 days on HSD, the number of infiltrating macrophages increased gradually to 1.4 ± 0.3 and 2.8 ± 0.3

macrophages/ mm^2 , respectively (Fig. 4A), and 85% expressed IL-1 β (1.2 ± 0.1 and 2.4 ± 0.3 , respectively; Fig. 4B). In the pancreas of CDr rats fed 30-day HSD, only a negligible number of macrophages (0.3 ± 0.1 macrophages/ mm^2) was





observed, and none of them expressed IL-1 β . CDs rats fed HSD+Cu (*study protocol 2*; Fig. 1) exhibited only a small number of macrophages (0.8 ± 0.2 macrophages/mm 2) (Fig. 4C), of which 50% (0.4 ± 0.1 macrophages/mm 2) expressed IL-1 β (Fig. 4D). The pancreas of CDs-RD (not shown) and CDr-HSD+Cu rats (*study protocol 2*; Fig. 1) exhibited only a negligible number of macrophages (Fig. 4, E and F). The intensity of insulin immunostaining was not different in islets of the CDs rats fed 30-day HSD (Fig. 4, A and B, *study protocol 1*), CDs or CDr rats fed 30-day HSD+Cu (Fig. 4, C-F, *study protocol 2*). Feeding hyperglycemic CDs rats HSD+Cu (*study protocol 3*, Fig. 1) reduced pancreatic lesions and the infiltration of fat and macrophages, as documented

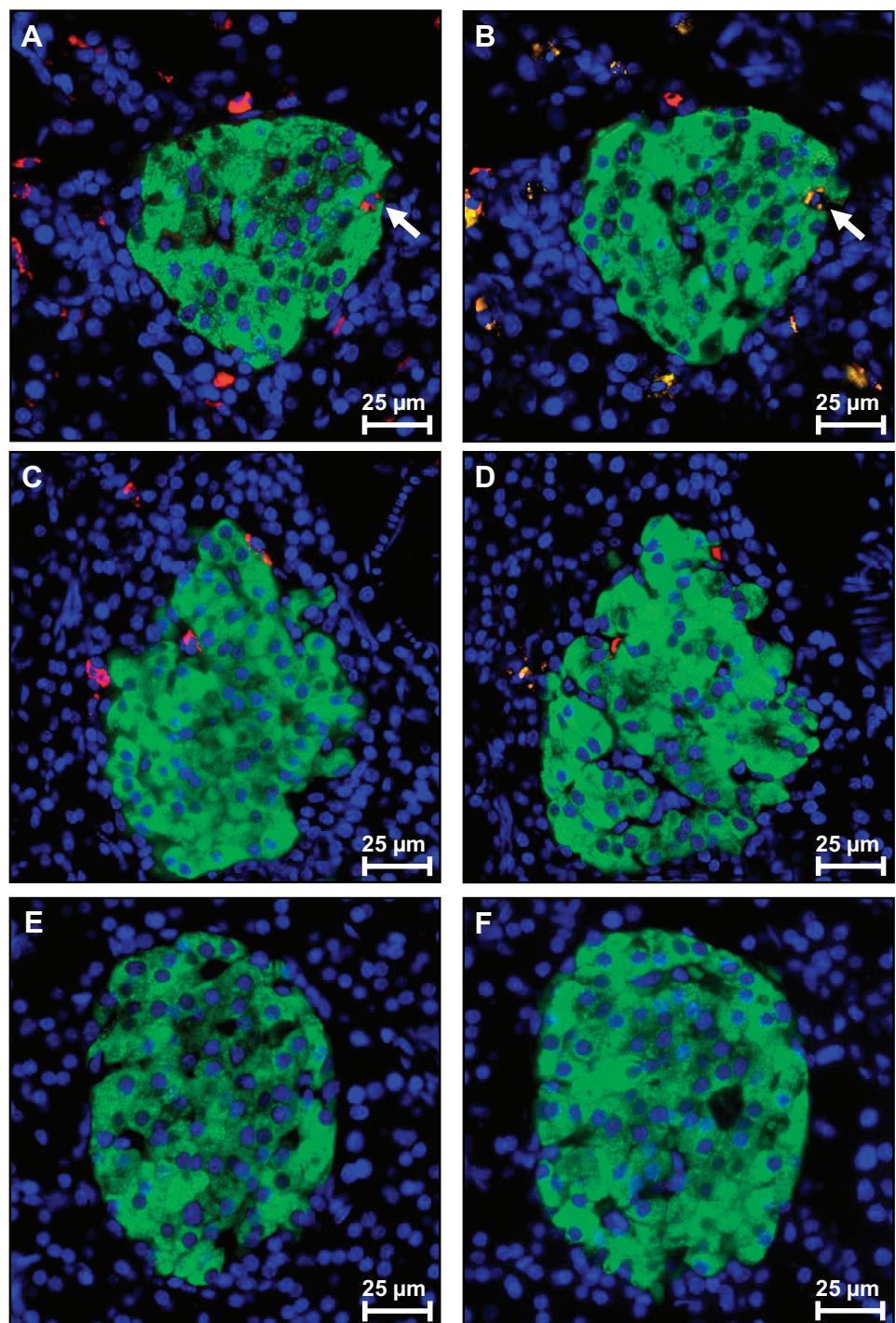
Fig. 3. Effect of the different dietary protocols on pancreas weight (A-E), blood NEFA (F), and pancreas TG concentrations (G). *Study protocol 1*: body weight (open bars) and pancreas weight (filled bars) of CDs (A), CDr rats (B) fed 10- ($n = 6$), 20- ($n = 6$), and 30- ($n = 6$) day HSD. *Study protocol 2*: body weight (open bars) and pancreas weight (filled bars) of CDs (C) and CDr rats (D) fed HSD or HSD+Cu ($n = 6$). *Study protocol 3*: E: body weight (open bars) and pancreas weight (filled bars) of CDs rats fed 30 days HSD and hyperglycemic CDs-HSD rats fed 20- ($n = 9$), and 40- ($n = 12$) days HSD+Cu. *Study protocol 2*: serum NEFA (mmol/l; F) and pancreatic TG content (mg/g tissue; G) of CDs rats fed 30-day HSD (open bars, $n = 6$), CDr rats fed 30-day HSD (filled bars, $n = 6$), and CDs rats fed 30-day HSD+Cu (gray bar, $n = 6$). Data are presented as means \pm SE.

through quantitative analyses. After only 10 days on HSD+Cu, the number of macrophages decreased by 46% (1.8 ± 0.3 macrophages/mm 2), and the degree of activation (IL-1 β expression) was reduced to 44% (1.2 ± 0.2 macrophages/mm 2 , $P < 0.05$ vs. CDs rats fed 30-day HSD). Following 20 days on HSD+Cu, the number of macrophages decreased to 50% (1.4 ± 0.2 macrophages/mm 2), and only 35% expressed IL-1 β (0.5 ± 0.1 macrophages/mm 2).

Ultrastructural Analysis of the Exocrine Pancreas and β -Cells
HSD gradually increased pancreatic damage (*study protocol 1*, Fig. 1). CDs rats fed 10-day HSD exhibited minor changes in the

Fig. 2. Glucose tolerance tests. Effect of the different dietary protocols on glucose-stimulated insulin secretion (GSIS). Blood glucose and serum insulin concentrations were measured in overnight-fasted rats before (-10 and/or 0 min) glucose administration and several times during 120 min of IVGTT (A-F and I) or OGTT (G and H). *Study protocol 1*: IVGTT of CDs (A and B) and CDr rats (C and D) fed 10- (\circ , $n = 6$), 20- (\square , $n = 6$), and 30- (\blacksquare , $n = 6$) day HSD, Blood glucose (mmol/l, A and C) and serum insulin (B and D, pmol/l) concentrations during 120 min of OGTT. *Study protocol 2*: E and F: IVGTT of CDs (\bullet , $n = 6$) and CDr (\blacksquare , $n = 6$) rats fed 30-day HSD+Cu and CDs fed 30-day RD (\circ , $n = 6$). Blood glucose (mmol/l) and serum insulin (pmol/l) were measured during 60 min of IVGTT until metabolic parameters returned to baseline levels. *Study protocol 3*: OGTT of normoglycemic CDs rats fed 30-day RD (\triangle , $n = 6$), hyperglycemic CDs rats fed 30-day HSD (\circ , $n = 19$), and hyperglycemic CDs rats fed 40-day HSD+Cu (\blacksquare , $n = 12$). Blood glucose (G; mmol/l) and serum insulin (H; pmol/l) were measured during 120 min of OGTT. I: IVGTT of hyperglycemic CDs rats fed 40-day HSD+Cu ($n = 5$). Blood glucose (\blacksquare) and serum insulin (\circ) levels were measured during 60 min of IVGTT until blood glucose concentration was reduced to 6 mmol/l (normoglycemic levels).

Fig. 4. Effect of different dietary protocols on immunostaining for insulin, macrophages, and IL-1 β . *A–F*: immunostaining for insulin (green), (*A*, *C*, and *E*) CD68 infiltrating macrophages (red) and (*B*, *D*, and *F*) IL-1 β in macrophages (orange-yellow) in pancreases of CDs rats fed 30-day HSD (*A* and *B*, *study protocol 1*), CDs rats fed 30-day HSD+Cu (*C* and *D*, *study protocol 2*), and CDr rats fed 30-day HSD+Cu (*E* and *F*, *study protocol 2*). *A–F*: counterstaining with DAPI (blue). Dense green insulin immunostaining was observed in the islets of rats in all groups. *A*: red immunostaining for infiltrating CD68 macrophages; *B*: orange-yellow immunostaining for IL-1 β expression. Macrophages were identified in the vicinity of the islets and in the pancreatic capillary system (white arrows) of CDs-HSD rats. *A* and *B*: macrophages expressing both CD68 and IL-1 β are marked in orange-yellow as an overlay of CD68 macrophage and IL-1 β staining in consecutive sections. *B* and *D*: only a few macrophages were observed in the pancreas of CDs-HSD+Cu rats (red), and very few (orange-yellow in *D*) expressed IL-1 β . *E* and *F*: macrophages were not found in the pancreas of CDr-HSD+Cu rats (*study protocol 2*).



pancreatic exocrine parenchyma (not different compared with CDs-RD; data not shown), preserving the structure of the cellular organelles including the mitochondria in exocrine pancreas and islets (not shown). After 20 days on HSD, the exocrine parenchyma of the CDs rats showed an advanced process of acinar degeneration and fat infiltration (not shown). After 30 days on HSD, macrophages showing ultrastructural signs of activation (i.e., increased cytoplasmic volume; Fig. 5C) and fat (Fig. 6C) were seen in the transition zone between exocrine and endocrine parenchyma. β -Cells in the islet periphery exhibited swollen mitochondria as a sign of cellular damage (Fig. 5D and

Ref. 50). The pancreas of CDr rats fed 30-day HSD did not show any signs of lesions in either exocrine parenchyma or in the islets (50). Feeding CDs rats 30-day HSD+Cu (*study protocol 2*) prevented these pancreatic lesions, preserving a normally appearing parenchyma (Fig. 5A) and intact cellular organelles including the mitochondria in the exocrine pancreas and in the β -cells (Fig. 5B). Copper supplementation (*study protocol 3*) reversed the deleterious effect seen in hyperglycemic CDs rats in a gradual manner depending on the time on HSD+Cu. After 10 days on HSD+Cu, the recovery of the exocrine parenchyma was underway, with complete restoration

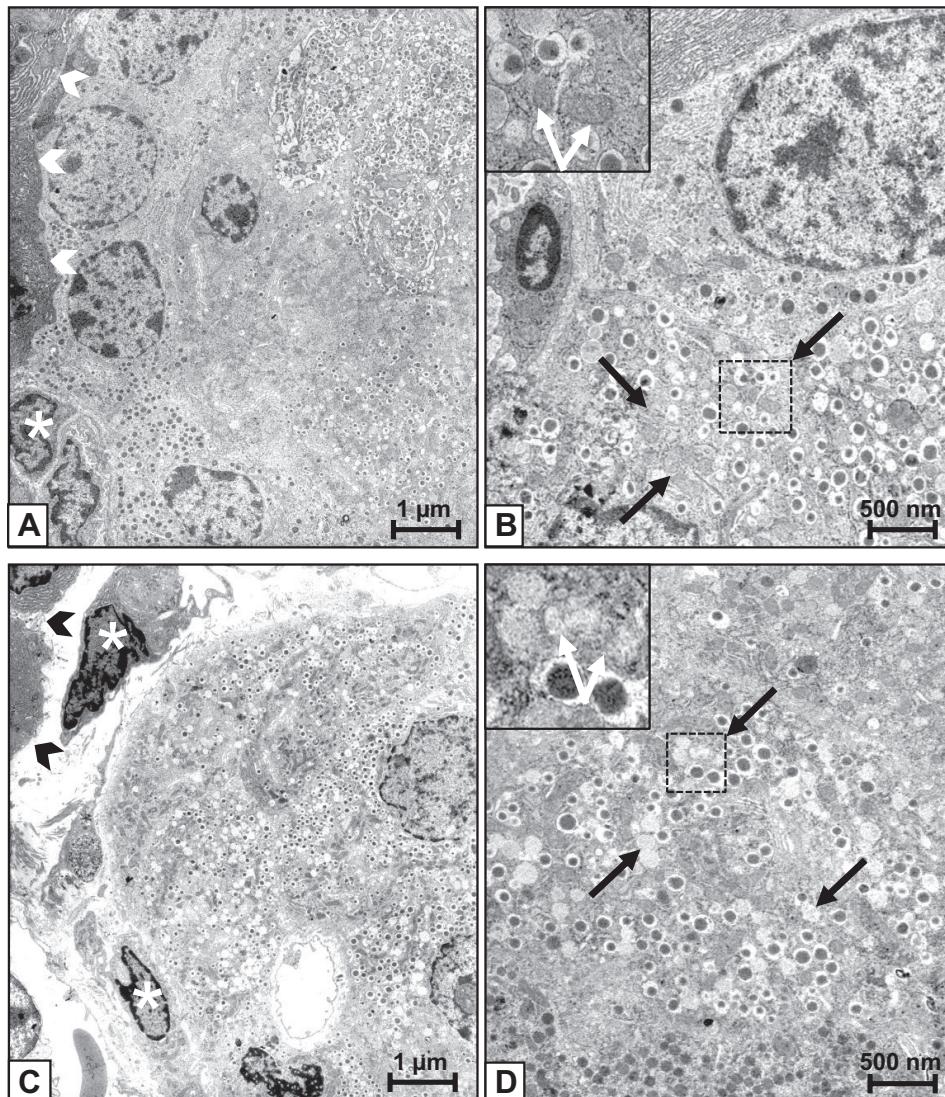


Fig. 5. Effect of *study protocols 1* and *2* on ultrastructure of the exocrine pancreas and β -cells. Ultrastructure of the pancreas of CDs rats fed 30-day HSD+Cu (*A* and *B*, *study protocol 2*) or 30-day HSD (*C* and *D*, *study protocol 1*). *A*: CDs rats fed HSD+Cu exhibited well-preserved exocrine pancreatic parenchyma (arrowheads). *B*: β -cell mitochondria showed normal structure (arrows in *B* and inset). Macrophages were rarely seen in the pancreas of these rats. Single macrophages found (asterisk in *A*), showed only mild signs of activation (cytoplasmic rim). *C*: exocrine pancreas of CDs-HSD rats exhibited atrophic parenchyma (arrowheads). Activated macrophages (large cytoplasmic volume, asterisks in *C*) were observed in the transition zone between the islets and exocrine pancreas. In the islet periphery, some β -cells exhibited swollen mitochondria (arrows in *D* and inset).

in a few of the acinar lobules (Fig. 6, *A* and *B*). The full recovery of both the exocrine parenchyma and β -cells was apparent after 20 days on HSD+Cu and persisted also after 40 days of HSD+Cu (Fig. 6, *D*, *G*, and *H*). Some cells in the restored areas of the exocrine parenchyma were still smaller compared with the controls, whereas all β -cells showed well-preserved cellular organelles, particularly the mitochondria (Fig. 6, *E* and *F*).

Blood NEFA Concentration and Infiltration of Adipose Tissue in the Exocrine Pancreas

The previously reported increase in blood NEFA concentration and in infiltration of adipose tissue in the exocrine pancreas of CDs rats fed 30-day HSD (50) was also demonstrated in the current study as a gradual increase. In CDs fed HSD (*study protocol 1*, Fig. 1), the proportion of pancreatic lesions and infiltration of adipose tissue increased in relation to time on HSD. Pancreatic TGs increased by 11 ± 4 , 23 ± 5 , and $36 \pm 3\%$ after 10, 20, and 30 days on HSD, respectively ($P < 0.05$ vs. CDs-RD). Copper supplementation (*study protocol 2*, Fig. 2) prevented the increase in blood NEFA and pancreatic TG

(Fig. 3, *F* and *G*) to levels that were comparable to those observed in CDs-RD pancreas (not shown). The proportion of adipose tissue observed in the exocrine parenchyma of hyperglycemic CDs rats decreased in relation to time on HSD+Cu (24 ± 3 , 18 ± 4 , and $5 \pm 1\%$ for 10, 20, and 40 days on HSD+Cu, respectively, $P < 0.05$ vs. 30 days on HSD, *study protocol 3*; Fig. 1). The pancreas of CDs-RD and CDr-HSD+Cu rats exhibited tiny amounts of TGs.

COX Activity

As copper intake seemed to be highly associated with GSIS capacity and with the pancreatic histopathology changes exhibited by CDs hyperglycemic rats, we chose to study whether there is a correlation between the diabetic phenotype in these rats and the activity of the copper-dependent, mitochondrial respiratory enzyme COX in β -cells. Islets isolated from hyperglycemic CDs-HSD rats (*study protocol 1*), exhibiting a markedly reduced GSIS during IVGTT (reduced insulin_{AUC} in Fig. 7A) demonstrated a 60% reduction in COX activity (COX measured in islet homogenates; Fig. 7, *B* and *C*), suggesting that low copper induced the reduction in COX activity. Likewise,

the histochemical enzyme staining of pancreatic sections of CDs rats fed HSD (*study protocol 1*) showed light-brown staining, indicating low COX activity (Fig. 7D) compared with dense-brown staining, indicative of high COX activity in islets of CDr-HSD-rats (Fig. 7E). The densitometric analysis of the histochemical enzyme staining confirmed the significantly

lower COX activity in the islets of CDs rats fed 30-day HSD compared with the islets of CDr rats fed 30-day HSD (61.8 ± 1.8 vs. 100.0 ± 2.7 , respectively, $n = 9$ islets in each study group, $P < 0.05$; Fig. 7, E and D). Hyperglycemic CDs rats fed 20-day HSD+Cu (*study protocol 3*) shown in previous sections of this study to exhibit a substantial recovery of their GSIS capacity and pancreatic exocrine damage also exhibited a substantial recovery of their islet COX activity (Fig. 7, A–C). Likewise, the histochemical enzyme staining of pancreatic sections of hyperglycemic CDs rats fed 20-day HSD+Cu (*study protocol 3*) exhibited dense-brown staining, indicative of high COX activity (Fig. 7F), suggesting that dietary copper supplementation may restore islet COX activity. The densitometric analysis of the histochemical enzyme staining confirmed the significant recovery of COX activity in islets of hyperglycemic CDs rats fed 20-day HSD+Cu (120.4 ± 2.5 vs. 61.8 ± 1.8 units in islets of hyperglycemic CDs rats, $P < 0.01$, $n = 20$ –40 islets per group; Fig. 7, D and F).

DISCUSSION

In the present study, we analyzed the mechanisms underlying β -cell dysfunction in genetically susceptible hyperglycemic CDs rats and the specific role of copper intake in this process. We demonstrated that exposure of CDs rats to a low-copper HSD induced a sequence of deleterious events resulting in inhibition of GSIS. Exposure to HSD markedly reduced the activity of the mitochondrial copper-dependent-enzyme COX, increased lipolysis, and the peri-islet infiltration of lipids and macrophages secreting IL-1 β . On the other hand, we showed that feeding CDs rats a copper-supplemented HSD prevented and reversed β -cell dysfunction and the vicious cycle of deleterious events.

As these deleterious events occurred exclusively in susceptible CDs rats and not in CDr rats fed the same diet (HSD), and considering that GSIS is dependent on mitochondrial ATP generation via COX (3, 29, 35), we hypothesize that 1) a latent inherited aberration in β -cell function, probably in COX activity, is stimulated in CDs rats following a short exposure to low-copper HSD; 2) longer exposure to the copper-deficient HSD leads to further reduction in COX activity, inducing the deterioration of GSIS and causing the concurrent exocrine pancreatic damage, increased lipolysis, pancreatic fat deposi-

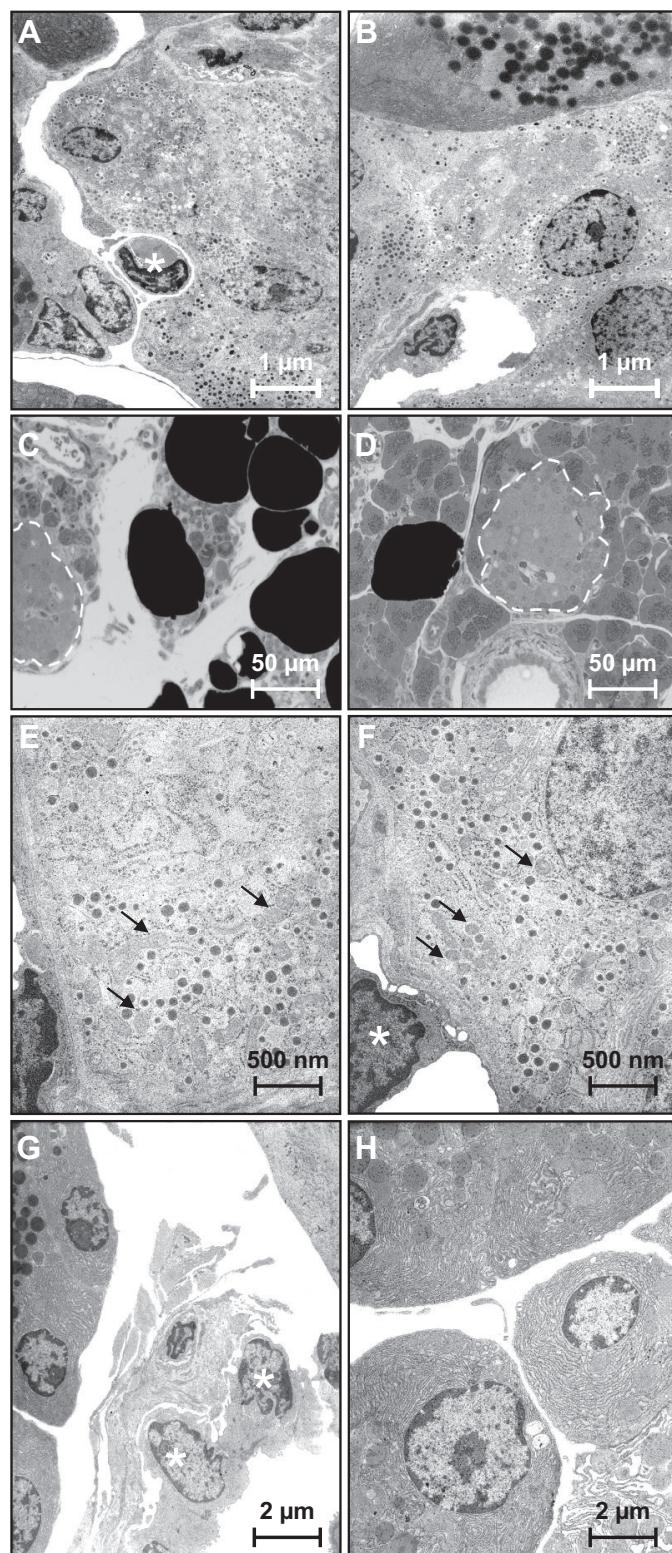


Fig. 6. Structure of exocrine parenchyma and islets of hyperglycemic rats fed HSD + Cu. *A* and *B*: ultrastructure of islets including β -cells and structural changes in exocrine parenchyma of hyperglycemic CDs rats fed 10-day HSD+Cu. *C*: CDs rats fed 30-day HSD. *D* and *E–H*: hyperglycemic CDs rats fed 20-day HSD+Cu. In hyperglycemic CDs rats fed 10-day HSD+Cu, recovery of the exocrine parenchyma was in process; macrophages (asterisks in *A*) were still present in the transition zone of the islets but were absent in areas (*B*) in which the exocrine pancreatic parenchyma was fully recovered. *D*: after 20 days of HSD+Cu, the exocrine pancreas of these rats exhibited full recovery of the exocrine parenchyma with only a small reminiscence of adipocyte tissue shown on a semithin section in the vicinity of the islets (dotted line). In these rats, β -cells in the periphery (*E*) and in the center (*F*) of the islets were ultrastructurally well preserved. Cellular organelles, especially mitochondria (arrows), had a normal shape. Single macrophages (asterisk in *F*) still remained, mostly in the interlobular region (*G*) of the exocrine parenchyma (asterisks in *G*). *H*: in these rats, some exocrine parenchymal cells were still decreased in size. *C*: in CDs rats fed 30-day HSD, a high degree of exocrine pancreatic atrophy was demonstrated as well as a large amount of adipose tissue in close vicinity to islets, as shown on this semithin section (dotted line).

* P<0.01 COX activity in isolated islets of CDs-HSD 30 days vs. CDs-HSD fed 20-days HSD+Cu and vs. CDr-HSD 30-days
P<0.01 COX activity in isolated islets of CDs-HSD fed 20-days HSD+Cu vs. CDs-HSD and CDr-HSD

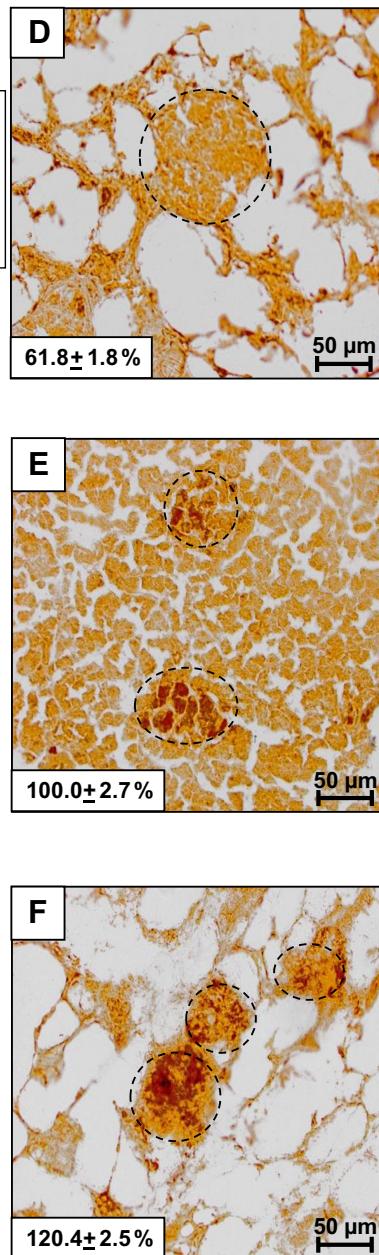
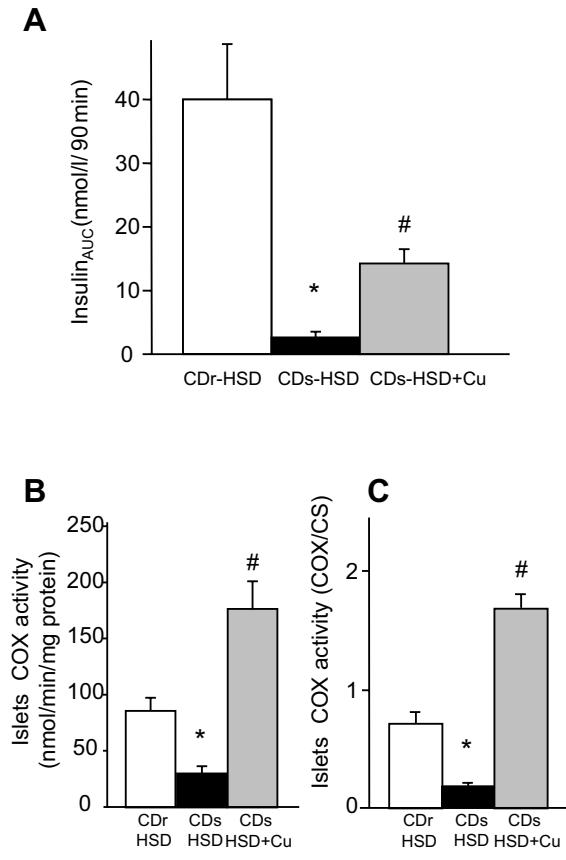


Fig. 7. Effect of copper concentration in HSD on cytochrome *c* oxidase (COX) activity in freshly isolated islets and in pancreas sections. A: insulin_{AUC} of the IVGTT curve and COX activity normalized (B) per protein and (C) per CS (citrate synthase, an ubiquitous mitochondrial matrix enzyme) in freshly isolated islets of CDr-HSD (open bars), CDs-HSD (filled bars), and hyperglycemic CDs rats fed 20-day HSD+Cu (gray bars). Comparable data were observed for COX activity calculated as activity per protein (B), normalized per CS (C) or measured by immunohistochemistry. Data are presented as means \pm SE. Brown enzyme histochemical staining COX activity in pancreatic cryostat sections of CDs-HSD (D), CDr-HSD (E), and hyperglycemic CDs rats (F) fed 20-day HSD+Cu. Islets are marked by dashed black circles. Results of densitometric measurements are expressed as gray values/ μ m² with the highest gray value of 68.4 \pm 4.3/ μ m² with CDr-HSD rats set as 100%.

tion, and macrophage infiltration and activation; and 3) the combined effect of reduced COX activity and macrophage infiltration establishes a deleterious microenvironment for pancreatic β -cells.

The first pathological event observed in CDs rats occurring after only 10 days on HSD was a reduction in GSIS that preceded hyperglycemia and all other deleterious events. The initial reduction in GSIS could therefore explain the particular sensitivity of CDs rats to developing hyperglycemia following longer exposure to low-copper HSD.

How may copper deficiency alter β -cell function? Copper is a necessary cofactor for several enzymes, including the mitochondrial respiratory chain enzyme COX (2–4, 17, 30, 36). We therefore assumed that copper deficiency could impair islet COX activity, oxidative phosphorylation, and ATP generation, thereby diminishing GSIS. The data presented in the current

study demonstrating a parallel decrease in COX activity and GSIS in islets isolated from hyperglycemic CDs rats fed HSD that was reversed when the rats were fed a copper-supplemented HSD support this assumption.

Other investigators also demonstrated a beneficial effect of copper supplementation on streptozotocin-diabetic mice preventing β -cell death and diabetes development (43). Copper deficiency has been shown to reduce COX activity in cardiac mitochondria of nondiabetic rats, associating copper insufficiency with disruption of mitochondrial architecture (8, 28, 32, 33, 52), impaired mitochondrial oxidative phosphorylation, and reduced mitochondrial respiration (2, 4, 19). The finding that mitochondrial dysfunction is a factor in the development of diabetes has been assigned higher importance in the last decade, since mitochondrial DNA mutations in humans and β -cell-specific deletions of mitochondrial genes in animal

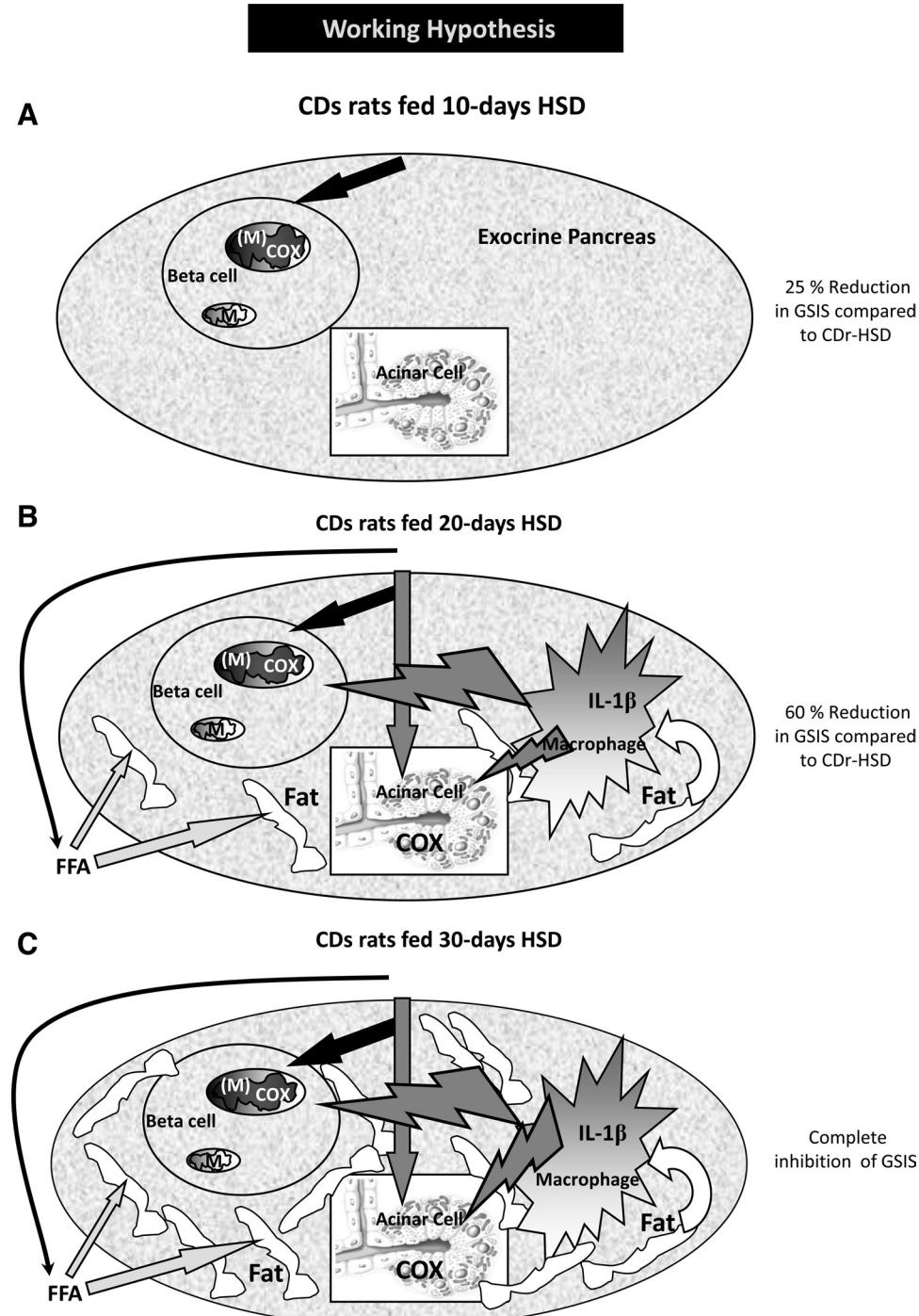
models have been shown to cause diabetes (35, 42, 44). It is of interest to note that the relationship of diabetes and mitochondrial dysfunction has recently also been reported in lymphocytes of type 2 diabetic patients exhibiting reduced COX activity, and reduced copper was observed in diabetic patients (1, 22, 35, 46).

Copper deficiency has also been implicated in lipolysis and fat accumulation in nonadipose tissues (7, 14, 25, 37, 45). A copper-deficient diet containing high concentrations of simple sugars such as sucrose and fructose has been reported to be associated with abnormal glucose tolerance, hypertriglyceride-

mia, and pancreatic atrophy (7, 13, 14). In previous studies performed in CDs rats, several lipogenic enzymes have been shown to be elevated in both liver and adipocytes (6). Consequently, increased lipolysis could be partially due to the marked decrease in insulin secretion, which could lead to the high levels of NEFA in the serum and the ectopic accumulation of TGs in nonadipose tissues, as suggested by others (23, 38) and demonstrated in the current study.

In close association with exocrine pancreatic damage and fat accumulation, we observed pancreatic local inflammation characterized by the infiltration of macrophages expressing exclu-

Fig. 8. Working hypothesis. A vicious sequence is triggered when CDs rats are exposed to low-copper HSD. A: after 10-days of HSD, CDs rats (compared to CDr progression 10-day rats) exhibited a 25% reduction in GSIS (black arrow) resulting from COX malfunction. At that time, the exocrine parenchyma was still unaffected (not shown). After 20 days of HSD, a larger reduction in GSIS (larger black arrows) and acinar apoptosis (dark gray arrow) were observed. Copper also induced an excess of NEFA in the blood, which probably results in accumulation of peri-islet fat (light gray arrows) and macrophages expressing IL-1 β . IL-1 β (gray flash arrow) caused the reduction in COX activity resulting in substantial reduction of GSIS and increased apoptosis of acinar cells. Thirty days of HSD induced a major GSIS reduction (larger black arrows), additional acinar apoptosis (larger gray arrow), perislet infiltration of adipose tissue, and further activation of macrophages expressing larger amounts of IL-1 β (larger gray flash arrow). Consequently, GSIS was completely inhibited. M, mitochondria.



sively IL-1 β (50). During the past decade, it has become clear that inflammation is a key feature of diabetes (9–11, 18, 31, 34). Cytokines released by both T-lymphocytes and activated macrophages, in particular IL-1 β , have been implicated as immunological effector molecules in the development of type 2 diabetes, reducing insulin secretion and causing β -cell dysfunction (9, 10, 12, 18). In our study, swollen mitochondria within the β -cells in the islet periphery and the infiltrating macrophages expressing exclusively IL-1 β (50) were gradually observed during copper deprivation (HSD diet) and prevented or reversed to normal with copper supplementation. The extent of the change in islet COX activity and GSIS at each period of time during diabetes progression and reversal paralleled the extent of pancreatic lesions and peri-islet infiltration of macrophages expressing IL-1 β . Similarly, copper supplementation has been shown to prevent the inhibitory effect of IL-1 β on GSIS of isolated rat β -cells (47). We assumed that exposure to IL-1 β alone, demonstrated in our study, has a substantial effect on β -cell function due to a particular vulnerability and sensitivity of the β -cells to oxidative stress (26, 27). Thus, our data in conjunction with observations from other studies support the assumption that the combination of reduced COX activity and the deleterious islet microenvironment caused by IL-1 β results in the inhibition of GSIS and hyperglycemia. IL-1 β can induce the production of the free radical nitric oxide (9, 16) that may cause endoplasmic reticulum stress and further inactivation of COX. The question of how IL-1 β originating from macrophages residing in the exocrine pancreas in the vicinity of the islets and in intraislet capillaries affects β -cells in the islets can be explained based on the literature (9) and on our data (50) by the ability of activated macrophages to affect β -cell function in a paracrine fashion (10, 12). The inflammatory element does suggest a close functional relationship between endocrine and exocrine pancreas, yet the exact nature of such interaction and the way in which it may affect β -cell function are important questions to be further clarified in future studies. Overall, the above support a link among low-copper HSD, local inflammation, mitochondrial dysfunction, and reduced GSIS.

Finally, we propose that glucose intolerance in the CDs rat resulted from the combined effect of genetic predisposition exacerbated by low-copper HSD. The genetic profile protected the CDr rat, enabling the maintenance of adequate COX activity despite the low-copper HSD, whereas the genetic sensitivity of CDs rats initiated the following sequence of events (Fig. 8, A–C). After 10 days on low-copper HSD, islet COX activity and GSIS were reduced (Fig. 8A). Following 20 days on HSD (Fig. 8B), COX was reduced in both islets and the exocrine tissue, inducing acinar apoptosis (50), additional reduction in GSIS, lipolysis, fat accumulation in the exocrine pancreas, and peri-islet macrophage infiltration and activation. Thirty days on HSD (Fig. 8C), GSIS is nearly abolished and acinar apoptosis is aggravated, while peri-islet fat accumulation and macrophage infiltration and activation are substantially increased. This vicious sequence of events resulted in a marked reduction of COX activity and complete inhibition of GSIS and hyperglycemia.

In conclusion, our new findings demonstrate the need for both genetic and environmental elements to cause marked β -cell dysfunction and hyperglycemia. We demonstrated a crucial-link among low dietary copper, reduced islet COX activity, pancreatic low-grade inflammation, and diminished

GSIS. We also demonstrated the beneficial effect of copper supplementation in preventing and restoring β -cell function, suggesting that dietary copper supplementation could hold promising potential for a therapeutic approach.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: S.W.-Z., L.T.-C., S.L., and I.R. conception and design of research; S.W.-Z., A.J., L.T.-C., F.V., G.A.-H., and A.S. performed experiments; S.W.-Z., A.J., L.T.-C., F.V., G.A.-H., and A.S. analyzed data; S.W.-Z., L.T.-C., F.V., G.A.-H., A.S., S.L., and I.R. interpreted results of experiments; S.W.-Z. and A.J. prepared figures; S.W.-Z. and A.J. drafted manuscript; S.W.-Z., F.V., G.A.-H., A.S., S.L., and I.R. edited and revised manuscript; S.W.-Z., A.J., L.T.-C., F.V., G.A.-H., A.S., S.L., and I.R. approved final version of manuscript.

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