IL-1β hampers glucose-stimulated insulin secretion in Cohen diabetic rat islets through mitochondrial cytochrome c oxidase inhibition by nitric oxide

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Weksler-Zangen S, Aharon-Hananel G, Mantzur C, Aouizerat T, Gurgul-Convey E, Raz I, Saada A. IL-1β hampers glucose-stimulated insulin secretion in Cohen diabetic rat islets through mitochondrial cytochrome c oxidase inhibition by nitric oxide. Am J Physiol Endocrinol Metab 306: E648–E657, 2014. First published January 14, 2014; doi:10.1152/ajpendo.00451.2013.—A high-sucrose, low-copper-diet (HSD) induces inhibition of glucose-sensitive rats (CDs) but not Cohen diabetes-resistant rats (CDr). Copper-supplemented HSD increased activity of the copper-dependent mitochondrial respiratory chain enzyme cytochrome c oxidase (COX) and reversed hyperglycemia. This study examined the mechanism by which interleukin-1β modulates GSIS and the role of COX in this process. We measured COX activity, ATP content, GSIS, iNOS expression, and nitrite production with and without IL-1β.

Mitochondrial cytochrome c oxidase (COX) is the terminal electron transport chain complex that catalyzes the transfer of electrons from cytochrome c to molecular oxygen. The electrochemical proton gradient formed by complexes I, III, and IV (COX) is utilized by complex V (ATP synthase) to generate ATP (25). COX activity is regulated and inhibited by numerous molecules, including potassium cyanide (KCN) and nitric oxide (NO), which reversibly competes with oxygen at the heme copper active site (8). Studies in diabetic animal models suggest that interleukin-1β (IL-1β) mediates β-cell dysfunction by inducing the formation of NO (6, 13, 22, 35). Mitochondrial dysfunction with subsequent OXPHOS impairment is thought to play a central role in the pathophysiology of type 2 diabetes mellitus (T2DM). The impact of mitochondrial DNA mutations may be most pronounced in tissues with a low mitotic rate, depending on high ATP production such, as β-cells. In humans, mitochondrial DNA (mtDNA) point mutations as well as pancreatic β-cell deletion of mitochondrial genes were associated with maternally inherited diabetes (32), whereas genes involved in OXPHOS show differential gene expression and DNA methylation in pancreatic islets from patients with T2DM compared with nondiabetic donors (28).

COX activity is dependent on copper as a cofactor (21, 38), and dietary copper deficiency was shown to decrease COX activity in hearts of nondiabetic rats (54), whereas copper supplementation was shown to protect streptozotocin-injected mice from diabetes (33). A study on isolated Wistar rat islets revealed that the inhibitory effect of IL-1β on insulin secretion and glucose oxidation was prevented by copper supplementation to the incubation medium (44). These reports supported a correlation between copper availability, IL-1β, and COX activation. However, the mechanism by which IL-1β modulates GSIS and the precise role of COX in this process have remained unclear. To clarify this process, we studied the Cohen diabetic rat model.

The Cohen diabetic rat is a genetic model of nutritionally induced diabetes (49, 50) consisting of two contrasting strains, the diabetes-sensitive rat (CDs) that develops hyperglycemia due to impaired GSIS when fed a high-sucrose, low-copper diet (HSD) but maintains normoglycemia on a regular diet and the diabetes-resistant rat (CDr), which maintains normoglycemia on HSD (49). We have observed previously that the development of hyperglycemia in CDs rats fed HSD is associated with peri-islet infiltration of fat and macrophages ex-

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pressing IL-1β (49). We also showed that copper supplementation to HSD improves GSIS, suggesting the involvement of COX (48). In the current study, we examine the mechanism by which interleukin-1β modulates GSIS to clarify the role of COX in GSIS of isolated islets from CDs and CDr rats.

**RESEARCH DESIGN AND METHODS**

**Animals and Diets**

Cohen diabetic rats were bred and maintained in the animal facility at the Hebrew University School of Medicine, Jerusalem, Israel. Animal studies were approved by the Animal Care Committee of Hebrew University. All studies were performed on 3-mo-old male rats fed one of three dietary protocols: 1) a regular diet (purchased from Teklad, 2018; Harlan Laboratories) containing adequate (16 ppm) copper (49); 2) HSD containing a low (1.2 ppm) copper concentration, 72% sucrose, 18% glucose and supplemented with 10% fetal calf serum (RPMI-1640 medium); 3) HSD supplemented with adequate amounts (16 ppm) of copper (48).

**Dietary Groups**

CDs and CDr male rats fed for 8 wk on a regular diet were either 1) maintained on a regular diet for 30 additional days, 2) switched to the diabetogenic HSD for 30 days (49), or 3) fed HSD for 30 days and then switched to HSD supplemented with copper (HSD + Cu) for an additional period of 20 days (48). Studies were performed on islets isolated from rats with one of these three diets.

**Islet Isolation, Culture, and Experimental Design**

Pancreatic islets were isolated from rats maintained on either one of the above-mentioned diets by pancreas distention/digestion with collagenase P (Roche Molecular Biochemicals, Indianapolis, IN). Islets were selected (hand-picked) and counted prior to experimentation. Batches of 200–300 islets were incubated at 37°C for 24 h under a 5% CO2 atmosphere counted prior to experimentation. Batches of 200–300 islets isolated from rats on 3-mo-old male rats fed either of the above-mentioned diets were transferred to 1-ml Eppendorf tubes and incubated in KRBB-BSA supplemented with either 1.7 or 16.7 mmol/l glucose for an additional 60 min (49). Insulin concentrations were determined in the islets (insulin content) and in the supernatant (insulin secretion) using a rat radioimmunoassay (RIA) commercial kit (Linco Research, St. Charles, MO). Insulin secretion was calculated as a percent of islet insulin content in the same experiment. Stimulated insulin secretion (16.7 mmol/l glucose) was calculated as the Δ-change from baseline (1.7 mmol/l) insulin secretion.

**Determination of Islet GSIS**

Islets from procedures A and B were preincubated at 37°C under a 5% CO2 atmosphere for 60 min in modified Krebs-Ringer buffer (KRBB-BSA) containing 20 mmol/l HEPES (Sigma) and 0.25% BSA (Sigma) supplemented with 17 mmol/l glucose. Subsequently, groups of five comparable-size islets (duplicates) were transferred to 1-ml Eppendorf tubes and incubated in KRBB-BSA supplemented with either 1.7 or 16.7 mmol/l glucose for an additional 60 min (49). Insulin concentrations were determined in the islets (insulin content) and in the supernatant (insulin secretion) using a rat radioimmunoassay (RIA) commercial kit (Linco Research, St. Charles, MO). Insulin secretion was calculated as a percent of islet insulin content in the same experiment. Stimulated insulin secretion (16.7 mmol/l glucose) was calculated as the Δ-change from baseline (1.7 mmol/l) insulin secretion.

**Enzymatic Chain Enzymatic Activities**

Enzymatic activity was determined in homogenates of 200–300 comparable-size islets from procedures A and B. Activity of the respiratory chain complexes II and IV (COX) and of the ubiquitous mitochondrial matrix enzyme citrate synthase (CS) serving as a mitochondrial control enzyme was determined by standard spectrophotometric methods (31). Briefly, complex II was measured as succinate dehydrogenase (SDH) based on the succinate-mediated phenazine methosulfate reduction of dichloroindophenol at 600 nm. COX activity was measured by following the oxidation of reduced cytochrome c at 550 nm. CS was measured in the presence of acetyl-CoA and oxaloacetate by monitoring the liberation of CoASH coupled to 5′-dithiobis(2-nitrobenzoic) acid at 412 nm. COX and SDH were expressed as a ratio normalized to the CS activity. Protein concentration was determined by the Lowry method and calculated according to a bovine serum albumin (BSA) standard curve.

**Islet ATP Content**

Using a 96-well plate containing 40 islets/well, islet ATP content from procedure A was determined by luciferin-luciferase using the ATPlite luminescence assay system (Perkin-Elmer, Waltham, MA) according to the manufacturer’s instructions and as described previously (31).

**GSIS of CDr Islets Following COX Inhibition by KCN**

Two-hundred to three-hundred islets were isolated from CDr rats fed a regular diet and incubated as described in procedure A. Subsequently, islets were incubated for 60 min in KRBB-BSA containing basal (1.7 mmol/l) or stimulatory (16.7 mmol/l) glucose with or without KCN (0, 0.4, 0.8, and 8 μmol/l) (Sigma). GSIS was determined as described above in Determination of Islet GSIS.
Determination of iNOS Expression in Islets

Two-hundred to three-hundred islets were isolated from CDs and CDr rats fed different diets and incubated as described in procedure A. Real-time RT-PCR for rat iNOS was done by QuantiTect SYBR Green technology (Qiagen). The reactions were performed by the DNA Engine Opticon Sequence Detection System (Biozym Diagnostik, Oldendorf, Germany), as described before (36). The purity of the amplified PCR products was verified by melting curves. Analyses of the real-time RT-PCR data were performed by the Opticon Monitor version 1.07 (MJ Research, Waltham, MA). The iNOS expression was normalized to β-actin.

Statistical Analysis

Statistical differences between the groups were calculated for each sample using the fold difference between COX activities, ATP production, and GSIS at 16.7 mmol/l glucose in the different study groups. Data were analyzed using two-way ANOVAs with Bonferroni’s posttest for comparison of multi-

RESULTS

Ex Vivo Studies on Islets Isolated From Rats on Different Diets

GSIS. We observed that islets of CDr rats constantly maintained higher GSIS compared with islets of CDs rats fed the same diet (Fig. 1A). Islets of normoglycemic CDs rats (fed a regular diet) exhibited a consistent partial reduction in GSIS compared with CDr islets (2.0 ± 0.04 vs. 3.3 ± 0.1 pmol/l respectively, \( P < 0.01 \); Fig. 1A) that was nevertheless adequate to maintain normoglycemia. Islets of hyperglycemic CDs rats were affected severely by the low-copper HSD feeding, exhibiting an extremely low GSIS whereas islets of CDr exhibited a lower decrease (1.0 ± 0.1 vs. 1.9 ± 0.1 pmol/l, respectively, \( P < 0.01 \)), reducing only to approximating the level found in islets of CDs rats fed a regular diet (2.0 ± 0.04 pmol/l) (Fig. 1A). Copper-supplemented HSD feeding restored islet GSIS of both hyperglycemic CDs and normoglycemic CDr rats to the respective initial value in islets of CDs and CDr rats fed a regular diet (Fig. 1A).

COX activity and ATP content. We found that the changes occurring in islet COX/CS activity and ATP content following exposure of CDs and CDr rats to different diets correlated with GSIS responses (Fig. 1, A and B, respectively). Islets of CDr rats constantly maintained higher COX activity and ATP content compared with islets of CDs rats fed the same diet. On a regular diet, islets of CDs rats exhibited lower COX activity and ATP content compared with islets of CDr rats (COX/CS: 1.4 ± 0.2 vs. 2.3 ± 0.2, \( P < 0.01 \); ATP: 6.2 ± 0.5 vs. 9.2 ± 0.4 pmol/islet, \( P < 0.01 \), respectively). COX activity and ATP content were significantly more reduced in islets of CDs rats fed HSD. COX activity decreased by ~86%, and ATP decreased by ~53% in islets isolated from hyperglycemic CDs rats compared with ~50 and ~30% reduction in normoglycemic-
CDr HSD islets, respectively (COX/CS: 0.2 ± 0.1 vs. 1.3 ± 0.1, P < 0.01; ATP: 3.1 ± 0.4 vs. 6.4 ± 0.3 pmol/islet, P < 0.01, respectively; Fig. 1, B and C). On a copper-supplemented HSD (HSD + Cu), COX activity and ATP content of both CDs and CDr islets were restored to their respective baseline (regular diet) levels concomitantly with an increase in GSIS (Fig. 1, A–C). These results demonstrate that the changes in islet GSIS correlate with the variation in COX activity and ATP content observed in islets of CDs and CDr rats fed different diets. In addition, these results show that exposure to HSD induces the most severe reduction in COX activity in islets of hyperglycemic CDs rats. Notably, COX activity is specifically affected independently of mitochondrial and protein content, as data were normalized to the activity of CS, a ubiquitous mitochondrial matrix enzyme marker.

To determine whether the change in COX activity is specific or part of a broad mitochondrial respiratory chain dysfunction, we measured the activity of SDH (complex II of the mitochondrial respiratory chain), a copper-independent respiratory chain enzyme. Importantly, we did not find any alteration in SDH activity in the islets of the different dietary groups (SDH/CS CDr on regular diet 0.4 ± 0.02, CDr on regular diet 0.3 ± 0.04, CDr-HSD 0.4 ± 0.02, CDs-HSD 0.3 ± 0.1, CDr-HSD + Cu 0.4 ± 0.03, CDs-HSD + Cu 0.5 ± 0.1).

**GSIS inhibition by KCN.** To provide further evidence for a direct link between COX activity inhibition and GSIS, we incubated CDr islets with KCN, a specific COX inhibitor (42, 45), and demonstrated a clear dose-dependent reduction in GSIS between 0.4 and 8 μM KCN (Fig. 2).

**iNOS expression in islets.** iNOS expression was somewhat higher in islets of CDs rats on regular diet compared with CDr islets, although these results did not reach statistical significance (Table 1). HSD feeding significantly increased iNOS gene expression in islets of CDs rats, which was reduced by copper supplementation (HSD + Cu; Table 1). CDs islets constantly maintained significantly higher iNOS expression on HSD and on HSD + Cu compared with islets of CDr rats fed the same diets (P < 0.05).

### In Vitro Studies of Islets Isolated From Rats Fed Regular Diet

**The effect of IL-1β on GSIS capacity.** We mimicked the effect of the pancreatic peri-islet IL-1β-expressing macrophages found in hyperglycaemic CDs rats fed HSD (49) by exposing islets isolated from CDs and CDr rats fed regular diets to different IL-1β concentrations. IL-1β exposure had no significant effect on basal insulin secretion. We observed that CDr islets maintained a significantly higher GSIS compared with CDs islets when exposed to increasing concentrations of IL-1β (Fig. 3). Low concentrations of IL-1β (2.5, 5 U/ml) markedly decreased the GSIS of CDH islets (0.7 ± 0.1 and 0.6 ± 0.09 pmol/l, respectively, vs. untreated CDH islets: 2.0 ± 0.04 pmol/l, P < 0.01; Fig. 3). The response of CDr islets to IL-1β differed significantly from CDs (Fig. 3). Exposure to 2.5 U/ml IL-1β caused an increase in insulin secretion, as reported in other studies (14, 26, 37). The dose of 5 U/ml IL-1β had no inhibitory effect, whereas 10 U/ml IL-1β partially reduced GSIS (2.1 ± 0.3 pmol/l vs. untreated CDr islets, 3.3 ± 0.1 pmol/l, P < 0.01; Fig. 3). Interestingly, the GSIS of CDr islets following 10 U/ml IL-1β was comparable with maximal GSIS of CDH islets without exposure to IL-1β. These results demonstrate that CDH islets are especially sensitive to low levels of IL-1β.

**The effect of IL-1β on nitrite production.** We hypothesized that the reduction in GSIS following exposure to IL-1β is mediated by NO formation with subsequent inhibition of COX activity (43). Therefore, we measured nitrite production in the medium of untreated islets and in islets exposed to IL-1β. The baseline nitrite production of untreated CDH islets was higher than untreated CDr islets (Fig. 4). Low levels of IL-1β (2.5, 5 U/ml) induced a substantial increase in nitrite production in both CDH and CDr islets. However, the production was higher in CDH islets compared with CDr islets. On the other hand, exposure to 10 U/ml IL-1β induced a comparable nitrite production from both CDH and CDr islets (Fig. 4).

**The effect of 2.5 and 10 U/ml IL-1β on COX activity and the protective effect of l-NOARG from the deleterious effect of IL-1β.** The concentration of 2.5 U/ml IL-1β was chosen, as it reduces COX activity (COX/CS, 1.1 ± 0.02 vs. 1.5 ± 0.1

### Table 1. Gene expression of iNOS in islets

| Diet       | CDH        | CDH
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<tr>
<td>Regular</td>
<td>0.05 ± 0.04 (3)</td>
<td>0.52 ± 0.13 (4)</td>
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<tr>
<td>HSD</td>
<td>0.30 ± 0.08 (7)</td>
<td>2.18 ± 0.52 (3)*</td>
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<tr>
<td>HSD + Cu</td>
<td>0.15 ± 0.09 (5)</td>
<td>1.00 ± 0.35 (6)*</td>
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Data are means (×1,000) ± SE. iNOS, inducible nitric oxide synthase; CDH, diabetes-resistant rats; CDH, diabetes-sensitive rats; HSD, high-sucrose low-copper diet; HSD + Cu, HSD supplemented with copper. RNA was isolated from rat islets. After reverse transcription, the expression of iNOS was measured by real-time PCR. The expression of iNOS was normalized to β-actin. The no. of samples per group is indicated in parentheses. Each sample represents a pool of islets isolated from 2 to 3 rats. *P < 0.01 vs. CDs, regular diet; #P < 0.05 vs. CDH, high sucrose; #P < 0.05 vs. CDH, high sucrose. ANOVA followed by Bonferroni.
without IL-1β, \(P < 0.05\) and causes a higher nitrite production in CDs islets. We then examined the effect of 10 U/ml IL-1β on COX activity that triggered a comparable NO production from CDs and CDr islets (Fig. 5) and found that it reduced COX activity in both CDs (COX/CS 0.6, ± 0.1 vs. 1.4 ± 0.2 without IL-1β, \(P < 0.01\)) and CDr islets (COX/CS, 1.7 ± 0.1 vs. 2.3 ± 0.2 without IL-1β, \(P < 0.01\)) (Fig. 5) but the effect on CDs islets was greater. We found this to be a specific effect of IL-1β on COX activity since SDH activity measured under the same conditions and in the same preparation was unaltered (SDH/CS, CDr islets 0.3 ± 0.02 vs. CDs islets 0.3 ± 0.03, with 10 U/ml IL-1β). To confirm that the inhibitory effect of IL-1β on GSIS and COX activity is mediated by NO, we repeated the above experiments in the presence of L-NOARG, a NO synthase inhibitor. We found that L-NOARG protected the islets of both strains from the inhibitory effects of IL-1β on GSIS (Fig. 3). Incubation with L-NOARG did not alter the baseline GSIS in CDs islets, but it did protect the islets from the inhibitory action of IL-1β on GSIS (Fig. 3). In CDr islets, L-NOARG increased the baseline GSIS and also provided protection from IL-1β (Fig. 3). In addition, L-NOARG exerted a comparable protective effect on COX activity. It prevented a large reduction in COX activity of both CDs and CDr islets following IL-1β exposure but failed to fully restore COX activity of CDs islets to its baseline level (Fig. 5). These observations provide additional confirmation for a major role of NO in the mechanism of IL-1β-induced inhibition of COX activity and GSIS.

The effect of islet incubation in vitro with Cu-GHL. We studied the effect of in vitro copper supplementation on GSIS and COX activity of islets exposed to IL-1β. Incubation with Cu-GHL induced a small increase in GSIS of CDs islets compared with CDs islets incubated without Cu-GHL (2.4 ± 0.1 vs. 2.0 ± 0.04 pmol/l, \(P < 0.01\); Fig. 3) but did not change GSIS of CDr islets (3.3 ± 0.1 vs. 3.2 ± 0.3 pmol/l; Fig. 3). COX activity of CDs and CDr islets did not change following
incubation with Cu-GHL (Fig. 5). Coincubation of CDs and CDr islets with IL-1β (10 U/ml) and Cu-GHL (0.4 μmol/l) prevented the decrease in GSIS and COX activity induced by IL-1β. GSIS of CDs islets coincubated with IL-1β and Cu-GHL was maintained at 2.2 ± 0.1 pmol/l (regular diet level), and the GSIS of CDr islets increased to 5.0 ± 0.2 pmol/l. COX activity was maintained at regular diet levels in both CDs (1.4 ± 0.2) and CDr islets (2.8 ± 0.4) following coincubation with IL-1β and Cu-GHL (Figs. 3 and 5). These findings suggest that in vitro copper supplementation may protect islets from the inhibitory effect of IL-1β on GSIS and COX activity.

**DISCUSSION**

Islets of CDs rats on regular diet exhibited a consistent partial reduction in COX activity, ATP content, and GSIS compared with CDr islets. Nevertheless, these were still adequate to maintain the CDs rats in a normoglycemic state. This partial reduction appears to be a constitutional change that defines the metabolic differences between the two strains that could explain the significant reduction in all the three parameters following exposure to HSD in islets of hyperglycemic CDs rats. Indeed, following exposure to HSD, the islets of both CDs and CDr rats exhibited a significant reduction in all three parameters, but the effect in islet COX activity of hyperglycemic CDs rats was especially evident. The latter exhibited only minimal residual COX activity, whereas islets of normoglycemic CDr rats fed HSD retained two-thirds of their baseline value, suggesting a crucial role for COX in islets/GSIS. In support of this, we recently found (preliminary unpublished data) that islet/COX protein was already reduced in normoglycemic CDs rats fed a regular diet, furthermore decreasing in islets of hyperglycemic CDs rats fed a HSD. Thus, the genetic susceptibility of the CDs islets sets COX activity, ATP content, and GSIS at a 50% lower level, which enables the development of diabetes when exposed to the deleterious environment created by the HSD. Moreover, these findings also demonstrate that the in vitro isolated islet model accurately reflects the previously described in vivo phenotype (49), and therefore, they provide a unique platform to further the investigation of the mechanisms underlying the marked deterioration of islet COX activity in CDs HSD rats, resulting in GSIS inhibition and sustained hyperglycemia.

Using this in vitro setup, we examined the hypothesis derived from our previous in vivo study that local exposure of CDs islets to IL-1β promotes GSIS inhibition (49). In the current in vitro study, we demonstrate that the pattern of response to IL-1β exposure differs in CDs and CDr islets. Exposure to low levels of IL-1β reduces COX activity and GSIS significantly in CDs islets but induces a stimulatory effect on GSIS of CDr islets, representing a normal response to extraneous stress, as was reported by others (14, 26, 37). Higher levels of IL-1β reduce GSIS and COX activity in both CDs and CDr islets, but the effect was more marked in CDs islets. Previously, the inhibitory effects of IL-1β were suggested by us and others to involve the production of NO and reactive nitrogen intermediates in pancreatic islets (4, 9, 10, 18, 19). Increased NO production may interact and inhibit the function of several components of the mitochondrial respiratory chain by reversible S-nitrosation. However, it is proposed that NO exerts some of its main physiological and pathological effects on cell functions by competing with oxygen binding, thereby inhibiting cytochrome c oxidase. Moreover, the interference with electron transport favors the production of reactive oxygen species, which by further interaction with NO form
peroxynitrite, inducing cellular damage (4, 6, 8–10, 18, 19). In β-cells, the overproduction of NO combined with weak anti-oxidative defense mechanisms renders these cells particularly vulnerable to redox insults (6, 22, 23). Our current data clearly demonstrate that IL-1β exposure substantially increases the level of nitrates in isolated islets and that this increase is associated with reduction in COX activity and GSIS. Moreover, treatment with the iNOS inhibitor Nω-nitro-l-arginine prevented the IL-1β-induced COX inhibition and GSIS reduction in isolated islets, whereas copper supplementation in vivo prevented the increase in iNOS expression and the development of hyperglycemia. These findings are reinforced by our previous studies showing that IL-1β-expressing macrophages infiltrating the exocrine pancreas of hyperglycemic CDs rats reduce COX activity and β-cell function, both of which were reversed by copper supplementation (48, 49). Thus, taken together, our previous and current data confirm the role of NO in IL-1β-mediated GSIS reduction by reducing COX activity below a certain critical level.

The notion that altering COX activity influences endogenous mitochondrial respiration when being reduced to a certain critical level was shown more than a decade ago by the exposure of intact cells to increasing concentrations of KCN (40, 41). We suggest that such a self-reinforcing cycle of progressive deterioration in mitochondrial function occurs when the CDs rats are fed a HSD, leading to a parallel decline in β-cell function below the “critical threshold,” resulting in mitochondrial dysfunction and ensuing diabetes. In our study, the hypothetical “critical COX activity threshold” was set at approximately “1”. Our data demonstrate that when COX/CS activity was maintained above “1”, hyperglycemia was prevented [i.e., COX activity in the islets of normoglycemic CDs rats was ~1.0 COX/CS (Fig. 1B), whereas in the islets of the hyperglycemic CDs rat on HSD it was reduced to ~0.3 COX/CS (Fig. 1B)]. COX activity in the islets of normoglycemic CDr rats on HSD was ~1.3 COX/CS (Fig. 1B) above the critical threshold of “1”, thereby preventing hyperglycemia. Such differences in COX activity reserves were also shown with respect to tissue specificity of mitochondrial diseases in various human cell types (43), and other studies showed that reduced OXPHOS and ATP content leads to impaired GSIS in β-cells of OPA-1-deficient mice, INS-1-derived clonal β-cell lines (24, 55), and islets of type 2 diabetic subjects (1).

Since COX activity, biogenesis, assembly, and stability are highly dependent on copper as an essential cofactor (21, 38), reduced copper intake may directly reduce islet/COX activity, leading to diminished GSIS, an effect observed by others in rat cardiomyocytes (56). In our previous in vivo study, low-copper HSD induced hyperglycemia in CDs rats, whereas copper-supplemented HSD reversed these phenomena (48, 49). Our current ex vivo experiments on isolated islets of rats fed copper-supplemented HSD as well as in vitro coinoculation of islets with the complex Cu-GHL (44) and IL-1β revealed that COX activity and GSIS similarly recovered upon copper supplementation. Interestingly, the protective effect of Cu-GHL and Nω-nitro-l-arginine on these parameters was similar, suggesting a comparable mode of protection for these two compounds. Indeed, the addition of copper ions was shown to reduce NO production of the murine macrophage cell line RAW 264.7 similarly to the effect of an iNOS inhibitor (39). Moreover, copper supplementation decreased blood glucose levels, lipid peroxidation, and mRNA expression of iNOS in

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**Fig. 6.** The following proposed scenario was designed on the basis of our previous in vivo and the current in vitro studies (black arrows) to explain glucose-stimulated insulin secretion (GSIS) inhibition of the Cohen CDs rat. The inherent partial decrease in COX activity demonstrated in the current in vitro study comprises the “first hit,” causing the initially reduced GSIS observed in normoglycemic CDs on regular diet. Low-copper HSD provides a “second hit,” further reducing COX activity through two parallel mechanisms: 1) directly by reducing the activity of the copper-dependent enzyme COX and 2) indirectly by inducing pancreatic acinar cell apoptosis, macrophage infiltration, and IL-1β secretion in the close vicinity of the islets, as suggested in our previous in vivo study (48, 49) and confirmed in the current in vitro study. In the current study, IL-1β was shown to promote the generation of nitric oxide (NO) and inhibit COX activity. An alternative pathway is also proposed (dashed arrows): the reduction in COX activity induced by exposure to low-copper HSD involves 1) increased ROS production and 2) decreased activity of the antioxidative enzymes Cu/Zn-SOD and glutathione peroxidase. The additive effect of these parallel pathways could lead to inhibited β-cell function and hyperglycemia. Black arrows represent findings confirmed by our data; dashed arrows represent findings extrapolated from our data but not directly proven in the current study.
C57BL6 mice following streptozotocin injection (33). Although the protective effect of copper supplementation in our in vitro studies may indeed derive from the reduced formation of NO, the exact mechanism by which copper protects COX activity warrants further investigation.

Our data support reduced COX activity and IL-1β-induced overproduction of NO as the leading causes of β-cell dysfunction in CDs rats. However, the dysfunction of the mitochondrial respiratory chain per se may also increase oxidative stress and cell damage, as demonstrated by others (12, 52). In studies of mitochondrial diseases, the increase in reactive oxygen species required a concurrent increase in the expression of the antioxidative enzymes superoxide dismutase and glutathione peroxidase to scavenge these reactive oxygen species and protect the cells from damage (3, 11). In this context, dietary copper restriction reducing the activity of these enzymes is likely to reduce antioxidant defense (2). Consequently, we propose that the inborn reduction of COX activity in CDs rats may produce an initial state of oxidative stress that is potentiated by the HSD due to the further reduction in activity of COX and of the antioxidative enzymes. In previous studies, we showed that the CDs rats have a genetically predetermined decreased clearance of reactive oxygen species by the enzymatic scavenging mechanism, which is aggravated by copper deficiency and diabetes (53). We also demonstrated that the 11- and 12-day-old embryos of CDs rats fed HSD, in contrast to the CDr embryos, did not respond to increased oxidative stress by an increase in the copper/zinc superoxide dismutase activity (30, 53). In addition, we found increased oxidative stress manifested by increased lipid peroxidation and protein carbonylation in the pancreas of the adult CDs diabetic animals (30, 53). All of the above support an alternative pathway that likely contributes to GSIS inhibition in the hyperglycemic CDs rat. These pathways as well as the potential significance of altered mitochondrial function in diabetes need further investigation.

Combining the data of the current in vitro and previous in vivo studies (48, 49), we suggest the following “hypothetical” scheme to explain the development of hyperglycemia in CDs rats fed HSD (Fig. 6). Congenitally reduced COX activity that correlated with reduced ATP content and GSIS is already present in islets isolated of normoglycemic CDs rats. These render the CDs rats particularly sensitive to environmental stresses such as low-copper HSD. The low-copper HSD promotes hyperglycemia by two closely interrelated mechanisms: 1) by further reducing the activity of the copper-dependent enzyme COX that is necessary for β-cell ATP production and insulin secretion and 2) by inducing pancreatic acinar cell apoptosis (46, 47) and infiltration of IL-1β-expressing macrophages. IL-1β inhibits COX activity via overproduction of NO, resulting in GSIS inhibition and hyperglycemia.

The potential significance of altered mitochondrial function in the development of diabetes in humans needs further investigation. Because T2DM is a very common disease, it is unlikely to be explained by rare mutation in a single gene, as demonstrated in the rare mitochondrial diabetes (29, 32). However, a critical role for mitochondria in normal β-cell function has become evident, and a recent study in young adult offspring of patients with T2DM demonstrated that those subjects with insulin resistance had a 30% reduction in mitochondrial phosphorylation compared with age-matched controls (28). Those investigators speculated that insulin resistance in the skeletal muscle of insulin-resistant offspring of patients with T2DM was due to an inherited defect in mitochondrial oxidative phosphorylation. Multiple confounding factors, including mtDNA variation, maternal environment or imprinting, variation in copy number, and tissue distribution of heteroplasmic mitochondrial DNA mutants, may be ascribed to explain the incomplete penetrance, making it difficult to unequivocally establish a role for mitochondrial dysfunction in diabetes (5, 16). Thus, the understanding of critical molecular mechanisms predisposing to β-cell damage may be important for both prevention and treatment of T2DM. The CDs rat is thus a good model for studying the naturally occurring variation in the mitochondrial function induced and prevented by dietary intervention. Using this model, we showed that a primary reduced GSIS may cause hyperglycemia and that could be linked directly to reduce OXPHOS. We also demonstrated for the first time a genetically determined threshold for COX activity that predisposes the CDs rats to diabetes and provides the explanation of cellular mechanisms by which IL-1β reduces COX activity below the threshold causing hyperglycemia.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS


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