Impaired β-cell-β-cell coupling mediated by Cx36 gap junctions in prediabetic mice

C. P. F. Carvalho,1,4 R. B. Oliveira,1 A. Britan,3 J. C. Santos-Silva,2 A. C. Boschero,2 P. Meda,3 and C. B. Collares-Buzato1

1Department of Histology and Embryology, Institute of Biology, University of Campinas, Campinas, São Paulo, Brazil; 2Department of Physiology and Biophysics, Institute of Biology, University of Campinas, Campinas, São Paulo, Brazil; 3Department of Cell Physiology and Metabolism, Medical School, University of Geneva, Geneva, Switzerland; and 4Department of Biosciences, Federal University of São Paulo, São Paulo, Brazil

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Gap junctional intercellular communication between β-cells is crucial for proper insulin biosynthesis and secretion. The aim of this work was to investigate the expression of connexin (Cx) 36 at the protein level as well as the function and structure of gap junctions (GJ) made by this protein in the endocrine pancreas of prediabetic mice. C57BL/6 mice were fed a high-fat (HF) or regular chow diet for 60 days. HF-fed mice became obese and prediabetic, as shown by peripheral insulin resistance, moderate hyperglycemia, hyperinsulinemia, and compensatory increase in endocrine pancreas mass. Compared with control mice, prediabetic animals showed a significant decrease in insulin-secretory response to glucose and displayed a significant reduction in islet Cx36 protein. Ultrastructural analysis further showed that prediabetic mice had GJ plaques about one-half the size of those of the control group. Microinjection of isolated pancreatic islets with ethidium bromide revealed that prediabetic mice featured a β-cell-β-cell coupling 30% lower than that of control animals. We conclude that β-cell-β-cell coupling mediated by Cx36 made-channels is impaired in prediabetic mice, suggesting a role of Cx36-dependent cell-to-cell communication in the pathogenesis of the early β-cell dysfunctions that lead to type 2-diabetes.

Cell-to-cell contacts mediated by GJs are also crucial for proper and fully mature secretory response of the pancreatic β-cells (7, 10, 11, 23). Recent data further indicate that GJs may also play a role in controlling the mass of these cells by modulating their growth and their resistance to proapoptotic conditions (20, 21). However, whether GJs participate in the β-cell dysfunction, which plays a central role in the pathogenesis of diabetes, is presently supported by only indirect data (7). Thus, the β-cells of connexin 36 (Cx36)-null mice are uncoupled and show impaired intercellular synchronization of [Ca2+]i transients, increased basal release of insulin, lack of glucose-stimulated insulin release in a normal pulsatile fashion, and a marked decrease in responsiveness to physiologically relevant glucose concentrations (30, 39), all alterations that are reminiscent of those observed in type 2 diabetes (T2DM). Prolonged exposure to high concentrations of glucose and fatty acids, as expected in diabetes, downregulates Cx36 expression (1, 2), and the human Cx36 gene is located on chromosome 15q14 (4), a locus that genome-wide scans have linked to diabetic phenotypes in different populations (13, 27). As yet, however, no study has investigated whether the function of Cx36 channels is impaired under conditions thought to initiate the chain of events that ultimately result in diabetic dysfunctions.

The aim of this work was to address this question by investigating whether alterations in Cx36 expression, distribution, and function are associated with the β-cell alterations seen in the prediabetes state (5, 15), which is thought to be an initial phase in the pathogenesis of T2DM and is characterized by systemic dysfunctions such as peripheral resistance to insulin, moderate hyperglycemia, and hyperinsulinemia. Using as a model mice fed a high-fat diet for 60 days, we have found that prediabetes was associated with a decreased Cx36 content and GJ size of β-cells, which impaired β-cell-β-cell coupling.

MATERIALS AND METHODS

Animals and diets. Male and female C57BL/6 mice were obtained from the breeding colonies maintained at the University of Campinas (UNICAMP, São Paulo, Brazil) and the University of Geneva (UNIGE, Geneva, Switzerland). The animals were housed at 22 ± 1°C on a 12:12-h light-dark cycle and had free access to water and food. When aged 4–5 mo, the animals were divided into two groups. One group was fed 60 days with a standard rodent diet containing 4.5 g% lipids, 53 g% carbohydrates, and 23 g% proteins. The other group was fed a high-fat (HF) diet containing 21 g% lipids, 50 g% carbohydrates, and 20 g% proteins. Most experiments were performed on both female and male mice except islet microinjections and freeze-fracture (FF) electron microscopy, which were performed only on males. No marked sex difference was observed with regard to body weight and metabolic parameters assessed in this work. In vivo
monitoring, insulin secretion, immunoblotting, and morphological studies were performed with mice of the Campinas colony. The Geneva mouse colony was used for FF and microinjection experiments. No significant difference was seen between the two colonies regarding the response to the HF diet, as assessed by the body weight gain and fasting glycemia. The animals were used in accordance with the guidelines of the Institutional Committee for Ethics in Animal Experimentation of the University of Campinas and the University of Geneva.

Blood glucose, insulin plasma levels, and body weight gain. All blood samples were collected between 9:00 and 11:00 AM. Blood glucose was determined in samples collected from the tail tip of Fig. 1. C57BL/6 mice become overweight and prediabetic after feeding a high-fat (HF) diet. a: HF diet for 60 days induced a significant increase ($P < 0.0001$) in body weight gain of mice vs. control mice fed regular chow diet (control, $n = 73$; HF, $n = 77$). b and c: fasting blood glucose (b: control $n = 37$, HF $n = 38$) and plasma insulin levels (c: control $n = 27$, HF $n = 26$) were also significantly increased ($P < 0.0001$) in HF-fed mice. d: insulin tolerance test (ITT) showed larger ($P = 0.0009$) area under curve (AUC) values in HF-fed than in control mice (control $n = 15$, HF $n = 16$). Data are expressed as means $\pm$ SE.

Fig. 2. HF-fed mice display increased $\beta$-cell mass. $a$ and $b$: immunostaining of insulin showed normal shape and prominent $\beta$-cell content of islets from chow-fed control ($a$) and HF-fed mice ($b$). Scale bar, 50 $\mu$m. $c$ and $d$: morphometric analysis showed that, compared with controls (open bars), HF-fed mice (filled bars) featured a significant increase in total islet ($c, P = 0.0020$) and $\beta$-cell mass ($d, P = 0.0007$). Bars represent means $\pm$ SE of 11 pancreases from each group (618–800 islets/group).
12-h-fasted mice, using an Accu-Chek Advantage II glucometer (Roche Diagnostic, Switzerland). Plasma insulin was also measured in tail blood samples of 12-h-fasted animals. Blood was collected in ice-cold heparinized tubes; plasma was separated by centrifugation and stored in aliquots at −20°C. Plasma insulin was determined by radioimmunoassay (RIA) using a rat insulin standard. All animals were weighed before starting the diet period, and at the end of it; values were expressed as percentage of body weight gain over the initial body weight.

**Insulin tolerance test.** For insulin tolerance test (ITT), fed mice were injected with 0.5 U/kg body wt ip human insulin (Biohulin R, Biobras, Brazil). Blood was taken immediately before insulin injection and 10, 15, 30, and 60 min thereafter. Blood glucose levels were measured as described above. Results were expressed as area under curve (AUC), which was measured using the GraphPad Prism version 4.00 for Windows (GraphPad Software).

**Pancreas histology and morphometry.** Pancreases of 11 mice per group were weighed and fixed for 18 h in either Bouin solution or 4% paraformaldehyde (in 0.05 M TBS, pH 7.4). Each pancreas was sectioned into five fragments, which were separately embedded in paraffin. Three blocks from each pancreas were serially sectioned (5 μm thickness). Two sections per block were randomly selected and processed for immunoperoxidase staining of insulin. After that, the sections were evaluated morphometrically, as previously described (6). To this end, all islets (defined as a cluster of ≥5 β-cells) were recorded in the six sections selected per animal (618 and 800 islets were scored in the control and HF-fed group, respectively) using a digital camera (Nikon FDX-35) coupled to a light microscope (Nikon Eclipse E800). Images were captured by an image analysis system (Image Pro Plus for Windows). The digitized images were analyzed using the free software ImageTool (http://ddsdx.uthscsa.edu/dig/idesc.html) to determine the total islet mass (ratio of islet to pancreas areas × weight of pancreas, expressed in mg), and the total β-cell mass (total islet mass × mean % islet area occupied by β-cells) (6).

**Immunofluorescence.** The cellular location of connexins and insulin was determined by indirect immunofluorescence staining of pancreas cryosections (8 μm thickness). The pancreases were frozen in n-hexane with liquid nitrogen. For Cx36 immunolabeling, the sections were permeabilized in acetone at −20°C for 3 min. For Cx43 detection, the sections were fixed in 2% paraformaldehyde (in PBS, pH 7.4) for 8 min and permeabilized with 0.1% Triton X-100 (in TBS, pH 7.4) for 5 min. All sections were then incubated for 1 h with 5% of dry skimmed milk (in TBS, pH 7.4) prior to incubation for 2 h at room temperature (RT) with one of the following primary antibodies: rabbit anti-Cx36 (Zymed) diluted 1:50 in 3% dry skimmed milk in TBS; rabbit anti-Cx43 (Sigma) diluted 1:1000 in 3% dry skimmed milk in TBS. After rinsing, sections were first incubated for 2 h at RT with a cognate FITC-conjugated secondary antibody (Sigma; dilutions 1:300 and 1:500 for Cx36 and Cx43 staining, respectively) and then with a guinea pig anti-insulin antibody (Dako; dilution 1:100) for 1.5 h followed by a final 2-h incubation at RT with a specific secondary antibody conjugated to TRITC (Sigma; dilution 1:150). All sections were scored in the control and HF-fed group, respectively using the free software ImageTool (http://ddsdx.uthscsa.edu/dig/idesc.html) to determine the total islet mass (ratio of islet to pancreas areas × weight of pancreas, expressed in mg), and the total β-cell mass (total islet mass × mean % islet area occupied by β-cells) (6).

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**Table 1. Morphometric analysis of GJs and TJs in freeze-fracture replicas of pancreatic islets from control and high-fat fed C57/BL6 mice**

<table>
<thead>
<tr>
<th>Group</th>
<th>β-Cell Membranes Examined</th>
<th>Junctions Found</th>
<th><strong>Type of Junctions</strong></th>
<th><strong>GJ Area, nm²</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>GJ</td>
<td>TJ + GJ</td>
</tr>
<tr>
<td>Control</td>
<td>50</td>
<td>122</td>
<td>17 (13.9%)</td>
<td>80 (65.6%)</td>
</tr>
<tr>
<td>HF</td>
<td>54</td>
<td>158</td>
<td>18 (11.4%)</td>
<td>97 (61.4%)</td>
</tr>
</tbody>
</table>

GJ, gap junction; TJ, tight junction. *4-6 freeze-fracture (FF) replicas of 100 islet pools were analyzed per group. †Data are no. of junctions (percentage of each junction type in relation to total junctions). ‡Values are means ± SE (no. of GJs analyzed). *P < 0.001 vs. control group (Student’s t-test).
of the Cx to the β-actin signals. The specificity of antibodies was confirmed in brain and heart homogenates, which were used as positive and negative controls for Cx36, respectively (11, 23).

**FF electron microscopy.** Pools of about 100 islets isolated from two mice from each experimental group were fixed for 60 min in a 2.5% glutaraldehyde solution and processed for FF using a BAF 60 Freeze Etching System (Balzers High Vacuum, Balzers, Liechtenstein), as previously described (11, 30). At least 50 β-cell membranes were randomly selected in four to six different replicas obtained from each experimental group. Replicas were examined with a Philips CM10 electron microscope. To restrict our evaluations to insulin-containing cells, we observed membranes only from cells containing secretory granules located at the center region of the islet (11, 25). All intercellular junctions (i.e., gap and tight junctions) were scored in these membranes, and most GJs photographed for further morphometric analysis. The area of GJ plaques was measured after digital enlargement of the micrographs using the Image Tool free software (http://ddsdx.uthscsa.edu/dig/itdesc.html, version 3.0) and expressed as square nanometers.

**Tracer microinjection.** Islets isolated from control and HF-fed mice were attached to Sylgard- and poly-t-lysine-coated dishes and transferred onto the heated stage (37°C) of a Universal Zeiss fluorescence microscope. Individual β-cells within the islet center were microinjected for 5 min using glass microelectrodes containing in the tip a 4% solution (in 150 mM LiCl, pH 7.2) of either Lucifer Yellow (LY) or ethidium bromide (EB), as previously described (11, 12). The injected field was photographed using an Axiocam digital camera (Carl Zeiss SMT, Oberkochen, Germany) coupled to the fluorescence microscope. The extent of coupling was determined by measuring the area labeled by each tracer on digitized photographs of the intact islets, using the Image Tool software (http://ddsdx.uthscsa.edu/dig/itdesc.html, version 3.0).

**Statistical analysis.** Results are expressed as means ± SE. Since we found no marked sex difference with regard to body weight and metabolic characteristics either at the beginning or at the end of the diet period (data not shown), pooled values obtained with similar numbers of male and female mice are shown. For analysis between groups, statistical significance was assessed by Student’s t-test. For multiple comparisons, statistical significance was assessed by ANOVA followed by the Bonferroni test. The significance level was set at P < 0.05. Statistical analyses were performed using GraphPad Prism version 4.00 for Windows.

**RESULTS**

**Mice fed a HF diet developed prediabetic alterations.** After 60 days, the HF diet feeding induced a significant increase (P < 0.0001) in the body weight gain of mice (Fig. 1a) compared with the control group (fed a regular chow diet). HF-fed mice also showed both higher fasting blood glucose (Fig. 1b, P < 0.0001) and plasma insulin levels (Fig. 1c) compared with the control group. The ITT resulted in slower plasma clearance of glucose in HF- than in control-fed mice, as revealed by the greater (P = 0.0009) AUC (Fig. 1d). HF mice also had higher postprandial glycemia and cholesterolemia than controls (data not shown). Histology showed that both HF and control mice had islets predominantly made of insulin-containing β-cells (Fig. 2, a and b). Morphometric analysis further revealed that the islets of HF mice were larger (P < 0.0001) than those of controls [HF: 10,430 ± 607.6 (800 islets) vs. control: 7,225 ± 431.6 (618 islets) μm²], resulting in a significant increase in both total islet area (Fig. 2c, P = 0.002) and β-cell masses (Fig. 2d, P = 0.0007). HF-fed mice also showed increased (P = 0.009) islet numbers per pancreas section compared with control mice [HF: 12.1 ± 0.8 (n = 11 animals) vs. control: 9.4 ± 0.5 (n = 11)]. All together,

![Image](http://ajpendo.physiology.org/DownloadedFrom/10.23073475807690)

Fig. 6. β-Cell-β-cell coupling is reduced in prediabetic mice. a–d: microinjection of islets from chow-fed control (a and c) and HF-fed mice (b and d) with Lucifer yellow (LY; a and b) or ethidium bromide (EB; c and d) resulted in variable transfer of tracers into adjacent β-cells. In the 2 groups of mice, the extent of EB transfer was greater than that of LY, consistent with presence of Cx36 but not Cx43 channels. Scale bar, 50 μm. e: quantitative analysis showed that EB transfer was significantly decreased in HF islets vs. control islets (P = 0.0395). f: no. of β-cell diameters labeled by EB revealed that HF islets also had more uncoupled cells (1 cell) and show fewer territories comprising more than 9 coupled β-cells than control islets. Data in e are mean ± SE of no. of islets indicated above the columns (n = 6–7 mice per group).
these findings show that HF-fed mice developed characteristics of prediabetes, including overweight, mild hyperglycemia, insulin resistance, hyperinsulinemia, and increased islet mass.

Islets of HF-fed mice show abnormal insulin secretion. Compared with islets from control mice, islets isolated from HF-fed mice showed increased (P = 0.0024) basal insulin secretion, expressed as a percentage of the insulin content of the islets, in the presence of 2.8 mM glucose (Fig. 3a). This change resulted in a lower (P = 0.003) fold increase in glucose-induced insulin secretion in HF than in control islets (Fig. 3b). No significant difference in insulin islet content was seen between the two experimental groups (control 93.3 ± 4.9 ng/islet vs. HF 87.4 ± 4.0 ng/islet). The data indicate that the HF diet altered the secretory functioning of β-cells independently of its effects on islet mass.

Islets of HF-fed mice featured reduced Cx36, GJ, and cell coupling. Immunofluorescence showed that Cx36 was distributed in a punctate pattern over the insulin-containing β-cell area in the center of both control and HF-fed islets (Fig. 4, a–d). However, exposure to the HF diet resulted in a significant (P = 0.01) decrease in the content of Cx36, as evaluated by Western blotting in islet protein extracts (Fig. 4, e and f).

FF electron microscopy showed that the β-cell membranes of both control and HF-fed mice displayed typical GJs, often associated with tight junction fibrils, as reported in both rodent and human islets (11, 25, 34) (Fig. 5). However, morphometric analysis of the P fracture faces of β-cell replicas revealed that the size of GJ plaques was significantly smaller (P < 0.001) in the HF-fed than in the control mice (Table 1).

The functional properties of Cx36-made channels were assessed by tracer microinjection (Fig. 6). Microinjection of LY showed that within 5 min the tracer diffused similarly between the coupled cells located in the central, β-cell-rich region of islets isolated from control and HF-fed mice (Fig. 6, a, b, and c). In both control and HF islets, this intercellular GJ coupling was larger when evaluated with EB (Fig. 6, c, d, and e). However, the intercellular transfer of EB was significantly (P = 0.04) reduced in the islets of HF-fed mice (Fig. 6, c, d, and e). These islets also displayed more frequently uncoupled β-cells and less frequently (P < 0.0001) territories comprising at least nine coupled β-cells (Fig. 6f).

To see whether exposure to HF diet specifically alters β-cell-associated Cx36 rather than inducing overall downregulation of islet Cxs, we assessed the protein level and cell distribution of Cx43, whose transcript is detected in intact pancreatic islets (33, 37) but extensively reported to be expressed by non-β-cells (mainly endothelial cells) of adult rodent at least in vivo conditions (11, 12, 30, 33, 37, 39). As shown in Fig. 7, the decreased levels of Cx36 protein observed in the prediabetic mice were not paralleled by significant changes in the protein levels of Cx43. These data indicate that the HF diet selectively altered the β-cell GJ and coupling dependent on Cx36.

DISCUSSION

The pathogenesis of T2DM is a complex process that largely depends on the functional state of the insulin-producing β-cells of the pancreas. T2DM often has an adulthood onset and is initially characterized by a moderate fasting hyperglycemia associated with marked peripheral insulin resistance, which is partially compensated for by β-cell hyperplasia (29, 31, 38). Later, decreased β-cell function and mass lead to dependence on insulin treatment (29, 31, 38). The cause of the β-cell dysfunction in T2DM is not completely understood. Insulin secretion is a multistep process that depends on several extra- and intracellular events, including membrane depolarization, controlled by ATP-dependent K⁺ channels, Ca²⁺ influx, controlled by opening of voltage-dependent Ca²⁺ channels, and intracellular increase of Ca²⁺ as well as the intercellular synchronization of the glucose-dependent Ca²⁺ oscillations, which are largely controlled by GJs (30, 36). Diabetic animals display alterations of several of these steps, in part due to chronic exposure to hyperglycemia and/or hyperlipidemia (14, 28, 29).

Here, we describe for the first time that changes in the functioning of Cx36-made channels found between β-cells are implicated in the early alterations in insulin secretion and resistance that characterize a prediabetic state. By feeding
C57/BL6 mice a HF diet for 60 days, we obtained animals displaying several defects that characterize this state, including insulin resistance, moderate hyperglycemia, and marked hyperinsulinemia associated with increased β-cell mass (5, 15). Islets from these prediabetic mice featured a significant decrease in the content of Cx36, the protein that forms β-cell GJs (33), but not of Cx43, another Cx isoform consistently shown to be expressed by non-β-cells (mainly endothelial cells) (11, 12, 30, 33, 37, 39). These data indicate that the HF diet selectively altered the β-cell GJ and coupling dependent on Cx36 rather than inducing an overall downregulation of islet Cx’s. Consistent with the selective Cx36 change, β-cells of prediabetic mice also displayed a smaller size of GJ plaques and reduced intercellular transfer of a Cx36 channel tracer, EB (12). This alteration is worth noting, inasmuch as GJ-mediated intercellular communication is crucial for proper insulin secretion (7, 10, 11, 35). The islets of HF-fed mice, which showed reduced Cx36 levels, displayed an impaired secretory response characterized by an increased release of insulin at basal glucose concentration, which is also observed in pancreatic islets of Cx36-null mice (30, 35, 39) and is one of the defects seen at the initial phase of T2DM development (3, 19). Interestingly, previous in vitro studies in both insulin-producing cells and intact islets have shown that expression of Cx36 is downregulated, through activation of a PKA-dependent pathway, after prolonged exposure to supraphysiological glucose concentrations (1) and elevated concentrations of saturated free fatty acids (2). Both hyperglycemia and hyperlipidemia were seen in mice after 60 days of HF diet. Therefore, chronic exposure to high blood glucose and lipids may explain the decrease in Cx36 levels and in the GJ-mediated β-cell-to-β-cell coupling observed herein.

Taken all together, our data suggest a link between T2DM development and impairment of GJ-mediated β-cell-to-β-cell coupling. This tentative conclusion is further supported by several pieces of experimental and genetic evidence, strengthening the idea of a pathogenetic role of GJs in diabetes. The human gene that codes for Cx36 is located on the 14q region of chromosome 15, which is a susceptible locus for T2DM (8, 13, 26, 27). Additionally, loss of Cx36 in mice and β-cell lines results in loss of the β-cell-to-β-cell synchronization of intracellular calcium changes, associated with deleterious disturbances of basal and/or stimulated insulin secretion (10, 30). It remains to be determined how the impairment of GJ function contributes to the altered insulin-secretory process and consequent metabolic disturbances reported here after a HF diet. The use of animal models such as KO-Cx36 (30, 35) and transgenic RIP-Cx36 mice (18, 21) offer valuable tools to further investigate this mechanism.

In conclusion, we have shown that the Cx36-dependent coupling of β-cells mediated by Cx36 is impaired in HF-fed prediabetic mice, supporting an important role of Cx36 in β-cell dysfunction. This role opens the possibility of taking advantage of this protein as a therapeutic target for diabetes.

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DISCLOSURES

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

AUTHOR CONTRIBUTIONS


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