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β-cell adaptation/dysfunction in an animal model of dyslipidemia and insulin resistance induced by the chronic administration of a sucrose-rich diet

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Glucose stimulated insulin secretion (GSIS) was different in rats chronically fed a sucrose-rich diet (SRD) for 3 or 30 wk. This work proposes possible mechanisms underlying insulin secretion changes from β-cell throughout these feeding periods. In isolated islets of rats fed the SRD or a control diet (CD) we examined: 1-the glucokinase and hexokinase activities and their protein mass expression; 2-pyruvate dehydrogenase activity; 3-uncoupling protein 2 (UCP2) and peroxisome proliferator-activated receptor γ (PPARγ) protein mass expression. At 3 wk on diet the SRD-fed rats showed: a marked increase in the first peak of GSIS; increased glucokinase protein mass expression without changes in glucokinase and hexokinase activities; increased PPARγ protein mass expression without changes in the UCP2 protein mass expression. No changes in either glucose oxidation and triglyceride content within the β-cell were observed. After 30 wk of feeding, a significant decrease of both glucokinase activity and its protein mass expression was accompanied by altered glucose oxidation, a triglyceride increase within the β-cell and a significant increase of PPARγ and UCP2 protein mass expression. Moreover GSIS depicted an absence of the first peak with an increase in the second phase. Finally, the SRD chronic administration altered GSIS by different mechanisms depending on the time on diet. At an early stage, the increased protein mass expression of the glucokinase and a fatty acid cooperative effect inducing PPARγ expression seem to be the mechanisms involved. At a late stage, glucolipotoxicity appears to be the cellular mechanism contributing to progressive β-cell dysfunction.

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Introduction

Modern life style with abundant nutrient supply and reduced physical activity has resulted in dramatic increases in the rates of metabolic syndrome-associated diseases. If the caloric intake is in excess of the energy expenditure, normoglycemia can be maintained at the expense of compensatory changes, including hyperinsulinemia, among others. When the compensatory response of the pancreatic β-cell to insulin resistance (IR) fails, hyperglycemia occurs.[1] While a healthy pancreatic β-cell responds rapidly and efficiently to acute changes in circulating nutrient availability to maintain metabolic homeostasis, the chronic exposure to both high glucose and fatty acid levels decreases the β-cell insulin response to an acute stimulus of glucose, the so called phenomenon of glucolipotoxicity.[2] Several mechanisms have been proposed that could contribute to the dysfunction of the β-cell under conditions of glucolipotoxicity such as changes in glucose phosphorylation and/or oxidation, increased triglyceride (Tg) content within the β-cell, downregulation of several genes including glucokinase (GK) and peroxisome proliferator-activated receptor alpha (PPARα) or stimulation of sterol regulatory element-binding protein-1c (SREBP-1c), PPARγ and uncoupling protein 2 (UCP2), among others.[3-6]

The administration of a sucrose-rich diet (SRD) to normal rats was shown to be an effective experimental model of insulin resistance and dyslipidemia that resembles some biochemical and hormonal aspects of the human metabolic syndrome. Our laboratory has demonstrated that the abnormal glucose homeostasis and insulin insensitivity that develop in the SRD fed rats depend on both the amount of carbohydrate and the length of time the diet is consumed. Moreover, in the presence of hypertriglyceridemia, plasma glucose and insulin evolve from normoglycemia and hyperinsulinemia after a short time (3 wk) to moderate hyperglycemia and normoinsulinemia with a long-term (15–40 wk) consumption of a SRD. In addition, a more pronounced increase in the plasma free fatty acid (FFA) levels, ectopic deposition of Tg in non-adipose tissues and visceral adiposity were observed in rats after a long-term SRD feeding.[7]

After 3 wk on a SRD, the biphasic pattern of glucose stimulated insulin secretion (GSIS) in the isolated pancreas showed a marked increase in the first peak of the hormone secretion which helped to maintain normoglycemia.[8] Chen et al.[9] demonstrated

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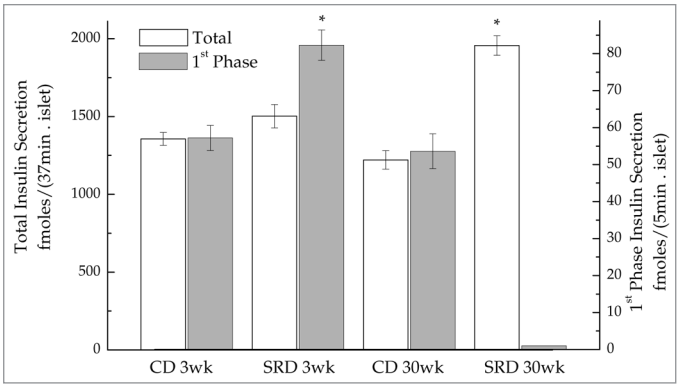

Figure 1. Total and first phase of insulin secretion were evaluated from isolated perifused islets of rats fed a control (CD) or a sucrose-rich (SRD) diet at the different times on diet. The area under total and first phase of insulin secretion by integrating the points between 3–40 min (total secretion) and 3 to 7 min (first phase); results are expressed as fmoles/37 min/islet or fmoles/5 min/islet, respectively. Values are expressed as mean ± SEM; n = 6. *p<0.05 SRD vs. CD at each time point of feeding.

that perifused islets from insulin resistant rats fed a sucrose-rich diet for 2 wk are more sensitive to the untoward effect of palmitate. Moreover, Maiztegui et al.[10] recently showed an increased activity of GK—the enzyme that plays a pivotal role upon islet glucose metabolism and insulin secretion-in normoglycemic hyperinsulinemic rats fed a standard diet in which fructose (10%) was added to the drinking water of the animals during 3 wk. This result suggests a major role of GK in the adaptative changes that lead to an increased release of insulin in response to IR.

During the length of time the SRD is consumed (30–40 wk), we demonstrated[8] an absence of the first peak with an increase in the second phase of the hormone secretion in the isolated perifused islets. This was accompanied by an increase in Tg storage within the β-cell concomitantly with a reduction of glucose oxidation (the pyruvate dehydrogenase complex (PDHc) was decreased).[11] The inhibition of PDHc limits the conversion of pyruvate derived from glycolisis to acetyl CoA, and decreases the oxidative glucose metabolism, a signal for insulin secretion and synthesis.[12] In this vein, Zhou et al.[13] showed that PDHc activity decreases while PDH kinase activity increases in the islets of obese diabetic mice. Besides, after a long-term SRD feeding (30 wk), the insulin secretion (IS) of perifused isolated islets under acute exposure to palmitic acid plus glucose was impaired.[11]

From the above information, we considered that it would be worthwhile to increase our previous knowledge by analyzing some mechanisms involved in the β-cell adaptation/dysfunction induced in the early and late state of the dyslipidemia and IR developed in the SRD fed rats. Therefore, the main aim of the present work was to examine isolated islets of rats fed a SRD for 3 or 30 wk in order to determine: 1-glucose phosphorylation and oxidation that play a key role in GSIS; 2-UCP2 protein mass expression, as an uncoupling protein that inversely correlates with β-cell ATP and GSIS; 3-PPARγ protein mass expression—since FFA act as natural ligands of PPARγ that have been shown to mediate the response of UCP2-. Besides, the Tg content of the islets was also quantified.

Results

Food consumption, body weight and plasma metabolites. As previously shown (8), during the first wks of feeding, body weight and energy intake were similar in both CD and SRD fed rats. Only a significant increase in both parameters was achieved at 30 wks in SRD-fed rats compared to control rats fed the CD for the same period of time. Data were as follows (mean ± SEM; n = 8) final body weight (g) 485 ± 17 in CD and 557 ± 21 in SRD ($p < 0.01$); energy intake (kJoule/day): 275 ± 21 in CD and 379 ± 22 in SRD ($p < 0.01$). Plasma Tg and FFA were significantly higher ($p < 0.01$) in rats fed SRD either at 3 and 30 wk, with a greater values at the end of the experimental period of feeding, compared with age-matched control rats fed a CD (data not shown). While a moderate increase in plasma glucose levels was only observed at 30 wk on the SRD, plasma insulin levels were significant increased in this dietary group just at 3 wk on diet. Values were as follows, (media ± SEM, n = 6) glucose (mmol/l) 6.57 ± 0.07 in the CD and 8.37 ± 0.10 in the SRD at 30 wk, $p < 0.05$ SRD vs. CD; Insulin (pmol/L): 373 ± 19 in CD and 577 ± 8 in SRD ($p < 0.05$) at 3 wk.

Triglyceride concentrations and PDH activity in isolated islets. No changes in Tg content and in the active form of PDHc (PDHa) activity were observed in rats fed CD and SRD for 3 wk. Values were as follows (mean ± SEM, n = 6) Tg (nmol/islet) 65.2 ± 6.1 in CD and 58.2 ± 5.4 in SRD (p = NS) and PDHa (% of total PDH): 69.6 ± 4.6 in CD and 73.7 ± 4.7 in SRD (p = NS). As previously described[11] during the length of feeding time, the rats fed a SRD for 30 wks showed an increase of islet Tg content. This was accompanied by a decrease in PDHa activity. Either at 3 or 30 wk of feeding, the total PDH activity (PDHt) did not differ among groups of rats (data not shown).

Perifusion of isolated islets. Confirming previous results, at 3 wk on the diet the SRD fed group showed a total amount of insulin secretion similar to that observed in the rats fed the CD, while the first peak was significantly higher ($p < 0.01$) than that observed in the control group (**Fig. 1**). When the diet was extended up to 30 wk, the insulin secretion from perifused islets of SRD fed rats, clearly differed from the classic pattern of GSIS observed in the CD fed rats. Although IS steadily increased throughout the time of islets perifusion (40 min), the first peak of IS was barely present and total IS (3 to 40 min perifusion) was higher ($p < 0.01$) than that observed in the age-matched rats fed the CD for the same period of time.

Glucokinase and hexokinase activities. Figure 2A depicts the GK activity of citosolic fraction of isolated islets of rats fed a SRD either at 3 or 30 wk compared to their respective CD fed rats. While no changes in GK activity was observed in SRD-fed rats compared to control rats at 3 wk of feeding, a significant decrease on the enzyme activity was detected when the SRD was administered for a long time (30 wk). No significant differences in HK activity were found between dietary groups either at 3 or 30 wk on diet (**Fig. 2A**). Consequently, the HK/GK activity ratio was significantly higher in the SRD-fed group only after 30 wk on the SRD. Values are expressed as mean ± SEM, n = 6: 3 wk CD 3.85

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± 0.29 and SRD 4.01 ± 0.39 (p = NS); 30 wk CD 3.48 ± 0.14 and SRD 5.47 ± 0.64 (p < 0.01).

Protein mass expression of glucokinase and hexokinase. The immunoblotting of isolated islets revealed a single 50 kDa band consistent with the GK and 109 kDa band for HK. Each gel contained equal number of samples from the CD, SRD groups at 3 or 30 wk of feeding, (**Fig. 2B**, upper part). After the densitometric analysis of immunoblots, the GK and HK of both CD groups were normalized to 100%. The SRD groups either at 3 or 30 wk on the diet were expressed relative to their respective age-matched controls. Qualitative and quantitative analysis of western blot showed that the relative abundance of GK was significantly increased in the citosolic fraction of isolated islets of rats fed a SRD during 3 wk when compared with rats fed a CD (**Fig. 2B**, lower part). When the SRD was extended up to 30 wk, a significant decrease of the relative abundance of GK was observed in the isolated islets compared to their respective age-matched control fed rats. No changes were observed in the protein mass expression of HK in each dietary group at both experimental feeding periods (**Fig. 2B**, lower part).

Protein mass expression of UCP2. The immunoblotting of isolated islets revealed a single 29 kDa band consistent with UCP2. Each gel contained equal number of samples from the CD and SRD groups at 3 or 30 wk (**Fig. 3A**, upper part). After the densitometric analysis of immunoblots, the UCP2 of both CD groups was normalized to 100%. The SRD groups either at 3 or 30 wk on the diet were expressed relative to their respective age-matched controls. Qualitative and quantitative analysis of western blot showed that the relative abundance of UCP2 was significantly increased in the isolated islets of the SRD-fed group when compared with rats fed a CD for 30 wk (**Fig. 3A**, lower part). On the contrary, no changes were observed in the UCP2 protein mass expression at 3 wk on the diet.

Protein mass expression of PPARγ. The immunoblotting of isolated islets revealed a single 67 kDa band consistent with PPARγ. Each gel contained equal number of samples from the CD and SRD groups, (**Fig. 3B**, upper part). After the densitometric analysis of immunoblots, the PPARγ of both CD groups was normalized to 100%. The SRD groups either at 3 or 30 wk were expressed relative to their respective age-matched controls. Qualitative and quantitative analysis of western blot showed that the relative abundance of PPARγ was significantly increased in the isolated islets of the SRD fed rats either at 3 or 30 wk of the feeding period when compared with rats fed a CD (**Fig. 3B**, lower part).

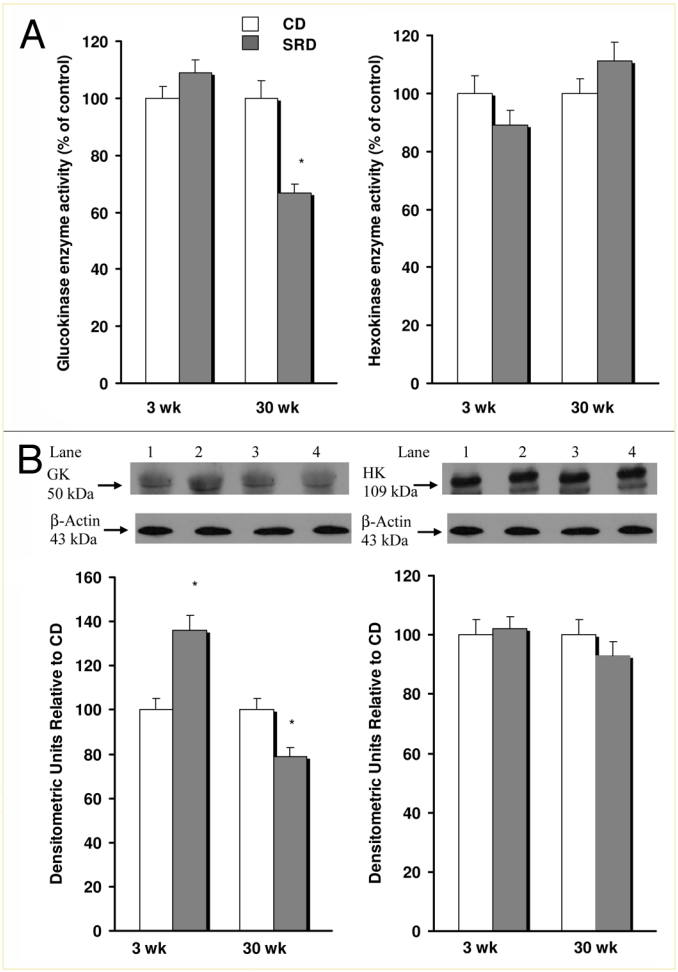

Figure 2. β-cell Glucokinase and Hexokinase activities and protein mass expression of rats fed a control (CD) or a sucrose-rich (SRD) diet. (A) GK and HK activities were measured in the citosolic fraction of isolated islets by fluorometric assay as described in materials and methods. Data are expressed as the percentage of enzyme activity measured in control fed rats. Values are expressed as mean ± SEM; n = 6. *p < 0.05 SRD vs. CD at 30 wk. (B) Upper part: immunoblots of GK and HK of isolated islets from CD and SRD rats. Molecular marker is shown on the right. Lane 1 CD 3 wk; lane 2 SRD 3 wk; lane 3 CD 30 wk; lane 4 SRD 30 wk. Lower part: densitometric immunoblot analysis of GK and HK protein mass in isolated islets of rats fed a CD or a SRD. Values are expressed as mean, with their errors depicted by vertical bars (six animals per group) and expressed as percentage relative of control diet (CD) corrected by β-actin. *p < 0.05 SRD vs. CD at each experimental time of feeding.

Discussion

Feeding rats a sucrose-rich diet induces dyslipidemia-increased levels of triglyceride and free fatty acids—and insulin resistance, associated with normoglycemia and hyperinsulinemia or hiperglycemia and normoinsulinemia, depending on the length of time on SRD administration. The present work provides new information on the time course of the development of altered insulin secretion and the demise of the β-cell of rats fed a SRD for either 3 or 30 wk. The major findings of the study are the following: 1-an increase of glucokinase protein mass expression without changes in glucokinase and hexokinase activity was observed in β-cell of rats fed a SRD for 3 wk. At this time, the protein mass expression of PPARγ increased without changes in the protein mass expression of UCP2. Besides, glucose oxidation and Tg content within the β-cell did not differ from that observed in the control group fed a CD; 2-after 30 wk of consuming the SRD, a significant decrease of glucokinase activity and its protein mass expression was accompanied by an altered glucose oxidation, an increase of Tg within the β-cell and a significant increase of both PPARγ and UCP2 protein mass expression.

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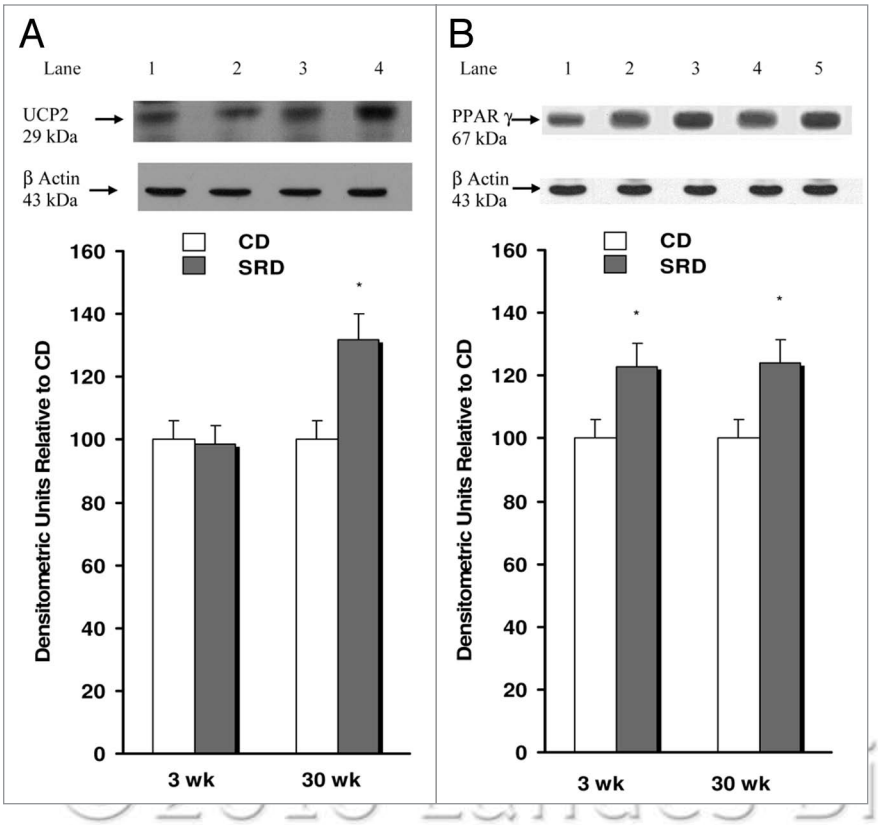

Figure 3. β-cell UCP2 and PPARγ protein mass expression of rats fed a control (CD) or a sucrose-rich (SRD) diet. (A) Upper part: immunoblots of UCP2 of isolated islets from CD and SRD rats. Molecular marker is shown on the right. Lane 1 CD 3 wk; lane 2 SRD 3 wk; lane 3: CD 30 wk; lane 4: SRD 30 wk. Lower part: densitometric immunoblot analysis of UCP2 protein mass in isolated islets of rats fed a CD or a SRD. (B) Upper part: immunoblots of PPARγ of isolated islets from CD and SRD rats. Molecular marker is shown on the right. Lane 1 skeletal muscle as positive control; lane 2 CD 3 wk; lane 3 SRD 3 wk; lane 4 CD 30 wk; lane 5 SRD 30 wk. Lower part: densitometric immunoblot analysis of PPARγ protein mass in isolated islets of rats fed a CD or a SRD. Values are expressed as mean, with their errors depicted by vertical bars (six animals per group) and expressed as percentage relative of control diet (CD) corrected by β-actin.

Confirming previous results,[8,14] after 3 wk on SRD pancreatic islets responded by increasing insulin secretion under the stimulus of glucose to maintain basal normoglycemia. The mechanisms of β-cell compensation involved in this process are not completely understood. We previously demonstrated that no morphological signs of β-cell reactivity were present at this period of feeding[15] on the SRD. However, different authors suggest that both expansion of β-cell mass and enhanced β-cell function are important features during the process of β-cell compensation.[16] Regarding the β-cell function, it is well known that glucose phosphorylation is the rate-limiting step for the beginning of GSIS. This process is accomplished by two enzymes, HK and GK. In addition, glucose is considered to be the main regulator that induces the activity and expression—both at posttranscriptional and posttranslational levels—of GK, as well as glucose-stimulated insulin release from β-cell.[17] Chen et al.[18] showed an increased GK activity and GSIS in spontaneously hypertensive rats with insulin resistance and normoglycemia, without changes in the GK protein mass expression. An increase in GK activity and GSIS was also observed in the short-term glucose infusion rat model[19] and in both ZF and Zucker diabetic fatty rats, compared with Zucker lean control rats.[20] On the other hand, in a hyperinsulinemic wild-type mouse after 20 wk on a high fat diet, Terauchi et al.[21] showed no changes in GK activity and a decreased GSIS by individual β-cells compared to mouse feeding the control diet. Hyperplasia was present in these animals islets; thus, the hyperinsulinemia could be explained only by the increased β-cell mass. Recently, Maiztegui et al.[10] demonstrated that the administration of 10% fructose in drinking water to normal Wistar rats for 3 wk induced an increase of both activity and protein mass expression of β-cell GK. This was associated with basal normoglycemia and an increased insulin release both in vivo and in vitro. These authors proposed that although glucose levels were relatively low, the permanent increase in postprandial glycemia would be sufficient to trigger GK protein mass expression and a subsequent increase in glucose-induced insulin secretion. Moreover, Gutman et al.[14] observed an increased GSIS in pancreas pieces from SRD-fed rats when they were incubated with glucose in the physiological range. Most of the studies cited above[18-21] were obtained after 60 min of islet incubation under the stimulus of glucose. However, in the present report GSIS was analyzed in perifused islets of both dietary groups. Under this experimental condition, hormone secretion was significantly higher in the first peak without significant changes in total insulin secretion. Therefore, the discrepancy observed in the results as to total hormone secretion could be due to the different methodologies employed. Regarding glucose phosphorylation, we were unable to observe any changes in both β-cell GK and HK activities after feeding the SRD during 3 wk. Therefore, a significant increase in the GK protein mass expression was present at the end of this time of feeding. At this point, it is important to mention that while GK certainly exerts a critical level of control on downstream events, other cytoplasmic and mithocondrial processes could also play an essential role in GSIS.[22] Besides after 3 wks on the SRD, rats showed normal plasma glucose levels and hyperinsulinemia associated with both altered i.v. glucose tolerance tests and insulin sensitivity.[7,8] Under these conditions, we cannot discard the possibility that rats have increased postprandial glycemia, enough to induce an increase of GK protein mass expression first, and later, of glucose-induced insulin secretion. We also demonstrated an increase in PPARγ protein mass expression in the SRD-fed rats. The role of PPARγ in pancreatic islets is still unclear, but evidence supports its direct action on genes involved in insulin-stimulated glucose disposal, such as GLUT2 and β-glucokinase.[23] Moreover at 3 wk in the

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SRD, plasma free fatty acids were significantly higher compared to CD fed rats. FFA are very important to the pancreatic β-cell for both its normal function and its capacity to compensate for insulin resistance.[23] It would be possible that the elevated FFA supply present in our experimental model could enhance GSIS as a compensatory mechanism to maintain normoglycemia, as proposed by Nolan et al.[24] Furthermore, both the Tg content within the β-cell and the glucose oxidation estimated by PDHa activity in rats fed SRD during 3 wk were similar to those observed in the CD-fed group.

Compensation processes from β cells observed at 3 wk on the SRD failed and hyperglycemia with normoinsulinemia became evident with the chronic SRD administration. We have previously shown[14] that SRD-fed rats at this time showed a significant increase in β-cell volume density and islet cell replication rate together with a decrease in β-cell apoptotic rate. It seems that the newly emerged β-cell mass has some sort of derangement with the increased insulin demand resulting from insulin resistance induced by the long-term SRD-feeding.[15] The prolonged exposure to moderate hyperglycemia and elevated levels of plasma FFA and Tg, could play a negative influence on the insulin secretory pattern observed "in vitro" in the long-term SRD fed animals. Poitout et al.[25] postulated that chronically elevated FFA levels do not harm the β-cell as long as blood glucose levels are normal, but profoundly affect β-cell function in the presence of concomitant hyperglycemia. The molecular mechanisms of glucolipotoxicity on the β-cell and the further glucose desensitization are still unclear. The decreased glucose phosphorylation (decreased both GK activity and protein mass expression) observed at 30 wk on the SRD could be involved in this process. In this regard, hyperglycemia in susceptible animals such as the DBA/2 mice would appear promoting the decreased GK protein mass expression and glucose induced insulin secretion.[26] Patane et al.[27] observed that the chronic exposure (72 h) of pancreatic islets to either glucose or FFA is associated with a decrease of glucose oxidation. In agreement, we also observed a low PDHa activity in the isolated islets of chronically SRD fed animals. This effect implies an altered signal for insulin secretion and synthesis; therefore, it could impair the GSIS. Moreover, Zhou et al.[13] reported a decrease of PDH activity in pancreatic islets exposed to high levels of FFA and suggested that this defect was related to the glucose-FFA cycle.

In the present study, a significant increase of Tg content in β-cell of 30 wk SRD fed animals was shown. Elevated glucose—as we observed in this experimental animal model-via AMPK/malonyl-CoA signaling network, could curtail FA oxidation and consequently the detoxification of fat, while at the same time promoting partitioning of FFAs into lipogenesis.[28] In isolated rat islets from GK rats fed a fat diet, GSIS was impaired only in the context of chronic hyperglycemia. These effects were not mediated by Tg accumulation within the islets but were associated with changes in the UCP2-expression.[29] Both fatty acids and their acyl-CoA derivates are important ligands for several classes of nuclear receptors involved in the β-cell function. In our experimental model, both increased levels of plasma FFA and UCP2 protein mass expression within the β-cell were observed in rats fed a SRD for 30 wk. The increment of UCP2 could result in mitochondrial uncoupling and altered insulin secretion. PPARγ could be involved in the relationship between chronic exposure of β-cell to high levels of plasma glucose and fatty acids and β-cell UCP2 protein mass expression.[30] Moreover, PPARγ antagonist prevents the increase of UCP2 expression and rescues insulin secretion.[31] In this regard, the increase of Tg stores within the β-cell observed in the SRD fed rats might serve as a pool of endogenously released fatty acids, which in turn could stimulate UCP2-overexpression via upregulation of PPARγ.

In conclusion, our data show that the chronic administration of SRD alters glucose-stimulated insulin secretion by different mechanisms: In the early stage (3 wk on the SRD), the increased insulin secretion to maintain normal glucose levels would be in part the result of an increased protein mass expression of the phosphorylating glucose enzyme-GK—and a cooperative effect of fatty acids inducing PPARγ that could influence the GK mass expression. At the late stage of SRD-feeding, the increased levels of glucose and FFA could contribute to the β-cell failure through at least some mechanisms of glucolipotoxicity, thus contributing to the progressive β-cell dysfunction in this nutritional model of dyslipidemia and insulin resistance.

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Materials and Methods

Animals and diets. Male Wistar rats initially weighing 180–200 g and purchased from the National Institute of Pharmacology (Buenos Aires, Argentina), were maintained under controlled temperature (22 ± 1°C), humidity and air flow condition, with a fixed 12 h light:dark cycle (light 7:00 AM to 7:00 PM). They were initially fed a standard nonpurified diet (Ralston Purina, St. Louis, MO). After 1 wk of acclimatizing, they were randomly divided into two groups of 120 rats (control and experimental). The experimental group received a purified sucrose-rich diet (SRD) containing by weight (g/100 g): 62.5 sucrose, 17 casein free of vitamins, 8 corn oil, 10 cellulose, 3.5 sal mixture (AIN-93M-MX), 1 vitamin mixture (AIN-93-VX), 0.2 choline chloride and 0.3 DL-methionine. The control group received the same purified diet but with sucrose replaced by cornstarch [high starch diet (CD)]. Details of the methodology used were described elsewhere.[8] The rats had free access to food and water and consumed their respective diets for 3 or 30 weeks. Both diets were isoenergetic and provided 16.3 kJ/g of food. Diets were prepared every week and stored at 4°C. The weight and energy intake of the rats were recorded twice each week during the experimental period. At the end of the 3 or 30 weeks feeding period, food was removed at the end of the dark period (7:00 AM) and experiments were performed between 9:00 and 12:00 AM. The experimental protocol was approved by the Human and Animal Research Committee of the School of Biochemistry, University of Litoral, Santa Fe, Argentina.

Analytical methods. At the end of the dark period, rats from each dietary group, after 3 or 30 wk period feeding, were decapitated. Blood samples obtained were rapidly centrifuged at 3,500x g for 15 min at 4°C and the plasma either immediately assayed or stored at -20°C and examined within 3 d. Plasma Tg,

FFA and glucose levels were determined by spectrophotometric methods and insulin was measured by an immunoreactive assay as previously described.[8] The immunoreactive insulin assays were calibrated against rat insulin standard (Novo Nordisk, Copenhagen, Denmark). Pancreases were rapidly removed from all rats and the islets were isolated by collagenase digestion and collected under a stereoscopic microscope as previously described.[8,11] Tg content and activity of the pyruvate dehydrogenase complex (PDHc) in the isolated islets were determined as previously described.[11]

Perifusion of isolated islets. Isolated islets as described above were washed twice with a Krebs-Ringer bicarbonate (KRB) buffer and groups of 30 to 40 islets isolated from each rat were loaded in a 13 mm chamber containing a 5 μm nylon membrane filter. Islets were perifused with KRB containing 3 mmol/L glucose, 250 mg/L essentially fatty acid-free bovine serum albumin, 40 mg/L dextran 70, pH = 7.4 at 37°C (constantly gassed with 95% O_2, 5% CO_2) at a flow rate of 0.9–1.2 mL/min. Complete details of the methodology used have been previously described.[8,11]

Glucokinase and hexokinase enzyme activities. GK and HK were extracted from isolated islets as described by Liang et al.[32] Approximately 300 islets per individual rat were washed twice and homogenized at 4°C with 300 μl of buffer K_2HPO_4 20 mM pH = 7.4 containing 5 mM dithiothreitol, 1 mM EDTA and 110 mM KCl. The homogenate was centrifuged at 12,000x g for 10 min and the supernatant fraction was used for the determination of GK and HK activities.[33] Briefly, 1 vol of extract was added to 25 vol of reaction buffer (50 mM Hepes/HCl, pH: 7.60, containing 100 mM KCl, 7.4 mM $MgCl_2$, 15 mM β-mercaptoethanol, 0.50 mM NAD^+, 0.05% BSA, 0.7 U/ml glucose-6-P dehydrogenase from *Leuconostoc mesenteroides,* 5 mM ATP and glucose at 0.5 or 100 mM). After 90 min at 30°C, the reaction was stopped with 250 vol of 500 mM $NaHCO_3$, pH 9.0 and glucose phosphorylation was determined by measuring glucose-6-phosphate formation by fluorometry. Duplicate samples were performed at each glucose concentration in parallel with reagent blanks (no tissue extracts) and a tissue blank (islet extract homogenate in reaction buffer without glucose). The reagent and tissue blanks were both subtracted from the total fluorescence of a corresponding complete reagent containing tissue. The standard curve was glucose-6-phosphate (0.4–8.3 nmol/ul) in a reaction buffer that contained 100 mM glucose. GK activity was determined by subtracting the activity of HK measured in the presence of 0.5 mM glucose from that measured at 100 mM glucose. Protein content in the supernatant was measured by the Lowry method.[34]

Western blot analysis of glucokinase and hexokinase protein mass expression. Approximately 200 isolated islets from each experimental group as described above were washed twice with ice-cold phosphate saline buffer (PBS) and lysed in cold lysis buffer containing 1% Igepal, 150 mM NaCl, 1 mM EDTA, 1 mM Na_3VO_4, 1 mM NaF, 50 mM Tris, pH 7.4, 1 mM PMSF, 1 μg/ml aprotinin, 1 μg/ml leupeptin and 1 μg/ml pepstatin as described by Shao et al.[35] The lysates were collected and centrifuged for 15 min at 12,000 g at 4°C. The pellets were discarded and protein content was measured by the Bio-Rad protein assay kit. The protein extract was stored at -80°C until the assay. Equal amounts of protein (80 μg) were subjected to 9% SDS-PAGE and transferred to PVDF membrane. After blocking the membranes with Tris-buffered saline (TBS) supplemented with 0.1% Tween (TBS-T) and 5% fat free milk, they were incubated overnight with 1:400 specific polyclonal rabbit anti-glucokinase antibody (Santa Cruz, Biotechnology, Santa Cruz, CA) or 1:800 specific polyclonal goat anti-hexokinase antibody (Santa Cruz, Biotechnology, Santa Cruz, CA) at 4°C. The membranes were washed extensively with TBS-T and incubated for 2 h at room temperature with goat horseradish peroxidase conjugated anti rabbit IgG 1:2,000 or mouse horseradish peroxidase conjugated anti goat IgG (1:2,000), respectively, and then washed wit TBS-T. The bands were visualized by enhanced chemiluminiscence (Super Signal West Pico Chemiluminiscent Substrate, Pierce Biotechnology, Rockford, IL) according to the manufacturers. GK and HK expression were normalized to β-actin. The intensity of the bands was quantified by the National Institute of Health (Bethedsa, MD) imaging software. Preliminary studies showed linearity of western blot assay from 20 to 100 μg of protein for both, GK and HK, respectively. The correlation between the amount of protein and the enhanced chemiluminiscence image intensity was 0.95 for GK and 0.98 for HK. The relationship between the amount of sample subjected to immunoblotting and the signal intensity observed was linear under the conditions described above.

Western blot analysis of peroxisome proliferator-activated receptor gamma (PPARγ) protein mass expression. Isolated islets (approximately 200 islets) were washed in ice-cold PBS and lysed for 10 min in cold lysis buffer containing 20 mM Tris, 150 mM NaCl, 1 mM EDTA, 1% Triton X and 1 ul/ml protease inhibitor cocktail.[36] After vortexes the protein extract was centrifuged at 13,000x g for 10 min at 4°C and the supernatant was collected and stored at -80°C. Protein content in the supernatant was determined by the Bio-Rad protein assay kit. Forty micrograms of islet proteins were subjected to 10% SDS-PAGE and the resolved proteins transferred to a PVDF membrane. The membranes, blocked as described above, were incubated overnight at 4°C with 1:300 specific polyclonal rabbit anti-PPARγ antibody (Santa Cruz, Biotechnology, Inc.). After being rinsed, the membranes were incubated for 2 h at room temperature with goat horseradish peroxidase-conjugated anti rabbit IgG (1:2,000). The bands were visualized by enhanced chemiluminiscence (Super Signal West Pico) and the intensity of the bands quantified as described for glucokinase and hexokinase expression. The PPARγ expression was normalized by the β-actin. The linearity of the western blot assay was from 20 to 80 μg of protein and the correlation coefficient was 0.93.

Western blot analysis of uncoupling protein 2 (UCP2) protein mass expression. Isolated islets (approximately 200 islets) were homogenized in a buffer containing 250 mM sucrose, 0.5 mM EGTA, 5 mM HEPES, with 1 μl/ml protease inhibitor cocktail and centrifuged at 800x g for 3 min at 4°C.[37] The supernatant was collected and stored at -80°C. Islet proteins were determined by the Bio-Rad protein assay kit. Equivalent amounts of proteins (80 μg) of the different experimental

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groups were subjected to 14% SDS-PAGE and electrophoretically transferred onto nitrocellulose membrane. The membranes were blocked as previously described, washed and incubated overnight at 4°C with 1:300 specific polyclonal goat anti-UCP2 antibody (Santa Cruz, Biotechnology, Santa Cruz, CA). After washing the membranes they were blotted with mouse horseradish peroxidase conjugated antigoat IgG (1:2,000) at room temperature for 2 h. The bands were visualized by enhanced chemiluminiscence (Super Signal West Pico) and the intensity of the bands quantified as described above. The UCP2 expression was normalized by the β-actin.

The linearity of western blot assay was from 20 to 100 μg of protein and the correlation coefficients was 0.92.

Statistical analysis. Results are expressed as means ± SEM. The significance between the two groups was determined by Student's t test. When appropriate, data were subjected to two-way ANOVA with diet and time as the main effects,[38] followed by inspection of all differences between pairs of means by Scheffe's test.[35] Differences with p values <0.05 were considered to be significant.

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