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Islet NADPH oxidase activity modulates β-cell mass and endocrine function in rats with fructose-induced oxidative stress

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ABSTRACT

Background: Islet NADPH oxidase activity is modulated by glucose and other insulin secretagogues and it might be part of the regulatory mechanism of insulin secretion. We studied its modulatory role of islet NADPH oxidase upon β-cell function in rats with fructose-induced oxidative stress.
Methods: Normal rats were fed for 3 weeks with a standard diet, a fructose-rich diet or both diets plus apocynin. We measured plasma glucose, insulin, triacylglycerol and lipid peroxidation levels and the homeostasis model assessment-insulin resistance (HOMA-IR) and HOMA-β indexes, and performed an oral glucose tolerance test. β-cell volume density and the number of islets per mm^2 were determined by immunomorphometric analysis of the pancreas. Insulin secretion, glucose metabolism, glucokinase and NADPH oxidase activities were studied in islets isolated from each experimental group.
Results: Fructose-fed rats had increased plasma triacylglycerol, insulin and lipid peroxidation levels associated with an insulin resistance state; the reactive higher secretion was unable to cope with the increased demand of insulin, leading to an impaired glucose tolerance. They also have a lower number of islets per area unit with a decreased β-cell volume density. All these alterations were prevented by blocking NADPH oxidase activity with apocynin.
Conclusion: Fructose-induced changes are partly mediated by modulation of NADPH oxidase activity.
General significance: The metabolic dysfunctions and enhanced oxidative stress measured in fructose-fed rats resemble those recorded in human prediabetes; thus, successful strategies employed in this model could be later used to prevent the progression of this state towards type 2 diabetes in human beings.

1. Introduction

The annual *per capita* consumption of fructose has drastically risen in the US in recent decades [1], and some authors consider that this consumption has actively contributed to the development of the current epidemics of obesity, type 2 diabetes and metabolic syndrome (MS) [2]. For these reasons, the US Dietary Guidelines recommend limiting calorie intake — which includes both added sugar and solid fat — to 13% of energy requirements [3].

On the other hand, several studies have demonstrated that administration of a fructose-rich diet (F) to normal rats for a relatively short period induces the development of multiple metabolic changes that resemble those observed in the human prediabetes state [4–9]. When these animals are fed with this diet for a longer period, they develop a stable type 2 diabetes syndrome [8]. Thus, this rat model becomes useful to gain knowledge on the pathophysiology of these clinical entities as well as to test potential prevention and treatment strategies.

In this context, these rats present indicators of increased β-cell function in vivo (hyperinsulinemia) and in vitro (increased glucose-stimulated insulin secretion [GSIS] and islet glucose metabolism), together with a decreased β-cell volume density (Vvi) and an increased oxidative stress (OS) [10–17]. OS is characterized by an excessive mitochondrial and/or extramitochondrial production of reactive oxygen species (ROS) [18], the latter mainly depending on the activity of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, an enzymatic complex widely distributed in the body, including pancreatic islets [19–21].

Islet NADPH oxidase activity is modulated by glucose and other insulin secretagogues, and might be part of the regulatory mechanism of insulin secretion [19,21–23]. In an attempt to gain some insights into the potential relationship among OS, NADPH oxidase activity and β-cell mass and function, we studied ROS production and NADPH oxidase activity together with glucose metabolism, glucokinase (GK) activity, insulin secretion in islets isolated from normal rats fed an F, with or without

Abbreviations: IR, insulin resistance; NADPH, nicotinamide adenine dinucleotide phosphate; HOMA, homeostasis model assessment; GSIS, glucose-stimulated insulin secretion; Vvi, volume density; OS, oxidative stress; ROS, reactive oxygen species; GK, glucokinase; HK, hexokinase; F, fructose; A, apocynin; TBARS, thiobarbituric acid reactive substances; MDA, malondialdehyde; OGTT, oral glucose tolerance test; KRB, Krebs-Ringer bicarbonate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; BSA, bovine serum albumin; G6P, glucose-6-phosphate; DPI, diphenylene iodonium; NBT, nitro-blue-tetrazolium; AUC, area under the curve

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co-administration of an NADPH oxidase inhibitor (apocynin) [24] and β-cell Vvi in pancreatic tissue.

2. Materials and methods

2.1. Chemicals and drugs

Collagenase was obtained from Serva Feinbiochemica (Heidelberg, Germany); bovine serum albumin (BSA, fraction V) and other reagents were from Sigma Chemical Co. (St. Louis, MO, USA). Fructose was obtained from Ciccarelli Reagents S.A. (Santa Fe, Argentina).

2.2. Experimental groups

Normal male Wistar rats (180–200 g bw) were divided into two groups: a control group with free access to a standard commercial diet and water (C), and another group that received the same diet plus 10% fructose (w/v) (F) in the drinking water for 3 weeks. Animals from C and F were randomly divided into two subgroups: untreated (C and F) and treated with 5 mM apocynin in drinking water (CA and FA). Animals were housed in a room with controlled temperature (25 °C) and lighting (12 h light–12 h darkness cycle). Water and food intake were measured daily, while individual body weight was recorded once a week. CA and FA solutions were prepared and replaced daily in order to minimize the risk of apocynin inactivation once in the solution. Each group was formed by 22 animals.

Animal experiments and handling were performed according to the "Ethical principles and guidelines for experimental animals" (3rd Edition 2005) of the Swiss Academy of Medical Sciences.

2.3. Plasma measurements

At the end of each dietary period, blood samples from non-fasted animals were collected from the retro-orbital plexus under light halothane anesthesia at 9:00 am to measure plasma glucose, triacylglycerol, lipid peroxidation (thiobarbituric acid reactive substances [TBARS]) and insulin levels. Glucose was measured with test strips (Accu-Chek Performa Nano System, Roche Diagnostics. Mannheim, Germany) and the triacylglycerol level was determined using commercial kits (BioSystems S.A., Buenos Aires, Argentina) implemented in an automated clinical analyzer. TBARS were determined by a fluorimetric assay [25] and results expressed as pmol of malondialdehyde (MDA) per mg of plasma protein, measured with the Bio-Rad Protein Assay kit (Bio-Rad Lab, RC, USA). Plasma insulin was measured by radioimmunoassay [26] using a specific antibody against rat insulin, rat insulin standard (Linco Research Inc., St. Charles, MO, USA) and highly purified porcine insulin labeled with ^{125}I [27]. Insulin resistance (IR) was assessed with the homeostasis model assessment-IR (HOMA-IR), using the formula [serum insulin (μ-units/mL) × fasting blood glucose (mM)] / 22.5. β-cell function was quantified with the HOMA-β, with the formula [serum insulin (μ-units/mL) × 20 / glucose (mM) − 3.5] [28]. Since these indexes were validated in humans but not in rodents, we compared the measured values in C with the other experimental groups instead of using a cut-off threshold value.

2.4. Oral glucose tolerance test (OGTT)

The OGTT was performed in 12-h fasted rats from each experimental group under light ketamine-midazolam anesthesia (80 and 5 mg/kg respectively). Glucose (1.1 g/kg in saline solution) was given through a gavage tube placed into the stomach and blood samples were obtained from the retro-orbital plexus at 0, 30, 60 and 120 min following the glucose load, using xylocaine (Lidocaína 4%, Scott Cassará, Buenos Aires) as topical anesthetics. We measured the glucose concentration with test strips (Accu-Chek Performa, Roche Diagnostics) at each time point and we calculated the area under the glucose curve (AUC), expressing it in mg/dL/120 min.

2.5. Immunohistochemical and morphometric studies of the pancreas

The whole pancreas of animals from each experimental group was carefully dissected and removed. It was then fixed in 10% formaldehyde and embedded in paraffin. Serial sections of fixed pancreas (5 μm) were cut from three different depths of the blocks with a rotatory microtome and mounted on silanized slides (3-amino-propyltriethoxysilane; Sigma Co.) [29]. Sections were thereafter deparaffinized, incubated for 30 min in 3% hydrogen peroxide in methanol to block the endogenous peroxidase activity and rehydrated in a descending ethanol series, followed by incubation in 2.5% porcine serum to reduce non-specific binding. The slides were then incubated for 24 h at 4 °C in a humidified chamber with our own anti-guinea pig insulin antibody (1:20,000). The final staining was performed by incubating the slides for 30 min with appropriately diluted streptavidin-biotin complex (1:40 and 1:20 respectively; Sigma); thereafter, the sections were stained with hematoxylin.

Morphometric analysis was performed by videomicroscopy using a Jenamed 2 Carl Zeiss light microscope and a RGB CCD Sony camera, together with the OPTIMAS software (Bioscan Incorporated, Edmons, WA, USA). We measured the number of islets per area unit (mm^2), and also estimated the Vvi of islet β-cell and extrainsular β-cells [30].

2.6. Perifusion of isolated islets

Islets from each experimental group were perifused as described previously [31,32]. Briefly, batches of 50 freshly isolated islets were placed in chambers containing 1 mL Bio-Gel P2 (100 to 200 mesh; Bio-Rad Laboratories, Richmond, CA) in Krebs-Ringer bicarbonate (KRB) buffer supplemented with 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 2 mg/mL BSA, 100 IU/mL penicillin, 100 μg/mL streptomycin, 400 kallikrein-inhibitor units (KIU)/mL aprotinin (Trasylol; Bayer, Leverkusen, Germany) and either 3.3 or 16.7 mM glucose under an atmosphere of 95% oxygen/5% CO_2 at pH 7.4. The perifusion chambers were installed in an isothermal box at 37 °C; each chamber was perifused with buffer at a flow rate of 0.25 mL/min, and fractions of the perfusate were collected at 1 to 10 min, 12, 14, 16, 20, 24, 28, 30, 32, 34, 36 and 38 min (flow rate was chosen to obtain sufficient insulin concentration for radioimmunoassay [31,32]).

2.7. Insulin secretion

Freshly isolated islets from each experimental group (C, CA, F and FA) were incubated for 60 min at 37 °C in KRB, pH 7.4, previously gassed with a mixture of CO_2/O_2 (5/95%), containing 1.0% (w/v) BSA and 3.3 or 16.7 mM glucose. In all cases, aliquots from the medium were taken after the incubation and kept frozen for insulin measurement by radioimmunoassay [26] using a specific antibody against rat insulin, rat insulin standard (Linco Research Inc., St. Charles, MO, USA) and highly purified porcine insulin labeled with ^{125}I [27].

2.8. Islet glucose oxidation

Groups of 20 islets were incubated in a small glass vial containing 40 μL of KRB buffer supplemented with 10 mM HEPES (pH 7.4), D-[U-^{14}C]-glucose (10 μCi/mL, [300 mCi/mmol]) in the presence of 3.3, 8.3 and 16.7 mM glucose. This vial was placed inside an airtight-sealed 20 mL glass scintillation vial containing a glass vial. After 2 h of incubation at 37 °C, the reaction was stopped by injecting 20 μL of 400 mM citric acid, 10 μM rotenone and 3 mg potassium cyanide (KCN), pH 4.9 into the incubation vial through the rubber seal; at the same time 250 μL hyamine was added to the empty tube. After a second incubation

for 60 min at 37 °C, the $^{14}CO_2$ fixed to hyamine was measured in vials containing 5 mL of scintillation liquid [33].

2.9. Measurement of islet hexokinase/glucokinase (HK/GK) activity

Groups of 20 isolated islets were homogenized (1 islet/μL) in 50 mM HEPES-NaOH buffer, pH 7.5; 20 μL of whole homogenate was then combined with another 20 μL of the reaction mixture (HEPES-NaOH, 10 mM ATP, 20 μCi/mL [300 mCi/mmol] D-[U-^{14}C]-glucose and 1 or 100 mM unlabelled D-glucose) and incubated for 60 min at 37 °C. The reaction was stopped by the addition of 1 mL iced water and the medium was then passed through a column of AG 1-X8 to separate D-[U-^{14}C]-G6P (glucose-6-phosphate) from D-[U-^{14}C]-glucose by ion exchange chromatography [34]. The hexose phosphate was eluted from the column with 1 M ammonium formate/0.1 M formic acid. The eluate was then mixed with 10 mL scintillation fluid (Ultima Gold XR, Packard), measuring its radioactivity in a liquid scintillation spectrophotometer. The same mixture but without islet tissue was used as a blank. Under these conditions, the G6P production obtained in the presence of 1 mM and 100 mM of unlabelled glucose corresponded to HK and GK activity, respectively.

2.10. Islet superoxide production and NADPH oxidase activity

Superoxide (O_2^-) generation was detected by nitro-blue-tetrazolium (NBT) assay [19,35]. Groups of freshly isolated islets (75 islets/375 μL KRB with 1% BSA) were incubated for 2 h with 0.2% NBT (37 °C, 95% O_2/5% CO_2) in the presence of 3.3 mM glucose, with or without 10 μM diphenylene iodonium (DPI), an NADPH oxidase inhibitor, in the incubation media. At the end of the incubation, islets were sonicated in 100 μL 50% acetic acid to dissolve the NBT-reduced formazan; thereafter, the absorbance of each sample was determined at 620 nm. The production of O_2^- ascribed to NADPH oxidase activity was expressed as the difference between the values measured in the absence and the presence of DPI in the reaction tube. Since Li et al. have recently shown that the presence of NBT in the incubation media triggers a negative effect upon GSIS, we used a different group of islets to measure GSIS and superoxide production [36].

2.11. Statistical analysis

All data are expressed as means ± SEM. Differences were analyzed using two-way ANOVA followed by either Tukey multiple comparison test or Tamhane test (data having normal distribution and equal variance), while data that failed for those conditions were analyzed by Friedman's test followed by Dunn's multiple comparison test. Statistical analyses were performed using SPSS program (version 15.0, SPSS, Inc, Chicago, IL). We considered differences as significant when P value < 0.05.

3. Results

3.1. Body weight, food and water intake

Water intake of F rats was significantly larger than that corresponding to C ones ($p < 0.05$). Since F and FA rats drunk a similar amount of water, their daily intake of fructose was also comparable (F and FA, 4.08 ± 0.40 and 3.29 ± 0.24 g/rat/day). On the other hand, CA and FA rats had a comparable apocynin intake (131.97 ± 6.15 and 164.30 ± 17.37 μmol/rat/day) (Fig. 1A). Solid food intake was significantly larger in C than in F rats (Fig. 1B; $p < 0.05$) and such consumption was not significantly modified by apocynin administration. This uneven intake of solid food and water resulted in a different percentage of daily nutrient consumption (Fig. 1D) but a comparable amount of calorie intake (Fig. 1C), that resulted in an also comparable body weight increase recorded during the study period (Fig. 1E).

3.2. Plasma glucose, triacylglycerol, TBARS and insulin levels

No significant differences in plasma glucose levels were recorded between C and F rats (Table 1), but the latter had significantly higher plasma insulin, triacylglycerol and TBARS levels as well as HOMA-IR and HOMA-β values than C rats ($p < 0.05$ in all cases), thus indicating

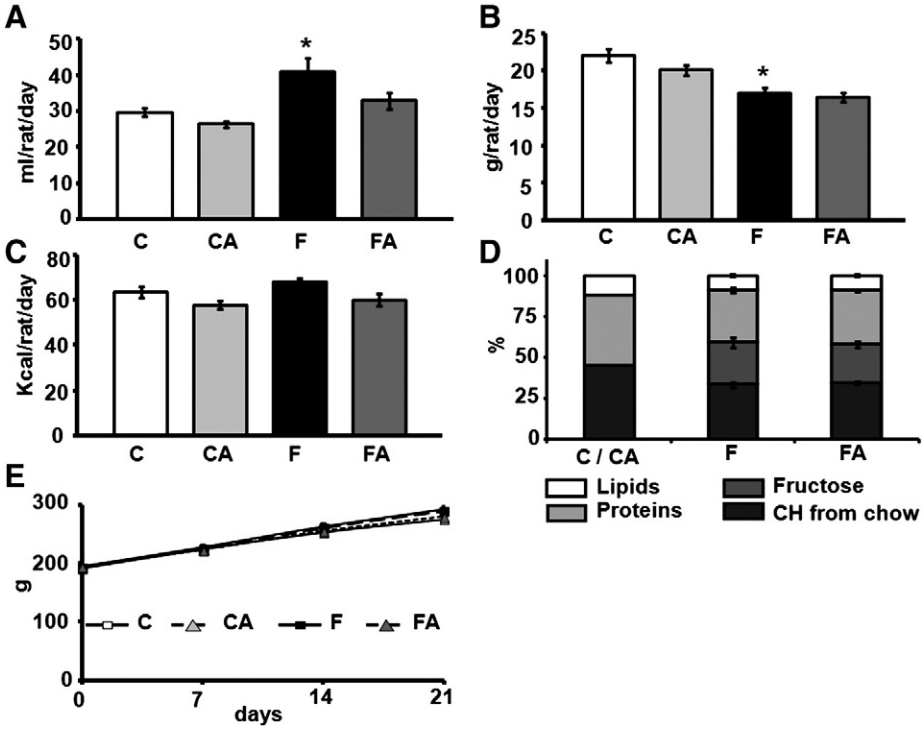

Fig. 1. Body weight and daily food intake. Daily intake of water (A), solid food (B) and total calories (C), and percentage of CH (carbohydrates), proteins and lipids (D) during the 3-week treatment period. Rat body weight was determined weekly during the treatment period (E). Values are means ± S.E.M. ($n = 22$ rats per group). *$p < 0.05$ compared with C.

Table 1
Metabolic and endocrine parameters measured in the different experimental groups.

Parameter	C	CA	F	FA
Glucose (mg/dL)	111.68 ± 1.59	103.32 ± 2.05	111.50 ± 3.67	108.05 ± 2.88
Insulin (ng/mL)	0.92 ± 0.13	0.84 ± 0.11	1.78 ± 0.14*	1.14 ± 0.19§
Triacylglycerol (mg/dL)	91.16 ± 6.34	86.13 ± 6.02	153.12 ± 6.89*	125.04 ± 9.58§
TBARS (pmol/mg prot)	58.92 ± 3.98	64.95 ± 4.16	73.24 ± 3.82*	61.58 ± 4.28§
HOMA-IR	6.47 ± 0.96	5.36 ± 0.83	12.21 ± 1.22*	7.89 ± 1.45§
HOMA-β	73.25 ± 10.64	73.72 ± 10.21	143.08 ± 11.78*	88.87 ± 15.12§

Values are means ± SEM ($n = 22$ rats per group). *$p < 0.05$ compared with C; §$p < 0.05$ compared with F.

the presence of an IR state in F rats. All these enhanced parameters were significantly reduced in F rats co-treated with apocynin ($p < 0.05$). Conversely, none of these parameters were affected when apocynin was co-administrated to C animals.

3.3. OGTT

Plasma glucose levels at 30 and 60 min were significantly higher in F compared with C animals ($p < 0.05$; Fig. 2A); the AUC was also significantly higher in F than in C rats ($p < 0.05$; Fig. 2B). Co-administration of apocynin to F rats prevented the increase of the AUC and of glucose levels at 30 and 60 min ($p < 0.05$). Apocynin co-administration to C rats did not affect the plasma glucose profile.

3.4. Morphometric analysis of the pancreas

F rats showed a significant decrease in both, the number of pancreatic islets per area unit (C vs. F: 1.55 ± 0.21 vs. 0.77 ± 0.07 islet/mm^2, $p < 0.05$; Fig. 3A and B) and the Vvi of islet β-cell (C vs. F: 0.95 ± 0.09 vs. 0.6 ± 0.05%, $p < 0.05$; Fig. 3C); no significant changes were recorded in the extrainsular β-cell Vvi (Fig. 3D). While co-administration of apocynin to C rats did not modify any morphometric parameter, it was sufficient to prevent the development of both alterations in F rats

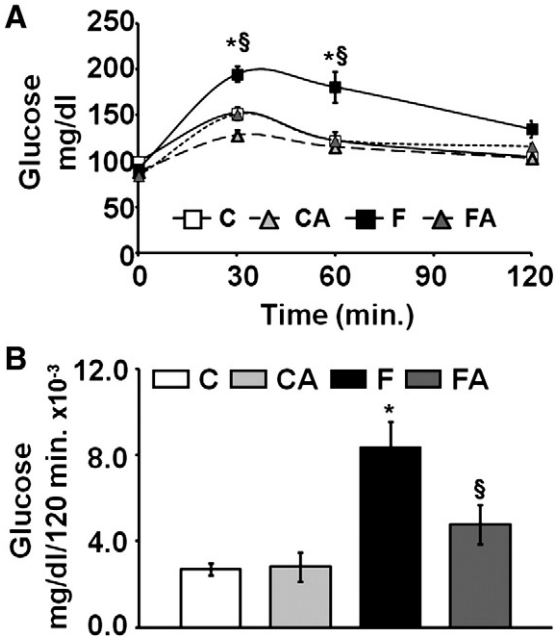

Fig. 2. Oral glucose tolerance test. (A) Blood glucose concentration at 0, 30, 60, and 120 min following an OGTT (1.1g/kg bw) in C, F, CA and FA rats. (B) AUC in mg/dL/120 min from C, CA, F and FA animals. In both panels, values are means ± S.E.M. ($n = 8$ rats per group). *$p < 0.05$ compared with C and §$p < 0.05$ for F vs. FA.

(F vs. FA: 0.77 ± 0.07 vs. 1.7 ± 0.1 islet/mm^2 and 0.6 ± 0.05 vs. 1.2 ± 0.07% of islet β cell Vvi; $p < 0.05$) (Fig. 3A, B and C, respectively).

3.5. Insulin secretion "in vitro"

Islets isolated from the different groups were perifused with 3.3 mM glucose until the insulin concentration in the perifusate attained constant values (data not shown). Thereafter, when the islets were challenged with 16.7 mM glucose, a biphasic release of insulin was recorded in all groups. We considered the first 10 min as the first phase and the following 28 min as the second phase of insulin secretion.

In islets isolated from F rats, the first phase of insulin secretion (Fig. 4A), its corresponding AUC, as well as that showing both phases together (Fig. 4B), were significantly higher than those from C rats ($p < 0.05$). Apocynin co-administration to C and F rats significantly decreased the GSIS in both groups ($p < 0.05$; Fig. 4B).

This insulin secretory pattern of the apocynin-treated C group did not strictly correlate with the changes induced by the inhibitor on serum insulin levels; thus, we decided to get a deeper insight into its action mechanism measuring GSIS for a longer period of time using a 60 min static incubation of freshly isolated islets. As shown in Fig. 4C, no significant differences were recorded in GSIS by islets isolated from C and CA rats. Conversely, islets isolated from F rats had a significantly larger GSIS ($p < 0.05$) and apocynin co-administration to these rats decreased the secretion to values comparable to those recorded in C and CA islets.

3.6. Glucose metabolism

The production of $^{14}CO_2$ from D-[U- ^{14}C]-glucose by islets isolated from all groups increased significantly when the glucose concentration in the incubation medium rose from 3.3 to 16.7 mM ($p < 0.05$). In the presence of 8.3 and 16.7 mM glucose, $^{14}CO_2$ production was significantly higher in F compared to C rats ($p < 0.05$). Apocynin decreased by 23% $^{14}CO_2$ production in islets from these rats (FA group), but this decrease was not statistically significant (Fig. 5A).

3.7. Islet GK/HK activity

A significantly higher GK activity was measured in homogenates of islets isolated from F compared with C rats ($p < 0.05$). Conversely, no significant differences in HK activity were found among all groups (data not shown). While apocynin co-administration to F rats decreased GK activity to values comparable to those measured in C rats ($p < 0.05$; Fig. 5B), it did not affect such activity in C rats.

3.8. Islet superoxide production and NADPH oxidase activity

Total O_2^- production was significantly higher ($p < 0.05$) in islets from F than C rats (Fig. 6); the O_2^- fraction ascribed to NADPH oxidase activity represented 48% and 20% of the total in F and C rats, respectively. Administration of apocynin decreased total O_2^- production as well as

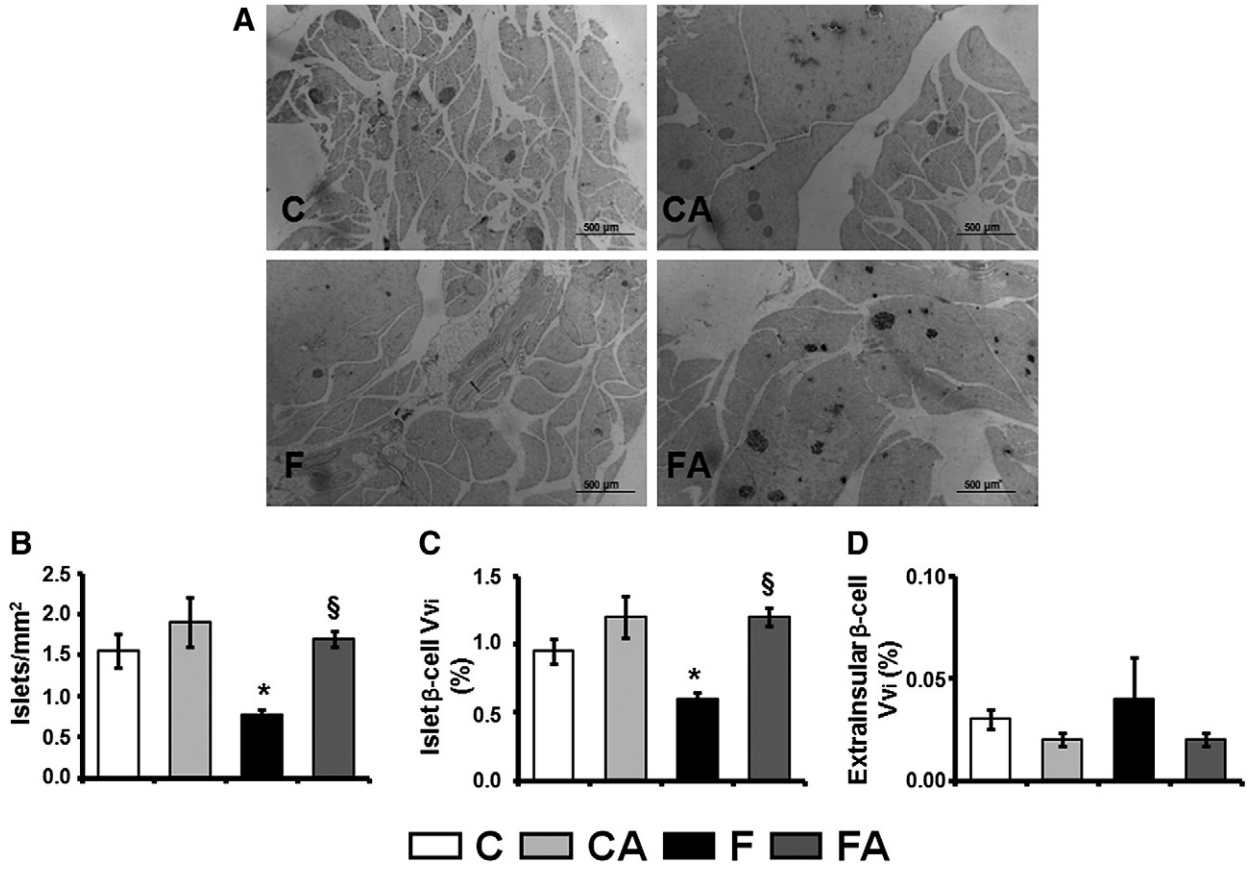

Fig. 3. Morphometric analyses. (A) Pancreas sections from C, CA, F and FA rats stained with insulin antibody. The results represent 3 different levels of the block from 3 animals of each group. (B) Number of pancreatic islets per mm^2. (C) Islet β-cell Vvi from all experimental groups measured as %. (D) Extrainsular β-cell Vvi measured in all groups as %. In all cases, bars are means ± S.E.M. from 3 replicate samples. $^*p < 0.05$ compared with C and §$p < 0.05$ for F vs. FA.

the fraction ascribed to NADPH oxidase (non detectable levels) in both C and F rats.

4. Discussion

We have previously shown that administration of a fructose-rich diet to normal rats for 3 weeks induces an increase of OS [11–17] as well as a dysfunction of adipose tissue [11–14], liver [15–17] and islet β-cells [10] which result in multiple metabolic and endocrine disorders. The current study focused on the islet changes occurring in this rat model and their possible prevention by co-administration of apocynin. As previously reported [10], F rats presented a simultaneous decrease in the number of islets (50.3%) and β-cell Vvi (36.8%) together with an increased rate of OS (high plasma TBARS levels) and an IR state (increased HOMA-IR) with compensatory hyperinsulinemia and higher HOMA-β. This hyperinsulinemia however, was not sufficient to cope with the IR state, since the animals had higher blood glucose (AUC) during the OGTT than C rats. All the changes registered in β-cell Vvi and function resemble those recorded in the pancreas of people with prediabetes [37–39].

Islets isolated from F rats had a higher GSIS, either measured using a dynamic or a static assay, together with increased GK activity (1.5 fold) and glucose metabolism (2.4 fold). These islets also showed an increased O^-_2 production (2.1 fold) and NADPH oxidase activity (4.9 fold), demonstrating the presence of a local OS that would depend, at least partly, on the higher activity of this enzyme complex. Thus, we could postulate the following association of events: the increased glucose phosphorylation by GK [40] increases glucose metabolism and, probably, NADPH production by the pentose-phosphate shunt [16, 41–43] leading to an increase of NADPH oxidase activity and ROS production. We cannot discard however, that the increased NADPH oxidase activity measured in F rats could also be partly conditioned by the higher 12-lipoxygenase activity and its reaction product (12-hydroxyeicosatetraenoic acid), with the consequent upregulation of NOX-1 in pancreatic β-cells [44].

Available evidence supports our assumption: inhibition of islet NADPH oxidase activity either by DPI or by the antisense oligonucleotide for p47 (PHOX) is accompanied by a decrease in glucose metabolism [45]. As shown in this and other studies reported by our group [14,17], apocynin co-administration prevented the development of the plurimetabolic alterations induced by fructose as well as those recorded in islet function. In fact, co-administration of apocynin to F rats prevented all the pancreas morphometric changes tested and induced a complete inactivation of islet NADPH oxidase with a parallel decrease of total O^-_2 production (47%), GK activity (26%) and static GSIS (23%), without changing the daily fructose consumption. Unexpectedly, in the dynamic assay apocynin markedly decreased GSIS in C animals (66%), mainly affecting the second secretory phase. This secretion pattern did not fit well neither with the effect induced by the inhibitor on serum insulin levels measured in vivo, nor with the recent publication by Koulajian et al. [46] in which they demonstrated that apocynin does not modify glucose homeostasis in control animals. However, the inhibitory effect of apocynin upon GSIS from islets isolated from C rats (CA group) was no longer observed when GSIS was measured in a static and longer incubation period. The uneven results recorded with apocynin in in vivo and in vitro conditions as well as in dynamics and static models could be explained by the fact that the dynamic model measures GSIS during a relatively short period (38 min) and the static

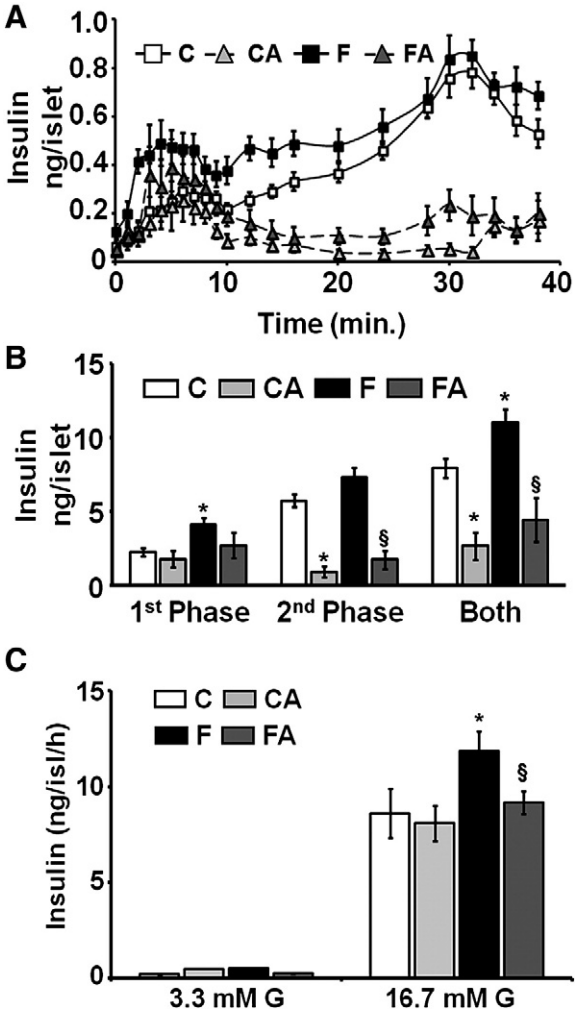

Fig. 4. Glucose stimulated insulin secretion. (A) Insulin secretion from perifused isolated rat islets with 16.7 mM glucose following a stabilization period with 3.3 mM glucose (not shown). (B) Total amount of insulin released (ng/islet) during the first and second phases of insulin secretion and the whole perifusion period with 16.7 mM glucose. Bars are means ± S.E.M. from 6 replicate samples. (C) Insulin secretion from isolated islets incubated for 60 min in the presence of 3.3 mM or 16.7 mM glucose (G). Values are expressed as ng/islet/hour (h). Bars are means ± S.E.M. from 3 experiments with 8 replicate samples each. *$p < 0.05$ compared with C; §$p < 0.05$ compared with F.

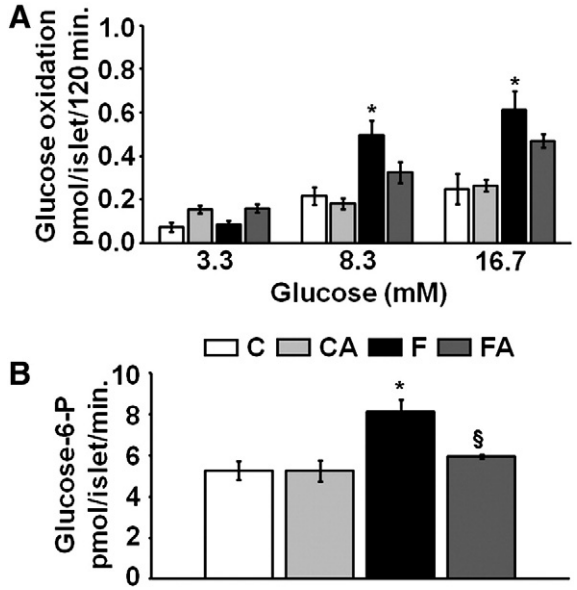

Fig. 5. Islet glucose metabolism and glucokinase activity. (A) Glucose oxidation measured as $^{14}CO_2$ produced from D-[U-^{14}C]-glucose in islets isolated from C, CA, F and FA rats. (B) Glucose phosphorylation activity of islet homogenates measured at 100 mM glucose (GK activity). In all cases, bars are means ± S.E.M. of 5 different experiments performed with islets isolated from each experimental group. *$p < 0.05$ compared with C; §$p < 0.05$ compared with F.

one does it for a longer time (60 min) allowing the process to get closer to the steady-state raised in the in vivo model.

It has been also reported that glucose increases intracellular accumulation of H_2O_2 and triggers insulin secretion in INS-1(832/13) cells and in isolated mouse islets, while exogenous H_2O_2 scavengers inhibit glucose-stimulated H_2O_2 accumulation and GSIS [47]. Other reports however, showed that islet ROS content decreases in the presence of high glucose concentrations [48–50]. These apparent controversial data as well as the one currently reported demonstrate that islet ROS content would play a key role in the fine tuning regulation of GSIS. It further suggests that a basal ROS level is necessary for the early β-cell response to glucose challenge (perifusion results with CA islets) but not when challenged for longer periods (longer static in vitro or in vivo model). In that context, our results suggest that NADPH oxidase is an important modulator of insulin secretion, mainly through the controlled production of ROS.

The short-term positive modulatory effect of ROS concentration and NADPH oxidase activity upon β-cell function, however, has been recently challenged by Li et al. [36], who found that NOX2 acts as a negative modulator of the β-cell secretory response in NOX2 knock-out mice. Although they claim that opposite data from other authors could be uncertain due to the lack of specificity of the inhibitors used (DPI or apocynin), they cannot explain their differences neither with models that used specific antisense oligonucleotide for p47PHOX [45] nor with the increased ROS concentration and NADPH oxidase activity measured in our F rats.

Concerning the claim that apocynin is not a specific NADPH oxidase inhibitor in view of its anti-inflammatory effects [51,52], its general ROS scavenger activity [36] and its recently reported capacity to inhibit other sources of superoxide anions in neurons [53], we cannot discard that some of the changes we found could be partially ascribed to such effects of the drug. Anyhow, apocynin has been used as an efficient inhibitor of the complex NADPH-oxidase in many experimental models involving phagocytic and nonphagocytic cells [54–57]. Consequently, the consistent decrease in ROS production and NADPH oxidase activity recorded both in vivo and in vitro plays more in favor of a specific effect rather than an artifact. The fact that the treatment of β-cells with apocynin or DPI reduced intracellular ROS production and β-cell dysfunction induced by pro-inflammatory cytokines in a dose-dependent manner [44], and that NADPH oxidase activity and glucose metabolism in the islets were inhibited by both DPI [58] and the antisense oligonucleotide for p47 (PHOX) [45] support this assumption.

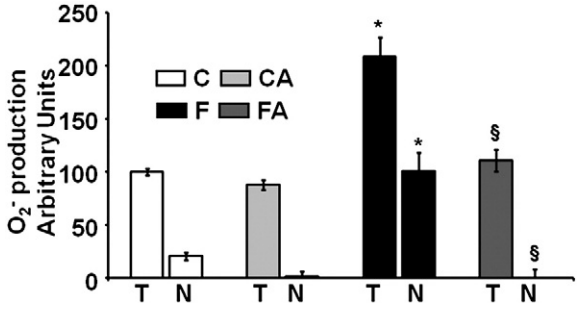

Fig. 6. Islet superoxide production and NADPH oxidase activity. Total O_2^- production (T) and O_2^- production depending on NADPH oxidase activity (N), measured in C, CA, F and FA rat islets. The last value was determined by subtracting the values of O_2^- production obtained in the presence of DPI to those measured in its absence. Bars are means ± S.E.M. from 4 different experiments. *$p < 0.05$ compared with C; §$p < 0.05$ compared with F.

Opposed to the current and previously reported evidence showing the enhancing effect of ROS production and NADPH oxidase activity upon insulin secretion, acute exposure of isolated mouse islets or INS-1(832/13) cells to different oxidative stressors decreased GSIS [20]. Taken together, these findings suggest a potential dual effect of OS upon β-cells: while an appropriate concentration of H_2O_2 derived from glucose metabolism seems to be essential for optimal GSIS [47,58], its increase, particularly in the long-run, would negatively affect β-cell function and mass [8,18,47].

In brief, we could assume that increased ROS production and plasma triacylglycerol levels induced by fructose administration trigger an IR state with a compensatory insulin hypersecretion (by affecting islet glucose metabolism), which was however insufficient to cope with the increased demand of the hormone, as demonstrated by the associated impaired glucose tolerance (IGT). Apocynin co-administration prevented the development of any change in β-cell mass, abolished islet NADPH oxidase activity and turned down the second phase of GSIS in both F and C rats. It also restored TBARS, insulin and triacylglycerol levels, HOMA indices, glucose metabolism and GK activity, and recovered IGT in F animals. Consequently, islet NADPH oxidase might play an important role in all the above-mentioned processes.

5. Conclusion

Changes recorded in β-cell Vvi and function of F rats are mediated, at least partly, through the modulation of NADPH oxidase activity. Since the metabolic dysfunctions and enhanced OS in F rats resemble those recorded in human prediabetes, such knowledge would help to develop appropriate strategies to prevent the progression of this metabolic state towards type 2 diabetes.

Author contribution

JJG conceived and designed the study. JJG and LEF evaluated the data obtained and drafted the manuscript. CLR, LEF, BM, HDZ and MAR handled the experimental animals, performed all the experimental work and carried out the statistical analyses. All authors read and approved the final manuscript.

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