Received: 21 December 2016 | Accepted: 13 March 2017

DOI: 10.1002/jcp.25908

ORIGINAL RESEARCH ARTICLE

WILEY Journal of Cellular Physiology

Reduced glucose-induced insulin secretion in low-protein-fed rats is associated with altered pancreatic islets redox status

Ana Paula G. Cappelli[1,2,3] | Claudio C. Zoppi[1] | Leonardo R. Silveira[1] | Thiago M. Batista[1] | Flávia M. Paula[1] | Priscilla M. R. da Silva | Alex Rafacho[1,4] | Helena C. Barbosa-Sampaio[1] | Antonio C. Boschero[1] | Everardo M. Carneiro[1]

[1] Department of Structural and Functional Biology, Cellular Biology and Physiology and Biophysics, Institute of Biology, University of Campinas (UNICAMP), Campinas, São Paulo, Brazil

[2] Laboratory of Experimental Physiology, Department of Physiological Sciences, Federal University of Maranhão (UFMA), São Luís, Maranhão, Brazil

[3] Department of Physiology and Biophysiology, Institute of Biomedical Sciences, University of Sao Paulo (USP), Sã, o Paulo, Brazil

[4] Department of Physiologic Sciences, Center of Biologic Sciences, Federal University of Santa Catarina (UFSC), Florianopolis, Santa Catarina, Brazil

Correspondence
Dr. Ana Paula Gameiro Cappelli, Department of Structural and Functional Biology, Institute of Biology, University of Campinas (UNICAMP), P.O. Box 6109, Campinas, SP. CEP: 13083-865, Brazil.
Email: apcappelli@gmail.com

Funding information
Fundação de Amparo à Pesquisa do Estado de São Paulo, Grant numbers: 06/58822-2, 07/50365-4; Conselho Nacional de Desenvolvimento Cientifico e Tecnológico (CNPq); Instituto Nacional de C&T: Obesidade e Diabetes

In the present study, we investigated the relationship between early life protein malnutrition-induced redox imbalance, and reduced glucose-stimulated insulin secretion. After weaning, male Wistar rats were submitted to a normal-protein-diet (17%-protein, NP) or to a low-protein-diet (6%-protein, LP) for 60 days. Pancreatic islets were isolated and hydrogen peroxide (H_2O_2), oxidized (GSSG) and reduced (GSH) glutathione content, CuZn-superoxide dismutase (SOD1), glutathione peroxidase (GPx1) and catalase (CAT) gene expression, as well as enzymatic antioxidant activities were quantified. Islets that were pre-incubated with H_2O_2 and/or N-acetylcysteine, were subsequently incubated with glucose for insulin secretion measurement. Protein malnutrition increased CAT mRNA content by 100%. LP group SOD1 and CAT activities were 50% increased and reduced, respectively. H_2O_2 production was more than 50% increased whereas GSH/GSSG ratio was near 60% lower in LP group. Insulin secretion was, in most conditions, approximately 50% lower in LP rat islets. When islets were pre-incubated with H_2O_2 (100 μM), and incubated with glucose (33 mM), LP rats showed significant decrease of insulin secretion. This effect was attenuated when LP islets were exposed to N-acetylcysteine.

KEYWORDS
antioxidant enzymes, insulin secretion, pancreatic islets, protein malnutrition, reactive oxygen species

1 | INTRODUCTION

Insulin release is known to be tightly coupled to ATP production. Briefly, the canonical theory of glucose-induced insulin secretion (GIIS) states that when blood glucose rises, islet β-cells stimulate ATP synthesis. ATP acts on ATP-dependent K^+ channels leading to membrane depolarization. This process is followed by opening of voltage-sensitive calcium channels; increasing cytosolic Ca^{2+} concentration and, consequently, insulin exocytosis (Aguilar-Bryan et al., 1995).

Although not yet fully understood, the discovery of ATP-independent K^+ channels mechanisms gave rise to several speculations about how glucose metabolism could regulate GIIS in addition to the solely ATP enhancement effect (Szollosi, Nenquin, Aguilar-Bryan, Bryan, & Henquin, 2007). In this sense, some pyruvate cycling pathways have been demonstrated to be coupled with insulin release and despite the well-knownmetabolic coupling factor (MCF) role displayed by ATP, emerging evidence points to other pyruvate cycling-induced MCFs production; such as NADPH, malonyl-CoA, glutamate and GTP, which in turn, might somehow control GIIS (Jitrapakdee, Wutthisathapornchai, Wallace, & MacDonald, 2010).

In addition, it was reported that GIIS is also regulated specifically by mitochondrial reactive oxygen species (ROS) production (Leloup et al., 2009), suggesting an important role of

β-cells intracellular redox state in this process. In agreement, it was reported that antioxidant enzymes including SOD and GPX were up regulated shortly after a glucose challenge (Oliveira, Curi, & Carpinelli, 1999), providing additive evidence for a GIIS redox modulation.

Protein malnutrition reduces insulin release in response to glucose and other secretagogues (Araujo et al., 2004; da Silva et al., 2010; Filiputti et al., 2008; Latorraca, Carneiro, Mello, & Boschero, 1999; Milanski et al., 2005). Our group reported reduced expression of signaling proteins such as, protein kinase A and protein kinase C in protein malnourished rats (Ferreira et al., 2003, 2004). Moreover, several genes involved in insulin production/secretion mechanisms have their expression altered as well (Delghingaro-Augusto et al., 2004). In addition, calcium uptake and insulin mRNA content were also reduced in undernourished rats (Carneiro et al., 1995; de Barros Reis et al., 2008; Latorraca et al., 1999). β-cells from rats fed with protein-deficient diet have decreased expression of protein kinase B/Akt, mechanistic target of rapamycin (mTOR), and the ribossomal p70S6 kinase (p70S6k) (Filiputti et al., 2010).

Similarly to obesity, intra-uterine and early life protein malnutrition leads to the development of metabolic syndrome and diabetes in adult life (Remacle et al., 2007) and this effect is attributed, among other factors, to islet mitochondrial malfunction-induced altered redox status (Theys, Clippe, Bouckenooghe, Reusens, & Remacle, 2009). Indeed, it was demonstrated that β-cells from fetal and adult rats fed with low protein diets are more susceptible to oxidative stress (Merezak et al., 2004; Merezak, Hardikar, Yajnik, Remacle, & Reusens, 2001). In this sense, it was recently reported increased ROS production in addition to reduced antioxidant enzymes gene expression and activity in offspring of intra-uterine low-protein-fed rats, predisposing pancreatic islets dysfunction (Tarry-Adkins, Chen, Jones, Smith, & Ozanne, 2010; Theys, Clippe, et al., 2009). However, the correlation among protein malnutrition-induced pancreatic islets redox imbalance and GIIS modulation has not been addressed.

Based on the above statement, we tested the hypothesis of altered intracellular redox status imposed by an isocaloric-low protein diet (6% of protein) would lead to impaired GIIS.

2 | MATERIALS AND METHODS

2.1 | Ethics statement

All the experiments described herein were approved by the State University of Campinas Committee for Ethics in Animal Experimentation and performed according to the "Principles of laboratory animal care" (NIH publication no. 85-23, revised 1985).

2.2 | Animals and diet

Soon after weaning male Wistar rats (21 day-old) from the breeding colony at UNICAMP were housed at 24°C on a 12 hr light/dark cycle. Rats were maintained in collective cages (five per cage), separated into two groups of isocaloric diets and treated for 8 weeks with: normo-protein diet (NP)—(17% protein) and low-protein diet (LP)—(6% protein) and water ad libitum. The two isocaloric diets were prepared according to AIN-93 guidelines (Reeves et al., 1993) and are detailed in Table 1.

2.3 | Whole blood and plasma measurements

Blood samples were collected from the tail in heparinized tubes in the day of euthanasia. Plasma was obtained by centrifugation at 1,800 rpm at 4°C, unless otherwise stated.After that, plasma samples were kept at −80°C until further analysis. Plasma total protein and albumin were measured using specific kit PROtal (Laborlab, Sao Paulo, SP, Brazil). Glycaemia was measured using the hand held glycosimeter Accu-Check Advantage II (Roche Ltd., Basel, Switzerland).

2.4 | Intraperitoneal glucose tolerance test and insulin tolerance test (ipGTT and ipITT)

IpGTT and ipITT were performed 1 week before euthanasia, as previously described (Rafacho, Cestari, Taboga, Boschero, Bosqueiro, 2009). Briefly, for the ipGTT and ipITT, rats were maintained in a fasted state for 12 hr. Fasting glucose was measured before 2 ml/kg glucose intraperitoneal injection, which was followed by glycaemia and insulinaemia measure at 15, 30, 60, 120, and 180 min after glucose infusion. For the ipITT, blood glucose was measured before insulin (0.75 U/kg) was administered intraperitoneally, and glycaemia was then measured at 2, 4, 8, and 12 min after insulin injection.

2.5 | Islets isolation

Islets were isolated by collagenase digestion of the pancreas in Hanks' balanced salt solution. Groups of approximately 300 freshly isolated islets were pelleted by centrifugation and then resuspended in 50–100 μl of homogenization buffer containing protease inhibitors, as previously described (Amaral et al., 2003) for enzyme activity assays. The islets were then sonicated for 15 s (Sonics & Materials, Newtown, CT), and total protein content was determined by the Bradford method.

2.6 | H_2O_2 and total glutathione measurement

Islets H_2O_2 production was measured using the Amplex Ultra Red probe (Invitrogen, Carlsbad, CA) according to manufacturers' instructions. Levels of GSSG and GSH were measured photometrically using the BIOXYTECH GSH/GSSG-412 kit (OxisResearch, Portland, OR) according to the manufacturer's guidelines.

2.7 | Pancreatic β-cell mass morphometry

To analyse β-cell mass, the endocrine pancreas was excised from four animals in each group and processed as previously described

TABLE 1 Detailed 17% normal protein (NP) and 6% low protein (LP) diets composition

Ingredient	Normal protein (17% protein) (g/kg)	Low protein (6% protein) (g/kg)
Casein (84% protein)	202.0	71.5a
Cornstarch	397.0	480.0a
Dextrinised cornstarch	130.5	159.0a
Sucrose	100.0	121.0a
Soybean oil	70.0	70.0
Fiber	50.0	50.0
Mineral mixb	35.0	35.0
Vitamin mixb	10.0	10.0
1-cystine	3.0	1.0
Choline chlorydrate	2.5	2.5

[a]Difference between the two isocaloric diets.
[b]For detailed composition see Reeves et al., 1993.

(Rafacho, Cestari, et al., 2009; Rafacho, Roma, Taboga, Boschero, & Bosqueiro, 2007). The relative and absolute β-cell mass as well as the islet number per pancreatic area were determined by point-counting morphometry on each pancreas section immunostained for insulin as previously reported (Rafacho, Cestari, et al., 2009; Rafacho et al., 2007). A minimum of 500 fields per pancreas were counted.

2.8 | RNA extraction and antioxidant enzymes quantitative real-time PCR

Freshly isolated islets were sonicated (Sonics & Materials, Newtown, CT) in TRIzol reagent (InVitrogen, São Paulo, SP, Brazil) for 30 s. After being cleared of debris by centrifugation at 6000 *g*, total RNA was isolated according to the manufacturer's guidelines and quantified by a spectrophotometer. The integrity of RNA was verified by agarose gel electrophoresis. Complementary DNA was prepared using 3 μg of total RNA and a reverse transcriptase. The genes analyzed and sequences of the primers are in Table 2. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a housekeeping gene. Real-time polymerase chain reaction was carried out in the StepOne thermocycler (Applied Biosystems, Foster City, CA). The polymerase chain reaction conditions were 95°C for 10 min, followed by 40 cycles at 95° for 10 s, and 60°C for 30 s. Real-time data were analyzed using the Sequence Detector System 1.7 (Applied Biosystems).

2.9 | Antioxidant enzymes activity assays

CAT activity was measured by monitoring the decrease in absorbance at 240 nm after the addition of 10 mM H_2O_2 (Aebi, 1984). SOD1 and GPx1 activities were measured photometrically as previously described (Winterbourn, Hawkins, Brian, & Carrell, 1975; Yoshikawa et al., 1993).

2.10 | Static insulin secretion

Groups of five islets were placed in 24 well plates containing 1 ml of Krebs-Hepes (KRBH) solution (pH 7.4) and pre-incubated with H_2O_2 0, 10, 100, 150, 200, 250, and 300 μM. In addition, islets were also pre-incubated with H_2O_2 150 μM plus NAC 10 mM. Perfusate solutions were gassed for 30 min with 95% O_2 and 5% CO_2 at 37°C. After that, islets were incubated with glucose 2.8, 8.3, 11, 22.2, or 33.3 mM for 60 min. After 60 min of incubation, supernatant was collected and insulin concentration was measured by radioimmunoassay (RIA) as previously described (Scott, Atewter, & Rojas, 1981).

2.11 | Statistical analysis

Results are expressed as means ± SEM. Statistical analyses were performed with help of Statistic 6.0 for Windows software, using Student-*t*-test and One-way ANOVA with student-Newman-Keuls post-hoc test. *p*-values <0.05 were considered statistically significant.

3 | RESULTS

3.1 | Body weight and blood analysis

As shown in Table 3, low protein fed rats (LP-6%) showed remarkable differences compared to normal protein fed control group (NP-17%). LP exhibited 50% lower body weight, an effect that was accompanied by reduced fast and fed total blood protein and blood albumin

TABLE 2 Real time-PCR primers used for mRNA quantification

mRNA	Genbank code	Primer sequences	Product size	Annealing temperature (°C)	Cycles
SOD1	NM_017050.1	Fw: 5′-TGAAGAGAGGCATGTTGGAGA-3′ Rv: 5′-TCATCTTGTTTCTCGTGGACC-3′	147	60	40
GPX1	NM_030826.3	Fw: 5′-CCCTCAAGTATGTCCGACCC-3′ Rv: 5′-GCAGGAAGGTAAAGAGCGGG-3′	105	60	40
CAT	NM_012520.1	Fw: 5′-GATGAAGCAGTGGAAGGAGC-3′ Rv: 5′-TGCCATCTCGTCGGTGAA A-3′	154	60	40
GAPDH	NM_017008	Fw: 5′-GGAGAAACCTGCCAAGTATGATG-3′ Rv: 5′-AACCTGGTCCTCAGTGTAGCCC-3′	97	60	40

Fw, forward; Rv, reverse.

TABLE 3 Values are presented as mean ± SEM for body weight (n = 10), fed total plasma protein (n = 9), fast total plasma protein (n = 5-6), fed albumin (n = 9), fast albumin (n = 10), fast glycaemia (n = 13), and fast insulin (n = 8–10)

	NP	LP
Body weight (g)	451.7 ± 12.173	262.7 ± 17.473*
Fed total protein (g/dl)	6,69 ± 0,21	5,78 ± 0,22*
Fast total protein (g/dl)	7,77 ± 0,33	6,55 ± 0,34*
Fed albumin (g/dl)	2,89 ± 0,05	2,72 ± 0,09*
Fast albumin (g/dl)	2,98 ± 0,10	2,65 ± 0,08*
Fast glucose (mg/dl)	69 ± 1.88	65.69 ± 2.00
Fast Insulin (ng/ml)	1,04 ± 0,12	0,71 ± 0,13*

Samples and body weight were obtained in the day of euthanasia.
*p < 0.05 (Student's-t-Test).

concentration. Fast blood insulin and glycaemia were not different between groups, however, LP showed a twofold improved glucose tolerance, as shown by ipGTT area under the curve values (Figure 1b), and reduced blood insulin observed during ipGTT (Figure 1d). The higher glucose tolerance showed by LP rats is in agreement with elevated insulin sensitivity reported by the ipITT, which showed a fourfold enhancement in kITT value (Figure 2a,b). Altogether, these results are in agreement with previous report from our group and others (Merezak et al., 2001, 2004; Rafacho, Cestari, et al., 2009; Remacle et al., 2007), showing reduced insulinaemia and enhanced insulin sensitivity, indicating that the protein undernourishment model employed here was effective.

3.2 | Antioxidant enzymes mRNA content and activity

After 60 days of protein malnourishment, it was detected altered mRNA content and activity specifically in superoxide dismutase (SOD) and catalase (CAT) in LP islets, whereas glutathione peroxidase (GPX) mRNA content and enzymatic activity remained unaltered (Figures 3 and 4a–c, respectively). LP CAT mRNA content showed twofold increase. SOD activity showed a significant higher activity as compared to NP islets (p < 0.05), and CAT activity was near 50% significantly lower than control values.

3.3 | H_2O_2 production and reduced-to-oxidized glutathione (GSH/GSSG) ratio

Islets H_2O_2 production was almost twofold higher in LP when incubated with both, basal (2.8 mM) and stimulating (33 mM) glucose concentrations (Figure 5a). In addition, GSH/GSSG was reduced by approximately 60% in LP as compared to NP, revealing altered redox status in LP group (Figure 5b).

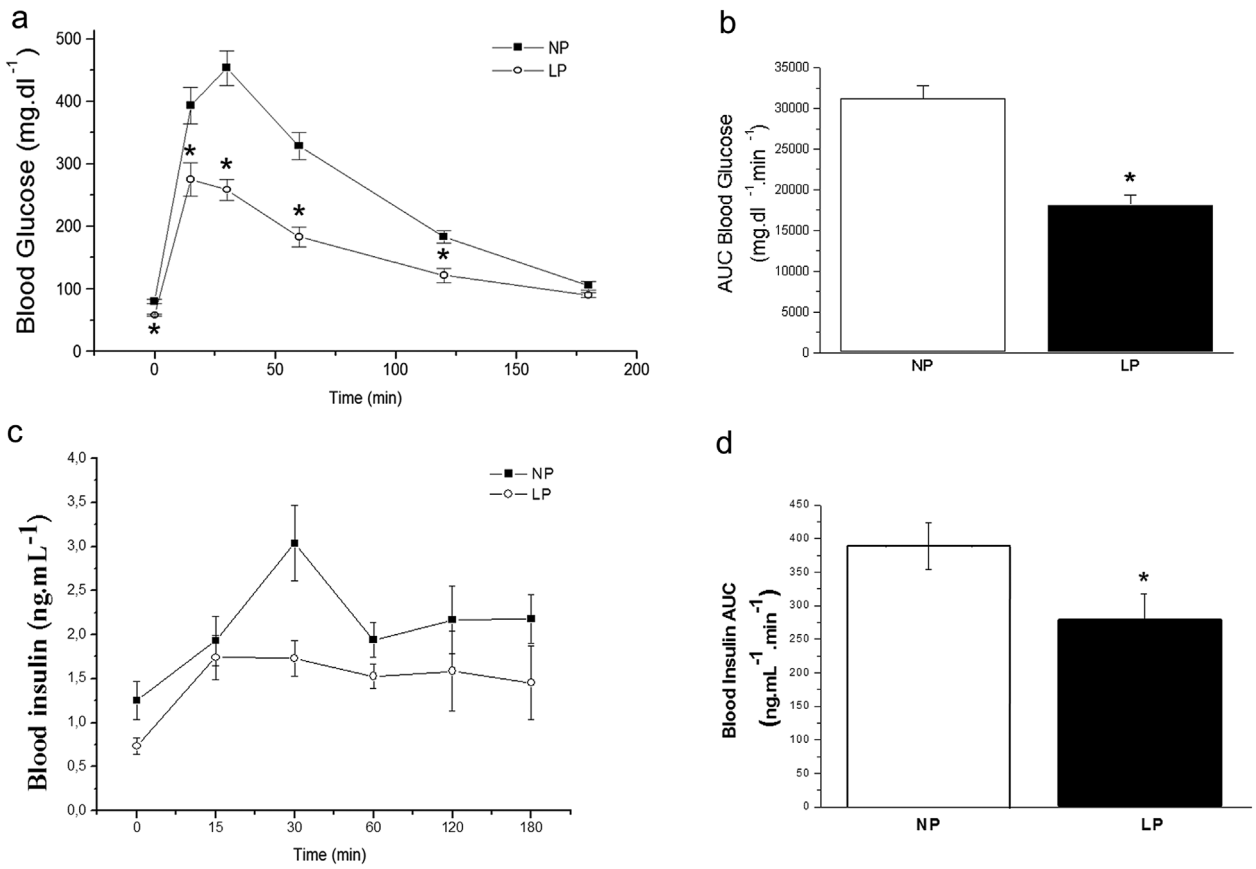

FIGURE 1 Intraperitoneal glucose tolerance test (ipGTT) glycaemia (a) and glycaemia area under the curve (AUC) (b), insulinaemia (c), and insulinaemia AUC (d) from control (NP, 17% protein) and low-protein-fed rats (LP, 6% protein). Values are presented as mean ± SEM (n = 10). *p < 0.05 (Student's-t-Test)

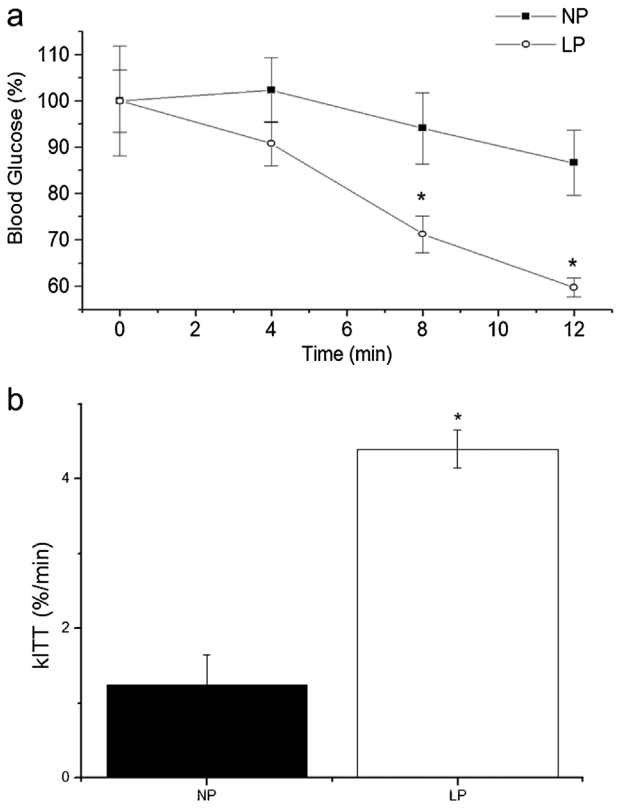

FIGURE 2 Intraperitoneal insulin tolerance test (ipITT) (a) and constant rate for blood glucose disappearance after intraperitoneal insulin administration (kITT) (b) from control (NP, 17% protein) and low-protein-fed rats (LP, 6% protein). Values are presented as mean ± SEM (n = 10). *p < 0.05 (Student's-t-Test)

3.4 | Decreased pancreatic β-cell mass in pancreas from LP rats

Morphometrical parameters revealed differences between LP and NP pancreatic islets. The total pancreas weight was significantly reduced in LP compared to NP rats (Figure 6a). Morphometric analysis revealed that the relative β-cell mass per pancreas area was similar in both groups (Figure 6b). However, a marked reduction in absolute β-cell mass was observed in LP compared to NP rats after correction by the pancreas weight (Figure 6c). As LP rats exhibited reduction of body weight, we next normalized the β-cell mass by the total body weight, which revealed a reduction in β-cell mass in LP compared to NP rats (Figure 6d). Finally, the number of islets per pancreatic area was similar in NP and LP rats (data not shown).

3.5 | Oxidant challenging conditions and GIIS

Similar to what was observed in vivo, isolated LP islets showed reduced GIIS. Thus, we investigated the effect of redox imbalance upon GIIS control in NP and LP. When NP and LP islets were pre-incubated with H_2O_2 (150 μM), and further incubated with glucose (22 and 33 mM), it was observed a decrease in the percent of insulin release in both groups compared to islets that were not pre-incubated with H_2O_2 (Figure 7).

Figure 8 shows the total insulin secretion, the percent to NP insulin secretion and the percent from maximum secreted insulin by each group

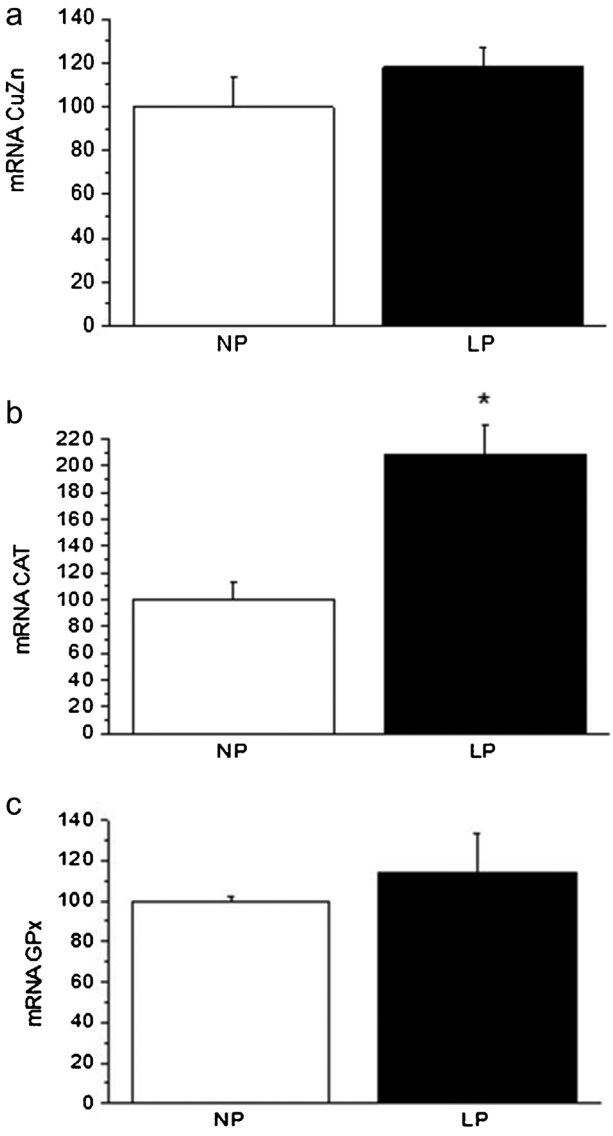

FIGURE 3 SOD1 (a), CAT (b), and GPX1 (c) messenger RNA (mRNA) in isolated islets from control (NP, 17% protein) and low-protein-fed rats (LP, 6% protein). Values are presented as mean ± SEM (n = 5–9) normalized by the internal control (GAPDH) from two independent experiments. *p < 0.05 (Student's-t-Test)

(a–c, respectively). When islets from NP and LP groups were pre-incubated with increased H_2O_2 concentrations and incubated with glucose 22 mM, it was observed that GIIS of both groups decreased, and when islets were pre-exposed to H_2O_2 concentrations above 150 μM, the GIIS returned to baseline levels (incubation with glucose 2.8 mM) (Figure 8a). Insulin secretion is lower in LP at all conditions, with an exception made to the condition of H_2O_2 300 μM (Figure 8b). GIIS fall profile was the same in NP and LP (Figure 8c). Based on these findings, which suggest no alterations between groups regarding redox status at each condition, we decided to enhance islets challenges and conducted the same experiments at a higher glucose concentration (33 mM).

Thus, when islets were pre-incubated with H_2O_2, and further challenged with glucose 33 mM, LP GIIS returned to baseline levels (incubation with glucose 2.8 mM) when it was previously pre-incubated with H_2O_2 100 μM, whereas NP showed the same

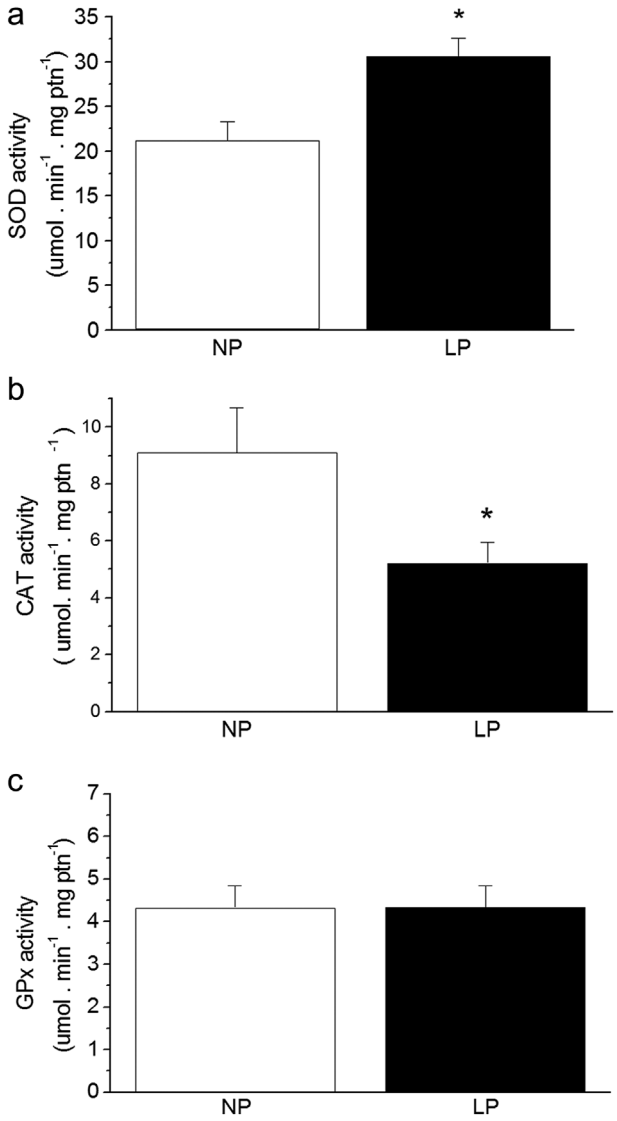

FIGURE 4 SOD1 (a), CAT (b), and GPX1 (c) activities in isolated islets from control (NP, 17% protein) and low-protein-fed rats (LP, 6% protein). Values are presented as mean ± SEM ($n = 4$). *$p < 0.05$ (Student's-*t*-Test)

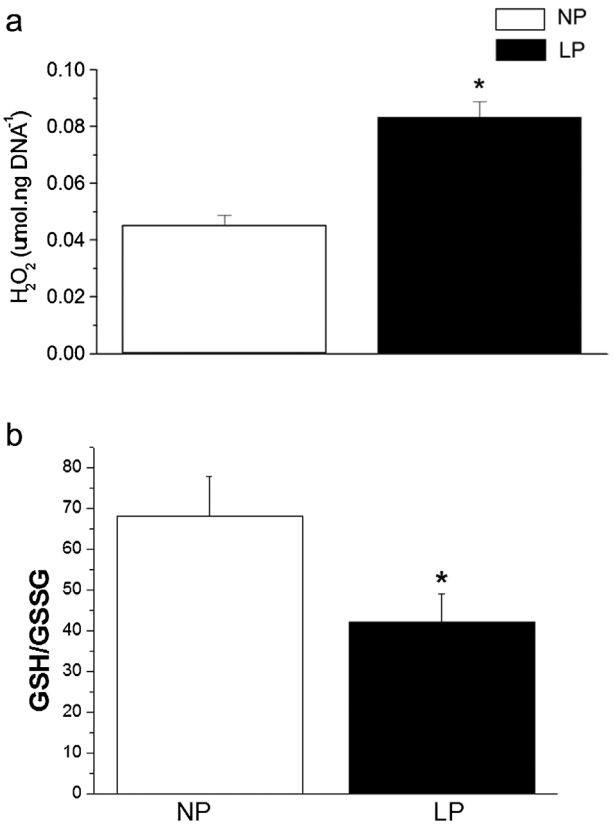

FIGURE 5 H_2O_2 production (a) and GSH-to-GSSG ratio (b) in isolated islets from control (NP, 17% protein) and low-protein-fed rats (LP, 6% protein). H_2O_2 concentration was normalized by islet DNA content, which was measured by SybrGreen method. Values are presented as mean ± SEM ($n = 5$). *$p < 0.05$ (Student's-*t*-Test) compared with respective control

behaviour only at H_2O_2 300 μM (Figure 9a). The absolute values of LP GIIS was lower than NP at all conditions as well (Figure 9b), and as showed by Figure 9c, LP insulin fall profile is more evident, reaching significant values ($p < 0.05$) already at H_2O_2 100 μM.

Interestingly, when the experiments were performed at the same condition above described, however, at absence of H_2O_2, NAC induced markedly GIIS reduction in both NP and LP groups, providing evidence of altered redox control of GIIS at this condition. The pre-incubation with NAC (10 mM) corroborates with the GIIS redox modulation proposal as it altered NP and LP insulin release (Figure 10). At conditions that did not induce GIIS curve returns to baseline levels, NAC reduced GIIS in both groups. However, at the condition that induced LP GIIS returns to baseline (pre-incubation with H_2O_2 150 μM plus incubation with glucose 33 mM), NAC significantly enhanced ($p < 0.05$) GIIS as compared to the same condition without H_2O_2 pre-incubation (Figure 11).

4 | DISCUSSION

Many studies with low-protein diet were performed after animal weaning, showing insulin secretion impairment in rodents (Batista et al., 2012; da Silva et al., 2012; Ferreira et al., 2003; Lippo et al., 2015; Rafacho, Giozzet, et al., 2009; Soriano et al., 2010). Soriano et al. (2010) showed that islets from LP mice exhibited a decreased level of coupling among beta-cells, and they suggested it was probably due to the low expression levels of connexin 36. It was shown that LP rats isolated islets presented reduced insulin secretion in response to increasing glucose concentrations, which was accompanied by a reduction in carbachol-induced internal Ca^{2+} mobilization (Batista et al., 2012). Those reports indicate that several alterations in the stimulus-secretion coupling of pancreatic β-cells might explain the diminished insulin secretion in this malnutrition state. Moreover, impaired GDH (glutamate dehydrogenase) function was associated with lower insulin release in LP islets, suggesting that metabolic enzymes could also be affected by LP treatment in rats (da Silva et al., 2012).

There is an extensive amount of data demonstrating the involvement of K^+_{ATP} channels in GIIS control. However, this mechanism does not explain the entire metabolic regulation of β-cells insulin secretion. Evidence of mitochondrial metabolism in the generation of MCF which may regulate insulin secretion is at constant growth (Jitrapakdee et al., 2010; Maechler,

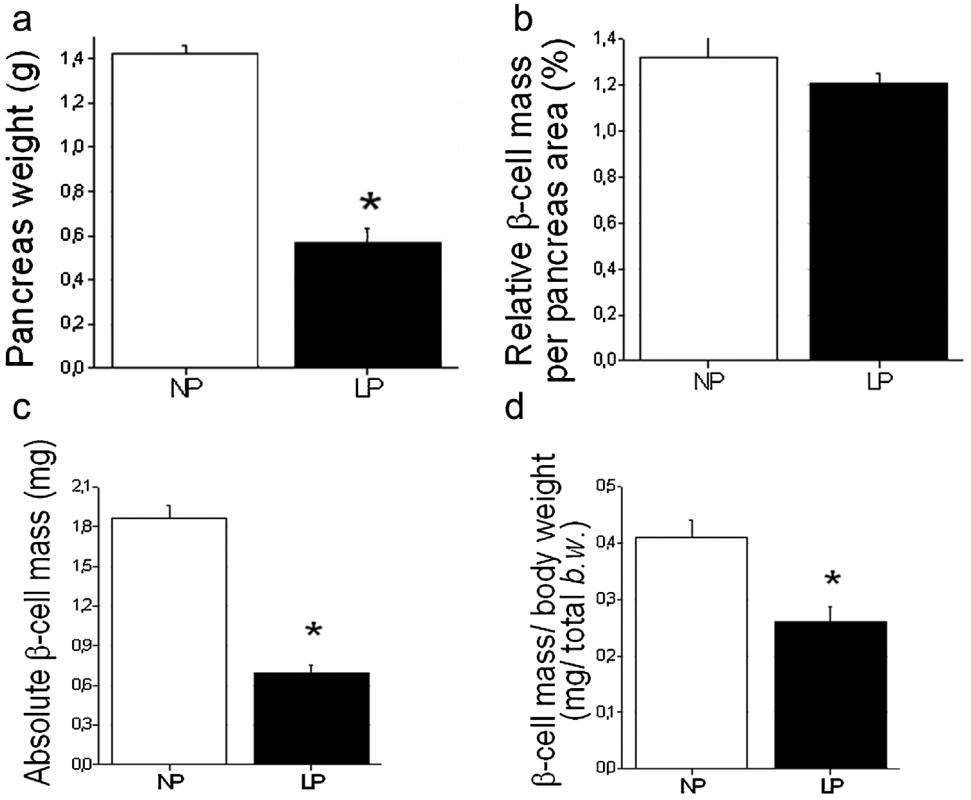

FIGURE 6 Pancreas weight (a), relative β-cell mass per pancreas area (b), absolute β-cell mass (c) and relative β-cell mass per total body weight (d) from control (NP, 17% protein), and low-protein-fed rats (LP, 6% protein). Values are presented as mean ± SEM (n = 4). *p < 0.05 (Student's-t-Test)

Carobbio, & Rubi, 2006). Redox imbalance and oxidative stress-induced β-cells dysfunction in conditions of fuel excess and glucolipotoxicity have been reported (Lenzen, 2008; Li, Frigerio, & Maechler, 2008). On the other hand, Leloup et al. (2009) provided direct evidence on physiological mitochondrial-produced ROS in GIIS control.

Protein malnutrition induces several β-cells alterations, leading to the reduction in insulin secretion. Nevertheless, information regarding redox status in post-weaning protein undernourished rats associated with GIIS control is still scarce. Intra-uterine protein malnutrition was reported to increase ROS and decrease ATP content (Theys, Clippe, et al., 2009). In agreement our findings show that post-weaning protein malnutrition also increases H_2O_2 production.

Despite findings showing increased SOD and GPX activities in response to a stimulating glucose challenge in regular fed rats (Oliveira et al., 1999), protein malnutrition alters antioxidant enzymes gene expression and activities, which may lead to impaired islet redox balance under this condition. In this sense, SOD and CAT seem to be the most sensible enzymes to protein malnutrition. Theys, Clippe, et al. (2009) reported enhanced SOD and reduced CAT activities, despite higher CAT mRNA content in islets of adult LP rats. In addition, it was also reported altered MnSOD and CuZnSOD gene expression in islets from offspring of protein malnourished dams (Tarry-Adkins et al., 2010). Most of our results agree with previous reports, showing altered SOD and CAT mRNA and activities, whereas the same variables regarding GPx remained relatively unaltered in post-weaning protein malnutrition. Reduced beta cell mass in LP adult rats was documented by Rafacho, Giozzet, et al. (2009), Swenne et al. (1992), and Tse et al. (1997), suggesting decreased beta cell mass regulation by LP diets. We believe that reduced beta cell mass could be involved with oxidative stress in LP rats. As previous reported, LP rat islets presented reduced FoxO1 phosphorylation (Rafacho, Giozzet, et al., 2009). FoxO1 was shown to modulate antioxidant enzymes mRNA content (Golson & Kaestner, 2016). These results corroborate with our data, where reduced beta cell mass was accompanied by increased catalase expression, probably as consequence of decreased FoxO1 phosphorylation in LP rats, leading to the higher mRNA levels of that antioxidant enzyme (Rafacho, Giozzet, et al., 2009).

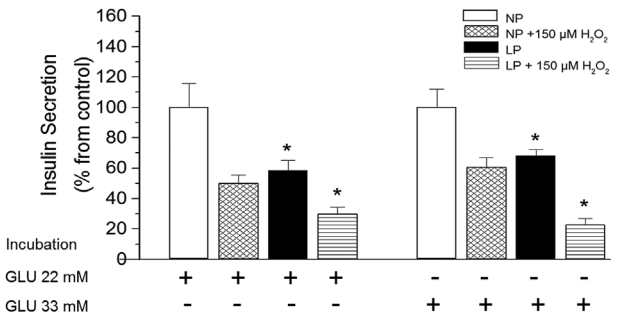

FIGURE 7 Isolated pancreatic islets static insulin secretion from control (NP, 17% protein) and low-protein-fed (LP, 6% protein) rats. Values are presented as mean ± SEM (n = 12-45). *p < 0.05 (Student's-t-Test) compared with respective control without H_2O_2 pre-incubation

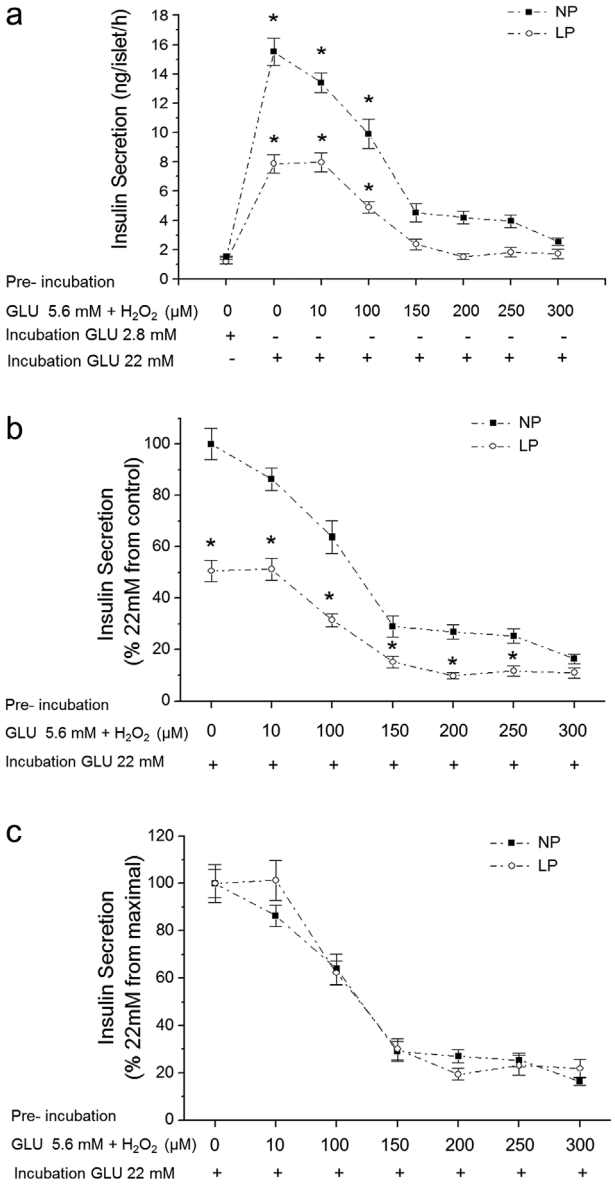

FIGURE 8 Isolated pancreatic islets absolute static insulin secretion, percent from control, and percent from maximal insulin secretion (a–c, respectively) in NP and LP rats. Values are presented as mean ± SEM (n = 7–20). *p < 0.05 (ANOVA one-way and student-Newman-Keuls post-hoc test) compared to glucose 2.8 mM condition from the same experimental group. #p < 0.05 (ANOVA one-way and student- Newman-Keuls post-hoc test) compared to NP

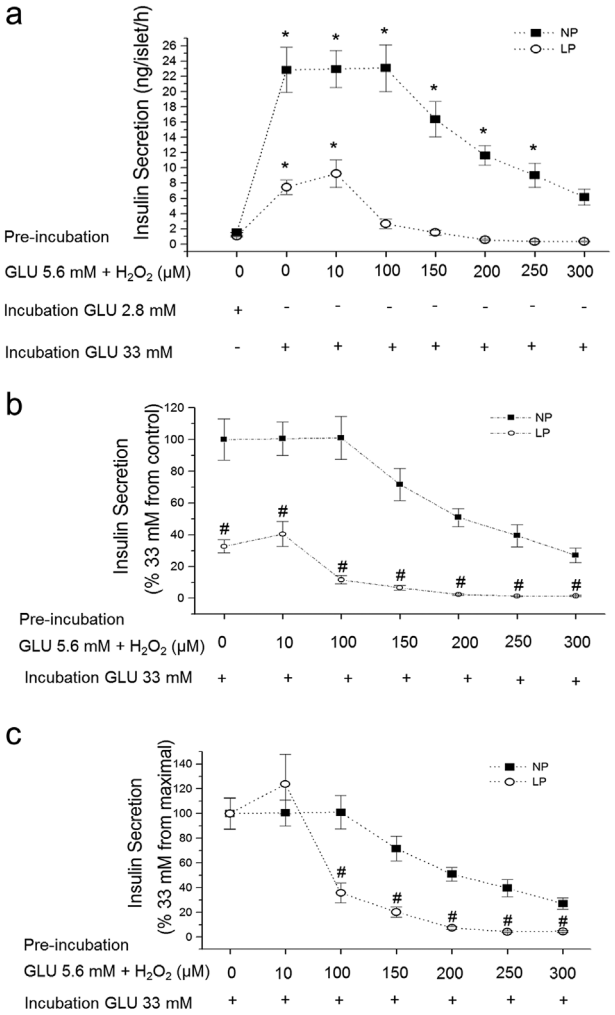

FIGURE 9 Isolated pancreatic islets static insulin secretion, percent from control, and percent from maximal insulin secretion (a–c, respectively) in NP and LP rats. Values are presented as mean ± SEM (n = 6–8). *p < 0.05 (ANOVA one-way and student-Newman-Keuls post-hoc test) compared to glucose 2.8 mM condition from the same experimental group. #p < 0.05 (ANOVA one-way and student- Newman-Keuls post-hoc test) compared to NP

As already reported to intra-uterine protein malnutrition, increased ROS production associated with reduced antioxidant enzymes activities induces pancreatic islets oxidative stress (Tarry-Adkins et al., 2010). Similarly, our protein malnutrition treatment in early life also induced islets to oxidative stress as demonstrated by reduced GSH/GSSG ratio. It was previously reported enhanced uncoupling protein 2 (UCP2) in protein malnourished rats (Theys, Bouckenooghe, Ahn, Remacle, & Reusens, 2009), probably occasioned by redox imbalance. Although UCP2 is thought to counteract to the increase in ROS production, it also reduces GIIS (Chan, Saleh, Koshkin, & Wheeler, 2004). Thus, the increased UCP2 expression might be a metabolic aspect associated with protein malnourishment-induced GIIS decrease.

Therefore, our model of post-weaning protein malnutrition, although apparently less severe than intra-uterine model, also presents imbalance between ROS production and its enzymatic antioxidant scavenger system. In addition, the reduction in GSH/GSSG reinforces this statement and provides evidence that early life protein malnutrition alters β-cells redox status, leading to oxidative stress.

The insulin secretion values reported by our results, showed near 50% reduction in LP GIIS, which is in agreement with other reports (Ferreira et al., 2003, 2004; Filiputti et al., 2010). In a recent review, we presented molecular and metabolic alterations induced by protein malnutrition such as: Reduced insulin mRNA, PDX-1 and protein kinases A and C expression, calcium influx, glucose oxidation, and anaplerotic

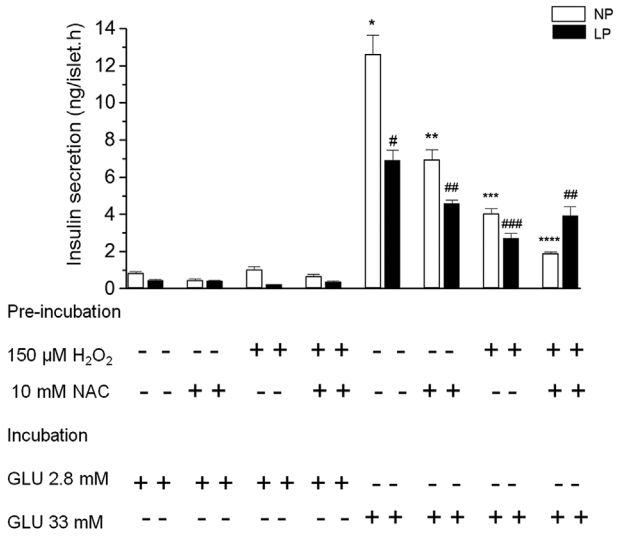

FIGURE 10 Isolated pancreatic islets static insulin secretion from control (NP, 17% protein) and low-protein-fed (LP, 6% protein) rats. Values are presented as mean ± SEM (n = 6–12). Different symbols indicate statistical significance ($p < 0.05$) (Student's-t-Test and ANOVA one-way with Student-Newman-Keuls post-hoc test) among different conditions

capacity, which, taken together, may partially explain the observed reduction in low-protein-fed state GIIS (Zoppi et al., 2010). However, the redox control of GIIS under protein undernourishment status remained unclear. It was demonstrated in INS-1 cell line and mice islets that, H_2O_2 is essential to stimulate GIIS (Pi et al., 2007). In addition, Leloup et al. (2009) demonstrated that the increase in GIIS at high glucose concentration is accompanied by a rise in mitochondrial ROS production and this production seems to be mandatory to insulin secretion signaling.

Based on the insulin secretion redox control and the reduction in CAT activity observed in our results, we investigated the effect of pre-exposing isolated pancreatic islets to H_2O_2, followed by the incubation with glucose upon GIIS profile from early life protein malnourished rats. In this sense, it was reported that H_2O_2 pre-incubation concentrations until 100 μM plus incubation with high glucose concentrations (22 or 33 mM), did not alter insulin secretion profile in NP and LP islets, evidencing that LP islets are able to handle even high oxidant challenges. However, pre-incubation with H_2O_2 (150 μM) followed by high glucose concentrations incubation reduced significantly GIIS as compared to intra-group glucose, but without H_2O_2 pre-incubation, showing imbalanced redox status in both NP and LP.

The observed increase in LP islets oxidative stress susceptibility at glucose 33 mM incubation might be due to a higher ROS production associated with this condition, in addition to the observed reduced H_2O_2 detoxifying capacity, which in turn, might alter redox status and impairs adequate GIIS signaling. Thus, it was observed that LP islets when pre-incubated with H_2O_2 100 μM, followed by the incubation with glucose 33 mM was enough to reduce GIIS to baseline values, whereas NP islets showed the same response only at H_2O_2 300 μM pre-incubation condition. In addition, GIIS decay was significantly different between groups, being LP fall more pronounced. Therefore, these data reveal that redox unbalance impairs protein malnourished rats' islets only at high oxidant challenges.

The higher LP susceptibility to redox unbalance is reinforced by islets insulin secretion data when pre-incubated with NAC. It was demonstrated that the pre-incubation with NAC (10 mM) reduced NP islets insulin secretion, whereas LP showed improved GIIS in a condition where it was previously abolished. The observed reduction in GIIS in both groups when pre-incubated with NAC suggests that when antioxidant capacity rises

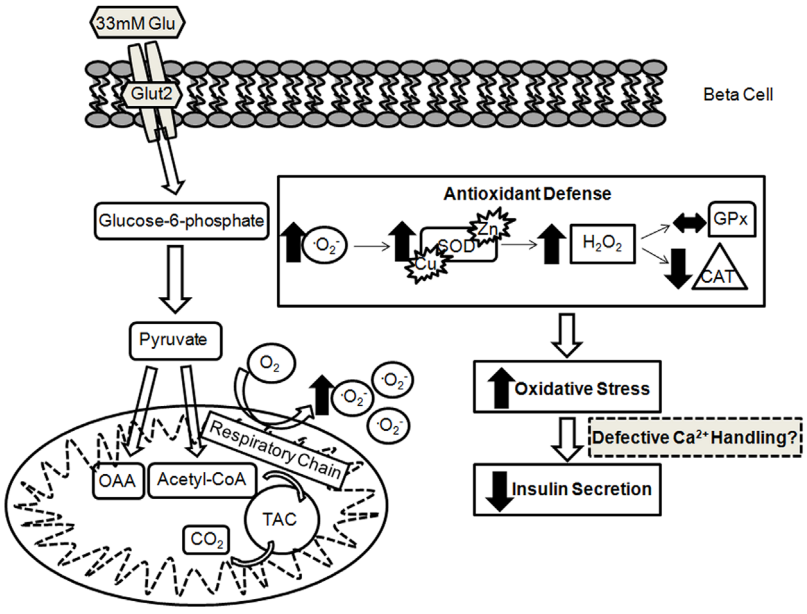

FIGURE 11 Schematic representation of protein malnutrition-induced redox balance alteration in pancreatic islets, reducing GIIS. Increased H_2O_2 production in addition to reduced antioxidant capacity might favor islet oxidative damage and somehow, compromise Ca^{2+} handling, which in turn, would impair GIIS. Filled arrows indicate positive, negativ,e or none alterations in concentration or function; empty arrows indicate the paths. Glu, glucose; Glut2, glucose transporter 2; OAA, oxaloacetate; TAC, tricarboxylic acid cycle

excessively, insulin secretion redox signaling is blunted. Otherwise, the attenuation observed in LP insulin secretion fall might be due to enhanced antioxidant capacity provided by NAC, ensuring adequate ROS content, coupling redox balance, and GIIS signaling maintenance.

Lower CAT activity, observed in our results, supports the statement that LP redox imbalance induced by the pre-incubation with H_2O_2 (150 μM), followed by the incubation with glucose (33 mM), might be due higher islets H_2O_2 production at this glucose concentration. Indeed, CAT have a pivotal role in H_2O_2 scavenging at high concentrations according to its lower affinity with H_2O_2 when compared to GPx (Eaton, 1991), which in turn, did not present altered activity in LP islets. Therefore, it suggests that while ROS production remained only at GPx detoxifying range, it was not observed alterations in GIIS profile between groups. However, when ROS production overwhelmed GPx capacity and reached CAT range, LP group demonstrated higher susceptibility to redox unbalance, leading to impaired GIIS.

In this sense, the possible link between redox status and GIIS might be Ca^{2+} metabolism. It was recently reported that Ca^{2+} oscillations were reduced in the presence of H_2O_2 and elevated glucose concentrations (Rebelato, Abdulkader, Curi, & Carpinelli, 2010). In addition, our group demonstrated that Ca^{2+} handling is altered in protein malnourished rats (Latorraca et al., 1999). Thus, based on previous evidences, we proposed that the link between metabolic redox status and GIIS alterations induced by protein malnutrition would be related to impaired Ca^{2+} handling.

In conclusion, our data suggest that short-term early life protein malnourishment induces reduction in redox GIIS signaling only at extreme oxidant challenges, elicited here by high H_2O_2 pre-incubation followed by elevated glucose concentration incubation, probably due to lower antioxidant scavenging capacity. However, the chronically lifetime effect of the reduced antioxidant capacity induced by protein deficient diets, leading to adult life β-cells dysfunction, deserves further investigations, and follow-up studies are required to answer the remaining questions.

ACKNOWLEDGMENTS

Fundação de Amparo a Pesquisa do estado de São Paulo (FAPESP), Conselho Nacional de Desenvolvimento Cientifico e Tecnológico (CNPq) and Instituto Nacional de C&T: Obesidade e Diabetes grants are acknowledged here for the financial support.

CONFLICTS OF INTEREST

The authors declared that they have no conflicts of interest.

REFERENCES

Aebi, H. (1984). Catalase in vitro. *Methods Enzymology*, *105*, 121–126.

Aguilar-Bryan, L., Nichols, C. G., Wechsler, S. W., 4th, Clement, J. P., 3rd, Boyd, A. E., González, G., … Nelson, D. A. (1995). Cloning of the beta cell high-affinity sulfonylurea receptor: A regulator of insulin secretion. *Science*, *268*, 423–426.

Amaral, M. E., Ueno, M., Carvalheira, J. B., Carneiro, E. M., Velloso, L. A., Saad, M. J., & Boschero, A. C. (2003). Prolactin-signal transduction in neonatal rat pancreatic islets and interaction with the insulin-signaling pathway. *Hormone Metabolism Research*, *35*, 282–289.

Araujo, E. P., Amaral, M. E., Filiputti, E., De Souza, C. T., Laurito, T. L., Augusto, V. D., … Carneiro, E. M. (2004). Restoration of insulin secretion in pancreatic islets of protein-deficient rats by reduced expression of insulin receptor substrate (IRS)-1 and IRS-2. *Journal of Endocrinology*, *181*, 25–38.

Batista, T. M., Ribeiro, R. A., Amaral, A. G., de Oliveira, C. A., Boschero, A. C., & Carneiro, E. M. (2012). Taurine supplementation restores glucose and carbachol-induced insulin secretion in islets from low-protein diet rats: Involvement of Ach-M3R, Synt 1 and SNAP-25 proteins. *Journal of Nutritional Biochemistry*, *23*(3), 306–312.

Carneiro, E. M., Mello, M. A. R., Gobatto, C. A., & Boschero, A. C. (1995). Low protein diet impairs glucose-induced insulin secretion from and 45 Ca uptake by pancreatic rat islets. *Journal of Nutritional Biochemistry*, *6*, 314–318.

Chan, C. B., Saleh, M. C., Koshkin, V., & Wheeler, M. B. (2004). Uncoupling protein 2 and islet function. *Diabetes*, *53*, S136–S142.

da Silva, P. M., Batista, T. M., Ribeiro, R. A., Zoppi, C. C., Boschero, A. C., & Carneiro, E. M. (2012). Decreased insulin secretion in islets from protein malnourished rats is associated with impaired glutamate dehydrogenase function: Effect of leucine supplementation. *Metabolism: Clinical and Experimental*, *61*(5), 721–732.

da Silva, P. R. M., Zoppi, C. C., Filiputti, E., Silveira, L. R., Quesada, I., Boschero, A. C., & Carneiro, E. M. (2010). Preliminary report: Leucine supplementation enhances glutamate dehydrogenase expression and restores glucose-induced insulin secretion in protein malnourished rats. *Metabolism: Clinical and Experimental*, *59*, 911–913.

de Barros Reis, M. A., Arantes, V. C., Cunha, D. A., Latorraca, M. Q., Toyama, M. H., Carneiro, E. M., & Boschero, A. C. (2008). Increased L-CPT-1 activity and altered gene expression in pancreatic islets of malnourished adult rats: A possible relationship between elevated free fatty acid levels and impaired insulin secretion. *Journal of Nutritional Biochemistry*, *19*, 85–90.

Delghingaro-Augusto, V., Ferreira, F., Bordin, S., do Amaral, M. E., Toyama, M. H., Boschero, A. C., & Carneiro, E. M. (2004). A low protein diet alters gene expression in rat pancreatic islets. *Journal of Nutrition*, *134*, 321–327.

Eaton, J. W. (1991). Catalases and peroxidases and glutathione and hydrogen peroxide: Mysteries of the bestiary. *Journal of Laboratory and Clinical Medicine*, *118*, 3–4.

Ferreira, F., Barbosa, H. C. L., Stoppiglia, L. F., Delghingaro-Augusto, V., Pereira, E. A., Boschero, A. C., & Carneiro, E. M. (2004). Decreased insulin secretion in islets from rats fed a low protein diet is associated with a reduced PK A alpha expression. *Journal of Nutrition*, *134*, 63–67.

Ferreira, F., Filiputti, E., Arantes, V. C., Stoppiglia, L. F., Araújo, R. P., Delghingaro-Augusto, V., … Carneiro, E. M. (2003). Decreased cholinergic stimulation of insulin secretion by islets from rats fed a low protein diet is associated with reduced protein kinase C alpha expression. *Journal of Nutrition*, *133*, 695–699.

Filiputti, E., Ferreira, F., Souza, K. L., Stoppiglia, L. F., Arantes, V. C., Boschero, A. C., & Carneiro, E. M. (2008). Impaired insulin secretion and decreased expression of the nutritionally responsive ribosomal kinase protein S6K-1 in pancreatic islets from malnourished rats. *Life Sciences*, *82*, 542–548.

Filiputti, E., Rafacho, A., Araujo, E. P., Silveira, L. R., Trevisan, A., Batista, T. M., … Carneiro, E. M. (2010). Augmentation of insulin secretion by leucine supplementation in malnourished rats: Possible involvement of PI3K/mTOR pathway. *Metabolism: Clinical and Experimental*, *59*, 635–644.

Golson, M. L., & Kaestner, K. H. (2016). Fox transcription factors: From development to disease. *Development*, *143*, 4558–4570.

Jitrapakdee, S., Wutthisathapornchai, A., Wallace, J. C., & MacDonald, M. J. (2010). Regulation of insulin secretion: Role of mitochondrial signaling. *Diabetologia*, *53*, 1019–1032.

Latorraca, M. Q., Carneiro, E. M., Mello, M. A., & Boschero, A. C. (1999). Reduced insulin secretion in response to nutrients in islets from

malnourished young rats is associated with a diminished calcium uptake. *Journal of Nutritional Biochemistry*, *10*, 37–43.

Leloup, C., Cuzin, C. T., Magnan, C., Leloup, C., Cuzin, C. T., Magnan, C., … Pénicaud, L. (2009). Mitochondrial reactive oxygen species are obligatory signals for glucose-Induced insulin secretion. *Diabetes*, *58*, 673–681.

Lenzen, S. (2008). Oxidative stress: The vulnerable beta-cell. *Biochemical Society Transaction*, *36*, 343–347.

Li, N., Frigerio, F., & Maechler, P. (2008). The sensitivity of pancreatic beta-cells to mitochondrial injuries triggered by lipotoxicity and oxidative stress. *Biochemical Society Transactions*, *36*, 930–934.

Lippo, B. R., Batista, T. M., de Rezende, L. F., Cappelli, A. P., Camargo, R. L., Branco, R. C., … Ferreira, F. (2015). Low-protein diet disrupts the crosstalk between the PKA and PKC signaling pathways in isolated pancreatic islets. *Journal of Nutritional Biochemistry*, *26*(5), 556–562.

Maechler, P., Carobbio, S., & Rubi, B. (2006). In beta-cells, mitochondria integrate and generate metabolic signals controlling insulin secretion. *International Journal of Biochemistry and Cellular Biology*, *38*, 696–709.

Merezak, S., Hardikar, A. A., Yajnik, C. S., Remacle, C., & Reusens, B. (2001). Intrauterine low protein diet increases fetal beta-cell sensitivity to NO and IL-1 beta: The protective role of taurine. *Journal of Endocrinology*, *171*, 299–308.

Merezak, S., Reusens, B., Renard, A., Goosse, K., Kalbe, L., Ahn, M. T., … Remacle, C. (2004). Effect of maternal low-protein diet and taurine on the vulnerability of adult Wistar rat islets to cytokines. *Diabetologia*, *47*, 669–675.

Milanski, M., Arantes, V. C., Ferreira, F., de Barros Reis, M. A., Carneiro, E. M., Boschero, A. C., … Latorraca, M. Q. (2005). Low-protein diets reduce PKAalpha expression in islets from pregnant rats. *Journal of Nutrition*, *135*, 1873–1878.

Oliveira, H. R., Curi, R., & Carpinelli, A. R. (1999). Glucose induces an acute increase of superoxide dismutase activity in incubated rat pancreatic islets. *American Journal of Physiology*, *276*, C507–C510.

Pi, J., Bai, Y., Zhang, Q., Wong, V., Floering, L. M., Daniel, K., … Collins, S. (2007). Reactive oxygen species as a signal in glucose-stimulated insulin secretion. *Diabetes*, *56*, 1783–1791.

Rafacho, A., Cestari, T. M., Taboga, S. R., Boschero, A. C., & Bosqueiro, J. R. (2009). High doses of dexamethasone induce increased beta-cell proliferation in pancreatic rat islets. *American Journal of Physiology*, *296*, E681–E689.

Rafacho, A., Giozzet, V. A., Boschero, A. C., Abrantes, J. L., Cestari, T. M., Carneiro, E. M., & Bosqueiro, J. R. (2009). Reduced pancreatic beta-cell mass is associated with decreased FoxO1 and Erk1/2 protein phosphorylation in low-protein malnourished rats. *Brazilian Journal of Medical and Biological Research*, *42*(10), 935–941.

Rafacho, A., Roma, L. P., Taboga, S. R., Boschero, A. C., & Bosqueiro, J. R. (2007). Dexamethasone-induced insulin resistance is associated with increased connexin 36 mRNA and protein expression in pancreatic rat islets. *Canadian Journal of Physiology and Pharmacology*, *85*, 536–545.

Rebelato, E., Abdulkader, F., Curi, R., & Carpinelli, A. R. (2010). Low doses of hydrogen peroxide impair glucose-stimulated insulin secretion via inhibition of glucose metabolism and intracellular calcium oscillations. *Metabolism: Clinical and Experimental*, *59*, 409–413.

Reeves, P. G., Nielsen, F. H., & Fahey, G. C., Jr (1993). AIN-93 purified diets for laboratory rodents: Final report of the American Institute of Nutrition ad hoc writing committee on the reformulation of the AIN-76A rodent diet. *Journal of Nutrition*, *123*, 1939–1951.

Remacle, C., Dumortier, O., Bol, V., Goosse, K., Romanus, P., Theys, N., … Reusens, B. (2007). Intrauterine programming of the endocrine pancreas. *Diabetes Obesity and Metabolis*, *9*, 196–209.

Scott, A. M., Atewter, I., & Rojas, E. (1981). A method for the simultaneous measurement of insulin release and B cell membrane potential in single mouse islets of Langerhans. *Diabetologia*, *21*, 470–475.

Soriano, S., Gonzalez, A., Marroquí, L., Tudurí, E., Vieira, E., Amaral, A. G., … Quesada, I. (2010). Reduced insulin secretion in protein malnourished mice is associated with multiple changes in the beta-cell stimulus-secretion coupling. *Endocrinology*, *151*(8), 3543–3554.

Swenne, I., Borg, L. A., Crace, C. J., & Schnell, L. A. (1992). Persistent reduction of pancreatic beta-cell mass after a limited period of protein-energy malnutrition in the young rat. *Diabetologia*, *35*, 939–945.

Szollosi, A., Nenquin, M., Aguilar-Bryan, L., Bryan, J., & Henquin, J. C. (2007). Glucose stimulates Ca2+ influx and insulin secretion in 2-week-old beta-cells lacking ATP-sensitive K+ channels. *Journal of Biological Chemistry*, *282*, 1747–1756.

Tarry-Adkins, J. L., Chen, J. H., Jones, R. H., Smith, N. H., & Ozanne, S. E. (2010). Poor maternal nutrition leads to alterations in oxidative stress, antioxidant defense capacity, and markers of fibrosis in rat islets: Potential underlying mechanisms for development of the diabetic phenotype in later life. *FASEB Journal*, *24*, 2762–2771.

Theys, N., Bouckenooghe, T., Ahn, M. T., Remacle, C., & Reusens, B. (2009). Maternal low protein diet alters pancreatic islet mitochondrial function in a sex-specific manner in the adult rat. *American Journal of Physiology*, *297*, R1516–R1525.

Theys, N., Clippe, A., Bouckenooghe, T., Reusens, B., & Remacle, C. (2009). Early low protein diet aggravates unbalance between antioxidant enzymes leading to islet dysfunction. *PLoS ONE*, *4*(7), e6110.

Tse, E. O., Gregoire, F. M., Reusens, B., Remacle, C., Hoet, J. J., & Johnson, P. R. (1997). Changes of islet size and islet size distribution resulting from protein-malnutrition in lean (Fa/Fa) and obese (fa/fa) Zucker rats. *Obesity Research*, *5*, 563–571.

Winterbourn, C. C., Hawkins, R. E., Brian, M., & Carrell, R. W. (1975). The estimation of red cell superoxide dismutase activity. *Journal of Laboratory and Clinical Medicine*, *85*, 337–341.

Yoshikawa, T., Naito, Y., Kishi, A., Tomii, T., Kaneko, T., Iinuma, S., … Kondo, M. (1993). Role of active oxygen, lipid peroxidation, and antioxidants in the pathogenesis of gastric mucosal injury induced by indomethacin in rats. *Gut 34*:732–737.

Zoppi, C. C., Silveira, L. R., Oliveira, C. A. M., Boschero, A. C., Curi, R., & Carneiro, E. M. (2010). Insulin release, peripheral insulin resistance and muscle contraction capacity in protein malnutrition: A role of tricarboxylic acid cycle anaplerosis. *British Journal of Nutrition*, *103*, 1237–1250.

How to cite this article: Cappelli APG, Zoppi CC, Silveira LR, et al. Reduced glucose-induced insulin secretion in low-protein-fed rats is associated with altered pancreatic islets redox status. *J Cell Physiol*. 2018;233:486–496. https://doi.org/10.1002/jcp.25908