



Early protein restriction increases intra-islet GLP-1 production and pancreatic β -cell proliferation mediated by the β -catenin pathway

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Abstract

Purpose In the present study, we investigated whether intra-islet GLP-1 production and its modulation have a role in apoptosis, proliferation or neogenesis that is compromised by protein restriction during the foetal and suckling periods.

Methods Exendin-4, a GLP-1 receptor agonist (treated groups), or saline (non-treated groups) was intraperitoneally administered for 15 days from 75 to 90 days of age in female adult rats consisting of offspring born to and suckled by mothers fed a control diet (control groups) and who had the same diet until 90 days of age or offspring born to and suckled by mothers fed a low-protein diet and who were fed the control diet after weaning until 90 days of age (protein-restricted group).

Results The β -cell mass was lower in the protein-restricted groups than in the control groups. Exendin-4 increased β -cell mass, regardless of the mother's protein intake. The colocalization of GLP-1/glucagon was higher in the protein-restricted rats than in control rats in both the exendin-4-treated and non-treated groups. The frequency of cleaved caspase-3-labelled cells was higher in the non-treated protein-restricted group than in the non-treated control group and was similar in the treated protein-restricted and treated control groups. Regardless of treatment with exendin-4, Ki67-labelled cell frequency and β -catenin/DAPI colocalization were elevated in the protein-restricted groups. Exendin-4 increased the area of endocrine cell clusters and β -catenin/DAPI and FoxO1/DAPI colocalization regardless of the mother's protein intake.

Conclusions Protein restriction in early life increased intra-islet GLP-1 production and β -cell proliferation, possibly mediated by the β -catenin pathway.

Keywords Early protein restriction · GLP-1 · β -Cell proliferation · β -Catenin · Exendin-4 · Islets

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Introduction

Protein restriction at critical developmental phases, such as during intrauterine life and lactation, causes morphological adaptations that continue to be expressed even with the recovery of nutritional status and results in a predisposition to the development of type-2 diabetes mellitus (T2DM) in adult life [1]. β -Cell mass is maintained by the balance between production (replication, neogenesis and transdifferentiation) and death (apoptosis, necrosis and autophagy), as well as by the number and size of β -cells [2, 3]. Replication refers to the expansion of preexisting adult β -cells through cell division, whereas neogenesis comprises β -cell differentiation from progenitors within the pancreas [4]. Proteins expressed at certain times in the cell cycle, such as Ki67, have been used as cell replication markers [2]. For neogenesis identification, the criterion most commonly used is islet hormone-positive cells that seem to be budding from the pancreatic duct epithelium or tiny clusters (one to three cells) of scattered insulin-positive cells [5]. Apoptosis is a cellular process characterized by membrane blebbing, cell shrinkage, condensation of chromatin, and fragmentation of DNA followed by rapid engulfment of the corpse by neighbouring cells [6]. The identification of highly condensed and/or fragmented nuclei (apoptotic bodies) with DNA dyes, such as propidium iodide, is a classic morphological method for apoptosis detection [2]. Another marker for apoptosis is cleaved caspase-3, the main effector caspase of the apoptotic cascade within cells [7]. The two mechanisms of β -cell formation from the embryo, neogenesis or differentiation from ductal precursor cells, and replication of a differentiated β -cell, are maintained postnatally and even in adult life [4]. In humans with T2DM and in a mouse model of T2DM, apoptosis is the major cause of β -cell death [8]. In adult rats that recover from protein restriction in the early stages of life, a reduction in β -cell mass is attributed to low proliferative capacity and elevated apoptosis [9].

Glucagon-like peptide-1 (GLP-1) is an important incretin hormone that increases β -cell mass by stimulating replication and neogenesis, as well as by reducing apoptosis by activation of several signalling pathways see [10]. Several transcription factors and signalling molecules are involved in the anti-apoptotic and proliferative actions of GLP-1. In MIN6 cells (a line cell of β -cells) exposed to hydrogen peroxide, activation of GLP-1 receptor (GLP-1R) by exendin-4 inhibits apoptosis, and this effect depends on 3',5'-cyclic adenosine monophosphate (cAMP) and phosphoinositide 3-kinase (PI3K) in association with the upregulation of β -cell lymphoma-2 (Bcl-2) [11]. Moreover, GLP-1 inhibits forkhead box transcription factor (Fox) subclass 1 (FoxO1), a transcriptional

effector of insulin signalling and insulin-like growth factor 1, promoting its phosphorylation and consequent nuclear exclusion. Inhibition of FoxO1 increases the expression of pancreatic and duodenal homeobox 1 (PDX-1), with antiapoptotic, neogenic and proliferative effects [12]. The Wnt signalling pathway, initially discovered for its role in tumourigenesis, has as its key effector the bipartite transcription factor β -cat/TCF, formed by free β -catenin (β -cat) and a TCF protein, such as transcription factor 7-like 2 (TCF7L2) [13]. Exendin-4 stimulates the Wnt/ β -cat/TCF7L2 pathway through cAMP/PKA signalling and increases cyclin D1 transcription, contributing to β -cell proliferation. Finally, GLP-1 induces the expression of transcription factors through the β -catenin signalling pathway, contributing to an increase in β -cell mass [14–16].

In intestinal L cells, GLP-1 is produced from the cleavage of proglucagon by the enzyme prohormone convertase 1/3 (PC1/PC3), whereas in pancreatic α -cells, proglucagon is cleaved into glucagon by the action of prohormone convertase 2 (PC2) [17]. In normal pancreatic α -cells, little or no PC1/PC3 is detected [18–21]. However, in stress conditions or during β -cell regeneration, PC1/PC3 expression is upregulated in α -cells [19–22]. There is a significant increase in PC1/PC3 and GLP-1 expression in α -cells from diabetic rats [20], in murine models of insulin resistance [*ob/ob*, *db/db*, NOD (nonobese diabetic)] and in situations typically associated with islet cell expansion (pregnancy or neonatal stage) [19]. The increased GLP-1 expression in islets under stress and/or from β -cell damage suggests that this hormone produced in the islet contributes to cell survival [16, 22–24]. Furthermore, the positive regulation of GLP-1 in α -cells that are undergoing expansion (pregnancy and neonatal phases) or regeneration (*ob/ob*, *db/db*, NOD) models of β -cells suggests that GLP-1 produced in pancreatic islet cells promotes cell proliferation [10, 25].

We have shown that female and male rats that are protein-restricted during intrauterine and lactation phases have a monophasic pattern of insulin secretion [26, 27]. However, in female rats, the first phase of insulin secretion is preserved and potentiated, while in males, it is abolished. Interestingly, GLP-1 potentiates the first phase of insulin secretion. Thus, we evaluated whether the intra-islet production of GLP-1 and its modulator effect on apoptosis, proliferation and neogenesis were altered by early protein restriction. Because the effects of GLP-1 are limited due its short half-life (1–2 min) as result of cleavage by the enzyme dipeptidyl peptidase 4 (DPP-4), we used a GLP-1R agonist, exendin-4, which is resistant to the action of this enzyme and is already used in the treatment of diabetic patients.

Material and methods

The experimental procedures were performed in accordance with the guidelines of the Brazilian Society for Science in Laboratory Animals (SBCAL) and were approved by the Ethics Committee at the Federal University of Mato Grosso (protocol number: 23108.015308/14-1).

Animals and diets

Male ($n=6$) and virgin female ($n=25$) Wistar rats (85–90 days old) were obtained from the Cuiabá University breeding colony. Pairing was performed by housing males with females overnight (1 male to 4 females), and pregnancy was confirmed based on the examination of vaginal smears for the presence of sperm. Pregnant females were separated at random and maintained from the first day of pregnancy until the end of lactation on isocaloric diets containing either 17% (control diet) or 6% protein (low-protein diet), constituting control (C, $n=12$ rats) and protein-restricted (PR, $n=13$ rats), respectively. Spontaneous delivery took place on day 22 of pregnancy, after which, at 3 days of age, large litters were reduced to eight pups to ensure a standard litter size per mother. At weaning (4 weeks after birth), female pups were divided into two groups: C, consisting of offspring born to and suckled by mothers fed a control diet and who were subsequently fed the same diet after weaning until 90 days of age; and PR, consisting of the offspring of mothers fed a protein-restricted diet and who were subsequently fed the control diet after weaning until 90 days of age. In this phase, rats were maintained in collective cages (three or four rats/cage).

The diets were isocaloric, as described in Table 1. Food intake was recorded three times/week during the experimental period. The pre-weighed diet was provided, and after 48 h, rats were briefly removed from their cages. The amount of food remaining, including any food on the bottom of the cages, was recorded. Food intake was calculated as the weight (in grams) of diet provided less that recovered. It is expressed in absolute or relative values.

Body weight was recorded at 3 and 28 days of age, and after weaning, the measurement of body weight was performed once a week.

At 75 days of age, the rats were divided into four groups according to treatment with the GLP-1 receptor agonist and the mother's protein intake: non-treated control (NTC, $n=11$ rats), consisting of rats receiving daily saline injections (0.9% sodium chloride solution); treated control (TC, $n=13$ rats), consisting of rats treated with the GLP-1 receptor agonist; non-treated protein restricted (NTPR, $n=13$ rats), consisting of rats from the protein-restricted

Table 1 Composition of the control and low-protein diets (g/kg)

Ingredient	Control diet (17% protein)	Low-protein diet (6% protein)
Casein (84% protein)	202.0	71.5
Cornstarch	397.0	480.0
Dextrinizedcornstarch	130.5	159.0
Sucrose	100.0	121.0
Soybeanoil	70.0	70.0
Fiber	50.0	50.0
Mineral mix (AIN-93G)	35.0	35.0
Vitaminmix (AIN-93G)	10.0	10.0
L-Cystine	3.0	1.0
Cholinechlorhydrate	2.5	2.5

See Reeves et al. [45] for more details

group receiving daily saline injections; and treated protein-restricted (TPR, $n=15$ rats), consisting of rats from the protein-restricted group treated with the GLP-1 receptor agonist. Throughout the treatment, rats were housed alone in a cage.

During the experimental period, rats had free access to food and water and were housed at 22 °C with a 12 h light/dark cycle.

Treatment

In the TC and TPR groups, the GLP-1 receptor agonist exendin-4 (Sigma-Aldrich, USA, E7144) was administered intraperitoneally at a dose of 0.3 µg/kg (1.1 mL/kg body weight) dissolved in 0.9% saline solution. The NTC and NTPR groups received 0.3 µg/kg (1.1 mL/kg body weight) of 0.9% saline solution. This procedure was performed daily at 5:00 pm for a period of 15 days.

Oral glucose-tolerance test (OGTT)

After a 12 h fast, glucose (200 g/L) was administered orally at a dose of 2 g/kg of body weight. Blood samples were obtained from the cut tip of the tail 0, 15, 30, 60 and 120 min later for the determination of blood glucose using an Accu-Chek glucometer and serum insulin concentration by ELISA. The stimulation index was calculated as the insulin concentration 15 min after glucose load divided by the insulin concentration before the glucose load.

Euthanasia, collection of biological material, biochemical and hormonal analyses

In the present study, we used two cohorts of rats. One was for the oral glucose tolerance test, measurement of pancreatic

insulin content and histological procedures. Another cohort of animals was used for real-time PCR. A representative sample of each cohort was used for serum biochemical analyses.

One day after the oral glucose tolerance test, rats were euthanized after a 12 h fast for collection of biological material. Initially, rats were narcotized in a CO₂ chamber and euthanized by decapitation. The pancreas was removed, dissected, and weighed for morphometric and immunohistochemical analyses or for measurement of insulin content. Pancreatic tissue was homogenized using buffer containing 20 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA and 1% Triton X 100 and stored at – 80 °C [25]. Samples of pancreas homogenate were diluted (1:10,000) for insulin determination. Pancreatic insulin was analysed by specific enzyme-linked immunoassays (Insulin Total ELISA, EZRMI-13K, Millipore, USA) following the manufacturer's instructions. All commercial assay kits have been shown to exhibit a high degree of specificity. For real-time PCR, the pancreases were removed, dissected and digested with collagenase.

Blood samples were collected in tubes without additives and without anticoagulants (BD Vacutainer, Becton and Dickinson, Brazil) and were centrifuged for 20 min at 2465 ×g. The serum was separated and stored at – 80 °C for the subsequent measurement of urea, alanine transaminase (ALT), aspartate transaminase (AST), and alkaline phosphatase (ALP) using commercial kits (Bioclin/Quibasa, Brazil).

Histological processing

Five rats from each group were used for the histological studies. Pancreatic tissues were fixed in 4% paraformaldehyde solution for 24 h at 4 °C, dehydrated in increasing concentrations of ethanol, clarified in xylol, and embedded in paraffin using a histological processor (MTP 100 Slee, Mainz, Germany). Tissue was serially sectioned at 3 μm thickness throughout its length using a microtome (Leica RM2125, Leica Biosystems Nussloch, Germany) and mounted on slides with an adhesive surface. Ten pancreatic sequential sections were deparaffinized in an oven at 60 °C for 2 h, followed by immersion in xylol and descending concentrations of ethanol. After, the slides were rehydrated and stained with haematoxylin and eosin for determination of pancreatic areas using a light microscope at 5× magnification (Axio Scope. A1, Carl Zeiss, Oberkochen, Germany). Pancreatic areas were used for determination of β- and α-cell masses as described previously [28]. For immunofluorescence, after antigen retrieval at 70 °C for 1 h using citrate buffer (pH 6.0), the sections were blocked using 5% albumin serum and incubated overnight at 4 °C with primary antibodies. In the same section were simultaneously

used (1) mouse monoclonal anti-Ki67 antibody (Dako, Carpinteria, USA; M7249; 1:50), guinea pig polyclonal anti-insulin antibody (Dako, Carpinteria, USA; A0564; 1:200) and rabbit polyclonal anti-glucagon antibody (Dako, Carpinteria, USA; A0565; 1:200); (2) mouse monoclonal anti-PC2 antibody (Santa Cruz Biotechnology Inc., Santa Cruz, USA; SC-374140; 1:100) and rabbit polyclonal anti-glucagon antibody; (3) mouse monoclonal anti-PC1, 3 antibody (Abcam plc, England; ab55543, 1: 33) and rabbit polyclonal anti-glucagon antibody; (4) mouse monoclonal anti-GLP-1 antibody (Santa Cruz Biotechnology Inc., Santa Cruz, USA; SC-80602; 1:200) and rabbit polyclonal anti-glucagon antibody; (5) rabbit polyclonal anti-GLP-1R antibody (Thermo Fisher Scientific, USA; PA5-72433, 1:100) and guinea pig polyclonal anti-insulin antibody; or (6) mouse monoclonal anti-β-catenin antibody (Abcam plc, England; ab6301, 1:100) and rabbit polyclonal anti-glucagon antibody. Rabbit monoclonal anti-FoxO1 antibody (Cell Signaling Technology, USA; #2880S, 1:100), mouse monoclonal anti-PDX-1 antibody (Santa Cruz Biotechnology Inc., Santa Cruz, USA; sc-390792, 1:120) and rabbit polyclonal anti-cleaved caspase-3 antibody (Cell Signaling Technology, USA; #9661S, 1:200) were used in sections separately. The sections were subsequently incubated for 1 h at room temperature with an appropriate secondary antibody (Alexa Fluor AF-555 goat anti-mouse, Alexa Fluor 647 goat anti-guinea pig IgG, or Alexa Fluor 488 goat anti-rabbit. Thermo Fisher Scientific, USA). 4',6-Diamidine-2'-phenylindole dihydrochloride (DAPI) (Sigma-Aldrich, USA, D9564, 1:3000) was used as a cell nucleus marker. The antibody dilution and methods were previously tested and validated in our laboratory.

Propidium iodide (PI) was used to identify morphologically altered nuclei, which are indicative of cell death, as previously described [29]. To this end, deparaffinized and rehydrated pancreas sections were incubated for 40 min in a dark, humidified chamber in a solution of PI (Sigma-Aldrich, USA; P4170, 20 μg/mL) and ribonuclease A (Sigma-Aldrich, USA; R6513, 100 μg/mL). The labelled apoptotic nuclei of endocrine cells in the pancreas sections were counted in digitized islet images.

At least 10 fields from each section were photographed to make morphometric measurements using an Axio Scope A1 microscope (Carl Zeiss, Germany). Image capture and analysis were performed by a blinded observer.

Morphometry

For determination of the islet and β-cell areas, entire pancreatic sections stained for insulin were imaged at 20× magnification using an Axio Scope A1 microscope (Carl Zeiss, Germany) equipped with a motorized stage. A tile image of the tissue section was generated using AxioVision version 4.8.2

software (Carl Zeiss). The areas of the pancreas stained positive for insulin were digitally quantified with a colour-based threshold using AxioVision software. The α -cell area was obtained by subtracting the β -cell area from the total islet area. The α - and β -cell relative areas were calculated by the ratio between the α - and β -cell areas and the pancreas area. The islet relative area was obtained by the ratio between the islet area and the total pancreatic tissue area and multiplying the result by 100. The α - and β -cell masses were calculated by multiplying the total β -cell volume (the product of the sum of the entire α - or β -cell and the total pancreas area) by the pancreas weight. The sizes of the β -cells were obtained by dividing the β -cell area by the number of β -cells. It is important to emphasize that this method overestimates size because the actual number of cells is probably greater than the number counted since not all cells are sectioned in their nuclei [30].

The endocrine cell proliferation frequency was assessed by dividing the total number of endocrine cells by the number of Ki67-positive endocrine cells in one islet and multiplying the result by 100. The ratio between proliferation and α - or β -cell mass was obtained by dividing the frequency of Ki67-positive endocrine cells by the α - or β -cell mass.

The index of apoptotic cells was obtained by dividing the number of apoptotic nuclei labelled with PI or apoptotic cells labelled with cleaved caspase-3 in one islet by the total number of endocrine cells in one islet and multiplying the result by 100. The ratio between apoptosis and α - or β -cell mass was obtained by dividing the frequency of cells labelled with cleaved caspase-3 by the α - or β -cell mass. The ratio between proliferation and apoptosis in endocrine cells was obtained by dividing the frequency of Ki67-positive endocrine cells by the number of endocrine cells labelled with cleaved caspase-3.

GLP-1 fluorescence was measured using AxioVision 4.9.1 software (Carl Zeiss). Colocalization analysis was carried out using the “JACoP” plugin in ImageJ and expressed as Pearson’s correlation coefficient. Pearson’s correlation coefficient measures the pixel-by-pixel covariance in the signal levels of two images, subtracting the mean intensity from each pixel’s intensity value. Hence, Pearson’s correlation coefficient is independent of signal levels and signal offset (background) [31]. A value near or equal to -1.0 represents no colocalization, while a value near or equal to 1.0 represents full colocalization of two antigens. Data from five to seven sections were averaged for each pancreas.

Islet isolation

The pancreas was removed from each rat and digested with collagenase (Boehringer Mannheim). Briefly, the pancreas was inflated with Hanks solution containing 0.7–0.9 g/L collagenase and then excised and maintained at 37 °C for

20 min. The tissue was harvested, and the islets were hand-picked under a stereomicroscope for quantitative real-time PCR. Pancreatic islet pools were used; each contained approximately 300 islets and was obtained from two rats.

RNA extraction and quantitative real-time PCR

Total RNA from 300 islets was extracted using TRIzol® (Invitrogen, Brazil) and homogenized for 1 min using a vortex. Total RNA was isolated and quantified using a spectrophotometer. Complementary DNA was prepared using 3 μ g of total RNA and reverse transcriptase and transcribed into cDNA using a TaqMan mRNA reverse transcription kit (Applied Biosystems, LTU, Ref: 4368814). The primers used in the experiments were standard TaqMan primers (Applied Biosystems, USA). GAPDH (glyceraldehyde-3-phosphate dehydrogenase) mRNA was used as an endogenous control (TaqMan Rodent GAPDH Control Reagents, Ref: 4352338E). The genes analysed were GLP-1R (GenBank Rn00562406_m1), Bcl-2 (GenBank Rn99999125-m1), caspase 3 (GenBank Rn00563902-m1) and Pdx-1 (GenBank Rn00755591-m1). Real-time polymerase chain reaction was carried out in a StepOne polymerase chain reaction cycler (Applied Biosystems, USA). The polymerase chain reaction conditions were 95 °C for 10 min, followed by 45 cycles at 95 °C for 10 s and 60 °C for 45 s. Real-time data were analysed using a Sequence Detector System 1.7 (Applied Biosystems, USA). Relative quantitation of gene expression was performed by comparing the efficiency of amplification of each gene of interest using the Δ Ct method, as described by the manufacturer (Applied Biosystems, USA). The results are expressed as fold inductions compared with endogenous controls.

Statistical analysis

The number of rats used was determined by the resource equation method [32]. The results are expressed as the means \pm SD for the number of indicated rats (n). Levene’s test for the homogeneity of variances was initially used to check the fit of the data to parametric assumptions. The Shapiro–Wilk test was used to test data normality. When necessary, data were transformed to correct for variance heterogeneity. Body weight at birth, cumulative food intake during the recovery phase, PC1/PC3/glucagon colocalization and PC2/glucagon colocalization from the C and R groups were compared with the unpaired t test. Changes in body weight during lactation and during the growth phase after weaning, as well as blood glucose and serum insulin concentration curves, were evaluated by two-way repeated-measures ANOVA. A two-way ANOVA (effects of nutritional status and exendin-4 treatment) was used to evaluate the *in vivo* data, mRNA expression, biochemical and hormonal data,

and histological and morphometric data. The relative frequency distribution of islet size was evaluated and classified as follows: small islets-area $\leq 10,000 \mu\text{m}^2$; medium islets-area $> 10,000$ and $\leq 25,000 \mu\text{m}^2$; and large islets-area $> 25,000 \mu\text{m}^2$. A three-way ANOVA was used to evaluate the effects of the factors tested (nutritional status (NS), exendin-4 treatment and islet size classification (Clas)) using a customized model (Clas, NS \times Clas, exendin-4 \times Clas, NS \times exendin-4 \times Clas). When necessary, these analyses were followed by Tukey's test to determine the significance of individual differences. The level of significance was set at

$P < 0.05$. The data were analysed using the Statistica software package (Statsoft).

Results

At 3 days of age, the body weights of the PR and C groups were similar, and at 28 days of age, both groups had higher body weights than at 3 days ($P < 0.0001$). However, the body weight of the PR group was lower than that of the C group at 28 days of age ($P < 0.0001$) (Fig. 1a). During

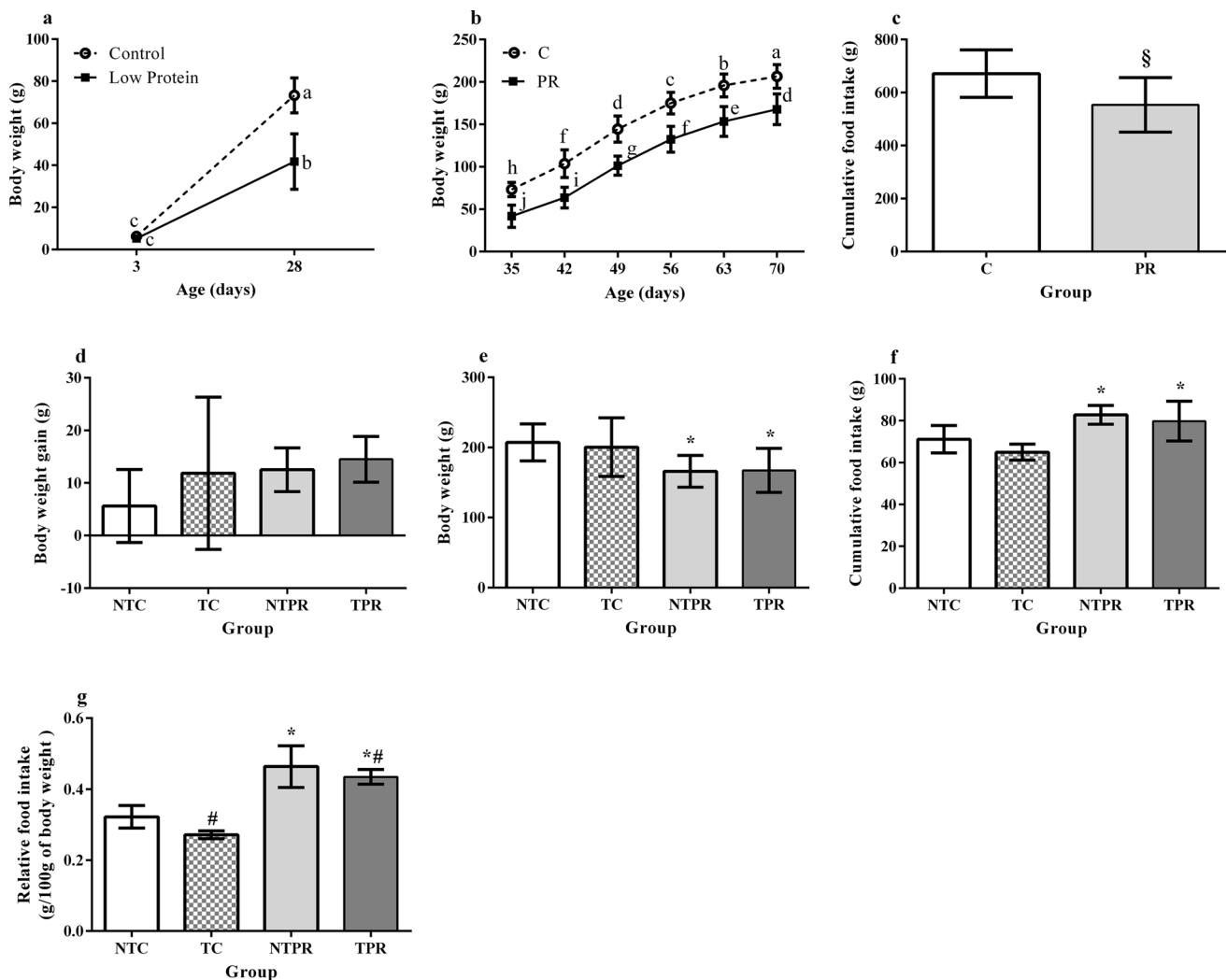


Fig. 1 **a** Changes in body weight during lactation of offspring born to and suckled by mothers fed a control ($n=20$ rats) or a low-protein diet ($n=26$ rats). **b** Changes in body weight and **c** cumulative food intake during the growth phase after the weaning of offspring born to and suckled by mothers fed a control diet and who were subsequently fed the same diet after weaning (C group, $n=20$ rats) or of offspring born to and suckled by mothers fed a low-protein diet and who were subsequently fed a control diet after weaning (PR group, $n=26$ rats). **d** Body weight gain, **e** body weight at 90 days age, **f** cumulative food

intake, and **g** relative food intake of non-treated and treated rats from the control (NTC group, $n=5$; TC group, $n=5$) and protein-restricted groups (NTPR group, $n=5$; TPR group, $n=5$). Values are the mean with standard deviation. *Indicates effect of nutritional status (two-way ANOVA, $P < 0.05$). #Indicates effect of treatment (two-way ANOVA, $P < 0.05$). §Indicates a significant difference (independent samples t test, $P < 0.05$). Different superscript letters denote significant differences (Tukey's test $P < 0.05$)

the phase of growth after weaning, the PR group showed a lower body weight compared to the C group at all times evaluated (Fig. 1b). The cumulative food intake from 28 days until 75 days was significantly lower in the PR than in the C rats ($P < 0.05$) (Fig. 1c). During the 15 days treatment with exendine-4 or saline, body weight gain did not differ among groups (Fig. 1d). At 90 days of age, protein-restricted rats showed lower body weight compared to control rats ($F_{1,42} = 15.91, P < 0.0003$) (Fig. 1e). In this phase, the cumulative food intake was significantly higher in the protein-restricted groups than in the control groups ($F_{1,16} = 20.81, P < 0.0003$) (Fig. 1f). Food intake relative to 100 g body weight was higher in the protein-restricted groups compared to the control groups ($F_{1,16} = 92.6, P < 0.00001$) but lower in the treated groups compared to the non-treated groups ($F_{1,16} = 6.33, P < 0.02$) (Fig. 1g).

The basal blood glucose and serum insulin concentrations did not differ among the groups (Fig. 2a–d). The blood

glucose concentration was similar in all groups after a glucose load (Fig. 2a, b). 15 min after the glucose load, the serum insulin concentration was similar in the NTPR and TPR groups but was higher in the TC group than in the NTC group (Fig. 2c, d). The stimulation index was significantly higher in the TC group than in the NTC group ($P < 0.03$) and in both groups this variable did not differ to the NTPR and TPR groups (Fig. 2e).

The ALP, AST and ALT concentrations were lower in the treated groups compared to the non-treated groups ($F_{1,16} = 5.90, P < 0.0273; F_{1,15} = 5.98, P < 0.02; F_{1,16} = 9.70, P < 0.0067$, respectively). The urea concentration did not differ among groups (Table 2).

The absolute pancreas weight was lower in the protein-restricted groups than in the control groups ($F_{1,16} = 10.79, P < 0.0047$). The pancreas weight relative to 100 g of body weight did not differ among the groups. The pancreatic insulin content per gram of pancreas and the content per

Fig. 2 **a** Blood glucose concentration curves of non-treated and treated rats from the control groups and **b** of non-treated and treated rats from the protein-restricted groups. **c** Serum insulin concentration curves of non-treated and treated rats from the control groups and **d** of non-treated and treated rats from the protein-restricted groups. **e** Stimulation index obtained from the glucose oral tolerance test of non-treated and treated rats from the control or protein-restricted groups. ^{a,b}Mean values are significantly different (Tukey’s test, $P < 0.05$)

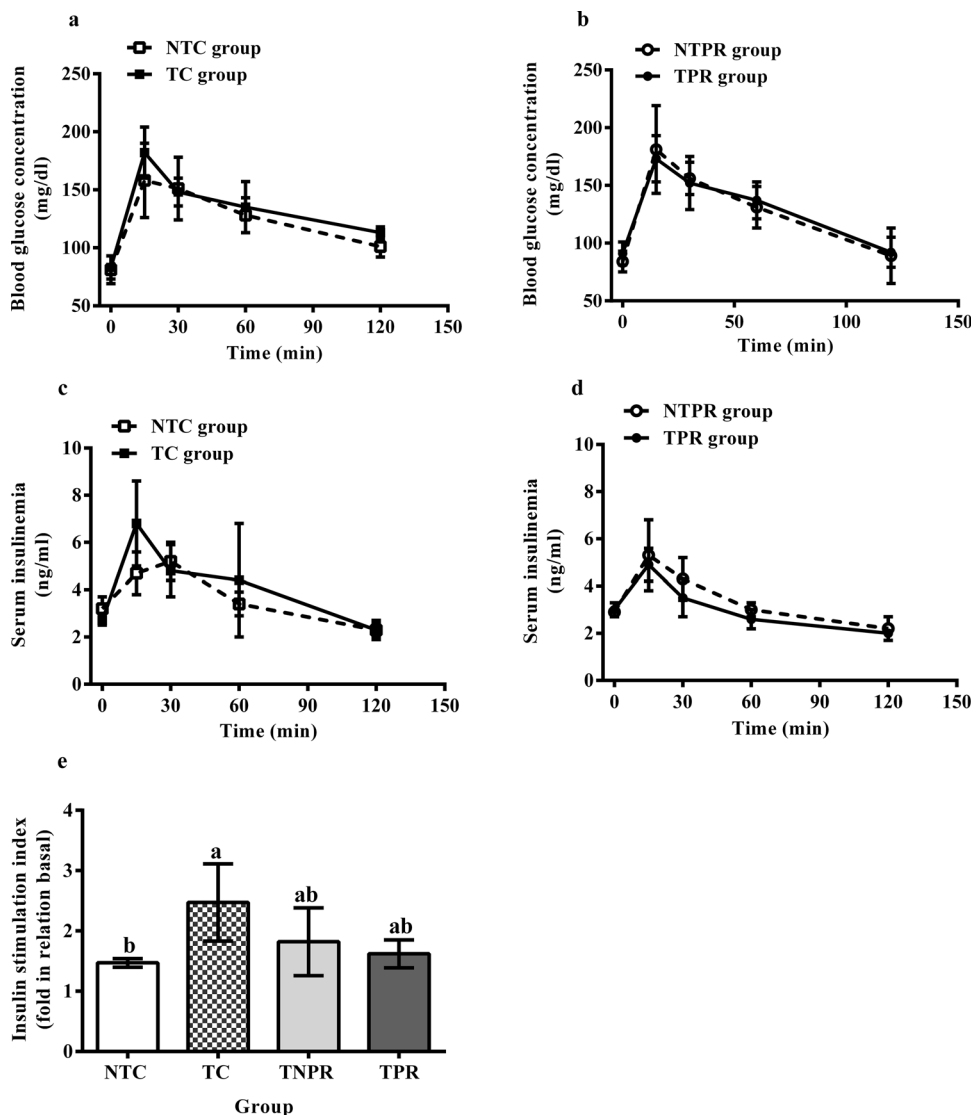


Table 2 Alkaline phosphatase (ALP), aspartate transaminase (AST), alanine transaminase (ALT) and urea concentrations from non-treated and treated rats from control (NTC and TC) or protein-restricted (NTPR and TPR) groups

Variable	Group			
	NTC	TC	NTPR	TPR
ALP (U/L)	18.5 ± 3.1 (5)	15.1 ± 1.4 [#] (5)	17.9 ± 3.2 (5)	15.8 ± 2.0 [#] (5)
AST (U/mL)	103.1 ± 25.8 (5)	94.2 ± 8.7 [#] (5)	89.8 ± 8.6 (4)	70.9 ± 13.8 [#] (5)
ALT (U/mL)	20.4 ± 8.8 (5)	6.6 ± 4.5 [#] (5)	19.8 ± 8.6 (5)	13.8 ± 5.4 [#] (5)
Urea (mg/dL)	27.9 ± 5.7 (5)	18.8 ± 1.7 (5)	21.5 ± 1.6 (4)	22.0 ± 5.9 (5)

Values are means ± SD for the number of rats shown in parentheses

[#]Indicates the effect of treatment (two-way ANOVA, $P < 0.05$)

milligram of protein was higher in the treated groups compared to the non-treated groups ($F_{1,12} = 4.81$, $P < 0.0488$; $F_{1,12} = 12.30$, $P < 0.0043$, respectively). The protein-restricted groups exhibited higher pancreatic insulin content per kilogram of body weight than the control groups ($F_{1,12} = 17.16$, $P < 0.0014$) (Table 3).

Regardless of the factors tested, 65% of the islets were small, 20% were medium, and 15% were large. ANOVA revealed significant effects of nutritional status (NS × classification) and the interaction between nutritional status and treatment with exendin-4 (NS × exendin-4 × classification) on the relative frequency distribution of islet size. Thus, nutritional status on its own and in conjunction with exendin-4 decreased the relative frequency of smaller islets

and increased the frequency of larger islets. However, the NS × exendin-4 × classification interaction showed that this pattern was observed only in the control group (Fig. 3a). The β -cell size was similar among groups (Fig. 3b). The β -cell area relative to the total pancreas area was higher in the treated groups than in the non-treated groups ($F_{1,9} = 5.14$, $P < 0.049$) (Fig. 3c). The β -cell mass was lower in the protein-restricted groups compared to the control groups ($F_{1,9} = 14.32$, $P < 0.0043$) but higher in the treated groups compared to the non-treated groups ($F_{1,9} = 10.43$, $P < 0.01$) (Fig. 3d). GLP-1 fluorescence was higher in the protein-restricted groups than in the control groups ($F_{1,16} = 27.23$, $P < 0.0001$) (Fig. 3e). The GLP-1 fluorescence/ α -cell mass ratio was higher in the protein-restricted groups than in the control groups, independent of treatment ($F_{1,15} = 10.74$, $P < 0.005$) (Fig. 3f). The islet and α -cell areas relative to the total pancreas area were similar among groups (data not shown).

PC1/PC3/glucagon colocalization was lower in the PR group than in the C group (Fig. 4a and Fig. 8 in ESM). The PR and C groups exhibited similar PC2/glucagon colocalization (Fig. 4b, Fig. 8 in ESM). GLP-1/glucagon colocalization was higher in islets from the NTPR group than in islets from the NTC group ($P < 0.001$) and was higher in the TPR group than in the TC group ($P < 0.02$). Moreover, GLP-1/glucagon colocalization was higher in islets from the TC group compared to the NTC group ($P < 0.0003$) and did not differ between the TPR and NTPR groups (Fig. 4c, Fig. 9 in ESM). The colocalization of GLP-1R/insulin did not differ among groups (Fig. 4d, Fig. 10 in ESM).

PDX-1/DAPI colocalization was similar in all groups (Fig. 5a, Fig. 11 in ESM). FoxO1/DAPI colocalization

Table 3 Pancreas weight and pancreatic insulin content in non-treated and treated rats from control (NTC and TC) or protein-restricted (NTPR and TPR) groups.

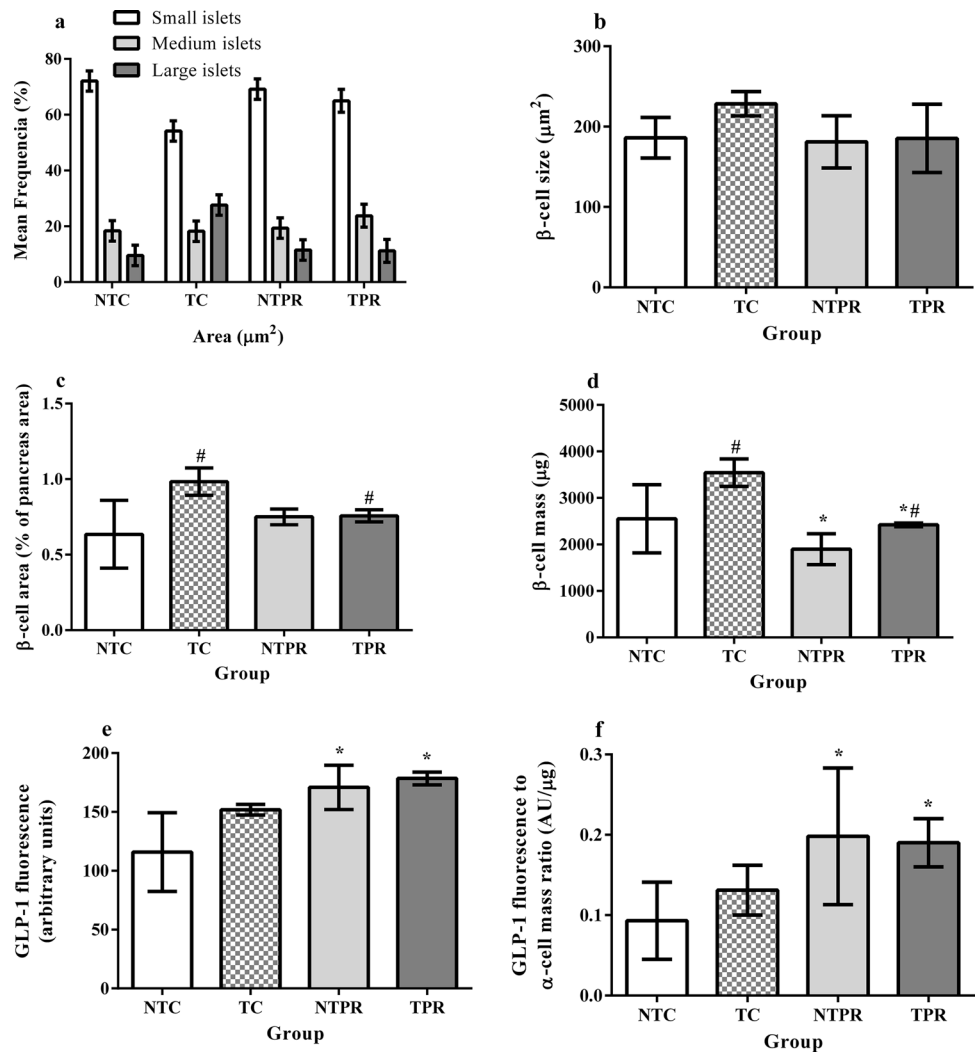
Variable	Group			
	NTC	TC	NTPR	TPR
Weight of pancreas				
mg	610 ± 60 (5)	630 ± 82 (5)	509 ± 68 ^a (5)	521 ± 73 ^a (5)
mg/100g body weight	276 ± 32 (5)	270 ± 23 (5)	283 ± 39 (5)	251 ± 27 (5)
Insulin (ng/mL)				
Per pancreas	6415 ± 214 (4)	6509 ± 367 (4)	6486 ± 345 (4)	6450 ± 124 (4)
Per g of pancreas	23469 ± 1121 (4)	29190 ± 9530 [#] (4)	25232 ± 2265 (4)	31895 ± 5515 [#] (4)
Per kg body weight	29168 ± 2232 (4)	27462 ± 1196 (4)	35812 ± 5706 ^a (4)	36678 ± 4434 ^a (4)
Per mg protein	0.70 ± 0.13 (4)	1.12 ± 0.32 [#] (4)	0.56 ± 0.21 (4)	1.24 ± 0.47 [#] (4)

Values are means ± SD for the number of rats shown in parentheses

[#]Indicates the effect of treatment (two-way ANOVA, $P < 0.05$)

^aIndicates difference in relation to control rats

Fig. 3 **a** Relative frequency distribution of islet size, **b** β -cell size, **c** area of β -cells, **d** β -cell mass, **e** GLP-1 fluorescence, and **f** GLP-1/ α -cell mass ratio of non-treated and treated rats from the control and protein-restricted groups. Values are means with standard deviations represented by vertical bars ($n=3-5$ rats). *Indicates the effect of nutritional status (two-way ANOVA, $P < 0.05$). #Indicates the effect of treatment (two-way ANOVA, $P < 0.05$)

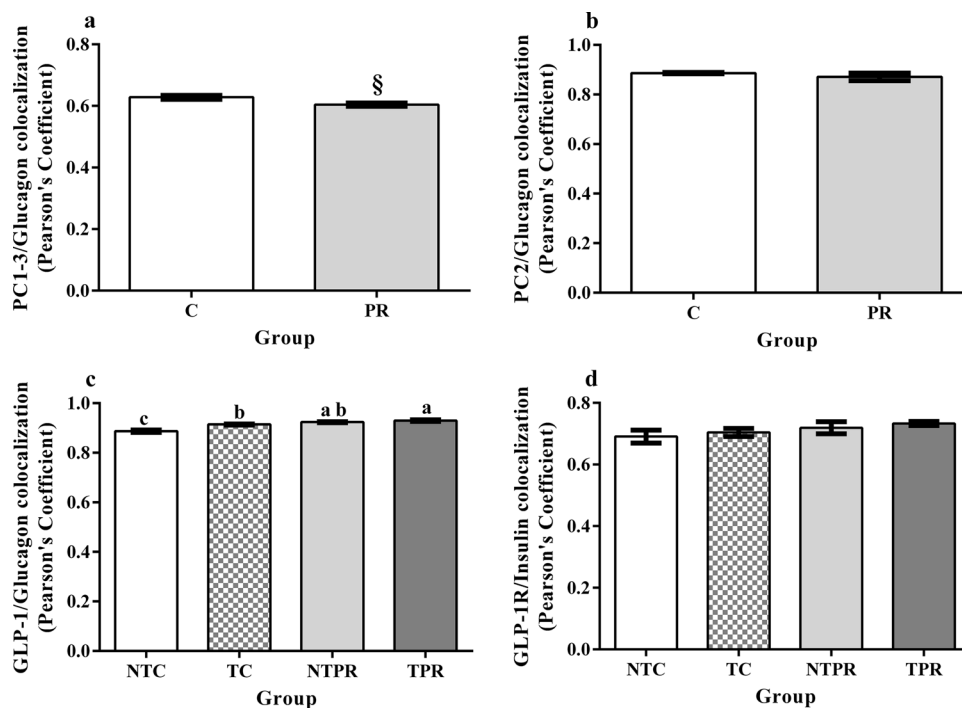


was higher in the treated groups than in the non-treated groups ($F_{1,15} = 7.60$, $P < 0.014$) (Fig. 5b, Fig. 12 in ESM). β -Catenin/DAPI colocalization was higher in the protein-restricted groups than in the control groups ($F_{1,16} = 12.47$, $P < 0.0028$), as well as in the treated groups compared to the non-treated groups ($F_{1,16} = 39.56$, $P < 0.0000$) (Fig. 5c, Fig. 13 in ESM).

The frequency of PI-positive endocrine cells was higher in the protein-restricted groups compared to the control groups ($F_{1,16} = 14.92$, $P < 0.0014$) but lower in the treated groups compared to the non-treated groups ($F_{1,16} = 9.02$, $P < 0.0084$) (Fig. 6a, Fig. 14 in ESM). The NTPR group showed a higher frequency of cleaved caspase-3-positive endocrine cells than the NTC group ($P < 0.0002$). The frequency of cleaved caspase-3-positive endocrine cells was lower in the TPR group compared to the NTPR group ($P < 0.0001$), but the frequency in the NTPR group was similar to that in the TC group. There was no difference in the frequency of cleaved caspase-3-positive endocrine cells between the TC and NTC groups (Fig. 6b, Fig. 14 in

ESM). The cleaved caspase-3/ α -cell mass ratio was higher in the NTPR group compared to the NTC, TC and TPR groups ($P < 0.029$, $P < 0.014$ and $P < 0.025$, respectively) (Fig. 6c). The cleaved caspase-3/ β -cell mass was higher in the protein-restricted groups compared to the control groups ($F_{1,15} = 4.86$, $P < 0.043$) but lower in the treated groups compared to the non-treated groups ($F_{1,15} = 24.01$, $P < 0.0002$) (Fig. 6d). The frequency of Ki67-positive endocrine cells was higher in the protein-restricted groups compared to the control groups ($F_{1,16} = 22.2$, $P < 0.0002$) (Fig. 6e, Fig. 15 in ESM). The Ki67/ α -cell mass ratio and Ki67/ β -cell mass ratio were higher in the protein-restricted groups than in the control groups ($F_{1,15} = 8.22$, $P < 0.011$; $F_{1,15} = 9.93$, $P < 0.0066$) (Fig. 6f, g). The Ki67/cleaved caspase-3 ratio was similar among the NTPR, NTC and TC groups, which all exhibited a lower Ki67/cleaved caspase-3 ratio compared to the TPR group ($P < 0.002$; $P < 0.002$; $P < 0.003$) (Fig. 6h). The cluster areas relative to the total pancreas area were higher in the treated groups compared to the non-treated groups ($F_{1,11} = 6.73$, $P < 0.025$) (Fig. 6i).

Fig. 4 **a** PC1/PC3/glucagon colocalization and **b** PC2/glucagon colocalization in islets from rats maintained on a control diet or low-protein diet during the intrauterine life and lactation. Values are means with standard deviation, represented by vertical bars ($n=5$ rats per group). §Indicates a significant difference (independent samples t test, $P<0.05$). **c** GLP-1/glucagon colocalization and **d** GLP-1R/insulin colocalization in the islets of non-treated and treated rats from the control (NTC=5 and TC=5) and recovered groups (NTR=5 and TR=5). ^{a-c}Mean values are significantly different (Tukey's test, $P<0.05$)



The Glp-1R and Bcl2 mRNA contents in the TPR, NTPR and TC groups were similar. However, these groups showed higher Glp-1R ($P<0.03$; $P<0.0005$; $P<0.007$, respectively) and Bcl2 mRNA contents ($P<0.021$; $P<0.02$; $P<0.03$, respectively) than the NTC group (Fig. 7a, b). The caspase-3 mRNA content was higher in islets from the protein-restricted groups compared to the control groups ($F_{1,11}=8.07$, $P<0.016$) (Fig. 7c). There was no difference among groups in Pdx-1 transcript (Fig. 7d).

Discussion

We evaluated the intra-islet GLP-1 production and its modulator effect on the regulator mechanisms of β -cell mass in adult female rats that were protein-restricted during the intrauterine and lactation stages or not. Although the protein-restricted group exhibited low PC1/PC3/glucagon colocalization, intra-islet GLP-1 production was increased in both protein-restricted groups compared to control groups, as shown by GLP-1 fluorescence, the GLP-1 fluorescence/ α -cell mass ratio and GLP-1/glucagon colocalization. There are at least two plausible explanations for this paradox: (1) low PC1/PC3 expression was counter-regulated by its enhanced activity, since PC1/PC3, through its various domains, is capable of controlling its own enzymatic activity [33]; and (2) there was decreased DPP4 activity, as shown by the presence of this enzyme within α -cells [34].

As expected, exendin-4 produced an anorexigenic effect in both the control and protein-restricted groups, but not

enough to interfere with body weight. It is interesting to note the difference in absolute food intake before vs. during treatment with exendin-4, especially in the protein-restricted groups. We attributed the increased dietary intake in protein-restricted groups during treatment to the synergistic effect of protein restriction on intrauterine life and lactation and the stress caused by the isolation of rats in individual cages. Both situations increase the expression of the neurotransmitter NPY in the hypothalamus [34, 36], a potent orexigenic peptide.

Based on the results from the oral glucose tolerance test, the control group treated with exendin-4 exhibited an elevated insulin stimulation index compared to the non-treated control group, despite the unaltered GLP-1 fluorescence in pancreatic islets. Curiously, the increase in the intra-islet GLP-1 did not produce the expected incretin effect in our protein-restricted groups. However, in a previous study [27], we verified that isolated islets from protein-restricted rats displayed a kinetics of insulin secretion characterized by a rapid increase after exposure to 11.1 mmol/L glucose (first-phase secretion), which resulted in a significant enhancement in the area under the insulin curve compared to the control rats. In the same study, an increase in glucose concentration from 2.8 to 11.1 mmol/L induced biphasic insulin secretion with a rapid first-phase release after exposure to high glucose, followed by a decrease and then an increase during the second phase in islets from control rats. The discrepancies between the results of stimulatory indexes evaluated in the present study and the kinetics of insulin secretion verified previously [27] are not surprising. In vivo, β -cells

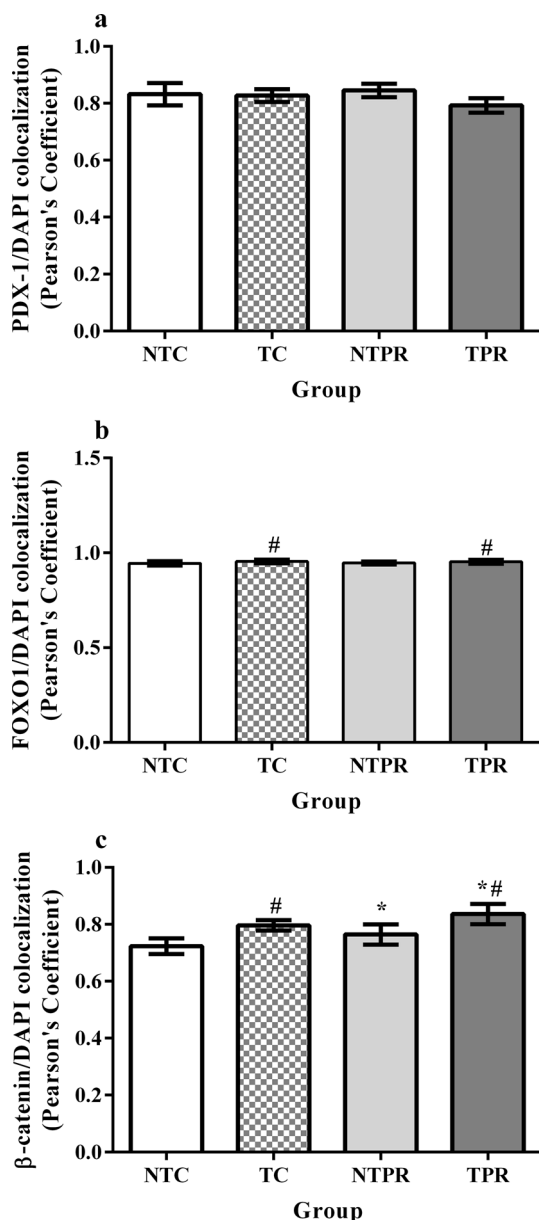


Fig. 5 a PDX-1/DAPI colocalization, b FoxO1/DAPI colocalization, and c β -catenin/DAPI colocalization in the islets of non-treated and treated rats from the control and protein-restricted groups. Values are means with standard deviation, represented by vertical bars ($n=3-5$ rats). *Indicates the effect of nutritional status (two-way ANOVA, $P<0.05$). #Indicates the effect of treatment (two-way ANOVA, $P<0.05$)

are under the influence of glucose and other nutrients, hormones, and neurotransmitters, whereas in *in vitro*, islets are stimulated by glucose alone. Moreover, changes in peripheral insulin levels do not exclusively reflect alterations in insulin secretion because they are influenced by liver insulin extraction rates [37]. Thus, the intra-islet GLP-1 production verified in our protein-restricted animals and the kinetics of insulin secretion previously observed corroborate the idea

that GLP-1 participates in making the first phase particularly prominent [38]. Although the increase in intra-islet GLP-1 content seems to have an impact on the increase of the first secretory phase, which is considered beneficial for metabolic health, the loss of the typical biphasic pattern in our model indicates that these islets are dysfunctional.

Interestingly, the protein-restricted rats exhibited reduced β -cell mass due to increased apoptosis, which was confirmed by an increase in caspase-3 transcripts, cleaved caspase-3 expression, cleaved caspase-3/ β -cell mass ratio and frequency of PI-positive endocrine cells. It is noteworthy that these effects were observed despite increased mRNA Glp-1R and Bcl2 expression, which could contribute to cellular survival. At least in relation to GLP-1R, alterations in GLP-1R/insulin colocalization were not observed. Although these results appear controversial, it is known that transcription and translation are not always correlated because there are many processes governed by these mechanisms that can be regulated by hormones, nutrients such as amino acids, and energy state [39]. Moreover, it is important to emphasize that mRNA Glp-1R expression was determined in pancreatic islets and that its content was assessed exclusively in β -cells. The elevated α -cell apoptosis observed in the non-treated protein-restricted group, as shown by the cleaved caspase-3/ α -cell mass ratio, was not reflected in the α -cell mass, possibly due to increased α -cell proliferation.

The proliferative effect of GLP-1 was observed in our protein-restricted animals, as shown by the elevated frequency of Ki67-positive endocrine cells, increased the ratios of Ki67/ α -cell mass and Ki67/ β -cell mass. This effect was possibly modulated by the β -catenin pathway, whose increase in nuclear localization contributes to the expression of genes that encode cyclins, contributing to the proliferation of β -cells [14]. Although studies have indicated that active β -catenin promotes the increase in FoxO1 in the nucleus and consequently decreases Pdx-1 transcription [40, 41], in the present study, these effects were not observed. Previous findings regarding elevated adenylate cyclase and PKA contents in islets from protein-restricted rats [42] support the hypothesis that β -catenin participates in the increased β -cell proliferation.

Exendin-4 treatment did not impair hepatic function; instead, it reduced the levels of hepatic enzymes related to insulin resistance, corroborating the report of its suppressor effect on hepatic glucose production [43]. Additionally, exendin-4 treatment increased the relative frequency of large islets, especially in the control group, and the relative area and absolute mass of β -cells in both treated groups. These effects resulted from the reduced apoptosis in the protein-restricted rats and increased neogenesis in both groups. Curiously, the activation of neogenesis was not mediated by PDX-1, since its transcription and nuclear localization were unaltered by exendin-4. In addition, in

Fig. 6 **a** Frequency of propidium iodide (PI)-positive endocrine cells, **b** frequency of cleaved caspase-3-positive endocrine cells, **c** cleaved caspase-3/ α -cell mass ratio, **d** cleaved caspase-3/ β -cell mass ratio, **e** frequency of Ki67-positive endocrine cells, **f** Ki67-positive endocrine cell/ α -cell mass ratio, **g** Ki67-positive endocrine cell/ β -cell mass ratio, **h** Ki67-positive endocrine cell/cleaved caspase-3 ratio, and **i** endocrine cell cluster area in relation to pancreas area in the islets of non-treated and treated rats from the control (NTC and TC) and protein-restricted (NTPR and TR) groups. Values are means with standard deviation, represented by vertical bars ($n=4-5$ rats). *Indicates the effect of nutritional status (two-way ANOVA, $P<0.05$). #Indicates the effect of treatment (two-way ANOVA, $P<0.05$). ^{a-c}Mean values are significantly different (Tukey's test, $P<0.05$)

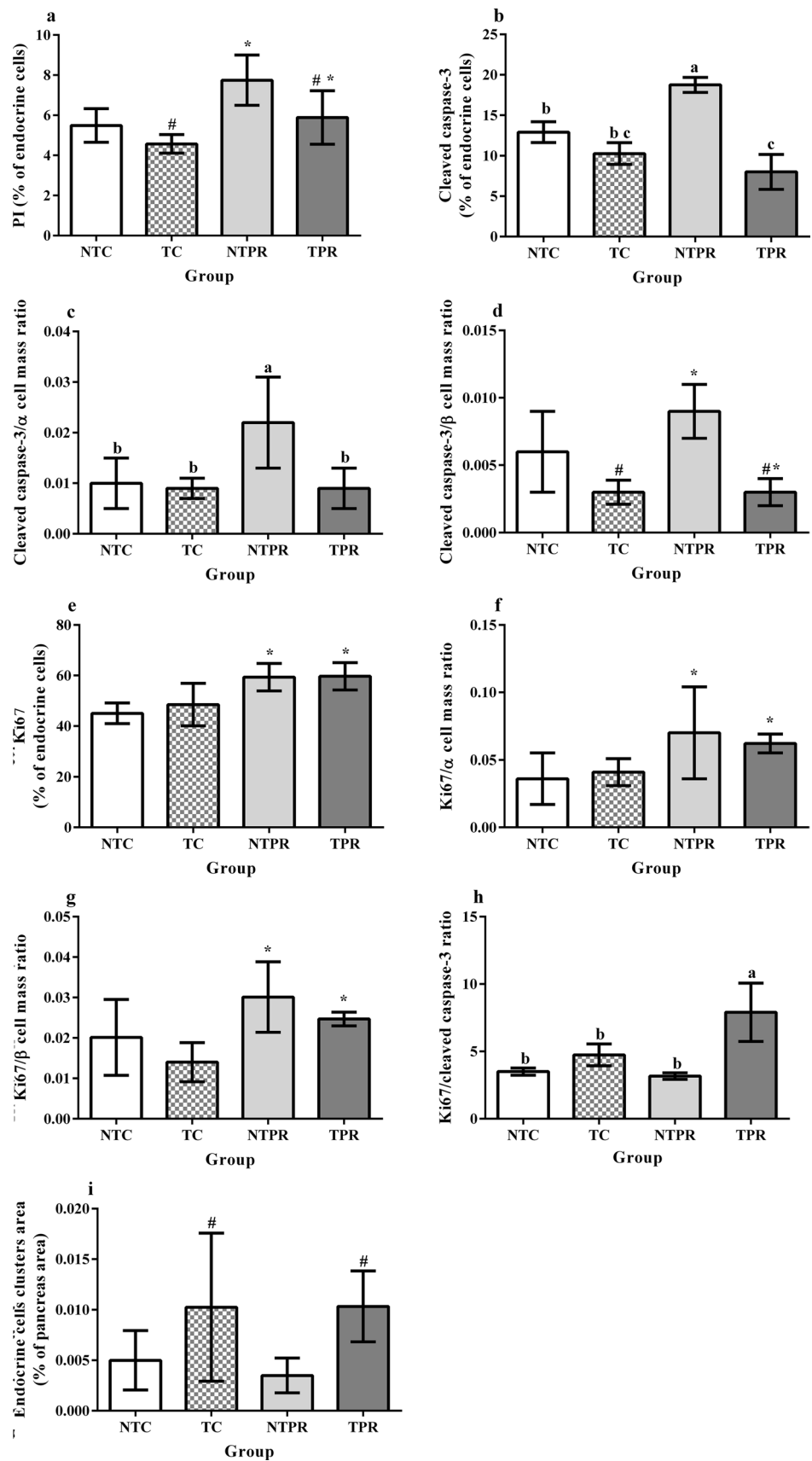
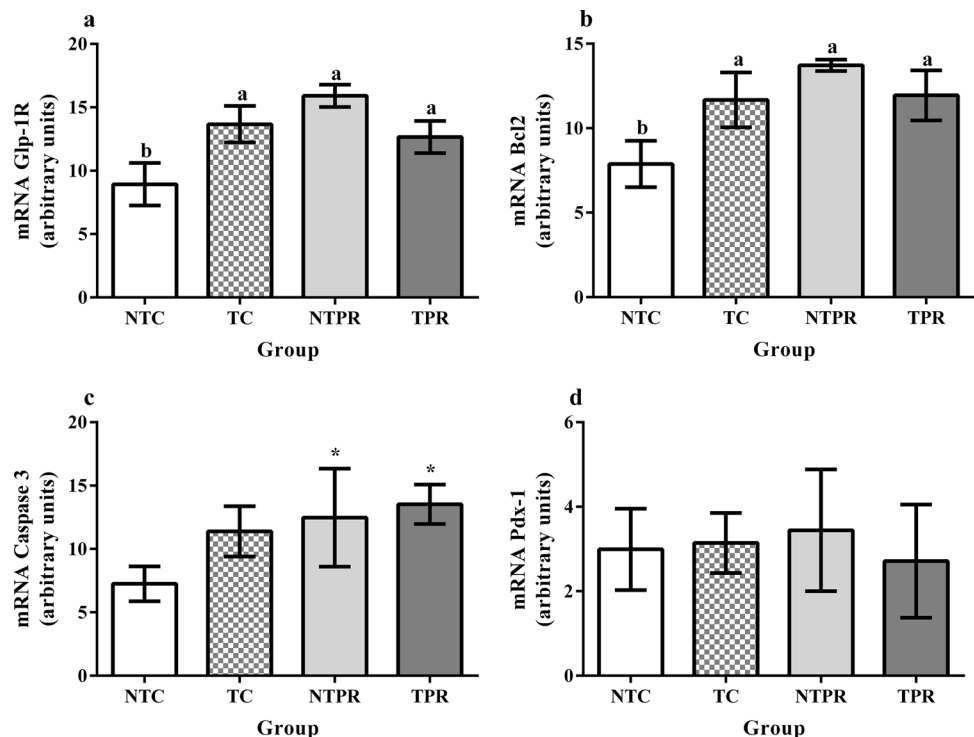


Fig. 7 **a** Glp-1R, **b** Bcl2, **c** caspase-3 and **d** Pdx-1 mRNA contents in the islets of non-treated and treated rats from control (NTC and TC) and protein-restricted (NTR and TR) groups. Values are means with standard deviation, represented by vertical bars ($n=4-5$ rats). *Indicates the effect of nutritional status (two-way ANOVA, $P<0.05$). ^{a,b}Mean values are significantly different (Tukey's test, $P<0.05$)



the present study, the increase in FoxO1 nuclear localization by exendin-4 did not correlate with apoptosis, contradicting the report that increased FoxO1 nuclear localization decreases cellular survival [44]. Although exendin-4 did not influence β -cell proliferation, its anti-apoptotic effect was able to increase the proliferation/apoptosis ratio only in the protein-restricted group, but not enough to restore the β -cell mass.

Taking into consideration the results obtained in the present study, we conclude that protein restriction in early life increased intra-islet GLP-1 production and β -cell proliferation, possibly mediated by the β -catenin pathway. Exendin-4 prevented apoptosis and increased neogenesis. Therefore, GLP-1 receptor analogues could be a therapeutic option to mitigate structural damage to pancreatic cells caused by adverse intrauterine and neonatal environments.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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