

Taspoglutide, a novel human once-weekly GLP-1 analogue, protects pancreatic β -cells *in vitro* and preserves islet structure and function in the Zucker diabetic fatty rat *in vivo*

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ABSTRACT (250 words)

Objective: Glucagon-like peptide-1 (GLP-1) has protective effects on pancreatic β -cells. We evaluated the effects of a novel, long-acting human GLP-1 analogue, taspoglutide, on β -cells *in vitro* and *in vivo*.

Research design and methods: Proliferation of murine pancreatic β (MIN6B1)-cells and rat islets in culture was assessed by imaging of 5-ethynyl-2'-deoxyuridine-positive cells after culture with taspoglutide. Apoptosis was evaluated with the transferase-mediated dUTP nick-end labelling (TUNEL) assay in rat insulinoma (INS-1E) cells and isolated human islets exposed to cytokines (recombinant interleukin-1 β , interferon- γ , tumour necrosis factor- α) or lipotoxicity (palmitate) in the presence or absence of taspoglutide. Islet morphology and survival, and glucose-stimulated insulin secretion in perfused pancreata were assessed 3–4 weeks after single-application of taspoglutide to prediabetic 6-week-old male Zucker diabetic fatty (ZDF) rats.

Results: Proliferation was increased concentration-dependently up to four-fold by taspoglutide in MIN6B1 cells and was significantly stimulated in isolated rat islets. Taspoglutide almost completely prevented cytokine- or lipotoxicity-induced apoptosis in INS-1E cells (control 0.5%, cytokines alone 2.2%, taspoglutide + cytokines 0.6%, $p < 0.001$; palmitate alone 8.1%, taspoglutide + palmitate 0.5%, $p < 0.001$) and reduced apoptosis in isolated human islets. Treatment of ZDF rats with taspoglutide significantly prevented β -cell apoptosis, preserved healthy islet architecture and insulin-staining intensity as shown in pancreatic islet cross-sections. Basal and glucose-stimulated insulin secretion of *in situ* perfused ZDF rat pancreata was normalized after taspoglutide treatment.

Conclusions: Taspoglutide promoted β -cell proliferation and prevented apoptosis *in vitro*, and exerted multiple β -cell protective effects on islet architecture and function *in vivo* in ZDF rats.

INTRODUCTION

Type 2 diabetes is characterized by a progressive loss of pancreatic β -cell function [1] that eventually necessitates the use of insulin therapy to control glycaemia in most patients.

Current pharmacological treatments for type 2 diabetes have not been demonstrated to halt or reverse β -cell dysfunction. Glucagon-like peptide-1 (GLP-1) is an incretin hormone released from L-cells of the distal intestine into the circulation in response to ingestion of carbohydrates or lipids, as reviewed by Holst [2]. GLP-1 exists as two equipotent bioactive forms; an amidated 30-mer peptide, hGLP-1(7-36)NH₂ and a glycine-extended 31-mer, hGLP-1(7-37).

GLP-1 enhances glucose-dependent release of insulin from β -cells [3-5], suppresses postprandial secretion of glucagon from pancreatic α -cells [6,7], inhibits gastric emptying [8,9] and elicits satiety [10-15]. *In vitro* studies in insulinoma cells and isolated islets, as well as *in vivo* rodent investigations, have shown that GLP-1 also stimulates β -cell proliferation and prevents β -cell apoptosis, effectively acting as a pancreatic β -cell growth factor, as reviewed elsewhere [16,17]. Similarly, GLP-1 mimetics or analogues have been shown to enhance β -cell function, inhibit β -cell apoptosis and increase β -cell mass *in vitro* or *in vivo* [18-23]. Although it is currently not possible to measure human β -cell mass *in vivo*, clinical therapy with GLP-1 receptor agonists improves surrogate measures of β -cell function [24-27]. Currently approved GLP-1 mimetics (exenatide) and analogues (liraglutide) are administered once or twice daily. Taspoglutide is a novel analogue of human GLP-1(7-36)NH₂ with enhanced biochemical stability and equivalent potency [28]. A sustained-release formulation of taspoglutide improved glycaemic control and reduced body weight dose-dependently in a clinical study [29], and has undergone phase 3 clinical studies as a once-weekly treatment for type 2 diabetes.

The aim of this study was to characterize the effects of taspoglutide *in vitro* and *in vivo* on proliferation and apoptosis of pancreatic β -cells, and on islet morphology and function in the Zucker diabetic fatty (ZDF) rat, a commonly used animal model of type 2 diabetes.

RESEARCH DESIGN AND METHODS

***In Vitro* Experiments**

Proliferation of murine clonal pancreatic β (MIN6B1)-cells and isolated rat islets.

MIN6B1 cells were cultured for 24 h in MIN6 medium (DMEM-based) pH 7.0 as described previously [30]. The medium was then replaced by serum-free and low glucose (5 mmol/l) MIN6 medium pH 7.0 containing 0, 0.1, 1, 10 or 100 nmol/l taspoglutide or human GLP-1(7-36)NH₂ (hGLP-1; Bachem, Switzerland). After incubation for 48 h, the medium was replaced by MIN6 medium pH 7.0 containing 10 nmol/l 5-ethynyl-2'-deoxyuridine (EdU) (Click-It EdU kit; Invitrogen, Basel, Switzerland) and incubation was continued for 30 min in the presence or absence of taspoglutide or hGLP-1, after which the cells were fixed, permeabilized and subjected to EdU assay (Click-It EdU kit; Invitrogen). For each experiment, ≥ 10 wells per treatment were quantified.

Islets from 7–9-week-old male Sprague-Dawley rats (Charles River Laboratories International, Inc., Wilmington, MA, USA) were isolated by digestion of pancreata with collagenase (Roche Diagnostics, Rotkreuz, Switzerland), followed by several suspension and centrifugation steps to remove exocrine tissue, and finally separated by a Histopaque 1077 gradient (Sigma-Aldrich, Buchs, Switzerland). Islets were collected from the inter-phase, handpicked and maintained overnight in 5.6 mmol/l glucose/RPMI-1640 (Invitrogen) pH 7.0 supplemented with 10% FCS, 60 mg/l penicillin, 100 mg/l streptomycin and 100 mg/l gentamicin (rat islet medium). Islets were cultured for 48 h with rat islet medium containing 5

μm EdU in the presence or absence of 10 nmol/l taspoglutide or hGLP-1, after which they were dispersed to single cells using trypsin (Invitrogen) and seeded in 8-well culture slides (BD Falcon, Basel, Switzerland) and fixed and permeabilized for EdU assay and immunofluorescence co-staining with guinea-pig anti-insulin (Dako, Switzerland) and mouse anti-glucagon (Sigma-Aldrich, Switzerland). The proliferation rate, expressed as the percent of EdU-positive islets in the total islet population, was determined using the Axioplan 2 fluorescence microscope (Carl Zeiss AG, Feldbach, Switzerland).

Apoptosis in rat insulinoma (INS-1E) cells and human islets. As MIN6B1 cells are very resistant to palmitate-induced apoptosis, we used a different β cell line, INS-1E, to study apoptosis (INS-1E cells were not used to study proliferation, as they have a very high background of basal proliferation). INS-1E cells were preincubated with or without 10 nmol/l taspoglutide or hGLP-1 for 30 min in RPMI-1640 (pH 7.0) containing 11 mmol/l glucose supplemented with 10 mmol/l HEPES (pH 7.3), 10% FCS, 50 $\mu\text{mol/l}$ β -mercaptoethanol, 1 mmol/l sodium pyruvate, 50 mg/l penicillin and 100 mg/l streptomycin. The culture was continued in the presence or absence of either cytokines (recombinant human interleukin [IL]-1 β : 1 $\mu\text{g/l}$ + interferon [IFN]- γ : 5 $\mu\text{g/l}$ + tumour necrosis factor [TNF]- α : 5 $\mu\text{g/l}$) for 6 h or palmitate (0.5 mmol/l) for 24 h. The transferase-mediated dUTP nick-end labelling (TUNEL) apoptosis assay was performed following the manufacturer's protocol (Roche Diagnostics).

Transplantation-grade human islets (80% purity) were isolated (by hand-picking) from three non-diabetic organ donors (approximately 3000 islets/donor) and maintained in CMRL-1066 (Invitrogen) pH 7.0 at 5.6 mmol/l glucose supplemented with 10% FCS, 60 mg/l penicillin, 100 mg/l streptomycin and 100 mg/l gentamycin for 2–4 days before experiments. Islets were pre-exposed to 10 nmol/l taspoglutide for 30 min before adding cytokines (recombinant human IL-1 β 1 $\mu\text{g/l}$, IFN- γ 5 $\mu\text{g/l}$, TNF- α 5 $\mu\text{g/l}$) and incubating at

37°C for 24 h, or palmitate 0.5 mmol/l followed by incubation at 37°C for 48 h. This experiment was approved by the University of Geneva ethics committee, and was conducted in adherence to all relevant laws and ethical guidelines regulating the collection, transfer and use of human tissue.

Opera high content screening: image acquisition and analysis. Spinning-disc confocal fluorescence microscopy of 96-well plates was performed on the high-throughput automated imaging system Opera™ QEHS (PerkinElmer Cellular Technologies, Hamburg, Germany). The nuclear stain (Hoechst or 4',6-diamidino-2-phenylindole [DAPI]), TUNEL (apoptosis marker, tetramethyl-rhodamine labelling) and EdU (proliferation marker, Alexa-488 labelling) were excited at 405 nm, 532 nm and 488 nm, respectively, with optimized intensity and duration (typically, 50 mW, 40–400 ms integration time). In each well, typically 12 pairs of scanning images were recorded through an Olympus UAPO 20x NA 0.7 water immersion objective lens (Optical Analysis Corporation, Nashua, NH, USA) and optimized filter sets.

Confocal micrographs were analyzed using proprietary image analysis software (Acapella 2.0; PerkinElmer Cellular Technologies). The intensity in the EdU or TUNEL stain image was quantified at the location of the nucleus disregarding any residual unspecific stain. A homogeneous population of well-separated nuclei was chosen for further analysis. After application of a suitable threshold, the percentage of proliferating or apoptotic cells was calculated by the number of EdU or TUNEL-positive cells and the total number of all identified cells.

In Vivo Study of Chronic Effects in ZDF Rats

Animals. Five-week-old male homozygous ZDF rats [*ZDF/GmiCrl fa/fa*] and lean male heterozygous ZDF (ZL) rats [*ZDF/GmiCrl fa/+*] (Charles River Laboratories, Sulzfeld, Germany) were given unrestricted access to a diabetogenic diet (Ssniff_ZDF [composition by

weight, 23.5% protein, 39.4% carbohydrate, 6.6% fat]; Ssniff, Spezialdiäten, Soest, Germany) and tap water. Rats were housed individually at approximately 21°C and 55–65% relative humidity, and a 12-h light, 12-h dark cycle was maintained. After delivery, animals were acclimatized to the facility for 6–7 days before experimental procedures were initiated. Six-week-old ZDF rats were randomized and treated with either a single subcutaneous dose of a sustained-release formulation of taspoglutide 1 mg (n = 5), or vehicle (n = 5) on day 0. All procedures were conducted in accordance with the ‘Principles of laboratory animal care’ (NIH publication no. 8523, revised 1985; <http://grants1.nih.gov/grants/olaw/references/phspol.htm>), and under authorization from the Swiss Federal Veterinary Office and the Association for Assessment and Accreditation of Laboratory Animal Care International.

Immunohistochemistry. Four hours prior to being killed, rats were administered an intraperitoneal injection of BrdU (Sigma-Aldrich) to label proliferating cells. Six-week-old ZDF and ZL rats and 9-week-old ZL rats were used as healthy age-matched controls (n = 5 each). Pancreata were rapidly dissected, fixed in formalin, dehydrated and embedded into paraffin. Thin slices (4 µm) were transferred onto Superfrost⁺ slides (Menzel Glaser, Braunschweig, Germany), dewaxed and processed in separate staining procedures. To visualize islet morphology, sections were incubated with guinea pig anti-swine insulin (Dako, Glostrup, Denmark) and mouse anti-glucagon (R&D Systems, Minneapolis, MN, USA) followed by goat anti-guinea pig Alexa Fluor 488 (Invitrogen) and donkey anti-mouse Alexa Fluor 555 (Invitrogen), then DAPI. To visualize proliferating cells, slides were incubated sequentially with mouse anti-BrdU-antibody (Dako), donkey anti-mouse Alexa Fluor 555 (Invitrogen), guinea pig anti-swine insulin (Dako), and goat anti-guinea pig 488 (Invitrogen) and DAPI. To visualize apoptotic cells, slides were incubated first with guinea pig anti-swine

insulin (Dako) and TUNEL assay solution (*in situ* cell death detection kit; Roche Diagnostics), then DAPI.

Digital imaging fluorescence microscopy was performed using the Zeiss Axioplan 2 fluorescence microscope equipped with a Zeiss Plan-NEOFLUAR 10/0.30 objective lens (Carl Zeiss AG) with detection at 350 nm for DAPI, 488 nm for insulin and 555 nm for glucagon, BrdU and TUNEL. Islets were imaged using a Leica DFC 420 camera (Leica Camera AG, Solms, Germany) connected to an imaging system (Adobe, San Jose, CA, USA). Images were analyzed with a custom-tailored rule set in Definiens Developer 7.0.6 (Definiens, München, Germany).

Pancreatic insulin content. Dissected pancreata from 6- and 9-week-old ZDF and ZL rats with or without treatment were lysed and total insulin content (ELISA; Mercodia AB, Uppsala, Sweden) was measured and calculated as the percent of total protein content (BioRad DC Protein Assay; BioRad Labs, Hercules, CA, USA).

Pancreas perfusion. Pancreas perfusion experiments were conducted 3 weeks after initiation of treatment (i.e., in 9-week-old ZDF rats treated with taspoglutide or vehicle at week 6), and also in untreated 6-week-old ZDF rats for comparison (n = 3). Pancreata were surgically isolated *in situ* in anaesthetized rats (buprenorphine 0.025 mg/kg followed by a 2 ml/kg intraperitoneal cocktail of ketamine 77 mg/kg and xylazine 11 mg/kg body weight) while retaining access to the abdominal aorta and the portal vein, both of which were cannulated. The rats were then placed in an isolated, temperature- and pressure-controlled perfusion system at 37°C and the pancreata were connected to infusion pumps via the abdominal aorta.

Glucose-stimulated insulin secretion was assessed as follows. Pancreata were first perfused with freshly prepared Krebs-Ringer solution (5 ml/min) containing 2.8 mmol/l glucose to stabilize basal insulin secretion. Perfusion was continued for 20 min with 16.7 mmol/l glucose to sensitize the pancreata. This cycle was repeated using a 40-min perfusion

of 2.8 mmol/l glucose followed by 20 min with 16.7 mmol/l glucose to stimulate full phase 1 and 2 insulin secretion. Elution fractions were collected in 96-well plates outside the perfusion system via a catheter introduced into the portal vein, immediately cooled to 4°C and subsequently stored at –20°C until analyzed. Fractions were assayed for insulin (ELISA; Mercodia AB) and glucose (GOD-PAP kit; Roche Diagnostics).

Plasma drug levels. Plasma concentrations of taspoglutide were measured using a highly sensitive, validated liquid chromatography-tandem mass spectrometry method [31].

Statistical Methods

For quantification of β -cell apoptosis in human islets, the 99.9% log-intensity quantile of the TUNEL image pixels was extracted from each image. The 10% log-intensity quantile was subtracted as background. The log-transformed background-corrected TUNEL quantiles were used for linear mixed-effect model analysis using R 2.8.1 (® The R Foundation of Statistical Computing). The response y was modeled by two interacting two-level factors, the pretreatment (cytokines or palmitate) and for the treatment (taspoglutide or vehicle), with the donors as an additive random effect: $y \sim \text{pretreatment} + \text{taspoglutide} + \text{pretreatment:taspoglutide} + \text{rnd}(\text{donor})$. In addition, the effect of taspoglutide addition on each pretreatment was tested separately, with Benjamini–Hochberg post hoc correction of the p values.

Data from other experiments were analyzed by one-way ANOVA followed by Dunnett's test to compare all groups using SAS®/JMP® for Windows (version 6.0.0; SAS Institute Inc, Cary, NC, USA). All values are presented as means \pm SEM if not stated otherwise.

RESULTS

***In Vitro* Effects of Taspoglutide**

Proliferation of murine clonal pancreatic β -cells and rat islets. Taspoglutide and hGLP-1 stimulated proliferation of MIN6B1 cells in a concentration-dependent manner with a four-fold increase at 100 nmol/l compared with control ($p < 0.001$). The results for taspoglutide and hGLP-1 are almost identical (figure 1a and c). Taspoglutide also stimulated proliferation in dispersed islet cells (figure 1b) and intact islets (figure 1d) isolated from Sprague-Dawley rats to a similar extent as hGLP-1 ($70.1 \pm 3.4\%$ and $66.0 \pm 1.9\%$ EdU-positive islets, respectively, both $p < 0.001$ compared with $8.3 \pm 0.3\%$ for control). As demonstrated by immunofluorescence staining in dispersed rat islets, over 90% of EdU-positive islet cells co-localized with insulin (figure 1b).

Reduction of apoptosis in rat pancreatic β -cells and human islets. In INS-1E cells, taspoglutide significantly reduced both cytokine-induced apoptosis (apoptosis rate [%]: control: 0.5 ± 0.2 , cytokines: 2.2 ± 0.4 , taspoglutide + cytokines: 0.6 ± 0.2 ; $p < 0.001$) and lipotoxicity-induced apoptosis (control: 0.5 ± 0.2 , palmitate: 8.1 ± 4.6 , taspoglutide + palmitate: 0.5 ± 0.2 ; $p < 0.001$); hGLP-1 had similar effects (figure 2). Taspoglutide also significantly reduced cytokine-induced (cytokines: $1.60 \pm 0.04\%$ vs. taspoglutide + cytokines: 1.21 ± 0.02 ; $p < 0.001$) or palmitate-induced (palmitate: 1.44 ± 0.07 vs. taspoglutide + palmitate, 1.23 ± 0.03 ; $p < 0.001$) apoptosis in isolated human islets (figure 3).

***In Vivo* Effects of Taspoglutide**

The *in vivo* effects of taspoglutide were investigated in ZDF rats by examining β -cell proliferation and apoptosis, islet morphology and pancreatic function (glucose-stimulated insulin secretion). Following the single dose of taspoglutide, plasma levels of the peptide remained in the range of 0.06–0.18 nmol/l (0.2–0.6 ng/ml) throughout the study.

Effects on pancreatic islets in the ZDF rat. Quantitative immunohistochemistry was used to study the effect of taspoglutide on β -cell proliferation, apoptosis, islet area expansion, α -cell infiltration from the periphery to the core of islets and insulin staining intensity in pancreatic sections from ZDF rats.

Animals treated with taspoglutide had significantly fewer proliferating pancreatic β -cells per islet cross-section than vehicle-treated rats (0.7 vs. 1.3, respectively; $p < 0.001$), and significantly fewer apoptotic β -cells per islet cross-section (0.06 vs. 0.19, respectively; $p < 0.001$) (figure 4). The magnitude of the suppressive effect of taspoglutide on both proliferation and apoptosis was generally similar regardless of the size of the islet (figures 4C and 4D).

Photomicrographs of pancreatic slices revealed that whereas healthy islets from 6- and 9-week-old ZL rats contained a dense core of insulin-positive β -cells surrounded by a peripheral ring of glucagon-positive α -cells, the architecture of vehicle-treated ZDF rat islets appeared increasingly disrupted during disease progression from weeks 6 to 9. In contrast, treatment with taspoglutide preserved a compact islet shape (figure 5). Quantitatively, taspoglutide treatment prevented islet area expansion compared with vehicle treatment (mean islet area: taspoglutide, $23\,680 \pm 1464 \mu\text{m}^2$ vs. vehicle, $43\,692 \pm 2356 \mu\text{m}^2$; $p < 0.001$) (figure 5B). The distribution of islet sizes in the taspoglutide-treated group was shifted to the left (fewer large islets, more small islets; figure 5C). Only 4% of islet cross-sections from taspoglutide-treated animals were $>120\,000 \mu\text{m}^2$ compared with 10% from the vehicle group. Similarly, only 11% of total islet cross-sections from taspoglutide-treated animals showed a mean islet area $50\,000$ – $120\,000 \mu\text{m}^2$ compared with 18% of islet cross-sections from the vehicle group. In contrast, 63% of islet cross-sections from the taspoglutide group were smaller than $15\,000 \mu\text{m}^2$ versus only 45% from vehicle-treated animals.

Treatment with taspoglutide also substantially reduced the number of α -cells in the islet core, referred to as central α -cells (figure 6). The islet core is defined here as the islet region at least 20 μm (approximate diameter of two adjacent α -cells) away from the periphery. The number of central α -cells per islet cross-section was 17.8 ± 1.4 and 7.2 ± 0.8 in the vehicle and taspoglutide ($p < 0.001$) groups, respectively, with relative increase compared with prediabetic 6-week-old ZDF rats of 282% (vehicle) and 53% (taspoglutide).

The loss of insulin content of pancreatic islets in animals treated with taspoglutide was significantly reduced compared with vehicle controls, as quantified by two independent methods, staining intensity and total pancreatic insulin content (figure 7). In the insulin-staining intensity experiments, mean pixel intensity in taspoglutide-treated rats was approximately 20% higher than in the vehicle controls and was comparable to that in the prediabetic 6-week-old ZDF rats. Similarly, total pancreatic insulin content normalized to total protein content of the pancreata was $0.52 \pm 0.08\%$ for the taspoglutide group versus $0.26 \pm 0.02\%$ for the vehicle group ($p < 0.05$).

Effects on pancreas function in the ZDF rat. The chronic effects of single administration of taspoglutide on pancreas function were evaluated by measuring insulin secretion during *in situ* perfusion of surgically isolated pancreata from 9-week-old rats. In these experiments, an initial stimulation with a high concentration of glucose (16.7 mmol/l) was used to prime the pancreas followed by a second stimulation to evaluate full pancreatic insulin response (figure 8A). Basal insulin secretion during second perfusion with a low concentration of glucose (2.8 mmol/l) was substantially and significantly reduced in rats treated with taspoglutide compared with vehicle (figure 8B). During the second perfusion with 16.7 mmol/l glucose, taspoglutide-treated rats exhibited a significant reduction in phase 1 insulin secretion compared with the vehicle-treated group (figure 8C). Additionally, there was a trend towards reduction in phase 2 insulin secretion in taspoglutide-treated animals (figure 8D).

DISCUSSION

This preclinical study shows beneficial effects of taspoglutide, a novel, metabolically stable analogue of GLP-1, on pancreatic β -cell health *in vitro* and *in vivo*.

In vitro, taspoglutide had similar protective effects on β -cells as human GLP-1 in several different pancreatic cell lines, including proliferative effects on murine clonal β -cells and isolated rat islets, and prevention of cytokine- or lipotoxicity-induced apoptosis in rat insulinoma cells and isolated human islets.

The ZDF rat [32,33] is an inbred strain in which males homozygous for a recessive mutation in the leptin receptor (*fa/fa*) develop obesity, hyperlipidaemia, fasting hyperglycaemia, hyperinsulinaemia and insulin resistance [33,34]. Insulin resistance and mild glucose intolerance are typically evident by 7 weeks of age, with compensatory hyperinsulinaemia occurring between weeks 7 to 10. However, pancreatic insulin secretion eventually declines, possibly due to lipotoxicity in the pancreas resulting from dietary triglyceride accumulation and/or defective transcription of the insulin gene in β -cells [35], and overt diabetes develops by around 12 weeks. The course of disease in homozygous male ZDF rats closely mimics that of type 2 diabetes in humans.

In our study, a single dose of a sustained-release formulation of taspoglutide administered to prediabetic 6-week-old ZDF rats protected pancreatic islet architecture over weeks 6–9 by reducing β -cell apoptosis, abnormal enlargement of islets and infiltration of α -cells into the islet core.

The beneficial effects of taspoglutide on pancreatic β -cells in the present study are consistent with previous preclinical studies of GLP-1 and other GLP-1 receptor agonists. In general, GLP-1 receptor agonism has trophic effects on β -cells *in vitro* and *in vivo*, stimulating their proliferation and differentiation, and preventing glucotoxicity- and

lipotoxicity-induced apoptosis, as reviewed elsewhere [17,36,37]. It appears that the β -cell trophic effects of GLP-1 receptor agonism are dependent on the presence of hyperglycaemia and insulin resistance [38,39]. The present study provides further evidence for this as, contrary to the taspoglutide-stimulated proliferation of β -cells *in vitro*, proliferation of β -cells *in vivo* was significantly reduced in ZDF rats receiving taspoglutide; this is likely to reflect their improved glucose homeostasis, as previously reported [40], compared with the hyperglycaemic state of the vehicle-treated animals. A similar phenomenon was seen in ZDF rats treated with liraglutide, where β -cell proliferation did not increase when treatment maintained normoglycaemia but did increase in treated hyperglycaemic animals [39].

Taspoglutide also exhibited beneficial effects on pancreatic function as demonstrated by the normalization of glucose-stimulated insulin secretion in pancreata perfused *in situ* with glucose. In this experiment, taspoglutide significantly reduced basal insulin secretion under low glucose concentration while maintaining a healthy profile of insulin secretion upon glucose stimulation (consisting of distinct phase I and phase II), thus ameliorating disease-associated hyperinsulinaemia and potentially inhibiting islet exhaustion. Overall, the glucose-stimulated insulin secretion profile of 9-week-old taspoglutide-treated rats resembled that of prediabetic 6-week-old rats rather than 9-week-old vehicle-treated diabetic rats. These data suggest that treatment with taspoglutide helps to maintain the function of the β -cells and slows disease progression in this model of type 2 diabetes.

The strengths of the present study are its use of several independent *in vitro* and *in vivo* experimental systems to assess the effects of taspoglutide on pancreatic β -cells, including a rodent disease model that closely resembles type 2 diabetes. In particular, the combination of large-scale image analysis of islet cross-sections to investigate islet morphology, proliferation and apoptosis with an *in vivo* study comprising *in situ* pancreas

perfusion, assessing β -cell function upon glucose challenge, provides a complex level of information on pancreatic β -cell health.

Limiting the interpretation of the observed improvements in pancreas structure and function in ZDF rats treated with taspoglutide is the inability to distinguish the relative contribution of enhanced glycaemic control (and thus lower glucotoxicity) versus direct β -cell protective effects. We previously demonstrated that taspoglutide improves glycaemic control and reduces body weight in the same animals that were used in the present study [40]. Ultimately, the importance of the beneficial effects of taspoglutide on pancreatic β -cell health observed in the present study depends on whether or not they occur in humans with type 2 diabetes. The current inability to non-invasively image β -cells *in vivo* in humans means that conclusions must be inferred from various indirect measures of β -cell function. Insulin secretion patterns and other indirect measures of β -cell function are being assessed in ongoing clinical studies of taspoglutide.

In conclusion, this preclinical study demonstrates that taspoglutide has multiple beneficial effects on pancreatic β -cell function *in vitro* and preserves islet integrity and function *in vivo*, suggesting that this novel GLP-1 analogue might provide substantial benefits in preventing the progression of type 2 diabetes.

AUTHOR CONTRIBUTIONS

S.U. researched data, contributed to discussion, reviewed/edited manuscript. H.W. researched data, reviewed manuscript. A.B. researched data, contributed to discussion, reviewed/edited manuscript. M.P. researched data, reviewed manuscript. M.B. researched data, reviewed manuscript. S.S. researched data, contributed to discussion, reviewed/edited manuscript. L.T. made substantial contributions to study conception, reviewed/edited manuscript. D.B. contributed to acquisition of data, reviewed manuscript. C.B.W. made substantial contributions to study design, reviewed/edited manuscript, contributed to discussion. C.M. researched data, contributed to discussion, reviewed/edited manuscript. E.S. designed the study, researched data, reviewed/edited manuscript, contributed to discussion. All authors approved the final version of the manuscript for submission.

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Conflict of Interest

S.U., H.W., A.B, M.P., M.B., S.S, C.M. and E.S. are employees and stockholders of F. Hoffmann-La Roche. L.T. is an employee of Ipsen Pharma. F. Hoffmann-La Roche and Ipsen are developing tasoglutide for pharmaceutical use. C.B.W. has been a member of advisory boards for F. Hoffmann-La Roche. D.B. has received research funding from F. Hoffmann-La Roche.

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FIGURES

Figure 1. Proliferation in MIN6B1 cells (*A, C*) and isolated rat islets (*B, D*). *A*: Representative confocal micrographs of MIN6B1 cells incubated with 100 nmol/l taspoglutide or 100 nmol/l hGLP-1, magnification 20 \times . Cells were labelled with EdU (green, proliferating cells) and DAPI (blue, all cells). *B*: Representative photomicrographs of dispersed pancreatic islets isolated from Sprague-Dawley rats and cultured in the presence or absence of 10 nmol/l taspoglutide or 10 nmol/l hGLP-1, magnification 10 \times . Cells were labelled with EdU (green, nuclei of proliferating cells), insulin (red, β -cells), and glucagon (blue, α -cells). *C*: Concentration response of taspoglutide (white bars) and hGLP-1 (black bars) on proliferation of MIN6B1 cells. Data are means \pm SD from three independent experiments. *D*: Percentages of intact islets with ≥ 1 Edu-positive cell in pancreatic islets isolated from Sprague-Dawley rats and cultured with 10 nmol/l taspoglutide (white bar) or 10 nmol/l hGLP-1 (black bar), or without (hatched bar). Data are means \pm SD of all images analyzed. In total, approximately 300 islets were analyzed. *** $p < 0.001$ compared with control.

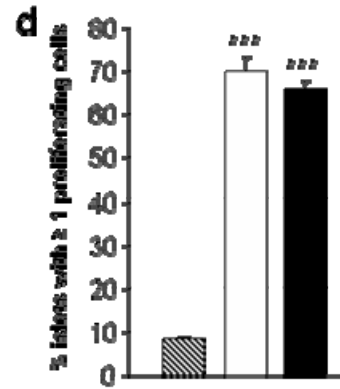
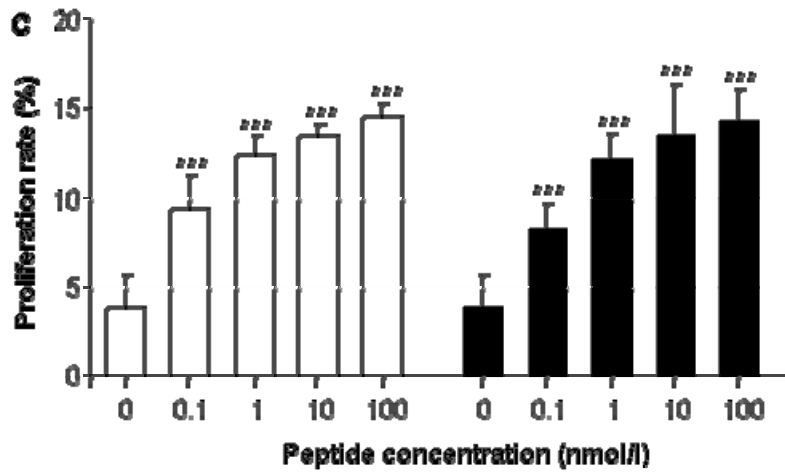
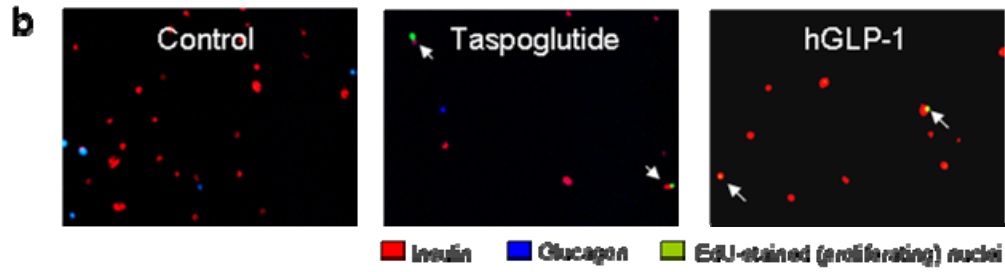
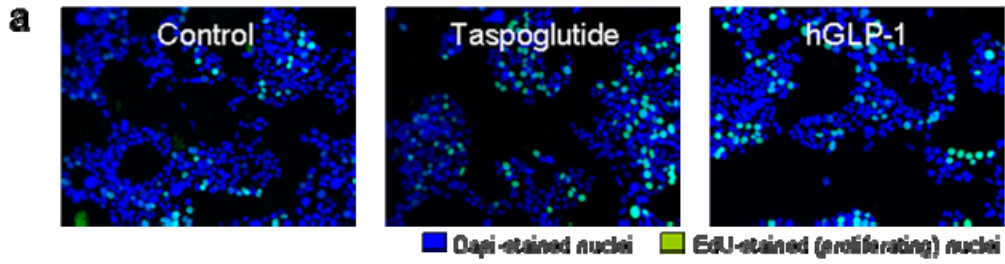
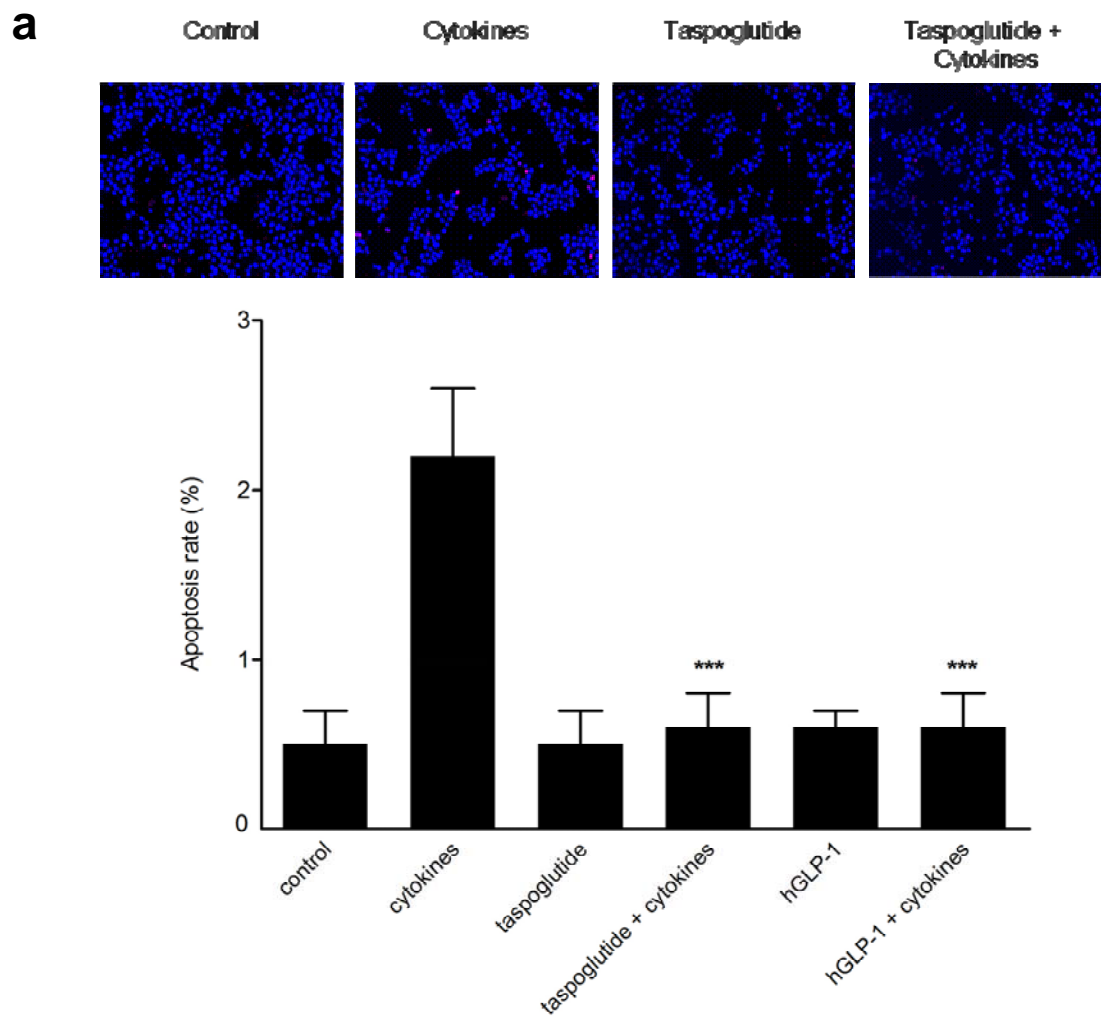


Figure 2. Apoptosis in INS-1E cells in the presence of 10 nmol/l taspoglutide or hGLP-1 and/or (A) cytokines (recombinant human IL-1 β 1 μ g/l, INF- γ 5 μ g/l, TNF- α 5 μ g/l) or (B) palmitate 0.5 mmol/l. Data are means \pm SD from three independent experiments ($***p < 0.001$ compared with apoptosis rate with cytokines or palmitate alone). Representative images show TUNEL-positive (apoptotic) nuclei in red and DAPI-stained (all) nuclei in blue, magnification 20 \times



b

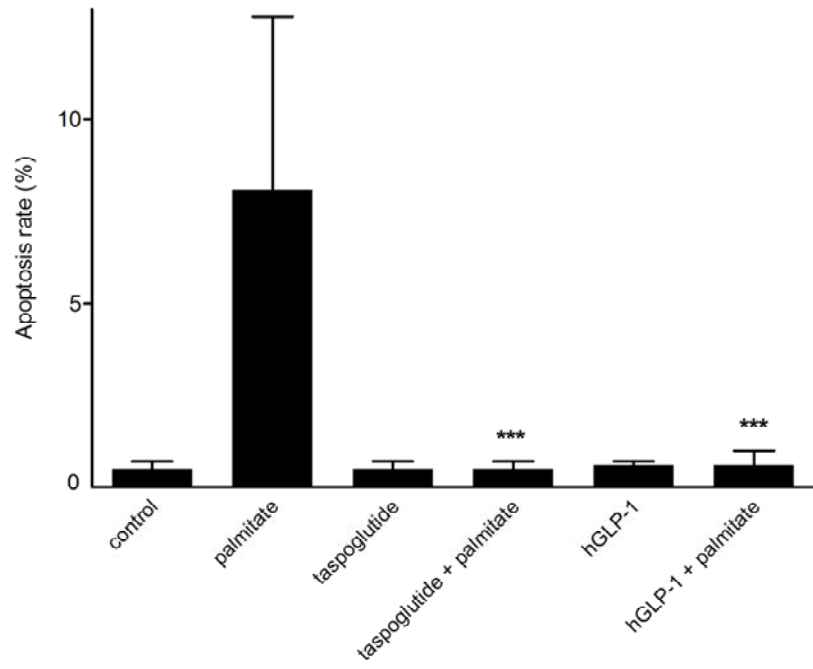
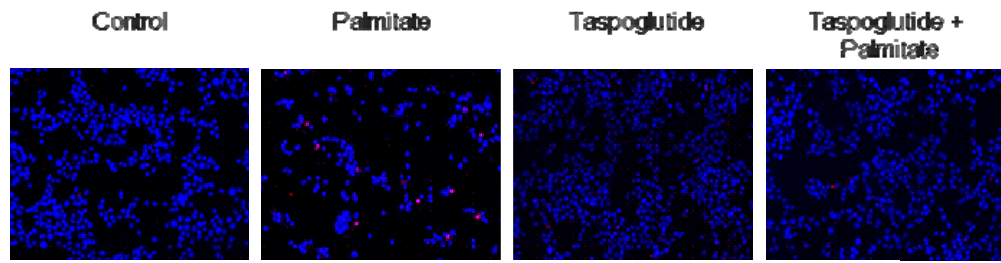


Figure 3. Apoptosis in human islets. (A) Representative photomicrographs of human islets after incubation with 10 nmol/l taspoglutide and/or cytokines (recombinant human IL-1 β 1 μ g/l, INF- γ 5 μ g/l, TNF- α 5 μ g/l) or palmitate 0.5 mmol/l. Magnification 40 \times . TUNEL-positive (apoptotic) nuclei are red, and insulin-stained cells are in green. B: TUNEL intensity scores extracted from randomly selected images of human islets after incubation with taspoglutide and/or cytokines or palmitate, respectively. In total, approximately 160 islets were analyzed. (***) $p < 0.001$. Circle, donor 1; +, donor 2; triangle, donor 3.

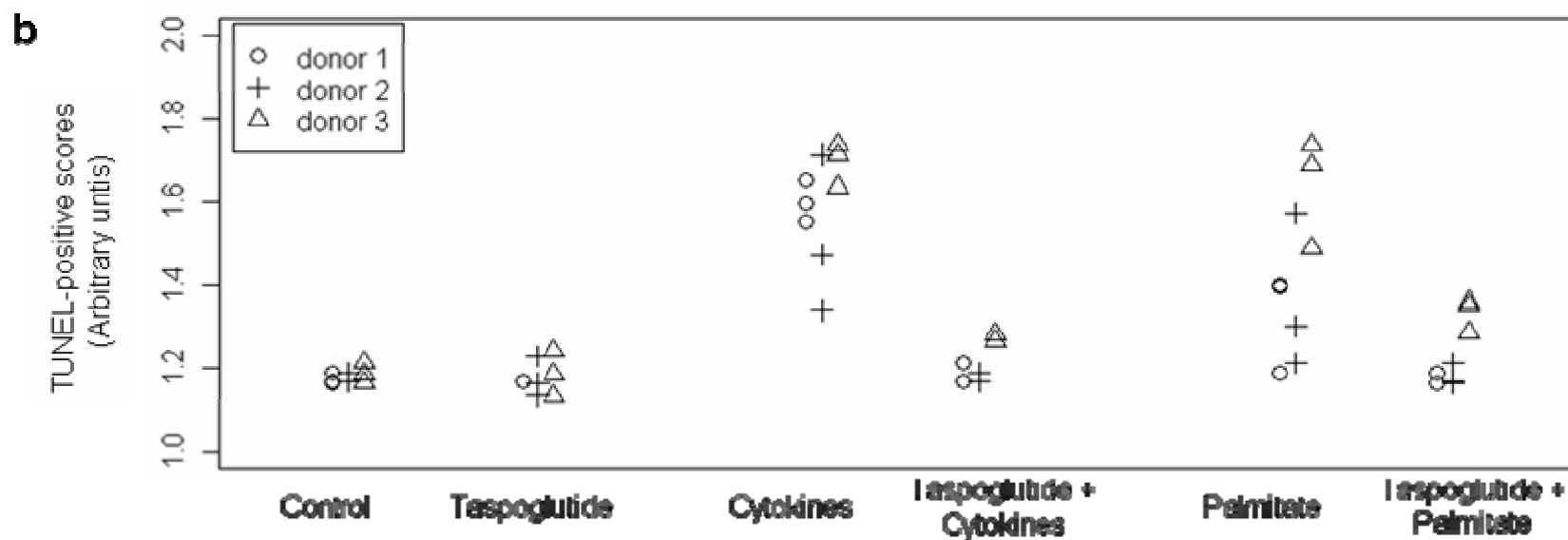
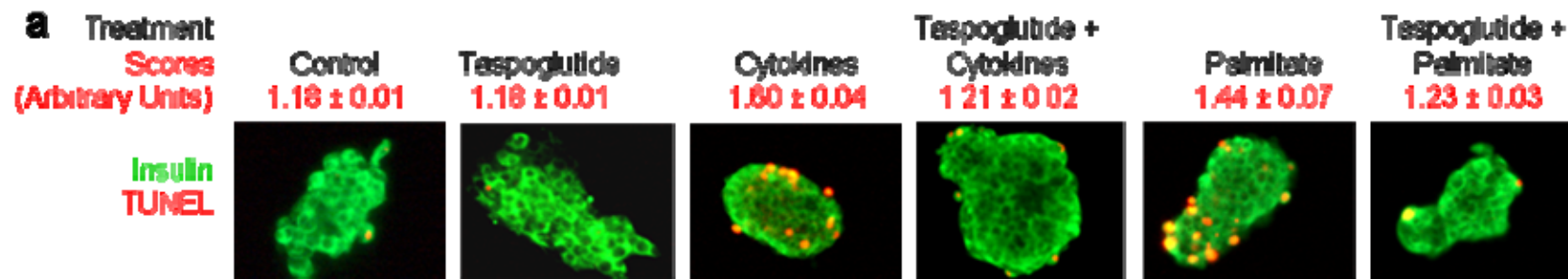


Figure 4. β -Cell proliferation (A) and β -cell apoptosis (B) in $n > 80$ (A) and $n > 430$ (B) islet cross-sections of control ZDF and ZL and treated ZDF rats (6 animals per group; $***p < 0.001$ vs. vehicle-treated 9-week-old ZDF rats). Distribution of the number of proliferating β -cells (C) or apoptotic β -cells (D) as a function of the number of β -cells per islet cross-section. 95% bivariate normal density ellipses are displayed for 6-week-old ZDF rats (grey squares and dotted line) and 9-week-old ZDF rats treated with vehicle (black circles and solid line) or tasopglutide (blue cross and dashed line).

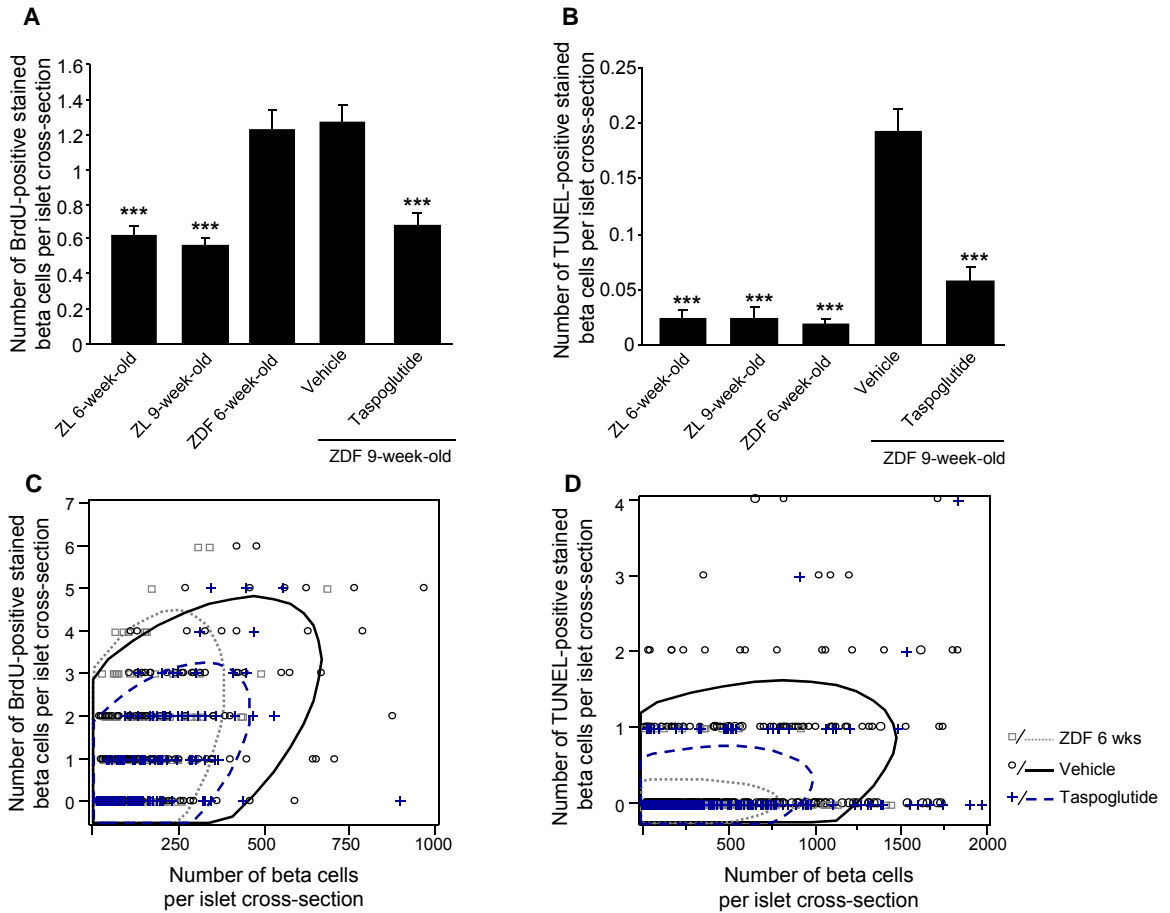


Figure 5. Islet morphology in treated and control rats. *A*: Representative photomicrographs of islet cross-sections, magnification 10×; α -cells were labelled with an anti-glucagon (red) antibody, β -cells with an anti-insulin (green) antibody, and all nuclei were stained with DAPI (blue). *B*: Islet area; $n > 320$ and < 580 islets per group, ($***p < 0.001$ vs. vehicle-treated 9-week-old ZDF rats). *C*: Islet size distribution; small, $<15\,000\ \mu\text{m}^2$ (white bars), medium, $15\,000\ \mu\text{m}^2$ to $<50\,000\ \mu\text{m}^2$ (striped bars), large, $50\,000\ \mu\text{m}^2$ to $<120\,000\ \mu\text{m}^2$ (checkered bars), extreme, $>120\,000\ \mu\text{m}^2$ (black); $n > 320$ and $n < 580$ islet cross-sections per group.

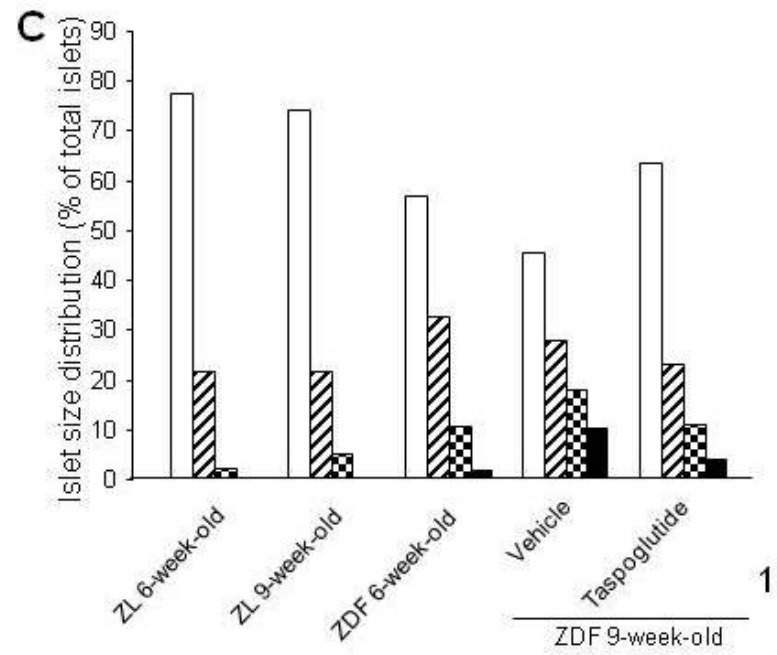
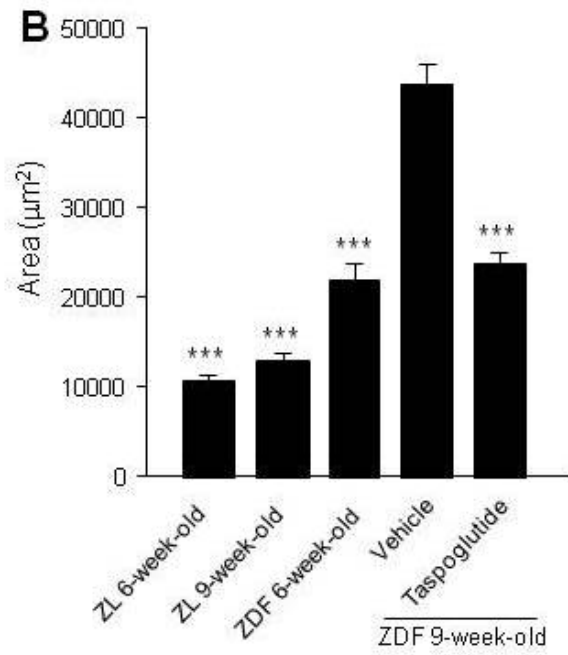
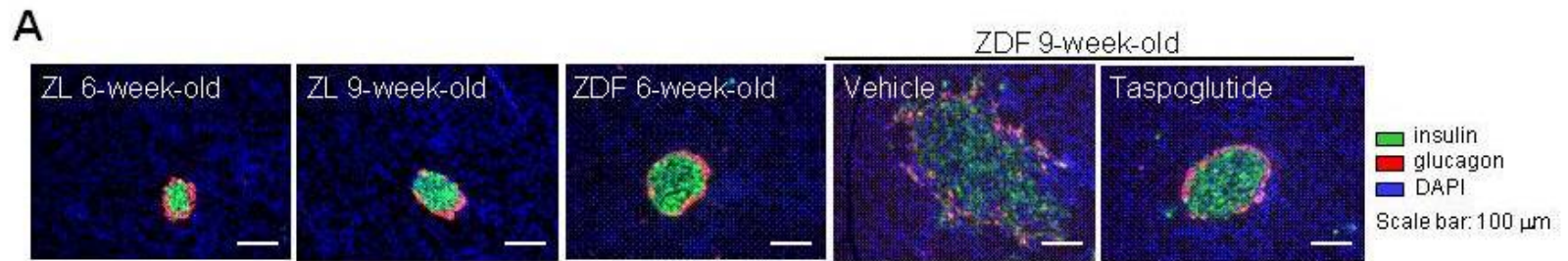


Figure 6. α -Cell distribution within the islet core in treated and control rats. $n > 309$ and $n < 513$ islet cross-sections per group; $***p < 0.001$ versus vehicle-treated 9-week-old ZDF rats.

A: Number of central α -cells per islet cross-section. *B:* Number of central α -cells as a function of islet area per islet cross-section. 95% bivariate normal density ellipses are displayed for 6-week-old ZDF rats (grey squares and dotted line) and 9-week-old ZDF rats treated with vehicle (black circle and solid line) or taspoglutide (blue cross and dashed line).

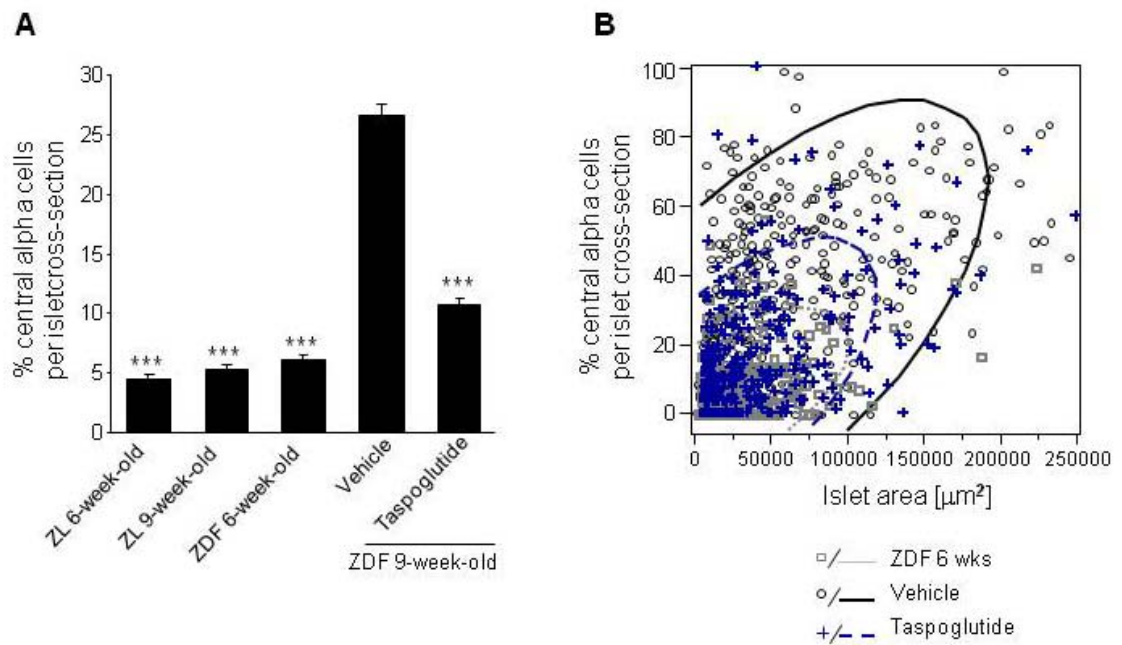


Figure 7. Pancreatic insulin content in treated and control rats. *A*: relative insulin staining intensity in pancreatic islet cross-sections; determined by the sum of insulin-positive pixel intensity values within the islet area divided by the number of pixels in the respective islet cross-section; $n > 320$ and $n < 580$ islet cross-sections per group ($***p < 0.001$ vs. vehicle-treated 9-week-old ZDF rats). *B*: total pancreatic insulin content, $n = 4-6$ rats per group ($*p < 0.05$, $**p < 0.01$ vs. vehicle-treated 9-week-old ZDF rats).

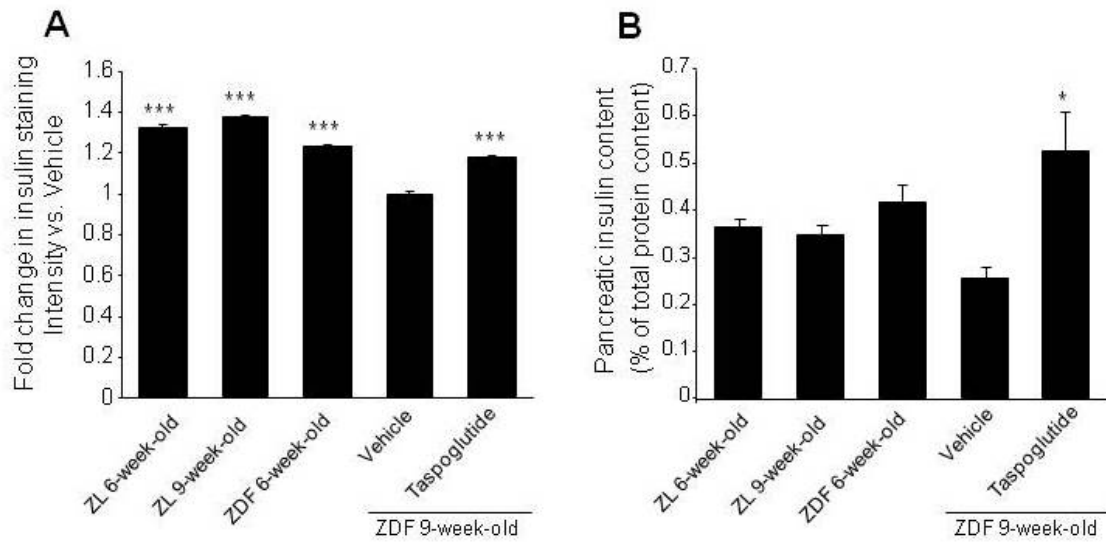


Figure 8. Insulin secretion during *in situ* pancreas perfusion in treated and control rats. *A*: Secretion profile during perfusion with glucose (black dashed line) of 6-week-old ZDF (grey line) and 9-week-old ZDF rats treated with vehicle for taspoglutide (black line) or taspoglutide (blue line). $n = 3-6$ rats per group. *B*: Basal insulin secretion during perfusion with a low concentration of glucose (2.8 mmol/l). *C*: Phase 1 insulin secretion during perfusion with a high concentration of glucose (16.7 mmol/l [Stim]). *D*: Phase 2 insulin secretion during perfusion with 16.7 mmol/l glucose. Figures B, C and D represent the quantification of the data presented in A. $n = 3-6$ rats per group ($*p < 0.05$, $**p < 0.01$ compared with vehicle-treated 9-week-old ZDF rats).

