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Metabolic effects of combined glucagon receptor antagonism and glucagon-like peptide-1 receptor agonism in high fat fed mice

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ABSTRACT

Ablation of glucagon receptor (GCGR) signalling is a potential treatment option for diabetes, whilst glucagon-like peptide-1 (GLP-1) receptor agonists are clinically approved for both obesity and diabetes. There is a suggestion that GCGR blockade enhances GLP-1 secretion and action, whilst GLP-1 receptor activation is known to inhibit glucagon release, implying potential for positive interactions between both therapeutic avenues. The present study has examined the ability of sustained GCGR antagonism, using desHis^{1}Pro^{4}Glu^{9}-glucagon, to augment the established benefits of the GLP-1 mimetic, exendin-4, in high fat fed (HFF) mice. Twice-daily injection of desHis^{1}Pro^{4}Glu^{9}-glucagon, exendin-4 or a combination of both peptides to groups of HFF mice for 10 days had no impact on body weight or energy intake. Circulating blood glucose and glucagon concentrations were significantly ($P < 0.05–0.01$) decreased by all treatment regimens, with plasma insulin levels elevated ($P < 0.001$) when compared to lean control mice. Intraperitoneal and oral glucose tolerance were improved ($P < 0.05–0.01$) by all treatments, despite lack of enhanced glucose-stimulated insulin secretion. Following exogenous glucagon administration, all HFF treatment groups displayed reduced ($P < 0.05–0.001$) glucose and insulin levels compared to HFF saline controls, although peripheral insulin sensitivity was largely unchanged across all animals. Interestingly, all treatments had tendency to increase pancreatic insulin content with pancreatic glucagon content significantly elevated ($P < 0.05$) by all interventions. These studies highlight the capacity of peptide-based GCGR inhibition, or GLP-1 receptor activation, to significantly improve metabolism in HFF mice but suggest no obvious additive benefits of combined therapy.

1. Introduction

The glucagon receptor (GCGR) has long been a target of interest for the development of antidiabetic agents. Initially, GCGR antagonism has been explored based on potential to directly oppose characteristic glucagon mediated elevations of blood glucose levels [1]. However, advances in our understanding of the pathways involved in energy homeostasis have since prompted investigation into the weight-lowering benefits of GCGR agonism, especially in combination with other glucose-lowering drugs [2]. Thus, co-activation of receptors for glucagon and the incretin hormone, glucagon-like peptide-1 (GLP-1), reveal significant improvements in body weight and metabolism in both the preclinical [2,3] and clinical [4] setting, with this treatment strategy currently progressing through clinical trials [5–7]. Therefore, somewhat of a dilemma exists as to whether activation or inhibition of GCGR represents the best strategy to promote antidiabetic actions. Although seemingly counterintuitive, a similar quandary also exists for the incretin hormone glucose-dependent insulinotropic polypeptide (GIP), where positive or negative receptor modulation leads to encouraging benefits on metabolic control [8–10]. Thus, both avenues may still hold promise for development of therapeutically relevant GCGR modulating drugs.

To date, clinical investigation of the antidiabetic efficacy of GCGR inhibition has relied on use of either small molecules [11], monoclonal antibodies [12] or antisense oligonucleotides [13]. Although glucose-lowering efficacy of each individual approach is indisputable, off-target side effects of these non-peptidic molecules

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appears to be a concern [14]. In that respect, preclinical studies in our laboratory using highly specific peptide-based GCGR antagonists, founded on the amino acid sequence of the parent peptide, reveal excellent antidiabetic effectiveness with no obvious safety concerns [15–18]. Indeed, other research groups now appear to be adopting a similar peptide-orientated approach in the pursuit of safe and effective GCGR antagonists [19]. Thus, as previously documented by Victor Hruby and Bruce Merrifield, His^1, Gly^4 and Asp^9 are essential amino acids of glucagon to exert agonist activity when bound to its receptor [20,21]. It follows that the structural modifications present in the well characterised glucagon analogue, desHis^{1}Pro^{4}Glu^9-glucagon, yield a highly effective peptidic GCGR antagonist [18].

Whilst positive effects of GCGR activation in combination with other glucose-lowering agents has been extensively explored in human studies, there has been relatively little investigation of possible benefits of GCGR blockade alongside other established antidiabetic drugs in man. We have previously shown that sustained GCGR blockade using a desHis^{1}Pro^{4}Glu^9-glucagon derivative, in combination with GIP receptor agonism, was an effective means of improving diabetic control in obese-diabetic high fat fed (HFF) mice [22]. The experimental motivation for those studies was based on the established glucagonotropic actions of GIP [23] and hypothesis that this would be effectively curtailed by concurrent GCGR antagonism to result in improved glucose homeostasis. In addition to this, co-administration of a GCGR antagonist with a dipeptidyl peptidase-4 (DPP-4) inhibitor, a drug that augments circulating levels of biologically active GIP and GLP-1, in diabetic mice significantly improved glycaemic control [24]. In that respect, more recent evidence suggests that the metabolic benefits of GCGR blockade are directly linked to upregulation of circulating GLP-1 levels by promoting intestinal L-cell proliferation [25] and inhibiting L-cell apoptosis [26]. Thus, combined GCGR blockade and GLP-1 receptor activation may represent a particularly attractive therapeutic strategy for diabetes [27,28].

Therefore, to probe this concept we have investigated the impact of sub-chronic twice-daily treatment with desHis^{1}Pro^{4}Glu^9-glucagon alongside the clinically approved GLP-1 mimetic, exendin-4, in HFF mice. Effects on food intake, body weight, circulating glucose, insulin and glucagon, as well as glucose tolerance, insulin sensitivity and pancreatic hormone content were assessed.

2. Materials and methods

2.1. Peptide synthesis

desHis^{1}Pro^{4}Glu^9-glucagon and exendin-4 were purchased from GL Biochem Ltd. (Shanghai, China) at greater than 95% purity. To confirm peptide characteristics in-house, purity was confirmed using high performance liquid chromatography (HPLC) analysis with molecular weight measured by matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry, as described previously [29].

2.2. Animals

Lean control male NIH Swiss mice (Envigo Ltd., UK) were provided with a standard rodent diet (10% fat, 30% protein, 60% carbohydrate; percent of total energy 12.99 kJ/g, Trouw Nutrition, Cheshire, UK) throughout. For HFF mice, these animals were maintained on a high fat diet (45% fat, 20% protein, 35% carbohydrate; percent of total energy 26.15 kJ/g; Special Diet Services (SDS), UK) from 8 weeks of age for 150 days. At this point, HFF mice presented with increased body weight and elevated non-fasting blood glucose when compared to lean control mice. All mice were singly caged and housed in an air-conditioned room maintained at 22 ± 2 °C with a 12 h light: 12 h dark cycle (08:00–20:00 h). Drinking water and respective diets was freely available. All animal experiments were conducted in accordance with the UK Animals (Scientific Procedures) Act 1986 and EU Directive 2010/63EU. All necessary steps were taken to prevent any potential animal suffering. The animal studies were approved by local Ulster Animal Welfare and Ethical Review Body (AWERB) committee.

2.3. In vivo experimental procedures

Over a 10-day treatment period, HFF mice (n = 8) received twice daily intraperitoneal (i.p.) injections of either desHis^{1}Pro^{4}Glu^9-glucagon alone (25 nmol/kg bw), exendin-4 alone (25 nmol/kg bw) or a combination of both peptides at the same doses. Control groups of lean and HFF mice received twice daily i.p. injections of saline vehicle (0.9%, w/v, NaCl). Food intake, body weight, and non-fasting blood glucose and plasma insulin were monitored at regular intervals throughout the study period. Intraperitoneal and oral glucose tolerance (18 mmol/kg bw), glucagon tolerance (25 nmol/kg bw) and insulin sensitivity (5 IU/kg bw) tests were performed at the end of the study period. At termination, blood was taken for measurement of plasma glucagon concentrations and pancreatic tissues excised to determine insulin and glucagon content following hormone extraction with 5 ml/g of ice-cold acid ethanol (750 ml ethanol, 235 ml water, 15 ml concentrated HCl).

2.4. Biochemical analysis

Blood samples were collected from the cut tip of the tail vein of conscious mice. Blood glucose was measured using an Ascencia Contour blood glucose meter (Bayer Healthcare, UK). Blood samples were collected into chilled fluoride/heparin glucose microcentrifuge tubes (Sarstedt, Numbrecht, Germany) for plasma insulin analysis. Plasma was separated by centrifugation (30 s at 13,000×*g*) using a Beckman microcentrifuge and stored at −20 °C prior to analysis. Insulin was determined using a modified dextran-coated charcoal radioimmunoassay as described previously [30]. Glucagon was measured by electrochemiluminescent immunoassay using a SECTOR™ Imager 2400 (Meso Scale Discovery, Maryland, USA).

2.5. Statistical analysis

Results are expressed as mean ± SEM and data compared using a one-way ANOVA, followed by the Student-Newman-Keuls *post-hoc* test. Analysis of area under the curve (AUC) were calculated using a trapezoidal rule with baseline subtraction. Groups of data were considered significantly different if $P < 0.05$.

3. Results

3.1. Effect of desHis^{1}Pro^{4}Glu^9-glucagon, exendin-4 or a combination of both peptides on metabolic status in HFF mice

Twice daily administration of desHis^{1}Pro^{4}Glu^9-glucagon, exendin-4 or a combination of both peptides to HFF mice had no effect on body weight, cumulative food intake or plasma insulin

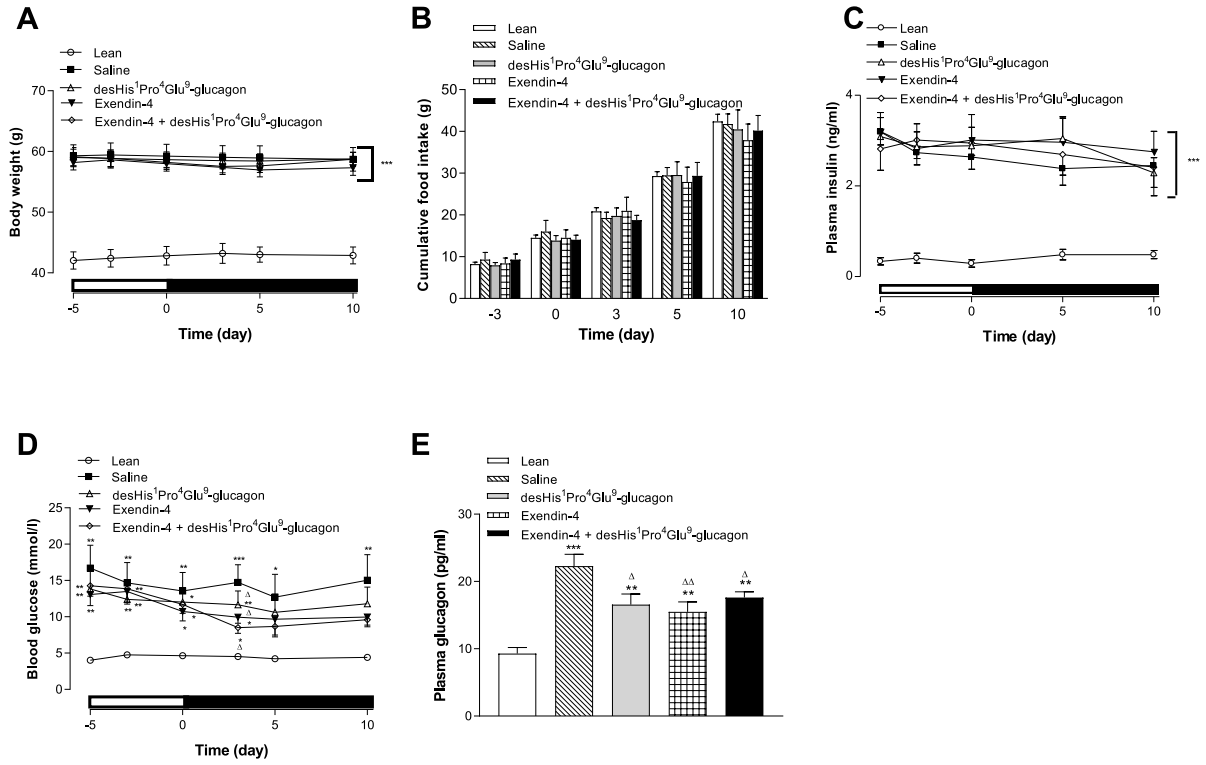

Fig. 1. Effects of twice daily administration of desHis^{1}Pro^{4}Glu^9-glucagon, exendin-4 or a combination of both peptides (each at 25 nmol/kg bw) for 10 days on body weight (A), cumulative food intake (B), plasma insulin (C), blood glucose (D) and terminal plasma glucagon (E). (A–D) Measurements were taken at regular intervals 5 days prior during the treatment period or (E) on day 10. (A,C,D) The treatment period is depicted by the horizontal black bar. Values are mean ± SEM (n = 8). *P < 0.05, **P < 0.01, ***P < 0.001 compared to lean controls. $^{\Delta}$P < 0.05, $^{\Delta\Delta}$P < 0.01, compared to HFF saline controls.

concentrations over the 10-day treatment period when compared to saline treated HFF controls (Fig. 1A–C). Moreover, body weight and plasma insulin levels were elevated (P < 0.001) in all HFF mice when compared to lean controls (Fig. 1A,C). However, significant (P < 0.05) decreases in circulating glucose levels were observed on day 3 in all treatment groups compared to HFF saline controls (Fig. 1D), and by the end of the study, glucose levels of all HFF treatment groups were not different when compared to lean control mice (Fig. 1D). Interestingly, all treatment interventions decreased (P < 0.05-P < 0.01) circulating glucagon concentrations, but these were still elevated (P < 0.01) when compared to lean control mice (Fig. 1E).

3.2. *Effect of desHis^{1}Pro^{4}Glu^9-glucagon, exendin-4 or a combination of both peptides on glucose tolerance as well as metabolic responses to exogenous insulin in HFF mice*

Twice daily administration of desHis^{1}Pro^{4}Glu^9-glucagon, exendin-4 or a combination of both peptides for 10 days significantly (P < 0.05-P < 0.01) improved glucose disposal following an i.p. glucose challenge in HFF mice (Fig. 2A), which was fully corroborated by 0–60 min AUC values (Fig. 2B). Moreover, the glycaemic profile in all treated HFF mice was comparable to that of lean control mice (Fig. 2A and B). A strikingly similar response to an oral glucose load was also observed in these HFF mice (Fig. 3A and B). Interestingly, in terms of glucose-stimulated insulin secretion, both i.p. (Fig. 2C and D) and oral (Fig. 3C and D) glucose resulted in decreased insulin output in all HFF treatment groups when compared to saline control mice, which was particularly pronounced following oral glucose administration (Fig. 3C and D). Peripheral insulin sensitivity was largely similar in all HFF mice (Fig. 4), but saline and exendin-4 treated HFF animals had elevated (P < 0.05) glucose levels when compared to lean control mice at the 60 min post-injection observation point (Fig. 4A). However, 0–60 min area above the curve (AAC) values that relate to the overall glucose-lowering effects of exogenous insulin injection were not different between all groups of mice (Fig. 4B).

3.3. *Effect of desHis^{1}Pro^{4}Glu^9-glucagon, exendin-4 or a combination of both peptides on glucagon tolerance as well as pancreatic insulin and glucagon content in HFF mice*

All treatment intervention groups of HFF mice were associated with diminished (P < 0.05-P < 0.001) hyperglycaemic action of 25 nmol/kg glucagon administration at the end of the study, both in terms of individual (Fig. 5A) and 0–60 min AUC values (Fig. 5B). Moreover, glucose levels were similar to those observed in lean control mice (Fig. 5A and B). Glucagon-mediated elevations in plasma insulin concentrations were increased (P < 0.01-P < 0.001) in all HFF mice compared to lean controls, but all treatments reduced (P < 0.05) insulin secretion when compared to HFF control mice (Fig. 5C and D). Pancreatic glucagon content was elevated (P < 0.05) by all treatment interventions when compared to HFF saline control mice (Fig. 6A), which was associated with a small increase in pancreatic insulin content (Fig. 6B).

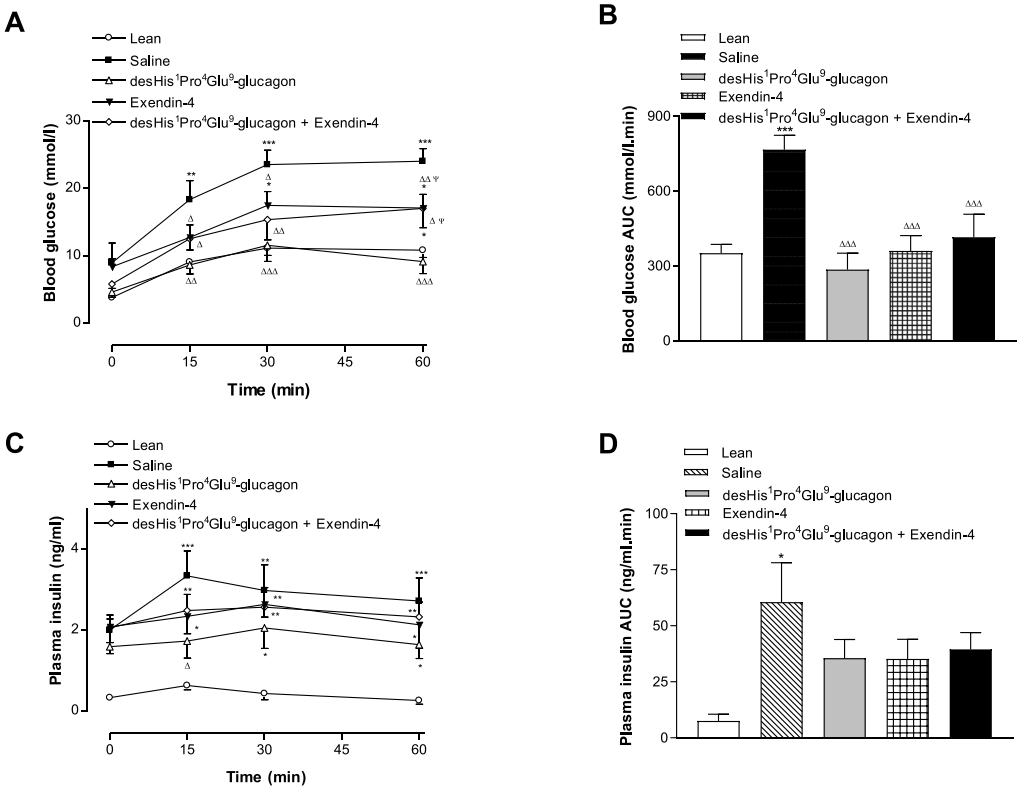

Fig. 2. Effects of twice daily administration of desHis^{1}Pro^{4}Glu^{9}-glucagon, exendin-4 or a combination of both peptides (each at 25 nmol/kg bw) for 10 days on intraperitoneal glucose tolerance. Glucose (18 mmol/kg bw) was injected at t = 0 min following 10 days of treatment. Blood glucose (A) and associated plasma insulin responses (C) are depicted, alongside respective 0–60 min area under the curve (B,D) data. Values are mean ± SEM (n = 8). *P < 0.05, **P < 0.01, ***P < 0.001 compared to lean controls. $^{\Delta}$P < 0.05, $^{\Delta\Delta}$P < 0.01, $^{\Delta\Delta\Delta}$P < 0.001 compared to HFF saline controls.

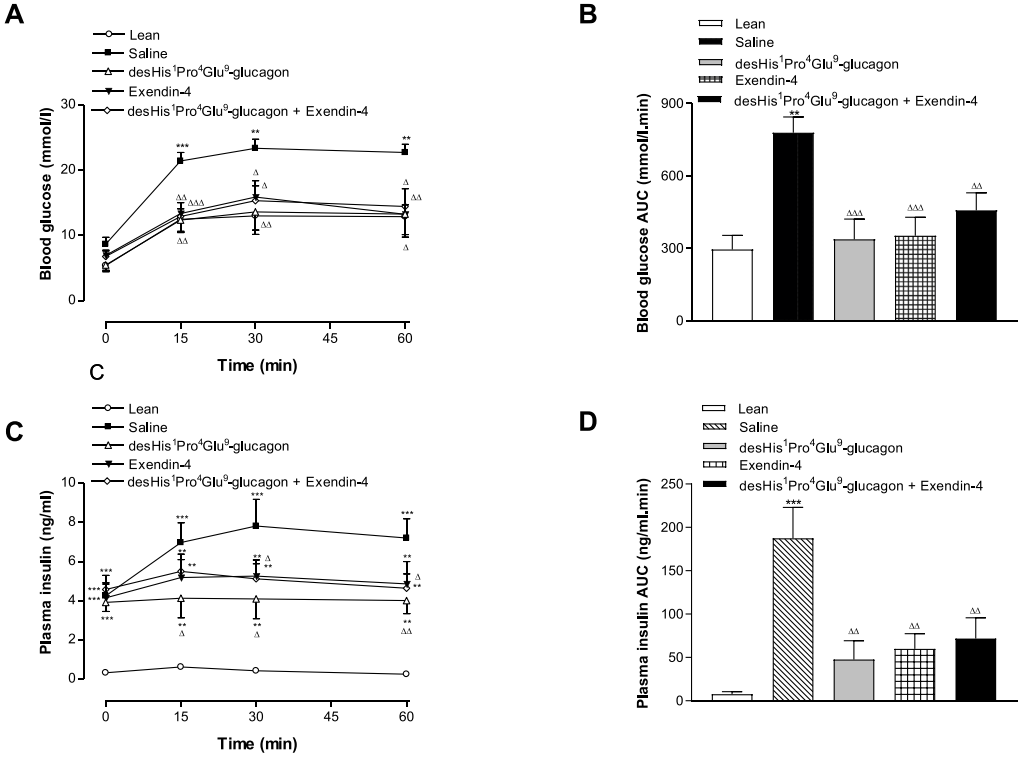

Fig. 3. Effects of twice daily administration of desHis^{1}Pro^{4}Glu^{9}-glucagon, exendin-4 or a combination of both peptides (each at 25 nmol/kg bw) for 10 days on oral glucose tolerance. Glucose (18 mmol/kg bw) was administered by gavage at t = 0 min following 10 days of treatment. Blood glucose (A) and associated plasma insulin responses (C) are depicted, alongside respective 0–60 min area under the curve (B,D) data. Values are mean ± SEM (n = 8). *P < 0.05, **P < 0.01, ***P < 0.001 compared to lean controls. $^{\Delta}$P < 0.05, $^{\Delta\Delta}$P < 0.01, $^{\Delta\Delta\Delta}$P < 0.001 compared to HFF saline controls.

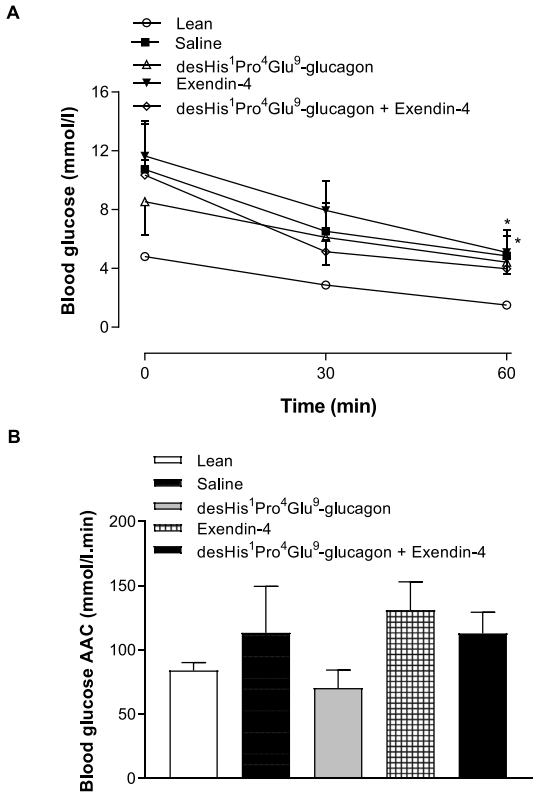

Fig. 4. Effects of twice daily administration of desHis[1]Pro[4]Glu[9]-glucagon, exendin-4 or a combination of both peptides (each at 25 nmol/kg bw) for 10 days on peripheral insulin sensitivity. Insulin (5 IU/kg/bw) was administered at t = 0 min in non-fasted mice. Blood glucose (A) and associated 0–60 min area above the curve (B) data are depicted. Values are mean ± SEM (n = 8). *P < 0.05 compared to lean controls.

4. Discussion

Variability in the aetiology and progression of type 2 diabetes in humans results in numerous pharmacological agents being used at different stages of the disease, with drug failure representing another major compounding factor [31]. Indeed, the importance of treatment stratification to better manage within-person responses to antidiabetic agents, and reduce failure rates, is becoming more apparent in recent times [32]. Another approach to improve the overall effectiveness of conventional diabetes treatment options is earlier initiation of multiple medications, particularly in relation to regulatory peptide hormone therapeutics where dual and triple acting unimolecular compounds are exhibiting pronounced and sustained metabolic benefits [33,34].

However, overall control of metabolism by the numerous regulatory hormones secreted into the bloodstream is highly complex. For example, on one hand antagonism of receptors for the incretin hormone GIP demonstrates therapeutic promise for obesity-diabetes, either alone [10,35] or in combination with GLP-1 receptor agonism [36,37]. Whereas on the other hand, activation of receptors for GIP also exhibits positive effects, both alone [38,39] and together with concurrent GLP-1 receptor activation [33,34,40]. The picture is similarly unclear for GCGR modulation, with both activation and inhibition evoking benefits in diabetes [2,18], although sound scientific justification does exist for both strategies [14]. To date, GCGR agonism combination therapy, especially alongside GLP-1 receptor mimetics, has revealed significant benefits in preclinical and clinical studies [2,41,42].

The potential beneficial impact of a GCGR antagonist in combination with a GLP-1 receptor agonist has not been fully investigated. One study has employed a dual-acting unimolecular GLP-1 receptor agonist/GCGR antagonist peptide, named PEG-DAPD [43]. However, earlier studies with PEG-DAPD suggest that this molecule can activate the GCGR [27], thus any observed benefits may be linked to the established positive effects of dual GLP-1 and glucagon receptor activation [34]. Therefore, to fully address the concept of additive actions of GCGR antagonism and GLP-1 receptor activation, we have employed 10-days twice-daily treatment with the well-characterised GCGR antagonist, desHis[1]Pro[4]Glu[9]-glucagon [15,16], together with the clinically approved GLP-1 mimetic, exendin-4, in HFF mice.

Sub-chronic administration of desHis[1]Pro[4]Glu[9]-glucagon, exendin-4 or a combination of both peptides to HFF mice for 10 days did not affect food intake or body weight. This contrasts with the satiety and energy mobilising actions of GLP-1 and glucagon receptor activation, respectively [44,45]. Increased palatability of the high fat diet could represent one explanation for this lack of effect, although it is known that neural circuits regulating satiety and energy balance are highly complex and exhibit inherent plasticity to help maintain homeostasis [46]. In addition, higher doses may be required to reveal the centrally mediated effects of peripherally administered peptides. Notwithstanding this, all treatment interventions significantly reduced circulating plasma glucose levels and improved glucose tolerance in response to both an oral and intraperitoneal glucose challenge. Improvements in glucose handling were not linked to significant augmentation of glucose-stimulated insulin secretion, despite obvious hyperinsulinaemia in all HFF mice. This contrasts with the well characterised insulinotropic actions of GLP-1 receptor activation [47] but may simply reflect the glucose dependency of the peptide together with the near restoration of normoglycaemia. It should also be noted that the hypoglycaemic actions of exogenous insulin were relatively similar in lean control and HFF mice which might reflect the regression of glucose toxicity in the HFF treatment groups mice [48].

Blocking the GCGR is believed to improve metabolism, in part, through augmenting GLP-1 secretion and action [14]. Thus, although we were unable to assess plasma GLP-1 levels due to limited volumes of blood that can be withdrawn from mice, co-administration of exendin-4 alongside desHis[1]Pro[4]Glu[9]-glucagon should enhance this reciprocal pathway. However, we did not observe any obvious benefits of combined therapy over the individual monotherapy approaches. This could reflect the good efficacy of each treatment alone as well as the duration of treatment regimens, or it may simply be that GCGR antagonism in combination with GLP-1 receptor agonism does not offer additive benefits. The recognised ability of GLP-1 to suppress glucagon secretion could also represent another contributing factor [49], thereby obviating the potential benefit of GCGR antagonism. In addition to this, native glucagon has been reported to bind with low affinity to the GLP-1 receptor [50], and it is therefore conceivable that desHis[1]Pro[4]Glu[9]-glucagon could partially impede GLP-1 receptor signalling. Indeed, if anything, the glycaemic responses to oral glucose were marginally less favourable inidesHis[1]Pro[4]Glu[9]-glucagon treated HFF mice when compared to an intraperitoneal glucose challenge. As such, the previously observed benefits of GCGR antagonism in combination with DPP-4 inhibition [24], may be linked to additive effects with GIP, rather than GLP-1, receptor activation [22]. Glucagon

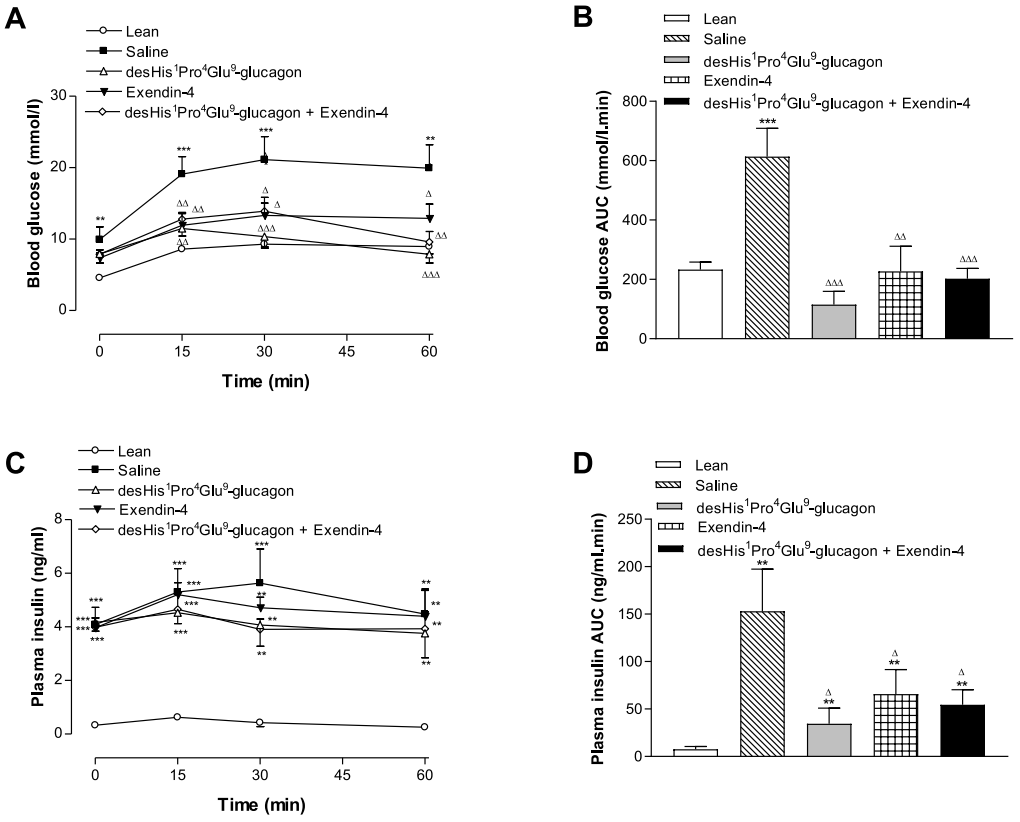

Fig. 5. Effects of twice daily administration of desHis[1]Pro[4]Glu[9]-glucagon, exendin-4 or a combination of both peptides (each at 25 nmol/kg bw) for 10 days on glucagon tolerance. Glucagon (25 mmol/kg bw) was injected at t = 0 min following 10 days of treatment. Blood glucose (A) and associated plasma insulin responses (C) are depicted, alongside respective 0–60 min area under the curve (B,D) data. Values are mean ± SEM (n = 8). **P < 0.01, ***P < 0.001 compared to lean controls. $^{\Delta}$P < 0.05, $^{\Delta\Delta}$P < 0.01, $^{\Delta\Delta\Delta}$P < 0.001 compared to HFF saline controls.

tolerance tests at the end of the study highlight lack of tachyphylaxis with twice-daily desHis[1]Pro[4]Glu[9]-glucagon administration, eliminating this as a potential reason for lack of additive effects with exendin-4.

As would be expected [49], plasma glucagon levels were reduced by exendin-4 treatment, but more intriguingly similar reductions in circulating glucagon were also evident in desHis[1]Pro[4]-Glu[9]-glucagon treated HFF mice. This is somewhat unexpected given previous work with small molecule GCGR antagonists [51], but our observations do have their counterparts in earlier studies employing peptide-based GCGR annulment in HFF mice [22]. Moreover, discontinuation of small molecule GCGR antagonist therapy has been documented to provoke rebound hyperglycaemia [52], likely as a result of elevated circulating glucagon. Thus, desHis[1]Pro[4]Glu[9]-glucagon may remove this potential drawback observed with non peptide-based GCGR antagonist therapies. However, there were clear elevations of pancreatic glucagon in desHis[1]Pro[4]Glu[9]-glucagon treated HFF mice, although this occurred in concert with a mild elevation of pancreatic insulin concentrations, leading to largely maintained overall pancreatic hormone balance. Moreover, exendin-4 therapy evoked similar changes suggesting this effect may be specific to this mouse model. Typically, GCGR antagonism is thought to increase pancreatic alpha-cell mass [53], but this has been suggested to be less prominent in adults [54]. Indeed, sub-chronic administration of desHis[1]Pro[4]Glu[9]-glucagon to normal adult mice had no obvious adverse effects on pancreatic morphology [16]. Comparable to the current setting, concurrent elevations in pancreatic alpha- and beta-cell mass have been demonstrated with GCGR antagonism in severely insulin resistant mice [55]. Moreover, recent observations on islet cell transdifferentiation events suggest that alpha-cells can act as precursors for mature functional beta-cells, particularly under situations of islet stress [56]. Thus, increased alpha-cell mass may simply represent natural plasticity of islet endocrine cells in response to dietary or drug intervention, in a bid to ultimately help maintain beta-cell mass and normal metabolic state. In agreement, a monoclonal GCGR antibody was recently shown to restore functional beta-cells in type 1 diabetic mice and enhance the overall secretory function of human islets [57], substantiating translatable benefits of GCGR blockade at the level of the endocrine pancreas.

In conclusion, GCGR antagonism is a proven strategy for improving glucose homeostasis in diabetic animals and patients, but overall safety does still need to be confirmed [58]. In that respect, peptide-based GCGR antagonists may offer comparable efficacy, but with a reduced side-effect profile, when compared to low molecular weight comparator drugs [16,18]. However, unlike others [43], we reveal limited evidence for benefits of sustained GCGR antagonism in combination with GLP-1 receptor activation. Taken together, the current study demonstrates that GCGR blockade is equally as effective as exendin-4 in terms of improving metabolism in HFF mice, but despite the differing modes of action of both compounds, there is no evidence for additive therapeutic benefits.

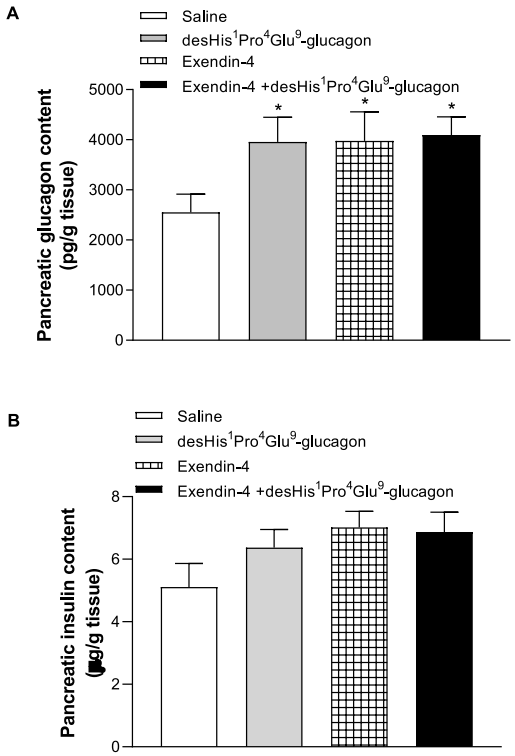

Fig. 6. Effects of twice daily administration of desHis^{1}Pro^{4}Glu^9-glucagon, exendin-4 or a combination of both peptides (each at 25 nmol/kg bw) for 10 days on pancreatic hormone content. Pancreatic glucagon (A) and insulin (B) levels were assessed in excised pancreatic tissue, following acid-ethanol hormone extraction, using a commercially available electrochemiluminescent immunoassay or an in-house radioimmunoassay, respectively. Values are mean ± SEM (n = 8), expressed in relation to total pancreas weight. *P < 0.05 compared to HFF saline controls.

Author contributions

NI and FOH conceived/designed the study. NI, RAL and PRF drafted the manuscript. LMMcS and ZJF and participated in the conduct/data collection and analysis and interpretation of data. All authors revised the manuscript critically for intellectual content and approved the final version of the manuscript.

Data availability statement

The authors declare that the data supporting the findings of this study are available within the article. Any additional raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Declaration of competing interest

The authors declare that no conflicting interests exist.

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