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Weight reducing, lipid lowering and antidiabetic activities of a novel AVP analogue acting at V1a and V1b receptors in high fat fed mice

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Short title: Antidiabetic actions of AVP analogues

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Abstract

Aim: Evidence suggests beneficial metabolic effects of the nonapeptide hormone, arginine vasopressin (AVP), on metabolism, as also observed recently with the closely related oxytocin peptide.

Materials and Methods: In the current study we have exchanged selected amino acids at position 3 and 8 of AVP, namely phenylalanine and arginine, with those of oxytocin to generate novel analogues with altered receptor selectivity. Secondary modification by N-terminal acetylation was used to impart stability to circulating endopeptidases. Analogues were screened for degradation, bioactivity in rodent/human clonal beta-cells and primary murine islets together evaluation of receptor activation profile.

Results: Analogue Ac3IV, which lacked effects at V2 receptors responsible for modulation of fluid balance, was selected as the lead compound for assessment of antidiabetic efficacy in high fat fed (HFF) mice. Twice daily administration of Ac3IV, or the gold-standard control exendin-4, for 22 days reduced energy intake as well as body weight and fat content. Both interventions decreased circulating glucose levels, enhanced insulin sensitivity and substantially improved glucose tolerance and related insulin secretion in response to an intraperitoneal or oral glucose challenge. The peptides decreased total- and increased HDL-cholesterol, but only Ac3IV decreased LDL-cholesterol, triglyceride and non-fasting glucagon concentrations. Elevations of islet and beta cell areas were partially reversed, accompanied by suppressed islet cell proliferation, decreased beta-cell apoptosis and, in the case of exendin-4, also decreased alpha-cell apoptosis.

Conclusion: AVP-based therapies that exclusively target V1a and V1b receptors may have significant therapeutic potential for the treatment of obesity and related diabetes, that merits further clinical exploration.

Keywords: Vasopressin, receptor selectivity, enzymatic stability, obesity, type 2 diabetes

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1. Introduction

The past 15 years have witnessed a substantial increase in the treatment options available for type 2 diabetes with the timely introduction of glucagon-like peptide-1 (GLP-1) mimetics, as well as dipeptidyl peptidase-4 (DPP-4) and sodium-glucose co-transporter-2 (SGLT-2) inhibitors [1]. In particular, the clinical success of GLP-1 mimetics has illustrated the benefit of simultaneous activation of multiple physiological pathways by a single peptide entity, conferring positive effects on insulin secretion and action, gut motility, appetite and body weight as well as cardiovascular and neuronal function [2]. Unimolecular dual or even triple acting peptides targeting GLP-1, GIP and glucagon receptors are now being developed to modulate multiple receptor sites [3,4]. Other peptides being explored include apelin, peptide YY (PYY), cholecystokinin (CCK-8), fibroblast growth factor-21 (FGF-21), oxyntomodulin, irisin, obestatin and xenin [5,6]. Given present imperfections in attempts to normalise blood glucose and prevent risk of diabetic complications [5], the search continues for other naturally occurring peptide hormones with beneficial action profiles that can be structurally modified to confer enzyme stability and long duration of action for potential exploitation in T2DM therapy [4].

Arginine vasopressin (AVP), also known as antidiuretic hormone (ADH) is a nonapeptide synthesised in the hypothalamus and secreted by the posterior pituitary gland [7]. Although the classically recognised physiological action of AVP is in the regulation of fluid balance and cardiovascular function [8], an increasing body of evidence suggests that AVP plays an important role in glucose homeostasis and metabolic control [9]. The biological effects of AVP are mediated through modulation of three separate GPCRs, namely *Avpr1a* (V1a), *Avpr1b* (V1b) and *Avpr2* (V2) [10]. Whilst the V2 receptor is responsible for the effects of AVP on the kidney and water retention [11], V1a and V1b receptors are expressed in metabolically active tissues [10]. Studies in AVP receptor knockout (KO)

rodents confirm a role for AVP signalling in metabolism. Thus, plasma glucose levels are elevated in V1a receptor KO mice [12] but reduced in V1b KO mice [13]. Mice with double V1a and V1b receptor KO present with unaltered circulating glucose levels, but this is accompanied by hyperinsulinaemia and glucose intolerance [14]. Furthermore, an inherent genetic mutation leading to lack of AVP production in rats results in reduced plasma insulin concentrations [15]. Genetic variation in AVP receptor expression in humans is also associated with obesity and increased prevalence of diabetes [16,17].

In addition, AVP receptors are present on pancreatic islets and their activation directly evokes insulin secretion and protects against beta cell loss [18]. AVP is also known to stimulate glucagon secretion [19], but AVP-induced insulin release only occurs in the presence of high glucose concentrations, whereas effects on glucagon secretion are only apparent when glucose levels are low [20]. This represents a physiologically important action in the maintenance of glucose homeostasis, linked to prevention of both hyper- and hypoglycaemia. Such a biological characteristic is also evident with the incretin hormone GIP [5,21], which is now considered to possess bona fide therapeutic promise for diabetes [22,23]. Taken together, these observations suggest that AVP may have untapped potential for exploitation in the treatment of diabetes and related metabolic disorders.

Interestingly, AVP has a strikingly similar structure to oxytocin with the two nonapeptides only differing at positions 3 and 8, with phenylalanine and arginine in AVP being replaced by isoleucine and leucine in oxytocin [24]. Although native oxytocin is best known for positive effects on mood and reproductive function [25], enzyme resistant analogues have recently been developed and shown to exert notable benefits on body weight, glucose homeostasis, lipid metabolism and pancreatic architecture in HFF mice [26,27]. We hypothesised that the similarity in structure between the two hormones provides a good foundation for the generation of novel and therapeutically interesting peptides that could

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positively interact with both oxytocin and AVP receptor subtypes. In an attempt to generate such compounds, we designed six novel AVP peptides where amino acids at positions 3 and 8 in AVP were replaced by those of oxytocin (Table 1). We also examined effects of removal of the characteristic disulphide bridge plus addition of N terminal acetyl group to impart enzyme resistance, as employed previously [27].

The AVP analogues were initially screened for enzymatic stability, insulin secretory responses *in vitro* and *ex vivo* together with receptor activation profile. The lead peptide emerging from these studies, namely Ac3IV, which lacked appreciable effects at V2 receptors, was progressed to antidiabetic efficacy testing in high fat fed (HFF) mice in head-to-head comparison with the clinically approved GLP-1 mimetic, exenatide. Our results suggest that such designer AVP analogues constitute a potentially exciting new drug class meriting further exploration for the treatment of obesity and type 2 diabetes.

2. Materials and Methods

2.1 Peptides

All peptides (Table 1) were obtained from Synpeptide Co. Ltd. (Shanghai, China) at 95% purity. Characterisation of the peptides was carried out in-house by HPLC and MALDI–ToF MS, as described previously [28]. Abbreviated names are used for AVP analogues, denoting as appropriate, the position and type of amino acid substitutions in AVP (where AVP is abbreviated to V), namely 3IV and 8LV, presence of N-acetyl group (abbreviated to Ac) and indication of reduced form without disulphide bridge by letter R.

2.2 Plasma stability

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To establish *in vitro* stability of the peptides, peptides (10 μg) were incubated with 5 μl of overnight (18 h) fasted murine plasma at 37ºC, with degradation profiles acquired using RP-HPLC and MALDI-ToF, as described previously [29].

2.3 *In vitro* and *ex vivo* insulin secretion

Rat BRIN BD11 and human 1.1B4 cells were utilised to investigate the influence of test peptides on insulin release. The origin and secretory characteristics of these clonal beta-cells have been detailed in full elsewhere [30,31]. Insulin secretory activity (20 min) of test peptides (10^{-12} – 10^{-6} M) at 5.6 and 16.7 mM glucose was determined as previously described [27]. In a separate series, BRIN BD11 cells were incubated at 16.7 mM glucose with test peptides (10^{-6} M) alone, or in combination with 10^{-6} M of either a selective oxytocin (L-351,257; Tocris), V1a (SR-49059, Sigma-Aldrich), V1b (Nelivaptan; SR -149415, Axon Medchem) or V2 (Tolvaptan, Sigma-Aldrich) receptor antagonist, and insulin secretion determined as described above. Given somewhat unexpected lack of effect of L-351,257 on Ac3IV mediated insulin secretion, insulin secretory effects of Ac3IV were examined in INS1 832/13 cells with CRISPR-Cas9 induced knockout (KO) of the oxytocin receptor. Full details on the generation and characterisation of the KO cell line have previously been reported [27]. For isolated islets studies, islets were obtained from male C57BL/6 mice by standard collagenase digestion, as described previously [32]. Fresh islets were preincubated for 30 min at 37°C in Krebs–Ringer bicarbonate buffer (pH 7.4) supplemented with 10 mM HEPES, 0.1% bovine serum albumin and 1.1 mM glucose, prior to conducting insulin secretion studies (n=4) over the following 60 min.

2.4 Animals

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Studies were conducted in 20-week old HFF male NIH Swiss mice (Envigo Ltd, UK) previously maintained on a high fat diet for 12 weeks (45% fat, 20% protein, 35% carbohydrate; percent of total energy 26.15 kJ/g; Dietex International Ltd., Witham, UK; catalogue number 824018) to evoke dietary induced obesity-diabetes. All mice were housed individually and kept in a temperature-controlled environment (22 ± 2 °C), with a 12 hour light/dark cycle. Experiments were carried out in accordance with the UK Animal Scientific Procedures Act 1986. All animal studies were approved by the University of Ulster Animal Welfare and Ethical Review Body (AWERB). No adverse effects were observed during the *in vivo* procedures.

2.5 *In vivo* experimental design

HFF mice were allocated into comparable groups (n=8) based on blood glucose and body weight and were administered either saline vehicle (0.9% (w/v) NaCl, i.p.), Ac3IV or exendin-4 (both at 25 nmol/kg, bw, i.p.) twice daily for 22 days. This peptide dose was chosen based on our previous studies assessing metabolic effects of AVP in mice [18]. An additional control group of HFF mice receiving either native AVP or oxytocin was not employed due to the short biological half-lives of these peptides [18,27]. Cumulative food and fluid intake, body weight, non-fasting glucose and insulin concentrations were monitored at regular intervals. At the end of the treatment period, plasma was collected for assessment of circulating glucagon and lipids. In addition, i.p. and oral glucose (18 mmol/kg) tolerance tests were performed in 18 h fasted mice, with peripheral insulin sensitivity (15 U/kg bw, i.p.) examined tests in non-fasted mice, at the end of the study. At termination, body composition was assessed by dual energy X-ray absorptiometry (DEXA) scanning using a PIXImus densitometer (GE Medical Systems, USA), prior to subsequent analyses detailed below [33].

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2.6 Terminal analyses

Following completion of DEXA analysis, pancreatic tissues were excised and processed for either histological analysis or hormone content. Briefly, pancreatic tissues were divided longitudinally with half snap frozen for hormonal content measurement as described previously [34], and the other half processed for immunohistochemical analysis using standard laboratory methods, as previously described from our laboratory [35].

2.7 Biochemical analysis

Blood samples were collected from the cut tip on the tail vein of conscious mice. Blood glucose was measured immediately using a hand-held Ascencia Contour blood glucose meter (Bayer Healthcare, Newbury, Berkshire, UK). For plasma insulin and glucagon, blood was collected in chilled fluoride/heparin coated micro-centrifuge tubes (Sarstedt, Numbrecht, Germany) and centrifuged using a Beckman micro-centrifuge (Beckman Instruments, Galway, Ireland) for 10 minutes at 12,000 rpm. Plasma was separated and stored at -20°C, until determination of plasma insulin by radioimmunoassay [36] and glucagon by a commercially available ELISA kit (EZGLU-30K, Merck Millipore). Plasma lipid profile was assessed by an ILab 650 Clinical Analyser (Instrumentation Laboratory, Warrington, UK).

2.8 Statistical analysis

Analyses were performed using GraphPad PRISM software (Version 5.0). Values are expressed as mean ± S.E.M. Comparative analyses between groups were carried out using a One-way ANOVA with Bonferroni's post hoc test or student's unpaired t-test, as appropriate. The difference between groups was considered significant if $P<0.05$.

3. Results

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3.1 Plasma degradation

AVP was fully degraded by a 4 hour incubation in plasma (Table 1). In addition, oxytocin and all non N-terminally acetylated AVP analogues were also degraded by more than 60% during the 4 hour incubation (Table 1). N-acetylation of the cysteine residue in 3IV or 3IVR generated AVP analogues that were completely stable (Table 1).

3.2 Effects of AVP analogues on *in vitro* insulin secretion

Representative dose-dependent insulin secretory responses of native AVP and oxytocin, as well as the two enzymatically stable analogues, namely Ac3IV and Ac3IVR, in both cell lines are depicted in Figure 1A-D. As expected, AVP induced prominent insulinotropic effects, with both AVP analogues also displaying clear dose-dependent actions in each cell line (Figure 1A-D). Full concentration-response curves could not be obtained, thus precluding calculation of EC_{50} values, as well as any comment about relative efficacy of these compounds in the different cell lines. Nonetheless, clear insulinotropic efficacy of Ac3IV was confirmed in murine islets at a concentration of 10^{-6} M (Figure 1E).

3.3 *In vitro* receptor activation profile of AVP analogues

As expected, AVP-induced insulin secretion was strongly linked to activation of oxytocin, V1a and V1b receptors, and to a lesser degree with V2 receptors (Table 1). Removal of the disulphide bridge in AVP completely abolished activity at V2 receptors (Table 1). Interestingly, the insulinotropic activity of 8LV was related to interaction with all oxytocin and vasopressin receptors subtypes, and especially V2 receptors (Table 1). Activity at all receptors, barring V1b, was dramatically decreased by disulphide bond removal (Table 1). Substitution of isoleucine for phenylalanine in AVP represented by 3IV, had minimal impact of receptor activation profile in BRIN BD11 cells (Table 1), but removal of the characteristic

AVP disulphide bridge, substantially decreased activity at the level of the oxytocin receptor and annulled all V2 receptor activity (Table 1). N-terminal acetylation of 3IV, to yield Ac3IV, diminished activity at oxytocin receptors, as evidence by the marked reduction in the ability of the oxytocin-receptor antagonist L-351,257 to reduce the insulin secretory response in BRIN BD11 cells (Table 1). When the disulphide bridge was removed from Ac3IV, oxytocin receptor activity was restored but activity at V1b, and particularly V1a, receptors reduced (Table 1). Given Ac3IV exhibited pronounced enzymatic stability and good insulinotropic efficacy in 1.1B4 cells, oxytocin receptor interaction was also examined in INS1 832/13 beta cells with CRISPR-Cas9 induced oxytocin receptor KO to corroborate findings with L-351,257. As shown in Figure 1F, insulin secretory effectiveness of Ac3IV was significantly ($P<0.05$) more prominent than AVP in oxytocin receptor KO beta cells.

3.4 Effects of twice daily administration of Ac3IV on body weight, energy intake, fluid intake and plasma glucose, insulin and glucagon in HFF mice

Based on enzymatic stability, impressive *in vitro* bioactive profile and lack of effect at V2 receptors which mediate renal actions of AVP, Ac3IV was selected for antidiabetic efficacy testing in HFF mice, and compared directly head-to-head against the clinically approved GLP-1 mimetic, exendin-4. Twice daily administration of 25 nmol/kg Ac3IV or exendin-4 resulted in significant ($P<0.05$ to $P<0.001$) reductions in body weight (Figure 2A), and importantly body fat content (Figure 2B), by day 22 when compared to HFF control mice. This was associated with significantly decreased ($P<0.05$ to $P<0.001$) cumulative energy intake in both treatment groups (Figure 2C). Fluid intake was similar in all groups with a small cumulative increase observed after 19 days of Ac3IV treatment (Figure 2D). Circulating blood glucose levels were also decreased in all treatment groups, culminating in significant ($P<0.01$) reductions when compared to HFF controls on day 22 (Figure 2E). This

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was accompanied by reduced overall glucose exposure in exendin-4 and Ac3IV mice during the entire treatment period (Figure 2E inset). Corresponding, daily plasma insulin concentrations were largely unaltered during the study, but there was a significant decrease (P<0.05) in this parameter in both treatment groups on day 16 (Figure 2F), as well as in overall insulin levels during the 22 days (Figure 2F inset). Plasma glucagon levels were not changed in exendin-4 treated HFF mice, but significantly reduced (P<0.05) by Ac3IV treatment (Figure 3A).

3.5 Effects of twice daily administration of Ac3IV on pancreatic hormone content, lipid profile and bone mineral density in HFF mice

Pancreatic insulin content was significantly reduced (P<0.05 to P<0.01) in both treatment groups when compared to HFF control mice (Figure 3B), whereas pancreatic glucagon content was not altered (Figure 3C). In terms of lipid profile, Ac3IV and exendin-4 therapy were both associated with reduced total cholesterol (Figure 3D) and increased HDL-cholesterol (Figure 3E) levels, but only Ac3IV significantly (P<0.05) decreased LDL-cholesterol and triglyceride concentrations (Figure 3F,G). Bone mineral density (P<0.01) and content (P<0.05) were increased on day 22 in both the HFF treatment groups of mice (Figure 3H,I).

3.6 Effects of twice daily administration of Ac3IV on glucose tolerance and insulin sensitivity in HFF mice

Administration of exendin-4 twice daily for 22 day significantly (P<0.05 to P<0.01) decreased individual and overall blood glucose levels following an i.p. glucose challenge (Figure 4A,B). Ac3IV treatment was also associated with significantly (P<0.05) decreased glucose 0-120 min AUC values when compared to HFF controls (Figure 4B). In addition,

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individual glucose-induced insulin concentrations were only increased (P<0.05 – P<0.01) by Ac3IV (Figure 4C), with both treatment regimens increasing overall AUC insulin values (Figure 4D). In response to an oral glucose challenge, the treatment interventions significantly decreased individual (P<0.05 to P<0.001) and overall (P<0.01) blood glucose levels when compared to saline controls (Figure 4E,F). There were no changes in plasma insulin concentrations at individual timepoints (Figure 4G), but exendin-4 and Ac3IV both increased (P<0.01 to P<0.001) 0-120 overall plasma insulin concentrations in response to an oral glucose load (Figure 4H). Furthermore, the glucose lowering activity of exogenous insulin was significantly increased (P<0.01 to P<0.001) by both treatments on day 22 (Figure 4I,J), which was supported by a significant decrease (P<0.01 to P<0.001) in HOMA-IR values in these groups of mice when compared to saline treated HFF controls (Figure 4K).

3.7 Effects of twice daily administration of Ac3IV on pancreatic islet morphology in HFF mice

Representation histological images of pancreatic islets stained for insulin and glucagon are shown in Figure 5A. High fat feeding was associated with insulin resistance and islet expansion, but both exendin-4 and Ac3IV significantly decreased islet and beta-cell areas when compared to HFF saline controls (Figure 5B,C), with effects being more prominent in Ac3IV treated mice. There was no significant change in alpha-cell area between the groups of HFF mice (Figure 5D). Proliferation rates of alpha and beta cells were decreased by both exendin-4 and Ac3IV treatment (Figure 5E,F). Apoptotic rates were also examined by co-staining glucagon or insulin, respectively, with TUNEL dye (Figure 5G,H). Both exendin-4 and Ac3IV reduced (P<0.01 – P<0.001) beta cell apoptosis rates when compared to saline control mice (Figure 5G), with exendin-4 treatment also increasing (P<0.05) the rate of alpha-cell apoptosis (Figure 5H).

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4. Discussion

The presence of AVP receptors on pancreatic beta-cells, as well as related positive effects of AVP on insulin secretion demonstrated many years ago [37], have since been confirmed by several laboratories [18,38,39]. AVP also has established satiety actions [40], as well as an ability to avert the onset of hypoglycaemia [9]. Indeed, hypothalamic AVP neurons regulating glucagon secretion are considered to function as metabolic glucose sensors [41], supporting an important role for AVP in glucose homeostasis. Such actions are believed to be largely mediated through activation and modulation of V1a and V1b receptor signalling [10]. Despite this knowledge, the potential therapeutic utility of AVP has been overlooked to date. The reason for this is likely two-fold; firstly, AVP has an extremely short half-life in the circulation and secondly, AVP also activates V2 receptors that are linked to aquaporin-mediated fluid reabsorption and concomitant elevation of blood pressure [42]. The present study was undertaken to directly overcome these drawbacks, through generation of enzymatically stable AVP peptides that possess a receptor activation profile more compelling to long-term benefits in obesity-diabetes.

Given the similarity in amino acid sequence of AVP and oxytocin [10], plus the established benefits of oxytocin receptor activation in diabetes [27], introduction of key oxytocin residues into the sequence of AVP analogues might be envisaged to generate peptides with additional beneficial oxytocin receptor activation properties. However, it should be noted that oxytocin has a much less prolonged and diminished effect on insulin secretion than AVP as observed in this study and elsewhere [37], highlighting the major importance of V1a and V1b receptor activity for beta-cell actions. Interestingly, AVP is also believed to play a role in somatostatin secretion [43], indicating a complex mode of action on islet cells. Although it has been suggested that the stimulatory effects of AVP on insulin

secretion are indirectly mediated by glucagon via paracrine islet interactions [20], we and others clearly show involvement of direct beta-cell stimulatory effects [12,18,39]. In agreement, we observed only moderate insulinotropic actions of the AVP analogues in clonal beta-cells, with much more prominent effects in isolated murine islets, confirming importance of paracrine islet cell signalling in this regard [20].

In the current study, native AVP and some related analogues modulated both oxytocin and AVP receptor signalling pathways in BRIN BD11 cells, but preservation V2 receptor activity and associated effects on the kidney and fluid retention were not considered favourable. Interestingly, reduced AVP exhibited bioactivity at oxytocin, V1a and V1b , with no interaction at V2 receptors, but this peptide was relatively unstable. It should also be noted that removing the disulphide bridge appeared to lead to slightly different effects on insulin secretory activity of native AVP and Ac3IV. This is likely to reflect distinct changes in the three dimensional structure of the peptides and their interaction with receptors on target cells. However, further studies and detailed structure/function analysis are required to clarify this aspect. In addition, investigation of specific downstream signalling pathways would also have been useful to help further confirm receptor selectivity of the AVP analogues. However, on closer inspection, our *in vitro* characterisation data for 3IV, and the daughter analogue Ac3IV, led to selection of the N-terminally acetylated analogue to evaluate the potential benefits of combined V1a and V1b receptor signalling in diabetes. This included careful consideration of enzymatic stability, receptor activation prolife and *in vitro* bioactivity in both rodent and human beta-cells. Key in this decision was the lack of effect of Ac3IV on V2 receptors and resistance to enzymatic degradation. In this regard, AVP is known to be rapidly degraded by vasopressinase enzymes in the circulation, as well as being subject to renal elimination [9]. Whilst our *in vitro* system suggests excellent enzymatic stability of Ac3IV, it did not assess the influence of kidney filtration on peptide bioactivity. To assist with more

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pragmatic quantification of the ability of Ac3IV to reverse metabolic dysregulation present in HFF mice, beneficial effects were compared directly against the approved antidiabetic drug, exendin-4. Importantly, there was no obvious change in mouse behaviour during, and gross tissue anatomy was unaltered at the end of, the Ac3IV treatment regimen suggesting lack of toxicity of this compound. Further longer-term studies are required to fully assess safety issues, but it is noteworthy that Ac3IV is structurally identical to native AVP barring addition of an N-terminal acetyl group and substitution of the third amino acid residue, phenylalanine for isoleucine, that already exists in the closely related peptide hormone oxytocin.

Both peptide interventions decreased circulating glucose, body weight and fat content in HFF mice, presumable in part linked to prominent satiety actions. This is in full agreement with established metabolic benefits of GLP-1 receptor activation [2], and in harmony with actions of V1a receptor activation to suppress appetite [12]. Studies in mice pair-fed to mimic Ac3IV induced reductions of food intake would be required to help establish if benefits on body weight and metabolism occurred independently of changes in energy intake. Consistent with lack V2 action which might otherwise cause fluid retention, Ac3IV had only marginal effects on water intake with even a small cumulative increase being apparent towards the end of the study, but further studies are required to fully assess effects of Ac3IV on renal function. Somewhat surprisingly, given the established glucagonostatic effects of GLP-1 receptor agonism [2] and reported glucagonotropic actions of AVP [20], circulating glucagon concentrations were reduced by Ac3IV, but not by exendin-4. This being in the face of increased pancreatic alpha-cell apoptotic rates in exendin-4 treated HFF mice, and a tendency for reduced pancreatic glucagon content. In relation to this, there was a reduction in islet- and beta-cell areas, as well as pancreatic insulin content, in both treatment groups of HFF mice, accompanied by decreased proliferation of both alpha- and beta-cells. Thus, changes in islet-cell proliferation rates, or in recently reported islet cell transitioning events [44], probably

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outweigh decreased beta-cell apoptosis observed with both peptides. Moreover, GLP-1 receptor signalling has recently been linked to positive effects on alpha- and beta-cell transdifferentaition [45]. Taken as a whole, it could be assumed that reductions in islet- and beta-cell areas are directly linked to the improved diabetic state evoked by both treatment regimens, leading to reduced metabolic demand.

In keeping with this, circulating insulin concentrations were largely unaltered and peripheral insulin sensitivity significantly improved in peptide treated HFF mice, in accordance with notable GLP-1 and V1b receptor mediated benefits on insulin action [14,33]. Moreover, both Ac3IV and exendin-4 reduced total- and increased HDL-cholesterol levels. However, only Ac3IV significantly reduced circulating LDL-cholesterol and triglyceride levels, highlighting this as a distinct advantage over exendin-4 given that diabetes and obesity represent key risk factors for cardiovascular disease [46]. In addition, obesity-driven forms of diabetes are also associated with increased occurrence of bone fragility fractures [47,48], and both Ac3IV and exendin-4 augmenting bone mineral content and density, implying another potentially important therapeutic benefit.

In spite of this, it should also be noted that increased levels of copeptin, a peptide co-secreted with AVP and considered as an excellent surrogate marker for assessing circulating AVP concentrations [49], is associated with metabolic dysregulation and onset of diabetes [9]. However, this effect could well be linked to AVP receptor desensitisation and inherent adaptations to increase AVP secretion, and as such requires further detailed study. Indeed, a very similar phenomenon is reported for the incretin hormone GIP in type 2 diabetes [50] that possesses significant parallels with the bioactive profile of AVP, and this issue now appears to be more than surmountable from a therapeutic viewpoint [23]. Moreover, both oral and intraperitoneal glucose tolerance were substantially improved by Ac3IV in HFF mice, and consistently linked to the glucose-dependent insulinotropic actions. Such positive effects are

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particularly notable given aforementioned reductions in beta-cell area and pancreatic insulin content. Interestingly, AVP has been shown to stimulate V1b receptor-dependent secretion of GLP-1 from mouse and human intestines [51], and this could be one potential reason for the observed similar benefits of Ac3IV and exendin-4 on glucose disposal. Furthermore, we and others have previously illustrated similarities in beta-cell signalling pathways for AVP and the other hormones including incretin hormones [18]. However, further studies would be required to confirm the potential positive impact of Ac3IV on the secretion and action of GLP-1 and other gut hormones.

In conclusion, combined sustained activation of V1a and V1b receptor pathways by Ac3IV exerts notable benefits on body weight and energy regulation, glucose homeostasis, insulin action and lipid metabolism, as well as reversing detrimental effects of high fat feeding on pancreatic architecture. Metabolic benefits were at least equivalent, and at times superior, to those exerted by the clinically approved GLP-1 mimetic, exendin-4. Future investigations into the therapeutic potential of AVP for the treatment of obesity and related diabetes may also want to consider further peptide optimisation and approaches capable of combining specific metabolic benefits of GLP-1 mimetics alongside V1a and V1b receptor activation. In this respect, it is not altogether uninteresting that effective strategies exist for the oral administration of both AVP and GLP-1 analogues [52].

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Author contribution

PRF and RCM conceived/designed the study. SM, NI and PRF drafted the manuscript. SM 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.

Conflict of interest

All authors declare no conflict of interest.

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References

1. Borse SP, Chhipa AS, Sharma V, Singh DP, Nivsarkar M (2020) Management of Type 2 Diabetes: Current Strategies, Unfocussed Aspects, Challenges and Alternatives. Med Princ Pract. Online ahead of print. doi: 10.1159/000511002.
2. Müller TD, Finan B, Bloom SR, D'Alessio D, Drucker DJ, Flatt PR, Fritsche A, Gribble F, Grill HJ, Habener JF, Holst JJ, Langhans W, Meier JJ, Nauck MA, Perez-Tilve D, Pocai A, Reimann F, Sandoval DA, Schwartz TW, Seeley RJ, Stemmer K, Tang-Christensen M, Woods SC, DiMarchi RD, Tschöp MH. (2019) Glucagon-like peptide 1 (GLP-1). Mol Metab. 30:72-130.
3. Brandt SJ, Götz A, Tschöp MH, Müller TD. (2018) Gut hormone polyagonists for the treatment of type 2 diabetes. Peptides 100:190-201.
4. Hasib A (2020) Multiagonist Unimolecular Peptides for Obesity and Type 2 Diabetes: Current Advances and Future Directions. Clin Med Insights Endocrinol Diabetes 13:1179551420905844.
5. Bailey CJ (2018) Glucose-lowering therapies in type 2 diabetes: Opportunities and challenges for peptides. Peptides 100:9-17.
6. Pathak V, Flatt PR, Irwin N (2018) Cholecystokinin (CCK) and related adjunct peptide therapies for the treatment of obesity and type 2 diabetes. Peptides 100:229-235.
7. Oliver G, Schafer EA (1895) On the Physiological Action of Extracts of Pituitary Body and certain other Glandular Organs: Preliminary Communication. J Physiol 18:277-279.
8. Guelinckx I, Vecchio M, Perrier ET, Lemetais G (2016) Fluid Intake and vasopressin: Connecting the dots. Annals of Nutrition and Metabolism 68:6-11.
9. Nakamura K, Velho G, Bouby N (2017). Vasopressin and metabolic disorders: translation from experimental models to clinical use. J Intern Med 282:298-309.
10. Koshimizu T, Nakamura K, Egashira N, Hiroyama M, Nonoguchi H, Tanoue A (2012) Vasopressin V1a and V1b Receptors: From Molecules to Physiological Systems. Physiol Rev 92:1813-1864.
11. Lolait SJ, O'Carroll AM, McBride OW, Konig M, Morel A, Brownstein MJ (1992) Cloning and characterization of a vasopressin V2 receptor and possible link to nephrogenic diabetes insipidus. Nature 357:336-339.
12. Aoyagi T, Birumachi J, Hiroyama M, et al. (2007) Alteration of glucose homeostasis in V1a vasopressin receptor-deficient mice. Endocrinology 148:2075-2084.
13. Fujiwara Y, Hiroyama M, Sanbe A, et al. (2007) Insulin hypersensitivity in mice lacking the V1b vasopressin receptor. J Physiol (Lond) 584:235-244.
14. Nakamura K, Aoyagi T, Hiroyama M, et al. (2009) Both V1A and V1B vasopressin receptors deficiency result in impaired glucose tolerance. Eur J Pharmacol 613:182-188.
15. Nakamura K, Yamashita T, Fujiki H, et al. (2011) Enhanced glucose tolerance in the Brattleboro rat. Biochem Biophys Res Commun 405:64-67.
16. Enhörning S, Sjögren M, Hedblad B, Nilsson PM, Struck J, Melander O (2016) Genetic vasopressin 1b receptor variance in overweight and diabetes mellitus. European Journal of Endocrinology 174:69-75.
17. Enhörning S, Leosdottir M, Wallstrom P, et al. (2009) Relation between human vasopressin 1a gene variance, fat intake, and diabetes. Am J Clin Nutr 89:400-406.

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18. Mohan S, Moffett RC, Thomas KG, Irwin N, Flatt PR (2019) Vasopressin receptors in islets enhance glucose tolerance, pancreatic beta-cell secretory function, proliferation and survival. Biochimie 158:191-198.
19. Yibchok-Anun S, Abu-Basha E, Yao CY, Panichkriangkrai W, Hsu WH (2004) The role of arginine vasopressin in diabetes-associated increase in glucagon secretion. Regul Pept 122:157-162.
20. Kim A, Knudsen JG, Madara JC, Benrick A, Hill T, Kadir LA, Kellard JA, Mellander L, Miranda C, Lin H, James T, Suba K, Spigelman AF, Wu Y, MacDonald PE, Asterholm IW, Magnussen T, Christensen M, Vilsboll T, Salem V, Knop FK, Rorsman P, Lowell BB, Briant LJB (2020) AVP-induced counter-regulatory glucagon is diminished in type 1 diabetes. bioRxiv. https://doi.org/10.1101/2020.01.30.927426.
21. Cassidy RS, Irwin N, Flatt PR (2008) Effects of gastric inhibitory polypeptide (GIP) and related analogues on glucagon release at normo- and hyperglycaemia in Wistar rats and isolated islets. Biol Chem 389:189-193.
22. Irwin N, Flatt PR (2015) New perspectives on exploitation of incretin peptides for the treatment of diabetes and related disorders. World Journal of Diabetes 6:1285-1285.
23. Frias JP, Nauck MA, Van J, et al. (2018) Efficacy and safety of LY3298176, a novel dual GIP and GLP-1 receptor agonist, in patients with type 2 diabetes: a randomised, placebo-controlled and active comparator-controlled phase 2 trial. Lancet 392:2180-2193.
24. Gimpl G, Fahrenholz F (2001) The Oxytocin Receptor System: Structure, Function, and Regulation. Physiol Rev 81:629-683.
25. Lee HJ, Macbeth AH, Pagani JH, Scott Young W (2009) Oxytocin: The great facilitator of life. Prog Neurobiol 88:127-151.
26. Snider B, Geiser A, Yu X, et al. (2019) Long-Acting and Selective Oxytocin Peptide Analogs Show Antidiabetic and Antiobesity Effects in Male Mice. Journal of the Endocrine Society 3:1423-1444.
27. Mohan S, McCloskey AG, McKillop AM, Flatt PR, Irwin N, Moffett RC (2020) Development and characterisation of novel, enzymatically stable oxytocin analogues with beneficial antidiabetic effects in high fat fed mice. Biochim Biophys Acta Gen Subj. 1865:129811.
28. Gault VA, Porter DW, Irwin N, Flatt PR (2011) Comparison of sub-chronic metabolic effects of stable forms of naturally occurring GIP(1-30) and GIP(1-42) in high-fat fed mice. J Endocrinol 208:265-271.
29. O'Harte FP, Franklin ZJ, Irwin N (2019). Two novel glucagon receptor antagonists prove effective therapeutic agents in high-fat-fed and obese diabetic mice. Diabetes Obes Metab. 16:1214-22.
30. McClenaghan NH, Barnett CR, Ah-Sing E, et al. (1996) Characterization of a novel glucose-responsive insulin-secreting cell line, BRIN-BD11, produced by electrofusion. Diabetes 45:1132-40.
31. McCluskey JT, Hamid M, Guo-Parke H, McClenaghan NH, Gomis R, Flatt PR (2011) Development and functional characterization of insulin-releasing human pancreatic beta cell lines produced by electrofusion. J Biol Chem 286:21982-21992.
32. Khan D, Vasu S, Moffett RC, Gault VA, Flatt PR, Irwin N (2017) Locally produced xenin and the neurotensinergic system in pancreatic islet function and beta-cell survival. Biol Chem. 399:79-92.
33. Hasib A, Ng MT, Khan D, Gault VA, Flatt PR, Irwin N (2018) A novel GLP-1/xenin hybrid peptide improves glucose homeostasis, circulating lipids and restores GIP sensitivity in high fat fed mice. Peptides 100:202-211.

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34. Vasu S, Moffett RC, Thorens B, Flatt PR (2014) Role of endogenous GLP-1 and GIP in beta cell compensatory responses to insulin resistance and cellular stress. PLoS ONE 9:e101005
35. Khan D, Vasu S, Moffett RC, Gault VA, Flatt PR, Irwin N (2017) Locally produced xenin and the neurotensinergic system in pancreatic islet function and β-cell survival. Biol Chem 399:79-92.
36. Flatt PR, Bailey CJ (1981) Abnormal plasma glucose and insulin responses in heterozygous lean (ob/+) mice. Diabetologia 20:573-577.
37. Lee B, Yang C, Chen TH, al-Azawi N, Hsu WH (1995) Effect of AVP and oxytocin on insulin release: involvement of V1b receptors. Am J Physiol 269:E1095-100.
38. Folny V, Raufaste D, Lukovic L, et al. (2003) Pancreatic vasopressin V1b receptors: characterization in In-R1-G9 cells and localization in human pancreas. American journal of physiology.Endocrinology and metabolism 285:E566-E576.
39. Oshikawa S, Tanoue A, Koshimizu T, Kitagawa Y, Tsujimoto G (2004) Vasopressin stimulates insulin release from islet cells through V1b receptors: a combined pharmacological/knockout approach. Mol Pharmacol 65:623-629.
40. Pei H, Sutton AK, Burnett KH, Fuller PM, Olson DP (2014) AVP neurons in the paraventricular nucleus of the hypothalamus regulate feeding. Molecular Metabolism 3:209-215.
41. Song Z, Levin BE, Stevens W, Sladek CD (2014) Supraoptic oxytocin and vasopressin neurons function as glucose and metabolic sensors. AJP: Regulatory, Integrative and Comparative Physiology 306:R447-R456.
42. Tamma G, Di Mise A, Ranieri M, et al. (2017) The V2 receptor antagonist tolvaptan raises cytosolic calcium and prevents AQP2 trafficking and function: an in vitro and in vivo assessment. J Cell Mol Med 21:1767-1780.
43. Gao Z, Gérard M, Henquin J (1992) Glucose- and concentration-dependence of vasopressin-induced hormone release by mouse pancreatic islets. Regul Pept 38:89-98.
44. Tanday N, Moffett RC, Gault VA, Flatt PR, Irwin N (2020) Effects of an enzymatically stable analogue of the gut-derived peptide xenin on beta-cell transdifferentiation in high fat fed and insulin-deficient Ins1(Cre) (/+) ;Rosa26-eYFP mice. Diabetes Metab Res Rev 37:e3384.
45. Tanday N, Flatt PR, Irwin N, Moffett RC (2020) Liraglutide and sitagliptin counter beta- to alpha-cell transdifferentiation in diabetes. J Endocrinol 245:53-64.
46. Padwal RS, Sharma AM (2010) Prevention of cardiovascular disease: obesity, diabetes and the metabolic syndrome. Can J Cardiol 26 Suppl C:18C-20C.
47. Mabilleau G, Chappard D, Flatt PR, Irwin N (2015) Effects of anti-diabetic drugs on bone metabolism. Expert Review of Endocrinology & Metabolism 10:663-675.
48. Vyavahare SS, Mieczkowska A, Flatt PR, Chappard D, Irwin N, Mabilleau G (2020) GIP analogues augment bone strength by modulating bone composition in diet-induced obesity in mice. Peptides 125:170207.
49. Enhörning S, Struck J, Wirfalt E, Hedblad B, Morgenthaler NG, Melander O (2011) Plasma copeptin, a unifying factor behind the metabolic syndrome. J Clin Endocrinol Metab 96:E1065-72.
50. Irwin N, Gault VA, O'Harte FPM, Flatt PR (2020) Blockade of gastric inhibitory polypeptide (GIP) action as a novel means of countering insulin resistance in the treatment of obesity-diabetes. Peptides 125:170203.
51. Pais R, Rievaj J, Meek C, et al. (2016) Role of enteroendocrine L-cells in arginine vasopressin-mediated inhibition of colonic anion secretion. J Physiol (Lond) 594:4865-4878.

52. Drucker DJ (2020) Advances in oral peptide therapeutics. Nature Reviews Drug Discovery 19:277-289.

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Table 1: Amino acid sequence, degradation profile, insulin release characteristics and receptor selectivity of AVP analogues

Peptide/ Analogues	Sequence	% degradation at 4 h	% reduction in insulin secretion compared to the control in the presence of receptor antagonists			
			OTRa	**V1aRa**	**V1bRa**	**V2Ra**
AVP	C<YFQNC>PRG-NH_2	100 ± 0.1	42 ± 5*	64 ± 3***	57 ± 6***	20 ± 10
AVPR	CYFQNCPRG-NH_2	65 ± 0.4	59 ± 6**	42 ± 5*	39 ± 5 *	0
Oxytocin	C<YIQNC>PLG-NH_2	65 ± 0.9	42 ± 5*	15 ± 7	62 ± 4***	1 ± 6
8LV	C<YFQNC>P**L**G-NH_2	67 ± 0.1	42 ± 11*	46 ± 5***	76 ± 5***	92 ± 2***
8LVR	CYFQNCP**L**G -NH_2	88 ± 0.7	27 ± 20	8 ± 15	58 ± 5 **	0
3IV	C<Y**I**QNC>PRG-NH_2	68 ± 0.2	68 ± 3***	82 ± 3***	45 ± 9*	22 ± 10
3IVR	CY**I**QNCPRG-NH_2	64 ± 0.4	13 ± 5	34 ± 11*	46 ± 13*	0
Ac3IV	**Ac-C**<Y**I**QNC>PRG-NH_2	0	17 ± 4	24 ± 3***	22 ± 5**	0
Ac3IVR	**Ac-C**Y**I**QNCPRG-NH_2	0	50 ± 4***	12 ± 5	15 ± 5	0

Replacement of amino acids in AVP with those from oxytocin in positions 3 and 8 are indicated in **bold underlined** text for each analogue. < > indicates presence of the disulphide bridge between the two cysteines at position 1 and 6. To assess enzymatic stability, peptides (n=3) were incubated with plasma for 4 h and degradation profile followed by RP-HPLC. For insulin secretory experiments, BRIN BD11 cells were incubated (n=8; 20 min) with peptides (10^{-6} M) in combination with 10^{-6} M of either selective oxytocin (OTRa, L-351,257), V1a (V1aRa , SR-49059), V1b (V1bRa , SR -149415) or V2 (V2Ra, Tolvaptan) receptor antagonists, and percentage inhibition of insulin secretion recorded. Values are expressed as mean ± SEM. *P<0.05, **P<0.01, ***P<0.001 compared with respective control. Abbreviations: Ac; acetyl.

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Figure legends

Figure 1. Effects of AVP, oxytocin and AVP analogues Ac3IV, Ac3IVR on insulin secretion and receptor selectivity. (A-D) Effects of AVP and related analogues (10^{-12} - 10^{-6} M) on insulin secretion (20 min) at (A,C) 5.6 and (B,D) 16.7 mM glucose from (A,B) rodent BRIN BD11 or (C,D) human 1.1B4 beta cells. (E) Isolated islets from male mice were incubated (60 min) at 16.7 mM glucose and the effects of AVP, oxytocin and Ac3IV (10^{-8} and 10^{-6} M) on insulin secretion examined. (F) Effects of AVP and Ac3IV on insulin secretion (20 min) from oxytocin receptor KO INS1 832/13 beta-cells at 16.7 mM glucose. Values are mean ± SEM (A-D,F) n=8 and (E) n=3. *P<0.05, **P<0.01, ***P<0.001 compared to respective glucose alone control. $^{\Delta}$P<0.05 compared to AVP at the same concentration.

Figure 2. Effects of twice daily administration of Ac3IV or exendin-4 on (A) body weight, (B) total body fat, cumulative (C) energy and (D) fluid intake, as well as (E) glucose and (F) insulin concentrations in HFF mice. (A,C-F) Parameters were measured at regular intervals prior to, and during, 22 days twice-daily treatment with test peptides (25 nmol/kg bw) in HFF mice. (B) Parameter was measured on day 22. (E,F) Insets depict AUC data for glucose and insulin over the 22 day treatment period. All values are expressed as mean ± SEM for 7 mice. *P<0.05, **P<0.01, ***P<0.001 compared with HFF-saline control mice.

Figure 3. Effects of twice daily administration of Ac3IV or exendin-4 on pancreatic circulating glucagon, hormone content, lipid profile and body composition in HFF mice. Parameters were measured after 22 days treatment with twice-daily injection of test peptides (25 nmol/kg bw) in HFF mice. (A) Plasma glucagon was determined using a commercially

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available ELISA kit. (B,C) Pancreatic tissue was isolated at the end of the study and (B) insulin or (C) glucagon content determined following acid-ethanol extraction. (D-G) Terminal non-fasted plasma (D) total cholesterol, (E) HDL-cholesterol (F) LDL-cholesterol and (G) triglycerides were measured by an ILab 650 Clinical Analyser. (H) Bone mineral density and (I) bone mineral content were measured by DXA. Values are mean ± SEM for 7 mice. *P<0.05, **P<0.01 compared with HFF-saline control mice.

Figure 4. Effects of twice daily administration of Ac3IV or exendin-4 on glucose tolerance and insulin sensitivity in HFF mice. Parameters were measured after 22 days treatment with twice-daily injection of test peptides (25 nmol/kg bw) in HFF mice. (A,E) Blood glucose and (C,G) plasma insulin were measured prior to and after (A,C) i.p. or (E,G) oral administration of glucose alone (18 mmol/kg) at t = 0 min in 18 h fasted mice. (B,D,F,H) Respective AUC data for 0-120 min post glucose injection are shown. (I,J) Blood glucose was measured after i.p. administration of insulin (15 U/kg bw) at t = 0 min in non-fasted mice. (K) HOMA-IR was calculated using fasting insulin (mUI/L) multiplied by fasting glucose (mmol/L) divided by 22.5 on day 22. All values are expressed as mean ± SEM for 7 mice. *P<0.05, **P<0.01, ***P<0.001 compared with HFF-saline control mice.

Figure 5. Effects of twice daily administration of Ac3IV or exendin-4 on pancreatic islet architecture as well as alpha- and beta cell proliferative and apoptotic rates in HFF mice. Parameters were measured after 22 days treatment with twice-daily injection of test peptides (25 nmol/kg bw) in HFF mice. (A) Representative images of islets showing insulin (red) and glucagon (green) immunoreactivity from each treatment group. (B-D) Islet architectural analysis included assessment of (B) total islet, (C) beta- and (D) alpha-cell areas. (E,G) Beta- and (F,H) alpha-cell proliferation or apoptotic rates were measured by co-

staining with insulin or glucagon, respectively, and (E,F) Ki-67 antibody or (G,H) TUNEL reagent, as appropriate. Values are mean ± SEM for 7 mice. *$P<0.05$, **$P<0.01$, ***$P<0.001$ compared with HFF-saline control mice.

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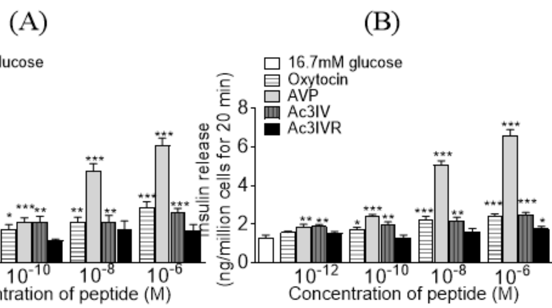
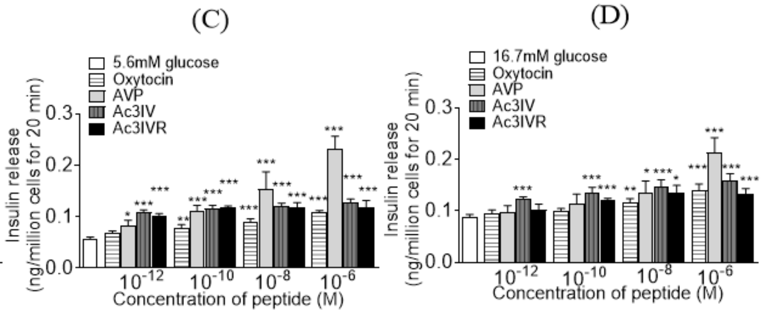
Figure 1

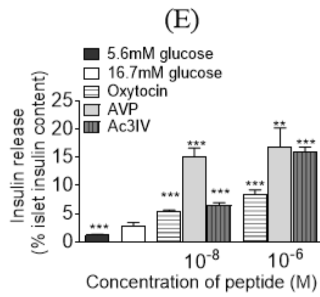
BRIN BD11 rodent beta cells



1.1B4 human beta cells



Isolated mouse pancreatic islets



OTR KO INS-1 823/13 cells

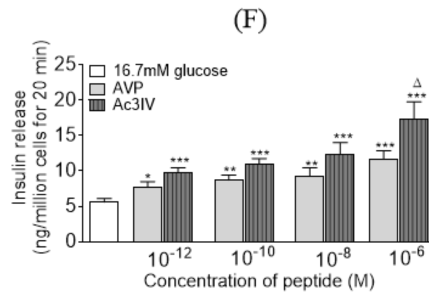


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Figure 2

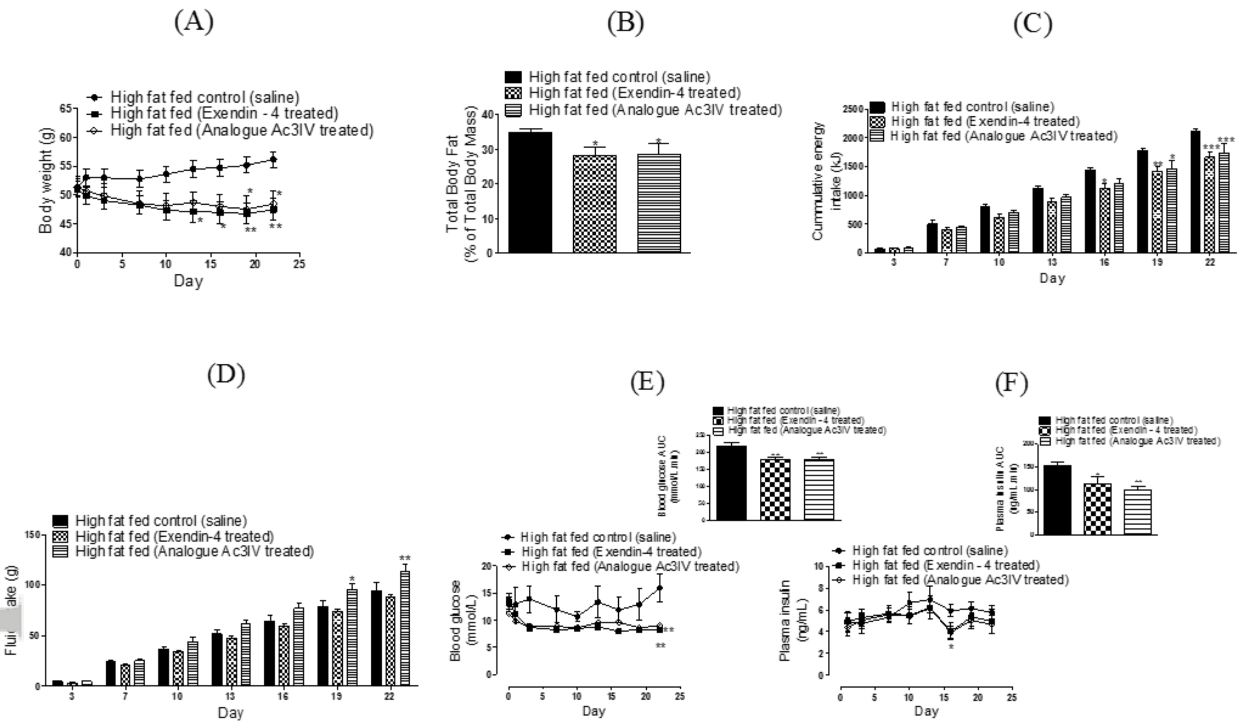

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Figure 3

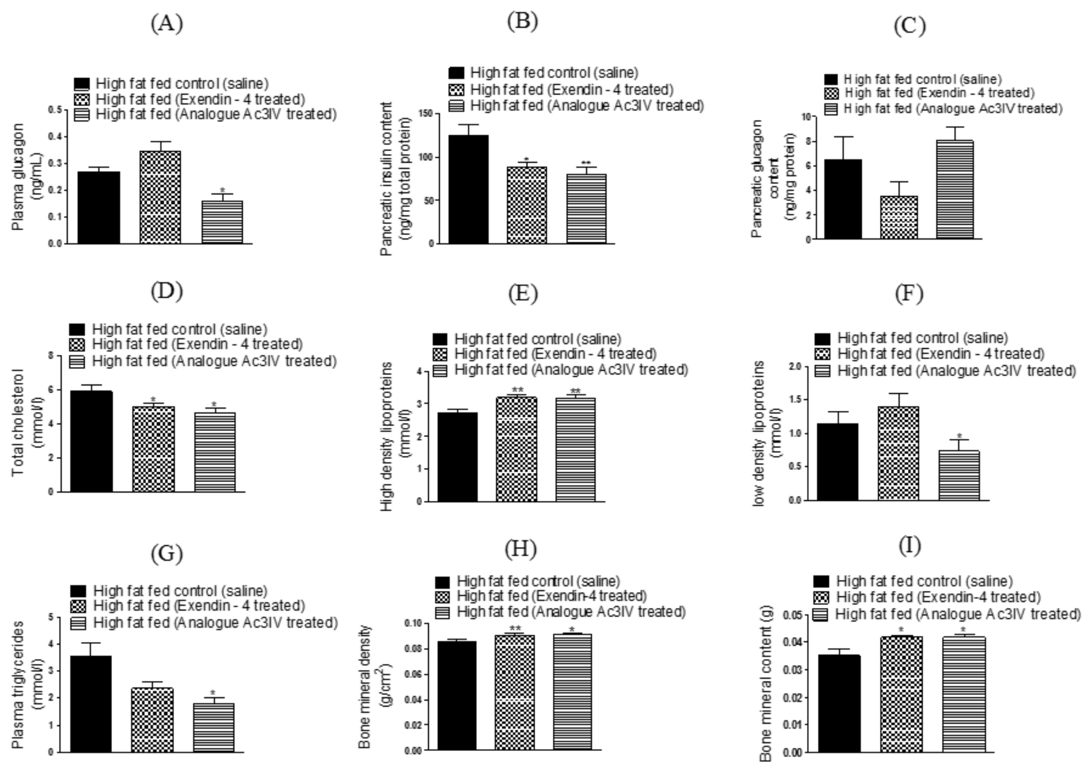

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Figure 4

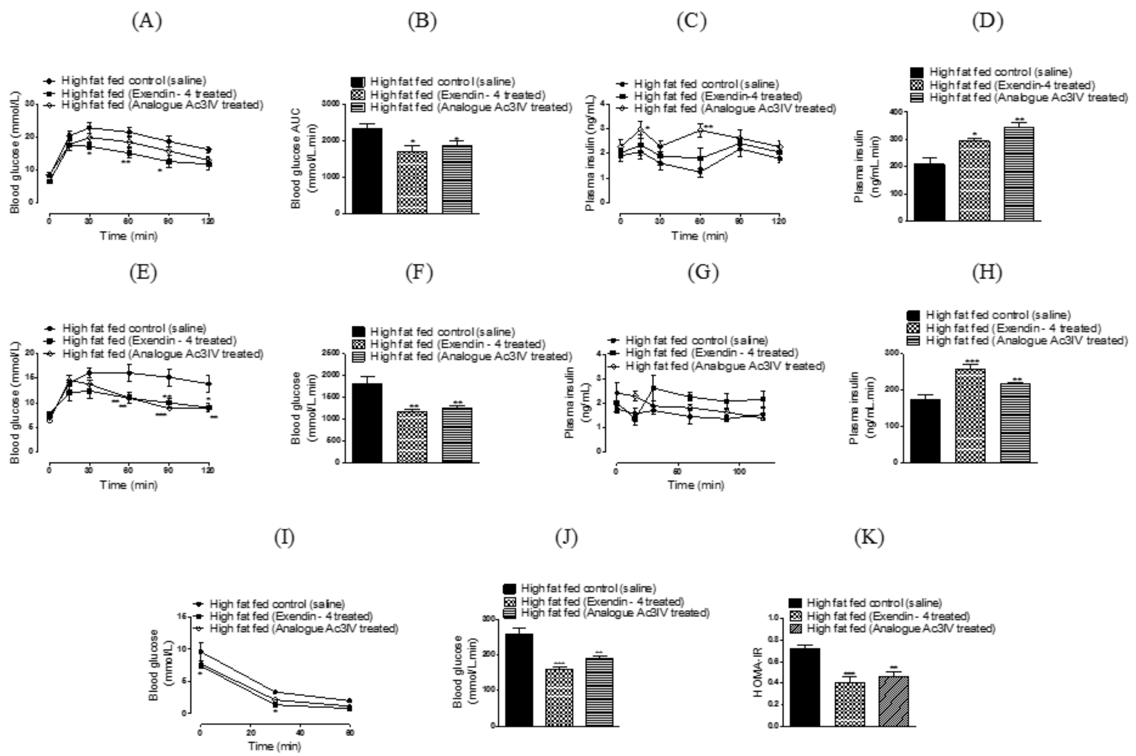
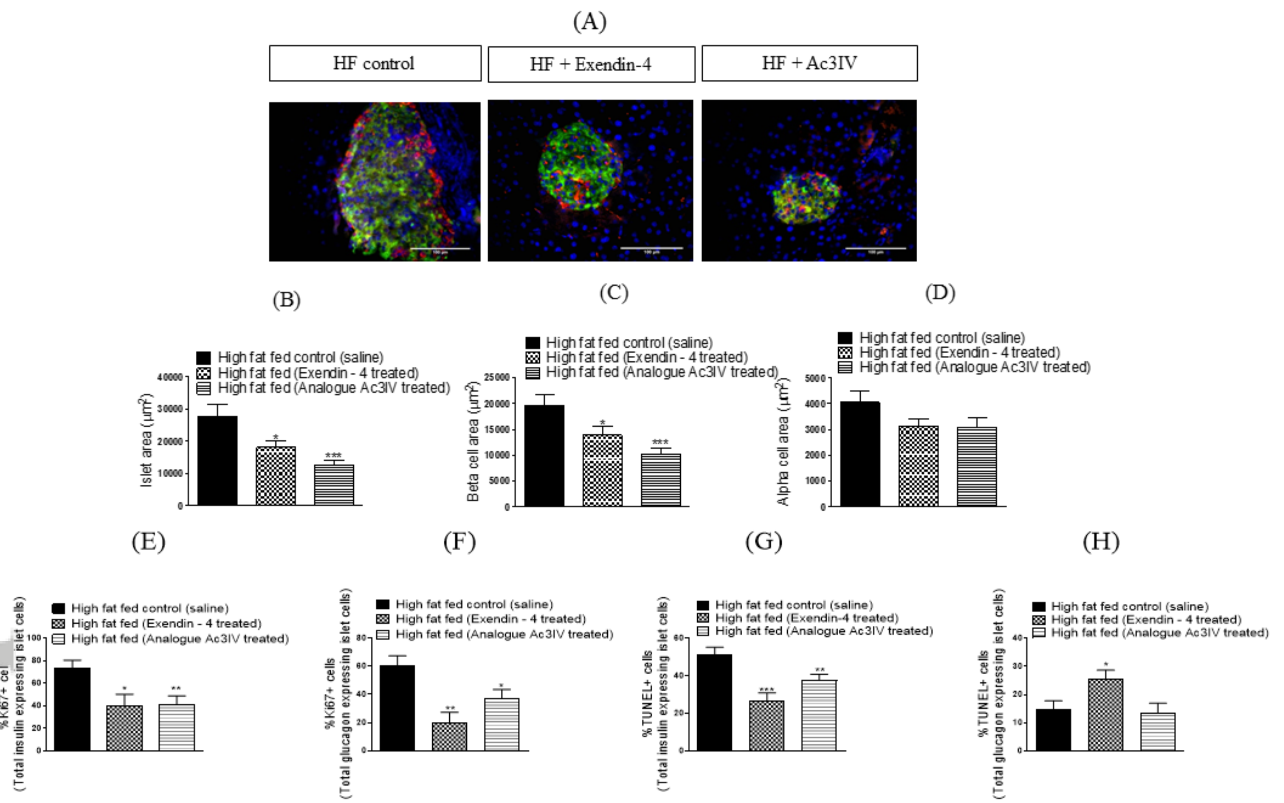


Figure 5



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