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(D-Ser^2)Oxm[mPEG-PAL]: A novel chemically modified analogue of oxyntomodulin with antihyperglycaemic, insulinotropic and anorexigenic actions

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ABSTRACT

Oxyntomodulin (Oxm) is a hormone which has been shown to exhibit a range of potentially beneficial actions for alleviation of obesity-diabetes. However, exploitation of Oxm-based therapies has been severely restricted due to degradation by the enzyme dipeptidylpeptidase-IV (DPP-IV). Thus, the aim of this study was to assess the glucose-lowering, insulin-releasing and anorexigenic actions of chemically modified, enzyme-resistant analogues of Oxm. Oxm, (D-Ser^2)Oxm and (D-Ser^2)Oxm[mPEG-PAL], were incubated with DPP-IV to assess enzyme stability and pancreatic beta-cells to evaluate insulin secretion. cAMP production was assessed using glucagon-like peptide-1 (GLP-1) and glucagon receptor transfected cells. *In vivo* effects of Oxm analogues on glucose homeostasis, insulin secretion, food intake and bodyweight were examined in obese diabetic (*ob*/*ob*) mice. (D-Ser^2)Oxm[mPEG-PAL] displayed enhanced DPP-IV resistance compared to (D-Ser^2)Oxm and Oxm. All peptides demonstrated similar *in vitro* cAMP and insulin-releasing actions, which was associated with dual action at GLP-1 and glucagon receptors. Acute administration of (D-Ser^2)Oxm[mPEG-PAL] and (D-Ser^2)Oxm reduced plasma glucose and food intake, whilst plasma insulin levels were elevated. Once-daily administration of (D-Ser^2)Oxm[mPEG-PAL] for 14 days to *ob*/*ob* mice decreased food intake, bodyweight, plasma glucose and increased plasma insulin. Furthermore, daily (D-Ser^2)Oxm[mPEG-PAL] improved glucose tolerance, increased glucose-mediated insulin secretion, pancreatic insulin content, adiponectin and decreased both visfatin and triglyceride levels. The ability of enzyme-resistant (D-Ser^2)Oxm[mPEG-PAL] to improve glucose homeostasis, insulin secretion, satiety, bodyweight and markers of fat metabolism suggests significant promise for Oxm-based therapies for obesity-diabetes.

1. Introduction

Oxyntomodulin (Oxm) is a 37 amino acid peptide hormone released from intestinal L-cells into the blood in response to feeding [1]. Oxm is a member of the proglucagon-derived peptide family comprising glucagon, glucagon-like peptide-1 (GLP-1) and glucagon-like peptide-2 (GLP-2) [2]. Despite the well-documented biological actions of glucagon, GLP-1 and GLP-2 [3], the precise biological role of Oxm remains unclear. Studies have demonstrated that Oxm increases core body temperature and heart rate in rodents [4] while in humans it has been shown to increase energy expenditure and voluntary physical activity [5]. Oxm reduces bodyweight in animal and human studies due possibly to suppression of pre-prandially released ghrelin [4,6]. Recent evidence has also suggested that Oxm may play a role in glucose homeostasis through long-term bodyweight reduction, most probably due to the causal link between obesity and type 2 diabetes [7] as well as enhancing glucose-dependent insulin secretion [8].

While separate receptors for other proglucagon-derived peptides have been characterised, a pharmacologically unique binding site for Oxm has yet to be identified [9]. It has been suggested that Oxm induces catabolic effects that favour weight loss through activation of the glucagon receptor while modulating glucose homeostasis through agonism of the GLP-1 receptor [10]. Indeed, the insulinotropic properties of GLP-1 and the regulation of metabolism in fasting through the glucagon receptor are well established [11,12]. Oxm has been shown to activate both GLP-1 and glucagon receptors, albeit with a 10- to 100-fold reduced potency relative to GLP-1 and glucagon [13]. This may point to the possibility of a dual action Oxm-based therapy aimed at both obesity and type 2 diabetes [10,14].

Despite their anti-diabetic and anti-obesity potential, Oxm-based therapies have not yet reached the clinic, due to the susceptibility of the native hormone to enzymatic degradation [15]. Proglucagon peptides are rapidly degraded by the

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ubiquitous enzyme dipeptidylpeptidase-IV (DPP-IV) [16]. However, many studies have demonstrated greatly increased bioactivity of other DPP-IV susceptible gut hormones through chemical modifications [17,18]. Notably, substitution of the naturally occurring L-Ala at position 8 in GLP-1 or position 2 in GIP results in DPP-IV resistance [19–22]. Furthermore, C-terminal PEGylation and fatty acid derivatisation of GIP and GLP-1 results in longer-acting and more potent analogues, due largely to reduced renal clearance through binding to plasma proteins [23–27]. Building on these observations and approaches to stabilise related gut hormones, we developed a novel Oxm analogue with D-Ser at position 2 and C-16 palmitic acid conjugated via a mini-PEG linker at the C-terminus, (D-Ser^2)Oxm[mPEG-PAL], and assessed its antihyperglycaemic, insulinotropic and anorexigenic properties. mPEG was used as a linker in view of previous reports that such linkers improve the actions of other acylated peptides such as GLP-1 [23].

2. Materials and methods

2.1. Peptide synthesis

Oxm, (D-Ser^2)Oxm, and (D-Ser^2)Oxm[mPEG-PAL] (each >97% purity) were purchased from GL Biochem Ltd. (Shanghai, China). (D-Ser^2)Oxm was created by substitution of Ser at position 2 with its D-isomer. (D-Ser^2)Oxm[mPEG-PAL] was generated similar to (D-Ser^2)Oxm with further addition of a C-16 fatty acid moiety attached to the C-terminus via a mini-PEG linker (Product: HK(mini-PEG-Pal)-38-NH_2; molecular formula: $C_{220}H_{347}N_{64}O_{65}S_1$). Observed molecular masses for Oxm, (D-Ser^2)Oxm, and (D-Ser^2)Oxm[mPEG-PAL] were 4445.7, 4449.8 and 4957.8 Da, respectively, corresponding closely to theoretical values and confirming structural identities.

2.2. Degradation of Oxm peptides by DPP-IV

Oxm, (D-Ser^2)Oxm, and (D-Ser^2)Oxm[mPEG-PAL] were incubated at 37 °C in 50 mM triethanolamine–HCl (pH 7.8; Sigma–Aldrich, Dorset, UK) with purified porcine DPP-IV (5 mU; Sigma–Aldrich, Dorset, UK) for 0, 2, 4, 8 and 24 h. Degradation profiles were obtained using HPLC analysis as described previously [24] and HPLC peak area data used to calculate percentage intact peptide remaining at various time points during the incubation. In addition, the established DPP-IV inhibitor Vildagliptin (10 mM; Novartis Pharma AG, Basel, Switzerland) was added to 2 h incubations with Oxm and native GLP-1.

2.3. In vitro cAMP production and insulin secretion

Effects of Oxm, (D-Ser^2)Oxm and (D-Ser^2)Oxm[mPEG-PAL] on cAMP production and insulin secretion were assessed in clonal pancreatic BRIN-BD11 cells [28]. Effects of peptide analogues, as well as native GLP-1 and glucagon on cAMP production were also evaluated using Chinese hamster lung (CHL) cells transfected with human GLP-1 receptor [29] and glucagon receptor transfected HEK2935 cells [30]. For cAMP studies, cells were seeded (100,000 cells per well) into 96-well plates (Nunc, Roskilde, Denmark) and washed with Hanks buffered saline (HBS) buffer prior to incubation with native or Oxm peptides (10^{-6} to 10^{-12} M) in the absence or presence of 10^{-6} M glucagon or GLP-1 receptor antagonists, DesHis^{1}DesPhe^{6}Glucagon-amide and exendin (9–39), respectively (in the presence of 200 μM IBMX for 20 min at 37 °C). After incubation, medium was removed and cells lysed prior to measurement of cAMP using HTS Immunoassay Kit (Millipore, Watford, UK). Percentage cAMP production was calculated from the maximal response induced by the agonist or antagonist peptides at 10^{-6} M. For insulin-release studies, BRIN-BD11 cells were seeded (150,000 cells per well) into 24-well plates (Nunc, Roskilde, Denmark) and allowed to attach overnight at 37 °C. Following 40 min pre-incubation (1.1 mM glucose; 37 °C), cells were incubated (20 min; 37 °C) in the presence of 5.6 mM glucose with a range of peptide concentrations. After 20 min incubation, buffer was removed from each well and aliquots (200 μl) were stored at −20 °C for measurement of insulin.

2.4. Animals

The majority of experiments were performed using obese diabetic (*ob/ob*) mice (20–22 weeks of age) derived from the Aston colony to assess *in vivo* biological properties of Oxm peptides [31]. Animals were age-matched, divided into groups and housed in an air-conditioned room at 22 ± 2 °C with a 12 h light/12 h dark cycle. Animals had free access to drinking water and normal laboratory chow (Trouw Nutrition, Cheshire, UK). Male NIH *Swiss* mice (6–8 weeks and 26–30 g; Harlan Ltd, Blackthorne, UK) had free access to drinking water and high-fat diet composed of 45% fat, 20% protein and 35% carbohydrate (percent total energy 26.15 kJ/g; Special Diet Services, Essex, UK) for 140 days prior to commencement of acute *in vivo* studies. This diet resulted in progressive body weight gain (54.8 ± 5.2 *vs.* 38.9 ± 2.9 g, $p < 0.05$) and hyperglycaemia (8.8 ± 0.5 *vs.* 7.2 ± 0.4 mM/l, $p < 0.05$) compared with age-matched controls on normal laboratory chow (data not shown). All animal experiments were performed in accordance with the 'Principles of Laboratory Animal Care' (NIH Publication No. 85-23, revised 1985) as well as UK Animals (Scientific procedures) Act 1986. No adverse effects were observed following administration of any of the peptides.

2.5. Acute and persistent effects of Oxm peptides on glucose-lowering, insulin release and food intake in vivo

In a first series of experiments, *ob/ob* mice and high-fat fed mice received glucose alone (18 mmol/kg bw; i.p.) or in combination with Oxm, (D-Ser^2)Oxm and (D-Ser^2)Oxm[mPEG-PAL] (each at 1400 nmol/kg bw; final volume 8 ml/kg bw). In a second series of experiments using *ob/ob* mice to assess duration of bioactivity, glucose (18 mmol/kg bw; i.p.) was administered 4, 8 and 12 h after injection of saline vehicle (0.9% (w/v) NaCl), Oxm, (D-Ser^2)Oxm or (D-Ser^2)Oxm[mPEG-PAL] (each at 1400 nmol/kg bw). In a final series of experiments, accumulated food intake was measured in 12-h fasted *ob/ob* mice and high-fat fed animals at 0 h, and additionally at 4, 8 and 12 h in *ob/ob* mice after injection of saline vehicle (0.9% (w/v) NaCl) Oxm, (D-Ser^2)Oxm or (D-Ser^2)Oxm[mPEG-PAL] (each at 1400 nmol/kg bw).

2.6. Sub-chronic effects of once-daily administration of (D-Ser^2)Oxm[mPEG-PAL] in ob/ob mice

Once-daily injections of (D-Ser^2)Oxm[mPEG-PAL] (1400 nmol/kg bw; i.p.) or saline vehicle (0.9% (w/v) NaCl) were administered at 16:00 h over a 14-day period. Food intake, bodyweight, plasma glucose and insulin were monitored at intervals of 2–4 days during the treatment period and for an additional 14 days following cessation of peptide administration. Glucose tolerance (18 mmol/kg bw; i.p.) and insulin sensitivity (50 U/kg bw; i.p.) tests were performed on days 14 and 28. At termination, blood for measurement of circulating triglycerides, adiponectin and visfatin was taken and pancreatic tissues were excised and processed for measurement of insulin following extraction with ice-cold ethanol (5 ml/g) as described previously [24].

2.7. Biochemical analyses

Blood samples were collected from the cut tip on the tail vein of conscious mice into chilled fluoride/heparin glucose micro-centrifuge tubes (Sarstedt, Numbrecht, Germany) at time points indicated in the figures. Samples were immediately centrifuged using a Beckman micro-centrifuge (Beckman Instruments, Galway, Ireland) for 30 s at 13,000 × *g*. Whole blood samples were used to measure plasma glucose values with an Ascensia® CONTOUR® Microfill Blood Glucose Meter and Ascensia® MICROFILL® test strips (Bayer Healthcare, Uxbridge, UK). Plasma and pancreatic insulin was assayed by a modified dextran-coated charcoal radioimmunoassay as described previously [32]. Plasma triglyceride levels were measured using a Hitachi Automatic Analyser 912 (Boehringer Mannheim, Germany). Circulating adiponectin and visfatin were measured using ELISA kits (Phoenix Pharmaceuticals Inc., Burlingame, CA, USA). All analyses were carried out according to the manufacturer's instructions.

2.8. Statistical analysis

Results are expressed as means ± SEM and data compared using the unpaired Student's *t*-test with chi-squared analysis for correlations. Where appropriate, data were compared using repeated measures ANOVA or one-way ANOVA, followed by the Student–Newman–Keuls post hoc test. Incremental area under the curve (AUC) analyses for plasma glucose and insulin were calculated using GraphPad Prism version 3.02. Groups of data were considered to be significantly different if $p < 0.05$.

3. Results

3.1. DPP-IV stability

As depicted in Fig. 1A, Oxm was progressively degraded over 24 h with an estimated *in vitro* half-life of 9.3 h. In contrast, (D-Ser^2)Oxm and (D-Ser^2)Oxm[mPEG-PAL] maintained a significantly higher percentage of intact peptide ($p < 0.001$) over the 4–24 h period with biological half-lives >24 h. A significantly greater percentage of intact (D-Ser^2)Oxm[mPEG-PAL] (60% increase; $p < 0.001$) was observed after 24 h compared with degradation of (D-Ser^2)Oxm (Fig. 1A). Incubation with Vildagliptin for 2 h completely inhibited DPP-IV mediated degradation of Oxm and native GLP-1 (data not shown).

3.2. cAMP production and insulin secretion in BRIN-BD11 cells

Oxm, (D-Ser^2)Oxm and (D-Ser^2)Oxm[mPEG-PAL] stimulated cAMP production in a concentration-dependent manner with similar potency in BRIN-BD11 cells with EC_{50} values of 1.84 ± 0.09, 1.81 ± 0.37, and 1.97 ± 1.21 nM, respectively (Fig. 1B). Similarly, all peptides stimulated insulin secretion in a concentration-dependent and equipotent manner with maximal insulin secretion observed at the highest peptide concentrations (40–150% increase; $p < 0.001$; Fig. 1C).

3.3. cAMP production in GLP-1R and GR transfected cells

Receptor transfected cells were used to assess specificity of effects of Oxm and novel analogues. Table 1 shows cAMP production stimulated by various concentrations of Oxm, (D-Ser^2)Oxm, (D-Ser^2)Oxm[mPEG-PAL] and either native GLP-1 or glucagon. All Oxm peptides stimulated cAMP production in a concentration-dependent manner in both GLP-1 and glucagon receptor transfected cells with approximately similar potencies. In the presence of GLP-1 and glucagon receptor antagonists, cAMP production induced by Oxm peptides was antagonised in both GLP-1 (65–70% maximal antagonism; $p < 0.01$ to $p < 0.001$) and glucagon receptor (73–78% maximal antagonism; $p < 0.001$) transfected cells compared to control (respective natural ligands).

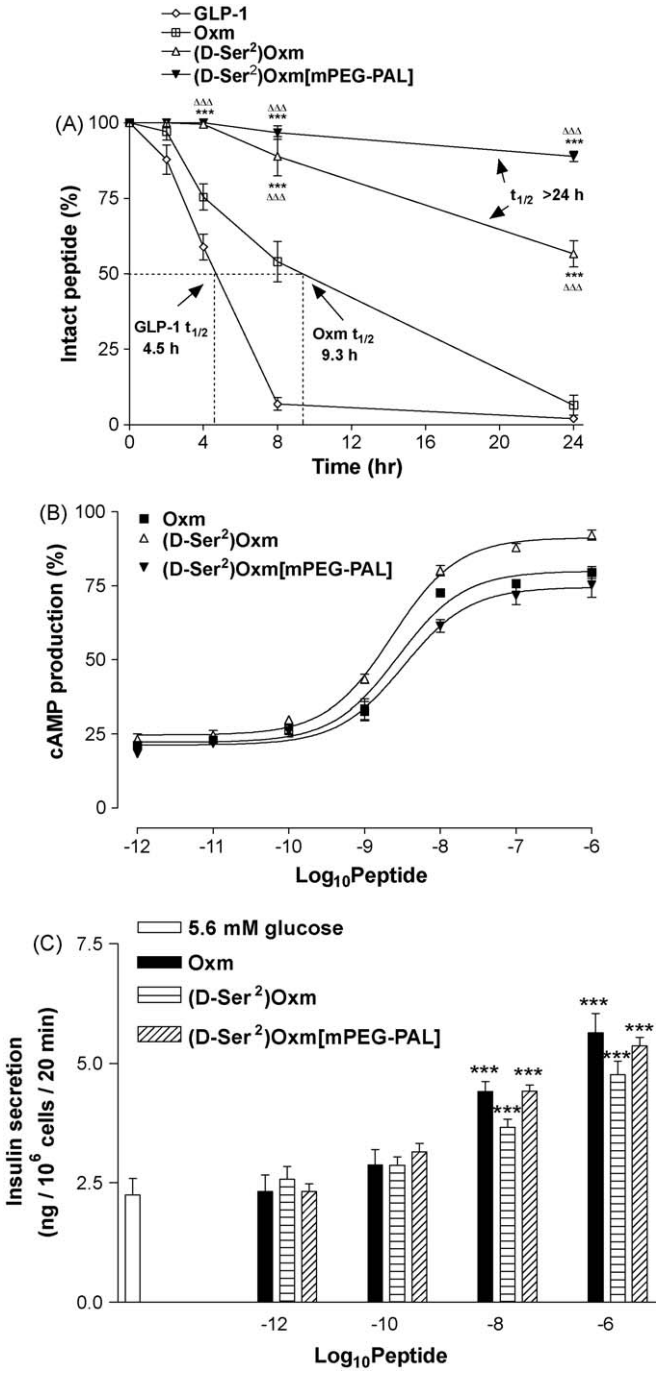

Fig. 1. Effects of Oxm analogues on (A) DPP-IV resistance, (B) cAMP production and (C) insulin secretion. (A) Resistance of Oxm, (D-Ser^2)Oxm, (D-Ser^2)Oxm[mPEG-PAL] and GLP-1 to degradation by DPP-IV (5 mU) was measured ($n = 3$) following 0, 2, 4, 8 and 24 h incubations. Reaction products were subsequently analyzed by HPLC and degradation expressed as a percentage of intact peptide. $^{***}p < 0.001$ compared with Oxm, $^{\Delta\Delta\Delta}p < 0.001$ compared with (D-Ser^2)Oxm. (B) BRIN-BD11 cells were exposed to various peptide concentrations for 20 min ($n = 4$) and cAMP production subsequently measured using ELISA. (C) BRIN-BD11 cells were incubated with a range of peptide concentrations for 20 min ($n = 8$) in the presence of 5.6 mM glucose and insulin release measured by radioimmunoassay. Data represent means ± SEM. $^{***}p < 0.001$ compared with 5.6 mM glucose control.

Table 1
Effects of Oxm analogues on cAMP production in GLP-1 and glucagon receptor transfected CHL cells.

Peptide	Agonist cAMP response (EC_{50}/nM)		Maximal cAMP antagonism in presence of 10^{-6} M antagonist (%)	
	GLP-1 R	Glucagon R	GLP-1 R	Glucagon R
GLP-1	0.05 ± 0.09	–	100.0	–
Glucagon	–	0.56 ± 0.08	–	100.0
Oxm	8.60 ± 0.53***	4.82 ± 0.81***	69.7 ± 1.2***	80.2 ± 3.8***
(D-Ser^2)Oxm	7.73 ± 0.62***	7.33 ± 0.52***	63.9 ± 3.2***	78.2 ± 1.9***
(D-Ser^2)Oxm[mPEG-PAL]	8.89 ± 0.43***	8.16 ± 1.23***	68.4 ± 6.3**	62.5 ± 2.2***

For agonist experiments, GLP-1 and glucagon receptor transfected CHL cells were exposed to various concentrations (10^{-12} to 10^{-6} M) of Oxm peptides or native GLP-1 or glucagon (as appropriate) for 20 min ($n = 4$) and cAMP production measured using ELISA. For antagonist studies, Oxm peptides or native GLP-1 or glucagon (as appropriate) (each at 10^{-6} M) were incubated in the presence of exendin (9–39)-amide (GLP-1 receptor antagonist) and DesHis^{1}DesPhe^{6}Glucagon-amide (glucagon receptor antagonist) each at 10^{-6} M. Data represent means ± SEM.
** $p < 0.01$ compared to natural ligands, GLP-1 and glucagon.
*** $p < 0.001$ compared to natural ligands, GLP-1 and glucagon.

3.4. Acute actions of Oxm, (D-Ser^2)Oxm and (D-Ser^2)Oxm[mPEG-PAL] on glucose-lowering and insulin secretion in vivo

All peptides significantly lowered plasma glucose concentrations (35–43% reduction; $p < 0.05$ to $p < 0.001$) compared to glucose alone in *ob/ob* mice (Fig. 2A). This effect was confirmed by AUC analysis (32–38% reduction; $p < 0.001$). Decreased glucose concentrations were associated with significantly increased plasma insulin levels (66–82% increase; $p < 0.01$ to $p < 0.001$) (Fig. 2B) which was confirmed by AUC analysis (58–66% increase; $p < 0.001$). As shown in Fig. 4A, Oxm peptides also significantly lowered plasma glucose (44–53% reduction; $p < 0.01$ to $p < 0.001$) following administration of glucose in high-fat fed mice. This effect was corroborated by plasma glucose AUC (35–48% reduction; $p < 0.01$ to $p < 0.001$).

3.5. Time-dependent effects of Oxm, (D-Ser^2)Oxm and (D-Ser^2)Oxm[mPEG-PAL] on glucose-lowering in vivo in ob/ob mice

As can be seen in Fig. 3A, animals treated with Oxm, (D-Ser^2)Oxm and (D-Ser^2)Oxm[mPEG-PAL] 4 h prior to a glucose load had significantly decreased plasma glucose (23–57% reduction; $p < 0.05$ to $p < 0.001$) compared to saline controls. This was corroborated by AUC analysis (25–57% reduction; $p < 0.05$). Compared with Oxm, both (D-Ser^2)Oxm and (D-Ser^2)Oxm[mPEG-PAL] demonstrated significantly improved glucose-lowering effects (15–32% reduction; $p < 0.05$) at 60 min post-glucose load. Eight hours post-administration, (D-Ser^2)Oxm and (D-Ser^2)Oxm[mPEG-PAL] had significantly reduced (25–42% reduction; $p < 0.05$ to $p < 0.01$; Fig. 3B) plasma glucose levels compared to both saline and Oxm (AUC: 25–34% reduction; $p < 0.05$ to $p < 0.01$). At 12 h post-administration, no significant differences were observed between any of the treatment groups (Fig. 3C).

3.6. Time-dependent effects of Oxm, (D-Ser^2)Oxm and (D-Ser^2)Oxm[mPEG-PAL] on feeding in vivo

Accumulative food intake was significantly reduced (13–56% reduction; $p < 0.05$ to $p < 0.01$) for (D-Ser^2)Oxm and (D-Ser^2)-Oxm[mPEG-PAL] at all time points up to 1400 min post-administration (Fig. 5A). Furthermore, (D-Ser^2)Oxm and (D-Ser^2)Oxm[mPEG-PAL] reduced food intake, 4 h (3–30% reduction; $p < 0.05$ to $p < 0.01$; Fig. 5B) and 8 h (7–10% reduction; $p < 0.05$; Fig. 5C) post-administration compared to saline controls. In contrast, Oxm-treated mice only demonstrated significant reductions in food intake when administered at 0 h (4–23% reduction; $p < 0.05$). At 12 h, no significant differences were observed between treatment groups (Fig. 5D). As shown in Fig. 4B, Oxm peptides also significantly inhibited food intake in high-fat fed mice relative to saline controls (33–53% reduction; $p < 0.05$ to $p < 0.001$). The two Oxm analogues were also more effective at inhibiting food intake during the latter stages of the experiment ($p < 0.01$ to $p < 0.001$).

3.7. Sub-chronic effects of once-daily administration of (D-Ser^2)Oxm[mPEG-PAL] on bodyweight, food intake, plasma glucose and insulin levels in ob/ob mice

Daily administration of (D-Ser^2)Oxm[mPEG-PAL] for 14 days significantly reduced bodyweight (11–15% reduction; $p < 0.05$ to $p < 0.01$; Fig. 6A) and food intake (11–29% reduction; $p < 0.05$ to $p < 0.001$; Fig. 6B). Similarly, non-fasting plasma glucose concentrations were significantly decreased (22–27% reduction; $p < 0.05$ to $p < 0.01$; Fig. 6C) and this was accompanied by significantly increased plasma insulin concentrations (45–54% increase; $p < 0.01$; Fig. 6D). No significant differences in any of the above parameters were observed 14 days following cessation of treatment.

3.8. Sub-chronic effects of once-daily administration of (D-Ser^2)Oxm[mPEG-PAL] on glucose tolerance and insulin response to glucose in ob/ob mice

Once-daily treatment with (D-Ser^2)Oxm[mPEG-PAL] for 14 days resulted in significantly reduced plasma glucose excursion (24–37% decrease; $p < 0.05$ to $p < 0.001$) following an i.p. glucose load (AUC: 26% decrease; $p < 0.01$, Fig. 7A). This improvement in glucose-lowering was associated with markedly improved glucose-mediated insulin responses (55–65% increase; $p < 0.05$ to $p < 0.01$; Fig. 7C), which were confirmed by AUC analysis (57% increase; $p < 0.01$). Following 14 days cessation of treatment, no significant differences in glucose tolerance or insulinotropic responses were noted (Fig. 7B and D).

3.9. Sub-chronic effects of once-daily administration of (D-Ser^2)Oxm[mPEG-PAL] on insulin sensitivity, pancreatic insulin content, circulating adiponectin, visfatin and triglycerides in ob/ob mice

Table 2 shows that (D-Ser^2)Oxm[mPEG-PAL] treated mice exhibited lower glucose concentrations following insulin ($p < 0.05$). Pancreatic insulin content was significantly increased (47%; $p < 0.001$). Furthermore, treatment with (D-Ser^2)Oxm[mPEG-PAL] significantly increased circulating adiponectin (37% increase; $p < 0.01$) and decreased circulating visfatin (23% reduction; $p < 0.01$) and triglycerides (28% reduction; $p < 0.01$). No significant differences were observed in any of these parameters following cessation of treatment (data not shown).

4. Discussion

Although being one of the less well-characterised members of the proglucagon family, Oxm has been shown to exhibit a range of potentially useful actions for the alleviation of obesity and diabetes [1,7,33]. These include bodyweight loss associated with decreased food intake and increased energy expenditure and voluntary physical activity [4,6,34]. Recently Oxm has been suggested to play a role in glucose homeostasis by lowering plasma glucose, most probably by improving pancreatic beta-cell function [8]. The cellular mechanisms responsible for these beneficial effects are poorly understood. However, recent evidence suggests a dual

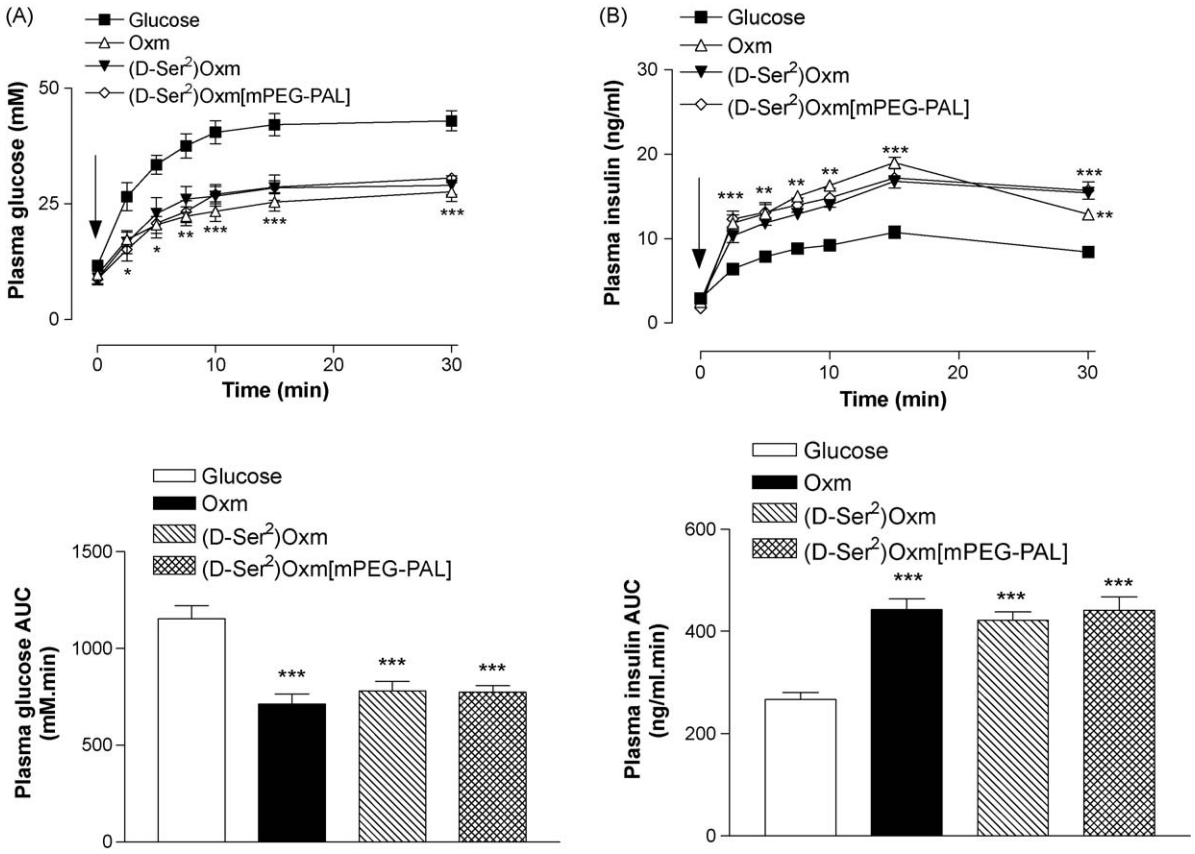

Fig. 2. Acute effects of Oxm analogues on plasma glucose (A) and insulin response (B) in *ob/ob* mice. Plasma glucose and insulin concentrations were measured prior to and after intraperitoneal administration of glucose alone (18 mmol/kg bw) or in combination with Oxm, (D-Ser^2)Oxm and (D-Ser^2)Oxm[mPEG-PAL] (each at 1400 nmol/kg bw). The time of glucose injection is indicated by the arrow (0 min). Plasma glucose and insulin AUC values for 0–30 min are also included. Data are expressed as means ± SEM for 6 mice. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared to glucose alone.

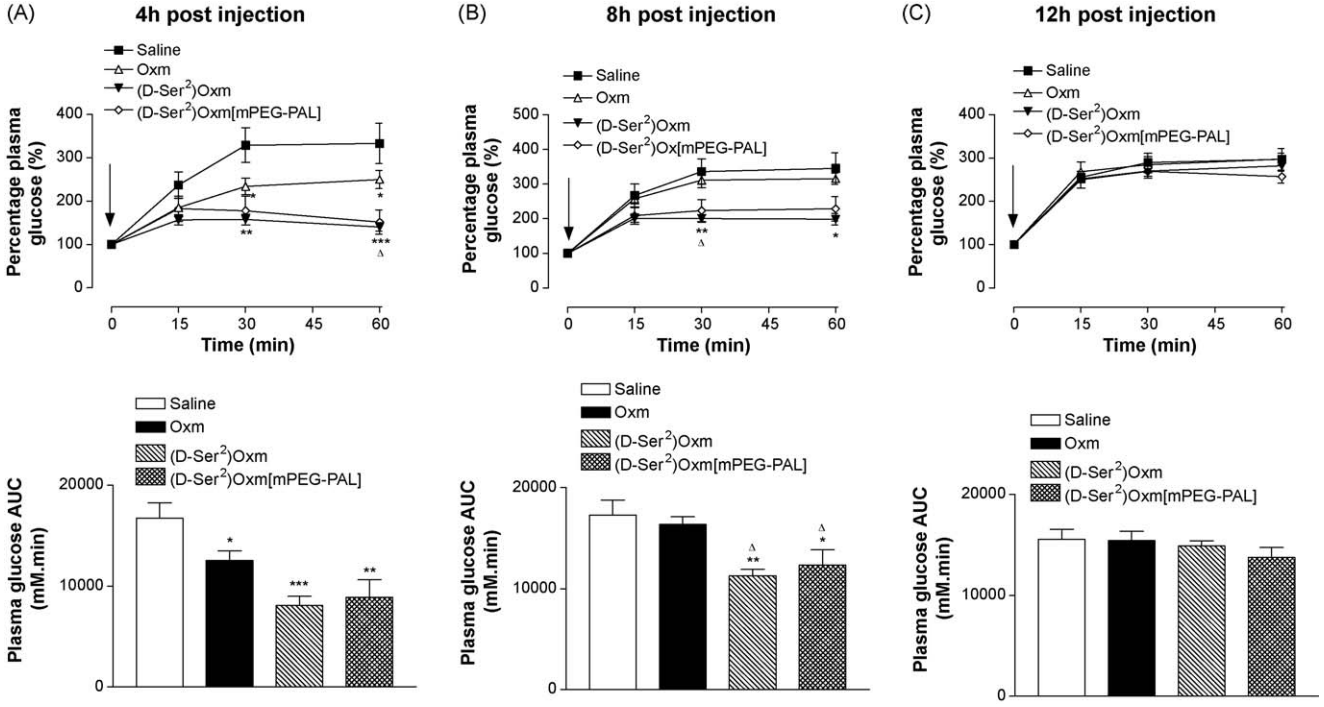

Fig. 3. Persistent effects of Oxm analogues on glucose-lowering in *ob/ob* mice. Plasma glucose concentrations were measured prior to and after intraperitoneal administration of glucose (18 mmol/kg bw) in animals injected 4 h (A), 8 h (B) and 12 h (C) previously with saline (0.9% (w/v), NaCl) or either Oxm, (D-Ser^2)Oxm or (D-Ser^2)Oxm[mPEG-PAL] (each at 1400 nmol/kg bw). The time of glucose injection is indicated by the arrow (0 min). Plasma glucose AUC values for 0–60 are included. Data represent means ± SEM for 6 mice. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared to saline control. $^{\Delta}p < 0.05$ compared with Oxm-treated mice.

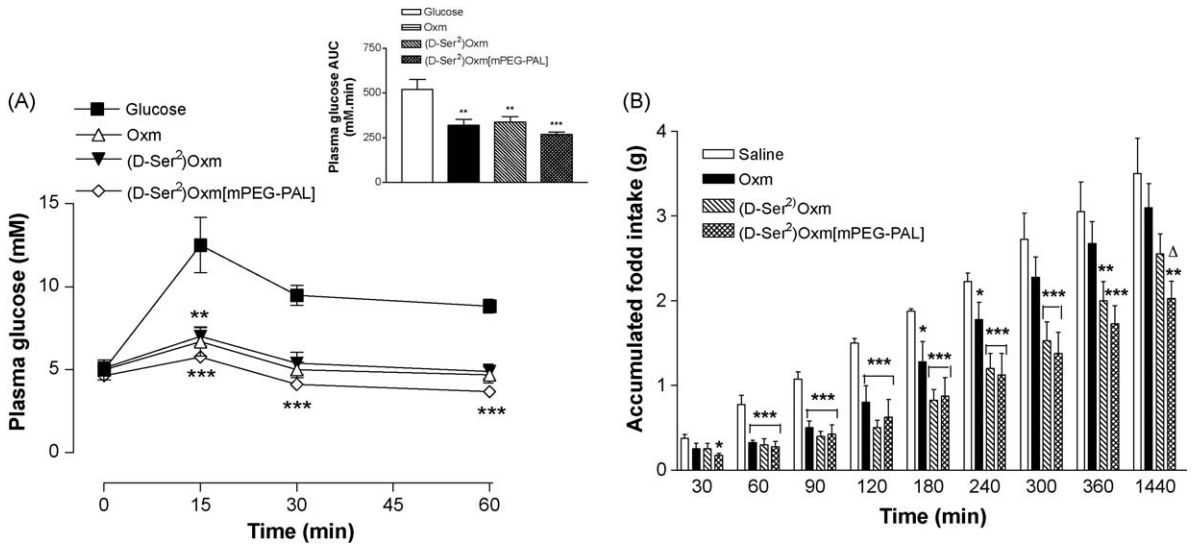

Fig. 4. Acute effects of Oxm analogues on plasma glucose (A) and food intake (B) in high-fat fed mice. Plasma glucose concentrations were measured prior to and after intraperitoneal administration of glucose alone (18 mmol/kg bw) or in combination with Oxm, (D-Ser^2)Oxm or (D-Ser^2)Oxm[mPEG-PAL] (each at 1400 nmol/kg bw). Plasma glucose AUC values for 0–60 min are also included. Food intake was measured in animals injected intraperitoneally at 0 h with saline vehicle or Oxm peptides (each at 1400 nmol/kg bw). Data are expressed as means ± SEM for 6 mice. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared to control animals. $^{\Delta}p < 0.05$ compared to Oxm-treated mice.

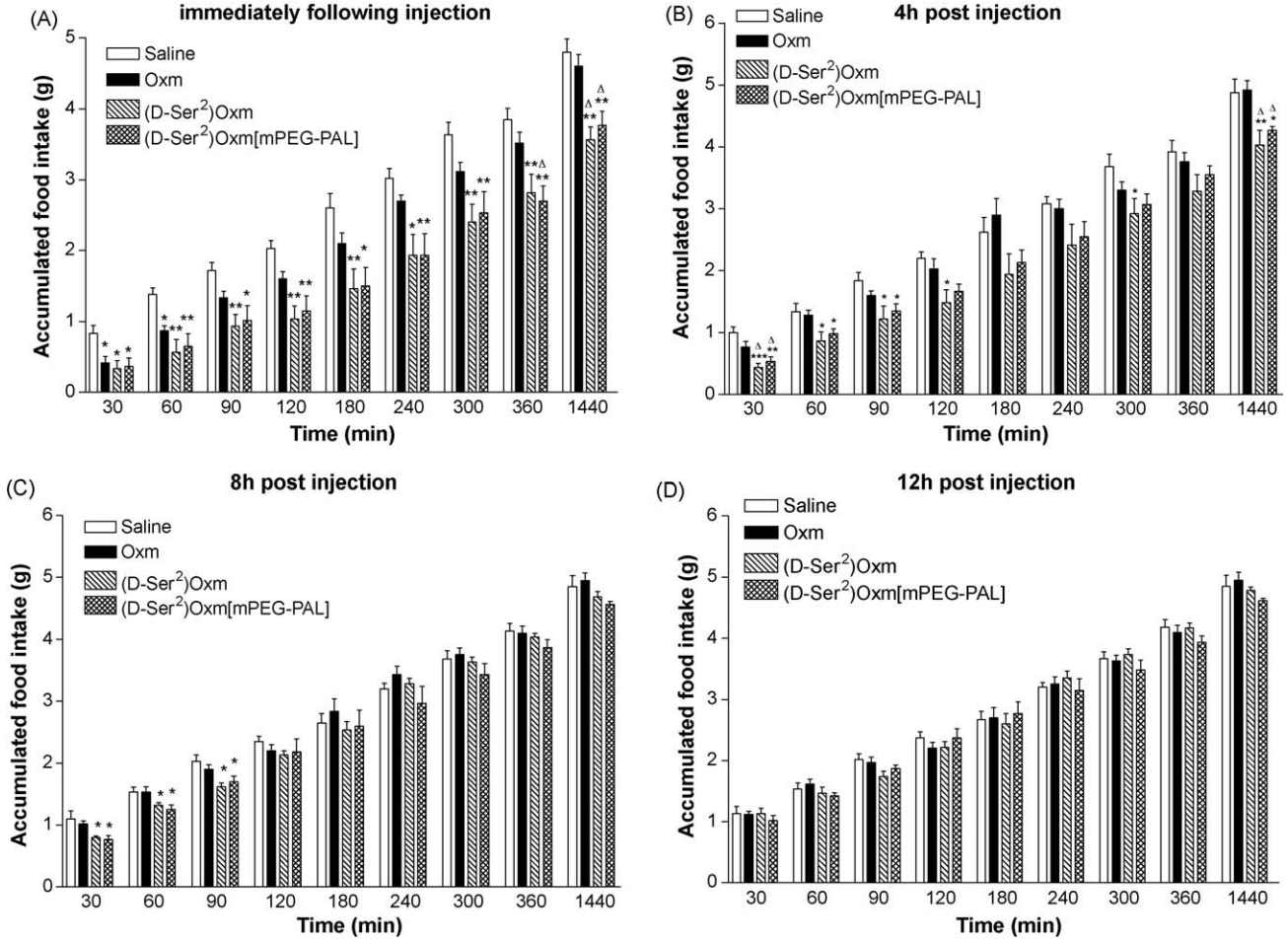

Fig. 5. Persistent effects of Oxm analogues on food intake in *ob/ob* mice. Food intake was measured in animals injected intraperitoneally at 0 h (A), 4 h (B), 8 h (C) and 12 h (D) previously with saline (0.9% (w/v), NaCl) or either Oxm, (D-Ser^2)Oxm or (D-Ser^2)Oxm[mPEG-PAL] (each at 1400 nmol/kg bw). Mice were fasted for 12 h prior to re-feeding. Data represent means ± SEM for 6 mice. $^{*}p < 0.05$, $^{**}p < 0.01$ compared to saline control. $^{\Delta}p < 0.05$ compared with Oxm-treated mice.

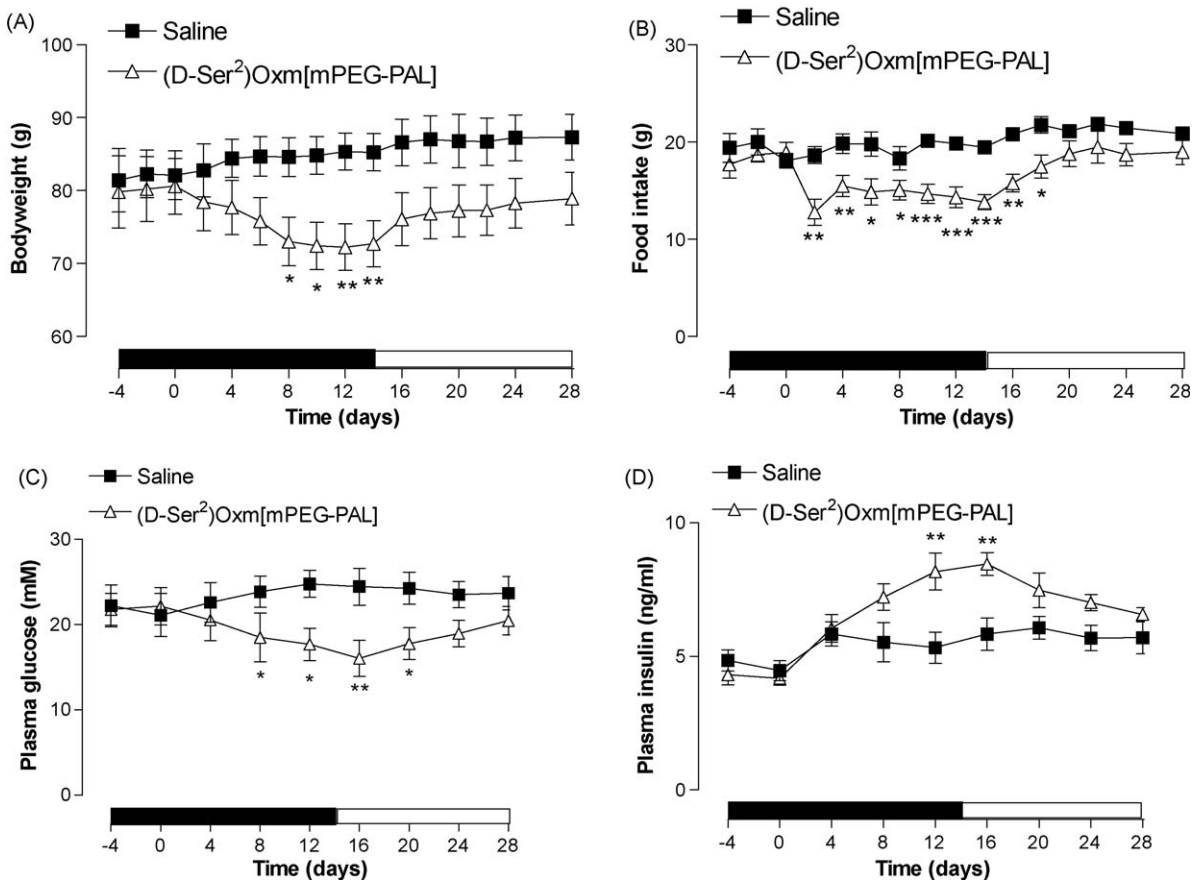

Fig. 6. Effects of once-daily administration of (D-Ser^2)Oxm[mPEG-PAL] on (A) body weight, (B) food intake, (C) plasma glucose and (D) plasma insulin in *ob/ob* mice. Parameters were measured prior to and 14 days during intraperitoneal treatment with (D-Ser^2)Oxm[mPEG-PAL] (1400 nmol/kg bw) or saline vehicle (0.9% (w/v) NaCl) and for 14 days following cessation of treatment. Data are expressed as means ± SEM for 9 mice. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared to saline-treated group.

mode of action mediated through activation of both GLP-1 and glucagon receptors [10,14]. This contrasts with some hybrid GLP-1–glucagon peptides which can have weak glucagon agonism in some tissues or even block glucagon sufficiently to exert beneficial effects [10].

The possible exploitation of therapies based around Oxm has been severely restricted due to proteolytic degradation of the peptide by the ubiquitous enzyme DPP-IV [15]. Improved biological efficacy of other proglucagon-derived peptides has been achieved through chemical modifications enabling DPP-IV resistance and prolonging biological half-life [17,18]. In the current paper, the effects of a novel chemically modified Oxm analogue, (D-Ser^2)Oxm[mPEG-PAL], has been examined on DPP-IV resistance, cellular cAMP production and insulin secretion, as well as acute and sub-chronic effects on glucose homeostasis, food intake and bodyweight regulation in obese diabetic (*ob/ob*) mice.

As expected from previous observations on DPP-IV resistance of N-terminally modified GLP-1, (D-Ser^2)Oxm and (D-Ser^2)Oxm[mPEG-PAL] remained almost completely intact throughout the 24 h incubation [35]. This contrasted sharply with Oxm which was progressively degraded over time with an *in vitro* half-life of 9.3 h. The *in vitro* half-life of Oxm is moderately longer than that of native GLP-1, consistent with previous observations that Oxm is less susceptible to degradation by DPP-IV. Furthermore, addition of the established DPP-IV inhibitor vildaglitpin in our assay system completely inhibited degradation of Oxm. (D-Ser^2)Oxm showed good but intermediate stability indicating that substitution with the D-isomer at Ser^2 is insufficient to confer total resistance to DPP-IV. However, the development of a specific assay to directly measure Oxm and related analogues in plasma would provide more precise details of endoprotease-mediated degradation and circulating half-life. Thus, the improved performance of (D-Ser^2)Oxm[mPEG-PAL] suggests that the C-terminal modification with C-16 fatty acid attached through a mini-PEG linker may completely mask the DPP-IV binding site. The addition of hydrophobic moieties might also have disrupted the hydro-affinity of (D-Ser^2)Oxm[mPEG-PAL] for DPP-IV as previously reported with incretin hormones [24,27]. Moreover, the *in vitro* DPP-IV assay used in this study does not exactly mimic the protease-laden *in vivo* situation nor rule out the possible involvement of other endoproteases [15].

(D-Ser^2)Oxm and (D-Ser^2)Oxm[mPEG-PAL] displayed similar concentration-dependent cAMP stimulation to Oxm with broadly similar EC_{50} values observed for the peptide family in BRIN-BD11, GLP-1 and glucagon receptor transfected cells. Consistent with dual agonism, all Oxm-derived peptides activated receptors for GLP-1 and glucagon receptors, but with less favourable EC_{50} values compared to native GLP-1 and glucagon. Consistent with this action, cAMP production by Oxm peptides was markedly reduced in the presence of GLP-1 and glucagon receptor antagonists. The equi-potency of (D-Ser^2)Oxm[mPEG-PAL] to other Oxm peptides indicates that the analogue retained full receptor affinity for both biochemical pathways. This view fits with observations that (D-Ser^2)Oxm[mPEG-PAL] enhanced insulin stimulation from BRIN-BD11 cells in a concentration-dependent manner similar to Oxm. Interestingly, other studies also point to a possible insulinotropic role for Oxm [8]. This maintenance of *in vitro* biological efficacy for (D-Ser^2)Oxm[mPEG-PAL] coupled with a strong resistance to DPP-IV supports the idea of improved potency of the peptide *in vivo*.

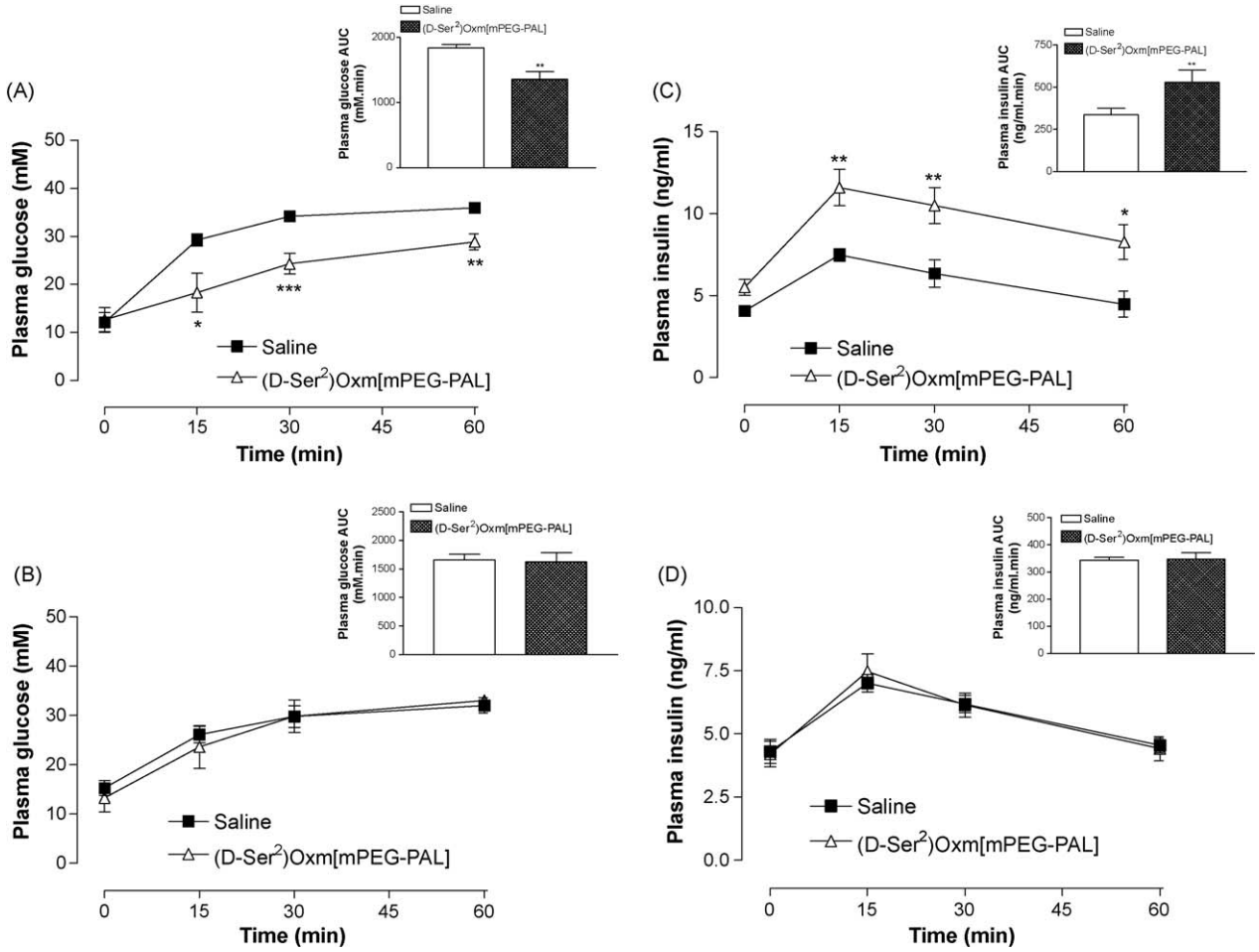

Fig. 7. Effects of once-daily administration of (D-Ser^2)Oxm[mPEG-PAL] on glucose tolerance and insulin response to glucose in *ob/ob* mice. Tests were conducted following 14 days intraperitoneal treatment (A and C) with (D-Ser^2)Oxm[mPEG-PAL] (1400 nmol/kg bw) or saline vehicle (0.9% (w/v) NaCl) and 14 days following cessation of treatment (B and D). Data are expressed as means ± SEM for 9 mice. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared to saline-treated group.

To assess the antihyperglycaemic, insulinotropic and anorexigenic effects of (D-Ser^2)Oxm[mPEG-PAL] *in vivo* we employed obese diabetic (*ob/ob*) mice [31]. These mice represent a well established model of obesity and type 2 diabetes, presenting with hyperphagia, obesity, moderate hyperglycaemia and severe hyperinsulinaemia. All Oxm peptides significantly reduced plasma glucose excursion following a glucose load which was associated with a strong increase in plasma insulin concentrations. This is consistent with a possible glucose-dependent stimulation of insulin secretion similar to that observed with other incretin hormones [36]. The antihyperglycaemic effect of native Oxm was not sustained beyond 4 h. In contrast, (D-Ser^2)Oxm and (D-Ser^2)Oxm[mPEG-PAL] elicited a glucose-lowering action for up to 8 h after initial administration, with (D-Ser^2)Oxm[mPEG-PAL] showing a trend towards reduced plasma glucose even up to 12 h post-treatment. Similarly, whereas inhibition of feeding by native Oxm did not persist for more than 60 min, (D-Ser^2)Oxm and (D-Ser^2)Oxm[mPEG-PAL] decreased food intake for much longer periods even when administered 4 or 8 h before feeding. This extended glucose-lowering and satiety action suggests a much longer plasma half-life of the Oxm analogues, similar to the findings of *in vitro* DPP-IV studies, providing a basis for use of (D-Ser^2)Oxm[mPEG-PAL] in longer-term studies. Observations of acute glucose-lowering and inhibition of feeding in high-fat fed mice with diet-induced obesity suggests that these effects of Oxm analogues extend to other animal models.

Table 2
Effects of once-daily administration of (D-Ser^2)Oxm[mPEG-PAL] on insulin sensitivity, pancreatic insulin content, adiponectin, visfatin and triglyceride levels in *ob/ob* mice.

Parameter	Saline vehicle	(D-Ser^2)Oxm[mPEG-PAL]
Insulin sensitivity (mM min)	1189.13 ± 14.21	1007.44 ± 8.5*
Insulin content (μg/g tissue)	4.31 ± 0.27	6.33 ± 0.23***
Adiponectin (ng/ml)	1.06 ± 0.09	1.45 ± 0.06**
Visfatin (ng/ml)	1.35 ± 0.06	1.02 ± 0.05**
Triglycerides (mmol/l)	6.39 ± 0.23	4.62 ± 0.45**

Tests were conducted following 14 days intraperitoneal treatment with (D-Ser^2)Oxm[mPEG-PAL] (1400 nmol/kg bw) or saline vehicle (0.9% (w/v) NaCl). Data are expressed as means ± SEM for 5 mice.
* $p < 0.05$ compared to saline-treated group.
** $p < 0.01$ compared to saline-treated group.
*** $p < 0.001$ compared to saline-treated group.

Daily administration of (D-Ser^2)Oxm[mPEG-PAL] for 14 days to *ob/ob* mice significantly reduced bodyweight and food intake. Thus despite probable half-life around 12 h, administration of peptide at 16:00 h, immediately before the dark period with associated feeding, induced clear-cut biological effects. Importantly, subchronic (D-Ser^2)Oxm[mPEG-PAL] treatment also reduced plasma glucose concentrations with an associated increase of plasma insulin. This was accompanied by improvements in glucose tolerance, insulin response to glucose and pancreatic insulin content, suggesting an important anti-diabetic action in this commonly employed animal model. Observed up-regulation of glucose homeostasis was also associated with moderate improvement of insulin sensitivity and lowered glucose concentrations.

These observations add to previous suggestions of enhanced beta-cell function and glucose homeostasis with use of Oxm-based therapies [8] and the general utility of enzyme-resistant acylated peptides [23,25,27]. Furthermore, sub-chronic (D-Ser^2)Oxm[mPEG-PAL] treatment significantly increased circulating adiponectin levels while decreasing visfatin and triglyceride concentrations, which are supported by studies using a dual GLP-1/glucagon agonist [10]. Although the precise mechanisms for these changes in adipokines are unclear, they suggest that the weight loss observed following (D-Ser^2)Oxm[mPEG-PAL] treatment may be at least partly attributable to reduction in adipose tissue. Further studies incorporating pair-fed groups assessing parameters such as locomotor activity, energy expenditure and respiratory quotient may prove useful to address this. Interestingly, all of these effects were reversed following 14 days of cessation of treatment, indicating that continuing peptide exposure is required for sustained effects. Oxm peptides were well tolerated and no noticeable changes in general behaviour or activity was observed following administration.

In conclusion, this study has demonstrated that N-terminal modification by substitution of L- to D-isomer at Ser^2 and addition of a C-16 palmitic acid conjugated via a mini-PEG linker at the C-terminus of Oxm substantially improved DPP-IV resistance without compromising cellular actions including cAMP production, stimulation of insulin secretion and inhibition of feeding. (D-Ser^2)Oxm[mPEG-PAL] clearly exhibited enhanced biological activity over native Oxm and (D-Ser^2)Oxm. Indeed, the positive acute and sub-chronic effects of (D-Ser^2)Oxm[mPEG-PAL] on glucose homeostasis, beta-cell insulin secretion, food intake, bodyweight and circulating markers of fat metabolism encourage further studies to progress Oxm-based therapies for obesity-diabetes.

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