Investigation of Structure-Activity Relationships of Oxyntomodulin (Oxm) Using Oxm Analogs

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Oxyntomodulin (Oxm) is an intestinal peptide that inhibits food intake and body weight in rodents and humans. These studies used peptide analogs to study aspects of structure and function of Oxm, and the sensitivity of parts of the Oxm sequence to degradation. Analogs of Oxm were synthesized and studied using receptor binding and degradation studies *in vitro*. Their effects on food intake and conditioned taste avoidance were measured *in vivo* in rodents. Oxm breakdown by the enzyme dipeptidyl peptidase IV (DPPIV) was demonstrated *in vitro* and *in vivo*. *In vitro* degradation was reduced and *in vivo* bioactivity increased by inhibitors of DPPIV. Modifications to the N terminus of Oxm modulated binding to the glucagon-like peptide (GLP)-1 receptor and degradation by DPPIV. Modifications to the midsection of Oxm modulated binding to the GLP-1 receptor and degradation by neutral endopeptidase. These modifications also altered bioactivity *in vivo*. The C-terminal octapeptide of Oxm was shown to contribute to the properties of Oxm *in vitro* and *in vivo* but was not alone sufficient for the effects of the peptide. Elongation and acylation of the C terminus of Oxm altered GLP-1 receptor binding and duration of action *in vivo*, which may be due to changes in peptide clearance. An Oxm analog was developed with enhanced pharmaceutical characteristics, with greater potency and longevity with respect to effects on food intake. These studies suggest that Oxm is a potential target for antiobesity drug design. (*Endocrinology* 150: 1712–1721, 2009)

Oxyntomodulin (Oxm) is an intestinal peptide from the preproglucagon family, which inhibits gastric acid secretion (1–3) and inhibits food intake (4). Oxm can bind to and activate the glucagon-like peptide (GLP)-1 receptor (4, 5), albeit relatively weakly (6). The anorectic effects of Oxm are blocked by the GLP-1 receptor antagonist exendin $_{(9-39)}$ and are absent in the GLP-1 receptor knockout mouse, suggesting that they are mediated by the GLP-1 receptor (7). However, it has been reported that similar doses of Oxm and GLP-1 are required to inhibit food intake to an equivalent degree, despite their differential affinity for the GLP-1 receptor (8). There is evidence that Oxm acts primarily in the hypothalamic arcuate nucleus, whereas GLP-1 acts principally via the brainstem (8), though other studies have not found these differences (7). The effects of Oxm and GLP-1 on nausea (9–12) and energy expenditure (7, 12–14) may also differ in both rodents and humans. However, whereas a receptor distinct from the GLP-1 receptor may mediate some Oxm effects

(13), this has not to date been convincingly demonstrated. Oxm and GLP-1 may differ in terms of central nervous system penetration or tissue-specific modification of receptor function but appear likely to both act via the GLP-1 receptor for at least some functions. Therefore, analysis of peptide binding at the GLP-1 receptor may give insight into biological function.

Determination of the roles of specific functional groups and structural properties of Oxm may provide insight into its mechanism of action (15). Furthermore, for peptides with potential therapeutic applications, clinical use is frequently limited by their short duration of action and the consequent need for repeated administration. Rational modifications of peptide sequence or secondary structure may enhance bioactivity and/or half-life (16).

The Oxm amino terminus may be involved in receptor binding and activation. Proglucagon-derived peptides are broken down at least partly by the enzyme dipeptyl peptidase IV (DPPIV) at the amino terminus (17–19), and rational changes to this part

Abbreviations: CTA, Conditioned taste avoidance; DPPIV, dipeptidyl peptidase IV; GLP, glucagon-like peptide; NEP, neutral endopeptidase 24.11; Oxm, oxyntomodulin; TFA, trifluoroacetic acid.

of the molecule may modulate susceptibility to DPPIV degradation (20–23). The midsection of Oxm may also be a target for degradative enzymes such as the ectopeptidases (24) and neutral endopeptidase 24.11 (NEP) (24, 25). Proglucagon-derived peptides adopt α-helical conformations in environments resembling the cell membrane (26). Evidence suggests that this helix may be required for receptor binding (27–29). Oxm differs from glucagon by an eight-amino acid sequence at the carboxy terminus. Although Oxm binds the glucagon receptor with an affinity 10- to 50-fold lower than glucagon (30, 31), both Oxm and glucagon can bind and activate the GLP-1 receptor, though with lower affinity than GLP-1 (5, 32). Oxm is 10–20 times more potent than glucagon at inhibiting stimulated gastric acid secretion in the rat, suggesting a role for the octapeptide extension in mediating this biological action (1). The octapeptide (also known as spacer peptide II) contains the minimal fragment necessary to inhibit acid secretion, albeit with a 100-fold reduced potency compared with full-length Oxm (2), and, thus, may have a physiological role. Its role in food intake is less clear.

We have designed a series of Oxm analogs with modifications to specific regions of the peptide as tools to investigate the effects of these regions on actions *in vivo* and *in vitro*. We have investigated their binding affinity for the GLP-1 receptor and their effect on food intake. These studies provide insights into the structure-function relationships of the Oxm molecule. The results have aided the development of an Oxm analog with enhanced bioactivity for exogenous administration and suggest that Oxm analogs may have utility as antiobesity agents.

Materials and Methods

Peptides

Peptides were obtained from Advanced Biotechnology Services, Imperial College London (London, UK) and BioMol International LP (Exeter, UK). Peptides were synthesized, purified, and analyzed using established protocols (33–35). Synthesis and purification used automated Fluorenyl-Methoxy-Carbonyl solid phase peptide synthesis (Fmoc SPSS). Peptides were synthesized on TentaGel resins, derivatized with one of a number of cleavable linkers, using an Fmoc/t-butyl-based solid-phase synthesis strategy. Temporary N-amino group protection was afforded by the Fmoc-group, with t-butyl ethers being used for protection of tyrosine, serine, and threonine hydroxyl side chains, whereas t-butyl esters protected the side chains of aspartic and glutamic acid residues. Histidine and lysine side chains were protected as their N- and N- Boc derivatives, respectively, cysteine as its S-trityl derivative, and arginine guanidine moiety as its Pbf derivative.

Rational peptide modifications can be used to influence the duration of peptide activity *in vivo*. For example, elongating a peptide chain can reduce susceptibility to degradation and clearance (25, 28), and addition of an acyl side chain can encourage a peptide to bind plasma proteins, thus reducing breakdown by endopeptidases and impeding metabolic clearance by the kidney (36). Where N-acylation of lysine was required, orthogonal protection was afforded by the incorporation of 1-(4,4-dimethyl-2,6-dioxocyclohexylidene)3-methyl-butyl lysine (Lys(ivDde)), which was deprotected on solid phase using 2% hydrazine in dimethylformaldehyde. Acylation was performed using diisopropylcarbodiimide/1-hydroxybenzotriazole hydrate. Upon completion of the synthesis, peptides were cleaved from the solid support, with removal of side chain protecting groups, by treatment with aqueous trifluoroacetic acid (TFA) containing triisobutylsilane as a scavenger. After removal of TFA and scavengers by evapora-

tion and trituration, peptide purification was performed by reversed-phase preparative HPLC, followed by lyophilization. The purified product was subsequently analyzed by reverse-phase HPLC and by mass spectrometry. The sequences of all peptide analogs used are shown in Fig. 1.

Acute feeding studies in mice

C57BL/6 mice (20–25 g; Harlan, Loughborough, UK) were singly housed, and maintained under controlled conditions of temperature (21–23 C) and light (lights on at 0700 h, lights off at 1900 h) with *ad libitum* access to RM1 diet (Special Diet Services, Witham, UK) (37). All animal procedures undertaken were approved by the British Home Office Animals (Scientific Procedures) Act 1986 (Project License 70/5516). Animals were acclimatized to laboratory conditions for at least 1 wk and handled daily before the first study, during which time they received two injections of saline to minimize stress on the study days. Intraperitoneal and sc injections were administered via a 1-ml syringe with a 28-gauge needle (maximum injection volume 0.2 ml). For each study, animals were fasted from 1600 h the preceding day and injected in the early light phase (0900–1000 h). All peptides were dissolved in 0.9% saline, and 0.9% saline was administered as a control injection. After injection, animals were returned to their home cages containing a preweighed amount of food that was reweighed at 1, 2, 4, 8, and 24 h after injection.

Receptor binding assay

GLP-1 receptor binding study

Plasma membranes from male Wistar rat lung tissue, known to be rich in GLP-1 receptors, were prepared and binding assays performed as previously described (4, 38). Rats were killed, lungs removed, and membranes prepared by homogenization and differential centrifugation. Membranes (200 μg) were incubated for 90 min in silanized polypropylene tubes together with [^{125}I]Ex-4 [500 Bq (100 pM)] and unlabeled competing peptides (as specified), at 4 C in binding buffer [50 mM HEPES (pH 7.4), 1 mM MgCl$_2$, 0.1% BSA and protease inhibitors (0.2 mM phenylmethylsulfonylfluoride; Sigma-Aldrich Corp., St. Louis, MO), 0.1 mM Diprotin A (Sigma-Aldrich), and 10 μM phosphoramidon (Sigma-Aldrich)] in a final assay volume of 0.5 ml. Peptides were iodinated as previously described (39) using ^{125}I from GE Healthcare Life Sciences (Amersham, Bucks, UK). Pelleted membranes were washed with assay buffer (0.5 ml, ice cold) and the membranes centrifuged (15,874 × g, 2 min, 4 C) as described to separate bound and free label. Bound radioactivity was measured using a γ-counter. Specific binding was calculated as the difference between the amount of [^{125}I]Ex-4 bound in the absence (total binding) and presence of 5 μM unlabeled competing peptide (nonsaturable binding). All curves were performed with points in triplicate. IC$_{50}$ values were calculated using the Prism 4 program (GraphPad Software Inc., San Diego, CA).

Glucagon receptor binding assay

Membranes were prepared as described (38) using the same method as that used in the GLP-1R binding assay except that liver was used as the tissue source. Binding assays were completed as for GLP-1r binding except that 100 ng membrane was used with [^{125}I]glucagon [500 Bq (100 pM)] label.

Degradation assays

DPPIV protease assay

Digest buffer [100 mM Tris-HCl (pH 8)] containing 15 μM peptide and 1 μg porcine kidney DPPIV (Sigma-Aldrich) was incubated at 37 C. The reaction was terminated at the specified time point by adding 10 μl 10% TFA, followed by reverse-phase HPLC on a Gemini C18 column (Phenomenex, Macclesfield, UK). The column was eluted with a linear gradient of 27–31% AcN over 50 min at 1 ml/min. Peptides and their degradation products were monitored by their absorbance at 214 nm. Percent degradation was quantified by integration of peak areas related to undigested peptide peaks and corrected for degradation in the absence of enzyme.

residue position	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42
Oxm	His	Ser	Gln	Gly	Thr	Phe	Thr	Ser	Asp	Tyr	Ser	Lys	Tyr	Leu	Asp	Ser	Arg	Arg	Ala	Gln	Asp	Phe	Val	Gln	Trp	Leu	Met	Asn	Thr	Lys	Arg	Asn	Arg	Asn	Asn	Ile	Ala					
Glucagon	His	Ser	Gln	Gly	Thr	Phe	Thr	Ser	Asp	Tyr	Ser	Lys	Tyr	Leu	Asp	Ser	Arg	Arg	Ala	Gln	Asp	Phe	Val	Gln	Trp	Leu	Met	Asn	Thr													
GLP-1	His	Ala	Glu	Gly	Thr	Phe	Thr	Ser	Asp	Val	Ser	Ser	Tyr	Leu	Glu	Gly	Gln	Ala	Ala	Lys	Glu	Phe	Ile	Ala	Trp	Leu	Val	Lys	Gly	Arg												
Exendin-4	His	Gly	Glu	Gly	Thr	Phe	Thr	Ser	Asp	Leu	Ser	Lys	Gln	Met	Glu	Glu	Glu	Ala	Val	Arg	Leu	Phe	Ile	Glu	Trp	Leu	Lys	Asn	Gly	Gly	Pro	Ser	Ser	Gly	Ala	Pro	Pro	Pro	Ser	NH2		
DHis1-Oxm	DHis	Ser	Gln	Gly	Thr	Phe	Thr	Ser	Asp	Tyr	Ser	Lys	Tyr	Leu	Asp	Ser	Arg	Arg	Ala	Gln	Asp	Phe	Val	Gln	Trp	Leu	Met	Asn	Thr	Lys	Arg	Asn	Arg	Asn	Asn	Ile	Ala					
Ala2-Oxm	His	Ala	Gln	Gly	Thr	Phe	Thr	Ser	Asp	Tyr	Ser	Lys	Tyr	Leu	Asp	Ser	Arg	Arg	Ala	Gln	Asp	Phe	Val	Gln	Trp	Leu	Met	Asn	Thr	Lys	Arg	Asn	Arg	Asn	Asn	Ile	Ala					
DHis1-Ala2-Oxm	DHis	Ala	Gln	Gly	Thr	Phe	Thr	Ser	Asp	Tyr	Ser	Lys	Tyr	Leu	Asp	Ser	Arg	Arg	Ala	Gln	Asp	Phe	Val	Gln	Trp	Leu	Met	Asn	Thr	Lys	Arg	Asn	Arg	Asn	Asn	Ile	Ala					
Oxm(ex15-18)	His	Ser	Gln	Gly	Thr	Phe	Thr	Ser	Asp	Tyr	Ser	Lys	Tyr	Leu	Glu	Glu	Glu	Ala	Ala	Gln	Asp	Phe	Val	Gln	Trp	Leu	Met	Asn	Thr	Lys	Arg	Asn	Arg	Asn	Asn	Ile	Ala					
Oxm(ex15-21)	His	Ser	Gln	Gly	Thr	Phe	Thr	Ser	Asp	Tyr	Ser	Lys	Tyr	Leu	Glu	Glu	Glu	Ala	Val	Arg	Leu	Phe	Val	Gln	Trp	Leu	Met	Asn	Thr	Lys	Arg	Asn	Arg	Asn	Asn	Ile	Ala					
Oxm(ex15-23)	His	Ser	Gln	Gly	Thr	Phe	Thr	Ser	Asp	Tyr	Ser	Lys	Tyr	Leu	Glu	Glu	Glu	Ala	Val	Arg	Leu	Phe	Ile	Gln	Trp	Leu	Met	Asn	Thr	Lys	Arg	Asn	Arg	Asn	Asn	Ile	Ala					
Oxm(ex15-24)	His	Ser	Gln	Gly	Thr	Phe	Thr	Ser	Asp	Tyr	Ser	Lys	Tyr	Leu	Glu	Glu	Glu	Ala	Val	Arg	Leu	Phe	Ile	Glu	Trp	Leu	Met	Asn	Thr	Lys	Arg	Asn	Arg	Asn	Asn	Ile	Ala					
Oxm(ex27-33)	His	Ser	Gln	Gly	Thr	Phe	Thr	Ser	Asp	Tyr	Ser	Lys	Tyr	Leu	Asp	Ser	Arg	Arg	Ala	Gln	Asp	Phe	Val	Gln	Trp	Leu	Lys	Asn	Gly	Gly	Pro	Ser	Ser	Asn	Asn	Ile	Ala					
Oxm(ex29-33)	His	Ser	Gln	Gly	Thr	Phe	Thr	Ser	Asp	Tyr	Ser	Lys	Tyr	Leu	Asp	Ser	Arg	Arg	Ala	Gln	Asp	Phe	Val	Gln	Trp	Leu	Met	Asn	Gly	Gly	Pro	Ser	Ser	Asn	Asn	Ile	Ala					
Oxm(ex30-33)	His	Ser	Gln	Gly	Thr	Phe	Thr	Ser	Asp	Tyr	Ser	Lys	Tyr	Leu	Asp	Ser	Arg	Arg	Ala	Gln	Asp	Phe	Val	Gln	Trp	Leu	Met	Asn	Thr	Gly	Pro	Ser	Ser	Asn	Asn	Ile	Ala					
Oxm(ex27-30)	His	Ser	Gln	Gly	Thr	Phe	Thr	Ser	Asp	Tyr	Ser	Lys	Tyr	Leu	Asp	Ser	Arg	Arg	Ala	Gln	Asp	Phe	Val	Gln	Trp	Leu	Lys	Asn	Gly	Gly	Arg	Asn	Arg	Asn	Asn	Ile	Ala					
Oxm19-37																			Ala	Gln	Asp	Phe	Val	Gln	Trp	Leu	Met	Asn	Thr	Lys	Arg	Asn	Arg	Asn	Asn	Ile	Ala					
Oxm30-37																														Lys	Arg	Asn	Arg	Asn	Asn	Ile	Ala					
Oxm-Ala38	His	Ser	Gln	Gly	Thr	Phe	Thr	Ser	Asp	Tyr	Ser	Lys	Tyr	Leu	Asp	Ser	Arg	Arg	Ala	Gln	Asp	Phe	Val	Gln	Trp	Leu	Met	Asn	Thr	Lys	Arg	Asn	Arg	Asn	Asn	Ile	Ala	Ala				
Oxm-Ala38,39	His	Ser	Gln	Gly	Thr	Phe	Thr	Ser	Asp	Tyr	Ser	Lys	Tyr	Leu	Asp	Ser	Arg	Arg	Ala	Gln	Asp	Phe	Val	Gln	Trp	Leu	Met	Asn	Thr	Lys	Arg	Asn	Arg	Asn	Asn	Ile	Ala	Ala	Ala			
Oxm-Ala38-42	His	Ser	Gln	Gly	Thr	Phe	Thr	Ser	Asp	Tyr	Ser	Lys	Tyr	Leu	Asp	Ser	Arg	Arg	Ala	Gln	Asp	Phe	Val	Gln	Trp	Leu	Met	Asn	Thr	Lys	Arg	Asn	Arg	Asn	Asn	Ile	Ala	Ala	Ala	Ala	Ala	Ala
Oxm-Lys38-Laur	His	Ser	Gln	Gly	Thr	Phe	Thr	Ser	Asp	Tyr	Ser	Lys	Tyr	Leu	Asp	Ser	Arg	Arg	Ala	Gln	Asp	Phe	Val	Gln	Trp	Leu	Met	Asn	Thr	Lys	Arg	Asn	Arg	Asn	Asn	Ile	Ala	Lys	LAUROYL			
Oxm-Lys38-Palm	His	Ser	Gln	Gly	Thr	Phe	Thr	Ser	Asp	Tyr	Ser	Lys	Tyr	Leu	Asp	Ser	Arg	Arg	Ala	Gln	Asp	Phe	Val	Gln	Trp	Leu	Met	Asn	Thr	Lys	Arg	Asn	Arg	Asn	Asn	Ile	Ala	Lys	PALMITOYL			
Oxm-Ala38,39-Lys40Laur	His	Ser	Gln	Gly	Thr	Phe	Thr	Ser	Asp	Tyr	Ser	Lys	Tyr	Leu	Asp	Ser	Arg	Arg	Ala	Gln	Asp	Phe	Val	Gln	Trp	Leu	Met	Asn	Thr	Lys	Arg	Asn	Arg	Asn	Asn	Ile	Ala	Ala	Ala	Lys	LAUROYL	
Oxm-Ala38,39-Lys40Palm	His	Ser	Gln	Gly	Thr	Phe	Thr	Ser	Asp	Tyr	Ser	Lys	Tyr	Leu	Asp	Ser	Arg	Arg	Ala	Gln	Asp	Phe	Val	Gln	Trp	Leu	Met	Asn	Thr	Lys	Arg	Asn	Arg	Asn	Asn	Ile	Ala	Ala	Ala	Lys	PALMITOYL	
DHis1-Ala2-Oxm(ex15-23)(ex27-33)-Ala38,39-Lys40-Lauroyl	DHis	Ala	Gln	Gly	Thr	Phe	Thr	Ser	Asp	Tyr	Ser	Lys	Tyr	Leu	Glu	Glu	Glu	Ala	Val	Arg	Leu	Phe	Ile	Gln	Trp	Leu	Lys	Asn	Gly	Gly	Pro	Ser	Ser	Asn	Asn	Ile	Ala	Ala	Ala	Lys	LAUROYL	

Key

■ D-amino acid   ■ Change to residue 2   ■ Exendin-like sequence   ■ Lys residue for acylation   ■ Alanine chain insertion   ■ Acyl side chain

FIG. 1. Peptide sequences for analogs used in studies.

Neutral endopeptidase (neprilysin) protease assay

The neprilysin protease assay was performed as described previously, but using digest buffer 50 mM Tris-HCl, 50 mM NaCl (pH 7.5), with 200 ng recombinant human neprilysin-2 (R&D Systems, Bad Nauheim, Germany). The incubated samples were run and analyzed as described, but with a linear gradient of AcN in 0.05% TFA (15–60% AcN over 30 min).

Conditioned taste avoidance (CTA)

Animals, diet, peptides, and chemicals were all as described in the preceding sections. Lithium chloride (127 mg/kg ip) was used as a positive control (40, 41). Fluid was given in specially designed bottles comprising a 10-ml pipette (Bibby Sterilin Ltd., Staffs, UK), nondrip sipper from 50 ml mouse feeder bottles (Classic Pet Products, Caldex Ltd., Halifax, UK), and Parafilm cap (American National Can Group Inc., Chicago, IL), with separate sets of bottles for water and novel flavor (sweetened Kool-Aid; Kraft Foods Co., Northfield, IL). Based on an established protocol using a single-bottle method (40), mice were acclimatized and trained to drink their full fluid requirement during a 1-h period each day. Once stabilized, during a test week, on d 1 and 3, 1 h access to a novel flavor was paired with an ip injection of saline or peptide. On d 5 (test day), the mice had access to the flavor for 1 h, and fluid intake was measured.

Statistical analysis

Statistical advice was obtained from the Imperial College Statistical Advisory Service (Imperial College London). Data are shown as mean and SEM. Food intake study data were analyzed by ANOVA with Bonferroni's *post hoc* test (Prism 4). In all cases $P \leq 0.05$ was considered statistically significant.

Results

The effect of Oxm on food intake in mice

To establish the dose of Oxm required to cause a robust reduction in food intake after ip administration, the effect of Oxm on food intake in C57BL/6 mice was examined (n = 8–10 per group). Food intake in the first hour was inhibited by doses of 1400 nmol/kg Oxm and above [food intake 0–1 h (g): saline 1.23 ± 0.05, Oxm 800 nmol/kg 1.22 ± 0.9, 1400 nmol/kg 0.95 ± 0.07, 2200 nmol/kg 0.76 ± 0.12, 3800 nmol/kg 0.35 ± 0.04; $P < 0.05$ for 1400 nmol/kg Oxm and above *vs.* saline]. There was no effect of Oxm on food intake in any group by 24 h (data not shown). Repeated experiments (n = 3, data not shown) showed that the dose of Oxm needed to reliably reduce food intake by 25–40% compared with saline control was 1400 nmol/kg.

Effect of DPPIV on the amino terminus of Oxm

Oxm degradation in vitro

After incubation of Oxm with DPPIV for 2 h, no HPLC peak corresponding to the original peptide was detected. The addition of the specific DPPIV inhibitor Diprotin A to the reaction (42, 43) completely prevented Oxm degradation (data not shown). The observed degradation can be compared with that seen with exendin-4 as a negative control, GLP-1 and glucagon as compar-

ators, and a degradation-prone modified Oxm (DHis1-Ala2-Oxm) as positive control (supplemental Fig. 1S, which is published as supplemental data on The Endocrine Society's Journals Online web site at http://endo.endojournals.org). After incubation of Oxm with DPPIV, mass spectroscopy was used to determine that the mass of the resulting fragment was in accord with that of Oxm3-37.

Oxm degradation in vivo

A total of 2700 nmol/kg ip Oxm (a high but not maximal dose for food intake inhibition) was administered alone or in conjunction with sc 75 mg/kg valine pyrrolidide (a DPPIV inhibitor obtained as a kind gift from Novo Nordisk, Crawley, UK) (44), and effects on food intake in mice were examined. Oxm alone reduced food intake for the first hour after injection. There was no effect of DPPIV inhibitor alone on food intake. Coadministration of DPPIV inhibitor with Oxm enhanced the reduction in food intake for 1 h after injection (Fig. 2), with no effects during later time periods (data not shown).

The effect of modifications to the amino terminus of Oxm on receptor binding and DPPIV degradation in vitro

Amino terminus modifications affect susceptibility of proglucagon-derived peptides and related peptides to degradation (2, 45). Oxm analogs were synthesized using similar principles to investigate effects on receptor binding and breakdown *in vitro* by DPPIV. Some changes reduced receptor binding affinity (Table 1), but several also inhibited DPPIV degradation *in vitro* (Table 2).

The effect of modifications to the amino terminus of Oxm on bioactivity in vivo

Peptide analogs DHis-Oxm, Ala2-Oxm, and DHis1-Ala2-Oxm (receptor binding and degradation shown in Tables 1 and 2) were administered to mice ip at 2700 nmol/kg, with native Oxm as a comparator (n = 9–10 per group). In the first hour after injection, Oxm reduced food intake, and DHis1-Oxm reduced food intake to a similar degree. Ala2-Oxm was less potent than Oxm and did not significantly reduce food intake compared with saline. DHis1-Ala2-

Oxm reduced food intake similarly to Oxm in the first hour [food intake 0–1 h (g) after saline 1.19 ± 0.06, Oxm 0.41 ± 0.06, $P <$ 0.001 *vs.* saline, D-His-Oxm 0.42 ± 0.11, $P < 0.001$ *vs.* saline, Ala2-Oxm 0.83 ± 0.05, $P < 0.001$ *vs.* saline and $P < 0.01$ *vs.* Oxm, and DHis1-Ala2-Oxm 0.27 ± 0.03, $P < 0.001$ *vs.* saline]. DHis1-Ala2-Oxm had a prolonged action, with a greater reduction in food intake than saline and Oxm-injected animals at 0–2 h [food intake 0–2 h (g) after saline 1.83 ± 0.09, Oxm 1.29 ± 0.07, $P > 0.05$ *vs.* saline, DHis1-Ala2-Oxm 0.47 ± 0.03, $P < 0.01$ *vs.* saline and $P < 0.01$ *vs.* Oxm].

Effect of DHis1-Ala2-Oxm on food intake

The dose-response effect of DHis1-Ala2-oxm on food intake in mice was investigated, using Oxm as comparator and looking at food intake over the first hour of feeding (n = 8 per group). Oxm 1400 nmol/kg reduced 0–1 h food intake. DHis1-Ala2-oxm significantly reduced food intake in the first hour at doses of 500, 1000, and 2000 nmol/kg, and, thus, was more potent than native Oxm [food intake 0–1 h (g): saline 1.01 ± 0.05, Oxm 1400 nmol/kg 0.60 ± 0.11, DHis1-Ala2-oxm 250 nmol/kg 0.84 ± 0.06, 500 nmol/kg 0.62 ± 0.11, 1000 nmol/kg 0.35 ± 0.10, 2000 nmol/kg 0.16 ± 0.05; $P \le 0.05$ for 500, $P \le 0.01$ for 1000 and 2000 nmol/kg DHis1-Ala2-oxm *vs.* saline].

Effects of alterations to the midsection of Oxm on receptor binding and endopeptidase degradation

Effects of substitution of short amino acid sequences in the midsection of Oxm on receptor binding, endopeptidase degradation, and bioactivity

The GLP-1 receptor agonist exendin-4 has a greater affinity for the GLP-1 receptor than Oxm or GLP-1 (Table 1) and is less susceptible to degradation by NEP than Oxm (46) (Table 2 and supplemental Fig. 2S). Both Oxm and exendin-4 are thought to have an α-helix structure. Oxm analogs were produced with putative degradation-resistant amino acid sequences from exendin-4 in the midsection: Oxm(ex15-18), Oxm(ex15-21), Oxm(ex15-23), and Oxm(ex15-24) (Fig. 1).

Insertion of a short exendin-4 sequence (ex15-18) slightly reduced affinity for the GLP-1 receptor, but longer sequence inserts (ex15-21) to (ex15-24) increased affinity for the GLP-1 receptor by approximately 10-fold (Table 1). Degradation of the peptides with the midsection inserts by NEP was reduced compared with native Oxm, with Oxm(ex15-23) the most degradation resistant of the group (Table 2).

The effects of these peptide analogs on food intake were assessed *in vivo*. Oxm 2700 nmol/kg reduced food intake significantly in the first hour, whereas the Oxm analogs had a more prolonged effect. The duration of action reflected the receptor binding data, by the 8- to 24-h interval, only the Oxm(ex15-23), which had the greatest affinity for the GLP-1, caused a significant reduction in food intake compared with saline (Fig. 3).

Effects of substituting short amino acid sequences in the midsection/octapeptide junction of Oxm on receptor binding, endopeptidase breakdown, and bioactivity

To investigate the role of the terminal octapeptide, Oxm analogs were produced with putative degradation-resistant
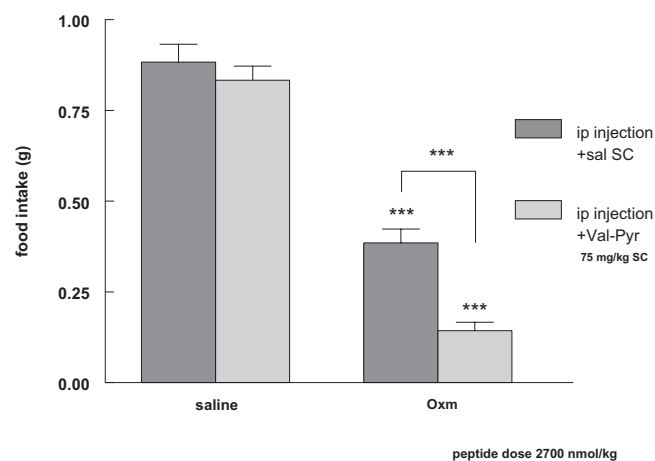

FIG. 2. Effects in mice of valine pyrrolidide (Val-Pyr) 75 mg/kg sc on the inhibition of food intake over 1 h resulting from the administration of Oxm 2700 nmol/kg ip (n = 12 per group). ***, $P \le 0.001$ compared with saline (sal) or comparison between groups indicated.

TABLE 1. Receptor binding affinity for Oxm and analogs for the GLP-1 receptor

Peptide/analog	Mean IC$_{50}$ (nM)	95% Confidence interval
Natural sequence peptides		
Oxm (shown for comparison)	33.1	27.2–49.0
Glucagon	<10,000	Not applicable
GLP-1 (shown for comparison)	0.34	0.27–0.41
Exendin-4 (shown for comparison)	0.27	0.21–0.35
N terminus modifications		
Des-His-Oxm	<10,000	Not applicable
Acetyl-Oxm	<10,000	Not applicable
DHis-Oxm	1,064	792–1,336
Ala2-Oxm	28.1	21.8–35.9
DHis1-Ala2-Oxm	544	398–744
DHis1-Val2-Oxm	<5,000	Not applicable
DHis1-Ala2-Glu3-Oxm	624	393–983
DHis1-Ser2-Glu3-Oxm	972	323–2,393
His1-Gly2-Gln3-Oxm	367	224–602
DHis1-Gly2-Gln3-Oxm	3,540	2,755–4,355
Midsection modifications		
Oxm(ex15-18)	59.0	34.0–99.0
Oxm(ex15-21)	4.0	3.0–5.3
Oxm(ex15-23)	2.2	1.4–3.5
Oxm(ex15-24)	4.6	3.0–7.1
Oxm(ex30-33)	199	128–309
Oxm(ex29-33)	4.8	2.3–10.1
Oxm(ex27-33)	9.6	5.5–16.8
Oxm(ex27-30)	10.3	6.7–16.3
Carboxy terminus fragments		
Oxm19-37	<10,000	Not applicable
Oxm30-37	<10,000	Not applicable
Carboxy terminus modifications		
Oxm-Ala38	194	129–301
Oxm-Ala38,39	20.8	16.0–26.7
Oxm-Ala38-42	39.0	25.0–58.0
Oxm-Lys38-Octanoyl	322	91.0–738
Oxm-Lys38-Lauroyl	476	178–1,200
Oxm-Lys38-Palmitoyl	538	255–1,100
Oxm-Ala38,39-Lys40-Lauroyl	36.0	21.5–60.3
Oxm-Ala38,39-Lys40-Palmitoyl	25.7	16.5–40.0
Combined N terminus, midsection, and carboxy terminus modifications		
DHis1-Ala2-Oxm(ex15–23)(ex27–33)-Ala38,39-Lys40-Lauroyl	0.79	0.59–1.0

IC$_{50}$ value calculated from a minimum of three separate experiments, each concentration tested in triplicate in each experiment.

amino acid sequences encroaching on the terminal octapeptide: Oxm(ex27-33), Oxm(ex29-33), Oxm(ex30-33), and Oxm(ex27-30) (Fig. 1). The modified peptides had greater affinity for the GLP-1 receptor than native Oxm, except (ex30-33), which showed reduced receptor binding (Table 1). Incubation of Oxm with NEP resulted in degradation of the peptide (Table 2) with multiple degradation products (supplemental Fig. 2S). The analogs were more resistant to NEP degradation, with the highest protection from degradation observed with Oxm(ex27-33) (Table 2).

The effect of two representative peptides on food intake in mice was studied. Oxm 2700 nmol/kg reduced food intake significantly in the first hour. Oxm(ex30-33) had a similar effect. Oxm(ex27-33) was significantly more bioactive than Oxm in the first hour ($P < 0.001$), and the effects on food intake lasted until the 4- to 8-h interval (Fig. 4).

The role of the carboxy terminus of Oxm in the bioactivity of Oxm on food intake *in vivo*

To investigate whether the terminal octapeptide is necessary for the effects of Oxm on food intake, Oxm and glucagon were compared. Oxm had greater affinity for the GLP-1 receptor than glucagon, which did not show specific binding at the highest doses tested (IC$_{50}$ 33.1 for Oxm and >10,000 for glucagon) (Table 1). At the rat glucagon receptor, Oxm bound with markedly lower affinity than glucagon [glucagon IC$_{50}$ 1.1 nM (0.84–1.4), Oxm IC$_{50}$ 18.6 nM (14–24)], whereas GLP-1 and exendin-4 showed no binding (IC$_{50}$ >10,000). When the effects of Oxm and glucagon on food intake in mice were compared, a dose of 1400 nmol/kg Oxm inhibited food intake, whereas the effect of glucagon was not statistically significant [food intake 0–1 h (g): saline 0.91 ± 0.07, Oxm 1400 nmol/kg 0.20 ± 0.05 ($P < 0.001$), glucagon 1400 nmol/kg 0.60 ± 0.10 ($P > 0.05$ *vs.* saline and $P < 0.01$ *vs.* Oxm)].

TABLE 2. Effect of rational modifications of the Oxm N terminal on DPPIV-induced peptide degradation and of the Oxm midsection and carboxy terminus on NEP-induced peptide degradation *in vitro*

Peptide	% Degradation with DPPIV at 2 h	% Degradation with NEP
Natural sequence peptides		
Oxm	100 (40% at 30 min)	77
GLP-1	100	100
Exendin-4	0	0
Glucagon	100	81
N terminus modifications		
Des-His-Oxm	0	Not tested
Acetyl-Oxm	0	Not tested
DHis-Oxm	0	Not tested
Ala2-Oxm	100 (95% at 30 min)	70
DHis1-Ala2-Oxm	0	Not tested
DHis1-Val2-Oxm	0	Not tested
DHis1-Ala2-Glu3-Oxm	0	Not tested
DHis1-Ser2-Glu3-Oxm	0	Not tested
His1-Gly2-Gln3-Oxm	51 (19% at 30 min)	Not tested
DHis1-Gly2-Gln3-Oxm	0	Not tested
Midsection modifications		
Oxm(ex15-18)	Not tested	38
Oxm(ex15-21)	Not tested	39
Oxm(ex15-23)	Not tested	0
Oxm(ex15-24)	Not tested	6
Oxm(ex30-33)	Not tested	8
Oxm(ex29-33)	Not tested	4
Oxm(ex27-33)	Not tested	0
Oxm(ex27-30)	Not tested	12
Carboxy terminus modifications		
Oxm-Ala38	Not tested	41
Oxm-Ala38,39	Not tested	24
Oxm-Ala38-42	Not tested	37

Because the minimally active fragment of Oxm for gastric acid secretion has been reported to lie in the carboxy terminus, the effects of Oxm carboxy-terminal fragments (Oxm 19-37 and Oxm 30-37) on food intake were evaluated. Neither Oxm 19-37 nor Oxm 30-37 displayed specific affinity for the GLP-1 receptor at the highest doses tested ($IC_{50} > 10,000$ nM) (Table 1). Fragments (Oxm19-37 and Oxm30–37; Fig. 1) were administered ip to mice. Oxm 1400 nmol/kg reduced food intake in the first hour after injection, but the fragments tested did not reduce food intake at this dose or at 10,000 nmol/kg (data not shown).

The carboxy terminus of Oxm may be susceptible to degradation. Complex additions to peptide sequences can reduce breakdown but may interfere with secondary structure and receptor binding. Oxm analogs were synthesized with the addition of alanine residues at the C terminal to investigate the effects of C-terminal elongation on food intake. The effect of the peptides Oxm-Ala38, Oxm-Ala38,39, and Oxm-Ala38-42 on receptor binding and food intake were compared with native Oxm. Oxm-Ala38 had lower binding to the GLP-1 receptor than Oxm, but Oxm-Ala38,39 had slightly greater affinity for the GLP-1 receptor. The affinity of Oxm and Oxm-Ala38-42 for the GLP-1 receptor was comparable (Table 1). Degradation of the extended peptides by NEP *in vitro* was reduced compared with that of native Oxm (Table 2).

When administered *in vivo* to mice, all three elongated peptides had a similar effect on food intake to native Oxm. Oxm-Ala38,39 had a slightly greater anorectic effect than Oxm, but this difference was not statistically significant [food intake 0–1 h (g) after saline 0.91 ± 0.05, Oxm 0.53 ± 0.13, $P < 0.05$ *vs.* saline, Oxm-Ala38 0.52 ± 0.11, $P < 0.05$ *vs.* saline,
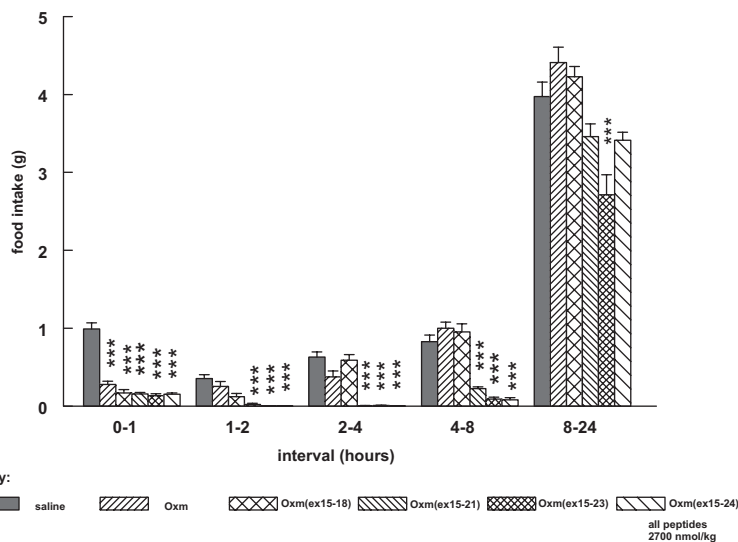

FIG. 3. Effects on 24 h food intake, after an overnight fast in mice, after ip administration of human Oxm 2700 nmol/kg, or Oxm analog peptides with sequence replacements with exendin sequence Oxm(ex15-18), Oxm(ex15-21), Oxm(ex 15-23), and Oxm(ex15-24) all at 2700 nmol/kg (n = 6–8 per group). ***, $P \leq 0.001$ compared with saline.
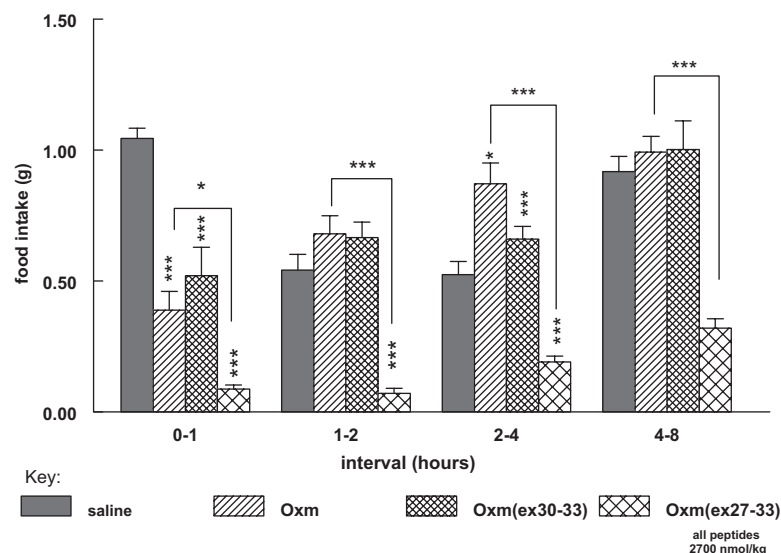
FIG. 4. Effects on food intake for 8 h after an overnight fast in mice, after ip administration of human Oxm 2700 nmol/kg, or Oxm analog peptides with sequence replacements with exendin sequence Oxm(ex30-33) and Oxm(ex27-33) all at 2700 nmol/kg (n = 9–10 per group). *, $P \le$ 0.05; ***, $P \le$ 0.001 compared with saline or comparison between groups indicated.

Oxm-Ala38,39 0.24 ± 0.04, $P < 0.001$ *vs.* saline, Oxm-Ala38-42 0.60 ± 0.07, $P > 0.05$ *vs.* saline]. Food intake for all groups returned to baseline after 1 h after injection (data not shown).

The effect of altered protein binding on bioactivity of Oxm

Addition of acyl groups to a peptide can increase peptide binding to albumin, and impair degradation and clearance (47, 48). Oxm analogs were synthesized with the C terminus extended by a lysine residue (Lys 38) to which an acyl group was attached, comprising eight, 12, or 16 residues. These peptides had reduced binding to the GLP-1 receptor (Table 1). When administered at 1400 nmol/kg *in vivo* (n = 8–10 per group), they had less effect on food intake in the first hour after administration than Oxm at the same dose, with only native Oxm and Oxm-Lys38 showing statistically significant re-
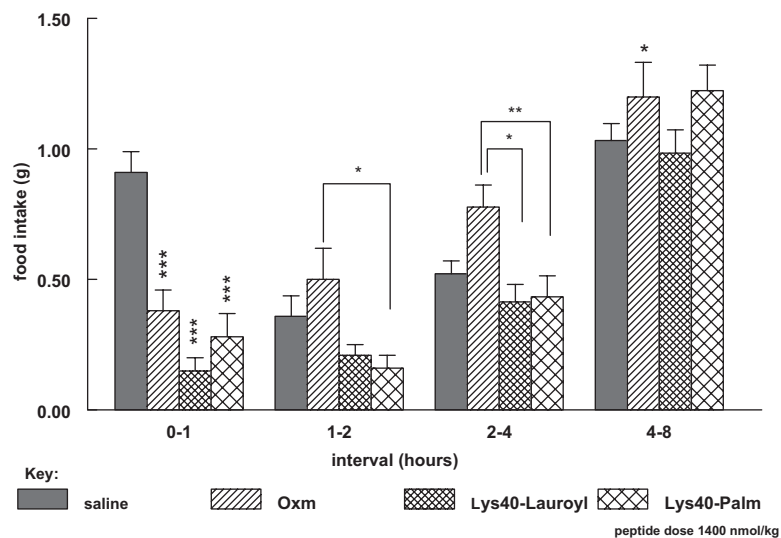
FIG. 5. Effects on food intake for 8 h after an overnight fast in mice, after ip administration of saline or human Oxm, Oxm-Ala38,39-Lys40-Lauroyl or Oxm-Ala38,39-Lys40-Palmitoyl at 1400 nmol/kg (n = 6–8 per group). *, $P \le$ 0.05; **, $P \le$ 0.01; ***, $P \le$ 0.001 for food intake compared with saline control or Oxm as indicated.

ductions in food intake compared with saline control ($P < 0.05$). The peptides did not affect food intake at later time points (data not shown).

With the aim of reducing acyl-group interference with receptor binding, further carboxy-terminal extended analogs were developed with the acyl side chain attached further from the putative α-helix region. Oxm-Ala38,39-Lys40-Lauroyl and Oxm-Ala38,39-Lys40-Palmitoyl bound to the GLP-1 receptor with affinities similar to that of native Oxm (Table 1). When administered to mice *in vivo*, these peptides reduced food intake in the first hour after administration and had a significantly more inhibitory effect than Oxm for up to 4 h after administration (Fig. 5), with a trend to correction in the 4- to 8-h period. There were no significant effects in the 8- to 24-h period (data not shown).

The contribution of peptide domains of Oxm to nausea and CTA

As discussed previously, in repeated experiments, Oxm 1400 nmol/kg caused a 30–40% reduction in food intake in mice in the first hour after injection, compared with saline control. Food intake studies were performed to establish dose-response curves for exendin-4 and for Oxm analogs with alterations in each of three domains [DHis1-Ala2-Oxm, Oxm(ex15–24) and Oxm(ex27–33)] (supplemental Fig. 3S). These studies were used to determine a dose predicted to reduce food intake by 30–40% in the first hour after administration: exendin-4 1 nmol/kg, DHis1-Ala2-Oxm 500 nmol/kg, Oxm(ex15–24) 7 nmol/kg, and Oxm(ex27–33) 150 nmol/kg.

A CTA study was performed to examine the effects of the native peptides GLP-1, Oxm, and exendin-4 at doses that either reduce food intake 30–40% at 1 h or by a 3-fold higher dose. Exendin-4 administered at the 3-fold higher dose caused CTA, whereas peptides administered at the lower dose did not (supplemental Fig. 4S).

Peptides were administered at doses 3-fold higher than those required to reduce food intake at 1 h by 30–40% (n = 6–8 per group). The doses administered were Oxm 4200 nmol/kg, exendin-43 nmol/kg, DHis1-Ala-Oxm 1500 nmol/kg, Oxm(ex15-24). The positive control, lithium chloride, reduced Kool-Aid intake significantly. Exendin-4 had a trend toward and Oxm reduced Kool-Aid intake compared with saline. The Oxm analogs all reduced Kool-Aid intake compared with saline, with no significant difference between the Oxm analogs [mean intakes 0–1 h (ml) 1.49 ± 0.06 for saline, 0.05 ± 0.03 for LiCl, $P <$ 0.001 *vs.* saline, 1.19 ± 0.2 for exendin-4, $P = 0.1$ *vs.* saline, 1.03 ± 0.1 ml for Oxm, $P < 0.05$ *vs.* saline, 0.69 ± 0.2 for DHis1-Ala2-Oxm, $P < 0.01$ *vs.* saline, 0.96 ± 0.1 for Oxm(ex15–24), $P < 0.05$ *vs.* saline, and 0.38 ± 0.1 for Oxm(ex27–33), $P < 0.01$ *vs.* saline].

When the Oxm analog peptides were administered at the doses that reduced food intake by 30–40% at 1 h, in a CTA paradigm (n = 7–8 per group), there were no significant effects on Kool-Aid intake. The

positive control, lithium chloride, reduced Kool-Aid intake significantly. None of the other peptides tested significantly reduced Kool-Aid intake compared with saline [mean intakes 0–1 h (ml) 1.93 ± 0.13 for saline, 1.13 ± 0.29 for LiCl $P < 0.05$ *vs.* saline, and 1.66 ± 0.15 for exendin-4, 1.68 ± 0.11 ml for Oxm, 1.61 ± 0.19 for DHis1-Ala2-Oxm, 1.55 ± 0.13 for Oxm(ex15–24), and 1.69 ± 0.07 for Oxm(ex27–33): all $P > 0.05$ *vs.* saline].

The effect of combining modifications to Oxm in a single analog on binding, degradation, and clearance

An Oxm analog was synthesized with changes in the different peptide regions combined in a single molecule (Fig. 1), and the effects on food intake were examined. The effect of peripheral administration of 10, 30, 100, and 300 nmol/kg DHis1-Ala2-oxm(ex15-23)(ex27-33)-Ala38,39-Lys40-Lauroyl on food intake was compared with that of Oxm 1400 nmol/kg. Oxm and all doses of the Oxm analog DHis1-Ala2-oxm(ex15-23)(ex27-33)-Ala38,39-Lys40-Lauroyl reduced food intake in the first hour after administration (Fig. 6). A dose of 10 nmol/kg DHis1-Ala2-oxm(ex15-23)(ex27-33)-Ala38,39-Lys40-Lauroyl reduced food intake significantly until 8 h after administration, and doses of 100 or 300 nmol/kg were still effective at 24 h. Thus, a much lower dose of this analog was required to reduce food intake than would be required of Oxm. The combination of changes together improved the binding to the GLP-1 receptor 10-fold compared with native Oxm (Table 1). The effects of this multiply modified peptide on CTA suggest that it may have a less marked effect on CTA than exendin-4 (supplemental Fig. 5S).

Discussion

Modifications to the structure of Oxm generated peptides with increased binding to the GLP-1 receptor, reduced enzyme deg-

FIG. 6. Effects on 24 h food intake after an overnight fast in mice after ip administration of saline or human Oxm 1400 nmol/kg, or Oxm analog DHis1-Ala2-oxm(ex15–23)(ex27-33)-Ala38,39-Lys40-Lauroyl with combined changes in several peptide regions at doses ranging from 10–300 nmol/kg. Graph shows food intake for defined intervals during the study (n = 6–8 per group). **, $P < 0.01$; ***, $P < 0.001$ for food intake compared with saline control or Oxm as indicated.

radation, and increased anorectic effects. This increase in bioactivity appears to reflect an increase in the magnitude of acute effects and/or an increase in the duration of effect.

The first series of experiments investigated the properties of the amino terminus of Oxm. DPPIV is a membrane-bound ectoenzyme believed important in the biological regulation of peptides, including GLP-1. DPPIV cleaves an NH_2-terminal dipeptide, particularly, but not exclusively, from peptides with a penultimate amino proline or alanine residue (17). DPPIV has degraded both GLP-1 and Oxm *in vitro* (49), although Oxm was less susceptible to degradation by the enzyme. We confirm that *in vitro*, Oxm and related peptides can be degraded by DPPIV. However, exendin-4 is resistant to degradation by DPPIV (Table 2 and supplemental Fig. 1S). As predicted, Oxm3–37 is the major breakdown product.

Unlike GLP-1, a role for DPPIV in the *in vivo* regulation of Oxm had not previously been demonstrated, although Oxm is a substrate for DPPIV *in vitro* (49). The greater reduction in food intake after coadministration of Oxm and a DPPIV inhibitor suggests that DPPIV is involved in Oxm breakdown *in vivo*. The greater food intake reduction at 1 h after administration is likely to represent a short extension of the half-life of Oxm within this first hour, rather than an increase in potency. Because DPPIV has strict substrate requirements (50), we synthesized analogs likely to have altered susceptibility to DPPIV cleavage (21, 22). Analogs for which *in vitro* experiments suggested resistance to DPPIV degradation, such as Des-His-Oxm, DHis1-oxm, and DHis1-Ala2-oxm reduced food intake *in vivo*, and DHis-1-Ala2-Oxm appeared to have increased potency and a more prolonged action compared with native Oxm. In contrast, the analog Ala-2-Oxm, which *in vitro* experiments predicted to be more susceptible to DPPIV degradation, was less effective on food intake *in vivo* than Oxm. These data suggest that DPPIV can influence Oxm breakdown.

In addition to DPPIV, Oxm degradation may involve endopeptidases. Specific modifications to either the midsection or the section including the octapeptide of Oxm were able to increase receptor binding affinity. NEP is a membrane-bound zinc metallopeptidase that cleaves peptides at the NH_2-terminal side of aromatic or hydrophobic amino acids. GLP-1 is a substrate for NEP *in vivo* (24, 25) with six potential NEP cleavage sites. Degradation of Oxm by NEP produces multiple cleavage products (supplemental Fig. 2S). Exendin-4, which has a much longer circulating half-life than GLP-1, lacks many of these cleavage sites (25). Exendin-4 also contains a nine-amino acid C-terminal sequence reported to improve receptor binding affinity (51). In contrast to the rapid metabolic clearance of GLP-1, the clearance of exendin-4 in humans is similar to the glomerular filtration rate (52), suggesting that the kidneys are important in exendin-4 clearance. Replacement of short peptide sequences of Oxm by exendin-4 sequence reduced degradation by NEP and led to a greater duration of action on food intake. These modified peptides had greater potency than Oxm on food intake but less than exendin-4 itself. These data suggest that, as in GLP-1, the midsection of Oxm plays a role in receptor binding and endopeptidase degradation. However,
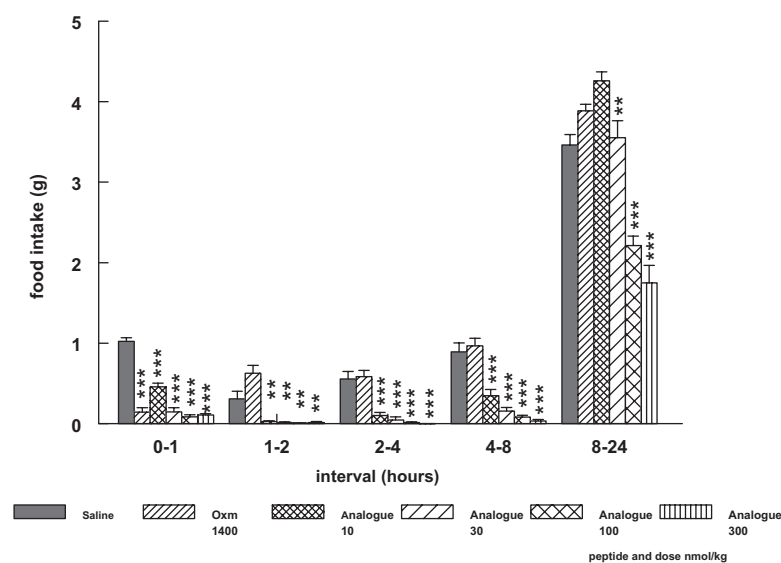
there are likely to be additional endopeptidases involved in the breakdown of both GLP-1 and Oxm *in vivo* in addition to DPPIV and NEP (53), and relative resistance to these as yet unidentified factors may contribute to the differences in bioactivity observed between the peptides *in vivo*.

Elongation of the Oxm molecule by two amino acids did not impair receptor binding and increased bioactivity. Addition of further residues did not further increase bioactivity. This could be due to steric interference with receptor binding. The more sustained action of acylated Oxm is in accord with possible reduction in renal clearance as proposed for acylated GLP-1 analogs (36). Oxm analogs with acyl groups on Lys38 showed reduced affinity for the GLP-1 receptor and caused only nonsignificant reductions in food intake. However, analogs of Oxm with acyl groups attached to Lys40 retained the receptor affinity of the native molecule, and the duration of action of the peptide on food intake was increased. This suggests that acylation of the peptide can increase the duration of action of Oxm *in vivo* but that the acyl group must be carefully positioned not to compromise receptor binding affinity.

The role of the carboxy terminus octapeptide of Oxm was studied by investigating the effects of C terminus Oxm fragments and of glucagon on GLP-1 receptor binding and on food intake. Glucagon given at the same dose as Oxm did not have a statistically significant effect on food intake in mice. The fragments of Oxm tested had no specific affinity for the GLP-1 receptor and did not affect food intake even at very high doses. Thus, glucagon and the C-terminal octapeptide of Oxm contribute to GLP-1 receptor binding and effects on food intake, but independently are insufficient to cause these effects. It is interesting that C-terminal fragments have previously been shown to have biological activity of other types such as gastric acid secretion (54, 55), and the discrepancy between this effect and effects on feeding is worthy of further investigation.

Our results did not demonstrate any effect of Oxm or modified analogs of Oxm when given at similar doses to those that reduce food intake by 30–40%. When administered at higher doses, all were capable of producing CTA; the specific modifications did not have differential effects. This is in accord with the suggestion that nausea (for which CTA is a surrogate) may lie at one end of the spectrum of normal satiation and, therefore, reflect the propensity of a substance to inhibit food intake (56). Further studies investigating the effects on CTA of Oxm analogs that are active at low doses to reduce food intake over long time periods may determine whether a smaller acute and longer chronic reduction in food intake may be associated with reduced avoidance.

Finally, studies using an analog of Oxm incorporating several domain changes and modifications suggested that these changes can have an additive effect on biological activity *in vivo*. Effects of this peptide with multiple modifications on CTA at different doses, compared with a high dose of exendin-4, suggest that the modified peptide may have a slightly less marked effect on CTA than exendin-4. However, these results are preliminary and assume a linear relationship between dose and avoidance, not proven for all appetite-regulating peptides (57).

Thus, these studies have provided information regarding structure-function relationships for particular domains of Oxm. In addition, Oxm has been proposed as a potential therapy for obesity (11). Using Oxm analogs could reduce the amount of peptide required and/or the frequency of administration while retaining the appetite and weight-reducing qualities of the peptide. Future studies will examine the effects of acute and chronic administration of Oxm analogs on feeding, body weight, insulin secretion, and energy expenditure. Further work is required to determine the potential utility of Oxm analogs as treatments for obesity.

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