Effect of Oxyntomodulin, Glucagon, GLP-1 and Combined Glucagon + GLP-1 Infusion on Food Intake, Appetite and Resting Energy Expenditure

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Context: The gut hormone, oxyntomodulin, is a proglucagon product with body weight-lowering potential. It binds to both the glucagon-like peptide-1 (GLP-1) receptor and the glucagon receptor, however the mechanism behind the body weight lowering effect remains elusive.

Objective: We wanted to delineate the contributions of separate and combined GLP-1 receptor and glucagon receptor activation to the body weight-reducing mechanisms of oxyntomodulin.

Design: Double blinded, randomized, crossover.

Setting: Specialized research unit.

Participants: Fifteen young healthy male volunteers (age: 22 (range 18–32) years; body mass index: 23 (21–26) kg/m^2; fasting plasma glucose (FPG): 5.1 (4.4–5.4) mmol/l; glycated hemoglobin A1c (HbA_{1c}): 40 (37–42) mmol/mol)

Interventions: Five four-hour liquid meal-tests during the infusion of saline, GLP-1 (1 pmol×kg^{-1}×min^{-1}), glucagon (0.86 pmol×kg^{-1}×min^{-1}), oxyntomodulin (3 pmol×kg^{-1}×min^{-1}) or glucagon+GLP-1 (same doses)

Main Outcome Measures: We evaluated resting energy expenditure (measured as oxygen uptake (O_2), gastric emptying (GE), composite appetite scores (CAS) and food intake,

Results: Oxyntomodulin, GLP-1 and GLP-1+glucagon slowed GE and reduced CAS whereas glucagon did not affect GE and CAS. All infusions caused a similar decrease in food intake compared to saline (Total intake (g [CI 95%]), saline: 811 [729, 892], GLP-1: 669 [586, 750], glucagon: 686 [604, 768], oxyntomodulin: 689 [608, 771], glucagon+GLP-1: 688 [606, 769]). O_2 did not change significantly from baseline in response to any peptide infusion compared to saline.

Conclusions: Oxyntomodulin, GLP-1 and glucagon decreased food intake, but with no additional effect of combining GLP-1 and glucagon.

Acute effects of the gut-derived peptide hormone, oxyntomodulin, include inhibition of gastric emptying (GE), gastric and pancreatic exocrine secretion and food intake (1, 2), which may translate into body weight loss upon repeated administration in obese subjects (3). Also, oxyntomodulin has been reported to increase resting energy expenditure (REE), potentially contributing to the body weight-lowering effect of the hormone (4). A specific receptor for oxyntomodulin has not been identified, but the peptide shows affinity for both the glucagon receptor

Abbreviations:

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and the glucagon-like peptide-1 (GLP-1) receptor (5–7). Like GLP-1, oxyntomodulin is derived from proglucagon and is released from intestinal L cells after meal ingestion, with increases in plasma concentrations being related to the calorie intake (6). The amino acid sequence of oxyntomodulin corresponds to the entire 29-amino acid sequence of the glucagon molecule plus a C-terminal extension of eight amino acids (8, 9). After secretion, the peptide is eliminated with a plasma half-life of approximately 12 minutes (1, 10). Oxyntomodulin activates the GLP-1 receptor with lesser potency than GLP-1 itself, but the two peptides seem to have similar effects on food intake (11–14). The food intake-lowering effect of intra-cerebro-ventricular administration of oxyntomodulin in rats can be blocked with the GLP-1 receptor antagonist exendin(9–39) (2). Consistent with this, oxyntomodulin has limited effect in preclinical studies with GLP-1 receptor knock-out mice (15), suggesting that the interaction with the glucagon receptor has limited importance. However, studies in humans have demonstrated that glucagon may reduce hunger measures, food intake (16–18) and body weight (17). Interestingly, an oxyntomodulin analog with increased affinity for the glucagon receptor demonstrated significant potency with regards to inhibition of food intake and body weight reduction in mice when compared to native oxyntomodulin (19). Studies by Pocai et al using oxyntomodulin analogs with or without affinity for the glucagon receptor indicated an essential role of glucagon signaling in the body weight-lowering effect of oxyntomodulin in rodents (20). With the present study, we aimed to evaluate the effects of oxyntomodulin on GE, composite appetite scores (CASs), REE determined by oxygen uptake (O_2) and food intake in young healthy men. Furthermore, we aimed to delineate the possible dual receptor agonistic effects of oxyntomodulin by comparing the effects of oxyntomodulin and saline infusions with separate and combined infusions of GLP-1 and glucagon.

Materials and Methods

The protocol was approved by the Scientific-Ethical Committee of the Capital Region of Denmark (registration no. H-4–2010-089), the Danish Data Protection Agency (registration no. 2010–41–5506) and registered at ClinicalTrials.gov (ID: NCT01232244). The study was conducted according to the principles of the Helsinki Declaration II. Oral and written informed consent was obtained from all participants before inclusion.

Subjects. Fifteen young males (mean age: 22 (range 18–32) years; body mass index (BMI): 23 (21–26) kg/m^2; fasting plasma glucose (FPG): 5.1 (4.4–5.4) mmol/l; glycated hemoglobin A1c (HbA_{1c}): 5.8 (5.5–6.0)% [40 (37–42) mmol/mol] were included. None had hypercholesterolemia, hypertension or impaired renal or liver function. All subjects were without family history of diabetes and had normal glucose tolerance according to a 75 g-oral glucose tolerance test (OGTT) performed immediately before inclusion in the study. None of the subjects used medication regularly.

Experimental design. All participants were studied in a recumbent position in the morning after an overnight (10 hours) fast and tobacco abstinence on five randomized occasions separated by at least 48 hours. A cannula was inserted into a cubital vein, and the forearm was placed in a heating box (55°C) throughout the experiment for collection of arterialized blood samples. Another cannula was inserted into a contralateral cubital vein for hormone infusions through separate infusion lines. The experimental protocol is outlined in Figure 1A.O_2 was measured 30 minutes before start of test infusion and test meal. The continuous infusions of 1) glucagon (0.86 pmol/kg/min), 2) GLP-1 (1 pmol/kg/min), 3) oxyntomodulin (3 pmol/kg/min), 4) glucagon (0.86 pmol/kg/min) + GLP-1 (1 pmol/kg/min), or 5) saline, were given in a double blinded fashion during four hours. At time 0 minutes, the infusion was started and a liquid test meal was ingested over 5 minutes. Blood samples were drawn 15 and 0 minutes before and 15, 30, 45, 60, 75, 90, 105, 120, 150, 180, 210, and 240 minutes after meal ingestion. During the 4 hour infusion, hunger scores were collected ten times using standardized visual analog scales (VAS), recording hunger, satiety, fullness and prospective food consumption (21) and further inquiry was made regarding general well-being, nausea and thirst. During the final 30 minutes of the experiment (from 210 to 240 minutes), O_2 was measured again, after which an ad libitum meal was served with recordings of palatability and impression of the meal using VAS (22). In order to measure renal nitrogen excretion for estimation of protein turnover the urinary bladder was emptied before each experiment, and total urine production during each experiment was collected.

Synthetic GLP-1 and oxyntomodulin (PolyPeptide Laboratories A/S, Hillerød, Denmark) and glucagon (GlucaGen®, Novo Nordisk A/S, Bagsværd, Denmark) were dissolved in sterilized water containing 2% human albumin (Statens Serum Institut, Copenhagen, Denmark), subjected to sterile filtration (followed by testing for sterility and pyrogens) and dispensed into coded frozen vials at the pharmacy of the Capital Region, Herlev, Denmark.

The meal test was ingested within 5 minutes and consisted of 200 mL chocolate-flavored Nutridrink® (Nutricia, Allerød, Denmark) (300 kcal: 55 g carbohydrates, 17 g fat and 18 g protein) to which was added 1.5 g of acetaminophen (paracetamol, Panodil®, Dungarvan Ltd., Dungarvan, Ireland) dissolved in 50 mL of water.

Arterialized blood was collected in chilled tubes containing EDTA, aprotinin (500 kIU/mL blood; Trasylol®; Bayer, Leverkusen, Germany) and a dipeptidyl peptidase 4 (DPP-4) inhibitor (Valine Pyrrolidide, final concentration 0.01 mmol/L, a gift from Novo Nordisk A/S, Bagsværd, Denmark) for analyses of GLP-1, glucagon and oxyntomodulin. Blood for analysis of acetaminophen, creatinine, creatine kinase (CK) and triglyceride was collected in lithium heparin tubes. The blood for analysis of insulin, C-peptide, nonesterified free fatty acids (NEFA) and fibroblast growth factor 21 (FGF21) was left to coagulate for 20 minutes at room temperature. All samples were centrifuged for 20 minutes at 1.200 *g* and 4°C. Plasma samples for GLP-1, glu-

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cagon, and oxyntomodulin analysis and serum samples for acetaminophen analysis were stored at –20°C, and serum samples for insulin, C-peptide, NEFA, FGF21 and plasma samples for triglyceride, creatinine and CK at – 80°C. For bedside measurement of plasma glucose (PG), blood was added to fluoride tubes and centrifuged immediately at 7400 *g* for 2 minutes at room temperature. The ad libitum meal served at all five occasions consisted of minced meat, pasta, corn, carrots, and green pepper (37% fat, 13% protein, and 50% carbohydrates, approximately: 1.5 kcal per g).

Analyses. Resting metabolic rate was measured by indirect calorimetry using a tight facemask connected to the calorimeter, which measures the gas exchange breath-by-breath via an O_2-alkali cell and an infrared CO_2 sensor (CCMexpress®, Medgraphics, Medical Graphics Corporation, St. Paul, Minnesota, USA). The calorimeter was calibrated immediately before every measurement session. Metabolic rates are represented as averages of measures carried out every 10th second within a 20 minutes period. Plasma concentrations of glucose were measured by the glucose oxidase method, using a glucose analyzer (Yellow Springs Instrument Model 2300 STAT plus analyzer; YSI Inc., Yellow Springs, Ohio, USA). Serum insulin and C-peptide concentrations were measured using a two-sided electro chemiluminescence immunoassay (ADIVA Centaur XP, Simens, Ballerup, Denmark). Plasma samples for total GLP-1 and glucagon analysis were extracted with 70% ethanol (final concentration) before RIA measurements. Total GLP-1was analyzed using an antibody (code no 89 390) specific for the amidated c-terminus (23), and glucagon was analyzed using a c-terminal glucagon-specific antibody (code no 4305) (24). The glucagon analysis showed no cross-reaction with oxyntomodulin. For measurements of oxyntomodulin we used a newly developed RIA using an antiserum (code no 9D645) raised in rabbits against a c-terminal fragment of oxyntomodulin. Human oxyntomodulin (Bachem, cat no H-6058) was used as standard and the same peptide was ^{125}I-labeled and used as tracer. Analysis of acetaminophen, creatinine kinase (CK), triglyceride and creatinine was carried out using enzyme-linked color shift reactions and liquid chromatography (Vitros 5.1 FS Ortho-Clinical Diagnostics, Rochester, New York, USA). NEFA was analyzed using an Acyl-CoA oxidase-linked assay (NEFA-HR, Wako Chemicals GmbH, BY, Germany). FGF21 was measured using ELISA (BioVendor.com, Bratislava, Slovakia).

Statistical analyses and calculations. Baseline, peak and area un-

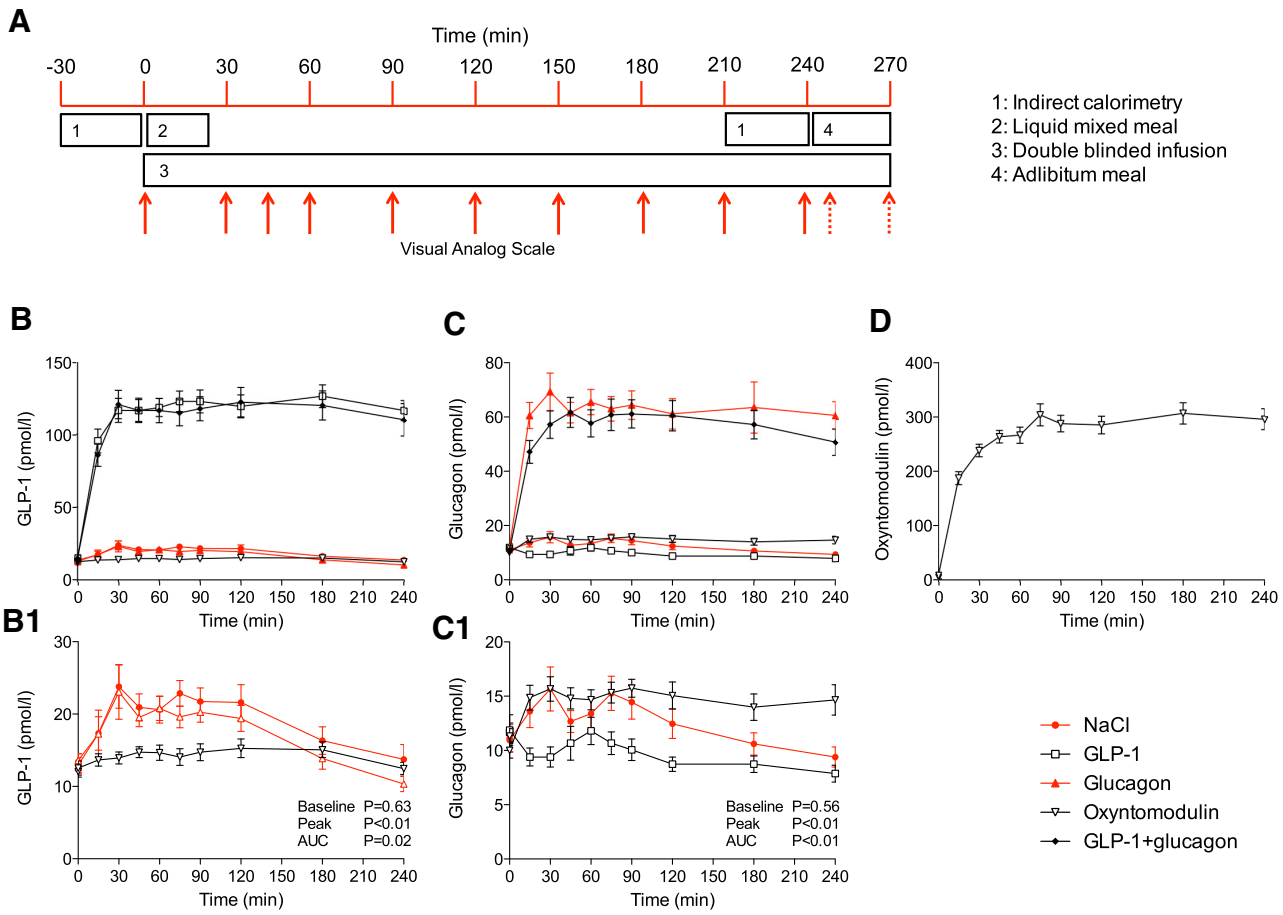

Figure 1. Diagram of experimental procedures (A). Arrows indicate time for appetite scoring (by visual analog scale (VAS)), broken arrows indicate time for assessment of palatability (by VAS). Plasma concentrations of GLP-1 (B), glucagon (C) and oxyntomodulin (D) during the infusion of saline (dot), GLP-1 (square), glucagon (upright triangle), oxyntomodulin (downright triangle) and GLP-1+glucagon (diamond), respectively. Postprandial responses of GLP-1 (B1) and glucagon (C1) are displayed in the lower panels as responses without the levels caused by infusion of the respective peptide along with the significance level of the overall model (P value). Red lines indicate rapid gastric emptying and black lines indicate slow gastric emptying as predicted by acetaminophen levels (Figure 2A).

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der the curve (AUC) values are expressed as mean and 95% confidence intervals (CI). Differences resulting in P values < 0.05 were considered significant. CAS was calculated from VAS assessment of appetite measures (hunger + prospective food consumption + (100-satiety) + (100-fullness) / 4) (25).

Steady-state levels of the infused peptides were determined graphically for each participant and presented as mean and CI of the mean.

Linear mixed effect modeling was used for analysis of longitudinal and repeated measures using statistical software R, with the "nlme" package. Data was transformed according to distribution pattern. We used a 'top-down' modeling strategy, with subject identity as random variable (26). A homogeneous or heterogeneous residual variance structure was chosen according to likelihood ratios. Results are presented as 95% CIs of the estimate. Insulin secretion rate (ISR) values were calculated using ISEC as described previously (27–29) and expressed as pmol insulin secreted per minute per kilogram body weight.

Results

Levels of infused peptides. Steady state plasma concentrations of GLP-1, glucagon and oxyntomodulin during the infusions (Figure 1B-D) were 120 [117, 123] and 117 [114, 121] pmol/L (GLP-1 infusions, mean level between 30 – 240 minutes), 63 [61, 66] and 58 [55, 61] pmol/L (glucagon infusions, mean level between 30 - 240 minutes), and 295 [283, 306] pmol/L (oxyntomodulin infusion, mean level from 75 - 240 minutes)

Acetaminophen. During the infusion of saline, the serum acetaminophen concentration increased briskly (mean time-to-peak [95% CI]; 80 [69, 90] min, Figure 2A) as an indirect measure of a fast GE rate of the liquid meal. During glucagon infusion the GE rate was almost identical to that of the saline infusion (time-to-peak 91 [-46, 24] min, whereas the infusions with GLP-1, oxyntomodulin, and glucagon+GLP-1, respectively, resulted in delayed GE (time-to-peak: 188 [-143, –73] (GLP-1), 146 [-101, –31] (oxyntomodulin), and 159 [-114, –44] min (glucagon+GLP-1)).

GLP-1. Plasma levels of GLP-1 after the initial liquid meal

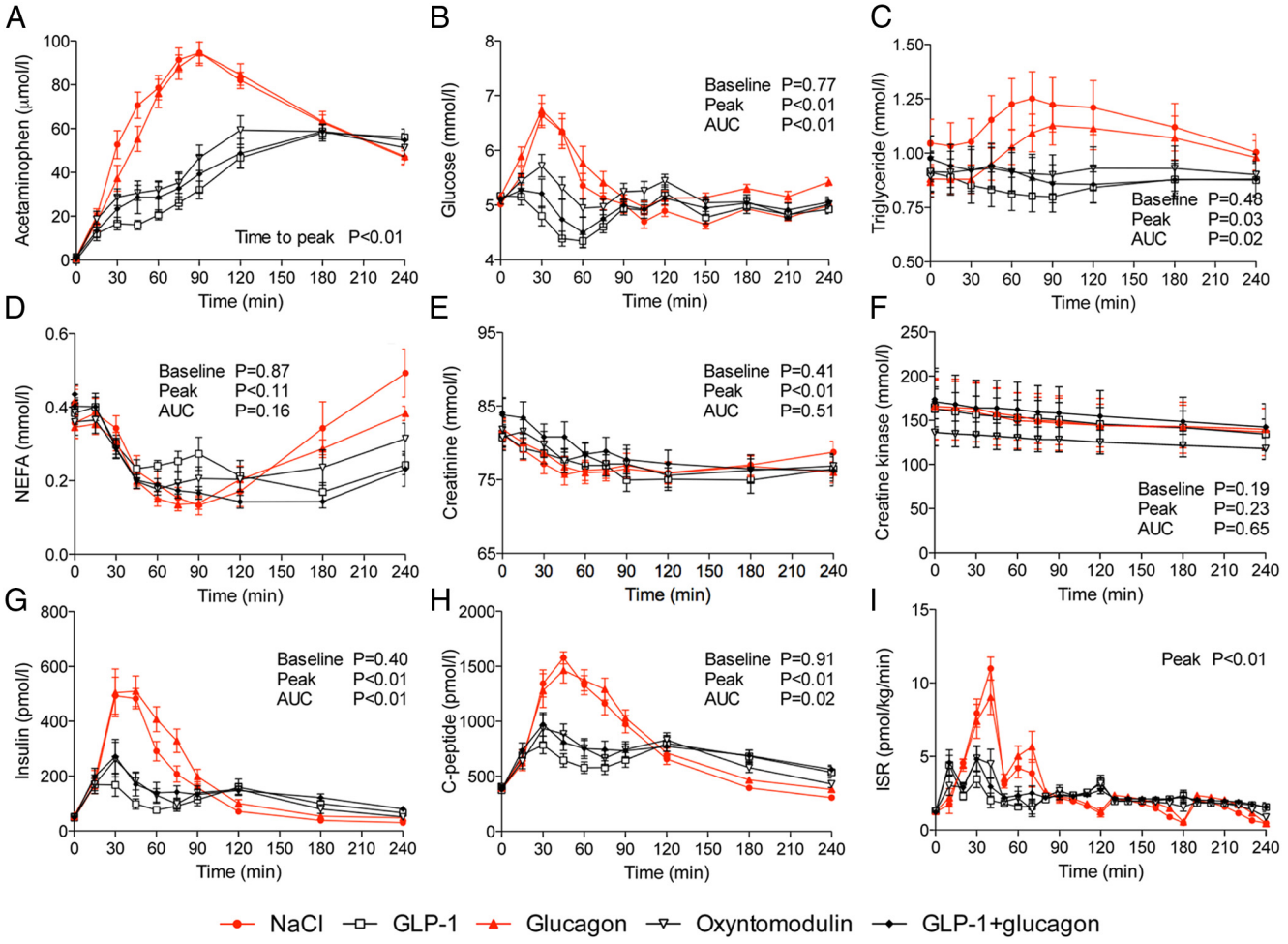

Figure 2. Postprandial plasma/serum excursions and the levels of significance from the overall models of acetaminophen (A), glucose (B), triglyceride (C), nonesterified fatty acids (D), creatinine (E), creatinine kinase (CK) (F), insulin (D), C-peptide (E) and insulin secretion rate (F), during intravenous (IV) infusions with saline (dot), GLP-1 (square), glucagon (upright triangle), oxyntomodulin (downright triangle) and GLP-1+glucagon (diamond), respectively. Red lines indicate rapid gastric emptying and black lines indicate slow gastric emptying as predicted by acetaminophen levels (Figure 2A).

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are shown in Fig. 1B1 and Table 1. During the infusion of saline and glucagon, plasma levels of GLP-1 doubled after meal ingestion. Plasma levels returned to baseline after three hours. We observed no changes during the oxyntomodulin infusion.

Glucagon. Plasma levels of glucagon after the initial liquid meal are shown in Fig. 1C1 and Table 1. Meal-induced rises in plasma levels of glucagon were observed during the infusions with saline and oxyntomodulin, respectively. However, during the saline infusion, plasma glucagon levels started to decline at 90 minutes and gradually returned to baseline after three hours, whereas plasma glucagon continued to be elevated during the four hours of oxyntomodulin infusion, probably caused by the reduced GE and the subsequently continued stimulation of the epithelium and absorption of amino acids. The GLP-1 infusion effectively reduced postprandial plasma glucagon levels (compared to saline infusion), which never rose above baseline plasma glucagon levels.

Glucose. Mean plasma glucose (PG) concentrations are displayed in Figure 2B. Baseline PG did not differ between study days (Table 1). The postprandial PG profiles were almost identical during saline and glucagon infusions although the AUC was significantly greater following the glucagon infusion (Table 1 and Figure 2B). Compared to the saline infusion, infusion of GLP-1 resulted in a clear reduction in PG after meal ingestion (during the first 60 minutes of the GLP-1 infusion), whereas infusion of oxyntomodulin induced a more moderate reduction in postprandial PG excursions (Table 1 and Figure 2B). The GLP-1+glucagon infusion resulted in a PG profile similar to what was seen with GLP-1 although with a slightly delayed lowering of PG (Figure 2B).

Triglycerides. Fasting plasma triglyceride concentrations did not differ between study days (Table 1). During the first hour of saline infusion, the postprandial triglyceride levels increased slowly, peaked at 75 minutes and then slowly decreased to baseline levels at 240 minutes (Table 1, Figure 2C). The glucagon infusion resulted in a similar postprandial response, whereas GLP-1 and GLP-1+glucagon infusions induced significantly lower postprandial triglyceride responses. Infusion of oxyntomodulin kept triglyceride levels constant during the entire experiment (Table 1 and Figure 2C).

NEFA. Baseline levels of NEFA did not differ between the test days (Table 1). Following meal ingestion, NEFA levels decreased during all experimental days (Figure 2D). The peptide infusions resulted in a delayed return to baseline compared to the saline infusion, although clearly the most following the GLP-1 and GLP-1+glucagon infusions.

Creatinine and CK. Only peak creatinine values differed significantly, related to minor difference in baseline levels rater than the infusions Figure 2E. We observed no other differences in the levels of creatinine or CK between the infusions. We observed steady decreases from baseline throughout all experiments (Figure 2E and 2F, Table 1).

Insulin, C-peptide and ISR. The ingestion of the liquid meal resulted in brisk and similar beta cell responses in all groups within the first 15 minutes (Figure 2D-F). Hereafter the excursions diverged in a manner closely related to the GE (Table 1, Figure 2G-I).

Appetite, thirst and comfort. In general, the VAS scores for appetite sensations decreased after ingestion of the liquid meal and then increased gradually for the remainder of the experiments (opposite for satiety sensations) (Figure 3A-E). We found an overall significant difference in the development in hunger, satiety, fullness and CAS scores between the infusions (Table 2). This overall difference seems to be driven primarily by the remarkable differences between scores obtained with the GLP-1 and glucagon infusion which is even greater than the difference between the GLP-1 and saline infusion (Table 2 and Figure 3A-C and E). Only with respect to CAS and fullness did the GLP-1 infusion result in values significantly different from those obtained after saline infusion (Table 2). We found no differences with respect to thirst, comfort or nausea (Figure 3F-H).

Food intake and palatability. Overall, the ad libitum meal was considered palatable with ingested food portions ranging from 347 to 984 g. A consistent and significantly decreased food intake was seen following all peptide infusions compared to the saline infusion (Table 2). No differences in food intake or palatability were observed between the different peptide infusions (differences from saline ranging from –121 to –141 g (Table 2)).

Calorimetry. Baseline O_2 was similar at all experimental days (Figure 4). At the second registration, the saline infusion was associated with a slight decrease in O_2 from baseline, whereas slight increases were observed in response to all peptide infusions (Figure 4) with increases ranging from 1% to 19%. However, there were no significant changes in O_2 between the baseline and final measurements on any day of the protocol. CO_2 reflected O_2 and we found no differences in urinary nitrogen excretion (data not shown).

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Table 1. Baseline, peak and AUC values Mean baseline, peak and area under the curve (AUC) values in response to the initial liquid mixed meal of GLP-1, glucagon, plasma glucose, triglyceride, non-esterified fatty acids (NEFA), insulin, C-peptide, creatinine and CK. Infused levels of GLP-1 and glucagon are not shown here but referred to in the text. First row are mean values and model adjusted 95% CI's of the mean in brackets. The following lines represents the post-hoc analysis as 95% CI's of the respective differences from the saline, GLP-1, glucagon and oxyntomodulin infusion (as predicted by the model)

		Saline	GLP-1	Glucagon	Oxyntomodulin	GLP-1+glucagon
GLP-1 (pmol/liter)	Baseline	13 [11, 15]		13 [11, 15]	12 [10, 14]	
Intercept	*Saline*			[-13, 16]%	[-19, 9]%	
	Glucagon				[-19, 9.]%	
	Peak	30 [24, 34]		29 [22, 32]	18 [15, 22]	
	Saline			[-25, 15]%	[-48, −21]%	
	Glucagon				[-44, −15]%	
(min×mmol/liter)	AUC	4446 [3709, 5015]		4049 [3382, 4573]	3467 [2897, 3918]	
	Saline			[-23, 9]%	[-34, −7]%	
	Glucagon				[-28, 2]%	
Glucagon (mmol/liter)	Baseline	11 [9, 12]	12 [9, 13]		11 [9, 13]	
Intercept	*Saline*		[-6, 23]%		[-11, 17]%	
	GLP-1				[-17, 9]%	
	Peak	19 [15, 21]	14 [11, 16]		18 [15, 21]	
	Saline		[-34, −14]%		[-12, 15]%	
	GLP-1				[17, 53]%	
(min×mmol/liter)	AUC	2974 [2485, 3408]	2288 [1785, 2708]		3576[3061, 3984]	
	Saline		[-1171, −228]		[105, 1048]	
	GLP-1				[805, 1747]	
Glucose (mmol/liter)	Baseline	5.0 [4.9, 5.2]	5.1 [5.0, 5.2]	5.1 [5.0, 5.2]	5.1 [5.0, 5.2]	5.1 [5.0, 5.2]
Intercept	*Saline*		[-0.1, 0.2]	[-0.1, 0.2]	[-0.1, 0.2]	[-0.1, 0.2]
	GLP-1			[-0.1, 0.1]	[-0.2, 0.1]	[-0.1, 0.1]
	Glucagon				[-0.2, 0.1]	[-0.2, 0.1]
	Oxyntomodulin					[-0.1, 0.1]
	Peak	7.0 [6.5, 7.4]	5.6 [5.3, 6.0]	7.2 [7.1, 7.5]	6.1 [5.7, 6.4]	5.9 [5.4, 6.1]
	Saline		[-24, −13]%	[-4, 10]%	[-18, −6]%	[-22, −11]%
	GLP-1			[18, 35]%	[1, 16]%	[-4, 10]%
	Glucagon				[-19, −8]%	[-24, −13]%
	Oxyntomodulin					[-12, 1]%
(min×mmol/liter)	AUC	1234 [1197, 1270]	1164 [1127, 1200]	1307 [1270, 1343]	1227 [1190, 1262]	1176 [1140, 1212]
	Saline		[-109, −32]	[34, 111]	[-46, 31]	[-97, −20]
	GLP-1			[105, 182]	[24, 101]	[-26, 51]
	Glucagon				[-119, 42]	[-170, −93]
	Oxyntomodulin					[-89, −12]
Triglyceride (mmol/liter)	Baseline	1.0 [0.8, 1.2]	0.9 [0.7, 1.0]	0.9 [0.7, 1.0]	0.9 [0.7, 1.0]	1.0 [0.7, 1.1]
	Saline		[-28, 8]%	[-30, 5]%	[-30, 5]%	[-23, 14]%
	GLP-1			[-20, 19]%	[-20, 19]%	[-13., 29]%
	Glucagon				[-18, 22]%	[-11, 33]%
	Oxyntomodulin					[-11, 33]%
	Peak	1.3 [1.1, 1.5]	1.0 [0.8, 1.2]	1.2 [1.0, 1.4]	1.1 [0.9, 1.3]	1.1 [0.9, 1.3]
	Saline		[-0.5, −0.1]	[-0.3, 0.1]	[-0.4, 0.0]	[-0.4, 0.0]
	GLP-1			[0.0, 0.4]	[-0.1, 0.3]	[-0.1, 0.3]
	Glucagon				[-0.3, 0.1]	[-0.3, 0.1]
	Oxyntomodulin					[-0.2, 0.2]
(min×mmol/liter)	AUC	273 [213, 308]	205 [160, 233]	250 [195, 283]	221 [171, 248]	218 [167 242]
	Saline		[-37,- 9]%	[-23, 10]%	[-33, −3]%	[-34, −6]%
	GLP-1			[1, 43]%	[-12, 28]%	[-14, 25]%
	Glucagon				[-27, 5]%	[-29, 3]%
	Oxyntomodulin					[-19, 17]%
NEFA (mmol/liter)	Baseline	0.4 [0.3, 0.5]	0.4 [0.3, 0.5]	0.4 [0.3, 0.4]	0.4 [0.3, 0.4]	0.4 [0.4, 0.5]
	Saline		[-0.1, 0.1]	[-0.1, 0.1]	[-0.1, 0.1]	[-0.1, 0.1]
	GLP-1			[-0.1, 0.1]	[-0.1, 0.1]	[-0.1, 0.1]
	Glucagon				[-0.1, 0.1]	[-0.1, 0.1]
	Oxyntomodulin					[0.0, 0.1]
	Peak	0.6 [0.5, 0.7]	0.4 [0.4, 0.5]	0.5 [0.4, 0.5]	0.5 [0.4, 0.5]	0.5 [0.4, 0.5]
	Saline		[-0.2, 0.0]	[-0.2, 0.0]	[-0.2, 0.0]	[-0.2, 0.0]
	GLP-1			[-0.1, 0.1]	[-0.1, 0.1]	[-0.1, 0.1]
	Glucagon				[-0.1, 0.1]	[-0.1, 0.1]
	Oxyntomodulin					[-0.1, 0.1]
(min×mmol/liter)	AUC	68 [51, 86]	57 [48, 66]	61 [52, 70]	58 [49, 68]	47 [39, 57]
	Saline		[-31, 9]	[-27, 13]	[-30, 10]	[-41, −1]
	GLP-1			[-10,16]	[-12, 14]	[-22, 3]
	Glucagon				[-15, 10]	[-27, −1]
	Oxyntomodulin					[-24, 2]
Creatinine (mmol/liter)	Baseline	81 [78, 84]	81 [78, 84]	82 [79, 85]	81 [78, 84]	84 [78, 89]
	Saline		[-3, 3]	[-2, 4]	[-2, 3]	[0, 6]
	GLP-1			[-2, 4]	[-2, 3]	[-1, 7]
	Glucagon				[-3, 2]	[-1, 6]
	Oxyntomodulin					[-1, 6]
	Peak	82 [78, 85]	82 [79, 86]	83 [79, 86]	83 [80, 86]	86 [83, 90]
	Saline		[-2, 3]	[-2, 4]	[-1,4]	[2, 8]
	GLP-1			[-2, 3]	[-2, 4]	[1, 7]
	Glucagon				[-2, 3]	[1, 7]
	Oxyntomodulin					[1, 6]
(min×mol/liter)	AUC	18.5 [17.8, 19.2]	18.3 [17.6, 19.0]	18.4 [17.7, 19.1]	18.5 [17.8, 19.2]	18.7 [18.0, 19.5]
	Saline		[-0.7, 0.3]	[-0.6, 0.5]	[-0.5, 0.6]	[-0.3, 0.8]
	GLP-1			[-0.3, 0.7]	[-0.3, 0.8]	[-0.1, 1.0]
	Glucagon				[-0.4, 0.6]	[-0.2, 0.8]
	Oxyntomodulin					[-0.3, 0.7]
CK (U/liter)	Baseline	163 [94, 194]	164 [109, 189]	166 [100, 186]	136 [96, 159]	171 [105 189]
	Saline		[-20, 44]%	[-26, 41]%	[-29, 21]%	[-23, 43]%
	GLP-1			[-25., 19]%	[-24, −1]%	[-21, 20]%

(*Continued*)

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Table 1. Continued

		Saline	GLP-1	Glucagon	Oxyntomodulin	GLP-1+glucagon
	Glucagon				[-26, −10]%	[-21, 32]%
	Oxyntomodulin					[-5, 35]%
	Peak	164 [95, 194]	166 [111, 192]	169 [102, 188]	139 [98, 162]	175 [107, 192]
	Saline	[-19, 45]%	[-26, 42]%	[-29, 23]%	[-22, 45]%	
	GLP-1		[-24, 19]%	[-25, −1]%	[-20, 20]%	
	Glucagon			[-25, 11]%	[-19, 33]%	
	Oxyntomodulin				[-5, 36]%	
(min×kU/liter)	AUC	35 [20, 43]	35 [24, 41]	36 [22, 39]	30 [21, 36]	37 [24, 41]
	Saline	[-21, 47]%	[-25, 39]%	[-31, 29]%	[-22, 46]%	
	GLP-1		[-21, 14]%	[-27, 6]%	[-17, 19]%	
	Glucagon			[-23, 26]%	[-13, 26]%	
	Oxyntomodulin				[-6, 36]%	
Insulin (pmol/liter)	0.40Baseline	48 [38, 55]	49 [39, 56]	55 [44, 63]	51 [39, 57]	54 [43, 61]
	Saline	[-15, 21]%	[-10, 38]%	[-15, 24]%	[-6, 34]%	
	GLP-1		[-5, 36]%	[-15, 22]	[-8, 32]%	
	Glucagon			[-25, 7]%	[-19, 16]%	
	Oxyntomodulin				[-10, 29]%	
	Peak	617 [469, 752]	256 [179, 286]	672 [493, 790]	345 [243, 389]	349 [235, 376]
	Saline	[-71, −49]%	[-21, 39]%	[-61, −31]%	[-62, −33]%	
	GLP-1		[108, 248]%	[3, 80]%	[-1, 74]%	
	Glucagon			[-63, −35]%	[-64, −37]%	
	Oxyntomodulin				[-27, 28]	
(min×nmol/liter)	AUC	35 [30, 40]	27 [22, 32]	43 [39, 48]	29 [24, 35]	34[28, 39]
	Saline	[-14, −2]	[2, 14]	[-11, 0]	[-7, 4]	
	GLP-1		[10, 22]	[-4, 8]	[1, 12]	
	Glucagon			[-20, −8]	[-15, −4]	
	Oxyntomodulin				[-1, 10]	
C-peptide (pmol/liter)	Baseline	386 [337, 435]	391 [342, 440]	395 [346, 443]	391 [342, 440]	373 [324, 422]
	Saline	[-44, 53]	[-40, 57]	[-44, 54]	[-61, 36]	
	GLP-1		[-45, 52]	[-49, 49]	[-67, 31]	
	Glucagon			[-53, 45]	[-70, 27]	
	Oxyntomodulin				[-67, 31]	
	Peak	1725 [1548, 1876]	962 [795, 1082]	1719 [1420, 1933]	1160 [944,1285]	1128 [917,1248]
	Saline	[-53, −36]%	[-17, 13]%	[-45, −25]%	[-46, 27]%	
	GLP-1		[67, 117]%	[-2, 44]%	[-5, 40]%	
	Glucagon			[-45, −19]%	[-47, −21]%	
	Oxyntomodulin				[-20, 18]%	
(min×nmol/liter)	AUC	178 [160, 195]	160 [142, 177]	189 [171, 207]	163[145, 180]	172 [155 180]
	Saline	[-36, 1]	[-7, 30]	[-33, 4]	[-24, 13]	
	GLP-1		[11, 48]	[-15, 22]	[-6, 31]	
	Glucagon			[-44, −8]	[-35, 2]	
	Oxyntomodulin				[-9, 27]	
ISR(pmol/kg/min)	Peak	12[11, 14]	6[5, 7]	12[10, 14]	7[5, 9]	7[5, 9]
	Saline	[-7, 5]	[-2, 2]	[-7, −3]	[-7, −3]	
	GLP-1		[4, 8]	[0, 3]	[0 3]	
	Glucagon			[-7, −2]	[-7, −2]	
	Oxyntomodulin				[-2, 2]	

FGF21. Baseline levels varied considerably (saline: 157(77–6039) (median with range in brackets) pg/mL GLP-1: 122(80–5853) pg/mL, glucagon: 142(75–5723) pg/mL, oxyntomodulin: 168(77–6382) pg/mL, GLP-1+glucagon: 110(76–6527) pg/mL), with no differences between the experimental days. After 240 minutes infusion, there were neither significant changes between the infusions nor differences compared to baseline (saline: 105(76–5953) pg/mL, GLP-1: 107(79–5836) pg/mL, glucagon: 107(75–5645) pg/mL, oxyntomodulin: 107(77–6250) pg/mL, GLP-1+glucagon: 105(75–6668) pg/mL).

Discussion

In the present study, we show that an infusion of oxyntomodulin and separate or combined infusions of GLP-1 and glucagon inhibited food intake similarly in young, lean, healthy male subjects, with no additive effect of the combined infusion. We confirm the inhibitory effects of oxyntomodulin and GLP-1, respectively, on GE observed previously, but by adding glucagon to the infusion of GLP-1, we found no additive effects. Surprisingly, glucagon alone had no effect on GE and appetite scores, but food intake decreased to the same extent as during oxyntomodulin, GLP-1 and GLP-1+glucagon infusions.

The positive effects of the peptide infusions reported here with respect to the second calorimetric measurement of O_2 are confounded by a residual meal-induced thermogenesis (driven by absorption and deposition nutrients (30)). Especially during the GLP-1, oxyntomodulin and the GLP-1+glucagon infusions, which all delayed GE, the measurement of O_2 was performed relatively soon after the serum acetaminophen peak, indicating that considerable nutrient absorption was still going on compared to saline. Flint et al previously concluded from a protocol very similar to the present that the observed increases in energy expenditure during GLP-1 infusions most likely were linked to the meal (13). However, robust reflections of the actual nutrient absorption ie, glucose levels, triglyceride levels and insulin responses were rather similar between the five infusions at the time of second calorimetry (31, 32), suggesting that the differences could be due to the

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different infusions. On the other hand, there were no significant changes in O_2 from baseline in any of the experiments (Figure 4). The lack of a clear effect on O_2 contrasts to recent reported results of infusions of glucagon and GLP-1 (33). But the dose of glucagon used in that particular study was more than 15 fold higher than ours and associated with large changes in glucose and insulin levels. Such increases are likely to influence REE and offer an explanation for the reported additive effect of combinations of GLP-1 and glucagon (33). Our observation that the peptides did not have consistent effects on energy expenditure is consistent with recent findings showing no increases after short term native GLP-1 infusions (34). Long-term treatments with the GLP-1 analog liraglutide using 24 hours chamber calorimetry has, so far, shown no differences in energy expenditure following the treatment (35, 36). In a single study, infusions of oxyntomodulin were associated with increased energy expenditure related to physical activity (as determined with the Actiheart device) in humans (but had no effect on basal metabolic rate). This finding is very difficult to translate to the general experience with peptide infusions, where decreases may be observed (because of malaise), but not increases (4). It has been suggested that stimulation of the production/levels of the metabolic regulator, FGF21, could constitute the link by activation of specific metabolic pathways such as improved glucose metabolism and activation of brown adipose tissue (19, 37). However, none of the peptide infusions tested in the present study lead to consistent changes in circulating FGF21. Our data do not support this link. This may, however, be due to insufficient statistical power, although we did use relatively high doses of the peptides.

In the present study, the infusion of glucagon did not change GE. This finding was unexpected since glucagon previously has been shown to inhibit bowel motility (38, 39). However, the dose used to inhibit bowel motility was more than 3000 fold higher than the dose used in the present study (38) and such doses might activate the GLP-1 receptor pathway (in vitro EC_{50} of glucagon on the

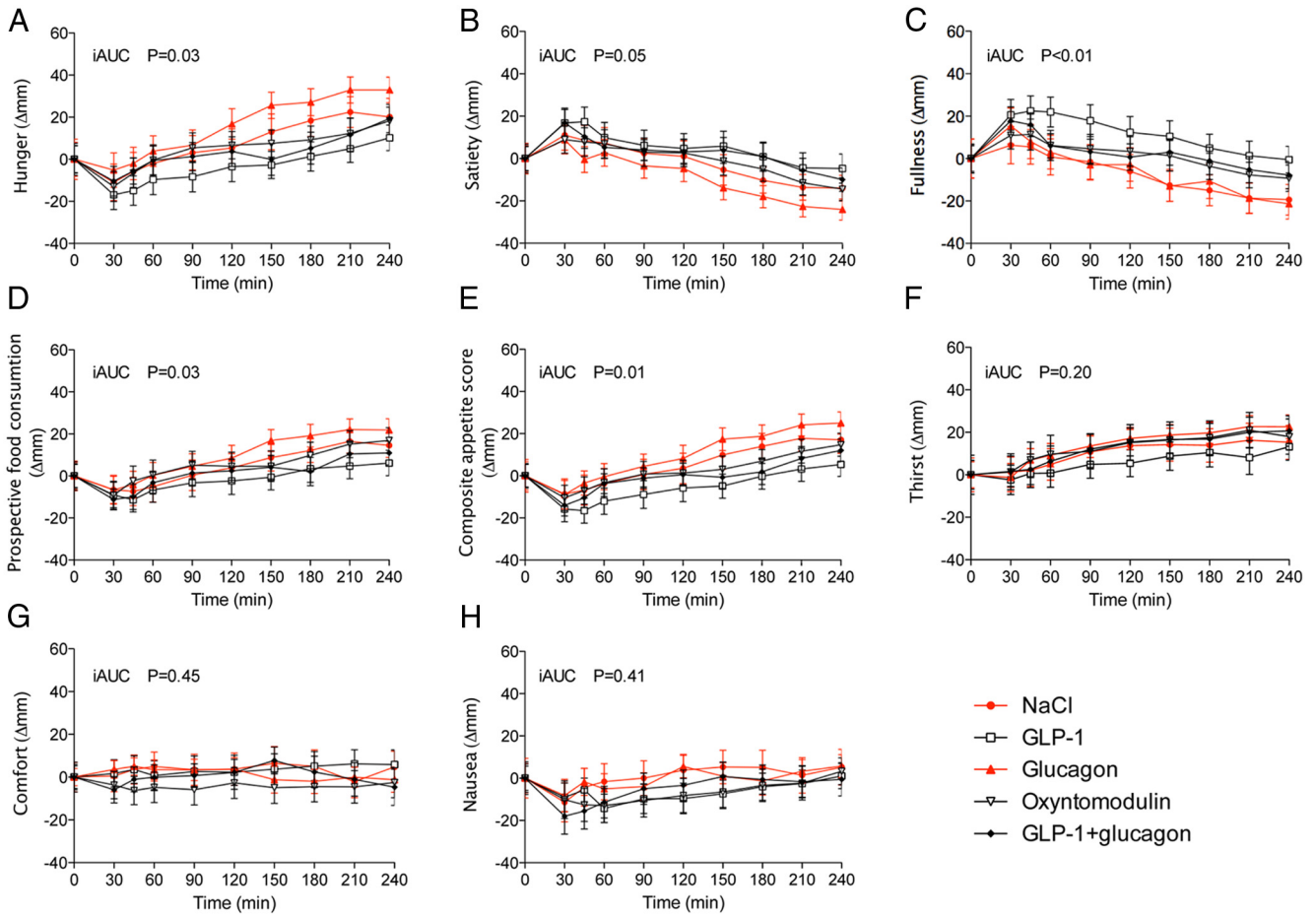

Figure 3. Baseline-subtracted postprandial visual analog scale (VAS) scores (measured in millimeters (mm)) as response over time and the levels of significance from the overall models during infusion of saline (dot), GLP-1 (square), glucagon (upright triangle), oxyntomodulin (downright triangle) and GLP-1+glucagon (diamond), respectively. A: hunger; B: satiety; C: fullness; D: prospective food consumption; E: composite appetite score; F: thirst; G: comfort; H: nausea. Red lines indicate rapid gastric emptying and black indicate slow gastric emptying as predicted by acetaminophen levels (Figure 2A).

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Table 2. Appetite scores and food intake Appetite score by VAS tabled by iAUC ((min×cm)/100) and food intake in weight (g). For each measure the first row are mean values and model adjusted 95% CIs of the mean in brackets. The following lines represents the post-hoc analysis as 95% CIs of the respective differences from the saline GLP-1, glucagon and oxyntomodulin infusion (as predicted by the model)

Infusion	NaCl	GLP-1	Glucagon	OXM	GLP-1+glucagon
Hunger ((min×mm)/100)	53 [31, 75]	32 [15,49]	67 [44, 89]	47 [30, 63]	39 [23, 55]
Saline		[-43, 2]	[-13, 41]	[-29, 16]	[-36, 9]
GLP-1			[12, 57]	[-3, 32]	[-10, 24]
Glucagon				[-42, 2]	[-50, −5]
Oxyntomodulin					[-24, 9]
Satiety	−12 [-28, 5]	2 [-16, 18]	−24 [-41, −8]	−4 [-21, 13]	−5 [-21, 12]
Saline		[-4, 31]	[-30, 5]	[-10, 25]	[-11, 24]
GLP-1			[-43, −8]	[-23, 12]	[-24, 11]
Glucagon				[3, 37]	[2, 37]
Oxyntomodulin					[-18, 17]
Fullness	−19 [-38, 1]	10 [-5, 24]	−21 [-41, −2]	−2 [-16, 13]	−6 [-21, 9]
Saline		[10, 47]	[-25, 20]	[-2, 36]	[-6, 31]
GLP-1			[-50, −12]	[-25, 2]	[-29, −2]
Glucagon				[1, 38]	[-3, 34]
Oxyntomodulin					[-17, 9]
Prospective Food Consumption	43 [24, 61]	29 [15, 43]	54 [40, 68]	45 [31, 59]	38 [24, 52]
Saline		[-33, 6]	[-8, 31]	[-17, 22]	[-24, 15]
GLP-1			[10, 41]	[1, 32]	[-6, 25]
Glucagon				[-24, 6]	[-31, −1]
Oxyntomodulin					[-22, 8]
CAS	46 [28, 64]	28 [15, 41]	57 [39, 75]	39 [26, 53]	37 [24, 50]
Saline		[-35, −2]	[-10, 31]	[-24, 10]	[-26, 8]
GLP-1			[12, 46]	[0, 23]	[-2, 21]
Glucagon				[-35, −1.]	[-37, −4]
Oxyntomodulin					[-14, 9]
Thirst	48 [33, 63]	40 [25, 55]	61 [46, 75]	52 [38, 67]	52 [37, 67]
Saline		[-26, 9]	[-5, 30]	[-13, 22]	[-13, 21]
GLP-1			[3, 38]	[-5, 30]	[-5, 30]
Glucagon				[-25, 9]	[-26, 9]
Oxyntomodulin					[-18, 17]
Comfort	26 [9, 43]	29 [11,46]	20 [3, 37]	14 [-3, 31]	27 [10, 44]
Saline		[-15, 20]	[-24, 11]	[-29, 5]	[-17, 18]
GLP-1			[-26, 9]	[-32, 3]	[-19, 16]
Glucagon				[-23, 12]	[-10, 24]
Oxyntomodulin					[-5, 30]
Nausea	37 [17, 57]	16 [-4, 36]	33 [14, 53]	21 [1, 40]	32 [13, 52]
Saline		[-47, 5]	[-29, 22]	[-42, 1 0]	[-30, 21]
GLP-1			[-8, 43]	[-21, 30]	[-9, 42]
Glucagon				[-38, 13]	[-26, 24]
Oxyntomodulin					[-13, 37]
Food intake (g)	811 [729, 892]	669 [586, 750]	686 [604, 768]	689 [608, 771]	688 [606, 769]
Saline		[-241, −44]	[-222, −27]	[-219, −24]	[-221, −25]
GLP-1			[-80, 116]	[-77, 119]	[-79, 118]
Glucagon				[-95, 101]	[-95, 99]
Oxyntomodulin					[-99, 96]

GLP-1 receptor is about 100-fold higher compared to GLP-1) (20). The rate of glucagon infusion used in our trial was specifically chosen to avoid marked increases in PG, but expected to be high enough to impact food intake (18). We did observe a small but significant increase in PG towards the end of the experiment with glucagon infusion compared to the other infusions. Nevertheless, the glucagon infusion did result in decreased food intake to the same extent as the other peptide infusions (despite having no impact on GE and appetite scores). These findings sug-

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gest that glucagon receptor agonists could be considered for the treatment of obesity. By adding a GLP-1 receptor agonist any deleterious effect of glucagon on glucose homeostasis (as observed in the present study; ie, slightly elevated PG levels after separate glucagon infusion) may be prevented. On the other hand, no additional effects on any measure were observed by adding glucagon to the GLP-1 infusion. The doses of both GLP-1 and oxyntomodulin used, have both (like glucagon) previously been shown to inhibit food intake (13, 40). Furthermore, the dose of oxyntomodulin was chosen to be sufficiently high to potentially impact both the glucagon and GLP-1 receptor (because oxyntomodulin has lower potency on both receptors compared to GLP-1 and glucagon, respectively) in order to be comparable to the combined infusion of glucagon and GLP-1. However, we did not find any significant superior effect of oxyntomodulin on any of the measures in the present protocol as was suggested by preclinical interventions in rodents (10, 19).

The present protocol focused on the effects of four hour continuous infusions of three potent peptides with short half-lives (2–12 minutes), and whether the observed effects would persist for days or even weeks as suggested by preclinical interventions in mice (19) cannot be concluded from this study. Nevertheless, we found a mean 180 kcal (120 g) difference in food intake following infusions of all the peptides compared to saline. This would roughly sum up to a body weight loss of 402 g per week (using the energetic value of fat 9.4 kcal/g), which is in the range of what previously has been found in overweight and obese humans with subcutaneous injections of oxyntomodulin (3). The relative weak signal observed in appetite scores when comparing saline and GLP-1 might reflect the limited number of participants included (21), though we did show highly significant differences between appetite scores during the glucagon and GLP-1 infusion. The dissociation between appetite scores and GE indicate differences between the mode of actions of GLP-1 and glucagon in relation to the inhibition of food intake.

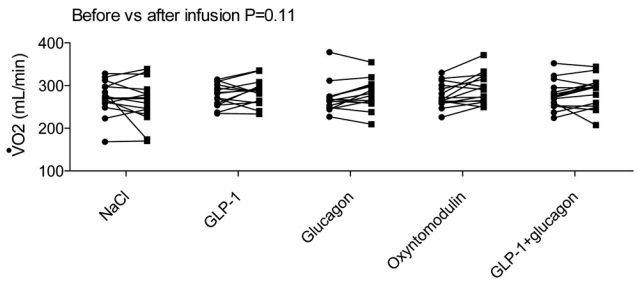

Figure 4. The measured gas exchange of O_2 ($\dot{V}O_2$)at steady state before infusion start (circles) and after 210 minutes of infusion (squares) during the five different experimental days. The gas exchange is displayed as the means from the 20 minutes measurement on the individual level linked with lines. The P-value is the significance level from the overall model testing the differences between before and after 210 minutes of infusion between the infusions.

In conclusion, infusion of oxyntomodulin, GLP-1 and glucagon reduced food intake similarly, but with no additional effect of adding glucagon to the GLP-1 infusion. Surprisingly, in these near physiological doses, glucagon lacked inhibitory effects on gastric emptying as well as appetite scores.

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AUTHOR CONTRIBUTIONS: JIB, JJH, FKK and TV designed the study; JIB preformed the experiments and the data analysis; JIB, JJH, FKK and TV wrote the manuscript; BH and BA contributed to the measurement of peptides, to the interpretation of the results and writing the manuscript. JIB and TV are the guarantors of this work and, as such, had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

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