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Quantification of the contribution of GLP-1 to mediating insulinotropic effects of DPP-4 inhibition with vildagliptin in healthy subjects and type 2-diabetic patients using exendin [9-39] as a GLP-1 receptor antagonist, Michael A. Nauck, Joachim Kind[3] Lars D. Köthe, Jens J. Holst, Carolyn F. Deacon, Matthias Broschag, Yan Ling He, Lise Kjems, James Foley

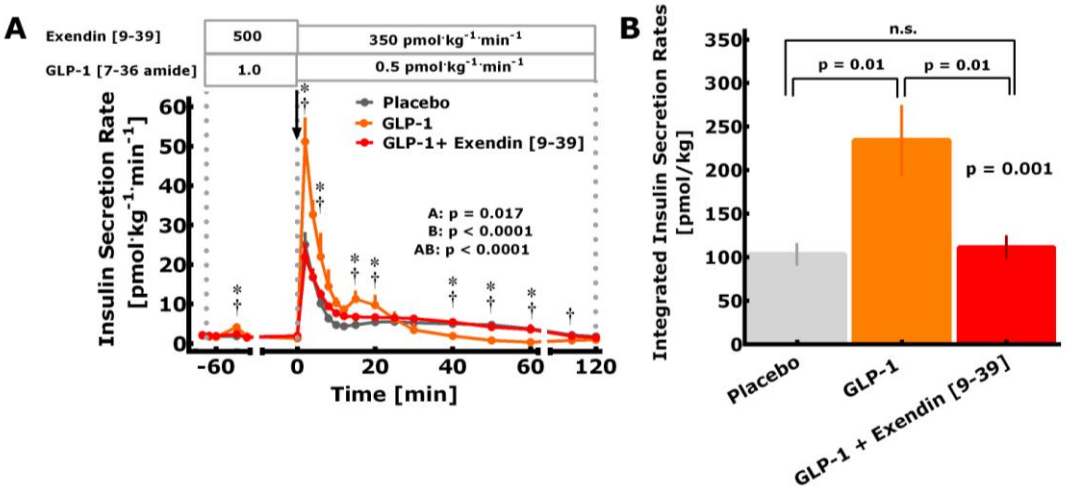
Pilot study: Exendin [9-39] as a GLP-1 receptor antagonist blocking insulinotropic activity of exogenous GLP-1

Healthy subjects (characteristics see table 1) participated in three examinations performed in the morning after an overnight fast. An intravenous glucose bolus (0.3 g/kg body weight as glucose 20 % in water) was injected at time 0 h. The subjects either received, in a single blind fashion, and in randomized order, (a) placebo, (b) GLP-1 [7-36] amide, or (c) GLP-1 [7-36]amide together with an excess of exendin [9-39]. GLP-1 [7-36] amide was administered at 1.0 $pmol \cdot kg^{-1} \cdot min^{-1}$ from -1 h to 0 h, and 0.5 $pmol \cdot kg^{-1} \cdot min^{-1}$ from 0 h to 2 h) by intravenous infusion. This dose is assumed to lead to high-physiological plasma concentrations (1; 2). Exendin [9-39] was administered at 500 $pmol \cdot kg^{-1} \cdot min^{-1}$ from -1 to 0 and at 350 $pmol \cdot kg^{-1} \cdot min^{-1}$ from 0 h to 2 h by intravenous infusion. This dose is slightly higher than reported in previous experiments using this GLP-1 receptor antagonist to block effects of endogenously secreted GLP-1 (3-5).

GLP-1 receptor blockade with exendin [9-39]

A glucose bolus injection in healthy subjects elicited a prominent first-phase and second-phase insulin secretory response (Figure 1). Exogenous GLP-1 leading to steady-state plasma levels of 53 ± 14 (total) or 5.8 ± 0.9 (intact) pmol/l (without exendin [9-39]) and 61 ± 5 (total) or 6.9 ±1.3 (intact) pmol/l GLP-1 (with exendin [9-39]) significantly augmented this glucose-induced insulin secretory response by more than two-fold. Exendin [9-39] at plasma concentrations of 167 ± 13 nmol/l completely antagonized the effect of GLP-1 on first-phase insulin secretion. This dosing regimen for exendin [9-39], therefore, was suitable to examine the contribution of endogenous GLP-1 to mediating gluco-regulatory effects of the DPP-4 inhibitor vildaglitpin.

Supplementary Figure 1. Insulin secretion rates derived by deconvolution analysis in response to a glucose bolus administration (time 0 min) with placebo (●–●), glucagon-like-peptide-1 infused intravenously from -75 to 120 min, (GLP-1: ●–●), and GLP-1 administered in combination with the GLP-1 receptor antagonist exendin [9-39] (●–●), in healthy subjects (A). Integrated incremental responses of insulin secretion rates from 0-10 after glucose stimulation are shown in panel B. Mean ± SEM. Statistical analysis: A. Repeated-measures analysis of variance (A: experiment, B: time, AB: interaction of experiment and time). Asterisks (*) indicate a significant ($p < 0.05$) stimulation of insulin secretion by GLP-1 (vs. placebo). Daggers (†) indicate a significant inhibition ($p < 0.05$) of insulin secretion by exendin [9-39] vs. GLP-1 alone. B. ANOVA, Duncan's post hoc test.



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Supplementary Table 1. DPP-4 activity and its inhibition by vildagliptin therapy

Treatment:	Vildagliptin	-	-	+	+	-	-	+	+
	Exendin [9-39]	-	+	-	+	-	+	-	+
Subjects/Patients:		Healthy subjects				Type 2-diabetic patients			
DPP-4 activity [$mU \cdot ml^{-1} \cdot min^{-1}$]									
Time [h][a]									
-1		8.4 ± 0.3	8.7 ± 0.3	3.1 ± 0.7	2.5 ± 0.5	9.1 ± 0.2	9.0 ± 0.3	3.6 ± 0.5	3.2 ± 0.5
0		8.5 ± 03	8.5 ± 0.3	0.9 ± 04	1.0 ± 04	9.4 ± 0.4	9.2 ± 0.4	0.7 ± 0.1	0.6 ± 0.1
2		8.7 ± 0.3	8.4 ± 0.4	0.8 ± 04	0.8 ± 05	9.6 ± 0.4	9.7 ± 0.7	0.4 ± 0.0	0.5 ± 0.0
DPP-4 activity [% inhibition vs. placebo]									
Time [h][a]		n.a.	n.a.			n.a.	n.a.		
-1		n.a.	n.a.	- 70.2 ± 6.0	- 77.2 ± 4.2	n.a.	n.a.	- 50.6 ± 6.8	- 50.6 ± 7.1
0		n.a.	n.a.	- 94.0 ± 0.6	- 94.1 ± 0.8	n.a.	n.a.	- 90.5 ± 1.1	- 90.8 ± 1.6
2		n.a.	n.a.	- 96.4 ± 0.2	- 94.5 ± 1.7	n.a.	n.a.	- 94.3 ± 0.3	- 92.3 ± 0.8

n.a.: Not applicable; [a]: Relative to the start of meal ingestion
DPP-4 activity was determined in serum specimens using the method described in (6).

Sample size calculation. Our original sample size calculation was based on the primary endpoint that we had pre-specified, glucagon. Glucagon was chosen, because we anticipated glucose-lowering effects either through the glucagonostatic effects of GLP-1, or through a deceleration of gastric emptying (which would also reduce meal-related glucagon increments), while insulinotropic and gastric emptying effects go in opposing directions and would potentially cancel each other out. With the finding of cross-reaction of exendin [9-39] in the glucagon immunoassay, we could no longer use the pre-specified primary endpoint and had to change the primary endpoint to the insulinogenic index as the primary endpoint.

Power calculation. For this endpoint, we performed a *post-hoc* power calculation, indicating that our study had a 95 % power to detect the smallest difference relevant for the interpretation of our study (effect of exendin [9-39] after vildagliptin treatment): insulin secretion rates divided by glucose increments with vildagliptin compared to vildagliptin/exendin [9-39] (mean difference, 0.6 [$pmol \cdot kg^{-1} \cdot min$]/[$mmol \cdot l^{-1} \cdot min$], SD of the difference, 0.07 [$pmol \cdot kg^{-1} \cdot min$]/[$mmol \cdot l^{-1} \cdot min$] already with 20 patients per group (nQuery advisor).

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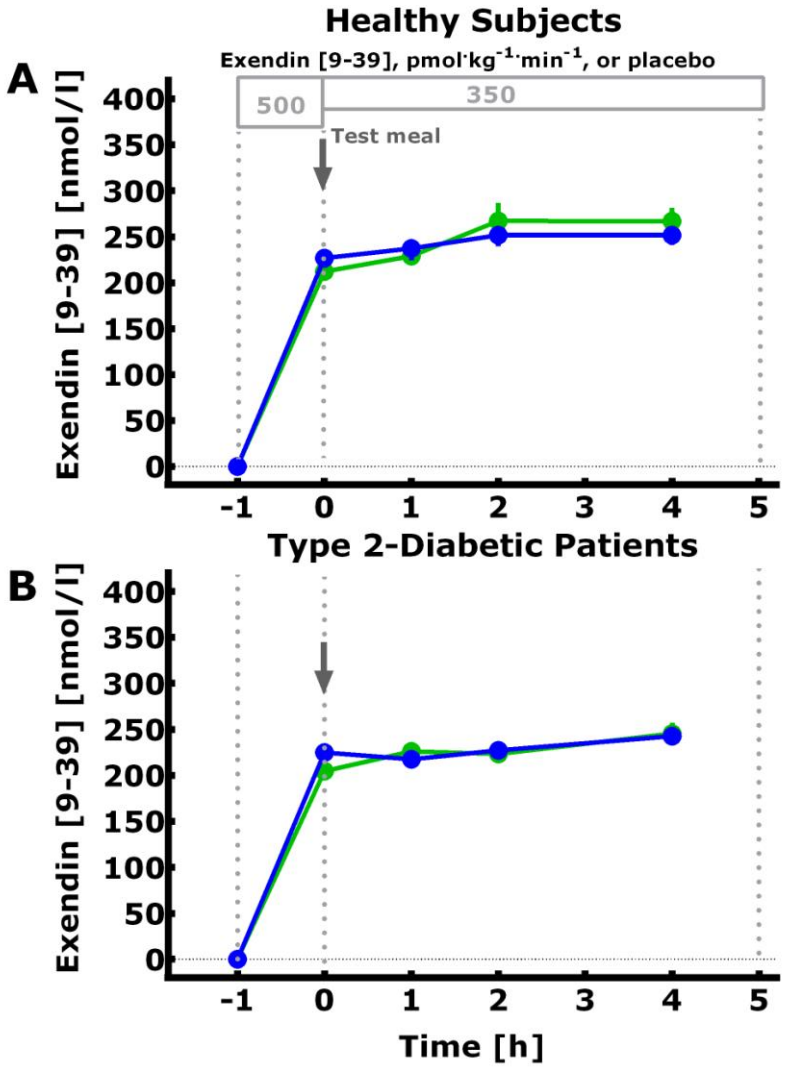
Supplementary Figure 2. Concentrations of exendin [9-39] (left panels), glucagon (middle panels) determined by radioimmunoassay using antibody 4505, and glucagon corrected for cross-reactivity of exendin [9-39] (right panels, details, see methods) in healthy control subjects (upper panels) and in type 2-diabetic patients (lower panels). Meal tests were performed after treatment with placebo, with (day 10; ●–●) or without (day 9; ●–●) the administration of the GLP-1 receptor antagonist exendin [9-39]; or with vildagliptin, with (day 10; ●–●) or without (day 9; ●–●) the administration of the GLP-1 receptor antagonist exendin [9-39]. Mean ± SEM. Statistical analysis: Repeated-measures analysis of co-variance reporting p-values for the independent variables V (vildagliptin), E (exendin [9-39]), T (time) and any significant interactions. Baseline concentrations/values with placebo were imputed as a co-variate. Asterisks in the bottom indicate time points, when the independent variable in question (V, E) was associated with a significant ($p < 0.05$) difference in the dependent variable.



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Mixed meal composition. The mixed meal contained one scrambled egg, a slice of ham, 10 g of butter, two slices of toast, 20 g strawberry jam, and 200 ml of unsweetened tea. The total caloric load of this test meal, which represents a typical European breakfast, was 541 kCal, with 53.5 % of the calories as carbohydrate, 22.4 % as protein, and 24.1 % as fat.

Measurement of gastric emptying. Because the liquid served with the meal did not contain nutrients, substrates or any ^{13}C-labeled material, the gastric emptying rates reported are those for the solid component of the meal. At intervals of 10 min during the initial 30 min, at 15 min intervals between 30 and 120 min, and at 30 min intervals up to 360 min, breath specimens were sampled into gas-tight plastic bags holding approximately 400 ml. Within 24 h, the $^{13}CO_2$-content of these samples was determined using near-infrared absorptiometry (Wagner Analysentechnik, Bremen, Germany). Total CO_2 and ^{13}C isotope enrichment were determined. The percent dose of ^{13}C-octanoic acid recovered with each sample was summed up to yield the cumulated percent dose (CUPCD).

Measuring and calculating gastric emptying. Using non-linear regression analysis (Graph PAD Prism, version 3, San Diego, CA), a curve was fitted according to the equation (7):

Equation 1 $$y(t) = m \cdot (1-e^{-kt})^{ß}$$

Y (t) was the cumulated percent dose recovered until time t. In this equation, m represents the level of y that is reached asymptotically with increasing time. k and ß are constants related to the velocity of gastric emptying (half-emptying time, $t_{1/2}$) and lag time(t_{lag}):

Equation 2 $$t_{1/2} = (-1/k) \cdot \ln(1-2^{-1/ß})$$

Equation 3 $$t_{lag} = \ln(ß)/k$$

Finally, gastric emptying was expressed as percent of the initial gastric contents (M = 100 %) by computing the difference to this initial value at each time point according to equation 3.

Equation 4 $$\text{Gastric content}(t) = (M-CUPCD(t))/M) \cdot 100\ [\%]$$

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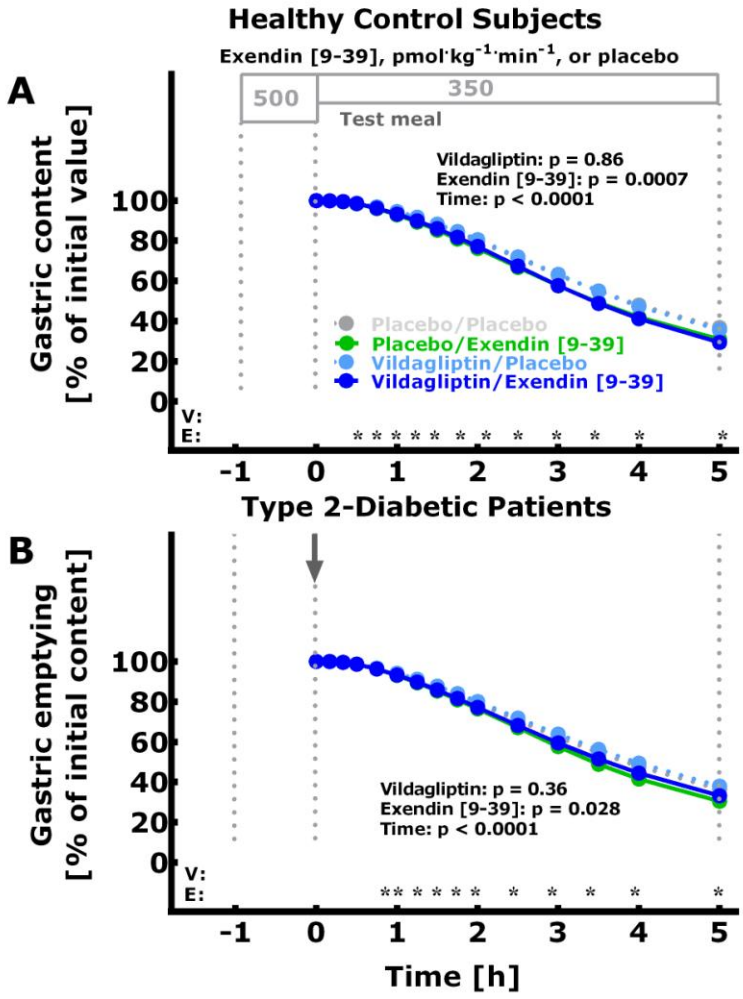
Supplementary Figure 3. Gastric emptying (octanoate ^{13}C-CO_2 breath tests) of a test meal administered at 0 min (gastric content = 100 %) in healthy control subjects (upper panel) and in type 2-diabetic patients (lower panel). Test were performed after treatment with placebo, with (day 10; ●–●) or without (day 9; ●–●) the administration of the GLP-1 receptor antagonist exendin [9-39]; or with vildagliptin, with (day 10; ●–●) or without (day 9; ●–●) the administration of the GLP-1 receptor antagonist exendin [9-39]. Mean ± SEM. Statistical analysis: Repeated-measures analysis of variance reporting p-values for the independent variables V (vildagliptin), E (exendin [9-39]), T (time) and any significant interactions. Subjects were imputed as a random variable. Asterisks in the bottom indicate time points, when the independent variable in question (V, E) was associated with a significant ($p < 0.05$) difference in the dependent variable.



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Supplementary Table 2. Parameters characterizing the velocity of gastric empyting in relation to vildagliptin treatment and to the administration of the GLP-1 receptor agonist exendin [9-39] in healthy control subjects and in type 2-diabetic patients, as determined with an octanoate ^{13}C-CO_2 breath test.

Conditions	Vildagliptin	No	No	Yes	Yes	Significance		
	Exendin [9-39]	No	Yes	No	Yes	Vilda-gliptin	Exendin [9-39]	Interaction
Parameter	Unit							
Healthy control subjects								
Gastric emptying $T_{1/2}$	H	4.19 ± 0.27	3.79 ± 0.27	4.03 ± 0.20	3.52 ± 0.11	0.34	0.012	0.72
Lag time	H	2.56 ± 0.12	2.32 ± 0.11	2.59 ± 0.10	2.30 ± 0.07	0.95	0.0008	0.76
M		70.2 ± 5.2	65.6 ± 5.5	65.3 ± 5.7	60.2 ± 3.7	0.36	0.31	0.95
K		0.37 ± 0.03	0.42 ± 0.03	0.39 ± 0.02	0.43 ± 0.02	0.45	0.015	0.87
ß		2.50 ± 0.11	2.56 ± 0.11	2.70 ± 0.13	2.70 ± 0.10	0.049	0.74	0.67
A		1.84 ± 0.18	2.17 ± 0.15	1.62 ± 0.10	2.07 ± 0.15	0.18	0.0088	0.45
B		1.44 ± 0.12	1.46± 0.08	1.59 ± 0.11	1.62 ± 0.08	0.06	0.78	0.92
C		0.19 ± 0.05	0.25 ± 0.04	0.22 ± 0.04	0.29 ± 0.03	0.28	0.096	0.87
Type 2-Diabetes								
Gastric emptying $T_{1/2}$	H	4.23 ± 0.31	3.75 ± 0.24	4.51 ± 0.40	4.19 ± 0.52	0.22	0.28	0.80
Lag time	H	2.59 ± 0.16	2.33 ± 0.10	2.76 ± 0.19	2.52 ± 0.22	0.15	0.17	0.94
M		69.5 ± 9.4	66.5 ± 6.0	68.1 ± 6.7	57.8 ± 3.6	0.40	0.30	0.37
K		0.38 ± 0.02	0.44 ± 0.04	0.37 ± 0.02	0.41 ± 0.03	0.29	0.052	0.43
ß		2.50 ± 0.10	3.35 ± 0.78	2.51 ± 0.09	2.52 ± 0.09	0.31	0.29	0.29
A		1.66 ± 0.17	2.24 ± 0.25	1.53 ± 0.13	1.92 ± 0.20	0.29	0.0021	0.27
B		1.30 ± 0.11	1.91 ± 0.46	1.28 ± 0.08	1.37 ± 0.09	0.21	0.19	0.32
C		0.16 ± 0.05	0.39 ± 0.15	0.11 ± 0.04	0.20 ± 0.04	0.15	0.069	0.33

Means ± SE; Statistics ANOVA (details, see methods); Parameters M, k, ß as well as a, b, and c were obtained from curve fitting procedures.

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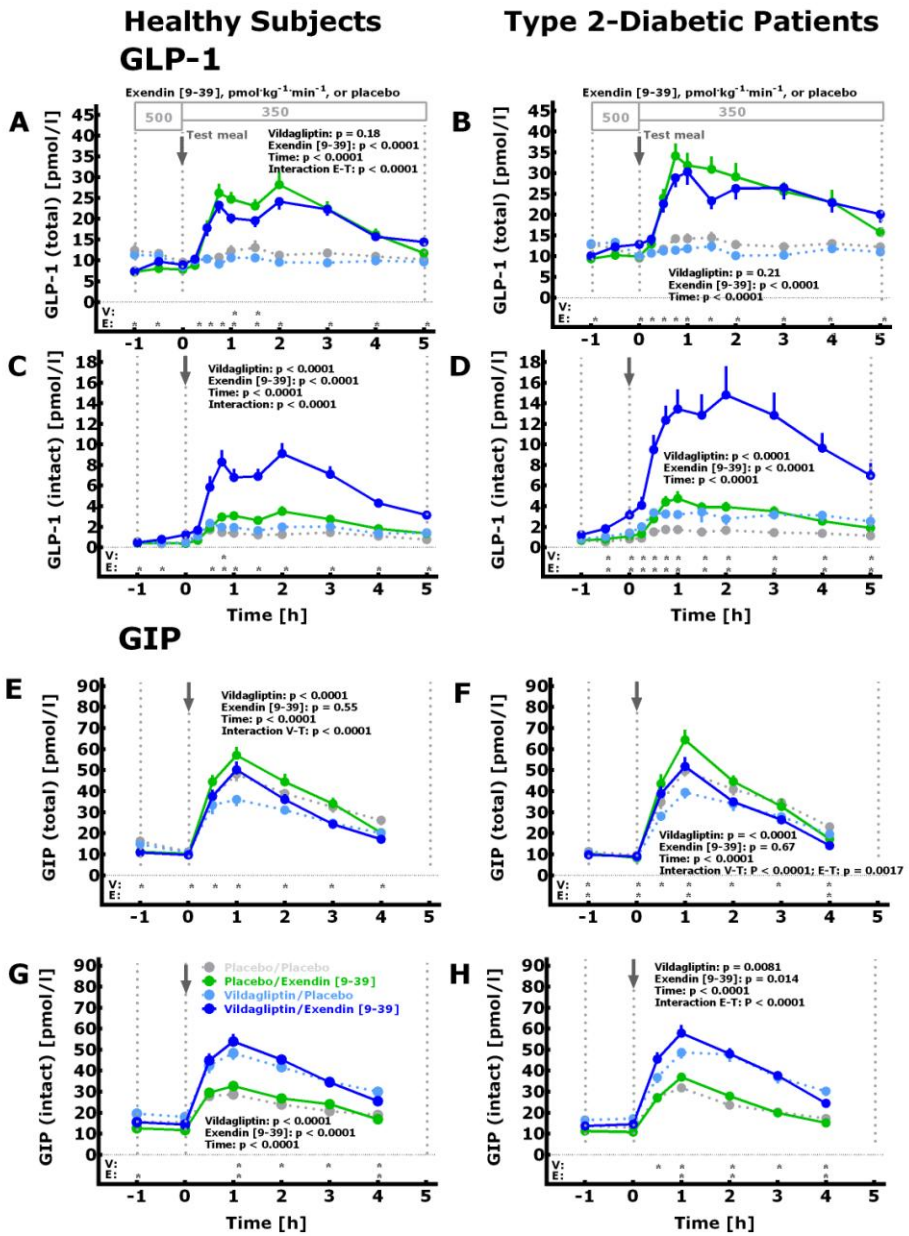
Supplementary Figure 4. Concentrations of total GLP-1 (including degradation products due to DPP-4 activity; upper panels; A, B) intact, biologically active GLP-1 (second row of panels; C, D), total GIP (third row of panels; E, F), and intact GIP (lower panels); G, H) in healthy control subjects (left panels; A, C, E, G) and in type 2-diabetic patients (right panels; B, D, F, H). Test were performed after treatment with placebo, with (day 10; ●–●) or without (day 9; ●–●) the administration of the GLP-1 receptor antagonist exendin [9-39]; or with vildagliptin, with (day 10; ●–●) or without (day 9; ●–●) the administration of the GLP-1 receptor antagonist exendin [9-39]. Mean ± SEM. Statistical analysis: Repeated-measures analysis of co-variance reporting p-values for the independent variables V (vildagliptin), E (exendin [9-39]), T (time) and any significant interactions. Baseline concentrations/values with placebo were imputed as a co-variate. Asterisks in the bottom indicate time points, when the independent variable in question (V, E) was associated with a significant ($p < 0.05$) difference in the dependent variable.



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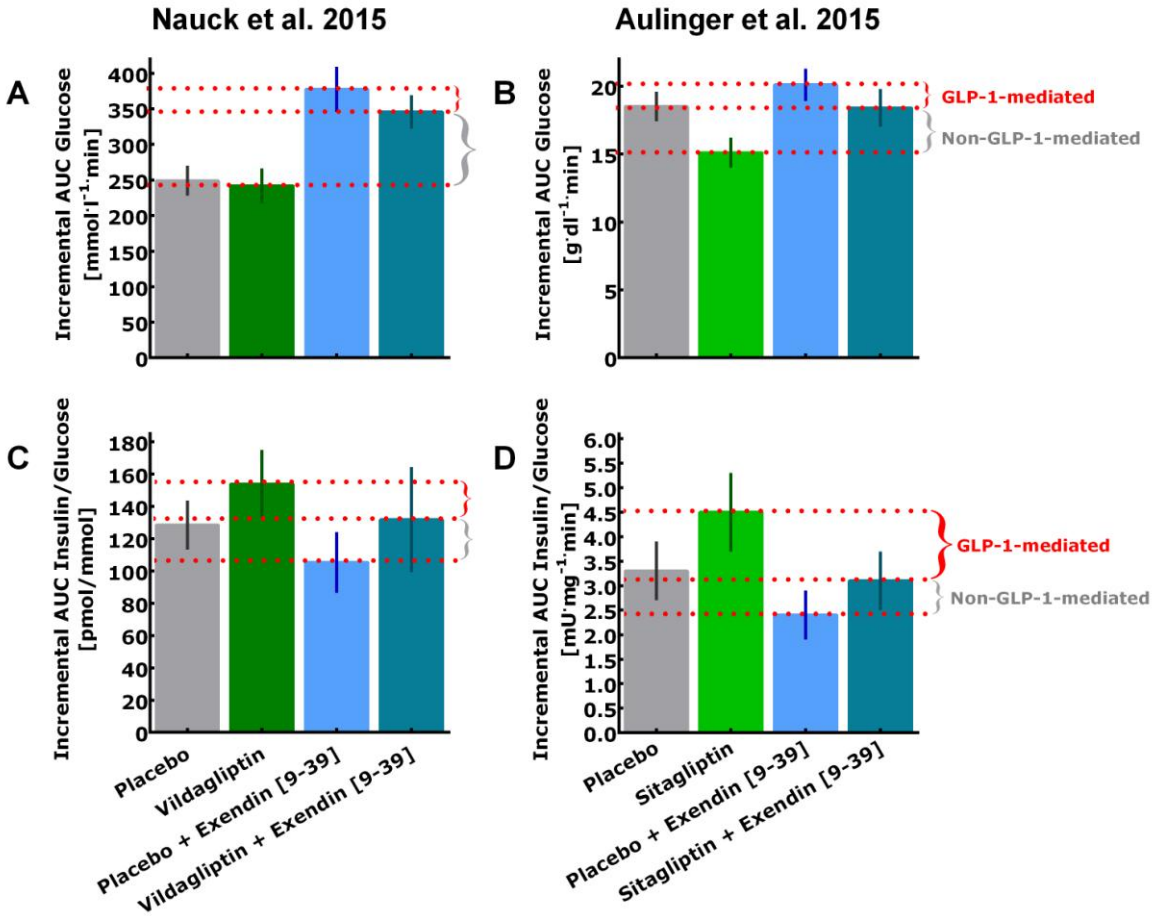
Supplementary Figure 5. Comparison of results reported by Aulinger et al. 2014 (8) and from the present study. The therapeutic effects of DPP-4 inhibition (vildagliptin in the present study, sitagliptin in the publication by Aulinger et al. (8)) on incremental integrated glucose concentrations (upper panels, A, B) or insulinogenic indices (based on integrated incremental glucose and insulin concentrations; lower panels, C, D) following oral glucose (Aulinger et al. (8)) or a mixed meal (present study) are shown. They were divided into GLP-1 mediated and non-GLP-1-mediated proportions based on the following reasoning: Placebo and exendin [9-39] define non-GLP-1-mediated effects in the absence of DPP-4 inhibitor treatment. DPP-4-inhibitor treatment (vildagliptin or sitagliptin) defines the maximum effect of treatment including a contribution mediated by GLP-1. The difference defines the potentially inhibitable proportion of the DPP-4 inhibitor-induced treatment effect. The experiment with DPP-4 inhibition and exendin [9-39] separates this range into the GLP-1-mediated and non-mediated proportion as indicated by the red and grey brackets, respectively.



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