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Clinical Research Article

GLP-1 Receptor Blockade Reduces Stimulated Insulin Secretion in Fasted Subjects With Low Circulating GLP-1

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Abstract

Context: Glucagon-like peptide 1 (GLP-1), an insulinotropic peptide released into the circulation from intestinal enteroendocrine cells, is considered a hormonal mediator of insulin secretion. However, the physiological actions of circulating GLP-1 have been questioned because of the short half-life of the active peptide. Moreover, there is mounting evidence for localized, intra-islet mediation of GLP-1 receptor (GLP-1r) signaling including a role for islet dipeptidyl-peptidase 4 (DPP4).

Objective: To determine whether GLP-1r signaling contributes to insulin secretion in the absence of enteral stimulation and increased plasma levels, and whether this is affected by DPP4.

Methods: Single-site study conducted at an academic medical center of 20 nondiabetic subjects and 13 subjects with type 2 diabetes. This was a crossover study in which subjects received either a DPP4 inhibitor (DPP4i; sitagliptin) or placebo on 2 separate days. On each day they received a bolus of intravenous (IV) arginine during sequential 60-minute infusions of the GLP-1r blocker exendin$_{[9\text{-}39]}$ (Ex-9) and saline. The main outcome measures were arginine-stimulated secretion of C-Peptide (C-P$_{Arg}$) and insulin (Ins$_{Arg}$).

Results: Plasma GLP-1 remained at fasting levels throughout the experiments and IV arginine stimulated both α- and β-cell secretion in all subjects. Ex-9 infusion reduced C-P$_{Arg}$ in both the diabetic and nondiabetic groups by ~14% ($P < .03$ for both groups). Sitagliptin lowered baseline glycemia but did not affect the primary measures of insulin secretion. However, a significant interaction between sitagliptin and Ex-9 suggested more GLP-1r activation with DPP4i treatment in subjects with diabetes.

Conclusion: GLP-1r activation contributes to β-cell secretion in diabetic and nondiabetic people during α-cell activation, but in the absence of increased circulating GLP-1. These results are compatible with regulation of β-cells by paracrine signals from α-cells. This process may be affected by DPP4 inhibition.

Key Words: insulin secretion, incretin effect, dipeptidyl peptidase 4, GLP-1, glucagon, GLP-1 receptor

Abbreviations: BMI, body mass index; DPP-4, dipeptidyl-peptidase 4; DPP4i, DPP4 inhibitor; Ex-9, exendin[9-39]; GLP-1, glucagon-like peptide 1; GLP-1r, GLP-1 receptor; GLP-1RA, GLP-1r agonist; IV, intravenous; T2DM, type 2 diabetes mellitus.

Glucagon-like peptide 1 (GLP-1) is a potent insulin secretagogue that has a central role in the incretin effect (1, 2). The widely held view of GLP-1 action is as an endocrine mediator, released during nutrient absorption by enteroendocrine L-cells into the circulation from whence it acts directly on target tissues like the β-cell that express the GLP-1 receptor (GLP-1r). However, this model has been questioned because of the relatively small dynamic range of postprandial GLP-1 secretion, and its rapid inactivation by the widely distributed enzyme dipeptidyl peptidase 4 (DPP-4) (3-5). We recently reported that GLP-1r signaling is necessary for normal glucose tolerance in mice, but that this effect is independent of circulating GLP-1 (6). Moreover, in studies of fasted healthy people and people with diabetes our group and others observed that blockade of the GLP-1r blunts intravenous (IV) glucose–stimulated insulin secretion (7, 8), suggesting an alternative to meal-stimulated GLP-1 as the sole mechanism for activating the β-cell GLP-1r. One such alternative is activation of the GLP-1r through paracrine signaling in the pancreatic islet (5, 9, 10). In this model, there is important α- to β-cell communication, namely proglucagon peptides produced by islet α-cells regulating secretion of neighboring β-cells (10-13). This construct is supported by reports of α-cell production of GLP-1 (10, 14-16), and insulinotropic actions of glucagon mediated through β-cell GLP-1r (13, 17, 18). Taken together, recent preclinical work supports intra-islet signaling by proglucagon peptides as a component of physiological regulation of insulin secretion that is independent of circulating GLP-1.

The GLP-1r signaling system has been the centerpiece of diabetes drug development in the past decade, and has provided 2 new classes of drugs: small molecule DPP4 inhibitors (DPP4i) that protect and prolong the action of endogenous GLP-1, and peptidergic GLP-1r agonists (GLP-1RAs) that are resistant to enzymatic cleavage by DPP4 (19). While studies in humans and animals indicate that GLP-1RAs act directly on GLP-1r accessible through the circulation, the mechanism

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by which DPP4is lower blood glucose is still not fully understood. The approximate doubling of active GLP-1 concentrations in the plasma of patients taking a DPP4i such as sitagliptin are dwarfed by the levels of GLP-1RA achieved after injection (20). Moreover, it has not been conclusively demonstrated that plasma GLP-1 in these amounts is actually sufficient to promote insulin secretion (21, 22). However, DPP4 has been detected in the endocrine pancreas of animals and humans (23-25), and protection of locally produced peptides from being metabolized may be a component of the islet regulatory system (10). If this were the case, the magnitude of α- to β-cell communication could be governed to some extent by DPP4 activity.

We hypothesized that insulinotropic GLP-1r signaling occurs independent of circulating concentrations of GLP-1, and that this process is amplified by DPP4 inhibition. We tested this hypothesis in a cohort of healthy volunteers and volunteers with type 2 diabetes mellitus (T2DM) using the GLP-1r antagonist exendin-[9-39] (Ex-9) to identify the actions of proglucagon peptides on stimulated insulin secretion.

Materials and Methods

Subjects

Twenty-two middle-aged men and women without chronic medical conditions and 18 comparably aged persons with T2DM were recruited by advertisement. One diabetic subject and 1 nondiabetic subject did not complete studies due to wheal/flare responses to subcutaneous skin tests, 1 nondiabetic subject did not complete their second study because IV access was lost, 1 diabetic subject did not complete studies following an unpleasant response to arginine administration, and 3 diabetic subjects could not schedule visits after screening because of time constraints. In total, 33 subjects completed studies: 20 nondiabetic subjects and 13 diabetic subjects (Table 1). Nondiabetic subjects had normal fasting glucose concentrations and hemoglobin A1c. Diabetic subjects had a definite diagnosis by blood glucose or A1c criteria, had current A1c levels ≤8.0%, and were treated with metformin alone (n = 4), sulfonylurea (n = 1), sitagliptin (n = 1), diet and exercise (n = 2), or combinations of metformin and sitagliptin (n = 2), metformin and dulaglutide (n = 1), metformin, sitagliptin, and sulfonylurea (n = 1), or empagliflozin and liraglutide (n = 1). Subjects in both cohorts were otherwise free of medical disease and did not use other medications that affect glucose or insulin regulation. The diabetic subjects stopped their usual medications for 3 days before each day of experimentation.

Protocols

All study procedures were conducted at Duke University and approved by the Duke Health Institutional Review Board. This study was registered at ClinicalTrials.gov under identifier NCT02683187. Subjects were recruited between September 2016 and February 2018. Each subject had 2, 3-hour studies in the Duke Molecular Physiology Institute Clinical Research Unit. Following enrollment, subjects were randomized to receive either sitagliptin or placebo first and the order of these treatments was randomized and counterbalanced across the diabetic and nondiabetic cohorts: in each group half of the subjects took sitagliptin on the first study day and half took sitagliptin on their second study day. Subjects, but not investigators, were blinded to the order of drug and treatments.

The study design is depicted in Fig. 1. Upon arrival to the unit, subjects received sitagliptin (100 mg orally) or placebo, and a skin test was performed to test for immediate sensitivity to Ex-9 solution (Ex-9 dissolved in 0.25% human serum albumin). Subjects had intravenous catheters placed in each forearm for (1) blood drawing and (2) infusion of test materials, and the arm used for blood draws was heated to maintain blood flow and maintain a stable degree of arterialized venous blood. Forty minutes after taking sitagliptin or placebo, fasting blood samples were taken at –10, –5, and 0 minutes prior to initiation of a 60-minute infusion of Ex-9 (750 pmol/kg/minute) or saline; half of the subjects had the Ex-9 infusion in the first part of the study and the other half had saline first. Blood samples were taken for baseline measurements at 10, 15, 20, and 25 minutes after infusion. At time 30, subjects received an infusion of 5 g of arginine over 1 minute and blood samples were taken at 32, 34, 36, 38, 40, 42, and 45 minutes. After 60 minutes, Ex-9 or saline infusion was stopped during a washout period. At 120 minutes, subjects received the alternate infusion of either saline or Ex-9 and baseline samples for the second period were taken at 130, 135, 140, and 145 minutes. At 150 minutes, the arginine bolus was repeated and blood was sampled at 152,

Table 1. Subject characteristics

	Nondiabetic group (n = 20)	Diabetic group (n = 13)	*P*
Age, years (SD)	48.9 (10.4)	54.8 (8.1)	.09
Range	(35.2-62.8)	(43.2-70.5)	
BMI, kg/m²	29.9 (4.8)	35.3 (7.9)	.02
Range	(23.3-42.1)	(23.2-52.9)	
Fasting glucose, mg/dL	89.6 (10.0)	130.5 (35.2)	<.001
Range	(75.0-109.3)	(92.2-211.6)	
Hemoglobin A1c, %	5.5 (0.4)	7.0 (0.9)	<.001
Range	(4.8-6.2)	(5.0-8.0)	
Estimated glomerular filtration, mL/minute/1.73 m²	89.4 (15)	85.0 (16.1)	.43
Range	(63-113)	(57-115)	
Male/Female	12/8	4/9	
Caucasian/African American	15/5	6/7	

Where indicated, *P* value results from a t-test.

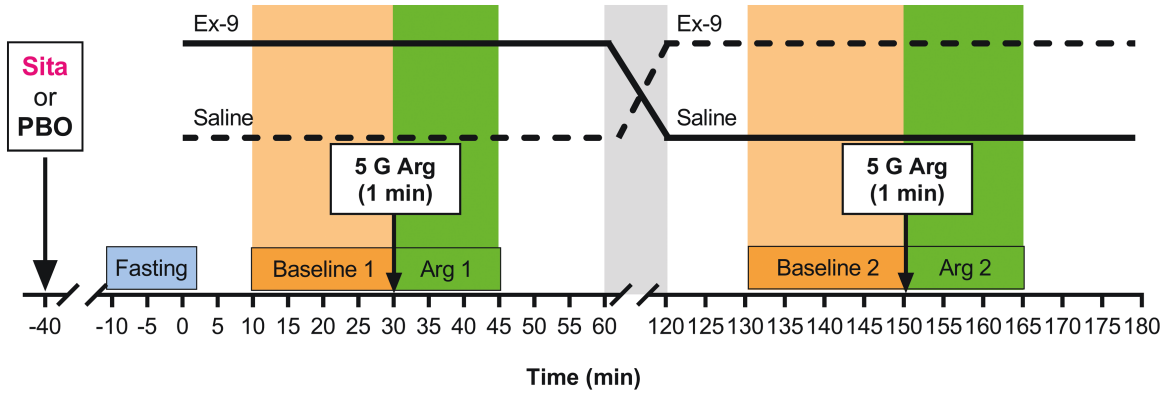

Figure 1. Study design for each study day. Subjects received sitagliptin (sita) or placebo (PBO) 40 minutes prior to receiving either Ex-9 (solid line) or saline (hatched line) infusion at time 0. Fasting and baseline data collection periods are indicated prior to arginine dose. Following IV administration of arginine (5 g over 1 minute), plasma samples were collected. Between 60 and 120 minutes, a washout period occurred. Infusion of the opposite infusate began at 120 minutes and data collection repeated.

154, 156, 158, 160, 162, and 165 minutes at which point the infusion was stopped, completing the experiment. The time between sitagliptin or placebo ingestion and the first arginine infusion was >60 minutes. The 2-by-2 design allowed the effects of GLP-1r blockade and DPP-4 inhibition on arginine-stimulated insulin secretion to be determined separately and together.

Assays

Blood samples were collected in tubes containing heparin for determinations of plasma insulin and blood glucose. Blood was also collected in tubes containing 50 mM EDTA, 500 kallikrein inhibitory units/ml aprotinin, and 0.1 M diprotin A for measurement of plasma C-peptide and proglucagon peptides. Blood glucose concentrations were determined by a glucose oxidase measurement (Stan Bio 1070125). Insulin (Alpco Diagnostics Cat# 80-INSHU-E01.1, RRID:AB_2801438), C-peptide (Alpco Diagnostics Cat# 80-CPTHU-E01.1, RRID AB_2904191), glucagon (Ansh Labs Cat# AL-157, RRID:AB_2783696), and total GLP-1 (Ansh Labs Cat# AL-172, RRID:AB_2783703) concentrations were determined using a commercial enzyme-linked immunosorbent assay, each according to the specifications of the manufacturer. Performance metrics of the GLP-1 and glucagon assays are described elsewhere (Tables 1 and 2 (26)).

Calculations and Analysis

Fasting values of blood glucose were computed as the average of the samples drawn from –10 to 0 minutes on the placebo study day. Baseline glucose, insulin, C-peptide, glucagon, and GLP-1 were the mean of the 3 or 4 samples taken in the 20 minutes before each arginine bolus. The primary outcome, arginine-stimulated β-cell secretion measured as plasma insulin (Ins_{Arg}) or C-peptide ($C\text{-}P_{Arg}$), was summed as the incremental increase in plasma concentrations for 10 minutes after arginine infusion over the baseline concentration. This approach was also used to measure secondary outcomes of arginine-stimulated glucagon (Gcg_{Arg}) and GLP-1 ($GLP\text{-}1_{Arg}$) responses. We computed a sample size estimate based on a previous study of healthy subjects who had insulin secretion following an arginine bolus (Ins_{Arg}) of 624 ± 84 pmol/L, with a coefficient of variation of 49% (27). Using this estimate of between subject variation, it was estimated that to detect a 25% difference between the Ex-9 and control groups with a power of 80% at a significance level of .05 would require 18 subjects.

Statistical Analysis

Subject data are presented as mean ± SD, experimental results in tables are presented as least squares mean ± 95% CI, and figures depict mean ± SEM. Clinical and demographic characteristics of the subjects were compared using unpaired t-tests. Data were tested for normality, and variables that did not pass the Shapiro–Wilks test for normality were transformed to achieve normality prior to statistical analysis. Only the Gcg_{Arg} was not normally distributed even after multiple approaches at normalization. A mixed linear model, with fixed effects of drug (sitagliptin, placebo), treatment (Ex-9, saline), and time (arginine 1, arginine 2), a repeated effect of arginine stimulation, and a random effect of subject, was performed using SAS (University Edition, Cary, NC). Initial analyses of variables utilized a full model with all interaction terms. If interactions were not significant, they were removed from the model. Least squares means were compared with a Tukey–Kramer post hoc test for multiple comparisons and statistical significance was determined as $P < .05$. Significant interactions are shown in figures and tables with main effect P values provided for reference in the tables. For data with significant interactions, post hoc pairwise comparisons and their significance are reported in both tables and figures.

Results

Subject Characteristics

Nondiabetic subjects who completed studies included 8 women and 12 men, 5 African-American and 15 Caucasian, with a mean body mass index (BMI) of 30 ± 5, A1c 5.5 ± 0.4%, and estimated glomerular filtration rate 89.4 ± 15 mL/minute. Volunteers with T2DM included 9 women and 4 men, 7 African American and 6 Caucasian, with a mean BMI of 35 ± 8, A1c 6.9 ± 0.9%, and estimated glomerular filtration 85 ± 16. Between the 2 groups, BMI, fasting glycemia, and hemoglobin A1c were significantly greater in subjects with diabetes, and these volunteers also tended to be older (Table 1).

Hypothesis testing in this study involved 2 overlapping interventions: sitagliptin/placebo (drug) and exendin-9/saline

(treatment). Because each subject received both pairs of interventions, and the principle comparison was within subjects, the primary (main) effects of drug and treatment were considered independently. The study was counterbalanced to spread the interventions (exendin-9/saline) equally between the first and second arginine stimulus. Because there was an observed effect of time on the primary outcome measure, this factor was added into the mixed model analysis. Interactions between main effects were evaluated and are reported where significant. Given that glycemia was a distinguishing inclusion criterion for the 2 groups, and since diabetic and nondiabetic volunteers were not matched for age, sex, and body weight, the 2 groups were analyzed separately to assess the primary outcome. However, since the 2 groups had similar results in this primary analysis they were combined for a secondary analysis to assess insulin, C-peptide, glucagon, and GLP-1 with a larger sample size; "group" (nondiabetic/diabetic) was included as a factor in this model (Tables 6 and 7 (26)). Another secondary analysis was undertaken to examine the main effects of treatment and drug in the first arginine stimulus only (Table 8 (26)).

Effects of Interventions on Blood Glucose

Glucose values prior to arginine infusions were modestly but significantly reduced on the day subjects from both the nondiabetic and diabetic groups took sitagliptin (Table 2). The time of the infusions was also significant, in that both diabetic and nondiabetic volunteers had lower preinfusion blood glucose before the second arginine administration. Infusion of Ex-9 had no effect on baseline glycemia.

Baseline and Arginine-stimulated β-Cell Secretion

In the nondiabetic volunteers, baseline C-peptide was increased with sitagliptin, and concentrations were lower prior to the second arginine bolus compared with the first. Infusion of arginine caused characteristic responses in C-peptide and insulin (Fig. 2A and 2C; Figure 1a, 2a (26)) that peaked within 5 minutes and dissipated over 15 minutes. Ins_{Arg} and $C\text{-}P_{Arg}$ were not different on days nondiabetic subjects took sitagliptin or placebo, but secretion of β-cell peptides was significantly reduced during the second arginine treatment (Table 3, and Fig. 3A and 3C; Figure 1a, 2a (26)). Infusion of Ex-9 significantly reduced arginine-stimulated insulin and C-peptide in this group by ~ 14% (Table 3). In addition, there was a significant interaction between drug and treatment on $C\text{-}P_{Arg}$ (Table 3 and Fig. 3A). Post hoc comparisons revealed that the suppressive effect of Ex-9 was apparent on the day subjects took placebo but not sitagliptin (Table 4 (26); Fig. 3A).

In the diabetic subjects sitagliptin increased, and Ex-9 decreased, baseline C-peptide (Table 5 (26)). The insulin and C-peptide responses to arginine in this group were qualitatively and quantitatively similar to the nondiabetic subjects (Fig. 2B and 2D; Figure 1b, 2b (26)). As with the nondiabetic subjects, Ex-9 significantly reduced $C\text{-}P_{Arg}$ by 13% compared with saline infusion, and there was also a significant reduction of stimulated β-cell peptides after the second arginine infusion (Fig. 2B and 2D and Table 3). However, Ins_{Arg} in this group did not differ by drug or treatment (Fig. 3E). There was a tendency for sitagliptin to raise stimulated $C\text{-}P_{Arg}$ ($P = .062$), an effect driven by the studies with saline more than Ex-9 infusion (Fig. 3B). There was also a drug by treatment interaction on $C\text{-}P_{Arg}$ among the diabetic subjects, but in this case the suppressive effect of Ex-9 was apparent on the day subjects took sitagliptin not placebo (Fig. 3B; Table 4 (26)).

In the secondary analysis of the combined groups (Fig. 4A and 4B; Tables 6, 7 and Figure 1c, Figure 2c (26)), subjects with diabetes had increased baseline insulin (140.8 vs 108.2, $P = .015$) and C-peptide (617.4 vs 435.8, $P = .022$, Table 6 (26)) compared with the nondiabetic group. However, there was no effect of group on arginine-stimulated β-cell secretion (Table 7 (26)). In this analysis the significant effects of Ex-9 and time to reduce Ins_{Arg} and $C\text{-}P_{Arg}$ were maintained, but there was no main effect of sitagliptin nor any interaction between drug and treatment (Fig. 4A and 4B). In the secondary analysis of the first arginine stimulus, we did not detect significant interactions between drug and treatment for nondiabetic or diabetic subjects. (Table 8 (26)). This cross-section of the data indicated that, in nondiabetic subjects, sitagliptin did not meaningfully effect arginine-stimulated beta cell secretion while exendin-9 decreased arginine-stimulated beta cell secretion. However, it does not detect significant responses in diabetic subjects that we report in the primary analysis. We believe this is due to a lack of power when only the first arginine stimulus is investigated.

Baseline and Arginine-stimulated α-Cell Secretion

Baseline glucagon and GLP-1 were not affected by exendin-9 or time in nondiabetic (Table 3 (26)) or diabetic subjects (Table 4 (26)), and did not differ between nondiabetic and diabetic subjects in the secondary analysis (Table 6 (26)). In response to arginine administration there was a 15-minute spike of α-cell secretion with significant elevations of plasma glucagon (Fig. 2E and 2F and Fig. 3D and 3F; Figure 3 (26)) and GLP-1 (Fig. 5) that did not differ between nondiabetic and diabetic subjects (Table 7 (26)). There was no effect of

Table 2. Baseline glucose concentrations in nondiabetic and diabetic subjects

Glucose (mg/dL)									
	Drug		Treatment		Time		*P* values		
	Sitagliptin	Placebo	Ex-9	Saline	Arg1	Arg 2	Drug	Treatment	Time
Nondiabetic subjects	88.4 (84.7-92.1)	91.0 (87.4-94.7)	90.5 (86.9-94.2)	88.9 (85.2-92.6)	91.1 (87.4-94.8)	88.4 (84.7-92.0)	.047	.203	.037
Diabetic subjects	117.6 (100.1-138.2)	123.3 (104.9-144.8)	121.6 (103.5-142.9)	119.2 (101.5-140.0)	132.8 (109.1-150.6)	117.6 (96.2-132.8)	.049	.383	<.001

Data presented are from the baseline period prior to arginine stimulation. Data are reported as LS mean ± 95% CI. *P* values are from type 3 tests of fixed effects. Interaction terms are shown.

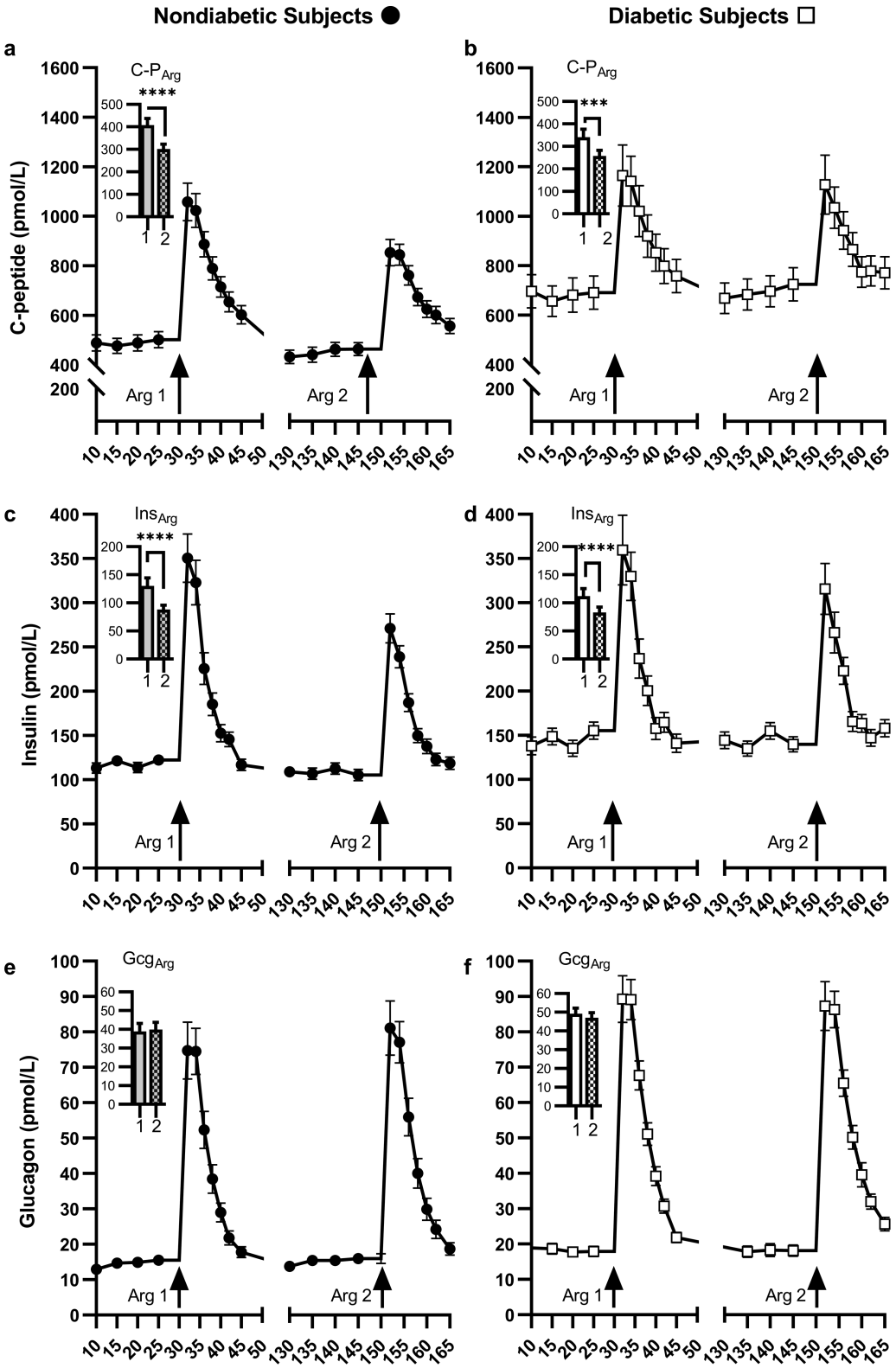

Figure 2. Effects of time on islet hormone secretion. Average C-peptide (A,B), insulin (C,D), and glucagon (E,F) concentrations for all conditions (drug and treatments combined) throughout the experiment for nondiabetic (A,C,E), and diabetic (B,D,F) subjects. Summed arginine responses are shown as inserts in graphs to show α- and β-cell hormone responses to the first and second arginine infusions. Arrows indicate time of arginine (5 g) infusion. Data are shown as mean ± SEM and compared with mixed model, $***P < .001$, $****P < .0001$.

either sitagliptin or Ex-9 on Gcg_{Arg} or GLP-1_{Arg} (Table 4). In contrast to the effect of time on β-cell secretion, α-cell secretion did not differ between the first and second arginine infusions in nondiabetic subjects; however, diabetic subjects had lower GLP-1_{Arg} following the second, relative to the first, arginine infusion (Fig. 2E and 2F and Table 4).

Table 3. Arginine-stimulated β-cell secretion

	Drug		Treatment		Time		*P* values			
	Sitagliptin	Placebo	Ex-9	Saline	Arg1	Arg 2	Drug	Treatment	Time	Drug* Treatment
Insulin$_{Arg}$ (pmol/L)										
Nondiabetic subjects	88.7	88.9	82.2	95.9	103.6	76.2	.975	.015	<.01	
	(67.9-116.0)	(68.0-116.3)	(62.9-107.5)	(73.4-125.4)	(79.2-135.4)	(58.3-99.6)				
Diabetic subjects	79.2	77.1	75.8	80.5	92.3	66.1	.811	.610	.006	
	(53.9-116.4)	(52.3-113.4)	(51.6-111.5)	(54.6-118.5)	(62.8-135.8)	(44.8-97.3)				
C-peptide$_{Arg}$ (pmol/L)										
Nondiabetic subjects	319.4	322.5	299.4	344.1	375.5	274.3	.840	.005	<.001	.004
	(266.6-382.7)	(269.1-386.4)	(249.9-358.7)	(287.1-412.3)	(313.4-450.0)	(228.9-328.7)				
Diabetic subjects	278.9	250.3	247.3	282.1	305.5	228.4	.062	.028	<.001	.020
	(215.2-361.4)	(193.0-324.5)	(190.8-320.5)	(217.6-366.0)	(235.7-396.1)	(176.1-296.3)				

Data are reported as least squares mean ± 95% CI. *P* values are from type 3 tests of fixed effects. Interaction terms are shown where significant interactions occurred. Drug*Treatment indicates interactions of these two variables.

Discussion

In the study described here we sought to determine whether signaling through the GLP-1r controls insulin release independent of intestinal secretion of GLP-1. To this end we assessed GLP-1r signaling in the fasting state, when gut derived plasma levels of GLP-1 were low and unchanging. In this setting GLP-1r antagonism with Ex-9 attenuated IV arginine-stimulated C-peptide secretion coincident with α-cell secretion of glucagon and GLP-1. This effect was comparable in diabetic and nondiabetic subjects, and involved an interaction with sitagliptin, although the nature of the drug by treatment interaction differed between the 2 groups. While the magnitude of suppression of C-P$_{Arg}$ by Ex-9 was 13% to 15%, the studies were conducted at basal glucose where the insulinotropic effects of GLP-1r activation are muted. These findings add to a growing body of evidence that challenge endocrine effects of GLP-1 as the sole mechanism for stimulating β-cells (3-5). Moreover, the demonstration that treatment with Ex-9 inhibits insulin release during α-cell but not L-cell stimulation suggests the islet as a source of GLP-1r ligands in humans. Taken in the context of recent preclinical studies (10-16), our results are consistent with a model of paracrine regulation whereby α-cell proglucagon peptides contribute to the β-cell response to acute stimulus through the GLP-1r.

We chose a potent but short-lived stimulus for insulin secretion to allow same-day comparisons of the insulin response with and without Ex-9. The use of C-peptide as well as insulin as a primary outcome mitigates any effects of differences in hepatic insulin clearance on the results. Arginine was selected because it gives a more reliable acute insulin response in people with diabetes than IV glucose and also because it stimulates α-cell secretion (28, 29). A group of subjects with well-controlled T2DM was recruited to enrich for people with retained β-cell function. Subjects were fasted for 10 to 12 hours to minimize nutrient stimulus of enteroendocrine L-cells that release GLP-1. Sitagliptin was given as an acute challenge and the time between drug administration and assessment of the first arginine bolus conforms with peak drug levels after an oral dose and the onset of pharmacologic effects (30). The effect of this single dose of sitagliptin on baseline glucose was similar to a previous study in which drug was administered for 3 days before study (31). To control for potential changes in the acute insulin response over a day of experiment, patients were allocated to experiments in a counterbalanced fashion, so half received Ex-9 and the other half saline as the first treatment on their 2 days of study. This control was important because in our subjects there was a significant impact of time of administration on the acute response of the β-cell to arginine, with the first arginine bolus consistently greater than to the second. However, our study protocol and statistical analysis allowed for separation of the impacts of DPP-4 inhibition, GLP-1r blockade, and repeated arginine stimulation on α-cell and β-cell secretion as well as the interplay of these factors.

The primary new observation in this study is the inhibition of stimulated β-cell secretion (C-P$_{Arg}$) by GLP-1r blockade in fasted subjects with low circulating concentrations of GLP-1. Over the course of the experiment total GLP-1 levels remained in the ~5 pmol/L range; the small increments in plasma GLP-1 after the arginine challenge were considerably lower than those demonstrated to stimulate insulin secretion in humans given

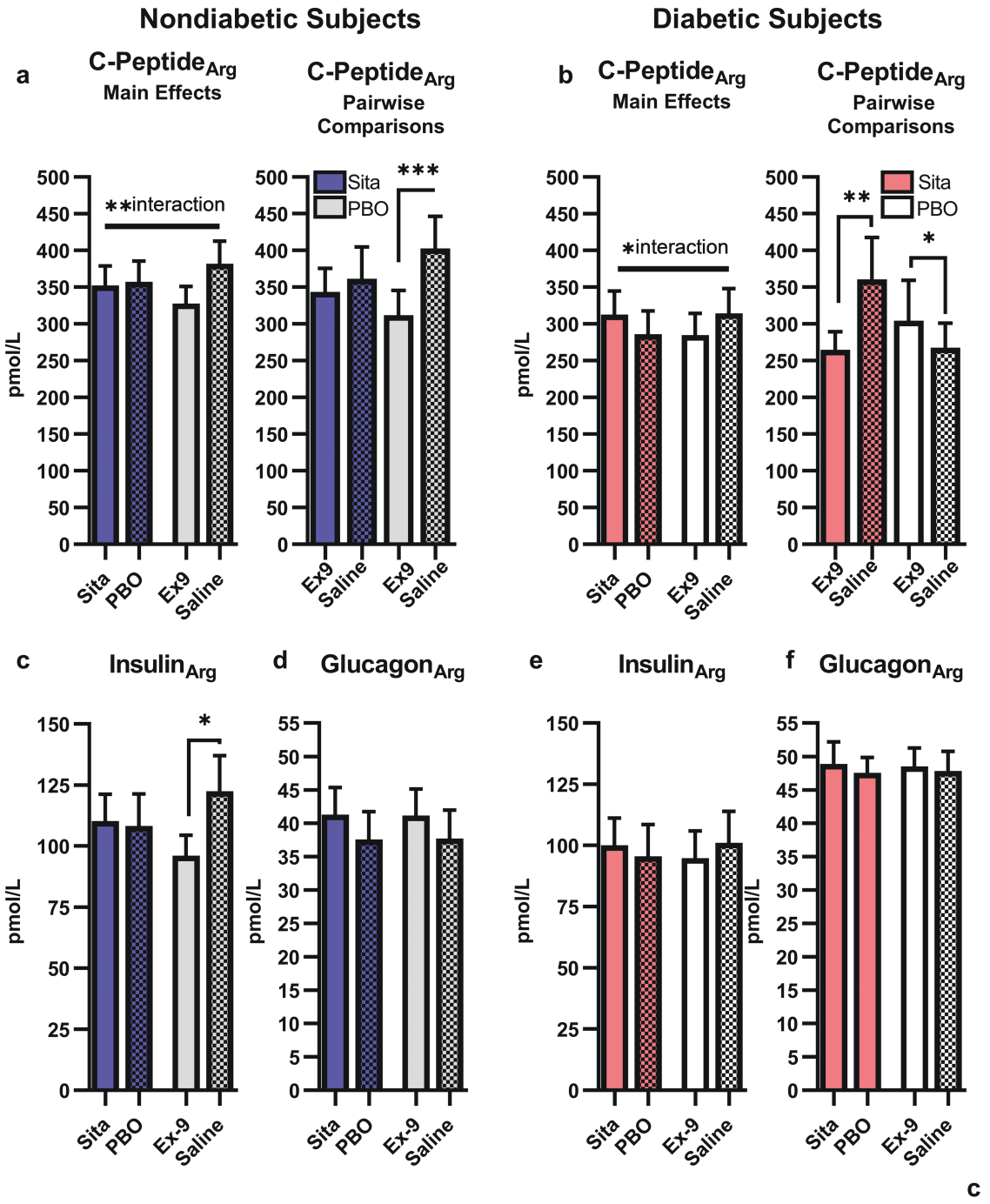

Figure 3. Effects of drug (sitagliptin/placebo) and treatment (Exendin-9/saline) on stimulated islet cell hormone release. Arginine-stimulated responses for C-peptide (A,B), insulin (C,E), and glucagon (D,F) for subjects without (A,C,D) and with (B,E,F) type 2 diabetes. Interactions between main effects of drug and treatment on C-peptide responses to arginine, and pairwise post hoc comparisons are shown. Data are presented as mean ± SEM and compared with a mixed model. $*P < .05$, $**P < .01$, $***P < .001$.

graded doses of IV GLP-1 (21, 22), and only 1 to 2 pmol/L greater than fasted levels. Previous papers have reported acute elevations of GLP-1 following arginine stimulation (32, 33), and although we measured total GLP-1, active GLP-1 has also been demonstrated to increase after a pulse of arginine in both diabetic and nondiabetic subjects (32). Given the temporal concordance with glucagon release, we presume the small increases of GLP-1 measured in our subjects was due to secretion by α-cells. This is consistent with previous work showing that arginine-stimulated GLP-1 accompanies other α-cell peptides (33), but not L-cell products such as PYY (31). And while stimulated levels of plasma glucagon and GLP-1 were relatively low, based on work with mouse islets it seems likely that intra-islet concentrations were much higher (13). Taken together, our findings suggest that the effects of GLP-1r antagonism in this study did not involve blockade of circulating GLP-1, and are more compatible with Ex-9 blocking GLP-1r ligands released locally in the islet to mediate β-cell secretion.

Ex-9 reduces the insulin response to hyperglycemia in persons with T2DM following ingestion of oral glucose or

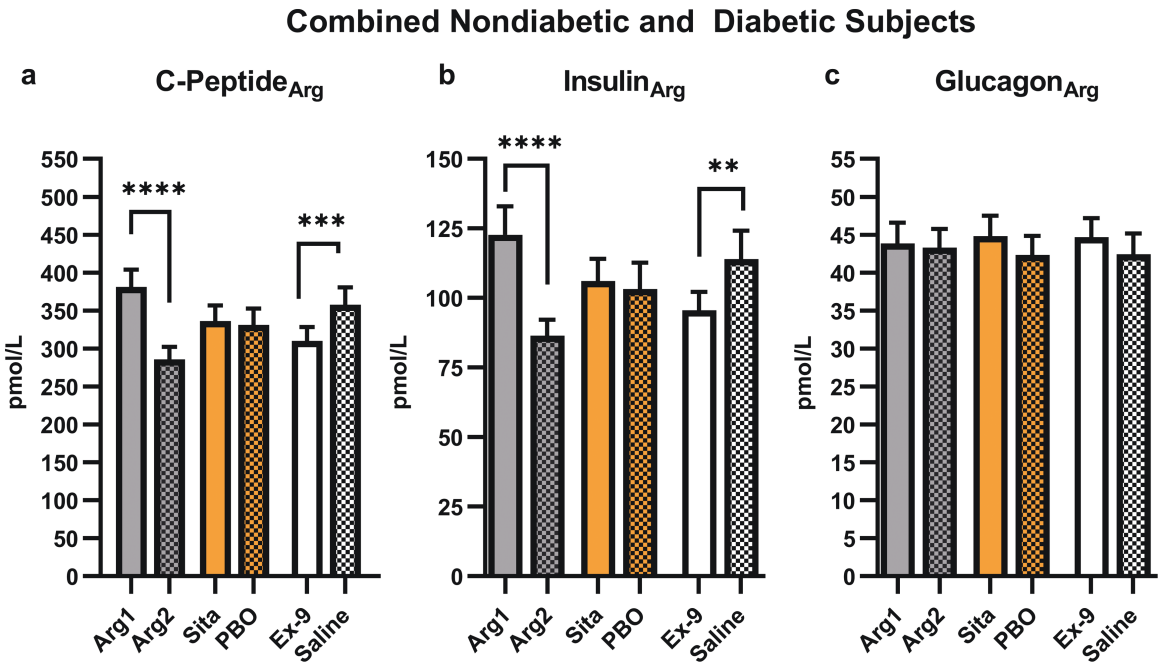

Figure 4. Secondary analysis of stimulated islet cell peptide responses to drug and treatment in the combined subjects with and without diabetes. Arginine-stimulated responses for C-peptide (A), insulin (B), and glucagon (C) in analysis where nondiabetic and diabetic subjects were combined. Data are shown as mean ± SEM and compared with a mixed model. $^{**}P < .01$, $^{***}P < .001$, $^{****}P < .0001$.

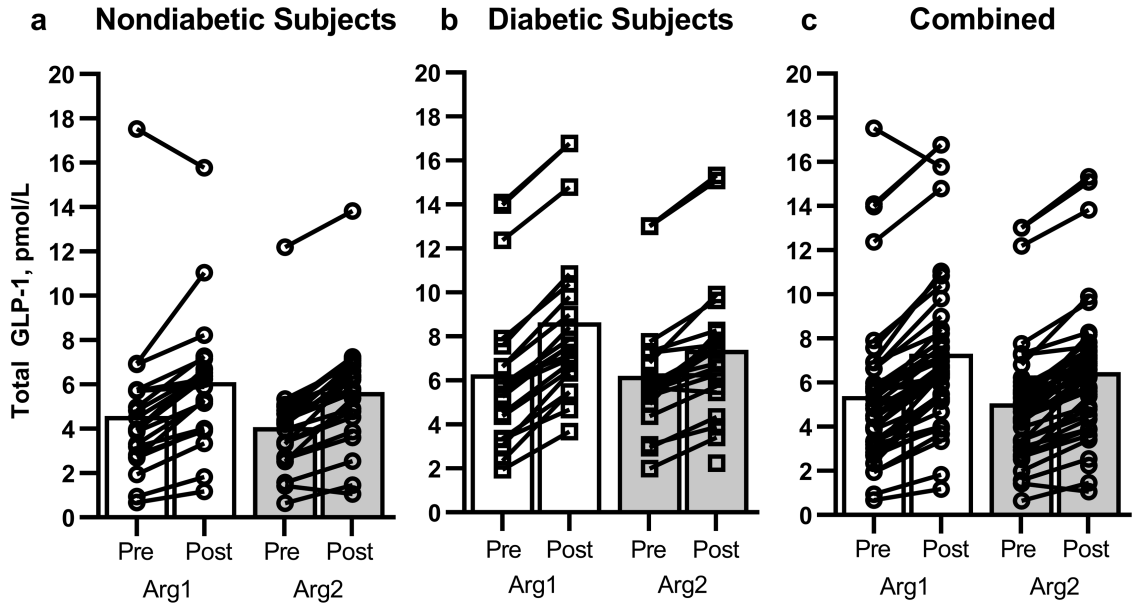

Figure 5. Arginine-stimulated plasma GLP-1 concentrations in subjects with and without diabetes and the groups combined. Average total GLP-1 concentrations before and after the first and second arginine infusions in nondiabetic (A), diabetic (B), and combined (C) subjects. Pre- and post-arginine values were compared using a paired t-test. $^{****}P < .0001$.

a mixed meal, settings in which gut secretion of GLP-1 is increased (31, 34). Our previous studies are in agreement with these in that we noted that Ex-9 reduced insulin secretion by ~30% during experimental hyperglycemia with mixed meal stimulation in diabetic, nondiabetic, and bariatric surgery subjects (7, 33). Based on the classic endocrine model of the incretin effect and the postprandial rise in GLP-1 in these studies (7, 31, 34, 35), these were the predicted outcomes. In contrast, our current results do not comport with entero-insular effects of GLP-1 because of the fasted status of our subjects, and the low circulating GLP-1 concentrations. Yet despite this important difference between the current and previous studies with Ex-9, we observed that acute GLP1r blockade reduced CP_{Arg} by ~ 15% suggesting a mechanism distinct from the classically described incretin effect. Moreover, we would note that the previous studies that used protein-containing meals (7, 34, 35) included both enteral and α-cell stimuli, raising the possibility that they induced stimulation by both local and systemic GLP-1r ligands. To date there has been no consideration of

Table 4. Arginine-stimulated α-cell secretion

	Drug		Treatment		Time		*P* values		
	Sitagliptin	Placebo	Ex-9	Saline	Arg1	Arg 2	Drug	Treatment	Arginine
Gcg_{Arg} (pmol/L)									
Nondiabetic subjects	41.3 (29.8-52.8)	37.6 (26.1-49.1)	41.1 (29.6-52.6)	37.7 (26.2-49.3)	39.1 (27.6-50.6)	39.8 (28.3-51.3)	.1749	.2146	.7991
Diabetic subjects	48.6 (41.0-56.1)	47.6 (40.0-55.1)	48.5 (41.0-56.1)	47.6 (40.0-55.2)	49.2 (41.7-56.7)	46.9 (39.4-54.5)	.6706	.697	.3539
$GLP\text{-}1_{Arg}$ (pmol/L)									
Nondiabetic subjects	1.6 (1.2-2.1)	1.3 (1.0-1.7)	1.4 (1.0-1.8)	1.6 (1.2-2.1)	1.4 (1.0-1.8)	1.6 (1.2-2.0)	.2277	.4099	.4634
Diabetic subjects	2.0 (1.3-3.1)	1.3 (0.8-1.9)	1.4 (0.9-2.1)	1.8 (1.2-2.8)	2.2 (1.5-3.5)	1.1 (0.7-1.8)	.0971	.3402	.0152

Data are reported as least squares mean ± 95% CI. *P* values are from type 3 tests of fixed effects.

the relative effects of endocrine and paracrine stimulation of β-cell GLP-1r. However, the results reported here add to longstanding proposals to revise the physiologic model of GLP-1 action (3).

Several investigators have reported evidence of DPP4 synthesis and action in animal and human islets (23-25), and the possibility has been raised that protection of GLP-1 in the islet may be part of the mechanism by which DPP4i have therapeutic effects in diabetes. We previously reported that 3 months of treatment with vildagliptin increased IV glucose–stimulated insulin secretion in fasted subjects with T2DM (36), consistent with an effect of DPP4 inhibition on β-cell function in the absence of acute stimulation by circulating GLP-1. In the present study, acute sitagliptin treatment increased fasting C-peptide and lowered blood glucose in diabetic and nondiabetic subjects. However, while these results indicate some of the expected drug effects, there was no independent effect of sitagliptin on arginine-stimulated β-cell secretion in our cohorts, although there was a trend for this in the T2DM subjects. However, the interactions of sitagliptin and Ex-9 on CP_{Arg} during the conditions of our study raise the possibility that islet DPP4 activity has a role in β-cell regulation. From the results here we cannot explain why a greater effect of Ex-9 was observed in the absence of sitagliptin in the nondiabetic group and the presence of sitagliptin in the T2DM subjects; one interesting possibility is differences in α-cell function in people with and without diabetes. A larger study would be necessary to discern what may be distinct drug by treatment effects in diabetic and nondiabetic people.

In these studies, repeated arginine administration elicited a significant ~25% decrease in the β-cell response ($C\text{-}P_{Arg}$ and Ins_{Arg}) during the second arginine bolus. While 2 groups previously reported that 3 or 4 pulses of arginine given over 90 minutes caused equivalent insulin responses (37, 38), there is also a description of arginine-stimulated insulin secretion decreasing over time with fasting (39). Our findings on $C\text{-}P_{Arg}$ and Ins_{Arg} are more in keeping with this latter finding and may be due to several additional hours of fasting in our study compared to early experiments (37, 38). While the significant effect of time to reduce the second arginine stimulus for β-cell secretion would be expected to obscure the action of drug or treatment, we were still able to identify specific effects; these actions might be more apparent with a different study design that mitigated the time effect. Indeed, in a cross-sectional study of the first arginine study, we observed similar trends in nondiabetic subjects, though we did not have enough power to detect differences in diabetic subjects. In contrast to the decline in acute β-cell responses over the course of the morning experiment, we noted similar glucagon responses with the serial arginine infusions in both groups, consistent with earlier studies (36), and demonstrating an interesting difference between α- and β-cell responses.

There are several shortcomings to this study that warrant comment. Due to dropouts our sample of T2DM subjects was less than optimal and several results in the separate analysis of this group were ambiguous. A lack of power may explain the absence of a significant effect of Ex-9 on Ins_{Arg}, the 1 discrepant outcome of our arginine tests. Nonetheless, the diabetic cohort had results in the primary analysis that were directionally comparable with the nondiabetic group and these similarities were apparent in the combined analysis. Our diabetic subjects were fairly homogeneous in glycemic parameters and so do not permit any insight into the possible effects of more advanced disease on our results. Moreover, since both groups were studied at euglycemic or only slightly higher blood glucose levels, the impact of GLP-1r signaling and the effect of Ex-9 were likely muted since these are glucose dependent. Finally, effects of sitagliptin on arginine-stimulated β-cell secretion with and without Ex-9 may have been greater with longer exposure to the drug.

In summary, blockade of the GLP-1r inhibits arginine-stimulated insulin secretion in fasted nondiabetic and diabetic subjects. This effect is apparent at basal glycemia and low plasma GLP-1, and coincident with stimulated α-cell secretion. Our findings corroborate previous incidental observations in humans (7, 8), and are consistent with new evidence from animal studies for local islet regulation of β-cell function by α-cells. Taken together, they indicate that even in the fasting state with low circulating GLP-1, β-cell GLP-1r are activated when α-cells are activated. We propose that this represents α-to-β-cell communication, an explanation that will require further experiments to validate. However, the results presented here add to a revised model of GLP-1 physiology that has emerged over the recent past, moving beyond

a simple endocrine mechanism of action to other modes of β-cell regulation.

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Disclosures

D.A.D. consults for Eli Lilly and Sun Pharmaceuticals. S.M.G., A.L.H., R.K., and C.A.S. have nothing to disclose.

Data Availability

Some or all datasets generated during and/or analyzed during the current study are not publicly available but are available from the corresponding author on reasonable request.

Clinical Trial Information

ClinicalTrials.gov Identifier: NCT02683187. Registered Feb 11, 2016.

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