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# Incretin and islet hormonal responses to fat and protein ingestion in healthy men

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**Carr RD, Larsen MO, Winzell MS, Jelic K, Lindgren O, Deacon CF, Ahrén B.** Incretin and islet hormonal responses to fat and protein ingestion in healthy men. *Am J Physiol Endocrinol Metab* 295: E779–E784, 2008. First published July 2, 2008; doi:10.1152/ajpendo.90233.2008.—Glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP) regulate islet function after carbohydrate ingestion. Whether incretin hormones are of importance for islet function after ingestion of noncarbohydrate macronutrients is not known. This study therefore examined integrated incretin and islet hormone responses to ingestion of pure fat (oleic acid; 0.88 g/kg) or protein (milk and egg protein; 2 g/kg) over 5 h in healthy men, aged 20–25 yr ($n = 12$); plain water ingestion served as control. Both intact (active) and total GLP-1 and GIP levels were determined as was plasma activity of dipeptidyl peptidase-4 (DPP-4). Following water ingestion, glucose, insulin, glucagon, GLP-1, and GIP levels and DPP-4 activity were stable during the 5-h study period. Both fat and protein ingestion increased insulin, glucagon, GIP, and GLP-1 levels without affecting glucose levels or DPP-4 activity. The GLP-1 responses were similar after protein and fat, whereas the early (30 min) GIP response was higher after protein than after fat ingestion ($P < 0.001$). This was associated with sevenfold higher insulin and glucagon responses compared with fat ingestion (both $P < 0.001$). After protein, the early GIP, but not GLP-1, responses correlated to insulin ($r^2 = 0.86$; $P = 0.0001$) but not glucagon responses. In contrast, after fat ingestion, GLP-1 and GIP did not correlate to islet hormones. We conclude that, whereas protein and fat release both incretin and islet hormones, the early GIP secretion after protein ingestion may be of primary importance to islet hormone secretion.

insulin; glucagon; glucagon-like peptide-1; glucose-dependent insulinotropic polypeptide; incretins; man

THE INTEGRATED ENDOCRINE RESPONSES TO FOOD INGESTION are dependent on both the size and the composition of a meal and include the postprandial release of the incretin hormones glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP) and the islet hormones insulin and glucagon (3, 5, 21, 32). Most studies have focused on responses to an oral glucose tolerance test, after which levels of GIP, GLP-1, and insulin rise, whereas glucagon levels are suppressed (4, 18, 20, 24). It is also known that fat and protein ingestion stimulate GLP-1 and GIP secretion (10, 14, 20, 27). Less is known, however, regarding relationships between the incretin responses and changes in insulin and glucagon levels after meal or noncarbohydrate macronutrient ingestions.

GLP-1 and GIP are rapidly degraded by dipeptidyl peptidase-4 (DPP-4), which cleaves the two $NH_2$-terminal amino acids of the peptides, making them largely inactive (9). Accurate estimation of the relationship between incretin hormone secretion and islet hormones therefore requires measurement of both the total and the active intact forms of the two incretins. How this is related to macronutrient ingestion is not known. Indeed, we recently showed in mice that protein ingestion increased intact incretin hormone levels compared with carbohydrate ingestion, and this was associated with reduced intestinal DPP-4 activity (17).

The aim of this study was to examine whether the incretin hormones contribute to changes in islet hormone secretion after noncarbohydrate macronutrient ingestion in humans. To that end, we investigated the relationship between incretins (both the active and total concentration of the two incretin hormones) and the islet hormones throughout a 5-h period after ingestion of pure fat or pure protein as noncarbohydrate macronutrients.

## MATERIALS AND METHODS

*Subjects.* Twelve healthy males being 20–25 yr old [mean age 22.0 ± 1.8 (SD) yr] were included in the study. They were all nonobese [body mass index (BMI) 20–25 kg/m$^2$, mean BMI 22.3 ± 1.2 kg/m$^2$] with normal fasting glucose (4.6 ± 0.2 mmol/l), no personal or family history of diabetes or gastrointestinal disease, and they were not taking any medication. They were recruited through advertisements in Lund, Sweden. All subjects underwent an oral glucose tolerance test (2 g/kg glucose) to exclude glucose intolerance (fasting glucose was 4.6 ± 0.2 mmol/l, range 3.7–5.4 mmol/l; 2-h glucose value was 5.2 ± 0.2 mmol/l; range 4.1–6.6 mmol/l). The study was approved by the ethics committee of Lund University, Sweden, and all subjects gave written informed consent before entrance into the study.

*Study protocol.* On three occasions, separated by at least four, and maximally eight, weeks overnight-fasted subjects were provided with an antecubital vein catheter. After two baseline samples at −5 and −2 min, oral isocaloric (560 kcal), isovolemic (400 ml) loads of *1*) oleic acid (0.88 g/kg body wt; water was ingested after the oil to reach the volume of 400 ml; Casa Oilio Sperlonga, Priverna, Italy); *2*) protein dissolved in water [Promax protein 85$^R$ (Global, Rødovre, Denmark); 2 g/kg body wt, consisting of milk and egg protein; 4.4% carbohydrate and 2% fat] or; *3*) pure water (400 ml) were ingested within 5 min. At 30 min before all tests, paracetamol (1 g; GlaxoSmithKline, Mölndal, Sweden) was administered for an indirect determination of gastric emptying; this method has shown good correlation when validated against the tracer techniques (22). The tests were undertaken in a

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randomized fashion. Blood samples were taken throughout a 300-min study period.

*Analyses.* Blood samples, collected in chilled tubes containing EDTA (7.4 mmol/l; final concn) and aprotinin (500 kallikrein inhibitor units/ml blood; Novo Nordisk, Bagsvaerd, Denmark), were immediately centrifuged at 4°C, and plasma was frozen at −20°C until analysis. Insulin and glucagon were analyzed with double-antibody radioimmunoassay (Linco Research, St. Charles, MO). Free fatty acids (FFAs) and triglycerides (Wako Chemicals, Neuss, Germany) and paracetamol (Cambridge Life Science, Ely, Cambridgeshire, UK) were analyzed by colorimetric assays. Blood samples for determination of intact and total GLP-1 and GIP were collected into chilled tubes containing EDTA and aprotinin as above, with addition of diprotin A (0.1 mmol/l final concn; Bachem, Bubendorf, Switzerland). Plasma was separated and stored at −20°C until analysis. Intact GLP-1 was determined by an $NH_2$-terminal specific assay using guinea pig anti-GLP-1 and $^{125}$I-labeled GLP-1 (Linco). Total GLP-1 was determined using the COOH-terminally directed antiserum 89390 (29). Total GIP concentrations were measured using the COOH-terminally directed antiserum R65, and intact GIP was measured using antiserum 98171, which is specific for the intact $NH_2$-terminus of GIP (8). DPP-4 activity was assessed kinetically using Gly-Pro-*p*-nitroaniline (1 mmol/l) as substrate (17).

*Statistics.* Means ± SE are shown, unless otherwise noted. Areas under curves (AUC) were calculated by the trapezoidal rule for the suprabasal responses of insulin, glucagon, intact and total GLP-1, and GIP for the early (0–30 min) and late (30–300 min) time period, whereas AUC for paracetamol was calculated for the 0- to 120-min time period. ANOVA with Tukey's post hoc test was used for tests of significance between variables obtained during ingestion of fat, protein, and water. Pearson's product-moment correlation coefficients were obtained to estimate linear or quadratic correlation. A Spearman correlation was performed between the early glucagon and early intact GIP responses and between the early insulin and early intact GIP responses.

## RESULTS

*Glucose, insulin, and glucagon responses to fat, protein, and water challenge.* Fasting glucose levels were 4.6 ± 0.2 mmol/l, and glucose levels did not change significantly during any of the tests. Fasting insulin levels were 55 ± 3 pmol/l. Insulin levels were unaltered after water ingestion, whereas they increased after fat and protein ingestion. The increased plasma insulin concentrations were seen between 30 and 240 min after fat ingestion ($P = 0.031$ vs. water) and between 15 and 240 min after protein ingestion ($P = 0.018$ vs. water). When compared with water ingestion, fat and protein ingestion both significantly increased early and late insulin responses (Table 1). These responses were more pronounced after protein than after fat ingestion ($P < 0.001$ for all). Fasting glucagon levels were 65 ± 3.7 ng/l. Glucagon levels were unaltered after water ingestion. In contrast, glucagon levels were increased by both fat and protein ingestion, with significant elevations from *minute 120* and onward after fat ingestion ($P = 0.019$ vs. water) and from *minute 30* and onward after protein ingestion ($P = 0.005$ vs. water). The late glucagon response was increased by fat ingestion, whereas, after protein ingestion, both early and late responses were significantly increased. As for insulin, early and late glucagon responses were higher after protein ingestion than after fat ingestion (both $P < 0.001$; Fig. 1).

*FFA and triglyceride responses to fat, protein, and water challenge.* Fasting FFA levels were 0.34 ± 0.04 mmol/l. Water or fat ingestion did not change plasma FFA levels, whereas they were markedly reduced by protein ingestion from *minute 60* and onward ($P < 0.001$). Fasting triglyceride levels were 0.61 ± 0.05 mmol/l and were unaffected by water or protein ingestion. In contrast, triglyceride levels increased following fat ingestion from *minute 60* and onward ($P = 0.012$; Fig. 1).

Table 1. *Early (0–30 min) and late (40–300 min) insulin, glucagon intact and total GLP-1, and intact and total GIP responses to water, fat, or protein ingestion in healthy volunteers*

| | Early | Late |
|---|---|---|
| Insulin response, nmol·l$^{-1}$·30 or 270 min | | |
| Water | −0.09±0.09 | −5.3±1.7 |
| Fat | 0.5±0.2* | 3.7±0.9* |
| Protein | 3.4±0.8† | 18.7±1.6‡ |
| Glucagon response, μg·l$^{-1}$·30 or 270 min | | |
| Water | 0.25±0.07 | −0.27±0.21 |
| Fat | 0.18±0.06§ | 6.3±1.2† |
| Protein | 1.2±0.2‡ | 22.1±1.8‡ |
| Intact GLP-1 response, nmol·l$^{-1}$·30 or 270 min | | |
| Water | −0.05±0.06 | −0.46±0.19 |
| Fat | 0.043±0.007† | 2.1±0.26‡ |
| Protein | 0.11±0.01‡ | 1.76±0.28‡ |
| Total GLP-1 response, nmol·l$^{-1}$·30 or 270 min | | |
| Water | 0.05±0.2 | −0.18±1.1 |
| Fat | 0.17±0.04* | 8.62±1.01‡ |
| Protein | 0.23±0.07* | 7.15±1.12† |
| Intact GIP response, nmol·l$^{-1}$·30 or 270 min | | |
| Water | −0.07±0.04 | −0.69±0.48 |
| Fat | 0.13±0.05† | 4.40±0.78‡ |
| Protein | 0.53±0.07‡ | 5.22±1.01† |
| Total GIP response, nmol·l$^{-1}$·30 or 270 min | | |
| Water | 0.04±0.04 | −0.54±0.59 |
| Fat | 0.39±0.06† | 17.2±1.89† |
| Protein | 1.72±0.27† | 16.7±1.98* |

Values are means ± SE; $n = 12$ men in each group. GLP-1, glucagon like peptide-1; GIP, glucose-dependent insulinotropic polypeptide. *$P < 0.05$, †$P < 0.01$, and ‡$P < 0.001$, probability level of random difference after ingestion of fat or protein vs. water ingestion. §Not significant, $P = 0.66$.

*Paracetamol responses.* The 120-min $AUC_{paracetamol}$ was 12.0 ± 1.4 mmol·l$^{-1}$·120 min$^{-1}$ after water ingestion. It was not significantly affected by protein ingestion (11.4 ± 1.1 mmol·l$^{-1}$·120 min$^{-1}$) but lower after fat ingestion (8.6 ± 0.8 mmol·l$^{-1}$·120 min$^{-1}$; $P = 0.010$).

*GLP-1 and GIP responses to fat, protein, and water challenge.* Fasting levels of intact and total GLP-1 were 3.9 ± 1.4 and 14.8 ± 1.4 pmol/l, respectively, and fasting levels of intact and total GIP were 15.1 ± 1.1 and 16.6 ± 4.3 pmol/l, respectively. Water ingestion did not change these levels, whereas intact and total GLP-1 and GIP levels were proportionally increased after fat and protein ingestion ($P < 0.001$ for both vs. water). With regard to the intact and total GLP-1 levels, the increases were similar after fat and protein ingestion. In regard to GIP, early (0–30 min) intact and total GIP responses were significantly higher after protein than after fat ingestion ($P < 0.001$ for both). In contrast, the late intact and total GIP responses were similar after protein and fat ingestion (Fig. 2 and Table 1).

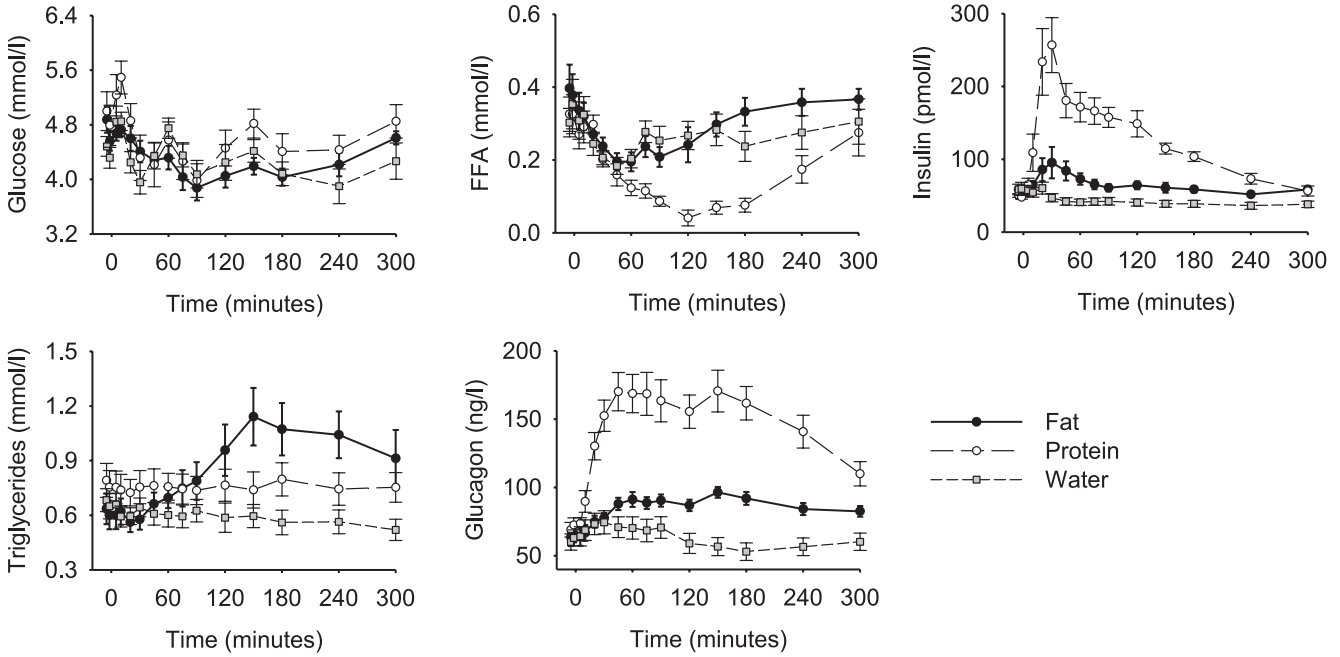


Fig. 1. Plasma levels of glucose, insulin, glucagon, free fatty acid (FFA), and triglycerides before and during 300 min after ingestion of fat, protein, or water in healthy male volunteers ($n$ = 12). Means ± SE are shown.

*DPP-4 activity.* Fasting plasma DPP-4 activity was 555 ± 33 mmol·min$^{-1}$·mg protein$^{-1}$. Plasma DPP-4 activity was not significantly changed after water, fat, and protein ingestion throughout the 5-h study period (Fig. 2).

*Correlation between incretin and islet hormone responses.* The early insulin responses to protein ingestion correlated to the early intact GIP response (Fig. 3). The relationship between early GIP and insulin responses was a quadratic regression ($r^2$ = 0.86; $P < 0.0001$), whereas the correlation between the early GIP and glucagon responses was not significant by Spearman analysis ($r^2$ = 0.32) (data not shown). No such correlations were seen for GLP-1 responses and insulin or glucagon responses.

## DISCUSSION

The term "incretin" refers to a gut hormone that is released by oral glucose and that potentiates glucose-stimulated insulin secretion (6). However, the term may be more general and apply to a gut factor stimulating the endocrine pancreas, even

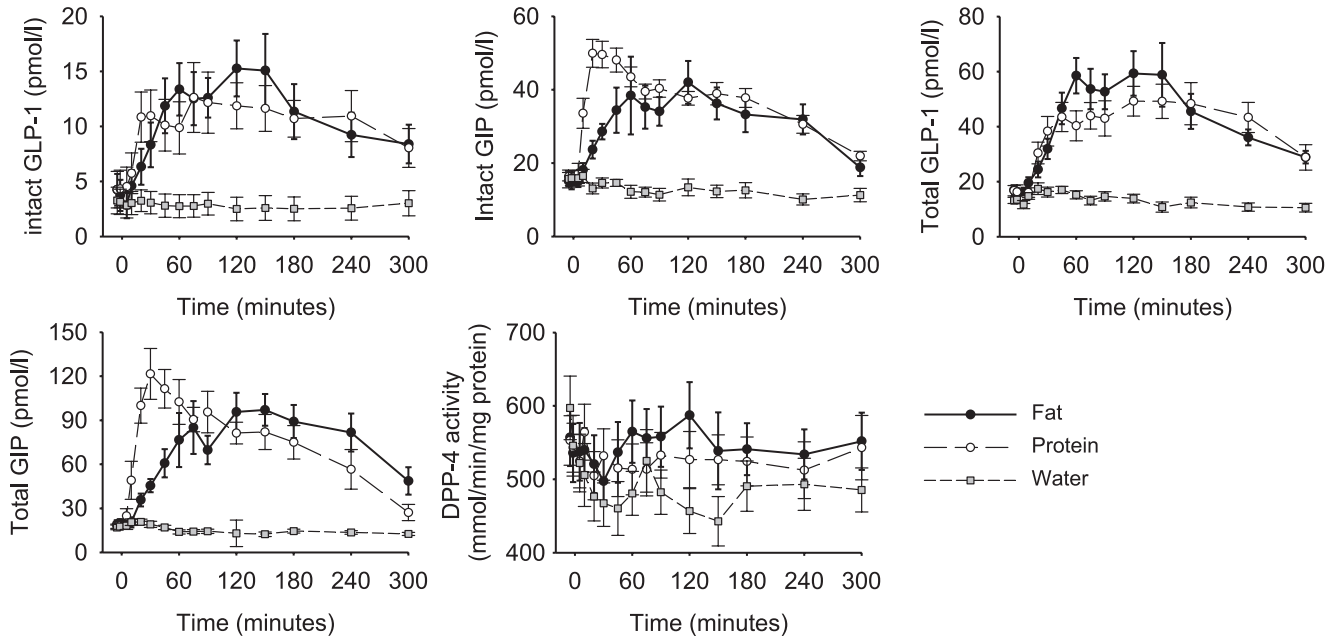


Fig. 2. Plasma levels of intact and total glucagon-like peptide-1 (GLP-1) and intact and total glucose-dependent insulinotropic polypeptide (GIP) before and during 300 min after ingestion of fat, protein, or water in healthy male volunteers ($n$ = 12). Means ± SE are shown.

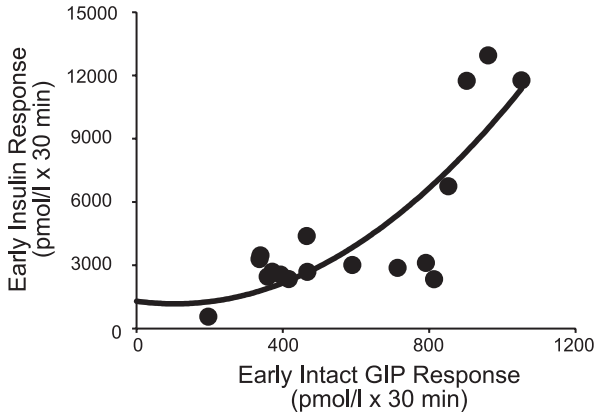


Fig. 3. Quadratic regression between early (30 min) increase in intact GIP vs. increase in insulin following ingestion of protein in healthy male volunteers ($n = 12$).

in the absence of a primary stimulation by glucose. Such stimulation may broaden the concept of regulation by incretins of islet function. The aim of the study was to examine the relationship between incretin and islet hormones after ingestion of noncarbohydrate macronutrients.

Several methodological precautions were employed in this study in an attempt to clarify interpretation of emergent data. First, water ingestion served as control for changes due to gastric distension alone and to time-dependent changes during the study period. Second, glycemia did not change after fat or protein ingestion, which allowed evaluation of glucose-independent actions of the incretin-islet axis, although a limitation of the present study was that we determined venous glucose levels only. Therefore, we cannot exclude a change in arterial glycemia. Third, blood sampling was continued for 5 h for comparison of long-term effects, i.e., much longer than in earlier studies (14, 27). For most of the parameters measured, use of this 5-h observation period enabled us to observe the entire endocrine response to macronutrient ingestion. Fourth, both intact and total levels of GIP and GLP-1 were determined. This is of importance considering that the total levels of the incretins reflect the secretion of the hormones, whereas the intact levels reflect the active form of GLP-1 and GIP (8).

We also determined plasma DPP-4 activity, since this enzyme is of relevance for the physiology of the incretin hormones (10). A noteworthy observation from this study is, therefore, that the plasma DPP-4 activity did not change during the 5-h studies following either water, protein, or fat challenge. The fact that plasma DPP-4 activity is stable over a long period in humans is an important finding considering the importance of DPP-4 as a regulator of incretin physiology, islet function and glucose tolerance, and its central role in development of novel therapeutics for diabetes (7). In a previous study in mice, we demonstrated differences in the GLP-1 response after ingestion of protein vs. fat, in that protein ingestion was associated with reduced intestinal DPP-4 activity and augmented responses of active GLP-1, whereas the plasma DPP-4 activity was unaltered (17). Whether similar differences exist in humans remains to be studied, since we determined plasma and not intestinal DPP-4 activity in this study.

Following fat or protein ingestion, GLP-1 and GIP were released, as evidenced by the increased total levels of GLP-1 and GIP. It has previously been shown that GLP-1 is released by both fat and protein, whereas for GIP, fat but not protein ingestion has been thought to be a strong stimulus for secretion (4, 14, 20, 24, 27). However, we now show that protein also has the ability to markedly stimulate GIP release. We cannot exclude that low levels of nonprotein components of the protein load [fat (2%) and carbohydrate (4.4%)] contributed to the GIP secretion, although this is unlikely to fully explain the large early GIP secretion seen after protein ingestion. Nevertheless, our results confirm a previous report that intraduodenal administration of amino acids stimulates GIP secretion in humans (30). We found that stimulation of GIP and GLP-1 secretion by fat and protein was a long-term event, since the plasma levels were still elevated after 5 h. When comparing the effect of fat vs. protein ingestion, it is evident that the two macronutrients had a similar effect on GLP-1 secretion. However, in regard to GIP secretion, a noteworthy difference was observed in that protein ingestion elicited a much higher early (0–30 min) response than fat ingestion, whereas the late responses were similar after the two macronutrients. Hence, protein elicits a more rapid GIP response than fat ingestion, whereas the same rapidity exists for the two macronutrients on GLP-1 secretion. The lower early GIP response to fat vs. protein ingestion may be partially explained by a reduced gastric emptying by fat ingestion, as determined indirectly by the paracetamol test. The same time patterns in the responses of total vs. intact GLP-1 and total vs. intact GIP responses were seen after both fat and protein ingestion. This is consistent with the finding that DPP-4 activity did not change.

The current study is observational; therefore, no clear mechanisms of the observed effects are established. Therefore, further studies are required to establish the mechanisms of the release of incretin hormones after macronutrient ingestion. However, the mechanism of release of GIP and GLP-1 after nutrient ingestion has been a matter of discussion. Most studies favor that a major component is the nutrients from the luminal side via engagement of luminal nutrient receptors (10, 11, 12, 20). This would explain the faster increase in GIP than in GLP-1 after protein ingestion, because GIP-producing K cells are in general localized more proximally in the gut than GLP-1-producing L cells. In fact, the proximal small intestine has been shown to be the major site for release of GIP in humans (31). However, additional mechanisms may contribute, because GIP secretion was more rapid after protein ingestion, whereas, after fat ingestion, there was no obvious dissociation in time pattern between GIP and GLP-1 secretion. Of interest, immunohistochemical evidence suggests that GIP and GLP-1 are colocalized in some cells in the human gut (25), which, presumably, allows for parallel secretion of the two hormones. Another point may be that GIP, released from the proximal cells, reaches the more distally located L cells via the circulation to stimulate the release of GLP-1. However, although this has been observed in rats (11), there is no indication of such a mechanism in humans (16). A third possibility is that neural factors, activated by oral macronutrient ingestion, stimulate the secretion of both GIP and GLP-1 (11).

An important aspect of this study was the differential temporal relationship between the incretins and the islet hormones after fat and protein ingestion. After fat ingestion, insulin levels were increased, with a peak during the first 30–60 min. This was a larger increase in insulin than in previous studies (15, 19)

and would perhaps be explained by lipids, which both directly and indirectly stimulate insulin secretion (28, 32). However, there was no increase in circulating FFA after fat ingestion, and triglyceride levels increased after 60 min, which was a later time point than the rapid insulin release. This suggests that mechanisms other than direct stimulation of insulin secretion by lipids underlie total insulin secretion, such as a contribution from the incretin hormones. However, the time pattern in the responses differed, since circulating insulin was raised almost immediately after fat ingestion, whereas the circulating incretin levels only began to rise after 30–60 min and lasted for up to 5 h, which is beyond the time of the insulin response. Furthermore, there was no correlation between incretin and islet hormones after fat ingestion. The fact that incretin levels reached their maximal levels and remained elevated after the timing of the insulin response to fat and protein calls into question their primary role as incretin hormones under these conditions, although counterregulatory responses could contribute at the later time points. Furthermore, besides activating insulin secretion through a direct action on β-cells, incretins may also stimulate insulin secretion through a neural effect via vagal efferent fibers innervating the pancreas (2, 26). Hence, other gut hormones or neural factors affecting insulin release after macronutrient ingestion need to be considered and explored in more detail.

Glucagon secretion was increased by fat ingestion. This novel finding suggests that lipids stimulate glucagon secretion. In fact, glucagon levels after fat ingestion remained elevated over a longer time period than insulin levels. This would support a role for the incretins in maintenance of euglycemia in the presence of non-carbohydrate-mediated insulin release. The large increase in GIP during the later time point may be involved in this effect, since GIP has been shown to stimulate glucagon secretion (23). GLP-1 may be involved as a modulatory factor, however, since GLP-1 inhibits glucagon secretion (23).

Insulin and glucagon secretion were increased by protein in association with a profound reduction in plasma FFA levels, which reflects the antilipolytic action of insulin. GIP and GLP-1 levels were also increased by protein, suggesting that the incretins might contribute to the islet response to fat and protein ingestion. This is corroborated by the tight correlation between the early increase in intact GIP levels and the early increase in insulin. However, this correlation, although of interest, does not establish a casual relationship and requires further examination. A conclusion that the raised GIP may contribute to the raised insulin after protein ingestion is, nevertheless, supported by in vitro studies demonstrating that GIP augments amino acid-stimulated insulin secretion (16). Although GIP has been reported to contribute to the glucagon response to oral protein (23), no such correlation between these two processes was observed in the present study. Again, modulatory effects of GLP-1 on glucagon secretion are limited to inhibitory effects if indeed they exist at normoglycemia (13). However, there might exist a negative feedback loop whereby the raised glucagon stimulates GLP-1 secretion; such a hypothesis needs to be studied in more detail.

In summary, this study on the relationships between GIP and GLP-1 responses and those of insulin and glucagon following fat and protein ingestion in humans has shown that *1*) fat and protein ingestion stimulate insulin, glucagon, GIP, and GLP-1 secretion independent from changes in glucose or lipids, *2*) the early GIP response to protein ingestion is more pronounced than the early GIP response to fat ingestion, whereas GLP-1 responses are similar after fat and protein ingestion, *3*) the release of GIP, but not that of GLP-1, correlates with the release of insulin and glucagon after protein (but not after fat) ingestion, and *4*) plasma DPP-4 activity is unchanged after fat or protein ingestion. Based on these findings, we conclude that the relationship between the plasma levels of GIP, GLP-1, and islet hormone secretion is complex following macronutrient ingestion, and, therefore, a primary role of GLP-1 and GIP as mediators of insulin release is questionable after pure fat and protein ingestion.

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