Effects of Euglycemic Hyperinsulinemia and Lipid Infusion on Circulating Cholecystokinin

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Aims: Functions of the gut hormone cholecystokinin (CCK) include an important role in the regulation of gastric emptying, postprandial glucose homeostasis, and postmeal satiety. Postprandial CCK responses are significantly blunted in type 2 diabetic patients by unknown mechanisms. We hypothesized that hyperinsulinemia and lipid infusion influence circulating levels of biologically active CCK.

Methods: Eleven healthy subjects were studied in a cross-over design after 10-h overnight fasts, using euglycemic-hyperinsulinemic clamps for 443 min, with an additional infusion of lipid-heparin (1.25 ml·min^{-1}) or saline (1.25 ml·min^{-1}) for the last 300 min after constant plasma glucose levels were achieved.

Results: Euglycemic-hyperinsulinemia resulted in a sustained, up to 5-fold increase of plasma CCK ($P < 0.001$). When adding lipid infusion instead of saline, CCK concentrations rapidly declined and returned to baseline levels ($CCK_{300\ min}$ 1.1 ± 0.2 *vs.* 3.3 ± 0.3 pmol/liter, $P < 0.001$). Partial intraclass correlation showed an independent correlation of plasma CCK with free fatty acids ($r_{ic} = -0.377$, $P < 0.001$) but not with serum insulin ($r_{ic} = 0.077$, $P = 0.32$). Whole-body insulin sensitivity decreased in lipid-exposed subjects (M value 7.1 ± 0.7 *vs.* 5.6 ± 0.9 mg·kg·min^{-1}, $P = 0.017$) but was not independently correlated with CCK ($r_{ic} = 0.040$, $P = 0.61$).

Conclusions: We report novel findings showing that circulating CCK markedly increased in the euglycemic-hyperinsulinemic state, possibly as a result of near-complete suppression of circulating free fatty acids. Moreover, raising blood lipids even moderately by lipid infusion rapidly and significantly interfered with this effect, suggesting that a negative feedback mechanism of blood lipids on circulating CCK might exist. **(*J Clin Endocrinol Metab* 93: 2328–2333, 2008)**

Patients with type 2 diabetes have lower postprandial plasma levels of cholecystokinin (CCK) (1, 2). Even though CCK was one of the first gut hormones discovered, interactions among CCK and various hormones, neuropeptides, and circulating factors are just beginning to be identified (3), partly explained by the lack of sensitive and specific assays until recently (4). CCK is a peptide hormone that is released from diverse tissues, including endocrine I cells in the small intestine, enteric nerves, and the central nervous system. CCK exerts various physiological effects, which include an important function in the regulation of meal termination and satiety by interacting through CCK-1 (CCK-A) receptors in the hindbrain, by inhibiting the expression of orexigenic peptides in the hypothalamus and preventing the stimulation of specialized neurons by ghrelin (3). CCK-1 receptor antagonists increase energy intake, whereas exogenous administration of CCK decreases the size of spontaneously ingested

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Abbreviations: AUC, Area under the curve; CCK, cholecystokinin; EHC, euglycemic hyperinsulinemic clamp; FFA, free fatty acid; M-value, insulin-mediated glucose uptake; r_{ic}, intraclass correlation; TG, triacylglycerol.

meals (5, 6). Administration of CCK, in physiological concentrations, also inhibits proximal gastric motility while increasing contractions in the antrum and pylorus, thereby delaying gastric emptying, slowing the delivery of glucose to the duodenum, and reducing postprandial hyperglycemia (7). CCK-8 infusion also exerts an antidiabetic action particularly in type 2 diabetic patients by increasing insulin secretion after a meal, most likely exerted through a direct islet effect (8). Further functions include the regulation of gallbladder contraction and meal-stimulated pancreatic enzyme secretion as well as functions in the regulation of anxiety, sexual behavior, sleep, memory, and intestinal inflammation (3). In man, mainly CCK-58 and also CCK-22, CCK-33, and CCK-8 are the biologically active forms in plasma (9, 10).

Food, particularly the ingestion of fat- and protein-rich food, is the most important known physiological stimulus to release CCK (9), resulting in an up to 5-fold postprandial increase in circulating CCK. However, there is paucity of knowledge regarding the mechanisms and hormonal factors that regulate CCK. It has been suggested that high plasma triacylglycerol (TG) levels lead to reduced gall bladder motility due to decreased sensitivity to CCK (11). Moreover, postprandial CCK responses have been reported to be significantly reduced in patients with type 2 diabetes (1, 2), which might have unfavorable effects on meal termination, satiety, and postprandial glucose responses in these subjects. Importantly, potential mechanisms leading to blunted CCK responses in diabetic patients are unknown (2, 3). In the present study, we hypothesized that euglycemic-hyperinsulinemia and lipid infusion could influence circulating levels of biologically active CCK.

Subjects and Methods

Study participants

The experimental protocol was approved by the local ethical committee. All participants gave written informed consent before starting the study. Eleven healthy subjects (five males, six females, aged 52.3 ± 4.2 yr, body mass index 23.8 ± 1.1 kg/m^{-2}) participated in this cross-over study and were investigated twice between August 2006 and September 2007. Normal fasting glucose and normal glucose tolerance were ensured before the study, using a 75-g oral glucose tolerance test. Exclusion criteria were menstrual irregularities, a history of smoking, or current intake of any medication. Fertile female subjects were studied in the early follicular phase of the menstrual cycle. Subjects were instructed to maintain normal physical activity for 3 d before all study days.

Study design/euglycemic hyperinsulinemic clamps (EHCs)

The design of the study is shown in (Fig. 1A). Subjects arrived at the metabolic unit between 0715 and 0800 h after 10-h overnight fasts. To reduce variance, all subjects consumed standardized liquid meals at 2200 h in the evening before all study days (two portions Biosorp; Pfrimmer Nutricia, Erlangen, Germany). Consumption of any other meals or drinks apart from tap water was not allowed within the last 12 h before the studies. After arrival, two iv catheters were inserted into contralateral forearm veins. The arm at which blood samples were drawn was placed into a heating box (65 C) throughout the studies.

After administration of an insulin bolus at −10 min (individually adjusted according to the body surface area of the participants), EHCs were performed at a constant insulin infusion rate of 40 mU·kg^{-1}·min^{-1}, as previously detailed (12). In brief, EHCs without lipid infusion was performed for at least 2 h until stable plasma glucose levels were achieved (Fig. 1A). Stable plasma glucose levels were assumed as soon as plasma glucose was kept constant for at least 30 min within the range of 4.4 ± 0.4 mmol/liter. Whole-body glucose disposal [expressed as insulin-mediated glucose uptake (M-value)] was calculated from the glucose infusion rate, which was constant during the last 30 min of the respective clamp periods. Thereafter, EHCs were continued for a further 300 min, and plasma free fatty acids (FFAs) and TG concentrations were raised using a constant lipid-heparin infusion (Deltalipid LCT 20%; Deltaselect, Pfullingen, Germany; 1.25 ml·min^{-1}; contents in 1.000 ml: soybean oil 200 g, glycerol 25.9 g, egg phospholipids 12 g, oleate 0.3 g; supplemented with Heparin-Natrium-25000-ratiopharm; Merckle, Blaubeuren, Germany; 0.4 IU kg^{-1}·min^{-1}) (Fig. 1A). The lipid solution was ordered in one batch and stored under identical conditions. For the control experiments, all subjects were reinvited and investigated using an identical protocol, apart from infusing saline (saline 0.9%; Fresenius Kabi, Bad Homburg, Germany; 1.25 ml·min^{-1}) instead of lipid-heparin (Fig. 1A). The time period until stable plasma glucose levels were reached was not different between the EHC-lipid and the EHC-saline experiments (143.6 ± 8.6 *vs.* 141.4 ± 8.2 min, $P = 0.80$). Plasma glucose was clamped at 4.4 mol/liter throughout all clamps. Blood samples were drawn at timed intervals from −30 to +300 min, immediately chilled, and centrifuged, and the supernatants were stored at −80 C until analysis.

Blood sampling and analyses

Plasma CCK was measured every 30 min, serum insulin and plasma FFAs were measured every 60 min, plasma glucose was measured every 5–10 min, and TG was measured at −30, +143, and +443 min.

For the measurements of CCK, blood was collected in EDTA tubes containing 200 kIU aprotinin (Bayer, Leverkusen, Germany) per milliliter of whole blood. Plasma CCK was determined by a sensitive and specific RIA, as prescribed previously (13). In brief, the antibody (CH40IX) was raised in rabbits specifically directed to the biologically active site of CCK, including the sulfated tyrosyl residue at position 7 from the C-terminal end. It shows no cross-reactivity with biologically inactive unsulfated CCK-8 and unsulfated gastrin-17/-34. The cross-reactivity to sulfated gastrin-17 is less than 1%. Plasma samples were subjected to an ethanol extraction procedure. CCK octapeptide was labeled with ^{125}I served as a tracer (PerkinElmer, Boston, MA). Standard curves prepared with sulfated CCK-8 showed a mean ID 50 (concentration that inhibits specific binding by 50%) of 2.7 ± 0.22 pmol/liter (n = 27). The mean minimal detectable concentration of CCK in extracted plasma samples was 0.3 ± 0.1 pmol/liter. The coefficients of variation for the intraassay variation were between 5.6% (0.7 pmol/liter) and 7.2% (15.1 pmol/liter). The coefficients of variation for interassay variation were between 12.3% (0.85 pmol/liter) and 15% (14.8 pmol/liter). Results for plasma CCK concentrations are given after calculating the recovery rate. Interference of lipid enriched plasma with the accuracy of the CCK assay was excluded. Circulating concentrations of serum insulin, plasma glucose, plasma FFAs, and plasma TG were measured as described previously (12).

Statistical analyses

Results are presented as means ± SE. Total area under the curve (AUC) was calculated using the trapezoid method. Data were compared using repeated-measures ANOVA with treatment and time as within-subject factors and the Huynh-Feldt-ε procedure as correction factor, which corrects for a positive biased F test for within-subject factors (14). General linear models were used to test the simultaneous dependence of variables on plasma CCK concentrations; partial intraclass correlations (r_{ic}) were calculated between CCK as the dependent variable and plasma FFAs, serum insulin, plasma glucose, and insulin sensitivity (M-value) as the independent variables and with simultaneous adjustment for lipid-heparin *vs.* saline treatment, time of insulin exposure, plasma FFAs, serum insulin, and plasma glucose levels and M-values. Pearson corre-

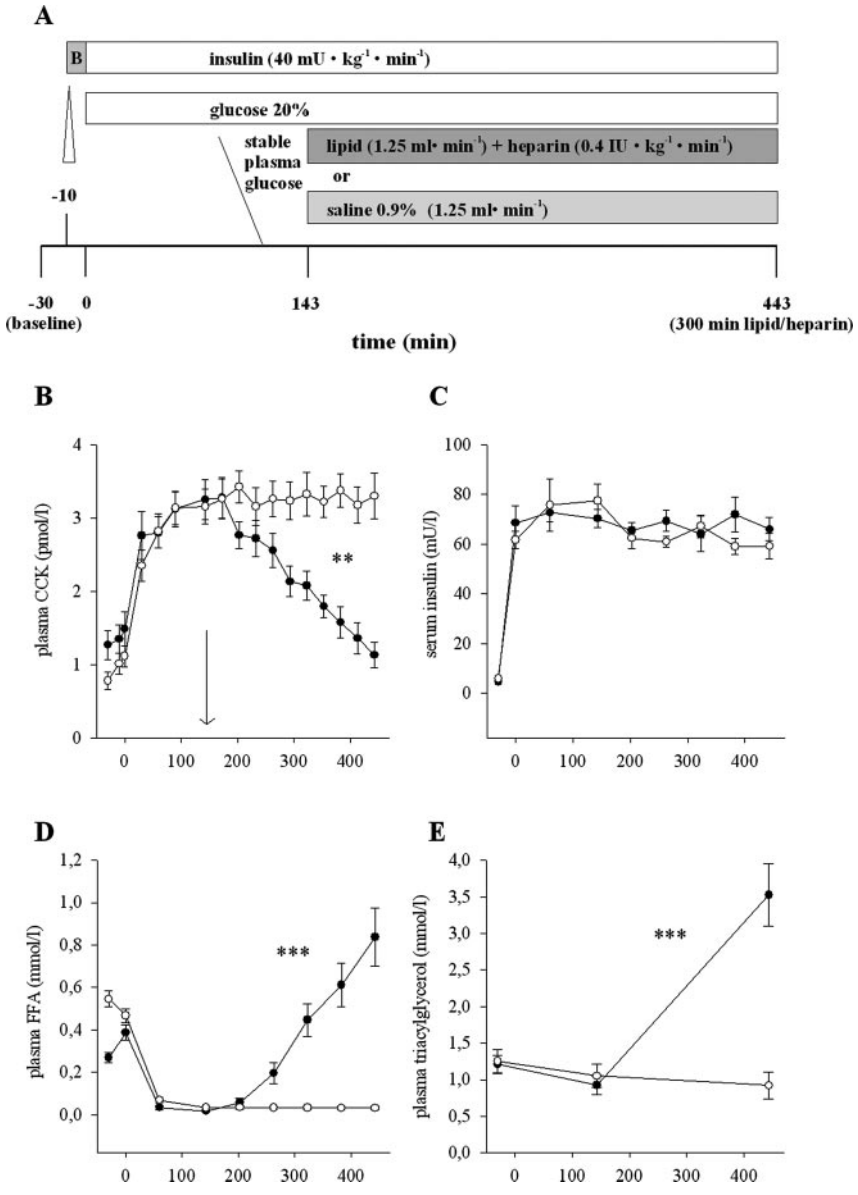

FIG. 1. A, Study design. After the administration of an insulin bolus at −10 min (B, *arrowhead*), EHCs were performed for at least 2 h, until stable plasma glucose levels were ensured. Thereafter (mean 143 ± 6 min), a constant infusion of lipid-heparin or saline was administered for a further 300 min. Arterialized plasma glucose was adjusted at 4.4 ± 0.4 mmol/liter throughout the lipid-heparin or saline infusion periods. Circulating concentrations of plasma CCK (B), serum insulin (C), plasma FFAs (D), and plasma TGs (E) during the EHCs with lipid-heparin (*black circles*) or saline (*white circles*) infusion (*arrow*, both 1.25 ml·min^{-1} for 300 min). Values are means ± SE for 11 subjects in each group. **, $P < 0.01$ *vs.* EHC-saline; ***, $P < 0.001$ *vs.* EHC-saline.

lation coefficients were further used to examine the relationships between variables. For the analysis of Pearson correlations, all time points from both study days were included in one correlation matrix. AUCs were compared using the two-tailed Student's *t* test for paired analysis. Statistical significance was defined as $P < 0.05$. Calculations were performed using SPSS version 14 (SPSS, Chicago, IL).

Results

CCK responses during EHCs, EHC-lipid, and EHC-saline experiments

CCK concentrations significantly increased after the start of the insulin bolus ($CCK_{-30\ min}$ *vs.* $CCK_{-10\ min}$, $P = 0.017$, Fig. 1B). The observed up to 5-fold increase in circulating CCK during euglycemic-hyperinsulinemia and before the start of lipid-heparin or saline was very similar at both study days and not significantly different between the two study days ($P = 0.34$, Fig. 1B).

When adding lipid infusion after stable plasma glucose levels were ensured, a rapid onset and significant decline of circulating CCK was observed, and baseline concentrations were reached after 270 min of lipid infusion. Importantly, lipid infusion did not lead to a further significant reduction of plasma CCK concentrations below baseline levels (CCK_{-30min} *vs.* $CCK_{443\ min}$, $P = 0.58$), even though plasma triglycerides and FFA levels markedly increased during the lipid infusion period.

$AUC_{143\ min}$ (measured from 0 min to +143 min) for CCK was not significantly different between the EHC-lipid and the EHC-saline experiments (405 ± 29 *vs.* 386 ± 27 pmol/liter·min; $P = 0.13$). However, after lipid-heparin or saline was added, $AUC_{143-443\ min}$ (measured from +143 to +443 min) for CCK was significantly different between groups (674 ± 41 *vs.* 980 ± 67 pmol/liter·min; $P = 0.002$).

Repeated-measures ANOVA showed a significant effect of the time to insulin exposure ($P = 0.006$), the lipid-heparin *vs.* saline treatment ($P < 0.001$), and a treatment-*vs.*-time interaction ($P < 0.001$) on circulating CCK levels, thereby excluding a time-dependent decline of CCK levels independent of the lipid treatment.

Insulin sensitivity

Whole-body insulin sensitivity expressed as M-value significantly decreased in lipid-exposed subjects, as expected (M-value 7.1 ± 0.7 for the period from 113 to 143 min *vs.* 5.6 ± 0.9 mg·kg·min^{-1} for the period from 413 to 443 min, $P = 0.017$) but not during the EHC-saline clamps (M-value 6.7 ± 0.8 for the period from 113 to 143 min *vs.* 6.9 ± 0.6 mg·kg·min^{-1} for the period from 413 to 443 min, $P = 0.66$). However, differences in M-values between lipid- and saline-exposed subjects failed to reach significance level ($P = 0.09$), which is most likely explained by the relatively low number of cases.

Other parameters

Plasma glucose concentrations were clamped at 4.4 ± 0.4 mmol/liter throughout the clamps. There was no difference between the lipid and saline treatment for serum insulin (treatment-*vs.*-time interaction, $P = 0.34$, Fig. 1C) and plasma glucose ($P =$

0.21) concentrations. Lipid infusion resulted in significantly increased concentrations of plasma FFAs (Fig. 1D; treatment-*vs.*-time interaction, $P = 0.003$) and plasma TG (Fig. 1E; treatment-*vs.*-time interaction, $P < 0.001$; $TG_{443\ min}$ *vs.* $TG_{-30\ min}$, $P < 0.001$) levels. $AUC_{473\ min\text{-}lipid}$ *vs.* $AUC_{473\ min\text{-}saline}$ for both FFA and TG concentrations were significantly different between treatments ($P < 0.001$).

Correlation analysis

When calculating Pearson correlation coefficients for all time points and both study days, a negative correlation of plasma CCK concentrations with plasma FFA ($r = -0.68$, $P < 0.0001$) and a positive correlation with serum insulin ($r = 0.38$, $P < 0.0001$) concentrations were detected. The correlation of CCK with insulin was driven mainly by the strong positive correlation of CCK with insulin during the early period of the clamps (−30 to +143 min), before the infusion of lipid-heparin or saline was started; lipid-heparin clamps: $r = 0.53$, $P < 0.0005$; saline clamps: $r = 0.61$, $P < 0.0001$). However, no significant correlations were observed between CCK and insulin after the start of the lipid-heparin or saline infusions. Moreover, partial intraclass correlations (r_{ic}) showed that only plasma FFA concentrations ($r_{ic} = -0.377$, $P < 0.001$) but not serum insulin concentrations ($r_{ic} = 0.077$, $P = 0.32$) were independently correlated with plasma CCK levels, also suggesting that the observed changes in circulating CCK were more likely to have been indirectly caused by insulin-induced suppression of FFAs rather than by a direct stimulatory effect of insulin on CCK levels. Circulating CCK was not independently correlated with insulin sensitivity expressed as M-value ($r_{ic} = 0.040$, $P = 0.61$). The adjusted R^2 for the total model was 0.753.

Accuracy of the CCK assay under hyperlipidemic conditions

Adding lipid solution (Deltalipid LCT 20%; Deltaselect) at molar concentrations of 0, 4, 8, and 16 mmol/liter to human plasma samples resulted in measured TG concentrations of 1.9, 3.8, 4.9, 8.2, and 13.4 mmol/liter. Corresponding measured CCK concentrations were 2.5, 2.3, 2.4, 2.0, and 1.8 pmol/liter, and corrected values after calculating the recovery rate were 3.1, 2.8, 3.0, 2.5, and 2.3 pmol/liter. Therefore, TG concentrations as observed *in vivo* in the present study were unlikely to relevantly interfere with the measured concentrations of CCK.

Discussion

The health consequences of obesity include insulin resistance, hyperlipidemia, and type 2 diabetes as well as gallbladder dysmotility and gallbladder stones. Several studies indicate that postprandially released CCK could play an important role in this context by being involved in the regulation of meal termination and satiety in humans as well as influencing gastric emptying, postprandial glucose homeostasis, and gallbladder contraction (3).

In the present study, we report novel findings showing that euglycemic-hyperinsulinemia in healthy humans resulted in a significant and sustained, up to 5-fold increase of circulating levels of biologically active CCK. The magnitude of this increase was comparable with the strongest known physiological stimulus of CCK, which is observed postprandially after the consumption of a fat- or protein-rich meal (15). Moreover, raising blood lipids within the range seen in obese subjects postprandially after a high-fat meal (16) completely abolished this effect, with circulating CCK concentrations returning to baseline levels within 270 min.

A further novel finding of the present study was that the increase of circulating CCK during the euglycemic-hyperinsulinemic-saline clamps was sustained over the observation period of more than 7 h, possibly because of near-complete suppression of circulating FFAs. Of interest, postprandially after a high-fat meal, both an increase of blood lipids (16) and a gradual decline of CCK to basal levels (9) is well established. Otherwise, endogenously derived FFAs are nearly completely suppressed by euglycemic hyperinsulinemia (17), as was also observed in the present study. These data suggest that a negative feedback mechanism might exist with raised blood lipids exerting a CCK suppressive effect in the later postprandial state. Both hyperinsulinemia due to insulin resistance and disturbances in lipid homeostasis are common findings in diabetic subjects. Notably, blood lipid levels within the range observed in the present study are frequently observed in insulin-resistant subjects particularly in the postprandial state due to inappropriately high hepatic production of very low density lipoproteins and reduced lipoprotein lipase activity (18, 19). Therefore, the observed rapid-onset interference of lipid infusion with circulating CCK levels in the present study could provide a potential explanation for the observed reduced postprandial CCK response in diabetic patients (1, 2). This hypothesis is further supported by the finding that TG-lowering therapy with fibrates of patients with marked hypertriglyceridemia improves gall bladder dysmotility, associated with a significant increase in postprandial CCK levels (11).

Another potentially involved mechanism leading to a decline of circulating CCK concentrations in lipid-exposed subjects might be related to lipid-induced insulin resistance. However, even though insulin sensitivity significantly decreased in lipid-exposed subjects, as expected (20), no independent association of insulin sensitivity with CCK concentrations was observed, and differences in insulin sensitivity after 300 min lipid-heparin *vs.* saline exposure failed to reach significance level. Finally, and most importantly, the rapid onset (60 min) of a significant decline in circulating CCK levels during the euglycemic-hyperinsulinemic-lipid clamps suggests that lipid-induced insulin resistance was unlikely to be the lead mechanism, considering that acute increases of plasma FFAs cause insulin resistance with a 3- to 4-h delay (21).

Several studies reported that, by unknown mechanisms, postprandial CCK responses to a standard meal are significantly lower in patients with type 2 diabetes, in comparison with healthy controls (1, 2). Reduced gallbladder emptying and disturbances of gastric motility in long-standing diabetes are also well known, even in patients with no clinical evidence

for autonomic neuropathy (2). One previous study reported delayed gastric emptying of a meal in healthy men exposed to euglycemic-hyperinsulinemia *vs.* the infusion of saline, without significantly affecting circulating CCK levels (22). However, authors also reported no increase of postprandial CCK levels on the consumption of a meal with 26% fat and 10% protein content, which is in conflict with other reports (9, 15), indicating that measured CCK concentrations need to be interpreted with caution. Lipid infusion did not affect basal plasma CCK and gallbladder volume in another study (23). This is in agreement with results from the present study because we also did not observe a further reduction of plasma CCK below baseline levels during lipid infusion. In another study that investigated effects of insulin on basal and cholecystokinin-stimulated gallbladder motility in humans, no significant changes in plasma CCK levels were observed during a basal clamp period of 120 min in healthy subjects (24). This finding is in conflict with the results of the present study, and it can only be hypothesized that differences in the used assays or differences in the preparation of CCK plasma samples might have led to diverse findings. Notably, in the present study CCK concentrations markedly increased in the euglycemic-hyperinsulinemic state, whereas even moderate elevations of plasma lipids significantly interfered with this effect. Therefore, both increased insulin levels in insulin-resistant states and elevated blood lipids could result in fluctuations of CCK, which might unfavorably affect gastric emptying and glucose homeostasis in patients with type 2 diabetes. Furthermore, interference of circulating insulin via the suppression of blood lipids with CCK could contribute to the known centrally satiating effects of insulin (25).

The exact mechanisms of how the regulation of CCK, insulin, and blood lipids are linked at a molecular level need to be investigated in future studies. Oral ingestion of lipid rich meals potently increases CCK secretion (9). Furthermore, FFAs have been shown to directly increase CCK secretion *in vitro* through the activation of the FFA receptor GPR120 (26). Therefore, direct inhibitory effects of circulating FFAs on CCK secretion appear unlikely. However, circulating FFAs might indirectly interfere with CCK secretion by increasing the release of factors that have been shown to inhibit CCK secretion, such as pancreatic proteases and bile acids (27). In addition, raised plasma FFAs might interact with factors that increase the degradation of circulating CCK. Repeating similar studies in obese and type 2 diabetic patients might be also of interest but face the problem that substantially higher insulin doses will be needed in these subjects, resulting in circulating insulin concentrations that clearly exceed physiologically observed postprandial insulin levels.

In summary, we report novel findings showing that plasma FFAs were independently and inversely associated with plasma CCK concentrations, suggesting that a negative feedback mechanism of blood lipids on circulating CCK might exist. Our data also suggest that pharmacological and nutritional measures aimed at reducing blood lipid levels might have the potential to restore postprandial CCK responses and thus beneficially affect the known disturbances of postmeal satiety, gastric emptying, glucose homeostasis, and gallbladder motility in type 2 diabetic patients.

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