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RESEARCH

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Ulrik Ø Andersen[1,2], Dijana Terzic[1,2], Nicolai Jacob Wewer Albrechtsen[1,2,3], Peter Dall Mark[1,2], Peter Plomgaard[1,4], Jens F Rehfeld[1], Finn Gustafsson[5] and Jens P Goetze[1,2]

[1]Department of Clinical Biochemistry, Rigshospitalet, Copenhagen, Denmark
[2]Institute of Biomedical Sciences, Faculty of Health and Medical Sciences, University of Copenhagen, Copenhagen, Denmark
[3]Novo Nordisk Foundation Centre for Protein Research, Faculty of Health and Medical Sciences, University of Copenhagen, Copenhagen, Denmark
[4]Department of Clinical Medicine, Faculty of Health and Medical Sciences, University of Copenhagen, Copenhagen, Denmark
[5]Department of Cardiology, Rigshospitalet, Copenhagen, Denmark

Correspondence should be addressed to J P Goetze: JPG@dadlnet.dk

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Abstract

Aims: Neprilysin degrades natriuretic peptides in circulation and is also suggested to degrade the gut hormones gastrin and cholecystokinin. Neprilysin inhibition has become a therapeutic strategy and thus a regimen in need of further testing in terms of other hormonal axes besides natriuretic peptides. The aim of this study was to examine whether acute inhibition of neprilysin affects meal-induced responses in gastrin and cholecystokinin concentrations in healthy individuals.
Methods and results: Nine healthy young men were included in an open-labelled, randomized cross-over clinical trial. The participants received a standardized meal (25 g fat, 26 g protein, 42 g carbohydrate) on two separate days with or without a one-time dosage of sacubitril ((194 mg)/valsartan (206 mg)). Blood pressure, heart rate and blood samples were measured and collected during the experiment. Statistical differences between groups were assessed using area under the curve together with an ANOVA with a Bonferroni *post hoc* test. Sacubitril/valsartan increased the postprandial plasma concentrations of both gastrin and cholecystokinin (80% ($AUC_{0\text{-}270\ min}$, $P = 0.004$) and 60% ($AUC_{0\text{-}270\ min}$, $P = 0.003$), respectively) compared with the control meal. No significant hemodynamic effects were noted (blood pressure, $AUC_{0\text{-}270\ min}$, $P = 0.86$, heart rate, $AUC_{0\text{-}270\ min}$, $P = 0.96$).
Conclusion: Our study demonstrates that sacubitril/valsartan increases the postprandial plasma concentrations of gastrin and cholecystokinin in healthy individuals. The results thus suggest that neprilysin-mediated degradation of gastrin and cholecystokinin is physiologically relevant and may have a role in heart failure patients treated with sacubitril/valsartan.

Introduction

Chronic heart failure is treated by inhibition of the sympathetic and the renin-angiotensin-aldosterone system (RAAS). More recently, sacubitril/valsartan has been introduced as treatment for patients with chronic heart failure. The drug is composed of valsartan, an angiotensin-II receptor 1 blocker, and sacubitril, which inhibits the peptide-degrading enzyme neprilysin (neutral endopeptidase 24.11) (1). Neprilysin degrades a plethora of peptide hormones, including atrial (ANP) and B-type (BNP) natriuretic peptides (2). Increased plasma

concentrations of natriuretic peptides counteract the detrimental circulatory effects of heart failure, as they improve cardiac pre- and afterload by enhancing renal excretion of sodium and water, and also via vasodilatory effects. Importantly, it has also been shown that sacubitril/valsartan reduces the mortality for patients with chronic heart failure (3).

Several other hormones, for instance glucagon-like peptide-1 (GLP-1) and substance P, are known substrates of neprilysin (1, 4). So far, only indirect experiments have suggested that neprilysin degrades gastrin and cholecystokinin (CCK) (5, 6, 7, 8, 9, 10). These hormones are secreted in parallel with GLP-1 from the endocrine gut after food intake. Gastrin regulates gastric acid secretion and growth of the fundic mucosa (11), whereas CCK regulates gallbladder contraction, satiety, and pancreatic exocrine and endocrine secretion (11, 12). Patients with chronic heart failure are now treated with sacubitril/valsartan on a long-term basis, and it is therefore important to fully acknowledge the effects on all gut hormones. We thus examined the possible effects of acute neprilysin inhibition on the postprandial plasma concentrations of gastrin and CCK in healthy individuals.

Methods

The study was designed as an open-labelled, randomized trial and performed at the Department of Clinical Biochemistry (Rigshospitalet, University of Copenhagen). The Ethical Committee of The Capital Region of Denmark approved the protocol (H-18000360) and the study conformed to the Declaration of Helsinki. Before entering the trial, all subjects were informed in both oral and written form of the study protocol and the potential risks. At a consultation, height and weight were measured, and blood was collected as a general health check. Based on the results of the consultation, the subjects were either included or excluded. Eligible subjects were young, lean, and healthy males, all Caucasian. The inclusion and exclusion criteria are shown in Table 1. The study protocol and patient characteristic has been reported previously (13).

Study design

The subjects were enrolled for two study days separated by a 2-week washout period. The entire study was carried out over a period of 3 months. The subjects were initially randomized to receive either sacubitril/valsartan or no treatment on the first day of examination. Regardless of randomization, subjects fasted overnight (10 h) and the tests were performed the following morning. The subjects were placed in a supine position and a peripheral venous catheter was inserted into the cubital vein. On the day of treatment, two tablets of sacubitril/valsartan, 97 mg/103 mg (C09DX04) (total dose of 194 mg sacubitril/206 mg valsartan) were administrated orally (t=0 min). On both days of examination, a standardized meal (a Big Mac burger containing 42 g carbohydrate, 25 g fat, and 26 g protein) (Big Mac, McDonalds; https://www.mcdonalds.com/dk/da-dk/product/big-mac.html) was ingested (t=30 min). The meal was consumed within 5 min on both trial days. The dose for sacubitril/valsartan was chosen from the suggested doses for patients with heart failure; the time between the consumption of the drug and the beginning of meal intake was chosen on the basis of the given information for peak concentration of sacubitrilat in plasma (1–2 h).

During each trial day, a total of 12 blood samples per subject were collected in 6 mL ethylene diamine tetra-acetate, and 4 mL lithium-heparin tubes at −30, 0, 15, 30, 45, 60, 75, 90, 120, 150, 210, and 270 min; see Fig. 1. The tubes were immediately centrifuged for 10 min at 2000 g at 4°C, and the plasma was transferred to tubes (Cryopure, ref. 72.379; Sarstedt, Nümbrecht, Germany) and false

Table 1 Criteria of inclusion and exclusion.

Inclusion criteria	Exclusion criteria
Healthy	Drugs consumption
Male	Weight loss cure
Aged 18–30 years	Donation of blood the past 2 weeks
BMI between 20 and 25 kg/m^2	Daily medicine
Non-smoker	Normal blood sample results at the first consultation
No use of daily medicine	Smoker
	Weekly alcohol intake above 21 units
	Pre-blood samples showing sign of disease

Table 1 list the in- and exclusion criteria of this study.
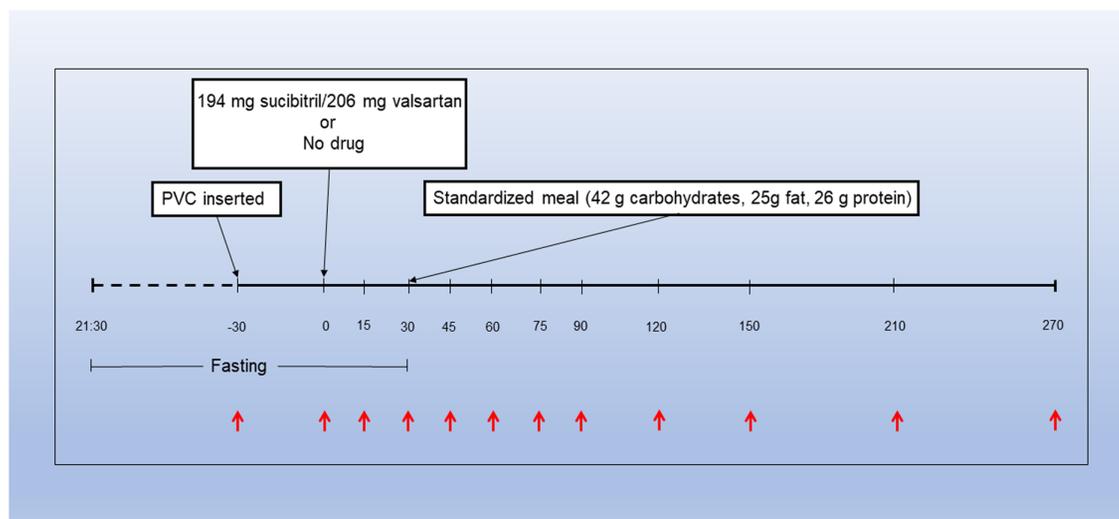
Figure 1

The subjects were fasting from 21:30 h the day before. A periphery venous catheter (PVC) was inserted, and baseline blood samples were collected before ingestion of sacubitril/valsartan (a total of 194 mg sacubitril/206 mg valsartan). The standardized meal (42 g carbohydrate, 25 g fat, 26 g protein) was consumed 30 min after ingestion of the drug. Red arrows indicate when blood samples, blood pressure (BP), and heart rate (HR) were collected and measured.

bottom tubes (ref. 60.614.010; Sarstedt) and stored at −80°C. During the study, the subjects were allowed to drink a maximum of 500 mL of water.

Biochemical analyses

The plasma from the inclusion examination was analysed for standard biochemical parameters (COBAS 8000, Roche). During the trial, blood pressure and heart rate (HR) were measured using an Edan M3A Vital sign monitor. Plasma glucose was measured on a Cobas 8000, c702 module. Radioimmunoassays (RIAs) developed in the department were used to quantitate gastrin and CCK concentrations in plasma. The analytical and clinical validity of the RIAs has previously been reported (14, 15, 16). In brief, gastrin and CCK were measured using antiserum 2604 and antiserum 92128, respectively, which bind all bioactive forms of the two hormones and with no cross-reactivity between the two.

Statistical analyses

Data are presented as mean values with s.e.m. if not otherwise indicated. In the statistical analyses, we compared the mean value of the outcomes between the two trial days. Area under the curve (AUC) was calculated using the trapezoidal method and used to conduct a paired *T*-test. In addition, a mixed model ANOVA was used to compare gastrin CCK and gastrin concentrations

with the control group followed by the Bonferroni *post hoc* test. The mixed-model ANOVA was chosen due to missing data at a few time-points. A two-way ANOVA followed by a *post hoc* Bonferroni was used to determine any potential differences of mean arterial pressure (MAP), HR, and glucose concentrations. For the statistical analysis, the program Prism 8 for Windows (Graphpad) was used. The level of significance was set at P value <0.05.

Results

Ten healthy male subjects were screened for inclusion in the study. One dropped out after the initial consultation due to problems with adhering to the timeline of the study. The remaining subjects completed both trial days. Prior to the study, all subjects underwent a medical examination with all results within reference ranges. For baseline characteristics, see Table 2.

Hemodynamic response

Baseline mean arterial pressure (MAP) was 91±2 mmHg in the control group and 88±2 mmHg in the sacubitril/valsartan group (two-way ANOVA, $P > 0.99$). In response to the ingestion of the meal, MAP decreased in both groups, but increased steadily 2 h after ingestion: There were no significant difference between the groups ($tAUC_{0-270\ min}$: −762±−1125 vs −652±-1089 min×mmHg, $P=0.86$). In

Table 2 Subject data.

	Reference	Mean	s.e.m.
Height (cm)	–	185	2
Weight (kg)	–	81	2
Age (years)	–	24	1
BMI (kg/m^2)	–	24	1
Hb (mmol/L)	8.3–9.5	9.3	0.2
K (mmol/L)	3.5–4.4	4.1	0.1
Creatinine (µmol/L)	60–105	85.8	4.6
Sodium (mmol/L)	137–145	140.2	0.7
Albumin (g/L)	36–48	42.2	0.8
CRP (mg/L)	<5	1.1	0.1
ALAT (U/L)	10–70	26.1	5.0
HbA1C (IFCC) (mmol/mol)	31–44	30.8	0.9
Glucose (mmol/L)[a]	4.2–6.3	5.3	0.1
Total cholesterol (mmol/L)	<5	4.2	0.3
HDL (mmol/L)	>1	1.3	0.1
LDL (mmol/L)	<3	2.8	0.4
Triglycerides (mmol/L)	<2	1.1	0.4

Table 2 list the anthropometrics and biochemical results at consultation before entering the study.

[a]Glucose reference interval is in a fasting state, but the blood samples were collected at a pre-interview in a not-fasting state.

parallel, heart rate (HR) decreased in both groups during the 30 min before ingestion of the meal. No response to ingestion of sacubitril/valsartan was observed, but in response to food intake, HR increased in both groups with a peak of 67 ± 3 bpm in the sacubitril/valsartan group vs 62 ± 3 bpm in the control group. No significant difference (tAUC$_{0\text{-}270 \text{ min}}$: 52 ± 63 sacubitril/valsartan group vs 46 ± 57 control group min × bpm, $P = 0.98$) was noted between trial the days.

Glucose concentration

There was no significant difference in glucose plasma concentration in response to ingestion of sacubitril/valsartan and a meal between the two groups (peak 6.7 ± 0.3 vs 6.7 ± 0.2 mmol/L, $P > 0.99$: tAUC$_{0\text{-}270 \text{ min}}$: 76 ± 93 vs 63 ± 81, $P = 0.67$).

Gastrin and CCK response

The gastrin concentration in plasma rapidly increased after consumption of the standardized meal. Combined with intake of sacubitril/valsartan, the increase was markedly enhanced; AUC was 80% greater compared with the control group (tAUC$_{0\text{-}270 \text{ min}}$: 1263 ± 56 vs 710 ± 77 min × pmol/L, $P = 0.004$, Fig. 2A). Ninety minutes after meal intake, the concentrations were similar in the two groups and they continued to be so throughout the remaining study period.

The baseline CCK concentrations in plasma did not differ between the trial days (mixed model ANOVA, $P > 0.99$). The plasma concentration increased after ingestion of a meal with and without sacubitril/valsartan. The AUC was 60% higher when subjects received sacubitril/valsartan (tAUC$_{0\text{-}270 \text{ min}}$: 487 ± 207 vs. 308 ± 115 min × pmol/L, $P = 0.003$); see Fig. 2B.

Discussion

The present study shows that acute inhibition of neprilysin with sacubitril/valsartan increases the postprandial plasma concentrations of gastrin and cholecystokinin (CCK) in healthy young men. Previous studies have suggested a proteolytic effect of neprilysin on gastrin and CCK using indirect methods (7, 8, 9, 17, 18, 19). Specifically, the effect of neprilysin was suggested through chromatographic comparison of endogenous peptide fragments with incubation of synthetic peptides and enzyme *in vitro*. A major difference between our study and the earlier *in vitro* studies was the former use of CCK and gastrin synthetic peptides together with neprilysin of non-human origin, which complicates the interpretation in relation to human physiology. One human study (9) did infuse human gastrin (gastrin-17) into healthy humans and evaluated the circulating fragments over time using gel chromatography. The study concluded on a possible degrading effect of neprilysin. However, no direct link was established to specific neprilysin degradation. The results of our human study show that targeted neprilysin inhibition immediately alters the gastrin and CCK concentrations after a meal in healthy young men.

The present report is mainly an exploratory study. The drug is indicated for patients with chronic heart failure and reduced ejection fraction, thus receiving daily drug doses for years. The long-term effect of neprilysin inhibition on gastrin and CCK concentrations is still unknown, but it seems reasonable to hypothesize that the plasma concentrations will also increase in patients with heart failure and during chronic therapy. Gastrin regulates proliferation and growth of the gastric mucosa and studies have indicated that long-term hypergastrinemia in humans induces hyperplasia and dysplasia of the gastric mucosa (20, 21, 22). It is unknown if the peaks of gastrin seen in this study is sufficient to induce hyperplasia in human. Whether hypergastrinemia will follow long-term treatment with sacubitril/valsartan needs to be examined, and if so, whether this increase is associated with an increase in gastric disorders such as dyspeptic pain and duodenal ulcers.
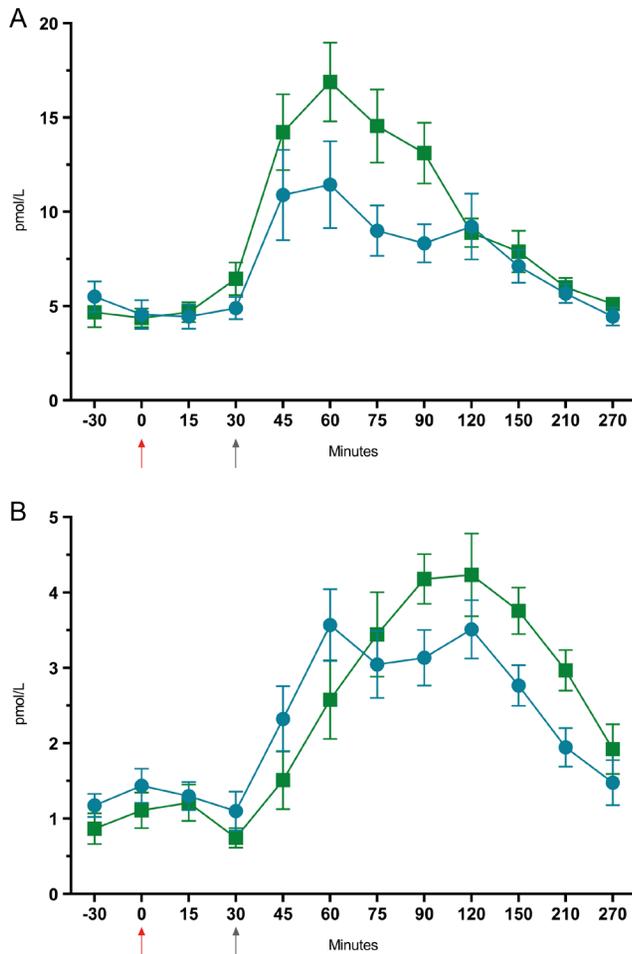
Figure 2
The subjects were randomized to not receive (blue circles) or to receive a single dose of two tablets of sacubitril/valsartan (a total of 194 mg sacubitril/206 mg valsartan) (green squares) and afterward a standardized meal. The red arrow represents the time sacubitril/valsartan was given and the grey arrow indicates administration of the standardized meal. Panel A and B shows the effect on gastrin and CCK concentrations, respectively.

A seminal study on sacubitril/valsartan treatment in heart failure patients has shown improved glycemic control in patients with both chronic heart failure and diabetes (23). While it remains unresolved exactly how sacubitril/valsartan improves the glycemic control, several mechanisms have been suggested including increased plasma concentrations of the incretin hormone GLP-1 (23). We have recently reported that sacubitril/valsartan increases total but not intact GLP-1 concentrations in healthy humans (13). Other hormonal contributors behind the decrease in HbA1c values observed in patients could be CCK and gastrin, as both are also incretin hormones. The endocrine pancreas expresses gastrin/CCK$_2$ receptors, and several studies have shown that

these hormones have an insulinotropic effect (24, 25, 26, 27). Another possibility could be that CCK inhibits gastric motility (28), which will delay gastric emptying. This could also contribute to reducing the postprandial hyperglycemia seen in type 2 diabetes. Whether the earlier observed decrease in HbA1c is mediated mainly by one hormone system or, rather, several operating in concert, needs to be examined further. Finally, it should be recapitulated that a treatment which improves hemodynamic function in heart failure also will have an (positive) impact in physical activity of the patients and thereby contribute to decrease in HbA1c.

Gastrin and CCK both circulate in multiple molecular forms (29). The differences between the molecular forms are mainly defined by the N-terminal part of their structuresa, whereas the active/receptor binding part of the hormones resides in the C-terminal sequence. In this study, we only used immunoassays specific for the receptor binding part of the hormones, and as such, we thus report on the concentrations of the bioactive parts of gastrin and CCK. Several proteases are involved in the elimination of gastrin and CCK peptides, and mainly the N-terminus of CCK peptides is subject to cleavage and degradation *in vivo* by aminopeptidases. In contrast, the predominant gastrin forms (gastrin-34 and gastrin-17) are protected against aminopeptidases by pyroglutamination. In perspective, examining the different molecular forms of gastrin and CCK in relation to NEP inhibition could prove worthwhile to pursue.

This exploratory study has several limitations. Treatment with sacubitril/valsartan is indicated for patients with chronic heart failure, and this study was conducted on young healthy male subjects. A second limitation is the chosen dosage. The recommended dosage of sacubitril/valsartan is 49 mg/51 mg through weeks of upscaling until an endpoint of 97 mg/103 mg, twice daily. In this study, the subjects received two tablets of 97 mg/103 mg, a total of 194 mg sacubitril/206 mg valsartan, once. This is two- to four-fold the recommended dosage, and it would, therefore, be relevant to examine if subjects receiving the clinical dosage have the same response as this study shows. Finally, this is an acute study of sacubitril/valsartan treatment, and whether the effect of long-term use of sacubitril/valsartan augments gastrin and CCK concentrations in circulation in patients with chronic heart failure is not clear.

In conclusion, this exploratory study shows that acute neprilysin inhibition with sacubitril/valsartan increases the meal-related concentrations of gastrin and CCK in plasma. Further studies including patients with chronic

heart failure receiving sacubitril/valsartan are now needed to clarify the potential effects and their possible association to stomach-related disease.

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