Neprilysin Inhibition Increases Glucagon Levels in Humans and Mice with Potential Effects on Amino Acid Metabolism

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**Clinical Trial Information:** ClinicalTrials.gov no. NCT03717688 and no. NCT01631864.

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**Disclosures:** SZ was previously supported by Novartis (until 2019) but unrelated to the current study. The remaining authors have no conflict of interest to declare.
Abstract

**Context:** Inhibitors of the protease nephrilysin (NEP) are used for treating heart failure, but are also linked to improvements in metabolism. NEP may cleave proglucagon-derived peptides, including the glucose and amino acid (AA)-regulating hormone glucagon. Studies investigating NEP inhibition on glucagon metabolism are warranted.

**Objective:** To investigate whether NEP inhibition increases glucagon levels.

**Subjects and methods:** Plasma concentrations of glucagon and AAs were measured in eight healthy men during a mixed meal with and without a single dose of the NEP inhibitor/angiotensin II type 1 receptor antagonist, sacubitril/valsartan (194 mg/206 mg). Long-term effects of sacubitril/valsartan (eight weeks) were investigated in individuals with obesity (n=7). Mass-spectrometry was used to investigate NEP-induced glucagon degradation, and the derived glucagon fragments were tested pharmacologically in cells transfected with the glucagon receptor (GCGR). Genetic deletion or pharmacological inhibition of NEP with or without concomitant GCGR antagonism was tested in mice to evaluate effects on AA metabolism.

**Results:** In healthy men, a single dose of sacubitril/valsartan significantly increased postprandial concentrations of glucagon by 228%, concomitantly lowering concentrations of AAs including glucagonotropic AAs. Eight weeks sacubitril/valsartan treatment increased fasting glucagon concentrations in individuals with obesity. NEP cleaved glucagon into five inactive fragments (in vitro). Pharmacological NEP inhibition protected both exogenous and endogenous glucagon in mice after an AA challenge, while NEP-deficient mice showed elevated fasting and AA-stimulated plasma concentrations of glucagon and urea compared to controls.
Conclusion: NEP cleaves glucagon, and inhibitors of NEP result in hyperglucagonemia and may increase postprandial AA catabolism without affecting glycemia.

Keywords: Entresto; Proglucagon-derived peptides, Metabolism
Introduction

The endopeptidase neprilysin (NEP) is widely expressed in humans (1, 2), and several peptide hormones including the vasoactive hormone, atrial natriuretic peptide (ANP), and the proglucagon derived incretin hormone, glucagon-like peptide 1 (GLP-1), have been identified as substrates (3-7). Inhibitors of NEP (sacubitril) combined with the angiotensin II type 1 receptor antagonist (valsartan) are currently used in the treatment of chronic heart failure (8), but have recently also been reported to improve metabolic function (7, 9). The proglucagon derived hormone glucagon is a substrate for NEP degradation in vitro (10, 11) and NEP degrades both exogenous and endogenous glucagon in pigs (12). Glucagon plays a central role in hepatic amino acid metabolism, both at transcriptional level and during increased amino acid availability (13, 14). Amino acids stimulate the secretion of glucagon, and glucagon in turn regulates hepatic amino acid catabolism by stimulating ureagenesis (13, 15-24). The metabolic implications of NEP inhibitors on endogenous glucagon levels and amino acid metabolism are currently unknown.

We hypothesized that NEP cleaves glucagon and that inhibitors of NEP increase amino acid catabolism in a glucagon receptor (GCGR)-dependent manner. To address this, we measured plasma concentrations of glucagon and amino acids in healthy young men and individuals with obesity after administration of the dual NEP inhibitor/angiotensin II type 1 receptor antagonist, sacubitril/valsartan, as sacubitril alone is not available for use in humans. Moreover, we examined the effects of pharmacological NEP inhibition as well as genetic deficiency of NEP activity in mice. To dissect the metabolic effects of NEP inhibition, we modified GCGR signaling, using a GCGR antagonist (GRA). To account for the potential effects of altered GLP-1 receptor (GLP-1R) signaling upon inhibition of NEP, we also studied mice treated with a GLP-1R antagonist (Ex9-39) or mice
lacking the GLP-1R (GLP-1R−/−). Finally, we assessed NEP-induced glucagon degradation in vitro, and examined potential agonistic properties of the generated glucagon fragments for GCGR activity in COS-7 cells transiently transfected with human or murine GCGR.

Materials and methods

Study approvals and ethical considerations

The study of healthy young males was approved by the scientific ethical committee of the Capital region of Denmark (H-18000360) and registered with Danish Data protection Agency (VD-2018-131) and ClinicalTrials.gov (NCT03717688). The long-term study investigating individuals with obesity was conducted by the Maastricht University Medical Centre and was approved by the institutional review board or the independent ethics committee for each participating center, and registered at clinicaltrials.gov (NCT01631864). Written and oral consent was obtained from all participants, and both studies were conducted in accordance with the principles of the Declaration of Helsinki. Primary outcomes from both cohorts have previously been reported (5, 25). Animal experiments were conducted with permission from the Danish Animal Experiments Inspectorate, Ministry of Environment and Food of Denmark (2018-15-0201-01397), VA Puget Sound Health Care system (VAPSHCS) Institutional Animal Care and Use Committee (IACUC) in Seattle, USA, and The Department of Experimental Medicine IACUC in Copenhagen, Denmark (Approval No. P19-147 and P19-475). All studies performed were in accordance with the European Union Directive 2010/63/EU.
Clinical study designs and participants

The acute effect of sacubitril/valsartan was investigated in healthy males as a crossover trial. Eight men received, in randomized order, a single dose (two tablets) of the NEP-inhibitor/angiotensin II type 1 receptor antagonist (194 mg sacubitril/206 mg valsartan) (Novartis Pharma GmbH, Nürnberg, Germany) 30 min before ingesting a solid mixed meal (42 g/34% carbohydrates, 25 g/45% fat, 26 g/21% protein) or the meal alone. Participants were studied in the morning after an overnight (10-hour) fast, and the meal was ingested over a 10-min period. A cannula was inserted into a cubital vein and blood samples were drawn at -60 min (fasting) and -30, -15, 0, 15, 30, 45, 60, 90, 120, 180 and 240 min relative to meal ingestion (at time 0 min). After completion of the study, three participants meeting the same inclusion criteria were administered valsartan (206 mg) alone 30 min before ingesting the same solid mixed meal described above or the meal alone (at time 0 min), with blood drawn at times -60, -15, 0, 30, 45, 90 and 180 min. Long-term effects of sacubitril/valsartan were investigated in individuals with hypertension and obesity in a multicenter randomized, double-blinded study. Participants were administered 8 weeks treatment of either sacubitril/valsartan (194 mg/206 mg once a day, n=7) or amlodipine, included in this study as a control group (10 mg once a day, n=7). Plasma samples were obtained after an overnight fast before and after 8 weeks treatment.

Animals and compounds

Female and male C57BL/6Jrj mice (20-25 g) were acquired from Janvier Laboratories (Saint-Berthevin Cedex, France). Female mice deficient of GLP-1R signaling (GLP-1R−/−; C57BL/6N-Glp1rtm1C(KOMP)MbpH) and wildtype (WT) littermates (GLP-1R+/+) (previously characterized (26)) were bred in-house. Experiments on male mice deficient of NEP activity (NEP−/−) (C57BL/6.NEP−/−),
acquired from Dr. B. Lu (Harvard Medical School, Boston, Massachusetts (27)) with subsequent establishment in Seattle, Washington (28), were conducted at the University of Washington, Seattle. All mice were housed in groups of 3-8 in individually ventilated cages, following a light dark cycle of 12 hours (lights on from 6 AM to 6 PM) with ad libitum access to standard chow (Altromin Spezialfutter, Lage, Germany) and water. Mice were acclimatized for a minimum of 1 week after transferal before being included in the experiments. A GCGR antagonist (GRA) (100 mg/kg BW) (25-2648; a gift from Novo Nordisk (29)) was administered in suspension by oral gavage as previously described (30, 31). The NEP inhibitor, sacubitril (dissolved in DMSO and diluted in PBS) was administered by oral gavage at a concentration (0.7 nmol/g BW) corresponding to 1000x the IC50 for sacubitril (32) after correcting for 60% bioavailability. PBS (0.7% DMSO) was administered by oral gavage as a sacubitril control. The GLP-1R antagonist, Ex9-39 (Bachem, Switzerland) was administered as a fixed dose of 100 µg by i.p. injection. Glucagon (96 ng/g BW; Bachem, Denmark), dissolved in DMSO and diluted in PBS + 0.1% BSA, was administered by i.p. injection. Amino acids were administered by oral gavage (Vamin 14 g/l Electrolyte Free; Fresenius Kabi, Copenhagen, Denmark).

Study design for mouse experiments

First, we investigated the effects of sacubitril on exogenous glucagon concentrations in female C57BL/6Jrj mice that received either sacubitril or vehicle 30 min prior to an injection of glucagon at time 0 min. Blood glucose concentrations were measured from tail bleeds using a handheld glucometer (Accu-Chek Mobile; Roche Diagnostics, Mannheim, Germany) and blood was collected in EDTA-coated capillary tubes (Micro Haematocrit Tubes; Vitrex Medical, Herlev, Denmark) at time points 0, 5, 15, 30 and 60 min. Next, we investigated the effects of the NEP inhibitor on
endogenous glucagon concentrations in female (n=7-10) and male (n=6) C57BL/6JRj mice (~22 g) treated with GRA vehicle (time -180 min), GRA (time -180 min) or Ex9-39 (time -15 min). Sacubitril or vehicle were administered at time -30 min followed by a fixed dose of amino acids (34 mg) at time 0 min. Blood glucose concentrations and blood samples were collected and stored as described above at times 0, 10, 15, 30, 60 and 180 min. The experiment was also repeated in female mice that received water instead of amino acids (n=4). GLP-1R−/− mice and GLP-1R+/+ mice were treated with sacubitril or vehicle at time -30 min followed by an amino acid challenge (34 mg), with measurement of blood glucose and collection of blood at times 0, 10, 15, 30, 60 and 180 min, as described above. Male NEP−/− mice and non-littermate controls (NEP+/+) (n=5-6) were administered a volume of amino acids equivalent to 1% of BW (corresponding to approx. 22 mg). Blood glucose concentrations were measured with a glucometer (Accu-Chek Aviva Plus; Roche, Basel, Switzerland) and blood was drawn in heparinized hematocrit tubes (Fisherbrand micro-hematocrit capillary tubes) at times 0, 15, 30 and 60 min. All blood samples from the above mentioned studies were collected from the retro bulbar plexus (~75 µl) and kept on ice until centrifugation with plasma being transferred to pre-chilled PCR tubes and stored at -20 or -80 °C for subsequent analyses. All mice were fasted for 3 hours prior to experiments with water freely accessible. Mice were euthanized by cervical dislocation.
In vitro degradation of glucagon by NEP and pharmacological characterization of glucagon degradation products

NEP-induced glucagon degradation was investigated in vitro using synthetic glucagon (Bachem, Denmark) and recombinant human or mouse (cat. no. 1182-ZNC-010 and 1126-ZNC-010; R&D Systems, Denmark) NEP. Ten ng recombinant NEP was added to 1 µg synthetic glucagon with or without the NEP inhibitor in the active form of the prodrug, sacubitril (sacubitrilat, 0.5 nmol, 10 % DMSO) (cas. no. SML2064, Merck, Denmark). Reactions were performed in 50 mM Tris, 0.05 % Brij-35, pH 9 (human derived NEP), or 50 mM Tris, pH 9 (mouse derived NEP) and incubated at 37°C. The reaction was stopped after 15, 30 or 60 min incubation by addition of an excess of 0.1% trifluoroacetic acid (TFA) and spotted onto a ground steel MALDI target plate using α-Cyano-4-hydroxycinnamic acid (HCCA) as a matrix. The mass spectrometer was calibrated for each reaction. Metabolite generation was assessed by a Bruker AutoFlex MALDI-TOF mass spectrometer with accompanying Compass version 1.4 FlexSeries software. Synthetic ANP (cat. no. 005-06.; Phoenix Pharmaceuticals) was included as a positive control, as this peptide is a known substrate for NEP (33, 34). A computation tool (web.expasy.org/compute) was used to find amino acid sequences corresponding to the molecular weight of the identified degradation products. Amino acid sequences were synthesized (CASLO ApS, Denmark) and tested on COS-7 cells transfected with human or mouse GCGR to assess potential activation of GCGR signaling, antagonism (evaluated as cAMP production) and binding affinity (measured by displacement of $[^{125}I]$-glucagon). COS-7 cells were cultured as previously described (35) and transiently transfected using the calcium phosphate precipitation method (36). To test for agonism, the glucagon degradation products were added in increasing concentrations (10 pM-10 µM) to the transfected COS-7 cells. Human glucagon (hGCG-NH$_2$) was used as a positive control. To test for antagonistic properties, COS-7
cells were pre-incubated with increasing concentrations of the degradation products (0.1 nM, 10 nM and 1 µM), with subsequent addition of the agonist (1 nM glucagon corresponding to 40% of the maximal cAMP accumulation response to glucagon). cAMP production was measured with HitHunter™ cAMP XS assay (DiscoverX, Herlev, Denmark) according to the manufacturer’s instructions. Finally, to test for competition binding, COS-7 cells were seeded in 96-well plates and incubated with 15-40 pM monoiodinated [125I]-labeled glucagon and different concentrations of the glucagon degradation products (ranging from 1 pM-10 µM). Specific binding was calculated by subtracting the non-specific binding from total binding. Samples were analyzed using the Wallac Wizard 1470 Gamma Counter and the output was calculated as the percent of specific binding. Unlabeled synthetic glucagon (0.1 pM-1 µM) (cat. no. H-6790; Bachem, Denmark) was used as a positive control.

**Biochemical analyses**

Plasma concentrations of glucagon were measured by a validated (37) two-site enzyme immunoassay (Mercodia Cat# 10-1281-01, RRID:AB_2783839), recognizing both COOH- and NH2-termini of the molecule, according to the manufacturer’s protocol, and by an in-house radioimmunoassay (RIA) specific for the COOH-terminus of glucagon, using antiserum (no. 4305) and monoiodinated [125I]-labeled glucagon, as previously described (38, 39). Plasma concentrations of insulin (Mercodia Cat# 10-1247-01, RRID:AB_2783837), L-amino acids (Abcam, Cambridge, UK) and urea (BioAssay Systems, Hayward, CA) were measured according to manufacturers’ protocol. Samples for measuring individual amino acids were derivatized with methyl chloroformate and measured using a slightly modified version of the previously described protocol (40), and processed as previously described (41).
Statistics

Incremental and total AUCs (iAUC and tAUC) were calculated using the trapezoidal rule, with iAUC calculated as positive peaks after adjusting for baseline values and tAUC calculated as positive peaks above 0. IC\textsubscript{50} and EC\textsubscript{50} values were determined by nonlinear regression. Significance was assessed by un-paired t-tests, paired t-tests, one-way ANOVA or two-way ANOVA/mixed effects analysis followed by Holm-Sidak post-hoc analysis to correct for multiple testing. P ≤ 0.05 is considered significant and P = 0.05-0.1 is considered a trend. One symbol indicates P ≤ 0.05, two symbols indicate P ≤ 0.01 and three symbols indicate P ≤ 0.001. Statistical calculations and graphs were made using GraphPad Prism (version 8.01 for Windows; GraphPad Software, La Jolla, CA).

Data in the main text are reported as mean ± SD if not otherwise indicated.

Additional information on the methods for pharmacological characterization of glucagon degradation products and biochemical analyses can be found in a digital research repository (42).

Results

Acute NEP inhibition increases plasma concentrations of glucagon and reduces amino acids in healthy young male individuals

To investigate the effect of NEP inhibition on postprandial glucagon metabolism, healthy young men (mean ± SD; 24 ± 2 years, BMI 23 ± 1 kg/m\textsuperscript{2}; n=8) were included in a crossover study involving ingestion of a standardized solid high-fat mixed meal with or without co-ingestion of a single dose of the NEP inhibitor/angiotensin II type 1 receptor antagonist (sacubitril/valsartan). Sacubitril/valsartan did not significantly alter fasting concentrations of glucagon compared to
control treatment (13 ± 8 vs 9 ± 3 pmol/L, P=0.21), but postprandial glucagon concentrations were significantly increased by 228% compared to controls as reflected by incremental AUC (iAUC, 2435 ± 1455 vs 742 ± 365 pmol/L x min, P=0.02). This was seen with both the sandwich ELISA (employing NH₂- and COOH-terminal wrapping antibodies; Figure 1a) and the RIA (using a COOH-terminal wrapping antibody; Supplemental Figure 1a (42). All supplemental material and figures are available in a digital research repository (42)). Fasting and postprandial plasma concentrations of glucose were similar between control and sacubitril/valsartan treatments (Supplemental Figure 1b (42)) (plasma glucose concentrations are previously published in (5)). Fasting amino acid concentrations were unchanged with sacubitril/valsartan (Figure 1b), but postprandial amino acid concentrations were reduced by 12% compared to control treatment as reflected by total AUC (tAUC, 464 ± 55 vs 526 ± 81 mmol/L x min, P=0.014), and plasma concentrations of glucagon (measured by ELISA) and amino acids were negatively correlated when evaluated by AUC (Supplemental Figure 1c (42), P=0.03). We also measured individual amino acids at time point 90 min and found reduced concentrations of several amino acids after treatment with sacubitril/valsartan (Figure 1c, n=6). Of particular note, levels of amino acids (alanine, asparagine and proline) known to be involved in the liver-alpha cell axis were reduced concomitantly with the increase in plasma concentrations of glucagon. As sacubitril is only available for clinical use in combination valsartan, valsartan alone (206 mg) was administered to a subset of individuals (n=3; Supplemental Figure 1d (42)) to ascertain that the effects on glucagon were due to the inhibition of NEP activity and not to the changes in the renin-angiotensin-aldosterone system. Valsartan alone had no effect on postprandial plasma concentrations of glucagon when compared to control treatment as reflected by iAUC (1680 ± 428 vs 1744 ± 524 pmol/L x min, P=0.55).
Long-term NEP inhibition increases fasting concentrations of glucagon in individuals with obesity and hypertension

The effect of long-term sacubitril/valsartan treatment on plasma concentrations of glucagon was investigated by analyzing plasma obtained before and after 8 weeks of treatment with sacubitril/valsartan in individuals with obesity and hypertension (1 woman, ~57 ± 7 years, BMI 33 ± 7 kg/m², 143/90 mmHg, n=7). Fasting concentrations of glucagon increased by 42% (9 ± 3 vs 13 ± 6 pmol/L, P=0.015) (Figure 1d), with no change in fasting concentrations of amino acids (P=0.63) (Figure 1e) or glucose (P=0.12) (Figure 1f), consistent with the findings in the healthy young men in the fasted state. Seven individuals treated with amlodipine (calcium channel antagonist) for 8 weeks were included as controls and showed no significant changes in plasma concentrations of glucose, glucagon or amino acids (Supplemental Figure 1e-g (42)).

Recombinant NEP cleaves native glucagon in vitro

To investigate whether NEP degrades glucagon directly, native glucagon (Gcg1-29) was incubated with recombinant NEP with or without sacubitrilat (the active compound of the NEP inhibitor prodrug sacubitril), and products of degradation were assessed by matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectrometry. As a control, we also analyzed glucagon in the absence of NEP and sacubitrilat, and observed that glucagon is stable over time (15, 30 and 60 min) (Figure 2a-b “0 min”) when incubated in buffer alone. Recombinant human (Figure 2a) and mouse NEP (Figure 2b) cleaved native glucagon after 15 min incubation, as shown by the generation of five glucagon fragments and the reduced peak of native glucagon. The inclusion of sacubitrilat prevented the formation of any fragments and preserved the peak size of intact glucagon. Following analysis of the MALDI-TOF spectra, we identified NEP cleavage sites.
between amino acids Asp21 and Phe22 (Gcg1-21), Ala19 and Gln20 (Gcg1-19), and Arg18 and Ala19 (Gcg1-18) in the COOH terminus of the glucagon molecule (Table 1). Two additional glucagon fragments were identified with recombinant mouse NEP with m/z values of 1738 and 1520, corresponding to amino acid sequences Gcg5-18 and Gcg1-13. These two glucagon fragments were not detectable following incubation of native glucagon with human recombinant NEP, probably due to a high signal-to-noise ratio at m/z < 2000. ANP, a well characterized substrate of NEP (34, 43), was included as a positive control (Supplemental Figure 2a-b (42)), and showed that NEP cleavage resulted in the formation of a known substrate (m/z 2585), which was prevented by the addition of sacubitrilat (44).

**Glucagon fragments generated by NEP-induced cleavage do not activate, bind to or antagonize GCGR signaling in vitro in COS-7 cells**

Having identified the glucagon fragments (Gcg1-21, Gcg1-19, Gcg1-18, Gcg5-18 and Gcg1-13) arising from NEP cleavage of glucagon, we next investigated whether these fragments were capable of modulating GCGR signaling. We therefore assessed their agonistic properties on GCGR activation (evaluated by increases in cAMP accumulation) using COS-7 cells transiently transfected with human or mouse GCGR. Native glucagon, included as a positive control, increased GCGR activity within an expected dynamic range from 0.1 nM-1 μM with EC_{50} values of 9.149e^{-10} M (n=3) and 1.185e^{-9} M (n=3) in COS-7 cells transfected with human (Figure 2c) or mouse (Supplemental Figure 3a (42)) GCGR, whereas none of the five glucagon fragments had any effect on either the human (Figure 2d) or mouse (Supplemental Figure 3b (42)) GCGR. To further evaluate their pharmacological properties, we assessed GCGR binding affinity and antagonistic properties of the glucagon fragments in the same cellular expression system on the human (Supplemental Figure 4
Native glucagon displaced \([^{125}\text{I}]\)-glucagon radioligand binding in a dose dependent manner with an IC\(_{50}\) of 8.16e\(^{-8}\) M (n=3) and 6.44e\(^{-8}\) M (n=3) in COS-7 cells transfected with human (Supplemental Figure 4a (42)) or mouse GCGR (Supplemental Figure 3c (42)). None of the glucagon fragments altered the binding of \([^{125}\text{I}]\)-glucagon, indicating their inability to bind to human (Supplemental Figure 4b (42)) and mouse GCGR (Supplemental Figure 3d (42)). The glucagon fragments were also devoid of any antagonistic properties at the human (Supplemental Figure 4c (42)) and mouse (Supplemental Figure 3e (42)) GCGR, when assessed in the presence of 1 nM native glucagon. Finally, we measured the glucagon fragments using the same immunoassays as were used in the human studies (NH\(_{2}\)- and COOH-directed sandwich ELISA and COOH-directed RIA), and found that none of the glucagon fragments derived by NEP cleavage cross-reacted with either of the two glucagon immunoassays used to measure glucagon in plasma.

*Exogenous and endogenous glucagon concentrations are increased in mice in response to pharmacological NEP inhibition*

The effect of NEP inhibition on exogenous glucagon was studied in female C57BL/6JRj mice by administering sacubitril (0.7 nmol/g BW, ~60 % inhibition of NEP activity in mice; data not shown) 30 min prior to a glucagon injection (96 ng/g BW). Sacubitril decreased the degradation of glucagon following exogenous glucagon administration (P=0.001) (Figure 3a) without affecting blood glucose concentrations (Figure 3b). We investigated the effect of NEP inhibition on endogenous glucagon concentrations by measuring plasma concentrations of glucagon in female C57BL/6JRj mice (and male mice in Supplemental Figure 5 (42)) after stimulation with oral administration of amino acids (amino acid composition shown in Supplemental Table 1 (42))
Sacubitril increased the glucagon response to amino acids by 41% (at time 10 min, P=0.007) (Figure 3d). The GLP-1R antagonist (Ex9-39) increased plasma concentrations of amino acid-stimulated glucagon compared to vehicle treatment (Figure 3f-g), while co-administration of Ex9-39 and sacubitril increased plasma concentrations of glucagon more when compared to Ex9-39 treatment alone (Figure 3e). Sacubitril did not, however, increase the glucagon response to amino acids in a model of genetic GLP-1R deficiency (GLP-1R−/− mice) (Supplemental Figure 6 (42)). The augmenting effects of sacubitril on amino acid-stimulated glucagon levels were only apparent in littermate controls (GLP-1R+/+ mice) (time point 15 min, P=0.02). Sacubitril increased blood glucose concentrations in response to an amino acid challenge in GLP-1R−/− mice (P=0.03), while no significant increase was observed in C57BL/6JRj mice after sacubitril (Figure 3f) despite increased plasma concentrations of glucagon. Similar experiments were performed in male C57BL/6JRj mice and comparable results to the findings in female C57BL/6JRj mice were observed (Supplemental Figure 5a-c (42)).

**NEP inhibition may increase amino acid-stimulated ureagenesis**

Since inhibitors of NEP increase both glucagon and GLP-1 concentrations in humans, we investigated whether the effects of sacubitril on amino acid catabolism and ureagenesis depend on GCGR and/or GLP-1R signaling. To address this, we treated female C57BL/6JRj mice with a GRA or Ex9-39 and investigated GLP-1R−/− mice (Supplemental Figure 6 (42)). As observed previously by our group, GRA increased plasma concentrations of glucagon and amino acids, while reducing blood glucose levels. NEP inhibition had no effect on plasma concentrations of amino acids when compared to vehicle treatment (Figure 3g) and co-administration of Ex9-39 and sacubitril as well as co-administration of GRA and sacubitril similarly had no effect on amino acid concentrations
when compared to Ex9-39 treatment and GRA treatment alone (Figure 3g). Overall, sacubitril did not appear to reduce plasma concentrations of amino acids in this setting contrary to what we found in healthy young men. In fact, sacubitril unexpectedly increased plasma concentrations of amino acids in GLP-1−/− mice as reflected by iAUC (376 ± 67 vs 255 ± 118, P=0.05). As expected, GRA treatment reduced fasting (6 ± 1 vs 8 ± 1 mmol/L, P<0.001) and amino acid-stimulated urea concentrations compared to both vehicle and Ex9-39 treated groups, supporting previous findings showing that GCGR signaling is important for urea production. A trend towards increased amino acid-stimulated urea levels with sacubitril compared to vehicle treatment was apparent when evaluated by iAUC (737 ± 190 vs 592 ± 97 mmol/L x min, P=0.08), and this effect was dependent on GCGR signaling as reflected by the significant difference in urea levels between sacubitril treatment and the combined treatment of sacubitril and GRA (P=0.01, Figure 3h-i). Plasma concentrations of urea were similar between groups that were co-administered sacubitril and Ex9-39 compared to sacubitril treatment alone (Figure 3i), indicating that the increase in urea with NEP inhibition is independent of GLP-1R signaling. Fasting (143 ± 63 vs 58 ± 21 pmol/L, P=0.0045) and postprandial insulin concentrations were decreased in Ex9-39 treated groups compared to vehicle treatment, and co-administration of Ex9-39 and sacubitril did not affect plasma concentrations of insulin compared to Ex9-39 treatment alone (Figure 3j). To assess potential gender differences, we performed an identical experiment in male C57BL/6JRj mice (n=6) and similarly to the cohort of female mice, sacubitril increased plasma concentrations of glucagon without altering blood glucose or plasma concentrations of amino acids (Supplemental Figure 5 (42)). However, sacubitril did not increase plasma concentrations of urea, contrary to what was observed in female mice. Finally, we repeated the study in female mice (n=3) that were administered water instead of amino acids (Supplemental Figure 7 (42)) and saw that plasma concentrations of glucagon were...
increased numerically in groups that were co-administered sacubitril with vehicle, GRA or Ex9-39 compared to vehicle, GRA or Ex9-39 alone.

**Mice deficient of NEP have increased plasma concentrations of glucagon and urea**

We challenged male NEP−/− mice and controls with amino acids by oral gavage (Figure 4a-j) to assess the effect of congenital NEP deficiency on endogenous glucagon concentrations. Fasting plasma concentrations of glucagon were 114% higher in NEP−/− mice compared to NEP+/+ mice, and this difference became even more pronounced after an amino acid challenge (P=0.02) (Figure 4a-b). Fasting blood glucose concentrations were 27% higher in NEP−/− mice compared to NEP+/+ mice (10.2 ± 0.7 vs 7.4 ± 1.3 mmol/L, P=0.009), while plasma concentrations of insulin were numerically increased (Figure 4c-d). NEP−/− mice were heavier than NEP+/+ mice (P=0.01) (Figure 4e) despite being age-matched. An amino acid challenge increased plasma concentrations of amino acids to a greater extent in NEP−/− mice than in NEP+/+ mice (Figure 4f-g). Fasting plasma urea concentrations were 22% higher in NEP−/− mice (9 ± 1 vs 7 ± 1 mmol/L, P=0.001) compared to NEP+/+ mice, and this difference was further augmented by an amino acid challenge (P=0.026) (Figure 4h-j).
Discussion

The present study demonstrates that the endopeptidase NEP plays an important role in glucagon metabolism in both humans and mice, and that pharmacological inhibition of NEP induces hyperglucagonemia, with potential effects on amino acid levels in humans but with negligible effects on glucose levels. The latter may potentially be due to increased GLP-1R signaling, as supported by the increase in postprandial glucose concentrations observed upon NEP inhibition in GLP-1R−/− mice as shown here and the finding that NEP also appears to affect plasma concentrations of GLP-1 in humans (5). Hyperglucagonemia may affect amino acid catabolism by increasing hepatic amino acid uptake (45) and ureagenesis (13, 46). The present data suggest that NEP inhibition may induce the effects on amino acid metabolism as seen phenotypically with hyperglucagonemia although species and gender differences were observed. The reason for these differences is not clear and warrant further investigation. Given that amino acid abnormalities are reported in patients with heart failure (47), it may be speculated that sacubitril/valsartan-induced hyperglucagonemia could affect metabolic function through altered amino acid catabolism.

First, we investigated the effect of NEP inhibition with sacubitril/valsartan on postprandial glucagon concentrations in healthy young male individuals, and found increased postprandial glucagon concentrations (identified identically by sandwich ELISA and by a COOH-terminal wrapping RIA), consistent with an earlier experimental study in pigs (12). These observations align with our current findings by mass spectrometry, demonstrating that glucagon is directly cleaved by NEP, resulting in the formation of glucagon fragments that are truncated in the COOH-terminal and therefore lose their immunoreactivity. We do consider the use of sacubitril/valsartan (as opposed to sacubitril alone, which is not available for use in humans) as a limitation to the study.
However, the finding that valsartan alone had no effect on glucagon levels in a subset of individuals (n=3), together with our in vitro and mouse data indicating that glucagon concentrations are altered in response to changes in NEP activity, lead us to suggest that the valsartan component of the drug is probably not responsible for the observed changes in the plasma concentrations of glucagon.

Sacubitril/valsartan increased plasma concentrations of glucagon in healthy young men and individuals with obesity without affecting glucose concentrations. However, long-term sacubitril/valsartan treatment is reported to improve glycemic control in individuals with obesity and type 2 diabetes (9, 25). While this may be counterintuitive in view of the data shown here, it is pertinent to recognize that NEP affects a wide range of additional peptide hormones, including ANP, through direct cleavage by NEP, as well as GLP-1 (5, 48) possibly through reduced DPP-4 activity (49) and/or direct cleavage (50). GLP-1 may affect glucose levels via its well-known insulinotropic effects (51), and ANP may also improve glycemia (52, 53). The unaltered plasma concentrations of glucose in the cohorts investigated here may, therefore, be due to increased GLP-1 and/or ANP receptor signaling counterbalancing glucagon’s effects on hepatic glucose production. Indeed, we find that sacubitril increases blood glucose concentrations in GLP-1R−/− mice compared to vehicle treatment, supporting the notion that increased GLP-1R signaling in response to NEP inhibition may counteract the effects of glucagon on glucose levels. It has also been shown that glucagon secretion – augmented by amino acids – stimulates the beta cell via their GLP-1R and thereby lower glycemia (54). At any rate, during protein intake, which stimulates both glucagon and insulin secretion, increases in plasma concentrations of glucagon do not necessarily lead to increases in plasma concentrations of glucose (55, 56).
In accordance with our own (13) and others’ studies reporting an acute role for glucagon on amino acid metabolism (14), the sacubitril/valsartan-induced increase in the postprandial concentrations of glucagon in healthy young men was accompanied by a reduction in the concentration of postprandial amino acids. In fact, amino acid concentrations did appear to correlate with glucagon concentrations (based on AUC values) supporting the relationship between glucagon and amino acid catabolism. On the other hand, this statistical correlation was lost when removing a single data point (for an individual with a very high glucagon AUC), which likely suggests a power issue regarding this sub-analysis. The concentration of some amino acids, including those that have been suggested to be involved in the liver-alpha cell axis (alanine and proline) (57) and to stimulate glucagon secretion (asparagine) (58), were significantly reduced following sacubitril/valsartan treatment. Reductions were, however, modest and not apparent for all amino acids. It should be noted that these analyses were done post hoc on samples which had not been stored optimally for these particular analyses, which may have confounded the results, so these data should be interpreted with caution. In line with our findings in healthy young men, fasting amino acid concentrations were unchanged after 8 weeks sacubitril/valsartan treatment in individuals with obesity and hypertension. A mixed meal tolerance test was not performed in these individuals before and after the intervention. This is a limitation, as glucagon-stimulated amino acid metabolism may be dependent on substrate availability, meaning that the metabolic implications of sacubitril/valsartan-induced hyperglucagonemia may only become apparent when substrate availability is high. Further studies are, therefore, needed to explore the consequence of any NEP-induced effect on amino acid catabolism.

To investigate whether the increase in glucagon concentrations following NEP inhibition occurs because of a direct effect to inhibit NEP cleavage of the glucagon molecule or reflects an indirect
mechanism (such as increased glucagon secretion or reduced glucagon clearance in tissues), we studied the degrading effects of recombinant NEP on glucagon in vitro using mass spectrometry. Recombinant human and mouse NEP rapidly (within 15 min) and extensively cleaved glucagon and generated several fragments. NEP has a strong preference towards hydrophobic amino acids with cleavage at the N-terminal side (P1) of the residue (59), and this was the case for 4 out of 6 cleavage sites shown here. NEP also targets neutral amino acids (60), and we show that at least one amino acid in every bond break was either neutral or hydrophobic. Pharmacological analyses showed that the glucagon fragments did not bind, agonize or antagonize either the human or mouse GCGR in transiently transfected COS-7 cells, indicating that NEP activity exclusively attenuates GCGR signaling by reducing plasma concentrations of native glucagon through direct cleavage. These data, therefore, suggest that hyperglucagonemia caused by NEP inhibition in vivo at least partly results from reduced enzymatic glucagon degradation, although our findings do not exclude reduced glucagon clearance in tissues (61) or that increased glucagon secretion by pancreatic alpha cells may contribute, as previously suggested (22, 62).

While NEP directly decreases circulating concentrations of its substrates by cleavage (63-65), we found that amino acid-stimulated glucagon concentrations were not increased with sacubitril in a model of congenitally-disrupted GLP-1R signaling. However, acute co-administration of the GLP-1R antagonist Ex9-39 and sacubitril markedly increased amino acid-stimulated glucagon concentrations compared to Ex9-39 treatment alone. The GLP-1R−/− mouse strain is a global and lifelong genetically modified mouse model, and has likely developed adaptive mechanisms (66). Other gene products regulated secondarily to GLP-1R signaling may have influenced results. We also propose that chronic hyperglycemia may have inhibited the secretion of glucagon, as shown previously (67-69). In our cohort of NEP−/− mice, fasting concentrations of glucose were also
increased, which is contrary to previous reports from this mouse strain (28, 49). There was a discrepancy between the effects on glycemia with the NEP inhibitor compared to NEP<sup>+/−</sup> mice, which is currently unexplained and warrants further investigation. However, given that higher glucagon concentrations did not affect glucose concentrations in our other models of reduced NEP activity, the elevated glucagon concentrations may not fully explain the elevated glucose concentrations in NEP<sup>−/−</sup> mice. Plasma concentrations of amino acids (post oral amino acid administration) were also increased in NEP<sup>+/−</sup> mice and whether this was due to reduced amino acid uptake in extrahepatic tissue, increased proteolysis or denotes another explanation is unknown. However, NEP inhibition may increase plasma concentrations of cortisol (70, 71), a steroid hormone that during prolonged elevation can lead to increased proteolysis (72), which may partially explain the increased levels of amino acids and glucose in NEP<sup>−/−</sup> mice.

We investigated the effects of sacubitril in C57BL/6JRj mice treated with either vehicle, a GRA or Ex9-39 to explore the underlying mechanisms for the reduction in postprandial amino acid responses seen with NEP inhibition in healthy young male individuals. Mice treated with Ex9-39 were included, as NEP inhibition has been shown to increase insulin secretion in mouse islets in a GLP-1R dependent manner (73). Plasma concentrations of urea were measured as an indicator of amino acid catabolism as we were unable to sample urine in our mouse studies. However, this is also a limitation to the study, as small changes in plasma urea levels may have been missed due to efficient renal urea excretion (74). Here we show that mice with a global deficiency of NEP have increased plasma concentrations of urea in response to an amino acid challenge, which we hypothesized to be dependent on GCGR signaling. Sacubitril numerically (P=0.08) increased amino acid-stimulated urea levels (as indicated by iAUC) in female C57BL/6JRj mice. Furthermore, amino acid-stimulated urea concentrations were reduced in mice that were co-administered GRA and
sacubitril compared to sacubitril alone in both males and females, indicating that the effects with NEP inhibition on amino acid catabolism may depend on GCGR signaling. However, since sacubitril did not significantly increase plasma levels of urea in female and male C57BL/6JRj mice, inhibitors of NEP may increase amino acid catabolism by GCGR independent mechanism(s). Amino acid-stimulated urea concentrations (as indicated by iAUC) were similar in mice that were co-administered Ex9-39 and sacubitril compared to sacubitril alone in both male and female mice, indicating that the effects of NEP inhibition on amino acid catabolism were independent of GLP-1R signaling.

Taken together, the present study demonstrates, by use of several different experimental approaches, that NEP inhibition reduces the degradation of endogenous glucagon in humans and mice due to the inhibition of NEP-mediated glucagon cleavage. In healthy young men, sacubitril/valsartan reduced postprandial amino acid concentrations, including some glucagonotropic amino acids, which may be a result of increased glucagon-stimulated, substrate-dependent ureagenesis, as we found increased amino acid-stimulated plasma concentrations of urea in mice deficient of NEP. NEP inhibitors are prescribed to patients with heart failure, and the reported metabolic improvements in these patients may be related to increased concentrations of proglucagon-derived peptides, as demonstrated here and by others (5, 12, 48). Furthermore, our results contribute to the emerging evidence suggesting that glucagon may contribute to the acute clearance of amino acids in a postprandial setting.
Acknowledgements

We thank Jesper Lau, Novo Nordisk A/S, Måløv, Denmark, for providing the GCGR antagonist 26–2548, Finn Gustafsson (Department of Cardiology, Rigshospitalet) for expert advice, and Maibritt Baggesen, Department of Biomedical Sciences, University of Copenhagen and Christine Rasmussen, Department of Clinical Biochemistry, Rigshospitalet for excellent technical assistance in the pharmacological experiments and the biochemical analyses. The graphical abstract and illustrations in figures 2 and 3, and supplemental figures 2, 3, 6, and 7 were created with Biorender.com.

Financial Support: This study was supported by NNF Project support in Endocrinology and Metabolism – Nordic Region (Application No. 34250), NNF Excellence Emerging Investigator Grant – Endocrinology and Metabolism (Application No. NNF19OC0055001), Novartis Pharma AG, Basel, Switzerland (grant to GHG and EEB.), the National Institutes of Health (grants DK-098506 to SZ and P30 DK-017047 University of Washington Diabetes Research Center), and the United States Department of Veterans Affairs. Novo Nordisk Foundation Center for Protein Research is supported financially by the Novo Nordisk Foundation (Grant agreement NNF14CC0001).

Clinical Trial Information: ClinicalTrials.gov no. NCT03717688 and no. NCT01631864.

Author Contributions: SASK and NJWA designed the study. SASK and LH designed, performed and interpreted the MALDI-TOF mass spectrometry experiments. SASK, SZ, NE, SM, MWS, JEH, HK, FRC and KDG designed, performed and interpreted the animal experiments. MMR designed, performed and interpreted the pharmacological experiments. DT, PDM, PP, JPG, GHG, EB, JJH, CFD and NJWA designed, performed and interpreted the clinical studies. SASK wrote the manuscript draft. All authors revised the manuscript and accepted the final edition.
Additional Information

Conflicts of interest: SZ has previously received funding from Novartis, which ended in 2019. The remaining authors have no conflict of interest to declare.

Data Availability: The data sets generated are not publicly available but can be made available from the corresponding author upon reasonable request. Supplementary figures to this article and a detailed description of the materials and methods section can be found online in a digital research repository (42).
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Figure 1: NEP inhibition with sacubitril/valsartan increases plasma concentrations of glucagon in lean and obese individuals and reduces postprandial amino acid concentrations. Plasma concentrations of (a) glucagon and (b) amino acids in healthy young males treated with sacubitril/valsartan (black circles) followed by a mixed meal (34 % carbohydrates, 45 % fat and 21 % protein), or the meal alone (controls, open circles) (cohort previously published in (5)). Sacubitril/valsartan was administered as a single dose (194 mg sacubitril/206 mg valsartan) 30 min prior to the meal (given at time 0 min). The same individuals completed the control and sacubitril/valsartan treatments in a randomized open-labeled crossover design (n=8). (c) Individual concentrations of amino acids in healthy men before control or sacubitril/valsartan treatment at time point 90 min after the meal (n=6). Asparagine was not included in the column graph due to the dissimilar y-axes. Plasma concentrations of (d) glucagon, (e) amino acids and (f) glucose in individuals with obesity treated with sacubitril/valsartan for 8 weeks (n=7) (cohort previously published in (25)). Data are presented as mean ± SEM (b and c) and are analyzed by repeated measures two-way ANOVA (a and b) or paired t-tests (c, d, e and f).

Figure 2: NEP degrades glucagon into metabolites (Gcg1-21, Gcg1-19, Gcg1-18, Gcg5-18 and Gcg1-13) that do not activate the GCGR. MALDI-TOF-identified m/z’s in the m/z spectra 1500-4500 after recombinant (a) human and (b) mouse NEP (10 ng) mixed with glucagon (1 µg) after 15, 30 and 60 min incubation, and after 60 min incubation with glucagon, NEP and the NEP inhibitor sacubitrilat (0.5 nmol, 10 % DMSO) (active form of the prodrug, sacubitrilat). (c) GCGR activation (dose-response curve) for native glucagon (Gcg1-29)-induced cAMP accumulation in COS-7 cells transiently transfected with human GCGR. (d) Dose-response curves for cAMP accumulation with glucagon degradation products in COS-7 cells transiently transfected with human GCGR (3 technical replicates).

Figure 3: NEP inhibition increases plasma concentrations of glucagon in female C57BL/6JRj mice, perturbing amino acid metabolism. Plasma concentrations of (a) glucagon and (b) blood glucose concentrations in female C57BL/6JRj mice (n=10) treated with either vehicle (PBS + 0.7 % DMSO, open circles) or sacubitril (0.7 nmol/g BW, black circles) at time point -30 min followed by an i.p. injection of glucagon (96 ng/g BW) at time 0 min. (c) Experimental design used in female C57BL/6JRj mice (n=7-10) treated with either vehicle (black lines and circles), GRA (100 mg/kg BW, blue lines and squares) or Ex9-39 (fixed dose of 100 µg in 100 µl, red lines and triangles) with co-administration of vehicle (open symbols) or sacubitril (colored symbols) followed by oral administration of amino acids (AAs, 34 mg) at time 0 min.
Plasma concentrations of glucagon in mice treated with (d) sacubitril or vehicle, and in mice treated with (e) GRA, Ex93-39, co-administration of GRA and sacubitril or co-administration of Ex9-39 and sacubitril. (f) Blood glucose concentrations, plasma concentrations of (g) amino acids, (h) delta urea with (i) corresponding iAUCs and (j) insulin. Data are presented as mean ± SEM and are analyzed by repeated measures two-way ANOVA (a and d) and unpaired t-tests (h).

Figure 4: NEP deficient mice have increased fasting and amino acid-stimulated glucagon and urea concentrations. Plasma concentrations of (a) glucagon and (b) corresponding iAUCs, (c) blood glucose concentrations, plasma concentrations of (d) insulin, (e) body weight, plasma concentrations of (f) amino acids and (g) corresponding iAUCs, plasma concentrations of (h) urea and (i) corresponding iAUCs and plasma concentrations of (j) delta urea in male NEP^/-^ mice (black circles) and NEP^+/+^ mice (open circles) challenged amino acids (equivalent to 1% of BW) at time 0 min (n=5-6). Data are presented as mean ± SEM and are analyzed by repeated measures two-way ANOVA (a, c, d, f, h and j) and unpaired t-tests (b, e, g and i). -/- indicates NEP^-/^- mice and +/- indicates NEP^-/+^ mice.
Table 1: Identified glucagon fragments (m/z’s) and corresponding amino acid sequences

| Calculated m/z | Observed m/z | Amino acid sequence                  | ID       | Bond break               |
|----------------|--------------|--------------------------------------|----------|--------------------------|
| 3482.8         | 3481.4       | HSQGTFTSDYSKYLDSSRAQDFVQWLMNT        | Gcg1-29  | Native glucagon          |
| 2462.6         | 2461.3       | HSQGTFTSDYSKYLDSSRAQD               | Gcg1-21  | Asp21-Phe22              |
| 2219.4         | 2220.3       | HSQGTFTSDYSKYLDSSRA                 | Gcg1-19  | Ala19-Gln20              |
| 2148.3         | 2148.3       | HSQGTFTSDYSKYLDSSRR                 | Gcg1-18  | Arg18-Ala19              |
| 1738.9         | 1738.4       | TFTSDYSKYLDSSRR                     | Gcg5-18  | Gly4-Thr5, Arg18-Ala19   |
| 1520.6         | 1520.4       | HSQGTFTSDYSKY                      | Gcg1-13  | Tyr13-Leu14              |
