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Neprilysin Deficiency Reduces Hepatic Gluconeogenesis in High Fat-Fed Mice

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Abstract

Neprilysin is a peptidase that cleaves glucoregulatory peptides, including glucagon-like peptide-1 (GLP-1) and cholecystokinin (CCK). Some studies suggest that its inhibition in diabetes and/or obesity improves glycemia, and that this is associated with enhanced insulin secretion, glucose tolerance and insulin sensitivity. Whether reduced neprilysin activity also improves hepatic glucose metabolism has not been explored. We sought to determine whether genetic deletion of neprilysin suppresses hepatic glucose production (HGP) in high fat-fed mice. *Nep*$^{+/+}$ and *Nep*$^{-/-}$ mice were fed high fat diet for 16 weeks, and then underwent a pyruvate tolerance test (PTT) to assess hepatic gluconeogenesis. Since glycogen breakdown in liver can also yield glucose, we assessed liver glycogen content in fasted and fed mice. In *Nep*$^{-/-}$ mice, glucose excursion during the PTT was reduced when compared to *Nep*$^{+/+}$ mice. Further, liver glycogen levels were significantly greater in fasted but not fed *Nep*$^{-/-}$ versus *Nep*$^{+/+}$ mice. Since gut-derived factors modulate HGP, we tested whether gut-selective inhibition of neprilysin could recapitulate the suppression of hepatic gluconeogenesis observed with whole-body inhibition, and this was indeed the case. Finally, the gut-derived neprilysin substrates, GLP-1 and CCK, are well-known

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AUTHOR CONTRIBUTIONS

S.Z. conceived and designed the study, analyzed and interpreted data, and wrote the manuscript. N.E., S.M.M., T.O.M. and B.M.B. performed experiments, analyzed and interpreted data. All authors revised the manuscript and approved the final version to be published.

CONFLICT OF INTEREST

There is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

CRediT authorship contribution statement

Nathalie Esser: Methodology, Investigation, Writing- Reviewing and Editing. **Stephen M. Mongovin:** Methodology, Investigation, Data curation, Writing- Reviewing and Editing. **Thomas O. Mundinger:** Methodology, Investigation, Writing- Reviewing and Editing. **Breanne M. Barrow:** Methodology, Investigation, Validation, Data curation, Writing- Reviewing and Editing. **Sakeneh Zraika:** Conceptualization, Methodology, Formal analysis, Visualization, Writing- Original draft preparation, Funding acquisition.

to suppress HGP. Having previously demonstrated elevated plasma GLP-1 levels in *Nep*$^{-/-}$ mice, we now measured plasma CCK bioactivity and reveal an increase in *Nep*$^{-/-}$ versus *Nep*$^{+/+}$ mice, suggesting GLP-1 and/or CCK may play a role in reducing HGP under conditions of neprilysin deficiency. In sum, neprilysin modulates hepatic gluconeogenesis and strategies to inhibit its activity may reduce HGP in type 2 diabetes and obesity.

Keywords

neprilysin; gluconeogenesis; cholecystokinin; glucagon-like peptide-1; glycogenolysis; liver

1 INTRODUCTION

Neprilysin is a ubiquitous peptidase known to cleave glucoregulatory peptides [1]. Neprilysin protein and activity are upregulated in type 2 diabetes (T2D), obesity and metabolic syndrome [2, 3], and have been associated with impaired glucose tolerance, insulin resistance and insulin secretory dysfunction [3–5].

We and others have shown that neprilysin ablation/inhibition exerts beneficial glycemic effects in the setting of increased dietary fat and/or diabetes [4, 6–11]. For example, high fat-fed neprilysin deficient mice have improved glucose tolerance, insulin sensitivity and insulin secretion [4]. Similarly, diabetic mice treated with the neprilysin inhibitor, sacubitril, exhibit lower fasting and fed glucose levels, which is accompanied by enhanced insulin secretion [9, 10]. In humans, neprilysin inhibitors have not been used alone due to an effect to increase angiotensin II levels, which can be deleterious. However, combination of sacubitril with an angiotensin II receptor blocker (valsartan) may improve glycemic control in humans with T2D [12–14]. Given that sacubitril/valsartan results in greater reductions in HbA1c levels when compared to valsartan or another renin-angiotensin system inhibitor alone [13, 14], it is likely that the added benefit observed with sacubitril/valsartan is mediated by neprilysin inhibition. In these human studies, it is important to note that glycemic control was not the primary endpoint and that two of the studies utilized a post hoc analysis, which can be biased by several factors. Thus, caution is warranted when interpreting the data, especially in light of another human study that directly tested the effect of sacubitril/valsartan on glycemic status in the setting of obesity/diabetes and showed no benefit [15]. An important distinction amongst these studies is that the absence of a beneficial effect is seen only with acute sacubitril/valsartan administration, suggesting that long-term neprilysin inhibition may be required to elicit glycemic benefits in humans.

While the beneficial glycemic effects of neprilysin ablation/inhibition may be mediated, in part, by enhanced glucose tolerance, insulin sensitivity and/or insulin secretion, it remains unknown whether suppression of hepatic glucose production (HGP) is also a contributing factor. The liver produces glucose via gluconeogenesis and glycogenolysis, both of which are increased in T2D and obesity [16–18]. Neprilysin can cleave substrates known to lower HGP, some of which are produced in the gut, like glucagon-like peptide-1 (GLP-1) [19, 20] and cholecystokinin (CCK) [21]. Indeed, plasma levels of active GLP-1 are increased in high fat-fed *Nep*$^{-/-}$ mice [4], and some glycemic benefits of neprilysin inhibition are mediated

via GLP-1 receptor (GLP-1R) signaling [9, 22]. Also, postprandial plasma concentrations of CCK are increased in humans upon acute inhibition of neprilysin with sacubitril/valsartan [23].

Thus, in the present study, we sought to determine whether global genetic deletion of neprilysin suppressed HGP in high fat-fed and/or diabetic mice. Given that gut-derived factors modulate HGP, we also examined whether gut-selective inhibition of neprilysin could recapitulate any effect to suppress HGP that occurs with whole-body ablation. Having previously demonstrated that GLP-1 levels are elevated in the setting of neprilysin deficiency [4], and considering that CCK can both enhance GLP-1 production/secretion and lower HGP, we also assessed CCK bioactivity in mice lacking neprilysin.

2. MATERIALS AND METHODS

2.1 Animal models and treatments

Ten-week old male *Nep*$^{+/+}$ and *Nep*$^{-/-}$ mice on a C57BL/6 background (originally from B. Lu, Children's Hospital, Harvard Medical School, Boston, MA, USA [24]) were fed a high fat diet (HFD, 60% kcal fat, D12492; Research Diets Inc; New Brunswick, NJ, USA) for 16 weeks, as previously described [4]. Weekly food intake throughout the 16 weeks was calculated on a per cage basis, with 2 mice/cage. Phenotypic characteristics of this model have been published, including data on glucose tolerance, islet beta-cell function and mass, and insulin sensitivity [4, 25]. Active GLP-1 levels in plasma from *Nep*$^{+/+}$ and *Nep*$^{-/-}$ mice fed HFD for 16 weeks have also been reported previously [4].

At the end of the 16-week HFD period, mice were subjected to an intraperitoneal pyruvate tolerance test (PTT) as described below. Fifteen minutes prior to the PTT, a subset of *Nep*$^{+/+}$ mice received by oral gavage a single dose of the neprilysin inhibitor DL-thiorphan (5 μg/kg body weight, Sigma-Aldrich, St. Louis, MO, USA) or vehicle (0.9% NaCl). The dose of thiorphan was selected based on a pilot study testing the effect of doses ranging from 0.005 to 0.05 mg/kg on intestinal and plasma neprilysin activities (data not shown). *Nep*$^{+/+}$ mice were studied in a crossover design, wherein thiorphan and vehicle administration in any one mouse was separated by 2 weeks. Blood was collected via the lateral saphenous vein before and 85 minutes after thiorphan or vehicle administration (i.e., at the 70-minute timepoint during the PTT) to measure plasma neprilysin activity. This subset of mice was immediately euthanized after the PTT, and intestines (duodenum and colon) and kidneys harvested, frozen in liquid nitrogen and stored at −80°C prior to protein extraction for neprilysin activity.

All mice were housed two per cage, with a 12-hour light/12-hour dark cycle, and food and water ad lib. The study was approved by the Institutional Animal Care and Use Committee of VA Puget Sound Health Care System.

2.2 Pyruvate tolerance test

Pyruvate tolerance tests were performed in conscious mice fasted for 16 hours, wherein tail-tip blood was sampled at −5, 10, 20, 30, 45, 60, 90 and 120 minutes relative to intraperitoneal pyruvate (2 g/kg) injection. Blood glucose levels were measured using

an Accu-Chek Aviva Plus glucometer (Roche, Basel, Switzerland). To determine glucose excursions during the PTT, percent change of glucose from the baseline value was calculated, and area under the curve (AUC) was computed.

2.3 Liver glycogen content and gallbladder weights

For glycogen measures, livers were collected from high fat-fed $Nep_{+/+}$ and $Nep^{-/-}$ mice that were either ad lib fed (HFD) or fasted for 16 hours. For gallbladder collection, high fat-fed $Nep^{+/+}$ and $Nep^{-/-}$ mice were fasted for 16 hours. Mice were euthanized without undergoing a PTT. Liver and gallbladder (including contents) were harvested and weighed. Liver tissue was homogenized in $HClO_4$, after which an aliquot of supernatant was removed. To the aliquot, $KHCO_3$ and amyloglucosidase was added, and the samples incubated for 2 h at 37°C to facilitate glycogen hydrolysis. Free glucose was measured in the liver tissue homogenates using the glucose oxidase method. Glucose was also measured in the amyloglucosidase-digested samples as an index of glycogen content. Glycogen content per liver weight for each mouse was determined by subtracting ‘free glucose’ from ‘glucose from glycogen’.

2.4 Plasma glucagon measurement

At the end of the 16-week HFD period, arterial blood was collected from $Nep^{+/+}$ and $Nep_{-/-}$ mice that had been fasted for 16 hours. Plasma was separated and stored at −80°C prior to assay. Plasma glucagon concentrations were measured using a glucagon ELISA kit (Mercodia, Upsala, Sweden).

2.5 Neprilysin activity assay

Plasma, duodenum, colon and kidneys were collected from $Nep^{+/+}$ mice that received neprilysin inhibitor by oral gavage to assess neprilysin activity using an established fluorometric enzyme method, as previously described [4]. Tissues were homogenized in protein lysis buffer (50 mM HEPES, 150 mM NaCl, 0.5% Triton X-100, 25 mM ZnCl2, pH 7.4) containing EDTA-free protease inhibitor cocktail and phosphatase inhibitors, using a Bullet Blender tissue homogenizer (NextAdvance, NY, USA). The protein concentration of each extract was quantified via the bicinchoninic acid assay (Pierce, Waltham, MA, USA). For neprilysin activity, each plasma or tissue sample was assayed in both the absence and presence of a specific neprilysin inhibitor (DL-thiorphan, Sigma-Aldrich, St Louis, MO, USA) to differentiate neprilysin activity from non-specific endopeptidase activity.

2.6 Real-time quantitative RT-PCR

For colon collection, high fat-fed $Nep^{+/+}$ and $Nep^{-/-}$ mice were ad lib fed (HFD), then euthanized. For liver collection, high fat-fed $Nep^{+/+}$ and $Nep^{-/-}$ mice were fasted for 16 hours, then euthanized. Colon and liver were homogenized in lysis buffer from the High Pure RNA Isolation Kit (Roche, Basel, Switzerland), using a Bullet Blender tissue homogenizer (NextAdvance). Total RNA was extracted using the same kit, then reverse transcribed to cDNA. Relative mRNA levels of *Gcg* and *Psck1* in colon, and *Ppargc1a, Pcx, Pck1, Fbp1, G6pc* and *Pygl* in liver were determined by real-time quantitative RT-PCR

using TaqMan probes (Table 1; Thermo Fisher Scientific, Waltham, MA, USA). TaqMan eukaryotic 18S rRNA was used as endogenous control.

2.7 *In vitro* intestinal cell culture

STC-1 cells were purchased from ATCC (# CRL-3254; Manassas, VA, USA) and maintained in Dulbecco's Modified Eagle Medium (DMEM) containing 5.5 mM glucose and 10% fetal bovine serum. 1×10^6 cells were grown to 50–70% confluency in T75 flasks. Cells were then cultured for 24 h in DMEM containing 25 mM glucose + 0.1 mM palmitate (complexed to BSA in a 5:1 molar ratio) with 20 μM thiorphan or vehicle. After 24 h, media was replaced with Earle's Balanced Salt Solution (EBSS) containing 50 mM KCl + 20 μM thiorphan or vehicle to stimulate CCK release, as previously described [26]. After 2 h incubation, EBSS was collected and stored at −20°C for amylase assay. Cells were lysed for measurement of neprilysin activity, as described above.

2.8 Amylase secretion assay

A well-established CCK bioassay [27–29] was used to quantify CCK bioactivity in plasma and EBSS from incubations of STC-1 cells. This bioassay was chosen over direct measurement of CCK via commercially available immunoassays because methodology for the latter relies on antibodies for detection of CCK, yet these antibodies cannot distinguish between intact CCK and any neprilysin-derived CCK fragments. In contrast, the bioassay measures the ability of a sample (e.g., plasma from $Nep^{+/+}$ and $Nep^{-/-}$ mice) to stimulate amylase secretion from primary pancreatic acini. Amylase secretion is CCK-1 receptor-dependent and proportional to the amount of CCK-8 present in the sample. To validate the bioassay, we included positive and negative controls, as well as different sample types that are expected to elicit opposing effects on CCK-stimulated amylase secretion to ensure we could detect increases and decreases in CCK bioactivity. Specifically, we incubated acini with synthetic CCK-8 or vehicle, plasma from fed or fasted mice, or EBSS samples comprising carbachol since the latter is known to inhibit CCK-induced stimulation of amylase secretion [30, 31].

For our measurements, we collected (i) plasma from a subset of high fat-fed $Nep^{+/+}$ and $Nep^{-/-}$ mice 120 minutes after an oral glucose (2 g/kg) bolus, and (ii) EBSS following incubation with STC-1 cells. Pancreatic acini were isolated from 8–12-week old standard chow-fed (PicoLab Rodent Diet 20 #5053, LabDiet, St. Louis, MO, USA) $Nep^{+/+}$ mice by collagenase digestion [32], then incubated for 2 h with plasma from $Nep^{+/+}$ and $Nep^{-/-}$ mice or EBSS from STC-1 cell incubations. Supernatant was collected for measurement of amylase levels using an Amylase Assay Kit (#ab102523, Abcam, Cambridge, MA, USA), and acini were lysed for protein quantification via the bicinchoninic acid assay (Thermo Fisher Scientific). Amylase levels were normalized to acini protein content.

2.9 Statistical analyses

Data are presented as mean ± SEM, with bar graphs also showing individual values. PTT data were analyzed via two-way analysis of variance (ANOVA), with time and genotype or treatment being the two variables. A Student's *t*-test or Mann-Whitney U test (if data were not normally distributed) was used to compare two groups. A $p<0.05$ was considered

statistically significant. Statistical analysis was performed using GraphPad Prism (v. 9.5.0 for Mac; GraphPad Software, San Diego, CA, USA).

3 RESULTS

3.1 Hepatic glucose production is reduced in high fat-fed *Nep*$^{-/-}$ mice

We previously demonstrated that *Nep*$^{-/-}$ mice fed a high fat diet for 16 weeks had reduced glucose levels when compared to *Nep*$^{+/+}$ mice on high fat diet [4]. To determine whether whole-body neprilysin ablation suppressed hepatic gluconeogenesis, a PTT was performed in high fat-fed mice that had been fasted for 16 hours. As shown in Figures 1A and 1B, glucose excursion during the PTT was significantly reduced in *Nep*$^{-/-}$ versus *Nep*$^{+/+}$ mice. In addition to HGP from pyruvate, glycogenolysis can also yield glucose from the breakdown of liver glycogen. Thus, we assessed liver glycogen content in fasted and fed mice. As expected, glycogen levels were increased in the fed versus fasted state in both genotypes (Figure 1C). Moreover, glycogen levels in the fasted state were significantly elevated in *Nep*$^{-/-}$ versus *Nep*$^{+/+}$ mice, whereas no difference was observed between genotypes in the fed state (Figure 1C). We also assessed liver mRNA expression of key genes involved in gluconeogenesis and glycogenolysis. Following a 16-hour fast, mRNA levels of *Ppargc1a* (p=0.038) and *Pcx* (p=0.054) were reduced, and *Pck1* (p=0.079) tended to be reduced in *Nep*$^{-/-}$ mice (Figure 1D). *Fbp1, G6pc* and *Pygl* mRNA levels were unchanged between genotypes (Figure 1D).

3.2 Gut-selective inhibition of neprilysin reduces hepatic gluconeogenesis in high fat-fed *Nep*$^{+/+}$ mice

Several gut-derived factors, including the neprilysin substrates GLP-1 and CCK, modulate hepatic gluconeogenesis. Thus, we determined whether selective inhibition of neprilysin in the gut would be sufficient to suppress hepatic gluconeogenesis in high fat-fed mice. Fifteen minutes prior to a PTT, *Nep*$^{+/+}$ mice received a single dose (5 μg/kg) of the neprilysin inhibitor, thiorphan, via oral gavage. Thiorphan did not alter neprilysin activity in the plasma or kidney, the latter a major site of neprilysin (Table 2). In contrast, thiorphan significantly reduced neprilysin activity in the gut, as demonstrated in proximal (duodenum) and distal (colon) gut segments (Table 2). During the PTT, glucose excursion was significantly reduced in mice that received thiorphan versus vehicle (Figures 2A, B).

3.3 CCK bioactivity is elevated under conditions of reduced neprilysin activity

Like GLP-1, CCK is also a gut-derived neprilysin substrate that can lower HGP [33]. To determine whether reduced neprilysin activity increases CCK levels, we first employed an *in vitro* model involving CCK-producing intestinal STC-1 cells cultured in high glucose/fat conditions, akin to the milieu in the mouse model we utilized. Following treatment with thiorphan, neprilysin activity in STC-1 cells was completely blunted (vehicle 5.0±0.4 vs thiorphan 0.0±0.0 pmol MNA/h/μg protein; n=3, p=0.007), and CCK bioactivity increased by ~50% (Figure 3A, measured as amylase secretion).

We next assessed whether plasma CCK bioactivity/levels were elevated in high fat-fed *Nep*$^{-/-}$ mice, a model in which we previously showed plasma levels of active GLP-1 levels

to be elevated [4]. Similar to data from STC-1 cells, CCK bioactivity was increased by ~50% in *Nep*$^{-/-}$ mice, relative to levels in *Nep*$^{+/+}$ mice (Figure 3B, measured as amylase secretion). Given that CCK can act as a satiety signal, we measured weekly food intake and found that it was comparable between *Nep*$^{+/+}$ and *Nep*$^{-/-}$ mice throughout the 16-week high fat feeding period (Figure 4A). In contrast, elevated CCK bioactivity in *Nep*$^{-/-}$ mice was accompanied by a trend for reduced gallbladder weight (*Nep*$^{-/-}$ 26±9 vs *Nep*$^{+/+}$ 48±6 mg; n=5–8, p=0.0876), which is consistent with CCK's ability to stimulate gallbladder contraction and bile acid delivery to the gut. Since the latter can trigger induction of GLP-1 levels in the gut [34, 35], we measured expression of prohormone convertase 1/3 (*Pcsk1*), which cleaves proglucagon to generate GLP-1 in L cells of the colon. *Pcsk1* mRNA levels were elevated in colon from *Nep*$^{-/-}$ versus *Nep*$^{+/+}$ mice, whereas *Gcg* mRNA levels were unchanged (Figure 4B). In addition, fasting plasma glucagon levels were unchanged in high fat-fed *Nep*$^{-/-}$ vs *Nep*$^{+/+}$ mice (1.28±0.10 vs 1.53±0.19 pM; n=9–10, p=0.3)

4 DISCUSSION

In this study, we show that neprilysin ablation/inhibition suppresses hepatic gluconeogenesis, demonstrated as reduced glycemic excursion in response to intraperitoneal administration of pyruvate in mice fed high fat diet for 16 weeks. This was observed in two settings: (i) whole-body genetic deletion of neprilysin, and (ii) acute pharmacological inhibition of neprilysin specifically in the gut. To our knowledge, these data are the first to demonstrate a role for neprilysin (and intestinal neprilysin *per se*) in modulating HGP.

Previously, we found that genetic [4] or pharmacological [9, 10] ablation of neprilysin activity in obese/diabetic mice was associated with reduced fasting and/or fed glucose levels. While this beneficial glycemic effect was accompanied by improved glucose tolerance, enhanced insulin secretion, and/or increased whole-body insulin sensitivity [4, 9, 10], an effect to lower glucose production by the liver was not investigated. Indeed, suppression of HGP may also contribute to improved glycemia in mice lacking neprilysin activity. Conversely, it is possible that the improved metabolic phenotype under conditions of neprilysin deficiency drives suppression of HGP, given that the liver participates in inter-organ crosstalk for its glucose metabolism. The liver produces glucose via gluconeogenesis and glycogenolysis. In addition to observing reduced gluconeogenesis (via PTT) in high fat-fed *Nep*$^{-/-}$ mice in the present study, liver glycogen stores in the fasting state were also elevated, raising the possibility that neprilysin deficiency limits glucose production from glycogenolysis. When we assessed liver mRNA expression of key genes involved in gluconeogenesis and glycogenolysis, we found that high fat-fed *Nep*$^{-/-}$ mice exhibited significantly lower levels of *Ppargc1a*, which is a key regulator of gluconeogenesis that acts by inducing expression of gluconeogenic enzymes. One of these enzymes is phosphoenolpyruvate carboxykinase 1 (encoded by *Pck1*), whose mRNA expression tended to be reduced in high fat-fed *Nep*$^{-/-}$ mice. Similarly, expression of the gene encoding the enzyme that catalyzes the first committed step for gluconeogenesis, namely *Pcx*, was reduced in high fat-fed *Nep*$^{-/-}$ mice. *Fbp1* and *G6pc* also encode for gluconeogenic enzymes, however their mRNA levels were unaltered in *Nep*$^{-/-}$ vs *Nep*$^{+/+}$ mice. Also, expression of *Pygl*, which encodes for the glycogenolytic enzyme glycogen phosphorylase, was comparable in *Nep*$^{-/-}$ vs *Nep*$^{+/+}$ mice. Together, these mRNA data suggest that some

but not all the effects observed with respect to glucose excursion during the PTT and liver glycogen content in *Nep*$^{-/-}$ mice may involve transcriptional changes in key genes within the liver.

Neprilysin is known to cleave substrates capable of lowering HGP. One of these substrates is GLP-1, wherein neprilysin cleaves and inactivates up to 50% of GLP-1 entering the circulation [8, 19, 20]. We and others have shown that active GLP-1 levels are elevated under conditions of neprilysin ablation/inhibition [4, 12, 36] and that GLP-1R signaling contributes to the beneficial glycemic and insulinotropic effects of neprilysin inhibition [9, 22]. GLP-1 suppresses HGP via different pathways. First, a gut-pancreas-liver axis in which GLP-1 secreted from enteroendocrine L cells potentiates insulin secretion, which in turn lowers HGP [37]. Second, a gut-brain-liver axis in which GLP-1 secreted from enteroendocrine L cells activates GLP-1Rs and PKC-δ in intestinal mucosal cells to trigger neuronal-dependent lowering of HGP – this occurs independent of changes in circulating insulin levels [38]. Third, bile acid-dependent activation of intestinal TGR5 (G protein-coupled bile acid receptor 1, GPBAR-1) stimulates GLP-1 production and release from enteroendocrine L cells [34, 35], which can ultimately act to lower HGP via the two pathways described above. It is possible that one, some or all these pathways are operative under conditions of neprilysin deficiency. In support of the gut-pancreas-liver axis, we have previously shown that mice with either genetic or pharmacological ablation of neprilysin activity have enhanced insulin release [4, 5, 9, 10].

Further insight into potential mechanisms contributing to suppression of HGP with neprilysin deficiency can be gleaned from our data showing that gut-selective inhibition of neprilysin activity is sufficient to lower HGP. Specifically, these data suggest that neprilysin localized to non-intestinal sites is not necessary for modulating HGP, and conversely, that signals emanating from the gut are critical players. Aside from GLP-1, CCK is another gut-derived hormone that is a neprilysin substrate [21] and can lower HGP [33]. We show that neprilysin inhibition in intestinal STC-1 cells cultured in high glucose/fat conditions increases CCK bioactivity/levels. Further, CCK bioactivity/levels are elevated in high fat-fed *Nep*$^{-/-}$ mice that exhibit reduced hepatic gluconeogenesis. Previously, it was established that CCK secreted from enteroendocrine I cells activates CCK1 receptors in the duodenum to trigger neuronal signaling and ultimately lower HGP via a gut-brain-liver axis – this occurs independent of changes in circulating insulin levels [33]. CCK can also bind CCK1 receptors located on the gallbladder to mediate its contraction for bile acid release into the gut. Bile acids then bind receptors including TGR5 and nuclear farnesoid X receptor (FXR). TGR5 activation induces expression of *Psck1* (which cleaves proglucagon to generate GLP-1) and production/secretion of GLP-1 [34, 35], whereas FXR activation induces fibroblast growth factor 15/19 (mouse FGF15, human FGF19) [39]. As described above, GLP-1 can act through multiple routes to lower HGP. On the other hand, FGF15/19 represses hepatic gluconeogenesis through a pathway involving inhibition of the CREB-PGC-1α signaling cascade in the liver [40]. Given that in high fat-fed *Nep*$^{-/-}$ mice we observe (i) a trend for reduced gallbladder weight, (ii) increased colon expression of *Psck1*, and (iii) elevated plasma levels of GLP-1 [4], we posit that the higher CCK bioactivity/levels in these mice trigger bile acid-dependent activation of TGR5. That said, it cannot be ruled out that FXR is also activated by CCK-mediated gallbladder contraction, and/or that CCK

drives gut-brain-liver signaling to reduce HGP – additional studies are needed to explore these possibilities. Another factor to consider is that the documented effect of CCK to induce satiety may contribute to suppression of HGP in high fat-fed *Nep*$^{-/-}$ mice; however, we feel this is unlikely since food intake did not differ between *Nep*$^{+/+}$ and *Nep*$^{-/-}$ mice throughout the 16-week high fat feeding period.

A limitation of our study is that we utilized the pyruvate tolerance test as a measure of hepatic gluconeogenesis. In interpreting results derived from this test, we assumed that the change in blood glucose in response to intraperitoneal pyruvate administration reflects glucose production mostly by the liver. While pyruvate is indeed converted into glucose primarily by the liver, it is also utilized by other tissues. The latter could affect blood glucose levels, though the contribution from extra-hepatic tissues is unlikely to explain the marked reductions in glucose excursion observed with neprilysin ablation/inhibition. Another consideration in interpreting our data is that neprilysin has broad substrate specificity, thus it is possible levels of substrates besides GLP-1 and CCK that are known to modulate HGP may have been elevated under conditions of neprilysin deficiency. One such example is glucagon [41, 42] whose plasma levels in humans treated acutely (single dose) or chronically (8 weeks) with sacubitril/valsartan were shown to be elevated [15, 41] While glucagon is well known to stimulate HGP, at least one study shows that the elevated glucagon levels observed in the setting of neprilysin inhibition did not affect glucose levels [41]. To explain such a finding, it has been proposed that increased GLP-1R signaling in neprilysin deficient states may counteract the effects of glucagon on glucose levels [41]. It is important to acknowledge that in some cases of neprilysin deficiency, increases in both glucagon and glucose levels have been observed [15, 41] – this includes in lean *Nep*$^{-/-}$ mice. However, our data show that in high fat-fed *Nep*$^{-/-}$ mice, fasting plasma glucagon levels are comparable to those observed in high fat-fed *Nep*$^{+/+}$ mice; thus, we feel glucagon is unlikely to play a major role in the effects we observed on HGP in high fat-fed *Nep*$^{-/-}$ mice. It is presently unclear whether high fat-fed *Nep*$^{+/+}$ mice treated acutely with oral thiorphan have elevated glucagon levels. Based on our published data, we know that the neprilysin inhibitor sacubitril does not alter fasting plasma glucagon levels in high fat-fed wild-type mice made diabetic with low-dose streptozotocin [9]. Thus, we feel glucagon is unlikely to be differentially modulating HGP in states with versus without neprilysin activity in the present study.

In conclusion, to our knowledge, our data provide the first evidence that neprilysin modulates HGP. That is, genetic deletion of neprilysin suppresses HGP in high fat-fed mice, and such an effect can be achieved by selectively targeting neprilysin in the gut. Thus, strategies to inhibit neprilysin activity may reduce HGP in type 2 diabetes and obesity.

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Abbreviations:

CCK	cholecystokinin
GLP-1	glucagon-like peptide-1
GLP-1R	GLP-1 receptor
HFD	high fat diet
HGP	hepatic glucose production
PTT	pyruvate tolerance test
T2D	type 2 diabetes

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HIGHLIGHTS

- Whole-body neprilysin deficiency reduces hepatic glucose production
- Gut-selective inhibition of neprilysin reduces hepatic glucose production
- Plasma cholecystokinin bioactivity is elevated with neprilysin deficiency

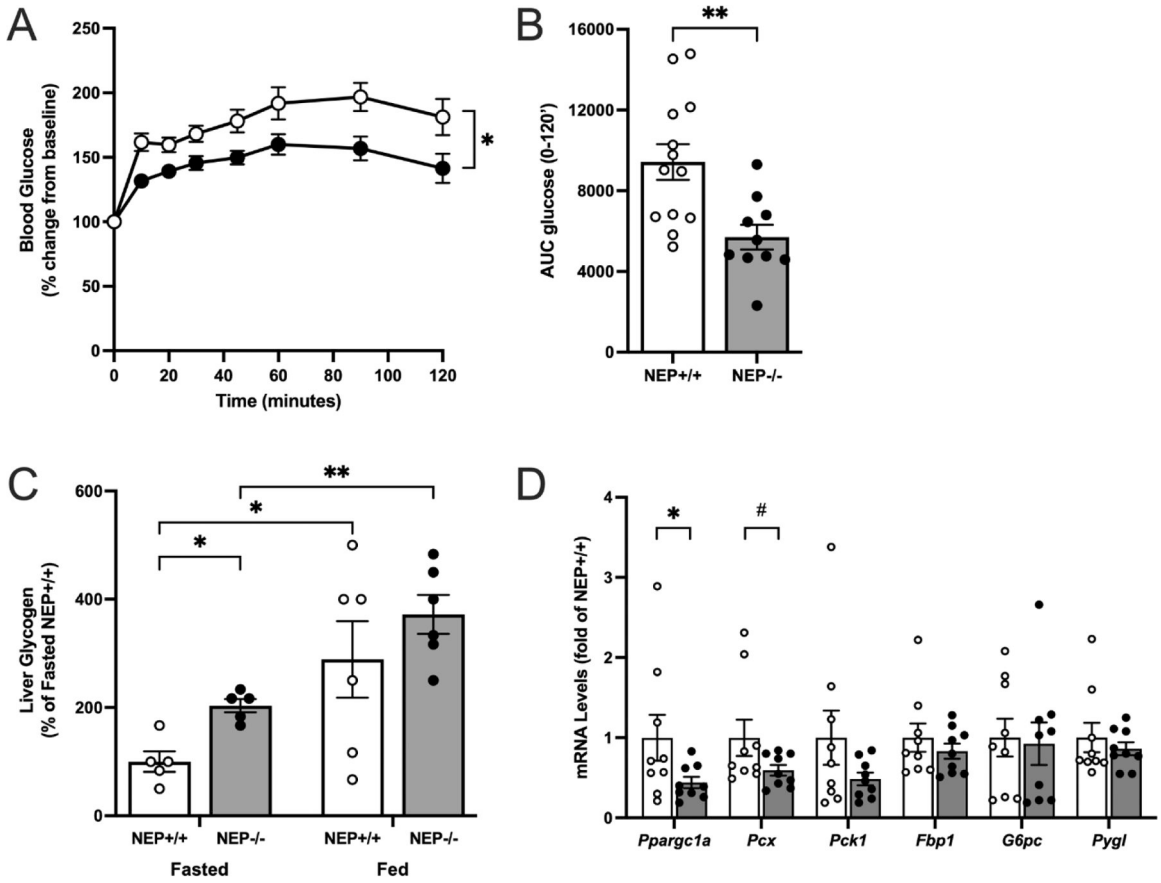

Figure 1: High fat-fed *Nep*$^{-/-}$ mice display decreased glucose excursion in response to pyruvate and increased liver glycogen content in the fasting state compared to *Nep*$^{+/+}$ mice.

(A) Blood glucose over time and (B) corresponding area under the curve (AUC) during a PTT in high fat-fed *Nep*$^{+/+}$ (n=13) and *Nep*$^{-/-}$ (n=10) mice. (C) Liver glycogen content in high fat-fed *Nep*$^{+/+}$ (n=5–6) and *Nep*$^{-/-}$ (n=5–6) mice following a 16 h fast or ad lib feeding. (D) mRNA levels of key gluconeogenic and glycogenolytic liver genes in high fat-fed *Nep*$^{+/+}$ (n=9) and *Nep*$^{-/-}$ (n=9) mice following a 16 h fast. *Nep*$^{+/+}$ mice, open circles; *Nep*$^{-/-}$ mice, closed circles. *p<0.05 (by ANOVA) in panel A, **p<0.01 in panels B and C, *p<0.05 in panels C and D, #p≤0.05 in panel D.

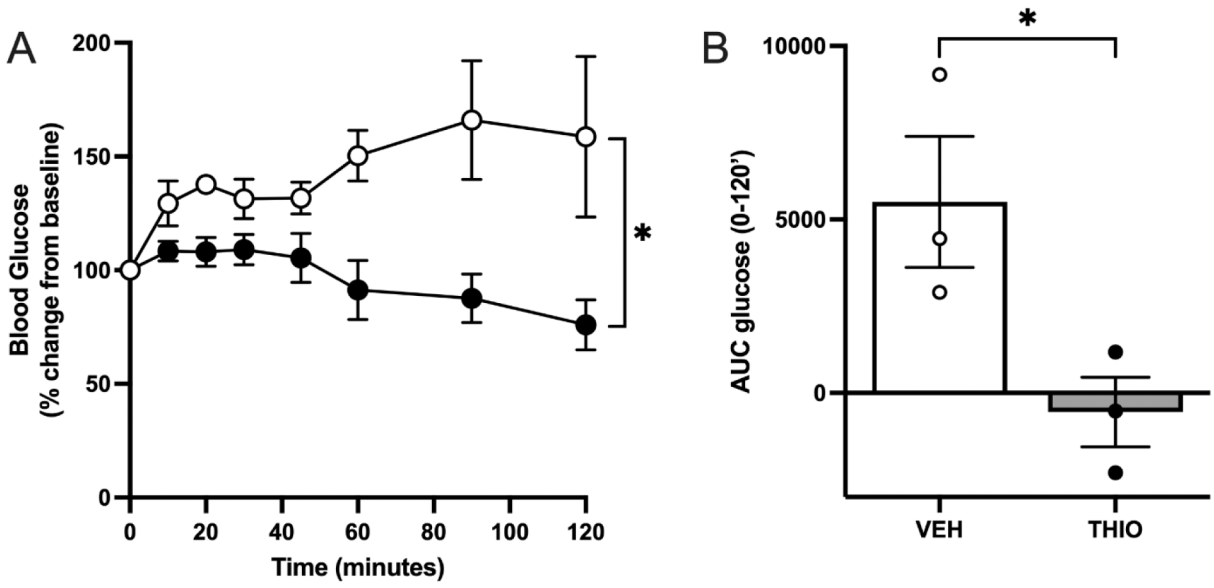

Figure 2: Gut-selective inhibition of neprilysin reduces glucose excursion in response to pyruvate in high fat-fed *Nep*$^{+/+}$ mice.

(A) Blood glucose over time and (B) corresponding area under the curve (AUC) during a PTT in high fat-fed *Nep*$^{+/+}$ mice that received vehicle (n=3) or thiorphan (n=3) via oral gavage. Vehicle (VEH), open circles; Thiorphan (THIO), closed circles. *p<0.05 (by ANOVA) in panel A, *p<0.05 in panel B.

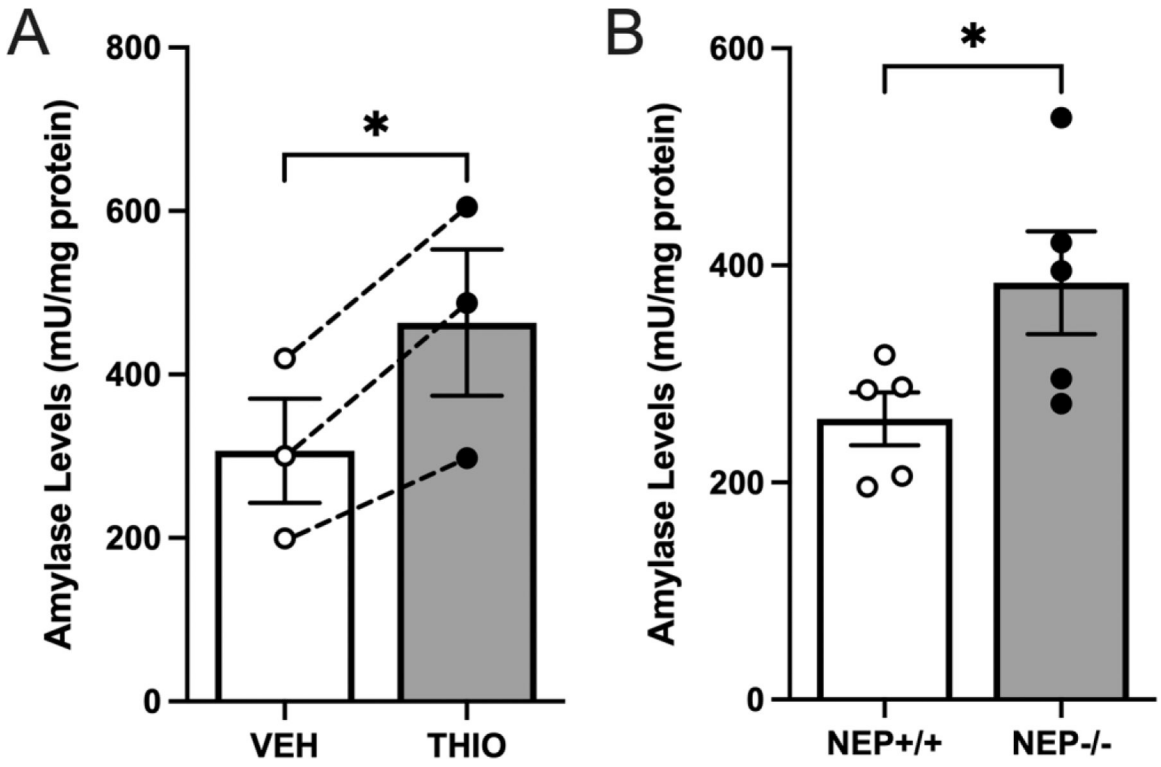

Figure 3: CCK bioactivity is elevated from immortalized intestinal cells treated with a neprilysin inhibitor and in plasma from high fat-fed *Nep*$^{-/-}$ versus *Nep*$^{+/+}$ mice.

A bioassay involving amylase secretion was utilized to assess CCK bioactivity/levels from (A) STC-1 cells cultured in 25 mM glucose and 0.1 mM palmitate with 20 μM thiorphan (n=3; THIO, closed circles) or vehicle (n=3; VEH, open circles), or (B) plasma from high fat-fed *Nep*$^{+/+}$ (n=5; open circles) and *Nep*$^{-/-}$ (n=5; closed circles) mice. Dashed lines in panel A indicate paired samples. *p<0.05 in panels A and B.

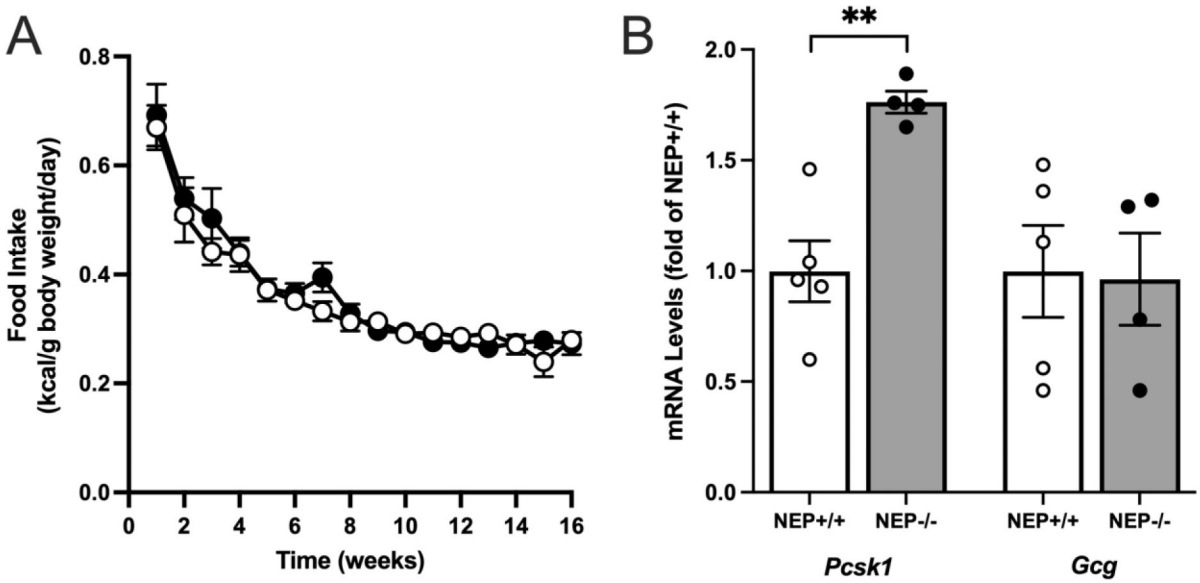

Figure 4: Food intake is unchanged in high fat-fed *Nep*$^{-/-}$ versus *Nep*$^{+/+}$ mice, whereas expression of the enzyme that cleaves proglucagon to generate GLP-1 in the intestine is increased.

(A) Weekly food intake throughout the 16 weeks in high fat-fed *Nep*$^{+/+}$ (n=12 cages) and *Nep*$^{-/-}$ (n=9 cages) mice, calculated on a per cage basis with 2 mice/cage. (B) mRNA expression of *Pcsk1* and *Gcg* in colon from high fat-fed *Nep*$^{+/+}$ (n=5; open circles) and *Nep*$^{-/-}$ (n=4; closed circles) mice. **p<0.01 in Panel B.

Table 1:

List of TaqMan probes used in real-time quantitative RT-PCR

Gene Symbol	Gene Name	TaqMan Assay Number
18S	eukaryotic 18S rRNA	Hs99999901_s1
Fbp1	fructose bisphosphatase 1	Mm00490181_m1
Gcg	glucagon	Mm00801712_m1
G6pc	glucose-6-phosphatase	Mm00839363_m1
Pck1	phosphoenolpyruvate carboxykinase 1	Mm01247058_m1
Psck1	proprotein convertase subtilisin/kexin type 1	Mm00479023_m1
Pcx	pyruvate carboxylase	Mm00500992_m1
Ppargc1a	peroxisome proliferative activated receptor, gamma, coactivator 1 alpha	Mm01208835_m1
Pygl	liver glycogen phosphorylase	Mm01289790_m1

Table 2:

Oral thiorphan selectively reduces neprilysin activity in the intestines of high fat-fed *Nep*+/+ mice.

Neprilysin activity (% of vehicle) in plasma and tissues from *Nep*$^{+/+}$ mice that received vehicle or a single dose of the neprilysin inhibitor thiorphan (5 μg/kg) via oral gavage. All values are mean±SEM. Plasma values are calculated as change from baseline in each mouse.

	Vehicle (n=3)	Thiorphan (n=3)	p value
Plasma	100±16	94±14	0.7932
Kidney	100±12	91±13	0.6360
Duodenum	100±4	18±4	0.0008
Colon	100±33	0±0	0.0401