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Brief Report

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Acute Inhibition of Intestinal Neprilysin Enhances Insulin Secretion via GLP-1 Receptor Signaling in Male Mice

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Abstract

The peptidase neprilysin modulates glucose homeostasis by cleaving and inactivating insulinotropic peptides, including some produced in the intestine such as glucagon-like peptide-1 (GLP-1). Under diabetic conditions, systemic or islet-selective inhibition of neprilysin enhances beta-cell function through GLP-1 receptor (GLP-1R) signaling. While neprilysin is expressed in intestine, its local contribution to modulation of beta-cell function remains unknown. We sought to determine whether acute selective pharmacological inhibition of intestinal neprilysin enhanced glucose-stimulated insulin secretion under physiological conditions, and whether this effect was mediated through GLP-1R. Lean chow-fed *Glp1r*$^{+/+}$ and *Glp1r*$^{-/-}$ mice received a single oral low dose of the neprilysin inhibitor thiorphan or vehicle. To confirm selective intestinal neprilysin inhibition, neprilysin activity in plasma and intestine (ileum and colon) was assessed 40 minutes after thiorphan or vehicle administration. In a separate cohort of mice, an oral glucose tolerance test was performed 30 minutes after thiorphan or vehicle administration to assess glucose-stimulated insulin secretion. Systemic active GLP-1 levels were measured in plasma collected 10 minutes after glucose administration. In both *Glp1r*$^{+/+}$ and *Glp1r*$^{-/-}$ mice, thiorphan inhibited neprilysin activity in ileum and colon without altering plasma neprilysin activity or active GLP-1 levels. Further, thiorphan significantly increased insulin secretion in *Glp1r*$^{+/+}$ mice, whereas it did not change insulin secretion in *Glp1r*$^{-/-}$ mice. In conclusion, under physiological conditions, acute pharmacological inhibition of intestinal neprilysin increases glucose-stimulated insulin secretion in a GLP-1R–dependent manner. Since intestinal neprilysin modulates beta-cell function, strategies to inhibit its activity specifically in the intestine may improve beta-cell dysfunction in type 2 diabetes.

Key Words: neprilysin, thiorphan, GLP-1, insulin secretion, mouse

Abbreviations: DPP-4, dipeptidyl peptidase-4; GLP-1, glucagon-like peptide-1; GLP-1R, GLP-1 receptor; OGTT, oral glucose tolerance test; QUICKI, quantitative insulin sensitivity check index.

Activity of the peptidase neprilysin is increased in obesity and type 2 diabetes (1, 2). Clinical studies have reported that use of a neprilysin inhibitor combined with an angiotensin II receptor blocker in obese individuals with or without type 2 diabetes improves insulin sensitivity (3) and glycemic control (4, 5). In mice, global deletion or systemic pharmacological inhibition of neprilysin has also been associated with improved islet beta-cell function (2, 6, 7).

Neprilysin cleaves and inactivates insulinotropic peptides, such as the incretin glucagon-like peptide-1 (GLP-1) (8, 9). In response to a meal, GLP-1 is produced by enteroendocrine L cells, mainly located in the distal ileum and colon. It modulates glucose homeostasis via endocrine and neuronal modes of action involving potentiation of insulin secretion and inhibition of glucagon secretion, as well as regulation of gastric emptying and satiety (10, 11). Of note, GLP-1 is also produced by islet alpha cells (12, 13). Islet-derived GLP-1 is also important for glucose homeostasis as it can enhance insulin secretion via paracrine signaling from alpha-to-beta cells (14-16). We have reported that inhibition of systemic or islet neprilysin improves beta-cell function via GLP-1R–dependent mechanisms (6, 17). While neprilysin is expressed in enterocytes (18), it is unknown whether intestinal neprilysin per se can modulate beta-cell function and whether any such effect occurs via GLP-1R signaling.

Here, we examine whether an acute selective pharmacological inhibition of neprilysin in the gut enhances oral glucose–stimulated insulin secretion in vivo in lean chow-fed mice. By using *Glp1r*$^{+/+}$ and *Glp1r*$^{-/-}$ mice, we assess whether any such effect is mediated through GLP-1R.

Materials Methods

Animals

Glp1r$^{+/-}$ mice were provided by Daniel Drucker (University of Toronto, Canada) to generate *Glp1r*$^{+/+}$ and *Glp1r*$^{-/-}$ mice on a C57BL/6J background at VA Puget Sound Health Care System (VAPSHCS). Mice were housed in a controlled environment with a 12 hours light/12 hours dark cycle, fed a chow diet containing 3% calories from fat and 21% from protein (PicoLab Rodent Diet 20 #5053, LabDiet, St. Louis, MO, USA), and had access to food and water ad libitum. The study was approved by the VAPSHCS Institutional Animal Care and Use Committee.

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Procedures

Age- and body weight–matched 10- to 16-week old male *Glp1r*$^{+/+}$ and *Glp1r*$^{-/-}$ mice were fasted for 6 hours, anesthetized with isoflurane, and then underwent catheterization of the left carotid artery. After a 60-minute stabilization period to clear the potential metabolic effects of acute surgery, mice received by oral gavage a single dose of the neprilysin inhibitor DL-thiorphan (0.05 mg/kg body weight, Sigma-Aldrich, St. Louis, MO, USA) or vehicle (0.9% NaCl), followed 30 minutes later by oral administration of glucose (2 g/kg). The dose of thiorphan was chosen based on a pilot study testing the effect of doses ranging from 0.005 to 50 mg/kg on intestinal and plasma neprilysin activities (data not shown). Mice then went on to receive 1 of the following procedures.

A first cohort of mice was allocated for assessment of plasma and intestinal neprilysin activity. Blood was collected via carotid catheter in heparinized tubes before and 40 minutes after thiorphan or vehicle administration to measure plasma neprilysin activity. Thereafter, mice were immediately euthanatized and intestines (distal ileum and colon) were harvested, frozen in liquid nitrogen and stored at −80 °C prior to protein extraction for neprilysin activity.

In another cohort of mice, an oral glucose tolerance test (OGTT) was performed 30 minutes after oral DL-thiorphan (0.05 mg/kg) or vehicle administration. Blood was collected via carotid catheter in heparinized tubes at 30 and 5 minutes before oral glucose and at 0, 10, 20, 30, 60, 90, and 120 minutes post oral glucose for measurement of glucose and insulin levels. Incremental area under the curve for glucose and insulin from 0 to 30 minutes were calculated. The quantitative insulin sensitivity check index (QUICKI) was calculated as follows: $1/[\log(I_0) + \log(G_0)]$, where I_0 and G_0 are fasting plasma insulin (µU/mL) and blood glucose (mg/dL) levels, respectively, measured immediately prior to oral glucose administration.

Blood collected via carotid catheter 10 minutes after glucose administration in both cohorts was used to measure active GLP-1 levels. Aprotinin (500 kU/mL), EDTA (3.2 mmol/L), and diprotin A (0.01 mmol/L) were added to the blood upon collection to avoid GLP-1 degradation.

All plasma samples were stored at −80 °C prior to assay.

Protein Tissue Extraction

Sections of frozen colon and ileum were homogenized in protein lysis buffer (50 mmol/L HEPES, 150 mmol/L NaCl, 0.5% Triton X-100, 25 mmol/L $ZnCl_2$, pH 7.4) containing EDTA-free protease inhibitor cocktail and phosphatase inhibitors, using a Bullet Blender Tissue Homogenizer (NextAdvance). The protein concentration of each extract was quantified via the bicinchoninic acid assay (Pierce, Waltham, MA, USA).

Glucose, Insulin, Active GLP-1, and Neprilysin Activity Levels Measurements

Blood glucose levels were measured using an Accu-Chek Aviva Plus glucometer (Roche, Basel, Switzerland). Plasma insulin and active GLP-1 levels were determined using a mouse ultrasensitive insulin enzyme-linked immunosorbent assay (Alpco, Salem, NH, USA; Cat#80-INSMSU-E01; RRID:AB_2792981) and active GLP-1 immunoassay (Meso Scale Discovery, Rockville, MD, USA; cat#K1503OD, RRID:AB_2935695), respectively. Neprilysin activity was assessed in plasma (5 µL) and intestine extracts (40 µg) by an established fluorometric enzyme method, as previously described (2, 6). Briefly, glutaryl-ala-ala-phe-4-methoxy-2-naphthylamine is broken down by neprilysin in samples to phe-4-methoxy-2 -naphthylamine and then the fluorescent product methoxy-2 -naphthylamine by aminopeptidase M. Each sample was assayed both in the absence or presence of thiorphan to differentiate neprilysin activity from nonspecific endopeptidase activity. Fluorescence was compared against a methoxy-2 -naphthylamine standard curve.

Statistical Analyses

Data are presented as mean ± SEM. Bar graphs also show individual values. Statistical significance was determined using Student's t-test or 2-way analysis of variance (with treatment and time or tissue being the 2 variables) with Bonferroni post hoc analysis. $P < .05$ was considered to be statistically significant. Statistical calculations and graphs were made using GraphPad Prism (v. 9.01 for Mac; GraphPad Software, La Jolla, CA, USA).

Results

Acute Intestinal Neprilysin Inhibition Increases Oral Glucose–Stimulated Insulin Secretion in Glp1r$^{+/+}$ Mice

To confirm selective intestinal neprilysin inhibition by low-dose thiorphan (0.05 mg/kg), neprilysin activity was assessed in plasma collected just prior to oral thiorphan or vehicle administration, and in plasma and intestine collected 40 minutes after administration of the drug or vehicle. In lean *Glp1r*$^{+/+}$ mice, a single low dose of thiorphan significantly inhibited neprilysin activity in the ileum and colon (Fig. 1A) without altering plasma neprilysin activity (Fig. 1B) when compared with vehicle. Thus, this low dose of thiorphan was used to selectively inhibit intestinal neprilysin in a separate cohort of lean *Glp1r*$^{+/+}$ mice undergoing an OGTT. Glucose and insulin profiles are shown in Figs. 1C and 1D, respectively. While there was no significant effect of thiorphan on glucose levels during the OGTT (Fig. 1C), thiorphan significantly increased insulin secretion (Fig. 1D) without altering basal insulin levels (Fig. 1D and 1E). Insulin levels were significantly higher in thiorphan-treated mice 10 minutes after oral glucose (Figs. 1D and 1E) and were significantly increased by 1.8-fold during the first 30 minutes of the OGTT (Fig. 1D, inset). The surrogate index of insulin sensitivity, QUICKI, was similar between thiorphan- and vehicle-treated mice (QUICKI: 0.37 ± 0.02 vs 0.39 ± 0.02 in thiorphan- vs vehicle-treated mice, n = 7 and 6, respectively; $P = .58$), suggesting acute inhibition of intestinal neprilysin did not alter insulin sensitivity. Finally, to assess whether circulating GLP-1 levels were increased with acute inhibition of intestinal neprilysin, systemic active GLP-1 levels were measured in plasma collected 10 minutes after oral glucose challenge. Low dose thiorphan did not significantly increase plasma active GLP-1 levels (Fig. 1F).

Acute Intestinal Neprilysin Inhibition Does Not Increase Oral Glucose-Stimulated Insulin Secretion in Glp1r$^{-/-}$ Mice

Similarly to what was observed in lean *Glp1r*$^{+/+}$ mice, a single low dose of thiorphan (0.05 mg/kg) significantly inhibited neprilysin activity in ileum and colon of *Glp1r*$^{-/-}$ mice (Fig. 2A) without altering neprilysin activity in plasma (Fig. 2B). Lean *Glp1r*$^{-/-}$ mice that underwent an OGTT after oral thiorphan administration did not display any change in glucose levels (Fig. 2C), basal insulin levels or glucose-stimulated insulin

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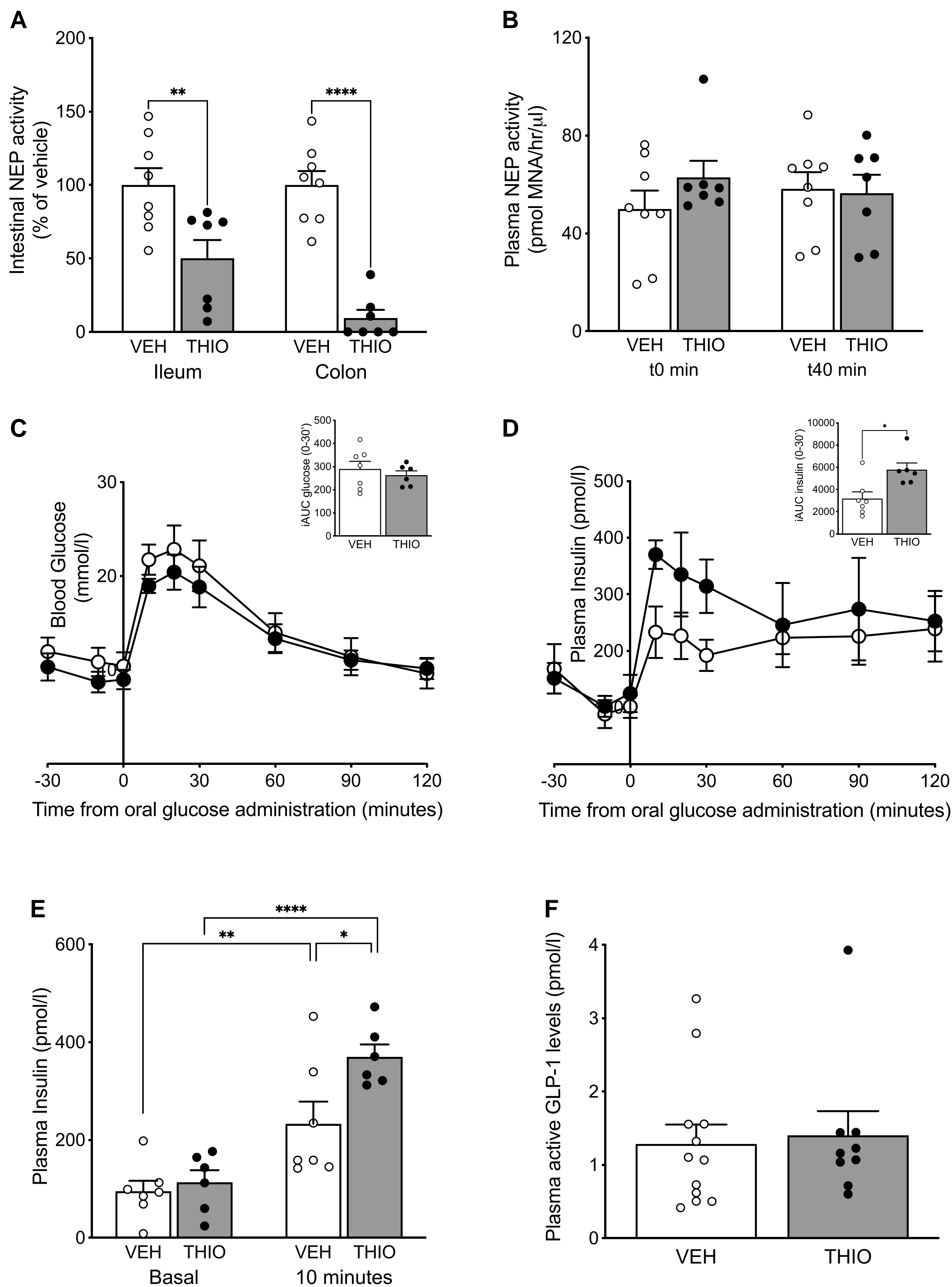

Figure 1. Acute intestinal neprilysin inhibition increases oral glucose–stimulated insulin secretion in *Glp1r*$^{+/+}$ mice. *Glp1r*$^{+/+}$ mice received a single oral dose of the neprilysin inhibitor thiorphan (THIO, 0.05 mg/kg, n = 7; filled circles, gray bars) or vehicle (VEH, 0.9% NaCl, n = 8; open circles, open bars). (A) Neprilysin activity was measured in (A) ileum and colon 40 minutes after the gavage, and (B) plasma just prior to and 40 minutes after the gavage. In another cohort of *Glp1r*$^{+/+}$ mice, an OGTT (2 g glucose/kg) was performed 30 minutes after oral thiorphan (THIO, 0.05 mg/kg, n = 6; filled circles) or vehicle (VEH, 0.9% NaCl, n = 7; open circles) administration. (C) Blood glucose and (D) plasma insulin levels during the OGTT. Insets represent incremental area under the curve (iAUC) from 0 to 30 minutes. (E) Plasma insulin levels at baseline and 10 minutes after oral glucose administration. (F) Plasma active GLP-1 levels 10 minutes after oral glucose administration in *Glp1r*$^{+/+}$ mice that had received a single dose of oral thiorphan (THIO, 0.05 mg/kg, n = 9; filled circles, gray bars) or vehicle (VEH, 0.9% NaCl, n = 12; open circles, open bars) 30 minutes before the glucose administration. Data are mean ± SEM. *$P < .05$, **$P < .01$, ****$P < .0001$.

secretion (Fig. 2D and 2E) when compared to mice that received vehicle. Insulin sensitivity was also similar between the 2 groups of mice (QUICKI: 0.36 ± 0.02 vs 0.37 ± 0.02 in thiorphan- vs vehicle-treated mice, n = 8 per group, $P = .88$). Finally, thiorphan did not increase systemic plasma active GLP-1 levels in *Glp1r*$^{-/-}$ mice (Fig. 2F).

Discussion

In this study, we show that selective inhibition of intestinal neprilysin in an acute, physiological setting increases insulin secretion in response to oral glucose, and that this effect occurs in a GLP-1R–dependent manner. To our knowledge, these

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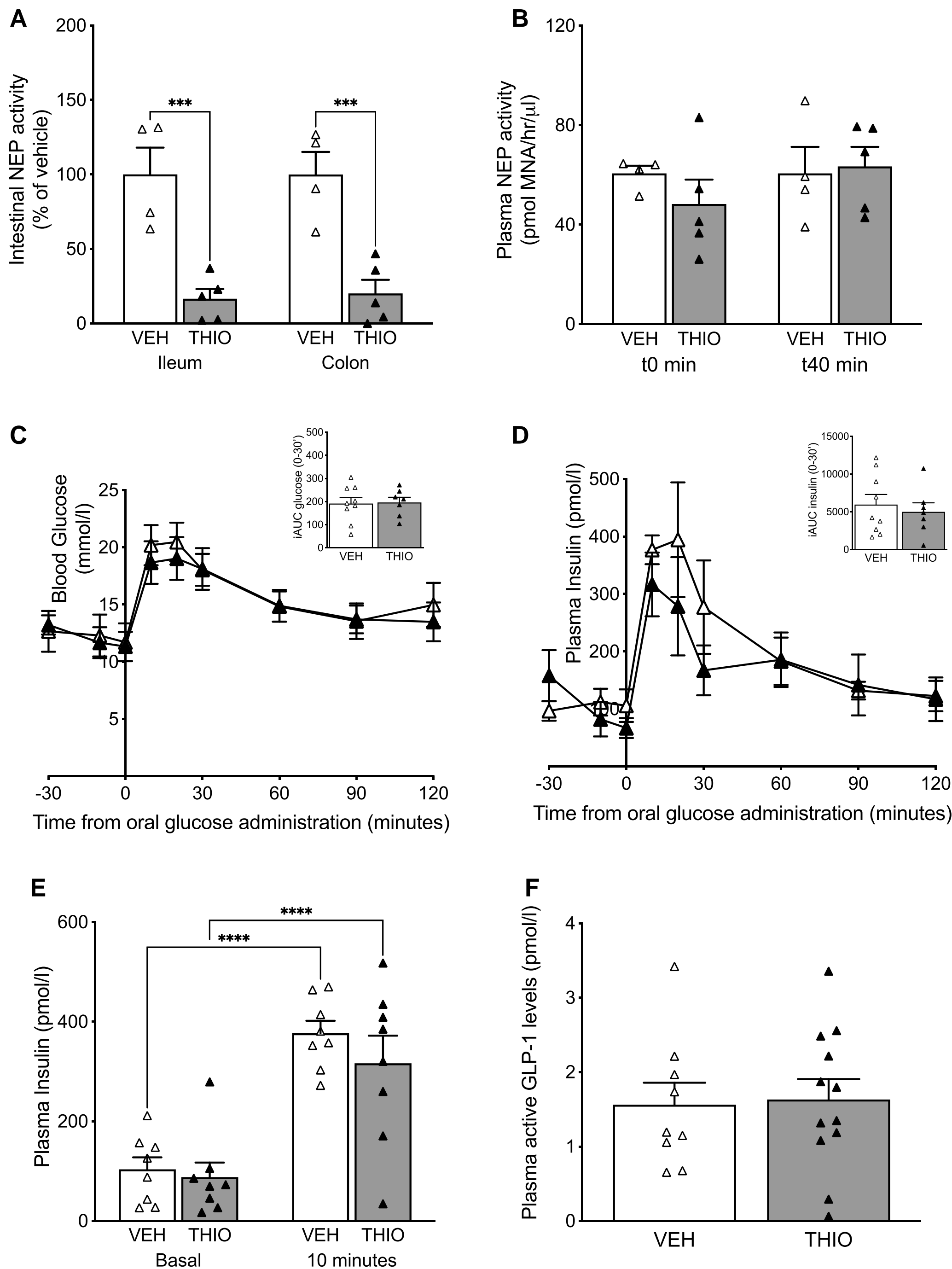

Figure 2. Acute intestinal neprilysin inhibition does not increase oral glucose–stimulated insulin secretion in *Glp1r*$^{-/-}$ mice. *Glp1r*$^{-/-}$ mice received a single oral dose of the neprilysin inhibitor thiorphan (THIO, 0.05 mg/kg, n = 5; filled triangles, gray bars) or vehicle (VEH, 0.9% NaCl, n = 4; open triangles, open bars). (A) Neprilysin activity was measured in (A) ileum and colon 40 minutes after the gavage, and (B) plasma just prior to and 40 minutes after the gavage. In another cohort of *Glp1r*$^{-/-}$ mice, an OGTT (2 g glucose/kg) was performed 30 minutes after oral thiorphan (THIO, 0.05 mg/kg, n = 8; filled triangles) or vehicle (VEH, NaCl 0.9%, n = 9; open triangles) administration. (C) Blood glucose and (D) plasma insulin levels during the OGTT. Insets represent incremental area under the curve (iAUC) from 0 to 30 minutes. (E) Plasma insulin levels at baseline and 10 minutes after oral glucose administration. (F) Plasma active GLP-1 levels 10 minutes after oral glucose administration in *Glp1r*$^{-/-}$ mice that had received a single dose of oral thiorphan (THIO, 0.05 mg/kg, n = 12; filled triangles, gray bars) or vehicle (VEH, 0.9% NaCl, n = 9; open triangles, open bars) 30 minutes before the glucose administration. Data are mean ± SEM. ***$P < .001$, ****$P < .0001$.

data are the first to demonstrate a role for intestinal neprilysin per se in modulating beta-cell function.

Systemic inhibition of neprilysin in mice and humans is associated with improved beta-cell function and glucose homeostasis (2, 4, 7, 17). Since the incretin GLP-1 is a neprilysin substrate (8, 9) and is produced by enteroendocrine L cells mainly located in the distal ileum and colon, we sought to understand the relative contribution of the intestinal compartment per se in the insulinotropic effects of neprilysin inhibition. In the present study, we used oral administration of a low dose of thiorphan to selectively target intestinal neprilysin. This technique of pharmacological inhibition of a

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peptidase selectively in the gut has been applied to studies of dipeptidyl peptidase-4 (DPP-4) (19, 20), another GLP-1 degrading enzyme. Similarly to selective inhibition of intestinal DPP-4, we found that reduced neprilysin activity in the ileum and colon (ie, proximal to the site of GLP-1 secretion) was associated with enhanced insulin secretion, suggesting that intestinal neprilysin also participates in modulating beta-cell function under physiological conditions.

We previously demonstrated the importance of GLP-1R signaling for the insulinotropic effects of systemic neprilysin inhibition in mice with genetic deletion of GLP-1R (17). At the level of specific tissues, we found that inhibition of islet neprilysin per se increased glucose-stimulated insulin secretion in vitro in a GLP-1R-dependent manner (6, 17). The mechanisms underlying increased insulin secretion with intestinal neprilysin inhibition also involve GLP-1R signaling, as demonstrated by our data from *Glp1r*$^{-/-}$ mice, wherein intestinal neprilysin inhibition had no effect on insulin secretion. It is now well established that activation of GLP-1R in the gut triggers insulin secretion via gut–brain–islet neural signaling (21–23). Although we do not have an index of activation of neural signaling in our study, it is a likely contributor to the insulinotropic effects of intestinal neprilysin inhibition. This notion is consistent with the fact that potentiation of insulin secretion occurred in the absence of any increase in systemic active GLP-1 levels. Such a finding was in fact expected since lack of an increase in plasma active GLP-1 levels was also observed with selective inhibition of intestinal DPP-4 (19) Indeed, systemically circulating GLP-1 can still be degraded by systemic DPP-4 and/or neprilysin. That is, we show low-dose thiorphan significantly decreased neprilysin activity in the ileum and colon, but not in plasma; thus, it is expected local (but not systemic) degradation of GLP-1 would be reduced, resulting in enhanced GLP-1R activation in the gut.

In contrast with our OGTT results, we previously reported pharmacological, systemic, inhibition of neprilysin improves intravenous but not oral glucose-mediated insulin secretion via GLP-1R signaling in mice, and we suggested that preservation of intra-islet GLP-1 rather than gut-derived GLP-1 may be a mechanism by which neprilysin inhibitors exert beneficial effect on beta-cell function (17). Unlike the present study involving use of lean healthy mice, it is important to note that our previous study utilized a mouse model of reduced insulin secretion (streptozotocin-induced diabetes) in which production of alpha-cell-derived GLP-1 was expected to be increased (16, 24). Further, contrasting findings between the 2 studies might also be explained by other significant differences in the experimental design (eg, the use of a higher dose of neprilysin inhibitor to obtain systemic rather than selective intestinal neprilysin inhibition and a more prolonged study period). Additional studies are therefore warranted to investigate the insulinotropic effect of selective intestinal neprilysin inhibition in the context of obesity and diabetes and/or in a more prolonged setting.

While acute selective intestinal neprilysin inhibition increased insulin secretion during the OGTT in *Glp1r*$^{+/+}$ mice, it was not associated with changes in oral glucose tolerance. On face value, this finding seems surprising, but it could occur for several reasons. First, changes in insulin output may not necessarily have a large impact on glucose levels due to the fact that insulin-independent glucose disposal is a major contributor to glucose tolerance in mice (25). Second, we cannot completely exclude that an increase in activity of the sympathetic nervous system or in glucagon levels (another neprilysin substrate (8, 26, 27)), both previously reported with systemic neprilysin inhibition (27-29), had prevented a decrease in glucose levels in our study. Finally, since insulin sensitivity impacts glucose tolerance, any thiorphan-mediated decrease in glucose levels may have been negated by a reduction in insulin sensitivity. Given that the QUICKI index was similar in mice treated with thiorphan vs vehicle, it is unlikely that the lack of change in glucose with thiorphan treatment was due to a decrease in insulin sensitivity.

Clinically, neprilysin inhibitors have not been used alone in humans due to the detrimental effects of increased angiotensin II levels following systemic neprilysin inhibition. Clinical trials have shown that combination of the neprilysin inhibitor sacubitril with the angiotensin II receptor blocker valsartan improves glycemic control and reduces initiation of insulin therapy in patients with heart failure and type 2 diabetes (4, 5). However, in another recent study, acute administration of sacubitril/valsartan in individuals with obesity and type 2 diabetes, but without heart failure, impaired rather than improved glucose tolerance during a meal tolerance test (28). The latter was attributed to hyperglucagonemia resulting from systemic neprilysin inhibition (28). Although our acute study was not conducted in the context of diabetes, it suggests that selective intestinal inhibition of neprilysin may help circumvent deleterious effects in non-intestine compartments that would occur with systemic inhibition (eg, increased levels of angiotensin II and hyperglucagonemia)—this is clearly an area that warrants further investigation.

Finally, neprilysin has a broad substrate specificity and can degrade gut-derived peptides other than GLP-1 that also have insulinotropic properties, such as the incretin gastric inhibitory peptide (8), cholecystokinin-8 (30, 31), peptide YY (32), and gastrin (31, 33). Therefore, GLP-1R–independent mechanisms could also be involved in the insulinotropic effects of intestinal neprilysin inhibition. Investigating the metabolic effect of these other neprilysin substrates using a similar study design is an area for future work.

In conclusion, this study delineates a role for intestinal neprilysin in modulating insulin secretion in response to oral glucose administration through GLP-1R. Thus, strategies to inhibit neprilysin specifically in the intestine may improve beta-cell function in type 2 diabetes.

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Author Contributions

N.E. conceived and designed the study, performed experiments, analyzed and interpreted data, and wrote the manuscript. T.O.M. and B.M.B. designed the study, performed experiments, analyzed and interpreted data, and edited the manuscript. S.Z. conceived and designed the study, analyzed and interpreted data, and edited the manuscript. All authors approved submission of the manuscript. S.Z. is the guarantor of this work and as such, had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Disclosures

The authors have nothing to disclose.

Data Availability

Some or all datasets generated during and/or analyzed during the current study are not publicly available but are available from the corresponding author on reasonable request.

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