NEPRILYSIN IS REQUIRED FOR ANGIOTENSIN-(1-7)'S ABILITY TO ENHANCE INSULIN SECRETION VIA ITS PROTEOLYTIC ACTIVITY TO GENERATE ANGIOTENSIN-(1-2)

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ABSTRACT

Recent work has renewed interest in therapies targeting the renin-angiotensin system (RAS) to improve β-cell function in type 2 diabetes. Studies show that generation of angiotensin-(1-7) by angiotensin converting enzyme 2 (ACE2) and its binding to the Mas receptor (MasR) improves glucose homeostasis, partly by enhancing glucose-stimulated insulin secretion (GSIS). Thus, islet ACE2 upregulation is viewed as a desirable therapeutic goal. Here, we show that although endogenous islet ACE2 expression is sparse, its inhibition abrogates angiotensin-(1-7)-mediated GSIS. However, a more widely expressed islet peptidase, neprilysin, degrades angiotensin-(1-7) into several peptides. In neprilysin-deficient mouse islets, angiotensin-(1-7) and neprilysin-derived degradation products, angiotensin-(1-4), -(5-7) and -(3-4), failed to enhance GSIS. Conversely, angiotensin-(1-2) enhanced GSIS in both neprilysin-deficient and wild-type islets. Rather than mediating this effect via activation of the G-protein-coupled receptor (GPCR), MasR, angiotensin-(1-2) was found to signal via another GPCR, namely GPCR family C group 6 member A (GPRC6A). In conclusion, in islets, intact angiotensin-(1-7) is not the primary mediator of beneficial effects ascribed to the ACE2/angiotensin-(1-7)/MasR axis. Our findings warrant caution for concurrent use of angiotensin-(1-7) compounds and neprilysin inhibitors as therapies for diabetes.

The renin-angiotensin system (RAS) is a major regulator of blood pressure and fluid balance, with its role in mediating other physiological effects recently being the subject of intense investigation. The classic view is that angiotensinogen is hydrolyzed by renin to produce angiotensin I, which is subsequently hydrolyzed by angiotensin converting enzyme (ACE) to generate angiotensin II. Angiotensin II is biologically active, primarily binding angiotensin II receptor type I (AT$_1$) to mediate its effects. This cascade is known as the ACE/angiotensin II/AT$_1$ axis.

Discovery of more components of the RAS has seen the classical model evolve, wherein the ACE2/angiotensin-(1-7)/Mas receptor (MasR) axis was proposed. This involves generation of angiotensin-(1-7) directly from angiotensin I or angiotensin II by angiotensin converting enzyme 2 (ACE2), or indirectly from angiotensin-(1-9) (1-3). Angiotensin-(1-7) binds the G-protein-coupled receptor (GPCR), MasR (4), to elicit responses that can counteract those of the ACE/angiotensin II/AT$_1$ axis.

Another peptidase capable of generating angiotensin-(1-7) is neprilysin (5-7). Kinetic studies of peptide cleavage showed that neprilysin more efficiently hydrolyzed angiotensin I to angiotensin-(1-7), compared to ACE2 (1). Also, angiotensin-(1−9) was cleaved preferentially by neprilysin to generate angiotensin-(1-7), which was further cleaved by neprilysin to several smaller peptides (1; 6; 8). Whether these small peptides bind the MasR remains unknown.

The existence of a local RAS has been reported in many tissues, including the pancreatic islet (9). The islet RAS plays important roles in regulating local blood flow, insulin biosynthesis and

secretion, and β-cell survival (10; 11). Previously, we demonstrated that neprilysin is expressed in islets (12). Despite this, angiotensin-(1-7)'s effects on islet function have been attributed to intact angiotensin-(1-7) *per se*, rather than neprilysin-derived degradation products. Moreover, emphasis has been on ACE2 as the primary enzyme governing generation of angiotensin-(1-7) and its beneficial effects; however, whether activity and localization of endogenous ACE2 within the islet is sufficient to promote insulin secretion is unclear. Here, we utilized islets from neprilysin-deficient mice to determine whether lack of neprilysin activity limits the ability of angiotensin-(1-7) to promote insulin secretion. We identify the cellular localization of endogenous islet ACE2, compare this to the known expression pattern of neprilysin, and establish whether ACE2 inhibition also affects angiotensin-(1-7) action.

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RESEARCH DESIGN AND METHODS

Animals

C57BL/6.NEP$^{-/-}$ mice (originally from Dr. Lu, Harvard Medical School, USA (13)) and C57BL/6.NEP$^{+/+}$ controls were from our colony at VA Puget Sound Health Care System (VAPSHCS) in Seattle. At the time of islet isolation, 16-hour fasting plasma glucose levels were similar in C57BL/6.NEP$^{+/+}$ versus C57BL/6.NEP$^{-/-}$ mice (male: 95.6±4.1 vs. 96.6±2.7 mg/dl; female: 85.6±6.3 vs. 94.5±3.1 mg/dl; n=8-11). Studies were approved by the VAPSHCS Institutional Animal Care and Use Committee.

Peptides, agonists and inhibitors

Angiotensin-(1-7) was purchased from Sigma-Aldrich (St. Louis, MO). Angiotensin-(5-7), -(1-2) and -(3-4) were from AnaSpec (Fremont, CA), and angiotensin-(1-4) from GenScript (Piscataway, NJ). The MasR agonist AVE0991 was from MedChem Express (Monmouth Junction, NJ) and antagonist A779 from Abcam (Cambridge, MA). The ACE2 inhibitor DX600 was from Phoenix Pharmaceuticals (Burlingame, CA).

Islet isolation and culture

Islets were isolated from 10-12-week old female and male mice as previously described (14). RPMI-1640 media (Gibco #22400; Life Technologies Corporation, Grand Island, NY) containing 11.1 mM glucose was used for all cultures. After overnight recovery, islets were cultured for 48 hours in media plus one of the following: angiotensin-(1-7), -(1-4), -(5-7), -(1-2), -(3-4) or vehicle. 1 nM was utilized as dose-response studies showed this to be the lowest effective concentration of each peptide (data not shown). A 48-hour culture period was chosen as

it is the typical paradigm used to mimic chronic exposure of islets to factors seen under normal/diabetic conditions. In some experiments, the ACE2 inhibitor DX600 was added to islets for 48-hour culture, at a dose (10 μM) previously shown to maximally inhibit ACE2 activity in mouse tissues (15). In other experiments, the MasR antagonist A779 (1 μM) was added to islets 20 minutes prior to addition of angiotensin-(1-2) or the MasR agonist AVE0991 (0.1 μM). For these A779 experiments, the culture period is as indicated in the Results section.

In a subset of experiments, freshly isolated islets were transfected with the following siRNA oligonucleotides: 10 nM or 25 nM si-Scramble (#4390843; Thermo Fisher, Waltham, MA), 10 nM si-GPRC6A (combination of 5 nM siRNA #s102233 + 5nM siRNA #s102235; Thermo Fisher), 25 mM si-MasR (#s69599; Thermo Fisher), or lipofectamine vehicle (Lipofectamine RNAiMAX Reagent; Invitrogen, Carlsbad, CA) in RPMI-1640 media containing 11.1 mM glucose. After 72 hours, islets were transferred to fresh media and recovered for up to 72 hours. After recovery, islets were cultured for 48 hours in the presence of 1 nM angiotensin-(1-2) or vehicle. This was followed by static insulin secretion measurements as described below. The degree of GPRC6A or MasR knockdown was similar immediately after 72-hour transfection versus at the time of insulin secretion assessment. Insulin secretion data from lipofectamine vehicle versus si-Scramble treated islets were similar.

Insulin secretion and content

Insulin secretion in response to 2.8 mM (basal) or 20 mM (stimulated) glucose was measured in static incubations as previously described (14), wherein both C57BL/6.NEP^{+/+} and C57BL/6.NEP^{-/-} islets treated with either vehicle or angiotensin peptide were studied

simultaneously for each *n*. Each angiotensin peptide was studied independently. For 48-hour cultured islets, 2.8 mM or 20 mM glucose-induced insulin secretion was measured in the absence of angiotensin peptides. In a subset of experiments, basal and glucose-induced insulin secretion was measured in the presence of 1 nM angiotensin-(1-7) or angiotensin-(1-2) in islets that were isolated and recovered without angiotensin peptide exposure overnight. Islet insulin content was measured after acid-ethanol extraction. Insulin concentrations were determined using the Insulin (Mouse) Ultrasensitive ELISA (Alpco, Salem, NH).

Real-time quantitative RT-PCR

Expression of islet GPRC6A, MasR and CasR mRNA was determined by real-time quantitative RT-PCR using the TaqMan system (ABI Prism 7500; Applied Biosystems, Carlsbad, CA), as previously described (12). GPRC6A (Mm00467618_m1), MasR (Mm00434823_s1) and CasR (Mm00443375_m1) gene expression mixes from Applied Biosystems were used with eukaryotic 18S rRNA (Hs99999901_s1) as endogenous control.

Western blotting

Islet protein (20-35 µg/well) separated by SDS-PAGE was transferred to PVDF membranes, which were probed with polyclonal rabbit anti-phospho-AKT antibody (1:500; Cell Signaling Technology, Davers, MA), anti-GPRC6A antibody (1:1000; MyBioSource, San Diego, CA), or anti-MAS1 (1:500; Abcam, Cambridge, MA). Stripped membranes were re-probed with polyclonal rabbit anti-AKT antibody (1:500; Cell Signaling Technology, Davers, MA) or anti-β-actin (1:2000; Sigma, St. Louis, MO). Secondary antibody was goat anti-rabbit IgG HRP (1:50,000; Dako, Carpinteria, CA). Bands were visualized on a FluorChem M system using

enhanced chemiluminescence and quantified using ImageJ software.

Mass spectrometry

Angiotensin-(1-7), angiotensin-(1-4) and A779 at 40 μM and recombinant human neprilysin (Reprokine Ltd, Valley Cottage, NY) at 0.8 μM, in 20 mM ammonium acetate buffer (pH 6.5) were combined in equal volumes to yield final concentrations of 20 μM peptide and 0.4 μM enzyme, and incubated at 37°C. Samples were taken at 0, 0.75, 1.5, 3, 6, 12, 24 and 48 hours, the reactions quenched by addition of trifluoroacetic acid (TFA; 0.5%) and analyzed by LC-TOF mass spectrometry. Digestion products were separated on a Zorbax-SB-C18 column by a sequence of increasingly steep linear gradients from 0.1% acetic acid/0.1% TFA in 98% water/2% methanol to 0.1% acetic acid/0.1% TFA in 4% water/96% methanol. Mass spectrometry was performed in singlicate on at least two independent occasions. Extraction ion current (EIC) area for each degradation product was computed and expressed as percent of total ion count at each time-point.

Histology

Frozen sections (7-10 μm) of OCT-embedded, formalin-fixed human pancreas from normal subjects were purchased from Zyagen (San Diego, CA). Frozen, OCT-embedded pancreas sections (5 μm) were obtained from C57BL/6.NEP$^{+/+}$ mice following perfusion fixation and overnight post-fixing in 10% neutral-buffered formalin. For both human and mouse pancreas, non-specific immunoreactivity was blocked with up to 10% normal goat and/or donkey serum. Sections were incubated overnight with rabbit polyclonal anti-ACE2 antibody (1:100; Santa Cruz Biotechnology, Dallas, TX). Either Cy3 conjugated donkey anti-rabbit IgG or biotinylated

goat anti-rabbit IgG followed by Alexa Fluor 488 streptavidin was then applied. To visualize β, α, δ and PP cells, sections were then incubated for an hour with either guinea pig anti-insulin (1:50; Dako, Carpinteria, CA), mouse anti-glucagon (1:2000; Sigma-Aldrich, St. Louis, MO), goat anti-somatostatin (1:250; Santa Cruz Biotechnology, Dallas, TX) or goat anti-PP (1:500; Novus Biologicals, Littleton, CO) antibodies, respectively. Sections were subsequently incubated for an hour with either Cy3 conjugated goat or donkey anti-IgG antibodies, or biotinylated donkey anti-goat IgG followed by Alexa Fluor 488 streptavidin. To visualize cell nuclei, sections were counterstained with Hoechst 33258 (2 μg/ml). Secondary antibody controls were performed by substituting primary antibodies with the same amount of normal serum from the same species.

Statistical analyses

Data are presented as mean ± standard error of the mean (SEM) for the number of experiments indicated. Statistical significance was determined using analysis of variance with post hoc analysis, the non-parametric Mann-Whitney U test or paired Student's *t*-test. A $p<0.05$ was considered statistically significant.

RESULTS

Neprilysin mediates the ability of angiotensin-(1-7) to promote insulin secretion

Angiotensin-(1-7) has been shown to enhance insulin secretion (16-18). To determine whether neprilysin activity is required for this effect, basal (2.8 mM glucose) and glucose-stimulated (20 mM glucose) insulin secretion was assessed following 48-hour culture of C57BL/6.NEP^{+/+} or C57BL/6.NEP^{-/-} islets in the absence and presence of angiotensin-(1-7). In C57BL/6.NEP^{+/+} islets, angiotensin-(1-7) exposure did not alter basal, but did potentiate glucose-stimulated insulin secretion (GSIS; Figure 1A). However, exposure of C57BL/6.NEP^{-/-} islets to angiotensin-(1-7) did not result in changes in basal or GSIS (Figure 1C). Islet insulin content was not different in either C57BL/6.NEP^{+/+} or C57BL/6.NEP^{-/-} islets cultured with angiotensin-(1-7) (Figures 1B,D).

Since neprilysin can cleave angiotensin-(1-7) into smaller peptides, and that one or more of these may be potentiating GSIS in C57BL/6.NEP^{+/+} islets, we performed mass spectrometry to identify the neprilysin-derived products of angiotensin-(1-7). When angiotensin-(1-7) was incubated with neprilysin for 48 hours, to mirror the period of islet culture, six peptide products were generated, corresponding to three cleavage sites (Figures 2A,B). Time-course experiments demonstrated that generation of the six products occurred rapidly (i.e. within 45 minutes) and the relative abundance of each peptide product did not change significantly over time (data not shown). The most abundant peptide was angiotensin-(5-7), consistent with previous reports that neprilysin primarily cleaves angiotensin-(1-7) between amino acids 4 (Tyr) and 5 (Ile) (8). The least abundant peptide was angiotensin-(1-3), accounting for <1.0% of the total ion count at 48 hours.

Angiotensin-(1-4), but not angiotensin-(5-7), enhances insulin secretion in the presence of neprilysin

To determine whether the neprilysin-derived products of angiotensin-(1-7) hydrolysis between residues 4 and 5 were insulinotropic, C57BL/6.NEP$^{+/+}$ or C57BL/6.NEP$^{-/-}$ islets were cultured in the absence and presence of angiotensin-(1-4) or -(5-7) for 48 hours, and insulin secretion assessed. With angiotensin-(1-4), basal insulin secretion was unaltered in C57BL/6.NEP$^{+/+}$ islets whereas GSIS was enhanced (Figure 3A), and in C57BL/6.NEP$^{-/-}$ islets both basal and GSIS were unaltered (Figure 3B). These data argue that further cleavage of angiotensin-(1-4) is required to promote GSIS. With angiotensin-(5-7), basal and GSIS were unchanged in both C57BL/6.NEP$^{+/+}$ (Figure 3C) and C57BL/6.NEP$^{-/-}$ (Figure 3D) islets.

Given that angiotensin-(1-4) enhanced GSIS only in islets expressing neprilysin, we determined by mass spectrometry whether it could be further cleaved by neprilysin into smaller bioactive products. After 48-hour incubation of angiotensin-(1-4) with neprilysin, two dipeptides were detected corresponding to cleavage of angiotensin-(1-4) between amino acids 2 (Arg) and 3 (Val) (data not shown).

Angiotensin-(1-2) enhances insulin secretion independent of islet neprilysin activity

Next, we tested whether either angiotensin-(1-2) and/or angiotensin-(3-4) were able to promote insulin secretion. Following 48-hour culture of C57BL/6.NEP$^{+/+}$ islets in the presence of angiotensin-(1-2), basal secretion was unchanged, whereas GSIS was increased by 60% (Figure 4A). Similarly in C57BL/6.NEP$^{-/-}$ islets, angiotensin-(1-2) enhanced GSIS by 60% without

altering basal secretion (Figure 4B). In contrast, neither basal nor GSIS were affected when C57BL/6.NEP$^{+/+}$ or C57BL/6.NEP$^{-/-}$ islets were cultured with angiotensin-(3-4) (Figures 4C,D).

Angiotensin-(1-2) does not signal through the MasR to promote insulin secretion

In order to establish whether angiotensin-(1-2) signals through the MasR to modulate insulin secretion, islets were pre-treated with the MasR antagonist A779 or siRNA oligonucleotides to knockdown MasR prior to culture with angiotensin-(1-2).

As the amino acid sequence of A779 is identical to angiotensin-(1-7) with the exception of a proline to D-alanine substitution at position 7, we first sought to determine whether it was similarly cleaved by neprilysin. Mass spectrometry revealed that after 48-hour incubation of neprilysin with A779, several smaller products were detectable indicating four cleavage sites (Table S1 and Figure S1). One of these products was angiotensin-(1-2); thus, we tested whether 48-hour incubation of C57BL/6.NEP$^{+/+}$ islets with A779 would enhance insulin secretion. Indeed, A779 increased GSIS by 30% (Figure S2). Additionally, A779 potentiated the insulin response of C57BL/6.NEP$^{+/+}$ islets to angiotensin-(1-2) (data not shown). Next we tested whether A779 would alter insulin secretion in the absence of neprilysin. Incubation of C57BL/6.NEP$^{-/-}$ islets with A779 resulted in increased GSIS after 48-hour incubation, but not after 12 hours (data not shown). Thus, experiments utilizing A779 and angiotensin-(1-2) were performed in C57BL/6.NEP$^{-/-}$ islets only, with a 12-hour culture period.

Following 12-hour culture of C57BL/6.NEP$^{-/-}$ islets with angiotensin-(1-2), we evaluated protein levels of phosphorylated AKT (pAKT) as a measure of signaling downstream of the MasR.

Diabetes

pAKT and total AKT levels were unchanged with angiotensin-(1-2) compared to vehicle treatment (Figure 5A), despite GSIS being increased (124.4±14.7 vs 87.8±10.6 pM/5 islets/h; p=0.02, n=9). Similarly, A779 pre-treatment of islets did not affect either pAKT or total AKT levels (Figure 5A). To confirm that we could detect an increase in pAKT upon MasR activation, we generated a positive control in which C57BL/6.NEP^{-/-} islets were treated with a known MasR agonist, AVE0991, for 12 hours. AVE0991 significantly increased pAKT levels (pAKT/AKT: vehicle 1.0±0.2 vs AVE0991 1.8±0.6 fold; p=0.04, n=6), an effect that could not be blocked by pre-treatment with A779. A potential explanation for the latter may be that a longer exposure to A779 is required; however, given the caveats of using A779 over a longer timeframe, we employed an alternate method to establish whether angiotensin-(1-2) signals through the MasR to increase GSIS.

We transfected islets for 72 hours with siRNA to knockdown MasR, then assessed GSIS following 48-hour exposure to angiotensin-(1-2). C57BL/6.NEP^{+/+} and C57BL/6.NEP^{-/-} islets transfected with si-MasR showed a ~40% decrease in MasR mRNA (Figures 5B,D) and ~30% decrease in MasR protein (si-Scramble 1.00±0.06 vs si-MasR 0.69±0.05 fold; p<0.01, n=8) levels, compared to si-Scramble. As expected, 48-hour exposure to angiotensin-(1-2) increased GSIS in si-Scramble treated C57BL/6.NEP^{+/+} and C57BL/6.NEP^{-/-} islets (Figures 5C,E). Knockdown of MasR failed to affect this increase in GSIS. Together, these data suggest angiotensin-(1-2) is not acting through the MasR to enhance insulin secretion.

Angiotensin-(1-2) signals through GPCR family C group 6 member A (GPRC6A) to promote insulin secretion

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Another GPCR shown to be involved in mediating insulin secretion in response to amino acids and small peptides is GPRC6A (19). We tested whether angiotensin-(1-2) signals through GPRC6A to promote GSIS. C57BL/6.NEP^{+/+} and C57BL/6.NEP^{-/-} islets were transfected with siRNA to knockdown GPRC6A as described above for MasR, resulting in GPRC6A mRNA and protein levels that were decreased by ~40% (Figures 6A,C) and ~30% (si-Scramble 1.00 ± 0.19 vs si-GPCR6A 0.69 ± 0.10 fold; $p<0.05$, n=9) respectively, compared to si-Scramble (Figures 6A,C). Again, 48-hour exposure to angiotensin-(1-2) increased GSIS in si-Scramble treated C57BL/6.NEP^{+/+} and C57BL/6.NEP^{-/-} islets (Figures 6B,D). In contrast, knockdown of GPRC6A attenuated the increase in GSIS seen with angiotensin-(1-2) in both genotypes, suggesting angiotensin-(1-2) acts, at least in part, via GPRC6A to enhance insulin secretion. Basal secretion was unchanged by siRNA treatment or angiotensin-(1-2) culture (Figures 6B,D). Further, GPRC6A knockdown *per se* (i.e. in the absence of angiotensin-(1-2)) did not alter GSIS following 48-hour culture, compared to si-Scramble. While off-target effects of GPRC6A knockdown cannot be completely excluded, mRNA levels of another closely related receptor, Ca^{2+}-sensing receptor (CaSR), in islets treated with si-GPRC6A were unchanged compared to si-Scramble (data not shown), suggesting any off-target effects may be minimal.

Acute exposure of islets to angiotensin-(1-7) or angiotensin-(1-2) does not potentiate insulin secretion

To determine whether angiotensin-(1-7) and angiotensin-(1-2) also enhance GSIS after short-term exposure, we performed static insulin secretion experiments wherein isolated and overnight recovered C57BL/6.NEP^{+/+} and C57BL/6.NEP^{-/-} islets were co-incubated with one of these peptides (1 nM) plus either 2.8 or 20 mM glucose for one hour. Following acute exposure,

neither basal nor GSIS was altered in islets co-incubated with 1 nM angiotensin-(1-7) or angiotensin-(1-2) versus vehicle (Figure S3).

ACE2 is not widely expressed in human or mouse islets, but may play a paracrine role in modulating angiotensin-(1-7)-mediated insulin secretion

Both neprilysin and ACE2 are capable of generating angiotensin-(1-7), and neprilysin can further cleave angiotensin-(1-7) into smaller peptides (1; 5; 6; 8). Previously, we showed that islet neprilysin is expressed in both β cells and non-β cells (12), and is thus ideally positioned to modulate insulin secretion. However, the expression pattern of islet ACE2 remains unclear. To determine the localization of ACE2 in islets, pancreas sections from normal, healthy humans and C57BL/6.NEP^{+/+} mice were immunostained for ACE2, insulin, glucagon, somatostatin and pancreatic polypeptide (PP). Figures 7A and B show that ACE2 expression was co-localized with glucagon, somatostatin and PP, the latter being seen in mouse islets only. Importantly, no co-localization was observed with insulin in either human or mouse islets, suggesting ACE2 is not expressed in β cells.

To determine whether ACE2 inhibition affects the ability of angiotensin-(1-7) to potentiate insulin secretion, we co-cultured C57BL/6.NEP^{+/+} islets with angiotensin-(1-7) and the ACE2 inhibitor DX600, then determined GSIS. As expected, 48-hour exposure to angiotensin-(1-7) increased GSIS, without changing basal insulin secretion (Figure 7C). However, co-culture of angiotensin-(1-7) with DX600 abrogated the effect of angiotensin-(1-7) to promote GSIS. Further, DX600 alone increased the insulin response to 20 mM glucose.

DISCUSSION

Emerging evidence suggests that modulation of pancreatic RAS flux can improve glycemic control in diabetic conditions. Specifically, the ACE2/angiotensin-(1-7)/MasR axis has been identified as an important player, wherein upregulation of ACE2 and/or angiotensin-(1-7) appears to be an attractive therapeutic strategy to treat type 2 diabetes (20). Here, we show that another RAS component, neprilysin, is critical in ensuring angiotensin-(1-7) is capable of eliciting beneficial effects with respect to insulin secretion.

In mouse islets lacking neprilysin, we found that angiotensin-(1-7) failed to enhance GSIS. Similarly, its neprilysin-derived degradation products angiotensin-(1-4) and -(5-7) did not increase GSIS in neprilysin-deficient islets. Mass spectrometry analysis revealed that both angiotensin-(1-7) and -(1-4) could be further cleaved by neprilysin to generate the dipeptide, angiotensin-(1-2), which was insulinotropic in islets with or without neprilysin expression. These data argue that the ability of angiotensin-(1-7) to evoke an insulin secretory response is critically dependent on its neprilysin-mediated degradation to the bioactive angiotensin-(1-2) dipeptide. To our knowledge, this is the first report to show that intact angiotensin-(1-7) is not the primary mediator of beneficial effects ascribed to the ACE2/angiotensin-(1-7)/MasR axis.

Neprilysin's role in the RAS is often overlooked. Like ACE2, it can hydrolyze angiotensins I and II to generate angiotensin-(1-7). In fact, neprilysin hydrolyzes angiotensin I to angiotensin-(1-7) with greater efficiency than ACE2 (1). In comparing the expression pattern of neprilysin versus ACE2 in islets, we (12) and others (21) demonstrate that neprilysin is more widespread, being in both β cells and non-β cells. We show ACE2 is limited to α, δ and PP cells, in keeping with a

recent report (22). Other histological reports do not clearly define the islet cell types expressing ACE2 (11; 23-25). One study showed ACE2 co-localized with both insulin and somatostatin, but rarely with glucagon and PP (26), while another showed ACE2 co-localized primarily with insulin (21). Inconsistency in descriptions of islet ACE2 localization could be due to species-specific differences in ACE2 expression. We believe our observations in human pancreas help to clarify this. Importantly, we show that although islet ACE2 expression is more sparse than neprilysin, it also plays a key role in modulating the β-cell response to exogenous angiotensin-(1-7). Specifically, when islet ACE2 activity is inhibited, angiotensin-(1-7) can no longer potentiate GSIS. To our knowledge, no studies have shown ACE2 cleaves angiotensin-(1-7) as neprilysin does, thus the molecular mechanism for its ability to promote angiotensin-(1-7)-mediated insulin secretion in a paracrine manner remains to be elucidated.

Few studies have interrogated the direct effect of angiotensin-(1-7) on modulating insulin secretion. In one study, acute exposure of mouse islets to angiotensin-(1-7) was shown to increase both basal and GSIS by 1.4-fold. However, when continuously infused in mice for 10 days, angiotensin-(1-7) failed to enhance the insulin response to oral glucose. Interestingly, when studied *ex vivo*, islets from these mice demonstrated increased GSIS even without further angiotensin-(1-7) exposure in culture (21). These findings were attributed to both MasR-dependent and -independent mechanisms modulating insulin secretion via increased cAMP. In other studies utilizing β cells co-cultured with islet endothelial cells (17), or diabetic rats (18), angiotensin-(1-7)-mediated improvements in GSIS were associated with enhanced islet endothelial cell function and pancreatic microcirculation. Given that our *in vitro* study design involved islet culture with peptides for up to 48 hours, and the majority of endothelial cells are

typically lost during this period in culture (27), it is unlikely that the islet microvasculature played a significant role in the increased insulin secretion we observed with angiotensin peptides.

Importantly, our findings suggest that angiotensin-(1-7) must be hydrolyzed by neprilysin to angiotensin-(1-2) in order to elicit an increase in insulin secretion. It has previously been shown that angiotensin-(1-7) can be cleaved to generate angiotensin-(1-4) and then angiotensin-(1-2) (5; 8). There are no published studies demonstrating the ability of angiotensin-(1-2) to bind and activate the MasR. While the effects of angiotensin-(1-7) have been proposed to be mediated mainly via the MasR (4), we did not find this to be the case with angiotensin-(1-2). Rather, we uncovered that another GPCR, GPRC6A, facilitates the insulinotropic actions of angiotensin-(1-2). GPRC6A is known to bind small peptides and L-α-amino acids, including L-arginine, to increase intracellular calcium concentrations (28-31) and insulin secretion (19; 28; 32). Thus, our findings contribute to the evolving model of the RAS (Figure 8), wherein we propose that neprilysin, angiotensin-(1-2) and GPRC6A mediate, at least in part, the secretory response of islets to angiotensin-(1-7). This mechanism requires chronic (>1 hour) exposure to angiotensin-(1-2), given that acute (1-hour) exposure failed to enhance GSIS. Chronic exposure raises the possibility that effects of angiotensin-(1-2) on GSIS may be due to non-receptor-mediated events; however, we feel this is unlikely given that GPRC6A knockdown abolished potentiation of GSIS by angiotensin-(1-2).

A caveat for interpretation of our data is that it is unclear as to whether angiotensin-(1-7) is indeed cleaved to angiotensin-(1-2) by endogenous neprilysin in our islet culture system. While our mass spectrometry data clearly show cleavage occurs with recombinant neprilysin in a 'test

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tube' setting, confirmation of this in islets is hindered by a lack of the resolution required to detect a dipeptide like angiotensin-(1-2) in a highly complex biological sample. Future work will be required to resolve this issue. Another consideration for data interpretation relates to the lack of GSIS potentiation by angiotensin-(1-7) in neprilysin-deficient islets. One potential explanation for these data is that under control/vehicle conditions, those islets secrete more insulin than wild-type islets. This may limit or mask the effect of angiotensin-(1-7) to potentiate GSIS, although we feel this is unlikely because other peptides like angiotensin-(1-2) are still able to increase GSIS in neprilysin-deficient islets above levels seen with vehicle treatment. Finally, our mass spectrometry data on cleavage of the MasR antagonist, A779, warrant caution for its use in conditions where neprilysin is active. Its effect to enhance GSIS independent of MasR antagonism in islets following 48-hour culture, even in those lacking neprilysin, coupled with inability to block MasR agonist-mediated increases in pAKT over 12 hours, limits its utility in studying the role of MasR in β-cell function.

In summary, we show that both neprilysin and ACE2 are required for angiotensin-(1-7) to enhance insulin secretion *in vitro*. Neprilysin cleaves angiotensin-(1-7) to generate the insulinotropic angiotensin-(1-2) dipeptide which acts, at least in part, through GPCR6A. With the recent development of therapeutic angiotensin-(1-7) and neprilysin inhibitors, our findings have significant clinical implications for use of these compounds concurrently and thus warrant further investigation.

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AUTHOR CONTRIBUTIONS

S.Z. conceived and designed the study, performed experiments, analyzed data and wrote the manuscript. G.S.B. and B.M.B. designed and performed experiments, analyzed data and edited the manuscript. M.W. performed experiments, analyzed data and edited the manuscript. R.G., E.C., B.R. and A.W. performed experiments and analyzed data. D.P.R. contributed analytic tools, analyzed data and edited 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.

The authors declare that there is no conflict of interest associated with this manuscript.

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FIGURE LEGENDS

Figure 1: Angiotensin-(1-7) promotes insulin secretion only in islets expressing neprilysin. Insulin secretion in response to 2.8 mM or 20 mM glucose and insulin content from C57BL/6.NEP$^{+/+}$ (A, B; n=8-9) and C57BL/6.NEP$^{-/-}$ (C, D; n=6) islets after 48-hour culture in the absence or presence of 1 nM angiotensin-(1-7). Data are mean ± SEM. *p<0.05 vs Vehicle.

Figure 2: Angiotensin-(1-7) is cleaved into several smaller peptides by neprilysin. Angiotensin-(1-7) and neprilysin were co-incubated and samples taken at various time-points up to 48 hours were analyzed by mass spectrometry. (A) Extraction ion current (EIC) areas for each peptide are expressed as percent of total ion count at 48 hours (n=2). (B) Based on mass spectrometry data, three neprilysin cleavage sites within angiotensin-(1-7) were identified. Data are mean ± SEM. *p<0.05 vs all other cleavage products.

Figure 3: Angiotensin-(1-4) but not -(5-7) promotes insulin secretion only in islets expressing neprilysin. Insulin secretion in response to 2.8 mM or 20 mM glucose from islets after 48-hour culture in the absence or presence of 1 nM angiotensin-(1-4) (A: C57BL/6.NEP$^{+/+}$, n=6; B: C57BL/6.NEP$^{-/-}$, n=8) or 1 nM angiotensin-(5-7) (C: C57BL/6.NEP$^{+/+}$, n=7; D: C57BL/6.NEP$^{-/-}$, n=7). Data are mean ± SEM. *p<0.05 vs Vehicle.

Figure 4: Angiotensin-(1-2) but not -(3-4) promotes insulin secretion in islets with or without neprilysin. Insulin secretion in response to 2.8 mM or 20 mM glucose from islets after 48-hour culture in the absence or presence of 1 nM angiotensin-(1-2) (A: C57BL/6.NEP$^{+/+}$, n=6;

B: C57BL/6.NEP$^{-/-}$, n=6) or 1 nM angiotensin-(3-4) (C: C57BL/6.NEP$^{+/+}$, n=6; D: C57BL/6.NEP$^{-/-}$, n=6). Data are mean $\pm$ SEM. *p<0.05 vs Vehicle.

Figure 5: Angiotensin-(1-2) does not signal through the MasR to promote insulin secretion. Phospho-AKT and total AKT protein levels (A: n=3-5) from C57BL/6.NEP$^{-/-}$ islets cultured in the absence or presence of 1 nM angiotensin-(1-2), with or without pre-treatment with 1 μM A779. Western blot images are representative, with quantification of all experiments shown in the graphs. MasR mRNA levels in C57BL/6.NEP$^{+/+}$ (B; n=9) and C57BL/6.NEP$^{-/-}$ (D; n=8) islets transfected with either si-Scramble or si-MasR for 72 hours. Insulin secretion in response to 2.8 mM or 20 mM glucose from transfected C57BL/6.NEP$^{+/+}$ (C; n=7) and C57BL/6.NEP$^{-/-}$ (E; n=5) islets after 48-hour culture in the absence or presence of 1 nM angiotensin-(1-2). Data are mean $\pm$ SEM. #p<0.001 vs si-Scramble, *p<0.05 vs Vehicle 20 mM glucose.

Figure 6: Angiotensin-(1-2) promotes insulin secretion via GPRC6A. GPRC6A mRNA levels in C57BL/6.NEP$^{+/+}$ (A; n=9) and C57BL/6.NEP$^{-/-}$ (C; n=10) islets transfected with either si-Scramble or si-GPRC6A for 72 hours. Insulin secretion in response to 2.8 mM or 20 mM glucose from transfected C57BL/6.NEP$^{+/+}$ (B; n=9) and C57BL/6.NEP$^{-/-}$ (D; n=10) islets after 48-hour culture in the absence or presence of 1 nM angiotensin-(1-2). Data are mean $\pm$ SEM. #p<0.0001 vs si-Scramble, *p<0.05 vs Vehicle 20 mM glucose.

Figure 7: ACE2 is localized to non-β cells and contributes to angiotensin-(1-7)-mediated potentiation of insulin secretion. Pancreas sections from (A) C57BL/6.NEP$^{+/+}$ mice and (B) humans were immunostained for ACE2, insulin (INS), glucagon (GLU), somatostatin (SS) and

pancreatic polypeptide (PP). Merged images show co-localization (yellow) of ACE2 with glucagon, somatostatin and PP, but not insulin. Scale bar: 50 μm. (C) Insulin secretion in response to 2.8 mM or 20 mM glucose from C57BL/6.NEP$^{+/+}$ islets cultured for 48 hours in the absence or presence of 1 nM angiotensin-(1-7), with or without the ACE2 inhibitor DX600 (10 μM). Data are mean ± SEM; n=5. *p<0.05 vs Vehicle 20 mM glucose.

Figure 8: Proposed NEP/angiotensin-(1-2)/GPRC6A axis of the RAS. Angiotensinogen is cleaved to angiotensin I, which is further cleaved to angiotensin II, angiotensin-(1-9) and angiotensin-(1-7) by previously identified enzymes. Angiotensin-(1-7) has been shown to bind the MasR to mediate beneficial effects. In islets, we show that angiotensin-(1-7) is cleaved by neprilysin to angiotensin-(1-4) and angiotensin-(5-7). Angiotensin-(1-4) is further cleaved to angiotensin-(1-2) and angiotensin-(3-4) by neprilysin. Angiotensin-(1-2) binds GPCR6A to elicit an increase in insulin secretion. ACE, angiotensin converting enzyme; ACE2, angiotensin converting enzyme 2; NEP, neprilysin.

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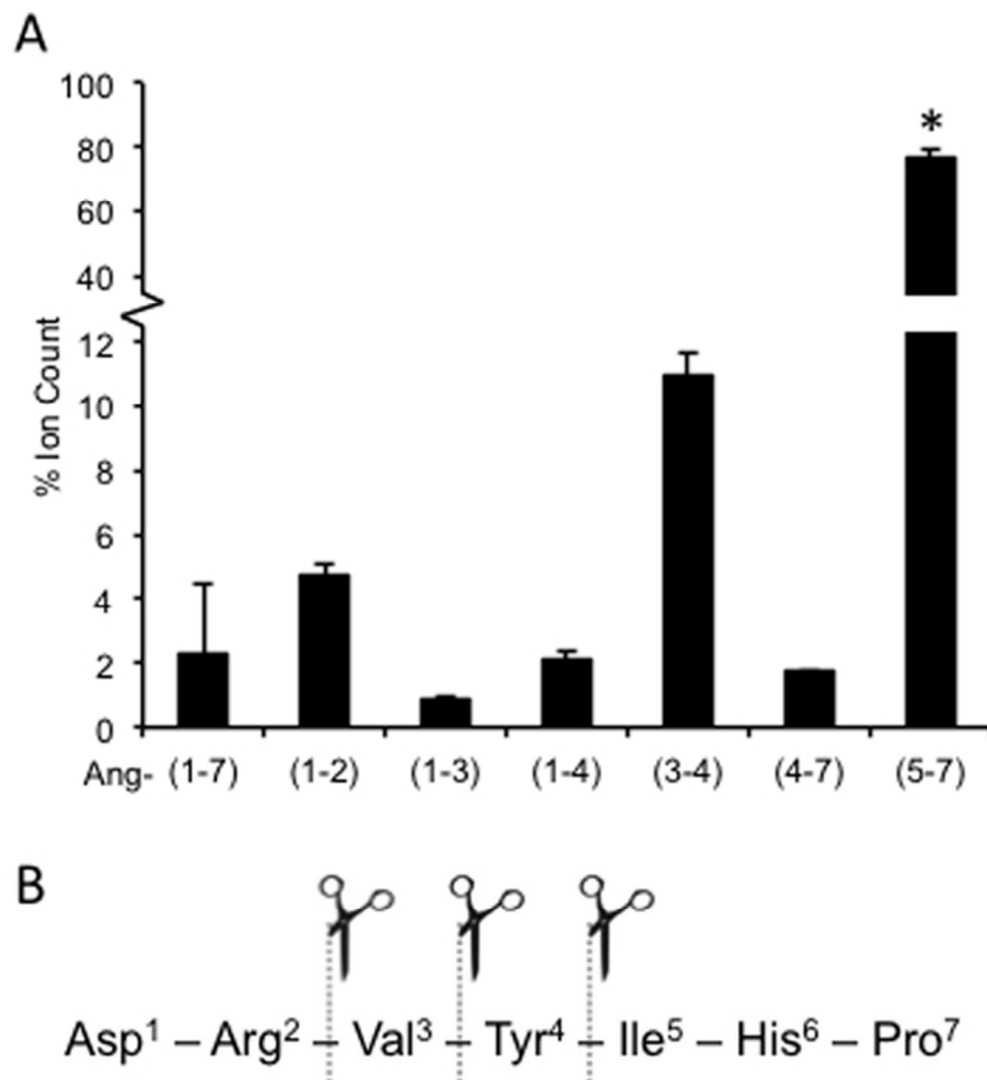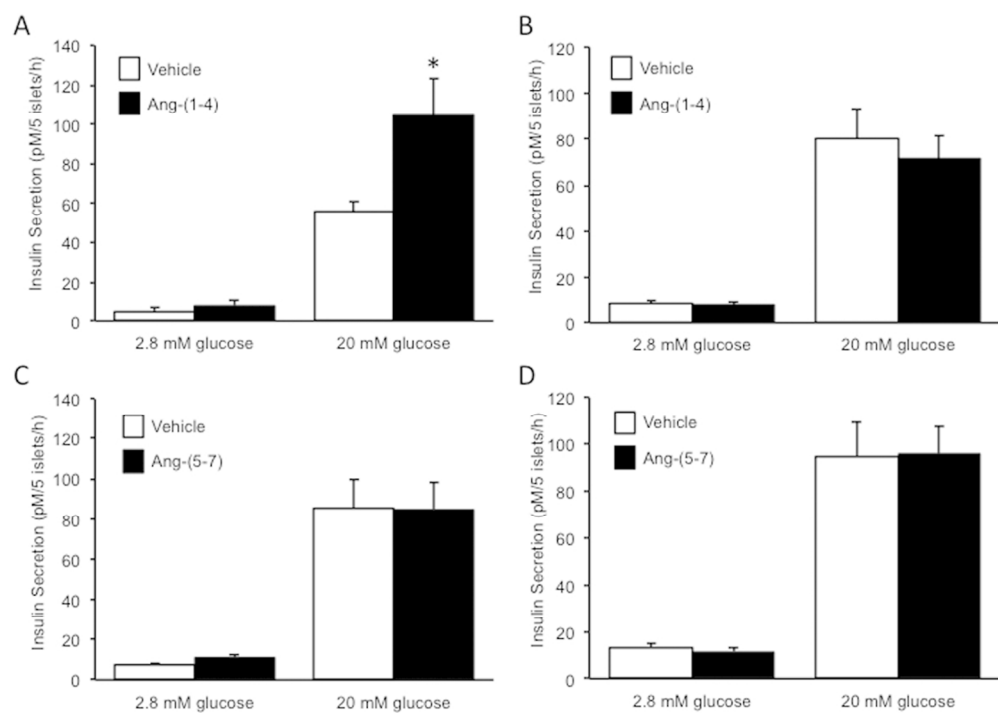
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50x56mm (300 x 300 DPI)

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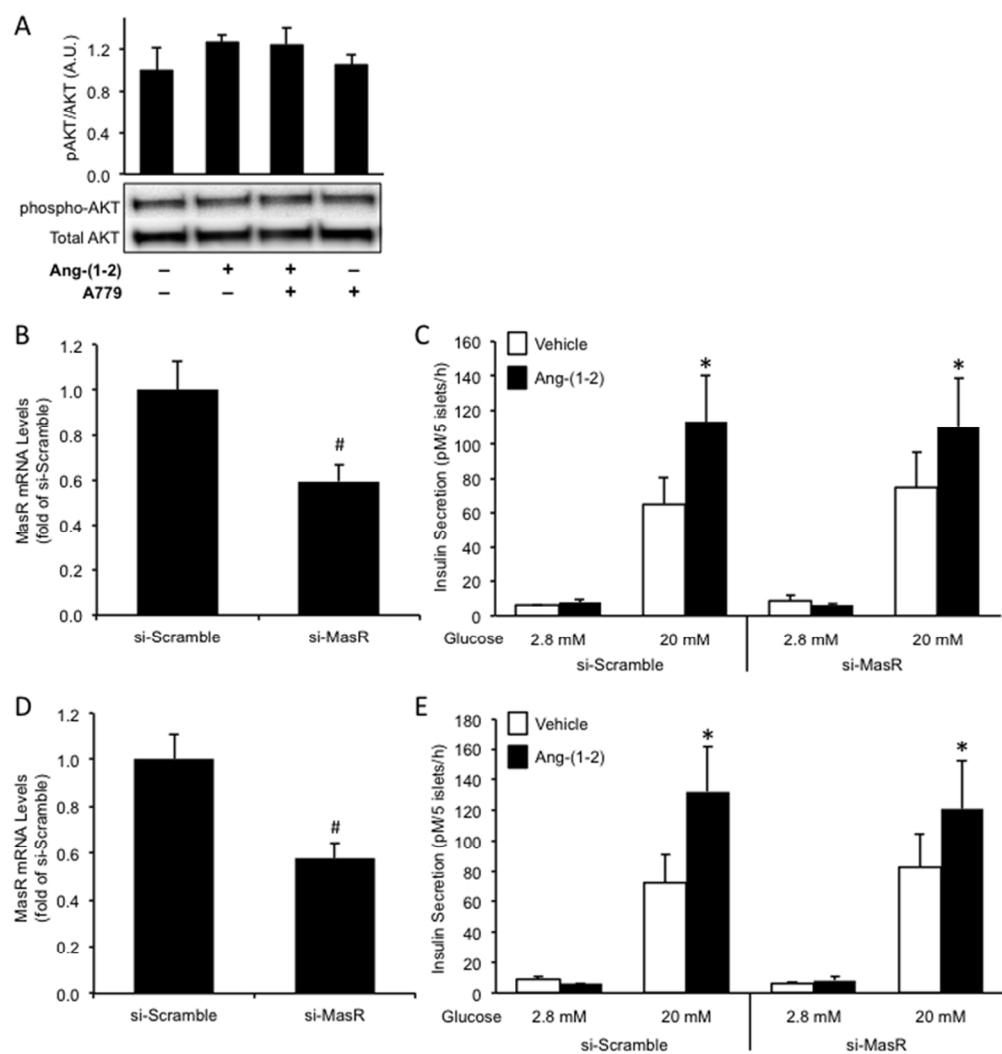
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76x53mm (300 x 300 DPI)

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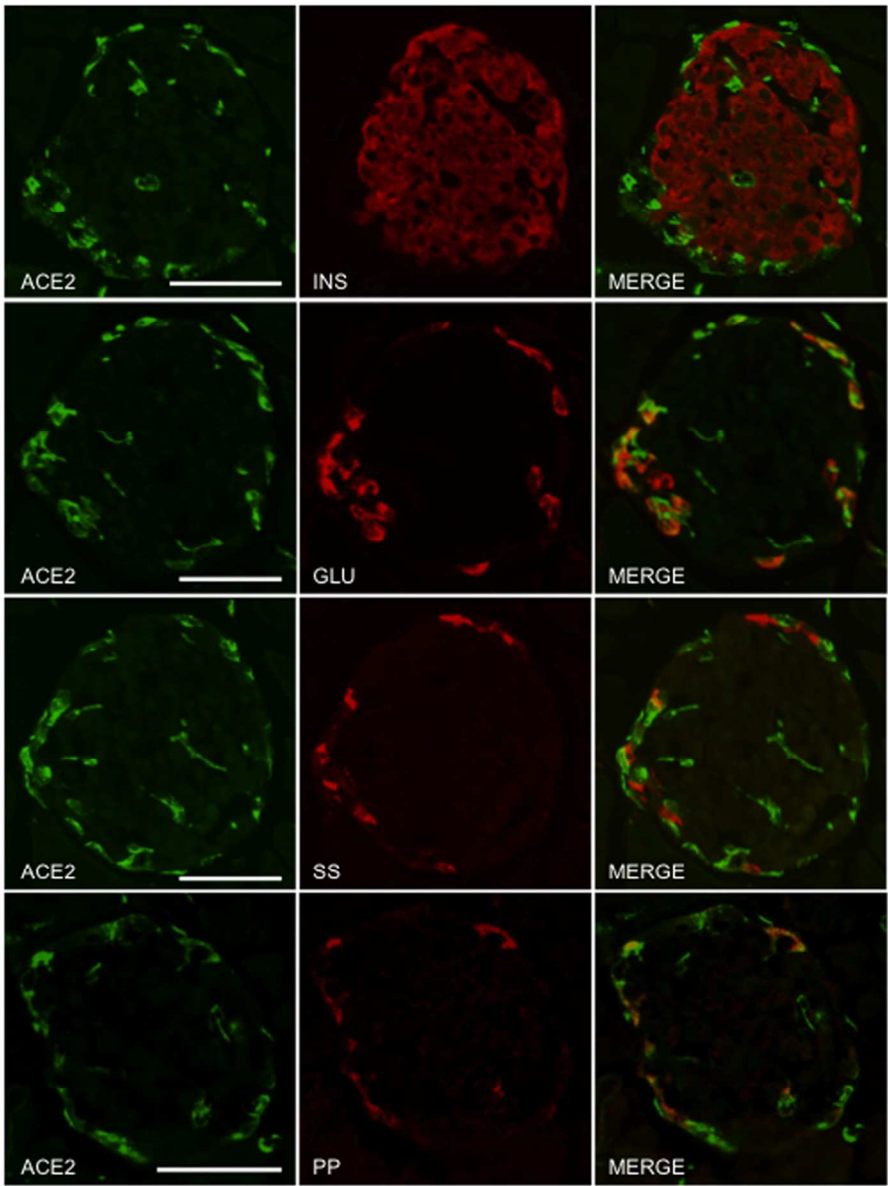
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76x57mm (300 x 300 DPI)

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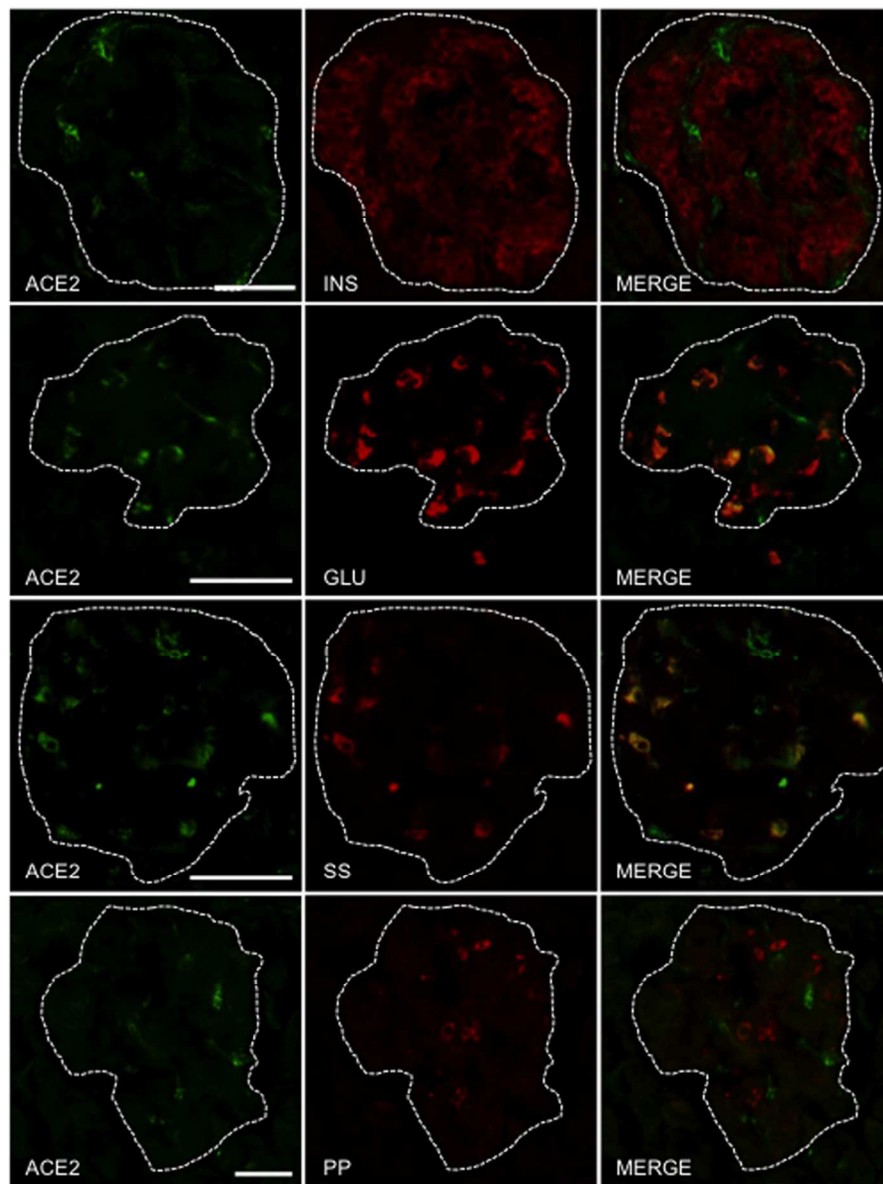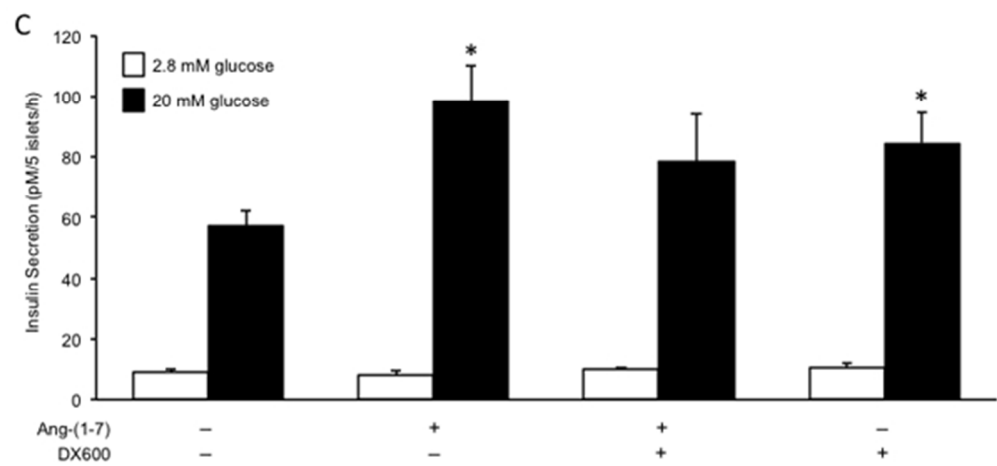
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76x102mm (300 x 300 DPI)

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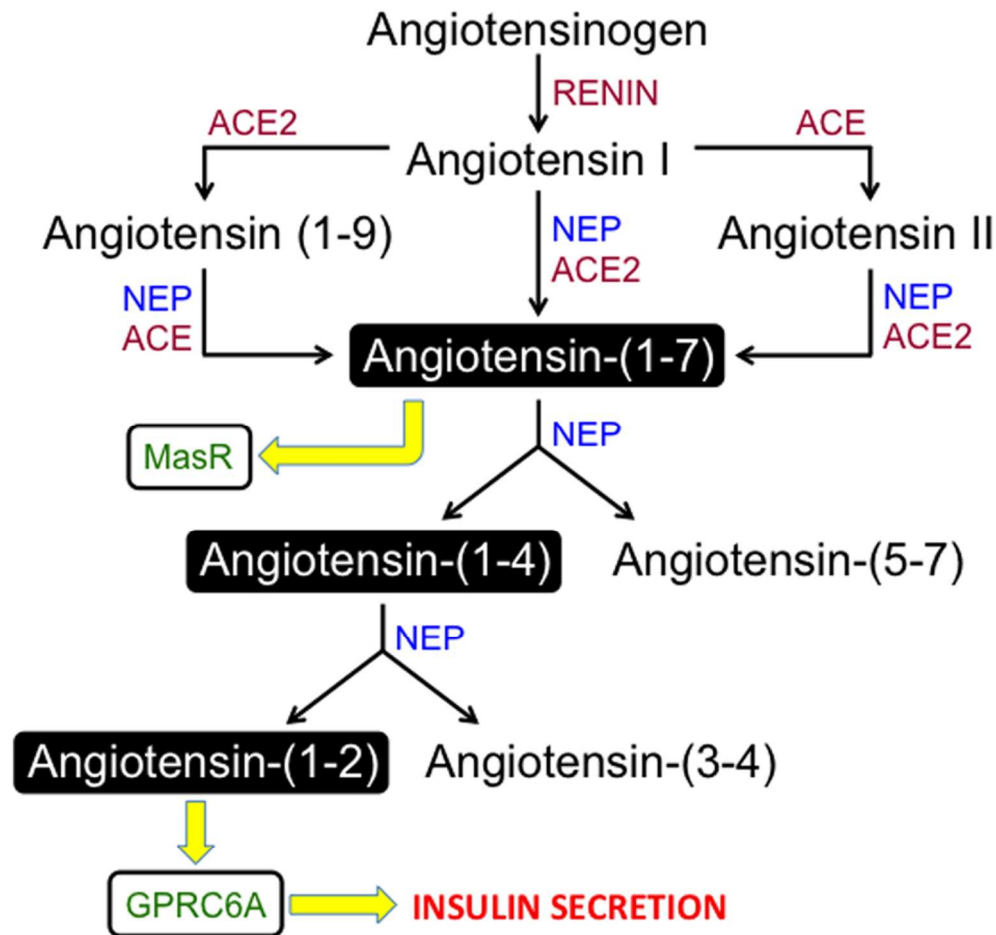
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76x72mm (300 x 300 DPI)

Online Supplemental Data

TABLE S1: Peptides generated after incubation of A779 with neprilysin for up to 48 hours.

	0 h	24 h	48 h
A779-(1-7)	100	7.2	0
A779-(1-2)	0	12.8	22.0
A779-(1-3)	0	0.6	0.2
A779-(1-4)	0	5.9	3.1
A779-(3-4)	0	18.5	23.3
A779-(4-7)	0	0.6	0.1
A779-(5-7)	0	53.9	50.8
A779-(6-7)	0	0.5	0.5

Data are extraction ion current (EIC) areas for each peptide expressed as percent of total ion count.

$Asp^1 - Arg^2 \mid Val^3 \mid Tyr^4 \mid Ile^5 \mid His^6 - D\text{-}Ala^7$
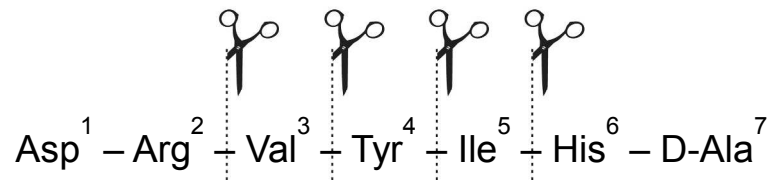
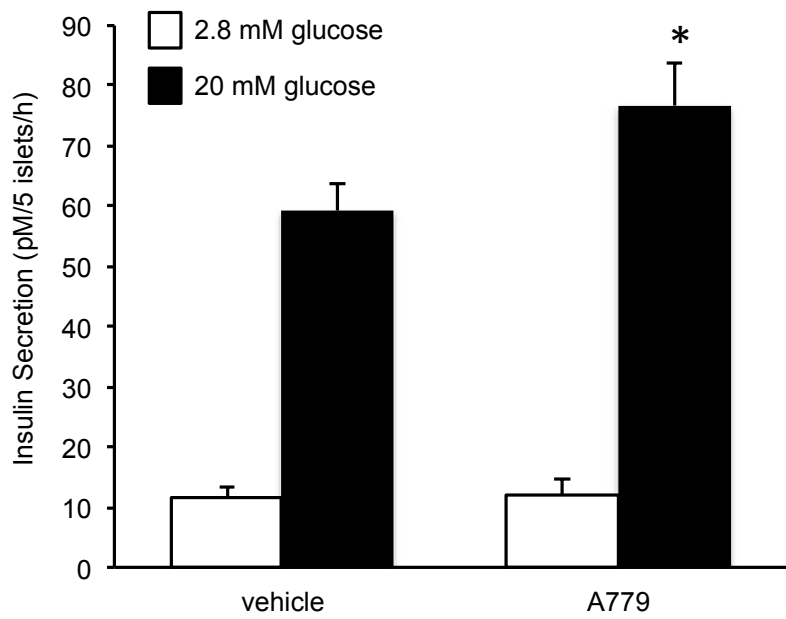**FIGURE S1: Neprilysin cleaves A779 at four sites.**

Figure S2: The MasR antagonist, A779, promotes insulin secretion in islets expressing neprilysin. Insulin secretion in response to 2.8 mM or 20 mM glucose from C57BL/6.NEP^{+/+} islets after 48-hour culture in the absence or presence of 1 μM A779. Data are mean $\pm$ SEM. *p<0.05 vs vehicle; n=11.
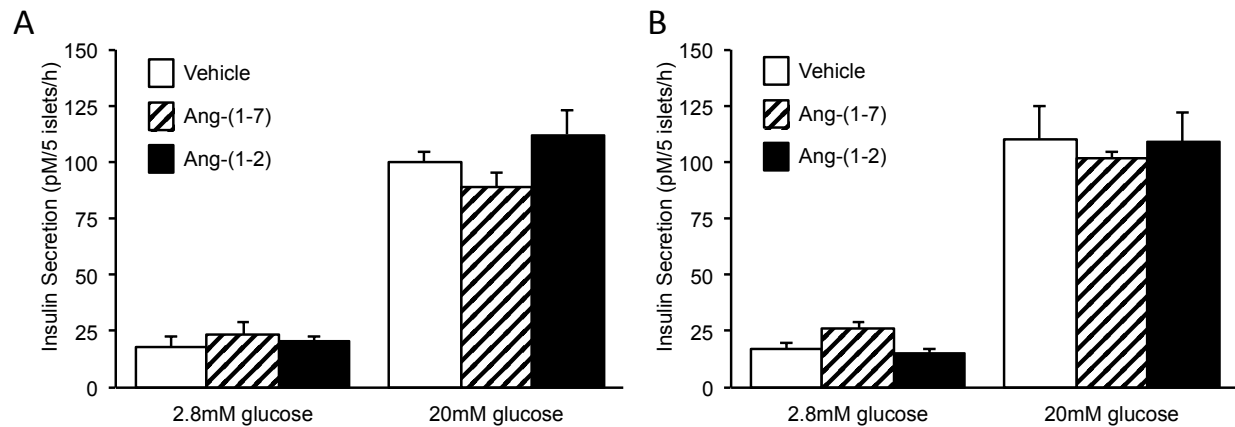
FIGURE S3: Short-term exposure to Ang-(1-7) or Ang-(1-2) does not potentiate GSIS.
Insulin secretion from $C57BL/6.NEP^{+/+}$ (A; n=6) and $C57BL/6.NEP^{-/-}$ (B; n=6) islets in response to 1-hour exposure to 2.8 mM or 20 mM glucose in the absence or presence of 1 nM angiotensin-(1-7) or 1 nM angiotensin-(1-2). Data are mean ± SEM.