

Peptidomics approach to elucidate the proteolytic regulation of bioactive peptides

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Edited by Peter G. Schultz, The Scripps Research Institute, La Jolla, CA, and approved April 18, 2012 (received for review February 23, 2012)

Peptide hormones and neuropeptides have important roles in physiology and therefore the regulation of these bioactive peptides is of great interest. In some cases proteolysis controls the concentrations and signaling of bioactive peptides, and the peptidases that mediate this biochemistry have proven to be extremely successful drug targets. Due to the lack of any general method to identify these peptidases, however, the role of proteolysis in the regulation of most neuropeptides and peptide hormones is unknown. This limitation prompted us to develop an advanced peptidomics-based strategy to identify the peptidases responsible for the proteolysis of significant bioactive peptides. The application of this approach to calcitonin gene-related peptide (CGRP), a neuropeptide associated with blood pressure and migraine, revealed the endogenous CGRP cleavage sites. This information was then used to biochemically purify the peptidase capable of proteolysis of CGRP at those cleavage sites, which led to the identification of insulin-degrading enzyme (IDE) as a candidate CGRP-degrading enzyme. CGRP had not been identified as an IDE substrate before and we tested the physiological relevance of this interaction by quantitative measurements of CGRP using IDE null (IDE^{-/-}) mice. In the absence of IDE, full-length CGRP levels are elevated *in vivo*, confirming IDE as an endogenous CGRP-degrading enzyme. By linking CGRP and IDE, this strategy uncovers a previously unknown pathway for CGRP regulation and characterizes an additional role for IDE. More generally, this work suggests that this may be an effective general strategy for characterizing these pathways and peptidases moving forward.

Bioactive peptides play a role in a broad range of physiology (1, 2), including pain sensation (3), blood pressure (4), and energy homeostasis (5, 6). The disruption of bioactive peptide signaling can lead to prevalent diseases such as diabetes (7). The regulation of bioactive peptides has therefore become an important area of research with implications for basic physiology as well as for medicine (3, 4, 8–11). Indeed, targeting the proteolytic pathways that regulate peptide levels has proven to be a successful strategy in the development of novel therapeutics (4, 8, 12). Dipeptidyl peptidase 4 (DPP4) inhibitors, for example, are a new class of antidiabetic drugs that prevent the degradation of glucagon-like peptide 1 (GLP-1), an insulinotropic peptide hormone, and thereby raise physiological insulin levels (8, 13, 14). The discovery of the peptidases that regulate other bioactive peptides will provide a deeper understanding of peptide regulation, identify new roles for enzymes, and may also reveal new opportunities in medicine.

The neuropeptide alpha calcitonin gene-related peptide (α CGRP, CGRP or CGRP_{1–37}) was discovered as an alternatively spliced form of the calcitonin RNA (15, 16). Shortly after its discovery, pharmacological studies revealed CGRP to be a potent vasodilator (17), suggesting a role in blood pressure regulation. Genetic studies have shown that mice lacking a functional CGRP receptor have elevated blood pressure (18), supporting the role for this peptide in regulating blood pressure. CGRP has also been shown to actively participate in the onset of migraines. Analysis of plasma from migraine sufferers revealed elevated CGRP levels in comparison with control patients (19). In addition, current

antimigraine therapeutics have been shown to lower CGRP levels (20). Together, these studies suggest that blockage of CGRP signaling could be of therapeutic benefit in treating migraine. The recent development of and clinical trials with CGRP-receptor antagonists support this hypothesis as patients taking CGRP-receptor antagonists have fewer migraines (21).

As a peptide with important biological and pathological roles, the underlying pathways that regulate CGRP levels and signaling are of great interest. Several aspects of CGRP regulation are understood—such as CGRP secretion following transient receptor potential cation channel subfamily V member 1 (TRPV1) activation (22)—but it is not known whether proteolysis controls physiological CGRP levels. There have been several unsuccessful attempts to identify peptidases responsible for CGRP proteolysis (23–26). *In vitro* experiments with recombinant enzymes, for example, have implicated several candidate peptidases (25, 26), but none of these enzymes have been shown to process CGRP *in vivo*. Immunoassays have also been used in an attempt to map the endogenous CGRP cleavage sites by identifying the endogenous CGRP fragments (23–25), but the inability of antibodies to distinguish proteolytic fragments (27, 28) limited this approach. The lack of a general approach to identify peptidases that regulate bioactive peptides and the desire to identify a CGRP-degrading enzyme inspired us to develop a peptidomics strategy to characterize a CGRP-degrading enzyme.

Results and Discussion

Peptidomics Analysis of Spinal Cords. Our strategy can be divided into three key steps: (i) the characterization of CGRP fragments in tissues using peptidomics, (ii) the identification of the peptidase capable of producing those fragments from full-length CGRP (CGRP_{1–37}), and (iii) the validation of the candidate CGRP-degrading enzyme through genetics in animal models (Fig. 1A). The distinguishing feature of our approach is the use of a liquid chromatography-tandem mass spectrometry (LC-MS/MS) peptidomics platform to detect CGRP fragments in tissues (29) and reveal the endogenous CGRP cleavage sites. Combining this peptidomics platform with biochemistry and genetics enables the discovery of the peptidases responsible for the proteolysis of important bioactive peptides.

Peptidomics analysis of mouse spinal cords detected a total of 10 CGRP fragments in addition to the full-length CGRP_{1–37} (Fig. 1B). Only two of these fragments, CGRP_{18–37} and CGRP_{19–37}, had previously been reported (24), which illustrates the dramatic

Author contributions: Y.-G.K., A.M.L., W.M.N., and A.S. designed research; Y.-G.K., A.M.L., and W.M.N. performed research; Y.-G.K., A.M.L., and W.M.N. contributed new reagents/analytic tools; Y.-G.K., A.M.L., W.M.N., and A.S. analyzed data; and Y.-G.K., A.M.L., W.M.N., and A.S. wrote the paper.

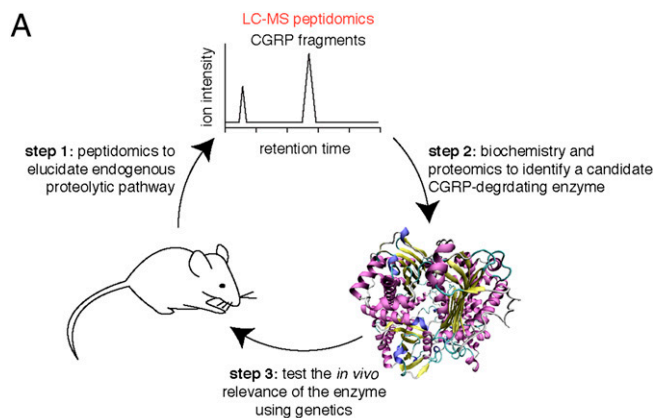
The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1203195109/-DCSupplemental.



B

Sequence	Fragment Number	% of Total CGRP Signal
full-length		
SCNTATCVTHRLAGLLSRSGGVVKNDFVPTNVGSEAF-NH ₂	CGRP ₁₋₃₇	22
N-terminal fragments		
SCNTATCVTHRLAGLLSRSGGVVKN	CGRP ₁₋₂₆	8
SCNTATCVTHRLAGLLS	CGRP ₁₋₁₇	8
C-terminal fragments		
FVPTNVGSEAF-NH ₂	CGRP ₂₇₋₃₇	17
VPTNVGSEAF-NH ₂	CGRP ₂₆₋₃₇	13
RSGGVVKNDFVPTNVGSEAF-NH ₂	CGRP ₁₈₋₃₇ *	8
SGGVVKNDFVPTNVGSEAF-NH ₂	CGRP ₁₉₋₃₇ *	14
GGVVKNDFVPTNVGSEAF-NH ₂	CGRP ₂₀₋₃₇	3
GVVKNDFVPTNVGSEAF-NH ₂	CGRP ₂₁₋₃₇	2
internal fragments		
VPTNVGSE	CGRP ₂₆₋₃₅	4
AGLLSRSGGVVKN	CGRP ₁₅₋₂₆	1

* previously reported



Fig. 1. Identifying endogenous CGRP cleavage sites. (A) Combination of peptidomics, biochemistry, and genetics provides a unique strategy to identify peptidases that process important bioactive peptides, such as CGRP. (B) Peptidomics analysis of mouse spinal cords reveals the largest number of CGRP fragments ever reported. These fragments were used to determine the endogenous CGRP cleavage sites. (Note: there is a disulfide between Cys₂ and Cys₇ for the full-length and N-terminal fragments). (C) Detection of the N-terminal CGRP fragments, CGRP₁₋₁₇ and CGRP₁₋₂₆, and the C-terminal fragments, CGRP₁₈₋₃₇ and CGRP₂₇₋₃₇, indicates that full-length CGRP₁₋₃₇ is cleaved at the Ser₁₇-Arg₁₈ and Asn₂₆-Phe₂₇ sites.

improvement in peptide coverage afforded by LC-MS/MS peptidomics. The two N-terminal fragments, CGRP₁₋₁₇ and CGRP₁₋₂₆, and two C-terminal fragments, CGRP₁₈₋₃₇ and CGRP₂₆₋₃₇, proved particularly useful in identifying the endogenous CGRP cleavage sites. These four peptides indicate that CGRP₁₋₃₇ has two cleavage sites *in vivo*; Ser₁₇-Arg₁₈ and Asn₂₆-Phe₂₇ (Fig. 1C). These CGRP cleavage sites had not previously been reported. In addition, testing some of these CGRP fragments reveals a loss of activity against the CGRP receptor, indicating that this proteolytic pathway can inactivate CGRP signaling (Table S1).

Tissue Lysate Experiments. Further support for these CGRP cleavage sites was obtained through *in vitro* experiments with tissue lysates. Upon incubation with mouse spinal cord lysates (soluble fraction), CGRP₁₋₃₇ is cleaved at Ser₁₇-Arg₁₈ and Asn₂₆-Phe₂₇ to produce the major endogenous CGRP fragments (i.e., CGRP₁₋₁₇, CGRP₁₈₋₃₇, CGRP₁₋₂₆, and CGRP₂₇₋₃₇) (Fig. S1 and Fig. 2). Further, we were interested in studying CGRP regulation

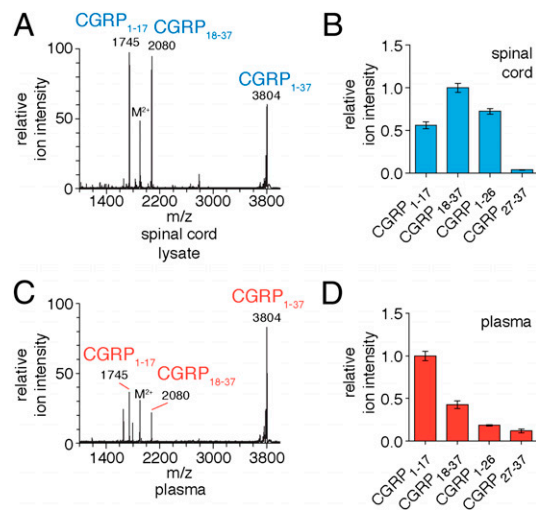


Fig. 2. Biochemical assays with CGRP₁₋₃₇ in spinal cord lysates and plasma. CGRP₁₋₃₇ was incubated with spinal cord lysate and analyzed by (A) MALDI-TOF MS and (B) LC-MS/MS. MALDI-TOF MS analysis identified CGRP₁₋₁₇ and CGRP₁₈₋₃₇ as the major breakdown products of CGRP₁₋₃₇, whereas the more sensitive LC-MS also identified CGRP₁₋₂₆ and CGRP₂₇₋₃₇. Incubation of CGRP₁₋₃₇ with plasma and subsequent analysis by (C) MALDI-TOF MS and (D) LC-MS/MS identified the same fragments in plasma. (Error bars show SEM.)

in the blood where CGRP is also found *in vivo*. Examination of CGRP₁₋₃₇ proteolysis in plasma demonstrated that the peptide is processed at Ser₁₇-Arg₁₈ and Asn₂₆-Phe₂₇, too (Fig. 2). The ratios of the CGRP fragments differ between the spinal cord lysates and plasma samples (Fig. 2B and D). We believe these differences are likely due to the differing relative stabilities of these fragments in spinal cord lysates and plasma after they are produced, which leads to the different fragment ratios observed. The identification of identical cleavage sites in plasma and spinal cord lysates suggests that the same unidentified peptidase may be responsible for CGRP proteolysis in both settings. To confirm this hypothesis, we proceeded to identify the peptidase.

Identification of Candidate Enzymes. A search of the peptidase database (MEROPS) (30) identified a number of candidate peptidases that would likely cleave the Ser₁₇-Arg₁₈ and Asn₂₆-Phe₂₇ sites of CGRP (Table S2). This list included peptidases and proteases from every class (serine, cysteine, aspartyl, and metallo), as well as soluble and membrane-bound forms of the enzyme. According to this list, the metallopeptidases thimet oligopeptidase (THOP) and matrix metalloprotease 19 (MMP19) can cleave CGRP at both cleavage sites, suggesting that a single enzyme may regulate CGRP. Because the MEROPS database cannot account for uncharacterized peptidases or proteases, we opted to biochemically purify the CGRP-degrading enzyme from tissues to ensure the identification of the correct enzyme.

To assay CGRP-degrading activity, we used LC-MS/MS to quantitatively measure the production of CGRP₁₋₁₇, CGRP₁₈₋₃₇, or CGRP₁₋₂₆ from full-length CGRP₁₋₃₇. First, we used this assay to determine the peptidase class (i.e., serine, cysteine, aspartyl, or metallo) responsible for CGRP proteolysis. The use of different protease inhibitors in spinal cord lysates and plasma revealed that the CGRP-degrading enzyme is a metallopeptidase, with CGRP₁₋₃₇ proteolysis inhibited by the presence of the metal chelators 1,10-phenanthroline or EDTA (Fig. 3 and Fig. S2). Metallopeptidase inhibitors also prevented the production of CGRP₁₋₂₆ (Fig. S3), which indicates that metallopeptidases are responsible for processing CGRP₁₋₃₇ at both cleavage sites.

To identify the peptidase, mouse spinal cord lysate was separated by anion exchange chromatography and fractions containing

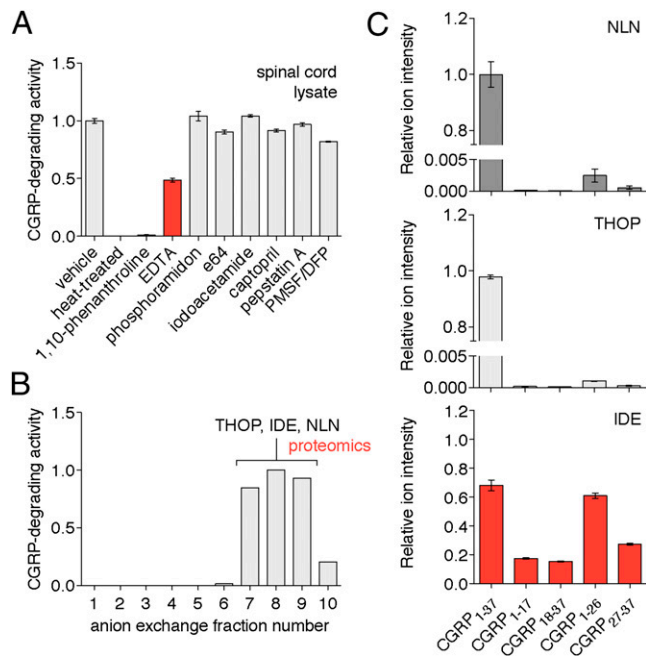


Fig. 3. Characterization of IDE as a candidate CGRP-degrading enzyme. CGRP-degrading activity was measured by quantitation of (A) CGRP₁₋₁₇ or (B) CGRP₁₈₋₃₇ produced from CGRP₁₋₃₇ by LC-MS (i.e., area under the curve for these peaks in the LC-MS chromatogram). (A) Biochemical assays using spinal cord lysates treated with class selective protease inhibitors (aspartyl, cysteine, metallo, or serine) showed that the peptidase responsible for CGRP proteolysis is a metallopeptidase. Control reactions included a vehicle-treated sample that retained full activity and a heat-treated sample that lacked any activity. Metallopeptidase inhibitors 1,10-phenanthroline and EDTA were the only two compounds tested that inhibited the CGRP degradation. (B) Identification of candidate peptidases was carried out by anion-exchange chromatography of the spinal cord proteome followed by proteomics of the fractions with the highest levels of CGRP-degrading activity. Proteomics identified three metallopeptidases in these fractions; THOP, NLN, and IDE. (C) IDE was identified as the only peptidase with CGRP-degrading activity by incubating CGRP₁₋₃₇ with recombinant murine THOP, NLN, and IDE followed by quantitation of CGRP₁₋₃₇, CGRP₁₋₁₇, CGRP₁₈₋₃₇, CGRP₁₋₂₆, and CGRP₂₇₋₃₇. THOP and NLN barely showed any activity against CGRP (two orders of magnitude less than IDE). (Error bars show SEM).

CGRP-degrading activity were analyzed by proteomics to identify any metallopeptidases (Fig. 3). The fractions with the highest CGRP-degrading activity contained the metallopeptidases neurolysin (NLN), thimet oligopeptidase (THOP) and insulin-degrading enzyme (IDE, insulysin). The addition of N-ethylmaleimide (NEM)—a cysteine-labeling reagent that inhibits IDE (31), THOP, and NLN (32)—to spinal cord lysates and plasma prevented CGRP proteolysis and supported a role for one or more of these enzymes in CGRP processing (Fig. S4). According to the MEROPS database, IDE should be able to cleave at the Ser₁₇-Arg₁₈ site, NLN at the Asn₂₆-Phe₂₇ site, and THOP at both sites (Table S2).

Identification of IDE as a CGRP-Degrading Enzyme Candidate. To determine whether any of these peptidases is a CGRP-degrading enzyme—none of them have been implicated in CGRP proteolysis previously—we performed a series of biochemical assays with pure enzymes. When CGRP₁₋₃₇ was incubated with each peptidase, only IDE cleaved CGRP₁₋₃₇ at Ser₁₇-Arg₁₈ and Asn₂₆-Phe₂₇ efficiently, whereas NLN and THOP barely cleaved CGRP₁₋₃₇ at all (Fig. 3C and Fig. S5). The discordance between these data and the predicted cleavage specificities is due to the fact that MEROPS only takes into account sequence. It is known that NLN and THOP prefer to cleave shorter peptides (<17

amino acids) (33). By contrast, IDE has been reported to cleave longer peptide substrates such as insulin (34, 35) and the beta amyloid peptide (A β) (34, 36) *in vivo*.

IDE Regulation of CGRP in TT Cells. The hydrolysis of CGRP₁₋₃₇ by pure IDE demonstrates that IDE is able to cleave CGRP₁₋₃₇ in a physiologically relevant manner, but it was still necessary to ascertain whether IDE is responsible for the CGRP-degrading activity in complex proteomes (i.e., cell culture, spinal cord lysate, or plasma). Toward this goal, CGRP₁₋₃₇ proteolysis was first analyzed in TT cells, which naturally produce CGRP (37). Overexpression of IDE in TT cells led to lower concentrations of CGRP₁₋₃₇ and elevated levels of CGRP₁₈₋₃₇ in conditioned media, demonstrating the IDE can mediate CGRP₁₋₃₇ proteolysis in cell culture (Fig. 4A). Importantly, these experiments demonstrate that CGRP and IDE can interact in a biologically relevant environment. Although IDE is predominantly an intracellular enzyme, recent studies have demonstrated that it can be secreted from cells through a noncanonical exosome-mediated pathway (38, 39). Thus, even though IDE does not have a signal sequence indicating export from the cell, it is secreted and can interact with CGRP in the extracellular environment.

To test that IDE is responsible for the majority of CGRP-degrading activity observed in tissue lysates, we performed *ex vivo* experiments using tissue lysates and plasma from IDE null (IDE^{-/-}) mice (34). In the absence of IDE, the majority of CGRP-degrading activity was lost, confirming this enzyme as the major source of CGRP-degrading activity in both spinal cord lysates and plasma (Fig. 4). This result supports the *in vitro* assignment of IDE as the CGRP-degrading enzyme and validates

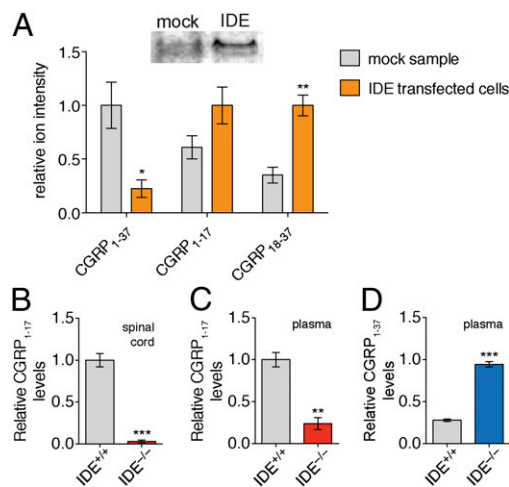


Fig. 4. IDE cleaves CGRP₁₋₃₇ in complex proteomes. (A) TT cell line naturally produces and secretes CGRP. By Western blot (*Inset, Top*) TT cells have very little or no IDE. To study the impact of IDE on CGRP levels in TT cells, we transfected these cells with an empty vector (mock) or a vector containing the IDE gene (IDE transfected cells). CGRP₁₋₃₇ levels were significantly lower in media from IDE transfected cells, whereas levels of the CGRP fragment, CGRP₁₈₋₃₇, were elevated in media expressing IDE, indicating increased CGRP₁₋₃₇ proteolysis. Incubation of CGRP₁₋₃₇ with (B) spinal cord lysate and (C) plasma revealed higher CGRP₁₋₁₇ in IDE^{+/+} samples, demonstrating that IDE is responsible for the majority of the CGRP-degrading activity in these lysates and plasma. (D) Incubation of CGRP₁₋₃₇ with plasma revealed that CGRP₁₋₃₇ is more stable in IDE^{-/-} plasma, demonstrating that IDE processing can regulate CGRP₁₋₃₇ levels *in vitro*. Relative quantification was accomplished by using the ion intensities of the different peptides in the LC-MS chromatogram. These ion intensities were then normalized to the largest peak to enable comparison of different peptides on the basis of relative changes in their abundance. (**P* value < 0.05, ***P* value < 0.01, ****P* value < 0.001; *P* values were derived from the two-tailed Student *t* test, *n* = 3. Error bars show SEM.)

the hypothesis that the same enzyme is responsible for the CGRP-degrading activity in spinal cord lysates and plasma. In addition, in these *ex vivo* experiments we find that CGRP levels are higher when incubated with plasma from IDE^{-/-} mice. Of course, *in vivo* proteolysis will compete with other processes, such as renal clearance or tissue uptake, and therefore the net effect of IDE proteolysis on endogenous CGRP levels had to be assessed in animals.

IDE Regulation of CGRP Levels in Plasma. To determine whether IDE is a CGRP-degrading enzyme *in vivo*, we measured the absolute levels of CGRP₁₋₃₇ in plasma from IDE^{+/+} and IDE^{-/-} mice using an isotope-dilution mass spectrometry (IDMS) approach (40). We selected plasma for these measurements because IDE and CGRP₁₋₃₇ are both present in blood and therefore they should be able to interact (22, 39). In addition, we compared CGRP₁₋₃₇ levels in mice treated with capsaicin, a TRPV1 agonist that promotes CGRP₁₋₃₇ secretion (22) (Fig. 4). The capsaicin experiments were included to establish whether IDE regulates CGRP₁₋₃₇ under different conditions, such as the rapid increase in plasma CGRP₁₋₃₇ levels associated with TRPV1 activation.

Comparison of plasma from IDE^{+/+} and IDE^{-/-} mice revealed significantly higher CGRP₁₋₃₇ levels in IDE^{-/-} mice (40% greater than IDE^{+/+} mice) (Fig. 5). These data establish IDE as an endogenous regulator of CGRP and demonstrate a role for proteolysis in CGRP regulation. As expected, capsaicin increased the absolute amounts of CGRP₁₋₃₇ in plasma regardless of genotype, but this increase did not impact the ability of IDE to regulate CGRP₁₋₃₇. In the capsaicin-treated cohort, CGRP₁₋₃₇ levels were 60% greater in plasma samples from IDE^{-/-} mice than in IDE^{+/+} plasma samples (Fig. 4B). Interestingly, some phenotypes associated with capsaicin administration such as vasorelaxation (22) are mediated by CGRP. The fact that IDE^{-/-} (red) samples had similar levels of CGRP₁₋₃₇ to capsaicin-treated IDE^{+/+} samples

(dark gray) (Fig. 5) suggests that IDE^{-/-} mice may also have similar phenotypic responses as capsaicin-treated IDE^{+/+} mice in certain assays.

Concluding Remarks. In conclusion, we have discovered an additional role for IDE as a CGRP-degrading enzyme and simultaneously elucidated a proteolytic pathway for CGRP regulation *in vivo*. CGRP is only the third substrate identified for IDE *in vivo*, along with insulin^{22,23} and A β ^{22,24}. The identification of insulin (34, 35) and A β (34, 36) as substrates for IDE helped explain the link between IDE and diabetes (41, 42) and Alzheimer's disease (43–45), respectively. Moreover, the identification of A β as an IDE substrate has led to the development of small-molecule IDE activators in an attempt to develop novel treatments for Alzheimer's disease (46). The identification of CGRP as an IDE substrate suggests a potential role for this enzyme in blood pressure as well as the etiology of migraine, two important processes regulated by CGRP. It will be interesting to test specific hypotheses regarding IDE and physiology as more tools [e.g., small-molecule IDE activators (46) and IDE inhibitors (47)] become available. More generally, this discovery indicates that our peptidomics-based strategy can reveal unknown pathways and peptidases that regulate significant bioactive peptides, such as CGRP. Given the success of peptidase inhibitors as drugs, the continued application of this approach may reveal new targets for the development of next-generation therapeutics.

Materials and Methods

LC-MS/MS Experiments to Detect CGRP Peptide Fragments. Aliquots of peptide extracts from spinal cords were injected onto a Waters nanoAcquity HPLC configured with in-house packed 75- μ m reverse-phase capillary trapping and analytical columns (New Objective) coupled to an LTQ-Orbitrap mass spectrometer (ThermoFisher Scientific). The liquid chromatography gradient proceeded from 3 to 33% acetonitrile/water (0.1% formic acid) over 180 min. Mass spectra from 395 to 1,600 *m/z* were acquired in the Orbitrap with a resolution of 60,000, and the six most abundant ions were targeted for concurrent MS/MS in the linear ion trap with relative collision energy of 30%, 2.5-Da isolation width, and recurring ions dynamically excluded for 60 s. Sequencing was done using the SEQUEST algorithm with differential modifications including methionine oxidation, amidation, acetylation, phosphorylation, and disulfide. Peptides were accepted within 3 ppm of the expected mass if they also met a series of custom filters on ScoreFinal (S_f), -10 Log P and charge state that attained an average peptide false discovery rate (FDR) of <2% across datasets. Manual inspection of spectra, FDR calculation, and protein inference were performed in Proteomics Browser Suite 2.23 (Thermo Fisher Scientific).

MALDI-TOF MS and LC-MS/MS Analysis of *In Vitro* and *In Vivo* Peptides. Quenched *in vitro* and *in vivo* peptides were desalted using a ZipTip C18 (Millipore; ZTC18S096) according to the manufacturer's protocol before MALDI-TOF MS and LC-MS/MS analysis. MALDI-TOF MS was performed with α -cyano-4-hydroxycinnamic acid as the matrix. Reaction products were also monitored using LC-MS/MS to confirm the peptides observed in the MALDI spectra. For LC-MS analysis, MALDI samples were diluted 10-fold into 0.1% formic acid in water. Aliquots were injected onto an Eksigent nanoLC-2D HPLC configured with a prepacked IntegraFrit trapping column (ProteoPep II C18, 300 \AA , 5 μ m) and an in-house packed reverse-phase 75- μ m picotip analytical column (New Objective) coupled to an LTQ mass spectrometer (ThermoFisher Scientific). The liquid chromatography gradient proceeded from 5 to 50% acetonitrile in water (0.1% formic acid) over 40 min. Mass spectra were acquired from 400 to 1,600 *m/z* followed by targeting the three most abundant ions for MS/MS. Sequencing was carried out using the SEQUEST algorithm with differential modifications including methionine oxidation, amidation, acetylation, phosphorylation, and disulfide.

Capsaicin Injection Experiments. For capsaicin injection experiments, 5- to 7-month-old male and female IDE^{-/-} and IDE^{+/+} mice ($n = 5$; 3 females and 2 males) were fasted overnight. Injections were performed *i.p.* with a 10 μ L/g injection of either vehicle or 0.1 mg/mL capsaicin in 5% Tween-80, 5% ethanol, and 90% saline (vol/vol) for a final dose of 1 mg/kg capsaicin. Mice were

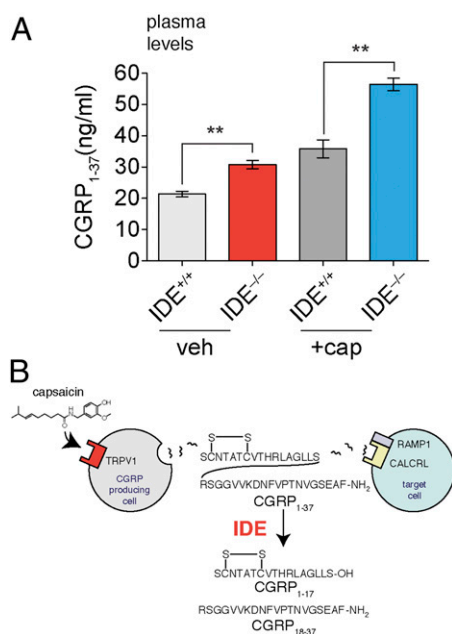


Fig. 5. IDE regulates CGRP₁₋₃₇ *in vivo*. (A) IDE^{+/+} and IDE^{-/-} mice were treated with vehicle or capsaicin, and absolute endogenous CGRP levels were measured by IDMS²⁹ using a synthetic d18-CGRP₁₋₃₇ internal standard. CGRP₁₋₃₇ levels were significantly higher in the IDE^{-/-} mice than in the IDE^{+/+} mice under both treatment conditions. (B) A unique model for CGRP regulation includes the IDE-mediated proteolysis pathway discovered here. (***P* value < 0.01; *P* values were derived from the two-tailed Student *t* test, $n = 3$. Error bars show SEM.)

allowed to return to their cages for 5 min and then plasma was collected as described below in *Quantitative CGRP Measurements from Plasma*.

Quantitative CGRP Measurements from Plasma. To prevent the breakdown of physiological peptides during standard blood processing, we used a modified version of the recently reported reduced temperatures, acidification, protease inhibition, isotopic exogenous controls, and dilution (RAPID) method (48). Mice were killed by CO₂ inhalation, decapitated, and their trunk blood collected into an EDTA-coated tube (BD Biosciences). This tube was quickly inverted five times and aliquots of blood (200 μ L) were rapidly transferred into ice-cold buffer (pH 3.6, 4 mL) containing 1 nM heavy-labeled CGRP₁₋₃₇ (d18-CGRP₁₋₃₇), 0.1 M ammonium acetate, 0.5 M NaCl, and enzyme inhibitors (1 μ g/mL of LAF-237, e-64, antipain, leupeptin, chymostatin, 1,10-phenanthroline, N-ethylmaleimide) and then immediately centrifuged at 3,000 \times g for 10 min at 4 $^{\circ}$ C. The supernatant was transferred into a new tube and frozen at -80° C for 24–72 h.

A Sep-Pak C18 cartridge 6 mL (Waters; WAT043395) was preconditioned by washing three times with acetonitrile (18 mL total) and four times with 0.1% TFA in water (24 mL total). Frozen supernatants from the RAPID plasma collection were thawed and 2 mL of this supernatant was applied to the Sep-Pak C18 cartridge. The loading step was repeated three times to ensure that all of the material bound to the column. The Sep-Pak was then washed with 0.1% TFA in water (24 mL total) and eluted with a 70:30 mixture of acetonitrile/water containing 0.1% TFA (2 mL total) into LoBind Eppendorf tubes.

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