Opinion

New horizons in specific hormone proteolysis

Jakob Triebel [iD],[1,*] Juan Pablo Robles [iD],[2] Magdalena Zamora [iD],[1,2]
Carmen Clapp [iD],[2] and Thomas Bertsch[1]

Proteolysis of protein hormones is primarily acknowledged in the context of breakdown and metabolic clearance by hepatorenal elimination. However, less explored is the specific proteolytic processing of large protein hormones, for which canonical signaling pathways were already established [e.g., prolactin (PRL)], to generate unique messengers that impact cellular functions via pathways unrelated to the receptors of their precursor molecules. Yet, the proteolysis of PRL to generate new messengers evolved under positive selection, and cleaved protein hormones regulate essential functions to maintain homeostasis at the organismal, tissue, or organ levels. The cleavage sites at which proteolysis occurs and the proteases with their determinants define a hormone–metabolism junction at which specific proteolytic cleavage, pathological alteration, and hepatorenal elimination occur.

Proteolytic breakdown and elimination of protein hormones: is that all there is?

The physiology of protein hormones is conventionally described by a sequence of events through which hormones are synthesized, are released into the circulation, act on primary target cell(s), and are then subjected to proteolytical breakdown and hepatorenal elimination. The plasma half-time of protein hormones is relatively short. For example, the protein hormone **PRL** (see Glossary), best known for its fundamental role in lactation, has a half-time of disappearance from the circulation of ~25–50 min [1], and its metabolic fate is proteolysis and clearance by the liver and kidney. A similar plasma half-time has also been determined for human **growth hormone (GH)**, essential for somatic growth [1,2], and for **placental lactogen** [**PL**; also known as **chorionic somatomammotropin hormone** (**CSH**1/2)], as determined by studies in animals [3,4]. Renal elimination is a cornerstone of the **metabolic clearance rate (MCR)** of these three protein hormones, as indicated by the direct correlation between their MCR and **glomerular filtration rate (GFR)** and the reduction in their MCR in patients with chronic renal failure. Clearing by glomerular filtration and urine excretion is further exemplified by the facts that filtered GH associates with low peritubular uptake and secretion, and only degradation products of GH return to the circulation [5,6]. In addition, di- or multimeric molecular complexes, formed by the binding of GH to GH-binding proteins or of PRL to immunoglobulin complexes, and post-translational modifications, such as glycosylation, significantly alter the renal MCR of these two hormones [7–10]. The main site of extrarenal disposal of all protein hormones is the liver, where cellular uptake by receptor-mediated endocytosis is followed by degradation in endocytotic vesicles via lysosomal interaction and ubiquitin–proteasome-associated pathways [11]. Inside vesicles and lysosomes, protein hormones are exposed to an acidic pH, whereupon they uncoil, and are cleaved by proteases, such as cathepsins. Moreover, proteolysis determines the removal of signal peptides from prehormones and the processing of inactive precursor molecules or prohormones. One of the best-known examples of the latter is that of proopiomelanocortin (POMC), so named because it encodes opioid, melanotropic, and corticotropic activities. POMC, a 226-amino acid precursor, is cleaved by prohormone-converting enzymes type 1 and 2 (PSK1 and PSK2) and carboxypeptidase E to generate adrenocorticotropic

[1]Institute for Clinical Chemistry, Laboratory Medicine and Transfusion Medicine, Nuremberg General Hospital and Paracelsus Medical University, Nuremberg, Germany
[2]Instituto de Neurobiología, Universidad Nacional Autónoma de México (UNAM), Campus UNAM-Juriquilla, Querétaro, México

*Correspondence:
Jakob.Triebel@gmx.de (J. Triebel).

hormone; α-, β-, and γ-melanocyte-stimulating hormone (MSH); and β-endorphin, and other protein hormones, respectively. The processing of POMC by proteolytic enzymes is tissue specific, depending on the differential distribution of responsible enzymes in the various tissues exposed to POMC. Another relevant example is insulin produced by pancreatic β cells, which is generated by the enzymatic cleavage of **proinsulin** and the subsequent proteolytic removal of the C peptide [1]. **Preproinsulin** and proinsulin are also cleaved by PSK1, PSK2, and carboxypeptidase E and secretory granules contain insulin and C peptide in equimolar amounts and only minute quantities of proinsulin and partially cleaved intermediates [1]. The circulatory half-life of endogenous insulin is ~3–5 min, and it is degraded mainly by insulinases in the liver and kidney. Similar proteolytic processing occurs during the maturation of numerous other protein hormones, such as glucagon and somatostatin.

Based on these findings, a rigid understanding of protein hormones developed, in which the cellular synthesis, release, endocrine, paracrine, and/or autocrine effects via a specific receptor in the target tissue, and the subsequent metabolic clearance by hepatorenal elimination, represent their essential physiology, with few or no exceptions. The role of proteolysis was largely confined to prohormone processing and degradation. This long-held intellectual framework prevented the exploration of other avenues of proteolysis with functional significance, such as of the specific proteolytic processing of protein hormones for which **canonical signaling** pathways were already established, such as PRL and GH, not for the purpose of metabolic clearance, but instead to generate additional unique messengers that impact cellular functions via pathways unrelated to the receptors of their precursor molecules. Interest focused on the detailed characterization of the canonical pathways and effects of PRL, GH, and PL, while the role of their cleaved products as relevant functional messengers was underestimated.

Canonical signaling of protein hormones: end of the story?

Protein hormones signal through seven-transmembrane domain (TMD) and single-TMD receptors, which are further subdivided into growth factor receptors, cytokine receptors, and guanylyl cyclase-linked receptors. The seven-TMD receptors depend on G protein transducers, and single-TMD receptors mostly harbor intrinsic tyrosine kinase activity. The transforming growth factor (TGF)-β family signals through serine/threonine kinase domains and guanylyl cyclase-linked receptors to induce the synthesis of cGMP. The critical components of PRL and GH signaling are single-TMD receptors, which, after ligand binding, activate Janus kinase tyrosine kinases, which phosphorylate cytoplasmatic signal transducer and activator of transcription proteins (the JAK/STAT signaling pathway). The different modes of canonical protein hormone signaling result in effects in which activation depends on systemic (endocrine), local (autocrine or paracrine), or intracellular (intracrine) routes of communication. These modes of operation are subject to regulation at the hypothalamic, pituitary, and/or the peripheral level, which thereby define three tiers of control in **endocrine axes**. However, proteolytically cleaved fragments of protein hormones activate **non-canonical signaling** pathways, which are not activated by their uncleaved precursor molecules. A prime example is **vasoinhibin**, a molecular fragment generated by the proteolytic processing of PRL, GH, and PL by proteases such as cathepsin D [12,13], matrix metalloproteases (MMPs) [14], bone morphogenetic protein 1 [15], thrombin [16], and plasmin [17]. In contrast to its parent molecule PRL, vasoinhibin is antiangiogenic and inhibits vasodilation and vasopermeability. It does not activate the PRL receptor, but instead interacts with a multicomponent complex formed by plasminogen activator inhibitor-1, urokinase plasminogen activator, and the urokinase plasminogen activator receptor [18] to block the activation of the Ras-Raf-MAPK pathway, the Ras-Tiam1-Rac1-Pak1 pathway, and the Ca^{2+}/calmodulin and PI3K/Akt activation of endothelial nitric oxide synthase induced by several proangiogenic and vasopermeability factors [vascular endothelial growth factor (VEGF), basic

Glossary

Canonical signaling: signaling via established, specific cell surface receptors.

Cleavage site: site at which a protein hormone is enzymatically cleaved; usually comprises eight amino acids.

Consensus sequence: sequence of amino acids at which the most efficient proteolytic cleavage occurs.

Diabetic retinopathy: pathological alterations in the retina of patients with diabetes mellitus.

Endocrine axis: hormone levels and effects controlled by hypothalamic, pituitary, and peripheral signals.

Exon junction: junction between two exons.

Glomerular filtration rate (GFR): volume of circulating blood plasma that is filtered by the kidney per time unit.

Growth hormone (GH): protein hormone essential for somatic growth.

Isoforms: molecules with the same effect but different structure (size).

Metabolic clearance rate (MCR): volume of circulating blood plasma that is cleared of a protein hormone per time unit.

Non-canonical signaling: signaling unrelated to specific cell surface receptors.

Peripartum cardiomyopathy: syndrome in pregnant women with cardiac insufficiency.

Placental lactogen or chorionic somatomammotropin hormone (PL/CSH): protein hormone secreted by the placenta.

Preeclampsia: syndrome in pregnant women with elevated blood pressure and proteinuria.

Preproinsulin, proinsulin: inactive insulin precursor molecules.

Prolactin (PRL): protein hormone essential for lactation.

Vasoinhibin: protein generated by the proteolytic cleavage of PRL, GH, and PL, with antiangiogenic effects.

fibroblast growth factor (bFGF), interleukin (IL)-1β, and bradykinin] [19,20]. The bioactive site of vasoinhibin is an HGR motif, a short linear motif at residues 74–76 (or residues 46–48 without the signal peptide) [21]. The generation of vasoinhibin, its bioactive domain, and specific signaling pathways exemplify how cleaved protein hormones convert into messengers with new functionality that act beyond the canonical signaling pathways of their uncleaved precursors (Figure 1 and Boxes 1 and 2).

Active protein hormone fragments: an accident of birth?

If proteolytic processing of protein hormones is considered as the consecutive breakdown of a longer chain of amino acids by one or more proteases, the enzymatic **cleavage sites** appear as arbitrary regions that are already exposed at either the solvent-accessible surface area of

The hormone-metabolism junction
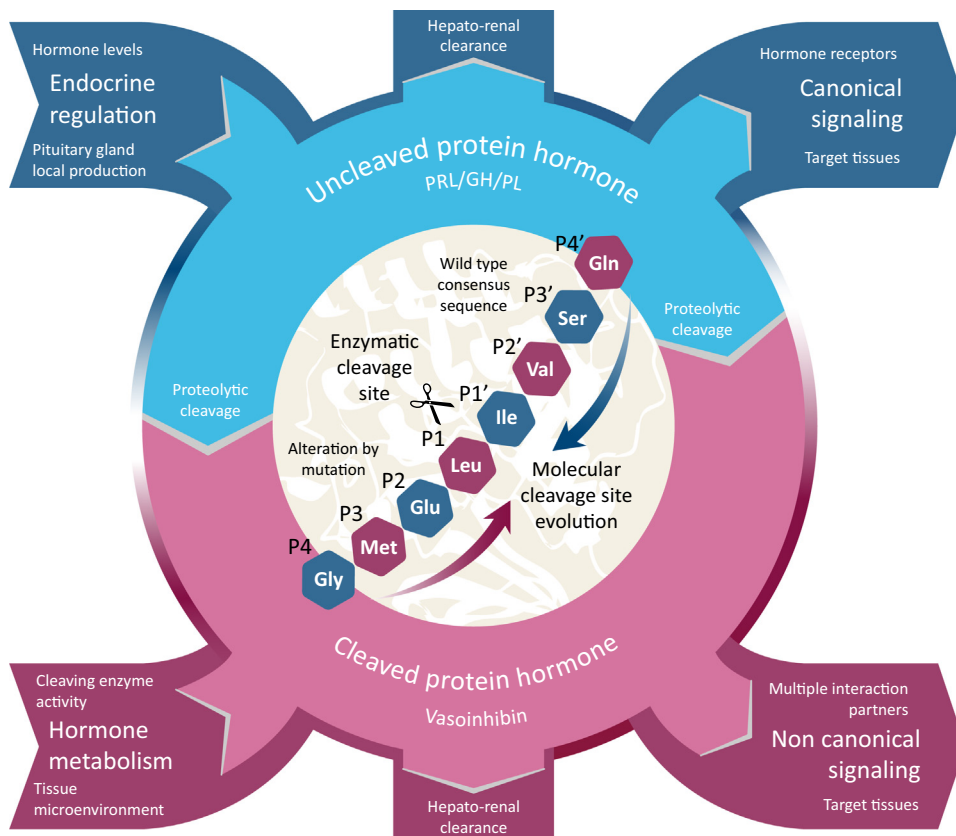
Figure 1. Circulating protein hormone levels and levels in target tissues are controlled by the hypothalamus, pituitary gland, and local production. Uncleaved protein hormones exert their effects via their canonical signaling pathways in the target tissues and are subject to hepatorenal clearance from the circulation. Specific proteolytic cleavage generates cleaved protein hormones, which signal via multiple interaction partners not related to the signaling pathways of their uncleaved precursors. Proteolytic cleavage occurs at the wild-type consensus sequence comprising eight amino acids and can be altered by mutation. The cleavage sites are conserved and have evolved under positive selection. The cleavage sites in the protein hormone at which proteolysis occurs and the proteases with their determinants define a hormone–metabolism junction at which specific proteolytic cleavage, pathological alteration, and hepatorenal elimination can occur. Abbreviations: GH, growth hormone; PL, placental lactogen; PRL, prolactin.

Box 1. Vasoinhibin

Vasoinhibin is generated by the proteolytic cleavage of PRL, GH, and PL.

Vasoinhibin isoforms vary in size, depending on the cleavage site and cleaving enzyme, ranging from 5.6 to 18 kDa.

Vasoinhibin interacts with plasminogen activator inhibitor-1 and blocks the activation of the Ras-Raf-MAPK pathway, the Ras-Tiam1-Rac1-Pak1 pathway, and the Ca^{2+}/calmodulin and PI3K/Akt activation of endothelial nitric oxide synthase induced by several proangiogenic and vasopermeability factors.

The bioactive site of vasoinhibin derived from PRL is a short motif of three residues (HGR).

Vasoinhibin inhibits angiogenesis, vasopermeability, and vasodilation.

The vasoinhibin receptor remains poorly defined.

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the folded intact protein precursor, regions that become exposed when the protein reorganizes its 3D structure after a first cleavage, or sites that become solvent accessible dependent on pH-induced structural changes. The cleavage sites of the substrates would then have features resembling natural substrates and act as active site intruders. Enzymes involved in protein catabolism would act catalytically promiscuously and generate fragments that are physiologically irrelevant. Along this line, the biological activity of a cleaved protein hormone fragment, such as vasoinhibin, would be an accident of birth. However, the contrary is suggested by recent evidence. Bioactive sites of protein hormone fragments and cleavage sites have evolved and are conserved; in addition, predicted proteolysis sites in large-scale protein analyses coincide with transcribed **exon junctions** [22]. For example, the HGR motif determining the bioactive site of vasoinhibin is conserved in mammals, birds, reptiles, amphibians, and fish [21]. In addition, the cleavage site responsible for the generation of a 15-kDa vasoinhibin **isoform** evolved in higher primates and has remained conserved [23]. Cleavage sites are usually defined as eight residues relevant for the recognition of the substrate, the formation of the transition state, and the subsequent disruption of the peptide bond between the residues. The eight residues within a given protein sequence are numbered P4, P3, P2, P1, P1', P2', P3', and P4', with cleavage occurring between P1 and P1' [24] (Figure 1). Proteases usually require a specific sequence of residues at the eight positions of the cleavage site (**consensus sequence**), and alterations in this sequence, such as point mutations, can either inhibit or facilitate the cleavage. It appears that there can be more than one cleavage site per protease, such that isoforms of various sizes are generated. For example, β-endorphin, a cleavage product of POMC, and an endogenous μ-opioid receptor agonist with pain-relieving effects may comprise 26, 27, or 31 amino acids, depending on where cleavage occurs. The variability of vasoinhibin isoforms is even larger and comprises isoforms between 5.6 and 18 kDa, which reflects cleavage by more than one protease at various cleavage sites and is linked not only to tissue-specific cleavage, but also to differential effects in various organic systems [25] (Boxes 1 and 2).

Box 2. Cleaved protein hormones

Cleaved protein hormones are generated by proteolytic cleavage of protein hormones for which canonical signaling pathways may already exist.

Cleaved protein hormones are subject to evolutionary conservation and positive selection.

Various isoforms exist, depending on the cleaving enzyme and tissue.

Cleaved protein hormones contain folded structures and intrinsically disordered regions.

Cleaved protein hormones can signal via short linear motifs and through more than one signaling pathway.

Targets include metabolic and organ system homeostasis.

When the rubber hits the road: clinical significance?

Dysfunction of the proteolytic processing of protein hormones is usually a disease modifier in multi-etiological endocrine and metabolic entities and syndromes, which is likely to have contributed to the systematic undervaluation of cleaved protein hormones and their physiological and pathological significance. However, it is now acknowledged that the whole chain of instances in which proteolytic processing of protein hormones occurs is susceptible to dysfunction. Such dysfunction may affect the proteolytic removal of signal peptides, prepro- and prohormone processing, as well as the generation of cleaved protein hormones from mature protein hormones with established canonical signaling, whereby 'dysfunction' relates to sequence aberrations in the substrate or cleaving enzyme, such as mutations, changes in enzyme activity or microenvironmental factors determining their activity, or alterations in the enzyme–substrate ratio. Consequences may affect the secretory pathway, protein conformation, and secreted or circulating hormone levels. For example, an Ala2Thr mutation in preproinsulin is projected to lead to a conformational change from an α-helix to β-sheet, severely compromising the efficiency of signal peptide cleavage of preproinsulin. Ala2Thr mutation carriers secrete insulin at only 50% of the levels of their unaffected relatives and the mutation co-segregates with diabetes mellitus whereas the unaffected relatives remain healthy. Therefore, the mutation appears as a pathogenic cause of maturity-onset diabetes of the young (MODY) with a defect in insulin biosynthesis, classified as INS/MODY 10 [26,27]. In addition, cleaving or converting enzymes may be affected. For example, mutations in the gene encoding carboxypeptidase E (*CPE*) were responsible for the manifestation of a syndrome characterized by obesity, intellectual disability, and hypogonadotropic hypogonadism in three siblings. Whole-exome sequencing revealed a homozygous nonsense c.405>A (p.Y135*) mutation in *CPE* that was present in the index case and their affected siblings. Their parents were phenotypically normal heterozygous mutation carriers [28]. Mutations in *PRL*, *CSH1/2,* or *GH1* or the cleaving enzymes responsible for the generation of vasoinhibin are possible but have not yet been investigated in conjunction with associated phenotypes or diseased conditions, such as **preeclampsia**, **diabetic retinopathy**, or **peripartum cardiomyopathy** [29]. However, for the latter two, clinical studies were conducted or are underway, in which the generation of vasoinhibin is pharmacologically stimulated or inhibited. For the treatment of diabetic retinopathy and diabetic macular edema, an ongoing clinical study is investigating the effect of levosulpiride, a selective dopamine D2 receptor antagonist, on retinal alterations [30]. The rationale behind this trial is that the levosulpiride-induced hyperprolactinemia promotes MMP-mediated conversion to vasoinhibin, which could lead to beneficiary outcomes in terms of vasoinhibin-mediated antagonization of diabetes-induced retinal alterations [31] (NCT03161652[i]). For the treatment of peripartum cardiomyopathy, a trial investigated the effect of bromocriptine, a dopamine D2 receptor agonist[ii]. The rationale of treatment with bromocriptine is the inhibition of vasoinhibin generation due to enhanced activity of cathepsin D by substrate depletion to prevent detrimental effects on myocardial microvascularization. The trial demonstrated that bromocriptine treatment was associated with a high rate of left ventricular recovery [32]; thus, adding bromocriptine to standard heart failure therapy has been included as a consideration in the guidelines for the management of cardiovascular diseases during pregnancy [33]. Both treatment rationales resemble concepts in the treatment of metabolic diseases, where substrate depletion or supplementation therapies are established. The generation of vasoinhibin depends on PRL levels, and hyperprolactinemia promotes the conversion of PRL to vasoinhibin by providing more substrate for cleaving proteases. Hyperprolactinemic mice overexpressing PRL in the liver have enhanced levels of circulating vasoinhibin associated with increased blood pressure [34]. Dopamine D2 receptor antagonist-induced hyperprolactinemia results in higher levels of vasoinhibin in ocular tissues and fluids of rats [35] and humans [31] and can protect against diabetic retinopathy and diabetic macular edema. These observations unravel interactions at the level of the PRL–vasoinhibin axis influencing the pathophysiology of vascular diseases and the development of new treatments.

Endocrine disease usually comprises a dysregulation of effector hormone levels, which is related to gland dysfunction due to autoimmunity and genetic causes, or cancer. Metabolic diseases are classified as disorders that give rise to intoxication (group 1), disorders involving energy metabolism (group 2), and disorders involving complex molecules (group 3) [36]. Disorders that give rise to intoxication include inborn errors of intermediary metabolism that lead to acute or progressive intoxication from the accumulation of small molecules proximal to the metabolic block, including errors of amino acid catabolism, such as phenylketonuria, tyrosinemia, and maple syrup urine disease. However, these entities affect the metabolism of single amino acids, such as phenylalanine (phenylketonuria) or tyrosine (tyrosinemia), or the branched chain amino acids leucine, isoleucine, and valine (maple syrup urine disease), and not the protein level. Nevertheless, the pathobiochemical basis of a dysregulation in specific hormone proteolysis resembles that of inborn errors of metabolism and of endocrine disease, because quantitative or qualitative alterations of the substrate or the cleaving enzymes can result in effector hormone levels that are too low or too high relative to what is required to maintain homeostasis. This demonstrates that disorders related to cleaved protein hormones fit neither into classic endocrine diseases nor smoothly into the established classification for metabolic diseases. Rather, the cleavage sites in the protein hormone at which proteolysis occurs and the proteases with their determinants define a hormone–metabolism junction at which specific proteolytic cleavage, pathological alteration, and hepatorenal elimination can occur (Figure 1). The stronger consideration of the various paths that can be taken at this junction leads to a deeper understanding of the associated pathologies.

Concluding remarks

Birds of a feather flock together and cleaved protein hormones share common features with respect to their evolution, generation, structure–function relationships, signal transduction, and effects (see Outstanding questions). Cleaved protein hormones are subject to evolutionary conservation and positive selection and are usually generated by more than one enzyme. Various isoforms exist depending on where the cleavage occurs, and the processing is tissue specific and depends on the expression of the cleaving enzyme, as well as on factors in the microenvironment. Cleaved protein hormones may not only be folded, but may also comprise intrinsically disordered regions and their bioactive site may consist of short linear motifs as small as three amino acids. Cleaved protein hormones signal through more than one receptor or signaling pathway, and their main target is metabolic and organ system homeostasis. In general, physiological relevance is to be anticipated for any product of hormone proteolysis, as suggested by the evolution and conservation of fragment-generating cleavage sites and the ever-expanding list of functions ascribed to protein hormone fragments, which were considered physiologically irrelevant or only resulting from proteolytic breakdown and metabolic clearance. Acknowledging this trend, combined with the emergence of new technologies to study protease–substrate relationships [37], yields new horizons in specific hormone proteolysis, the exploration of which will lead to a deeper understanding of diseases related to hormone metabolism.

Declaration of interests

The authors declare the following competing interests: J.T., J.P.R., M.Z., C.C., and T.B. are inventors of submitted Mexican (MX/E/2019/079075) and multinational (PCT/ EP2020/069154) patent applications. The Universidad Nacional Autónoma de México (UNAM) and J.T. and T.B. are owners of pending patents.

Outstanding questions

Which phenotypes are associated with genetic alterations in protein hormone cleavage sites and cleaving enzymes?

Which evolutionary forces drive the emergence of cleaved protein hormones and their cleavage sites?

Which factors control the generation of cleaved protein hormones in target tissues?

What are the principles determining the interplay between local and systemic effects of cleaved protein hormones?

What are the best tools to study the generation and effects of cleaved protein hormones?

Resources

[i]https://clinicaltrials.gov/ct2/show/NCT03161652.

[ii]https://clinicaltrials.gov/ct2/show/NCT00998556.

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