Is the metalloendopeptidase EC 3.4.24.15 (EP24.15), the enzyme that cleaves luteinizing hormone-releasing hormone (LHRH), an activating enzyme?

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Abstract

LHRH (GNRH) was first isolated in the mammalian hypothalamus and shown to be the primary regulator of the reproductive neuroendocrine axis comprising of the hypothalamus, pituitary and gonads. LHRH acts centrally through its initiation of pituitary gonadotrophin release. Since its discovery, this form of LHRH (LHRH-I) has been shown to be one of over 20 structural variants with a variety of roles in both the brain and peripheral tissues. LHRH-I is processed by a zinc metalloendopeptidase EC 3.4.24.15 (EP24.15) that cleaves the hormone at the fifth and sixth bond of the decapeptide (Tyr⁵-Gly⁶) to form LHRH-(1–5). We have previously reported that the auto-regulation of LHRH-I (*GNRH1*) gene expression and secretion can also be mediated by itself and its processed peptide, LHRH-(1–5), centrally and in peripheral tissues. In this review, we present the evidence that EP24.15 is the main enzyme of LHRH metabolism. Following this, we look at the metabolism of other neuropeptides where an active peptide fragments is formed during degradation and use this as a platform to postulate that EP24.15 may also produce an active peptide fragment in the process of breaking down LHRH. We close this review by the role EP24.15 may have in regulation of the complex LHRH system.

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Introduction

LH-releasing hormone (also referred to as GNRH or LHRH) is the central regulator of reproduction via its action upon the hypothalamic–pituitary axis. Neurons that synthesise LHRH secrete their products into the hypothalamo-hypophyseal portal blood to stimulate the release of LH and FSH from the anterior pituitary. The release of LH and FSH, in turn, acts on the gonads to stimulate follicular maturation and eventual ovulation in the female, or sperm maturation in the males (Gore 2002). The anatomical distribution of this hypothalamic hormone is however, much broader than first thought, with expression demonstrated in both neural and non-neural peripheral tissues (Millar 2005). Following processing in specialised hypothalamic neurons, LHRH-I is released in a pulsatile manner and transported to the anterior pituitary via the hypothalamic–hypophyseal portal circulation. Here, LHRH acts via a specific G-protein-coupled receptor (GPCR), the LHRH-receptor (LHRH-R, GNRHR), to control the synthesis and release of the gonadotrophins, LH and FSH (Gore 2002).

In addition to this role, evidence is accumulating for a direct regulatory role in a number of peripheral and non-pituitary neural tissues.

Many laboratories including those of Drs Samuel McCann, Andrew Schally and Roger Guillemin were involved in the characterisation of this neurohormone. The studies from these laboratories led to the elucidation of its structure and functions (Gore 2002, Limonta *et al.* 2003).

First, discovered and characterised in mammals, the hypophysiotropic form of the peptide (pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH₂) is part of a much larger family of decapeptides. Over 20 forms of naturally occurring LHRHs have been described in a variety of mammalian and non-mammalian species (Millar 2005). Their nomenclature is originally based upon the species from which they were first described. Furthermore, within each species, there may be one or more LHRH forms where some of these peptide structures are common across species. In this particular review that focuses on mammalian biology and clinical literature, the

mammalian LHRH is designated LHRH-I as recommended by others (Wray 2002, Schneider *et al.* 2006).

In the adult, LHRH-I neurons are widely distributed along the rostrocaudal axis in the basal forebrain region around the preoptic area. The brain and pituitary localisation of LHRH has been extensively reviewed elsewhere (Wu *et al.* 1997, Gore 2002). Studies conducted in a variety of species have identified LHRH-I production along with its binding site and cognate receptor, the LHRH-R1, in non-hypothalamic tissue (Leung *et al.* 2003, Ramakrishnappa *et al.* 2005, Walters *et al.* 2008), suggesting a role for this neuroendocrine hormone beyond the pituitary. The wide and varied distribution of LHRH-I underscores the complexity of its function. Much of its biology remains to be elucidated.

Following its release, LHRH is degraded by intracellular peptidases. It has been suggested that the zinc metalloendopeptidase EC 3.4.24.15 (also known as EP24.15 or thimet oligopeptidase) is the main mediator of this process in both the brain and periphery. Other intracellular peptidases, such as the angiotensin-converting enzyme (ACE), in the process of degradation produce peptide fragments with biological activity. When degrading LHRH, EP24.15 produces the pentapeptide fragment LHRH-(1–5). Work is emerging and suggesting that this peptide fragment has biological activity separate from that of its parent peptide. Biologically, its effects may be different to or even antagonise those of its parent peptide.

In this review, we present the evidence that EP24.15 is the main enzyme of LHRH metabolism. Following this, we look at the metabolism of other neuropeptides where an active peptide fragments is formed during degradation and use this as a platform to postulate that EP24.15 may also produce an active peptide fragment in the process of breaking down LHRH. Evidence is accumulating for clinically important direct peripheral effects of LHRH and LHRH agonists are being widely used for a number of clinical applications. In light of this it is interesting to note that the LHRH agonists in current use are not substrates for EP24.15. We close this review by the role EP24.15 may have in regulation of the complex LHRH system.

LHRH-I is processed by a metalloendopeptidase

Processing of neuropeptide

Neuropeptides form following translation of mRNA in nerve cells to form pro-peptide. Intracellular processing results in an active peptide that is secreted, for example, the pro-hormone of LHRH-I, is converted into a mature decapeptide in secretory vesicles prior to release. In some cases, several neuropeptides may be cleaved from the same large precursor. For example, proopiomelanocortin can be cleaved to form ACTH, α MSH and β -endorphin. The cascade of intracellular processes

within nerve cells that results in the formation of active neuropeptides not only regulates the quantity of neuropeptide produced but also its bioactivity. The end result of cleavage of a large precursor molecule is a set of neuropeptides that may have similar, opposite, different or more selective properties. Following secretion into the extracellular area, these neuropeptides may be further processed. The peptide fragments formed following their degradation may provide an additional layer of regulation, demonstrating different effects to their parent peptides.

Zinc metalloendopeptidase EP24.15 has a crucial role in the processing of LHRH-I

The 75 kDa EP24.15 is the main enzyme in the metabolism of LHRH-I. This endopeptidase was first identified in the soluble fraction of rat brain homogenates (Orlowski *et al.* 1983) and is widely distributed in a variety of cell and tissue types (Müller *et al.* 1997, Tullai *et al.* 2000, Grundker *et al.* 2001, Tiong *et al.* 2004, Ramakrishnappa *et al.* 2005). Enzymatic activity is dependent upon phosphorylation by protein kinase A (PKA; Tullai *et al.* 2000) and as a thermolysin like metalloendopeptidase, the enzyme is zinc dependent (Dong *et al.* 1993). Substrate specificity is dependent upon peptide size (<17 amino acids), with no clear preference for amino acid sequence (Ray *et al.* 2004). However, a preference for a hydrophobic amino acid residue in the P1 and P2 positions along with a bulky hydrophobic residue in the P3' position has been demonstrated (Swanson *et al.* 2004).

The spatial and temporal location of EP24.15 demonstrates its important role in the processing of LHRH-I. Confocal microscopy has demonstrated EP24.15 in the external layer of the median eminence and preoptic area, where it can be seen co-localised with LHRH-I axon terminals (Wu *et al.* 1997). For a processing enzyme to play an important physiological role in neuropeptide processing and regulation it must be localised extra-cellularly (Kim *et al.* 2003). Many enzymes involved in neuropeptide degradation are either anchored to the extracellular membrane or free in the extracellular fluid. EP24.15, however, is predominantly found in the cytoplasm and is secreted into the extracellular space via poorly understood mechanisms. EP24.15 does not contain a membrane-anchoring motif yet it has been localised to the extracellular surface of the plasma membrane (Crack *et al.* 1999) as well as the exofacial leaflet of the lipid raft microenvironment (Jeske *et al.* 2004). Peripherally, EP24.15 is found in tissues under the control of LHRH-I regulation, the highest known levels of being found in the testis. Here, the enzyme's activity levels rise in a linear fashion with age and is thought to have a role in spermatogenesis (Pierotti *et al.* 1991, Pineau *et al.* 1999). Levels in the rat ovary are similar to those seen in the brain and increase sharply during puberty (before falling

away in adulthood; Pierotti *et al.* 1991). In addition, activity levels fluctuate during the proestrous period in the rat; with peak activity coinciding with the ascending phase of the LH surge (Wu *et al.* 1997). A regulatory role of circulating steroid hormones in the activity levels of this enzyme has therefore been suggested. Physiological data adds further weight to the idea that EP24.15 is the *in vivo* processing enzyme of LHRH-I. I.c.v. administration of a specific inhibitor of EP24.15 leads to an eightfold increase in the half-life of LHRH-I (Lasdun *et al.* 1989) and its peripheral administration augments the LHRH-I dependent surge of LH in the rat (Wu *et al.* 1997).

Activity of EP24.15 is regulated by phosphorylation of serine residue 644 by PKA. Phosphorylation of this conserved site reduces enzyme affinity for binding LHRH-I. However, once LHRH-I is bound the phosphorylated enzyme can rapidly turn over large quantities of substrate. The ability of phosphorylated EP24.15 to handle large quantities of substrate before saturating adds further evidence for the important regulatory role this enzyme has in the pulsatile LHRH-I waveform (Tullai *et al.* 2000). Therefore, while other enzymes may also play a role in LHRH-I metabolism, it is proposed that EP24.15 is the prime mediator of LHRH-I degradation in both the brain and periphery. Consequently, regulation of this enzymes activity may provide an additional mechanism of control of reproductive tissue.

Processed peptides have biological activity

EP24.15 is not the only intracellular peptidase thought to have activities beyond degradation. Other metallo-peptidases such as the ACE are known to, in the process of cleavage, activate peptides allowing them to profoundly affect physiology. Some examples to support the concept that active neuropeptides are converted by peptidases to fragments that are biologically active are shown in Table 1.

There are four roles for processed peptides exemplified. Firstly, the processed peptide may be degraded to a peptide demonstrating similar yet weaker action to its parent peptide. For example, angiotensin III has weaker hypertensive properties than angiotensin II. Secondly, processed peptides may show separate actions to their parent peptides. The fragment AVP-(4–9), for example, has no effect on diuresis instead being implicated in social learning and memory (de Wied *et al.* 1993, Nakayama *et al.* 2000). Thirdly, processing can result in more specific activity. For example, bradykinin-(1–5) inhibits platelet aggregation in a similar manner to its parent peptide, bradykinin. However, unlike its parent it does not produce vasodilatation making it a potential novel therapeutic (Murphey *et al.* 2006). Finally, processed peptides can provide counterbalance within a system. This is exemplified by the action of ACE2 on angiotensin II, producing angiotensin-(1–7) (Santos *et al.* 2008). This peptide opposes the action of angiotensin II,

formed by ACE from angiotensin I. Depending on the ratio of activity of these two enzymes; the renin angiotensin system may have vasoconstrictor/proliferative effects (mediated by angiotensin II) or vasodilator/antiproliferative effects (mediated by angiotensin-(1–7)). Again, the therapeutic modulation of this ratio could have great implications in the treatment of cardiovascular disease.

LHRH-I is metabolised by a two-step process

The mechanism of metabolism of LHRH suggests that EP24.15 converts rather than degrades the peptide. EP24.15 cleaves LHRH at the Tyr⁵-Gly⁶ bond producing its major degradation product, LHRH-(1–5). Interestingly, kinetic analysis has shown that LHRH is a poor substrate for EP24.15. However, LHRH-(1–9), a peptide fragment produced by the action of prolyl endopeptidase on LHRH, has a much greater *K_m* (Lew *et al.* 1994). It is therefore suggested that the conversion of LHRH to its major product LHRH-(1–5) occurs via a two-step mechanism. Firstly, LHRH-(1–9) is generated by prolyl endopeptidase. Conversion by EP24.15 to LHRH-(1–5) then occurs. It is suggested that the carboxy terminal glycine that is removed in the first metabolic step significantly hinders the ability of EP24.15 to cleave LHRH-I.

The two-step mechanism of LHRH metabolism highlights the substrate specificity of EP24.15 suggesting it has a role above simple inactivation. In addition, it has been suggested that a free carboxyl terminus may be required for binding to neutral endopeptidases such as EP24.15 (Orlowski *et al.* 1983), a similar mechanism seen with the degeneration of LHRH-I and bradykinin by endo-oligopeptidase A. This enzyme will cleave the Tyr-Gly bond only after the removal of glycine from the COOH terminus (De Camargo *et al.* 1982). Substituting an amide for the carboxy group reduced hydrolysis, as carboxypeptidases do not cleave alpha amidated peptides efficiently. Therefore, it can be postulated that the carboxy terminal amidation seen in the metabolism of LHRH-I may protect this neuropeptide from non-specific degradation (Lew *et al.* 1994).

EP24.15 is an activating enzyme

EP24.15 is known to degrade a number of important bioactive peptides in addition to LHRH-I, for example bradykinin and neurotensin. Classically, EP24.15, as an endopeptidase is thought to degrade neuropeptides terminating their physiological function. It was initially classified as a metabolising enzyme that terminated function through hydrolysis, rendering its substrate unable to bind to the cognate receptor (Orlowski *et al.* 1989, Dahms & Mentlein 1992, Montiel *et al.* 1997). Current thinking is that EP24.15 has a number of

Table 1 Many neuropeptides have alternative biological activity following processing.

Substrate	Substrate action	Processing enzymes	Active cleavage peptide	Cleaved peptide actions
Substance P	Nociceptive can lead to chronic pain (Zubrzycka & Janecka 2000) Potentiates response to opioid withdrawal (Sharpe & Jaffe 1989) Vasodilation during the inflammatory response (Lembeck & Holzer 1979) Implicated in control of aggression (Shaikh <i>et al.</i> 1993) Associated with depression (Kramer <i>et al.</i> 1998) Agonist at the κ opioid receptor producing dysphoria Hyperalgesia via the ORL1 receptor (Meunier 1997)	ACE (Skidgel <i>et al.</i> 1984) NEP (Skidgel <i>et al.</i> 1984) SP endopeptidase (Nyberg <i>et al.</i> 1991)	Substance P (1–7)	Anti-nociceptive (Stewart <i>et al.</i> 1982) Depresses response to opioid withdrawal (Kreeger & Larson 1996) Opposes SP induced vasodilation (Wiktelius <i>et al.</i> 2006) Enhances memory (Huston & Hasenöhrl 1995) Anxiolytic (Barros <i>et al.</i> 2002)
Dynorphin A, B and α neoeendorphin Nociceptin/orphanin FQ		DCE (Nyberg & Silberring 1990) Aminopeptidase N EP24.15 (Montiel <i>et al.</i> 1997) Unknown	Leu-enkephalin Nociceptin (1–7)	Agonist at the μ/δ opioid receptor producing euphoria (Koob 1996) Anti-nociceptive (Rossi <i>et al.</i> 1997)
CGRP α and β	Mediates neurogenic inflammation (Nohr <i>et al.</i> 1999)	Unknown	CGRP 8–37	Antagonises inflammation mediated by CRGP (Mimeault <i>et al.</i> 1992)
AVP	Antidiuresis Behaviour – learning, memory, reward	Aminopeptidase (Wang & Burbach 1986)	AVP 4–9	Facilitates social learning and memory (de Wied <i>et al.</i> 1993, Nakayama <i>et al.</i> 2000)
AVP	Antidiuresis Behaviour – learning memory, reward		DGLVP	Minor pressor and antidiuretic effect Behaviour – learning, memory, rewards (de Wied <i>et al.</i> 1993)
Oxytocin	Let down reflex Uterine contraction Monogamous bonding and maternal behaviour		OXT 4–9	Attenuates passive avoidance behaviour (de Wied <i>et al.</i> 1993)
LHRH	Controls release of gonadotrophins from the pituitary Decreases expression of LHRH gene in the GT1 cell line (Krismanovic <i>et al.</i> 1999) Increases expression of LHRH and LHRH1 Receptor in endometrial cancer cells Antiproliferative in peripheral tissues	EP24.15	LHRH 1–5	Facilitates lordosis in the female rat (Wu <i>et al.</i> 2006) Stimulates expression of LHRH gene in the GT1 cell line (Wu <i>et al.</i> 2005) Increases expression of LHRH II and LHRH II receptor in endometrial cancer cells (Baldwin <i>et al.</i> 2007) Proliferative in endometrial cancer cells (Walters <i>et al.</i> 2007)
Bradykinin	Vasodilation Inhibition of thrombin induced platelet aggregation (Hasan <i>et al.</i> 1996)	ACE (Murphey <i>et al.</i> 2000)	Bradykinin 1–5	Does not cause vasodilation (Murphey <i>et al.</i> 2006) Inhibition of thrombin induced platelet activation (Hasan <i>et al.</i> 1996, 1999) and aggregation (Murphey <i>et al.</i> 2006)
Angiotensin I	Inactive	ACE I	Angiotensin II	Vasoconstriction AVP release Renal sodium retention

Angiotensin II	Vasoconstriction Cell proliferation Cellular hypertrophy Cardiac remodeling – reduction in cardiac performance (Lijnen & Petrov 2003)	ACE II (Warner <i>et al.</i> 2004) EP24.15	Ang 1–7	Vasodilation Antiproliferative Antihypertrophic (Santos <i>et al.</i> 2008) Reduction in cardiac remodelling – increased cardiac performance (Sampaio <i>et al.</i> 2003, Botelho-Santos <i>et al.</i> 2007)
Angiotensin II	Vasoconstriction AVP release Vasoconstriction	Aminopeptidase A	Angiotensin III (ang II (3–7))	Vasoconstriction AVP release Cognitive enhancement (Gard 2008)
Angiotensin II	Vasoconstriction	Aminopeptidase N	Angiotensin IV (angII (3–8))	Cardioprotective in isoproterenol induced ischaemic injury (Jia <i>et al.</i> 2006)
Intermedin	Vasodilation, hypotension		Intermedin 1–53	Skin vasodilation in normothermia, attenuates vasodilation in hyperthermia (Bennett <i>et al.</i> 2003)
VIP	Skin vasodilation		VIP 10–28	

SP, substance P; ACE, angiotensin converting enzyme; NEP, neutral endopeptidase; DCE, dynorphin converting enzyme; ORL1, orphan receptor 1; CGRP, calcitonin gene related peptide; AVP, arginine vasopressin; VIP, vasoactive intestinal peptide; DGLVP, desGly9-Lys8-vasopressin.

alternative physiological functions (Kim *et al.* 2003). Firstly, it can transform an inert precursor neuropeptide into an active peptide. This is seen in the activation of neoeendorphin (to Leu-enkephalin) by EP24.15 (Acker *et al.* 1987). Secondly, it acts as a convertase, changing one bioactive peptide into a different peptide that either binds to a different receptor or binds to the same receptor but conveys different downstream messages. For example, EP24.15 can convert the decapeptide LHRH-I to the pentapeptide LHRH-(1–5). This fragment does not bind to LHRH-RI instead it has antagonistic properties at the *N*-methyl-D-aspartic acid (NMDA) receptor (Bourguignon *et al.* 1994). Finally, EP24.15 has a biomodulating action, forming a peptide product that opposes the action of its parent peptide. This is seen with the conversion of angiotensin I to the hypotensive fragment angiotensin-(1–7) by EP24.15 rather than to the hypertensive fragment angiotensin-(1–8) (angiotensin II) by ACE (Chappell *et al.* 2004).

The many roles of EP24.15 in the metabolism of neuropeptides suggest that this enzyme has a role in cell regulation. Using an inactive EP24.15 'substrate capture' assay in rat brain tissue Cunha *et al.* (2008) identified nine novel peptides that bound to the enzyme. They subsequently demonstrated that three of these peptides when introduced into CHO-S and HEK293 cells were able to alter GPCR signal transduction. The modulation of GPCR activity was shown to involve the proteins interacting with EP24.15. Adding these peptides intracellularly or overexpression of the enzyme modulated cell functioning suggesting physiological consequences of this signalling effect. This data suggest that EP24.15 and the peptides this enzyme may cleave are regulators of GPCR signal transduction.

Possible role for a metabolite of LHRH-I, LHRH-(1–5)

Others and our laboratory have hypothesised and shown that LHRH-(1–5), a metabolite of LHRH-I composed of the first five amino acids, may be bioactive (Bourguignon *et al.* 1994, Wu *et al.* 2005, 2006, Baldwin *et al.* 2007, Walters *et al.* 2007). Previous studies in our laboratory showed that this metabolite stimulates LHRH-I (*GNRH1*) mRNA expression in the neuronal GT_{1–7} cell line (Wu *et al.* 2005). LHRH-I treatment, on the other hand, had a negative regulatory effect on its own expression in the same cells, indicating that these effects are not mediated through the LHRH-RI. Instead it is suggested that LHRH-(1–5) acts via the NMDA receptor (Bourguignon *et al.* 1994). Therefore, it is proposed that the specific metabolite LHRH-(1–5) is biologically active and acts through a distinct mechanism when compared to its parent molecule (Table 1).

LHRH-(1–5) has also been shown to affect sexual behaviour (Wu *et al.* 2006). Specifically, i.c.v. administration of LHRH-(1–5) facilitated the lordosis response in ovariectomised female rats. It is interesting to also

note that LHRH-(1–5) has proliferative effects in the Ishikawa endometrial cell line (Walters *et al.* 2007) and a number of ovarian surface epithelial cell lines such as those previously shown to be responsive to direct LHRH-I effect (K Walters & T J Wu, unpublished observations). The ability of LHRH-(1–5) to stimulate proliferation appears to be linked to suppression of caspase-3/7 and ERK-1/2 expression (Walters *et al.* 2007). It is possible that EP24.15 expression is altered in cancer, resulting in an increase in production of LHRH-(1–5). This in turn may contribute to greater growth and the diminishing role of LHRH-I in regulating the extracellular matrix and activating the apoptosis pathway.

LHRH-I locally regulates healthy reproductive and non-reproductive tissue

In addition to its central role as a regulator of reproduction there is now much evidence that LHRH-I also regulates reproduction at a local level. Given its short half life (2–4 min) in the peripheral circulation (Bennett & McMartin 1979, Handelsman & Swerdloff 1986), it is unlikely that LHRH-I from the hypothalamus has a direct effect on peripheral tissue. However, local production of LHRH-I and regulation via an autocrine or paracrine mechanism has been suggested. Metabolism of LHRH by EP24.15 either to LHRH-(1–5) or alternate degradation products presents itself as an additional layer of regulation of this complex system.

Historically, LHRH-I is a regulator of reproduction

Throughout evolution, the LHRH-I system has been shown to provide a link between the nervous and reproductive systems (Tsai 2006). It has been suggested that the LHRH-I neurons seen in non-chordate invertebrates, who lack a hypothalamic–pituitary–gonadal axis (HPG axis), secreted LHRH-I directly into the blood stream from where it had a direct action on the gonads (Powell *et al.* 1996, Rastogi *et al.* 2002). LHRH-I may have developed as an early neuropeptide with a generalised neurotransmitter or neuromodulatory action in simple unicellular and multicellular organisms. As these organisms evolved, LHRH-I could have been recruited by the nerve cells and used to activate reproduction. First by direct action on the gonads and later through the regulation of the pituitary gland (Millar 2005). Early elaboration of LHRH-I function may have involved sensitising the neuroepithelium to pheromones as is seen in bony fish. Therefore, it is suggested that whilst LHRH-I was primarily recruited to regulate reproduction via the HPG axis it may maintain a regulatory role in peripheral reproductive and non-reproductive tissues.

LHRH and its receptor are present in peripheral reproductive tissues

LHRH-I and its cognate receptor have been co-localised in a number of tissues, mainly of the reproductive system (e.g. breast (Kottler *et al.* 1997, Limonta *et al.* 2003, Harrison *et al.* 2004), gonads (Clayton *et al.* 1992, Bull *et al.* 2000, Choi *et al.* 2006), prostate (Limonta *et al.* 1999, Tieva *et al.* 2001), endometrium (Murdoch 1995, Raga *et al.* 1998), oviduct (Casan *et al.* 2000) and placenta (Wolfahrt *et al.* 1998, Chou *et al.* 2004)). For example, in healthy breast tissue RT-PCR has been used to identify both LHRH-I and LHRH-RI (Kottler *et al.* 1997) while in the ovary, *in situ* hybridisation and RT-PCR have been employed to identify LHRH mRNA in the granulosa cells of primary, secondary and tertiary follicles and the mRNA of LHRH-RI in human granulosa luteal cells (Ramakrishnappa *et al.* 2005). Interestingly, tumours originating from these tissues (e.g. ovarian (Ohno *et al.* 1993, Irmer *et al.* 1995, Arencibia & Schally 2000, Kang *et al.* 2000), endometrial (Irmer *et al.* 1994), prostatic (Limonta *et al.* 1993, Bahk *et al.* 1998, Lau *et al.* 2001) and breast (Harris *et al.* 1991, Kottler *et al.* 1997)) also contain a high level of LHRH-RI. As previously described, EP24.15 has a similarly wide distribution in reproductive tissues.

Evidence for the presence of *LHRH-I* gene transcripts, binding sites and mRNA have been demonstrated in the rat ovary. Although data from human ovaries has been more variable, LHRH-I and LHRH-RI mRNA has been demonstrated using RT-PCR in both the fresh and cultured granulosa luteal cells of women undergoing IVF (Peng *et al.* 1994, Minaretzis *et al.* 1995). More specifically *in situ* autoradiography has shown high affinity binding of LHRH-I to the granulosa cells of the dominant follicle with low affinity binding sites seen in corpus luteum homogenates (Bramley *et al.* 1985, Latouche *et al.* 1989).

LHRH has direct antiproliferative effects in reproductive tissues

LHRH-I is known to induce apoptosis in healthy rat ovarian granulosa cells via unknown mechanisms (Yano *et al.* 1997). In ovarian cancer cells LHRH-I induces apoptosis via stimulation of Fas ligand (Imai *et al.* 1998). In the pituitary, activation of the GPCR LHRH-RI results in activation of MAPK cascades via tyrosine kinase, calcium and protein kinase C-dependent mechanisms. These cascades alter growth factor signalling via ERK and apoptosis via the p38 MAPK kinase. It is unknown whether downstream signalling of the peripheral LHRH-RI resembles that of the pituitary LHRH-R. In human gynaecological tumours, LHRH-I signalling neutralises epidermal growth factor tyrosine kinase activity and down regulates its receptors via the activation of phosphotyrosine phosphatase (Imai *et al.* 1996).

This occurs via coupling to the G α i protein, one explanation of this differing effect (Wu *et al.* 2009). The emerging evidence regarding LHRH-(1–5) suggests that this active peptide fragment has a proliferative effect on tumour cells. The downstream signalling pathway of this effect is as yet unknown but one could speculate as to a balanced regulatory system with proliferation being influenced by varying levels of LHRH and LHRH-(1–5). The enzyme EP24.15 and regulation of its activity could therefore have a role in cellular proliferation of reproductive tissues.

LHRH agonists are not degraded by EP24.15

The LHRH analogues (e.g. buserelin, goserelin, leuprolide, nafarelin and triptorelin) are used in the treatment of a number of hormonally responsive tissues. Injections of such analogues result in downregulation of pituitary LHRH-I receptors and desensitisation of gonadotroph cells, inducing a pharmacological castration (Conn & Crowley 1994). LHRH released from the hypothalamus is not present in the peripheral circulation and it is possible that peripheral production of LHRH is altered in pathological tissues; however, depot injection of an LHRH analogue has been shown to produce measurable circulating levels of LHRH. These 'super agonists' have increased receptor affinity and a high biological stability due to modification of the Tyr⁵-Gly⁶ residues. Interestingly, this modification renders these agonists resistant to cleavage by EP24.15. Additionally, incubation of EP24.15 with varying concentrations of LHRH-I analogue before assessing enzyme function revealed

that most of the LHRH-I analogues used in clinical practice are inhibitory to this enzyme's activity (Fig. 1). Increasing the size and hydrophobic nature of the residue in position 6 accentuated this inhibition (Cummins *et al.* 1999). Evidence for an additional role of these analogues, where effect comes from a direct interaction of analogue and LHRH receptors within diseased tissue is growing. Scrutiny of this evidence adds a further dimension to possible activating role of EP24.15, as while LHRH-1 and LHRH-(1–5) are likely to act via different pathways, the fact that LHRH-(1–5) is not produced by these analogues may go some way to explaining the differing effects of LHRH and LHRH agonists.

The LHRH system is active in peripheral tissue

Anatomical data has demonstrated LHRH-I and its receptor in the majority of reproductive tissue cancers. Receptor transcripts have been shown in around 80% of primary ovarian and endometrial tumours (Imai *et al.* 1994) with 50% of breast cancers expressing LHRH-I binding sites (Fekete *et al.* 1989). The coding for this receptor in breast and ovarian tumours is identical to the pituitary LHRH-RI (Kakar *et al.* 1994). Production of LHRH-I has been demonstrated in human breast, prostatic, ovarian and endometrial cancer cell lines (Emons & Schally 1994, Schally 1999, Schally *et al.* 2001). Additionally, the LHRH-I system has been seen in hormonally unresponsive malignancies (Jungwirth *et al.* 1998, Moretti *et al.* 2002, Keller *et al.* 2005). Whilst the function and downstream effect following activation of

Figure 1 A diagram showing LHRH-I in the folded conformation with the Tyr⁵-Gly⁶ bond available for hydrolysis by EP24.15. Substitutions with D-amino acids in the sixth position (Gly) (labelled D-AA in the figure) increases binding affinity to the LHRH-R but prevents hydrolysis. EP24.15 cleaves LHRH-I to produce LHRH-(1–5), whereas it does not cleave LHRH-I agonists containing substitutions of D-amino acids in the sixth position.

this receptor remain contentious, one can speculate about a local regulatory system based around LHRH-I. LHRH-RI activation in cultured tumour cells has anti-proliferative and pro-apoptotic effects (Hsueh & Jones 1982, Ikeda *et al.* 1996, Pineau *et al.* 1999, Limonta *et al.* 2003) led to the proposal that the LHRH-R system may act as a local negative regulator of tumour growth.

Prostate tissue

LHRH-I analogues have been used in the treatment of prostate cancer since the early 1980's (Labrie *et al.* 1982, Plosker & Brogden 1994). In the androgen-dependent phase of the disease their major effect is via suppression of the HPG axis, with an early worsening of symptoms (e.g. pain from bone metastasis) frequently observed prior to agonist induced suppression of pituitary function, an effect that can be reversed with use of anti-androgens (Sogani & Fair 1987). Evidence for an additional direct effect comes from *in vitro* experiments where LHRH-I agonists exert an inhibitory effect on the androgen induced proliferation of prostate cancer cells in a dose-dependent manner (Limonta *et al.* 1992, 1999). When the cell line ALVA-31 was xenografted into surgically castrated male mice, treatment with leuprolide inhibited its growth (Loop *et al.* 1995). These results suggest an alternative mechanism of LHRH-I action independent of the suppressive effect on the HPG axis. Prostate cancer tends to progress to a stage of androgen independence. It has been suggested that in this phase LHRH-I agonists may have a direct anti-tumour effect. In androgen insensitive prostate cancer cells, treatment with LHRH-I results in blockade of the fibroblast growth factor induced proliferation and invasion only in tumour cells with very high expression levels of LHRH-RI (Gnanapragasam *et al.* 2005). LHRH-I agonist therapy was then shown to improve survival only in patients with a high expression of LHRH-IR, an effect not seen when this subset of patients underwent surgical castration.

Mammary tissue

LHRH-I agonists are used as adjuvant therapy in oestrogen receptor (ER) or progesterone receptor positive pre-menopausal breast cancer as well as in salvage therapy for advanced disease. Interestingly, the combined effect of ER blockade (with tamoxifen) and LHRH-I agonist treatment was shown to be superior to either individual treatment in early breast cancer (Klijn *et al.* 2001). Whether this is due to suppression of the tamoxifen induced stimulation of pituitary function or due to a direct effect of the LHRH-I analogue on breast cancer cells remains to be determined. Evidence for the latter explanation comes from *in vitro* studies on the

oestrogen insensitive breast cancer cell line, MDA-MB 231, in which LHRH-I was anti-proliferative. This effect remained when the cells were xenografted into mice (Vincze *et al.* 1991). The absence of active ERs on this cell line suggests an effect of LHRH-I above suppression of circulating levels of sex steroids.

LHRH-I produced peripherally acts via its cognate receptor on a number of peripheral reproductive tissues. This direct local effect on both healthy and diseased tissue has a number of clinical implications. Further understanding of the regulation of this system is needed before the full scope of their potential clinical use can be described. Specifically, there is the possibility of an additional level of regulation, via the downstream processing of this peripherally produced LHRH by EP24.15.

Summary

The classical action of LHRH-I analogues is depression of pituitary gonadotrophs with consequent suppression of gonadal function. The evidence presented here suggests that in addition LHRH-I analogues may have direct effects on both healthy and diseased reproductive tissues where it is seen to regulate cellular growth. LHRH-I was named after its first known function, control of pituitary release of LH. Subsequently, this neuropeptide has been shown to have a diversity of location and action that extends far beyond its current nomenclature.

It is known that pro-hormone precursors are cleaved to form variety of neuropeptides often with different actions. In this review an additional layer of regulation is proposed; that is that following secretion neuropeptides are further cleaved to form additional active peptide fragments. LHRH-I is processed by the endopeptidase, EP24.15, to form LHRH (1–5), a fragment that has contrasting biological activity to its parent peptide. Levels of EP24.15 may be used to balance the action of the different peptides, both centrally and peripherally. Disruption of this regulation may lead to disordered cellular proliferation with consequent pathology.

The LHRH-I agonists in current clinical use are not substrates for EP24.15. Their use may shift the balance of LHRH-I activity in peripheral tissues towards anti-proliferation. This is a potential additional explanation for the peripheral actions of these drugs. In addition further description of the action and receptor of LHRH (1–5) may present a possible therapeutic target for antiproliferatives.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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