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Protease Nexin-1 in Reproductive Tissues: a Review*

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Abstract

Price CA. Protease nexin-1 in reproductive tissues: a review. ARBS Annu Rev Biomed Sci 2008;10:75-83. The Serpins comprise a family of proteins that bind irreversibly to and inactivate serine proteases such as thrombin and plasminogen activators. This review summarizes the current knowledge of one Serpin, SerpinE2 (also known as protease nexin-1, PN-1) in the reproductive system. By virtue of the ability to regulate the activity of secreted proteases, PN-1 is most likely involved in tissue remodeling and extracellular matrix formation and degradation. Accordingly, PN-1 is expressed in the developing hypothalamus and testis, both sites of significant cell migration. PN-1 is also upregulated during tissue remodeling in the ovarian follicle (at ovulation) and in the placenta during implantation, and downregulated in the mammary gland during pregnancy and lactation. PN-1 has been shown to be essential for formation of the copulatory plug in rodents. The physiological role of PN-1 is not clear, but given the fairly widespread expression of this protein in reproductive tissues, this protein clearly deserves greater attention from reproductive biologists.

Keywords: protease, serpin, extracellular matrix, ovary, uterus

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1. Introduction

Proteases have long been known to impact reproductive physiology, particularly those involved in tissue remodeling. These include the plasminogen activators (PA), urokinase (uPA) and tissue-type plasminogen activator (tPA). The PAs are key determinants of proteolytic activity, as they cleave the zymogen plasminogen to the active protease plasmin, and among the many actions of plasmin is the activation of a number of matrix metalloproteinases (MMP) (Rundhaug, 2005). The MMPs and PAs are important mediators of tissue remodeling, and the roles of MMPs and PAs in several reproductive tissues have been reviewed (Ny *et al.*, 2002; Curry & Osteen, 2003; Le Magueresse-Battistoni, 2007).

The proteolytic activity of plasmin and PAs is regulated in part by inhibitors that associate with the cell surface and extracellular matrix (ECM). These inhibitors belong to the serine protease inhibitor (serpin) superfamily. This family contains proteins that occur in all animal, plant and prokaryote genera, and there are currently 36 known serpin genes in humans (Law *et al.*, 2006). Mammalian serpins are subdivided into clades A to I, and numbered within clades. A well-known PA inhibitor (PAI) is PAI-1, which is the first member of clade E and has the gene symbol SERPINE1. This serpin has been implicated in tissue remodeling that occurs in the ovary at ovulation (see below).

Owing to increasing use of gene profiling experiments, including differential display and microarray approaches, more serpins are being identified as potentially important in reproductive physiology. One of these is protease nexin-1 (PN-1), also known as SERPINE2. The expression and role of PN-1 in a number of reproductive tissues is unclear and much work remains to be done. The purpose of this review is to summarize the structure and function of PN-1, and to describe the expression of PN-1 in the reproductive system.

2. Structure and Activity

PN-1 was discovered in nervous tissue, and is secreted mainly by astrocytes. PN-1 inhibits protease activity close to the cell surface and in the ECM. PN-1 inhibits several proteases, including thrombin, plasmin and the PAs. One of the first known functions of PN-1 was as a neurite outgrowth factor released from glial cells, and PN-1 was originally named glial-derived nexin (Zurn *et al.*, 1988). Stimulation of neurite outgrowth is achieved by inhibiting the actions of thrombin, which is a known inhibitor of neurite growth (Cunningham & Gurwitz, 1989). PN-1 also inhibits neuron migration, and this is mediated through inhibition of the migration-stimulating activity of plasmin and tPA (Seeds *et al.*, 1992; Seeds *et al.*, 1999). PN-1 is now believed to play a significant role in nerve injury (Meier *et al.*, 1989; Lino *et al.*, 2007) and pathologies including Alzheimer's disease (Choi *et al.*, 1995).

The SERPINE2 gene is located on chromosome 2q33q35 in humans, and contains 9 coding exons resulting in mRNA of 2104 bp. The protein contains 398 amino acids and is approximately 44 kDa in size. The protein possesses a signal peptide, and elegant videomicroscopy studies have indicated that PN-1 is constitutively secreted from glial cells (Giau *et al.*, 2005).

The serpins inhibit protease activity by irreversibly binding to the protease in a 1:1 stoichiometry, with the so-called 'suicide' mechanism of inhibition. The three-dimensional structure of several serpins and their precise mechanism of action have been elucidated, and the reader is encouraged to consult recent reviews for details and excellent figures (Huntington, 2006; Law *et al.*, 2006; Whisstock & Bottomley, 2006). Although the structure and mechanism have not been defined for every serpin, including PN-1, the general principles are believed to apply to all. Briefly, the serpin protein is folded into several β-sheets connected by α-helices. Between two of the sheets lies a stretch of 20 amino acids that are held in a loop above the main structure of the protein; this is the reactive center loop (RCL) that is accessible to and acts as 'bait' for target proteases. In a crude sense, the serpin molecule can be likened to a baited mousetrap, with the RCL being the cheese. The target protease associates with the RCL of a serpin and initiates a proteolytic attack as it would for any other target protein, but in this case proteolysis does not run to completion. As the catalytic serine residue of the protease forms ester bonds with the RCL (the

mouse biting the cheese), there is a marked conformational change in the serpin molecule; the RCL is withdrawn into the body of the serpin between two β-sheets, pulling with it the reactive site of the protease. This distorts the structure of the protease and traps it within the serpin structure (the analogy with the mousetrap is obvious). The 'sprung' serpin molecule is highly stable, more so than free serpin, and binding is irreversible. One advantage of these complex conformational changes may be in the regulation of serpin clearance: unbound, native serpins (and proteases) exhibit very slow clearance rates, whereas the serpin-protease complex is rapidly cleared through a low-density lipoprotein receptor-mediated event (Huntington, 2006).

3. PN-1 in Reproduction

Although PN-1 is typically associated with nerve cells, it is also expressed in several other cell types, including neural and non-neural cells of the reproductive system. The first recorded instance was the expression in that most peripheral of reproductive tissues, the human foreskin (Eaton & Baker, 1983).

3.1. Hypothalamus

As PN-1 is secreted mainly from nerve cells, it is logical to propose that any action of PN-1 in reproduction would be observed in the hypothalamus. PN-1 was not localized to GnRH secreting neurons in the mouse (Drapkin *et al.*, 2002), but PN-1 immunoactivity was localized to neurons of the olfactory system in embryonic mice (Drapkin *et al.*, 2002). The importance of this is that during embryogenesis GnRH neurons arise from the olfactory epithelium and migrate along the olfactory nerve into the forebrain. To test whether PN-1 expression during embryogenesis alters GnRH neuron migration, Drapkin *et al.* (2002) placed PN-1 or trypsin-coated beads into the olfactory epithelium of chick embryos; trypsin increased whereas PN-1 inhibited the number of GnRH neurons entering the forebrain, strongly suggesting a role for PN-1 in regulating development of GnRH neurons.

There are no reports of PN-1 expression or activity in the pituitary gland.

3.2. Gonads

During sexual development, PN-1 is first expressed in the primitive testis during development of the testis cords (from 11.5 days post-coitus in the mouse; (Grimmond *et al.*, 2000), and was detected in Sertoli cells in fetal and 3-week old mice (Grimmond *et al.*, 2000; Odet *et al.*, 2006). Interestingly, expression was not detected in the mouse ovary prior to overt gonadal differentiation (Grimmond *et al.*, 2000). In agreement with the data from the developing hypothalamus, these results suggest a role for PAs and PN-1 during migration of cells, and potentially during formation of the basal lamina surrounding the seminiferous tubules.

PN-1 is, however, expressed in the adult ovary. PN-1 mRNA and protein were detected in rat and mouse granulosa cells by in-situ hybridization and immunohistochemistry, and relative amounts increased during follicle growth to reach a maximum at the onset of ovulation and rapidly decreased thereafter (Hägglund *et al.*, 1996; Hasan *et al.*, 2002). Similar results have been described in cattle; PN-1 was expressed by granulosa cells but not theca cells, and expression was higher in large, dominant bovine follicles compared to small growing follicles or periovulatory follicles (Liu *et al.*, 1987; Shen *et al.*, 1997; Bédard *et al.*, 2003; Cao *et al.*, 2004; Fayad *et al.*, 2004; Liu, 2004). In-situ hybridization and immunohistochemical studies have demonstrated the expression of PN-1 in granulosa and theca cells of preovulatory follicles in the Rhesus monkey (Zhang H. *et al.*, 2007).

A more detailed study of changes in PAs and inhibitors after an ovulatory dose of hCG in cattle revealed that PN-1 expression was initially increased by hCG but then decreased to pre-injection levels (Cao *et al.*, 2006b), a pattern that was similar to the changes in thecal PAI-1 (Dow *et al.*, 2002; Cao *et al.*, 2006b). The role of PN-1 at this time is most likely to prevent precocious proteolytic breakdown of the follicle wall before ovulation. Combining the above data with those from rodents and human studies of PA and PAI-1 (Liu *et al.*, 1987; Shen *et al.*, 1997; Liu, 2004), the following sequence of events may occur in large follicles as a consequence of the preovulatory LH surge: there is an increase in expression

of PA, thecal PAI-1 and granulosal PN-1 in the follicle wall after the LH surge but before follicle rupture, followed by a reduction in PAI-1 and PN-1 expression, but not of PA expression, just before ovulation. This would allow a narrow window of increased PA activity in both thecal and granulosal compartments of the follicle wall that results in degradation of the basal lamina, follicle rupture and ovulation. Indeed, increased follicular plasmin activity is evident in sheep and cattle after the LH surge and leading up to ovulation (Murdoch, 1998; Cao *et al.*, 2006b).

PN-1 expression in follicles may be developmentally regulated. Secretion of tPA from granulosa cells of large bovine follicles increases with time in culture whereas that of PN-1 decreases (Cao *et al.*, 2004), which is consistent with the periovulatory changes described above. However, granulosa cells from small or medium sized follicles do not show this divergent pattern of PA and PN-1 secretion (Cao *et al.*, 2004), suggesting that the regulation of PN-1 differs between large potentially ovulatory follicles and smaller non-ovulatory follicles. By extension, this also suggests that the biological role of PN-1 may change during follicle development. In a study of follicles obtained from an abattoir and classed as healthy or atretic based on follicular estradiol and progesterone concentrations, we have demonstrated that PN-1 protein levels were higher in healthy compared to atretic follicles and, conversely, plasmin activity was lowest in healthy follicles (Cao *et al.*, 2006b). As plasmin is implicated in ECM remodeling, it is noteworthy that changes in specific ECM components occur during atresia in ruminant follicles (Huet *et al.*, 1998), and among the earliest morphological changes in atresia is apoptosis and loss of granulosa cells (Huet *et al.*, 1998; Irving-Rodgers *et al.*, 2001). Going one step further, tPA has been shown to cause apoptosis and detachment of adherent Chinese hamster ovary fibroblasts, an effect which is inhibited by PN-1 (Rossignol *et al.*, 2004). Therefore the following scenario can be proposed: within the granulosa cell layer of healthy growing follicles, proteolytic induction of apoptosis is held in check by local secretion of PN-1. As the follicle enters the atretic pathway, granulosa cell PN-1 secretion decreases and as a consequence plasmin activity increases. This leads to degradation of ECM components within the granulosa layer and subsequent apoptosis of granulosa cells. This leads to the interesting question of whether decreased PN-1 secretion is a cause or effect of early atresia.

If PN-1 is associated with atresia, it would be expected that factors promoting granulosa cell survival and activity may also promote PN-1 expression/secretion. This was investigated in cultured granulosa cells from small bovine follicles in serum-free medium. The major folliculogenic and anti-apoptotic hormones are FSH and IGF-1, and these stimulated granulosa cell proliferation and the secretion of estradiol and PN-1 (Cao *et al.*, 2006a). Bone morphogenetic protein (BMP)-7 also stimulated cell proliferation and secretion of estradiol and PN-1. In contrast, epidermal growth factor suppressed cell proliferation and secretion of estradiol and PN-1 (Cao *et al.*, 2006a). The close correlation between estradiol and PN-1 secretion is interesting, as a loss of estradiol secretion is an early sign of follicle atresia (Ireland & Roche, 1983; Badinga *et al.*, 1992; Price *et al.*, 1995), although estradiol itself appears not to regulate PN-1 secretion (Cao *et al.*, 2006a). These data collectively support the hypothesis that PN-1 may be a mediator of atresia in bovine follicles.

A caveat to this discussion of the role of PN-1 in atresia and ovulation is the observation that PN-1 knock-out female mice are fertile (Murer *et al.*, 2001). This suggests that redundant mechanisms for the regulation of proteolysis may exist, which is likely given the sheer number of serpin family members, or that there are species differences between polyovulatory mice and monovulatory ruminants.

3.3. Uterus

Extensive tissue remodeling occurs in the uterus during the primate menstrual cycle, and during pregnancy and parturition of all mammals, and a considerable amount of information is available about PAs and MMPs in this tissue (Curry & Osteen, 2003; Zhang & Nothnick, 2005). The uterus is a site of production of a novel member of the serpin superfamily, named uterine serpin, however this serpin is not a typical serpin in that the major biological activity appears to be inhibition of lymphocyte proliferation rather than inhibition of serine proteases (Peltier *et al.*, 2000; Tekin *et al.*, 2006). Much less attention has been given to PN-1. In rats, PN-1 expression occurs in the endometrial stroma, and expression is highly upregulated at the time of implantation (6.5 d post-coitus in the rat - Kim *et al.*, 2001). No expression was detected in other uterine cell types or in the nonpregnant uterus. In an in-situ hybridization

study of early-pregnant Rhesus monkeys, the same group reported very low levels of PN-1 expression from 12 – 26 days of pregnancy (post-implantation in this species) (Lin *et al.*, 2006). Whether the differences between these studies is due to species or methodological differences remains to be determined.

In humans, PN-1 mRNA and protein were reported in the placenta (White *et al.*, 1993), and suppressive subtractive hybridization studies indicated significant upregulation of PN-1 in first-trimester placental villi in response to short-term experimental hypoxia in vitro (Mondon *et al.*, 2005). Utero-placental hypoxia is associated with preeclampsia in women, although PN-1 expression was not significantly elevated in preeclamptic placentas (Vaiman *et al.*, 2005; Chelbi *et al.*, 2007).

Collectively, these data suggest a role for PN-1 in modulating tissue remodeling during implantation. There are likely to be considerable species differences in PN-1 expression in the uterus given the different patterns of implantation across species.

3.4. Mammary gland

The mammary gland is another organ that undergoes considerable tissue remodeling, not only for growth and differentiating during pregnancy but also for involution after weaning. Like the uterus, the mammary gland is a major site of production of an atypical serpin, maspin (mammary serine protease inhibitor). This serpin has no known protease inhibitory activity and is thought to be a tumor suppressing factor (Bailey *et al.*, 2006).

The expression of several PA and MMP genes has been investigated in the mammary gland (Fata *et al.*, 2004; Rabot *et al.*, 2007), but little is specifically known about PN-1 expression. In a recent study of mice, microarray data indicated down-regulation of PN-1 expression during pregnancy and lactation compared to nonpregnant controls, although no changes in other PA or serpin genes were reported (Huh *et al.*, 2007). In other studies, peaks of mammary tPA expression have been described during pregnancy and involution concomitant with increased plasmin activity (Sorrell *et al.*, 2005; Flint *et al.*, 2006), thus decreased PN-1 and increased tPA expression during pregnancy/lactation may be important for normal mammary development and function.

3.5. Male accessory glands

High levels of PN-1 expression were reported in mouse seminal vesicle and epididymis by RNase-protection assay, with lower levels in prostate and vas deferens (Vassalli *et al.*, 1993). Expression and secretion of PN-1 in the seminal vesicle is regulated by testosterone, as castration decreased and testosterone replacement restored PN-1 expression (Vassalli *et al.*, 1993). The seminal vesicle secretes large amounts of uPA, therefore the function of PN-1 in this tissue is likely to inhibit PA activity and consequently proteolysis in seminal secretion. This has essentially been demonstrated in the PN-1 knock-out mouse. Males produced normal spermatozoa, but upon mating they generated small, soft copulatory plugs that failed to retain the ejaculated spermatozoa within the female tract (Murer *et al.*, 2001). The plugs from knock-out mice contained more proteolytic activity than those of wild-type mice, and demonstrated degradation of proteins important for formation of a normal copulatory plug. Human semen also coagulates, but forms a soft gel rather than the more solid plug seen in rodents (Suarez & Pacey, 2006). Interestingly, PN-1 protein levels in semen were higher in men with abnormal seminal vesicle secretion than in fertile men (Murer *et al.*, 2001). Thus abnormally low or high PN-1 secretion by the seminal vesicles can have deleterious effects on male fertility.

3.6. Cancer

Breast cancer is a significant concern for women's health, and like all cancers, ECM remodeling occurs during cell migration and metastasis. The potential roles of PAs and MMPs in breast cancer have been reviewed (Almholt *et al.*, 2007). The sole report of PN-1 in breast cancer to date showed that the majority of tumors examined displayed higher levels of PN-1 expression compared to normal tissue, and that this pattern paralleled that of PAI-1 and uPA, also known to be over-expressed in breast cancer (Candia *et al.*, 2006).

Gene profiling studies have also identified a number of proteases that are differentially regulated in various cancers, and PN-1 expression is lower in serous ovarian tumors compared to borderline (of low malignant potential) tumors [online data supplemental to (Gilks *et al.*, 2005) www.gpec.ubc.ca/index.php?content=papers/papers.php].

In prostate cancer, the serine protease prostasin is down-regulated relative to normal prostate epithelia, and prostasin is thought to be a tumor suppressor (Takahashi *et al.*, 2003). The major inhibitor of prostasin is PN-1 (Chen *et al.*, 2004), therefore modified PN-1 activity may influence the progression of prostate cancer.

4. Concluding Remarks

It is clear from the above that PN-1 is expressed in a number of reproductive tissues. There has been a focus on the role of PN-1 in the ovary, where it likely modulates PA activity and thus tissue remodeling during ovulation. Similar actions may occur in the uterus during implantation and in the mammary gland during development and involution, but relatively little work has been done in these areas. PN-1 largely has a regulatory function in females, as knock-out female mice are fertile. The function of PN-1 in these mice is likely assumed by other members of the serpin family. PN-1 is obligatory in males, however, for formation of the copulatory plug. This study has demonstrated one function of PN-1 in males - the regulation of proteolysis. While this is the assumed function in females, this has not been convincingly demonstrated. As PN-1 interacts with non-classical serine proteases such as prostasin, and expression is altered in certain cancers, research is required to determine the mechanism(s) of action of PN-1 in the different parts of the reproductive tract.

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