

Theme Issue Article

Protein C inhibitor, a serpin with functions in- and outside vascular biology

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Summary

Human protein C inhibitor (PCI), a serpin-type protease inhibitor originally described as an inhibitor of activated protein C, has broad protease reactivity. In addition to its activities within the blood clotting and fibrinolytic cascades, it seems to par-

ticipate in several biological processes including reproduction and tumor growth. This review summarizes the current understanding of PCI function, regulation, and potential biological role.

Keywords

Serpins, phospholipids, proteases

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Protein C inhibitor (PCI), a member of the serpin family

Serpins (*serine protease inhibitors*) are a family of closely related glycoproteins, which includes inhibitors of serine proteases and non-inhibitory members with other biological functions [for review see (1)]. Serpins are widely distributed in nature. They do not only occur in mammals, but also in birds, insects, plants, viruses, and bacteria (1–5). Serpins exhibit a conserved secondary structure comprised of three β -sheets (A, B, C) and at least seven α -helices (A–I). The serpin family has been divided into clades (A–P) based on their phylogenetic relationship (1). PCI is an inhibitory member of the serpin family (6). It belongs to the serpin clade A (alpha-1-antitrypsin clade) and its gene symbol is *SERPINA5* (1).

Serpin-type protease inhibitors function as suicide substrates. They interact with the active site of their target proteases via their “reactive site”, which is held on an exposed loop. Upon this interaction the serpin undergoes a conformational change including the insertion of the reactive site loop into β -sheet A, the cleavage of the reactive site peptide bond (P1-P1’), and the formation of a covalent enzyme-serpin complex. The complexed protease is enzymatically inactive. Depending on the serpin and enzyme involved, the complex can also dissociate and release active enzyme and cleaved inactive serpin. The specificity of a serpin is in general determined by the amino acid forming the reac-

tive site bond and the sequence of the adjacent amino acids.

The human serpin family includes inhibitors of the blood coagulation system such as antithrombin III (7) or heparin cofactor II (8), inhibitors of the fibrinolytic system such as the plasminogen activator inhibitors PAI-1 (9) and PAI-2 (10), or alpha-2 plasmin inhibitor (11), and other protease inhibitors (12–14). As mentioned above, the serpin family also has non-inhibitory members with other biological functions. Non-inhibitory members of the serpin family include the hormone precursor angiotensinogen (15), the hormone-binding proteins CBG (16) and TBG (17), the tumor suppressor maspin (18), and the molecular chaperone HSP47 (19). So far very little is known about to which extent inhibitory serpins have additional non-inhibitory functions.

PCI is a multifunctional protease inhibitor

PCI has originally been described in plasma as an inhibitor of the anticoagulant serine protease activated protein C (20, 21). Since then we and several other groups have shown that PCI inactivates a variety of other proteases including blood coagulation factors (22, 23), fibrinolytic enzymes (24), tissue kallikreins (25, 26), and the sperm protease acrosin (27, 28). As far as tissue kallikreins are concerned, PCI seems to be the most efficient inhibitor (26).

In blood coagulation PCI has procoagulant as well as anticoagulant activities: PCI inhibits the anticoagulant, vitamin

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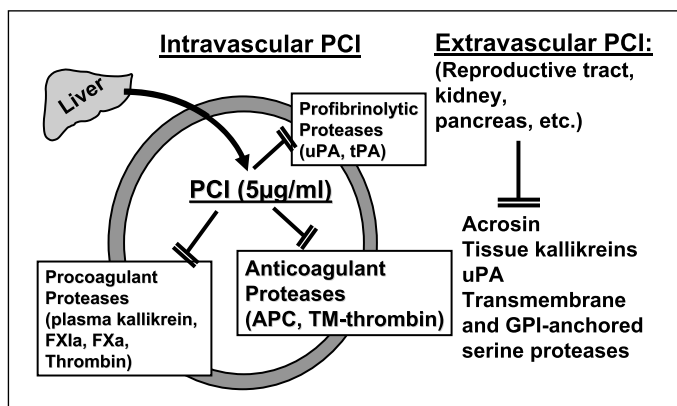


Fig. 1: Intra- and extravascular target proteases of protein C inhibitor (PCI).

K-dependent serine protease activated protein C (APC) (20–22, 29) as well as the generation of APC by the thrombin/thrombomodulin complex (30). APC has not only anticoagulant activity by inactivating factors Va and VIIIa, but also anti-inflammatory activity (31). Therefore, by inhibiting APC, PCI could also influence inflammatory pathways. PCI has anticoagulant activity by directly inhibiting thrombin, factor Xa, factor XIa, and plasma kallikrein (22, 23). Recently it has been shown that PCI cannot only inactivate secreted, extracellular serine proteases, but also serine proteases that are anchored in the cell membrane. Membrane-anchored serine proteases that can be inhibited by PCI are either type-2 transmembrane serine proteases (32, 33) or GPI-anchored serine proteases (32). Type-2 transmembrane serine proteases are integrated in the membrane via an amino terminal transmembrane domain. GPI-anchored serine proteases are attached to the membrane via a glycosyl-phosphatidylinositol linkage. Both expose their active site on the cell surface. PCI can therefore be considered a non-specific protease inhibitor (Fig. 1).

PCI is a heparin-binding serpin (29, 34–37), and heparin and other glycosaminoglycans can stimulate the interactions of PCI with many of its target proteases. On the other hand glycosaminoglycans interfere with the inhibition of tissue kallikrein by PCI, and high concentrations of glycosaminoglycans completely block the interaction of PCI with this extravascular protease (25, 39). In the presence of more than one target protease heparin can also change the specificity of PCI (39). Therefore, *in vivo* the specific environment may change not only the activity but also the target enzyme specificity of PCI.

Also certain phospholipids (e.g. phosphatidylethanolamine) have been described to bind PCI and to increase its inhibitory activity (40). Therefore it is possible that *in vivo* PCI might be a much better protease inhibitor than estimated from experiments performed in purified systems.

Human PCI is expressed in many tissues

Human PCI is a plasma protein (plasma concentration: ~100 nM), but is also present in many body fluids and secretions. The highest PCI concentrations (3–4 µM) have been reported in seminal plasma (41). Seminal plasma PCI has been described to

be derived from seminal vesicles (42). However, PCI is also synthesized in the testis and in the prostate (43, 44). PCI is also present in urine, sweat, saliva, tears, milk, and cerebrospinal fluid (41). Urinary PCI seems to be derived from endogenous synthesis in tubular cells of the kidney (45) and not from filtration of plasma PCI. So far synthesis of PCI has been shown in organs of the male and female reproductive tract (testis, prostate, ovary) (41), in the kidney (45), in the skin (46), and in megakaryocytes (38). Also in the spleen, in the pancreas, in skeletal muscles, and in the heart PCI synthesis has been shown by Northern blot analysis (43). Complexes of PCI with target proteases have been described to be present in body fluids (47, 48), indicating that PCI interacts with these proteases not only *in vitro* but also *in vivo*. In plasma free PCI has a half-life of ~23 hours, whereas APC-PCI complexes are cleared with a half-life of ~20 minutes (49). The clearance mechanism for APC-PCI complexes is not known. However, the lipoprotein receptor-like protein (LRP), which is involved in the clearance of other serpin-protease complexes (50) could play a role.

Non-inhibitory functions of PCI

Based on the fact that members of the serpin family (corticosteroid-binding globulin, thyroxin-binding globulin) function as carriers of hydrophobic hormones (16, 17), we have studied possible interactions of inhibitory serpins with steroid hormones and retinoic acid (51). We were able to show a specific interaction between PCI and retinoic acid, while no other serpin analyzed (PAI-1, antithrombin III, heparin cofactor II) bound to any of the hormones used (estradiol, progesterone, testosterone, aldosterone, cortisol, or retinoic acid). Kinetic analysis of the interaction of PCI and retinoic acid revealed an apparent K_d of 1.43 µM and 0.8 binding sites per molecule of PCI. An interaction of retinoic acid and PCI was not only seen in purified systems, but also in seminal plasma. We have observed that retinoic acid preferentially associated with a high-molecular-weight form of PCI (>200 kD) as judged from gel-filtration chromatography. The binding of retinoic acid to PCI did not influence its inhibitory activity towards APC or tissue kallikrein. Further studies are needed to determine the role of PCI in retinoid metabolism.

Animal models for PCI function

So far the biological role of PCI has not been defined. Therefore, we and other groups developed transgenic mouse models (43, 44, 52) to study the consequences of PCI deficiency or the effect of expression of human PCI in mice. One major result of preparatory studies performed for this project was the finding that there are major differences in the tissue-specific expression between mouse and human PCI. While human PCI is expressed in many organs and is present in many body fluids, mouse PCI is almost exclusively expressed in the male and female reproductive tracts of adult animals. Only trace amounts of PCI mRNA are occasionally detectable in other organs (44). Consistently, PCI is not present in mouse plasma. Therefore, Waagenar et al. (52) and Hayashi et al. (43) used the mouse as a model animal that has no endogenous plasma PCI. Waagenar et al. (52) generated trans-

genic mice that expressed human PCI in the liver and secreted a functionally active protein into the circulation. These mice should therefore be suitable to study the role of PCI in blood coagulation. Hayashi et al. (43) have developed transgenic mice expressing human PCI with an organ distribution similar to that seen in humans. These mice should allow the analysis of PCI functions also outside the vascular system. However, in mice, different, so far not identified, serpins could substitute for PCI and thereby limit the use of mice expressing human PCI as a model to determine the biological role of PCI.

It was our approach to inactivate the PCI gene in mice (44). We cloned and characterized the mouse PCI gene (53). These studies revealed that on the amino-acid level mouse PCI shows 63% identity with human PCI (6, 54, 55). Especially, functionally important domains, such as the reactive site, the heparin-binding site, and the “hinge” region, which is important for the conformational change upon protease binding, are highly homologous in mouse and human PCI. In order to characterize the mouse PCI protein, we expressed mouse PCI in *E. coli* and purified the recombinant protein. Mouse and human PCI are also functionally very similar as far as the inhibition of proteases and the affinity for heparin are concerned (Zechmeister-Machhart et al., unpublished data). Preliminary unpublished data furthermore suggest that also retinoid binding is similar in human and mouse PCI.

PCI-deficient mice were generated by targeted disruption of the PCI gene using homologous recombination in embryonic stem cells (44). Heterozygous (PCI^{+/-}) as well as homozygous (PCI^{-/-}) animals appeared morphologically normal at birth. All mice developed normally and looked healthy. However, male homozygous PCI-deficient mice were infertile. All females as well as young heterozygous males reproduced normally. Infertility in PCI^{-/-} mice was associated with abnormal morphology of sperm and testes. Some sperm had no flagella, most were degenerated. Some had also malformed heads. Histological analysis of testes from PCI^{-/-} mice revealed that the seminiferous tubules were filled with cells in different stages of spermatogenesis. The cytoplasm of Sertoli cells contained vacuoles and appeared necrotic. The Sertoli cell barrier was disrupted. Additionally, there was increased amidolytic activity in testis extracts of PCI^{-/-} mice as compared to wild-type and heterozygous animals. These findings suggest that increased/unopposed proteolytic activity might be the cause for the observed abnormalities. However, further studies are needed to clarify the mechanism responsible for the infertility of PCI-knockout mice.

PCI in human diseases

As described in more detail below, high or low PCI levels have been described in association with certain diseases. However, so far there has been no clear correlation of elevated PCI levels or PCI-deficiency with human medical conditions.

PCI in haemostasis and vascular biology

As outlined above, PCI can inhibit procoagulant as well as anticoagulant proteases. Its overall effect could therefore be antithrombotic as well as prothrombotic. Very few studies have been performed to determine the role of PCI in haemostasis and in

vascular biology. We have shown previously that PCI plasma levels are elevated in a group of survivors of myocardial infarction (56). Elevated PCI levels were significantly associated with the number of acute coronary events. High PCI-plasma concentrations can furthermore be considered as a risk factor for reinfarction (56). Meijers et al. analyzed the role of PCI in thrombosis (57). PCI levels were assessed in the Leiden Thrombophilia Study, a case-control study of more than 450 patients with a first deep vein thrombosis and age- and sex-matched controls. Data obtained in this study indicate that high levels of PCI may constitute a mild risk factor for venous thrombosis. Sayinalp et al. described elevated plasma PCI concentrations in patients with immune thrombocytopenic purpura (58), possibly due to the release of PCI from activated platelets. Nevertheless, the net effect of PCI in haemostasis and its role in cardiovascular diseases still remains to be determined.

Activation of the protein C pathway is a very sensitive marker for the activation of the coagulation system, and generated APC in plasma forms complexes with PCI, which is present in excess over APC. Therefore, the presence of APC-PCI-complexes in plasma could be a suitable marker for the detection of intravascular thrombin formation. Kölbel et al. (59) have determined APC-PCI-complex levels in different groups of patients with atherosclerotic disease. These patients exhibited significantly elevated concentrations of APC-PCI complex, particularly patients with aortic aneurisms. The authors therefore suggest that measuring APC-PCI could be a suitable screening assay for the detection of ongoing thrombin formation.

PCI in reproduction

So far the only biological system, in which PCI seems to play a fundamental role, is the male reproductive system, at least as judged from the results obtained in knockout mice (44). There has also been a report on two cases of human male infertility, which was associated with a lack of PCI activity in seminal fluid (60). Seminal plasma samples from these patients exhibited normal PCI antigen concentrations, but no PCI activity. The authors therefore suggested that unopposed uPA and/or tPA activity in seminal plasma could be responsible for the damage of spermatozoa (60). However, recently generated double-knockout mice, lacking both PCI and either tPA or uPA, do not support this hypothesis, since they were still infertile (Uhrin et al., manuscript submitted).

There is additional evidence from in-vitro studies that PCI is involved in processes related not only to sperm development and maturation, but also to fertilization itself. In-vitro studies with human spermatozoa have shown that exogenously added PCI as well as anti-PCI-IgG inhibit sperm-egg binding (61, 62). Also peptides corresponding to the conserved core region of PCI, a serpin region generally responsible for the interaction with target proteases, blocked human sperm-egg binding (63).

PCI in cancer

Proteolytic systems, in particular the uPA/plasmin system, are involved in the degradation of extracellular-matrix molecules and in the promotion of tumor-cell invasion and metastasis (64, 65). Inhibitors of urokinase, such as PCI, could therefore influence the benign or malign behavior of a tumor. By mRNA ex-

pression profiling using microarray analysis PCI has in fact been recently identified as one of two key regulators for benign behavior of serous borderline tumors of the ovary (66). In ovarian serous carcinomas PCI is downregulated as compared to serous borderline tumors. The authors suggest that protease inhibition by PCI possibly represents a mechanism which restrains these tumors from overexpressing tumor-associated proteases, despite an activated mitogenic signal. PCI can therefore be considered a putative tumor suppressors. This could be accomplished not only by inhibition of urokinase, but also by inhibition of other proteases such as tissue kallikreins, e.g. kallikrein 5, a serine protease potentially involved in cancer progression (67). Also in two other reports low PCI expression was associated with malignant behavior (68, 69)

Glasscock et al. (70) analyzed PCI expression in benign and malign prostate tissue, in prostate cancer cell lines and in the CWR22 prostate cancer xenograft model. These authors did not find any difference in PCI expression between normal and malignant prostate tissues, nor did they find an androgen depen-

dence of PCI expression in the CWR22 xenograft model. Therefore, according to them PCI does not seem to act as a tumor suppressor, although it may balance various tumor-associated serine proteases.

Conclusions

Human PCI is a serpin-type protease inhibitor with broad protease reactivity and wide tissue distribution. PCI has affinity for glycosaminoglycans and certain phospholipids, which may cause local accumulation of PCI *in vivo*. Additionally, these compounds can modify PCI activity and target enzyme specificity. There are several indications for the participation of PCI in biological processes. PCI seems to be required for PCI in spermatogenesis; however, PCI could also play a role in processes related to thrombin generation and/or tumor growth. Nevertheless, it is difficult to determine the biological role of PCI, especially since studies in mice are limited by the fact that in mice PCI is not a plasma protein, but exclusively expressed in the reproductive system.

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