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Immunopharmacology 36 (1997) 279–284

Immunopharmacology

Protein C inhibitor (PCI) and heparin cofactor II (HCII): possible alternative roles of these heparin-binding serpins outside the hemostatic system

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Keywords: Serpins; Protein C inhibitor; Heparin cofactor II; Serine proteases; Reproductive tract; Hormone binding

1. Introduction

Serpins (serine protease inhibitors) are a family of single chain glycoproteins that inactivate serine proteases by a suicide substrate mechanism, namely. by forming covalent, enzymatically inactive 1:1 enzyme–inhibitor complexes. Upon complex formation, the active site of the serine protease binds to the reactive site of the serpin and the reactive site peptide bond of the serpin is cleaved by the protease. Depending on the serine protease and on the serpin involved, the complex can be very stable or dissociate slowly resulting in the release of the active enzyme and the cleaved, inactive serpin. Several of these inhibitory serpins have been shown to be involved in the regulation of protease cascades, in plasma, such as the blood coagulation and fibrinolytic systems (for review see Harper and Carrell, 1994). In addition to these proteins with protease inhibitory activity, the serpin family also includes members that have lost protease inhibitory activity and have developed other specialized roles: thyroxin binding globulin (Flink et al., 1986) and corticosteroid binding globulin (Hammond et al., 1987) act as hormone carriers; angiotensinogen acts as a hormone precursor (Tanaka et al., 1984); maspin is a non-inhibitory serpin with tumor suppressor activity (Zou et al., 1994).

1.1. Heparin cofactor II (HCII)

HCII ($M_r = 66.000$) is a member of the serpin family (Blinder et al., 1988) and is, besides antithrombin III (formerly called heparin cofactor), another heparin dependent inhibitor of thrombin (Bringinshaw and Shanberge, 1974; Tollefsen and Blank, 1981). HCII is present in plasma at concentrations between 0.45 and 1.4 μM (Pratt et al., 1989). The only target protease, in plasma, for HCII described so far, is thrombin. Additionally, HCII has been shown to exhibit some inhibitory activity towards chymotrypsin (Church et al., 1985) and leukocyte cathepsin G (Parker and Tollefsen, 1985). Thrombin inhibition by HCII is stimulated by heparin and by dermatan sulfate (Pratt et al., 1989). It has also been shown that fibroblasts and vascular smooth muscle cells were able to stimulate the inhibitory activity of HCII (McGuire and Tollefsen, 1987). This stimulation of activity has been attributed to cell derived dermatan sulfate containing

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PII S0162-3109(97)00033-7

proteoglycans. These findings led to the hypothesis, that HCII might be an important thrombin inhibitor in the extravascular compartment, where dermatan sulfate is normally found, while antithrombin III is the main intravascular thrombin inhibitor. However, so far no data have been published on that subject and it is especially unclear whether at all HCII is present in the extravascular space. A distinct function has been shown for the N-terminal peptide of HCII, which is generated by neutrophil elastase cleavage of HCII and which has been shown to be a chemoattractant for neutrophils and monocytes (Church et al., 1991; Hoffman et al., 1991).

1.2. Protein C inhibitor (PCI)

PCI ($M_r = 57.000$) is a serpin (Suzuki et al., 1987, Meijers and Chung, 1991) that has been purified from human plasma as an inhibitor of the anticoagulant serine protease activated protein C (Suzuki et al., 1983). In the meantime it has been shown that PCI inactivates a variety of plasma proteases, which are involved in blood coagulation and fibrinolysis (e.g. thrombin (España et al., 1989), factor Xa (España et al., 1989), factor XIa (Meijers et al., 1988), plasma kallikrein (Meijers et al., 1988), thrombin–thrombomodulin complex (Rezaie et al., 1995), urokinase (Geiger et al., 1989) and tissue plasminogen activator (España et al., 1989)). In plasma, PCI is present at a concentration of ~ 70–90 nM (Suzuki et al., 1983). Plasma PCI seems to be derived from the liver, since patients with liver diseases have lower PCI plasma levels as compared to healthy controls (Suzuki, 1985). PCI, like heparin cofactor II, belongs to the subgroup of heparin binding serpins and heparin and other glycosaminoglycans stimulate many PCI/target protease interactions (Pratt and Church, 1992; Geiger et al., 1991). Complexes between PCI and some of its target proteases have been shown to occur in plasma in some diseases (e.g. activated protein C/PCI-complexes in patients with disseminated intravascular coagulation and deep venous thrombosis (España et al., 1989) and urokinase/PCI complexes in patients receiving intravenous urokinase for thrombolysis after myocardial infarction (Geiger et al., 1989)). However, so far there is no evidence for a major role of PCI in the physiological regulation of these plasma proteases. As will be shown later, PCI is present not only in plasma, but occurs in many other body fluids and secretions. Furthermore, these body fluids also contain proteases that have been shown to interact with PCI. It is therefore likely that PCI has a regulatory function in these extravascular systems.

2. Aims

So far, the serpins PCI and especially HCII have mainly been studied with respect to their potential roles in the regulation of processes related to hemostasis. For PCI it has been shown that it is a non-specific inhibitor, and that the inhibition rate constants for most plasma proteases are rather low, even in the presence of stimulating glycosaminoglycans. Therefore, these proteases seem to be more efficiently inhibited by other serpins present in plasma. HCII, on the other hand, seems to be a very specific and efficient inhibitor of thrombin. However, also for HCII a definitive role in hemostasis has not been demonstrated. As outlined in the introduction, serpin-type inhibitors could have other functions in addition to the regulation of protease activities in plasma. It has therefore been our aim to analyze HCII and PCI with respect to possible functions outside the hemostatic system. In order to address this question we have studied (a) the tissue localization of PCI and HCII, (b) the existence of possible alternative target proteases of PCI and HCII and (c) possible alternative (non-inhibitory) functions of PCI and HCII.

2.1. Tissue localization

In humans, PCI synthesis has been shown in the liver (Laurell et al., 1992), in proximal tubular cells of the kidney (Radtke et al., 1994), in the pancreas (Zechmeister-Machhart et al., 1996) and in organs of the male reproductive tract (Laurell et al., 1992). PCI antigen is present in many human body fluids and secretions, the highest concentrations have been described in seminal plasma (~ 3–4 μM; España et al., 1991; Laurell et al., 1992). Analyzing the tissue specific expression of PCI in mice, we found that PCI mRNA was exclusively localized in the reproductive tract (testis, seminal vesicle, ovary). All other

organs analyzed, including the liver, were negative for PCI mRNA (Zechmeister-Machhart et al., 1996), suggesting that PCI might have different functions in these two species.

As far as the tissue localization of HCII is concerned, we so far, have not been able to detect HCII antigen in extravascular tissues. Preliminary, unpublished data from our group as well as preliminary data from another group (Loh et al., 1996) suggest that HCII antigen could be associated with macrophages.

2.2. Possible alternative target proteases

We have shown previously that PCI inactivates tissue kallikrein with an apparent second order rate constant of 2.3×10^4 M^{-1} s^{-1} in a reaction that is inhibited by heparin (Ecke et al., 1992). In the meantime in vivo formed PCI/tissue kallikrein complexes have been shown in urine and in semen (España et al., 1995), suggesting that PCI might in fact play an important role in the regulation of the tissue kallikrein.

The finding that seminal plasma contains much more PCI than any other body fluid analyzed so far (España et al., 1991; Laurell et al., 1992) prompted us and other groups to search for (a) target protease(s) of PCI in the male reproductive tract. Christensson and Lilja (1994) and España et al. (1991) have shown that PCI forms complexes with prostate specific antigen (PSA) in seminal plasma. However, it seems unlikely that this complex formation is of any importance for the regulation of PSA activity, since the concentration of PSA in seminal plasma exceeds the concentration of PCI by far. On the other hand, complex formation with PSA and subsequent cleavage of PCI by PSA might play a role for the regulation of PCI activity. We (Zheng et al., 1994a) and others (Hermans et al., 1994) have studied a possible inhibition of the sperm protease acrosin by PCI and have shown that in a purified system human PCI inhibited boar acrosin. We have also studied the kinetics of the inhibition of boar acrosin by PCI and by other human serpins (i.e. α_1-antitrypsin, antithrombin III, α_2-antiplasmin, plasminogen activator inhibitor-1, plasminogen activator inhibitor-2 and heparin cofactor II) and determined the concentrations of these serpins in human seminal plasma

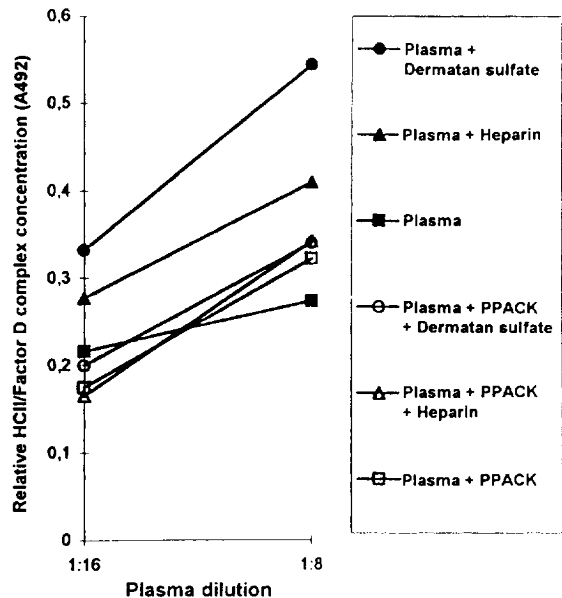

Fig. 1. Complex formation between complement factor D and HCII in plasma. Pooled plasma (5 mM EDTA) from 10 healthy volunteers was diluted 1:8 or 1:16 in phosphate buffered saline containing 1% bovine serum albumin and pre-incubated for 10 min in the absence or presence of the protease inhibitor PPACK (10 μM final concentration). Thereafter heparin (50 μg/ml final concentration), dermatan sulfate (50 μg/ml), or buffer alone was added to the samples and samples were incubated for 2 h at 37°C in wells of a microtiter plate coated with sheep anti factor D-IgG (20μg/ml). Bound HCII/factor D complexes were quantified by incubating the wells with rabbit anti HCII-IgG, followed by peroxidase linked donkey anti rabbit-Ig and a peroxidase substrate.

(Zheng et al., 1994b). These experiments revealed that from the serpins analyzed PCI seems to be the best candidate to function as an acrosin inhibitor in vivo as well. In a mouse system active human PCI inhibited sperm-egg-binding and in vitro fertilization, while heat inactivated PCI had no effect (Zheng et al., 1996).

Searching for new possible target proteases for HCII we found that HCII in plasma forms complexes with complement factor D, a serine protease that has been shown recently to be identical to the adipocyte derived protease, adipsin (White et al., 1992). Complex formation of HCII and factor D is stimulated by heparin and dermatan sulfate and does not occur with active site blocked complement factor D (Fig. 1).

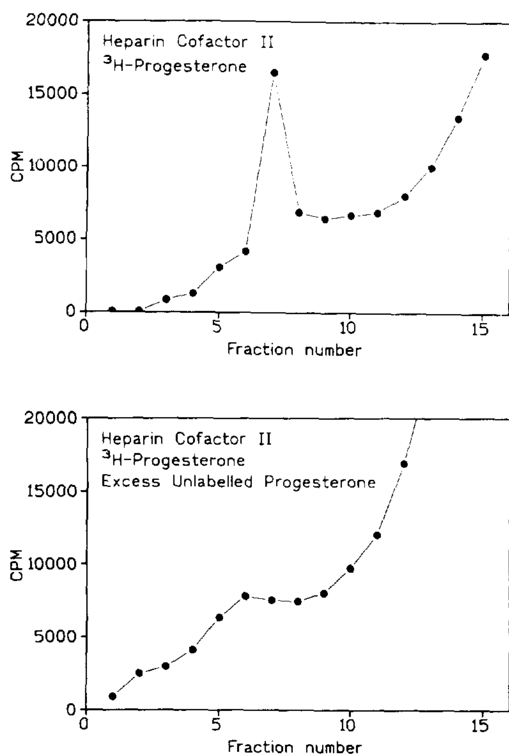

Fig. 2. Binding of progesterone to HCII. HCII (20 μg/ml final concentration) was incubated with ^{3}H-progesterone (140 nM final concentration) for 1 h at 37°C in the absence (upper panel) or presence of 14 μM (final concentration) unlabelled HCII. Thereafter samples were subjected to chromatography on Sephadex LH-20. Fractions were collected, and ^{3}H-radioactivity present in each fraction was determined in a liquid scintillation counter.

2.3. Possible alternative functions

In addition to the regulation of serine protease activities serpin-type inhibitors might have additional, non-inhibitory functions also. This hypothesis does not seem to be unlikely since other members of the serpin family act, for example, as hormone carriers (Hammond, 1995), as hormone precursors (Tanaka et al., 1984), or as tumor suppressors (Zou et al., 1994). So far inhibitory members of the serpin family have not been studied with respect to other possible functions. We therefore started analyzing binding of steroid hormones to inhibitory members of the serpin family. Preliminary studies revealed that HCII seems to specifically bind progesterone, since two peaks of radioactivity were obtained when HCII was preincubated with ^{3}H-progesterone, and subsequently subjected to size exclusion chromatography on Sephadex LH-20 (Fig. 2). When the preincubation of HCII with ^{3}H-progesterone was performed in the presence of an excess of unlabelled hormone, the first peak of radioactivity markedly decreased. Under the same conditions other steroid hormones (i.e. testosterone, estradiol, cortisol, aldosterone) did not bind to HCII (not shown).

3. Conclusions

In this report, we summarize results from previous studies on the physiological roles of the heparin binding serpins HCII and PCI. We describe also our recent approaches to analyze these serpins with respect to possible alternative biological roles. Preliminary data obtained in these studies revealed that both, HCII and PCI, may have more complex roles besides being involved in the regulation of the hemostatic system. Furthermore, our data raises the possibility that the inhibition of serine proteases may not be the only function of the so called 'inhibitory serpins'.

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