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Potentiation of C1-esterase inhibitor by heparin and interactions with C1s protease as assessed by surface plasmon resonance

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ABSTRACT

Background: Human C1-esterase inhibitor (C1-INH) is a multifunctional plasma protein with a wide range of inhibitory and non-inhibitory properties, mainly recognized as a key down-regulator of the complement and contact cascades. The potentiation of C1-INH by heparin and other glycosaminoglycans (GAGs) regulates a broad spectrum of C1-INH activities *in vivo* both in normal and disease states.
Scope of research: We have studied the potentiation of human C1-INH by heparin using Surface Plasmon Resonance (SPR), circular dichroism (CD) and a functional assay. To advance a SPR for multiple-unit interaction studies of C1-INH we have developed a novel (consecutive double capture) approach exploring different immobilization and layout.
Major conclusions: Our SPR experiments conducted in three different design versions showed marked acceleration in C1-INH interactions with complement protease C1s as a result of potentiation of C1-INH by heparin (from 5- to 11-fold increase of the association rate). Far-UV CD studies suggested that heparin binding did not alter C1-INH secondary structure. Functional assay using chromogenic substrate confirmed that heparin does not affect the amidolytic activity of C1s, but does accelerate its consumption due to C1-INH potentiation.
General significance: This is the first report that directly demonstrates a significant acceleration of the C1-INH interactions with C1s due to heparin by using a consecutive double capture SPR approach. The results of this study may be useful for further C-INH therapeutic development, ultimately for the enhancement of current C1-INH replacement therapies.

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1. Introduction

Human C1-INH is an acute-phase plasma protein that belongs to the serpin superfamily, the largest class of serine protease inhibitors that also includes antithrombin (ATIII), α_1-proteinase inhibitor (α_1-PI) and many other proteins that share a high structural similarity and regulate diverse physiological systems [1,2]. C1-INH is a multifunctional plasma protein with a wide range of inhibitory and non-inhibitory properties. It is mainly recognized as a major down-regulator of the complement and contact (kallikrein–kinin) amplification cascades [3]. Physiological level of functional C1-INH in plasma is ~240 μg/mL (~3 μM), but may double during inflammation [4]. The importance of C1-INH is underlined by its deficiency which is considered a cause of the hereditary angioedema (HAE) [5,6]. Whereas most C1-INH research still focuses on understanding the mechanism and treatment of HAE, the physiological and pharmacological activities of C1-INH are much broader. In plasma C1-INH is shared by several proteases of the complement, contact, coagulation and fibrinolytic systems that are functionally closely related. Therefore, a lack of the functional C1-INH in plasma not only triggers an inappropriate activation of the kallikrein–kinin pathway leading to a high level production of bradykinin and to angioedema [7,8], but has also a profound effect on the complement cascade and other systems. It is known that *in vivo* activities of many serpins including ATIII and C1-INH are modulated by GAGs, particularly by heparin, [9–11]. In turn, the pharmacological activity of heparin is mediated by ATIII, C1-INH and other serpins. C1-INH is the only known regulator of an early stage of the complement activation due to inhibition of the C1r and C1s proteases of the first component of complement system. Since 1975, when it was first reported that C1-INH binding to GAGs may increase its binding to the complement proteins C1s and C1r [12, 13],

Abbreviations: Anti-C1-INH ab, anti-C1-esterase inhibitor antibody; α_1-PI, α_1-proteinase inhibitor; ATIII, antithrombin; C1-INH, C1-esterase inhibitor; CD, circular dichroism; DMSO, dimethyl sulfoxide; GAG, glycosaminoglycan; L/P, ligand-to-protein molar ratio; PBS, phosphate buffer saline; RCL, reactive center loop; SA, Streptavidin; SPR, Surface Plasmon Resonance

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the potentiation of C1-INH by heparin has been under intensive research [14–16].

Investigation of C1-INH potentiation by heparin is essential for a better understanding of the mechanism of C1-INH potentiation. Moreover, it is important for a regulation of the C1-INH potentiation as it may result in an accelerated consumption of the activated C1-INH by multiple proteases, thus leading to a deficiency of the functional C1-INH level which is essential for an accurate regulation of the complement and contact pathways. It is also important for the development of tools that may enhance the efficiency of the currently available pharmaceutical preparations of C1-INH (*e.g.*, inhibitory activity of ATIII is being enhanced up to 4000-fold by heparin [17,18].

SPR has been previously used to study the binding of complement proteins to heparin [19, 20], however, to our knowledge this is a first report in which the interactions of C1-INH with C1s and the role of heparin in the enhancement of these interactions have been demonstrated by SPR. We have developed a unique double capture SPR method to investigate the C1-INH binding to C1s with and without heparin. To visualize the effect of each individual interactant, we have immobilized C1s, C1-INH and heparin one at a time to further understand the role of the heparin in the enhancement of C1-INH to C1s binding. The data presented here for three different SPR experimental designs consistently showed a strongly marked (from 5- to 11-fold) increase of the association rate between C1-INH and C1s due to the potentiation of C1-INH by heparin. The SPR data for the reference experiments without heparin are in agreement with earlier reports on the kinetics of C1-INH with C1s as assessed by functional assay [21].

2. Materials and methods

2.1. Materials

Complement C1-INH and activated C1s were obtained from EMD chemicals USA (Gibbstown, NJ). Anti-Human C1s was purchased from American Research Products, Inc. (Belmont, MA). Porcine intestinal heparin (sodium salt) and biotinylated heparin were from Sigma (St. Louis, MO). Sensor chip SA (Streptavidin) and sensor chip CM5, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC), N-hydroxysuccinimide (NHS), ethanolamine–HCl, HBS-P buffer (0.01 M HEPES, pH 7.4, 0.15 M NaCl, 0.005% surfactant P20), Phosphate buffer saline, 10× (PBS) (0.1 M phosphate buffer with 27 mM KCl and 1.37 M NaCl, pH 7.4), Acetate buffer (pH 5.0), Glycine (pH 2.5), NaOH (50 mM), NaCl (4 M) $MgCl_2$ (5 M) and deionized water were from GE Healthcare (Piscataway, NJ). Technochrom® C1-INH test kit was from Technoclone GmbH (Vienna, Austria).

2.2. Methods

2.2.1. Immobilization of C1s on CM5 chip

C1s in sodium acetate 3 μM (pH 5) was immobilized on the surface of CM5 sensor chip using amine coupling. Approximately 6000 RU of C1s was immobilized on CM5 chip surface. The surface was then blocked by injecting 1 M ethanolamine. 50 mM NaOH was injected to wash off non-covalently bound C1s. Flow cell 1 was similarly treated with buffer in the absence of C1s (control).

2.2.2. Immobilization of anti-human C1-INH on CM5 chip

Anti-human C1-INH was diluted in 10 mM sodium acetate, pH 5.0 to the concentration 250 μg/mL and immobilized on sensor chip CM5 using amine coupling. Approximately 10,000 RU of the antibody was immobilized on CM5 chip surface. The surface was then blocked by injecting 1 M ethanolamine. 50 mM NaOH was injected to wash off non-covalently bound antibody. Flow cell 1 was similarly treated with buffer in the absence of the antibody (control).

2.2.3. Immobilization of biotinylated heparin on SA chip

A sensor chip SA was pretreated with three injections, 5 μL each, of 50 mM NaOH in 1 M NaCl, to remove any nonspecifically bound contaminants. A 20-μL injection of biotinylated heparin (500 μg/mL) in HBS-P running buffer (flow rate, 10 μL/min) was made in flow cell 2, followed by a 10-μL injection of 2 M NaCl. Flow cell 1 was similarly treated with buffer in the absence of biotinylated heparin (control). Approximately 66 RU of biotinylated heparin was immobilized in flow cell 2.

2.2.4. SPR assessment of the binding kinetics

Biacore 3000 instrument (GE Healthcare) was programmed to conduct SPR experiments and kinetic analysis. The sensorgram was recorded as a plot of binding response (resonance unit) versus time. All the sensorgrams were processed using the double referencing method to eliminate the nonspecific binding from background contribution and buffer artifacts by subtracting signals from the reference flow cell and from buffer blank injections [22]. The data for all binding proteins were analyzed and fitted to 1:1 Langmuir binding using the Biaevaluation software, version 4.1.1, supplied by GE Healthcare.

2.2.5. C1s/C1-INH binding kinetics with and without heparin using immobilized C1s

C1-INH and C1-INH incubated with heparin in PBS buffer (pH 7.4) were injected separately over C1s-immobilized CM5 surface (prepared as described in Section 2.2.1) and reference flow cells for 3 min at a flow rate of 30 μL/min over a range of concentrations. At the end of the sample injection, the running buffer (PBS, pH 7.4) was flowed for 5 min over the sensor surface to allow dissociation.

2.2.6. C1s/C1-INH binding kinetics with and without heparin using immobilized anti-human C1-INH antibody

A double capture kinetics method was used to study the interaction of C1-INH with C1s in the absence and presence of heparin using anti-human C1-INH antibody immobilized on CM5 chip as described above in Section 2.2.2. Briefly, C1-INH at 6 μM was first injected over the surface for 3 min. Approximately 60 RU of C1-INH was captured. For heparin-related experiment, heparin at 50 mg/mL was then injected for 3 min, resulting in 30 RU of captured heparin on the surface. 90 μL of C1s over the range of concentration was injected. At the end of the sample injection, the running buffer (PBS, pH 7.4) was flowed for 5 min over the sensor surface to allow dissociation. Finally, the surface was regenerated using 2 M $MgCl_2$ and Glycine (pH 2.5) for 30 s.

2.2.7. C1s/C1-INH binding kinetics using immobilized heparin

A single capture method was used to study the binding of C1-INH with C1s on SA chip with immobilized heparin (as described in Section 2.2.3). Briefly, C1-INH at 1 μM was injected over the heparin surface for 3 min. Approximately 300 RU of C1-INH was captured. 90 μL of C1s over the range of concentrations was injected. At the end of the sample injection, the running buffer (PBS, pH 7.4) was flowed for 5 min over the sensor surface to allow dissociation. The surface was regenerated using 2 M $MgCl_2$, 2 M NaCl and 5 mM NaOH for 1 min. A direct kinetics between heparin and C1-INH was also performed, in which 90 μL C1-INH over the range of concentration was injected on heparin surface then the running buffer was flowed for 5 min to allow dissociation. The surface was regenerated using the same conditions as in the single capture method. For a direct kinetics between heparin and C1s, 90 μl of C1s over the range of concentrations was injected on heparin surface then the running buffer was flowed for 5 min to allow dissociation. The surface was regenerated using 1 M NaCl for 30 s.

2.2.8. SPR data analysis

Resonance signals were corrected for nonspecific binding by subtracting the background of the control flow cell. Binding isotherms were analyzed and binding constants K_D and K_A were calculated