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REGULAR ARTICLE

Heparin chain-length dependence of factor Xa inhibition by antithrombin in plasma☆

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Abstract Heparin anticoagulants function by enhancing the inhibition of coagulation proteases by the serpin antithrombin (AT). A direct evaluation of the specific anti-factor Xa (fXa) activity of therapeutic heparins in the physiologically relevant plasma-based clotting assays has not been feasible since thrombin, the final protease of the cascade, is the primary target for inhibition by AT in the presence of heparin. To circumvent this problem, we developed an assay in which the native AT in plasma was replaced with an AT mutant which exhibits identical affinity for heparin and near normal reactivity for fXa, but does not react with thrombin and other coagulation proteases in either the absence or presence of heparin. This assay was used to distinguish the anti-fXa activity of different molecular weight heparins from their anti-thrombin activity in clotting assays which were initiated by the triggers of either the extrinsic or intrinsic coagulation pathway. The results suggest that the acceleration of fXa inhibition by AT exhibits a marked heparin chain-length dependence, with fondaparinux (a pentasaccharide) having the lowest and unfractionated heparin having the highest effect. Interestingly, comparative studies revealed that the fondaparinux-catalyzed acceleration of thrombin inhibition by AT also contributes to the prolongation of the clotting time, possibly suggesting that the anticoagulant function of the therapeutic pentasaccharide is mediated though the inhibition of both fXa and thrombin.

Introduction

Heparins of different molecular sizes are the most common anticoagulant drugs used in cardiovascular medicine. The anticoagulant function of heparin is primarily mediated through its ability to accelerate

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the inhibition of blood coagulation proteases by antithrombin (AT) [1,2]. Heparin accelerates the AT inhibition of coagulation proteases by up to 3—4 orders of magnitude [3—5]. The mechanism and the extent of this acceleration have been thoroughly investigated for the AT inhibition of thrombin and factor Xa (fXa) [6—8]. It has been established that a template mechanism is primarily responsible for the catalytic effect of heparin in the AT inhibition of thrombin [8,9]. In this mechanism, high molecular weight heparins containing a specific AT binding pentasaccharide and at least an additional 13 saccharides accelerate the AT inhibition of thrombin by simultaneously binding to both the protease and the serpin, thereby enhancing the rate of the encounter complex formation between the two proteins (bridging effect) [9,10]. In the case of fXa inhibition, a pentasaccharide-induced conformational change in the reactive site loop of AT is thought to primarily account for the rate-accelerating effect of heparin in the reaction [6,8,11]. However, we recently identified a heparin-binding exosite on fXa and demonstrated that high molecular weight heparins can bind to this site of fXa with a high affinity in the presence of physiological concentrations of Ca^{2+}, thereby making a significant additional contribution to the acceleration of the protease inhibition by AT by a template mechanism [5,12].

A synthetic high affinity AT-binding pentasaccharide (fondaparinux) has been approved for treatment of deep vein thrombosis and thromboprophylaxis after major orthopedic surgery [13—15]. Although it was initially thought that the pentasaccharide is a specific anti-fXa drug, recent results have indicated that it also enhances the acceleration of factor IXa (fIXa) inhibition by AT ~300—600-fold [16—18], and can also enhance the inhibition of other coagulation proteases by the serpin at varying degrees [19]. Furthermore, it is known that the pentasaccharide also enhances the AT inhibition of thrombin ~2-fold [8,19], however, the pentasaccharide-catalyzed inhibition of thrombin by AT has not been considered important for the anticoagulant function of the drug [13].

Despite an overwhelming number of studies related to understanding the mechanism of the anticoagulant function of different molecular size heparins, the exact mechanism by which these compounds exert their therapeutic effect is a matter of controversy. A major factor that has contributed to this problem is that no specific and reliable plasma assay systems exist that can directly measure the extent to which the inhibition of individual proteases of the clotting cascade contributes to the anticoagulant function of heparin during the process of thrombin generation, as occurs in an ongoing thrombosis. This is because both the enzymes and products of the coagulation activation complexes that are responsible for generation of thrombin are themselves targets for rapid inhibition by AT in the presence of heparin. To overcome this problem, we recently prepared an AT mutant in which the reactive site loop of the serpin from the P4 to P4′ site (nomenclature of Schechter and Berger [20]) has been replaced with the identical site of the second fXa cleavage site (Ile^{319}-Asp-Gly-Arg-Ile-Val-Glu-Gly^{326}) in prothrombin [21]. This mutant has been fully characterized; it is folded properly, has a normal affinity for heparin and rapidly inhibits fXa, but not thrombin in the presence of heparin [21,22]. We previously used this mutant to show that prothrombin protects fXa from inhibition by AT in complex with both low and high molecular weight heparins in the purified system [22]. In this study, we supplemented AT immuno-depleted human plasma with this mutant and developed a simple clotting assay which is suitable for the evaluation of the heparin chain-length dependence of fXa inhibition by AT during the process of thrombin generation. Since the AT mutant is specific for fXa, the assay is also suitable for distinguishing the extent of the anti-fXa activity of therapeutic heparins from their anti-thrombin activity. The results of clotting assays suggest that, similar to thrombin, the anti-fXa activities of therapeutic heparins are dependent on their molecular weights, with fondaparinux having a minimal and unfractionated heparin having a maximal inhibitory effect. Moreover, comparative clotting studies using plasma supplemented with either wild-type or mutant AT suggest that the fondaparinux inhibition of thrombin may contribute significantly to the anticoagulant function of the drug.

Materials and methods

Proteins and other reagents

The construction and expression of wild-type and an AT mutant in which the P4—P4′ residues of the reactive site loop of the serpin have been replaced with the corresponding residues of the second fXa cleavage site in prothrombin (Ile^{319}-Asp-Gly-Arg-Ile-Val-Glu-Gly^{326}, named AT/Proth-2) have been described previously [21]. Recombinant thrombin was prepared as described [23]. Therapeutic heparins: fondaparinux sodium (MW = 1.728 kDa) was from Organon Sanofi-Synthelabo (France); enoxaparin sodium (average MW ~4.5 kDa) was from Aventis pharmaceuticals Products Inc. (UK); dalteparin sodium (average MW ~5.0 kDa) and unfractio-