Homozygous Deficiency of Heparin Cofactor II
Relevance of P17 Glutamate Residue in Serpins, Relationship With Conformational Diseases, and Role in Thrombosis

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Background—Heparin cofactor II (HCII) is a hepatic serpin with significant antithrombin activity that has been implicated in coagulation, inflammation, atherosclerosis, and wound repair. Recent data obtained in mice lacking HCII suggest that this serpin might inhibit thrombosis in the arterial circulation. However, the clinical relevance and molecular mechanisms associated with deficiency of HCII in humans are unclear.

Methods and Results—We studied the first family with homozygous HCII deficiency, identifying a Glu428Lys mutation affecting a conserved glutamate at the hinge (P17) of the reactive loop. No carrier reported arterial thrombosis, and only 1 homozygous HCII-deficient patient developed severe deep venous thrombosis, but she also had a de novo Glu100Stop nonsense truncation in the antithrombin gene.

Conclusions—Our results confirm the key structural role of the P17 glutamate in serpins. The same mutation causes conformational instability and polymerization in 3 serpins: Drosophila necrotic, human α1-antitrypsin, and human HCII, which explains their plasma deficiency. In the family under study here, however, plasma HCII deficiency was not associated with a significant clinical phenotype. (Circulation. 2004;110:1303-1307.)

Key Words: coagulation ■ genetics ■ proteins ■ risk factors ■ thrombosis

Thrombin plays a key role in several biological processes. Besides its central role in the formation of the fibrin clot, it is involved in the stimulation of platelet reactivity, causes mitogenesis of fibroblasts, is chemotactic for macrophages, is involved in the regulation of proliferation of endothelial cells, and inhibits neurite outgrowth.1 The activity of thrombin in the vasculature is finely controlled by 2 glycosaminoglycan-activated proteins, antithrombin (AT) and heparin cofactor II (HCII). Both proteins are members of the serpin family of serine protease inhibitors with closely homologous structures,2 and both are synthesized in the liver. Whereas a deficiency of AT is associated with an increased risk of venous thrombosis, there is as yet no evident linkage of the deficiency of HCII with thrombosis or other pathologies.3

Human HCII is a 66-kDa secretory protein composed of 480 amino acid residues that is present in adult plasma at 1.2±0.2 μmol/L.4 The human gene, located in chromosome 22q11, spans 15.8 kb and includes 5 exons.5 Only 4 cases of heterozygous HCII deficiency have been analyzed at the molecular level.6–9 We reported the first homozygous deficiency of HCII in an extended family.10 Here, we identify the molecular mechanism of this HCII deficiency as being the result of a mutation at P17 at the base of the hinge of the mobile reactive center loop of the serpin molecule. Our results support the structural relevance of this P17 residue and demonstrate the sensitivity of HCII, as a new member of the serpin family, to conformational mutations with relevant consequences.

Methods

Patients
The family study was previously reported in detail.10 Briefly, the proband is a 33-year-old white woman with early and recurrent venous thromboembolisms. Biochemical and genetic thrombophilic factors were evaluated, and all parameters except AT and HCII were normal. The proband had a severe reduction in antigenic concentration and activity of HCII (3% and 11%, respectively) and a moderate decrease in AT (48% antigen and 51% functional activity). Her asymptomatic sister (37 years of age) also had a severely decreased HCII antigen and activity (3% and 13%, respectively). The other 25 members of this family had reduced (heterozygous) or normal levels of HCII, and all had normal levels of AT. Neither homozygous nor heterozygous subjects had abnormal biochemical, cardiological, gynecological and obstetric, or hematological data.

Additionally, we measured the plasma levels of hepatic-origin serpins in 1 patient with emphysema, homozygous for the α1-antitrypsin G1342L mutation, who did not report hepatic failure. We also studied 25 healthy blood donors.
Patients and control subjects were fully informed of the aim of this study, which was performed according to the Declaration of Helsinki as amended in Edinburgh in 2000.

**Blood Samples**

Blood samples were collected into vacuum tubes that contained 3.8% trisodium citrate. Samples were centrifuged at 2000g for 15 minutes to obtain platelet-poor plasma, which was stored at −70°C until tested.

**Genetic Analysis**

Promoter, exons I through 5, and flanking regions of the HCII gene were amplified by genomic polymerase chain reaction (PCR). Primers and conditions used for PCR are shown in Table 1. All exons and flanking regions of the AT gene were amplified as described elsewhere with modifications.11

The PCR products were isolated and purified from 0.8% to 1.5% agarose gels with Ultraclean Gel Spin (MoBio). Sequencing was performed with the ABI Prism Big Dye Terminator Cycle sequenc-

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### TABLE 1. Oligonucleotides Used for Amplification and Sequence Analysis

<table>
<thead>
<tr>
<th>HCII</th>
<th>Primers (5'-3')</th>
<th>Nucleotide</th>
<th>PCR Size, bp</th>
<th>AT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Promoter</td>
<td>CATGCCACAAATCCCTTATTC</td>
<td>−518/−498</td>
<td>629</td>
<td>65</td>
</tr>
<tr>
<td>Exon I</td>
<td>GTTGCCATCGATTTAACC</td>
<td>92/111</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Promoter</td>
<td>CATGCCACAAATCCCTTATTC</td>
<td>−518/−498</td>
<td>361</td>
<td>65</td>
</tr>
<tr>
<td>Promoter</td>
<td>ATCTCCGATCTGAGCAGCT</td>
<td>−180/−158</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Exon II</td>
<td>ATATGTTGTGGTGTTGTGTGG</td>
<td>5135/5155</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Exon III</td>
<td>CAGCTATCTGAGGCTTAT</td>
<td>9757/9788</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Exon IV</td>
<td>CGTGGCGATATCAATAACAGGTG</td>
<td>11850/11880</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Exon V</td>
<td>CCTCAGCCTTTATCATAC</td>
<td>13695/13715</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

AT indicates annealing temperature.

### TABLE 2. Nucleotide Differences Identified in the HCII Gene

<table>
<thead>
<tr>
<th>Position</th>
<th>Nucleotide</th>
<th>Amino Acid</th>
<th>References</th>
<th>Deficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Promoter, −181</td>
<td>A</td>
<td>...</td>
<td>15, This study</td>
<td></td>
</tr>
<tr>
<td>Exon 1, 36</td>
<td>A</td>
<td>...</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Exon 2, 5536</td>
<td>Ins T</td>
<td>Frameshift</td>
<td>6</td>
<td>Type I (Awajii)</td>
</tr>
<tr>
<td>Exon 2, 5718</td>
<td>T</td>
<td>Gly149</td>
<td>5, 6, 14, This study</td>
<td></td>
</tr>
<tr>
<td>Exon 2, 5837</td>
<td>G</td>
<td>Arg189</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td>Exon 2, 5924</td>
<td>A</td>
<td>Lys218</td>
<td>5, 6, 14, This study</td>
<td></td>
</tr>
<tr>
<td>Exon 5, 12809</td>
<td>G</td>
<td>Glu428</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td>Exon 5, 12915</td>
<td>T</td>
<td>His463</td>
<td>5–7, 15, This study</td>
<td></td>
</tr>
<tr>
<td>Exon 5, 12896–99</td>
<td>DelTT</td>
<td>Frameshift</td>
<td>7</td>
<td>Type I (Rimini)</td>
</tr>
<tr>
<td>Exon 5, 12854</td>
<td>C</td>
<td>Pro443</td>
<td>Type I (Tokushima)</td>
<td></td>
</tr>
</tbody>
</table>

Ins indicates insertion; Del, deletion.
ing kit on an automated sequencer type 377 (Perkin-Elmer Applied Biosystems).

Confirmation of nucleotide changes was performed by allele-specific restriction assay (ASRA). HCII–181 genotype was determined by nested PCR with the mutagenic primer ATCTCCAGTTCTTAGAGCTGAGCA (Table 1) and Alw211 restriction. The exon 5 mutation was recognized by restriction with MnlI. AT Glu100Stop mutation was identified by restriction with Alw211. Digestion was performed with 3 U PCR and 1 U restriction enzyme (Fermentas) during 6 hours. Restriction fragments were analyzed in 5% to 7% acrylamide gels stained with silver.

Antigen and Functional Determination

AT antigen was measured by radial immunodiffusion (Behringwerke-AG). AT activity was assayed with the Coamatic Antithrombin Kit (Chromogenix-AB). HCII antigen was measured by an ELISA (Enzyme Research Laboratory). HCII activity was determined quantitatively with the Stachrom HCII assay provided by Diagnostica Stago. Antithrombin and C1-inhibitor levels were determined by an immunoturbidimetric assay using antibodies and reagents provided by Dade-Behring. a2-Antiplasmin activity was determined by the IL Test Plasmin Inhibitor Kit (Instrumentation Laboratory).

Results

Genetic Characterization of HCII Valencia

As shown in Table 2, comparison of the proband sequence with previously reported cDNA12,13 and genomic HCII sequences5–7,14 showed 6 nucleotide differences: G-181A, A36C, T5718C (Gly149), A5924G (Lys218Arg), G12809A (Glu428Lys), and T12915C (His463) (nucleotide numbering according to Herzog et al).5 The genetic change T12915C (His463), found in heterozygous state in the proband, has been previously described as a common polymorphism.7 The proband presented a homozygous genotype at positions 36A, 5718T (Gly149), and 5924A (Lys218). These alleles were also identified by other authors.5,6,13,14 These data suggest that the nucleotides identified in our study are the common alleles of polymorphisms with low frequency or that the sequence identified by Blinder et al12 contained some mistakes.

Of greater interest is the homozygous 181A genotype identified in the proband because it is located in the promoter region of the HCII gene, close to the TATA-like sequence (TTATTTA67/61) and the inverted CCAAT sequence (ATTGG75/71).5 Remarkably, 1 study identified the same 181A allele in a control subject,14 whereas another reported a G at –181.5 These data suggest the existence of a polymorphism at this position. Thus, we analyzed the frequency of this modification by nested PCR-ASRA in 25 healthy subjects and its distribution in the affected family. Our results support the conclusion that the G–181A change is a frequent polymorphism affecting the HCII gene (–181A allele frequency, 0.395). Moreover, distribution of this polymorphism in members of the affected family indicated that this change was not responsible for the HCII deficiency.

Finally, the homozygous 12809A genotype was the clearly significant mutation found in the proband (Figure, A). This genetic change causes a single amino acid substitution,
TABLE 3. Plasmatic Determination of Hepatic Serpins Among Subjects With Distinct HCII 428 Genotype

<table>
<thead>
<tr>
<th>Disease</th>
<th>HCII 428 Genotype</th>
<th>HCII, Ag %</th>
<th>AT, Ag %</th>
<th>α1-AT, mg/dL</th>
<th>C1-I, mg/dL</th>
<th>α2-AP, Act %</th>
</tr>
</thead>
<tbody>
<tr>
<td>VT</td>
<td>Lys/Lys</td>
<td>3.8</td>
<td>48</td>
<td>197</td>
<td>17.3</td>
<td>105</td>
</tr>
<tr>
<td>Healthy</td>
<td>Lys/Lys</td>
<td>3.2</td>
<td>89</td>
<td>129</td>
<td>16.4</td>
<td>89</td>
</tr>
<tr>
<td>Healthy</td>
<td>Glu/Lys</td>
<td>41</td>
<td>98</td>
<td>106</td>
<td>18.5</td>
<td>101</td>
</tr>
<tr>
<td>Healthy</td>
<td>Glu/Lys</td>
<td>35</td>
<td>99</td>
<td>128</td>
<td>15.0</td>
<td>86</td>
</tr>
<tr>
<td>Healthy</td>
<td>Glu/Glu</td>
<td>93</td>
<td>120</td>
<td>121</td>
<td>17.9</td>
<td>109</td>
</tr>
<tr>
<td>Healthy</td>
<td>Glu/Glu</td>
<td>70</td>
<td>99</td>
<td>96.5</td>
<td>17.9</td>
<td>100</td>
</tr>
<tr>
<td>Emphysema α1-AT</td>
<td>Lys342Lys</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VT</td>
<td>Glu/Glu</td>
<td>107</td>
<td>110</td>
<td>22.8</td>
<td>23.4</td>
<td>96</td>
</tr>
</tbody>
</table>

Normal range... 62–130 69–129 110–220 15–35 70–120

Ag indicates antigen; C1-I, C1-inhibitor; AP, antiplasmin; Act, activity; and VT, recurrent venous thrombosis.

Glu428Lys, affecting a glutamate located at P17, which is conserved in all serpins (Figure, A). To confirm the mutation and its association with the HCII deficiency, we performed PCR-ASRA in all members of the affected family and 25 healthy blood donors. Representative restriction pattern of the 3 possible genotypes is shown in panel A of the Figure. The mutation was not found in healthy subjects, and the genotype completely matched the phenotype of HCII deficiency in the family (Figure, B).

Sequence of the AT Gene
The proband sequence showed a previously undescribed C2762T mutation in heterozygous state. This mutation causes a Glu100Stop nonsense truncation that explains the classic type I deficiency displayed by the patient. PCR-ASRA analysis confirmed the presence of this mutation in the patient but its absence in all available members of her family. This result and the normal levels of AT displayed by her parents supported the hypothesis that the proband experienced a de novo mutation in the AT gene.

Plasma Levels of Hepatic Serpins
We determined the plasma level of 5 hepatic serpins (HCII, AT, α1-antitrypsin, C1-inhibitor, and α2-antiplasmin) in 6 subjects of the family with HCII deficiency (2 homozygous, 2 heterozygous, and 2 normal with respect to the Glu428Lys mutation) (Table 3). Apart from the previously indicated differences in HCII and AT, we observed no significant differences in the level of α1-antitrypsin, C1-inhibitor, and α2-antiplasmin in relation to the HCII genotype. Moreover, except for α1-antitrypsin, the levels of these serpins in 1 patient with homozygous Z-α1-antitrypsin were in the normal range (Table 3). Finally, the plasma AT and HCII levels (antigen and activity) were unchanged during 5 years of observation in both HCII homozygous subjects.

Discussion
The highly conserved structure of the serpin family ensures that the study of the effects of a mutation in 1 member of the family is directly relevant to homologous mutations in other serpins. There is now a large collection of such mutations in which molecular consequences are well understood and that result in a diversity of diseases: thrombosis with AT, angioedema with C1-inhibitor, and dementia with neuroserpin. The observation that such dysfunctional mutations affect predominantly the mobile regions of the molecule introduced the concept of the conformational diseases, disorders that result from conformationally driven protein aggregation and tissue deposition. The archetypal example of a conformational disease is the deficiency of the plasma serpin α1-antitrypsin as a result of a missense polymorphism (Glu342Lys) at the critical hinge of a mobile peptide loop 16-residue distance (P17) from the reactive center at P1. The consequence is an instability of folding so that the reactive loop of 1 molecule can insert into a β sheet of another to give sequentially the formation of long beadlike polymers of the abnormal α1-antitrypsin. The combination of the misfolding and intracellular accumulation of polymers results in a grossly reduced secretion of the protein and hence plasma deficiency.

Although the molecular pathogenesis of α1-antitrypsin deficiency has been well studied, there is still much that is not fully understood. Unexpected insights, however, are coming from the finding of the same P17 mutation in other serpins. A surprising example came with the recent demonstration that a disease in the fruit fly Drosophila was due to a P17Lys mutation in a serpin that controls the fly’s immune response. Studies of the affected flies confirm that the mutation results in polymer formation and provide strong support for the previous tentative deduction in humans: that this polymerization is temperature dependent. Here, we show that the occurrence of the same P17Lys mutation in human HCII results in a plasma deficiency of 50% in heterozygotes and an almost complete deficiency in homozygotes (Figure, B). But is there any disadvantage associated with HCII aggregates comparable to those now being observed with the P17 mutation of α1-antitrypsin? The levels of synthesis in the liver of HCII is only a fraction of that of α1-antitrypsin, so it is not surprising that its misfolding and accumulation in hepatocytes has no apparent effect on liver function or release rate of other serpins, supporting the idea that a conformational abnormality of 1 serpin does not affect the hepatic expression and secretion of others (Table 3). Therefore, the conformational consequences of this mutation in HCII seem to be restricted to the grossly reduced secretion of the protein and hence its deficiency in plasma.
The puzzle with HCII is the inability to demonstrate that its deficiency has any significant pathological consequences. Unlike complete AT deficiency, which is associated with embryonic lethality in mice and humans,20 the absence of HCII in mice21 and humans is not lethal. Moreover, HCII deficiency does not disturb normal hematopoiesis, does not modify the risk of hemorrhagic disorders, and does not affect normal gestation in either the mouse knockout model21 or in the human family with homozygous deficiency. The role of HCII deficiency in venous thrombosis is unclear.9 The HCII-deficient mice21 and our data argue against a significant role of HCII deficiency in venous thrombosis. Only 1 homozygote developed early, severe, and recurrent venous thrombosis, but she also presented with AT deficiency. Certainly, the occurrence of a de novo nonsense mutation in the AT gene causing type I deficiency in the proband could explain the higher venous thrombotic risk15 but the simultaneous role of HCII deficiency in venous thrombosis. Only 1 HCII-deficient mice21 and our data argue against a significant role of HCII deficiency in venous thrombosis. The murine model, however, suggests that HCII might protect against thrombosis in the arterial circulation.21 It is of interest that 2 other deficiency variants of HCII have been associated with coronary artery disease6 and multiple atherosclerotic lesions.9 We were not able to confirm this effect in the family studied here. Homozygous or heterozygous patients reported no arterial ischemic episodes, and they do not show carotid lesions. However, those with almost complete absence of HCII are relatively young, and extensive follow-up is required to check the contribution of HCII to maintaining blood flow after injury to the arterial endothelium.

Addendum

During the revision of this manuscript, Takamori et al22 demonstrated that patients with high plasma HCII activity showed a reduced incidence of in-stent restenosis, pointing out the possible relevance of HCII in the development of atherosclerosis and restenosis after percutaneous coronary intervention.

Acknowledgments

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