Functional Analysis of Two Haplotypes of the Human Endothelial Protein C Receptor Gene

P. Medina,* S. Navarro,* E. Bonet, L. Martos, A. Estellés, R.M. Bertina, H.L. Vos, F. España

Objective—To confirm the effect of the endothelial protein receptor gene (PROCR) haplotypes H1 and H3 on venous thromboembolism (VTE), to study their effect on endothelial protein C receptor (EPCR) expression in human umbilical vein endothelial cells, and to investigate the functionality of H1 tagging single-nucleotide polymorphisms in an in vitro model.

Approach and Results—Protein C (PC), activated PC, and soluble EPCR (sEPCR) levels were measured in 702 patients with VTE and 518 healthy individuals. All subjects were genotyped for PROCR H1 and H3. Human umbilical vein endothelial cells isolated from 111 umbilical cords were used to study the relation between PROCR haplotypes, PROCR mRNA, cellular distribution of EPCR, and rate of PC activation. Finally, the functionality of the intragenic PROCR H1 single-nucleotide polymorphisms was analyzed using a luciferase-based method. We confirmed that individuals carrying H1 have reduced VTE risk, increased plasma activated PC levels, and reduced plasma sEPCR levels and that individuals with the H3H3 genotype have an increased VTE risk and increased plasma sEPCR levels. In cultured human umbilical vein endothelial cells, H1 is associated with increased membrane-bound EPCR, increased rate of PC activation, and reduced sEPCR in conditioned medium, but does not significantly influence PROCR mRNA levels. In contrast, H3 is associated with reduced membrane-bound EPCR and increased sEPCR in human umbilical vein endothelial cell–conditioned medium, higher levels of a truncated mRNA isoform, and a lower rate of PC activation. Finally, we identified the g.2132T>C single-nucleotide polymorphism in intron 1 as an intragenic H1-specific functional single-nucleotide polymorphism.

Conclusions—These results support a protective role of PROCR H1 against VTE and an increased risk of VTE associated with the H3 haplotype. (Arterioscler Thromb Vasc Biol. 2014;34:684-690.)

Key Words: gene expression  ■  human umbilical vein endothelial cells  ■  receptors, cell surface  ■  venous thromboembolism
H3 is tagged by the minor allele of g.4600A>G (Ser219Gly; rs867186) and has been associated with the risk of venous4–15,18,19 and arterial thrombosis,19–23 but with contradictory results. The presence of H3 results in increased plasma sEPCR levels,8–13,18,20,22,24 which is largely explained by a Ser219Gly substitution, which renders EPCR more susceptible to cleavage by metalloproteinases such as tumor necrosis factor-α converting enzyme/ADAM17.7 Another mechanism that could link H3 to high plasma levels of sEPCR is its association with a truncated form of PROCR mRNA lacking the transmembrane and intracellular domains.25 Recently, PROCR H3 has also been found to be associated with increased plasma levels of PC.20,26

Therefore, we aimed to verify the effects of H1 and H3 on VTE risk in a case–control study to investigate their effects on EPCR expression in human umbilical vein endothelial cells (HUVECs) and to identify the functional SNP that mediates the protection of H1 against VTE using luciferase constructs.

In the present study, we confirm previous reports on a protective effect of PROCR H1 against VTE8–11,15 and show, for the first time, that it is probably mediated by the observed increase in functional membrane-bound EPCR in HUVEC carrying the H1 haplotype and the resulting enhanced rate of PC activation. As reported earlier, the H3H3 genotype was associated with increased sEPCR and increased VTE risk,9 and we demonstrate, for the first time, that it is also associated with reduced functional membrane-bound EPCR levels. Using a modified luciferase reporter system, we could identify the g.2532T>C (rs2069948) SNP in intron 1 as a functional H1-specific SNP, although there are probably more.

Materials and Methods
Materials and Methods are available in the online-only Supplement.

Results
Clinical Characteristics of the Subjects
The clinical characteristics of the study subjects are presented in Table 1. About 34% of the patients had spontaneous thrombosis, which is defined as thrombosis in the absence of known triggering factors (use of oral contraceptives, pregnancy, surgery, trauma, or immobilization). The prevalence of classical prothrombotic polymorphisms was similar to that described in other series.

PROCR Haplotypes and VTE Risk
The 702 patients and 518 control subjects were successfully genotyped for PROCR haplotypes H1 and H3. Table 2 shows the genotype distributions in patients and controls. To analyze the associations between genotypes and VTE, we performed logistic regression analyses. In univariate analysis, we confirmed that the presence of the H1H1 genotype significantly reduces the risk of VTE.5,11–15 Adjustment for sex, age, and the presence of thrombophilic defects in a multivariate analysis did not significantly modify the odds ratio. We also confirmed that the presence of the H3H3 genotype is associated with an increased risk of VTE.9

APC, sEPCR, and PC Levels
Because variations in the PROCR gene contribute to circulating plasma PC,20,26 APC,8,11–13 and sEPCR,8–13,18,20,22,24 levels, we measured these levels in 462 patients with VTE (excluding those undergoing coumarin therapy) and 510 healthy individuals. As previously reported,17 the levels of circulating APC were lower in patients with VTE (1.03±0.37 ng/mL) than in controls (1.25±0.40 ng/mL; P<0.001; Table 3). Both in patients and controls, APC levels increased with the number of H1 alleles (P<0.001), whereas they tended to decrease with increasing number of H3 alleles (Table 3).

Plasma sEPCR levels were similar in patients and controls (Table 3). When sEPCR levels were distributed according to genotypes, sEPCR levels strongly increased when the number of H3 alleles increased (P<0.001), confirming previous results.8–13,18,20,22,24 There was no obvious effect of the H1 allele.

In agreement with previous reports,20,26 both in patients and controls, antigen PC levels were significantly higher in carriers of the PROCR H3 allele than in noncarriers. Thus, PC levels were 101±17%, 103±19%, and 102±16% in controls carrying the HxHx, HxH1, and H1H1 genotypes, respectively, and 114±19% and 116±24% in controls carrying the HxH3 and H1H3 genotypes, respectively (P<0.001). In patients with VTE, PC levels were 100±21%, 103±20%, and 106±26% in carriers of the HxHx, HxH1, and H1H1 genotypes, respectively, and 122±17%, 110±18%, and 138±9% in carriers of the HxH3, H1H1, and H3H3 genotypes, respectively (P<0.001). These data indicate that the increased circulating APC levels observed in H1 carriers cannot be explained by an increase in PC levels in these individuals.

EPCR Protein and mRNA Levels in HUVECs
To further investigate whether the PROCR haplotypes H1 and H3 are functional and to identify the underlying mechanism, we used passage 2 HUVECs derived from 111 human umbilical cords from white newborns. Thirty-four HUVEC cultures were HxHx, 36 were HxH1, 51 H1H1, 8 HxH3, and 1 was H3H3. Figure 2 shows the concentration of EPCR in cell lysates and in conditioned medium (CM). EPCR levels in cell lysates increased (P<0.001), and sEPCR levels in the HUVEC-CM slightly decreased (P=0.024) with the number of H1 alleles. The total amount of EPCR measured in cell lysates and HUVEC-CM combined also increased with the number of H1 alleles (Figure 2C). In contrast, the presence of the H3 allele was associated with reduced levels of EPCR in cell lysates and, as expected, with increased levels in CM. This clearly establishes that the H3 allele leads not only to
increased levels of sEPCR, but also to significantly reduced levels of EPCR on the cell membrane.

We also measured the PROCR full-length and truncated mRNA levels in 90 HUVEC samples for which mRNA isolated from passage 1 confluent cultures was available. Figure 3 shows the results obtained. There was a nonsignificant decrease in PROCR full-length mRNA levels with increasing number of H1 alleles. Similarly, there was a trend to lower PROCR full-length mRNA levels when the number of H3 alleles increased, although these differences were again not significant (Figure 3A). As previously reported, a truncated mRNA form was detected in HUVECs, representing 0.3% to 0.5% of the total PROCR mRNA form was detected in HUVECs, representing 0.3% to 0.5% of the total PROCR mRNA form was detected in HUVECs not carrying H3 and from 1% to 2.3% in HUVECs carrying PROCR mRNA in HUVECs from 0.3% to 0.5% of the total PROCR mRNA form was detected in HUVECs, representing 0.3% to 0.5% of the total.

Table 1. Characteristics of the Study Subjects

<table>
<thead>
<tr>
<th></th>
<th>Patients</th>
<th>Controls</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>702</td>
<td>518</td>
<td></td>
</tr>
<tr>
<td>Age, y, median</td>
<td>42 (33–53)</td>
<td>42 (32–52)</td>
<td>0.359</td>
</tr>
<tr>
<td>Age at first onset, y, median</td>
<td>39 (30–50)</td>
<td>…</td>
<td></td>
</tr>
<tr>
<td>Male sex, n (%)</td>
<td>381 (54)</td>
<td>283 (55)</td>
<td>0.796</td>
</tr>
<tr>
<td>Pulmonary embolism, n (%)</td>
<td>175 (25)</td>
<td>…</td>
<td></td>
</tr>
<tr>
<td>Recurrent thrombosis, n (%)</td>
<td>161 (23)</td>
<td>…</td>
<td></td>
</tr>
<tr>
<td>Familial thrombosis, n (%)</td>
<td>147 (21)</td>
<td>…</td>
<td></td>
</tr>
<tr>
<td>Spontaneous thrombosis, n (%)</td>
<td>239 (34)</td>
<td>…</td>
<td></td>
</tr>
<tr>
<td>FV Leiden, n (%)</td>
<td>604 (86)</td>
<td>497 (66)</td>
<td>…</td>
</tr>
<tr>
<td>+/- and +/+</td>
<td>96 and 2 (14)</td>
<td>16 and 0 (3)</td>
<td>0.001</td>
</tr>
<tr>
<td>PT G20210A, n (%)</td>
<td>639 (91)</td>
<td>487 (94)</td>
<td></td>
</tr>
<tr>
<td>GT and AA</td>
<td>63 and 4 (9)</td>
<td>31 and 0 (6)</td>
<td>0.001</td>
</tr>
</tbody>
</table>

H3 haplotypes, we selected passage 2 HUVECs which were homozygous wild type for the THBD g.1418C>T SNP (1418CC). The rate of PC activation increased with the number of H1 alleles, whereas it decreased in the presence of H3. Preincubation of HUVECs with monoclonal antibody RCR-379 to block EPCR-dependent PC activation drastically reduced PC activation (Figure 4). In the same experiment, part of the HUVECs was used to measure the amount of EPCR in the cytosolic and cell membrane fractions. As seen in the top of Figure 4, HUVECs carrying the H1 haplotype have more membrane-bound EPCR than HUVECs not carrying the H1 haplotype, and HUVECs carrying the H3 haplotype have less membrane-bound EPCR than noncarriers. Cytosolic fractions did not contain detectable EPCR (<0.3 ng/mL).

**PC Activation on HUVECs**

PC activation on HUVECs carrying different combinations of haplotypes was compared (Figure 4) to test the functionality of the membrane-bound EPCR. Because the thrombomodulin gene (THBD) g.1418C>T polymorphism has been associated with the rate of PC activation in cultured HUVECs, we also measured the PROCR full-length and truncated mRNA levels in 90 HUVEC samples for which mRNA isolated from passage 1 confluent cultures was available. Figure 3 shows the results obtained. There was a nonsignificant decrease in PROCR full-length mRNA levels with increasing number of H1 alleles. Similarly, there was a trend to lower PROCR full-length mRNA levels when the number of H3 alleles increased, although these differences were again not significant (Figure 3A). As previously reported, a truncated mRNA form was detected in HUVECs, representing 0.3% to 0.5% of the total PROCR mRNA form was detected in HUVECs not carrying H3 and from 1% to 2.3% in HUVECs carrying H3 (Figure 3B). This small amount of the truncated isoform does not explain the large increase in sEPCR levels observed in H3 carriers.

**Table 1.** Distribution of *PROCR* Haplotypes in 702 Patients and 518 Control Subjects

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Cases, n (%)</th>
<th>Controls, n (%)</th>
<th>OR (95% CI)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>HxHx</td>
<td>150 (21.4)</td>
<td>100 (19.3)</td>
<td>1†</td>
</tr>
<tr>
<td>H1x</td>
<td>293 (41.7)</td>
<td>192 (37.1)</td>
<td>1.02 (0.74–1.39)</td>
</tr>
<tr>
<td>H1H1</td>
<td>136 (19.4)</td>
<td>138 (26.6)</td>
<td>0.66 (0.46–0.93)</td>
</tr>
<tr>
<td>H1H3</td>
<td>53 (7.5)</td>
<td>48 (9.3)</td>
<td>0.75 (0.47–1.19)</td>
</tr>
<tr>
<td>HxH3</td>
<td>62 (8.8)</td>
<td>40 (7.7)</td>
<td>1.03 (0.64–1.66)</td>
</tr>
<tr>
<td>H3H3</td>
<td>8 (1.1)</td>
<td>0 (0)</td>
<td>12.07 (0.69–211.75)</td>
</tr>
</tbody>
</table>

CI indicates confidence interval; and OR, odds ratio.

*Adjusted for age, sex, and presence of thrombophilic defects (factor V Leiden, prothrombin 20210A, protein C deficiency, protein S deficiency, and antithrombin deficiency).

†x=1 and 3.

‡Reference group.
**PROCR** H1 intron 1 showed a significant reduction of 45% in luciferase activity compared with constructs containing H2 intron 1 (P=0.029). Constructs containing H1 intron 2 showed a 27% reduction in luciferase activity (P=0.071), and constructs containing the H1 3′ untranslated region and downstream flanking region showed a 14% increase in luciferase activity (P=0.383) compared with that of H2 (Figure 5).

![Figure 2](image-url)  
**Figure 2.** Endothelial protein C receptor (EPCR) levels in cell lysates and conditioned media from human umbilical vein endothelial cells (HUVECs), according to their **PROCR** haplotype. Passage 2 HUVECs were grown to 80% to 100% confluence and exposed for 3 hours to serum-free culture medium (50 μL/well). Thereafter, EPCR was measured in conditioned medium and membrane fraction as indicated in Materials and Methods in the online-only Data Supplement. A, Membrane fraction. B, Conditioned media. C, Membrane fraction plus conditioned media. Cytosolic fractions did not contain detectable EPCR (<0.3 ng/mL). Values are expressed as means±SEM. HxHx and H3. *Kruskal–Wallis test.

![Figure 3](image-url)  
**Figure 3.** **PROCR** mRNA levels in human umbilical vein endothelial cells (HUVECs) according to **PROCR** haplotypes. Total RNA was isolated from passage 1 HUVEC confluent cultures, and real-time quantitative polymerase chain reaction was performed. Figures represent the relative level of **PROCR** mRNA compared with TBP mRNA (means±SEM). HxHx and H3. A, **PROCR** full-length mRNA levels. B, **PROCR** truncated mRNA levels. *Kruskal–Wallis test.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>n</th>
<th>Patients</th>
<th>n</th>
<th>Controls</th>
<th>P Value</th>
<th>n</th>
<th>Patients</th>
<th>n</th>
<th>Controls</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>All</td>
<td>462</td>
<td>1.03±0.37</td>
<td>510</td>
<td>1.25±0.40</td>
<td>&lt;0.001</td>
<td>462</td>
<td>1.01 (83–122)</td>
<td>510</td>
<td>1.00 (79–125)</td>
<td>0.216</td>
</tr>
<tr>
<td>HxHx</td>
<td>101</td>
<td>0.94±0.34</td>
<td>100</td>
<td>1.14±0.33</td>
<td>&lt;0.001</td>
<td>101</td>
<td>0.86 (74–104)</td>
<td>100</td>
<td>0.88 (74–108)</td>
<td>0.278</td>
</tr>
<tr>
<td>HxH1</td>
<td>186</td>
<td>1.03±0.36</td>
<td>188</td>
<td>1.23±0.35</td>
<td>&lt;0.001</td>
<td>186</td>
<td>0.98 (84–110)</td>
<td>188</td>
<td>0.91 (78–110)</td>
<td>0.463</td>
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<tr>
<td>H1H1</td>
<td>93</td>
<td>1.22±0.41</td>
<td>134</td>
<td>1.33±0.47</td>
<td>0.004</td>
<td>93</td>
<td>0.97 (81–111)</td>
<td>134</td>
<td>0.91 (75–112)</td>
<td>0.342</td>
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<tr>
<td>K–W test</td>
<td>&lt;0.001</td>
<td></td>
<td>&lt;0.001</td>
<td></td>
<td></td>
<td>0.335</td>
<td></td>
<td>0.523</td>
<td></td>
<td></td>
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<tr>
<td>H1H3</td>
<td>34</td>
<td>0.98±0.32</td>
<td>48</td>
<td>1.36±0.44</td>
<td>&lt;0.001</td>
<td>34</td>
<td>0.236 (207–300)</td>
<td>48</td>
<td>0.246 (196–282)</td>
<td>0.851</td>
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<tr>
<td>HxHx</td>
<td>101</td>
<td>0.94±0.34</td>
<td>100</td>
<td>1.14±0.33</td>
<td>&lt;0.001</td>
<td>101</td>
<td>0.86 (74–104)</td>
<td>100</td>
<td>0.88 (74–108)</td>
<td>0.255</td>
</tr>
<tr>
<td>HxH3</td>
<td>41</td>
<td>0.97±0.29</td>
<td>40</td>
<td>1.20±0.31</td>
<td>&lt;0.001</td>
<td>41</td>
<td>0.261 (212–295)</td>
<td>40</td>
<td>0.279 (211–326)</td>
<td>0.408</td>
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<tr>
<td>H3H3</td>
<td>7</td>
<td>0.64±0.25</td>
<td>7</td>
<td>…</td>
<td></td>
<td>7</td>
<td>0.491 (410–516)</td>
<td>7</td>
<td>0. …</td>
<td></td>
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<tr>
<td>K–W test</td>
<td></td>
<td>0.031</td>
<td></td>
<td>0.176</td>
<td></td>
<td>&lt;0.001</td>
<td>0.001</td>
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</tr>
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</table>

**Table 3.** Levels of APC (mean±SD) and sEPCR (Median and 25th–75th Percentiles) in 462 Patients (Without Coumarin Therapy) and 510 Controls According to the **PROCR** Haplotypes

* APC indicates activated protein C; K–W, Kruskal–Wallis; and sEPCR, soluble endothelial protein C receptor.
Figure 4. The rate of protein C (PC) activation increases with the number of PROCR H1 present. Passage 2 human umbilical vein endothelial cells (HUVECs) from 2 HxHx, 2 HxH1, 2 H1H1, and 2 HxH3 carriers were incubated with PC and thrombin at 37°C for 30 minutes as described in the Materials and Methods in the online-only Data Supplement. The activated PC (APC) generated/2×10⁴ cells is plotted against the corresponding genotype. The results represent the mean±SEM of 2 separate experiments performed in duplicate. Solid bars represent PC activation in the absence of anti–endothelial cell protein C receptor (EPCR) monoclonal antibody (mAb), RCR-379. Open bars represent PC activation in the presence of anti–EPCR mAb, RCR-379. In the absence of cells, 0.2 nmol/L of APC was generated. Top, Numbers represent the mean concentration of membrane-bound EPCR in the 2 HUVECs with the corresponding genotype. *Kruskal–Wallis test.

These results indicate that PROCR intron 1 might contain a functional SNP, and therefore, we generated, by site-directed mutagenesis, 4 sets of constructs in which we cloned each H1 SNP individually in the H2 background and again performed transfection experiments in EC-RF24 cells. As shown in Figure 6, only the construct containing the 2532C allele (rs2069948) showed a significant 34.4% reduction in luciferase activity compared with the construct containing H2 (P=0.003), a reduction that is similar to that obtained when all 4 H1-specific SNPs are included compared with H2 (36.7%; P=0.006). Therefore, the g.2532T>C SNP seems to be the functional SNP responsible for the PROCR H1 effect observed in this in vitro model.

Discussion

In the present study, we confirmed that carriers of the H1H1 genotype in the PROCR gene have normal plasma sEPCR levels, higher levels of circulating APC, and a lower risk of VTE than those carrying other genotypes. We have shown for the first time that HUVECs carrying the H1 allele have significantly higher levels of membrane-bound EPCR and slightly lower sEPCR levels in HUVEC-CM than noncarriers. In agreement with this finding is the observation that the EPCR-dependent thrombin-mediated PC activation on intact HUVECs, as calculated from the experiments adding anti-EPCR (Figure 4), was higher in H1 carrying cells than in non-H1 carrying cells. Together, these findings indicate that the reduced risk of VTE of H1H1 carriers is associated with an increased concentration of functional EPCR on the endothelial cells. The latter might be responsible for the association of the H1 allele with increased plasma APC concentrations. In contrast, the PROCR full-length mRNA levels were similar in HUVECs from H1 carriers and non-H1 carriers, which suggests that the increase in functional membrane-bound EPCR associated with H1 is not caused by an increase in gene expression.

Several studies have investigated the association between PROCR haplotypes and venous and arterial thrombosis, with conflicting results. Some studies showed a reduced risk of VTE in carriers of the H1H1 genotype, whereas others did not find such an effect. An explanation for at least part of these discrepancies may lie in differences in study design and patient populations. Our present study is the largest case–control study performed with this aim.

We observed that the H3H3 genotype was associated with increased sEPCR levels and an increased risk of VTE, with
higher sEPCR levels in HUVEC-CM and, for the first time, with reduced functional membrane-bound EPCR levels. This reduction in functional membrane-bound EPCR seems not to be attributable to reduced gene transcription because the levels of PROCR full-length mRNA did not vary in the presence of H3. As expected, the levels of PROCR truncated mRNA significantly increased with the number of H3 alleles. It is however unlikely that this is the cause of the large increase in sEPCR levels observed in H3 carriers.25

The increased risk of VTE associated with the H3H3 genotype is in agreement with previous reports.9,12,15 However, its mechanism is not fully understood. Our data suggest that it could be mediated by the increased EPCR shedding from the endothelial membrane, leading to significantly lower EPCR levels on the cell membrane and to a large increase in sEPCR levels. This effect of the H3 haplotype might be explained by the Ser219Gly substitution (rs867186), which would render levels on the cell membrane and to a large increase in sEPCR

mechanism is not fully understood. Our data suggest that it might be functional. Interestingly, the g.2532T>C SNP is located 16 bases from the branch site. The 2532C allele is phylogenetically reduced protein expression most likely by influencing splic-
ing efficiency. The g.2532T>C SNP is located 16 bases from the intron 1 as a plausible functional SNP in this part of the H1 haplotype. In our reporter system, this SNP is associated with reduced protein expression most likely by influencing splicing efficiency. The g.2532T>C SNP is located 16 bases from the intron 1-exon 2 splicing site in the branch point consensus sequence YTNAY, with 2532T/C at the position of the first pyrimidine (C or T) residue and the consensus A being used for the branch site. The 2532C allele is phylogenetically well conserved and is the ancestral allele, which suggests that this SNP might be functional. Interestingly, the g.2532T>C SNP reduced protein expression in our in vitro model system, which is opposite of what might be expected based on the observed association between H1 and EPCR expression in HUVECs and on the predictions of an in silico analysis using the Human Splicing Finder29 (data not shown). On the contrary, it might be in agreement with the trend of reduced PROCR mRNA expression with increasing number of H1 alleles. Definite answers await further functional analysis of the H1-specific SNPs outside the PROCR gene itself.

In the present study, we have contributed to the further elucidation of the mechanism by which PROCR haplotypes 1 and 3 influence the risk of VTE. Our results show that the protective effect of H1 is associated with increased levels of functional membrane-bound EPCR, which will lead to an enhanced rate of PC activation. In contrast, the increased risk of VTE associated with H3 may be explained by the decrease in functional membrane-bound EPCR observed in HUVECs carrying the H3 haplotype.

Acknowledgments
We thank Marta Gurria, Silvana Aniorte, Eugenia Romaguera, and Elisabet Rodriguez for collecting the umbilical cords, Ursula Salinas and Josefa Llorens for technical assistance, and Haidy van der Putten for assistance in the in vitro functional experiments.

Sources of Funding
This work was supported by the Plan Nacional de I+D+i 2008 to 2011 (ISCIII PS09/00610 and PI12/00027), Fondo Europeo de Desarrollo Regional and Redes Red de Enfermedades Cardiovasculares (RD06/0014/0004), and Red de Investigación Cardiovascular (RD12/0042/0029), Consellería de Sanidad, Generalitat Valenciana (Prometeo 2011/027), and Instituto de Investigación Sanitaria La Fe, Spain. Pilar Medina is a Miguel Servet researcher (ISCIII CP09/00065).

Disclosures
None.

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11. Medina P, Navarro S, Estellés A, España F. Polymorphisms in the endo-
We have contributed to further elucidation of the mechanism by which PROCR haplotypes influence the risk of VTE.

EPCR haplotypes influence the risk of VTE. PROCR haplotypes have been associated with venous thromboembolism (VTE) risk, therefore we aimed to investigate the effects of PROCR haplotypes on plasma levels of activated protein C. We have demonstrated that H1H1 individuals have increased plasma levels of activated protein C and reduced VTE risk. In human umbilical vein endothelial cells, we demonstrated that H1 is associated with increased levels of membrane-bound EPCR and an enhanced rate of protein C activation. In contrast, H3H3 carriers have increased plasma soluble EPCR levels and an increased VTE risk. Likewise, in cultured human umbilical vein endothelial cells, we demonstrated a decrease in the levels of functional membrane-bound EPCR, which might explain increased VTE risk associated with the H3H3 genotype. Finally, we identified the g.2132T>C single-nucleotide polymorphism in intron 1 as an intragenic H1-specific functional single-nucleotide polymorphism. With our investigation, we have contributed to further elucidation of the mechanism by which PROCR haplotypes influence the risk of VTE.
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Arterioscler Thromb Vasc Biol. 2014;34:684-690; originally published online January 16, 2014;
doi: 10.1161/ATVBAHA.113.302518
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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Material and methods

Patients and controls

The study included 702 unrelated Caucasian patients with VTE. Patients with an objectively confirmed episode of VTE who consecutively entered the anticoagulation clinic in our hospital for thrombophilic study between 1997 and 2012, were enrolled. Objective diagnoses of VTE and pulmonary embolism were made by clinical probability, D-dimer levels, compression ultrasonography, ventilation perfusion lung scan and, when necessary, phlebography or pulmonary angiography. Patients with known malignant disorders were excluded. The control group included 518 unrelated healthy subjects with no history of thromboembolic disease. Controls were randomly selected to match cases by age, gender and geographic distribution.

All subjects gave their informed consent to enter the study, which was approved by the Ethics Committee of our institution, and was performed according to the declaration of Helsinki, as amended in Edinburgh in 2000.

Blood collection

Blood was collected at least 6 months after the acute event. At the time of blood collection 240 patients were still on oral anticoagulants. For the thrombophilic study and the measurement of sEPCR, blood was collected in vacuum tubes containing 0.129 M trisodium citrate. For the measurement of circulating APC, blood was collected as previously reported. Blood was centrifuged at 1500 x g for 30 min at 4 °C. Plasma was frozen and stored at -72 °C until testing. Tubes containing EDTA were used to collect blood for DNA studies.

Plasma sEPCR, protein C and APC

Levels of sEPCR in plasma were measured with the Asserachrom sEPCR ELISA kit (Diagnostica Stago, Asnières-sur-Seine, France). The intra- and inter-assay coefficients of variation were less than 7%.

Plasma protein C and circulating APC levels were measured as previously reported.

Isolation of HUVECs

HUVECs from 111 umbilical cords were obtained by collagenase digestion and grown to confluence in T-75 flasks, as previously described.

The biochemical and cellular behaviors of HUVECs may be altered after several passages. Over 5-7 passages, cells gradually start to increase in size, to grow more slowly and to lose specific functions. Therefore, we used passage 1 HUVECs for DNA isolation and mRNA quantification, and passage 2 HUVECs for EPCR measurement and protein C activation experiments.
**EPCR measurement in HUVECs**

For measurement of EPCR in HUVEC conditioned medium (CM) and cell membrane and cytosolic fractions, cells were plated in 96-well culture plates, at a density of approximately $20 \times 10^3$ cells/well, and grown to reach 80-100% confluency in culture medium as previously described\(^4\). Cells were treated with 50 µL of lysis buffer pH 7.4 per well (10 mM Tris, 0.5% dithiothreitol, 10% glycerol, 1.5 mM EDTA), during 2 hours at 4ºC, with constant shaking. After centrifugation at 126,000 x g for 15 min at 4ºC, the supernatants (cytosolic fractions) were stored at -72ºC. The pellets were solubilized with 50 µL per well of 20 mM Tris-HCl, 125 mM NaCl, 1% Triton X-100, pH 7.4, vortexed for 5 min at 4ºC and centrifuged at 126,000 x g during 15 min at 4ºC. The supernatants (membrane fractions) were stored at -72ºC.

**Protein C activation on HUVECs**

Protein C activation on HUVECs was studied as described before\(^4\). Briefly, 100 nM protein C and 2 U/ml bovine thrombin (final concentrations) were added to confluent HUVECs in 96-well plates. After 30 min at 37 ºC, the reactions were stopped by addition of 50 µL of hirudin (50 U/ml) and 75 µL aliquots of the supernatants were transferred into 96-well microplates where the amidolytic activity of APC was measured by adding 15 µL of 8 mM SB2366. Under the conditions used in this study, <10% of the PC was activated during the incubation. All measurements were performed in duplicate. Where indicated, HUVECs were pretreated with 50 µg/ml of rat monoclonal anti-EPCR antibody RCRB379 (Abcam, Cambridge, UK) for 15 min before the addition of PC and thrombin. This antibody blocks the ability of EPCR to enhance protein C activation by thrombomodulin.

**Genotyping of the PROCR H1 and H3 haplotypes**

Genomic DNA was isolated from patients, controls and HUVECs using the Wizard Genomic DNA purification kit (Promega, Madison, WI), following the manufacturer’s instructions.

PROCR H1 and H3 are tagged by the rs9574 and rs867186 SNPs, respectively, and these SNPs were genotyped as previously described\(^5,6\).

**mRNA isolation and real-time quantitative PCR analysis**

Isolation of total RNA from HUVECs and synthesis of first strand cDNA was carried out as previously reported\(^4\).

Quantification of the two PROCR mRNA transcripts (full-length and truncated mRNA) was performed following the previously reported protocol\(^7\). The results were normalized using the TATA-binding protein (TBP) transcript. Primer sequences are available on request. RT-qPCR was carried out using semi-automatic equipment (Light-Cycler Real-Time PCR Detection System, Roche, Mannheim, Germany). Each 15 µL reaction contained 2 µL of 1/10 diluted cDNA, 1.5 µL of 10X LightCycler® FastStart DNA Master Mix, 3 mM MgCl₂, 5 pmol µL\(^{-1}\) of the PROCR primer mix or 6 pmol µL\(^{-1}\) of the TBP primer mix. The amplification reaction for the PROCR cDNA was initially incubated at
95 °C for 10 minutes, followed by 40 cycles of 5 seconds at 95 °C, 10 seconds at 60 °C, and 6 seconds at 72 °C. The amplification reaction for the TBP cDNA was initially incubated at 95 °C for 8 minutes, followed by 40 cycles of 15 seconds at 95 °C, 5 seconds at 59 °C, and 10 seconds at 72 °C.

**PROCR H1 in vitro functional study**

To study the functionality of the PROCR H1-specific SNPs in intron 1, intron 2 and the 3′UTR with the downstream flanking sequence, we amplified these PROCR regions from homozygous carriers of H1 and H2 (intron 3 is identical in both haplotypes), using the *Pfu Ultra* Hotstart DNA Polymerase (Stratagene). H2 was used as the reference sequence, because it contains the common allele of all SNPs. The fragments were cloned into a modified pGL3-Basic vector (Promega). This pGL3-Basic vector was adapted by engineering SexAI and EcoRV restriction sites around the original position of the firefly luciferase intron 1, without affecting the encoded amino acids, to generate a system for testing intronic variants (van der Putten HH, Bertina RM, Vos HL, unpublished data). Introns 1 or 2 were cloned in the position of the first intron of the original firefly luciferase gene using hybrid primers that contained a modified luciferase sequence containing the relevant SexAI or EcoRV restriction sites followed by the ends of the relevant human PROCR introns. The DNA fragments with the 3′UTR and the downstream flanking region containing all sequences required for polyadenylation were cloned downstream from the luciferase cDNA (using XbaI and BamHI restriction sites), thereby replacing the SV40 late polyadenylation site normally present in pGL3-Basic. The CMV promoter was cloned upstream of the luciferase gene to drive its transcription. Primer sequences and cycling conditions are available upon request. Plasmid DNA was isolated using the PureYield Plasmid Maxiprep System (Promega).

The constructs were transiently transfected into the human endothelial cell line EC-RF24 (a kind gift from Dr. Hans Pannekoek) by electroporation. In all cases, the pRL-CMV vector encoding the Renilla luciferase was co-transfected to correct for differences in transfection efficiency. Briefly, cells were trypsinized at 60-80% confluency and resuspended in RPMI 1640 with 10% fetal bovine serum. 550,000 cells were mixed with 2 µg of the construct of interest and 100 ng of pRL-CMV control vector DNA, and the mixture was electroporated at 220V and 1075 µF in a GenePulserII electroporator (Bio-Rad). The cell suspension was distributed in 3 wells of a 12-well plate containing complete M199 medium. After 48 h culture, cells were lysed mechanically by constant shaking for 15 min at room temperature in 200 µl passive lysis buffer (Promega) followed by a freeze-thaw cycle at -72°C. Subsequently, firefly and Renilla luciferase activities were measured with the Dual-Luciferase Reporter Assay System (Promega) in a GLOMAX 96 microplate Luminometer (Turner Biosystems). Activities were expressed as the ratio of firefly luciferase activity to Renilla luciferase activity.

The construct containing PROCR intron1 H1 seemed to contain a functional SNP, because of a difference in luciferase activity between the H1 and H2 constructs, therefore it was investigated in more detail. By using the Quikchange Site-Directed Mutagenesis Kit (Stratagene) we generated 4 sets of constructs each containing one of the 4 H1-specific SNPs in intron 1 in an H2-
background. Then we performed a new set of transfections as previously described.

**Statistical analysis**

The allelic distribution was analyzed with the HaploView program. Other statistical analyses were conducted using the SPSS for Windows release 11.5 statistical software (SPSS Inc., Chicago, IL). Data are reported as medians and interquartile ranges (25th - 75th percentiles) or means ± SEM. Allele frequencies were calculated by gene counting. The Chi-squared test was used to compare percentages. Parameter levels were compared with the Mann-Whitney U-test or the Kruskal-Wallis One ANOVA test. Correlations were assessed by the Spearman test. Logistic regression analysis was performed to identify the associations between genotypes and VTE risk. Multivariate analysis was performed using multiple logistic regression by including all the significant covariates in a single step. Odds ratios (ORs) and 95% confidence intervals (95% CI) were calculated from the logistic model. As none of the control individuals carried the H3H3 genotype, the OR for the PROCR H3-containing genotypes was calculated by assigning 0.5 to the number of controls with this genotype. Transfection results of each set of constructs were expressed as mean ± SEM of 3-5 experiments, in which 2 or more DNA preparations of each construct were transfected in triplicate. Finally, the expression level of the H1-derived constructs was calculated relative to that of the H2-derived construct, which was set as 100%. Luciferase activities of wt and mutant constructs were compared with an unpaired t-test using the GraphPad Prism® Software. Any differences with a two-tailed P value of <0.05 were considered statistically significant.

**References**


