FVII, FVIIa, and Downstream Markers of Extrinsic Pathway Activation Differ by EPCR Ser219Gly Variant in Healthy Men

Helen A. Ireland, Jackie A. Cooper, Fotios Drenos, Jayshree Acharya, Jacqueline P. Mitchell, Ken A. Bauer, James H. Morrissey, M. Peter Esnouf, Stephen E. Humphries

Objective—The purpose of this study was to determine the effect of a variant in EPCR (Ser219Gly), previously shown to affect EPCR shedding, on plasma FVII, FVIIa, and downstream markers of activated coagulation.

Methods and Results—Statistical analysis was undertaken in ~2000 healthy middle aged men (NPHSII). Higher soluble EPCR levels were confirmed for Gly allele carriers (P<0.0001). Significantly higher levels of FVII, FVIIa, and downstream markers of activated coagulation in the extrinsic pathway (FIX activation pep [FIXpep]; FX activation pep [FXpep]), and prothrombin F1 +2 (F1 +2) were identified in baseline samples, in Gly carriers compared to Ser/Ser (P<0.04 for trend). In repeat samples collected for up to 5 years, levels of FVII and F1 +2 were higher in Gly allele carriers compared to Ser/Ser by (FVII; 6.9% CI 5.5 to 8.4 in Ser/Gly; and 23.4% CI 16.3 to 30.8 in Gly/Gly, P<0.0001), (F1 +2: 8.1% CI 5.2 to 11.1 in Ser/Gly; 25.2% CI 11.8 to 40.3 in Gly/Gly, P<0.04), confirming reproducibility of findings at baseline. Molar ratios for FIXpep, FXpep, and F1 +2 to FVIIa were constant in Ser/Ser and Ser/Gly but tended to be higher in Gly/Gly, reaching statistical significance for FIXpep:FVIIa (P=0.04).

Conclusions—These data suggest that higher levels of FVII and FVIIa circulate when EPCR shedding is greatest. Furthermore, these results suggest consequences for activation of extrinsic coagulation. (Arterioscler Thromb Vasc Biol. 2009;29:00-00.)

Key Words: endothelial protein C receptor ▪ FVIIa ▪ extrinsic coagulation ▪ FIX activation peptide ▪ FX activation peptide ▪ prothrombin F1 +2

Endothelial protein C receptor (EPCR) plays an important role in the inhibition of thrombin generation by its participation in the PC pathway. As an integral endothelial membrane protein it captures circulating PC and presents it to an adjacent thrombin-thrombomodulin complex which activates the PC with a consequent inhibition of coagulation factors Va and VIIIa, resulting in reduced thrombin generation. We and others have previously reported altered functional characteristics associated with an amino acid change within the membrane spanning region of EPCR (Ser219Gly), both by in vitro analysis and in data derived from ex vivo plasma samples. Increased thrombin generation, as determined by prothrombin F1 +2 (F1 +2) levels, was found in those with the EPCR 219Gly allele in a large study of patients with type 2 diabetes and in healthy individuals within the large prospective study of heart disease, Northwick Park Health Study II (NPHSII). A large difference in soluble EPCR levels across EPCR Ser219Gly genotype groups is now well established both in health and disease, with at least 70% of the variance of soluble EPCR levels in plasma being caused by the EPCR Ser219Gly variant. In vitro analysis has confirmed increased shedding of EPCR in those with the Gly allele. Recent in vitro analysis by surface plasmon resonance of FVIIa passed over soluble EPCR bound to the surface, and cell surface EPCR binding to both FVII and FVIIa has suggested that EPCR also acts as a cellular receptor for activated factor VII (FVIIa) and FVII on endothelium. FVIIa and FVII bound to EPCR with a similar affinity compared to binding of PC and activated PC to EPCR. Furthermore, FVIIa that had bound to EPCR was unable to accelerate either FVIIa activation of FX or to activate protease activated receptors. FVIIa endocytosis was also facilitated by its binding to EPCR. EPCR binding is therefore suggested as a clearance mechanism for FVIIa. An in vitro study has also shown that blocking the FVII binding site of EPCR on the endothelial surface increased the generation of FXa by FVIIa, further suggesting a physiological hemostatic consequence of
EPCR-FVIIa binding.\(^9\) Soluble EPCR was shown to dose-dependently inhibit the activation of FX by the FVIIa-tissue factor (TF) complex.\(^9\) These findings suggest, in terms of the FVII/FVIIa binding, that higher levels of endothelial or soluble EPCR may shift hemostatic balance toward anticoagulant activity, although it is difficult to predict the relative effects of these processes in vivo.

Within NPHSII, markers of coagulation activity have been measured at several steps along the extrinsic or tissue factor pathway. A sensitive assay to the peptide liberated (FIXpep) with the generation of activated factor IX (FIXa)\(^{10}\) has demonstrated that FIXa generation in vivo results mainly from activity of the tissue factor pathway, rather than the contact or intrinsic pathway (factor XII, prekallikrein, high-molecular-weight kininogen, factor XI). Levels of FIXpep were shown to be reduced in hereditary FVII or FIX deficiency.\(^{10}\) Similarly, levels of FXpep were shown to be lower in patients with FVII deficiency.\(^{11}\)

In a recent study of NPHSII, a higher ratio of FIXpep: FVIIa was found to be an independent risk for heart disease\(^{12}\) and was as good as or better than the classic and inflammatory risk factors used in risk algorithms. In the current analysis of this large study of middle-aged men, healthy at the time of sampling, measurements of FVII and FVIIa\(^{13}\) have been assessed by EPCR Ser219Gly genotype, and the consequences of this on activation of coagulation has been studied using activation markers within the extrinsic coagulation pathway (FIXpep and FXpep). Measurements of activated factor XII (FXIIa)\(^{14}\) and F1+2,\(^{15}\) were used also to determine any effect on coagulation outside the extrinsic pathway, and thrombin generation, respectively.

### Methods

#### Study Cohort

NPHSII is a prospective study of heart disease currently with 15 years follow-up. The study involved 2997 men who gave written informed consent, and was approved by the institutional ethics committee. Subjects did not fast before blood collection but were instructed to avoid heavy meals. They were also requested not to smoke or to take vigorous exercise from midnight before sampling.

#### EPCR Genotyping

EPCR genotyping was determined using PCR, followed by restriction digest, and visualized using microarray diagonal gel electrophoresis, as reported previously.\(^2\)

#### Measurements of Coagulation Factors and Markers of Activated Coagulation

For the current analysis, only samples without a questionable venepuncture performance were included, following a scoring system by predetermined criteria.\(^{16}\) FVII levels were measured by coagulant (FVIIc) and antigen (FVIIag) levels. Markers of an activated extrinsic pathway (FIXpep,\(^{10}\) FXpep,\(^{11}\) FVIIa\(^{13}\)), and a coagulation marker outside the extrinsic coagulation pathway (FXIa, Axis-Shield)\(^{14}\) together with a marker of thrombin generation (F1+2)\(^{15}\) were measured as described previously, in baseline samples.\(^{17}\) Numbers for those with genotype data corresponding to EPCR Ser219Gly and each coagulation marker, in baseline samples are as follows: FVIIc (n=2427), FVIIag (n=2273), FVIIa (n=1086), FIXpep (n=1259), FXpep (n=1120), FXIa (n=1970), F1+2 (n=2188).

In addition, FVIIc levels had been measured in samples collected at 1, 2, 3, 4, and 5 years and F1+2 levels had been measured at 1, 2, 3, and 4 years. These data were used to determine the reproducibility of findings in baseline samples. Only those who had both genotyping data and coagulation markers for each year were included in this analysis (FVIIc n=1564; F1+2 n=1147).

#### Measurement of sEPCR Levels

sEPCR levels were measured specifically for this study using an ELISA assay (Diagnostica Stago). Baseline (n=84) or 1-year (n=15) samples from both individuals who did and did not develop a CHD event and within each genotype group were randomly selected for assay. For those homozygous for the rare allele, all available samples were used (n=11). Eleven samples would have 90% power to detect a 1.5-fold difference between those homozygous for the rare allele compared to those with both common alleles. Previous findings have shown a 3.9-fold difference between sEPCR levels between these 2 groups. All samples were measured in duplicate, the mean of each pair being recorded. sEPCR levels were also measured in samples at 2 (n=99) and 5 years (n=99) for the same individuals. Interassay coefficient of variation for the sEPCR assay was 9.7%, 9.9%, and 13.1% for 3 different control samples.

#### Statistical Analysis

Levels of coagulation markers were log-transformed. Geometric mean levels and approximate SD were used throughout. Effect of genotype on baseline levels was tested using both a codominant and an additive genetic model. Adjustments were made by analysis of covariance. Coagulation markers by genotype group and across years were used repeated measures analysis of variance using those with complete data for all years. Follow-up visit treated was as a categorical variable, and an additive model was used for genotype to give a test for trend across the 3 genotype groups. B coefficients were exponentiated to obtain an estimate of the percentage increase for each category. Differences in the effect of genotype over time were assessed by fitting an interaction term.

### Results

#### Levels of FVIIc, FVIIag, and FVIIa in Baseline Samples by EPCR Ser219Gly Variant

All 3 measurements showed a statistically significant increasing trend from lowest levels in Ser/Ser to higher levels in Ser/Gly with the highest levels in those who were Gly/Gly (P<0.04, Figure 1). Similarly, for all 3 measurements, levels were significantly higher in a combined group with at least 1 Gly allele compared to those with only Ser alleles (P<0.04, Figure 1). The significance of the associations with EPCR Ser219Gly after adjustment for confounders (center, age, smoking, creatinine, CRP) were: P<0.0001 for FVIIc; P<0.0001 for FVIIag; P=0.05 for FVIIa, confirming the significance of each association.

#### Markers of Activated Coagulation Downstream From FVIIa, in Baseline Samples, by EPCR Ser219Gly Variant

To determine whether an increase in circulating FVII and FVIIa could have consequences for downstream events in the extrinsic coagulation pathway, FIXpep, FXpep, and thrombin generation (F1+2) were analyzed. Both FXpep (P=0.04) and FIXpep (P=0.01) were elevated in those who were heterozygous and homozygous for EPCR 219Gly compared to non-carriers and showed a statistically significant trend (P<0.04, Figure 2). FXIIa however, a coagulation marker outside the extrinsic system, showed no significant difference in levels by variant group (P=0.73, Figure 2). The previously reported significantly higher F1+2 levels by EPCR genotype in these healthy middle-aged men\(^2\) are included here also for
completeness. F1+2 levels were significantly higher in the EPCR 219Gly carrier groups ($P=0.001$) and by trend ($P=0.0001$, Figure 2). These results show slightly different geometric mean levels to those in the previous publication because of removal, in the current study, of those individuals whose blood sampling was thought to be of inadequate quality. This did not, however, change the statistical significance of the results. The significance of the associations with EPCR variant site after adjustment for potential confounders, (center, age, smoking, creatinine, CRP) were: $P=0.12$ for...
Alhtough levels were considerably higher in those who were Ser/Gly or Gly/Gly compared to the other variant groups, there was no significant increase in either FVIIc or F1\textsubscript{2} levels from baseline to year 5 (Table). FVIIc and F1\textsubscript{2} levels increased significantly over time in those who were complete data for all time points, by EPCR Ser219Gly variant. Although the association with fragments intermediate in the tissue-factor pathway became statistically borderline, after adjustment, the marker of thrombin generation F1\textsubscript{2} remained highly statistically significant.

**Reproducibility of FVIIc and F1\textsubscript{2} Levels, Across 4 to 5 Years, by EPCR Ser219Gly Variant**

Levels of FVIIc and F1\textsubscript{2} across 5 years and 4 years, respectively, in those with complete data for all time points, were analyzed by EPCR Ser219Gly variant. FVIIc and prothrombin F1\textsubscript{2} levels increased significantly over time in those who were Ser/Ser (P<0.0001) and Ser/Gly (P<0.0005). Although levels were considerably higher in those who were Gly/Gly compared to the other variant groups, there was no significant increase in either FVIIc or F1\textsubscript{2} levels from baseline to 4 or 5 years (P>0.42). Results were remarkably consistent across the years by the EPCR variant site for both FVIIc and F1\textsubscript{2}, with levels by variant site being highly statistically significant at each year (P<0.0001 for FVIIc, Table 1A; P<0.04 for F1\textsubscript{2}, Table 1B). The effect of EPCR variant was consistent over time, as no significant interaction for genotype with time was identified (P=0.68 for FVIIc and P=0.85 for F1\textsubscript{2}). When the effect over all years was estimated by repeated measures ANOVA, with year of follow-up fitted as a categorical variable, levels of FVIIc and F1\textsubscript{2} were higher according to the number of Gly alleles carried (P<0.0001). After adjustment for the differences between years, levels of FVIIc in Ser/Gly were 6.9% (95% CI: 5.5 to 8.4) higher than in Ser/Ser and in Gly/Gly were 23.4% (95% CI: 16.3 to 30.8) higher (Table 1A). Similarly after adjustment F1\textsubscript{2} levels were 8.1% (95% CI: 5.2 to 11.1) higher in Ser/Gly than in Ser/Ser and 25.2% (95% CI: 11.8 to 40.3) higher in Gly/Gly (Table 1B).

**Molar Ratios of Coagulation Activation Markers, in Baseline Samples, by EPCR Ser219Gly Variant**

In line with the other coagulation activation markers, which were reported as molar concentrations, FVIIa was converted to a molar concentration using a molecular weight estimation of 51.6 kDaltons. Molar ratios for FXpep:FVIIa, FIXpep: FVIIa, and F1\textsubscript{2}:FVIIa were calculated to determine whether they differed by EPCR variant. Although levels of the 3 extrinsic pathway activation markers (FVIIa, FIXpep, and FXpep) and a marker of thrombin generation (F1\textsubscript{2}) were higher in carriers of the Gly allele, molar ratios for these 3 activation markers to FVIIa levels were constant in Ser/Ser and Ser/Gly (Table 3). However, although numbers were small, there was a tendency toward higher molar ratios in those who were Gly/Gly, which reached statistical significance for the ratio of FXpep:FVIIa (1.8-fold higher than in noncarriers, P=0.04), and >1.3-fold higher than in noncarriers for the other 2 ratios (P=0.20 for F1\textsubscript{2}:FVIIa; P=0.10 for FXpep:FVIIa).

**Levels of sEPCR Across Ser219Gly Genotype**

To confirm previous results that sEPCR levels differ by Ser219Gly genotype, baseline (or year 1) samples were assessed across the genotype groups. No significant difference could be found between the samples at baseline (n=84).
and year 1 (n = 15, \(P = 0.52\)), and accordingly, these were assessed as 1 group. Significantly higher levels were identified for those with the Gly allele, as expected (\(P < 0.0001\), both unadjusted and adjusted for those who later had a CHD event). Geometric mean levels in Ser/Ser were 82.6 (SD 34.6, \(n = 99\); year 1, 149.3 [102.4] \(n = 34\)).

### Figure 3. Box-plots show median levels and interquartile range for molar ratios of FXIIIa:FVIIa (A), FXI:FVIIa (B), F1+2:FVIIa (C) by EPCR Ser219Gly (S/G) variant in baseline samples. Below are shown geometric mean levels (± SD) and statistical analysis for the same data.

Significantly higher levels were identified also with each extrinsic pathway activation marker (FVIIa were 2-fold higher in Ser/Gly and 4-fold higher in Gly/Gly compared to noncarriers of Gly.2–6 The current analysis has confirmed the higher levels of sEPCR identified for the Gly allele. The large variability in levels for the “Gly” allele, which can be seen in the current study by the large SD for the Gly allele, has been suggested to be caused at least in part by alternative splicing of the mRNA for this allele, resulting in a longer protein that is expressed, but which does not associate with the membrane. A minor portion of plasma sEPCR has been identified as this alternatively spliced variant in carriers of the Gly allele.18 It has been suggested that the plasma level of the alternatively spliced variant may be related to inflammatory processes.18 The levels of sEPCR in the current study were measured in health and, as the alternatively spliced variant would be expected to be present in only minor amounts, would not be expected to account for the findings in the current study.

The current data would support the concept that reduced amounts of EPCR on the surface of endothelium, attributable to increased shedding, in EPCR Gly carriers, has a consequence for reduced binding of FVII and FVIIa, resulting in higher circulating levels of these coagulation pathway proteins. These findings extend those reported for binding of FVII and FVIIa to surface expressed EPCR by in vitro analysis.7,8,9 and suggest that binding of FVII and FVIIa may have physiological significance. Analysis of FVIIC levels
over 5 years in a large number of samples confirmed the effect of the EPCR variant site on levels and gives strong evidence for the reproducibility of the results.

The finding of higher levels of downstream activation markers for the extrinsic pathway (FIX:pep, FX:pep) but not for a coagulation marker outside of the extrinsic pathway (FXIIIa) suggests that the reduced binding of FVIIa in those with the Gly allele may have consequences for activation of these coagulant proteins, resulting in an increase in thrombin generation (F1+2). Again, as for FVIII levels over 5 years, the consistent effect of the variant site on F1+2 levels over 4 years gives strong evidence for the reproducibility of these results.

The question arises as to whether the increased activation through the extrinsic pathway is directly related to the lower FVII and FVIII binding to the endothelial cell surface or via another mechanism. In this regard, it may be expected that reduced amounts of EPCR on the surface of endothelium would impact on PC binding also, leading to reduced activated PC generation in Gly carriers. The consequence of this would be reduced inhibition of the thrombin feedback loop leading potentially to an increase in FIX:pep, FX:pep, and F1+2 levels. FVIIIa levels, however, would not be expected to increase in this case, as it has been suggested that thrombin is probably not a physiologically important activator of FVII in vivo. To disentangle the potential consequences of increased FVIIa level per se on downstream activation of the extrinsic coagulation system, compared to the consequences of reduced amounts of activated PC generation, a molar ratio of each downstream activated coagulation marker to FVIIa was calculated.

Although the increase in FVIIIa levels in Ser/Gly was statistically significant compared to Ser/Ser, the percentage increase was small. For this small increase in FVIIa level in heterozygotes, the molar ratio of each activation marker in the extrinsic coagulation system to FVIIa was constant, compared to each ratio in Ser/Ser, suggesting that the higher FVIIa level in carriers of one Gly allele leads to an equally high “knock-on” effect of downstream activation.

The tendency toward an increased molar ratio for FIX:pep:FVIIa (1.8-fold), FX:pep:FVIIIa (1.6-fold), and F1+2:FVIIIa (1.3-fold) in those homozygous for Gly, however, does suggest that further deregulation of activated coagulation downstream from FVIIIa is likely in those with the highest levels of EPCR shedding. Tissue factor pathway inhibitor (TFPI) is the most important regulator of the FVIIIa-tissue factor complex, but differences in levels of TFPI would not be expected to differ by EPCR variant and account for these results. The altered ratio may represent the added effect of a deregulated PC pathway, with reduced inhibition of the thrombin feedback loop, but requires further study. Collectively, the findings suggest 2 physiological roles for endothelial EPCR in the regulation of thrombin generation: binding and clearance of FVIIIa, in addition to regulation of the thrombin feedback loop.

In vitro analysis has suggested that FVIII and FVIIIa will bind soluble EPCR in addition to endothelial-bound EPCR and that soluble EPCR-bound FVIIIa has reduced ability to activate FX. The higher soluble EPCR levels associated with the Gly allele, therefore, may be expected to compensate for the reduced endothelial located EPCR, but it is not clear what the importance of this mechanism would be in vivo. This finding is in contrast to activated PC binding to soluble EPCR, which unlike membrane-bound EPCR has been suggested to inhibit the functional activity of activated PC, rather than promote it. In mice, it has been suggested that only supraphysiologic levels of soluble EPCR could influence PC activation and exaggerate the coagulant response. This is a complicated dynamic physiological process, therefore, and it is difficult to predict what would actually happen in vivo, from in vitro studies. The current study has not addressed the relative contribution of the endothelial versus circulating levels of soluble EPCR to bind FVIII and FVIIIa, nor the relative effects of the membrane versus circulating compartments of EPCR-FVIIIa complex on downstream hemostasis. However, the results have suggested that in health, when EPCR shedding is high (Gly/Gly), the balance of these processes leads to relatively higher downstream activation of extrinsic coagulation, determined by molar ratios of the downstream activation markers to FVIIIa, than was observed for the other 2 genotype groups. At an intermediate level of EPCR shedding (Ser/Gly), whereas increased FVIIIa levels paralleled an increased activation of extrinsic coagulation, the relative amounts of FVIIIa and downstream activation markers were constant (determined by molar ratios), compared to those who have the lowest level of EPCR shedding.

These findings, in Ser/Ser compared to Gly/Gly, help the understanding of the 3-fold elevation in risk for heart disease for Gly/Gly identified previously in NPHSII and extend the findings for the association of a higher FIX:pep:FVIIIa in those with heart disease without reference to genotype in NPHSII also. The small increase in extrinsic coagulation markers measured in health in heterozygotes for Gly in the current study does not detrimentally affect clinical outcome for heart disease. In these heterozygotes, the molar ratio of FIX:pep:FVIIIa did not increase above that seen for Ser/Ser and further suggests the usefulness of this molar ratio in prediction of risk for heart disease.

As stated previously, analysis of NPHSII has shown a higher molar ratio for FIX:pep:FVIIIa in baseline samples in those who went on to have a heart disease event. This ratio was attributable to both higher FIX:pep levels and lower FVIIIa levels in those who later developed a heart disease event. The potential explanation for these results was through increased amounts of intravascular tissue factor, with the lower FVIIIa levels suggested to arise because of binding of FVIIIa to tissue factor and loss of circulating measurable FVIIIa. In contrast, although the current analysis would suggest that homozygotes for EPCR 219Gly are hemostatically compromised by an increased FIX:pep:FVIIIa molar ratio and were previously associated with heart disease in NPHSII, we have shown FVIIIa levels to be higher in Gly/Gly. So these results indicate 2 situations associated with risk for development of heart disease, in healthy individuals: High FIX:pep, low FVIIIa, high FIX:pep:FVIIIa ratio across all individuals in NPHSII; and high FIX:pep, high FVIIIa, high FIX:pep:FVIIIa ratio in EPCR 219Gly homozygotes.
In summary, we have extended previous in vitro analysis which showed that FVII and FVIIa bind endothelial and sEPCR, by providing evidence that a functional variant site in EPCR has consequences both for plasma levels of FVIIa and for downstream activation of the extrinsic coagulation pathway. The stability of these results across 4 to 5 years, shown for FVIIc and F1+2 levels in this very large study, provides strong evidence for the variant effect. These results have also extended a previous study of NPHSII, suggesting that an increased ratio of FIXp:FVIIa is associated with future development of heart disease. The consequences of the findings for the EPCR variant site for heart disease and other conditions associated with increased inflammatory or thrombotic processes, require further study.

Limitations of the Study

The ability to measure activated PC in this study would have been useful but is not possible as samples require collection into a specific anticoagulant cocktail.

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