Acquired APC resistance and oral contraceptives: differences between two functional tests

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Received 13 August 1998; accepted for publication 6 January 1999

Summary. Resistance to activated protein C (APC) is often associated with a mutation in factor V (factor V Leiden). Individuals without factor V Leiden who exhibit a response in functional APC-resistance tests similar to that of carriers of factor V Leiden are considered to be acquired APC resistant. This phenomenon is particularly observed in women using oral contraceptives (OC).

In the present study we compared the response to APC in plasma from normal individuals, carriers of factor V Leiden and women who use OC using functional tests that either quantify the effect of APC on the endogenous thrombin potential (ETP) or on the activated partial thromboplastin time (aPTT).

Both tests discriminated equally well between individuals with and without factor V Leiden who were not using OC. In contrast to the aPTT-based test, the ETP-based assay yielded significant differences in sensitivity to APC between non-OC users and OC users and between users of second and third generation OC. Since there was no correlation between APC-sensitivity determined with both assays in non-carriers of factor V Leiden and in women who use OC and a poor correlation in carriers of factor V Leiden, we propose that other plasma components differentially modulate the response to APC in the aPTT- and ETP-based APC-resistance tests and that OC change the level of plasma protein(s) that modulate the effect of APC on thrombin formation initiated via the extrinsic coagulation pathway.

Keywords: APC resistance, venous thrombosis, factor V Leiden, oral contraceptives.

Prolongation of the activated partial thromboplastin time (aPTT) by activated protein C (APC) was reported to be considerably less in a large group of patients with venous thrombosis than in a control group of healthy individuals (Dahlbäck et al., 1993). This defect, called APC resistance, appears to be a common hereditary risk factor for venous thrombosis (Griffin et al., 1993; Koster et al., 1993; Rosendaal et al., 1995; Svensson & Dahlbäck, 1994), which in the majority of the cases is associated with a single point mutation in factor V (Bertina et al., 1994; Greengard et al., 1994; Voorberg et al., 1994; Zöller & Dahlbäck, 1994). This mutation, often referred to as factor V Leiden, causes the replacement of an amino acid (Arg506 → Gln) at a predominant APC cleavage site which renders the activated form of factor V, factor Va, less susceptible to proteolysis by APC (Aparicio & Dahlbäck, 1996; Heeb et al., 1995; Kalafatis et al., 1995; Nicolaes et al., 1995).

On the basis of the original observations of Dahlbäck et al. (1993) several functional tests were developed for the diagnosis of APC resistance. However, screening methods that are based on measurement of the effect of APC on the aPTT of undiluted plasma do not fully discriminate between carriers and non-carriers of the factor V Leiden mutation (Engel et al., 1996; Rosen & Sturk, 1997; Zöller et al., 1994). In particular, women who are using oral contraceptives (Henkens et al., 1995; Olivieri et al., 1995; Rosing et al., 1997) or who are pregnant (Meinardi et al., 1997) appear to be less sensitive to the anticoagulant action of APC and hence are considered to have acquired APC resistance.

Recently, we developed an APC resistance test (Nicolaes et al., 1997) that is based on measurement of the effect of APC on the endogenous thrombin potential (ETP), i.e. the time integral of thrombin generated in plasma in which coagulation is initiated via the extrinsic pathway (Hemker & Beguin, 1995). This so-called ETP-based assay not only enabled...
Acquired APC Resistance and Oral Contraceptives

...detection of an abnormal anticoagulant response of plasma to APC (e.g. the presence of factor V\textsubscript{Leden}), but it also appeared that the plasma of women using oral contraceptives (OC) exhibited considerable resistance to APC when analysed by this procedure (Rosing et al., 1997).

The observation that women who were using so-called third-generation oral contraceptives were more resistant to APC than women using second-generation oral contraceptives attracted much attention (Alexandre & Brandberg, 1997; Lidegaard & Milsom, 1996; Vandenbroucke et al., 1997) in the discussion about the different risks for venous thrombosis between second and third generation OC users (Jick et al., 1995; W.H.O., 1995a. However, the impact of our observations for ‘pill thrombosis’ was also questioned (Balsach, 1997; Schramm & Heinemann, 1997; Spitzer, 1997; Winkler, 1998). Amongst other things it was argued that the APC resistance test developed in our laboratory has not yet been clinically validated (Balsach, 1997; Spitzer, 1997; Winkler, 1998) and that the different effects of OC in our APC resistance test might have been affected by the selection of patients or may be due to an ex-vivo effect (Schramm & Heinemann, 1997).

In order to gain more insight in possible differences between the aPTT- and ETP-based APC resistance tests we present in this article a comparison of APC sensitivity ratios of a large plasma collection of normal individuals, women who use OC and carriers of factor V\textsubscript{Leden} determined with both tests.

MATERIALS AND METHODS

Materials. The aPTT-based APC-resistance test (Coatest\textsuperscript{W}, APC\textsuperscript{TM}) and \textsuperscript{125}I-pro-Glu-Pro-Arg-pNA (S2238) and \textsuperscript{125}I-pyroGlu-Pro-Arg-pNA (S2366) were supplied by Chromogenix, Mölndal, Sweden. Ancrod was purchased from the W.H.O. International Laboratory for Biological Standards, Hertfordshire, U.K., and Arwin\textsuperscript{W} was from Knoll, Germany. Relipidated recombinant tissue factor was obtained from Dade, U.S.A. The APC used in the ETP-based APC resistance test was purified human APC (a kind gift of Immuno A.G., Vienna, Austria) or human APC (Enzyme Research Laboratories) purchased from Kordia Laboratory Supplies, Leiden, The Netherlands. APC was quantitated as described by Sala et al. (1984). Immuno-depleted factor V-deficient plasma was obtained from Organon Technica. Phospholipids were from Avanti Polar Lipids, Alabaster, Alabama, U.S.A. Small unilamellar phospholipid vesicles, composed of a mixture of 1,2-dioleoyl-sn-glycero-3-phosphoserine (DOPS), 1,2-dioleoyl-sn-glycero-3-phosphatidylethanolamine (DOPE) and 1,2-dioleoyl-sn-glycero-3-phosphatidylycholine (DOPC) (20/20/60, M/M/M), were prepared by mixing appropriate quantities of phospholipids dissolved in CHCl\textsubscript{3}/CH\textsubscript{3}OH (9/1, v/v) in a glass tube. After drying under a mild flow of N\textsubscript{2}, the phospholipids were suspended in 2 ml buffer (25 mM Hepes, 175 mM NaCl, pH 7.5) and vigorously vortexed for 1 min. The phospholipid suspension was subsequently sonicated for 10 min at 0°C using a MSE Soniprep 150 ultrasonic disintegrator set at 7-5 \mu m peak to peak amplitude. Phospholipid concentrations were determined by phosphate analysis (Böttcher et al., 1961).

Collection and handling of plasma samples. This study was performed according to a protocol approved by our institutional ethics committee.

Nine pairs of blood from consenting volunteers were collected in one part of 0·13 M trisodium citrate (pH 7·8). Platelet-poor plasma was obtained by centrifugation for 25 min at 3000 \textit{g} at room temperature followed by centrifugation for 25 min at 20,000 \textit{g} at 4°C and was snapfrozen in liquid nitrogen in small aliquots and stored at -80°C until analysis. Normal pooled plasma from healthy volunteers (40 females not using OC, 50 males, mean age 35 years) was the same as used before (Rosing et al., 1997).

Some of the plasma samples used in this study were also used in an earlier paper in which we reported the effect of OC use on the ETP-based normalized APC sensitivity ratio (nAPC-sr) (Rosing et al., 1997). Plasmas no longer available for determination of the aPTT-based nAPC-sr were excluded from the current analysis. Plasmas of women using triphasic OC, treated as a separate group earlier (Rosing et al., 1997), were reclassified as second (n = 23) or third-generation OC (n = 6) depending on the kind of progestagen. When sufficient plasma was available, the ETP-based nAPC-sr was redetermined and the average of the original and newly determined value was used in this analysis. When insufficient plasma was available to redetermine the ETP-based nAPC-sr, the original value (Rosing et al., 1997) was used. Compared to the earlier study the plasma collection was extended with 101 new samples from individuals recruited within our own institute.

The final plasma collection contained samples of: male volunteers (n = 46, 18–48 years, mean age 30 years, n = 21), women not using OC (n = 62, 17–41 years, mean age 31 years, new n = 15), women using second-generation OC containing levonorgestrel or lynestrel (n = 62, 20–41 years, mean age 29 years, duration OC use 0·3–20 years, average duration OC use 9 years, n = 14), women using third-generation contraceptives containing desogestrel or gestodene (n = 64, 18–46 years, mean age 28 years, duration OC use 0·3–20 years, average duration OC use 6 years, new n = 24), men heterozygous for the factor V\textsubscript{Leden} mutation (n = 21, 14–78 years, mean age 40 years, new n = 13) of which four with previous venous thrombotic embolism (VTE), 14 were asymptomatic relatives of a propositus with VTE and three were obtained by random sampling, women heterozygous for the factor V\textsubscript{Leden} mutation not using OC (n = 26, 17–80 years, mean age 42 years, new n = 12) of which nine had a history of previous VTE, 13 asymptomatic relatives and three obtained by random sampling. Women heterozygous for the factor V\textsubscript{Leden} mutation using second or third generation OC (n = 7, 20–35 years, mean age 29 years, duration OC use 3–15 years, average duration OC use 10 years, new n = 2) of which none with previous VTE, two asymptomatic relatives and five obtained by random sampling. Compared to our previous study (Rosing et al., 1997), the group specifics, i.e. range and average of age and duration of OC, had not changed significantly.

Women who had ceased OC therapy for >6 months were considered non-users. The OC user group consisted of...
women who were using the same OC for at least 3 months. We further excluded pregnant women and individuals with known hereditary risk factors for venous thrombosis other than factor V Leiden, with a previous episode of venous thrombosis, a chronic or intercurrent acute disease or with medication known to interfere with blood coagulation.

Diagnosis of the presence of the factor V Leiden mutation. The occurrence of heterozygosity for factor V Leiden was established by determination of the sensitivity of plasma factor Va for APC (Nicolaes et al, 1996) or by DNA analysis (Beauchamp et al, 1994).

Determination of nAPCsr with the ETP-based and the aPTT-based APC-resistance tests. Normalized APC sensitivity ratios (nAPCsr) were determined with the ETP-based APC-resistance test as described before (Rosing et al, 1997). The normalized APC sensitivity ratio (nAPCsr) was defined as the ratio of a2M-IIa determined in the presence and absence of APC divided by the ratio determined in the normal plasma pool (cf. de Ronde & Bertina, 1994): nAPCsr = (a2M-IIa/ APC / a2M-IIa/normal plasma) / (a2M-IIa/ APC / a2M-IIa/normal plasma).

The aPTT-based APC resistance test was performed in undiluted plasma as described by the supplier (Chromogenix, Mölndal, Sweden). Clotting times were determined on an ACL 300R coagulation analyser (Instrumentation Laboratory, Milan, Italy). The normalized APC sensitivity ratio (nAPCsr) was defined as the ratio of the clotting times (aPTT) determined in the presence and absence of APC normalized by division through the same ratio determined in the normal plasma pool (de Ronde & Bertina, 1994): nAPCsr = (aPTT / aPTT/ normal plasma) / (aPTT / aPTT/ normal plasma).

Statistics. Statistical analysis was performed after logarithmic transformation of the data which resulted in normally distributed nAPCsr values. 95% confidence intervals of the mean are given in Tables I and II. P values shown in the figures were obtained by comparison of the groups using Student's t test. The association between both tests (Pearson's correlation and P values) were obtained by regression analysis using SPSS.

RESULTS

Comparison of APC sensitivity ratios of normal individuals and carriers of factor V Leiden in the aPTT- and ETP-based APC-resistance tests

Normalized APC sensitivity ratios (nAPCsr) of healthy individuals (men and women not using oral contraceptives) and heterozygous carriers of the factor V Leiden mutation were evaluated in the aPTT- and ETP-based APC-resistance test in undiluted plasma (Fig 1). Due to the calculation procedure, APC-resistant plasmas will give a decrease of the nAPCsr in the aPTT-based (A) and an increase in the ETP-based assay (B). At cut-off ratios of 0.76 for the aPTT-based APC-resistance test and 2.08 for the ETP-based APC-resistance test (95% specificity) the sensitivity of the tests were 83% and 89%, respectively.

Table I summarizes the data together with the results for men and women, separately. In both tests women appeared slightly more resistant to APC than men, confirming earlier reports (De Stefano et al, 1996; Henkens et al, 1995; Koster et al, 1993; Rosing et al, 1997), but only in the ETP-based assay did the gender difference reach statistical significance (P < 0.05, Table I).

Effect of OC use on nAPCsr determined in aPTT- and ETP-based APC-resistance tests

Fig 2 and Table II summarize the effects of OC use on nAPCsr obtained in both tests for plasmas of normal healthy women who do not carry the factor V Leiden mutation and of women heterozygous for the factor V Leiden mutation. Increased resistance to APC in OC users was observed in both tests, but the differences between non-users and OC users in the aPTT-based test appeared rather small. The effects of OC use on nAPCsr determined

Fig 1. Comparison of nAPCsr of carriers and non-carriers of factor V Leiden determined with the aPTT- and ETP-based APC-resistance tests. APC sensitivity ratios were determined in the same plasma collection of 109 non-carriers (46 men and 62 women) and 47 carriers of the factor V Leiden mutation (21 men and 26 women) with the aPTT- and ETP-based APC-resistance tests as described under Methods. (A) aPTT-based APC-resistance test; (B) ETP-based APC-resistance test.
Acquired APC Resistance and Oral Contraceptives

Table I. nAPCsr of men and women determined in aPTT- and ETP-based APC-resistance tests.

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<th>aPTT-based test</th>
<th>ETP-based test</th>
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<tr>
<td></td>
<td>n</td>
<td>Median</td>
</tr>
<tr>
<td>Men and women</td>
<td>108</td>
<td>0·94</td>
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<tr>
<td>Men and women FV Leiden</td>
<td>47</td>
<td>0·67</td>
</tr>
<tr>
<td>Men</td>
<td>46</td>
<td>0·95</td>
</tr>
<tr>
<td>Men FV Leiden</td>
<td>21</td>
<td>0·71</td>
</tr>
<tr>
<td>Women</td>
<td>62</td>
<td>0·92</td>
</tr>
<tr>
<td>Women FV Leiden</td>
<td>26</td>
<td>0·64</td>
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Table II. Effects of OC use on nAPCsr determined in aPTT- and ETP-based APC-resistance tests.

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<th>aPTT-based test</th>
<th>ETP-based test</th>
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<tr>
<td></td>
<td>n</td>
<td>Median</td>
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<tr>
<td>Women</td>
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<tr>
<td>No OC</td>
<td>62</td>
<td>0·92</td>
</tr>
<tr>
<td>Second-generation OC</td>
<td>62</td>
<td>0·86</td>
</tr>
<tr>
<td>Third-generation OC</td>
<td>64</td>
<td>0·86</td>
</tr>
<tr>
<td>FV Leiden, no OC</td>
<td>26</td>
<td>0·64</td>
</tr>
<tr>
<td>FV Leiden using OC</td>
<td>7</td>
<td>0·56</td>
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in the ETP-based were much more pronounced and significant differences were observed between non-users and pill users independent of the kind of OC used (P < 0·0001) and between users of second and third generation OC (P < 0·0001). Women who used third generation OC had nAPCsr in the range of heterozygous female carriers of factor V Leiden who were not using OC (Fig 2B). The effect of OC and the factor V Leiden mutation on the nAPCsr appear to amplify each other, since female heterozygous carriers of factor V Leiden who used OC were considerably more resistant to APC than carriers of the mutation who did not use OC (P < 0·0001), a phenomenon that was not observed with the aPTT-based APC-resistance test (Fig 2A).
Correlation between nAPCsr determined in the aPTT- and ETP-based APC-resistance tests

Fig 3 shows a correlation plot of nAPCsr determined with the aPTT-based and ETP-based APC-resistance tests. There appears to be a rather poor correlation between the nAPCsr determined with the two tests in the case of non-carriers of factor V Leiden \( (r = -0.37, \text{Fig 3A}) \) and in women using oral contraceptives \( (r = -0.12, \text{Fig 3B}) \). The correlations were also low (data not shown) within the populations of men \( (r = -0.36) \), women not using OC \( (r = -0.34) \), women using second-generation OC \( (r = -0.13) \) and women using third-generation OC \( (r = 0.06) \).

A slightly higher correlation \( (r = -0.46) \) was observed for heterozygous carriers of factor V Leiden. This means that, despite the fact that both assays have approximately equal sensitivity and specificity for the diagnosis of factor V Leiden, the correlation between the nAPCsr obtained in both tests is rather low. These data, taken together with the fact that OC use shows differential effects in both tests, indicates that the nAPCsr is subject to differences in modulation by other plasma components in the two APC-resistance tests. This is illustrated by the experiment presented in Fig 4 which shows the effect of the factor V Leiden mutation per se on values of the nAPCsr of plasma in which the levels of other coagulation factors are the same. In this experiment factor V-deficient plasma was reconstituted with varying amounts of purified normal factor V and factor V Leiden at a constant level of 20 nM total factor V. nAPCsr were determined with aPTT- and ETP-based APC-resistance tests as described under Methods. In the ETP-based APC-resistance test, 50 nM APC was required to reduce thrombin formation to 10% in factor V-deficient plasma reconstituted with 20 nM normal factor V. The nAPCsr of plasmas with varying amounts of factor V and factor V Leiden was normalized against plasma with 100% normal factor V. The correlation coefficient and \( P \) value are given in the figure.

**DISCUSSION**

An impaired response to APC in functional clotting tests is associated with an increased risk for venous thrombosis (Griffin et al., 1993; Koster et al., 1993; Rosendaal et al., 1995; Svensson & Dahlback, 1994). The defect, called APC resistance, is often caused by a mutation (Bertina et al., 1994; Greengard et al., 1994; Voorberg et al., 1994; Zoller & Dahlback, 1994) in factor V (factor V Leiden) at a predominant APC cleavage site \( \text{Arg}^{506} \rightarrow \text{Gln} \). APC resistance associated with factor V Leiden is a hereditary defect that increases the risk for venous thrombosis some 7-fold in the case of heterozygosity (Koster et al., 1993; Rosendaal et al., 1995) and 80-fold in the case of homozygosity (Rosendaal et al., 1995).
In contrast to assays performed in factor V-deficient plasma (Sun et al., 1994), commercially available aPTT-based APC-resistance tests on undiluted plasma (Engel et al., 1996; Rosen & Sturk, 1997; Zoller et al., 1994) are generally not 100% specific and 100% sensitive for the detection of factor V<sub>Leiden</sub>. Depending on the test used, there appears to be a variable number of individuals who do not carry the mutation but who show a response to APC that is comparable to that observed for heterozygous carriers of factor V<sub>Leiden</sub>. When no other reason for this impaired response to APC is known, these individuals are considered to be acquired APC resistant.

Acquired APC resistance particularly occurs in plasma of women who use oral contraceptives (Henkens et al., 1995; Olivieri et al., 1995; Rosing et al., 1997) or who are pregnant (Meinardi et al., 1997) and is particularly observed in a functional test in which the ability of APC to down-regulate coagulation is quantitated by measuring its effect on thrombin generation initiated in plasma via the extrinsic pathway (Rosing et al., 1997).

Since the effects of oral contraceptives on APC sensitivity ratios determined with other APC-resistance tests appeared to be marginal (Henkens et al., 1995; Olivieri et al., 1995; Schramm & Heinemann, 1997), it was argued (Schramm & Heinemann, 1997) that our earlier observations (Rosing et al., 1997) in the ETP-based assay may have been caused by the selection of OC users or by an ex vivo effect on the plasma collection. The data presented here, however, show that our plasma collection yields results that are in agreement with earlier publications in which rather small effects of OC use on the aPTT-based APCsr were reported (Henkens et al., 1995; Olivieri et al., 1995; Schramm & Heinemann, 1997).

Therefore we propose that the different observations in the two APC-resistance tests are due to the fact that levels of other plasma proteins differentially modulate the response to APC in the ETP-based test (effect of APC on thrombin generation initiated via the extrinsic pathway) and in the aPTT-based test (effect of APC on clotting times after initiation of the intrinsic pathway).

Differences in modulation of the nAPCsr determined in the two tests by other plasma proteins becomes evident after correlation of nAPCsr determined with the aPTT- and ETP-based test (effect of APC on clotting times after initiation of the intrinsic pathway).


determined via the extrinsic coagulation pathway. At present we have no information about possible proteins that might be responsible for such a phenomenon.

Another major question is the clinical significance of acquired APC resistance. There are as yet no reports that directly associate acquired APC resistance with an increased risk for venous thrombosis. To investigate this possibility we are presently comparing nAPCsr of populations of patients with a previous unexplained venous thrombosis and proper controls.

ACKNOWLEDGMENT

This study was supported by Program Grant 900-526-192 from the Dutch Organization for Scientific Research (NWO).

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