Oral contraceptives and venous thrombosis: different sensitivities to activated protein C in women using second- and third-generation oral contraceptives


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Summary. Epidemiological studies have shown that women who use third-generation oral contraceptives (OC) containing desogestrel, gestodene or norgestimate have a higher risk of venous thrombosis than women who use second-generation OC containing levonorgestrel. It is also known that a mutation in factor V (factor VLeiden), which results in resistance to activated protein C (APC) and which is the most common cause of hereditary thrombophilia, potentiates the prothrombotic effect of OC.

Effects of APC on thrombin generation in the plasma of women using OC were compared to the response to APC in non-OC users and in individuals that were heterozygous or homozygous for factor VLeiden. The response towards APC was evaluated on basis of the ratio (APC-sr) of the time integrals of thrombin formation determined in the presence and absence of APC.

Compared with women not using OC, women who used OC exhibited a significantly decreased sensitivity to APC (P<0.001), independent of the kind of OC used. Women who used third-generation monophasic OC were significantly less sensitive to APC than women using second-generation OC (P<0.001) and had APC-sr that did not significantly differ from heterozygous female carriers of factor VLeiden who did not use OC. Women who were heterozygous for factor VLeiden and used OC had APC-sr in the range of homozygous carriers of factor VLeiden. Two women who started OC therapy had significantly elevated APC-sr within 3d.

Acquired APC resistance may explain the epidemiological observation of increased risk for venous thrombosis in OC users, especially in women using third-generation OC.

Keywords: venous thrombosis, oral contraceptives, APC resistance.
Although the higher risk of idiopathic venous thromboembolic events, deep-vein thrombosis and/or pulmonary embolism in association with the use of OC is well established, no clear causal relations with alterations within the haemostatic system have been reported (Speroff & DeCherney, 1993; Newton, 1995). In general, the effects of OC therapy on coagulation variables are modest, and in several reports it has been suggested that the use of OC induces changes in the procoagulant and anticoagulant pathways that may counterbalance each other (Speroff & DeCherney, 1993; Newton, 1995).

In this paper we report the change of a haemostatic variable that may be indicative for a substantial disturbance of the haemostatic balance and for the existence of a prothrombotic state in OC users. We measured the effect of APC on thrombin generation in plasma and have shown that women who use OC are much less sensitive to APC than non-users, and that differences in sensitivity to APC between women who use second- and third-generation OC correlate with the reported higher risk of thrombosis in third-generation OC users.

MATERIALS AND METHODS

This study was performed according to a protocol approved by our institutional ethics committee. The participants in this study were selected from general practitioners’ offices using criteria described below.

Study cohorts. The following plasmas were used in our study: a normal plasma pool prepared from plasma from healthy volunteers (40 females not using OC and 50 males, mean age 35 years), individual plasma samples from female volunteers (n = 25; range 18–38 years old, mean age 28 years), women not using OC (n = 53; 19–39 years, mean age 31 years), women using triphasic OC (n = 24; 19–37 years, mean age 28 years; duration of OC use 0.5–17 years; average duration of OC use 8 years), women using second-generation monophasic contraceptives containing levonorgestrel or lynestrenol (n = 32, 20–39 years, mean age 29 years; duration of OC use 0.5–20 years; average duration of OC use 9 years), women using third-generation monophasic contraceptives containing desogestrel, gestodene or norgestimate (n = 40; 18–36 years, mean age 29 years; duration of OC use 1–20 years; average duration of OC use 7 years) and women heterozygous for the factor V Leiden mutation not using OC (n = 17; 16–79 years, mean age 41 years).

Women who had ceased therapy on OC for more than 6 months were considered as non-users. The OC user group consisted of women who had been using the same OC for >3 months. With the exception of carriers of the factor V Leiden mutation, individuals with a previous episode or a known familial history of venous or arterial thrombosis (~10% of our population), having a chronic or intercurrent acute disease, taking medication that may interfere with coagulation, and pregnant women, were excluded from our study.

Collection and handling of plasma samples. Nine parts 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 g at room temperature, followed by centrifugation for 25 min at 20000 g at 4°C and stored in small aliquots at −80°C until analysis.

The occurrence of heterozygous and homozygous APC resistance was established by determination of the sensitivity of plasma factor Va for APC (Nicolae et al., 1996) and by DNA analysis (Beauchamp et al., 1994).

Determination of thrombin generation in plasma. Thrombin generation curves were determined at 37°C in defibrinated plasma (Hemker & Beguin, 1995) containing 15 μM phospholipid vesicles (Nicolae et al., 1996, 1995) (dioloyl-phosphatidylserine/diisoyl phosphatidylethanolamine/diisoylphosphatidylcholine, 20/20/60, M/M/M; Avanti Polar Lipids, U.S.A.), 0.1 ng/ml relipidated tissue factor (Recombinant S. Dade, U.S.A.), and 15 μM added CaCl2, with or without 5 nm APC (Immuno AG, Vienna, Austria). At regular intervals, samples were withdrawn to determine the generation of amidolytic activity (Hemker & Beguin, 1995) (thrombin plus α2-macroglobulin–thrombin complex [α2M-Ila] with t-Phe-pipocyl-Arg-pNA (S2238, Chromogenix, Sweden). Correction of the time course of generation of amidolytic activity for the contribution of α2M-Ila yielded the free thrombin concentration as a function of time (Hemker et al., 1986). These values were used to calculate the endogenous thrombin potential (ETP), which is defined as the time-integral of free thrombin concentration in a thrombin generation test (Hemker et al., 1986; Hemker & Beguin, 1995).

Since ETP values of a particular plasma sample are directly proportional to the residual levels of amidolytic activity (α2M-Ila) (Duchemin et al., 1994), we quantitated the effect of APC on thrombin generation (i.e. on the ETP) by measuring its effect on the final level of α2M-Ila. The APC sensitivity ratio (APC-sr) was defined as the ratio of α2M-Ila determined in the presence and absence of APC divided by the ratio determined in the normal plasma pool (cf. de Ronde & Bertina, 1994): APC-sr = (α2M-Ila APC/α2M-Ila APC plasma sample)/(α2M-Ila APC/α2M-Ila APC normal plasma). Statistical. Statistical analysis was performed after logarithmic transformation of the data which resulted in normally distributed APC-sr values. P values were obtained by pairwise comparison of the groups using Student’s t-test and were corrected for comparison of multiple groups according to the Bonferroni procedure (Altman, 1991).

RESULTS

Effect of APC on thrombin generation. Fig 1 shows thrombin generation curves (closed symbols) obtained after initiating thrombin formation via the extrinsic pathway in plasma pools of women not using OC, women using third-generation OC, and heterozygous APC-resistant men and women who did not use OC. APC strongly inhibited thrombin formation in the plasma pool of women not taking OC (open symbols) and the residual ETP was 9.5% of that determined in the absence of APC. Thrombin generation in the plasma pool of women taking third-generation OC was much less sensitive to APC (residual ETP = 28.0%) and exhibited a response towards APC that is similar to that
observed in a pool of heterozygous APC-resistant individuals (residual ETP 29–0%). Thrombin formation in the plasma pools of women using triphasic OC or second-generation OC was also considerably less affected by APC than in women who did not use OC (Table I).

**Effects of OC therapy and factor V<sub>Leiden</sub> on APC-sensitivity ratios**

Since determination of the APC-sr in a large number of plasmas is precluded by the fact that measurement of complete thrombin generation curves is very time consuming, we routinely determined the APC-sr from the residual levels of the α2M-IIa complex obtained in the presence and absence of APC. This procedure yields APC-sr that are identical to values calculated from the ratio ETP<sub>¹</sub>/ETP<sub>₁</sub> (Duchemin <i>et al</i>, 1994). It should be emphasized that APC-sr calculated by this method exhibit trends that are the reverse of APC-sr determined with APTT-based clotting assays (de Ronde & Bertina, 1994; Koster <i>et al</i>, 1993; Dahlback <i>et al</i>, 1993) (APC-sr = APTT<sub>₁</sub>/APTT<sub>₁</sub>-APC). Since APC inhibits thrombin generation and prolongs the APTT, decreased sensitivity for APC (APC resistance) yields higher APC-sr in ETP-based assays and lower APC-sr in APTT-based assays.

**Table I. Effect of APC on thrombin generation in pooled plasmas.**

<table>
<thead>
<tr>
<th>Pooled plasmas&lt;sup&gt;*&lt;/sup&gt;</th>
<th>n</th>
<th>Mean age (years)</th>
<th>ETP&lt;sub&gt;₁&lt;/sub&gt;-APC&lt;sup&gt;†&lt;/sup&gt; (nM min)</th>
<th>ETP&lt;sub&gt;₁&lt;/sub&gt;-APC&lt;sup&gt;‡&lt;/sup&gt; (nM min)</th>
<th>ETP&lt;sub&gt;₁&lt;/sub&gt;-APC&lt;sup&gt;‡&lt;/sup&gt; (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal plasma</td>
<td>90</td>
<td>35</td>
<td>458 ± 6</td>
<td>43 ± 4</td>
<td>9-4</td>
</tr>
<tr>
<td>Men</td>
<td>23</td>
<td>28</td>
<td>442 ± 5</td>
<td>39 ± 1</td>
<td>8-8</td>
</tr>
<tr>
<td>Women</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No contraceptive</td>
<td>27</td>
<td>29</td>
<td>473 ± 29</td>
<td>45 ± 2</td>
<td>9-5</td>
</tr>
<tr>
<td>Triphasic Monophasic</td>
<td>28</td>
<td>28</td>
<td>533 ± 10</td>
<td>105 ± 3</td>
<td>19-7</td>
</tr>
<tr>
<td>Second generation Monophasic</td>
<td>28</td>
<td>29</td>
<td>483 ± 5</td>
<td>85 ± 1</td>
<td>17-6</td>
</tr>
<tr>
<td>Third generation Monophasic</td>
<td>25</td>
<td>29</td>
<td>554 ± 4</td>
<td>155 ± 13</td>
<td>28-0</td>
</tr>
<tr>
<td>Heterozygous factor V&lt;sub&gt;Leiden&lt;/sub&gt; §</td>
<td>23</td>
<td>45</td>
<td>486 ± 5</td>
<td>141 ± 9</td>
<td>29-0</td>
</tr>
</tbody>
</table>

<sup>*</sup> Plasma pools were prepared from individual plasma samples described under Methods. <sup>†</sup> Thrombin formation was quantified by calculating the time integral of the thrombin generation curve (ETP) as described under Methods. Values are means ±SE. <sup>‡</sup> Per cent of value determined without APC. § Men (n = 8) plus women (n = 15).

**Table II. Effect of OC therapy and factor V<sub>Leiden</sub> on APC-sr.**

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Mean age (years)</th>
<th>APC-sr (median)</th>
<th>5th–95th percentile</th>
</tr>
</thead>
<tbody>
<tr>
<td>Men</td>
<td>25</td>
<td>28</td>
<td>0.96</td>
<td>0.65–1.28</td>
</tr>
<tr>
<td>Women</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No OC</td>
<td>53</td>
<td>31</td>
<td>1.22</td>
<td>0.87–1.15</td>
</tr>
<tr>
<td>No OC, heterozygous FV&lt;sub&gt;Leiden&lt;/sub&gt;</td>
<td>17</td>
<td>41</td>
<td>3.10</td>
<td>1.90–6.60</td>
</tr>
<tr>
<td>No OC, homozygous FV&lt;sub&gt;Leiden&lt;/sub&gt;</td>
<td>5</td>
<td>41</td>
<td>4.75</td>
<td>3.91–6.02</td>
</tr>
<tr>
<td>Triphasic OC</td>
<td>24</td>
<td>28</td>
<td>1.93</td>
<td>1.26–3.29</td>
</tr>
<tr>
<td>Second-generation monophasic</td>
<td>32</td>
<td>29</td>
<td>1.81</td>
<td>1.19–2.87</td>
</tr>
<tr>
<td>Third-generation monophasic</td>
<td>40</td>
<td>29</td>
<td>2.59</td>
<td>1.91–3.88</td>
</tr>
<tr>
<td>Heterozygous FV&lt;sub&gt;Leiden&lt;/sub&gt; using OC</td>
<td>5</td>
<td>31</td>
<td>5.41</td>
<td>4.53–6.45</td>
</tr>
</tbody>
</table>

The APC-sr of individual plasma samples of men, women not using OC, women on OC therapy, and heterozygous carriers of the factor V Leiden mutation (women not using OC) determined as described above are summarized in Fig 2 and Table II. It appeared that men had significantly lower APC-sr than women who were not using OC, confirming that men are more sensitive to APC than women (Henkens et al., 1995; Koster et al., 1993). Plasma from women using different kinds of OC had APC-sr that were considerably higher than those of non-OC users. Independent of the type of OC used, significant differences were observed between the APC-sr of OC users and female non-users (Fig 2). The APC-sr of plasma from women who take third-generation monophasic OC were almost equal to the APC-sr of heterozygous APC-resistant women not using OC and was significantly higher than the APC-sr of women who used triphasic (P < 0.005) or second-generation monophasic OC (P < 0.001).

For heterozygous APC-resistant women not using OC, the APC-sr defined by the 5th and 95th percentile fell in the range 2.09–6.60. No men and 3/53 (6%) of the women not using OC had an APC-sr within this range. Among women using OC, 12/32 (37%) taking second-generation monophasic OC, 8/24 (33%) using triphasic OC, and 32/40 (80%) on third-generation monophasic OC therapy had APC-sr values within the 5th and 95th percentile of the heterozygous APC-resistant population.

Fig 2. APC-sr of men, women not using OC, women using OC, and heterozygous female carriers of factor V Leiden. Medians (indicated in the figure) and 5th and 95th percentiles are given in Table II. MP2 = second-generation monophasic OC users; TP = triphasic OC users; MP3 = third-generation monophasic OC users.

Fig 3. Effect of OC therapy on the APC-sr. APC-sr were determined in plasma from two healthy women, age 26 years (○) and 34 years (□), who had not taken oral contraceptives for more than 8 years and started OC therapy on day 0 with a third-generation monophasic OC (150 μg desogestrel/30 μg ethinylestradiol). OC-free periods are indicated by shaded bars.
During our study we identified five women who were using OC and were also heterozygous carriers of the factor V<sub>Leiden</sub> mutation. These women had an APC-sr (4-52–6-40) of the levels normally observed for homozygous APC-resistant individuals (Table II).

Changes of APC-sr after starting OC therapy

The sensitivities to APC of two healthy women, who had not used OC for >8 years and who started OC therapy, were followed over a 4-month period. Prior to the use of OC the women had an APC-sr within the normal range (Fig 3). Within 3 d of starting OC therapy the APC-sr had significantly increased, reaching values ≥3 in the third week of OC use. During the seven OC-free days the APC-sr significantly decreased, rising again during the second and third treatment cycles.

DISCUSSION

In vivo down-regulation of thrombin formation is achieved by plasma protease inhibitors (e.g. antithrombin III and α<sub>2</sub>-macroglobulin) and by the proteins of the anticoagulant pathway (APC and protein S). The physiological importance of these pathways is demonstrated by the association of venous thrombosis with congenital deficiencies in anti-thrombin III (Hirsch et al, 1989), protein C (Griffin et al, 1981) or protein S (Schwarz et al, 1984) and by the occurrence of hereditary thrombophilia in individuals with a genetic defect (Bertina et al, 1994; Dahlbäck et al, 1993) that makes factor Va less sensitive to proteolytic inactivation by APC (APC resistance).

An increased risk for venous thromboembolism has also been associated with the use of OC by healthy women. Although large retrospective and prospective studies have associated OC therapy with a 4-9-fold increased incidence of venous thrombosis with congenital deficiencies in anti-thrombin III (Hirsch et al, 1989), protein C (Griffin et al, 1981) or protein S (Schwarz et al, 1984) and by the occurrence of hereditary thrombophilia in individuals with a genetic defect (Bertina et al, 1994; Dahlbäck et al, 1993) that makes factor Va less sensitive to proteolytic inactivation by APC (APC resistance).

In this paper we report a substantial change of a haemostatic variable (the APC-sr) in women using OC which appears to be much more sensitive and that may be indicative for the existence of a prothrombotic state in OC users. We have shown that measurement of the effect of OC on thrombin formation in plasma provides information on the sensitivity of an individual to APC. Screening a population of women who use or do not use OC shows that OC therapy induces acquired APC resistance. Significant differences in sensitivities to APC were observed between women not using OC and women on OC therapy, independent of the type of OC used. Within the group of OC users, women taking third-generation monophasic OC were significantly less sensitive to APC than triphasic or second-generation monophasic OC users and exhibited a decreased sensitivity to APC comparable to that observed in heterozygous APC-resistant women who did not use OC (Fig 2).

We propose that decreased sensitivity to APC (acquired APC resistance) in OC users may, at least in part, explain the higher risk of thrombotic disease of women taking OC. The fact that users of OC with third-generation progestagens, for whom a higher risk of venous thromboembolism has been reported (Spitzer et al, 1995; W.H.O., 1995a, b), exhibit significantly lower sensitivities to APC than users of second-generation OC, reinforces this hypothesis. Until now, the increased risk for venous thrombosis during OC therapy has always been linked to effects of oestrogen on the coagulation system (Speroff & DeCherney, 1993; Newton, 1995). Our study provides the first example of a biological effect of the progestagen component on haemostasis and shows that, with respect to the risk of venous thrombosis, the role of progesterone cannot be ignored.

Whereas women who use third-generation OC have APC-sr similar to heterozygous carriers of factor V<sub>Leiden</sub> not using OC (Fig 2), the APC response in women who take OC and who also carry the factor V<sub>Leiden</sub> mutation is further impaired and similar to that observed in homozygous APC-resistant individuals (Table II). Together with the fact that, compared with non-carriers non-users, the increased risk for deep-vein thrombosis of non-carriers who use third-generation OC (7–8-fold) (Spitzer et al, 1996; W.H.O., 1995a, b) is similar to that of heterozygous carriers not using OC (7-fold) (Koster et al, 1993), and that heterozygous carriers of factor V<sub>Leiden</sub> who use OC exhibit a risk increase (30–50-fold) (Bloomankenamp et al, 1995) that approaches that of homozygous carriers not using OC (80-fold) (Vandenbroucke et al, 1994; Rosendaal et al, 1995), this supports our proposal that the increased incidence of venous thrombosis in congenital APC-resistant individuals and in women using OC originate from a defect in the same physiological pathway. The observations that the effect of OC on the APC response takes place within a few days after starting OC therapy and that the APC-sr drops in pill-free periods (Fig 3) contains valuable information about the underlying mechanism. Unfortunately, our present data do not allow further conclusions regarding changes in the haemostatic system that may link the impaired APC response and the increased thrombotic risk of OC users. It is unlikely, however, that the decreased sensitivity to APC is due to the reported decreased levels of protein S in OC users (Speroff & DeCherney, 1993; Newton, 1995), since addition of protein S to the plasma of OC users did not normalize the APC-sr (data not shown). We confirmed that measurement of sensitivity for APC in APTT-based clotting assays yields marginal differences between women using and not using OC (Olivieri et al, 1995; Henkens et al, 1995). The major difference between ETP-based and APTT-based methods is that initiation of coagulation in the case of the ETP occurs via the extrinsic pathway of coagulation (tissue factor/factor VIIa), whereas in APTT-based systems coagulation is initiated via the intrinsic coagulation pathway. This indicates that the basis of the impaired APC response in women using OC may have to be sought in the activity and/or regulation of the extrinsic pathway of coagulation.

It is well known that normal pregnancy is also associated with an increased thrombotic risk and induces changes in
the haemostatic system that are comparable to, but more pronounced than, those observed in women using OC (Greer, 1994). Preliminary experiments performed in our laboratory show that pregnancy is accompanied by changes in sensitivity to APC that even exceed that of women using OC. This indicates that plasma of pregnant women will probably be the preferred system to study the aetiology of the increased risk of thrombosis during pregnancy and OC therapy.

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REFERENCES


