Hyperprothrombinaemia-induced APC resistance: Differential influence on fibrin formation and fibrinolysis

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Summary
The prothrombin gene mutation G20210A is a common risk factor for thrombosis and has been reported to cause APC resistance. However, the inhibition of thrombin formation by APC not only limits fibrin formation but also stimulates fibrinolysis by reducing TAFI activation. We evaluated the influence of prothrombin G20210A mutation on the anticoagulant and fibrinolytic activities of APC (1 µg/ml). Thirty-two heterozygous carriers and 32 non carriers were studied. APC anticoagulant activity was assessed by aPTT prolongation whereas APC fibrinolytic activity was determined by a microplate clot lysis assay. APC-induced aPTT prolongation was markedly less pronounced in carriers than in non carriers. On the contrary, fibrinolysis time was shortened by APC to a comparable extent in both groups. Accordingly, prothrombin levels were strongly correlated with APC-induced prolongation of lysis time. The addition of purified prothrombin to normal plasma (final concentration 150%) caused APC resistance in the clotting assay over the whole range of tested APC concentrations (0.125–1.5 µg/ml). In the fibrinolytic assay, instead, prothrombin supplementation made the sample resistant to low but not to high concentrations of APC (>0.5 µg/ml). Thrombin and TAFIa determination in the presence of 1 µg/ml APC revealed that hyperprothrombinemia, although capable of enhancing thrombin generation, was unable to induce detectable TAFIa formation. It is suggested that APC resistance caused by hyperprothrombinaemia does not translate in impaired fibrinolysis, at least in the presence of high APC levels, because the increase in thrombin formation is insufficient to activate the amount of TAFI required to inhibit plasminogen conversion. These data might help to better understand the relationship between thrombin formation and fibrinolysis down-regulation.

Keywords
Protein C, prothrombin G20210A, TAFI, thrombin generation, thrombosis

Introduction
The prothrombin gene mutation G20210A is a common genetic disorder associated with venous thrombosis (reviewed in 1). The presence of the 20210A allele is accompanied by elevated prothrombin levels, and hyperprothrombinaemia has been shown to be an independent risk factor for thrombosis (2). As concerns the underlying mechanism predisposing to thrombosis, available evidence suggests that prothrombin elevation upregulates thrombin generation (3, 4), thereby promoting excessive fibrin formation. Moreover, we have recently shown that the fibrinolysis time of hyperprothrombinemic plasma is significantly prolonged as compared to normal plasma (5) because of an increase in the activation of TAFI (thrombin activatable fibrinolysis inhibitor), a plasmatic procarboxypeptidase that, once converted into the active enzyme, TAFIa, removes the plasminogen binding sites from partially degraded fibrin, thereby reducing plasmin formation (6). Since thrombin is considered the main physiological activator of TAFI, it is conceivable that the down-regulation of fibrinolysis is ascribable to the increase in thrombin formation associated with the G20210A mutation. Another mechanism that might upregulate thrombin formation in carriers of the prothrombin mutation is the impairment of the anticoagulant protein C pathway. Indeed, an APC resistant phenotype has been
documented in these patients (7, 8), which is probably due to a direct inhibitory effect of prothrombin on APC-mediated inactivation of factor Va (9). In the light of these observations it can be anticipated that the combined effect of hyperprothrombinemia on prothrombin activation and APC activity might result in a greater generation of thrombin and thus in a greater inhibition of fibrinolysis. This study was undertaken to test this hypothesis.

Materials and methods

Patients
Thirty-two carriers of the prothrombin G20210A mutation (all heterozygous) and 32 non carriers were studied. Blood was collected by vacuum collection tubes (Vacutainer, Becton Dickinson, Meylan, France) containing 1/10 volume of 105 mM trisodium citrate. Citrated blood was centrifuged for 15 minutes at 2,500 g (room temperature), and the supernatant plasma was collected, snap-frozen in liquid nitrogen and stored at –70°C until testing.

Reagents and laboratory assays
Human prothrombin and the TAFIa/TAFIai ELISA kit were from American Diagnostica, [Greenwich, CT, USA (courtesy of Dr. D. Santo, Instrumentation Laboratory, Milan, Italy)]. APC was provided by Immuno AG (Vienna, Austria); t-PA was from Boehringer Ingelheim (Florence, Italy); human thrombin and hippuryl-Arg were from Sigma (Milan, Italy); thromboplastin (Recombiplastin) and aPTT reagent (Synthafax) were from Instrumentation Laboratory; phospholipid vesicles (Unichrom) were purchased from Cabru (Peregallo di Lesmo, Milan, Italy). PPACK (D-Phe-Pro-Arg- chloromethylketone, HCl) was from Calbiochem (Darmstadt, Germany). Prothrombin levels were measured using S2238 (Instrumentation Laboratory) as chromogenic substrate and Echis Carinatus (Sigma) as activator (10). Detection of the G20210A mutation in the prothrombin gene was performed as described by Poort, et al. (2). aPTT, in the absence and in the presence of APC (1 µg/ml plasma, unless otherwise stated), was performed by the manual (tilt tube) technique using reagents from Instrumentation Laboratory, as reported (11). APC sensitivity was expressed as aPTT ratio between values obtained in the presence and in the absence of APC. Due to sample volume limitations, aPTT ratio could not be determined in two normal subjects and in one 20210A carrier. Prothrombin-enriched plasma (150% final concentration) was obtained by adding purified prothrombin to plasma samples from subjects not carrying the G20210A mutation.

Clot lysis experiments
The fibrinolytic activity of APC was evaluated using a previously described t-PA-induced clot lysis assay (5), modified as follows: 100 µl citrated plasma, 10 µl phospholipid vesicles, 5 µl t-PA (25 ng/ml, final concentration), 10 µl APC (1 µg/ml final plasma concentration, unless otherwise stated) or vehicle, 50 µl buffer, 5 µl thromboplastin (1/1,000 final dilution), and 50 µl 0.04 M CaCl₂ were added to a microplate well. In some experiments, the clot lysis mixture was changed by replacing thromboplastin and phospholipid vesicles with 10 µl Synthafax and 5 µl buffer. Afterwards, the plate was incubated at 37°C, and the changes in OD at 405 nm were monitored every 5 min for up to 3 h. Clot lysis time was defined as the time from the midpoint of clear to maximum turbid transition to the midpoint of the maximum turbid to clear transition. The fibrinolytic activity of APC is measured by the shortening of the lysis time and is expressed as APC clot lysis ratio which is obtained by dividing the lysis time in the absence of APC by the lysis time in the presence of APC. Thus, the higher the APC clot lysis ratio, the higher the fibrinolytic effect of APC.

Assay of thrombin and TAFIai generation
The profile of thrombin and TAFIa generation was determined under conditions similar to those used for clot lysis assay but using defibrinated plasma (12) instead of normal plasma. In these experiments, the lysis mixture was prepared in a 5 ml tube (final volume 1 ml) and incubated at 37°C. For thrombin assay, aliquots of 50 µl were taken at predetermined intervals and transferred to a prewarmed tube containing 100 µl of human fibrinogen (6 mg/ml) dissolved in citrate-Tris buffer (0.38% sodium citrate). The clotting time was determined by the manual (tilt tube) technique and thrombin activity was calculated by reference to a calibration curve constructed with purified human thrombin. For TAFIai determinations, we used a specific TAFIa/TAFIai ELISA recently developed (13). This assay is based on the selective binding of TAFIa and of its inactive form (TAFIai), generated by conformational change (14), to CPI (carboxypeptidase inhibitor from potato tuber) which is used as capture molecule. Aliquots of 60 µl were withdrawn from the lysis mixture at fixed intervals and transferred to a refrigerated tube preloaded with 90 µl assay buffer (provided by the manufacturer) containing PPACK (50 µM) and trisodium citrate (0.38%) in order to stop both thrombin formation and activity. Samples were kept on melting ice until beginning of the assay, which was carried out according to the manufacturer’s instructions. Results were expressed as percent of total TAFI by reference to a calibration curve constructed with serial dilutions of plasma pretreated for 5 min at 37°C with thrombin (5 U/ml) plus thrombomodulin (1 µM) in order to convert all TAFI to TAFIai (15). Under our experimental conditions the concentration of TAFIai detected in the starting samples (time zero) was negligible (<0.5 %).

Statistical analysis
All analyses were carried out using the GraphPad Prism software (San Diego, CA, USA). Unless otherwise stated, data are expressed as mean ± SD. The differences between carriers and non carriers of prothrombin G20210A mutation were assessed by the Mann-Whitney test; correlations between variables were evaluated by the Pearson correlation coefficient. The differences between normal plasma and prothrombin-enriched plasma were assessed by paired t-test. Differences in thrombin and TAFIa generation were assessed by comparing the areas under the enzyme generation curves.

Results
Influence of G20210A mutation on APC anticoagulant activity
Basal aPTT values were similar in the two groups of subjects (34.0 ± 4.6 s vs. 34.8 ± 4.5 s). Upon addition of APC, mean aPTT
was prolonged to 105.8 ± 44.8 s in controls and to 73.4 ± 16.4 s in carriers, giving rise to APC sensitivity ratios (aPTT ratio) of 3.15 ± 1.37 and 2.11 ± 0.41, respectively (p<0.0001, Fig. 1A). As previously reported (7), prothrombin levels were significantly correlated with aPTT ratio (r=–0.529, p<0.0001) (Fig. 1C).

Influence of G20210A mutation on APC fibrinolytic activity
According to our previous findings (5), mean fibrinolysis time of plasma clots from prothrombin 20210A carriers was significantly prolonged as compared with controls (73.0 ± 11.2 vs. 62.8 ± 13.7 min, p<0.002). Upon APC addition, mean lysis time was reduced to 38.8 ± 11.1 min in carriers and to 37.1 ± 12.9 min in controls, and the difference between the two groups was no longer evident. Corresponding APC clot lysis ratios were 2.0 ± 0.5 and 1.81 ± 0.45, respectively (Fig. 1B). In line with these data, no correlation was found between prothrombin level and APC clot lysis ratio (r=–0.02, p=0.87) (Fig. 1D).

Influence of prothrombin supplementation on APC responses
To directly assess the influence of hyperprothrombinaemia on APC responses, we added purified human prothrombin to normal plasma in order to raise the prothrombin level to 150% and evaluated the changes in aPTT and plasma fibrinolysis induced by different concentrations of APC (0.125 to 1.5 µg/ml of plasma). As shown in Figure 2 (top panel), prothrombin addition had no influence on the starting aPTT value (i.e. in the absence of APC) but attenuated the anticoagulant response to all tested APC concentrations as witnessed by the clearly divergent aPTT curves obtained with normal and prothrombin-enriched plasma. In agreement with our previous data (5), prothrombin supplementation caused a prolongation of the fibrinolysis time (Fig. 2, middle panel). However, upon addition of increasing concentrations of APC, the difference between normal and hyperprothrombinaemia plasma became progressively smaller and disappeared at APC concentrations above 0.5 µg/ml. When the data were expressed as clot lysis ratio (Fig. 2, bottom panel), the prothrombin-enriched sample was less sensitive than normal plasma to the fibrinolytic effects of low concentrations of APC (below 0.5 µg/ml) but displayed a normal or even increased response to APC when the latter was present at greater concen-
trations. Similar results were obtained when plasma clots were produced by replacing thromboplastin and phospholipids with the aPTT reagent (not shown). Moreover, it has to be noted that the resistance to the anticoagulant effect of APC caused by prothrombin addition was also evident during the fibrinolysis experiments. Indeed, the clotting time of the fibrinolytic mixture containing prothrombin-enriched plasma was markedly shorter that containing normal plasma at all APC concentrations (not shown).

**Effect of prothrombin supplementation on thrombin and TAFIa generation in the absence and in the presence of APC**

Given that the inhibition of thrombin-dependent TAFI activation is considered the main mechanism by which APC stimulates fibrinolysis (16), we evaluated the influence of hyperprothrombinaemia on APC response by determining thrombin and TAFIa generation. Experimental conditions were similar to those used for clot lysis experiments with the only exception that plasma samples were defibrinated in order to avoid clot formation. As shown in Figure 3, thrombin generation as well as TAFIa activation were significantly increased in prothrombin-enriched plasma both in the absence (Fig. 3A, D) and in the presence of 0.25 µg/ml APC (Fig. 3B, E). However, in the presence of 1 µg/ml APC (Fig. 3C, F), while thrombin formation was still enhanced in prothrombin-enriched as compared to normal plasma, TAFIa accumulation was almost completely inhibited in both samples.

**Discussion**

The protein C system plays an important role in the maintenance of blood fluidity by controlling thrombin formation (17). By this, APC not only reduces fibrin formation but also stimulates fibrinolysis by inhibiting thrombin-induced TAFI activation (16, 18). Accordingly, APC resistance, associated with homozygous FV Leiden mutation or with the presence of autoantibodies that protect FVa from cleavage by APC, has been reported to down-regulate plasma fibrinolysis through a TAFI-mediated mechanism (18, 19). Hyperprothrombinaemia has also been shown to be associated with APC resistance (7–9), and therefore we evaluated the influence of prothrombin G20210A on the fibrinolytic activi-
itivity of APC. In agreement with previous data (7, 8), plasma samples from 20210A carriers displayed a marked and significant resistance to APC in the aPTT assay. Concerning the fibrinolytic assay, it should be noted that, in the absence of APC, the lysis time in G20210A carriers was significantly longer than in non carriers, an effect previously shown to be attributable to a greater TAFIa generation resulting from an increase in thrombin formation and unrelated to the occurrence of TAFI variants characterised by an extended functional half-life (5, 20). This notwithstanding, upon APC addition the lysis time in G20210A carriers was shortened very markedly and brought to a value that was similar to that recorded in non carriers, indicating that the prothrombin mutation has no impact on the fibrinolytic activity of APC, at least under our experimental conditions. The different influence of prothrombin concentration on APC responses is supported by the observation that aPTT ratio was strongly correlated with prothrombin levels, whereas APC clot lysis ratio was not.

Similar results were obtained when the prothrombin concentration of normal plasma was artificially raised to 150% by the addition of purified prothrombin. Indeed, prothrombin-enriched plasma displayed a reduced anticoagulant response to APC over the whole range of tested APC concentrations and the difference in aPTT values between normal and hyperprothrombinaemic plasma increased in parallel with the APC concentration. In the fibrinolytic assay the results were almost the opposite, inasmuch as the prolongation of fibrinolysis time caused by prothrombin supplementation decreased progressively upon addition of increasing amounts of APC and disappeared when the APC concentration exceeded 0.5 µg/ml. Calculation of APC sensitivity by means of APC clot lysis ratio indicated that hyperprothrombinaemia attenuates the fibrinolytic response to concentrations of APC below 0.5 µg/ml but enhances the response to concentrations above this value.

APC has been shown to stimulate fibrinolysis through two main mechanisms: i) neutralization of PAI-1 (21), and ii) inhibition of TAFI activation (16). Under our experimental conditions, the profibrinolytic effect of APC is almost entirely TAFI-dependent. Indeed, APC is unable to shorten the lysis time of clots generated from TAFI-depleted plasma as shown by Bajzar, et al. (16) and by ourselves (unpublished data). This implies that APC accelerates fibrinolysis by virtue of its anticoagulant activity (22). It is therefore surprising that hyperprothrombinaemia, while reducing the anticoagulant response to APC, is unable to impair the profibrinolytic effect of this clotting inhibitor, at least when it is present at concentrations above 0.5 µg/ml. However, it has to be considered that aPTT only measures the initial thrombin formation, whereas the activation of TAFI is likely dependent on thrombin generated after clot formation (23, 24). Therefore, we evaluated the effect of hyperprothrombinaemia on the anticoagulant activity of APC by determining thrombin generation under conditions similar to those used for clot lysis experiments. In this experiment we tested two representative concentrations of APC, i.e. 0.25 and 1 µg/ml. As expected (3, 4, 8), hyperprothrombinaemia enhanced thrombin generation both in the absence and in the presence of the two tested APC concentrations. Such an increase in thrombin formation, however, did translate in enhanced TAFIa generation in the absence and in the presence of the low concentration of APC but not in the presence of the high APC concentration, under which condition TAFI activation was negligible in both normal and prothrombin-enriched plasma. Although it cannot be excluded that the TAFIa assay was not sensitive enough to unmask a small difference in TAFI activation between normal and hyperprothrombinaemic plasma exposed to 1 µg/ml APC, we can safely conclude that the concentration of TAFIa in both samples was low and very likely below the level required to inhibit the fibrinolytic process (25, 26). This suggests that detectable amounts of TAFIa are formed only when the concentration of thrombin overcomes a critical threshold level and that the greater thrombin formation observed in prothrombin-enriched plasma at APC concentrations above 0.5 µg/ml, while enhancing fibrin formation, has no effect on fibrinolysis, because the concentration of thrombin does not reach the level required to activate significant amounts of TAFI.

In conclusion, our study suggests that the effect of hyperprothrombinaemia on fibrinolysis need not necessarily be detrimental, provided that the amount of APC generated is sufficiently high to dampen thrombin formation below the concentration required to inhibit the fibrinolytic process. It is difficult to say which amount of APC is needed in vivo to abrogate the antifibrinolytic effect of hyperprothrombinaemia and whether such an amount can be formed locally at the level of the nascent fibrin plug or thrombus. We do know that APC formation is greatly enhanced in vivo by thrombomodulin and EPCR on the intact endothelium (27), and studies in dogs (28) and rabbits (29) indicate that more than 20–30% of circulating PC (corresponding to about 1 µg/ml of APC) may be activated following the infusion of thrombin at a dose as low as 1 U/kg/min. Therefore, even assuming that the extent of protein C activation in G20210A carriers is similar to that of non carriers, as suggested by the measurement of circulating APC-PCI (protein C inhibitor) complexes under basal conditions (30), it might be anticipated that the concentration of APC reached locally at the site of blood clotting activation will be sufficiently high. On the other hand, it has to be considered that thrombomodulin also facilitates the activation of TAFI by thrombin (6) and, thus, the threshold thrombin concentration required to generate significant amounts ofTAFIa in vivo is expected to be lower than in vitro. However, given that the activation of protein C occurring upon tissue factor-induced clotting activation has been shown to inhibit the activation of TAFI when the concentration of thrombomodulin is above 10 nM (31), it can be speculated that, at least under certain conditions, thrombin generation is accompanied by a net profibrinolytic effect.

So far, the only conditions associated with a clear in vitro refractoriness to the fibrinolytic activity of APC are those characterised by a strong APC resistance and a high risk of thrombosis (18, 19). Therefore, the concept that the fibrinolytic response to APC may be normal in carriers of the G20210A mutation harmonises better with the relatively low risk of thrombosis associated with this genetic disorder. Our data, on the other hand, might help to better understand the relationship between thrombin formation and inhibition of fibrinolysis.
anism characterized by poor anticoagulant response to activated protein C: prediction of factor to...

References


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