The Effect of Fibrinogen Genotype on Fibrinogen Levels after Strenuous Physical Exercise

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Summary

We have examined the effect of two β-fibrinogen gene promoter polymorphisms (-455G>A and -854G>A) on the fibrinogen response to severe exercise in a group of male army recruits undergoing basic training. Fibrinogen was measured pre-training and again serially after severe 48 h final military exercise (FME). Out of 884 subjects, 762 completed training of whom 250 were selected for post-FME study. Fibrinogen levels (g/l) were significantly elevated over baseline levels 2, 48 and 96 h after FME, representing increases of 15.7%, 3.4% and 7.6% (p < 0.005; p = 0.05 and p < 0.005 respectively), with higher levels in -455A allele carriers than genotype -455GG: 3.17 ± 0.05 vs. 2.94 ± 0.05 (p < 0.001), 2.86 ± 0.05 vs. 2.60 ± 0.05 (p < 0.0005) and 2.98 ± 0.06 vs. 2.69 ± 0.06 (p < 0.0005) at 2, 48 and 96 h respectively. There was no effect of the -854G>A polymorphism on fibrinogen, even after taking into account β-fibrinogen -455 genotype. Thus the fibrinogen -455G>A polymorphism influences fibrinogen levels following exercise. The effect of genotype might be clinically relevant at times of hyperfibrinogenaemia such as following an acute inflammatory response.

Introduction

Fibrinogen is a hepatically derived acute phase protein whose levels rise markedly in response to stimuli as diverse as infection, smoking and trauma (1-3). Elevated levels are an independent risk factor for coronary vascular events amongst both healthy individuals (4-6) and those with documented coronary artery disease (CAD) (7), correlating with angiographic disease severity and with the transition from stable disease to acute coronary syndromes (7, 9). Similar risk extends to the development of peripheral arterial (5, 10) and cerebrovascular disease (5, 11). Overall, an increase in fibrinogen concentration of just 0.1 g/l corresponds to an increase in cardiovascular risk of approximately 15% (12), making fibrinogen as powerful a risk factor as hypercholesterolaemia, smoking and hypertension (6, 13, 14).

Severe exercise drives an acute phase response similar to that seen in sepsis (15), but more readily quantified and prospectively studied (16). This response is associated with a rise in interleukin-6 (IL6) levels (17-19) driving the associated and well-defined rise in fibrinogen concentration (17, 20-24). Just as in sepsis, the magnitude of this rise depends upon both the magnitude of environmental stimulus and upon individual genetic variation.

The 340KD-fibrinogen molecule is composed of two identical sub-units, each comprising three polypeptide chains α, β and γ (25) encoded by separate genes on chromosome 4 (26). As production of the β-chain is the rate-limiting step in fibrinogen synthesis (27), variation in its promoter sequence might be expected to influence the scale of the acute-phase fibrinogen response. Specifically, an IL6-responsive element has been identified in the promoter region of the β-fibrinogen gene (28), variation in which might be expected to alter the magnitude of fibrinogen response. Several common β-fibrinogen gene promoter variants have been described. In particular, the -455G>A polymorphism is consistently associated with effects on fibrinogen levels (29-34). Varying degrees of linkage disequilibrium have been observed between the -455G>A polymorphism and other β-fibrinogen variants. There is essentially complete allelic association with -148C>T and a weaker association with -854G>A variant (35-38). Data from electromobility shift assays have shown that both the -455 and -854 polymorphisms result in differences in nuclear protein binding and in allelic specific differences in the rate of fibrinogen transcription (38), supporting their in vitro functionality. While it is possible that the -455G>A polymorphism is itself functional and is associated with a direct effect on gene transcription, clearly the -455G>A sequence change might also be a marker for the -148C>T or -854G>A functional changes.

We have previously shown the β-fibrinogen -455G>A promoter polymorphism to be associated with differences in the plasma fibrinogen response to acute severe intensive exercise (24). In addition it has also been demonstrated that the -455G>A polymorphism is associated with a more rapid increase in fibrinogen following coronary bypass surgery (39, 40). However, such data apply to relatively small numbers, and require confirmation. In addition, the role of the -854G>A polymorphism in influencing the fibrinogen acute-phase response has yet to be examined. We have thus examined the association of the -455G>A polymorphism on the acute-phase response to severe exercise, and have investigated the impact of combined fibrinogen -455G>A and -854 promoter genotype on this response.

Materials and Methods

We have previously validated the use of intense exercise amongst military recruits as a model for studying gene-environment interaction on fibrinogen synthesis (24). Every fortnight, groups of soldiers start an 11-week period of basic training designed to emphasise physical fitness. At the end of this period, recruits embark on an intensive 48 h final military exercise (FME), after which further serial blood samples are taken. During FME recruits had free access to water to prevent dehydration. With appropriate ethics committee approval, and with informed written consent from all participants, the study was performed at the Army Training Regiment, Basingbourn, UK. Between January 2000 and...
January 2001, consecutive Caucasian recruits were asked to participate. From those who agreed, an additional blood sample (10 ml EDTA) was drawn concurrently with those taken at their initial routine medical examination. From this, DNA was extracted and fibrinogen -455G>A and -854G>A genotype determined.

Constraints on recruit availability meant that only a maximum of 6 individuals could be accurately and prospectively studied at the latter time points during basic training. Hence all comers were initially invited to participate in the study, with the goal of enriching the final study sample such that half the final cohort would carry at least one -455A-allele. In this way it was possible to choose 5-6 individuals from each entry troop (approximately 35 soldiers) in order to take serial blood samples following final military exercise, without disrupting the troop's training program.

**Blood Sampling Protocol and Serum Analysis**

Venous blood (4.5 ml citrated sample) was drawn from the antecubital vein at rest in a sitting position. Samples were drawn at induction (pre-training sample), and again 2, 48 and 96 h after returning from FME-times chosen to “bracket” the peak of the fibrinogen acute-phase response, as determined previously (24). Samples were centrifuged (3000 × g, 10 min) and the serum transported (2 h, 4° C) then immediately frozen at –20° C. Fibrinogen concentration was determined (semi-automated Clauss assay: MDA-180 coagulometer, Organon Teknika, Cambridge), using the manufacturer’s reagents and calibrated with the 7th British Standard (NIBSC).

**DNA Extraction and Genotyping**

DNA was extracted from peripheral blood by the salting out method (41). Genotyping for the fibrinogen -455G>A and -854G>A polymorphisms was performed by staff blind to subject data, using the methods previously described (32, 38).

**Statistical Analysis**

All data were analysed using SPSS for Windows version 9 (SPSS Inc. Chicago, Illinois). Differences in fibrinogen levels between genotypes were assessed by analysis of variance (ANOVA) and by Student’s t-tests for paired data. One-way analysis of covariance (ANCOVA) was performed to test whether genotype was associated with differences in fibrinogen using age, smoking and BMI (kg/m²) as co-variates. Allele frequencies were estimated by gene counting. A χ² test was used to compare the observed numbers of each genotype with those expected for a population in Hardy-Weinberg equilibrium. Linkage disequilibrium between sites in pairwise combination was estimated using the method of Chakravarti et al. (42). P-values less than 0.05 were considered to be statistically significant.

**Results**

A total of 1901 recruits passed through ATR Bassingbourn whilst study selection was in progress. Nine hundred and seventy-seven

<table>
<thead>
<tr>
<th>Number</th>
<th>Overall Cohort</th>
<th>Final Study Cohort</th>
<th>Non participants</th>
<th>P value</th>
</tr>
</thead>
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<tr>
<td></td>
<td>[977]</td>
<td>[250]</td>
<td>[727]</td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>19.4±2.1</td>
<td>19.1±2.4</td>
<td>19.2±2.3</td>
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<tr>
<td>BMI</td>
<td>22.3±2.6</td>
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<tr>
<td>Current Smokers</td>
<td>442 [45.2%]</td>
<td>119 [47.6%]</td>
<td>323 [44.3%]</td>
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</tr>
<tr>
<td>Systolic BP</td>
<td>122±12</td>
<td>122±10</td>
<td>122±13</td>
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<tr>
<td>Diastolic BP</td>
<td>71±9</td>
<td>71±8</td>
<td>71±8</td>
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<th>Genotype-455G&gt;A</th>
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<th>48 hours</th>
<th>96 hours</th>
<th>ANOVA p value</th>
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<tr>
<td>GG</td>
<td>2.58±0.05</td>
<td>2.94±0.05</td>
<td>2.60±0.05</td>
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<td>GA</td>
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<tr>
<td>AA</td>
<td>2.70±0.08</td>
<td>3.17±0.08</td>
<td>2.90±0.07</td>
<td>2.98±0.09</td>
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<table>
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<tr>
<th>Genotype-854G&gt;A</th>
<th>Baseline</th>
<th>2 hours</th>
<th>48 hours</th>
<th>96 hours</th>
<th>ANOVA p value</th>
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<tr>
<td>GG</td>
<td>2.67±0.04</td>
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<td>2.74±0.04</td>
<td>2.86±0.05</td>
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<tr>
<td>GA</td>
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<td>2.75±0.07</td>
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<tr>
<td>AA</td>
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<td>2.89±0.29</td>
<td>2.51±0.14</td>
<td>2.84±0.34</td>
<td>0.72</td>
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Table 1 Baseline demographics for the overall study group and final study cohort. Statistical comparison has been made between those who were selected for the final study cohort and those who did not participate.

Table 2 Mean fibrinogen levels (± SE) at baseline and at different times after FME by -455G>A and -854G>A genotype (numbers of subjects).
(51.3%) agreed to participate, of whom 884 were successfully genotyped for the -455G>A polymorphism [572 (64.7%) GG, 283 (32%) GA, 29 (3.3%) AA]. Genotype distribution was consistent with Hardy-Weinberg equilibrium, with an -455A allele frequency 0.19 (95% confidence intervals 0.17-0.21) similar to that previously reported in a Caucasian British population (32).

Of the 977 in the initial group, 762 (78%) completed training of whom 250 were selected by -455G>A genotype: [126 (50.4%) GG, 105 (42.0%) GA, 19 (7.6%) AA] for post-FME study. This final cohort was also genotyped for the -854G>A polymorphism: 187 (77.6%) were -854GG, 51 (21.2%) -854GA and 3 (1.2%) -854AA. The -854G A allele frequency was 0.12 (95% CI 0.09-0.15) and genotype distribution did not differ from that expected of a population in Hardy-Weinberg equilibrium. Although -854A allele frequency was lower than in Sweden (38) it was not significantly different from that found in a representative sample of British Caucasians, (unpublished data). As shown previously (38) the -455G>A and -854G>A polymorphisms were found to be in negative allelic association ($\Delta = -0.21, P = 0.04$).

Baseline characteristics of those studied (Table 1) did not differ from those who failed to complete training or from those who did not volunteer for serial blood testing. Likewise, genotype distribution was similar amongst those who did and did not complete the final stages of the study.

**Fibrinogen Response**

Prior to training mean fibrinogen levels (2.60 ± 0.04 mg/dl) were similar to those in our initial study (Table 2) (24). As expected, carriers of one or more -455A-allele showed a trend towards higher baseline fibrinogen levels than subject with genotype -455GG, however this difference was not statistically significant ($p = 0.11$). There was no effect of -854G>A genotype on enrolment fibrinogen levels.

Following the 48 h final military exercise (FME), fibrinogen levels were significantly elevated over baseline levels at the 2, 48 and 96 h time points (Fig. 1), consistent with the expected acute phase response, showing increases of 15.7%, 3.4% and 7.6% ($p <0.005$; $p = 0.05$ and $p <0.005$ respectively). The magnitude of this rise in fibrinogen was -455G>A genotype dependent (Table 2). Post-FME fibrinogen levels were similar in -455G>A heterozygotes and those subjects who were homozygous for the rare -455A allele. For this reason -455A allele carriers were combined with -455A homozygotes for further analysis. Fibrinogen levels were significantly higher in these individuals than in those of genotype -455GG, with values 8.3% higher 2 h after FME, 11.8% after 48 h and 11.4% higher 96 h after exercise. The difference in mean fibrinogen levels was statistically significant throughout recovery from FME (Fig. 2a), an effect that was not diminished by multivariate analysis. Furthermore, whilst at baseline fibrinogen -455 genotype only accounted for only 1.1% of the total variance of fibrinogen, this effect was much larger after exercise, accounting for 4.8%, 6.6% and 6.9% of the total variance in fibrinogen 2, 48 and 96 h after FME.

In contrast, there was no effect of the -854G> polymorphism on either baseline or post-exercise fibrinogen levels (Fig. 2b) even after taking -455 genotype into consideration. In the 115 subjects with genotype -455GG baseline fibrinogen for genotype -854GG was 2.62 (± 0.06) vs. 2.45 (± 0.09) for -854GA+AA, $p = 0.13$. There was no significant difference in fibrinogen revealed after FME, or after analysis of the effect of -854 genotype in subjects carrying one or more -455A-allele.

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**Fig. 1** The impact of 48-h final military exercise (FME) on mean fibrinogen levels (± SE). * $p = 0.05$, ** $p <0.005$

**Fig. 2** a) The effect of fibrinogen -455G>A genotype on post-exercise fibrinogen response. b) The effect of fibrinogen -854G>A genotype on post-exercise fibrinogen response. *P-values are quoted comparing fibrinogen levels of the GG wild type with carriers of one or more A-allele.
Discussion

In a sample of 250 subjects these data confirm our initial observation of an association between fibrinogen -455G>A genotype and the fibrinogen response to exercise (24). Carriers of one or more -455A-allele had significantly higher fibrinogen levels after exercise than -455GG homozygotes. We were unable to confirm previous reports that the -854A-allele is associated with higher resting fibrinogen levels (36, 38), nor have we identified any difference in the degree of acute-phase response to exercise.

It should be noted that we were unable to adjust for alterations in fibrinogen concentration related to differences in exercise-related haemoconcentration. However, all subjects were permitted free access to fluids throughout the FME. In addition, any variation in intravascular volume would have minimized the identified impact of the fibrinogen genotype, unless the two were themselves directly associated. This seems biologically implausible. Therefore the (unlikely) presence of substantial variation in hydration status would only have lessened the potential to identify a fibrinogen gene association – thus strengthening confidence in our observations.

The magnitude of maximal increase in fibrinogen in the current study (15.7%, 3.4% and 7.6% increases over baseline levels 2, 48 and 96 h after FME) is lower than first reported, where peak fibrinogen increased by 37% 2 days after exercise (24). These findings likely reflect changes in the content of the current final exercise, which is less intense than when first studied, with reduced emphasis on exercises combining heavy lifting with endurance running. The absence of such exertion toward the end of FME may also account for the earlier fibrinogen peak [on return, rather than at 48 h (24)] now seen. Finally, the newly observed subsidiary rise at 96 h may relate to 3 km endurance run in the first two days after the end of FME (23).

The present study improves our original observations by the study of an expanded cohort, in whom serial samples were obtained, rather than paired samples at staggered time points with no individual giving more than one post-exercise sample. Secondly, enrichment of the final cohort allowed study of 19 subjects (rather than the original three) of the rare -455AA genotype. Interestingly, our original finding that fibrinogen levels were related to the number of -455A-alleles is not replicated here, where values were similar regardless of the presence of one or more -455A-allele. Similar discrepancies have been described in the effect of -455G>A genotype on the fibrinogen response to coronary artery bypass surgery, with Gardemann demonstrating only an effect of -455AA genotype, whilst Cotton reported a co-dominant effect of the -455A-allele (39). The mechanism underlying such study variation is unclear.

Although fibrinogen levels rose significantly following exercise, all values remained within the normal healthy range. However, such changes may yet be of biological significance. Regular low-level physical activity is associated with a reduction in both fibrinogen and in cardiovascular risk (43-46). However, the benefits seem reduced if the regular exercise taken is more severe (43), suggesting that repetitive exposure to fibrinogen elevation might carry risk. Such risk might be mediated through small changes in plasma viscosity (6, 47), as fibrinogen accounts for nearly 50% of its predictive value. By whatever mechanism, fibrinogen may mediate the association of inflammatory cytokines such as IL-6 with atherosclerosis. The β-fibrinogen gene promoter contains an IL6 responsive element, which is situated close to the -148C>T polymorphism and which, in Caucasians, is in complete allelic association with the -455G>A polymorphism (37). Thus IL6 production induced by inflammatory stimuli such as smoking, trauma and indeed exercise will in turn result in increased fibrinogen synthesis.

Our data suggest that the β-fibrinogen -455G>A polymorphism may represent a powerful tool for investigating the association between fibrinogen levels and CAD. However several studies have not shown the fibrinogen -455A-allele to be a genetic cardiovascular risk factor (30-32, 34, 36). Whilst the -455G>A polymorphism may not predispose towards CAD under all circumstances, the -455G>A polymorphism might assume greater importance under situations of inflammatory stress, such as in smokers and after surgical trauma (39, 40). Thus a causal role for the fibrinogen -455A-allele in mediating CAD risk associated with inflammation should be explored amongst individuals exposed to a similar inflammatory stimulus rather than amongst a “mixed population” at basal state. In the first instance, one might advocate studies of allele frequency amongst those exposed to a chronic inflammatory burden, such as low-grade chronic infection with Chlamydia or Helicobacter pylori (HP). One such study has demonstrated an additive effect of the B2 allele of the Bcl fibrinogen polymorphism on fibrinogen in subjects who were HP seropositive (48). Furthermore HP infection showed a stronger effect on the risk of myocardial infarction in B2 allele carriers. Clearly similar studies are warranted of the fibrinogen -455G>A polymorphism.

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