Short communication

Postprandial factor VII metabolism: the effect of the R353Q and 10 bp polymorphisms

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Elevated levels of coagulation factor VII activity (FVIIc) are associated with increased risk of CHD. FVIIc is strongly determined by two polymorphisms (R353Q and 0/10 base pairs (bp)) and plasma triacylglycerol (TAG) concentrations. The Q and 10 bp polymorphisms show strong linkage disequilibrium and have been associated with lower levels of fasting FVII, but there has been little investigation of the effect of these genotypes on the postprandial FVII metabolism. The present study demonstrated that fasting activated factor VII (FVIIa) and factor VII antigen (FVIIag) levels were significantly lower in the heterozygotes carrying the Q and 10 bp alleles (*n* 12), than in the R/0 bp homozygotes (*n* 12) (43·0 (SE 4·8) *v.* 23·9 (SE 6·5) mU/ml and 85·7 (SE 5·4) *v.* 71·6 (SE 7·5) % respectively). During postprandial lipaemia there was a significant increase in FVIIa in R/0 bp homozygotes but not in the heterozygotes carrying the Q and 10 bp alleles. The proportion of FVIIa (FVIIa : FVIIag) increased in the homozygotes but not in the heterozygotes (2·04 (SE 0·35) *v.* 1·20 (SE 0·26) respectively). Therefore possession of the relatively common Q and 10 bp alleles is not associated with postprandial activation of FVII, which may in turn have a protective effect against CHD.

Factor VII: Triacylglycerols: Genetic polymorphism

Elevated levels of coagulation factor VII activity (FVIIc) are independently associated with increased risk of fatal CHD (Meade *et al.* 1993). A number of genetic and environmental factors determine the plasma levels of the factor VII zymogen (FVIIag) and its derivative, the serine-protease-activated factor VII (FVIIa), both of which contribute to FVIIc. A common polymorphism (R353Q) of the factor VII gene, whereby a single base change in exon 8 of the factor VII gene results in the replacement of arginine (R) at position 353 with glutamine (Q) in the protein product (Green *et al.* 1991), strongly determines FVIIc. Several studies have demonstrated that levels of FVIIc and FVIIag are 20–25 % lower in heterozygotes carrying the Q allele (Bernardi *et al.* 1996). Possession of the Q allele was associated with reduced risk of myocardial infarction in the study of Iacoviello *et al.* (1998) but a similar study did not show a significant effect of the Q allele on risk (Lane *et al.* 1996). Another common polymorphism of the factor VII gene, a decanucleotide (10 base pair (bp)) insertion at −323 in the promoter of the FVII gene, is also associated with lower levels of FVIIc and FVIIag (Humphries *et al.* 1996). There is strong linkage disequilibrium between the R and 0 bp alleles and the Q and 10 bp alleles in European populations (de Maat *et al.* 1997).

Plasma triacylglycerol (TAG) concentrations are an important environmental determinant of FVIIc. Several studies have shown strong positive associations between plasma TAG concentrations and FVIIc. It is proposed that this effect is due at least in part to the ability of TAG-rich lipoproteins (TRL) to promote the cleavage of the inactive single-chain zymogen of FVII to FVIIa (Silveira *et al.* 1996). The postprandial phase of lipid metabolism is an

Abbreviations: bp, base pair; FVIIa, activated factor VII; FVIIag, factor VII zymogen; FVIIc, total coagulation factor VII activity; PCR, polymerase chain reaction, TAG, triacylglycerol; TRL, triacylglycerol-rich lipoprotein.

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important determinant of FVII metabolism. Following the ingestion of a meal containing fat, there is an increase in the concentration of TRL and there is a concomitant increase in levels of FVIIc and FVIIa (Larsen *et al.* 1997; Roche & Gibney, 1997; Mennen *et al.* 1998; Roche *et al.* 1998). Despite the fact that postprandial TAG metabolism significantly contributes to the diurnal variation in FVIIa concentration, there is a relative paucity of studies which have investigated the effects of FVII polymorphisms on the interactions between TAG and FVII metabolism in the postprandial state (Silviera *et al.* 1994; Bentzen *et al.* 1996; Ghaddar *et al.* 1998). Silveira *et al.* (1994) investigated the interaction between the R353Q polymorphism and postprandial TAG metabolism in a group of hypertriacylglycerolaemic, post-infarction males with premature CHD. The study demonstrated that the Q allele was associated with lower fasting and postprandial levels of FVIIc and individuals with the R/R genotype had a greater proportion of FVII molecules in the active form (FVIIa : FVIIag ratio). Sanders *et al.* (1999) demonstrated that fasting and postprandial FVIIa levels were significantly lower in subjects with moderately elevated non-fasting TAG concentrations, but the postprandial increase in FVIIa concentration was independent of the R353Q polymorphism. The present study was designed to investigate how the R353Q and 10 bp promoter polymorphisms affect postprandial TAG and FVII metabolism in normotriacylglycerolaemic subjects. Postprandial FVII metabolism was investigated in a group of heterozygotes carrying both the Q and 10 bp alleles and a group of R/0 bp homozygotes who demonstrated an equimolar postprandial TAG response to a standard test meal. Therefore we could measure the effect of polymorphisms of the factor VII gene under identical conditions of postprandial TAG metabolism.

Materials and methods

Study design

This study was approved by the Ethics Committee of the Federated Dublin Voluntary Hospitals. Genotyping for the R/Q and 10 bp polymorphisms of factor VII and the postprandial TAG response was investigated in sixty-four individuals; twelve heterozygotes (seven males and five females) carrying both the Q and 10 bp alleles were identified and matched with twelve homozygotes (nine males and three females) for both polymorphisms, who demonstrated an equimolar postprandial TAG response. All subjects were healthy, free-living individuals. Postprandial levels of FVIIa and FVIIag were then measured in both groups.

Genotyping

Blood for DNA isolation was collected in 2 ml vacutainers containing EDTA, DNA isolation was performed using the DNA isolation procedure designed by Frosst *et al.* (1995). Polymerase chain reaction (PCR) was used to amplify segments of the FVII gene containing the polymorphisms. The PCR cocktail contained 1·25 mmol/l each deoxyATP, -CTP, -GTP, -TTP, 50–100 ng primer, 60 ml/l Mg^{2+} PCR buffer, 6 units Taq and 1 μl DNA solution. The factor VII genomic sequence (O'Hara *et al.* 1987) was used to design forward and reverse primers for use in amplifying a 214 bp fragment containing the R353Q polymorphism. The forward primer GGA GTA CAT GTT CTG TGC CGG and reverse primer GTG GCT CTG AGA GCT were synthesized on the PCR-MATE (391 DNA synthesizer; Applied Biosystems, Cheshire, UK) oligonucleotide synthesis machine. A 10 mM-Mg^{2+} PCR buffer was added to make a 25 μl PCR cocktail. The PCR programme began with a 7 min denaturation at 94°, then thirty cycles of 94° for 1 min, 55° for 1 min and 72° for 1 min 12 s, ending in a final extension for 5 min at 72° (Hybaid OmniGene, Middlesex, UK) (Marchetti *et al.* 1993). Digestion of 10 μl of the PCR product with MspI (New England Biolabs, Herts, UK) for 4 h at 37° resulted in fragments of 194 and 18 bp for the R allele and 125, 67 and 18 bp for the Q allele. The DNA fragments were visualized on a 20 g/l agarose gel. Primer 1 (AGG CTC TCT TCA AAT AAT TAC ATC) and primer 3 (CGG GCT GGC TCC TGG ATT T) were synthesized (GibcolBRL, Paisley, UK) to amplify the region containing the promoter polymorphism, a 20 mM-Mg^{2+} PCR buffer was added to make a 50 μl PCR cocktail and the programme was run as previously described (Marchetti *et al.* 1993). The DNA fragments were separated on 30 g/l agarose gel to show 439 and 133 bp fragments for the 0 bp allele and 439 and 143 bp fragments for the 10 bp allele.

Postprandial investigations

The study was conducted in the Nutrition Laboratory at Trinity College Medical School, St James's Hospital. The pre-prandial conditions, and timing and nature of the postprandial investigations were conducted according to a standard protocol (Roche & Gibney, 1997). All subjects completed the postprandial investigations in random order, between 08.00 and 09.00 hours following a 12 h overnight fast. The test meal was composed of a milk shake, consisting of 40 g sunflower oil (Flora; Unilever, Croydon, Surrey, UK) and 30 g dried skimmed milk powder (Marvel; Premier Beverages, Stafford, Staffs., UK) mixed with 150 ml skimmed milk. This provided 2·16 MJ energy, 15·8 g protein, 40·3 g fat and 23·4 g carbohydrate. It was consumed under supervision, within a 20 min period. Postprandial blood samples were drawn every 2 h for 8 h. Blood samples were immediately centrifuged at room temperature, the plasma was harvested, divided into portions, snap frozen (factor VII samples only) and stored at −70° until subsequent batch analysis.

Plasma TAG (TAG PAP, bioMerieux SA, Lyon, France), cholesterol (Cholesterol PAP, bioMerieux SA) and non-esterified fatty acid (NEFA, Randox Laboratories, Co. Antrim, UK) concentrations were determined in duplicate using enzymic colorimetric assays on a Technicon RA-XT analyser (Bayer, Dublin, Republic of Ireland). The TRL were isolated as previously described (Roche *et al.* 1998). The TAG inter-assay CV for 1·3 mmol/l and 3·1 mmol/l were 1·2 % and 1·1 % respectively, the cholesterol inter-assay CV for 4·0 mmol/l was 1·1 % and the non-esterified fatty acid inter-assay CV for 1·0 mmol/l was 8·1 %. Plasma LDL-cholesterol concentrations were estimated

using the Friedewald formula (Friedewald *et al.* 1972). Fasting, 4 and 8 h FVIIa concentrations were assessed using the Staclot VIIa-rTF kit (Diagnostica Stago, Paris, France), as previously described (Roche *et al.* 1998). The inter-assay CV for 37·5 mU/ml was 5·4 %. FVIIag concentrations at 0, 4 and 8 h were determined by an ELISA using specific rabbit anti-human factor VII antibody (Diagnostica Stago). The inter-assay CV for 77·6 % was 6·6 %.

Statistical analysis

Statistical analyses were completed with the Apple Macintosh compatible statistical package Data Desk 4.1 (Data Description Inc., Ithaca, NY, USA). TAG and FVII data were transformed to the natural log (ln). Repeated measures ANOVA investigated significant differences in the postprandial TAG and FVII responses. The postprandial data were also expressed in summary form, i.e. postprandial area under the curve and maximum postprandial levels. Pooled *t* tests investigated significant differences of these variables between genotypes. FVII genotype linkage analysis was completed using the Haplotype Program (Xie & Ott, 1993).

Results

Table 1 presents the main results of the study. There were no significant differences in the age, body weight and plasma lipid concentrations between the two groups, with the exception of plasma and LDL-cholesterol concentrations which were significantly greater in the R/0 bp homozygotes. Both genotypes demonstrated equimolar postprandial plasma and TRL-TAG concentrations. Fasting levels and the postprandial areas under the curves for FVIIa, FVIIag and FVIIa : FVIIag were significantly greater in the R/0 bp homozygotes. The incremental areas under the curves for FVIIa and FVIIag were greater (not significant) in the R/0 bp homozygotes than in the heterozygotes. Fig. 1 presents values for coagulation FVIIa and FVIIa : FVIIag during postprandial lipaemia. FVIIa increased significantly in the R/0 bp homozygotes, but there was no increase in postprandial FVIIa concentration in the heterozygotes. Repeated measures ANOVA demonstrated that FVIIa concentrations were significantly ($P \leq 0{\cdot}0001$) different between groups, and the genotype × time interaction approached significance ($P = 0{\cdot}06778$). The FVIIa : FVIIag value was significantly ($P = 0{\cdot}0003$) lower in carriers of the Q/10 bp alleles. The FVIIa : FVIIag value increased during postprandial lipaemia in the R/0 bp homozygotes but not in the heterozygotes, a difference which was almost significant ($P = 0{\cdot}0574$). Pearson product moment correlation analysis demonstrated that fasting and postprandial plasma TAG levels were significantly correlated with FVIIa (r 0·611, $P = 0{\cdot}035$; r 0·587, $P = 0{\cdot}045$) in the R/0 bp homozygotes but not in the heterozygotes carrying the Q/10 bp alleles.

Discussion

It has been proposed that up to 40 % of the phenotypic variation in plasma FVIIc can be explained by polymorphisms of the FVII locus (Bernardi *et al.* 1996). The R353Q and 10 bp polymorphisms of the FVII gene are of importance because they are relatively common and they are associated with significantly lower FVIIc levels, which may

Table 1. Age, body weight, fasting and postprandial lipid concentrations and factor VII levels in subjects homozygous for the R353R/0 bp polymorphisms of the factor VII gene and heterozygotes carrying the R353Q and 10 bp variants

(Mean values and standard deviations)

	R353Q/10 bp genotype (*n* 12)		R353R/0 bp genotype (*n* 12)		Statistical significance of difference between means
	Mean	SD	Mean	SD	
Age (years)	29·00	9·35	35·25	8·93	NS
Weight (kg)	73·18	11·47	82·46	17·12	NS
Fasting variables					
Plasma TAG (mmol/l)	0·98	0·41	0·99	0·41	NS
TRL TAG (mmol/l)	0·56	0·20	0·60	0·32	NS
Plasma cholesterol (mmol/l)	4·69	0·80	5·70	0·94	$P = 0{\cdot}01$
LDL-cholesterol (mmol/l)	3·41	0·81	4·29	0·85	$P = 0{\cdot}02$
Plasma NEFA (mmol/l)	0·45	0·16	0·51	0·20	NS
FVIIa (mU/ml)	23·9	6·5	43·0	4·8	$P \leqslant 0{\cdot}0001$
FVIIag (%)	71·6	7·7	85·7	5·4	$P \leqslant 0{\cdot}0001$
FVIIa : FVIIag	0·33	0·08	0·50	0·05	$P \leqslant 0{\cdot}0001$
Postprandial variables					
TAG C_{max} (mmol/l)	1·46	0·55	1·39	0·51	NS
TAG AUC (mmol/l per 8 h)	9·35	3·68	8·97	3·43	NS
TRL TAG AUC (mmol/l per 8 h)	6·31	3·15	5·785	3·02	NS
FVIIa AUC (mU/ml per 8 h)	194·0	58·4	396·4	74·7	$P \leqslant 0{\cdot}0001$
FVIIa IAUC (mU/ml per 8 h)	2·3	41·89	39·2	57·89	$P = 0{\cdot}0877$
FVIIag AUC (% per 8 h)	581·3	79·2	666·5	35·9	$P = 0{\cdot}0026$
FVIIag IAUC (% per 8 h)	8·3	38·8	–19·0	28·9	$P = 0{\cdot}0637$
FVIIa : FVIIag AUC	1·20	0·26	2·04	0·35	$P \leqslant 0{\cdot}0001$

TAG, triacylglycerol; TRL; TAG-rich lipoproteins; NEFA, non-esterified fatty acids; FVIIa, activated factor VII; FVIIag, factor VII zymogen; C_{max}, maximum postprandial concentration; AUC, area under the curve; IAUC, incremental area under the curve.

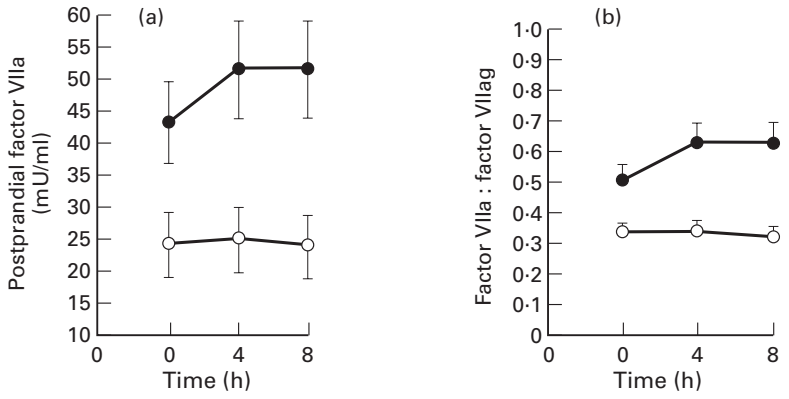

Fig. 1. (a) Postprandial activated factor VII (factor VIIa) concentrations and (b) the ratio factor VIIa : factor VII zymogen (factor VIIag) in subjects with the R353Q/10 bp (○) and R353R/0 bp (●) polymorphisms of the factor VII gene following ingestion of a fat-rich test meal. Values are untransformed group means for twelve subjects, with their standard errors represented by vertical bars. For details of subjects and procedures, see pp. 468–469.

protect against acute thrombotic events and CHD mortality. The frequency of the Q and 10 bp alleles (0·2) in this screened population of healthy Caucasians, from which subjects for postprandial investigations were drawn, was greater than the frequencies of the Q (0·11) and 10 bp (0·12) alleles which were previously reported in Europeans (Bernardi *et al.* 1997; De Maat *et al.* 1997). There is strong linkage disequilibrium between the polymorphisms, with the R and 0 bp alleles being on the same chromosome in 96 % of cases, and Q and 10 bp being on the same chromosome in 90 % of cases (Humphries *et al.* 1994). The subjects in the present study population also showed strong linkage (0·875) between the R and 0 bp alleles.

The present study demonstrated that fasting FVIIa and FVIIag concentrations were lower (44·3 % and 16·4 % respectively) in individuals carrying the Q and 10 bp alleles. This is in agreement with European data which demonstrated that heterozygotes for both polymorphisms had lower levels of FVIIa and FVIIag (39 % and 4·9%; Bernardi *et al.* 1997). It is unclear whether the R353Q and/or the 10 bp promoter polymorphisms determine factor VII status and how TRL interact with either or both of these polymorphisms. Two population-based studies strongly support the view that the 10 bp promoter polymorphism is most important (Humphries *et al.* 1996; De Maat *et al.* 1997). It has been proposed that TRL either directly or indirectly increase the rate of transcription from the 0 bp but not the 10 bp promoter allele, thereby affecting the synthesis of FVIIag mRNA and protein. This is supported by *in vitro* transactivation studies which demonstrated that the 10 bp polymorphism reduced promoter activity by 30 % (Pollak *et al.* 1996). However, *in vitro* transfection assays with FVII cDNA containing the base substitution resulting in the Q allele and wild-type factor VII cDNA in COS-1 cells demonstrated that the concentration of FVIIag was significantly reduced in the media of the COS-1 cells expressing the Q allele but the concentration of FVIIag was similar in the cell lysates (Hunault *et al.* 1997). Therefore the amino acid substitution as result of the R353Q polymorphism may result in reduced FVIIag secretion from the hepatocyte. Whilst it is unresolved whether the R353Q and/or 10 bp promoter polymorphisms determine fasting FVIIag concentration, it is probable that lower FVIIag concentrations partly account for the lower fasting levels of FVIIa in individuals carrying the Q and 10 bp alleles.

The present study provides new data in relation to the effects of genetic polymorphisms of the factor VII gene on the interactions between TAG and factor VII metabolism in the postprandial state in normolipaemic subjects. In this study heterozygotes, carrying both the Q and 10 bp alleles, were matched with R/0 bp homozygous subjects who demonstrated an equimolar postprandial TAG response. Whilst the R/0 bp homozygous subjects were slightly older and heavier, this difference was not significant. Plasma cholesterol concentrations were significantly greater in the homozygotes, but since the relationship between cholesterol and FVII is inconsistent (Mennen *et al.* 1996), it is unlikely that this would account for the difference between genotypes. The present study demonstrated that FVIIa increased significantly during postprandial lipaemia in the homozygotes but not in the heterozygotes carrying both the Q and 10 bp alleles. There was no significant change in FVIIag during postprandial lipaemia in either group. The proportion of activated factor VII, as measured by the ratio FVIIa : FVIIag, was lower in carriers of the Q/10 bp alleles compared with the R/0 bp homozygotes. During postprandial lipaemia the FVIIa : FVIIag value increased in the homozygotes but not in the heterozygotes. Whilst the precise molecular nature of the effect of TRL on factor VII metabolism is unknown, it is probable that events during lipoprotein lipase-mediated TRL lipolysis stimulate the conversion of FVII to its activated form FVIIa (Marckmann *et al.* 1998). The amino acid substitution resulting from the R353Q polymorphism could affect the conformation and reactivity of the FVII protein. It may be that the zymogen in those carrying the Q allele may be less reactive or that greater TRL concentrations are required to activate the FVII in individuals carrying the Q allele. Silveira *et al.* (1994) investigated the effect of the R353Q polymorphism,

but not the promoter polymorphism, on postprandial FVII metabolism in hypertriacylglycerolaemic men and showed that the postprandial increment of FVIIa in subjects carrying the Q allele was less than half of that demonstrated by the homozygotes. It is important to note that the subjects in that study were hypertriacylglycerolaemic and received almost twice the amount of fat, which would generate greater concentrations of postprandial TRL. This may explain why the hypertriacylglycerolaemic Q allele carriers showed attenuated postprandial factor VII activation, but the normolipaemic subjects carrying the Q allele in the present study demonstrated no increase in FVIIa during postprandial lipaemia. Sanders *et al*. (1999) also showed that FVIIa levels were significantly increased during postprandial lipaemia, an effect that was independent of R353Q genotype. It is of note that these subjects had greater postprandial TAG concentrations than in the present study. In another study, Ghaddar *et al*. (1998) failed to demonstrate any increase in FVIIa during postprandial lipaemia, irrespective of the R353Q polymorphism. The absence of an effect may be related to the fact that postprandial measures were taken at 3·5 h only. Clearly the mechanism(s) which determines FVIIa concentration and the complex interaction between genotype and plasma TAG level are not resolved. The present study provides important data, demonstrating that possession of the common Q and 10 bp alleles is associated with no significant increase in postprandial FVIIa concentration. This may represent a potential protective effect against CHD, whereby a normal postprandial lipaemic response is not associated with a pro-thrombotic state.

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