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ORIGINAL DATA

Effects of Different Protein Sources on Plasminogen Inhibitor-1 and Factor VII Coagulant Activity Added to a Fat-Rich Meal in Type 2 Diabetes

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■ Abstract

BACKGROUND: Exaggerated postprandial triglyceride concentration is believed to be atherogenic, and to influence the risk of thrombosis. Both elevated plasminogen inhibitor 1 (PAI-1) and increased factor VII coagulant activity (FVIIc) are potential important contributors to the increased risk of cardiovascular disease in type 2 diabetes. **AIM**: We aimed to investigate the effect of adding four different protein types (i.e. casein, whey, cod, and gluten) to a fat-rich meal on postprandial responses of PAI-1 and FVIIc in type 2 diabetic patients. **METHODS**: Twelve type 2 diabetic patients ingested four isocaloric test meals in random order. The test meals contained 100 g of butter and 45 g of carbohydrate in combination with 45 g of casein (Cas-meal), whey (Whe-meal), cod (Cod-meal), or gluten (Glu-meal), respectively. Plasma concentrations of PAI-1 and FVIIc were measured before meal, and at regular intervals for 8-h postprandially. **RESULTS**: The postprandial PAI-1 concentration decreased significantly by 49% to 56% in response to the four test meals. There were no significant differences between the outcomes from the four test-meals. The FVIIc levels decreased by 8% to 11% after the meals. Again, we observed no significant differences in outcomes between the four protein-enriched meals. **CONCLUSIONS**: The four proteins casein, whey, cod, and gluten, added to a fat-rich meal, all decreased the PAI-1 and FVIIc concentrations postprandially in type 2 diabetic subjects. However, postprandial levels of PAI-1 and FVIIc were not acutely influenced by the protein source.

Keywords: type 2 diabetes · coagulation · fibrinolysis · plasminogen inhibitor-1 · dietary protein · postprandial

Introduction

Coronary heart disease (CHD) is a major cause of morbidity and mortality in type 2 diabetic patients. It is assumed that diabetic patients carry the same cardiovascular risk as non-diabetics who had a prior myocardial infarction [1]. The substantially increased risk of CHD in type 2 diabetes is not fully explained by traditional risk factors, such as dyslipidemia, hypertension, obesity, and smoking [2]. Due to the importance of acute thrombosis in the pathogenesis of CHD, alterations in coagulation and fibronolytic function have emerged as strong predictors of CHD. Therefore, these effects have been recognized as part of the metabolic syndrome.

Plasminogen activator inhibitor-1 (PAI-1) is the primary physiologic inhibitor of plasminogen activation in blood. Therefore, it is the predominant inhibitor of the fibrinolytic system. Considerable evidence indicated that a high level of PAI-1 is a risk factor of CHD [3, 4]. In type 2 diabetes, PAI-1 expression in the arterial wall is higher than in CHD patients without diabetes [5, 6], and elevated

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PAI-1 concentrations can suppress fibrinolysis [7]. Therefore, increased PAI-1 production enhances the risk of vascular diseases in diabetes.

Abbreviations:
ANOVA - analysis of variance
BMI - body mass index
Cas - casein
CHD - coronary heart disease
Cod - cod
CV - coefficients of variation
FVII - coagulation factor VII
FVIIc - factor VII coagulant activity
Glu - gluten
PAI-1 - plasminogen inhibitor 1
SD - standard deviation
SEM - standard error of mean
tAUC - total area under the curve
Whe - whey

Coagulation factor VII (FVII) is the first enzyme involved in the extrinsic pathway of blood coagulation. High levels of FVII may lead to a prothrombotic state. Interestingly, the Northwick Park Heart study found a significant association between elevated FVII coagulant activity (FVIIc) levels and the incidence of fatal cardiac events [8]. Higher Levels of FVIIc have been found in both the first degree relatives of individuals with type 2 diabetes [9], and in subjects with type 2 diabetes [10].

Postprandial triglyceridemia was found to associate with CHD, increased PAI-1, and increased FVIIc levels [11, 12]. Postprandial lipemia may increase the risk of CHD, at least in part by increasing PAI-1 activity [13], and FVIIc [14].,Both are influenced by diet. Thus, high-fat meals acutely induce a dose-dependent elevation in FVIIc [14-19], whilst the impact on PAI-1 has been found to be inconsistent. Some research groups have found increased PAI-1 levels [13, 20], but others have found the opposite [21, 22].

Observational studies have indicated that dietary protein sources influence the risk of CHD [23]. Thus, high red meat intake apparently increases the risk of CHD, while CHD risk may be reduced by shifting sources of protein to dairy, poultry, and fish protein [23]. We have recently shown that whey protein, as compared with protein from casein, cod, and gluten, reduced triglyceride responses to a fat-rich meal in type 2 diabetic patients [24]. To the best of our knowledge, no investigations have been made to determine whether protein quality, or source, affects coagulation and fibrinolysis in type 2 diabetes. Therefore, we investigated whether different proteins, e.g. from whey, casein, cod, or gluten, added to a fat-rich meal, differentially influence postprandial responses of PAI-1 and FVIIc in type 2 diabetic subjects.

Methods

Subjects

Twelve type 2 diabetic patients (6 women and 6 men) were recruited from our outpatient clinic. They all had been diagnosed with type 2 diabetes for more than one year (4 ± 3.8 yr). The clinical characteristics of the subjects are shown in Table 1. Five patients were treated by diet only, 3 by metformin, 1 by sulfonylurea, and 3 by a combination of metformin and sulfonylurea. Insulin treatment was an exclusion criterion. All subjects continued their regular medication with unchanged dosage during the entire study period, except their antidiabetic medication, which was paused for 24 h, prior to each study day.

After oral and written information, all subjects gave their written, informed consent to participate in the study. The Central Denmark Region Committee on Biomedical Research Ethics approved the study.

Study design

According to a cross-over design, all subjects were examined on four separate occasions at intervals of ≥2 weeks between the four different isocaloric test-meals. The study design has been reported previously [24]. In brief, patients were randomly assigned to the sequence of the test meals using a balanced Latin square design. To reduce effects of diets ingested before the study, patients received a standard diet for 24 h before each test meal. This diet had the following energy distribution: 56% of energy as carbohydrate, 24% of energy as fat, and 20% of energy as protein. The energy density was 7000 kJ and 9000 kJ for female and male participants, respectively.

Subjects were instructed not to drink alcohol, or to perform hard physical activities, on the day before a test meal. Following a 12-h fasting period, the patients arrived at the clinic at 7:30 a.m. after a minimum of physical activity. A catheter was inserted into an antecubital vein. After 15 min of rest, a fasting blood sample (baseline) was drawn. Then, the test meal was ingested within 20 min. For the rest of the test day, the patients only consumed water, and rested at the clinic.

Table 1. Baseline characteristics of the type 2 diabetic patients

Variable	Mean ± SD	Range
Age (yr)	64.6 ± 3.3	60.0-69.0
Weight (kg)	83.4 ± 9.3	59.5-94.7
BMI (kg/m^2)	28.9 ± 3.7	21.7-35.0
Waist (cm)	102.5 ± 9.0	78.0-113.0
HbA1c (%)	6.8 ± 0.6	5.9-7.9
Fasting P-glucose (mmol/l)	7.9 ± 1.2	6.3-10.2
Fasting P-triacylglycerol (mmol/l)	1.4 ± 0.6	0.7-2.4

Legend: Values are mean ± SD. BMI: body mass index. n = 12 (6 women/6 men).

During an 8-h period, blood samples were drawn at 1, 2, 4, 6, 7, and 8 h after beginning of the meal for PAI-1 and FVIIc measurement. Blood was collected in citrated tubes. Plasma was immediately separated by centrifugation at 2000 x *g* for 20 min at 4ºC for the PAI-1 samples, and at 20ºC for the FVIIc samples. The Plasma samples were rapidly frozen and stored at -80º C.

The study has been registered on www.clinicaltrials.gov, ID: NCT00817973.

Test meals

Four isocaloric test meals were given to the subjects in a randomized order. All test meals consisted of an energy free soup enriched by 100 g of butter (Lurpak; Arla Foods amba, Viby J, Denmark), corresponding to 80 g of fat (68% of energy as saturated fat). 45 g of carbohydrate was added as white wheat bread (Läntmann Schulstad A/S, Hvidovre, Denmark). Finally, either 45 g casein protein (Cas-meal), 45 g whey protein (Whe-meal), 45 g cod protein (Cod-meal), or 45 g gluten protein (Glu-meal) was added to the test meal. The energy content and macronutrient composition of the test meals are shown in Table 2.

In all meals, butter was added to the soup, and heated in a micro wave oven to 60°C. Raw sliced leek (25 g) was added to the soup to make it more palatable. The casein protein used was calcium caseinate (Miprodan 40, kindly provided by Arla Foods Ingredients amba, Viby J, Denmark), a spray-dried pure milk protein. Half of the casein protein was added to the soup and the other half was dissolved in 200 g of water. The spray-dried whey protein (Lacprodan DI-9224, kindly provided by Arla Foods Ingredients amba, Viby J, Denmark) was dissolved in 200 g of water. For the Cod-meal, we used frozen cod fillet from the local market (Coop torskefilet, Royal Greenland A/S, Aalborg, Denmark). The cod fillet was minced and heated in the soup. The gluten protein (Gluvital 21000, kindly provided by Cerestar Scandinavia A/S, Charlottenlund, Denmark) was a dried, insoluble protein fraction separated from whole wheat with pure water. The gluten protein was added to the soup. All the test meals were served with water. The amount of water, both in food stuffs and added for preparation of meals, reached 675 g in all test meals. The test meals were ingested within 20 min.

Blood sampling and analysis

The protein concentration of PAI-1 was assessed by a commercially available enzyme-linked immunosorbent assay (TriniLIZE PAI kit from Trinity Biotech, Bray, Ireland). This assay measured PAI-1 in active state, inactive (latent) state, and in a form complexed to tPA and uPA. The coefficients of variation (CV) were 2.9% at 40 ng/ml, and 1.9% at 20 ng/ml.

FVIIc was determined using the ACL 9000 autoanalyzer from Instrumentation Laboratory Systems (ILS), Milan, Italy. FVIIc is a functional assay of FVII activity that depends on the concentration of the zymogen FVII antigen and the concentration of FVII circulating in the activated state. The plasma sample was diluted in FVII-depleted plasma obtained from ILS. Recombinant rabbit tissue factor (from ILS) containing $CaCl_2$ was added, and the coagulation time was recorded. The procedure is described in detail in Bladbjerg *et al*. [15].

Statistical analysis

The postprandial response data are shown as total area under the curve (tAUC), i.e. the total in-

Table 2. Energy and calculated nutrient content of the four test meals

Energy content	Cas-meal	Whe-meal	Cod-meal	Glu-meal
Total energy (kJ)	4850	4812	4855	4817
Fat (energy %)	68	68	68	68
Carbohydrate (energy %)	15	15	15	15
Protein (energy %)	17	17	17	17

Legend: Meals: energy-free soup plus 100 g of butter and 45 g of carbohydrate consumed with 45 g of casein (Cas-meal), 45 g of whey (Whe-meal), 45 g of cod (Cod-meal), or 45 g of gluten (Glu-meal).

Table 3. Concentration of plasminogen activator inhibitor 1 (PAI-1) and plasma factor VII coagulant activity (FVIIc) before and after ingesting the test meals

Variable	Cas-meal	Whe-meal	Cod-meal	Glu-meal
PAI-1 (ng/ml)				
Fasting	25.5 ±6.9	24.7 ± 10.0	26.8 ± 9.9	24.3 ± 7.6
Postprandial decrease (%)[§]	55 ± 13	56 ± 14	49 ± 10	50 ± 13
tAUC (% of standard · 480 min)	8332 ± 1735	8254 ± 2488	9877 ± 3525	8732 ± 3133
FVIIc (% of standard)				
Fasting	103 ± 28	98 ± 33	99 ± 32	103 ± 31
Postprandial decrease (%)[§]	9 ± 6	10 ± 6	8 ± 4	11 ± 7
tAUC (ng/ml · 480 min)	47165 ± 12853	44638 ± 13172	46273 ± 13639	46520 ± 14600

Legend: Values are mean ± SD. Test meals: energy-free soup plus 100 g butter and 45 g carbohydrate consumed with either 45 g casein (Cas-meal), 45 g whey (Whe-meal), 45 g cod (Cod-meal), or 45 g gluten(Glu-meal). n = 12 (6 females/6 males). Repeated measures ANOVA. No significant differences identified between the protein sources. [§] Difference between fasting and non-fasting nadir values in % of the fasting value. tAUC: total area under the curve.

crease above zero and the postprandial decrease (difference between fasting and non-fasting nadir values in percent of the fasting value). The trapezoidal rule was used to calculate the tAUC [25]. Comparisons between the four test meals were made by repeated measurement one-way analysis of variance (ANOVA). Normality was tested using the Shapiro-Wilk test. Where data were not normally distributed, the Friedman analysis of variance on ranks was applied. In cases of statistical differences between test meals, Tukey's multiple comparisons test was used (SigmaStat for Windows version 11; Systat software Inc. San Jose, USA). A p-value of <0.05 was considered significant.

Results

All subjects completed the study and ingested the test meals without any problems. We found no significant differences in fasting concentrations between test days (Table 3).

PAI-1

The concentrations of PAI-1 are shown in Figure 1. All four test meals induced a decrease in the postprandial PAI-1 response. After an 8-h observation period the PAI-1 concentration was reduced by about 50% compared with baseline PAI-1 concentration. We observed no significant differences in tAUC and the postprandial decrease among the test meals (Table 3).

FVIIc

The postprandial FVIIc responses are shown in Figure 2. The FVIIc concentration decreased slightly below baseline, after consumption of all four test meals. They all reached nadir within the first 4 hours, and approached baseline values after 8 h postprandial. We found no significant differences in tAUC and the postprandial decrease between the four test meals (Table 3).

Discussion

There is a growing interest in physiological and metabolic changes occurring during the postprandial phase, including the changes in the hemostatic system. This is relevant, since humans spend most of the time in the postprandial state, and coagulation factors are important protagonists in the development of CHD.

Our results indicate that four different protein sources, i.e. whey, casein, cod and gluten, derived from milk, fish, and vegetables, and added to a fat-rich meal, caused a postprandial decrease in both PAI-1 and FVIIc levels in type 2 diabetic subjects. There were no significant differences between the impacts of the four protein sources on postprandial PAI-1 and FVIIc levels. This indicated that the postprandial levels of PAI-1 and FVIIc were independent of the protein source. We found that PAI-1 decreased postprandially by 49-56% when protein was added. This shows a notably positive effect following the addition of protein to a fat-rich meal. However, some of the decrease could be

due to diurnal variation, as PAI-1 concentrations usually have a circadian rhythm, with a peak in the morning and a low in the late afternoon and evening [17, 26]. It has been proposed that the morning peak of PAI-1 relates to the higher incidence of myocardial infarction in the early morning hours [27].

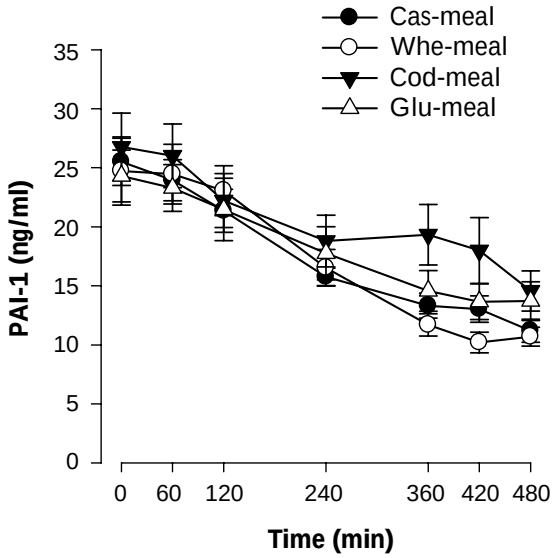

Figure 1. Plasminogen activator inhibitor 1 (PAI-1) responses to a test meal in 12 subjects with type 2 diabetes. The test meal consisted of an energy-free soup plus 100 g of butter and 45 g of carbohydrate consumed with either 45 g of casein (Cas-meal), 45 g of whey (Whe-meal), 45 g of cod (Cod-meal), or 45 g of gluten (Glu-meal). Data are mean ± SEM.

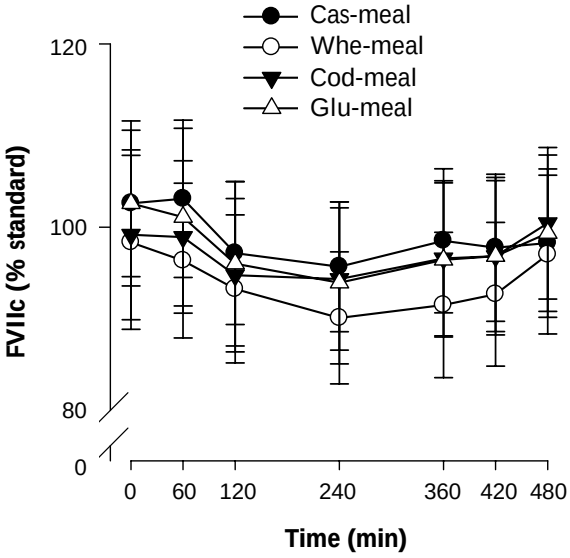

Figure 2. Plasma factor VII coagulant activity (FVIIc) responses to a test meal in 12 subjects with type 2 diabetes. The test meal consisted of an energy-free soup plus 100 g of butter and 45 g of carbohydrate consumed with either 45 g of casein (Cas-meal), 45 g of whey (Whe-meal), 45 g of cod (Cod-meal), or 45 g of gluten (Glu-meal). Data are mean ± SEM.

Our finding of decreased postprandial PAI-1 corroborates with previous studies. Sanders *et al.* compared meals containing 50 g fat with three different fatty acid compositions, and found that PAI-1 activity declined similarly with all three meals [22]. Oakley *et al.* studied the intake of 95 g fat and 41 g protein, which is comparable to the protein and fat content applied in our study [28]. Likewise, healthy non-diabetic subjects exhibited a similar decrease in PAI-1 activity after test meals with different fatty acid compositions. In line with these results, PAI-1 antigen has been found to decline postprandially [21, 29]. In contrast, Carroll and Schade reported a postprandial increase in PAI-1 activity after a high fat meal (70 g fat) in type 2 diabetic subjects [20]. These conflicting results may be explained by disparities in study design. Carroll and Schade conducted their study in the evening [20]. Thus, the difference may in part be explained by diurnal variations. However, in another study, an increase in PAI-1 antigen was even found after a high-fat meal (130 g fat) during daytime [13].

Regarding FVIIc, we expected a postprandial increase, since a transient rise in FVIIc after a fat-rich meal has consistently been shown previously [16, 22, 28-30]. The response is generally detectable 2-3 h postprandially, persisting for at least 8 h, and possessing dose-response characteristics [18]. Surprisingly, contrary to the previous results, we found a decline in the FVIIc concentration after all four protein supplements. This divergence from earlier studies may be due to different study populations. While earlier studies were carried out in healthy subjects, our study was conducted in type 2 diabetic subjects. However, the difference may reflect a direct effect of protein on FVIIc levels. Previously, we have demonstrated that whey protein causes a lower triglyceride increase than

casein, cod, and gluten proteins [24]. Increased triglyceride levels activate FVII, because this molecule adheres to the surface of large triglyceride-rich lipoprotein particles [31, 32]. Therefore, one might have expected a different effect. However, this could not be confirmed; but it should be noted that the FVIIc curve to whey tended to represent the lowest concentrations.

A postprandial increase in FVIIc appeared to be due to a rise in the plasma concentration of activated FVII [14,17,19]. Sanders *et al*. did not detect activation of FVII until fat intake was increased to 90 g or more [18]. We cannot exclude the possibility that the 80 g of fat (68% of energy from fat) in our test meals were too low to induce a lipemic response sufficient to activate FVII. However, Tholstrup *et al*. observed an increase in both FVIIc and activated FVII until 6 h postprandial using 50.6% of energy from fat [29]. Also, in a study by Silveira *et al.*, conducted in healthy human subjects, and in patients with manifested CHD, an increase in FVIIc was observed within 3 h of oral fat tolerance test consisting of 4180 kJ in total energy content, which accounted for 60.2% of energy from fat [19].

FVIIc measured in one-stage assays, as in the present study, reflects a mixture of FVII zymogen and activated FVII. Earlier studies have shown that the sensitivity to activated FVII depends on the origin of thromboplastin used in the assay [33-36]. We used recombinant rabbit tissue factor, also known as rabbit brain thromboplastin, to measure FVIIc in this study. Controversies exist regarding the sensitivity of activated FVII, when rabbit brain thromboplastin is used in one-stage clotting assays. With this assay, some detected a high sensitivity [33], whereas others found a low sensitivity [34, 35]. The reason for the discrepancy may be related to different rabbit brain thromboplastins, as demonstrated by Poggio *et al*. [36].

Anti-diabetic medication differed between the study subjects. Therefore, we cannot exclude the possibility that differences in medication could have influenced the results. However, the cross-over design of the study may have cleared this potential problem. Also, we ensured that all anti-diabetic medication was paused for 24 h preceding each study day.

The hemostatic variables were not among our primary effect variables in the power calculation (which was based on changes in triglyceride concentration) [24]. Therefore, we cannot exclude the possibility that a type 2 error caused the absence of a significant difference between the four protein types.

There is conflicting data regarding acute and chronic effects of fat composition on FVIIc levels. On the one hand, it is possible that the activation of FVIIc is not acutely influenced by dietary fat compositions [14, 16]. On the other hand, different background diets influenced postprandial FVIIc levels differentially [30, 37, 38]. Thus, it would be of interest to investigate the long term effects of different proteins on hemostatic variables.

In conclusion, our study showed that the four proteins casein, whey, cod, and gluten added to a fat-rich meal all suppressed PAI-1 and FVIIc concentrations postprandially in type 2 diabetic subjects. Long-term studies in type 2 diabetes are needed to clarify how different protein sources over time influence PAI-1 concentrations, which in turn reflect the fibrinolytic system, and the FVIIc concentrations related to the prothrombotic state.

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