Increased synthesis rate of fibrinogen as a basis for its elevated plasma levels in obese female adolescents

PRABHAKARAN BALAGOPAL,1 SHAWN SWEETEN,1 AND NELLY MAURAS2

1Nemours Research Programs and 2Division of Endocrinology, Department of Research, Nemours Children’s Clinic, Jacksonville, Florida 32207

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Balagopal, Prabhakaran, Shawn Sweeten, and Nelly Mauras. Increased synthesis rate of fibrinogen as a basis for its elevated plasma levels in obese female adolescents. Am J Physiol Endocrinol Metab 282: E899–E904, 2002.—Increased concentrations of plasma fibrinogen, an independent risk factor for cardiovascular disease (CVD), in obese children have been reported. The underlying mechanism for this, however, remains to be defined. In the current study, we measured the fractional synthesis rates (FSR) of plasma fibrinogen in six healthy postpubertal obese girls (body mass index [BMI]: 36.6 ± 1.8 kg/m²; age: 16.6 ± 0.5 yr) and six age-matched lean normal control girls (BMI: 20.8 ± 0.7 kg/m²; age: 16.4 ± 0.4 yr) during a primed, continuous infusion of L-[1-13C]leucine in the postabsorptive state. The method involved purification of plasma fibrinogen by use of immunoaffinity chromatography followed by measurement of [13C]leucine enrichment using gas chromatography-combustion-isotope ratio mass spectrometry. The FSR of fibrinogen in obese girls (35.06 ± 2.61%/day) was almost double that in lean girls (17.02 ± 1.43%/day), and this increase was associated with a relative increase in plasma concentration of fibrinogen as well as BMI in the subjects studied. Obese subjects had high fasting insulin levels (138 ± 0.3 mmol/l) compared with lean subjects (54 ± 11 pmol/l), whereas their glucose concentrations were similar (4.5 ± 0.3 mmol/l in obese and 4.4 ± 0.4 mmol/l in lean subjects), suggesting insulin resistance. The doubling of the FSR of fibrinogen provides novel insight into the mechanism of elevated levels of plasma fibrinogen and suggests a primary role for increased synthesis in producing hyperfibrinogenemia associated with obesity. This finding may have important implications in the design of therapies for modulating plasma fibrinogen levels in obesity and/or CVD in childhood.

cardiovascular disease; fractional synthesis rate; metabolism; obesity

OBESITY CAUSES AND/OR AGGRAVATES many health problems, both independently and in association with other diseases. In particular, it is reported to be associated with the development of cardiovascular disease (CVD) (11, 12, 23, 30). Factors that predispose individuals to CVD are thought to develop during childhood (4, 5). Besides being an acute-phase reactant protein, fibrinogen plays an important role in promoting atherogenesis and thrombogenesis. Recent studies have demonstrated that elevated plasma concentration of fibrinogen is an independent risk factor for CVD (8, 22, 24, 40). Plasma fibrinogen concentrations were even more strongly associated with cardiovascular death than plasma cholesterol (29). Several previous studies have clearly shown increased plasma fibrinogen concentrations in uncontrolled diabetes, vascular disease, and obesity (11, 30); however, the mechanisms that regulate plasma fibrinogen levels in vivo in these conditions are poorly understood.

There are several pathways by which acute or chronic increase in fibrinogen levels may lead to atherosclerotic and cardiovascular events. These include infiltration of the vessel wall by fibrinogen, rheological effects due to increased blood viscosity, increased platelet aggregation and thrombus formation, and increased fibrin formation. In a recent meta-analysis (13), a series of factors such as cigarette smoking, positive energy balance, diabetes mellitus, obesity, pregnancy, high dietary fat intake, increasing age, menopause, inflammation, thrombin, and vascular damage have all been identified to affect plasma fibrinogen concentrations. Genetic and environmental determinants are other important factors contributing to the changes in plasma concentrations of fibrinogen (22, 35). The concentration of plasma fibrinogen represents an imbalance between the production (synthesis) and the disposal (breakdown) rates of this protein. Although currently there is no viable method for the measurement of fibrinogen breakdown in humans, the ability to measure changes in synthesis rate of this protein could offer insight into the mechanisms that regulate this risk factor for CVD.

In humans, fibrinogen FSR was increased by acute insulin deficiency in type 1 diabetes (9) and was decreased by short-term insulin infusion in both normal subjects and subjects with type 1 diabetes (9, 10). Additionally, Tessari et al. (42) showed an acute stimulation of fibrinogen synthesis by glucagon. In a very recent study, Hunter et al. (21) reported a primary role for increased synthesis in producing hyperfibrinogenemia associated with smoking. Fu and Nair (16), how-

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ever, observed no increase in the synthesis rate of fibrinogen in older people compared with young subjects despite increased levels of plasma fibrinogen levels in the former group. They concluded that the elevated levels of plasma fibrinogen found in the older population are likely due to reduced breakdown of this hepatic protein. Many of the risk factors for CVD, including obesity, begin to develop in childhood. Understanding the impact of the fibrinogen synthesis rate on the elevation of plasma fibrinogen levels may better enable the development of therapeutic research toward alleviating this independent risk factor for CVD. The objective of the present study was to better understand the nature of changes in plasma fibrinogen concentration by measuring the FSR of fibrinogen in obese and lean adolescent females.

RESEARCH DESIGN AND METHODS

Materials

1-[1-13C]leucine [99% atom percent excess (APE)] was purchased from Cambridge Isotope Laboratories. Purchased lots of stable isotopes were tested for chemical, isotopic, and optical purity by gas chromatography-mass spectrometry (GC-MS). Solutions of the sterile and bacteria-free isotopes were passed through a 0.22-µm filter and stored in sterile, sealed containers <24 h before each infusion and kept at 4°C until used.

Study Subjects and Design

Six obese (BMI >30 kg/m²) and six healthy lean control adolescent girls (age >15 yr <18 yr; Tanner stage IV) were studied. These subjects were part of a larger study protocol that assessed whole body protein metabolism in these subjects, and some of their data have been published previously (28). The study protocol was approved by the Nemours Children’s Clinic Research Review Committee and Baptist Medical Center/Wolfson Children’s Hospital Institutional Review Committee. The subject characteristics are given in Table 1. All subjects consumed a weight-maintaining diet for ≥3 days before they were admitted to the Clinical Research Center (CRC) at the Wolfson Children’s Hospital. All studies were performed in the postabsorptive state with the constant infusion method proposed by Matthews et al. (26). During a primed (4.2 µmol/kg), continuous (4.2 µmol·kg⁻¹·h⁻¹) infusion of 1-[1-13C]leucine for 4 h, blood and breath samples were collected every 20 min from 180 min onward.

Analytical Procedures

Precursor pool enrichment. The plasma enrichment of α-[13C]ketoisocaproate ([13C]KIC), an index of intracellular leucine enrichment, was determined by GC-MS with selected ion monitoring and electron impact ionization of a t-butyldimethylsilyl derivative, as previously described (27, 38).

Purification of fibrinogen and measurement of [13C]leucine enrichments. Incorporation of labeled leucine into fibrinogen, produced by the liver and subsequently secreted into plasma, was measured after isolation (15), subsequent hydrolysis, and GC-combustion-isotope ratio mass spectrometry (GC-Com-IRMS) (2, 49). By use of the precursor-product relationship, the fractional synthesis rate (FSR) was calculated (46, 48). Briefly, the affinity column of fibrinogen antibody was prepared by coupling monoclonal anti-human fibrinogen antibodies (the anti-human fibrinogen antibody was generously supplied by Dr. S. Nair, Mayo Clinic, Rochester, MN) onto the Affi-gel 10 (Bio-Rad). The separation of the protein was carried out using an automated biological system (model ES-1, Bio-Rad), as described previously. The system allowed automatic control of buffer change, loading speed, and elution and collection. Approximately 500 µl of plasma were passed through the affinity column containing anti-human fibrinogen antibody. The bound protein was eluted with elution buffer (0.1 M sodium acetate with 2 N urea, pH 3.7) and collected for further treatment. The entire process was performed at room temperature. The purity of the protein separated was always ascertained by analytical SDS electrophoresis followed by silver staining. The separated proteins were precipitated with 10% trichloroacetic acid. The proteins were recovered by centrifugation and then hydrolyzed using 1 ml of 6 N HCl at 110°C for ~24 h. The hydrolysates were further purified by passing them through a cation exchange column containing 1 ml of AG 50W-X8, 100–200 mesh, H⁺ form. The dried amino acids were reconstituted in 0.01 M HCl and stored at ~80°C until MS analysis.

Table 1. Physical and clinical characteristics

<table>
<thead>
<tr>
<th>Group</th>
<th>Lean</th>
<th>Obese</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>6</td>
<td>6</td>
<td>NS</td>
</tr>
<tr>
<td>Age, yr</td>
<td>16.4 ± 0.4</td>
<td>16.6 ± 0.5</td>
<td>NS</td>
</tr>
<tr>
<td>Height, cm</td>
<td>159.7 ± 2.2</td>
<td>164.2 ± 2.8</td>
<td>NS</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>20.8 ± 0.7</td>
<td>36.6 ± 1.8</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>FFM</td>
<td>30.9 ± 1.4</td>
<td>55.9 ± 2.2</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>%Fat</td>
<td>31.0 ± 1.4</td>
<td>43.1 ± 0.8</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Glucose, mmol/l</td>
<td>4.5 ± 0.3</td>
<td>4.4 ± 0.4</td>
<td>NS</td>
</tr>
<tr>
<td>Insulin, pmol/l</td>
<td>54 ± 11</td>
<td>138 ± 47</td>
<td>0.05</td>
</tr>
<tr>
<td>FFA, mmol/l</td>
<td>0.41 ± 0.05</td>
<td>0.61 ± 0.04</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Albumin, g/l</td>
<td>4.57 ± 0.26</td>
<td>3.93 ± 0.11</td>
<td>0.04</td>
</tr>
<tr>
<td>Fibrinogen, g/l</td>
<td>1.95 ± 0.06</td>
<td>2.39 ± 0.07</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

Values are means ± SE. BMI, body mass index; FFM, Fat-free mass; FFA, free fatty acids; NS, not significant.

Body weight and height were measured using stadiometers and calibrated scales. BMI was calculated as body weight in kilograms divided by height in meters squared, and the 1994 clinical guidelines (19) were used to define obesity (BMI ≥30 kg/m²). The percent fat mass (%FM) was measured using the sum of the thickness of four skinfolds taken with calipers (βTechnology, Cambridge, MA). The concentration of fibrinogen was determined immunologically by automated laser rate nephelometry. Serum albumin concentrations were measured colorimetrically by the bromocresol green method with a kit from Sigma Diagnostics (St. Louis, MO). Free fatty acids (FFA) were also measured by colorimetric methods with a kit from Sigma Diagnostics. Plasma glucose was measured by a glucose oxidase method with a Beckman glucose analyzer (Beckman Instruments, Palo Alto, CA). Plasma insulin concentrations were measured by RIA at Endocrine Sciences Laboratories (Calabases Hills, CA).
Calculations

The FSR (expressed in %/24 h) of plasma fibrinogen was calculated by dividing the regression slope of isotope enrichment from 180 to 240 min of isotope infusion by the plasma plateau enrichments of $[^{13}C]KIC$ according to the precursor-product relationship

$$\text{FSR} = 24 \times 100 \times \frac{(E_{\text{fibrinogen}})}{(E_{\text{KIC}})}$$

where $E_{\text{fibrinogen}}$ is the slope of the $^{13}$C enrichment in the bound leucine residues in plasma fibrinogen from 180 to 240 min of isotope infusion (APE/h). $E_{\text{KIC}}$ is the plateau value of $^{13}$C enrichment of the precursor pool (plasma KIC and or plasma leucine), and 100 and 24 convert the result to a percentage and daily rate, respectively (46, 48). The absolute synthesis rate (ASR) of fibrinogen in the two groups was calculated as the product of FSR and the intravascular mass of fibrinogen (16, 21). The intravascular fibrinogen pool size was calculated by multiplying plasma fibrinogen concentration (in mg/l) by the plasma volume (in liters), and plasma volume was calculated by multiplying body weight by 0.045 (18, 31). The ASR is then expressed as milligrams per kilogram of body weight.

RESULTS

Subjects: Physical and Clinical Characteristics

Table 1 shows the physical and clinical characteristics of the study subjects. Each group, obese and lean control, consisted of six age-matched adolescent girls. By design, the obese subjects had significantly higher BMI (BMI > 35 kg/m$^2$; range 34.1–44.4 kg/m$^2$) than lean controls (BMI < 25 kg/m$^2$; range 19.4–22.9 kg/m$^2$). Moreover, height was similar between the groups. The %FM and fat-free mass (FFM) values were higher in the obese group ($P < 0.01$) compared with the lean control group (%FM range: 40.1–46.3 kg for obese and 28.0–35.2 kg for the lean; FFM range: 49.2–65.9 kg for obese and 33.1–42.4 kg for lean). The obese and lean control groups had similar levels of fasting glucose concentrations, whereas the fasting insulin levels were significantly higher in the obese group ($P < 0.05$). The concentration of plasma fibrinogen was significantly higher in the obese compared with the lean group (2.36 ± 0.07 g/l in the obese and 1.85 ± 0.06 g/l in the lean group; $P < 0.01$), and there was significant correlation between fibrinogen concentration and BMI ($r = 0.81; P = 0.003$). Similarly, FFA level was higher in the obese group than in the lean control group (0.61 ± 0.05 mmol/l for the obese and 0.43 ± 0.05 mmol/l for the lean controls; $P < 0.01$). The albumin concentration, however, was lower in the obese subjects than in lean control subjects (3.9 ± 0.3 g/l in obese vs. 4.6 ± 0.3 g/l in lean; $P = 0.04$).

FSR and ASR Fibrinogen

Figure 1B depicts the increase in tracer enrichment in fibrinogen between 180 and 240 min of isotopic infusion in the obese and lean control groups, showing a linear increment of isotope enrichment in fibrinogen in both groups. The regression slope of the line correlating the change of fibrinogen-bound leucine enrichment vs. time was consistently higher in the obese group than in the lean control group. The plateau value of $[^{13}C]KIC$ was used as the precursor pool enrichment (Fig. 1A) for the calculation of FSR of fibrinogen.

Figure 2A shows the FSR values of fibrinogen in the obese and lean groups. The FSR of fibrinogen in the control group, 17.02 ± 1.43%/day (range between 12 and 21%/day) on the basis of calculation with plasma KIC as the precursor pool, was less than that reported (~28%/day) in young adults (16) but close to that reported by Hunter et al. (21). The mean FSR of fibrinogen in the obese subjects, 35.06 ± 2.61%/day (range 22–44%/day), was almost double that in the lean control group. The FSR of fibrinogen showed statistically significant correlation between BMI ($r = 0.79; P = 0.002$) and plasma concentration of fibrinogen ($r = 0.74; P = 0.009$) in the subjects we studied (Fig. 3, A and B).

The ASR of fibrinogen was higher in the obese subjects (Fig. 2B) compared with the lean controls (37.6 ± 2.9 mg·kg$^{-1}$·day$^{-1}$ in obese children and 14.0 ± 1.2 mg·kg$^{-1}$·day$^{-1}$ in lean; $P < 0.01$). The intravascular fibrinogen pool size was also higher ($P < 0.01$) in the obese group than in the lean group.

DISCUSSION

The current study demonstrates an upregulation of the synthesis rate of fibrinogen in obese postpubertal young females compared with lean age-matched con-
controls. The almost doubling of the FSR of fibrinogen along with significant increase in the plasma fibrinogen concentration suggests a primary role for augmented synthesis of fibrinogen in producing the hyperfibrinogenemia in obese female adolescents. This increase in the FSR was directly associated with BMI and plasma fibrinogen concentration. In addition, the obese subjects had highly elevated fasting insulin levels, whereas the glucose levels were comparable in both groups. This suggests that the increased fibrinogen synthesis may likely be mediated through a mechanism involving insulin resistance. The observed increase in the FSR of fibrinogen in obese adolescent females provides new insight into the mechanism of elevated levels of plasma fibrinogen in these subjects and its potentially detrimental role as a CVD risk factor later in life.

Several studies have established the connection between elevated levels of plasma fibrinogen, obesity, and CVD (4, 6, 8, 14, 24, 25). However, it remains unclear whether an elevated plasma level of fibrinogen is a consequence of increased fibrinogen synthesis or decreased fibrinogen degradation. In addition to genetic and environmental factors (22, 35), impaired fibrinolytic capacity in CVD and obesity may contribute to hyperfibrinogenemia by limiting endogenous fibrinolysis (36, 37). It may, however, be noted that the substantially elevated level of plasma fibrinogen in obesity and CVD is not fully accounted for by this mechanism. The results from the current study, demonstrating higher rates of fibrinogen synthesis in obese than in lean control girls along with its increased plasma concentration, support the hypothesis that increased synthesis of fibrinogen plays an important role in the regulation of plasma levels of fibrinogen in childhood obesity, at least in females. Of interest, we found an increase of fibrinogen ASR (ASR = FSR × intravascular fibrinogen pool) in the obese subjects. However, in contrast to the direct measurement of FSR, the ASR of fibrinogen is an estimated value based on several assumptions. The ASR values are likely influenced by differences in body composition in the two groups, and it is possible that plasma volume is overestimated in the obese group. Additionally, because the ASR of fibrinogen is normalized for body weight, it potentially introduces an error, because the obese group obviously has higher amounts of fat. Regardless of these limitations, the increased ASR and FSR values in the obese group suggest that an upregulation of the synthesis of fibrinogen has a major role in increasing the plasma pool of fibrinogen in the obese subjects. The increase in the FSR of plasma fibrinogen was also significantly correlated with plasma fibrinogen concentration and BMI (Fig. 3, A and B).

The current study, however, is limited, only in postpubertal girls, and needs to be established and validated in a larger population, including both genders. The absolute values reported here are different from the previously reported values of FSR of fibrinogen in adult subjects (16, 44). The current study was performed in female adolescents, and there are no reference values available for FSR of fibrinogen in children with which to compare. However, previous studies have suggested that there are no gender-related differences in the fibrinogen synthesis rate in either young or old populations (16, 44). Although Fu and Nair (16) did not notice any gender effect on fibrinogen synthesis rate, they showed that there was an age-dependent

Fig. 2. A: fractional synthesis rate (FSR) of fibrinogen in the lean and obese groups. The FSR of fibrinogen in the obese group was almost double that in the lean group. B: absolute synthesis rate (ASR) of fibrinogen in the lean and obese groups. *P < 0.01.

Fig. 3. A: relationship between FSR of fibrinogen and body mass index (BMI, kg/m²); B: relationship between fibrinogen FSR and plasma concentration of fibrinogen (g/l). Fibrinogen FSR was significantly correlated with BMI and plasma fibrinogen concentration.
increase in the concentration of plasma fibrinogen that was not associated with an increase in the synthesis rate of fibrinogen. This is suggestive of a decrease in the breakdown of fibrinogen with advancing age. In contrast to the current study, the study by Fu and Nair was performed in a healthy aging population and not in obese young people, which may explain the difference. Additionally, it is unlikely that insulin, glucagon, and glucose homeostasis had a role in the regulation of fibrinogen synthesis in the study in the elderly population, as there was no significant difference in blood concentrations of insulin, glucagon, and glucose observed in the study by Fu and Nair. This is contrary to the apparent insulin resistance observed in the current study of obese girls.

Several assumptions were made for the calculation and interpretation of the data. We assumed that plasma α-KIC is in equilibrium with intrahepatic α-KIC. Previous studies have shown that plasma α-KIC enrichment is in reality close to the very low density lipoprotein apoB-100-bound leucine at plateau (7, 43, 45) and also to the leucyl t-RNA (1). Therefore, plasma α-KIC enrichment can be used as a valid surrogate precursor pool for the calculation of FSR of hepatic proteins. A recent study by Barazzoni et al. (5) has also demonstrated that hepatic venous leucine enrichment (likely to represent intracellular leucine in liver) is not different from circulating KIC enrichment. Therefore, on the basis of various studies cited (1, 5, 7, 43, 45), it is likely that the calculation of FSR of fibrinogen based on plasma α-KIC as the precursor pool enrichment is appropriate.

From basic mechanistic as well as therapeutic considerations, the results from this study have important implications. This study for the first time provides evidence to show that increased FSR of plasma fibrinogen has a major role in regulating the plasma concentration of this liver protein in obese postpubertal girls. The underlying molecular mechanism for these changes, however, remains unclear. Although it is well known that obesity is associated with resistance to insulin’s effects on glucose metabolism, it is not clear whether it is resistant to its effects on protein metabolism also. Conditions such as Cushing’s syndrome (41), old age (3, 17), and non-insulin-dependent diabetes (39, 47) are not associated with increased postabsorptive protein turnover. On the other hand, we observed that obesity in young postpubertal females is associated with insulin resistance in both peripheral carbohydrate and protein metabolism (28). In our study, the glucose concentrations during an intravenous glucose tolerance test (IVGT) in the obese vs. the lean controls were superimposable, similar to a glucose clamp, whereas the insulin concentrations during an IVGT were markedly different (28). This clearly demonstrates marked insulin resistance in the obese group that we studied. The increased FSR of fibrinogen in the obese subjects observed in the current study may thus be related to insulin resistance. Additionally, elevated levels of FFA, along with low levels of albumin, have been observed in the obese subjects compared with the lean subjects in the current study. Because albumin binds to FFAs and FFAs are strong stimuli of hepatic synthesis of fibrinogen, as shown in animal studies (20, 32, 34), changes in the FFA-to-albumin ratio may also contribute to the regulation of the synthesis of fibrinogen (20, 33, 34). Obviously, this needs to be studied further. It is also important to study further whether reported genetic variations in fibrinogen gene expression are important in modulating the response to specific environmental factors.

In conclusion, data from the present study show that, in obese adolescent females, the levels of plasma fibrinogen are elevated primarily because of the increased synthesis rate of this protein. This is an important step in elucidating the pathways through which fibrinogen levels are increased in obese adolescent females. This may contribute to the risk of CVD observed in obese patients and may be a consequence of insulin resistance. It is important to consider this aspect in the optimization therapies to control this independent risk factor in obesity and CVD. Future studies that investigate the effects and mechanisms of pharmacological and nonpharmacological interventions in the modulation of fibrinogen levels (along with other risk factors) in obese children and adults are warranted.

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