Correlation between Plasma Fibrinogen and Serum Lipids in Rats with Hyperlipidemia Induced by Cholesterol Free-High Fructose or High Cholesterol Diet

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(Received April 21, 1994)

Summary We studied the coagulative and fibrinolytic activity in intrinsic or extrinsic hyperlipidemia using 4-week-old male Wistar rats. Intrinsic hyperlipidemia was induced by a cholesterol-free high-fructose diet (HFD) and extrinsic hyperlipidemia, by a high-cholesterol diet (HCD) for 14 days. In intrinsic hyperlipidemic rats fed on the HFD, serum lipids were significantly increased as compared with the levels in control rats fed on a standard diet. An apparent increase in plasma fibrinogen level and coagulant factor XIII activity was also observed in HFD rats. In extrinsic hyperlipidemic rats fed on the HCD, significant increases in plasma fibrinogen level compared with that of control rats were found with the increases in serum lipids. Activities of antithrombin III and α₂-plasmin inhibitor in HFD-fed rats significantly increased compared with those of control and HFD rats. There was a significant positive correlation between plasma fibrinogen and serum total cholesterol, free cholesterol, or phospholipid in diet-induced hyperlipidemia (p < 0.01). Because of the increase in coagulant XIII activity in HFD-fed rats and the increase in α₂-plasmin inhibitor activity in HCD-fed rats, both diet-induced hyperlipidemic rats were shown to have enhanced coagulative activity compared with the control rats. These results suggest that the HFD as well as the HCD causes a pre-hypercoagulative state due to the increase in plasma fibrinogen level and activities in other coagulative and fibrinolytic factors.

Key Words fibrinogen, cholesterol free-high fructose diet, high cholesterol diet, hyperlipidemia, coagulation, fibrinolysis, rats

There is now strong epidemiological evidence that increased fibrinogen can be a significant risk factor for stroke, myocardial infarction, and ischemic heart disease (1,2). Fibrinogen increases progressively with the severity of coronary atheroscle-
rosis, and the plasma fibrinogen levels can serve as an independent indicator of the progression of coronary atherosclerosis (3). Cucuianu et al. reported that hyperlipidemic subjects had an increased concentration of plasma fibrinogen. The values were significantly higher in atherosclerotic patients than in hyperlipidemic subjects who did not show clinical atherosclerosis (4). All of these phenomena could be related to the role of fibrinogen in predisposing subjects to atherosclerosis. However, the role of fibrinogen and/or fibrin in the beginning and development of atherosclerosis is still uncertain. What we recognize from clinical papers is that hyperlipidemia can easily induce atherosclerosis and many fatty streaks are observed in the earlier stages of aortic atherosclerosis in humans (5).

On the other hand, abundant evidence exists from studies in laboratory animals that dietary cholesterol raises serum cholesterol concentrations in many species (6). Atherosclerosis induced by a high fat diet has been reported in several species including nonhuman primates (7–10). Therefore, diet composition plays a crucial role in the development of hyperlipidemia. However, there have been few experimental reports to reveal the correlation between plasma fibrinogen and serum lipid levels in diet-induced hyperlipidemic models, especially when a high fructose diet was used. Thus, we were urged to examine whether plasma fibrinogen levels increased or not in different types of diet-induced hyperlipidemia. Evidence that dietary cholesterol is highly atherogenic in many species including primates has bolstered the view that high cholesterol intake may also be atherogenic in humans. In addition to this extrinsic hyperlipidemic model fed on a high cholesterol diet (HCD) (11), intrinsic hyperlipidemia can be induced by feeding rats with a cholesterol-free high-fructose diet (HFD) (12, 13).

In the present work, we fed rats with an HFD and an HCD for 14 days to induce intrinsic and extrinsic hyperlipidemic models, respectively. To study the correlation between plasma fibrinogen and serum lipid levels, the coagulative and fibrinolytic activities were examined in both types of diet-induced hyperlipidemic rats and the involvement of diet-induced hyperlipidemia in the pathophysiological mechanisms of atherosclerosis is discussed.

MATERIALS AND METHODS

Animals and diets. Four-week-old male Wistar rats (Sankyo Labo Service Co., Japan) were housed individually under standard laboratory conditions (23 ± 2°C, 55 ± 15% humidity) and with 12-h light: dark cycle (lights on 0600–1800 h). The experimental protocol and the use of laboratory animals were in compliance with those provided by the Japanese Pharmacological Society. After prefeeding with a standard diet (MF, Oriental Yeast Co., Japan) for 5 days, rats were randomly divided into three groups (11–15 rats per group) and fed three kinds of diets for 14 days. Control rats were fed on a standard diet which contained 90 mg of cholesterol/100 g diet (MF, Oriental Yeast Co., Japan). Intrinsic hyperlipidemia was induced by feeding of a cholesterol-free high-fructose diet which contained 68
Table 1. Composition of the cholesterol-free high-fructose diet.

| Ingredient        | g/100 g |
|-------------------|---------|
| Fructose          | 68.0    |
| Casein            | 23.7    |
| DL-Methionine     | 0.3     |
| Corn oil          | 1.0     |
| Minerals¹         | 4.0     |
| Vitamins¹         | 1.0     |
| Cellulose         | 2.0     |
| Choline chloride  | 0.01    |

¹Minerals and vitamins are provided according to Iwata's method (13).

Table 2. Composition of the high-cholesterol diet.

| Ingredient        | g/100 g |
|-------------------|---------|
| Sucrose           | 50.0    |
| Casein            | 20.0    |
| Coconut oil       | 12.0    |
| Minerals¹         | 4.0     |
| Cholic acid       | 1.0     |
| Vitamins¹         | 0.5     |
| Cellulose         | 4.0     |
| Whitefish meal    | 7.5     |
| Cholesterol       | 1.0     |

¹Minerals and vitamins are provided according to Nakayama's method (11).

g of fructose/100 g diet (HFD: Oriental Yeast Co., Japan), and extrinsic hyperlipidemia was induced by a high cholesterol diet which contained 1 g of cholesterol/100 g diet (HCD: Oriental Yeast Co., Japan). The compositions of the HFD and the HCD are shown in Tables 1 and 2, respectively. Rats were given free access to tap water and each diet. We determined all items after fasting for 17 h.

Blood collection and preparation. Blood specimens were taken from the inferior vena cava with a plastic syringe and a silicon-coated needle under pentobarbital anaesthesia (40 mg/kg, i.p.). Whole blood from rats was mixed with a 3.2% sodium citrate solution in a volume ratio of nine to one, and then centrifuged at 3,000 rpm for 15 min at 4°C. The supernatant was used for examination of citrated plasma. Serum was also separated and used for lipid analysis.

Chemical assays. Activities of glutamic oxaloacetic transaminase (GOT) and glutamic pyruvic transaminase (GPT) in rat serum were determined by the method of POP-TOOS using a commercial kit (Transaminase C-II Test : Wako Pure Chemical Ind., Ltd., Japan). Total cholesterol, free cholesterol, phospholipid, and triglyceride contents in the serum were determined enzymatically (Cholesterol C-II
Test, Free Cholesterol C Test, Phospholipid B Test, and Triglyceride G Test, respectively). Nonesterified fatty acid was determined by the method of Duncombe \((14)\). These assays of serum lipids were performed using commercial kits (Wako Pure Chemical Ind., Ltd., Japan).

**Assay of coagulative and fibrinolytic activities.**

1) **Fibrinogen level:** The fibrinogen level was measured by a method previously reported \((15)\). Citrated plasma was mixed with \(\text{CaCl}_2\) and tranexamic acid (Sigma Chemical Co., St. Louis, MO, U.S.A.) and the mixture was incubated at \(37^\circ\text{C}\) for 30 min. After removal of the non-clottable proteins from the diluted plasma clot by centrifugation, the protein content of the fibrin precipitate was determined by the method of Lowry et al. \((16)\).

2) **Coagulant factor XIII and antithrombin III:** The activity of factor XIII was determined on the basis of the intensity of fluorescence from the dansylcadaverine complex by a fluorescence spectrofluorometer (650-10M Fluorescence Spectrophotometer, Hitachi, Japan). Antithrombin III activity was determined by synthetic chromogenic substrates. These assays were performed using commercial kits (F.XIII or Chromolate ATIII, respectively, Iatron Laboratories, Inc., Japan); these activities were shown as a ratio compared with those in normal human plasma.

3) **Plasminogen and \(\alpha_2\)-plasmin inhibitor:** Plasminogen assay was conducted by the chromogenic method using the synthetic chromogenic substrate (Kabi Diagnostica, Daiich Chemical Pharmacy Co., Ltd., Japan). We used urokinase (Mochida Pharmaceutical Co., Ltd., Japan) as the plasminogen activator. Activity of \(\alpha_2\)-plasmin inhibitor was determined by a method using the same synthetic chromogenic substrate mentioned above. These activities were shown as a ratio compared with those in normal human plasma.

**Statistical analysis.** Results are expressed as \(M \pm SD\) of 11 to 15 rats per group. Statistical significance was evaluated using Student's \(t\)-test and differences were judged significant when a \(p\) value was less than 0.05. The correlation between variables was assessed by a simple linear regression.

**RESULTS**

**Body weight and other biochemical determination in serum**

The body weight of all rats increased daily, but the body weight gain of rats fed on the HFD and the HCD was lower than that of the rats fed a standard diet. Therefore, the body weights of HFD- and HCD-fed rats were significantly lower than those of the control rats (Table 3), most likely resulting from the decrease in the daily intake of food in HFD- and HCD-fed rats (12–15 g/day) compared with that of the control rats (16–19 g/day). Serum GOT and GPT levels, which indicate liver injury, did not change significantly in rats fed the HFD and the HCD. In HCD rats, total protein and albumin in the serum were significantly higher compared to those of the control and HFD rats. However, the hematocrit value did

*J. Nutr. Sci. Vitaminol.*
DIET-INDUCED HYPERLIPIDEMIA AND FIBRINOGEN

Table 3. Comparison of animal weight, serum levels of GOT, GPT, total protein, and albumin between rats fed a standard diet, HFD, and HCD.

|                  | Control       | HFD           | HCD           |
|------------------|---------------|---------------|---------------|
| Body weight (g)  | 207.65±16.53  | 192.64±10.86* | 182.94±18.02* |
| GOT (KU)         | 69.67±12.97   | 68.03±13.62   | 71.92±14.06   |
| GPY (KU)         | 15.33±3.89    | 16.05±2.65    | 18.03±5.01    |
| Total protein (g/dl) | 4.67±0.34  | 4.89±0.19     | 5.59±0.45***  |
| Albumin (g/dl)   | 3.40±0.22     | 3.54±0.21     | 4.09±0.36***  |

Values are M±SD for 11-15 rats fed a standard diet (control), high-fructose diet (HFD), and high-cholesterol diet (HCD), respectively. *,**Significant difference from the control rat with p<0.05 and p<0.01, respectively. ***Significant difference from the HFD rat with p<0.01. Abbreviations used: GOT, glutamic oxaloacetic transaminase; GPT, glutamic pyruvic transaminase; KU, karmen unit.

Table 4. Serum total cholesterol, free cholesterol, triglyceride, phospholipid, and nonesterified fatty acid in rats fed a standard diet, HFD, and HCD.

|                  | Control       | HFD           | HCD           |
|------------------|---------------|---------------|---------------|
| Total cholesterol| 51.98±19.89   | 100.61±10.35* | 99.89±26.41*  |
| Free cholesterol | 7.99±2.51     | 16.87±1.85*   | 30.27±8.89**,* |
| Triglyceride     | 68.16±25.73   | 144.25±22.40* | 54.69±18.21** |
| Phospholipid     | 100.66±14.21  | 147.62±15.05* | 136.21±38.70* |
| Nonesterified fatty acid | 15.29±4.37 | 17.91±2.66   | 10.68±2.25**,* |

Values are M±SD for 11-15 rats fed a standard diet (control), HFD, and HCD, respectively. *Significant difference from the control rat with p<0.01. **Significant difference from the HFD rat with p<0.01.

Serum lipids

Table 4 shows serum lipid levels in rats fed the HFD and the HCD. In HFD rats, total cholesterol, free cholesterol, triglyceride, and phospholipid levels significantly increased compared with those of the control rats. In HCD rats, the apparent increase in total cholesterol, free cholesterol, and phospholipid except for triglyceride was observed, while nonesterified fatty acid decreased compared with that of the control rats.

Coagulation and fibrinolysis

Table 5 shows plasma fibrinogen levels and other coagulative and fibrinolytic activities in rats fed the HFD and the HCD. Fibrinogen levels in both hyperlipidemic rats significantly increased compared with those in the control rats (p<0.01 and p<0.001, respectively). The activity of coagulant factor XIII increased in HFD treated rats (216.49±79.12%) compared with that in the control rats.
Table 5. Coagulative and fibrinolytic activities in plasma in rats fed a standard diet, HFD, and HCD.

|                                  | Control          | HFD             | HCD             |
|----------------------------------|------------------|-----------------|-----------------|
| Fibrinogen (mg/dl)               | 373.63±104.55    | 493.21±83.89**  | 564.18±86.89****|
| Factor XIII (%)                  | 140.25±71.20     | 216.49±79.12*   | 122.0±33.27*****|
| Antithrombin III (%)             | 110.92±14.93     | 110.45±13.31    | 126.48±13.37****,****|
| Plasminogen (%)                  | 92.37±34.19      | 111.83±52.15    | 88.80±32.62     |
| α2-Plasmin inhibitor (%)         | 128.17±29.01     | 123.12±29.39    | 158.84±18.36****,****|

Values are M±SD for 11–15 rats fed a standard diet (control), HFD, and HCD, respectively. The activities of factor XIII, antithrombin III, plasminogen, and α2-plasmin inhibitor are shown as a ratio compared with those in normal human plasma. **,***,**** Significant difference from the control rat with p<0.05, p<0.01, p<0.001, respectively. **** Significant difference from the HFD rat with p<0.01.

Fig. 1. Correlation between plasma fibrinogen levels and serum lipid contents in rats fed a standard diet (control), HFD, or HCD. Plasma fibrinogen levels are shown as mg/dl (horizontal line) and serum total cholesterol (A), free cholesterol (B), triglyceride (C), and phospholipid (D) are also shown as mg/dl (vertical line). r, correlation coefficient; N.S., not significant.

(140.25±71.20%). In HCD-treated rats, antithrombin III activity increased from the levels of the control and HFD rats. No apparent change in plasminogen levels in either hyperlipidemic rats was observed, but α2-plasmin inhibitor activity was lower.
Fig. 2. Correlation between plasma fibrinogen levels and plasma antithrombin III and $\alpha_2$-plasmin inhibitor activities in rats fed a normal diet (control), or HCD. Plasma fibrinogen levels are shown as mg/dl (horizontal line) and plasma activities antithrombin III and $\alpha_2$-plasmin inhibitor are shown as a ratio compared with those in normal human plasma (vertical line). $r$, correlation coefficient.

significantly increased in HCD-treated rats (158.84±18.36%) compared with that in the control rats (128.17±29.01%).

Correlation between fibrinogen and lipids and other factors

In the control group and each diet-induced hyperlipidemic group, we observed no apparent correlations between serum lipids and plasma fibrinogen levels. In Fig. 1, the correlations between serum lipids and plasma fibrinogen levels in three groups of rats are shown. We found significant and positive linear correlations between plasma fibrinogen levels and serum total cholesterol ($r=0.437$, $p<0.01$), free cholesterol ($r=0.563$, $p<0.001$), and phospholipid ($r=0.453$, $p<0.01$), but not between the fibrinogen levels and triglyceride or nonesterified fatty acid. Figure 2 shows correlations between plasma fibrinogen levels and activities of other factors. Antithrombin III activity increased progressively with an increase in plasma fibrinogen level ($r=0.424$, $p<0.01$). We recognized that $\alpha_2$-plasmin inhibitor activity also increased with the increase in plasma fibrinogen level ($r=0.463$, $p<0.01$).

DISCUSSION

In general, the development of atherosclerotic lesions is induced by a high-fat diet in many mammalian species including rats (9), rabbits (7), and swine (8). In this paper, we investigated the effect of diet-induced hyperlipidemia on coagulative and fibrinolytic activities in rats. Our results showed that the elevation of plasma...
fibrinogen levels correlated with serum cholesterol among control and diet-induced hyperlipidemic rats. We also found that the activity of coagulant factor XIII in intrinsic hyperlipidemia and the activities of $\alpha_2$-plasmin inhibitor and antithrombin III in extrinsic hyperlipidemia increased. All of these results except the antithrombin III increase might provide some insight into the pathophysiological mechanisms involved, that is, diet-induced hyperlipidemia induces the pre-hypercoagulative state in experimental models using rats.

We used HFD-fed rats as the intrinsic hyperlipidemic model and HCD-fed rats as the extrinsic hyperlipidemic model. Both model rats showed no apparent liver injury as seen from GOT, GPT, total protein, and albumin levels in the serum. In a previous study, Nakayama et al. reported that HCD-treated rats showed increases in liver lipids (total cholesterol, triglyceride, and phospholipid) and fatty degeneration in their liver after 14 days’ feeding (11). In addition, recent investigation in HFD-treated rats showed no increase in liver lipids and a fatty liver (17). In this paper, we observed that HFD-fed rats showed significant increases in serum total cholesterol, free cholesterol, triglyceride, and phospholipid. Because the HFD contains no cholesterol, the increase in serum total cholesterol was due to the enhancement of its synthesis in the liver (12). Various nutrients affect serum triglyceride levels. Dietary carbohydrate stimulates the production of VLDL-triglycerides in the liver and raises the serum triglyceride level (6). Several reports suggest that serum triglyceride concentrations are affected differently depending on the type of dietary carbohydrate. In baboons, a high intake of sucrose or fructose raises triglyceride levels more than glucose does (18). The ratio of the serum triglyceride level in rats fed a fructose diet to that in rats fed a glucose diet was 2.16–2.27 (12). In rats fed the HCD which contained 1% cholesterol, we could see a significant increase in serum total cholesterol, free cholesterol, and phospholipid levels but not triglyceride. Stewart-Phillips et al. reported that serum triglyceride decreased in cholesterol-fed mice (10). It is well established that the liver regulates plasma levels of cholesterol and triglyceride by secretion and transport of these lipids in the VLDL and by removal of lipoproteins by receptor-mediated endocytosis (19). When the excess cholesterol was given to rats, an autoregulation system in lipid metabolism is sure to operate.

Fibrinogen is not only a clotting factor but also an acute phase protein. It is an essential co-factor for platelet aggregation. In addition, a high level of fibrinogen is known to be an important risk factor in cardiovascular disease (1, 20). Several studies have demonstrated that fibrinogen and fibrin are associated with thrombus formation and atherosclerosis. Fibrinogen and degradation products of fibrinogen and fibrin are present in all samples of human aortic intima, and fibrin is a major component of many plaques (21). Additionally, fibrin migrates into smooth muscles cells and provides a source of fibrin degradation products. Fibrinogen and fibrin-derived products have been shown to stimulate the release of endothelial-derived growth factor that was mitogenic for both endothelial cells and fibroblasts (22). Using the Watanabe heritable hyperlipidemic rabbit, Mori et al.
concluded that increases in clotting factor VIII and fibrinogen may be significantly related to the progression of thromboatherosclerosis (23). Both pathological and experimental studies suggest that fibrinogen and fibrin may be multifactorial stimulating factors for atherogenesis.

In this paper using diet-induced hyperlipidemic models, HFD rats showed a pre-hypercoagulable state due to an increase in fibrinogen level and XIII factor activity that could facilitate fibrin clot formation. In hyperlipidemic subjects, the fibrin stabilizing factor XIII was found to have increased (24). On the other hand, HCD rats showed an increase in plasma fibrinogen levels with an increase in the activity of $\alpha_2$-plasmin inhibitor that inactivates the plasmin effect to dissolve fibrin clots. Both increases eventually induce the hypercoagulable state in HCD rats. However, HCD rats simultaneously induced a mild increase in antithrombin III activity. Since antithrombin III has an inhibitory effect on the activities of the coagulant factors of IXa, Xa, and thrombin, the effect of thrombin to convert fibrinogen to fibrin becomes weak. The increase in antithrombin III activity in HCD rats may have resulted from hemostatic balance regulations. A previous clinical study also reported that plasma antithrombin III levels were positively correlated with serum cholesterol and with plasma fibrinogen levels in hyperlipidemic patients (4); this is consistent with our data from three groups of rats (Fig. 2). In the present paper, since HCD-induced hyperlipidemic rats showed no significant increase in triglyceride, we can conclude that the increase in fibrinogen in hyperlipidemic rats is mainly due to the increase in serum total cholesterol and free cholesterol. In another clinical study, it was reported that the fibrinogen level was correlated to serum cholesterol, not to triglyceride concentration in Swedish men aged 54 years (25). However, our previous study showed that the increase in plasma fibrinogen level also was related to the increase in triglyceride concentration in Triton WR-1339-induced hyperlipidemic rats (26). Some studies have suggested that the increase in plasma triglyceride accompanied with the increase in cholesterol may be important in the pathogenesis of ischemic heart disease (27). Simpson et al. reported that severe hypertriglyceridemia had significantly higher concentrations of plasma fibrinogen that did a normolipidemic comparison group (28). From these results, cholesterolemia with high levels of triglyceride may be associated with a hypercoagulable state. It is possible that HFD-induced hyperlipidemia induces thromboatherosclerosis as well as HCD-induced hyperlipidemia. Studies involving observation for a longer period with both types of diet-induced hyperlipidemic animals are necessary to elucidate the relationship between fibrinogen and atherosclerotic lesion formation.

It is not yet clear what mechanisms caused hyperlipidemia to induce an increase in plasma fibrinogen levels. Whatever the mechanisms might be, our data showed that the HFD and the HCD induced hyperlipidemia both intrinsically and extrinsically, respectively, and caused the pre-hypercoagulative state due to the increase in plasma fibrinogen level and activities of other coagulative and fibrinolytic factors. We can hypothesize that arterial fibrin from fibrinogen and lipid
deposition are the essential features of the pathogenesis of atherosclerosis.

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*J. Nutr. Sci. Vitaminol.*
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