Enhancement of the Coagulation System in Spontaneously Hypertensive and Hyperlipidemic Rats

Hidetoshi Amagasa¹, Masako Okazaki¹, Shinichi Iwai¹, Toshio Kumai², Shinichi Kobayashi², and Katsuji Oguchi¹

¹ Department of Pharmacology, Showa University School of Medicine, Tokyo, Japan.
² Department of Pharmacology, St. Marianna University School of Medicine, Kawasaki, Japan.

As a risk factor for cardiovascular and cerebrovascular disease, hypertension and hyperlipidemia are believed to provoke vascular damage leading to a hypercoagulative state. The aim of the present study was to investigate the coagulative and fibrinolytic activity and hepatic mRNA expression of the coagulative factors in spontaneously hypertensive and hyperlipidemic female rats (SHHR: > 150 mmHg of systolic blood pressure, > 150 mg/dl of plasma cholesterol). Plasma levels of fibrinogen, thrombin-antithrombin III (ATIII) complexes and ATIII in the SHHR at 9 months of age increased significantly compared with those of age-matched Sprague-Dawley rats (SD). In the SHHR, the hepatic mRNA expression of the α- and β-chains, but not the γ-chain of fibrinogen and prothrombin was significantly enhanced. Therefore, the hyperfibrinogenemia in the SHHR was demonstrated to be due to the increase in hepatic mRNA expression of α- and β-chains of fibrinogen. The pathological findings of the aortic arch from the 9-month old SHHR were cytoplasmic vacuolization and intimal thickening in the endothelium. These results suggest that hypercoagulation concomitant with the increase in hepatic mRNA expression of fibrinogen components may contribute to the development of atherosclerosis in the SHHR. J Atheroscler Thromb, 2005; 12: 191–198.

Key words: Fibrinogen, mRNA, New animal model, SHHR

Introduction

Much attention has been directed to life-style related diseases such as hypertension, hyperlipidemia and obesity, which have the probability of developing into atherosclerosis. Several epidemiologic studies have shown that hypertension and hyperlipidemia are the most important risk factors for cerebrovascular and cardiovascular diseases, for example stroke and ischemic disease (1, 2). Atherosclerotic vascular disease is responsible for the majority of cases of coronary heart disease, cerebrovascular disease and peripheral arterial disease in both developing and developed countries (3). Furthermore, there is a close correlation between cerebral and myocardial infarction and the thrombosis of a major supply vessel with atherosclerotic change (4). Fibrinogen is a well-known risk factor for cardiovascular disease as well as hyperlipidemia and hypertension, and the plasma fibrinogen level is predictive of coronary events, for instance sudden death, myocardial infarction or angina pectoris (5–7). Clinical studies have reported that patients with hypertension and hyperlipidemia happened to have strokes and demonstrated that improving lipid metabolism and lowering blood pressure were the most effective means of preventing cardiovascular events in hypertensive patients with dyslipidemia (8–10). However, there are few reports using animal models with hypertension and hyperlipidemia, because there is no suitable
animal model. Therefore, the development of an animal model will help us to study the effects of therapeutic and/or prophylactic agents, and to investigate the mechanisms of the induction and promotion of cardiovascular and cerebrovascular diseases in hypertensive and hyperlipidemic patients. To develop a new combination hypertension and hyperlipidemia model, Kumai et al. crossbred spontaneously hypertensive rats (SHR) with spontaneously hyperlipidemic rats (HLR) (11), and a new strain of spontaneously hypertensive hyperlipidemic rats (SHHR) was established at generation 10 through selective mating of brothers and sisters (12). SHHR had a systolic blood pressure (SBP) about 150 mmHg and a plasma cholesterol concentration of over 150 mg/dl. They reported that plasma levels of chatecholamine and low-density lipoprotein expression were higher in SHHR than in crossbred Wistar Kyoto rats (WKY) and Sprague-Dawley rats (SD), but coagulation and fibrinolysis in this model was not examined. Therefore, we investigated the plasma levels of coagulative and fibrinolytic activity with morphological changes in the aortic arches of the SHHR. In addition, the hepatic mRNA levels of the component chains of fibrinogen and prothrombin in this model were also examined. The purpose of this study was to examine the potential role of hypercoagulation in the SHHR as an atherosclerotic model.

Materials and Methods

Experimental animals and blood collection
Female spontaneously hypertensive hyperlipidemic rats (SHHR) at 9 months of age were used as experimental animals, because our preliminary experiments showed that plasma concentrations of total cholesterol (TC) and triglyceride (TG) were higher in female than male SHHR, and we already reported that a hyperlipidemic model resulted in hypercoagulation involving an increase in the plasma fibrinogen concentration (13–15). Eight-week old male spontaneously hyperlipidemic rats (HLR) that were originally raised from the SD at St. Marianna University School of Medicine, and the female SHR were interbred, and the SHHR were generated in the breeding colony by brother-sister mating. All procedures were performed according to the guiding principles for the care and use of laboratory animals of The Japanese Pharmacological Society. The rats were housed in a semi-barrier system under controlled room temperature (23 ± 1°C), and lighting (from 6:00 am to 6:00 pm). They were fed on a normal diet (CE-2, Clea, Tokyo, Japan) and tap water. SBP was 122 ± 3 mmHg for the SD and 160 ± 2 mmHg for the SHHR at 9 months of age. Blood specimens were taken from the inferior vena cava under pentobarbital anesthesia and mixed with a 3.2% sodium citrate solution at a ratio of 9:1. The citrated plasma supernatant was used for chemical assays and assays of coagulation and fibrinolysis.

Plasma biochemical assays
TC and TG levels in the plasma were measured using a colorimetric method (Cholesterol-B Test and Triglyceride-G Test: Wako Pure Chemical Ind., Tokyo, Japan). Plasma levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were determined using the Transaminase C-II Test (Wako Pure Chemical Ind.). Plasma levels of lipid hydroperoxides (LPO) were determined using a modified version of Yagi’s assay (16) with a commercially available kit (Kyowa Medex, Tokyo, Japan).

Plasma fibrinogen levels were measured as previously reported (13). Briefly, citrated plasma was mixed with CaCl₂ and trans 4-aminomethyl cyclohexane carbonylic acid (Sigma-Aldrich St. Louis, MO, USA) and the mixture was incubated at 37°C. After removal of the non-clottable proteins from the diluted plasma clot by centrifugation, the protein content of the fibrin precipitate was determined. Plasma levels of antithrombin III (ATIII), α₂-plasmin inhibitor (α₂-PI) and plasminogen were determined using a synthetic chromogenic substrate assay (Daiichi Chemical Pharmacy Co., Ltd., Tokyo, Japan). We used urokinase (Mochida Pharmaceutical Co., Ltd., Tokyo, Japan) as the plasminogen activator. The activity of plasminogen activator inhibitor (PAI) was determined using a commercially available kit (Biopool AB, Umeå, Sweden).

Reverse transcription-polymerase chain reaction (RT-PCR)
Total RNA was extracted from the median lobe of the liver. Approximately 150 mg of liver tissue was excised from a similar site on each lobe and homogenized with Isogen (Nippon Gene Co. Ltd., Tokyo, Japan) on ice. The yield of total RNA was between 2 and 6 mg/g of the wet liver tissue. Sense and antisense primers for rat genes of fibrinogen and prothrombin were selected by a computer program for PCR primer optimization using the published complete cDNA sequences. Each sequence of the three fibrinogen chains, prothrombin and β-actin is shown in Table 1. The RT reaction was performed in a 50 µl volume of reaction mixture containing 2.5 µg of total liver RNA, 125 U of murine leukemia virus (MuLV) reverse transcriptase (Perkin-Elmer Co., Branchburg, NJ, USA), 2.5 µM random hexamer and all four deoxynucleotide phosphates (dNTPs; 1 mM each) in PCR buffer at pH 8.3, consisting of 50 mM KCl, 10 mM Tris-HCl, 3.5 mM MgCl₂, and 50 U of RNase inhibitor. An initial mixture of total RNA, H₂O, and random hexamer was heated at 70°C for 10 min and chilled on ice. A complete reaction mixture composed of PCR buffer, dNTPs, MgCl₂, MuLV reverse transcriptase and RNase inhibitor was incubated at 42°C for 15 min, then heated at 99°C for 5 min before the RT reaction was stopped. The 25 µl PCR mixture was com-
posed of 5 µl of cDNA, 0.4 µM of each primer, 2 µl of each dNTP, and 1 U of Taq polymerase (Amply Taq®; Sigma-Aldrich) in a PCR buffer of 40 mM KCl, 8 mM Tris-HCl (pH 8.3), and 0.5 mM MgCl₂.

PCR of the α-chain and β-chain of fibrinogen mRNA was performed with a Takara PCR thermal cycler (Takara Shuzo, Tokyo, Japan) for 23 cycles at 94°C for 30s, 60°C for 30s and 72°C for 60s. For the γ-chain of fibrinogen mRNA, 26 cycles of 94°C for 30s, 53°C for 60s and 72°C for 60s were used. For the prothrombin mRNA, 28 cycles of 94°C for 30s, 55°C for 30s, and 72°C for 60s were used. As an internal standard, β-actin mRNA was amplified from a cDNA template and detected using a 26 cycle PCR with settings of 94°C for 30s, 60°C for 30s, and 72°C for 60s. A 10-µl volume from each reaction was resolved by 2% agarose gel electrophoresis, stained with ethidium bromide and photographed under ultraviolet light (Atto Corp., Tokyo, Japan). The photographic images were analyzed by computerized densitometric scanning using IPLab Spectrum P 3.1 software (Signal Analystics, Vienna, VA, USA). The level of target RNA from the liver of the SD and the SHHR was expressed as a ratio to that of the β-actin RNA.

Data are presented as the mean ± SEM. Comparisons between the SD and SHHR groups were made using a two-tailed Student’s t-test for unpaired observations. Differences with p values of less than 0.05 were considered to be significant.

Table 1. Sequences of oligonucleotide primers for RT-PCR.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence</th>
<th>Length</th>
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<tbody>
<tr>
<td>β-actin</td>
<td>sense 5'-TTGTAACCAAACGGCGAGATATGG-3'</td>
<td>764 bp</td>
</tr>
<tr>
<td></td>
<td>anti-sense 5'-GATCTTGGATCTTCATGAGTCTAGG-3'</td>
<td></td>
</tr>
<tr>
<td>α-Fibrinogen</td>
<td>sense 5'-TCCTGATACATCACAACCG-3'</td>
<td>604 bp</td>
</tr>
<tr>
<td></td>
<td>anti-sense 5'-TCCTACCTGCTGCCTCATCTGC-3'</td>
<td></td>
</tr>
<tr>
<td>β-Fibrinogen</td>
<td>sense 5'-CAGTACGCGACGATCCCTTCC-3'</td>
<td>743 bp</td>
</tr>
<tr>
<td></td>
<td>anti-sense 5'-CAGTACCACGATCCCTTCC-3'</td>
<td></td>
</tr>
<tr>
<td>γ-Fibrinogen</td>
<td>sense 5'-CATGCCATAGGACTGAGA-3'</td>
<td>411 bp</td>
</tr>
<tr>
<td></td>
<td>anti-sense 5'-CATGGTGTTTCCCTTCCAGG-3'</td>
<td></td>
</tr>
<tr>
<td>Prothrombin</td>
<td>sense 5'-CAAGCCTCGAACCAGA-3'</td>
<td>402 bp</td>
</tr>
<tr>
<td></td>
<td>anti-sense 5'-TCCTCGGCTGGGTGCATTCC-3'</td>
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Table 2. Background data on Sprague-Dawley rats (SD) and spontaneously hypertensive hyperlipidemic rats (SHHR) at 9 months of age.

<table>
<thead>
<tr>
<th></th>
<th>SD</th>
<th>SHHR</th>
</tr>
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<tbody>
<tr>
<td>BW (g)</td>
<td>311.6 ± 3.2</td>
<td>305.3 ± 12.3</td>
</tr>
<tr>
<td>L/BW (× 10⁻²)</td>
<td>3.6 ± 0.1</td>
<td>4.5 ± 0.1*</td>
</tr>
<tr>
<td>TC (mg/dl)</td>
<td>53.1 ± 5.2</td>
<td>179.2 ± 11.6*</td>
</tr>
<tr>
<td>TG (mg/dl)</td>
<td>261.2 ± 23.6</td>
<td>450.2 ± 51.2*</td>
</tr>
<tr>
<td>AST (IU/l)</td>
<td>52.6 ± 9.8</td>
<td>139.7 ± 14.4*</td>
</tr>
<tr>
<td>ALT (IU/l)</td>
<td>6.5 ± 0.8</td>
<td>16.5 ± 1.4*</td>
</tr>
<tr>
<td>LPO (nmol/ml)</td>
<td>8.64 ± 8.14</td>
<td>11.62 ± 2.01</td>
</tr>
</tbody>
</table>

Values are the mean ± SEM for 8 to 17 rats per group. * shows a significant difference compared to SD at p < 0.01 (Student’s t-test). BW: body weight, L/BW: liver weight/body weight ratio, TC: total cholesterol, TG: triglyceride, AST: asparatate aminotransferase, ALT: alanine aminotransferase, LPO: lipid hydroperoxide

Results

Bio-physiological parameters in plasma

Table 2 shows the body weight (BW), ratios of liver weight/body weight (L/W/BW), and plasma levels of TC, TG, AST, ALT and LPO in the SD and the SHHR at 9 months of age. BW in the SD and the SHHR did not show any differences, while the ratio of L/W/BW and plasma levels of TC and TG were significantly higher in the SHHR than SD. Plasma levels of AST and ALT were mildly but significantly higher in the SHHR than SD. LPO did not differ between the SD and the SHHR at 9 months of age.

Coagulative and fibrinolytic activity

Figure 1 shows plasma levels of fibrinogen, TAT and
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ATIII in the SD and the SHHR at 9 months of age. The fibrinogen level in the SHHR increased significantly to 2.6-fold that in the SD. The TAT and ATIII levels in the SHHR increased significantly to 2.4-fold and 2.0-fold that in the SD, respectively. Figure 2 shows plasma levels of plasminogen, α2-PI and PAI in the SD and the SHHR at 9 months of age. Activities of plasminogen and α2-PI are shown as a ratio relative to normal human plasma. Values are the mean ± SEM. * and ** show significant differences compared to the SD at p < 0.05 and p < 0.01, respectively (Student’s t-test).

Fibrinogen and prothrombin mRNA levels in rat liver

Figure 3 shows the standard concentration curves of β-actin, prothrombin and α-, β- and γ-fibrinogen. PCR products for β-actin and target RNA were quantified by measuring fluorescence intensity. The fluorescence intensity increased with an increase in cycle numbers of PCR. The quantification of PCR products of β-actin and target RNA during the reaction was performed by measuring the fluorescence intensity. Figure 4 shows the hepatic mRNA expression of α-, β- and γ-chains of fibrinogen and prothrombin in the SD and the SHHR at 9 months of age. The hepatic mRNA expression of α-chain fibrinogen increased significantly to 146.5% in the SHHR compared with that in the SD (Fig. 4A). Hepatic mRNA expression of the β-chain of fibrinogen significantly increased in the SHHR to 146.3% of that in the SD rats (Fig. 4B). However, the hepatic mRNA levels of γ-chain fibrinogen did not increase in the SHHR compared with those in the SD (Fig. 4C). Prothrombin mRNA levels tended to increase mildly, but not significantly (Fig. 4D).
Hypercoagulation in SHHR

Morphological observation

As shown in Fig. 5B, light micrographs of the livers from the 9-month old SHHR showed many lipid droplets, which reflected fatty degeneration, compared with livers of the SD (Fig. 5A). Light micrographs of the aortic arch from the 9-month SHHR showed cytoplasmic vacuolization (Fig. 5C) and intimal thickening in the endothelium (Fig. 5D) but this was not the case in the SD.

Discussion

As a risk factor for cardiovascular and cerebrovascular disease, hypertension and hyperlipidemia are believed to provoke vascular damage that progresses to a hypercoagulative state. We found that the SHHR showed hypercoagulation due to a significant increase in the plasma levels of fibrinogen, TAT, ATIII and α2-PI with atheromatic lesions in the aortic arch compared with the SD. We also observed a increase in plasma plasminogen but not PAI activity in the SHHR. These data findings can be considered to be due to the hemostatic balance against the hypercoagulative state in the SHHR, because hypercoagulation is concomitant with an activation of fibrinolytic factors, such as the plasin-α2-PI complex and tissue plasminogen activator (17). We have already reported similar findings that mice and rabbits fed on a high-fat diet for 3 or 6 months showed an increase in plasma plasminogen compared with those fed a normal diet (14, 15). Thus, the SHHR were characterized as having coagulative and fibrinolytic homeostasis, despite an enhancement of blood coagulation.

Fibrinogen, as the major clotting factor involved in the hemostatic process, is a complex consisting of three different chains (α-, β- and γ-chains) encoded by three different genes (18, 19). During the synthesis of fibrinogen, the individual chains are translated, processed, assembled, and eventually secreted into the plasma as a mature fibrinogen molecule. Therefore, we investigated not only the plasma levels of the coagulative factors containing fibrinogen, but also the hepatic mRNA levels of α-, β- and γ-fibrinogen and prothrombin in the SHHR. We found, that the hepatic expression of mRNA for the α- and β-chains of fibrinogen was increased significantly in the SHHR compared with the SD. Imbalances in the intracellular levels of the α-, β- and γ-chains have been observed in hepatocytes from several species (18). In HepG2 cells, an enhancement of β-fibrinogen synthesis causes an increase in production of the other two chains of fibrinogen and surplus amounts of α- and γ-chains are maintained intracellularly (20). In addition, data from our laboratory suggest that the initial steps in fibrinogen assembly involve the formation of αγ and βγ dimers linked by disulfide bonds, because the mRNA levels of β-chain fibrinogen increased significantly at 1 hr and then the α-chain but not γ-chain increased at 3 hr after the defibrinogenation with batroxobin as a thrombin-like enzyme (21). Thus, the synthesis of the β-chain of fibrinogen is considered to be the rate limiting step in the synthesis of fibrinogen (19, 21). In the SHHR, a significant increase in hepatic mRNA levels of α- and β-chain fibrinogen and a mild increase in prothrombin were observed. The present study demonstrated that the hypercoagulation in the SHHR is implicated in the hyperfibrinogenemia due to the increase in the level of liver transcripts of the component chains of fibrinogen, which can directly induce hyperviscosity, red cell aggregation and thrombogenesis (17).

The increased fibrinogen concentration can be regarded as a predictor of cardiovascular risk and plays a key role in cerebrovascular and cardiovascular events (4–7). We found cytoplasmic vacuolization and intimal thickening in the endothelial cells of the aortic arch from the 9-month old SHHR. Most lipids derived from plasma low-density lipoproteins (LDL) are considered to be deposited in the atherosclerotic lesion, and the intravascular internalization and accumulation of cholesterol and its esters probably induces severe endothelial damage (22). Normal rats
Amagasa et al. are considered to be atherosclerosis-resistant species, because they have difficulty in maintaining high concentrations of plasma lipids, expressing plasma LDL and forming atheromas in their blood vessels due to feeding on a high-fat diet. However, not only the female SHHR, but also the male SHHR at 9 months of age showed a significant increase in plasma lipids compared with the SD at the same age. That is, plasma levels of TC were $215.9 \pm 13.6$ mg/dl in the female SHHR and $178.1 \pm 5.3$ mg/dl in the male SHHR, and plasma levels of TG were $450.2 \pm 51.2$ mg/dl in the female SHHR and $181.2 \pm 7.7$ mg/dl in the male SHHR. A Kumai et al. demonstrated a moderate but apparent expression of plasma LDL in the SHHR compared with the SD (12). Therefore, the SHHR is an atherosclerotic rat model with early vascular lesions characterized by a plasma lipoprotein pattern and hyperfibrinogenemia.

High blood pressure is known to be one of the most important factors promoting vascular dysfunctions such as vascular wall hyperplasia, decreased sensitivity to vasodilative substances and increased expression of adhesion molecules and cytokines in animal models (1). Joensuu et al. reported that systolic blood pressure, as well as serum total cholesterol, is the most important determinant of early atherosclerosis in the carotid artery (2). There was a significant positive correlation between hypertension and the fibrin monomer polymerization velocity, and hypertension and fibrinogen (23). High concentrations of fibrinogen have been found in developing atherosclerotic lesions and the plasma concentration plays an important role in the potential rate of fibrin formation. An elevated fibrin monomer polymerization rate is associated with ischemic cerebrovascular disease and is an independent risk factor for this disease (24). Sawada et al. reported that the plasma levels of thrombomodulin, which is localized to endothelial cells as a receptor of thrombin, decreased due to endothelial injury in deoxycorticosteron acetate-treated hypertensive rats (25). In addition, the blood prothrombin time was significantly shorter in SHR than WKY (26). Therefore, hypertension is suggested to injure endothelial cells and to induce a hypercoagulable state in rats. In clinical studies, the use of lipid-lowering treatments may significantly improve blood pressure control in subjects with both hypercholesterolemia and hypertension (9, 27). Since atherosclerotic vascular disease is an enormous public health problem, the SHHR model may be useful for the study of various kinds of therapeutic strategies for hypertension and hyperlipidemia-induced disease.

In this study using the female SHHR, we showed the presence of hyperfibrinogenemia with a moderate but significant increase in plasma ALT and AST levels and a severely fatty liver compared with that in the SD. However, these attributes in the SHHR were considered to

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**Fig. 5.** Light micrographs of the liver and the aortic arch from Sprague-Dawley rats (SD) and spontaneously hypertensive hyperlipidemic rats (SHHR) at 9 months of age. A and B are livers from the SD and the SHHR (HE × 100). C and D are the aortic arch from the SHHR (C: HE staining × 200, D: Oil Red O staining × 200). Arrows show cytoplasmic vacuolization (C) and intimal thickening in the endothelium (D).
be insufficient to suppress the function of liver cells, because our previous report showed that coagulation activity in vulnerable vulnerable liver cells isolated from streptozotocin-treated rats is retained and actually enhanced (28). In both men and women, most risk factors contribute to coronary heart disease, but the impact of individual risk factors may be different (29). In patients with angina pectoris, the levels of fibrinogen and von Willebrand factor antigen are independent predictors of subsequent acute coronary syndromes. Low fibrinogen concentrations characterize patients at a low risk of coronary events despite increased serum cholesterol levels (3, 4). Thus, trials with fibrinogen-lowering measures and/or antihypertensive treatment may be recommended using SHHR, which are a good model for investigating the initial changes during arterial disease and for examining the effects of therapeutic and prophylactic agents in atherosclerosis due to hypertension and hyperlipidemia.

In conclusion, the hypercoagulation due to the increase in the plasma levels of fibrinogen, TAT, ATIII and α2-PI was observed to be greater in the SHHR than the SD. In addition, the hyperfibrinogenemia and the increase in hepatic mRNA expression of fibrinogen components may contribute to the hypercoagulative state and the development of atherosclerosis in the SHHR.

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References


