Involvement of IL-6 and IL-6 Receptor in Fibrinogen Synthesis in the Liver of Triton WR-1339-induced Hyperlipidemic Rats

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Abstract. To clarify the mechanisms by which Triton WR-1339 causes an elevation in the plasma fibrinogen level, we studied the time courses of hepatic mRNA expression for β-chain fibrinogen, interleukin-6 (IL-6) and IL-6 receptor (R) by RT-PCR. After intravenous injection of Triton WR-1339 (150, 300 and 500 mg/kg) in Sprague-Dawley rats, the plasma level of fibrinogen and MCP-1 significantly and dose-relatedly increased from 12 to 24 hours (h). At 3 and 6 hours dose dependent increases were found in hepatic IL-6 and IL-6R mRNA expression with increases in the hepatic mRNA expression for β-chain fibrinogen as the rate-limiting step in fibrinogen synthesis. These results suggest that the increase in the plasma fibrinogen level is followed by the enhancement of hepatic mRNA expression of β-chain fibrinogen, IL-6 and IL-6R, that is, IL-6 and IL-6R may partly regulate the plasma fibrinogen level in Triton WR-1339-induced hyperlipidemic rats.

Hyperlipidemia and hyperfibrinogenemia have been reported as risk factors for cardiovascular disease (1). A great deal of evidence suggests that inflammation plays a role in the development of coronary heart disease (2,3). Triton WR-1339 is a nonionic surfactant that induces hyperlipidemia in rats (4). Triton WR-1339 causes hyperlipidemia via inhibition of lipolysis of triglyceride (TG)-rich lipoprotein (5-7) and increase in hepatic cholesterol synthesis by enhancing the activity of 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) reductase as the rate-limiting enzyme in cholesterol synthesis (8). Previous studies have shown that Triton WR-1339 could produce rapid dose-related increases in plasma lipids and fibrinogen (9). Moreover, we investigated the expression of the α-, β- and γ-chains of fibrinogen genes by RT-PCR. Triton WR-1339 caused a change in the mRNA expression of each chain of fibrinogen (10). However, the mechanism of the increase in the plasma fibrinogen level in acute hyperlipidemic rats induced by Triton WR-1339 remains unknown.

Elevated plasma levels of several markers of the inflammatory cascade have been shown to predict future risk of coronary heart disease (3). Recently, circulating interleukin-6 (IL-6) was assessed as one of the markers of coronary heart disease (2, 11). IL-6 has a pivotal role in stimulating the acute-phase response, which elevates the circulating concentrations of several plasma proteins including fibrinogen and C-reactive protein (CRP). IL-6 exerts its action via a plasma membrane receptor consisting of two subunits, a ligand-binding 80-kDa glycoprotein and a 130-kDa glycoprotein involved in signal transduction (12, 13). In a preliminary study, we determined the plasma IL-6 level to examine its involvement in hyperfibrinogenemia and showed the increase in the plasma IL-6 level in this model. This result suggests that IL-6 may be involved in the hyperfibrinogenemia in Triton WR-1339-induced hyperlipidemic rats.

In the present study, we aimed to investigate the involvement of IL-6 and IL-6R in the mechanism of hyperfibrinogenemia in Triton WR-1339-induced hyperlipidemic rats. Therefore, we studied the time-course changes of the hepatic expression of IL-6 and IL-6R genes with β-chain fibrinogen considered to be a rate-limiting chain for synthesizing fibrinogen. In addition to the in vivo study, we investigated the effects of Triton WR-1339 on the production of fibrinogen and IL-6R using HepG2 cells and demonstrated the direct effect of Triton WR-1339 on hepatocytes.

Materials and Methods

In vivo experiment. Animals and blood collection. Male Sprague-Dawley rats, 6 weeks old with a body weight of 170 – 190 g, were fed a stock diet (MR stock; Nihon Noso Ind., Kanagawa, Japan) and tap water ad libitum under standard laboratory conditions (21±2°C, 55±15% humidity). The experimental protocol and the use of
laboratory animals were in compliance with the guidelines provided by the Japanese Pharmacological Society. The rats were divided into four groups: a saline control group (n=30) and Triton WR-1339 (150, 300 and 500 mg/kg)-treated groups (n=90). Triton WR-1339 (p-isoctylpolyoxy-ethylenephenol formaldehyde polymer) was purchased from Nakai Chemical Ind., Ltd., Kyoto, Japan. After fasting for 18 h, each animal was administered Triton WR-1339 and a corresponding volume of saline solution via tail vein injection. At 3, 6, 12 and 24 h after Triton WR-1339 administration, blood specimens were taken from the inferior vena cava under pentobarbital anesthesia (40 mg/kg, i.p.) and mixed with 3.2% sodium citrate solution at a volume ratio of nine to one. The citrated plasma supernatant was used for chemical and cytokine assays.

Chemical and cytokine assays. The plasma fibrinogen level was measured as described previously (14). Briefly, citrated plasma was incubated with CaCl₂ and trans 4-aminomethyl cyclohexane carboxylic acid (Sigma Chemical Co., St. Louis, MO, USA) at 37°C to form fibrin. After removal of the non-clottable proteins, the protein content of the formed fibrin precipitate was determined by the method of Lowry et al. (15). Total cholesterol (TC) and triglyceride (TG) levels in the plasma were measured by a colorimetric method (Cholesterol-B Test and Triglyceride-G Test: Wako Pure Chemical Ind., Tokyo, Japan). The level of aspartate aminotransferase (AST) in plasma was determined using the Transaminase C-II Test (Wako Pure Chemical Ind.). IL-6, TNF-α and MCP-1 levels were determined using polyclonal antibodies specific to IL-6, TNF-α and MCP-1 using both immobilized and secondary antibodies from a rat IL-6 ELISA kit, TNF-α ELISA kit and MCP-1 ELISA kit (Bio Source International, Inc., Camarillo, CA, USA), respectively.

RT-PCR. Total RNA was extracted from the median lobe of rat liver. Approximately 150 mg of liver tissue was excised from a similar site on each lobe and homogenized with Isojen (Nippon Gene Co. Ltd., Tokyo, Japan) on ice. Sense and antisense primers for each mRNA were designed using a computer program for PCR primer optimization using published complete cDNA sequences. The sequence of the sense-primer was CGT-CAA-CTG-CAA-CAT-CCC-G and the antisense-primer was GAG-TAC-CAT-CAT-CCC-TTC-C. The rat RNA specific primers of IL-6 and IL-6R were synthesized. The sense-primer sequence of IL-6 was ATG-AAG-TTT-CTC-CCC-GCA-A and the antisense-primer was TAG-GCA-TAG-AGC-AGT-ATT (16). The sense-primer sequence of IL-6R was CTC-TCA-AGC-TAT-CCT-CTC-AG and the antisense-primer was CAG-AAG-TAG-AGA-GAG-GAG-GG (17). The sense-primer sequence of β-actin was TTG-TAA-CCA-ACT-GGG-ACG-ATA-TGG and the antisense-primer was CAG-AAG-TAG-CTC-AG and the antisense-primer was CAG-GTA-AGT-GCT-AGG (Clontec Laboratories, Inc., Palo Alto, CA, USA). Reverse transcription was performed in 50 μl of a reaction mixture containing 1 μg of total liver RNA, 50 μl of murine leukemia virus (MuLV) reverse transcriptase (Perkin-Elmer Co., Branchburg, NJ, USA), 2.5 mM random hexamer and 1 mM deoxynucleotide phosphate mix (dNTPs) in PCR buffer (pH 8.3), consisting of 50 mM KCl, 10 mM Tris-HCl, 5 mM MgCl₂ and 20 U of RNase inhibitor. An initial mixture of total RNA, H₂O and random hexamer was heated at 70°C for 10 min (10 min) and cooled. Then a complete reaction mixture composed of PCR buffer, dNTPs, MgCl₂, MuLV reverse transcriptase and RNase inhibitor was incubated at 42°C for 20 min. Heating at 99°C for 5 min stopped the RT reaction. For the amplification of cDNA, 25 μl of the PCR reaction mixture contained 5 μl of cDNA, 0.4 μM of each primer, 2 μl of 1 mM dNTPs mixture, 1 U of Ampli Taq ® DNA polymerase (Sigma Chemical Co.) in PCR buffer of 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 0.5 μl of 25 mM MgCl₂, PCR to detect the β-chain of fibrinogen mRNA was performed using 26-cycle PCR with a Takara PCR thermal cycler (Takara Shuzo Co. Ltd., Tokyo, Japan), with a cycle setting of 94°C for 30 sec, 60°C for 30 sec and 72°C for 60 sec. For IL-6 mRNA, we used 40-cycle PCR with a cycle setting of 95°C for 60 sec, 60°C for 60 sec and 72°C for 120 sec. For IL-6R mRNA, we used 33-cycle PCR with a cycle setting of 94°C for 30 sec, 54°C for 30 sec and 72°C for 60 sec. As an internal standard, β-actin mRNA was amplified from cDNA by 30-cycle PCR with a cycle setting of 94°C for 30 sec, 60°C for 30 sec and 72°C for 60 sec. From each reaction, 10 μl of PCR products were electrophoresed in an ethidium bromide-stained agarose gel with Tris-acetate/EDTA buffer (pH8.0) and visualized by ultraviolet transillumination (Atto Corp., Tokyo, Japan). Photographed images were analysed using the computer IPLab program (Signal Analytics, Vienna, VA, USA). The level of target RNA from the liver of Triton WR-1339-treated rats was expressed as the ratio to β-actin RNA. We confirmed that each band for β-actin was expressed stably.

In vitro experiment. Culture of HepG2 cells and Triton WR-1339 treatment. The human hepatoblastoma cell line, HepG2 cell was purchased from the Institute of Physical and Chemical Research (Saitama, Japan). HepG2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin G sodium, 100 μg/ml streptomycin sulfate and 25 μg/ml amphotericin B as fungizone in 0.85% saline (Intronig Corp., Carlsbad, CA, USA). At 37°C in a humified atmosphere of 5% CO₂ in air. After the cells had reached confluence (1x10⁶ cells per well), the medium was exchanged for FBS-free DMEM with or without Triton WR-1339 and left for 6, 12 or 24 h. The Triton WR-1339 was used at four concentrations (0.12 μg/ml, 1.2 μg/ml, 12 μg/ml and 120 μg/ml) for 6, 12 and 24 h. Following treatment, the medium was collected and the cells were scraped into 1 ml of phosphate-buffered saline (PBS). The protein concentrations of medium and cells were determined using the protein assay reagent (Bio-Rad Lab. Hercules, CA, USA).

Cytotoxicity assay. Activities of alanine amino transferase (ALT) were determined in the medium secreted from the Triton WR-1339-treated HepG2 cells using the commercial kit (Transaminase C-II Test: Wako Pure Chemical Ind.). In addition, we examined the effects of Triton WR-1339 on HepG2 cell survival by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrasodium bromide] assay (Chemicon International Inc., Temecula, CA, USA), which measures the cytotoxicity of various chemicals in cultured cells (18). Fibrinogen and human soluble IL-6R (hsIL-6R) analysis. Fibrinogen concentrations in the HepG2 cells medium were measured by enzyme-linked immunosorbent assay (ELISA) (19). Microtiter plates (96-well) were coated overnight at 4°C with a rabbit anti-fibrinogen antibody (Dako A/S, Glostrup, Denmark) diluted 1:800 in 0.05 M barbitone buffer (pH8.6). Bound protein was detected using a peroxidase-conjugated rabbit anti-fibrinogen antibody.
Table I. Changes in plasma lipids and AST levels in rats treated with Triton WR-1339.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>T-WR 150 mg/kg</th>
<th>T-WR 300 mg/kg</th>
<th>T-WR 500 mg/kg</th>
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<tbody>
<tr>
<td></td>
<td>TC (mg/dl)</td>
<td>61.4±11</td>
<td>61.4±30.9*</td>
<td>78.8±3.0*</td>
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<tr>
<td></td>
<td>TG (mg/dl)</td>
<td>38.0±2.8</td>
<td>118.4±57.0*</td>
<td>419.0±85.1*</td>
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<tr>
<td></td>
<td>AST (IU/l)</td>
<td>40.6±2.1</td>
<td>48.1±4.4</td>
<td>48.5±3.7</td>
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</tbody>
</table>

Each value is the mean ± S.E.M. of 6-8 specimens. Lipid (TC: total cholesterol, TG: triglyceride) content is given in mg/dl and AST is IU/l. *,** Significantly different from the saline control value at p<0.05 or p<0.01, respectively. All results were analyzed using Student’s t-test.
of the control values, respectively. Thereafter, at 6 and 12 h the expression of IL-6R mRNA decreased gradually. At 24 h after 150, 300 and 500 mg/kg of Triton WR-1339 administration, the expression of IL-6R mRNA had re-increased significantly to 1.1-fold, 1.5-fold and 1.6-fold of the control value, respectively.

In vitro experiment. Cytotoxicity of Triton WR-1339 in cultured HepG2 cells. Table III shows the effects of Triton WR-1339 treatment at 0.12, 1.2, 12 and 120 μg/ml for 6, 12 and 24 h on the HepG2 cell membranes by the leakage of ALT into the medium. Following treatment with 120 μg/ml of Triton WR-1339 for 12 and 24 h, the ALT level increased by

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**Table II. Changes in plasma IL-6 and MCP-1 levels in rats treated with Triton WR-1339.**

<table>
<thead>
<tr>
<th>T-WR</th>
<th>Control</th>
<th>3 h</th>
<th>6 h</th>
<th>12 h</th>
<th>24 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-6 (pg/ml)</td>
<td>39.8±0.3</td>
<td>41.6±0.5*</td>
<td>41.4±0.7*</td>
<td>41.6±0.9*</td>
<td>40.1±0.5</td>
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<td></td>
<td>300 mg/kg</td>
<td>42.8±1.0**</td>
<td>45.0±2.3*</td>
<td>42.1±1.4*</td>
<td>41.2±0.6*</td>
</tr>
<tr>
<td></td>
<td>500 mg/kg</td>
<td>42.5±0.3**</td>
<td>40.0±0.4**</td>
<td>47.2±2.6**</td>
<td>40.6±1.0</td>
</tr>
<tr>
<td>MCP-1 (pg/ml)</td>
<td>6.6±0.9</td>
<td>7.3±1.2</td>
<td>9.3±1.0*</td>
<td>12.1±3.4*</td>
<td>11.1±0.9**</td>
</tr>
<tr>
<td></td>
<td>300 mg/kg</td>
<td>10.0±1.9</td>
<td>16.5±2.6**</td>
<td>17.9±1.9**</td>
<td>21.6±3.9**</td>
</tr>
<tr>
<td></td>
<td>500 mg/kg</td>
<td>11.7±4.6</td>
<td>18.2±4.0**</td>
<td>38.3±9.7**</td>
<td>43.6±7.8**</td>
</tr>
</tbody>
</table>

T-WR: Triton WR-1339. Each value is the mean ± S.E.M. of 6-8 specimens. IL-6 and MCP-1 are given in pg/ml. *,** Significantly different from the saline control value at p<0.05 or p<0.01, respectively. All results were analyzed using Student’s t-test.

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Figure 1. Changes of plasma fibrinogen levels in rats after intravenous injection of Triton WR-1339. Rats were divided into four groups: a saline control group ( ) and 3 Triton WR-1339-treated groups ( □ 150 mg/kg, ■ 300 mg/kg, ■ 500 mg/kg). Values represent means±S.E.M. of 6-8 rats per group. *, ** significantly different from saline control rats at p<0.05 and p<0.01, respectively (Student’s t-test).
approximately 150% over the untreated controls. Treatment with the other three concentrations of Triton WR-1339 for 24 h caused a significant increase in ALT in the medium compared to the untreated cells. The MTT assay was performed to examine the cytotoxic effects of Triton WR-1339 on HepG2 cells. As shown in Table III, 120 ìg/ml of Triton WR-1339 for 24 h decreased the cell viability to 73% of the non-treated controls. From these results, experiments to examine the effects of Triton WR-1339 on fibrinogen synthesis in HepG2 cells were performed at concentrations of 0.12, 1.2 and 12 ìg/ml.

Effect of Triton WR-1339 on fibrinogen and hsIL-6R synthesis in HepG2 cells. Table IV shows ELISA results of a time-course for fibrinogen and hsIL-6R levels in the medium of HepG2 cells following treatment with Triton WR-1339. The fibrinogen levels were corrected for the equivalent untreated control. At 6-h treatment with all doses of Triton WR-1339, no significant changes in the fibrinogen levels in the medium were detected, but after a 12-h treatment with 1.2 and 12 ìg/ml of Triton WR-1339 the levels of fibrinogen in the medium were decreased. In contrast, the 24-h treatment with 0.12, 1.2 and 12 ìg/ml of Triton WR-1339 resulted in significantly increased fibrinogen levels in the medium of 141.2%, 111.3% and 120.9%, respectively compared to the equivalent control. The effect of Triton WR-1339 on hsIL-6R levels was determined in the medium of the HepG2 cells by ELISA. Triton WR-1339 treatment
with 0.12, 1.2 and 12 μg/ml for 24 h produced a slight increase in hsIL-6R levels in the medium.

**Discussion**

In the present study, we showed that the plasma fibrinogen level significantly increased dose-relatedly between 12 and 24 h and that the hepatic mRNA levels for β-chain fibrinogen peaked at 3 h, gradually decreased at 12 h and then tended to recover at 24 h after administration with 300 and 500 mg/kg Triton WR-1339. These results suggest that the increase in β-chain mRNA is the rate-limiting step before assembly of the peptide chain of fibrinogen, which is consistent with our previous findings (10). Additionally, the time-course changes in the hepatic mRNA levels for IL-6 and IL-6R determined by RT-PCR showed the expression of IL-6R mRNA increased at 3 h, decreased gradually at 12 h and then increased again at 24 h after injection of Triton WR-1339. On the other hand, strong expression of IL-6 mRNA was found with a peak at 6 h after injection of Triton WR-1339 compared to one of the control rats, in which only a small expression of IL-6 mRNA was observed. So the present study demonstrated that the hepatic mRNA level of IL-6R correlated with the mRNA level of β-chain fibrinogen in Triton WR-1339-induced hyperlipidemic rats.
Plasma IL-6 is mainly secreted by lymphocytes, macrophages, endothelia and fibroblasts. IL-6 modulates the transcription of acute phase response genes during acute and chronic inflammatory states (20, 21). Recently, evidence in support of the role of inflammatory processes in acute coronary syndromes comes from findings showing an increase in the IL-6 level (3). Jung et al. demonstrated that IL-6 mRNA expression in the liver parallels the elevation of the plasma IL-6 level (16). In untreated liver cells, the expression of IL-6 mRNA is slight, but it is promoted by hepatic stimuli such as the inflammatory response and infections. In Triton WR-1339-induced hyperlipidemic rats, a significant but small increase in the plasma IL-6 level was observed, but the hepatic expression of IL-6 mRNA was strongly enhanced, which suggests the possibility that IL-6, after being released into plasma, combines with IL-6R instantly.

Mackiewicz et al. demonstrated that soluble hepatic IL-6R was involved in the modulation of the liver response in acute and chronic inflammatory processes using HepG2 cells (12). Castell et al. demonstrated that IL-6 promoted fibrinogen synthesis in a dose- and time-dependent manner using human hepatocytes (22). In this in vitro study, we showed that Triton WR-1339 induced an increase in the level of hsIL-6R and fibrinogen secreted from HepG2 cells. In the in vivo study using Triton WR-1339-treated rats, the hepatic expression of IL-6 and IL-6R mRNA was enhanced significantly compared with that of control rats.
The simultaneous increases in the hepatic expression of IL-6 and IL-6R mRNA with the increase in β-chain fibrinogen suggest the possibility that IL-6 and/or IL-6R regulate the synthesis of fibrinogen as an acute phase protein. IL-6 was suggested to combine with the specific ligand-binding chain as IL-6R (80-kDa glycoprotein), which consists of the membrane-bound form and the soluble form. The complex of IL-6/IL-6R activates the intracellular signal cascade after the detection of Triton WR-1339 as a nonionic surfactant function. At the same time, the direct cytotoxicity of Triton WR-1339 was determined by MTT assay, which detects changes in the mitochondrial electron transport system. In the liver, the cytotoxicity of Triton WR-1339 was due to the direct injury of the hepatic cell membrane as a nonionic surfactant function. At the same time, the direct cytotoxicity of Triton WR-1339 was determined by MTT assay, which detects changes in the mitochondrial electron transport system (26). The high dose of Triton WR-1339 significantly increased mildly but significantly. So, Triton WR-1339 directly injured the hepatic cell membrane as a nonionic surfactant function. On the other hand, Nagorney et al. suggested that Triton WR-1339 accumulated in rat hepatocyte lysosomes and induced lysosomal fragility (25). We observed hemolysis and hemourine in rats after the injection of 300 and 500 mg/kg of Triton WR-1339. In the in vivo and in vitro study for the estimation of membrane integrity of the hepatocytes, AST in rat plasma and ALT in the HepG2 cell medium were increased mildly but significantly. So, Triton WR-1339 directly injured the hepatic cell membrane as a nonionic surfactant function. Therefore, the direct effect of Triton WR-1339 is likely to induce the temporal inflammation and/or the synthesis of fibrinogen as the acute-phase protein.

In summary, the increase in plasma fibrinogen in Triton WR-1339-induced hyperlipidemic rats is due to the increase in the hepatic expression of fibrinogen β-chain, which is associated with the increase in the hepatic mRNA level of IL-6 and IL-6R. The finding in the in vitro experiment using HepG2 cells complements our present in vivo study. These results suggest the mRNA expression of β-chain as the rate-limiting step before the assembly of the peptide chains of fibrinogen is associated with changes in hepatic IL-6 and IL-6R mRNA expression, that is, IL-6 and IL-6R may partly regulate plasma fibrinogen movement in Triton WR-1339-induced hyperlipidemic rats.
References


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