Nonalcoholic fatty liver disease (NAFLD) can develop into end-stage disease that includes cryptogenic cirrhosis and hepatocellular carcinoma. Bacterial endotoxin, for example lipopolysaccharide (LPS), plays an important role in the pathogenesis of NAFLD. The aim of this study was to assess the role of LPS in the development of NAFLD. Twenty-one male Zucker (fa/fa) rats were divided into three groups: rats fed for twelve weeks on a diet rich in disaccharide (D12 group), rats similarly managed but treated with LPS (LPS group), and those on the same diet for 24 weeks (D24 group). Histological examination demonstrated that this protocol induced hepatic steatosis in the LPS and D24 groups. Significant, marked accumulation of lipid droplets was observed in the LPS group, compared with the D24 group. Rats from the LPS group showed a decrease in plasma adiponectin levels, an increase in plasma leptin levels, and greater expression of FAS and SREBP-1c mRNA in the liver, compared with rats from the D24 group. These findings coincided with histological findings. We therefore suggest that LPS may accelerate the progression of hepatic steatosis.

**Key Words:** nonalcoholic fatty liver disease (NAFLD), lipopolysaccharides (LPS), disaccharide, hepatic steatosis, adiponectin

Nonalcoholic fatty liver disease (NAFLD) is a major health problem worldwide, and has emerged as the major cause of chronic liver injury.\(^1\)\(^-\)\(^3\) The spectrum of NAFLD ranges from simple fatty liver to nonalcoholic steatohepatitis (NASH) and cirrhosis. The pathogenesis of NAFLD is unclear and therapeutic options are limited, although studies have found several factors that were associated with the development of NAFLD.\(^6\)\(^-\)\(^7\) Not all patients with steatosis will progress to NASH, a fact that strongly suggests the influence of other factors on the progression of NAFLD. Lipopolysaccharide (LPS), referred to as endotoxin, is considered a potent inducer of hepatic inflammation. Many studies have reported this role of LPS, as both peripheral and portal endotoxin levels were elevated in patients with NASH and animal models.\(^8\)\(^-\)\(^10\) In addition, probiotics diminish the development of NAFLD, suggesting a relationship between the enteric flora and liver injury.\(^11\)\(^-\)\(^13\)

In this study, we attempted to evaluate the role of LPS in the development of NAFLD. As obesity and type 2 diabetes are strongly associated with the pathogenesis of NAFLD, we used obese, diabetic Zucker (fa/fa) rats. We employed a synthetic diet rich in disaccharides (synthetic diet: 12.1 cal% disaccharide) to induce hepatic steatosis and metabolic abnormalities in rats. Following this, the rats were treated with LPS, is bacterial endotoxin in bowel bacteria flora.

**Materials and Methods**

**Animals.** Six-week-old male Zucker (fa/fa) rats were purchased from Nippon SLC Co. Ltd. (Shizuoka, Japan). Rats were housed in plastic cages for 12 or 24 weeks on a 12 h light/12 h dark cycle under conditions of controlled temperature (22 ± 1°C) and humidity (50 ± 10%), with ad libitum access to food and tap water.

**Experimental design.** Rats were fed a diet rich in disaccharides (sucrose and lactose diet; CLEA Japan Inc., Tokyo Japan) for 12 or 24 weeks. Diet composition is shown in Table 1. The calories per gram in this diet were the same as in a regular diet, and the only difference between the regular diet and this diet was the content of disaccharides. It has been reported that feeding Zucker (fa/fa) rats a diet rich in disaccharides for 1 or 3 weeks induces simple hepatic steatosis.\(^14\)\(^-\)\(^16\) The constituents of this diet induce gradual onset of hepatic steatosis compared with previously reported diets.\(^14\) Rats were divided into three experimental groups: (i) rats fed the diet for 12 weeks (D12 group, \(n = 7\)), (ii) rats fed the diet for 12 weeks and treated with LPS (LPS group, \(n = 7\)), (iii) rats fed the diet for 24 weeks (D24 group, \(n = 7\)). Rats in the LPS group were injected intraperitoneally (i.p.) with LPS (Sigma Chemical Co., St. Louis, MO) at a dose of 100 μg/kg once daily for 2 weeks. These rats were fed for 12 weeks and treated with LPS for the last 2 weeks under the same conditions. After 12 or 24 weeks of the diet, the rats were sacrificed after fasting overnight, and blood and liver samples were collected for analysis.

All surgical and experimental procedures were performed according to the guidelines for the care and use of animals approved by the Osaka Medical College.

**Measurement of plasma biochemical parameters and adipokine parameters.** Blood samples were collected via inferior vena cava puncture under terminal anesthesia. Levels of plasma alanine aminotransferase (ALT), free fatty acids (FFA), glucose, insulin, adiponectin, leptin, and concentrations of LPS were measured by a local laboratory that does clinical analyses.

**Table 1. Diet composition**

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<thead>
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<th>Regular diet (calorie%)</th>
<th>Diet rich in disaccharide (calorie%)</th>
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<tbody>
<tr>
<td>Protein</td>
<td>20.7</td>
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<tr>
<td>Fat</td>
<td>12.5</td>
<td>9.7</td>
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<tr>
<td>Nitric free extract</td>
<td>66.8</td>
<td>69.6</td>
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<tr>
<td>Disaccharide</td>
<td>0.16</td>
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Measurement of hepatic triglycerides. The liver from each animal was perfused via the portal vein with PBS to remove the blood. Hepatic tissues were homogenized with a Janke & Kunkel Polytron homogenizer (ULTRA-TURRAX T24/1051; IKA-Labortechnik, Staufen, Germany) in buffer (pH 7.4) containing 20 mM Tris HCl, 1 mM EGTA, 2 mM EDTA, and treated with protease inhibitor (2 μg/ml, leupeptin cocktail). Hepatic tissue triglyceride (TG) levels were measured by SRL Co., Ltd. (Tokyo, Japan).

Histochemical analysis. The liver from each animal was fixed in formalin, embedded in paraffin, and cut into 4 µm-thick sections. After deparaffinization, hematoxylin and eosin (H.E.) staining were performed and evaluated in a blinded manner by a pathologist.

Measurement of mRNA levels in the liver. Total RNA was extracted using TRIzol® reagent (QIAGEN, Tokyo, Japan) according to the manufacturer’s protocol. cDNA was synthesized from 1 µg of isolated RNA using a first-strand cDNA Synthesis Kit for real-time quantitative polymerase chain reaction (RT-PCR) Avian Myeloblastosis Virus (AMV+) (Roche Diagnostics, Mannheim, Germany). RT-PCR was performed for quantitative assessment of mRNA using a LightCycler (Roche Diagnostics) according to the manufacturer’s protocol. The sequences primers used were: Tumor necrosis factor (TNF)-α (forward, 5'-CAA-ACGGGAGATGGGAGACGT-3'; reverse, 5'-TTTCATCTGGAT-TCAAGGCT-3'); Medium-chain acyl-CoA dehydrogenase (MCAD) (forward, 5'-CAAGAGACCTGGAAGACTT-3'; reverse, 5'-CTCCCAAGATTTGCTC-3'); Long-chain acyl-CoA dehydrogenase (LCAD) (forward, 5'-AAGGATTATAAGGGCAGAAGACG-3'; reverse, 5'-GGAAAGGACCAGGGAGGC-3'); ATP-citrate lyase (ACL) (forward, 5'-CCATGTCAGCCAAGGCAAATG-3'; reverse, 5'-CATGCTGGTGAACCTTCCA-3'); Acetyl-CoA carboxylase (ACC) (forward, 5'-CAAGGCCGCCGTCA-GCAACAGTA-3'; reverse, 5'-TGAGTCTGAGCGGTGCTCA-3'); Fatty acid synthase (FAS) (forward, 5'-AGGTGCTAGAGGCAAGGCGCAAA-3'; reverse, 5'-TCCAGAGGCAATGACAGACAG-3').

Relative levels of target mRNAs were determined using a standard curve method recommended in the LightCycler Software ver. 3.5 (Roche Diagnostics). PCR reactions and analyses were performed according to manufacturer’s protocols (Roche Diagnostics).

Statistical analysis. Values are expressed as means ± SD and compared using the Mann-Whitney U test and ANOVA, followed by the Bonferroni-Dunn’s post hoc test, respectively. A p value of <0.05 was considered statistically significant.

Results

Based on our hypothesis that LPS plays a role in development of NAFLD, we evaluated the mechanism of progression in the development of NAFLD. Each group of Zucker (fa/fa) rats was fed a diet rich in disaccharides for 12 or 24 weeks, and the LPS group was in addition treated with LPS for the final 2 weeks.

Liver/body weight ratio. The liver/body weight ratio increased significantly in the LPS group compared to the other groups (p < 0.05) (Table 2).

Plasma and hepatic biochemical parameters. The LPS group showed significantly higher levels of plasma glucose and insulin on comparison with all groups (p < 0.05) (Table 2). To determine whether the approach used in the LPS group induced liver damage and hepatic steatosis, we quantified plasma ALT, FFA levels, and hepatic TG accumulation. Plasma ALT levels were not significantly different among the groups (Table 2). Plasma FFA and hepatic TG levels in the LPS group were significantly higher than in the other groups (p < 0.05) (Table 2). Plasma levels were below the lower limit of detection in all groups (data not shown).

Histochemical analysis. Severe hepatic steatosis was observed in the LPS group (Fig. 1). These findings were in keeping with biochemical examination of blood and liver samples. Some livers from the LPS and D24 groups showed development of mild hepatic fibrosis (data not shown).

Plasma adipokine parameters. The plasma adiponectin level is decreased in patients with obesity or type 2 diabetes, a hallmark of NAFLD as adiponectin prevents hepatic steatosis and fibrosis. The level of plasma adiponectin in the LPS group was the lowest of all the groups (Fig. 2). Leptin is an adipokine, and has been reported to promote insulin resistance and activation of the TGF beta signaling pathway, resulting in the development of hepatic fibrosis via activation of hepatic stellate cells (HSCs). The LPS group had the highest levels of plasma adiponectin among all groups, and this achieved statistical significance (Fig. 2).

Hepatic LPS-related mRNA expression. TNF-α has been suggested to cause obesity-related metabolic disorders such as insulin resistance. LPS activates Kupffer cells to produce cytokine, such as TNF-α. In addition, TLR4 receptor specifically responds to LPS derived from Gram-negative bacteria. A report has shown that TLR4, an important role in the activation of Kupffer cells. When hepatic TNF-α and TLR4 mRNA were examined, these mRNA levels were relatively increased in the LPS group (Fig. 3).

Hepatic lipogenic-related mRNA expression. The observed histological findings and plasma adipokine parameters suggest expression of cytokines involved in the development of NAFLD. Hepatic steatosis is developed as a result of abnormally enhanced de novo lipid synthesis and fat delivery. ACL is an important lipogenic enzyme that regulates the flow of glucose carbons to cytosolic acetyl-coenzyme A (CoA). In de novo fatty acid synthesis, Fatty acids are subsequently synthesized from malonyl-CoA by FAS. MCAD and LCAD are involved in

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<th>Table 2. Liver/Body weight ratio, plasma and hepatic parameters of three experimental groups</th>
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<td>D12 group (n = 7)</td>
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<tr>
<td>Liver/Body weight ratio (%)</td>
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<tr>
<td>Glucose (mg/dL)</td>
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<td>Insuline (ng/mL)</td>
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<td>FFA (μEQL)</td>
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<td>ALT (IUL)</td>
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<td>Hepatic TG (mg/dL)</td>
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*p < 0.05 vs Diet for 24 weeks. D12 group; a diet rich in disaccharides for 12 weeks-fed Zucker (fa/fa) rats, LPS group; a diet rich in disaccharides for 12 weeks-fed Zucker (fa/fa) rats treated with LPS, D24 group; a diet rich in disaccharides for 24 weeks-fed Zucker (fa/fa) rats.
mitochondrial fatty acid β-oxidation.\(^{25}\) Furthermore, one of the most important lipogenic factors is SREBP-1c.\(^{26,27}\) SREBP-1c-responsive gene include those of ACL, ACC and FAS. When these mRNAs were examined, hepatic ACC, FAS and SREBP-1c mRNA levels were relatively increased in the LPS group (Fig. 4D, E and F). However, hepatic ACL, MCAD and LCAD mRNA levels were relatively decreased in the LPS group, compared with the D24 group (Fig. 4A, B and C).

**Hepatic fibrogenic mRNA expression.** Since some rats showed the development of mild hepatic fibrosis, we examined profibrogenic factors. TGF-β1 is produced by Kupffer cells and infiltrating inflammatory cells, and activates HSCs that play a role in fibrogenesis in the liver.\(^{28,29}\) Since TGF-β1 and α-SMA (a marker of HSCs) are associated with hepatic fibrosis, their mRNA expression were examined. Levels of TGF-β1 and α-SMA mRNA were significantly increased in the D24 group (Fig. 5). On the other hand, expression of these mRNA transcripts in the LPS group was higher than in the group simply fed a disaccharide-rich diet for the same period (Fig. 5).

**Discussion**

In view of the notable incidence of NAFLD in obese patients and those with type II diabetes mellitus, this study was conducted to investigate effects of the bacterial endotoxin LPS on hepatic steatosis. Our animal model involved induction of NAFLD by...
Fig. 3. Expressions of TNF-α and TLR4 mRNA in specimens of hepatic tissue. TNF-α mRNA expression (A) and TLR4 mRNA expression (B) was evaluated by quantitative RT-PCR. The results shown represent mean ± SD of relative levels of target mRNA of five rats per group. *p<0.05 vs D24 group.

Fig. 4. Expressions of lipogenic-related mRNA in specimens of hepatic tissue evaluated by quantitative RT-PCR. A, MCAD; B, LCAD; C, ACL; D, ACC; E, FAS; F, SREBP-1c. The results shown represent mean ± SD of relative levels of target mRNA of five rats per group. *p<0.05 vs D24 group.
feeding Zucker rats a diet rich in disaccharide with subsequent injection of LPS.

Histological examination revealed the most severe hepatic steatosis in the LPS group compared with the other groups. In an attempt to account for the histological differences, we determined plasma markers of disordered liver function and hepatic lipids, including the examination of TG concentration in the liver. Results of these assays failed to reveal any significant difference in plasma ALT level among the groups, however, the plasma FFA level and hepatic TG concentration, both parameters of hepatic steatosis, were highest in the LPS group. Elevation of plasma insulin and glucose, indicating aggravation of so-called insulin resistance, was evident in the LPS group. A previous report showed that the gut-derived endogenous endotoxin, such as LPS may an important factor in the upregulation of TLR4 gene expression and activation of Kupffer cells. This is coincided with the upregulation of hepatic TNF-α and TLR4 expression in the present study. These results, which are consistent with the histological findings in the liver, are concordant with previous reports that suggest involvement of LPS in the development of NAFLD.

The finding of greatest interest among the results obtained from this study was that the administration of LPS after feeding for 12 weeks produced more severe hepatic steatosis in half the time, when compared to that induced in rats fed for 24 weeks. The results suggest possible involvement of various cytokines. Treatment with ethanol consumption increases gut permeability to LPS, resulting in activated Kupffer cells to produce inflammatory cytokines, such as TNF-α. TNF-α is widely known to be a cytokine that has bearing upon the progression of NAFLD, and SREBP-1c is a factor related to lipid synthesis. Both these factors are considered essential in the progression of NAFLD. The administration of LPS gave rise to increased TNF-α expression in the liver and also SREBP-1c expression in the liver. These results are consistent with previous reports that purport to demonstrate that expression of TNF-α stimulated expression of SREBP-1c, suggesting the possibility that LPS may play an important role in the aggravation of hepatic steatosis.

Hepatic steatosis occurs as result of abnormally enhanced lipid synthesis and fat delivery, as well decreased fatty acid oxidation and lipid export. In the present study, we examined the expression level of FAS, ACC, ACL, MCAD and LCAD in lipogenic pathway. Expressions of these enzymes in LPS group are higher than D12 group. These results suggested that LPS may be involved in peroxisomal and mitochondrial fatty acid β-oxidation.

Another finding of interest in the present study is that the plasma level of adiponectin, known to have a protective effect on liver, was lowered in the LPS group. As adiponectin is secreted from adipocytes, this finding suggests that the injected LPS may affect adipocytes, reducing adiponectin secretion.

The Zucker rats used in this study are an animal model that expresses hyperphagia and obesity, associated with genetic leptin receptor deficiency. It has recently been reported that leptin-dependent STAT3 and CD14 signal transduction has a bearing on the development of NASH, and that leptin intensifies reactions to bacterial endotoxins. The plasma leptin level significantly increased in the LPS group after feeding for 12 weeks. As leptin is a hormone secreted from adipocytes, as is adiponectin, it has been suggested that LPS may be involved in the progression of NAFLD by acting upon adipocytes. Further study of the implications of this effect on adipocytes is thus needed.

It seems unlikely that the etiology of NAFLD depends solely on a single factor because of the peculiar pathophysiology of this disease. Currently, the “two-hit” or “multiple-parallel-hits” theories are usually invoked as the cause of NAFLD. The present study suggests that bacterial endotoxin derived from the intestine, LPS, is involved in the secretion not only of leptin but also of adiponectin by acting upon adipocytes, in addition to lipid synthesis and fatty acid oxidation. As these factors were put together, severe hepatic steatosis may have resulted in LPS group. The present results provide further support for the finding previously obtained that the intestinal bacterial flora has bearing upon the progression of NAFLD, and may contribute substantially to future investigations into development of NAFLD.

Acknowledgments

The authors would like to thank Yukio Nakahira, at the Osaka Medical College, for providing them with technical support.

Abbreviations

<table>
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<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tr>
<td>ALT</td>
<td>Alanine aminotransferase</td>
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<td>FFA</td>
<td>Free fatty acid</td>
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<td>H.E.</td>
<td>Hematoxylin and eosin</td>
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<td>HSCs</td>
<td>Hepatic stellate cells</td>
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<td>LPS</td>
<td>Lipopolysaccharide</td>
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<td>NAFLD</td>
<td>Nonalcoholic fatty liver disease</td>
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<td>NASH</td>
<td>Nonalcoholic steatohepatitis</td>
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<tr>
<td>SREBP</td>
<td>Sterol regulatory element binding protein</td>
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<td>TG</td>
<td>Triglycerides</td>
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<td>TGF</td>
<td>Transforming growth factor</td>
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<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
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Conflict of Interest

No potential conflicts of interest were disclosed.
References


