

## Role of p38 Mapk in Development of Acute Hepatic Injury in Long-Evans Cinnamon (LEC) Rats, an Animal Model of Human Wilson's Disease

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**ABSTRACT.** The Long-Evans Cinnamon (LEC) rat, an animal model of human Wilson's disease, spontaneously develops fulminant hepatitis associated with severe jaundice at about 4 months of age. In this study, we examined the changes in gene expression during progression of acute hepatic injury. When levels of gene expression in the liver of LEC rats at 13 weeks of age were compared to those in rats at 4 weeks of age using oligonucleotide arrays, 1,620 genes out of 7,700 genes analyzed showed more than 2-fold differences. Expression levels of 11 of 29 genes related to stress-activating protein kinase (SAPK) changed by more than 2-fold in the liver of LEC rats, but none of the SAPK-related genes showed changes in expression levels in the liver of control rats. Activity of p38 mapk in the liver of LEC rats at 13 weeks of age was about 8.1-fold higher than that in rats at 4 weeks of age. When LEC rats were administered SB203580, a p38 mapk-specific inhibitor, by *s.c.* injection twice a week from 10 to 13 weeks of age, activities of p38 mapk in the liver, activities of AST and ALT and concentrations of bilirubin in sera of rats administered SB203580 significantly decreased compared to those in rats not administered. These results showed that the increase in activities of p38 mapk was related to the occurrence of acute hepatic injury in LEC rats.

**KEY WORDS:** hepatic injury, LEC rat, p38 mapk, SB203580.

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An inbred strain of the Long-Evans Cinnamon (LEC) rat was established as a mutant strain that spontaneously develops fulminant hepatitis associated with severe jaundice at about 4 months of age [10, 23]. The LEC rat has a mutation in a gene homologous to the human Wilson's disease gene, *ATP7B* [3]. A defect in the final product (Cu-binding P-type ATPase) of the gene, *Atp7b* [19, 21, 24, 29], results in abnormal copper metabolism, which is characterized by hepatic copper accumulation in LEC rats [16, 17]. Therefore, LEC rats provide a useful experimental model for human Wilson's disease [22, 27, 28].

It is thought that accumulation of copper in the liver causes acute hepatic injury in patients with Wilson's disease and in LEC rats. It is well known that excessive copper induces the production of large amounts of reactive oxygen species (ROS) [11, 25]. ROS are thought to be related to various human diseases [6]. Furthermore, ROS induce several types of DNA damage such as base alterations and DNA strand breaks [11, 26, 34]. It has been reported that the amount of 8-hydroxydeoxyguanosine (oh<sup>8</sup>dG) in DNA, a marker of ROS-derived DNA damage, was increased in the livers of LEC rats at 15 weeks of age compared with the amounts in the livers of LEC rats at 5 and 10 weeks of age and was 1.9-fold larger than the amounts in control rats [32]. DNA strand breaks were also produced in hepatic cells of LEC rats

in an age-dependent manner from 4 to 13 weeks of age [7, 8]. Furthermore, it has been reported that lipid peroxidation was induced in the liver of LEC rats [33].

Klein *et al.* [12] analyzed gene expression using an oligonucleotide array covering 1,031 genes and reported that during the progression of hepatic injury, genes related to oxidative stress, DNA damage, apoptosis and inflammation with acute-phase reaction were up-regulated. Thus, oxidative stress caused by copper accumulation leads to changes in gene expression in development of acute hepatic injury. However, underlying mechanisms leading to the development of hepatic injury have not been fully elucidated, though Fong *et al.* [5] suggested that TNF- $\alpha$  plays a role in pathogenesis of copper-induced acute hepatitis in LEC rats.

In this study, we examined the levels of gene expression in the liver of LEC rats at 4 and 13 weeks of age using oligonucleotide arrays including about 7,700 genes and showed that increase in activity of p38mapk was related to the occurrence of acute hepatic injury in LEC rats.

### MATERIALS AND METHODS

*Rats and treatment with SB203580:* Inbred strains of LEC and WKAH/Hkm (WKAH) rats were obtained from the Institute for Animal Reproduction Inc. and the Institute for Animal Experimentation, School of Medicine, Hokkaido University, respectively. LEC rats were bred in our breeding facility. All research protocols were approved by the Animal Research Committee of Rakuno Gakuen University. The rats were housed at a temperature of 25  $\pm$  2°C and exposed to a daily cycle of 14-hr light and 10-hr darkness. A solid diet (MF-Food, Oriental Yeast Co., Ltd., Tokyo, Japan) and water

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were provided *ad libitum*. Twenty four male LEC and 12 male WKAH rats were used in the present study. Although Long-Evans Agouti (LEA) strain rats have been established as a sibling strain of LEC rats, LEA rats show some genetic abnormalities such occurrence of diabetes mellitus (N. Kasai, Tohoku University, personal communications). In the present study, WKAH rats were used as control rats.

SB203580 (4-[4-fluorophenyl]-2-[4-methylsulfinyl]-5-[4-pyridyl]1H-imidazole), a p38 mitogen-activated protein kinase (p38 mapk)-specific inhibitor, was purchased from Wako Chemicals (Osaka, Japan) and was dissolved in dimethyl sulfoxide (DMSO). SB203580 inhibits selectively an activation of p38 mapk, but not c-Jun N-terminal kinase (JNK) and p48 MAPK. LEC rats were administered SB203580 by *s.c.* injection at doses of 2.5 and 5 mg/kg body weight twice a week from 10 to 13 weeks of age. An equal volume of DMSO was injected as a vehicle control.

**RNA purification:** Rats at 4 and 13 weeks of age were anesthetized with pentobarbital, and livers were flushed with ice-cold phosphate buffered saline (PBS). Total RNA was isolated from liver tissue using a Polytron homogenizer (Quiagen, Hilden, Germany) and TRI reagents (Sigma-Aldrich, St. Louis, MO, U.S.A.) and purified using RNeasy columns (Quiagen).

**Preparation of cRNA:** Total RNA (3  $\mu$ g) was used as the starting material for cDNA preparation. cDNA synthesis and labeling of aRNA were performed using an amino-allyl RNA amplification kit ver.2 (Sigma-Aldrich) and Cy3 and Cy5 Mono-reactive Dye Pack (Amersham Bioscience, Uppsala, Sweden) according to the manufacturers' instructions. Labeled aRNA was purified using Microcon-YM30 (Millipore, Billerica, MA, U.S.A.). The quality of the labeling products was tested with Nano Drop ND-1000 (NanoDrop Technology, Rockland, ME, U.S.A.) before hybridization to the oligonucleotide array.

**Array hybridization and scanning:** cRNA was fragmented at 95°C for 15 min in a fragmentation buffer and hybridized to a Panorama Rat Micro Array (Sigma-Aldrich) in a humid chamber (Sigma-Aldrich) at 60°C for 16 hr. The probe array was washed with 4 X standard saline citrate (SSC) containing 0.1% sodium dodecyl sulfate (SDS) at 50°C for 5 min, followed by washing with 2 X SSC containing 0.1% SDS at 50°C for 5 min and 2 X SSC at 50°C for 5 min. The probe arrays were scanned by Scanning service of Sigma-Aldrich. In the comparison of two arrays (LEC rats at 4 vs 13 weeks or WKAH rats at 4 vs 13 weeks), the original output was signal log ratio to log base 2, which was subsequently converted to linear fold changes by Lowess standardization. The expression level normalized at 4 weeks of age was 1.0.

**Assay for JNK and p38 mapk:** Small pieces of freshly resected rat liver were lysed in cell lysis buffer (Cell Signaling, Danvers, MA, U.S.A.) using a sample grinding kit (GE Healthcare Bioscience, Uppsala, Sweden) at 4°C. Protein concentrations of the lysates were determined by the method of Lowry *et al.* [18]. Measurements of activities of JNK and p38 mapk were carried out using a JNK Assay kit (Cell Signaling) and p38 map kinase kit (Cell Signaling) according to the manufacturer's instructions. After SDS polyacrylamide

gel electrophoresis and dry electrotransfer (iBlot Dry blotting System, Invitrogen, Carlsbad, CA, U.S.A.) to nitrocellulose paper (Invitrogen), equivalent protein loading was confirmed by staining with amido black. The nitrocellulose paper was incubated with primary antibodies to phosphorylated ATF-2 rabbit IgG for p38 mapk and to phosphorylated c-Jun rabbit IgG for JNK. The proteins were detected with an antibody to HRP-anti rabbit IgG and LumiGLO. Chemical luminescence was analyzed using Lumi Vision PRO (Aisin, Kariya, Japan).

**Measurements of activities of ALT and AST and concentrations of bilirubin in sera:** Samples for measurements of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities and concentrations of bilirubin were frozen with liquid nitrogen and stored at -80°C until used. The activities of AST and ALT and concentrations of bilirubin in sera were determined using an Automatic analyzer TBA-120 FR (Toshiba, Tokyo, Japan).

**Statistical analysis:** All data are expressed as means  $\pm$  SD. Differences between means were analyzed statistically by one-way ANOVA followed by Mann-Whitney U test. Values of  $P < 0.05$  and  $P < 0.01$  were considered significant.

## RESULTS

**Changes in gene expression analyzed by oligonucleotide arrays:** When the levels of gene expression in the livers of LEC rats at 13 weeks of age were compared to those in the livers of rats at 4 weeks of age using oligonucleotide arrays covering 7,700 genes, 764 genes were up-regulated and 856 genes were down-regulated at 13 weeks more than 2-fold compared to at 4 weeks (Table 1). In the case of livers of WKAH rats, 390 genes were up-regulated, and 449 genes were down-regulated. Although expression levels of many genes were different at 4 and 13 weeks of age in the livers of both LEC and WKAH rats, only 25 and 30 genes that were up-regulated and down-regulated were overlapped in LEC and WKAH rats at 13 weeks of age (Fig. 1). Gene ontology was followed according to classification of manufacturer's software (Sigma-Aldrich). Figure 2 shows the number of genes that were up-regulated and down-regulated more than 2-fold in some groups of gene ontology in the livers of LEC and WKAH rats at 13 weeks of age compared to the levels in livers of rats at 4 weeks of age. Many genes related to oxidative stress, DNA damage, apoptosis and inflammation with acute-phase reaction were up-regulated and down-regulated in the livers of LEC rats at 13 weeks of age (Fig. 2). These results are in good agreement with results obtained by Klein *et al.* [12]. Expression levels of 11 of 29 genes related to SAPK changed more than 2-fold in the livers of LEC rats at 13 weeks of age (Fig. 2). However, there were no SAPK-related genes that showed changes in expression levels more than 2-fold in the livers of WKAH rats at 13 weeks of age compared to the levels in the livers of rats at 4 weeks of age.

**SAPK activity in the liver of LEC rats:** To examine the activities of SAPK in the liver of LEC rats at 13 weeks of age, the activities of p38 mapk and JNK were measured. Although the activities of p38 mapk in the livers of LEC rats at

Table 1. Numbers of up-regulated and down-regulated genes in the livers of LEC and WKAH rats at 13 weeks of age compared to those at 4 weeks of age

| Rats | No. of up-regulated genes |         |         | No. of down-regulated genes |         |         |
|------|---------------------------|---------|---------|-----------------------------|---------|---------|
|      | >2-fold                   | >3-fold | >4-fold | >2-fold                     | >3-fold | >4-fold |
| LEC  | 764                       | 272     | 129     | 856                         | 294     | 130     |
| WKAH | 390                       | 79      | 33      | 449                         | 90      | 23      |

Numbers of genes that showed differences in expression levels by more than 2, 3 and 4 fold in the livers of LEC and WKAH rats at 13 weeks of age from those at 4 weeks of age.

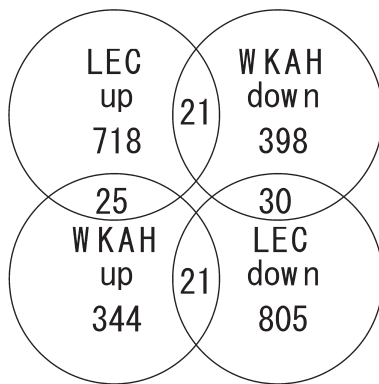


Fig. 1. Number of genes for which expression levels changed by more than 2-fold in livers of LEC and WKAH rats at 13 weeks of age compared to the levels in livers of rats at 4 weeks of age. Up and Down represent up-regulated and down-regulated genes in expression levels showing more than 2-fold differences in the livers of LEC and WKAH rats at 13 weeks of age compared to the levels in livers of rats at 4 weeks of age.

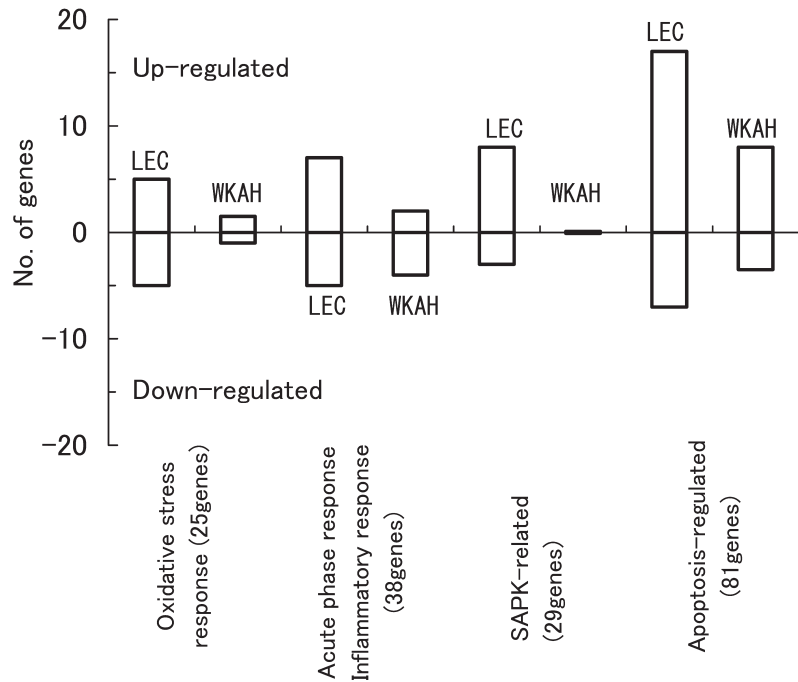


Fig. 2. Number of genes for which expression levels in some groups of gene ontology changed by more than 2-fold in the livers of LEC and WKAH rats at 13 weeks of age compared to the levels in the livers of the rats at 4 weeks of age. Number in parenthesis is the number of genes analyzed in each group of ontology.

13 weeks of age were  $8.1 \pm 2.1$ -fold higher than those in rats at 4 weeks of age (Fig. 3a), there was no significant difference between the activities of JNK in the livers of LEC rats at 4 and 13 weeks of age (data not shown). When LEC rats were administered SB203580, a p38 mapk-specific inhibitor, by *s.c.* injection at doses of 2.5 and 5 mg/kg body weight twice a week from 10 to 13 weeks of age, p38 mapk activities in the livers of LEC rats at doses of 2.5 and 5 mg/kg were  $0.45 \pm 0.12$ - and  $0.54 \pm 0.15$ -fold lower, respectively, than those in livers of rats not administered SB203580 (Fig. 3b and 3c).

*Effects of SB203580 on acute hepatic injury in LEC rats:* Activities of AST and ALT and concentrations of total bilirubin in sera of LEC rats at 13 weeks of age significantly increased compared to those in rats at 4 weeks of age (Table 2). There were no significant differences in activities of AST and ALT and concentrations of total bilirubin in sera of

WKAH rats at 4 and 13 weeks of age. Activities of AST and ALT in sera of LEC rats rapidly and significantly increased from 10 to 13 weeks of age ( $P < 0.01$ ). These results showed that acute hepatic injury with jaundice occurred in LEC rats at 13 weeks of age. When LEC rats were administered SB203580, activities of AST and ALT and concentrations of bilirubin in sera significantly decreased compared to those in rats not administered SB203580 (Table 2).

## DISCUSSION

Since hepatic copper contents and frequencies in DNA strand breaks in hepatic cells in LEC rats were maximized at 13 weeks of age under our experimental conditions [7, 8], the levels of gene expression in the livers of LEC rats at 13 weeks of age were compared to those in the livers of rats at 4 weeks of age. Although expression levels of many genes

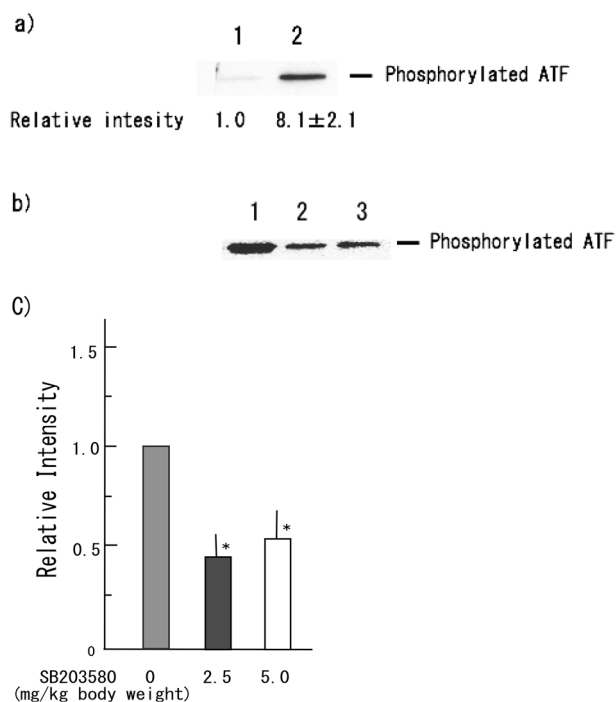


Fig. 3. Relative activities of p38 mapk in the liver of LEC rats analyzed by Western blotting. (a) Relative activities of p38 mapk in the livers of LEC rats not administered SB203580 at 4 (lane 1) and 13 weeks (lane 2) of age. Relative activity of p38 mapk normalized to the band intensity of rats at 4 weeks of age is 1.0. The relative activities represent an average of 3 to 4 separate experiments with standard deviations. The activities at 13 weeks of age were significantly higher than those at 4 weeks of age ( $P < 0.01$ ). (b) Activities of p38 mapk in the livers of LEC rats not administered (lane 1) and administered SB203580 at 2.5 (lane 2) and 5 (lane 3) mg/kg body weight at 13 weeks of age. (c) Relative activity of p38 mapk normalized to the band intensity of non-administered rats is 1.0. The activities represent an average of 3 to 4 separate experiments with standard deviations. \* represents statistically significant difference from non-administered control ( $P < 0.05$ ).

differed by more than 2-fold in LEC and WKAH rats at 4 and 13 weeks of age, only 25 and 30 genes that were up-regulated and down-regulated were overlapped in the livers of LEC and WKAH rats. These results suggested that copper accumulation in the liver of LEC rats induced expression of different genes from those in WKAH rats.

In this study, we showed that the activities of p38 mapk in the livers of LEC rats at 13 weeks of age were  $8.1 \pm 2.1$ -fold higher than those in rats at 4 weeks of age. To determine whether the increase in activity of p38 mapk is related to the occurrence of acute hepatic injury in LEC rats, LEC rats were administered SB203580, a p38 mapk-specific inhibitor. Activities of AST and ALT and concentrations of total bilirubin in sera of LEC rats administered SB203580 significantly decreased compared to those in rats not administered SB203580. The p38 mapk activities in the livers of LEC rats administered SB203580 were also significantly lower than those in rats not administered SB203580. Activities of AST and ALT in sera of LEC rats rapidly increased from 10 to 13 weeks of age (Table 2). Therefore, inhibition of activation of p38 mapk by SB203580 resulted in attenuation of the progression of acute hepatic injury in LEC rats.

It is well known that SAPK is activated by a variety of stressors such ROS, ultraviolet and ionizing radiation [1, 13, 14]. Excessive copper induces the production of large amounts of ROS [11, 26]. Therefore, it is thought that ROS produced by accumulated copper activated p38 mapk in the liver of LEC rats. Activation of p38 mapk regulates a variety of cellular processes such inflammation, cell cycle arrest and apoptosis in a cell type-specific manner [4, 29, 31]. Indeed, expression levels of many genes that regulate apoptosis and inflammatory response changed in the livers of LEC rats at 13 weeks of age compared to those at 4 weeks of age. Since it has been reported that activation of p38 mapk induces apoptosis [20, 31], SB203580 might inhibit the induction of apoptosis of hepatic cells in LEC rats. The study concerning a role of apoptosis in occurrence of acute hepatic injury in LEC rats is now in progress. Furthermore, activation of p38 mapk increases expression of inflammatory cytokines including TNF- $\alpha$  [30]. Since Fong *et al.* [4] suggested that

Table 2. Activities of AST and ALT, and concentrations of total bilirubin in sera of rats

| Rats | Weeks of age | Concentrations of SB203580 (mg/kg) | Activities                   |                              | Concentrations of total bilirubin (mg/dl) |
|------|--------------|------------------------------------|------------------------------|------------------------------|---|
|      |              |                                    | AST (U/l)                    | ALT (U/l)                    |   |
| LEC  | 4            | 0                                  | 53 $\pm$ 12                  | 121 $\pm$ 15                 | 0.27 $\pm$ 0.07                           |
|      | 10           | 0                                  | 149 $\pm$ 20*                | 155 $\pm$ 32                 | —   |
|      | 13           | 0                                  | 573 $\pm$ 129*. <sup>!</sup> | 548 $\pm$ 154*. <sup>!</sup> | 6.4 $\pm$ 3.7*                            |
|      | 13           | 2.5                                | 286 $\pm$ 144 <sup>#</sup>   | 295 $\pm$ 155 <sup>#</sup>   | 0.29 $\pm$ 0.17 <sup>##</sup>             |
|      | 13           | 5.0                                | 219 $\pm$ 92 <sup>#</sup>    | 363 $\pm$ 114 <sup>#</sup>   | 3.26 $\pm$ 1.91 <sup>#</sup>              |
| WKAH | 4            | 0                                  | 44 $\pm$ 10                  | 72 $\pm$ 11                  | 0.20 $\pm$ 0.08                           |
|      | 13           | 0                                  | 54 $\pm$ 15                  | 66 $\pm$ 15                  | 0.15 $\pm$ 0.09                           |

The activities of AST and ALT, and concentrations of total bilirubin represent an average of 3 to 4 separate experiments with standard deviations. \* and ! represent statistically significant differences from 4 and 10 weeks of age, respectively ( $P < 0.01$ ). # and ## represent statistically significant differences from the control not administered at 13 weeks of age ( $P < 0.05$  and  $P < 0.01$ , respectively).



TNF- $\alpha$  plays a role in pathogenesis of copper-induced acute hepatitis in LEC rats, inhibition of p38 mapk activation might cause decrease in expression of inflammatory cytokines.

Although administration of LEC rats with SB203580 lowered activation of p38 mapk, the activities of p38 mapk at 13 weeks of age were about 4-fold higher than those at 4 weeks of age. Therefore, occurrence of hepatic injury might not be completely inhibited under the conditions in this study. There was no significant difference in activities of ALT and AST in the sera of LEC rats administered SB203580 between 2.5 and 5 mg/kg. The reason why dose-dependency was not shown remains unclear. Administration of rats with high concentration of SB203580 might inhibit other protein kinase such phosphatidylinositol 3-kinase/protein kinase B [15]. It has been reported that SB203580 also directly inhibits thromboxane synthase, cyclooxygenase-1 and cyclooxygenase-2 [2]. Furthermore, it has been reported that SB203580 activates extracellular-regulated protein kinase and JNK in primary human hepatocyte [9]. Our preliminary results showed that the protective effects by SB203580 at 10 mg/kg body weight on acute hepatic injury reduced in LEC rats compared to rats administered at 2.5 mg/kg body weight (data not shown). Therefore, activation or inhibition of other protein kinases might affect the occurrence of hepatic injury in LEC rats administered SB203580 at high concentrations.

In the present study, it was shown that the increase in activities of p38 mapk was related to the occurrence of acute hepatic injury in LEC rats. An inhibitor of p38 mapk may be a therapeutic agent for attenuation of acute hepatic injury in patients with Wilson's disease.

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