MEK/ERK and NFκB pathways, which are activated by the membrane-bound CD14 and toll-like receptors (TLR)-4 [13].

The aim of the present study was to evaluate *in vivo* changes in the plasma C4BP and PS–C4BP complex antigen levels, and in the liver expression of C4BPα and C4BPβ mRNA after treatment of rats with lipopolysaccharide (LPS) and IL-6. In addition, we evaluated the *in vitro* effect of LPS and IL-6 on C4BP production and the C4BPα and C4BPβ mRNA expression in hepatocytes isolated from normal rats, and the signal transduction pathways that mediate the effect of LPS and IL-6 in hepatocytes.

## Materials and methods

### *Materials*

LPS (from *Escherichia coli* 026: B6) was purchased from Sigma Chemical (St Louis, MO, USA). Collagenase was from Wako Pure Chemical Industries, Osaka, Japan, and fetal bovine serum (FBS) from BioWhittaker, Walkersville, MD, USA. Trypsin inhibitor, William's medium E and SuperScript First Strand cDNA Synthesis System kit were from Invitrogen, Carlsbad, CA, USA. Type I collagen-coated dishes and 12-well plates were from Becton Dickinson Labware, Bedford, MA, USA. Protein A-Sepharose FF was from Amersham Bioscience, Uppsala, Sweden. RNAzol B for extraction of total RNA from rat whole liver and hepatocytes was from TEL-TEST, Friendswood, TX, USA. Taq DNA polymerase was from Roche Diagnostics, Basel, Switzerland. Streptavidin-horseradish peroxidase (POD) conjugate was from GE Healthcare, Piscataway, NJ, USA. MPL + TDM + CWS Emulsion was from RIBI Immunochemical Research, Hamilton, MT, USA. ACTICLOT protein S was purchased from American Diagnostica, Stamford, CT, USA. Rat IL-4, IL-6, IL-10 and protein kinase C (PKC) inhibitor, Calphostin C, were from Wako Pure Chemical Industries. Rat IL-13 was from R&D System, Minneapolis, MN, USA. NFκB inhibitor and NFκB control were from Santa Cruz Biotechnology, Santa Cruz, CA, USA. Anti-p44/42 MAPK (MEK), anti-phosphorylated p44/42 MAPK, anti-STAT-3 and anti-phosphorylated STAT-3 (Ser727) antibodies were from Cell Signaling, Boston, MA, USA. BCA protein assay reagents were from Therma Fisher Scientific Inc., Rockford, IL, USA. Peroxidase (POD)-conjugate antirabbit IgG goat IgG was from BIO-RAD, Hercules, CA, USA. Immobilon-P, polyvinylidene fluoride (PVDF) membrane was from Millipore Corp, Bedford, MA, USA. MEK inhibitor, PD98059, and STAT-3 inhibitor were from CALBIOCHEM, Darmstadt, Germany. Jun kinase (JNK) inhibitor, SP600125, was from Tocris Bioscience, Ellisville, MO, USA. All other chemicals and reagents were of the highest quality commercially available.

### *Proteins and antibodies*

C4BP was purified from rat plasma as described previously [14]. Antiserum against C4BP was prepared by subcutaneous injection of a mixture of rat C4BP antigen with MPL + TDM + CWS Emulsion into rabbits every two weeks for two months. Rabbit anti-C4BP IgG was isolated from antisera of rabbit using Protein A-Sepharose FF according to the manufacturer's instruction. Rabbit antirat PS IgG was also obtained as described previously [14].

### *Animals and LPS or IL-6 treatment to the rats*

The male Wistar–Hannover rats used in this study were purchased from CLEA Japan (Osaka, Japan). The animals were housed under a constant light and dark cycle, and allowed free access to standard food and water. The experiments were approved by the Mie University Review Board for animal experiments and were conducted according to the guidelines of the National Institute of Health. LPS and IL-6 were dissolved in sterile, pyrogen-free saline at concentrations of 1 mg $mL^{-1}$ and 10 μg $mL^{-1}$, respectively. To examine the effect of LPS or IL-6 injection on C4BP expression in the liver and plasma levels of C4BP, PS–C4BP complex, total PS and free PS, LPS (2 mg $kg^{-1}$) and IL-6 (10 μg $kg^{-1}$) were injected in rats intraperitoneally and intravenously, respectively. Under anesthesia (intraperitoneal injection of sodium pentobarbital; 10 mg $kg^{-1}$), blood samples were collected by cardiac puncture after performing thoracotomy into plastic tubes containing a 1:10 volume of 3.8% sodium citrate at each time point from three rats (time 0, 2, 4, 6, 8, 12, and 24 h after LPS or IL-6 injection). Citrated plasma was separated by centrifugation and stored at −30 °C until use. Liver samples for preparation of total RNA for real-time polymerase chain reaction (PCR) analyses were also collected at each time point, immediately frozen in liquid $N_2$, and stored at −80 °C until use.

### *Isolation and culture of hepatocytes from rats*

Hepatocytes were isolated from male rats (weight 200–220 g) using the two-step collagenase perfusion method as described previously [13]. Primary culture of hepatocytes was performed using 60-mm culture dishes or 12-well plates coated with type I collagen. After isolation, hepatocytes were plated at $10^5$ $cm^{-2}$ cell density, and maintained in William's medium E containing penicillin G (100 μg $mL^{-1}$), kanamycin (10 μg $mL^{-1}$), and insulin (2.0 U $L^{-1}$), supplemented with 10% FBS [13]. Hepatocytes were then cultured at 37 °C in a moist incubator under 5% $CO_2$ atmosphere. Every experiment was performed after 12 h of cell culture to facilitate cell spreading, and with immediate substitution of fresh serum-containing media. After 24 h of culture with or without LPS, cytokine or varying signal transduction inhibitors, expression of C4BPα and C4BPβ mRNA in hepatocytes, or C4BP antigen level in the medium were determined.

### *Enzyme-linked immunosorbent assay*

The C4BP antigen level in plasma and in culture medium was determined by enzyme-linked immunosorbent assay

(ELISA) using polyclonal antirat C4BP IgG, biotinylated antirat C4BP IgG, and streptavidin-horseradish peroxidase conjugate as described previously [13]. To measure PS-C4BP complex, antirat PS IgG was used as a coating antibody and biotinylated antirat C4BP IgG as a detection antibody, following the procedure as described previously [13]. The total and free PS antigens in plasma were determined by ELISA as described previously [13]. When the free PS level in plasma was measured, plasma was mixed with the same volume of 10% polyethyene glycol, centrifuged, and the supernatant after centrifugation was used as the sample for measuring the PS antigen [15]. The mean value of plasma C4BP, PS-C4BP complex, total PS or free PS level in 10 normal rats was measured using these ELISAs and taken as 100%.

### *Assay of APC cofactor activity of rat plasma*

The APC cofactor activity of plasma obtained from normal, and LPS- or IL-6-treated rats was determined by the activated partial thromboplastin time (APTT) assay [15] using ACTI-CLOT Protein S. The clotting time was measured using a coagulometer CA-50 (Sysmex, Kobe, Japan).

### *Isolation of total RNA*

Total RNA was extracted from hepatocytes and whole liver by a modification of the method of Chomczynski and Sacchi [16] using RNAzol B, and then quantitated spectrophotometrically. Aliquots of RNA were electrophoresed on formaldehyde agarose gels and stained with ethidium bromide to confirm the amount and quality of total RNA.

### *Real-time polymerase chain reaction*

Total RNA (5 μg) extracted from hepatocytes or whole liver was used for first-strand cDNA synthesis using the SuperScript First Strand cDNA Synthesis System kit according to the manufacturer's instructions. To evaluate the expression of C4BPα and C4BPβ mRNA in hepatocytes and whole liver, real-time PCR [17] was performed using rC4BPα-F (5′-ACCAGCAGCTCCACAGTGTAAA-3′; residue 1359–1380) and rC4BPβ-F (5′-TCCTGTCCATGGCTATTTTGAAG-3′; residue 689–710) as forward primers, rC4BPα-R (5′-TCCTGCTCACATCTGGGCACCT-3′; residue 1559–1538) and rC4BPβ-R (5′-TGCAAAGGTCCTTACTCTCCTG-3′; residue 917–896) as reverse primers, and rC4BPα-TaqmanP (FAM-CCAAAGCATCACTTGTTCGGAGAATG-TAMRA; residue 1497–1422) and rC4BPβ-TaqmanP (FAM-ACACAGAAGGTGCAGTGCAGTGATGG-TAMRA; residue 776–801) [18] as TaqMan probes. A TaqMan rodent glyceraldehyde-3-phosphate dehydrogenase (GAPDH) control reagents VIC probe (Applied Biosystems, Foster City, CA, USA) was used to determine GAPDH mRNA expression, and this was used for correction of C4BPα and C4BPβ mRNA expression. The resulting relative increase in reporter fluorescence dye emission was monitored by i-cycler (BIO-RAD). Thermal cycling was initiated by 2-min incubation at 50 °C, followed by a preliminary denaturation step at 95 °C for 15 min and 40 cycles of 95 °C for 15 s and 60 °C for 1 min.

### *Sodium dodecylsulfate polyacrylamide gel electrophoresis and Western blot analysis*

Evaluation of total and phosphorylated p44/42 MAPK, or total and phosphorylated STAT-3 was performed by Western blot analysis using specific antibodies. After rat hepatocytes were treated with LPS (100 μg mL$^{-1}$) in the presence or absence of MEK inhibitor (PD98059; 50 μg mL$^{-1}$) for 10 min, or with IL-6 (100 IU mL$^{-1}$) in the presence or absence of STAT-3 inhibitor (10 μmol L$^{-1}$) for 10 min, they were washed twice with phosphate-buffered saline (PBS) and treated with lysis buffer (20 mM Tris–HCl, pH7.5, 5 mM ethylene glycol-bis (β-amino-ethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid, 0.5% Triton X-100, 50 mM 2-glycerophosphate, 6 mM dithiothreitol, 0.1 mM NaF, 1 mM $Na_3VO_4$, 0.01% leupeptin, 1 mM phenylmethylsulforyl fluoride). Cell lysates were centrifuged at 15 000 *g* for 15 min to discard cell debris, and the protein concentration of each supernatant was determined using the BCA protein assay. Equal amounts of proteins were subjected to sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) followed by transfer to PVDF membrane. Then, p44/42 MAPK proteins were detected using anti-p44/42 MAPK antibody or antiphosphorylated p44/42 MAPK antibody as a first antibody, and POD-conjugate antirabbit IgG goat IgG as the second antibody, while STAT-3 proteins were detected using anti-STAT-3 antibody or antiphosphorylated STAT-3 antibody as the first antibody, and POD-conjugate antirabbit IgG goat IgG as a second antibody. Finally, protein bands were visualized by chemiluminescence using Chemilumi One reagent (Nacalai Tesque, Kyoto, Japan), and the intensity of the bands was quantified by densitometric analysis using LAS-1000 Image Analyzer (Fujifilm, Tokyo, Japan).

### *Effect of inhibitors of NFκB, JNK, MEK, p38 MAPK, and PKC on LPS-induced decreased expression of C4BP in hepatocytes*

Hepatocytes isolated from normal rats were treated with LPS (10 μg mL$^{-1}$) to reduce expression of C4BP, and were co-incubated with NFκB control (5 and 50 μg mL$^{-1}$), NFκB inhibitor (5 and 50 μg mL$^{-1}$), JNK inhibitor SP600125 (5 and 50 μmol L$^{-1}$), MEK inhibitor PD98059 (5 and 50 μmol L$^{-1}$), p38 MAP kinase inhibitor SB203580 (5 and 50 μmol L$^{-1}$) or PKC inhibitor Calphostin C (5 and 50 nmol L$^{-1}$) for 24 h. Culture medium was collected and centrifuged at 15 000 × *g* for 10 min to exclude cell debris before determination of C4BP antigen levels by ELISA as described above. Total RNA was extracted from the cells, and C4BPα and C4BPβ mRNA expression was determined by real-time PCR analysis as described above.

### *Effect of STAT-3 inhibitor on IL-6-induced increased expression of C4BPβ in hepatocytes*

Hepatocytes isolated from normal rats were treated with IL-6 (10 and 100 IU $mL^{-1}$) in the presence of STAT-3 inhibitor (10 μmol $L^{-1}$) for 24 h. Total RNA was extracted from the cells, before determining C4BPα and C4BPβ mRNA expression by real-time PCR analysis as described above.

### *Statistical analysis*

All values were expressed as mean ± standard deviation of the mean. All experiments were repeated at least three times. Differences between the means of two groups were determined by Student's *t*-test, and differences for multiple comparisons by analysis of variance with post hoc analysis. Values of $P < 0.05$ were considered as statistically significant.

## Results

### *C4BP and PS antigen levels in plasma and C4BP mRNA expression in the liver of LPS-treated rats*

Plasma C4BP antigen level in LPS-treated rats was transiently decreased until 12 h but was increased 24 h after LPS injection (Fig. 1A) while injection of the saline vehicle had no effect on plasma C4BP antigen level (data not shown); the plasma PS-C4BP complex level remained unchanged (Fig. 1B). However, the plasma total PS level was significantly altered, with the free PS level being drastically decreased in LPS-treated rats (Figs. 1C and 1D); in addition, a decrease of plasma APC cofactor activity was observed even at 2 h after LPS treatment (data not shown). Real-time PCR analysis revealed that C4BPα mRNA level in the liver was transiently decreased from 2 to 8 h after LPS injection and then recovered 12 h after LPS injection; C4BPβ mRNA level did not change 4 h after LPS injection, and then increased up to 24 h after LPS injection (Fig. 1E).

### *Effect of LPS on C4BP expression in hepatocytes from normal rats*

The direct effect of LPS on C4BP expression was examined in hepatocytes isolated from normal rats. LPS dose-dependently decreased C4BP antigen levels in medium from cultured hepatocytes (Fig. 2A). Real-time PCR analysis showed that LPS dose-dependently decreased both C4BPα and C4BPβ mRNA expression in hepatocytes; interestingly, with a stronger reduction in the mRNA level of C4BPα than of C4BPβ (Fig. 2B).

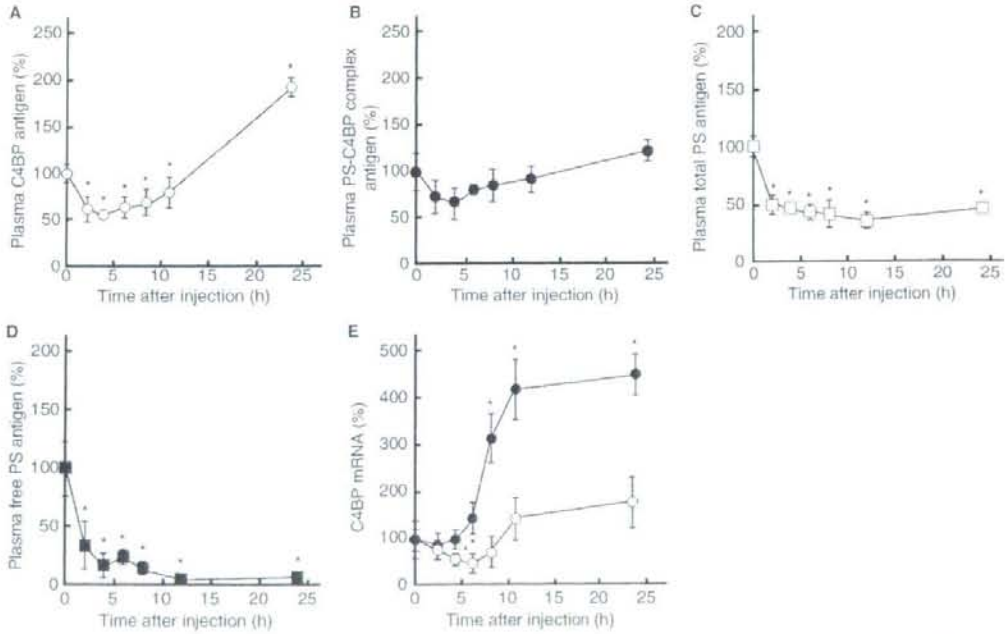


**Fig. 1.** Changes in plasma levels of C4b-binding protein (C4BP), protein S (PS)-C4BP complex, total PS and free PS antigen and liver mRNA expression of C4BPα and C4BPβ in rats treated with lipopolysaccharide (LPS). (A) Plasma C4BP antigen (open circle), (B) PS-C4BP complex (closed circle), (C) total PS (open square) and (D) free PS (closed square) levels in citrated rat plasma obtained from three LPS-treated rats (2 mg $kg^{-1}$) at each time point were measured using ELISAs. Data are expressed as the mean ± SD. (E) Rat C4BPα (open circle) and C4BPβ (closed circle) mRNA expressions were determined by real-time polymerase chain reaction. Data are expressed as the mean ± SD ($n = 3$). *$P < 0.05$ vs. time 0.

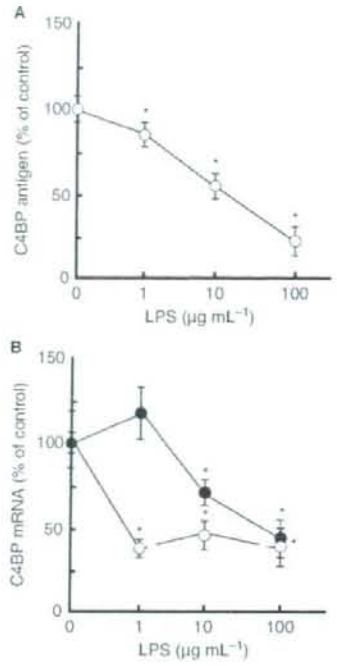


**Fig. 2.** The effect of lipopolysaccharide (LPS) on C4b-binding protein (C4BP) expression in hepatocytes isolated from normal rats. Hepatocytes isolated from normal rats were treated for 24 h with the indicated concentrations of LPS in the presence of fetal bovine serum. (A) C4BP antigen levels in the culture medium and (B) mRNA expression of C4BPα (open circle) and C4BPβ (closed circle) in hepatocytes after 24 h of LPS treatment. $^{*}P < 0.05$ vs. 0 μg mL$^{-1}$ LPS (control).

### *C4BP and PS antigen levels in plasma and C4BP mRNA expression in the liver of IL-6-treated rats*

The IL-6 level was transiently increased 1 h after IL-6 injection (data not shown). As shown in Fig. 3A, the plasma C4BP level increased gradually after IL-6 injection. The PS–C4BP complex also gradually increased in proportion to the increase in the plasma level of C4BP, with the maximum increase observed 8 h after IL-6 injection (Figs. 3A and 3B). However, the plasma total PS level in IL-6-treated rats was unchanged (Fig. 3C), and the free PS level gradually decreased after IL-6 injection (Fig. 3D). Furthermore, as shown in Fig. 3E, C4BPα expression remained unchanged, but C4BPβ expression was significantly increased 24 h after IL-6 injection. In addition, saline injection as control did not affect the plasma C4BP levels.

### *APC cofactor activity of plasma from rats treated with IL-6*

APC cofactor activity of plasma isolated from rats treated with IL-6 was also evaluated by APTT. As shown in Fig. 3F, plasma obtained from rats 24 h after IL-6 treatment prolonged the APTT significantly less than plasma from control rats.

### *Effect of IL-6 on C4BP expression in hepatocytes from normal rats*

IL-6 dose-dependently increased C4BP antigen level in culture medium of hepatocytes (Fig. 4A). IL-6 also dose-dependently increased C4BPβ mRNA expression, but exerted no effect on C4BPα mRNA expression in hepatocytes (Fig. 4B). However, anti-inflammatory cytokines (such as IL-4, IL-10, and IL-13) had no effect on C4BP expression in rat hepatocytes (data not shown).

### *Effect of NFκB inhibitor on C4BP expression in hepatocytes*

The effect of NFκB inhibitor on LPS-induced decreased expression of C4BP in hepatocytes was evaluated. As shown in Fig. 5A, C4BP expression in hepatocytes from normal rats decreased after LPS treatment, and this decrease was suppressed by the NFκB inhibitor, but not by the NFκB control. Real-time PCR analysis showed that LPS decreased both C4BPα and C4BPβ mRNA expression in hepatocytes; the decrease of both mRNAs was blocked by the NFκB inhibitor (Fig. 5B) but not by the NFκB control (data not shown).

### *Effect of inhibitors of JNK, MEK, p38MAPK, and PKC on LPS-induced decreased C4BP expression in hepatocytes*

We examined the effect of the JNK inhibitor (SP600125), the MEK inhibitor (PD98059), p38 MAPK inhibitor (SB203580), and the PKC inhibitor (Calphostin C) on C4BP expression in hepatocytes treated with LPS. The inhibitors themselves showed no effect on C4BP expression in hepatocytes (data not shown). As shown in Fig. 6A, SP600125, SB203580, and Calphostin C did not affect the LPS-induced decreased expression of C4BP; however, PD98059 significantly blocked LPS-induced decreased production of C4BP in hepatocytes. Real-time PCR analysis showed that the MEK inhibitor blocks LPS-mediated decreased mRNA expression of both C4BPα and C4BPβ, and that the recovery of expression of C4BPβ mRNA is greater than that of C4BPα (data not shown). In addition, Western blot analysis showed that phosphorylation of MEK in rat hepatocytes is observed after LPS treatment, and that this LPS-induced phosphorylation of MEK is inhibited by PD98059 (Figs. 6B and 6C).

### *Effect of STAT-3 inhibitor on IL-6-induced increased C4BP expression in hepatocytes*

The effect of a STAT-3 inhibitor on IL-6-induced increased C4BPβ expression in hepatocytes was examined. First, we confirmed using Western blot analysis that phosphorylation of STAT-3 occurs after IL-6 treatment of rat hepatocytes, and that this IL-6-induced phosphorylation of STAT-3 is inhibited by STAT-3 inhibitor (Figs. 7A and 7B). Subsequently, as shown in Fig. 7C, neither IL-6 nor the STAT-3 inhibitor affected C4BPα mRNA expression; in addition, the STAT-3 inhibitor did not affect C4BPα mRNA expression in

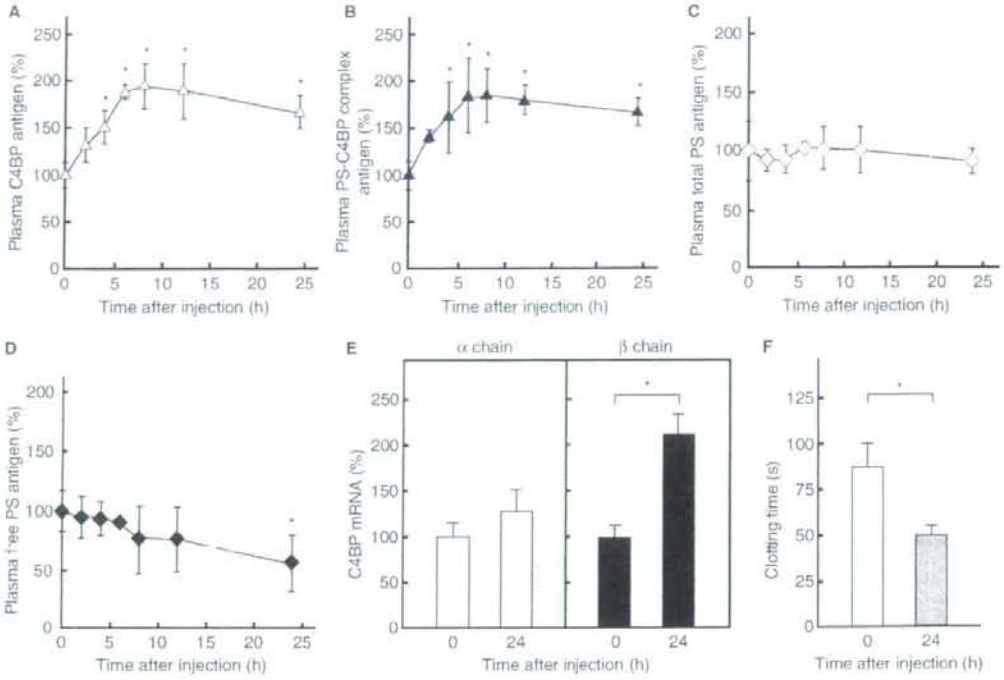


**Fig. 3.** Changes in plasma levels of C4b-binding protein (C4BP), protein S (PS)–C4BP complex, total PS and free PS antigen, liver mRNA expression of C4BPα and C4BPβ, and anticoagulant activity of plasma PS in rats treated with interleukin (IL)-6. (A) Plasma C4BP antigen (open triangle), (B) PS–C4BP complex (closed triangle), (C) total PS (open diamond) and (D) free PS (closed diamond) levels in citrated rat plasma obtained from three IL-6-treated rats (10 μg kg$^{-1}$) at each time point were measured using ELISAs. Data are expressed as the mean ± SD. (E) C4BPα (open bar) and C4BPβ (closed bar) mRNA expression in the liver of rats 24 h after IL-6 treatment (10 μg kg$^{-1}$) were determined by real-time polymerase chain reaction. Data are expressed as the mean ± SD. ($n = 3$). *$P < 0.05$ vs. time 0. (F) PS activity in plasma of rats treated with IL-6. Data are expressed as the mean ± SD. ($n = 3$). *$P < 0.05$ vs. time 0.

IL-6-treated hepatocytes. However, IL-6 increased C4BPβ mRNA expression in hepatocytes, which was decreased by STAT-3 inhibitor treatment.

## Discussion

C4BP is an important cofactor of serine protease FI for degradation of C4b in the classic complement pathway. In addition, C4BP also regulates the anticoagulant activity of PS, because PS bound to C4BP is unable to serve as a cofactor for APC, or to inhibit FXa [6,9,10]. Sepsis and oral contraceptives have been reported to decrease plasma PS leading to a thrombotic tendency. Plasma C4BP levels increased in patients with an acute phase response [19–21] and the increased plasma concentration of C4BP containing β chain correlated with PS–C4BP complex level [19]; however, it is unknown whether the increase in C4BP containing β chains affects PS–C4BP complex levels and hence the APC cofactor activity of PS. Also, under acute phase condition, levels of various cytokines were elevated, and among these cytokines, IL-6 and TNF-α increased C4BP expression in human and rat hepatoma cell lines [22–25]. Whether C4BP expression is directly affected by LPS, and how C4BPα and C4BPβ mRNA expression change during sepsis had not been explored. In the present study, we examined the effect of LPS and IL-6 on C4BP expression *in vivo* in the liver of rats and *in vitro* in isolated hepatocytes. The results have shown that LPS transiently decreases the plasma level of C4BP antigen; the maximum decrease was between 4 and 6 h after LPS injection, which recovered by 24 h after LPS injection. C4BPα mRNA levels transiently decreased, and the C4BPβ mRNA level remained unchanged until 4 h and then increased. These results suggest that the early decrease of plasma C4BP is caused by a direct effect of LPS, and that the relatively late increase in the plasma level of C4BP is caused by IL-6. However, the total and free PS antigen levels in plasma were decreased immediately after LPS treatment and the plasma APC cofactor activity was also decreased even at 2 h after LPS treatment (data not shown). These results indicate that LPS-induced decrease of plasma PS is also an important mechanism of thrombotic tendency observed in patients with sepsis. Subsequently, we

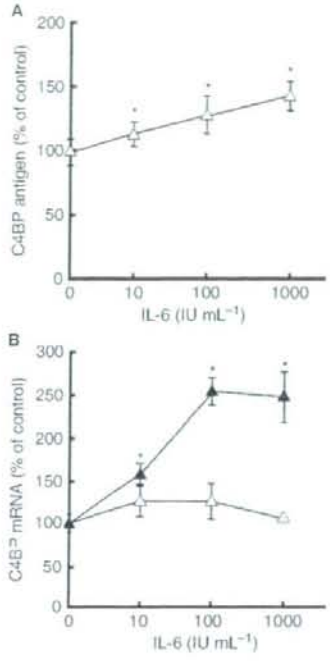


**Fig. 4.** The effect of interleukin (IL)-6 on C4b-binding protein (C4BP) expression in hepatocytes isolated from normal rats. Hepatocytes isolated from normal rats were treated for 24 h with the indicated concentrations of IL-6 in the presence of fetal bovine serum. (A) C4BP antigen levels in culture medium and (B) mRNA expression of C4BPα (open triangle) and C4BPβ (closed triangle) in hepatocytes after 24 h of IL-6 treatment. $^{*}P < 0.05$ vs. 0 IU mL$^{-1}$ IL-6 (control).

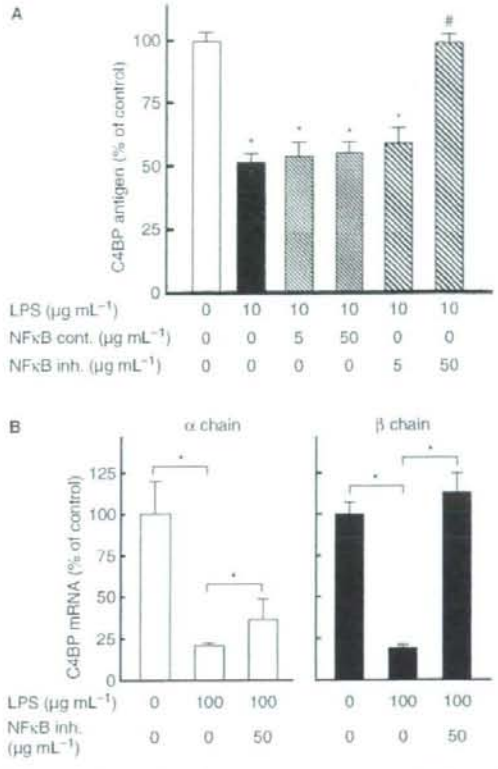


**Fig. 5.** The effect of NFκB inhibitor on lipopolysaccharide (LPS)-induced decreased C4b-binding protein (C4BP) expression in hepatocytes isolated from normal rats. (A) Hepatocytes isolated from normal rats were incubated with medium alone (control), LPS (10 μg mL$^{-1}$), LPS (10 μg mL$^{-1}$) + NFκB control (5 or 50 μg mL$^{-1}$), or LPS (10 μg mL$^{-1}$) + NFκB inhibitor (5 or 50 μg mL$^{-1}$) for 24 h. After incubation, culture medium was collected and C4BP antigen level was determined using a specific ELISA. $^{*}P < 0.05$ vs. control. $^{\#}P < 0.05$ vs. 10 μg mL$^{-1}$ LPS. (B) Hepatocytes isolated from normal rats were incubated with medium alone (control), LPS (10 μg mL$^{-1}$), or LPS (10 μg mL$^{-1}$) + NFκB inhibitor (50 μg mL$^{-1}$) for 24 h. After incubation, mRNA expression of C4BPα (open bar) and C4BPβ (closed bar) in hepatocytes was evaluated by real-time polymerase chain reaction analysis. $^{*}P < 0.05$.

examined the effect of LPS on C4BPα and C4BPβ mRNA expression in hepatocytes isolated from normal rats, and found that LPS directly decreases the mRNA expression of both C4BPα and C4BPβ, and this decrease might be the cause of the decreased C4BP production by hepatocytes.

Recently, it has been reported that hepatocytes express CD14 and TLR-4, and that the expression of both proteins depends on NFκB activation induced by LPS [13,26,27]. We also found that LPS decreases PS expression from hepatocytes via CD14- and TLR-4-mediated NFκB activation [13]. In the present study, we found that NFκB activation also mediates LPS-induced decreased expression of C4BP in hepatocytes similarly to PS. These findings suggest that LPS downregulates both PS and C4BP in hepatocytes, and that this effect occurs by NFκB activation. Further, inhibitors of JNK, p38 MAP kinase, and PKC did not affect expression of C4BP in hepatocytes, but a MEK inhibitor blocked the decreased in C4BP expression induced by LPS. These results are consistent with previous studies showing that in hepatocytes LPS induces MEK activation, which subsequently leads to NFκB activation [27,28]. These results indicate that the PKC pathway is not linked to the LPS signaling pathway in rat hepatocytes, and that the MEK/ERK pathway mediates the LPS-induced NFκB activation with a subsequent decrease of C4BP expression. Further studies are required to clarify whether NFκB directly decreases C4BP expression in hepatocytes.

In our rat model of endotoxemia, the plasma concentration of LPS rapidly increased after LPS injection followed by increased circulating levels of inflammatory cytokines such as TNF-α, IL-6, and IFN-γ [13]. The present study has demonstrated that IL-6 specifically increases C4BPβ mRNA in the liver, resulting in increased plasma concentrations of C4BP, more PS-C4BP complex, less availability of free PS

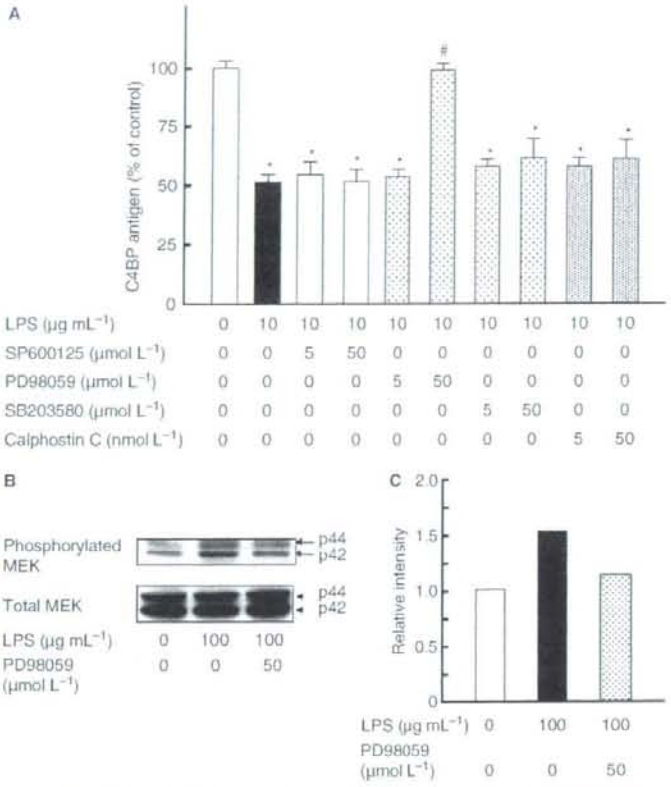


**Fig. 6.** The effect of inhibitors of JNK, MEK, p38 MAPK, and PKC on lipopolysaccharide (LPS)-induced decreased expression of C4b-binding protein (C4BP) in hepatocytes isolated from normal rats, and MEK activation by LPS. (A) Hepatocytes isolated from normal rats were incubated in the presence of medium alone (control), LPS (10 μg mL$^{-1}$), LPS (10 μg mL$^{-1}$) + SP600125 (5 or 50 μmol L$^{-1}$), LPS (10 μg mL$^{-1}$) + PD98059 (5 or 50 μmol L$^{-1}$), LPS (10 μg mL$^{-1}$) + SB203580 (5 or 50 μmol L$^{-1}$), or LPS (10 μg mL$^{-1}$) + Calphostin C (5 or 50 nmol L$^{-1}$) for 24 h. After incubation, cultured medium was collected and the C4BP antigen level was determined by specific ELISA. Data are expressed as the mean ± SD ($n$ = 3). *$P$ < 0.05 vs. control. #$P$ < 0.05 vs. 10 μg mL$^{-1}$ LPS. (B) The levels of total and phosphorylated MEK were determined by Western blot analysis using whole cell lysates prepared 10 min after LPS (100 μg mL$^{-1}$) or LPS (100 μg mL$^{-1}$) + PD98059 (50 μmol L$^{-1}$) treatment. (C) Individual band intensities were determined by densitometric analysis and expressed as the ratio of phosphorylated MEK to total MEK. Band intensity ratio in the absence of LPS was considered as 1.

and therefore decreased APC cofactor activity of PS, which may explain the increased thrombotic tendency observed in patients with sepsis. It is unknown whether synthesis of C4BPβ is the rate-limiting step in C4BP secretion. Based on our results, we hypothesized that some C4BP lacking C4BPβ constitutively exists in plasma, and that when C4BPβ expression is increased, C4BP containing C4BPβ concomitantly increased, thus affecting the APC cofactor activity of PS. Further investigations are needed to elucidate this subject.

The IL-6-induced increase in the expression of C4BPβ in hepatocytes was significantly blocked by a STAT-3 inhibitor, suggesting that IL-6 induces C4BPβ expression via the STAT-3 pathway. Although IL-6 has been shown to exert its effects on cells via the STAT-3 transduction pathway [29,30], this is the first report showing that IL-6 specifically increases C4BPβ mRNA via the STAT-3 pathway.

In brief, the results of the present study show that LPS directly decreases C4BP expression in hepatocytes, and that this effect is mediated via the MEK and NFκB pathways while IL-6 specifically increases C4BPβ mRNA expression via the STAT-3 pathway. Increased C4BPβ expression increases formation of the PS–C4BP complex leading to an enhanced thrombotic tendency.

## Addendum

M. Kishiwada measured C4BPα and C4BPβ mRNA levels in the liver and hepatocytes, and wrote the first draft of the manuscript. T. Hayashi designed the study. H. Yuasa

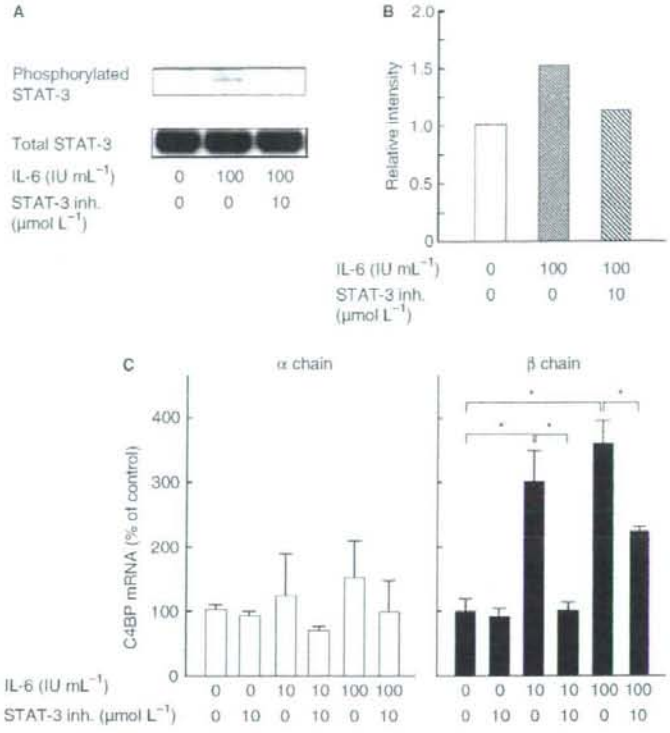


**Fig. 7.** STAT-3 activation by interleukin (IL)-6, and the effect of STAT-3 inhibitor on IL-6-induced increased C4b-binding protein (C4BP)β expression in hepatocytes isolated from normal rats. (A) The levels of total and phosphorylated STAT-3 were determined by Western blot analysis using whole cell lysates prepared 10 min after IL-6 (100 IU mL$^{-1}$) or IL-6 (100 IU mL$^{-1}$) + STAT-3 inhibitor (10 μmol L$^{-1}$) treatment. (B) Individual band intensities were determined by densitometric analysis and expressed as ratio of phosphorylated STAT-3 to total STAT-3. Band intensity ratio in the absence of IL-6 was considered as 1. (C) Hepatocytes isolated from normal rats were incubated in the presence of medium alone (control), STAT-3 inhibitor (10 μmol L$^{-1}$), IL-6 (10 IU mL$^{-1}$ or 100 IU mL$^{-1}$), or IL-6 (10 or 100 IU mL$^{-1}$) + STAT-3 inhibitor (10 μmol L$^{-1}$) for 24 h. After incubation, mRNA expression of C4BPα (open bar) and C4BPβ (closed bar) in hepatocytes were evaluated by real-time polymerase chain reaction analysis. *$P < 0.05$.

and K. Fujii measured C4BP antigen levels in plasma and culture medium. J. Nishioka purified rat C4BP and prepared antirat C4BP IgG. N. Akita and H. Tanaka isolated and cultured rat hepatocytes. T. Okamoto injected LPS into rats and collected blood samples from the rats. M. Ido measured plasma cytokine levels in LPS-treated rats. E. C. Gabazza and S. Isaji made intellectual contributions to the manuscript. K. Suzuki coordinated all of the studies.

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## Disclosure of Conflict of Interests

The authors state that they have no conflict of interest.

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