

• BASIC RESEARCH •

Lipopolysaccharide induced synthesis of CD14 proteins and its gene expression in hepatocytes during endotoxemia

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Abstract

AIM: To observe synthesis of CD14 protein and expression of CD14 mRNA in hepatic tissue and hepatocytes of rats during endotoxemia.

METHODS: The endotoxemia model of Wistar rat was established by injection of a dose of lipopolysaccharide (LPS) ($5\text{mg}\cdot\text{kg}^{-1}$, *Escherichia coli* O111:B4) via the tail vein, and then the rats were sacrificed after 3, 6, 12 and 24 h in batches. Hepatocytes were isolated from normal and LPS-injected rats by in situ collagenase perfusion technique and were collected to measure the expression of CD14 mRNA and synthesis of CD14 protein by reverse transcript-polymerase chain reaction (RT-PCR) or Western blot analysis. The binding of fluorescein isothiocyanate (FITC)-CD14 polyclonal antibody to isolated hepatocytes was also assessed by flow cytometric analysis (FCM).

RESULTS: In the rats with endotoxemia, the expressions of CD14 mRNA in hepatic tissue and isolated hepatocytes were stronger at 3, 6, and 12 h than that in control rats (3.48 ± 0.15 , 5.89 ± 0.62 , 4.33 ± 0.18 , vs 1.35 ± 0.14 in hepatic tissue, $P<0.01$; 4.12 ± 0.17 , 6.24 ± 0.64 , 4.35 ± 0.18 , vs 1.87 ± 0.15 in hepatocytes, $P<0.01$). The synthesis of CD14 protein in hepatic tissue and isolated hepatocytes increases also obviously in 6 and 12 h when compared to that in control rats (13.27 ± 1.27 , 17.32 ± 1.35 , 11.42 ± 1.20 , vs 7.34 ± 0.72 in hepatic tissue, $P<0.01$; 14.68 ± 1.30 , 17.95 ± 1.34 , 11.65 ± 1.19 , vs 7.91 ± 0.70 in hepatocytes, $P<0.01$). FCM showed that mean fluorescence intensity (MFI) and numbers of FITC-CD14 positive cells in the rats with endotoxemia increased obviously at 3, 6, 12 and 24 h when compared with normal control group (43.4%, 70.2%, 91.4%, 32.6% vs 4.5%, $P<0.01$).

CONCLUSION: LPS can markedly promote the synthesis of CD14 protein and up-regulate the expression of CD14 mRNA in isolated hepatocytes and hepatic tissue. Liver might be a main source for soluble CD14 production during endotoxemia.

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INTRODUCTION

CD14 is a glycosylphosphatidylinositol-anchored lipopolysaccharide (LPS) receptor, and first reported to be a differentiation marker expressed on the surface of macrophages, neutrophils and other myeloid lineage cells^[1,2]. Recent works have shown that the CD14 antigen is expressed in many types of cell and tissues^[3-8]. But it is not yet clear whether hepatocytes can express CD14 protein and gene. Hepatocytes are the major source of most acute-phase proteins. If in fact soluble CD14 (sCD14) is an acute-phase protein, then hepatocytes might be expected to express CD14, which is upregulated during endotoxemia^[9-11]. Furthermore, hepatocytes isolated from endotoxemic animals exhibit markedly enhanced responses to LPS, raising the possibility that these cells may express CD14^[3,4,12-14]. To determine whether hepatocytes express CD14, our experiments were to observe the synthesis of CD14 protein and expression of CD14 mRNA in hepatocytes and hepatic tissue of rats during endotoxemia and to verify hepatocytes as a main source for soluble CD14 (sCD14) production.

MATERIALS AND METHODS

Reagents

LPS (*Escherichia coli* O111:B4) and type IV of collagenase were purchased from Sigma Chemical Company (St. Louis, Mo.). An anti-CD14 polyclonal antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, Calif.). SP Reagent boxes and fluorescein isothiocyanate (FITC)-IgG were purchased from Zhongshan Biotechnology Company (Beijing, China).

Animals

Male Wistar rats, which were pathogen-free and weighed approximately 250 g each, were purchased from the Animal Center of Chongqing University of Medical Science. The rats were exposed each day 12 h of light and darkness. Rodent chow and water were provided ad libitum. Experimental protocols were approved by the Institutional Care and Use Committee of the Chongqing university of Medical Science.

The endotoxemia model of animals

The acute endotoxemia model of Wistar rat was established as described by Li SW, *et al*^[4]. In brief, animals were injected with a dose of LPS ($5\text{mg}\cdot\text{kg}^{-1}$, *Escherichia coli* O111:B4) via the tail vein, and then the rats were sacrificed at 3, 6, 12 and 24 h respectively. There were six rats at each time point, and other six animals were used as controls (0 h).

Hepatocyte Isolation

Hepatocytes were isolated from normal and LPS-injected rats by an in situ collagenase perfusion technique, modified as described previously^[15, 16]. In brief, livers were removed after a portal vein

perfusion with Hanks' balanced salt solution (HBSS) and the homogenate was digested in a solution of $0.5\text{g}\cdot\text{L}^{-1}$ collagenase. Hepatocytes were separated from the nonparenchymal cells by two cycles of differential centrifugation (50g for 2 min) and further purified over a 30% Percoll gradient. Hepatocyte purity exceeded 90% as assessed by light microscopy, and viability was typically greater than 95% as determined by trypan blue exclusion assay.

RNA Isolation and Complementary DNA Synthesis

Total RNA was isolated from rat liver tissue and hepatocytes by using the TRIZOL Reagent (Life Technologies, USA). The quality of RNA was controlled by the intactness of ribosomal RNA bands. A total of 0.5mg of each intact total RNA samples was reverse-transcribed to complementary DNA (cDNA) by using the reverse transcription-polymerase chain reaction (RT-PCR) kit (Roche, USA). cDNA was stored at -70°C until polymerase chain reaction (PCR) analysis.

Determination of CD14 mRNA by RT-PCR

The PCR primers used were CD14: sense ($5'$ -CTCAACCTAGAGCCGTTTCT- $3'$), anti-sense ($5'$ -CAGGATTGTCAGACAGGTCT- $3'$); β -actin: sense ($5'$ -ACCACAG-CTGAGAGGGAAATCG- $3'$), anti-sense ($5'$ -AGAGGTCTTTACGGATGTCAACG- $3'$). The sizes of the amplified PCR products were 267 bp for CD14 and 281 bp for β -actin. The conditions for amplification were as follows: denaturation at 93°C for 1 min, annealing at 57°C for 1 min, and extension at 70°C for 2 min for 30 cycles. The PCR products were electrophoresed in $20\text{g}\cdot\text{L}^{-1}$ agarose gels, and the gels were ethidium bromide stained and video photographed on an ultraviolet transilluminator, and the results were showed with the relative absorbance (Ar: relative optical density, ROD).

Western blot analysis of CD14 protein

Cultured hepatocytes were washed twice with phosphate-buffered saline (PBS), pelleted by centrifugation. Cell pellets were resuspended in 50 μl of lysis buffer containing 20 $\text{mmol}\cdot\text{L}^{-1}$ HEPES (pH 7.9), 25% glycerol, $0.42\text{ mmol}\cdot\text{L}^{-1}$ NaCl, $15\text{ mmol}\cdot\text{L}^{-1}$ MgCl_2 , $0.2\text{ mmol}\cdot\text{L}^{-1}$ EDTA, $0.5\text{ mmol}\cdot\text{L}^{-1}$ phenylmethylsulfonyl fluoride (PMSF) and $0.5\text{ mmol}\cdot\text{L}^{-1}$ dithiothreitol (DTT). After three freeze-thaw cycles, cell lysates were centrifuged at 12000g for 30 min, and the supernatant was saved. The liver tissue was homogenized with homogenizer before the freeze-thaw lysis, as described above for cultured hepatocytes. For Western blot analysis, samples (20 μg per lane) were separated on an SDS- $100\text{g}\cdot\text{L}^{-1}$ polyacrylamide gel and transferred to nitrocellulose membrane. The membrane was sequentially blocked in PBS-Tween ($1\text{g}\cdot\text{L}^{-1}$) containing $50\text{ml}\cdot\text{L}^{-1}$ milk and then incubated with 5 μg of anti-rat CD14 polyclonal antibody per mL, washed three times, and further incubated with a goat anti-rabbit immunoglobulin G horseradish peroxidase-conjugated secondary antibody. Blocking and antibody incubations each lasted 1 h at room temperature. After several washes, the membrane was developed with DAB reagent, and the results were showed with relative absorbance Ar (relative optical density, ROD).

Flow cytometric analysis (FCM)

Expression of CD14 protein in hepatocytes was examined by FCM. In brief, hepatocytes were incubated with the anti-CD14 polyclonal antibody ($0.1\text{mg}\cdot\text{L}^{-1}$) after washing, cells were incubated with goat anti-rabbit immunoglobulin G labeled with FITC, after being washed three times, and 10000 cells were analyzed by flow cytometry (Coulter, USA).

Statistical Analysis

All results were expressed as $\bar{x}\pm s$. Statistical difference between means were determined by using Student's t test. A P value of <0.01 was considered significant.

RESULTS

Expression of CD14 mRNA in liver tissue and hepatocytes

We postulated that hepatocytes and hepatic tissue could express CD14 gene which could be upregulated during endotoxemia. Rats were injected with LPS and total RNA was extracted from freshly isolated and purified hepatocytes and hepatic tissue at different time points indicated. RT-PCR analysis showed that hepatocytes and liver tissue from controls had low but detectable levels of CD14 mRNA. LPS treatment showed steady-state CD14 mRNA levels in hepatocytes, inducing a threefold elevation by as early as 3 h after LPS treatment. The levels increased with times, reaching a maximum of six-fold by 6 h after treatment, and subsequently declined to near baseline levels by 24 h. We also examined the CD14 mRNA levels in RNA isolated from hepatic tissue during endotoxemia and found that the pattern of CD14 mRNA induction by LPS was similar to that of the isolated hepatocytes, indicating that the upregulation of CD14 mRNA was not likely to be simply a consequence of the hepatocyte isolation procedure (Figure 1).

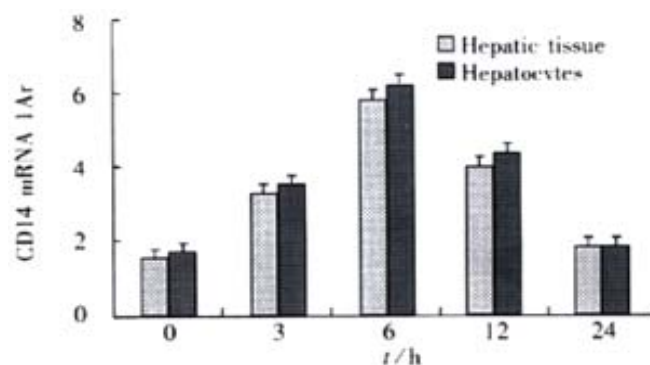


Figure 1 Expression of CD14 mRNA in hepatic tissue and hepatocytes $P<0.01$, vs 0 h.

CD14 protein expression in hepatocytes and hepatic tissue

To determine if the upregulation of CD14 expression could also be appreciated in protein levels, Western blot analysis was performed on both hepatocytes and hepatic tissue sample from LPS-treated animals or control animals. In hepatocytes extracts, increases of CD14 protein were seen 6 h after LPS treatment, peaked at 12 h, and declined thereafter. A similar increase of CD14 protein in hepatic tissue was observed. there were significantly different when compared with control animals ($P<0.01$, Figure 2).

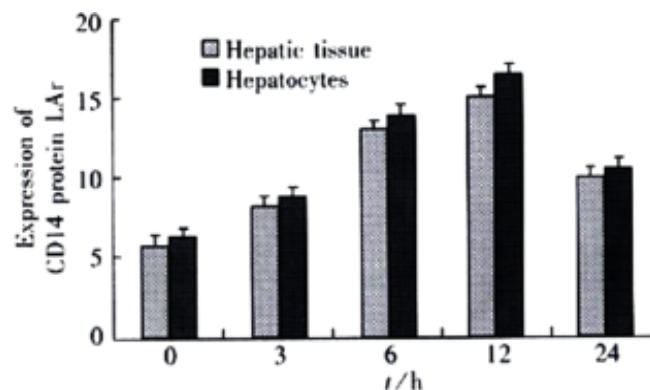


Figure 2 Expression of CD14 protein in hepatic tissue and hepatocytes $P<0.01$, vs 0 h

Binding of FITC to Hepatocytes

To confirm the expression of CD14 on hepatocytes, we also examined the binding of FITC to the cells. FITC-CD14 positive cells were 4.5% in rats of normal group. But in rats with endotoxemia, the mean fluorescence intensity (MFI) increased, the numbers of FITC-CD14 positive cells were 43.4%, 70.2%, 91.4%, and 32.6%, respectively in 3, 6, 12 and 24 h after stimulation of LPS. There was significant difference when compared to normal group animals ($P < 0.01$, Figure 3).

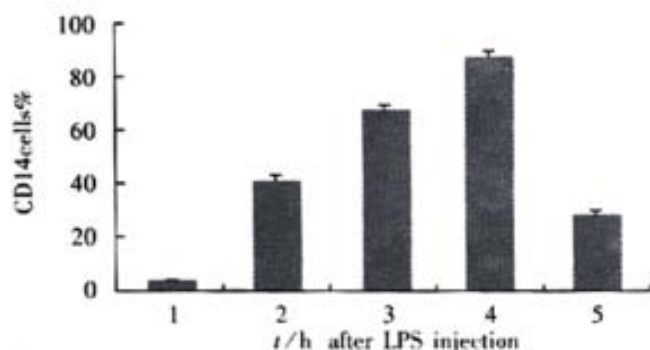


Figure 3 Percentage of CD14 positive cells $P < 0.01$, vs 0 h.

DISCUSSION

CD14 was first described as a myeloid differentiation antigen in 1980^[1]. It is a 55-kDa glycoprotein with multiple leucine-rich repeats and is encoded on chromosome (5q) together with growth factors, such as granulocyte macrophage colony stimulating factor. CD14 has been identified as a receptor for complexes of LPS and LPS-binding protein but it also binds other bacterial products^[17-22]. The LPS-binding region within the CD14 molecule is remarkably conserved across species with a high degree of gene sequence homology, and it has therefore been suggested that CD14 is a pattern recognition receptor^[17,23-25]. CD14 as a key LPS signaling molecule was reported to be expressed mainly in the monocyte-macrophages system^[1,26-29]. Recent works have shown that the CD14 antigen is expressed in many types of cells and tissues^[5-8,12]. But it is not yet clear whether hepatocytes express CD14. Although shedding from leukocytes has been proposed as the major source of sCD14 in blood, it is likely that other sources exist^[3,5,30-32]. Some reports suggested that sCD14 behaves like other acute-phase proteins^[9-11,33-36]. Hepatocytes are the major source of most acute-phase proteins. So we think if sCD14 is an acute-phase protein, then hepatocytes might be expected to express CD14 gene and synthesize CD14 protein, which is upregulated during endotoxemia^[37-40].

To determine whether hepatocytes synthesize CD14 protein and express CD14 gene, we measured steady-state CD14 protein and its mRNA both *in vivo* and *in vitro*. We found that: ① isolated hepatocytes and liver tissue could synthesize basal levels of CD14 protein and express basal levels of CD14 gene and that synthesis and expression of CD14 were markedly upregulated by LPS during endotoxemia; these results are in agreement with previous report^[41,42]; ② synthesis of CD14 protein and expression of CD14 mRNA in both Hepatocytes and liver tissue indicated that such synthesis and expression were not likely to be simple a consequence of hepatocyte isolation procedure; ③ in liver, besides hepatocytes, nonparenchymal cells such as Kupffer cells, endothelial cells, neutrophils and other cells can also express CD14 gene and synthesize CD14 protein^[4,43-47], but the fact that both isolated hepatocytes and hepatic tissue expressed CD14 protein and its mRNA indicated that the nonparenchymal cells could hardly have any effect on such expression in liver tissue.

Although we do not provide direct evidence here that sCD14 in plasma originates from hepatocytes during endotoxemia, our results showed that there was the possibility that the liver is an important source for sCD14 during endotoxemia. Pan *et al*^[5] found that the liver is one of the major organs for the production of soluble CD14. Liu *et al*^[41,42] reported also that CD14 transcription rates were significantly increased in hepatocytes from LPS-treated rat, indicating that the upregulation in CD14 mRNA levels observed in rats hepatocytes after LPS treatment was dependent, in part, on increased transcription, and their observations supported the idea that sCD14 would be an acute-phase protein and hepatocytes might be a source for circulating sCD14 production^[3,4,8,48-52]. Our data indicate that hepatocytes from LPS-stimulated rats express higher amounts of CD14 gene and CD14 protein, and may release more sCD14. Further investigation of the expression of CD14 in hepatocytes is actively ongoing in our laboratory.

In summary, our *in vitro* and *in vivo* data indicate that hepatocytes can synthesize CD14 protein and express CD14 mRNA and their synthesis and expression are upregulated effectively by LPS during endotoxemia. Liver is a main source for sCD14 production during sepsis or endotoxemia.

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