The potential role of transcription factor aryl hydrocarbon receptor in downregulation of hepatic cytochrome P-450 during sepsis

MIAN ZHOU, SUBIR R. MAITRA and PING WANG

Department of Surgery, North Shore University Hospital and Long Island Jewish Medical Center and The Feinstein Institute for Medical Research, Manhasset, NY 11030, USA

Received September 10, 2007; Accepted October 22, 2007

**Abstract.** We recently demonstrated that the hepatic cytochrome P-450 (CYP) isoform 1A2 is downregulated in sepsis, which appears to play an important role in the inflammatory response and liver injury. However, the mechanism responsible for the decreased CYP1A2 remains unknown. Since the transcription factor aryl hydrocarbon receptor (AhR) regulates the expression of CYP1A2 and the disruption of the AhR gene causes liver injuries, we hypothesized that downregulation of AhR plays an important role in the reduced hepatic CYP1A2 during sepsis. Adult male rats were subjected to sepsis by cecal ligation and puncture (CLP). Hepatic tissues were collected at 5, 10, and 20 h after CLP or sham-operation. The gene expression of AhR was assessed by RT-PCR technique. Its protein was determined by Western blot analysis. In addition, subcellular localization of AhR was examined by immunohistochemical staining. The results indicate that hepatic AhR gene expression decreased at 5 h and remained downregulated at 10-20 h after CLP. AhR protein levels were significantly reduced at 10-20 h after CLP. Immunohistochemical examination showed that AhR was mainly located in hepatocyte cytoplasm in sham animals. The translocation of AhR from the cytoplasm to the nucleus was observed in septic animals. The downregulation of hepatic AhR and CYP1A2 observed in septic animals does not appear to be due to the elevated endotoxin levels since administration of polymyxin B (an endotoxin-binding agent) did not affect AhR and CYP1A2 gene expression. However, proinflammatory cytokines tumor necrosis factor-α and interleukin-1β decreased AhR and CYP1A2 expression. As AhR activates the specific gene expression by binding to the target genes, the translocation of AhR to the nucleus in sepsis would suggest that alterations at AhR binding sites may also contribute to the downregulated CYP1A2 expression in sepsis. Since AhR gene expression decreased earlier than the occurrence of depression of CYP1A2 (CYP1A2 decreased at 10-20 h post CLP), the decreased AhR may play an important role in downregulating hepatic CYP1A2 during the progression of sepsis.

**Introduction**

The incidence of sepsis and septic shock has increased significantly despite improvement in the management of septic patients with systemic antibiotics, surgical intervention, aggressive fluid resuscitation, and careful monitoring (1,2). There is a progressive deterioration of cell and organ functions and even death in the septic host, which might be delayed or even prevented by intervention with pharmacological agents against and/or modulating various pro-inflammatory mediators. The animal model of polymicrobial sepsis induced by cecal ligation and puncture (CLP) mimics various features of clinical sepsis-peritonitis. The CLP model of sepsis is associated with an early, hyperdynamic phase (characterized by increased cardiac output and tissue perfusion, and decreased vascular resistance) followed by a late, hypodynamic phase (characterized by reduced cardiac output and tissue perfusion, as well as increased vascular resistance) (3-5). This model of sepsis has been used extensively to study the pathophysiological and immunologic alterations in sepsis (6).

Cytochrome P-450 (CYP) enzymes are a superfamily of heme proteins which is responsible for the metabolic activation or inactivation of most clinically used drugs and many toxins. CYP isoforms can be found in most tissues, but the largest concentrations of P-450 are found in the liver. The expression of P-450 enzymes is regulated by a variety of factors such as genetic polymorphism, drugs, hormones, development and diet (7). Inflammatory mediators can cause changes in the activities and expression of the P-450 enzyme system. A large number of reports have shown that CYP isoforms and their activities are, for the most part, suppressed in animal models of endotoxemia as well as in cultured hepatocytes stimulated by endotoxin (7-11). In this regard, a

**Correspondence to:** Dr Ping Wang, Laboratory of Surgical Research, The Feinstein Institute for Medical Research, 350 Community Drive, Manhasset, NY 11030, USA
E-mail: pwang@nshs.edu

**Key words:** liver, hepatoma cells, cecal ligation and puncture, tumor necrosis factor-α, interleukin-1β, hepatic cytochrome P-450 isoform 1A2
recent study from our laboratory showed that hepatic CYP1A2, the most predominant CYP isoform in the rat liver (12), is downregulated during the progression of polymicrobial sepsis induced by CLP (13).

Transcriptional activation of CYPs involves the aryl hydrocarbon receptor (AhR), a ligand-activated transcription factor as well as its nuclear translocator (Arnt) and a chaperone protein, heat shock protein 90 (HSP90) (14,15). Using a Kupffer cell-hepatocyte co-culture system, we recently showed that reduction in nuclear receptor AhR, due to the increased production of Kupffer cell-derived pro-inflammatory cytokines, tumor necrosis factor (TNF)-α and interleukin (IL)-1β, plays an important role in suppressing hepatic CYP1A2 expression in sepsis (16). However, the mechanism responsible for suppression of hepatic CYP1A2 in polymicrobial sepsis warrants additional investigations. Therefore, the objective of the present study was to further study the underlying mechanism responsible for the downregulation of CYP1A2 during polymicrobial sepsis. Various experiments were performed to determine whether sepsis-induced CYP1A2 downregulation was associated with and possibly caused by the reduction of AhR expression under such conditions.

Materials and methods

Animal model of sepsis. Male Sprague-Dawley rats (275-325 g), purchased from Charles River Laboratories (Wilmington, MA), were housed in a temperature-controlled room on a 12-h light/dark cycle and fed a standard Purina rat chow diet. Prior to induction of sepsis, rats were fasted overnight but allowed water ad libitum. Rats were then anesthetized with isoflurane inhalation, and the abdomen was shaved and washed with 10% povidone iodine. Cecal ligation and puncture (CLP) was performed to induce sepsis, rats were fasted overnight but allowed water ad libitum. After the onset of CLP, rats were then anesthetized with isoflurane inhalation, and the abdomen was shaved and washed with 10% povidone iodine. Cecal ligation and puncture (CLP) was performed as previously described (4,17), and the animals were randomly assigned to various experimental groups. Hepatic tissues were collected at 5, 10, and 20 h after CLP or sham-operation for determination of CYP1A2 and AhR expression. In separate experimental groups, polymyxin B (PMB, an endotoxin-binding agent) was administered to observe its effect on hepatic AhR and CYP1A2 gene expression. At 0.5 h before CLP as well as 9 h after CLP, PMB (Sigma, St. Louis, MO) was administered intraperitoneally at a total dose of 4000 U/kg (2000 U/kg each injection). This dosage was determined as optimal according to our previous experiment (18). Hepatic tissues were collected at 20 h after the onset of sepsis or sham-operation (5-6 rats/group). PMB is an endotoxin-binding antibiotic, which binds and detoxifies lipid A. In vitro studies have confirmed that PMB neutralizes E. coli endotoxin activity (19). All animal experiments were performed in accordance with the NIH guidelines for the use of experimental animals. This project was approved by the Institutional Animal Care and Use Committee of the Feinstein Institute for Medical Research.

Determination of AhR and CYP1A2 gene expression. The gene expression of AhR and CYP1A2 was determined using RT-PCR technique. The primers were as follows: 5’ GGG ATC GAT TTC GAA GAC ATC AG 3’ (sense), 5’ AAC GGC GAG CCT GGA ATC TC 3’ (antisense) for AhR (NM-013149); and 5’ GAA TCG GTG GCT AAC GTC AT 3’ (sense), 5’ CCA GAA GAT GCC TGT TGT GA 3’ (antisense) for CYP1A2 (NM-012541). Rat glyceraldehyde 3-phosphate dehydrogenase (G3PDH, M17701: sense, TGA AGG TCG GTG TCA ACG GAT TTG GC and antisense, CAT GTA GCC CAT GAG GTC CAC CAC) served as a housekeeping gene. PCR was carried out in a Bio-Rad thermal cycler. PCR cycles were carefully tested in order to avoid the plateau phase of the amplification. Following the RT-PCR procedure, the reaction products were separated in 1.6% TBE-agarose gel containing 0.22 μg/ml ethidium bromide by electrophoresis. The gel was then photographed and the band density was determined by a digital image system (Bio-Rad, Hercules, CA). Target/housekeeping gene band density ratios were then calculated.

Immunohistochemistry. The liver tissues were collected 20 h after CLP or sham-operation (n=4 rats/group) and immediately fixed in the neutralized 4% paraformaldehyde solution overnight. They were then dehydrated and embedded in paraffin through the standard histology procedure. The paraffin sections were dewaxed and rehydrated followed by the microwave antigen retrieval procedures. To retrieve the antigen, slides were soaked in 20% citric acid buffer, pH 6.0 (Vector Labs, Burlingame, CA) and kept at 95°C for 15 min. The slides were then washed in TBS for 5x10 min. Blots were incubated with rabbit anti-rat AhR polyclonal antibody (1:600; Biomol Research Laboratories Inc., Plymouth Meeting, PA), or goat anti-rat CYP1A2 serum (1:600; Daiichi Pure Chemicals, Tokyo, Japan) overnight at 4°C, respectively. The blots were then washed in TBST for 5x10 min. Blots were incubated with HRP labeled anti-rabbit (1:10,000) or anti-goat (1:4000) IgG for 1 h at room temperature. The blots were then washed in TBST for 5x10 min. To reveal the reaction bands, the membrane was reacted with the ECL Western blot detection system (Amersham, Piscataway, NJ) and exposed on X-ray film. The Bio-Rad GS-800 Calibrated Densitometer analysis system was used to quantitate the Western blots. This system selects the contour of the band, subtracts the background and calculates the density.
Hepatoma H-4-II-E cell culture and stimulation. Rat hepatoma cell line H-4-II-E cells (ATCC, Manassas, VA) were cultured in Eagle’s minimum essential medium (ATCC) with 10% fetal bovine serum and 1% penicillin-streptomycin (Invitrogen, Carlsbad, CA). H-4-II-E cells were stimulated with proinflammatory cytokines, TNF-α (5.0 ng/ml) or IL-1β (2.5 ng/ml) (both from Biosource International, Camarillo, CA) for 48 h. Proteins isolated from these cells were used for Western blot analysis.

Statistical analysis. Data are presented as means ± SE. One-way analysis of variance (ANOVA) and Tukey’s test were utilized for the comparison among different groups. The difference was considered significant at P≤0.05.

Results

Alterations in hepatic AhR expression in septic animals. The expression of both the AhR gene and protein in the liver was assessed at 5, 10, and 20 h after the onset of sepsis. The results indicated that hepatic AhR gene expression decreased by 43% at 5 h (P<0.05), and remained significantly reduced at 10-20 h after CLP (Fig. 1). Similarly, AhR protein levels were also significantly reduced at 5, 10, and 20 h after CLP (by 30-59%, Fig. 2). The finding that the AhR gene and protein expression decreased significantly as early as 5 h after the onset of sepsis suggests that the downregulation may have occurred even earlier than 5 h post CLP. Our previous studies showed that hepatic CYP1A2 expression decreased at 10-20 h after CLP (13). The fact that AhR downregulation occurs earlier than that of CYP1A2 may suggest a cause and effect relationship. Immunohistochemical examination showed that AhR protein staining was mainly located in the hepatocyte cytoplasm in sham animals (Fig. 3A). The translocation of AhR from the cytoplasm to the nucleus was
observed in septic animals at 20 h after CLP (Fig. 3B). As AhR activates the specific gene expression by binding to the target genes, the translocation of AhR to the nucleus in sepsis would suggest that alterations at AhR binding sites may also contribute to the downregulated CYP1A2 expression in sepsis.

**Effects of endotoxin neutralization on hepatic AhR and CYP1A2 gene expression in sepsis.** To determine whether endotoxin plays a role in downregulating AhR and CYP1A2 expression in sepsis, PMB was administered to neutralize circulating endotoxin in septic animals. The gene expression of AhR and CYP1A2 was assessed in the liver at 20 h after the onset of sepsis. The results indicated that AhR gene expression decreased significantly 20 h after CLP, and PMB treatment did not affect the diminished expression (Fig. 4A). Likewise, the downregulated CYP1A2 gene expression after sepsis was not affected by PMB treatment (Fig. 4B). These results suggest that AhR and CYP1A2 downregulation in sepsis may not be directly caused by the increased endotoxin.

**Effects of TNF-α and IL-1β on AhR and CYP1A2 in hepatoma cell culture.** Since administration of PMB in sepsis did not prevent the downregulation of hepatic AhR and CYP1A2 gene expression, we hypothesized that the reduced AhR and CYP1A2 expression may be caused by proinflammatory cytokines. To study this, hepatoma cells (H-4-II-E) were cultured with or without proinflammatory cytokine TNF-α (5.0 ng/ml) or IL-1β (2.5 ng/ml) for 48 h. The results indicated that incubation of the hepatoma cells with TNF-α decreased AhR protein expression by 36% (P<0.05, Fig. 5) and CYP1A2 protein expression by 40% (P<0.05, Fig. 6). Similarly, incubation of the hepatoma cells with IL-1β for 48 h significantly reduced AhR (Fig. 5) and CYP1A2 protein expression (Fig. 6). These results suggest that the elevated levels of proinflammatory cytokines in sepsis may play an important role in producing the downregulation of hepatic AhR and CYP1A2 expression.

**Discussion**

By using an animal model of polymicrobial sepsis, we demonstrated that the AhR gene and protein expression in the liver decreased significantly after the onset of sepsis (i.e. 5-20 h after CLP). It was also observed that the translocation of AhR from the cytoplasm to the nucleus occurred in septic animals. Furthermore, the downregulation of hepatic AhR and CYP1A2 observed in septic animals did not appear to be due to the elevated endotoxin levels, but appeared to be caused by proinflammatory cytokines TNF-α and IL-1β. As AhR gene expression decreased earlier than the occurrence of depression of CYP1A2 (CYP1A2 decreased at 10-20 h post CLP), the decreased AhR may play an important role in the downregulation of hepatic CYP1A2 in polymicrobial sepsis.

Nuclear receptors such as AhR represent a superfamily of ligand-modulated transcription factors. They mediate a variety of physiologic responses to steroids, retinoids, thyroid hormones, and various xenobiotics (20). These receptors play a key role in development and cell differentiation, as well as...
inflammatory cytokines such as TNF-α can be partially explained by the observation that pro-inflammatory cytokines, but not the direct effects of endotoxin, play an important role in downregulating hepatocellular CYP1A2 expression in sepsis. The reduction of AhR expression may be the underlying mechanism responsible for such downregulation.

Acknowledgements

This investigation was supported by National Institutes of Health (NIH) grants R01 GM053008 and R01 GM057468 (P. Wang). The authors sincerely thank Dr H. Hank Simms and Dr Yan J. Jiang for their excellent assistance.

References

15. Waxman DJ: P450 gene induction by structurally diverse xenoc- 
exchimicals: central role of nuclear CAR, PXR, and PPAR. Arch 
via AhR pathway: The central role of proinflammatory 
17. Wu R, Cui X, Lim YP, Bendelja K, Zhou M, Simms HH and 
Wang, P: Delayed administration of human inter-alpha 
hinhibitor proteins reduces mortality in sepsis. Crit Care Med 32: 
18. Yang S, Zhou MN, Chaudry IH and Wang P: The role of 
lipopolysaccharide in stimulating Adrenomedullin production 
during polymicrobial sepsis. Biochim Biophys Acta 1537: 
19. Morrison DC and Jacobs DM: Binding of polyoxymyxin B to the 
lipid A portion of bacterial lipopolysaccharides. Immunochemistry 
20. Beato M, Herrlich P and Schultz G: Steroid hormone receptors: 
Umesono K, Blumberg B, Kastner P, Mark M and Chambon P: 
The nuclear receptor superfamily: the second decade. Cell 83: 
22. Forman BM, Chen J and Evans RM: The peroxisome 
proliferators-activated receptors: ligands and activators. Ann 
NY Acad Sci 804: 266-275, 1996.
24. Xiao W, Zhou MN, Chaudry IH and Wang P: The role of 
lipopolysaccharide in stimulating Adrenomedullin production 
during polymicrobial sepsis. Biochim Biophys Acta 1537: 
25. Hankinson O: The aryl hydrocarbon receptor complex. Annu 
26. Waxman DJ: P450 gene induction by structurally diverse xenoc- 
exchimicals: central role of nuclear receptors CAR, PXR and 
27. Hankinson O: The aryl hydrocarbon receptor complex. Annu 
28. Bobrowski M, McGuire J, Picard D, Gustafsson JA and 
Poellinger L: Heat shock protein hsp90 regulates dioxin receptor 
29. Spatzenegger M, Horsmans Y and Verbeck RK: Differential 
activities of CYP1A isozymes in hepatic and intestinal 
microsomes of control and 3-methylcholanthrene-induced rats. 
30. Morgan ET: Regulation of cytochromes P450 during 
inflammation and infection. Drug Metab Rev 29: 1129-1188, 
1997.
32. Nicholson TE and Renton KW: Role of cytokines in the 
lipopolysaccharide-evoked depression of cytochrome P450 in 
The complex pattern of cytokines in sepsis: Association 
between prostaglandins, cachectin and interleukins. Ann Surg 
34. Milosevic N, Schwalder H and Maier P: Kupffer cell-mediated 
differential down-regulation of cytochrome P450 metabolism in 
35. Piscitelli SC, Reiss WG, Figg WD and Petros WP: Pharmacokinetic studies with recombinant cytokines. Scientific issues and 