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ScienceDirect
Biochemical and Biophysical Research Communications 353 (2007) 679–685
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KLF6 and HSF4 transcriptionally regulate multidrug resistance transporters during inflammation

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Received 7 December 2006
Available online 20 December 2006

Abstract

Endotoxin-induced inflammation alters the hepatic expression of the drug efflux transporter genes *mdr1b* (Abcb1b) and *mrp3* (Abcc3) in rats. In this study, we identified a novel kruppel-like zinc finger protein 6 (KLF6) *cis*-element on the rat *mdr1b* promoter which is important for basal activity and IL-1β and endotoxin-mediated induction in gene transcription. Interestingly, KLF6 also functioned as a negative transcriptional regulator, inhibiting TNF-α-mediated induction of *mdr1b*. Furthermore, novel CCAAT/enhancer binding protein β (C/EBPβ) and heat shock factor 4 (HSF4) transcription binding sites were identified on the rat *mrp3* promoter. Deletion of the HSF4 element significantly increased transcriptional activity of the *mrp3* gene when exposed to TNF-α. Endotoxin treatment significantly affected transcriptional activity only in C/EBPβ and HSF4 double deletion *mrp3* promoter constructs. In summary, KLF6 and HSF4 are stimuli-specific regulatory elements which may be important in the control of the rat *mdr1b* and *mrp3* genes during health and disease.

Keywords: Endotoxin; LPS; IL-1β; IL-6; TNF-α; Transcription; P-glycoprotein; Multidrug resistance-associated protein; EMSA; CAT assay

It has been reported that endotoxin-induced inflammation alters the plasma concentration and toxicity of various drugs [1,2]. Administration of bacterial endotoxin (lipopolysaccharide; LPS) results in the release of several pro-inflammatory cytokines including interleukin-1β (IL-1β), interleukin-6 (IL-6), and tumor necrosis factor-α (TNF-α). These cytokines elicit the activation of several transcription factors and cell signaling pathways resulting in alterations in the expression of numerous hepatic drug metabolizing enzymes and transporters.

The liver expresses numerous drug transporters that can regulate the uptake and efflux of xenobiotics. The canalicular membrane drug transporter p-glycoprotein (pgp), encoded by *mdr1a* (Abcb1a) and its isoform *mdr1b* (Abcb1b) in rodents [3], is capable of effluxing a diverse group of lipophillic organic cations [4]. On the other hand, the multidrug resistance-associated protein 3 (mrp3), which is encoded by the *mrp3* (Abcc3) gene, is located on the basolateral membrane and is primarily involved in the transport of anionic substrates and their glucuronate conjugates [5]. There are conflicting results pertaining to the alteration in the expression of these drug transporters during endotoxemia. It has been observed that endotoxin causes a significant induction in the hepatic expression of *mdr1b* in rats [6,7], whereas a significant suppression of *mdr1b* mRNA and protein levels is seen in the livers of endotoxin-treated mice [8,9]. Likewise, in the case of *mrp3*, studies have shown that endotoxin mediates an induction of *mrp3* in the rat liver [7,10], while in mice, endotoxin mediates a significant down-regulation [9,11]. Overall, these results suggest that the transcriptional regulation of *mdr1b* and *mrp3* expression during endotoxemia is species-specific.

Several transcription factors have been implicated in hepatic gene regulation during inflammation and other stress-evoking conditions. Bacterial endotoxin and the pro-inflammatory cytokines have been shown to dramatically induce CCAAT/enhancer binding protein β (C/EBPβ) and signal transducers and activators of transcription 3 (STAT3)

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0006-291X/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2006.12.090

[12]. In addition, increasing studies are suggesting that the kruppel-like zinc finger protein family (KLF) may be involved during tissue injury and the inflammatory response [13]. Elevated expression of heat shock transcription factors (HSFs), members of the diverse family of proteins involved in the heat shock response, have also been detected during endotoxemia [14]. Currently, no studies have analyzed the transcriptional regulation of the rat *mdr1b* and *mrp3* genes by KLF or HSF during an inflammatory response. Hence, our objective was to examine the transcriptional pathways involved in endotoxin and cytokine-mediated regulation of *mdr1b* and *mrp3*.

Materials and methods

Materials. DMEM, trypsin:EDTA, fetal bovine serum, streptomycin/penicillin were purchased from Invitrogen™ (Burlington, ON). Sodium dodecyl sulfate, Hepes, phenylmethylsulfonyl fluoride, Nonidet® P-40, glycerol, NaCl, $MgCl_2$, dithiothreitol (DTT), benzadamine, pepstatin, leupeptin, aprotinin, Tris–HCl, poly(dI-dC) poly(dI-dC), recombinant cytokines (IL-1β, IL-6, TNF-α and LPS (from *Escherichia coli* serotype 055:B5) were purchased from Sigma–Aldrich Chemical Company (St. Louis, MO).

Nuclear protein extraction and the electrophoretic mobility shift assay (EMSA). Male Sprague–Dawley rats (250–275 g) were injected intraperitoneally with saline or bacterial endotoxin (LPS, 5 mg/kg). Livers were collected at various timepoints (2, 4, 6, 12, and 24 h post-injection) and nuclear proteins were extracted from 100 to 200 mg of liver tissue according to previously described procedures [15]. Isolated nuclear proteins were stored at −80 °C until further use. EMSA was performed using 5 μg of nuclear protein and is described in the Supplementary Material. The double stranded oligonucleotide probes used for EMSA are listed in Supplementary Table 1.

Plasmids, transient transfections and the chloramphenicol acetyl transferase (CAT) assay. Generation of the rat *mdr1b* and *mrp3* wild-type and deletion CAT constructs (−449mdr1bWT-CAT, −449mdr1bDEL-CAT, −2407mrp3WT-CAT, −2407mrp3DEL1-CAT, −2407mrp3DEL2-CAT, and −2407mrp3DEL3-CAT) are described in the Supplementary Material. Promoter sequences for *mdr1b* and *mrp3* are based on GenBank Accession Nos. L16546 and AY039030, respectively. The human hepatoma cell line, Huh-7, was maintained in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin. The cells were grown at 37 °C in a humidified incubator equilibrated with 5% CO_2. Huh7 cells were plated onto 24-well tissue culture plates and transfected using Lipofectamine™ 2000 reagent (Invitrogen, Carlsbad, CA) with 0.8 μg of plasmid DNA following manufacturer's instructions. PSV-β-galactosidase control vector (Promega, Madison, WI) was co-transfected with reporter constructs as an internal control. After 24 h, cells were washed twice with 1× PBS and replaced with fresh media containing various concentrations of LPS (10–1000 ng/mL) or cytokines (IL-6: 2.0–40 ng/mL; TNF-α: 0.05–1.0 ng/mL, IL-1β: 0.05–1.0 ng/mL). Twenty-four hour post-treatment, cells were lysed with 1× Reporter Lysis Buffer (Promega, Madison, WI) and CAT activity was determined using the CAT assay system (Promega, Madison, WI) which utilizes liquid scintillation counting (Beckman LS5000TD, Beckman, CA). Results were normalized to β-galactosidase activity, which was determined using the β-galactosidase enzyme assay system (Promega, Madison, WI) spectrophotometrically at 420 nm. Cells transfected with an empty pCAT®3 basic vector (no promoter insert) treated with cytokines and endotoxin were also included as controls.

Protein isolation and characterization. The unknown transcription factor was isolated from hepatic nuclear fractions of LPS-treated rats using the DNA-Binding Protein Purification Kit (Roche Applied Science, Laval, QB), following manufacturer's instructions. The purity and molecular weight of the protein was determined by SDS–PAGE. Gel image was captured using the Fluorchem™ 8800 (Alpha Innotech, San Leandro, CA). The partial amino acid sequence of the protein was determined by the Edman degradation method (The Hospital for Sick Children, Advanced Protein Technology Centre, Toronto, ON).

Results

Time-dependent effects of endotoxin on nuclei protein binding to the rat mdr1b promoter

To study the effects of endotoxin treatment on transcription factor binding activity, hepatic nuclear extracts from LPS-treated and saline control rats were examined in combination with a segment of the rat *mdr1b* promoter containing a putative over-lapping C/EBPβ/STAT3 binding site (as determined by Transfac®). EMSA revealed a time-dependent induction in protein binding to the *mdr1b* promoter, with maximal binding occurring at 6 h of LPS treatment, which thereafter, binding decreased (Fig. 1A). These results suggest a transient induction or activation in transcription factor binding activity. In addition, the formation of two DNA–protein complexes was detected. Competition experiments using 100-fold unlabeled probe (Fig. 1A) resulted in a complete loss of DNA–protein complex 2 and a slight decrease in band intensity of DNA–protein complex 1. This suggests that complex 2 is our product of interest and that complex 1 is a result of non-specific interaction. No supershift was observed with the addition of C/EBPβ and/or STAT3 antibodies to the reaction mixture. These results indicate that the rat *mdr1b* fragment of interest (nt −291/−278) contains a specific binding site for an unknown LPS-induced transcription factor. Further studies were required to isolate and identify this unknown interacting transcription factor.

KLF6 is the unknown transcription factor interacting with the rat mdr1b promoter

To determine the identity of the transcription factor which interacted with the *mdr1b* promoter fragment between nt −291/−278, the protein was isolated based on its specific DNA-binding sequence. Isolation of the protein was successful as confirmed by SDS–PAGE (Fig. 1B). A clean, single band appeared around ~60 kDa indicating that the transcription factor is not likely to be C/EBPβ (20 or 35 kDa) [16] nor STAT3 (80 or 89 kDa) [17]. Partial N-terminal amino acid sequencing determined that the protein contained the sequence "SEVKILN", which when searched on the protein-protein BLAST database, was homologous to the human KLF protein. Supershift EMSA studies using specific antibodies with crude nuclear extract or the isolated protein further confirmed that the "unknown" factor was KLF6 (Fig. 1C). Antibody directed against C/EBPβ was included as a negative control. Literature revealed that KLF6 has a molecular weight of 56 kDa [18], which is very close to the predicted molecular weight, as determined by SDS–PAGE.