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Acute endotoxemia is associated with upregulation of lipocalin 24p3/Lcn2 in lung and liver

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Abstract

Acute endotoxemia is associated with production of acute phase proteins which regulate inflammatory responses to tissue injury. Consistent with DNA microarray experiments, we found that acute endotoxemia, induced by administration of lipopolysaccharide (LPS) to mice (1 mg/kg) or rats (5 mg/kg), resulted in increased expression of the hepatic acute phase protein, lipocalin 24p3, which was evident within 4 h and persisted for 24–48 h. Increases in 24p3 expression were also observed in the lung after LPS administration, as well as in isolated liver and lung macrophages, and Type II alveolar epithelial cells. The actions of LPS are dependent, in part, on Toll-like receptor (TLR) proteins. Macrophages from C3H/HeJ mice, which possess a nonfunctional TLR-4, expressed low levels of 24p3 mRNA when compared to cells from control C3H/OuJ mice. Whereas LPS administration increased 24p3 expression in lung and liver macrophages from control C3H/OuJ mice, minimal effects were observed in TLR-4 mutant mice demonstrating that TLR-4 is important in regulating 24p3 expression during acute endotoxemia. Promoters for genes encoding lipocalin proteins including 24p3 contain consensus sequences for transcription factors including NF- κ B, and C/EBP. Acute endotoxemia resulted in NF- κ B nuclear binding activity in both alveolar macrophages and Type II cells. In contrast, C/EBP activation was evident only in Type II cells, suggesting differential effects of LPS on these cell types. These data suggest that the acute phase response to acute endotoxemia involves induction of 24p3 in both the lung and liver. This protein may be important in restoring tissue homeostasis following LPS-induced injury.

Keywords

Lcn2; 24p3; LPS; Alveolar macrophage; Type II cell

Introduction

Lipopolysaccharide (LPS) is a major component of the cell wall of Gram negative bacteria. Exposure of humans and animals to excessive amounts of LPS causes injury to various organs including the lung and liver (Deitch, 1992). Tissue damage induced by LPS is

mediated, in part, by inflammatory products released from macrophages (Saluk-Juszczak and Wachowicz, 2005; Van Amersfoort et al., 2003). Whereas alveolar macrophages play a central role in initiating this process in the lung (Laskin and Pendino, 1995; Sibille and Reynolds, 1990), Kupffer cells mediate LPS-induced inflammatory responses in the liver (Dhainaut et al., 2001; Kmiec, 2001). Type II alveolar epithelial cells are also activated by LPS and may participate in pulmonary inflammatory processes (dos Santos et al., 2004; Farberman et al., 2004; Schulz et al., 2002; Sunil et al., 2002).

The acute phase response is a complex reaction to infection or tissue injury and is known to be induced by LPS (Berczi, 1998; Desiderio and Yoo, 2003). It is characterized by fever, leukocytosis, changes in vascular permeability, altered metabolic responses and activation of nonspecific host defenses (Baumann and Gauldie, 1994). Many of these responses are mediated by acute phase proteins released in large part, from hepatocytes (Tilg et al., 1997). One acute phase protein of particular interest is lipocalin 24p3, also known as uterocalin (Liu et al., 1997), SIP24 (Davis et al., 1991; Nilsen-Hamilton et al., 1982), or N-GAL (Kjeldsen et al., 1994) and identified in the genomic database as Lcn2. This protein has been implicated not only in the regulation of inflammatory responses, but also in control of cell growth and development, tissue involution, and apoptosis (Akerstrom et al., 2000; Devireddy et al., 2001; Flo et al., 2004; Nilsen-Hamilton et al., 2003; Yanagisawa et al., 2004; Zerega et al., 2000). 24p3 has also been reported to be secreted by mouse macrophages, neutrophils, and various other cell types (Kjeldsen et al., 1993; Meheus et al., 1993; Nantermet et al., 2005). Lipocalins bind both carbohydrate and protein receptors and can function as iron transporters (Bratt et al., 1999; Flower, 2000; Yang et al., 2003).

In the present studies, we characterized expression of 24p3 RNA and protein in liver and lung of mice and rats during acute endotoxemia induced by LPS administration. The role of TLR-4 in 24p3 expression was also examined. Understanding factors that regulate the synthesis of acute phase proteins like 24p3 may be important in the development of new therapies to abrogate inflammation associated with infections.

Materials and methods

Animals and treatment

Female specific pathogen-free Sprague–Dawley rats (200–225 g, 6–8 weeks) were purchased from Taconic (Germantown, NY). C57BL/6 female mice (15–20 g, 6–8 weeks) were purchased from Jackson Laboratory (Bar Harbor, Maine). C3H/OuJ and C3H/HeJ mice (15–20 g, 6–8 weeks) were from Jackson Laboratories (Bar Harbor, ME). Animals were housed in microisolator cages and maintained on sterile food and pyrogen-free water *ad libitum*. All animals received humane care in compliance with the institution's guidelines, as outlined in the *Guide for the Care and Use of Laboratory Animals*, published by the National Institutes of Health. Acute endotoxemia was induced by intravenous injection of rats with 5 mg/kg or intraperitoneal injection to mice with 1 mg/kg *Escherichia coli* LPS (serotype 0128:B12, Sigma Chemical Co., St. Louis, MO).

Tissue collection

Animals were euthanized by intraperitoneal injection of Nembutal (250 mg/kg for rats and 200 mg/kg for mice). The lungs were perfused with 50 ml (rats) or 10 ml (mice) of warm (37 °C) HBSS containing 25 mM HEPES, 0.5 mM EGTA, and 4.4 mM NaHCO₃, pH 7.3, followed by perfusion with Ca²⁺/Mg²⁺-free HBSS containing 22 mM HEPES, 4.2 mM NaHCO₃, pH 7.3 at a rate of 22 ml/min for rats and 10 ml/min for mice. Lung and liver were then removed, excised, and immediately frozen in liquid nitrogen.

Cell isolation

Rat and mouse alveolar macrophages were isolated from perfused lung by bronchoalveolar lavage 5–6 times with HBSS. Cells were washed 4 times with HBSS containing 2% FBS. Cell viability was 98% as determined by trypan blue dye exclusion, and cell purity greater than 97% as determined morphologically after Giemsa staining.

Rat Type II cells were isolated from lavaged lung as previously described (Sunil et al., 2002). Briefly, after washing twice with 10 ml of buffer (140 mM NaCl, 5 mM KCl, 2.5 mM Na₂HPO₄, 1.3 mM MgSO₄·7H₂O, 2 mM CaCl₂, 10 mM HEPES, pH 7.4), 30 ml of elastase (4.2 U/ml, Worthington Biochemicals, NJ) was infused into the lung using a 60 cc syringe. The tissue was then incubated at 37 °C for 20 min, the trachea and major bronchi removed, the lung minced in the presence of 4 ml DNase (1 µg/ml), and then digested for 5 min at 37 °C with 10 ml of elastase. The reaction was stopped by the addition of 5 ml FBS. The tissue was then sequentially filtered through 220, 60 and 15 µm nylon mesh. Cells were collected, washed and purified by negative selection (1 h, 37 °C) on IgG coated plates. Non-adherent cells were collected and washed with DMEM containing 10% FBS. Cell purity, assessed by modified Papinicolou staining, was 95% and viability, determined by trypan blue dye exclusion, was greater than 98%.

Hepatic macrophages were isolated as described previously with some modifications (Morio et al., 2000). The liver was perfused with Ca²⁺/Mg²⁺-free HBSS containing 0.5 mM EGTA, followed by perfusion with Leibovitz L-15 medium containing 100 U/ml collagenase type IV (Sigma-Aldrich Corp, St. Louis, MO). The liver was excised and combed; the resulting suspension filtered through a 220 µm nylon filter and washed 3 times (50×g, 1 min) to remove contaminating hepatocytes. The nonparenchymal cells were recovered by centrifugation of the supernatants (300×g, 7 min). Cells were purified using a Beckman J-6 centrifuge (Beckman Instruments Inc., Fullerton, CA) equipped with a centrifugal elutriation rotor with a pump speed of 35 ml/min and a rotor speed of 2500 rpm. Cells were further purified by differential centrifugation on a Nycodenz gradient (1.077 g/ml density, 1:2 gradient: sample; 450×g for 2 min). The purity of the cells, assessed by Giemsa staining was greater than 90%.

Microarray analysis

Microarray experiments were designed and run by Dr. Christopher A. Bradfield's laboratory (University of Wisconsin, Madison, WI) with poly A purified RNA isolated from whole livers of mice at various times after administration of LPS (1 mg/kg) using Cy3/Cy5 and a custom designed chip. The genes were analyzed on the chip and the data were kindly made available on the Bradfield laboratory website (<http://edge.oncology.wisc.edu/edge.php>).

Relative reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was isolated from whole tissue or cells using an RNeasy Mini kit (Qiagen, CA) following the manufacturer's instructions. Briefly, for first strand synthesis, RNA (200 ng) in 9 µl water was denatured at 65 °C for 4 min, rapidly cooled on ice, and then resuspended in a 20 µl final volume containing 250 mM Tris-HCl pH 8.3, 375 mM KCl, 15 mM MgCl₂, 100 mM DTT, 10 mM dNTP, 200 µM random hexamers and 200 U Superscript II RNase H⁻ reverse transcriptase (Invitrogen, Carlsbad, CA). After 1 h incubation at 37 °C, RNase H⁻ (2 U) was added and the samples incubated for an additional 20 min. The samples were then denatured at 95 °C for 5 min and chilled on ice. RNA concentration was determined by absorbance at 260 nm and converted to cDNA using 0.1–0.2 mg RNA. Initially, the linear range of amplification for 24p3 mRNA was determined using mouse and rat primers (IDT) with tissue and cells. For mouse and rat, 23–29 and 22–28 cycles, respectively, fell within the linear range for each control and experimental sample. In order to normalize each

primer, the 18S primer/competimer ratio was adjusted to produce an amplification signal in the linear range of the 24p3 products. A ratio of 1:9 was found to be appropriate to normalize the products. A rat 24p3 primer pair (5'-GTT TTC AGA GGT CTT CTG GA-3'; 5'-ACC ATC TAT GAG CTA CAG GA-3'), or a mouse 24p3 primer pair (5'-GCC CAG GAC TCA ACT CAG AA-3'; 5'-GAC CAG GAT GGA GGT GAC AT-3') (IDT, Coralville, IA), and an 18 S rRNA primer (Ambion, Austin, TX) with 1–2 µl of cDNA was amplified in a total volume of 20 µl, containing 10X PCR buffer, 1 µl tissue or cell reverse transcribed cDNA, 5 µM rat or mouse 24p3 primer, 1:9 ratio of 18S competimers/primers, 10 mM dNTP, 0.2 µl α[³²P] dCTP (10 mCi/ml; >3000 Ci/mmol) and 0.5 units of Taq DNA polymerase (Invitrogen) using GeneAmp PCR System 9600 (Perkin Elmer). Amplification was initiated at 94 °C for 1 min, continued with 25 and 27 cycles for rat and mouse respectively, at 94 °C for 15 s, 56 °C for 30 s and 72 °C for 90 s. The amplified PCR products (20 µl total) were mixed with 10 µl of loading buffer (95% formamide, 10 mM EDTA, 0.1% xylene cyanol, 0.1% bromophenol blue) and 6 µl aliquots were then run on a 5% denaturing polyacrylamide gel. The gel was dried and radioactive bands from the PCR products excised and counted by the Cerenkov method in a scintillation counter. Amplifications for all samples were performed at the same time and run on the same gel to minimize variability.

Preparation of cytoplasmic and nuclear extracts

Cytoplasmic extracts for Western blotting and nuclear extracts for electrophoretic mobility shift assays (EMSA) were prepared from cells using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce, Rockford, IL). Briefly, freshly isolated cells were resuspended in ice-cold Cytoplasmic Extraction Reagent (CER I) buffer, vortexed for 15 s and incubated on ice for 10 min. Cells were lysed in ice-cold CER II for 1 min, vortexed and centrifuged at 16,000×g for 5 min. Supernatants containing cytoplasmic extracts were transferred to fresh tubes. Ice-cold Nuclear Extraction Reagent (NER) buffer was added to the pellet and incubated on ice for 40 min with intermittent vortexing. Samples were then centrifuged at 16,000×g for 10 min and supernatants containing nuclear extracts transferred to fresh tubes. Aliquots of cytoplasmic and nuclear extracts were frozen at –70 °C until analysis.

To prepare cytoplasmic extracts from tissues, lung or liver tissue was homogenized in lysis buffer (150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 10.8 mM sodium phosphate pH 7.4, 0.1 mM PMSF, 5 mg/ml aprotinin) and centrifuged for 15 min at 16,000×g at 4 °C. Supernatants were collected and protein concentrations assayed using the BCA Protein Kit (Pierce) with bovine serum albumin as the standard.

Western blot analysis

Proteins (10–100 µg) were fractionated on 15% SDS polyacrylamide gels and transferred to nitrocellulose membranes. Non-specific binding was blocked by incubation of the blot for 30 min at room temperature or 4 °C overnight with 5% non-fat dry milk in Enhanced Chemi-Luminescence (ECL) buffer (Amersham Life Sciences, Arlington Heights, IL). Blots were incubated overnight at 4 °C with anti-uterocalin/NRL (1:500–1:1000) antibody prepared as described previously (Davis et al., 1991). After 4 washes in ECL buffer, blots were incubated in anti-rabbit HRP-conjugated secondary antibody (1:5000–1:10,000) for 30 min at room temperature and the bands visualized using an ECL detection system (Amersham).

Electrophoretic mobility shift assay (EMSA)

EMSA was performed as described previously (Sunil et al., 2002) with some modifications. Binding reactions were carried out at room temperature for 20 min in a total volume of 10–15 µl containing 10 µg of nuclear extracts, 3–6 µl of 5× gel shift binding buffer (20%

glycerol, 5 mM MgCl₂, 2.5 mM EDTA, 2.5 mM DTT, 250 mM NaCl, 50 mM Tris-HCl, pH 7.5), 2 µg poly dI-dC and 3×10⁴ cpm/µl γ[³²P]ATP (3000 Ci/mmol at 10 mCi/ml)-labeled C/EBP (TGC AGA TTG CGC AAT CTG CA), or CREB (AGA GAT TGC CTG ACG TCA GAG AGC TAG) consensus oligonucleotide (Santa Cruz Biotechnology, Santa Cruz, CA). Protein-DNA complexes were separated on 7% non-denaturing polyacrylamide gels run at 250 V in 0.5× TBE and visualized after the gels were dried and autoradiographed. For supershift assays, 10–12 µg of antibodies to C/EBP subunits (β and δ) (Santa Cruz Biotechnology) were added to the reaction mixtures and incubated on ice for 2 h prior to the addition of labeled oligonucleotide. For competitor reactions, 50-fold excess of unlabeled C/EBP oligonucleotide was added to the mixture prior to the addition of the labeled probe.

Immunohistochemistry

Lungs were perfused with buffer, removed, fixed in 3% paraformaldehyde in PBS for 4 h on ice and transferred to 50% ethanol. Tissue sections (6 µm) were deparaffinized and incubated overnight at 4 °C with rabbit serum, anti-mouse uterocalin/NRL (1:300) (Davis et al., 1991; Nilsen-Hamilton et al., 2003), rabbit IgG, or rabbit anti-rat C/EBPβ (1:200) antibody (Santa Cruz Biotechnology) followed by a 30 min incubation with biotinylated secondary antibody (Vector Labs, Burlingame, CA). Binding was visualized using a Vectastain Elite ABC kit (Vector Labs).

Results

Effects of acute endotoxemia on 24p3 mRNA expression in the liver

Analysis of microarray data obtained from livers of C57BL/6J mice revealed that at least 16 genes were altered 3-fold or more, 6 h following administration of LPS (Table 1). This included increases in genes encoding various acute phase proteins (24p3, serum amyloid A), and decreases in genes involved in hepatic drug metabolism (CYP2A5, alcohol dehydrogenase, flavin containing monooxygenase 5, hydro-xysteroid transferase). These data are consistent with previous studies on the hepatic effects of acute endotoxemia in mice (Desiderio and Yoo, 2003). The most prominent change noted was in expression of 24p3 (utc/NRL), which increased 15-fold 6 h after LPS treatment of the mice and remained elevated (23-fold) for at least 24 h. To confirm these findings, we assessed the effects of LPS on expression of 24p3 mRNA in mouse liver by RT-PCR. As observed in the microarray experiments, 24p3 mRNA was expressed at low levels in livers of control mice (Fig. 1, left panel). LPS administration caused a time-dependent increase in 24p3 mRNA expression which was evident within 4 h and persisted for at least 24 h. This was associated with a time-related rise in 24p3 protein expression in mouse liver, as measured by Western blotting, which peaked 4–24 h after LPS administration. For comparative purposes, we also evaluated 24p3 mRNA and protein expression in rats treated with LPS. As observed in mice, 24p3 mRNA was expressed at low levels in livers of untreated rats (Fig. 1, right panel). In addition, LPS treatment of rats caused a time-related increase in hepatic 24p3 mRNA and protein expression. However, the response was more prolonged in rats when compared to mice, persisting for at least 48 h.

Macrophages play a critical role in the hepatic inflammatory response to LPS (Kmiec, 2001). We next assessed 24p3 expression in rat liver macrophages. Low levels of 24p3 protein were detectable in macrophages isolated from livers of control rats (Fig. 2). LPS administration caused a rapid increase in 24p3 expression which was observed within 1.5 h and persisted for at least 24 h before declining. These data are generally consistent with our findings in whole rat liver.

Effects of LPS on 24p3 expression on lung tissue and cells

The lung is also known to be highly sensitive to LPS (Chaby et al., 2005; Thorn, 2001). In further studies, we analyzed the effects of LPS administration to mice and rats on 24p3 expression in the lungs. Constitutive 24p3 mRNA was detectable in the lungs of control animals (Fig. 3). LPS treatment of both mice and rats resulted in a rapid (within 1–4 h) but transient increase in 24p3 mRNA. Whereas in mice maximal induction of 24p3 was observed 4 h after LPS administration decreasing to control levels by 24–48 h, peak expression of 24p3 in rats was delayed for 24 h. Low levels of 24p3 protein were also constitutively expressed in lungs of control mice and rats. In both species, LPS administration resulted in increased 24p3 protein expression in the lung (Fig. 3, bottom panel), which was maximal at 4 h.

We next determined if 24p3 mRNA and protein also increased in lung macrophages as shown in Fig. 2 for rat liver macrophages. Alveolar macrophages from control mice and rats expressed low levels of 24p3 mRNA (Fig. 4). Treatment of both animals with LPS caused an increase in 24p3 mRNA expression in alveolar macrophages, a response that was generally similar to whole lung. In both mice and rats, mRNA levels for 24p3 decreased 24–48 h after LPS administration. Type II alveolar epithelial cells are also known to become activated in response to LPS (Sunil et al., 2002). Like alveolar macrophages, Type II cells from control animals expressed low levels of 24p3 mRNA and protein (Figs. 4 and 5). LPS administration caused a time-related increase in 24p3 mRNA and protein expression in Type II cells which peaked at 4–24 h. More 24p3 protein was expressed in macrophages when compared to Type II cells (Fig. 5). In Type II cells (Fig. 5), as well as in liver macrophages (Fig. 2), at least 2 bands appeared in the protein gels. At the present time it is unclear if this is due to degradation, alternative structures or a variation in post translational modification such as glycosylation of 24p3 (Davis et al., 1991). This has also been observed in other studies of 24p3 extracted from tissues (Liu et al., 1997; Ryon et al., 2002). Changes in expression of 24p3 protein were confirmed by immunohistochemical staining of lung sections. Following administration of LPS to rats, a time-dependent increase in 24p3 expression was observed in both alveolar macrophages and Type II cells within 1 h, which persisted for at least for 24 h (Fig. 6).

Role of TLR-4 in 24p3 expression in macrophages

The actions of LPS depend, in part, on members of the Toll-like receptor (TLR) protein family (Politorak et al., 1998). In further experiments, we determined if TLR-4 is involved in induction of 24p3 during acute endotoxemia. For these experiments, we used C3H/HeJ mice that possess a nonfunctional mutated TLR-4 receptor (Politorak et al., 1998). We found that alveolar and hepatic macrophages from control C3H/OuJ mice, which possess a functional TLR-4 receptor, express low levels of 24p3 mRNA (Fig. 7). As observed with C57BL/6 mice (Fig. 4), LPS treatment of C3H/OuJ mice caused a significant induction of 24p3 mRNA, which was maximum at 12 h in alveolar macrophages and at 3–12 h in hepatic macrophages. In contrast, very low constitutive 24p3 mRNA expression was detected in macrophages from TLR-4 mutant C3H/HeJ mice. Moreover, although LPS upregulated 24p3 mRNA expression in lung and liver macrophages, this was only observed at relatively low levels 3 h post treatment of the mice (Fig. 7). Small increases in 24p3 at 3 h may be due to activation of signaling pathways by LPS that are not dependent on TLR-4 (Wong et al., 2000). These data indicate that TLR-4 plays a role in LPS-induced alterations in 24p3 gene expression.

Regulation of 24p3 expression

The promoter region for murine 24p3 and the human homolog of 24p3, N-GAL, contains consensus sequences for several different transcription factors including NF- κ B, C/EBP and

CREB, which may be important in controlling expression of the gene (Cowland and Borregaard, 1997; Shen et al., 2006). Recent studies also demonstrate that cytokines including IL-17 utilize NF- κ B and C/EBP to activate the 24p3 promoter (Shen et al., 2006). We previously reported that LPS administration to rats readily induces NF- κ B DNA binding activity in alveolar macrophages and Type II cells (Sunil et al., 2002). In further studies we analyzed the effects of LPS on the activity of C/EBP and CREB using mobility shift assays. Constitutive C/EBP DNA binding activity was evident in both alveolar macrophages and Type II cells from control rats (Fig. 8). Whereas LPS administration had no major effect on C/EBP activity in alveolar macrophages, an increase (within 1 h) in activity was observed in Type II cells which persisted for at least 24 h. In alveolar macrophages, antibody to C/EBP β , but not C/EBP δ , caused a supershift in DNA binding activity. In contrast, in Type II cells, antibody to both β and δ subunits caused a supershift in DNA binding activity. This suggests that different subunits of C/EBP are activated in alveolar macrophages and Type II cells following LPS treatment of animals. A 50-fold excess of unlabeled probe blocked C/EBP binding demonstrating the specificity of the probe (Fig. 8). Similar results were observed in histological sections stained with antibody to C/EBP protein. Thus, both alveolar macrophages and Type II cells from untreated rats were found to express low levels of C/EBP β (Fig. 9). Whereas LPS increased the expression of this protein in Type II cells, there was no significant change in its expression in alveolar macrophages. We also observed constitutive DNA binding activity for CREB in both alveolar macrophages and Type II cells, which was not altered by LPS in either cell type (data not shown).

Discussion

During the acute phase response the liver synthesizes a number of proteins that contribute to both the initiation and resolution of inflammatory processes. This latter activity is particularly important in reestablishing homeostasis following injury or infection (Gabay et al., 2001; Logdberg and Wester, 2000). Bacterially-derived LPS is a potent inducer of the acute phase response (Desiderio and Yoo, 2003). In addition to stimulating hepatic acute phase protein production, LPS activates liver macrophages or Kupffer cells, the primary cell population responsible for clearance of LPS from the portal circulation (Callery et al., 1991). Excessive amounts of LPS however, can overwhelm Kupffer cell clearance capacity leading to activation of other macrophage populations of the reticuloendothelial system including alveolar macrophages in the lung (Kalmovarin et al., 1991). These cells are highly responsive to LPS and studies have suggested that they may also contribute to the acute phase response (Deitch, 1992; Hernandez-Pando et al., 1998; Welbourn and Young, 1992). In the present studies we analyzed the effects of acute endotoxemia induced by administration of LPS to rodents on expression of 24p3, an acute phase protein important in modulating inflammatory responses in the liver and in the lung. Signaling molecules potentially involved in regulating 24p3 were also examined.

Microarray analysis revealed that LPS administration to mice caused a time-dependent increase in expression of the 24p3 gene in the liver. This was confirmed using techniques in RT-PCR and Western blotting. Thus, we found increases in hepatic expression of 24p3 mRNA and protein in mice, as well as in rats. Whereas the hepatic response peaked 4–24 h following LPS administration in mice, it was more prolonged in rats, persisting for at least 48 h. This may be due to differences in LPS clearance capacity between rat and mouse Kupffer cells (McCuskey et al., 1984). Since the lung is also highly sensitive to the effects of LPS (Chaby et al., 2005; Thorn, 2001), for comparative purposes we analyzed 24p3 expression in lungs of treated animals. As observed in the liver, LPS was found to induce 24p3 mRNA and protein expression in the lungs of both mice and rats. These findings suggest that the lung may also play an important role in the acute phase response to LPS (Kalmovarin et al., 1991; Wilson et al., 2005). Our findings are in accord with previous

reports demonstrating that intranasal or intratracheal administration of LPS to mice induces 24p3 mRNA expression in the lung (Nilsen-Hamilton et al., 2003; Sun et al., 2005; Yanagisawa et al., 2004). LPS has also been reported to induce expression of α -1 acid glycoprotein, another acute phase protein, in both the lung and the liver, confirming that both tissues are highly sensitive to the inflammatory effects of this bacterially-derived product (Crestani et al., 1998; Geller et al., 1994). The human homolog of 24p3, N-GAL, has been shown to bind gelatinase, a potent neutrophil chemoattractant that degrades extracellular matrix proteins and promotes inflammation (Kjeldsen et al., 1993). These data suggest that 24p3 may function to down-regulate the inflammatory response by binding and/or sequestering chemokines thereby reducing their biological activity. 24p3 has also been reported to stimulate apoptosis, which may contribute to its anti-inflammatory activity (Ryon et al., 2002).

Macrophages play an important role in the inflammatory response to LPS. Whereas initially these cells release proinflammatory and cytotoxic mediators that destroy invading pathogens and recruit additional inflammatory cells, subsequently they generate mediators that down-regulate the inflammatory response and initiate tissue repair (Kmiec, 2001). Our studies demonstrate that 24p3 is expressed constitutively in both hepatic and alveolar macrophages, suggesting that these cells are primed to participate in the acute phase response. This is supported by our findings that 24p3 is rapidly upregulated in both macrophage populations after LPS administration. Our results are in accord with previous reports that 24p3 is expressed in rat peritoneal macrophages, as well as in macrophage cell lines treated with LPS (Meheus et al., 1993; Pawluczyk et al., 2003).

Induction of acute endotoxemia also resulted in a persistent induction of 24p3 mRNA and protein in Type II alveolar epithelial cells. LPS has been shown to up-regulate α -1 acid glycoprotein in Type II cells (Crestani et al., 1998). These findings suggest that Type II cells may also be important in the pulmonary acute phase response. Increases in N-GAL have been detected in human lung A549 epithelial cells, and in human bronchial epithelial cells following stimulation with IL-1 β , but not LPS (Cowland et al., 2003). The lack of response of these cells to LPS may be due to differences in the activity of primary and transformed epithelial cells and/or differential responses of human and rat cells to LPS. We found that LPS-induced expression of 24p3 was greater in macrophages when compared to Type II cells. This is consistent with the reduced capacity of Type II cells to release inflammatory mediators relative to macrophages (Punjabi et al., 1994; Wizemann et al., 1994). Tissue remodeling and repair is important in the resolution of inflammatory responses. Members of the lipocalin family are known to participate in tissue remodeling, during development, and during the acute phase response (Descalzi Cancedda et al., 2000; Gwira et al., 2005; Liu et al., 1997; Mallbris et al., 2002; Nilsen-Hamilton et al., 2003; Ryon et al., 2002). Following damage to the lower lung, Type II epithelial cells differentiate into Type I cells restoring the pulmonary architecture (Castranova et al., 1988; Uhal et al., 1991). Upregulation of 24p3 in Type II cells during acute endotoxemia may also be important in this process (Liu et al., 2005).

The actions of LPS are mediated, in part, by members of the TLR protein family including TLR-4 (Poltorak et al., 1998). We found that macrophages from control C3H/OuJ mice express significantly greater amount of 24p3 mRNA after LPS administration when compared to TLR-4 mutant C3H/HeJ mice. These findings are in accord with reports of decreased 24p3 levels in serum, and reduced 24p3 protein production by peritoneal cells following LPS administration to TLR-4 $^{-/-}$ mice, when compared to wild type mice (Flo et al., 2004). The inability of LPS to induce 24p3 in A549 epithelial cells is also thought to be due to defects in TLR-4 (Cowland et al., 2003). Our studies demonstrate that LPS-induced expression of 24p3 in alveolar and hepatic macrophages also requires functional TLR-4.

Activation of the transcription factor C/EBP is correlated with expression of a number of inflammatory and acute phase response genes, including 24p3, in the liver and cells of the myelomonocytic lineage as well as in non-immune cell types (Cortes-Canteli et al., 2004; Poli, 1998; Poli et al., 1990; Shen et al., 2006), suggesting that C/EBP regulates 24p3 expression. C/EBP β activity has also been detected in the lung during the acute phase response (Alam et al., 1992; Sugahara et al., 1999). Our studies show that both alveolar macrophages and Type II cells display constitutive C/EBP DNA binding activity. Interestingly, whereas LPS treatment of rats activated C/EBP in Type II cells, no major effects were observed in alveolar macrophages. These findings suggest that C/EBP may play a unique role in regulating Type II cell activity during endotoxemia. Similar activation of C/EBP has been previously described in Type II cells following LPS administration to rats (Sugahara et al., 1999). In contrast to our findings, LPS has been reported to induce C/EBP- β activity in peritoneal macrophages (Sonoki et al., 1997), and in cultured J744.2 macrophages (Tengku-Muhammad et al., 2000). These differences may be due to the distinct origins of cell populations examined. Consensus sequences for the transcription factors, CREB and NF- κ B, have also been identified in the promoter region of the 24p3 gene (Cowland and Borregaard, 1997; Cowland et al., 2003; Sousa et al., 2005). Shen et al. (2006) also demonstrated that NF- κ B was essential in activating 24p3 in murine fibroblasts and ST2 cells, a stromal cell line derived from the bone marrow. We have previously demonstrated that LPS induces NF- κ B nuclear binding activity in both alveolar macrophages and Type II cells (Sunil et al., 2002). In contrast, the present studies showed no major effects of LPS on CREB binding activity in these cells. These data indicate that distinct signaling pathways are activated by LPS in macrophages and epithelial cells during the acute phase response.

In summary, the present studies demonstrate that LPS administration to both mice and rats induces expression of the acute phase protein 24p3, in the liver and the lung. Moreover, this is evident in macrophages, as well as in Type II alveolar epithelial cells. The precise role of 24p3 in the acute phase response is not known. We speculate that it functions to down-regulate the inflammatory response and to protect the lung and liver from excessive damage induced by endotoxin. Our studies also suggest that TLR-4 and transcription factors C/EBP and NF- κ B may be involved in regulating 24p3 expression in the lung and liver. These data are consistent with earlier observations demonstrating that these transcription factors are critical in regulating cytokine-induced 24p3 expression (Shen et al., 2006). Moreover, over expression of C/EBP or the p65 subunit of NF- κ B has been shown to drive 24p3 promoter expression (Shen et al., 2005). Further studies are required to determine the precise signaling pathways that control expression of 24p3 in the liver and the lung and its relative contribution to the acute phase response to LPS.

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Fig. 1.

Effects of LPS on expression of 24p3 mRNA and protein in mouse and rat livers. RNA and protein were extracted from livers of control (C) and treated animals 1–48 h after LPS administration. Upper panels, 24p3 mRNA expression. Middle panels, radioactive bands from RT-PCR gels were counted and data presented as the ratio of 24p3:18S mRNA. Lower panels, western blot showing 24p3 protein expression.

Fig. 2. Effects of LPS on expression of 24p3 protein in hepatic macrophages. Cells were isolated from livers of control (C) and treated rats 1.5–48 h after LPS administration. Cell lysates were prepared and analyzed for 24p3 expression by western blotting.

Fig. 3.

Effects of LPS on expression of 24p3 mRNA and protein in lungs of mice and rats. RNA and protein were extracted from lungs of control (C) and treated animals 1–48 h after LPS administration. Upper panels, 24p3 mRNA expression. Middle panels, radioactive bands from RT-PCR gels were counted and data presented as the ratio of 24p3:18S mRNA. Lower panels, 24p3 protein expression was determined by western blotting. Densitometry was performed using One DScan (Scanalytics, Billerica, MA) and data presented as integrated optical density (Integrated OD).

Fig. 4. Effects of LPS on expression of 24p3 in alveolar macrophages and Type II cells. Cells were isolated from control (C) and treated mice and rats 1–48 h after LPS administration. Left panels, 24p3 RT-PCR gels. Right panels, radioactive bands from RT-PCR gels were counted and data presented as the ratio of 24p3:18S mRNA.

Fig. 5. Effects of LPS on expression of 24p3 protein in alveolar macrophages and Type II cells. Cells were isolated from control (C) and treated rats 1–48 h after LPS administration. Cell lysates were prepared and analyzed for 24p3 expression by western blotting.

Fig. 6. Effects of LPS on 24p3 protein expression in rat lung. Tissue sections, prepared 1–48 h after administration of LPS or control (C) were stained with antibody to 24p3 or IgG (Serum). Arrows, Type II cells. Arrowheads, alveolar macrophages.

Fig. 7. Role of TLR-4 in LPS-induced expression of 24p3 mRNA. Alveolar macrophages (AM) and hepatic macrophages (HM) were isolated from control (C) and treated C3H/OuJ and TLR-4 mutant C3H/HeJ mice 3–48 h after LPS administration. Upper panels, 24p3 mRNA expression. Lower panels, radioactive bands from RT-PCR gels were counted and data presented as the ratio of 24p3:18S mRNA.

Fig. 8.

Effects of LPS on C/EBP nuclear binding activity. Alveolar macrophages (AM) and Type II cells (T II) were isolated from control (C) and treated rats 1–48 h after LPS administration. Nuclear extracts were analyzed for C/EBP binding activity by EMSA. Extracts prepared from cells collected 48 h after LPS administration were pre-incubated with antibodies to C/EBP β (β), C/EBP δ (δ), or 50-fold excess unlabeled C/EBP competitor probe (Comp).

Fig. 9. Effects of LPS on expression of C/EBP in rat lung. Tissue sections, prepared 1–48 h after administration of LPS or control (C) were stained with antibody to C/EBP β or IgG (from 1 h LPS). Arrows, Type II cells. Arrowheads, alveolar macrophages.

Table 1

Summary of microarray data from livers of mice treated with LPS

Gene	Time following LPS			
	6 h	12 h	24 h	48 h
Mouse 24p3 (utc/NRL)	14.9	31.74	23.08	1.24
Mouse serum amyloid A	14.62	11.37	11.02	1.55
Mouse endogenous leu virus envelope	5.39	1.08	-2.41	1.32
Mouse endogenous leu virus gp70	5.14	1.07	-2.6	1.44
Mouse endogenous virus envelope	4.9	-1.03	-2.38	1.36
Mouse TGF β 3	4.79	1.98	-1.13	1.02
Mouse MHC III	3.91	1.23	-2.25	1.32
~Rat α fibrinogen	3.77	1.93	1.41	-1.29
Mouse II GP	3.46	1.73	1.33	-1.31
Mouse α 2 anti plasmin	3.37	1.75	1.19	-1.51
Mouse flavin-containing monooxygenase 5	-3.01	-2.24	-2.31	1.3
Mouse phenylalanine hydroxylase	-3.09	-2.64	-1.46	1.18
Mouse class I alcohol dehydrogenase	-3.12	-2.15	-1.02	1.02
~Rat hydroxysteroid sulfotransferase a	-3.36	-2.74	-1.92	1.04
Mouse CYP2A5	-4.1	-2.21	-2.58	1.12
~Rat hydroxysteroid sulfotransferase	-4.19	-2.65	-2.05	1.38

Data from genes changing 3-fold or more, 6 h following LPS treatment of mice are included. Fold changes in gene expression were obtained by comparison to vehicle treated controls. -, decrease in gene expression.