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The Leptospira Outer Membrane Protein LipL32 Induces Tubulointerstitial Nephritis-Mediated Gene Expression in Mouse Proximal Tubule Cells

CHIH-WEI YANG,* MAI-SZU WU,* MING-JENG PAN,† WANG-JU HSIEH,† ALAIN VANDEWALLE,† and CHIU-CHING HUANG*

**Department of Nephrology, Chang Gung Memorial Hospital, Taipei, Taiwan, Republic of China; †Graduate Institute of Veterinary Medicine, National Taiwan University, Taipei, Taiwan, Republic of China; and ‡National Institute of Health and Medical Research (INSERM), Unit 478, Faculty of Medicine Xavier Bichat, Paris, France.*

Abstract. Tubulointerstitial nephritis is a main renal manifestation caused by pathogenic leptospira that accumulate mostly in the proximal tubules, thereby inducing tubular injury and tubulointerstitial nephritis. To elucidate the role of leptospira outer membrane proteins in tubulointerstitial nephritis, outer membrane proteins from pathogenic *Leptospira shermani* and nonpathogenic *Leptospira patoc* extracted by Triton X-114 were administered to cultured mouse proximal tubule cells. A dose-dependent increase of monocyte chemoattractant protein–1 (MCP-1), RANTES, nitrite, and tumor necrosis factor–α (TNF-α) in the culture supernatant was observed 48 h after incubating *Leptospira shermani* outer membrane proteins with mouse proximal tubule cells. RT competitive-PCR experiments showed that *Leptospira shermani* outer membrane proteins (0.2 μg/ml) increased the expression of MCP-1, nitric oxide synthase (iNOS), RANTES, and TNF-α mRNA by 3.0-, 9.4-, 2.5-, and 2.5-fold, respectively, when compared with untreated cells. Outer membrane proteins extract from avirulent *Leptospira patoc* did not induce significant effects. The pathogenic outer membrane proteins extract contain a major component of a 32-kD lipoprotein (LipL32), which is absent in the nonpathogenic leptospira outer membrane. An antibody raised against LipL32 prevented the stimulatory effect of *Leptospira shermani* outer membrane proteins extract on MCP-1 and iNOS mRNA expression in cultured proximal tubule cells, whereas recombinant LipL32 significantly stimulated the expression of MCP-1 and iNOS mRNAs and augmented nuclear binding of nuclear factor–κB (NF-κB) and AP-1 transcription factors in proximal tubule cells. An antibody raised against LipL32 also blunted the effects induced by the recombinant LipL32. This study demonstrates that LipL32 is a major component of pathogenic leptospira outer membrane proteins involved in the pathogenesis of tubulointerstitial nephritis.

Leptospirosis is a widespread and reemerging zoonosis and has become an important global human and veterinary health problem (1). The infection is transmitted via either direct or indirect contact with leptospira, the highly invasive pathogenic spirochetes, via infected animals or contaminated environment (1). Clinical manifestations range from mild to severe forms of infection, the latter culminating in multiorgan failure and mortality (Weil's syndrome). Among the many organs affected, the most commonly involved is the kidney. Pathogenic leptospira in the infected host disseminate hematogenously to the kidney. The leptospira further colonize and multiply in proximal tubule cells, shedding into the urine and transmitting to new hosts. Direct invasion of the organism into the kidney through the effect of leptospira endotoxin and immunologic response may induce tubulointerstitial nephritis (1,2).

Leptospiral antigens are found in several sites of the kidney, including the proximal tubule cells and macrophages, and in the form of large extracellular clumps in the interstitium (3). They are associated with marked interstitial edema, infiltrates of lymphocytes, monocytes, plasma cells, and neutrophils leading to tubulointerstitial nephritis (4). A leptospiral glycolipoprotein, a component of leptospiral endotoxin that parallels tubulointerstitial nephritic changes, has been found to be expressed in infected renal tubules and vascular lumen of the interstitium (5). The association of leptospira with membrane-bound protein droplets in proximal tubules may thus be important in the induction of tubulointerstitial nephritis and of chronic carrier state in rat leptospirosis, where diffuse interstitial nephritis and fibrosis may be initiated (6).

Leptospiral outer membrane proteins are likely to be relevant to host-pathogen interactions. Leptospiral endotoxins containing leptospiral outer membrane proteins and lipopolysaccharide are located on the outer membrane and appear to be the major antigens that confer immunity to leptospira and may cause renal dysfunction (7). We have previously demonstrated

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Correspondence to Dr. Chih-Wei Yang, Chang gung Memorial Hospital, Department of Nephrology, 199, Tun-Hwa North Road, Taipei 105, Taiwan. Phone: 88633285386; Fax: 88633282173; E-mail: cwyang@ms1.hinet.net

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that pathogenic leptospiral outer membrane proteins activate nuclear factor–κB (NF-κB) and downstream gene expression in mouse medullary thick ascending limb cells (8). The leptospiral outer membrane protein components may therefore elicit a disturbed tubular function and inflammation. The identification of one component of the outer membrane proteins should represent an important step in understanding the pathogenesis of tubulointerstitial nephritis caused by invasive spirochetal pathogens (9).

To date, only a few leptospiral outer membrane proteins have been characterized in detail, including a porin, OmpL1 (10), and several lipoproteins, such as LipL36 (11), LipL41 (10), and LipL32 (12). Zuerner *et al.* (13) have shown that extraction of outer membrane with the nonionic detergents Triton X-100 or Triton X-114 permitted to solubilize the 32-kD LipL32 lipoprotein representing the most prominent antigen identified by infection-derived swine sera in the Triton X-114 detergent phase (14).

Immunohistochemical analyses of *Leptospira kirschneri*–infected hamster kidneys have demonstrated intense LipL32 reactivity in proximal tubule cells and the interstitium (9). LipL32 is also a prominent immunogen during human leptospirosis in which anti-LipL32 reactivity displayed the greatest sensitivity and specificity in acute and convalescent-phase sera of human leptospirosis (15). The sequence and expression of LipL32 is highly conserved among pathogenic leptospira, whereas it is absent in the outer membrane of nonpathogenic avirulent leptospira (12). However, the role of proximal tubule in responding to leptospiral infection and in initiating tubulointerstitial nephritis by LipL32 is not yet understood. In this study, outer membrane proteins extract from a common pathogenic leptospira in Taiwan, *Leptospira shermani* were incubated with cultured mouse proximal tubule cells (16), having kept the main features of the parental cells from which they were derived (17) to study the activation of the transcription factor and downstream gene expression in relation to tubulointerstitial nephritis. The main results from this study strongly suggest that LipL32 is the main pathogenic *Leptospira shermani* outer membrane protein responsible for proximal tubule cell proinflammatory response, which may lead *in vivo* to tubulointerstitial nephritis.

Materials and Methods

Preparing Outer Membrane Proteins Extract of Leptospira by Triton X-114

A commonly encountered pathogenic leptospira serovar *Leptospira shermani* and a nonpathogenic *Leptospira biflexa* serovar *patoc* were obtained from ATCC (Gaithersburg, MD) and cultured in 10% EMJH leptospiral enrichment medium (Difco, Detroit, MI). Leptospira cells were cultured for 5 to 7 d at 28°C until they attained a cell density of 10^8/ml and were then enumerated by dark-field microscopy as described (18). The outer membrane proteins from *Leptospira shermani* were extracted with 1% Triton X-114 by a modification of the method described previously (13,14). In brief, cultured leptospira cells were washed in phosphate buffered saline (PBS) and 5 mM $MgCl_2$ and then extracted in the presence of 1% Triton X-114 (Sigma, St. Louis, MO), 10 mM Tris (pH 8), and 1 mM ethylenediaminetetraacetic acid (EDTA) at 4°C. The insoluble material was removed by centrifuge at 17,000 × *g* for 10 min. The Triton X-114 concentration in the supernatant was increased to 2%. Phase separation was performed by warming the supernatant to 37°C and subjecting it to centrifugation for 10 min at 2000 × *g*. The detergent and aqueous phases were separated and precipitated with acetone. Protein concentration was measured by the Bradford method (Protein Assay; Bio-Rad Laboratories, Hercules, CA). More than 90% of outer membrane proteins could be extracted in the Triton X-114 detergent phase. For comparison, a 0.04% sodium dodecyl sulfate (SDS) extract method for *Leptospira shermani* outer membrane proteins was also used as described previously (8).

Cultured Proximal Tubule Cells

The PKSV-PR cells used in this study have been derived from microdissected proximal tubules from the kidney of a L-PK/Tag1 transgenic mouse as described earlier (16). Cells were cultured in 1:1 DMEM-HAM F12 (vol/vol), 30 nM sodium selenate, 5 μg/ml transferrin, 2 mM glutamine, 5 μg/ml insulin, 50 nM dexamethasone, 5 nM triiodothyronine, 10 ng/ml epidermal growth factor, 20 mM D-glucose, 2% heat-inactivated fetal calf serum (FCS), and 20 mM D-glucose at 37°C in a 5% CO_2–95% atmosphere (16). Cells were seeded in either 35-mm or 60-mm Petri dishes precoated with collagen. The medium of all samples was changed every 2 d. Experiments were performed on exponentially growing or confluent cells between the 40th and 55th passages. Cells were shifted to a serum-free medium 24 h before the experiment. Outer membrane proteins extract or recombinant LipL32 from leptospira was added to the cell culture medium for 48 h. In blocking experiments, 50 μl of antiserum to *Leptospira shermani* outer membrane proteins extract or to LipL32 was preincubated with outer membrane proteins extract or recombinant LipL32 at 37°C for 30 min then added to culture cells containing 2 ml of medium. Total RNA was extracted for RT-PCR, and supernatant was collected for nitrite and protein measurement. Nuclear protein was extracted for electrophoresis mobility shift assay. All measurements were done at least in triplicate.

Cytotoxicity

Cell viability was assessed by a tetrazolium-based 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (19) to determine the nonspecific cytotoxicity of the outer membrane proteins extract of *Leptospira shermani* to proximal tubule cells. Cells were plated and grown in 96-well plates (Corning Co., Corning, NY) for 3 d. Cells were then exposed to various concentrations (0.1 to 1 μg/ml) of outer membrane proteins extract for 48 h, and 40 μl of 5 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide was added to each well. After 2 h at 37°C, the cells were lysed by adding 100 μl of 20% (wt/vol) SDS and 50% (vol/vol) N, N-dimethylformamide (pH 4.7) and incubated overnight at 37°C. The absorbance at 570 nm was measured for each well with a Dynex microplate reader. Experiments were performed in duplicates and cell viability was expressed as the percentage of viable cells compared with that of untreated cells.

Enzyme-Linked Immunosorbent Assay (ELISA)

The peptide levels of the supernatant from cultured mouse proximal tubule cells were measured after exposure to 0.1 to 1 μg/ml outer membrane proteins of *Leptospira shermani* for 48 h and in the case of blocking experiments. Mouse monocyte chemoattractant protein–1 (MCP-1), RANTES, and tumor necrosis factor–α (TNF-α) peptide levels were measured by the ELISA (Quantikine; R&D systems, Minneapolis, MN).

Nitrite Production Assay

Accumulation of nitrite (NO_2^-), employed as an index of nitric oxide production in the culture supernatant, was determined by a Griess reagent as described previously (8).

Competitive RT-PCR Assay

RNA was extracted from confluent proximal tubule cells with the RNA-zol (Cinna/Biotecx Laboratories International Inc., Friendwood, TX) as described reference 8. RNA (1 μg) was reverse transcribed with avian myeloblastosis virus reverse transcriptase (RT AMV, Boehringer Mannheim, Mannheim, Germany) at 42°C for 60 min. Complementary DNA was amplified for 30 to 42 cycles in 100-μl total volume containing 50 mM KCl, 20 mM Tris-HCl (pH 8.4), 10 mM dNTP, 1.5 to 3.0 mM $MgCl_2$, 1 unit *Taq* polymerase, and 10 picomoles of specific PCR primers. The thermal cycling protocol was as follows: 94°C for 1 min, 60°C for 1 min, and 72°C for 3 min. Amplification products were separated on a 4% agarose gel with ethidium bromide and then photographed. The sets of primers used for RT-PCR were as described previously (20,21). The nitric oxide synthase (iNOS) primer pair was 5' GTG TTC CAC CAG GAG ATG TTG 3' (sense) and 5' TCT GGT CGA TGT CAT GAG CAA AGG 3' (antisense), yielding a 508-bp PCR product. The MCP-1 primer pair was 5' AGG TCC CTG TCA TGC TCC TGG 3' (sense) and 5' GTC ACT CCT ACA GAA GTG CTT G 3' (antisense), yielding a 424-bp PCR product. The RANTES primer pair was 5' CCC TGC TGC TTT GCC TAC CTC TCC 3' (sense) and 5' CGG TCG GAG CGG ATG GAG ATG CCG 3' (antisense), yielding a 133-bp PCR product. The TNF-α primer pair was 5' ATG AGC ACA GAA AGC ATG ATC CGC 3' (sense) and 5' CCA AAG TAG ACC TGC CCG GAC TC 3' (antisense), yielding a 692-bp PCR product. The β-actin primer pair was 5' TCT AGG CAC CAA GGT GTG 3' (sense) and 5' TCA TGA GGT AGT CCG TCA GG 3' (antisense), yielding a 460-bp PCR product. The PCR products were analyzed initially by amplification at the exponential phase. Competitive PCR assays were performed for more accurate quantitation for those mRNA levels that displayed changes in the ratio to β-actin mRNA by optical density obtained by scanning densitometer. Competitive PCR was performed to measure MCP-1, iNOS, TNF-α, and β-actin as described previously (8). The test template for all PCR reactions was an aliquot of cDNA collected from cell culture. To quantitate test cDNAs, various amounts of mutant cDNA templates were added to compete with test cDNA on an equimolar basis, as described in references 21 and 22. For MCP-1, iNOS, TNF-α, and β-actin, deletion cDNA mutant templates were developed to create 87-, 39-, 124-, and 103-bp deletions in the middle of the molecules, resulting in mutant cDNAs of 339, 469, 568, and 357 bp, respectively. After agarose gel electrophoresis, amplification bands stained by ethidium bromide were quantitated from the film negative by scanning densitometry. As previously reported (23,24), the ratio of mutant to wild-type band density was calculated for each lane and plotted as a function of the amount of initial mutant template added to the reaction. The amount of cDNA was derived from linear regression analysis with duplicate or triplicate assays. The mean values for assays were expressed as a percentage change to the control. Conventional PCR was performed to measure RANTES mRNA because of the short sequence, which precluded performing competitive PCR mutant DNA. Semiquantitation was done by serial dilution of the input cDNA to measure RANTES mRNA.

Gel Electrophoresis and Immunoblotting by SDS-PAGE

Leptospiral samples for SDS-PAGE were solubilized in a final sample buffer composed of 62.5 mM Tris hydrochloride (pH 6.8), 10% glycerol, 5% 2-mercaptoethanol, and 2% SDS. Proteins were separated on a 12% SDS and stained with Coomassie brilliant blue or were transferred to nitrocellulose (Schleicher & Schuell, Keene, NH) for immunoblotting. For antigenic detection on immunoblots, the nitrocellulose was blocked with 5% nonfat dry milk in PBS–0.1% Tween 20 (PBS-T) and incubated for 2 h with antiserum in PBS-T. The immunoblot was initially probed with rabbit antiserum specific for leptospiral outer membrane protein LipL32 (1:5000) followed by anti-rabbit IgG conjugated to alkaline phosphatase to allow colorimetric detection.

Cloning of LipL32 and LipL41

Standard recombinant DNA procedures were performed to clone LipL32 as described earlier by Haake *et al.* (12). *Leptospira shermani* genomic DNA was used as a template, and PCR was used to amplify the portion of the LipL32 gene encoding the mature protein, beginning with the first residue after the amino-terminal cysteine. The 5' oligonucleotide contained the nucleotide sequence encoding for the six amino acids after the amino-terminal cysteine of mature LipL32, including a *Xho*I restriction endonuclease site: 5'-TTA CCG CTC GAG GTG CTT TCG GTG GTC TGC-3'. The 3' oligonucleotide contained the nucleotide sequence encoding for the five carboxy-terminal amino acids and the LipL32 stop codon, including a *Sma*I restriction endonuclease site: 5'-TGT TAA CCC GGG TTA CTT AGT CGC GTC AGA-3'. The 782-bp amplified LipL32 gene was digested with *Xho*I and *Sma*I and ligated into pRSETc (Invitrogen, Groningen, The Netherlands) digested with *Xho*I and *Pvu*II. The resulting construct, pRSETc-LipL32, was transformed into *Escherichia coli* BL21(DE3)pLysS (Novagen, Madison, WI) for expression. The expression of the His6-LipL32 fusion protein was achieved by isopropyl-D-thiogalactopyranoside (IPTG, Sigma) induction. After solubilization in PBS and purification by affinity chromatography with Ni_2^+-nitrilotriacetic acid-agarose (Qiagen, Chatsworth, CA), the His6-LipL32 fusion protein was stored in PBS. LipL41, another minor outer membrane protein initially described by Haake *et al.* (25) and encoding for a 41-kD protein from pathogenic leptospira, was also cloned into the expression vector using the same methods as described above to generate recombinant LipL41. In all cases, the protein concentration was measured by the Bradford method (Protein Assay, Bio-Rad Laboratories).

Antisera

Antiserum against 1% Triton X-114 outer membrane proteins extract of *Leptospira shermani* was prepared in New Zealand white rabbits by immunization as described previously (26). Outer membrane proteins (400 mg) were mixed with Freund's complete adjuvant and injected intradermally into various sites along the back of the rabbit. A booster of the same dose was given 2 wk later. The animals were bled 2 wk after the last inoculation. The titer of the antiserum was determined by a microscopic agglutination test, which revealed a titer of 1:10,000.

Antiserum to LipL32 was prepared as described previously (12). Briefly, New Zealand White rabbits were immunized with purified His6-LipL32 fusion proteins expressed by *Escherichia coli* BL21(DE3)pLysS transformed with the pRSET plasmid containing LipL32 gene (12). Next, 150 μg of protein was mixed with Freund's complete adjuvant and inoculated intramuscularly into a New Zealand White male rabbit. Additional immunizations with approximately 150 μg of His6-LipL32 fusion protein in Freund's incomplete adjuvant were given at 4 and 8 wk after primary immunization. The rabbit was bled 10 wk after the primary immunization.

Nuclear Protein Extraction

Nuclear proteins were prepared according to Satriano and Schlondroff (27) with slight modifications (8). After the medium was re-

moved, mouse proximal tubule cells in 75-cm^2 flasks were harvested with trypsin and pelleted after centrifugation. Cell pellets from the cells were washed with ice-cold PBS and resuspended with buffer A containing 10 mM Hepes (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 3.5 mM dithiothreitol (DTT), H_2O, and protease inhibitor (Complete, Boehringer Mannhein). Cells were incubated on ice for 10 min and centrifuged for 5 min at 650 × *g*. The pellets were resuspended with the same buffer A containing 0.5% Nonidet P-40, lysed by vortexing, and allowed to swell on ice for 20 min. The nuclear fractions were pelleted for 5 min at 6000 × *g* and resuspended with buffer B containing 5 mM Hepes (pH 7.9), 26% glyceral, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 0.4 M NaCl, H_2O, and Complete. After incubation on ice for 30 min, the nuclear fractions were centrifuged for 10 min at 12,000 × *g*. The supernatants containing nuclear protein were divided into aliquots and stored at −80°C for subsequent use. Protein concentrations were determined by a Bradford method using the Bio-Rad protein assay.

Electrophoretic Mobility Shift Assay (EMSA)

Whole cell nuclear extracts are subjected to assays for NF-κB binding activity employing NF-κB consensus oligonucleotide (5'-AGT TGA GGG GAC TTT AGG C-3'; Promega, Madison, WI) using Digoxigenin EMSA kit (Roche, Sommerville, NJ). A total of 10 μg of nuclear protein was incubated with Digoxigenin labeled NF-κB oligonucleotide in a binding mixture (50 mM Hepes [pH 7.9], 20% glycerol, 5 mM $MgCl_2$, 2.5 mM EDTA, 2.5 mM DTT, 250 mM NaCl, 0.5 μg of poly dI-dC [Pharmacia Biotech, Uppsala, Sweden], and H_2O) to a final volume of 15 μl. After 20-min incubation at room temperature, the protein-DNA complexes were resolved on native 4% polyacrylamide gel in 0.5× Tris-borate-EDTA buffer system and run at 200 V for 1.5 h in a 4°C cold room. Gels were transferred to Whatmann paper, dried, and exposed to XR5 film (Eastman Kodak, Rochester, NY) in film holders for 4 to 16 h at −80°C. Specific competition control of unlabeled oligonucleotide at 100-fold excess was added to the binding reaction mixture for 10 min before the labeled NF-κB probe was added.

Statistical Analyses

All measurements were performed at least in triplicate experiments, and results were expressed as mean ± SEM. Differences between groups were analyzed by unpaired *t* test or ANOVA when suitable. $P < 0.05$ was considered significant.

Results

Cytotoxicity

Cell viability, employed as an index of cell injury (19), was analyzed for *Leptospira shermani*-treated (0.1 to 1 μg/ml Triton X-114 *Leptospira shermani* outer membrane proteins extract for 48 h at 37°C) and untreated mouse proximal tubule cells. The percentage of viable cells did not differ significantly between treated and untreated cells (ranging between 104 ± 7% and 111 ± 6%; $n = 4$ *versus* control). Confluent mouse proximal tubule cells remained viable after 48 h incubation with *Leptospira shermani* outer membrane extracts. Untreated mouse proximal tubule cells were also incubated in either serum-free or serum-enriched culture medium for 24 h to evaluate cell viability. Under these conditions, the serum did not influence the cell viability and growth of the mouse proximal tubule cells (data not shown).

Effects of Pathogenic Leptospira shermani Outer Membrane Proteins Extract on MCP-1, RANTES, iNOS, and TNF-α Expression

The addition of *Leptospira shermani* outer membrane proteins (0.2 μg/ml for 48 h) to cultured mouse PKSV-PR cells increased the expression of MCP-1 (3.0-fold; $P < 0.01$), RANTES (2.5-fold; $P < 0.05$), iNOS (9.4-fold; $P < 0.001$), and TNF-α (2.5-fold; $P < 0.05$) mRNAs compared with that of untreated cells (Figure 1). Consistent with these results, a 48-h incubation with of *Leptospira shermani* outer membrane proteins (0.1 to 1 μg/ml) caused a dose-dependent increase in MCP-1, RANTES, nitrite, and TNF-α recovered in the supernatant from cultured mouse PKSV-PR proximal tubule cells (Figure 2).

Identification of LipL32 as a Major Component of the Pathogenic Leptospira shermani Outer Membrane

The outer membrane proteins extract from a nonpathogenic serovar *Leptospira patoc* was added to the mouse proximal tubule cells for a comparison study with pathogenic *Leptospira shermani*. Outer membrane proteins extract (0.2 μg/ml) was applied to mouse PKSV-PR cells for 48 h. Thereafter, MCP-1, iNOS, and β-actin mRNA expressions were analyzed. Significant changes in the levels of MCP-1 and iNOS mRNAs were found in cells incubated with the outer membrane proteins from pathogenic *Leptospira shermani* but not in those from *Leptospira patoc.* Competitive RT-PCR revealed that *Leptospira shermani* outer membrane proteins induced a 3.0-fold increase in MCP-1 mRNA expression ($P < 0.01$) and a 9.4-fold increase in iNOS mRNA expression ($P < 0.001$). In contrast, no significant changes in any of the analyzed mRNA

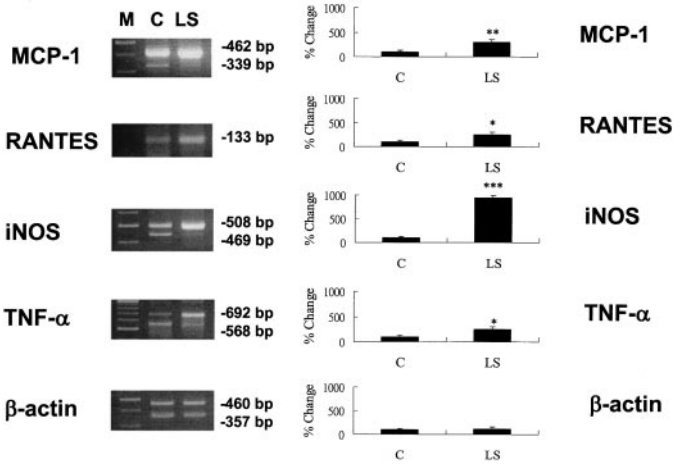


Figure 1. Expression of monocyte chemoattractant protein–1 (MCP-1), RANTES, nitric oxide synthase (iNOS), tumor necrosis factor–α (TNF-α) mRNAs analyzed by reverse transcriptase–PCR (RT-PCR) in proximal tubule stimulated by leptospiral outer membrane proteins extract (0.2 μg/ml) for 48 h. As control, β-actin was used as an internal standard. The expression of MCP-1, RANTES, iNOS, and TNF-α mRNAs significantly increased as compared with untreated cells. The mean values for assays were expressed as percent changes of untreated values (control). M, marker; C, control; LS, *Leptospira shermani*; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

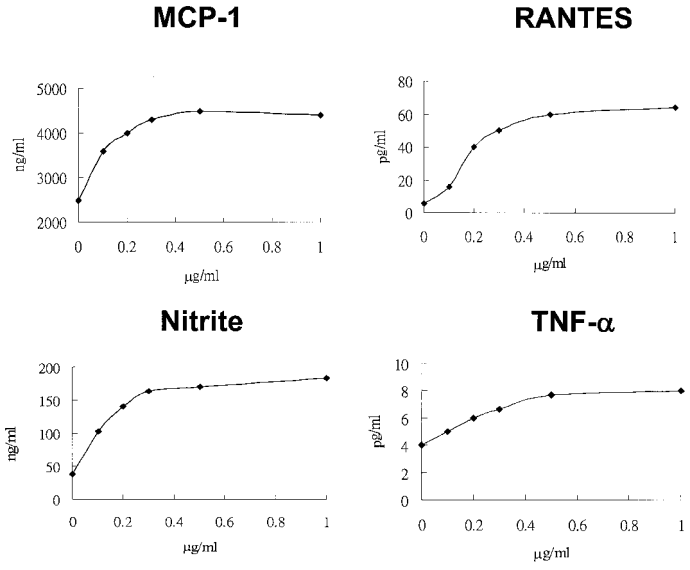


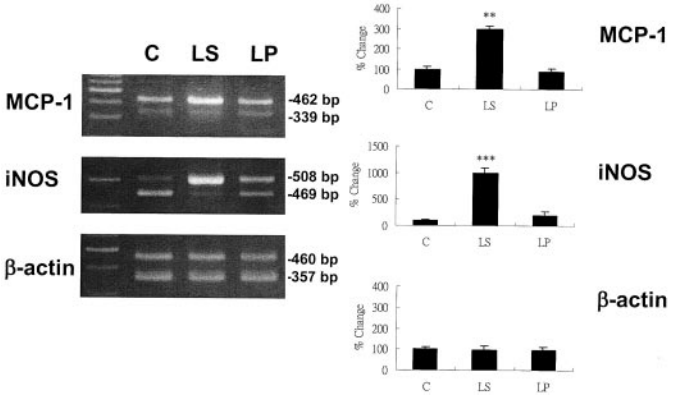
Figure 2. Culture supernatants from proximal tubule cells stimulated with varying amounts of leptospiral outer membrane proteins extracted (0.1 to 1 μg/ml) by Triton X-114 for 48 h displayed a dose-dependent increase as measured by the enzyme-linked immunosorbent assay (ELISA) and Griess method. (A) MCP-1; (B) RANTES; (C) nitrite; (D) TNF-α.

were detected when proximal tubule cells were incubated with *Leptospira patoc* outer membrane proteins extract (Figure 3).

Outer membrane proteins extracted by Triton X-114 were subjected to SDS-PAGE. Protein and antigenic profiles of the pathogenic *Leptospira shermani* were compared with those of the nonpathogenic *Leptospira patoc*. Coomassie blue-stained gels revealed several protein bands, one of 32 kD and the other of 41 kD, and several minor protein bands mainly of higher molecular weights in the pathogenic *Leptospira shermani* but not in nonpathogenic *Leptospira patoc* outer membrane proteins extract (Figure 4A). Extraction of *Leptospira shermani* outer membrane proteins by Triton X-114 and SDS methods were then compared by SDS-PAGE gel analysis. The results also revealed a 32-kD protein band and a 41-kD protein band corresponding to LipL32 and LipL41, respectively (Figure 4B). To demonstrate that this major protein band effectively corresponded with LipL32, immunoblot analysis was performed on outer membrane proteins extract from *Leptospria shermani* by using an antiserum from a rabbit immunized with purified recombinant LipL32. The resulting LipL32 antiserum revealed a single 32-kD band, which indicates that LipL32 is the major pathogenic component of the leptospira outer membrane (Figure 4C).

Effects of an Antibody Raised against LipL32 and Recombinant LipL32

Cultured proximal tubule cells were incubated with *Leptospira shermani* outer membrane proteins and antisera raised against LipL32 (anti-LipL32) and against the detergent phase of Triton X-114 extract (anti-LS) to evaluate LipL32's role in the inducement of tubulointerstitial nephritis. Preincubation of *Leptospira shermani* outer membrane proteins with the anti-LipL32 antibody prevented the increase of MCP-1 and iNOS mRNAs. The anti-LipL32 antibody decreased the expression of MCP-1 and iNOS mRNA by 92% ($P < 0.01$) and 48% ($P < 0.01$), respectively. On the other hand, the anti-LS antibody decreased the expression of MCP-1 and iNOS mRNA by 79% ($P < 0.01$) and 95% ($P < 0.001$), respectively. The anti-LipL32 antibody also significantly decreased by 63% ($P < 0.05$) and 82% ($P < 0.05$) the amount of MCP-1 peptide and nitrite levels, respectively, recovered in the culture supernatants from cells incubated with *Leptospira shermani* outer



Figuure 3. Effects of pathogenic and nonpathogenic leptospira outer membrane proteins in the induction of MCP-1 and iNOS mRNA expression in proximal tubule cells. As controls, β-actin was used as internal standard. In contrast to *Leptospira shermani* and *Leptospira patoc* outer membrane proteins extract did not induce any significant changes in MCP-1 and iNOS expressions. The mean values for assays were expressed as percent changes of untreated values (control). M, marker; C, control; LS, *Leptospira shermani*; LP, *Leptospira patoc*; $^{**}P < 0.01$; $^{***}P < 0.001$ *versus* control untreated.

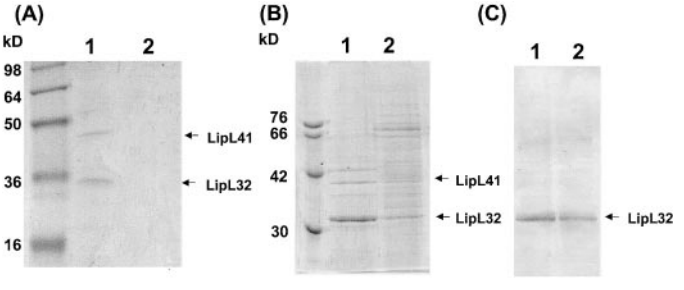


Figure 4. (A) SDS-PAGE of Triton X-114–treated outer membrane proteins extract from pathogenic *Leptospira shermani* (lane 1) revealed 32-kD and a 41-kD protein bands that were not detected in nonpathogenic *Leptospira patoc* extracts (lane 2). (B) SDS-PAGE of Triton X-114–treated (lane 1) and SDS-treated (lane 2) outer membrane proteins extract from pathogenic *Leptospira shermani* both revealed the 32-kD and 41-kD protein bands. (C) Immunoblot of *Leptospira shermani* outer membrane proteins extracted by Triton X-114 (lane 1) and SDS (lane 2) using the anti-LipL32 antibody revealed that the 32-kD band corresponded to LipL32. Note that extraction by Triton X-114 revealed a higher amount of LipL32 protein band than the SDS extraction.

membrane proteins extract. Similarly, the anti-LS antibody also decreased by 52% ($P < 0.05$) and 93% ($P < 0.01$) the amount of MCP-1 peptide and nitrite, respectively, recovered in the culture supernatants**,** whereas no significant inhibitory effect was observed when preimmune rabbit serum (PIS) replaced the antibodies (Figure 5). These results provided lines of strong evidence that LipL32 largely transduces the induction of the tubulointerstitial nephritis–mediated gene expression by leptospira outer membrane proteins. Cultured mouse proximal tubule cells were incubated with the recombinant LipL32 for 48 h to further elucidate the role of LipL32. mRNAs for MCP-1, iNOS, and β-actin were then measured by RT-competitive PCR, and the results were expressed as fold-change of controls. Increasing concentrations of the recombinant protein (0.6, 1.2, 2.4, 6, and 12 ng/ml) increased in a dose-dependent manner the expression of MCP-1 (4.0-, 9.0-, 11.3-, 11.4-, and 11.8-fold, respectively) as compared with those untreated cells ($P < 0.001$). iNOS expression also displayed 7.4-, 11.5-, 15.0-, 15.6-, and 14.9-fold increases for 0.6, 1.2, 2.4, 6, and 12 ng/ml, respectively, as compared with untreated cells ($P < 0.001$) (Figure 6A). In contrast, the recombinant LipL41 did not induce any changes in the levels of MCP-1 and iNOS expressions when compared with equivalent doses of recombinant LipL32 (Figure 6B).

Cultured proximal tubule cells were then incubated with the recombinant LipL32 (2.4 ng/ml) and the antiLipL32 antiserum. Recombinant LipL32 significantly enhanced the MCP-1 (11.3-fold; $P < 0.001$) and iNOS (15-fold; $P < 0.001$). In contrast, a preincubation with the anti-LipL32 antiserum prevented the increase in MCP-1 and iNOS by 81% ($P < 0.01$) and 78% ($P < 0.01$), respectively. The recombinant LipL32 protein significantly increased the amount of MCP-1 (2.4-fold, $P < 0.05$) and nitrite (2.2-fold, $P < 0.05$) recovered in the culture supernatants. In contrast, preincubation of the recombinant LipL32 with the anti-LipL32 antiserum reduced the increase in MCP-1 and nitrite by 78% ($P < 0.05$) and 95% ($P < 0.01$), respec-

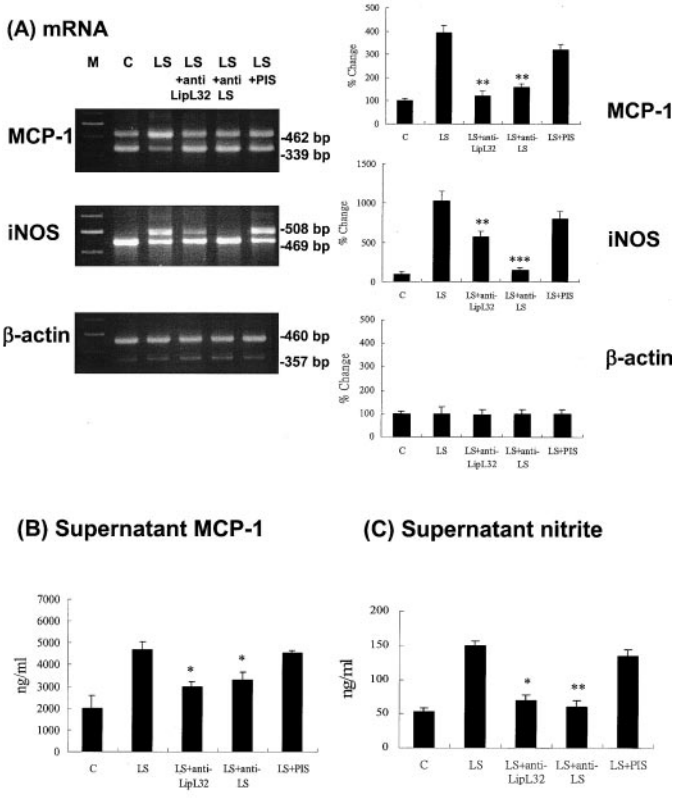


Figure 5. RT competitive-PCR for MCP-1, iNOS, and β-actin mRNAs in proximal tubule cells stimulated by leptospiral outer membrane proteins: inhibition by anti-LipL32 (anti-LipL32) and anti-Triton X-114 detergent phase extract (anti-LS) antisera. Incubation of the anti-LipL32 antiserum with *Leptospira shermani* outer membrane proteins extract induced a marked decrease in proximal tubule MCP-1 and iNOS mRNAs expression. Adding anti-LS antiserum also inhibited MCP-1 and iNOS mRNAs expression (A). MCP-1 peptide (B) and nitrite (C) levels measured in culture supernatants showed a similar pattern of stimulation by outer membrane proteins extract (LS) that was almost completely prevented by anti-LipL32 and anti-LS antisera. No inhibition effect was observed when preimmune rabbit serum (PIS) was used to replace antisera. Results are the mean ± SEM from triplicate experiments. C, control untreated; LS, *Leptospira shermani*; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ *versus* LS.

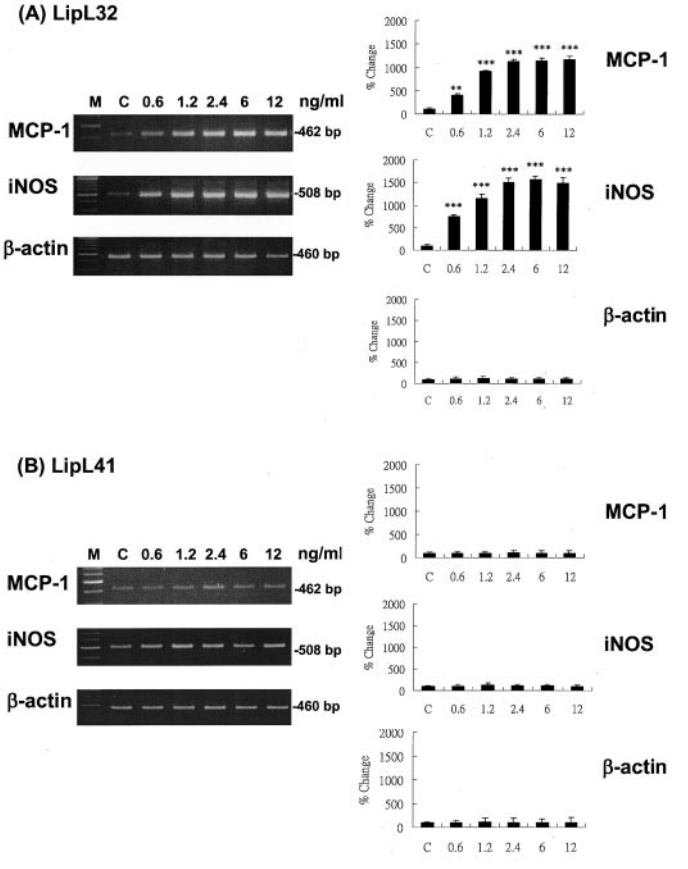


Figure 6. RT competitive-PCR results of MCP-1, iNOS, and β-actin mRNAs in proximal tubule cells stimulated with various concentrations of recombinant LipL32 (A) or recombinant LipL41 (B). Results are the mean ± SEM from three separate experiments. M, marker; C, control untreated; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ *versus* control untreated cells.

tively. As controls, no inhibitory effects were detected when cells were preincubated with the PIS (Figure 7). Altogether, these findings indicate that LipL32 significantly induces an increase in tubulointerstitial nephritis–mediated gene expression in proximal tubule cells.

Effects of LipL32 on NF-κB, AP-1 Nuclear DNA Binding

Significant proximal tubule nuclear DNA binding of transcriptional factor NF-κB was observed when cells were incubated for 15 to 120 min with the recombinant LipL32. A similar nuclear DNA binding of AP-1 transcriptional factor was also observed under the same conditions of incubation (Figure 8). These results and the fact that a 100-fold excess of cold NF-κB probe inhibited the NF-κB nuclear DNA binding confirm the specificity of the LipL32 recombinant protein.

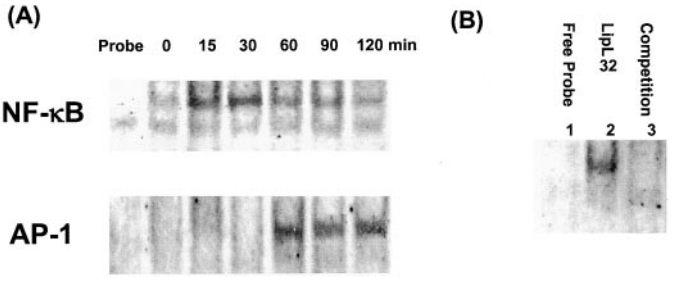


Figure 8. (A) Induction of nuclear factor–κB (NF-κB) and AP-1 nuclear factors by recombinant LipL32. Illustration of the time-dependent induction of NF-κB and AP-1 in cultured mouse proximal tubule cells incubated with recombinant LipL32 (8 ng/ml). (B) NF-κB stimulated by LipL32 was not detected with a free-probe (lane 1) or when proximal tubule cells were incubated with LipL32 plus a 100-fold excess of cold NF-κB probe (lane 3).

Discussion

The results from this study indicate that LipL32, a major protein constituent of the *Leptospira shermani* outer membrane, is certainly involved in the pathogenicity of leptospirosis leading to tubulointerstitial nephritis. Pathogenic *Leptospira* outer membrane may determine virulence and be the main target for immunity. Many of the most abundant proteins in the spirochetal outer membrane are lipoproteins, which may lead to a possible mechanism for pathogenesis. Previous studies have suggested that the leptospiral outer membrane has a relatively complex protein profile (13,14,28). The search for the pathogenic protein components of the leptospiral outer membrane represents an important approach in the understanding of the pathogenesis of leptospiral renal disease.

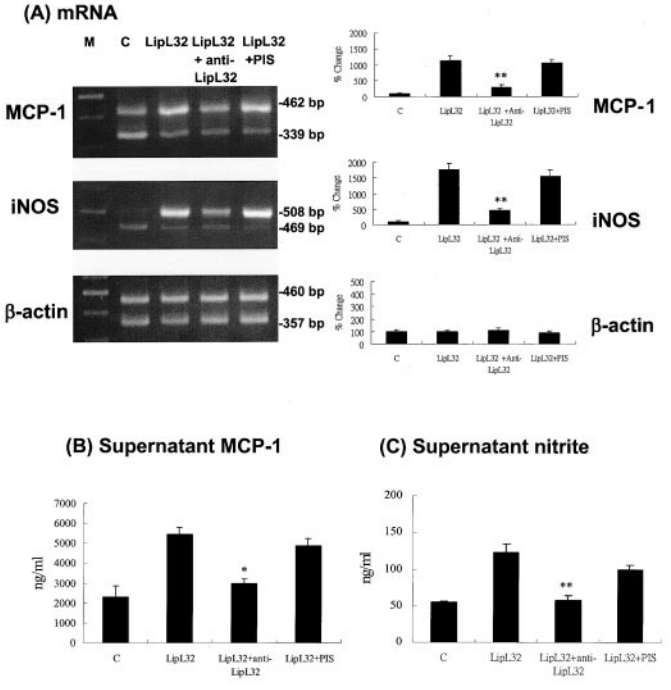


Figure 7. RT competitive-PCR results of MCP-1, iNOS, and β-actin mRNAs in proximal tubule incubated with LipL32 alone or with LipL32 plus the anti-LipL32 antibody. Preincubating outer membrane proteins with the anti-LipL32 antibody significantly reduced ($P < 0.01$) the increase of MCP-1 and iNOS mRNAs (A). MCP-1 peptide (B) and nitrite (C) levels measured in culture supernatants showed similar pattern of stimulation by LipL32 that was almost completely prevented by anti-LipL32 antisera. No inhibition effect was observed when preimmune rabbit serum (PIS) was used to replace antibody. Results are the mean ± SEM from three experiments. M, marker; C, control untreated; LS, *Leptospira shermani*; anti-LipL32, antiserum to recombinant LipL32; PIS, preimmune rabbit serum; $*P < 0.05$; $**P < 0.01$.

The results from the present study provide lines of evidence on the determinant role of LipL32 in the onset of inflammatory tubule cell response. The sequence and expression of LipL32 are highly conserved among all pathogenic leptospiral species; therefore, outer membrane proteins extracted from pathogenic *Leptospira shermani* containing LipL32 or the recombinant LipL32 have been tested on cultured mouse proximal tubule cells. The outer membrane proteins extract containing LipL32 induced the production of chemokine MCP-1, RANTES, iNOS, and TNF-α at both the mRNA and the protein levels in mouse proximal tubule cells. LipL32 markedly stimulated the production of MCP-1 and iNOS, and both antisera to whole outer membrane proteins extract or recombinant LipL32 largely prevented the inflammatory response of proximal tubule cells induced by outer membrane proteins extract. These two facts strongly suggest that LipL32 is the major outer membrane component that may initiate *in vivo* leptospira-related tubulointerstitial nephritis. The role of LipL32 in the pathogenesis of leptospirosis may deserve further detailed investigation because LipL32 has been also shown to induce TNF-α induction in the human monocytic cells (29). In contrast, the outer membrane protein LipL41 in the pathogenic leptospira does not induce changes in the levels of gene expression related to tubulointerstitial nephritis. This result indicates that LipL41 may not be involved in the tubular injury and inflammation induced by pathogenic leptospira.

Chemokines are a family of chemotactic cytokines that induce the migration of various cell types, particularly those of lymphoid origin. Chemokines are upregulated during various

forms of glomerular and interstitial injuries (30,31). Among those entities, CC chemokines are molecules that most likely recruit mononuclear leukocytes to the kidney. Because most forms of tubulointerstitial nephritis, including leptospirosis, cause the mononuclear leukocytes to infiltrate the tubule and interstitium (6), leptospira outer membrane extract were added to cultured mouse proximal tubule cells to reproduce the *in vivo* condition. The increased expression of CC chemokines MCP-1 and RANTES associated with the increased production of iNOS and TNF-α indicate the propensity of leptospiral outer membrane proteins to stimulate inflammatory response of proximal tubule cells and perhaps to recruit infiltrating cells in the event of tubulointerstitial nephritis. These results also suggest that multiple CC chemokines may play a role in the recruitment of leukocytes in leptospirosis renal disease. Adding outer membrane proteins from pathogenic *Leptospira shermani* upregulated chemokines in cultured proximal tubule cells, whereas adding nonpathogenic *Leptospira patoc* outer membrane proteins did not induce such an upregulation. A pathogenic leptospiral outer membrane protein may therefore conceivably initiate tubulointerstitial nephritis through the components contained in the outer membrane. This finding is the first evidence that demonstrates a role of pathogenic leptospira outer membrane proteins in the pathogenesis of tubulointerstitial nephritis related to proximal tubule cells.

Proximal tubule is one of the major sources of nitric oxide production under both basal and stimulated conditions (32). A series of *in vivo* and *in vitro* studies (32) have revealed nitric oxide's significant role in proximal tubule physiology and pathophysiology. It is generally agreed that nitric oxide inhibits proximal tubule fluid and sodium reabsorption. In this study, outer membrane proteins from *Leptospira shermani* markedly stimulated nitric oxide production by proximal tubule cells. The production of nitric oxide may play an important role in regulating renal oxygenation and preventing hypoxic injury. On the other hand, nitric oxide displays potent proinflammatory effects in stimulated states. Nitric oxide may further contribute to tissue injury by interacting with superoxide and the generating peroxynitrite (33). Nitric oxide also interacts with other regulatory mechanisms. Enhanced nitric oxide production may downregulate Na^+-K^+-ATPase, Na^+/H^+ exchangers, and paracellular permeability of proximal tubule cells, which may contribute to its effect on proximal tubular transport (32). Here, leptospira outer membrane proteins markedly stimulate iNOS production. As shown by Younes-Ibrahim *et al.* (34), the addition of leptospira glycolipoproteins extracted from *Leptospira interrogans* inhibits the Na^+-K^+-ATPase activity in renal epithelial cells. It cannot therefore be excluded that the increased iNOS expression may alter the regulation of ion transport in proximal tubule cells.

Pathogenic leptospira outer membrane proteins also stimulate TNF-α expression in proximal tubule cells, which is associated with an increase in nuclear DNA binding capacity of NK-κB. TNF-α is a proinflammatory cytokine that is produced by monocytes/macrophages (35) and by resident renal cells (36). The TNF-α gene contains a NF-κB binding sequence in its promoter (37). TNF-α itself stimulates NF-κB activation (38) and may increase TNF-α synthesis through further NF-κB activation in an autocrine amplification fashion. It has been shown that the outer membrane peptidoglycan of *Leptospira copenhageni* induces the release of TNF-α from peripheral blood mononuclear cells (39). Similar induction occurs with TNF-α when leptospira outer membrane proteins extract is added to medullary thick ascending limb cells in culture (8).

The eukaryotic cells contain a multitude of pathways coupling environmental stimuli to the specific regulation of gene expression. Two early-response transcriptional complexes, NF-κB and AP-1, appear to respond to environmental stressors by inducing the expression of specific downstream genes. The importance of NF-κB activation has been demonstrated in various nephritis models. This activation plays a central role of inflammation, both in glomerulonephritis and in tubulointerstitial nephritis (38). We have previously demonstrated that leptospira outer membrane proteins induce in cultured thick ascending limb cells a significant nuclear DNA binding of NF-κB by *Leptospira shermani* but not by the avirulent nonpathogenic *Leptospira patoc.* The results from this study show similar activation of LipL32 on nuclear DNA binding of NF-κB in proximal tubule cells.

In conclusion, the results from this study provide strong evidence that among *Leptospira shermani* outer membrane proteins, LipL32 represents the major pathogenic protein that can induce proximal tubular inflammation through the NF-κB–associated gene expression. These findings may serve as an important step in understanding how a microorganism may initiate tubulointerstitial nephritis related to its outer membrane components via an inflammatory injury pathway.

Acknowledgments

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