The role of Toll-like receptor proteins (TLR) 2 and 4 in mediating inflammation in proximal tubules

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Corticosteroids are the mainstay of treatment for nephrotic syndrome in patients with minimal change disease, focal segmental glomerulosclerosis, and lupus nephritis (7). However, immunosuppressive therapy is also an important part of the management of patients with end-stage renal disease (64). Therefore, investigations into the mechanisms underlying renal inflammation may provide new insight into novel therapeutic targets for patients with diabetic nephropathy.

Toll-like receptors (TLRs) are germline-encoded receptors which are central to the innate immune system. To date, 11 members of the TLR family have been identified in humans (18). They function as important pathogen recognition receptors activated by conserved structural motifs known as pathogen-associated molecular patterns, which are expressed by bacteria, viruses, and fungi (44). Upon activation by exogenous (pathogen derived) or endogenous (tissue derived) ligands, all TLRs are able to activate the NF-κB pathway, which leads to the synthesis of proinflammatory cytokines and chemokines (14).

Among the 11 human TLRs, experimental studies have provided compelling evidence that TLR2 and 4 are actively involved in the development of kidney diseases such as renal ischemia-reperfusion injury (22, 34, 40), transplant rejection (8, 11, 30), infection (38), nephrotoxicity (45), and glomerulonephritis (3). Furthermore, elevated expressions of TLR2 and 4 and their functional activation have been demonstrated in monocyes of patients with type 2 diabetes (7), suggesting a molecular link between inflammation and diabetes. However, tubulointerstitial inflammation is a hallmark of diabetic nephropathy, and to date the precise role of TLR2 and 4 in regulating inflammation in proximal tubular cells remains unknown.

Endogenous ligands to TLR2 and 4 such as high-mobility group box-1 (HMGB1), fibropectin, and heat shock proteins are upregulated in the presence of high glucose and ischemia-hypoxia, which are central to the pathophysiology of diabetic nephropathy (20, 32, 35). HMGB1 was initially identified as a highly conserved nuclear protein that binds to DNA and regulates transcription (25). It was later discovered to be a cytokine which is passively released during cell damage by necrotic, apoptotic, and damaged cells or actively secreted by monocytes and macrophages in response to injury (2, 6, 25, 36). Independent of the release mechanism, extracellular HMGB1 is able to induce a signaling cascade that activates NF-κB, leading to the synthesis of proinflammatory cytokines (21, 43).

diabetic nephropathy; Toll-like receptor 2; Toll-like receptor 4; NF-κB; HMGB1
The role HMGB1 has been well characterized in many inflammatory diseases, with an increasing number of studies documenting its contribution to the development of diabetes. Elevated levels of circulating HMGB1 have been demonstrated in the serum of patients with type 1 and 2 diabetes, and its increased expression in pancreatic cells has been shown to play a role in the onset and disease progression in patients with type 1 diabetes (7, 9, 16). It is considered that elimination of circulating HMGB1 is largely via renal excretion, which may in part explain the elevated circulating levels of HMGB1 in patients with diabetic nephropathy (4). HMGB1 is also implicated in renal ischemia-reperfusion injury, with its release from damaged renal epithelial and endothelial cells proposed to be a trigger for TLR4-induced activation of leukocytes and macrophages (5). However, little is known about the role of HMGB1 in transducing tubulointerstitial inflammation, which is critical in the pathogenesis of diabetic nephropathy.

We have also previously demonstrated that high-glucose conditions upregulate fibronectin expression in proximal tubules and stimulate profibrotic and proinflammatory pathways (32). However, expression of cellular fibronectin in proximal tubules and its regulation of TLR2 and 4 with exposure to mild/moderate glucose levels are yet to be determined.

Therefore, in the present study, we hypothesized that activation of TLR2 and 4 in the presence of moderately high glucose by the release of endogenous ligand HMGB1 in proximal tubules may provide a mechanistic link between hyperglycemia and inflammation within the kidney.

MATERIALS AND METHODS

Ethics statement. Experiments in this study were approved by the Animal Care and Ethics Committee of Royal North Shore Hospital and were performed according to the recommendations of the Australian Council for Animal Care.

In vivo experiments. Briefly, inbred male enos knockout mice on a C57BL/6 background were purchased from Jackson Laboratory and were maintained under standard animal house conditions. Diabetes was induced in the mice by intraperitoneal injections of streptozotocin (STZ) at a dose of 55 mg/kg in sodium citrate buffer (pH 4.5) for 5 consecutive days at 7–8 wk of age. Diabetes was defined by fasting blood glucose >250 mg/dl 2 wk after the first STZ injection. Control animals received citrate buffer injections (pH 4.5). Twenty-four hours after STZ injections, all mice were euthanized, and their kidneys were obtained for further analyses.

Cell culture. Immortalized human renal proximal tubules, HK2 cells (American Type Culture Collection), were grown in keratinocyte serum-free media (KSFM) supplemented with bovine pituitary extract (20–30 µg/ml) and epidermal growth factor (0.1–0.2 ng/ml; Gibco). Cell culture media was changed every 48 h. These cells were grown at 37°C in a humidified 5% CO2 incubator and were subcultured at 50–80% confluence using 0.05% trypsin, 0.02% EDTA (Gibco). Culture conditions upregulate fibronectin expression in proximal tubules and stimulate profibrotic and proinflammatory pathways (32). However, expression of cellular fibronectin in proximal tubules and its regulation of TLR2 and 4 with exposure to mild/moderate glucose levels are yet to be determined.

Experimental protocol. When 50% confluent, HK2 cells were exposed to three different glucose conditions for 72 h. Experiments were performed at the conclusion of the 72-h experimental protocol. The three different glucose levels are 1) 5 mM glucose (control), 2) 30 mM glucose (high), and 3) 11.2 mM glucose (moderately high).

Small interfering RNA silencing. Small interfering RNA (siRNA) was purchased to specifically target TLR2 mRNA (Thermo Scientific, Pittsburgh, PA). HK2 cells were seeded onto six-well plates, and after 24 h of culture were transfected with 0.1 µM TLR2 siRNA (AGUAGGAAUGCAAUAAUCUA) using Ribojuice (Novagen, Darmstadt, Germany) as per the manufacturer’s instructions. All siRNA experiments included nonspecific control transfection of cells (nonspecific control siRNAs, GenePharma). TLR2 knockdown was confirmed by Western blotting. Subsequent experiments were conducted to determine the effect of TLR2 silencing on NF-κB activation.

TLR 4 signaling inhibition. Resatorvid, ethyl (6R)-6-[N-(2-chloro-4-fluorophenyl)sulfamoyl]cyclohex-1-ene-1-carboxylate (TAK-242) was synthesized at Takeda Pharmaceutical (Osaka, Japan). The IC50 of ligand-induced NF-κB activation of TAK-242 is 110 nM. In this study, TAK-242 was dissolved in dimethyl sulfoxide. HK2 cells were seeded onto six-well plates. After culture for 24 h, cells were incubated with TAK-242 for 2 h at 5 µM and vehicle control. To upregulate the expression of NF-κB, cells were exposed to 50 ng/ml recombinant HMGB1 (ProteinOne) for 2 h. Thereafter, the nuclear extract was harvested and NF-κB activity was assessed.

Western blot analysis. Cells collected were 95% confluent, and the cell pellet was resuspended in cell lysis buffer containing 50 mM Tris-HCl, 150 mM NaCl, 5 mM EDTA (pH 7.4), 0.5% Triton X-100, and protease inhibitors (Roche Diagnostics, Mannheim, Germany). The cell lysate was spun at 13 000 rpm at 4°C for 5 min and stored at −20°C. Protein quantification (Bio-Rad, Hercules, CA) was carried out to determine the protein concentration of the cell lysate. Fifty micrograms of total cell protein was mixed with 6 µL Laemmlı sample buffer containing β-mercaptoethanol and heated at 95°C for 10 min. Samples were then analyzed by SDS-PAGE and electroblotted to Hybond nitrocellulose membranes (Amersham Pharmacia Biotech, Bucks, UK). Membranes were blocked in Tris-buffered saline containing 0.2% Tween 20 (TBST) in 5% skim milk for 2 h and then incubated overnight at 4°C with the following antibodies: TLR2, 1:500 (Imgenex, San Diego, CA); TLR4, 1:125 (Invitrogen); peroxisome proliferator-activated receptor-γ (PPAR-γ; 1:300 (Santa Cruz Biotechnology, Santa Cruz, CA); fibronectin, 1:1,000 (Sigma-Aldrich, St. Louis, MO); HMGB1, 1:500 (Abcam), and NF-κB p65, 1:500 (Santa Cruz Biotechnology). Membranes were washed with TBST and incubated with horseradish peroxidase-conjugated secondary antibody. Proteins were visualized using the enhanced chemiluminescence (ECL) detection system (Amersham Pharmacia Biotech). All membranes were probed with β-actin 1:300 (Santa Cruz Biotechnology), and results were corrected for actin as a loading control and analyzed using ImageJ software (Java-based software program, National Institutes of Health).

Nuclear extraction and EMSA for NF-κB. Nuclear extracts were prepared using a NucBuster Protein Extraction Kit (Novagen) as per the manufacturer’s instructions. The DIG Gel Shift Kit (Roche Applied Science, Indianapolis, IN) was used in the EMSA. Twenty-five micrograms of nuclear extract were incubated with 1 µg poly d [I-C] as the nonspecific competitor, 1 µg poly l-lysine in a binding buffer [100 mM HEPES, pH 7.6, 5 mM EDTA, 50 mM (NH4)SO4, 5 mM DTT, Tween 20, 1% wt/vol, 150 mM KCl] and digoxigenin (dig)-labeled NF-κB (5′-AGT TGA GGG GAC TTT CCC AGG C-3′) consensus oligonucleotide (Promega) for 30 min at room temperature. The reaction mixture was electrophoresed through 6% polyacrylamide gels, transferred onto nylon positively charged membrane (Roche Applied Science), and then cross-linked using a UV-transilluminator for 3 min. The membrane was subjected to immunological detection using an anti-dig-AP conjugate and chemiluminescence. Results were analyzed using Image J software, and shift bands were quantified.

ELISA. Cells were seeded onto six-well plates and exposed to the experimental conditions as defined above in triplicate. After treatment, the supernatant was removed and centrifuged at 13,000 rpm for 5 min. Protein concentrations of MCP-1 and IL-8 were determined using commercially available ELISA kits (Invitrogen) as per the manufacturer’s instructions. The optical density (OD) at 450 nm was then read using a microplate reader. Cell lysate protein concentration from corresponding wells was determined by protein assay (Bio-Rad). MCP-1 and IL-8 levels were corrected for protein content per well.

Real-time PCR. RNA was extracted using an RNeasy minikit (Qiagen, Valencia, CA) according to the manufacturer’s instructions. RT-PCR was performed with the SuperScriptIII One-step RT-PCR system.
System and Platinum Taq DNA polymerase (Invitrogen). cDNA was generated by reverse transcribing 1 μg of total RNA in a reaction volume of 20 μl using Vilo cDNA synthesis kits (Invitrogen). One microliter (50 ng) of cDNA was used as a template in a 20-μl PCR reaction. Quantitative real-time PCR was performed using the ABI Prism 7900 HT Sequence Detection System (Applied Biosystems, Foster City, CA) with Taqman Gene Expression Master Mix (Applied Biosystems) and gene-specific expression assays containing two unlabeled PCR primers. Reactions were performed in at least triplicate and were analyzed by relative quantitation using RQ Manager software, version 1.2 (Applied Biosystems). The following primers were used for mRNA detection: PPAR-γ: forward 5′-CAAAGCAGAAGCAGGAGG, reverse 5′-ACGGAGCGAAGACTGG, MCP-1: forward 5′-CCAAAGAAGCTGTGATCTTCAA, reverse 5′-TGGAAATCCTTGAAACCACCTTC, and IL-8: forward 5′-AGCCTTCATGATTCTGACGGCT, reverse 5′-AATTTCCTGTGTGCGCAATGTTGA. All data are presented as fold-change compared with control after normalization to β-actin.

**Immunohistochemistry.** Formalin-fixed paraffin-embedded sections of 5-μm thickness were deparaffinized and boiled for 10 min in 10 mM sodium citrate buffer (pH 6.0). Immunohistochemistry was performed using the following antibodies: goat anti-mouse TLR4 (Santa Cruz Biotechnology), rabbit anti-mouse TLR2 (Imgenex), and rabbit anti-mouse HMGB1 (Abcam). Concentration-matched rabbit or goat IgG was used as an isotype-negative control. The sections were blocked with Dako for 10 min and incubated with primary antibodies overnight. Sections were exposed to 8% H₂O₂ for 5 min to quench endogenous peroxidases, and for detecting TLR4, an avidin/biotin blocking kit (Dako) was used. The slides were then incubated with biotinylated secondary antibody anti-goat or horseradish peroxidase anti-rabbit Envision+ system followed by a 3,3′-diaminobenzidine (DAB) substrate-chromogen solution (Dako) and counterstained with Harris hematoxylin. The tissue specimens were examined by brightfield microscopy using a Leica photomicroscope linked to a DFC 480 digital camera.

The quantitation of TLR2, TLR4, and HMGB1 staining was performed by capturing 10 random nonoverlapping fields from stained sections. Areas of brown staining reflecting TLR2, TLR4, and HMGB1 were highlighted using a selective color tool for their color ranges, and the proportional area of their tissue with their respective ranges of color was then quantified. Calculation of the proportional area stained brown was then determined using the automated cellular imaging system Image J.

**Statistical analyses.** Normalized results are expressed as a percentage of the means ± SE of control values or as stated. Experiments were performed at least in triplicate or as detailed in the text. Statistical comparisons between groups were made by ANOVA or unpaired t-tests where appropriate. Analyses were performed using the software package Statview version 5.0 (Abacus Concepts, Berkeley, CA). P values <0.05 were considered significant.

**RESULTS**

**Maximal increase in the expression of TLR2 and 4 with exposure to 11.2 mM glucose.** To evaluate the effect of high and moderate glucose concentrations on TLR2 and 4 expression, HK2 cells were exposed to 5 mM glucose, 30 mM glucose, and 11.2 mM glucose for 72 h. Maximal increase in NF-κB activation with exposure to 11.2 mM glucose with no increase observed with 30 mM glucose (Fig. 3A). Conversely, TLR4 expression was reduced with 30 and 11.2 mM glucose concentrations by 7 days, being 65.9 ± 13.2% (P < 0.05) and 72.2 ± 11.4% (P < 0.05) of control values, respectively (Fig. 2B).

By 7 days, TLR2 expression was sustained with ongoing exposure to 30 and 11.2 mM glucose. Maximal expression of TLR2 expression was induced by 11.2 mM glucose, being 159.7 ± 13.0% of control (P < 0.01; Fig. 2A). Although exposure to 30 mM glucose for 72 h showed no significant increase in TLR2 expression, after 7 days’ exposure to 30 mM glucose cells exhibited a significant increase in TLR2 expression to 125.0 ± 6.8% (P < 0.05) of control values.

Maximal increase in NF-κB activation with exposure to 11.2 mM glucose. We next identified the effects of high and moderate glucose concentrations on nuclear NF-κB p65 translocation and NF-κB-DNA binding affinity in HK2 cells. Western blot analysis showed that the levels of NF-κB p65 protein exposed to 11.2 mM glucose increased to 133.5 ± 9.6% (P < 0.05) of control values, respectively (Fig. 1B).

**Fig. 1.** Protein expression of Toll-like receptors TLR2 and 4 with exposure to moderate and high glucose concentrations for 72 h. Western blot analysis shows maximal increase in TLR2 (A) and TLR4 expression (B) in cells exposed to 11.2 mM glucose. Representative image shows nonsequential lanes from the same gel. Normalized results are expressed as means ± SE; n = 5. *P < 0.05 vs. HK2 cells cultured with 5 mM glucose.
Using EMSA, we showed that exposure to 11.2 mM glucose resulted in increased NF-κB-DNA binding to 175.0 ± 13.0% (P < 0.01) of control values at 72 h with no increase observed with 30 mM glucose (Fig. 3B).

At 7 days, consistent with a sustained increase in TLR2 expression, nuclear NF-κB p65 subunit expression was increased to 268.8 ± 21.2 (P < 0.01) and 288.4 ± 36.7% (P < 0.01) with exposure to 30 and 11.2 mM glucose, respectively (Fig. 3C). NF-κB-DNA binding activity was also increased to 249.6 ± 22.6% (P < 0.01) with 30 mM glucose and 273.2 ± 30.1% (P < 0.01) with 11.2 mM glucose (Fig. 3D) as assessed with EMSA, respectively.

Fibronectin expression is induced with exposure to 11.2 and 30 mM glucose. Fibronectin, a known ligand for TLRs, is an extracellular protein which is implicated in increased extracellular matrix, characteristic of renal pathology. Hence, fibronectin expression was determined in the presence of moderate and high glucose concentrations. An increase in protein expression with exposure to 30 and 11.2 mM glucose for 7 days. Longer term exposure to 30 and 11.2 mM glucose showed a greater increase in TLR2 expression with 11.2 mM glucose than with 30 mM glucose (A) and showed a significant reduction in TLR4 expression with both 30 and 11.2 mM glucose (B). Normalized results are expressed as means ± SE; n = 5. *P < 0.05 vs. HK2 cells cultured with 5 mM glucose.

**Increase in HMGB1 secretion with exposure to 11.2 and 30 mM glucose.** To determine whether exposure to 30 and 11.2 mM glucose increased HMGB1, the proposed relevant endogenous ligand for both TLR2 and 4 in diabetic nephropathy, we determined its presence in the supernatant of cells cultured with 30 and 11.2 M glucose concentrations for 72 h. These studies confirmed an upregulation in high glucose-induced HMGB1 secretion with 30 and 11.2 mM glucose to 283.2 ± 17.4 (P < 0.01) and 218.3 ± 25.7% (P < 0.05) of control values, respectively (Fig. 5).

**NF-κB is activated with recombinant HMGB1 stimulation.** To confirm the role of HMGB1 in mediating NF-κB activation, HK2 cells were stimulated with 50 ng/ml recombinant HMGB1 for 2 h. We showed an increase in the nuclear expression of NF-κB p65 subunit to 124.9 ± 6.8% of control (P < 0.01) (Fig. 6A) with a concurrent increase in NF-κB-DNA binding in the presence of recombinant HMGB1 to 268.3 ± 17.3% (P < 0.01) of control values (Fig. 6B).

**Silencing of TLR2 attenuates downstream NF-κB binding.** TLR2 expression was determined with exposure to 11.2 mM glucose following transfection of TLR2 siRNA. As previously shown, 11.2 mM glucose was used to upregulate maximal TLR2 expression. Silencing of TLR2 with specific siRNA suppressed its expression to 33.09 ± 7.4% (P < 0.01) of control values. TLR2 siRNA was specific as it had no effect on TLR4 expression (Fig. 7A). The activation of NF-κB by HMGB1-induced TLR2 signaling was confirmed by silencing TLR2 in HK2 cells. In the presence of TLR2 siRNA, nuclear expression of NF-κB p65 was abrogated to 67.1 ± 7.4% (P < 0.01) of control (Fig. 7B). Similarly, HMGB1-induced NF-κB-DNA binding was also reduced to 33.4 ± 18.4% (P < 0.01) compared with nonspecific siRNA controls (Fig. 7C).

**Inhibition of TLR4 attenuates downstream NF-κB binding.** TAK-242 was used as a selective inhibitor of TLR4 intracellular signaling. TAK-242 binds to the Toll/IL-1 receptor (TIR) domain of TLR4 and prevents signal transduction by disrupting interactions between TLR4 and its adaptor molecules. Functional assays have demonstrated that TAK-242 exhibits a specific binding to TLR4 among the 11 TLRs (19, 26). We have demonstrated that TAK-242 inhibited the increase in HMGB1-induced NF-κB binding in a concentration-dependent manner (data not shown). In this study, the role of TLR4 in HMGB1-induced NF-κB signaling was determined by inhibiting TLR4 signaling with TAK-242 (5 μM). Inhibition of TLR4 signaling with TAK-242 attenuated the nuclear expression of NF-κB p65 to 89.8 ± 3.5% (P < 0.05) (Fig. 8A) and significantly reduced NF-κB-DNA binding to 23.1 ± 7.9% (P < 0.01) of control (Fig. 8B), confirming that both TLR2 and 4 participate in signaling.

**Upregulation of MCP-1 and IL-8 by glucose in proximal tubules is mediated through TLR2 and 4.** Monocyte chemoattractant protein-1 (MCP-1) and Interleukin-8 (IL-8) are known to be upregulated in proximal tubules in diabetic nephropathy. We examined the effects of moderate and high glucose on MCP-1 and IL-8 expression (Fig. 9A). Normalized results are expressed as means ± SE; n = 5. *P < 0.05 vs. HK2 cells cultured with 5 mM glucose.
IL-8 secretion by exposing HK2 cells to 30 and 11.2 mM glucose for 72 h and determining their transcription and secretion into the milieu with real-time PCR and ELISA, respectively.

A 1.81 ± 0.25-fold (P < 0.05) increase in MCP-1 transcription was observed with 11.2 mM glucose. Increased IL-8 transcription was also observed with 30 and 11.2 mM glucose, being a 2.03 ± 0.22 (P < 0.05)- and 2.49 ± 0.10-fold (P < 0.01) increase, respectively (Fig. 9A).

In addition, we assessed for the activation of MCP-1 and IL-8 with exposure to moderate and high glucose concentrations by determining with ELISA the protein secretion into the milieu after 72 h. We observed an increase of 162.8 ± 20.9% (P < 0.05) in MCP-1 secretion when cells were exposed to 11.2 mM glucose. However, an insignificant increase in MCP-1 was recorded with 30 mM glucose. Increased IL-8 secretion was also observed with 30 and 11.2 mM glucose, being 135.6 ± 7.7 (P < 0.01) and 122.0 ± 9.9% (P < 0.05) of control values, respectively (Fig. 9B). To summarize, both the proinflammatory chemokines were significantly upregulated in HK2 cells with exposure to 11.2 mM glucose, with IL-8 also upregulated by 30 mM glucose.

We next explored the role of TLR2 and 4 in mediating the activation of chemokines in the presence of TLR2 silencing or inhibition of TLR4 signaling. With respect to protein secretion, TLR2 siRNA attenuated MCP-1 secretion to 59.7 ± 10.9% (P < 0.05) of control values and a reduction of 64.4 ± 2.8%...
(P < 0.01) in IL-8 secretion compared with control values (Fig. 10A), whereas in the presence of TAK-242 we also observed a reduction of MCP-1 and IL-8 protein secretion to 78.6 ± 5.0 (P < 0.01) and 73.09 ± 9.0% (P < 0.05) of control values (Fig. 10B).

**(PPAR-γ expression.** We assessed the gene expression of the anti-inflammatory nuclear receptor PPAR-γ at a 24-h time point to determine mechanistically the reasons, at 72 h, 30 mM glucose failed to elevate TLR2 and 4 expression with downstream activation of NF-κB and proinflammatory chemokines. Exposure to 30 mM glucose induced PPAR-γ transcription by 1.44 ± 0.199-fold of control (P < 0.05). Interestingly, there was a significant reduction in PPAR-γ gene expression by 0.55 ± 0.074-fold with exposure to 11.2 mM glucose (P < 0.01 vs. 30 mM glucose) (Fig. 11), which is also consistent with a proinflammatory effect.)
Increased expression of TLR2 and HMGB1 in the kidneys of diabetic mice. As shown in Fig. 12, TLR2, TLR4, and HMGB1 are constitutively expressed in control mice kidneys. In the diabetic model, TLR2 expression was significantly upregulated, particularly in the damaged tubules compared with control. However, there was no significant difference in the cortical tubular expression of TLR4 in both control and diabetic kidneys. Since HMGB1 is an endogenous ligand to both TLR2 and 4, the level of HMGB1 was also determined. An upregulation of renal cortical HMGB1 with increased nuclear and cytoplasmic staining of the tubules in the diabetic kidney was observed.

DISCUSSION

There is increasing clinical and experimental evidence demonstrating that inflammatory processes play a significant role in the pathogenesis of diabetic nephropathy. However, the mechanisms remain incompletely understood. This study clearly demonstrates that TLR2 and 4 are upregulated in the presence of moderate increases in glucose with a concurrent increase in their ligand, HMGB1. Hence, our data suggest a role for HMGB1 in regulating tubulointerstitial inflammation in diabetic nephropathy through TLR2 and 4 signaling. We have demonstrated that fibronectin, a key extracellular matrix protein which increases in the development of renal fibrosis (32), is also increased in proximal tubules following exposure to moderate increases in glucose. Given that fibronectin is a ligand for TLRs (12, 37), it is likely that fibronectin may be transducing signaling through the TLR pathway, thereby mechanistically linking the inflammatory and fibrotic processes in diabetic nephropathy.

TLR2 and 4 are constitutively expressed at low levels within the kidney. However, recent studies have implicated their involvement in the pathogenesis of renal diseases (3, 11, 22). TLR2 and 4 have also been shown to increase in immune cells such as macrophages and monocytes with exposure to high
glucose, and their proinflammatory role has been well documented in diabetes (7). However, their expression and regulation with exposure to high glucose in the pathogenesis of diabetic nephropathy is yet to be elucidated. In this study, we exposed cells to 11.2 mM glucose, which approximates the blood concentration required for the clinical diagnosis of diabetes mellitus (33), and have demonstrated greater TLR2 and 4 expression than in the presence of 30 mM glucose, which is more commonly used to simulate elevated glucose in in vitro studies pertaining to diabetes and its complications. In a recent study, Lin et al. (24) have demonstrated that short exposure to high-glucose conditions (15–30 mM) for up to 24 h had induced the transcription of TLR4 but not TLR2 in vitro in human proximal tubular epithelial cells. We provide incremental evidence that exposing HK2 cells to 11.2 mM glucose for a longer period of 72 h significantly increases the protein expression of TLR2 and 4 with concomitant NF-κB activation and the induction of inflammatory cytokines like MCP-1 and IL-8.

Moreover, we prove that with a prolonged exposure of 7 days to 11.2 mM glucose, TLR2 expression is sustained in HK2 cells with the activation of the inflammatory pathway. Although Lin et al. did not study TLR2 expression in their diabetic in vivo model, they showed that TLR4 cortical expression in their diabetic animals was not significantly different from the wild-type controls, which is consistent with our data shown here.

This is consistent with a recent study which showed prominent expression of TLR2 in the kidneys of diabetic rats with an associated increase in inflammatory infiltration (23). To date, enhanced expression of TLRs and downstream proinflammatory markers studied in in vivo models have been usually attributed to the increase in infiltrating immune cells, including the study by Devaraj et al. (10), who discussed the increase in TLR2 expres-

Fig. 8. Effect of TLR4 intracellular signaling inhibitor TAK-242 on NF-κB binding in cells exposed to recombinant HMGB1. In the presence of TAK-242 (5 μM), nuclear translocation of NF-κB p65 was attenuated (A) and increased upregulation of NF-κB-DNA binding with exposure to 50 ng/ml HMGB1 was reduced (B). Normalized results are expressed as means ± SE; n = 7. *P < 0.05 vs. HK2 cells treated with recombinant HMGB1 in the absence of TAK-242 (control). **P < 0.01 vs. HK2 cells treated with recombinant HMGB1 in the absence of TAK-242 (control).

Fig. 9. Increased transcription and secretion of downstream MCP-1 and IL-8 with exposure to moderate and high glucose concentrations for 72 h. Maximal transcription of MCP-1 and IL-8 occurred with exposure to 11.2 mM glucose (A), and maximal increase in MCP-1 secretion into the milieu was observed with exposure to 11.2 mM glucose, whereas an increase in IL-8 secretion was observed with exposure to both 30 and 11.2 mM glucose after 72 h (B). Normalized results are expressed as means ± SE; n = 3. **P < 0.01 vs. HK2 cells treated with 5 mM glucose.
intracellular signaling with an inhibitor attenuated this binding, indicating the regulation of a NF-κB-dependent inflammatory pathway through TLR2 and 4. TAK-242, a selective inhibitor of TLR4, has been shown to be potent in suppressing ligand-dependent and ligand-independent signaling of TLR4 (19). TAK-242 binds to Cys 747 in the intracellular domain of TLR4 and prevents the association of TLR4 with its adapter proteins, thereby inhibiting TLR4 signal transduction and its downstream signaling events (26). Therefore, a blockade of TLR2 and 4 may abrogate the proinflammatory cascade contributing to diabetic nephropathy.

With the upregulation of TLR2 and 4 expression, we observed a concomitant increase in the NF-κB p65 subunit and NF-κB-DNA binding with exposure to 11.2 mM glucose. NF-κB, an important nuclear transcription factor regulated by TLRs, initiates transcription of genes associated with immune responses and inflammation (43, 44). With an upregulation of NF-κB activation, we detected an increase in the production of proinflammatory chemokines MCP-1 and IL-8. In addition, transcription of these chemokines was reduced when TLR2 expression and 4 signaling were abrogated, which further supports the role of TLR2 and 4 in transducing inflammation within HK2 cells in diabetic nephropathy. Reduction in TLR2 receptor expression also resulted in a greater reduction in the secretion of MCP-1 and IL-8 in contrast to the inhibition of TLR4 signaling, suggesting that TLR2 was the more predominant receptor in mediating the transcription of inflammatory chemokines.

We have uniquely demonstrated that the expression of TLR2, 4, and downstream transcriptional markers of inflammation in HK2 cells are more significantly induced by moderate rather than high glucose concentrations. Since exposure to high glucose for 72 h had minimal cellular inflammatory consequences, we assessed for the presence of PPAR-γ, well recognized for its role in limiting high glucose-induced inflammation (31). We have previously demonstrated that short-term (24 h) exposure of high glucose (30 mM glucose) induced tubular production of PPAR-γ and gene expression with exposure to 11.2 mM glucose. Normalized results are expressed as means ± SE; n = 3. *P < 0.05 vs. HK2 cells treated with nonspecific (NS) siRNA. **P < 0.01 vs. HK2 cells treated with nonspecific (NS) siRNA.

Stimulation with HMGB1 induced NF-κB binding in HK2 cells, and the gene silencing of TLR2 or the blockade of TLR4 expression and its signal transduction in peritoneal and kidney macrophages. However, it is critical to determine the role of the kidney in mediating tubular damage and renal atrophy to alleviate inflammation in diabetic nephropathy. In this study, we have demonstrated an increase in TLR2 expression in human proximal tubular cells in vitro and in a diabetic animal model, thereby implicating the direct involvement of the tubulointerstitium in transducing the inflammatory pathway through the activation of NF-κB in the presence of high glucose.

We next assessed the tubular expression of HMGB1, an endogenous ligand to TLR2 and 4. HMGB1 is a nuclear protein that acts as a cytokine when secreted by activated immune cells into the extracellular milieu (1). It is released in response to injury, infection, or other inflammatory stimuli to mediate systemic inflammatory responses. A study by Bruchfeld et al. (4) demonstrated an increase in circulating HMGB1 levels in patients with chronic kidney disease. We have uniquely demonstrated the increase in HMGB1 release by proximal tubules in the presence of moderately high glucose, implicating its role in the progression of TLR-induced inflammation in diabetic nephropathy. However, TLR2 and 4 have a wide range of endogenous ligands which may also be involved in their activation of the inflammatory cascade (46).

Stimulation with HMGB1 induced NF-κB binding in HK2 cells, and the gene silencing of TLR2 or the blockade of TLR4-dependent inflammatory signaling with an inhibitor attenuated this binding, indicating the regulation of a NF-κB-dependent inflammatory pathway through TLR2 and 4. TAK-242, a selective inhibitor of TLR4, has been shown to be potent in suppressing ligand-dependent and ligand-independent signaling of TLR4 (19). TAK-242 binds to Cys 747 in the intracellular domain of TLR4 and prevents the association of TLR4 with its adapter proteins, thereby inhibiting TLR4 signal transduction and its downstream signaling events (26). Therefore, a blockade of TLR2 and 4 may abrogate the proinflammatory cascade contributing to diabetic nephropathy.

With the upregulation of TLR2 and 4 expression, we observed a concomitant increase in the NF-κB p65 subunit and NF-κB-DNA binding with exposure to 11.2 mM glucose. NF-κB, an important nuclear transcription factor regulated by TLRs, initiates transcription of genes associated with immune responses and inflammation (43, 44). With an upregulation of NF-κB activation, we detected an increase in the production of proinflammatory chemokines MCP-1 and IL-8. In addition, transcription of these chemokines was reduced when TLR2 expression and 4 signaling were abrogated, which further supports the role of TLR2 and 4 in transducing inflammation within HK2 cells in diabetic nephropathy. Reduction in TLR2 receptor expression also resulted in a greater reduction in the secretion of MCP-1 and IL-8 in contrast to the inhibition of TLR4 signaling, suggesting that TLR2 was the more predominant receptor in mediating the transcription of inflammatory chemokines.

We have uniquely demonstrated that the expression of TLR2, 4, and downstream transcriptional markers of inflammation in HK2 cells are more significantly induced by moderate rather than high glucose concentrations. Since exposure to high glucose for 72 h had minimal cellular inflammatory consequences, we assessed for the presence of PPAR-γ, well recognized for its role in limiting high glucose-induced inflammation (31). We have previously demonstrated that short-term (24 h) exposure of high glucose (30 mM glucose) induced tubular production of PPAR-γ and gene expression with exposure to 11.2 mM glucose. Normalized results are expressed as means ± SE; n = 3. *P < 0.05 vs. HK2 cells treated with nonspecific (NS) siRNA. **P < 0.01 vs. HK2 cells treated with nonspecific (NS) siRNA.

![Fig. 10. Secretion of MCP-1 and IL-8 when TLR2 expression is silenced or TLR4 signaling is inhibited. Protein secretion of MCP-1 and IL-8 with exposure to 11.2 mM glucose for 72 h was reduced in the presence of TLR2 siRNA (A). Protein secretion of MCP-1 and IL-8 secretion were also reduced in the presence of TAK-242 following upregulation with recombinant HMGB1 (B). Normalized results are expressed as means ± SE; n = 3. *P < 0.05 vs. HK2 cells treated with recombinant HMGB1 in the absence of TAK-242 (control). **P < 0.01 vs. HK2 cells treated with nonspecific (NS) siRNA.](http://ajprenal.physiology.org/)

![Fig. 11. Transcription of peroxisome proliferator-activated receptor-γ (PPAR-γ) with exposure to moderate and high glucose for 24 h. An increase in PPAR-γ transcription was induced with exposure to 30 mM glucose. However, there was a significant reduction in PPAR-γ gene expression with exposure to 11.2 mM glucose. Normalized results are expressed as means ± SE; n = 3. *P < 0.05 vs. HK2 cells exposed to 5 mM glucose. †P < 0.01 vs. HK2 cells treated with nonspecific (NS) siRNA.](http://ajprenal.physiology.org/)
Fig. 12. Expression of TLR2, TLR4, and HMGB1 in control and diabetic mice. Immunohistochemistry analyses were conducted for TLR2 (A and B), TLR4 (C and D), and HMGB1 (E and F) expression in control and streptozotocin (STZ)-induced diabetic C57BL/6J (eNOS-KO) mice. TLR2 cortical tubular expression increased in diabetic mice compared with control (G). No significant change was observed in TLR4 expression (H). However, there was an increase in the intensity of nuclear and cytoplasmic staining of HMGB1 compared with control (I). *P < 0.05 vs. control mice. **P < 0.01 vs. control mice.
a reduction in MCP-1 (31), which we have previously suggested may be a compensatory “cytoprotective” effect. In this study, PPAR-γ was assessed at an earlier time point (24 h), since its activation has been shown to repress downstream NF-κB signaling (32). We observed a greater reduction in PPAR-γ trans- 
scription following exposure to moderate rather than high glucose, which is likely to be responsible for the exaggerated inflammatory response under these conditions.

We have also clearly demonstrated expression of TLR2, TLR4, and HMGB1 in the kidneys of a diabetic mouse model. There is an increase in the expression of TLR2 in the tubules and an upregulation of nuclear and cytoplasmic staining of HMGB1 in STZ-induced diabetic kidneys. This is consistent with the findings of Li et al. (23), who demonstrate an upregulation of renal TLR2 in diabetic rats and human biopsy samples. Taken together, this suggests that TLR2 may be the integral receptor in transducing inflammation in diabetic nephropathy. In vivo, we observed HMGB1 secretion from the nucleus into the cytoplasm in diabetic mice. This was in keeping with our in vitro studies in which we demonstrated the secretion of HMGB1 into the supernatant with exposure to high and moderate levels of glucose. We therefore postulate that, in the presence of hyperglycemia, nuclear HMGB1 is secreted into the cytoplasm where it may possibly interact with cytoplasmic TLR2 to potentiate inflammation in the kidney. However, further investigations are necessary to delineate the HMGB1-mediated TLR signaling pathway in the pathogenesis of diabetic nephropathy. On the contrary, the increase in HMGB1 expression did not induce an increase in TLR4 expression in the tubulointerstitium, indicating that the inflammatory pathway in the diabetic kidney may be occurring independently of TLR4 signal transduction. In keeping with our findings, a study conducted by Devaraj et al. (10) proves that in a STZ-induced diabetes model, TLR2 knockout mice demonstrated reduced inflammation independently of TLR4 since TLR4 levels and its signaling proteins remained unaltered. A recent study has implicated TLR4 in modulating inflammatory processes in the mesangium (17) which warrants further investigations into the role of TLR4 in perpetuating diabetic vascular complications.

Taken together, our data suggest that HMGB1 is involved in the pathogenesis of diabetic nephropathy by mediating the activation of TLR2 and 4. Furthermore TLR2, with its ability to sustain the NF-κB-mediated inflammatory pathway in vitro and with its increased tubular expression in diabetic mice, may serve as the more potent receptor in transducing inflammation in diabetic nephropathy in the chronic setting. Since HMGB1 has the unique ability to self-amplify and is able to prolong the inflammatory response as a late mediator of inflammation (29), therapeutic interventions aimed at targeting the inflammatory component through interruption of HMGB1-induced TLR2 signaling may prove to be a novel strategy, useful in the prevention and treatment of tubulointerstitial injury in diabetic nephropathy.

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