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Nod1 and Nod2 Are Expressed in Human and Murine Renal Tubular Epithelial Cells and Participate in Renal Ischemia Reperfusion Injury

Alana A. Shigeoka,* Amanpreet Kambo,* John C. Mathison,* Andrew J. King,† Wesley F. Hall,‡ Jean da Silva Correia,* Richard J. Ulevitch,* and Dianne B. McKay*

Nucleotide-binding oligomerization domain (Nod) 1 and Nod2 are members of a family of intracellular innate sensors that participate in innate immune responses to pathogens and molecules released during the course of tissue injury, including injury induced by ischemia. Ischemic injury to the kidney is characterized by renal tubular epithelial apoptosis and inflammation. Among the best studied intracellular innate immune receptors known to contribute to apoptosis and inflammation are Nod1 and Nod2. Our study compared and contrasted the effects of renal ischemia in wild-type mice and mice deficient in Nod1, Nod2, Nod(1 × 2), and in their downstream signaling molecule receptor-interacting protein 2. We found that Nod1 and Nod2 were present in renal tubular epithelial cells in both mouse and human kidneys and that the absence of these receptors in mice resulted in protection from kidney ischemia reperfusion injury. Significant protection from kidney injury was seen with a deficiency of Nod2 and receptor-interacting protein 2, and the simultaneous deficiency of Nod1 and Nod2 provided even greater protection. We conclude that the intracellular sensors Nod1 and Nod2 play an important role in the pathogenesis of acute ischemic injury of the kidney, although possibly through different mechanisms. The Journal of Immunology, 2010, 184: 2297–2304.

The innate immune system is responsible for an organism’s initial response to potentially dangerous stressors, such as pathogens or tissue injury, and thus plays an essential role in the pathogenesis of many inflammatory disease processes. The innate response is Ag-independent and uses both membrane-bound (e.g., TLRs) and intracytoplasmic (e.g., nucleotide-binding oligomerization domain-like receptors [NLRs]) pattern recognition receptors (PRRs) to recognize conserved molecular motifs. Intracytoplasmic PRRs, such as the NLRs, are putative cytoplasmic ligands for molecules released by injured cells and as such are important initial participants in cellular injury (1). Ligation of NLRs by molecules released from injured cells has been shown to ignite a cascade of molecular signaling events that ultimately lead to cell death and inflammation (2, 3).

One clinically important disease process that is known to involve activation of PRRs is acute kidney injury, which is modeled experimentally by kidney ischemia reperfusion (IR) injury. IR injury of the kidney is a complex pathophysiological process that involves both cell death and inflammation. Kidney IR injury is known to involve the TLR family of innate immune receptors (4–6), but little is known about the role of NLRs in IR injury. This study is the first to our knowledge to describe the role of the intracellular NLRs in kidney IR injury.

The NLRs are a family of PRRs expressed in both immune cells and epithelial cells. These molecules are multidomain proteins that serve as scaffolds for the assembly of signaling platforms, triggering the activation of molecules involved in apoptosis and inflammation. Among the NLR family, Nod1 and Nod2 have been identified as key detectors of intracellular microbes (they sense peptidoglycan, a heterogeneous polymer found in the cell walls of bacteria) (7–10) and possibly danger signals (molecules released from dead and dying cells) (11). Nod1 is expressed widely in many cell types, and Nod2 has been found in macrophages (12), dendritic cells (13), Paneth cells (14), keratinocytes (15), epithelial cells of the intestine (16), lung (17), and oral cavity (18), and osteoblasts (19).

Nod1 and Nod2 share structural and functional characteristics. Both contain ligand-binding regions (C-terminal leucine-rich repeats), a central Nod, and N-terminal caspase recruitment domains (CARDs) that bind downstream signaling molecules (2). Binding of Nod ligands results in oligomerization of the nucleotide-binding oligomerization domains, which results in CARD binding of downstream proteins, such as receptor-interacting protein (RIP)-like interacting caspase-like apoptosis-regulatory protein kinase (a serine-threonine kinase also known as Rip2) (2). Rip2 is important for Nod-induced NF-κB activation as well as apoptotic signaling through association with members of the TNFR-associated factor family and members of the inhibitor of apoptosis protein (IAP) family, cIAP-1 and cIAP-2 (3). IAP family proteins play prominent roles in regulating programmed cell death by virtue of their ability to bind caspases, intracellular cysteine proteases responsible for apoptosis. Stimulation of Nod1 or Nod2 also results in the secretion of proinflammatory cytokines and chemokines (IL-6, CXCL8/IL-8, CXCL1 [KC], and MIP2), and Nod1 activation induces neutrophil recruitment in vivo (20). There is mounting evidence that Nod1 and Nod2 participate in renal IR injury. This study is the first to our knowledge to describe the role of the intracellular NLRs in kidney IR injury.
evidence that Nod1 and Nod2 signals are likely to contribute to a variety of human diseases, such as Crohn's disease (21), Blau syndrome (22), sarcoidosis (23), and possibly asthma (24).

To begin to define the role of Nod1 and Nod2 in kidney disease, we examined human and mouse kidneys for intracellular Nod1 and Nod2 expression. To ascertain a linkage to disease, we tested the response to kidney IR injury using a well-established murine model. Using this model, we examined the dependence of kidney injury and cytokine/chemokine secretion on Nod1 and Nod2 as well as on their dependent downstream signaling kinase Rip2. We also determined the contributions of Nod1 and Nod2 to tubular epithelial apoptosis and to inflammation associated with kidney IR injury.

Materials and Methods

Mice

All of the mice used in the experiments were housed in the vivarium at The Scripps Research Institute (La Jolla, CA) and approved for use by the Laboratory Animal Care and Use Committee of the Animal Research Center at The Scripps Research Institutes. All mice used were handled according to the standards of the Association for the Assessment and Accreditation of Laboratory Animal Care. Nod-deficient [Nod1−/−, Nod2−/−, and Nod1 (1 × 2)−/−] mice and Rip2−/− mice were obtained from R. Ulevitch (The Scripps Research Institute). All Nod- and Rip2-deficient mice used in these experiments were bred onto a C57BL/6 background by >10 generations.

Detection of Nod1 and Nod2 in mouse and human kidneys

Nod1 and Nod2 mRNA was detected in rat renal tubular epithelium (RTE) by semiquantitative PCR. To detect Nod1 and Nod2 in murine RTE, the same protocol for isolation of human RTE was used as noted below (Detection of Nod1 and Nod2 in human RTE), except that collagenase D was used instead of collagenase A for murine RTE. The first strand of cDNA of each sample was synthesized from 1 μg total RNA using a Quantitect reverse transcription kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. For reverse transcription PCR, 1 μg total cellular RNA was reverse-transcribed, and cDNA was amplified. The expression of Nod1 and Nod 2 mRNA in renal tissue was detected by semiquantitative PCR with 5'-AAAGCATTCTGCATCCCGAG-3' as a forward primer and 5-AAA GACATGGTCCGGTTCAC-3' as a reverse primer for Nod1 mRNA and with 5' -CATCTGGTCACCAACATCTGG-3' as a forward primer and 5'-GA GTAGCAGAAGGCGGTCAC-3' as a reverse primer for Nod2 mRNA. The level of GADPH mRNA in each sample was also determined by PCR using 5'-ACCACAGTCTCATCCATC-3' as a forward primer and 5'-TCCACCATGCATTGGT-3' as a reverse primer. For detection of Nod1 in human RTE, the forward primer was 5'-TCCCAAAGGCAAACGCAAGCT-3', and the reverse primer was 5'-CAGCATTGAGCAAGC-3'. The level of GADPH mRNA in each sample was also determined by PCR using 5'-CACCATCTCATGAGCCAC-3' as a forward primer and 5'-TCCACACCACGTGACTGTA-3' as a reverse primer. For detection of Nod1 in human RTE, the forward primer was 5'-TCCAAAAGGCAACGCAAGCT-3', and the reverse primer was 5'-CAGCATTGAGCAAGC-3'. The GADPH housekeeping gene was detected in the human cells by the forward primer 5'-CACCATCT CATCCAGACG-3' and the reverse primer 5'-CATAGGTCTCTCCACGATACC-3'. The PCR products were separated on a 1% agarose gel, and digital photographs were taken on a transilluminator.

Isolation of human RTE

Human kidney tissue was obtained from discarded nephrectomies for renal cell carcinoma (IRB: 08-5054, Scripps Clinic and Green Hospital). Immediately after the nephrectomy, the renal pathologist removed a wedge section of normal tissue (distal from the site of the tumor). After transport to the laboratory in sterile media, the renal capsule was removed, and the cortex was dissected from the medulla. The cortex was then finely minced and placed in collagenase A (Sigma-Aldrich, St. Louis, MO) and incubated with 5×-g/ml supplements, hydrocortisone (Sigma-Aldrich) (0.05 μM), epidermal growth factor (Sigma-Aldrich) (10 ng/ml), triiodothyronine (32 ng/ml), penicillin/streptomycin (1 ml), and HEPES (15 mM) to promote growth of tubular epithelial cells but not other cell types. Within a few days, a cobblestone morphology characteristic of cultured RTE was evident. Confirmation of RTE was made by positive staining for Lotus tetragonolobus lectin (27) (Vector Laboratories, Burlingame, CA).

Induction of in vivo IR injury

All of the experimental mice were matched for age (8–12 wk); only male mice were used. As published previously (4, 28), the following methods were used to induce nonlethal IR injury. The mice were anesthetized with isoflurane and injected i.p. with ketamine (100 mg/kg)/xylazine (8 mg/kg) in saline. Core body temperatures were maintained between 36 and 37.5°C during surgery by continuous monitoring with a rectal thermometer and an automatic heating blanket. Both kidneys were exposed with bilateral flank incisions, and ischemia was induced by clamping both renal arteries with nontraumatic microvessel clamps (S&K, Neuhausen, Switzerland) for 25 min. Renal veins remained unoccluded. Cessation of blood flow was documented by visual inspection. After 25 min of ischemia, the clamps were released, and reflow was verified by visual inspection of the kidneys. All of the mice received 200 μl saline dripped over the open flanks during surgery to keep the tissue moist and 30 μl saline per gram body weight injected s.c. after surgery to replenish for fluid loss.

Measurement of renal function

Measurement of renal function was conducted at the indicated times following reperfusion. In all of the cases, the mice were anesthetized prior to sacrifice, and blood was collected from the inferior vena cava into a syringe prefilled with 3.8% sodium citrate. Sham controls also were anesthetized prior to sacrifice, and blood was collected in an identical manner. Plasma was isolated by centrifugation at 4000 × g for 10 min at 4°C. Renal function was assessed using the Sigma Diagnostics creatinine kit (Sigma-Aldrich), running all of the samples in triplicate and repeating measurements three times for each sample. Baseline (2 wk before laparotomy) and terminal (at the time of sacrifice) serum creatinine levels were measured in all of the animals.

Histological assessments

Histological injury and inflammation. To assess renal histology, kidneys were harvested 24 h after kidney IR injury or sham surgery, fixed in a solution of zinc formalin, and paraffin-embedded. Sections (4 μm) were stained with H&E or periodic acid Schiff stain. The tissue slides were blind-labeled and reviewed by a renal pathologist who had no knowledge of the experimental groups. A histologic score system, adapted from Kelly et al. (29), was used. The percentage of tubules in the outer medulla that showed epithelial cell necrosis was estimated, and a numerical score was assigned to represent the degree of tubule injury: 0 (no injury); 1 (0–10%); 2 (11–20%); 3 (21–40%); 4 (41–60%); 5 (61–75%); 6 (>75%). The scores represented the pathologist’s impression of the severity of tubular injury (including loss of proximal tubule brush border, cell blebbing or vacuolization, and pyknosis). The histologic score of the renal cortex was also evaluated using a similar scoring system. The scores represented the degree of inflammation: 0 (no inflammation); 1 (microscopic); 2 (light); 3 (moderate); 4 (severe). The scores represented the degree of inflammation: 0 (no inflammation); 1 (microscopic); 2 (light); 3 (moderate); 4 (severe).

Measurement of renal cortical necrosis. The renal cortex was dissected from the medulla. The cortex was then finely minced and placed in collagenase A (Sigma-Aldrich) and incubated with 5×-g/ml supplements, hydrocortisone (Sigma-Aldrich) (0.05 μM), epidermal growth factor (Sigma-Aldrich) (10 ng/ml), triiodothyronine (32 ng/ml), penicillin/streptomycin (1 ml), and HEPES (15 mM) to promote growth of tubular epithelial cells but not other cell types. Within a few days, a cobblestone morphology characteristic of cultured RTE was evident. Confirmation of RTE was made by positive staining for Lotus tetragonolobus lectin (27) (Vector Laboratories, Burlingame, CA).

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measured in tissue homogenates using commercial ELISA kits (R&D Systems, Minneapolis, MN) according to the manufacturer’s instructions. The detection limits were 16 pg/ml for IL-6, KC, IL-1β, and MIP2 and 31 pg/ml for TNF-α and IFN-γ. Plasma concentrations of mouse IL-6 also were measured using ELISA kits (limited to 16 pg/ml). To control for the ability to produce the indicated cytokines and chemokines, wild-type (WT) and Nod(1 × 2)−/− mice were injected i.p. with LPS from Salmonella minnesota R595 (Alexis Biochemicals, Plymouth Meeting, PA) at a concentration of 10 ng/kg body weight. WT and Nod(1 × 2)−/− serum and kidneys were harvested at serum time points. Nonmanipulated controls also were sacrificed to compare baseline cytokine and chemokine levels.

**Bone marrow transplantation**

To examine the role of inflammation in kidney IR injury, Nod(1 × 2)−/− versus WT bone marrow was transplanted into WT mice. Male WT mice were lethally irradiated with two doses of 5 Gy, separated by 3 h, using a 137Cs GammaCell 40 Extractor irradiator (MDS Nordion, Ottawa, ON, Canada). The next day, bone marrow was collected from Nod(1 × 2)−/− or WT mice by flushing femurs and tibia with sterile 10K media (RPMI containing t-glutamin [2 mM], FBS [10%], penicillin [100 U/ml], streptomycin [100 μg/ml], 2-ME [0.05 mM], and HEPES [10 mM]). The bone marrow cells then were washed with sterile PBS, and 5 × 10⁹ Nod(1 × 2)−/− or WT bone marrow cells in sterile PBS were injected into the tail vein of recipient irradiated mice. The mice were kept in microisolator cages for 6 wk to complete engraftment with donor bone marrow and were given trimethoprim sulfa-enriched water until induction of IR injury. Confirmation of Nod(1 × 2)−/− engraftment after bone marrow transplantation was performed on bone marrow cells of chimeric WT mice engrafted with either Nod(1 × 2)−/− bone marrow or WT bone marrow. To detect the Nod1 mutation, the WT forward and reverse primers were 5′-CCTGCTCTCCTTGTCTAGT-3′ and 5′-ACTGCTGCTTGGCTTTATCTC-3′, respectively. The Nod2 mutant forward and reverse primers were 5′-TTGGTGGTCGAATGGGCAGGTA-3′ and 5′-CGCGGCTTCTC- CCTTCTCTCA-3′, respectively. The Nod2 WT forward and reverse primers were 5′-ACAGAGATGGCCGACACTGAGCTG-3′ and 5′-TTGGAGAAGGTT-TGGAGAAGGTCTGCAGGATGTC-3′, respectively. The Nod2 mutant forward and reverse primers were 5′-TGACTGTGGCTTATGCTTCTTGG-3′ and 5′-TCTCCTTTGTCAGCTTC-3′, respectively.

**Results**

**Nod expression within mouse and human kidney**

Nod proteins are activated by endogenous ligands and are thought to be sentinel intracellular receptors for molecules released by dead and dying cells. To evaluate the role of these innate immune receptors in renal IR injury, we first determined whether Nod1 and Nod2 genes were expressed in RTE cells of human and mouse kidneys. As seen in Fig. 1, using semiquantitative PCR, we found that Nod1 and Nod2 were expressed highly in RTE cells from both species. Nod1 signaling and renal IR injury

Because Nod1 and Nod2 were expressed in RTE, we next investigated whether these intracellular innate immune sensors contributed to the syndrome of kidney IR injury in a murine model. Using WT and mutant mice bred for >10 generations to the same genetic background (C57BL/6), we induced nonlethal IR injury in WT, Nod1−/−, Nod2−/−, and Nod(1 × 2)−/− mice, using our model described previously (4).

Once activated, Nod1 and Nod2 recruit a serine-threonine kinase called Rip2 (also known as RIP-like interacting caspase-like apoptosis-regulatory protein kinase or caspase recruitment domain-containing serine/threonine kinase RIP2) through homotypic CARD–CARD interactions (2). Because Nod1 and Nod2 were highly expressed in renal epithelium, we also asked whether the absence of Rip2, their putative downstream signaling protein, influenced renal IR injury. Rip2 is essential for not only NF-κB activation but also for the activation of JNK pathways and induction of cell death through apoptosis (3, 30). Fig. 2 shows serum creatinine levels 2 wk before injury for WT mice and 24 h after injury for each group of mice. Preinjury creatinine levels are listed in the figure legend for Nod1−/−, Rip2−/−, Nod2−/−, and Nod(1 × 2)−/− mice; none of the preinjury creatinine levels of the mutant mice differed statistically from those of the WT mice. As seen in Fig. 2, there was no significant difference in renal injury induced by this kidney IR injury model between WT and Nod1−/− (p = 0.14) mice. There was however a significant difference in injury between WT and Rip2−/− (p = 0.01), Nod2−/− (p = 0.0008), and Nod(1 × 2)−/− (p = 0.0007) mice. Sham-operated mice showed no difference in serum creatinine levels from the baseline, as expected (data not shown). Interestingly, the functional data show that a deficiency in Nod2 and Rip2 provided more protection from injury than a deficiency in Nod1, despite the presence of Nod1 in the murine kidney.

Histological injury was assessed in each group by a pathologist who was blinded to the experimental groups (Fig. 3). With a modified standard histologic scoring system (29), tissue injury was scored 24 h after the initial ischemic insult (Fig. 3A); the means of each group are shown in the figure legend. WT and Nod1−/− kidneys showed no injury. Representative photomicrographs are shown for FIGURE 1. Nod1 and Nod2 in mouse and human RTE. Three representative panels depicting Nod1 and Nod2 genes as well as a housekeeping gene (GADPH) control (listed as Nod1, Nod2, and GADPH below each panel) are shown. Each panel illustrates gene expression, detected by semiquantitative PCR in RTE of either WT (C57BL/6) mice, Nod1(1 × 2)−/− mice, or human tubular epithelial cells extracted from normal human kidney tissue (hTEC). The results are representative of four mice per group and three different human kidneys extracted for renal tubular cells with identical results.

![FIGURE 2](image-url) Kidney injury following IR injury. WT, Nod-deficient, and Rip2-deficient mice were subjected to 25 min of ischemia and 24 h of reperfusion, and serum was obtained at 24 h to detect creatinine levels from WT (n = 11), Nod1−/− (n = 11), Rip2−/− (n = 6), Nod2−/− (n = 11), and Nod(1 × 2)−/− mice (n = 11). Preinjury creatinine levels were obtained 2 wk earlier for each group of mice (WT preinjury values are shown in the figure). Preinjury creatinine levels of WT mice did not differ significantly from those of Nod1−/− (0.3 ± 0.032), Rip2−/− (0.5 ± 0.059), Nod2−/− (0.35 ± 0.12), and Nod(1 × 2)−/− (0.43 ± 0.057) mice. Shams had no significant change from baseline preinjury levels (data not shown). Error bars represent SDs of creatinine levels, and statistical significance was determined with a two-tailed Student t test.
and sham (n = 8), Nod1−/− (n = 8), Nod2−/− (n = 10), Nod1 × 2−/− (n = 7), and sham (n = 3) mice that were subjected to 25 min of ischemia and 24 h of reperfusion. The top panel shows the blinded histologic score (see Materials and Methods for details of scoring), and the bottom panel shows a representative micrograph of each group of mice (periodic acid Schiff stain, original magnification ×200). Means of histological scores are as follows: WT (m = 4.5); Nod1−/− (m = 3.8; p = 0.14); Nod2−/− (m = 3.0; p = 0.17); Nod1 × 2−/− (m = 1.4; p = 0.04); sham (m = 0). B. Neutrophil infiltration into kidneys of WT versus Nod-deficient mice after bilateral renal artery clamping. This figure shows neutrophil infiltration following 25 min of ischemia and 24 h of reperfusion in WT (n = 8), Nod1−/− (n = 8; p = 0.11), Nod2−/− (n = 8; p = 0.013), Nod1 × 2−/− (n = 8; p = 0.002), and sham (n = 3) mice. The blinded score (see Materials and Methods for details of scoring) of neutrophil infiltration is shown. Statistical significance was determined with a two-tailed Student’s t test.

Cytokine and chemokine expression after murine IR injury

In vitro studies have shown that upon ligand recognition Nod1 and Nod2 activation result in the transcription of a large repertoire of genes, many of which are dependent on NF-κB activation (2). In dendritic cells, macrophages, and monocytes, activation of Nod1 and Nod2 lead mainly to the production of proinflammatory cytokines (IL-6, IL-8/KC, TNF-α, and IL-1β) and expression of costimulatory molecules and adhesion molecules (2, 11). In epithelial cell lines, triggering of the Nod pathway induces the production of proinflammatory mediators (TNF-α, IL-6, IL-8/KC, MIP2, and MCP-1) (2). These cytokines and chemokines help to recruit and activate inflammatory effector cells and establish an appropriate immune response to the IR injured kidney.

Proinflammatory cytokines and chemokines known to participate in the inflammatory response to kidney IR injury (4, 32) were investigated 24 h after IR injury in WT and Nod1 × 2−/− mice (Fig. 5). As shown in Fig. 5, there was significantly less IL-6, KC, and TNF-α 24 h following IR injury in Nod1 × 2−/− mice. KC has been validated as an early biomarker for renal IR injury (32, 33), serum IL-6 levels are correlated highly with the severity of tissue injury (34–36), and TNF-α plays an important role in KC-induced neutrophil infiltration (37). There was no significant difference in IFN-γ, IL-1β, MIP2, and MCP-1 levels at 24 h following renal injury between the two groups of mice.
FIGURE 5. Cytokine and chemokine expression in WT versus Nod(1 × 2)−/− mice. The top row shows peak levels of the indicated cytokines and chemokines (24 h after IR injury). IL-6 levels were detected in serum, whereas KC and TNF-α were detected in tissue homogenates. The bottom row shows peak levels of the indicated cytokines and chemokines after i.p. injection with LPS (10 mg/kg). Peak levels of serum IL-6 were noted at 6 h and KC at 24 h after LPS injection, whereas the serum peak for TNF-α was seen at 1 h after injection. The pre bars represent unmanipulated controls, whereas the post bars represent after either IR injury or LPS injection. The data represent six mice per group. Error bars represent SDs, and statistical significance was determined with a two-tailed Student t test.

Biochemical and functional studies have revealed that Nod2-deficient mutants exhibit a reduced ability to activate NF-κB in response to the Nod2-specific ligand muramyl dipeptide, but they maintain a normal response to LPS stimulation (10, 38). To confirm the ability of WT and Nod-deficient mice to produce the tested cytokines and chemokines, mice were injected i.p. with ultrapure LPS (from S. minnesota R595), and serum was tested at a range of intervals after the injection of LPS (0, 1, 3, 6, and 24 h) to obtain the peak of cytokine and chemokine expression; TNF-α peaked at 1 h, IL-6 peaked at 6 h, and KC peaked at 24 h. There were no significant differences in production of the tested cytokines and chemokines between the LPS-injected WT and Nod(1 × 2)−/− mice.

WT mice transplanted with Nod(1 × 2)−/− bone marrow are protected only modestly from renal IR injury

Ischemic renal injury is a complex process that involves not only direct damage to RTE but also inflammation in response to the tissue injury. To assess the role of Nod1 and Nod2 in systemic (inflammation) injury following renal IR injury, we replaced the bone marrow of WT mice with Nod(1 × 2)−/− bone marrow (Fig. 6). As seen in Fig. 6A, there was a small difference (p = 0.05) between WT mice replaced with syngeneic bone marrow and WT mice replaced with Nod(1 × 2)−/− bone marrow, suggesting that inflammation might play a role, albeit small, in the primary protection from kidney IR injury offered by the absence of Nod receptors in this model. Blinded analysis of histologic and neutrophil infiltration though did not show significant differences between the groups (Fig. 6A, histology score and neutrophil score). Analysis of proinflammatory cytokine and chemokine secretion (IL-6, KC, and TNF-α) also showed no differences between the two groups (data not shown). Fig. 6B confirms that the bone marrow of WT mice indeed was replaced by the indicated mutant marrow. Future studies are ongoing to define the role of the Nod receptors on various inflammatory cells known to participate in the syndrome of kidney IR injury (39–41).

Discussion

The pathogenesis of kidney IR is complex, and many causative mechanisms converge to produce the pathomorphologic findings of RTE cell apoptosis and necrosis and postinjury inflammatory cell infiltration (42–44). A complex network of cross talk occurs among injured RTE cells, endothelial cells, and inflammatory cells; each cell type generates and responds to a variety of cytokines and chemokines. Despite this complexity, a growing body of evidence highlights a significant role for innate PRRs in both the induction of ischemic kidney injury and the inflammatory response to the ischemic tissue. Because renal tubular apoptosis and inflammation are cardinal features of kidney IR injury, the investigations in this study focused on a family of intracellular innate immune receptors known to play a role in apoptosis and inflammation, the NLR family members Nod1 and Nod2.

Our studies are the first, to our knowledge, to demonstrate that Nod1 and Nod2 receptors are expressed within RTE cells in both murine and human kidneys. The finding of renal epithelial cell expression is not surprising, because Nod1 has been described already in various human epithelial cells (17) and Nod2 has been found in intestinal epithelium (Paneth cells) (16), murine and canine ophthalmic epithelial cells (45), skin (15), lung epithelium (17), human endometrium (46), and human dental pulp tissue (47). Nod1 is considered generally to be expressed in all cell types (2), whereas Nod2 expression is restricted to specialized epithelium and inflammatory cells. The expression within RTE cells and inflammatory cells positions these innate intracellular proteins as sentinel participants in responses to endogenous danger signals associated with kidney IR injury.

To further define the role of Nod1 and Nod 2 in the murine kidney, we compared and contrasted kidney IR injury induced in WT mice to injury in Nod-deficient mice. Interestingly, we found that despite the presence of both Nod1 and Nod2 in RTE cells a deficiency in Nod2 provided greater protection than a deficiency in Nod1 for kidney IR injury. Because much is yet to be learned...
about the in vivo role of Nod receptor molecules, there are likely many potential reasons for the differential sensitivity observed in this study, including tissue-specific ligand processing or even tissue-specific expression of Nod-dependent regulatory signaling molecules. Nod1 and Nod2 ligands that are produced by bacteria enter the cell via endocytosis and epithelial transporters. Different epithelial transporters for Nod ligands have been identified already in tissue (48, 49), and recent reports suggest differential tissue distributions and polymorphisms in these transporters (50, 51). Future studies will need to address the role of these peptide transporters in Nod1- versus Nod2-specific responses in the kidney. The molecular details of Nod downstream signaling mediators are not yet completely understood, and additional explanations for differential susceptibility to kidney IR injury will likely come from clarification of the downstream Nod1 and Nod2 regulatory proteins (52–57).

Consistent with the finding that Nod2-deficient mice were protected from kidney IR injury, we also found that Rip2-deficient mice also were protected. Upon oligomerization of Nod1 or Nod2, the serine-threonine kinase Rip2 is recruited through homophilic CARD–CARD interactions. Rip2 in turn is cross-activated to transduce downstream signals leading to NF-κB activation and to apoptotic signaling pathways (3). Rip2 had been shown previously to contribute to TLR2 signaling (58), although more recently has been found to be a mediator of Nod1 and Nod2 signaling independent of TLRs (59). Notably, we have shown already that TLR2-deficient mice are protected from kidney IR injury (4), suggesting there might be an as yet undefined role for Rip2 in mediating several PRR responses in the kidney.

The greatest protection from kidney IR injury was offered by a simultaneous deficiency of Nod1 and Nod2, even surpassing the protection offered by Nod2 alone. These data suggest that although functional protection was not seen per se in the Nod1-deficient mice, Nod1-mediated signals might play a less obvious role in renal IR injury. One possible role for Nod1 in our model might be through regulation of inflammation in response to the ischemic tissue. Nod1 has been shown to be important for neutrophil infiltration in vivo (20), and neutrophil accumulation quickly follows IR injury in the kidney (60, 61). There were fewer neutrophil infiltrates in the injured Nod1-deficient mice than in the injured WT mice. Because Nod1 activation induces neutrophil recruitment in vivo (20), it is possible that the combined effect of Nod1 and Nod2 deficiency produced more complete protection because mechanisms that were operative in the presence of Nod1 (e.g., neutrophil recruitment) were inhibited more completely in the absence of both Nod proteins. Arguing against this theory though is the finding that there were no significant differences in histologic injury, neutrophil infiltration, or proinflammatory cytokine secretion in WT mice transplanted with Nod1-deficient bone marrow versus Nod1-/−/− bone marrow. Future studies will need to more extensively analyze neutrophil function as well as the role of other inflammatory mediators in the two groups of animals.

The pathophysiology of kidney IR injury is complex and involves both direct renal tubular injury as well as extension of the injury through inflammation. Nod1 and Nod2 are known participants in cellular stress responses, and we found that mice simultaneously deficient in Nod1 and Nod2 were protected from renal tubular apoptosis and inflammation. The primary protection appeared to be protection from apoptosis, because Nod(1 × 2)-/−/− bone marrow did not offer chimeric WT mice complete protection from kidney IR injury. The protection afforded by a deficiency in Nod1...
and Nod2 positions these receptors as potential therapeutic targets for prevention and treatment of ischemic kidney injury. Future studies will likely illuminate an important role of the Nod family of PRRs in normal and stress-related renal tubular cell responses and will better define tissue-specific expression of not only the Nod receptors but also their downstream signaling molecules.

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Disclosures

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References


