Expression of TLR2, NOD1, and NOD2 and the NLRP3 Inflammasome in Renal Tubular Epithelial Cells of Male versus Female Mice

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Keywords

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Abstract

Background: Gender-biased outcomes are associated with acute kidney injury (AKI) and human and animal studies have shown that females are preferentially protected from renal ischemia. However, the reason for this is not known. One clue might lie with pattern recognition receptors (PRRs), which are triggers of ischemic injury when ligated by molecules in the ischemic milieu. Several PRR families are expressed by renal tubular epithelial cells (RTEs) and incite cell death signaling and production of pro-inflammatory molecules. Blockade of specific PRRs (e.g., TLR2, NOD1, NOD2, and NLRP3) provides highly significant protection from ischemic RTE injury. As a first step to understand gender-biased outcomes of AKI, we tested whether constitutive gender-based differences exist in expression of these PRRs in RTEs. **Methods:** To determine whether PRR expression differences exist, primary RTEs isolated from male and female WT kidneys were examined by FACS, qPCR, and Western Blot for expression of TLR2, NOD1, NOD2, and NLRP3 inflammasome components. **Results:** No RTE gender-based differences in TLR2,

NOD1, NOD2, NLRP3, or ASC were found. RTEs from female kidneys had approximately half the mRNA, but the same protein concentration of pro-caspase-1 compared to RTEs isolated from male kidneys. **Conclusions:** Our findings indicate that intrinsic gender differences in RTE expression of TLR2, NOD1, NOD2, NLRP3, and ASC are not responsible for the gender-biased outcomes observed in ischemia/reperfusion injury. The lower caspase-1 mRNA expression in RTEs from females warrants further exploration of additional upstream signals that might differentially regulate caspase-1 in male vs. female RTEs.

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Introduction

Acute kidney injury (AKI) occurs commonly and is a major cause of morbidity and mortality in hospitalized patients [1, 2]. Ischemic renal injury is studied in experimental models of ischemia/reperfusion injury (IRI), where it has been shown that renal tubule cells located in regions of the kidney most susceptible to low blood flow experience the greatest injury [3, 4]. Recent data have shown that after an ischemic insult, renal tubular epithelial cells (RTEs) release their intracellular contents, containing molecules with structural motifs recognized by

pattern recognition receptors (PRRs). Ligation of these receptors results in the production of pro-inflammatory cytokines and/or the induction of cell death signaling, further propagating injury to the kidney [5].

It is well known that female mice are resistant to IRI and, in fact, many experimental models exclude female mice in IRI research [6–8]. The gender-specific effect demonstrated in experimental models is paralleled by observations from human trials showing that outcomes for men are worse than those for females after AKI; and males are more likely to develop CKD after AKI [9, 10].

The mechanisms behind the gender differences in susceptibility to renal IRI are not yet known, and many investigative studies to date have focused on the role that sex hormones play in renal injury. While conflicting reports exist, there is evidence to suggest that estrogen has protective effects, while androgens seem to worsen IRI [6, 11, 12].

Data from our lab and other sources have shown that mice with targeted deletions in TLR2, the NLRP3 inflammasome, and the cytosolic NOD2 receptor are highly protected from renal IRI [13–16]. This protection appears to be specific to the renal tubule epithelium, as WT mice transplanted with bone marrow deficient in these PRRs experience the same injury as mice with WT bone marrow cells [14]. Deletions in other PRRs (e.g., TLR4) have also been associated with protection from renal AKI, and they exert additional effects on renal endothelium and leukocytes [17, 18].

The National Institutes of Health has recently recognized the importance of considering gender in experimental study design and now requires gender and gender inclusion plans in preclinical research [19]. Understanding the differences or similarities in expression of TLRs and NLRs in RTEs of males vs. females is an area of IRI research that has not previously been addressed. In this study, we probe whether there are intrinsic differences in baseline or stimulated expression of TLR2, NOD1, NOD2, and the NLRP3 inflammasome; PRRs that we have found to have a primary effect on RTEs in renal IRI. Studying the RTEs isolated from male vs. female kidneys provided a first step to understanding how gender influences renal responses to ischemia.

Methods

Mice and Isolation of RTEs

Female and male C57BL/6 mice were purchased from Jackson Laboratories and housed at UCSD vivarium. Handling and use of animals were in accordance with the standards of the Association

for the Assessment and Accreditation of Laboratory Animal Care. All mice used for experiments were age 6–8 weeks of age. RTEs were isolated from the murine kidneys and cultured *ex vivo*, as has been previously described [14, 15]. RTEs were analyzed at approximately 5 days post-isolation, when cells had reached approximately 70% confluence.

Stimulation of RTEs

Cells were cultured on collagen plates in a monolayer then washed twice with 3 mL of DMEM:F12 (Life Technologies, Carlsbad, CA, USA), trypsinized and 4×10^5 cells stimulated with either 1 μ g/mL of Pam3Cys (Alexis Biochemical, Farmingdale, NY, USA) dissolved in DMEM:F12 or DMEM:F12 alone and placed in a water bath at 37°C for 3 h with gentle agitation every 20 min. After 3 h, the cells were pelleted at 1200 RPM for 5 min, media removed, and washed with 1 mL of sterile PBS (Life Technologies, Carlsbad, CA, USA), and cell pellets stored at –80°C until RNA isolation could be performed.

Stimulation for Western Blot

The stimulation procedure was identical to the above-mentioned procedure, except for the following changes: (1) 5×10^5 cells were added to each tube; (2) cells were stimulated for 1 h with 10 μ g/mL of Pam3Cys; (3) cells were washed with protease inhibitor-containing wash buffer instead of PBS. The wash buffer contained 0.4 mM Sodium-Orthovanadate + 5 mM NaF + 4.46 mg/mL sodium pyrophosphate (Sigma, St. Louis, MO, USA) in PBS.

FACS Analysis

RTEs were grown in collagen plates as indicated above. Cells were trypsinized and counted. 1×10^6 were added to FACS tubes and spun down at 1200 RPM for 5 min. Media was removed and cells resuspended in 400 μ L of FACS wash buffer. Cells were then divided evenly into individual wells of a Costar round bottom 96-well plate (Corning, Corning, NY, USA) and stained with TruStain FcX (Biolegend; San Diego, CA, USA) in FACS wash buffer at 1:50 dilution and placed on ice for 5 min incubation. Fifty microliter of PE-conjugated anti-TLR2 (eBiosciences; San Diego, CA, USA) in FACS wash buffer (1:50 dilution) was then added to cell suspensions (final dilution of 1:100) and placed on ice away from light for 45 min incubation. Stained cells were washed 2 times with FACS wash buffer and placed in 1.2 mL micro titer tubes on ice (Fischer Scientific; Hampton, NH, USA) for FACS analysis. Cells were run on the BD Biosciences LSRIII and analysis performed on FlowJo software.

Western Blot

Equal amounts of protein were added to each well along with 3 μ L of TNF-treated and TNF-non treated cell extracts (Cell Signaling, Danvers, MA, USA, #9243 and #9243s, respectively) to act as positive and negative controls. Blots were blocked over night with 5% milk powder in TBST. Primary antibody staining was performed over 2 h, while secondary antibody staining was performed over 1 h. Antibodies for protein detection of NLRP3, ASC, procaspase-1, pro-IL-1b, NOD1, NOD2, and RIP2 proteins were purchased from the following sources with listed catalogue numbers: NLRP3 (R&D systems, Minneapolis, MN, USA, #MAB7578); ASC (ENZO Life Sciences, Farmingdale, NY, USA, #ADI-905-173-100); pro-caspase-1 (EMD Millipore, Temecula, CA, USA, #EMD06-503); pro-IL-1b (Cell Signaling, #12426); NOD1 (Cell

Fig. 1. Baseline expression of TLR2 on primary unstimulated RTEs isolated from male vs. female mice. **a** Histogram showing cell surface expression of TLR2 on primary unstimulated RTEs isolated from male vs. female mice (dotted lines, male; solid line female). The data represent 1 of 3 identical experiments comparing RTEs from 3 males and 3 females per group. **b** Baseline mRNA expression of TLR2 comparing primary unstimulated RTEs isolated from male vs. female mice. Male mice were the standard to which females were compared (relative expression). Error bars represent standard deviation ($n = 6$ males and 5 females). No significant differences were detected between the groups. The data represent 6 identical experiments.

Signaling, #12426); NOD2 (Santa Cruz, Dallas, TX, USA, #sc56168; RIP2 (Cell Signaling, #4142). Both pNF- κ B (Ser536) and I κ B α (L35A5) antibodies were purchased from Cell Signaling (#3033s and #4814 respectively). Blots were analyzed using Syngene PXi gel imager (Syngene, Frederick, MD, USA) and manual band quantification performed on GeneSys Gene Tools software.

RNA, cDNA Isolation, and qPCR

RNA was isolated using the Zymo Research Quick-RNA Mini-Prep (Zymo research corp, Irvine, CA, USA) based on the manufacturer's instructions. cDNA was isolated using the Invitrogen First-Strand cDNA synthesis kit based on the manufacturer's instructions. All primers for the qPCRs were purchased from Genecopoeia Biotechnology Company (Rockville, MD, USA) or Qiagen (Hilden, Germany). cDNA was standardized to 300 ng/ μ L and then 2 μ L of cDNA was added to 4 μ L of master mix containing 1 μ L primer:3 μ L SSO Advanced Universal SYBRgreen (Bio-Rad, Irvine, CA, USA). qPCR was performed on the Illumina Eco Real-Time PCR system with GAPDH as the housekeeping gene. In order to calculate relative expression at baseline, the ΔC_q values for the male no ligand treatment were first averaged. This average was then considered the reference to which all of the female mice were compared. $\Delta\Delta C_q$ values were then calculated by subtracting ΔC_q (female) - ΔC_q (male average). This process was also performed with females as the reference, and there was no difference in the results. Calculations for relative expression were performed as follows:

$$\begin{aligned} \text{Baseline expression} & \quad \text{TLR2 stimulation} \\ \text{Relative expression} &= 2^{\Delta\Delta C_q} \quad \text{Relative expression} = 2^{\Delta\Delta C_q} \\ \Delta\Delta C_q &= \Delta C_q(\text{female}) - \Delta C_q(\text{male avg}) \quad \Delta\Delta C_q = \\ \Delta C_q(\text{Pam3Cys}) - \Delta C_q(\text{no ligand}) \\ \Delta C_q &= C_q(\text{gene of interest}) - C_q(\text{GAPDH}) \quad \Delta C_q = C_q(\text{gene of} \\ \text{interest}) - C_q(\text{GAPDH}) \end{aligned}$$

Statistics

The numbers of male and female mice used for each experiment are described in the figure legends. Relative expression of mRNA was compared using a 2-tailed student *t* test (Graph-

Pad Software, Inc. La Jolla, CA, USA), with $p < 0.05$ considered significant and >0.05 indicating no difference between the 2 groups.

Results

Female mice are resistant to renal IRI and therefore models of AKI often employ male mice [6–8]. These observations prompted us to probe whether there were constitutive differences in PRR expression between male vs. female RTEs.

Baseline PRR Expression

Cell surface expression of TLR2 is highest in the areas of the kidney that are most susceptible to ischemic injury, primarily the proximal tubular epithelial cells located in the outer medulla and cortex of the kidney [13]. Blockade of TLR2 has been shown to prevent AKI and prevent renal tubular apoptosis and necrosis in murine models of IRI [13, 20–22]. As shown in Figure 1a, we found equivalent baseline TLR2 cell surface expression on primary RTEs isolated from male vs. female kidneys (Fig. 1a, male – dotted line; female – solid line; and Panel B, male – black square; and female – grey square).

We next checked whether other PRRs shown to be important in renal tubules after IRI were differentially expressed in male vs. female RTE cells. Work from our lab and others has shown that blockade of the NLRP3 inflammasome provides highly significant protection from renal IRI, and that production of pro-inflammatory cytokines IL-1b and IL-18 in RTEs decreases significantly in the absence of NLRP3 [15, 23]. To evaluate the expression

Fig. 2. Baseline expression of NLRP3 inflammasome components on primary unstimulated RTEs. **a** Baseline mRNA expression of NLRP3 inflammasome components NLRP3, ASC, and pro-caspase-1 and downstream targets pro-IL-1b and pro-IL-18. Unstimulated primary RTEs isolated from male vs. female mice were compared (black box male, grey box female). Male mice were the standard to which females were compared (relative expression). The

data represents 6 identical experiments. Error bars represent SE of the mean. No statistical differences were detected, except in pro-caspase-1 ($p = 0.02$). **b** Baseline protein expression of NLRP3 inflammasome components NLRP3, ASC, pro-caspase-1, and pro-IL-1b. The corresponding densitometry is shown in a bar graph accompanying the Western Blots. No statistical differences were detected. The blot represents 6 identical experiments.

of the NLRP3 inflammasome, we examined the relative expression of NLRP3, its co-receptor molecule ASC, and its downstream targets pro-caspase-1, pro-IL-1b, and pro-IL-18 mRNA in resting RTE cells from male vs. female mice (Fig. 2, males – black square; females – grey square). Shown in Figure 2a, no differences were detected in baseline mRNA expression of NLRP3, ASC, pro-IL-1b, or pro-IL-18 between female and male mice. There was, however, a statistically significant difference in the expression of pro-caspase-1 mRNA in RTEs between males and females with females having half the expression of pro-caspase-1 as males (Fig. 2a, caspase-1). Confirming the mRNA expression, we also found that there were no

differences in the protein expression of NLRP3, ASC, or pro-IL-1b in lysates of RTEs from male vs. female mice and interestingly there was equivalent protein expression of pro-caspase-1 in male and female RTEs (Fig. 2b).

To further evaluate PRRs found to be important in renal IRI, we next investigated baseline mRNA expression of the best-characterized cytosolic NLRs, NOD1, and NOD2. As was the case with the inflammasome components, we found no differences in constitutive NOD1 or NOD2 expression between male and female RTE cells (Fig. 3a, NOD1, NOD2; male – black square; female – grey square). We also analyzed for baseline mRNA expression of RIP2, a downstream NOD signaling mediator [24–26],

Fig. 3. Baseline mRNA expression of NOD1, NOD2, RIP2, and IL-6. **a** Unstimulated primary RTEs isolated from male vs. female mice were compared (black box male, grey box female). Male mice were the standard to which females were compared. In each group, 6 males were compared to 6 females. Error bars represent SE of the mean. No statistical differences were detected between male vs.

female groups. **b** Baseline protein expression of NOD1, NOD2, and RIP2. The corresponding densitometry is shown in a bar graph accompanying the Western Blots. No statistical differences were detected between male vs. female groups. The blot represents 6 identical experiments.

and found no significant differences between males and females (Fig. 3a, RIP2, male – black square; female – grey square). There was also no difference between baseline IL-6 expression between male vs. female RTEs (Fig. 3a, IL-6, male – black square; female – grey square). We also found no differences in protein expression of NOD1, NOD2, or RIP2 between male and female mice (Fig. 3b).

Upregulation of Inflammasome Components in RTEs after Stimulation

Stimulation of TLR2 has been shown to upregulate several cytoplasmic PRRs, including NLRP3 as well as its substrates pro-IL-1b and pro-IL-18 [27–29]. To deter-

mine whether NLRP3 inflammasome components, or downstream targets pro-IL-1b and pro-IL-18 were differentially regulated between male vs. female RTEs, we stimulated RTE cells with the ultrapure synthetic ligand for TLR2, Pam3Cys. We found that pro-IL-1b and NLRP3 were upregulated rapidly after Pam3Cys exposure, as has been previously reported [28]. There was a trend toward lower expression of pro-IL-1b in female mice, but it did not reach statistical significance, and no significant difference was seen in TLR2-induced NLRP3 expression in RTEs between male and female kidneys (Fig. 4a, pro-IL-1b, Nlrp3, male – black square; female – grey square). We also analyzed ASC, pro-caspase-1, and pro-IL-18 mRNA

Fig. 4. TLR2-induced expression of the cytoplasmic innate immune receptors NLRP3 and NOD1/NOD2 family members. **a** TLR2-induced mRNA expression of NLRP3 inflammasome components (NLRP3 and ASC, caspase-1) and the downstream targets IL-1b, IL-18. Primary RTEs from male (black box) vs. female (grey box) mice were compared after anti-TLR2 stimulation.

b TLR2-induced mRNA expression of NOD1, NOD2, RIP2, and IL-6 in primary RTEs isolated from male (black box) vs. female (grey box), after anti-TLR2 stimulation. In both panels, error bars represent SE of the mean. No statistical differences were detected between male vs. female groups. The data represent 6 identical experiments.

expression and found no TLR2-induced differences between male vs. female RTEs (Fig. 4a, male – black square; female – grey square).

Upregulation of NLRs, RIP2, and IL-6 in Response to TLR2 Stimulation

Significant crosstalk exists between TLR2 and NOD1 and NOD2 signaling pathways [24, 25, 30], which prompted us to determine whether TLR2 stimulation induced differential upregulation of these NLRs in RTEs from male vs. female mice. NOD2 was upregulated by 2.5-fold after TLR2 stimulation, but there were no significant differences in the induced expression in RTEs between the 2 groups (Fig. 4b, NOD1, NOD2; male – black square; female – grey square). NOD1 mRNA expression was not induced by TLR2 stimulation in either males or females. RIP2 has been shown to be upregulated by TLR2 in other organs [31, 32]. Interestingly we found that RIP2 was significantly upregulated by TLR2 stimulation in murine RTEs, which is the first time to our knowledge that TLR2-induced RIP2 expression has been shown in RTE cells (Fig. 4b, IL-6; male – black square; female – grey square). There was, however, no difference in RIP2 expression between TLR2 stimulated RTEs isolated from male vs. fe-

male mice. IL-6 mRNA was also upregulated by TLR2 activation, and again there were no differences between male vs. female RTEs (Fig. 4b, IL-6, male – black square; female – grey square).

TLR2 activation induces downstream NF- κ B transcription [33] and we asked whether stimulation with Pam3Cys produced differences in NF- κ B activation in RTEs from male vs. female kidneys. As shown in Figure 5, there was no appreciable difference between TLR2-induced NF- κ B activation in male vs. female RTEs (Fig. 5a, pNF- κ B, I κ Ba and Fig. 5b, male – black square; female – grey square).

Discussion

Our study found no gender-biased differences in baseline or induced expression of TLR2, NOD1, NOD2, or NLRP3 inflammasome components in RTEs isolated from male vs. female kidneys. These data suggest that differences in expression of these PRRs do not account for the natural protection afforded to the female mice from ischemic renal injury.

Renal ischemia reperfusion injury causes significant morbidity and mortality in hospitalized patients and con-

Fig. 5. TLR2-induced activation of NF- κ B in primary RTEs from male vs. female mice. **a** Western blot of pNF- κ B and its inhibitory regulator I κ B α after TLR2 stimulation, comparing primary male vs. female RTEs. The data represent one of 2 identical experiments with one male and one female per group. **b** Band quantification

ratio of pNF- κ B to its inhibitor I κ B α . Higher values indicate greater activation. No stimulation (-); Stimulation with the TLR2 ligand PAM3CSK (+). TNF-treated extracts are shown as controls; TNF-treated (+) or untreated (-) cell extract.

tributes to delayed graft function in transplant recipients [1, 2, 34]. Female gender is protective for IRI in both humans and animal models, and therefore female mice are generally excluded from experimental models that are used to study ischemic renal injury [6–8]. The mechanisms behind the renal protection experienced by the female gender are not known. One hypothesis is that sex hormones confer protection, and indeed many studies to date have focused on the role of sex hormones in renal protection. In studies of organ ischemia, estrogen has been shown to have protective effects [11, 12, 35, 36], while androgens worsen injury [6, 11, 12]. However, contradictory data exist even within studies, highlighting the complex nature of a potential hormonal influence.

An alternative hypothesis is that males and females have intrinsic differences in the expression of intracellular signaling molecules that are important for injury responses in the kidney. Arnold et al. [37] showed that cardiac IRI was worsened in mice with 2 X chromosomes as compared to those with one X chromosome, irrespective of which sex organs were present, indicating that IRI can be influenced by genetic factors unrelated to hormones. This observation is contrary to the observation that renal IRI is worse in males; however, it highlights the importance of considering intrinsic, non-hormonal differences between males and females.

In this study, we analyzed whether intrinsic differences exist in TLR2, NOD1, NOD2, or NLRP3 inflammasome components between RTEs isolated from male vs. female kidneys. PRRs are constitutively present on RTEs

and are ligated by molecules released during renal ischemia, thereby propagating renal injury after an ischemic insult by causing the upregulation of inflammatory cytokines and by activating cell death signaling pathways [5, 25, 38, 39]. Knockout models have shown that mice lacking specific PRRs, such as TLR2, the NLRP3 inflammasome, and NOD2, experience significant renal protection from IRI [13–15, 40–42]; however, mice defective in NLRP3 are also protected from rhabdomyolysis-induced AKI [43] but not from cisplatin-induced AKI [42]. A reasonable question to ask is whether males have higher expression levels of these molecules in their kidneys or whether they upregulate them more robustly, resulting in a gender-biased predisposition to renal IRI. Many studies have shown that sex hormones influence PRR expression in inflammatory cells. For instance, Jitprasertwong et al. [44] showed that TLR2 is downregulated in monocytes by both β -estradiol and progesterone. Specific TLR transcripts in platelets have been found to be more abundant in women and to have distinct associations with cardiovascular risk and inflammatory biomarkers that vary by gender [45]. Coxsackie B infection causes an upregulation of TLR2 in female (and TLR4 in male) splenic lymphocytes and this differential expression contributes to the disease resistance in females and disease susceptibility in males [46]. Gender-dependent differences have also been shown in the NLRP3 and AIM2 inflammasomes; NLRP3 is hyperactivated in macrophages of both male and female systemic lupus erythematosus patients, but the mechanisms underlying NLRP3 hyperactivation might

be different between the genders, and it was postulated that the AIM2 inflammasome might also contribute to the female gender bias of SLE pathogenesis and severity [47].

The conclusion of this study was that no differences were detected in TLR2 cell surface expression, NLRP3 inflammasome components, or NOD1 and NOD2 cytosolic expression in male vs. female RTEs. These findings suggest that intrinsic differences in RTE expression of these PRRs between males and females are not responsible for the gender-biased outcomes observed in IRI. We did however find that RTEs from female kidneys had about half the mRNA expression of pro-caspase-1 than those from male kidneys. This is intriguing because caspase-1 inhibition has been known for some time to have a protective effect on hypoxic injury of proximal tubular cells in rodent models [48] and caspase-1^{-/-} mice are protected from both cisplatin-induced AKI and ischemic AKI [49, 50]. Our data showed no difference in terms of the pro-caspase-1 protein between male and female RTEs, suggesting that additional studies are needed to sort out the reason for the differential expression between the mRNA and the protein forms of pro-caspase-1 in male vs. female RTEs. Pro-caspase-1 is cleaved to its active form by the NLRP1, NLRC4, or AIM2 inflammasomes [51] and so future studies will also examine the role of these inflam-

masomes, or their interacting proteins (e.g., NLRP3/MAVs [52]), in male vs. female caspase-1 expression. Future studies will also be conducted to explore the role of sex hormones on PRR expression.

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Statement of Ethics

All procedures performed in studies involving animals were in accordance with the ethical standards of the University of California San Diego and were conducted under approval from the Institutional Animal Care and Use Committee (IACUC) approval (IACUC approval number S12261). This article does not contain any studies with human participants performed by any of the authors.

Disclosure Statement

The authors have no conflicts of interest to report that have relevance to the data reported in this manuscript.

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