Resolvin D1 Protects Lipopolysaccharide-induced Acute Kidney Injury by Down-regulating Nuclear Factor-kappa B Signal and Inhibiting Apoptosis

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Abstract

**Background:** Resolvin D1 (RvD1) is a newly found anti-inflammatory bioactive compound derived from polyunsaturated fatty acids. The current study aimed to explore the protective effect of RvD1 on lipopolysaccharide (LPS)-induced acute kidney injury (AKI) and its possible mechanism.

**Methods:** Both in vivo and in vitro studies were conducted. Male BALB/c mice were randomly divided into control group (saline), LPS group (LPS 5 mg/kg), RvD1 group (RvD1 5 μg/kg + LPS 5 mg/kg), and blockage group (Boc-MLP 5 μg/kg + RvD1 5 μg/kg + LPS 5 mg/kg). Boc-MLP is a RvD1 receptor blocker. The mice were intraperitoneally injected with these drugs and recorded for general condition for 48 h, while the blood and kidneys were harvested at 2, 6, 12, and 24 h time points, respectively (n = 6 in each group at each time point). Human proximal tubule epithelial cells (HK-2) were randomly divided into control group (medium only), LPS group (LPS 5 μg/ml), RvD1 group (RvD1 10 ng/ml + LPS 5 μg/ml), and blockage group (Boc-MLP 10 ng/ml + RvD1 10 ng/ml + LPS 5 μg/ml). The cells were harvested for RNA at 2, 4, 6, 12, and 24 h time points, respectively (n = 6 in each group at each time point). Blood creatinine was tested by using an Abbott i-STAT portable blood gas analyzer. Tumor necrosis factor-α (TNF-α) level was detected by ELISA. Kidney pathology was observed under hematoxylin and eosin (HE) staining and transmission electron microscope (TEM). We hired immune-histological staining, Western blotting, and fluorescence quantitative polymerase chain reaction to detect the expression of RvD1 receptor ALX, nuclear factor-kappa B (NF-κB) signaling pathway as well as caspase-3. Kidney apoptosis was evaluated by TUNEL staining.

**Results:** RvD1 receptor ALX was detected on renal tubular epithelials. Kaplan–Meier analysis indicated that RvD1 improved 48 h animal survival (80%) compared with LPS group (40%) and RvD1 blockage group (60%), while RvD1 also ameliorated kidney pathological injury in HE staining and TEM scan. After LPS stimulation, the mRNA expression of toll-like receptor 4, myeloid differentiation factor 88, and TNF-α in both mice kidneys and HK-2 cells were all up-regulated, while RvD1 substantially inhibited the up-regulation of these genes. Western blotting showed that the phosphorylated-IκBα/IκBα ratio in LPS group was significantly higher than that in the control group, which was inhibited in the RvD1 group. RvD1 could inhibit the up-regulation of cleaved-caspase-3 protein stimulated by LPS, which was prohibited in RvD1 blockage group. RvD1 group also had a lower proportion of apoptotic nuclei in mice kidney by TUNEL staining compared with LPS group.

**Conclusion:** In LPS-induced AKI, RvD1 could decrease TNF-α level, ameliorate kidney pathological injury, protect kidney function, and improve animal survival by down-regulating NF-κB inflammatory signal as well as inhibiting renal cell apoptosis.

**Key words:** Acute Kidney Injury; Apoptosis; Lipopolysaccharide; Nuclear Factor-kappa B; Resolvin D1

Introduction

Acute kidney injury (AKI) is characterized as the rapid decline in renal function due to various reasons, which is a critical clinical condition affecting multiple organs and systems. Sepsis and septic shock are the major causes of AKI in the critical illness. It is reported that sepsis accounts...
for more than 50% of AKI in intensive care units. Septic AKI has become an important medical issue confronted by clinical practitioners. Despite the remarkable progression in medical care service, the mortality of septic AKI remains high, which calls for a more profound understanding on the pathophysiology toward this disease, as well as searching for new potential therapeutic targets.

Resolvins (RVs) are a class of anti-inflammatory bioactive small molecules with conformational specificity which are derived from polyunsaturated fatty acids enzymatically in natural condition. According to their origins, RVs could be categorized into E-series RVs, D-series RVs, and Aspirin-triggered RVs. RVs can promote the resolution of inflammation by way of inhibiting inflammatory cell infiltration, down-regulating cytokine excretion, promoting neutrophil apoptosis, etc. Resolvin D1 (RvD1) is one of the most extensively and intensively studied RVs, which is found to be a beneficial compound in different animal disease models including acute lung injury (ALI), insulin resistance, peritonitis, wound infection, atherosclerosis as well as ischemia-reperfusion injury. In the fields of nephrology, RvD1 has been reported to protect against ischemia-reperfusion injury, adriamycin-induced nephropathy, obstructive nephropathy, and kidney transplant rejection, of which inhibition of nuclear factor-kappa B (NF-κB) signaling pathway and cytokines has been proved to play an important role. Nowadays, the distribution of RvD1 receptors in the kidney has not been clearly investigated. There have been few studies focusing on the potential protection of RvD1 on septic AKI and its possible mechanism.

Lipopolysaccharide (LPS) can induce early clinical manifestations of sepsis including AKI, which has been widely used in establishing sepsis animal model for its simplicity to use and quantify. The current study aimed to explore in LPS-induced AKI, whether RvD1 could protect renal function and improve animal survival by inhibiting NF-κB signaling pathway and regulating renal cell apoptosis, so as to provide potential insights of therapeutic targets to septic AKI.

**Methods**

Reagents

LPS (Escherichia coli, 0111:B4) was purchased from Sigma (St. Louis, MO, USA). RvD1 was obtained from Cayman (Ann Arbor, MI, USA). Tert-butoxycarbonyl Met-Leu-Phe peptide (Boc-MLP, RvD1 receptor blocker), anti-phosphorylated-1xB alpha (rabbit monoclonal), anti-1xB alpha (rabbit monoclonal), and anti-lipoxin A4 receptor (ALX, RvD1 receptor, and rabbit polyclonal) were from Santa Cruz (Dallas, TX, USA). Anti-cleaved caspase-3 (rabbit polyclonal) was obtained from Abcam (Cambridge, UK). The real-time polymerase chain reaction (PCR) kit was purchased from Takara Bio (Shiga, Japan). In situ cell death fluorescein (TUNEL) kit was purchased from Roche (Basel, Switzerland). Tumor necrosis factor-α (TNF-α) ELISA kit was from R&D (Minneapolis, MN, USA).

**Animals and cells**

Specific pathogen free (SPF) male BALB/c mice, 6–8 weeks old, weighing 24–26 g were purchased from Genetically Engineered Animal Experiment Platform of West China Medical Center of Sichuan University. Human proximal tubular epithelial cell line (HK-2) was provided by the Key Laboratory of Transplantation and Immunology of Ministry of Health.

**Experimental protocols**

**In vivo study**

AKI is defined as doubling of blood creatinine from the control group. We gave SPF level BALB/c male mice intraperitoneal (i.p.) injection of LPS at an amount of 5 mg/kg to establish an animal model of AKI. In the first set of in vivo study, forty BALB/c mice were randomly divided into four groups according to a computer program generated allocation number, which were (1) control group (saline i.p.), (2) LPS group (LPS 5 mg/kg i.p.), (3) RvD1 group (RvD1 5 μg/kg + LPS 5 mg/kg i.p.), and (4) blockade group (Boc-MLP 5 μg/kg + RvD1 5 μg/kg + LPS 5 mg/kg i.p.). In RvD1 group, mice were pretreated i.p. with RvD1 30 min prior to LPS administration. In RvD1 blockade group, mice were sequentially pretreated i.p. with Boc-MLP and RvD1 60 min and 30 min prior to LPS administration, respectively. The mice were monitored every 4 h for general condition and survival for 48 h and survival curve was drafted. In the second round of in vivo study, 120 BALB/c mice were randomly divided into four groups according to a computer program generated allocation number into four groups as previously described. Blood and kidneys were harvested at 2, 6, 12, 24, and 48 h, respectively, with the mice anesthetized by chloral hydrate and then sacrificed humanely (n = 6 in each group at each time point). The study was approved by the Animal Care and Use Committee of West China Hospital.

**In vitro study**

HK-2 cells were randomly divided into four groups with specific drug concentrations in culture medium, which were (1) control group (medium only), (2) LPS group (LPS 5 μg/ml), (3) RvD1 group (RvD1 10 ng/ml + LPS 5 μg/ml), and (4) blockade group (Boc-MLP 10 ng/ml + RvD1 5 μg/ml + LPS 5 μg/ml). The cells were harvested at 2, 4, 6, 12, and 24 h, respectively (n = 6 in each group at each time point). The drug dosage was determined according to literature report and our previous pilot experiments. The experiments were performed in the State Key Laboratory of Biotherapy, Sichuan University, China.

**Parameters and measurements**

**Histology**

Kidney samples were obtained at specific time points after LPS challenge. Tissues were fixed in 10% neutral-buffered formalin and subsequently embedded in paraffin. Sections
(5 μm thick) were stained with hematoxylin and eosin (HE) using a standard protocol and analyzed by optical microscopy. The kidney samples embedded with Epon 812 were observed by transmission electron microscope (TEM) (Hitachi, Tokyo, Japan) under uranyl acetate and lead citrate double staining.

**Immunohistochemistry**

Paraffin-embedded sections were stained for ALX and cleaved caspase-3, using debritylated secondary antibodies. We used 3,39-diaminobenzidine for visualization as well as hematoxylin as a counterstain. According to the Fromowitz score system, we randomly selected six high magnification fields (>400) in each kidney section. The proportions of positively stained cells out of the total cell counts were recorded. According to the percentage of positive cells, <25% was measured as 0 point, 26–50% for 1 point, 51–75% for 2 points, and >75% for 3 points. According to the intensity of staining, negative was classified as 0 point, light yellow as 1 point, while 2 points for yellow, and 3 points for brown. The sum of these scores, 0 point for negative, 1–2 for slightly positive, 3–4 points for moderately positive, and 5–6 points for strongly positive.

**TUNEL staining**

Apoptotic cell death in kidney sections was detected by using the TUNEL kit. Six randomly selected high-power fields (>400) of juxtamedullary cortex were counted to determine the number of apoptotic nuclei.

**Serum creatinine and tumor necrosis factor-α**

Serum creatinine was measured by CHEM8+ cartridge of Abbott i-STAT System (Abbott, USA). The TNF-α concentrations were calculated according to the optical density (OD) values detected by spectrophotometer after the samples were processed using ELISA kit.

**Western blotting**

Mice kidneys were homogenized in buffer supplemented with protease inhibitors. Samples with equal amounts of total protein (50 mg/ml) were processed for Western blotting as described previously[19] using antibodies against ALX, IκB, phosphorylated-IκB, and cleaved caspase-3 with diluting ratio varying from 1:200 to 1:1000.

**Quantitative real-time polymerase chain reaction**

The mRNA was extracted from mice kidney or cultured HK-2 cells, and the first-strand cDNA was prepared after mRNA was treated with DNase to eliminate potential gDNA contamination. Quantitative amplification of the cDNA was performed on a BioRad Chromo 4 Real-Time PCR System. Results were normalized to GAPDH content, and relative mRNA levels were expressed as fold change compared with the control group. The reaction system, conditions as well as primers are listed in Supplementary Material.

**Statistical analysis**

Continuous data are expressed as mean ± standard deviation (SD). Categorical data are expressed as percent (%). Multiple comparisons of means among groups were examined by one-way analysis of variance (ANOVA) test, while between-group comparisons of means were analyzed by Least Significant Difference (LSD) t-test or Student–Newman–Keuls test where appropriate. In the case of nonnormality, the data were normalized or underwent Kruskal–Wallis H-test. Survival data were compared with a Kaplan–Meier curve and log-rank test. A P < 0.05 was considered as statistically significant. We used SPSS version 19.0 (SPSS Inc., Chicago, USA) for statistical analyses.

**RESULTS**

**Resolvin D1 improves animal survival after lipopolysaccharide stimulation**

Kaplan–Meier survival analysis indicated that LPS group had the earliest death event at 12 h with the lowest 48 h survival (40%); RvD1 group had the latest onset of death event at 36 h with the highest 48 h survival (80%) while blockage group had both parameters (first death event at 28 h point, 48 h survival 60%) in between. The control group experienced no death event. The difference in survival curve of LPS group versus control group (χ² = 8.293, P = 0.005), blockage group versus control group (χ² = 4.764, P = 0.030), and LPS group versus RvD1 group (χ² = 4.039, P = 0.044) were all of statistical significance, while RvD1 group versus control group had similar survival (χ² = 2.110, P = 0.147). The difference between LPS group and blockage group is of no statistical significance (χ² = 1.364, P = 0.243) [Figure 1a].

**Resolvin D1 protects renal function and ameliorates kidney pathological injury in lipopolysaccharide-related acute kidney injury**

The blood creatinine level of LPS group (9.8 ± 1.0 mg/L) and blockage group (8.0 ± 0.9 mg/L) were significantly increased at 6 h (t = 3.838, 3.210, P = 0.001, 0.005, respectively), doubling the creatinine level in control group (2.3 ± 0.4 mg/L), which indicated successful establishment of AKI, while the 6 h time point creatinine level in RvD1 group (3.1 ± 0.5 mg/L) remained comparable to control group (t = 1.705, P = 0.070). The peak concentrations of blood creatinine (6.0 ± 1.5 mg/L) in RvD1 group were statistically lower than that of LPS group and blockage group (all P < 0.05) [Figure 1b]. HE staining of mice kidney was shown in Figure 2a-2d. Tubular epithelial vacuolation/flatness, loss of brush border/epithelials, exposure of epithelial nucleus, and dilation of tubular lumen were observed in LPS group and blockage group, while in RvD1 and control group, we recorded little pathological injury. TEM found notable mitochondria swelling, vacuolization, chromatin margination, and nuclear shrinkage in LPS group and blockage group. In RvD1 group, mitochondria swelling and vacuolization could also be noticed, but the morphology of nucleus remained intact [Figure 2e-2h].

**Resolvin D1 receptor ALX distributes on renal tubular epithelial cells**

According to immune-histology, ALX mainly distributed on tubular epithelial cells, with its amount increased under LPS stimulation and resumed by RvD1 [Figure 2i-2l]. PCR
showed the mRNA expression of ALX could be detected in both mice renal tissue and human proximal tubular epithelial cells. LPS stimulation could remarkably boost its expression, while RvD1, on the contrary, promoted resume of gene expression back to normal level. Blockage group showed a similar trend of LPS group in receptor gene expression [Figure 3a and 3b]. Western blotting also revealed an accordant change in tissue ALX protein level among different groups, with RvD1 significantly prohibited the up-regulation of kidney ALX secondary to LPS challenge [Figure 3c].

**Resolvin D1 inhibits up-regulation of nuclear factor-kappa B pathway under lipopolysaccharide stimulation**

The results of PCR indicated that after LPS stimulation, the mRNA expression of toll-like-receptor-4 (TLR4), MyD88, and TNF-α were all up-regulated. RvD1 could substantially inhibit the up-regulation of these genes, while RvD1 receptor blocker could partially reverse the inhibition of RvD1 on these molecules [Figure 4]. The Western blotting OD ratio of p-IκB/IκB in LPS group was significantly higher than the control group \((t = 3.431, P = 0.003)\), and the RvD1 group was even lower than the control group \((t = 2.355, P = 0.020)\), revealing that RvD1 could inhibit the activation of NF-κB signal induced by LPS [Figure 5a and 5b]. In the mice serum, the TNF-α level of both LPS and RvD1 blockage groups were significantly improved as compared with control group \((P < 0.05)\), but for RvD1 group, the fluctuation was less obvious than the other two groups [Figure 1c], which showed that RvD1 had a down-regulation on the cytokines downstream to NF-κB, such as TNF-α.

**Resolvin D1 regulates renal cell apoptosis in lipopolysaccharide-related acute kidney injury**

As shown in Western blotting analysis, RvD1 suppressed the up-regulation of cleaved-caspase-3 protein in LPS challenged mice, while RvD1 blockage could partially reserve the protection effect [Figure 5c and 5d]. In the histochemistry staining of mice kidney slice, cleaved-caspase-3 was barely shown in the control group, while heavily expressed in LPS group. The distribution of cleaved-caspase-3 was ameliorated in RvD1 group [Figure 6a-6d]. Quantitative analysis confirmed similar findings that RvD1 could inhibit the up-regulation of cleaved-caspase-3 stimulated by LPS, which was prohibited in RvD1 blockage group [Figure 6e]. In the TUNEL staining, apoptotic cell nuclei were observed in kidney slice of LPS and RvD1 blockage group, but we found few apoptotic cell nuclei in RvD1 group, indicating that RvD1 could inhibit the renal cell apoptosis in LPS related AKI [Figure 6f-6i]. Quantitative analysis of TUNEL staining confirmed that the proportion of apoptotic nuclei was significantly higher in LPS group \((t = 2.972, P = 0.009)\) and blockage group \((t = 2.155, P = 0.030)\) as was compared to control group, but there was no statistically significant difference between RvD1 group and control group \((t = 1.360, P = 0.110)\) [Figure 6j].

**DISCUSSION**

RvD1, one of the most studied RVs, has been reported to play a protective role in a variety of animal disease models including ALI, insulin resistance, peritonitis, wound infection, atherosclerosis as well as ischemia-reperfusion injury.[13,20,21] The role of RvD1 in septic AKI has yet to be described. In this study, we employed i.p. LPS injection to establish murine septic AKI model, so as to observe whether RvD1 could be protective to this disease and reveal its possible mechanism. We observed the protection effects of RvD1 against septic AKI by inhibition of NF-κB signal and renal cell apoptosis.

So far, two receptors of RvD1 have been identified: ALX and GPR32. ALX could also be the receptor of lipoxins, while GPR32 being an orphan. Scholars have detected ALX on surfaces of respiratory tract epithelial, vascular smooth muscle cell, and polymorphonuclear cell, and GPR32 has been found to be expressed in airway epithelial, polymorphonuclear cell, fat cell, vascular smooth muscle cell, macrophage as well as renal interstitial fibroblast.[22] However, previous researches have not described the distribution of RvD1 receptors in other kidney cells. In our research, we detected the existence of ALX on tubular epithelial cells, rather than glomerulus or renal interstitial tissue. Literature reported an up-regulation of ALX on human monocyte under zymosan or granulocyte/monocyte stimulating factor stimulation.[22] Similarly, we also observed the up-regulated ALX expression on LPS challenged renal tubular epithelials, indicating that this receptor might be involved in the feedback regulation in inflammatory response.
RvD1 has been found to down-regulate cytokine level and improve animal survival in various inflammatory disease models such as sepsis after cecal ligation/puncture and LPS related ALI. In the present experiment, we also noted that RvD1 could ameliorate LPS induced symptoms including immobility, chills, piloerection and excessive secretions, as well as improving survival. By blocking ALX with Boc-MLP, the protecting effects of RvD1 was partially reversed, reflected as the exacerbation in mice general conditions and increased mortality, indicating RvD1 impose its anti-inflammatory effects via ALX in LPS-induced AKI model, which was in consistency with previous reports. RvD1 could also preserve renal function and inhibit fibrosis in multiple kidney diseases such as obstructive nephropathy, adriamycin-induced nephropathy, and ischemia-reperfusion kidney injury. Our research confirmed the therapeutic effects of RvD1 on LPS-induced AKI, and similarly, this trend could also be partially reversed by blocking ALX. We noticed that after blocking ALX, the protective effect of RvD1 was not completely abolished, which is probably due to the existence of another unaltered RvD1 receptor GRP32 as described previously. Our study also recorded the pathological changes in mice kidney. Optical microscopy showed in the HE staining, the mice challenged by LPS presented with tubular epithelial vacuolization, flattening, loss of brush border, lumen dilation, and even loss of epithelial cells. By TEM scan in LPS group and ALX blockage group, remarkable mitochondria swelling and vacuolization were observed, along with chromatin margination and nuclear shrinkage, but RvD1-treated group suffered little nuclear involvement.

NF-κB is a classic inflammatory signal mediated by TLR4, the major LPS receptor on cellular surface, and their compound LPS-MD2-TLR4 could activate NF-κB via either MyD88 dependent or independent pathway, so as to finally increase the expression of a variety of cytokines including TNF-α. For aspirin-triggered RvD1, NF-κB was proved to be inhibited for the treatment of endotoxin-induced AKI. Accordingly, several studies also indicated a role of NF-κB inhibition as a possible mechanism of RvD1’s anti-inflammatory effects. Zhang et al. found RvD1 could down-regulate the expression of IkB-α in human vascular endothelials under LPS stimulation, while NF-κB inhibitor pyrrolidine dithiocarbamate (PDTC) imposed synergistic protection with RvD1 on LPS-induced vascular endothelial injury; extracellular regulated protein kinases 1/2 inhibitor PD98059 showed no similar synergism. Xu et al. first reported RvD1 suppressed the microglia inflammatory activation treated by LPS by inhibiting NF-κB signal. In LPS-induced AKI model, RvD1 could activate peroxisome proliferator-activated receptor-γ (PPARγ), inhibit NF-κB, and mitogen-activated protein kinase (MAPK) pathway. Under LPS stimulation, an up-regulation of NF-κB was identified in both mice kidney and HK-2 cells in our experiment. Pretreatment of RvD1 was able to inhibit the overexpression of NF-κB, while Boc-MLP, the blocker of ALX, partially reversed the inhibition, which we observed in LPS-induced AKI.

By analyzing the upstream and downstream signal pathways of NF-κB, we revealed that other signal pathway components, including TLR4, MyD88, and TNF-α, all presented similar trend of up or down regulation in different mice groups, which might imply the whole pathway from TLR4, MyD88, NF-κB to TNF-α were all inhibited by the treatment of RvD1 in LPS-induced AKI. Meanwhile, since similar results were observed in in vitro PCR tests, RvD1 is likely to play a direct role on proximal tubular epithelials, which was supported by our observation of RvD1 receptor ALX distribution on...
tubular epithelial cells. In 2013, Lee et al. reported that LPS remarkably inhibited efferocytosis of mice macrophages, while RvD1 promoted the phosphorylation of p65/p50 heterodimer into p50/p50 homodimer which has a low bio-activity, so as to inhibit the production of TNF-α. Gene knockout of p50, on the contrary, eliminated the protective effects of RvD1. We observed the expression of cleaved-caspase-3 distribution at both glomerular capillary and renal tubules, which accords literature report. The exact apoptotic pathway upstream of caspase-3 which was involved in the anti-apoptotic effects of RvD1 during septic AKI awaits more exploration.

Apoptosis has been proved to be involved in the pathophysiology of LPS-induced AKI. In our experiment, LPS could stimulate the expression of caspase-3, which is a well-defined common downstream effect molecule in a variety of apoptosis pathways, as was depicted by biochemistry staining and Western blotting of caspase-3 protein. In some AKI models such as ischemia-reperfusion kidney injury and drug-induced AKI, tubular necrosis and apoptosis mainly account for the AKI pathophysiology. Studies also suggested that apart from tubular epithelial apoptosis, glomerular endothelial apoptosis also participated during septic AKI.

We used only ALX blocker, while another receptor GRP32 was left altered, so the blockage might not be complete. Other candidate signal pathways such as PPARγ and MAPK, as reported in various models, have not yet been explored in the present experiment. Microdissection separating cortex from medulla would also be needed in our future research to provide information at tissue-specific level.

In summary, the present study observed a protective effect of RvD1 to LPS-induced AKI in preserving renal function.
function, ameliorating pathological injury, and improving mice mortality, which was achieved by the inhibition of NF-κB inflammatory signal and renal cell apoptosis. RvD1 as a promising therapeutic compound for septic AKI needs further research to shed light on its more detailed mechanism in anti-inflammatory and anti-apoptotic pathways.

Supplementary information is linked to the online version of the paper on the Chinese Medical Journal website.

Financial support and sponsorship
This research was supported by a grant of the National Natural Science Foundation of China (No. 81270818).

Conflicts of interest
There are no conflicts of interest.

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