RasGRP Exacerbates Lipopolysaccharide-Induced Acute Kidney Injury Through Regulation of ERK Activation

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**Background.** Excessive inflammatory activities are reported to be the primary cause of sepsis-induced acute kidney injury (AKI). Ras guanyl nucleotide-releasing protein (RasGRP) could prevent inflammatory response. However, its role in the regulation of inflammatory response in sepsis-associated AKI remains unclear.

**Methods.** Wild-type or RasGRP1-deficient mice were treated with lipopolysaccharide intraperitoneally in combination with D-galactosamine to establish a mouse model of sepsis-associated AKI. Serum inflammatory cytokines were measured using enzyme-linked immunosorbent assay. The messenger RNA (mRNA) levels of interleukin 6, tumor necrosis factor, nitric oxide synthase 2, and interleukin 1β were measured using quantitative reverse-transcription polymerase chain reaction. The morphological change in kidney tubule was determined by hematoxylin-and-eosin staining. The protein levels of RasGRP, extracellular signal-regulated kinases 1 and 2 (ERK1/2), and c-Jun N-terminal kinase (JNK) were determined using Western blot.

**Results.** RasGRP1 mRNA and protein levels were significantly increased in patients with sepsis-related AKI compared to those in healthy subjects. RasGRP knockout markedly reduced inflammatory cytokines induced by AKI in sepsis when compared with wild-type mice. Additionally, RasGRP deficiency inhibited the phosphorylation of ERK1/2 without altering JNK expression. In conclusion, we demonstrate that RasGRP1 plays a pivotal role in sepsis-associated AKI. Downregulation of RasGRP1 could significantly inhibit inflammatory response by inhibiting the activation of ERK1/2 and mitogen-activated protein kinase pathway, thereby reducing AKI induced by sepsis.

**Conclusions.** Our data suggest that RasGRP exacerbates lipopolysaccharide-induced acute kidney injury through regulating ERK activation, which reveals a potential therapeutic target for the treatment of sepsis-induced AKI.

**Keywords.** acute kidney injury; ERK/MAPK pathway; inflammation; RasGRP; sepsis.

Sepsis is the most common cause of acute kidney injury (AKI) and is associated with increased risks of poor prognosis [1–4]. Prolonged kidney dysfunction and limited functional renal reserve may result in impairment of ability of the kidney to repair [5]. In clinical, sepsis AKI diagnosis is mainly based on specific conditions. Azotemia and oliguria are still the key criteria for diagnosis [6]. However, AKI is a major factor of mortality in patients with sepsis [1]. The pathogenesis of septic AKI is still lacking, and development of critical care medicine is urgently needed. It has been shown that the excessive release of inflammatory mediators and maladaptive responses to inflammation are considered the primary cause of sepsis-related AKI [7]. Therefore, inhibiting inflammation could serve as an effective strategy to improve sepsis-related AKI. Systematic study of the signaling pathways involved in sepsis-induced kidney damage allows us to gain insights into the pathophysiology of sepsis-related AKI.

The mitogen-activated protein kinase (MAPK) is responsible for transducing extracellular signals into the cell. MAPK pathway transmits cell signaling through the form of a 3-level kinase cascade, thereby regulating cell proliferation and differentiation, apoptosis, inflammatory response and vascular development, and other biological functions [8]. The RAS-RAF-MAPK/ERK kinase (MEK)–extracellular signal-regulated kinase (ERK), also known as the MAPK/ERK pathway, is one of the most characterized MAPK pathways. Mechanistically, the RAS guanosine triphosphatase (RAS GTPase) is a down-stream effector of receptor tyrosine kinase (RTK) [9]. The activated RAS binds to the N-terminal domain of RAF kinase to activate RAF; then the activated RAF can further bind to and activate the downstream MEK [9]. Subsequently, ERK is activated by MEK. As a result, the activated ERK translocates into the nucleus and causes a series of physiological and biochemical reactions. Ras is a GTP-binding protein and has been an important target for cancer research so far [10, 11]. Studies have
shown that the inhibition of MEK significantly mitigates AKI [12]. However, little is known on the anti-inflammatory role of RAS in the setting of AKI, particularly sepsis. Thus, this study aims to use Ras guanyl nucleotide-releasing protein (RasGRP) knockout mice to explore its function in reducing inflammatory response in sepsis-induced AKI.

MATERIALS AND METHODS

Patient Consent Statement
Written informed consent was obtained from each participant. The design of the work was approved by the People’s Hospital of Xinjiang Uygur Autonomous Region.

Sepsis Animal Model
Female C57BL/6 wild-type and RasGRP C57BL/6 mice at 8 weeks old were purchased from Cyagen Biosciences (Suzhou, China). Mice were housed in a specific pathogen–free facility, and all animal experiments were carried out according to the protocol approved by the Institutional Animal Care and Use Committee in the People’s Hospital of Xinjiang Uygur Autonomous Region.

The mice were injected intraperitoneally with lipopolysaccharide (LPS; 0.1 μg per mouse) plus D-galactosamine (0.5 mg/g body weight). Animals were monitored every hour for 24 hours. Blood was obtained from challenged mice at various times, and the serum concentrations of tumor necrosis factor α (TNF-α), interleukin 1β (IL-1β), and interleukin 6 (IL-6) were measured by enzyme-linked immunosorbent assay (ELISA; R&D Biosystems, Minneapolis, Minnesota).

Immunoblot Assay
The samples were homogenized in radiolabeled precipitation buffer (10 mM Tris-HCl, pH 7.0, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, and 0.1% sodium dodecyl sulfate). Following centrifugation, the supernatants were saved and the protein concentrations were measured using Bradford BCA (Bio-Rad, Hercules, California). Antibodies targeting ERK (K-23, 1:5000), pERK (E-4, 1:3000), and JNK1 (C-17, 1:1000) were from Santa Cruz Biotechnology (Dallas, Texas). Antibodies targeting pJNK (Thr180/Tyr185, 1:1000) were from Cell Signaling Technology (Danvers, Massachusetts). Antibodies targeting RasGRP (ab96293, 1:1000) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (ab9485) were purchased from Abcam (Shanghai, China).

ELISA and Quantitative Reverse-Transcription Polymerase Chain Reaction
Supernatant cell cultures were collected and analyzed using ELISA (eBioScience, San Diego, California). Total RNA was extracted using TRizol reagent (Qiagen, Valencia, California). Complementary DNA synthesis was performed using RNAse H-reverse transcriptase (Invitrogen, Waltham, Massachusetts) and oligo (dT) primers. Quantitative reverse-transcription polymerase chain reaction (qRT-PCR) was performed with iQTM SYBR Green Supermix (Bio-Rad, Hercules, California) using the iCycler Sequence Detection System (Bio-Rad). The expression of target genes was quantified by a standard curve method and normalized to Actb.

The primers were as follows: IL-6: 5′-CTGATGCTGTGACACAC-3′ (forward), 5′-CAGACTCCATTGCAAC-3′ (reverse); TNF: 5′-CATCCTTCATAATCCAGTGCAAC-3′ (forward), 5′-CCAGCCTCCTCCTAC-3′ (reverse); IL-1β: 5′-TGGACCTTCCAGGAGGACA-3′ (forward), 5′-GTTCATCTCGGACCTGTAGT-3′ (reverse); Nos2: 5′-GAGACAGGGAAGTCTGAAGCA-3′ (forward), 5′-CCAGCAGTTGCTGTACCTTC-3′ (reverse); and RasGRPI: 5′-CTTCACACGCTGTGGCTTG-3′ (forward), 5′-GGACAGAGGACTTCATCGAC-3′ (reverse).

Statistical Analysis
Statistical analysis was performed using Prism software (GraphPad Prism version 6.01). Two-tailed unpaired Student t tests were performed, and data are presented as mean ± standard error of the mean. One-way analysis of variance, where applicable, was performed to determine whether an overall statistically significant change existed before Student t test to analyze the difference between any 2 groups. A P value < .05 was considered statistically significant.

RESULTS

The Protein and Messenger RNA Level of RasGRP Is Increased in Patients With Sepsis
To evaluate whether RasGRP is involved in the inflammatory response caused by sepsis, we tested the messenger RNA (mRNA) and protein levels of RasGRP in the peripheral blood mononuclear cells (PBMCs) of healthy people and patients with sepsis using qRT-PCR and Western blot. PBMCs were obtained from 37 healthy people and 37 patients with sepsis. We found that in patients with sepsis, the expression level of RasGRP was significantly increased (Figure 1A and 1B). Consistently, we found that after LPS stimulation, the level of RasGRP continued to increase over time in mouse macrophages (Figure 1C and 1D). These results suggest that RasGRP plays a key role in response to inflammation.

RasGRP Is Required for the Induction of Various Proinflammatory Cytokines in Bone Marrow–Derived Macrophages Induced by Toll-Like Receptor Stimulation
To further explore the function of RasGRP in sepsis-induced inflammation, we measured cytokines, such as IL-6, TNF-α, Nos2, and IL-1β, using a RasGRP knockout mice model of sepsis. The results showed that bone marrow–derived macrophages from RasGRP knockout mice showed lower mRNA expression of...
all inflammatory cytokines mentioned above under LPS stimulation (Figure 2A). Similarly, significantly elevated levels of cytokines were detected after cytosine-phosphate-guanosine (CpG) stimuli, which were abrogated in RasGRP-deficient mice (Figure 2B). Furthermore, we found that the protein levels of IL-6 and TNF-α were significantly lower in RasGRP-deficient compared to the control mice after LPS stimulation (Figure 2C).

RasGRP Deficiency Impaired LPS-Induced Kidney Function and Pathological Changes in Mice

To verify the regulation effect of RasGRP on inflammation and sepsis-induced kidney injury in vivo, LPS (0.1 μg per mouse) plus D-galactosamine (0.5 mg/g body weight) were used to induce a mouse model of acute sepsis. Hematoxylin-and-eosin staining showed a significant kidney injury with LPS treatment in wild-type mice, which was notably reduced in RasGRP-deficient mice (Figure 3A). Serum creatinine (SCr) and blood urea nitrogen (BUN) are widely used indicators of AKI. As shown in Figure 3B, SCr and BUN were significantly increased at 12 hours after LPS treatment, and this elevation continued through 24 hours in the wild-type mice. Remarkably, SCr and BUN levels were significantly reduced in RasGRP-deficient mice after LPS treatment when compared with wild-type mice (Figure 3B). Furthermore, we evaluated the effects of RasGRP on serum levels of inflammatory cytokines after LPS treatment using ELISA. The results showed that RasGRP knockout mice significantly inhibited LPS-induced inflammatory response. Correspondingly, the levels of inflammatory cytokines IL-6, TNF-α, and IL-1β in the serum were markedly reduced in RasGRP knockout mice compared to control animals (Figure 3C). The above results suggest that RasGRP may serve as a potential new drug target for the treatment of sepsis and renal injury.

RasGRP Positively Regulated the ERK Pathway

To determine the molecular mechanism of RasGRP regulating inflammation, we analyzed via the STRING Interaction Network and found that RasGRP molecules can bind to K-Ras protein and Ras-related protein R-Ras (RRAS) (Figure 4A). Therefore, we hypothesize that RasGRP also functions via regulation of the phosphorylation and activity of ERKs. We further assessed the mechanism of RasGRP. Western blot detection showed that RasGRP deletion significantly inhibited the activation of the ERK1/2 signaling pathway downstream of Toll-like receptor 4 (TLR4); however, the phosphorylation of other MAPK branches such as c-Jun N-terminal kinase (JNK) was not affected (Figure 4B). We used ERK inhibitors to verify our findings, and the results showed that ERK inhibitors not only inhibit the expression of proinflammatory cytokines, but also remove the phenotype caused by RasGRP deletion (Figure 4C).
DISCUSSION

Sepsis-associated AKI is a significant cause of prolonged hospitalization and increases the risk of mortality [6]. There is an urgent need to develop an effective strategy to reduce sepsis-associated AKI. In this study, we assessed the alteration of RasGRP in healthy subjects and patients with sepsis-associated AKI. Furthermore, to confirm the protective effects of RasGRP deficiency in AKI, we used a RasGRP knockout mice model of LPS-associated AKI to determine the involvement of the RAS-ERK pathway in reducing inflammatory response during AKI.

RasGRP is mainly expressed in blood cells, contributing to facilitate GTP hydrolysis and activate Ras [13–16]. In this study, the mRNA and protein levels of RasGRP1 were significantly elevated in PBMCs obtained from patients with sepsis compared with healthy subjects. It was found that RasGRP1 binds to the orphan nuclear receptor transcription factor Nurr1 and regulates the inflammatory response [17]. Moreover, upregulated RasGRP1 has been associated with less inflammation in patients with cancer [18, 19]. However, the effect of RasGPR1 on the inflammatory response in kidney remains unknown. Therefore, we examined the serum RasGRP1 level in a mice model of inflammation induced by LPS; we found that the RasGRP1 level was increased after 1 hour of LPS stimulation and continuously increased through 6 hours of LPS treatment by utilizing a mouse model of LPS-induced AKI. These findings indicate that RasGRP1 is closely related to the inflammatory response.

Inflammatory response is a primary factor causing complications during sepsis-associated AKI [20–24]. It was found that RasGRP1 could prevent an inflammatory response. For example, it was shown that suppressing RasGRP1 inhibited neuroinflammation [25]. Additionally, Baars et al showed that decreased RasGRP1 reduced active inflammatory disease [26]. However, the impact of RasGRP1 on sepsis-induced AKI is still unclear. To determine the role in regulating inflammatory response in sepsis-associated AKI, we measured mRNA and protein levels of inflammatory mediators including IL-6, TNF-α, Nos2, and IL-1β. We found that the protein levels of inflammatory cytokines were significantly increased in sepsis-associated AKI in wild-type mice but markedly inhibited in RasGRP1 knockout mice.

**Figure 2.** Ras guanyl nucleotide-releasing protein (RasGRP) is required for the induction of various proinflammatory cytokines in bone marrow–derived macrophages (BMDMs) induced by Toll-like receptor stimulation. A and B, Expression of lipopolysaccharide (LPS; 100 ng/mL)– or CpG (5 nM)–induced cytokines of wild-type and RasGRP knockout BMDMs was measured by quantitative reverse-transcription polymerase chain reaction (n = 6). C, Enzyme-linked immunosorbent assay of LPS-induced cytokines in the supernatants of wild-type and RasGRP knockout BMDMs for 24 hours (n = 6). All data are presented as fold relative to the Actb messenger RNA level. Data are presented as mean ± standard error of the mean and representative of at least 3 independent experiments. Statistical analyses represent variations in experimental biological replicates. Two-tailed Student t tests were performed. ∗P < .05; ∗∗P < .01. Abbreviations: CpG, cytosine-phosphate-guanosine; IL-1β, interleukin 1β; IL-6, interleukin 6; LPS, lipopolysaccharide; Nos2, nitric oxide synthase 2; RasGRP, Ras guanyl nucleotide-releasing protein; TNF-α, tumor necrosis factor alpha.
knockout mice. In line with the protein data, the mRNA levels of inflammatory cytokines appeared to show similar trends. These results suggest that downregulation of RasGRP1 could effectively reduce inflammation induced by sepsis.

To confirm the protective role of RasGRP1 in sepsis-induced AKI, we used a mouse model of sepsis-associated AKI by treatment with LPS in combination with D-galactosamine. First, the kidney damage was confirmed by histological staining. Morphological changes in glomerular and kidney tubule demonstrated a marked injury in wild-type mice, and less injury was observed in RasGRP1 knockout mice. Consistently, SCr and BUN, commonly used clinical biomarkers of kidney function, showed that renal injury was significantly augmented in wild-type mice. In contrast, RasGRP effectively reduced renal injury to the level of that of controls. These data imply that RasGRP1 plays a crucial role, in particular, in sepsis-associated AKI.

RasGRP plays an essential role in regulating MAPK pathways in response to inflammation. To explore the mechanisms of RasGRP in sepsis-related AKI, we carried out STRING Interaction Network analysis. We found that RasGRP could bind to KRAS and RRAS proteins, thereby regulating ERK/MAPK pathway. Notably, Western blot data showed that RasGRP deficiency significantly inhibited the activation of TLR4 and the downstream mediator ERK1/2. Interestingly, no significant change was observed in JNK phosphorylation. In line with our findings, Molineros et al reported that abnormally expressed RasGRP1 is responsible for the phosphorylation of the ERK/MAPK pathway in systemic lupus erythematosus [27]. Ko et al further suggested that RasGRP regulates the ERK1/2/MAPK pathway through activating the H-Ras isoform of Ras [28]. Overall, these findings indicate that RasGRP1 deficiency inhibits the activation of the MAPK pathway by suppressing the phosphorylation of ERK1/2 in sepsis-associated AKI.

There are limitations to this study. First, the role of other isoforms of RasGRPs in sepsis-associated AKI have not been studied. The mechanisms of other isoforms of RasGRP need to be studied in future research. Second, whether RasGRP1 deficiency affects cell apoptosis and kidney function, such as glomerular filtration rate and urinary protein, will need to be included in future studies.

In conclusion, we demonstrated that RasGRP1 plays a pivotal role in sepsis-associated AKI. Downregulation of RasGRP1 could significantly inhibit inflammatory response by inhibiting
the activation of the ERK1/2 and MAPK pathways, thereby reducing AKI induced by sepsis. Our data reveal the potentiality of targeting RasGRP to develop a novel approach for treatment of sepsis-induced AKI.

Notes

Potential conflicts of interest. All authors: No reported conflicts of interest.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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