Research Paper

iRhom2 loss alleviates renal injury in long-term PM2.5-exposed mice by suppression of inflammation and oxidative stress

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ABSTRACT

Particulate matter (PM2.5) is a risk factor for organ injury and disease progression, such as lung, brain and liver. However, its effects on renal injury and the underlying molecular mechanism have not been understood. The inactive rhomboid protein 2 (iRhom2), also known as rhomboid family member 2 (Rhibd2), is a necessary modulator for shedding of tumor necrosis factor-α (TNF-α) in immune cells, and has been explored in the pathogenesis of chronic renal diseases. In the present study, we found that compared to the wild type (iRhom2+/+) mice, iRhom2 knockout (iRhom2−/−) protected PM2.5-exposed mice from developing severe renal injury, accompanied with improved renal pathological changes and functions. iRhom2−/− mice exhibited reduced inflammatory response, as evidenced by the reduction of interleukin 1 (IL-1β), IL-6, tumor necrosis factor-α (TNF-α) and IL-18 in kidney samples, which might be, at least partly, through inactivating TNF-α converting enzyme/TNF-α receptors (TACE/TNFRs) and inhibitor of α nuclear factor κ B (IκBα/NF-κB) signaling pathways. In addition, oxidative stress was also restrained by iRhom2−/− in kidney of PM2.5-exposed mice by enhancing heme oxygenase/nuclear factor erythroid 2-related factor 2 (HO-1/Nrf-2) expressions, and reducing phosphorylated c-Jun N-terminal kinase (JNK). In vitro, blockage of HO-1 or Nrf-2 rescued the inflammatory response and oxidative stress that were reduced by iRhom2 knockout in PM2.5-incubated RAW264.7 cells. Similar results were observed in JNK activator-treated cells. Taken together, our findings indicated that iRhom2 played an essential role in regulating PM2.5-induced chronic renal damage, thus revealing a potential target for preventing chronic kidney diseases development.

1. Introduction

Exposure to fine particulate matter (PM2.5) is related to the development of cardiovascular health impacts, including elevated risk of irregular heartbeat and pulmonary embolism [1]. A new longitudinal study supplies early evidence that PM2.5 exposure is associated with reduced kidney function, and an elevated rate of kidney function decline over time [2,3]. Kidney injury molecule-1 (KIM-1), a recently discovered transmembrane protein, is undetectable in normal kidneys, but it is significantly induced in renal injury including acute kidney injury (AKI) and chronic kidney disease (CKD) [4–6]. Many studies indicate that KIM-1 is a sensitive and specific marker of kidney injury as well as a predictor of prognosis [7,8]. Blood urea nitrogen (BUN) and creatinine (Cr) are essential parameters associated with the loss of renal functions [9,10]. Proteinuria in patients with kidney injury is common [11]. Proteinuria promote the infiltration of renal macrophages, leading to the generation of proinflammatory cytokines and ROS, contributing to renal damage [12,13]. However, the relationship between PM2.5 and renal function, an independent cardiovascular risk factor, as well as the underlying molecular mechanism, is poorly understood.

PM2.5 is a significant promoter of systemic inflammation and enhances circulating levels of inflammatory cytokines [14]. After

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Mechanically, iRhom2 deficiency restrained TACE/TNFRs and 1κBα/NF-κB signaling pathways, leading to the reduction of pro-inflammatory cytokines. Further, oxidative stress was repressed in iRhom2−/− mice with PM2.5 exposure, accompanied by the enhanced anti-oxidants, while the reduced oxidants and JNK activation. Significantly, blocking the expression of anti-oxidants or activating JNK rescued inflammation and oxidative stress in iRhom2-knockdown RAW264.7 cells exposed to PM2.5. Together, our data demonstrated that iRhom2 and its regulated pathways play a critical role in modulating renal inflammation and oxidative stress induced by long-term PM2.5 exposure.

2. Materials and methods

2.1. Animals and culture

Male, 6 to 8 week old wild type mice were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). The iRhom2-knockout (iRhom2−/−) based on C57BL/6 background mice weighed 20–25 g were used in this study. They were housed in a specific pathogen-free (SPF), temperature and humidity-controlled environment (25 ± 2 °C, 50 ± 5% humidity) with a standard 12 h light/12 h dark cycle with food and water in cages. After adaptation, mice were exposed to concentrated PM2.5 (150.1 ± 2.5 μg/m3, flow rate of 65 L/min) or filtered air (Con) for 6 h/day, 5 times a week in a mobile exposure system-automatic nose and mouth type inhalation exposure system [16]. The components of PM2.5 were shown in Supplementary table 1. After PM2.5 exposure for 24 weeks, all mice were sacrificed for blood collection. The renal tissue was isolated from mice for further study. All procedures were performed in accordance the Regulations of Experimental Animal Administration issued by the Ministry of Science and Technology of the People’s Republic of China. The Institutional Animal Care and Use Committee at Chongqing Key Laboratory of Medicinal Resources in the Three Gorges Reservoir Region of Chongqing University of Education (Chongqing, China) approved the animal study protocols.
2.2. Cells and culture

Human Embryonic Kidney 293 (HEK293) cells, mouse RAW264.7 macrophages, human proximal tubule epithelial cell line (HK-2), were purchased from American Type Culture Collection (ATCC, Manassas, VA). Mouse podocytes (MPC5) were purchased from Rantai Company (Shanghai, China). Bone marrow derived macrophages (BMDM) cells were isolated according to the procedures as described previously [25,26]. All cells were incubated in DMEM or RPMI 1640 medium (Hyclone, USA) supplemented with 10% fetal bovine serum (Hyclone) [25,26]. Mouse podocytes (MPC5) were purchased from Rantai Company (Shanghai, China). Bone marrow derived macrophages (BMDM) cells purchased from American Type Culture Collection (ATCC, Manassas, VA). Mouse podocytes were purchased from Rantai Company (Shanghai, China). Bone marrow derived macrophages were isolated according to the procedures as described previously [25,26]. All cells were incubated in DMEM or RPMI 1640 medium (Hyclone, USA) supplemented with 10% fetal bovine serum (Hyclone) [25,26]. Mouse podocytes (MPC5) were purchased from Rantai Company (Shanghai, China). Bone marrow derived macrophages (BMDM) cells were isolated according to the procedures as described previously [25,26]. All cells were incubated in DMEM or RPMI 1640 medium (Hyclone, USA) supplemented with 10% fetal bovine serum (Hyclone)

2.3. Plasmids transfection

The iRhom2 plasmid and empty vector (EV), as well as NF-κB, TACE, TNFR2 and TNFR1 luciferase reporter plasmids were constructed as previously described by standard molecular biology techniques [27–29]. All constructs were confirmed by DNA sequencing. Plasmids were transfected into mouse RAW264.7 cells with a mouse macrophage nucleofector kit (Lonza) following the instructions of the manufacturer.

2.4. Real time-quantitative PCR analysis (RT-qPCR)

Total RNA was isolated using Trizol reagent (Invitrogen) in accordance with its instructions. First strand cDNA was synthesized using Reverse EasyScript One Step gDNA Removal and cDNA Synthesis SuperMix (TAKALA, Dalian, China). The RNA expression levels were determined using SYBR® Green mixture (Qiagen) reagent on ABI PRISM 7900HT detection systems (Applied Biosystems, USA). The primer sequences were provided in Supplementary table 2 and 3. GAPDH was considered as an internal control and the gene expressions were assessed using the 2-ΔΔCt method.

2.5. Western blot analysis (WB)

Total protein was extracted from renal tissue samples or cells using RIPA lysis buffer (Solarbio, Beijing, China). Then, the final liquid supernatants were harvested by centrifugation at 13,500 rpm for 30 min. Protein concentrations were calculated using Pierce™ Rapid Gold BCA Protein Assay Kit (Thermo, USA). Next, the protein samples were subjected to 10% or 12% SDS-PAGE and transferred into PVDF membranes (Millipore, USA). The membranes were blocked in 5% non-fat milk, then incubated with specific primary antibodies at 4°C overnight (Supplementary table 4). The membranes were incubated with HRP-conjugated secondary antibody. After washing, protein bands were visualized using Super ECL Detection Reagent (Yeassen Biotech Co., Ltd., Shanghai, China) and exposed to Kodak (Eastman Kodak Company, USA) X-ray film. Corresponding protein expression will be determined as grey value (ImageJ, Version 1.4.2b, National Institutes of Health, USA) and standardized to housekeeping gene (GAPDH) and expressed as a fold of control.

2.6. Biochemical measurements

Serum blood urea nitrogen (BUN) and kidney injury molecule 1 (KIM1) levels were detected using corresponding kits that were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Proteinuria was determined by evaluating the urine albumin/creatinine ratio with Albuewell M Test Kit and Creatinine Companion Kit (Exocell, Philadelphia, PA). ELISA detection for TNF-α (catalog MTA00B), TNFR1 (catalog MRT10) and TNFR2 (catalog MRT20) levels in serum were tested according to the manufacturer’s introductions. NADH oxidase (NOX), ROS in kidney, superoxide dismutase (SOD), xanthine oxidase (XO), nitric oxide synthase (NOS), malondialdehyde (MDA), hydrogen peroxide (H2O2), and total antioxidant capacity (TAC) levels were detected using corresponding kits from commercially available kits (Beyotime Institute of Biotechnology, Haimen, China) or Nanjing Jiancheng Bioengineering Institute. Total ROS generation in cell was also determined using the fluorogenic probe 2',7'-dichlorodihydrofluorescein diacetate (DCFH2-DA, KeyGEN BioTECH, nanjing, China).

2.7. Immunohistochemistry analysis (IHC)

Kidney samples from each group of mice were fixed in 10% v/v formalin/PBS, and then embedded in paraffin and sectioned at 4 μm for staining with hematoxylin and eosin (H&E) and Masson trichrome staining. Images were obtained using a microscope. All sections were detected by 3 histologists without knowledge of the treatment procedure. IHC analysis for phosphorylated JNK (ab131499, 1:200, Abcam, USA), phosphorylated NF-κB (ab86299, 1:200, Abcam) and TACE (ab2051, 1:200, Abcam) were performed as previously described [30]. In brief, renal tissue sections were incubated in 3% H2O2 to block endogenous peroxidase activity for 10 min 5% bovine serum albumin (BSA, Shanghai Boao Biotechnology Co., Ltd., Shanghai, China) was used for blocking non-specific binding for 1 h. Then, tissues were incubated with primary antibodies. After immunostaining, tissue sections were counterstained with hematoxylin.

2.8. Data analysis

Data were expressed as mean ± standard error of the mean (SEM). Statistical analyses were performed using GraphPad PRISM (version 6.0; GraphPad Software, USA) by analysis of variance with Dunnet’s least significant difference post hoc tests. A p value < 0.05 was considered significant.

3. Results

3.1. Suppression of iRhom2 negatively regulates inflammatory response in mouse macrophages RAW264.7 cells

As shown in Fig. 1A-D, PM2.5 dose- and time-dependently up-regulated iRhom2 and TACE mRNA levels in macrophages or renal cell lines, including mouse RAW264.7, BMDM, HEK-293, MPC5 and HK-2.
Fig. 3. iRhom2 deficiency alleviates PM2.5-induced renal dysfunction. (A) H&E, and Masson Trichrome staining of renal tissue sections from the indicated groups of mice. Quantification of (B) renal score and (C) collagen contents based on histological staining. (D) WB analysis of NPSH2. (E) Determination of serum BUN, proteinuria and KIM1. Data are represented as mean ± SEM (n = 8). * p < 0.05, ** p < 0.01 and *** p < 0.001 versus the iRhom2+/+/Con group. + p < 0.05, ++ p < 0.01 and +++ p < 0.001 versus the iRhom2+/+/PM2.5 group.
The in vitro results indicated the potential role of iRhom2 in regulating renal injury. Further, over-expressing iRhom2 enhanced PM2.5-induced NF-κB, TACE, TNFR2 and TNFR1, as well as the mRNA levels of pro-inflammatory cytokines (IL-1β, IL-6, TNF-α and IL-18) in mouse RAW264.7 cells (Fig. 2A and B). Inversely, iRhom2 knockdown inhibited PM2.5-induced transcription of iRhom2, TACE, TNFR2, IL-1β, IL-6, TNF-α and IL-18 in RAW264.7 cells (Fig. 2C). Similarly, iRhom2 silence reduced the expression levels of iRhom2, phosphorylated IκBα and NF-κB in PM2.5-treated cells (Fig. 2D). The findings demonstrated that iRhom2 played an essential role in PM2.5-induced inflammatory response in mouse macrophages.

3.2. iRhom2 deficiency alleviates PM2.5-induced renal injury by reducing inflammatory infiltration

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3.2. iRhom2 deficiency alleviates PM2.5-induced renal injury by reducing inflammatory infiltration

Histological analysis indicated that compared to the control group of iRhom2+/+ mice, long term PM2.5-exposure led to pathological alterations in renal tissue sections, accompanied with significant collagen accumulation. Conversely, in the iRhom2−/− mice, these histologic changes were markedly alleviated in renal tissue samples from mice after PM2.5 challenge (Fig. 3A-C). Western blot analysis demonstrated that iRhom2−/− improved the decrease of NPHS2 in kidney of PM2.5-treated mice, along with the reduction of serum BUN, proteinuria and KIM1, indicating the rescued renal functions (Fig. 3D and E). IHC staining and qPCR analysis showed the increased level of TACE in kidney of iRhom2+/+ mice exposed to long-term PM2.5, which were, however, decreased in kidney of mice lacking iRhom2 (Fig. 4A). Compared to iRhom2+/−/PM2.5 group, PM2.5-induced higher levels of TNF-α and TNFR2 in serum of mice were down-regulated by iRhom2-shortage, while no significant difference was observed in the change of TNFR1 (Fig. 4B). Consistently, iRhom2+/−/PM2.5 mice exhibited lower mRNA levels of IL-1β, IL-6, TNF-α and IL-18 in kidney than that of iRhom2+/+/PM2.5 mice (Fig. 4C). We observed a significant reduction of phosphorylated NF-κB and IκBα in renal tissue samples of PM2.5-treated mice with iRhom2 deficiency, which was comparable to the iRhom2+/−/PM2.5 group (Fig. 4D and E). Finally, PM2.5 exposure markedly elevated iRhom2 mRNA expressions in kidney of iRhom2+/+ mice (Fig. 4F).

3.3. iRhom2 inhibition reduces oxidative stress and JNK activation in PM2.5-induced renal injury in vitro and in vivo

Exposure of PM2.5 led to oxidative stress in mouse RAW264.7 cells, evidenced by the up-regulated ROS production, H2O2, MDA, iNOS, and XO levels, while the down-regulated SOD and TAC contents; however, the process was abolished by the knockdown of iRhom2 (Fig. 5A and B). WB analysis showed a significant reduction of HO-1 and Nrf-2 in RAW264.7 cells exposed to PM2.5, and a remarkable elevation of the two proteins was observed in iRhom2-knockdown cells. Conversely, iRhom2-silence markedly decreased Keap-1, XO and phosphorylated JNK expressions in PM2.5-stimulated cells (Fig. 5C-E). In vivo, we also found that long-term exposure of PM2.5 caused an obvious promotion of total ROS, MDA, iNOS, and XO levels in renal tissue samples, whereas being reduced by iRhom2-deficiency. SOD activity, however, exhibited an inversed trend (Fig. 6A and B). Moreover, PM2.5-induced decrease of HO-1 and Nrf-2 was restored by iRhom2-knockout. In contrast, over-expression of Keap-1, XO and phosphorylated JNK induced by PM2.5 was markedly decreased in iRhom2−/− mice (Fig. 6C-E).
3.4. PM2.5-induced renal injury via iRhom2-regulated oxidative stress and inflammation

The findings above illustrated that iRhom2 could regulate PM2.5-induced renal injury via mediating inflammation and oxidative stress. Here, HO-1 was markedly inhibited by using its suppressor of SnPP. Nrf-2 expression was successfully knock down by Nrf-2 siRNA (Fig. 7A and B). Also, JNK phosphorylation was elevated by ANI, a JNK activator (Fig. 7C). We found that reducing HO-1, Nrf-2 or promoting JNK activation markedly recovered TACE, TNFR2 and TNFR1 expressions in PM2.5-exposed RAW264.7 cells with iRhom2 knockdown (Fig. 7D). Consistently, the mRNA levels of pro-inflammatory cytokines reduced by iRhom2 suppression in PM2.5-treated cells were significantly regained by restraining HO-1 or Nrf-2 expression, or by activating JNK (Fig. 7E). Finally, we also observed that iRhom2 inhibition-triggered decrease of oxidative stress was also rescued by the treatment of SnPP, Nrf-2 siRNA or ANI in RAW264.7 cells challenged with PM2.5 (Fig. 7F). Collectively, the findings above indicated that iRhom2 inhibition-ameliorated renal injury could be abrogated by ROS production.

4. Discussion

In the present study, long-term exposure of PM2.5 led to kidney damage in murine animals. We established a pivotal role of iRhom2, an essential regulator of TACE, in the pathogenesis of renal injury. We found that PM2.5-exposed mice lacking iRhom2 were protected from progressive kidney damage induced by PM2.5, as evidenced by the reduced pathological alterations and the rescued NPHS2 expressions in renal tissue sections. NPHS2 plays a critical role in the regulation of glomerular permeability and functions probably as a linker between the plasma membrane and the cytoskeleton [31]. The attenuated processes were also accompanied with reduced BUN, proteinuria and KIM1 levels. KIM1 is a biomarker of acute and chronic kidney injury and renal cell carcinoma [4-8,32]. Further, iRhom2-knockout resulted in the blockage of TACE/TNFRs and IκBα/NF-κB signaling pathways, which led to the suppression of pro-inflammatory cytokines. Moreover, iRhom2−/−/PM2.5 mice showed attenuated oxidative stress, associated with the increase of HO-1/Nrf-2 and decrease of JNK activation. Notably, suppressing HO-1, Nrf-2 or enhancing JNK activity restored PM2.5-triggered inflammatory response and oxidative stress in mouse RAW264.7 cells with iRhom2 knockdown. Therefore, we for the first time supplied the proof that iRhom2 could be a promising target to prevent renal function or injury.

Exposure to PM2.5 could lead to chronic systematic inflammation [3,14-17]. It has been wildly demonstrated that PM2.5 is associated with inflammatory cytokines whereby it stimulates the over-expression of various transcription factor genes and inflammation-related cytokine genes, contributing to inflammatory injury [33-35]. Recently, we reported that long-term exposure to PM2.5 resulted in severe inflammation in brain and liver, along with over-release of pro-inflammatory...
cytokines, such as IL-1β, IL-6, and TNF-α [16,17]. TACE is an important enzyme responsible for TNF-α release and is needed for the cleavage of other ligands [36]. TACE could cleave TNFR to dissociate the TNFR-binding complex and terminates the TNFR-regulated signal transduction [23]. As reported before, ectodomain shedding of TNFR by TACE leads to termination of cellular response to TNF-α [37,38]. Consistently, in the present study, PM2.5 exposure caused circulating and renal inflammation, as evidenced by the enhanced expression of TNF-α, TNFR2, IL-1β, IL-6 and IL-18 in serum or renal tissue samples. Also, activated IκBα/NF-κB signaling pathway was observed in kidney of mice with PM2.5-exposure. Accordingly, blockade of iRhom2 has the advantage of specifically suppressing production of soluble TNF-α and thus primarily targeting the pro-inflammatory pathway [39]. Here, we found that iRhom2−/− attenuated renal inflammatory response, which was, at least partly, through the suppression of TACE/TNF-α and IκBα/NF-κB signaling pathways, consequently leading to the reduction of the secretion of pro-inflammatory cytokines. Similar results were observed in mouse RAW264.7 cells incubated with PM2.5 that knockdown of iRhom2 restrained inflammatory response via inactivating TACE/TNF-α and IκBα/NF-κB pathways. Therefore, we hypothesized iRhom2−/−-alleviated kidney damage was attributed to the inhibition of inflammation, which was in line with our previous study.

Earlier studies indicated that the free radicals, metal and the organic components of PM2.5 could induce free radical production to oxidize lung cells, which may be the primary cause of body injury [40,41]. Oxidative stress is definitely caused by an imbalance between the production of various ROS and the antioxidant defense. PM2.5 could cause excessive production of free radicals or ROS and reduce the antioxidant capacity of cells, contributing to the peroxidation of lipids on the cell membrane and the enhancement of intracellular Ca2+ concentrations, which result in inflammation or cell damage [42,43]. Oxidative stress is the result of an imbalance in the pro-oxidant/antioxidant homeostasis [44]. Recently, our previous study has indicated that PM2.5-induced CNS injury was partly attributed to oxidative stress, associated with the increase of oxidants and the decrease of anti-oxidants. SOD1, HO-1, and Nrf-2 reduction was involved in PM2.5-triggered oxidative stress in CNS [16]. Here, both in vitro and in vivo studies demonstrated that PM2.5 exposure caused ROS generation, along with the reduction of HO-1/Nrf-2. JNK could be activated by oxidative stress, which modulates cellular functions. JNK MAPK has been indicated to be of great importance in regulating oxidative stress [45–47]. iRhom2 was suggested to regulate MAPKs (ERK1/2) in kidney damage [48–50]. Here, JNK was markedly activated by PM2.5. Intriguingly, iRhom2 suppression reduced JNK phosphorylation, whereas enhanced HO-1/Nrf-2 expressions in PM2.5-treated animals or cells. Importantly, the in vitro results indicated that blocking HO-1 or Nrf-2 expression, or promoting JNK
activation apparently rescued inflammation and oxidative stress in PM2.5-incubated cells lacking of iRhom2 expressions. Therefore, we supposed that iRhom2 deficiency-attenuated chronic renal injury was largely attributed to the repression of oxidative stress.

Collectively, for the first time we provided evidence that iRhom2 was crucial for the progression of renal injury induced by PM2.5 exposure, which was mostly likely through activating TACE/TNFRs and IκB/NF-kB signaling pathways. Moreover, iRhom2 blocked HO-1/Nrf-2 pathway, and activated JNK MAPK to promote oxidative stress in renal tissues of mice or cells with PM2.5 exposure (Fig. 8). Therefore, blockage of iRhom2 supplied a potential therapeutic approach to chronic renal injury. However, further study is still necessary in future to comprehensively explore the effects of iRhom2 on kidney injury, including its influence on fibrosis formation.

Fig. 7. PM2.5-induced renal injury via iRhom2-regulated oxidative stress and inflammation. (A) WB analysis of HO-1 in mouse RAW264.7 cells after 5 μM SnPP pre-treatment for 3 h. (B) WB analysis of Nrf-2 in mouse RAW264.7 cells after transfected with NC or Nrf-2 siRNA for 24 h. (C) WB analysis of phosphorylated JNK in mouse RAW264.7 cells after 5 μM ANI pre-treatment for 3 h. (D) Relative TACE mRNA abundance, which was most likely through activating TACE/TNFRs and IκB/NF-kB signaling pathways. Moreover, iRhom2 blocked HO-1/Nrf-2 pathway, and activated JNK MAPK to promote oxidative stress in renal tissues of mice or cells with PM2.5 exposure (Fig. 8). Therefore, blockage of iRhom2 supplied a potential therapeutic approach to chronic renal injury. However, further study is still necessary in future to comprehensively explore the effects of iRhom2 on kidney injury, including its influence on fibrosis formation.

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Fig. 8. Model showing the role of iRhom2 as a positive regulator of PM2.5-induced renal injury. iRhom2 played an essential role in regulating the progression of renal injury in PM2.5-exposed mice, most likely through activating TACE/TNFRs and IkBα/NF-κB signaling pathways to promote inflammation. In addition, iRhom2 inactivated HO-1/Nrf-2 pathway, whereas activated JNK expression to enhance oxidative stress, thus exacerbating renal injury.

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.redox.2018.08.009.

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