TRPM2 Plays a Minor Role in AKI and Kidney Fibrosis

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Key Points
- TRPM2 is a Ca\(^{2+}\)-permeable cationic channel and serves as an oxidative stress sensor.
- TRPM2 deletion was harmful in renal ischemia-reperfusion injury, whereas TRPM2 deletion mitigated kidney fibrosis.
- Our findings suggest the role of TRPM2 in kidney diseases is context dependent.

Introduction
TRPM2 is a Ca\(^{2+}\)-permeable, nonselective cationic channel and is widely expressed in various tissues and cells. TRPM2 activation allows the influx of cations (Ca\(^{2+}\), Na\(^{+}\), and K\(^{+}\)) into cells and TRPM2 plays essential roles in the susceptibility to oxidative stress (1). Reactive oxygen species (ROS) including H\(_2\)O\(_2\) activate TRPM2 (2). Due to its high sensitivity to ROS, TRPM2 can serve as an oxidative stress sensor. Oxidative stress plays an important role in both acute and CKD. Ischemia-reperfusion injury (IRI) is characterized by tissue damage mediated by ROS generation (3). Renal IRI is one of the most common causes of AKI. CKD is characterized by irreversible interstitial fibrosis, and oxidative stress is also implicated in the development of renal fibrosis (4,5). Considering these findings, TRPM2 is a promising therapeutic target for kidney diseases. In this study, we investigated the role of TRPM2 in AKI and kidney fibrosis by using TRPM2-knockout (KO) mice subjected to bilateral IRI or unilateral ureteral obstruction (UUO).

Materials and Methods
Animal Studies
Male wild-type (WT) C57BL/6J mice purchased from CLEA Japan (Tokyo, Japan) and TRPM2-KO mice with the C57BL/6J background, which were gifted from Dr. Y. Mori (1) at the age of 7–8 weeks, were allocated to different experimental groups in a randomized manner. All animal experiments were approved by the ethics committee of the Graduate School of Medicine, The University of Tokyo, and performed in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and the guidelines established by the Committee on the Ethical Animal Care and Use at the University of Tokyo. In the bilateral IRI, mice were anesthetized by intraperitoneal administration of the mixture including medetomidine, midazolam, and butorphanol. Both kidney pedicles were clamped for the indicated duration (20, 25, or 30 minutes). Body temperature was maintained at 37°C during the procedure. Mice were euthanized 1 day after IRI. In the UUO model, the left ureter was ligated at two points and cut between them. Mice were sacrificed 7 days after the procedure.

Histology and Immunohistochemistry
Formalin-fixed and paraffin-embedded sections of the kidney were stained with Periodic Acid–Schiff and Sirius Red. Kidney tissues were also stained by immunohistochemistry with rabbit polyclonal anti–kidney injury molecule 1 (Kim-1) antibody (#NBP1-76701) and rabbit polyclonal anti–alpha smooth muscle actin (αSMA) antibody (#ab5694). Quantification of Sirius Red staining and Kim-1 and αSMA immunostaining was performed using ImageJ. Tubular injuries were blindly graded semiquantitatively (0–4) on cortical fields of Periodic Acid–Schiff stained biopsies (6).

Quantitative Real-time PCR
Total RNA of the kidney was isolated and reverse-transcribed. Complementary DNA was subjected to quantitative real-time PCR (qPCR). Data were analyzed using the ΔΔCt method. Relative expression values were normalized by Rpl32 levels.

ELISA
IL-1β and CCL2 levels in kidney homogenates were measured by ELISA using commercially available kits (#MLB00C and #MJE00B) according to the manufacturer’s instructions.

Statistics
GraphPad Prism version 8.4.3 software was used to analyze the data. The results were described by the mean and standard error. Continuous variables were tested using the unpaired, two-sided \(t\) test to compare the two groups. Multiple groups were compared by one-way ANOVA with a post hoc Tukey’s test. Ordinal

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Figure 1. TRPM2 deletion has no protective effect on renal ischemia-reperfusion injury (IRI). (A) Renal mRNA expression of Trpm2 normalized to Rpl32 expression was analyzed by quantitative PCR (qPCR). (B and C) Plasma creatinine and BUN levels 1 day after bilateral IRI. (D and E) Renal mRNA expression of Kim1 and Ccl2 normalized to Rpl32 expression were analyzed by qPCR. (F) Periodic Acid–Schiff (PAS) staining of renal tissue sections of knockout (KO) and wild-type (WT) mice 1 day after bilateral IRI. Representative images of WT or KO mice in different ischemia time groups. Scale bars: 100 μm. (G) Quantification of tubular injury score on a scale of 0–5 for WT or KO mice. (H) Kidney injury molecule 1 (Kim-1) staining of kidney tissue sections. Each %area was calculated. Scale bars: 100 μm. *P<0.05; **P<0.01.
variables were analyzed by Kruskal–Wallis test with a post hoc Dunn’s multiple comparisons test. Statistical significance was set at P<0.05.

Results
To examine the effects of TRPM2 on acute ischemic kidney disease, we first created a bilateral IRI mice model with different ischemia times (20, 25, or 30 minutes). TRPM2 deletion was confirmed by qPCR (Figure 1A). There were no significant differences in renal function between WT and KO mice in the 20- and 25-minute groups, whereas in the 30-minute group the plasma creatinine level was significantly increased in KO mice (Figure 1, B and C). As for renal mRNA levels of kidney injury and inflammatory markers, Kim1 in the 25- and 30-minute groups and Ccl2 in the 20- and 25-minute groups were significantly higher in KO mice (Figure 1, D and E). In the histologic analysis, the kidney tubular injury score was similar between WT and KO mice in all ischemia duration groups (Figure 1, F and G). Kim-1 immunohistochemistry revealed no significant difference between WT and KO mice (Figure 1H).

To investigate the role of TRPM2 in kidney fibrosis, we subjected mice to UUO, a representative model of renal fibrosis. There were no significant differences in renal function between WT and KO mice (Figure 2, A and B). The expression levels of fibrosis-related genes in the kidney were evaluated by qPCR (Figure 2, C–H). Tgfβ and Ctgf were significantly reduced in KO mice, and Colα1 and Col3α1 were also decreased in KO mice, although not significant. In the histologic analysis, Sirius Red staining revealed attenuated fibrosis in KO mice (Figure 2O), whereas there was no significant difference in αSMA staining (Figure 2P). We also evaluated inflammation-related gene expression (Figure 2, I–L). There was no significant difference except for Il1β: Il6 was decreased in KO mice, although not significant. We also measured IL-1β and CCL2 protein levels in the kidney by ELISA, and there was no significant difference (Figure 2, M and N).

Discussion
In this study, we explored the role of TRPM2 in the pathophysiology of AKI and kidney fibrosis. In contrast to our speculation of a protective effect of TRPM2 deletion in the IRI model, TRPM2 KO was harmful as demonstrated by an increase in plasma creatinine and increased mRNA expression levels of Kim1 and Ccl2 in some ischemia time groups. In the report by Gao and et al. (7), genetic and pharmacological TRPM2 inhibition confers renoprotection in the bilateral IRI model. This study also showed that TRPM2 in kidney parenchymal cells interacted with the small GTPase Rac1 to promote ROS generation. Although IRI procedures used in our study and Gao’s study are largely the same, the differences are anesthetics (mixed anesthetics vs pentobarbital) and ischemia time (20, 25, or 30 minutes vs 28 minutes). Regarding ischemia time, we experimented with three different times and confirmed that 25 and 30 minutes of ischemia induced the same extent of kidney damage as in Gao’s report in terms of plasma creatinine concentration. TRPM2 KO mice used in our study were generated by deletion of the exon encoding the transmembrane segment 5 and the linker between segments 5 and 6, which are necessary for creating Ca2+ pore of TRPM2 (8,9). The previous study confirmed that Ca2+ influx in response to H2O2 was nearly lost in monocytes from this TRPM2 KO mouse (8). TRPM2 KO mice used in Gao’s study had also the same background as ours, with the deletion of exons encoding transmembrane 5 and 6, and cardiac myocytes from the KO mice exhibited decreased Ca2+ influx on H2O2 exposure (7), suggesting no apparent functional difference between these TRPM2 KO mice. In the UUO model, we observed partial attenuation of fibrosis in KO mice as shown by decreased Sirius Red–positive area and decreased Tgfb and Ctgf expression. Wang et al. demonstrated that TRPM2 KO mice were resistant against kidney fibrosis after UUO, and TRPM2 deficiency also alleviates UUO-induced inflammation (10). This study showed that TRPM2 promoted TGF-β1-induced JNK phosphorylation and subsequent NF-κB, leading to fibrosis and inflammation. In contrast to the findings from Wang et al. in which TRPM2 deletion significantly alleviates kidney dysfunction and inflammation, we failed to demonstrate these protective effects.

Although the reason why our results contradicted the prior studies is unknown, recent studies suggest that TRPM2 has a Janus-faced role in pathologic conditions. To date, there has been accumulating evidence supporting the harmful effects of TRPM2 on oxidative stress–induced injury in various organs, such as the brain (11), heart (12), and kidney (7,13). However, some recent reports challenge this concept and conversely suggest the protective role of TRPM2 in pathologic conditions (9,14). Di et al. showed that phagocyte TRPM2 inhibited the activity of NADPH oxidase and ROS production by induction of plasma membrane depolarization, resulting in reduced lung inflammation after LPS administration (14). As for cardiac IRI, Miller and colleagues have presented a series of findings (9,15) suggesting the beneficial role of TRPM2 in cardiac ischemic injury. They showed that TRPM2 KO deteriorated cardiac contractility after IRI and that TRPM2-mediated Ca2+ influx reduced ROS by inducing expression of HIF-1α, forkhead box Os (FoxO1 and FoxO3a), and their downstream superoxide dismutases (SOD1 and SOD2) (9). Moreover, in supplemental findings from Gao’s study (7), TRPM2 KO does not afford protective effects in cisplatin-induced AKI, and rather seems to be harmful considering increased BUN and creatinine levels in the KO group. Taken together, the role of TRPM2 in pathologic conditions is not consistent and can be either protective or detrimental depending on the setting. There are several limitations of this study. First, we only evaluated UUO-induced kidney fibrosis. Although the UUO model is a well-established fibrosis model, other fibrosis models need to be investigated. Second, we used global KO mice instead of conditional KO mice, which enable spatial and temporal control of a target gene. Therefore, this study left the possibility that compensatory responses might affect the phenotype.

In conclusion, although our results showed the protective effects of TRPM2 deletion in kidney fibrosis to some extent, we failed to demonstrate the favorable effects of TRPM2 KO in kidney IRI. The conventional concept that TRPM2 plays a harmful role in pathologic conditions is not
Figure 2. | TRPM2 deletion partially mitigated fibrosis in unilateral ureteral obstruction (UUO). (A and B) Plasma creatinine levels (A) and BUN levels (B) 7 days after UUO. (C–L) Renal mRNA levels of Acta2, Fn1, Col1a1, Col3a1, Tgfb1, Ctgf, Il6, Tnfa, Ccl2, and Il1b were measured by qPCR, which were normalized to Rpl32 expression. (M and N) CCL2 and IL-1β protein levels in the kidney were measured by ELISA. (O and P) Sirius Red staining and alpha smooth muscle actin (αSMA) staining of kidney tissue sections, and each %area was calculated. Scale bars: 100 μm. *P<0.05.
robust, and the role of TRPM2 seems to be context dependent.

Disclosures
H. Cernebeck and F. Eitner report being employed by Bayer. M. Nangaku reports receiving research funding from Astellas, Bayer, Chugai, Daiichi-Sankyo, JT, Kyowa-Kirin, Mitsubishi Tanabe, and Torii; reports receiving honoraria from Astellas, AstraZeneca, BI, Chugai, Daiichi-Sankyo, GSK, JT, Kyowa-Kirin, and Mitsubishi Tanabe; and reports being a scientific advisor or member of Akebia Kyowa-Kirin, Astellas, Bayer, BI, Daiichi-Sankyo, GSK, JT, and Mitsubishi Tanabe. T. Tanaka reports having consultancy agreements with AstraZeneca and Torii; reports receiving research funding from Chugai, Daiichi-Sankyo, and Kyowa-Kirin; and reports receiving honoraria from Astellas, AstraZeneca, Bayer, Kyowa-Kirin, Mitsubishi Tanabe, and Torii. The remaining author has nothing to disclose.

Funding
This work was supported by a Bayer Pharmaceuticals Research and Development grant.

Acknowledgments
We thank Dr. Y. Mori for providing TRPM2 KO mice.

Author Contributions
H. Cernebeck, F. Eitner, Y. Kurata, M. Nangaku, and T. Tanaka designed the study; Y. Kurata performed experiments and wrote the original draft; M. Nangaku and T. Tanaka provided supervision.

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Received: August 24, 2021 Accepted: October 22, 2021