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Key Points:
*TRPM2 is a Ca2+ 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 that the role of TRPM2 in kidney diseases is context-dependent.

Abstract:

Disclosures: H. Cernecka and F. Eitner are employed by Bayer AG. T. Tanaka reports the following: Consultancy Agreements: AstraZeneca, Torii; Research Funding: Chugai, Daiichi-Sankyo, Kyowa-Kirin; Honoraria: Astellas, AstraZeneca, Bayer, Kyowa-Kirin, Mitsubishi-Tanabe, Torii. M. Nangaku reports the following: Research Funding: Kyowa-Kirin, Daiichi-Sankyo, Astellas, Mitsubishi-Tanabe, JT, Chugai, Torii, Takeda, Bayer; Honoraria: Kyowa-Kirin, Astellas, Astra Zeneca, GSK, Daiichi-Sankyo, Tanabe-Mitsubishi, Chugai, JT, BI; Scientific Advisor or Membership: Kyowa-Kirin, Astellas, JT, Mitsubishi-Tanabe, Daiichi-Sankyo, BI, GSK, Bayer, Akebia. The remaining author has nothing to disclose.

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TRPM2 plays a minor role in acute kidney injury and kidney fibrosis

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Key Points

- TRPM2 is a Ca2+ 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 that the role of TRPM2 in kidney diseases is context-dependent.

Introduction

TRPM2 is a Ca^{2+}-permeable non-selective 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_2O_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 chronic kidney diseases. Ischemia-reperfusion injury (IRI) is characterized by tissue damage mediated by ROS generation (3). Renal IRI is one of the most common causes of acute kidney injury (AKI). Chronic kidney disease 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 renal 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 min). Body temperature was maintained at temperature 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 (IHC)

Formalin-fixed and paraffin-embedded sections of the kidney were stained with Periodic Acid Schiff (PAS), and Sirius Red. Kidney tissues were also stained by IHC 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 semi-quantitatively (0-4) on cortical fields of PAS-stained biopsies (6).

Quantitative real-time PCR (qPCR)

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.

Enzyme-linked immunosorbent assay (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 Student t-test to compare the two groups. Multiple groups were compared by one-way analyses of variance (ANOVA) with a post hoc Tukey's test. Ordinal variables were analyzed by Kruskal-Wallis test with a post hoc Dunn's multiple comparisons test. Statistical significance was set at the level of $P < 0.05$.

Results

To examine the effects of TRPM on acute ischemic kidney disease, we first created a bilateral IRI mice model with different ischemia times (20, 25, or 30 min). TRPM2 deletion was confirmed by qPCR (Fig. 1a). There were no significant differences in renal function between WT and KO mice in the 20 and 25 min groups, whereas in the 30 min group the plasma creatinine level was significantly increased in KO mice (Fig. 1b and c). As for renal mRNA levels of kidney injury and inflammatory markers, *Kim1* in the 25 and 30 min groups and *Ccl2* in the 20 and 25 min groups were significantly higher in KO mice (Fig. 1d and e). In the histological analysis, the kidney tubular injury score was similar between WT and KO mice in all ischemia duration groups (Fig. 1f and g). Kim-1 IHC revealed no significant difference between WT and KO mice (Fig. 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 (Fig. 2a and b). The expression levels of fibrosis-related genes in the kidney were evaluated by qPCR (Fig. 2c-h). *Tgfb* and *Ctgf* were significantly reduced in KO mice, and *Cola1* and *Col3a1* were also decreased in KO mice, although not significant. In the histological analysis, Sirius red staining revealed attenuated fibrosis in KO mice (Fig. 2o), whereas there was no significant difference in αSMA staining (Fig. 2p). We also evaluated inflammation-related gene expression (Fig. 2i-l). There was no significant difference except for *Il6*: *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 (Fig. 2m 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 v.s. pentobarbital) and ischemia time (20, 25, or 30 min v.s. 28 min). Regarding ischemia time, we experimented with 3 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 Ca^{2+} pore of TRPM2 (8, 9). The previous study confirmed that Ca^{2+} influx in response to H_2O_2 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 Ca^{2+} influx upon H_2O_2 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

following 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 pathological 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 pathological 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 Ca^{2+} 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 doesn't 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 pathological conditions is not consistent and can be

either protective or detrimental depending on the setting. There are several limitations of the present 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, the present 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 robust and the role of TRPM2 seems to be context-dependent.

Disclosures

H. Cernecka and F. Eitner are employed by Bayer AG. T. Tanaka reports the following: Consultancy Agreements: AstraZeneca, Torii; Research Funding: Chugai, Daiichi-Sankyo, Kyowa-Kirin; Honoraria: Astellas, AstraZeneca, Bayer, Kyowa-Kirin, Mitsubishi-Tanabe, Torii. M. Nangaku reports the following: Research Funding: Kyowa-Kirin, Daiichi-Sankyo, Astellas, Mitsubishi-Tanabe, JT, Chugai, Torii, Takeda, Bayer; Honoraria: Kyowa-Kirin, Astellas, Astra Zeneca, GSK, Daiichi-Sankyo, Tanabe-Mitsubishi, Chugai, JT, BI; Scientific Advisor or Membership: Kyowa-Kirin, Astellas, JT, Mitsubishi-Tanabe, Daiichi-Sankyo, BI, GSK, Bayer, Akebia. The remaining author has nothing to disclose.

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Author Contributions

Y. Kurata, T. Tanaka, H. Cernecka, F. Eitner, and M. Nangaku designed the study. Y. Kurata performed experiments and wrote the original draft. T. Tanaka and M. Nangaku supervised Y. Kurata. All authors reviewed and approved the manuscript.

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Figure legends

Figure 1. TRPM2 deletion has no protective effect on renal IRI

(a) Renal mRNA expression of *Trpm2* normalized to *Rpl32* expression was analyzed by 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) PAS staining of renal tissue sections of KO and WT mice 1 day after bilateral IRI. Representative images of WT or KO mice in different ischemia time groups. Scale bars: 100µm.

(g) Quantification of tubular injury score on a scale of 0-5 for WT or KO mice.

(h) Kim-1 staining of kidney tissue sections. Each %area was calculated. Scale bars: 100µm.

*P<0.05; **P<0.01.

Figure 2. TRPM2 deletion partially mitigated fibrosis in UUO

(a and b) Plasma creatinine levels (a) and BUN levels (b) 7 days after UUO.

(c-l) Renal mRNA levels of *Acta2*, *Fn1*, *Col1α1*, *Col3α1*, *Tgfb1*, *Ctgf*, *Il6*, *Tnfa*,*Ccl2*, *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 αSMA staining of kidney tissue sections, and each %area was calculated. Scale bars: 100µm.

*P<0.05.

Figure 1

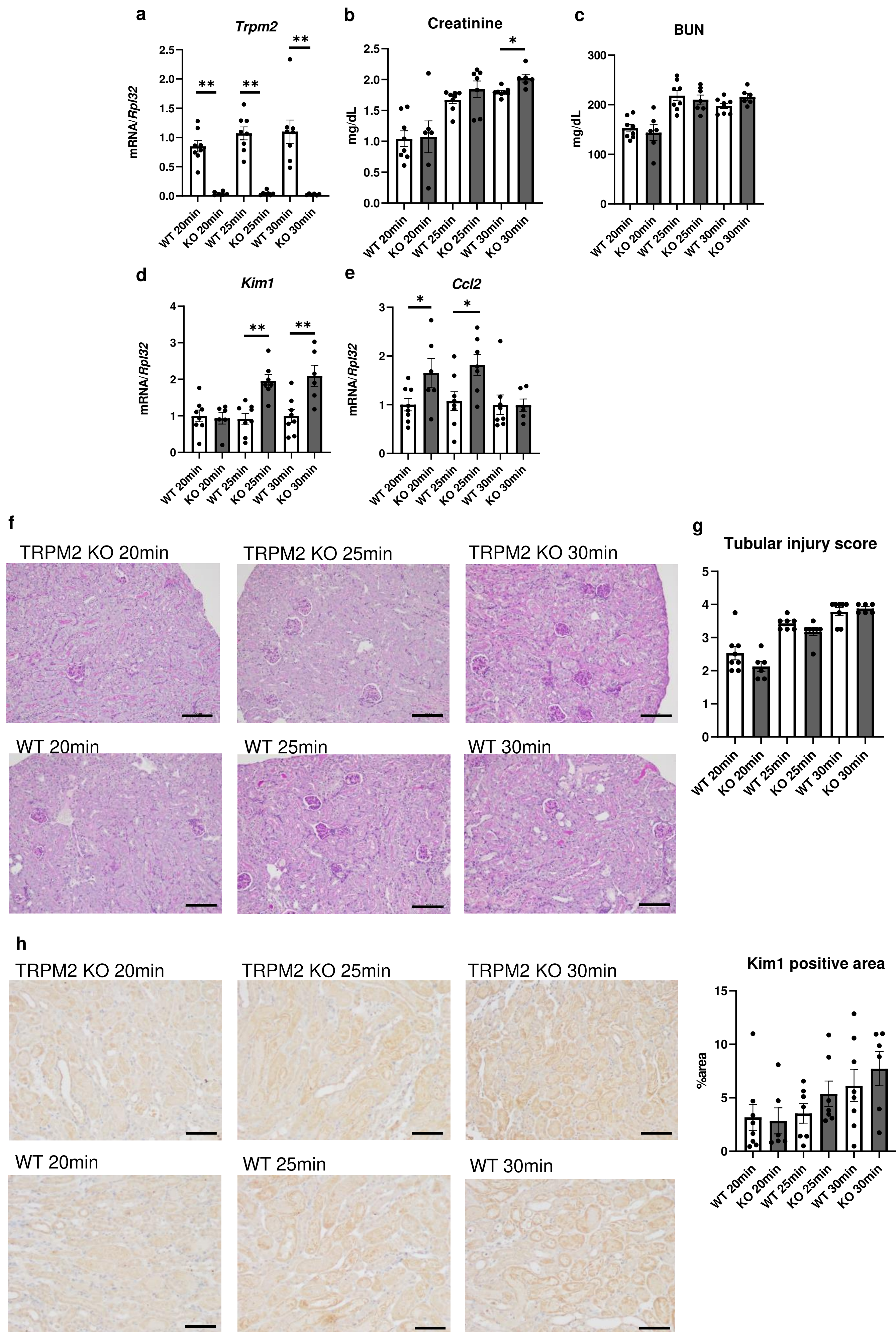

Figure 2