SUPPORTING INFORMATION

Appendix S1 | Supporting information on materials and methods

Animals

Male non-diabetic C57BL/6J (BL6) and diabetic KK.Cg-*A*^{y}/TaJcl (KK-Ay) mice (4 weeks old; CLEA Japan, Tokyo, Japan) were randomly assigned to one of the five groups (*n* = 12, respectively): 1) BL6 mice treated with saline (BL6); 2) BL6 mice treated with a moderate-dose of suramin (BL6(S)); 3) KK-Ay mice treated with saline (KK-Ay); 4) KK-Ay mice treated with a low-dose of suramin (KK-Ay(LS)); and 5) KK-Ay mice treated with a moderate-dose of suramin (KK-Ay(S)). Low (0.01 mg/kg) and moderate (1 mg/kg) doses of suramin (Sigma-Aldrich, St Louis, MO, USA) were injected i.p., once every 2 weeks for a period of 8 weeks. KK-Ay mice, model mice of type 2 diabetes, are produced by transferring the yellow obese gene (*Ay* alle) into KK mice [1]; however, KK mice also exhibit insulin resistance, obesity, albuminuria and accumulation of mesangial matrix [1, 2]. Therefore, we used BL6 mice as controls for safety assessment of suramin because BL6 mice are widely used as control mice for KK-Ay mice in recent studies [1, 3-5]. Metabolic parameters were measured and creatinine clearance (mL min^{-1} kg^{-1}) was calculated as described previously [6]. All mice were killed at 12 weeks of age and their kidneys were harvested. Glomeruli were isolated using a previously reported technique [6]. All animal studies were approved by the Animal Care and

Use Committee of Okayama University, and all procedures were performed according to the Policy on the Care and Use of the Laboratory Animals at Okayama University.

Quantification of glomerular size and mesangial matrix area

Periodic acid-Schiff (PAS)-stained sections were analyzed after modifications to the procedure described previously [7]. To evaluate glomerular size and mesangial matrix area, 20 randomly selected glomeruli from each mouse were analyzed in a blinded manner using Lumina Vision (MITANI CORPORATION, Fukui, Japan).

Immunoperoxidase staining

Immunoperoxidase staining was performed following modification to a protocol described previously [6, 8]. Primary antibodies used: F4/80 rat antibody (ab6640, 1:100; Abcam, Cambridge, UK) and caspase-1 (p10) mouse antibody (AG-20B 0044, 1:100; AdipoGen Life Sciences, San Diego, CA, USA). A mouse on mouse (M.O.M.) immunodetection kit (Vector Laboratories, Burlingame, CA, USA) was used to block the caspase-1 (p10) mouse antibody. Secondary antibodies used: biotin-labelled rabbit anti-rat IgG antibody (BA-4000, 1:200; Vector Laboratories) and M.O.M kit. Images were obtained using an optical microscope (BX51; Olympus, Tokyo, Japan). Intraglomerular F4/80 or caspase-1 positive cells were analyzed in a blinded manner in 20 randomly selected glomeruli from each mouse.

Immunofluorescence staining

Immunofluorescence staining was performed using the protocol described previously [6]. Antibodies used: primary antibody, type IV collagen rabbit antibody (LB-1403, 1:150; Cosmo Bio, LSL, Tokyo, Japan), podocin goat antibody (sc-22298, 1:100; Santa Cruz Biotechnology, Dallas, TX, USA), and NPHS1 rabbit antibody (A3048, 1:50; ABclonal Technology, Woburn, MA, USA); secondary antibody, Alexa-Fluor 488-labelled goat anti-rabbit IgG antibody (A11034, 1:200; Thermo Fisher Scientific, Waltham, MA, USA), Alexa-Fluor 594-labelled donkey anti-goat IgG antibody (A11058, 1:200; Thermo Fisher Scientific), and Alexa-Fluor 546-labelled donkey anti-rabbit IgG antibody (A10040, 1:200; Thermo Fisher Scientific). Micrographic fluorescence images were obtained using a fluorescence microscope (BX51). The type IV collagen, podocin and nephrin index was calculated using the formula {[*X* (density) × positive area (μm^2)]/glomerular total area (μm^2)} and analyzed in a blinded manner in 10 randomly selected glomeruli from each mouse.

Double immunofluorescence staining

Primary antibodies: P2X4 rabbit (APR-002, 1:200; Alomone Labs, Jerusalem, Israel), P2X7 rabbit (TA328994, 1:100; OriGene, Rockville, MD, USA), and CD90 mouse (MCA47R, 1:200; Bio-Rad Laboratories, Hercules, CA, USA) antibodies. Secondary antibodies: Alexa-Fluor 488-labelled goat anti-rabbit IgG (A11034, 1:200) and 594-labelled goat anti-mouse IgG (A11005, 1:200; Thermo Fisher Scientific). Nuclei were stained with DAPI and the sections were observed under a fluorescence microscope (BZ-8000; Keyence, Osaka, Japan).

RNA extraction and quantitative real-time RT-PCR (qPCR)

Total RNA was extracted from each sample (renal cortex, glomeruli, and cells) using RNeasy

Plus Mini kit (Qiagen, Venlo, Netherlands). Single-strand cDNA was synthesized from individual samples (1 μg of total RNA) using an iScript cDNA Synthesis kit (Bio-Rad Laboratories). qPCR was performed using StepOnePlus (Thermo Fisher Scientific) and SYBR Premix-Ex-Taq (Takara Bio, Shiga, Japan). For the primer sequences used for qPCR, refer to Table S1.

Immunoblotting

Proteins extracted from the renal cortex or cellular lysates were separated on 4-12% or 12% Bis-Tris gels and transferred onto PVDF membranes. The following antibodies were used: NLRP3 rabbit antibody (ab214185, 1:100; Abcam), NLRP3 mouse antibody (AG-20B 0014, 1:200; AdipoGen Life Sciences), caspase-1 rabbit antibody (NBP1-45433, 1:200; Novus Biologicals, Centennial, CO, USA), IL-18 rabbit antibody (sc-7954, 1:100; Santa Cruz Biotechnology), P2X4 rabbit (APR-002, 1:2000; Alomone Labs), P2X7 rabbit (APR-004, 1:1000; Alomone Labs) and β-actin mouse antibody (A5441, 1:5000; Sigma-Aldrich). Anti-Rabbit IgG, peroxidase-linked whole antibody (NA934V, 1:200; GE Healthcare, Chicago, IL, USA) and anti-mouse IgG, peroxidase-linked whole antibody (NA931V, 1:200; GE Healthcare) were used as secondary antibodies. Can Get Signal (TOYOBO, Osaka, Japan) was used as a buffer for the primary and secondary antibodies when immunoblotting for IL-18. Chemiluminescence was detected using exposure film or ImageQuant LAS 4000 mini (GE Healthcare).

BN-PAGE and two-dimensional polyacrylamide gel electrophoresis (2D-PAGE)

BN-PAGE was performed using a NativePAGE Bis-Tris Gel System (Thermo Fisher Scientific), according to the manufacturer's instructions. Cell lysates were prepared using a NativePAGE Sample Prep Kit and run on a NativePAGE 3-12% Bis-Tris Gel. The proteins were then transferred onto PVDF membranes. The primary antibody was NLRP3 mouse antibody (AG-20B 0014, 1:200; AdipoGen Life Sciences). Anti-mouse IgG, peroxidase-linked whole antibody (NA931V, 1:200; GE Healthcare) was used as a secondary antibody. The blots were visualized using ImageQuant LAS 4000 mini. We also performed 2D-PAGE (BN-PAGE as the first dimension and SDS-PAGE as the second dimension) using a 4-12% Bis-Tris ZOOM Gel (Thermo Fisher Scientific). Strips from the (first dimension) BN-PAGE gels were excised and treated according to the manufacturer's instructions. Each lane strip was loaded on the ZOOM Gel, and SDS-PAGE was performed (second dimension) using the immunoblotting protocol as described earlier.

siRNA transfection of MMCs

Transfection of MMCs with P2X4 and P2X7 siRNA (Horizon Discovery, Cambridge, UK) was performed using a Lipofectamine RNAiMAX transfection reagent (Thermo Fisher Scientific) according to the manufacturer's instructions. MMCs were plated in 6-well cell plates and

transfected with P2X4 and/or P2X7 siRNA (100nM). We also used a negative control siRNA pool (Horizon Discovery), which does not interfere with any known cellular mRNA. After 24 h of incubation, the cell culture medium of MMCs was replaced with DMEM supplemented with 0.5% (vol./vol.) FBS. The MMCs were treated with 5mM ATP and 1μM suramin, or vehicle as described earlier. After 24 h of incubation, the MMCs were collected and subjected to qPCR and western blotting.

References

1. Kitada M, Ogura Y, Koya D. Rodent models of diabetic nephropathy: their utility and limitations. Int J Nephrol Renovasc Dis 2016;9:279-290.

2. Okazaki M, Saito Y, Udaka Y et al. Diabetic nephropathy in KK and KK-Ay mice. Exp Anim 2002;51(2):191-196.

3. Mizuno Y, Yamamotoya T, Nakatsu Y et al. Xanthine Oxidase Inhibitor Febuxostat Exerts an Anti-Inflammatory Action and Protects against Diabetic Nephropathy Development in KK-Ay Obese Diabetic Mice. Int J Mol Sci 2019;20(19).

4. Liu X, Xu C, Xu L et al. Empagliflozin improves diabetic renal tubular injury by alleviating mitochondrial fission via AMPK/SP1/PGAM5 pathway. Metabolism 2020;111:154334.

5. Zhang J, Zhang Y, Yang X et al. Diabetic bladder dysfunction in T2D KK-Ay mice and its changes in the level of relevant gene expression. Biomed Pharmacother 2020;131:110706.

6. Kodera R, Shikata K, Kataoka HU et al. Glucagon-like peptide-1 receptor agonist ameliorates renal injury through its anti-inflammatory action without lowering blood glucose level in a rat model of type 1 diabetes. Diabetologia 2011;54(4):965-978.

7. Miyamoto S, Hsu CC, Hamm G et al. Mass Spectrometry Imaging Reveals Elevated

Glomerular ATP/AMP in Diabetes/obesity and Identifies Sphingomyelin as a Possible Mediator. EBioMedicine 2016;7:121-134.

8. Zhao J, Miyamoto S, You YH et al. AMP-activated protein kinase (AMPK) activation inhibits nuclear translocation of Smad4 in mesangial cells and diabetic kidneys. Am J Physiol Renal Physiol 2015;308(10):F1167-1177.

Fig.S1

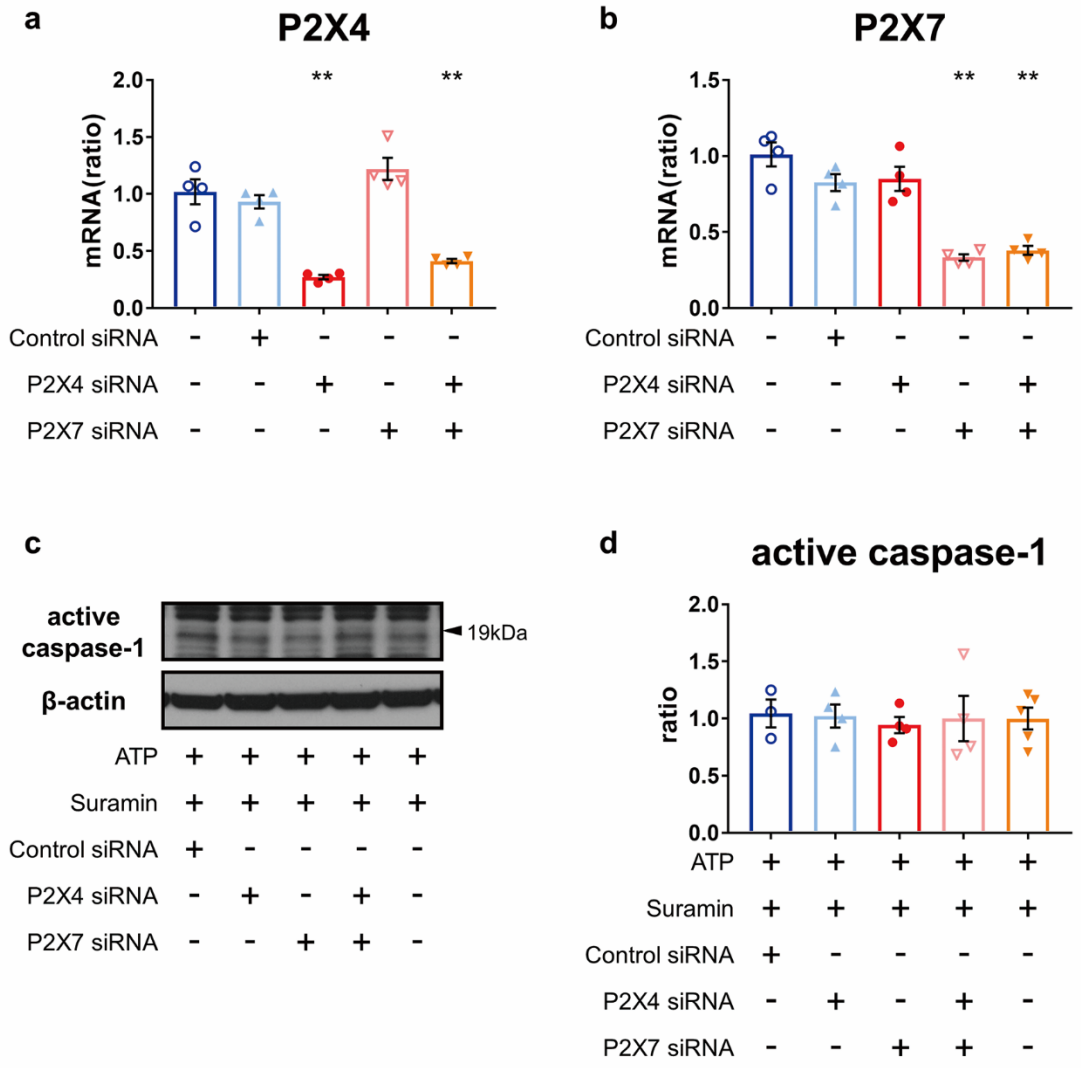

Figure S1 | No additional effect on active caspase-1 is observed by the knockdown of P2X4 or P2X7 in both ATP and suramin treated MMCs. (a, b) Quantification of P2X4 (a) and P2X7 (b) mRNA expression in MMCs by real-time RT-PCR (n = 4). (c-d) Western blotting images (c) and quantified data (d) for caspase-1 protein expression in MMCs (n = 3-5). Data are presented as means ± SEM. **p < 0.001 vs. the group not treated with siRNA. MMCs, mouse mesangial cells.