Appendix

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Cultured cells. C127, which is a fibroblastic cell line derived from mouse mammary tissue, was obtained from the American Type Culture Collection and cultured in High Glucose Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 200 mg/L penicillin (Meiji Seika, Tokyo, Japan), 100 mg/L streptomycin (Meiji Seika), and 10% fetal bovine serum (FBS) (PAA Laboratories, Pasching, Austria). Mouse neuroblastoma C1300 cells were purchased from RIKEN BRC (Tsukuba, Japan) and grown in DMEM supplemented with 5% FBS (PAA Laboratories). Human embryonic kidney HEK293T cells were grown in DMEM supplemented with 10% FBS (Biowest, Nuaille, France).

Nano LC-MS/MS analysis of bleb membrane proteins. Proteins of fraction-7 (F7: ~50 μg), which were obtained after liquid-phase isoelectric focusing of total bleb membrane proteins and found to display the maximal activity of Maxi-Cl, were subjected to SDS-PAGE electrophoresis. The gel was stained with Coomassie brilliant blue R-250 and divided into 26 pieces, and then each piece was destained and dehydrated. The proteins were digested with trypsin (10 μg/mL) in 40 mM ammonium bicarbonate at 37°C overnight. After digestion, the peptides were extracted with a solution containing 50% acetonitrile and 5% trifluoroacetic acid. The digested samples were concentrated by speed vac. Nano LC-MS/MS analysis was conducted using a Waters nanoACQUITY UPLC Chromatographic System (Waters Corp., Milford, MA, USA) connected to a Waters Q-TOF Premier Instrument with Electrospray Ionization System (Waters Corp.). Chromatographic separation was performed

using a Waters nanoACQUITY Trap Column (0.18 mm x 20 mm) and a Waters nanoACQUITY UPLC BEH C18 Column (70 μm x 100 mm) with maintaining the temperature of the column oven at 30°C. Separation was performed by gradient elution with a mobile phase consisting of 0.1% formic acid/H_2O (solution A) and 0.1% formic acid/CH_3CN (solution B). The injection volume of the test sample and the flow rate were set at 5 μL and 0.5 μL /min, respectively. The nano-LC eluate was introduced into the mass spectrometer via an electrospray ionization (ESI) interface at a capillary voltage of 3.5 kV. The mass range was set from m/z 350 to m/z 1800, where z is the charge of the ionized particles, the capillary voltage was at 3.5 kV, the sample cone voltage was at 40 V, and the nanoflow gas pressure was at 0.4 bar. Protein identification was conducted using the Mascot Search Engine Platform with additional searches in Kyoto Encyclopedia of Genes and Genomes (KEGG) and International Protein Index (IPI) databases. The list of 439 protein hits is provided in the Dataset S1. The spectral counts are expressed as emPAI (Exponentially Modified Protein Abundance Index).

Expression vectors and transfection. The full coding sequence of *Slco2a1* (NM_033314.3; NP_201571.2) with the Kozak sequence for the initiation of translation was amplified by PCR from cDNAs of C127 cells (primer set; forward: 5'-cacaaccatggggctcctgcccaagc-3', reverse: 5'-tcagatgaggcccgaggcattc-3') and cloned into a pGEM-T easy vector. To produce the expression vectors for mammalian cell lines, wild-type *Slco2a1* and its mutants were inserted into bicistronic vectors (CMV-pIRES2-EGFP or CMV-pIRES2-dsRED2: Clontech, Mountain View, CA, USA) or into a pCMV-(DYKDDDDK)-C vector (Clontech) and tagged with the FLAG sequence at the C-terminus. Vectors were transfected into C127 or HEK293T cells using Lipofectamine 2000 or Lipofectamine 3000 Transfection Reagent (Thermo Fisher Scientific) according to the manufacturer's instructions. Transfected cells were used for experiments within 24–48 h after transfection. We also generated GFP-tagged WT- or mutant-*Slco2a1* expression vectors (#2, #17, #18 vector: Appendix Table S2) to observe the localization of SLCO2A1 proteins in living cells under a confocal laser microscope (A1Rsi: Nikon, Tokyo, Japan) or conventional fluorescence microscope.

RT-PCR analysis of mRNA expression. Molecular expression of the genes and their knockdown by gene-specific siRNAs were monitored by RT-PCR. Total RNA was isolated from cultured cells or from mouse tissues using Sepasol RNA I reagent (Nacalai Tesque, Kyoto, Japan) according to the manufacturer's instructions. Total RNA was treated with DNase I (Takara Bio Inc., Otsu, Japan) to remove genomic DNA. cDNA was synthesized using an oligo-dT primer (Thermo Fisher Scientific) and GoScript^{TM} Reverse Transcription System (Promega, Madison, WI, USA) or PrimeScript II 1st-Strand cDNA Synthesis Kit (Takara Bio Inc.). PCR products were analyzed on a 1.5% agarose gel to confirm the sizes expected from the known cDNA sequences. To amplify the *Slco2a1* fragment, two primer sets were used (forward: 5'- tctccacgtctcgctccccatag-3', reverse: 5'-gtgtgggagggtgaggacaaagg-3', product size 484 bp; or forward: 5'-attaaacggttcccccgcat-3', reverse: 5'- tcatcctggttcaccacacg-3', product size 710 bp). *Gapdh* was also amplified as an internal control (forward: 5'-atggtctacatgttccagt-3', reverse: 5'-ccttccacaatgccaaag-3', product size 392 bp; or forward: 5'-gcacagtcaaggccgagaat-3', reverse: 5'-ttcaccaccatggagaaggc-3', product size 151 bp).

Purification of recombinant proteins. HEK293T cells were grown in 225 cm^2 flasks until 50−60% confluent and transfected with 75 μg of vectors containing C-terminally FLAG-tagged *Slco2a1* and K613G mutant (#7 and #8 vectors: Appendix Table S2). On the following day, the cells were washed with PBS once, and bleb formation was induced by treatment with 2.5 μM latrunculin B for 10 min in PBS followed by a single wash with PBS, and then by exposure to a hypoosmotic solution (10 mM HEPES-Tris, 1 mM EDTA, pH 7.4) supplemented with a protease inhibitor cocktail (10 μL/mL) for 5 min. The cells were then harvested by scraping, thoroughly pipetted, and vortexed for 3 min. After centrifuging at 4°C and 550 *g* for 20 min, the supernatant was centrifuged at 2°C and 100,000 *g* for 60 min. The pellet was solubilized by vortexing in 1x Wash Buffer provided in the FLAG M Purification Kit (CELLMM2: Sigma-Aldrich) supplemented with 1% DDM and a protease inhibitor cocktail (10 μL/mL). After removing the insoluble contaminants by centrifugation at 2°C and 24,000 *g* for 40 min, the solubilized membrane proteins were loaded onto the anti-FLAG affinity column pre-equilibrated with 1x Wash Buffer. The column was washed with 10-fold

volumes of the 1x Wash Buffer supplemented with 0.25% DDM (5 mM) and 0.02% NaN_3. The bound SLCO2A1 protein was eluted with the same buffer in which DDM was replaced with 0.5% CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate) and additionally containing 150 μg/mL 3X FLAG peptide (F4799: Sigma-Aldrich). The elution was performed in three steps, and the eluates were analyzed by SDS-PAGE followed by silver staining. The protein-rich fractions were pooled and concentrated with an Amicon Ultra Centrifugal Filter for 10 kD (C86533: Merk Millipore, Billerica, MA, USA).

Western blotting. Membrane proteins of wild-type C127 cells and #47-9-8-2 cells were isolated using ProteoExtract® Subcellular Proteome Extraction Kit (Calbiochem®/Merck, Darmstadt, Germany). We also isolated membrane proteins from wild-type HEK293T cells and SLCO2A1-overexpressing HEK293T cells. The proteins were suspended in Tris-Glycine SDS sample buffer (2x) (Thermo Fisher Scientific). As the sample for SLCO2A1 detection, an aliquot of the suspension was boiled at 95°C for 5 min, whereas as the sample for Na^+/K^+ ATPase α1, another aliquot of the same suspension was incubated at 37°C for 30 min without boiling, because this protein was not well detected in the boiled samples. 3-5 μL of these samples was loaded on to a 12% precast gel (Mini-protean® TGX™: BIO-RAD, Hercules, CA, USA) and transferred to a polyvinylidene difluoride membrane (Merck Millipore) in Towbin buffer. Membranes were blocked using 5% dry non-fat milk in TBS. To decrease the non-specific binding, the anti-SLCO2A1 polyclonal antibody (1/1,000, a kind gift from Prof. K. Hosoya, Univ. Toyama) was preabsorbed by incubation with wild-type HEK293T-protein-transferred membrane for 30 min. SLCO2A1 proteins were then detected with appropriate HRP-labeled secondary antibody and ECL™ Prime Western Blotting Detection Reagent (Amersham™/GE Healthcare, Buckinghamshire, UK). The anti-Na^+/K^+ ATPase α1 monoclonal antibody (Santa Cruz Biotech., Dallas, Texas, USA) was used as the internal control.

Luciferin-luciferase ATP assay. For the ATP-release assay, we used isotonic 50-mM NaCl Ringer solution containing (in mM): 50 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 5 Na-HEPES, and 6 HEPES (pH 7.4; 290 mOsm/kg-H_2O adjusted with mannitol). In hypotonic 50-mM NaCl

solution, only the amount of mannitol was varied, thereby osmolality could be changed while keeping the ionic strength at a constant level, enabling reproducible ATP measurements. Hypotonicity-induced ATP release was quantified by a luciferin-luciferase assay system (ATP Luminescence Kit AF-2L1: DKK-TOA, Tokyo, Japan), as described previously (Fahlke *et al*, 2016). Briefly, the cells were cultured to confluence in 24-well plates. After fully replacing the culture medium with isotonic 50-mM NaCl Ringer solution (1000 µL and 500 µL for 12- and 24-well plates, respectively), the cells were incubated at 37°C for 60 min. To measure the background release of ATP, a 100 µL aliquot of the extracellular isotonic solution was collected and used as a reference sample. An osmotic challenge (124 mOsm/kg-H_2O) was then initiated by gently removing most of the remaining extracellular solution (875 µL and 300 µL for 12- and 24-well plates, respectively), adding the hypotonic 50-mM NaCl Ringer solution (1000 and 400 µL for 12- and 24-well plates, respectively), and maintaining the plates on a plate heater at 37°C. After 30 min of incubation, the plates were carefully rocked to ensure homogeneity of the extracellular solution, and samples (50 µL and 20 µL for 12- and 24-well plates, respectively) were collected from each well for the luminometric ATP assay. To the 20-µL samples that were collected, 30 µL of the 50-mM NaCl hypotonic solution were added to adjust the final volume to 50 µL, and 500 µL H_2O was added for the final volume of 550 µL. After adding 50 µL of the luciferin-luciferase reagent, the ATP concentration in the samples, all of equal ionic strength, was measured with an ATP analyzer (Model AF-100: DKK-TOA). For pharmacological studies, bromosulfophthalein (BSP) was added to the isotonic and hypotonic solution. The assay was calibrated using a standard 100-nM ATP solution in the absence and presence of the drug. In experiments with $GdCl_3$, 20 µL of the chelating solution containing 50 mM EDTA and 10 mM $MgCl_2$ (pH 7.4) were added to 20 µL of sample before performing the luciferin-luciferase assay. ATP release, after siRNA-mediated gene knockdown, was measured using similar methods. After the luminescence measurements, the cells were trypsinized, detached by thorough pipetting, and counted manually in a cell counting chamber. The total luminescence intensity for each well was normalized by the cell number in order to assess the variability in growth rate after siRNA treatment. To examine the effects of SLCO2A1 overexpression on ATP release in HEK293T, the cells were cultured on a collagen coated 24-well plate (code 4820-010, Asahi

Glass, Tokyo, Japan) for 1 day and then transfected with CMV-*Slco2a1*-pIRES2-EGFP (#1 vector: Appendix Table S2) and CMV-*Slco2a1* K613G-pIRES2-EGFP (#6 vector: Appendix Table S2) using Lipofectamine 3000. As a mock control, an empty vector (#13: Appendix Table S2) was transfected. The concentration of the plasmids in this experiment was 1/10 (50 ng/well) of other transfections to reduce damage of cells and leakage of ATP therefrom induced by the overexpression of SLCO2A1. ATP- release measurements were performed 1 day after the transfection.

Langendorff-perfusion of mouse hearts. The experimental protocol was approved by the Ethics Review Committee for Animal Experimentations of the National Institute for Physiological Sciences and by the Animal Care and Use Committee of Shiga University of Medical Science. Female mice (C57BL/6J, 9–10 weeks old) weighing 16 to 20 g were anaesthetized by an intraperitoneal injection of sodium pentobarbital (50 mg/kg) with heparin (8000 U/kg). Hearts were isolated and retrogradely perfused by the Langendorff technique, as described previously (Reichelt *et al*, 2009). After mounting, the hearts were perfused at a constant perfusion pressure of 80 mmHg with O_2-bubbled normal Tyrode solution containing (in mM): 140 NaCl, 5.4 KCl, 1.8 $CaCl_2$, 0.5 $MgCl_2$, 0.33 NaH_2PO_4, 5.5 glucose, and 5 HEPES (pH 7.4, 37°C), for 30 min for stabilization. Then, samples of coronary effluent were taken every 2 min, sample volume was measured by gravimetry, and the ATP concentration was assayed by mixing the 50 μL aliquot with an equal volume of luciferin-luciferase reagent and measuring the luminescence with a luminometer (Compactlumi VS501: Yamato Science, Tokyo, Japan). The oxygen-glucose deprivation (OGD) was conducted by perfusing the heart with N_2-bubbled Tyrode solution in which glucose was replaced with an equimolar concentration of 2-deoxyglucose. Calibration of the luminometric ATP assay was performed separately for every experimental condition in the absence and presence of the applied drug. The amount of the released ATP (pmol) was calculated as a product of the ATP concentration (nM) and the volume of coronary effluent (mL). The total release of ATP was calculated for basal conditions (for 6 min before OGD), during OGD period (for 6 min) and upon reperfusion (for 6 min after OGD), and values were normalized to the mass of the hearts. OGD/reperfusion-induced ATP release to the coronary effluent was reproducibly observed

with increasing the effluent ATP concentration from 0.30 ± 0.03 nM to 1.24 ± 0.22 nM (n = 11).

Data analysis. The single-channel amplitudes were measured manually by placing a cursor at the open and closed channel levels. After subtracting the background currents, the mean patch currents were measured at the beginning (first 25–30 ms) of current responses to voltage steps in order to minimize the contributions of voltage-dependent current inactivation and channel occupancy in the sub-conductance states. The mean number of channels open, nP_o (where P_o and n represent the open channel probability and the number of active channels, respectively), was calculated by dividing the mean macro-patch current by the single-channel amplitude. The reversal potentials were calculated by fitting *I-V* curves to a second order polynomial.

Data were analyzed in OriginPro 7 or 8.6 (Origin Lab Corporation, Northampton, MA, USA). Plotted data are provided as means ± SEM of n observations. Statistical differences of the data were evaluated by ANOVA and the paired or unpaired Student's t test where appropriate and considered significant at $P<0.05$.

The dimensions of PGE2 and NMDG were calculated using Molecular Modeling Pro computer software (Norgwyn Montgomery Software Inc., North Wales, PA, USA).

2. Appendix Table S1. List of genes for targeted siRNA screening.

#	Symbol	Name	Accession number
1	*Slc15a4*	solute carrier family 15, member 4	NM_133895
2	*Slc25a3*	solute carrier family 25, member 3	NM_133668
3	*Slc25a4*	solute carrier family 25, member 4	NM_007450
4	*Slc25a5*	solute carrier family 25, member 5	NM_007451
5	*Slc25a11*	solute carrier family 25, member 11	NM_024211
6	*Slc33a1*	solute carrier family 33, member 1	NM_015728
7	*Slc44a1*	solute carrier family 44, member 1	NM_133891
8	*Slc44a2*	solute carrier family 44, member 2	NM_152808
9	*Slco2a1*	solute carrier organic anion transporter family, member 2a1	NM_033314
10	*Tmem62*	transmembrane protein 62	NM_175285
11	*Tmem65*	transmembrane protein 65	NM_175212
12	*Tmem97*	transmembrane protein 97	NM_133706
13	*Tmem167b*	transmembrane protein 167B	NM_026198
14	*Tmem189*	transmembrane protein 189	NM_145538
15	*Tspan31*	tetraspanin 31	NM_025982

3. Appendix Table S2. List of expression vectors used in this study.

#	Vector construction	Used for
1	pCMV-*Slco2a1*-pIRES2-EGFP	forced expression, patch-clamp
2	pCMV-*Slco2a1*-GFP-fusion vector	observations of protein localization
3	synonymous variant of *Slco2a1*-pIRES2-dsRED2	rescue experiments in a stably *Slco2a1*-knockdown cell line
4	synonymous variant of *Slco2a1*K613G-pIRES2-dsRED2	rescue experiments in a stably *Slco2a1*-knockdown cell line
5	pCMV-*Slco2a1*R560N-pIRES2-GFP	forced expression, patch-clamp
6	pCMV-*Slco2a1*K613G-pIRES2-GFP	forced expression, patch-clamp
7	pCMV-*Slco2a1*-(DYKDDDDK)-C	observations of localization, protein reconstitution
8	pCMV-*Slco2a1*K613G-(DYKDDDDK)-C	observations of localization, protein reconstitution
9	pCMV-*Slco2a1*R560N-(DYKDDDDK)-C	observations of localization
10	pcDNATM6.2-GW/EmGFP-miR-neg control	negative control for miR RNAi experiments
11	pcDNATM6.2-GW/EmGFP-miR72	transient or stable knockdown of *Slco2a1* expression
12	pcDNATM6.2-GW/EmGFP-miR73	transient knockdown of *Slco2a1* expression
13	pCMV-pIRES2-EGFP	mock transfection
14	pCMV-(DYKDDDDK)-C	mock transfection
15	pCMV-*Slco2a1* P219L-pIRES2-GFP	forced expression, patch-clamp
16	pCMV-*Slco2a1* G222R-pIRES2-GFP	forced expression, patch-clamp
17	pCMV-*Slco2a1* P219L-GFP-fusion vector	observations of protein localization
18	pCMV-*Slco2a1* P222L-GFP-fusion vector	observations of protein localization

References

Reichelt ME, Willems L, Hack BA, Peart JN, Headrick JP (2009) Cardiac and coronary function in the Langendorff-perfused mouse heart model. *Exp Physiol* 94: 54 – 70