Supporting Information

Kheradpezhouh et al. 10.1073/pnas.1322657111

SI Materials and Methods

Chemicals. Acetaminophen; clotrimazole; chlorpromazine; *N*-(*p*-amylcinnamoyl)anthranilic acid (ACA); ADP ribose (ADPR); 3,4-dihydro-5-[4-(1-piperidinyl)butoxy]-1(2H)-isoquinoline; pleuronic acid; SDS; coumaric acid; and a luminal and bicinchoninic acid assay kit were purchased from Sigma-Aldrich. DMEM, penicillin/streptomycin, and trypsin-EGTA were purchased from GIBCO. Fura2-AM, TRIzol, DNase, and RT enzyme were purchased from Invitrogen. All of the primary and secondary antibodies were purchased from Abcam; FBS was purchased from Bovogen, and Tris and glycerin were purchased from Amresco.

Solutions. The Krebs–Ringer–Hepes (KRH) solution (in mM) was as follows: NaCl 136, KCl 4.7, $CaCl_2$ 1.3, $MgCl_2$ 1.25, glucose 10 and Hepes 10 with the pH adjusted to 7.4 by sodium hydroxide (NaOH). Control bath solution for patch clamping (in mM): NaCl 140, CsCl 4, $CaCl_2$ 2, $MgCl_2$ 2, and Na·Hepes 10, adjusted to pH 7.4 with NaOH. Pipette solution for patch clamping (in mM) was as follow: cesium glutamate 130, $MgCl_2$ 5, $CaCl_2$ 5, and EGTA 10 (120 nM free Ca^{2+}), adjusted to pH 7.3 with NaOH. PBS (in mM) was as follows: 137 NaCl, 2.7 KCl, 8 disodium phosphate, 1.46 monopotassium phosphate. Lysis buffer (in mM) was as follows: 50 Tris·HCl (pH 7.4), 150 NaCl, 3 EDTA (pH 8.2), plus Triton X-100 1% (vol/vol), and protease inhibitor mixture.

RT-PCR. Total RNA was extracted from 2×10^6 hepatocytes using TRIzol reagent. The contaminating DNA was removed by DNA-free DNase and the amount of RNA was measured using NanoDrop ND-8000 (Thermo Scientific). Conventional PCR was performed using sense and antisense primers to detect Transient Receptor Potential Melanostatine 2 (TRPM2) mRNA and to determine the TRPM2 subtypes expressed in hepatocytes (Table S1).

Cell Transfections. Hepatocytes cultured on glass coverslips for 4 h after isolation were transfected with 125 nM of either siRNA specific for TRPM2 or control (nonsilencing) siRNA (Ambion) using HighPerFect transfection reagent (Qiagen) according to the manufacturer's instructions. To achieve a sufficient level of TRPM2 knockdown, transfected hepatocytes were cultured for 36–48 h before experiments.

Western Blot Analysis. Isolated hepatocytes (1×10^6 to 2×10^6 cells) were lysed by incubation with 50 µL lysis buffer for 20 min on ice. The supernatant-containing protein was extracted by centrifuging the lysate at $5{,}000 \times g$ for 15 min at 4 °C. Western blot analysis was performed using a polyclonal antibody to TRPM2 (1:750; Abcam ab63015) and a horseradish peroxidase (HRP) secondary antibody (1:5,000; Abcam ab97130) by standard procedures.

Cell Viability Assay. Hepatocytes cultured on glass coverslips for 4 h after isolation were treated with 10 mM acetaminophen or 1 µM ACA, or a combination of 10 mM acetaminophen and 1 µM ACA for 16 h. The proportion of damaged cells was determined by Trypan Blue exclusion using an Olympus BX51 microscope at 10× magnification.

Immunofluorescence. Hepatocytes on cultured on glass coverslips were fixed by methanol at −20 °C for 5 min and blocked by 20% FBS in PBS (vol/vol) for 15 min. Intracellular levels of poly-ADPR were determined by using poly-ADPR–specific primary antibodies (1:200; ab14460) and FITC secondary antibody (1:1,500; ab6877). Immunofluorescent images were captured using BX51WI microscope with an UPLSAPO60xW objective, Olympus XM10 camera, and 490:518 nm excitation:emission filter set. Images were analyzed using cellˆB software (Olympus).

In Vivo Acetaminophen Toxicity. TRPM2 knockout (KO), (wild-type) WT, and Het mice were separated into six groups as follows: (*i*) control WT; (*ii*) vehicle WT; (*iii*) control TRPM2 KO; (*iv*) WT, acetaminophen i.p. injection (500 mg/kg); (*v*) TRPM2 KO, acetaminophen i.p. injection (500 mg/kg); and (*vi*) TRPM2 Het, acetaminophen i.p. injection (500 mg/kg). Acetaminophen dissolved in vehicle solution [propylene glycol:water, 50–50% (vol/vol)] or vehicle solution was injected i.p. after induction of anesthesia using isoflurane. Twenty-four hours postinjection each mouse in each group was anesthetized with ketamine–xylazine (50 and 8 mg/kg, respectively, i.p. injection) immediately after short-course sedation with inhaled isoflurane. After induction of surgical anesthesia, the abdomen was opened vertically, the anterior part of the chest was removed, and 400 µL of blood was taken from the left ventricle of the heart and transferred to heparinized tube for further analysis. The liver was immediately perfused with KRH solution to remove the blood, harvested, and fixed in buffered formaldehyde (10% in PBS, vol/vol) for histopathology. Fixed liver tissue was blocked in paraffin, sliced with microtome (5 µm thick), stained with hematoxalin/eosin (H&E), and mounted on glass slides using a mounting reagent.

Blood Liver Enzymes Assay. Alanine transaminase (ALT) and aspartate transaminase (AST) were measured spectrophotometrically using a Roche Modular Automatic Analyzer and standard Roche–Hitachi methodology and are reported in units per liter.

Histopathology. All H&E-stained liver sections were examined at 40×, 200×, and 400× magnifications by two observers using a Bright-Field Olympus microscope BX53 with an Olympus DP72 camera. The necrotic and normal areas and the total area of tissue were quantified using CellSense software (Olympus).

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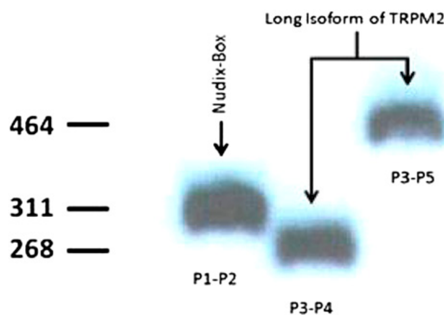

Fig. S1. Expression of TRPM2L isoform in rat in hepatocytes. Presence of Nudix-Box located on the C terminus of TRPM2L was detected using P1 and P2 primers (lane 1: note the absence of the 255-bp product characteristic of TRPM2ΔC). Primers designed to discriminate between TRPM2ΔN and TRPM2L (P3–P4 and P3–P5) confirmed expression of TRPM2L and the absence of TRPM2ΔN in rat hepatocytes.

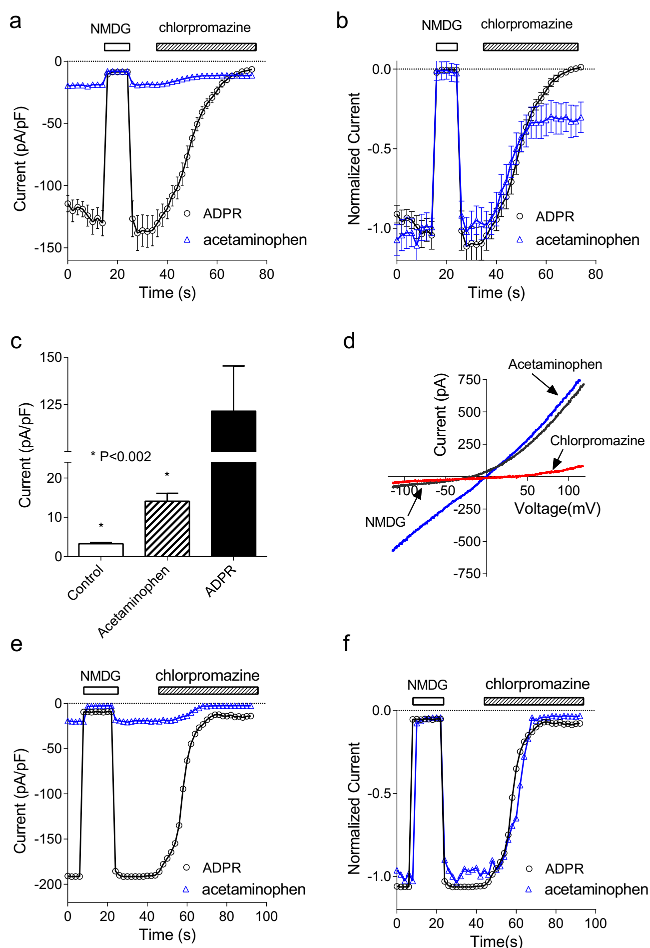

Fig. S2. Chlorpromazine blocks ADPR- and Acetaminophen-activated TRPM2 current in rat hepatocytes and HEK293T cells. (*A*) The time course of inhibition of ADPR- and acetaminophen-activated TRPM2 current in rat hepatocytes by 100 μM chlorpromazine. Each data point represents amplitude of the current at −100 mV. (*B*) The same data as in *A*, normalized to the maximum current. (*C* and *F*) HEK293T cells grown on glass coverslips were transfected with a pCIneo plasmid containing hTRPM2 cDNA (a gift from Yasuo Mori) using TrueFect transfection reagent (United BioSystems) according to the manufacturer's instructions. (*C*) The amplitude of the current inhibited by the replacement of NaCl in the bath solution by NMDGCl in hTRPM2 transfected cells. Control: untreated cells ($n = 6$). Acetaminophen: cells were treated with 10 mM acetaminophen for 40 min before current measurements ($n = 6$). ADPR: cells were patch clamped using pipette solution containing 100 μM ADPR ($n = 7$). Cells treated with acetaminophen exhibited significantly larger nonselective cation current compared with untreated cells ($P < 0.002$). (*D*) Current-voltage (I-V) plots recorded in a representative cell treated with acetaminophen under control conditions (Acetaminophen), after replacement of NaCl in the bath solution with NMDGCl (NMDG) and in the presence of 100 μM chlorpromazine in the bath. (*E* and *F*) The time course of chlorpromazine-induced (100 μM) inhibition of the inward current activated by either intracellular ADPR or preincubation with 10 mM acetaminophen. Each data point represents current amplitude measured at −100 mV. Data presented as means ± SEM.

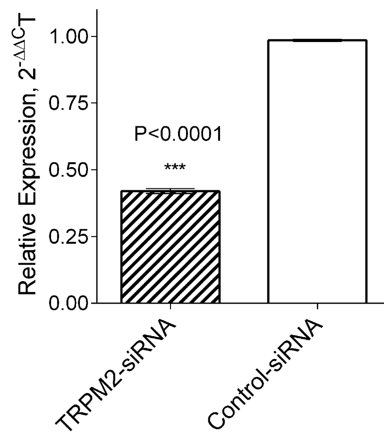

Fig. S3. The effect of TRPM2-specific siRNA on TRPM2 mRNA levels in rat hepatocytes. Cell transfections and mRNA extraction were performed as described in *SI Materials and Methods*. Average data from three separate transfections are shown.

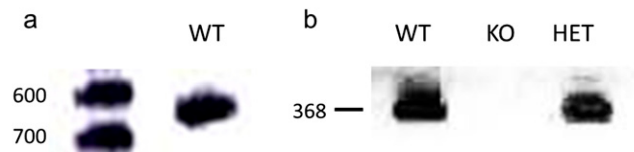

Fig. S4. TRPM2 expression in mouse hepatocytes by PCR. (*A*) Presence of Nudix-Box located on the C terminus of TRPM2L was detected in WT mouse hepatocytes using the P1 and P2 primers listed in Table S3. The left lane shows part of the ladder. (*B*) Presence of the sequence corresponding to the TRPM2 pore region was detected in hepatocytes isolated from WT and TRPM2 Het mice, but not in TRPM2 KO mice (primers P3 and P4 in Table S3).

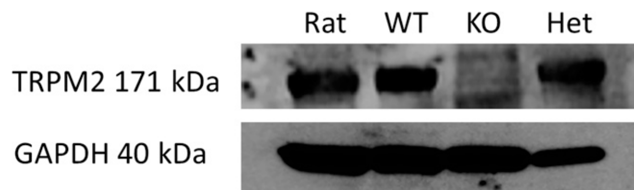

Fig. S5. TRPM2 expression in mouse hepatocytes by Western blotting. Western blot analysis was performed using a polyclonal antibody to TRPM2 (1:750; ab63015) and an HPR secondary antibody (1:5,000; ab97130) following standard procedures. Note the absence of the TRPM2 band in the KO mouse.

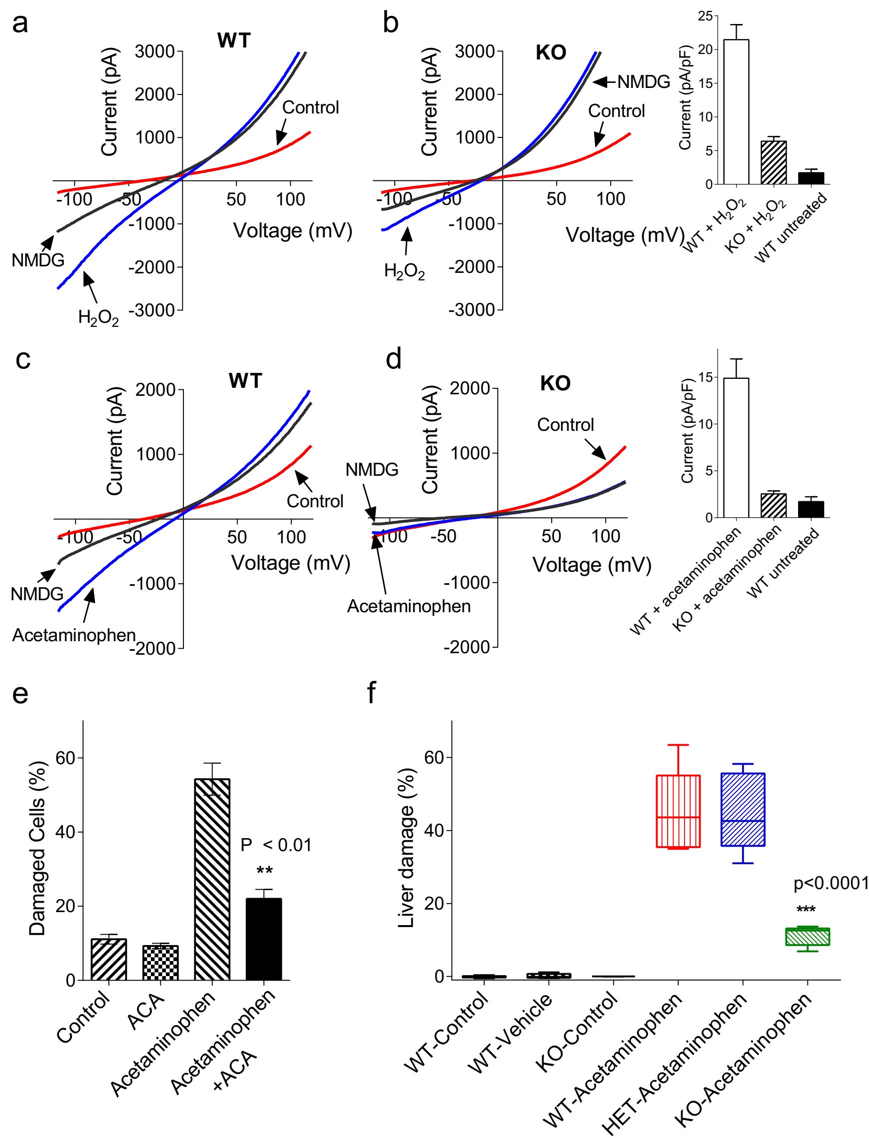

Fig. S6. (*A*–*D*) Acetaminophen and H_2O_2 activate a nonselective cation current in mouse hepatocytes. Averaged current–voltage plots of membrane currents measured in untreated WT (*A* and *C*) and KO (*B* and *D*) hepatocytes (Control), and hepatocytes treated with either 0.5 mM H_2O_2 for 30 min (*A* and *B*) or 10 mM acetaminophen for 60 min (*C* and *D*). *Insets* show the amplitude of the cation current (at −100 mV) inhibited by the replacement of 140 mM NaCl in the bath solution with 140 mM NMDGCl in WT and KO mouse hepatocytes treated with either H_2O_2 (0.5 mM for 30 min) or acetaminophen (10 mM for 60 min) (n = 17–20 for each condition). (*E* and *F*) Rat hepatocytes treated by the TRPM2 inhibitors and TRPM2 KO mice are protected against acetaminophen-induced damage. (*E*) The percentage of damaged cells determined by Trypan Blue staining of isolated rat hepatocytes cultured for 16 h in the presence of 1 μM ACA, 10 mM acetaminophen, or both. (*F*) The percentage of necrosis in TRPM2 WT, KO, and Het liver sections is expressed as a percentage of the total area. The data in each group was averaged from six mice. Data in insets and in panels *E* and *F* are presented as means ± SEM.

Table S1. List of primers used to determine the expression of TRPM2 isoforms in rat hepatocytes

Primer	Sequence (5′–3′)	Position	Size, bp	Isoform detected
Sense (P2)	5′-AGCTGAGAAGAAGGATGCGAC-3′	3825–3845	311	TRPM2L, TRPM2ΔN
Antisense (P1)	5′-GAGGCAACTTCATGACCAG-3′	4135–4117	255	TRPM2ΔC
			None	TRPM2S
Sense (P4)	5′-AAGGTGCTGGCAGAAGAGCATG-3′	1609–1630	268	TRPM2L, TRPM2ΔC, TRPM2S
Antisense (P3)	5′-TGAGAAGATCGCGGACTGG-3′	1876–1858	None	TRPM2ΔN
Sense (P5)	5′-CTTCACTGATGAGTGGCAGT-3′	1413–1432	464	TRPM2L, TRPM2ΔC, TRPM2S
Antisense (P3)	5′-TGAGAAGATCGCGGACTGG-3′	1876–1858	406	TRPM2ΔN

TRPM2L, long isoform of TRPM2 with full length C and N termini (1); TRPM2S, short isoform of TRPM2 (1); TRPM2ΔC, splice variant of the long isoform of TRPM2 with a 34-aa deletion (1292–1325) in the C terminus, which does not respond to ADP ribose, but can be activated by H_2O_2 (1); TRPM2ΔN, splice variant of the long isoform of TRPM2 with a 20-aa deletion (538–557) in the N terminus, which does not respond to either ADP ribose or H_2O_2 (1).

1. Wehage E, et al. (2002) Activation of the cation channel long transient receptor potential channel 2 (LTRPC2) by hydrogen peroxide. A splice variant reveals a mode of activation independent of ADP-ribose. *J Biol Chem* 277(26):23150–23156.

Table S2. Primers for TRPM2 quantitative RT-PCR

Primer	Sequence (5′–3′)	Position	Size, bp	Isoform detected
Sense	5′-GAAGGAAAGAGGGGGTGTG-3′	936–954	100	All, except TRPM2S
Antisense	5′-CATTGGTGATGGCGTTGTAG-3′	1036–1017		

Table S3. List of primers used to determine the expression of TRPM2 in mouse hepatocytes

Primer	Sequence (5′–3′)	Position	Size, bp	Sequence detected
Sense (P2)	5′-TGCCAACATCCTGCTGCTTA-3′	3413–3432	651	TRPM2-NUDT9-H
Antisense (P1)	5′-ATGCGGGCATTGGGATAGAG-3′	4063–4044		
Sense (P4)	5′-CATCCCAGCGACGCTGTAC-3′	2969–2987	368	TRPM2 pore
Antisense (P3)	5′-CAGGACACTTAGGCTTGTAG-3′	3336–3317		