SUPPLEMENTAL MATERIAL

Methods

Reagents and chemicals. Endothelial growth medium (EBM-2) was obtained from Clonetics (San Diego, CA), and the Dulbecco's modified Eagle's medium (DMEM) from Invitrogen (Grand Island, NY). His^6-tagged TRPM2-S cDNA construct was made by modification of the cDNA encoding the green fluorescent protein-fused TRPM2-S (GFP-TRPM2-S) kindly provided by Dr. Barbara A. Miller (Pennsylvania State University College of Medicine, Hershey, PA). Trypsin, Hank's balanced salt solution (HBSS), molecular cellular and developmental biology (MCDB) media 131, TRIzol reagent, AmplexR Red glucose/glucose oxidase assay kit and Superscript II were obtained from Invitrogen (Carlsbad, CA). FuGENE HD transfection reagent and TUNEL assay kit were obtained from Roche Applied Science (Indianapolis, IN); and TransIT-TKO Mirus transfection reagent from Mirus Bio (Madison, WI). Fura-2/acetoxymethyl ester (AM) was obtained from Molecular Probes (Eugene, OR). The myristoylated PKCα peptide inhibitor Myr-RFARKGALRQKNV was from Promega (Madison, WI). H_2O_2, myristoylated PKCβII inhibitor Myr-SLNPEWNET (PKCβIIi), penicillin, lipopolysaccharides (LPS) and peptides and chemicals were from Sigma Chemical Co. (St. Louis, MO). Matrigel, Dynabeads M-450 and the platelet/endothelial cell adhesion molecule-1 (PECAM-1) were purchased from BD Bioscience (San Jose, CA). Anti-TRPM2 antibodies (one against the 171-kDa TRPM2 long isoform [27] and the other recognizing both TRPM2 and TRPM2-S isoforms) were purchased from Abcam (Cambridge, MA) [5]. His^6, PKCα and phospho-Ser antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA).

Isolation of endothelial cells from mouse lungs. Mice at age 6-8 weeks were deeply anesthetized (2.5% isoflurane in a bell jar), and heparin (50 U/mouse) was injected into the jugular vein. A thoracotomy was carried out and the pulmonary artery was cannulated. Krebs-Henseleit solution supplemented with bovine serum albumin (5 g/100 mL) was infused to remove blood. Lungs were removed and placed inside a culture hood. Lung tissue slices from 3 mice were prepared, washed, and suspended in HBSS. Excess HBSS was aspirated, and the tissue slices were minced and transferred to a 15-mL sterile tube. The minced tissues were suspended in 10 mL of collagenase A (1.0 mg/mL in HBSS) and digested for 60 minutes at 37°C with gentle shaking. The released cells were collected by centrifugation, resuspended, filtered and incubated in buffer containing 1.5ug/mL anti-mouse PECAM-1 antibody at 4°C for 30 minutes with gentle shaking. After washings, cells were incubated with Dynabeads M-450 (Sheep anti-rat IgG) for 30 minutes at 4°C. After this incubation period, the cell suspension was attached to a magnetic column and the unbound cells were aspirated. Cells bound to the magnetic beads were washed with HBSS and digested with trypsin for 3 minutes at 22°C. Endothelial cells released from the magnetic beads were washed and suspended in growth medium (EGM-2 supplemented with 10% fetal bovine serum), then plated on Matrigel-coated 35-mm culture dish and allowed to grow to confluence for 10 days. Cells were then harvested from the Matrigel plates by dispase (BD Bioscience) for 60 to 90 minutes. Cells were washed after dispase treatment once with growth medium and plated on 0.1% gelatin coated culture dish. Cells passaged between 3 and 4 times were used in experiments. Endothelial cells were characterized by their cobblestone morphology, platelet/endothelial cell adhesion molecule-1 (PECAM-1 or CD31) expression, and Dil-Ac-LDL uptake.

Endothelial cell culture and transfection.

*Isolation of endothelial cells from murine tissue***:** Endothelial cells were isolated from lungs of WT, *PKCα*$^{-/-}$ (obtained from *Dr* Jeffrey D. Molkentin [3], University of Cincinnati, Cincinnati, OH) and *TRPM2*$^{-/-}$ (obtained from GlaxoSmithKline [4]) mice as described [5]. Cells were cultured in gelatin-coated flasks using DMEM/F12 medium supplemented with endothelial growth factor plus 10% fetal bovine serum, and used in experiments between passages 2–5.

Transfections: Human pulmonary artery endothelial cells (HPAE; Clonetics, La Jolla, CA) were cultured in gelatin-coated flasks using endothelial basal medium 2 (EBM2) supplemented with bullet kit additives plus 10% fetal bovine serum, and used in experiments between passages 3–6. Human TRPM2-S short splice variant, tagged on its carboxy-terminus with poly-His (His^6-TRPM2-S), was inserted into pcDNA3 expression vector (Invitrogen). Phosphorylation-defective TRPM2-S was generated by alanine substitution (S39A) and phosphorylation-mimetic TRPM2-S was generated by aspartic substitution (S39D). Point mutation was introduced in His^6-TRPM2-S construct using the QuikChange site-directed mutagenesis protocol (Stratagene), and was verified by sequencing. HPAE cell cultures, grown to 60-80% confluency,

were transfected with 1 μg/ml each of His^6-(S39A)TRPM2-S or His^6-(S39D)TRPM2-S cDNA, or with vector alone (control cells) using fuGENE HD and in the presence of protease inhibitor cocktail (Sigma Aldrich) to prevent degradation of the transfected protein. In some experiments, cells were co-transfected with PKCα siRNA and TRPM2-S mutant cDNA using X-tremeGENE siRNA Transfection Reagent (Roche) and maintain in culture medium containing protease inhibitor cocktail (Sigma Aldrich) to prevent degradation of the transfected protein and caspase 9 inhibitor (Ac-LEHD-CHO, 20 mol/L) to prevent apoptosis. Successful transfection of cells with (S39A)TRPM2-S or (S39D)TRPM2-S and depletion of PKCα was verified by Western Blot.

*Stable transfection of human HEK293 cells***:** HEK293 cells grown at 37°C with 5% CO2 in DMEM supplemented with 10% fetal bovine serum were transfected with 1 μg/ml each of the human long variant of TRPM2, tagged on its carboxy-terminus with poly-His (His^6-TRPM2) and inserted into pcDNA6 expression vector (Invitrogen) and either His^6-TRPM2-S or His^6-(S39A)TRPM2-S plasmids using the FuGENE HD transfection reagent. The sucessfully transfected cells were then selected with Geneticin (G418, 100 μg/mL) and Blasticidin (100 μg/mL).

Small interfering RNA transfection: HPAEs were transiently transfected with 100 nmol/L of TRPM2 or PKCα pre-designed small interfering RNAs (siRNAs, Santa Cruz Biotechnology, Santa Cruz, CA) using TransIT-TKO transfection reagent (Mirus, Madison, WI) according to manufacturer's instructions. As control, we used commercially available nonspecific (NS) siRNA (Ambion, Austin, TX). Protein silencing was verified by Western Blots analysis. Transfection efficiency was at least 75%.

[Ca^{2+}]i measurements.

Ratiometric Ca^{2+} *measurements using Fura-2/AM*: Control or transfected HPAE cells (see above) grown to confluence on 25-mm glass coverslips were loaded with Fura-2/AM (2 μmol/L) for 20 min at 37 °C. Cells received two washes with Hank's balanced salt solution and were placed in an experimental chamber containing 200 μl of buffer. We measured Fura 2 fluorescence using Attoflor Ratio Vision digital fluorescence microscope (Atto Instruments, Rockville, MD) equipped with F-Fluar 40 x oil-immersion objectives with a numerical aperture of 1.3. Excitation wavelengths used were 340 and 380 nm, and emission wavelength was 510 nm. Intracellular Ca^{2+} levels are given as fluorescent ratio F340/F380 representing bound/free Ca^{2+}.

Ca^{2+} measurements were also made using FlexStation scanning fluorometer. Mouse endothelial cells were grown to confluence in clear-bottom 96-well assay plates. Assays utilized the FLIPR (Fluorometric Imaging Plate Reader) Calcium Plus kit (Molecular Devices, Sunnyvale, CA). Cells were loaded with the FLIPR Ca^{2+}-sensitive fluorescence indicator and incubated for 1 h at 37 °C according to the manufacturer's protocol. The addition of agonists was robotically controlled, and monolayer fluorescence in each well was read by the FlexStation data acquisition system (Molecular Devices) at 0.1 Hz. Cells were excited at 485 nm and monitored at 515 nm.

Western blotting. Endothelial monolayers were washed in PBS, lysed in Tris buffer (containing 1% Triton X-100, 1 mmol/L phenylmethylsulfonyl fluoride [PMSF], and protease-inhibitor cocktail), and sonicated (20 s). Protein was separated by electrophoresis (4-12% SDS gradient polyacrylamide gel) and transferred to nitrocellulose membranes for Western blotting with antibodies (TRPM2, PKCα, His^6, phospho-Ser, or actin). Band intensity was determined by densitometry using Image J (NIH).

Immunoprecipitation and phosphorylation studies. Untransfected and His^6-(S39A)TRPM2-S transfected HPAE cultures in six-well culture dishes were treated with 300μM H_2O_2 for various times at 37°C. In some of the experiments, cells were pretreated with the Poly(ADPR) polymerase inhibitor (DPQ) or PKC inhibitors described above 30 min prior to the assay. In other experiments, cells were previously transduced with siRNA to selectively suppress expression of TRPM2 or PKCα. Following H_2O_2 challenge, cells were washed with ice-cold PBS and lysed with 0.4 ml 0.5% deoxycholate buffer (pH 7.5) containing 1% NP-40, 0.1% SDS, 1 mmol/L PMSF, 50 mmol/L Tris, 150 mmol/L NaCl, and 10 μl protease inhibitor mixture. After shaking for 10 min at 4°C, lysates were sonicated and then centrifuged for 15 min at 16,000 *g* and 4°C. Supernatants were collected and diluted with 390 μl of 50 mmol/L Tris buffer (pH 7.5) containing 150 mmol/L NaCl and protease inhibitors. Samples were then incubated with 1 μg of antibody (rabbit anti-TRPM2 or anti-PKCα) overnight at 4°C. TRPM2 or PKCα immune complexes were precipitated with protein A-Sepharose beads (Sigma) at 4°C for 2 h. The beads were then washed five times with lysis buffer, and the precipitated proteins were eluted by boiling the beads in sample buffer [80 mmol/L Tris (pH 6.8), 3%

SDS, 15% glycerol, 0.01% bromophenol blue, 5% DTT]. Proteins were then separated on a 4–12 % SDS-PAGE gradient gel.

His-Tagged TRPM2 protein purification using Ni-NTA beads: His^6-TRPM2-S/ His^6-TRPM2 transfected HEK cells pelleted from 50 ml tissue culture were resuspended in 8 ml of native binding buffer (50 mM NaH2PO4, 0.5 M NaCl, pH 8.0), supplemented with protease and phosphatase inhibitors. Cells were lysed by two freeze-thaw cycles. The lysates preparation were passed through an 18-gauge needle to shear the DNA, then centrifuged at 3,000 × g for 15 minutes to pellet the cellular debris. The supernatant (8 mL) was transferred to a 15-mL purification column containing 1 ml of 50% slurry of Ni-NTA beads (Qiagen, Valencia, CA) at 4°C for 2 h. Beads were washed twice with native buffer containing 20 mM imidazole and His^6-tagged TRPM2 proteins were eluted with native buffer containing 250 mM imidazole. The eluted proteins were stored at−20°C.

***In vitro* phosphorylation assay**. For TRPM2 phosphorylation by PKCα, 5µg of the TRPM2 channel proteins (short and long) purified from transfected HEK cells fraction were incubated for 1 h at 30 °C in the absence or presence of 0.045 pg of PKCα in a buffer containing 25 mM Hepes (pH 7.4), 1 mmol/L DTT, 10 mmol/L MgCl2, 0.2 mmol/L Na3VO4, 1.7 mmol/L $CaCl_2$, 5 mM beta-glycerophosphate. Phosphorylation was initiated by the addition of ATP at a concentration of 50 µmol/L. The reaction was terminated by the addition of sample buffer [0.35 mol/L Tris-HCl (pH 6.8), 10.28% (w/v) SDS, 36% (v/v) glycerol, 0.6 mol/L DTT, 0.012% (w/v) bromphenol blue] and boiling for 5 min. Proteins were separated by SDS-PAGE and analyzed by immunodetection with an anti-phosphoserine antibody after transfer to nitrocellulose.

Measurement of hydrogen peroxide released. The amount of H_2O_2 released after the action of glucose oxidase was measured spectrophotometrically based on the generation of resorufin (absorbance at 565 nm) using the extinction coefficient at 58,000 M-1cm-1 and the height of a 150 µl column of solution in a typical 96-well plate. The concentration of glucose oxidase used in experiments was calculated to produce a continuous flow of 320 nmol/L H_2O_2/min in cells.

The amount of H_2O_2 generated in the mouse lungs 4 h following lipopolysaccharides (LPS, 40 mg/kg) stimulation was measured with a horseradish peroxide-linked Amplex Red assay (Molecular Probes, Carlsbad, CA). Lungs of mice untreated, injected intraperitoneally with LPS and treated with Tempol (100 mg/kg, IP) 30min before injection of LPS were homogenized in PBS containing protease inhibitors and the Amplex Red dye; H_2O_2 was determined spectrophotometrically (absorbance at 570 nM) using a microplate reader and corrected for total protein content assessed using the Bradford assay.

Analysis for apoptotic cell death. Apoptosis was identified by double fluorescence staining with PE Annexin V-FITC (to detect apoptotic cells) and 7-AAD (to detect dead cells). Apoptotic cells translocate phosphatidylserine from the internal face of the plasma membrane to the outer surface, and therefore stain with Annexin V-PE which binds with high affinity to phosphatidylserine, resulting in red fluorescence when excited at 450–480 nm. Confluent endothelial monolayers in 6-well culture dishes, untreated or treated to inhibit PKCα activation, were exposed to 300 µM H_2O_2 for 6 or 24 h at 37°C. Following H_2O_2 challenge, cells were washed twice with PBS and trypsinized; cell samples (1×10^6 cells per sample) were incubated with 5 µl of PE-labeled Annexin V and 5 µl of 7-AAD (BD bioscience, Rockville, MD) for 20 min at 24 °C in the dark and then analyzed with a Beckman Coulter CyAn II cytometer (Beckman Coulter, Miami, FL) within 1 h of Annexin V-PE labeling.

Apoptosis in lung endothelium by immunofluorescence and Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay*:* Lungs of *TRPM2*$^{-/-}$ and wild-type mice were perfused (2 ml/min, 37ºC) for 3 hr with a recirculating volume of RPMI 1640 solution containing H_2O_2 (300 µmol/L) or glucose oxidase (that produced 320 nmol/L/min H_2O_2). Lungs were removed, inflated and frozen using an OCT matrix solution. Frozen lungs were cut (5 µmol/L) and fixed in 3.7% paraformaldehyde, then permeabilized in 0.2% tritonX-100 containing buffer for 5min. Tissue sections were block in 10% FBS and incubated with the goat anti-VE-cadherin and the rabbit antibody raised against the 89 kDa cleaved-PARP (1:200 dilution) overnight at 4ºC. The immunofluorescence assay was performed by incubation with secondary antibodies conjugated to Alexa Fluor 594 and Alexa Fluor 488 (Invitrogen). Nuclei were visualized by 4,6-diamidino-2-phenylindole staining (DAPI, Sigma-Aldrich, Saint Louis, MO). Alternatively to cleaved PARP antibody, TUNEL staining was performed according to the manufacturer's protocol (*Roche* Diagnostics Corp.; Indianapolis, IN). Slides were analyzed under Zeiss fluorescence microscope with ApoTome attachment (Axio Imager Z1 stand) and equipped with Axiocam camera and the AxioVision software. Apoptotic cells in the alveolar area were identified by double staining (PARP + VE-cadherin).

Bone marrow transplantation: Bone marrow transplantation was performed as previously described [6]. Recipient mice were lethally irradiated with 9.5 Gy and received an i.v. injection of 4 million donor bone marrow cells (isolated from WT mice) under ketamine / xylazine (100/5 mg/kg IP) anesthesia 24 hr after irradiation. To determine the transplantation efficiency, bone marrow cells were immunoblotted with anti-TRPM2 antibody. LPS injection and survival studies were performed 8 weeks after bone marrow transplantation.

Murine model of endotoxin-mediated mortality: Eight weeks following bone marrow transplantion, mice were challenged with LPS (30 mg/kg body weight) *via* intraperitonial injection. This LPS concentration (30 mg/kg BW) was established to be lethal. Mice were observed for feeding, movement and activity, grooming (smooth and shiny coats versus dull and ruffled coats) and survival for 96 h.

Statistical analysis. Mean values ± S.E.M were calculated for each experiment and statistical comparisons were made with the two-tailed Student's *t*-test. The significance of differences between groups was determined with a two-tailed *t*-test.

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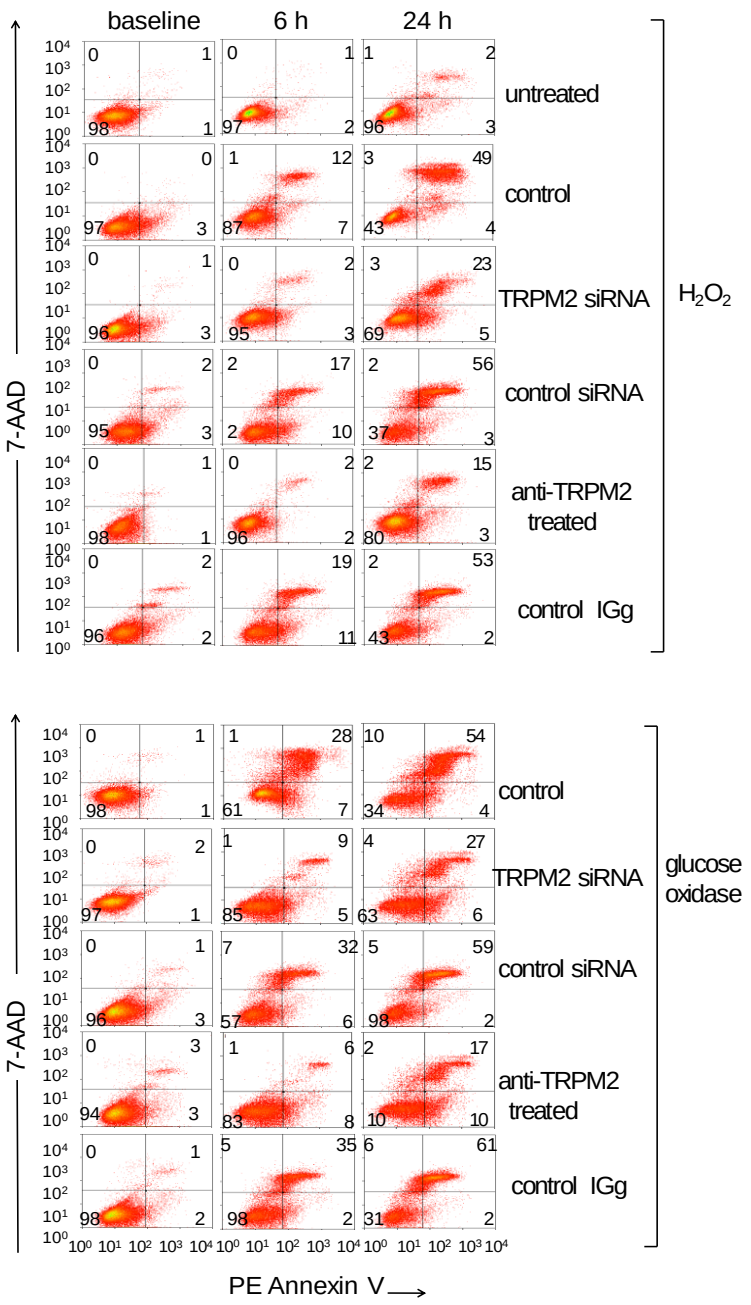
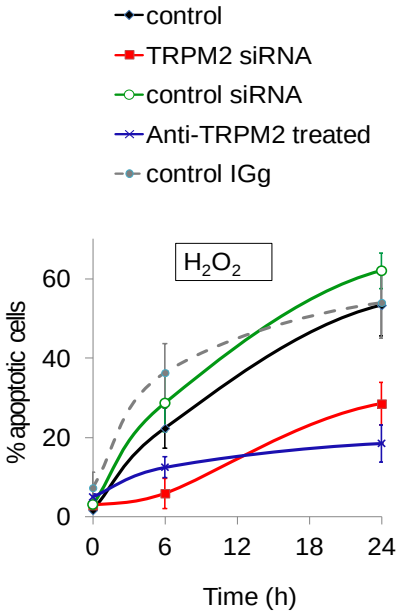
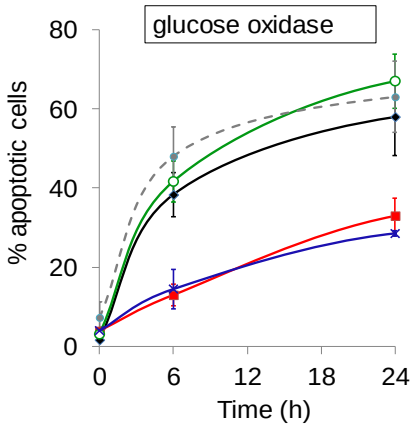
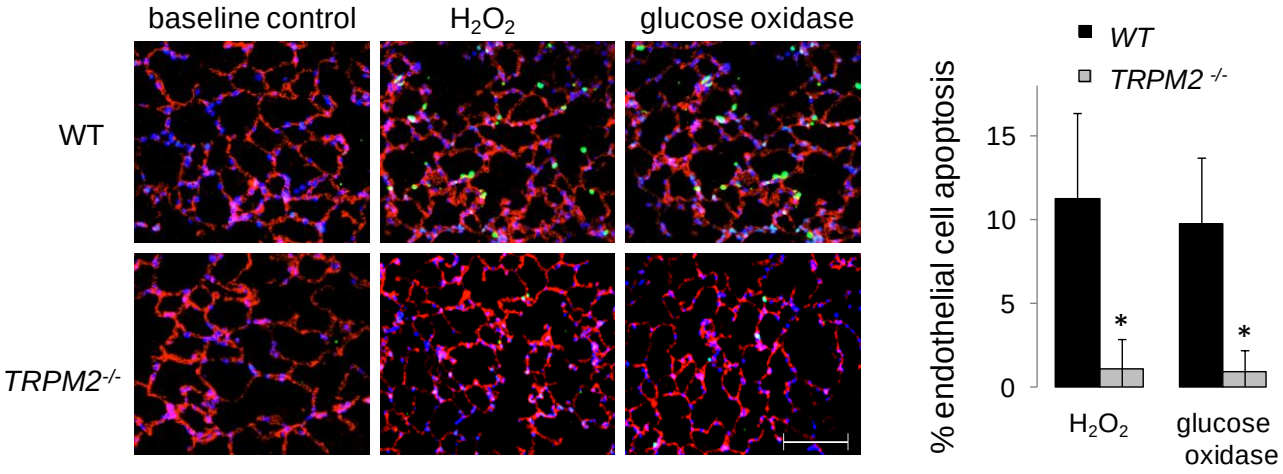


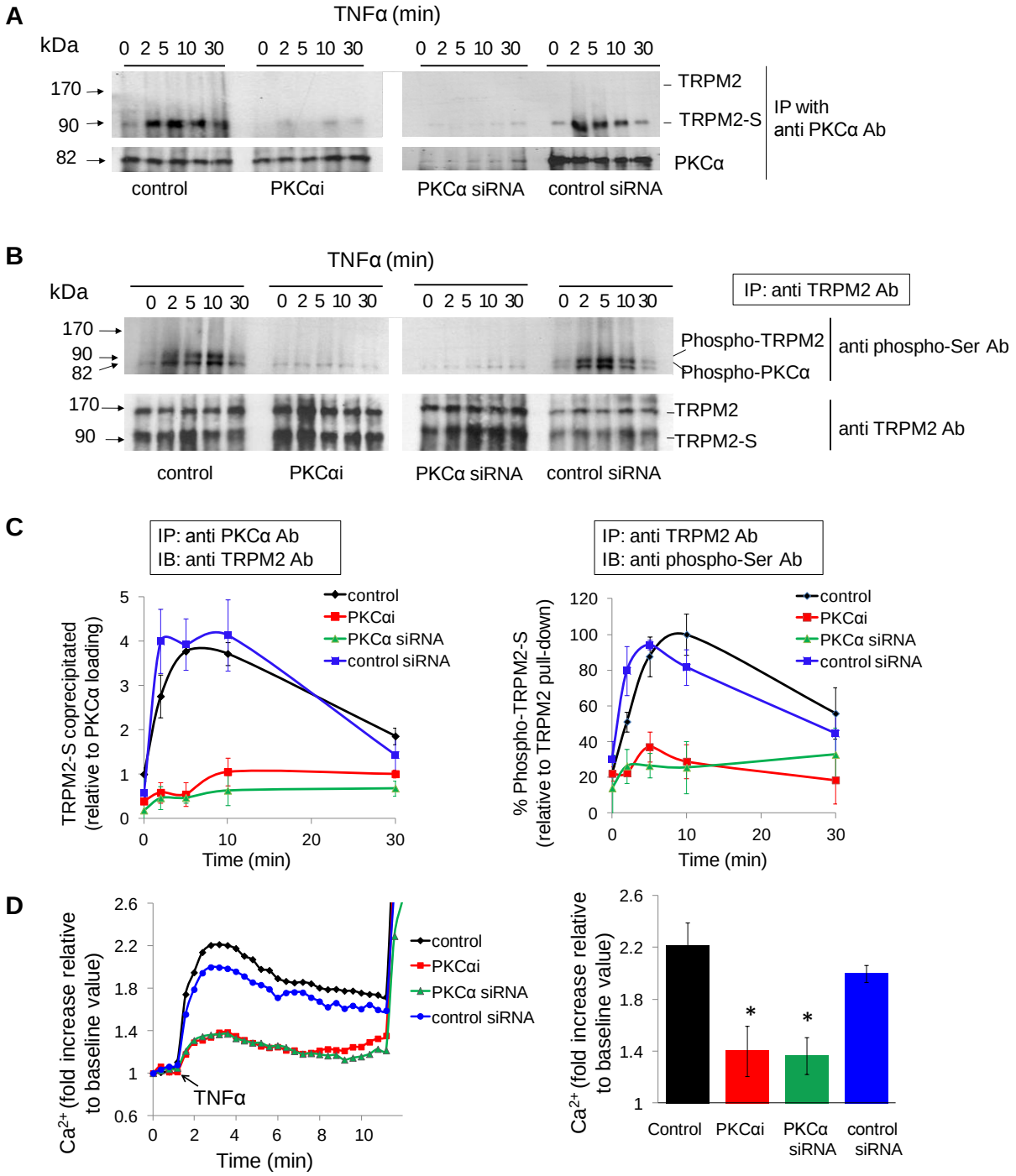




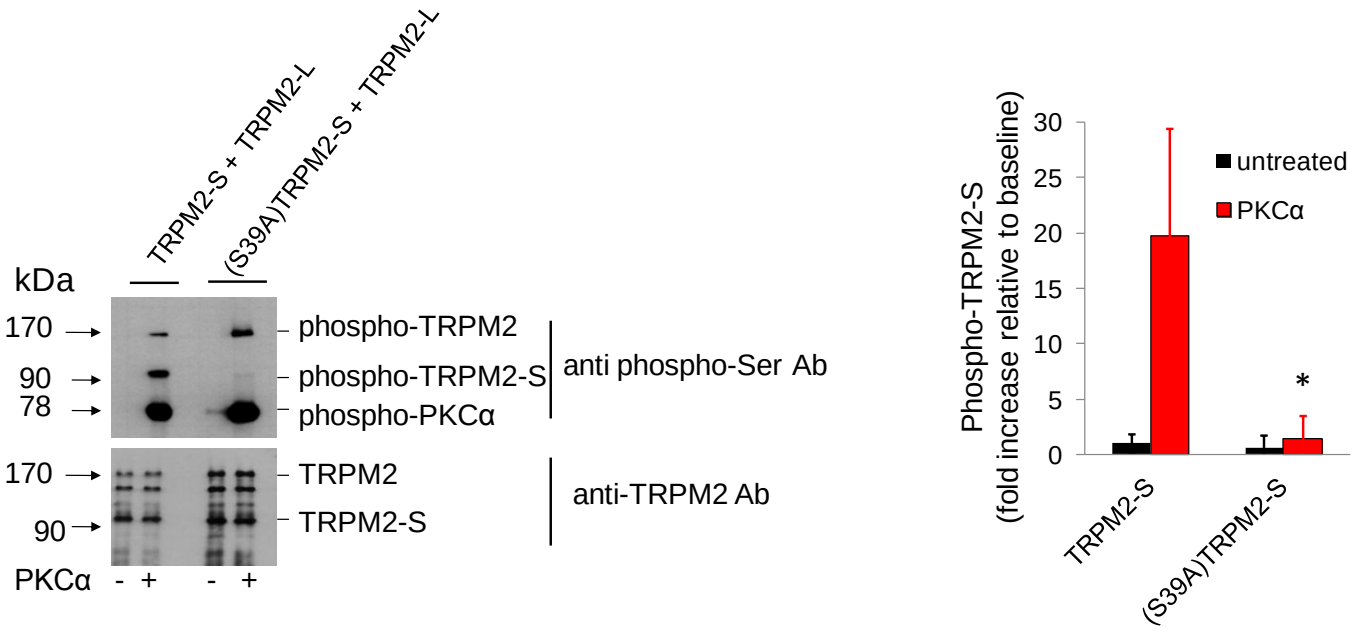
Online Figure I: TRPM2 mediates H_2O_2-induced apoptosis of endothelial cells. *Left*, representative flow cytometry histograms at 0, 6, or 24 h after exposure to 300 µmol/L H_2O_2 or glucose oxidase/glucose (90 min), with or without prior TRPM2 silencing or inhibition with TRPM2 blocking antibody. *Right panel*, mean percentage of apoptotic cells at 0, 6, or 24 h after H_2O_2 treatment (± SEM, n=3). The baseline values were not significantly altered by TRPM2 silencing or TRPM2 blocking Ab.



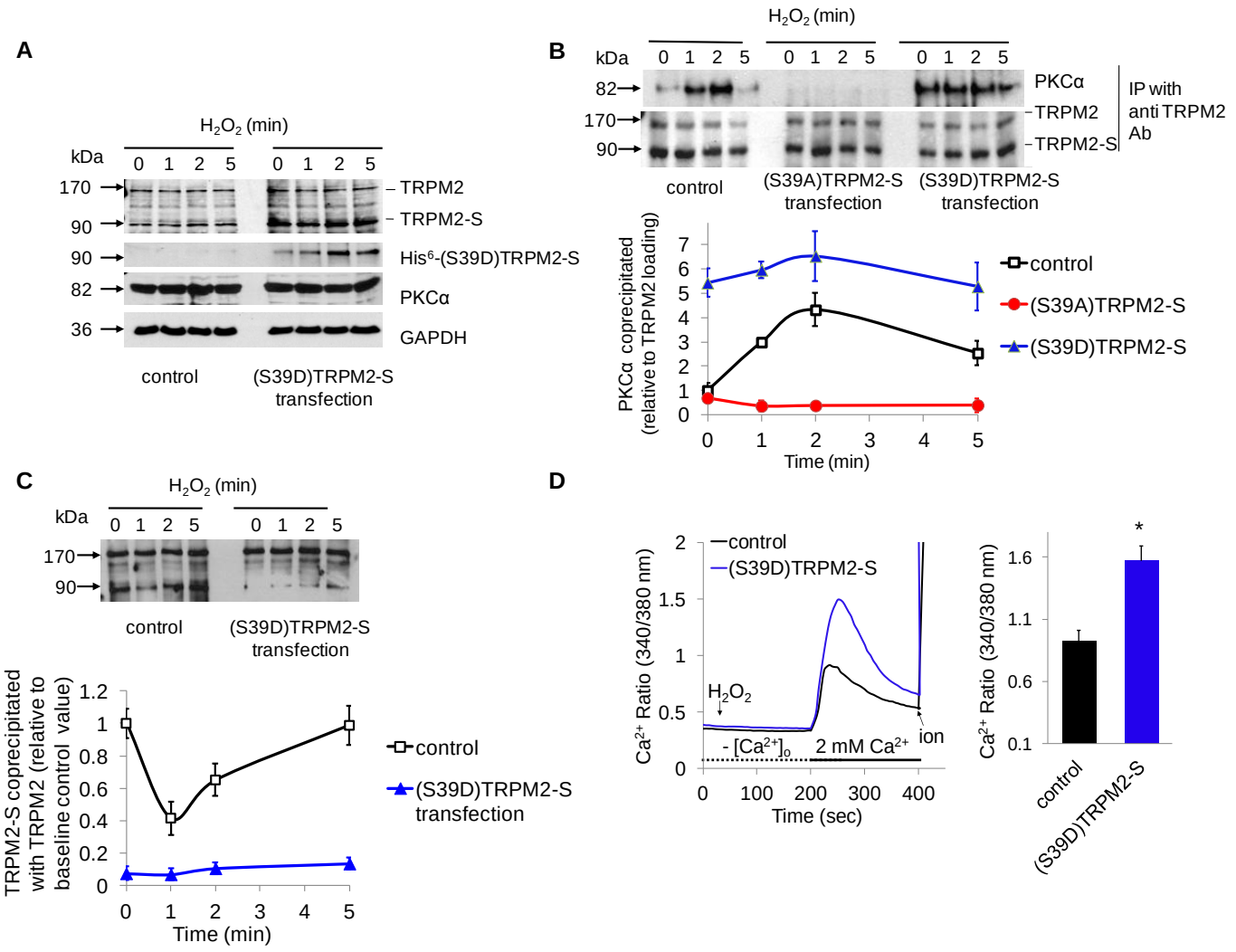
Online Figure II: **Apoptosis in lungs of *TRPM2*$^{-/-}$ vs. wild-type (WT) mice.** Apoptosis in lungs of *TRPM2*$^{-/-}$ and wild-type (WT) mice measured 3 h after perfusion with a solution containing H_2O_2 (300 µmol/L) or glucose oxidase/glucose (75 min). ***(A)*** Representative photomicrographs of TUNEL staining in the lung. TUNEL-positive cells are shown in green, VE-cadherin is counterstained in red (Alexa 594) and nuclear in blue (DAPI), (n=3). Scale bar: 50 µm. ***(B)*** percentagd apoptotic-positive endothelial cells; percentages were obtained from 2 fields/slide X 6 (±SEM; n=6). * p = 0.001 compared with control (*t-test*). Deletion of TRPM2 markedly reduced endothelial apoptosis in lungs of mice challenged with H_2O_2 or glucose oxidase/glucose compared to WT.



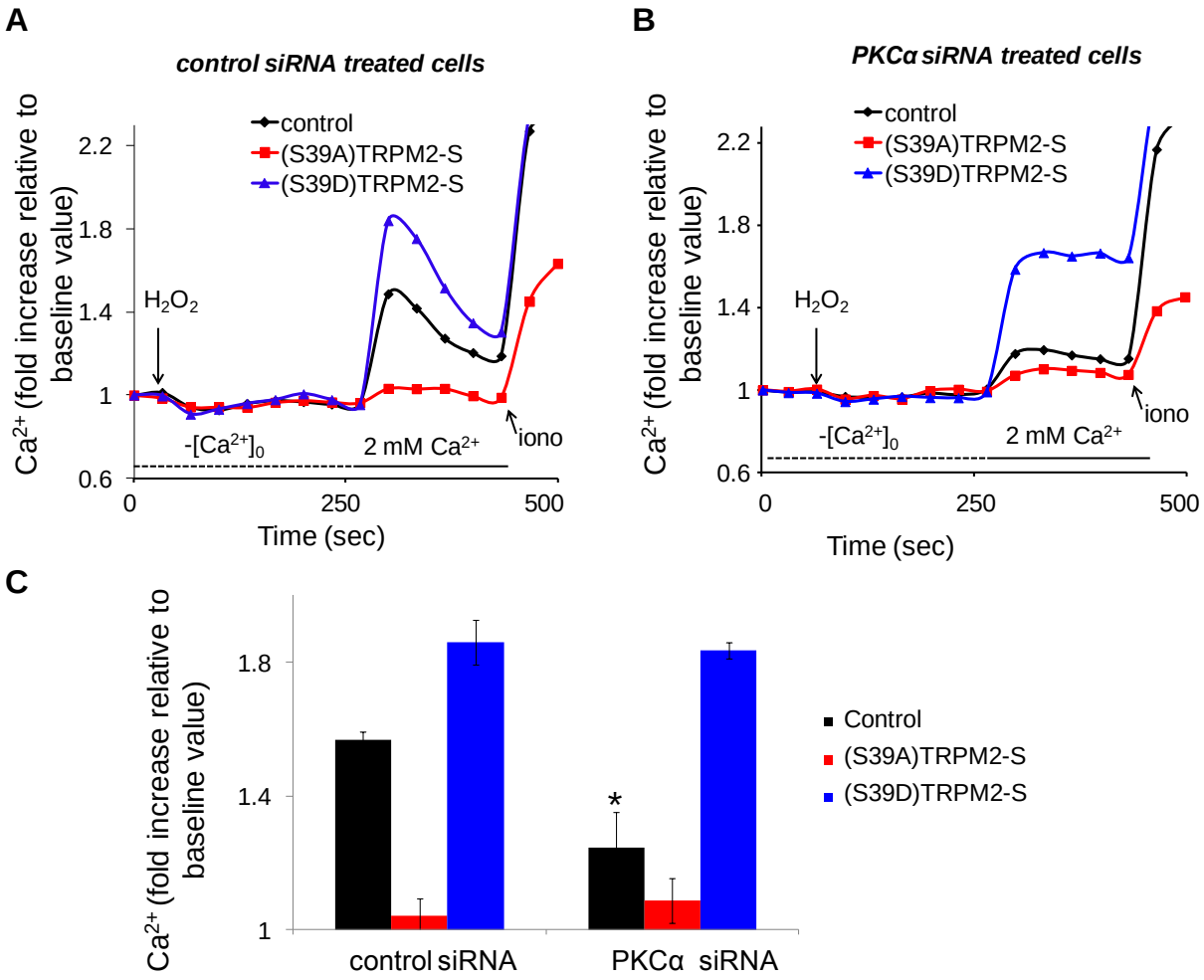
Online Figure III: TNFα induces PKCα phosphorylation of TRPM2-S and TRPM2-dependent Ca^{2+} entry in endothelial cells. HPAECs transduced with PKCα siRNA or pretreated with PKC inhibitors (100 nmol/L Gö6976, 1 µmol/L PKCαi) were challenged with 20 ng/mL TNFα for the indicated times at 37ºC. (**A**) TNFα induced the association of PKCα and TRPM2-S. PKCα was immunoprecipitated from cell lysates and co-immunoprecipitated TRPM2 was detected by Western blotting using an antibody recognizing both TRPM2 and TRPM2-S. TRPM2-S associated with PKCα following TNFα exposure whereas inhibition of PKCα prevented the association. (**B**) TNFα induced PKCα-dependent phosphorylation of 90kDa TRPM2-S splice variant. TRPM2 was immunoprecipitated from same cell lysates using an Ab that recognizes either TRPM2 isoform. Top panel: Blots showing phosphorylation of TRPM2 using monoclonal anti-phospho-Ser antibody. Successful immunoprecipitation of TRPM2 was verified using an anti-TRPM2 Ab (lower panel). (**C**), Mean densitometric values (± SEM; n=3-4) obtained in **A-B** showing that PKCα inhibition prevented TNFα-induced association of PKCα with TRPM2-S and phosphorylation of TRPM2-S. **(D)**, *Left,* Ca^{2+} mobilization assay using "the Fluor-3 Ca^{2+} indicator. *Right*, Summary of mean ratiometric data (± SEM) for the peak intracellular $[Ca^{2+}]_i$ (n = 6). *P ≤ 0.0005 vs. control (*t*-test). PKCα inhibition or deletion abrogated TNFα-elicited Ca^{2+} transients.



Online Figure IV: PKCα mediates phosphorylation of TRPM2-S at serine 39. His^6-tagged TRPM2-S or (S39A) TRPM2-S, and His^6-TRPM2 channel proteins purified from transfected-HEK cells were incubated with recombinant PKCα (active, 0.5µg) in a reaction buffer containing 50 µmol/L ATP for 30 min at 30 °C. **(A)** Representative Western blot for PKCα-dependent phosphorylation of these channel proteins analyzed using a specific anti-phospho serine antiboby. **(B)** Mean densitometric values of TRPM2-S and (S39)TRPM2-S serine phosphorylation relative to baseline untreated controls (± SEM; n =3). * p = 0.0001 compared with PKCα treated control (t-test).



Online Figure V. TRPM2-S Ser 39 phospho-mimetic mutant fails to bind TRPM2 and enhances H_2O_2-induced Ca^{2+} entry. The predicted PKCα phosphorylation site on TRPM2-S N-terminus at Ser 39 was mutated by Asp (phospho-mimetic substitution). HPAE monolayers transduced with mutant TRPM2-S (tagged on its carboxy-terminal end with poly-His residues) were grown to confluence and prepared for Western blot analysis (A through C) or intracellular Ca^{2+} measurements using fura-2 (D). (**A-C**)**.** Cells were exposed to 300 μM H_2O_2 for the indicated times. (**A**) Western blots for TRPM2, PKCα, and GAPDH expression in cells transduced with phosphomimetic construct. TRPM2 and anti-His^6 Abs confirmed expression of mutant TRPM2-S construct. (**B**) TRPM2 was immunoprecipitated from cell lysates using an Ab recognizing both forms of TRPM2 and co-immunoprecipitated PKCα protein was detected with an Ab. *Graph in B*, mean densitometric values (± SEM; n=3-4). Mutation of Ser 39 with Ala in TRPM2-S prevented TRPM2-S association with PKCα while mutation with Asp increased it. (**C**) TRPM2 was immunoprecipitated from cell lysates with an anti-TRPM2 Ab recognizing a region present only on the long isoform. The co-immunoprecipitated short isoform was then detected using an Ab that recognizes both TRPM2 and TRPM2-S. *Graph in C,* density of co-immunoprecipitated TRPM2-S was quantified as ratio to TRPM2 and plotted relative to the zero time value of untransfected control cells (mean ± SEM; n = 3). S39 phosphomimetic mutation of TRPM2-S prevented association of TRPM2-S with TRPM2 at time zero and following H_2O_2 addition. (**D**) Ca^{2+} mobilization assays were carried out using the "Ca^{2+} add-back" protocol. Transduction of phosphomimetic TRPM2-S mutant showed enhanced H_2O_2-induced Ca^{2+} entry (mean ± SEM; n = 6). * p = 0.0001 compared with control (*t*-test).



Online Figure VI. Expression of TRPM2-S phospho-mimetic mutant restores H_2O_2-induced Ca^{2+} entry in PKCα-depleted cells. HPAEC co-transduced with the mutant TRPM2-S and (A) control siRNA or (B) PKCα siRNA were prepared for intracellular Ca^{2+} measurements using "Ca^{2+} add-back" protocol with the Fluor-3 Ca^{2+} indicator. (**A)** In control cells, transduction of phosphomimetic TRPM2-S mutant showed enhanced H_2O_2-induced Ca^{2+} entry while transduction of the phospho-defective mutant decreased it (mean ± SEM; n = 6). (**B**) Depletion of PKCα decreased Ca^{2+} entry elicited by H_2O_2 compared to control cells; however, the peak Ca^{2+} transient was restored by expression of the (S39D)TRPM2-S but not (S39A)TRPM2-S. *(***C**) Summary of mean ratiometric data (± SEM) for the peak intracellular $[Ca^{2+}]i$ obtained in (A-B) (n = 5 to 6). *P ≤ 0.0005 *vs*. control siRNA (t-test).