Cooperative Interaction of *trp* Melastatin Channel Transient Receptor Potential (TRPM2) With Its Splice Variant TRPM2 Short Variant Is Essential for Endothelial Cell Apoptosis

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**Rationale:** Oxidants generated by activated endothelial cells are known to induce apoptosis, a pathogenic feature of vascular injury and inflammation from multiple pathogeneses. The melastatin-family transient receptor potential 2 (TRPM2) channel is an oxidant-sensitive Ca\(^{2+}\)-permeable channel implicated in mediating apoptosis; however, the mechanisms of gating of the supranormal Ca\(^{2+}\) influx required for initiating of apoptosis are not understood.

**Objective:** Here, we addressed the role of TRPM2 and its interaction with the short splice variant TRPM2 short variant (TRPM2-S) in mediating the Ca\(^{2+}\) entry burst required for induction of endothelial cell apoptosis.

**Methods and Results:** We observed that TRPM2-S was basally associated with TRPM2 in the endothelial plasmalemma, and this interaction functioned to suppress TRPM2-dependent Ca\(^{2+}\) gating constitutively. Reactive oxygen species production in endothelial cells or directly applying reactive oxygen species induced protein kinase C-\(\alpha\) activation and phosphorylation of TRPM2 at Ser 39. This in turn stimulated a large entry of Ca\(^{2+}\) and activated the apoptosis pathway. A similar TRPM2-dependent endothelial apoptosis mechanism was seen in intact vessels. The protein kinase C-\(\alpha\)-activated phosphoswitch opened the TRPM2 channel to allow large Ca\(^{2+}\) influx by releasing TRPM2-S inhibition of TRPM2, which in turn activated caspase-3 and cleaved the caspase substrate poly(ADP-ribose) polymerase.

**Conclusions:** Here, we describe a fundamental mechanism by which activation of the *trp* superfamily TRPM2 channel induces apoptosis of endothelial cells. The signaling mechanism involves reactive oxygen species–induced protein kinase C-\(\alpha\) activation resulting in phosphorylation of TRPM2-S that allows enhanced TRPM2-mediated gating of Ca\(^{2+}\) and activation of the apoptosis program. Strategies aimed at preventing the uncoupling of TRPM2-S from TRPM2 and subsequent Ca\(^{2+}\) gating during oxidative stress may mitigate endothelial apoptosis and its consequences in mediating vascular injury and inflammation. (Circ Res. 2014;114:469-479.)

**Key Words:** apoptosis • capillary permeability • endothelium • inflammation

Melastatin-like transient receptor potential 2 (TRPM2) is an oxidant-sensitive Ca\(^{2+}\)-permeable channel expressed in many cells, including neurons, \(1,2\) microglia, \(3,4\) multiple lung cell types, \(5,6\) pancreas \(\beta\) cells, \(7,9\) hematopoietic and immune cells, \(10,11\) and vascular endothelial (VE) cells. \(5\) However, the function of TRPM2 remains enigmatic. TRPM2 is activated by the generation of reactive oxygen species (ROS), such as H\(_2\)O\(_2\) and production of adenosine diphosphate ribose (ADPR) after DNA damage and activation of the enzyme poly(ADPR) polymerase. \(6,12\) TRPM2 has been implicated in mediating of oxidant-induced apoptosis secondary to Ca\(^{2+}\) influx that may initiate apoptosis program via the caspase pathway. \(1,13,14\) Although apoptosis is important in normal biological processes and development, apoptosis of endothelial cells, which have low turnover in vessels, \(15\) is a fundamental pathogenic feature of inflammatory and vascular diseases, such as acute lung injury \(16\) and sepsis. \(17\) Our studies have demonstrated a key role of TRPM2 in mediating oxidative injury of the endothelium, \(5\) resulting in disruption of endothelial barrier and tissue edema. \(18-20\) A component of endothelial disruption seen in these studies may well have been because of TRPM2-induced apoptosis.

TRPM2 channel opening after exposure to H\(_2\)O\(_2\) and other ROS is induced by the binding of ADPR to the Nudix box sequence motif (nucleoside diphosphate type motif 9 protein) in the carboxyl-terminal domain of TRPM2. \(5,6,10,12,21-23\)
new mechanism of endothelial apoptosis involving ROS-induced dant-induced apoptosis in endothelial cells. The study presents a with TRPM2 and how the component cooperated to signal oxi-
ruption of the interaction of TRPM2 with TRPM2-S and opening
a
interest is the short splice variant (TRPM2-S) that functions as
ating oxidant-induced apoptosis remains obscure. Of particular
isoform TRPM2 and the Ca2+-permeable pore present in TRPM2.28
which itself lacks both the carboxyl terminus present in the long
action of the apoptosis program.

Nonstandard Abbreviations and Acronyms

<table>
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<th>Abbreviation</th>
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<tr>
<td>ADPR</td>
<td>adenosine diphosphate ribose</td>
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<tr>
<td>FACS</td>
<td>fluorescence-activated cell sorting</td>
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<td>HPAEC</td>
<td>human pulmonary artery endothelial cells</td>
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<td>PARP</td>
<td>poly(ADPR) polymerase</td>
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<tr>
<td>PKCα</td>
<td>protein kinase C-α</td>
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<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
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<tr>
<td>S39A</td>
<td>serine 39 mutated to alanine</td>
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<tr>
<td>TNF-α</td>
<td>tumor necrosis factor-α</td>
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<tr>
<td>TRPM2</td>
<td>melastatin-like transient receptor potential 2</td>
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<tr>
<td>TRPM2-S</td>
<td>TRPM2 short variant</td>
</tr>
<tr>
<td>VE</td>
<td>vascular endothelial</td>
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\( \text{H}_2\text{O}_2 \) produced in the cell also also activated the production of ADPR, which functioned by binding to the TRPM2 Nudix motif. In addition, other mechanisms of TRPM2 activation, such as direct oxidative modification of the channel, have been proposed.26

Besides TRPM2, several splice variants of TRPM2 associated with TRPM2 in the plasma membrane have also been identified.3,4 Their role in regulating TRPM2 function and mediating oxidant-induced apoptosis remains obscure. Of particular interest is the short splice variant (TRPM2-S) that functions as a dominant-negative to inhibit TRPM2 channel activity but which itself lacks both the carboxyl terminus present in the long isoform TRPM2 and the Ca2+-permeable pore present in TRPM2.28 In cells in which both isoforms are expressed, TRPM2-S interact with TRPM2 to inhibit formation of functional homomerotropic channels.14 Here, we investigated the interaction of TRPM2-S with TRPM2 and how the component cooperated to signal oxidant-induced apoptosis in endothelial cells. The study presents a new mechanism of endothelial apoptosis involving ROS-induced and protein kinase C (PKC)-α phosphorylation-dependent disruption of the interaction of TRPM2 with TRPM2-S and opening of the channel to allow sufficient Ca2+ entry required for activation of the apoptosis program.

Methods

An expanded Materials and Methods is available in the online Data Supplement.

Endothelial Cell Culture and Transfection

Isolation of Mouse Endothelial Cells

Endothelial cells were isolated from lungs of wild-type (WT), PKCcα- (obtained from Dr Jeffrey D. Molkentin, University of Cincinnati, Cincinnati, OH) and TRPM2-/- mice (GlaxoSmithKline). The cells were used between passages 2 and 5.

Transfections

Human pulmonary artery endothelial cells (HPAEC; Clonetics, La Jolla, CA) were cultured in gelatin-coated flasks and used between passages 3 and 6. Human TRPM2-S splice variant, tagged with poly-His (His6-TRPM2-S), was inserted into a 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). Transfection of TRPM2-S constructs using fuGENE HD was verified by Western blotting. Control cells received vector alone.

siRNA Experiments

HPAECs were transiently transfected with TRPM2 or PKCα siRNAs (100 nmol/L; Santa Cruz Biotechnology, Santa Cruz, CA) using TransIT-TKO transfection reagent (Mirus, Madison, WI); nonspecific siRNA served as control (Ambion, Austin, TX). Transfection efficiency was >75%.

Immunoprecipitation and Phosphorylation Studies

Untransfected, His6-(S39A)TRPM2-S and His6-(S39D)TRPM2-S–transfected HPAEC cultures were treated with 300 μmol/L H2O2 for indicated times (37°C). In some experiments, cells were pretreated with 3,4-dihydro-5-[4-(1-piperidinyl)butoxy]-1(2H)-isouquinoline or PKC inhibitors 30 minutes before the assay. In other experiments, cells first received siRNA to suppress TRPM2 or PKCα expression. TRPM2 or PKCα immune complexes were precipitated with protein A-Sepharose beads (Sigma) for 2 h at 4°C as described.

Generation of H2O2 Using Glucose Oxidase/Glucose

H2O2 production in vitro was induced by glucose (1 mmol/L) and glucose oxidase (GO; 1–2.5 mU/mL) and was measured spectrophotometrically from the generation of resorufin (absorbance, 565 nm; extinction coefficient, 58,000 mol/L per cm). GO produced H2O2 at a constant rate (320 nmol/L/H2O2/min).

Analysis for Apoptosis

Apoptosis was identified by double-fluorescent staining with phycoerythrin annexin V-fluorescein isothiocyanate and 7-aminoactinomycin D, which detected apoptotic and dead cells, respectively. Confluent endothelial monolayers, without or with PKC inhibition or silencing, were incubated in 300 μmol/L H2O2 for 6 or 24 hours (37°C). Cells were washed twice with PBS and trypsinized; samples of 1×10^6 cells were incubated with 5 μL of phycocerythrin-labeled annexin V and 5 μL of 7-aminoactinomycin D (BD Bioscience, Rockville, MD) for 20 minutes at 24°C in the dark and analyzed with a Beckman Coulter CyAn II cytometer (Beckman Coulter, Miami, FL). We also assessed apoptosis in intact lung vascular endothelium by immunofluorescence and terminal deoxynucleotidyl transferase dUTP nick-end labeling assay. Lungs of TRPM2-/- and WT mice were perfused (2 mL/min; 37°C) for 3 hours with recirculation of Roswell Park Memorial Institute medium 1640 (5 mL) containing H2O2 (300 μmol/L) or GO. Lungs were removed and frozen by the optimal cut-
ting temperature method. Frozen lungs, sectioned (5 μm), were permeabilized in 3.7% formaldehyde, were permeabilized with 0.2% triton X-100. Tissue sections were blocked with 10% fetal bovine serum and incubated with goat anti–VE-cadherin and rabbit anti–poly(ADP-ribose) polymerase (PARP; ie, the cleaved 89-kDa fragment; 1:200 dilution) overnight (4°C). The sections were incubated with secondary antibodies conjugated to Alexa Fluor 488 and 594 (Invitrogen). As an alternative to cleaved PARP antibody, terminal deoxynucleotidyl transferase dUTP nick-end labeling was performed according to the manufacturer’s protocol (Roche Diagnostics Corp, Indianapolis, IN). Nuclei were visualized by 4,6-diamidino-2-phenylindole (Sigma-Aldrich, Saint Louis, MO). Slides were analyzed under a Zeiss fluorescence microscope using AxioVision software. Apoptotic cells were identified by double staining (PARP+VE-cadherin or terminal deoxynucleotidyl transferase dUTP nick-end labeling+VE-cadherin).

Statistical Analysis

Statistical comparisons were made with the 2-tailed Student t test. The significance level was P<0.05.

Institutional Study Approval

All studies were conducted after review by the Institutional Animal Care and Use Committee at the University of Illinois at Chicago where the work was performed and in accordance with the Policy on the Care, Welfare, and Treatment of Laboratory Animals.

Methods in Supplement

Methods for 
\([\text{Ca}^{2+}]_i\) measurements, TRPM2 protein purification, in vitro phosphorylation assay, bone marrow transplantation, murine
model of endotoxin-mediated mortality, and Western blotting are given in the Online Data Supplement.

Results

TRPM2 Is Required for H₂O₂-Induced Endothelial Cell Apoptosis

Apoptosis was determined by staining endothelial cells with annexin V-phycocerythrin and 7-aminoactinomycin D followed by fluorescence-activated cell sorting assessment (Figure 1A and 1B). H₂O₂ in a concentration-dependent manner induced apoptosis within 24 hours with an EC₅₀ value of 136±6 μmol/L (Figure 1A). Inhibition of TRPM2 by an anti-TRPM2 blocking antibody or TRPM2 siRNA silencing prevented the apoptosis (Figure 1B). The flow cytometry dot plot data demonstrating apoptosis are shown in Online Figure I. The sustained generation of H₂O₂ (320 nmol/L per minute for 90 minutes) by GO with glucose substrate also induced endothelial apoptosis, which was blocked by TRPM2 silencing or inhibition of channel activity (Figure 1B). To address in vivo relevance, we also examined apoptosis in endothelial cells of lung vessels in WT and TRPM2 knockout mice perfused with blood containing TRPM2-S, but not with TRPM2, and the response persisted up to 5 minutes (Figure 2B). Immunoprecipitation was reduced when PKCα activation was inhibited (Figure 2B). Treatment with PKCβII inhibitory peptide, used as control for nonspecific effects of Gö6976 in blocking activation of both PKCα and PKCβII, did not modify the H₂O₂-induced TRPM2-S association with PKCα (Figure 2B). PKCα-TTRPM2-S immunoprecipitation was also suppressed predictably by TRPM2 silencing (Figure 2B). Control experiments showed that transfection of endothelial cells with TRPM2 siRNA significantly reduced the expression of both TRPM2 and TRPM2-S (Figure 2A). Control experiments confirmed that siRNA effectively suppressed PKCα expression (Figure 2B). Treatment of cells with PKCα inhibitors (Gö6976 or PKCα inhibitor peptide) and PKCβII blocking peptide did not modify PKCα expression (Figure 2B).

Because H₂O₂ induces PKCα phosphorylation of TRPM2-S

We next determined the role of TRPM2 and its binding partner TRPM2-S in the mechanism of apoptosis. Western blotting showed that both TRPM2-S and TRPM2 (90 and 171 kDa, respectively) were basally expressed in HPAECs (Figure 2A) and in multiple other endothelial cells examined (unpublished observations). TRPM2 and TRPM2-S expressions were not modified by H₂O₂ exposure per se (Figure 2A). Using the motif scanning graphic software (Merck Genome Research Institute), we identified a putative high-affinity binding site for PKCα near the N terminus of TRPM2-S, at Ser 39. This site was predicted as a possible domain that could be phosphorylated by PKCα. We observed that PKCα was basally expressed in these cells, and its expression was not modified by H₂O₂ (Figure 2A). Depleting PKCα using siRNA did not modify the expression of either TRPM2 or TRPM2-S (Figure 2A), whereas TRPM2 depletion as expected suppressed the expression of its splice variants (Figure 2A).

To address whether H₂O₂ was involved in the phosphorylation at Ser39 on TRPM2-S, we used HPAEC monolayers treated with PKCα inhibitors (Gö6976 or PKCα blocking peptide [PKCα inhibitor peptide]) or transfected with siRNA to knockdown PKCα expression. PKCα was immunoprecipitated from lysates of cells exposed to H₂O₂, and immunoprecipitated TRPM2 and TRPM2-S were detected using an antibody recognizing each form (Figure 2B). H₂O₂ rapidly induced the association of PKCα with TRPM2-S, but not with TRPM2, and the response persisted up to 5 minutes (Figure 2B). Immunoprecipitation was reduced when PKCα activation was inhibited (Figure 2B). Treatment with PKCβII inhibitory peptide, used as control for nonspecific effects of Gö6976 in blocking activation of both PKCα and PKCβII, did not modify the H₂O₂-induced TRPM2-S association with PKCα (Figure 2B). PKCα-TRPM2-S immunoprecipitation was also suppressed predictably by TRPM2 silencing (Figure 2B). Control experiments showed that transfection of endothelial cells with TRPM2 siRNA significantly reduced the expression of both TRPM2 and TRPM2-S (Figure 2A). Control experiments confirmed that siRNA effectively suppressed PKCα expression (Figure 2B). Treatment of cells with PKCα inhibitors (Gö6976 or PKCα inhibitor peptide) and PKCβII blocking peptide did not modify PKCα expression (Figure 2B).

Because the above studies dealt with the role of H₂O₂ in activating the phosphorylation of TRPM2-S, we next examined whether key alterations could be replicated using a physiological stimulus to generate oxidants. Here, we used tumor necrosis factor-α (TNF-α), a generator of intracellular oxidants and potent inducer of endothelial cell apoptosis. We observed that TNF-α induced the association of PKCα with TRPM2-S, whereas suppressing PKCα activity prevented the association (Online Figure IIIA).

To determine whether PKCα was responsible for phosphorylating TRPM2-S after H₂O₂ challenge, in other studies TRPM2 proteins in cell lysates were precipitated using antibodies recognizing TRPM2 and TRPM2-S, and phosphorylated proteins were visualized using antiphospho-Ser antibody. Western blotting demonstrated that only TRPM2-S was phosphorylated, which occurred within 1 minute of H₂O₂ exposure with maximum response seen at 2 minutes, whereas there was no phosphorylation of TRPM2 (Figure 2C). Western blotting also showed that TRPM2-S but not TRPM2 was phosphorylated within the same time frame after TNF-α exposure (Online Figure IIIB). Phosphorylation of PKCα (82 kDa) was detected as a comigrating band on the gel (seen in top blot of Figure 2C; Online Figure IIIB), an indication that the kinase was in the active state. We next determined phosphorylation of PKCα using an antibody recognizing phosphorylated on Ser 657, the crucial PKCα catalytic domain. H₂O₂ rapidly induced phosphorylation of PKCα at this site and the phosphorylated PKCα co-migrated with TRPM2 (middle blot; Figure 2C). Treatment with Gö6976 (but not with control PKCβII inhibitor peptide) inhibited not only PKCα phosphorylation but also H₂O₂-induced phosphorylation of TRPM2-S (Figure 2C). These results thus show time-dependent and reversible association between TRPM2-S and PKCα induced by PKCα activation (Figure 2D).
Phosphorylation of TRPM2-S Activates TRPM2 and Supranormal Ca\(^{2+}\) Influx

We used the Fura-2 dye to study the Ca\(^{2+}\) entry response activated by TRPM2 interaction with TRPM2-S, in addition, we used the Ca\(^{2+}\) add-back protocol to rule out any indirect effects of \(\text{H}_2\text{O}_2\) on Ca\(^{2+}\) entry secondary to Ca\(^{2+}\)-store depletion. In the absence of extracellular Ca\(^{2+}\), \(\text{H}_2\text{O}_2\) did not produce a Ca\(^{2+}\) transient (Figure 3A and 3B), indicating that \(\text{H}_2\text{O}_2\) did not deplete intracellular Ca\(^{2+}\) stores. By contrast, extracellular Ca\(^{2+}\) repletion in the continued presence of \(\text{H}_2\text{O}_2\) elicited a sharp and marked increase in intracellular Ca\(^{2+}\) concentration secondary to Ca\(^{2+}\) entry (Figure 3A and 3B). TRPM2 knockdown markedly suppressed the Ca\(^{2+}\) transient (Figure 3A and 3B), showing that \(\text{H}_2\text{O}_2\)-induced Ca\(^{2+}\) entry required TRPM2. G66976 significantly decreased the amplitude of Ca\(^{2+}\)-repletion-dependent transients by 66±9%, and PKC\(\alpha\) inhibitor peptide reduced Ca\(^{2+}\) transient by 46±10% (Figure 3A and 3B). PKC\(\alpha\) silencing also reduced Ca\(^{2+}\) entry...
by 43±6% (Figure 3A and 3B). Treatment of cells with control PKCβII peptide inhibitor, however, did not modify H2O2-activated Ca2+ entry via TRPM2 channels (Figure 3A and 3B). Along the same lines, TNFα-induced Ca2+ entry in endothelial cells (Online Figure IID) was also decreased by inhibiting PKCα.

We next addressed the role of the S39 phosphoswitch on TRPM2-S in mediating TRPM2 channel activity. Here, we determined whether mutation of TRPM2-S at Ser 39 (S39A), the PKCα phosphorylation site disrupted Ca2+ signaling. The mutant was tagged on its C terminus with a poly-His fusion protein. Transfected HPAECs showed protein expression of the (S39A)-TRPM2-S mutant (Figure 4A). Western blotting showed that S39A mutation of TRPM2-S abrogated the migration of TRPM2-S with PKCα on the gel (Figure 4B) and phosphorylation of TRPM2-S by PKCα after H2O2 challenge (Figure 4C). PKCα also did not migrate with (S39A)-TRPM2-S mutant (Figure 4C).

We next determined the functional significance of the failure of PKCα to bind to and phosphorylate TRPM2-S on the TRPM2-mediated Ca2+ entry. Intracellular Ca2+ transient elicited by H2O2 was markedly reduced in cells transduced
with the TRPM2-S phosphodefective mutant (Figure 4D).

To validate the finding that PKCα was indeed responsible for phosphorylation of TRPM2-S at S39, we treated an extract of native protein with recombinant active PKCα. We observed that active PKCα induced phosphorylation of WT TRPM2-S but not of S39A mutant (Online Figure IV).

Figure 3. Protein kinase C-α (PKCα) and melastatin-like transient receptor potential 2 (TRPM2) cooperation mediates Ca²⁺ entry in endothelial cells required for signaling apoptosis. A, Ca²⁺ repletion transients generated by Ca²⁺-add-back in the presence of H₂O₂. Cultured human pulmonary artery endothelial cells (HPAECs) were loaded with Fura-2 Ca²⁺ dye, washed, and transferred to Ca²⁺-free medium. In control cells, H₂O₂ (100 µmol/L) elicited a marked Ca²⁺ transient on Ca²⁺ repletion (red trace). Ca²⁺ transients were blocked by TRPM2 silencing (gray trace) and reduced by Gö6976 (100 nmol/L; green trace), PKCα inhibitor peptide (PKCαi; 1 µmol/L; cyan trace), or after PKCα silencing (yellow trace); PKCβII (1 µmol/L, navy trace) and control siRNA (purple trace) had no effect. Ordinate gives [Ca²⁺]i as 340:380 nm ratio. B, Summary of mean ratiometric data (±SEM) for the peak intracellular [Ca²⁺]i obtained in (A) (n=3–5). *P≤0.0002 vs control (t test).

Figure 4. Protein kinase C-α (PKCα) binds melastatin-like transient receptor potential 2 short variant (TRPM2-S) at Ser 39 and activates apoptosis-inducing Ca²⁺ entry signal in endothelial cells. The sole predicted PKCα phosphorylation site near the TRPM2-S N terminus at Ser 39 was mutated by Ala substitution (resulting in phosphodefective mutant). Human pulmonary artery endothelial cell monolayers transduced with mutant TRPM2-S (tagged on its carboxy-terminal end with poly-His residues) were grown to confluence for Western blot analysis (A–C) or intracellular Ca²⁺ measurements using fura-2 (D). A–C, Cells were exposed to 300 µmol/L H₂O₂ for the indicated times. A, Western blots for TRPM2, PKCα, and GAPDH expression in cells transduced with phosphodefective construct. Transfected protein was detected with an anti-His6 Ab confirming the expression of mutant TRPM2-S construct. B, PKCα was immunoprecipitated from cell lysates with an antibody and coimmunoprecipitated TRPM2 protein was detected using an Ab recognizing both forms of TRPM2. Graph in B shows mean densitometric values (±SEM; n=3–4). Mutation of Ser 39 with Ala in TRPM2-S prevented PKCα association with TRPM2-S. C, TRPM2 was immunoprecipitated from the same lysates and phosphorylated TRPM2 was detected using antiphospho-Ser Ab. Graph in C shows mean densitometric values (±SEM; n=3–4). Ala substitution at Ser 39 abrogated H₂O₂-induced phosphorylation of TRPM2-S confirming the importance of the PKCα phosphorylation site on TRPM2-S at Ser 39. D, Ca²⁺ mobilization assay was performed using the Ca²⁺-add-back protocol. Transduction of phosphodefective TRPM2-S mutant suppressed H₂O₂-induced Ca²⁺ entry. *P=0.0001 compared with control (t test). (n=3 per bar); error bars, ±SEM.
Regulated TRPM2 Gating and Apoptosis

PKCα Phosphorylation of TRPM2-S Induces TRPM2-S Dissociation From TRPM2

We next determined whether PKCα phosphorylation of TRPM2-S in some manner interfered with TRPM2-S association with TRPM2, thus permitting TRPM2 to gate Ca2+ at sufficient level to activate the apoptosis program. Because TRPM2 variants generated by alternative splicing differed only in their C terminal,28 we immunoprecipitated TRPM2 from cell lysates using an anti-TRPM2 antibody, recognizing the region present solely in TRPM2 form. TRPM2-S, which in the plasma membrane basally associated with TRPM2, dissociated within minutes from TRPM2 after H2O2 addition (Figure 5A).

Inhibition of PKCα activation suppressed this TRPM2-S dissociation from TRPM2 (Figure 5A). S39A mutation of TRPM2-S also suppressed the dissociation of TRPM2 from TRPM2-S (Figure 5A). PKCα-dependent phosphorylation of TRPM2-S at Ser 39 blocked the interaction of TRPM2-S with TRPM2 (summarized in Figure 5B). These results show that phosphorylation of TRPM2-S at Ser 39 was responsible for releasing the TRPM2-S inhibition of TRPM2 and thus mediated the increased Ca2+ entry needed for apoptosis.

To reinforce the crucial role of TRPM2-S S39 phosphorylation in mediating TRPM2 channel activity, we mutated TRPM2-S S39 to aspartate (S39D) to mimic the effects of phosphorylation. This poly-His tagged phosphomimetic mutant was expressed in HPAECs (Online Figure VA), and we examined its ability to associate with TRPM2, and influence Ca2+ entry. Western blotting showed that phosphomimetic of TRPM2-S promoted TRPM2-S interaction with PKCα (Online Figure VB), but impaired its association with TRPM2 (Online Figure VC) under basal condition in the absence of H2O2 and after H2O2 challenge. Moreover, S39D mutation of TRPM2-S enhanced TRPM2-mediated Ca2+ entry after H2O2 challenge (Online Figure VD) consistent with the key role of PKCα phosphorylation of TRPM2-S in activating TRPM2 channel activity.

To elucidate the mechanism of PKCα regulation of TRPM2 channel activity further, we next coexpressed the TRPM2-S phosphorylation mutants with either PKCα siRNA or control siRNA in endothelial cells (Online Figure VI). Expression of (S39A)-TRPM2-S phosphodeficient mutant in control siRNA-transduced cells as expected inhibited H2O2-elicted Ca2+ entry when compared with control cells, whereas expression of phosphomimetic mutant enhanced this response. The decreased H2O2-activated Ca2+ entry caused by depletion of PKCα was restored by expression of the (S39D)-TRPM2-S but not the (S39A)-TRPM2-S mutant, consistent with the essential role of PKCα phosphorylation of TRPM2-S in activating TRPM2 channel activity.

PKCα Mediates H2O2-Induced Apoptosis Through Activation of TRPM2

Fluorescence-activated cell sorting analysis showed that inhibition of PKCα activation or its silencing protected the cells from H2O2-induced apoptosis (Figure 6A and 6B). The role of PKCα in regulating TRPM2-mediated apoptosis was also seen in endothelial cells transduced with the (S39A)-TRPM2-S mutant (Figure 6A). Using lung endothelial cells cultured from TRPM2 or PKCα knockout mice to validate the above studies in human endothelial cells, we observed normal expression of TRPM2 in endothelial cells from PKCα knockout mice and normal expression of PKCα in endothelial cells from TRPM2 knockout mice (Figure 6A and 6B). The H2O2-induced Ca2+ entry was virtually abolished in TRPM2-null cells and was reduced by 40% in PKCα-null cells (Figure 6C). As in the human cells, H2O2-mediated apoptosis in WT mouse endothelial cells was concentration dependent (Figure 6D). Deletion of TRPM2 caused 2.4-fold rightward shift in the concentration–response curve for H2O2-induced apoptosis (EC50 shift, 213–553 µmol/L), indicating the crucial role of TRPM2 in mediating H2O2-induced apoptosis (Figure 6D). Deletion of the PKCα gene similarly inhibited apoptosis (EC50 shift, 213–512 µmol/L; Figure 6D).

Deletion of PKCα Gene in Mice Reduces TRPM2-Induced Endothelial Cell Apoptosis Improves Survival in Endotoxia

To address the pathophysiological significance of PKCα phosphorylation of TRPM2 channel activity in mediating apoptosis in vivo, we examined the apoptosis response in mouse lung endothelial cells and survival of mice after intraperitoneal challenge with lipopolysaccharide (30 mg/kg), the Gram-negative bacterial endotoxin, which produces ROS in endothelial cells.34,35 Because TRPM2 and PKCα expressed in myeloid cells may also play a role in ROS production and apoptosis,36 we generated chimeric mice in which the PKCα- and TRPM2-deficient mice were transplanted with bone marrow cells from WT mice. These mice showed comparable TRPM2 and PKCα protein expression as WT (Figure 7A). We observed that either TRPM2 or PKCα...
deletion markedly reduced endothelial cell apoptosis in lungs 4 hours after lipopolysaccharide treatment when compared with WT mice (Figure 7B). In a positive control experiment, administration of the oxidant scavenger Tempol in mice 30 minutes before lipopolysaccharide also reduced oxidant-mediated lipopolysaccharide-induced apoptosis (Figure 7B). In addition, deletion of either TRPM2 or PKCα significantly improved survival rate of lipopolysaccharide-challenged mice (Figure 7C).

Discussion

In the present study, we addressed the role of the ROS-activated TRPM2 channel in mediating endothelial cell apoptosis. We identified that the interaction of the 171-kDa TRPM210,12,27,37 with its 90-kDa splice variant TRPM2-S28 in the endothelial cell plasma membrane. This interaction functioned constitutively to restrain TRPM2 Ca2+ entry. However, ROS-induced activation of PKCα and resulting phosphorylation of TRPM2-S at Ser39 released the TRPM2-S inhibition of TRPM2 to induce the large Ca2+ influx required for activation of the caspase apoptosis program.

PKCα phosphorylation of TRPM2-S and the dissociation of TRPM2-S from TRPM2 increased the Ca2+ concentration in endothelial cells to 4-fold the baseline levels within the range of the Ca2+ burst required to signal apoptosis, which has the intracellular Ca2+ concentration threshold of 200 to 500 nmol/L.38 Inhibition of PKCα by preventing the phosphorylation of TRPM2-S reduced Ca2+ entry through TRPM2 by half this level, well below the Ca2+ threshold required for activation of caspase-mediated apoptosis.

A stop codon (TAG) on the TRPM2 gene is located at the splice junction between exons 16 and 17; hence, alternative splicing resulted in deletion of the 4 C-terminal transmembrane domains in TRPM2-S, the putative Ca2+-permeable pore region.28 The observation that TRPM2-S served as a negative regulator of endothelial cell apoptosis. H2O2 exposure or oxidants generated by mediators, such as TNF-α, induced the interaction between PKCα and TRPM2-S permitting the channel to open for Ca2+ entry. Thus, TRPM2-S only functioned to induce apoptosis when PKCα was activated and induced TRPM2-S phosphorylation. That TRPM2-S mediated Ca2+ gating through heterodimerization with TRPM2 is reminiscent of the finding in melanocytes that another splice variant member of the trp gene family TRPM1 interacted with full-length long form of TRPM1 to suppressed its activity.39

PKCα activation was shown to be crucial for the mechanism of H2O2-induced apoptosis through its binding to and phosphorylation of TRPM2-S. Mutation of the sole PKCα phosphorylation site on Ser39 of TRPM2-S N terminus to
Ala resulted in failure of PKCα to phosphorylate TRPM2-S. This mutation in turn prevented PKCα to phosphorylate TRPM2-S and hence the apoptosis-inducing Ca2+ entry signal. Both K562 myeloid leukemia cell line that do not express the TRPM2-S14 and Jurkat t-lymphocyte cell line that expresses the short isoform at very low levels also did not undergo apoptosis secondary to TRPM2 activation, consistent with the critical role of TRPM2-S as the apoptosis-suppressing partner of TRPM2. Although PKCα activation contributed to TRPM2-induced endothelial apoptosis in the present through phosphorylation of TRPM2-S, it is important to note that PKCα signaling in other signaling pathways can induce endothelial injury. We have shown that PKCα phosphorylation of p120-catenin mediates dissociation of p120-catenin from VE-cadherin that resulted in disassembly of adherens junctions and disruption of VE barrier function. These studies showed the role of PKCα in mediating phosphorylation of p120-catenin in response to endotoxin and resultant increased lung vascular permeability. Thus, PKCα can function in a complex matter at multiple levels to induce endothelial dysfunction either via injury or through activating the apoptosis program.
blebbing, cell shrinkage, and apoptotic body formation. PARP is part of a protective mechanism involved in repair of DNA damage and DNA stability.29 Inactivation of PARP by cleavage of the enzymatic domain after oxidative activation of TRPM2 also caused apoptosis similar to that seen with PKCα-induced uncoupling of TRPM2-S from TRPM2 in the present study.

We have uncovered in these studies a novel mechanism of TRPM2 activation resulting in Ca2+ entry secondary to PKCα-induced phosphorylation of TRPM2-S. A question arises about the relationship of this mechanism with TRPM2 activation induced by the generation of ADPR after the activation of poly(ADPR) polymerase.6,12 It is possible that both mechanisms function to activate TRPM2 secondary ROS stimulation (see Model Figure 8). ADPR generation after activation of poly(ADPR) polymerase may help to amplify the Ca2+ entry response. However, in the event that both TRPM2-S and TRPM2 are coexpressed as they are in endothelial cells, it is likely as the present results show that TRPM2-S functions by restraining the activity of TRPM2 (and hence suppresses apoptosis). However, when TRPM2-S is not expressed or poorly expressed, ADPR binding to Nudix box sequence would by default be the primary mechanism of TRPM2 activation, but it is not clear whether Ca2+ entry by this mechanism is sufficient to activate the proapoptotic caspases.

In summary, we identified a fundamental relationship between oxidant-activated TRPM2 channel and its associated short splice variant TRPM2-S in the gating of large Ca2+ influx and the critical role of loss of this interaction in mediating oxidant-induced apoptosis of endothelial cells. We demonstrated that apoptosis induced by this mechanism contributed to the mortality seen in endotoxin-challenged mice. PKCα functioned to induce phosphorylation of TRPM2-S, which prevented its association with TRPM2, and thereby activated Ca2+ gating and caspases. Thus, disabling TRPM2-S and TRPM2 interaction such as by inhibiting PKCα activation represents a novel strategy for abrogating apoptosis and resultant vascular injury and inflammation associated with apoptosis in diseases, such as acute lung injury and vascular inflammation.

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Disclosures

None.

References

Novelty and Significance

**What Is Known?**

- Oxidants induce injury to the vascular endothelium resulting in endothelial denudation, permeability alterations, edema formation, and inflammation.
- Endothelial cell loss via apoptosis is a crucial feature of vascular injury and is implicated in the mechanism of lung injury induced by sepsis and other vascular diseases.
- Activation of the cation (primarily Ca\(^{2+}\)) permeable melastatin transient receptor potential 2 (TRPM2) channel during oxidative stress is linked to cell death.

**What New Information Does This Article Contribute?**

- Oxidant activation of TRPM2 mediates lung endothelial cell apoptosis and is critically regulated by protein kinase C-\(\alpha\) (PKC\(\alpha\)).
- TRPM2 in endothelial cells is normally impermeable to Ca\(^{2+}\) because of its binding to the short splice variant TRPM2 short variant (TRPM2-S).
- Oxidants induce PKC\(\alpha\) phosphorylation of TRPM2-S at Ser 39, which functions by releasing TRPM2-S inhibition of TRPM2 channel and thereby induces Ca\(^{2+}\) entry and sequestration to activate the apoptotic program.

Endothelial cell apoptosis is a crucial feature of vascular injury that leads to disruption of the endothelial barrier and to inflammation. Understanding the molecular mechanisms regulating apoptosis is vital for identifying novel targets for treating vascular injury and inflammatory disorders. We have uncovered a role of PKC\(\alpha\) phosphorylation of the short splice variant of TRPM2, TRPM2-S, which acts as a phosphoswitch to regulate channel activity and results in calcium overload. Phosphorylation of TRPM2-S at Ser 39 caused release of TRPM2-S inhibition of TRPM2 and thereby activated Ca\(^{2+}\) gating. This unique mechanism of TRPM2 channel activation was crucial for induction of oxidant-mediated apoptosis of endothelial cells. Thus, oxidant-induced gating of Ca\(^{2+}\) via TRPM2 and apoptosis are critically dependent on PKC\(\alpha\) phosphorylation of TRPM2-S at a specific site identifying a novel potential anti-inflammatory target.
Cooperative Interaction of trp Melastatin Channel Transient Receptor Potential (TRPM2) With Its Splice Variant TRPM2 Short Variant Is Essential for Endothelial Cell Apoptosis
Claudie M. Hecquet, Min Zhang, Manish Mittal, Stephen M. Vogel, Anke Di, Xiaopei Gao, Marcelo G. Bonini and Asrar B. Malik

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**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 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/acetoxyethyl ester (AM) was obtained from Molecular Probes (Eugene, OR). The myristoylated PKCa peptide inhibitor Myr-RFARKGALRQKNV was from Promega (Madison, WI). H$_2$O$_2$, 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 $^2$ and the other recognizing both TRPM2 and TRPM2-S isoforms) were purchased from Abcam (Cambridge, MA) $^5$. His$^6$ PKCa 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, PKCa$^{-/-}$ (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$^5$-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$^5$-TRPM2-S construct using the QuikChange site-directed mutagenesis protocol (Stratagene), and was verified by sequencing. HPAE cell cultures, grown to 60-80% confluence,
were transfected with 1 μg/ml each of His<sup>6</sup>-(S39A)TRPM2-S or His<sup>6</sup>-(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 PKCa 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 PKCa was verified by Western Blot.

**Stable transfection of human HEK293 cells:** HEK293 cells grown at 37°C with 5% CO<sub>2</sub> 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<sup>6</sup>-TRPM2) and inserted into pcDNA6 expression vector (Invitrogen) and either His<sup>6</sup>-TRPM2-S or His<sup>6</sup>-(S39A)TRPM2-S plasmids using the FuGENE HD transfection reagent. The successfully 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 PKCa 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<sup>2+</sup>]i measurements.**

*Ca<sup>2+</sup>* 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<sup>2+</sup>-sensitive fluorescence indicator and incubated for 2 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, PKCa, His<sup>6</sup>, phospho-Ser, or actin). Band intensity was determined by densitometry using Image J (NIH).

**Immunoprecipitation and phosphorylation studies.** Untransfected and His<sup>6</sup>-(S39A)TRPM2-S transfected HPAE cultures in six-well culture dishes were treated with 300 μM H<sub>2</sub>O<sub>2</sub> 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 PKCa. Following H<sub>2</sub>O<sub>2</sub> 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-PKCa) overnight at 4°C. TRPM2 or PKCa 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% bromphenol 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<sup>6</sup>-TRPM2-S/ His<sup>6</sup>-TRPM2 transfected HEK cells pelleted from 50 ml tissue culture were resuspended in 8 ml of native binding buffer (50 mM NaH<sub>2</sub>PO<sub>4</sub>, 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 x 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<sup>6</sup>-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 Hapes (pH 7.4), 1 mmol/L DTT, 10 mmol/L MgCl<sub>2</sub>, 0.2 mmol/L Na<sub>3</sub>VO<sub>4</sub>, 1.7 mmol/L CaCl<sub>2</sub>, 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<sub>2</sub>O<sub>2</sub> 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<sup>–1</sup>cm<sup>–1</sup> 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<sub>2</sub>O<sub>2</sub>/min in cells.

The amount of H<sub>2</sub>O<sub>2</sub> 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) 30 min before injection of LPS were homogenized in PBS containing protease inhibitors and the Amplex Red dye; H<sub>2</sub>O<sub>2</sub> 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<sub>2</sub>O<sub>2</sub> for 6 or 24 h at 37°C. Following H<sub>2</sub>O<sub>2</sub> challenge, cells were washed twice with PBS and trypsinized; cell samples (1 x 10<sup>6</sup> 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<sup>−/−</sup> and wild-type mice were perfused (2 ml/min, 37°C) for 3 h with a recirculating volume of RPMI 1640 solution containing H<sub>2</sub>O<sub>2</sub> (300 μmol/L) or glucose oxidase (that produced 320 nmol/L/min H<sub>2</sub>O<sub>2</sub>). 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 5 min. 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 4°C. 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. 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 transplantation, mice were challenged with LPS (30 mg/kg body weight) via intraperitoneal 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.

References


Online Figure I: TRPM2 mediates H$_2$O$_2$-induced apoptosis of endothelial cells. Left, representative flow cytometry histograms at 0, 6, or 24 h after exposure to 300 µmol/L H$_2$O$_2$ 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$_2$O$_2$ 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 H₂O₂ (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 H₂O₂ 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 PKCai) were challenged with 20 ng/mL TNFα for the indicated times at 37°C. (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+}] (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\textsuperscript{6}-tagged TRPM2-S or (S39A) TRPM2-S, and His\textsuperscript{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 antibody. (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$_2$O$_2$-induced Ca$^{2+}$ entry. The predicted PKC$\alpha$ 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 $\mu$M H$_2$O$_2$ for the indicated times. (A) Western blots for TRPM2, PKC$\alpha$, 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 PKC$\alpha$ protein was detected with an Ab. Graph in B, mean densitometric values ($\pm$ SEM; n=3-4). Mutation of Ser 39 with Ala in TRPM2-S prevented TRPM2-S association with PKC$\alpha$ 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 $\pm$ SEM; n = 3). S39 phosphomimetic mutation of TRPM2-S prevented association of TRPM2-S with TRPM2 at time zero and following H$_2$O$_2$ 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$_2$O$_2$-induced Ca$^{2+}$ entry (mean $\pm$ SEM; n = 6). * $p = 0.0001$ compared with control (t-test).
Online Figure VI. Expression of TRPM2-S phospho-mimetic mutant restores H$_2$O$_2$-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$_2$O$_2$-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$_2$O$_2$ 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$^{2+}$] obtained in (A-B) (n = 5 to 6). *P ≤ 0.0005 vs. control siRNA (t-test).