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, Asrar B. Malik

**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 \(\text{H}_2\text{O}_2\) 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,\(^{1,5}\) 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.\(^{16-20}\) A component of endothelial disruption seen in these studies may well have been because of TRPM2-induced apoptosis.

TRPM2 channel opening after exposure to \(\text{H}_2\text{O}_2\) 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.\(^{5,6,10,12,21-23}\)

Original received August 13, 2013; revision received December 10, 2013; accepted December 11, 2013. In November 2013, the average time from submission to first decision for all original research papers submitted to *Circulation Research* was 14.6 days.

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The online-only Data Supplement is available with this article at http://circres.ahajournals.org/lookup/suppl/doi:10.1161/CIRCRESAHA.114.302414/-/DC1.

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*Circulation Research* is available at http://circres.ahajournals.org DOI: 10.1161/CIRCRESAHA.114.302414
Nudix motif. 6,10,12,24,25 In addition, other mechanisms of oxidant-induced apoptosis remain obscure. Of particular interest is the short splice variant (TRPM2-S) that functions as a dominant-negative to inhibit TRPM2 channel activity.14,28 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 interacted with TRPM2-S to inhibit formation of functional homotetrameric channels.14 Here, we investigated the interaction of TRPM2-S with TRPM2 and how the component cooperated to signal oxidant-induced apoptosis. Besides TRPM2,5,27 several splice variants of TRPM2 associated with TRPM2 in the plasma membrane have also been identified.28 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.14,28 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 interacted with TRPM2-S to inhibit formation of functional homotetrameric 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), PKCα−/− (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 (HPAE; 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.

H2O2 produced in the cell also activated the production of ADPR,6,10,23,24 which functioned by binding to the TRPM2 Nudix motif.6,10,12,24,25 In addition, other mechanisms of oxidant-induced apoptosis remain obscure. Of particular interest is the short splice variant (TRPM2-S) that functions as a dominant-negative to inhibit TRPM2 channel activity.14,28 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 interacted with TRPM2-S to inhibit formation of functional homotetrameric 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.

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model of endotoxin-mediated mortality, and Western blotting are given in the Online Data Supplement.

Results

TRPM2 Is Required for $H_2O_2$-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). $H_2O_2$ in a concentration-dependent manner induced apoptosis within 24 hours with an EC$_{50}$ 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 $H_2O_2$ (320 nmol/L per minutes 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 a solution containing $H_2O_2$ or GO/glucose (this generated 320 nmol/L per minute $H_2O_2$).

Because Ca$^{2+}$ signaling activates the apoptotic pathway through activation of caspase-3 followed by cleavage of caspase substrates, such as 113-kDa PARP$^{29,30}$ we also determined expression of the 17- and 20-kDa caspase-3 fragments and 89- or 24-kDa caspase cleavage fragments of PARP$^{29,30}$ Data from lungs showed that cleaved PARP characteristically colocalized with the endothelial cell marker VE-cadherin, whereas deletion of TRPM2 (TRPM2$^{-/-}$ mice) markedly reduced endothelial apoptosis (Figure 1C). Western blotting confirmed the activation of caspase-3 and cleaved PARP in lungs of WT and not TRPM2$^{-/-}$ mice (Figure 1D). Using another apoptosis assay, terminal deoxynucleotidyl transferase dUTP nick-end labeling staining, we observed fewer endothelial cells undergoing apoptosis after $H_2O_2$, or GO/glucose infusion in TRPM2$^{-/-}$ mice when compared with WT (Online Figure II).

$H_2O_2$ Induces PKC$\alpha$ 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 $H_2O_2$ exposure per se (Figure 2A). Using the motif scanning graphic software (Merck Genome Research Institute), we identified a putative high-affinity binding site for PKC$\alpha$ near the N terminus of TRPM2-S, at Ser 39. This site was predicted as a possible domain that could be phosphorylated by PKC$\alpha$. We observed that PKC$\alpha$ was basally expressed in these cells, and its expression was not modified by $H_2O_2$ (Figure 2A). Depleting PKC$\alpha$ 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 $H_2O_2$ was involved in the phosphorylation at Ser39 on TRPM2-S, we used HPAEC monolayers treated with PKC$\alpha$ inhibitors (Gö6976 or PKC$\alpha$ blocking peptide [PKC$\alpha$ inhibitor peptide]) or transfected with siRNA to knockdown PKC$\alpha$ expression. PKC$\alpha$ was immunoprecipitated from lysates of cells exposed to $H_2O_2$, and coimmunoprecipitated TRPM2 and TRPM2-S were detected using an antibody recognizing each form (Figure 2B). $H_2O_2$ rapidly induced the association of PKC$\alpha$ with TRPM2-S, but not with TRPM2, and the response persisted up to 5 minutes (Figure 2B). Immunoprecipitation was reduced when PKC$\alpha$ activation was inhibited (Figure 2B). Treatment with PKC$\beta$ inhibitory peptide, used as control for nonspecific effects of Gö6976 in blocking activation of both PKC$\alpha$ and PKC$\beta$, did not modify the $H_2O_2$-induced TRPM2-S association with PKC$\alpha$ (Figure 2B). PKC$\alpha$-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 PKC$\alpha$ expression (Figure 2B). Treatment of cells with PKC$\alpha$ inhibitors (Gö6976 or PKC$\alpha$ inhibitor peptide) and PKC$\beta$ blocking peptide did not modify PKC$\alpha$ expression (Figure 2B).

Because the above studies dealt with the role of $H_2O_2$ in activating the phosphorylation of TRPM2-S, we next examined whether key alterations could be replicated using a physiological stimulus to generated oxidants. Here, we used tumor necrosis factor-$\alpha$ (TNF-$\alpha$), a generator of intracellular oxidants and potent inducer of endothelial cell apoptosis.$^{32}$ We observed that TNF-$\alpha$ induced the association of PKC$\alpha$ with TRPM2-S, whereas suppressing PKC$\alpha$ activity prevented the association (Online Figure IIIA).

To determine whether PKC$\alpha$ was responsible for phosphorylating TRPM2-S after $H_2O_2$ 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 $H_2O_2$ 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-$\alpha$ exposure (Online Figure IIIB). Phosphorylation of PKC$\alpha$ (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 PKC$\alpha$ using an antibody recognizing phosphorylated on Ser 657, the crucial PKC$\alpha$ catalytic domain.$^{33}$ $H_2O_2$ rapidly induced phosphorylation of PKC$\alpha$ at this site and the phosphorylated PKC$\alpha$ co-migrated with TRPM2 (middle blot; Figure 2C). Treatment with Gö6976 (but not with control PKC$\beta$II inhibitor peptide) inhibited not only PKC$\alpha$ phosphorylation but also $H_2O_2$-induced phosphorylation of TRPM2-S (Figure 2C). These results thus show time-dependent and reversible association between TRPM2-S and PKC$\alpha$ induced by PKC$\alpha$ activation (Figure 2D).
S39 Phosphorylation of TRPM2-S Activates TRPM2 and Supranormal Ca2+ Influx

We used the Fura-2 dye to study the Ca2+ entry response activated by TRPM2 interaction with TRPM2-S. In addition, we used the Ca2+ add-back protocol to rule out any indirect effects of H2O2 on Ca2+ entry secondary to Ca2+-store depletion. In the absence of extracellular Ca2+, H2O2 did not produce a Ca2+ transient (Figure 3A and 3B), indicating that H2O2 did not deplete intracellular Ca2+ stores. By contrast, extracellular Ca2+ repletion in the continued presence of H2O2 elicited a sharp and marked increase in intracellular Ca2+ concentration secondary to Ca2+ entry (Figure 3A and 3B). TRPM2 knockdown markedly suppressed the Ca2+ transient (Figure 3A and 3B), showing that H2O2-induced Ca2+ entry required TRPM2. G06976 significantly decreased the amplitude of Ca2+-repletion-dependent transients by 66±9%, and PKCα inhibitor peptide reduced Ca2+ transient by 46±10% (Figure 3A and 3B). PKCα silencing also reduced Ca2+ entry.

Figure 1. Melastatin-like transient receptor potential 2 (TRPM2) is required for H2O2-mediated apoptosis of endothelial cells.
A and B, Confluent endothelial cell monolayers (human pulmonary artery endothelial cells) challenged with H2O2 or glucose oxidase/glucose were labeled with phycoerythrin (PE) annexin V-fluorescein isothiocyanate and 7-aminoactinomycin D (7-AAD) and analyzed by flow cytometry. A, Concentration–response curve for H2O2-induced apoptosis after 24-hour period of H2O2 exposure. Mean apoptotic values (±SEM) obtained by flow cytometry (top) are plotted as % apoptotic cells vs [H2O2] (EC50, 136 µmol/L; bottom; n=3). B, Left. Representative flow cytometry histograms 6 or 24 hours after exposure to 300 µmol/L H2O2 or glucose oxidase/glucose (90 minutes), with or without prior TRPM2 silencing or inhibition with blocking TRPM2 antibody. Right. Mean percent apoptotic cells at 0, 6, or 24 hours after H2O2 treatment (±SEM; n=3). Baseline values were not significantly altered by TRPM2 silencing or TRPM2 blocking Ab. C and D, Apoptosis in lungs of TRPM2−/− and wild-type (WT) mice measured 3 hours after perfusion with solution containing H2O2 (300 µmol/L) or glucose oxidase/glucose (75 minutes). C, Right, Immunofluorescent staining of frozen lung sections using vascular endothelial-cadherin (red) and cleaved-poly(ADPR) polymerase (PARP; green) antibodies and 4,6-diamidino-2-phenylindole (blue; n=3). Scale bar, 50 µm. Left, Quantification of apoptotic endothelial cells (±SEM; n=3), *P≤0.007 vs WT lungs (t test). D, Western immunobots (Right) and quantification of inactive or cleaved caspase-3 and active or cleaved PARP in lung homogenates (Left). GAPDH was used as loading control. (±SEM; n=3), *P≤0.002, **P≤0.009 vs WT perfused-lungs.
Regulated TRPM2 Gating and Apoptosis

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 IIID) 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...
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 Ca\(^{2+}\) entry in endothelial cells required for signaling apoptosis. A, Ca\(^{2+}\) repletion transients generated by Ca\(^{2+}\)-add-back in the presence of H\(_2\)O\(_2\). Cultured human pulmonary artery endothelial cells (HPAECs) were loaded with Fura-2 Ca\(^{2+}\) dye, washed, and transferred to Ca\(^{2+}\)-free medium. In control cells, H\(_2\)O\(_2\) (100 µmol/L) elicited a marked Ca\(^{2+}\) transient on Ca\(^{2+}\) repletion (red trace). Ca\(^{2+}\) 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); PKCγIII (1 µmol/L, navy trace) and control siRNA (purple trace) had no effect. Ordinate gives [Ca\(^{2+}\)]\(_i\) as 340:380 nm ratio. B, Summary of mean ratiometric data (±SEM) for the peak intracellular [Ca\(^{2+}\)]\(_i\) 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 Ca\(^{2+}\) 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 Ca\(^{2+}\) measurements using fura-2 (D). 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 TRPM2-S association with PKCα. 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 H\(_2\)O\(_2\)-induced phosphorylation of TRPM2-S confirming the importance of the PKCα phosphorylation site on TRPM2-S at Ser 39. D, Ca\(^{2+}\) mobilization assay was performed using the Ca\(^{2+}\)-add-back protocol. Transduction of phosphodefective TRPM2-S mutant suppressed H\(_2\)O\(_2\)-induced Ca\(^{2+}\) entry. *P=0.0001 compared with control (t test). (n=3 per bar); error bars, ±SEM.
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 phosphodefective 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 Endotoxemia

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 TRPM2 with its 90-kDa splice variant TRPM2-S 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. 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. The observation that TRPM2-S served as a dominant-negative for TRPM2, and its uncoupling from TRPM2 required for the full gating of Ca²⁺ identifies TRPM2-S as an important intrinsic 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 suppress its activity.

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

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**Figure 6.** H₂O₂-induced endothelial cell apoptosis resulting from melastatin-like transient receptor potential 2 (TRPM2)–mediated Ca²⁺ influx. Human pulmonary artery endothelial cells (HPAECs; A) and mouse lung endothelial cells (B–D) challenged with H₂O₂ were labeled with phycoerythrin (PE)-annexin/7-aminoactinomycin D (7-AAD). A, Flow-cytometry histograms (left) and summary plots of apoptosis (right) 0, 6, and 24 hours after challenge with H₂O₂ (300 µmol/L) or glucose oxidase/glucose to generate 320 nmol/L H₂O₂/min for 90 minutes, as a function of PKCα inhibition or silencing (±SEM; n=5). B–D, Mouse endothelial cells were isolated from lungs of TRPM2–/–, PKCα–/–, and wild-type (WT) mice. B, Western blot verifying absence of PKCα expression in PKCα–/– cells and of TRPM2 expression in TRPM2–/– cells. C, Left, Ca²⁺ mobilization assay using Ca²⁺ add-back protocol with the fluor-3 Ca²⁺ indicator. Right, Mean ratiometric values (±SEM) for steady-state [Ca²⁺]i (n=6). *P<0.0001 vs WT cells (t test). D, Dose–response curve for H₂O₂-induced apoptosis in mouse endothelial cells detected by flow cytometry. Deletion of PKCα or TRPM2 caused ≈2.5-fold rightward shift in the dose–response curve.
Ala resulted in failure of PKC\textalpha to phosphorylate TRPM2-S. This mutation in turn prevented TRPM2-S dissociation from TRPM2 and hence the apoptosis-inducing Ca\textsuperscript{2+} entry signal. Both K562 myeloid leukemia cell line that do not express the TRPM2-S\textsuperscript{14} and Jurkat t-lymphocyte cell line that expresses the short isoform at very low levels\textsuperscript{14,40} also did not undergo apoptosis secondary to TRPM2 activation,\textsuperscript{14,40} consistent with the critical role of TRPM2-S as the apoptosis-suppressing partner of TRPM2.

Although PKC\textalpha activation contributed to TRPM2-induced endothelial apoptosis in the present through phosphorylation of TRPM2-S, it is important to note that PKC\textalpha signaling in other signaling pathways can induce endothelial injury. We have shown that PKC\textalpha phosphorylation of p120-catenin mediates disassociation of p120-catenin from VE-cadherin that resulted in disassembly of adherens junctions and disruption of VE barrier function.\textsuperscript{41} These studies showed the role of PKC\textalpha in mediating phosphorylation of p120-catenin in response to endotoxin and resultant increased lung vascular permeability.\textsuperscript{41} Thus, PKC\textalpha can function in a complex manner at multiple levels to induce endothelial dysfunction either via injury or through activating the apoptosis program.

The signaling pathway downstream of Ca\textsuperscript{2+} entry leading to cell death involves the activation of intrinsic executioner caspases (caspase-9) and extrinsic caspases (caspase-8) that activate the effector caspase-3 and caspase-7.\textsuperscript{7,14,42,43} These cleave cellular substrates, disrupting survival pathways and inducing membrane

Figure 7. Protein kinase C-\textalpha (PKC\textalpha) interaction with melastatin-like transient receptor potential 2 (TRPM2) in mice is required lung endothelial cell apoptosis in response to lipopolysaccharide (LPS) and contributes to mortality. WT, TRPM2\textsuperscript{–/–}, and PKC\textalpha\textsuperscript{–/–} mice were transplanted with bone marrow cells isolated from WT mice 8 weeks before Western blotting (A), apoptosis (B and C), and survival (D) studies. A, Representative Western blots verifying expression of TRPM2, PKC\textalpha, and \beta-actin in bone marrow of transplanted mice (molecular masses of 171, 82, and 45 kDa, respectively). Left, Protein was quantified by densitometry. TRPM2 and PKC\textalpha densities were normalized to \beta-actin and plotted as percentage of untreated control (mean±SEM for n=3). Endothelial apoptosis (B) and oxidant production (C) were determined in lungs of mice 4 hours after intraperitoneal injection of LPS (40 mg/kg) and in lungs of mice treated with the oxidant scavenger Tempol (100 mg/kg, IP) 30 minutes before injection of LPS. B, Right, Immunofluorescent staining of frozen lung sections using vascular endothelial-cadherin (red) antibody, terminal deoxynucleotidyl transferase dUTP nick-end labeling (green), and 4,6-diamidino-2-phenylindole (DAPI; blue; n=3). Scale bar, 50 \mu m. Left, Quantification of apoptotic endothelial cells (±SEM; n=3), *P≤0.001 and \#P≤0.005 vs LPS-treated wild-type (WT) lungs. TRPM2 and PKC\textalpha deletion significantly reduced LPS-induced endothelial apoptosis. C, Lungs were homogenized and assayed for H$_2$O$_2$ accumulation using the horseradish peroxidase–linked Amplex Red assay. H$_2$O$_2$ was determined spectrophotometrically from its absorbance at 570 nmol/L and corrected for total protein (±SEM; n=3). *P≤0.005 vs LPS-treated control lungs. D, Deletion of either TRPM2 or PKC\textalpha reduced LPS-induced lethality in mice. LPS (30 mg/kg) was injected intraperitoneally and survival was assessed every 12 hours during the experiment (WT, n=16; TRPM2\textsuperscript{–/–}, n=16; and PKC\textalpha\textsuperscript{–/–}, n=12). Statistical analysis was performed using the log-rank test. *P≤0.03 and \#P≤0.05 vs WT cells.

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blebbing, cell shrinkage, and apoptotic body formation. PARP is part of a protective mechanism involved in repair of DNA damage and DNA stability. Inactivation of PARP by cleavage of the enzymatic domain after oxidant 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. 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.

Acknowledgments

We thank M. Ushio-Fukai for her insights. We also thank B.A. Miller (Pennsylvania State University School of Medicine) for kindly supplying the GFP-TRPM2-S construct, and GlaxoSmithKline for providing the Trpm2−/− C57BL/6 mice used in these experiments. We are also grateful to Dr Jeffrey Molkentin for PKCα−/− mice.

Sources of Funding

This work was supported by National Institutes of Health grant P01 HL077806-07 to A.B. Malik and by grant 10SDG2610057 from American Heart Association Midwest affiliate to C.M. Hecquet.

Disclosures

None.

References


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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Circ Res. 2014;114:469-479; originally published online December 11, 2013;
doi: 10.1161/CIRCRESAHA.114.302414
Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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Data Supplement (unedited) at:
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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$^5$-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 PKC$\alpha$ peptide inhibitor Myr-RFARKGARQKNV was from Promega (Madison, WI). H$_2$O$_2$, myristoylated PKC$\beta$II inhibitor Myr-SLNPEWNET (PKC$\beta$III), 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$^5$, PKC$\alpha$ 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 growth medium (EGM-2 supplemented with 10% fetal bovine serum), then plated on Matrigel-coated 35-mm culture dish and allowed to confluence for 10 days. Cells were then harvested from the Matrigel plates by dispensing (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, PKC$\alpha$$^-$ (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 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<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 Blastocidin (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<sup>2+</sup>]i measurements.**

**Ratiometric Ca<sup>2+</sup> measurements using Fura-2/AM:** Control or transfected HPAE cells<sup>2</sup> (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<sup>2+</sup> levels are given as fluorescent ratio F340/F380 representing bound/free Ca<sup>2+</sup>.

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 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<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 300µM 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 PKCα. 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-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% 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 × 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) bromophenol 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) 30min 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 × 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 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 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 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.

References


Online Figure I: TRPM2 mediates H₂O₂-induced apoptosis of endothelial cells. Left, representative flow cytometry histograms at 0, 6, or 24 h after exposure to 300 μmol/L H₂O₂ 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 H₂O₂ 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<sup>-/-</sup> vs. wild-type (WT) mice. Apoptosis in lungs of TRPM2<sup>-/-</sup> and wild-type (WT) mice measured 3 h after perfusion with a solution containing H<sub>2</sub>O<sub>2</sub> (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)** Percentaged 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<sub>2</sub>O<sub>2</sub> 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. His⁶-tagged TRPM2-S or (S39A) TRPM2-S, and His⁶-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 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$_2$O$_2$ 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$_2$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 ± 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 phospo-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$^{2+}$]i obtained in (A-B) (n = 5 to 6). *P ≤ 0.0005 vs. control siRNA (t-test).