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Cooperative Interaction of *trp* Melastatin Channel TRPM2 with its Splice Variant TRPM2-S is Essential for Endothelial Cell Apoptosis

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Abstract

Rationale: Oxidants generated by activated endothelial cells are known to induce apoptosis, a pathogenic feature of vascular injury and inflammation from multiple etiologies. The melastatin-family transient receptor potential 2 (TRPM2) channel is an oxidant-sensitive Ca²⁺ permeable channel implicated in mediating apoptosis; however, the mechanisms of gating of the supra-normal Ca²⁺ influx required for initiating of apoptosis are not understood.

Objective: Here we addressed the role TRPM2 and its interaction with the short splice variant TRPM2-S in mediating the Ca²⁺ 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 constitutively suppress TRPM2-dependent Ca²⁺ gating. ROS production in endothelial cells or directly applying ROS induced PKC α activation and phosphorylation of TRPM2 at Ser 39. This in turn stimulated a large entry of Ca²⁺ and activated the apoptosis pathway. A similar TRPM2-dependent endothelial apoptosis mechanism was seen in intact vessels. The PKC α -activated phospho-switch opened the TRPM2 channel to allow large Ca²⁺ 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 describes a fundamental mechanism by which activation of the *trp* super-family TRPM2 channel induces apoptosis of endothelial cells. The signaling mechanism involves ROS-induced PKC α activation resulting in phosphorylation of TRPM2-S that allows enhanced TRPM2-mediated gating of Ca²⁺ and activation of the apoptosis program. Strategies aimed at preventing the uncoupling of TRPM2-S from TRPM2 and subsequent Ca²⁺ gating during oxidative stress may mitigate endothelial apoptosis and its consequences in mediating vascular injury and inflammation.

Keywords

Apoptosis; oxidant-activated cation channel; endothelium; vascular permeability; inflammation; oxidant signaling

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Disclosures
None.

Introduction

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 β cells⁷⁻⁹, hematopoietic and immune cells^{10,11}, and vascular endothelial cells⁵. However, the function of TRPM2 remains enigmatic. TRPM2 is activated by the generation of ROS such as H_2O_2 and production of adenosine diphosphate ribose (ADPR) following 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 vessels¹⁵, is a fundamental pathogenic feature of inflammatory and vascular diseases such as acute lung injury¹⁶ and sepsis¹⁷. Our studies have demonstrated a key role of TRPM2 in mediating oxidative injury of the endothelium⁵ resulting in disruption of endothelial barrier and tissue edema¹⁸⁻²⁰. A component of endothelial disruption seen in these studies may well have been due to TRPM2-induced apoptosis.

TRPM2 channel opening after exposure to H_2O_2 and other ROS is induced by the binding of ADPR to the Nudix box sequence motif (NUDT9-H) in the carboxyl-terminal domain of TRPM2^{5,6,10,12,21-23}. H_2O_2 produced in the cell⁵ 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 TRPM2 activation such as direct oxidative modification of the channel have been proposed²⁶.

Besides TRPM2^{5,27}, several splice variants of TRPM2 associated with TRPM2 in the plasma membrane have also been identified²⁸. Their role in regulating TRPM2 function and mediating oxidant-induced apoptosis, remains obscure. Of particular interest is the short splice variant (TRPM2-S), which 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 as well as the Ca^{2+} -permeable pore present in TRPM2²⁸. In cells in which both isoforms are expressed, TRPM2-S interact with TRPM2 to inhibit formation of functional homotetrameric channels¹⁴. 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 PKC α phosphorylation-dependent disruption of the interaction of TRPM2 with TRPM2-S and opening of the channel to allow sufficient Ca^{2+} entry required for activation of the apoptosis program.

METHODS

An expanded Materials and Methods section is available in the online data supplement at <http://circres.ahajournals.org>.

Isolation of mouse endothelial cells

Endothelial cells were isolated from lungs of WT, *PKC α ^{-/-}* (obtained from Dr Jeffrey D. Molkenin, University of Cincinnati, Cincinnati, OH) and *TRPM2^{-/-}* mice (GlaxoSmithKline). The cells were used between passages 2–5.

Transfections

Human pulmonary artery endothelial cells (HPAEC; Clonetics, La Jolla, CA) were cultured in gelatin-coated flasks and used between passages 3–6. 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 PKC α 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 μ mol/L H₂O₂ for indicated times (37°C). In some experiments, cells were pretreated with DPQ or PKC inhibitors 30 min prior to the assay. In other experiments, cells first received siRNA to suppress TRPM2 or PKC α expression. TRPM2 or PKC α immune complexes were precipitated with protein A-Sepharose beads (Sigma) for 2 h at 4°C as described⁵.

Generation of H₂O₂ using glucose oxidase/glucose

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

Analysis for apoptosis

Apoptosis was identified by double-fluorescent staining with Phycoerythrin (PE) Annexin V-FITC and 7-Aminoactinomycin D (7-AAD), which detected apoptotic and dead cells, respectively. Confluent endothelial monolayers, without or with PKC α inhibition or silencing were incubated in 300 μ mol/L H₂O₂ for 6 or 24 h (37°C). Cells were washed twice with PBS and trypsinized; samples of 1 \times 10⁶ cells were incubated with 5 μ l of PE-labeled Annexin V and 5 μ l of 7-AAD (BD Bioscience, Rockville, MD) for 20 min 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 TUNEL assay. Lungs of *TRPM2*^{-/-} and wild-type mice were perfused (2 ml/min, 37°C) for 3 h with recirculation of RPMI 1640 (5 ml) containing H₂O₂ (300 μ mol/L) or glucose oxidase. Lungs were removed, and frozen by the OCT method. Frozen lungs, sectioned (5 μ m) and fixed in 3.7% formaldehyde, were permeabilized with 0.2% triton X-100. Tissue sections were blocked with 10% FBS and incubated with goat anti-VE-cadherin and rabbit anti-PARP (i.e., 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, TUNEL (Terminal deoxynucleotidyl transferase dUTP nick end labeling) staining was performed according to the manufacturer's protocol (Roche Diagnostics Corp., Indianapolis, IN). Nuclei were visualized by 4,6-diamidino-2-phenylindole (DAPI; 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 TUNEL + VE-cadherin).

Statistical analysis

Statistical comparisons were made with the two-tailed Student's *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 $[Ca^{2+}]_i$ 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-PE and 7-AAD followed by FACS assessment (Figure 1A-B). H₂O₂ in a concentration-dependent manner induced apoptosis within 24 h with an EC₅₀ value of $136 \pm 6 \mu\text{mol/L}$ (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/min for 90 min) by glucose oxidase 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 wild-type and TRPM2 knockout mice perfused with a solution containing H₂O₂ or glucose oxidase/glucose (this generated 320 nmol/L/min H₂O₂).

As Ca²⁺ signaling activates the apoptotic pathway through activation of caspase-3 followed by cleavage of caspase substrates, such as 113-kDa poly(ADP-ribose) polymerase (PARP)^{29,30}, we also determined expression of the 17-kDa and 20-kDa caspase-3 fragments and 89-kDa or 24-kDa caspase cleavage fragments of PARP^{29,30}. Data from lungs showed that cleaved-PARP characteristically co-localized 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 wild-type and not *TRPM2*^{-/-} mice (Figure 1D). Using another apoptosis assay, TUNEL staining, we observed fewer endothelial cells undergoing apoptosis after H₂O₂ or glucose oxidase/glucose infusion in *TRPM2*^{-/-} mice compared to WT (Online Figure II).

H₂O₂ induces PKC α 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) as well as in multiple other endothelial cells examined (unpublished observations). TRPM2 and TRPM2-S expression was 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 PKC α near the N-terminus of TRPM2-S, at Ser 39. This site was predicted as a possible domain that could be phosphorylated by PKC α . We observed that PKC α was basally expressed in these cells and its expression was not modified by H₂O₂ (Figure 2A).

Depleting PKC α 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 PKC α inhibitors (Gö6976 or PKC α blocking peptide, PKC α i) or transfected with siRNA to knockdown PKC α expression. PKC α was immunoprecipitated from lysates of cells exposed to H₂O₂, and co-immunoprecipitated TRPM2 and TRPM2-S were detected using an antibody recognizing each form (Figure 2B). H₂O₂ rapidly induced the association of PKC α with TRPM2-S, but not with TRPM2, and the response persisted up to 5 min (Figure 2B). Immunoprecipitation was reduced when PKC α activation was inhibited (Figure 2B). Treatment with PKC β II inhibitory peptide, used as control for non-specific effects of Gö6976 in blocking activation of both PKC α and PKC β II³¹, did not modify the H₂O₂-induced TRPM2-S association with PKC α (Figure 2B). PKC α -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 α expression (Figure 2B). Treatment of cells with PKC α inhibitors (Gö6976 or PKC α i) and PKC β II blocking peptide did not modify PKC α expression (Figure 2B).

Since 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 alpha (TNF- α), a generator of intracellular oxidants and potent inducer of endothelial cell apoptosis³². We observed that TNF- α induced the association of PKC α with TRPM2-S whereas suppressing PKC α activity prevented the association (Online Figure IIIA).

To determine whether PKC α was responsible for phosphorylating TRPM2-S following 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 anti-phospho-Ser antibody. Western blotting demonstrated that only TRPM2-S was phosphorylated, which occurred within 1 min of H₂O₂ exposure with maximum response seen at 2 min, while 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 following TNF α exposure (Online Figure IIIB). Phosphorylation of PKC α (82 kDa) was detected as a co-migrating band on the gel (seen in top blot of Figure 2C and Online Figure IIIB), an indication that the kinase was in the active state.

We next determined phosphorylation of PKC α using an antibody recognizing phosphorylated on Ser 657, the crucial PKC α catalytic domain³³. H₂O₂ rapidly induced phosphorylation of PKC α at this site and the phosphorylated PKC α co-migrated with TRPM2 (middle blot) (Figure 2C). Treatment with Gö6976 (but not with control PKC β IIi) inhibited not only PKC α phosphorylation but also H₂O₂-induced phosphorylation of TRPM2-S (Figure 2C). These results thus show time-dependent and reversible association between TRPM2-S and PKC α induced by PKC α activation (Figure 2D).

S39 phosphorylation of TRPM2-S activates TRPM2 and supra-normal Ca²⁺ influx

We used the Fura-2 dye to study the Ca²⁺ entry response activated by TRPM2 interaction with TRPM2-S. In addition, we employed the "Ca²⁺ add-back" protocol to rule out any indirect effects of H₂O₂ on Ca²⁺ entry secondary to Ca²⁺-store depletion⁵. In the absence of extracellular Ca²⁺, H₂O₂ did not produce a Ca²⁺ transient (Figure 3A, B), 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, B). TRPM2 knockdown markedly suppressed the Ca²⁺ transient (Figure 3A, B), showing that H₂O₂-induced Ca²⁺ entry required TRPM2. Gö6976 significantly decreased the amplitude of Ca²⁺repletion-dependent transients by 66 ± 9 % and PKC α i reduced Ca²⁺ transient by 46 ± 10 % (Figure 3 A, B). PKC α silencing also reduced Ca²⁺ entry by 43 ± 6 % (Figure 3A, B). Treatment of cells with control PKC β II peptide inhibitor, however, did not modify H₂O₂ -activated Ca²⁺ entry via TRPM2 channels (Figure 3A, B). Along the same lines, TNF α -induced Ca²⁺ entry in endothelial cells (Online Figure IIID) was also decreased by inhibiting PKC α .

We next addressed the role of the S39 phospho-switch on TRPM2-S in mediating TRPM2 channel activity. Here we determined whether mutation of TRPM2-S at Ser 39 (S39A), the PKC α 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 PKC α on the gel (Figure 4B) as well as phosphorylation of TRPM2-S by PKC α after H₂O₂ challenge (Figure 4C). PKC α also did not migrate with (S39A)-TRPM2-S mutant (Figure 4C).

We next determined the functional significance of the failure of PKC α 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 phospho-defective mutant (Figure 4D). To validate the finding that PKC α was indeed responsible for phosphorylation of TRPM2-S at S39, we treated an extract of native protein with recombinant active PKC α . We observed that active PKC α induced phosphorylation of WT TRPM2-S but not of S39A mutant (Online Figure IV).

PKC α phosphorylation of TRPM2-S induces TRPM2-S dissociation from TRPM2

We next determined whether PKC α 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. As TRPM2 variants generated by alternative splicing differed only in their C terminal²⁸, 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 following H₂O₂ addition (Figure 5A). Inhibition of PKC α 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). PKC α -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 Fig 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 PKC α (Online Figure VB), but impaired its association with TRPM2 (Online Figure VC) under basal condition in the absence of H₂O₂ as well as 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 PKC α phosphorylation of TRPM2-S in activating TRPM2 channel activity.

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

PKC α mediates H₂O₂-induced apoptosis through activation of TRPM2

FACS analysis showed that inhibition of PKC α activation or its silencing protected the cells from H₂O₂-induced apoptosis (Figure 6A and B). The role of PKC α 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 PKC α knockout mice to validate the above studies in human endothelial cells, we observed normal expression of TRPM2 in endothelial cells from PKC α knockout mice as well as normal expression of PKC α in endothelial cells from TRPM2 knockout mice (Figure 6A and B). The H₂O₂-induced Ca²⁺ entry was virtually abolished in TRPM2-null cells and was reduced by 40% in PKC α -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 μ mol/L) indicating the crucial role of TRPM2 in mediating H₂O₂-induced apoptosis (Figure 6D). Deletion of the PKC α gene similarly inhibited apoptosis (EC₅₀ shift = 213-512 μ mol/L) (Figure 6D).

Deletion of PKC α gene in mice reduces TRPM2-induced endothelial cell apoptosis improves survival in endotoxemia

To address the pathophysiological significance of PKC α phosphorylation of TRPM2 channel activity in mediating apoptosis *in vivo*, we examined the apoptosis response in mouse lung endothelial cells and survival of mice following intraperitoneal (ip) challenge with lipopolysaccharide (LPS, 30 mg/kg), the Gram-negative bacterial endotoxin, which produces ROS in endothelial cells^{34,35}. Because TRPM2 and PKC α expressed in myeloid cells may also play a role in ROS production and apoptosis³⁶, we generated chimeric mice in which the PKC α - and TRPM2-deficient mice were transplanted with bone marrow cells from wild-type mice. These mice showed comparable TRPM2 and PKC α protein expression as wild-type (Fig 7A). We observed that either TRPM2 or PKC α deletion markedly reduced endothelial cell apoptosis in lungs 4 h after LPS treatment compared to wild-type mice (Figure 7B). In a positive control experiment, administration of the oxidant scavenger Tempol in mice 30 min prior to LPS also reduced oxidant-mediated LPS-induced apoptosis (Figure 7B). In addition, deletion of either TRPM2 or PKC α significantly improved survival rate of LPS-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^{10,12,27,37} with its 90 kDa splice variant TRPM2-S²⁸ in the endothelial cell plasmalemma. This interaction functioned constitutively to restrain TRPM2 Ca²⁺ entry. However, ROS-induced activation of PKC α 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.

PKC α 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 200-500 nmol/L³⁸. Inhibition of PKC α 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 region²⁸. The observation that TRPM2-S served as a dominant negative for TRPM2 and its uncoupling from TRPM2 was 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 PKC α and TRPM2-S permitting the channel to open for Ca²⁺ entry. Thus, TRPM2-S only functioned to induce apoptosis when PKC α 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 form of TRPM1 to suppress its activity³⁹.

PKC α 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 PKC α phosphorylation site on Ser39 of TRPM2-S N-terminus to Ala resulted in failure of PKC α to phosphorylate TRPM2-S. This mutation in turn prevented TRPM2-S dissociation from TRPM2 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^{14,40} also did not undergo apoptosis secondary to TRPM2 activation^{14,40}, consistent with the critical role of TRPM2-S as the apoptosis-suppressing partner of TRPM2. Although PKC α activation contributed to TRPM2-induced endothelial apoptosis in the present through phosphorylation of TRPM2-S, it is important to note that PKC α signaling in other signaling pathways that can induce endothelial injury. We have shown that PKC α phosphorylation of p120-catenin mediates disassociation of p120-catenin from VE-cadherin that resulted in disassembly of adherens junctions (AJs) and disruption of vascular endothelial barrier function⁴¹. These studies showed the role of PKC α in mediating phosphorylation of p120-catenin in response to endotoxin and resultant increased lung vascular permeability⁴¹. Thus, PKC α can function in a complex matter at multiple levels to induce endothelial dysfunction either via injury or through activating the apoptosis program.

The signaling pathway downstream of Ca²⁺ entry leading to cell death involves the activation of intrinsic “executioner” caspases (caspase-9) and extrinsic caspases (caspase-8) that activate the effector caspases -3 and -7^{14,42,43}. These cleave cellular substrates, disrupting survival pathways and inducing membrane blebbing, cell shrinkage, and apoptotic body formation. PARP is part of a protective mechanism involved in repair of DNA damage⁴⁴ and DNA stability²⁹. Inactivation of PARP by cleavage of the enzymatic domain following oxidant activation of TRPM2 also caused apoptosis¹⁴ similar to that seen with PKC α -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 PKC α -induced phosphorylation of TRPM2-S. A question arises about the relationship of this mechanism with TRPM2 activation induced by the generation of ADPR following 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 following activation of poly(ADPR) polymerase may help to amplify the Ca^{2+} entry response. However, in the event that both TRPM2-S and TRPM2 are co-expressed 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 Ca^{2+} entry by this mechanism is sufficient to activate the pro-apoptotic 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 Ca^{2+} 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. PKC α functioned to induce phosphorylation of TRPM2-S, which prevented its association with TRPM2, and thereby activated Ca^{2+} gating and caspases. Thus, disabling TRPM2-S and TRPM2 interaction such as by inhibiting PKC α 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.

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 PKC α .
- TRPM2 in endothelial cells is normally impermeable to Ca^{2+} because of its binding to the short splice variant TRPM2-S.
- Oxidants induce PKC α 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 α phosphorylation of the short splice variant of TRPM2, TRPM2-S, which acts as a phospho-switch 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 α phosphorylation of TRPM2-S at a specific site identifying a novel potential anti-inflammatory target.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Nonstandard Abbreviations and Acronyms

ADPR	adenosine diphosphate ribose
DPQ	3,4-Dihydro-5[4-(1-piperindinyl)butoxy]-1(2H)-isoquinoline
FACS	Fluorescence-activated cell sorting
GO	glucose oxidase
HPAEC	human pulmonary aortic endothelial cells
NUDT9-H	nucleoside diphosphate type motif 9 protein
PE-Annexin	phycoerythrin Annexin V
PARP	poly(ADPR) polymerase
PKCα	protein kinase C-α
PKCαi	protein kinase C-α inhibitor peptide
PKCβII	protein kinase βII
PKCβIIi	protein kinase βII inhibitor peptide
ROS	reactive oxygen species
S39A	serine 39 mutated to alanine
TRPM2	Melastatin-like transient receptor potential 2
TRPM2-S	Melastatin-like transient receptor potential 2 short variant
VE-cadherin	vascular endothelial cadherin
WT	wild-type
7-AAD	7-Aminoactinomycin D

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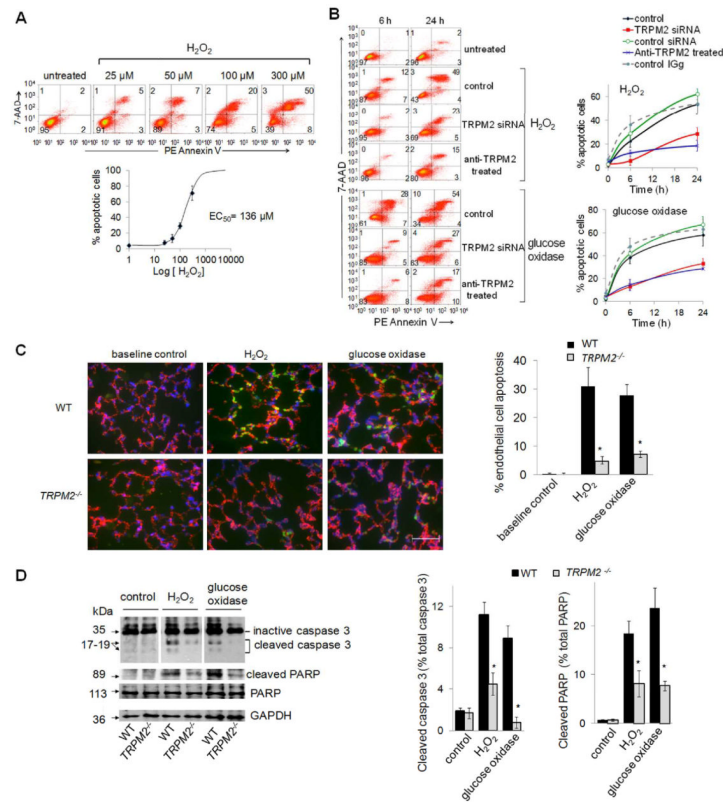


Figure 1. TRPM2 is required for H₂O₂-mediated apoptosis of endothelial cells

(A-B) Confluent endothelial cell monolayers (HPAECs) challenged with H₂O₂ or glucose oxidase/glucose were labeled with PE Annexin V-FITC and 7-AAD and analyzed by flow cytometry. (A) Concentration-response curve for H₂O₂-induced apoptosis after 24-h period of H₂O₂ exposure. Mean apoptotic values (± SEM) obtained by flow cytometry (*top panel*) are plotted as % apoptotic cells vs. [H₂O₂] (EC₅₀ = 136 μmol/L ; *bottom panel*) (n=3). (B) *Left*, representative flow cytometry histograms 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 blocking TRPM2 antibody. *Right panel*, mean percent apoptotic cells at 0, 6, or 24 h after H₂O₂ treatment (± SEM, n=3). Baseline values were not significantly altered by TRPM2 silencing or TRPM2 blocking Ab. (C-D) Apoptosis in lungs of TRPM2^{-/-} and wild-type (WT) mice measured 3 h after perfusion with solution containing H₂O₂ (300 μmol/L) or glucose oxidase/glucose (75 min). (C) *Right*, immunofluorescent staining of frozen lung sections using VE-cadherin (red) and cleaved-PARP (green) antibodies and DAPI (blue)(n=3). Scale bar: 50 μm. *Left*, quantification of apoptotic endothelial cells (±SEM; n=6), * p 0.007 vs. WT lungs (*t*-test). (D) Western immunoblots (*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.

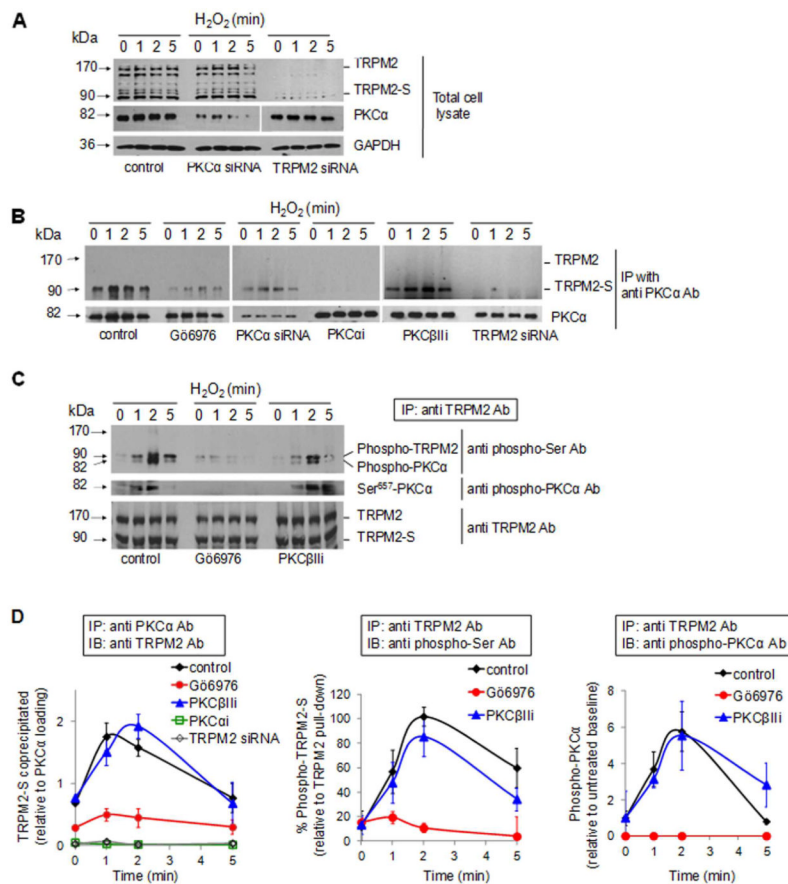


Figure 2. PKC α binding and phosphorylation of TRPM2-S
 HPAECs were transduced with siRNA to suppress the expression of TRPM2 or PKC α or pretreated with PKC inhibitors (100 nmol/L G66976, 1 μ mol/L PKC α i or 1 μ mol/L PKC β IIi). Cells were challenged with 300 μ mol/L H₂O₂ for the indicated times at 37°C. (A) Western blots from HPAEC lysates identifying expression of TRPM2 isoforms and PKC α ; GAPDH was used as loading control. Endothelial cell expression of TRPM2-S (90 kDa) and two TRPM2 splice variants (140 and 171 kDa) were suppressed by TRPM2 silencing and PKC α protein expression (82 kDa) was blocked by PKC α silencing. (B) H₂O₂ induced association of PKC α and TRPM2-S. PKC α was immunoprecipitated from cell lysates. Following SDS-PAGE and electrophoretic transfer, co-immunoprecipitated TRPM2 was detected using an antibody recognizing both TRPM2 and TRPM2-S. TRPM2-S associated with PKC α immediately after H₂O₂ exposure whereas inhibition of PKC α activation prevented the association. (C) H₂O₂ induced PKC α -dependent phosphorylation of 90kDa TRPM2-S splice variant. TRPM2 was immunoprecipitated from same cell lysates using an Ab that recognized either TRPM2 isoform. Top panel: Blots showing phosphorylation of TRPM2 using monoclonal anti-phospho-Ser antibody. Membrane was reblotted with anti-phospho-PKC α (Ser 657) Ab to check the amount of phosphorylated PKC α in the complex (middle panel). Immunoprecipitation of TRPM2 was verified using an anti-TRPM2 Ab (lower panel). (D) Mean densitometric values (\pm SEM; n=3-4) obtained in B-C showing that PKC α inhibition prevented H₂O₂-induced association of PKC α with TRPM2-S and phosphorylation of TRPM2-S

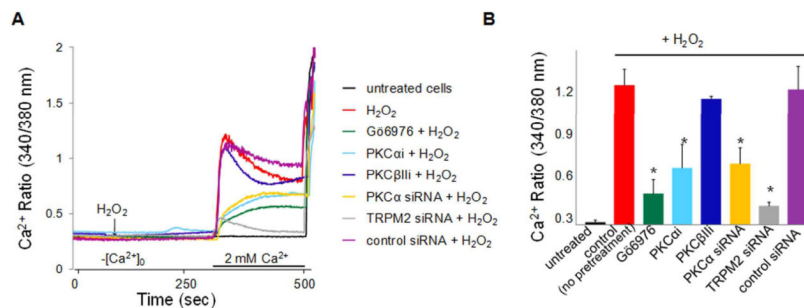


Figure 3. PKC α and TRPM2 cooperation mediates Ca²⁺ entry in endothelial cells required for signaling apoptosis

(A) Ca²⁺ repletion transients generated by “Ca²⁺-add-back” in the presence of H₂O₂. Cultured HPAECs were loaded with Fura-2 Ca²⁺ dye, washed, and transferred to Ca²⁺-free medium. In control cells, H₂O₂ (100 μ M) elicited a marked Ca²⁺ transient on Ca²⁺ repletion (red trace). Ca²⁺ transients were blocked by TRPM2 silencing (grey trace) and reduced by Gö6976 (100 nmol/L; green trace), PKC α i (1 μ mol/L; cyan trace), or after PKC α silencing (yellow trace); PKC β IIIi (1 μ mol/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 (\pm SEM) for the peak intracellular [Ca²⁺]_i obtained in (E) (n = 3 to 5). *P = 0.0002 vs. control (*t*-test).

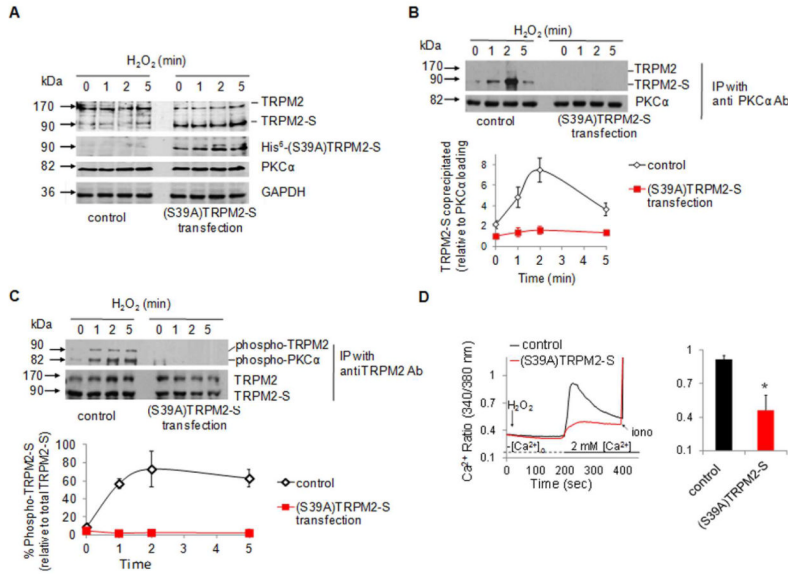


Figure 4. PKC α binds TRPM2-S at Ser 39 and activates apoptosis-inducing Ca $^{2+}$ entry signal in endothelial cells

The sole predicted PKC α phosphorylation site near the TRPM2-S N-terminus at Ser 39 was mutated by Ala substitution (resulting in phosphodeficient mutant). HPAEC 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 through C) or intracellular Ca $^{2+}$ measurements using fura-2 (D). (A-C) Cells were exposed to 300 μ M H $_2$ O $_2$ for the indicated times. (A) Western blots for TRPM2, PKC α , and GAPDH expression in cells transduced with phosphodeficient construct. Transfected protein was detected with an anti-His 6 Ab confirming the expression of mutant TRPM2-S construct. (B) PKC α was immunoprecipitated from cell lysates with an antibody and co-immunoprecipitated TRPM2 protein was detected using an Ab recognizing both forms of TRPM2. Graph in B shows mean densitometric values (\pm SEM; n=3-4). Mutation of Ser 39 with Ala in TRPM2-S prevented TRPM2-S association with PKC α . (C) TRPM2 was immunoprecipitated from the same lysates and phosphorylated TRPM2 was detected using anti-phospho-Ser Ab. Graph in C shows mean densitometric values (\pm SEM; n=3-4). Ala substitution at Ser 39 abrogated H $_2$ O $_2$ -induced phosphorylation of TRPM2-S confirming the importance of the PKC α phosphorylation site on TRPM2-S at Ser 39. (D) Ca $^{2+}$ mobilization assay was carried out using the “Ca $^{2+}$ add-back” protocol. Transduction of phosphodeficient 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, \pm SEM.

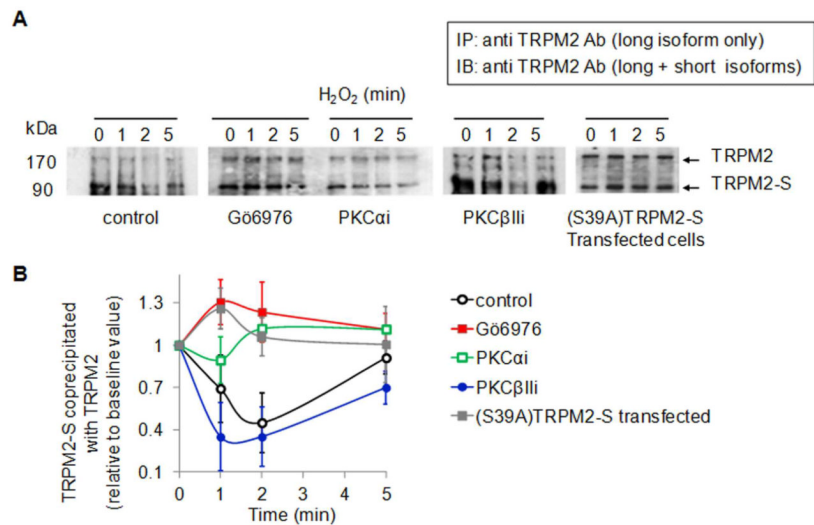


Figure 5. PKC α phosphorylation of TRPM2-S mediates TRPM2-S dissociation from TRPM2 resulting apoptosis-inducing Ca²⁺ entry signal

HPAEC monolayers were pretreated with PKC α and PKC β II (control) inhibitors 45 min prior to experiments. **(A)** TRPM2 was immunoprecipitated from cell lysates with an anti-TRPM2 antibody recognizing the region present only on the long isoform following H₂O₂ (300 μ M) exposure for indicated times. Co-immunoprecipitated short isoform was then detected using an Ab that recognizes both TRPM2 and TRPM2-S. At 0 time, TRPM2 in plasma membrane was associated with its short isoform, and H₂O₂ application induced rapid dissociation of TRPM2-S from TRPM2. H₂O₂-mediated dissociation was suppressed by PKC α inhibition and did not occur when Ser 39 of TRPM2-S was substituted by Ala. **(B)** Western blots were quantified by densitometry. Co-immunoprecipitated TRPM2-S was quantified as ratio to TRPM2 and plotted relative to zero time value (mean \pm SEM; n = 3)..

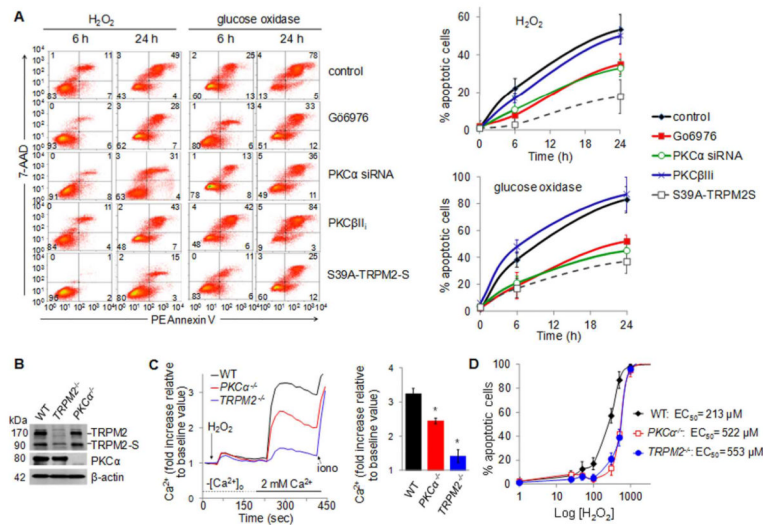


Figure 6. H₂O₂-induced endothelial cell apoptosis resulting from TRPM2-mediated Ca²⁺ influx HPAECs (**A**) and mouse lung endothelial cells (**B-D**) challenged with H₂O₂ were labeled with PE-Annexin/7-AAD. (**A**) Flow-cytometry histograms (*Left*) and summary plots of apoptosis (*Right*) 0, 6, and 24 h after challenge with H₂O₂ (300 μmol/L) or glucose oxidase/glucose to generate 320 nmol/L H₂O₂/min for 90 min, as a function of PKCα inhibition or silencing (± SEM, n=5). (**B-D**) Mouse endothelial cells were isolated from lungs of TRPM2^{-/-}, PKCα^{-/-}, and 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.

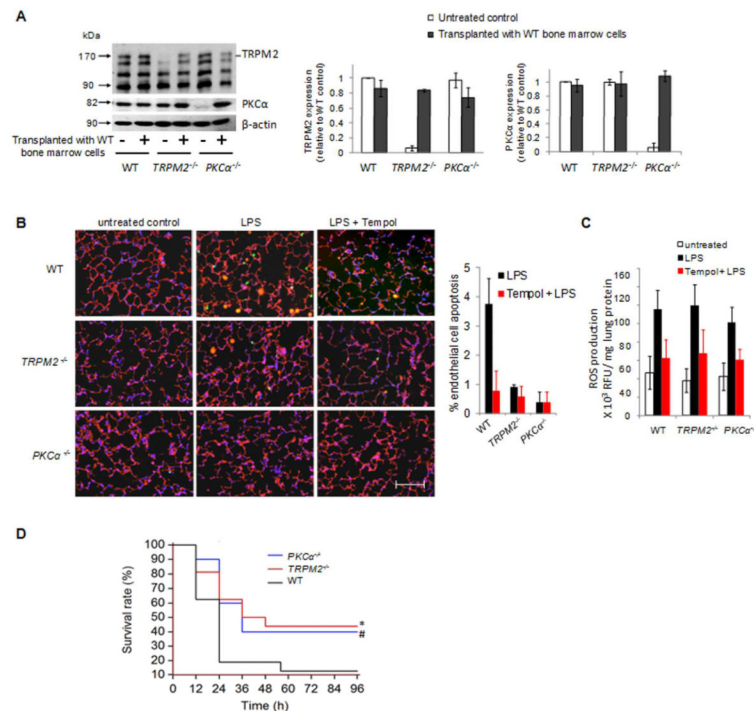


Figure 7. PKC α interaction with TRPM2 in mice is required lung endothelial cell apoptosis in response to LPS and contributes to mortality

WT, TRPM2^{-/-} and PKC α ^{-/-} mice were transplanted with bone marrow cells isolated from WT mice 8 weeks prior to Western blotting (A), apoptosis (B-C) and survival (D) studies. (A) Representative Western blots verifying expression of TRPM2, PKC α , and β -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 α densities were normalized to β -actin and plotted as percentage of untreated control (mean \pm SEM for n=3). (B-C) Endothelial apoptosis (B) and oxidant production (C) were determined in lungs of mice 4h after intraperitoneal injection of lipopolysaccharide (LPS, 40 mg/kg) and in lungs of mice treated with the oxidant scavenger Tempol (100 mg/kg, IP) 30 min before injection of LPS. (B) *Right*, immunofluorescent staining of frozen lung sections using VE-cadherin (red) antibody, TUNEL (green), and DAPI (blue)(n=3). Scale bar: 50 μ m. *Left*, quantification of apoptotic endothelial cells (\pm SEM; n=3), * p 0.001 and # p 0.005 vs. LPS-treated WT lungs. TRPM2 and PKC α deletion significantly reduced LPS-induced endothelial apoptosis. (C) Lungs were homogenized and assayed for H₂O₂ accumulation using the horseradish peroxidase-linked Amplex Red assay. H₂O₂ was determined spectrophotometrically from its absorbance at 570 nM and corrected for total protein (\pm SEM, n=3). * p 0.005 vs. LPS-treated control lungs (D) Deletion of either TRPM2 or PKC α reduced LPS-induced lethality in mice. LPS (30 mg/kg) was injected intraperitoneally and survival was assessed every 12 h during the experiment (WT, n=16; TRPM2^{-/-}, n= 16; and PKC α ^{-/-}, n=12). Statistical analysis was performed using the log-rank test. *p 0.03 and #p 0.05 vs. WT cells.

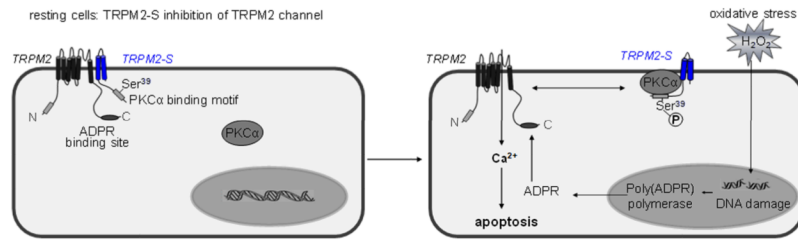


Figure 8. PKC α -induced phosphorylation of TRPM2-S mediates oxidant-induced TRPM2 channel opening and supra-normal Ca²⁺ entry required for activation of the apoptosis program
 In resting cells, TRPM2-S associates with TRPM2 to restrict Ca²⁺ entry (the channel is in a closed conformation state). Oxidative stress induces PKC α activation resulting in binding and phosphorylation of TRPM2-S on Ser 39. TRPM2-S uncoupling from TRPM2 in turn opens the channel and Ca²⁺ entry resulting in activation of executioner caspases and endothelial apoptosis. In this model the generation of the endogenous TRPM2 agonist, ADPR may also contribute to opening TRPM2 channel.