Role of TRPM2 Channel in Mediating \( \text{H}_2\text{O}_2 \)-Induced \( \text{Ca}^{2+} \) Entry and Endothelial Hyperpermeability

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Abstract—Oxidative stress through the production of oxygen metabolites such as hydrogen peroxide (\( \text{H}_2\text{O}_2 \)) increases vascular endothelial permeability. \( \text{H}_2\text{O}_2 \) stimulates ADP-ribose formation, which in turn opens transient receptor potential melastatin (TRPM)2 channels. Here, in endothelial cells, we demonstrate transcript and protein expression of TRPM2, a \( \text{Ca}^{2+} \)-permeable, nonselective cation channel. We further show the importance of TRPM2 expression in signaling of increased endothelial permeability by oxidative stress. Exposure of endothelial cell monolayers to sublytic concentrations of \( \text{H}_2\text{O}_2 \) induced a cationic current measured by patch-clamp recording and \( \text{Ca}^{2+} \) entry detected by intracellular fura-2 fluorescence. \( \text{H}_2\text{O}_2 \) in a concentration-dependent manner also decreased trans-monolayer transendothelial electrical resistance for 3 hours (with maximal effect seen at 300 \( \mu \text{mol/L} \) \( \text{H}_2\text{O}_2 \)), indicating opening of interendothelial junctions. The cationic current, \( \text{Ca}^{2+} \) entry, and transendothelial electrical resistance decrease elicited by \( \text{H}_2\text{O}_2 \) were inhibited by siRNA depleting TRPM2 or antibody blocking of TRPM2. \( \text{H}_2\text{O}_2 \) responses were attenuated by overexpression of the dominant-negative splice variant of TRPM2 or inhibition of ADP-ribose formation. Overexpression of the full-length TRPM2 enhanced \( \text{H}_2\text{O}_2 \)-mediated \( \text{Ca}^{2+} \) entry, cationic current, and the transendothelial electrical resistance decrease. Thus, TRPM2 mediates \( \text{H}_2\text{O}_2 \)-induced increase in endothelial permeability through the activation of \( \text{Ca}^{2+} \) entry via TRPM2. TRPM2 represents a novel therapeutic target directed against oxidant-induced endothelial barrier disruption. (Circ Res. 2008;102:347-355.)

Key Words: transient receptor potential channels \( \bullet \) \( \text{Ca}^{2+} \) influx \( \bullet \) endothelial vascular barrier \( \bullet \) permeability \( \bullet \) lung injury

Reactive oxygen species (ROS) are important mediators of vascular barrier dysfunction in settings such as acute respiratory distress syndrome, ischemia/reperfusion, and hyperoxia. Evidence suggests that oxidants increase \( \text{Ca}^{2+} \) permeability of endothelial cell membrane. The resulting elevation of intracellular \( \text{Ca}^{2+} \) could contribute to barrier disruption because \( \text{Ca}^{2+} \) entry into endothelial cells is recognized to promote interendothelial gap formation. The molecular mechanisms of oxidant-induced change in endothelial \( \text{Ca}^{2+} \) permeability remains an important area of inquiry.

Transient receptor potential melastatin (TRPM2) is an oxidant-activated channel belonging to the TRP family of cation channels. TRPM2, first named TRPC7 and later LTRPC-2, is a nonselective cation channel widely expressed in mammalian tissues, including the brain, peripheral blood cells such as neutrophils, bone marrow, spleen, heart, and liver, and lungs. TRPM2 opening after exposure to oxidants is induced by the binding of the intracellular second messenger adenosine diphosphoribose (ADP-ribose) or related molecules to the Nudix box sequence motif NUDT9-H in its carboxyl-terminal domain. Because the Nudix box has significant homology with a pyrophosphatase, NUDT9 ADP-ribose hydrolase, TRPM2 was dubbed a “chanzyme.” Besides free ADP-ribose primarily generated from poly(ADP-ribose) polymerase (PARP) activity, NAD (possibly through conversion to ADP-ribose) and cyclic adenosine diphosphoribose (cADP-ribose) may also activate TRPM2.

Hydrogen peroxide (\( \text{H}_2\text{O}_2 \)) produced in the cytosol during oxidative stress stimulates ADP-ribose formation in the nucleus and mitochondria. TRPM2 channels may thus participate in signaling oxidative stress–induced \( \text{Ca}^{2+} \) entry, thereby eliciting \( \text{Ca}^{2+} \)-dependent cellular processes. Although most investigators can demonstrate an indirect action of \( \text{H}_2\text{O}_2 \) in stimulating ADP-ribose formation in nuclei and mitochondria, direct agonist action of \( \text{H}_2\text{O}_2 \) on TRPM2 is also proposed for myeloid cells.

In addition to full-length functional TRPM2 (TRPM2-L), several TRPM2 isoforms have been identified in human hematopoietic cells, including a short splice variant (TRPM2-S). TRPM2-S (short) lacks the entire carboxyl terminus of the long variant, 4 of 6 carboxyl-terminal transmembrane domains, including the putative \( \text{Ca}^{2+} \)-permeable pore, and functions in a dominant-negative fashion to inhibit TRPM2-L activity. TRPM2-S directly interacts with TRPM2-L to...
suppress H$_2$O$_2$-induced Ca$^{2+}$ influx through TRPM2-L in transfected 293T cells.$^{24}$ Thus, TRPM2-S is an important isoform of TRPM2 that may modulate channel activity and cell death induced by oxidative stress activation of TRPM2-L.$^{24}$

Here, we demonstrate that H$_2$O$_2$ at noncytolytic concentrations elicits marked Ca$^{2+}$ influx via TRPM2 channels, which thereby signals increased endothelial permeability. Inhibition of endogenous TRPM2 expression and function in endothelial cells by RNA silencing, a specific TRPM2-blocking antibody, overexpression of TRPM2-S isoform, or inhibition of ADP-ribose generation significantly decreased H$_2$O$_2$-induced increase in [Ca$^{2+}$], and the resulting increase in endothelial permeability. These data demonstrate a critical role of TRPM2 in the mechanism of endothelial barrier disruption following oxidative stress.

Materials and Methods

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

Endothelial Cell Culture and Transfection

Human pulmonary artery endothelial (HPAE) cells (Clonetics, La Jolla, Calif) 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 and 6.

Human full-length TRPM2, tagged on its carboxyl terminus with the blue fluorescent protein (BFP)–TRPM2-L, was subcloned into pQBI50 (QbioGene, Carlsbad, Calif). Short splice variant, tagged on its carboxyl terminus with the green fluorescent protein (GFP)–TRPM2-S, was inserted into pTracer-CMV (Invitrogen). HPAE cells grown to 60% to 80% confluence were transfected for 4 hours with 1 µg/mL each BFP–TRPM2-L or GFP–TRPM2-S cDNA or with vector alone (control cells) using LipofectAMINE Plus.$^{26}$ Cells transfected by TRPM2-L were susceptible to apoptosis; therefore, we added caspase 9 inhibitor (Ac-LEHD-CHO, 20 µM) to the medium. After 48 hours, transfected or control cells forming confluent monolayers were used for experiments. Successful transfection of cells with TRPM2-S or TRPM2-L was verified by detection of GFP (excitation, 478 nm; emission, 535 nm) or BFP (excitation, 380 nm; emission, 435 nm) with laser-scanning confocal microscope (Zeiss LSM 510). Transfection efficiency was 80% to 90%.

Small Interfering RNA Transfection

HPAE cells were transiently transfected with 50 nmol/L each 2 predesigned small interfering (si)RNAs using TransIT-TKO transfection reagent according to the instructions of the manufacturer. siRNAs were targeted to exon 1 or 4 of the TRPM2 mRNA sequence. We cotransfected both siRNAs for maximal knockdown of TRPM2 expression. As a control, we used commercially available nonspecific siRNA. Experiments and RNA extractions were performed at 48 hours after transfections. Transfection efficiency was >75%.

Whole-Cell Patch-Clamp Recording in Endothelial Cells, [Ca$^{2+}$]$_i$ Measurements, Transendothelial Electrical Resistance Measurement, and Western Blotting Analyses

Methods are detailed in the online data supplement.

Detection of TRPM2 by RT-PCR

Two-step RT-PCR was performed using the Eppendorf Mastercycler gradient system (Eppendorf, New York) and real-time PCR using the ABI Prism 7700 Sequence Detection System (Perkin-Elmer, Applied Biosystems). PCR was performed for 36 cycles (denaturation at 94°C for 15 seconds, annealing at 56°C for 30 seconds, and extension at 72°C for 1 minute for 36 cycles). The primers used to amplify the TRPM2-L transcript targeted its carboxyl-terminal region. The forward sequence used was 5'-TCCGACCCACCACACGCTGTA-3', and the reverse sequence was 5'-CGCATCTCQGTCCTGGAAGTG-3'. Primers targeting both transcripts (TRPM2-L and -S) were: forward, 5'-GAAGACATTTTCCGCAGA-3'; reverse, 5'-GCTGCTGCCCTTGCCTGTF-3'. We used coamplified human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a reference. The amplified products were separated on 1.4% agarose gels.

Results

H$_2$O$_2$ and ADP-Ribose Analog 3-Deaza-cADP-Ribose Activate TRPM2 Ionic Current in Endothelial Cells

To record the ionic current induced by oxidants in single HPAE cells, we voltage clamped the cells at −50 mV after achieving a whole-cell configuration. Addition of H$_2$O$_2$ (300 µmol/L, Figure 1A) rapidly elicited an inward current above background. Isosmotic replacement of all external Na$^+$ by the impermeant organic cation N-methyl-D-glucamine (NMDG$^+$) abolished the H$_2$O$_2$-induced inward current. Repletion of external Na$^+$ restored the current (Figure 1A). TRPM2 knockdown using siRNA abrogated the H$_2$O$_2$ effects (Figure 1B), indicating that expression of TRPM2 channels is required. Overexpression of TRPM2-L, which we verified as described (see Materials and Methods), augmented the H$_2$O$_2$-induced current (Figure 1C versus Figure 1A). The current–voltage relationship for cationic current was linear over a wide potential range (−80 to +80 mV) and passed through the origin. This current–voltage characteristic is typical of TRPM2 channels studied in expression systems.$^{24}$ Transduction of TRPM2-L, which caused overexpression of the functional channel (see below), increased the H$_2$O$_2$-induced current at all clamp potentials; the current–voltage curve was steeper in slope but unaltered in linearity or reversal potential. By contrast, TRPM2 silencing, which we also verified (see below), markedly reduced the slope. As a functional assay for TRPM2 channels, we tested a nonhydrolysable cADP-ribose analog (3-deaza-cADP-ribose, 10 nmol/L). Control experiments showed that saline vehicle elicited no current (Figure 1E). Internal application of the analogue via the patch pipette induced an inward current (Figure 1F), implying the presence of TRPM2 channel activity in HPAE cells. To further test the involvement of TRPM2 in the H$_2$O$_2$-induced inward current, we pretreated cultures with a cell-permeable inhibitor of PARP {100 µmol/L 3,4-dihydro-5-[4-(1-piperidinyl)butoxy]-1(2H)-isoquinolinone (DPQ) for 45 minutes} that prevents the generation of ADP-ribose$^{27}$ without directly blocking TRPM2 channel.$^{16}$ H$_2$O$_2$ was inactive in the presence of inhibitor (Figure 1G), indicating that endogenous TRPM2 activators ADP-ribose and cADP-ribose mediate effects of H$_2$O$_2$ in HPAE cells. The results, summarized in Figure 1H, show TRPM2 expression and cADP-ribose generation to be essential for elicitation of inward cationic current by H$_2$O$_2$.

Action of H$_2$O$_2$ on Ca$^{2+}$ Entry

Because the electrophysiological observations suggest that H$_2$O$_2$ can stimulate Ca$^{2+}$ entry by activating the Ca$^{2+}$-permeable channel TRPM2-L, we next measured intracellular
Ca\(^{2+}\) responses to H\(_2\)O\(_2\) in HPAE cells. We used a “Ca\(^{2+}\) add-back” protocol designed to rule out indirect effects of H\(_2\)O\(_2\) on Ca\(^{2+}\) entry via Ca\(^{2+}\)-store depletion (Figure 2). In the absence of extracellular Ca\(^{2+}\), H\(_2\)O\(_2\) application (\(\approx\)300 \(\mu\)mol/L) did not produce a Ca\(^{2+}\) transient, although in the same experiment, Ca\(^{2+}\) repletion (in the continued presence of H\(_2\)O\(_2\)) elicited a brisk Ca\(^{2+}\) response. Control experiments showed that Ca\(^{2+}\) repletion per se (no H\(_2\)O\(_2\) added) was completely ineffective in evoking Ca\(^{2+}\) transients (Figure 2). The amplitude of Ca\(^{2+}\)-repletion transients depended on the concentration of H\(_2\)O\(_2\) (EC\(_{50}\), 58.6 \(\mu\)mol/L; Figure 2, inset).

Figure 2. Concentration-dependent effects of H\(_2\)O\(_2\) on Ca\(^{2+}\) entry. HPAE cells in culture were loaded with fura-2, washed, and transferred to Ca\(^{2+}\)-free medium. H\(_2\)O\(_2\) (0 to 500 \(\mu\)mol/L) was added at the arrow and CaCl\(_2\) (2.0 \(\mu\)mol/L) was repleted at the fifth minute; the resulting Ca\(^{2+}\)-repletion transient reflects Ca\(^{2+}\) entry. The Ca\(^{2+}\) ionophore ionomycin (ion) was added at the end of the experimental recordings for calibration purposes. Each tracing is the average response of 60 to 99 cells in HPAE monolayers. The abscissa indicates time in seconds; the ordinate, relative [Ca\(^{2+}\)] level. Experiments were repeated 3 to 5 times with similar results. The inset displays the dose–response curve of best fit for the calcium-repletion transient (EC\(_{50}\), 58.6 \(\mu\)mol/L). The data points are mean values (n=3 per point), and the bars indicate \(\pm\)SEM. At >300 \(\mu\)mol/L, H\(_2\)O\(_2\) mobilized stored intracellular Ca\(^{2+}\).

**Figure 1.** H\(_2\)O\(_2\)- and 3-deaza-cADP-ribose-induced currents in HPAE cells. Subconfluent cultures of HPAE cells were prepared for whole-cell patch-clamp recording as described (Materials and Methods) and voltage-clamped to a standard holding potential (−50 mV). A through C, H\(_2\)O\(_2\) (300 \(\mu\)mol/L) addition to the bath (arrow) induced an inward current in control cells, which was reversibly blocked by isosmotic substitution of Na\(^{+}\) for NMDG\(^{-}\), an impermeant organic cation (A). TRPM2 silencing suppressed the current (B) in normal medium, and TRPM2-L overexpression enhanced it (C). D, Current–voltage relationship for membrane current induced by H\(_2\)O\(_2\) in untransfected cells, TRPM2 siRNA–transfected cells, and in TRPM2-L–overexpressing cells. E. Addition of control buffer to bath fluid had no effect above background. F, 3-Deaza-cADP-ribose (10 nmol/L) added to the pipette solution induced an inward current on establishing the whole-cell configuration. G, Pretreatment for 45 minutes with DPQ (D\(_{2}\); 100 \(\mu\)mol/L), an inhibitor of PARP, prevented the H\(_2\)O\(_2\)-induced (300 \(\mu\)mol/L) current. H, Bar graph quantifying peak currents (in pA) obtained in A through F. Results are given as means ±SEM (n=3 to 4 HPAE cells). *Significant difference in current amplitude from untreated control.

**TRPM2 Expression in Endothelial Cells**

RT-PCR and Western Blot analysis gave evidence of TRPM2 channel expression in HPAE cells (Figure 3A and 3B). TRPM2 transcript was detected in untreated wild-type HPAE cells and to an even greater extent in TRPM2-L transfected cells (Figure 3A). The channel was not detected in TRPM2 siRNA–treated cells, and in TRPM2-L–overexpressing cells. Western blot analysis showed TRPM2-L protein expression as a prominent band at 171 kDa. The level of TRPM2 detected in untreated HPAE cells was significantly reduced in TRPM2 siRNA–transfected cells; treatment with control siRNA was ineffective. Cells overexpressing TRPM2-L had a significantly intensified band at 171 kDa by densitometric analysis, confirming specificity of the antibody used.
TRPM2 Regulates H$_2$O$_2$-Induced Ca$^{2+}$ Entry in Endothelial Cells

Monolayers of HPAE cells adhering to glass coverslips had submicromolar levels of intracellular Ca$^{2+}$ in Ca$^{2+}$-containing medium (Figure 3C, inset). Addition of H$_2$O$_2$ (300 μmol/L) produced Ca$^{2+}$ transients (Figure 3D, inset). TRPM2 knockdown (siRNA-transfected HPAE cells) nearly abolished the H$_2$O$_2$ effect (Figure 3E, inset). Anti-TRPM2 blocking antibody (Bethyl Laboratories, Montgomery, Tex) also prevented the H$_2$O$_2$-induced Ca$^{2+}$ transients (Figure 3F, inset). We added back Ca$^{2+}$ after first depleting extracellular Ca$^{2+}$ for less than 5 minutes to rule out indirect effects of H$_2$O$_2$ via Ca$^{2+}$-store depletion. This alone without H$_2$O$_2$ had no effect on intracellular Ca$^{2+}$ levels (Figure 3C). In Ca$^{2+}$-free medium, H$_2$O$_2$ addition of released no intracellular Ca$^{2+}$, whereas Ca$^{2+}$ repletion in the continued presence of H$_2$O$_2$ elicited a Ca$^{2+}$ transient (Figure 3D), which represented the Ca$^{2+}$ entry stimulated by H$_2$O$_2$. A similar Ca$^{2+}$-repletion-dependent transient did not occur on TRPM2 depletion with siRNA (Figure 3E) or anti-TRPM2 blocking antibody (5 μg/mL for 16 hours) (Figure 3F). Preincubation of cells with control IgG did not modify Ca$^{2+}$ entry induced by H$_2$O$_2$ (see the legend of Figure 7), confirming the role of TRPM2 channels in H$_2$O$_2$-evoked Ca$^{2+}$ entry. The experiments were repeated 3 to 5 times with similar results. The abscissa indicates time in seconds; the ordinate, relative [Ca$^{2+}$] level. G, Mean ratiometric values (±SEM) for steady-state [Ca$^{2+}$] ($n=3$ to 4) obtained in C through F. Note that inhibition of TRPM2 activity prevents H$_2$O$_2$-mediated Ca$^{2+}$ entry.

TRPM2 Depletion Reduces H$_2$O$_2$-Mediated Endothelial Hyperpermeability

Increased intracellular Ca$^{2+}$ causes opening of interendothelial junctions, which is detectable as reduction in transendo-
thelial electrical resistance (TER). Because H₂O₂ increased intracellular Ca²⁺, we tested H₂O₂ for its ability to decrease TER. HPAE cells were grown to confluence on gold microelectrodes and treated with H₂O₂ (300 to 600 µmol/L), and changes in TER were followed for 4 hours. Recordings of TER in Figure 4A show that H₂O₂ decreases TER. The effect was transitory; at <500 µmol/L, H₂O₂-mediated response recovered to basal levels within 2 hours. Results of quantification of the peak TER response at each H₂O₂ concentration are displayed with curve of best fit (EC₅₀ of 254 µmol/L; Figure 4A).

We next compared TER responses to H₂O₂ with or without TRPM2 silencing. To show the effect of siRNAs on TRPM2 protein expression, we transduced HPAE cultures with BFP-tagged TRPM2-L, a fluorescent fusion protein forming functional channels. RT-PCR analysis showed the specificity of siRNAs. TRPM2 siRNA transfection reduced TRPM2 transcript expression by 75% without affecting expression of TRPM7 or GAPDH (Figure 4B). Control siRNA had no effect on TRPM2 expression (Figure 4B). We determined the percentage of fluorescent cells by confocal imaging. Without siRNA, 85% to 90% of cells were fluorescent (Figure 4C, left). TRPM2 siRNAs greatly reduced this percentage and the fluorescent intensity (Figure 4C, center). Nonspecific siRNA was ineffective (Figure 4C, right). HPAE cells were plated to confluence on gold electrodes without or with siRNA, and TER was measured. TRPM2 silencing reduced the TER response to H₂O₂ (300 µmol/L) by 42% relative to control (no siRNA transfection) or negative control group (scrambled siRNA; n=4 per group). The absissa indicates time in hours; the ordinate, normalized resistance. Right, Mean value (±SEM) of peak TER responses to H₂O₂.

**Figure 4.** H₂O₂-induced increase in endothelial barrier permeability depends on TRPM2-L expression. A, Concentration-dependent action of H₂O₂ on endothelial barrier function. HPAE cells were grown to confluence on gold microelectrodes, the cells were treated with H₂O₂ (concentration indicated), and TER was followed for 4 hours. Each tracing is the average response of 4 wells. Experiments were repeated 3 times with similar results. The inset shows the corresponding dose–response curve (n=12; bars, ±SEM). H₂O₂ (0 to 600 µmol/L) caused a rapid, dose-dependent decrease in TER with an EC₅₀ of 254 µmol/L. At <500 µmol/L, H₂O₂ effects were transitory. B, Real-time RT-PCR showing specificity of the siRNA used toward TRPM2 but not TRPM7 or GAPDH in confluent HPAE cultures transfected by TRPM2-L. C, Influence of TRPM2 siRNA in TRPM2-L–transduced cells. In these experiments, HPAE cells were transfected with a fluorescent form of TRPM2 (BFP–TRPM2-L). Confocal image of control cells expressing the construct is shown (left). Cotransfection with TRPM2-silencing RNA (siRNA) eliminated expression of the fusion protein (center). Cotransfection with scrambled siRNA (negative control) was ineffective (right). D, TER decrease on H₂O₂ exposure (300 µmol/L). Left, Note that TRPM2 silencing inhibits H₂O₂ responses relative to the untreated group (no transfection) or the negative control group (scrambled siRNA; n=4 per group). The absissa indicates time in hours; the ordinate, normalized resistance. Right, Mean value (±SEM) of peak TER responses to H₂O₂.

**Transduction of TRPM2-S Inhibits H₂O₂-Induced Ca²⁺ Entry and Endothelial Hyperpermeability**

The short splice variant of the functional channel, TRPM2-S, acts in a dominant-negative fashion to inhibit TRPM2-L activity. We overexpressed TRPM2-S in HPAE cells to determine its effects on H₂O₂-mediated responses. To quantify the expression level of TRPM2-S, we transfected HPAE cells with the fluorescent fusion protein GFP–TRPM2-S. We divided the cells for use in parallel determinations of intracellular Ca²⁺ and TER changes. Confocal imaging showed that 80% of cells expressed GFP–TRPM2-S (Figure 5A). RT-PCR analysis demonstrated that construct overexpression did not alter expression of endogenous TRPM2-L isofrom
Endothelial Barrier Disruption

Materials and Methods

We next tested the blocking effect of a TRPM2 antibody (see Materials and Methods) on the TER response to H₂O₂. TRPM2-L isoform (ie, the channel-forming isoform). C, Left, Ca²⁺ entry assay as described in Materials and Methods (see also the legend of Figure 2). Right, Mean ratiometric values (±SEM) for steady-state concentration does not alter endogenous level of TRPM2-L isoform (GFP–TRPM2-S). A, Confocal images of HPAE monolayer expressing GFP–TRPM2-S. B, RT-PCR analysis showing that construct expression does not influence the expression of GAPDH. C, Right, Summary of TER data. Note that the overexpression of GFP–TRPM2-S attenuates H₂O₂-induced (300 μmol/L) resistance changes. *Significance at P<0.05.

Overexpression of TRPM2-L Augments H₂O₂-Induced Ca²⁺ Entry and Endothelial Hyperpermeability

If the balance between short and long TRPM2 isoforms determines channel activity, overexpression of TRPM2-L should enhance H₂O₂ effects; thus, we transfected HPAE cells with the fluorescent BFP–TRPM2-L isoform (see Figure 4) and monitored H₂O₂ responses (Figure 6). H₂O₂ addition increased Ca²⁺ entry above the vector control by 29±14% (Figure 6A; n=4) and also increased the TER response above the control by 151±7% (Figure 6B; n=4).

TRPM2 Blocking Antibody Prevents H₂O₂-Induced Endothelial Barrier Disruption

We next tested the blocking effect of a TRPM2 antibody (see Materials and Methods) on the TER response to H₂O₂. Confluent HPAE monolayers were treated overnight with either blocking antibody (5 μg/mL) or isotype-matched control antibody (5 μg/mL). Treatment with the specific antibody markedly reduced Ca²⁺ entry evoked by 300 μmol/L H₂O₂ compared with control (Figure 7A; n=3 for each group). TRPM2 antibody also diminished the TER response to H₂O₂ by 44±9% (Figure 7B; n=3); control antibody had no effect.

Inhibition of PARP Suppresses H₂O₂-Mediated Ca²⁺ Entry and Permeability Increase

TRPM2-L is activated by the binding of ADP-ribose to its binding cleft in TRPM2 carboxyl terminus. H₂O₂ stimulates PARP to generate ADP-ribose, whereas inhibitors of PARP prevent agonist formation. Therefore, we tested 2 PARP inhibitors, DPQ and 3-aminobenzamide (3-AB), for their ability to prevent H₂O₂ responses. HPAE cells were treated with 3-AB (1 mmol/L) or DPQ (100 μmol/L) for 45 minutes, and intracellular Ca²⁺ was measured in response to H₂O₂ (300 μmol/L) using the Ca²⁺ add-back protocol. Both 3-AB and DPQ significantly reduced Ca²⁺-repletion transients (60±11% and 62±9%, respectively) compared with untreated cells (Figure 8A). We obtained similar results in Ca²⁺-containing media on stimulating cells with H₂O₂ (300 μmol/L) (see Figure 8A, inset). In parallel experiments

Figure 5. Transduction of TRPM2-S ("short") dominant-negative splice variant of TRPM2 inhibits H₂O₂-induced Ca²⁺ entry and increase in endothelial permeability. The short splice variant of the channel, TRPM2-S, lacking the putative Ca²⁺-permeable pore, acts in a dominant-negative fashion to inhibit TRPM2-L activity. HPAE cells were transfected by a fluorescently tagged TRPM2-S isoform (GFP–TRPM2-S). A, Confocal images of HPAE monolayer expressing GFP–TRMP2-S. B, RT-PCR analysis showing that construct expression does not alter endogenous level of TRPM2-L isoform (ie, the channel-forming isoform). C, Left, Ca²⁺ entry assay as described in Materials and Methods (see also the legend of Figure 2). Right, Summary of TER data. Note that the overexpression of GFP–TRPM2-S attenuates H₂O₂-induced (300 μmol/L) resistance changes. *Significance at P<0.05.

(Figure 5B). Using the Ca²⁺ add-back protocol, we observed that overexpression of GFP–TRPM2-S inhibited H₂O₂-mediated (300 μmol/L) Ca²⁺ entry by 83±13% (Figure 5C). Overexpression of GFP–TRPM2-S also attenuated the peak TER response to H₂O₂ (300 μmol/L) by 31±10% and markedly reduced its duration (Figure 5D; n=4).

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also express functional TRPM2 channels. Under physiological conditions, TRPM2 channels are Ca\(^{2+}\)/H\(_{11005}\) sensitive activators of TRPM2 channels, suggesting that TRPM2 channels mediate approximately half of the permeability-increasing effect of H\(_{2}O_{2}\). The residual effect of H\(_{2}O_{2}\), which appears to be independent of Ca\(^{2+}\) entry, remains to be elucidated.

We showed that pathophysiologically relevant H\(_{2}O_{2}\) concentrations of 100 to 300 \(\mu\)mol/L did not release Ca\(^{2+}\) from intracellular stores but did significantly stimulate extracellular Ca\(^{2+}\) entry, indicating that H\(_{2}O_{2}\) (<300 \(\mu\)mol/L) does not activate store-operated channels in HPAE cells. We determined whether oxidants (≤300 \(\mu\)mol/L) activated Ca\(^{2+}\) entry via TRPM2 channels by suppressing TRPM2 expression or activity by various means, eg, siRNAs, TRPM2-blocking antibody, overexpression of TRPM2-S, and PARP inhibitors that prevent generation of ADP-ribose. All of these interventions abolished H\(_{2}O_{2}\)-induced Ca\(^{2+}\) entry, indicating the crucial role of activation of TRPM2 channels in mediating Ca\(^{2+}\) entry caused by H\(_{2}O_{2}\) in endothelial cells.

Discussion

The present study demonstrates the expression and function of TRPM2-L channels in endothelial cells. Western blot analysis indicated the presence of the channel protein. Bath-applied H\(_{2}O_{2}\) or internally applied ADP-ribose (the endogenous activator of TRPM2 channels) elicited inward currents consistent with opening of nonselective cation channels. The ability to abolish H\(_{2}O_{2}\)-induced current by blocking cellular generation of ADP-ribose suggests that TRPM2 channel is responsible for the observed electrophysiological effects of H\(_{2}O_{2}\). TRPM2-silencing studies confirmed that TRPM2 is requisite for H\(_{2}O_{2}\)-induced currents. The linearity of the current–voltage curve for TRPM2 channels and reversal potential obtained agree with findings in microglia, which also express functional TRPM2 channels. Under physiological conditions (140 mmol/L Na\(^{+}\), 2 mmol/L Ca\(^{2+}\)), Na\(^{+}\) was the main carrier of inward current via endothelial TRPM2 channels, because NMDG substitution for Na\(^{+}\) abolished the current. Ca\(^{2+}\) entry via the channel was also detected using the fura-2 method. Because oxidant-activated TRPM2 channels are Ca\(^{2+}\)-permeable, we hypothesized their involvement in the oxidant-induced rise in intracellular Ca\(^{2+}\) and the resulting increase in paracellular permeability.

We observed that pathophysiologically relevant H\(_{2}O_{2}\) concentrations of 100 to 300 \(\mu\)mol/L did not release Ca\(^{2+}\) from intracellular stores but did significantly stimulate extracellular Ca\(^{2+}\) entry, indicating that H\(_{2}O_{2}\) (<300 \(\mu\)mol/L) does not activate store-operated channels in HPAE cells. We determined whether oxidants (≤300 \(\mu\)mol/L) activated Ca\(^{2+}\) entry via TRPM2 channels by suppressing TRPM2 expression or activity by various means, eg, siRNAs, TRPM2-blocking antibody, overexpression of TRPM2-S, and PARP inhibitors that prevent generation of ADP-ribose. All of these interventions abolished H\(_{2}O_{2}\)-induced Ca\(^{2+}\) entry, indicating the crucial role of activation of TRPM2 channels in mediating Ca\(^{2+}\) entry caused by H\(_{2}O_{2}\) in endothelial cells.

We also determined the role of TRPM2 in mediating the H\(_{2}O_{2}\)-induced decrease in TER resulting from opening of interendothelial junctions. Suppression of TRPM2 activity caused only a 50% reduction in the TER response of H\(_{2}O_{2}\), suggesting that TRPM2 channels mediate approximately half of the permeability-increasing effect of H\(_{2}O_{2}\). The residual effect of H\(_{2}O_{2}\), which appears to be independent of Ca\(^{2+}\) entry, remains to be elucidated.

We showed that TRPM2-S overexpression suppressed Ca\(^{2+}\) entry and TER responses to H\(_{2}O_{2}\) in endothelial cells. Importantly, the cells normally expressed both isoforms because the relative expression levels observed by real-time RT-PCR were greater using the primer targeting both isoforms, as opposed to the primer specifically targeting the long form. TRPM2-S in human hematopoietic cells is generated by alternative splicing of the full-length protein.
by Ca2++ entry and permeability increase. HPAE cells were pretreated with inhibitors of ADP-ribos e polymerase (1 mmol/L 3-AB or 100 μmol/L DPQ) for 45 minutes. A and B. In response to H2O2 challenge (300 μmol/L), we measured intracellular Ca2++ response to H2O2 may well depend on the cell current of 125 pA (at 50 mV), we calculated that polymerase inhibitor significantly reduced Ca2++ rise induced by H2O2 (n = 3). B. Left, Recordings of TER in the absence or presence of polymerase inhibitors. Right, ADP-ribose polymerase inhibitors suppressed the increase in barrier permeability caused by H2O2 (n = 12). These observations are consistent with known properties of TRPM2 channel that is selectively activated by H2O2 by the intracellular ligand ADP-ribose.

cells have also demonstrated direct interaction between short and long TRPM2 isoforms, resulting in suppression of H2O2-induced current and Ca2++ entry via TRPM2-L.24 Thus, endothelial responses to H2O2 may well depend on the relative abundance of the 2 isoforms.

Our electrophysiological observations suggest that a small population of TRPM2 channels can account for the whole-cell current induced by H2O2. Given an estimated single-channel conductance of 67 pS,20,32 and our observed whole-cell current of 125 pA at −50 mV, we calculated ~40 functional channels per cell. However, this apparent value presumably underestimates the actual expression of TRPM2 in the endothelial cell membrane because of the presence of the inhibitory short isoform TRPM2-S.

Lung endothelial injury, particularly in the setting of sepsis, is the result of oxidant generation by endothelial cells themselves and neutrophils and other inflammatory cells adherent to vessels.33,34 The generally held belief is that the resulting oxidants, including H2O2, directly damage endothelium.1−4 Our observations demonstrate a novel mechanism of H2O2-mediated disruption of endothelial barrier function that, in part, is attributable to a rise in intracellular Ca2++ mediated by Ca2++ entry through oxidant-sensitive TRPM2 channels. In this regard, increased microvessel endothelial permeability secondary to ischemia/reperfusion and neutrophil adhesion and activation may depend on H2O2-mediated activation of TRPM2 and influx of Ca2++. Therefore, inhibition of TRPM2 may provide a useful therapeutic strategy for the treatment of endothelial barrier dysfunction and vascular inflammation.

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Disclosures
None.

References


Role of TRPM2 Channel in Mediating H$_2$O$_2$-Induced Ca$^{2+}$ Entry and Endothelial Hyperpermeability

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Expanded Materials and Methods

Reagents and chemicals: Endothelial growth medium (EBM-2) was obtained from Clonetics Corp. (San Diego, CA). cDNAs encoding functional fusion proteins of human TRPM2 channels and blue or green fluorescent proteins (i.e., TRPM2-BFP and sTRPM2-GFP) were donated by Dr. Barbara A. Miller (Pennsylvania State University College of Medicine, Hershey, PA). Myc-tagged TRPM2-L cDNA construct was made by us. Trypsin, Hank’s balanced salt solution, molecular cellular and developmental biology (MCDB) media 131, TRIzol reagent, and Superscript II were obtained from Invitrogen (Carlsbad, CA). Superfect transfection reagent was obtained from Qiagen (Valencia, CA); and TransIT-TKO Mirus transfection reagent from Mirus Bio (Madison, WI). Fura-2/acetoxyethyl ester (AM) was obtained from Molecular Probes (Eugene, OR) and caspase 9 inhibitor (Ac-LEHD-CHO) was from AG Scientific (San Diego, CA). H2O2, penicillin, and peptides and chemicals were purchased from Sigma Chemical Co. (St. Louis, MO). TRPM2 pre-designed and custom-made SiRNAs were obtained from Ambion, (Austin, TX). Two TRPM2 antibodies (for blocking and Western blots) were purchased from Bethyl Laboratories (Montgomery, TX) and Abcam (Cambridge, MA), respectively.

Endothelial cell culture and transfection. 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.

Transfection of HPAE cells. Human full length TRPM2, tagged on its carboxy-terminus with BFP (BFP-TRPM2-L), was sub-cloned into pQBI50 (QbioGene, Carlsbad, CA). Short splice-
variant, tagged on its carboxy-terminus with GFP (GFP-TRPM2-S), was inserted into pTracer-CMV (Invitrogen). HPAE cells were grown to 60-80% confluency. The cultures were transfected for 4 hr with 1 µg/ml each of BFP-TRPM2-L or GFP-TRPM2-S cDNA or with vector alone (control cells) using LipofectAMINE Plus. Cells transfected by TRPM2-L were susceptible to apoptosis; therefore, we added caspase 9 inhibitor (Ac-LEHD-CHO, 20 µmol/L) to the medium. After 48 hr, transfected or control cells forming confluent monolayers were used for experiments. Successful transfection of cells with TRPM2-S or TRPM2-L was verified by detection of GFP (excitation, 478 nm; emission, 535 nm) or BFP (excitation, 380 nm; emission 435 nm) with laser-scanning confocal microscope (Zeiss LSM 510). Transfection efficiency was 80-90%.

**siRNA transfection.** HPAEs were transiently transfected with 50 nM each of two pre-designed small interfering RNAs (siRNAs) using TransIT-TKO transfection reagent (Mirus Bi, Madison, WI) according to manufacturer's instructions. siRNAs were targeted to exon1 (siRNAex1) or exon 4 (siRNAex4) of TRPM2 mRNA sequence. We co-transfected both siRNAs for maximal knock-down of TRPM2 expression. As control, we used commercially available nonspecific (NS) siRNA (Ambion, Austin, TX). Experiments and RNA extractions were performed at 48 hr after transfections. Transfection efficiency was over 75%.

**Whole cell patch clamp recording in endothelial cells.** Patch clamp recording was performed on individual HPAE cells adhering to the coverslip. TRPM2 channel opening was induced with H₂O₂ (300 µmol/L) in the bath solution or cyclic ADP ribose analog (3-deaza-cADP-ribose, 10 nmol/L) in the pipette solution. TRPM2 currents were recorded at the holding potential (–50 mV); currents were normalized by the cell capacitance. In experiments with the ADP-ribose polymerase inhibitor (3,4-dihydro-5-[4-(1-piperidinyl)butoxy]-1(2H)-isoquinolinone, DPQ),
HPAE cells were pretreated with the blocker 45 min prior to experiments. All experiments were performed at room temperature.

**[Ca^{2+}]_{i} measurements.** Control or transfected HPAE cells (see above) grown to confluence on 25-mm 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 filled with 200 µl of buffer. We measured Fura 2 fluorescence using Attoflor Ratio Vision digital fluorescence microscope (Atto Instruments, Rockville, MD) equipped with a Zeiss Axiovert S100 inverted microscope and F-Fluar 40 x oil-immersion objective, with a numerical aperture of 1.3. Excitation wavelengths used were 340 and 380 nm, and emission wavelength was 510 nm. Intracellular Ca^{2+} levels are given as fluorescent ratio F_{340}/F_{380} representing bound/free Ca^{2+}.^{2}

**Transendothelial electrical resistance measurement.** Transendothelial electrical resistance, reflecting the paracellular permeability of endothelial monolayers, was measured using established protocols.^{3} Control or transfected HPAE cells that over-expressed BFP-TRPM2-L or GFP-TRPM2-S (see above) were grown to confluence on gelatin-coated gold electrodes. Cells were placed in reduced serum MCDB131 medium (1% fetal bovine serum) for 1 hr and baseline TER measurements was recorded. Monolayers were exposed to H_{2}O_{2} and subsequent changes in TER were determined. Data are presented as change in monolayer resistance (i.e, the in-phase component of monolayer impedance) normalized to resistance value at zero time. In experiments in which drugs were used to block H_{2}O_{2} responses, cultures received the inhibitor 1 hr prior to H_{2}O_{2} challenge.

**RNA extraction and detection by RT-PCR.** Total RNA was isolated from HPAE cultures using TRIzol. two-step RT-PCR was performed using the Eppendorf Mastercycler gradient system
(Eppendorf, New York, NY), and real-time PCR using the ABI Prism 7700 Sequence Detection System (Perkin-Elmer, Applied biosystems). PCR was performed for 36 cycles (denaturation at 94 °C for 15 s, annealing at 56 °C for 30 s, and extension at 72 °C for 1 min for 36 cycles). The primers used to amplify the TRPM2-L transcript targeted its carboxy-terminal region. The forward sequence used was 5’-TCGGACCCAACCACACGCTGTA-3’ and reverse sequence was 5’-CGTCATTCTGGTCTGGGAAGTG-3’. Primers targeting both transcripts (TRPM2-L&S) were: forward, 5’ - GAAGAGCATTTTCCGCAGA - 3’ and reverse, ATGAGCTCGCCTTCCCTTG. We utilized co-amplified human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) for reference. The amplified products were separated on 1.4 % agarose gels.

**Western Blot Analysis.** HPAE monolayers were washed with 1x phosphate-buffered saline, lysed in Tris buffer (supplemented with 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, and protease-inhibitor cocktail), and then sonicated for 20 sec. Proteins from each lysate were separated by electrophoresis on a 4-12% gradient of SDS polyacrylamide gel, and transferred to nitrocellulose membrane for Western blotting with TRPM2 (Abcam, Cambridge, MA) or actin antibodies. Band intensity was determined by densitometry with the aid of MetaMorph software (Molecular Devices Corp., Downingtown, PA).

**Statistical analysis.** Mean values ± S.E. were calculated for each experiment and significance of differences was tested by one-way analysis of variance using Excel® software (Microsoft, Redmond, WA).
References

