Cardiovascular Research (2011) **91**, 677–684
doi:10.1093/cvr/cvr135

Transient receptor potential melastatin 4 inhibition prevents lipopolysaccharide-induced endothelial cell death

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Received 11 January 2011; revised 7 April 2011; accepted 9 May 2011; online publish-ahead-of-print 11 May 2011

Time for primary review: 26 days

Aims Endothelial dysfunction is decisive in the progression of cardiovascular diseases. Lipopolysaccharide (LPS)-induced reactive oxygen species (ROS)-mediated endothelial cell death is a main feature observed in inflammation secondary to endotoxaemia, emerging as a leading cause of death among critically ill patients in intensive care units. However, the molecular mechanism underlying LPS-induced endothelial cell death is not well understood. Transient receptor protein melastatin 4 (TRPM4) is an ion channel associated with cell death that is expressed in endothelium and modulated by ROS. Here, we investigate the role of TRPM4 in LPS-induced endothelial cell death, testing whether suppression of the expression of TRPM4 confers endothelial cell resistance to LPS challenge.

Methods and results Using primary cultures of human umbilical vein endothelial cells (HUVEC), we demonstrate that TRPM4 is critically involved in LPS-induced endothelial cell death. HUVEC exposed to LPS results in Na^+-dependent cell death. Pharmacological inhibition of TRPM4 with 9-phenanthrol or glibenclamide protects endothelium against LPS exposure for 48 h. Furthermore, TRPM4-like currents increase in cells pre-treated with LPS and inhibited with glibenclamide. Of note, suppression of TRPM4 expression by siRNA or suppression of its activity in a dominant negative mutant is effective in decreasing LPS-induced endothelial cell death when cells are exposed to LPS for 24–30 h.

Conclusion TRPM4 is critically involved in LPS-induced endothelial cell death. These results demonstrate that either pharmacological inhibition of TRPM4, suppression of TRPM4 expression, or inhibition of TRPM4 activity are able to protect endothelium against LPS injury. These results are useful in sepsis drug design and development of new strategies for sepsis therapy.

Keywords Endothelium • Inflammation • TRPM4 • Cell death • Lipopolysaccharide

1. Introduction

Endotoxaemia-derived sepsis is the most important cause of death among critically ill patients in intensive care units, resulting from a systemic enhanced response to severe bacterial infection or trauma.[1,2] Bacterial infections expose endothelial cells to lipopolysaccharide (LPS), a major component of the outer membrane in gram-negative bacteria. LPS exerts beneficial effects by inducing a controlled cellular response to bacterial infection.[3] However, large quantities of LPS are deleterious due to immune hyperactivation causing cellular and systemic damage. Progression of sepsis includes activation of a systemic inflammatory response in which the sepsis development is determined by the host response to infection.[4,5] Oxidative stress increase is a central feature observed in the sepsis physiopathology. LPS and several cytokines enhance oxidative stress through reactive oxygen species (ROS) production promoting an oxidative-mediated cellular injury.

Endothelium dysfunction and endothelial cell death have been observed in sepsis as well as in other deadly cardiovascular diseases such as hypertension, atherosclerosis, and diabetic vasculopathy.[6,7] During endotoxaemia, interaction between LPS and endothelial cells is unavoidable. Thus, LPS exposed to endothelial cells promotes ROS-dependent cell death in endothelial cells.[8] However, the molecular

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mechanism underlying the LPS-induced endothelial cell death is not well understood.

The transient receptor potential (TRP) family of ion channels includes over 30 mammalian members that conduct generally mono and divalent cations. TRP melastatin 4 (TRPM4) is a non-selective cation channel activated by a high intracellular Ca^{2+} concentration impermeable to divalent cations.[9,10] TRPM4 has been linked to diverse physiological functions, such as protection against Ca^{2+} overload by cell membrane depolarization, modulation of Ca^{2+} oscillations controlling cytokine production in T lymphocytes and mast cells, and dendritic cell migration.[9,11–13]

Recently, TRPM4 have been associated with pathological processes. TRPM4 expression and activation are critical events responsible for secondary haemorrhage in spinal cord injury, suggesting a leading role for TRPM4 in endothelium lesions.[14] TRPM4 expression has been detected in endothelial cells from different vascular regions, such as rat brain endothelial cells,[14,15] mouse aortic endothelial cells,[16] and human lung artery endothelial cells.[17] In addition, endogenous TRPM4-like currents have been detected in human umbilical vein endothelial cells (HUVEC),[18] human macrovascular endothelial cells,[19] and rat brain capillary endothelial cells,[20] suggesting that TRPM4 mRNA expression detected in endothelium is translated into a functional protein.

More recently, we reported that oxidative stress-induced cell death is decisively modulated by TRPM4 activation.[21] Cells exposed to hydrogen peroxide showed a predominantly necrotic-like cell death, while cells in which TRPM4 was down-regulated failed to show significant cell death. Concordantly, cells overexpressing TRPM4 exhibited vulnerability against an oxidative stress challenge. Furthermore, oxidative stress promoted a persistent TRPM4 activation resulting in a continuous inward Na^+ current and cell volume increase. Thus, this unrestricted influx of sodium promotes cell swelling resulting eventually in oncotic cell death.[21]

Despite the finding that TRPM4 has been linked to oxidative stress-induced cell death, participation of this protein in LPS-induced endothelial cell death is unknown. Therefore, our aim was to study the role of TRPM4 in LPS-induced endothelial cell death. To our knowledge, this is the first report to show that TRPM4 is critically involved in LPS-induced endothelial cell death. Using pharmacological and molecular biology-based approaches, we demonstrate that TRPM4 is crucial to LPS-induced cell death in HUVEC. Our data show a novel target for drug development in the line of sepsis treatment.

2. Methods

2.1 Cell culture

HUVEC were obtained from fresh umbilical cord veins from normal pregnancies, after patient's informed consent. The Commission of Bioethics and Biosafety of Universidad Andrés Bello approved all experimental protocols. The investigation also conforms with the principles outlined in the Declaration of Helsinki. HUVEC-derived endothelial cell line, EA. hy926 (EA cells) were kindly providing by Edgell *et al.*[22] Details of procedures are provided in the Supplementary material online.

2.2 Cell death and cell viability determination

2.2.1 LDH release

Cellular death was monitored by measuring lactate LDH released into culture medium (Valtek-Diagnostic, Santiago, Chile). LDH release was defined as the ratio of LDH activity in the medium to the LDH activity observed after total cell death according to the manufacturer's protocol and was expressed as percent of total LDH release.

2.2.2 MTT assay

Cell viability was evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay (Invitrogen, Oregon, USA), in which cell viability was quantified by the amount of MTT reduction. Data were expressed as percentage of cell death.

2.2.3 Trypan blue exclusion method

Cell viability was performed using the trypan blue exclusion method. Cells were quantified by light microscopy using a haematocytometre chamber and expressed as percentage of cell viability.

Details of procedures are provided in the Supplementary material online.

2.3 Intracellular Na^+ measurement, cell depolarization, and cell volume changes

Intracellular Na^+ measurement cells were used CoroNa Green-AM (Molecular Probes), a specific Na^+ dye which exhibits an increase in the fluorescent emission intensity upon binding Na^+.[23] Cell depolarization measurements cells were loaded with the cell membrane depolarization indicator bis-(1,3-dibutylbarbituric acid) trimethine oxonol ($DiBAC_4(3)$).[24,25] Cell volume was examined measuring changes in the forward-light-scattering properties of the cells or using calcein-AM (Molecular Probes). Details of procedures are provided in the Supplementary material online.

2.4 Electrophysiology

Whole-cell patch-clamp experiments on EA cells were performed as previously described,[26] 24–30 h after LPS treatment. Free [Ca^{2+}] of the solutions was calculated using the programme WinMaxc, version 2.50 (www.stanford.edu/~cpatton/maxc.html) with the appropriate Ca^{2+} buffers. Solution composition and further information are provided in the Supplementary material online.

2.5 Small interfering RNA against TRPM4, dominant negative against TRPM4 and transfections

SiGENOME SMARTpool siRNA against TRPM4 ($siRNA^{TRPM4}$) were purchased from Dharmacon (Dharmacon, Lafayette, CO, USA). Transfections were performed using DharmaFECT 4 transfection reagent (Dharmacon) used according to the manufacturer's protocols. Deletion of the NH_3-end of TRPM4 (ΔN-TRPM4) was made according to Launay *et al.*[12] EA cells were transfected using Lipofectamine 2000 transfection reagent (Invitrogen) according to the manufacturer's protocol. After LPS treatments, cells were incubated with propidium iodide (PI) and fixed. Transfected cells were identified by green fluorescence. Details of procedures are provided in the Supplementary material online.

2.6 Cell death determination by PI incorporation

Cells which incorporated PI were visualized in red. Healthy cells showed the green label only, while the non-viable exhibited both, green and red labels. After counting 100–300 transfected cells, percentage of non-viable cells were determined. Furthermore, structural changes in cells were observed using reflected light. Details of procedures are provided in the Supplementary material online.

2.7 Quantitative PCR, reverse transcriptase-PCR

Quantitative PCR (QPCR) was performed to measure TRPM4 and TRPM7 mRNA levels in EA cells. QPCR was performed using SYBR Green PCR Master Mix (AB Applied Biosystems, Foster City, CA, USA). Data are presented as relative mRNA levels of the gene of interest normalized to relative levels of either GADPH or 28S mRNA. Reverse Transcriptase-PCR amplification was performed to detect TRPM2, -4, -5, and -7 expression. Primers sequences, PCR protocols, and further details see the Supplementary material online.

2.8 Western blotting for TRPM4

Cells transfected with siRNA^{CTRL} or siRNA^{TRPM4} were lysed and proteins were extracted. Whole-cell extracts were subjected to 12% SDS–PAGE. Resolved proteins were transferred to a nitrocellulose membrane, blocked, and then incubated ON with the anti-TRPM4 antibody (Origene, Rockville, MD, USA). Tubulin was used as a loading control (Sigma). HEK293 cells overexpressing the human TRPM4 were used as a positive control. TRPM4 protein content was determined by densitometric scanning of immunoreactive bands and intensity values were obtained by densitometry of individual bands compared with tubulin and normalized against siRNA^{CTRL}. Details of procedures are provided in the Supplementary material online.

2.9 Data analysis

All results are presented as mean ± SD. Student's *t*-test and ANOVA followed by the Bonferroni or Dunns *post hoc* tests were used and considered significant at $P < 0.05$.

3. Results

3.1 LPS-induced extracellular Na^+-dependent endothelial cell death

Previously we have shown that HUVEC exposed to LPS 10 μg/mL for 48 h exhibit extensive endothelial cell death.[8] In agreement with this, HUVEC exposed to LPS showed a broad cell detachment suggesting cell death (*Figure 1A*). HUVEC exposed to 5, 10, 20, and 30 μg/mL LPS for 48 h showed a dose-dependent increase in endothelial cell death evaluated by two different approaches, LDH release (*Figure 1B*) and MTT assay (*Figure 1C*). Concurrently, a decrement in endothelial cell viability was measured using the trypan blue exclusion method (*Figure 1D*). The range of LPS concentration used was chosen to achieve endothelial cell death. Similar results were found in the HUVEC-derived endothelial cell line EA. hy926 cells (EA cells; Supplementary material online, *Figure S1*).

To test whether LPS-induced endothelial cell death was dependent on extracellular Na^+ influx, we studied the effect of the absence of extracellular Na^+ on LPS-induced cell death in HUVEC. For that purpose, we prepared a culture medium depleted of Na^+, keeping osmolarity and tonicity constant, replacing Na^+ with the non-permeant cation $NMDG^+$. As depicted in *Figure 2A* and *B*, absence of extracellular Na^+ decreased endothelial cell death compared with regular $[Na^+]_o$ conditions in HUVEC exposed to 10 and 20 μg/mL LPS. In agreement with this, using the trypan blue exclusion approach, we obtained concordant results (*Figure 2C*).

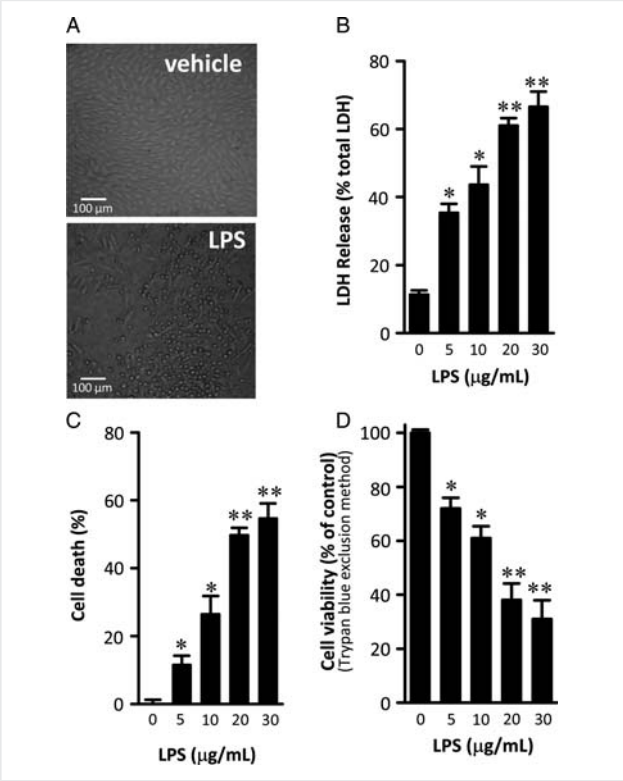

Figure 1 LPS-induced endothelial cell death. (*A*) Representative phase-contrast images from separates experiments of HUVEC exposed to either vehicle alone (upper image) or 20 μg/mL LPS (lower image) for 48 h. (*B*) and (*C*) Changes in cell death determined by LDH release (*B*) and MTT assay (*C*) of HUVEC exposed to 0, 5, 10, 20, 30 μg/mL LPS for 48 h. (*D*) Changes in cell viability measured by the trypan blue exclusion method of HUVEC exposed to 0, 5, 10, 20, 30 μg/mL LPS for 48 h. Statistical differences were assessed by one-way analysis of variance (ANOVA) (Kruskal–Wallis) followed by Dunns *post hoc* test. *: $P < 0.05$ and **: $P < 0.01$ against 0 μg/mL LPS. Graph bars show the mean ± SD ($N = 6–9$).

3.2 LPS-induced endothelial Na^+ overload, cell depolarization, and cell volume increase

Given that extracellular Na^+-dependence for LPS-induced endothelial cell death should be associated with LPS-induced Na^+ influx, we tested whether HUVEC increased $[Na^+]_i$ in response to LPS exposure. In control conditions, HUVEC behaved as a uniform fluorescent population of the cell-permeant specific Na^+ dye, CoroNa (*Figure 3A*, left panel). In contrast, endothelial cells exposed to LPS for 48 h showed a significant increase in the proportion of cells displaying higher levels of CoroNa fluorescence (*Figure 3A*, rigth panel and B). Further, we investigated the onset of LPS-induced $[Na^+]_i$ increase. Increased CoroNa green fluorescence was first detected ~3 h after LPS-treatment (*Figure 3C*).

In view of the finding that increased Na^+ influx may cause cell depolarization, we studied the effect of LPS exposure on cell membrane potential, using the membrane potential fluorescent indicator $DiBAC_4(3)$. As depicted in *Figure 3D* and *E*, HUVEC treated with LPS for 48 h showed a significant increase in levels of $DiBAC_4(3)$ fluorescence, suggesting that LPS-induced cell membrane depolarization.

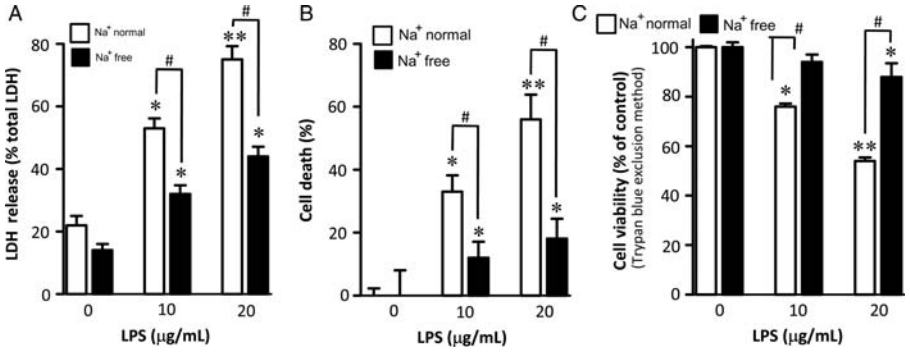

Figure 2 Extracellular Na^+-dependent LPS-induced endothelial cell death. HUVEC were cultured in medium with normal extracellular NaCl (open bars) or modified medium (Na^+-free) in which NaCl was replaced with NMDG-Cl (closed bars). (*A*) and (*B*), Changes in cell death determined by LDH release (*A*) and MTT assay (*B*) of HUVEC exposed to 0, 10, 20 μg/mL LPS for 48 h. (*C*), Changes in cell viability measured by the trypan blue exclusion method of HUVEC exposed to 0, 10, 20 μg/mL LPS for 48 h. Statistical differences were assessed by one-way ANOVA (Kruskal–Wallis) followed by Dunns *post hoc* test. *: $P < 0.05$ and **: $P < 0.01$ against 0 μg/mL LPS, and two-way ANOVA followed by Bonferroni *post hoc* test. #: $P < 0.05$ against modified mediun. Graph bars show the mean ± SD ($N = 4–8$).

Unrestricted Na^+ influx is frequently followed by water diffusion promoting cell swelling. Therefore, we investigated whether LPS treatment induces changes in cell volume. HUVEC treated with LPS for 48 h showed an increase in cell volume, measured as a change in light scattering (*Figure 3F* and *G*) or change in calcein fluorescence (*Figure 3H*). The onset of LPS-induced cell volume increase was detected nearly 5 h after LPS treatment (*Figure 3H*).

As expected, similar intracellular Na^+ increase, cell depolarization, and cell volume increase was observed in EA cells (Supplementary material online, *Figures S2–S4*).

3.3 LPS-induced endothelial cell death is inhibited by the TRPM4 inhibitors 9-phenanthrol and glibenclamide

Since ion channels are the main pathways to conduct ions across the cell membrane, the Na^+ influx observed in LPS-treated HUVEC could be mediated by cationic channels. Taking into account the proposed role of TRPM4 in endothelial physiopathology, we tested whether TRPM4 was involved in LPS-induced endothelial cell death. Although no specific blockers are available for TRPM4, the hydroxytricyclic compounds 9-phenanthrol produces a rapid inhibition of TRPM4 current without effects on its homologous TRPM5.[27] Furthermore, the sulfonylurea glibenclamide blocks with high affinity and specificity the receptor–channel complex SUR1/TRPM4.[28] HUVEC treated with either 1 μmol/L 9-phenanthrol or 100 μmol/L glibenclamide simultaneously with 0, 10, and 20 μg/mL LPS showed a significant inhibition of cell death measured by LDH release (*Figure 4A*), MTT assay (*Figure 4B*), and trypan blue exclusion method (*Figure 4C*).

Although TRPM4 expression has been detected in various types of endothelial cells,[14–17] there are no data relative to TRPM4 expression specifically in HUVEC. Using RT–PCR, we observed TRPM4 mRNA expression in HUVEC, while TRPM5 was not detected (*Figure 4D*). Furthermore, in our experimental conditions, TRPM7, but not TRPM2 mRNA expression, both of which have been linked to oxidative stress-induced cell death, was detected (*Figure 4E*). An identical mRNA expression profile was detected in EA cells (Supplementary material online, *Figure S5*).

Next, we investigated whether endothelial cells have a TRPM4-like current and whether LPS is able to activate such current. EA cells show a small basal current, with a reversal potential (E_{rev}) close to −24 ± 4 mV (*Figure 4F* and *H*). However, EA cells exposed to 30 μg/mL LPS for 24–30 h showed an increased outwardly rectifying current density (*Figure 4G* and *H*), with an E_{rev} closer to 0 mV (−7 ± 2 mV). As expected for a Ca^{2+}-dependent cationic-current, when Ca^{2+} was omitted from the intracellular solution, no current was observed (not shown). Moreover, the current evoked by LPS and the E_{rev} shift was totally abolished using 100 μmol/L glibenclamide (−27 ± 4 mV), suggesting that the LPS-induced current but not the basal current is attributable to TRPM4 (*Figure 4F–H*). Previous results have shown that TRPM4 is modulated by ROS,[21] therefore, we explored whether the reducing agent dithiothreitol (DTT) was able to prevent the TRPM4-like current evoked by LPS. As shown in *Figure 4F–H*, in cells incubated with LPS (30 μg/mL) and DTT (1 mmol/L) the current observed did not differ from cells treated only with DTT, suggesting that LPS-mediated ROS production is responsible for the increase in the outwardly rectifying current density.

3.4 TRPM4 is a key modulator of LPS-induced endothelial cell death

In spite of the pharmacological evidence, the use of a molecular biology strategy for TRPM4 expression knockdown was necessary to prove unequivocally the participation of TRPM4. Thus, to demonstrate the participation of TRPM4 in LPS-induced endothelial cell death, HUVEC were transfected with a specific small interference RNA (siRNA) against human TRPM4. Forty-eight hours after siRNA transfection, cells were exposed to LPS. Then, cell death was analysed by PI incorporation in individual transfected cells.

The siRNA efficiency in the TRPM4 expression knockdown was demonstrated by qPCR (*Figure 5A*) and western blot (*Figure 5B* and *C*). The $siRNA^{TRPM4}$ effect is specific since it did not affect the mRNA expression of TRPM7 (Supplementary material online, *Figure S6*).

Figure 5D–K depicts images of HUVEC transfected with either non-targeting siRNA used as a control ($siRNA^{CTRL}$) (*Figure 5D–G*) or siRNA against TRPM4 ($siRNA^{TRPM4}$) (*Figure 5H–K*) exposed to

20 μg/mL LPS for 24–30 h. LPS treatment in HUVEC transfected with the siRNA^{TRPM4} created resistance to cell injury whereas cells transfected with siRNA^{CTRL} shown cell swelling, blebbing, and membrane leakage, resulting in nuclear labelling by PI consistent with a process of cell death (*Figure 5L*). Furthermore, using a well-proven similar mRNA knockdown strategy,[12,21] we obtained comparable results in EA cells transfected with either a short hairpin RNA against TRPM4 (shRNA^{TRPM4}) or a scrambled sequence used as a control (shRNA^{SCR}) in cells exposed to 30 μg/mL LPS for 24–30 h (Supplementary material online, *Figure S7*).

Deletion of the first 177 amino acids in the NH_3-terminus of TRPM4 (ΔN-TRPM4) produced a mutant able to reach the cell membrane but failing in ion conductance, suggesting a dominant negative effect on endogenous TRPM4.[12] Using this mutant, consistent results were obtained in EA cells transfected with either a mock vector used as a control (mock) (*Figure 6A–D*) or ΔN-TRPM4 (*Figure 6E–H*) exposed to 30 μg/mL LPS for 24–30 h. These several separate experiments are summarized in the *Figure 6I*.

The TLR-4-dependence for LPS-induced endothelial cell death[8] prompts to us to hypothesize that LPS is able to promote cell death not only in endothelium but also in all cells that express TRPM4 as well as TLR-4. HeLa cells are a non-endothelial-derived cell line that express both TRPM4 (Supplementary material online, *Figure S8*) and TLR-4. HeLa cell clones stably expressing either shRNA^{SCR} or shRNA^{TRPM4} cultured in the absence or presence of different concentrations of LPS for 48 h showed a marked dose-dependent increase in cell death in the shRNA^{SCR} HeLa cells clone compared with shRNA^{TRPM4} HeLa cells clone that were significantly resistent to LPS-induced cell death (Supplementary material online, *Figure S9*).

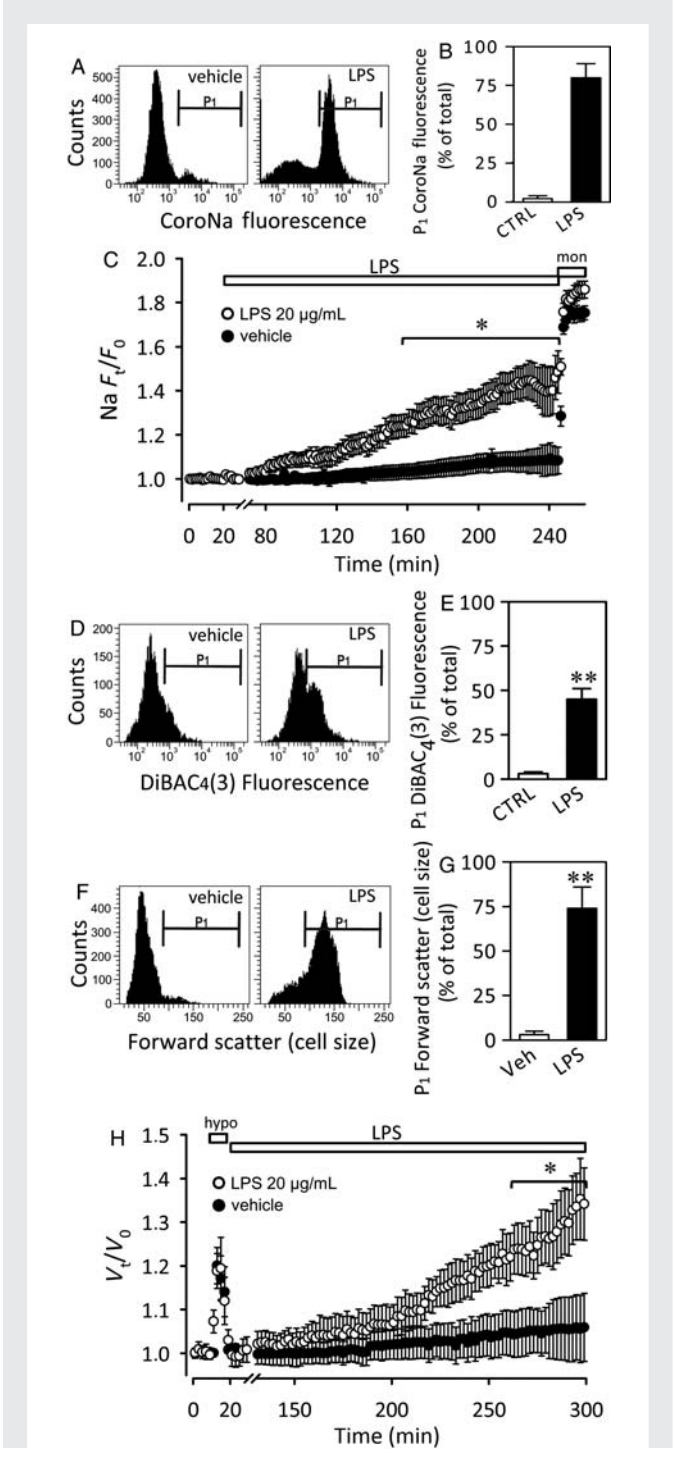

Figure 3 LPS-induced endothelial Na^+ overload, cell depolarization, and cell volume increase. Representative histograms for CoroNa fluorescence (*A*), $DiBAC_4(3)$ fluorescence (*D*), and forward scatter light (*F*) from separated experiments of HUVEC exposed to either vehicle alone (left image) or 20 μg/mL LPS (right image) for 48 h. Graph bars show the percentage (mean ± SD) of CoroNa green fluorescence (*B*), $DiBAC_4(3)$ fluorescence (*E*), and forward scatter light (*G*) increase in P_1 population obtained from at least three independent experiments as those depicted in (*A*), (*D*), and (*F*), respectively. Statistical differences were assessed by student's *t*-test (Mann–Whitney). **: $P < 0.01$ against 0 μg/mL LPS. Graph bars show the mean ± SD. Representative experiment showing changes on CoroNa (*C*) and calcein fluorescence (*H*) in HUVEC exposed to either vehicle alone (closed circles) or 20 μg/mL LPS (open circles). Monensin (20 μmol/L) was added as a positive control in (*C*). For calibration purposes, cells were briefly exposed to a 15% hypotonic solution in (*H*). Each point represents the mean ± SD of values obtained from 10 to 15 cells from at least three independent experiments. Statistical differences were assessed by one-way ANOVA (Kruskal–Wallis) followed by Dunns *post hoc* test. *: $P < 0.05$ against 0 μg/mL LPS.

4. Discussion

An important aspect of LPS exposure during sepsis is the dysfunction and death of the endothelium—characterized by hyperpermeability, oedema progression, imbalance in nitric oxide production, and hypotension—which explains in part why sepsis is the main cause of mortality in critically ill patients. Therefore, it is important to understand the molecular bases underlying LPS-induced endothelial cell death to improve the existing rather ineffective sepsis treatment.

In this study, we demonstrate that the ion channel TRPM4 is critically involved in LPS-induced endothelial cell death. Our results showed that down-regulation of TRPM4 significantly decreases LPS-induced endothelial cell death, increasing cell viability of the endothelium. Furthermore, our data show a novel target which could be useful for drug design to improve the current sepsis therapy.

We previously demonstrated that LPS triggers an intracellular ROS increase resulting in LPS-induced endothelial cell death[8] and recently

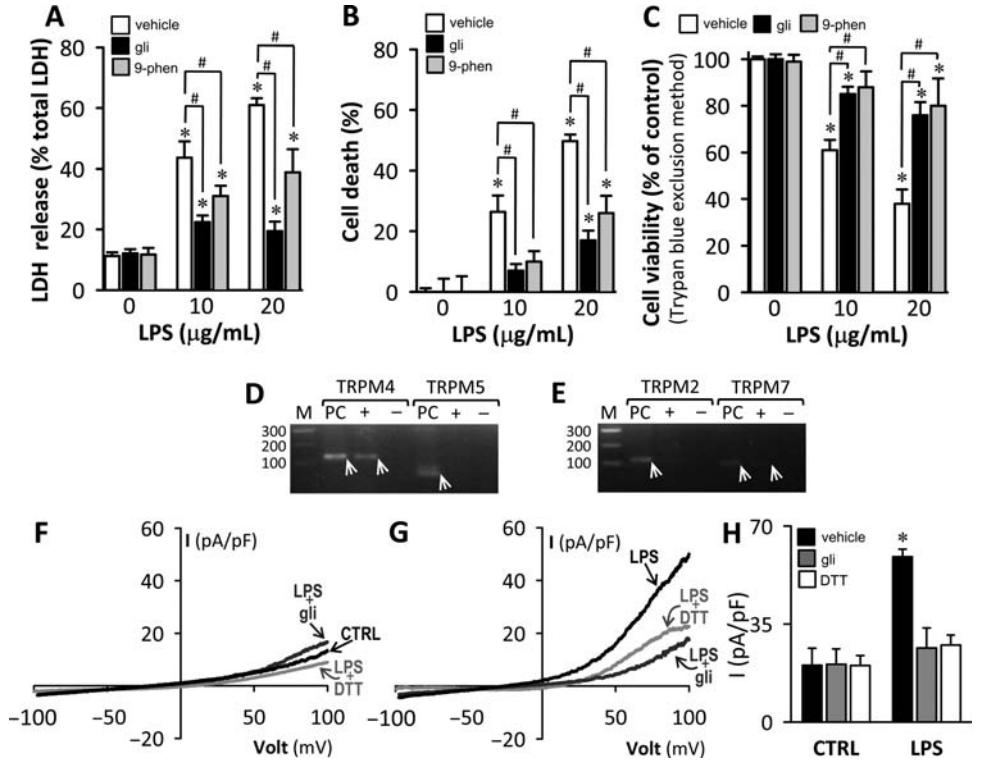

Figure 4 Pharmacological inhibition of TRPM4 decreases LPS-induced endothelial cell death. Changes in cell death determined by LDH release (*A*) and MTT assay (*B*), and changes in cell viability measured by the trypan blue exclusion method (*C*) of HUVEC treated by vehicle (open bars), 100 μmol/L glibenclamide (closed bars), and 1 μmol/L 9-phenanthrol (grey bars) exposed to 0, 10, 20 μg/mL LPS for 48 h. Statistical differences were assessed by one-way ANOVA (Kruskal–Wallis) followed by Dunns *post hoc* test. *: $P < 0.05$ and **: $P < 0.01$ against 0 μg/mL LPS, and two-way ANOVA followed by Bonferroni *post hoc* test. #: $P < 0.05$ against either glibenclamide or 9-phenanthrol. Graph bars show the mean ± SD ($N = 4–8$). (*D*) and (*E*) mRNA detection of TRPM4 and TRPM5 (*D*), and TRPM2 and TRPM7 (*E*) by reverse transcriptase-PCR in HUVEC. In all panels: *lane 1*, PC: positive control (TRPM4 overexpression in HEK, HeLa, TRPM2 overexpression in HEK, and HeLa cells were used as positive control for TRPM4, TRPM5, TRPM2, or TRPM7, respectively); *lane 2*, +: HUVEC sample; *lane 3*, −: non-RT sample. M: 100 bp DNA ladder. Arrows depict transcripts expression. Current–voltage relationship recorded in EA cells control (*F*) or treated with 30 μg/mL LPS for 24–30 h (*G*) in absence (black line) or presence of either 100 μmol/L glibenclamide (dark grey line) or 1 mmol/L DTT (grey line) derived by a voltage-ramp protocol ranging from −100 to 100 mV (800 ms duration). (*H*) Bars showing current density (mean ± SEM, $N > 4$) in absence (CTRL) or presence (LPS) of 30 μg/mL LPS with vehicle alone (filled bars) or either 100 μmol/L glibenclamide (dark grey bars) or 1 mmol/L DTT (empty bars). Statistical differences were assessed by one-way ANOVA (Kruskal–Wallis) followed by Dunns *post hoc* test. *: $P < 0.05$.

reported that TRPM4 is an ion channel involved in cell death modulated by intracellular ROS.[21] These data, in addition to those reported here, prompted us to suggest that the role played by TRPM4 in LPS-induced endothelial cell death is triggered by LPS-induced intracellular ROS generation. The expression of enzymes that produce ROS, such as NAD(P)H oxidase (NOX), has been reported in endothelial cells.[29–31] In addition, direct interaction between TLR-4 and NOX-4 has been reported,[32] suggesting that LPS could be able to activate the oxidase directly.

Extracellular Na^+ dependence for LPS-induced endothelial cell death was correlated with an increase in $[Na^+]_i$ as well as with cell depolarization. As we expected, an LPS-induced cell volume increase was detected. Unrestricted influx of Na^+ could promote water entry resulting in cell swelling, loss of cell shape, cell detachment, plasma membrane disintegration, and finally oncotic cell death. All these findings are compatible with necrotic cell death features.[8,33,34] However, both Na^+ dependence and depolarization are also linked to the apoptotic cell death process. In myocytes, Na^+ influx via NSCC resulting in accumulation of Na^+ in the cytosol is involved in caspase-3-mediated apoptosis.[35] Inhibition of Na^+ influx produces a complete reduction of Fas-induced apoptosis in Jurkat cells.[36] Furthermore, plasma membrane depolarization is linked to the early stages of apoptosis.[25,37] In agreement with this, LPS-induced apoptotic endothelial cell death has been reported,[38–41] suggesting that our results could be compatible with necrotic as well as apoptotic cell death.

Considering the TRPM4 activation mechanism, all stimuli able to produce an increase in intracellular ROS should be able to activate TRPM4 to promote a cell death process. However, intracellular ROS increase do not seem to be a sufficient stimulus to activate TRPM4.[21] Regarding intracellular Ca^{2+} dependence for TRPM4 activation,[13] it is plausible that both ROS and intracellular Ca^{2+} increases are necessary for TRPM4 activation. Since normal levels of intracellular nucleotides such as ATP block TRPM4,[42] a decrease in ATP is also required for TRPM4 activity. Therefore, in addition to ROS production and an increase of intracellular Ca^{2+}, a decrease in intracellular ATP should be needed to activate TRPM4.

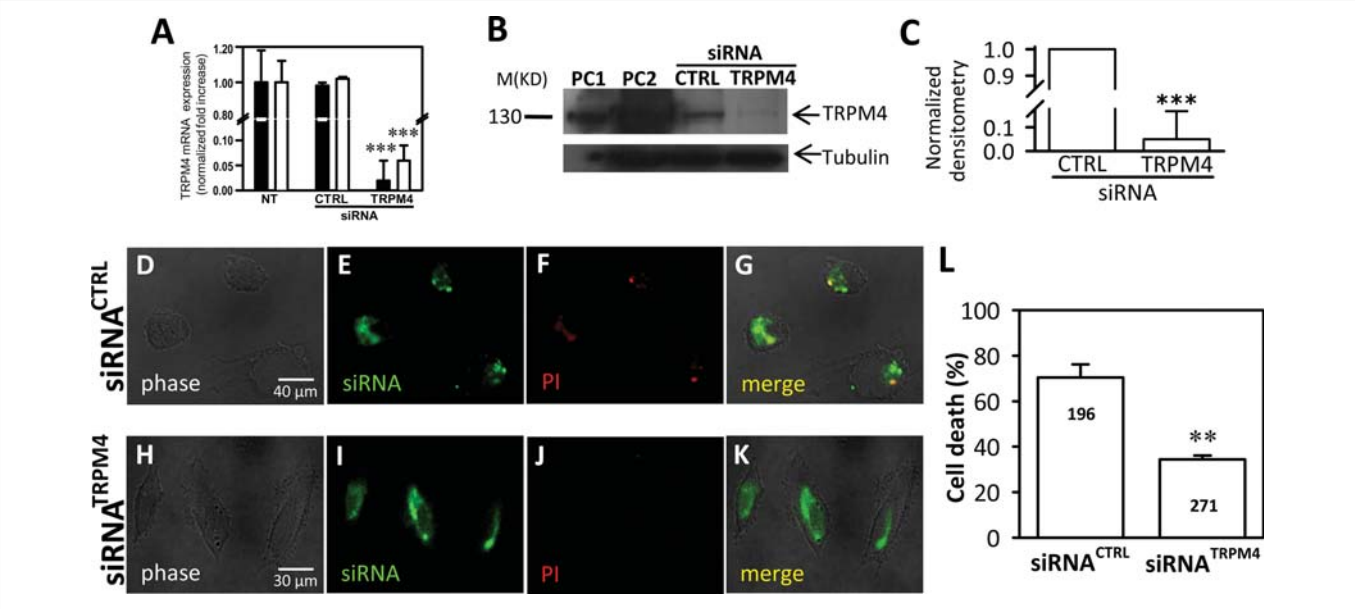

Figure 5 Small interference RNA against TRPM4 protects HUVEC from to LPS-induced cell death. (*A*) Determination of the efficiency of siRNA by qPCR. Determinations were done in EA cells in triplicates and results are expressed normalized as relative levels to 28S (closed bars) or GADPH (open bars) mRNA expression. Statistical differences were assessed by one-way ANOVA (Kruskal–Wallis) followed by Dunns *post hoc* test. ***: $P < 0.001$. (*B* and *C*) determination of the efficiency of siRNA by westernblot (*B*). HEK cells overexpressing TRPM4 were used as a positive control. PC1: positive control 1 (minimal protein amount loading), PC2: positive control 2 (high protein amount loading). Determinations were done in EA cells in triplicates and results are expressed normalized against tubulin and relative to $siRNA^{CTRL}$ (*C*). (*D*) Quantification of PI-positive cells exposed to 20 µg/mL LPS for 24–30 h in HUVEC transfected with non-targeting siRNA ($siRNA^{CTRL}$) or with siRNA against TRPM4 ($siRNA^{TRPM4}$). Values represent the percentage of the transfected cells (green) with nuclear PI labelling (red). Statistical differences were assessed by student's *t*-test (Mann–Whitney). **: $P < 0.01$. Graph bars show the mean ± SD ($N = 3–4$, ≈70 cells/experiment). Numbers in bars denotes the total number of cells assessed in each condition. (*E–L*) Representative images from experiments of cells exposed to 20 µg/mL LPS for 24–30 h in HUVEC transfected with non-targeting siRNA ($siRNA^{CTRL}$, upper images) or with siRNA against TRPM4 ($siRNA^{TRPM4}$, lower images). Phase-contrast images (*E* and *I*), transfected siRNA green indicator (*F* and *J*), nuclear labelling with PI (*G* and *K*), and merged images (*H* and *L*). Cells showed were fixed and mounted. Graph bars show the Mean ± SD. NT, non-transfected. Statistical differences were assessed by student's *t*-test (Mann–Whitney). ***: $P < 0.001$.

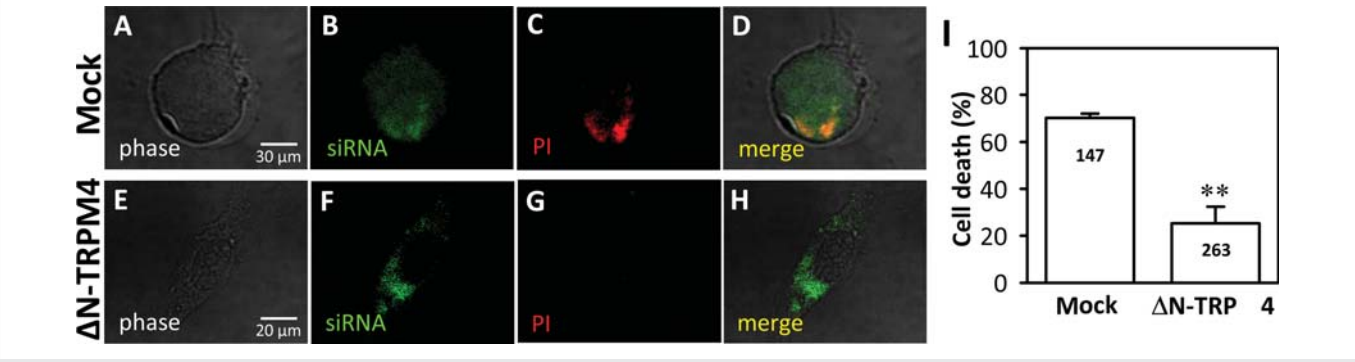

Figure 6 Dominant negative against TRPM4 protects EA cells from to LPS-induced cell death. (*A–H*) Representative images from experiments of cells exposed to 30 µg/mL LPS for 24–30 h in HUVEC transfected with mock vector (mock, upper images) or with the dominant negative against TRPM4 (ΔN-TRPM4, lower images). Cells showed were fixed and mounted. Phase-contrast images (*A* and *E*), transfected siRNA green indicator (*B* and *F*), nuclear labelling with PI (*C* and *G*), and merged images (*D* and *H*). (*I*) Quantification of PI-positive cells exposed to 30 µg/mL LPS for 24–30 h in EA cells transfected with mock or ΔN-TRPM4. Values represent the percentage of the transfected cells (green) with nuclear PI labelling (red). Statistical differences were assessed by student's *t*-test (Mann–Whitney). **: $P < 0.01$. Graph bars show the mean ± SD ($N = 4–8$, ≈35 cells/experiment). Numbers in bars denotes the total number of cells assessed in each condition.

Gerzanich *et al.* have reported that TRPM4 is overexpressed in capillary endothelial cells of the spinal cord following spinal cord injury as a mechanism for TRPM4-mediated capillary fragmentation, petechial haemorrhage development, and secondary haemorrhage.[14] Spinal cord injury produces an inflammatory reaction similar to the one seen in endothelial cells exposed to LPS, and TRPM4 current is observed to increase in HUVEC exposed to LPS. Therefore, it is plausible to suggest that in our experimental LPS exposure approach increased TRPM4 expression is likely. Furthermore, it is possible to suggest that the endothelial death produced in our endothelial sepsis model and the one observed in the spinal injury model in Gerzanich's work are produced by the same ROS-dependent inflammatory pathways. However, further experiments are needed to elucidate this speculation.

In conclusion, these findings demonstrate that TRPM4 plays a crucial role in LPS-induced endothelial cell death. Our results raise the possibility of developing novel drugs to produce selective and transient inhibition of TRPM4 that could be beneficial for the treatment of sepsis.

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

Conflict of interest: none declared.

Funding

This work was supported by research grants from Fondo Nacional de Desarrollo Científico y Tecnológico—FONDECYT 11080119 to F.S. and 11080019 to D.V. This work was supported by MECESUP UAB 08-02. The authors are grateful to Unidad Medicina Materno-Fetal, Departamento Obstericia y Ginecología, Hospital Clínico Universidad de Chile. This work received institutional support from grant Fondecyt-FONDAP 15010006.

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