A gain-of-function SNP in TRPC4 cation channel protects against myocardial infarction

Carole Jung†, Gemma G. Gené†, Marta Tomás‡, Cristina Plata†, Jana Selent‡, Manuel Pastor‡, César Fandos†, Mariano Senti†,2, Gavin Lucas‡, Roberto Elosua‡,4, and Miguel A. Valverde†*

1Laboratory of Molecular Physiology and Channelopathies, Universitat Pompeu Fabra, Parc de Recerca Biomédica de Barcelona, Room 343, C/ Dr. Aiguader 88, Barcelona 08003, Spain; 2Cardiovascular Epidemiology and Genetics Research Group, IMIM Institut de Recerca Hospital del Mar, Barcelona 08003, Spain; 3Biomedical Informatics Program, Department of Experimental and Health Sciences, Universitat Pompeu Fabra, Barcelona, Spain; and 4Centro de Investigación Biomédica en Red en Epidemiología y Public Health, Barcelona, Spain

Received 15 November 2010; revised 16 March 2011; accepted 17 March 2011; online publish-ahead-of-print 22 March 2011

Time for primary review: 42 days

Aims

The TRPC4 non-selective cation channel is widely expressed in the endothelium, where it generates Ca\(^{2+}\)+ signals that participate in the endothelium-mediated vasodilatory response. This study sought to identify single-nucleotide polymorphisms (SNPs) in the TRPC4 gene that are associated with myocardial infarction (MI).

Methods and results

Our candidate-gene association studies identified a missense SNP (TRPC4-I957V) associated with a reduced risk of MI in diabetic patients [odds ratio (OR) = 0.61; confidence interval (CI), 0.40–0.95, \(P = 0.02\)]. TRPC4 was also associated with MI in the Wellcome Trust Case–Control Consortium’s genome-wide data: an intronic SNP (rs7319926) within the same linkage disequilibrium block as TRPC4-I957V showed an OR of 0.86 (CI, 0.81–0.94; \(P = 10^{-4}\)). Functional studies of the missense SNP were carried out in HEK293 and CHO cells expressing wild-type or mutant channels. Patch-clamp studies and measurement of intracellular \([Ca^{2+}]_i\) in response to muscarinic agonists and direct G-protein activation showed increased channel activity in TRPC4-I957V-transfected cells compared with TRPC4-WT. Site-directed mutagenesis and molecular modelling of TRPC4-I957V suggested that the gain of function was due to the presence of a less bulky Val-957. This permits a firmer interaction between the TRPC4 and the catalytic site of the tyrosine kinase that phosphorylates TRPC4 at Tyr-959 and facilitates channel insertion into the plasma membrane.

Conclusion

We provide evidence for the association of a TRPC4 SNP with MI in population-based genetic studies. The higher Ca\(^{2+}\)+ signals generated by TRPC4-I957V may ultimately facilitate the generation of endothelium- and nitric oxide-dependent vasorelaxation, thereby explaining its protective effect at the vasculature.

Keywords

Myocardial infarction • TRP ion channels • Calcium • Genetics of cardiovascular diseases • Gene polymorphisms

1. Introduction

The discovery of the TRP family of non-selective ion channels has facilitated our understanding of how animals detect diverse sensory inputs, but also how calcium signalling is generated in a broad range of cellular systems. Different cellular, molecular, and animal model studies have highlighted the relevance of TRP channels in the vasculature whereby shaping Ca\(^{2+}\)+ signals and modulating cell membrane potential play important roles in the regulation of vascular tone, remodelling, and integrity. However, from the genetic point of view, only a few reports have studied the relevance of TRP channels at the population level. For example, a single-nucleotide polymorphism (SNP) that generates a de novo binding site for nuclear factor \(\kappa\)B in the promoter region of TRPC6 is associated with pulmonary hypertension; an SNP-inducing loss of activity in TRPV4 has been associated with hyponatraemia, and a loss-of-function SNP in TRPV1 has been associated with lower risk of respiratory symptoms in childhood asthma.
Among the TRP channels, TRPC4 channel is widely expressed in the vasculature, where it participates in the generation of intracellular Ca2+ signals that regulate functions such as endothelial permeability, smooth muscle proliferation, and, of most importance to this study, endothelium- and nitric oxide (NO)-dependent vasorelaxation. TRPC4 gating is not completely understood. It occurs downstream of both tyrosine kinase and G-protein-coupled receptor activation, with the participation of phosphatidylinositol 4,5-bisphosphate (PIP_2) inhibition only relevant to the long TRPC4α-splicing isoform. Together, these observations make the TRPC4 channel an interesting target in evaluating contributors to the pathogenesis of human cardiovascular diseases.

Coronary artery disease (CAD) is the leading cause of death in the developed world. The pathophysiology of myocardial infarction (MI), one of the clinical manifestations of CAD, involves a reduced blood supply to the myocardium. This blood supply is inversely related to the resistance in the myocardial vascular bed, which in turn depends on a calcium signal generated in the vascular smooth muscle (VSM). Through modulation of the calcium signal, several VSM ion channels participate in setting the level of coronary arterial tone and a few of them have shown a genetic association with CAD, its treatment, or cardiovascular diseases that are risk factors for CAD.

Endothelial cells modulate VSM tone following the release of vasomotor regulators or electrical coupling between endothelial and VSM, mechanisms in which endothelial ion channels are also involved, including TRPC4.

MI is a complex disease whose heritability component has been extensively reevaluated in the last few years by genome-wide association studies. Novel loci have appeared, although in most cases, the functional connection between loci and phenotype remains to be elucidated. One exception seems to be an SNP in 1p13 that alters lipid metabolism by generating a new transcription factor-binding site. Therefore, identification of functional genetic variants associated with MI is still necessary to improve our knowledge of the disease pathophysiology. The present study sought to identify SNPs in the TRPC4 that are associated with MI.

2. Methods

2.1 Gene polymorphism identification

The 11 exons of human TRPC4 were amplified from genomic DNA using PCR and analysed by direct sequencing in 50 healthy subjects. A novel SNP was found in exon 11 (A3104G, later included in SNP databases as rs73184536, NM_016179), corresponding to an isoleucine to valine substitution. Mutants were verified by sequencing with ABIPrism BigDye Terminator 3.0; Applied Biosystems) and confirmed by sequencing with ABIPrism 3.1 BigDye kit.

2.2 MI case–control study

We used a case–control design. Between January 1996 and December 2002, we recruited 1025 patients (188 women, 18.3%) with a first MI consecutively admitted to the only reference coronary unit in the catchment area in Girona, Spain. The 3899 participants from two cross-sectional studies in the same area served as controls and were judged free of angina or MI by medical records, physical examination, and electrocardiography. Standardized smoking and diabetes mellitus questionnaires and details of recruitment and measured variables are already described.

2.3 Cell culture and transfection

Chinese hamster ovary (CHO) fibroblasts (from the European Collection of Cell Cultures) were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% foetal bovine serum. Culture media contained 1% streptomycin with penicillin. Cells were incubated at 37°C in an atmosphere of 95% air–5% CO₂. The CHO cells were transiently transfected with 0.3 μg pEGFP and 3 μg of either pcDNA3-hTRPC4 WT, pcDNA3-hTRPC4 I957V, or hTRPC4 Y959F using 8 equivalents of polyethylenimine (Fermentas MBI). Cells were used 24–48 h after transfection. Human TRPC4-I957V mutant was generated by site-directed mutagenesis with primers 5'-GAAGAGACTCTAGTGAGCTATGAG TCTAAGAACCCTCC-3' and 5'-GAAGGTTGATCATGCTACACTA GAGTCTCTTTCTC-3'. With the Quick Change Site Directed Mutagenesis kit (Stratagene), TRPC4-I957V double mutants were generated using primers 5'-GAA GAG GAC TCT AGT AGA TCA GAC TTT GAT CTA AAC CTC CCA GAC-3' and 5'-GTC TAT ACT AGA AGA TGA GGT GAT TAG TAC AAA GTG TAT ACT AGA GTG CTC TTC-3' for TRPC4-Ile-957 variant and primers 5'-GAA GAG GAC TCT AGT AGA TCA GAC TTT GAT CTA AAC CTC CCA GAC-3' and 5'-GTC TAT ACT AGA AGA TGA GGT GAT TAG TAC AAA GTG TAT ACT AGA GTG CTC TTC-3' for TRPC4-Ile-Val-957 variant (changes underlined). Mutants were verified by sequencing with ABIPrism 3.1 BigDye kit.

2.4 Patch-clamp recordings

Cells were plated in 35 mm plastic dishes and mounted on the stage of an Inverted Olympus IX70 microscope and the whole-cell currents were recorded with an Axon Instruments 9300A amplifier as described previously. The bath solution contained either (in mM) 140 NaCl, 2.5 KCl, 1.2 CaCl₂, 0.5 MgCl₂, 5 glucose, 10 HEPES, or 12 glucose; pH was adjusted to 7.4 with Tris, 305 mOsm/L. The pipette solution contained (in mM) 80 CsCl, 1 MgATP, 10 EGTA, 10 HEPES, 5 glucose, 500 μM CaCl₂ (calculated free Ca²⁺ concentration was 67.5 nM), and 88 μM GTPγS, a G-protein-activating analogue of GTP; pH was adjusted to 7.4 with CsOH and 300 mOsm. Patch electrodes of 3–5 MΩ were used. Membrane potential was held at 0 mV and ramps from −110 to +100 mV (400 ms) were applied at a frequency of 0.2 Hz. Data were acquired at 10 kHz and low-pass filtered at 1 kHz. Single-channel recordings were carried out using a bath solution containing (in mM): 140 NaCl, 2.5 KCl, 0.5 EGTA, 0.5 MgCl₂, 5 glucose, and 5 HEPES (pH 7.4 with Tris). The pipette solution contained (in mM) 20 CsCl, 1 MgATP, 10 EGTA, 10 HEPES, 5 glucose, 500 μM CaCl₂ (calculated free Ca²⁺ concentration was 67.5 nM), and 88 μM GTPγS, a G-protein-activating analogue of GTP; pH was adjusted to 7.4 with CsOH and 300 mOsm. Patch electrodes of 3–5 MΩ were used.

2.5 Measurement of intracellular [Ca²⁺]

Cells were plated onto glass cover slips, loaded with 5 μM of Fura-2-AM for 30 min at room temperature, washed out thoroughly, and bathed in an isotonic solution containing (in mM): 140 NaCl, 2.5 KCl, 1.2 CaCl₂, 0.5 MgCl₂, 5 glucose, and 10 HEPES (305 mOsm/L, pH 7.4 with Tris). Ca²⁺-free solutions were obtained by replacing CaCl₂ with an equal amount of MgCl₂ plus 0.5 mM EGTA. Carbamylcholine chloride (Cch, Sigma), a muscarinic receptor agonist, was added to the bath solution.
as indicated in the legends for Figures 1 and 3. All experiments were carried out at room temperature as described previously. \(^\text{35}\) AquaCosmos software (Hamamatsu Photonics) was used to capture the fluorescence ratio at 505 nm, obtained following excitation at 340 and 380 nm. Images were computed every 5s.

2.6 Molecular modelling of TRPC4–Fyn kinase interaction

We used molecular modelling techniques to investigate how a single-point mutation at position –2 (aa 957) from Tyr-959 in the human TRPC4 channel could affect the interaction between a tyrosine kinase and the short sequence fragment containing the target tyrosine (see Supplementary material online for further details). We used the structural data available for the Fyn kinase that phosphorylates and modulates TRPC4. An overview of the model obtained for the FYN kinase in complex with [I\(_2\)Dy]-DLN is shown in Supplementary material online, Figure S2 and subsequent figures. Additional details of methods are included in the Supplementary material online.

2.7 Statistical analysis

Deviation from the Hardy–Weinberg equilibrium was assessed using a \(\chi^2\) test with one degree of freedom. Adjusted odds ratios (ORs) of MI risk and their 95% confidence intervals (CIs) were estimated for Val-carriers (ValVal + IleVal genotypes) vs. Ilele genotype by unconditional logistic regression analysis. Interaction between the genetic variant and age, sex, or diabetes was also assessed for impact on MI risk.

Functional data were expressed as means ± SEM of \(n\) (number of cells analysed). Statistical analysis was performed with Student’s unpaired \(t\)-test or one-way analysis of variance (ANOVA) with Tukey’s test for post hoc comparison of means, using SigmaPlot or OriginPro software. The criterion for a significant difference was a final value of \(P < 0.05\).

3. Results

3.1 Genetic association studies

We identified an SNP in TRPC4 (A3104G, later renamed as rs73184536, NM_016179) corresponding to an isoleucine to valine mutation at position 957 of the protein (957V). The frequency of the Val allele was 0.07 and the genotype frequencies in the representative random population sample of 3899 participants were 86.2, 13.4, and 0.4% for the Ile/Ile, Ile/Val, and Val/Val genotypes, respectively. The observed genotype frequencies fitted the Hardy–Weinberg equilibrium. The Val/Val homozygote and Ile/Val heterozygote subjects were analysed together because of the low prevalence of the former.

We evaluated the association of TRPC4-957V SNP with MI risk in a case–control study. The main characteristics of the sample are shown in Table 1. We observed a statistically significant interaction \((P = 0.049)\) between the genetic variant and diabetes on MI risk. Multivariate analysis showed a protective effect of the 957V allele against MI risk only in diabetic subjects (adjusted \(OR = 0.61; 95\% CI, 0.40–0.95; P = 0.02\); Table 2).

The association of TRPC4 with MI was also tested in the Wellcome Trust Case–Control Consortium (WTCCC), which included 1988 cases with CAD and 5380 healthy controls. The non-synonymous rs73184536 SNP (TRPC4-I957V) was not included in the chip used in this study. Therefore, we analysed SNPs within or near the TRPC4 chromosomal region (13q13.37:108-37.341). One SNP within the TRPC4 gene (rs7319926), belonging to the same linkage disequilibrium block as TRPC4-I957V, was associated with a lower prevalence of CAD \((OR = 0.87; CI, 0.81–0.94; P = 0.0002)\).

### Table 1 Main characteristics of the sample

<table>
<thead>
<tr>
<th></th>
<th>Controls ((n = 3899))</th>
<th>Cases ((n = 1025))</th>
<th>(P)-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)(^a)</td>
<td>52.2 (13.8)</td>
<td>59.7 (10.3)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Sex (women)</td>
<td>2041 (52.3%)</td>
<td>188 (18.3%)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>BMI (kg/m(^2))(^b)</td>
<td>27.3 (4.58)</td>
<td>27.8 (4.21)</td>
<td>0.008</td>
</tr>
<tr>
<td>SBP (mmHg)(^a)</td>
<td>131 (20.9)</td>
<td>116 (17.3)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>DBP (mmHg)(^a)</td>
<td>78.9 (10.5)</td>
<td>66.2 (11.0)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Smoking(^c)</td>
<td>955 (24.9%)</td>
<td>476 (47.0%)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>543 (14.5%)</td>
<td>344 (44.3%)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Antihypertensive treatment</td>
<td>614 (15.9%)</td>
<td>402 (41.4%)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

\(^a\)Mean (standard deviation). \(^b\)BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure. \(^c\)Current smoker or former <1year.

### Table 2 Adjusted odds ratio of MI risk for 957V-carriers in the whole sample and stratified by diabetes mellitus status

<table>
<thead>
<tr>
<th>Model</th>
<th>Subjects included</th>
<th>OR (95% CI)</th>
<th>(P)-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Model 1 All</td>
<td>0.83 (0.64–1.08)</td>
<td>0.16</td>
<td></td>
</tr>
<tr>
<td>Stratified by diabetes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Model 2 Non-diabetes</td>
<td>0.99 (0.71–1.36)</td>
<td>0.93</td>
<td></td>
</tr>
<tr>
<td>Model 2 Diabetes</td>
<td>0.61 (0.40–0.95)</td>
<td>0.02</td>
<td></td>
</tr>
</tbody>
</table>

Model 1: adjusted by age, sex, hypertension, antihypertensive therapy, and diabetes mellitus. Model 2: adjusted by age, sex, hypertension, and antihypertensive therapy.

3.2 Functional analysis of TRPC4-I957V variant

To evaluate the mechanism by which TRPC4-I957V might be protective against cardiovascular disease, we expressed and functionally tested wild-type (TRPC4-WT) and mutant channels (TRPC4-I957V) in CHO cells. Similar levels of expression were observed between TRPC4-WT and TRPC4-I957V channels (Supplementary material online, Figure S1). We measured cytosolic \(\text{Ca}^{2+}\) using the fura-2 sensor and cationic currents using the whole-cell and single-channel modes of the patch-clamp technique. TRPC4 is a non-selective cation channel that responds to the muscarinic agonist carbachol (Ch), epidermal growth factor, and other stimuli that use G-protein-mediated signalling.\(^{14,15}\)

Figure 1A shows representative \(\text{Ca}^{2+}\) traces obtained from CHO cells transfected with control GFP, TRPC4-WT, or TRPC4-I957V and stimulated with 100 \(\mu\text{M}\) Ch using an add-back \(\text{Ca}^{2+}\) protocol. The first peak reflects \(\text{Ca}^{2+}\) entry, is shown in Figure 1B. Following ER \(\text{Ca}^{2+}\) depletion by thapsigargin and subsequent activation of store-operated calcium entry mechanisms, no difference in \(\text{Ca}^{2+}\) entry was observed between cells transfected...
with GFP, TRPC4-WT, and TRPC4-I957V (Figure 1C and D), consistent with the current view that TRPC4 is not a key player in store-operated calcium entry in the endothelium. Mutant channel was also evaluated using electrophysiological techniques. Gating of TRPC4 currents with 88 mM GTPγS in transfected cells occurred within a few minutes following the whole-cell configuration and showed the characteristic TRPC4 double rectification current/voltage (I/V) curves (Figure 2A and B). In agreement with the [Ca2+] data, TRPC4-I957V channels showed higher response than TRPC4-WT channels. The mean channel response (at $-100 \text{ mV}$) is shown in Figure 4C. Interestingly, TRPC4-I957V protein appears to exert a dominant positive effect on the WT protein, as co-expression of both TRPC4-WT and TRPC4-I957V proteins in the same cell produces an increase in current density similar to that of TRPC4-I957V protein alone (Figure 4C). TRPC4 activity is regulated by tyrosine kinase phosphorylation, a process that may be engaged via cross-activation secondary to muscarinic receptor activation. Ile-957 resides close to a tyrosine (Tyr-959) that, when phosphorylated, regulates TRPC4 activity by inserting more channels into the plasma membrane. In fact, single-channel analysis using the cell-attached mode of the patch-clamp technique showed a three-fold increase in the number of active TRPC4-I957V channels compared with TRPC4-WT channels following carbachol stimulation (Figure 3). The mean single-channel currents were similar to that reported previously for mouse TRPC4 (20) and no differences were observed between WT and I957V channels ($2.7 \pm 0.2$ and $2.8 \pm 0.2$ pA for WT and mutant channel, respectively; $n = 4$). This observation is consistent with a higher insertion of mutant channels into the membrane, although we cannot fully discard that mutant channels also presented an increased open probability, thereby facilitating the occurrence of multiple channels within a membrane patch.

The presence of different amino acid residues within $\pm 5$ positions from the target tyrosine modifies phosphorylation rates. It might occur that I957V substitution affects channel phosphorylation and insertion into the membrane, which would offer an explanation for the higher activity of TRPC4-I957V. Prediction of the phosphorylation rate of the peptide I(V)-D-Y959-D-L-N (corresponding to the TRPC4 sequence around Y959) using the NetPhos 2.0 server suggests an increase in the phosphorylation rate from 0.7 to 0.8 with the I957V change. Besides, molecular dynamics simulations over 1 ns, using the structural data available for the Fyn kinase that phosphorylates and modulates TRPC4 and I(V)-D-Y959-D-L-N peptide as the substrate, showed that the presence of a less bulky Val permits the substrate to approach Lys-431 of the Fyn kinase more closely and to establish a firmer interaction (Supplementary material online, Figures S2–S6).

To further investigate at the molecular level the link between gain of channel function and tyrosine phosphorylation, we replaced...
Figure 2 Whole-cell TRPC4 currents. Time course of whole-cell cationic currents at –100 mV in CHO cells transfected with TRPC4-WT (A) or TRPC4-I957V (B) and dialysed with a pipette solution containing 88 μM GTPγS. Top plots show the current–voltage relationship of peak whole-cell cationic currents recorded at the time indicated by the grey and black symbols in the bottom graphs.

Figure 3 Single-channel recordings of TRPC4-WT and TRPC4-I957V channels. (A) Unitary currents of human TRPC4-WT channels recorded in cell-attached patches of transfected CHO cells before (top) and after the addition of 100 μM carbachol (bottom) using a pipette potential of +60 mV. (B) Unitary currents of human TRPC4-I957V channels recorded under identical conditions as in (A). Traces showed a maximum of two active channels for cells expressing TRPC4-WT and five active channels in the case of cells expressing TRPC4-I957V. Dash line indicates the channel close state; dotted lines indicate the unitary current levels of different channels (up to five). (C) Mean increase in the number of active channels in response to 100 μM carbachol obtained in cells expressing TRPC4-WT and TRPC4-I957V.
Tyr-959 by the non-phosphorylated phenylalanine (Phe-959) in both TRPC4-WT and TRPC4-I957V. Y959F substitution decreased the activation of TRPC4-I957V to levels similar to those obtained with TRPC4-WT (Figure 4A and B). Mean responses are shown in Figure 4C. We also evaluated the sensitivity of TRPC4-WT and TRPC4-I957V channels to the tyrosine kinase inhibitor PP2, finding that PP2 reduced channel activity by 50% in TRPC4-WT, without a significant effect on TRPC4-I957V (Figure 4D). The non-active analog of PP2 (PP3) did not affect either WT or I957V channels (n = 4, results not shown). Finally, the direct effect of I957V substitution on TRPC4 phosphorylation was evaluated (Supplementary material online, Figure S7). HEK293 cells transfected with FLAG-TRPC4-WT or FLAG-TRPC4-I957V were stimulated with epidermal growth factor and TRPC4 immunoprecipitated with an anti-FLAG antibody. Immunoprecipitated TRPC4 was assessed for tyrosine phosphorylation by immunoblotting with anti-phosphotyrosine antibody (PY20). Tyrosine phosphorylation of TRPC4-I957V showed a two-fold increase compared with TRPC4-WT.

4. Discussion

We have identified a genetic variation in TRPC4 (I957V) that links increased channel activity to a protective effect against MI. This gain of function determines higher Ca^{2+} signals that may facilitate the generation of endothelium- and NO-dependent vasorelaxation mediated by TRPC4. The enhanced channel activity of the genetic variant appears to be related to a firmer interaction of the channel with a regulatory tyrosine kinase that phosphorylates and regulates channel insertion into the membrane and its activity. This observation may also offer a clue to the interpretation of the protective effect of TRPC4-I957V against MI in diabetic patients, who present endothelial dysfunction typically associated with a reduced NO-dependent vasorelaxation. Therefore, it is conceivable that the association of the TRPC4-I957V polymorphism with protection against MI in diabetic patients might be due, at least in part, to the phosphorylation-mediated gain of function of the mutated channel in the endothelium. The higher Ca^{2+} signals generated by TRPC4-I957V may ultimately facilitate the generation of endothelium- and NO-dependent vasorelaxation, which would compensate the loss of tyrosine phosphorylation and subsequent reduction in NO-mediated vasodilatation described in diabetic vessels.

Supplementary material

Supplementary material is available at Cardiovascular Research online.

Acknowledgements

We thank M. Schaefer (University of Leipzig) for TRPC4 plasmid. This study makes use of data generated by the Wellcome Trust Case–Control Consortium. A full list of the investigators who contributed to the generation of the data is available from www.wtccc.org.uk.

Conflict of interest: none declared.
Funding
This work was supported by Fondo de Investigación Sanitaria (Red HERACLIES RD06/0009, CIBERSESP) Fondos Europeos de Desarrollo Regional (FEDER), Plan E; the Spanish Ministry of Science and Innovation (SAF2009-09848); Generalitat de Catalunya (SGR09–1369); Fundació la Marató de TV3 (080403, 080431); and Beatriu Pins Grant from the Generalitat de Catalunya to M.T. M.A.V. is the recipient of an Institució Catalana de Recerca i Estudis Avançats (ICREA) Academia Award.

References
11. Serban DN, Nilius B, Vanhoutte PM. The endothelial saga: the past, the present, the future. Pflugers Arch 2010;459:787–792.
13. Serban DN, Nilius B, Vanhoutte PM. The endothelial saga: the past, the present, the future. Pflugers Arch 2010;459:787–792.
14. Serban DN, Nilius B, Vanhoutte PM. The endothelial saga: the past, the present, the future. Pflugers Arch 2010;459:787–792.