Cardiovascular Research (2008) **80**, 445–452
doi:10.1093/cvr/cvn207

Role of cytochrome P450-dependent transient receptor potential V4 activation in flow-induced vasodilatation

Annemarieke E. Loot[1], Rüdiger Popp[1], Beate Fisslthaler[1], Joris Vriens[2], Bernd Nilius[2], and Ingrid Fleming[1]*

[1]*Vascular Signalling Group, Institut für Kardiovaskuläre Physiologie, Goethe-Universität, Theodor-Stern-Kai 7, D-60590 Frankfurt am Main, Germany; and* [2]*Department of Molecular Cell Biology, Division of Physiology, Laboratory of Ion Channel Research, Katholieke Universiteit Leuven, Leuven, Belgium*

Received 28 January 2008; revised 18 July 2008; accepted 28 July 2008; online publish-ahead-of-print 5 August 2008

Time for primary review: 38 days

KEYWORDS
Cytochrome P450 epoxygenase;
Transient receptor potential channel V4;
Fluid shear stress;
Mechanotransduction;
Endothelium

Aims Fluid shear stress elicits endothelium-dependent vasodilatation via nitric oxide and prostacyclin-dependent and -independent mechanisms. The latter includes the opening of Ca^{2+}-operated potassium channels by cytochrome P450 (CYP) epoxygenase-derived epoxyeicosatrienoic acids (EETs) leading to endothelial hyperpolarization. We previously reported that EETs activate the transient receptor potential (TRP) V4 channel in vascular endothelial cells and that Ca^{2+} influx in these cells in response to mechanical stimuli is dependent on the activation of CYP epoxygenases. We therefore hypothesized that the TRPV4 channel is involved in the flow-induced vasodilatation attributed to the endothelium-derived hyperpolarizing factor (EDHF).

Methods and results In the presence of N^{ω}-nitro-L-arginine methyl ester and diclofenac, precontracted mouse carotid arteries displayed a considerable vasodilatation in response to step-wise increases in luminal flow. The EDHF-mediated, flow-induced vasodilatation could be inhibited by the epoxygenase inhibitor MS-PPOH, was abolished after down-regulation of CYP epoxygenases in tissue culture, and could be restored by viral expression of CYP2C9 in the endothelium. The TRPV4-channel inhibitor ruthenium red (RuR) inhibited the EDHF-mediated flow response, but the combination of MS-PPOH and RuR had no further effect. RuR also inhibited the response in CYP2C9-overexpressing vessels. Moreover, TRPV4-deficient mice demonstrated a blunted EDHF-mediated response to increases in luminal flow in comparison to their wild-type littermates, and the addition of MS-PPOH was without effect in these mice (up to $38 \pm 3\%$ in $TRPV4^{-/-}$ vs. $57 \pm 6\%$ in $TRPV4^{+/+}$, $P < 0.01$). In cultured human endothelial cells, exposure to fluid shear stress induced the translocation of the TRPV4 channel from a perinuclear localization to the cell membrane.

Conclusion We conclude that the TRPV4 channel is involved in flow-induced, endothelium-dependent vasodilatation of murine carotid arteries. Moreover, the activation of the TRPV4 channel by flow requires an active CYP epoxygenase and the translocation of the channel to the cell membrane.

1. Introduction

Fluid shear stress is the physiologically most relevant stimulus of the endothelial layer, and endothelial responses to fluctuations in shear stress acutely modulate tone as well as having long-term effects on vascular homeostasis. The exposure of endothelial cells to shear stress triggers the release of vasoactive mediators, and although nitric oxide (NO) is referred to as the primary endothelium-derived relaxing factor, increased luminal flow induces vasodilatation partly by stimulating hyperpolarization of the endothelial cell plasma membrane.[1] Several mechanisms have been reported to underlie such endothelial cell hyperpolarization, and in some, but not all, arteries, flow-induced vasodilatation has been linked to the generation of an endothelium-derived hyperpolarizing factor (EDHF).[2–4]

The epoxyeicosatrienoic acids (EETs) are arachidonic acid epoxides generated by cytochrome P450 (CYP) enzymes that can act as EDHFs by activating Ca^{2+}-activated K^+ (K_{Ca}) channels in vascular smooth muscle[5,6] as well as in endothelial cells.[7] CYP epoxygenase activity has also been implicated in flow-induced vasodilatation since both epoxygenase inhibitors and blockers of endothelial K_{Ca} channels reduce flow-induced, EDHF-dependent vasodilatation.[8] The molecular events through which EETs activate K_{Ca} channels are unclear, but most likely involve a $G_{\alpha s}$-mediated mechanism[9] and/or an elevation in intracellular $[Ca^{2+}]_i$.[10–12]

* Corresponding author. Tel: +49 69 6301 6972; fax: +49 69 6301 7668.
E-mail address: fleming@em.uni-frankfurt.de

We recently demonstrated that EETs are able to enhance Ca^{2+} influx into bradykinin-stimulated endothelial cells at the same time as stimulating the translocation of the transient receptor potential (TRP) C3 and TRPC6 channels to the plasma membrane.[12] This led to the hypothesis that the EET-mediated activation of a TRP channel could also contribute to flow-induced vasodilatation. Endothelial cells express members of several TRP channel families,[13] including TRPV4; a channel that has previously been implicated in mechanotransduction and flow-induced vasodilatation.[14,15] How exactly the open probability of TRPV4 channels is regulated is not entirely clear (for review, see ref. 16), but we previously demonstrated that in endothelial cells 5,6- and 8,9-EET can directly stimulate the TRPV4 channel leading to enhanced Ca^{2+} influx and that the intracellular generation of EETs by CYP enzymes in response to a mechanical stimulus, i.e. hypotonic cell swelling, leads to TRPV4 activation.[17]

The aim of the present study was to assess the role of the TRPV4 channel in the flow-induced, NO- and prostacyclin (PGI_2)-independent (i.e. EDHF-mediated) vasodilatation of the murine carotid artery and to study the molecular mechanisms involved in its activation. We therefore assessed flow-induced vasodilatation in carotid arteries from wild-type and TRPV4-deficient ($TRPV4^{-/-}$) mice as well as fluid shear stress-induced changes in the subcellular localization of the TRPV4 channel.

2. Methods

2.1 Materials

The epoxygenase inhibitor *N*-methylsulfonyl-6-(2-propargyloxyphenyl) hexanamide (MS-PPOH)[18] was kindly provided by John R. Falck (Dallas, TX, USA). All other chemicals were purchased from Sigma (Taufkirchen, Germany).

2.2 Animals

Male C57BL/6 mice, 6–9 weeks of age, were obtained from Charles River (Sulzfeld, Germany). $TRPV4^{-/-}$ mice,[19] kindly provided by Dr Makoto Suzuki (Tochigi, Japan), and their wild-type littermates were bred and genotyped at the Division of Physiology at the KU Leuven. These animals have normal blood pressures compared with their wild-type littermates.[20] The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

2.3 Adenoviral infection of carotid arteries

Adenoviruses encoding either GFP or GFP and CYP2C9 were delivered to the endothelial layer of mouse carotid arteries as described previously.[21] In short, the mouse was euthanized with isoflurane, and the carotid arteries were cleared of surrounding tissue but left *in situ*. Both arteries were filled with 3×10^7 pfu of virus in MCDB culture medium containing 2% FCS. The arteries were isolated and incubated at 37°C in culture medium for 48 h.

2.4 Flow-induced vasodilatation

Freshly isolated or adenovirus-treated murine carotid arteries were cannulated and placed in a video-monitored perfusion system (Living Systems Instruments, Burlington, VT, USA). Flow-induced vasodilatation was studied as described.[21] Briefly, the vessels were continuously perfused at 10 μL/min and intraluminal pressure was maintained at 80 mmHg. The arteries were constricted with phenylephrine (0.1–3 μmol/L) to ~80% of the baseline internal diameter. Intraluminal flow was increased step-wise from 10 to 800 μL/min and changes in internal diameter were recorded. After flow was restored to 10 μL/min and the diameter had returned to baseline, vascular responsiveness to acetylcholine (ACh, 1 μmol/L) and sodium nitroprusside (SNP, 10 μmol/L) was tested. To measure the responses to acute intraluminal delivery of 4α-phorbol 12,13-didecanoate (4αPDD, 1 μmol/L), intraluminal flow was gradually increased to 200 μL/min and a 10-times concentrated solution was infused upstream of the artery at 20 μL/min with a syringe-infusion pump.

Where indicated, N^{ω}-nitro-L-arginine methyl ester (L-NAME, 300 μmol/L), diclofenac (10 μmol/L), or the CYP epoxygenase inhibitor, MS-PPOH (10 μmol/L), were present in both the intraluminal and extraluminal perfusate throughout the experiment. The TRPV4 channel blocker ruthenium red (RuR, 1 μmol/L) was added to the intraluminal perfusate only.

After the experiments, adenoviral infected vessels were lysed in SDS buffer, boiled for 5 min, and the expression of CYP2C was assessed by SDS–PAGE as described.[22]

2.5 Immunohistochemistry and cell culture

Small sections of carotid arteries from $TRPV4^{-/-}$ mice and their wild-type littermates were embedded in Tissue Tek (Sakura Finetec), frozen, cut into sections (10 μm), fixed with 4% paraformaldehyde (PFA), permeabilized with 0.03% Triton X-100, and incubated with antibodies against TRPV4 (Alomone Labs, Jerusalem, Israel) and murine CD31 (BD Pharmingen, Franklin Lakes, NJ, USA).

Human umbilical vein endothelial cells were isolated as described.[22] The use of human material in this study conforms to the principles outlined in the Declaration of Helsinki.[23] The cells were passaged once and seeded on glass cover slips coated with fibronectin. Confluent cells were incubated in MCDB 131 medium containing 2% FCS overnight before subjecting the cells to shear stress (12 dynes/cm^2) for 24 h. Experiments were performed in the presence of 1-adamantyl-3-cyclohexylurea (1 μmol/L) and diclofenac (10 μmol/L) to prevent breakdown of endogenously produced EETs by the soluble epoxide hydrolase and cyclooxygenase (COX), respectively. Experiments were stopped by the addition of 4% PFA, and cells were permeabilized with Triton X-100 and incubated with antibodies against human TRPV4 (MBL International, Woburn, MA, USA). Cells and tissue sections were subsequently incubated with Alexa Fluor-labelled secondary antibodies and phalloidin (Invitrogen Corp., Carlsbad, CA, USA). The samples were mounted with fluorescence mounting medium (DAKO) and viewed using a confocal microscope (LSM 510 META, Zeiss).

2.6 Statistics

Data are expressed as mean ± SEM. Statistical evaluation was performed with Student's *t*-test for unpaired data, one-way ANOVA followed by a Bonferroni *t*-test, or ANOVA for repeated measures where appropriate. Values of $P < 0.05$ were considered statistically significant.

3. Results

3.1 Flow-induced vasodilatation is largely nitric oxide- and prostacyclin-independent and sensitive to cytochrome P450 inhibition

Phenylephrine-precontracted murine carotid arteries responded to step-wise increases in luminal flow (from 10 to 800 μL/min) with a gradual vasodilatation (up to 39 ± 2 μm; *Figure 1A*). NOS inhibition with L-NAME partially attenuated the flow-induced vasodilatation but the addition of diclofenac did not result in further inhibition. The remaining NO- and PGI_2-independent response to flow was, however, abolished

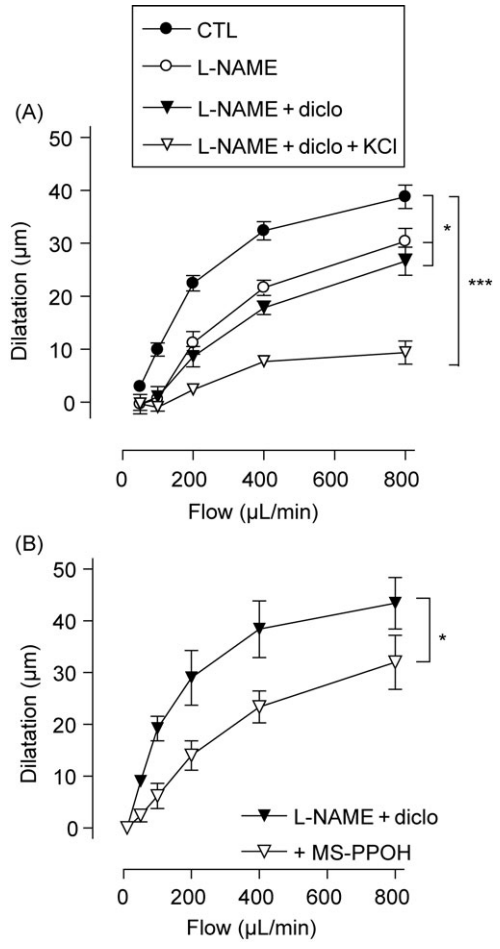

Figure 1 Contribution of nitric oxide, prostacyclin, and endothelium-derived hyperpolarizing factor (EDHF) to the flow-induced vasodilatation of mouse carotid arteries. (*A*) Freshly isolated arteries were exposed to incremental increases in flow (10–800 μL/min) in the absence (CTL) or presence of N^{ω}-nitro-L-arginine methyl ester (L-NAME) (300 μmol/L), L-NAME and diclofenac (diclo; 10 μmol/L), or L-NAME, diclofenac, and KCl (20 mmol/L). (*B*) Flow-induced, EDHF-mediated vasodilatation was assessed in the presence of both L-NAME and diclofenac and in the absence and presence of the CYP epoxygenase inhibitor MS-PPOH (10 μmol/L). The graphs summarize data from three to 10 experiments; $^{*}P < 0.05$, $^{***}P < 0.001$ vs. CTL.

by elevating the extracellular K^+ concentration from 5 to 20 mmol/L in both the intraluminal and the extraluminal perfusate. In a separate set of experiments, we found that the NO- and PGI_2-independent, i.e. EDHF-mediated, flow-induced vasodilatation was significantly attenuated by the CYP epoxygenase inhibitor MS-PPOH (10 μmol/L; *Figure 1B*), while MS-PPOH was without effect in the absence of L-NAME and diclofenac. None of the pharmacological treatments employed influenced either the basal internal diameter or the level of precontraction (see Supplementary material online, Table S1).

3.2 Cytochrome P450 epoxygenase activity expression plays a crucial role in flow-induced vasodilatation of the mouse carotid artery

As no specific CYP epoxygenase has been identified that contributes to the CYP-dependent EDHF responses in mice, we used an alternative approach to demonstrate the essential role of CYP-derived EETs in the flow-induced EDHF-like vasodilatation. Briefly, as we have previously demonstrated that CYP expression decreases rapidly in freshly isolated endothelial cells[6] as well as in isolated arteries maintained under static conditions,[24] we reassessed flow-induced vasodilatation after maintaining mouse carotid arteries for 48 h in organ culture. Consistent with our previous results such vessels, i.e. maintained under control conditions and/or infected with a control (GFP) virus, exhibited poor EDHF-mediated (i.e in the presence of L-NAME and diclofenac) vasodilatations and the responses observed were no longer sensitive to epoxygenase inhibition (*Figure 2A*). Adenoviral overexpression of CYP2C9 selectively targeted the endothelium of the carotid arteries studied (*Figure 2B*). Although the overexpression of CYP2C9 did not influence the EDHF-mediated response to ACh or relaxation to SNP (data not shown), this approach restored the EDHF-mediated vasodilatation elicited by increased flow (*Figure 2C*). Moreover, the addition of MS-PPOH reduced the latter responses to the level observed in vessels overexpressing only GFP.

3.3 Transient receptor potential V4 channels mediate the cytochrome P450-dependent flow-induced vasodilatation

As TRPV4 has been implicated in endothelial cell mechanotransduction[14,15] and can be activated by CYP-derived EETs,[17] we assessed the effects of the non-selective TRPV4 blocker ruthenium red on the EDHF-mediated responses to increased flow. In freshly isolated murine carotid arteries, the addition of ruthenium red (1 μmol/L) to the intraluminal perfusate had no effect on vasodilator responses to flow under control conditions or in the presence of L-NAME alone (*Figure 3A* and *B*). However, in the presence of both L-NAME and diclofenac, ruthenium red significantly attenuated flow-induced vasodilatation (*Figure 3C*). The TRP-channel blocker was unable to inhibit vasodilatation in arteries pretreated with the CYP epoxygenase inhibitor MS-PPOH, indicating a requirement for CYP activation in the EDHF responses observed (*Figure 3D*).

To demonstrate the interdependence of the flow-induced EDHF-dependent vasodilatation on CYP epoxygenase activity and TRPV4 expression, we repeated experiments in which arteries were maintained in organ culture to down-regulate endogenous CYP epoxygenase activity. As stated above, the maintenance of arteries under static conditions was associated with the loss of an EDHF-like flow-induced vasodilatation. In these arteries, ruthenium red also failed to affect flow-induced vasodilatation (*Figure 4A*). However, the TRPV4 antagonist clearly attenuated responses in arteries overexpressing CYP2C9 (*Figure 4B*).

3.4 Effect of transient receptor potential V4 deletion on flow-induced endothelium-derived hyperpolarizing factor-mediated vasodilatation

As ruthenium red exerts numerous effects including blockade of mitochondrial Ca^{2+} uptake[25] and ryanodine receptors,[26] we next assessed flow-induced vasodilatation in arteries from $TRPV4^{-/-}$ mice. Immunohistochemical analysis of carotid arteries from these mice showed selective expression of TRPV4 in the vascular endothelium that was absent in the $TRPV4^{-/-}$ mice (*Figure 5A*). Moreover, in the presence of L-NAME and diclofenac, the TRPV4 activator 4αPDD relaxed arteries from $TRPV4^{+/+}$ mice but not from their $TRPV4^{-/-}$ littermates (*Figure 5B*). There was no

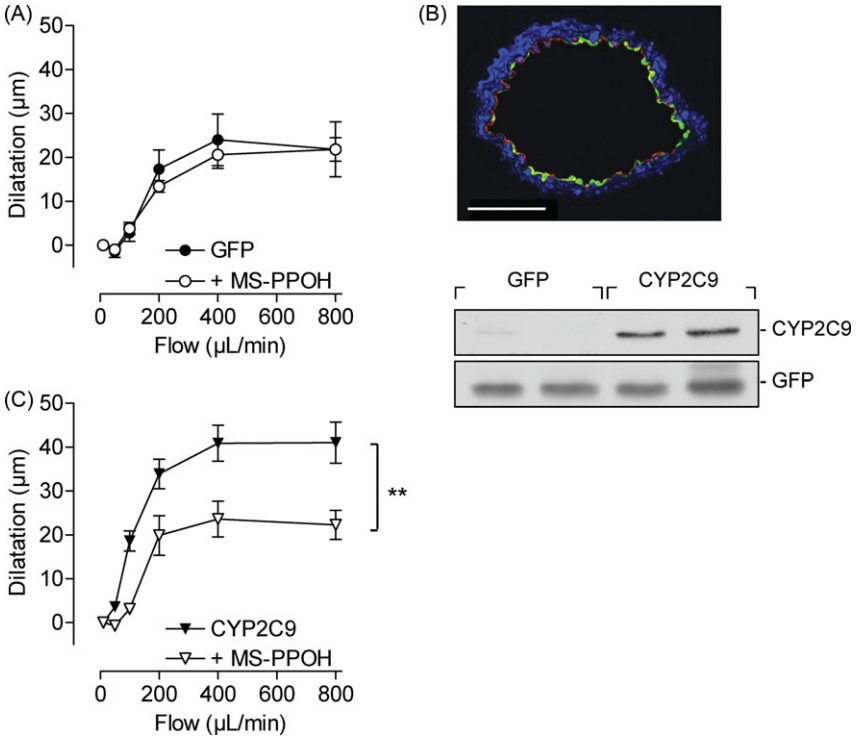

Figure 2 Flow-induced, endothelium-derived hyperpolarizing factor (EDHF)-mediated vasodilatation depends on CYP epoxygenase expression. Carotid arteries were infected with viruses expressing either GFP (*A* and *B*) or GFP and CYP2C9 (*B* and *C*) and maintained in culture medium for 48 h, and flow-induced vasodilatation was assessed in the presence of both N^{ω}-nitro-L-arginine methyl ester and diclofenac but in the absence (CTL) or presence of MS-PPOH (10 μmol/L). (B) Immunohistochemical analysis (bar = 100 μm) showing the selective endothelial expression of GFP and western blot showing the expression of GFP and CYP2C9 in vessels infected with the respective adenoviruses. The graphs summarize data from seven to eight experiments; $^{**}P < 0.01$ vs. CTL.

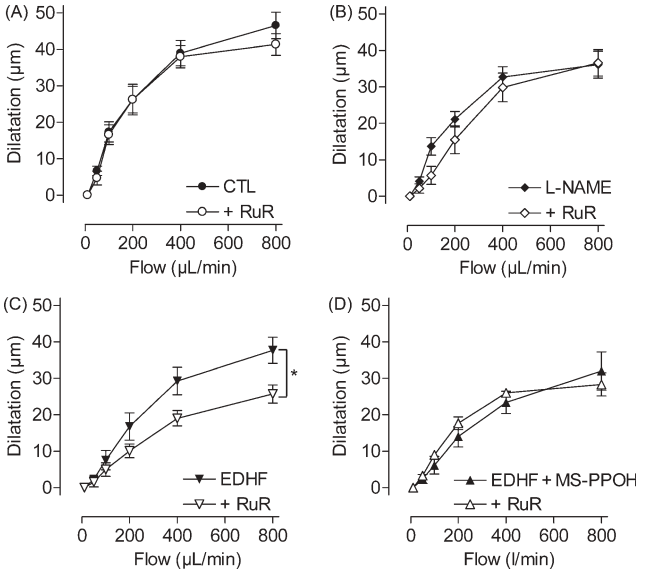

Figure 3 Effect of ruthenium red on flow-induced, nitric oxide and endothelium-derived hyperpolarizing factor (EDHF)-mediated vasodilatation in freshly isolated carotid arteries. Flow-induced vasodilatation was assessed in the absence and presence of ruthenium red (RuR, 1 μmol/L) in the luminal perfusate in the presence of (*A*) solvent (CTL) to assess NO-dependent responses, (*B*) in the presence of N^{ω}-nitro-L-arginine methyl ester (L-NAME), (*C*) in the presence of L-NAME and diclofenac to study EDHF-dependent responses, or (*D*) in the presence of L-NAME, diclofenac and MS-PPOH (10 μmol/L) to determine the contribution of CYP epoxygenases. The graphs summarize data from seven to 10 experiments; $^{*}P < 0.05$ vs. EDHF.

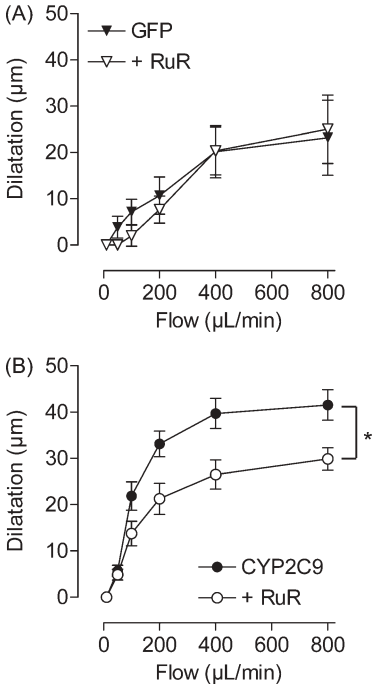

Figure 4 Effect of ruthenium red on the flow-induced vasodilatation of CYP2C9-expressing carotid arteries. Carotid arteries were infected with adenoviruses expressing either (*A*) GFP or (*B*) CYP2C9 and maintained in tissue culture for 48 h. Flow-induced vasodilatation was assessed in the absence and presence of ruthenium red (RuR, 1 μmol/L) in the luminal perfusate and in the continuous presence of N^{ω}-nitro-L-arginine methyl ester and diclofenac. The graphs summarize data from six to eight experiments. $^{*}P < 0.05$ vs. CYP2C9.

detectable difference in the responses elicited by the endothelium-dependent but CYP-independent vasodilator ACh or the endothelium-independent vasodilator SNP

(*Figure 5C*). Consistent with the effect of RuR, carotid arteries from TRPV4$^{-/-}$ mice showed a normal response to flow under control circumstances, i.e. in the absence of NOS and COX inhibitors (*Figure 6A*). The EDHF-mediated, flow-induced vasodilatation, however, was severely blunted in TRPV4$^{-/-}$ mice and was insensitive to MS-PPOH (*Figure 6B*).

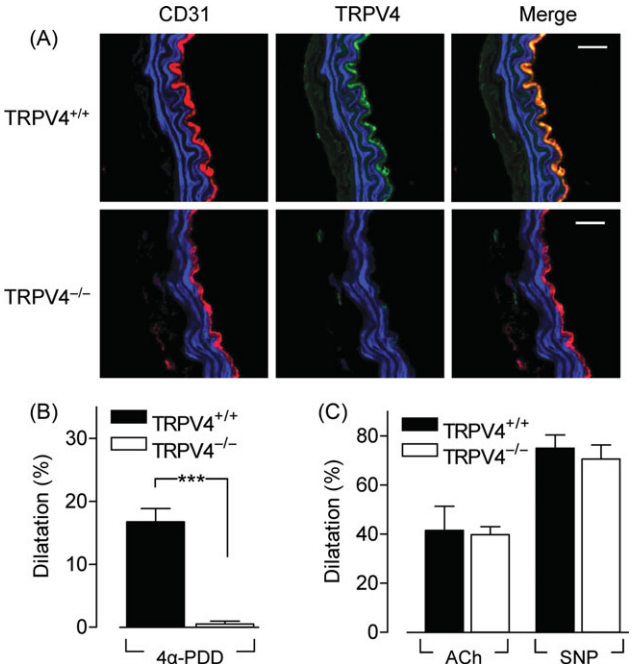

Figure 5 Vascular reactivity in TRPV4$^{-/-}$ mice. (*A*) Immunohistochemical analysis of TRPV4 expression in carotid arteries from wild-type (TRPV4$^{+/+}$) and TRPV4-deficient (TRPV4$^{-/-}$) mice. Representative pictures from three experiments. (*B* and *C*) The vasodilator responses of carotid arteries from TRPV4-deficient (TRPV4$^{-/-}$) or wild-type (TRPV4$^{+/+}$) mice were assessed in response to (B) 4αPDD (1 μmol/L) and (C) acetylcholine (ACh, 1 μmol/L) and sodium nitroprusside (SNP, 10 μmol/L). All experiments were performed in the continuous presence of N^{ω}-nitro-L-arginine methyl ester and diclofenac. The graphs summarize data from four to eight experiments; ***$P <$ 0.001 vs. TRPV4$^{+/+}$.

3.5 Fluid shear stress and epoxyeicosatrienoic acids stimulate the membrane insertion of transient receptor potential V4 channels

We previously reported that EETs can induce the translocation of TRPC channels to caveolae[12] at the cell membrane and therefore assessed the subcellular localization of TRPV4 in endothelial cells. In human endothelial cells cultivated under static conditions, TRPV4 was mainly localized in the perinuclear Golgi apparatus, but after the exposure of endothelial cells to fluid shear stress (12 dynes cm^{-2}, 24 h) to induce CYP epoxygenase expression,[24] TRPV4 was enriched at the cell membrane, a step required to allow Ca^{2+} influx (*Figure 6C*).

4. Discussion

The results of the present study indicate that the flow-induced, NO- and PGI_2-independent vasodilatation of the murine carotid artery is sensitive to a CYP epoxygenase inhibitor as well as to a TRPV4 channel blocker. Moreover, EDHF-responses and sensitivity to ruthenium red could be restored by the overexpression of a CYP epoxygenase (CYP2C9) in arteries maintained in organ culture. These observations together with the finding that shear stress induced the translocation of TRPV4 to the endothelial cell membrane suggest that the activation of the channel by CYP-derived EETs underlies the EDHF component of flow-induced vasodilatation.

Flow-induced vasodilatation is an integrated response involving the release of several endothelium-derived

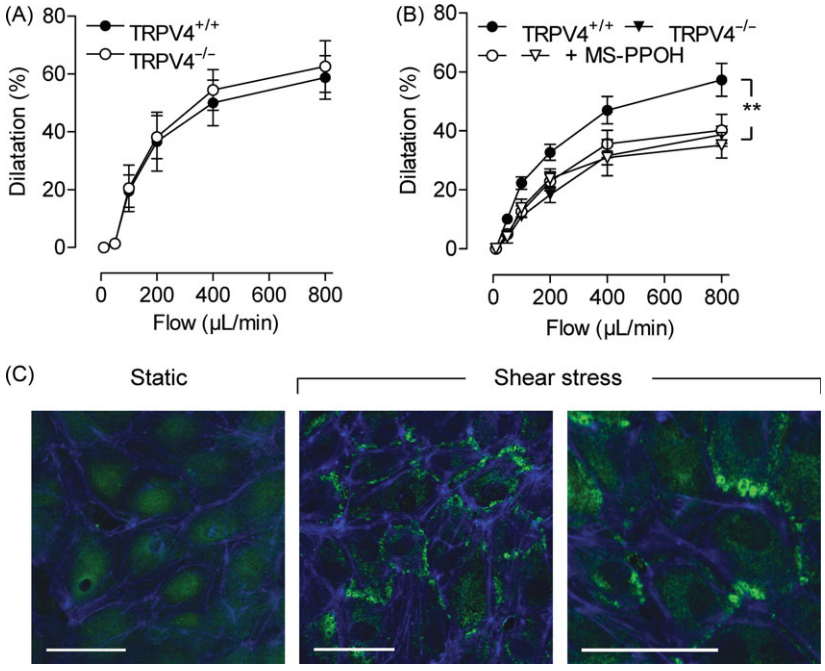

Figure 6 Selective attenuation of endothelium-derived hyperpolarizing factor-mediated flow-induced vasodilatation in TRPV4$^{-/-}$ mice. The response to flow was assessed in carotid arteries from TRPV4$^{+/+}$ and TRPV4$^{-/-}$ littermates (*A*) in the absence of inhibitors, or (*B*) continuous presence of N^{ω}-nitro-L-arginine methyl ester and diclofenac but in the absence (closed symbols) or presence (open symbols) of MS-PPOH. **$P < 0.01$. (*C*) Immunofluorescent staining for TRPV4 (green) and phalloidin (blue) of human endothelial cells cultured under static conditions or exposed to fluid shear stress (12 dynes/cm^2) for 24 h (bar = 50 μm).

vasodilator autacoids. Although NO is generally described as the primary endothelium-derived relaxing factor, we found that in the presence of NOS and COX inhibitors the mouse carotid artery still exhibited a robust response to flow. As the latter was sensitive to increased extracellular [K^+] and to the epoxygenase inhibitor MS-PPOH, it appeared that this component of flow-induced vasodilatation can be attributed to a CYP epoxygenase-dependent EDHF. EETs produced by endothelial CYP epoxygenases are recognized as hyperpolarizing factors,[5,6] and although the majority of studies assessing their contribution to the regulation of vascular tone have concentrated on the responses observed following the application of receptor-dependent agonists, a number of studies have demonstrated EET production in response to mechanical stimuli such as increased luminal flow and cyclic stretch.[27–29] CYP epoxygenase expression decreases rapidly in cultured endothelial cells[6] as well as in isolated coronary arteries,[27] making detailed studies of the molecular actions of the EETs relatively difficult. In the present study, we observed that maintaining isolated murine carotid arteries in culture medium under static conditions led to the loss of the MS-PPOH-sensitive component of the response to flow. Although we cannot exclude that static organ culture influences various intracellular processes in isolated vessels, we previously observed that the eNOS-dependent pathway remains fully intact,[21] suggesting a more or less specific loss of the EDHF/CYP-dependent pathway. Moreover, the overexpression of CYP2C9 in these arteries largely restored flow-induced EDHF-mediated responses as well as sensitivity to MS-PPOH, providing further evidence that CYP epoxygenase activity makes a significant contribution to endothelium-dependent vasodilatation.

A lot is now known about the mechanisms by which EETs elicit vasodilatation, and in addition to the activation of large conductance K_{Ca} channels BK_{Ca} in smooth muscle cells,[5] these eicosanoids can also affect inter-endothelial gap junctional communication.[30] However, perhaps, the most important event in the initiation/generation of EDHF-mediated responses is the rapid hyperpolarization of endothelial cells; an event that precedes the hyperpolarization of vascular smooth muscle cells (for review, see ref. 7). Taking recent evidence together, it seems that EETs can act intracellularly to modulate endothelial cell hyperpolarization and it is not essential that EETs diffuse to vascular smooth muscle cells to activate BK_{Ca} channels in order to induce EDHF-dependent vasodilatation. Indeed, although CYP metabolites can be released from the cells in which they are generated to exert paracrine effects,[31] the majority is either not usually present in a free form or is rapidly metabolized by the soluble epoxide hydrolase.[32] In endothelial cells, EETs have been linked to the activation of protein kinase A,[12,33] and the modulation of Ca^{2+} influx,[10–12] as well as to the activation of small and intermediate K^+_{Ca} channels.[34] Following on from our recent observation that in response to agonist stimulation, CYP2C9-derived EETs are able to amplify endothelial cell hyperpolarization by enhancing Ca^{2+}-influx through TRPC channels (TRPC6 and TRPC3) and reports of the involvement of TRPV4 channels in shear stress-induced vasodilatation,[14,15] we hypothesized that the activation of a CYP epoxygenase followed by that of TRPV4 may underlie flow-induced, EDHF-mediated vasodilatation.

The TRP channel family encompasses a broad variety of widely expressed cation channels, several of which facilitate Ca^{2+} entry and thus contribute to the opening of K_{Ca} channels. Of the TRP channels, TRPV4 has been frequently implicated in mechanotransduction but direct evidence of an actual role in mediating endothelium-dependent vasodilatation is lacking. As with most TRP channels, the various hypothesis about the mechanisms involved in regulating TRPV4 channels have arisen chiefly from observations made in heterologous expression systems and *in vitro* studies (see review[35]). However, circumstantial evidence exists to link endothelial CYP epoxygenases with TRPV4 activation. For example, both shear stress and hypo-osmotic stretch induce Ca^{2+} influx into TRPV4 overexpressing HEK cells,[36] and the hypo-osmotic stretch-induced Ca^{2+} influx into endothelial cells is sensitive to CYP inhibition, can be mimicked by exogenously applied 5,6-EET (but not 11,12-EET), and is abrogated in cells from $TRPV4^{-/-}$ mice.[17] Moreover, flow-induced vasodilatation is attenuated in arteries from $TRPV4^{-/-}$ animals.[14,15] In the present study, we were able to confirm that flow-induced, EDHF-mediated vasodilatation of the isolated mouse carotid artery was attenuated by the TRPV4 blocker, ruthenium red, and severely impaired in arteries from $TRPV4^{-/-}$ mice. In accordance with a role for TRPV4 in endothelial hyperpolarization, but at odds with previous publications,[14,15] we did not observe an effect of ruthenium red or of TRPV4 knockout in the absence of NOS and COX inhibition. This discrepancy can most probably be attributed to differences in methodology since rather than acutely elevating the viscosity of the perfusate, we studied vasodilatation in response to more gradual and maintained increases in luminal flow. We were, however, able to establish a solid link between EET production and TRPV4. For example MS-PPOH and ruthenium red both attenuated flow-induced responses to the same extent in freshly isolated arteries, but had no cumulative effect. Moreover, in arteries maintained in culture for up to 48 h to decrease endothelial CYP expression, the sensitivity to ruthenium red was lost but could be reinstated by the overexpression of CYP2C9. And finally, responses in $TRPV4^{-/-}$ mice were insensitive to CYP epoxygenase inhibition.

Transient receptor potential channels are activated by very diverse stimuli, involving distinct activating pathways.[37] One factor critically regulating TRP-channel function is their insertion into the plasma membrane, and intracellular trafficking is recognized as an important mechanism for modulating the activity of several channels.[38] Given that the subcellular localization of TRPV4 appears to be tightly regulated,[39] we studied the subcellular localization of TRPV4 after exposure of cells to fluid shear stress. Our data suggest that prolonged shear stress leading to the re-expression of CYP epoxygenases promotes the membrane translocation of TRPV4. The enhanced membrane localization of TRPV4 is reminiscent of the previously described translocation of TRPC6 to endothelial cell caveolae upon agonist stimulation or 11,12-EET application.[12] It was, however, not possible to take the same approach, i.e. to monitor the movements of a TRPV4 fusion protein between different cell compartments, as the overexpression of TRPV4 consistently induced endothelial cell death as a consequence of Ca^{2+} overload. The exact molecular mechanism(s) by which EETs induce the membrane translocation of TRP channels remains to be elucidated. Moreover, although we found that channels of the TRPC and TRPV families can be affected by EETs, it should be stressed that

these channels generally respond to very distinct stimuli as well as to distinct EETs. For example, TRPV4 has been linked to mechanotransduction and can be activated by 5,6- and 8,9-EET but not 11,12-EET,[17] whereas TRPC6 is unaffected by shear and hypo-osmotic stress as well as by 5,6-EET (unpublished observation) but both its translocation and Ca^{2+} influx are stimulated by 11,12-EET.[12] Thus, the spectrum of EET regioisomers generated in response to a given stimulus would be expected to determine which TRP channels can be affected. On the basis of the above-mentioned data, we speculate that 5,6-EET underlies the flow-induced activation of TRPV4; a speculation supported by the observation that the inclusion of diclofenac to inhibit COX rendered carotid arteries sensitive to CYP epoxygenase as well as TRPV4 inhibition. Indeed, while the bioavailability of 8,9-, 11,12-, and 14,15-EET is largely determined by the activity of the soluble epoxide hydrolase, the intracellular concentration of 5,6-EET is determined by COX.[40]

The mouse carotid artery is a useful model to study the molecular mechanisms involved in flow-induced vasodilatation since it can easily be manipulated by adenoviral infection. This system does of course have other limitations as the importance of the NO- and PGI_2-independent flow response is only unmasked when eNOS and COX are inhibited in healthy carotid arteries. Generally, the contribution of CYP and EDHF to vascular responses is thought to be more significant in the smaller sized vessels (e.g. mesenteric and cerebral resistance vessels) in which TRPV4 expression and function have previously been demonstrated.[41,42] Thus, while it is tempting to speculate that the CYP-TRPV4 pathway is crucial for the dynamic regulation of organ perfusion by contributing to flow responses in resistance vessels, this will have to be addressed in detail in future studies using smaller arteries or isolated vascular beds.

Taken together, our data indicate that the role of TRPV4 in flow-induced vasodilatation is not linked to the generation of either NO or PGI_2 but to the activation of a CYP epoxygenase. The epoxygenase product (most probably 5,6-EET) is then able to elicit vasodilatation by a mechanism involving the intracellular translocation of TRPV4 channels and their subsequent activation.

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

Funding

This work was supported by the Deutsche Forschungsgemeinschaft (SFB 553 B5 and FI 830/2-3) and by the European Vascular Genomics Network, a Network of Excellence supported by the European Community's sixth Framework Programme (Contract No. LSHM-CT-2003-503254) and by grants (to B.N.) from the Fund for Scientific Research-Flanders F.W.O. (G. 0508.05; F.W.O G.0172.03), the Human Frontiers Science Programme (HFSP Research Grant Ref. RGP 32/2004), the Belgian Federal Government (Interuniversity Poles of Attraction Program, Prime Ministers Office IUAP Nr. 3P4/23), the Onderzoeksraad KU Leuven (GOA 2004/07, Centre of Excellence Financing EF/95/010). J.V. is a postdoctoral fellow of the F.W.O.

Acknowledgements

The authors are indebted to Ingrid Kempter and Claudia Grosser for expert technical assistance.

Conflict of interest: none declared.

References

1. Busse R, Fleming I. Regulation of endothelium-derived vasoactive autacoid production by hemodynamic forces. *Trends Pharmacol Sci* 2003; **24**:24–29.
2. Takamura Y, Shimokawa H, Zhao H, Igarashi H, Egashira K, Takeshita A. Important role of endothelium-derived hyperpolarizing factor in shear stress-induced endothelium-dependent relaxations in the rat mesenteric artery. *J Cardiovasc Pharmacol* 1999;**34**:381–387.
3. Huang A, Sun D, Carroll MA, Jiang H, Smith CJ, Connetta JA *et al*. EDHF mediates flow-induced dilation in skeletal muscle arterioles of female eNOS-KO mice. *Am J Physiol Heart Circ Physiol* 2001;**280**:H2462–H2469.
4. Watanabe S, Yashiro Y, Mizuno R, Ohhashi T. Involvement of NO and EDHF in flow-induced vasodilation in isolated hamster cremasteric arterioles. *J Vasc Res* 2005;**42**:137–147.
5. Campbell WB, Gebremedhin D, Pratt PF, Harder DR. Identification of epoxyeicosatrienoic acids as endothelium-derived hyperpolarizing factors. *Circ Res* 1996;**78**:415–423.
6. Fisslthaler B, Popp R, Kiss L, Potente M, Harder DR, Fleming I *et al*. Cytochrome P450 2C is an EDHF synthase in coronary arteries. *Nature* 1999; **401**:493–497.
7. Busse R, Edwards G, Feletou M, Fleming I, Vanhoutte PM, Weston AH. EDHF: bringing the concepts together. *Trends Pharmacol Sci* 2002;**23**: 374–380.
8. Sun D, Huang A, Koller A, Kaley G. Endothelial K_{Ca} channels mediate flow-dependent dilation of arterioles of skeletal muscle and mesentery. *Microvasc Res* 2001;**61**:179–186.
9. Li PL, Campbell WB. Epoxyeicosatrienoic acids activate K^+ channels in coronary smooth muscle through a guanine nucleotide binding protein. *Circ Res* 1997;**80**:877–884.
10. Hoebel BG, Kostner GM, Graier WF. Activation of microsomal cytochrome P450 mono-oxygenase by Ca^{2+} store depletion and its contribution to Ca^{2+} entry in porcine aortic endothelial cells. *Br J Pharmacol* 1997; **121**:1579–1588.
11. Graier WF, Simecek S, Sturek M. Cytochrome P450 mono-oxygenase-regulated signalling of Ca^{2+} entry in human and bovine endothelial cells. *J Physiol (Lond)* 1995;**482**:259–274.
12. Fleming I, Rueben A, Popp R, Fisslthaler B, Schrodt S, Sander A *et al*. Epoxyeicosatrienoic acids regulate trp channel dependent Ca^{2+} signaling and hyperpolarization in endothelial cells. *Arterioscler Thromb Vasc Biol* 2007;**27**:2612–2618.
13. Nilius B, Owsianik G, Voets T, Peters JA. Transient receptor potential cation channels in disease. *Physiol Rev* 2007;**87**:165–217.
14. Hartmannsgruber V, Heyken WT, Kacik M, Kaistha A, Grgic I, Harteneck C *et al*. Arterial response to shear stress critically depends on endothelial TRPV4 expression. *PLoS ONE* 2007;**2**:e827.
15. Köhler R, Heyken WT, Heinau P, Schubert R, Si H, Kacik M *et al*. Evidence for a functional role of endothelial transient receptor potential V4 in shear stress-induced vasodilatation. *Arterioscler Thromb Vasc Biol* 2006;**26**:1495–1502.
16. Nilius B, Vriens J, Prenen J, Droogmans G, Voets T. TRPV4 calcium entry channel: a paradigm for gating diversity. *Am J Physiol Cell Physiol* 2004; **286**:C195–C205.
17. Vriens J, Owsianik G, Fisslthaler B, Suzuki M, Janssens A, Voets T *et al*. Modulation of the Ca^{2+} permeable cation channel TRPV4 by cytochrome P450 epoxygenases in vascular endothelium. *Circ Res* 2005;**97**:908–915.
18. Falck JR, Belosludtsev YY, Reddy KK, Reddy KM, Shortt MF, Chauhan K *et al*. Eicosanoid biosynthesis: differential inhibition of cytochrome P450 epoxygenase and ω-hydroxylase. *Bioorg Med Chem Lett* 1997;**7**:3053–3056.
19. Suzuki M, Mizuno A, Kodaira K, Imai M. Impaired pressure sensation in mice lacking TRPV4. *J Biol Chem* 2003;**278**:22664–22668.
20. Mizuno A, Matsumoto N, Imai M, Suzuki M. Impaired osmotic sensation in mice lacking TRPV4. *Am J Physiol Cell Physiol* 2003;**285**:C96–C101.
21. Dixit M, Loot AE, Mohamed A, Fisslthaler B, Boulanger CM, Ceacareanu B *et al*. Gab1, SHP2, and protein kinase A are crucial for the activation of the endothelial NO synthase by fluid shear stress. *Circ Res* 2005;**97**: 1236–1244.

22. Fleming I, Fisslthaler B, Dixit M, Busse R. Role of PECAM-1 in the shear-stress-induced activation of Akt and the endothelial nitric oxide synthase (eNOS) in endothelial cells. *J Cell Sci* 2005;**118**:4103–4111.
23. World Medical Association. World Medical Association Declaration of Helsinki: recommendations guiding physicians in biomedical research involving human subjects. *Cardiovasc Res* 1997;**35**:2–3.
24. Fisslthaler B, Popp R, Michaelis UR, Kiss L, Fleming I, Busse R. Cyclic stretch enhances the expression and activity of coronary endothelium-derived hyperpolarizing factor synthase. *Hypertension* 2001;**38**:1427–1432.
25. Dedkova EN, Ji X, Lipsius SL, Blatter LA. Mitochondrial calcium uptake stimulates nitric oxide production in mitochondria of bovine vascular endothelial cells. *Am J Physiol Cell Physiol* 2004;**286**:C406–C415.
26. Imagawa T, Smith JS, Coronado R, Campbell KP. Purified ryanodine receptor from skeletal muscle sarcoplasmic reticulum is the Ca^{2+}-permeable pore of the calcium release channel. *J Biol Chem* 1987;**262**:16636–16643.
27. Popp R, Fleming I, Busse R. Pulsatile stretch in coronary arteries elicits release of endothelium-derived hyperpolarizing factor: a modulator of arterial compliance. *Circ Res* 1998;**82**:696–703.
28. Miura H, Wachtel RE, Liu Y, Loberiza FR Jr, Saito T, Miura M *et al*. Flow-induced dilation of human coronary arterioles: important role of Ca^{2+}-activated K^+ channels. *Circulation* 2001;**103**:1992–1998.
29. Huang A, Sun D, Jacobson A, Carroll MA, Falck JR, Kaley G. Epoxyeicosatrienoic acids are released to mediate shear stress-dependent hyperpolarization of arteriolar smooth muscle. *Circ Res* 2005;**96**:376–383.
30. Popp R, Brandes RP, Ott G, Busse R, Fleming I. Dynamic modulation of interendothelial gap junctional communication by 11,12-epoxyeicosatrienoic acid. *Circ Res* 2002;**90**:800–806.
31. Gauthier KM, Edwards EM, Falck JR, Reddy DS, Campbell WB. 14,15-Epoxyeicosatrienoic acid represents a transferable endothelium-dependent relaxing factor in bovine coronary arteries. *Hypertension* 2005;**45**:666–671.
32. Newman JW, Morisseau C, Hammock BD. Epoxide hydrolases: their roles and interactions with lipid metabolism. *Prog Lipid Res* 2005;**44**:1–51.
33. Imig JD, Inscho EW, Deichmann PC, Reddy KM, Falck JR. Afferent arteriolar vasodilation to the sulfonimide analog of 11,12-epoxyeicosatrienoic acid involves protein kinase A. *Hypertension* 1999;**33**:408–413.
34. Weston AH, Feletou M, Vanhoutte PM, Falck JR, Campbell WB, Edwards G. Bradykinin-induced, endothelium-dependent responses in porcine coronary arteries: involvement of potassium channel activation and epoxyeicosatrienoic acids. *Br J Pharmacol* 2005;**145**:775–784.
35. Liedtke W. TRPV channels' role in osmotransduction and mechanotransduction. *Handb Exp Pharmacol* 2007;**179**:473–487.
36. Wu L, Gao X, Brown RC, Heller S, O'neil RG. Dual role of the TRPV4 channel as a sensor of flow and osmolality in renal epithelial cells. *Am J Physiol Renal Physiol* 2007;**293**:F1699–F1731.
37. Vriens J, Watanabe H, Janssens A, Droogmans G, Voets T, Nilius B. Cell swelling, heat, and chemical agonists use distinct pathways for the activation of the cation channel TRPV4. *Proc Natl Acad Sci USA* 2004;**101**:396–401.
38. Ambudkar IS. Trafficking of TRP channels: determinants of channel function. *Handb Exp Pharmacol* 2007;**179**:541–557.
39. Cuajungco MP, Grimm C, Oshima K, D'hoedt D, Nilius B, Mensenkamp AR *et al*. PACSINs bind to the TRPV4 cation channel. PACSIN 3 modulates the subcellular localization of TRPV4. *J Biol Chem* 2006;**281**:18753–18762.
40. Chiamvimonvat N, Ho CM, Tsai HJ, Hammock BD. The soluble epoxide hydrolase as a pharmaceutical target for hypertension. *J Cardiovasc Pharmacol* 2007;**50**:225–237.
41. Saliez J, Bouzin C, Rath G, Ghisdal P, Desjardins F, Rezzani R *et al*. Role of caveolar compartmentation in endothelium-derived hyperpolarizing factor-mediated relaxation: Ca^{2+} signals and gap junction function are regulated by caveolin in endothelial cells. *Circulation* 2008;**117**:1065–1074.
42. Earley S, Heppner TJ, Nelson MT, Brayden JE. TRPV4 forms a novel Ca^{2+} signaling complex with ryanodine receptors and BK_{Ca} channels. *Circ Res* 2005;**97**:1270–1279.