Stimulation of the EP_3 receptor causes lung oedema by activation of TRPC6 in pulmonary endothelial cells

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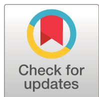

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EP_3 activation triggers pulmonary oedema *via* G_i-dependent activation of PLC and subsequent tyrosine phosphorylation of TRPC6. In PAF-induced lung oedema this TRPC6 activation coincides with ASMase-dependent caveolar recruitment of TRPC6. https://bit.ly/34P3d13

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Abstract

Background Prostaglandin E_2 (PGE_2) increases pulmonary vascular permeability by activation of the PGE_2 receptor 3 (EP_3), which may explain adverse pulmonary effects of the EP_1/EP_3 receptor agonist sulprostone in patients. In addition, PGE_2 contributes to pulmonary oedema in response to platelet-activating factor (PAF). PAF increases endothelial permeability by recruiting the cation channel transient receptor potential canonical 6 (TRPC6) to endothelial caveolae *via* acid sphingomyelinase (ASMase). Yet, the roles of PGE_2 and EP_3 in this pathway are unknown. We hypothesised that EP_3 receptor activation may increase pulmonary vascular permeability by activation of TRPC6, and thus, synergise with ASMase-mediated TRPC6 recruitment in PAF-induced lung oedema.

Methods In isolated lungs, we measured increases in endothelial calcium (ΔCa^{2+}) or lung weight (Δweight), and endothelial caveolar TRPC6 abundance as well as phosphorylation.

Results PAF-induced ΔCa^{2+} and Δweight were attenuated in EP_3-deficient mice. Sulprostone replicated PAF-induced ΔCa^{2+} and Δweight which were blocked by pharmacological/genetic inhibition of TRPC6, ASMase or Src-family kinases (SrcFK). PAF, but not sulprostone, increased TRPC6 abundance in endothelial caveolae. Immunoprecipitation revealed PAF- and sulprostone-induced tyrosine-phosphorylation of TRPC6 that was prevented by inhibition of phospholipase C (PLC) or SrcFK. PLC inhibition also blocked sulprostone-induced ΔCa^{2+} and Δweight, as did inhibition of SrcFK or inhibitory G-protein (G_i) signalling.

Conclusions EP_3 activation triggers pulmonary oedema *via* G_i-dependent activation of PLC and subsequent SrcFK-dependent tyrosine phosphorylation of TRPC6. In PAF-induced lung oedema, this TRPC6 activation coincides with ASMase-dependent caveolar recruitment of TRPC6, resulting in rapid endothelial Ca^{2+} influx and barrier failure.

Introduction

Regulated endothelial permeability is critical for lung fluid homeostasis, and its failure causes permeability-type lung oedema [1]. Tight control of endothelial permeability is ensured, among others, by

the fact that only few mediators such as platelet-activating factor (PAF) or prostaglandin E_2 (PGE_2) directly increase endothelial permeability in the intact lung without the help of inflammatory cells [2]. Once these mediators activate their respective endothelial receptors, vascular permeability increases rapidly yet transiently within minutes, thus limiting the extent and duration of barrier leakage [3, 4].

Lung endothelial permeability in response to relevant triggers of vascular leak including PAF [5] or endotoxin [6] is critically regulated by transient receptor potential canonical 6 (TRPC6), a polymodal unselective cation channel which mediates endothelial calcium (Ca^{2+}) entry with subsequent activation of endothelial myosin light chain kinase and endothelial cell contraction [7]. Functional activity of TRPC6 is controlled by its subcellular trafficking and the phosphorylation state of several regulatory threonine and tyrosine residues [8]. In previous work we have shown that stimulation by PAF triggers the recruitment of TRPC6 into caveolae of pulmonary vascular endothelial cells by a process that requires the activity of acid sphingomyelinase (ASMase) [5, 9] (supplementary figure S1). ASMase converts sphingomyelin into ceramide, which promotes the formation of new ceramide-rich caveolae and the compositional change of existing caveolae [10]. Following PAF, these effects enhance the recruitment of caveolin 1 (cav-1) into caveolae, which interacts with endothelial nitric oxide synthase (eNOS) [11], and TRPC6, which in turn become enriched in the caveolae [5, 12]. The interaction of eNOS with cav-1 at the plasma membrane silences eNOS activity, thus leading to a sudden drop in NO production [12] and subsequent disinhibition of TRPC6, which under resting conditions is kept dormant by a NO/cyclic guanosine monophosphate (cGMP)-dependent mechanism. It may be speculated that this regulation involves serine/threonine-phosphorylation of TRPC6 by protein kinase G (PKG) [13, 14], yet this concept remains to be tested for PAF stimulation. In line with a functional role for eNOS inhibition in this context, PAF-induced oedema can be effectively mitigated by exogenous NO [12].

However, PAF-induced oedema is not fully explained by caveolar recruitment of eNOS and TRPC6. In addition to ASMase, PAF also activates cyclo-oxygenase (COX)-1 [15] which generates PGE_2, which in turn contributes to oedema formation by activation of the prostaglandin E receptor 3 (EP_3) [4, 9]. Consistent with a detrimental role of EP_3 receptor activation, the EP_3>EP_1 receptor agonist sulprostone, which is clinically used in the treatment of pre-eclampsia and atonic uterine haemorrhage, has been associated with the development of pulmonary oedema as a serious side-effect [16–19]. The clinical relevance of this COX-dependent pathway is highlighted by an observational clinical study which reported that aspirin (acetylsalicylic acid (ASA)) therapy was associated with reduced intensive care unit mortality in patients with acute respiratory distress syndrome [20].

However, the cellular mechanisms underlying the effects of PGE_2/EP_3 signalling in the regulation of lung endothelial permeability, and its interaction with the ASM/eNOS/TRPC6 axis are so far unclear (supplementary figure S1). Such knowledge would be essential for a better understanding of permeability-type pulmonary oedema and the clinical side-effects of sulprostone. The EP_3 receptor has several splice variants that all couple to inhibitory G-protein (G_i), but some of which may also couple to G_{12} or stimulatory G-protein (G_s) [21, 22]. In the present context, activation of G_s seems unlikely, as PAF decreases pulmonary cAMP levels [23]. A more probable pathway relates to the G_i-mediated mobilisation of Ca^{2+} *via* phospholipase C (PLC) [24–26]. Indeed, TRPC6 has been shown to become activated either by the PLC product diacylglycerol (DAG) independent of protein kinase C (PKC) [27, 28], and/or by tyrosine-phosphorylation of the channel [29–31].

Putting together these findings we hypothesised that EP_3 receptor activation may increase pulmonary vascular permeability by a PLC-dependent tyrosine phosphorylation of TRPC6 that may act in synergism with the ASMase-mediated recruitment of the channel to caveolae and TRPC6 disinhibition due to loss of NO synthesis. In order to exclude effects of activated inflammatory cells on endothelial permeability, and because endothelial cells in culture regulate permeability through distinctly different pathways than pulmonary endothelial cells in the intact lung [2], we tested this hypothesis in isolated perfused lungs from rats or from mice deficient in either TRPC6, EP_3 or ASMase. Our findings demonstrate that EP_3 activation triggers endothelial Ca^{2+} influx through TRPC6 which becomes activated by PLC-mediated tyrosine phosphorylation. For PAF-induced oedema formation, these findings suggest a triple activation mechanism in that PAF 1) stimulates the ASMase-dependent recruitment of TRPC6 to caveolae, where these channels are concomitantly 2) disinhibited by a drop of NO levels and 3) activated *via* EP_3-mediated phosphorylation of their tyrosine residues.

Methods

Experiments were performed in isolated lungs of Sprague Dawley rats or mice deficient in EP_3 (*Ptger3*$^{-/-}$), TRPC6 (*Trpc6*$^{-/-}$) or ASMase (*Smpd1*$^{-/-}$), and their wild-type controls. The study was approved by the

animal care and use committee of the local government authorities, and experiments were performed in accordance with the guidelines [32].

Rat and mouse lungs were prepared, ventilated and perfused in constant pressure mode [5]. Drugs were used at concentrations as given in the figure legends. Real-time imaging of endothelial $[Ca^{2+}]_i$ and NO production was performed in isolated perfused lungs as described [5]. Membrane fractions from pulmonary endothelial cells of isolated perfused lungs were prepared by silica bead isolation [12]. Endothelial caveolar fractions were separated by density gradient centrifugation, and used for immunoblotting and immunoprecipitation. $[Ca^{2+}]_i$ and NO data are given as mean±SEM and were compared by Mann–Whitney U-test and Kruskal–Wallis test. All other data are shown as individual data points with mean±SD and were analysed by Tukey test. Statistical significance was assumed at $p<0.05$.

Methods are described in detail in the supplementary methods.

Results

To probe for the contribution of the COX/PGE_2/EP_3 axis in PAF-induced endothelial barrier failure and its potential role in the activation of TRPC6 channels, we first compared the effects of the COX inhibitor ASA and the TRPC6 inhibitor larixol acetate [33] on PAF-induced oedema formation. In isolated perfused rat lungs, the PAF-induced weight gain (figure 1a), as well as the corresponding increase in filtration coefficient (figure 1b) were reduced by both ASA and larixol acetate pre-treatment. However, the effects of both inhibitors were not additive, indicating that they act through a common pathway. Additionally, ASA blocked the PAF-induced $[Ca^{2+}]_i$ increase in lung endothelial cells *in situ* (figure 1c), but not the concomitant cessation of NO production (figure 1d). This finding is in line with a COX-dependent regulation of TRPC6, as we had shown previously that the PAF-induced endothelial $[Ca^{2+}]_i$ response, but not the inhibition of NO production, is mediated by TRPC6 [5]. ASA did not affect the PAF-induced recruitment of TRPC6 into endothelial caveolae (figure 1e), a process mediated by ASMase [5]. These findings suggest that COX products regulate lung endothelial permeability *via* a TRPC6-dependent mechanism, yet without affecting the ASMase-dependent recruitment of the channel to caveolae or its regulation *via* the NO/cGMP axis.

Notably, PAF-induced caveolar recruitment of TRPC6, as well as the subsequent endothelial $[Ca^{2+}]_i$ and permeability response could be replicated *in vitro* in both pulmonary artery and lung microvascular endothelial cells (supplementary figure S2), yet TRPC6 recruitment was absent in pulmonary arterial smooth muscle cells (supplementary figure S3). These findings indicate that this pathway is unique to the endothelium, but not restricted to a specific compartment of the pulmonary vasculature.

We previously identified PGE_2 as a candidate COX product that mediates the endothelial permeability response to PAF and showed that the pertinent receptor is probably EP_3 [4]. In isolated perfused mouse lungs, we now provide direct proof for this concept by demonstrating that lungs of *Ptger3*$^{-/-}$ mice are largely protected from weight gain (figure 2a) and endothelial $[Ca^{2+}]_i$ increase (figure 2b) in response to PAF. To gain further insights into the endothelial responses mediated *via* EP_3 and their connection to TRPC6, we next studied the effects of the EP_3 agonist sulprostone. In isolated lungs of wild-type mice, sulprostone caused a marked increase in lung weight that was absent in lungs of *Trpc6*$^{-/-}$ mice (figure 2c). Sulprostone also induced a moderate yet distinct increase in endothelial $[Ca^{2+}]_i$ that was blocked in the presence of larixol acetate (figure 2d) and absent in lungs of *Trpc6*$^{-/-}$ mice (figure 2e). Conversely, the sulprostone-induced $[Ca^{2+}]_i$ response could be reproduced by a second, structurally distinct specific EP_3 receptor agonist, ONO-AE-248 [34] (supplementary figure S4). Notably, and in agreement with the lack of effect of ASA on NO production, sulprostone did not affect basal endothelial NO production (figure 2f).

To further elucidate the relationship between EP_3 and TRPC6, we probed whether sulprostone could replicate the changes in caveolar composition evoked by PAF [5, 12]. However, probing of endothelial caveolar fractions from sulprostone-stimulated lungs for proteins typically increased upon PAF treatment, namely TRPC6, cav-1, eNOS and ASMase, revealed no change in their caveolar abundance (figure 3a–e), although the applied dose of sulprostone had proven effective to induce lung oedema in a TRPC6-dependent manner (figure 2c). This finding is in agreement with the lack of effect of ASA on PAF-induced TRPC6 recruitment to caveolae (figure 1e), in that it suggests that the stimulation of TRPC6-mediated Ca^{2+} signalling by EP_3 receptor activation is independent of the ASMase-mediated recruitment of TRPC6. In line with this notion, ASMase and sulprostone, when given sequentially, triggered an additive increase in endothelial $[Ca^{2+}]_i$ that exceeded the effect of either ASMase or sulprostone alone (figure 3f).

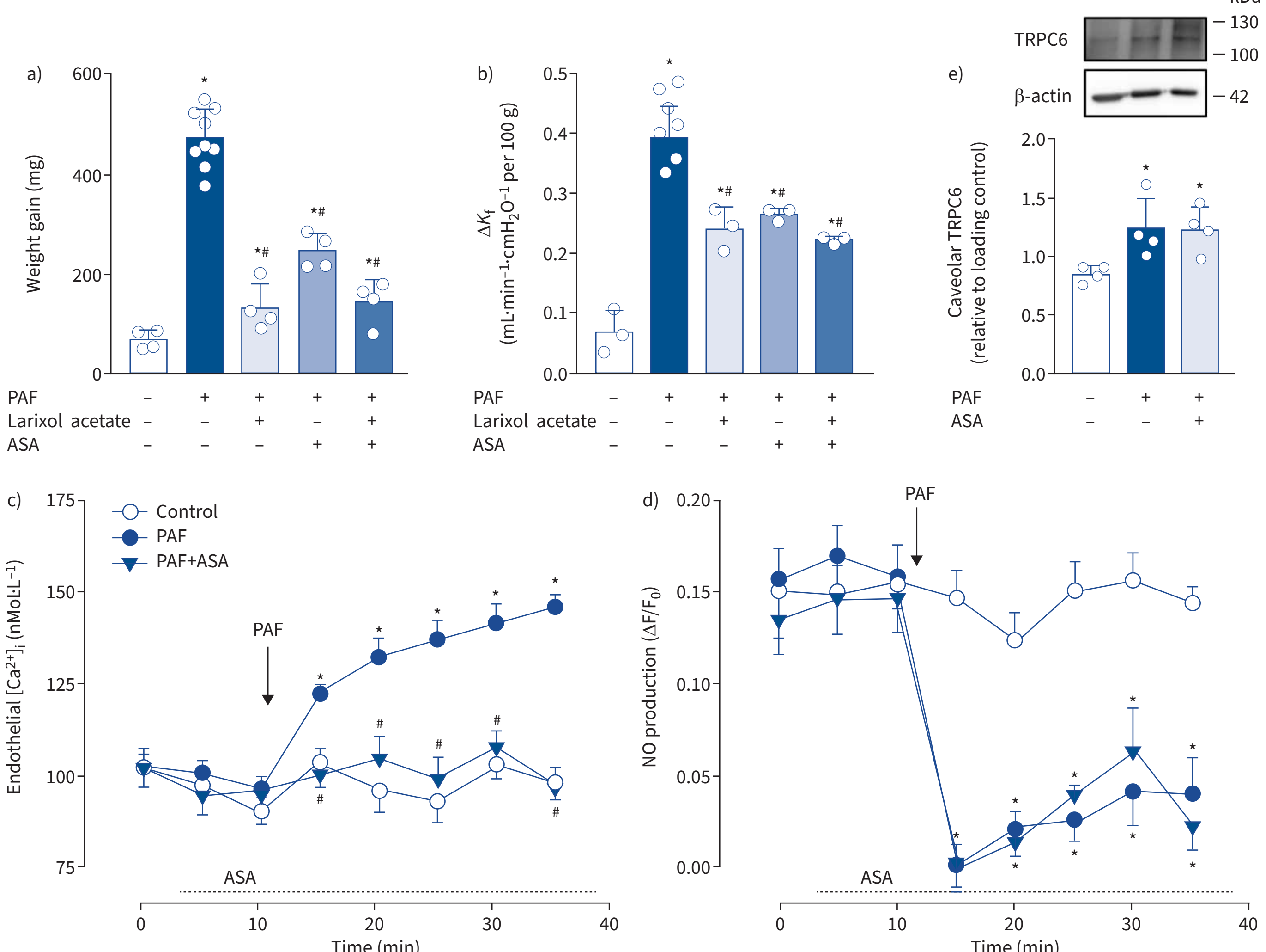

FIGURE 1 The cyclo-oxygenase-dependent part of platelet-activating factor (PAF)-induced oedema is mediated *via* transient receptor potential canonical 6 (TRPC6). a) Weight gain (n=4–9 each) and b) increase in lung vascular filtration coefficient (ΔK_f) (n=3–12 each) determined 10 min after bolus infusion of either vehicle (0.9% sodium chloride) or PAF (5 nmol bolus) in larixol acetate (5 µmol·L^{-1}) or acetyl salicylic acid (ASA) (500 µmol·L^{-1}) perfused rat lungs. c) Mean endothelial $[Ca^{2+}]_i$ and d) endothelial nitric oxide (NO) production shown as 5-min averages in rat lungs at baseline and after bolus infusion of either vehicle (control) or PAF (arrow) in the absence or presence of ASA (n=5 each). e) Caveolae from endothelial cells were prepared and probed with TRPC6 antibodies. Shown are representative immunoblots and group data for densitometric measurements of TRPC6 band intensities normalised to β-actin in perfused rat lungs treated with vehicle, PAF or ASA/PAF (n=4 each). $\Delta F/F_0$: change in fluorescence intensity relative to the corresponding baseline as a measure of NO production. *: p<0.05 *versus* untreated controls; #: p<0.05 *versus* PAF alone.

As EP_3 may couple to G_i-proteins that act *via* PLC, we next probed for the effects of the PLC inhibitor U73122 on the lung endothelial response to sulprostone. U73122, but not the chemically similar but inactive control compound U73343, blocked the increases in lung weight gain (figure 4a) and endothelial $[Ca^{2+}]_i$ (figure 4b) triggered by sulprostone. U73122 also mitigated the PAF-induced lung weight gain (figure 4c). Notably, these effects were not attributable to potential off-target effects of U73122 on ASMase, as demonstrated by a fluorescence resonance energy transfer based sphingomyelinase activity assay (supplementary figure S5). The endothelial $[Ca^{2+}]_i$ increase in response to both PAF (figure 4d) or sulprostone (figure 4e) and the sulprostone-induced lung weight gain (figure 4f) were similarly inhibited by pre-treatment with the G_i inhibitor pertussis toxin (PTX) [35], corroborating the view that EP_3 activation increases endothelial permeability *via* G_i-mediated activation of PLC.

Based on the fact that sulprostone and ASMase increase endothelial $[Ca^{2+}]_i$ in an additive manner (figure 3f), we speculated next that the EP_3 pathway acts independent of ASMase. To test this notion, we pre-treated

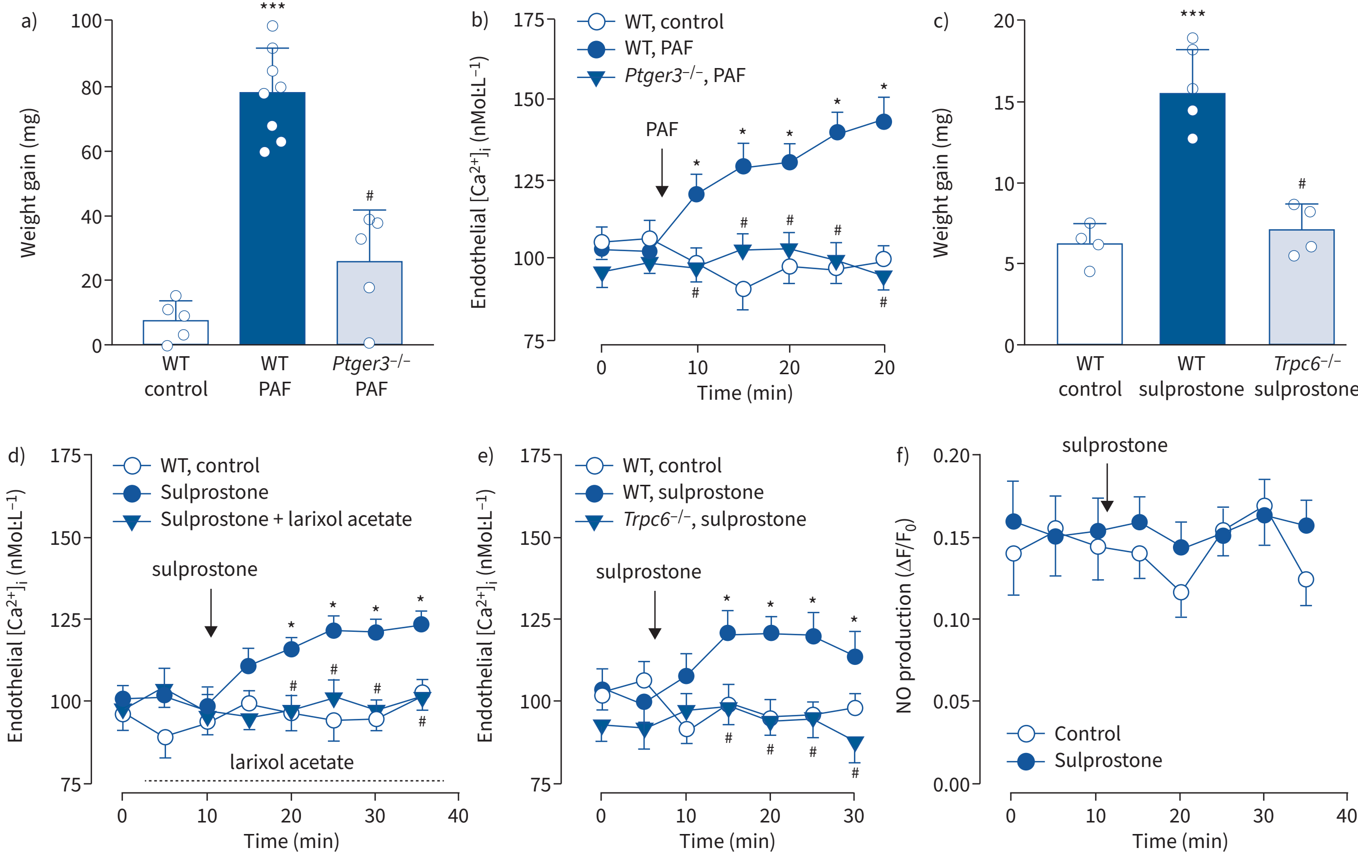

FIGURE 2 Role of the prostaglandin E_2 (PGE_2) receptor 3 (EP_3) and transient receptor potential canonical 6 (TRPC6) in platelet-activating factor (PAF)- and sulprostone-induced oedema, respectively, and the endothelial $[Ca^{2+}]_i$ response. a) Weight gain and b) mean endothelial $[Ca^{2+}]_i$ in isolated mouse lungs from wild-type (WT) mice treated with vehicle (0.9% sodium chloride (NaCl); control) or with PAF (5 nmol bolus), or lungs from EP_3-deficient *Ptger3*$^{-/-}$ mice treated with PAF (n=5–8 each). c) Weight gain in isolated perfused mouse lungs from WT mice treated with vehicle (0.9% NaCl) or with sulprostone (1 µmol·L^{-1}), or lungs from *Trpc6*$^{-/-}$ mice treated with sulprostone (n=4 each). d) Mean endothelial $[Ca^{2+}]_i$ in isolated rat lungs treated with vehicle, sulprostone or sulprostone plus larixol acetate (5 µmol·L^{-1}; n=5 each). e) Mean endothelial $[Ca^{2+}]_i$ in isolated mouse lungs from WT mice treated with vehicle or sulprostone, or in mouse lungs from *Trpc6*$^{-/-}$ mice treated with sulprostone (n=5 each). f) Endothelial nitric oxide (NO) production shown as 5-min averages in rat lungs at baseline or in the presence of sulprostone (n=5 each). $\Delta F/F_0$: change in fluorescence intensity relative to the corresponding baseline as a measure of NO production. *: p<0.05 *versus* untreated controls; ***: p<0.001 *versus* untreated controls; #: p<0.05 *versus* PAF or sulprostone.

lungs with Arc39, a recently discovered enzymatic ASMase inhibitor [36]. Unexpectedly, Arc39 reduced both lung weight gain (figure 5a) and endothelial $[Ca^{2+}]_i$ increase (figure 5b) in response to sulprostone. The latter result was confirmed in ASMase-deficient (*Smpd1*$^{-/-}$) mice (figure 5c). These effects were not attributable to a direct effect of ASMase on EP_3-PLC signalling, as Arc39 did not attenuate endothelial DAG formation in response to sulprostone (figure 5d). We then tested whether ASMase activity may not only be required for the high caveolar abundance of TRPC6 after PAF stimulation, but also for the basal presence of TRPC6 in caveolae. Indeed, Arc39 also reduced TRPC6 abundance in endothelial caveolae in the absence of PAF (figure 5e), suggesting a critical role of ASMase in the basal supply of TRPC6 to caveolae as a prerequisite for subsequent activation of TRPC6-mediated Ca^{2+} influx by sulprostone.

Next, we probed for the ability of PAF or sulprostone to activate TRPC6 by phosphorylation of regulatory residues. Immunoprecipitation of TRPC6 and immunoblotting for phosphotyrosine moieties revealed a marked increase in TRPC6 tyrosine phosphorylation in response to PAF (figure 6a). Similarly, lung perfusion with sulprostone stimulated overall phosphorylation (supplementary figure S6) as well as tyrosine phosphorylation (figure 6b) of TRPC6. Sulprostone-induced tyrosine phosphorylation was blocked by the PLC-inhibitor U73122, but not by its inactive derivative U73343 (figure 6c). In addition, PAF reduced threonine phosphorylation of TRPC6 (supplementary figure S7a), an effect that was mimicked by the NO synthase inhibitor L-nitro-arginine-methyl-ester (L-NAME) (supplementary figure S7b). As TRPC6

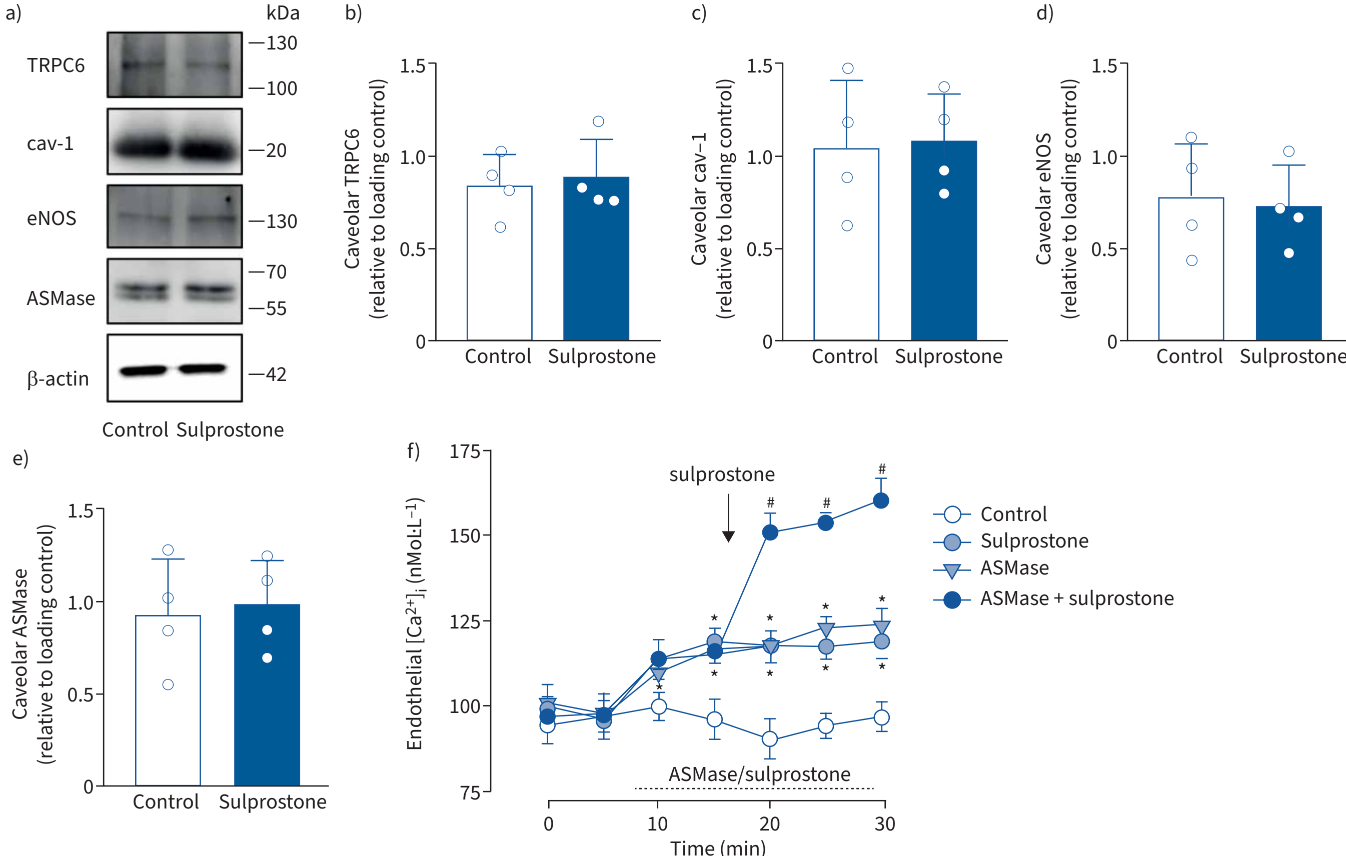

FIGURE 3 The effects of sulprostone are independent from the recruitment of transient receptor potential canonical 6 (TRPC6), acid sphingomyelinase (ASMase), caveolin-1 (cav-1) and endothelial nitric oxide synthase (eNOS), as well as from the activity of ASMase. a–e) Isolated perfused rat lungs were perfused for 10 min with vehicle (0.9% sodium chloride (NaCl); control) or sulprostone (1 µmol·L^{-1}) before caveolae from endothelial cells were prepared and probed with antibodies for TRPC6, cav-1, eNOS and ASMase. Shown are a) representative immunoblots and b–e) group data for densitometric measurements of band intensities normalised to β-actin (n=4 each). f) Mean endothelial $[Ca^{2+}]_i$ in isolated rat lungs perfused with vehicle, sulprostone, ASMase (1 U·mL^{-1}) and ASMase plus sulprostone. *: p<0.05 *versus* untreated controls; #: p<0.05 *versus* ASMase alone; n=6 each.

activation by the PLC product DAG has been shown previously to be prevented by tyrosine kinase inhibition, and specifically by the Src-family kinase inhibitor PP2 [29, 30], we tested for the effects of PP2 on TRPC6 phosphorylation and endothelial barrier failure and showed that PP2 prevented TRPC6 tyrosine phosphorylation, endothelial $[Ca^{2+}]_i$ increase and lung weight gain (figure 6d–f) in response to sulprostone, as well as the corresponding endothelial barrier response to PAF (supplementary figure S8).

Discussion

Our work identifies important signalling pathways in the regulation of pulmonary vascular permeability under both resting and inflammatory conditions. One major control hub appears to be TRPC6, which is regulated by at least three distinct mechanisms: it is activated by G_i/PLC-mediated tyrosine phosphorylation; it is inactivated by NO-dependent threonine phosphorylation; and its density in caveolae is controlled in an ASMase-dependent manner. While the present work has focused on the regulation of TRPC6, endothelial Ca^{2+} and oedema formation by EP_3 receptors, it also provides novel insights for the regulation of vascular permeability under resting conditions as well as during PAF-induced oedema formation. The conclusions from our findings are summarised graphically in figure 7.

Resting conditions

Blockade of ASMase activity in otherwise untreated lungs reduced the caveolar abundance of TRPC6 below baseline, indicating constant trafficking of TRPC6 to lung endothelial caveolae at rest in an ASMase-dependent manner (figure 7a). This notion is supported by our previous studies where treatment with imipramine or Arc39 reduced ASMase activity and the amount of caveolin-1 in caveolar fractions

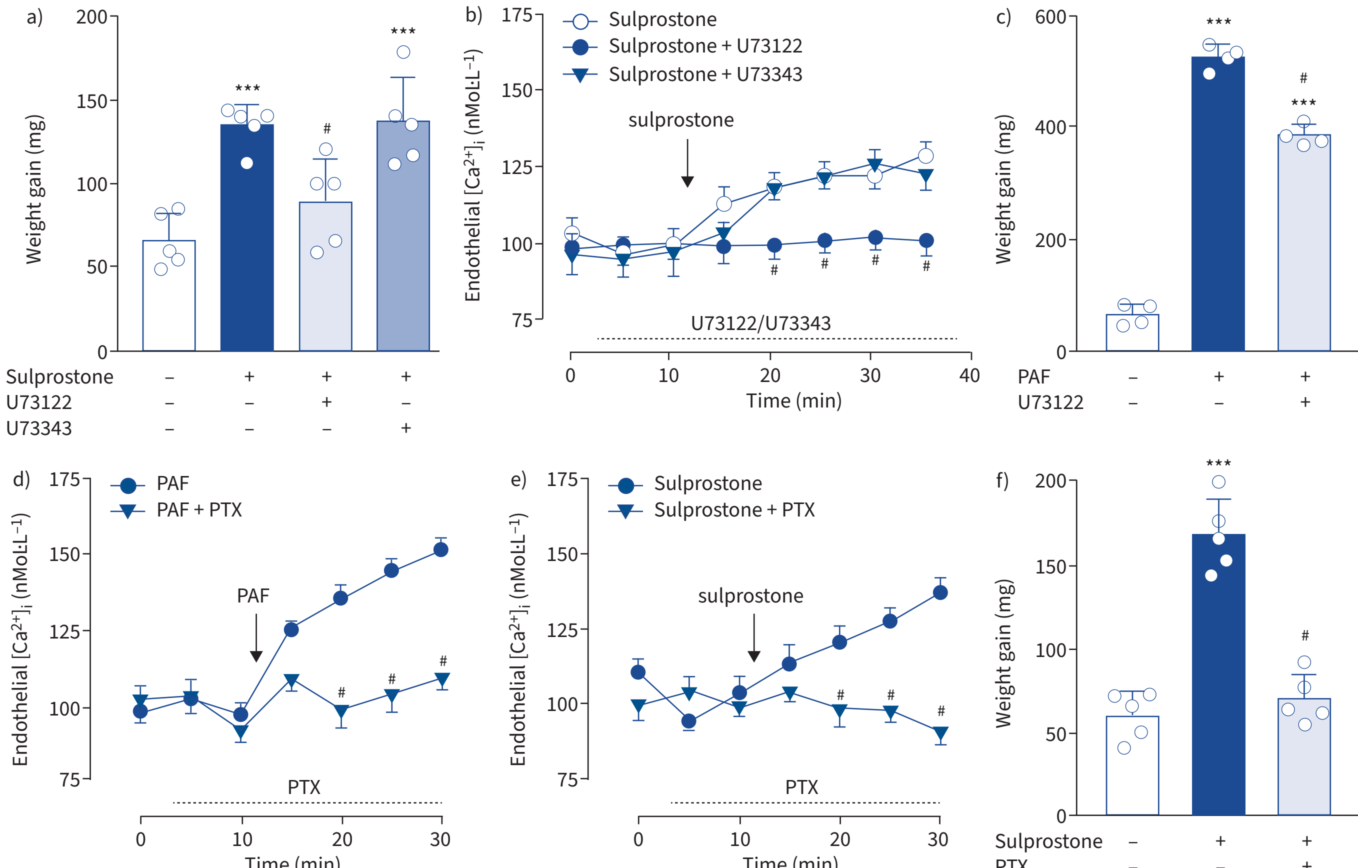

FIGURE 4 Role of phospholipase C (PLC) in the platelet-activating factor (PAF)- and sulprostone-induced $[Ca^{2+}]_i$ response and oedema formation. a) Weight gain in perfused rat lungs after bolus infusion of vehicle (0.9% sodium chloride (NaCl); control), sulprostone (1 µmol·L^{-1}), sulprostone plus the PLC inhibitor U73122 (10 µmol·L^{-1}) or sulprostone plus the control drug U73342 (10 µmol·L^{-1}; n=5 each). b) Mean endothelial $[Ca^{2+}]_i$ in isolated rat lungs perfused with vehicle, sulprostone plus U73122 or sulprostone plus U73342 (n=5 each). c) Weight gain in perfused rat lungs after bolus infusion of vehicle (0.9% NaCl), PAF (5 nmol bolus) or PAF plus U73122 (n=4 each). d) Mean endothelial $[Ca^{2+}]_i$ in isolated rat lungs perfused with PAF or PAF plus pertussis toxin (PTX) (1 nmol L^{-1}; n=4 each). e) Mean endothelial $[Ca^{2+}]_i$ in isolated rat lungs perfused with vehicle, sulprostone or sulprostone plus PTX (n=5 each). f) Weight gain in isolated rat lungs perfused with vehicle, sulprostone or sulprostone plus PTX (n=5 each). ***: p<0.001 *versus* untreated controls; #: p<0.05 *versus* PAF or sulprostone.

below baseline [37]. Thus, similar to the closely related TRPC3 [38], TRPC6 also seems to cycle continuously in and out of the plasma membrane.

For control of endothelial permeability, it is mandatory that the constantly recruited TRPC6 channels remain silenced under baseline conditions. This is apparently achieved by threonine phosphorylation (probably at Thr69 [14]) *via* eNOS-derived NO (supplementary figure S7). This arrangement guarantees that TRPC6 is readily available for swift regulation of vascular permeability, either by phosphorylation of tyrosine residues (see later) or by dephosphorylation of threonine residues when endothelial NO production is suddenly blocked, as is the case following stimulation by PAF or L-NAME [12].

Regulation of TRPC6 by EP_3 receptors

PGE_2 increases intracellular Ca^{2+} through EP_3-mediated activation of PLC [24–26], in a manner that is either G_i-dependent (PTX-sensitive) [21, 24, 39–44] or G_i-independent [21, 25, 26, 43] (figure 7b); a diversity which might be explained by the multiple splice variants of the EP_3 receptor [45] and/or biased signalling [46]. Here, the EP_3-mediated Ca^{2+} increase in pulmonary endothelial cells appeared to be entirely G_i-dependent (figure 4d–f). Several studies have demonstrated previously that the EP_3/G_i-protein can couple to PLC-isoenzymes to augment intracellular Ca^{2+} levels [21, 24, 44], but the related Ca^{2+} channel had not yet been identified.

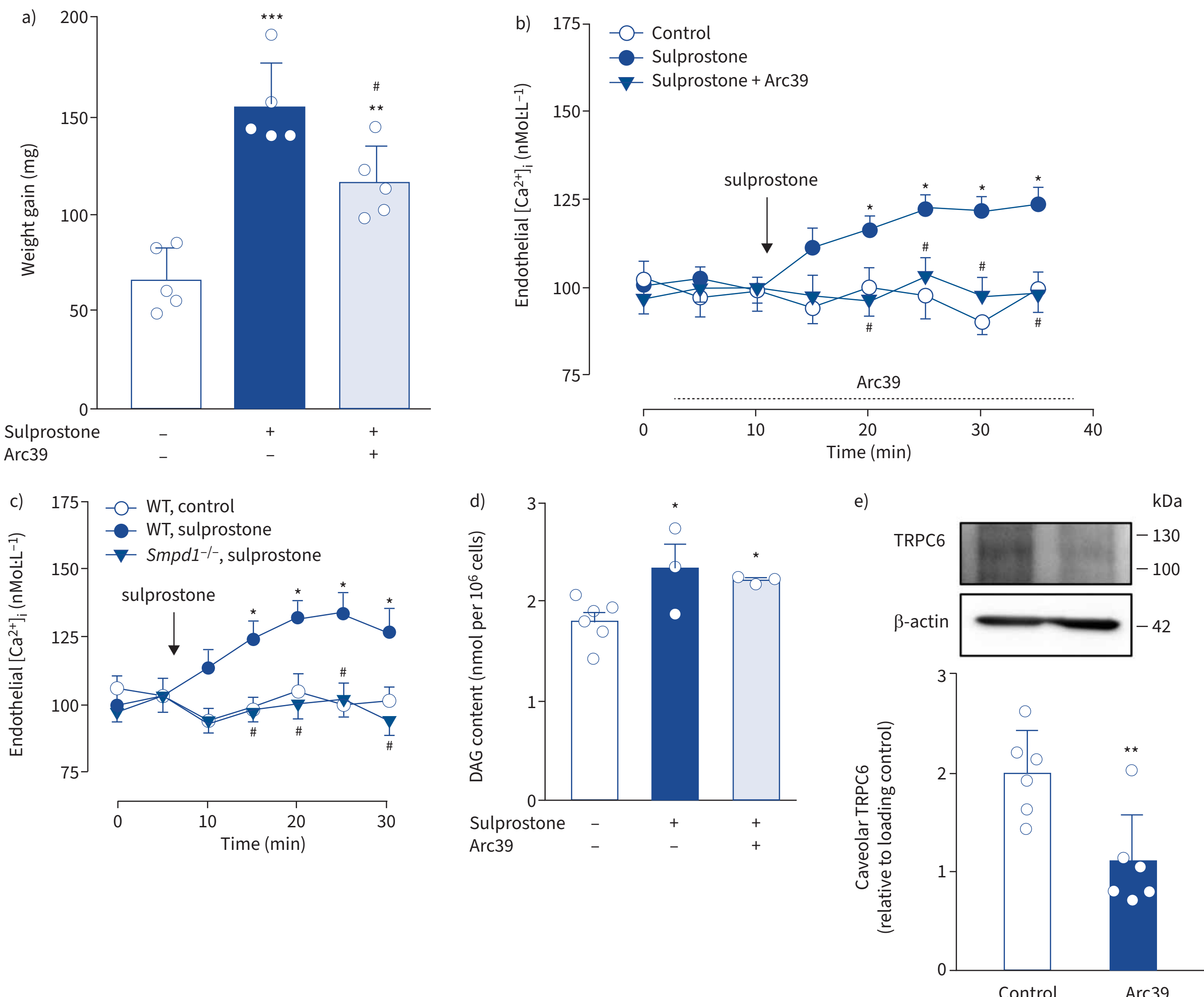

FIGURE 5 Role of acid sphingomyelinase (ASMase) in the sulprostone-induced $[Ca^{2+}]_i$ response and oedema formation, and for baseline transient receptor potential canonical 6 (TRPC6) levels. a) Weight gain in perfused rat lungs after bolus infusion of vehicle (0.9% sodium chloride (NaCl); control), sulprostone (1 µmol·L^{-1}), or sulprostone plus the ASMase inhibitor Arc39 (10 µmol·L^{-1}; n=5 each). b) Mean endothelial $[Ca^{2+}]_i$ in isolated rat lungs perfused with vehicle, sulprostone or sulprostone plus Arc39 (n=5 each). c) Mean endothelial $[Ca^{2+}]_i$ in isolated mouse lungs from wild-type (WT) mice treated with vehicle or sulprostone, or in mouse lungs from ASMase-deficient *Smpd1*$^{-/-}$ mice treated with sulprostone (n=5 each). d) Diacylglycerol (DAG) content of human pulmonary microvascular endothelial cells treated with either dimethyl sulfoxide (control) or sulprostone (1 µmol·L^{-1}) in the absence or presence of Arc39 (10 µmol·L^{-1}) (n=3–6 each). e) Isolated perfused rat lungs were perfused for 10 min without (control) or with Arc39, before caveolae from endothelial cells were prepared and probed with antibodies for TRPC6 and β-actin. Shown are representative immunoblots and group data for densitometric measurements of band intensities normalised to β-actin (n=6 each). *: p<0.05 *versus* untreated controls; **: p<0.01 *versus* untreated controls; ***: p<0.001 *versus* untreated controls; #: p<0.05 *versus* sulprostone.

Our findings suggest that this Ca^{2+} channel is TRPC6, with G_i/PLC-dependent TRPC6 activation inducing Ca^{2+} entry and oedema. To our knowledge, G_i-dependent activation of TRPC6 has not been reported previously. However, activation of TRPC6 by PKC-derived DAG is well established [27]. While DAG may activate the channel directly [27], there is evidence that TRPC6 may also be activated by Src-family kinase dependent tyrosine phosphorylation, since TRPC6 activation or tyrosine phosphorylation in response to DAG analogues or epidermal growth factor could be blocked by the Src-family tyrosine kinase inhibitor PP2 [29, 30]. Here, we show that sulprostone enhanced tyrosine phosphorylation of TRPC6 in a PLC-dependent and PP2-sensitive manner. The functional relevance of this phosphorylation was

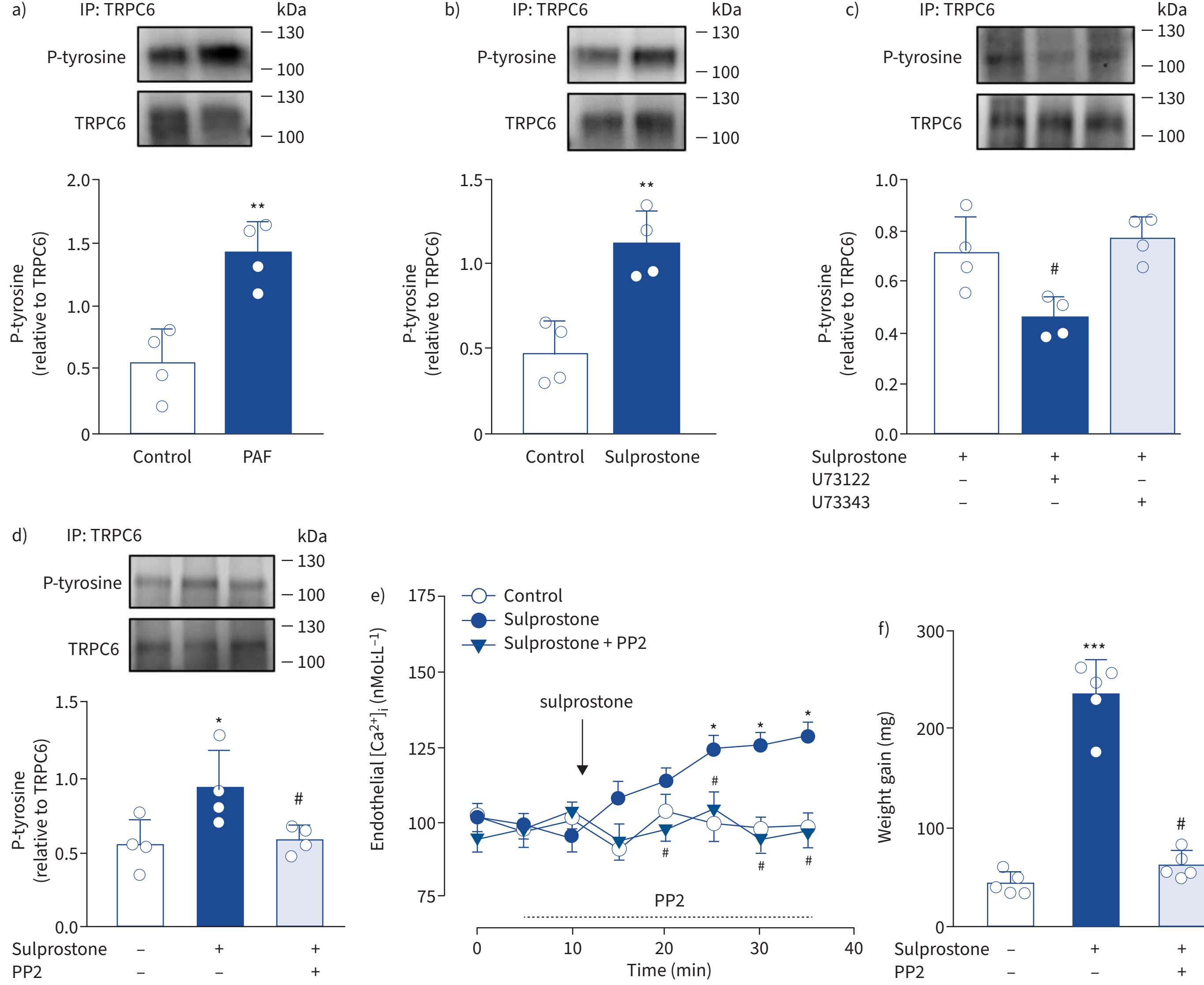

FIGURE 6 Platelet-activating factor (PAF) and sulprostone cause tyrosine phosphorylation of transient receptor potential canonical 6 (TRPC6). Caveolar fractions freshly isolated from rat lungs 10 min after infusion of either vehicle (0.9% sodium chloride (NaCl); control), a) PAF (5 nmol bolus) or b–d) sulprostone (1 µmol·L^{-1}) were immunoprecipitated (IP) with TRPC6 antibodies and precipitates were probed for phosphotyrosine (n=4 each). c) Lungs were pre-treated with either U73122 or U73343 (10 µmol·L^{-1} each). d) Lungs were pre-treated with either vehicle (control) or PP2 (20 µmol·L^{-1}). Shown are representative immunoblots and group data for densitometric measurements of band intensities normalised to TRPC6 (n=4 each). e) Mean endothelial $[Ca^{2+}]_i$ and f) weight gain in control and sulprostone-treated lungs in the presence or absence of PP2 (20 µM·L^{-1}; n=5 each). *: p<0.05 *versus* untreated controls, **: p<0.01 *versus* untreated controls; ***: p<0.001 *versus* untreated controls; #: p<0.05 *versus* sulprostone.

demonstrated by the effective inhibition of the sulprostone-induced increase in TRPC6 tyrosine phosphorylation, endothelial $[Ca^{2+}]_i$, and lung weight by PP2, yet the exact site of the phosphorylated tyrosine residues remains to be determined. Based on these findings we propose that activation of the EP_3/G_i/PLC axis leads to Src-family kinase-dependent tyrosine phosphorylation of TRPC6. Notably, EP_3–TRPC6 signalling may not only play a critical role in scenarios of PAF-mediated barrier failure (see later), but also relates to direct oedematogenic effects of PGE_2 as reported for the mouse paw [47].

PAF-induced lung oedema

Several lines of evidence based on our present data suggest that PAF-induced oedema formation is caused by at least three different signalling events that all culminate on TRPC6 (figure 7c). The first pathway has been described in detail here and involves EP_3-dependent tyrosine phosphorylation of TRPC6. The

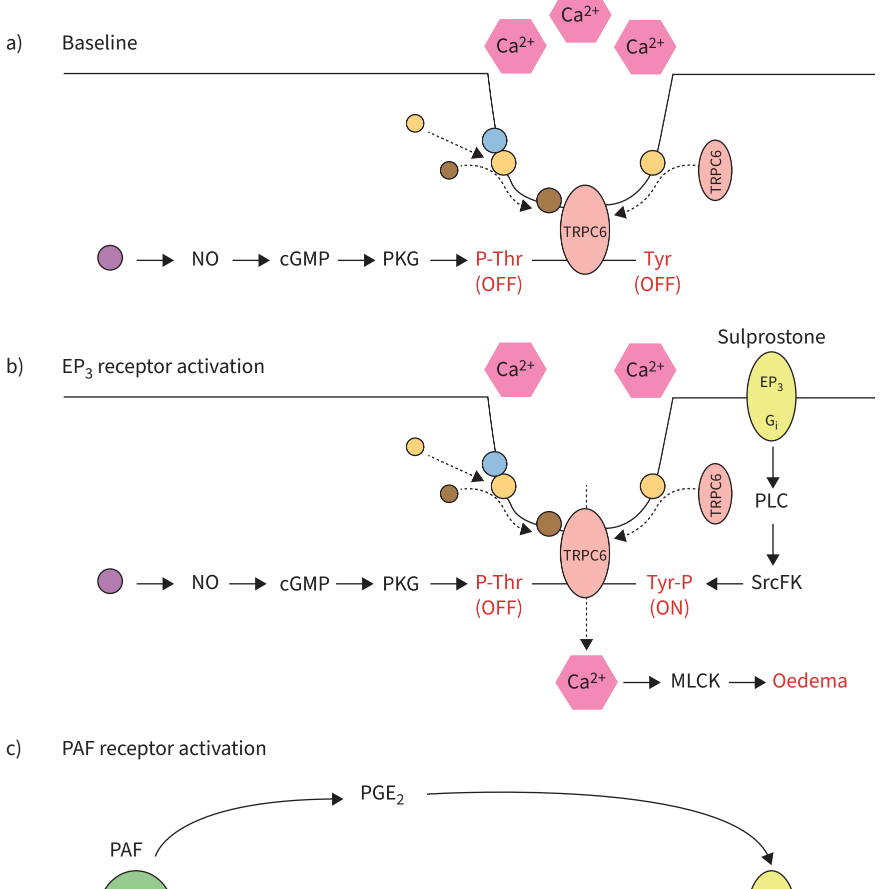

FIGURE 7 Schematic concept of the control of vascular permeability. a) At baseline, there is constant acid sphingomyelinase (ASMase)-dependent recruitment of caveolin-1 (cav-1) and transient receptor potential canonical 6 (TRPC6) to caveolae. However, TRPC6 is kept dormant by threonine (Thr) phosphorylation due to physiological nitric oxide (NO) production in endothelial cells. b) Activation of the prostaglandin E_2 (PGE_2) receptor 3 (EP_3) receptor leads to inhibitory G-protein (G_i)/phospholipase C (PLC)/Src-family kinase (SrcFK)-dependent tyrosine (Tyr) phosphorylation of TRPC6, which now becomes permeable. However, the resulting calcium (Ca^{2+}) influx is modest, since the inhibitory Thr phosphorylation is maintained and because only relatively few TRPC6 channels are available in the caveolae. c) Activation of the platelet-activating factor (PAF) receptor (PAFR) liberates PGE_2, which activates the EP_3 signalling pathway as described in b). In addition, PAF triggers two ASMase-dependent effects: 1) the recruitment of additional TRPC6 into caveolae and 2) a decrease in endothelial NO levels, which leads to the dephosphorylation of inhibitory threonine moieties on TRPC6. The combined effect of TRPC6 recruitment to caveolae, TRPC6 activation *via* tyrosine phosphorylation and TRPC6 disinhibition *via* threonine dephosphorylation results in a prominent Ca^{2+} signal that drives endothelial barrier failure and formation of overt lung oedema. For further details see the text. cGMP: cyclic guanosine monophosphate; eNOS: endothelial NO synthase; MLCK: myosin light chain kinase; PKG: protein kinase G.

importance of this pathway is illustrated by the findings that PAF increases production of PGE_2 [4], that antibodies directed against PGE_2 reduce PAF-induced oedema [4], that isolated perfused lungs from EP_3-deficient *Ptger3*$^{-/-}$ mice are protected from PAF-induced increases in endothelial $[Ca^{2+}]_i$ as well as oedema formation (this study) and that *Ptger3*$^{-/-}$ mice are protected from PAF-induced oedema *in vivo* [4].

The second pathway releases a physiological brake from TRPC6. At baseline, TRPC6 is inactivated by phosphorylation of what is most likely threonine-69 *via* a process that is mediated by NO, soluble guanylate cyclase and protein kinase G [48, 49]. Consistent with such a mechanism, we have shown recently that PAF leads to a sudden drop in endothelial NO levels while administration of exogenous NO attenuates PAF-induced oedema [12]. These findings already suggested that PAF should lead to diminished threonine phosphorylation of TRPC6 by blocking of NO production. Here (supplementary figure S7), we provide direct proof for this pathway by showing decreased threonine phosphorylation of TRPC6 in caveolae in response to PAF and L-NAME, respectively. Interestingly, dephosphorylation of TRPC6 appears to mainly serve as a sensitising mechanism, because inhibition of endothelial NO-production either by L-NAME or in eNOS-deficient mice produced a noteworthy phenotype, in that it increased vascular permeability, yet without causing overt lung oedema [12, 50].

The third pathway is dependent on the activation of ASMase above baseline levels in order to translocate more TRPC6 into caveolae. The link between ASMase, TRPC6 and pulmonary oedema formation has been firmly established [5, 51], yet how exactly ASMase leads to the recruitment of TRPC6 to caveolae remains to be shown. That notwithstanding, in the presence of PAF these newly recruited channels are now activated by tyrosine phosphorylation and concomitantly disinhibited by the removal of phospho-threonine residues, resulting in profuse Ca^{2+} influx, endothelial barrier failure, and ultimately, lung oedema formation.

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