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Resolvin E₁ normalizes contractility, Ca²⁺ sensitivity and smooth muscle cell migration rate in TNF-α- and IL-6-pretreated human pulmonary arteries

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Hiram R, Rizcallah E, Marouan S, Sirois C, Sirois M, Morin C, Fortin S, Rousseau E. Resolvin E₁ normalizes contractility, Ca²⁺ sensitivity and smooth muscle cell migration rate in TNF-α- and IL-6-pretreated human pulmonary arteries. Am J Physiol Lung Cell Mol Physiol 309: L776 –L788, 2015. First published August 28, 2015; doi:10.1152/ajplung.00177.2015.—Pulmonary hypertension (PH) is a rare disease in which pathophysiology is characterized by an increase in proinflammatory mediators, chronic endothelial dysfunctions, and a high migration rate of smooth muscle cells (SMC). Over the course of the last decade, various treatments have been proposed to relax the pulmonary arteries, none of which have been effective in resolving PH. Our hypothesis is that artery-relaxing drugs are not the long-term solution, but rather the inhibition of tissue inflammation, which underlies human pulmonary artery (HPA) dysfunctions that lead to abnormal vasoconstriction. The goal of the present study was to assess the anti-inflammatory effects of resolvin E₁ (RvE₁) with concomitant effects on SMC migration and on HPA reactivity. The role and mode of action of RvE₁ and its precursor, monoacylglyceride eicosapentaenoic acid were assessed on HPA under proinflammatory conditions, involving a combined pretreatment with 10 ng/ml TNF-α and 10 ng/ml IL-6. Our results show that TNF-α and IL-6 treatment induced hyperreactivity and Ca²⁺ hypersensitivity in response to pharmaco-mechanical stimuli, including 80 mM KCl, 1 μM phorbol 12-13-dibutyrate, and 30 nM U-46619. Furthermore, the proinflammatory treatment increased the migration rate of SMC isolated from HPA. The phosphorylation level of regulatory contractile proteins (CPI-17, MYPT-1), and proinflammatory signaling pathways (c-Fos, c-Jun, NF-κB) were also significantly increased compared with control conditions. Conversely, 300 nM RvE₁ was able to normalize all of the above abnormal events triggered by proinflammation. In conclusion, RvE₁ can resolve human arterial hyperreactivity via the resolution of inflammatory markers.

RvE₁; RVD₁; CPI-17; NF-κB; overreactivity; pulmonary hypertension

PULMONARY HYPERTENSION (PH) is a multifactorial disorder of the lung vasculature, characterized by an abnormal blood pressure of 25 mmHg due to severe remodeling of the arterial wall, pulmonary arterial vasoconstriction (6, 43, 45), and an overall marked component of inflammation-mediated damages (19, 43). Inflammation is, thus, suspected to be a key component of the induction and exacerbation of the pathogenesis of various chronic lung diseases (24, 40). Several proresolving compounds have recently been identified, including lipoxin A₄, which is derived from arachidonic acid (19), as well as D- and E-series resolvins, which are derived from specific omega-3 fatty acids, such as docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), respectively. Dietary supplementations with omega-3 polyunsaturated fatty acids (ω-3 PUFAs) have been shown to confer beneficial effects on many of these pathologies (4). Indeed, ω-3 PUFAs and several of their metabolites interact with specific receptors and several enzymes, thus limiting the conversion of arachidonic acid into proinflammatory eicosanoids (41), thereby inducing the generation of a range of specialized resolvin mediators (SPM). These mediators display positive effects on various intracellular and nuclear signaling pathways involved in the pathophysiology of a number of diseases, including cardiovascular (30) and neurodegenerative disorders (44). EPA and DHA have been described as essential fatty acids and are obtained through dietary sources such as fish oils (13). These compounds or their derivatives are able to modulate the expression and synthesis of various proinflammatory molecules such as TNF-α (49), NF-κB (23), and IL-1α, IL-1β, IL-6, and IL-13 (7). Hence, several of these cytokines are, in part, responsible for the remodeling of the arterial wall in PH, a central event that increases the severity of this lung disease (43).

Specifically, remodeling of the arterial wall involved increased migration and proliferation of endothelial cells, which results in an obstruction of the artery that enhances the vascular resistances and dramatically increases the blood pressure. Proliferation of pulmonary artery smooth muscle (PASM) cells, also called muscularization, severely increases the thickness of the artery wall. PASM cells in PH have been described to present various differential properties, such as dysfunctions of voltage-dependent potassium channels (29), enhanced expression of TMEM16A (10), and increased Ca²⁺ sensitivity (25), which, together, are associated with abnormal vasoconstriction.

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Ca\(^{2+}\) sensitization mechanisms involve specific agonists, such as endothelin-1 (ET-1) or thromboxane A2 (TXA2), which activate the specific regulatory proteins of the contractile machinery. Their stimulation induces the rise of cytosolic calcium concentration, but also an increase in Ca\(^{2+}\) sensitivity due to the activation of PKC/PI-17 and Rho-kinase pathways, which, in turn, allows it to maintain the phosphorylated state of the myosin light chain, resulting in an abnormal tension of the vascular smooth muscles of the media, which increases the tone of the pulmonary arteries (PA). This phenomenon is summarized under the term of “human pulmonary artery (HPA) hyperreactivity”.

Over the past decade, our laboratory has developed an in vitro model of induced proinflammatory conditions resulting in a hyperreactivity of HPAs (26, 27). Monoacylglycerides of DHA (MAG-DHA) and EPA (MAG-EPA), moreover, have been synthesized and used as neutral precursors of DHA and EPA to assess their ability to play beneficial effects on this HPA model of induced hyperresponsiveness, in vitro.

Recent results have shown that the treatments with MAG-DHA and its derived products via 15-lipoxygenase (15-LOX) and 5-lipoxygenase (5-LOX), for example, RvD1 (a D-series resolvins), were able to normalize HPA hyperresponsiveness induced by pretreatment with proinflammatory compounds (TNF-\(\alpha\) and IL-6) and by ET-1, a potent vasoconstrictor agent (14).

EPA metabolism has also been documented to yield a number of anti-inflammatory compounds, more specifically, when aspirin was used to block cyclooxygenase 2 (COX-2) and produce aspirin-triggered compounds (36). As a matter of fact, EPA metabolism by COX-2 favors the production of some anti-inflammatory (PGE3, LTB5) and several proinflammatory mediators (PGE2, LTB4) (8). However, under the condition in which aspirin blocks COX-2 activity, EPA was shown to be metabolized into 18 R-HEPE, which, in turn, was metabolized by 5-LOX into E-series resolvins, including the 5S,12R,18R-trihydroxy-6Z,8E,10E,14Z,16E-ecosapentaenoic acid, also called resolvin E1 (RvE1) (46). RvE1 has already been reported to attenuate inflammatory pain (48) and has been identified in plasma; its anti-inflammatory action has been confirmed via its specific receptor, chemerin receptor 23 (ChemR23) (11). However, it is not clear how RvE1 could impact the arterial tone. Moreover, as described above, the migration and proliferation of the PASM cells are crucial during the arterial remodeling, which leads to abnormal vasoconstriction. The role of RvE1 and its receptor ChemR23 on these events are not well understood yet, in the context of PH.

We hypothesized that ChemR23 may be expressed in human pulmonary arterial smooth muscle cells and that a proinflammatory status triggered by the combination of TNF-\(\alpha\) and IL-6 in vitro on HPA could, in turn, be normalized by curative treatments with RvE1. The aim of the present work was, therefore, to demonstrate that RvE1 and its precursor MAG-EPA induce a powerful resolution of inflammatory markers, as well as normalize HPA hyperresponsiveness and PASM cell migration through the inhibition and/or modulation of various proinflammatory intracellular signaling pathways.

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**Materials and Methods**

**Drugs and Chemical Reagents**

Resolvin E1 (RvE1), U-46619, and MK886 were obtained from Cayman Chemical (Ann Arbor, MI), Serotonin (5-HT), IL-6, type IV collagenase, and type IV elastase were obtained from Sigma-Aldrich (St. Louis, MO). Endothelin-1, phorbol 12–13-dibutyrate (PDBu), Anti-CPI-17, anti-phospho-CPI-17, anti-phospho-c-Jun (Ser-63), anti-phospho-c-Fos (Ser-32), anti-COX-2, anti-IkBx, anti-NF-\(\kappa\)B, and anti-phospho-NF-\(\kappa\)B were purchased from Cedarlane (Burlington, ON, Canada). TNF-\(\alpha\), anti-TNF-\(\alpha\), and anti-TMEM16A were purchased from Abcam (Toronto, ON, Canada). Antibodies against PKC\(\zeta\), MYPT-1, and P-MYPT-1 (phosphorylated form of MYPT-1 on Thr-696) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Eicosapentaenoic acid monoacylglyceride (MAG-EPA) was synthesized and purified by SCF-Pharma (Rimouski, Québec, Canada). DMEM:F-12 and penicillin-streptomycin were obtained from Gibco Invitrogen (Burlington, Ontario, Canada).

**Culture of Human Pulmonary Arteries**

This study was approved by our institutional Ethics Committee (protocol no. 05-088-S1–R4). Patients hospitalized for lung carcinoma were recruited after providing informed consent, and lung tissue samples were obtained from the Service of Thoracic Surgery via the Department of Pathology (12). Tissue samples confirmed as "tumor-free" by the pathologist were transferred to our laboratory bathed in fresh sterile Krebs solution at pH 7.4. Given that recruited patients did not have pulmonary hypertension, tissue samples were, hence, deemed to be obtained from control patients (n= 49). HPA were dissected under a binocular microscope, and arterial segments of 4 mm in length and 0.5–0.8 mm in diameter were cultured for 24 h in DMEM: nutrient mixture F-12 (DMEM-F12) containing 1% penicillin/streptomycin (Pen-Strep). To mimic proinflammatory conditions, HPA were cultured in either control conditions (untreated) or with 10 ng/ml TNF-\(\alpha\) and 10 ng/ml IL-6, in the absence or presence of 300 nM RvE1, for 24 h at 37°C in 5% CO\(_2\) (12). The optimal concentration to observe an effect has been chosen, according to previous reports that have performed concentration-response curve experiments to assess the effects of resolvins (38, 46).

**Isolation of Pulmonary Arterial Smooth Muscle Cells**

HPA segments were incubated for 1 h at 37°C under 5% CO\(_2\) in HBSS (GIBCO) supplemented with 1% Pen-Strep, 0.2% type IV collagenase, and 0.05% type IV elastase (29). The quality of the smooth muscle cell preparation was assessed exactly, as previously described (29) and in which 98% of cells were positive for \(\alpha\)-smooth muscle actin, a fairly specific biomarker of vascular smooth muscle cells.

**Cell Migration Assay**

Migration was assayed in a transwell filter system (6.5-mm diameter, 8-µm pore size; Costar, Corning, NY). PASM cells were seeded in upper transwells (20 × 10\(^3\) cells/well) in 160 µl DMEM/F12 in the absence of FBS or other agents, and incubated at 37°C under 5% CO\(_2\). In the lower chamber, 600 µl of DMEM/F12 + 1% PEN/StREP + 5% FBS were then added in the absence (control) or presence of TNF-\(\alpha\) + IL-6 (10 ng/ml of each cytokone), both conditions in the absence or presence of RvE1 (according to the combined treatment described below in the RESULTS). After a 24-h incubation period under the various experimental conditions, cells in the upper area of the membrane were removed with a dry Q-tip, while the cells that had migrated to the lower surface were fixed in absolute methanol and stained with 0.25% crystal violet for cell counting under a confocal microscope. Averages of five random fields were analyzed per condition.
Isometric Tension Measurements

Tension measurements were performed on prepared HPA rings, as previously described (28). A basal 0.8-g tension was applied to each cultured ring. Pharmacologically induced contractile responses by specific agonists, Resolvins and MAG-EPA were assessed using transducer systems coupled to Polyspy software (Grass-Astro-Med, West Warwick, RI) enabling data acquisition and analysis. The amplitudes of maximum tensions are expressed for a given agonist concentration (1 μM PDBu or 30 nM U-46619) normalized to the control response to 80 mM KCl for each tested HPA ring.

β-Escin Permeabilization and Ca²⁺ Sensitivity

Arterial rings were mounted in organ baths, as previously described (37). Active tone developed by the permeabilized arterial rings were subsequently measured at 37°C according to free-Ca²⁺ concentrations expressed in terms of pCa (pCa = −log [Ca²⁺]). Reproducible concentration-response curves to free Ca²⁺ concentrations induced by step increases in free Ca²⁺ (from pCa = 9.0 to 5.3) indicated successful permeabilization.

SDS-PAGE and Western Blot Analyses

Western blots were performed using specific antibodies against PKC α, CPI-17, P-CPI-17, MYPT-1, P-MYPT-1, TMEM16A, c-Fos, c-Jun, COX-2, IkBa, NF-kB, VEGF, MMP9, TNF-α and β-actin proteins. Blot immunostainings were revealed on Kodak film and digitized by using a Xerox GPD PS V3.4377.6.0 set to 600 dpi and analyzed by use of Image J software (28). Photomicrographs were taken with the RS image software 1.9.2 on a Nikon TE-300 inverted microscope and framed with Photoshop CS5 12.0 before insertion into a PowerPoint file (MS Professional Plus 2010, V14.0.7).

Data presentation and Statistical Analysis

Results are expressed as means ± SE, with n indicating the number of rings for each set of experiments and N the number of subjects involved. Western blot analyses are depicted with n indicating the number of experiments. Statistical analyses were performed using a Student’s t-test or one-way or two-way ANOVA, as appropriate, with Sigma Plot 12.0 and SPSS 14.0 software (SPSS-Science, Chicago, IL). EC₅₀ values were determined from data curve fittings performed with Sigma Plot 12.0 algorithm. Differences were considered statistically significant when *P < 0.05.

RESULTS

Effect of MAG-EPA on HPA-Induced Hyperresponsive and Ca²⁺ Hypersensitivity

PDBu is a permeable compound previously shown to activate PKC-dependent pathways in vascular smooth muscle cells and to increase Ca²⁺ sensitivity and tone (18). Figure 1A represents the cumulative concentration-response curves (CCRC) to PDBu of HPA in control (no treatment and cultured for 24 h) or treated conditions, either with 10 ng/ml TNF-α + 10 ng/ml IL-6 alone or combined with 1 μM MAG-EPA or 1 μM MAG-EPA + 100 μM aspirin. A 24-h pretreatment with TNF-α combined with IL-6 induced a significant overreactivity (+62% of normalized tension) to PDBu, with an apparent EC₅₀ value of 0.8 μM, comparatively to 1.2 μM in control conditions. Treatment with 1 μM MAG-EPA or with 1 μM MAG-EPA + 100 μM aspirin significantly reduced the overreactivity induced by the proinflammatory treatment, decreasing the apparent EC₅₀ values to 1.1 μM and 1.4 μM, respectively. To ascertain the changes in Ca²⁺ sensitivity of myofilaments upon proinflammatory or proresolving treatments, the tonic responses of β-escin-permeabilized HPA were recorded and analyzed. Figure 1B displays the Ca²⁺ sensitivity of myofilaments following the specific treatments described above in permeabilized arteries as a function of pCa. CCRC to free Ca²⁺ concentrations revealed that 10 ng/ml TNF-α + 10 ng/ml IL-6 induced a significant increase in Ca²⁺ sensitivity [corresponding to a leftward shift of the pCa response curve (open circles)] compared with control conditions (solid circles). This observation was consistent with the recorded apparent EC₅₀ values of 0.17 μM and 1.12 μM for treated and untreated (control) conditions, respectively. The combined treatment of TNF-α with either 1 μM MAG-EPA or 1 μM MAG-EPA +
100 μM aspirin normalized the Ca²⁺ sensitivity initially induced by the proinflammatory condition, with EC₅₀ values of 1.07 μM and 1.10 μM, similar to the control EC₅₀ value of 1.12 μM. These consistent data suggest that MAG-EPA and its aspirin-triggered metabolites normalize HPA-induced overactivity and Ca²⁺ sensitivity induced by proinflammatory conditions.

Effects of MAG-EPA in the Presence of Specific Inhibitor and Receptor Antagonists

Aspirin (acetyl salicylic acid, ASA) has previously been reported to modify the metabolism of EPA and to promote proresolving metabolites from MAG-EPA (5). However, ASA alone basically has no significant effects on control conditions (5, 46). In the following set of experiments, MK886 was used to inhibit the 5-lipoxygenase activating protein enzyme while two specific peptide blockers were used to block the resolving receptors GPR120 [the EPA receptor (31)] and ChemR23 [the receptor for chemerin and resolvin E1 (11)], respectively.

Figures 2, A and B show that in response to distinct stimuli, either 80 mM KCl or 30 nM U-46619, HPA previously treated for 24 h with 10 ng/ml TNF-α + 10 ng/ml IL-6 displayed a significant overreactivity compared with controls (untreated). While 1 μM MAG-EPA alone had no effect in the control condition, the same concentration of MAG-EPA in the presence of 10 ng/ml TNF-α + 10 ng/ml IL-6 during 24 h significantly decreased the overreactivity (~62%). This tonic response was further normalized when 1 μM MAG-EPA was combined with 100 μM aspirin despite the presence of TNF-α and IL-6.

To block 5-LOX activation and EPA metabolite production, 1 μM MK886 was used in combination with 1 μM peptide blocker against GPR120 under proinflammatory conditions in the presence of 1 μM MAG-EPA. Despite a small, but significant, decrease in overreactivity following blockade of GPR120, there was a significant loss of resolving effect in the presence of the specific Chemr23 blocking peptide. Together, these results suggest that MAG-EPA or one of its metabolites displays coherent resolving effects that are partially antagonized by the use of GPR120 and ChemR23 receptor antagonists. These latter findings, furthermore, suggest that an EPA metabolite (such as RvE1) likely plays a key role in this resolving process.

RvE1 Inhibition of HPA-Induced Hyperresponsiveness and Ca²⁺ Hypersensitivity

The effects of RvE1 were next quantified in vitro on HPA in control and pretreated conditions. In Fig. 3A, while normalized responses to 30 nM U-46619 were observed under control conditions, 24-h pretreatment with 10 ng/ml TNF-α + 10 ng/ml IL-6 induced an overreactivity (solid column), which was largely prevented by 300 nM RvE1 treatment. Of note, 300 nM RvE1 alone had no effect on control conditions. Furthermore, in TNF-α and IL-6 pretreated HPA, the presence of 300 nM RvE1 and 300 nM ChemR23-blocking peptide largely abolished the proresolving effect induced by RvE1.

CCRC to free Ca²⁺ concentrations in Fig. 3B reveal increased Ca²⁺ sensitivity after pretreatment of HPA with TNF-α plus IL-6 compared with control (with EC₅₀ values of 0.09 μM and 1.09 μM, respectively), while 300 nM RvE1
Fig. 3. Effect of resolvin E1 under control and proinflammatory conditions in the absence and presence of Chem23 blocking peptide. A: bar graph displaying the mean contractile amplitudes induced by 30 nM U-46619 on 24-h cultured HPA either in the Control condition or pretreated with 10 ng/ml TNF-α + 10 ng/ml IL-6, 300 nM RvE1 alone, 10 ng/ml TNF-α + 10 ng/ml IL-6 + 300 nM RvE1, or 10 ng/ml TNF-α + 10 ng/ml IL-6 + 300 nM RvE1 + 300 nM ChemR23 peptide blocker (n = 18, *P < 0.05). B: Ca2+ sensitivity of myofilaments on HPA permeabilized with 50 μM β-escin after 24 h in culture under the same conditions as described above (n = 12, *P < 0.05). Reduced Ca2+ sensitivity induced by the proinflammatory pretreatment with an apparent EC50 value of 1.4 μM, similar to the EC50 value (1.09 μM) observed in control conditions. However, when RvE1 was combined with the ChemR23 blocking peptide, no change in Ca2+ sensitivity was observed compared with the effect of proinflammatory treatment (TNF-α plus IL-6), as confirmed by the apparent EC50 values 0.13 and 0.09 μM, respectively. These data reveal that RvE1, via its specific receptor ChemR23, can prevent TNF-α and IL-6-induced overreactivity and hypersensitivity in HPA in vitro.

Role of RvE1 on the Regulation of the Contractile Machinery of PASM Cells Cultured under Proinflammatory Conditions

Regulation of the contractile machinery is central for the normal activity of pulmonary artery smooth muscle (PASM) cells from HPA. Overreactivity and enhanced Ca2+ sensitivity are the result of a dysregulation of pharmaco-mechanical coupling and putative changes in phosphorylation levels of regulatory proteins involved in the control of contraction-relaxation processes. In Fig. 4A, Western blot analyses performed on microsomal fractions from PASM cells demonstrate that TMEM16A, a calcium-dependent chloride channel and recognized marker of pulmonary hypertension, was highly expressed in PASM cells cultured in the presence of 10 ng/ml TNF-α + 10 ng/ml IL-6 (lane 2) comparatively to control (lane 1). However, the addition of 1 μM MAG-EPA (lane 3) or 300 nM RvE1 (lane 4) inhibited the enhanced expression of TMEM16A induced by proinflammatory conditions. Note that selective blocking peptides for ChemR23 and GPR32 abolished the effects of RvE1 and RvD1, respectively (Fig. 4A lanes 5 and 6).

Figure 4B represents a Western blot analysis of the detection of PKCζ performed on cytosolic fractions from PASM cells treated under the same experimental conditions as described above. Compared with control, TNF-α + IL-6 induced an increase in expression of PKCζ, while MAG-EPA or its exogenously added metabolite, RvE1, largely inhibited the induced overexpression. Conversely, the independent blocking of respective EPA and RvE1 receptors induced a loss of the inhibitory effect of MAG-EPA and RvE1.

The phosphorylation levels of CPI-17 and MYPT-1 were next quantified on cytosolic fractions from the same experimental conditions and compared with control. TNF-α + IL-6 induced a major increase in phosphorylation levels of both CPI-17 and MYPT-1 in PASM cells (Fig. 4, C and D, respectively), which was well correlated with the extensive vasoconstriction of HPA under the same proinflammatory conditions (Fig. 1A). Pretreatments with either MAG-EPA or RvE1 basically normalized the phosphorylation levels of CPI-17, whereas the blockade of EPA or RvE1 receptors resulted in a loss of this inhibitory effect. Altogether, these data suggest that proinflammatory treatments give rise to an important and abnormal increase in the activation of the contractile machinery of PASM cells which, in turn, is responsible of the abnormal increase in HPA vasoconstriction. This inflammatory-induced event is conversely inhibited and resolved in the presence of proresolving mediators such as RvE1 and its precursors.

Comparative Analysis of RvE1 and RvD1 Treatments on Proinflammatory Markers

The activation of signaling pathways involved in the inflammation process is relatively complex and involves various proteins and nuclear factors. Among these, NF-kB, which is under the control of TNF-α receptors, is known to be phosphorylated prior to its translocation into the nucleus, where it activates the transcription of several genes, including VEGF or MMP9, both of which participate in artery wall remodeling (27). Figure 5A demonstrates that, compared with control (untreated), the phosphorylation level of NF-kB was increased in the nuclear fraction of PASM cells treated for 24 h under proinflammatory conditions with 10 ng/ml TNF-α + 10 ng/ml
IL-6. In contrast, 300 nM RvE1 or 300 nM RvD1 (a D-series Resolvin) abolished the increase in normalized phosphorylation ratio. Moreover, the specific inhibition of RvE1 or RvD1 binding on their respective receptors resulted in a complete loss of their apparent resolving properties.

IκBα plays an important role in the ability of NF-κB to be phosphorylated and translocated into the nuclei. Of interest, normalized IκBα levels in the cytoplasm depicted in Fig. 5B was inversely proportional to p-NF-κB detection in the nuclei when assessed under the same conditions described in Fig. 5A.

One of the most relevant inhibitors of proinflammatory effects is PPARγ 28. The expression of PPARγ was detected in the homogenates from HPA and expressed as a function of β-actin (Fig. 5C, top). Upon proinflammatory treatment with 10 ng/ml TNF-α + 10 ng/ml IL-6, the relative detection level of PPARγ was significantly decreased in the cytoplasm (Fig. 5C, bottom). However, this reduced detection was significantly enhanced to values similar to that in controls upon addition of 300 nM RvE1 or 300 nM RvD1 to the proinflammatory condition. Conversely, the presence of specific peptide blockers against both ChemR23 and GPR32 receptors eradicated the beneficial effects of their respective RvE1 or RvD1 ligands.

Lastly, COX-2, which is usually expressed in the endoplasmic reticulum membrane and nuclear envelope, becomes inducible during inflammation to produce prostaglandin intermediates involved in abnormal cell growth and inflammation severity. As depicted in Fig. 5D, Western blot analysis of microsomal fractions obtained from HPA reveals that, compared with the control, the expression level of COX-2 was significantly increased in intracellular compartments following 24 h proinflammatory treatment with 10 ng/ml TNF-α + 10 ng/ml IL-6. However, in the presence of either RvE1 or RvD1, COX-2 detection...
was basically abolished, whereas the blockade of ChemR23 and GPR32 receptors with specific peptide blockers antagonized the respective effect of RvE1 and RvD1 (Fig. 5D). Taken together, these data demonstrate that, under stringent proinflammatory conditions, E- or D-series resolvins are instrumental in reversing these inflammatory effects, which are consistently antagonized by the specific blocking peptides.

**RvE1 and RvD1-Controlled Regulation of Inflammatory Marker Expression in Smooth Muscle Cells**

Phosphorylation of c-Fos and c-Jun is generally increased under inflammatory conditions (1). Together, these two protein subunits form the AP-1 complex, which translocate into the nucleus and activates the transcription of specific genes such as MMP9 (16) and VEGF (25), both of which are known to promote pulmonary artery wall remodeling and an exponential increase in cell migration (15). As seen in Fig. 6, A and B, proinflammatory conditions (TNF-α + IL-6) induced an enhanced phosphorylation of c-Fos and c-Jun in the nuclear fraction, which was completely abolished by the addition of 300 nM RvE1 or RvD1. However, upon inclusion of specific blocking peptides, RvE1 and RvD1 were unable to bind to their respective receptor, resulting in the loss of phosphorylation of both protein subunits.

**Effect of RvE1 on PASM Cell Migration Rate**

The migration rate of primary PASM cells isolated from HPA was assessed under six experimental conditions fol-
lowing 24-h treatment: Control (untreated) proinflammatory conditions with 10 ng/ml TNF-α and 10 ng/ml IL-6; proresolving conditions with 10 ng/ml TNF-α and 10 ng/ml IL-6 supplemented with 1 μM MAG-EPA or 300 nM RvE1; and finally with 10 ng/ml TNF-α and 10 ng/ml IL-6 in the presence of 1 μM GPR120 peptide blocker or 300 nM ChemR23 peptide blocker. Representative images of the lower side of the transwells (Fig. 7A) and the corresponding quantitative analysis based on crystal violet colorimetry (Fig. 7B) reveal that proinflammatory treatment induced a large increase in the migration rate of PASM cells compared with control. Treatment with either MAG-EPA or RvE1 significantly limited the cell migration rate similar to that observed in control conditions. However, the blockade of either the EPA receptor by the GPR120 peptide blocker or the RvE1 receptor by the ChemR23 peptide blocker resulted in a loss of this inhibition of cell migration (Fig. 7B). These results suggest that RvE1 and its precursor are able to normalize the abnormal migration rate triggered by the proinflammatory condition and typically observed during HPA wall remodeling.

Effect of RvE1 and RvD1 on the Overexpression of VEGF and MMP9 in Inflammatory Conditions

During inflammation, the activation of intracellular proinflammatory pathways leads to the activation of the transcription of various genes that encode for proteins involved in the control of multiple events leading to tissue dysfunctions, including abnormal cell growth, cell differentiation, cell migration (i.e., VEGF), and degradation of the extracellular matrix (i.e., MMP9). These events play important roles in the pathology of pulmonary artery wall remodeling in an experimental or clinical context of PAH. Immunoblotting analyses of PASM cells were, therefore, performed to assess the expression levels of VEGF (Fig. 8A) and MMP9 (Fig. 8B) following a 24-h culture under identical experimental conditions, as described above. Results revealed that proinflammatory treatment increased the expression levels of both VEGF and MMP9 compared with control, whereas RvE1 or RvD1 largely inhibited this induced overexpression. This inhibition was, however, lost when specific peptide blockers of resolving receptors were combined to their ligand in the presence of TNF-α and IL-6. These data clearly demonstrate that E or D series resolvins modulate the expression of VEGF, as well as MMP9 in primary isolated smooth muscle cells derived from HPA.

DISCUSSION

In the present study, we investigated the ability of resolvins to prevent the abnormal increase in pharmacological reactivity and Ca2+ sensitivity induced by proinflammatory conditions on short-term cultured human pulmonary arteries. Our experimental design, aimed at enhancing the contractile properties of HPA using a combined treatment of TNF-α and IL-6 in the absence or presence of RvE1, enabled us to show that the normal functional, as well as biochemical properties, were largely maintained upon either RvE1 or RvD1 treatment in the presence of proinflammatory conditions. These lipid mediators, derived from EPA and DHA metabolism, represent new bioactive trihydroxylated omega-3 derivatives, which have previously been reported to reverse dermal inflammation, dendritic cell migration, and interleukin production (11). Results herein further demonstrate that MAG-EPA, an EPA donor and RvE1 precursor, is also able to prevent human arterial overreactivity, enhanced Ca2+ sensitivity, and PASM cell migration, suggesting a putative role of these compounds in limiting media remodeling typical of pulmonary hypertension. Of importance, these proresolving conditions appear to override proinflammatory signaling.
presence of proinflammatory mediators were normalized upon addition of the ω-3 derivatives RvE1 or MAG-EPA, TNF-α and IL-6, which bind to their specific receptors TNF-R and CD126, respectively, induce an activation of the PKC ζ pathway leading to the phosphorylation of CPI-17. Thereafter, phosphorylation of MYPT1 is increased, and contraction of PASM cells is enhanced. In the presence of pharmacological stimuli, additional effects could be observed. For instance, TXA2 (U-46619) activates an IP3-induced increase of cytosolic Ca2+ concentration. TXA2 also activates Rho A-Rho kinase pathway, while PDBu directly stimulates PKC/CPI-17 pathway. Together, these cellular events contribute to increase the arterial reactivity in response to proinflammatory treatments.

The resulting effect is an overreactivity of HPA, which has often been targeted in PH treatments. However, even if vessel relaxations were observed, such treatments failed to resolve the hypertension, likely, in part, because these treatments did not specifically tackle the inflammatory process. Our current data are consistent with the hypothesis that inflammation plays a central role in the induction of PH and may, thus, contribute in increasing the severity of this disease. Herein, we demonstrate that under proinflammatory conditions, RvE1, as well as its precursor MAG-EPA, is able to normalize the arterial tone and the phosphorylation level of the proteins that regulate the contractile machinery of PASM cells. Interestingly, these effects were either partially or largely abolished when RvE1 and EPA receptors were blocked with specific antagonists. The direct link between inflammatory conditions and overreactivity of HPA, while often suggested, has never been clearly assessed. This study confirms that inflammation can lead to an increased reactivity and that RvE1 and MAG-EPA can prevent the abnormal pharmacological reactivity. The present data are consistent with previous reports assessing the effect of MAG-DHA in an in vivo rat model of monocrotalin-induced pulmonary hypertension (27) or the effects of RvD1 on tissue and intracellular signaling in HPA (14).

**Proresolving Effects of RvE1 in HPA**

The combined use of proinflammatory molecules such as TNF-α and IL-6 leads to the activation and upregulation of various signaling pathways in PASM cells (3). The present data show that the detection of their functional transducers, such as PKC ζ, P- CPI-17, P-MYPT1, and TMEM16A are downregulated in the presence of RvE1 and MAG-EPA.

**Role of RvE1 and RvD1 on NF-κB pathway: the combined stimulation of TNF-R1.** and CD126 induces the activation of IKKα, which phosphorylates IκBα, which, in turn, phosphorylates NF-κB in the cytosol. Phospho-NF-κB translocates into the nucleus to promote specific gene transcriptions. The canonical role of NF-κB has not been assessed in PASM cells in the context of PH. However, in a rat model of the disease, NF-κB was found to activate FGF2 and monocyte chemotactic protein-1, thereby promoting PASM cell migration and proliferation (33), both events of which are central and typical in pulmonary artery hypertension. Our results clearly demonstrate that nanomolar concentrations of RvE1 and RvD1 (16) down-regulate the phosphorylation level of NF-κB in HPA.

**Role of RvE1 and RvD1 on AP-1 signaling pathway.** TNF-α-mediated activation of AP-1 has been reported to play an essential role in the genesis of PH, mainly via the accumulation

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**Fig. 7.** Migration rate of human PASM cells under proinflammatory and proresolving conditions. A: photomicrographs of migration assays performed on HPA smooth muscle cells from the underside of a Boyden chamber membrane. Cells were seeded and allowed to migrate during 24 h from the upper side to the underside of the transwell. The upper side contained DMEM-F12 with 0.3% FBS and 1% PEN/STREP alone (Control) (1) or supplemented with either 10 ng/ml TNF-α and 10 ng/ml IL-6 (2) alone or combined with 1 μM MAG-EPA (3); 300 nM RvE1 (4); 1 μM MAG-EPA + 1 μM GPR120 peptide blocker (5); or 300 nM RvE1 + 300 nM ChemR23 peptide blocker (6). (B) Bar graphs displaying the quantitative analysis of migrating cells as a function of the conditions described above (*n = 20, *P < 0.05).
Moreover, Steiner et al. (42) suggested that the upregulation of cytokines such as IL-6 contributes to the development of PH. Accordingly, when TNF-R and CD126 are stimulated, the combined activation of PKCζ and STAT3 pathways leads to activation of ERK and JNK (Fig. 9) to phosphorylate c-Fos and c-Jun, respectively (14). A complex called AP-1 is, henceforth, formed and translocated into the nucleus. AP-1, thereafter, binds to specific DNA sequences to promote the transcription of various genes involved in matrix degradation, collagen expression, cell growth, and migration. Our current data demonstrate that nanomolar concentrations of either RvE1 or RvD1 are able to lower the detection of AP-1 subunits into the nucleus of PASM cells, a key observation further explaining the events occurring during the PASM cell migration assay and during hyperplasia of the media in chronic PH (45).

Resolvins accentuate the effects of anti-inflammatory proteins: example of PPARγ. In 2003, Ameshima et al. (2) reported that PPARγ expression is decreased in the lung of patients with PH. The anti-inflammatory properties of PPARγ have been demonstrated in which the latter reduces cytokine production by monocytes (17) and inhibits NF-κB and AP-1

![Fig. 8. Relative detection of VEGF and MMP9 in human pulmonary arteries. Western blot analyses were performed on HPA homogenates, using monoclonal antibodies against VEGF and MMP9, respectively. Both transcription products are typical markers of pulmonary hypertension, as well as proinflammatory conditions (5). HPA were pretreated for 24 h, under the same conditions as described in Figs. 5 and 6. Immunoactive bands were quantified and expressed as relative VEGF/β-actin and MMP9/β-actin ratios (n = 6; *P < 0.05).](http://ajplung.physiology.org/doi/10.1152/ajplung.00177.2015)
transcription pathways. Conversely, the lack of PPARγ expression is associated with abnormal endothelial cell growth (due an increase in VEGF) (2), inhibition of apoptosis and angiogenesis, and increased cell migration (due to accumulation of MMP-9) (21). Our current data are consistent with the results of these studies, as witnessed by the substantial reduction in PPARγ expression under proinflammatory conditions (TNF-α, IL-6), whereas this downregulation is reversed in the presence of RvE1 and RvD1. These observations suggest that all proteins for which expression depends on the activation of NFκB and/or AP-1 are also likely to be downregulated in the presence of RvE1 and RvD1.

These effects induced by resolvins could also result in a decrease or halting of arterial wall remodeling putatively induced by proinflammatory signaling pathways. While further studies are needed to assess this hypothesis, the present findings provide new insight regarding the expression of key proteins involved in arterial wall hyperplasia.

Resolvins Curb the Expression of Proteins Involved in Arterial Wall Remodeling in PH

The transcription rate of various proteins involved in a large range of disorders is increased under proinflammatory conditions. For example, COX-2 leads to the synthesis of prostaglandins from arachidonic acid (36). VEGF triggers an increase and abnormal growth of endothelial cells concomitant with an enhanced migration of macrophages, monocytes, PASM, and endothelial cells (33). MMP9 is involved in the deterioration of the extracellular matrix (9) and typically induces pathological arterial wall remodeling and hypertrophy due to activation of cell migration (20). The present data attest that treatment of HPA with RvE1, as well as RvD1 prevents and blocks the detection of these biomarkers of arterial wall remodeling. Data also confirm that during inflammation, the ensuing tissue modifications promote the severity of this disease, leading to obstruction of the arteries and enhancement of pulmonary arterial tension. Consistent with these observations and according to previous reports, whereby PASM cell migration plays a crucial role in PH (33), assessment of PASM cell migration rate in this study confirmed the beneficial impact of resolvins under proinflammatory conditions. Indeed, PASM cell migration was increased in response to inflammation and is likely maintained in PH, hence, contributing to the severity of the disease. However, both RvE1 and its precursor MAG-EPA, via the activation of their respective receptors, were able to curb the migration of PASM cells triggered by inflammation. Further studies are, nonetheless, needed to clarify the pathways involved in this regulation. For example, Ca2+-dependent aquaporin called AQP1, which is present in the cell membrane of PASM cells, has been reported to be involved in this mechanism (22).

Limitations of the Study

The present study was essentially performed on lung samples obtained from patients devoid of PH. Pulmonary arteries and isolated cells from these patients were, therefore, treated in vitro to develop the characteristics of the disease targeted in this study (inflammation, overreactivity, and Ca2+ hypersensitivity). It would obviously be of interest to perform the present analysis directly on lung samples from patients with PH. Furthermore, it would be of interest to investigate whether the expression of ChemR23 and GPR32 would be altered on lung tissues affected by PH. However, such tissues are very rare and difficult to obtain. For the time being, we do not have access to fresh tissues from PH patients. However, efforts are currently under way for future collaborations with the Tissue Data Bank of the Respiratory Health Network of the FRQS to overcome this issue. Lastly, studies from Potus et al. (34) have reported a key role of miR204 in healing PH in a mouse model, while Recchiti et al. have reported that RvD1 regulates specific miRNAs involved in the resolution of inflammation (35). It would, thus, be of key interest to investigate the effects of RvE1 on miRNA expression under proinflammatory conditions in HPA. Moreover, the inhibitory role of short-chain fatty acids on histone deacetylase was reported by Vinolo et al. (47) in 2011. According to these observations, further studies would be required to explain the wide range of RvE1 and RvD1 effects observed on PASM cells.

Conclusion

The present study attests that RvE1 and RvD1 prevent HPA overreactivity in response to pharmacological challenges under proinflammatory conditions. These findings demonstrate that inflammation is a process that can induce tonic responses, which are potentially relevant in the context of PH. These observations open new perspectives about the key role of RvE1, which could be further assessed in future studies using a well-known model of MCT-induced PH on rats in vivo, following a curative experimental protocol. RvE1 and its analogs display resolving effects, which suggest that these SPM (11) could be used as new therapeutic tools in the prevention and successful treatment of proinflammatory induced overreactivity in HPA.

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DISCLOSURES

Samuel Fortin is the owner and CEO of SCF Pharma, which has an exclusive worldwide license of the patented compositions and uses of MAG-DHA. Otherwise, the authors declare no conflicts of interests.

AUTHOR CONTRIBUTIONS

Author contributions: R.H. and E. Rousseau conception and design of research; R.H., E. Rizcallah, S.M., C.S., M.S., C.M., and S.F. performed experiments; R.H. and E. Rousseau analyzed data; R.H. and C.M. interpreted results of experiments; R.H. and E. Rousseau prepared figures; R.H. drafted manuscript; R.H., C.M., and E. Rousseau edited and revised manuscript; R.H., E. Rizcallah, S.M., C.S., M.S., C.M., S.F., and E. Rousseau approved final version of manuscript.

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