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Resolvin D1 reverses reactivity and Ca^{2+} sensitivity induced by ET-1, TNF- α , and IL-6 in the human pulmonary artery

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Hiram R, Rizcallah E, Sirois C, Sirois M, Morin C, Fortin S, Rousseau E. Resolvin D1 reverses reactivity and Ca^{2+} sensitivity induced by ET-1, TNF- α , and IL-6 in the human pulmonary artery. *Am J Physiol Heart Circ Physiol* 307: H1547–H1558, 2014. First published October 3, 2014; doi:10.1152/ajpheart.00452.2014.—Pulmonary hypertension (PH) is a rare and progressive disease characterized by an inflammatory status and vessel wall remodeling, resulting in increased pulmonary artery resistance. During the last decade, treatments have been proposed; most of them target the endothelial pathways that stimulate smooth muscle cell relaxation. However, PH remains associated with significant morbidity. We hypothesized that inflammation plays a crucial role in the severity of the abnormal vasoconstriction in PH. The goal of this study was to assess the effects of resolvin D1 (RvD1), a potent anti-inflammatory agent, on the pharmacological reactivity of human pulmonary arteries (HPAs) via an in vitro model of induced hyperreactivity. The effects of RvD1 and monoacylglyceride compounds were measured on contractile activity and Ca^{2+} sensitivity developed by HPAs that had been pretreated (or not) under proinflammatory conditions with either 10 ng/ml TNF- α or 10 ng/ml IL-6 or under hyperreactive conditions with 5 nM endothelin-1. The results demonstrated that, compared with controls, 24-h pretreatment with TNF- α , IL-6, or endothelin-1 increased reactivity and Ca^{2+} sensitivity of HPAs as revealed by agonist challenges with 80 mM KCl, 1 μM serotonin (5-hydroxytryptamine), 30 nM U-46619, and 1 μM phorbol 12,13-dibutyrate. However, 300 nM RvD1 as well as 1 μM monoacylglyceride-docosapentaenoic acid monoglyceride strongly reversed the overresponsiveness induced by both proinflammatory and hyperreactive treatments. In pretreated pulmonary artery smooth muscle cells, Western blot analyses revealed that RvD1 treatment decreased the phosphorylation level of CPI-17 and expression of transmembrane protein member 16A while increasing the detection of G protein-coupled receptor 32. The present data demonstrate that RvD1, a trihydroxylated docosahexaenoic acid derivative, decreases induced overreactivity in HPAs via a reduction in CPI-17 phosphorylation and transmembrane protein member 16A expression.

pulmonary hypertension; docosapentaenoic acid monoacylglyceride; endothelin; resolvin D1; CPI-17; endothelin-1; tumor necrosis factor- α ; interleukin-6

PULMONARY HYPERTENSION (PH) is a rare, progressive, and multifactorial disorder. Typically, PH is characterized by a progressive increase in pulmonary vascular resistance associated

with an increase from 10 to 25 mmHg in pulmonary arterial blood pressure and a significant proliferation of smooth muscle cells into the media of human pulmonary arteries (HPAs) (14). The increase in vascular resistance is partially due to endothelial dysfunction leading to a considerable remodeling of the pulmonary artery wall. A complementary hypothesis has suggested that these alterations are induced by an increase in proinflammatory mediators released by dendritic cells, natural killer cells, macrophages, mast cells, and T and B lymphocytes. These mediators include various cytokines such as ILs (namely, IL-1 α , IL-1 β , and IL-6), TNF- α , and chemokines such as chemokine (C-X-C motif) ligands 2 and 6 (16). Moreover, the impact of inflammation in HPAs has been assessed by the positive effects of anti-inflammatory treatments (42).

The genesis of PH pathophysiology is poorly understood, although several studies have shown that patients with PH present a higher level of endothelium-secreted endothelin (ET)-1 detected in the plasma and lung. ET-1 is a powerful vasoconstrictor and mitogen for smooth muscle. ET-1 activates two receptors: ET_A receptors, expressed in smooth muscle cells, and ET_B receptors, found in endothelial cells and smooth muscle cells. Stimulation of ET_A and ET_B receptors in smooth muscle cells mediates the abnormal increase in vasoconstriction and cell proliferation typically observed in PH patients (5). While a number of different treatments have been proposed since the early 1980s, PH remains associated with serious vascular injury, right ventricular failure, and higher morbidity (45).

Several clinical trials, including diet supplementation with polyunsaturated fatty acids (*n*-3 PUFAs), have shown beneficial effects in various cardiovascular diseases (31). Among *n*-3 PUFAs of interest, eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), and monoacylglyceride (MAG)-docosapentaenoic acid monoglyceride (DPA) have been reported to modulate the expression of various anti-inflammatory genes and to exert inhibitory effects on thrombocyte aggregation as well as on lipogenesis (32). EPA and DHA also reduce the expression of proinflammatory genes such as TNF- α or NF- κB (4). Over the last 20 yr, the development of in vitro models as well as several studies conducted by various groups, including our laboratory (25), have demonstrated that cytochrome *P*-450 EPA and DHA metabolites are highly active in smooth muscle cells from both vascular (1) and bronchial beds (28). For instance, the mode of action of 19,20-epoxydocosapentaenoic acid and MAG-DHA has been assessed, and their ability to decrease Ca^{2+} sensitivity in human pulmonary explants has

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been demonstrated (23, 27). These observations allowed us to put forward the hypothesis that a spectrum of long-chain *n*-3 PUFA derivatives may be putatively able to reduce local inflammation, wall remodeling, and typical overreactivity in HPAs. Within inflammatory exudates, *n*-3 PUFA-derived mediators, including resolvin (Rv)E1 from EPA as well as RvD1 and protectin D1 from DHA, are produced (13).

The metabolic cascade of MAG-DHA (Fig. 1) in putative cells is relatively well known (34). The first metabolic step is the hydrolysis of the ester bond by phospholipase A₁, in which MAG-DHA is hydrolyzed to DHA and glycerol. DHA molecules are subsequently transformed into 17-*S*-hydroperoxy-DHA by 15-lipoxygenase (LOX) (17) and subsequently into Rv molecules upon action of 5-LOX. An alternative pathway involves the specific epoxydation of one of the double carbon bonds and localized hydroxylations resulting in the production of protectins, as previously reported (17).

The potent anti-inflammatory and proresolving effects of these lipid mediators have been recently assessed and demonstrated (39). RvD1 has been shown to decrease eosinophils and proinflammatory mediators and to promote macrophage clearance of allergens in allergic airway responses (38) as well as display a specific ability to resolve inflammation in chronic inflammatory diseases such as asthma and pathological retinal angiogenesis (40). Peripheral and central RvD1 have also been shown to attenuate pain inflammation (46). From a biochemical standpoint, RvD1 is a trihydroxylated derivative of DHA.

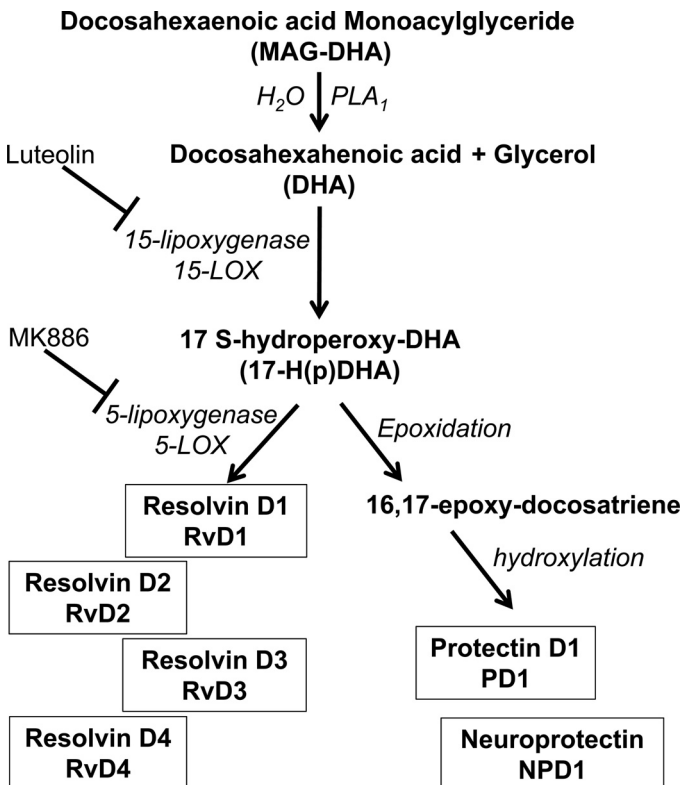


Fig. 1. General scheme of the metabolic cascade of monoacylglyceride (MAG)- docosahexaenoic acid (DHA) and DHA into various metabolites, including resolvins (Rvs) and protectins. Note that 15-lipoxygenase (LOX) and 5-LOX represent key enzymes to produce the Rvs of the D series, whereas protectins and neuroprotectins (NPDs) require epoxydation and hydroxylation. PLA₁, phospholipase A₁.

RvD1 via its interactions with its receptors, either ALX or G protein-coupled receptor 32 (GPR32), likely controls the expression of various microRNAs, transcription factors, and major proinflammatory enzymes, such as LOXs (8). These effects lead to the modulation of various molecular and cellular functions in resolving inflammation, a process correlated with antiproliferative activity (36).

Contraction in smooth muscle cells can be initiated by electrical, mechanical, and chemical stimuli. In fact, passive stretching of smooth muscle cells causes contraction. Depolarization of smooth muscle cells induces the opening of voltage-dependent Ca²⁺ channels, which cause contraction as a result of the increase in intracellular Ca²⁺ concentration. Numerous chemical stimuli, such as ANG II (via ANG II type 1 receptors), ET-1 (via ET_A and ET_B receptors), and thromboxane A₂ (via TP receptors), lead to the activation of different signaling pathways, such as inositol 1,4,5-trisphosphate (IP₃), PKC/CPI-17, and Rho kinase, all of which converge to increase Ca²⁺ release and sensitivity of the contractile machinery. Free Ca²⁺ binds calmodulin, and the Ca²⁺-calmodulin complex activates myosin light chain (MLC) kinase (MLCK), which phosphorylates MLCs, leading to smooth muscle cell contraction.

Regulation of the contractile machinery has previously been demonstrated to involve the PKC-CPI-17 signaling pathway. In fact, the lower the CPI-17 phosphorylation level, the lower the Ca²⁺ sensitivity, due to a lower level of phosphorylation of MLC phosphatase (MLCP), which, in turn, results in a higher dephosphorylation rate of MLC and reduced tone (16).

Our hypotheses are that a proinflammatory status may induce hyperreactivity in HPAs and that RvD1 may induce an inhibition of the local inflammatory component in HPAs. Therefore, RvD1 could become a potential mediator in reversing the induced rise in pulmonary arterial stiffness. The aim of the present study was to assess the effects of RvD1 and long-chain MAG precursors on pharmacologically induced tone and proinflammatory conditions.

In the present study, we report the first evidence that, in vitro, RvD1 prevents arterial wall overreactivity in HPAs. This effect appears to be related to a decrease in Ca²⁺ sensitivity, lower transmembrane protein member 16A (TMEM16A) expression, and a reduced phosphorylated (p-)CPI-17-to-total CPI-17 ratio.

MATERIALS AND METHODS

Culture of HPAs. This study was approved by our institutional Ethics Committee (protocol no. 05-088-S1-R4). Consenting patients hospitalized for lung carcinoma were recruited, and human lung tissues were obtained from the operating room via the Department of Pathology (12, 25). Tissue samples, declared “tumor free” by the pathologist, were transported to our laboratory bathed in sterile Krebs solution (pH 7.4). Note that none of the patients were suffering from PH, so all tissues were recovered from control patients. Distal HPAs were dissected under binocular control, and artery segments of 4 mm in length and 0.5–0.8 mm in diameter were cultured for 24 h in DMEM-F-12 containing 1% penicillin-streptomycin. HPAs were either untreated (control) or treated with 5 nM ET-1 or 5 nM ET-1 combined with either 300 nM RvD1 or MAGs and then incubated at 37°C in 5% CO₂ (12). Alternatively, to mimic the effects of proinflammatory conditions, HPAs were cultured for 24 h in either control conditions (untreated) or with either 10 ng/ml TNF-α or 10 ng/ml IL-6 in the absence or presence of 300 nM RvD1.

Isolation of pulmonary arterial smooth muscle cells. HPA rings were incubated 1 h at 37°C and 5% CO₂ in HBSS (GIBCO) supple-

mented with 1% penicillin-streptomycin, 0.2% collagenase type IV, and 0.05% elastase type IV (30). To assess the quality of the smooth muscle cell preparation, immunofluorescence staining with a monoclonal anti-mouse α -smooth muscle actin antibody and a goat anti-mouse IgG coupled to Alexa fluor 488 as a secondary antibody was performed and revealed that 95% of cells were positive for this specific biomarker.

Isometric tension measurements. Tension measurements were performed exactly as previously described (29, 30). A basal tension of 0.8 g was applied to each HPA ring. The pharmacologically induced contractile responses by specific agonists and eicosanoids were assessed on active tensions using transducer systems coupled to Polyview software (Grass-Astro-Med, West Warwick, RI), which enabled us to perform data acquisition and analysis. The amplitudes of maximum tensions were expressed for a given agonist concentration [1 μ M serotonin/5-hydroxytryptamine (5-HT), 1 μ M phorbol 12,13-dibutyrate (PDBu), or 30 nM U-46619] normalized to the control response to 80 mM KCl for each tested HPA ring.

β -Escin permeabilization and Ca^{2+} sensitivity. Arterial rings were mounted in organ baths to which 0.8-g tension was applied. After the contractile response elicited by 80 mM KCl in isosmotic Krebs solution had been measured, rings were incubated for 25 min in low- Ca^{2+} relaxing solution (pCa $^{2+}$ 9) and permeabilized at 25°C with 50 μ M β -escin. Tension developed by permeabilized arterial rings was measured at 37°C according to free Ca^{2+} concentrations expressed in terms of pCa [pCa = $-\log(Ca^{2+}$ concentration)]. Step increases in free Ca^{2+} (from pCa = 9.0 to 5.3) induced reproducible concentration response curves to free Ca^{2+} concentrations, indicating successful permeabilization under these conditions (30, 37).

SDS-PAGE and Western blot analyses. Western blots were performed using specific antibodies against CPI-17, p-CPI-17, TMEM16A, GPR32, and β -actin proteins. Blot immunostainings were revealed on Kodak film and digitized and analyzed using ImageJ software or Lab-Image software 2.7-2 (29).

Drugs and chemical reagents. RvD1, U-46619, and MK-886 were obtained from Cayman Chemical (Ann Arbor, MI). 5-HT, IL-6, and luteolin were obtained from Sigma-Aldrich (St. Louis, MO). ET-1, PDBu, anti-CPI-17, and anti-p-CPI-17 were purchased from Cedarlane (Burlington, ON, Canada). TNF- α and anti-TMEM16A were purchased from Abcam (Toronto, ON, Canada). Myosin phosphatase target subunit 1 (MYPT-1) and p-MYPT-1 (phosphorylated form of MYPT-1 on Thr 696) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). MAG-DHA and MAG-DPA were purified by SCF-Pharma (Rimouski, QC, Canada). DMEM-F-12 and penicillin-streptomycin were obtained from GIBCO Invitrogen (Burlington, ON, Canada).

Data presentation and statistical analysis. Results are expressed as means \pm SE, with n indicating the number of rings for each set of experiments. Western blot analyses are expressed with n indicating the number of experiments. Statistical analyses were performed using Student's t -test or one-way or two-way ANOVAs with Sigma Plot 12.0 and SPSS 14.0 software (SPSS-Science, Chicago, IL). Differences were considered statistically significant at $P < 0.05$. EC $_{50}$ values were determined from data curve fittings performed with the Sigma Plot 12.0 algorithm.

RESULTS

Effects of MAG-DHA and RvD1 on Ca^{2+} sensitivity and cumulative concentration-response curves to U-46619 and PDBu. Two major pathways have been demonstrated to play major roles as effectors on the contractile machinery: 1) a G protein-coupled receptor-dependent signaling pathway that enhances contractility by increasing intracellular Ca^{2+} release from IP $_3$ -dependent stores (21) and 2) a PKC-dependent CPI-17 pathway, which inactivates MLCP and modulates Ca^{2+}

sensitivity of the contractile machinery in HPA smooth muscle cells (21). U-46619, a thromboxane A $_2$ analog that stimulates TP receptors, is a pharmacological agonist known to increase HPA tone. Figure 2A shows cumulative concentration-response curves (CCRCs) to U-46619 in control and 5 nM ET-1-treated HPAs in the absence or presence of either 300 nM RvD1 or 1 μ M MAG-DHA. CCRCs demonstrated that 24-h

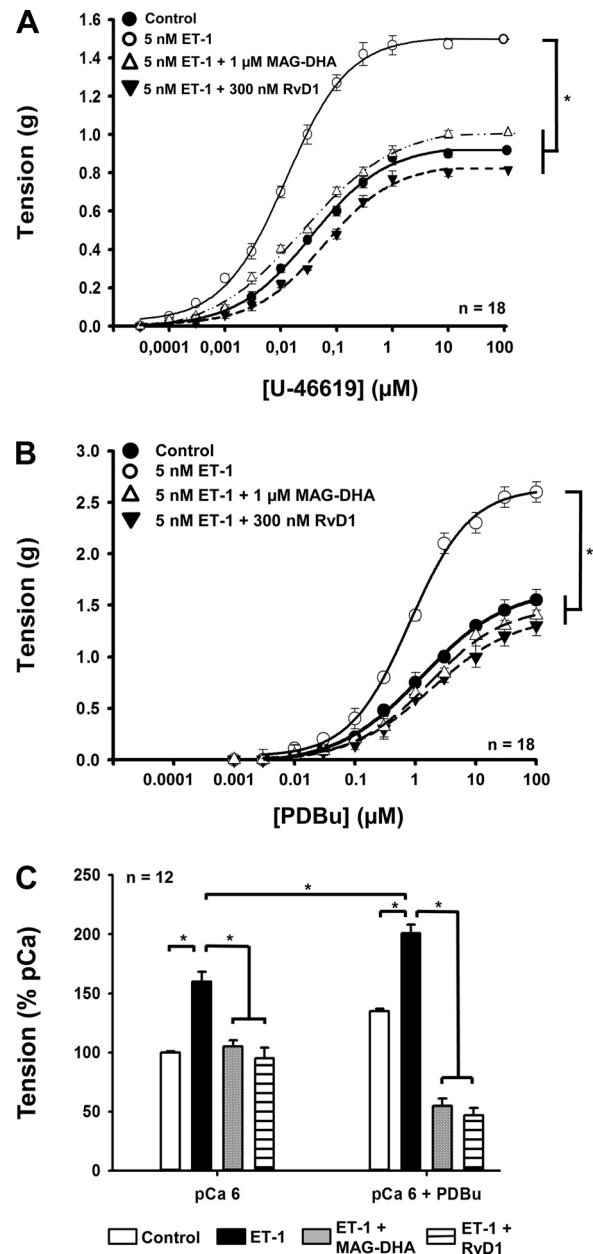


Fig. 2. Effect of MAG-DHA and RvD1 on Ca^{2+} reactivity and on U-46619- and phorbol 12,13-dibutyrate (PDBu)-induced tension in human pulmonary arteries (HPAs). A: cumulative concentration-response curves (CCRCs) to U-46619 on HPAs under control (untreated) conditions or pretreated with 5 nM endothelin (ET)-1 in the absence or presence of 1 μ M MAG-DHA or 300 nM RvD1. $n = 18$. * $P < 0.05$. B: CCRCs to PDBu on HPAs either under control (untreated) conditions or pretreated with 5 nM ET-1 in the absence or presence of 1 μ M MAG-DHA or 300 nM RvD1. $n = 18$. * $P < 0.05$. C: contractile responses to Ca^{2+} sensitization (pCa 6) in the presence or absence of 1 μ M PDBu under the same experimental conditions as described above. $n = 12$. * $P < 0.05$.

pretreatment with 5 nM ET-1 induced a hyperresponsiveness to U-46619 with an apparent EC_{50} value of 0.01 μ M compared with 0.04 μ M under control conditions. Conversely, treatment with 300 nM RvD1 or 1 μ M MAG-DHA significantly reduced the induced reactivity with apparent EC_{50} values of 0.05 and 0.03 μ M, respectively.

CCRCs to PDBu showed that, compared with control conditions, 24-h pretreatment with 5 nM ET-1 significantly increased the responsiveness to the PKC activator (Fig. 2B). However, 300 nM RvD1 or 1 μ M MAG-DHA significantly reduced the concentration-dependent effect of PDBu on ET-1-pretreated tissues, returning mean tension to that of control values with apparent EC_{50} values of 1.31, 1.33, 0.78, and 1.20 μ M, respectively. These data suggest that both RvD1 and MAG-DHA modulate HPA hyperresponsiveness via inhibition of two complementary pathways controlling the contractile machinery in HPAs.

The mean tonic response of β -escin-permeabilized explants to a single Ca^{2+} step from pCa 9.0 to 6.0 was assessed in the absence and presence of PDBu under four independent experimental conditions. PDBu has, in effect, been used to stimulate the PKC-CPI-17 pathway to enhance tone. Figure 2C shows that 5 nM ET-1 pretreatment significantly increased the tension response to pCa 6 compared with control conditions. The addition of 1 μ M PDBu further enhanced the mean amplitude of the active tensions, whereas 300 nM RvD1 or 1 μ M MAG-DHA normalized the responses to pCa 6. Moreover, in the presence of PDBu, RvD1 and MAG-DHA significantly decreased tonic responses and had an inhibitory effect even under control conditions. Taken together, these results suggest that RvD1 as well as MAG-DHA treatments impair Ca^{2+} reactivity via a PKC-dependent pathway.

Proresolving effect of MAG-DHA in the absence of enzymatic inhibitors. Tension measurements were performed on β -escin-permeabilized preparations to assess the effect of RvD1 and MAG-DHA on Ca^{2+} sensitivity on ET-1-pretreated HPAs. The Ca^{2+} sensitivity of myofilaments was evaluated after five treatment conditions in permeabilized arteries as shown by CCRCs to free Ca^{2+} concentrations (Fig. 3A). The results revealed that 5 nM ET-1 treatment ($EC_{50} = 0.08 \mu$ M) induced hypersensitivity to free Ca^{2+} concentration compared with control conditions ($EC_{50} = 1.02 \mu$ M). In contrast, 5 nM ET-1 plus 300 nM RvD1 or 1 μ M MAG-DHA induced a shift in the half-maximal effective concentration value toward higher Ca^{2+} concentrations with EC_{50} values of 2.2 and 1.54 μ M, respectively, and thereby reduced Ca^{2+} sensitivity induced by ET-1 pretreatment. No significant differences were quantified in Ca^{2+} sensitivity developed from control, 5 nM ET-1 + 300 nM RvD1-treated, and 5 nM ET-1 + 1 μ M MAG-DHA-treated HPAs. Combined treatment with 15-LOX and 5-LOX inhibitors (0.6 μ M luteolin and 1 μ M MK-886, respectively) prevented the effect induced by 1 μ M MAG-DHA on Ca^{2+} sensitivity of HPAs with an EC_{50} value of 0.13 μ M. These results suggest that MAG-DHA is metabolized by LOX enzymes in mediating its regulatory effects on human pulmonary arterial tone.

Further experiments were performed to ascertain whether MAG-DHA needs to be metabolized to normalize the contractile responses in ET-1-pretreated HPAs. After various pretreatments, cultured HPAs were stimulated with 30 nM U-46619, after which mean responses were compared (Fig. 3B). ET-1

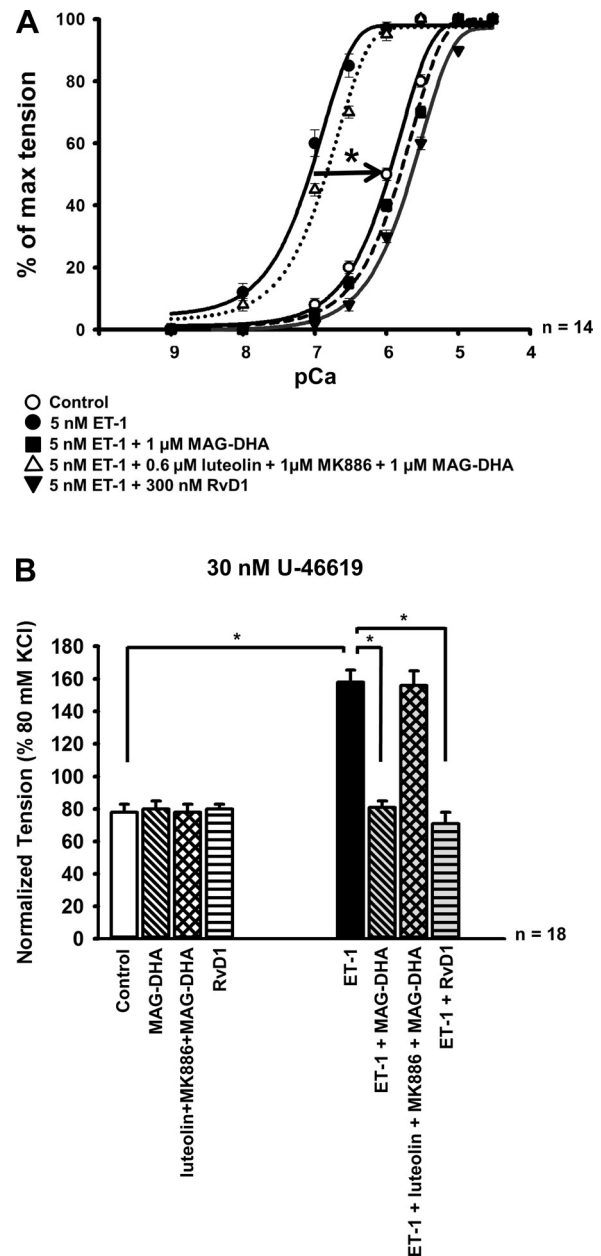


Fig. 3. Normalizing effects of MAG-DHA and RvD1 on HPA reactivity. *A*: CCRCs to free Ca^{2+} concentration steps on β -escin-permeabilized HPAs after 24-h culture in control (untreated) conditions or pretreated with 5 nM ET-1 in the absence or presence of 1 μ M MAG-DHA or 300 nM RvD1 or 1 μ M MAG-DHA + 0.6 μ M luteolin + 1 μ M MK-886. $n = 14$. $*P < 0.05$. *B*: bar graph showing mean contractile amplitudes induced by 30 nM U-46619 on 24-h cultured HPA either in the absence (control) or presence of 5 nM ET-1 alone or with 1 μ M MAG-DHA or 1 μ M MAG-DHA + 0.6 μ M luteolin + 1 μ M MK-886 or 300 nM RvD1. Note that the effect of MAG-DHA was blunted in the presence of luteolin and MK-886. $n = 18$. $*P < 0.05$.

pretreatment had a significant effect on the mean contractile response compared with control HPAs. Of note, 1 μ M MAG-DHA and 300 nM RvD1 essentially exhibited similar inhibitory effects on the overreactivity triggered by 30 nM U-46619. However, the inhibitory effect of MAG-DHA was blunted in the presence of 0.6 μ M luteolin and 1 μ M MK-886 (15-LOX and 5-LOX inhibitors, respectively). This result suggests that MAG-DHA must be metabolized to induce its inhibitory ef-

fects, which was further reproduced with a lower concentration of RvD1. Of interest, RvD1, MAG-DHA, or MAG-DHA in the presence of luteolin and MK-886 had no effect on the contractile response under control conditions (Fig. 3B).

Effect of RvD1 on the pharmacologically induced tone of HPA. The influence of RvD1 on the reactivity of HPA was assessed *in vitro* on cultured tissues under three specific conditions: HPA were cultured for 24 h either under control

condition (untreated) or in the presence of 5 nM ET-1 or with 5 nM ET-1 + 300 nM RvD1. Figure 4, A and B, shows the normalized response to 80 mM KCl. Pretreatment with 5 nM ET-1 induced an overreactivity that was abolished by 300 nM RvD1 treatment. Figure 4C shows that RvD1 had a similar effect upon acute stimulation with 1 μ M 5-HT. Finally, the addition of 300 nM RvD1 significantly decreased the mean response induced by 5 nM ET-1 pretreatment (Fig. 4D), lead-

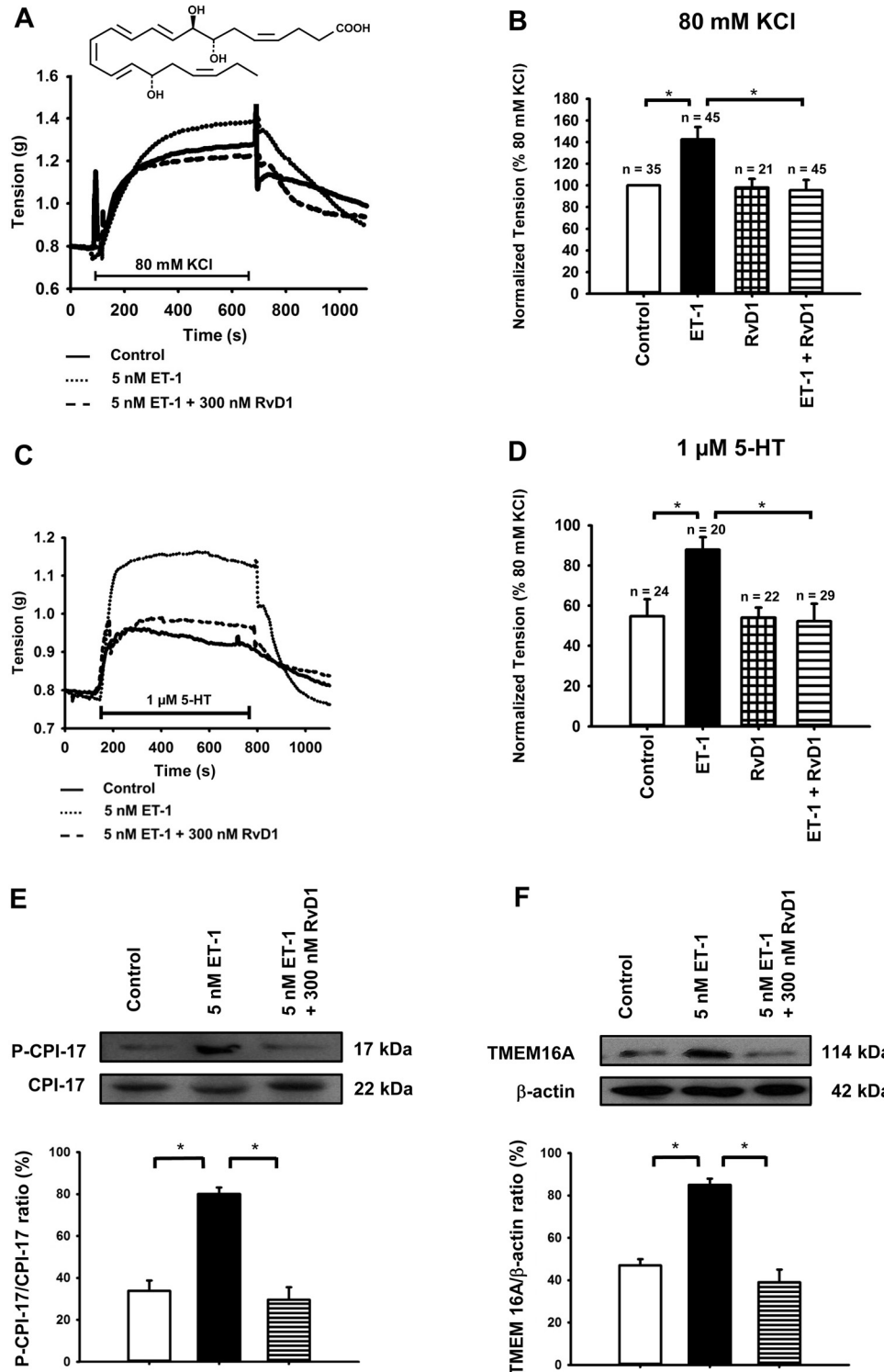


Fig. 4. Effects of RvD1 on HPAs in response to pharmacologically induced tone and on two putative effectors. *A*: representative recordings of the contractile activity induced by 80 mM KCl and RvD1 in the absence or presence of 5 nM ET-1. The chemical structure of RvD1 is shown in the *inset*. *B*: corresponding mean bar graph showing mean contractile amplitudes induced by 80 mM KCl either under control (untreated) conditions or after 5 nM ET-1 pretreatment in the absence or presence of 300 nM RvD1. *C*: typical recordings of contractions induced by 1 μ M serotonin [5-hydroxytryptamine (5-HT)] on cultured HPAs under the same experimental conditions. *D*: mean responses to 1 μ M 5-HT corresponding to experimental conditions as described above. * P < 0.05. *E*: Western blot analyses of the phosphorylated (p)-CPI-17-to-total CPI-17 ratio in assessing the putative effects of 5 nM ET-1 and 5 nM ET-1 + 300 nM RvD1 on pulmonary artery smooth muscle cell (PASMC) lysates compared with control conditions. n = 9. * P < 0.05. *F*: Western blot analyses using transmembrane protein member 16A (TMEM16A) antibodies. Densities of the immunoreactive bands are expressed as a function of the β -actin-immunoreactive band. n = 9. * P < 0.05.

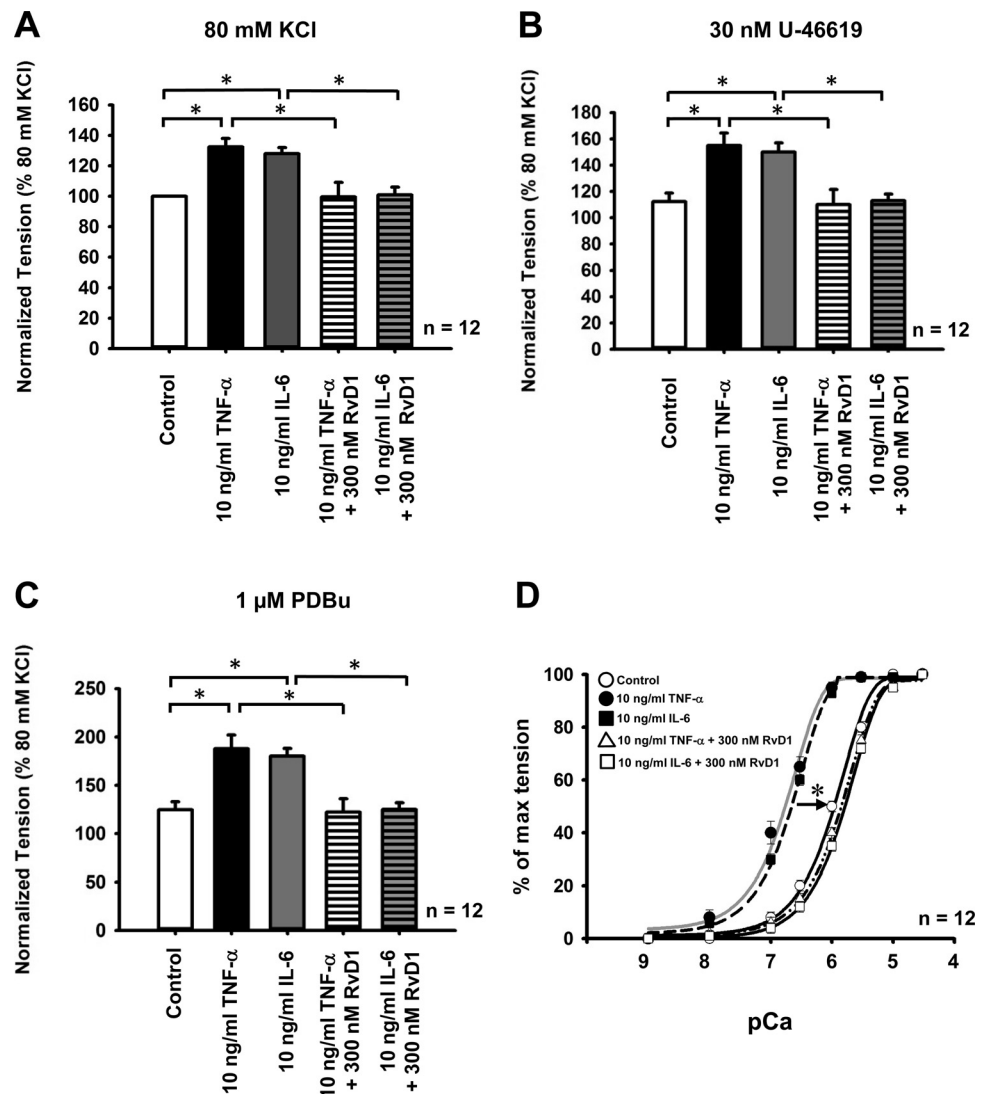
ing to a normalized tone in response to 1 μ M 5-HT. Of note, regardless of the pharmacological stimulus used, RvD1 had basically no effect under control conditions. Taken together, these data suggest that RvD1, a trihydroxylated derivative of DHA, was able to prevent the overreactivity induced by KCl and 5-HT on ET-1-pretreated HPAs.

A well-known effector between PKC and the contractile machinery is the CPI-17 protein (34a). Thus, expression and phosphorylation of CPI-17 were assessed by Western blot analyses in pulmonary artery smooth muscle cell (PASMC) lysates under control conditions and with 5 nM ET-1 or 5 nM ET-1 + 300 nM RvD1 pretreatment conditions. Figure 4E shows that 5 nM ET-1 significantly increased the phosphorylation of CPI-17 compared with control conditions. However, RvD1 significantly decreased CPI-17 phosphorylation compared with the level observed in 5 nM ET-1-treated HPAs (Fig. 4C, bottom). Finally, the activated state of CPI-17 has previously been shown to modulate the Ca^{2+} sensitivity of PASMCs (23). Furthermore, TMEM16A, a Ca^{2+} -dependant Cl^- channel reported to play an important role in enhancing tone of PASMCs, was assessed by Western blot analyses. Figure 4F shows that 5 nM ET-1 significantly increased the expression of

TMEM16A compared with control conditions, whereas RvD1 reversed this ET-1-induced overexpression. Taken together, these results suggest that RvD1, whose anti-inflammatory effect has been established, also displays strong inhibitory effects on CPI-17 phosphorylation and TMEM16A expression and thus could result in the lower reactivity of HPAs upon ET-1 stimulation (26), a condition mimicking typical HPA hyperreactivity observed in severe PH.

Inhibitory effect of RvD1 on TNF- α - and IL-6-pretreated HPAs. To assess whether a proinflammatory condition may lead to HPA overreactivity, tissue sections were treated under various experimental conditions in which pharmacomechanical properties were measured as previously assessed on human bronchi (29). HPAs were cultured for 24 h either under control conditions (untreated) or in the presence of 10 ng/ml TNF- α or 10 ng/ml TNF- α + 300 nM RvD1 or in the presence of 10 ng/ml IL-6 or 10 ng/ml IL-6 + 300 nM RvD1. Figure 5A shows the normalized response to 80 mM KCl. Compared with control conditions, pretreatment with 10 ng/ml TNF- α or 10 ng/ml IL-6 induced a significant overreactivity, as revealed by the \sim 35% increase in the mean amplitude of the contractile response. Despite the presence of TNF- α or IL-6, 300 nM

Fig. 5. Effect of RvD1 on TNF- α - and IL-6-induced reactivity and Ca^{2+} sensitivity in HPAs. *A*: bar graph of the contractile activity induced by 80 mM KCl on 24-h cultured HPAs in control (untreated) conditions or after 10 ng/ml TNF- α or 10 ng/ml IL-6 pretreatments in the absence or presence of 300 nM RvD1. *n* = 12. **P* < 0.05. *B*: bar graph showing mean contractile amplitudes induced by 30 nM U-46619 on 24-h cultured HPAs either under control conditions or with 10 ng/ml TNF- α or IL-6 in the absence or presence of 300 nM RvD1. *n* = 12. **P* < 0.05. *C*: bar graph showing mean contractile amplitudes induced by 1 μ M PDBu on HPAs under the same experimental conditions as described above. *n* = 12. **P* < 0.05. *D*: CCRCs to free Ca^{2+} concentrations on β -escin-permeabilized cultured HPAs under the same experimental conditions. Note that TNF- α and IL-6 pretreatments consistently increased the Ca^{2+} sensitivity (\bullet and \blacksquare , respectively), which was abolished by the combined addition of TNF- α and RvD1 (Δ) or IL-6 and RvD1 (\square). RvD1 also normalized the Ca^{2+} sensitivity with an EC_{50} value close to that recorded under control conditions (\circ). *n* = 12. **P* < 0.05.



RvD1 treatments abolished this overreactivity, leading to normalized tension. Figure 5B shows mean responses to acute stimulation with 30 nM U-46619 on cultured HPAs. Compared with control conditions, TNF- α and IL-6 (10 ng/ml) consistently induced an overreactivity (+47%), whereas, in contrast, 300 nM RvD1 abolished the overreactivity induced by U-46619 on TNF- α - and IL-6-pretreated HPAs. Figure 5C shows the similar effect upon acute stimulation with 1 μ M PDBu. Compared with control conditions, 10 ng/ml TNF- α and 10 ng/ml IL-6 pretreatments significantly increased (+52%) the mean contractile response to an acute stimulus, whereas 300 nM RvD1 treatments significantly decreased the mean response induced by 1 μ M PDBu. Given the results obtained with PDBu, we furthermore assessed the putative change in Ca²⁺ sensitivity.

Figure 5D shows CCRCs to free Ca²⁺ concentrations in control, 10 ng/ml TNF- α , 10 ng/ml IL-6, 10 ng/ml TNF- α + 300 nM RvD1, and 10 ng/ml IL-6 + 300 nM RvD1 conditions. The results demonstrate that TNF- α and IL-6 pretreatment increased HPA Ca²⁺ sensitivity compared with control (with EC₅₀ values of 0.18, 0.2, and 1.08 μ M, respectively), whereas RvD1 reduced the Ca²⁺ sensitivity induced by TNF- α and IL-6 pretreatment with an apparent EC₅₀ of 1.40 μ M, a value in proximity to the EC₅₀ value observed under control conditions. Taken together, these data suggest that RvD1 was able to prevent the overreactivity and Ca²⁺ hypersensitivity triggered by TNF- α on cultured HPAs.

Effect of MAG-DPA on HPA active tone. The above results provide evidence of the positive impact of the MAG-DHA metabolite RvD1 to reverse hyperreactivity in HPAs. Recently, a novel monoglyceride with a molecular structure similar to MAG-DHA has been synthesized and its properties investigated in an in vivo rat model of PH (26). The following series of experiments was designed to delineate the effects of this docosapentaenoic acid (22:5n-3) monoglyceride (MAG-DPA), a long-chain n-3 PUFA presumed to yield proresolving molecules, on the overreactivity induced by ET-1 (43) in response to various pharmacological agents.

As shown in Fig. 6A, HPAs were challenged with 5-nM ET-1 or 5-nM ET-1 + 1 μ M MAG-DPA in the presence of 1 μ M 5-HT. The HPA responses confirmed that ET-1 treatment induced a larger response than control (untreated) conditions, whereas mean contractile responses were reduced upon combined ET-1 + MAG-DPA treatment.

Twenty-four-hour treatment with 5 nM ET-1 induced a consistent overreactivity to 30 nM U-46619 (Fig. 6B), whereas 1 μ M MAG-DPA treatment significantly disrupted the effect of ET-1 and maintained the agonist-induced tone to that of normal tension values. These results are consistent with the previously reported effects of MAG-DPA on carcinoma cells as well as in a rat model of PH (26, 27). Of note, combined treatment with 5 nM ET-1, 0.6 μ M luteolin, and 1 μ M MK-886 in the presence of 1 μ M MAG-DPA abolished the effect of the MAG compound on the U-46619-induced tonic response.

Relative sensitivity of the myofilaments to Ca²⁺ was also quantified on β -escin-permeabilized HPAs under the same experimental conditions. Figure 6C shows CCRCs to free Ca²⁺ concentrations in control, 5 nM ET-1, and 5 nM ET-1 + 1 μ M MAG-DPA conditions. The results demonstrate that ET-1 pretreatment increased Ca²⁺ sensitivity compared with con-

trols (with EC₅₀ = 0.08 and 1.08 μ M, respectively), whereas MAG-DPA reduced the Ca²⁺ sensitivity induced by ET-1 pretreatment on HPA tissues with an apparent EC₅₀ value of 0.63 μ M, a value near the EC₅₀ value recorded under control conditions.

To test the potential curative effects of MAG-DPA and RvD1 on preestablished hyperreactivity, HPAs were first treated in the absence (control) or presence of 5 nM ET-1 followed by treatment 12 h later with a combination of 5 nM ET-1 + 1 μ M MAG-DPA or 5 nM ET-1 + 300 nM RvD1 during 12 h (Fig. 6D). Compared with controls, ET-1 treatment significantly increased the reactivity in response to 80 mM KCl, 1 μ M 5-HT, and 1 μ M PDBu. However, delayed treatment with 1 μ M MAG-DPA or 300 nM RvD1 was able to significantly reverse this overreactivity and maintain the agonist-induced tone near that of normal tension values.

The same treatment design was performed under proinflammatory conditions (Fig. 6E), where HPAs were first treated with 10 ng/ml TNF- α or 10 ng/ml IL-6 and treated 12 h later with 10 ng/ml TNF- α (or 10 ng/ml IL-6) + 1 μ M MAG-DPA or with 10 ng/ml TNF- α (or 10 ng/ml IL-6) + 300 nM RvD1. Compared with controls, TNF- α and IL-6 treatment induced a significant increase in reactivity in response to 80 mM KCl, 1 μ M 5-HT, and 1 μ M PDBu. However, 12-h treatment with 1 μ M MAG-DPA or 300 nM RvD1 was sufficient to significantly reverse the observed overreactivity and reestablish normal agonist-induced tone values.

Detection of TMEM16A, p-CPI-17, and p-MYPT-1 expression under MAG-DPA treatments. Western blot analyses were performed to assess whether or not MAG-DPA was able to downregulate the surface membrane biomarker TMEM16A in PASM lysates under control or pretreated experimental conditions with either 5 nM ET-1, 5 nM ET-1 + 0.01 μ M MAG-DPA, 5 nM ET-1 + 0.1 μ M MAG-DPA, or 5 nM ET-1 + 1 μ M MAG-DPA (Fig. 7A). The results showed that, compared with controls, 5 nM ET-1 significantly increased staining of a specific immunoreactive band consistent with the relative molecular weight of TMEM16A (114 kDa), whereas 1 μ M MAG-DPA incurred a significant inhibition of this upregulation induced by ET-1 pretreatment. CPI-17 phosphorylation in PASM lysates was also detected under the same experimental conditions (Fig. 7B). The quantitative results suggest that MAG-DPA induced a concentration-dependent decrease in the p-CPI-17-to-total CPI-17 ratio compared with the enhanced ratio detected upon 5 nM ET-1 treatment.

The final step of all the convergent pathways that lead to the contraction of smooth muscle cells is the phosphorylation of MLC. Phosphorylation levels of MLC were therefore tested under hyperreactive conditions as well as under the influence of MAG-DPA and RvD1. Western blot analyses were performed on PASM lysates and HPA homogenates to detect p-MYPT-1, which is the myosin-binding subunit of MLCP (Fig. 7C). The results revealed that 5 nM ET-1 significantly increased the level of the p-MYPT-1-to-MYPT-1 ratio, whereas 1 μ M MAG-DPA or 300 nM RvD1 reversed the effect of ET-1 treatment.

Taken together, these data indicate that submicromolar concentrations of MAG-DPA exhibit strong inhibitory effects on the hyperreactivity and Ca²⁺ sensitivity induced by ET-1. Furthermore, the results suggest that these functional effects are likely mediated by a metabolic byproduct since

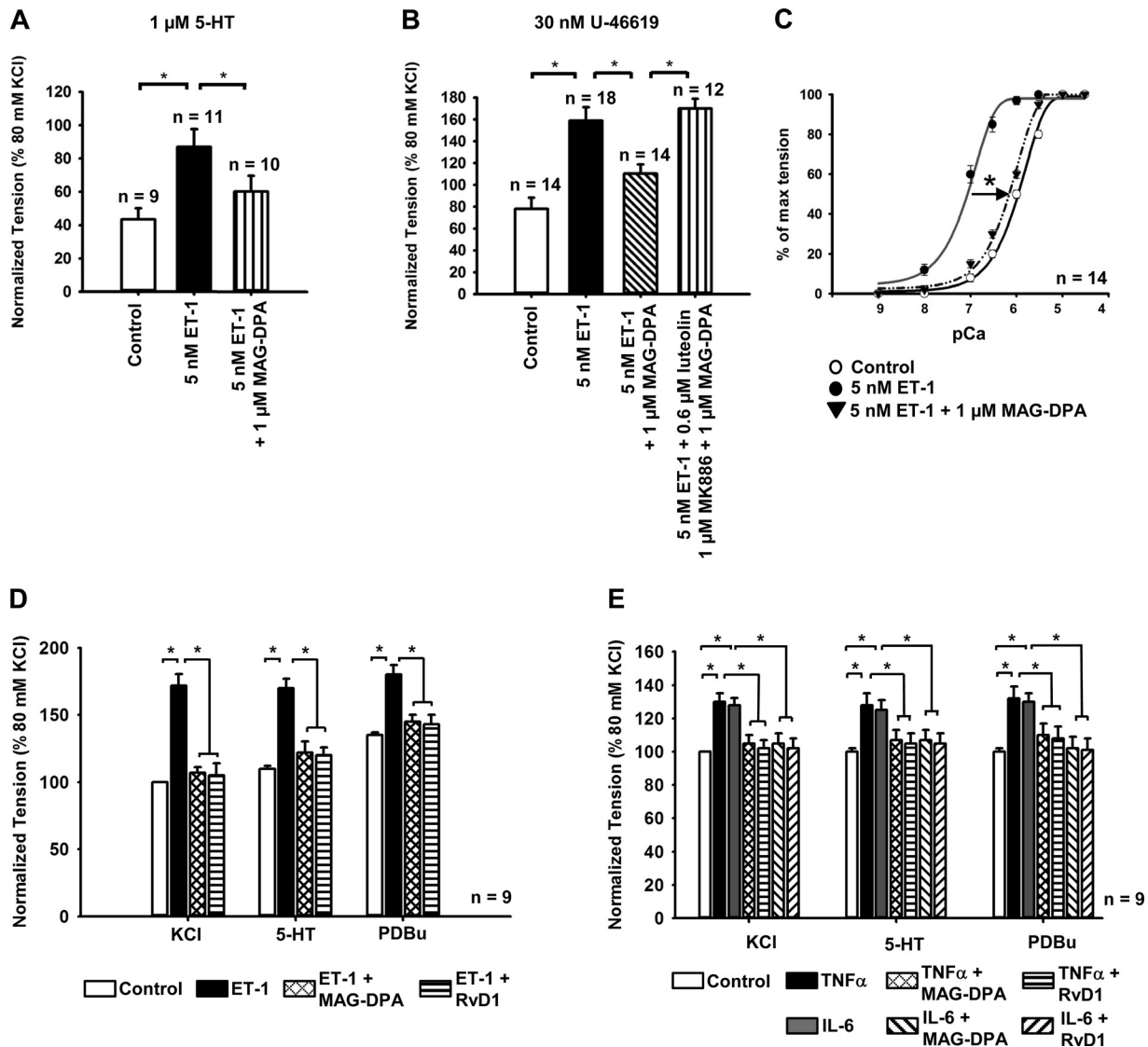


Fig. 6. Effect of MAG- docosapentaenoic acid monoglyceride (DPA) on the active tone of HPAs. *A*: bar graph of the contraction induced by 1 μ M 5-HT on 24-h cultured HPAs under control (untreated) conditions or after 5 nM ET-1 pretreatment in the absence or presence of 1 μ M MAG-DPA. $*P < 0.05$. *B*: bar graph showing mean contractile amplitudes induced by 30 nM U-46619 on 24-h cultured HPAs either under control conditions or with 5 nM ET-1 in the absence or presence of 1 μ M MAG-DPA or 1 μ M MAG-DPA + 0.6 μ M luteolin + 1 μ M MK-886. $*P < 0.05$. *C*: CCRCs to free Ca^{2+} steps on β -escin-permeabilized cultured HPAs either under control conditions or pretreated with 5 nM ET-1 in the absence or presence of 1 μ M MAG-DPA. Note the increase in Ca^{2+} sensitivity by ET-1 pretreatment (\bullet), which was abolished after the combined addition of ET-1 and MAG-DPA (\blacktriangle). $n = 14$. $*P < 0.05$. *D*: bar graph showing mean contractile amplitudes induced by 80 mM KCl, 1 μ M 5-HT, and 1 μ M PDBu on HPAs cultured either in the absence (control) or presence of 5 nM ET-1 alone during 12 h and then cultured for an additional 12 h in the presence of 5 nM ET-1 + 1 μ M MAG-DPA or 5 nM ET-1 + 300 nM RvD1. $n = 9$. $*P < 0.05$. *E*: bar graph of the contraction induced by 80 mM KCl, 1 μ M 5-HT, and 1 μ M PDBu on HPAs cultured either in the absence (control) or presence of 10 ng/ml TNF- α or 10 ng/ml IL-6 alone during 12 h and then cultured for an additional 12 h in the presence of 10 ng/ml TNF- α (or 10 ng/ml IL-6) + 1 μ M MAG-DPA or 10 ng/ml TNF- α (or 10 ng/ml IL-6) + 300 nM RvD1. $n = 9$. $*P < 0.05$.

in the presence of the enzymatic inhibitors 5-LOX and 15-LOX, the same concentration of MAG-DPA was no longer effective, as shown in Fig. 6*B*. Given the comparable structures of MAG-DPA and MAG-DHA, it could be suggested that this MAG-DPA effector metabolite is also a RvD1-like product, of which potential applications remain to be determined.

Detection of the RvD1 receptor GPR32. Finally, Western blot analyses performed on lysate preparations of PASMC cultures after pharmacological pretreatments (Fig. 7*D*) demonstrated that 24-h pretreatment with 5 nM ET-1 did not significantly modulate the expression of GPR32. However, 300 nM

RvD1 and 1 μ M MAG-DPA significantly enhanced the detection and membrane expression of GPR32, as shown in Fig. 7*D* (right lanes).

DISCUSSION

In the present study, we investigated the ability of RvD1 and its precursors to reverse the overreactivity and enhanced Ca^{2+} sensitivity triggered by ET-1, TNF- α , and IL-6 pretreatments on smooth muscle cells in a human model of organ cultured pulmonary arteries. This *n*-3 PUFA trihydroxyl derivative is well known for its anti-inflammatory and proresolving proper-

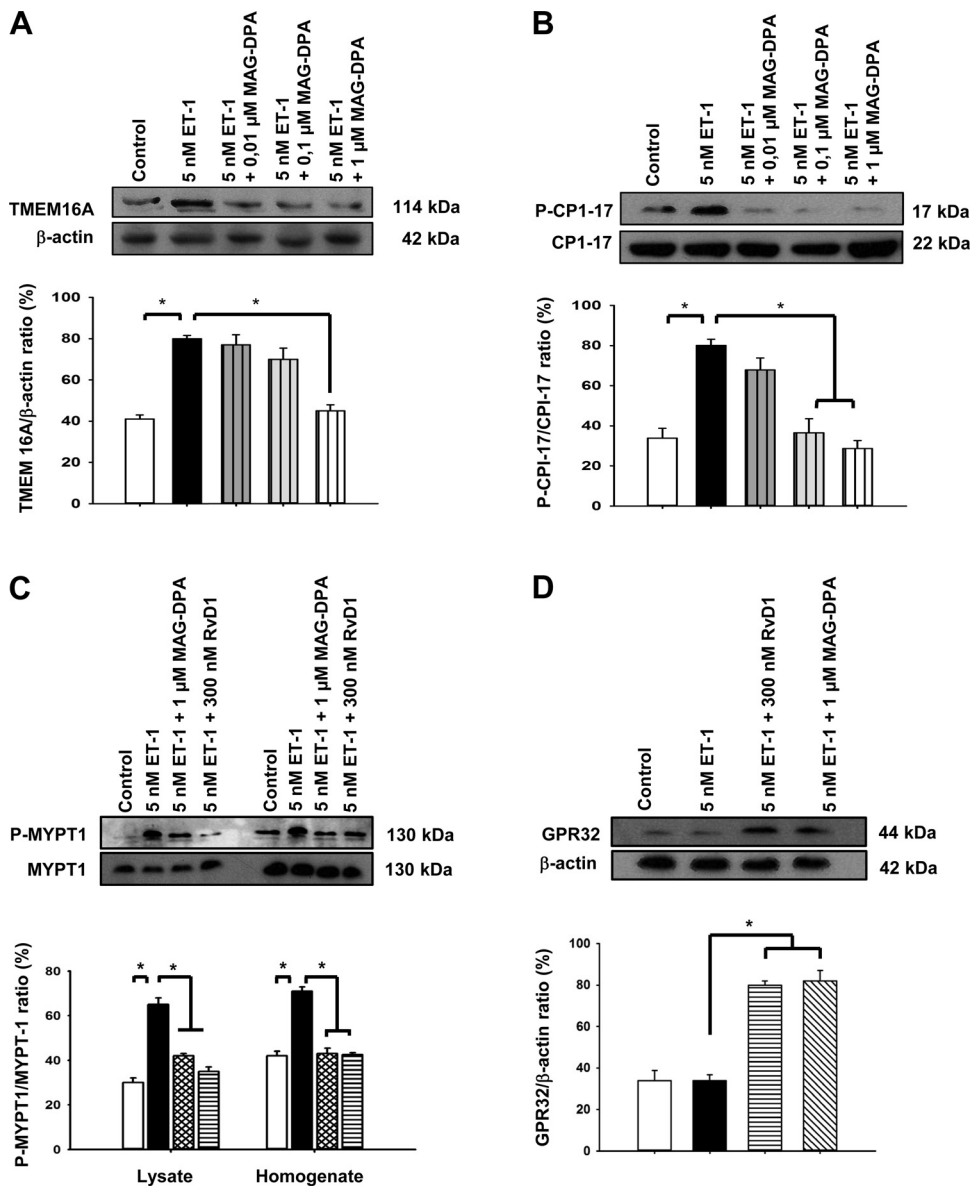


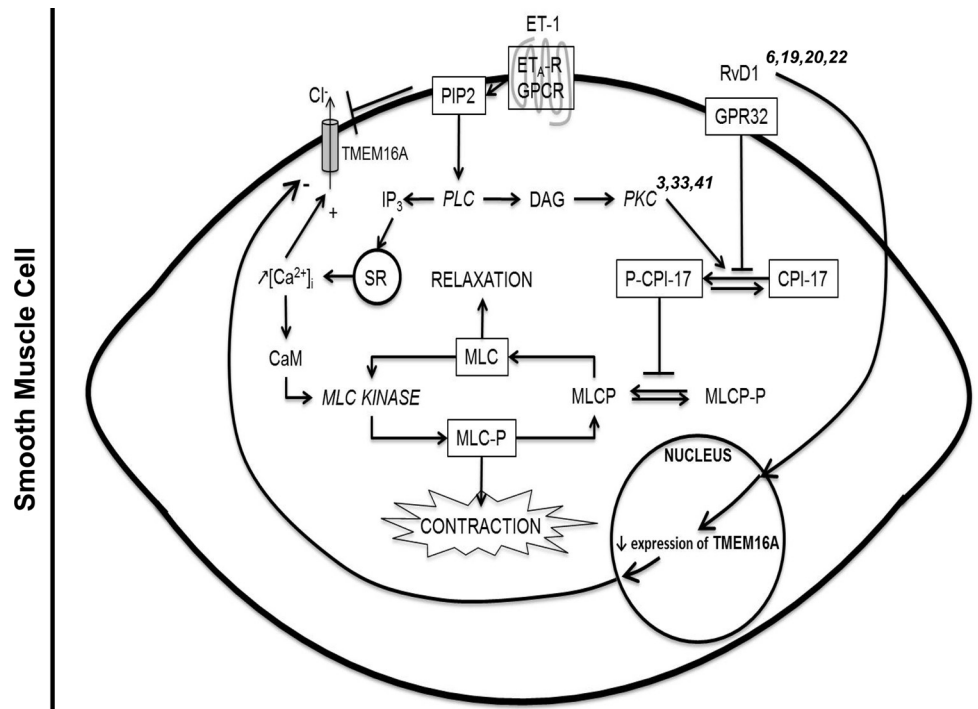
Fig. 7. Detection of TMEM16A, p-CPI-17, p-myosin phosphatase target subunit 1 (p-MYPT-1), and G protein-coupled receptor 32 (GPR32). **A**: TMEM16A-immunoreactive bands (114 kDa) in cell lysates were expressed as the relative TMEM16A-to- β -actin ratio under control condition or with 5 nM ET-1 or 5 nM ET-1 in the presence of increasing concentrations of MAG-DPA (0.01–1 μ M). $n = 9$. $*P < 0.05$. **B**: Western blot analyses were performed using antibodies against p-CPI-17 and total CPI-17. The relative density ratio of p-CPI-17 to total CPI-17 was used to quantify the comparative effects of increasing concentrations of MAG-DPA (0.01–1 μ M). $n = 9$. $*P < 0.05$. **C**: Western blot analyses were performed using antibodies against p-MYPT-1 and total MYPT-1. The relative density ratio of p-MYPT-1 to MYPT-1 was used to quantify the effects of 1 μ M MAG-DPA and 300 nM RvD1 on 5 nM ET-1 treatment in cell lysates and HPA homogenates. $n = 6$. $*P < 0.05$. **D**: Western blot analyses using specific antibodies against GPR32 and β -actin. The immunoreactive bands as well as the calculated density ratio attest to the presence of GPR32 in PSMCs. $n = 6$. $*P < 0.05$.

ties in blood cells and bronchi (38, 39). Given that we wanted to address the putative role of inflammation in pulmonary arteries, we therefore decided to challenge HPAs with either nanomolar concentrations of ET-1, TNF- α , or IL-6 and test contractile responses in the absence and presence of RvD1 or its MAG precursors.

RvD1 and MAG compounds reverse the overreactivity induced by ET-1 in HPAs. ET-1 has previously been shown to be overexpressed and released in the lung of patients with PH (9) due to endothelial dysfunctions. Upon binding to its receptor, ET-1 activates phospholipase C, which hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP₂) into IP₃ and diacylglycerol. IP₃ activates the release of Ca²⁺ from endoplasmic reticulum stores, whereas diacylglycerol has been reported to activate PKC, which, in turn, phosphorylates CPI-17 (Fig. 8). The latter has also been shown to inactivate MLCP through phosphorylation of MYPT-1, its catalytic subunit (27). A third pathway, which is activated by the ET-1 receptor, is the RhoA/Rho kinase pathway (44). This specific pathway is recognized to

phosphorylate CPI-17, which, in turn, phosphorylates and inactivates the catalytic subunit of MLCP (Fig. 8) (16). These regulatory pathways result in an increased contractile reactivity and Ca²⁺ sensitivity. Nevertheless, pharmacological options for PH treatment remain limited despite the recent use of ET-1 receptor antagonists (10). In the present study, RvD1 was found to abolish the hyperresponsiveness induced by short-term (24 h) ET-1 pretreatment of HPA explants. Indeed, in the presence of submicromolar concentrations of RvD1, contractile responses to pharmacological agonists such as 5-HT and U-46619 were maintained near the level recorded under control conditions (cultured and untreated HPAs). The present data also show that treatment with MAG-DHA or MAG-DPA resulted in consistent inhibition of the pharmacological responses enhanced by ET-1, thus confirming that, among others, micromolar concentrations of Rv precursors were also able to induce a similar effect. In contrast, these effects were abolished in the presence of 5-LOX and 15-LOX inhibitors, thus confirming that both MAG compounds had to be metabolized to

Fig. 8. Schematic diagram of the molecular and cellular events summarizing the inhibitory effect of RvD1 on active arterial tone and Ca^{2+} sensitivity induced by the activation of ET-1 receptors in a virtual PASM. This scheme is consistent with the data reported in the present work and in previous studies (3, 6, 15, 19, 20, 33, 41, 41a). ET_A R, ET_A receptor; GPCR, G protein-coupled receptor; PIP₂, phosphatidylinositol 4,5-bisphosphate; PLC, phospholipase C; DAG, diacylglycerol; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; SR, sarcoplasmic reticulum; CaM, Ca^{2+} /calmodulin; MLC, myosin light chain; MLCP, MLC phosphatase.



generate significant effects on HPAs. These observations are consistent with other reports along these lines of evidence (27).

Change in Ca^{2+} sensitivity. Ca^{2+} -sensitizing mechanisms are also primed under pathophysiological conditions, with ET-1 previously shown to largely enhance the Ca^{2+} sensitivity of myofilaments in smooth muscle cells, including PASMCS (23). This increase in Ca^{2+} sensitivity is transduced into an amplified contractility in each PASM, leading to a stronger tone in HPAs. A better understanding of the modulation of the Ca^{2+} sensitivity is thus of key interest in PH, a disease marked by an increased tonicity of the artery wall. Control and reversal of this Ca^{2+} sensitivity would therefore represent a non-negligible benefit in the treatment of this type of anomaly. In the present study, RvD1 considerably reduced the Ca^{2+} sensitivity induced by ET-1, thereby suggesting that the activation of GPR32 (RvD1 receptor) results in a strong modulation of intracellular Ca^{2+} signaling. The present data also demonstrate that submicromolar concentrations of MAG-DPA were also able to reverse the hyper Ca^{2+} sensitivity induced by ET-1 on HPAs in vitro, implying the perspective that one or more Rv-type metabolites from MAG-DPA could exert this beneficial effect.

RvD1 abolishes the resulting effects induced by proinflammatory conditions. The effect of TNF- α and IL-6 pretreatments on pharmacological reactivity and Ca^{2+} sensitivity was also investigated on HPAs to further assess the putative effects of RvD1. The present findings demonstrate that a proinflammatory status, induced by TNF- α or IL-6 treatment, enhanced the reactivity and Ca^{2+} sensitization of the contractile machinery regardless of the agonist used. Inflammation has indeed been shown to be one of the critical events in the genesis of PH (42). Accordingly, patients treated with anti-inflammatory medications present notable improvement, although very little is known regarding the mechanisms involved. Of significant interest here is the demonstration that a low concentration of

TNF- α or IL-6 consistently induced an increase in arterial tone as well as a log shift in Ca^{2+} sensitivity. Both effects were abolished by 300 nM RvD1 despite the presence of TNF- α or IL-6, strongly suggesting a complete resolution of the molecular events related to the proinflammatory condition.

Mode of action of RvD1 on smooth muscle cells. Our data also suggest that RvD1 displays its effects through a decrease in the activation level of the CPI-17 regulatory protein (lower phosphorylation state), which was also targeted by MAG-DHA and MAG-DPA in independent sets of experiments, as reported above. The ability of RvD1 to decrease the responses to PDBu as well as the data obtained from Western blot analyses attest that the PKC-CPI-17 pathway was likely involved in the downregulation of the isotonic reactivity of HPA smooth muscle myofilaments. Hence, this mode of action, which has previously been delineated in airway smooth muscle cells and HPA tissues, therefore opposed the sensitizing effects of IL-6, TNF- α (29), and ET-1 (34).

The PKC-CPI-17 pathway directly impacts and regulates the contractile machinery, due to an observed higher dephosphorylation rate of MLC when the level of CPI-17 phosphorylation is low (16). These events are translated into a significant decrease in HPA tension.

These results further suggest that RvD1 decreases both the pharmacological responsiveness as well as Ca^{2+} sensitivity of HPAs. Moreover, the present data are consistent with a previous report (24) demonstrating that DHA was able to decrease VEGF production as well as CPI-17, MYPT-1, and ERK1/2 phosphorylation levels in HPAs, which clearly opposed ET-1 effects. According to current knowledge, a change in MYPT-1 phosphorylation is likely to modulate tension, whereas a lower ERK1/2 phosphorylation level is related to a lower proliferation rate (15).

RvD1 and MAG-DPA normalize the expression of TMEM16A. The Ca^{2+} -activated Cl^- channel TMEM16A has been proposed to be a biomarker of PH, since its expression

and activity are upregulated in this disorder (11). Taking into account the role of TMEM16A, we endeavored to identify a cascade of events that could lead to PH. PIP₂ has recently been shown to be an inhibitor of TMEM16A (35). When PIP₂ is hydrolyzed as a result of activation of G protein-coupled receptors by specific agonists such as ET-1, there is an increase in tone. There is also a concomitant downregulation of TMEM16A due to a decrease in PIP₂ in the internal membrane leaflet (35). Under these conditions, TMEM16A would nevertheless be more active, because of the increase in intracellular Ca²⁺ induced by IP₃ from intracellular Ca²⁺ stores. Enhanced cytosolic Ca²⁺ concentrations would activate TMEM16A; the ensuing higher open probability of Cl⁻ channels would increase the anionic membrane conductance and facilitate membrane depolarization (2). Depolarized cell membrane would, in turn, activate voltage-dependent Ca²⁺ channels, allowing Ca²⁺ entry into the smooth muscle cell, thus resulting in a tone increase. Even a slight increase in Ca²⁺ flux would likely increase tone due to the concomitant increases in Ca²⁺ sensitivity in this in vitro model. Interestingly, our findings show that RvD1 decreased the level of TMEM16A expression initially enhanced by ET-1 pretreatment. According to our data, we propose that RvD1 plays an indirect inhibitory role on the expression of this channel protein, although it does not necessarily modify its activation. Hence, Rv treatments could result in putative antihypertensive effects. These observations warrant further study to assess the putative electrophysiological effects of RvD1.

Thus, synthetic compounds such as MAG-DHA and MAG-DPA are of potential interest as biochemical precursors of low cytotoxicity, with the ability to produce Rvs of the D series (Fig. 1) and thereby neutralize or minimize the inflammatory component and overreactivity developed by HPAs under pathophysiological conditions.

Limitations. The present study was performed in vitro on pulmonary arteries from control patients that did not present disease and from which the PH profile (inflamed and hyperreactive vessels) was pharmacologically induced. This can be viewed as a relative limitation since this study could not be performed on tissues from patients with PH given that lobectomy on PH patients is not a recommended procedure. Despite a previous report by Serhan et al. (39), we did not assess the effects of RvD1 on the expression of specific microRNA in HPA tissues, which is therefore a limitation of the present study. Indeed, microR-204 has recently been demonstrated to be involved in the proliferative and antiapoptotic phenotype of PSMCs (7).

Conclusions. Here, we report the first evidence that RvD1 is a potent modulator of triggered pharmacological responsiveness in HPAs in vitro. This biological agent and its precursors were able to reduce Ca²⁺ sensitivity in HPA smooth muscle cells, likely via a reduction in phosphorylation levels of CPI-17 protein and negative regulation of MLCP. RvD1 also decreased the expression of TMEM16A, a specific marker of PH. Taken together, these data provide new insights into the modulating effects and proresolving properties potentially occurring within the pulmonary artery wall. RvD1 and its receptor may therefore represent new prospective and protective clinical targets in countering the overreactivity of inflammatory components in human PH.

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DISCLOSURES

S. Fortin is the CEO of SCF-Pharma and may declare an apparent conflict of interest. He provided us with MAG derivatives (MAG-DHA and MAG-DPA). All other coauthors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

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

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