

Docosapentaenoic acid monoacylglyceride reduces inflammation and vascular remodeling in experimental pulmonary hypertension

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¹SCF Pharma, Ste-Luce, Quebec, Canada; ²Department of Physiology and Biophysics, Faculty of Medicine and Health Sciences, Université de Sherbrooke, Sherbrooke, Quebec, Canada; and ³Department of Biology, Université du Québec à Rimouski, Rimouski, Quebec, Canada

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Morin C, Hiram R, Rousseau E, Blier PU, Fortin S. Docosapentaenoic acid monoacylglyceride reduces inflammation and vascular remodeling in experimental pulmonary hypertension. *Am J Physiol Heart Circ Physiol* 307: H574–H586, 2014. First published June 14, 2014; doi:10.1152/ajpheart.00814.2013.—n-3 Polyunsaturated fatty acids (n-3 PUFA) have been shown to reduce inflammation and proliferation of pulmonary artery smooth muscle cells under pathophysiological conditions. However, the anti-inflammatory effect of the newly synthesized docosapentaenoic acid monoacylglyceride (MAG-DPA) on key signaling pathways in pulmonary hypertension (PH) pathogenesis has yet to be assessed. The aim of the present study was to determine the effects of MAG-DPA on pulmonary inflammation and remodeling occurring in a rat model of PH, induced by a single injection of monocrotaline (MCT: 60 mg/kg). Our results demonstrate that MAG-DPA treatment for 3 wk following MCT injection resulted in a significant improvement of right ventricular hypertrophy (RVH) and a reduction in Fulton's Index (FI). Morphometric analyses revealed that the wall thickness of pulmonary arterioles was significantly lower in MCT + MAG-DPA-treated rats compared with controls. This result was further correlated with a decrease in Ki-67 immunostaining. Following MAG-DPA treatments, lipid analysis showed a consistent increase in DPA together with lower levels of arachidonic acid (AA), as measured in blood and tissue samples. Furthermore, in MCT-treated rats, oral administration of MAG-DPA decreased NF- κ B and p38 MAPK activation, leading to a reduction in MMP-2, MMP-9, and VEGF expression levels in lung tissue homogenates. Altogether, these data provide new evidence regarding the mode of action of MAG-DPA in the prevention of pulmonary hypertension induced by MCT.

docosapentaenoic acid; inflammation; pulmonary hypertension; nuclear factor- κ B; tumor necrosis factor- α

PULMONARY HYPERTENSION (PH) is primarily a disease of the small pulmonary arteries characterized by vascular proliferation, remodeling, and a progressive increase in pulmonary vascular resistance, ultimately leading to right ventricular failure and death (22, 24). PH results from many different underlying causes, and its exact pathophysiology remains unknown. Increasing evidence suggests that inflammation plays a significant role in primary PH. Pathological specimens from patients with PH reveal a mononuclear cell inflammatory infiltration surrounding the plexiform lesions, including macrophages, dendritic cells, T and B lymphocytes, and mast cells (8, 14, 22). Moreover, circulating levels of cytokines, including TNF α , IL-1 β , IL-6, and IL-8 (22, 49), and chemokines such as monocyte chemoattractant protein-1 and RANTES are en-

hanced in patients with PH (3), all of which may correlate with a high morbidity.

The effect of inflammation in PH development has been further confirmed by the fact that clinical improvements have been observed in patients after steroid treatment or immunosuppressor administration (5, 44). However, the potential benefit of anti-inflammatory therapies in PH still remains to be confirmed and requires further investigation.

Clinical assessment of dietary supplementation of ω -3 polyunsaturated fatty acids (n-3 PUFAs), including eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), has shown their beneficial impact in a wide range of cardiovascular diseases (10). n-3 PUFAs have been demonstrated to alter the transcription of specific genes involved in lipogenesis, glycolysis, synthesis of glucose transporters, inflammatory mediators, early response genes, and genes coding for cell adhesion molecules (10). EPA and DHA reduce the expression of genes for interleukin (IL-6, IL-8, and IL-1 β), vascular cell adhesion molecule-1, intracellular adhesion molecule-1, endothelial adhesion molecule, and E-selectin (9, 13, 52). However, docosapentaenoic acid (DPA; 22:5n-3) has not been studied extensively because of the limited availability of the pure compound (27). DPA is an elongated metabolite of EPA and is an intermediary product between EPA and DHA. The available data suggest that DPA displays beneficial health effects. DPA was found to be as effective as EPA and DHA in inhibiting *ex vivo* platelet aggregation in female subjects (43). Moreover, DPA binds and induces the expression of peroxisome proliferator-activated receptor- α , resulting in the reduction lipogenic gene expressions (20, 29). In cell culture models, DPA treatment was shown to reduce expression of inflammatory genes such as tumor necrosis factor- α (TNF α) (30). A clinical study demonstrated that 7-day supplementation with highly purified DPA increased the proportions of DPA in plasma phospholipids (PL) and triacylglycerol (TAG) fractions (28, 37) as well as the proportions of EPA and DHA in TAG fractions (28, 37). Recently, our group has synthesized a new docosahexaenoic acid monoacylglyceride (MAG-DPA) and further demonstrated that in colorectal carcinoma cells MAG-DPA exerts an inhibitory activity on the NF- κ B signaling pathway, thus contributing toward reducing cell proliferation and enhancing apoptosis (42). However, to our knowledge, the effects of DPA in free fatty acid or monoacylglyceride form or of putative epoxy- or polyhydroxy derivatives on key components of PH pathogenesis have never been tested. The aim of the present study was thus to evaluate the effects of MAG-DPA on pulmonary inflammation and remodeling occurring in PH. Fatty acids in monoacylglyceride form are generally recognized as safe and are widely used as emulsifying agents in the

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food industry (40). Moreover, PUFA monoacylglycerides have been demonstrated to increase the oral bioavailability of PUFA compared with commercially available marine oil (15, 16, 40). For the purposes of this study, an *in vivo* model of PH induced by monocrotaline (MCT) was used as experimental setting. Indeed, PH induced by monocrotaline (a plant-derived alkaloid) is a well-established experimental model that is highly similar to human pathology caused by vascular remodeling, proliferation of pulmonary artery smooth muscle cells, endothelial dysfunction, upregulation of inflammatory cytokines, and leukocyte infiltration (44). Herein, we report the first evidence that MAG-DPA given in prevention decreases the level of inflammatory mediators and reduces pulmonary vascular remodeling in an *in vivo* rat model of PH.

MATERIALS AND METHODS

Synthesis of *n*-3 PUFA monoacylglycerides. MAG-DPA, MAG-DHA, and MAG-EPA were synthesized as described previously using highly purified corresponding ethyl ester as starting material. In the resulting molecule, DPA/DHA/EPA is attached at the sn-1 position of glycerol (15, 16).

Animal model of PH. Adult (10 wk) male Wistar rats weighting 200–250 g were obtained from Charles River Laboratories (Montreal, QC, Canada). Rats were housed in our animal facilities in a 12:12-h light-dark cycle at $22 \pm 1^\circ\text{C}$ ambient temperature and maintained on normal rodent chow and tap water *ad libitum*. Rats were acclimated for 7 days before starting the experiments. All studies involving animals were approved by the Institutional Animal Care Committee of the Université du Québec à Rimouski (protocol no. CPA-49-12-105). Rats were randomly assigned into three groups: control (untreated), MCT, and MCT + MAG-DPA treated ($n = 6/\text{group}$). For the induction of PH, rats were injected with a single intraperitoneal dose of MCT (60 mg/kg; MCT rats) and used 3 wk later. MCT (Sigma, St. Louis, MO) was dissolved in an equal volume of HCl (1 M) and NaOH (1 M). Control rats were injected with the same volume of vehicle. The rats were assessed for development of PH 21 days after injection. MAG-DPA (231 mg/kg) was given orally directly to the back of the mouth with a pipette tip. MAG-DPA treatments were administered daily following MCT injection. The oral dose of 231 mg/kg was chosen according to Health Canada Draft Guidelines to obtain a human equivalent dose of 3.0 g/day (maximum allowed by Health Canada) (39, 40, 42, 45). Control and MCT rats were received a sham dose each day. On *day 22*, all three groups of rats were euthanized by lethal dose of pentobarbital sodium, and blood was collected by cardiac puncture. The heart was excised, and both ventricles were weighed. The right ventricular free wall was subsequently dissected, and the remaining left ventricular wall and ventricular septum were weighed. Right ventricular hypertension was assessed as Fulton's Index (ratio of right ventricular weight to the left ventricular + septum weight) and as the ratio of right ventricular weight (mg) to total body weight (g). The lung tissues were harvested for histological analysis and for homogenate preparation for Western blot experiments, as described previously (42).

Fatty acid composition of plasma and tissues using gas chromatography/flame ionization detector. Plasma and tissue fatty acid compositions were measured using a modified direct transesterification method in which toluene was used instead of benzene and acetyl chloride was replaced by sulfuric acid (32). Fatty acids were chromatographed as methyl esters on a 60-m fused silica column with an internal diameter of 0.25 mm. The column was wall-coated with 0.15 mm DB-23. Analysis was performed on a Thermo scientific Trace GC Ultra gas chromatograph equipped with a flame ionization detector. Helium was used as carrier gas and nitrogen as a makeup gas with a split ratio of 20:1. Injection port and detector temperatures were 230 and 280°C , respectively. The oven temperature was programmed to

initiate at 50°C for 1 min, after which the temperature was first raised to 140°C at a rate of $25^\circ\text{C}/\text{min}$ and then raised to 195°C at a rate of $3^\circ\text{C}/\text{min}$ and subsequently maintained for 5 min and finally increased to 225°C at a rate of $4^\circ\text{C}/\text{min}$ and maintained at this final level for 5 min. The gas chromatograph was calibrated using a standard mixture (Supelco 37 Component FAME Mix).

On-line solid-phase extraction-LC-MS/MS analysis of DPA metabolites. DPA metabolites were detected in lungs homogenates using the method described by Kortz et al. (31). An 1100 series HPLC system coupled with a tripleQuad 6410 mass spectrometer (Agilent) with electrospray ionization in negative ion mode was used. RvD5_{n-3} DPA (11) was quantified by multiple reaction monitoring (MRM) using the transition ($361.2 \rightarrow 143.2$).

Histological analysis. The rats' lungs were fixed in 10% buffered formalin and paraffin embedded, after which thin sections (3 μm thick) were stained with hematoxylin and eosin according to standard protocols (39). Images were acquired with a Hamamatsu ORCA-ER digital camera attached to a Nikon Eclipse TE-2000 inverted microscope (Nikon-Canada, Mississauga, ON, Canada). Images were obtained ($\times 20$ objective) from lung sections derived from control, MCT, and MCT + MAG-DPA-treated rats. To reveal vascular remodeling, artery wall thickness was determined by image analyses of lung tissue sections derived from control and treated animals.

Western blot analysis. Western blots using specific antibodies against the phosphorylated forms of p65 NF- κB (p-p65 NF- κB) and p38 MAPK (p-p38 MAPK), as well as NF- κB , p38 MAPK, $\text{I}\kappa\text{B}\alpha$, metalloproteinase (MMP)-2 and MMP-9, vascular endothelial growth factor (VEGF), and β -actin proteins, were performed on homogenate fractions or cell lysates, as described previously (38, 39, 41). Immunostaining of the blots were digitized and analyzed with Lab Image software 2.7.

ELISA assays. Measurements of inflammatory mediators were determined in serum by specific ELISA assays for TNF α and IL-8 according to the manufacturer's instructions (R & D Systems, Minneapolis, MN).

Immunofluorescence. Lung samples were obtained from control, MCT, and MCT + MAG-DPA-treated rats. Tissues were fixed in 10% paraformaldehyde-buffered solution for 24 h and embedded in paraffin. Multiple 3- μm -thin sections from each block were prepared. After removal of paraffin and rehydration, tissue sections were processed according to classical histological procedures. Sections were incubated with anti-Ki-67 (Abcam) overnight at 4°C . After washes, slides were incubated for 60 min at room temperature with a secondary conjugated anti-IgG antibody coupled with 570 nm of Alexa Fluor. Following 4,6-diamidino-2-phenylindole (DAPI) counterstaining, slides were then mounted with coverslips using Vectashield fluorescence mounting media for subsequent observations. Images were acquired with a Hamamatsu ORCA-ER digital camera attached to a Nikon Eclipse TE-2000 inverted microscope (Nikon-Canada) equipped for epi-illumination. Image analyses were performed for immunopositive Ki-67 staining in lung sections using MetaMorph 7.6 software (Molecular Devices, Silicon Valley, CA). Pixel size was unrestricted, and the automatic find function was set to search for immunopositive pixels using smooth edges. Images were obtained ($\times 20$ objective) from lung sections derived from control, MCT, and MCT + MAG-DPA-treated rats.

Cell culture. Primary human pulmonary endothelial cells were obtained from Lonza (Lonza, Allendale, NJ). Human pulmonary artery endothelial cells (HPAEC) were maintained in EGM-2 medium (Lonza). Cells were grown in a 5% CO_2 incubator at 37°C . Cells were untreated or treated with either MAG-DPA in the absence or presence of TNF α (10 ng/ml).

Isolation and culture of rat pulmonary artery myocytes. Rat pulmonary arteries were dissected and placed in Hanks' balanced salt solution containing antibiotics (100 U/ml penicillin and 100 $\mu\text{g}/\text{ml}$ streptomycin). Vessels were cut longitudinally and dissected into small fragments. Tissues were transferred in a tube containing Hanks'

balanced salt solution supplemented with 0.1% type IV collagenase and 0.05% type IV elastase at 37°C during 45 min. The solution was then centrifuged and the pellet suspended in 5 ml of DMEM-F-12 medium supplemented with 10% fetal bovine serum and 0.3% antibiotics. Culture flasks were placed in a 37°C incubator (5% CO₂). To validate the quality of the smooth muscle cell preparation, α -actin smooth muscle staining was performed and revealed that 95% of cells were positive for this marker (38).

Cell proliferation and apoptosis assay. Cell proliferation analyses were performed using the BrdU cell proliferation assay kit according to the manufacturer's instructions (New England BioLabs, Pickering, ON, Canada). This kit detects the level of 5-bromo-2'-deoxyuridine (BrdU) incorporated into cellular DNA during cell proliferation using an anti-BrdU antibody. Apoptosis analyses were performed using a cleaved caspase-3 ELISA assay performed on a cell lysate derived from control and TNF α -treated cells in the absence and presence of MAG-DPA according to the manufacturer's instructions (New England BioLabs).

Data analysis and statistics. Results are expressed as means \pm SE, with *n* indicating the number of experiments. Statistical analyses were performed using Sigma Plot 11 and SPSS 14.0 (SPSS-Science, Chicago, IL) via one-way ANOVA followed by Dunnett's post hoc test. Differences were considered statistically significant when *P* < 0.05.

RESULTS

Effect of MAG-DPA on right ventricular hypertrophy and lipid analyses. To test the effect of MAG-DPA treatment in our MCT-induced pulmonary hypertension model, we first assessed right ventricular hypertrophy (RVH) using Fulton's Index (FI) and the ratio of right ventricular weight to total body weight. Results showed that FI increased significantly from 0.24 ± 0.01 in control rats to 0.46 ± 0.03 at 3 wk following MCT exposure (Fig. 1A). Treatment with MAG-DPA during the 3 wk following MCT exposure resulted in significant improvement in RVH and a reduction in FI (0.22 ± 0.01). Similar findings were observed when RVH was assessed as the ratio of right ventricular weight to total body weight (Fig. 1B). Moreover, after 21 days, rats in the MCT groups had significantly lower body weights (326.2 ± 12.6 g) compared with control animals (384.0 ± 9.3 g, *P* = 0.002). However, MAG-DPA treatment following MCT exposure significantly recovered the loss in body weight observed in untreated MCT rats (374.1 ± 6.2 vs. 326.2 ± 12.6 g, *P* = 0.04; Fig. 1C).

Effect of MAG-DPA on relative fatty acid content in blood and tissues. Pulmonary and cardiovascular diseases are known to be associated directly with an increase in ω -6 and a reduction in ω -3 fatty acid levels in blood circulation and tissues (48). Fatty acid content was thus determined by gas chromatography/flame ionization detector in plasma, red blood cell, lung, and heart samples derived from control, MCT-treated, and MCT + MAG-DPA-treated animals. Lipid analyses revealed an increase in arachidonic acid (AA) ω -6 level in plasma and red blood cells derived from MCT-treated rats compared with the level found in control animals (Fig. 2, A and B). However, a decrease in AA and an increase in DPA levels in plasma and red blood cells derived from MCT + MAG-DPA-treated rats was quantified compared with levels found in MCT-treated animals (Fig. 2, A and B). Lung and heart tissue analyses demonstrated an increase in AA level in tissues derived from MCT-treated animals compared with tissues from control rats (Fig. 2, C and D). Lipid analyses of lung and heart tissues also demonstrated a decrease in AA accompanied by an

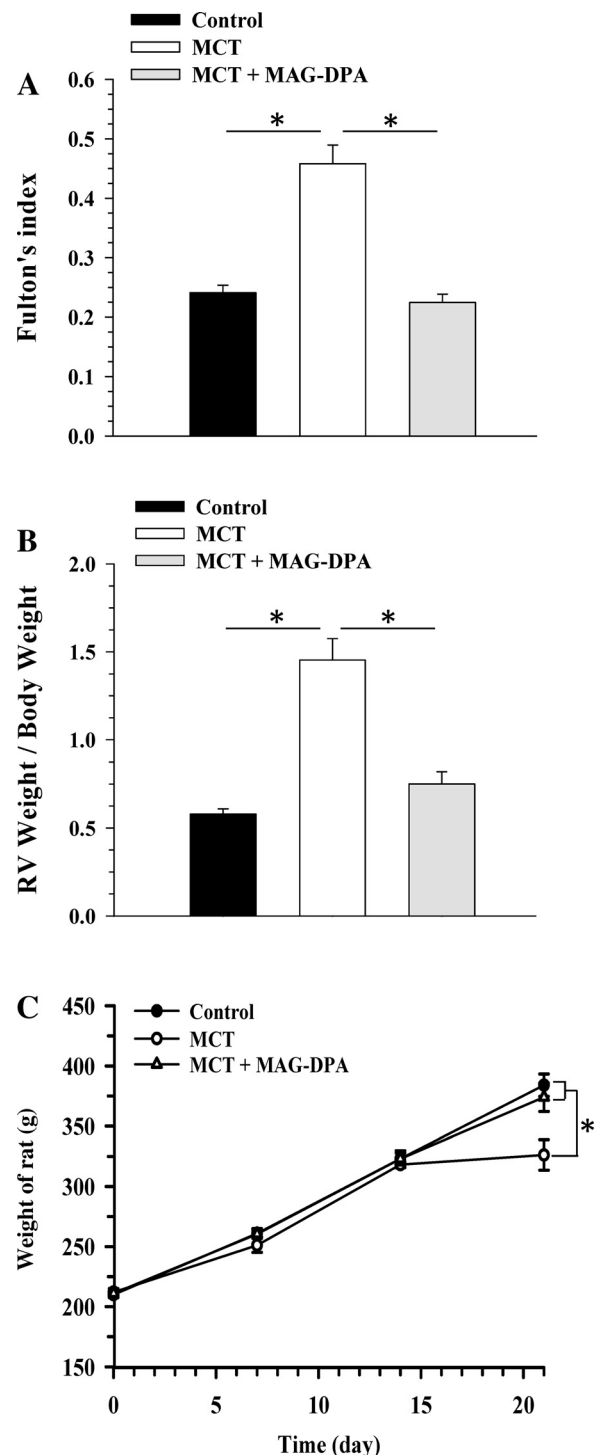


Fig. 1. Effect of docosapentaenoic acid monoacylglyceride (MAG-DPA) on right ventricular hypertrophy (RVH) in a monocrotaline (MCT)-treated rat model of pulmonary hypertension. A and B: RVH was assessed by Fulton's index (ratio of right ventricular weight to left ventricular + septal weight; A) and as the ratio of right ventricular weight to total body weight (B). Data represent means \pm SE from 6 rats/experimental group. *Values from MCT-treated rats significantly different (*P* \leq 0.05) from corresponding values in control (vehicle-treated) rats. C: weight (g) as a function of time (day) was measured in control, MCT-treated, and MCT + MAG-DPA-treated rats. Results represent means \pm SE (*n* = 6/group).

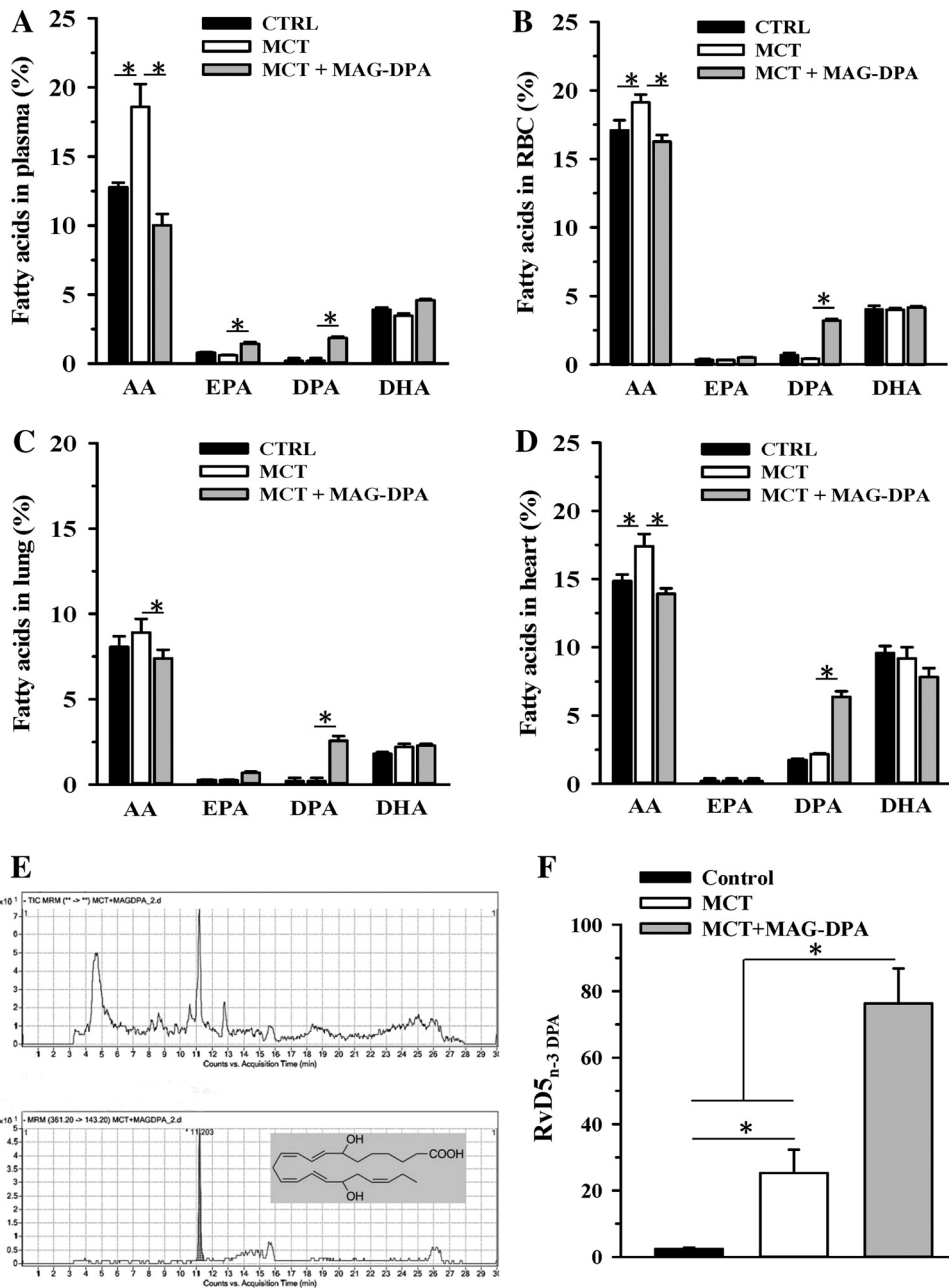


Fig. 2. Effect of MAG-DPA on blood and tissue lipid content in a MCT-treated rat model of pulmonary hypertension (PH). Quantitative analysis of relative fatty acid content (%) in plasma (A), red blood cells (RBC; B), lung (C), and heart (D) derived from control (CTRL), MCT-induced PH-treated, and MCT-induced PH + MAG-DPA-treated rats ($n = 6/\text{group}$). E: representative chromatogram of the total ion current (TIC) and extracted multiple reaction monitoring (MRM) chromatogram of the transition ($361.2 \rightarrow 143.2$) used for the quantification of RvD5_{n-3} DPA. These 2 traces were derived from a lung tissue sample of a MCT + MAG-DPA-treated animal. F: quantitative analysis of RvD5_{n-3} DPA found in lung tissue derived from CTRL, MCT-treated, and MCT + MAG-DPA-treated rats ($n = 6$). * $P \leq 0.05$. AA, arachidonic acid; EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid; DHA, docosahexaenoic acid.

increase in DPA fatty acid levels in tissues derived from MCT + MAG-DPA-treated rats compared with samples from MCT-treated animals (Fig. 2, C and D). Of note is that no difference was quantified in DHA and EPA fatty acid levels in MCT + MAG-DPA-treated rats. Together, these results demonstrate that following absorption of MAG-DPA, consistent increases in DPA levels as well as decreased AA levels were systematically observed in blood and tissue samples. DPA metabolites were determined by solid-phase extraction (SPE)-LC-MS/MS in lung tissue derived from control, MCT-treated, and MCT + MAG-DPA-treated rats. Figure 2E illustrates a representative chromatogram of the total ion current and extracted MRM chromatogram of the transition ($361.2 \rightarrow 143.2$) used for the quantification of resolvin RvD5_{n-3} DPA. These two traces were derived from lung tissue samples of MCT + MAG-DPA

treated animals. SPE-LC-MS/MS analyses revealed the presence of 7-hydroxy-DPA ($345.2 \rightarrow 143.2$), 17-hydroxy-DPA ($345.2 \rightarrow 247.2$), protectin PDI_{n-3} DPA ($361.2 \rightarrow 183.2$), and RvD5_{n-3} DPA ($361.2 \rightarrow 143.2$) in lung tissues derived from the three animal groups. Quantitative analysis revealed a significant increase in RvD5_{n-3} DPA concentration in lung tissues derived from MCT + MAG-DPA-treated rats (76.37 ± 10.51 pg/mg tissue) compared with the levels found in control (2.41 ± 0.44 pg/mg tissue) and MCT (25.27 ± 7.06 pg/mg tissue) animals (Fig. 2F).

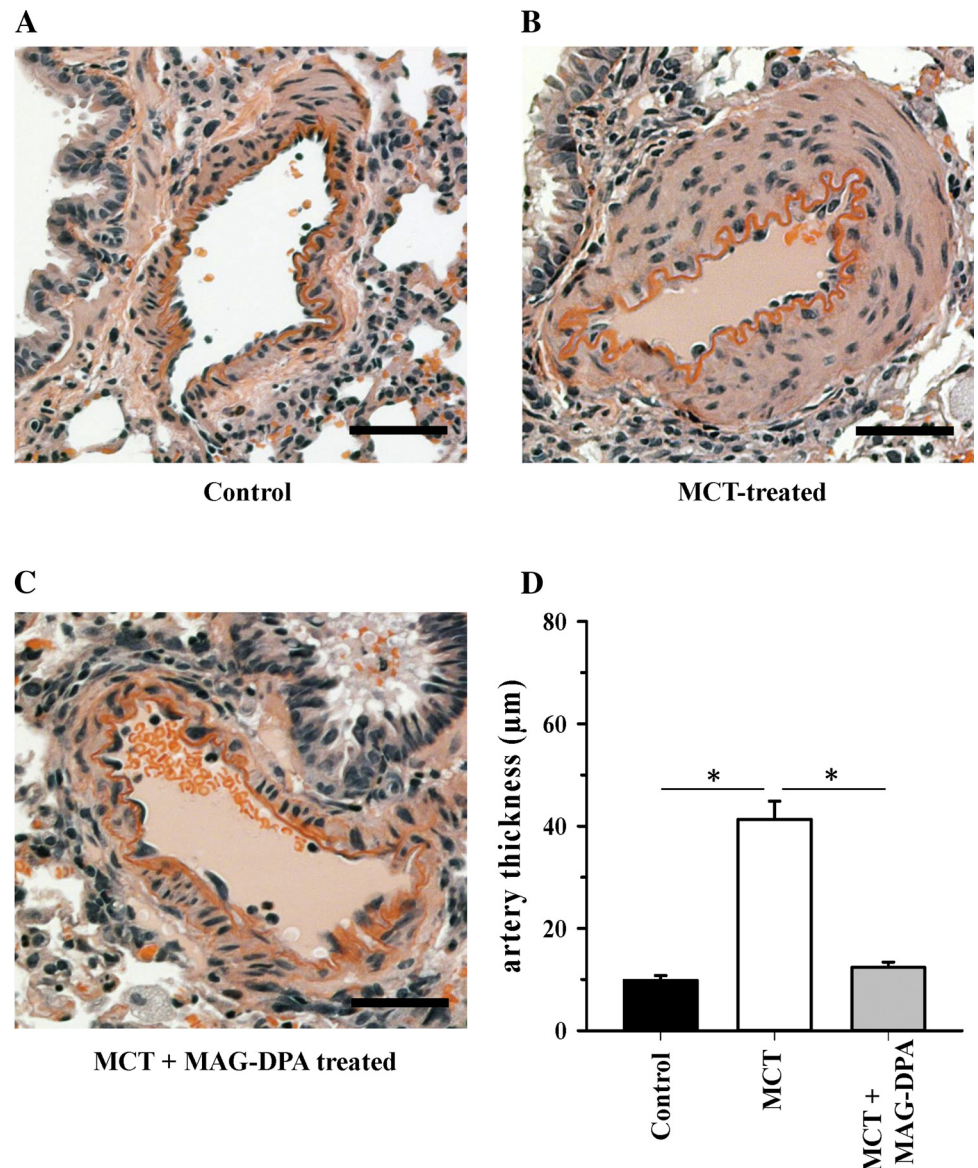
Effect of MAG-DPA on pulmonary artery thickness in MCT-treated rats. Increased wall thickness of pulmonary arterioles is a key structural feature of MCT-induced PH, as evidenced by the remodeling of small pulmonary arteries, vascular cell proliferation, and obliteration of the pulmonary

microvasculature (22). To define the level of pulmonary vascular remodeling, morphometric analysis was performed on lung tissue sections derived from all three experimental groups. Histological analyses on lung sections stained with hematoxylin and eosin revealed an increase in arteriole media and adventitia thickness in MCT-treated rats compared with control rats (Fig. 3, *A* and *B*). In contrast, in MCT rats treated with MAG-DPA, pulmonary vascular remodeling was markedly reduced, and arteriole wall thickness was comparable with that of control samples (Fig. 3, *A–C*). Quantitative morphometric analyses confirmed that pulmonary arteriole wall thickness was significantly greater in MCT-exposed rats compared with vehicle-treated control animals (Fig. 3*D*). However, 3-wk MAG-DPA treatment following MCT injection significantly attenuated the wall thickness of pulmonary arterioles (Fig. 3*D*).

Effect of MAG-DPA on NF- κ B activation and proinflammatory mediator expression. Substantial evidence suggests that inflammation plays a significant role in PH. Several studies

have revealed the existence of inflammatory cells surrounding pulmonary remodeled arteries and correlated with increased levels of proinflammatory mediators such as TNF α , IL-1 β , IL-6, and IL-8 in patients with PH and in experimental animal models of PH. To determine the molecular pathways involved in MAG-DPA intervention in MCT-induced pulmonary hypertension, protein levels of I κ B α and NF- κ B (both total and phosphorylated forms) were analyzed in lung homogenates by Western blotting. Activation of NF- κ B is usually correlated with a reduction in I κ B α due to extensive ubiquitination and proteosomal degradation of this inhibitory subunit, thereby resulting in increased nuclear translocation of the p65 NF- κ B subunit (26). Western blot and quantitative immunoblot analyses revealed that MCT injection resulted in I κ B α degradation and appearance of phosphorylated p65 subunit staining in lung fractions compared with preparations derived from control rats (Fig. 4*A*). However, MAG-DPA treatments prevented I κ B α degradation and decreased the phosphorylation level of p65 NF- κ B in lung tissues comparatively with that observed in the

Fig. 3. Effect of MAG-DPA on pulmonary artery wall thickness in MCT-treated rats. Representative lung tissue sections stained with hematoxylin and eosin obtained from control (*A*), MCT-treated (*B*), and MCT + MAG-DPA-treated rats (*C*). Scale bar, 50 μ m. Images are representative of 6 lungs from each group ($n = 6$ /group). *D*: quantitative morphometric analysis of wall thickness of pulmonary arteries derived from control, MCT-treated, and MCT + MAG-DPA-treated animals. Fifteen pulmonary arteries from 6 rats/experimental group were analyzed ($*P \leq 0.05$).



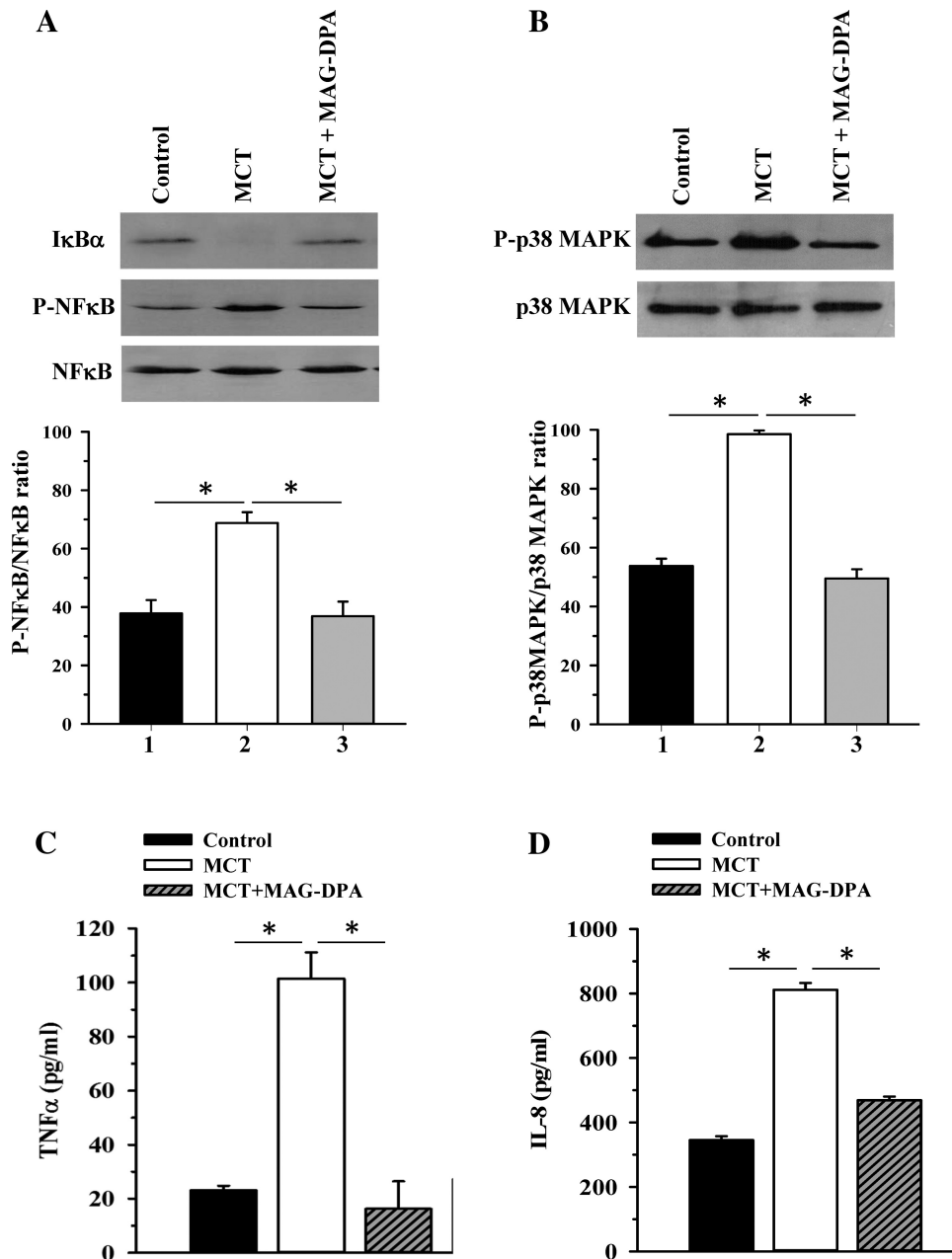


Fig. 4. Effect of MAG-DPA on p65 NF- κ B, p38 MAPK, TNF α , and IL-8 expression in lung tissue. *A*: typical Western blot and subsequent quantitative analysis of lung homogenate fractions derived from control, MCT-treated, and MCT + MAG-DPA-treated rats using specific antibodies against the phosphorylated form of p65 NF- κ B (p-NF κ B) and total NF- κ B. Staining densities in the homogenates were expressed as a function of NF- κ B signals for p-NF- κ B ($n = 6$ for each experimental condition). *B*: Western blot and quantitative analysis of lung homogenates derived from control, MCT, and MCT + MAG-DPA using specific antibodies against the phosphorylated form and total form of p38 MAPK ($n = 6$ for each experimental condition). *C* and *D*: TNF α (*C*) and IL-8 levels (*D*) in plasma were assessed using ELISA, as described in MATERIALS AND METHODS. MAG-DPA significantly decreased circulating TNF α and IL-8 levels compared with mean levels in the MCT-treated group. Results are expressed as means \pm SE of 6 animals for each group. * $P \leq 0.05$.

MCT-treated group (Fig. 4A). A significant reduction of 53% was quantified in MAG-DPA-treated rats following comparative analysis of the phosphorylated form of p65 NF- κ B (p-NF- κ B)/total NF- κ B ratio following normalization of identical immunoblot membrane areas (Fig. 4A).

Emerging evidence points to an important role of p38 MAPK activation in vascular inflammation, endothelial dysfunction, vascular remodeling, and smooth muscle contraction in hypertensive animal models (4, 53, 56). Therefore, we have examined the phosphorylation level of p38 MAPK in lung homogenate derived from control, MCT-treated and MCT + MAG-DHA-treated rats. Western blot analysis revealed an increased phosphorylation of p38 MAPK in preparations derived from MCT rats compared with the level detected in lung homogenates from control rats (Fig. 4B). However, p38 MAPK phosphorylation level was decreased in preparation derived

from the MCT + MAG-DHA group compared with the MCT-treated group (Fig. 4B). A significant reduction of $39 \pm 2.1\%$ was quantified in MAG-DHA-treated rats following comparative analysis of p-p38 MAPK/total p38 MAPK ratio after normalization of identical immunoblot membrane areas (Fig. 4B, bottom).

The level of TNF α and IL-8, also known to play a pivotal role in PH and pulmonary vascular remodeling (14, 23), was determined by specific ELISA in the three animal groups. As can be seen in Fig. 4C, MAG-DPA prevented the MCT-induced increase in TNF α level found in sera compared with the level detected in the MCT group. Moreover, no significant difference was quantified between MCT + MAG-DPA-treated and control animals (Fig. 4C). As shown in Fig. 4D, MAG-DPA treatments significantly decreased the level of IL-8 in sera by $\sim 36\%$ compared with that

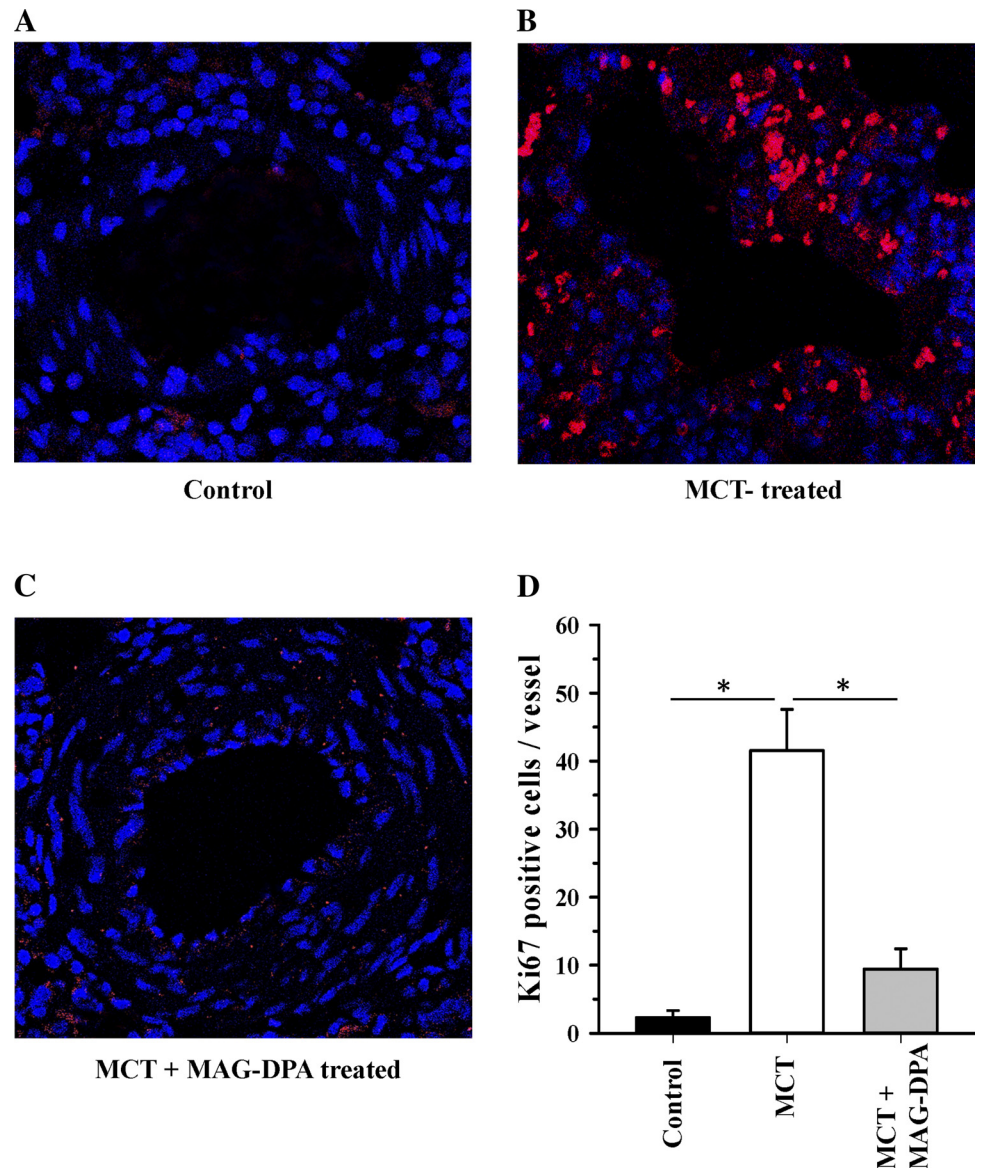
detected in the sera of MCT animals. Taken together, these data further demonstrate that MAG-DPA treatment is able to reduce pulmonary inflammation induced by MCT in a rat model of PH.

Effect of MAG-DPA on pulmonary artery cell proliferation. To elucidate the mechanism underlying the MAG-DPA-associated reduction in vascular remodeling, immunostaining with Ki-67, a nuclear proliferation marker, was performed to assess cell proliferation on lung thin sections derived from control, MCT-treated and MCT + MAG-DPA-treated animals. Image analyses of fluorescence microscopy following Ki-67 (red) and DAPI counterstaining (blue) revealed increased levels of proliferative cells in lung tissues derived from MCT-exposed rats compared with the level found in control rat lung tissues (Fig. 5, A and B). On the other hand, MAG-DPA treatment reduced the number of proliferative cells in pulmonary arteries following MCT exposure (Fig. 5C). Moreover, a similar level of proliferative cells was observed in lung tissues derived from MCT + MAG-DPA-treated and control animals (Fig. 5, A and

C). Quantitative analysis revealed $9.4 \pm 2.9\%$ of Ki-67-positive cells per vessel in lung sections derived from MCT + MAG-DPA-treated rats compared with $41.5 \pm 6.1\%$ in lung sections from MCT-treated rats and $2.5 \pm 0.4\%$ in control rats (Fig. 5D).

Effect of MAG-DPA on metalloproteinase and VEGF protein expression in the lung. Matrix metalloproteinases such as MMP-2 and MMP-9 are known biomarkers in PH. Moreover, increased expression of both these markers is found in pulmonary vascular remodeling. Therefore, experiments were performed to assess the effect of MAG-DPA treatment on MMP-2 and MMP-9 expression levels in lung homogenates derived from control, MCT-treated, and MCT + MAG-DPA-treated rats. Western blot analyses revealed a reduction in the expression levels of MMP-2 and MMP-9 following MAG-DPA treatment when compared with MCT-treated rats (Fig. 6A). Quantitative analysis of MMP/ β -actin ratio after normalization of identical immunoblot membrane areas revealed a significant

Fig. 5. Effect of MAG-DPA on cell proliferation in pulmonary arteries in a MCT-treated rat model of pulmonary hypertension. A–C: fluorescence microscopy following Ki-67 (red) and 4,6-diamidino-2-phenylindole counterstaining (blue) was performed on lung sections derived from control (A), MCT-treated (B), and MCT + MAG-DPA-treated rats (C). Images are representative of 6 experiments on multiple thin sections for each preparation (scale bar, 20 μ m). D: bar graph displaying the percentage of positive Ki-67 cells per vessel calculated for control, MCT-treated, and MCT + MAG-DPA-treated rats. ($n = 6$ /group). * $P \leq 0.05$.



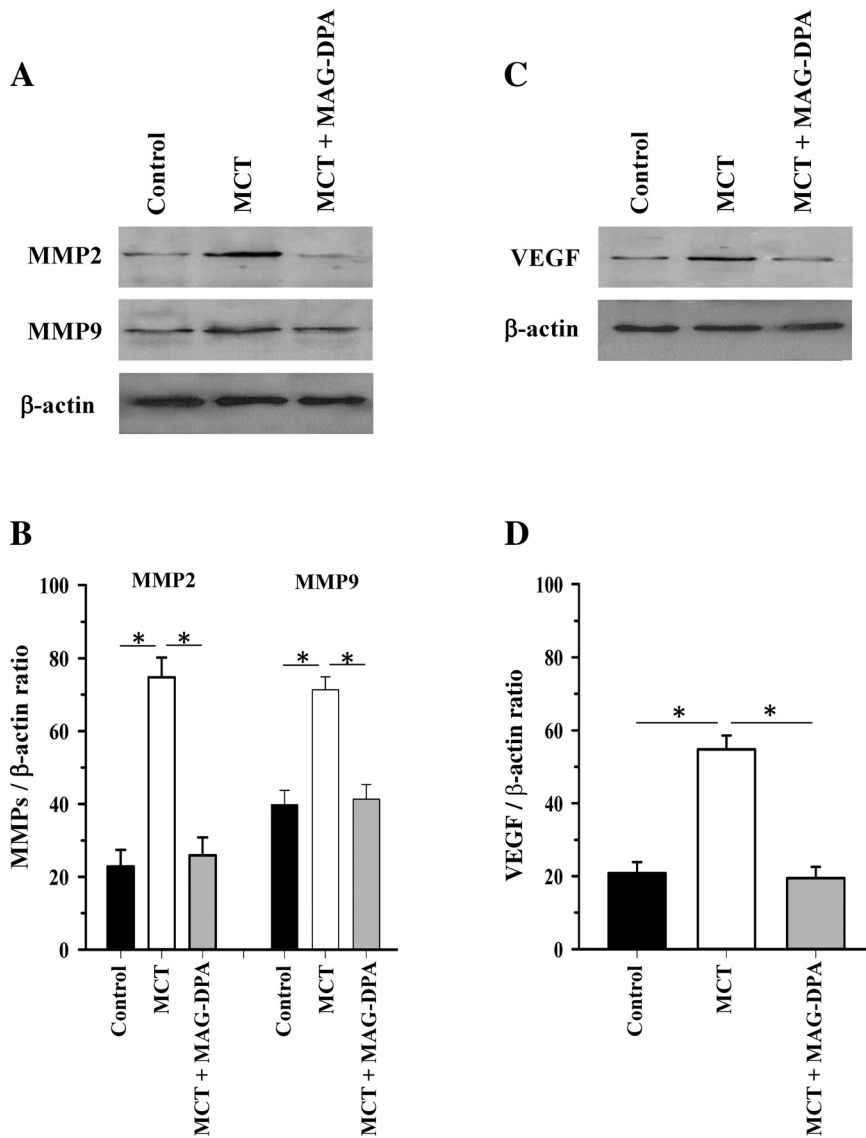


Fig. 6. Effect of MAG-DPA on metalloproteinase (MMP)-2, MMP-9, and VEGF expression in lung tissue. *A*: Western blot analysis of lung homogenate protein fractions derived from control, MCT-treated, and MCT + MAG-DPA-treated rats, using specific antibodies against MMP-2, MMP-9, and β -actin. *B*: quantitative analysis of MMP-2/ β -actin and MMP-9/ β -actin density ratios as a function of experimental conditions ($n = 6$). *C*: Western blot analysis of lung homogenate protein fractions derived from control, MCT-treated, and MCT + MAG-DPA-treated rats, using specific antibodies against VEGF and β -actin. *D*: quantitative analysis of VEGF/ β -actin density ratios as a function of experimental conditions ($n = 6$). * $P \leq 0.05$.

reduction of 66% in MMP2/ β -actin ratio and 42% in MMP9/ β -actin ratio (Fig. 6B).

Since VEGF is known to act as a potent mitogen for endothelial and smooth muscle cells, Western blot analyses were also performed to assess the expression level of VEGF in lung homogenates derived from control, MCT-treated, and MCT + MAG-DPA-treated rats. Analyses revealed a reduced VEGF expression level in lung homogenates derived from MCT + MAG-DPA-treated rats compared with MCT-treated rats (Fig. 6C). β -Actin staining remained constant under the different experimental conditions (Fig. 6, A and C). Upon quantitative comparative analysis of VEGF/ β -actin ratio after normalization of identical immunoblot membrane areas (Fig. 6D), the results showed that MAG-DPA decreased VEGF expression levels significantly, thereby suggesting a role for MAG-DPA in the reduction of cell proliferation and remodeling of pulmonary arteries.

Effect of MAG-DPA on proliferation of HPAEC and pulmonary artery smooth muscle cells. Complementary experiments were performed to assess the effects of MAG-DPA treatments

on HPAEC and rat pulmonary artery smooth muscle (PASM) cells. The cells were treated for 48 h with increasing concentrations of MAG-DPA (0.003, 0.01, 0.03, 0.1, 0.3, 1, 3, 10, and 30 μ M), after which cell proliferation rate was assessed by BrdU incorporation assay. Cumulative concentration response curves revealed that MAG-DPA decreased the proliferation rate of HPAEC and PASM cells, with IC_{50} values of 0.83 ± 0.04 and 0.51 ± 0.03 μ M, respectively (Fig. 7A). Complementary experiments were performed to evaluate the effect of 100 nM RvD5_{n-3} DPA on proliferation of HPAEC and PASM cells. The results demonstrated that RvD5_{n-3} DPA treatments decreased TNF α -induced proliferation of HPAEC and PASM by 63 and 50%, respectively. The effect of MAG-DPA treatment on caspase-3 activation has been quantified by ELISA. This assay identified the level of cleaved caspase-3 detected. Results demonstrated no significant difference in caspase-3 activation following 0.3- and 3- μ M MAG-DPA treatments; however, 30 μ M MAG-DPA induced the activation of caspase-3 in HPAEC and PASM cells (Fig. 7B). Etoposide was used as a positive control to induce caspase-3 activation in both cell lines. West-

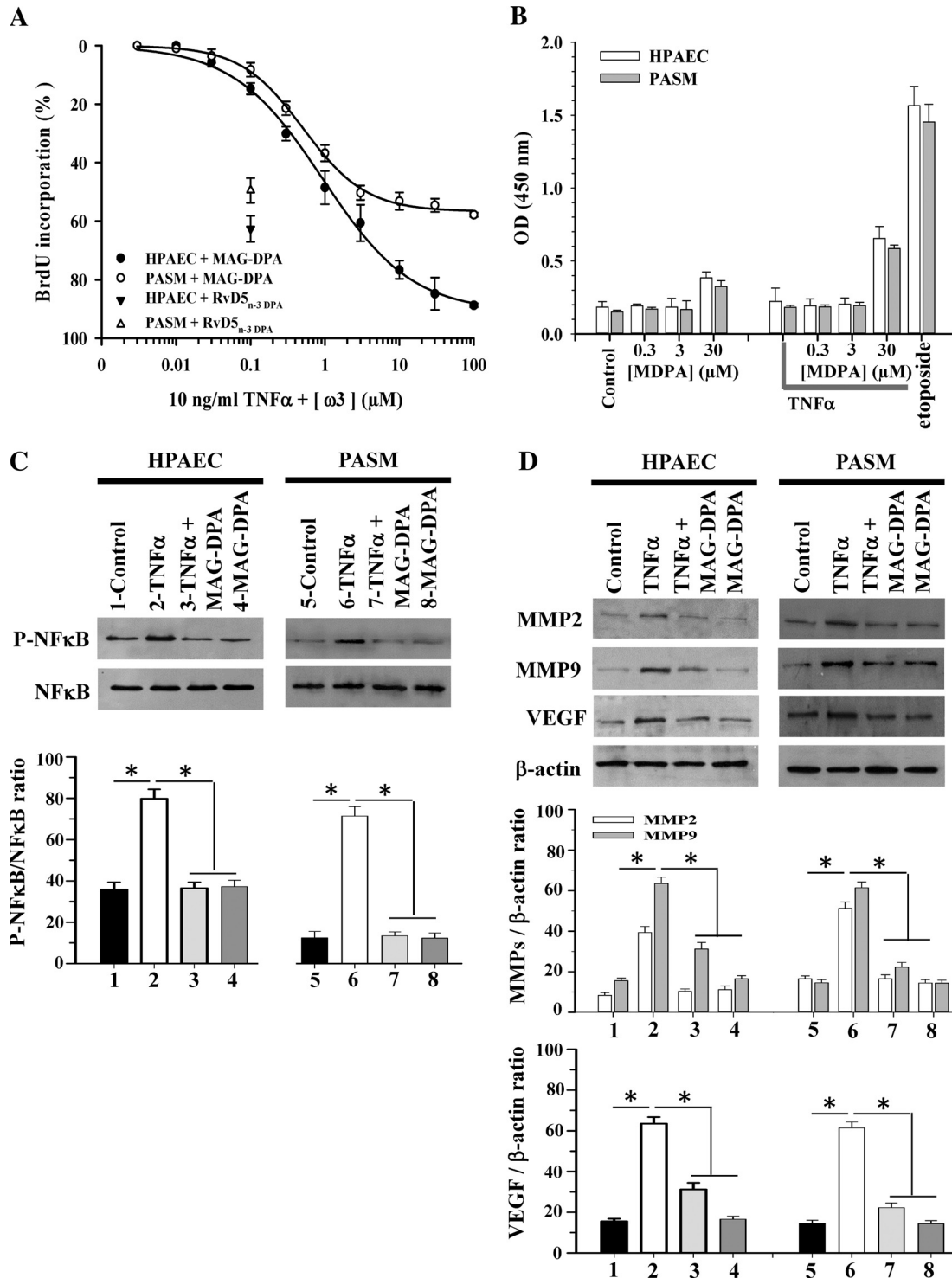


Fig. 7. Effect of MAG-DPA treatments on cell proliferation, p65 NF- κ B phosphorylation, and MMP-2 and MMP-9 protein expression in human pulmonary artery endothelial cells (HPAEC) and rat pulmonary artery smooth muscle (PASM) cells. *A*: cumulative concentration-response curves displaying the inhibitory effects induced by MAG-DPA and 100 nM RvD5_{n-3}DPA [following 5-bromo-2'-deoxyuridine (BrdU) proliferation assay in 10 ng/ml TNF α -treated HPAEC and PASM ($n = 6$ for each experimental condition)]. *B*: cleaved caspase-3 level was assessed using an ELISA assay. There was no significant difference in caspase-3 activation following 0.3- and 3- μ M MAG-DPA treatments in either untreated or TNF α -treated HPAEC and PASM cells. However, 30 μ M MAG-DPA induced the activation of caspase-3 in untreated and TNF α -treated HPAEC and PASM cells ($n = 6$ for each group). *C*: typical Western blots and subsequent quantitative analysis of HPAEC and PASM cell homogenate fractions derived from control, TNF α -treated, TNF α + MAG-DPA-treated, and MAG-DPA-treated cells, using specific antibodies against the phosphorylated form of p65 NF- κ B (p-NF- κ B) and total NF- κ B. Staining densities in the homogenates were expressed as a function of NF- κ B signals for p-NF- κ B ($n = 6$). *D*: Western blot analysis of HPAEC and PASM cell homogenate protein fractions derived from control, TNF α -treated, TNF α + MAG-DPA-treated, and MAG-DPA-treated cells using specific primary antibodies against MMP-2, MMP-9, VEGF, and β -actin. Quantitative analysis of MMP-2/ β -actin, MMP-9/ β -actin, and VEGF/ β -actin density ratios as a function of experimental conditions ($n = 6$). Nos. 1-4 and 5-8 represent the same experimental conditions depicted above the blots in *C*. * $P \leq 0.05$.

ern blot analyses performed on cell homogenates derived from control, TNF α (10 ng/ml), TNF α + 1 μ M MAG-DPA, and 1 μ M MAG-DPA-treated cells using antibodies against total and phosphorylated forms of p65 NF- κ B revealed that MAG-DPA treatment of TNF α -stimulated cells decreased the phosphorylation level of p65 NF- κ B significantly compared with expression levels in TNF α -treated HPAEC only (Fig. 7C, left). In rat PASM cells, the same concentration of MAG-DPA also reduced the phosphorylation level of p65 NF- κ B compared with TNF α -treated condition (Fig. 7C, right). Expression levels of total p65 NF- κ B were fairly constant from one preparation to the other. Western blot analyses performed on cell homogenates derived from similar preparations using antibodies against MMP-2 revealed that MAG-DPA treatments decreased the expression levels of MMP-2 and MMP-9 significantly in TNF α -treated HPAEC and PASM cells compared with the expression levels in TNF α -treated cells only (Fig. 7D). Moreover, TNF α + MAG-DPA treatment resulted in a reduction in the expression level of VEGF in both cell types compared with its expression in the corresponding TNF α -treated cells (Fig. 7D).

DISCUSSION

In the present study, we investigated the ability of a 3-wk per os treatment of MAG-DPA to prevent pulmonary inflammation and remodeling in an in vivo model of pulmonary hypertension induced by MCT. The resulting data demonstrated that MAG-DPA treatment markedly reduced pulmonary artery remodeling and reversed right ventricular hypertrophy induced by MCT. MAG-DPA was also found to reduce pulmonary artery cell proliferation via a reduction in TNF α , IL-8, MMP-2, MMP-9, and VEGF expression levels likely mediated via the inactivation of the NF- κ B pathway. Thus, we propose that MAG-DPA is able to fine-tune the transcription processes of regulatory proteins involved in the modulation of pulmonary artery inflammation and remodeling typical of pulmonary hypertension.

Inflammation and pulmonary hypertension. The link between pulmonary inflammation and vascular remodeling observed in PH pathology is not well understood. Anti-proliferative and anti-inflammatory agents that suppress vascular remodeling should in essence provide long-term beneficial effects in pulmonary hypertension (46). Nevertheless, present therapeutic options for PH remain limited despite the introduction of prostacyclin analogs, endothelin-1 receptor antagonists, and phosphodiesterase 5 inhibitors over the past 15 years (36, 46). Indeed, these interventions predominantly address the endothelial and vascular dysfunction associated with the condition but merely delay the progression of the disease rather than offer a cure (36). n-3 PUFAs have been demonstrated to alter the transcription of specific genes involved in lipogenesis, glycolysis, synthesis of glucose transporters, inflammatory mediators, early response genes, and genes for cell adhesion molecules (25). n-3 PUFA may directly or indirectly trigger anti-inflammatory effects on the pattern of inflammatory cytokines (i.e., TNF α , IL-1 β , IL-6, IL-8, and interferon- γ) produced by different cell types at sites of inflammation (19, 25, 33). However, levels of n-3 PUFA are decreased in certain airway illnesses characterized by excess airway inflammation, such as severe asthma and cystic fibrosis (17). In this study, we

evaluated the ability of MAG-DPA to prevent the inflammation and vascular pulmonary artery remodeling in an in vivo model of pulmonary hypertension induced by MCT. Fatty acids in monoacylglyceride form confer increased bioavailability of ω -3 and are generally recognized as safe as well as widely used as emulsifying agent in the food industry. The biochemical effects of n-3 DPA have not been extensively studied due to their limited availability and the high cost of pure compound. In light of the present literature, however, it can be speculated that the physiological consequences of using DPA in tissues may be related to the production of DPA and EPA and to inhibition of AA metabolism given the change in the ω -3/ ω -6 ratio (7, 21, 51).

Biological role of long-chain PUFAs. n-3 DPA has been reported to be effective (to a larger extent than EPA and DHA) in decreasing platelet aggregation from rabbit blood (1). Moreover, the beneficial role of DPA in cardiovascular health is supported by studies investigating its metabolism, showing that it is highly incorporated in heart PL in the same sn-2 position as EPA (21, 28). However, further research is needed to better assess the biological effects of this long-chain PUFA.

MCT-induced PH, similar to the human form of disease, is characterized by infiltration of inflammatory cells and significant remodeling of small-size pulmonary arteries as well as right ventricular hypertrophy and failure (46, 55). The present data reveal that, in animals developing drug-induced PH, MAG-DPA treatment markedly inhibits the growth of cells involved in pulmonary vascular remodeling and results in a reduction in right ventricular hypertrophy and vascular remodeling of pulmonary arteries.

Mode of action of MAG-DPA. In an attempt to elucidate the mechanism by which MAG-DPA given in prevention mediates its anti-inflammatory and anti-proliferative effects in the present PH model, we determined whether this monoacylglyceride (or ones of its metabolites) modulates NF- κ B and p38 MAPK signaling by analyzing lung homogenates derived from untreated and MAG-DPA-treated rats.

NF- κ B and p38 MAPK are activated in response to various growth factors, inflammatory stimuli, and pro-oxidants and are known to regulate the expression of gene products associated with inflammation, proliferation, invasion, and angiogenesis (4, 26). Constitutively activated NF- κ B and p38 MAPK pathways can often be observed in animal models of PH and in clinical pathology (4, 26, 53–56). In the MCT model used herein, MAG-DPA upregulated the basal protein levels of I κ B α while downregulating the phosphorylated form of p65 NF- κ B, thus conferring stabilization to the cytosolic I κ B/NF- κ B complex and decreasing p65 translocation to the nucleus. Moreover, MAG-DPA treatments reduce the phosphorylated forms of p38 MAPK in our rat model of pulmonary hypertension. Activated NF- κ B and p38 MAPK induce the transcription of a range of proinflammatory and proliferative genes, including those that encode cyclooxygenase-2 (COX-2), intercellular adhesion molecule-1, vascular cell adhesion protein 1, E-selectin, TNF α , IL-1 β , IL-8, IL-6, VEGF, MMPs, and inducible nitric oxide synthase (4, 35). Our data show that MAG-DPA downregulated the MCT-stimulated expression of TNF α , IL-8, VEGF, MMP-2, and MMP-9 in the present in vivo model of PH. Nonetheless, our findings strongly indicate that NF- κ B and p38 MAPK might be important molecular mediators for the biological actions of MAG-DPA in MCT-

induced PH. Moreover, in a recent study, we have shown that per os MAG-DPA treatment decreases COX-2 and VEGF and increases PTEN expression related to the inhibition of the NF- κ B pathway in a colorectal cancer mouse xenograft model (42).

The present results also demonstrate that per os MAG-DPA supplementation increases the level of DPA in plasma and red blood cells as well as in lung and heart tissues, suggesting a high DPA bioavailability, and thereby likely contributes to reducing inflammation and MCT-induced smooth muscle cell remodeling. This endogenous rise in DPA also lowered the level of ω -6 arachidonic acid found in blood circulation and tissue samples (Fig. 2), thus resulting in an increased DPA/AA ratio (data not shown). These observations may be of clinical relevance since it has been shown that the ratio of dietary ω -3 PUFA to ω -6 may be an important factor in pulmonary illnesses characterized by excess airway inflammation (17, 18). However, the exact mechanism explaining the absorption and incorporation into cell membranes of MAG-DPA in lung and heart tissues was not assessed in the present work. A lipidomic study of airway and cardiac cell membranes should prove useful (6) and will be performed in the near future.

Role of putative derivatives. Dietary supplementation with n-3 PUFA enhances the conversion of EPA and DHA into bioactive metabolites, such as resolvins and protectins, both of which can reduce cellular inflammation through specific receptors (48). Enzymes involved in the biosynthesis of these lipid mediators include 5-lipoxygenase (LO), 12-LO, and 15-LO (48). Moreover, the combination of n-3 PUFA and aspirin reduces the clinical symptoms of many inflammatory disorders, such as arthritis, cardiovascular diseases, asthma, and cancer (50). In addition, EPA and DHA have been shown to trigger anti-inflammatory effects by 1) inhibiting the metabolism of membrane AA to proinflammatory mediators, 2) producing anti-inflammatory and proresolving (resolvins and protectins) molecules, and 3) blocking the synthesis of proinflammatory enzymes and cytokines by interfering with NF- κ B pathways (35). n-3 PUFA and their derivatives also display beneficial effects in animal models of asthma (2, 34) and cystic fibrosis (54). Indeed, in recent studies, we have demonstrated that n-3 PUFA monoacylglycerides such as MAG-DHA and MAG-EPA display anti-inflammatory effects in animal models of asthma. These effects were likely related to the production of resolvins and protectins (38, 39, 40, 42). Moreover, it has been shown that n-3 DPA and MAG-DPA are likely metabolized by 5-LO, 15-LO, and CYP450 epoxygenase pathways in mediating these effects (12, 27, 42).

In this study, our data revealed that MAG-DPA administration resulted in the production of resolvin RvD5_{n-3} DPA, and an elevated level of this metabolite was found in lung tissue following MAG-DPA treatment of MCT rats. Overall, these data suggest that the anti-proliferative and anti-inflammatory effects of MAG-DPA were likely related to the production of RvD5_{n-3} DPA. In a previous study, we also demonstrated that 7(S),17(S)-dihydroxy-DPA and 17(S)-hydroxy-DPA, two active lipid mediators of MAG-DPA derived from 15-LO pathways, display anti-inflammatory and anti-proliferative effects in carcinoma cells (42). One drawback of these bioactive lipids, however, is that they contain several double bonds and hydroxyl groups that are highly sensitive to metabolic inactivation. Novel approaches are thus required to overcome such

structural and metabolic features of unstable epoxy metabolites, further justifying the use of biochemical precursors or stable analogs such as MAG-DPA.

Study limitations. There were several limitations in the present study. The MCT model of PH has been thoroughly described and validated in the rat (55, 57), and thus we have not performed intrapulmonary artery tension measurements. This study attests of the ability of MAG-DPA to prevent MCT-induced PH, in which inflammatory mechanisms related to pulmonary vascular endothelial dysfunction may contribute to the development and progression of pathological conditions. However, no experiment has been performed to verify the ability of MAG-DPA to reverse the established disease. This issue has been assessed by another study in which the tested compound was given 4 wk following MCT injection (57). A reversal protocol will be necessary to attest the efficacy of MAG-DPA in the treatment of PH. Furthermore, the present analysis would need to be repeated in multiple models of PH (chronic hypoxia, Sugen 5416 + hypoxia, monocrotaline + pneumonectomy, rats with an endothelin B receptor deficiency) using MAG-DPA in prevention and treatment, because none of the PH rodent models recapitulates the characteristics of human PH. The precise mechanism responsible for the reduction of proliferation and inflammation induced by MAG-DPA is not fully understood. We assumed that it was likely due to a decrease in NF- κ B and p38 MAPK activation, which in turn would inhibit the expression of proinflammatory cytokines, metabolic enzymes, and growth factors (4, 8, 26, 56). However, several pathways known to regulate both proliferation and inflammation, such as STAT3, and bone morphogenetic protein receptor 2, might be involved as MAG-DPA effectors. Hence, it has been demonstrated that MAG-DPA metabolism generates resolvins, which are known for their anti-inflammatory properties (2, 12, 47). Complementary *in vitro* experiments would help to delineate the exact mechanisms by which resolvins exert their beneficial effects.

In conclusion, we propose that DPA monoacylglyceride given in prevention is able to modify the ω -3/ ω -6 ratio, thus providing anti-inflammatory and anti-proliferative properties to DPA metabolites. Furthermore, when administered per os, MAG-DPA represents a stable compound that could serve as a precursor to generate a variety of PUFA-derived mediators, such as resolvins and protectins known to directly mediate anti-inflammatory and proresolving effects through specific receptors (47). Consequently, MAG-DPA could provide a safe and interesting new approach for the prevention of PH.

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DISCLOSURES

Only S. Fortin declares a potential conflict of interest, since he is the owner of SCF Pharma, including a worldwide exclusive license on patented MAG-DPA.

AUTHOR CONTRIBUTIONS

C.M., E.R., P.U.B., and S.F. conception and design of research; C.M., R.H., and S.F. performed experiments; C.M., E.R., and S.F. analyzed data; C.M., E.R., and S.F. interpreted results of experiments; C.M. prepared figures; C.M. drafted manuscript; C.M., E.R., P.U.B., and S.F. edited and revised manuscript; C.M., E.R., P.U.B., and S.F. approved final version of manuscript.

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