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ω3 and ω6 CYP450 Eicosanoid Derivatives: Key Lipid Mediators in the Regulation of Pulmonary Hypertension

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1. Introduction

Pulmonary arterial hypertension (PAH) is a multi-factorial, progressive disease with substantial mortality and morbidity. Despite recent improvements in treatment, mortality associated with PAH remains high, with a two-year survival rate after diagnosis of approximately 85% (Thenappan *et al.*, 2007). Although advances in the understanding of disease development and treatment have been achieved, the pathogenesis of PAH is still not clearly understood (Humbert et al., 2004). Therapeutic options remain limited despite the introduction of prostacyclin analogues, endothelin receptor antagonists and phosphodiesterase 5 inhibitors within the last 15 years. Moreover, these interventions predominantly address the endothelial and vascular dysfunction associated with the condition, and thus merely delay the progression of the disease rather than offer a cure (McLaughlin *et al.*, 2009a, McLaughlin *et al.*, 2009b).

PAH is a subset of pulmonary hypertensive syndromes, defined by a resting mean pulmonary artery pressure (PAP) >25mmHg, pulmonary vascular resistance (PVR) >3 Wood units and pulmonary wedge pressure <15mmHg, in the absence of other causes of PAH (Archer *et al.*, 1998). PAH is primarily a disease of the small pulmonary arteries, characterised by vascular proliferation, remodelling and progressive increases in PVR, ultimately leading to right ventricular failure and death (Voelkel *et al.*, 2006). These increases in PVR are attributed in part to endothelial dysfunction resulting in vasoconstriction, remodelling of the pulmonary vessel wall and thrombosis *in situ* (Budhiraja *et al.*, 2004). The role of inflammation in the development of PAH has been suggested (Voelkel et al., 1998). Inflammatory cells, including macrophages and

lymphocytes, are increased in the plexiform lesions of hypertensive pulmonary vessels (Tuder et al., 1994). Elevated levels of macrophage inflammatory protein-1a, interleukin-1b and interleukin-6 are also found in patients with severe pulmonary hypertension (Humbert et al., 1995, Fartoukh et al., 1998). However, the haemodynamic aberrations represent only one aspect of PAH such that enhanced proliferation, decreased apoptosis and a shift to glycolytic metabolism in pulmonary artery smooth muscle cells, fibroblasts and endothelial cells are now recognised as central to the pathogenesis of the disease. The causes and clinical consequences of PAH have recently been reviewed by a number of international scientists and clinicians (Galie et al., 2009a). While intracellular calcium plays a pivotal role in controlling vascular tone and remodelling of the arterial wall, it became of prime interest to search for alternative biochemical or pharmacological targets in order to better understand the haemodynamic and pathophysiological shifts in the functional properties of this specific vascular bed.

Endothelial dysfunction appears to play an integral role in mediating the vasoconstriction and structural changes in pulmonary vasculature (Budhiraja *et al.*, 2004). The endothelium releases diverse growth factors, vasoactive compounds and lipidic mediators, which regulate the physical and biochemical properties of the pulmonary vessels and affect vascular contractility and cell growth, thus modifying distal pulmonary artery resistance and compliance (Budhiraja *et al.*, 2004). An altered production of various endothelial vasoactive mediators, such as NO, prostacyclin, endothelin-1 (ET-1), serotonin and thromboxane, has been increasingly recognized in patients with PAH (Giaid et al., 1993). Because most of these mediators affect the growth of smooth muscle cells, an alteration in their production may facilitate the development of pulmonary vascular hypertrophy and the structural remodelling characteristic of PAH (Galie et al., 2009a). In addition to the potential consequences of an imbalance in the endothelial production of various mediators, injury to the endothelium may expose the underlying vascular media tissue to diverse blood-borne factors that may further promote pathological changes (Galie et al., 2009a, Saouti et al., 2010). Endothelial dysfunction may also have adverse consequences on pulmonary vascular haemostasis by altering the production of anticoagulant factors. Recent reports of genetic mutations in endothelial cells of patients with PAH further underscore the role of these cells in disease pathogenesis (Galie et al., 2009a).

Several studies have demonstrated that Ca^{2+} sensitizing mechanisms of the contractile proteins may also be primed under pathophysiological conditions by various vasoactive and lipid mediators and that this Ca^{2+} sensitization process is involved in hypertension (Uehata et al., 1997, Hata et al., 2011). In vascular smooth muscle (VSM), ET-1 has been shown to enhance Ca^{2+} sensitivity through the activation of Rho-kinases and PKC-dependent phosphorylation of the 17 kDa myosin phosphatase inhibitor protein (CPI-17) pathways (Hersch et al., 2004). Contraction of VSM occurs via two related mechanisms: i) a rise in cytosolic calcium concentration ($[Ca^{2+}]_i$) which results in the formation of calcium/calmodulin complexes and activation of the myosin light chain kinase (MLCK). The activated MLCK in turn phosphorylates the 20 kDa myosin light chain (MLC) (Kimura et al., 1996), resulting in vascular smooth muscle (VSM) cell contraction; ii) a second Ca^{2+}-independent mechanism which requires the activation of Rho-kinase as well as PKC-dependent phosphorylation of myosin phosphatase inhibitor protein of 17 kDa (so called

CPI-17) to maintain tone (Somlyo et al., 2003). The calcium sensitization mechanism occurs when an agonist, which stimulates the activation of Rho-kinase or the PKC/CPI-17 pathway, results in the inhibition of MLC phosphatase (MLCP) (Uehata et al., 1997, Somlyo et al., 2003, Kitazawa et al., 2010). Rho-kinase inhibits MLCP activity by phosphorylating the myosin-binding subunit of MLCP. Alternatively, CPI-17 phosphorylation also results in an inhibition of MLCP activity, which in turn maintains steady state tension in VSM (Somlyo et al., 2003, Kitazawa et al., 2010). Hence, CPI–17 de-phosphorylation facilitates relaxation as reported previously in bronchial smooth muscle tissues (C. Morin, 2008).

Omega-6 and omega-3 poly-unsaturated fatty acids (PUFA) such as arachidonic acid (AA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), respectively, can be metabolized by cytochrome P-450 (CYP450) enzymes into several classes of oxygenated and hydroxylated metabolites (Funk 2001, Fer et al., 2008). Several of these CYP450-derived eicosanoids are also known for their ability to modulate vascular (Roman 2002) and airway smooth muscle tone (Morin et al., 2009, Mouchaers et al., 2010). 20-hydroxyeicosatetraenoic acid (20-HETE) has been shown to induce relaxation of pulmonary arteries in several species including humans (Birks et al., 1997). Meanwhile, epoxyeicosatrienoic acid (EET) regioisomers have been shown to activate large conductance calcium-activated potassium channels in vascular smooth muscle cells and are considered as leading candidates for endothelium-derived hyperpolarizing factor (EDHF) in the coronary and systemic circulation (Zeldin et al., 1996, Fisslthaler et al., 1999, Node et al., 1999, Capdevila et al., 2000). Specific CYP450 epoxygenase isoforms are involved in EPA metabolism and produce 17(18)-epoxyeicosatetraenoic acid (17(18)-EpETE, see Fig.1B) (Lauterbach et al., 2002, Nguyen et al., 1999, Schwarz et al., 2004). Epoxy-docosapentaenoic acids are CYP450-dependent DHA metabolites (Fig. 1C) (Fer et al., 2004, Arnold et al., 2010, Lucas et al., 2010) and recent studies have demonstrated that these metabolites display potent vasodilatory activities on coronary arteries (Konkel et al., 2011, Wang et al., 2011). Metabolic pathways and chemical structures of CYP450-dependent AA, EPA and DHA metabolites known to modulate vascular tone are illustrated in Figure 1 A-C. Recently, pharmacological inhibitors of CYP450 epoxygenases and hydroxylases, such as N-methylsulfonyl-6-(2-propargyloxyphenyl)-hexanamide (MS-PPOH) and Dibromo-dodecenyl-methylsulfimide (DDMS), were developed to assess the specific role of these metabolites in cardiovascular circulation (Nithipatikom et al., 2006).

In this chapter, we will summarize the role and the effect of ω6 CYP-450 metabolites, such as EET regioisomers and 20-HETE, as well as their putative involvement in the control of pulmonary arterial tone. Moreover, we will review recent reports regarding the mode of action of epoxy-ω3 derivatives, such as EpETE and EpDPE, on pulmonary arteries. Although some progress has been made for the diagnosis and treatment of PAH, more effective treatments need to be developed. The omega 3 polyunsaturated fatty acids (n-3 PUFA) have recently attracted much attention in various research fields especially in cardiovascular research (McLaughlin *et al.*, 2009a). Accordingly, we will summarize the effect of a new DHA-derivative on human pulmonary arteries. Potential involvement of this DHA derivative as a novel and prospective medicinal compound will be discussed as compared to present treatments used in the management of pulmonary arterial hypertension.

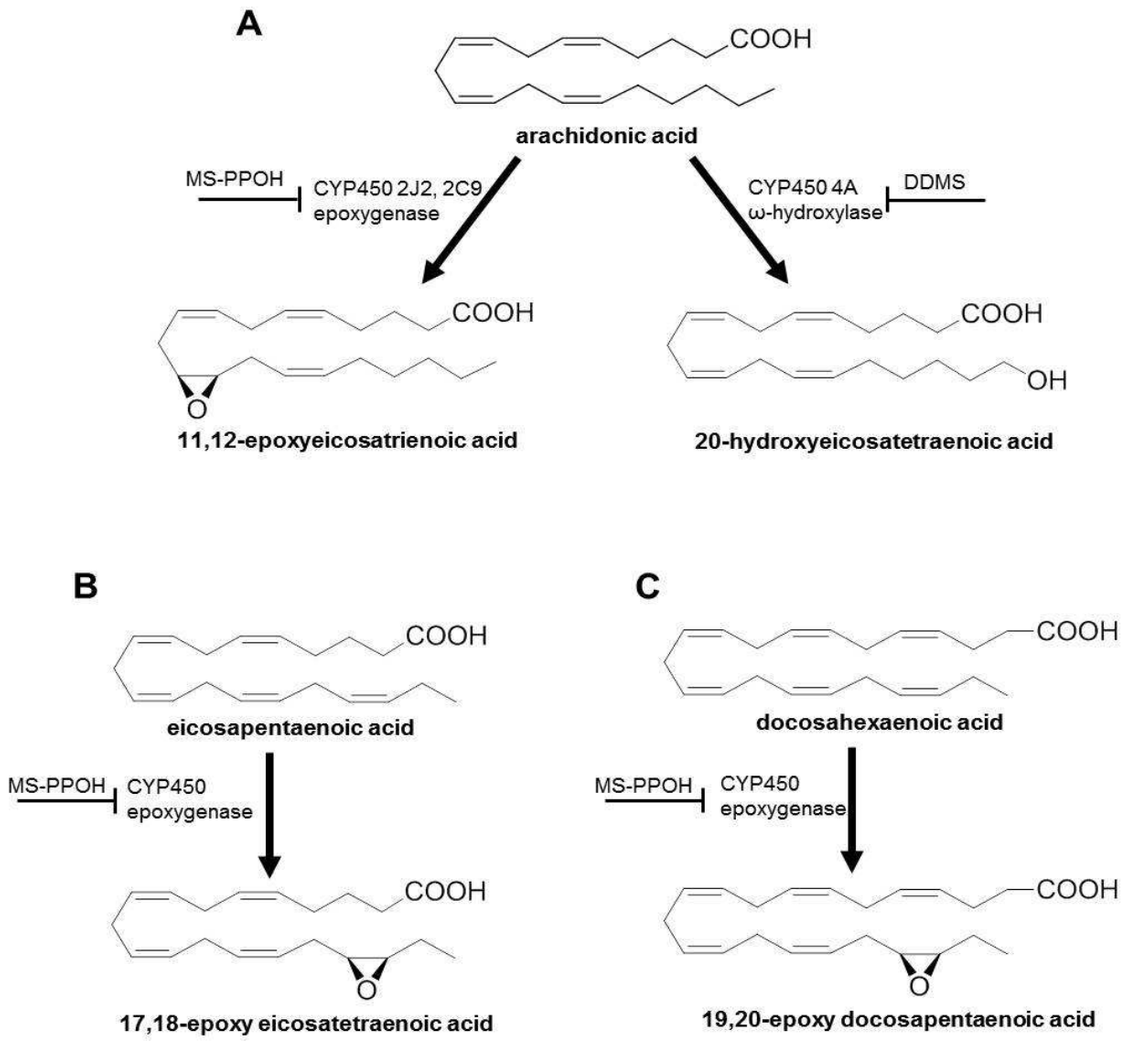


Fig. 1. Omega-3 and omega-6 metabolites produced by CYP450 enzymes in lung tissues. A: CYP450 epoxygenase and ω-hydroxylase enzymes metabolize arachidonic acid into two major active compounds: epoxyeicosatrienoic acid (EET) and 20-hydroxyeicosatetraenoic acid (20-HETE), respectively. MS-PPOH and DDMS are known specific inhibitors of CYP450 enzymes. B: 17,18-epoxyeicosatetraenoic acid (17,18-EpETE) is the major active metabolite produced by the action of CYP450 epoxygenase enzymes on eicosapentaenoic acid (EPA). C: docosahexaenoic acid (DHA) serves as a substrate for CYP450 epoxygenase enzymes to induce the formation of 19,20-epoxy docosapentaenoic acid (19,20-EpDPE).

2. Role of CYP450-dependent arachidonic acid metabolites in pulmonary arteries

Arachidonic acid is metabolized via cyclooxygenases, lipoxygenases, and cytochrome *P*-450 (CYP) enzymes to generate a range of bioactive eicosanoids. CYP enzymes, with an important role in cardiovascular function, are epoxygenases of the CYP2C and 2J gene families, which form four regioisomeric epoxyeicosatrienoic acids (5,6-, 8,9-, 11,12-, and

14,15-EET) and the ω-hydroxylases of the CYP4A family, which generate hydroxyeicosatetraenoic acids (19- and 20-HETE) (Roman,. 2002).

In the systemic vasculature, EETs are most commonly described as vasodilators. The mechanism of EET-induced vasodilation has been studied extensively and is dependent on the hyperpolarisation of vascular smooth muscle cells (Gebremedhin et al., 1992, Hecker et al., 1994, Campbell et al., 1996, Eckman et al., 1998, Morin et al., 2007a) resulting from EET-induced opening of smooth muscle cell potassium channels (Campbell et al., 1996, Zou et al., 1996, Eckman et al., 1998, Gebremedhin et al., 1998, Hayabuchi et al., 1998, Morin et al., 2007a). Although EETs also reduce tension in non-resistant extralobar pulmonary artery (PA) segments (Stephenson et al., 1998, Stephenson et al., 2003), all four EET regioisomers have been observed to be vasoconstrictors in small-diameter intralobar PA segments in rabbits and rats (Yaghi et al., 2001, Zhu et al., 2000). Because EETs also increase vascular resistance (and decrease compliance) in isolated perfused rabbit lungs (Stephenson et al., 2003), the predominant activity of EETs in lung vessels appears to be vasoconstriction. These data are supported by the observation that inhibition of epoxygenase activity with specific pharmacological inhibitors attenuates PA tension to phenylephrine (Zhu et al., 2000a), suggesting that endogenous EETs participate in α-adrenergic contraction of the pulmonary vasculature. Zeldin and co-workers reported that the most abundant EET regioisomer formed in the rabbit lung was 5,6-EET (Zeldin et al., 1995). Of the four EET regioisomers, 5,6-EET is also the most potent, albeit labile, constrictor of rabbit pulmonary arteries (Zhu et al., 2000b). The 5,6-EET-induced contraction of rabbit PA requires intact endothelium, cyclooxygenase activity, and activation of the thromboxane/endoperoxide (TP) receptor (Zhu et al., 2000b, Stephenson et al., 2003). Moreover data suggest that 5,6-EET induces contraction in intralobar PA by increasing Rho-kinase activity, thus phosphorylating MLC and increasing Ca^{2+} sensitivity of the contractile apparatus in rabbit lung (Losapio et al., 2005).

On the other hand, 20-hydroxyeicosatetraenoic acid (20-HETE), a CYP-450 4A metabolite of arachidonic acid, constricts renal, cerebral, coronary and mesenteric arteries via inhibitory effects on Ca^{2+}-activated K^+ channels, increased Ca^{2+} entry and modulation of Rho-kinase activity (Roman, 2002, Randriamboavonjy et al., 2003). However, 20-HETE has been shown to be an eicosanoid product of human lung tissue that acts as a potent vasodilator of isolated pressurized pulmonary arteries (Birks et al., 1997, Morin et al., 2008). Furthermore, cytochrome *P*-450 4A (CYP4A) protein is expressed in the pulmonary vasculature, a finding based on Western blots of PA microsomes (Zhu et al., 1998), the conversion of arachidonic acid into 20-HETE by dispersed vascular smooth muscle cells (Zhu et al., 1998), and immunohistochemistry localizing CYP4A to rabbit pulmonary capillary endothelium (Roman et al., 1993). These observations raise the possibility that products of CYP4A may contribute to the control of PA tone. However, the role of 20-HETE or other *P*-450 metabolites of AA in pulmonary circulation is not completely understood. In the acute hypoxic vasoconstrictive response in isolated blood-perfused rabbit lungs, 20-HETE was shown to relax rabbit PA rings, while an inhibitor of 20-HETE synthesis, DDMS, shifted the concentration response of PA rings to phenylephrine (PE) to the left, consistent with the loss of a pro-relaxing metabolite (Zhu et al., 2000b). Moreover, the inhibition of 20-HETE formation by pharmacological agents such as 17-oxydecanoic acid (17-ODYA) and DDMS

enhanced the acute hypoxia-induced increase in pulmonary perfusion pressures (Zhu et al., 2000b).

In light of these findings, we postulated that, as a vasodilator, 20-HETE may counter observed increases in human pulmonary artery (HPA) tone upon serotonin (5-HT) and α-adrenergic stimulations. To perform these experiments, human lung tissues from patients undergoing surgery for lung carcinoma and distant from the malignant lesion were obtained. Pulmonary arteries of similar weight and length (inner diameter of 0.5 - 0.8 mm) were micro-dissected and placed in culture for 24 hours in a humidified incubator at 37°C under 5% CO_2 as previously reported (Guibert et al., 2005, Morin et al., 2008). Resulting data demonstrated that 20-HETE acts as a potent vasodilator on HPA pre-contracted with 5-HT and phenylephrine (PE) (Figure 2A, Morin C et al., 2008). 20-HETE displayed potent relaxing effects on HPA, with IC_{50} values in the sub-micromolar range (≈ 0.3 μM) on both resting and active tone. In HPA, the relaxing effect induced by 20-HETE was partially abolished by 30 % in the presence of iberiotoxin (IbTx) (Morin et al., 2008), suggesting that the eicosanoid activates large conducting Ca^{2+}-activated potassium (BK_{Ca}) channels which usually results in membrane hyperpolarisation. Indeed, microelectrode measurements demonstrated that 20-HETE hyperpolarizes human ASM cells, an effect abolished by 10 nM IbTx (Morin et al., 2007b). Thus, the mode of action of this compound may be related to its molecular interactions with specific ionic channels of the surface membrane, as previously demonstrated in guinea pig and human smooth muscles (Cloutier et al., 2003, Morin et al., 2007b).

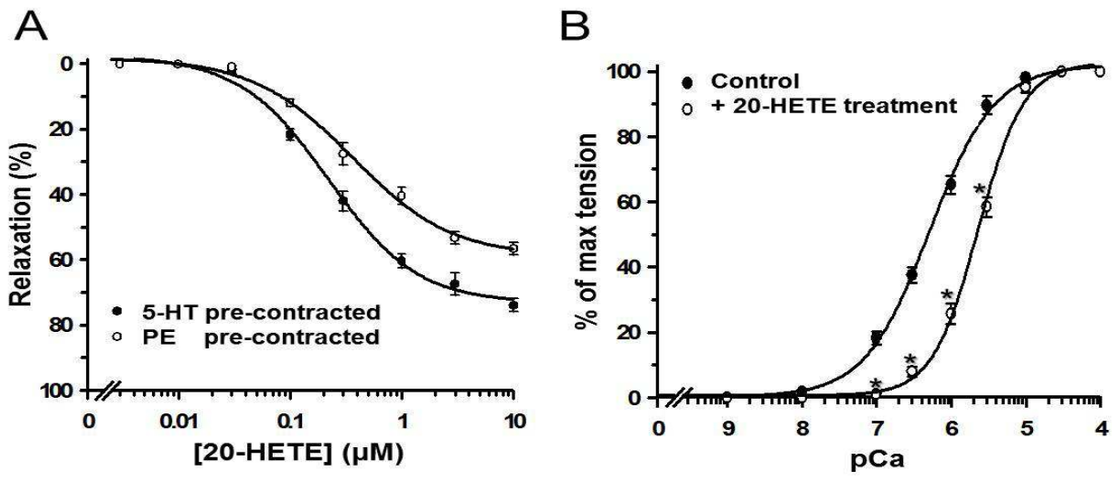


Fig. 2. Omega-6 ω-hydroxylase metabolite modulates pulmonary arterial tone.
A: Concentration-dependent relaxing responses induced by 20-HETE on distal human pulmonary arteries pre-contracted with either 1 μM 5-HT or 1 μM PE. Each point represents the mean ± S.E.M. with n=16 and n=14 for each experimental condition, respectively. B: Cumulative Concentration Response Curve (CCRC) to free [Ca^{2+}] obtained from β-escin-permeabilized pulmonary artery rings in control conditions (closed circles, n =16) and in the presence of 1 μM 20-HETE (open circles, n = 18).

The role of various ion channels in acute and chronic hypoxia in pulmonary vasculature has already been described as well (Weir et al., 2006, Guibert et al., 2007). Hence, from a physiological standpoint, it is noteworthy to emphasize the relaxing effects displayed by 20-HETE on both HPA and airway smooth muscle (Birks et al., 1997, Jacobs et al., 1999, Morin et al., 2008).

The inherent Ca^{2+} sensitivity of the myosin light chain kinase, resulting in MLC phosphorylation and contraction and subsequent de-phosphorylation by MLCP, is an important mechanism in the regulation of vascular smooth muscle tone (Somlyo et al., 2003, Koga et al., 2005). Modulation of this mechanism by 20 HETE could explain its overall effects on HPA. As shown in figure 2B, 20 HETE significantly reduces Ca^{2+} sensitivity in permeabilized preparations, suggesting that this eicosanoid modulates enzymatic systems such as Rho-Kinase and/or PKC/CPI-17 as well as down-stream MLCP activity (Morin et al., 2007a). Several studies have suggested that Ca^{2+} sensitizing mechanisms may also be primed under pathophysiological conditions and especially in PAH (Uehata et al., 1997, Somlyo et al., 2003). It was therefore of potential clinical interest to find a lipid mediator that would be able to shift the Ca^{2+} activation curve toward higher concentrations. Moreover, our group demonstrated that 20-HETE decreases CPI-17 phosphorylation levels and increase the expression of p116Rip, thus supporting the view that this eicosanoid downregulates PKC/CPI-17 and Rho kinase dependent pathways (Morin et al., 2008).

Despite the fact that CYP450 ω-hydroxylase has been identified in various lung tissues (Zeldin et al., 1995, Zhu et al., 1998, Jacob et al., 1999, Roman RJ, 2002, Miyata et al., 2005), a key issue has been to demonstrate the endogenous involvement of 20-HETE in human pulmonary arteries. Using a pharmacological ω-hydroxylase inhibitor such as DDMS, (Jacob et al., 2006, Nithipatikom et al., 2006) which minimizes the endogenous production of 20-HETE, it was now possible to amplify tonic responses to various vasoconstrictive agents. This experimental strategy enabled us to evaluate the putative role of this eicosanoid in human lung vascular tissues. Hence, it was hypothesized that 20-HETE could play the role of a paracrine mediator in the HPA wall whereby its basal production would facilitate PA dilation and help maintain low blood pressure (12-15 mm Hg) in this specific segment of the vascular apparatus. However, the difference in reactivity to 20-HETE between conduit and resistance arteries has not been addressed. In fact, 20-HETE is thought to play an important role in regulating tone in distal HPA (Diameter < 500 μm). In contrast, consistent contractions have been measured and reported in rodents such as guinea pig bronchi (Cloutier et al., 2003). Nevertheless, the relaxations induced by 20-HETE may be of pharmacological interest in pulmonary arterial hypertension, since it could be used to treat this critical clinical condition known to be refractory to classical treatments. Hence it would be relevant to analyse the expression of several genes encoding specific proteins, such as the CYP450 isoforms in the HPA wall and parenchyma. It has been reported that CYP450 4A and other isozymes represent ω-hydroxylases that are responsible for 20-HETE production in several human tissues (Gebremedhin et al., 1998), and for which their expression could be downregulated in patients diagnosed with PAH (Pierson et al., 2000). This hypothesis lends further support to the benefit of studying the functional implication of 20-HETE at both the cellular and molecular levels, despite the fact that such studies were initiated some 15 years ago in rabbit pulmonary tissues (Zeldin et al., 1995).

3. Key role of CYP450 epoxygenase-dependent metabolites derived from EPA and DHA in pulmonary hypertension

It is widely accepted that n-3 PUFA, rich in fish oils, protect against several types of cardiovascular diseases such as myocardial infarction, arrhythmia, atherosclerosis, as well as hypertension and inflammatory conditions (Abeywardena et al., 2001, Kris-Etherton et al., 2002). EPA, DHA or their derivatives may represent active biological components mediating these effects. Although the precise cellular and molecular mechanisms underlying these beneficial effects are not well understood, the protective effects of PUFA are likely related to their direct effects on VSM cells (Mizutani et al., 1997, Hirafuji et al., 2003). It has been shown that these PUFA activate K^+_{ATP} channels and inhibit specific types of Ca^{2+} channels (Ye et al., 2002). These reports suggest that modulation of VSM cell function contributes to the beneficial effects of PUFA in the systemic vascular system. EPA may also serve as an alternative substrate in CYP450-dependent epoxygenation and hydroxylation reactions as shown in rat hepatic and renal microsomes (Van Rollins et al., 1988). The CYP450-dependent EPA metabolites include the epoxyeicosatetraenoic acid regioisomers 5(6)-, 8(9)-, 11(12)-, 14(15)- and 17(18)-EpETE (Lauterbach et al., 2002). Specific CYP450 epoxygenase isoforms are involved in EPA metabolism which produce (17(18)-EpETE), and include CYP1A (Schwarz et al., 2004), CYP4A1, CYP4A3 (Nguyen et al., 1999, Lauterbach et al., 2002,) and CYP4A12A. An additional potential source for 17(18)-EpETE are endothelial CYP450 isoforms of the 2C and 2J subfamilies that otherwise produce EET from AA. Recent studies have demonstrated that EPA epoxides share and even exceed the ability of AA epoxides to stimulate large conducting Ca^{2+}-activated potassium (BK_{Ca}) channels (Lauterbach et al., 2002) and mediate vasodilatation (Zhang et al., 2001a).

However, several questions remain to be addressed as to the mode of action of EPA metabolites in human pulmonary arteries. Our group demonstrated that 17(18)-EpETE induced a relaxing effect of smooth muscle from distal human pulmonary arteries (HPA), with IC_{50} values in the sub-micromolar range on both HPA resting and active tone. To our knowledge, there are only few publications addressing the effects of 17(18) -EpETE in rodents and in cultured VSM cells (Lauterbach et al., 2002, Hercule et al., 2007). Our data showed that the relaxing effect induced by 17(18)-EpETE in HPA under normal external K^+ concentration was abolished in the presence of IbTx and glyburide (Glyb), suggesting that the eicosanoid activates BK_{Ca} and K_{ATP} channels, hence resulting in membrane hyperpolarisation. Indeed, intracellular microelectrode measurements revealed that 17(18)-EpETE induced significant hyperpolarisation of human pulmonary artery smooth muscle cells (Figure 3A). Since IbTx and Glyb prevented these hyperpolarizing effects, thereby reducing the relaxation induced by 17(18)-EpETE, BK_{Ca} and K_{ATP} channel activation thus appears to be a key determinant in the control of both HPA membrane potential and tone (Figure 3B). Moreover, it has been demonstrated that EPA epoxides share and even exceed the ability of AA epoxides to stimulate BK_{Ca} channels (Lauterbach et al., 2002) and to mediate vasodilatation in canine and porcine coronary microvessels (Zhang et al., 2001a). Using patch clamp measurements, 17(18)-EpETE has also been demonstrated to stimulate K^+ outward currents, displaying typical characteristics for BK_{Ca} channel activation in systemic VSM cells. Moreover, this effect is abolished by TEA, a BK_{Ca} channel blocker (Lauterbach et al., 2002). Recently, the BKα subunit, the pore-forming subunit of octameric BK_{Ca} channels, was shown to represent the main molecular target of 17(18) -EpETE in systemic VSM cells isolated from rat cerebral and mesenteric arteries (Hercule et al., 2007).

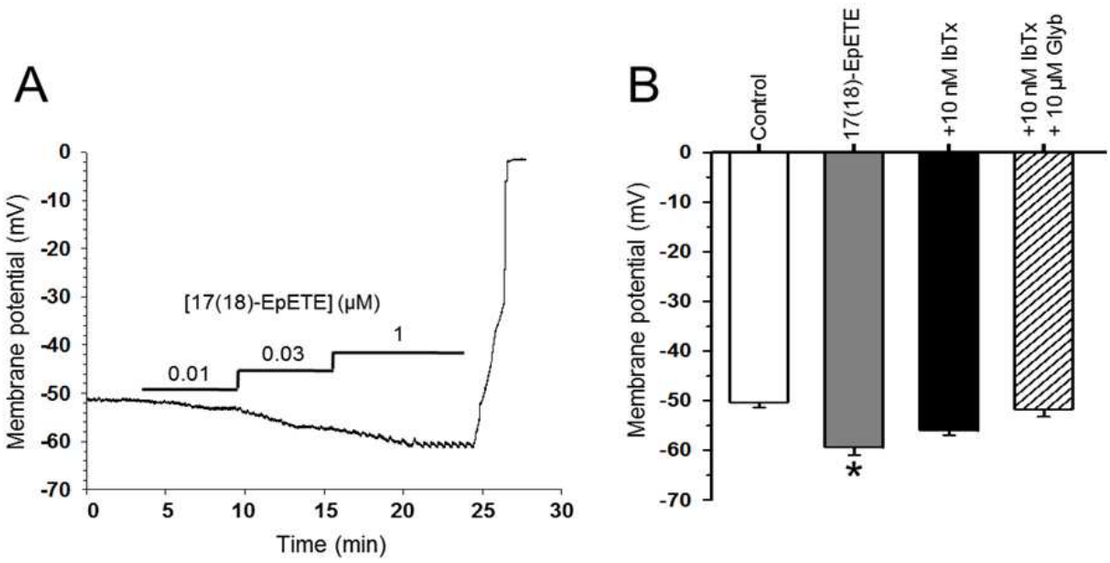


Fig. 3. EPA-derived metabolite hyperpolarisation of pulmonary arterial smooth muscles.
A: Recording of the membrane potential from pulmonary artery in control and following addition of cumulative concentrations of 17(18)-EpETE. At the end of each recording, the microelectrode was removed from the pulmonary artery smooth muscle cell to validate the electrophysiological measurements. B: Mean resting membrane potential values determined for 1 μM 17(18)-EpETE and following addition of either 10 nM IbTx or 10 nM IbTx plus 10 μM glyburide. (n = 7 for each condition).

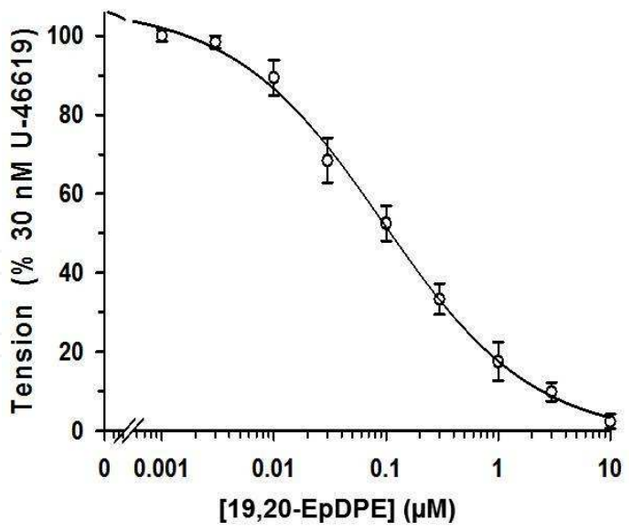


Fig. 4. DHA CYP450 epoxygenase metabolite-induced relaxation of pulmonary arterial smooth muscle.
A: Cumulative concentration response curve displaying the mean tension induced by 30 nM U46619 in control and after 24h-treatments with increasing concentrations of 19,20-EpDPE (0.001-10 μM), n=12 for each experimental condition.

CYP450-dependent DHA metabolites include the epoxy-docosapentaenoic acid regioisomers 4(5)-, 7(8)-, 10(11)-, 13(14)-, 16(17)- and 19(20)-EpDPE (Fer et al., 2008, Arnold et al., 2010, Lucas et al., 2010). Several epoxygenases have been identified in lung tissues, including 2J2, 2C8 2C9 and 1B1 (Fer et al., 2008). Recent studies have demonstrated that CYP450 epoxygenase metabolites of DHA display potent vasodilatory activities on coronary arteries. These epoxy-eicosanoids have been reported to be more potent than EET and EPA in activating BK_{Ca} channels (Konkel et al., 2011, Wang et al., 2011). In our laboratory, experiments were designed to assess the relaxing effect of 19,20-EpDPE on human pulmonary arteries pre-contracted with 30 nM U-46619, a TP receptor agonist. HPA were cultured for 24 hours in the absence or presence of increasing concentrations of 19,20-EpDPE. The tissues were then subjected to 0.6 grams of basal tone and thereafter challenged with 30 nM U-46619. The cumulative concentration response curve (CCRC) to 19,20-EpDPE (0.01-100 μM) revealed a concentration-dependent inhibitory effect on active tone, with an EC_{50} value of 0.11 μM (Figure 4).

4. Current treatments and new therapeutic strategies using omega-3 monoglyceride for PAH

Significant advances in the treatment of PAH have been made in the last 15 years. These agents target the prostacyclin pathway, the nitric oxide pathway and the endothelin pathway. A number of vasoactive substances are involved in the pathogenesis of PAH, including a major imbalance observed between prostacyclin and thromboxane. Both of these substances are by-products of arachidonic acid metabolism by endothelial cells. Prostacyclins increase intracellular cyclic AMP and reduce intracellular Ca^{2+} (Clapp et al., 2002). Transcription factors and cell cycle progression are dependent on $[Ca^{2+}]_i$. Prostacyclins also inhibit platelet activation, promote vasodilatation and inhibit smooth muscle proliferation. Thromboxane, which is also produced by endothelial cells, antagonizes the effects of prostacyclin. The imbalance between prostacyclin and thromboxane may result from a combination of genetic factors and aberrant response to certain forms of injury to endothelial cells. Intravenous prostacyclin was first introduced in the treatment of primary pulmonary arterial hypertension in the early 1980s. A cohort analysis of patients receiving intravenous prostacyclin has shown benefits in New York Heart Association (NYHA) class III and IV patients with regard to survival (McLaughlin et al., 2002b, Sitbon et al., 2002). In addition to idiopathic pulmonary arterial hypertension, epoprostenol has been successfully used in the treatment of pulmonary hypertension resulting from left to right shunt, portal hypertension and HIV infection (Rosenzweig et al., 1999, Nunes et al., 2003). While epoprostenol has shown a definite role in the treatment of primary and other forms of pulmonary hypertension, it remains expensive and very cumbersome to use. Administration of this medication requires a central line and a pump. Treatment with epoprostenol causes several side effects, with central line infection being a serious side effect (Palmer et al., 1998, Humbert et al., 1998). Other stable prostacyclins such as Treprostinil and Iloprost are also used in the treatment of PAH. However, side effects are similar to those exhibited by epoprostenol (Hoeper et al., 2000, Olschewski et al., 2002).

Endothelin-1 is produced by the vascular endothelium and serves as a vasoconstrictor and smooth muscle mitogen. While its action on endothelin-A receptors results in vasoconstriction through activation of protein kinase and an increase in $[Ca^{2+}]_i$, its effect on endothelin-B receptors results in vasodilatation secondary to prostacyclin and nitric oxide

release in addition to aiding in its clearance. Endothelin receptor antagonists are also currently used in the treatment of PAH. For example, bosentan is a non-selective endothelial receptor antagonist. Endothelin A receptor stimulation results in vasoconstriction and smooth muscle proliferation, while endothelin B receptor stimulation results in endothelin clearance as well as induction of NO and prostacyclin by endothelial cells. Two randomized double-blind placebo-controlled class III and IV trials have evaluated bosentan in patients with pulmonary arterial hypertension. Study results showed a significant improvement in 6 min walk test and haemodynamics in the bosentan group. In addition, an improvement in the time to clinical worsening such as death, lung transplantation and hospitalization was also noted (Channick et al., 2001, Rubin et al., 2002). Bosentan is metabolized by the liver and an increase in transaminases has been noted during treatment with this medication. Hence, it is mandatory to perform periodic liver function tests in patients taking bosentan and ambrisentan are other examples of selective ETA receptor antagonists. Ambrisentan does not seem to have toxic effects on the liver, while bosentan induces hepatotoxicity (Barst et al., 2001, Barst et al 2006).

Often viewed as an endothelial disease, PAH has already been related i) to changes in membrane receptor and ionic channel expression (Guibert et al., 2007), ii) to a decrease in NO production, due to a lower nitric oxide synthase (NOS) activity (Guibert, 2010), and iii) to a downstream decrease in cGMP related to lower guanylate cyclase activity, which in turn activates potassium channels and hyperpolarizes VSM cells (Guibert, 2010). This process results in an inactivation of Ca^{2+} channel activity and decreases intracellular free Ca^{2+}, leading to vasodilatation. In fact, patients with PAH show impaired NO synthase activity. Impaired synthesis of endothelium-derived nitric oxide and enhanced production of vasoconstrictor endothelin have also been implicated in the pathogenesis of PAH (Giaid et al., 1993, Giaid A et al., 1995). Nitric oxide treatment is very cumbersome and is mainly used in the management of pulmonary hypertension in the neonatal intensive care unit (ICU) and occasionally in adult ICU (Giaid et al., 1993).

High-doses of Ca^{2+} channel blockers have been shown by uncontrolled studies to prolong survival in patients with PAH (Fuster et al., 1984, Rich et al., 1992), with approximately 10% of such patients belonging to this group. In a large retrospective study of 557 patients with PAH, less than 7% of patients responded to Ca^{2+}-channel blockers. Among these, patients who had a significant vasodilator response exhibited a long-term response to Ca^{2+}-channel blockers. Long-term therapy with Ca^{2+}-channel blockers is not recommended when there is no acute vasodilator response (Fuster et al., 1984).

Pulmonary vasculature contains substantial amounts of phosphodiesterase type-5 (PDE-5) enzyme and inhibition of this enzyme results in vasodilatation through the NO/cGMP pathway. Hence, the potential clinical benefit of phosphodiesterase type-5 inhibitors has been investigated in PAH (Pauvert et al., 2004). In addition, phosphodiesterase type-5 inhibitors exert anti-proliferative effects (Galie et al., 2009b). Sildenafil, a PDE-5 inhibitor, increases the levels of cyclic GMP in smooth muscle cells and causes vasodilatation. Tadalafil is a long acting PDE-5 inhibitor which has also been used in the management of PAH. The major landmark trial involving sildenafil was conducted in the pulmonary arterial hypertension (SUPER) study. Two hundred seventy-eight patients with functional class II–IV PAH were randomized to three different doses of sildenafil, namely 20, 40, or 80 mg, three times daily or to placebo. The study extended over a period of 12 weeks and, based on the convincing results, the FDA subsequently approved a thrice-daily dose of

20 mg sildenafil in the treatment of PAH (Galie et al., 2009b). The PHIRST Trial studied the efficacy and safety of the long acting phosphodiesterase-type five inhibitor (PDE 5-I) tadalafil. The study extended over a period of 16 weeks and doses of tadalafil used were 2.5, 10, 20, and 40 mg. Based on the positive responses, the FDA approved the use of tadalafil 40 mg once a day (Galie et al., 2009b). However these phosphodiesterase-5 inhibitors also induce common side effects and, in some cases, quite severe reactions such as sudden blindness, myocardial infarction, stroke and sudden cardiac death secondary to ventricular arrhythmias have been recognized.

Hence, despite the introduction of prostacyclin analogues, endothelin receptor antagonists and phosphodiesterase 5 inhibitors within the last 15 years, therapeutic options for pulmonary hypertension remain limited. To date, these interventions predominantly address the endothelial and vascular dysfunction associated with the condition, and thus merely delay the progression of the disease rather than offer a cure (McLaughlin et al., 2009). Clinical assessment of dietary supplementation of omega-3 (n-3) polyunsaturated fatty acids (PUFA) including EPA and DHA has shown their beneficial impact in a wide range of cardiovascular diseases (Connor, 2000). One explanation for these beneficial effects is that n-3 PUFA competes with arachidonic acid (AA) for enzymatic conversion by COX, LOX and CYP450 enzymes. This competition can lead to reduced formation of vasoactive AA metabolites while alternative PUFA-metabolites originating from DHA and EPA are increased. In a recent study, the DHA metabolite 16,17-EpDPE was shown to mediate vasodilatation of coronary arteries by the activation of BK channels (Wang et al., 2011). These n-3 PUFA effects are also mediated by a variety of mechanisms that involve both indirect (by eicosanoids and hormones) and direct genomic effects. In addition, EPA and DHA have been reported to reduce the expression of genes for interleukins, vascular cell adhesion molecule-1, intracellular adhesion molecule-1, endothelial adhesion molecule and E-selectin (Clarke et al., 1993, De Caterina et al., 1994, Tagawa et al., 1999). Recently our group has synthesized a new DHA monoglyceride derivative (Figure 5A, Fortin, 2008) in order to assess the effect of n-3 PUFA on pulmonary arterial tone and their involvement in key components of PAH pathogenesis. Fatty acids in monoglyceride form are generally recognized as safe and are widely used as emulsifying agents in the food industry. Pharmacokinetic experiments were thus performed on rats treated with an oral dose of 309 mg/kg of either DHA monoglyceride (MAG-DHA), DHA triglycerides (DHA-TG) or DHA ethyl ester (DHA-EE), based on the recommended daily dose of DHA for humans following dose translation from human to rat by the equation described by Reagan-Shaw et al., 2008. Blood samples from different groups were taken at 0, 0.5, 4, 8 and 24 h post-drug administration and DHA concentration (%) in plasma was determined by high performance liquid chromatography (HPLC). Figure 5B demonstrates that DHA monoacylglyceride increases the oral bioavailability of DHA compared to commercially available marine oil (Fortin, 2008).

The Rho-kinase pathway participates in vasoconstriction elicited by numerous agents involved in PAH, including TXA_2, ET-1 and 5-HT (Rodat-Despoix et al., 2009; Rodat-Despoix et al., 2008; Connolly & Aaronson, 2011). Rho is a small monomeric GTPase which activates Rho-associated kinase (ROCK) which in turn phosphorylates and inhibits myosin light chain phosphatase, leading to prolonged, refractory vasoconstriction. Rho kinase is considered to be a major determinant of arterial tone, through its essential role in the regulation of Ca^{2+} sensitivity of the contractile machinery in smooth muscle cells (Somlyo et al., 2003). Moreover, Rho kinase regulates a variety of cellular functions including motility, proliferation, apoptosis,

contraction and gene expression. Amongst promising targets recently identified is the Rho-kinase pathway (Loirand et al., 2006). Recent pharmacological studies suggest that activation of the small G protein RhoA and its target Rho kinase is a critical shared mechanism in the pathogenesis of PAH (Loirand et al., 2006). *In vivo*, potent effects of treatment with Rho-kinase inhibitors (Y-27632 or fasudil) have been demonstrated in several animal models of PAH (Abe et al., 2004, Fagan et al., 2004, Nagaoka et al., 2006). Furthermore, acute intravenous administration of low dose fasudil has been shown to reduce PVR and Ppa in patients with PAH (Fukumoto et al., 2005, Ishikura et al., 2006, Fujita et al., 2010).

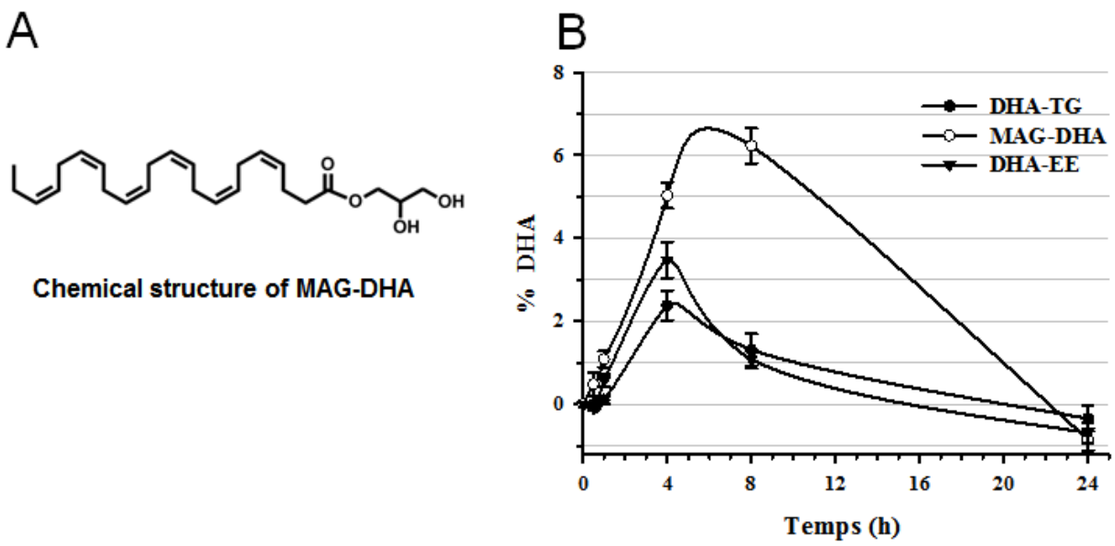


Fig. 5. Chemical structure and oral absorption of DHA monoacylglyceride (MAG-DHA). A: MAG-DHA was synthesized from highly purified DHA attached to a monoglycerol in sn-1 position. B: Pharmacokinetic experiments showing DHA concentration (%) in plasma derived from rats treated with an oral dose (309 mg/kg) of either DHA triglycerides (TG-DHA), DHA monoglyceride (MAG-DHA) or DHA ethyl ester (DHA-EE). Blood samples were taken at 0, 0.5, 4, 8 and 24 h post-drug administration, (n=6 for each experimental condition).

To investigate the potential usefulness of MAG-DHA, complementary approaches were used in our laboratory to assess putative changes in Ca^{2+} sensitivity and to determine the activation of RhoA in human pulmonary arteries. Comparative analyses were performed on β-escin-permeabilized human pulmonary arterial rings to assess the effect of MAG-DHA pre-treatments on Ca^{2+} sensitivity. Figure 6A illustrates CCRC to free Ca^{2+} concentrations on permeabilized HPA rings obtained from control and treated tissues. When compared to control (untreated) condition (Fig. 6A, dashed line), addition of the TP receptor agonist U-46619 to the organ bath enhanced Ca^{2+} sensitivity to pre-calibrated Ca^{2+} step increases in HPA explants (Fig. 6A, open circles). However, MAG-DHA pretreatment resulted in a marked inhibitory effect on Ca^{2+}-sensitivity (right shift) developed by U-46619-treated explants. Data analysis demonstrated that MAG-DHA treatment induced a shift in EC_{50} values (0.94 μM) toward higher Ca^{2+} concentrations when compared to untreated tissues challenged with U-46619 (0.13 μM) (Fig. 6A). However, the difference in Ca^{2+} sensitivity between control HPA and tissues pre-treated with 3 μM MAG-DHA was not significant, with EC_{50} values of 0.65 μM and 0.51 μM, respectively (Figure 6A). Furthermore, the

involvement of the Rho-kinase pathway was examined using a RhoA pull down assay to evaluate RhoA activity following MAG-DHA treatment in HPA-derived homogenates. Thus, in order to investigate whether these observations were correlated with a modulation status of contractile proteins, RhoA activity level was assessed in HPA following MAG-DHA treatment in the absence or presence of MSPPOH, a CYP450 epoxygenase inhibitor and in 19,20-EpDPE-treated tissues alone. Western blot and quantitative analyses of GTP-RhoA/RhoA ratio revealed that MAG-DHA treatment reduced the activity of RhoA induced by U-46619, while an increased RhoA activity level was observed in the presence of MS-PPOH. Hence, 19,20-EpDPE pre-treatment resulted in a reduction in RhoA activity level when compared to the activity level detected upon U-46619 treatment (Figure 6B). These data suggest that MAG-DHA can be metabolized by CYP450 epoxygenase enzymes, leading to the sequential production of 19,20-EpDPE. This CYP450 metabolite in turn decreases RhoA activity level leading to an inhibition of Rho-kinase activation, thus resulting in a lower Ca^{2+}-sensitivity of HPA.

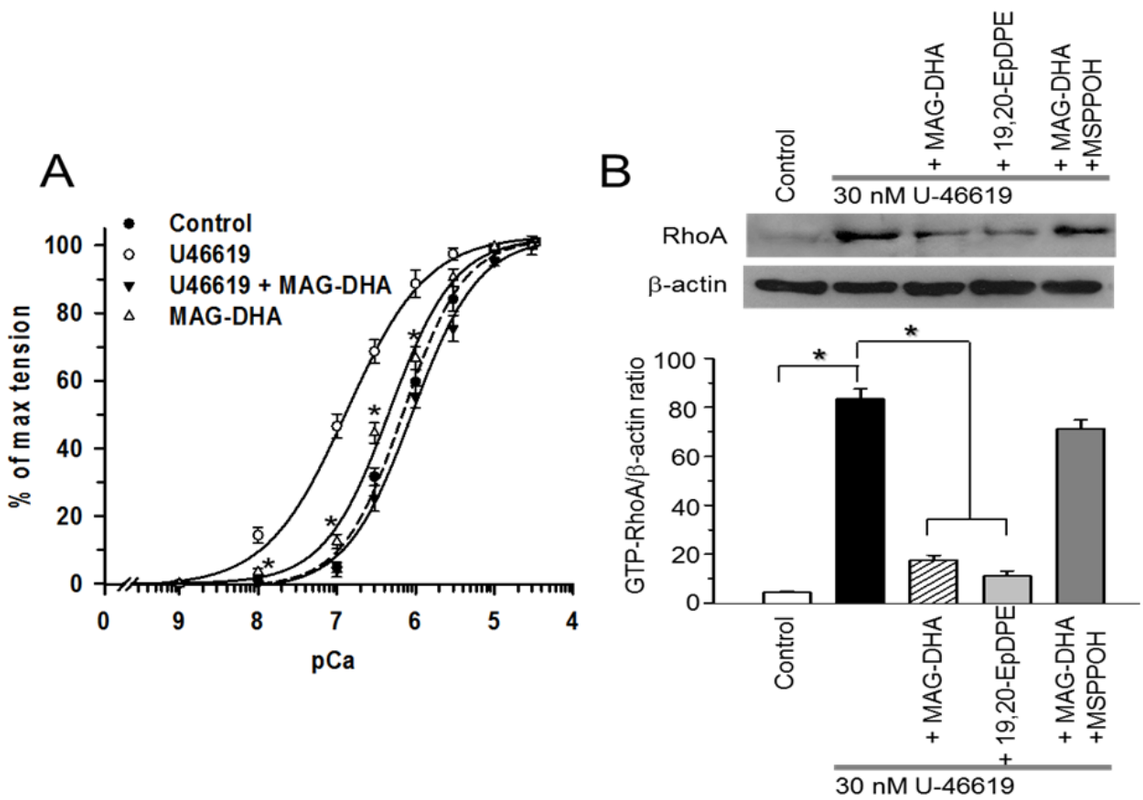


Fig. 6. MAG-DHA modulates Ca^{2+} sensitivity and Rho A activity in human pulmonary arteries.

A: Cumulative Concentration Response Curve to free [Ca^{2+}] obtained in control (untreated HPA) and after short term (20 min) stimulation with 30 nM U-46619; as well as on 3 µM MAG-DHA-treated HPA alone and after 30 nM U46619 challenge. Each point represents the mean ± s.e.m., n= 12 for each experimental condition, * $p < 0.05$. B: RhoA activity was quantified in human pulmonary artery homogenates by measurement of RhoA-GTP using a Rhotekin pull-down assay, (n=8 for each experimental condition, * $p < 0.05$).

Several studies have demonstrated that the use of Rho-kinase inhibitors reduces PAH in many animal models (Oka et al., 2007, Mouchaers et al., 2010). Y-27632 inhaled at 10 – 100 mM was shown to reduce mean pulmonary arterial pressure without altering systemic arterial pressure in a hypoxic rat model of hypertension (Nagaoka et al., 2004). In the monocrotaline model, fasudil 30 or 100 mg/kg/day per os improved survival, pulmonary hypertension, right ventricular hypertrophy as well as pulmonary vascular lesions (Abe et al., 2004). In Fawn-hooded rats exhibiting a raised pulmonary arterial pressure (PAP), inhaled fasudil reduced PAP to 55 mmHg without altering mean systemic arterial pressure (Nagaoka et al., 2006). In humans, Rho-kinase inhibition with fasudil has also been shown to bring about an immediate, albeit modest, reduction in PVR, although this Rho kinase inhibitor must be administrated by nebulisation to avoid systemic hypotension (Fujita et al., 2010, Ishikura et al., 2006). Our data attest that MAG-DHA, a newly synthesized DHA derivative, targets the Rho-kinase pathway to reduce U-46619-induced tension in HPA. Moreover, we were able to demonstrate that MAG-DHA treatment reduced RhoA activation which in turn inactivated the Rho-kinase pathway resulting in a reduction in U-46619-induced Ca^{2+} sensitivity of human pulmonary arterial smooth muscle cells. Finally, our data with the newly synthesized DHA derivative, MAG-DHA, could represent a new pharmacological agent of clinical interest in the management of PAH. Further investigations using *in vivo* models, such as a hypoxic rat model or a monocrotaline model of hypertension would however be required to determine the efficacy of MAG-DHA *per os* treatments in reversing pulmonary hypertension. Furthermore, the identification of CYP450 epoxygenase metabolites using high performance liquid chromatography coupled to tandem mass spectrometry (HPLC/MS/MS) may prove useful to explain the effect of MAG-DHA in these *in vivo* hypertension models.

5. Conclusion

Long-chain PUFAs are metabolized by CYP450 epoxygenase and ω-hydroxylase enzymes in the lung which induce the production of several bioactive eicosanoids, such as the EET regioisomers, 20-HETE, 17,18-EpETE and docosanoids, such as 19,20-EpDPE. These epoxy- and hydroxyl-derivatives are able to modulate the electrophysiological and mechanical properties of human pulmonary arterial smooth muscle. In addition, these vasoactive metabolites induce the activation of K^+ channels and reduce the Ca^{2+} sensitivity of the contractile apparatus in preparations derived from human pulmonary arteries. The main limitation of these compounds is their relative instability, further justifying the use of biochemical precursors or stable analogues (Roman, 2002). Thus we propose that PUFA derivatives, possessing selective pulmonary vasodilation capabilities, may provide an interesting new approach for various forms of PAH. Moreover, we have evaluated and summarized the effects of DHA monoacylglyceride on the reactivity of pulmonary arterial smooth muscle. Data revealed that MAG-DHA is likely metabolized into 19,20-EpDPE by the action of CYP450 epoxygenases in human lung. This epoxy-metabolite prevents U-46619-induced vasoconstriction through a decrease in RhoA activation thus leading to a reduction in Ca^{2+} sensitivity of smooth muscle cells. Since the activated RhoA/Rho kinase pathway is associated with both acute pulmonary vasoconstriction and chronic pulmonary artery remodelling, it is of potential clinical interest to find a lipid mediator able to reduce the activation of Rho-kinase. Moreover, animal and clinical studies have demonstrated that Rho-kinase inhibitors could inhibit signal transductions initiated by many vasoactive drugs;

hence it is possible that MAG-DHA may exert broader beneficial effects as compared to single receptor antagonists due to its mode of action on intracellular targets. In this respect, it is postulated that DHA-monoacylglyceride leads to the production of bioactive metabolites which represent new and prospective pharmacological compounds of low toxicity and medicinal interest in modulating pulmonary vasoconstriction. Collectively, the present review provides new insight regarding the mode of actions of specific epoxy and hydroxy eicosanoids and docosanoids, which represent biochemical compounds of putative clinical relevance in pulmonary hypertension.

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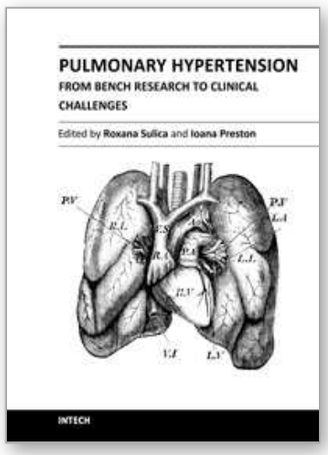
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The textbook "Pulmonary Hypertension - From Bench Research to Clinical Challenges" addresses the following topics: structure and function of the normal pulmonary vasculature; disregulated cellular pathways seen in experimental and human pulmonary hypertension; clinical aspects of pulmonary hypertension in general; presentation of several specific forms of pulmonary hypertension, and management of pulmonary hypertension in special circumstances. The textbook is unique in that it combines pulmonary and cardiac physiology and pathophysiology with clinical aspects of the disease. First two sections are reserved for the basic knowledge and the recent discoveries related to structure and cellular function of the pulmonary vasculature. The chapters also describe disregulated pathways known to be affected in pulmonary hypertension. A special section deals with the effects of hypoxia on the pulmonary vasculature and the myocardium. Other three sections introduce the methods of evaluating pulmonary hypertension to the reader. The chapters present several forms of pulmonary hypertension which are particularly challenging in clinical practice (such as pulmonary arterial hypertension associated with systemic sclerosis), and lastly, they address special considerations regarding management of pulmonary hypertension in certain clinical scenarios such as pulmonary hypertension in the critically ill.

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