Role of the soluble epoxide hydrolase and cytochrome P450-derived epoxyeicosatrienoic acids in hypoxic pulmonary vasoconstriction and hypoxia-induced pulmonary vascular remodelling

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To Gila

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1. Introduction

1.1. Hypoxic pulmonary vasoconstriction

Hypoxic pulmonary vasoconstriction (HPV) is unique to the pulmonary circulation and redistributes blood flow from areas of low oxygen partial pressure to areas of normal or relativity high oxygen availability, thus optimising the matching of perfusion and ventilation and preventing arterial hypoxemia.[1,2] HPV was first described by Von Euler and Liljestrand in 1946 who ventilated cats with an hypoxic gas mixture and recorded an increase in pulmonary artery pressure (PAP).[3] The strength of HPV is now known to depend on species, age, gender, pCO_2 and pH and the precapillary smooth muscle cells of resistance pulmonary arteries have been identified as effector cells. The exact molecular identity of the pulmonary oxygen sensor and the signalling cascade leading to hypoxic pulmonary vasoconstriction, however, remain to be fully elucidated.[4] HPV is initiated within seconds of moderate hypoxia and reverses quickly on restoration of normoxic ventilation.[5] Longer periods of hypoxia elicit a biphasic response consisting of a rapid constriction (phase 1) followed by a temporary vasodilation and a secondary slowly developing sustained contraction (phase 2) which can last for hours or even days if hypoxia is maintained.[6] At present it is not clear whether the acute hypoxic vasoconstriction and the prolonged response to hypoxia are regulated by identical or different mechanisms.[7]

In clinical situations, HPV may occur either as an acute episode during rapidly progressive critical illnesses or as a sustained response with pulmonary hypertension in progressive lung diseases.[8] Widespread alveolar hypoxia occurs as a result of many lung illnesses (e.g. asthma, inhalation of foreign objects, chronic obstructive pulmonary disease, emphysema), acute lung damage (e.g. pneumonia, acute respiratory distress syndrome) or as a consequence of movement to high altitude. Chronic hypoxia results in a sustained increase in pulmonary artery pressure which in turn leads to structural changes in the walls of the pulmonary vasculature (pulmonary vascular remodelling).

1.2. Hypoxia-induced pulmonary vascular remodelling

Under normal conditions, the thickness of the vascular wall is maintained at an optimal level by a fine balance between proliferation and apoptosis of the resident cell types. If this balance is disturbed in favour of proliferation, the vascular wall thickens and eventually obliterates the vessel lumen, leading to increased resistance. It is accepted that hypoxia is a cause of pulmonary vascular proliferation and vascular remodelling, but the mechanism remains unclear. Generally it is believed that the contribution of vasoconstriction is greatest early in the disease process and that structural remodelling of the pulmonary vascular bed becomes progressively important with time.[9] Remodelling processes include an increase in the extent of pulmonary artery muscularisation which is attributed to the increased proliferation and decreased apoptosis of pulmonary artery smooth muscle cells, media hypertrophy and eccentric intimal fibrosis,[10,11] and is thought to result from an alteration in the balance between the effects of vasodilators and antiproliferative agents and vasoconstrictors and mitogenic factors.[12]

Hypoxia activates a cascade of intracellular signalling mechanisms, including receptor and nonreceptor tyrosine kinases, mitogen-activated protein kinases (MAPK), protein kinase C (PKC), phosphotidylinositol 3-kinase (PI3K), SMAD phosphorylation, calcium (Ca^{2+}) entry and Rho kinase, which collectively act to control smooth muscle cell contractility, growth, differentiation, and matrix synthesis.[13] Changes in receptor expression or function as well as in Ca^{2+} handling have been observed in smooth muscle cells from pulmonary hypertensive patients.[14] Chronic hypoxia is also reported to downregulate the expression of K_v channels and upregulates the expression of canonical transient receptor potential (TRPC) channels in pulmonary artery smooth muscle cells[14,15] causing an increase in intracellular Ca^{2+} leading to pulmonary vasoconstriction. Furthermore, K_v downregulation is reported to be proliferative and anti apoptotic.[15,16]

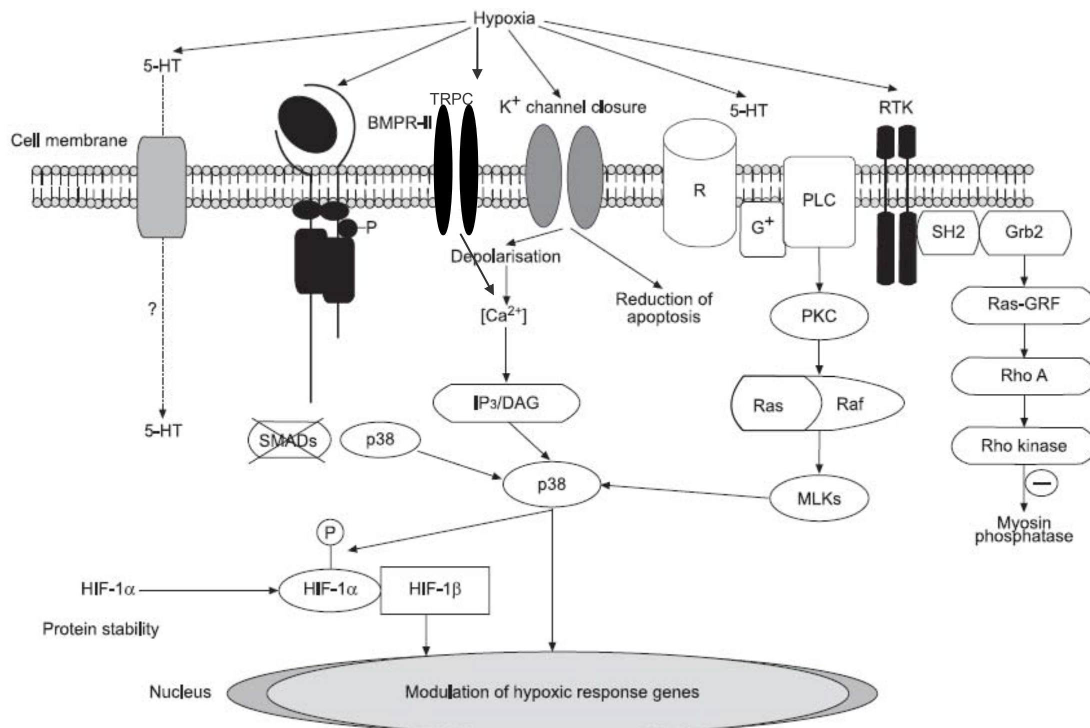

Figure 1: Scheme illustrating the hypoxic signalling pathways that lead to vascular remodelling of the pulmonary artery. 5-HT: 5-hydroxytryptamine; BMPR-II: bone morphogenetic protein receptor type II; RTK: receptor tyrosine kinase; P: phosphate; R: receptor; G: G protein; PLC: phospholipase C; SH: Src homolgy; Grb: growth factor receptor-bound protein; GRF: guanine nucleotide releasing factor; PKC: protein kinase C; IP_3: inositol-1,4,5-trisphosphate; DAG: diacylglycerol; MLK: mixed lineage kinase; HIF: hypoxia-inducible factor; TRPC: canonical transient receptor potential channels (modified from Pak 2007).[9]

The severity of chronic hypoxic pulmonary hypertension and remodelling is largely determined by the extent of the structural changes in the media of the pulmonary arterial wall. Diverse molecular mechanisms are involved in hypoxia-induced pulmonary vascular remodelling (Figure 1). Chronic exposure to hypoxia is also associated with a number of changes in the production and release of potent vasoactive substances by the endothelium which can exert significant effects not only on the contractile state of smooth muscle cells but on their proliferative state as well.[13] For example, decreased production of nitric oxide (NO) and prostacyclin (PGI_2) have been reported[17] as well as increased production of platelet activating factor (PAF), 5-hydroxytryptamine (5-HT), leukotrienes, endothelin, platelet derived growth factor (PDGF), and transforming growth factor-β (TGF-β).[13,18] 5-HT is a vasoactive and mitogenic factor and several studies have shown a role for the 5-HT

transporter (5-HTT) and 5-HT_1 and 5-HT_{2B} receptors in chronic hypoxia-induced pulmonary vascular remodelling.[12,19-23]

1.3. *Oxygen sensors*

Although the importance of HPV for the optimisation of pulmonary gas exchange was recognised relatively early, the molecular identity of the pulmonary oxygen sensor and the signalling cascade that initiates pulmonary artery smooth muscle contraction have not yet been identified. Current thinking tends to attribute the role of O_2 sensor to the mitochondria or the NADPH oxidase. Effectors are thought to be potassium channels, Ca^{2+} channels and the Rho kinase. Moreover, we recently reported that Cytochrome P450 (CYP) epoxygenase-derived epoxyeicosatrienoic acids (EETs) are involved in mediating HPV (Figure 2).[24]

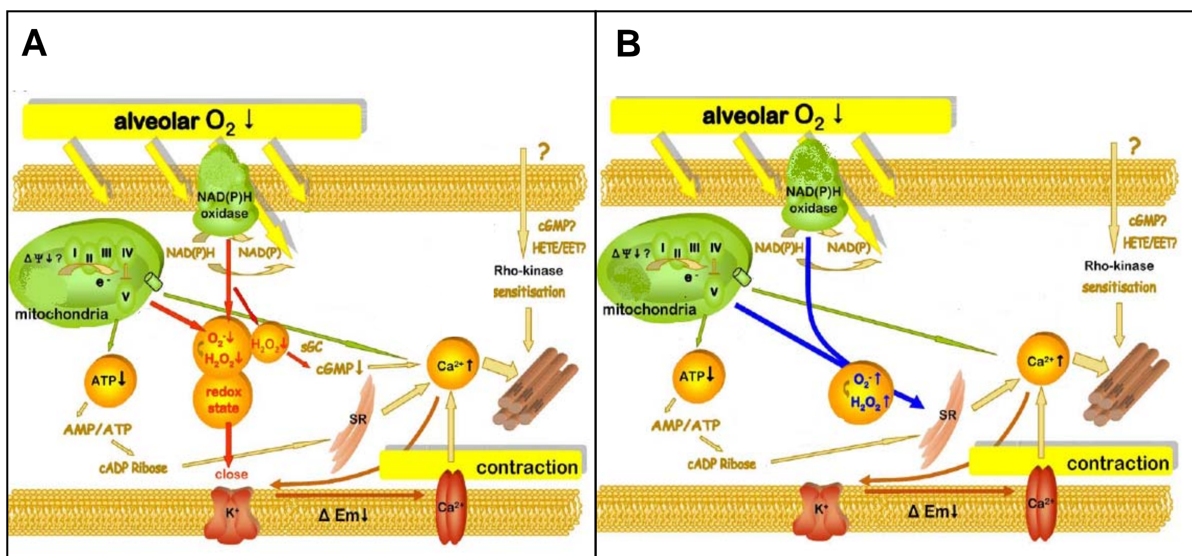

Figure 2: Current concepts of the oxygen sensing of hypoxic pulmonary vasoconstriction. Possible oxygen sensors are shown in green, mediators of hypoxic pulmonary vasoconstriction (HPV) in yellow. pO_2: oxygen partial pressure; Δψ: mitochondrial membrane potential; SR: sarcoplasmic reticulum; HETE: hydroxyeicosatetraenoic acid; EET: epoxyeicosatrienoic acid; Em: cellular membrane potential. (A) Includes those concepts that comprise a decrease in reactive oxygen species (ROS) as a trigger for HPV (red lines), (B) those that comprise an increase in ROS as a trigger for HPV (blue lines; modified from Weißmann 2006).[4]

The mitochondria are the main site of cellular oxygen consumption as well as energy generation and determine the cellular redox state. There are additional arguments that favor a role of the mitochondria as primary oxygen sensors. For example, inhibition of the mitochondrial electron transport chain blocks HPV, and pulmonary artery smooth muscle cells (PASMC) that lack a functional electron chain fail to show hypoxic-specific responses.[25-27] Different theories exist concerning the role for mitochondria in HPV.[28] Several groups propose that hypoxia decreases mitochondrial reactive oxygen species (ROS) production thus shifting the cellular redox state to a more reduced state and stimulating the closure of K_v channels which leads to depolarisation of the PASMC membrane potential.[26,29-32] Other investigators have provided evidence indicating that in PASMCs mitochondrial radical production increases during hypoxia and triggers the release of intracellular Ca^{2+} thus causing HPV.[6,27,33] A role for adenosine triphosphate (ATP) as a second messenger for HPV has also been suggested since the electron transport chain is the main oxygen consumption site in the cell. Indeed a recent theory postulates that hypoxia decreases ATP production, thus increasing the adenosine monophosphate (AMP)/ATP ratio in PASMCs. The latter imbalance results in the activation of the AMP-activated kinase and increases cyclic adenosine diphosphate (cADP)-ribose which then releases Ca^{2+} through ryanodine-sensitive Ca^{2+} stores.[34,35] However, a link between HPV and the latter signalling cascade still needs to be definitely demonstrated. Mitochondria also play a role in PASMC cytosolic Ca^{2+} homeostasis through a Ca^{2+} uniporter that is driven by the mitochondrial membrane potential, and the concentration of cytosolic Ca^{2+}.[36] Indeed, a decrease in membrane potential due to hypoxia-impaired respiration induces a mitochondrial Ca^{2+} release thus triggering HPV.[28]

Given the link between hypoxia and ROS production, it is not surprising that another potential O_2 sensor are the NADPH oxidases (NOX). The latter are a family of superoxide anion (O_2^-)-generating enzymes, of which several NOX isoforms have been identified.[37] Interest in the concept of that the NOXs could act as oxygen sensors for HPV emerged following reports that NOX inhibition, using diphenyleneiodonium or apocynin, two relatively unspecific NOX inhibitors, blocked HPV.[38,39] Currently two diverging concepts regarding the role of NOX in oxygen

sensing exist. The first postulates that hypoxia upregulates NOX-derived O_2^- production triggering the release of intracellular Ca^{2+} in PASMCs thus causing pulmonary vasoconstriction. The second assumes that hypoxia decreases NOX-derived O_2^- production and subsequent H_2O_2 generation which in turn attenuates the activity of the soluble guanylate cyclase (sGC) leading to a decrease in PASMCs cGMP-levels and consequently vasoconstriction.[4]

1.4. Cytochrome P450 enzymes

Cytochrome P450 enzymes are membrane-bound, heme-containing terminal oxidases and are part of a multi-enzyme system that also includes an FAD/FMN-containing NADPH-CYP reductase and a cytochrome b_5. CYP enzymes oxidise, peroxidise and/or reduce cholesterol, vitamins, steroids, xenobotics and different pharmacological substances in an oxygen and NADPH-dependent manner.[40] CYP enzymes are typical monooxygenases in that the enzymatic cleavage of molecular oxygen is followed by the insertion of a single atom of oxygen into the substrate while the remaining oxygen is released as water (Figure 3).[41]

Cytochrome P450 enzymes are ubiquitously expressed and are widely distributed in plants, insects and animal tissues. While in animals, most CYP enzymes are mainly expressed in the liver, lower levels of CYP enzymes are expressed in extra-hepatic tissues, such as the heart, the vasculature, the gastrointestinal tract and the lung.[42-46]

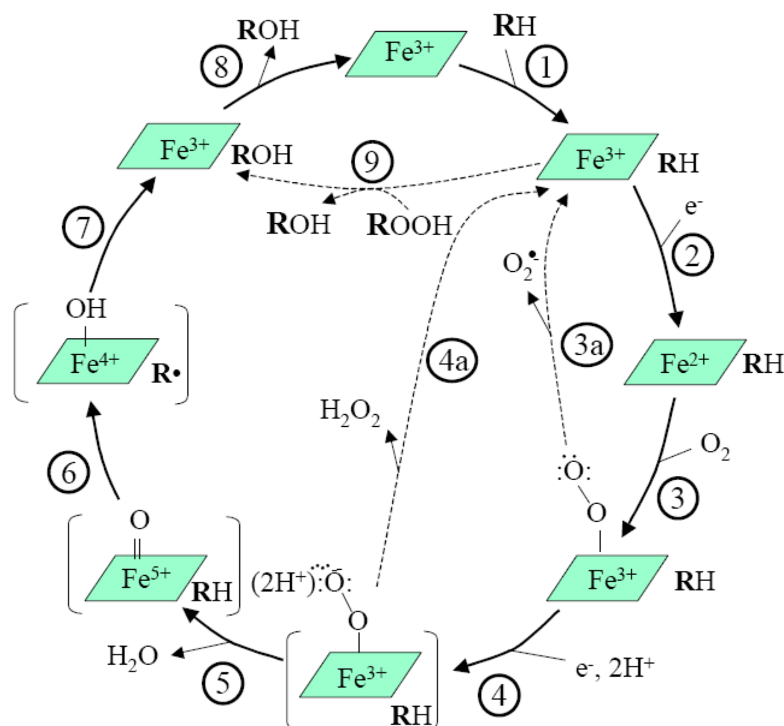

Figure 3: Catalytic cycle of cytochrome P450 enzymes. (1) Substrate (R) binding (Fe^{3+}-RH). (2) Cytochrome P450 reductase-dependent one electron reduction of the heme iron (Fe^{2+}-RH). (3-4) Binding of molecular oxygen, oxidation of the heme iron (Fe^{3+}) and transfer of the second electron to the complex. (3a) During this step generation of superoxide anions can occur. (5) Cleavage of the O-O bound with the concurrent incorporation of the distal oxygen atom into a molecule of water and the formation of a reactive iron-oxo species. (6-7) Oxygen atom transfer from the oxo complex to the substrate and dissociation of the product (8). (9) Peroxide shunt: hydroxylation of peroxides by CYP enzymes (from Davydov 2001).[47]

To-date more than 500 CYP isoforms have been identified.[48] The nomenclature of the various enzymes can be quite confusing. Proteins with approximately more than 40% homology in their sequence are included in the same family which is designated by an Arabic number. Proteins with more than 55% identity are grouped together in the same subclass as designated by a capital letter, the last number identifies specific gene products.[40]

Since it was recognised that many CYP isoforms are capable of metabolizing arachidonic acid to biologically active products, CYP enzymes have also been described as the third pathway of arachidonic acid metabolism; i.e., in addition to cyclooxygenases and lipoxygenases. CYP enzymes with an important role in

cardiovascular function are the epoxygenases of the CYP2C and 2J gene families and the ω-hydroxylases of the CYP4A family.

1.5. Cytochrome P450-derived arachidonic acid metabolites

CYP epoxygenases metabolise arachidonic acid to four regioisomeric epoxyeicosatrienoic acids (5,6-, 8,9-, 11,12-, and 14,15-EET) (Figure 4). The ratio of the produced EET isomers is dependent on the CYP isoform. The CYP2C8 isoform produces 14,15- and 11,12-EET in a ratio of 1.25:1. CYP2C9 produces 14,15-, 11,12-, and 8,9-EET in a ratio of 2.3:1:0.5, whereas the CYP2J2 isoform produces 5,6-, 8,9-, 11,12-, and 14,15-EETs with equal efficiency.[45,49] In contrast to the epoxygenases, the ω-hydroxylases metabolise arachidonic acid sub-terminally and ω-terminally to hydroxyeicosatetraenoic acids (HETE).[50] Other CYP enzymes for example the rat CYP4A2 and 4A3 are able to generate both EETs and HETEs.[51]

After their generation EETs can be incorporated into phospholipids, mainly phosphatidylcholine- and phosphatidylinositol-phospholipids.[52-54] These phospholipids are thought to be an intracellular reservoir for EETs.[55] The magnitude of endogenous EET levels was thought to depend on CYP activity. As inhibition of the phospholipase A_2 attenuated the CYP-dependent endothelium-derived hyperpolarising factor (EDHF) responses, CYP-activity was thought to be determined by the availability of its substrate (arachidonic acid).[40] However, phospholipases are not the only enzymes that control intracellular levels of CYP-derived metabolites. EETs can also be metabolised to various products. 5,6-EET is the chemically least stable arachidonic acid epoxide and is also metabolised by the cyclooxygenase,[56] whereas all other EET regio-isomers are degraded to their less active dihydroxyeicosatrienoic acids (DHET) by epoxide hydrolases (EH) (Figure 4).[57] Epoxide hydrolases exist as microsomal (mEH) and as soluble (sEH) isoforms. The sEH metabolises EETs most efficiently and therefore inhibition or deletion of the sEH increases EET levels.[58] There are alternative, less efficient ways of controlling cellular EET levels; for example β-oxidation or C2 elongation.[59]

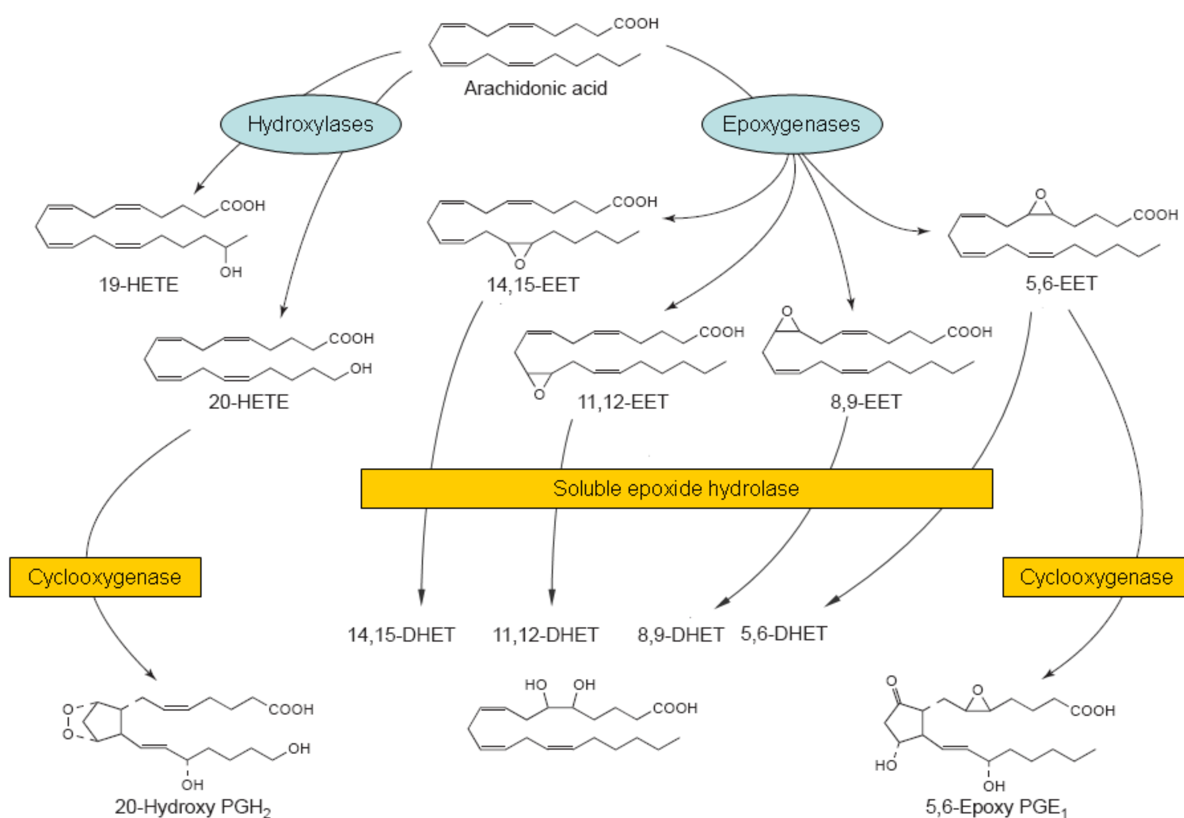

Figure 4: Overview of the pathways for regulating cellular levels of cytochrome P450-derived metabolites of arachidonic acid (modified from Quilly 2000).[60]

In the systemic circulation, 20-HETE and the EETs play an important role in the regulation of vascular tone.[48] EETs act as anti-inflammatory mediators and EDHF mediating NO and PGI_2-independent but endothelium-dependent vasodilatation in some vascular beds.[61,62] A number of studies have examined the mechanism by which EETs reduce vascular tone. The first well documented effect was the activation of Ca^{2+}-activated K^+ (K^+_{Ca}) channels on vascular smooth muscle cells to elicit hyperpolarisation and relaxation.[40,61] Although several groups have demonstrated that all of the EET regioisomers are potent vasodilators in bovine and porcine coronary arteries[63,64] this is not the case in all vascular beds. For example, in feline cerebral arteries, the vasodilator response to 11,12-EET is greater than seen with 8,9- or 5,6-EET[65] and in rat cerebral and caudal arteries 5,6-EET is a more effective dilator than the other regioisomers.[66,67] The molecular mechanisms involved in mediating the EET-induced activation of K^+_{Ca} channels are not entirely clear. EETs

can affect the activity of at least two different classes of TRP channels (TRPV (Vanilloid) and TRPC). For example, 5,6- and 8,9-EET but not 11,12-EET were reported to modulate the activity of TRPV4, a channel implicated in mechanotransduction.[68] Moreover, our group recently demonstrated that 11,12-EET modulates the agonist-induced hyperpolarisation of endothelial cells by a mechanism related to cyclic adenosine monophosphate (cAMP)/protein kinase A (PKA) activation and TRPC6 translocation.[69] Thus the diversity in EET regioisomer-induced responses would be expected to depend on the TRP channels expressed. 20-HETE, on the other hand, constricts renal, cerebral, coronary and mesenteric arteries by inhibiting K^{+}_{Ca} channels, thereby promoting Ca^{2+} entry by depolarising vascular smooth muscle cells, by increasing the conductance of L-type Ca^{2+} channels and by activating the Rho kinase.[48,70]

1.6. *Effects of hydroxyeicosatetraenoic and epoxyeicosatrienoic acids in the lung*

Although, data from numerous groups have implicated CYP-derived metabolites as modulators of vascular tone and other biological effects in the nonpulmonary tissue, the role of CYP-derived eicosanoids in the lung is incompletely understood.

CYP epoxygenase-derived metabolites have been reported to modulate airway epithelial ion transport,[71,72] activate K^{+}_{Ca} channels in airway smooth muscle cells[73] and dilate guinea pig bronchi.[74] The bronchomotor effect of 20-HETE seems to be complicated as 20-HETE dilated rabbit and human bronchi, but exerted the opposite effect in guinea pig bronchi.[42,75-77] Such contradictory reports seem to characterise the field at the moment as divergent findings have been published regarding a given metabolite dependent on the species, the size of the vessel and the model used.

20-HETE was found to elicit a dose-dependent and indomethacin-sensitive vasodilation of isolated small human pulmonary arteries,[78] while in the rabbit it relaxed phenylephrine-constricted pulmonary artery rings. Inhibition of CYP4A enhanced the acute HPV in isolated buffer-perfused rabbit lungs and shifted the phenylephrine concentration-response curve to the right.[79] In the ovine fetal lung

inhibition of 20-HETE formation decreased the myogenic response[80] and in piglet pulmonary resistance arteries 20-HETE caused a potent vasoconstriction, a response that has been linked to the cyclooxygenase prostaglandin pathway.[81]

5,6-EET caused a relaxation of isolated rabbit pulmonary artery rings and lungs a response that has been linked to NO and prostaglandin production.[82,83] Whereas others have reported that all EET regioisomers constrict isolated pressurised rabbit pulmonary artery rings and that inhibition of rabbit pulmonary CYP epoxygenases shifted the phenylephrine concentration-response curve to right.[79,84] The signalling pathway underlying EET-induced vasoconstriction seems to involve the Rho kinase as the 5,6-EET-induced contraction of intralobar rabbit pulmonary arteries was reportedly sensitive to Rho kinase inhibition.[85] In piglets, 5,6-EET was reported to dilate pulmonary resistance arteries via the activation of small and intermediate K^+_{Ca} channels.[86] Others have reported that 5,6-EET reduces the thromboxane A_2 analogue U46619-mediated increase in pulmonary vascular resistance in the isolated canine lung,[87] and relaxes canine pulmonary venous rings, an effect that was sensitive to cyclooxygenase inhibition.[88] In the rat, all EET regioisomers elicit an increase in wall tension of isolated pulmonary artery rings[89] and 11,12-EET and 14,15-EET are reported to enhance pulmonary resistance.[90]

Recently, our group demonstrated that a CYP epoxygenase is implicated in hypoxia-induced pulmonary vasoconstriction and pulmonary remodelling in the mouse.[24] In the latter study it was possible to demonstrate that the mediator involved in acute and chronic hypoxia-induced pulmonary vasoconstriction and chronic hypoxia-induced pulmonary vascular remodelling was a CYP epoxygenase-derived product, as inhibition of this enzyme decreased the acute and chronic HPV response and attenuated chronic hypoxia-induced pulmonary remodelling.[24] Moreover, as inhibition of the sEH enhanced the acute HPV response the potential mediator might be one of the EETs. Furthermore, hypoxia up to 1 week enhanced pulmonary levels of 8,9-, 11,12- and 14,15-EET without affecting 5,6-EET concentrations. Furthermore, lung specific gene transfer of the human CYP2C9 significantly enhanced right ventricular systolic pressure and total pulmonary resistance and both effects were sensitive to the specific CYP2C9 inhibitor sulfaphenazole.[24]

In the lung, more precisely in endothelial cells and human bronchi EETs display anti-inflammatory properties. Indeed, the overexpression of CYP2J2 in endothelial cells or application of 11,12-EET to uninfected endothelial cells decreased cytokine-induced endothelial cell adhesion, molecule expression and prevented leukocyte adhesion to the vascular wall by a mechanism involving inhibition of the transcription factor nuclear factor κB (NFκB) and the inhibitor of κB (IκB) kinase.[91] In tumor necrosis factor-α (TNF-α) stimulated human bronchi 14,15-EET also displayed anti-inflammatory effects, again attributed to the inhibition of the IκB degradation, suggesting a lower activation of NFκB.[92]

1.7. Soluble epoxide hydrolase

The soluble epoxide hydrolase which metabolises fatty acid epoxides (EETs, leukotriene A_4; LTA_4, epoxyoctadecenoic acids; EpOME, and epoxystearic acid; EpO) to their corresponding diols belongs to the α/β-hydrolase enzyme family (Figure 5). The enzyme was termed cytosolic and later soluble epoxide hydrolase because of its localisation in the soluble fractions of the cell.

In mammals the sEH is a homodimer composed of two 60kDa monomers joined by a proline rich bridge and is the product of the EPHX2 gene.[93-95] Each monomer has an N-terminal domain which displays lipid phosphatase activity and a larger C-terminal which processes classical α/β-hydrolase activity.[95,96] Although the biological role of the N-terminal lipid phosphatase activity remains unclear, the loss of sEH activity results in increased circulating levels of EETs and a decrease in DHET production. Tissues with high sEH expression are the liver, the kidney and the retina, but the enzyme is also expressed by some endothelial cells, smooth muscle cells, pancreatic islets and leucocytes.[97,98] There are different factors that regulate sEH expression including gender, hormones and genetic background. For example, sEH activity is increased in male mice versus female mice and rats, and castration decreased sEH activity and expression; a phenomenon restored by testosterone supplementation.[99,100] Moreover, there is a clear link to cardiovascular diseases as renal microsomes from spontaneously hypertensive rats displayed an increased sEH expression[101] and inhibition of the sEH in spontaneously hypertensive rats blunted

the development of hypertension.[101-103] Interestingly, angiotensin II increases vascular sEH expression and pharmacological sEH inhibition prevented or reversed angiotensin II-induced hypertension in mice and rats.[104,105]

Substrate	Absolute conformation	K_m (μM)	V_{max} (μmol/min/mg)	V_{max}/K_m
14,15-EET[a]	14(*R*),15(*S*)	4	9.03	2.3
	(±)	–	4.53	–
	14(*S*),15(*R*)	5	1.36	0.27
11,12-EET[a]	11(*S*),12(*R*)	4	3.02	0.76
	(±)	–	1.65	–
	11(*R*),12(*S*)	3	0.82	0.27
8,9-EET[a]	8(*S*),9(*R*)	5	3.10	0.62
	(±)	–	1.45	–
	8(*R*),9(*S*)	41	0.83	0.020
5,6-EET	(±)	–	<0.1	–
12,13-EpOME	(±)	6.2	2.67	0.43
9,10-EpOME	(±)	5.2	1.86	0.36
9,10-EpO	(±)	11	3.5	0.31
14,15-LTA_4[d]	(±)	11	0.90	0.081
14,15-LTA_4[b]	(±)	48	1.5	0.031
11,12-LTA_4[b,c]	(±)	18	2.4	0.13
5,6-LTA_4[b]	(±)	25	2.1	0.084
5,6-LTA_4	(±)	5	0.55	0.11

Figure 5: Activity of mammalian sEH with various fatty acid epoxides. EET: epoxyeicosatrienoic acid; LTA_4: leukotrien A_4; EpOME: epoxy octadecenoic acids; EpO: epoxystearic acid (from Newman 2005).[106]

There are several reported polymorphisms in the sEH gene that affect the enzymatic function[107] and that have been linked to cardiovascular disease.[108,109] For example, individuals carrying the K55R polymorphism demonstrated higher soluble epoxide hydrolase activity *in vivo* and a significantly higher risk of coronary heart disease.[110] On the other hand, three polymorphisms in the EPHX2 promotor that resulted in decreased sEH mRNA expression and activity were associated with the increased incidence of stroke in the rat;[111] an observation not entirely consistent with a predominantly anti-inflammatory effect of the sEH substrates. Another interesting observation is the link between sEH expression and cigarette smoking, a well known risk factor for cardiovascular diseases, and which increases sEH expression.[112] More recently, EPHX2 was characterised as a heart failure susceptibility gene in the

spontaneously hypertensive rat and ablation of the gene in the mouse protected from pressure overload-induced heart failure and cardiac arrhythmias.[113] The latter findings could be correlated with EPHX2 downregulation in human failing hearts which could be interpreted as an adaptive transcriptional process aimed at maintaining high levels of cardioprotective EETs.[113,114]

1.8. TRP channels

Transient receptor potential (TRP) channels have been linked to hypoxic pulmonary vasoconstriction[115] and can be activated by specific EETs.[69,116,117] To-date the TRP family of ion channels consists of nearly 30 mammalian members,[118] all belonging to the superfamily of cation channels formed by tetramers of six transmembrane domains which enclose a pore near the C-terminal and function as voltage-independent, non-selective cation channels, i.e., permeable to Na^+, K^+, Cs^+, Li^+, Ca^{2+}, and Mg^{2+}.[119,120] Different TRP channels gate in response to different stimuli (e.g. cold or hot temperatures, natural chemical compounds, mechanical stimuli, or changes in the composition of the lipid bilayer). They play a crucial role in numerous physiological processes e.g. photoreception, pheromone sensing, taste perception, thermosensation, pain perception, mechanotransduction, perception of pungent compounds, renal Ca^{2+}/Mg^{2+} handling, smooth muscle tone and blood pressure regulation.[121]

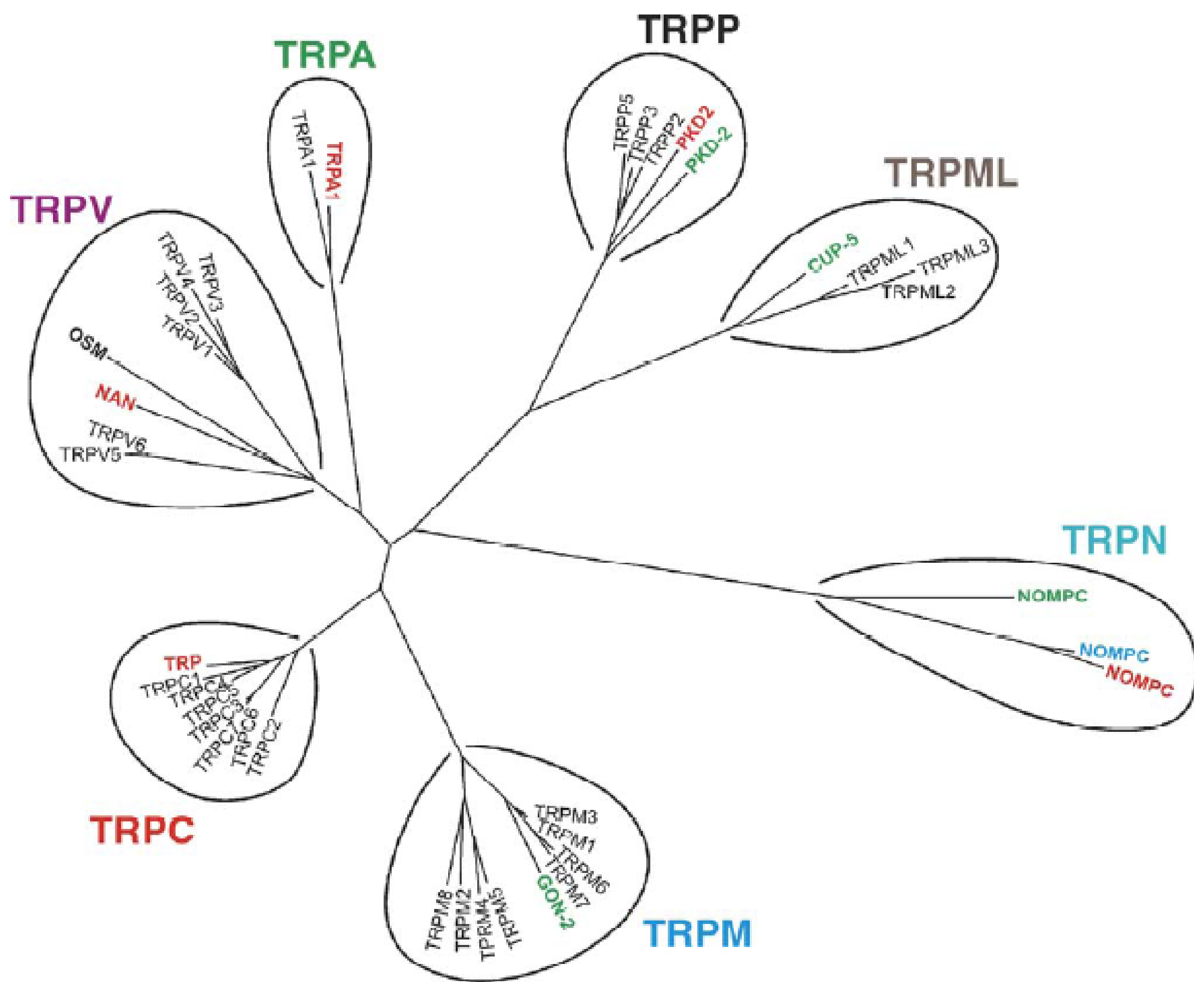

Figure 6: Phylogenetic tree of the TRP superfamily. Based on amino acid homology, TRP channels can be divided into seven subfamilies: TRPC (Canonical, TRPC1-TRPC7), TRPM (Melastatin, TRPM1-TRPM8), the TRPV (Vanilloid) subfamily comprises six members (TRPV1-TRPV6), the TRPML (Mucolipin) familiy comprises three members, the TRPP (Polycystin) family three channel-like and five non-channel members, the TRPA (Ankyrin, A1) and the TRPN (only detected in C. elegans, Drosophila and zebra fish; from Pedersen 2005).[122]

Based on their amino acid homology TRP channels can be divided into seven subfamilies (Figure 6). The TRPC (Canonical) family consists of seven different channels (TRPC1-TRPC7) while the TRPM (Melastatin) channels consist of eight different proteins (TRPM1-TRPM8). The TRPV (Vanilloid) subfamily comprises six members (TRPV1-TRPV6) while the TRPML (Mucolipin) familiy has currently three members and the TRPP (Polycystin) family three channel-like and five non-channel-like members. The TRPA (Ankyrin) has one member and the TRPN (no

mechanoreceptor potential C) has only been detected in C. elegans, Drosophila and Zebrafish.[122]

TRPC channels are broadly expressed and one cell type can contain multiple TRPC channels.[119,122] On the basis of sequence homology and functional similarities members of the mammalian TRPC family can be divided into four subfamilies; TRPC1, TRPC2, TRPC3/6/7 and TRPC4/5.[123] A functional TRPC channel is formed by association of four TRPC proteins and can form as homotetramers or as heterotetramers. For example, TRPC1 can form heteromers with TRPC4 and 5, and the TRPC subfamilies TRPC4/5, and TRPC3/6/7 can form heteromers among themselves.[122] Although the molecular organisation is complex, the evidence that TRPC channels are linked to HPV is convincing and using pharmacological agents (SKF-96365, Ni^{2+} and La^{3+}) to block Ca^{2+} influx through non-selective cation channels in PASMCs a potent inhibition of HPV was demonstrated at concentrations that did not affect voltage-gated Ca^{2+} channels.[124] Along the same lines, TRPC1 overexpression enhances pulmonary artery contraction to cyclopiazonic acid and small interfering RNAs targeted to mRNA encoding TRPC4 suppresses store-operated Ca^{2+} influx in pulmonary artery myocytes in culture, without having an effect on background $[Ca^{2+}]_i$ or Ca^{2+} release signals.[125,126] Moreover, TRPC6 was found to be upregulated in hypoxia-induced as well as idiopathic chronic pulmonary hypertension[14,127] and acute hypoxic vasoconstriction was completely absent in $TRPC6^{-/-}$ mice, whereas the response to chronic hypoxia was unaltered.[115]

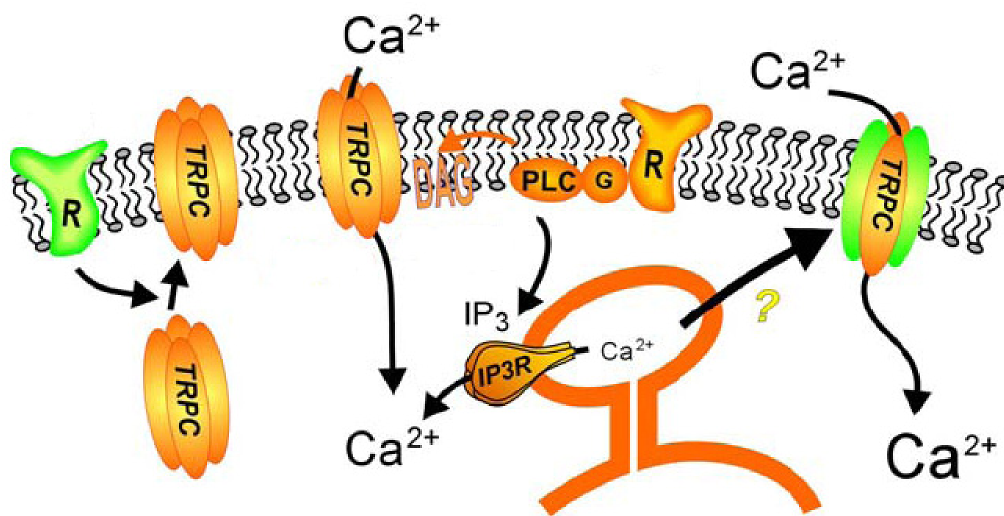

Figure 7: Activation mechanism for canonical transient receptor potential (TRPC) channels. TRPC channels can be activated in any of three distinct ways. From left: Channels sequestered in a vesicular compartment can be translocated to the plasma membrane. TRPC channels can be activated by diacylglycerol (DAG), formed as a result of activation of phospholipase C (PLC) by a G protein-coupled (G) pathway. Activation of PLC leads to the production of inositol-trisphosphate (IP_3), which activates the IP_3 receptor (IP_3R) causing release of Ca^{2+} from a critical component of the endoplasmic reticulum. This can in turn activate TRPC channels through the poorly understood capacitative or store-operated pathway (from Putney 2005).[123]

The TRPC channels can be activated by several mechanisms (Figure 7). They are activated downstream of phospholipase C and are sensitive to diacylglycerol (DAG) and inositol-trisphosphate (IP_3).[122] Initially TRPC channels were thought to be the long sought store-operated channels that account for capacitative Ca^{2+} entry. However, although a number of studies have reported that store depletion activates TRPC channels and that knockout or knockdown of TRPCs reduces store-operated Ca^{2+} entry.[123] Recent studies have revealed novel components of the capacitative Ca^{2+} entry, namely the stromal interacting molecule (STIM) and Orai proteins.[128,129] While STIM1 has been suggested to be the endoplasmatic reticulum Ca^{2+} sensor protein relaying the signal to the plasma membrane for activation of capacitative Ca^{2+} entry, Orai1 is reported to be the pore-forming component of store operated Ca^{2+}channels.[130] TRPC channels are however obviously intimately involved in Ca^{2+} homeostasis as the store-operated and receptor-operated Ca^{2+} entry mechanism in HEK 293 cells are composed of heteromeric complexes that include TRPCs and Orai

proteins that are controlled by STIM1.[131] Another mechanism for the activation of TRPC channels involves the regulation of their trafficking and insertion into the plasma membrane. It has been reported that TRPC1 translocates to the membrane in response to thrombin, TRPC6 and 3 in the presence of carbachol and TRPC4 and 5 upon activation of the epidermal growth factor receptor.[132-137] Recently our group reported that 11,12-EET can affect endothelial Ca^{2+}-signalling by stimulating the intracellular translocation of TRPC6 to caveolae in a cAMP/PKA-dependent manner.[69]

1.9. 5-Hydroxytryptamine (serotonin)

5-Hydroxytrymtamine (5-HT), a potent pulmonary vasoconstrictor, is reported to activate TRP channels.[138-140] Several studies have suggested a role for 5-HT in the pathogenesis of pulmonary hypertension.[141-144] 5-HT is an endogenous vasoactive indolamine substance mainly found in enterochromaffin tissue, brain and platelets that exerts its multiple physiological effects through diverse receptors. At least fourteen different 5-HT receptors, each encoded by a separate gene, are known in humans.[145] The latter are divided into seven groups (5-HT_1-5HT_7) and with exception of 5-HT_3, they are G protein-coupled receptors. The 5-HT_{2A} receptor couples primarily via the G protein $G\alpha_q$ to the activation of PLC and is widely expressed in the human vasculature, on the arterial smooth muscle cells that mediate vasoconstriction.[145]

Pulmonary vasoconstriction to 5-HT is normally mediated via 5-HT_{2A} receptors.[146] However, chronic hypoxia is reported to increase the 5-HT-induced pulmonary arterial contraction, a response linked to chronic hypoxia-induced upregulation of 5-HT_{1B} receptors.[146] 5-HT has been linked to acute HPV as the molecule itself, dexfenfluramine, as well as serotonin transporter inhibition or deletion are reported to increase acute HPV.[19-21,147] In addition, appetite suppressant drugs, mainly fenfluramines and 5-HT, have been associated with pulmonary arterial hypertension[19,144] and the secretion of large amounts of 5-HT from pulmonary neuroendocrine cells and neuroepithelial bodies occurs in response to airway hypoxia.[148] Recent reports underscored the importance of 5-HTT in pulmonary

hypertension by demonstrating that overexpression of the serotonin transporter increases chronic hypoxia-induced pulmonary hypertension in mice whereas mice lacking the 5-HTT or treated with 5-HTT inhibitors were protected against pulmonary hypertension induced by chronic hypoxia.[21,23,149]

1.10. Aim of the study

The aim of the present investigation was to analyse in detail the role of the sEH and CYP-derived EETs on acute and chronic HPV, pulmonary artery pressure and hypoxia-induced pulmonary vascular remodelling. Therefore a series of specific tools to inhibit CYP activity (CYP epoxygenase inhibitors), antagonize the actions of EET (14,15-epoxyeicosa-5(Z)-enoic acid), or to prolong their half-life (sEH inhibitors) were used. The molecular mechanisms involved in mediating the hypoxia- and 11,12-EET-induced pulmonary vasoconstriction and pulmonary remodelling described were addressed using a combination of cultured pulmonary smooth muscle cells, promoter activity assays and genetically modified animals (sEH$^{-/-}$ mice and TRPC6$^{-/-}$ mice).

2. Materials and Methods

2.1. Materials

Media, enzymes and buffer for cell culture were obtained from GIBCO Life Technology (Karlsruhe, Germany), ketamin from Pfizer (Karlsruhe, Germany), rompun from Bayer (Leverkusen, Germany) and heparin from Ratiopharm (Ulm, Germany). The sEH inhibitors 1-adamantyl-3-cyclohexylurea (ACU) and 1-adamantan-1-yl-3-{5-[2-(2-ethoxyethoxy)ethoxy]pentyl]}urea (AEPU or IK-950) were kindly provided by Bruce D. Hammock (University of California, Davis, USA).[24,150] The EET antagonist 14,15-epoxyeicosa-5(Z)-enoic acid (14,15-EEZE) and the CYP epoxygenase inhibitor MSPPOH were from John R. Falck (University of Texas, Dallas, USA).[151] 11,12-EET, 14,15-EET, 11,12-DHET and arachidonic acid were obtained from Cayman Chemicals (Massy, France), NADPH from Applichem (Darmstadt, Germany), U46619 from Alexis (Lörrach, Germany) and Y27632 dihydrochloride from Tocris (Ellisville, USA). Fenbendazole, 5-HT and all other substances were purchased from Sigma (Deisenhofen, Germany).

2.2. Animals

sEH$^{-/-}$ mice were obtained from Dr. Frank Gonzalez (National Institutes of Health, Bethesda, USA) and crossbred for 8 generations onto the C57BL/6 background. TRPC6$^{-/-}$ and TRPC6$^{+/-}$ mice, generated as described,[152] were bred by the animal facility at the University of Marburg. C57BL/6 mice (6-8 weeks old) were purchased from Charles River (Sulzfeld, Germany). Tie2-CYP2J2 and Tie2-CYP2C8 mice were obtained from Darryl Zeldin (National Institute of Environmental Health Sciences, Durham, USA). Animals were housed in conditions that conform to the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health (NIH publication no. 85-23). Both the University Animal Care Committee and the Federal Authority for Animal Research at the Regierungspräsidium Darmstadt (Hessen, Germany) approved the study protocol (# F28/14).

2.3. Isolated buffer-perfused mouse lung system

Mice were anesthetised intraperitoneally using ketamin (80 mg/kg body weight) and rompun (10 mg/kg body weight) and anticoagulated with heparin (1000 U/kg). Animals were then intubated via a tracheostoma and ventilated with room air using a positive pressure pump (Mini-Vent Type 845, Hugo Sachs Elektronik, March-Hugstetten, Germany; 300 µL tidal volume, 90 breath/min and 2 cmH_2O positive end-expiratory pressure). Then mice were transferred to a lung chamber (IL1 Type 839, Hugo Sachs Elektronik, March-Hugstetten, Germany). Midsternal thoracotomy was followed by insertion of catheters into the pulmonary artery and left atrium. As perfusion fluid a Krebs-Henseleit buffer containing 123.1 mmol/L NaCl, 4.4 mmol/L KCl, 1.1 mmol/L KH_2PO_4, 2.5 mmol/L $CaCl_2$, 1.3 mmol/L $MgCl_2$, and 13.5 mmol/L glucose as well as hydroxyethyl starch (200/0.5; 50 g/L) was used. Using a peristaltic pump (ISM834 MS-4/8, Ismatec, Wertheim-Mondfeld, Germany), buffer perfusion via the pulmonary artery was initiated at 4°C and a flow of 0.2 mL/min. In parallel with the onset of artificial perfusion, ventilation was changed from room air to a pre-mixed gas (21% O_2 5.3% CO_2, balanced with N_2). After rinsing the lungs with 20 mL buffer, the perfusion circuit was closed for recirculation and left arterial pressure was set to 2 mmHg. The flow was slowly increased from 0.2 to 2 mL/min and the entire system was heated to 37°C. Pressures in the pulmonary artery, the left atrium and the trachea were registered via blood pressure transducers (MLT0670, ADInstruments, Bella Vista, Australia) and monitored using a PC coupled bridgeAmp-Powerlab system (ML119, ML880, ADInstruments, Bella Vista, Australia). For hypoxic ventilation, a gas mixture containing 1% O_2, 5.3% CO_2, balanced with N_2 was used. Ten-minute periods of hypoxic ventilation (1% O2) were alternated with 15 min normoxic periods (Figure 8).

For measuring lung weight changes, mouse lungs were removed from the chest in deep anaesthesia, artificially ventilated, and perfused blood-free analogous to the isolated rabbit lung as described. [153]

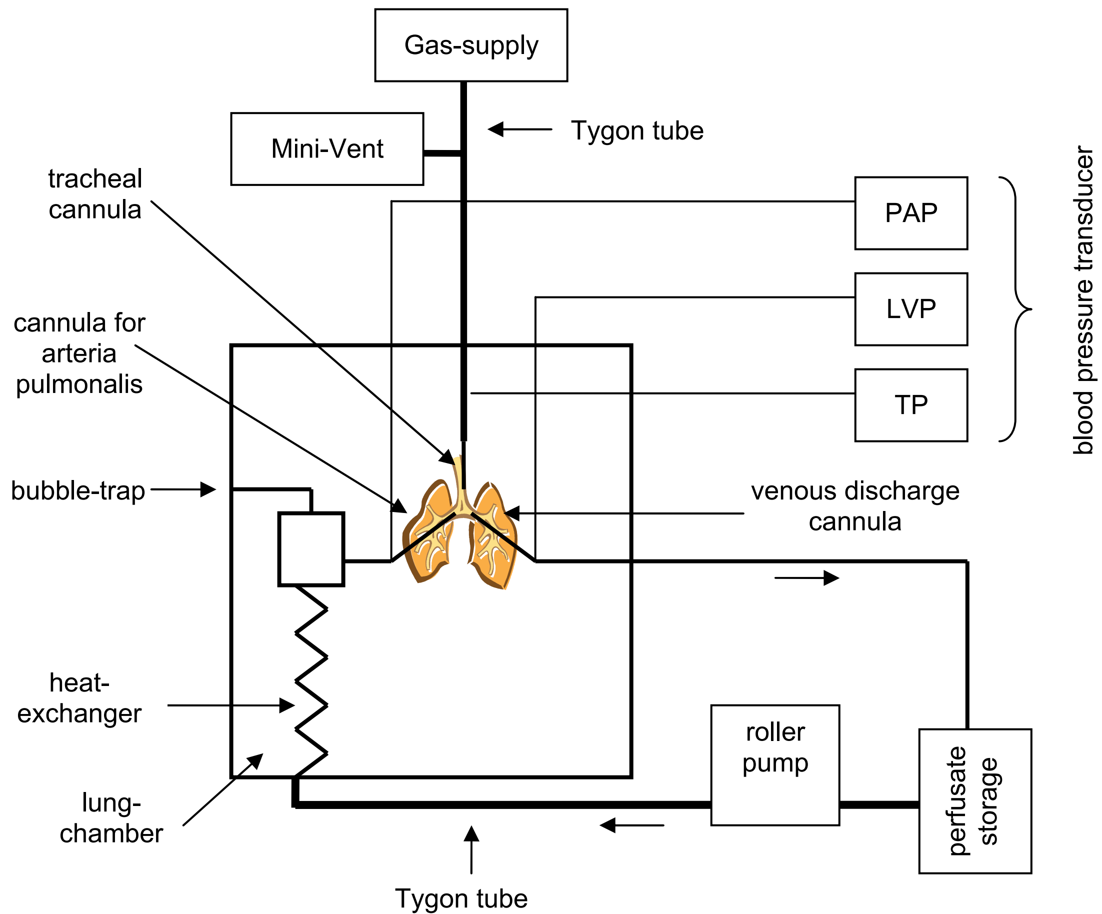

Figure 8: Isolated buffer-perfused mouse lung system. PAP: pulmonary artery pressure; LVP: left ventricle pressure, TP: tracheal pressure.

2.4. Cell culture

Rat pulmonary artery smooth muscle cells were isolated as described[154] and cultured in M199, supplemented with 10% FCS, penicillin (50 U/mL) and streptomycin (50 µg/mL).

2.5. RhoA activation assay

Isolated buffer-perfused lungs from wild-type mice were treated with solvent or 11,12-EET (3 µmol/L, 15 minutes) then snap frozen in liquid N_2. Lungs were then

homogenised and RhoA activity was determined using a specific G-LISA assay (Cytoskeleton, Denver, USA).

2.6. Protein isolation

Myosin light chain (MLC) 20 and α-smooth muscle actin: Rat pulmonary artery smooth muscle cells were maintained under normoxic conditions, treated with U46619 (1 µmol/L, 10 minutes) or exposed to hypoxia for 30 minutes. Cells were then immediately treated with trichloroacetic acid (15% w/v) and frozen in liquid N_2. After 30 minutes on ice, the suspension was centrifuged (4°C, 14000g, 30 minutes), and the pellet washed 3 times with water-saturated diethyl ether. Air-dried samples were solubilised in an ice cold Triton X-100 lysis buffer for 10 minutes, then centrifuged (4°C, 10000g, 10 minutes) and the protein concentration of the supernatant was determined (Bradford 1976).

CYP2C11 and sEH: Cells were washed with PBS and solubilised using ice cold Triton X-100 lysis buffer for 10 minutes, then centrifuged (4°C, 10000g, 10 minutes) and the protein concentration of the supernatant was determined (Bradford 1976).

2.7. Western blot analysis

Protein samples were separated via electrophoresis on a denaturating polyacrylamide gel in a buffer solution consisting of 190 mmol/L glycine, 0.1% SDS and 25 mmol/L TRIS-HCl and transferred on a nitrocellulose membrane (60 minutes at 250 mA). Afterwards the membranes were transferred to a solution of 3% bovine serum albumin in TRIS buffered saline with Tween (TBST, consisting of 50 mmol/L TRIS/HCl, pH 7.5, 150 mmol/L NaCl, 0.3% Tween-20) to block unspecific binding sites on the membrane. After incubation with the according primary antibody overnight, multiple washing steps with TBST and another blocking step, membranes were incubated with a horseradish peroxidase conjugated secondary antibody followed by repeated washing. Proteins were finally detected by applying an x-ray

film on the membrane that was subjected to a chemiluminescence reaction via the ‘Enhanced Chemiluminescence’ system (Amersham/Pharmacia).

The utilised antibodies were directed against CYP2C11 (Acris, Hiddenhausen, Germany), sEH (Prof. Michael Arand, Zürich, Switzerland), the phosphorylated form of the myosin light chain 20 (Cell signalling, Danvers, USA) and α-smooth muscle actin (Sigma; Saint Louis, USA).

2.8. Immunohistochemistry

sEH: Mice were euthanised by an intraperitoneal overdose of pentobarbital sodium. After intubation, a midsternal thoracotomy was performed, catheters were placed in the pulmonary artery and the left atrium, and the vasculature was flushed with 20 mL saline at a pulmonary artery pressure of 22 cmH_2O and a tracheal pressure of 12 cmH_2O. Thereafter, the pulmonary vasculature was perfused with Zamboni´s fixative for 30 minutes at the same pressures. After ligation of the pulmonary artery, veins, and the trachea, lungs were removed and placed in the Zamboni´s fixative for a further 6 hours at room temperature, followed by incubation in phosphate buffer (0.1 mol/L, 12 hours, 4°C). Tissues were then dehydrated and infiltrated with paraffin in an automated vacuum tissue processor (Leica TP1050, Bensheim, Germany). After deparaffinisation and rehydration of 3 µm sections, endogenous peroxidase was blocked in 3% hydrogen peroxide. Slides were incubated with trypsin (Digest All, Zytomed, Berlin, Germany) for 10 minutes for retrieval of the antigen and unspecific binding sites were blocked using horse serum (Alexis, Grünberg, Germany). The sections were incubated overnight (4°C) with a polyclonal sEH antibody (dilution 1:2000) raised against a recombinant murine sEH produced in the baculovirus expression system and then purified to apparent homogeneity by affinity chromatography. The ImmPRESS anti-rabbit IgG Peroxidase kit (Vector/Linaris, Wertheim-Bettingen, Germany) was then used according to the manufacturer’s protocol and the sEH visualised using the Nova Red chromogen kit for horseradish peroxidase (Vector, Linaris, Wertheim-Bettingen, Germany). Nuclear counterstaining was performed with hematoxylin (Zytomed, Berlin, Germany).

TRPC6: Translocation of the TRPC6 channel was assessed in cultured rat pulmonary smooth muscle cells infected with an adenovirus encoding a V5-tagged TRPC6 fusion protein (TRPC6-V5) as described.[69] Following stimulation, samples were fixed (4% paraformaldehyde in PBS), permeabilised with Triton X-100 and incubated with phalloidin (Molecular Probes, Leiden, Netherlands) and specific antibodies to V5 (Invitrogen) or caveolin-1 (BD Biosciences). The preparations were mounted and viewed using a confocal microscope (LSM 510 META, Zeiss).

2.9. Preparation of lung microsomes

Lungs were minced and homogenised in 5 vol. of ice-cold 50 mM Tris/HCl buffer (pH 7.4) containing 0.25 M sucrose, 150 mM potassium chloride, 2 mM EDTA, 2 mM dithiothreitol (DTT), 1 µmol/L FAD and FMN, and 0.25 mM PMSF in a motor-driven homogeniser. After differential centrifugation (10 minutes, 1000 g; 20 minutes, 10000 g; 90 minutes, 100000 g), the microsomes were suspended and homogenized in 50 mM Tris/HCl buffer (pH 7.7) containing 20% (v/v) glycerol, 5 mM EDTA and 1 mM DTT. Aliquots were snap-frozen in liquid nitrogen and stored at -80 °C.

2.10. Eicosanoid measurements by LC-MS/MS

Lung microsomes were prepared as described above. Microsomal protein (50 µg) was incubated in 100 µL of potassium phosphate buffer (100 mmol/L, pH 7.2) containing arachidonic acid (10 µmol/L) in the absence or presence of fenbendazole (100 µmol/L) and MSPPOH (10 µmol/L) for 15 minutes. To determine hypoxia-induced EET generation, microsomal proteins were incubated with either an hypoxic (1% O_2) or normoxic Krebs-Henseleit buffer containing arachidonic acid (10 µmol/L) for 10 minutes. Reactions were started by addition of NADPH (1 mmol/L final concentration) and terminated after 20 minutes by placing on ice. The reaction products were extracted twice using ethyl acetate, evaporated under nitrogen, resuspended in methanol/water (v/v, 1:1). To determine acute hypoxia-induced EET generation in intact cells, rat PASMCs were incubated with arachidonic acid (100 nmol/L). After 5 hours, cells were washed and after an additional hour PASMCs were

incubated with either a hypoxic (1% O_2) or normoxic cell culture medium for 10 minutes. Reactions were terminated by placing them on ice. Cells were harvested by scraping and reaction products were extracted as described.[155] The activity of the sEH was determined using cytosolic cell lysates generated as described.[156] Reactions were performed at 37°C for 20 minutes in 100 µl of 100 mmol/L potassium phosphate buffer (pH 7.2) containing 5 µg protein. Reactions were started by the addition of 14,15-EET (10 µmol/L) and stopped on ice and immediately extracted twice with ethyl acetate (0.7 mL). One tenth of the sample was spiked with a deuterated internal standard (14,15-EET-d8). After evaporation of the solvent in a vacuum block under a gentle stream of nitrogen the residues were reconstituted with 50 µL of methanol/water (v/v, 1:1). Eicosanoid profiles were determined with a Sciex API4000 mass spectrometer operating in multiple reaction monitoring (MRM) mode as described.[155]

2.11. Chronic hypoxia

For chronic hypoxia exposure, mice were kept under normobaric hypoxia (10% O_2) in a ventilated chamber for up to 21 days. The level of hypoxia was held constant by an autoregulatory control unit (BioSpherix, Lacona, USA) supplying either nitrogen or oxygen and CO_2 was continuously removed by soda lime. Cages were opened once per day for cleaning as well as for food and water supply. Control mice were exposed to normoxia (21% O_2) under identical conditions for 21 days.

2.12. RNA isolation and reverse transcriptase polymerase chain reaction

Total RNA from murine wild-type lungs was isolated as described.[157] Homogenised samples were incubated in Tri®Reagenz at room temperature to permit the complete dissociation of the nucleoprotein complex. Afterwards the solution containing RNA as well as DNA was mixed with chloroform (20% v/v). Samples were shaken vigorously by hand for 15 seconds and incubated at room temperature for 2 to 3 minutes. The

aqueous and organic phases were separated by centrifugation for 30 minutes at 12000g. The aqueous phase containing the RNA was transferred to a fresh tube and the nucleic acid was precipitated by adding an equal volume isopropanol (100%). Thereafter samples were incubated at room temperature for another 10 minutes, centrifuged, washed with ethanol (75%) and diluted in RNase-free water. RNA concentration was determined by photometric measurement at 260 nm.

For the reverse transcriptase-polymerase chain reaction 1 µg RNA was used. Incubation with reaction buffer (Invitrogen, Karlsruhe, Germany), desoxynucleosid-5'-triphophate (175 µmol/L of dATP, dCTP, dGTP and dTTP), dithiothreitol (1 mmol/L), random hexanucleotides and reverse transcriptase (Superscript III, Invitrogen, Karlsruhe, Germany) for 60 minutes at 50ºC resulted in cDNA-synthesis. To avoid false positive results by contamination a negative control without reverse transcriptase was used.

For the amplification of cDNA during the PCR (15 minutes at 95°C followed by 40 cycles à 30 seconds at 95°C, 1 minute at 60°C and 30 seconds at 72°C in a Mx4000 multiplex quantitative PCR system (Stratagene, Heidelberg, Germany)) the following primers were used:

sEH: forward 5'- AAGTAATCTGAAGCCAGCCCGT-3'

sEH: reverse 5'- AGAGCCATGTTCCACACCATCA-3'

To ensure that equal amounts of cDNA were used, 18S RNA was amplified by qPCR (Assay on Demand, Applied Biosystems, Darmstadt, Germany) and the amount of cDNA in the samples was calculated on the basis of the amplification of a serial dilution of a plasmid (sEH) or the serial dilution of the cDNA (18S RNA). The sEH levels were normalised to that of 18S. At least two RT reactions were performed using each RNA preparation and at least two PCR reactions were performed with each cDNA sample.

2.13. sEH promoter activity

HEK 293 cells were transiently co-transfected with the 4 kb sEH promoter or the 1.5 kb sEH promoter (Kindly provided by D. Hammock) together with a LacZ construct.

After 24 h, the cells were lysed, and luciferase activity was assayed according to the manufacturers protocol (Promega, Mannheim, Germany). The values were corrected for transfection efficiency by measuring β-galactosidase activity (Tropix, Bedford, MA).

2.14. Hematocrit and right heart hypertrophy determination

Prior to artificial perfusion, an aliquot of blood was drawn from the left atrial catheter and spun in an Adams autocrit centrifuge (Clay Adams, Parsippany, USA) for hematocrit determination. In some animals, the right ventricle was dissected from the left ventricle and septum, dried and weighed to obtain the right to left ventricle plus septum ratio

2.15. Vessel morphometry

Morphometric analysis of the pulmonary vasculature was performed as described.[158] Briefly, for determination of the degree of muscularisation, vessels were classified as nonmuscular (no smooth muscle cells detectable with α-actin staining), partially muscularised (at least one smooth muscle cell up to 75% circumference with α-actin staining), and fully muscularised (>75% of circumference with α-actin staining) This analysis was performed for at least 80 small vessels (20–70 µm diameter), 15 medium vessels (>70–150 µm diameter) and 5 large vessels (>150–1000 µm diameter) of each section. One section from each, the left upper, right upper, and left lower lobe was analysed. All morphometric analysis were done in a blinded fashion.

2.16. Statistical Analysis

Data are expressed as mean±SEM and statistical evaluation was performed using Student's t-test for unpaired data or 1-way ANOVA followed by a Bonferroni t-test where appropriate. Values of $P<0.05$ were considered statistically significant.

3. Results

3.1. Effect of 11,12-EET on pulmonary arterial pressure

In lungs from wild-type mice, the application of 11,12-EET (10 nmol/L to 3 µmol/L) to the pulmonary perfusate rapidly and concentration-dependently increased pulmonary artery pressure (Figure 9) while 14,15-EET and the solvent DMSO (0.3 ‰) were without significant effect (Figure 9B). 11,12-DHET also failed to affect pulmonary artery pressure (Figure 9B).

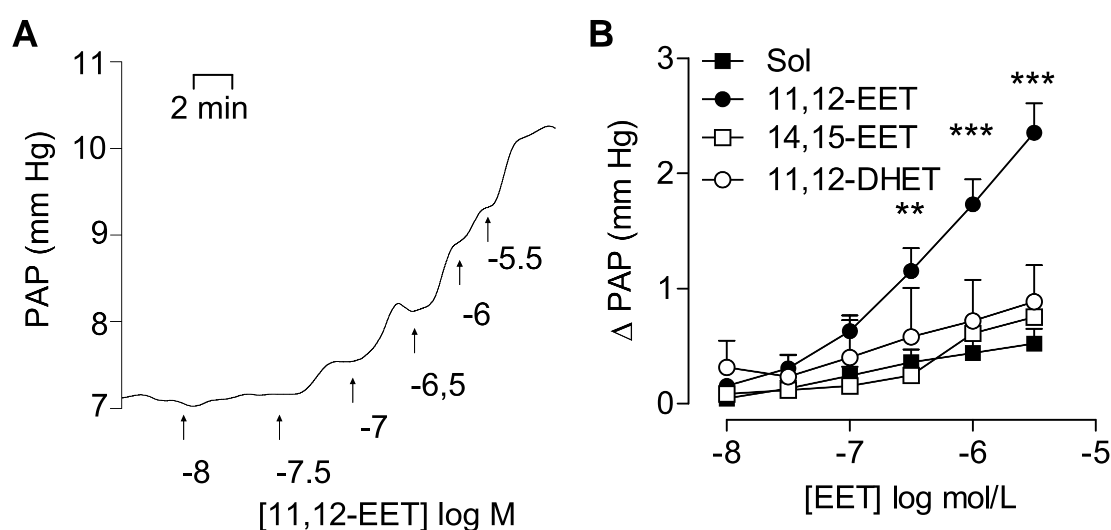

Figure 9: Effect of 11,12-EET, 11,12-DHET and 14,15-EET on pulmonary arterial pressure in lungs from wild-type mice. (A) Original tracing showing the effect of 11,12-EET on pulmonary arterial pressure in lungs from wild-type mice. (B) Effect of 11,12-EET (10 nmol/L to 3 µmol/L), 11,12-DHET EET (10 nmol/L to 3 µmol/L) and 14,15-EET (10 nmol/L to 3 µmol/L) versus their solvent (Sol, 0.3 ‰ DMSO) on pulmonary arterial pressure in lungs from wild-type mice. The graph summarises data obtained using 4-6 animals per group; **$P<0.01$, ***$P<0.001$ versus solvent.

3.2. Effect of sEH and CYP epoxygenase inhibition on acute hypoxic pulmonary vasoconstriction in isolated buffer-perfused mouse lungs

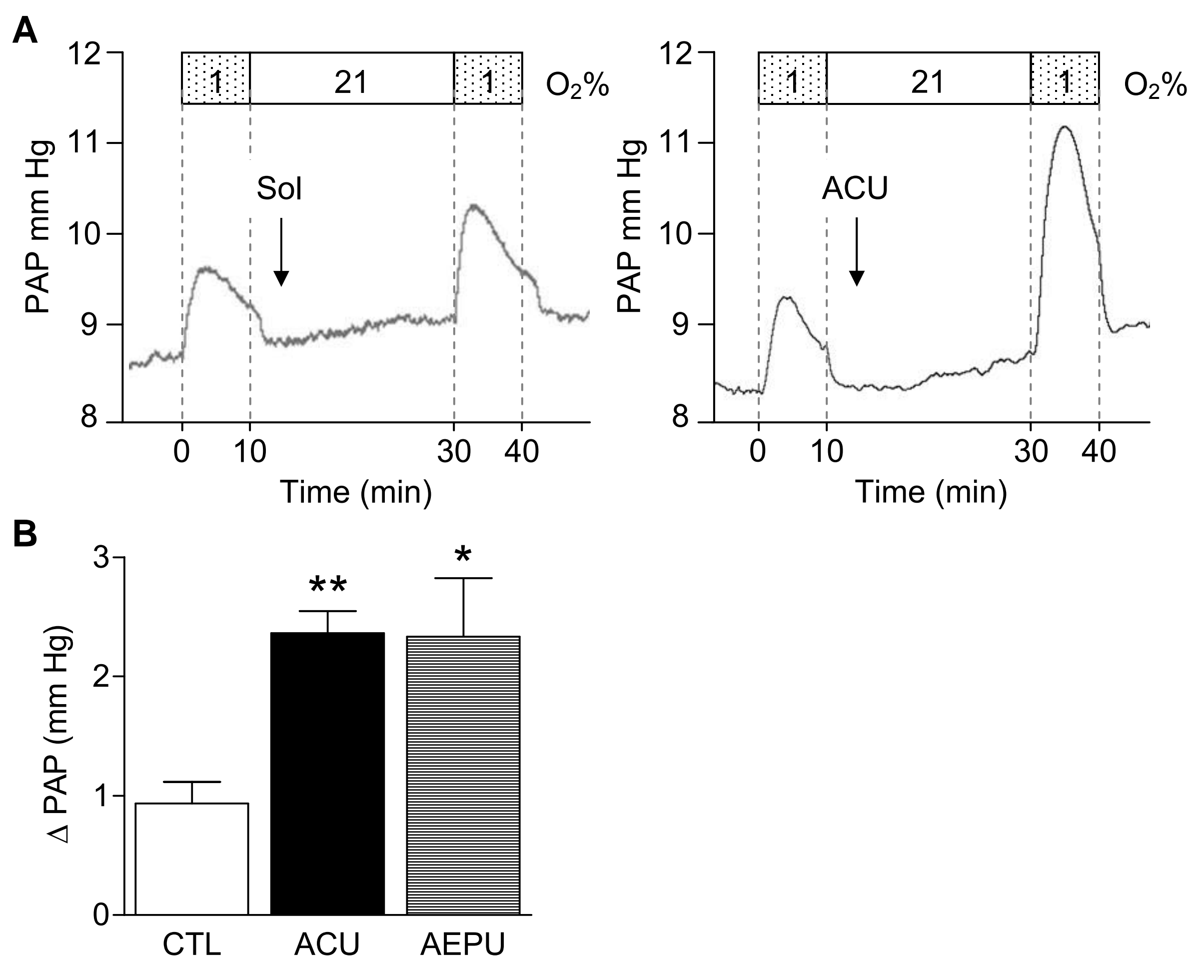

Figure 10: Effect of sEH and CYP inhibition on hypoxia-induced vasoconstriction in the isolated perfused mouse lung. (A) Original tracings showing the effect of acute hypoxia (1% O_2) on pulmonary arterial pressure before and after administration of solvent or the soluble epoxide hydrolase inhibitor, ACU (3 µmol/L). (B) Hypoxic pulmonary vasoconstriction in lungs from mice treated with solvent (CTL), ACU or AEPU (3 µmol/L). The bar graph summarises data obtained in 5-6 independent experiments; *P<0.05, **P<0.01 versus solvent.

In lungs from wild-type mice hypoxic ventilation (FiO_2 = 0.01) resulted in an acute increase in pulmonary artery pressure (Figure 10A) that is attributable to acute HPV. Repeated stimulation (up to five times) resulted in quantitatively similar responses.

To analyse the role of endogenous EETs in the pulmonary circulation, lungs from wild-type mice were incubated with sEH inhibitors, thereby preventing the metabolism of EETs to their less active DHETs and enhancing endogenous EET levels. Following application of the sEH inhibitor, ACU, to the pulmonary perfusate, the acute hypoxic vasoconstrictor response was significantly augmented (Figure 10A&B). A similar response was observed using a second sEH inhibitor, AEPU,[24] which has a comparable potency but markedly different physical properties (Figure 10B).

Previous reports revealed that sEH activity is greater in male than in female mice and rats and that castration decreased sEH activity and expression; a phenomenon restored by testosterone supplementation.[99,100] Moreover, as female animals demonstrated a more pronounced response to sEH inhibition in our previous study[24] we assessed the acute HPV response in castrated, ovarectomised and sham operated male and female C57BL/6 mice. In contrast to previous observations in Swiss Webster mice, HPV did not differ between the different subgroups and inhibition of the sEH potentiated the acute HPV to the same level in all of the groups analysed (Figure 11).

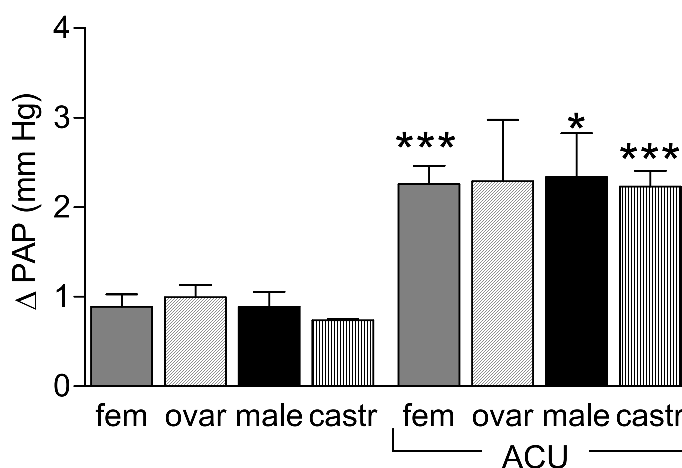

Figure 11: Effect of gender on acute hypoxic pulmonary vasoconstriction in the isolated mouse lung. Hypoxic pulmonary vasoconstriction in lungs from female sham-operated (fem) and ovarectomised (ovar) mice, as well as male sham-operated and castrated (castr) mice treated with solvent (CTL) or ACU (3 µmol/L). The bar graph summarises data obtained in 4-6 independent experiments; *P<0.05, ***P<0.001 versus the corresponding solvent treated group.

To determine whether or not CYP-derived EETs are involved in acute hypoxic pulmonary vasoconstriction responses experiments were re-assessed in animals treated with fenbendazole (4% in chow) for two weeks. CYP inhibition by fenbendazole was demonstrated in murine lung microsomes by determining the conversion of arachidonic acid to EET. Fenbendazole was equally effective as the epoxygenase inhibitor MSPPOH in attenuating the generation of 11,12- and 14,15-EET without affecting the generation of either 5,6- or 8,9-EET (Figure 12A). Treatment of mice with fenbendazole was without significant effect on the magnitude of acute HPV (Figure 12B) but significantly attenuated the potentiation of vasoconstriction induced by sEH inhibition (Figure 12B).

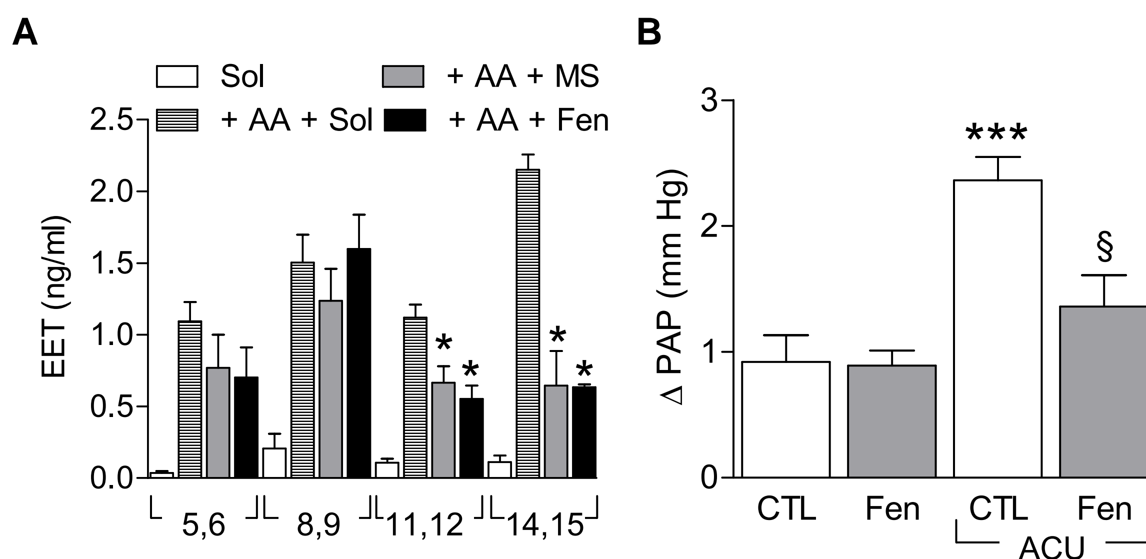

Figure 12: Effect of fenbendazole on CYP epoxygenase activity and acute hypoxic vasoconstriction. (A) Comparison of the effects of the CYP epoxygenase inhibitors MSPPOH and fenbendazole on arachidonic acid epoxide production in microsomes prepared from murine lungs. Epoxygenase activity was assessed by monitoring EET production following the addition of arachidonic acid (AA, 10 µmol/L, 20 minutes). Experiments were performed in the presence of either solvent (Sol), MSPPOH (MS, 10 µmol/L) or fenbendazole (Fen, 100 µmol/L). The graph summarises data obtained in 4 independent experiments; *P <0.05 versus AA + solvent. Reaction mixtures lacking AA served as a negative control. (B) Effect of fenbendazole (Fen; 4%, 14 days) on acute hypoxic pulmonary vasoconstriction. The bar graphs summarise data obtained in 5-6 independent experiments; ***P<0.001 versus solvent; §P<0.05 versus ACU/CTL.

In a second protocol the consequences of acute CYP epoxygenase inhibition and EET antagonism on hypoxic vasoconstriction were tested. MSPPOH and 14,15-EEZE had only a marginal effect on their own but both substances completely prevented the increased constriction induced by sEH inhibition (Figure 13).

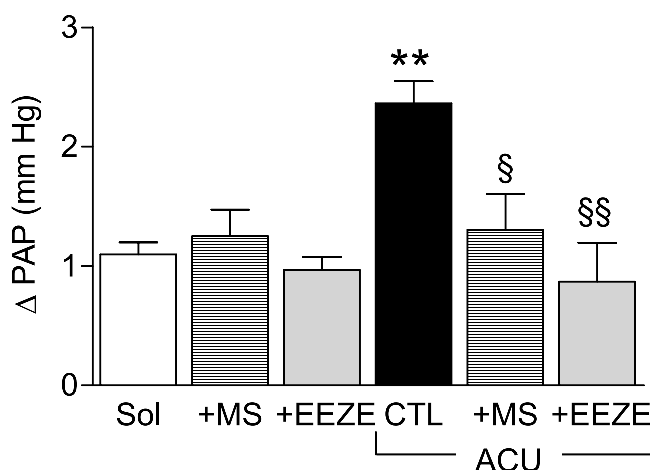

Figure 13: Effect of sEH and CYP inhibition on hypoxia-induced vasoconstriction in the isolated perfused mouse lung. Hypoxic pulmonary vasoconstriction was assessed in the presence of solvent (Sol), MSPPOH (MS, 10 µmol/L), or 14,15-EEZE (EEZE, 10 µmol/L) and in the absence and presence of ACU (3 µmol/L). The bar graph summarises data obtained in 3-6 independent experiments; **P<0.01 versus solvent; §<0.05, §§P<0.01 versus ACU/CTL.

3.3. Acute hypoxic pulmonary vasoconstriction in lungs from sEH$^{-/-}$ mice

Although the sEH is expressed in the carotid artery endothelium,[104] this is not the case in the lung where the enzyme was selectively expressed in vascular smooth muscle cells (Figure 14A). No sEH was detected in lungs removed from sEH$^{-/-}$ mice (Figure 14A).

Next the acute HPV response in lungs from wild-type mice and lungs from sEH$^{-/-}$ mice was assessed. As before, in lungs from wild-type mice, sEH inhibition enhanced the acute hypoxic vasoconstriction (Figure 14B). Lungs from sEH$^{-/-}$ mice displayed significantly enhanced hypoxic vasoconstriction responses compared to solvent

treated lungs from wild-type mice (Figure 14B). As expected, the sEH inhibitor had no effect on acute hypoxic vasoconstriction in lungs from $sEH^{-/-}$ mice (Figure 14B). However CYP epoxygenase inhibition and EET antagonism returned hypoxic responses to levels observed in lungs from wild-type animals (Figure 14B), indicating that the enhanced response to hypoxia was dependent on the activation of a CYP epoxygenase and the generation of an EET.

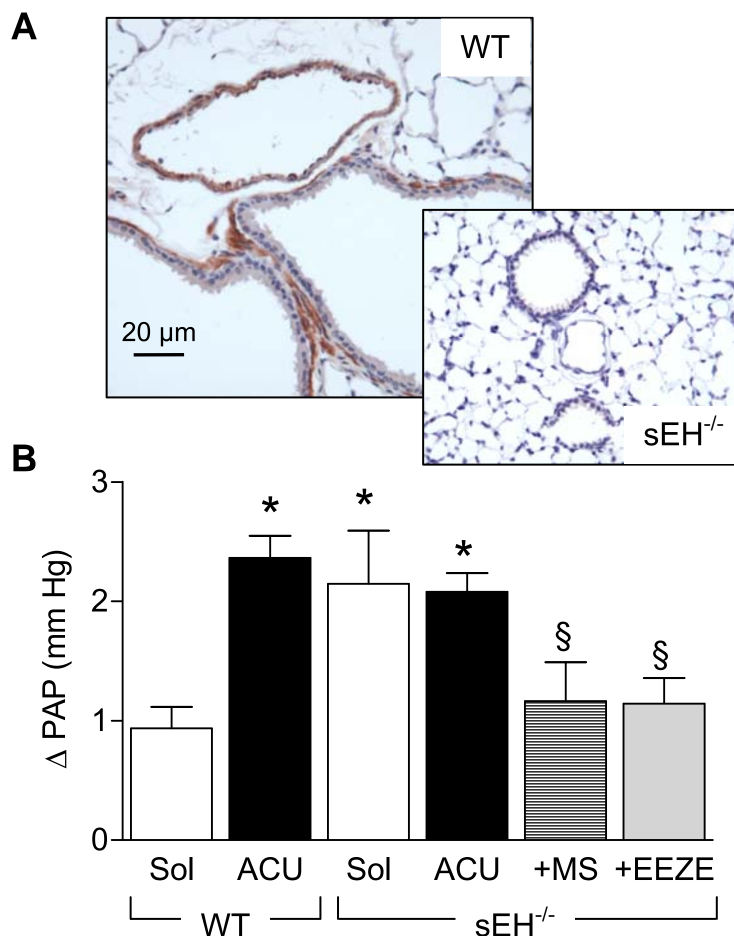

Figure 14: Hypoxic pulmonary vasoconstriction in lungs from $sEH^{-/-}$ mice. (A) Immunohistochemical analysis showing the expression of the sEH (in brown) in lungs from wild-type (WT) and $sEH^{-/-}$ mice. (B) Hypoxic pulmonary vasoconstriction in lungs of WT and $sEH^{-/-}$ mice treated with solvent (Sol) or ACU (3 µmol/L) in the absence and presence of MSPPOH (MS, 10 µmol/L) or 14,15-EEZE (EEZE, 10 µmol/L). The bar graph summarises data from 3-6 independent experiments; *P<0.05 versus Sol WT; §P<0.05 versus Sol $sEH^{-/-}$.

Moreover, in microsomes prepared from the lungs of wild-type mice, hypoxia (10 minutes) elicited a significant increase in EET production. As expected, EET levels in microsomes from the lungs of $sEH^{-/-}$ mice were greater than those detected in samples from wild-type animals and were also increased in response to hypoxia (Figure 15A-C). In intact rat PASMCs hypoxia (10 minutes) significantly increased 11,12-EET production (Figure 15D) but had no effect on 8,9-EET and 14,15-EET generation (data not shown).

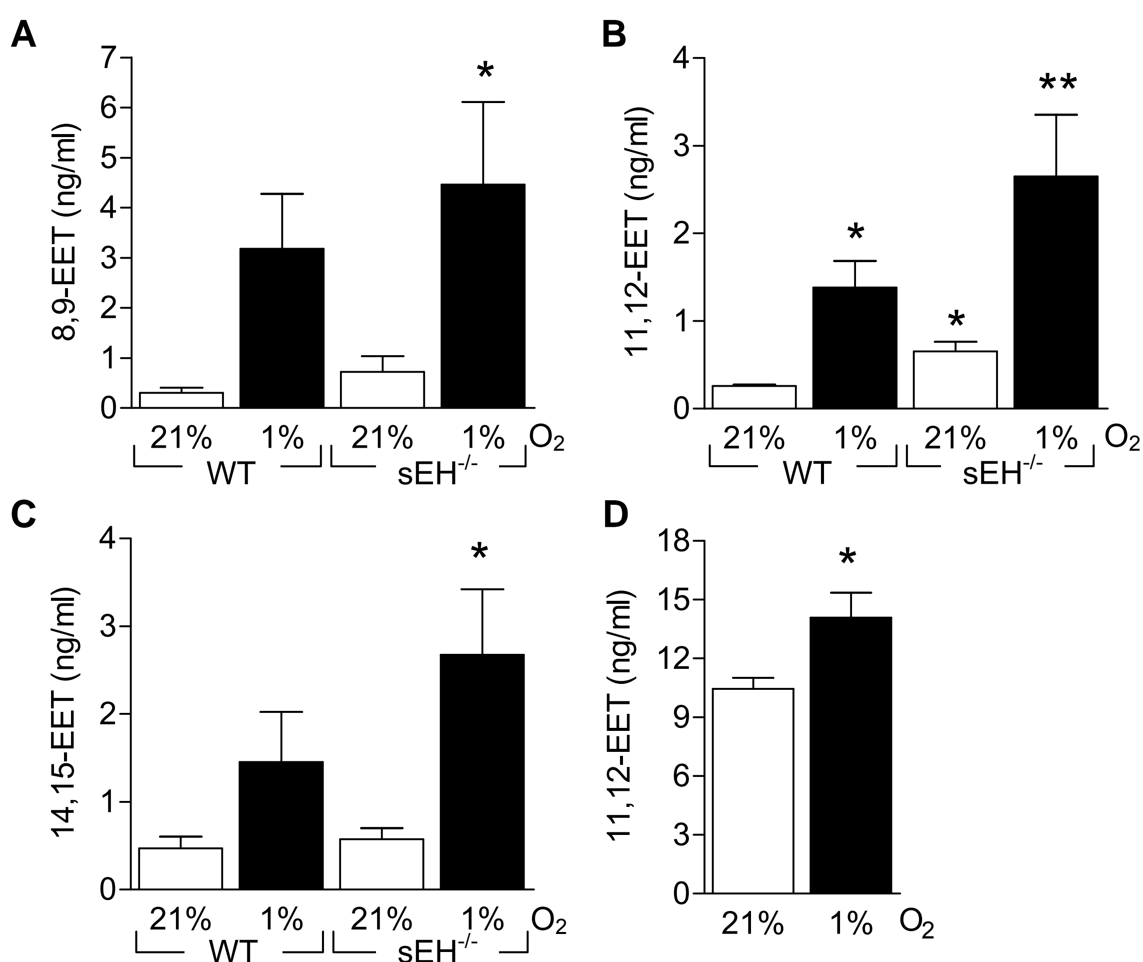

Figure 15: Effect of hypoxia on EET levels in murine lung microsomes and rat PASMCs. Microsomal protein from wild-type (WT) and $sEH^{-/-}$ mice (A-C) or rat PASMCs (D) were either maintained under normoxic conditions (21% O_2) or exposed to 1% O_2 for 10 minutes. EET levels were determined using LC-MS/MS. The bar graphs summarise data from 4-5 independent experiments; *$P<0.05$, **$P<0.01$ versus normoxic wild-type microsomes; *$P<0.05$ versus normoxic PASMCs.

3.4. Effect of CYP2C8 and CYP2J2 overexpression on acute hypoxic pulmonary vasoconstriction

In the systemic circulation the activation of CYP epoxygenases in endothelial cells is an important step in the NO and PGI_2-independent vasodilation of several vascular beds.[40,159-161] An initial step in such EDHF-mediated responses is endothelial cell hyperpolarisation and recently, overexpression of CYP2C9 in human umbilical vein endothelial cells has been reported to potentiate bradykinin-induced Ca^{2+} influx and hyperpolarisation.[69] Given this modulatory role of CYP overexpression on the vascular cells and the above-demonstrated involvement of CYP epoxygenase-derived products in hypoxia-induced vasoconstriction, the acute HPV response was assessed in lungs from mice overexpressing CYP2C8 or CYP2J2 in the endothelium (i.e. under the control of the Tie-2 promotor) and their corresponding wild-type littermates.

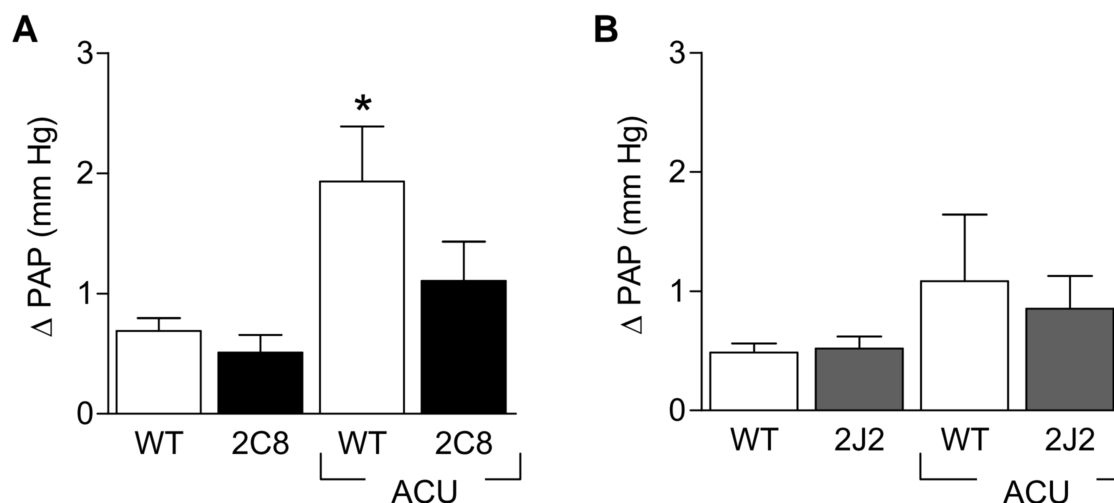

Figure 16: Effect of endothelial specific (A) CYP2C8 and (B) CYP2J2 overexpression on acute hypoxic pulmonary vasoconstriction. The acute hypoxic pulmonary vasoconstriction response was assessed in lungs from CYP2C8 (2C8) or CYP2J2 (2J2) overexpressing mice and their corresponding wild-type littermates (WT). The graphs summarise data obtained in 4 independent experiments; *P<0.05 versus WT.

Acute HPV was similar in lungs from wild-type, CYP2C8 and CYP2J2 mice (Figure 16). Inhibition of the sEH enhanced the acute HPV in lungs from wild-type littermates

(Figure 16) while in lungs from CYP2C8 and CYP2J2 overexpressing animals the ACU-dependent increase in hypoxic vasoconstriction was less pronounced (Figure 16). Although the effects failed to reach statistical significance, these data suggest that endothelium-derived EETs attenuate pulmonary vasoconstriction and that the hypoxia-sensitive CYP enzyme that contributes to HPV most probably is localised in pulmonary artery smooth muscle cells.

3.5. Effect of hypoxia and 11,12-EET on the RhoA/Rho kinase pathway

Hypoxia-induced pulmonary vasoconstriction has been attributed to activation of the Rho kinase.[162,163] Given that the contraction elicited by the CYP-derived eicosanoid, 20-HETE, in the systemic circulation is linked to the Rho kinase-dependent phosphorylation MLC[70] the effects of hypoxia, ACU and 11,12-EET on Rho kinase activity were compared using rat PASMCs and isolated lungs. For these analysis PASMCs were used as a functional tool as they expressed both, CYP2C11 and sEH (Figure 17)

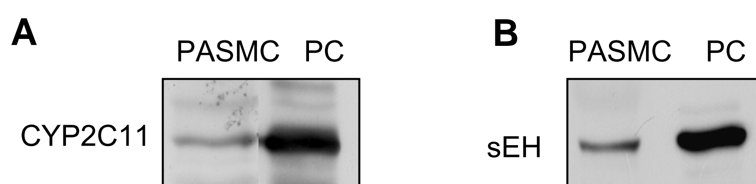

Figure 17: **Representative Western blots demonstrating the expression of CYP2C11 and the sEH in rat pulmonary artery smooth muscle cells (PASMC).** PC = positive control i.e. in the case of CYP2C11 CYP2C11-supersomes and the recombinant human protein in the case of the sEH.

In pulmonary artery smooth muscle cells, hypoxia stimulated the phosphorylation of MLC-20, an effect that was significantly potentiated by ACU and largely prevented by the Rho kinase inhibitor Y27632 (Figure 18A). The exogenous application of 11,12-EET had no significant effect on MLC-20 phosphorylation (not shown) but increased Rho kinase activity (measured by G-LISA) in the isolated perfused lung (Figure 18B).

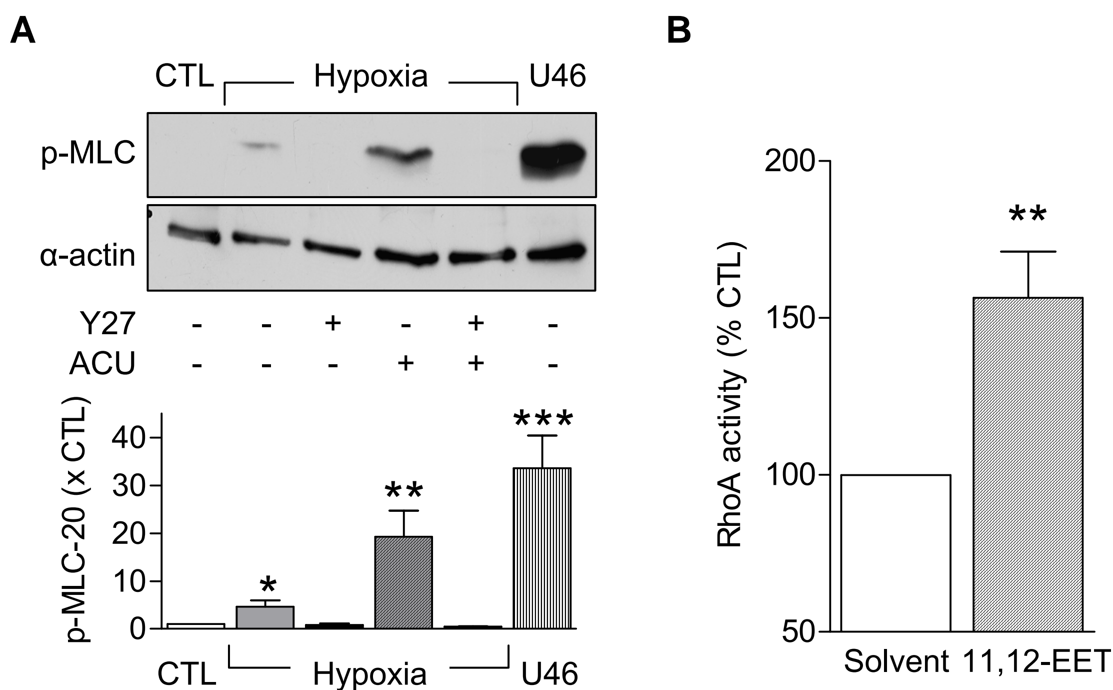

Figure 18: Effect of hypoxia, ACU and 11,12-EET on Rho kinase activity. (A) Representative Western blots and corresponding bar graph showing the effects of hypoxia on the phosphorylation of MLC-20 (p-MLC) in rat pulmonary artery smooth muscle cells. Experiments were performed in the absence or presence of ACU (3 µmol/L) and Y27632 (Y27, 10 µmol/L); cells were stimulated with U46619 (1 µmol/L) as positive control. (B) Effect of 11,12-EET (3 µmol/L, 15 minutes) on RhoA activity (measured by G-LISA) in isolated murine lungs. The graphs summarise data obtained in 4 independent experiments; *P<0.05, **P<0.01, ***P<0.001 versus CTL or solvent.

In a next step the effect of Rho kinase inhibition on the 11,12-EET-induced increase in pulmonary artery pressure and ACU-induced increases in HPV were assessed. In lungs from wild-type mice the increase in pulmonary artery pressure elicited by 11,12-EET was significantly inhibited by Y27632, a Rho kinase inhibitor (Figure 19A). Moreover, 11,12-EET enhanced the acute hypoxic pulmonary vasoconstriction response to same level as ACU and both effects were sensitive to Rho kinase inhibition (Figure 19B).

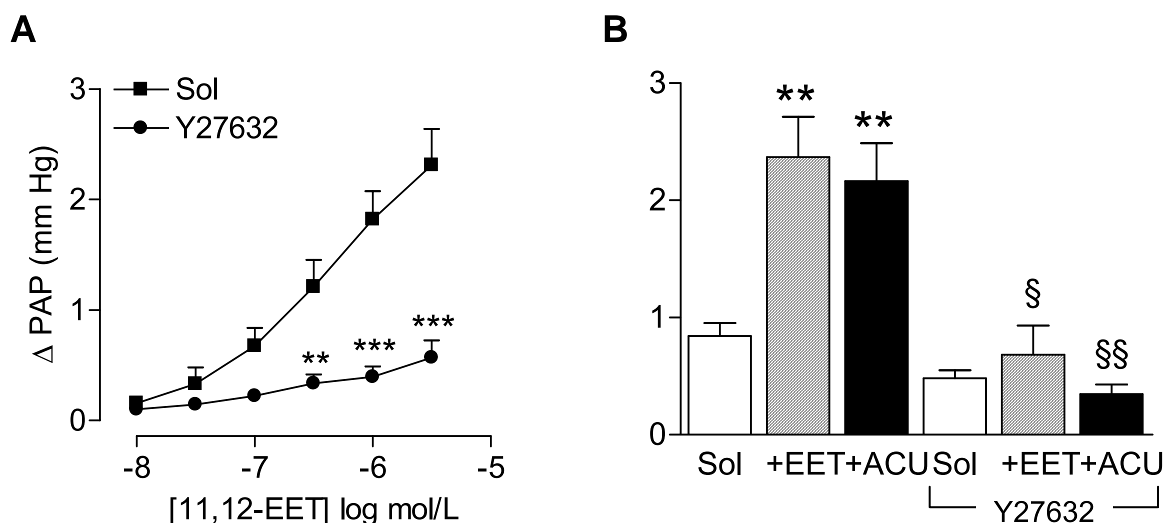

Figure 19: Sensitivity of the effects of 11,12-EET and ACU to Rho kinase inhibition. Effect of the Rho kinase inhibitor Y27632 (1 µmol/L) on (A) the 11,12-EET-induced increase in pulmonary artery pressure and (B) the 11,12-EET (3 µmol/L) or ACU-induced potentiation in hypoxic vasoconstriction in lungs from wild-type mice. The graphs summarise data obtained in 4-5 independent experiments; *P<0.05, **P<0.01, ***P<0.001 versus CTL or solvent; §P<0.05 versus EET alone and §§P<0.01 versus ACU alone.

3.6. Role of the 5-HT$_{2A}$ receptor in 11,12-EET-induced contraction and acute hypoxic pulmonary vasoconstriction

As 5-HT is undoubtedly a modulator of HPV[164] and the 5-HT$_{2A}$ receptor is mainly expressed in pulmonary artery smooth muscle cells[145] we assed the role of the 5-HT$_{2A}$ receptor on the ACU-dependent increase in HPV and the EET-induced contraction in murine lungs.

In lungs from wild-type mice, preincubation with ketanserin or ritanserin (5-HT$_{2A}$ receptor inhibitors) abrogated the effect of sEH inhibition on HPV (Figure 20A). Furthermore, in lungs from wild-type mice, ritanserin slightly decreased the basal HPV and significantly inhibited the 11,12-EET-induced increase in acute HPV (Figure 20B).

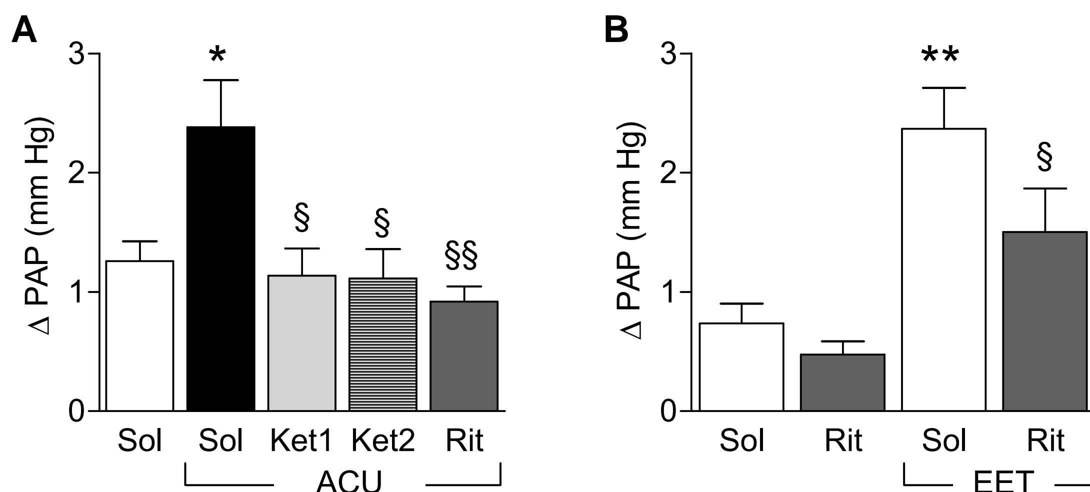

Figure 20: Effect of 5-HT_{2A} receptor inhibition on the ACU-induced or 11,12-EET-induced potentiation of hypoxic vasoconstriction. (A) Hypoxic pulmonary vasoconstriction was assessed in the presence of solvent (Sol), ketanserin (Ket1, 1 µmol/L or Ket2, 10 nmol/L), or ritanserin (Rit, 10 nmol/L) and in the absence and presence of ACU (3 µmol/L). (B) Effect of ritanserin (Rit, 10 nmol/L) on the 11,12-EET-induced potentiation in hypoxic vasoconstriction in lungs from wild-type mice. The graphs summarise data obtained in 3-5 independent experiments *P<0.05, **P<0.01 versus Sol alone; §P<0.05, §§P<0.01 versus ACU alone or Sol + EET.

Given that sEH inhibition enhanced the acute HPV, through EETs, and that this effect was sensitive to 5-HT_{2A} receptor antagonism, the effect of 5-HT_{2A} receptor inhibition on 11,12-EET-induced increase in pulmonary artery pressure was assessed. Thirty minutes preincubation of murine lungs with ritanserin significantly shifted the 11,12-EET concentration-response curve to right (Figure 21).

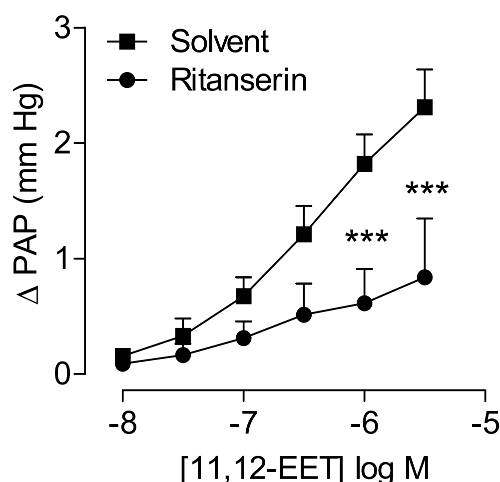

Figure 21: Effect of 5-HT_{2A} receptor inhibition on the 11,12-EET-induced increase in pulmonary artery pressure. Lungs from wild-type mice were treated with solvent or ritanserin (10 nmol/L) and the 11,12-EET concentration-response curve was assessed. The graph summarises data obtained in 3-4 independent experiments; ***P<0.001 versus solvent.

3.7. *Effect of hypoxic preconditioning on the 5-hydroxytryptamine-induced pulmonary vasoconstriction*

5-HT is known to be a potent pulmonary vasoconstrictor and others recently demonstrated that chronic hypoxia enhances the 5-HT response in murine pulmonary resistance arteries.[165] Moreover, in rat PASMCs 5-HT induced a $[Ca^{2+}]_i$ ncrease that was sensitive to CYP-epoxygenase inhibition.[140] Therefore,the effects of hypoxia and sEH inhibition or sEH deletion on the 5-HT dose response curve were assessed.

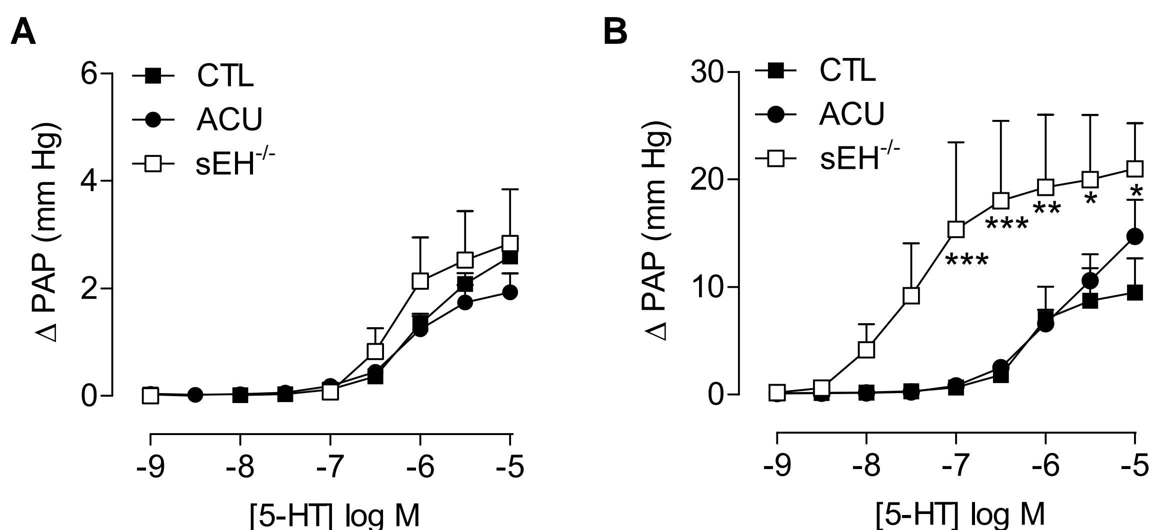

Figure 22: Effect of 5-HT on pulmonary artery pressure. (A) Effect of 5-HT on pulmonary artery pressure of lungs from wild-type mice in the presence or absence of ACU and on lungs from $sEH^{-/-}$ mice. (B) Effect of hypoxic preconditioning (two hypoxic periods prior the 5-HT concentration-response curve) on pulmonary artery pressures of wild-type lungs and $sEH^{-/-}$ lungs. The graphs summarise data obtained in 3-5 independent experiments. *P<0.05, **P<0.01,***P<0.001 versus CTL.

In lungs from wild-type and $sEH^{-/-}$ mice, 5-HT concentration-dependently increased pulmonary artery pressure (Figure 22A). sEH inhibition had no effect on the 5-HT concentration-response curve to 5-HT (Figure 22A) and the EC_{50} values to 5-HT were similar in all three groups ($LogEC_{50}$ in mol/L, CTL: -5.96±0.08; ACU: -6.13±0.12; $sEH^{-/-}$: -6.32±0.2). Interestingly, in lungs from wild-type and $sEH^{-/-}$ mice, hypoxic preconditioning (two hyoxic periods prior to the 5-HT concentration-response

curve) enhanced the sensitivity to 5-HT ($LogEC_{50}$ in mol/L, -6.2±0.18; Figure 22B). ACU further increased the hypoxia-induced 5-HT-hypersensitivity in wild-type mice ($LogEC_{50}$ in mol/L, -5.72±0.34). After hypoxic preconditioning, the $sEH^{-/-}$ lungs displayed a striking leftward shift in the 5-HT response ($LogEC_{50}$ in mol/L, -7.46±0.38; Figure 22B).

3.8. *Identification of TRPC6 as an effector of 11,12-EET-induced pulmonary vasoconstriction*

TRP channels are known to be activated by $G\alpha_q$-coupled receptors, such as the $5\text{-}HT_{2A}$ receptor.[166] Because TRPC6 has been linked to HPV,[115] and our group recently reported that 11,12-EET can affect endothelial Ca^{2+}-signalling by stimulating the intracellular translocation of TRPC6 to caveolae,[69] the effect of 11,12-EET on pulmonary arterial pressure and acute HPV in lungs from $TRPC6^{-/-}$ mice and their heterozygous ($TRPC6^{+/-}$) littermates was analysed.

In lungs from control ($TRPC6^{+/-}$) mice, acute hypoxia elicited a vasoconstriction that was potentiated by 11,12-EET (Figure 23A). Moreover, 11,12-EET (10 nmol/L to 3 µmol/L) elicited a concentration-dependent increase in pulmonary artery pressure that was similar to that observed in wild-type mice (Figure 23B). Hypoxia failed to elicit an acute increase in pulmonary pressure in lungs from $TRPC6^{-/-}$ mice (Figure 23A). Moreover, in the latter animals, 11,12-EET failed to significantly potentiate the response to hypoxia or elicit an increase in pulmonary artery pressure on its own (Figure 23).

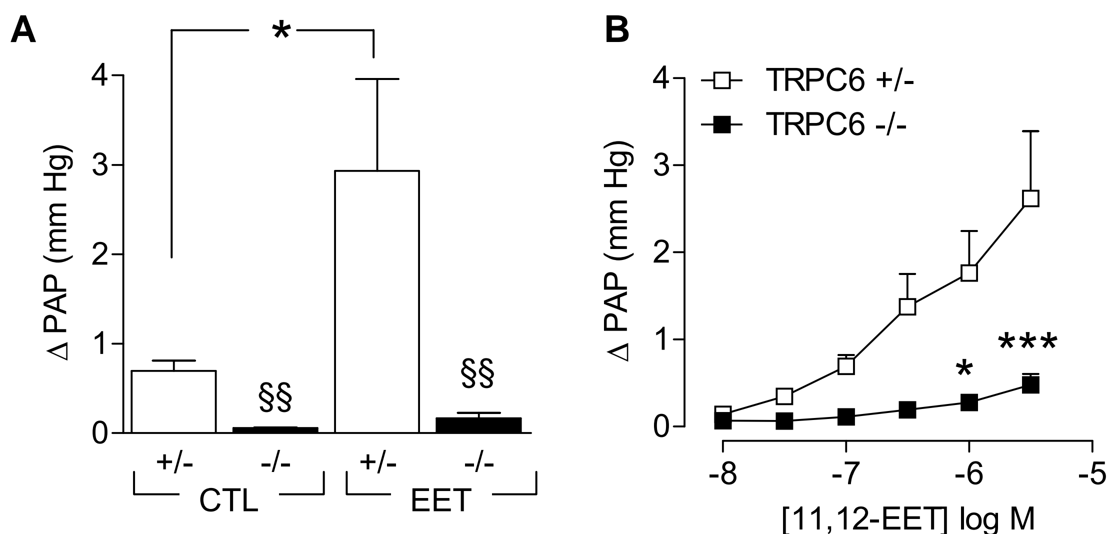

Figure 23: Identification of TRPC6 as an effector of 11,12-EET-induced pulmonary vasoconstriction. (A) Hypoxic pulmonary vasoconstriction in buffer-perfused lungs from $TRPC6^{+/-}$ and $TRPC6^{-/-}$ mice before (CTL) and after treatment with 11,12-EET (EET, 3 µmol/L). (B) Concentration-dependent effect of 11,12-EET on pulmonary arterial pressure in lungs isolated from $TRPC6^{+/-}$ and $TRPC6^{-/-}$ mice. The graphs summarise data obtained in 5-6 independent experiments; *P<0.05 versus control and §§ P<0.001 versus $TRPC6^{+/-}$ + 11,12-EET.

To assess the consequences of 11,12-EET on the intracellular localisation of TRPC6 a TrpC6-V5 fusion protein, in a double cassette together with GFP, was overexpressed in rat PASMCs. In cells maintained under basal conditions, the TRPC6-V5 was localised to the peri-nuclear Golgi apparatus as was the majority of the GFP signal (Figure 24A-C).

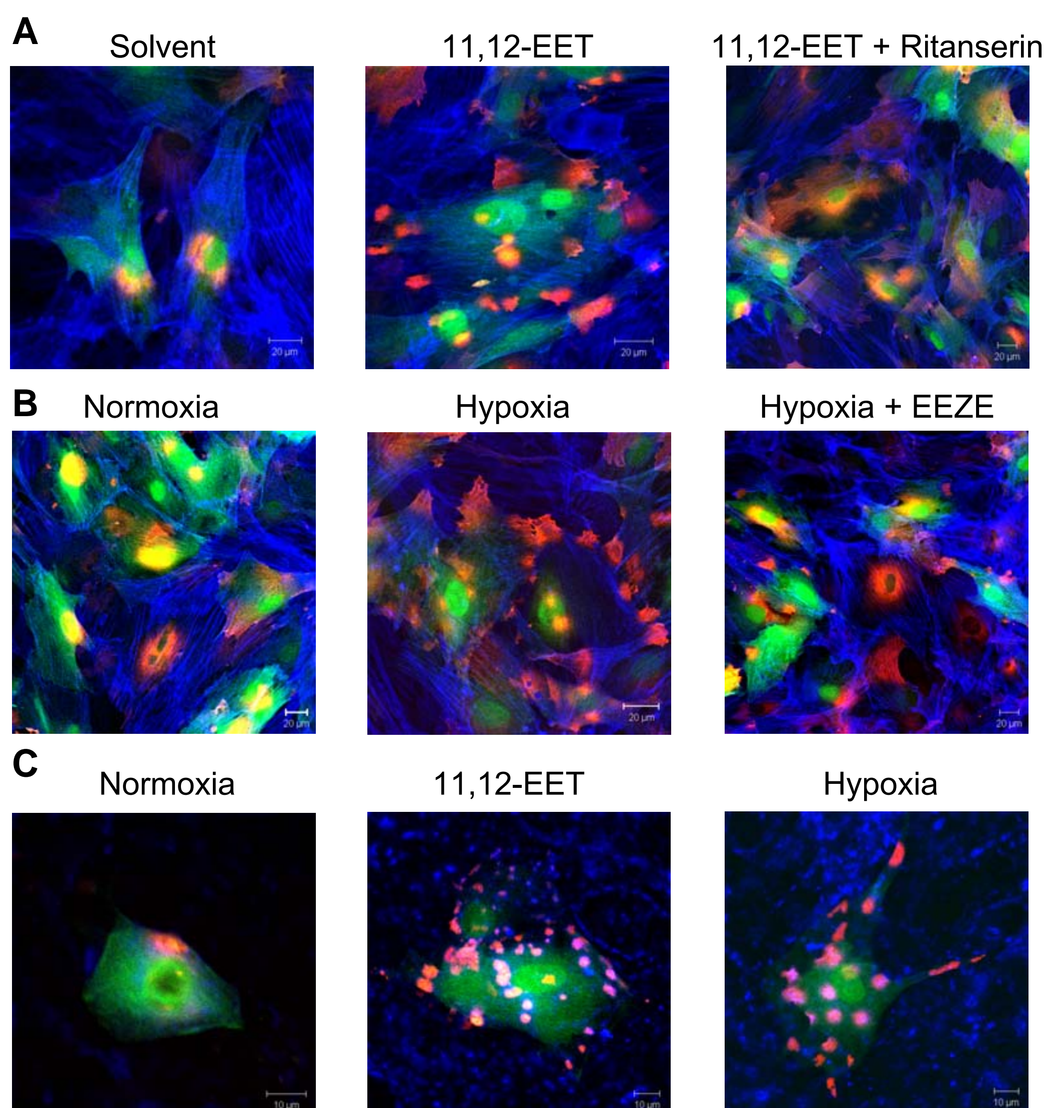

Figure 24: Effect of 11,12-EET or hypoxia on TRPC6-V5 translocation. Rat pulmonary smooth muscle cells were infected with TrpC6-V5 adenoviruses 24 hours prior to stimulation with (A) 11,12-EET (3 µmol/L, 5 minutes) in the absence and presence of ritanserin (10 nmol/L) or (B) hypoxia (1% O_2, 5 minutes) in the absence and presence of 14,15-EEZE (EEZE, 10 µmol/L). (A&B) Green=GFP, red=V5, blue=phalloidin. (C) Effect of 11,12-EET (3 µmol/L, 5 minutes) and hypoxia (1% O_2, 5 minutes) on the translocation of TrpC6-V5 to caveolae. Green=GFP, red=V5, blue=caveolin-1. Identical results were obtained in three additional experiments, each using a different cell batch.

Following the application of 11,12-EET (3 µmol/L, 5 minutes) the channel translocated to the plasma membrane (Figure 24A) more specifically to membrane domains enriched with the caveolae marker, caveolin-1 (Figure 24C). This effect was prevented by the 5-HT_{2A} receptor inhibitor ritanserin (Figure 24A) whereas the Rho kinase inhibitor Y27632 had no effect (data not shown). Exposure of cells to 1% O_2

(5 minutes) also stimulated the intracellular translocation of TRPC6-V5 to caveolae (Figure 24B&C), an effect not observed in cells pretreated with the EET antagonist, 14,15-EEZE (Figure 24B).

EETs have been suggested to increase lung vascular permeability by the activation of endothelial TRPV4 channels.[167] To exclude that the effects of exogenous (and potentially endogenous) 11,12-EET were in part attributable to interstitial/alveolar oedema formation causing a “secondary” HPV, EET-induced changes in lung permeability were assessed by measuring lung weight changes. However, although 14,15-EET has been linked to permeability changes, we found no effect of 11,12-EET on permeability in the isolated mouse lung (Figure 25).

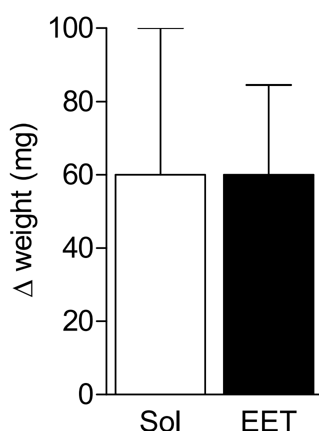

Figure 25: Effect of 11,12-EET on lung weight gain. 11,12-EET- (EET, 10 nmol/L to 3 µmol/L, ~30 minutes) and solvent-induced (Sol, 0.3 ‰ DMSO) changes in lung permeability by maintaining lung weight. The graph summarises data obtained in 5 animals.

3.9. Effect of chronic hypoxia on hematocrit, right heart hypertrophy and pulmonary vascular resistance

Chronic exposure to hypoxia is a major stimulus for the development of pulmonary hypertension and elevated pulmonary vascular resistance, which is associated with architectural remodelling. Chronic hypoxia in human subjects living at high altitude induces erythropoiesis,[168,169] an effect that was also observed following arachidonic acid administration.[170] Excessive erythrocytosis is known to be associated with a high

risk of cardiovascular complications and has been suggested to contribute to the development of pulmonary hypertension.[171,172]

When kept in normoxic conditions, hematocrit levels of wild-type and $sEH^{-/-}$ mice did not differ (Figure 26). However, when exposed to chronic hypoxia (10% O_2) for 3 weeks, the hematocrit increased in wild-type mice and $sEH^{-/-}$ mice compared to the respective normoxic control (Figure 26). In $sEH^{-/-}$ mice however, the hematocrit was significantly greater than that of wild-type mice following chronic exposure to hypoxia (Figure 26).

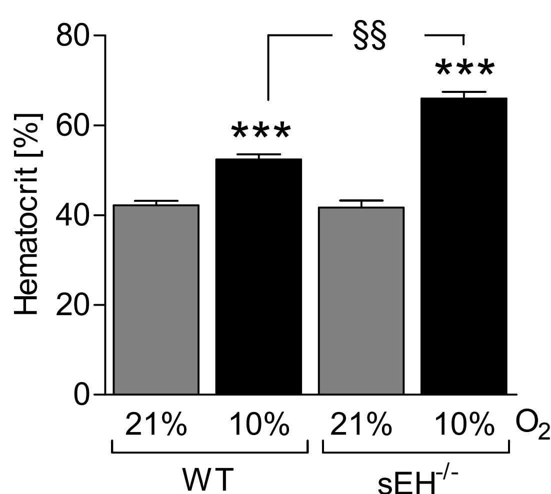

Figure 26: Effect of chronic hypoxia on the hematocrit in wild-type and $sEH^{-/-}$ mice. Hematocrit is given for wild-type (WT) as well as for $sEH^{-/-}$ mice either kept in normoxic conditions (21% O_2) or exposed to chronic hypoxia (10% O_2) for 21 days. The graph summarises data obtained in 5-6 animals per group; ***$P<0.001$ versus the corresponding normoxic control and §§$P<0.01$ versus WT chronic hypoxia.

Elevated pulmonary vascular resistance and sustained pulmonary hypertension put an excessive burden on the right ventricle and ultimately lead to right heart failure. Therefore, the effect of chronic hypoxia on right heart hypertrophy was assessed. The ratio of the right and left ventricle in normoxic conditions did not differ between wild-type and $sEH^{-/-}$ mice. Exposure to chronic hypoxia induced a right heart hypertrophy in both, wild-type and $sEH^{-/-}$ mice, as assessed by the ratio of the right ventricle /(left ventricle + septum). Compared to chronic hypoxic hearts from wild-type

mice $sEH^{-/-}$ mice displayed a slightly but not significantly enhanced right/(left ventricle + septum) ratio (Figure 27).

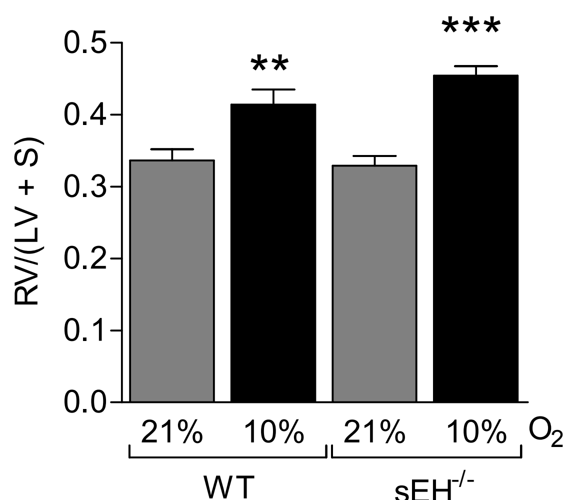

Figure 27: Effect of chronic hypoxia on right heart hypertrophy. Ratio of the right ventricular wall (RV) and the left ventricular wall+septum (LV + S) weight from hearts of wild-type and $sEH^{-/-}$ mice. Animals were kept under normoxic conditions (21% O_2) or exposed to hypoxia (10% O_2) for 21 days. The graph summarises data obtained in 10 independent experiments; **$P<0.01$, ***$P<0.001$ versus the corresponding normoxic control.

In the isolated buffer-perfused mouse lung the quantification of baseline pulmonary artery pressures is a measurement that directly reflects pulmonary vascular resistance. Baseline pulmonary artery pressures did not differ between normoxic lungs from wild-type mice and $sEH^{-/-}$ mice (Figure 28). Exposure of animals to 21 days hypoxia significantly enhanced baseline pulmonary artery pressures in lungs from wild-type mice and $sEH^{-/-}$ mice to the same extent (Figure 28).

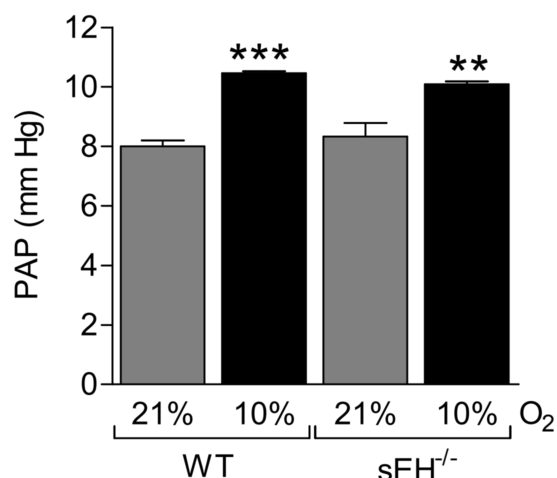

Figure 28: Effect of chronic hypoxia on pulmonary artery pressure measured in artificially perfused lungs. Baseline pulmonary artery pressure was assessed in lungs from wild-type (WT) and sEH$^{-/-}$ mice after the initial steady state period of lung perfusion. Lungs were perfused at a flow rate of 2 mL/min. Animals were kept under normoxic conditions (21% O_2) or pre-exposed to hypoxia (10% O_2) for 21 days. The graph summarises data obtained in 5-8 independent experiments; **$P<0.01$, ***$P<0.001$ versus the corresponding normoxic control.

3.10. Morphometric analysis of the pulmonary vasculature

To analyse the role of the sEH in chronic hypoxia-induced pulmonary vascular remodelling a morphometric analysis of the pulmonary vasculature of lungs from wild-type mice and sEH$^{-/-}$ mice was performed.

When dividing the pulmonary vessels into 3 categories (small: 20–70 µm diameter, medium: >70–150 µm diameter and: >150–1000 µm diameter) and analysing the degree of muscularisation (non-muscularised, partially muscularised, fully muscularised) within the different categories of vessel diameter, it appeared that under normoxic conditions the percentage of muscularised vessels was greater in sEH$^{-/-}$ than in wild-type mice (Figure 29). In wild-type mice kept under chronic hypoxia, the number of non-muscularised vessels decreased while the number of partially and fully muscularised vessels increased (Figure 29), an effect also observed in sEH$^{-/-}$ mice. When the degree of muscularisation of chronic hypoxic lungs was compared in wild-type mice and sEH$^{-/-}$ mice the latter animals displayed an enhanced muscularisation index. The effect was most prominent in the medium vessel size category (Figure 29).

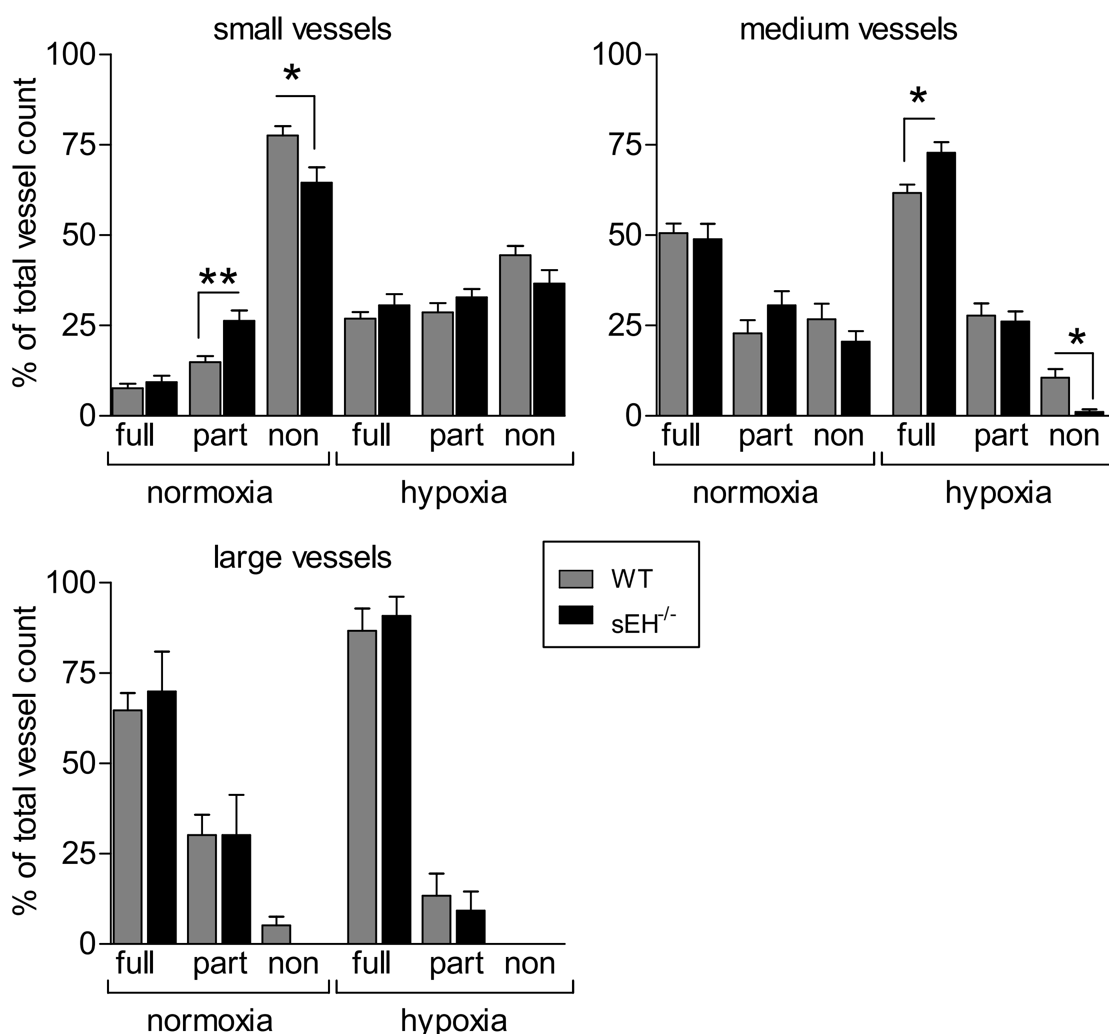

Figure 29: Effect of chronic hypoxia on pulmonary artery muscularisation in wild-type and sEH$^{-/-}$ mice. The degree of muscularisation (non: non-muscularised; part: partially-muscularised; full: fully-muscularised) is given for the following categories of vessel diameter: small: 20–70 µm diameter, medium: >70–150 µm diameter and: >150–1000 µm diameter. Data are given for wild-type and sEH$^{-/-}$ mice either exposed to chronic hypoxia (21 days, 10% O_2; hypoxia) or kept under normoxic conditions (normoxia). The graph summarises data obtained in 6 animals for each group. *P<0.05, **P<0.01 indicate significant differences.

3.11. Chronic hypoxia and soluble epoxide hydrolase expression/activity

Chronic hypoxia alters gene expression via the activation of different transcription factors.[173] Recently, our group reported that a CYP epoxygenase is implicated in chronic hypoxia-induced pulmonary hypertension and pulmonary vascular

remodelling.[24] The results of the present investigation indicate a role of the sEH on acute HPV and a vasoconstrictor role of EETs in the pulmonary vasculature. Therefore, the influence of hypoxia on the expression of the sEH and acute HPV was assessed.

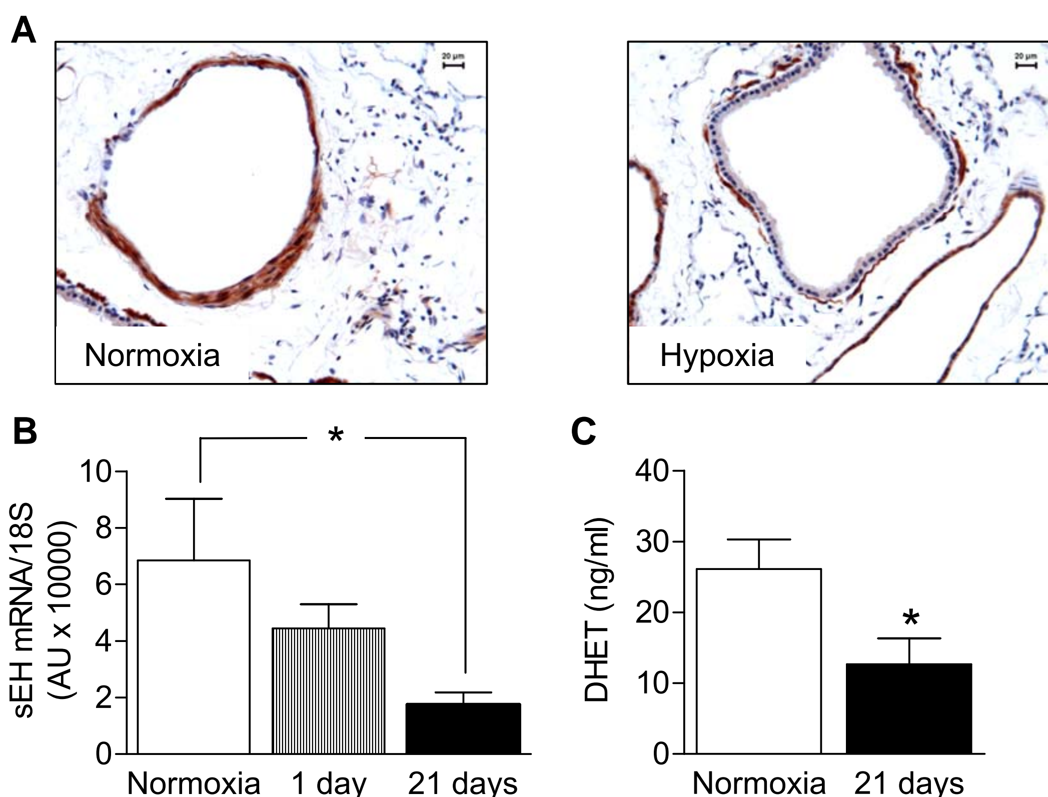

Figure 30: Effect of chronic hypoxia on the expression and activity of the sEH. (A) Immunohistochemical analysis showing the expression of the sEH (in brown) in lungs from wild-type mice kept under normoxic conditions or after exposure to 21 days hypoxia (10% O_2). (B) RT-PCR analysis of normoxic lungs from wild-type mice and of lungs from wild-type mice exposed to hypoxia (10% O_2) for 1 day and 21 days. (C) Effect of chronic hypoxia on the generation of 14,15-DHET from 14,15-EET by lung homogenates. Wild-type mice were kept under normoxic conditions (Normoxia) or exposed to 21 days hypoxia (10% O_2). The bar graph summarises data obtained in 4-16 independent experiments; *$P<0.05$ versus normoxia.

As mentioned above the sEH is expressed in pulmonary vascular smooth muscle cells. Using immunohistochemistry it could be demonstrated that chronic exposure to hypoxia (10% O_2) for 21 days decreased the expression of the sEH in lungs from wild-type mice (Figure 30A). Moreover, in lungs from wild-type mice short term (1

day) hypoxia decreased the sEH expression and exposure to 21 days hypoxia further decreased sEH levels (detected using RT-PCR, Figure 30B). Homogenates prepared from normoxic lungs hydrolysed 14,15-EET to 14,15-DHET, as determined by LC-MS/MS. The latter reaction was attenuated in lung homogenates prepared from animals exposed to chronic hypoxia (Figure 30C).

3.12. Effect of hypoxia on sEH promoter activity

To determine whether or not hypoxia could directly affect the expression of the sEH, we assessed the effect of hypoxia (1% O_2) on the activity of the sEH promoter using a luciferase gene-based reporter assay. As reported previously,[174] significant luciferase activity was detected in HEK 293 cells transfected with the 4 kb sEH promoter. Hypoxia rapidly (within 4 hours) decreased promoter activity, an effect that was maintained after 24 hours (Figure 31). Similar results were obtained using a 1.5 kb promoter fragment (Figure 31).

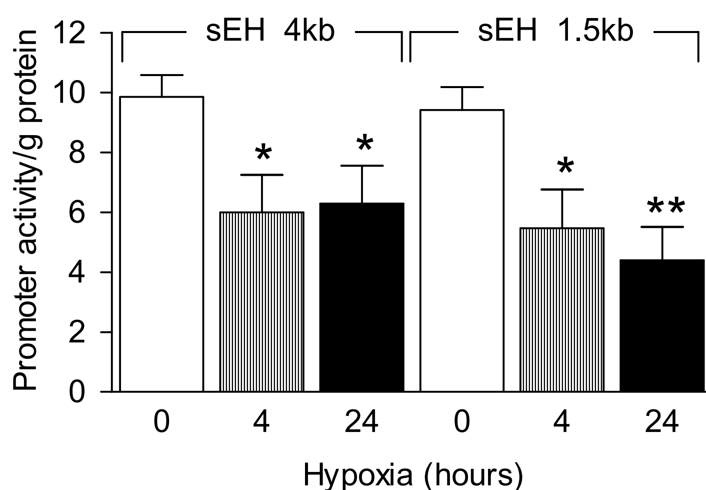

Figure 31: Effect of hypoxia (1% O_2) on sEH promotor activity. HEK 293 cells were transfected with the 4 kb sEH promoter or the 1.5 kb sEH promoter. Promoter activity was assessed using a luciferase gene based reporter assay. The graph summarises data obtained in 4 independent experiments; *P<0.05, **P<0.01 versus normoxia.

3.13. Effect of chronic hypoxia on acute hypoxic pulmonary vasoconstriction and pulmonary vasoreactivity

After chronic exposure to hypoxia, an increased, decreased or unchanged acute HPV response has been observed depending on species, subspecies and age.[31,175-180] However, in the mouse lung chronic exposure to hypoxia is reported to enhance acute HPV.[158] Given the results of the present study, modulation of the HPV response due to the activity of the sEH, down regulation of the sEH after exposure to chronic hypoxia and enhanced pulmonary artery muscularisation in $sEH^{-/-}$, the effects of chronic hypoxia on the acute HPV response were assessed in lungs from wild-type and $sEH^{-/-}$ mice.

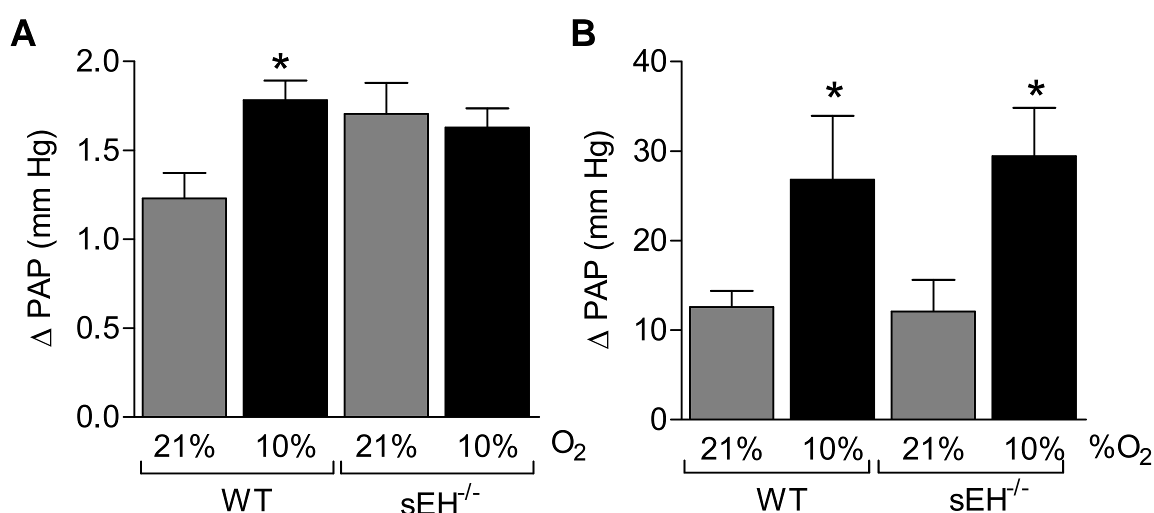

Figure 32: Comparison of the effects of normoxia and chronic hypoxia on pulmonary vascular responsiveness. Mice were kept under normoxic (21% O_2) conditions or exposed to hypoxia (10% O_2) for 21 days. (A) Acute hypoxic pulmonary vasoconstriction response to 1% O_2 (10 minutes) in lungs from wild-type (WT) and $sEH^{-/-}$ mice. (B) Maximum increase in pulmonary artery pressures following the application of U46619 (100 nmol/L) to the pulmonary perfusate. The bar graphs summarise data obtained in 5-8 independent experiments; *$P<0.05$ versus normoxic WT.

As before, challenging lungs with acute hypoxic ventilation for 10 min (1% O_2) resulted in an acute increase in pulmonary artery pressure (Figure 32A), pre-exposure to hypoxia (21 days) potentiated the acute HPV response (Figure 32A). Lungs from $sEH^{-/-}$ mice displayed an enhanced acute HPV, compared to that

observed in chronic hypoxic lungs from wild-type mice. However, chronic exposure to hypoxia did not further potentiate the responses (Figure 32A). Indicating, that the chronic hypoxia-induced amplification of the acute HPV in wild-type lungs is linked to sEH expression.

In lungs from normoxic wild-type mice, the application of the thromboxane mimetic U46619 elicited a potent vasoconstriction (Figure 32B). Pre-exposure of wild-type mice to chronic hypoxia for 21 days enhanced the U46619-induced vasoconstriction (Figure 32B). Normoxic lungs from $sEH^{-/-}$ displayed an U46619-induced contraction that was similar compared to response observed in normoxic lungs from wild-type mice (Figure 32B). Chronic exposure of $sEH^{-/-}$ mice to hypoxia also potentiated the U46619 response (Figure 32B), indicating, that the events initiated by chronic exposure to hypoxia did not alter the vasoreactivity to all pulmonary vasoconstrictors.

3.14. Effect of chronic hypoxia on the ACU-dependent increase in acute hypoxic pulmonary vasoconstriction

Given the sEH inhibition- and chronic hypoxia-induced potentiation of acute HPV response in wild-type mice, the absence of the latter effect in lungs from $sEH^{-/-}$ mice, the non-responsiveness of ACU in lungs from normoxic $sEH^{-/-}$ mice and the decrease of pulmonary sEH expression after chronic hypoxia, we assessed the effects of chronic hypoxia on the ACU-dependent increase in acute HPV.

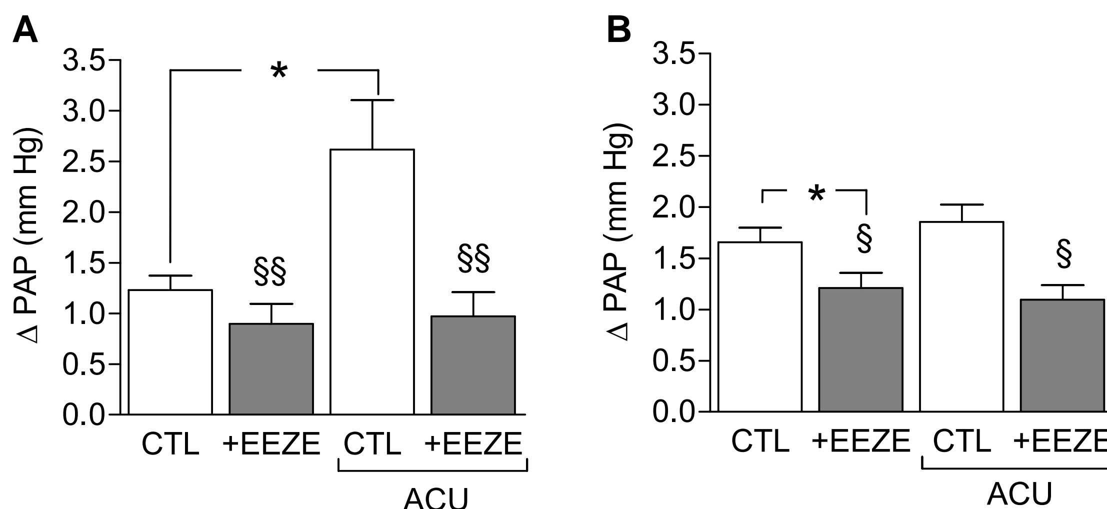

Figure 33: Effect of sEH inhibition and EET antagonism on acute hypoxia-induced vasoconstriction in isolated perfused mouse lungs. Wild-type mice were kept (A) under normoxic conditions or exposed to (B) hypoxia (10% O_2) for 21 days. Hypoxic pulmonary vasoconstriction was assessed in the presence or absence of 14,15-EEZE (EEZE, 10 µmol/L), and ACU (3 µmol/L). The bar graphs summarise data obtained in 5-8 independent experiments; *P<0.05 versus CTL and §P<0.05, §§P<0.01 versus ACU.

In normoxic lungs from wild-type mice inhibition of the sEH enhanced the acute HPV (Figure 33A). The EET antagonist, 14,15-EEZE, had only a marginal effect on its own but it completely prevented the increased constriction induced by sEH inhibition (Figure 33A). These data confirmed observations already presented in Figure 13. As before, chronic hypoxic lungs from wild-type mice displayed an enhanced acute HPV (Figure 32). However, sEH inhibition had no effect on acute HPV in lungs from chronic hypoxic wild-type mice (Figure 33B) whereas the EET-antagonist 14,15-EEZE significantly decreased the enhanced responses in the presence of solvent and after ACU treatment (Figure 33B).

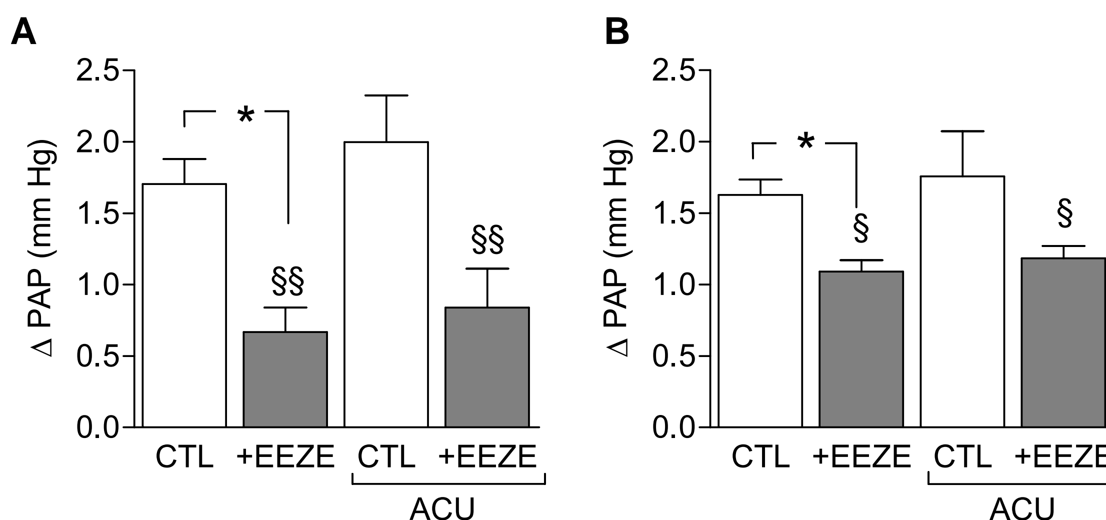

Figure 34: Effect of sEH inhibition, EET antagonism on acute hypoxic pulmonary vasoconstriction in isolated buffer-perfused mouse lungs. $sEH^{-/-}$ mice were kept under (A) normoxic conditions or exposed to (B) hypoxia (10% O_2) for 21 days. Hypoxic pulmonary vasoconstriction was assessed in lungs from $sEH^{-/-}$ mice in the presence or absence of 14,15-EEZE (EEZE, 10 µmol/L), or ACU (3 µmol/L). The bar graphs summarise data obtained in 4-7 independent experiments; *P<0.05 versus CTL and §P<0.05, §§P<0.01 versus ACU.

As expected, in normoxic and chronic hypoxic lungs from $sEH^{-/-}$ mice ACU did not further potentiate the acute HPV (Figure 34A&B). However, responses returned to levels observed in normoxic lungs from wild-type animals in the presence of 14,15-EEZE (Figure 34). Indicating that the chronic hypoxia-induced downregulation of the sEH is implicated in the increased HPV response in chronic hypoxic lungs from wild-type and that the mediator involved might be one of the EET isomers.

3.15. sEH expression and pulmonary hypertension

Pulmonary hypertension is a disabling disease with high mortality characterised by sustained elevation in pulmonary artery pressure and pulmonary vascular remodelling due to proliferation and migration of pulmonary artery smooth muscle cells.[181] Imbalance of vasodilatory and vasoconstrictive mediators have been implicated in these changes and immunohistological studies showed reduced expression of PGI_2 synthase in pulmonary vessels from patients with pulmonary

hypertension.[182] However, little is known about the expression of the sEH in patients with pulmonary hypertension. Immunohistochemistry demonstrated an extensive expression of the sEH in the medial wall of pulmonary arteries in lungs from healthy donors (Figure 35). sEH expression was, however, diminished in samples from pulmonary hypertension patients (Figure 35)

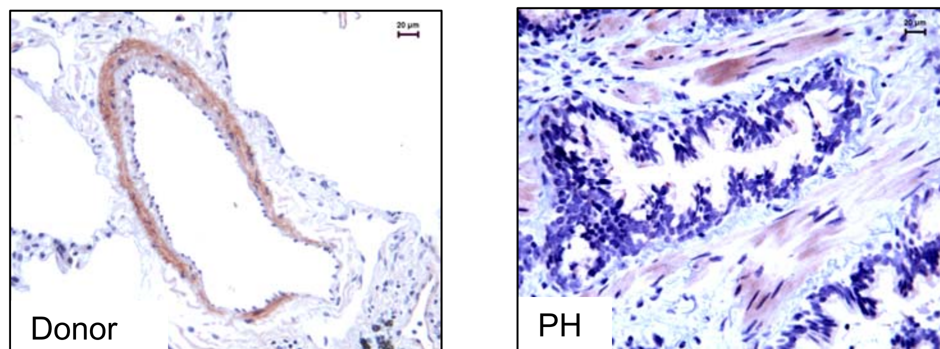

Figure 35: sEH expression and pulmonary hypertension. Immunohistochemical analysis showing the expression of the sEH (in brown) in samples from human donors or pulmonary hypertension (PH) patients.

4. Discussion

4.1. The role of the sEH and EETs in acute hypoxic pulmonary vasoconstriction

The results of the present investigation indicate that the activity/expression of the sEH plays an important role in determining the magnitude of acute hypoxia-induced pulmonary vasoconstriction. The mechanism involved appears to be related to the generation of CYP epoxygenase-derived epoxides because both the epoxygenase inhibitor and the EET antagonist abrogated the effects of sEH inhibition and the exogenous application of 11,12-EET elicited a rapid increase in pulmonary perfusion pressure. Both hypoxia and 11,12-EET were able to elicit the activation of the 5-HT_{2A} receptor, as responses were sensitive to 5-HT_{2A} antagonists, as well as the Rho kinase. Moreover, both hypoxia and 11,12-EET stimulated the translocation of TRPC6 channels within pulmonary smooth muscle cells indicating that the EET-induced activation of TRPC6 underlies the phenomenon observed. As EET-induced translocation of TRPC6 channels was sensitive to 5-HT_{2A} receptor but not to Rho kinase inhibition, activation of the latter either occurs downstream or independently of the TRPC6 channel.

In the systemic circulation one function of CYP epoxygenase-derived EETs is to elicit the hyperpolarisation of endothelial and vascular smooth muscle cells and thus vasodilation. CYP-dependent vasoconstriction, on the other hand, is generally attributed to metabolites such as 20-HETE,[183] which play an important role in the myogenic tone. The biological role of CYP-derived metabolites in the pulmonary circulation remains unclear and completely contradictory findings have been published. Although classed as vasodilators in the systemic circulation EETs have previously been linked with constriction in the pulmonary circulation.[84,89,90] However, vasodilatation seems to be restricted to larger conduit arteries as smaller resistance arteries contract in response to the exogenous application of EETs.[184] Thus, in order to analyse the role of EETs in the pulmonary circulation, the isolated buffer-perfused mouse lung seems a more suitable model than isolated arterial rings to study global effects of the arachidonic epoxides on resistance in the pulmonary microcirculation.

Biological activity has been attributed to all of the EET regioisomers generated in the lung. However, our study highlights the importance of 11,12-EET as exposure to hypoxia acutely increased the production of 11,12-EET in murine lung microsomes and in intact rat pulmonary artery smooth muscle cells; a response also recently observed in the rabbit lung.[185] Moreover, two CYP epoxygenase inhibitors, MSPPOH and fenbendazole, attenuated the generation of 11,12- and 14,15-EET without affecting that of either 5,6- or 8,9-EET and abrogated the effects of sEH inhibition on hypoxic vasoconstriction. For these reasons, the direct effects of 11,12- and 14,15-EET on pulmonary artery pressure in the isolated mouse lung were assessed. Exogenously applied 11,12-EET elicited a pronounced concentration-dependent increase in pulmonary perfusion pressure while 14,15-EET was surprisingly without effect. Generation of the corresponding diol is also unlikely to account for the observations made as 11,12-DHET had no effect on pulmonary artery pressure. Our observation that 11,12-EET is a potent vasoconstrictor within the pulmonary vasculature is at odds with reports that EETs cause vasodilation in the pulmonary circulation.[83,86,87] Such contradictory observations suggest that the pulmonary effects of EETs are not straightforward and appear to depend on factors such as species, concentration, size of the vessel (as mentioned above), model, and EET isomer. Indeed, all of the studies demonstrating EET-dependent pulmonary vasodilation were focused on 5,6-EET. In the present investigation only 11,12-EET and not 14,15-EET increased pulmonary artery pressure whereas other investigators found that 11,12- and 14,15-EET increased pulmonary resistance and constricted isolated pulmonary artery rings to the same magnitude.[84,89]

As we reasoned that more information about the biological actions of pulmonary-derived EETs can be obtained from experiments in which their metabolism was prevented than from experiments looking at responses to exogenously applied eicosanoids, our investigation focused on the importance of the sEH in modulating the acute hypoxic pulmonary vasoconstriction. Our results clearly show that inhibition of the sEH potentiated acute hypoxic vasoconstriction in lungs from wild-type mice and that this potentiating effect was not observed when CYP epoxygenases were inhibited. The two compounds used, ACU and AEPU, are both potent sEH inhibitors with IC_{50} values in the low nmol/L range for both the human and the murine

recombinant enzymes. However, their structures and physical properties are quite different[24] and the fact that these divergent compounds led to very similar pulmonary responses supports the conclusion that their action was due to the inhibition of the sEH. In keeping with these observations the acute increase in pulmonary perfusion pressure induced by hypoxia was markedly elevated in lungs from $sEH^{-/-}$ mice underlining the importance of the sEH and CYP epoxygenase-derived EETs in the acute hypoxic pulmonary vasoconstriction response. In $sEH^{-/-}$ mice, which demonstrate elevated circulating and pulmonary levels of EETs and low DHET levels,[186] neither of the sEH inhibitors tested were able to affect the acute pulmonary vasoconstriction induced by hypoxia, whereas a CYP epoxygenase inhibitor and the EET antagonist decreased the acute HPV response in lungs from $sEH^{-/-}$ mice up to levels observed in lungs from wild-type mice. Thus, the sEH inhibitor-induced increase in pulmonary vasoconstriction that was dependent on the activity of a CYP epoxygenase, seems to be attributable to 11,12-EET. Furthermore, an indirect effect of 11,12-EET on interstitial/alveolar oedema, which could elicit a “secondary” hypoxic vasoconstriction, could be ruled out, as we found no evidence to suggest that 11,12-EET affected endothelial cell permeability.

The involvement of CYP enzymes in the regulation of acute hypoxic pulmonary vasoconstriction has been proposed previously on the basis of earlier investigations reporting that CYP inhibition decreased acute hypoxic pulmonary vasoconstriction in the isolated rabbit lung,[187] and depressed venular contractions induced by decreased pO_2.[188] Recently a collaborative study between our group and others implicated a CYP epoxygenase in the acute pulmonary vasoconstriction induced by hypoxia in anaesthetised mice.[24] In the latter study it was also possible to demonstrate that the mediator involved was metabolised by the sEH, as inhibition of this enzyme potentiated the vasoconstrictor response.[24] One apparent difference between the present investigation in the isolated lung and the previous study in anaesthetised animals was the sensitivity of the hypoxic vasoconstriction *per se* to CYP epoxygenase inhibition.[24] This difference can most likely be attributed to the methods used to assess pulmonary function as pressures measured in the isolated lung model are generally lower than those measured *in vivo*. Further support for a prominent role of an arachidonic acid product in the pulmonary vasoconstrictor response to hypoxia

has been obtained using animals lacking the cytosolic phospholipase (cPL) A_2 that releases arachidonic acid from phospholipids in cell membranes.[189] In the latter study, hypoxic pulmonary vasoconstriction induced by left main stem bronchus occlusion was detectable in wild-type but not in $cPLA_2^{-/-}$ mice and could be restored in these animals by the exogenous application of arachidonic acid. Moreover, inhibition of the $cPLA_2$ in wild-type animals resulted in a complete loss in hypoxic pulmonary vasoconstriction. However, all of the studies that have addressed a possible role of CYP in acute hypoxic pulmonary vasoconstriction observed that CYP inhibition resulted in decreased and not in a total loss of acute hypoxic vasoconstriction. As deletion of the $cPLA_2$ resulted in a complete loss in HPV, it seems that other arachidonic acid metabolising enzymes and arachidonic acid-derived-products are involved in regulating pulmonary vascular tone.

One point that this study did not address in detail relates to the cellular localisation of the enzymatic machinery involved. Although, still to be confirmed experimentally by immunohistochemistry and in-situ hybridisation, we propose that the CYP2C enzymes and the sEH that mediate HPV are localised in vascular smooth muscle cells. Certainly these cells express the sEH *in vivo* in mice and the cultured rat pulmonary artery smooth muscle cells we used to demonstrate hypoxia induced translocation of TRPC6 expressed both CYP2C11 and the sEH. Evidence against an endothelial location of the hypoxia-sensitive CYP enzyme was obtained in experiments looking at responses in transgenic mice which overexpressed either CYP2C8 or 2J2 specifically in endothelial cells. In the latter animals we failed to observe an enhanced hypoxic pulmonary vasoconstriction even in the presence of ACU. Our supposition that the CYP and sEH enzymes are extra-endothelial fits with reports in the literature that pulmonary smooth muscle cells contain the primary oxygen sensor and mediators.[5,11]

sEH activity is increased in male mice versus female mice and castration is reported to decrease sEH activity and expression; a phenomenon restored by testosterone supplementation.[99,100] Thus, one would assume that female or castrated male mice display higher EET levels compared to normal male mice resulting in an exaggerated hypoxic pulmonary vasoconstriction and sensitivity to sEH inhibition. Although we previously observed greater sensitivity to sEH inhibition in female Swiss Webster

mice[24] this finding could not be reproduced in C57BL/6 mice and there were no differences in hypoxia-induced pulmonary vasoconstriction in male, male castrated, female or female ovarectomised animals. However, we did not make a detailed comparison of sEH expression in the different groups to determine whether the differential sEH expression reported in the ventral prostate, liver and kidneys were also apparent in the lung. Certainly, there are examples of sexual hormone regulated enzymes (e.g. the murine cytosolic glutathione S-transferase[190]) that are modified in the heart, liver and kidneys but not in the pulmonary system.

Contraction of smooth muscle cells occurs via Ca^{2+}-dependent mechanisms requiring an increase in intracellular Ca^{2+} as well as via Ca^{2+}-independent mechanisms related to the activation of the Rho kinase.[191-194] Hypoxic vasoconstriction has been linked to Rho kinase activation in a number of studies[162,163,195] as has the 20-HETE-induced contraction of coronary arteries.[70] Given that the actions of 11,12-EET and 20-HETE seem to be reversed in the lung,[196,197] we determined whether or not hypoxia, sEH inhibition and/or 11,12-EET were able to affect the activity of the Rho kinase. Our results demonstrate that sEH inhibition increases the hypoxia-induced phosphorylation of MLC-20 and that 11,12-EET is able to stimulate Rho kinase activity in the murine lung. Moreover, Rho kinase inhibition decreased the basal acute hypoxic pulmonary vasoconstriction an effect that was also observed by other investigators.[162,163] Furthermore, Rho kinase inhibition prevented not only the sEH inhibitor-induced potentiation of hypoxic vasoconstriction but also the increase in pulmonary artery pressure elicited by exogenous 11,12-EET.

Rho kinase activation is linked to G protein-coupled receptors, mainly via $G\alpha_{12/13}$-coupled receptors.[198] However, activation of the Rho kinase can also occur via a $G\alpha_q$-coupled mechanism[199] and seems to be dependent on the activation of PKC.[200-202] As 5-HT is one of the best studied modulators of the HPV response[11,164] and the 5-HT$_{2A}$ receptor, a $G\alpha_q$-coupled receptor, is mainly expressed on pulmonary artery smooth muscle cells,[145] we determined whether or not hypoxia and/or 11,12-EET can activate the 5-HT$_{2A}$ receptor. Our results showed that inhibition of the 5-HT$_{2A}$ receptor decreased the basal acute hypoxic vasoconstriction by approximately 30% and completely prevented the EET induced potentiation of the HPV response. Moreover the sEH inhibitor-induced potentiation of HPV was sensitive to two different 5-HT$_{2A}$

receptor inhibitors. Both of the compounds used, ketanserin and ritanserin, are potent inhibitors of the 5-HT_{2A} receptor and the concentration used in the present investigation were close to the IC_{50} values (approximately 1nmol/L). Ketanserin is a relatively selective 5-HT_{2A} receptor antagonist. It also binds less potently to 5-HT_{2C}, 5-HT_{2B}, 5-HT_{1D}, alpha-adrenergic, and dopamine receptors.[203] Ritanserin is more selective for the 5-HT_{2A} receptor but also binds in higher concentrations to the 5-HT_{2C} receptor.[204] However, the fact that both substances lead to similar responses on the sEH inhibitor-induced potentiation of acute hypoxic vasoconstriction and that ritanserin in low doses inhibited the 11,12-EET-induced actions indicate that hypoxia, sEH inhibition, and 11,12-EET signal via the 5-HT_{2A} receptor.

There are numerous agonists (e.g. bradykinin, angiotensin II) known to activate G protein-coupled receptors that also increase endogenous CYP activity and EET production but none seem to be as exquisitely sensitive to EET modulation as the 5-HT_{2A} receptor. Furthermore, given that the effects described were sensitive to the EET antagonist 14,15-EEZE we speculate a much closer link between EETs and the 5-HT_{2A} receptor than previously proposed. In fact, as the 5-HT_{2A} receptor antagonist was able to prevent the 11,12-EET-induced translocation of TRPC6 in pulmonary smooth muscle cells it may be possible that 11,12-EET can activate the 5-HT_{2A} receptor directly. Although a specific “EET receptor” on the cell surface remains to be identified its existence has been suggested on the basis of data showing a specific EET binding site on mononuclear cells.[205-207] The authors of the latter studies proposed that EET-signalling begins with the binding of the EET to its receptor and is followed by an increase in intracellular cAMP levels and the activation of PKA.[205-207] The cAMP/PKA pathway is also involved in the 11,12-EET-induced translocation of the TRPC6 in endothelial cells,[69] indicating that in monocytes and endothelial cells a $G\alpha_s$- coupled receptor is likely to act as a potential EET receptor. In the present study the 11,12-EET- and/or sEH inhibition-induced responses in pulmonary artery pressure were sensitive to 14,15-EEZE. The later is a so called “EET antagonist” a fact that makes the possibility that 11,12-EET signals via a receptor more likely. A more detailed investigation of the link between 11,12-EET and the 5-HT_{2A} receptor is ongoing.

Although the Rho kinase can be activated in the absence of a sustained increase in intracellular Ca^{2+},[208,209] there is a wealth of evidence indicating that Ca^{2+} plays a pivotal role in pulmonary vasoconstriction. While the activation of several types of Ca^{2+} channels can affect $[Ca^{2+}]_i$ in smooth muscle cells, a lot of attention has been focused on the role played by the TRP family of non-selective cation channels. To identify the potential mechanism of the hypoxia-induced, EET-mediated pulmonary vasoconstriction we concentrated on the TRPC6 channel. EETs can affect the activity of at least 2 different classes of TRP channels (i.e. TRPV and TRPC channels) but there were several reasons for singling out TRPC6 as an effector for 11,12-EET. Firstly, although CYP epoxygenases can modulate the activity of TRPV4, a channel implicated in mechanotransduction, these effects have been attributed to 5,6- and 8,9-EET but not 11,12-EET.[68] Secondly, while TRPC6 is unaffected by shear stress, hypo-osmotic stress and by 5,6-EET, both its membrane translocation and Ca^{2+} influx are influenced by 11,12-EET.[69] It is likely that the spectrum of EET regioisomers generated in response to a given stimulus determines which TRP channels can be affected. Indeed, TRPC6 can also be activated by CYP-derived 20-HETE in HEK cells overexpressing the channel.[210] Moreover, although relatively few studies have addressed the role of hypoxia in the regulation of TRP channels, the expression of TRPC6 and store- as well as receptor-operated Ca^{2+} entry into pulmonary artery smooth muscle cells are elevated in response to chronic (3 weeks) hypoxia,[14] and acute hypoxic vasoconstriction is almost abolished in the lungs of TRPC6$^{-/-}$ mice.[115] The results generated during the preparation of this thesis demonstrated that neither hypoxia nor 11,12-EET were able to stimulate an increase in pulmonary perfusion pressure in lungs from TRPC6$^{-/-}$ mice. The lack of responsiveness to hypoxia and 11,12-EET appears to be a specific phenomenon as the responsiveness of the lungs from TRPC6$^{-/-}$ mice to U46619 was normal[115] and aortic rings from these animals even demonstrate an elevated contractile response to phenylephrine.[152] Given that 11,12-EET has been demonstrated to increase Ca^{2+} entry and elicit the translocation of the TRPC6 channel from the peri-nuclear Golgi apparatus to caveolae,[69] we determined whether or not hypoxia could induce the translocation of the TRPC6 channel in pulmonary smooth muscle cells and whether or not this was an EET-dependent process. Indeed, hypoxia stimulated the

membrane translocation of a TRPC6-V5 fusion protein to membrane domains enriched with caveolin-1, an effect that was mimicked by 11,12-EET and markedly attenuated in the presence of the EET antagonist. Moreover, the 11,12-EET-induced TRPC6-V5 translocation was diminished upon 5-HT_{2A} receptor inhibition and unaffected by the Rho kinase inhibitor. Although our data suggest that the hypoxia and/or 11,12-EET-induced TRPC6 channel translocation depends on the activation of the 5-HT_{2A} receptor, the intermediate steps in the translocation process e.g. eventual involvement of PKA, remains to be determined. However, it seems safe to conclude that the EET-induced activation of RhoA/Rho kinase either occurs downstream of the TRPC6 channel or independently of the TRPC6 channel-induced increase in $[Ca^{2+}]_i$.

The exact mechanism by which translocation of TRP channels occurs remains unclear. For the TRPC1, TRPC3, TRPC4, TRPC6 and TRPV6 channels caveolae have been implicated[69,211-214] and for TRPC1 the activation of a G protein coupled receptor is assumed.[132] In endothelial cells, the EET-induced translocation of TRPC6 to caveolae was dependent on a signalling cascade including cAMP/PKA.[69] In rat pulmonary artery smooth muscle cells, the 5-HT_{2A} receptor coimmunoprecipitates with caveolin-1 and the K_V1.5 channel which is another potential hypoxia target.[215] Moreover, K_V1.5 channels were internalised when cells were stimulated with 5-HT,[215] thus it will be interesting to determine whether EETs stimulate the association of 5-HT_{2A} with TRPC6 or interfere with the activation/inactivation of this complex.

G protein-coupled receptors are involved in multiple ways in the activation or translocation of TRP channels. Recently, thrombin was reported to bind to and cleave protease-activated receptor-1 in human endothelial cells leading to the $G\alpha_q$ receptor-dependent activation of the TRPC6 channel.[202] Further, the TRPC6-induced rise in $[Ca^{2+}]_i$ activated PKCα thus causing the activation of the Rho kinase.[202] However, in the latter study it was not determined whether thrombin induced the translocation of TRPC6 to the plasma membrane and the authors linked the activation of the TRPC6 channel to increased levels of DAG.[202] It is however possible that a similar pathway could link the activation of the 5-HT_{2A} receptor to TRPC6-dependent Ca^{2+} influx. There are also hints in the literature that the activation of small G proteins occurs upstream of TRP channel translocation. For example, the thrombin

induced translocation of TRPC1 channels in endothelial cells requires active RhoA,[132] the translocation of TRPC5 channels in hippocampal neurons is dependent on the activation of the Rho GTPase Rac1[136] and the trafficking of TRPV5 and V6 channels to the plasma membrane in epithelial cells depends on the activation of Rab11.[216] Other factors that are discussed to be involved in the translocation of the TRP channel are proteins like VAMP2, Homers, RGA and PASCIN3.[217]

Taken together, our results indicate that the activity of the sEH is an important determinant of the magnitude of hypoxic pulmonary vasoconstriction by inactivating vasoconstrictor CYP-derived EETs. These eicosanoids appear to be important modulators of pulmonary vascular tone and can elicit contraction by targeting TRPC6 channels to the plasma membrane as well as by activating the Rho kinase. The exact molecular mechanism(s) by which EETs induce the membrane translocation of TRPC6 channels and the activation of the Rho kinase also remains to be elucidated but seems to involve the activation of the 5-HT_{2A} receptor.

4.2. The sEH in chronic hypoxia-induced pulmonary hypertension and pulmonary vascular remodelling

From the initial part of this study it seems clear that the expression of the sEH plays an important role in modulating responsiveness to acute hypoxia. The second part of this study was therefore aimed at investigating a potential link between chronic hypoxic alterations in lung responsiveness and morphology and the expression/activity of the sEH. Our results indicate that deletion of the sEH mimics, to a certain extent, the pathophysiological changes induced in the lung by chronic (21 days) hypoxia. Certainly, even under normoxic conditions the muscularisation index of the $sEH^{-/-}$ mice differed from that of wild-type mice and the acute hypoxia-induced pulmonary vasoconstriction was significantly elevated. Moreover, in wild-type animals, chronic hypoxia decreases pulmonary sensitivity to sEH inhibition at the same time as decreasing sEH mRNA and protein expression as well as activity. While it was not possible to demonstrate a causative link between sEH expression levels and the development of pulmonary hypertension, we routinely failed to detect

the sEH in lungs from patients with pulmonary hypertension even though the enzyme was expressed in vascular smooth muscle cells in lungs from healthy donors.

Pulmonary hypertension is a disabling disease with a high mortality characterised by sustained elevation in pulmonary artery pressure and pulmonary vascular remodelling that results from the proliferation and migration of pulmonary artery smooth muscle cells.[181] Chronic alveolar hypoxia is a major stimulus for the development of pulmonary hypertension and the subsequent changes in the architecture of the pulmonary vasculature.[218] Among the signalling intermediates implicated to-date are cyclooxygenase and lipoxygenase products of arachidonic acid as wells as the nitric oxide pathway. Surprisingly little is known about the role, if any played by CYP epoxygenases in the homeostasis of the pulmonary circulation[218,219] even though these enzymes were recently implicated in the pulmonary vascular remodelling induced by prolonged (7 days) hypoxia and CYP epoxygenase inhibition attenuated remodelling.[24]

It is well established that hypoxia-induced pulmonary hypertension increases right ventricular after load thus resulting in right heart hypertrophy and failure.[220] Although the muscularisation ratio was affected by the loss of the sEH even in normoxic conditions and the pulmonary vasoconstriction elicited by acute exposure to hypoxia was elevated by sEH deletion and inhibition, there was no evidence of an accompanying right heart hypertrophy and failure in the $sEH^{-/-}$ animals maintained under normoxic conditions. Moreover, chronic hypoxia elicited comparable changes in right ventricular mass in both wild-type and $sEH^{-/-}$ mice, even though responses were slightly more pronounced in $sEH^{-/-}$ animals. Similarly, baseline pulmonary artery pressure (measured in the isolated lung) was not significantly different in lungs from the two strains following exposure to hypoxia. Interestingly, there was a marked increase in the hematocrit of the hypoxic $sEH^{-/-}$ mice versus the wild-type animals, suggesting that the former are particularly sensitive to hypoxia, however, a more detailed investigation of the link between erythropoiesis and the activity of the sEH needs to be performed. Certainly, given the link between excessive erythrocytosis and cardiovascular risk as well as the development of pulmonary hypertension[171,181] and the fact that arachidonic acid administration also induces erythropoiesis[170] may implicate the cytochrome P450 pathway in this process.

Our finding that the muscularisation of small-sised (20-70 µm) arteries was greater in sEH$^{-/-}$ mice even in normoxia suggests that hypertrophic mechanisms are activated in these animals. Certainly, EETs have the potential to promote such a response as these epoxides are reported to enhance cell survival[221,222] as well as to elicit the phosphorylation of MAP kinases, particularly p38 MAP kinase and ERK1/2, all of which can contribute to pulmonary smooth muscle cell hypertrophy.[223,224] In this context, it is interesting to note that 11,12-EET promoted capillary muscularisation and maturation in a Matrigel plug assay *in vivo*,[225] although the exact cellular mechanisms that underlie the increase in vascular muscular mass need to be determined experimentally. Whether or not TRPC6 channels are involved in these processes is also unclear, since although channel expression was increased in hypoxia-induced, as well as idiopathic chronic pulmonary hypertension,[14,127] the development of pulmonary hypertension did not differ in TRPC6$^{-/-}$ and wild-type mice.[115]

The molecular pathways by which chronic hypoxia causes vasoconstriction and pulmonary vascular remodelling are just beginning to be identified. However, given that 11,12-EET, which is an sEH substrate, can elicit pulmonary vasoconstriction as well as vascular maturation,[225] we hypothesise that the development of pulmonary hypertension might be linked to a change in the CYP epoxygenase/sEH pathway. Certainly, hypoxia has been previously reported to increase pulmonary CYP epoxygenase expression and EET levels.[24,185] The latter activation would however not be expected to elicit a biological effect should the activity of the sEH be increased in parallel. The results of the present investigation indicate that hypoxia effectively decreased the expression of the sEH *in vivo* in animals exposed to hypoxia for as short as one day and decreased the activity of the sEH promoter in cells cultured under hypoxic conditions for only a few hours. It has been possible to link the sEH with pathology as enzyme expression is increased in spontaneously hypertensive and stroke-prone rats as well as in Wistar rats following the administration of angiotensin II.[226] However, little is known about the mechanisms regulating the expression of the sEH although both the binding of SP-1[174] and c-Jun[226] to the sEH promoter have been reported to regulate activity. Irrespective of the mechanisms involved, the decrease in EET metabolism resulting from the hypoxia-induced

downregulation of the sEH would be expected to enhance pulmonary EET levels, thus causing pulmonary vasoconstriction. There are however alternative, less efficient ways of controlling cellular EET levels; for example β-oxidation or C2 elongation[59,227] that may limit the pathology observed.

Nothing is known about the expression of CYP epoxygenases or the sEH in pulmonary hypertension patients. We observed that sEH expression was low to absent in samples from pulmonary hypertension patients whereas an extensive sEH expression was detected in the medial wall from donor lungs. The loss of a vasoconstrictor metabolising enzyme in human pulmonary hypertension is consistent with the findings in the animal experiments and fits in the hypothesis that the sEH and EETs are implicated in the development of pulmonary hypertension and pulmonary vascular remodelling.

Hypoxic pulmonary vasoconstriction consists of two phases: the first is the so-called acute hypoxic vasoconstriction that generally develops within minutes of exposure and is followed by a temporary vasodilatation. The second phase is sustained and can develop twenty to thirty minutes after challenge and last for hours or days if hypoxia is maintained.[6] Following chronic exposure to hypoxia, an increased, decreased or unchanged acute hypoxic pulmonary vasoconstriction has been observed depending on species, subspecies and age.[228] In the mouse lung chronic exposure to hypoxia has been reported to enhance the acute response,[158] a finding confirmed by this study. Moreover, we observed that the pulmonary vasoconstriction elicited by acute exposure to hypoxia in lungs from wild-type mice exposed to hypoxia for 21 days was largely abolished in the presence of 14,15-EEZE, implicating an EET in the response. Chronic exposure of $sEH^{-/-}$ mice to hypoxia however did not potentiate the acute hypoxic pulmonary vasoconstrictor response. We interpret this finding in the light of the chronic hypoxia-induced decrease in sEH expression as providing evidence of a functional consequence for a change in sEH activity in the pulmonary vasculature.

At this stage it is important to note that, although we have concentrated on 11,12-EET as the sEH substrate with the potential to alter pulmonary function and morphology, the enzyme can hydrolyse a number of substrates including

leukotrienes and EpoMEs to their corresponding diols. Our reasons for focusing on 11,12-EET were related to our findings regarding the effects of hypoxia on CYP expression and the fact that that the acute hypoxic vasoconstriction was significantly attenuated by the EET antagonist 14,15-EEZE. However, it is certainly possible that one of the non-EET substrates of the sEH might be implicated in the pulmonary changes recorded in $sEH^{-/-}$ mice. Certainly, EpoMEs have been reported to cause pulmonary contraction and induce lung injury[229,230] and thus might be implicated in the observed pathology. Leukotrienes, on the other hand, do not participate in hypoxic pulmonary vasoconstriction, and are reported to exert anti-proliferative rather than pro-proliferative actions.[231-233] Furthermore, the leukotriene diol is an important mediator of the adult respiratory distress syndrome, and sEH inhibition in this situation has been clearly linked with beneficial effects.[234,235]

In summary, the results of the present investigation support a role for the sEH and CYP derived EETs in the development of pulmonary hypertension and pulmonary vascular remodelling. It was possible to demonstrate that hypoxia decreases the expression of the sEH and that deletion of the sEH enhances chronic hypoxia-induced vascular remodelling. Moreover, we were unable to detect the sEH in samples from lungs obtained from patients with pulmonary hypertension. An aspect that is important to highlight is that, based on experimental data showing that sEH inhibitors attenuate hypertension in spontaneously hypertensive rats[101] and in angiotensin II-treated mice,[104] sEH inhibitors are currently being developed for the treatment of human hypertension and inflammation/atherosclerosis. Given the apparent involvement of CYP epoxygenases in pulmonary remodelling, in particular in response to hypoxia,[24] these compounds may even promote the development of pulmonary hypertension.

5. Summary

Hypoxic pulmonary vasoconstriction (HPV) redistributes pulmonary blood flow from areas of low oxygen partial pressure to areas of normal or relativity high oxygen availability, thus optimising the matching of perfusion to ventilation and preventing arterial hypoxemia. Generalised alveolar hypoxia results in a sustained increase in pulmonary artery pressure which in turn leads to structural changes in the walls of the pulmonary vasculature (pulmonary vascular remodelling). Recent findings have indicated a role for cytochrome P450 (CYP) epoxygenase-derived epoxyeicosatrienoic acids (EETs) in hypoxia-induced pulmonary vasoconstriction. Given that the intracellular concentration of EETs is determined by the soluble epoxide hydrolase (sEH), which metabolises EETs to their less active dihydroxyeicosatrienoic acids (DHETs), we assessed the influence of the sEH and EETs on pulmonary artery pressure, acute and chronic HPV, and pulmonary vascular remodelling in the mouse lung.

In isolated lungs from wild-type mice, acute HPV was significantly increased by sEH inhibition, an effect abolished by pre-treatment with CYP epoxygenase inhibitors and the EET antagonist 14,15-EEZE. The acute hypoxia-induced vasoconstriction and EET production were greater in lungs from $sEH^{-/-}$ mice than from wild-type mice and sEH inhibition had no further effect on HPV in lungs from the former animals, while MSPPOH (CYP epoxygenase inhibitor) and 14,15-EEZE decreased the response. Exogenous application of 11,12-EET increased pulmonary artery pressure in a concentration-dependent manner and enhanced acute HPV in wild-type lungs, while 14,15-EET and 11,12-DHET were without significant effect on pulmonary artery pressure. $5\text{-}HT_{2A}$ receptor antagonism or Rho kinase inhibition shifted the EET concentration-response curve to the right and abrogated the EET- and sEH inhibition-induced potentiation of acute hypoxic vasoconstriction. In lungs from wild-type and $sEH^{-/-}$ mice, hypoxic preconditioning (hypoxic ventilation for 10 minutes) enhanced the 5-HT response. 1-Adamantyl-3-cyclohexylurea (ACU), a sEH inhibitor, further amplified the hypoxia-induced 5-HT-hypersensitivity in wild-type mice. However, after hypoxic preconditioning, the $sEH^{-/-}$ lungs displayed a striking leftward shift in the 5-HT response. 11,12-EET can activate TRPC6 channels in endothelial

cells by eliciting its translocation to the plasma membrane, more specifically to membrane domains enriched with the caveolae marker caveolin-1. This effect was also observed in rat pulmonary artery smooth muscle cells overexpressing the channel. Exposure of the latter cells to acute hypoxia also stimulated the intracellular translocation of TRPC6 to caveolae, an effect that was sensitive to the EET antagonist. The EET-induced translocation of TRPC6 channels was prevented by a 5-HT_{2A} receptor antagonist but not by a Rho kinase inhibitor. Moreover, while acute hypoxia and 11,12-EET increased pulmonary pressure in lungs from $TRPC6^{+/-}$ mice, lungs from $TRPC6^{-/-}$ mice did not respond to either stimuli. These results indicate that the sEH and CYP-derived EETs are involved in acute HPV and that EET-induced pulmonary contraction under normoxic and hypoxic conditions involves a TRPC6 channel, a 5-HT_{2A} receptor-dependent pathway and Rho kinase activation.

In the second part of the study the role of the sEH in the development of pulmonary hypertension and vascular remodelling induced in mice by exposure to hypoxia (10% O_2) for 21 days was analysed. In wild-type mice, chronic hypoxia decreased the pulmonary expression/activity of the sEH, induced right heart hypertrophy and erythropoiesis, and increased the number of partially and fully muscularised pulmonary resistance arteries (by 3-fold). Moreover, in HEK 293 cells, hypoxia (1% O_2 up to 24 h) decreased sEH promoter activity by 50%. In isolated lungs, pre-exposure to chronic hypoxia significantly increased baseline perfusion pressures and potentiated the acute HPV. While an sEH inhibitor, ACU, potentiated acute HPV in lungs from mice maintained in normoxic conditions, it had no effect on HPV in lungs from mice exposed to hypoxia. The EET antagonist, 14,15-EEZE, abolished the sEH inhibitor-dependent increase in acute HPV in normoxic lungs and decreased HPV in chronic hypoxic lungs. Hypoxia-induced right heart hypertrophy and erythropoiesis were more pronounced in $sEH^{-/-}$ than in wild-type mice. Under normoxic and hypoxic conditions the muscularisation of resistance pulmonary arteries was greater in lungs from $sEH^{-/-}$ mice than in lungs from wild-type mice. $sEH^{-/-}$ mice also displayed an enhanced acute HPV, compared to that observed in wild-type mice and chronic exposure to hypoxia did not further potentiate acute HPV. However, in the presence of 14,15-EEZE responses returned to levels observed in normoxic lungs from wild-type animals. Furthermore, immunohistochemistry demonstrated an extensive

expression of the sEH in the medial wall of pulmonary arteries from human donor lungs. Whereas sEH expression was not detectable in samples from pulmonary hypertension patients, indicating that the sEH is involved in hypoxia-induced pulmonary vascular remodelling and hypoxic pulmonary vasoconstriction.

Taken together, the results presented in this thesis indicate that the expression/activity of the sEH is an important determinant of the magnitude of acute and chronic hypoxia-induced pulmonary vasoconstriction and pulmonary vascular remodelling by inactivating vasoconstrictor CYP-derived EETs. As sEH inhibitors are currently being developed for the treatment of human systemic hypertension, it should be noted that these compounds may even promote the development of pulmonary hypertension.

6. Zusammenfassung

Die akute hypoxische pulmonale Vasokonstriktion (HPV) ist ein physiologischer Mechanismus, der es erlaubt, den lokalen Blutfluss der Lunge an alveolaren O_2-Mangel anzupassen. Durch die Hypoxie-bedingte Widerstandserhöhung besteht die Möglichkeit, die Durchblutung schlecht ventilierter Lungenbezirke einzuschränken und den Blutstrom in gut ventilierte Gebiete zu leiten. Die HPV wurde erstmals im Jahr 1946 durch von Euler und Liljestrand beschrieben und ist bis heute Gegenstand intensiver Forschung. Dennoch sind die Signaltransduktionswege und die Sauerstoff-Sensoren, die in die HPV involviert sind, weitgehend unbekannt. Als Sensoren werden unter anderem die Mitochondrien und die NADPH-Oxidasen diskutiert. Allerdings konnten neuere Studien zeigen, dass die glatten Muskelzellen der pulmonalen Widerstandsgefäße Effektorzellen darstellen. Des Weiteren konnte nachgewiesen werden, dass Mediatoren - wie 5-Hydroxytryptamin (5-HT) und Endothelin-1 - die akute HPV modulieren und ein nicht selektiver Kationen-Kanal (TRPC6-Kanal) sowie die Aktivierung der Rho-Kinase wesentlich an der akuten HPV beteiligt sind.

Bei chronischer Hypoxie, wie sie z. B. bei dauerhaftem Aufenthalt in großen Höhen entsteht, kommt es zu einer globalen Vasokonstriktion der Lunge. Die Folge ist ein pulmonaler Bluthochdruck mit einer Hypertrophie der glatten Muskulatur der Pulmonalgefäße („pulmonales vaskuläres *Remodelling*“). Hinzu kommt, dass eine dauerhafte Hypoxie zur Produktion von endothelialen Substanzen führt, die sowohl die Kontraktilität als auch die Proliferation der glatten Muskelzellen beeinflussen. Es konnte in mehreren Studien nachgewiesen werden, dass eine chronische Hypoxie zu einer verminderten Bildung von Stickstoffmonoxid und Prostazyklinen sowie zu einer vermehrten Produktion von 5-HT, Endothelin-1 und verschiedenen Wachstumsfaktoren führt. Über eine Beteiligung des 5-HT-Transporters, der TRPC-Kanäle und der Rho-Kinase in der chronischen Hypoxie-bedingten pulmonalen Hypertension wird ebenfalls diskutiert.

Enzyme der Cytochrom P450 (CYP)-Epoxygenase-Familie werden extrahepatisch vor allem im Herz, dem Gefäßsystem, dem Gastrointestinaltrakt, der Niere und der

Lunge exprimiert. Diese liefern durch Metabolisierung von Arachidonsäure vier verschiedene Isomere der Epoxyeicosatriensäuren (5,6-, 8,9-, 11,12-, und 14,15-EETs), die sowohl den Gefäßtonus modulieren als auch antiinflammatorische und angiogenetische Eigenschaften haben. Nach ihrer Synthese stehen den EETs zwei Wege offen: Zum einen können sie in Phospholipide inkorporiert, zum anderen durch die Cyclooxygenase (5,6-EET) und die lösliche Epoxidhydrolase (sEH; 8,9-, 11,12- und 14,15-EET) zu ihren weniger aktiven Dihydroxyeicosatriensäuren (DHETs) metabolisiert werden. In Koronar- und Nierenarterien unterschiedlicher Spezies stellen EETs den sogenannten endothelialen hyperpolarisierenden Faktor dar, der zur Hyperpolarisation und Relaxation der glatten Gefäßmuskulatur führt. Kürzlich konnte unsere Arbeitsgruppe nachweisen, dass 11,12-EET die Agonisten-induzierte endotheliale Hyperpolarisation durch Translokation des TRRPC6-Kanals in mit Caveolin-1 angereicherte Membrangebiete, erhöht. Des Weiteren legen verschiedene Untersuchungen eine Verbindung zwischen der sEH und kardiovaskulären Erkrankungen nahe, da eine Hemmung dieses Enzyms der Entstehung einer Hypertonie entgegenwirkt.

Dagegen ist die Rolle von CYP-Epoxygenase-generierten EETs und der sEH in der pulmonalen Zirkulation weitgehend unbekannt. Hierzu wurden in den letzten Jahren widersprüchliche Ergebnisse publiziert. Jedoch konnte unsere Arbeitsgruppe vor kurzem nachweisen, dass eine CYP-Epoxygenase in die HPV und das chronische Hypoxie-induzierte pulmonale vaskuläre *Remodelling* involviert ist. Ziel der vorliegenden Arbeit war es daher, ausführlich die Rolle der sEH und der CYP-Epoxygenase-generierten EETs in der akuten und chronischen HPV sowie im Hypoxie-bedingten pulmonalen vaskulären *Remodelling* zu analysieren. Dazu wurden CYP-Epoxygenase-Inhibitoren, ein EET-Antagonist und sEH-Inhibitoren verwendet. Um den molekularen Mechanismus der Hypoxie- und EET-induzierten pulmonalen Vasokonstriktion und des pulmonalen vaskulären *Remodelling* zu untersuchen, wurden kultivierte pulmonale glatte Muskelzellen, Promotor-Aktivitäts-Assays und genetisch veränderte Tiere ($sEH^{-/-}$ und $TRPC6^{-/-}$ Mäusen) verwendet.

Zunächst wurde die Rolle der sEH und EETs in der akuten HPV anhand der isolierten perfundierten Mauslunge untersucht. In Lungen von Wildtyp-Mäusen führte eine Hemmung der sEH zu einer deutlichen Steigerung der Amplitude der akuten

HPV. Darüber hinaus konnte nachgewiesen werden, dass eine vorherige Inkubation mit CYP-Epoxygenase-Inhibitoren oder dem EET-Antagonisten 14,15-EEZE den Effekt der sEH-Inhibitoren auf die akute HPV signifikant reduzierte. Im Vergleich zu Lungen von Wildtyp-Mäusen zeigten $sEH^{-/-}$-Lungen eine deutlich erhöhte akute Hypoxie-bedingte Vasokonstriktion. Erwartungsgemäß hatte eine Hemmung der sEH in Lungen von $sEH^{-/-}$-Mäusen keine Wirkung, während MSPPOH, ein CYP-Epoxygenase-Inhibitor, und 14,15-EEZE die gesteigerte HPV signifikant reduzierten. Weiterhin konnte in der vorliegenden Arbeit bewiesen werden, dass eine hypoxische Inkubation (1% O_2, 10 Minuten) von Maus-Lungenmikrosomen die EET-Produktion erheblich steigerte. Dieser Effekt war deutlich potenziert in Lungenmikrosomen von $sEH^{-/-}$-Mäusen. Eine exogene Applikation von 11,12-EET in der isolierten perfundierten Mauslunge steigerte signifikant den basalen pulmonalarteriellen Druck und die akute HPV, während 14,15-EET und 11,12-DHET keine Effekte auf den pulmonalarteriellen Druck ausübten. Eine Inhibition des $5\text{-}HT_{2A}$-Rezeptors oder der Rho-Kinase führte sowohl zu einer deutlichen Reduktion der 11,12-EET-induzierten Vasokonstriktion, als auch zu einer signifikanten Hemmung der durch die EET und sEH-Inhibition gesteigerten akuten HPV. In isolierten Lungen von Wildtyp- und $sEH^{-/-}$-Mäusen führte eine hypoxische Präkonditionierung (hypoxische Ventilation der isolierten Lunge für 10 Minuten) zu einer Verstärkung der 5-HT-induzierten Vasokonstriktion. Der Verlust der sEH-Aktivität, entweder durch Einsatz spezifische Hemmstoffe oder durch genetische Deletion, führte zu einer weiteren deutlichen Verstärkung der 5-HT Dosiswirkungskurve nach hypoxischer Vorbehandlung. In pulmonalarteriellen glatten Muskelzellen, die TRPC6 überexprimieren, konnte nachgewiesen werden, dass 11,12-EET diesen Kanal durch Translokation in mit Caveolin-1 angereicherte Membrangebiete, aktiviert. Darüber hinaus führte eine akute Hypoxie in TRPC6-überexprimierenden glatten Muskelzellen ebenfalls zu einer TRPC6-Kanal-Translokation. Dieser Effekt wurde durch den EET-Antagonisten deutlich gehemmt. Ferner konnte gezeigt werden, dass die EET-induzierte TRPC6-Translokation durch einen $5\text{-}HT_{2A}$-Rezeptor-Antagonisten reduziert wurde, wogegen ein Rho-Kinase-Inhibitor ohne Effekt war. Außerdem bewirkte eine akute Hypoxie und die Zugabe von 11,12-EET einen Anstieg des pulmonalarteriellen Drucks in isolierten perfundierten Lungen von $TRPC6^{+/-}$-Mäusen. $TRPC6^{-/-}$-Mäuse

reagierten auf keinen der beiden Stimuli. Diese Befunde zeigen, dass die Aktivität der sEH und CYP-Epoxygenase-generierte EETs die akute HPV modulieren und die EET-induzierte Kontraktion unter normoxischen und hypoxischen Bedingungen über einen TRPC6-Kanal-, einen 5-HT_{2A}-Rezeptor-abhängigen Signaltransduktionsweg und die Aktivierung der Rho-Kinase verläuft.

Ein weiteres Ziel der vorliegenden Arbeit war, die Rolle der sEH in der Entwicklung einer Hypoxie-bedingten pulmonalen Hypertension und des pulmonalen vaskulären *Remodelling* aufzuklären. Dafür wurden Mäuse 21 Tage in Hypoxie-Kammern hypoxischen Bedingungen (10% O_2) ausgesetzt oder unter normoxischen Bedingungen gehalten. Eine chronische Hypoxie führte in Wildtyp-Mäusen zu einer verminderten pulmonalen Expression und folglich auch Aktivität der sEH. Zusätzlich induzierte eine dauerhafte Hypoxie eine Rechtsherzhypertrophie und Hämatopoese in Wildtyp-Mäusen und erhöhte signifikant die Anzahl der teil- und vollmuskularisierten pulmonalen Widerstandsgefäße. In isolierten perfundierten Lungen aus chronisch hypoxischen Mäusen war der Basisdruck signifikant erhöht und die akute HPV deutlich potenziert. Während der sEH-Inhibitor 1-Adamantyl-3-Cyclohexylurea die akute HPV in Lungen von Tieren, die unter normoxischen Bedingungen gehalten wurden, steigerte, hatte er keinen Effekt auf die Antwort in Lungen von chronisch hypoxischen Mäusen. Der EET-Antagonist 14,15-EEZE hemmte signifikant den sEH-Inhibitor-induzierten Anstieg der akuten HPV in normoxischen Lungen und reduzierte die potenzierte akute HPV in Lungen von chronisch hypoxischen Tieren. $sEH^{-/-}$-Mäuse zeigten im Vergleich zu Wildtyp-Mäusen eine verstärkte Hypoxie-induzierte Rechtsherzhypertrophie und Hämatopoese. Darüber hinaus konnte in Lungen von hypoxischen und normoxischen $sEH^{-/-}$-Mäusen eine erhöhte Muskularisierung der pulmonalen Widerstandsgefäße nachgewiesen werden. Außerdem zeigten $sEH^{-/-}$-Lungen eine verstärkte akute HPV. Eine vorherige hypoxische Exposition hatte keinen potenzierenden Effekt auf die akute HPV. Allerdings reduzierte 14,15-EEZE die akute HPV in $sEH^{-/-}$-Lungen auf das Niveau der HPV von normoxischen Wildtyp-Lungen. Überdies führte Hypoxie (1% O_2, 24 Stunden) in humanen embryonalen Nierenzellen (HEK 293), die mit sEH Promotor-Konstrukten transfiziert wurden, zu einer verminderten sEH-Promotor-Aktivität. Über die Expression der sEH in Lungen von Patienten mit pulmonaler

Hypertonie ist bisher wenig bekannt. In pulmonalen Gewebeschnitten des lungengesunden Kontrollkollektivs konnte eine starke sEH-Expression in der Media der Pulmonalarterien nachgewiesen werden, während in Gewebeschnitten von Patienten mit pulmonaler Hypertonie keine sEH-Expression detektiert werden konnte. Zusammenfassend legen die Resultate des zweiten Teils dieser Arbeit nahe, dass die sEH in die Entstehung der Hypoxie-bedingten pulmonalen Hypertension und des daraus folgenden pulmonalen vaskulären *Remodellings* involviert ist.

Die Ergebnisse der vorliegenden Arbeit zeigen, dass die Aktivität/Expression der sEH die akute hypoxische Vasokonstriktion sowie die chronische HPV und die damit einhergehenden Veränderungen der pulmonalen Gefäße beeinflusst. Ihren Effekt vermittelt die sEH über den Abbau der vasokonstringierenden EETs - vor allem ist hier 11,12-EET relevant - zu weniger aktiven Diolen. Außerdem konnte hier nachgewiesen werden, dass an der EET-induzierten Vasokonstriktion unter normoxischen und unter akut hypoxischen Bedingungen der TRPC6-Kanal, der 5-HT_{2A}-Rezeptor und die Rho-Kinase an der Signaltransduktion beteiligt sind. Wie chronische Hypoxie die Aktivität/Expression der sEH und folglich auch die Entwicklung einer pulmonalen Hypertension und des pulmonalen vaskulären *Remodelling* beeinflusst, bleibt offen. Gleiches gilt für eine Involvierung des für die akute HPV beschriebenen Signaltransduktionsweges (5-HT_{2A}-Rezeptor, TRPC6-Kanal) in diese Prozesse. In verschiedenen Studien wurde nachgewiesen, dass TRPC6-Kanäle in pulmonaler Hypertension verstärkt exprimiert werden. TRPC6$^{-/-}$-Mäuse zeigten im Vergleich zu Wildtyp-Mäusen jedoch keine Unterschiede in der Entwicklung einer chronisch Hypoxie-induzierten pulmonalen Hypertension. sEH-Inhibitoren werden momentan für den Einsatz in der Therapie der Hypertonie entwickelt. Angesichts der Tatsache, dass eine CYP-Epoxygenase, EETs und die Aktivität/Expression der sEH in die HPV und in das chronische Hypoxie-induzierte pulmonale vaskuläre *Remodelling* involviert sind, besteht die Möglichkeit, dass diese Substanzen die Entwicklung einer pulmonalen Hypertension fördern.

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Abbreviations

14,15-EEZE	14,15-epoxyeicosa-5(Z)-enoic acid
5-HT	5-hydroxytryptamine
5-HTT	5-HT transporter
ACU	1-adamantyl-3-cyclohexylurea
AEPU	1-adamantan-1-yl-3-{5-[2-(2-ethoxyethoxy)ethoxy]pentyl]}urea
AMP	adenosine monophosphate
ATP	adenosine triphosphate
BMPR-II	bone morphogenetic protein receptor type II
Ca^{2+}	calcium
cADP	cyclic adenosine diphosphate
cAMP	cyclic adenosine monophosphate
$cPLA_2$	cytosolic phospholipase A_2
CYP	cytochrome P450
DAG	diacylglycerol
DHET	dihydroxyeicosatrienoic acids
DMSO	dimethyl sulfoxide
DTT	dithiothreitol
EDHF	endothelium-derived hyperpolarizing factor
EDTA	ethylenediaminetetraacetic acid
EET	epoxyeicosatrienoic acid
Em	cellular membrane potential
EpO	epoxystearic acid
EpOME	epoxyoctadecenoic acids
ERK	extracellular signal regulated kinase
FAD	flavin adenine dinucleotide
FMN	flavin mononucleotide
G	G protein
GFP	green fluorescent protein
Grb	growth factor receptor-bound protein
GRF	guanine nucleotide releasing factor;
HETE	hydroxyeicosatetraenoic acid
HIF	hypoxia-inducible factor
HPV	hypoxic pulmonary vasoconstriction

IgG	immunoglobulin G
IP_3	inositol-1,4,5-trisphosphate;
IκB	inhibitor of κB
LC-MS/MS	liquid chromatography tandem mass spectrometry
LTA_4	leukotriene A_4
LV + S	left ventricle and septum
LVP	left ventricle pressure
MAPK	mitogen-activated protein kinase
mEH	microsomal epoxide hydrolase
MLC	myosin light chain
MLK	mixed lineage kinase
MSPPOH	methyl sulphonyl propargyloxyphenyl hexanamide
NADPH	nicotinamide adenine dinucleotide phosphate
NFκB	nuclear factor κB
NO	nitric oxide
NOX	NADPH oxidase
O_2^-	superoxide anion
P	phosphate
PAP	pulmonary artery pressure
PASMC	pulmonary artery smooth muscle cell
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDGF	platelet derived growth factor
PGI_2	prostacyclin
PI3K	phosphotidylinositol 3-kinase
PKA	protein kinase A
PKC	protein kinase C
PLC	phospholipase C
PMSF	phenylmethanesulphonylfluoride
pO_2	oxygen partial pressure
ROS	reactive oxygen species
RTK	receptor tyrosine kinase
RV	right ventricle
SDS	sodiumdodecylsulfatepolyacrylamide
sEH	soluble epoxide hydrolase
sGC	soluble guanylate cyclase

SR	sarcoplasmic reticulum
STIM	stromal interacting molecule
TBST	TRIS buffered saline with Tween
TGF-β	transforming growth factor-β
TNF-α	tumor necrosis factor-α
TP	tracheal pressure
TRIS	trishydroxymethylaminomethane
TRP	transient receptor potential

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