Role of endothelial Cytochrome P450 epoxygenases in the regulation of angiogenesis

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To Paul & my parents

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1. Introduction

1.1 Cytochrome P450 enzymes and CYP-derived metabolites of arachidonic acid

Cytochrome P450 (CYP) enzymes are membrane-bound heme enzymes named for the absorption band at 450 nm of their carbon monoxide (CO)-band or complexed form. They are involved in a number of vital processes including carcinogenesis and drug metabolism as well as the biosynthesis of steroids or lipids.

The most common reaction catalysed by CYP enzymes is a monooxygenase reaction, e.g. insertion of one atom of oxygen into a substrate while the other oxygen atom is reduced to water (Figure 1). The heme-containing enzymes are part of a multi-enzyme complex that also consists of cytochrom b5 and a NADPH cytochrome reductase and have a variety of functions. Some CYPs are substrate specific, but most can metabolize multiple substrates, and many can catalyze multiple reactions, which accounts for their central importance in metabolizing an extremely large number of endogenous and exogenous molecules. Even though most of the CYP enzymes are expressed in the liver where their substrates include drugs and toxic compounds as well as metabolic products such as bilirubin, they are also present in many other tissues of the body including the mucosa of the gastrointestinal tract, and play important roles in hormone synthesis and breakdown (including estrogen and testosterone synthesis and metabolism), cholesterol synthesis, and vitamin D metabolism.

CYP enzymes have been described in a number of different contexts since their discovery at the beginning of the 1960's and were subdivided into families and subfamilies according to their homology. At 55% homology enzymes are classified as the same subfamily that is indicated by a letter. Starting at a homology of 40% enzymes are classified as a family that is indicated by an Arabic numeral.

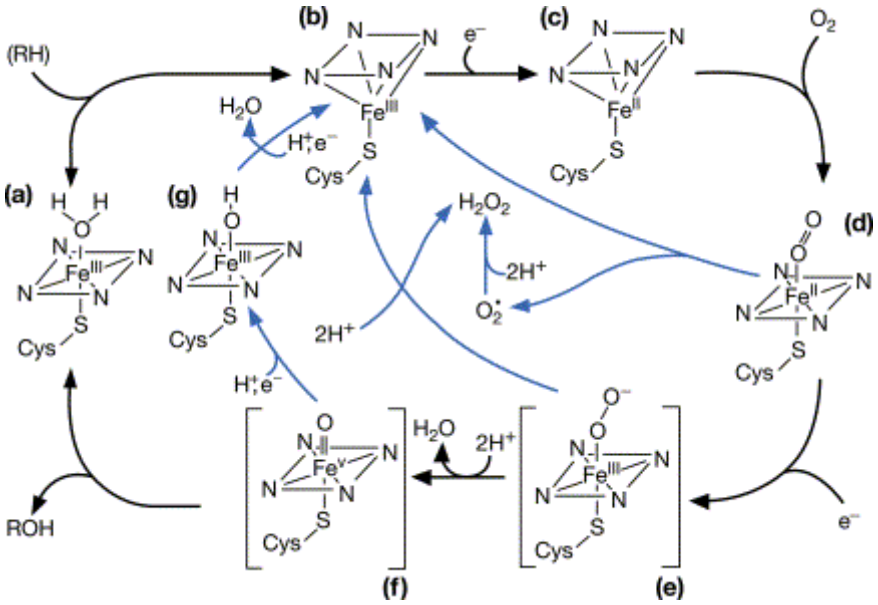

Figure 1. The catalytic cycle of CYP monoxygenases. At the start of the reaction cycle the substrate binds to the active centre close to the ferric ion of the central heme group (A). The ferric ion is reduced to the ferrous ion via electron transfer by the CYP NADPH reductase (B) in order to be in a state for molecular oxygen to be attached (C). After this oxidation of the substrate the dioxygen bond is destabilised by attachment of a second reductase-derived electron (D) and oxygen is separated in form of a water molecule (E). After water formation a number of instable intermediate products are formed (F-G) resulting in the separation of the oxidised substrate. From Zangar et al., 2004.

CYP-derived epoxides of arachidonic acid, such as 5,6-, 8,9, and 11,12-epoxyeicosatrienoic acid (EET) that are responsible for the cyclooxygenase-independent renal vasodilatation in rats (Pomposiello et al., 2003), play an important role in the regulation of vascular tone and homeostasis (for review see Fleming, 2001)) and have originally been linked to vascular smooth muscle cell hyperpolarisation and relaxation (Rosolowsky and Campbell, 1993; Campbell et al., 1996). These ecosanoids are also important intracellular signalling molecules that modulate much more than membrane potential. Multiple CYP enzymes metabolize arachidonic acid to EETs in a number of species and tissues (Table 1).

Isoform	Species	Product(s)	Tissues	Inducer
2C11	Rat	EETs	K, L, B	
2C12	Rat	EETs?	LG, K, B, L	
2C23	Rat	EETs	K	High-salt diet
2C24	Rat	EETs	K	
2B1	Rat	EETs	LG, L	Barbiturates
2B2	Rat	EETs	L	Barbiturates
2D18	Rat	EETs	B	
2J3	Rat	EETs, 19-HETE	H, L, K, P, LG	
2J4	Rat	EETs	K, L, H, LG, GI	
2J10	Rat	EETs	K	
1A2	Rat	EETs	L	Hydrocarbons
2B6	Human	EETs	L	
2C8	Human	EETs	L, LG, V	
2C9	Human	EETs	L, LG, V	
2C19	Human	EETs	L	
2J2	Human	EETs	H, K, GI, P	
1A2	Human	EETs	L, LG	Hydrocarbons
2B19	Mouse	EETs, 11-, 12-, 15-HETE	S	
2C29	Mouse	EETs	L, K, B	
2C38	Mouse	EETs	L, K, B	
2C39	Mouse	EETs	L, K, B	
2C40	Mouse	EETs	L, GI	
2J5	Mouse	EETs	K, L	
2J6	Mouse	EETs	H	
2C1	Rabbit	11,12-, 14,15-EET	S	
2C2	Rabbit	11,12-, 14,15-EET	L	
2C4	Rabbit	EETs, 19-HETE	L, K	
2CAA	Rabbit	EETs	L	
2B4	Rabbit	11,12-, 14,15-EET	L	Barbiturates
2B5	Rabbit	EETs	LG, L	

K, kidney; L, liver; V, vasculature; B, brain; LG, lung; P, pancreas; GI, gastrointestinal tract; S, skin.

Table 1. Formation of EETs from arachidonic acid by different CYP isoforms. From Roman, 2002

Arachidonic acid is a polyunsaturated fatty acid that is present in the phospholipids of membranes of cells and is freed from this phospholipid molecule via cleavage by the enzyme phospholipase A_2. Arachidonic acid can be metabolised via three main pathways, namely via cyclooxygenases (COX), lipoxygenases (LOX) and CYP epoxygenases (Figure 2) to generate biologically active fatty acid metabolites (eicosanoids).

In addition to its role in the metabolism of xenobiotics, the arachidonic acid pathway was of interest for vascular biologists because of its effect on vascular function (Aiken, 1974); initially mainly on renal physiology (McGiff et al., 1970). Furthermore, COX-2 was shown to reduce angiogenesis and specific inhibitors, e.g. celecoxib, are effective in cancer treatments (Kawamori et al., 1998). Likewise, LOX levels are upregulated in certain cancers such as prostate carcinoma (Gao et al., 1995). More recently CYP

enzymes have also been reported to play a role in the pathogenesis of a variety of human cancers by for example promoting the neoplastic cellular phenotype (Jiang et al., 2005).

CYP epoxygenases produce different regio- and stereoisomeric epoxides (5,6-; 8,9-; 11,12- and 14,15 epoxyeicosatrienoic acid, EET), whereas the ratio of EET-isomers produced is dependent on the specific CYP isomer studied. For example, in the endothelium CYP2C9 generates 14,15-EET, 11,12-EET and 8,9-EET at a ratio of 2,3:100:0,5. Despite its 80% homology the CYP2C8 isomer generates substantial amounts of 11,12-EET and 14,15-EET, but hardly any 8,9-EET (Daikh et al., 1994). In contrast to the epoxygenases, the ω-hydroxylases, metabolise arachidonic acid to hydroxyeicosatrienoic acids (HETEs) (Guengerich et al., 1995). Furthermore, there are some enzymes e.g. CYP 4A2 and 4A3, but also CYP2C9 that generate 11,12-EETs as well as 20-HETE (Nguyen et al., 1999). Of the CYP isoforms expressed in human ω-hydroxylases of the 4A family are primarily found in smooth muscle cells (Roman, 2002), whereas CYP2C8, CYP2C9 and CYP2J2 are mainly expressed in the endothelium (Fisslthaler et al., 1999). While CYP2C9 expression appears to be predominant in the endothelium of aorta and coronary arteries (Delozier et al., 2007), CYP2C8 and CYP2J2 are mainly found in the human heart.

Once synthesised EETs can be incorporated into phospholipids, especially into phosphatidylcholine and phosphotidylinositol (Capdevila et al., 1981; VanRollins et al., 1993). The physiological significance of this process is not yet understood, but some observations hint at the possibility that these lipids may be intracellular EET stores that can release the metabolites independently if required (Weintraub et al., 1997). On the other hand, EETs are mainly metabolised by the soluble epoxide hydrolase (sEH) as well as a microsomal form of the enzyme (mEH) to generate the biologically less active dihydroxyeicosatrienoic acids (DHETs). Only 5,6-EETs are chemically less stable and preferentially metabolised by COX (Oliw et al., 1981; Chacos et al., 1983).

Originally DHETs were assumed to be simply biologically inactive metabolites. However, recently several groups have demonstrated that they have vasorelaxating properties in porcine coronary arteries and other species (Oltman et al., 1998), and are also able to selectively activate the peroxisome-proliferator activated receptor PPARα (Fang et al., 2006).

While the sEH is the biologically most important EET regulating enzyme, EETs can be transformed either to shorter (via β-oxidation) or longer (via C2-attachment) derivatives.

However, these alternative metabolic routes do not seem to be be able to completely compensate for the loss of sEH activity as EET levels are chronically increased in sEH-deficient (sEH-/-) animals (Sinal et al., 2000).

20-HETE as well as the EETs play an important role in the regulation of vascular tone. 20-HETE is a potent vasoconstrictor produced in vascular smooth muscle cells that depolarizes vascular smooth muscle cells by attenuating the open probability of Ca^{2+}-activated K^{+}-channels (Miyata and Roman, 2005). 20-HETE also plays a role in angiogenesis, and is reported to be a component of the FGF-2-activated signalling pathway. It has for example been shown that FGF-2 is able to activate phospholipase A_2 resulting in arachidonic acid production and subsequent 20-HETE release by CYP4A (Sa et al., 1995). In vivo, in the corneal neovascularisation model, CYP4A inhibitors have been shown to abrogate the angiogenic response to VEGF, FGF-2 and EGF (Chen et al., 2005).

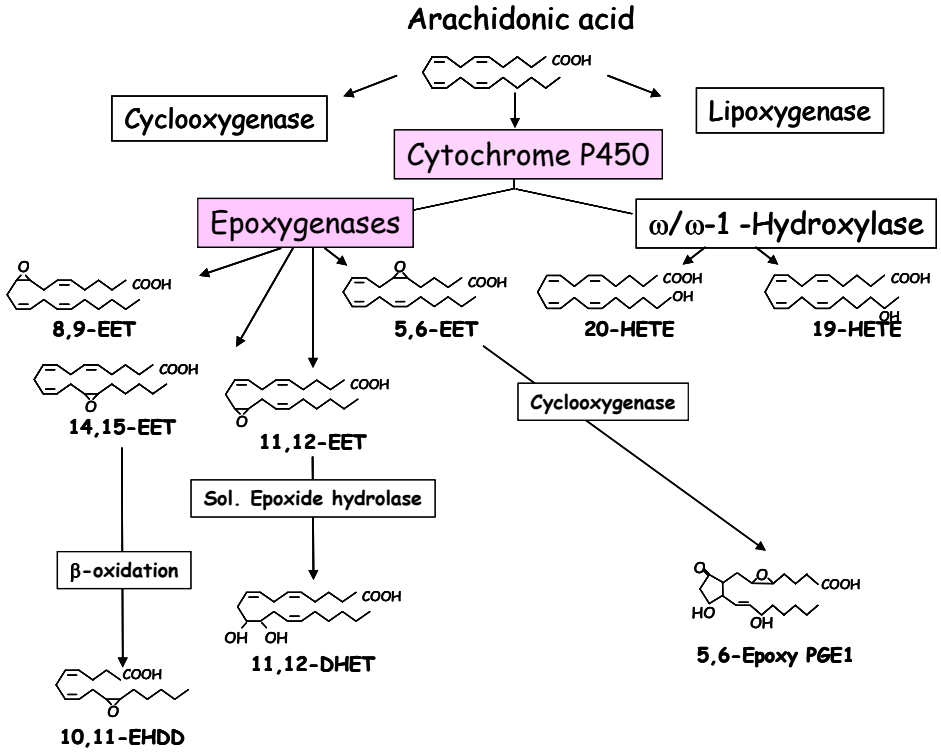

Figure 2. The arachidonic acid metabolism. Arachidonic acid metabolism by CYP epoxygenases results in the formation of different regioisomers of epoxyeicosatrienoic acids and hydroxyeicosatetranoic acids. Arachidonic aid is metabolised by three main pathways: via COX, LOX and CYP ω/ω-hydroxylases and epoxygenases. CYP epoxygenases generate different regioisomers of epoxyeicosatrienoic acid (5,6-, 8,9-, 11,12-, 14,15-EETs) that can be further metabolised by cyclo-oxygenases (5,6-EET), via β-oxidation (8,9-, and 14,15-EETs) or by soluble epoxide hydrolase (11,12-EET). From Fleming, 2001.

1.2 Epoxyeicosatrienoic acids

Interest in EETs and their vascular actions was originally linked to their identification as an endothelium derived hyperpolarizing factor (EDHF). Indeed, EETs stimulate the opening of calcium-activated potassium channels (K^+_{Ca}) (Hu and Kim, 1993; Campbell et al., 1996) and the hyperpolarisation of endothelial cells in addition to activating the Na^+-K^+-ATPase. This results in an endothelium-dependent hyperpolarisation of smooth muscle cells, which may also involve direct electrical coupling through myo-endothelial junctions especially in small arteries and/or the accumulation of K^+ ions in the sub-endothelial space (Edwards et al., 1998; Busse et al., 2002). Exogenously applied EETs activate iberiotoxin-sensitive, large-conductance calcium-sensitive K^+ channels (BK_{Ca}). However, the EDHF-mediated relaxation of porcine coronary arteries that is sensitive to the CYP2C9 inhibitor sulfaphenazole and that can be attenuated by antisense oligonucleotides directed against CYP2C, is insensitive to iberiotoxin (a selective inhibitor of BK_{Ca}-channels), but sensitive to charybdotoxin (a nonselective inhibitor of BK_{Ca} and IK_{Ca} channels) and apamin (a nonspecific inhibitor of SK_{Ca}) (Fisslthaler et al., 1999). This suggests on the one hand that the role of EETs in the EDHF phenomenon may not simply be related to the activation of BK_{Ca} channels. On the other hand the EET antagonist 14,15-epoxyeicosa-5(Z)-enoic acid (14,15-EEZE) attenuates the bradykinin-induced hyperpolarisation of native porcine coronary artery endothelial cells and this has been associated with the activation of endothelial intermediate- and small-conductance Ca^{2+}-sensitive K^+ channels (SK_{Ca} and IK_{Ca}) channels (Weston et al., 2005).

Physiologically relevant concentrations of nitric oxide (NO) attenuate EDHF-mediated dilatations via a decreased formation of this hyperpolarizing factor (Bauersachs et al., 1996). It is however known that NO inhibits CYP and this would not only explain the reduced EDHF answer in the presence of NO, but also the observation that in vessels that generate NO the EET-mediated hyperpolarisation is only of minor importance under physiological conditions. In contrast under pathophysiological conditions, such as endothelial dysfunction where the bioavailability of NO is reduced, EDHF/EET may represent important endogenous signalling mechanisms to compensate for loss of NO.

Keeping in mind the effects of EDHF/EETs on vascular tone, the pharmacological inhibition of the sEH is a potential approach to enhance EET-mediated vascular

protection and may be useful as a treatment for coronary artery disease and hypertension.

EETs elicit a number of other effects that can not be attributed to their role as an EDHF and it is now generally accepted that arachidonic acid epoxides are much more than vasodilators/vasoconstrictors. In fact, they induce a large number of effects other than those associated with K^+_{Ca} channels suggesting that the intracellular second messenger role of EETs may be their most important function. However, the signalling pathways that are involved seem to be largely dependent on species, the type of endothelium and the regioisomers that are involved. Furthermore, it has been shown that certain structural requirements, such as for example the location of the double bond, are essential for the mechanism of action of the molecule (Falck et al., 2003). In human umbilical vein endothelial cells (HUVECs) in particular 11,12- and 14,15-EETs activate several signalling molecules including tyrosine kinases and phosphatases (Fleming et al., 2001a), the p38 mitogen-activated protein (MAP) kinase, extracellular regulated protein kinase 1 and 2 (ERK1/2), MAP kinase phosphatases and inhibit other signalling molecules such as c-Jun N-terminal kinase. Furthermore EETs affect endothelial cell proliferation and angiogenesis, an effect at least partly attributable to transactivation of the epidermal growth factor (EGF) receptor (Chen et al., 1999; Michaelis et al., 2003). In murine pulmonary endothelial cells the responses reported are a bit more diverse, and while 5,6- and 11,12-EET stimulate proliferation through a phosphatidylinositol-3 kinase (PI3K)-pathway, the effects mediated by 8,9- and 11,12-EET are dependent on the p38 MAP kinase pathway (Pozzi et al., 2005).

1.3 Expression of CYP-derived epoxyeicosatrienoic acids *in vitro* and *in vivo*

As mentioned above the different CYP-derived EET-regioisomers have for a number of years been widely accepted to play a role in vascular smooth muscle cell hyperpolarisation and dilatation, but their role as second messengers that modulate a variety of processes other than membrane potential have been overlooked. Indeed their role in angiogenesis, which will be elucidated in more detail later on in the text and in the course of this study, has been largely overlooked. One of the main reasons for

this has certainly been a lack of appropriate experimental tools and strategies that take into account the rather labile nature of the enzymes in culture. Thus, although CYP epoxygenases can be detected without problems in native tissues, expression levels rapidly decrease following cell isolation. As a matter of fact, porcine coronary endothelial cells that clearly express CYP2C *in situ*, rapidly downregulate protein expression and 48 hours after cell isolation, the RNA of the enzyme is only detectable by RT-PCR (Fisslthaler et al., 2000a). Therefore, under static cell culture conditions, i.e. lacking the hemodynamic stimuli to which the native endothelium is continually exposed, transcriptional processes seem to play an important role in the regulation of CYP2C expression levels and may account for the labile nature of proteins. This also fits well with observations that physiological stimuli, such as cyclic stress (Fisslthaler et al., 2001) result in a significant increase in CYP2C protein expression levels as well as in endothelial EET production.

Consequently, in practice when investigating the consequences of CYP2C activation in cells cultured the enzyme needs to be upregulated. This can be achieved by the application of shear stress or cyclic stretch or by incubation with pharmacological stimuli, the so called 'CYP-inducers' such as nifedipine and cortisol (Fisslthaler et al., 2000b ; Bauersachs et al., 2002). Of course one further possibility is to overexpress the enzyme of interest using either a transfection or adenoviral approach.

1.4 Vasculogenesis and angiogenesis

The cardiovascular system is the first organ system to develop and reach a functional state in the vertebrate embryo. The initial steps consist of 'vasculogenesis', which basically means the in situ differentiation of endothelial cell precursors, the angioblasts, from the hemangioblasts to form a network of primitive tubules (Risau and Flamme, 1995). The juvenile vascular plexus develops from the primary capillary plexus by proliferation of endothelial cells, subsequent pruning and reorganization of endothelial cells in the process called 'sprouting angiogenesis' (Risau, 1997) to form more mature appearing vascular patterns seen in the adult organism (Figure 3). This sprouting of new vessel segments follows a well-defined program; degradation of basement membrane, endothelial cell proliferation, formation of sprouts of endothelial cells

connecting a neighbouring vessel and restructuring of the sprout into a lumen lined by endothelial cells and integrated in the vascular network.

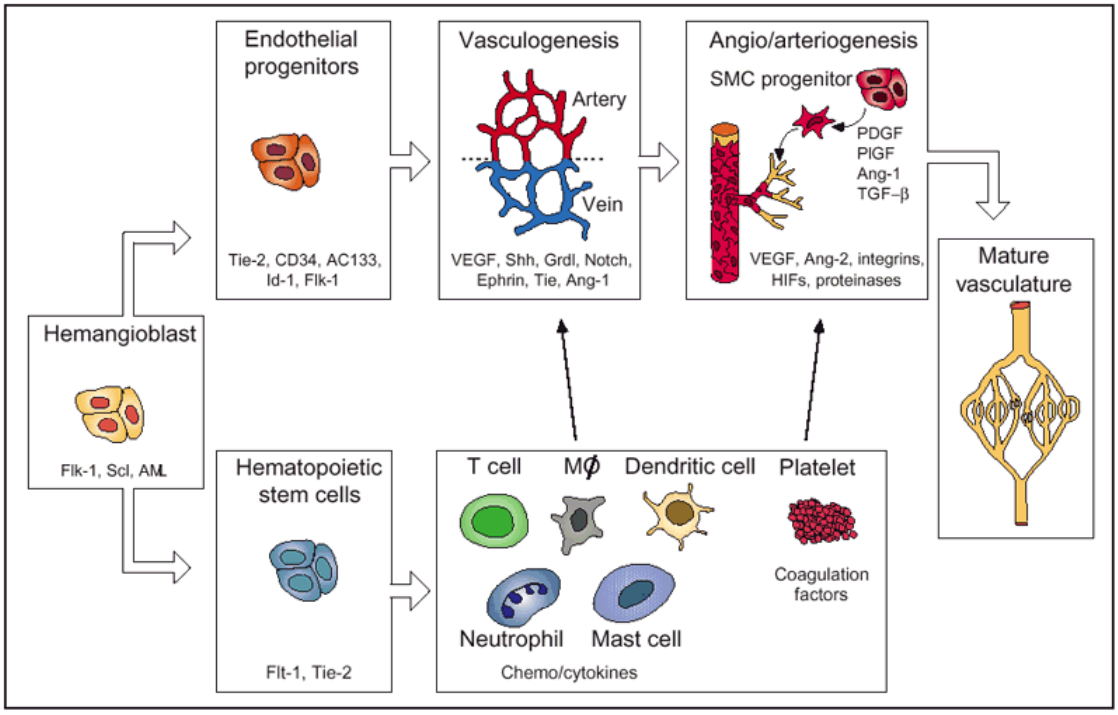

Figure 3. The formation of the vascular network. Hemangioblasts in the embryo differentiate into hematopietic stem cells and endothelial progenitors that then differentiate to arterial and venous endothelial cells and assemble in a primitive capillary plexus (vasculogenesis). Vessels then sprout and become stabilized by smooth muscle cells that differentiate from their progenitors. Furthermore hematopoietic stem cells contribute to angiogenesis directly and indirectly, by differentiating to leukocytes or platelets. Appreviations: Shh, Sonic hedgehog; Grdl, Gridlock; MΦ, macrophage; AML, acute myeloid leukemia; Scl, stem cell leukemia. From Carmeliet, 2003.

Alternatively during 'intussusceptive angiogenesis', also known as 'splitting angiogenesis', the key event is the formation of transluminal tissue pillars. The process begins with the protrusion of opposing capillary walls into the vessel lumen (Figure 4). After establishing an interendothelial contact, the endothelial bilayer and the basal membranes are perforated centrally and the newly formed pillar increases in girth after being invaded by fibroblasts and pericytes. The capillary wall expands to the lumen to split a single vessel in two (Kamei et al., 2006; Bianco et al., 2007).

Sprouting angiogenesis has great advantages, because it is invasive and can therefore bridge vascular gaps as for example required during wound healing. On the other hand intussuseption is much faster, appears to be metabolically and energetically more

effective and represents a unique way to expand and increase the complexity of the vascular tree (for review see Burri et al., 2004).

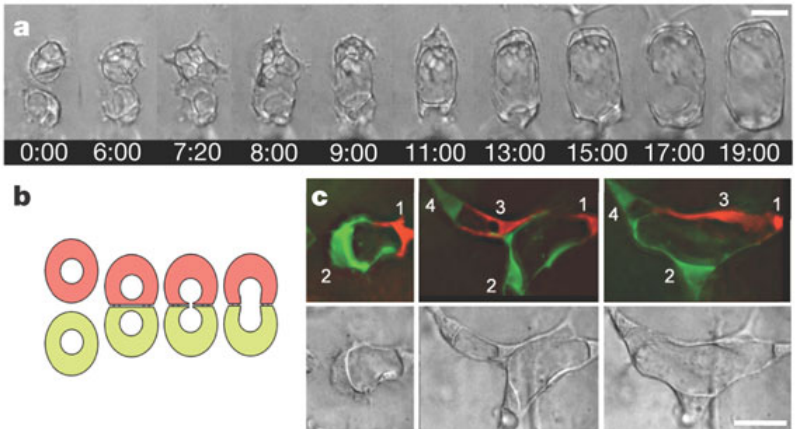

Figure 4. Endothelial tubes assemble in vivo. A and B, time-lapse images and schematic view of the fusion process of intracellular vacuoles of two endothelial cells to form a single luminal space. C, cytoplasm of cultured endothelial cells is labelled in green and red. Intercellular fusion process is not accompanied by cytoplasmatic mixing which indicates that the formation of a common luminal space occurs by exocytosis of intracellular vacuoles into junctional spaces between two neighbouring cells. From Kamei et al., 2006

Our view of processes that contribute to the formation of new vessels in adult animals and humans has also been modified by the realisation that the incorporation of bone-marrow-derived endothelial precursor cells contributes to vessel growth, a process that complements the sprouting of resident endothelial cells (Asahara et al., 1997).

In the adult, development of the vasculature is essential for tissue repair and reproductive functions such as control of ovulation and menstruation. On the other hand uncontroled angiogenesis or abnormal vascular remodelling contributes to the pathologenesis of disorders such as cancer, arthritis and psoriasis, but also obesity, asthma and infectious diseases. The formation of vessels is a complex process, requiring a finely tuned balance between numerous stimulatory and inhibitory signals, such as integrins, angiopoietins, chemokines, junctional molecules, oxygen sensors, endogenous inhibitors and many others (Carmeliet, 2003).

The latter phase of vascular development also involves the sprouting and penetration of vessels into previously avascular regions of the embryo, and also the differential

recruitment of associated supporting cells such as pericytes, as well as fibroblasts to different segments of the vasculature. These interactions between endothelial cells and mural cells (pericytes and vascular smooth muscle cells) in the blood vessel have recently attracted a great deal of attention as central processes in the regulation of vascular formation, stabilization, remodelling and function. Failure of these interactions between the two cell types results in severe and often lethal cardiovascular defects in numerous genetic mouse models. Therefore, establishment of a functional vascular network requires that nascent vessels mature into durable vessels. Insufficient recruitment of mural cells would result in exaggerated endothelial cell growth, permeability, fragility, subsequent vessel enlargement, bleeding and impaired perfusion. The absence of arteriogenic factors results in naked, leaky vessels that are not perfusable and therefore not subject to flow which ultimately results in vessel regression (Figure 5).

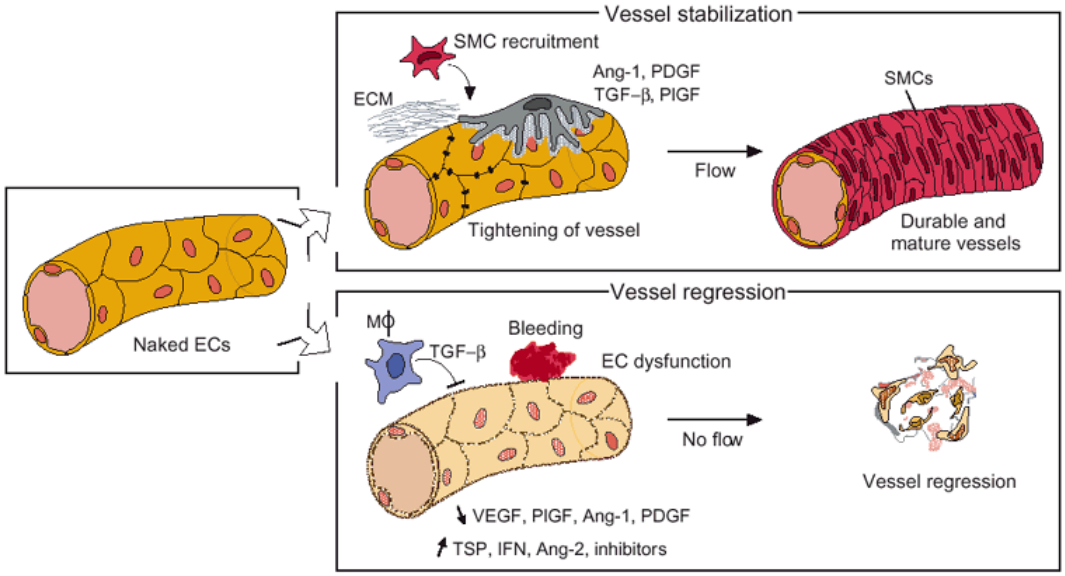

Figure 5. The initial phase of vascular development involves formation of endothelial cell tubes. Upper panel: vessel maturation and subsequent stabilisation requires a mix of angio- and arteriogenic factors for a sufficient duration. As result endothelial cells can tighten up and become firstly covered by smooth muscle cells (SMC) and extracellular matrix (ECM) and then maintained and further stabilised by flow. Lower panel: as a consequence of insufficient angio- and arteriogenic factors (e.g. VEGF, PIGF, And-1, PDGF) and abudent inhibitors (i.e. TSP, IFN, Ang-2) Endothelial tubes remain naked and leaky. Those fragile structures are easily ruptured and bleed which leads to reduced flow and results in vessel regression. From Carmeliet, 2003.

1.5 Angiogenesis in health and disease

Excessive, insufficient or abnormal angiogenesis directly contribute to numerous malignant, ischemic, inflammatory, infectious and immune disorders. Mammalian cells are always located within 100 to 200 µm of blood vessels and multicellular structures such as for example tumours must recruit new blood vessels to survive. The proximity is also crucial for tumour survival and growth as without the supply of sufficient nutrients and oxygen via the blood stream tumours are not able to grow beyond a certain point or to form metastases. This implies that in various diseases, especially cancer, the tumour strives to optimise its blood supply and the balance of pro-and antiangiogenic molecules is derailed. The observation that angiogenesis increasingly occurs around tumours was made over 100 years ago by Goldman, but it took until 1971 for Folkman to propose that tumour growth and metastasis are angiogenesis-dependent and that blocking vessel growth is a possible strategy to arrest tumour growth. Leading on from those findings, Gullino showed that cells in pre-cancerous tissue acquire angiogenic capacity on their way to become cancerous (Gullino, 1978) and that this might be the basis for a successful cancer strategy. Since then it has become widely accepted that the 'angiogenic switch' is 'off' when the pro-angiogenic molecules are balanced by the anti-angiogenic molecules and a number of signals have been identified that trigger this switch. It is well established that there are a number of molecular players involved in these mechanisms of vascular growth (Carmeliet, 2000) such as VEGF (Figure 6).

Normal tissue function is also dependent on an adequate supply of oxygen through the blood vessels and unravelling the mechanisms that may be involved in disturbing or facilitating this maintenance of oxygen supply would offer therapeutic options to ameliorate or even cure a number of disorders such as cancer that are now leading causes of mortality. Some of the main factors involved in this non-cancerous angiogenesis are certainly hypoxia and inflammation. Hypoxia, via activation of hypoxia-inducible transcription factors (HIF), induces the expression of a number of pro-angiogenic factors/stimuli such as VEGF, NO, PDGF and EphB4 (Vihanto et al., 2005).

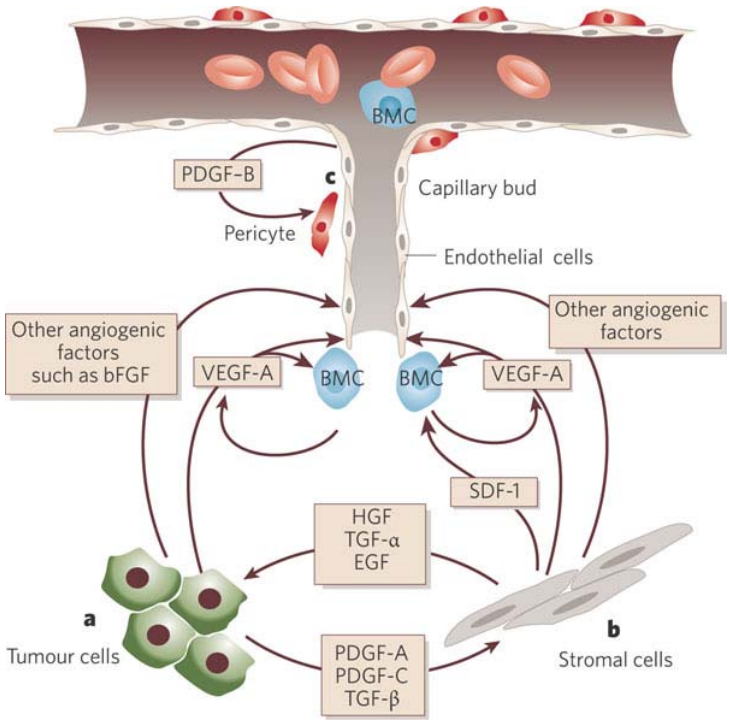

Figure 6. Key players in the microvascular (tumour) environment. A, Tumour cells produce proangiogenic factors such as VEGF, bFGF, angiopoietins, interleukin-8 and PlGF that result in endothelial cell migration and proliferation. B, Furthermore stromal cells such as fibroblastic, inflammatory and immune cells release angiogenic factor (e.g. chemokines such as SDF-1 and VEGF). These factors as well as VEGF-A and PlGF may also recruit bone-marrow-derived angiogenic cells (BMC) and produce growth and survival factor for tumour cells such as EGFR ligands and hepatocyte growth factor (HGF). c, PDGF-B that is produced by endothelial cells activates PDGFR-β resulting in pericyte recruitment in the microvasculature. From Ferrara and Kerbel, 2005.

Although endothelial cells certainly initiate angiogenesis and are therefore essential for vascular development, it is the periendothelial structures that are crucial to complete vascular maturation by inhibiting endothelial cell proliferation and migration while producing the extracellular matrix in order to stabilize nascent vessels. In this context another determinant that should certainly not be neglected is the influence of flow. As a result of higher flow in the capillaries proximal to the aorta, coronary arteries become covered by smooth muscle cells earlier than veins (Vrancken Peeters et al., 1997). In pathophysiological angiogenesis that is often induced by some degree of inflammation, monocytes/macrophages, platelets, mast cells and other leucocytes play a vital role as they are 'attracted' to sites of would healing or inflammation. This is at least in part mediated by angiogenic factors such as bFGF, IGF-1, PDGF or VEGF (Pinedo et al.,

1998) which then in turn produce angiogenic chemokines that attract amongst others endothelial cells and smooth muscle cells (Sunderkotter et al., 1994; Coussens and Werb, 2001).

1.6 EETs, proliferation and angiogenesis

Currently, relatively little is known about the molecular mechanisms underlying CYP epoxygenase/EET-induced proliferation and angiogenesis. As mentioned above, one of the most detailed analyses of the mechanisms involved reported is the transactivation of the EGF receptor as a consequence of the matrix metalloproteinases-induced release of heparin-binding EGF-like growth factor (HB-EGF) from the extracellular membrane. The EET-mediated activation of the EGF receptor leads in turn to the activation of the kinase Akt and an enhanced expression of cyclin D1 (Figure 7). All four EET-regioisomers regulate endothelial cell proliferation by increasing Akt phosphorylation in endothelial cells, but for example in murine endothelial cells only the effects seen with 5,6- and 14,15-EETs appear to be dependent on PI3K (Pozzi et al., 2005). Recently it has been suggested that 11,12-EET can activate Akt/ PI3K by means of sphingosine kinase 1 (SK1) (Yan et al., 2008) in endothelial cells. Other signalling pathways also contribute to the increase in cyclin D1 expression including the MAP kinase phosphatase-1 which decreases JNK activity (Potente et al., 2002). Activation of Akt by EETs also induces the phosphorylation and therefore inhibition of the forkhead factors FoxO1 and FoxO3a and subsequently a decrease in the expression of the cyclin-dependent kinase inhibitor $p27^{Kip1}$ (Potente et al., 2003).

Furthermore, in human dermal microvascular endothelial cells 14,15-EET have been found to induce angiogenesis via Src-dependent increase in STAT-3-mediated VEGF expression (Cheranov et al., 2008).

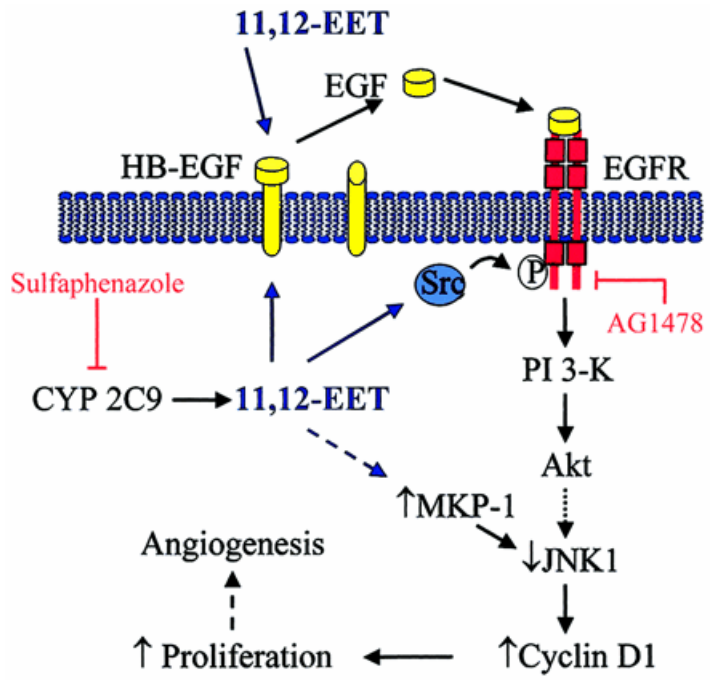

Figure 7. Angiogenic pathways activated by CYP2C-derived EETs in endothelial cells. EETs are able to transactivate the EGF receptor via a mechanism that involves matrix metalloprotease (MMP)-dependent heparin-binding EGF-like growth factor (HB-EGF) release. EET-mediated activation of the EGF receptor results in Akt-activation and subsequent cyclin D1 expression. Furthermore MAP kinase phosphatase-1 (MKP-1) activation which decreases JNK activity contributes to increased cyclinD1 expression. From Michaelis et al., 2003.

1.7 Growth factors acting via endothelial cell-specific receptor tyrosine kinases

The development of a functional vascular network requires a remarkable degree of coordination between different cell types undergoing complex changes and is dependent upon signals exchanged between these cell types. A number of signal transduction systems and molecules, such as VEGF, are involved in these processes and during the subsequent maturation of the developing vasculature amongst others the PDGF-B/PDGF-β pathway is essential for the differentiation of mural cells into vascular smooth muscle cells or pericytes (Betsholtz et al., 2004). Other lipid mediators have been implicated in this later maturation process and sphingosine-1-phosphate (S1P) has been reported to play a critical role in the recruitment of vascular smooth muscle

cells by endothelial cells as downregulation of one of its receptors results in defects in mural vessel coverage (Allende et al., 2003).

The present study focused on two main factors that are known to be very important for the development of the vascular network: VEGF as one of the most potent inducers of vascular permeability and angiogenesis and EphB4 because of its high relevance for the guidance of vascular sprouts (Munarini et al., 2002).

1.7.1 Vascular endothelial growth factor (VEGF)

The VEGF family provided the first example of a growth factor specific for the endothelium. During the early 1960s the human VEGF receptors were structurally characterized by cDNA cloning (Shibuya et al., 1990; Millauer et al., 1993). The VEGF family currently consists of seven members (VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E, VEGF-F and placental growth factor, PIGF), all of which have different physical and biological properties and act through specific tyrosine kinase receptors (VEGF-R1, VEGF-R2 and VEGF-R3). Although these receptors are structurally highly related, they display interesting differences in their ability to respond to ligand binding with increased kinase activity and the spectrum of transduced biological responses ranges from the regulation of physiological to pathophysiological angiogenesis. VEGFR-2 has strong tyrosine kinase activity and transduces the major signals of angiogenesis via the phospholipase C protein kinase C pathway to activate MAP kinases and DNA synthesis. One of the most important roles of VEGFR-2 is stimulation of endothelial cell survival and angiogenesis. Its gene inactivation results, for example, in death at embryonic day 8.5 and 9 due to a failure in vasculogenesis (Shalaby et al., 1995). Furthermore, overactive VEGFR-2 signaling results in pathological angiogenesis including diabetic retinopathy and cancer. VEGFR-1 plays a dual role, VEGFR-1 -/- mice die between embryonic day 8 and 9 (Fong et al., 1995), and this negative regulatory role is most likely due to trapping VEGF-A resulting in prevention of VEGFR-2 activation by its ligand (Hiratsuka et al., 1998). However in the adult VEGFR-1 is a positive regulator (Shibuya and Claesson-Welsh, 2006) of vascular development.

1.7.2 EphB4

The very large family of ephrins that act via endothelium-specific receptors known as Ephs have recently been identified and studied in vascular development and angiogenesis (Gale and Yancopoulos, 1999; Tallquist et al., 1999). Eph receptors are a family of receptor tyrosine kinases that play a critical role in embryonic patterning, neuronal targeting, vascular development and adult neovascularisation. Although first described due to their function in the nervous system, signalling via Eph receptors mediates critical steps of angiogenesis, including juxtacrine cell-cell contacts, cell adhesion to extracellular matrix, cell migration and proliferation.

Ephs were first discovered in a human cDNA library screen for sequences homologous to the viral oncogene vfps (Hirai et al., 1987). Unlike other families of RTK, which bind to soluble ligands, Eph receptors interact with cell surface-bound ephrin ligands which attach to the cell membrane either through a glycosylphosphatidyl inositol (GPI) anchor or a transmembrane domain. Based on how they attach to the cell membrane, Ephs have been divided in two subclasses, A and B, with EphB4 being the most prominent representative in vascular development. Moreover these receptor-ligand interactions activate signalling pathways in a bi-directional fashion, through both the Eph receptors and ephrin ligands. For example, mutant animals lacking EphB4 show a phenotype of disrupted vessel formation and early embryonic lethality (Gerety et al., 1999), but the exact role of EphB4 in angiogenesis is still largely unclear. Downstream signalling of Eph is very complex and involves amongst others activation of Abelson kinase and Src family kinases as well as Ras and Rho (Kullander and Klein, 2002). This is mirrored to some extent in the versatility that is involved when it comes to the Eph/ephrin system (Figure 8). Depending on the exact setting as well as on spatial gradients in receptor and ligand distribution cell signalling ranges from adhesion to repulsion and makes prediction of certain scenarios challenging.

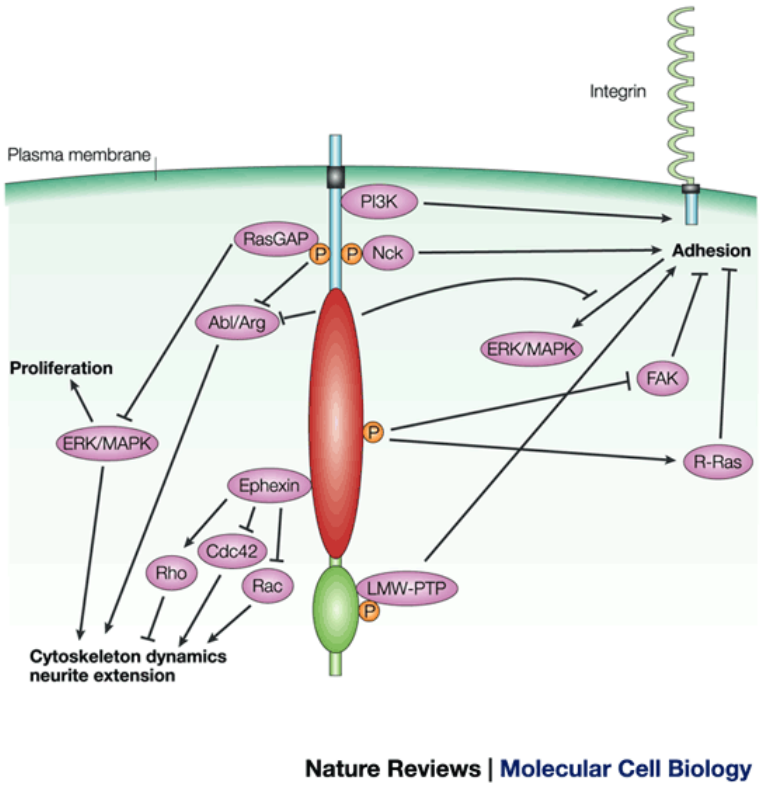

Figure 8. Downstream Eph signalling. Abelson (Abl) and Abl-related gene (Arg) bind the juxtamembrane region of EphB through a phosphorylation-independent interaction with EphB. Activated Eph receptors suppress the extracellular-signal regulated kinase (ERK)/mitogen-activated protein kinase (MAPK) pathway. Ephexin interacts with the Eph-receptor kinase domain and its activation affects a number of Rho GTPases-related processes. GAP, GTPase-activating protein; PI3K, phosphatidylinositol 3-kinase; FAK, focal adhesion kinase. LMW-PTP, low-molecular-weight protein tyrosine phosphatase; Nck, SH2–SH3 adaptor protein. From Kullander and Klein, 2002.

1.8 Aim of the study

Our group has previously reported that CYP2C-derived EETs are involved in the regulation of endothelial cell homeostasis and angiogenesis. The molecular mechanisms that account for the observed effects have only been partially elucidated and therefore the aim of this study was to further clarify the mechanisms involved in the angiogenic response mediated by CYP2C9-derived EETs in more physiologically-relevant conditions than those addressed in previous studies.

The first part of the study concentrates on the role played by EETs in EphB4 and VEGF signalling while the latter experiments were aimed at identifying new EET effector

pathways and the influence of the epoxides on cell-cell communication e.g. pericyte recruitment.

2. Materials and Methods

2.1 Materials

All culture media, enzymes and buffer solutions as well as antibiotics for cell culture were from Invitrogen (Karlsruhe, Germany), cell culture plates and falcon tubes were from BD Biosciences (Heidelberg, Germany). 11,12-EET was purchased from Cayman Chemicals (Massy, France), growth factor reduced basement membrane matrix (Matrigel) was obtained from BD Biosciences (Heidelberg, Germany), thrombin was from Haemochrom Diagnostica GmbH (Essen, Germany). Sulfaphenazole and all other substances were from Sigma (Deisenhofen, Germany). VEGF and bFGF were from PeproTech (Hamburg, Germany).

14,15-Epoxyeicosa-5(Z)-enoic acid (14,15-EEZE) and MS-PPOH were synthesized as described (Gauthier et al., 2002) and provided by J.R. Falck (Department of Biochemistry, University of Texas Southwestern Medical Center, Dallas, Texas, USA). Protein A Sepharose for the immunoprecipitation was from GE Healthcare (Freiburg, Germany) and Protein G Sepharose and dynabeads that were used for the isolation of murine lung endothelial cells were from Invitrogen (Karlsruhe, Germany).

The following antibodies were used in the course of this study:

Antibody	**Company**	**Method**
α-actin	Sigma, Deisenhofen, Germany	Matrigel immunohistochemistry
β-actin	Sigma, Deisenhofen, Germany	Western blot
Akt	Cell signaling, Danvers, MA, USA	Western blot
Phospho-Akt	Cell signaling, Danvers, MA, USA	Western blot
AMPK	New England Biolabs, Ipswich, MA, USA	Western blot
Phospho-Thr174AMPK	New England Biolabs, Ipswich, MA, USA	Western blot
CYP2C9	Acris, Hiddenhausen, Germany	Western blot
EphB4	R&D Systems, Wiesbaden, Germany	In situ immunohistochemistry
EphB4	Zymed, San Fransisco, USA	Western blot
ephrinB2	R&D Systems, Wiesbaden, Germany	Western blot
PECAM-1 (clone MEC 13.3)	Santa Cruz Biotechnology, Heidelberg, Germany	Immunohistochemistry
PECAM-1	BD Bioscience, Wiesbaden, Germany	Matrigel immunohistochemistry
Phosphotyrosine	Santa Cruz Biotechnology, Heidelberg, Germany	Immunoprecipitation

2.2 Cell culture

Human endothelial cells were either isolated from human umbilical veins (HUVEC) of fresh umbilical cords as described (Busse and Lamontagne, 1991) or purchased from Promocell if used for transfection. For the VEGF experiments HUVECs tested for the VEGF-signalling pathway were obtained from Cell Applications (San Diego, USA).

HUVECs were cultivated in MCDB 131 containing 8% fetal calf serum (FCS), L-glutamine (10 mmol/L), basic fibroblast growth factor (bFGF, 1 ng/mL), epidermal growth factor (EGF, 0.1 ng/mL), 'endothelial cell growth supplement' with bovine brain-derived heparin (ECGS/H, 0.4 %), penicillin (50 U/mL) and streptomycin (50 µg/mL).

Porcine aortic endothelial cells (PAEC) were isolated by excising porcine aortas segments from freshly slaughtered pigs as described (Popp et al., 1996). Under sterile conditions the aortas were cleaned of fat and connective tissue and sliced open longitudinally between the intercostal vessels. Vessels were mounted between a plastic plate and a frame with the endothelial side facing upwards. Endothelial cells were separated by incubating for 40 minutes at 37ºC with dispase followed by repeated pipetting. Afterwards cells were diluted with M199 containing 0.1 % BSA, recovered by centrifugation (100g, 5 minutes) and resuspended in a 1:1 mixture of MCDB 131 and M199 (with 14% FCS, 6 mmol/L L-glutamine, 0.5 ng/mL bFGF, 0.05 ng/mL EGF, 0.2 % bovine brain-derived ECGS/H, 50 U/mL penicillin and 50µg/mL streptomycine) and seeded on culture dishes coated with fibronectin (BD Bioscience, Heidelberg, Germany).

The murine lung microvascular endothelial cells were isolated from 3–4 month old animals as described (Fleming et al., 2005). Briefly peripheral lung tissue was minced and digested for 1 h at 37°C in 0.1% collagenase-A (Boehringer Mannheim). The digest was passed through a blunt 14-gauge needle and filtered through a 130 µm steel mesh. Cells were pelleted at 1200 rpm and resuspended in murine lung endothelial cell (MLEC) medium (37°C) containing 20% FCS, 35% DMEM, 35% F-12, 50 µg/mL endothelial mitogen (Biomedical Technologies, Stoughton, MA, USA), 2 mmol/L L-glutamine, 100 µg/mL heparin, and 100 U/100 µg/mL penicillin-streptomycin. To the suspended cells, magnetic beads (Dynabeads® Sheep anti-rat IgG) were added and and incubated for 1 h at 4°C. Cells were washed and selected in a magnetic field. Cultures were grown to confluence and selected twice before being plated for experiments.

First passage HUVECs were used throughout. Porcine aortic and murine lung endothelial cells were used up to passage five. HUVECs prescreened for VEGF and HUVECs purchased from Promocell were used up to passage seven.

2.3 Transfection of endothelial cells

HUVECs were transiently transfected using Gene Trans II (MobiTec, Göttingen, Germany), a lipophilic transfection reagent. One day after they were seeded on fibronectin coated culture dishes (at approximately 80% confluence) cells were incubated for 4 hours with the GeneTrans-DNA complex in the smallest possible volume of MCDB with 0.1% BSA. Afterwards, cells were left to recover in MCDB with 2% FCS, L-glutamine (10 mmol/L), bFGF (0.5 ng/mL), EGF (0.05 ng/mL), ECGS/H (0.2%), penicillin (50 U/mL) and streptomycin (50 µg/mL). Transfection efficiency was approximately 30-40%.
Murine lung endothelial cells were transfected accordingly using TransPass^{TM} HUVEC Transfection Reagent (New England BioLabs, Ipswich, MA, USA) and left to recover in DMEM/F12 with 2.5% FCS, 35% DMEM, 35% F-12, 50 µg/mL endothelial mitogen (Biomedical Technologies, Stoughton, MA, USA), 2 mmol/L L-glutamine, 100 µg/mL heparin, and 100 U/100 µg/mL penicillin-streptomycin.

2.4 Adenoviral infection of endothelial cells

In some experiments endothelial cells were treated with adenoviral vectors to increase transgene expression. The recombinant adenovirus expressing CYP2C9 sense was generated following transfection of the plasmid into human embryonic kidney cells (HEK 293) according to the AdEasy system. The CYP2C sense gene was incorporated into pAdTrack-CMV vector and the CYP2C antisense gene into pShuttle-CMV vector. The AMPK viruses used were from K. Walsh (School of Nursing, Midwifery and Nutrition, James Cook University, Cairns QLD, Australia). For the infection endothelial cells (80% confluent) were first serum-starved for 10 hours and then incubated with the recombinant adenovirus (10 pfU/cell) in MCDB 131 with 0.1% BSA for 4 hours. Afterwards, cells were left to recover in MCDB 131 with 2% FCS, L-glutamine (10 mmol/L), bFGF (0.5 ng/mL), EGF (0.05 ng/mL), ECGS/H (0.2%), penicillin (50 U/mL) and streptomycin (50 µg/mL). As described previously (Michaelis et al., 2005a) infection efficiency was between 90 and 100% and those cells infected with CYP2C9 sense adenovirus generated approximately two-fold more 11,12- and 14,15-EET under

basal conditions than cells infected with the control (CYP2C9 antisense) virus. This increase in EET production was sensitive to the CYP2C inhibitor miconazole.

2.5 EET measurements by LC-MS/MS

HUVECs were treated as described in the results section, harvested by scraping and the pellets (from approximately $8x10^6$ cells) were suspended in 100 µL potassium phosphate buffer (0.1 mol/L, pH 7.2), hydrolyzed for 1 hour in NaOH (0.5 N) and neutralized with HCl (2 mol/L) before deuterated internal standards (5-HETE-d_8, 12-HETE-d_8, 15-HETE-d_8, 20-HETE-d_6, 8,9-EET-d_8, 11,12-EET-d_8 and 14,15-EET-d_8 (Cayman, Massy, France)) were added. A liquid-liquid-extraction was performed twice using ethyl acetate (0.5 mL). After evaporation of the solvent in a vacuum block under a gentle stream of nitrogen, samples were reconstituted with 50 µL of methanol/water (1:1, v/v) and eicosanoids were determined with a Sciex API4000 mass spectrometer operating in the multiple reaction monitoring (MRM) mode. Chromatographic separation was performed on a Gemini C18 column (150 x 2 mm I.D., 5 µm particle size, Phenomenex, Aschaffenburg, Germany).

2.6 Transfection with antisense oligonucleotides

In order to downregulate protein expression in HUVEC an antisense approach was used. Endothelial cells were transiently transfected using Gene Trans II (MobiTec, Göttingen, Germany), a lipophilic transfection reagent. One day after they were seeded on fibronectin coated culture dishes (at approximately 80% confluence) cells were incubated for 4 hours with the GeneTrans-DNA complex (DNA concentration: 2 µmol/L) in the smallest possible volume of MCDB with 0.1% BSA. Afterwards cells were left to recover in MCDB with 2% FCS, L-glutamine (10 mmol/L), bFGF (0.5 ng/mL), EGF (0.05 ng/mL), ECGS/H (0.2%), penicillin (50 U/mL) and streptomycin (50 µg/mL).
Cells were treated with the following oligonucleotides (Biospring GmbH, Frankfurt am Main, Germany):

EphB4 antisense oligonucleotides:	5'-ATGGAGGCCTCGCTCAGAAA-3'
EphB4 scrambled oligonucleotides:	5'-TACCTGAAGGTCAGGCGCAC-3'

CYP2C antisense:	5’-TCC ATT GAA GCC TTC TCT TCT T-3’
CYP2C sense:	5’-AAG AAGAGA AGG CTT CAA TGG A-3’

The sequence of the CYP2C oligonucleotides spans the ATG and is 100% identical with human CYP2C8 and contains one mismatch to the other 3 human CYP2C isoforms.

2.7 Downregulation by RNA interference

In mouse lung endothelial cells RNA was downregulated by transfecting cells with one of three siRNAs directed against EphB4 using TransPass^{TM} HUVEC Transfection Reagent (New England Biolabs, Ipswich, MA, USA).

EphB4siRNA1:	5’-AAG-UAG-GUC-AAG-UUC-GUG-3’
EphB4siRNA2:	5’-UGU-CUC-CUA-UGU-CAA-GAU-3’
EphB4siRNA3:	5’-AAU-CUU-GAC-AUA-GGA-GAC-3’

One day after they were seeded on fibronectin coated culture dishes (at approximately 80% confluence) cells were incubated with the TransPass^{TM} HUVEC transfection reagent –DNA complex. After 24 hours cells were left to recover in DMEM/F12 with 2.5% FCS, L-glutamine (10 mmol/L), bFGF (0.5 ng/mL), EGF (0.05 ng/mL), ECGS/H (0.2%), penicillin (50 U/mL) and streptomycin (50 µg/mL).

2.8 Reporter gene assay

For maxi plasmid DNA preparation commercially available kits were used from Qiagen (Düsseldorf, Germany) and the DNA isolation was performed according to the manufacturer’s protocol.

PAECs were transiently co-transfected with the EphB4 promoter luciferase construct (amplified by PCR and cloned in *Kpn*I and *Xho*I sites of the pGL3 enhancer luciferase vector (Promega, Mannheim, Germany)) as described above together with either pcDNA 3.1 or a plasmid encoding for CYP2C9. After 36 hours, the cells were lysed in luciferase buffer (K_2HPO_4 and KH_2PO_4, pH 7.8, 0.5% TritonX-100, Dithiothreitol DTT 0.5 mmol/L) on ice. Membrane fractions were separated by centrifugation (13000 rpm, 10 minutes, 4ºC) and protein concentration in the lysate was determined photometrically at 595 nm using Bradford solution; a solution of increasing BSA concentration was used as a reference for calibration. Samples were equalised to a protein concentration of 1 µg/µL, mixed with equal amounts of luciferase substrate and luciferase activity was measured according to the manufacturer's protocol (Promega, Mannheim, Germany) in a luminometer.

Endothelial cells expressing either VEGF-R1 or VEGF-R2 were transiently transfected with the non-coding 5' region (-2088 to +21; kindly provided by Dr. P. Maurel, Montpellier, France) of CYP2C9 subcloned into pGL3basic (Promega, Mannheim, Germany). After 12 hours, the cells were treated with either solvent (PBS, 140 mmol/L NaCl, 2.68 mmol/L KCl, 10 mmol/L Na_2HPO_4, 1.47 mmol/L KH_2PO_4; pH 7.0) or VEGF (30 ng/mL) for 6 hours. Thereafter, the cells were lysed in luciferase buffer and luciferase activity was assayed according to the manufacturer's protocols (Promega, Mannheim, Germany). Promoter activity was determined as luciferase activity relative to protein content.

2.9 Protein isolation

Cells were washed with phosphate-buffered saline solution (PBS) and lysed on ice with a buffer containing 20 mmol/L TRIS-HCl, pH 7.5, 1% TritonX-100, 25 mmol/L β-glycerolphosphate, 150 mmol/L NaCl, 10 mmol/L Na pyrophosphate, 20 mmol/L NaF, 2mmol/L Na orthovanadat, 10 mmol/L okadaic acid and a protease inhibitor mix (2 µg/ml antipain, 2 µg/mL aprotinin, 2 µg/mL chymostatin, 2 µg/mL leupeptin, 2 µg/mL pepstatin, 2 µg trypsinhibitor and 40 µg/mL phenylmethysulfonyllfluoride (PMSF)). Insoluble fractions were separated by centrifugation (13000 rpm, 10 minutes, 4ºC) and protein concentration in the lysate was determined.

2.10 Immunoprecipitation

Cells were washed with PBS and lysed on ice in modified RIPA-buffer (150 mmol/L NaCl, 1 mmol/L EDTA, 1% Nonidet, 0.5% Na deoxycholate, 0.1% SDS, 20 mmol/L Tris, pH 8.0) containing a protease inhibitor mix (2 µg/mL antipain, 2 µg/mL aprotinin, 2 µg/mL chymostatin, 2 µg/mL leupeptin, 2 µg/mL pepstatin, 2 µg trypsinhibitor and 40 µg/mL PMSF and 1 mmol/L sodium orthovanadate) and the insoluble fractions were separated by centrifugation (13000 rpm, 10 minutes, 4ºC). Afterwards protein lysates (30 µg) were incubated with 2 µL of a phosphotyrosine antibody (Santa Cruz Biotechnology, Heidelberg, Germany) for 90 minutes at 4ºC and then 30 µL of a mixture of Protein A and G Sepharose (GE Healthcare, München, Germany) was added. After an additional 90 minutes immunoprecipitates were washed three times with RIPA buffer and were resuspended in sodium dodecylsulfate (SDS)-buffer. The samples were separated by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS PAGE).

2.11 Immunoblotting

Following lysis, protein samples were separated by SDS-PAGE in a buffer solution consisting of 190 mmol/L glycine, 0, 1% SDS and 25 mmol/L TRIS-HCl. The separated proteins were then transferred for 90 minutes at 250 mA (for 2 gels) in a buffer solution composed of 190 mmol/L glycine, 25 mmol/L TRIS-HCl and 20% methanol. Afterwards the membranes were incubated in 3% BSA in TRIS buffered saline with 0,3% Tween-20 (TBST, 50 mmol/L TRIS/HCl, 150 mmol/L NaCl) to block unspecific binding sites on the membrane. After incubation with the according primary antibody diluted in 3% BSA overnight, multiple washing steps with TBST and another blocking step, membranes were incubated with a horseradish peroxidase conjugated secondary antibody diluted with TRIS buffered saline followed by repeated washing. Membranes were subjected to a chemiluminescence reaction via the ‘Enhanced Chemiluminescence (ECL)’ system by mixing ECL-solution I (100 mM TRIS/Cl, pH 8.8, 2.5 mM luminol and 0.4 mM coumarin acid) and ECL-solution II (100 mM TRIS/Cl, pH 8.8 and 30% H_2O_2) and proteins detected by the first and secondary antibody were exposed to X-ray film in the darkroom.

2.12 RNA-Isolation and reverse transcriptase polymerase chain reaction (RT-PCR)

Total RNA from cultivated endothelial cells was isolated as described (Chomczynski and Sacchi, 1987). Endothelial cells were washed with PBS and lysed using phenol and guanidine isothiocyanate (Tri®Reagenz, Sigma, Deisenhofen, Germany). Homogenized samples were incubated for 5 minutes at room temperature to permit the complete dissociation of the nucleoprotein complex. Afterwards the solution containing RNA as well as DNA was mixed with 0.2 ml of chloroform per 1 ml of Tri®Reagenz. Samples were shaken vigorously by hand for 15 seconds and incubated at room temperature for 2 to 3 minutes. The two phases were separated by centrifugation for 30 minutes at 12000g to dilute RNA in the upper, aqueous phase while the DNA stays in the lower organic phase. RNA was transferred to a fresh tube and precipitated by adding isopropanol (100%). Thereafter samples were incubated at room temperature for another ten minutes, centrifuged, washed with ethanol (75%) and diluted in water. RNA concentration was defined 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), desoxynukleosid-5'-triphophate (175 µmol/L of dATP, dCTP, dGTP and dTTP), dithiothreitol (1 mmol/L), oligo (dT) and reverse transcriptase (200 U, Invitrogen, Karlsruhe, Germany) for 60 minutes at 37ºC 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 60 cycles à 30 secondes at 95°C, 1 minute at 60 °C and 30 secondes at 72°C in a Mx4000 multiplex quantitative PCR system (Stratagene, Heidelberg, Germany)) the following primers were used:

18 S: forward 5' –CTTTGGTCGCTCGCTCCTC-3'

18 S: reverse 5'- CTGACCGGGTTGGTTTTGAT-3'

CYP2C8: forward: 5'-GGACTTTATCGATTGCTTCCTG-3',

reverse: 5'-CCATATCTCAGAGTGGTGCTTG-3'

To ensure equal amounts of cDNA were used, Pol2 RNA was amplified by qPCR (Assay on Demand, Applied Biosystems) and the amount of cDNA in the samples was calculated on the basis of the amplification of a serial dilution of a plasmid (CYP2C8) or the serial dilution of the cDNA (18S RNA). The CYP2C8 levels were normalized to that of Pol2. 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 *In vitro* angiogenesis assays

2.13.1 Fibrin gel:

To generate a fibringel, fibrinogen (1.5 mg/mL) was dissolved for 2 hours in MCDB 131 supplemented with 0.1% BSA with penicillin (50 U/mL) and streptomycin (50 µg/mL), filtered through a sterile filter and portioned into a 24 well culture plate (250 µL per well). The gel was polymerized by the addition of thrombin (0.5 U/mL). After 30 minutes at room temperature 1 ml MCDB 131 with 0.1% BSA was added to each well and the gels were equilibrated in a cell incubator at 37ºC overnight. Thereafter, HUVEC in MCDB with 4% FCS, L-glutamine (10 mmol/L), bFGF (0.5 ng/mL), EGF (0.05 ng/mL), ECGS/H (0.2%), penicillin (50 U/mL) and streptomycin (50 µg/mL) were seeded onto the gel and stimulated. After 24 hours angiogenesis was quantified in three randomly chosen fields of view by measuring tube length with a computer-assisted microscope.

2.13.2 Spheroid assay:

For the three dimensional spheroid assay spheroids containing 400 cells were generated by the hanging drop method. Therefore, 25 µl of cell suspension (containing 400 cells) in endothelial growth medium (EGM, Clonetics (Cambrex Bio Science, Wakersville, Inc. USA)) containing 10% FCS, human epidermal growth factor (hEGF), bovine brain extract (BBE), penicillin (50 U/mL) and streptomycin (50 µg/mL) and 20% carboxymethyl cellulose (Sigma, Deisenhofen, Germany) was dispensed on cell culture plates to form individual drops, inverted and incubated at 37ºC overnight. After 24 hours, trays were uprighted and the drops containing spheroids of a defined cell number were harvested by flushing the tray with 5 mL of PBS containing 10% FCS. A collagen

stock solution was prepared prior to use by mixing 8 vol. of acidic collagen extract of rat tails (Collagen Type I, Rat Tail, BD Biosciences, Heidelberg, Germany, equilibrated to 2 mg/mL) with Medium 199 (Sigma, Deisenhofen, Germany), HEPES (Invitrogen; 1 vol.) and 0.1 N NaOH (approx. 1 vol.) to adjust the pH to 7.4. This stock solution (0.5 ml) was mixed with 0.5 ml room temperature endothelial basal medium (EBM, Clonetics, Cambrex Bio Science, Wakersville, Inc. USA)) containing 40% FCS and 0.5% (w/v) carboxymethylcellulose to prevent sedimentation of spheroids prior to polymerization of the collagen gel and 50 spheroids. The spheroid-containing gel was rapidly transferred into prewarmed (37ºC) 24 well plates and allowed to polymerize (30 minutes). Thereafter, 250 µl EGM containing 2% FCS and stimulants were pipetted on top of the gel and were incubated at 37°C in 5% CO_2 at 100% humidity. After 24 hours, angiogenesis was quantified by measuring the cumulative length of all of the capillary like sprouts originating from the central plain of an individual spheroid using a computer-assisted microscope. At least 5 spheroids per experimental group and experiment were analyzed. This analysis takes into consideration that the angiogenic response induced by a specific substance is more appropriately reflected by the length of individual capillary-like sprouts as well as the number of capillary like sprouts.

2.14 *In vivo* angiogenesis assays

Female C57BL/6 mice (8 weeks old) were lightly anesthetized with chloralhydrate (200 µL of a 4% solution, s.c.). When mice were asleep hair on the back was removed with an electric razor to allow a better visibility during the implantation of the Matrigel. Afterwards, 0.5 mL of Matrigels were impregnated with heparin (0.0025 units/mL s.c.), 11,12-EET (10 µmol/L) containing either a control siRNA (GFP; 2 µmol/L) or siRNA directed against EphB4 (siRNA1; 2 µmol/L), VEGF (150 ng/mL), EEZE (100 µmol/L) or a combinations of VEGF and EEZE and were injected along the dorsal midline on each side of the spine. When the gel solidified, mice were transferred to their cage and put on a heated blanket to avoid hypothermia. After seven days the mice were sacrificed, the Matrigel plugs removed, embedded in Tissue Tek and frozen at -20ºC. Plugs were then either cryo-sectioned (10 µm) and processed for staining for PECAM-1 (BD Biosciences, Heidelberg, Germany), α-actin (Sigma, Deisenhofen, Germany) and isolectin (Sigma, Deisenhofen, Germany) as described above or harvested and washed

with PBS. The latter samples were mixed with water (200 µL) on ice, homogenized with an electric mixer and centrifuged at 14000 rpm for 15 minutes to remove particulate material. Afterwards 100 µL of Matrigel lysate and 300 µL Drabkins solution (Sigma, Deisenhofen, Germany) were mixed and incubated for 15 minutes at room temperature. Drabkins solutions alone served as the blank value and the concentration of the hemoglobin contained in the plugs was determined at 595 nm by analysing 1 µl of sample in a spectrophotometer (NanoDrop Technologies, Fisher Scientific GmbH, Schwerte, Germany); murine haemoglobin was used for calibration. In some experiments Matrigel implants were fixed in zinc fixative (0.1M TRIS-buffer, calcium-acetate, zinc acetate and zinc chloride) overnight, dehydrated using alcohol and isopropanol and processed for paraffin sectioning as described in the immunohistochemistry section. EphB4 expression in the Matrigel plugs was determined using a polyclonal goat anti-mouse EphB4 antibody in combination with an enhanced detection method (Envision™, DAKO), DAB and Mayers Hematoxylin Solution (Sigma, Deisenhofen, Germany).

In order to demonstrate perfusion in the Matrigel plug isolectin was injected through the tail vein 30 minutes before animals were sacrificed.

Vessel formation was quantified manually as well as using a computer-assisted program (Nikon NIS-Elements) analyzing at least five sections per plug. Infiltration of endothelial cells (vessel formation index) and smooth muscle cells (stabilised vessel formation index) were scored blindly by four non-biased observers according to a predetermined scoring system. The scores were as follows: 0 no effect, 1 isolated endothelial cells, 2 clusters of endothelial cells but no distinct tube formation, 3 endothelial tube formation with little or no coating by α-actin positive cells and 4 complete coating of endothelial cells with α-actin positive cells. To facilitate comparison between the different groups the scores were normalized with respect to the effects observed in the control group.

Mesenteric arteries were isolated from male adult NMRI mice and incubated for 18 hours in DMEM supplemented with 15% FCS and either solvent (0.1% DMSO) or 11,12-EET (10 µmol/L). Blood vessels were then fixed in zinc overnight, dehydrated using alcohol and isopropanol as described above and processed for paraffin sectioning. Staining for EphB4 was performed using paraffin sections (4 µm) and the polyclonal anti-mouse EphB4 in combination with Envision™ and tyramide-Cy3 (Perkin Elmer, Rodgau, Germany) according to the manufacturer's instructions. EphB4 staining

intensity was determined by using the Cell^R software analysing at least two different sections per experimental group and animal. Exposure times during digital imaging were kept constant.

2.15 Immunohistochemistry

For immunohistochemistry Matrigel Plugs were removed and either imbedded in Tissue Tek (Sakura Finetec, Torrance, USA) and frozen at -80ºC or fixed in zinc fixative overnight and embedded in paraffin.

Tissue Tek embedded samples were cryo-sectioned (10 µm) and put on glass coverslips. Afterwards sections were fixed with 4% paraformaldehyde in PBS, washed with PBS and 0.2% glycine, treated with blocking buffer (10% horse serum in TBST) and incubated with the primary antibody (diluted in PBS 1:100 to 1:500) overnight. On the next day, sections were washed with PBS to remove excessive primary antibody and incubated with the FITC- or Cy3-coupled secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA, USA). Thereafter, sections were repeatedly washed with PBS and finally with deionised water and imbedded with fluorescence mounting medium (DAKO, Carpinteria, CA, USA). Sections were analysed with a confocal microscope at a wavelength of 633, 564 and 488 nm.

Paraffin embedded sections were dehydrated in a series of xylol, isopropanol, 99-96% ethanol, 85% ethanol, 70% ethanol and destillated water for 5 minutes each. They were first rinsed in 3% H_2O_2 for 10 minutes and afterwards in H_2O and treated with blocking buffer (0.25% casein, 15 mmol/L sodium azide and 0.1% bovine serum albumin in 50 mmol/L TRIS-HCl pH 7.6) for 30 minutes. Afterwards sections were incubated with goat anti-mouse EphB4 (1:200) (R&D Systems, Wiesbaden, Germany) in blocking buffer at 4ºC overnight. The next day sections were repeatedly washed in TBST, incubated with secondary antibody diluted (1:100) in blocking solution for 1 hour, washed with TBST and incubated with DAKO-Envision™ (DAKO, Carpinteria, CA, USA) enhanced detection method in combination with EphB4 antibody for 30 minutes at room temperature. After washing with TBST to remove excess fluid, sections were developed with 3, 3'-diaminobenzidine enhanced liquid substrate system solution A and B (DAB), Sigma) for 1-5 minutes. After the development process was stopped with H_2O, sections

were incubated with Mayers Hematoxylin Solution (Sigma, Deisenhofen, Germany) for 30 seconds and rinsed for 5-10 minutes under running tap water. After a series of wash steps with H_2O, 70% ethanol, 85% ethanol, 99-96% ethanol, isopropanol, xylol for 5 minutes each, sections were embedded in DePex mounting medium (Sigma, Deisenhofen, Germany).

2.16 Contrast enhanced sonography

For in vivo imaging with contrast enhanced sonography mice were anaesthetized with oxygen and isoflurane (3%). Ultrasonography was performed when at 14 MHz at low mechanical index in a pulse inversion mode on a clinical ultrasound scanner (Sequoia, Siemens/ Acuson, USA). After i.v. injection of 100 µl contrast media (Sono Vue, Bracco, Germany) the change of signal intensity (in dB) in the respective Matrigel plugs was recorded over time. Relative peak enhancement of the signal-intensity-time curves in the left and right Matrigel plugs of the same animal was determined with an integrated software tool of the scanner.

2.17 Statistical analysis

Data are expressed as mean ± s.e.m (standard error of mean). Statistical analysis of Matrigel plugs analyzed by ultrasound was performed using a paired t test. For all other statistical comparisons evaluation was performed with Student's t test for unpaired data or one-way ANOVA followed by a Bonferroni t test. Values of $P<0.05$ were considered statistically significant.

3. Results

3.1 Effect of CYP2C9 on EphB4 expression

Although CYP2C protein is expressed in native endothelial cells, mRNA as well as protein expression levels decrease rapidly following cell isolation (Fisslthaler et al., 1999). Already after the second passage protein levels can no longer be detected (Vernia et al., 2001). Consequently in order to analyse effects and the biological function of CYP2C-derived EETs in vitro the protein needs to be upregulated either by pharmacological stimuli or hemodynamic stimuli (e.g. shear stress), by hypoxia as described (Michaelis et al., 2005a) or by using adenoviral constructs to overexpress CYP2C.

To determine whether or not a link exists between CYP2C9 and the expression of the tyrosine kinase EphB4, HUVECs were infected with either a control (CYP2C9 antisense) adenovirus or CYP2C9 encoding virus. While low levels of EphB4 were detected in the endothelial cells employed, CYP2C9-overexpression resulted in a 4-fold increase in EphB4 expression within 24 hours compared to control virus-treated cells (Figure 9A). There was no effect on CYP2C9 expression in solvent treated cells (data not shown). The increase in EphB4 expression could be attributed to elevated CYP epoxygenase activity as the CYP2C9-induced increase in EphB4 expression was inhibited in the presence of the epoxygenase inhibitor, MSPPOH (10 µmol/L; Figure 9B).

Binding of the receptor EphB4 to its ligand ephrinB2 and subsequent signalling results in its phosphorylation. In order to address whether or not increased CYP2C levels also results in increased in EphB4 phosphorylation, HUVECs were treated with either control or the CYP2C9 virus and a immunoprecipitation of tyrosine phosphorylated receptor proteins was performed (Figure 10). CYP2C9 overexpression resulted in tyrosine phosphorylation of EphB4 as assessed by Western blotting using an antibody directed against EphB4.

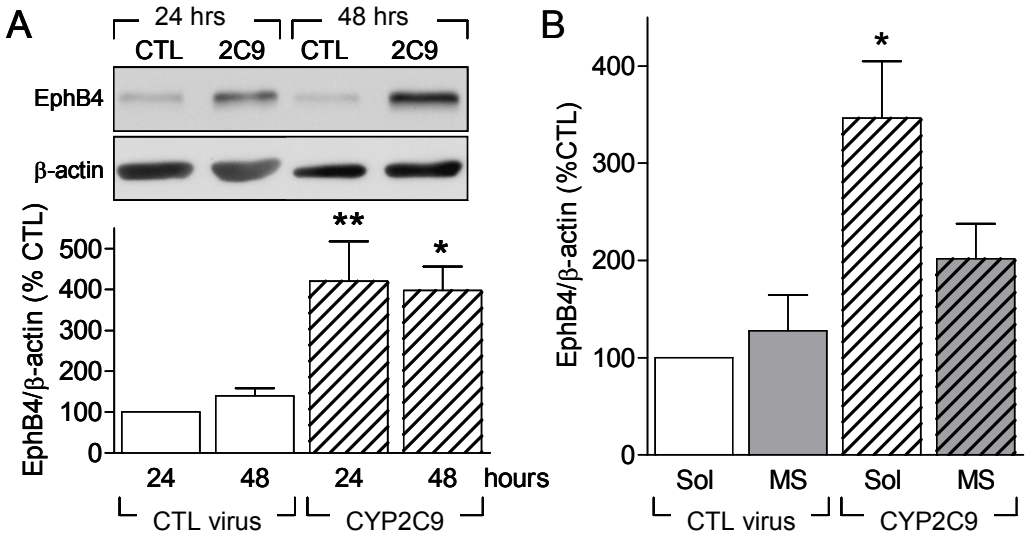

Figure 9. Effect of CYP2C9 overexpression on EphB4 expression in vitro. HUVECs were infected with either a control (CTL) adenovirus or CYP2C9 encoding virus for overexpression (CYP2C9). Experiments were performed in the absence (A) and presence (B) of MSPPOH (MS) (10µmol/L). The bar graphs summarize data obtained in 3-9 independent experiments; *P<0.05, **P<0.001 versus the respective CTL/Sol.

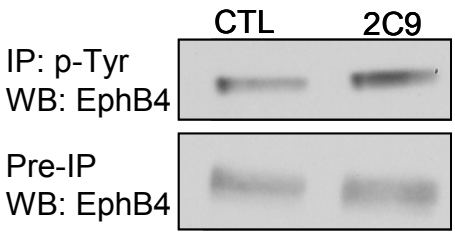

Figure 10. Effect of CYP2C9 overexpression on the tyrosine phosphorylation of EphB4 in HUVEC. Cells were treated with either control (CTL) or CYP2C9 adenoviruses 48 hours before harvesting and the immunoprecipitation (IP) of tyrosine phosphorylated (p-Tyr) proteins. The tyrosine phosphorylation of EphB4 as well as EphB4 expression in the lysate pre-IP was then assessed by Western blotting using an antibody directed against EphB4. Similar results were obtained in 2 additional experiments.

The activation of CYP epoxygenases not only results in the generation of EETs, but also in a series of other biologically active metabolites, e.g. HETEs as well as in production of reactive oxygen species. To ensure that the increase in EphB4 expression can be attributed to elevated EET levels the effect of exogenously applied 11,12-EET on EphB4 expression was assessed. Exogenously applied 11,12-EETs also

elicited a time- (Figure 11A), and concentration-dependent increase in endothelial EphB4 expression (Figure 11B).

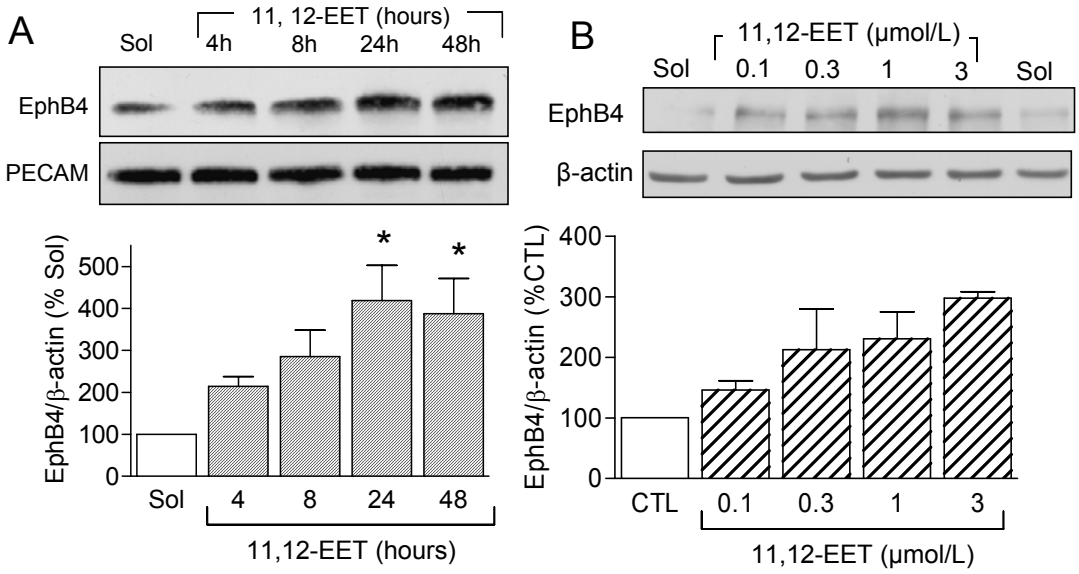

Figure 11. 11,12-EET-induced increase in EphB4 expression. Stimulation of HUVECs with 11,12-EET (1μmol/L) results in a time- and concentration-dependent increase in EphB4 expression. EphB4 expression was assessed after 4, 8, 24 and 48 hours (A) and at 24 hours for the dose response curve at 0.1, 0.3, 1 and 3 μmol/L (B). The bar graphs summarize data obtained in 3-9 independent experiments; *P<0.05, **P<0.001 versus the respective CTL/Sol.

EphrinB2 is the main ligand for EphB4 but is also capable of acting as a signal-transducing molecule itself, a process referred to as "reverse signaling". Traditionally, the ligand has been reported to promote adhesion, migration, chemotaxis, capillary network formation, and sprouting angiogenesis (Füller et al., 2003; Hamada et al., 2003). To clarify whether there is also a link between CYP2C9 and ephrinB2 expression that could account for the increase in EphB4 activation, murine lung endothelial cells were infected with control or CYP2C9. EphrinB2 was expressed by the endothelial cells used, but there was no detectable change in the expression of the protein following the overexpression of CYP2C9 (Figure 12).

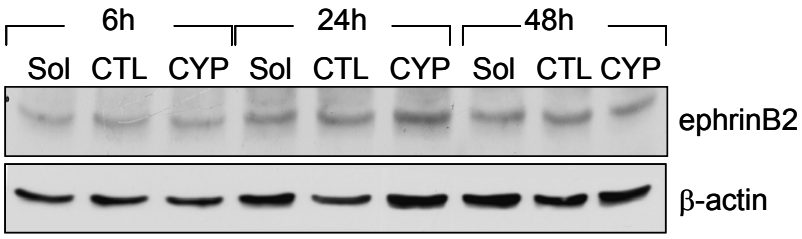

Figure 12. Effect of CYP2C9 overexpression on ephrinB2 expression *in vitro*. Murine lung endothelial cells were treated with solvent (Sol), control virus (CTL) or a CYP2C9 overexpressing virus (CYP) for 6, 24 and 48 hours and ephrinB2 expression levels were assessed by Western blot analysis. Similar results were obtained in 2 additional experiments.

3.2 Role of CYP2C9-induced EphB4 expression in angiogenesis *in vitro*

The results of the *in vitro* experiments showed clearly an involvement of CYP2C in the EphB4 mediated signalling cascade. However, the exact role of EphB4 was unclear.

As mentioned earlier crucial steps during angiogenesis are proliferation and differentiation of endothelial cells to form vascular tubes. Endothelial cell migration and the degradation of the extracellular matrix are essential steps in this angiogenic process. Prior to migration endothelial cells have to degrade the surrounding matrix which consists mainly of collagens, elastin, fibronectin and proteoglycans. These processes can be investigated *in vitro* in two-or three dimensional angiogenesis assays that are based on growth matrices similar to the *in vivo* situation (i.e. collagen, fibrin and Matrigel). To assess the impact of EphB4 on CYP2C9-mediated angiogenesis *in vitro*, a modified Matrigel sprouting assay was used. In an initial step we established an antisense oligonucleotide protocol to down regulate EphB4 levels in the endothelial cells studied. To this end endothelial cells were treated with scrambled or antisense oligonucleotides directed against EphB4. While the scrambled oligonucleotides were without effect, the EphB4 antisense oligonucleotides decreased basal EphB4 protein levels in HUVEC and completely prevented the CYP2C9-induced increase in protein expression (Figure 13A).

Next endothelial cells were incubated with EphB4 antisense oligonucleotides before generating spheroids and sprouting was assessed in the collagen based three-dimensional angiogenesis assay. A significantly attenuated CYP2C9-induced (24

hours) sprout formation was observed in the presence of the EphB4 antisense oligonucleotides (Figure 13B) compared to control cells treated with scrambled oligonucleotides.

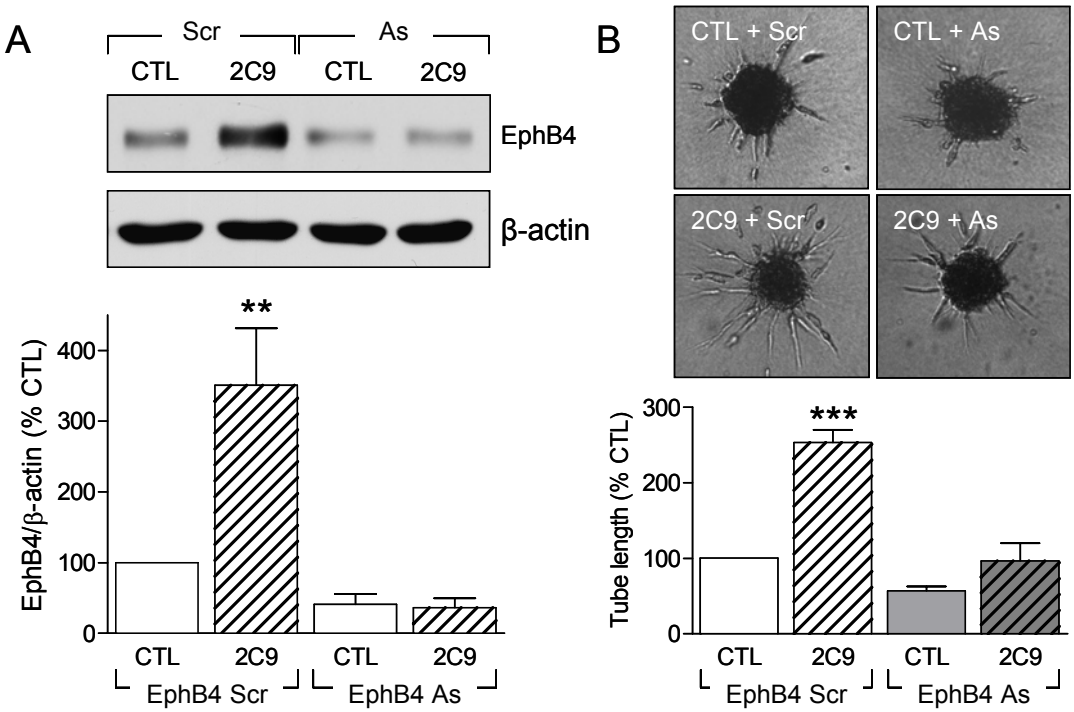

Figure 13. Effect of EphB4 antisense oligonucleotides in CYP2C9 overexpressing cells. (A) HUVECs were first infected with either a control (CTL) adenovirus or CYP2C9 encoding virus for overexpression (CYP2C9) and then transfected with EphB4 antisense oligonucleotides (As) or control (scrambled) oligonucleotides (Scr). (B) Endothelial cell sprouting was assessed in a collagen based spheroid assay. HUVECs were infected with either control (CTL) or CYP2C9 adenoviruses and transfected with EphB4 antisense (As) or scrambled (Scr) oligonucleotides. After 24 hours, spheroids were formed and seeded into a collagen gel after an additional 24 hours. Sprouting was assessed after 48 hours in the gel. The bar graphs summarize data obtained in 3-10 independent experiments; **P<0.01, ***P<0.001 versus CTL.

3.3 Role of the PI3K signalling pathway in CYP2C-induced angiogenesis

So far relatively little is known about the signalling pathways upstream of EphB4 and most of the proteins identified in the Eph receptor downstream signalling pathways have

been implicated in regulating cell morphology, attachment and motility (Cheng et al., 2002). The PI3K can be activated by a variety of extracellular signals and is involved in a number of cellular processes including cell proliferation, survival, protein synthesis, and tumour growth. As CYP2C-derived EETs, in particular 11,12- and 14,15-EET, have been reported to activate the PI3K/Akt signaling pathway in endothelial cells (Fleming et al., 2001a), the effects of CYP2C9 overexpression and the consequences of PI3K inhibition on this response were assessed.

Initially the effect of overexpression of CYP2C9 on EphB4 promoter activity was characterized. CYP2C9 increased EphB4 promoter activity compared to that detected in cells transfected with pcDNA (Figure 14A). To study whether or not this increase in EphB4 promoter activity was mediated by PI3K the effect of the PI3K inhibitors LY294002 (1.5 µmol/L) and wortmannin (100 nmol/L) on this increase in luciferase activity was investigated. Treatment with both inhibitors resulted in similar inhibitory effects on EphB4 promoter activity (Figure 14A) as well as at protein level (Figure 14B).

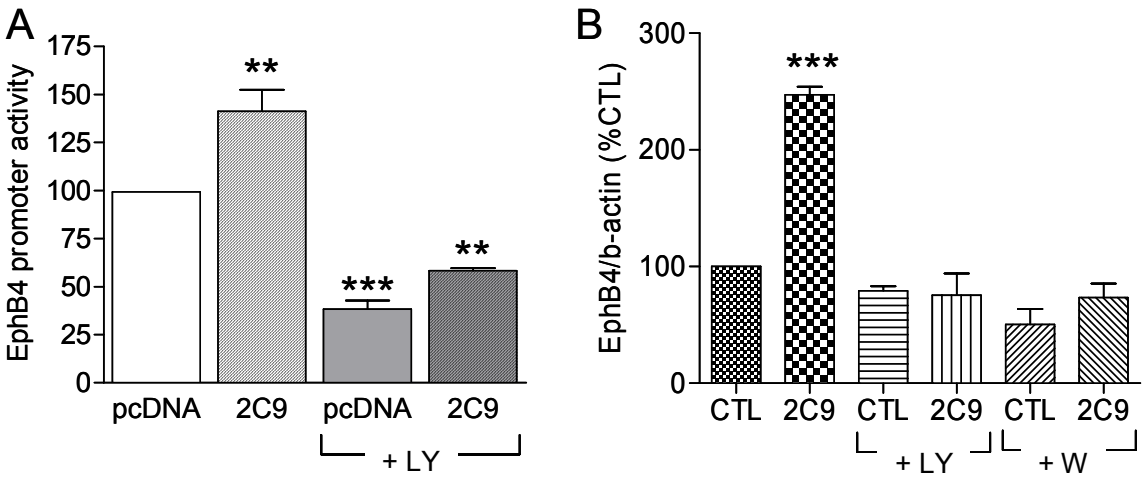

Figure 14. Effect of PI3K/Akt pathway inhibition on EphB4 promoter activity and protein expression in cells lacking or overexpressing CYP2C9. (A) Endothelial cells were either transfected with CYP2C9 or a control plasmid (cDNA) and the EphB4 promoter and promoter activity was assessed in the absence and presence of LY294002 (LY) (1.5 µmol/L). (B) HUVECs were infected with either a control (CTL) adenovirus or CYP2C9 encoding virus for overexpression (CYP2C9) was assessed in the absence and presence of LY294002 (LY) (1.5 µmol/L) and wortmannin (W) (100 nmol/L). The bar graphs summarize data obtained in 3-6 independent experiments; *P<0.05, **P<0.01, ***P<0.001 versus the respective CTL.

Wortmannin and LY294002 are not completely specific for the PI3K (Knight and Shokat, 2007). Therefore we confirmed that CYP2C9 overexpression increased Akt phosphorylation (Figure 15A) and determined whether or not a dominant negative Akt mutant could inhibit the CYP2C9-induced increase in EphB4 expression. As before, EphB4 expression increased in cells treated with CYP2C9 adenoviruses. However, no increase in EphB4 was observed in cells overexpressing CYP2C9 and transfected with the dominant negative Akt mutant (Figure 15B).

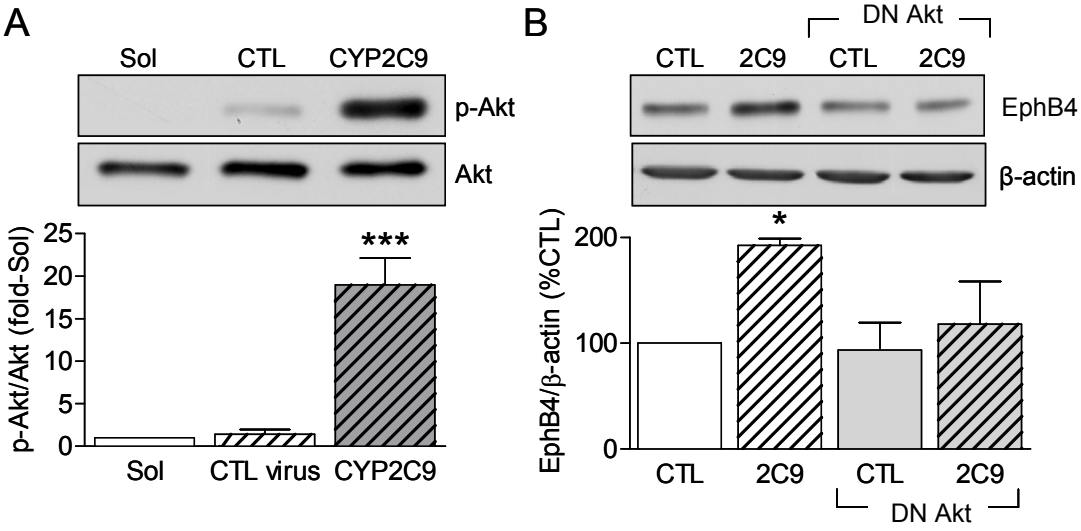

Figure 15. Effect of CYP2C9 on Akt phosphorylation and effect of a dominant DN Akt on the CYP2C9-induced expression of EphB4. HUVECs were infected with either a control (CTL) adenovirus or CYP2C9 encoding virus for overexpression (CYP2C9) and transfected with a DN Akt. Akt phosphorylation in CYP2C9-overexpressing cells (A) as well as the effect of DN Akt on EphB4 expression in CYP2C9 overexpressing cells was analysed (B) by Western blot. The bar graphs summarize data obtained in 3-4 independent experiments; *P<0.05, **P<0.01, ***P<0.001 versus the respective CTL.

3.4 Role of EETs in cell proliferation and angiogenesis *in situ*

The results of the studies detailed above clearly describe a correlation between CYP2C-derived EETs and EphB4 expression *in vitro*. It should be noted that ephrinB2 is classified as an arterial marker whereas EphB4 expression was thought to be restricted to venous endothelial cells (Gerety et al., 1999; Shin et al., 2001). In this study EphB4 expression was confirmed in venous endothelial cells, but to further characterize the (patho)physiological relevance of such effects and eventually be able to transfer these

observations to the *in vivo* situation, EET-mediated EphB4 expression was further characterized *in situ.*

Isolated mesenteric arteries and veins were incubated with either solvent (DME medium containing 15% FCS) or 11,12-EET (1 µmol/L) and EphB4 was assessed by immunofluorescence after 18 hours. Quantification of fluorescence staining confirmed the EET-induced increase in EphB4 levels in mesenteric arteries (Figure 16A). Although EphB4 is reported to be preferentially expressed in veins (Wang et al., 1998) a much weaker response was detected in mesenteric veins treated with 11,12-EET (Figure 16B).

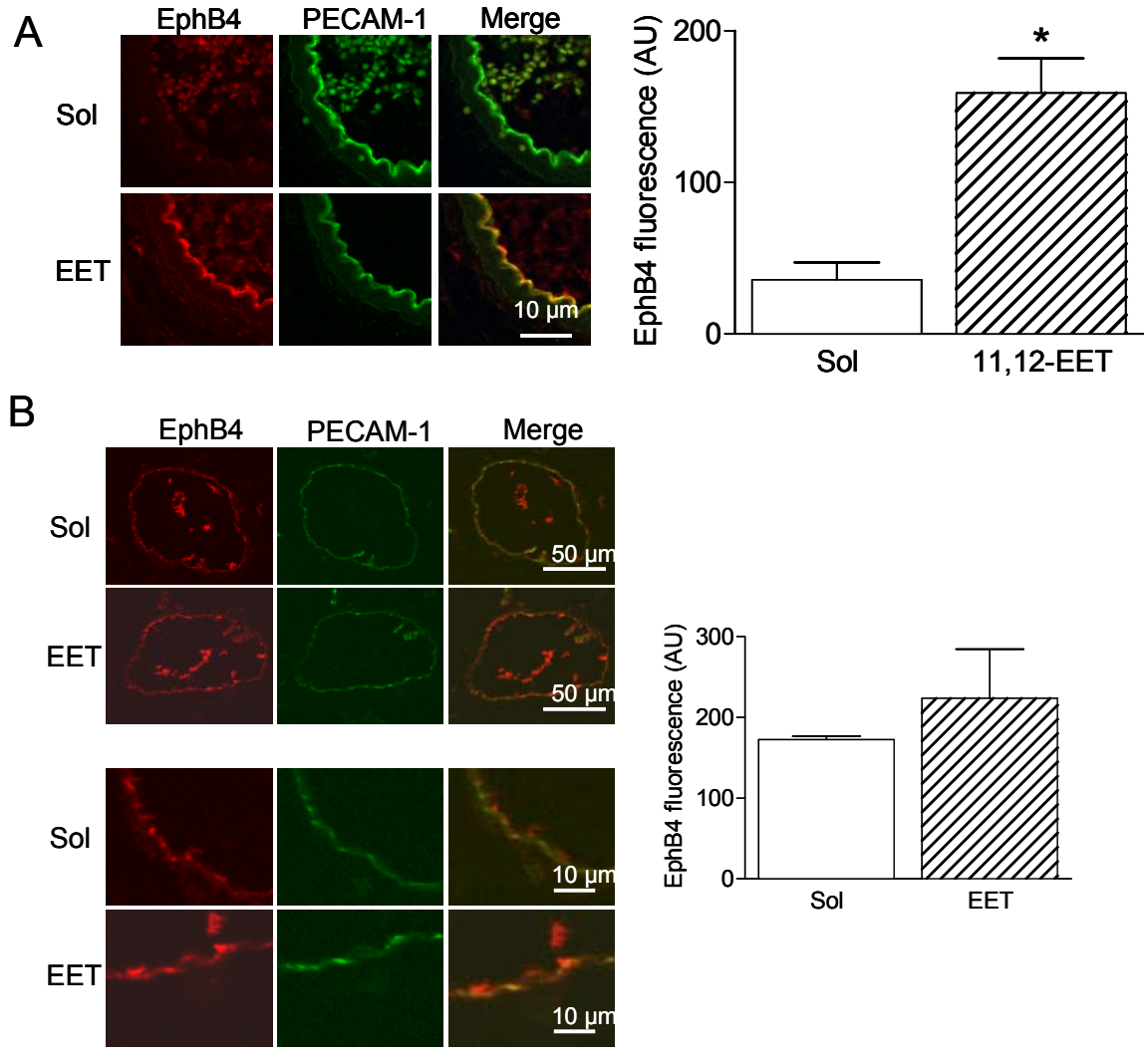

Figure 16. Effect of 11,12-EET on EphB4 expression *in situ*. Mesenteric arteries (A) and veins (B) were incubated with solvent (Sol) or 11,12-EET and the expression of EphB4 (red) and PECAM-1 (green) was assessed. The bar graph summarizes data obtained using vessels from 3 different animals; *P<0.05 versus Sol.

3.5 Role of CYP-induced EphB4 expression *in vivo*

The *in vitro* angiogenesis assay revealed a link between EphB4 and CYP2C-derived EETs, but even though an increase in EphB4 expression after EET-stimulation could also been confirmed *in situ* the ultimate physiological relevance needs to be assessed *in vivo*. Therefore the next crucial step was to evaluate and further specify the effects seen *in vitro* in an *in vivo* angiogenesis assay.

To perform studies targeting EphB4 expression in endothelial cells invading Matrigels implanted into mice it was initially necessary to develop an siRNA approach that downregulated murine EphB4 levels. Therefore, murine lung endothelial cells were treated with three different siRNAs directed against EphB4 and EphB4 levels assessed under control conditions and following CYP2C9 overexpression. The overexpression of CYP2C9 in murine lung endothelial cells increased the expression of EphB4 as detected in HUVECs. Two of the siRNA tested (siRNA1 and siRNA2) prevented the CYP2C9-induced increase in EphB4 expression but only one of them (siRNA1) also decreased basal levels of the protein (Figure 17).

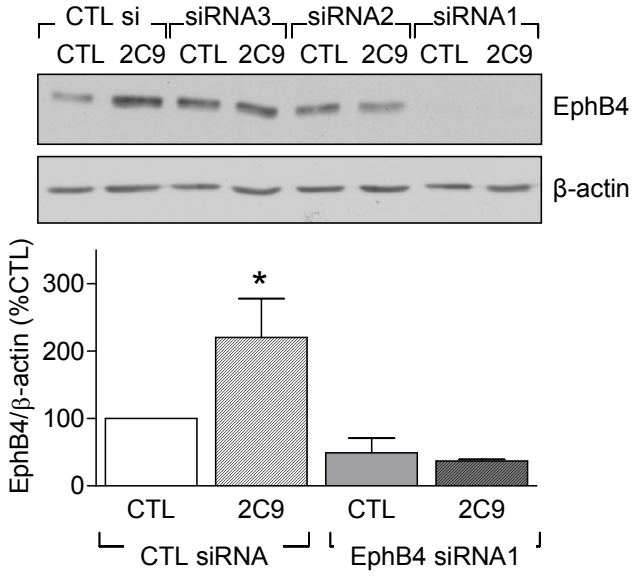

Figure 17. SiRNA-mediated downregulation of EphB4 in murine endothelial cells. Mouse lung endothelial cells were infected with either control (CTL) or CYP2C9 (2C9) adenoviruses and transfected with three different siRNAs directed against EphB4. The bar graph summarizes data obtained in 3 independent experiments; *P<0.05 versus CTL.

Having identified an effective siRNA, the next step was to impregnate Matrigels with solvent or 11,12-EET in the absence and presence of either a control siRNA (GFP) or the siRNA1 against EphB4. These Matrigels were then implanted in 8 week old female C57/BL6 mice (two per animal, one on each side of the spine) for a total of 7 days. Thereafter, the infiltration of endothelial and smooth muscle cells into the Matrigel plugs was assessed by immunohistochemistry. While PECAM-1 positive endothelial cells could be detected in plugs treated with solvent, fully formed vessels (i.e. PECAM-1 positive tube-like structures covered by α-actin positive cells) were detected in plugs impregnated with 11,12-EET (Figure 18A).

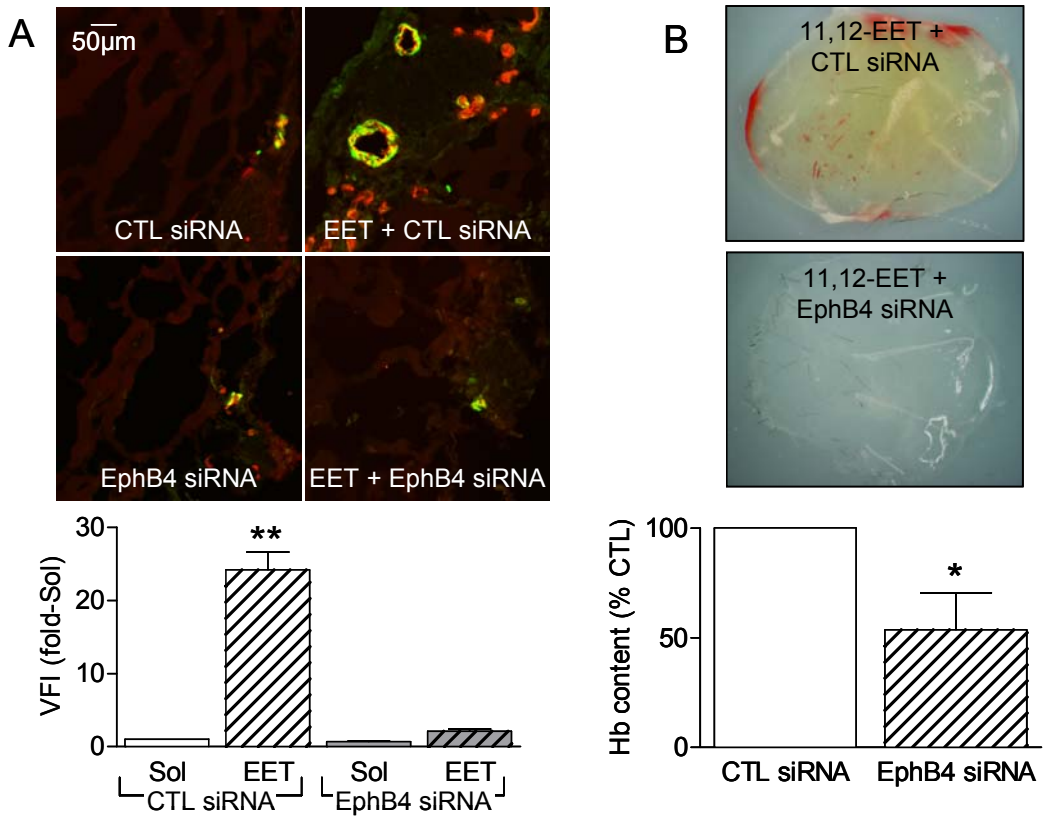

Figure 18. Effect of downregulating EphB4 on angiogenesis *in vivo*. Matrigel was impregnated with solvent (Sol) or 11,12-EET (10 µmol/L) together with either a control siRNA or EphB4 (siRNA1) and injected subcutaneously dorsally in mice. After 14 days (A) cryosections of the Matrigel plugs were stained for PECAM-1 (red) and α-actin (green) cells. (B) General appearance and hemoglobin (Hb) content of 11,12-EET-impregnated Matrigel plugs also containing either the CTL or EphB4 siRNA. The bar graphs summarize data from 3-4 animals per group; *P<0.05, **P<0.01 versus CTL siRNA-containing plugs.

These plugs were also blood perfused as they not only had a reddish colour suggesting that vessels invaded the plug, but also contained measurable levels of haemoglobin (Figure 18B). The inclusion of EphB4 siRNA1 in the Matrigel plugs completely prevented 11,12-EET-mediated infiltration of endothelial and smooth muscle cells into the plug and the accumulation of haemoglobin.

Apart from demonstrating how crucial EphB4 is to the EET-mediated angiogenic response these data highlight that the impregnation of Matrigel with 11,12-EET results in recruitment of endothelial cells as well as smooth muscle cells to generate functional vessels. It is however not known if EETs also have an effect on the recruitment of pericytes, the vascular mural cells embedded within the vascular basement membrane of blood microvessels, where they make specific focal contacts with the endothelium. Staining for desmin, a specific pericyte marker, also revealed a role of EETs in the recruitment of pericytes (Figure 19). This EET-mediated increased infiltration of desmin-positive structures was abolished by siRNA directed against EphB4, whereas control siRNA had no effect.

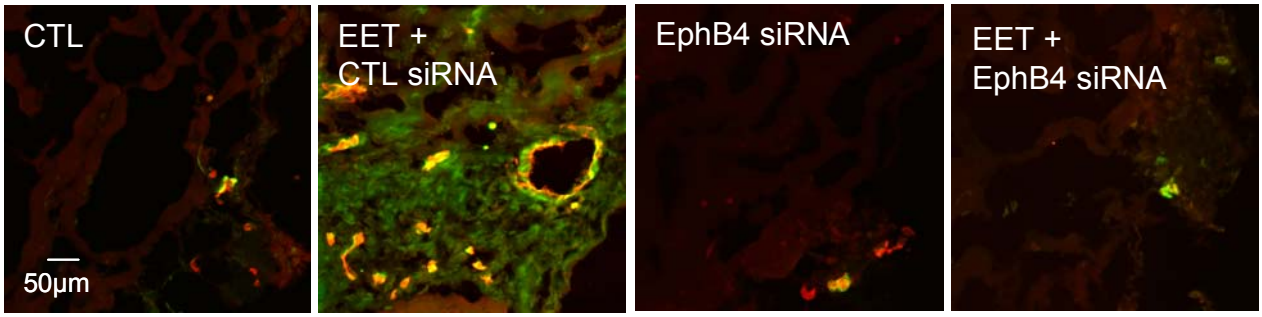

Figure 19. Effect of CYP2C9-derived EETs on EphB4-induced pericyte recruitment. Representative pictures showing desmin (green) and PECAM-1 (red) expressing cells in the Matrigel plug after treatment with solvent (Sol) or 11, 12-EET and with or without siRNA directed against murine EphB4.

The results of the staining for PECAM-1, smooth muscle cells and pericytes clearly suggests that EphB4 is involved in the EET-mediated angiogenic response, but at this stage this does not provide any evidence that EphB4 is present in vessels formed after stimulation with EET. Hence EET-impregnated Matrigel plugs were stained for EphB4. Compared to control plugs that were treated with solvent the vessel like structures that formed within those plugs expressed EphB4 (Figure 20).

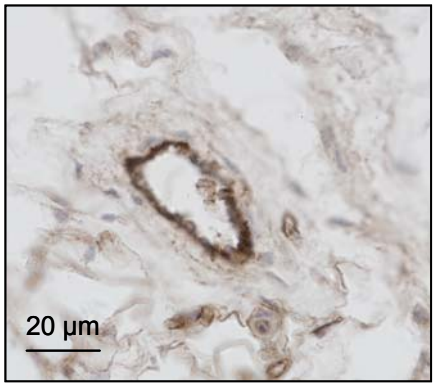

Figure 20. Effect of 11,12-EET on EphB4 expression in the Matrigel Plug. Representative section showing EphB4 expression in a Matrigel plug impregnated with 11,12-EET (10 µmol/L) 7 days after subcutaneous injection in mice. Similar pictures were taken from plugs of three additional mice.

3.6 Effect of VEGF on CYP2C expression

VEGF is a key regulator of physiological and pathological angiogenesis. *In vitro*, VEGF induces endothelial cell proliferation, migration as well as being an endothelial cell survival factor. Since the expression of VEGF is dependent on hypoxia (Levy et al., 1996) and it has recently been shown that CYP2C8 is implicated in hypoxia-induced angiogenesis (Michaelis et al., 2005b) we postulated a link between VEGF- and CYP epoxygenase signalling.

Stimulation of endothelial cells with VEGF increased CYP2C mRNA (Figure 21A) and protein levels (Figure 21B) in primary cultures of human endothelial cells.

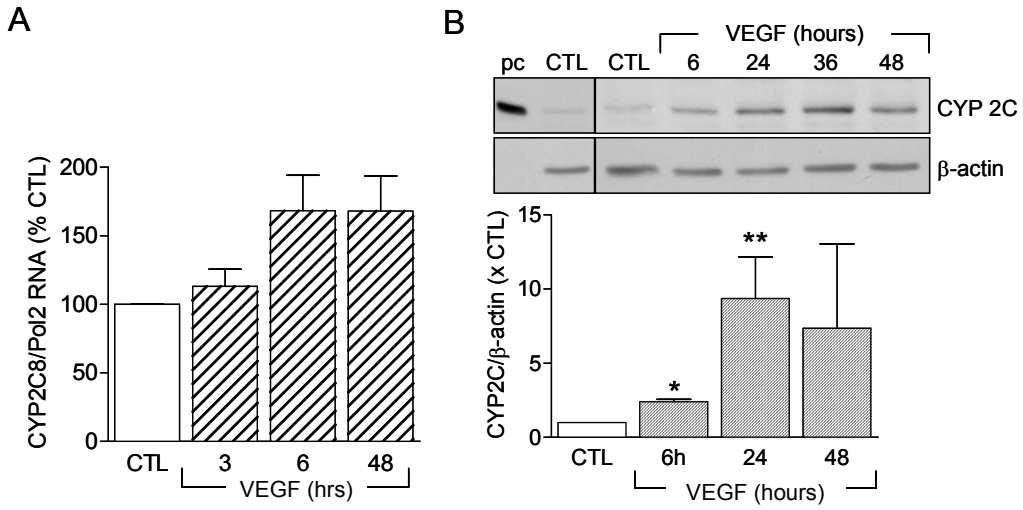

Figure 21. Effect of VEGF on the expression and activity of CYP2C in endothelial cells. Time course showing the effect of VEGF (30 ng/mL, 3 to 48 hours) on the expression of (A) CYP2C mRNA and (B) CYP2C protein in HUVECs The bar graphs summarize data obtained in 7-13 independent experiments; *P<0.05, **P<0.01, ***P<0.001 versus control (CTL).

VEGF also regulates CYP2C9 at the transcriptional level as stimulation with VEGF increased CYP2C9 promoter activity in cells expressing VEFR-1 or VEGFR-2 (Figure 22).

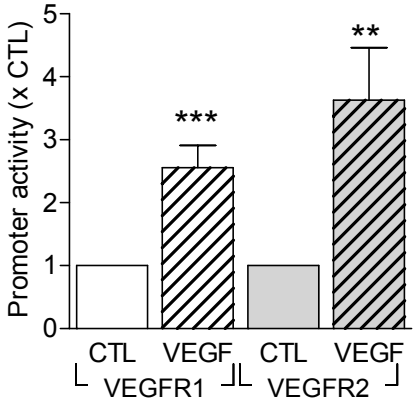

Figure 22. Effect of VEGF on the CYP2C9 promoter activity. Porcine endothelial cells overexpressing VEGF-R1 or VEGF-R2 were transfected with the CYP2C promoter construct 14 hours prior to stimulation with VEGF (30 ng/mL, 6 hours). The bar graphs summarize data obtained in 7-13 independent experiments; *P<0.05, **P<0.01, ***P<0.001 versus control (CTL).

The CYP2C9 promoter was used, because we were unable to generate a CYP2C8 promoter construct, which seems to be the more relevant CYP in endothelial cells. Consequently we had to rely on data from the homologus 2C9 enzyme.

To confirm that as a direct consequence of the increase in CYP epoxygenase expression after VEGF stimulation 11,12-EET levels would increase, endothelial cells were stimulated with VEGF and 11,12-EET levels were measured by LC/MSMS. VEGF resulted in elevated EET levels, an effect that was sensitive to the CYP epoxygenase inhibitor, miconazole (Figure 23).

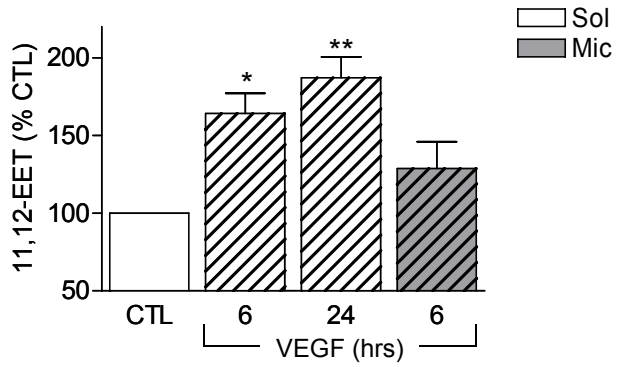

Figure 23. Effect of VEGF on 11,12-EET production in HUVECs. Cells were stimulated with VEGF (30 ng/ml), in some experiments in the presence of miconazole (Mic, 3 µmol/L) and after 24 hours 11,12-EET levels were measured by LC-MS/MS. The bar graphs summarize data obtained in 3-10 independent experiments; *P<0.05, **P<0.01 versus control (CTL).

3.7 Role of the AMP-activated protein kinase (AMPK) in CYP2C-induced angiogenesis

The AMP-activated protein kinase (AMPK) was originally recognized as a regulator of cellular metabolism in response to hypoxia or vigorous contraction in muscle (Hayashi et al., 1998), but has recently been reported to play a role in the regulation of hypoxia- and VEGF-induced angiogenesis (Nagata et al., 2003b). Interestingly, the AMPK has also been implicated in the induction of CYP expression by phenobarbital (Rencurel et al., 2005). We therefore determined the involvement of the AMPK in the VEGF-induced upregulation of CYP2C expression.

To study the possible involvement of the AMPK in the VEGF-mediated increase in CYP2C expression HUVECs cells were stimulated with VEGF. VEGF induced a rapid (within 5 minutes) phosphorylation of the AMPK on Thr172. The response was biphasic with an initial peak at 10 minutes followed by a return to basal levels and a secondary but sustained AMPK activation after 24 hours (Figure 24A).

To further characterize the involvement of VEGF on CYP2C expression cells were infected with the constitutively active and a dominant-negative AMPKα2 mutants. CYP2C RNA levels were increased in endothelial cells overexpressing the AMPKα2 while the overexpression of a dominant negative AMPKα2 mutant prevented the VEGF-induced increase in CYP2C RNA (Figure 24B), and protein (Figure 24C). The CYP inducer phenobarbital (Schuetz, 2001) was used as a positive control to increase endothelial CYP2C expression (Figure 24C). Phenobarbital also elicited an increased phosphorylation of AMPK in endothelial cells (Figure 24A).

To downregulate AMPK expression in endothelial cells invading Matrigels implanted into mice an siRNA approach to downregulated murine AMPK levels was developed. Therefore, murine lung endothelial cells were treated with two different siRNAs directed against AMPK, a mixture of both siRNAs or scrambled siRNA and AMPK levels were assessed under control conditions. Both siRNA tested as (siRNA1 and siRNA2) prevented AMPK expression (Figure 25A).

Having identified two siRNAs to downregulate AMPK expression, Matrigels were impregnated with VEGF (150 ng/mL) in the absence and presence of either a control siRNA (GFP) or a mixture of siRNA1 and 2 against AMPK. These Matrigels were then implanted in 8 week old female C57/BL6 mice (two per animal, one on each side of the spine) for a total of 7 days. Thereafter, the infiltration of endothelial into the Matrigel plugs was assessed by immunohistochemistry (Figure 25B).

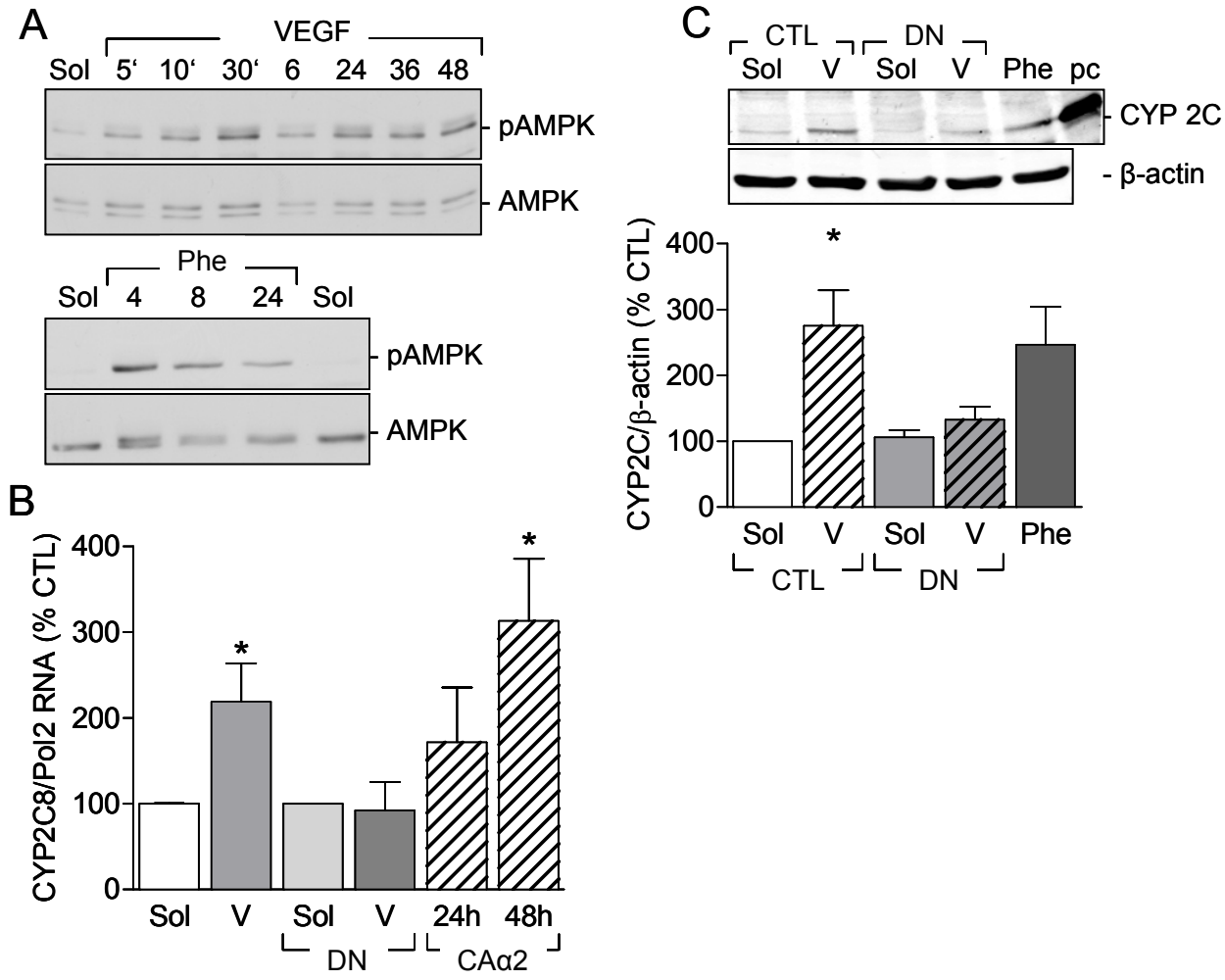

Figure 24. Involvement of the AMPK in VEGF-induced CYP2C expression. HUVECs were stimulated with VEGF (30 ng/mL, 5 minutes to 48 hours) (A) Representative Western blots showing the time-dependent effect of VEGF on AMPK phosphorylation on Thr172. Identical results were obtained in two additional experiments. (B) Effect of AMPK mutants on CYP2C RNA expression. HUVECs were infected with either a control virus or a dominant negative AMPK (DN) adenovirus 48 hours prior to stimulation with either solvent (Sol) or VEGF (30 ng/mL) for 6 hours. CYP 2C mRNA was assessed by RT-PCR. In some experiments endothelial cells were infected with a constitutively active AMPKα2 mutant (CAα2) and CYP2C RNA levels were assessed after 24 and 48 hours. (C) Effect of the dominant negative AMPK mutant on the VEGF-induced increase in CYP2C protein expression. The bar graphs summarize data obtained in 3-4 independent experiments; *P<0.05 versus CTL.

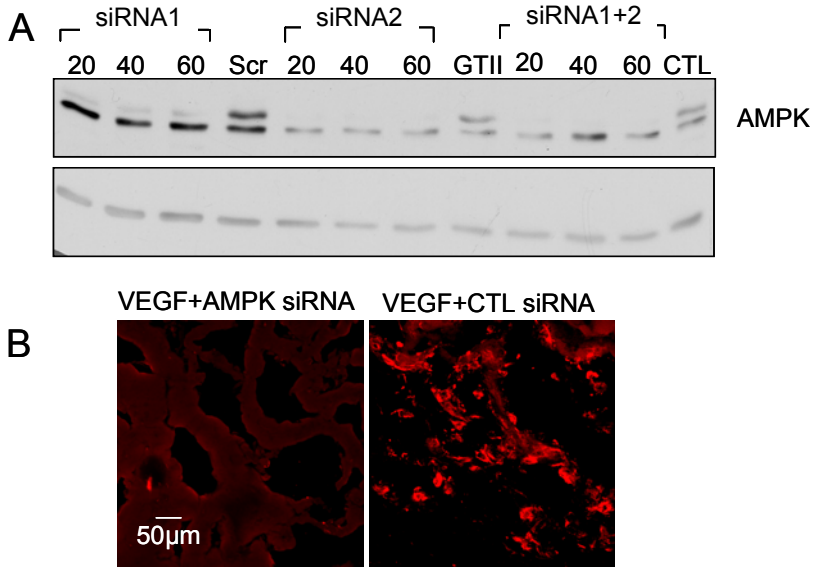

Figure 25. SiRNA-mediated downregulation of AMPK in murine endothelial cells and *in vivo*. (A) Mouse lung endothelial cells were transfected with different concentrations (20, 40 or 60 pM/L) of two different siRNAs, a mixture of both siRNAs directed against AMPK, scrambled siRNA (Scr) or treated with solvent (CTL) or GeneTrans transfection agent (GTII). (B) Matrigel was impregnated with VEGF (150 nmol/mL) together with either a control siRNA or a mixture of siRNA 1 and 2 directed against AMPK and injected subcutaneously dorsally in mice. After 7 days cryosections of the Matrigel plugs were stained for PECAM-1 (red). Similar pictures were obtained in Matrigel plugs from 3 additional animals.

3.8 Role of VEGF-induced CYP2C expression in cell proliferation and angiogenesis *in vitro*

To assess the involvement of CYP epoxygenases in VEGF-mediated tube formation *in vitro* endothelial cell tube formation was assessed in an angiogenesis assay. HUVECs were seeded on cell culture dishes coated with fibronectin and were treated with VEGF (30 ng/mL, 48 hours) in the absence and presence of the EET antagonist 14,15-EEZE (10 µmol/L), or the CYP epoxygenase inhibitor, MSPPOH (10 µmol/L). While endothelial cell tubes were apparent after 48 hours in the VEGF-treated cultures, the number of tubes formed was significantly attenuated in cells treated with either the EET antagonist or the CYP inhibitor (Figure 25A).

bFGF is also known to play a role in the development and maintenance of the vascular system (Lee et al., 2000). As one of the major growth factors involved in angiogenesis it is generally assumed to have very similar effects as VEGF, but it is unknown if

CYP2C is activated by one or several growth factors. Therefore, to determine whether or not the effects observed were specific to VEGF-activated signalling an fibrin gel based angiogenesis assay was performed using bFGF as an angiogenic stimulus. In the latter case, interfering with CYP generation and EET activity was without effect on the ability of the growth factor to stimulate tube formation (Figure 26A).

To determine the specificity of the effects described we ensured that EEZE did not interfere directly with VEGF signalling. We assessed the effect of the EET-antagonist, 14,15-EEZE, on the phosphorylation of the VEGF receptor-2. Stimulation with VEGF resulted in the rapid and pronounced tyrosine phosphorylation of the VEGF receptor as assessed by Western blotting, but EEZE had no effect on either the basal- or VEGF-stimulated phosphorylation of the receptor (Figure 26B).

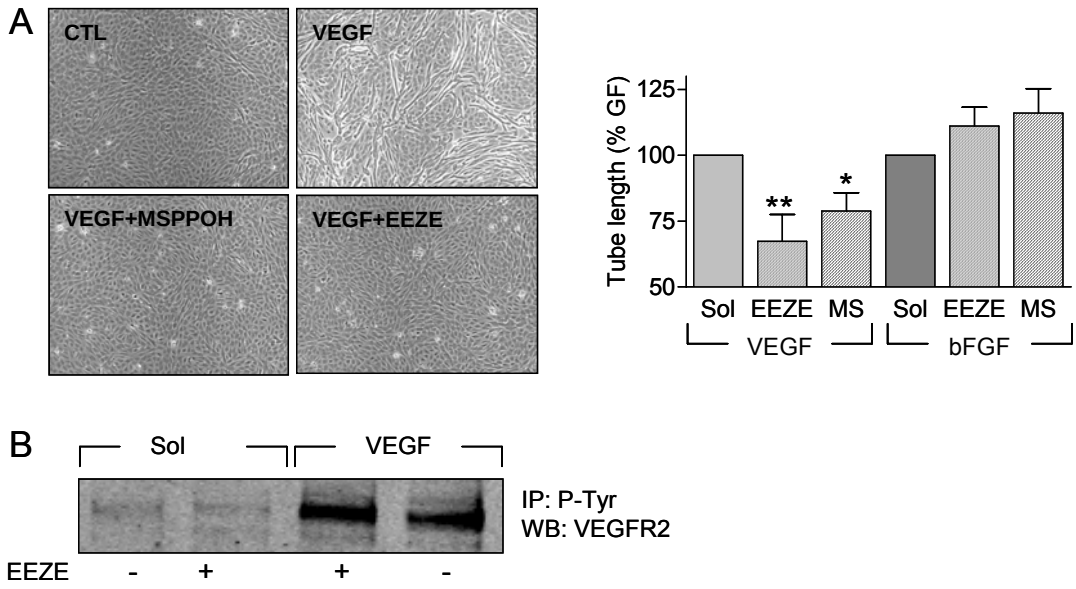

Figure 26. Effect of CYP inhibition and EET antagonism on VEGF-induced endothelial cell tube formation and receptor phosphorylation. (A) Endothelial cell tube formation was assessed in response to VEGF (30 ng/mL) or bFGF (30 ng/mL) in the absence and presence of solvent (Sol; DMSO, 0.01%), the EET antagonist 14,15-EEZE (EEZE, 10 µmol/L) or the CYP2C inhibitor MSPPOH (MS, 10 µmol/L). Four representative photographs showing the effect of VEGF in the absence and presence of EEZE and MSPPOH are shown. The bar graph summarizes data obtained in 3-7 independent experiments; *P<0.05, **P<0.01 versus Sol. (B) Lack of effect of EEZE on the basal VEGF (30 ng/mL, 20 minutes)-induced phosphorylation of the VEGFR2 receptor in phospho-tyrosine immunoprecipitates (IP) from VEGFR2 overexpressing porcine endothelial cells.

The results obtained show that EETs are indeed involved in the angiogenic response mediated by VEGF and that this response seems to be specific and not to be mimicked

by bFGF. However to more directly assess the impact of CYP2C on VEGF-induced endothelial cell sprouting *in vitro* a collagen-based spheroid assay was performed and combined with a previously described antisense oligonucleotide approach (Fisslthaler et al., 1999). In this three-dimensional *in vitro* angiogenesis assay antisense oligonucleotides were used to downregulate CYP2C expression in endothelial cells that were treated at the same time with VEGF. As expected VEGF (30 ng/mL, 24 hours) stimulated endothelial cell sprouting. However, transfecting endothelial cells with CYP2C antisense oligonucleotides 14 hours before generating the spheroids markedly attenuated VEGF-induced sprouting (Figure 27). To exclude any unspecific effects mediated by the transfection reagent CYP2C sense oligonucleotides were used as a control and did not interfere with the response observed in untransfected cells.

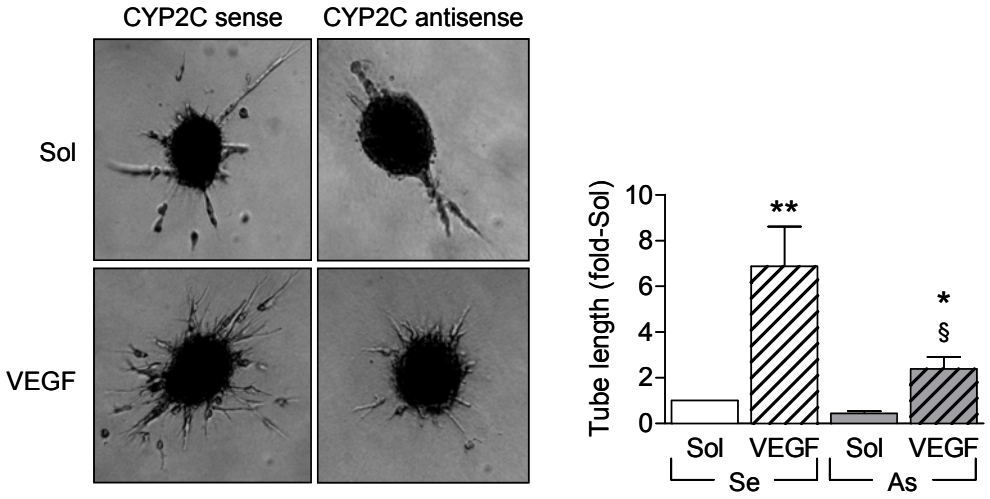

Figure 27. Effect of inhibiting CYP2C expression on VEGF-induced endothelial cell sprouting. Sprouting activity in endothelial cell spheroids treated with solvent (PBS, 0.3%) or VEGF (30 ng/mL, 24 hours). HUVECs were infected with either control (CTL) or CYP2C9 adenoviruses and transfected with CYP2C sense (Se) or antisense (As) oligonucleotides. After 24 hours, spheroids were formed and seeded into a collagen gel after an additional 24 hours. Sprouting was assessed after 48 hours in the gel. The bar graph summarizes data obtained in 3-5 independent experiments; *P<0.05, **P<0.01 versus Sol, $P<0.05 versus VEGF stimulation in cells treated with Se oligonucleotides.

3.9 Role of VEGF-induced CYP-derived EETs in angiogenesis *in vivo*

To assess the role of CYP-derived EETs on VEGF-induced angiogenesis *in vivo*, we used Matrigel plugs impregnated either with solvent, VEGF, the EET antagonist, 14,15-EEZE or a combination of both and injected one dorsally on each side of the spine in 8 week old female C57/BL6 mice. As described earlier cryo sections of frozen plugs were stained for endothelial cells with PECAM-1 and smooth muscle cells with α-actin.
Under the experimental conditions employed, endothelial cells (PECAM-1) and a few sparse smooth muscle cells/pericytes (α-actin) were detected in the VEGF-impregnated plugs after 14 days, only a few endothelial cells and no smooth muscle cells/ pericytes were detected in the solvent- or 14,15-EEZE-containing plugs. However, 14,15-EEZE almost completely abolished the angiogenic effect of VEGF when both were applied in combination (Figure 28).

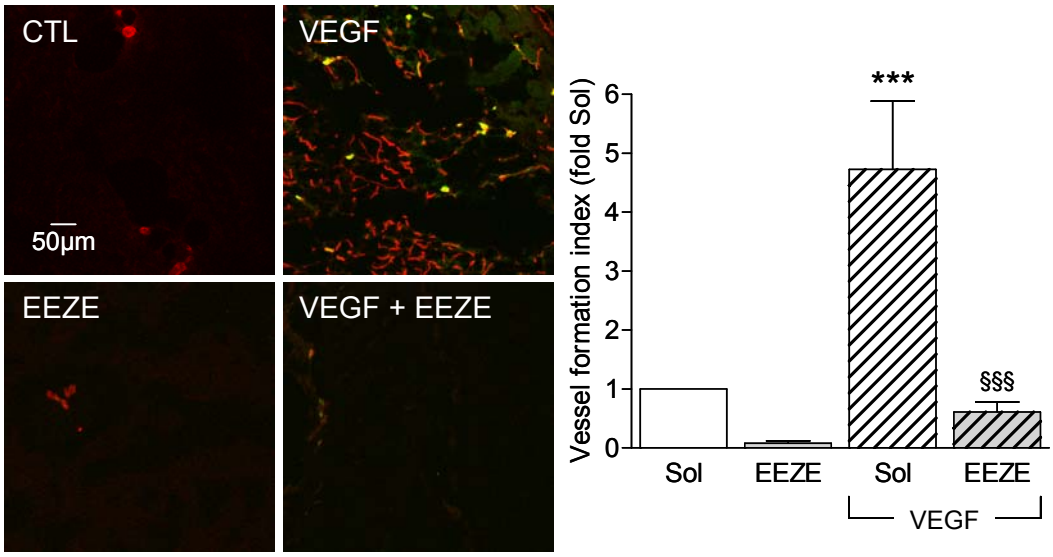

Figure 28. Effect of 14,15-EEZE on VEGF-induced angiogenesis in vivo. Endothelial cell migration into Matrigel plugs impregnated with either solvent (DMSO, 0.1%, CTL), VEGF (150 ng/mL), the EET antagonist 14,15-EEZE (100 µmol/L) or the combination of both VEGF and 14,15-EEZE and injected subcutaneously in mice. Images were taken after sectioning and immunostaining for PECAM-1 (red) and α-actin (green). The bar graphs summarize data obtained in 4-10 independent experiments; *P<0.05, ***P<0.001 versus the appropriate solvent (Sol) in the absence of EEZE, §§§P<0.001 versus VEGF.

Our previous *in vitro* data indicated that the EET antagonist 14,15-EEZE selectively interfered with VEGF activated angiogenesis. However, *in vitro* angiogenesis assays

focus on the proliferative and migratory processes of the angiogenic response where only endothelial cells, but no pericytes/smooth muscle cells are involved. To demonstrate that the effect of 14,15-EEZE was also specific for VEGF *in vivo*, the same assay was repeated using Matrigel plugs impregnated with bFGF alone or in combination with 14,15-EEZE. While bFGF clearly induced endothelial cell invasion of the plugs, this effect was insensitive to the EET antagonist (Figure 29).

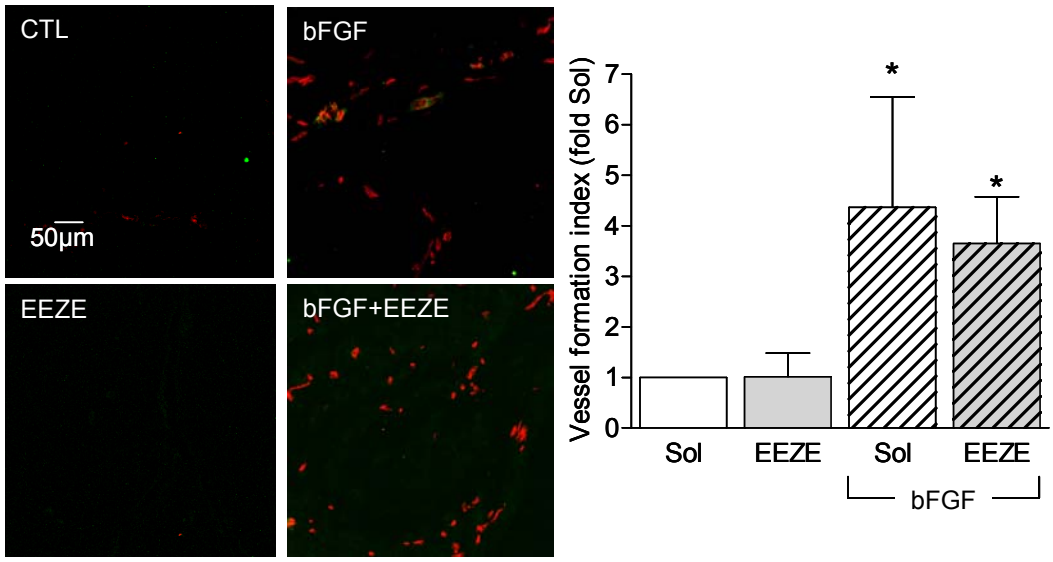

Figure 29. Effect of 14,15-EEZE on bFGF-induced angiogenesis in vivo. Endothelial cell migration into Matrigel plugs impregnated with either solvent (Sol), bFGF (150 ng/mL), the EET antagonist 14,15-EEZE (100 µmol/L) or the combination of both bFGF and 14,15-EEZE injected subcutaneously in mice. Images were taken after sectioning and immunostaining for PECAM-1 (red) and α-actin (green) and scored as the vessel formation index (see methods). The bar graphs summarize data obtained in 4-10 independent experiments; *P<0.05, ***P<0.001 versus the appropriate solvent (Sol) in the absence of 14-15-EEZE.

At this point it was unclear if impregnation of Matrigel plugs with EETs could mimic the effect seen with VEGF. Therefore to verify the role of EETs in angiogenesis *in vivo* and to compare the consequence of VEGF and 11,12-EET in the generation of vessel-like structures, the Matrigel plug assay was repeated. In plugs that contained 11,12-EET, tube-like structures of endothelial cells covered with smooth muscle cells were detectable, whereas in the VEGF-impregnated plugs only endothelial tubes were detectable. Moreover the latter were not covered by smooth muscle cells (Figure 30) or pericytes.

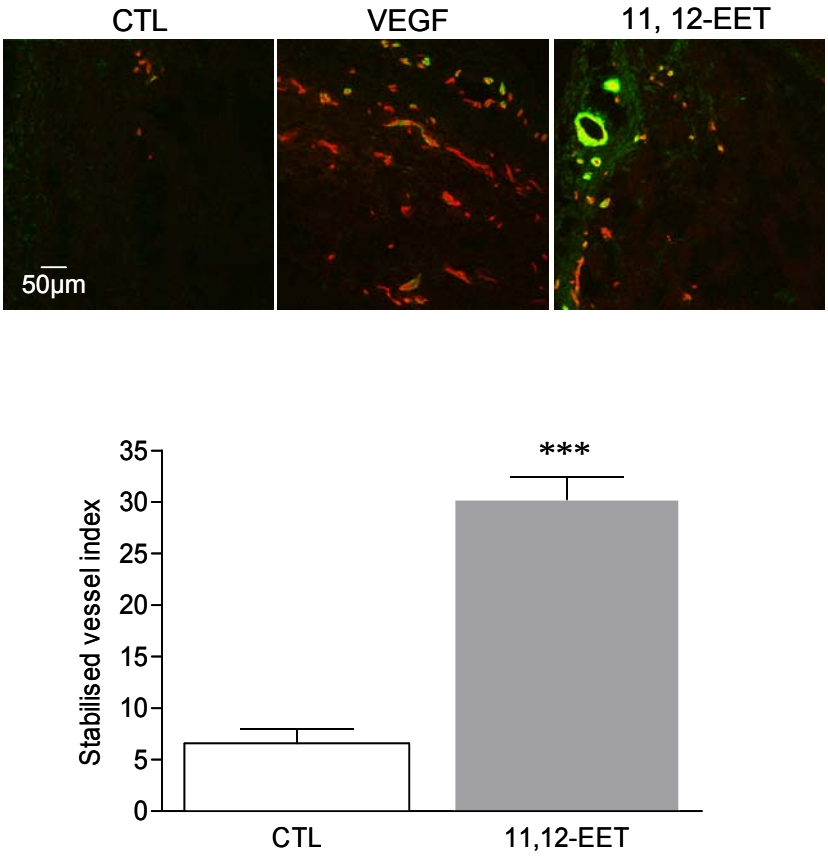

Figure 30. Comparison of the effects of VEGF and 11,12-EET on vessel stabilization in vivo. Matrigel plugs were injected subcutaneously in mice. After 14 days plugs impregnated with either VEGF (150 ng/mL) or 11,12-EET (10 µmol/L) were harvested and processed for immunostaining. Representative images showing endothelial cell (PECAM-1 staining, red) and smooth muscle/pericyte (α-actin, green) content and scored as the stabilized vessel formation index (see methods). The bar graphs summarize data obtained in 4-10 independent experiments; *P<0.05 versus the appropriate control (CTL).

As smooth muscle cell covered tube-like structures were detectable in EET-impregnated plus and recruitment of mural cells is essential for vessel stabilization an obvious point to address was the functionality (i.e. perfusion) of these structures.

Therefore, to assess whether or not those vessel like structure were functional isolectin was injected through the tail vein of mice before they were sacrificed. As reported above, the addition of VEGF to the Matrigel significantly increased the number of endothelial cells detected in the plugs after 14 days, and, again little or no smooth muscle cells/pericytes could be detected. Consistent with the formation of unstable endothelial tubes, we found no evidence of perfusion of the VEGF-treated plugs as we

were unable to detect significant isolectin in the plugs. The situation was clearly different in plugs impregnated with 11,12-EET in which we detected a significant number of endothelial cell-containing structures surrounded by α-actin expressing cells. Moreover, isolectin was detected in all of the 11,12-EET-containing plugs following injection into the tail vein. No isolectin signal was detected in the control plugs recovered from the same animals (Figure 31). These findings confirm dat presented in figure 18 in which we reported significant haemoglobin levels in EET-containing plugs.

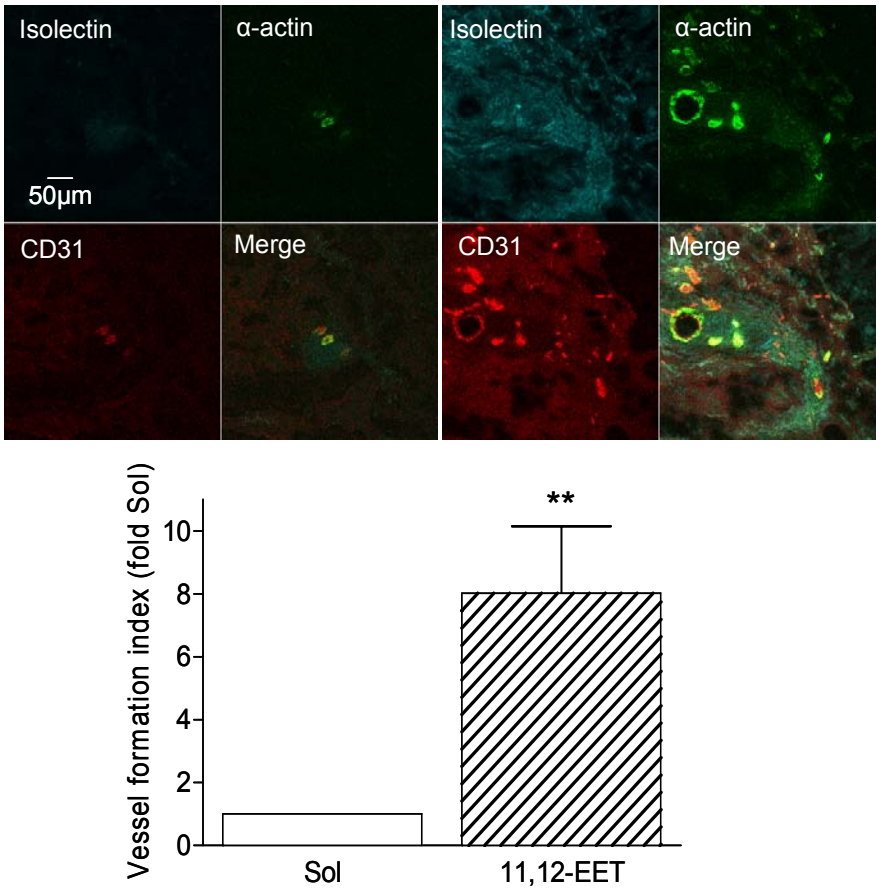

Figure 31. Demonstration of functional vessels in 11,12-EET-containing Matrigel plugs. Images were made after injection of isolectin through the tail vein, sectioning and immunostaining for isolectin (turquoise), PECAM-1 (red) and α-actin (green). The number of perfused vessels in each plug was scored as the vessel formation index (see methods). The bar graph summarizes data obtained in plugs from 3 (5 images per plug) mice per stimulation; **P<0.01 versus Sol.

To verify these observations the plugs were subjected to blood flow analysis using contrast-enhanced ultrasound measurements. Looking at the time-dependent

accumulation of contrast agent, significantly higher peak enhancement was found in 11,12-EET-impregnated plugs than in control plugs in the same animals (Figure 32).

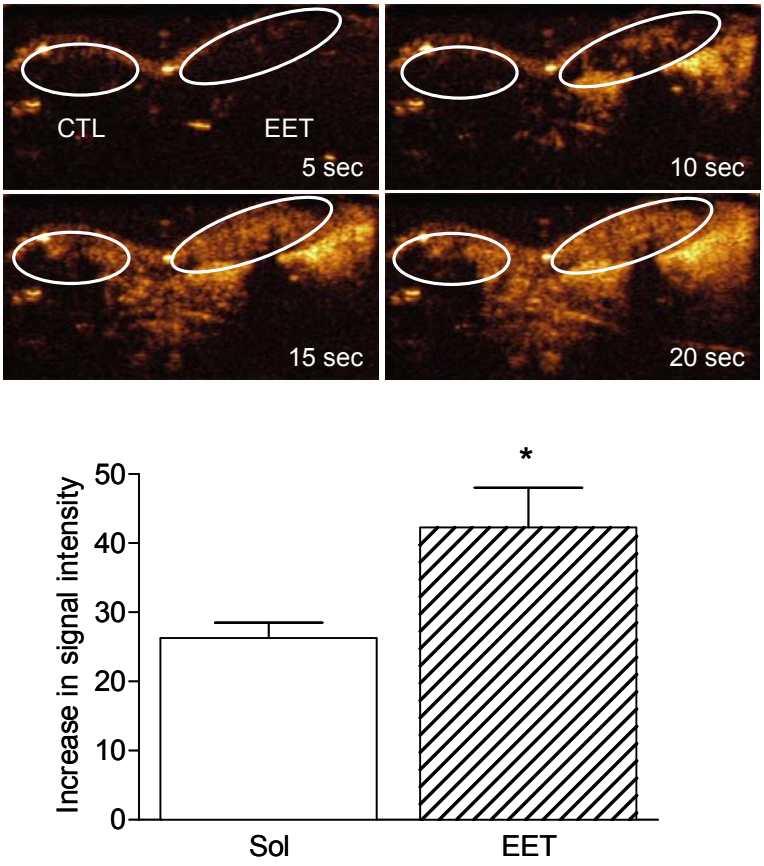

Figure 32. Ultrasound analysis of Matrigel perfusion in vivo. Solvent (Sol, DMSO, 0.01%) and 11,12-EET (10 µmol/L)-impregnated Matrigel plugs were implanted into mice (dorsal, one plug on each side of the spine) and blood flow was determined by ultrasound after 14 days. The representative images show the accumulation of contrast agent in the Sol- and EET-containing plugs 5, 10, 15 and 20 seconds after injection. The bar graph summarizes data obtained using 4 mice; *P<0.05 versus Sol.

4. Discussion

The results of the present investigation document the importance of CYP2C-derived EETs at different stages of the angiogenic response and highlight the intimate relationship between EET and key components of the regulation of proliferation and angiogenesis, i.e. EphB4 and VEGF.

We found that CYP2C9 overexpression and enhanced EET production lead to EphB4 upregulation via the Akt/PI3K pathway and that this effect *in vitro* as well as *in situ* is mimicked by the epoxygenase metabolite 11,12-EET. Moreover, moving away from overexpression studies, we found that VEGF stimulation increases CYP2C9 expression and subsequent EET-production by a mechanism that relies on the activation of the AMPK. Furthermore, it was possible to demonstrate *in vitro* that EphB4 as well as VEGF play a critical role in the angiogenic response initiated by CYP2C9 as inhibition of both pathways resulted in decreased endothelial sprouting. *In vivo* the significance of the *in vitro* findings was assessed in a Matrigel plug assay in which the downregulation of EphB4 as well as the use of an EET-antagonist resulted in decreased infiltration of endothelial cells as well as smooth muscle cells into the EET-impregnated plug. Thus, our findings characterize 11,12-EET not only as an angiogenic stimulus that plays a role in the modulation of signalling pathways and events that lie down as well as upstream of CYP2C activation, but also as a second messenger for a growth factor, i.e. VEGF, already well known to be involved in the angiogenic response. Moreover, our findings indicate that CYP2C-derived EETs have the potential to do much more than promote cell proliferation and tube formation and reveal an important role of EETs in the vascular maturation process that could have potential significance in the treatment of a number of diseases such as cancer and diabetic retinopathy.

4.1 Role of EphB4 and VEGF in CYP2C-induced angiogenesis

In general very little is known about the exact mechanisms involved in the angiogenic response initiated by CYP2C-derived EETs. It has for example been shown that CYP-derived EETs are able to stimulate matrix metalloproteases and thereby transactivate the EGF receptor resulting in the activation of the serine threonine kinase Akt and an

enhanced expression of cyclin D1 (Michaelis et al., 2003). However, no studies have yet elucidated in any great detail the involvement of any other major growth factors.

Eph receptor tyrosine kinases and their ligands, the ephrins, are involved in a number of processes that are crucial for several molecular and cellular mechanisms that control the formation of the vascular network and its remodelling (Holder and Klein, 1999). Up till now their function has mostly been studied in the nervous system where the Eph/ephrin system is involved in the development of both the central and peripheral neural system (O'Leary and Wilkinson, 1999), but recently a number of studies also demonstrated a role of Ephs and their ligands in the development of the cardiovascular system (Wang et al., 1998; Adams et al., 1999). Although the exact consequences of Eph-ephrin interactions and the associated forward and reverse signalling on endothelial cell function in adult animals are not clear, it has been proposed that the Eph-ephrin signals are likely to play a role in the spatial organization of developing/remodelling vasculature (Füller et al., 2003).

Our findings indicate that CYP2C9-overexpression results in an upregulation of EphB4 in HUVECs as well as in the Matrigel plug in mice *in vivo*. The fact that this upregulation is a direct consequence of increased CYP2C expression was confirmed using the epoxygenases inhibitor MSPPOH. However, metabolism by CYP epoxygenases not only results in the generation of EETs, but also in the production of reactive oxygen species (Fleming et al., 2001b). Although it has been shown that EphB4 is involved in tumorigenesis (Noren and Pasquale, 2007) and also that reactive oxygen species play a role in the signalling pathways involved in tumour progression (Wu, 2006), the increase in EphB4 expression elicited by CYP2C9 could be mimicked by one of the archidonic acid epoxides, i.e. 11,12-EET. Further evidence was provided *in situ* where stimulation of mesenteric arteries and veins with 11,12-EETs also resulted in increased EphB4 expression, making it unlikely that CYP2C-derived reactive oxygen species underlie the effects observed.

Regulation of Eph receptor expression occurs at many levels that include induction of transcription and internalization of the receptor-ligand complex. CYP2C9-mediated upregulation could be detected at the transcriptional level as the EphB4 promoter was activated in CYP2C9 overexpressing cells. One of the transcription factors that has emerged a major regulator of EpB4 expression is homeobox A9 (Bruhl et al., 2004). However, CYP2C9 overexpressing cells activated the EphB4 full length promoter construct to the same extent as a promoter construct with mutation of the putative

HoxA9 binding site (data not shown), indicating that homeobox A9 is not essential for the CYP2C-mediated activation of EphB4.

A number of EET-mediated effects *in vitro* and *in vivo* can be attributed to an increase in the intracellular concentration of cAMP and the subsequent activation of PKA. It has for example been shown that 11,12-EETs elicit afferent arteriolar vasodilation via activation of adenylate cyclase and PKA (Imig et al., 1999). Furthermore interendothelial communication via gap-junctions is mediated by a similar mechanism (Popp et al., 2002). In endothelial cells overexpression of CYP increases cAMP levels, stimulates the cAMP-response element-binding protein, and enhanced cyclooxygenase-2 (COX-2) promoter activity which contributes to the EET-mediated angiogenic response (Michaelis et al., 2005). However, in the present study a specific PKA inhibitor had no effect on CYP2C-induced EphB4 expression, indicated that another signalling mechanism must be responsible.

The protein kinase Akt is a central signalling molecule involved in cell survival, proliferation, tumour growth and angiogenesis (Jiang and Liu, 2008) and activation of the Akt/PI3K pathway has been reported to play a role in the regulation and signalling of Eph receptors. For example, EphB4 mediated proliferation and migration of microvascular endothelial cells is mediated by the Akt/PI3K-pathway (Steinle et al., 2002) and PI3K activity can be increased in HUVECs by Eph stimulation (Maekawa et al., 2003). One EET-activated pathway certainly linked to angiogenesis is the transactivation of the EGF receptor and subsequent Akt activation (Michaelis et al., 2003). Although, EETs probably also activate Akt via alternative mechanisms, the consequences are the same and 11,12-EET induces the phosphorylation and therefore inhibition of the forkhead factors FoxO1 and FoxO3a and subsequently a decrease in the expression of the cyclin-dependent kinase inhibitor $p27^{kip1}$ (Potente et al., 2002). The findings of the present study confirm activation of Akt by the CYP2C-metabolite 11,12-EET and link this to the expression of EphB4 as CYP2C-overexpression resulted in both increased Akt phosphorylation and this was paralleled by an increase in EphB4 expression. In addition, inhibition of the Akt/PI3K -pathway as well as suppression of Akt activity using a dominant negative mutant resulted in decreased EphB4 expression levels. However, the exact mechanisms by which CYP2C-derived EETs activate the Akt/PI3K pathway resulting in increased EphB4 expression still need to be elucidated. The p85 regulatory subunit of PI3K has been reported to be constitutively associated and phosphorylated by other growth factor receptors such as VEGFR-2, resulting in

increased PI3K and Akt activities *in vivo* (Gerber et al., 1998; Ilan et al., 1998) and it certainly cannot be excluded that a similar mechanisms might be relevant in the EET-mediated angiogenic response.

The differential expression of Ephs and ephrins in arteries and veins as well as the differential biological actions in vascular development raise a number of questions and are discussed very controversially. Traditionally, ephrinB2 was thought to be expressed in arterial endothelial cells whereas EphB4 was restricted to venous endothelial cells (Gerety et al., 1999; Shin et al., 2001). Although a role for EphB4 in arterial endothelial cell migration and tube formation has been proposed by others (Salvucci et al., 2006) these findings are somewhat controversial. Indeed, EphB4 is still predominantly classed as a negative regulator of blood vessel branching and vascular network formation, and is generally accepted to switch the vascularisation program from sprouting angiogenesis to circumferential vessel growth (Erber et al., 2006). This implies that large vessels with few branches would be expected to form in vessels expressing EphB4. However, the unconditional classification of ephrinB2 as an arterial and EphB4 as a venous marker no longer appears to be strictly the case, and although the arterial expression of ephrinB2 has been confirmed in many different models (Shin et al., 2001) EphB4 expression has also been repeatedly detected in arterial endothelial cells, (Hall et al., 2002; Diehl et al., 2005). For example EphB4 expression has been detected in remodelling rat mesenteric microvessels and particularly in capillary sprouts (Taylor et al., 2007), suggesting that the classical view of receptor ligand distribution may have to be adjusted. In the present study, atypical EphB4 expression was detected and the strongest EET-induced expression of EphB4 was observed in isolated murine mesenteric arteries, even though there was no detectable expression of the protein in the endothelium of freshly isolated vessels. In contrast, EphB4 was abundantly expressed in mesenteric venous endothelial cells but was not upregulated by EET treatment. In endothelial cells, it seems fair to state that expression of the EphB4 ligand ephrinB2 is determined by microenvironmental conditions and by stimulation with VEGF rather than being an intrinsic endothelial cell differentiation marker (Korff et al., 2006). Based on the results of this investigation it could be speculated that under certain conditions, for example in situations of increased CYP2C9 expression or direct application of the metabolite 11,12-EET, EphB4 expression may no longer be solely determined as the venous endothelial cell phenotype, but can rather be influenced by alternative determinants. Arteriovenous differentiation has been

characterized as a primarily genetically driven process during development (Moyon et al., 2001), but the findings of this study stress the importance of the control of vascular homeostasis by microenviromental cues.

EphrinB2 forward signalling through its receptor EphB4 induces cell detachment and inhibits cell spreading, migration, and proliferation, whereas EphB4 reverse signalling induces cell attachment, spreading, and migration (Hamada et al., 2003). EphrinB2-expressing angiogenic endothelial cells that are activated by EphB4 promote migration and this reverse ephrinB2 signalling therefore acts pro-adhesively and induces sprouting angiogenesis (Füller et al., 2003). EphrinB ligands are known to be activated by several growth factors *in vitro* and *in vivo* such as the fibroblast growth factor receptor (FGFR) (Chong et al., 2000), but it still remains to be clarified how this can affect reverse signalling and Eph receptor activation. As EETs can affect a number of angiogenic signalling cascades, and stimulation of endothelial cells with the CYP metabolites results in receptor phosphorylation, EET are certainly another potential candidate for EphB4 ligand activation. Classically, phosphorylation and signalling occurs after binding of the ligand ephrinB2 to its receptor. It would therefore be expected that an alteration in EphB4 expression patterns by EETs would also be accompanied by altered ephrinB2 expression. It has for example been shown that hypoxia leads to upregulation of the ligand as well as its receptor in a mouse skin flap model (Vihanto et al., 2005). However, even though CYP2C overexpression in endothelial cells resulted in EphB4 phosphorylation and therefore activation, ephrinB2 expression levels were not altered. Therefore, in the experimental settings used EphB4 expression was independent of the expression of its ligand, but dependent on EET in a concentration-and time-dependent manner. Consequently, increased reverse ligand signalling cannot be accounted for the increased angiogenic activity, because under the conditions employed in our study CYP activation does not result in increased ephrinB2 expression. Thus, in specific situations such as EET-mediated angiogenesis, EphB4 also seems to be implicated in sprouting angiogenesis. Indeed, in a collagen based tube formation assay the downregulation of EphB4 expression in CYP2C9-overexpressing cells markedly attenuated the angiogenic response. Furthermore, *in vivo* the vessels that formed within Matrigel plugs impregnated with 11,12-EET expressed EphB4, and endothelial cell infiltration into Matrigel was prevented by its downregulation. Taken together, our observations indicate that EphB4 may also be implicated in capillary sprouting in specific situations, such as angiogenesis associated

with inflammation. Certainly, there are a number of preliminary observations, e.g. in the mouse ear (T. Korff, unpublished data, 2007), that support this hypothesis.

The regulation of angiogenesis by hypoxia is an important component of homeostatic mechanisms that link the vascular oxygen supply to cellular and systemic responses (Hickey and Simon, 2006) and an important stimulus for expansion of the vascular bed. Cells are oxygenated by simple diffusion of oxygen, but when diffusion distances from the existing vascular supply increase and tissue size expands beyond the limit of oxygen diffusion, hypoxia triggers vessel growth. These mechanisms are in part governed by the activation of the hypoxia-inducible transcription factors HIF-1 and HIF-2. The promoter region of the CYP2C8/9 genes contain hypoxia-responsive elements and a number of CYP2C isoforms are sensitive to changes in oxygen tension (Marden et al., 2003; Pokreisz et al., 2006; Michaelis et al., 2008). Our group has previously reported that CYP induction by hypoxia contributes to cell proliferation and angiogenesis *in vitro* as well as *in vivo* (Michaelis et al., 2005a). Therefore, it seemed logical to hypothesize a potential link between VEGF- and EET-signalling since the induction of VEGF by hypoxia and the role of the latter growth factor in hypoxia-induced cell migration is well documented (Ferrara and vis-Smyth, 1997).

Stimulation of endothelial cells with VEGF increased CYP2C expression and EET production and preventing the induction or inhibiting the activity of CYP2C significantly attenuated the angiogenic response to VEGF. These observations go in a similar direction as the recent report indicating that another CYP-derived metabolite of arachidonic acid, namely 20-HETE, can, at least acutely, be activated by VEGF in pulmonary arteries (Guo et al., 2007).

The relevance of VEGF and its receptors in vascular development has been demonstrated in various knockout animals. For example, the deletion of either VEGFR1 (Shalaby et al., 1995) or VEGFR2 (Fong et al., 1995) has proven to be lethal due to abnormalities in vessel formation. Consequently, it appears both receptors are essential for the development of the vasculature in the mouse embryo. Analysis of VEGFR signalling has led to the conclusion that although affinity for VEGF binding is approximately ten-fold higher for VEGFR1 than for VEGFR2, it is the latter protein that conveys the VEGF-mediated effects in endothelial cells (Gille et al., 2001). However, in our studies VEGF was able to increase the activity of the CYP2C9 promoter in cells expressing either VEGFR1 or VEGFR2, indicating that the VEGF-mediated CYP2C

activation does not seem to be restricted to one of the two receptors and that under the conditions employed activation of both receptors is essential for the VEGF-mediated angiogenic response.

One of the most important functions of VEGF is certainly its essential role *in vivo* in embryonic vasculogenesis and the promotion of angiogenesis by stimulating endothelial cell survival and growth (Carmeliet et al., 1996; Ferrara et al., 1996). In the Matigel plug assay in mice, VEGF resulted in increased infiltration of endothelial cells and a few sparse smooth muscle cells into the plug. This is in line with the *in vitro* findings in a collagen gel spheroid assay and confirms the (patho)physiological significance of VEGF for endothelial cell sprouting and angiogenesis.

VEGF has previously been demonstrated to activate the AMPK in endothelial cells (Reihill et al., 2007) and activation of the α2 subunit of the kinase has been linked to endothelial cell migration and angiogenesis (Ouchi et al., 2004). Moreover, AMPK signaling has been identified as a regulator of angiogenesis that is specifically required for hypoxia-induced endothelial cell migration and differentiation (Nagata et al., 2003a). Interestingly, activation of the AMPK by phenobarbital is reported to increase the expression of CYP2B in chicken and human hepatocytes (Blattler et al., 2007). Therefore, the relevance of AMPK in the VEGF-induced increase in CYP2C expression was assessed. Consistent with the previous reports (Reihill et al., 2007; Shindo et al., 2007), VEGF and phenobarbital both stimulated the phosphorylation of the AMPK in endothelial cells and both stimuli increased CYP2C expression. Moreover, the overexpression of a dominant negative AMPK mutant significantly reduced VEGF-induced CYP2C expression. Although the involvement of the AMPK in the VEGF-induced expression of CYP2C could clearly be demonstrated, the complete molecular mechanism is still unclear. It is however likely that LKB1, one of the upstream kinases of AMPK might be involved (Blattler et al., 2007) since it phosphorylates and activates the AMPK subunit at Thr-172 as recently shown in human hepatoma cells (Rencurel et al., 2005), as well as in primary mouse and human hepatocytes (Rencurel et al., 2006). Another potential candidate is the TGFβ-activated kinase 1 (TAK1) that has been shown to play an important role in AMPK activation in the heart (Xie et al., 2006). Indeed, TAK1 mutant embryos have defects in the developing vasculature of the embryo (Jadrich et al., 2006). As it has been shown that TAK1 signals through the MAPK pathway (Shirakabe et al., 1997; Hanafusa et al., 1999) that activates several

signalling molecules, including p38 and JNK, and CYP2C influences JNK expression levels (Potente et al., 2002), regulation of TAK may also be involved in the CYP2C-mediated angiogenic response mediated by VEGF.

Although EETs have been suggested to act as second messengers in the EGF-activated signalling cascade (Chen et al., 1999), not all growth factors are able to upregulate CYP epoxygenases. For example, although the EET antagonist, 14,15-EEZE, almost abolished VEGF-induced endothelial tube formation *in vitro* and *in vivo*, it did not affect the angiogenic response induced by bFGF. VEGF and FGF both exert their effects via specific binding to cell surface-expressed receptors and their activiation results in similar downstream signal transduction pathways. Furthermore, VEGF and FGF share a number of downstream targets including MAPK and PI3K that have also been identified in signalling cascades activated by EETs. The two angiogenic growth factors, bFGF and VEGF, can for example be distinguished on the basis of their requirement for Src kinase activity (Eliceiri et al., 1999). While Src family kinases seem to serve compensatory roles during embryogenesis and angiogenesis, VEGF, but not bFGF-mediated angiogenesis requires its activity for endothelial cell survival. Therefore the AMPK-mediated upregulation of CYP2C via VEGF seems to be another growth factor specific phenomenon. Furthermore, to date a bFGF mediated activation of AMPK has not been reported. Consequently, VEGF and bFGF might potentiate somewhat different biological effects during blood vessel formation. This would also tie in with observations that blood vessel formation and regeneration in different organs may differ as for example neovascularisation in the retina has been linked to VEGF expression (Miller et al., 1994), while cutaneous wound repair rather seems to be bFGF-dependent (Takenaka et al., 1997).

The VEGF-activated signalling cascade leading to angiogenesis has previously been linked to an increase in the formation of reactive oxygen species (Colavitti et al., 2002). Although the NADPH oxidase has been implicated as the source of these radicals (Ushio-Fukai et al., 2002), it should be noted that the activation of CYP2C epoxygenases also results in the generation of superoxide anions (O_2^-) in sufficient amounts to alter the bioavailability of nitric oxide and the expression of adhesion molecules (Fleming et al., 2001b). To verify whether EETs rather than CYP2C-derived reactive oxygen species were involved in mediating VEGF-induced angiogenesis, the EET antagonist, 14,15-EEZE was employed. In contrast to the epoxygenase inhibitors MS-PPOH and sulfaphenazole, as well as the CYP2C antisense treatment, which

attenuate the production of all the CYP2C products (EETs, 20-HETE and O_2^-), 14,15-EEZE antagonizes only the effects of EETs without interfering with those of 20-HETE (Gauthier et al., 2002) or the production of O_2^- by CYP2C enzymes (U.R. Michaelis, unpublished observations). We found that 14,15-EEZE inhibits VEGF-induced tube formation *in vitro* and angiogenesis *in vivo*, indicating that EETs and not reactive oxygen species were responsible for the effects observed. Moreover, there was no detectable effect of VEGF on 20-HETE formation, indicating that 20-HETE does not contribute to angiogenesis under the conditions studied.

4.2 Role of EETs in vessel maturation

Angiogenesis consists of endothelial cell sprouting as well as vascular maturation, which includes the recruitment of perivascular cells, smooth muscle cells for large and pericytes for small vessels. In recent years, extensive efforts have been made to gain understanding into the molecular mechanisms of this process. Several angiogenic growth factors that regulate endothelial sprouting have been identified, but little attention has been focused on vascular maturation. One prerequisite for the formation of a functional vasculature system is regulated by communication between endothelial cells and smooth muscle cells (Hirschi and D'Amore, 1996; Hirschi and D'Amore, 1997; Hungerford and Little, 1999). Interaction between these two cell types in the blood vessel wall has a critical role in the regulation of vascular formation, stabilization, remodelling and function. Equally, failure of these interactions has been demonstrated in a number of mouse models resulting in severe and often lethal vascular defects (Armulik et al., 2005). Abnormal interactions of endothelial cells and pericytes are involved in a number of human pathological conditions, including tumour angiogenesis, diabetic microangiopathy, ectopic tissue calcification, stroke and the dementia syndrome cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy) (CADASIL) (Bergers et al., 2003; Hammes, 2005).

In the present study, we demonstrated that 11,12-EET activates several signaling molecules (i.e. AMPK and PI3K) that are involved in processes resulting in the recruitment of pericytes/smooth muscle cells and stabilization of blood vessels. In the Matrigel Plug assay *in vivo* impregnation of plugs with 11,12-EET stimulated the

formation of smooth muscle cell stabilized and perfused blood vessels. This was evidenced by the injection of isolectin into the tail vein which resulted in the accumulation of isolectin close to vessels in the Matrigel plug. Moreover, by ultrasound measurement, blood flow could be demonstrated through the EET-impregnated plugs as there was a significant accumulation of contrast agent compared to the control plug. In contrast, but as expected on the basis of previous reports (Zacchigna et al., 2007), VEGF stimulated endothelial cell recruitment into the matrix material but only a few α-actin-expressing cells could be detected. Moreover, it was not possible to detect isolectin staining in VEGF-containing plugs.

In endothelial cells, gap junctions are important for homotypic (endothelial to endothelial) and heterotypic (endothelial to e.g. smooth muscle cell) communication (Dejana et al., 1995). Those gap junctional communications, at least between endothelial cells, can be activated by a CYP2C and cAMP-dependent pathway (Popp et al., 2002). Because it has been shown that junctional coupling may be important for the coordination of migration and repair during angiogenesis (Pepper et al., 1989) heterotypic gap junctional communication might be one possible underlying mechanism for the recruitment of smooth muscle cells/pericytes to endothelial cell tubes in EET-impregnated plugs. This would be consistent with reports that the connexin43 knockout mouse dies at birth due to severe defects in coronary vasculogenesis/angiogenesis (Walker et al., 2005).

Pericytes are believed to be able to transdifferentiate into vascular smooth muscle cells and vice versa in conjunction with vessel growth and remodelling (Nehls and Drenckhahn, 1993). Much of the current knowledge about the signalling between endothelial cells and pericytes/smooth muscle cells comes from genetically modified mouse models. In those studies it has been demonstrated that the different cell types are interlinked to a great extent and this means that defects in one cell type have mandatory consequences for the other resulting in increasingly intricate signalling pathways where all cell types involved have to be taken into consideration. It has for example been shown that endothelium-derived TGF-β is important for pericyte differentiation and that activation of latent TGF-β is dependent on endothelial-pericyte contact (Carvalho et al., 2004).

Although there has been substantial progress in the identification of the molecular details of endothelial – pericyte/smooth muscle cell interactions, many aspects of the process are still incompletely understood. In the course of this study 11,12-EETs were

identified as positive regulators of mural cell recruitment. Evidence from Matrigel plug assays suggested that EETs are indeed a critical determinant of pericyte/smooth muscle cell recruitment as mature, perfusable endothelial tubes covered by pericytes/smooth muscle cells were not detectable in plugs impregnated with other growth factors, such as VEGF. This was at first sight surprising as we have also shown that VEGF stimulation resulted in increased EET-production. However, in the course of this study it was not investigated if VEGF had any effect on the activation of sEH, the enzyme metabolising EETs. Moreover, in contrast to the impregnation of a Matrigel plug which creates an EET- gradient, constant EET production by VEGF might not be high enough to elicit pericyte recruitment.

Although our data indicate a role for EETs in vascular maturation, it is still not clear if this effect is directly mediated by CYP2C9-derived EETs or if the epoxides stimulate secondary responses that involve other signalling molecules. There are a number of signalling molecules that have been shown to be essential for the recruitment of pericytes/smooth muscle cells to endothelial tubes. For example, the G protein-coupled receptor $S1P_1$ is expressed both in smooth muscle and endothelial cells and is known to act within the latter to regulate vascular maturation (Allende et al., 2003). The receptor may well be relevant for CYP-mediated angiogenesis as a recent report suggests that the activation of sphingosine kinase-1, a key enzyme catalyzing the phosphorylation of sphingosine to sphingosine-1-phosphate, mediates the induction of endothelial cell proliferation and angiogenesis by EETs (Yan et al., 2008). In conjunction with the findings of this study as well as data that S1P-mediated endothelial cell hyperpolarization can be abolished by the CYP epoxygenases inhibitor sulfaphenazole (unpublished data), this suggests a possible role of $S1P_1$ as a second messenger in the recruitment of smooth muscle cells by EETs. Although this hypothesis is interesting we have been unable to detect any substantial role of $S1P_1$ receptor signalling in our experimental models (unpublished data). There are a number of other signalling cascades, such as angiopoietin-Tie 2 and PDGF-B/PDGF-β, which have been implicated in mural cell development that could also play a role in EET-stimulated vascular maturation. 11,12-EET can for example inhibit the PDGF-induced migration of rat aortic smooth muscle cells (Sun et al., 2002), and this phenomenon correlates with increased intracellular cAMP levels. Even though it is known that a number of effects observed in endothelial cells involve elevated cAMP levels, the effect observed in smooth muscle cells might not only be due to CYP-derived 11,12-EETs, but possibly to

one of the other regioisomers generated by the enzyme. Responses also appear to be cell type-dependent as 11,12-EET stimulates migration in human kidney epithelial cells as well as in HUVECs (Cheng et al., 1999; Michaelis et al., 2005).

4.3 The putative EET-receptor and the role of endogenous EET production and exogenous EET application in angiogenesis

EETs are lipophilic molecules that can be released from the cells in which they are generated to exert paracrine effects. However, in most cases the CYP metabolite is either conjugated or rapidly metabolized by the sEH (Newman et al., 2005). Consequently, extracellular EET levels are usually fairly low and it can be assumed that the metabolite exerts most of its effects as an intracellular second messenger. This assumption certainly fits well with the role of EETs in regulating intracellular calcium concentration in endothelial cells (Fleming et al., 2007) and the fact that downregulation of CYP2C by antisense oligonucleotides markedly reduced the proangiogenic effect of VEGF.

Even though we assume that the intracellular effects of EETs are the most important in the regulation of endothelial cell biology, it is clear that endothelial cells do release EETs under certain conditions and that endothelial cell-derived EETs can affect other cell types. Autocrine responses are mediated by EETs that are either released from arachidonic acid or from intracellular phospholipids by PLA_2 and can be recovered in the extracellular fluid. They probably stay long enough within or in the close vicinity of cells to initiate the respective response. Observations that radiolabeled EETs that are initially present in the endothelium accumulate in the extracellular fluid suggest that some of these EETs remain in the cytosol before they are released to the medium. Similarly EETs from the extracellular fluid can initiate paracrine effects. They can for example be reincorporated into vascular smooth muscle cells or converted to DHETs in the cytosol (Zeldin et al., 1993).

The initial step responsible for EET signalling is still largely unknown. One potential mechanism might be activation of a putative EET receptor. Other alternatives are less direct effects on lipid fluidity as EETs incorporate into the plasma membrane in order to associate with other molecules as for example PKA (Imig et al., 1999) or the ADP

ribosyltransferase (Li et al., 1999). In favour of the concept of a specific EET receptor are reports that physiological actions of the epoxide are dependent on specific structural requirements, such as defined locations of the double bond. Such 'structure-activity' interdependencies are certainly an indicator for a specific receptor and in bovine coronary arteries (Falck et al., 2003) modification of the epoxy oxygen or the carboxyl group had profound effects on the ability of the epoxides to induce relaxation (Gauthier et al., 2002c). The identification of a functional EET antagonist (i.e. 14,15-EEZE) also fueled speculation on the existence of an EET receptor. Perhaps the most exciting evidence to support the concept was the demonstration of a higly highly specific binding site in radioligand binding studies with labelled EETs in guinea pig monocytes (Wong et al., 2000). Although the latter studies confirmed a previous report in U937 cells (Wong et al., 1997) which contain a cell surface protein with a stereoselective binding site for 14(R)-15(S)-EET, there has been disturbingly little progress in this area. Recent effort has concentrated on finding a binding partner for the EETs among the orphan receptors. Indeed, there is evidence that 11,12- and 14,15-EET binding activates the adenylyl cyclase, increases intracellular cAMP levels and activate PKA and the cAMP response element binding protein (Node et al., 2001; Spector and Norris, 2007); in other words all the 'classic' elements of a $G\alpha_S$-coupled receptor signalling cascade. In addition, it was shown that the EET-stimulated activation of BK_{Ca}-channels can be blocked by a $G\alpha_S$-specific G protein inhibitor (Li and Campbell, 1997). These data suggest that EETs might initiate their signalling cascade through a membrane, $G\alpha_S$-coupled receptor. However, while some acute effects of EETs are PKA-dependent, in the present study neither the signaling pathway involved in the EET-mediated increase in EphB4 expression nor in the VEGF-mediated CYP2C upregulation involved the activation of PKA via increased cAMP levels.

Alternatively it has been proposed that EETs can enter cells, either as a result of uptake, hydrolysis of EET-containing phospholipids or CYP-mediated production from arachidonic acid, and interact directly with intracellular effectors such as the fatty acid binding proteins, ion channels or transcription factors (e.g. PPARs) that elicit the functional response. However, a lot of the data supporting this mechanism have been generated biochemically or in cell culture and up till now no conclusive evidence of intracellular interaction with such effector molecules has been generated *in vivo*.

4.4 Relevance of this study

In the past decade, effort has been made to further develop what has been termed 'therapeutic angiogenesis', i.e. therapeutic strategies to promote the revascularization of ischemic tissues, or to inhibit angiogenesis in several diseases such as cancer, ocular, joint or skin disorders. However, up till now most of the clinical trials testing the pro-angiogenic potential of VEGF or bFGF have been disappointing as they failed to provide clear proof of effectiveness (Rajagopalan et al., 2003). Furthermore, animal studies involving VEGF gene therapy have revealed that major complications can be associated with a growth factor monotherapy, for example VEGF gene therapy in mice and human leads to hemangioma (i.e. visible red skin lesion) and vascular leakage (Springer et al., 1998). Recent clinical studies with VEGF inhibitors have raised a number of questions regarding the selectivity and efficiency of these compounds and it is now clear that targeting endothelial cells and/or the principal growth factor VEGF alone may not be sufficient to eradicate malignant tumours or cure growth factor-related diseases. Consequently, a more attractive approach would maybe be either a combined treatment of anti-angiogenic agents with distinct complementary mechanisms or a 'master gene therapy'; that is via genes capable of activating multiple angiogenic pathways and stimulating the expression of factors that promote not only angiogenesis, but also vessel maturation. One of those master genes could be CYP2C as our data indicate the importance of 11,12-EET on (patho)physiological angiogenic processes. At this point it is important to point out that CYP enzymes are not only expressed in endothelial cells and CYP2C as well as CYP2J enzymes have been detected in different tumour tissues (Yokose et al., 1999) and can induce tumour growth as well as promote metastasis (Jiang et al., 2005; Jiang et al., 2007). It is therefore tempting to speculate that CYP enzymes might represent a new target for the treatment of tumour growth and therefore cancer therapy. The findings of this study, together with a number of properties attributed to CYP2C9 may be a potential target for future gene therapy.

CYP2C-derived EETs may play important roles in promoting invasion and metastasis of cancer through several mechanisms. They are involved in the activation of many signalling pathways that can have both short term (cell proliferation, invasion and adhesion) and long term (gene expression) effects on carcinoma cells. By interfering with molecules that have been demonstrated in other studies to be involved in carcinogenesis, such as EphB4 and VEGF, modulation of CYP2C expression may

involve down-regulation of metastatic suppressor genes, the up-regulation of metastasis enhancer genes and the activation of a variety of signalling cascades such as Akt/PI3K. Furthermore, the recent emphasis on the importance of the role of interactions between pericytes and endothelial cells raises a number of questions regarding many aspects of endothelial-pericyte communication. It is for example far from clear how pericytes stabilize vessels and why this process fails in tumour vessels. In the course of this study it became evident that EETs play a role in these interactions, but the precise molecular mechanisms as well as the exact relevance for physiology and pathology is still unclear. Pharmacological inhibitors of some CYP isoforms have been identified as promising anti-cancer agents (Chun and Kim, 2003; Chong et al., 2007). However, the majority of work published to-date has focused on the consequences of CYP inhibition on the bioavailability of anti-cancer agents, rather than determining the consequences of CYP inhibition *per se*.

Beyond its potential use in oncology, there is certainly a role of EETs in the treatment of vascular diseases. As mentioned previously, the EET/EDHF pathway is assumed to be a backup vasodilator mechanism that ensures vasodilatation in case of decreased NO bioavailability. Consequently, cardiovascular diseases and CYP epoxygenases might be linked at two levels. Although there is currently very little known regarding a potential role of CYP-derived EETs in human diseases, at least in animal models epoxygenase levels have been linked to hypertension (Spector and Norris, 2007). It has for example been shown in studies focused on spontaneously hypertensive rats that EETs and sEH inhibition can affect blood pressure (Sellers et al., 2005). Another point that should not be ignored is that most patients, especially those treated for cardiovascular diseases, receive more than one pharmacological agent over a long time period. This is relevant since there are a number of drugs, such as the Ca^{2+} antagonist nifedipine and the HMG CoA reductase inhibitors cerivasatin and fluvastatin (Fisslthaler et al., 2003) that stimulate an increase in CYP2C expression (Fisslthaler et al., 2000a) and might therefore interfere with other treatments given at the same time. Another important aspect when looking at the role of altered EET levels in cardiovascular diseases is the sEH, the enzyme that metabolizes EETs to the less biologically active DHETs. Blockade of sEH leads to increased endogenous EET levels. In recent years there has been considerable interest in sEH as a potential target for antihypertensive therapy as specific inhibitors have been shown to be very effective at preventing as well as reversing the hypertensive effects of angiotensin II (Imig, 2005; Jung et al., 2005). In

this context sEH inhibition could potentially result in increased expression of for example EphB4 and this might explain side effects that occur during treatment.

Over the course of the present investigation it was possible to further clarify the molecular mechanisms involved in the CYP2C-mediated regulation of vascular homeostasis. At least two additional signalling molecules that have been reported to play an important role in the regulation of angiogenesis have been identified as being crucial for the angiogenic answer mediated by CYP2C i.e., VEGF and EphB4. Identification of the latter pathways as being modulated by altered CYP2C activity significally expands and complements the current understanding of the mechanisms involved in EET-induced angiogenesis and emphasises the central importance of CYP2C in the regulation of vascular tone and homeostasis.

5. Summary

Cytochrome P450 epoxygenases of the 2C family (CYP2C) are highly expressed in the endothelium and metabolize arachidonic acid to different regioisomers of epoxyeicosatrienoic acids (EET). They have a number of roles in the regulation of vascular tone and homeostasis by activating different signal transduction pathways and have recently been reported to be involved in proliferation and angiogenesis. However, the exact mechanisms by which epoxygenases regulate angiogenesis are still unclear. In particular the involvement of important endothelial growth factors and to what extent they are involved in CYP2C-mediated vessel formation needs to be elucidated. Therefore, the initial aim of the present study was to characterize the relevance of major signalling molecules that are involved in angiogenesis and to investigate possible signalling pathways involved.

Initially the effect of CYP2C9 overexpression on expression levels of EphB4, a tyrosine kinase that plays a role in a number of developmental processes, was investigated. EphB4 protein expression was increased in CYP2C9 overexpressing cells without any effects on expression levels of its ligand ephrinB2. The effect was induced by the metabolite 11,12-EET and was abolished by the CYP epoxygenase inhibitor MSPPOH as well as by the PI3K inhibitor LY 294002 and simultaneous transfection with a dominant negative mutant of Akt. Moreover, endogenous EphB4 expression was down-regulated in cells transfected with CYP2C9 antisense oligonucleotides. To clarify whether EphB4 is a critical determinant of CYP2C9-induced angiogenesis, endothelial cell sprouting was assessed using a collagen gel-based *in vitro* angiogenesis assay. Following transfection with EphB4 antisense or scrambled oligonucleotides, capillary-like structures were clearly present after 24 hours in cells overexpressing CYP2C9, while EphB4 downregulation abolished CYP2C9-induced sprouting.

In addition stimulation of human umbilical vein endothelial cells with VEGF resulted in an increase in CYP2C expression and a subsequent increase of 11,12-EET production; an effect that was abolished by the CYP epoxygenases inhibitor MSPPOH as well as when cells were infected with a dominant negative mutant of AMPK. Furthermore, it was shown that CYP2C is involved in VEGF-induced tube formation while there was no effect of the EET-antagonist 14,15-EEZE when cells were treated with bFGF.

Moreover, this study aimed at specifying the relevance of the *in vitro* findings for the angiogenic process and confirming the role of those findings *in vivo*. One crucial step in vessel formation is proliferation and differentiation of endothelial cells to form vascular tubes. *In vivo* 11,12-EET treatment increased EphB4 expression in mesenteric arteries as well as in Matrigel plugs; an effect that was abolished when plugs were impregnated at the same time with small interfering RNA (siRNA) for EphB4. Furthermore, impregnation of Matrigel plugs with VEGF resulted in endothelial cell and smooth muscle cell recruitment into a Matrigel plug and this effect was mediated by CYP2C9-derived EETs as it was prevented by 14,15-EEZE.

When infiltration of EET impregnated plugs with endothelial cells and pericytes/smooth muscle cells *in vivo* was compared to the effects seen in VEGF treated plugs, it was apparent that only EET treatment resulted in the formation of tube like structures that were covered by smooth muscle cells. Therefore, the final aim of the study was to further define the consequences of EET signalling *in vivo* as well as to characterize its physiological relevance. This hypothesis could be assessed by isolectin injection through the tail-vein where isolectin was taken up only by the EET-impregnated plug. Moreover ultrasound measurements revealed accumulation of contrast agent in EET impregnated plugs compared to control plugs.

Taken together our findings emphasize the *in vitro* as well as *in vivo* relevance of CYP2C and CYP-derived EETs in angiogenesis and vascular maturation. CYP2C plays a crucial role in the vessel formation process by modulating the effects mediated by two important control elements of the angiogenic response, namely VEGF and EphB4. It was shown that CYP2C-derived EETs play a critical role in modulating the activity of these growth factor receptors and failure to do so results in defective signalling. Consequently, CYP2C-derived EETs not only participate as second messengers in the angiogenic response, but have the potential to influence much more than angiogenesis by enhancing smooth muscle cell/pericyte recruitment to endothelial cell tubes to promote vascular maturation. However the exact steps resulting in EET-mediated pericyte/smooth muscle cell recruitment still need to be elucidated.

6. Zusammenfassung

Im nativen Endothel exprimierte Cytochrom P450 Epoxygenasen der 2C Familie (CYP2C) synthetisieren aus Arachidonsäure verschiedene Regioisomere der Epoxyeicosatriensäure (5,6-; 8,9-; 11,12- und 14,15 EET), welche eine wichtige Rolle bei der Regulation des vaskulären Tonus und der Homöostase spielen. Desweitern sind EETs wichtige intrazelluläre Signaltransduktionsmoleküle, die eine Reihe von Funktionen erfüllen, die über die Regulation des Membranpotentials und ihre Rolle als ‚endothelium derived hyperpolarizing factor' hinausgehen. Hierbei ist die genaue Wirkung der CYP-Metaboliten nicht nur abhängig von den daran beteiligten Regioisomeren, sondern auch von Gewebetyp und Spezies. Besonders im Endothel aktivieren vor allem 11,12- und 14,15-EET eine Reihe von Tyrosinkinasen- und phosphatasen wie die „p38 mitogen-activated protein (MAP) kinase", die „extracellular regulated protein kinase" 1 und 2 (ERK1/2) und die MAP Kinase Phosphatase. Andererseits hemmen sie jedoch auch eine Reihe von Signaltransduktionsmolekülen wie z.B. die c-Jun N-terminal Kinase. Obwohl schon beschrieben wurde, dass Enzyme der CYP2C Familie an Prozessen der Proliferation und Angiogenese beteiligt sind, indem sie zum Beispiel den „epidermal growth factor (EGF)" transaktivieren, ist der genaue molekulare Mechanismus ihrer Wirkung noch weitestgehend ungeklärt. Deswegen war das primäre Ziel der vorliegenden Arbeit die Identifizierung von Signaltransduktionsmolekülen, die an der CYP-induzierten Angiogenese beteiligt sind, sowie die nachfolgende Charakterisierung möglicher darin involvierter Signaltransduktionskaskaden.

Zunächst wurde der Einfluss von CYP2C9 auf die Expression von EphB4 untersucht. EphB4 gehört zur Familie der erst kürzlich identifizierten Gruppe von Eph Rezeptoren, die eine entscheidende Rolle in der vaskulären Entwicklung und Angiogenese spielen. Eph Rezeptoren sind eine Gruppe von Tyrosinkinasen, die ursprünglich als Leitmoleküle bei der Führung von Nerven identifiziert wurden, aber auch entscheidend für verschiedene Prozesse der Angiogenese sind, wie z.B. die Ausbildung juxtakriner Zell-Zell Kontakte, die Adhäsion an extrazelluläre Matrices, die Zellmigration und –proliferation. Im Gegensatz zu anderen Rezeptortyrosinkinasen, die an lösliche Liganden binden, interagieren Eph Rezeptoren mit an die Zelloberfläche gebundenen Ephrin Liganden. Diese sind entweder über einen Glycosylphosphatidylinositolanker

oder über eine transmembrane Domäne mit der Zellmembran verbunden. Eph Rezeptoren können in zwei Unterklassen, A und B , unterteilt werden, wobei für die vaskuläre Entwicklung der Rezeptor EphB4 der wichtigste Vertreter ist. Es konnte zum Beispiel unter anderem gezeigt werden, dass Mausmutanten, denen EphB4 fehlt, einen Phänotyp mit gestörter Gefäßbildung und einer verfrühten embryonalen Letalität aufweisen. Eine weitere Besonderheit dieser Signalmoleküle besteht darin, dass Eph-ephrin Signalübermittlung bidirektional, also sowohl durch Ligand als auch Rezeptor, erfolgt.

Obwohl CYP2C Protein im nativen Endothel in ausreichender Menge nachweisbar ist, nimmt die Expression sowohl von mRNA als auch Protein nach Zellisolation rapide ab und kann bereits in der zweiten Passage bei Kultivierung nicht mehr nachgewiesen werden. Folglich muss bei der Untersuchung von Effekten und der biologischen Bedeutung der von CYP2C-stammenden EETs *in vitro* das Protein entweder durch pharmakologische oder hämodynamische Stimuli (z.B. Schubspannung), durch Hypoxie oder durch den Einsatz von adenoviralen Konstrukten (CYP-Überexpression) hochreguliert werden. Um einen Zusammenhang zwischen der Expression der Tyrosinkinase EphB4 und CYP2C9 zu untersuchen, wurden daher humane Nabelschnurzellen entweder mit einem Kontrollvirus (CYP2C antisense) oder einem CYP2C9-kodierenden Adenovirus infiziert. In CYP2C9-überexprimierenden Zellen war die EphB4-Proteinexpression bereits nach 24 Stunden im Vergleich zur Kontrolle erhöht ohne einen Einfluss auf die Expression des Liganden ephrinB2 zu haben. Dieser Effekt konnte einer erhöhten CYP Epoxygenaseaktivität zugeschrieben und durch den CYP Epoxygenaseinhibitor MSPPOH verhindert werden. Eine Phosphorylierung und damit Aktivierung des Rezeptors durch erhöhte CYP2C-Spiegel konnte ebenfalls nachgewiesen werden, denn die CYP2C-Überexpression resultierte in der Tyrosinphosphorylierung von EphB4. Desweiteren konnte gezeigt werden, dass dieser Effekt direkt EET-vermittelt ist und nicht durch einen der anderen CYP-Metaboliten oder durch reaktive Sauerstoffspezies verursacht wurde, denn auch die direkte Gabe von 11,12-EET führte zu erhöhter EphB4 Expression.

Folglich konnte eine Bedeutung von CYP2C in der EphB4-vermittelten Signaltransduktion nachgewiesen werden, aber die genaue Bedeutung von EphB4 in der CYP2C-induzierten Bildung von Gefäßen war noch nicht erörtert. Ein entscheidender erster Schritt für die Blutgefäßbildung ist die Proliferation und Differenzierung von Endothelzellen, was zur Bildung von vaskulären röhrenartigen

Gebilden (engl. „Tubes“) führt. Dabei muss vor der Migration von Endothelzellen erst die umliegende Matrix, die vor allem aus Kollagen, Elastin, Fibronektin und Proteoglykan besteht, abgebaut werden. Die hierbei ablaufenden Vorgänge können *in vitro* in zwei- oder dreidimensionalen Angiogenese-„Assays“ untersucht werden, wobei die *in vitro* verwandten Wachstumsmatrices, wie z.B. Kollagen, Matrigel oder Fibrin, den *in vivo* Gegebenheiten nachempfunden sind,. Um den Einfluss von EphB4 auf die CYP2C-vermittelte Angiogenese zu untersuchen, wurde ein auf Kollagen basierender dreidimensionaler Sprossungs-(engl. „sprouting“)„Assay“ verwendet. Um hierbei die Expression von EphB4 zu verhindern, wurden Endothelzellen zunächst mit EphB4 antisense Oligonukleotiden bzw. mit Kontrolloligonukleotiden (EphB4 scrambled) behandelt. Danach wurden Endothelzellspheroide gebildet und das Ausmaß der Sprossung mikroskopisch analysiert. Es zeigte sich eine verminderte Sprossung von Endothelzellspheroiden unter Einfluss von EphB4-antisense Oligonukleotiden im Vergleich zu Spheroiden, die vorher mit „scrambled“ Oligonukleotiden behandelt wurden.

Bisher ist noch sehr wenig über die Signaltransduktionswege, die zur Expression von EphB4 führen, bekannt. PI3K kann durch eine Reihe von extrazellulären Signalen aktiviert werden und ist an vielen zellulären Vorgängen, wie z.B. Zellproliferation, Zellüberleben, Proteinsynthese und Tumorwachstum, beteiligt. Da beschrieben ist, dass EETs, insbesondere 11,12-und 14,15-EETs, den PI3K/Akt Signaltransduktionsweg in Endothelzellen aktivierten, wurde der Einfluss dieses Signalweges auf die CYP2C9-vermittelte EphB4 Expression und mögliche biologische Konsequenzen untersucht. Eine Hemmung der PI3K mit dem spezifischen Inhibitor LY 294002 führte sowohl zu einer geringeren Aktivierung des EphB4 Promotors in CYP2C-überexpremierenden Zellen als auch zu einer verminderten EphB4 Proteinexpression. Auch die gleichzeitige Infektion mit einer dominant negativen Akt-Mutante hob die erhöhte EphB4 Expression in CYP2C9-überexprimierten Zellen auf.

Um nun aufzuklären, ob die *in vitro* Befunde auch eine *in vivo* Relevanz aufweisen, wurde zunächst der Einfluss von EETs im Matrigel-Plug-„Assay“ untersucht. Die Imprägnierung solcher Plugs mit 11,12-EET führte zu einem erhöhten Einwandern von Endothelzellen und Perizyten/glatten Muskelzellen in das Matrigel im Vergleich zu Kontroll-Plugs. Dies konnte durch den gleichzeitigen Einschluss von siRNA gegen EphB4 verhindert werden. Unter EET-Einfluss bildeten sich im Plug röhrenförmigen Strukturen aus, die mit Perizyten/glatten Muskelzellen umkleidet waren. Außerdem

konnte in diesen röhrenförmigen Strukturen nur nach Behandlung mit EET, nicht aber in der Kontrolle, die Expression von EphB4 eindeutig nachgewiesen werden.

Desweiteren sollte ein Zusammenhang zwischen „vascular endothelial growth factor“(VEGF)- und CYP-vermittelten Signaltransduktionswegen untersucht werden. Die Familie der VEGFs ist eine sehr heterogene Gruppe mit einer Vielzahl von Untergruppen. VEGF ist als einer der Schlüsselsubstanzen bei der Regulation von sowohl physiologischer als auch pathologischer Angiogenese von entscheidender Bedeutung. Da bekannt ist, dass die Expression von VEGF unter hypoxischen Bedingungen zunimmt und, dass CYP2C an der hypoxievermittelten Angiogenese beteiligt ist, postulierten wir einen möglichen Zusammenhang zwischen VEGF- und CYP-Expression.

Die Stimulation von Endothelzellen humaner Nabelschnurvenen mit VEGF führte zu einer Steigerung der CYP2C-Expression und konnte durch den Epoxygenaseinhibitor MSPPOH gehemmt werden. Außerdem kam es unter VEGF-Einfluss zur vermehrten 11,12-EET Bildung. VEGF reguliert die CYP2C9 Expression auch auf Transkriptionsebene, denn Stimulation mit VEGF führte zu einer Erhöhung der CYP2C Promoteraktivität in VEGF-Rezeptor exprimierenden Endothelzellen, nicht aber in Zellen ohne VEGF-Rezeptor.

Die AMPK-aktivierte Proteinkinase spielt eine bedeutende Rolle bei der Regulation des Zellstoffwechsels unter hypoxischen Bedingungen und bei der Muskelkontraktion. Da sie außerdem sowohl an der Regulation der Hypoxie- und VEGF-vermittelten Angiogenese, als auch an der durch Phenobarbital ausgelösten Induktion von CYP beteiligt ist, wurde ein Zusammenhang zwischen AMPK und der VEGF-vermittelten CYP2C Expression untersucht. Es konnte gezeigt werden, dass eine Stimulation mit VEGF zu einer schnellen Phosphorylierung der AMPK führte. Wurden Endothezellen aber gleichzeitig mit einer dominant negativen Mutante der AMPK transfiziert, war sowohl die VEGF-vermittelte CYP2C-Expression als auch die Phosphorylierung der AMPK aufgehoben.

Darüber hinaus sollte auch hier die physiologische Bedeutung dieser *in vitro* Befunde *in vivo* weiter konkretisiert werden und die Relevanz für den Angiogeneseprozess erörtert werden. In einem Fibrin-basierten Angiogenese-„Assay“ konnte gezeigt werden, dass CYP2C an der VEGF-, nicht aber an der bFGF-vermittelten Ausbildung von röhrenförmigen Strukturen beteiligt ist, da der EET-Antagonist 14,15-EEZE beispielsweise in bFGF-behandelten Zellen keinerlei Effekt hatte. Desweiteren

verhinderte die gleichzeitige Transfektion der Endothelzellen mit einer dominant negativen AMPK-Mutante die Ausbildung dieser Strukturen. *In vivo* führte die Imprägnierung eines Matrigel-Plugs mit sowohl VEGF als auch bFGF zwar zur Rekrutierung von Endothelzellen (und in geringerem Ausmaß auch zur Rekrutierung von glatten Muskelzellen bzw. Perizyten), aber diese Rekrutierung konnte nur in den VEGF imprägnierten Plugs durch den spezifischen EET-Antagonisten 14,15-EEZE verhindert werden. Außerdem führte gleichzeitige Behandlung des Plugs mit der dominant negativen Mutante der AMPK zur deutlich verminderten Einwanderung von Endothelzellen in das Matrigel.

In der vorliegenden Arbeit wurde auch der Einfluss von EETs auf die Reifung von Gefäßen untersucht. Dabei zeigte sich, dass die Einwanderung von Endothelzellen und Pericyten/glatten Muskelzellen *in vivo* in einen EET-imprägnierten Plug vergleichbar war mit der Einwanderung in VEGF-behandelte Plugs. Allerdings führte nur die EET-Behandlung zur Ausbildung von vaskulären „Tubes“, die auch mit glatten Muskelzellen ausgekleidet waren. Um zu klären, ob diese röhrenartigen Strukturen auch perfundiert wurden, wurde kurz vor Dislokation der Wirbelsäure Isolektin in die Schwanzvene der Mäuse injiziert. Nur durch EET-imprägnierte Plugs, nicht aber durch die mit VEGF imprägnierten Plugs, wurde Isolektin aufgenommen. Um die Funktionalität der „Tubes“ weiterhin zu bestätigen, wurden die Matrigel Plugs in der lebenden Maus Ultraschallmessungen unterzogen. Hierbei konnte nur ein erhöhtes Anfluten von Kontrastmittel im EET-behandelten Plug, nicht aber im Kontrollplug gemessen werden, was ein weiterer Hinweis für die Ausbildung von perfundierten, funktionsfähigen Gefäßstrukturen ist.

Zusammenfassend lässt sich sagen, dass EETs über die Modulation zweier wichtiger Stellglieder der angiogenen Antwort, nämlich VEGF und EphB4, eine Rolle bei der Regulation von Rezeptoren und Signaltransduktionsmolekülen spielen, die essentiell für die normale Blutgefässentwicklung und Gefäßreifung sind. Die von CYP2C-stammenden EETs sind nicht nur als sekundäre Botenstoff an der angiogenen Antwort, die durch eine Vielzahl von Faktoren ausgelöst wird, beteiligt, sondern haben zudem das Potential sowohl Endothelzellen als auch Perizyten/glatte Muskelzellen zu rekrutieren, und leisten so ein entscheidender Beitrag zum vaskulären Reifungsprozess.

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8. Abbreviations

AMPK	AMP activated protein kinase
Ang-1	Angiotensin 1
BSA	Bovine serum albumine
Ca^{2+}	Calcium
CYP	Cytochrome P450
DHET	Dihydroxyeicosatrienoic acids
ECM	Extracellular matrix
EDHF	Endothelium-derived hyperpolarizing factor
EET	Epoxyeicosatrienoic acid
EEZE	14,15-Epoxyeicosa-5(Z)-enoic acid
EGF	Epidermal growth factor
FCS	Fetal calf serum
bFGF	Basic fibroblast growth factor
Flk-1	Fetal liver kinase receptor
Flt-1	Fms-like tyrosine kinase
HETE	Hydroxyeicosatetranoic acids
HGF	Hepatocyte growth factor
HPLC	High pressure liquid chromatography
HUVEC	Human umbilical vein endothelial cells
Mic	Miconazole
MLEC	Mouse lung endothelial cells
MS	Mass spectronomy
MSPPOH	N-methanesulfonyl-6-(2-proparyloxyphenyl)hexanamide
NADPH	Nicotinamidadeninnucleptide phosphate
NO	Nitric oxide
PBS	Phosphate buffered saline
PAEC	Porcine aortic endothelial cells
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
PECAM-1	Platelet and endothelial cell adhesion molecule-1

PIGF	Placental growth factor
PGI_2	Prostacyclin
PI3K	Phosphatidylinositol-3 kinase
PKA	Protein kinase A
RTK	Receptor Tyrosine Kinase
SDS	Sodium dodecyl sulphate
SMC	Smooth muscle cells
TGF-β	Transforming growth factor β
TRIS	Tris-hydroxymethyl-aminomethan
VEGF	Vascular endothelium growth factor

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