CHAPTER 1: INTRODUCTION

1.1 The endothelin system

1.1.1 Endothelin peptides

The endothelins (ETs) comprise a family of three structurally related peptides (ET-1, ET-2 and ET-3) produced by the endothelium and various other cell types. Endothelial cells (EC) cover the entire inner surface of the vascular system, and secrete various potent vasoconstricting and vasodilating mediators that regulate vasomotor function, trigger inflammatory processes, and affect hemostasis (Lüscher TF, 1990; Boulanger CM et al, 1998). Many endothelium-derived substances are functionally active, mutually antagonistic and some are apparantly redundant (Rubanyi GM et al, 1993). Besides being a physical interface between the circulating blood and the tissues, the normal functional endothelium modulates the tone of underlying vascular smooth muscle, maintains a nonadhesive luminal surface, and mediates homeostasis, cellular proliferation, and inflammatory and immune mechanisms in the vascular wall (Rubanyi GM et al, 1994). The functional endothelium secretes various factors, hormones and peptides into the circulation. Among the three peptides of the endothelin family, endothelin-1 (ET-1) was the first to be identified in 1988 in the supernatant of cultured porcine aortic EC (Yanagisawa et al, 1988) and is recognized as one of the most potent vasoconstrictors known (Maguire JJ et al, 2002), secreted by vascular EC and other cell types. Besides EC, ETs are expressed by epithelial cells, macrophages, fibroblasts, astrocytes, endometrial cells, hepatocytes and cardiomyocytes (Miyauchi T et al, 1999). The ETs are known to elicit their biological effects through activation of receptors belonging to the superfamily of G-protein coupled receptors (GPCR). Of all the three ET isoforms, the best studied is ET-1.

ET-1 consists of 21 amino acids (Fig. 1). This 21-amino-acid peptide is produced by the intact and dysfunctional vascular endothelium and is known to be a potent vasoconstrictor (Yanagisawa M et al, 1988) *in vivo* and *in vitro* (Hirata Y et al, 1989). ET-1 is derived from a precursor protein, termed preproET-1 which is a 212 amino-acid peptide cleaved by a furin-like peptidase (Fig. 2). This generates big endothelin-1 (big ET-1) consisting of 39 amino acids, which is biologically inactive. Subsequently, specific endothelin-converting enzymes (ECEs) cleave big ET-1 between Trp21 and Val/Ile22, thereby producing mature ET-1 (Xu D et al, 1994). ECE-1 is classified as a membrane-bound type II metalloprotease, which is phosphoramidon-sensitive. ET-1 is mainly

secreted at the abluminal membrane of EC, towards the smooth-muscle layer of the wall of the blood vessel, where it binds to specific ET receptors on the vascular smooth muscle cells (VSMCs). The biological effects of ET-1 are mediated via activation of specific receptors, endothelin A receptor (ET_A) and endothelin B receptor (ET_B).

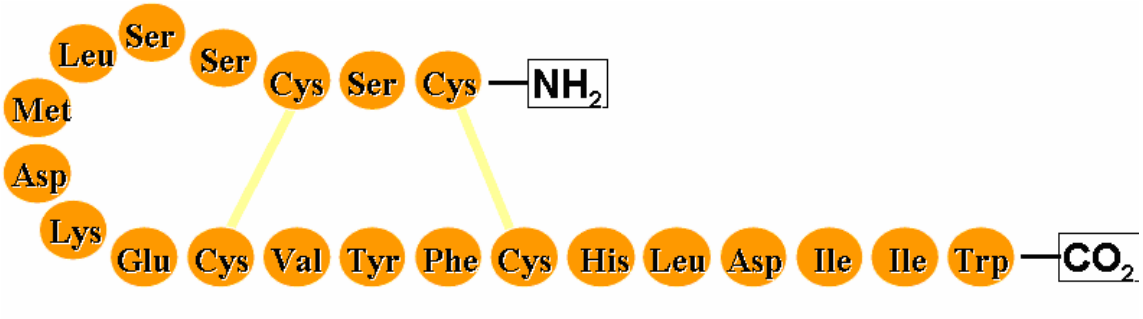

Fig. 1. Schematics of the molecular structure of ET-1

1.1.2 The endothelin receptors

The ET_A receptor shows the following descending receptor affinity for the three ET isopeptides: ET-1 ≥ ET-2 >> ET-3 (Sakurai T et al, 1992). This receptor subtype is mainly expressed on the large and small blood vessels (Hori S et al, 1992). The vascular system is richly endowed with GPCRs which form the largest protein family of receptors in the body of vertebrates. The majority of transmembrane signal transduction in response to hormones and neurotransmitters is mediated by GPCRs (Ji TH et al, 1998). All GPCRs have a common structure comprising of seven transmembrane alpha helices, an extracellular N-terminal segment and an intracellular C-terminal segment. This class of membrane proteins responds to a wide range of agonists. Some agonists bind to an extracellular loop of the receptors, others may penetrate into the transmembrane region. Both endothelin receptors, i.e ET_A and ET_B, are GPCRs. While on the VSMC the predominant ET receptor is of the ET_A subtype (Aria H et al, 1990; Takase H et al, 1995), contributing to potent and long-lasting vasoconstriction, EC express the ET_B receptor which mediates the formation of nitric oxide (NO) and prostacyclin (Hirata Y et al, 1993; Warner TD et al, 1993), inducing transient vasodilatation. Endothelial ET_B receptors also largely contribute to the pulmonary clearance of circulating ET-1 (Fukuroda T et al, 1994) as well as to the reuptake of ET-1 by EC. Intravenous administration of ET-1 causes a rapid and transient decrease in blood pressure followed by a strong and long-lasting increase in blood pressure (Yanagisawa M et al, 1988).

In 1990 (Arai H et al, 1990; Sakurai T et al, 1990) reported cloning of the ET_A receptor from bovine and of the ET_B receptor from rat species, respectively. The human sequence of the specific receptor subtypes was isolated and characterized a little later (Hosoda H et al, 1992; Arai et al, 1993). As stated earlier, ET_A receptors are primarily located on the VSMCs and mediate long lasting vasoconstriction by an increase in intracellular calcium [$(Ca^{2+})i$] concentration (see Fig. 2 for details) (Seo B et al, 1994). This increase in [$(Ca^{2+})i$] is due to the release of calcium (Ca^{2+}) from [$(Ca^{2+})i$] stores presumably, sarcoplasmic reticulum (SR), and Ca^{2+} influx from extracellular spaces. ET binding to ET_A and ET_B leads to activation of phospholipase C (PLC) via G-protein (Gαq or Gα11 subunits). PLC hydrolyses phosphoinositol-4,5- bisphosphate (PIP_2) into inositol-1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). IP3 acts as a second messenger to release Ca^{2+} via IP_3 sensitive Ca^{2+} channels located in the SR which contributes to the contraction. ET contraction also depends on the influx of Ca^{2+} from extracellular sites, and it appears that the Ca^{2+} influx may occur via voltage-sensitive L-type Ca^{2+} channels also known as voltage operated calcium channels (VOCC) and other pathway(s) whose exact nature are not known.

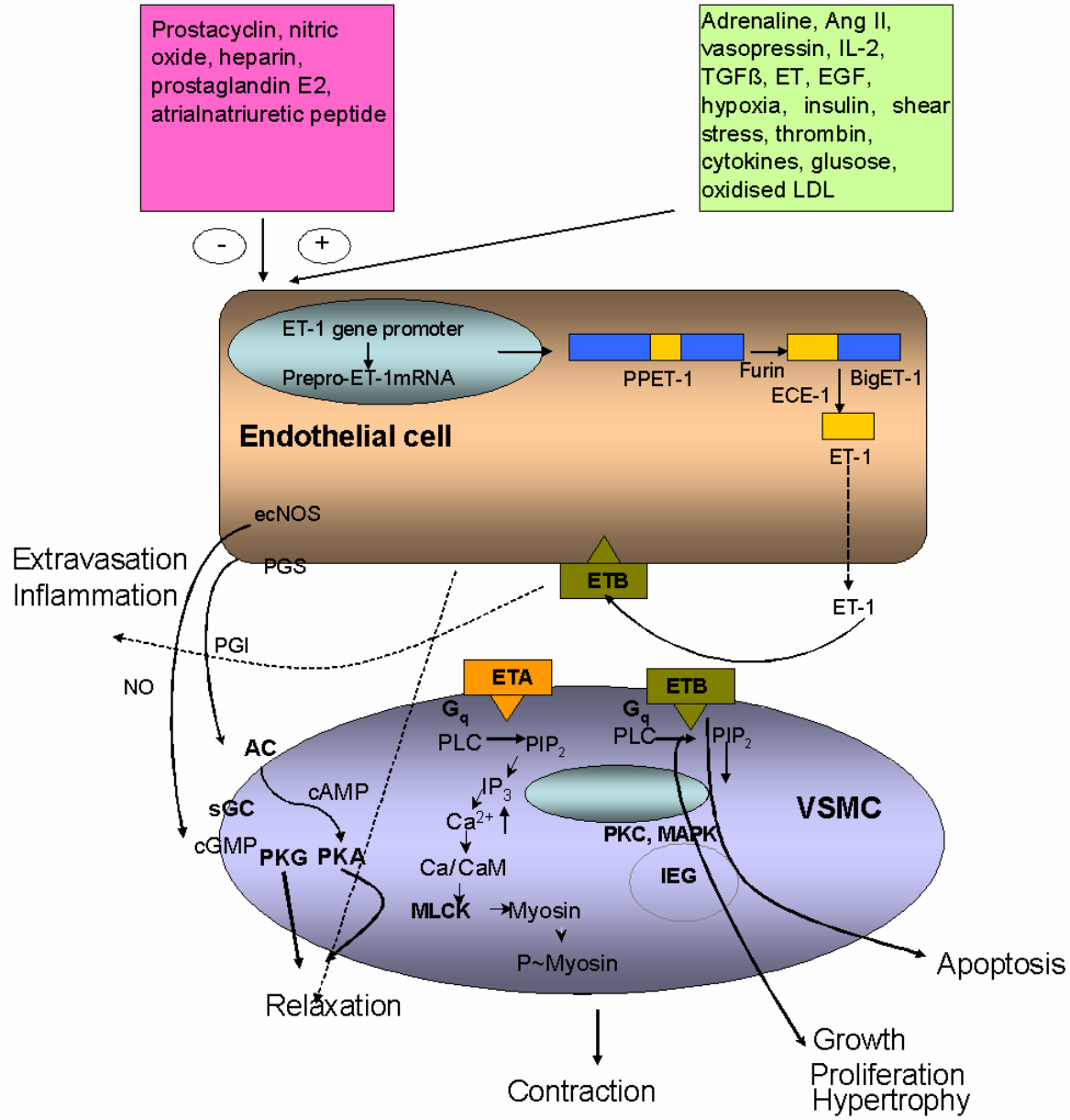

Fig. 2. Schematics of biosynthesis of ET-1, its regulation, and signal transduction pathway of ET-1 after binding to ET receptors and actions on vascular smooth muscle cells and on endothelial cells. ET-1 synthesis is regulated by various factors; Stimulators are highlighted in light green, and inhibitors in light maroon. ET-1 is produced from a precursor, named preproendothelin (PPET-1). After removal of a signal peptide, the precursor is selectively processed by an enzyme (a furin-like peptidase to yield a biologically inactive intermediate called big-endothelin-1 (bigET-1). BigET-1 is further converted into active ET by endothelin-converting enzyme-1 (ECE-1). Two ET receptors have been identified: ET_A and ET_B. ET_A receptors are predominantly expressed on vascular smooth muscle cells and mediate vasoconstriction, whereas ET_B receptors are mainly found on endothelial cells and induce vasodilatation through NO. ET_B receptors on smooth muscle cells can elicit vessel contraction. PKC, Protein kinase C; PLC, phospholipase C; cGMP, cyclic guanosine 3',5'-monophosphate, PIP2, phosphoinositol bisphosphate; AC, adenylate cyclase; NO, nitric oxide; IP3, Inositol triphosphate; Ca/CaM, calcium calmodulin complex; PGI, prostacyclin; PGS, prostaglandin synthase; MLCK, myosin light chain kinase; Gq, G-protein alpha q; sGC, soluble guanylate cyclase; IEG, Immediate early genes.

1.1.3 Tissue distribution of endothelin and its receptors

Endothelin-binding studies have revealed widespread distribution of ET receptors within virtually every organ system by virtue of ET's presence within the vascular endothelium. Much of the complexity surrounding the study of ET function is related to the fact that there is considerable variability in the distribution of receptor subtypes between different vascular beds and among various species. Endothelin receptors of both the ET_A and especially the ET_B subtype are found on many other cell types aside from vascular endothelium and smooth muscle, including nonvascular structures within the heart, kidney, lung, and nervous system. Table 1 summarizes the tissue distribution of endothelin receptors briefly. Both ET_A and ET_B receptors have been identified on the VSMC from human coronary vessels using RT-PCR and *in situ* hybridization and membrane binding techniques (Davenport AP et al, 1993; Davenport AP et al, 1995). Both receptor subtypes have been identified as mediating ET-1 induced contraction in the coronary circulation, although a high degree of species variability appears to exist. ET_{B2} but not ET_{B1} receptors have been pharmacologically identified in the porcine coronary vessels (Seo B et al, 1994), whereas canine coronary arteries possess both ET_{B1} and ET_{B2} (Teerlink JR et al, 1994). In human coronary arteries some groups have reported non-ET_A-induced vasoconstriction (Godfraind T, 1993; Bax WA et al, 1994), whereas others suggest that ET-1-induced constriction is predominantly mediated by the ET_A subtype (Davenport AP et al, 1994).

In addition to vascular endothelium, cells of renal tubular, mesangial and interstitial origin have been shown to synthesize and release ET in culture. The function of ET within the renal medulla appears to involve inhibition of ADH-induced changes in water permeability (Oishi R et al, 1991; Schnermann J et al, 1992) that occurs via interactions with ET_B receptors located on inner-medullary collecting duct cells (Kohan D E et al, 1993; Edwards RM et al, 1993). Pulmonary tissue contains some of the highest concentrations of ET-1 of any organ (Matsumoto H et al, 1989). Though both ET_A and ET_B receptors have been found throughout the pulmonary vasculature, airways and alveoli, ET_B receptors in the lungs play an important role in the clearance of circulating ET-1. Nakamichi K et al, 1992, have also shown in porcine pulmonary tissues that blood vessels and bronchi are rich in ET_A receptors and the lung parenchyma is rich in ET_B receptors (Inui T et al, 1994). These ET_B receptors in the lungs are responsible for the clearance of circulating ET-1 (Dupuis J et al, 1996).

In the gastrointestinal tract, ET-1 and its receptors have been localized within the mucosal layer of the rat colon, intestine and stomach (Takahashi K et al, 1990). ET-2, also known as vasoactive intestinal contractor, is found in high concentrations within the gastrointestinal tract and is a potent constrictor of visceral smooth muscles. Similarly, components of the ET system have been identified through out the central and peripheral nervous system. The predominant ET isoform within the brain is thought by many to be ET-3, particularly within the hypothalamus and cerebellum (Matsumoto H et al, 1989; Shinmi O et al, 1990), which could explain why the ET_B receptor is the predominant subtype found within such areas as the cerebellum (Elshourbagy NA et al, 1992).

Tissue/organ	Cell source of ET	Effect	ET receptor	References
Vasculature	Endothelial	Transient vasodilation	ET_B	Yanagisawa et al, 1988
		Sustained vasoconstriction	ET_A	
Heart	Endothelial	Increased coronary vascular resistance	ET_A	Miller et al, 1989
	Smooth muscle	Decreased cardiac output	ET_A	
	Cardiomyocytes	Hypertrophy of cardiomyocytes	ET_A	Ito et al, 1991
	Fibroblasts	Hypertrophy/ proliferation of fibroblasts	ET_A, ET_B	Spieker et al, 2001
Kidney	Endothelial	Renal vasoconstriction	ET_A	King et al, 1989
	Smooth muscle	Increased glomerular afferant and efferent arteriolar tone	ET_A	Edwards et al, 1990
	Mesangial	Mesangial cell proliferation	ET_A, ET_B	Badr et al, 1989
		Natriuresis, diuresis	ET_A, ET_B	Ischimura et al, 1991
Colon	Enteric ganglia	Vasodilatation	ET_B	Takahashi K et al, 1990
	Resistance mesenteric arteries	Vasoconstriction	ET_A	
Lung	Endothelial	Pulmonary artery	ET_A	Fagan et al, 2001
	Airway epithelial	Mucous gland hypersecretion	ET_A, ET_B	Goldie and Fernandes, 2000
Brain	Endothelial	Constriction of cerebral arteries	ET_A	Encabo et al, 1992
	Neurons	Activation of	ET_A, ET_B	Lisovoski et al, 1994
	Astrocytes	Inhibition of generation of second messangers by neurotransmitter catecholamines	ET_B	Levin et al, 1992

Table. 1. Tissue and organ distribution of ET_A and ET_B receptors. Physiological effects mediated by endothelin in the vasculatures (Highsmith RF, 1998).

1.1.4 Vascular smooth muscle cells

VSMCs are among the most plastic of all cells in their ability to respond to different growth factors. Specifically, VSMCs may proliferate (hyperplasia with an increase in cell number), hypertrophy [an increase in cell size without change in deoxyribonucleic acid (DNA) content], endoreduplicate (an increase in DNA content and usually size), and undergo apoptosis. VSMC may also migrate and dedifferentiate with increased matrix and collagen synthesis. Among the mechanisms utilized by VSMC to mediate these varying cellular responses are autocrine and paracrine growth pathways (Berk BC et al, 2001). VSMC in the vessel wall are continuously exposed to mechanical forces that modulate function. It has become clear that, in addition to regulating vessel tone, these physical forces modulate vessel architecture by changing VSMC gene expression. Similar to angiotensin-II (Ang-II), ET-1 causes a delayed mitogenesis of cultured VSMC that is related to the accumulation of growth factors such as epiregulin and TGF-ß. Addition of exogenous ET-1 also may promote VSMC hyperplasia *in vivo* (Hirata Y et al, 1989)

The degree of contraction of VSMCs controls the diameter of small arteries and thereby organ perfusion. Contraction is triggered by a rise in Ca^{2+} ion concentration in the cytoplasm leading to phosphorylation of myosin filaments. Contraction of VSMCs, in contrast to cardiac or skeletal smooth muscle cells (SMC) is characterized by a large degree of shortening, at slow velocity, sustained in nature, and low at energy cost (1/300 of the energy expenditure of striated muscle). The basic mechanism of contraction to agonist/receptor induced constriction to substances, such as Ang-II, ET-1, serotonin and norepinephrine (NE), might be slightly different but the different signaling pathways together leading to vasoconstriction may be more or less pronounced depending on the type of agonist, but also on the artery type and size of artery. Receptor-coupled stimulation of vascular contractility involves elevating smooth muscle [$(Ca^{2+})i$] levels and also sensitizing the myofilaments to the Ca^{2+} increase (Somlyo AP et al, 2000).

VSMCs are basically of heterogenous origin. The first explanation is that developmentally, the vessel media layer includes cells of different embryonic origins and/or vascular smooth muscle stem cells may differentiate into cells with varying morphology and function. Thus autocrine growth responses will be highly dependent on the nature of VSMC heterogeneity. This heterogeneity is mainly responsible for obvious

varied responses to different growth factors. A basic question is whether this is due to differences in origin or to spatiotemporal heterogeneity in expression of differentiation markers due to local environmental and hormonal factors. It is already known that both, developmental and environmental factors influence VSMC heterogeneity. Heterogeneity within the vessel wall may be related to alterations in the local environment. Firstly, hemodynamic variations may modify local gradients in substances (e.g., increased residence time of lipids) or local metabolic requirements (e.g., increased energy metabolism or altered cytoskeleton arrangements) (Ku DN et al, 1983; Davies PF et al, 1988; Davies PF et al, 1989). Hemodynamic flow variations are also known to influence shear stress (the dragging frictional force of blood on the vessel luminal surface) which is maintained within the narrow range of 10-20 dyne/cm^2 in the arteries. Turbulent blood flow leads to loss of oscillatory flow patterns. Intimal proliferation occurs most commonly in these areas of turbulent flow, such as the human carotid bulb. One explanation for intimal proliferation at these sites is related to alterations in EC-derived factors; specifically, there may be a decrease in factors that inhibit VSMC growth and an increase in factors that stimulate VSMC growth (Traub O et al, 1998). Another explanation is that blood-borne factors are better able to influence VSMC in these regions of disturbed flow. Secondly, variation in matrix composition may be important, as illustrated by the fact that fibronectin is thought to be growth promoting and laminin growth inhibiting. There are clear interactions between matrix and the ability of VSMC to respond to growth factors (Wilson E et al, 1995). Reports show that the reactivity of the vascular smooth muscle to different factors and growth mediators depends primarily on the concentration, (paracrine or autocrine) of the mediator in the milieu (Wilson E et al, 1993). Thirdly, variations in physical forces at a particular site as a consequence of vessel architecture and flow pattern may modulate VSMC function. Changes in mechanical strain have been shown to induce many VSMC growth factors, including platelet derived growth factor (PDGF), basic fibroblast growth factor (bFGF), insulin like growth factors IGF-I, and transforming growth factor- ß TGF-ß (Wilson E et al, 1993; Calara F et al, 1996; Cheng GC et al, 1996; Hu Y et al, 1998, Li Q et al, 1998; Wilson E et al, 1998; Zou Y et al, 1998). In addition, mechanical strain may make VSMC more sensitive to the mitogenic actions of other factors such as ET-1 and Ang-II (Sudhir K et al, 1993). In conclusion, various redundant growth mechanisms influencing VSMC growth may depend not on one factor but on a plethora of pathophysiological conditions and large number of mediators and their concentrations in the vascular milieu.

1.1.5 Physiological role of endothelin in the vasculature

Until the discovery of urotensin II (Maguire JJ et al, 2002), ET-1 was recognized as the strongest vasoconstrictor peptide (Levin ER et al, 1995), showing its effects both in arteries and veins and in the arterioles of the microcirculation (Yanagisawa et al, 1988). ET-1 injected into forearm of humans at slow rate (0.57 ng/100 ml forearm tissue/min) causes vasodilatation in forearm microcirculation of normal humans whereas at high infusion rates (25 and 50 ng/100 ml forearm tissue/min) it determines vasoconstriction (Kiowski W et al, 1991). ETs mediate their vasoconstrictor action via ET_A receptors. The overall vascular effects of endothelin on the vascular tone derive from the balance between direct vasoconstrictor effect via ET_A and ET_B receptors on the VSMC and vasodilatation by NO or prostacyclin released by activation of endothelial ET_B receptors (Haynes WG et al, 1998). Activation of the ET_B receptor expressed in EC mediates the release of NO and prostacyclin thereby causing transient vasodilation (De Nucci et al, 1988). Vasoconstriction persists after ET-1 is removed from the receptor (Clarke JG et al, 1989). Thus, it is supposed that ET-1 and ET_A receptors play a role in the maintenance of basal vasomotor tone. NO shortens the duration of vasoconstrictive effect by accelerating the return of [(Ca^{2+})i] to its basal concentration (Goligorsky et al, 1994). In the kidney, ET_A receptors, which are predominantly expressed in the vasa recta and arcuate arteries, regulate the renal circulation (Ferrario et al, 1989), whereas ET_B receptors are involved in natriuresis and diuresis. The natriuretic action occurs via the inhibition of the amiloride-sensitive sodium channel. In the lung, ET_A receptors mediate pulmonary vasoconstriction and ET_B receptors-bronchoconstriction. In astrocytes, ET_B receptors mediate the ability of the ET-1 and ET-3 to stimulate the synthesis of DNA and to inhibit the generation of second messengers by other neurotransmitters (Levin ER et al, 1992).

1.1.6 Plasma levels of endothelins

Endothelin is regarded mainly as a para/autocrine acting peptide which is reflected by very low ET plasma concentrations, ranging in healthy human adults from 1 to 5 fmol/ml (Wagner OF et al, 1992). It was estimated that approximately 80% of the endothelin synthesized is secreted abluminally towards the smooth muscle layer (Wagner OF et al, 1992), and circulating levels must, therefore, be regarded as spillover into the blood stream. This is in agreement with the increasing understanding of ET-1 as a hormone that acts in an autocrine or paracrine fashion. Indeed, endothelin concentrations in

plasma are below the threshold concentrations thought to be necessary to significantly activate the ET receptors. Moreover, it has also been shown (Frelin C et al, 1994) that under conditions of stoichiometric binding, virtually the entire ligand is bound to the receptor at relatively low concentrations of the ligand. Thus, the free concentration of the ligand does not necessarily reflect the biological activity of the system. Therefore, significant biological actions may occur despite the fact that the concentration of ET is below the value which would be predicted for initiating significant pathophysiological effects.

Furthermore, plasma ET-1 was shown to be degraded and cleared rapidly, with a half-life of approximately 1 min (Sirvio ML et al, 1990). Thus, it is not surprising that in general, increase in circulating levels of ET-1 is found only in pathophysiological conditions associated with severe vascular damage (Schiffrin EL et al, 1995). The observation that most studies have failed to demonstrate significant differences in ET-1 plasma levels thus, therefore explains the controversial role of ET in human hypertension (Schiffrin EL et al, 1991).

1.2 Pathophysiological role of endothelin in the vasculature

1.2.1 Human hypertension

ET-1 is one of the most potent vasoconstrictors yet identified and thus its potential role in the development and/or maintenance of hypertension has been studied extensively. It is known that ET-1 levels are higher in patients suffering from severe hypertension (Stewart DJ et al, 1991; Cody RJ et al, 1993; Bushuev VI et al, 2006) and African Americans (Ergul S et al, 1996). On the other hand, plasma levels of patients with essential hypertension were reported to be not significantly higher (Davenport AP et al, 1990; Kohno M et al, 1990; Schiffrin EL et al, 1991). In contrast to these reports a more recent study has reported significantly higher plasma ET-1 levels in early essential hypertensive patients with no signs of end-organ damage (Schneider MP et al, 2000). Chronic elevation of endogenous endothelin in man can result in hypertension as observed in two patients with malignant hemangioendothelioma resulting in dramatically increased circulating endothelin levels. In these patients, hypertension disappeared or reoccurred, depending on the presence of tumor and elevated plasma endothelin (Yokokawa K et al, 1991).

There also appear to be racial differences in plasma ET-1 concentrations in individuals with hypertension. Both, male and female black hypertensives have nearly fourfold

higher endothelin plasma levels compared to whites (Ergul S et al, 1996). Diabetes mellitus is well known for its association with microvascular damage. ET-1 concentrations were also reported to be elevated in non-insulin-dependent-diabetes-mellitus patients with pre-proliferative retinopathy (Letizia C et al, 1997). Plasma endothelin levels also appear to be higher in obese individuals and particularly in obese hypertensives (Cardillo C et al, 2004). In preeclampsia, elevated plasma concentrations of ET-1 have been reported (Ajne G et al, 2005). The ET-1 plasma levels have been shown to be significantly increased in patients exhibiting pregnancy-induced hypertension (pre-eclampsia) (Dekker GA et al, 1991) and this rise occurs together with an increase in the concentration of von Willebrand factor, which is a well-known marker of endothelial damage (Greer IA et al, 1991; Deng L et al, 1994). This suggests that endothelial damage in preeclampsia could contribute, via endothelin secretion, to blood pressure elevation. Cyclosporin A stimulates ET-1 secretion, and in patients receiving the drug after heart, liver, or kidney transplantation, ET-1 concentration in plasma was found to be elevated (Edwards BS et al, 1991). ET-1 can thus play a role in hypertension associated with cyclosporin treatment. Patients with chronic renal failure and on hemodialysis receive, as part of the treatment of anemia, administration of human recombinant erythropoietin, and this is associated with elevated ET-1 plasma levels which contribute to further hypertension (Kang DH et al, 1998; Stefanidis I et al, 2001; Shimada N et al, 2003).

Therefore, it is still unclear whether endothelin acts as a causative or an aggravating factor in the development of hypertension. It is thus certain that whereas the elevation of plasma ET-1 occurs in patients with end-organ disease manifested by damage to the endothelium, the causative role of ETs in the pathogenesis of essential hypertension and elevation of plasma ET-1 is still ambiguous.

1.2.2 Endothelin in animal models of hypertension

Studies measuring plasma ET-1 in experimental animal models of hypertension have also provided equivocal results. Whereas deoxycorticosterone acetate (DOCA)-salt, (Lariviere R et al, 1993; Li JS et al, 1994) DOCA-salt-treated spontaneously hypertensive rats (SHR) (Schiffrin EL et al, 1995), and Dahl salt-sensitive rats (Doucet J et al, 1996) showed significantly increased tissue and plasma ET-1 levels, ET-1 levels were not significantly altered in SHR and rats treated with N-nitro-L-arginine methyl ester (L-NAME). Moreover, chronic administration of an ET_A-selective endothelin

receptor antagonist A-127722 did not induce any significant lowering of blood pressure in the rats where hypertension was induced by chronic NOS inhibition by L-NAME (Sventek P et al, 1997), suggesting that endothelin does not appear to participate to an important degree in mechanisms leading to elevated blood pressure after NO synthesis inhibition with L-NAME in SD rats.

Though an activated vascular endothelin system has consistently been demonstrated in all these models, plasma levels of endothelin were fairly different. Long-term infusion of exogenous endothelin in dogs leads to a chronic increase in blood pressure (Wilkins FC et al, 1993) and overexpression of human prepro-ET-1 in the rats hepatocytes (Niranjan V et al, 1996) leads to sustained elevations in blood pressure. Studies evaluating endothelin receptors in rat models of experimental hypertension have also provided controversial data. Both receptor types have been shown to be reduced in mesenteric vessels of SHR rats. Binding studies performed using [125I]ET-1 in the presence of increasing concentrations of ET-1, BQ-123, or IRL-1620 (ET_B selective agonist) have shown reduced binding to ET_A and ET_B receptors in the mesenteric vessels of SHR rats (Schiffrin EL et al, 1995). Accordingly, unlike other models mentioned above the endothelin system appears not to be activated to an important degree in SHR (Larivière R et al, 1993; Schiffrin EL et al, 1995; Larivière R et al, 1995; Li JS et al, 1994; Li JS et al, 1995).

Activation of the vascular endothelin system is associated with exaggerated growth of the media of resistance arteries (Li J-S et al, 1994; Schiffrin E et al, 1995), and administration of endothelin antagonists not only lowered blood pressure but also induced regression of hypertrophic arterial remodeling (Barton M et al, 1998). In some of these hypertensive models, the effects of endothelin in the kidney may contribute to hypertension by water and sodium retention and renal vasoconstriction since ET-1 expression was reported to be increased in the vasculature and glomeruli of the kidney of DOCA-salt hypertensive rats (Deng LY et al, 1996). Administration of endothelin antagonists to DOCA-salt-treated SHR, which develop malignant hypertension and vascular and glomerular fibroid necrosis, results in amelioration of the renal lesions (Li JS et al, 1994). In rats infused with Ang-II, a known stimulant of ET-1 expression, endothelin antagonists lowered blood pressure (Rajagopalan S et al, 1997; Moreau P et al, 1997) and reduced cardiac and small artery hypertrophic remodeling (Moreau P et al, 1997). From these models it can be concluded that the endothelin system is activated in low-renin, salt-sensitive and severe forms of hypertension and whereas the

role of endothelin in salt-sensitive or salt-induced hypertension is well established, the role of this peptide and its receptors in other forms of human and experimental hypertension is still ambiguous.

1.3 Vascular interactions of the endothelin receptors

ET-1 is known to interact with various other vasoactive agonists. A two way interaction has been reported a long time ago between humoral factors in the regulation of vascular tone. ET-1 increases the production of thromboxane A2 (TXA2) in the kidney (Stier et al, 1992) and may regulate the release of prostaglandin H2 (PGH2) and TXA2 in blood vessels (Moreau et al, 1996). U46619, a TXA2 mimetic has also been shown to stimulate ET-1 production in cultured EC (Spatz M et al, 1994). Interaction has also been shown for ET-1 and Ang-II.

Ang-II potently stimulates ET-1 synthesis/release (Scott-Burden T et al, 1991) and ET-1 dose dependently increases Ang-II production by an enalapril-sensitive mechanism (Kawaguchi H et al, 1990; Kawaguchi H et al, 1991). In addition, ET-1 release might be stimulated by exogenous infusions of Ang-II, and therefore presumably under certain conditions by upregulated endogenous Ang-II as well. However, in two-kidney one-clip Goldblatt hypertension, a classical model of renin-Ang-II dependent hypertension, the endothelin system is not activated (Sventek P et al, 1996), and bosentan does not lower blood pressure (Li JS et al, 1996). This suggests that the relationship between Ang-II and the endothelin system may be more complex. It has also been demonstrated that Ang-II may stimulate the production of endothelin in SHR vessels, perhaps to a greater degree than in normotensive control vessels (Dohi Y et al, 1992). ET-1 can also stimulate the release of aldosterone (Cozza EN et al, 1992). The adrenal gland expresses ET-1 mRNA and contains a large amount of immunoreactive ET-1 (Zeng Z et al, 1998). Endothelin may amplify the pressor effects of the renin-angiotensin system and interact with adrenergic pathways (Rossi GP et al, 1999). Also subpressor concentrations of ET-1 are known to enhance NE induced vasoconstrictor responses in various vascular tissues (Yang ZH et al, 1990; Henrion D et al, 1993). Therefore, it is always very important to look into other vasoactive systems when analysing the effect of a potent vasoconstrictor like ET-1. This suggests that ET-1 might exaggerate or attenuate responses to other vasoconstrictor substances under different pathological conditions.

1.4 Transgenic animal models

1.4.1 Transgenic technology

The ultimate experimental model to study cardiovascular regulation and pathophysiology is the living animal model. Complementary to pharmacological studies, transgenic (tg) technology is now recognized as a powerful tool to address the physiological and pathophysiological function of so-called candidate genes in the cardiovascular system (Paul M et al., 1994; Bader M et al, 2000). The phenotypic impact of the tg approach was previously demonstrated when rats tg for the murine renin-2 gene were reported to develop fulminant hypertension (Mullins JJ et al, 1990).

The production of tg animals requires stable genetic manipulation of the germ line for the targeted deletion (knockout) or overexpression of the gene of interest. More refined tg models apply transgene constructs under control of cell-specific promoters to address the biological function of the gene of interest more specifically. The most widely used method for the generation of tg animals is the pronuclear microinjection method after induction of superovulation by gonadotropins. The protocol of this method is reported in detail elsewhere (Popova E et al, 2004). Pronuclear microinjection technique was used for the generation of the tg animals analyzed in this work.

1.4.2 Transgenic model of the human endothelin receptor

To achieve vascular smooth-muscle restricted expression of the tg human ET_A receptor, a 1,343 bp fragment of the murine SM22α promoter was used to generate the tg models analyzed in this work as described elsewhere in detail (Zollmann, FS. MD thesis, Freie Universität Berlin, 2003). The same construct was also used to generate a tg rat model of the human ET_B receptor (Zollmann FS et al, 2002).

SM22α, also called transgelin, is expressed abundantly and specifically in vascular and visceral SMCs in adult mice (Li L et al, 1996; Lawson D et al, 1997). SM22α is a SMC-specific 22-kDa Ca^{2+} binding protein with structural homology to the vertebrate thin filament myofibrillar regulatory protein calponin and the Drosophila muscle protein mp20 (Ayme-southgate A et al, 1989). SM22α is expressed in cardiac, smooth and skeletal muscle lineages during mouse embryogenesis and in adult SMC. Previous studies have suggested that SM22α gene product is expressed exclusively in smooth muscle-containing tissues of adult animals and is one of the earliest markers of differentiated SMC (Lees-Miller J P et al, 1987).

Mice tg for SM22-LacZ gene have shown specific expression of the transgene in arterial SMC cells but not in venous or in visceral SMC (Li L et al, 1996). It was demonstrated that the expression of the SM22α continues to persist in the adult vasculature of the mice at the age of 6 to 8 weeks (in the aorta, the pulmonary trunk and the right pulmonary artery) as well in the vessels of intestine and uterus but lacking transgene expression in the visceral SMC and in the veins (Moessler H et al, 1996).

1.4.2 Transgenic models of endothelin-1 and of endothelin-2

Overexpression of the human ET-1 gene under the control of its natural promoter in mice is associated with a pathological renal phenotype characterized by age-dependent development of interstitial fibrosis and glomerulosclerosis, leading to a progressive decrease in glomerular filtration rate without alterations of blood pressure (Hocher B et al, 1997). This blood-pressure independent fibrotic remodelling of the kidney occurred with rather moderate overexpression of ET-1 by about 50%, indicating that ET-1 is a very potent profibrotic peptide hormone *in vivo*, at least in the kidney. A blood-pressure independent fibrotic remodelling of the kidney was also seen in tg rats overexpressing the human ET-2 gene under control of its promoter. However, the extent of kidney fibrosis was less pronounced and mainly restricted to the glomeruli (Hocher B et al, 1996; Liefeldt L et al, 1999). This is most probably due to the preferential expression of the transgene within the glomeruli in ET-2 tg rats as shown by *in situ* hybridization (Hocher B et al, 1996), whereas the transgene is ubiquitously expressed within the entire kidney of ET-1 tg mice (Hocher B et al, 1997). In summary, both tg models of ET peptides provide interesting monogenetic animal models for the analysis of the effect of an activated renal ET system. However, these models were not useful in demonstrating the vascular role of ET due to the primary extravascular sites of transgene expression. Therefore, models with vascular-targeted overexpression are required to fully elucidate the pathobiological role of the endothelin system in the vascular system and, specifically, its pathophysiological role in hypertension.

AIMS OF THIS DISSERTATION

Pharmacological studies of rat models of experimental hypertension have revealed contradictory results with respect to the role of the endothelin system in arterial hypertension. Importantly, the pathophysiological significance of the ET_A receptor has been addressed by pharmacological inhibitors only in these models.

To address a putatively causal role of the ET_A receptor in hypertension and vascular hypertrophy, tg rat models were generated which were designed to overexpress the human ET_A receptor under control of a smooth-muscle specific promoter.

The principal hypothesis underlying the generation of the present tg model was that overexpression of the ET_A receptor will induce hypertension and/or vascular hypertrophy. To prove this hypothesis, the vascular phenotype in tg rats was analyzed in the present dissertation. The experiments included blood pressure recordings under basal and stimulated conditions, structural analysis of the blood vessel wall and pharmacological studies in isolated perfused mesenteric arteries.