CHAPTER 4: DISCUSSION

The family of biologically active peptides known as the endothelins has been in the focus of considerable research since the isolation of an endothelial cell derived constricting factor (EDCF) (Hickey KA et al, 1985) and the subsequent cloning of PreproET-1 cDNA (Yanagisawa M et al, 1988). Studies in certain rat models of hypertension have suggested a role for ET-1 and the ET_A receptor in hypertension and associated vascular hypertrophy whereas others failed to demonstrate a contribution of the endothelin system (Schiffrin EL et al, 2005). Previous tg models of endothelin peptides in rat and mouse did not show hypertension or associated vascular hypertrophy (Hocher B et al, 1996; Hocher B et al, 1997) which could, however, be attributed to insufficient trangene expression in the vascular wall. Specifically, direct experimental evidence of a role for the vascular expressed ET_A in vascular hypertrophy or hyperplasia has not yet been provided.

In the current dissertation work, the phenotypic characterization (functional and structural) of tg rats co-expressing the human ET_A (hET_A) receptor under the control of a VSMC-specific promoter (derived from the murine SM22α gene) was performed. We decided to establish models with ET_A overexpression in VSMC to directly address the biological role of this receptor at its primary site of physiological expression in the vascular system, namely the VSMC. Furthermore, overexpression of the receptor rather than overexpression of ET-1 would avoid problems with interpretation of data since in an ET-1 overexpression model the observed phenotype could be related to any ET receptor expressed in the vascular wall (endothelial ET_B and smooth muscle ET_A and ET_B, respectively). The rationale to co-express the human ET_A instead of rat ET_A was to establish a model which may be more useful in future pharmacological testing of ET_A antagonists in the context of human vascular disease treatment. Second, coexpresssion of human and rat ET_A would facilitate differential mRNA expression analysis to address whether expression of the human receptor may eventually influence expression of the endogenous rat receptor. It is important to note that, as to the current state of our knowledge, the affinity of ET-1 to rat and human ET_A receptor is not significantly different as analysed in heart homogenates with K_d value of 0.32 nM for rET_A and 0.92 for hET_A receptor (Wiley KE et al, 2004). Also, binding of ET-1 to a high affinity receptor site presumably ET_A in A10 rat VSMC cells with a K_d value of 0.5 nM has previously been reported (Lin HY et al, 1991).

4.1 Blood pressure phenotype of the transgenic lines

In vivo radiotelemetry is the technique of choice to invasively monitor blood pressure variables in non-anesthetized and non-restrained animals (Ning G et al, 2006). Among all blood pressure variables analyzed, only a small but nevertheless significant increase in the SAP was observed by telemetry in tg rats of L6351 as compared to their non-tg littermates at day 35/36 (see Tab. 2). The blood pressure phenotype was analysed primarily in one month old rats because of maximum mRNA expression of the tg hET$_A$ (Kliesch, S. MD thesis, Freie Universität Berlin; to be submitted). L6878 rats at the age of 6 weeks showed significantly increased MAP of 107 $\pm$ 5 mm Hg under PB anesthesia compared to age-matched non-tg SD rats showing 83 $\pm$ 7 mm Hg which is consistent with a moderately hypertensive phenotype. Telemetric blood pressure measurements, though desirable were not performed in this line due to high costs. The observed difference in the blood phenotype between both lines was concordant with total ET$_A$ transcript levels (human tg ET$_A$ and endogenous rat ET$_A$) detected in mesenteric arteries which were only marginally increased (1.1 fold) in mesenteric arteries of L6351 tg rats but 2.1-fold increased in L6878 tg rats (Kliesch, S. MD thesis).

Tail vein plethysmography under diethyl ether anesthesia was chosen as a non-invasive complementary method to further characterize the blood pressure phenotype. Using this method SD rat of 1 month of age showed a decrease in SAP by 14 mm Hg when compared to SAP under PB anesthesia. L6878 exhibited a reduction in SAP by 35 mm Hg at the age of 1 month when compared to SAP measured under PB anesthesia at the age of 6 weeks (Tab. 4b). This could be due to the reason that hypertensive tg animals from L6878 are more sensitive to anesthetic diethylether. The observed lowering of SAP in L6878 is regarded as a specific bp-lowering effect of the anesthetic diethylether. In rats, diethyl ether is known to produce potent arterial vasodilatation in the splanchnic circulation, which, although partially compensated by increased cardiac output, resulted in hypotension (Walsh GM et al, 1979; Vidt DG et al, 1959). Therefore it is concluded that diethylether mediated a significant splanchnic vasodilatation in both tg L6878 as well as in SD rats. The SAP in L6878 under ether though nonsignificant at 1 month is in line with the PB measurements at 6 weeks.

To analyse the functional phenotype with increasing age, blood pressure analysis was performed at the age of 3 months by tail vein plethysmography. The absolute difference in the SAP in L6878 compared to SD at 1 month of age; 13 mm Hg, under ether anesthesia was more conspicuous than at 3 months of age; 5 mm Hg. The reason for

this could be due to counteractive mechanisms which lowered the tg expression with age progression. The difference could also be due slow loss of tg expression with age, which was maximum at the age of 1 month, as was demonstrated by quantitative PCR in L6878. This also confirms findings from reports where counteractive mechanisms downregulate or shut down the expression of the tg in question or other vasoactive receptors (Lassegue B et al, 1995; Nickenig G et al, 1997). Another possibility for a lower blood pressure with increasing age could be the physiological adaptability of the tg hET$_A$ receptor with the endogenous ET$_A$ receptor.

A

Groups	L6351	L6878	SD	Non-tg Littermates
Telemetry 1 month	93 ± 3	NI	NI	90 ± 2
PB 6 weeks	NI	108 ± 5	83 ± 7	NI

B

Groups	L6351	L6878	SD	Non-tg Littermates
Telemetry 1 month	113	NI	NI	109
PB 6 weeks	NI	142.8 + 14	109.1 + 10	NI
Ether	90.1 + 3	107.8 + 18	95.2 + 5	NI

Tab. 4. Blood pressure in rat lines under study. **(A)** Table shows the MAP in tg lines L6351, and L6878, SD and non-tg littermates. Age of animals is depicted under each group. **(B)** Table shows SAP in tg lines L6351, L6878, SD and non-tg littermates. Data expressed as mean ± sd. PB group N=5; Ether group N= 6 in SD, N=9 in L6351 and N=8 in L6878; Telemetry group N=5; PB= Pentobarbital sodium: NI= not investigated.

4.2 Blood pressure phenotype in L6351 under NO inhibition

L-NAME is an L-arginine analogue which blocks NO synthesis. Inhibition with L-NAME is associated with elevation of blood pressure in rats (Deng LY et al, 1993; Li JS et al, 1994; Schiffrin EL; 1995). L-NAME treatment of L6351 rats for three days resulted in significantly increased blood pressure variables in tg rats and in their non-tg littermates. A significant difference in the blood pressure increase was not observed between tg and non-tg rats. An overactivated (endothelial) NO synthase in tg rats which may have compensated a hypothetical blood pressure effect of ET$_A$ overexpression is, therefore, regarded as unlikely. Treatment with L-NAME and LU 302146 combination for 6 days did not significantly decrease blood pressure in either group. This result is in line with previous reports where L-NAME mediated hypertension was not reversed by ET$_A$ receptor antagonist treatment in SD rats (Sventek P et al, 1997).

4.3 Structural phenotype

Histomorphometric analysis of cross-sectional areas of carotid arteries of L6351 tg rats of 1 month age showed a significantly increased NA of the media consistent with medial hypertrophy. The finding of carotid artery medial hypertrophy in L6351 is in line with the finding of a 2.5-fold increase in ET_A-encoding transcript levels in the carotid artery of 1 month old L6351 rats compared to non-tg littermates (Kliesch, S. MD thesis). This finding is of particular interest because it strongly supports a blood-pressure independent hypertrophic effect of ET_A since tg L6351 rats are normotensive (except for a slight increase in SAP only). Therefore, our hET_A tg L6351 can be regarded as the first *in vivo* model linking ET_A overexpression directly to vascular hypertrophy. Tg L6878 rats also showed a moderate, but non-significant increase in the NA of the carotid artery media associated with 1.7-fold increase in ET_A mRNA expression which may either reflect increased ET_A function or an adaptive response to the moderate hypertension observed in this line.

We did, however, not assess whether medial increase is related to muscle cell hypertrophy (increase in individual cell size) or to an increase in the number of VSMC (i.e., hyperplasia). Studies of primary cultures of porcine coronary artery and aortic VSMC have revealed that ET-1 acts as a co-mitogen (Assender JW et al, 1996) and stimulates collagen type I synthesis by coronary VSMC. This is inhibited by BQ123, an ET_A receptor antagonist suggesting a role of ET_A receptor in vascular remodelling and cellular proliferation (Rizvi MA et al, 1996). However, we did not analyze collagen content of arteries in our model. The co-mitogenic effect of ET_A has been demonstrated to involve transactivation of receptor tyrosine kinases, such as epidermal growth factor receptor (EGFR) and PDGF receptor (Iwasaki H et al, 1999). A recent *in vivo* study has shown that ET can act both as a trophic and as a mitogenic factor in the vascular wall depending on its local concentration (Dao HH et al, 2006). In this report it was shown that i.p. infusion of ET-1 mediates hypertrophy (assayed as leucine incorporation) at low and hyperplasia (assayed as thymidine incorporation) at high concentrations of ET-1.

Our histomorphometric analysis of aorta cross-sectional areas revealed similar increases in the NA of the aortic media in both tg lines (L6351: +18%, p<0.02; L6878: +21%, p<0.02) at the age of 1 month, which is consistent with 2-fold (L6351) and 1.6-fold (L6878) ET_A mRNA overexpression. However, receptor binding studies with aortic membranes (Tab. 3) revealed, however, only a minor increase in ET_A-specific binding in L6351 and no change in L6878. Whereas the small increase in ET_A binding may

account for the growth of the aortic media in L6351, medial growth in L6878 aorta may be explained solely as an adaptive response to the hypertensive phenotype.

For a long time it has been suggested that ET-1 plays a major role in abnormal vascular function and remodeling of blood vessels (Schiffrin EL et al, 1995). Previous studies have shown that vascular ET-1 mRNA levels and the level of immunoreactive ET-1 are elevated in certain models of experimental hypertension, particularly in DOCA-salt hypertensive rats (Deng LY et al, 1992; Larivière R et al, 1993; Larivière R, 1995), in DOCA-salt SHR (Schiffrin EL et al, 1995), and 1K1C Goldblatt hypertensive rats. All these models have been shown to develop hypertension and associated vascular hypertrophy which respond to treatment with mixed or ET_A-selective receptor antagonists. Since the phenotype of L6351 for the first time provides direct evidence of a vascular growth-promoting effect of ET_A *in vivo,* it can be assumed that regression of vascular hypertrophy by ET receptor blockade in rat hypertension models can be, at least in part, attributed to direct growth-inhibiting effects of ET_A antagonists independent of their blood pressure lowering effect.

In light of our data, the SHR model is of particular interest. SHR have a SAP of 175 mm Hg, while our tg line L6878 showed a SAP of 142 mm Hg under PB anesthesia. Although the SHR model develops a blood pressure phenotype comparable to that of DOCA-salt rats or other models showing increased vascular ET-1, it shows only moderate vascular hypertrophy. This difference is most likely due to an additional growth-promoting effect of vascular endothelin via vascular ET_A in DOCA-salt rats which is absent in the SHR model in which vascular hypertrophy may merely reflect the adaptive response to hypertension. According to our tg model, lower vascular ET-1 levels as observed in SHR as compared to DOCA-salt rats are assumed to cause less ET_A activation and limited vascular hypertrophy. In contrast, high vascular ET-1 levels may promote media thickening by enhanced ET_A activation state even in the absence of significant blood pressure elevation.

L6351 did not show a significant increase in SMA wall thickness which is consistent with only minute increase in ET_A mRNA transcripts in the mesenteric bed of this line. Limited ET_A receptor binding data obtained with SMA membrane preparations of this tg line suggest, however, a clear increase in ET_A binding which is in conflict with the mRNA data and with an apparent lack of SMA hypertrophy.

In contrast, analysis of right and left wall thickness of pressurized SMA of L6878 revealed significantly increased normalized wall thickness (+27%, $p<0.005$) when

compared to age-matched SD controls. The increase in wall thickness is associated with a 2.1-fold overexpression of ET_A mRNA in this line at the age of 1 month. It cannot be determined to what extent ET_A overexpression and the adaptive response to moderate hypertension in L6878 contributed to mesenteric artery hypertrophy. Unfortunately ET_A binding experiments, though desirable, have not yet been performed in this line. The medial hypertrophy of L6878 SMA is not completely compensated by an increase in total vessel diameter therefore resulting in lumen loss as observed. The volume flow rate of blood flowing through a blood vessel is defined by Hagen and Poiseuille's law, which is defined by the equation Q_v blood = $\Delta P \pi R^4$ / 8nblood x L, where Q_v is the flow rate, n is viscosity, L is length of the vessel, R is the radius of vessel and ΔP is the pressure difference upstream and downstream of the vessel. n, L and ΔP being constant, the flow rate or vascular resistance depends upon fourth power of the radius or the vessel diameter. Therefore, a lumen loss of 8% would lead to a reduction in flow by 28%. Assuming that the flow through the vessel in the circulatory system is constant, the tangential resistance on the vascular wall will be higher, thereby increasing vascular resistance. This explains that a relatively small loss in the lumen will lead to significantly increased peripheral vascular resistance in L6878 associated with the development of high blood pressure. A recent study reported that significantly increased endothelial specific ET-1 expression in a tg mouse model was associated with increased media thickness in mesenteric arteries (Amiri F et al, 2004). Moderately but not significantly increased blood pressure may have contributed to mesenteric arterial hypertrophy in this tg model.

4.4 Heart and kidney morphology

Locally synthesized ET-1 has been reported to mediate hypertrophy of cardiomyocytes via ET_A (Ito H et al, 1993; Yamazaki T et al, 1999). The finding that LVW in 1 month old tg rats of L6351 is normal is consistent with reported inactivation of the SM22α promoter in left ventricular cardiomyocytes after day E10.5 during murine embryonic development (Li L et al, 1996). The minute SAP increase in L6351 was overtly not sufficient to cause adaptive increase in LVW. Thus, the hET_A mRNA detected by RT-PCR most likely reflects exclusive expression in heart vessels. The non-significant increase in L6878 at the age of 4 months may reflect a residual state due to increased afterload by high blood pressure which was detected in 1 month old rats.

One month old tg rats of L6351 exhibited slightly reduced kidney weights compared to non-tg controls, which were overtly related to slightly decreased body weight of this line. This finding is concordant with a recent report which has also reported lower kidney weights (age 10 weeks) in tg mice expressing human ET-1 specifically in the endothelium (Amiri F et al, 2004). Kidneys of tg rats of all three tg lines did not show overt macroscopic or histologic kidney pathologies. Based on these findings we would, therefore, rule out a significant alteration in renal perfusion by ET_A overexpression in renal arteries or aberrant expression of the construct in mesangial cells which are functionally related to VSMC. We did, however, not analyze renal expression of the transgene in detail. Previous reports using tg models of ET peptides have reported significant renal pathology independent of blood pressure phenotype (Hocher B et al, 1996; 1997).

4.5 Induction of acute hypertension in young transgenic rats by S-ketamine/xylazine anesthesia

After inducing anesthesia using S-K/X, tg rats of both lines exhibited significant arterial hypertension (recorded invasively) at the age of 1 month with MAP (L6351, 130 $\pm$ 15; L6878, 129 $\pm$ 13 mm Hg) when compared to age-matched SD controls (MAP, 81 $\pm$ 6). Association of hypertension induction with K/X anesthesia was unequivocal in young L6351 rats since only minute increases in the systolic arterial pressure were recorded by radiotelemetry (compare 3.1.2.1). Association with acute hypertension was also likely in tg rats of L6878 since blood pressure recorded under K/X anesthesia was significantly higher compared to blood pressure recorded in PB anesthesia (compare 3.1.2.2). Knowledge of anesthetic agents, alone or in combination with other preanesthetics, is a prerequisite to understand hemodynamic effects in anesthetized animals. Each drug has its own peculiarities and it acts differently when combined with another (Gonzales G et al, 2003). As reported in results, SD rats of 1 month showed normotension after K/X anesthesia, while hypertension was confined to young tg rats from both lines in K/X group (refer 3.1.2.3). It has to be noted that our 2 month old SD rats also developed hypertension with MAP of 140 mm Hg after induction of K/X anesthesia (data not shown). The phenotype of K/X-induced arterial hypertension showed two aspects: earlier manifestation and more pronounced blood pressure increase compared to non-tg controls.

To achieve anesthesia we administered S-ketamine at a dose of 43.5 mg/kg i.p. Published literature in rat anesthetic protocol must theroretically be considered since this stereoisomeric form is suggested to be twice as potent as the racemic mixture (Ihmsen H et al, 2001). The bioequivalent dose of S-ketamine which was administered for anesthesia was 43.5 mg/kg i.p. where S-ketamine was supplied as 25 mg/ml. The MAP of our SD 1 month old tg rats under S-K/X anesthesia was 81 ± 6 mm Hg. In contrast arterial blood pressure measured in adult female SD rats was reported to rise from 125 ± 4 to 163 ± 6 mm Hg after rats were anesthetized with a combination of ketamine (43.5 mg/kg; i.p.) and xylazine (13 mg/kg; i.p) (Bouairi E et al, 2004) thus confirming the hypertensive effects of S-K/X as we observed in our adult SD rats. Induction of hypertension using K/X seems to depend also on the administration schedule. Another study (Hatzopoulos S et al, 2002) showed that fractionated application of K/X to about 2 month old rats did not induce hypertension.

Adminstration of ketamine in dogs is associated with a hemodynamic profile characterized by increase in blood pressure and depression of baroreflex control (Traber DL et al, 1970a; Traber DL et al, 1970b). The exact mechanism how ketamine increases blood pressure is not clear. Published literature suggests that ketamine raises blood pressure in rats via increased release of catecholamines into the peripheral circulation, indicating that the agent stimulates both neurosympathetic and adrenomedullary functions (Carruba MO et al, 1987). Also it is suggested that it acts as a stimulatory agent on the cardiovascular system, increases blood pressure and cardiac output primarily by speeding HR and maintaining stroke volume (White PF et al, 1982; Schaefer CF et al, 1984). The release of catecholamines is believed to be centrally mediated since ganglionic blockade or spinal transection completely counteracts ketamine-induced release of catecholamine levels (Carruba MO et al, 1987). In this report, catecholamine levels after ketamine anesthesia (60 mg/kg; i.v.) were more than two-fold increased and associated with increased MAP by ~34 mm Hg from 98 to 132 mm Hg.

When we administered S-ketamine i.p. to 2 month old L6878 rats preanesthetized using pentobarbital (60 mg/kg; i.p.) only a minor increase in MAP of 9 mm Hg from baseline MAP of 77 mm Hg was observed. This observation is confirmed by a previous report showing that ketamine administration was associated with less cardiovascular stimulation when adminstered after diazepam than it did when adminstered alone (Haskins SC et al, 1985; Haskins SC et al, 1986). Since PB and diazepam both act as

gamma-amino butyric acid A ($GABA_A$) agonists, it appears conclusive that $GABA_A$ stimulation inhibits the hemodynamic stimulation by ketamine. In contrast, co-administration with α_2-agonists (xylazine, dexmedetomidine) together with ketamine overtly potentiated the hemodynamic effects of ketamine.

To find out if catecholamines were involved in K/X-induced hypertension, *in vivo* blood pressure measurements were conducted using prazosin, a α_1-adrenoceptor receptor antagonist. Infusion of prazosin (3 mg/kg; i.a.), immediately normalized acute hypertension in tg rats of L6888 of 2 months of age (Fig. 13 left). In contrast BQ123, a ET_A receptor antagonist bolus dose (0.4 nmoles/kg) in 2 to 3 month old tg L6878 rat did not normalise the blood pressure within 15 min. Unfortunately, our preliminary measurements of catecholamine levels in the plasma after K/X and PB anesthesia respectively, are still inconclusive. The prazosin experiment strongly, although only indirectly, suggests that hypertension in young tg rats induced by S-K/X anesthesia is catecholamine mediated. The mechanism of exaggerated blood pressure response to S-ketamine in tg rats remains to be clarified.

4.6 Contractile responses of isolated perfused mesenteric arteries

4.6.1 SMA contractile response to KCl

High extracellular concentration of KCl causes membrane depolarization which triggeres Ca^{2+} influx via VOCC and thus mediates vasoconstriction of the vascular membrane by increasing [$(Ca^{2+})i$] concentrations (Wellman GC et al, 2001). Pressurized mesenteric arteries of L6878 tg rats anesthetized using PB did not exhibit significant changes in the DRC to KCl when compared to non-tg controls (Fig. 16). In contrast, mesenteric arteries isolated from tg rats of both lines, L6351 and L6878, anesthetized using K/X before artery preparation, exhibited significantly decreased contractile sensitivity to KCl (rightward shift of the DRC) compared to non-tg littermate controls (Fig. 23). The maximum contractile response was, however, not different. The contractile response of a blood vessel (here mesenteric artery) depends on the interplay of many ion channels and influx of Ca^{2+} via different pathways (Wellman GC et al, 2003). The theoretical peak plasma concentration achieved after i.p. injection of S-ketamine was estimated to be in the range of about 10^{-2} M. Since S-ketamine was injected as i.p. bolus we expected even 10-fold higher local concentration in the peritoneal space. Also S-ketamine may, therefore, diffuse locally to the wall of the mesenteric artery and inhibit VOCC as reported in the literature. It has been shown

more than two decades back that pretreatment of feline mesenteric arteries using ketamine at concentrations higher than 5×10^{-5} M attenuated KCl induced contractions (Fukuda S et al, 1983). Additionally, ketamine has also previously been shown to induce relaxation of rat resistance SMA VSMC by reducing $[(Ca^{2+})i]$ concentrations resulting in decreased contractility (Akata T et al, 2001). The exact mechanism by which ketamine inhibits L-type VOCC is currently unknown. Since SMA of non-tg L6878 littermates anesthetized using K/X did not show any difference when compared to those anesthetized using PB the decrease in KCl-induced contraction is apparently transgene dependent. The mechanism by which the tg state facilitates the postulated inhibition of VOCC by ketamine remains to be clarified.

4.6.2 Contractile response to ET-1

Our *in vivo* measurements exhibited non-significant differences in maximum blood pressure increase following ET-1 injection (refer 3.1.2.3). Pressurized SMA of tg animals of all lines (L6351, L6878, L6888) anesthetized using PB before artery dissection exhibited significantly increased sensitivity to exogenous ET-1 compared to non-tg SD controls. Based on the ET_A mRNA expression level detected in mesenteric artery homogenates one would expect a less pronounced leftward shift in the ET-1 DRC in L6351 compared to L6878. The interpretation of the functional data of L6351 is further complicated by the preliminary results of our receptor binding analysis suggesting strongly enhanced binding to ET_A and, unexpectedly to ET_B, in mesenteric artery homogenates of tg L6351 rats (Figs. 29 and 30). This suggests that ET_A receptor transcript levels cannot be translated into protein in a simple linear manner. ET receptor function is subject to extensive posttranslational mechanisms, as previously reported for ET_B (Okamoto Y et al, 1998). Additionally, ET_A/ET_B heterodimersation may occur and affect ligand binding and receptor signalling in our tg lines (Gregan B et al, 2004). It must also be considered that while vessel function was analyzed in isolated 1st order mesenteric arteries, mRNA expression and receptor binding were analyzed in homogenates of the complete mesenteric artery vascular bed which may, at least to some degree, account for differences between functional and expression data.

After induction of anesthesia using K/X and in contrast to PB anesthesia, isolated perfused mesenteric arteries of rats of both tg lines, L6351 and L6878, showed significantly increased contractile responses only with very low ET-1 concentrations (Fig 24). It has been clearly demonstrated that ET-1 induced arterial constriction is Ca^{2+}

dependent (Sarria B et al, 1990; Huang XN et al, 1991; Klockner U et al, 1991). However, ET-1 stimulates Ca^{2+} influx through multiple types of Ca^{2+} channels (Kawanabe Y et al, 2001). These Ca^{2+} channels differ in ion selectivity, tissue distribution, and mechanisms of activation (Neylon CB, 1999). Among these, VOCC seem to play a limited role since non-selective cation channels (NSCC) and store-operated Ca^{2+} channels (SOCC) are involved as well (Kawanabe Y et al, 2001). Voltage-gated L-type Ca^{2+} channels are one type of voltage channels which allow influx of extracellular Ca^{2+} into the cytosol to mediate further release of $[(Ca^{2+})i]$. Taking into consideration the complexity of Ca^{2+} currents activated by ET-1 and the inhibitory effect of S-ketamine on VOCC as discussed in 4.6.1 we hypothesize that at low ET-1 concentrations VOCC (e.g., NSCC or SOCC) are activated by ET-1 which are not sensitive to S-ketamine inhibition. At higher ET-1 concentrations, VOCC may be activated and inhibition by S-ketamine due to S-K/X anesthesia would diminish the contractile response as observed. More specific investigation of Ca^{2+} channels is required to further address this issue.

4.6.3 Contractile response to phenylephrine

Our *in vivo* blood pressure measurements exhibited attenuated maximum response to PE injection (refer 3.1.2.3) in S-K/X anesthetized rats. This *in vivo* observation is concordant with the functional analysis of isolated perfused SMA showing significantly decreased responses to PE when rats were anesthetized using S-K/X. This effect was clearly related to this anesthetic since SMA of tg L6878 rats anesthetized using PB did not show any significant difference compared to non-tg controls, neither in sensitivity nor in maximum attainable response to PE (Fig. 19). Moreover, SMA of non-tg littermate control rats anesthetized using S-K/X showed moderately decreased PE responsiveness compared to control rats anesthetized using PB (Fig. 25). PE primarily acts through the α_1 adrenergic receptor. Stimulation of α_1 receptor leads via G_q-coupling to activation of PLC, hydrolysis of the PIP2 to form IP3 which activates Ca^{2+} release from intracellular stores and DAG which activates plasma membrane Ca^{2+} channels resulting in contraction of the VSMC (Curtis BM et al, 1984; Curtis BM et al, 1985; Hosey MM et al, 1987; Seraskeris S et al, 2001). Inhibition of VOCC by locally (i.p.) administered S-ketamine may explain the decreased contraction observed in mesenteric arteries of tg rat anesthetized using K/X. Another possible explanation of the observed decrease in PE-induced contractions is the known desensitisation of α_1-

adrenergic receptors by catecholamine release evoked by ketamine (Lurie KG et al, 1985; Tsujimoto G et al, 1987; Wakabayashi I et al, 1989).

As stated before, ketamine increases plasma catecholamine levels by centrally mediated activation of sympathetic neuronal and adrenomedullary functions (Carruba MO et al, 1987). On VSMC, catecholamines activate α_1-adrenergic receptors which mediate increase of DAG as mentioned before. DAG can activate PKC and putatively induce α_{1B} adrenoceptor phosphorylation and desensitization (Garcia-Sainz JA et al, 1999). It was also shown that ET_A activation in rat-1 fibroblasts stably expressing transfected α_{1B} adrenoceptors induced marked and rapid desensitization of these receptors (Vazquez-prado J et al, 1997). It is, therefore, conceivable that ET_A overexpression in mesenteric arteries of tg rats effects enhanced desensitization of α_1-adrenoceptors on VSMC resulting in loss of responsiveness to PE after K/X-induced stimulation. Details of the signalling mechanisms involved in putative ET_A/α_1 adrenergic receptor interaction were not analyzed in this work.

Moreover, xylazine, an α_2 adrenergic receptor agonist with muscle relaxant and sedative-hypnotic properties, may also be involved in the inhibition of PE-evoked mesenteric artery constriction. Xylazine mediates its sedative-hypnotic action through stimulation of α_2 adrenoceptors in the brain stem resulting in central sympathicolysis associated with decreased release of catecholamines (NE) thus counteracting the effects of ketamine. The vascular actions of peripheral α_2 adrenergic receptors are diverse and associated with opposing effects: (1) inhibition of NA release from sympathetic nerve endings via presynaptic α_2 adrenergic receptors, (2) vasoconstriction via postsynaptic α_2 adrenergic receptors expressed on VSMC (Van Meel JC et al, 1981; Medgett IC et al, 1984; Carrier GO et al, 1985), and (3) endothelium-dependent vasodilatation through α_2 receptors on ECs (Agrawal KD et al, 1985; Miller VM et al, 1985; Nakane T et al, 1987; Matsuda H et al, 1985; Bockman CS et al, 1996). Analogous experiments using PB as anesthetic are required to better assess whether decreased maximum response to PE was due to the ET-1 dose administered, the acute hypertensive state *per se* induced by K/X, inhibition of ET_A signalling by K/X or posttranlational modification of ET_A function.

4.6.4 Contractile response to U46619

Prostaglandins and thromboxanes are the metabolites of the metabolic degradative pathway of phospholipids in the cell membrane, arachidonic acid being the intermediate

product (Dusting GJ et al, 1979). PGH2 and TXA2 act on endoperoxide/thromboxane receptors in VSMC to induce vasoconstriction (Halushka PV et al, 1989; Coleman RA et al, 1994). TXA2, formed in this pathway is a potent vasoconstrictor which mediates platelet aggregation and activation (Hamberg M et al, 1975) and is also involved in mediating hypertrophy and hyperplasia of VSMC in rat via PKC (Craven PA et al, 1996). Using the stable TXA2 mimetic U46619 (9,11-Dideoxy-9a,11a-methanoepoxyprostaglandin $F_{2\alpha}$), it was shown that TXA2 vasoconstrictor action could be related to its inhibition of calcium-activated potassium channels (K_{Ca}) (Scornik FS et al, 1992). Since the DRC to U46619 in both tg lines did not show any significant differences compared to non-tg controls (neither in maximum response nor sensitivity) an effect of ET_A overexpression on the function of VSMC K_{Ca} channels is unlikely. In contrast to experiments using agonists activating VOCC (ET-1, PE) an inhibitory effect of K/X pretreatment was not observed with U46619 confirming that VOCC are not involved in TXA2 mediated vasoconstriction.

4.7 Vasodilative mechanisms

Vascular endothelium has a very important function in inducing or maintaining vascular dilatation (Furchgott RF, 1980). Animals and humans with endothelial dysfunction were found to have reduced capacity to generate potent vasodilators like NO and PGI2. These subjects presented with exaggerated oxidative stress (Tomasian D et al, 2000) and exhibit attenuated response to ACh mediated vasodilatation (Kugiyama K et al, 1990). It was shown that in arteries isolated from animal models of different forms of hypertension endothelium-dependent relaxation is reduced (Vanhoutte PM et al, 1989; Vanhoutte PM et al, 1995). Endothelium-dependent vasodilatation was analyzed in tg rat L6351 by establishing DRC to increasing concentrations of ACh in SMA preconstricted using the TXA2 mimetic U46619. We used U46619 for preconstriction as it is known that equimolar concentration of PE and U46619 evoke contractions in the rat mesenteric arterial bed via Ca^{2+} influx differently. While PE-mediated contraction is rapid, followed by a tonic or rapid diameter oscillations, U46619 mediates Ca^{2+} influx rather in a slow and sustained manner (Shaw L et al, 2004). Tg rats of L6351 at the age of 7 weeks showed significantly decreased (by 40%, $p < 0.001$) vasodilatation to ACh, (Fig. 21) as compared to age-matched SD controls suggesting significant endothelial dysfunction. It is worth noting that ET-1 mediated vascular dysfunction has previously been reported in a tg mouse model of human ET-1 (Amiri F et al, 2004). It was shown in

this work that increased arterial ET-1 levels resulted in NADPH oxidase activation and superoxide generation. Published reports also suggest that ET-1 induced oxidative damage may be mediated via both ET_A (Galle J et al, 2000) and ET_B receptors (Dong F et al, 2005). In contrast endothelial-independent vasodilatation induced by nitrodilator SNP was not altered in either tg line. This suggests that ACh which requires intact endothelium to mediate vasodilatation, was dysfunctional in tg line, while the total turnover of cGMP mediated vasodilatation to nitrovasodilator was unchanged.

4.8 Expression of the transgenic human ET_A receptor is not induced by vascular injury

A physiological role for ET-1 in experimental balloon injury models has been suggested by several previous studies. Upregulation of ET-1 and its receptors was reported in balloon-injured arteries of rat and rabbit, suggesting that endothelin may contribute to neointima formation after vascular injury (Wang X et al, 1995, Azuma H et al, 1994, Azuma H et al, 1995; Viswanathan M et al, 1996)). Medial SMC proliferate and migrate to form a neointima in response to arterial balloon injury (Schwartz SM et al 1986) which may be promoted by ET_A. We were, therefore, interested in analyzing whether vascular injury of the carotid artery would induce mRNA expression of the tg ET_A in L6351 rats at the age of 4 months when basal tg expression was largely downregulated. The current hypothesis was prompted by the previous observation of SM22α expression in neointimal cells (Ribault S et al, 2001). In case of induction of tg expression, L6351 would have provided an injury-triggered model of ET_A overexpression allowing to study the immediate biological effect of the ET_A in this setting. However, qualitative RT-PCR analysis of hET_A transcript levels in injured carotid arteries at day 7 post injury excluded any significant difference compared to the noninjured contralateral artery of the same animal. Therefore, It can be concluded that the SM22α promoter fragment which drives expression of the hET_A is not activated during the highly proliferative phase at day 7 post injury which may be due to lack of enhancer elements to which injury-activated transcription factors bind. Since only the transcript level was analyzed in this study, we cannot exclude with certainty alterations in the protein expression level in the injured artery, nor was neointima formation analyzed histomorphometrically at later time points.