CHAPTER 3: RESULTS

The temporal expression of the transgene and endogenous ET_A receptor was analyzed in great detail on the transcript level in our group (Kliesch, S. MD thesis, to be submitted; Kliesch, S. et al., 2005). Since knowledge of the expression is necessary to fully appreciate the phenotypic findings presented in this work, the main findings of the quantitative expression analysis performed by Kliesch, S. are briefly summarized. Distinct expression of transgene hET_A receptor was demonstrated in each of the three tg lines established in our group (i.e., L6351, L6878, and L6888) at the age of 1 month. Quantitative mRNA expression analysis indicated that in 1 month young tg rats, total aortic ET_A transcript levels (i.e., hET_A and rat ET_A) are increased about 2 fold in L6351 and 1.6-fold in L6878 (compared to non-tg controls). In contrast, mesenteric artery ET_A transcripts are only modestly increased in L6351 rats, whereas they show more than 2.1 fold increased levels in L6878. Tg animals of L6351 have virtually lost vascular transgene expression at the age of one year and tg animals of L6878 showed considerably reduced expression. Qualitative analysis suggested that there was apparently decreased expression at the age of 4 months in L6351.

3.1 Blood pressure

3.1.1 Noninvasive blood pressure measurements

At the age of one month which is associated with maximal expression of tg hET_A as analyzed by quantitative RT-PCR, systolic arterial pressure (SAP) was assessed in ether anesthetized rats of lines L6351 and L6878 noninvasively using tail cuff plethysmography. Analysis of the SAP revealed slightly lower SAP (by 5 mm Hg) in L6351 at the age of 1 month compared to control (SD) rats ($p<0.05$). In contrast, SAP of 1 month old L6878 tg rats was non-significantly increased by 12 mm Hg (Fig. 7). At the age of 3 months, SAP of tg rats did not differ from age-matched SD rats. While tg ET_A line L6351, exhibited a non-significant decrease of 4 mm Hg, L6878 showed a non-significant increase of 5 mm Hg. Compared to the blood pressure at 1 month, SAP measured in 3 months old rats (about 120 mm Hg) was increased in all groups.

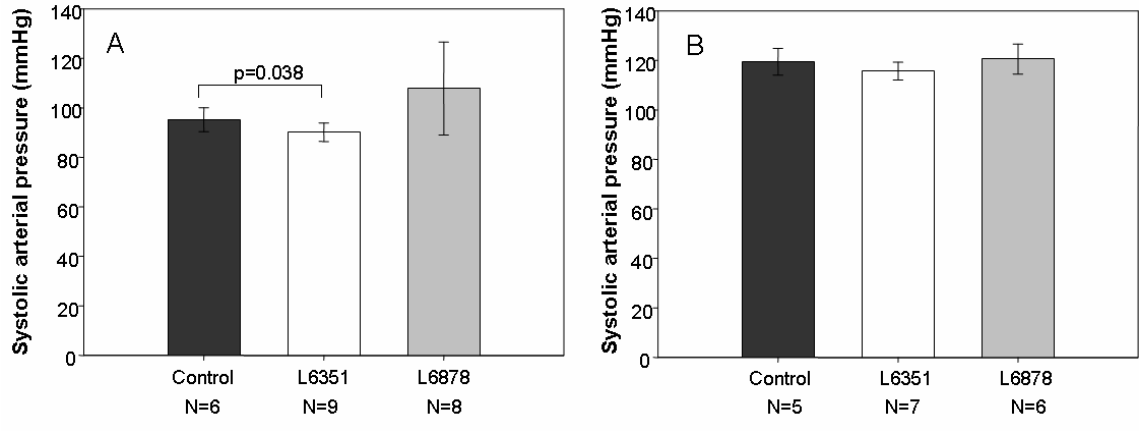

Fig. 7. Systolic arterial pressure in transgenic rats. SAP was measured using tail plethysmography in ether-anesthesized tg rats of L6351 and L6878 at the age of 1 month **(A)** and 3 months **(B).** SD rats served as non-tg controls. Data expressed as means ± sd.

3.1.2 Invasive blood pressure measurements

3.1.2.1 Radiotelemetric blood pressure analysis in L6351

In vivo basal blood pressure parameters were assessed in L6351 rats by radiotelemetry (transmitters implanted at day 28). After recovery from surgery, the tg rats showed a small but significant increase of 4 mm Hg in the SAP at day 35/36 and, according to unchanged DAP, a small increase (+3.3 mm Hg) in RR amplitude (Tab 2.).

Treatment with the NO synthase inhibitor L-NAME was started at day 42 and at day 45/46 rats showed clearly elevated blood pressure, but no significant differences between tg rats and their non-tg littermates were observed. Combined treatment with L-NAME and the ET_A specific antagonist LU 302146 resulted in only marginally decreased blood pressure in both groups which was statistically not significant. After day 45/46 administration of LU 302146 was continued and blood pressure assessed at day 58/59 was similarly decreased (MAP decrease of about 8 mm Hg) in both groups. HR was not different between tg and non-tg rats from day 35/36 and showed the expected adaptive decrease to blood pressure elevation by L-NAME to the same extent. The following Table 2 summarizes the results.

	Line 6351 (n=5)					
	Control – transgenic negative (n=5)					
Parameter	SAP	MAP	DAP	Pulse pressure	RR variability	HR
24 h post OP Day 29 (1)	111,9	88,4	67,5	44,4*	7,2*	479**
	114,5	87,4	65,8	48,7	9,5	460
Basal Day 35/36 (7/8)	113,8*	93,1	72,9	40,9**	7,1	484
	109,7	90,0	72,1	37,6	7,2	497
06:00-18:00 Day 36 (8)	116,0*	95,4	75,1	40,9**	6,7	492
	111,2	92,1	74,0	37,3	6,4	505
18:00-06:00 Day 35/36 (7/8)	113,8*	92,6	71,8	41,9**	6,9	488
	109,0	89,1	70,6	38,3	6,9	493
L-NAME Day 45/46 (17/18)	142,9	113,0	84,1	58,8	9,4*	407
	142,0	114,7	86,2	55,8	10,0	401
L-NAME + LU 302146 Day 51/52 (23/24)	142,8	111,7	83,6	59,1*	10,5	401
	139,0	111,6	86,1	52,9	10,4	390
LU 302146 Day 58/59 (30/31)	132,3	104,2	80,9*	51,4*	7,9	405
	130,1	106,1	85,1	45,0	7,7	401

Tab. 2. Telemetric blood pressure analysis in L6351. Invasive telemeteric blood pressure measurements in 4 to 8 week old freely moving male rats were performed using telemetric probes surgically inserted in rats, non-tg littermates served as controls. Data expressed as means $\pm$ sd. N=5 for each group. Systolic arterial pressure, (SAP), mean arterial pressure, (MAP), diastolic arterial pressure (DAP) and HR represents heart rate. Statistical significance of differences between tg (white) and non-tg rats (grey) was calculated (*p< 0.05, ** p< 0.01).

3.1.2.2 Blood pressure analysis in pentobarbital-anesthetized rats

Invasive systemic blood pressure analysis in 6 week old rats of L6878 anesthetized using pentobarbital (PB) showed significant increases in all blood pressure variables [SAP: +33 mm Hg, p<0.001; MAP: +25 mm Hg, p<0.001; DAP: +17 mm Hg, p<0.17] compared to age-matched non-tg SD rats (Fig. 8). HR measured under PB anesthesia in 6 weeks old animals of line L6878 was 365 $\pm$ 25 min^{-1} which are significantly higher when compared to those measured in the same line under K/X anesthesia (compare Fig. 10).

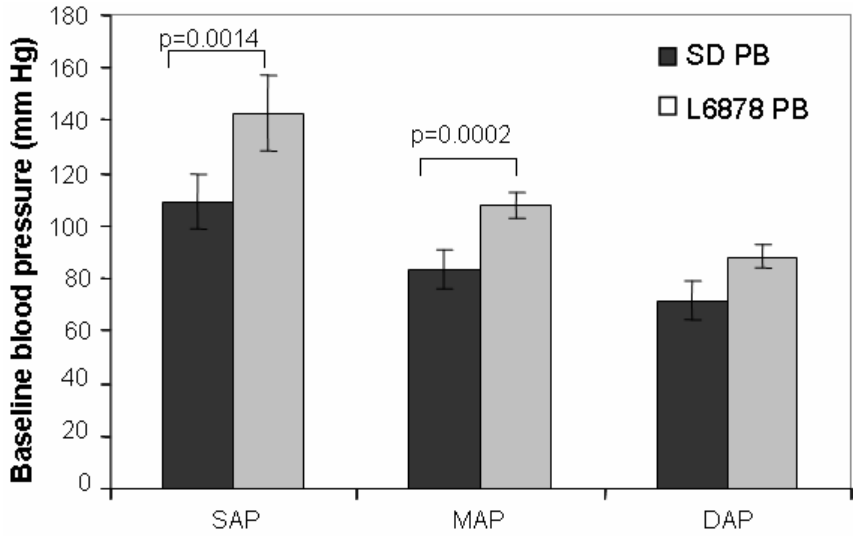

Fig. 8. Blood pressure in L6878 transgenic rats at the age of 6 weeks. Blood pressure was invasively recorded in cannulated rats (via carotid artery) under pentobarbital anesthesia. SAP, systolic arterial pressure, MAP, mean arterial pressure. DAP, diastolic arterial pressure. SD rats served as non-tg controls. Data expressed as means ± sd. N= 6 in each group.

3.1.2.3 Induction of hypertension in young tg rats by ketamine/xylazine anesthesia

Baseline blood pressure in L6351 and L6878 recorded at the age of 1 month showed significantly increased SAP, MAP and DAP under K/X anesthesia (Fig. 9). L6351 tg rats showed significantly increased blood pressure variables [SAP: 182 + 17 mm Hg (+40.1%), $p<0.0001$; MAP: 130 + 15 mm Hg (+38%), $p<0.0001$; DAP: 95 + 6 mm Hg (+30%), $p<0.0001$]. Similarly, L6878 tg rats also showed significantly increased basal blood pressure variables [SAP: 195 + 21 mm Hg (+45%), $p<0.003$; MAP: 129 + 13 mm Hg (+37%), $p<0.0001$; DAP: 101 + 10 mm Hg (+34%), $p<0.0001$] when compared to SD rats as non-tg controls.

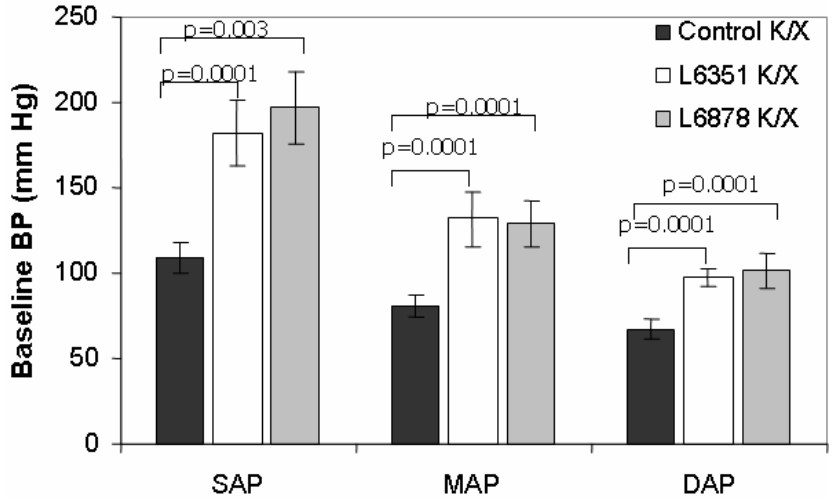

Fig. 9. Blood pressure in S-ketamine/xylazine anesthesized rats L6351 and L6878 at the age of 1 month. Basal blood pressure variables SAP, MAP and DAP were measured invasively in tg rats and controls at 1 month of age. SD rats served at transgenic negative control. Data expressed as means $\pm$ sd. N=6 in each group.

HR recordings under K/X anesthesia showed a distinct and statistically highly significant decrease in both tg lines compared to non-tg controls (Fig. 10). Whereas mean HR was 324 $\pm$ 47 min^{-1} in normotensive SD controls, hypertensive rats of lines 6351 and 6878 showed HR of 242 $\pm$ 19 min^{-1} and 236 $\pm$ 12 min^{-1} respectively.

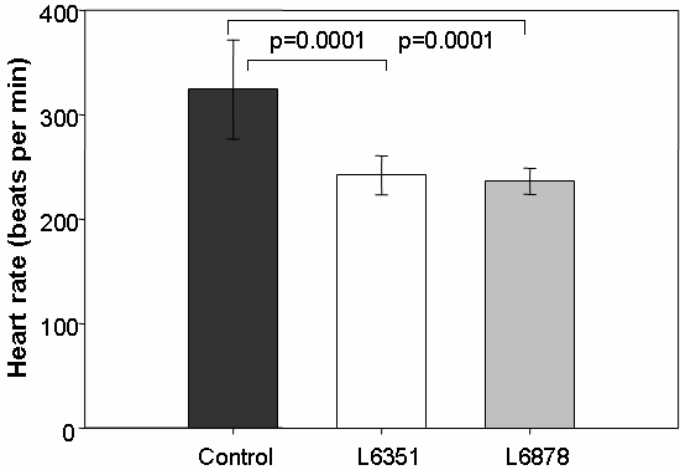

Fig. 10. Heart rates in L6351 and L6878 at age 1 month under S-ketamine/xylazine anesthesia. SD rats served as control. Data are expressed as mean $\pm$ sd of the decrease in heart beats/min at baseline immediately after cannulation. Data expressed as mean $\pm$ sd. N=6 in each group.

Exogenous ET-1 was injected as bolus of 0.3 nmol/kg intraarterially to analyse the maximal attainable response in L6351 and L6878 at the age of 1 month (Fig. 11). Maximal increase in blood pressure in tg rats of both lines was non-significantly different as compared to age-matched controls, maximum increase being 61 mm Hg and 60 mm Hg in L6351 and L6878, respectively, and 68 mm Hg in SD controls.

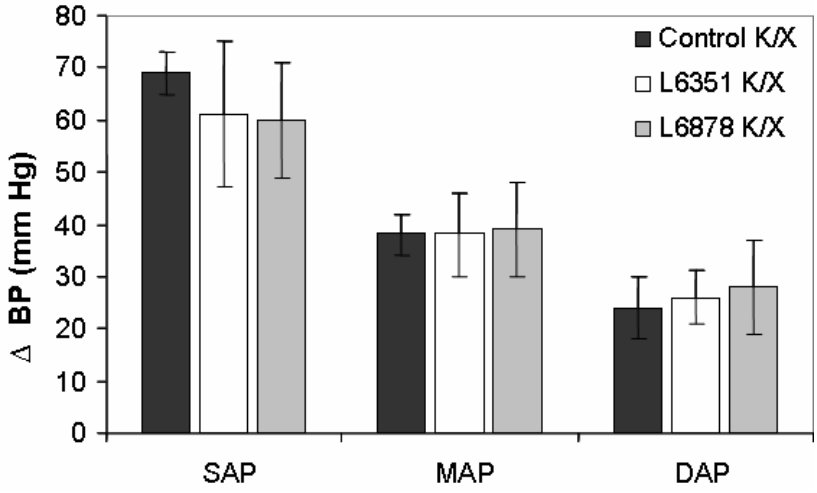

Fig. 11. Maximal blood pressure increase after ET-1 bolus injection. ET-1 (0.3 nmol/kg) was injected intraarterially in 1 month old tg rats of L6351 and L6878 under K/X anesthesia. SD rats served as negative controls. Systolic arterial pressure, (SAP), mean arterial pressure, (MAP), diastolic arterial pressure (DAP) and maximal change in blood pressure after ET-1 bolus (ΔBP) were recorded. Data expressed as means ± sd of maximal increase from baseline after bolus. N=6 in each group.

Before ET-1 injection, the hemodynamic response to the α_1-selective adrenergic agonist phenylephrine (PE) in K/X-anesthetized rats was also studied. A representative recording of the blood pressure showing typical SAP, MAP and DAP and the HR in 1 month old control (SD) rat after injection of PE bolus and, after a wash-out phase and a bolus injection with ET-1 is shown in (Fig. 12).

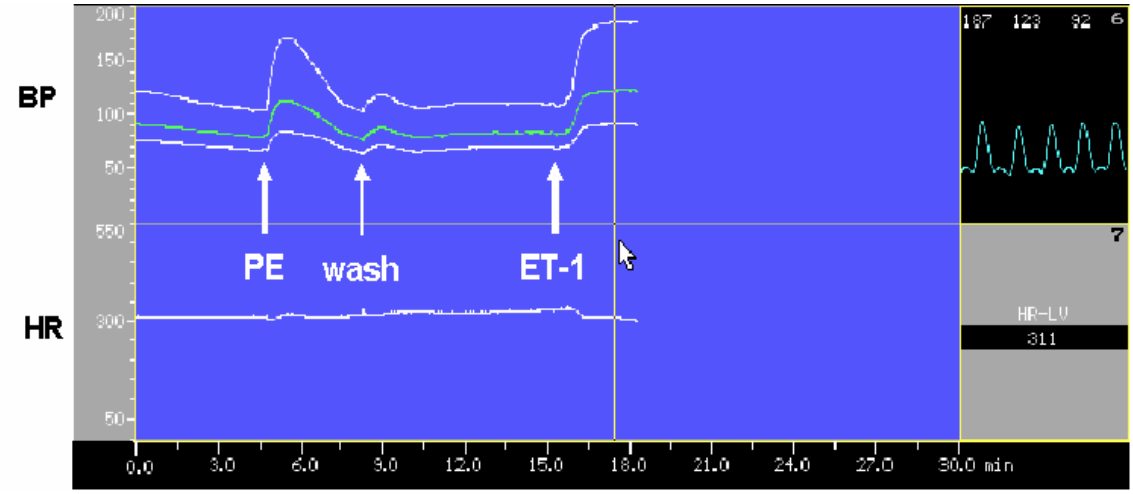

Fig. 12. Representative snap shot showing invasive blood pressure recording. Arrows indicate time points of phenylephrine (PE) and ET-1 bolus injections. BP, blood pressure; HR, heart rate.

Tg rats of L6888 at the age of 2 months were treated with (3 mg/kg; i.a.) prazosin (an alpha 1 receptor antagonist) which lowered elevated blood pressure immediately, which did not increase 10 min approximately up til ET-1 bolus was given (Fig. 13 left). A saline control (10^{-3} M) in L6878 of same age did not decrease high blood pressure which immediately reached the basal values (Fig. 13 right). We also used a second control to confirm this reduction in blood pressure variables. Injection of (a ET_A receptor antagonist) at a dose of 0.4 nmoles/kg did not immediately normalize the blood pressure, rather a slow reduction in SAP was observed over 10-15 min by 40-50 mm Hg (data not shown).

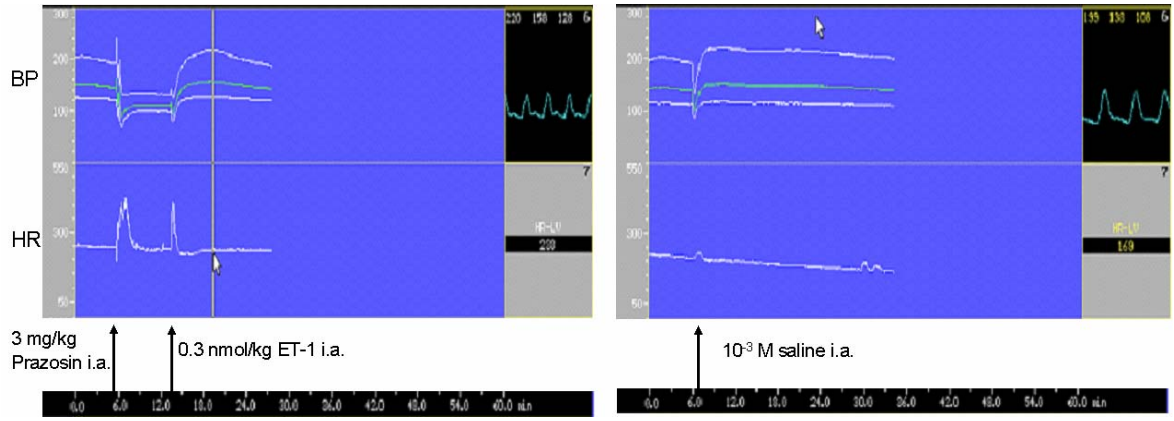

Fig. 13. Snap shot showing lowering of blood pressure variables after treatment with Prazosin in rats of line L6888 at the age of 2 months **(left).** Prazosin was injected intraarterially (3 mg/kg), equimolar strength of saline (10^{-3} M) serves as control in another ET_A line L6878 of same age **(right)**. BP, blood pressure; HR, heart rate.

PE was injected intraarterially (10 µg/kg) and the maximum increase in blood pressure variables was analysed. Tg L6351 rats showed significant decreases in the maximal attainable blood pressure variables after PE bolus, i.e [(-25 mm Hg, p<0.003; SAP,(-48%)], [(-10 mm Hg, p<0.003; MAP, (-34%)], and [(-5 mm Hg, p<0.17; DAP,(-26%)], while line L6878 also exhibited a significant blunted pressor response with [(-36 mm Hg, p<0.004; SAP,(-64%)], [(-18 mm Hg, p<0.005; MAP,(-57%)], [(-8 mm Hg, p<0.02; DAP,(-47%)] decrease as compared to age-matched SD control (Fig. 14).

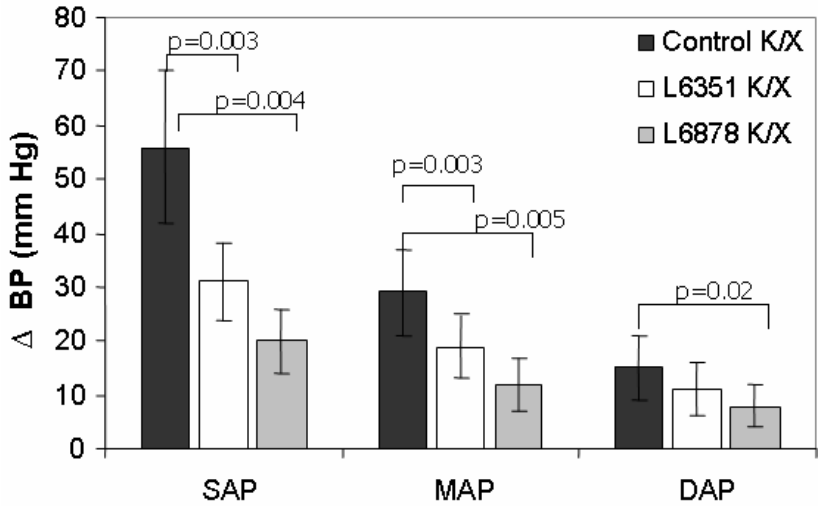

Fig. 14. Maximal blood pressure increase after PE bolus injection. PE (10µg/kg) was injected intraarterially into K/X-anesthetized 1 month old L6351 and L6878 tg rats. SD rats served as non-tg controls. Δ BP, is the maximum increase in BP after PE bolus. Systolic arterial pressure, (SAP), mean arterial pressure, (MAP), diastolic arterial pressure (DAP). Data expressed as means ± sd. N=6 in each group.

3.2 Analysis of *ex vivo* mesenteric artery function

Isolated 1st order SMA were pressurized (70 mm Hg) and DRC were established to various vasoconstrictor agonists to determine the vessel function in ET_A tg lines**.**

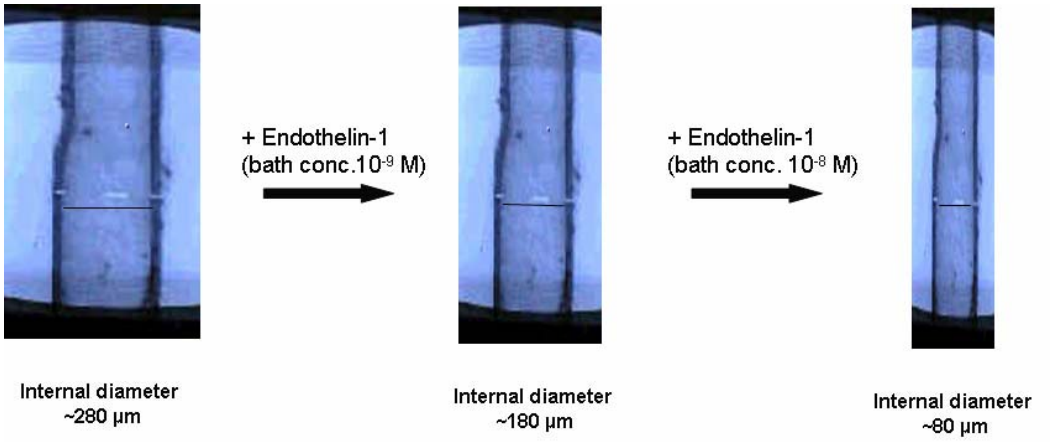

Fig. 15. Representative snap shot of the contractile response of a pressurized first order small mesenteric artery to ET-1.

3.2.1 Mesenteric artery contractile response to KCl

SMA of tg line L6878 of 1 month of age dissected from animals anesthetized with PB did not show a significant difference either in the sensitivity or in maximum attainable constriction to KCl (Fig. 16)

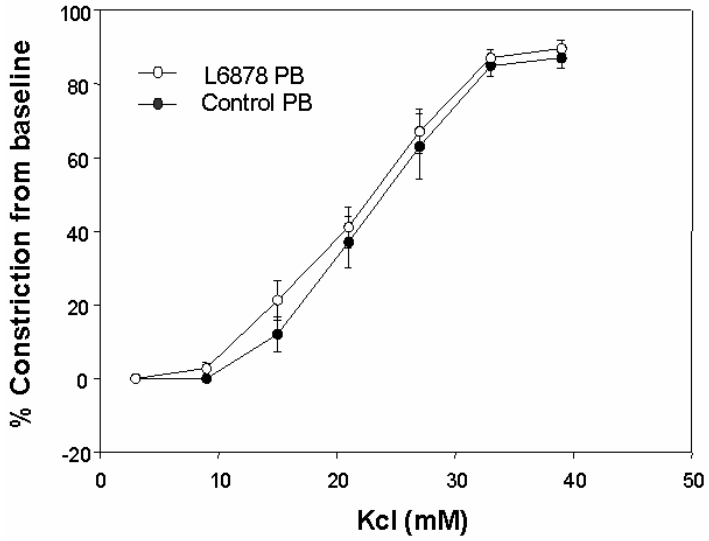

Fig. 16. KCl dose response curves of pressurized mesenteric arteries in L6878. Isolated 1st order mesenteric arteries of 1 month old L6878 were pressurized (70 mm Hg) and the contractile response to KCl was measured. Animals were anesthetized using pentobarbital (PB) before the gastrointestinal tract was removed. Age matched non-tg littermates served as controls. Results expressed as mean ± sd. N=6 in each group.

3.2.2 Mesenteric artery contractile response to ET-1

Dose response curves (DRC) of SMA with increasing concentrations of ET-1 showed a significant leftward shift up to 5.62 x 10^{-8} M ET-1 in 7 weeks old tg rats of L6878, but, unexpectedly, also to a similar degree, in L6351 rats as compared to control SD rats (Fig. 17). EC_{50} was calculated 3 x 10^{-9} M in SD control rats compared to 1.8 x 10^{-9} M in tg rats. However, at supraphysiological ET-1 concentrations (10^{-8} M and 1.8 x 10^{-8} M) mesenteric arteries showed slightly, but significantly decreased contractile response compared to non-tg controls.

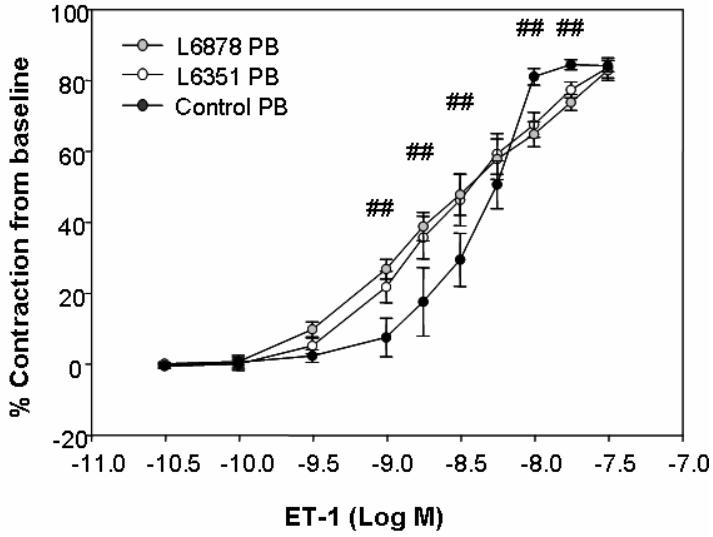

Fig. 17. ET-1 dose response curves of pressurized mesenteric arteries. Isolated 1st order mesenteric arteries of L6878 (age 7 weeks) and L6351 tg rats (age 1 month) were pressurized (70 mm Hg) and contractile responses to ET-1 were measured showing increased sensitivity to exogenous ET-1 up to 5.62 x 10^{-8}M. Animals were anesthetized using pentobarbital (PB) before gastrointestinal tract was removed. SD rats (age 1 month) served as non-tg controls. Results expressed as mean $\pm$ sd. N=6 in each group. ##, $p < 0.01$.

ET-1 DRC were also established in SMA dissected from 7 week old L6888 tg rats, which also showed a significant leftward shift to increasing concentrations of ET-1 (5.62 x 10^{-8}M) when compared to SD rats (Fig. 18).

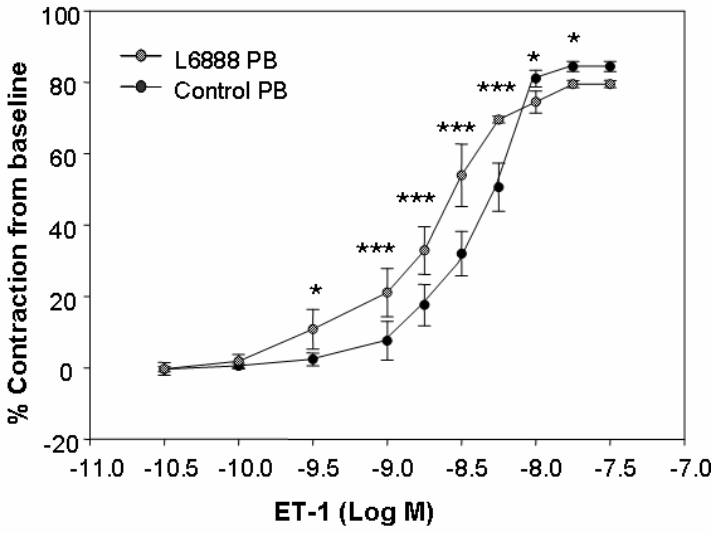

Fig. 18. ET-1 dose response curves of pressurized mesenteric arteries in L6888. Isolated 1st order mesenteric arteries of 7 weeks (L6888) were pressurized (70 mm Hg) and contractile responses to ET-1 were measured showing increased sensitivity to exogenous ET-1 up to 5.62 x 10^{-8}M. Animals were anesthetized using pentobarbital (PB) before gastrointestinal tract was removed. SD rats (age 1 month) served as non-tg controls. Data expressed as mean ± sd. N=6 in each group. *, p< 0.05, ***, p< 0.001.

3.2.3 L6878 mesenteric artery contractile response to phenylephrine

DRC to PE were also established in SMA dissected from 1 month old rats of L6878 anesthetized using PB. Only a slight, but non-significant decrease in PE-induced constriction was observed in tg rats compared to non-tg littermate controls (Fig. 19)

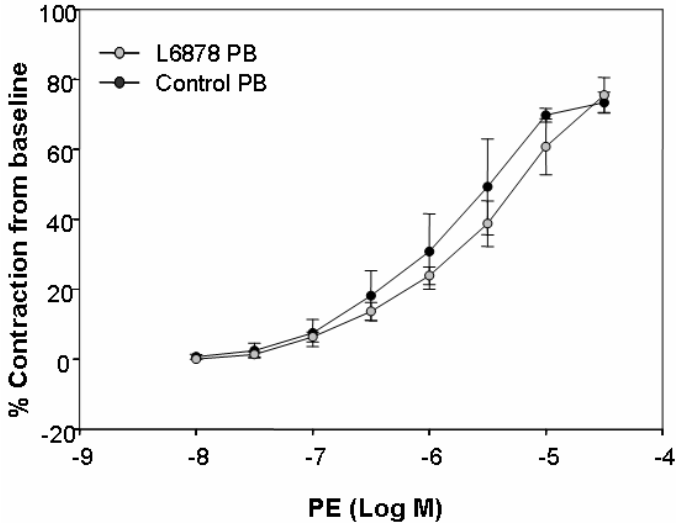

Fig. 19. Dose response curves to phenylephrine (PE) in L6878. Graph shows the maximum percentage constriction from baseline to increasing concentrations of PE in ET_A tg L6878 at the age of 1 month. Non-tg littermates of the same line served as controls. Date expressed as mean ± sd. N=6 in both groups.

3.2.4 Mesenteric artery contractile response to TXA2 mimetic in L6351

DRC to the TXA2 mimetic U46619 were established in SMA mesenteric arteries dissected from 7 weeks old rats of tg line L6351. Compared to non-tg control rats, the contractile response of L6351 arteries was slightly decreased, but this difference was statistically significant only at the highest concentration of the TXA2 agonist (Fig. 20)

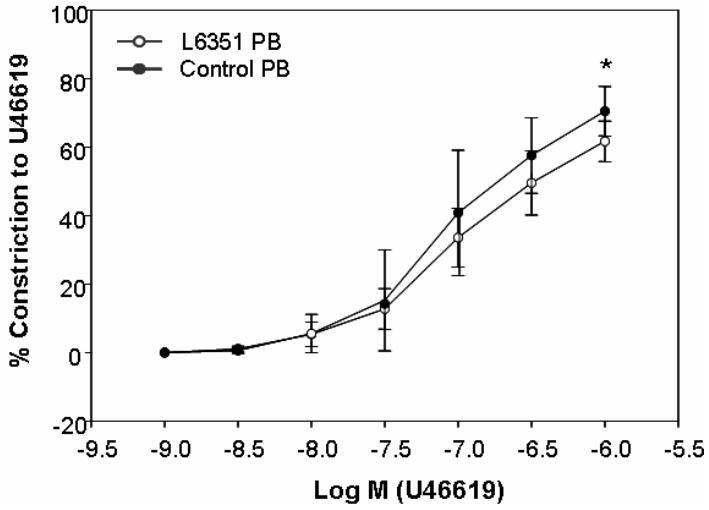

Fig. 20. Mesenteric artery contractile response to U46619 in L6351. Pressurized mesenteric arteries of 7 week old tg rats were subjected to increasing U46619 concentrations and contractile response was recorded. SD rats served as controls. Data expressed as mean ± sd. N=6 in both groups. *, $p< 0.05$.

3.2.5 Endothelium-dependent and -independent vasodilatation

Using acetylcholine (ACh), endothelium-dependent vasodilatation was analysed in the tg rat models. DRC were established using isolated perfused SMA dissected from 7 week old tg L6351 rats anesthetized with PB before gastrointestinal tract was removed. In this experiment, arteries were preconstricted with 10^{-6} M TXA2 receptor agonist U46619. ACh (1 nM to 1 µM) induced concentration-dependent relaxation which was significantly decreased by 40% in arteries from L6351 tg rats compared to arteries from SD rats (Fig. 21).

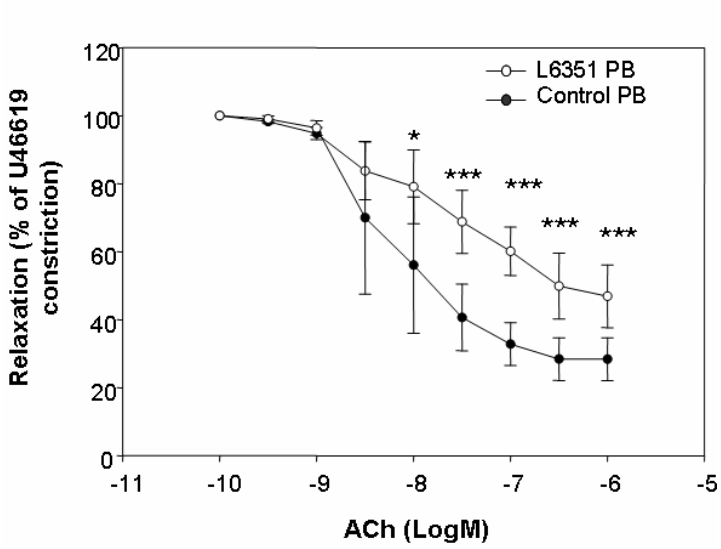

Fig. 21. Effect of acetylcholine in U46619 preconstricted mesenteric arteries of L6351. Isolated 1st order mesenteric arteries of rats of L6351 at the age of 7 weeks were pressurized (70 mm Hg) and maximal dilatation to ACh was observed after preconstriction with U46619. Animals were anesthetized using pentobarbital before gastrointestinal tract was removed. SD served as controls. Data expressed as mean ± sd. N=6 in each group. *, p< 0.05; ***, p< 0.001.

To assess endothelium-independent vasorelaxation, the dilatative response to 10^{-3} M SNP in mesenteric arteries dissected from 1 month old L6878 tg rats was studied. As with ACh, arteries were preconstricted using 10^{-6} M U46619. The SNP-induced vasorelaxation was slightly, but non-significantly enhanced in L6878 arteries compared to non-tg littermates which served as controls (Fig. 22).

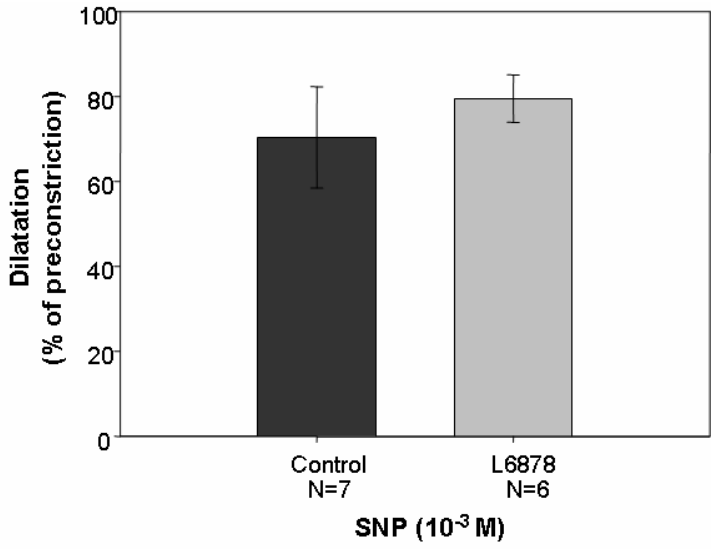

Fig. 22. Effect of sodium nitroprusside in pressurized mesenteric arteries in L6878. Isolated 1st order mesenteric arteries of 1 month old L6878 rats were pressurized (70 mm Hg) and maximal dilatation to SNP was observed after preconstruction with U46619. Animals were anesthetized using pentobarbital before gastrointestinal tract was removed. Non-tg littermates of the same line served as controls. Data expressed as mean ± sd.

3.3. Effect of S-ketamine/xylazine on mesenteric artery contractile response

3.3.1 Contractile response to KCl

Contractile response to KCl was also investigated in pressurized SMA dissected from animals under K/X anesthesia. SMA of L6351 and L6878 showed significantly decreased contractile responses to KCl (distinct rightward shift in the corresponding DRC) compared to non-tg littermates as controls (Fig. 23).

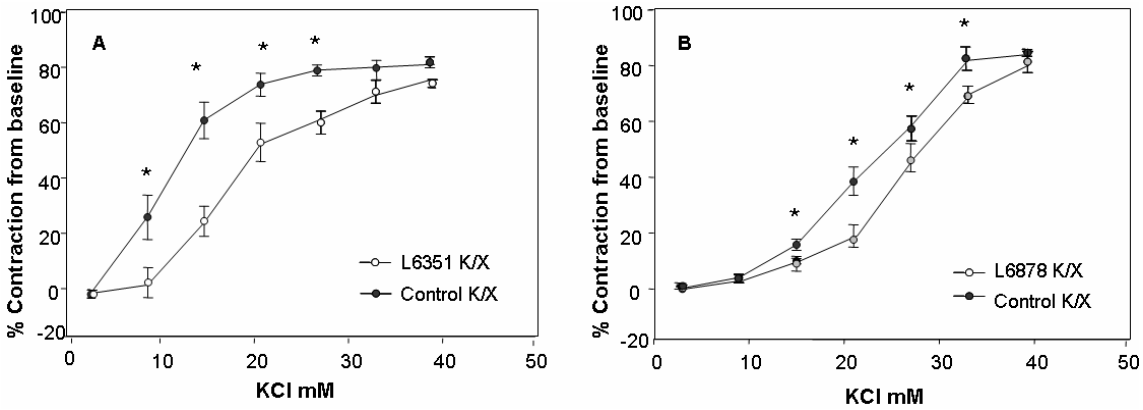

Fig. 23. Effect of S-ketamine/xylazine anesthezia on mesenteric artery contractions to KCl. Animals of L6351 and L6878 (age 1 month) were anesthetised using S-ketamine/xalyzine (S-K/X) before removal of the gastrointestinal tract. Pressurized mesenteric arteries were subjected to increasing KCl concentrations and contractile response was recorded. Non-transgenic littermates of respective lines served as controls. Data expressed as mean ± sd. N=6 minimum in each group. *, $p < 0.05$.

3.3.2 Contractile response to ET-1 - Effect of S-ketamine/xylazine

DRCs for ET-1 were also established for mesenteric arteries of tg lines L6351 and L6878 anesthetized with K/X to analyse whether this anesthetic pretreatment would also (as shown for KCl contractions before) affect the contractile response to ET-1. Compared to non-tg littermates of L6351, contractile response to ET-1 in both lines was increased only at very low concentrations of ET-1 but was not different from controls with increasing ET-1 concentrations (Fig. 24).

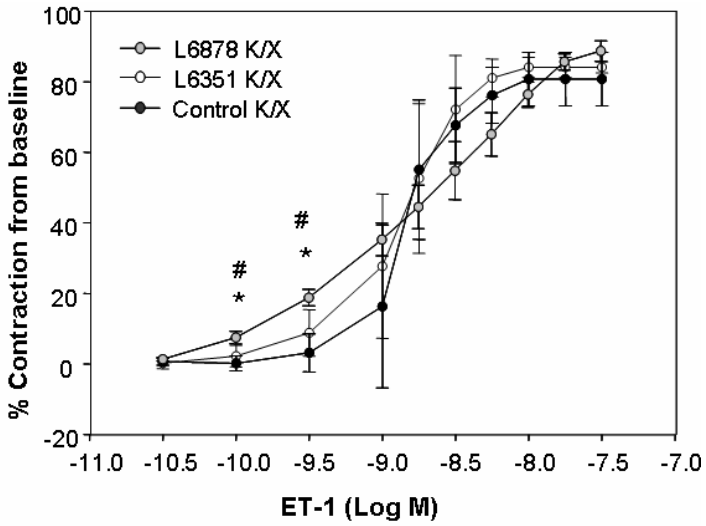

Fig. 24. Effect of ketamine/xylazine anesthetic pretreatment on mesenteric artery contractions to ET-1. Animals of L6351 and L6878 (age 1 month) were anesthetized using K/X before removal of the gastrointestinal tract. Pressurized mesenteric arteries were subjected to increasing ET-1 concentrations and contractile response was recorded. Non-tg littermates of L6351 are shown as non-tg controls. N=6 minimum in each group. (# means $p< 0.05$ when L6351 compared to non-tg control; * means $p<0.05$ when L6878 compared to non-tg control).

3.3.3 Contractile response to phenylephrine - Effect of S-ketamine/xylazine

Since we observed a reduction of the PE-induced blood pressure increase in tg rats of L6351 and L6878 under K/X anesthesia we also analysed the contractile response to PE in pressurized mesenteric arteries dissected from rats anesthetized using K/X. We found a significantly decreased contraction to PE in both tg lines (Fig. 25). Maximum constriction achieved with $3x10^{-5}$ M PE was around 15-20% in tg SMA as compared to 75-85% in non-tg littermate SMA serving as controls. In contrast, the PE response of L6878 mesenteric arteries preexposed to PB was unaffected (see also Fig. 19).

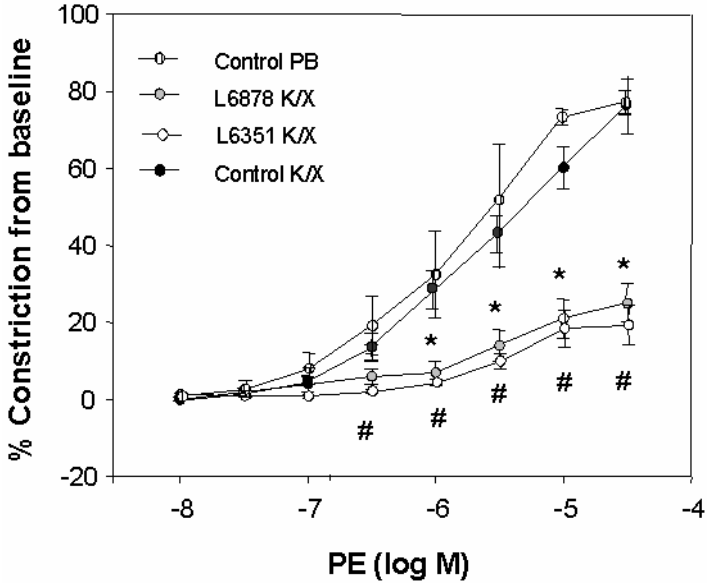

Fig. 25. Effect of ketamine/xylazine anesthetic pretreatment on mesenteric artery contractions to PE. Animals of L6351 and L6878 (age 1 month) were anesthetised using ketamine/xalyzine (K/X) before removal of the gastrointestinal tract. Pressurized mesenteric arteries were subjected to increasing PE concentrations and contractile response was recorded. Controls were non-transgenic littermates of L6351. N=6 to 9 in all groups. Data expressed as mean $\pm$ sd. (# means $p < 0.05$ when L6351 compared to non-tg control; * means $p<0.05$ when L6878 compared to non-tg control).

3.3.4 Contractile response to TXA2 agonist

Mesenteric artery contraction to TXA2 agonist U46619 (10^{-9} M to 10^{-6} M) was also analysed in tg rats (1 month old, L6351) pretreated with K/X. The slight decrease observed in L6351 rats anesthetized using PB (Fig. 20) was not observed after K/X anesthesia (Fig. 26). In contrast, tg rats of L6878 showed slightly decreased sensitivity to U46619 after K/X anesthesia, but the differences did not reach statistical significance (Fig. 27).

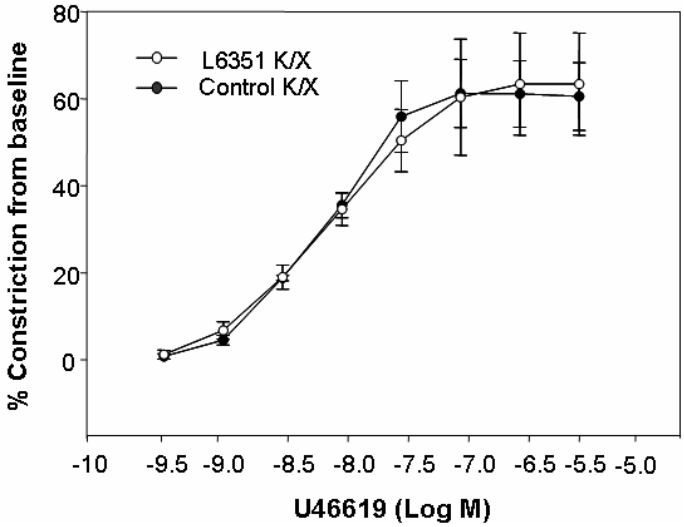

Fig. 26. Contractile response to U46619 in ET_A tg line L6351 at the age of 1 month. Animals were anesthetized using ketamine/xylazine (K/X) before removal of the gastrointestinal tract. Pressurized mesenteric arteries were subjected to increasing U46619 concentrations and contractile response was recorded.. Non-tg littermates of same line served as controls. Data expressed as mean ± sd. (Control N=8, L6878 N=6).

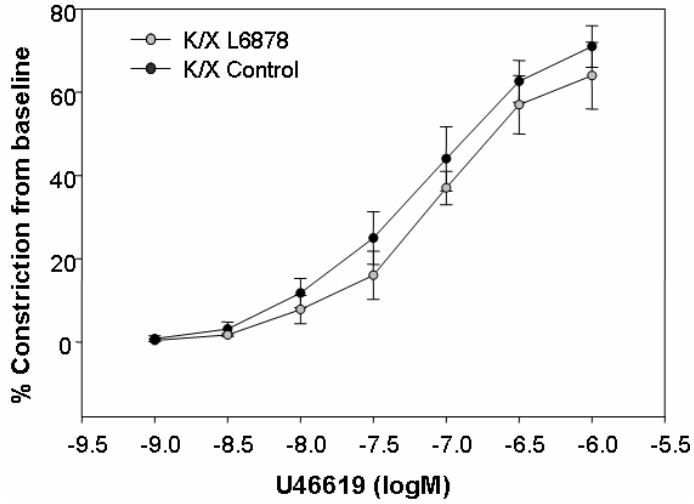

Fig. 27. Contractile response to U46619 in ET_A tg line L6878 at 1 month of age. Pressurized mesenteric arteries were subjected to increasing U46619 concentrations and contractile response was recorded. Controls were non-transgenic littermates of L6878. Data expressed as mean ± sd. N=6 in all groups.

3.3.5 Endothelium-independent vasodilatation - Effect of S-ketamine/xylazine

Endothelial-independent vasodilatation using 10^{-3} M SNP as exogenous NO donor was also studied in mesenteric arteries dissected from 1 month old tg rats of both lines anesthetized with K/X before gastrointestinal tract was removed. Vasodilatation caused

by SNP was slightly more in the non-tg littermate controls of line L6878 (Fig. 28B). No statistically significant differences were observed in the maximum dilatation in L6351 or L6878 rats compared to non-tg littermates of respective lines as controls (Fig. 28).

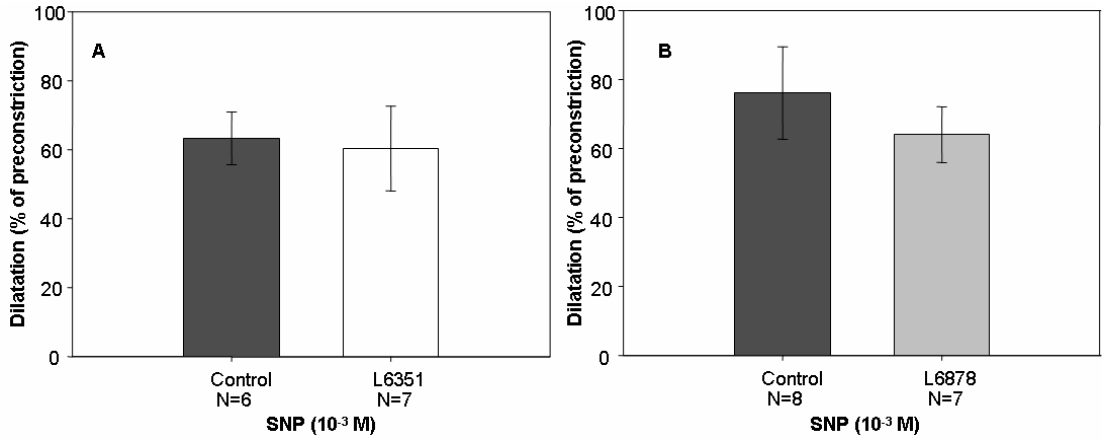

Fig. 28. Effect of sodium nitroprusside in pressurized mesenteric arteries in ET_A tg line L6351 (A) and L6878 (B) at the age of 1 month. Non-transgenic littermates of respective lines served as controls. Data expressed as mean ± sd.

3.3.6 Vascular ET_A and ET_B receptor binding

Preliminary receptor binding studies (saturation binding and displacement binding) were performed using ^{125}I-ET-1 and sub-type specific receptor ligands (BQ-123, IRL-1620) in membrane protein preparations isolated from aorta or mesenteric arteries. Compared to non-tg littermates as controls, aortic membrane preparations of tg L6351 rats of 1 month age showed 15% increased ET_A binding, whereas no difference was detected in L6878 (Table. 3). Interestingly, in both tg lines ET_B-specific binding appeared to be slightly increased (L6351: +19%; L6878: +10%). Due to small sample numbers statistical analysis was not performed.

Aorta	L6351	L6878	Control
ET_A	310	263	270
ET_B	108	100	91

Tab. 3. Saturation binding assay in L6351 and L6878. Membrane preparations of aorta from L6351 and L6878 were analyzed at the age of 1 month for ET_A and ET_B receptor binding. Values expressed as fmol/mg of protein. Data expressed as mean (N=3).

Mesenteric artery membrane preparations of 1 month old tg L6351 rats showed apparently increased ET_A specific binding (Fig. 29). Due to small sample numbers statistical analysis was not performed. However, the result suggested upregulated ET_A receptor protein in the mesenteric bed in this tg line which clearly exceeded the only modest increase in ET_A transcripts quantified in these vessels.

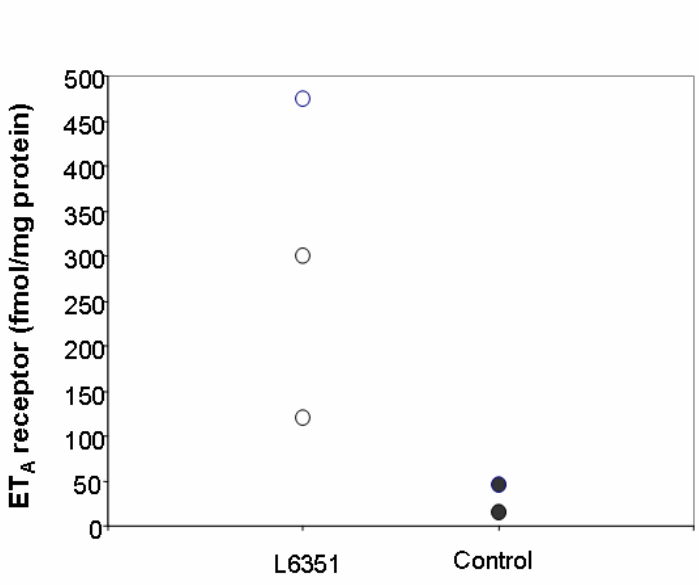

Fig. 29. Saturation binding assay showing ET_A receptor binding in the mesenteric arteries of L6351. Membrane preparations of mesenteric arteries from L6351 at the age of 1 month were analyzed for ET_A specific receptor binding. Non-tg littermates served as controls. (N=3, L6351; N=2 Control). Values expressed as fmol/mg protein.

Most strikingly, ET_B binding data suggested increased ET_B receptor binding in mesenteric artery protein preparations of tg line L6351 (Fig. 30).

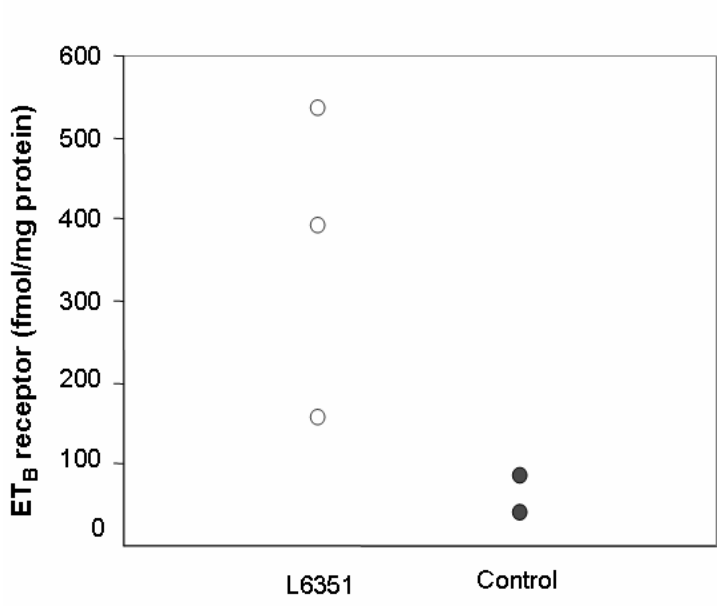

Fig. 30. Saturation binding assay showing ET_B receptor binding in the mesenteric arteries of L6351. Membrane preparations of mesenteric arteries from L6351 at the age of 1 month were analyzed for ET_B specific receptor binding. (N=3, L6351; N=2 Control). Values expressed as fmol/mg of protein.

3.4 Histomorphometric analysis

3.4.1 Morphometric analysis of histologic artery sections

Histological analysis of carotid artery cross sections of 1 month old tg rats of L6351 showed a significant increase (+24%, $p<0.0007$) in the normalized area (NA) of the media when compared to non-tg littermates as controls, whereas the carotid artery of L6878 showed only a slight (+8%) non-significant increase (Fig. 31).

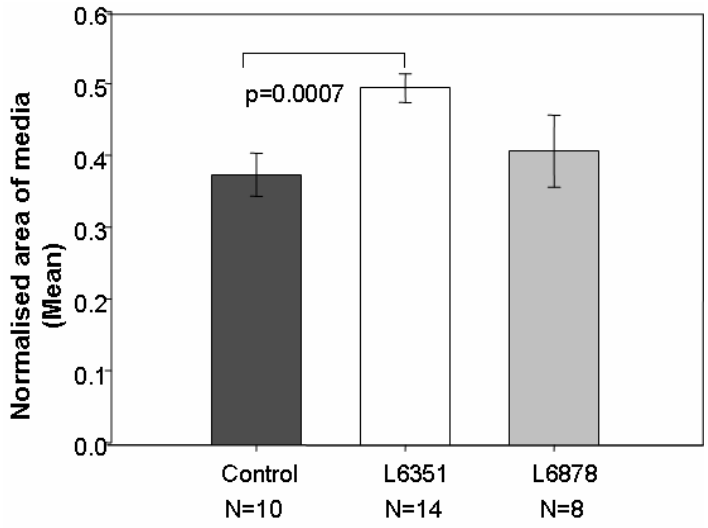

Fig. 31. Normalized area of the carotid artery media in L6351 and L6878. Carotid artery cross-sectional areas of 1 month old tg rats of L6351 and L6878 were analyzed histomorphometrically. Normalized area (NA) of the media was calculated as described in Materials and Methods. Non-tg littermates served as controls. Data expressed as means ± sem.

Histomorphometric analysis of cross-sectional aortic areas also showed significantly increased NA of media in L6351 (+18%, p=0.016) and in L6878 (+21%, p=0.012) as compared to controls (non-tg and SD) (Fig. 32).

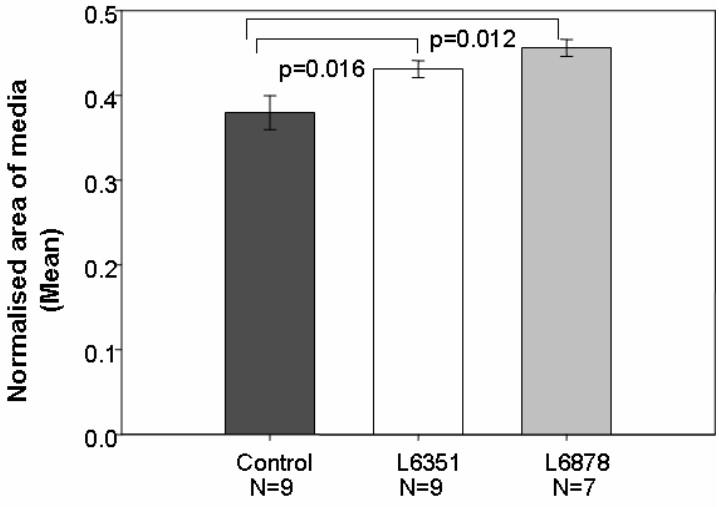

Fig. 32. Normalized area of aorta media in L6351 and L6878. Aorta cross-sectional areas of 1 month old tg rats of L6351 and L6878 were analyzed histomorphometrically. Normalized area (NA) of the media was calculated as described in Materials and Methods. Non-tg littermates served as controls. Data expressed as means ± sem.

No other apparent structural or cellular changes were observed in these vessels microscopically in HE stained paraffin sections. Fig. 33 shows photo micrographs depicting representative artery sections.

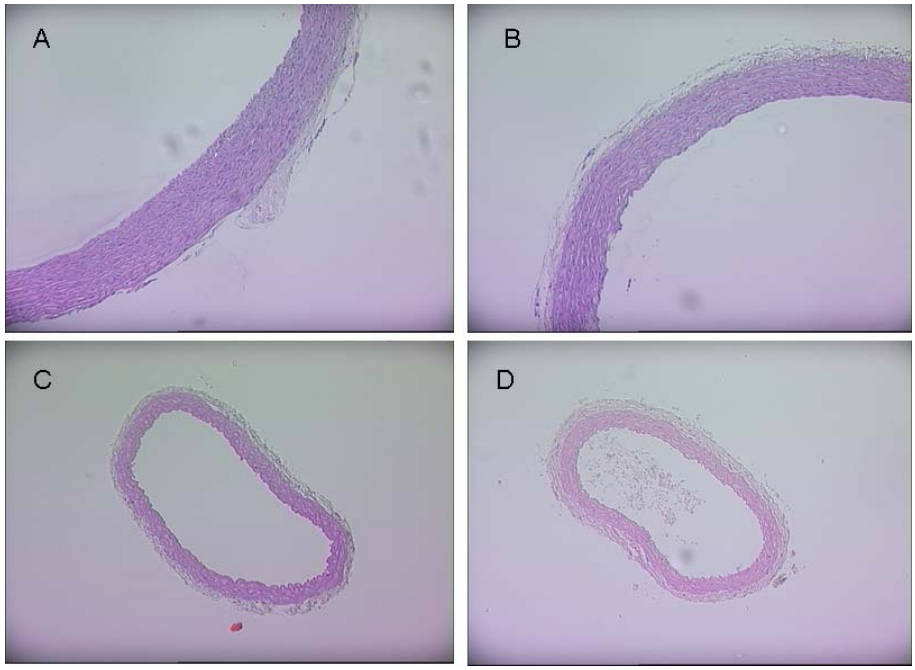

Fig. 33. Representative photomicrographs of HE stained aorta (A and B) and carotid artery (C and D). Normalized area (NA) of the media was measured in ET_A transgenic line L6878 aorta (A) and its non-tg control (B), and also in another ET_A transgenic line L6351 carotid artery (C) and its non-tg control (D). Objective magnification used was 2.5-fold.

3.4.2 Morphometric vessel wall analysis of isolated perfused mesenteric arteries

Pressurized mesenteric arteries from 40 day old tg rats of L6351 and L6878 were analyzed for vessel wall thickness and lumen diameter. The combined left and the right wall thickness (total wall thickness) normalized to total vessel diameter showed a significant increase (+27%) of mesenteric artery wall thickness in L6878 compared to age-matched SD controls (Fig. 34). In contrast, mesenteric arteries of L6351 rats only showed slightly increased wall thickness which was statistically non-significant compared to SD controls.

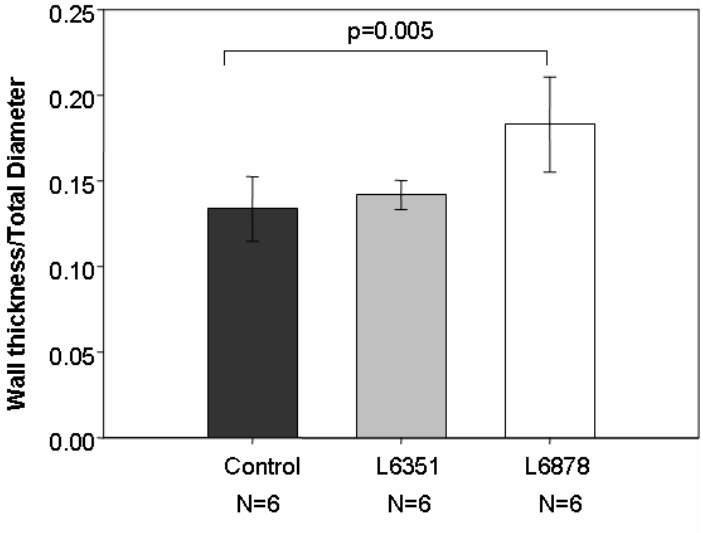

Fig. 34. Mesenteric artery wall thickness in L6351 and L6878 at the age of 40 days. Vessel wall thickness (left wall and right wall) was assessed in 40 day old tg rats and then normalized to total vessel diameter in pressurized mesenteric arteries. Data expressed as mean ± sd.

Vascular lumen was slightly decreased in L6351 which was statistically non-significant. Vascular lumen was significantly reduced (-8%) in L6878 mesenteric arteries (Fig. 35) and the total vascular diameter was also higher in L6878 when compared to SD controls, i.e. SD: 289 ± 24; L6351: 283 ± 22; and L6878: 302 ± 35 which was however statistically non-significant.

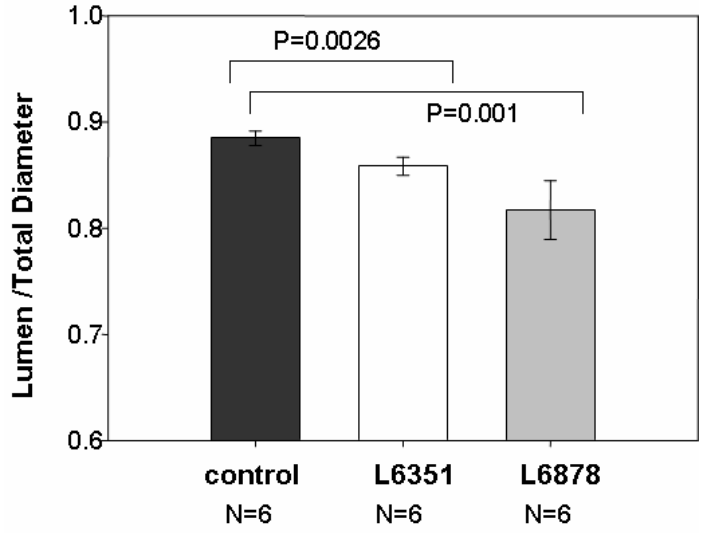

Fig. 35. Mesenteric artery lumen analysis in L6351 and L6878 at the age of 40 days. Total lumen loss was calculated by normalizing lumen to total vessel diameter of pressurized mesenteric arteries. Data expressed as mean ± sd.

3.4.3 Heart weight

Left ventricular weights (LVW) were assessed at the age of 1 month in lines L6351 and at age 4 months in L6878. While L6351 did not show any difference in the LVW (Fig. 36A), LVW in L6878 at the age of 4 months (Fig. 36B) exhibited a non-significant trend towards increase as compared to non-tg littermate controls.

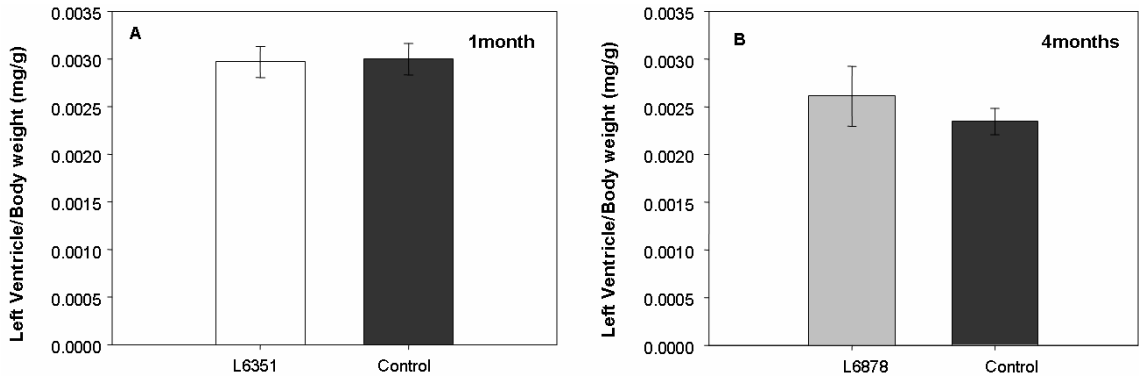

Fig. 36. Analysis of left ventricle weight in L6351 and L6878. Left ventricular weights normalized to body weights in tg line L6351 at 1 month (**A**), and in L6878 at the age of 4 months (**B**). Non-transgenic littermates served as controls. Data expressed as mean ± sd. N= 6 in L6878 and its control; N=5 for L6351 and its control.

3.4.4 Morphometric analysis of kidney weight

Morphometeric analysis of the kidneys of tg rats of lines L6351 and L6878 did not show macroscopic changes of the kidney. Similarly, no changes were observed in the kidney-to-body-weight ratios of line L6351 at the age of 1 month (Fig. 37B) when compared to its non-tg littermates. Also, at the age of 1 year pooled kidney weights from kidneys of tg rats of L6878 and L6888 demonstrated no significant changes when compared to non-tg littermates (L6878) as controls (Fig. 38).

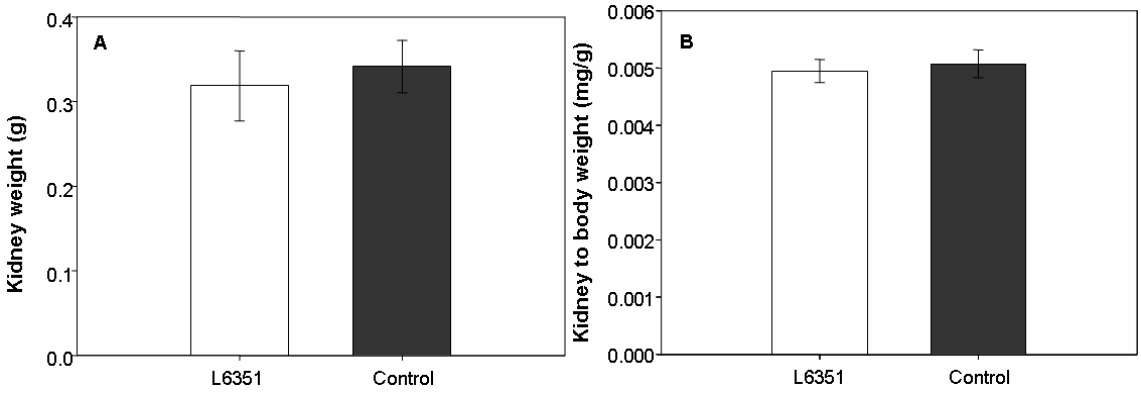

Fig. 37. Analysis of kidney weights in transgenic animals of L6351. Kidney weights (**A**), and normalized ratio of kidney-to-body-weight were analyzed in L6351 at the age of 1 month (**B**). Non-transgenic littermates of L6351 served as negative controls. Data expressed as mean ± sd. N=6 in each group.

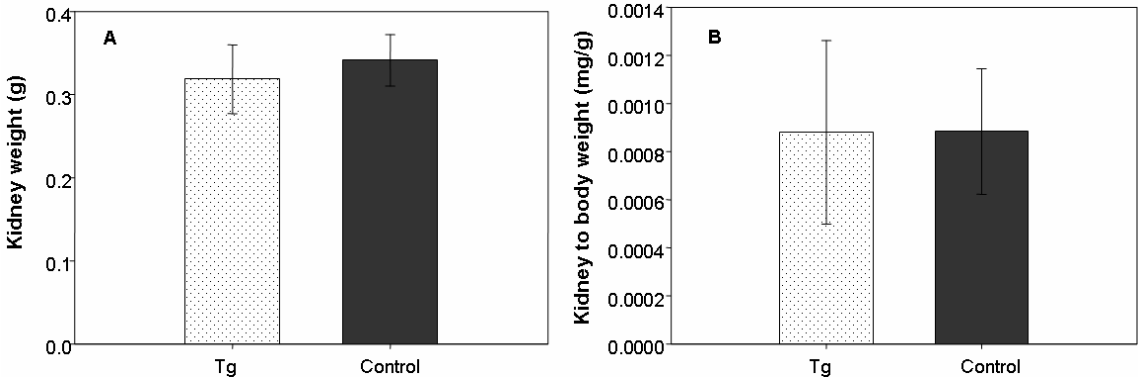

Fig. 38. Analysis of kidney weights in transgenic animals of L6878 and L6888. Kidney weight (**A**) and normalized ratio of kidney-to-body-weight were analyzed at the age of 1 year (**B**)**.** Kidneys were pooled from L6878 and L6888 and compared to non-transgenic littermates of L6878 as controls. Data expressed as mean ± sd. N=6 in each group.

3.5 Expression of the transgene was not induced by vascular injury

Animals of tg line L6351 show significant downregulation of hET_A receptor mRNA levels in carotid arteries and in other vessels at 4 months of age (Kliesch, S. MD thesis). Previous studies have shown that ET_A mRNA receptor expression was increased following balloon catheter injury of carotid arteries (Viswanathan M et al, 1996). To analyze whether expression of the downregulated tg construct may be reinduced by mechanical injury, carotid arteries of L6351 animals were injured using a balloon catheter and hET_A mRNA receptor expression was analyzed at day 7 post injury. mRNA

expression was analyzed qualitatively by RT-PCR showing unaltered mRNA levels in injured compared to non-injured contralateral artery of the same animal which excluded a significant activation of the SM22α promoter which was used in the tg construct in the neointimal tissue (Fig. 39).

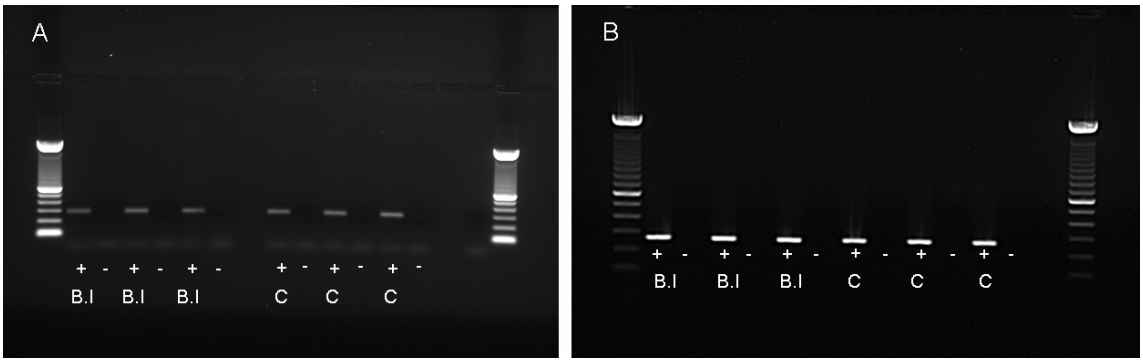

Fig. 39. Transgenic hET_A receptor mRNA expression after injury. Carotid arteries of 4 month old L6351 tg rats were balloon-injured and RNA was extracted 7 days later. Agarose gel shows RT-PCR products (35 cycles) specific for hET_A transgene **(A)** and of beta-actin (35 cycles) serving as house-keeping gene control **(B)**. B.I, balloon injury; C, contralateral vessel; +/-, with/without reverse transcriptase.