CHAPTER 2: MATERIALS AND METHODS

2.1 Materials

2.1.1 Chemicals and kits

100 base pair DNA Ladder	Invitrogen, Karlsruhe, Germany
Acetylcholine	Sigma-Aldrich Chemicals, Steinheim, Germany
Agarose	Carl Roth, Karlsruhe, Germany
Angiotensin-II	Sigma-Aldrich Chemicals, Steinheim, Germany
Aprotinine	Merck, Darmstadt, Germany
Bacitracin	Merck, Darmstadt, Germany
Benzamidine	Sigma-Aldrich Chemicals, Steinheim, Germany
Bovine serum albumin	Sigma-Aldrich Chemicals, Steinheim, Germany
BQ-123 (ET_A receptor antagonist)	Merck, Darmstadt, Germany
Buffer EB (Elution buffer)	Qiagen, Hilden, Germany
Calcium chloride	Sigma-Aldrich Chemicals, Steinheim, Germany
Chloroform	Merck, Darmstadt, Germany
Dithiothreitol (DTT)	Sigma-Aldrich Chemicals, Steinheim, Germany
dNTP	Invitrogen, Karlsruhe, Germany
Endothelin-1	Merck, Darmstadt, Germany
Ethanol	Carl Roth, Karlsruhe, Germany
Ethidium bromide	Merck, Darmstadt, Germany
Ethylendiamine-glycol-bis-(ß-amino ethylether)-tetraacetic acid (EGTA)	Carl Roth, Karlsruhe, Germany
Ethylendiamine-tetraacetic acid (EDTA)	Sigma-Aldrich Chemicals, Steinheim, Germany
Formaldehyde	Carl Roth, Karlsruhe, Germany
Glacial acetic acid	Sigma-Aldrich Chemicals, Steinheim,

	Germany
Glucose	Merck, Darmstadt, Germany
Hydrochloric acid	Carl Roth, Karlsruhe, Germany
Isopropanol	Carl Roth, Karlsruhe, Germany
Vulcite M	Fina, Karlsruhe, Germany
IRL1620 (ET_B receptor agonist)	Alexis, Läufelfingen, Switzerland
Ketanest (S-ketamine)	Parke-Davis, Freiburg, Germany
Magnesium chloride	Merck, Darmstadt, Germany
Magnesium sulphate heptahydrate	Merck, Darmstadt, Germany
2-Mercaptoethanol	Sigma-Aldrich Chemicals, Steinheim, Germany
MOPS	Carl Roth, Karlsruhe, Germany
Penicillin/streptomycin	Sigma-Aldrich Chemicals, Steinheim, Germany
Phenol	Sigma-Aldrich Chemicals, Steinheim, Germany
Phenol-Chloroform-Isoamylalcohol	Carl Roth, Karlsruhe, Germany
Phenylephrine	Sigma-Aldrich Chemicals, Steinheim, Germany
Phenylmethylsulfonyl fluoride	Carl Roth, Karlsruhe, Germany
Potassium chloride	Sigma-Aldrich Chemicals, Steinheim, Germany
Primer synthesis	TIB MOLBIOL, Berlin, Germany, and Proligo primers and probes, Paris, France
Proteinase K	Carl Roth, Karlsruhe, Germany
RNeasy Mini Kit	Qiagen, Hilden, Germany
Sodium acetate	Merck, Darmstadt, Germany
Sodium chloride	Merck, Darmstadt, Germany
Sodium dihydrogen phosphate monohydrate	Merck, Darmstadt, Germany
Sodium dihydrogen phosphate dihydrate	Merck, Darmstadt, Germany
Sodium hydrogen phosphate	Merck, Darmstadt, Germany

heptahydrate	
Sodium dodecylsulphate	Carl Roth, Karlsruhe, Germany
Sodium Hydroxide	Carl Roth, Karlsruhe, Germany
Sodium nitroprusside	Sigma-Aldrich Chemicals, Steinheim, Germany
Sodium pyruvate	PAA Laboratories, Germany
Tris(hydroxymethyl)aminomethane	Merck, Darmstadt, Germany
Trizol Reagent	Invitrogen, Karlsruhe, Germany
U46619	Merck, Darmstadt, Germany
Xylazinhydrochloride	Sanofi/Ceva, Düsseldorf, Germany

2.1.2 Solutions and buffers

Proteinase K stocks solution (10mg/ml)

Proteinase K	100 mg
ddH_2O q.s. to	10 ml

Endothelin-1 stocks solution (0.1 mM)

Endothelin-1	10^{-4} M	0.1 mg
5% glacial acetic acid q.s to		401 µl

Phenylephrine stocks solution (1 mM)

Name of the salt	final concentration	mg added for 100 ml
Phenylephrine	1 M	20.4 mg
ddH_2O q.s. to		100 ml

Sodium chloride solution (0.9%)

Name of the salt	final concentration	g added for 500 ml
NaCl	0.9%	4.5 g
ddH_2O q.s. to		500 ml

Tail buffer solution

Name of the salt	final concentration	g added for 1000 ml
Tris	50 mM	6.06 g

EDTA	100 mM	37.2 g
NaCl	100 mM	5.8 g
SDS	1%	10 g
ddH_2O q.s to		1000 ml

4% Formaldehyde solution in phosphate buffer (pH 7.4)

Name of the salt	final concentration	g added for 1000 ml
$Na_2HPO_4 \cdot 7H_20$		10.0 g
$NaH_2PO_4 \cdot 2H_20$		1.6 g
Formaldehyde 36-40%		100 ml
ddH_2O q.s to		1000 ml

Krebs-Henseleit solution stock solution

Name of the salt	final concentration	g added for 500 ml
NaCl	145 mM	84.74 g
KCl	4.7 mM	3.5 g
$CaCl_2$	2 mM	2.94 g
$MgCl_2$	1.2 mM	2.96 g
NaH_2PO_4	1.2 mM	1.66 g
Glucose	5 mM	9.0 g
Sodium pyruvate	2 mM	200 ml

The stock solution was stored frozen at -20°C in 100 ml aliquots. To each 100 ml stock aliquots the following substances were added to constitute a 2 litre final Krebs-Henseleit solution:

EDTA	3 mM	1.25 mg
MOPS	1 g/l	2 g
Bovine serum albumin	0.02 g	0.0149 g

Phosphate buffer saline (PBS)

4.5 g Na_2HPO_4

500 ml ddH_2O

Reagents for protein binding analysis

1 N HCl solution		
Tris-ME	Tris HCl	50 mM
	EGTA	2 mM
	MgCl2	10 mM pH7.2
Bacitracin stock solution	Bacitracin	2.13 g
	H_2O	ad 10 ml
		(1hr at 70°C)
Aprotinine stock solution	Aprotinine	0.15 g
	H_2O	ad 10 ml
Tris-BAME buffer	Tris-ME	500 ml
	Bacitracin stock solution	500 µl
	Aprotinine stock solution	500 µl
PMSF stock solution	PMSF	40 mM (in Ethanol)
Protease inhibitors mix	Benzamidine	100 mM
	Trypsin inhibitor	2 µg/ml
	Aprotinine	1 µg/ml
PBSI	PBS	10 ml
	PMSF stock solution	125 µl
	protease inhibitors mix	80 µl

2.1.3 Technical equipments and instruments

2-F Fogarty balloon catheter	Edwards Lifesciences, Unterschleissheim, Germany

Centrifuge (Varifuge 3.0 R)	Heraeus, Hanau, Germany
Cell harvester	Inotech AG, Dottikon, Switzerland
3CCD camera (MC-3255)	Sony, Japan
Digital camera C-5050 Zoom,	Olympus Europe, Hamburg, Germany
Electronic balance	Sartorius, Berlin, Germany
Glass douncers	Wheaton Science products, New Jersey, USA
Histokinette	Reichert-Jung, Germany
Micro forceps 5 BD 331R	Aesculap, Tuttlingen, Germany
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Microtome (HM 355 S)	Microm International, Walldorf, Germany.
Mini Centrifuge	Eppendorf AG, Hamburg, Germany
Pellet pestle	Kontes glass company, New Jersey, USA
Peltier Thermal Cycler (PTC-100)	Biozym Diagnostic, Hameln, Germany
Spectrophotometer UV-VIS 1202	Shimadzu, Duisburg, Germany
Spring microscissors	Aesculap, Tuttlingen, Germany
Surgical needles	Ethicon, Johnson & Johnson, Belgium
Surgical threads	Ethicon, Johnson & Johnson, Belgium
Ultra Turrax T25	Janke & Kunkel, Staufen, Germany
Vortexer	Janke and Kunkel, staufen, Germany

Tail vein plethysmography

Pressure cuff	Technical and Scientific Equipments, Bad Homburg, Germany
Process control unit series 209000	Technical and Scientific Equipments, Bad Homburg, Germany
Pulse transducer (ID=9 mm)	Technical and Scientific Equipments, Bad Homburg, Germany

Pressure myograph system

CCD camera	Hitachi, Japan
Circulating water bath	Therma Hake, Germany

Glass cannulas	Living Systems Instrumentation, Burlington, USA
High precision multichannel dispenser IPC	Ismatec, Zurich, Switzerland
Pressure servo control pump PS/200	Living Systems Instrumentation, Burlington, USA
Vessel chamber CH/2/A	Living Systems Instrumentation, Burlington, USA
Video dimension analyser V94	Living Systems Instrumentation, Burlington, USA
Video monitor VM 1202E	Hitachi, Bodgau-Jugesheim, Germany

Invasive blood pressure monitoring

Polyethylene cannulas	BD Adsyte pro-cannulation catheter, Madrid, Spain
Transducer series B980052	Datex-ohmeda, Germany
Universal transducer amplifier series 804700	Technical and Scientific Equipments, Bad Homburg, Germany

2.1.4 Software

for tail vein plethysmography	BP-monitor VM 1.0 Technical and Scientific Equipments, Bad Homburg, Germany
for small vessel myograph	Windaq/lite, Dataq Instruments, USA
for scientific graphs	Sigma Plot 8
for statistical calculations (version 3.5)	Quick statistics for optometrists version 3.5, CCSTAT, India
for histomorphometry	Scion image 1.6, Scion corporation, Frederick, MD, USA
for invasive hemodynamic monitoring	BIA Biosys, Technical and Scientific Equipments, Bad Homburg, German

2.1.5 Animals

All animal experiments were conducted in accordance with local institutional guidelines for the care and use of laboratory animals. Animals were procured from different tg projects approved by local authorities (G 0161/99, G 0151/04, T 0309/00, G 0236/01). The animals were kept under controlled environment (20°C, 64% humidity). Animals were maintained on a light-dark cycle (12/12 hours) and received food (standard rat diet) and tap water ad libitum. Two anesthetic protocols were applied to anesthetise animals. Animals were anesthetized using either 43.5 mg/kg S-ketamine and 13 mg/kg xylazine (S-K/X), or 60 mg/kg pentobarbital sodium (PB). All anesthetics were injected intraperitoneally (i.p.).

2.2 Methods

2.2.1 Generation of transgenic rats

Tg rats for the human ET_A receptor were generated by pronuclear microinjection of a purified 3 kb restriction fragment containing the human ET_A cDNA under control of a 1.3 kb fragment of the murine SM22α promoter and the polyA signal of the bovine growth hormone gene (bGH) (Fig. 3). Details of the plasmid construction are described elsewhere in detail (Zollmann FS, Dissertation Freie Universität Berlin, 2003). Microinjections were performed in close collaboration with the group of M. Bader at the Max-Delbrueck-Center, Berlin-Buch and the protocol has been described in great detail elsewhere (Popova E et al, 2004). Potential founder animals and their offspring were screened for stable integration of the transgene into the genome using genomic PCR.

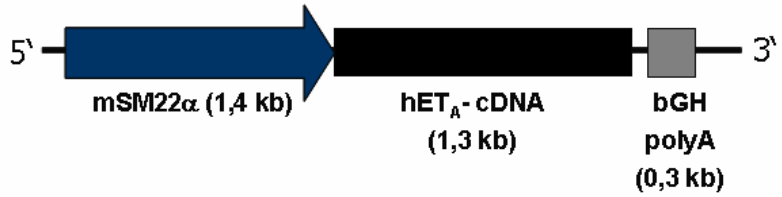

Fig. 3. Schematic representation of the transgenic hET_A construct. mSM22α**,** murine SM22alpha promoter (1.4 kb), hET_A, human endothelin receptor cDNA (1.3 kb); bGH, bovine growth hormone as polyadenylation signal (0.3 kb).

2.2.2 Genotype analysis

Total genomic DNA for genotyping was extracted from frozen tails which were digested at 55°C overnight in 750 µl tail buffer containing 50 µl proteinase K solution (10 mg/ml). The lysate was extracted using 500 µl phenol:chloroform: isoamylalcohol (25:24:1) and

centrifuged at 14,000 rpm at 4°C for 20 min. DNA was precipitated by adding the supernatant in 750 µl ethanol in 75 µl of sodium acetate (pH 7) and kept at -20°C for 1 hour. The precipitated DNA was centrifuged at 10000 rpm for 20 min at 4°C, the DNA pellet was washed with 80% ethanol, allowed to air dry, dissolved in 50 µl of DNA elution buffer and kept frozen at -20°C. DNA was analyzed photometrically at 260 nm and 280 nm and DNA concentration was calculated using the formula OD (260nm) x dilution factor x 50/1000 µg/µl.

100 ng genomic DNA was amplified in Hot Star Taq reaction buffer (final volume 25 µl) containing 0.6 µM of each sense (SP6) and antisense (pcDNA3 4462) primer, 0.2 µM of each dNTP, 0.75 U Hot Star Taq DNA polymerase and Q-solution. Primer sequences were as follows: SP6: 5'- ATA GTG TCA CCT AAA TGC TAG AGC -3'; pcDNA3 4462: 5'- ATA GAG CCC ACC GCA TCC CC -3'.
Enzymatic DNA amplification was performed using a step-down protocol in a PTC-100 thermal cycler (MJ Research). After initial denaturation (94°C, 15 min), the first annealing temperature was 67°C, which was then stepwise decreased (1°C per cycle) to the final annealing temperature of 62°C. Final cycling conditions were 94°C for 10 s, 62°C for 30 s, 72 °C for 10 s. After a total of 35 cycles, final extension was carried out for 10 min.

2.2.3 Hemodynamic monitoring using radiotelemetry

Blood pressure was measured by radiotelemetry in freely moving males of tg line 6351 between 4 to 8 weeks of age and their non-tg littermates as controls at the Max Delbrueck Center for Molecular Medicine (MDC), Berlin-Buch in cooperation with the group of Dr Michael Bader. Briefly, radiotelemetric implantable mouse blood pressure transmitters (Mills PA et al, 2000) were implanted in the abdominal cavity of 28 day old rats as described (Burckle CA et al, 2006), with the transducer-connected capillary tubing anchored in the lumen of the abdominal aorta. Animals were allowed to recover for 7 days before blood pressure was recorded. Basal blood pressure parameters were assessed at days 35 and 36. Hypertension was induced in both (tg and non-tg littermates) groups by oral treatment with the NO-inhibitor L-NAME (20 mg kg^{-1} d^{-1} in the drinking water) from day 42 to 53. Rats were orally treated with the specific ET_A receptor antagonist LU 302146 (30 mg/kg bw/day) between day 47 and 59. The chosen dose of the ET_A-receptor blocker LU 302146 was reported to completely block ET_A

without significantly affecting ET_B (Mulder P et al, 1997). LU 302146 was finally adminstered separately later and the hemodynamic variables were recorded on days 58 and 59.

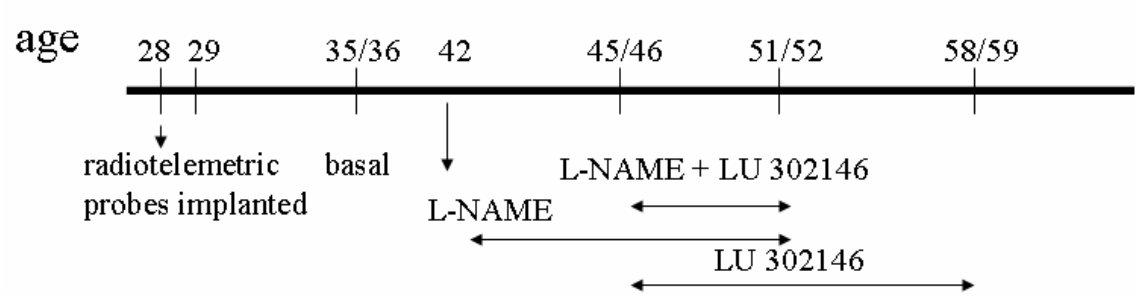

Fig. 4. Schematics of the radiotelemetric analysis and various pharmacological agents given at different time points. Age is shown in days.

2.2.4 *In vivo* hemodynamic measurements

Following induction of anesthesia using either ketamine-xylazine (K/X) or pentobarbital sodium (PB) as mentioned in 2.1.5, rats were wiped with water or alcohol to reduce inconvenience of loose hairs. A small incision was made on the ventral side of the neck which was extended to the upper part of thorax. After initial preparation of the carotid artery, cannulation of the right external carotid artery was performed. A BD adsyte Pro Violon IV catheter with injection valve 0.9 x 25 mm (36 ml/min) was used to cannulate the artery which was tranversed to the aortic bifurcation. Blood pressure and heart rate (HR) recorded by the transducer were transferred to a series 804700 universal transducer amplifier (TSE Systems). The amplified signals (blood pressure and HR) were processed using BIA BioSys software. BIA BioSys software run on a personal computer under MS-DOS and systolic arterial pressure (SAP), mean arterial pressure (MAP), diastolic arterial pressure (DAP) and HR were calculated. Baseline blood pressure and the maximal increment in blood pressure variables were recorded following administration of various pharmacological agonists given intraarterially. After attainment of an almost straight baseline (not more than 5 mm Hg change in 3 min time interval), bolus dose of various vasoconstrictor agonists i.e. ET-1 (0.3 nmol/kg), PE (10 µg/Kg) was administered and maximum response to the agonist from baseline was recorded.

2.2.5 Non-invasive blood pressure monitoring (tail vein plethysmography)

Ether anesthesia was induced by placing the rat in a closed glass jar with a hood over the bottom of the jar containing diethyl ether. After induction of light ether anesthesia, the animals were allowed to equilibrate for 30 seconds and, thereafter, additional anesthetic was added by holding a plastic cone containing cotton soaked in ether above the rat's nose. A tail pressure cuff for inflating the tail artery (ID 13 mm) was rapped around the tail of the rat. The rat was placed on the measurement table lying on one side (not supine) and the cuff was inflated using a process control unit version 209000 (TSE systems) to monitor the SAP. Pulse pressure signals generated by inflating the cuff were recorded using pulse transducer (ID 9 mm). Set points were displayed on the monitor. The principle is based on the standard sphygmomanometer whereby application of the external pressure by the inflated cuff blocks blood flow which is recorded as blood pressure. The two set points on the monitor displaying the SAP of the rat under analysis were selected manually. The SAP was taken in an appropriate range between the first descent and the first ascent of SAP when the external pressure is equal to the pressure in the artery.

2.2.6 Analysis of *ex vivo* mesenteric artery function

After deep anesthesia was achieved, using either PB or K/X, rats were wiped with water or alcohol to reduce inconvenience of loose hairs. The skin near the lower abdomen was picked up with forceps and a mid line ventral incision was made. The abdominal wall was cut laterally along both posterior margins of the rib bones and then the diaphragm was separated from the muscles on the ventral lines of the thoracic wall. The ribs were fixed with the help of fine needles on to the thermocol basement. The animal was killed by exsanguination by puncturing the lower caval vein. The gastrointestinal tract with the entire mesenterium clearly visible was carefully separated, excised and immediately transferred into Krebs-Henseleit buffer. The gastrointestinal tract with the mesenterium was spread and fixed on a Petri dish containing congealed silicon. Under a dissecting microscope first-order branches of the small mesenteric artery (SMA) were dissected very carefully from the surrounding perivascular tissue using microforceps and spring micro scissors without stretching the vessel. Dissected arteries were left in Krebs-Henseleit buffer until being mounted.

All tubings and cannulas were flushed with Krebs-Henseleit buffer before the start of the experimental protocol. The SMA was tied to one end of the glass cannula (internal diameter 125-150 µM) and secured by fine (19 µm) nylon threads. This end of the cannula was connected by tubing to a reservoir that contained Krebs-Henseleit buffer for vessel perfusion via peristaltic pressure pump. To carefully remove the blood from the vessel, the peristaltic pump was switched from manual to flow mode and blood was flushed out from the lumen of the vessel which was still uncannulated at the other end. The flow pressure was kept below 10 mm Hg. Thereafter, the other end of the vessel was also secured on to the glass cannula. The extreme end of this cannula was blocked using a stop cock. Pressure was developed to 70 mm Hg in the artery by turning the knob on the process control unit from manual to automatic. Leakage was detected by lack of development of pressure and the rotation of the peristaltic pump attached to the plastic tubing for generating a defined pressure. Only vessels without leakage were used in the experiments. After pressure was adjusted the vessel was stretched until any buckling disappeared. Krebs-Henseleit solution which recirculated in the vessel chamber was maintained at 37°C using a heat exchanger and an external water bath (Therma Hake, Germany). Buffer pH was checked regularly. Before establishing dose response curves (DRC), each vessel was allowed to equilibrate for 30 minutes. The perfusion chamber was positioned on the stage of an inverted microscope (with magnification 10 x objective). The chamber was positioned down on to the CCD camera (Hitachi, Japan) together with the microscope to visualize the artery. The amplified image was transmitted to a video monitor via a video dimension analyzer (Living Systems Instrumentation, USA), enabling lumen diameter, left and right wall thickness and total vessel diameter to be measured. To test for viability, arteries were preconstricted with 3 mM to 39 mM KCl. DRCs were generated by superfusing the vessel with increasing concentrations of ET-1 (3×10^{-11} M to 5.62×10^{-8} M), PE (10^{-8} M to 3×10^{-5} M) and the TXA2 agonist U 46619 (10^{-9} M to 10^{-6} M). The sequence of agonists added was as stated just before. At the end of the sequential analysis with various vasoconstrictor agonists, maximum endothelium-independent vasodilation was assessed by adding 10^{-3} M sodium nitroprusside (SNP) after preconstriction using 10^{-6} M U46619.

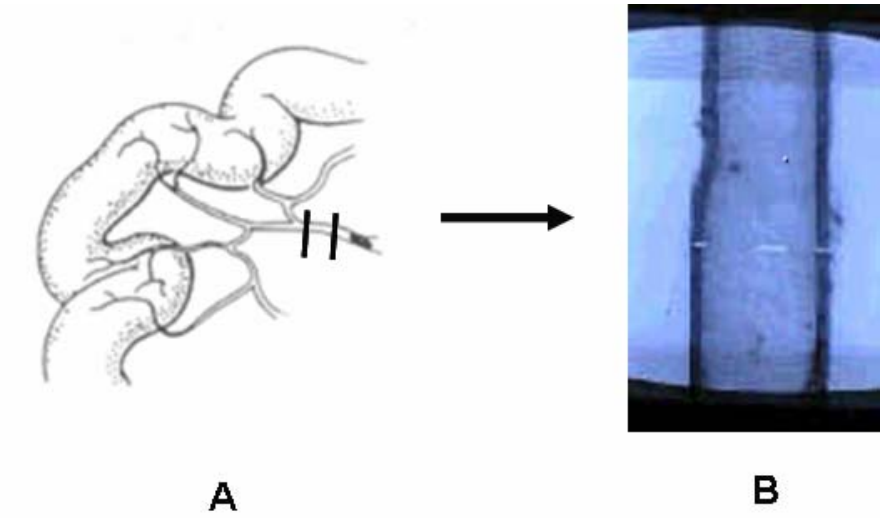

Fig. 5. Scheme of the dissected gastrointestinal tract of the rat with mesenterium (**A**) and snap shot of the prepared first order small mesenteric artery mounted on the vessel perfusion system for arteiograph as viewed on the monitor (**B**).

2.2.7 Histologic analysis of arteries

Animals were killed by exsanguination after anesthetization with PB and vessels were carefully dissected and kept in 4% paraformaldehyde PBS for a maximum of 48 hours. The paraformaldehyde was then replaced by 70% alcohol until the vessels were processed further. Thereafter, alcohol concentration was increased to 96% (absolute ethanol), which was replaced by xylene followed by isopropanol and then by Vulcite M2, a C12 carbohydrate compound. All steps were performed at room temperature. Thereafter the vessels were immersed in 50% vulcite M2/50% paraffin solution at (56°C), and then kept in liquid paraffin at (60°C), for a minimum of 24 hours using a histokinette (Reichert Jung), before processing the samples further. The vessels were then left in molten paraffin jar (60°C) overnight. Thereafter the vessels were embedded in the liquid paraffin in separate molds and allowed to congeal on the next day. From paraffin embedded vessels, 3 µM sections were cut using a microtome. For histological analysis, sections were transferred to glass slides and subsequently stained with hematoxylin and eosin (H&E). Histologic analysis was performed using a standard light microscope.

Histomorphometric analysis of H&E stained sections were performed using a light microscope (objective magnification 2.5) and a 3CCD camera (Sony, Japan). Lumen area and areas defined by internal and external elastic lamina were analyzed. Media of the vessel was defined by the internal elastic lamina subtracted from the external elastic

lamina. NA was defined by the ratio of the calculated media normalized to external elastic lamina. Quantitative image analysis was performed using Scion image 1.6 (Scion Corp.) run on a Macintosh personal computer.

2.2.8 Carotid artery balloon Injury

Neointima was induced by balloon injury (Clowes AW et al, 1983) in male tg rats (L6351) of around 350 to 450 g body weight. Briefly, rats were anesthetized using (S)-Ketamine and xylazine hydrochloride and a 2-F Fogarty balloon catheter was inserted through an incision made in the external carotid artery and advanced along the length of the common carotid artery to the aortic arch. The inner carotid artery was temporarily ligated during the procedure to stop the backflow of blood through the incision in the common carotid artery using surgical threads. The balloon was then inflated and passed three to four times along the length of the common carotid artery up to the aorta to induce sufficient amount of mechanical injury to the vessel wall (Fig. 6). The balloon catheter was then deflated slowly, withdrawn and the external carotid artery was permanently ligated using sutures. Injured and contralateral vessels were excised 7 days post injury and immediately frozen in liquid nitrogen for mRNA expression analysis. The contralateral vessel of the same animals served as control.

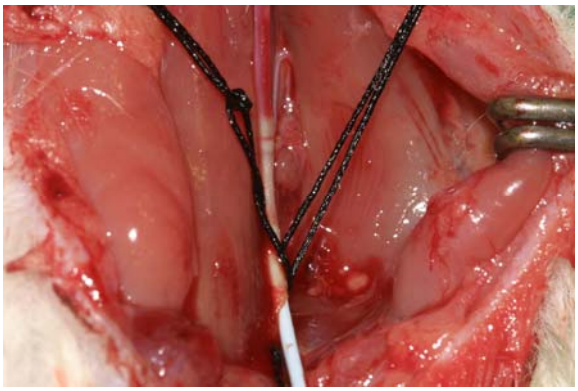

Fig 6. Balloon catheter inserted in the left common carotid artery during balloon injury procedure.

2.2.9 Membrane preparation for receptor binding analysis

Animals were sacrificed and aortae and SMA were removed quickly and frozen in liquid nitrogen. The superior and the inferior mesenteric arteries were isolated from the aortic bifurcation and carefully dissected along the entire length of the artery. Vessels from 3 animals were pooled and stored at -70°C until used. For preparation of membranes,

vessels were homogenized in 1 ml PBSI using an Ultra Turrax T25 at 24,000 rpm for 10 sec. After addition of 1 ml PBSI, the sample was then centrifuged at 3,000 x g for 10 min to remove debris and other cytoplasmic organelles. The supernatant was then transferred to a 2 ml reaction tube and centrifuged at 38,000 x g for 35 min at 4°C. The pellet containing the crude membrane fraction was resuspended in Tris-BAME buffer (pH 7.3), and again centrifuged at 38,000 x g for 35 min at 4°C. The final pellet was resuspended in 100 µl Tris-BAME and stored at -70° C until use.

2.2.10 Saturation binding analysis

Binding analysis was performed in close collaboration with the group of Dr. Alexander Oksche at the Forschungsinstitut of Molekulare Pharmakologie, Berlin-Buch, as described recently (Grantcharova E et al, 2002). Membrane protein (1 to 3 µg) was incubated in a final volume of 200 µl Tris/BAME buffer with increasing concentrations of ^{125}I-ET1 (1.56 to 200 pM) with or without excess of unlabeled non-radioactive ligand (ET-1, IRL1620 or BQ123) to determine total and non-specific binding, respectively. For determination of total ET receptor binding sites, 10 µM ET-1 was used as competing ligand, for determination of ET_A or ET_B receptor binding sites 100 µM BQ123 and 10 µM IRL1620, respectively, were used. The samples were incubated for 2 h at 25° C at 300 rpm in a shaking water bath, transferred onto GF/C filters (Whatman International Ltd., Maidstone, UK), pretreated with 0.1 % (w/v) polyethylenimine, and washed rapidly twice with PBS using a cell harvester (Inotech, Switzerland). Filters were finally transferred into 5 ml glass vials and radioactivity was determined in a liquid scintillation counter. Data were analyzed with RadLig Software 4.0 (Cambridge, UK).

2.2.11 Isolation of RNA

Frozen tissue was weighed, transferred into a small porcelain mortar filled with liquid nitrogen and crushed using a pestle. The tissue was triturated with Trizol (1 ml of Trizol per 50 to 100 mg of tissue), transferred to 1.5 ml reaction tubes and further minced using small plastic crushers (Kontes, USA). To the lysate 200 µl of chloroform was added, shaken vigorously for 15 seconds and incubated on ice for 15 min. The lysate was centrifuged at 12,000 x g for 15 min at 4°C and the RNA was precipitated from the aqueous supernatant phase by adding 500 µl of isopropanol per 1 ml of initial Trizol. Samples were incubated at 15 to 30°C for 10 min and centrifuged at 12,000 x g for 10 min at 2-8°C. The RNA pellet was washed with 75% ethanol and then centrifuged again

at 7,500 x g for 5 min at 4°C. The RNA pellet was air dried and dissolved in 50 µl of RNase-free water and stored at -80°C. Trizol-extracted RNA was further purified using RNeasy (Qiagen) and digested with DNase according to the manufacturer's protocol. The purified, DNase-digested RNA was immediately frozen and stored at -80°C. Before freezing, an aliquot was removed for photometric determination of purity and concentration. The RNA concentration was measured using a UV spectrophotometer using the formula OD (260 nm) x dilution factor x 40 / 1000 µg/µl.

2.2.12. Reverse transcription and polymerase chain reaction

For cDNA synthesis, 400 ng RNA was used as template to which was added 1 µl of dNTPs (10 mM) and 1 µl of random hexamers. The mixture was incubated in the cycler for 5 min at 65°C and then put on ice. 4 µl of 5 x first strand buffer, 2 µl of 0.1 M DTT and 1 µl of RNasin (40 U/µl) were then added. The solutions were mixed and 1 µl of superscript II reverse transcriptase (for RT+) or aqua (for RT-) was added. The reactions were then incubated at 42°C for 50 min and finally inactivated at 70°C for 10 min.

1 µl of the RT reaction (cDNA) was amplified in Hot Star Taq reaction buffer (final volume 25 µl) containing 0.5 µM of each sense (hET-A-SS) and antisense (hET-A-AS) primer, 0.2 mM of each dNTP, 0.75 U of Hot Star Taq DNA polymerase and Q-solution. Primer sequences were as follows: hET_A sense: 5'- GCT GGC ACT GGT TGG ATG T -3'; hET_A antisense: 5'- TCT GsGT AAA TGA TCC TGA GCA -3'. Enzymatic DNA amplification was performed in a PTC 100 thermal cycler (MJ Research). After initial denaturation (95°C, 15 min), final cycling conditions were 95°C for 10 s, 60°C for 60 s. After a total of 35 cycles, final extension was carried out for 10 min.

For beta-actin PCR, ß-actin primers were used: ß-actin sense, 5'- CAT TGA ACA CGG CAT TGT CAC -3'; ß-actin antisense, 5'- CCG TCT CCG GAG TCC ATC AC -3'. Enzymatic DNA amplification was performed with the following cycling conditions: 10 s at 95°C, 45 s at 65°C, and 60 s at 72°C for. After a total of 35 cycles, final extension was carried out for 10 min.

2.2.13 Statistical analysis

Statistical analysis was performed using CCSTAT (version 3.5). All data are expressed as the mean +/- SD. One way analysis of variance (ANOVA) in conjunction with

student's *t* test was performed to assess the differences between the different animal groups. Differences were considered significant at a p value less than 0.05. Graphs were drawn using Microsoft powerpoint and Microsoft excel and Sigma plot (version 8).