SMOOTH MUSCLE ENDOTHELIN B RECEPTORS REGULATE BLOOD PRESSURE BUT NOT VASCULAR FUNCTION OR NEOINTIMAL REMODELLING

Supplementary Information

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Short Title: ET_B deletion, BP and arterial remodelling

Animals

Mice with VSMC selective deletion of ET_B receptors were generated based on a strategy described previously to produce EC ETB KO mice[1]. Homozygous (Flox/Flox) ET_B mice (background: 50% 129/Ola and 50% BKW) were crossed with SM22-Cre transgenic mice and backcrossed to C57Bl/6J for 4-6 generations. As for previous studies[1,2] floxed, Cre-negative littermates ($ET_B^{f/f}$) were used as experimental controls for SM-specific ET_B deficient mice (SM ET_B KO). Genotyping to identify wild type and recombined alleles was performed by PCR[1] and the SM22-Cre transgene was detected as described[3]. All mice were given free access to tap water and standard mouse chow. Mice were housed according to United Kingdom Home Office recommendations at 22°C with 12-hour diurnal light/dark cycles. All procedures were performed under the provisions of the Animals Scientific Procedures Act (1986) and with the approval of the local ethics committee.

Autoradiography

After euthanasia, SM ET_B KO mice and controls (n=3/group) were rapidly frozen at -70°C, torsos were mounted in a cryostat and consecutive 30μm longitudinal sections were cut to encompass the major thoracic and abdominal organs. Sections were thaw-mounted onto gelatin-coated slides[4] and ligand binding assays performed as described[5]. Briefly, consecutive sections were incubated with 0.25nM of the ET_A selective ligand [^{125}I]PD151242 or 0.25nM of the ET_B selective ligand [^{125}I]BQ2030 (Amersham Bioscience, GE Healthcare, UK). Non-specific binding was determined by co-incubating adjacent sections with the ligand and corresponding excess unlabelled peptide. Slides with calibrated standards were exposed to Kodak MR-1 autoradiography film for 4 days before being developed.

Immunohistochemistry

Immunohistochemical identification of ET_B receptors was performed as previously described[6]. Site directed antisera were raised in rabbits to the sequence ET_B(302-313), as described[7]. Briefly, whole body tissue sections (15 μm) were dried overnight at room temperature and fixed in ice-cold acetone for 10 minutes. Three knock-out and three control mice were examined.

Slides were incubated with 5% non-immunised donkey serum (DS) in phosphate-buffered saline (PBS) for 1 hour at room temperature to block non-specific protein interactions and then incubated overnight at 4 °C with primary rabbit anti-ET_B (1:50) antiserum and primary goat anti-von Willebrand factor (1:50) PBS/0.1% Tween-20/3% DS. Slides were then washed (3×5 minutes) in cold 1% PBS/0.1% Tween-20 before incubation for 1 hour at room temperature with Alexa Fluor 488 conjugated donkey anti-rabbit (1:200), Alexa Fluor 568 conjugated donkey anti-goat (1:100) secondary antibodies and Hoechst (1:100) diluted in 1% PBS/0.1% Tween-20/3% DS. Tissue sections were washed again (3×5 minutes) in cold 1% PBS/0.1% Tween-20 and mounted with ProLong Gold (Invitrogen). Confocal imaging was performed using a Leica TCS-NT-UV confocal laser-scanning microscope (Leica Microsystems, Heidelberg,Germany).

Ex vivo *analysis of ET_B-mediated contraction*

Functional analyses were performed using isolated mouse trachea, aorta, femoral arteries, and 1st order mesenteric arteries and veins, as described[8]. The endothelium was removed from some aortic rings by rubbing the luminal surface with a wire. Some arteries and veins were incubated in serum-free medium (DMEM) for 1-5 days before functional analysis to induce ET_B-mediated contraction, after the method of Adner *et al.*[9]. Briefly, rings (~2mm in length) of trachea, femoral artery, or mesenteric artery or vein were suspended on two intraluminal

40 µm tungsten wires in a myograph (model 610M Multi-myograph; JP Trading, Aarhus, Denmark) chamber. These rings were equilibrated at their optimum resting force (trachea 2mN; aorta 7.36mN, femoral artery, 8mN; mesenteric artery 3mN; mesenteric vein, 1mN) in physiological salt solution (PSS; 119 mM NaCl, 14.9 mM $NaHCO_3$, 4.7 mM KCl, 1.18 mM KH_2PO_4, 1.17 mM $MgSO_4$, 1.6 mM $CaCl_2$, 0.026 mM EDTA, 5.5 mM glucose), aerated (95% O_2, 5% CO_2) and maintained at 37°C. Each ring was then exposed to a high potassium (125mM) PSS (KPSS. Cumulative concentration-response curves were obtained to phenylephrine (PE, $1x10^{-9}$-$3x10^{-3}$M), ET-1 ($1x10^{-11}$-$3x10^{-5}$M), acetylcholine ($1x10^{-9}$-$3x10^{-3}$M) or sarafotoxin 6c (S6c; $1x10^{-11}$-$3x10^{-5}$M), as required. Some rings were incubated with an ET_A antagonist (BQ123; 100nM), an ET_B antagonist (A192621; 100nM) or a mixture of the two antagonists 20 min before acquiring cumulative concentration-response curves. All responses were measured and recorded with Powerlab software.

Measurement of BP

Male SM ET_B KO mice and age-matched controls (n=8/ group) were caged singly and maintained on standard mouse chow (7 days) before measurement of BP and heart rate using telemetry, as described previously[1]. Briefly, under isoflurane anesthesia, a telemetry catheter was inserted into the left carotid artery and the transmitter device (Data Sciences) secured in the left flank. Mice were allowed to recover and were maintained on standard chow (7 days), high (7.6%) salt diet (7 days), then high salt plus ET_B antagonist (SB192621; 30/mg/kg/day in drinking water, 7 days). Systolic and diastolic BP and heart rate were recorded in unrestrained mice (for 5 min every 30 min) as described previously[1] and analyzed using the Powerlab data acquisition system. Average blood pressures over each 5 min period (48 measurements/ day) were used to calculate the 24 h average BP.

Femoral artery injury

Intra-luminal injury was performed as described[2]. Briefly, under inhaled isoflurane anaesthesia (induction 5%, maintenance 2-3%) a 0.014" diameter straight-sprung angioplasty guide wire was advanced ~1.5cm proximally into the isolated femoral artery through an arteriotomy in the popliteal branch. After withdrawal, the popliteal branch was ligated to allow re-perfusion of the injured femoral artery. Non-denuding injury was achieved by ligation of the right femoral artery at the femero-popliteal bifurcation[2]. Peri-operative analgesia was provided by administration of buprenorphine (0.05mg/kg buprenorphine s.c.; Alstoe Animal Health, UK). Mice were then allowed to recover (28 days) to allow lesion development.

Perfusion fixation

After the recovery period mice were killed by perfusion fixation. Under terminal anaesthesia (sodium pentobarbital, Ceva Animal Health, UK, 60 mg/kg; i.p.), thoracotomy and transverse sternotomy were performed to allow introduction of a 23-gauge needle into the left ventricle. Phosphate buffered saline containing heparin (Leo Laboratories, UK, 10 U/ml) was administered (6 ml/min) via the left ventricle and an incision was made in the right ventricle to allow perfusate to wash through. Once blood was washed out, 10% neutral buffered formalin (Sigma, UK) was perfused until adequate fixation occurred (indicated by the development of rigidity of the body). Following perfusion fixation, femoral arteries, liver, heart and kidneys were removed. Organs were weighed and all tissues were left in formalin for a further 48h before processing to paraffin for histological assessment.

Optical projection tomography

Non-destructive 3-dimensional assessment of lesions was performed using optical projection tomography (OPT), as described[10,11]. Briefly, vessels were embedded in agarose and optically cleared in benzyl alcohol/ benzyl benzoate. Intrinsic fluorescent emission was imaged (excitation filter: 425/40 nm; emission filter: 475 nm low pass) using a Bioptonics 3001 tomograph. Data were reconstructed by filtered back projection using NRecon software (Skyscan, Belgium) and volumetric measurements generated by semi-automated tracing of the internal elastic lamina and the neointima distinguished from the lumen using a grey level threshold.

Histological assessment of neointimal lesions
Sections (4 µm) were cut from paraffin-embedded femoral arteries at 80 µm intervals with a Leitz 1512 microtome (Leica microsystems, Germany), and mounted onto Superfrost glass slides. Every tenth slide was selected for staining (Shandon Varistain Gemini automated slide stainer) with United States Trichrome, as described[12]. Images were taken using an Axioskop KS300 stage microscope (Carl Zeiss Inc., UK) and a CCD camera (photometrics USA) with a liquid crystal filter (MicroColour, LRI, Inc, USA). Image analysis was performed using MCID basic 7.0 software (Imaging Research, USA). The location of the maximal lesion was determined and serial sections used for compositional analysis, including picro-sirius staining for the quantification of collagen content.

Immunohistochemistry
De-waxed and re-hydrated sections were blocked with goat serum before incubation with primary antibodies to α-smooth muscle actin (1:400; 30 min; Sigma, UK) and Mac2 (1:6000; overnight; Cedarlane, USA). Sections were then washed and incubated with a secondary antibody (goat anti-mouse or goat anti-rat, respectively; 1:400, 30 min; Vector Labs, UK). This was followed by incubation with streptavidin-conjugated horseradish peroxidase Extravidin; 30 min; Sigma, UK). Slides were developed by addition of 3,3-diaminobenzidine (DAB peroxidase staining kit, Vector Lab, UK) for 1 min. Images were taken as before and analysed with Image J software.

References

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Supplementary Table S1. Deletion of ET_B from smooth muscle did not alter body or organ weights.

Age & Weights	Wild Type	SMET$_B$ KO
Age (weeks)	20.0 ± 1.4	20.8 ± 1.6
Body Weight (g)	31.6 ± 0.8	32.1 ± 0.6
Heart (% Body Weight)	0.56 ± 0.03	0.56 ± 0.02
Liver (% Body Weight)	5.08 ± 0.15	4.88 ± 0.18
Right Kidney (% Body Weight)	0.72 ± 0.03	0.74 ± 0.03
Right Kidney (% Body Weight)	0.68 ± 0.02	0.71 ± 0.03
Lung (% Body Weight)	0.54 ± 0.03	0.56 ± 0.02

Data are mean ± s.e. mean, n=7.

Supplementary Table S2. Impact of endothelial cell removal on function of aorta and femoral arteries from control mice.

Drug	Measurement	Aorta (Intact)	Aorta (Denuded)	Femoral Artery (Intact)	Femoral Artery (Denuded)
PE	N	3	3	3	3
	pD_2	7.1 ± 0.2	7.3 ± 0.2	5.8 ± 0.1	6.5 ± 0.1*
	Emax (%)	80.3 ± 5.5	199.8 ± 11.4*	100.3 ± 6.2	94.6 ± 4.5
	Emax (mN/mm)	3.92 ± 0.34	3.48 ± 0.19	2.35 ± 0.20	1.61 ± 0.14
ACh	N	*3*	*3*	*3*	*3*
	$-logIC_{50}$	7.4 ± 0.4	7.9 ± 1.0	7.4 ± 0.2	6.5 ± 6.1
	Emax (%)	62.7 ± 7.1	31.5 ± 7.3	108.6 ± 5.7	19.5 ± 11.8*
ET-1	N	3	3	3	3
	pD_2	8.5 ± 0.4	8.0 ± 0.2	8.2 ± 0.2	8.4 ± 0.1
	Emax (%)	53.4 ± 8.4	74.3 ± 4.7	95.2 ± 4.5	89.6 ± 4.2
	Emax (mN/mm)	1.78 ± 0.42	1.23 ± 0.20	2.51 ± 0.36	2.35 ± 0.46
KPSS	N	4	4	4	4
	Emax (mN/mm)	2.91 ± 0.58	2.03 ± 0.43	3.21 ± 1.36	2.56 ± 1.23

Data are mean±s.e.mean. ACh, acetylcholine, ET-1, endothelin-1, KPSS, high (125mM) potssium physiological salt solution. PE, phenylephrine. *P<0.05 compared with intact artery. Emax, maximum contraction; pD_2, -log EC_{50}.

Supplementary Table S3 Impact of deletion of ET_B from vascular smooth muscle on functional responses of femoral arteries.

Drug	Measurement	Wild Type (Intact)	Wild Type (Denuded)	SM ET_B KO (Intact)	SM ET_B KO (Denuded)
PE	N	6	7	7	7
	pD_2	5.7 ± 0.1	6.0 ± 0.1	5.6 ± 0.1	6.1 ± 0.1
	Emax(%)	88.9 ± 3.2	94.2 ± 3.2	91.9 ± 4.4	102.8 ± 3.8
	Emax (mN/mm)	3.99 ± 0.35	2.05 ± 0.26	3.48 ± 0.25	2.37 ± 0.20
ACh	N	5	6	6	6
	$-logIC_{50}$	8.0 ± 0.6	$6.8 \pm 0.4^{\dagger}$	7.9 ± 0.5	7.2 ± 0.6*
	Emax(%)	120.1 ± 10.2	57.8 ± 6.4*	120.9 ± 9.6	$22.5 \pm 3.2^{\ddagger}$
ET-1	N	6	7	7	7
	pD_2	7.8 ± 0.2	7.8 ± 0.3	7.9 ± 0.2	8.0 ± 0.2
	Emax(%)	97.4 ± 7.9	112.9 ± 3.5	105.4 ± 5.8	130.3 ± 5.4
	Emax (mN/mm)	4.03 ± 0.42	2.46 ± 0.40	4.04 ± 0.33	2.99 ± 0.27
KPSS	N	6	7	7	7
	Emax (mN/mm)	4.44 ± 0.81	1.99 ± 0.64*	3.84 ± 0.52	2.38 ± 0.54

Data are mean±s.e.mean. SM ETB KO, selective deletion of the endothelin B receptor from smooth muscle. ACh, acetylcholine, ET-1, endothelin-1, KPSS, high (125mM) potassium physiological salt solution. PE, phenylephrine. *P<0.05, [†]P<0.01, [‡]P<0.005 compared with intact artery. Emax, maximum contraction; pD_2, -log EC_{50}.

Supplementary Table S4. Impact of endothelin receptor antagonism on endothelin-1-mediated contraction of murine mesenteric arteries.

Measurement	Vehicle	Antagonist		
		ET_A	ET_B	$ET_{A/B}$
N	7	3	3	3
pD_2	8.2 ± 0.5	7.4 ± 0.2*	8.0 ± 0.1	7.0 ± 0.1*
Emax (mN/mm)	2.4 ± 0.7	2.4 ± 1.3	3.4 ± 0.5	2.3 ± 1.0
Emax (% KPSS)	105 ± 17	102 ± 21	111 ± 10	75 ± 30

Data are mean$\pm$s.e.mean. *P<0.05 compared with Vehicle. Emax, maximum contraction; pD_2, -log EC_{50}.

Supplementary Table S5. Incubation induces ET_B-mediated contraction in mouse mesenteric arteries.

Drug	Measurement	Incubation		
		Day 0	**Day 1**	**Day 5**
KPSS	N	7	7	3
	Emax (mN/mm)	2.47$\pm$0.80	2.42$\pm$0.68	1.40$\pm$1.03
PE	N	7	7	3
	pD_2	5.90$\pm$0.25	5.71$\pm$0.24	5.65$\pm$0.44
	Emax (mN/mm)	2.33$\pm$0.70	2.89$\pm$1.30	1.04$\pm$1.24
	Emax (% KPSS)	97.8$\pm$15.6	114.1$\pm$25.1	111.7$\pm$91.7
S6c	N	7	7	3
	pD_2	---	8.75$\pm$0.19	8.73$\pm$0.05
	Emax (mN/mm)	0.13$\pm$0.16	1.13$\pm$1.08*	0.82$\pm$0.60†
	Emax (% KPSS)	6.4$\pm$7.6	40.2$\pm$32.6*	94.5$\pm$75.4†
ET-1	N	7	5	3
	pD_2	8.24$\pm$0.53	8.50$\pm$0.40	8.54$\pm$0.25
	Emax (mN/mm)	2.45$\pm$0.73	3.79$\pm$0.88*	1.35$\pm$1.22*
	Emax (% KPSS)	105.4$\pm$17.8	164.1$\pm$16.4*	132.8$\pm$87.6

Data are mean$\pm$s.e.mean.. ET-1, endothelin-1, KPSS, high (125mM) potassium physiological salt solution, PE, phenylephrine, S6c, sarafotoxin s6c. *P<0.05 compared with Day 0. †P<0.05 compared with Day 1. Emax, maximum contraction; pD_2, -log EC_{50}.

Supplementary Table S6. Impact of endothelin receptor antagonism on sarafotoxin s6c-mediated contraction of murine mesenteric arteries.

Timepoint	Measurement	Vehicle	Antagonist ET$_A$	ET$_B$	ET$_{A/B}$
Day 0	N	7	3	3	3
	pD$_2$	NC	NC	NC	NC
	Emax (mN/mm)	0.13$\pm$0.16	0.01$\pm$0.01	0.03$\pm$0.03	0.04$\pm$0.04
	Emax (% KPSS)	6.4$\pm$7.6	0.90$\pm$1.3	1.0$\pm$06	1.2$\pm$1.2
Day 1	N	7	3	3	3
	pD$_2$	8.75$\pm$0.19	8.41$\pm$0.17*	NC	NC
	Emax (mN/mm)	1.13$\pm$1.08	2.63$\pm$0.90	0.01$\pm$0.01	0.00$\pm$0.01
	Emax (% KPSS)	40.2$\pm$32.6	73.3$\pm$17.75	0.7$\pm$0.4*	0.4$\pm$0.4*

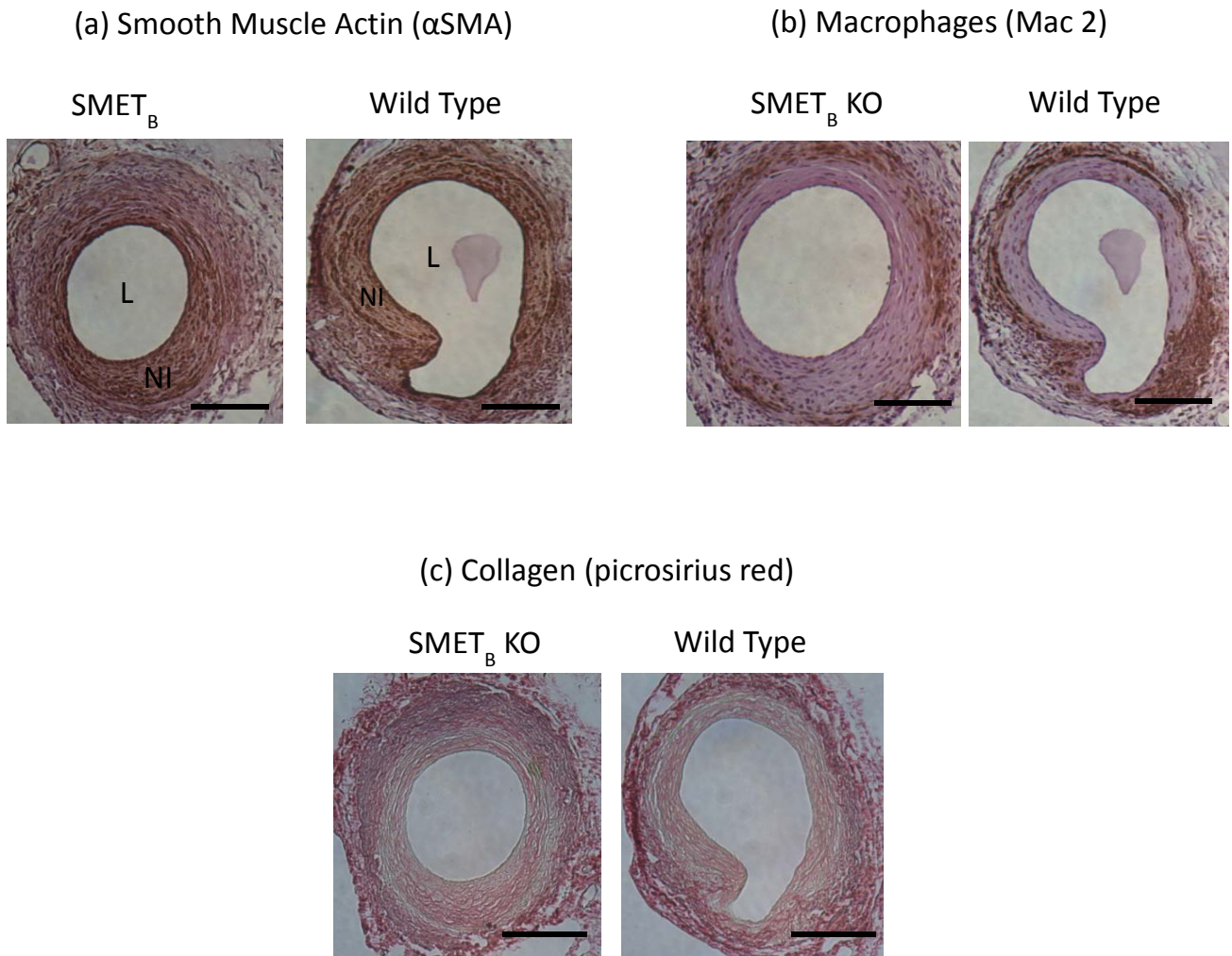Day 0, fresh arteries; Day 1, 24 h incubation. Data are mean$\pm$s.e.mean.. NC, not calculated. *P<0.05 compared with Vehicle. Emax, maximum contraction; pD$_2$, -log EC$_{50}$.

Supplementary Table S7. The impact of vascular cell selective deletion of murine vascular ET_B receptors (compared with wild type controls).

Measurement	EC ET_B KO*	SMC ET_B KO
Body Weight	NSD[1, 2, 3, 4]	NSD
Organ Weight	NSD[1, 2, 4]	NSD
Plasma [ET-1]		
Basal	Increased[2]	NSD
High salt diet + ET_B antagonist		NSD
Blood Pressure (basal)		
Basal	NSD[2]	Small (4mmHg) Increase
High salt diet	NSD[2]	Small (4mmHg) Increase
High salt diet + ET_B antagonist.	NSD[2]	NSD
Heart Rate	NSD[2]	Reduced
Neointimal Proliferation	NSD[1, 4]	NSD
ET_B-mediated contraction		
Trachea	NSD[1, 2, 4]	Reduced
Mesenteric Vein	N/A	Abolished
Mesenteric Artery	N/A	Induction Abolished
Femoral Artery	N/A	N/A
ACh-mediated relaxation		
Aorta	Impaired[2]	
Femoral Artery	NSD[1, 4]	NSD
ET-1-mediated contraction		
Femoral Artery	NSD[1, 4]	NSD
PE-mediated contraction		
Femoral Artery	NSD[1,4]	NSD

From previous investigations. [1]Kirkby *et al.*, 2012, [2]Bagnall *et al.*, 2006; [3]Kelland *et al.*, 2010; [4]Kirkby, N.S. PhD Thesis Edinburgh 2009. ACh, acetylcholine; ET-1, endothelin-1; PE, phenylephrine; ET_B, endothelin B receptor; NSD, No significant difference compared with Wild Type; N/A, not assessed.

Supplementary Figure S1

(a) Smooth Muscle Actin (αSMA)

SMET$_B$ — Wild Type

(b) Macrophages (Mac 2)

SMET$_B$ KO — Wild Type

(c) Collagen (picrosirius red)
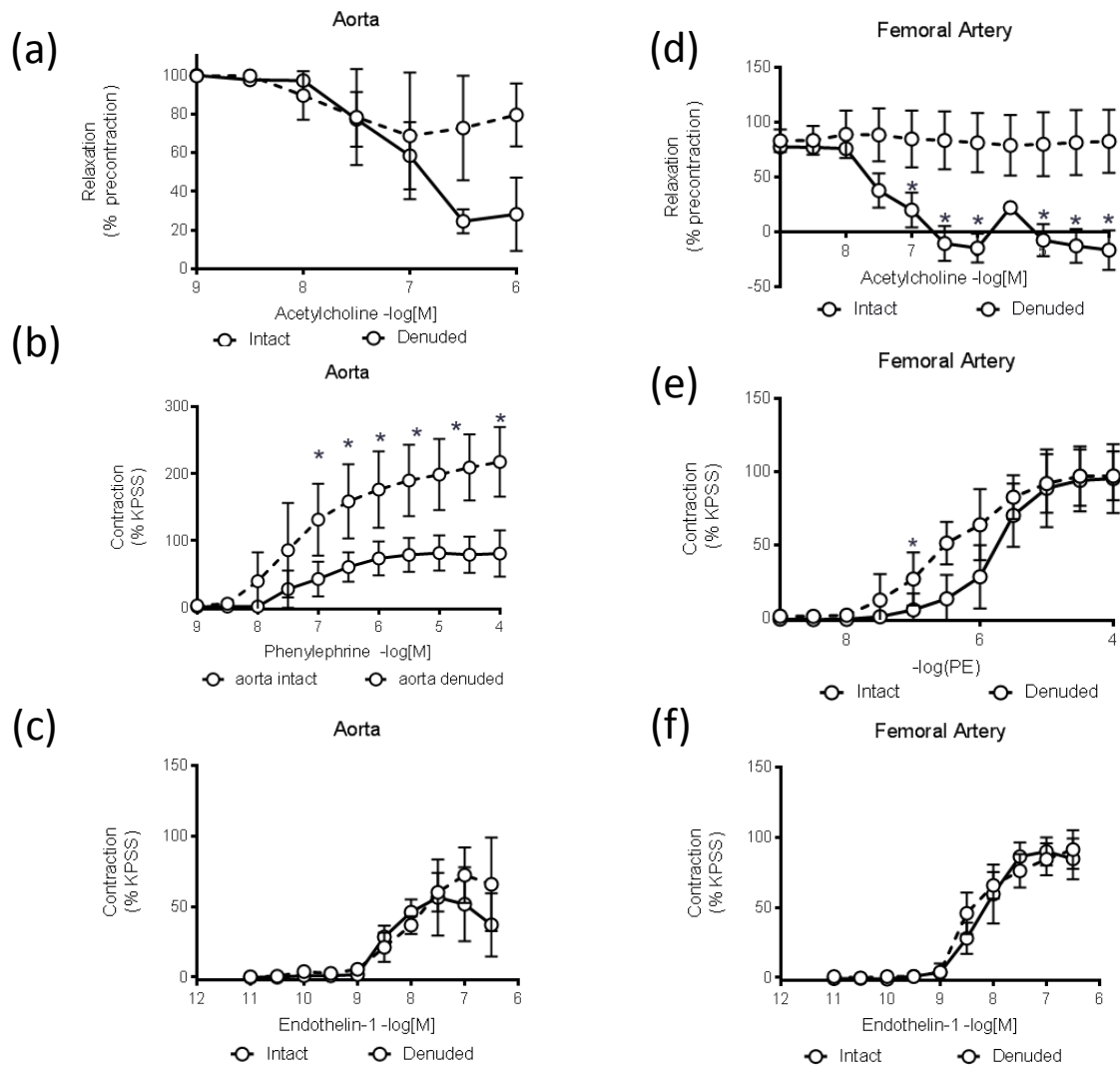
SMET$_B$ KO — Wild Type

Supplementary Figure S1. Identification of smooth muscle, macrophages and collagen in neointimal lesions.

Compositional analysis of neointimal lesions induced by wire injury in smooth muscle selective ET$_B$ KO (SMET$_B$KO) and wild type mouse femoral arteries showing the presence of immunoreactivity for (a) Smooth muscle actin (brown) and (b) Macrophages (Mac 2; brown). (c) Picrosirius red staining (pink) identified collagen in the neointima and media. Scale bar = 100µm. L, Lumen, NI, neointima.
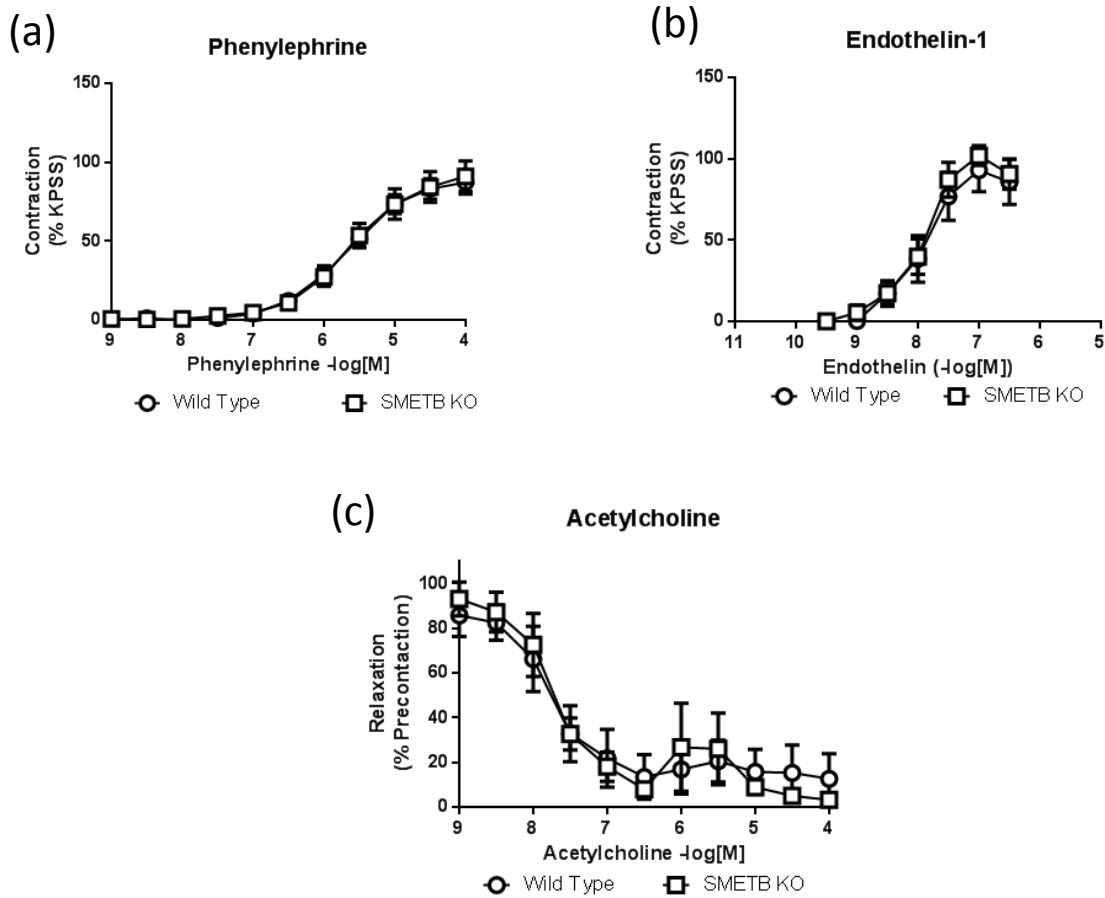
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Supplementary Figure S2. Impact of endothelial cell removal on functional responses of murine aorta and femoral artery.

Aorta (a, b, c) and femoral arteries (d, e, f) from adult male C57Bl/6j mice relaxed in response to acetylcholine (ACh) (a, d) and contracted in response to phenylephrine (PE) (b, e) and endothelin-1 (ET-1) (c, f). Removal of the endothelium abolished ACh-mediated relaxation and substantially increased aortic, but not femoral arterial, contraction to PE. It did not, however, alter ET-1 mediated contraction in aorta or in femoral artery. Symbols represent mean+s.e.mean for n= 3 mice. *P<0.05; **P<0.01; ***P<0.001.

Miller *et al.*

Supplementary Figure S3

(a)

Phenylephrine

Wild Type   SMETB KO

(b)

Endothelin-1

Wild Type   SMETB KO

(c)

**Acetylcholine**

Wild Type   SMETB KO

Supplementary Figure S3. Selective deletion of ET_B from the smooth muscle does not alter femoral artery function.

Concentration-response curves for (a) phenylephrine (PE), (b) endothein-1 (ET-1) and (c) acetylcholine (ACh) were generated in femoral arteries from control (FF--) and SM ET_B knockout (FFSm22Cre) mice. Responses to ACh were obtained following sub-maximal contraction with PE. Deletion of ET_B had no effect on the responses produced by these agonists. Symbols represent mean$\pm$s.e.mean for n=6-7mice.

Miller *et al.*
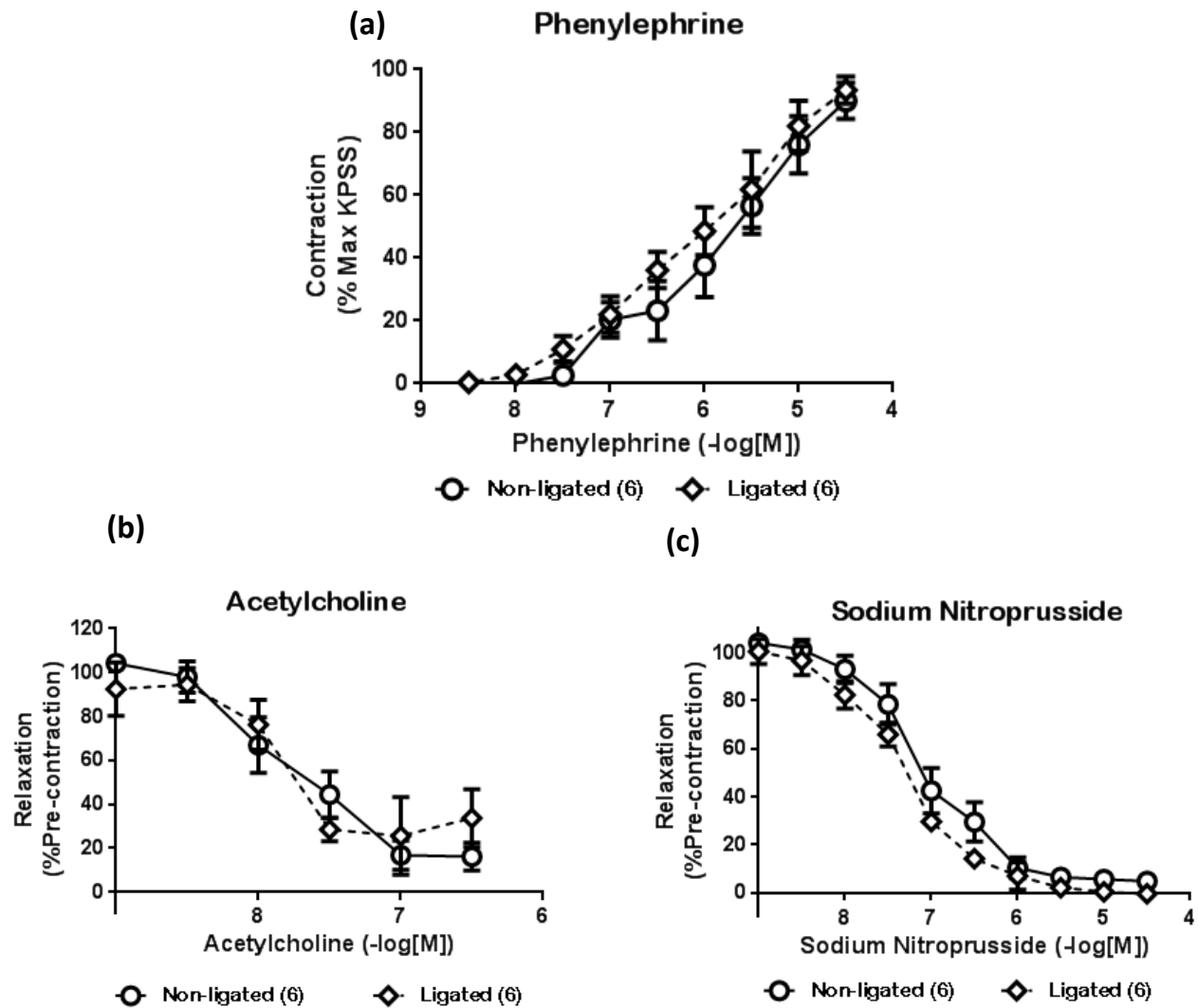
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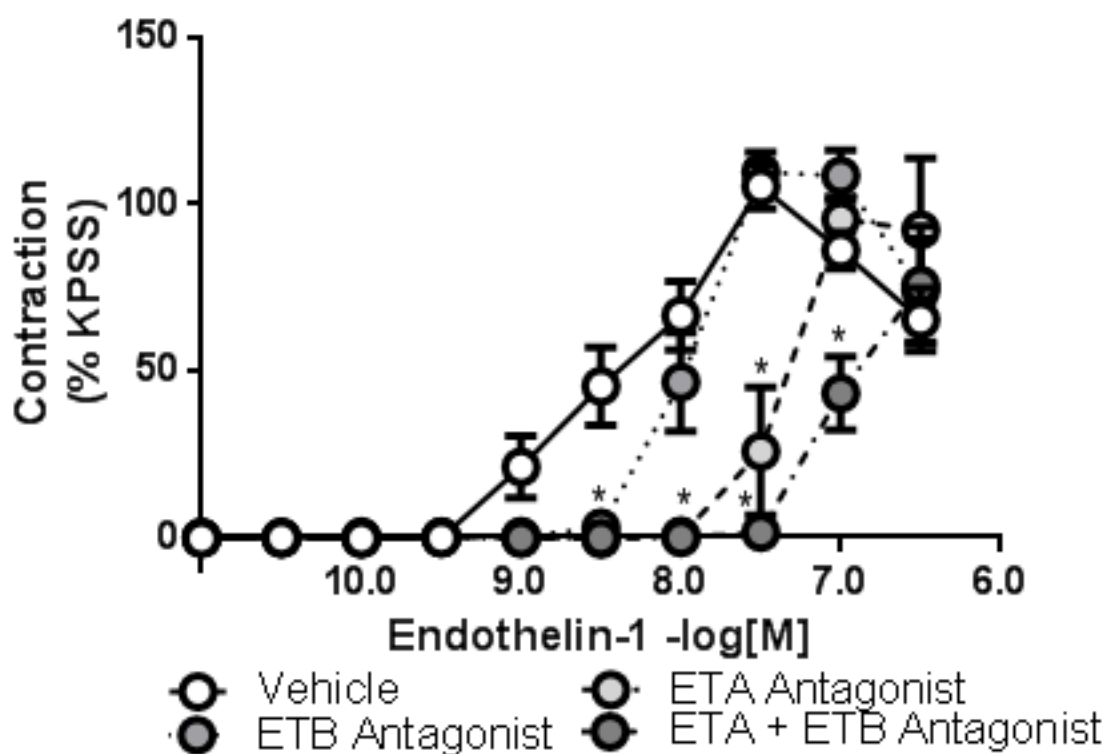
Supplementary Figure S4. Endothelin-1-mediated contraction of murine mesenteric arteries is mediated by ET$_A$ receptors.

In murine mesenteric arteries endothelin-1 (ET-1)-mediated contraction was shifted dramatically to the right by incubation with selective ET$_A$ (BQ123; 100nM) or mixed ET$_{A/B}$ antagonism, but selective ET$_B$ antagonism (A-192621; 100nM) had a much smaller effect. Symbols represent mean±s.e.mean, n=3-7. *P<0.05 compared with Vehicle.

Miller *et al*

Supplementary Figure S5

(a) Phenylephrine
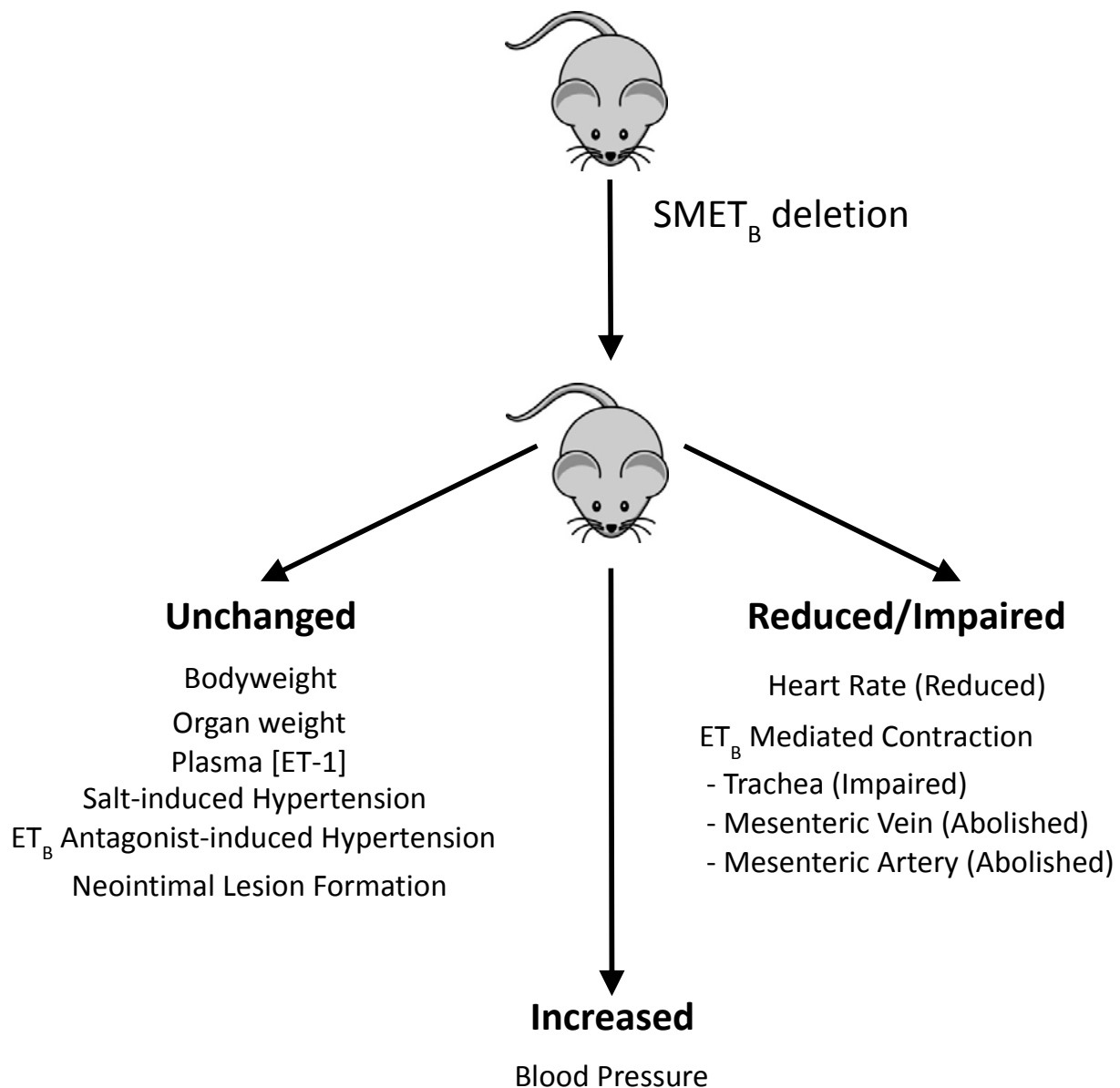
(b) Acetylcholine

(c) Sodium Nitroprusside

Supplementary Figure S5. Neointimal lesion formation does not alter functional responses of mouse femoral artery.

Mouse femoral arteries isolated 28 days after ligation showed unaltered responses to (a) phenylephrine (PE), (b) acetylcholine (ACh) or, (c) sodium nitroprusside (SNP) Data are mean±s.e.mean, n=3-6.

Miller *et al.*

Supplementary Figure S6

SMET$_B$ deletion

Unchanged

Bodyweight
Organ weight
Plasma [ET-1]
Salt-induced Hypertension
ET$_B$ Antagonist-induced Hypertension
Neointimal Lesion Formation

Reduced/Impaired

Heart Rate (Reduced)
ET$_B$ Mediated Contraction
 - Trachea (Impaired)
 - Mesenteric Vein (Abolished)
 - Mesenteric Artery (Abolished)

Increased

Blood Pressure

Supplementary Figure S6. Schematic summary of the effects of smooth muscle cell specific deletion of the Endothelin B receptor (SM ET$_B$ KO) in mice.

Miller *et al.*