

# Smooth Muscle Endothelin B Receptors Regulate Blood Pressure but Not Vascular Function or Neointimal Remodeling

Eileen Miller, Alicja Czopek, Karolina M. Duthie, Nicholas S. Kirkby, Elisabeth E. Fransen van de Putte, Sibylle Christen, Robert A. Kimmitt, Rebecca Moorhouse, Raphael F.P. Castellan, Yuri V. Kotelevtsev, Rhoda E. Kuc, Anthony P. Davenport, Neeraj Dhaun, David J. Webb, Patrick W.F. Hadoke

**Abstract**—The role of smooth muscle endothelin<sub>B</sub> (ET<sub>B</sub>) receptors in regulating vascular function, blood pressure (BP), and neointimal remodeling has not been established. Selective knockout mice were generated to address the hypothesis that loss of smooth muscle ET<sub>B</sub> receptors would reduce BP, alter vascular contractility, and inhibit neointimal remodeling. ET<sub>B</sub> receptors were selectively deleted from smooth muscle by crossing floxed ET<sub>B</sub> mice with those expressing cre-recombinase controlled by the transgelin promoter. Functional consequences of ET<sub>B</sub> deletion were assessed using myography. BP was measured by telemetry, and neointimal lesion formation induced by femoral artery injury. Lesion size and composition (day 28) were analyzed using optical projection tomography, histology, and immunohistochemistry. Selective deletion of ET<sub>B</sub> was confirmed by genotyping, autoradiography, polymerase chain reaction, and immunohistochemistry. ET<sub>B</sub>-mediated contraction was reduced in trachea, but abolished from mesenteric veins, of knockout mice. Induction of ET<sub>B</sub>-mediated contraction in mesenteric arteries was also abolished in these mice. Femoral artery function was unaltered, and baseline BP modestly elevated in smooth muscle ET<sub>B</sub> knockout compared with controls (+4.2±0.2 mm Hg; *P*<0.0001), but salt-induced and ET<sub>B</sub> blockade-mediated hypertension were unaltered. Circulating endothelin-1 was not altered in knockout mice. ET<sub>B</sub>-mediated contraction was not induced in femoral arteries by incubation in culture medium or lesion formation, and lesion size was not altered in smooth muscle ET<sub>B</sub> knockout mice. In the absence of other pathology, ET<sub>B</sub> receptors in vascular smooth muscle make a small but significant contribution to ET<sub>B</sub>-dependent regulation of BP. These ET<sub>B</sub> receptors have no effect on vascular contraction or neointimal remodeling. (*Hypertension*. 2017;69:275-285. DOI: 10.1161/HYPERTENSIONAHA.115.07031.) • [Online Data Supplement](#)

**Key Words:** autoradiography ■ endothelin-1 ■ hypertension ■ neointima ■ vasoconstriction

Endothelin-1 (ET-1), released by vascular endothelial cell (EC) and inner medullary collecting duct cells (and other cells under pathological conditions), stimulates endothelin<sub>A</sub> (ET<sub>A</sub>) and endothelin<sub>B</sub> (ET<sub>B</sub>) receptor subtypes.<sup>1,2</sup> ET<sub>A</sub> are present on vascular smooth muscle cells (VSMCs), predominantly mediating contraction<sup>3</sup> and regulating blood pressure (BP).<sup>4</sup> They also influence mitogenesis,<sup>5</sup> generation of reactive oxygen species, and adhesion molecule expression.<sup>6,7</sup> ET<sub>A</sub> receptors on leucocytes mediate cytokine release and cellular chemotaxis.<sup>8</sup> Many of these processes contribute to vascular

remodeling, and ET-1 clearly drives arterial lesion formation (including neointimal proliferation after injury).<sup>7</sup> This can be inhibited by selective ET<sub>A</sub> antagonism.<sup>9,10</sup>

Regulation of arterial function, BP, and arterial lesion formation by ET<sub>B</sub> receptors is likely to be more complex because they are expressed in EC, VSMC, and the kidney where they mediate physiologically antagonistic responses. ECET<sub>B</sub> receptors mediate production of vasodilator, antiproliferative, and anti-inflammatory molecules (eg, nitric oxide [NO])<sup>11,12</sup>; clearance of ET-1 from the circulation<sup>13,14</sup>; and regrowth of damaged

Received December 18, 2015; first decision January 2, 2016; revision accepted November 30, 2016.

From the University/BHF Centre for Cardiovascular Science, University of Edinburgh, United Kingdom (E.M., A.C., K.M.D., N.S.K., E.E.F.v.d.P., R.A.K., R.M., R.F.P.C., N.D., D.J.W., P.W.F.H.); University of Basel, Switzerland (S.C.); Centre for Functional Genomics, Skolkovo Institute of Science and Technology, Russian Federation (Y.V.K.); and Division of Experimental Medicine and Immunotherapeutics, Addenbrooke's Hospital, Cambridge, United Kingdom (R.E.K., A.P.D.).

Current address for N.S. Kirkby: National Heart and Lung Institute, Imperial College London, United Kingdom.

Current address for E.E.F. van de Putte: Department of Surgical Oncology, Division of Urology, The Netherlands Cancer Institute, Antoni van Leeuwenhoek, Amsterdam, The Netherlands.

The online-only Data Supplement is available with this article at <http://hyper.ahajournals.org/lookup/suppl/doi:10.1161/HYPERTENSIONAHA.115.07031/-/DC1>.

Correspondence to Patrick W.F. Hadoke, University/BHF Centre for Cardiovascular Science, The Queen's Medical Research Institute, University of Edinburgh, 47 Little France Crescent, Edinburgh EH16 4TJ, Scotland, United Kingdom. E-mail Patrick.Hadoke@ed.ac.uk

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EC.<sup>15</sup> VSMC ET<sub>B</sub> can mediate vascular contraction, similar to the ET<sub>A</sub> subtype,<sup>16</sup> and may compensate for ET<sub>A</sub> receptor dysfunction.<sup>17</sup> ET<sub>B</sub> upregulation in VSMC may mediate vasoconstriction and proliferation in cardiovascular disease.<sup>18,19</sup>

ET<sub>B</sub>-dependent regulation of BP is demonstrated by the sustained hypertension caused by ET<sub>B</sub> receptor antagonism in mice.<sup>20</sup> The importance of receptor distribution in this response is indicated by increased BP after deletion of ET<sub>B</sub> receptors in the renal collecting duct<sup>21</sup> but not after deletion of ECET<sub>B</sub>.<sup>22</sup> The influence of VSMC ET<sub>B</sub> on BP has not been established but, given their potential to mediate vasoconstriction, deletion or antagonism of VSMC ET<sub>B</sub> would be predicted to reduce BP.

Despite the influence of ET-1 in vascular remodeling,<sup>23</sup> the role of ET<sub>B</sub> is less clear. ET<sub>B</sub> activation in EC (NO release) and kidney (reduced BP) would be predicted to inhibit arterial remodeling, thus favoring selective ET<sub>A</sub> antagonism for reducing neointimal proliferation.<sup>9</sup> Certainly, global deletion of ET<sub>B</sub> receptors increases vascular lesion size.<sup>10,24</sup> However, selective ECET<sub>B</sub> deletion did not influence lesion formation, suggesting that the protective role was mediated by ET<sub>B</sub> receptors in other tissues.<sup>9</sup> If ET<sub>B</sub> receptors in VSMC contribute to lesion formation, mixed ET<sub>A/B</sub> antagonists might have advantages over ET<sub>A</sub> selective compounds, although recent investigations<sup>9,10,24</sup> favor the latter.

We generated novel smooth muscle ET<sub>B</sub> receptor knockout (SMET<sub>B</sub> KO) mice to address the hypothesis that loss of these receptors would impair arterial contraction, lower BP, and reduce neointimal lesion formation in response to vascular injury.

## Methods

Mice with VSMC-selective ET<sub>B</sub> receptor deletion were generated by crossing homozygous floxed ET<sub>B</sub> mice with SM22cre transgenic mice, which express cre-recombinase in the heart and smooth muscle, (then backcrossed to a C57Bl/6J background for 4–6 generations), as described for ECET<sub>B</sub> KO.<sup>22</sup> Controls were Cre-negative littermates (ET<sub>B</sub><sup>fl/fl</sup>). Genotyping was performed using ear clips.<sup>22,25</sup> Wild-type (WT) C57Bl/6J mice were from Charles River (United Kingdom). Mice were housed according to the UK Home Office recommendations (22°C; 12-hour light/dark cycles) with free access to water and chow. Procedures were performed under the provisions of the Animals Scientific Procedures Act (1986) and approved by the local Ethics Committee.

Selective SMET<sub>B</sub> deletion was demonstrated in organs and in isolated aortic smooth muscle cells (SMCs) using polymerase chain reaction, autoradiography,<sup>14,26</sup> immunohistochemistry,<sup>27</sup> and functional (myographic) investigation of isolated trachea, arteries, and veins.<sup>28,29</sup>

The impact of SMET<sub>B</sub> KO on BP was assessed using radiotelemetry<sup>22</sup> in conscious, unrestrained male SMET<sub>B</sub> KO mice and age-matched controls (n=8 per group), fed on chow (7 days), high (7.6%) salt diet (7 days), then high salt plus ET<sub>B</sub> antagonist (SB192621; 30 mg<sup>-1</sup> kg<sup>-1</sup> day<sup>-1</sup> in drinking water, 7 days). ET-1 concentrations in plasma from WT C57Bl/6J, controls, and SMET<sub>B</sub> KO were measured after exposure to chow or to high salt diet plus ET<sub>B</sub> antagonist, by ELISA (Endothelin-1 Quantikine ELISA kit; R&D Systems, Oxford, United Kingdom).

Intraluminal (left) or nondenuding (right) femoral artery injury was achieved by insertion of an angioplasty guidewire or ligation, respectively, as described.<sup>9</sup> After 28 days, arteries were retrieved (after perfusion fixation) and analyzed using optical projection tomography, histology, and immunohistochemistry.<sup>9,30</sup>

## Statistics

Results are mean±SEM, for n mice. Group sizes were chosen to detect 5%, 20%, and 20% differences in BP (n=7), lesion size (n=7), and

maximum responses to vasoactive agents (n=6) with >90% power. Investigations were performed by operators blinded to treatment. Components of lesions were expressed as a percentage of the neointimal area. Analyses were performed with GraphPad Prism using Student *t* test, 1-way or 2-way ANOVA with a Tukey post hoc test, as indicated. Significance was assumed for *P*<0.05.

Detailed methods are in the [online-only Data Supplement](#).

## Results

### Identification of SMET<sub>B</sub> KO

Genotyping for SM22cre, WT, and delta band alleles (Figure 1A) identified SMET<sub>B</sub> KO (positive for SM22cre, floxed, and delta band and negative for WT allele) and controls (SMET<sub>B</sub><sup>fl/fl</sup> cre-negative littermates; negative for WT allele, positive for floxed allele, and negative for SM22cre and delta band). SMC isolated from the aorta of SMET<sub>B</sub> KO mice expressed the cre, delta, and flox bands, whereas controls did not express the cre and the delta bands (Figure 1B).

Autoradiography (Figure 1C) identified ET<sub>B</sub> receptors in the gut lining, lung, and kidney. This signal was not diminished after SMET<sub>B</sub> deletion. ET<sub>B</sub> expression (real-time polymerase chain reaction) was not altered in the colon, heart, or gastrocnemius muscle of SMET<sub>B</sub> KO mice (Figure S1 in the [online-only Data Supplement](#)). Confocal imaging of immunofluorescence (Figure 1D) clearly showed ET<sub>B</sub> receptors localizing to the endothelium (von Willebrand factor positive) in SMET<sub>B</sub> KO coronary artery. ET<sub>B</sub> staining in medial SM remained at background levels. This confirms maintained ET<sub>B</sub> receptor expression in the endothelium of SMET<sub>B</sub> KO mice.

### Functional Confirmation of SMET<sub>B</sub> KO

SMET<sub>B</sub> KO mice were healthy with normal body and organ weights (Table S1).

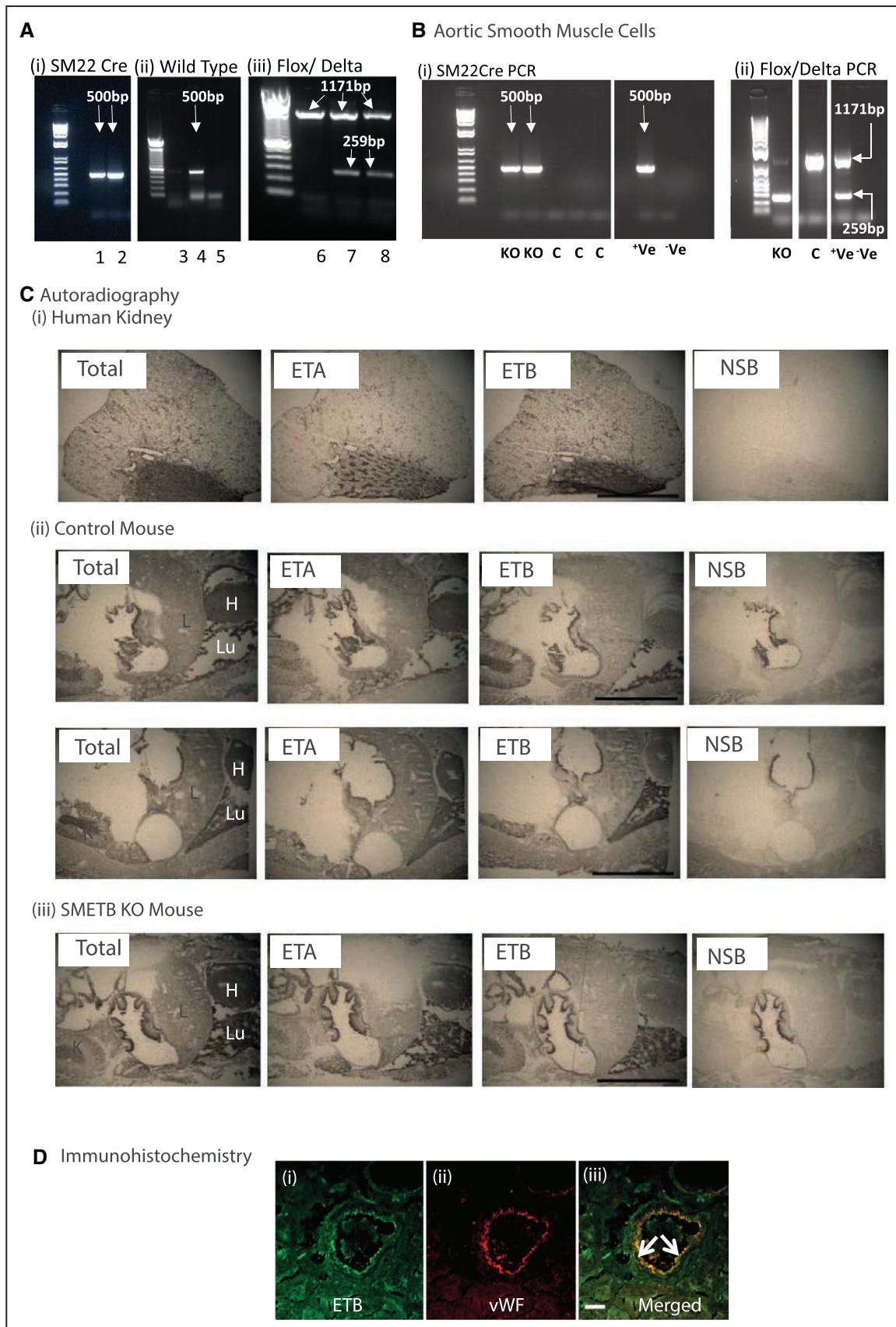
Sarafotoxin S6c (S6c)-mediated contraction in tracheas (which express ET<sub>B</sub> receptors on SM)<sup>22</sup> from controls was abolished by incubation with the selective ET<sub>B</sub> antagonist A192621 (Figure 2A).<sup>22</sup> In SMET<sub>B</sub> KO mice, S6c-mediated contraction was reduced (≈30%), but not abolished. The residual contraction was blocked by ET<sub>B</sub> antagonism. S6c-mediated contraction of mesenteric veins was abolished by selective deletion of SMET<sub>B</sub> (Figure 2B).

### SMET<sub>B</sub> KO and BP

Control and SMET<sub>B</sub> KO mice demonstrated a clear diurnal rhythm in BP (Figure 3A). Mean 24-hour BP was higher in SMET<sub>B</sub> KO mice than in controls (107.1±0.3 versus 102.8±0.5 mmHg; n=7; *P*<0.0001; Figure 3B). Systolic BP was not different between groups (123.5±0.6 versus 124.8±0.5 mmHg; *P*=0.09; Figure 3C), but SMET<sub>B</sub> KO mice had an increased diastolic BP (98.2±0.3 versus 92.2±0.4 mmHg; *P*<0.0001; Figure 3D). BP elevation occurred despite reduced heart rate (515±3 versus 538±5 bpm; *P*=0.004; Figure 3E). High salt increased BP in controls with a further increase induced by ET<sub>B</sub> antagonism (Figure 4A). These responses were similar in SMET<sub>B</sub> KO.

### SMET<sub>B</sub> KO and Circulating ET-1

Plasma ET-1 concentrations were similar in SMET<sub>B</sub> KO and control mice (Figure 4B) and consistent with levels in WT C57Bl/6J (1.14±0.08; n=6). The combination of high salt diet



**Figure 1.** Selective endothelin<sub>B</sub> (ET<sub>B</sub>) receptor deletion from smooth muscle. **A**, Mice were genotyped for (i) SM22cre (band at 500 bp), (ii) wild-type (band a 500 bp), and (iii) flox (band at 1171 bp)/delta (band at 259 bp) alleles in ear clip DNA. (i) Samples 1 and 2 are cre-positive, (ii) sample 4 is positive for the wild-type allele; samples 3 and 5 are not, (iii) samples 7 and 8 are positive for both the flox (*Continued*)

**Figure 1 Continued.** and the delta band; sample 6 has only the flox band. **B**, Polymerase chain reaction (PCR) for cre and flox/delta bands in murine aortic smooth muscle cells isolated from smooth muscle ET<sub>B</sub> receptor knockout (SMET<sub>B</sub> KO) and control (C) mice. Control mice lacked cre and delta alleles, whereas SMET<sub>B</sub> KO expressed all 3. Standard DNA ladders have band sizes 1500–100 bp. **C**, Autoradiography showing maintained ET<sub>B</sub> ligand binding in SMET<sub>B</sub> KO lung and kidney (representative of n=3 mice/genotype). **D**, Confocal images of a coronary artery from an SMET<sub>B</sub> KO mouse stained for (i) ET<sub>B</sub> receptor (green) or (ii) the endothelial cell marker von Willebrand factor (vWF; red). Merged images (iii) show clear colocalization of ET<sub>B</sub> with the endothelium (arrows). There is no ET<sub>B</sub> staining in medial smooth muscle. Scale bar=50 μm. +Ve, positive control; -Ve, negative control; ET<sub>A</sub> indicates endothelin<sub>A</sub>; H, heart; K, kidney; L, liver; Lu, lung; and NSB, nonspecific binding.

and ET<sub>B</sub> antagonism increased plasma ET-1 to a similar extent in control-type and SMET<sub>B</sub> KO mice (Figure 4C).

### SMET<sub>B</sub> KO and Neointimal Remodeling

Wire injury of the left femoral artery generated neointimal lesions (Figure 5A).<sup>9</sup> Optical projection tomography demonstrated that SMET<sub>B</sub> KO altered neither the lesion volume (Figure 5B) nor cross-sectional narrowing (Figure 5C). Histological analysis showed a trend toward reduced cross-sectional narrowing in SMET<sub>B</sub> KO (Figure 5D). Ligation of the right femoral artery generated lesions<sup>9</sup> with similar volume

(Figure 5E) and maximal cross-sectional area (Figure 5F) in SMET<sub>B</sub> KO mice and control mice.

Immunohistochemistry (Figure S2) showed that SMET<sub>B</sub> KO did not differ from controls in the amount of macrophage (Mac-2; SMET<sub>B</sub> KO 2.7±0.9% versus Control 2.6±0.7% lesion area), α-smooth muscle actin (SMET<sub>B</sub> KO 14.8±4.1% versus Control 19.9±3.8% lesion area), or collagen (SMET<sub>B</sub> KO 9.7±3.1% versus Control 14.9±3.2% lesion area) staining in the neointimal lesions.

### SMET<sub>B</sub> KO and Vascular Reactivity

In WT C57Bl/6J mice, EC removal from aortic rings abolished acetylcholine-mediated relaxation and enhanced the contractile response to phenylephrine but not to ET-1. EC removal from femoral arteries also abolished acetylcholine-mediated relaxation but had no effect on phenylephrine or ET-1 (Figure S3; Table S2). SMET<sub>B</sub> KO had no effect on contractile responses to phenylephrine or ET-1, or acetylcholine-mediated relaxation in femoral arteries (Figure S4; Table S3).

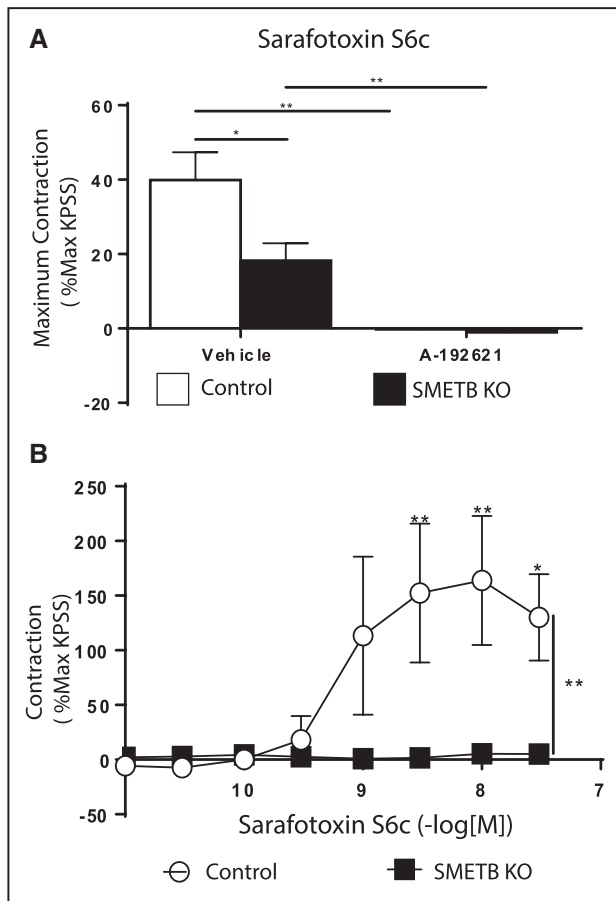
### Induction of ET<sub>B</sub>-Mediated Contraction in Isolated Mesenteric Arteries

ET-1-mediated contraction in mesenteric arteries from WT C57Bl/6J mice was shifted to the right by mixed ET<sub>A/B</sub>, or selective ET<sub>A</sub>, antagonism, but not by ET<sub>B</sub> selective antagonism (Figure S5; Table S4). Unlike mesenteric veins (Figure 6A), mesenteric arteries freshly isolated from WT C57Bl/6J mice did not contract in response to S6c (Figure 6B).

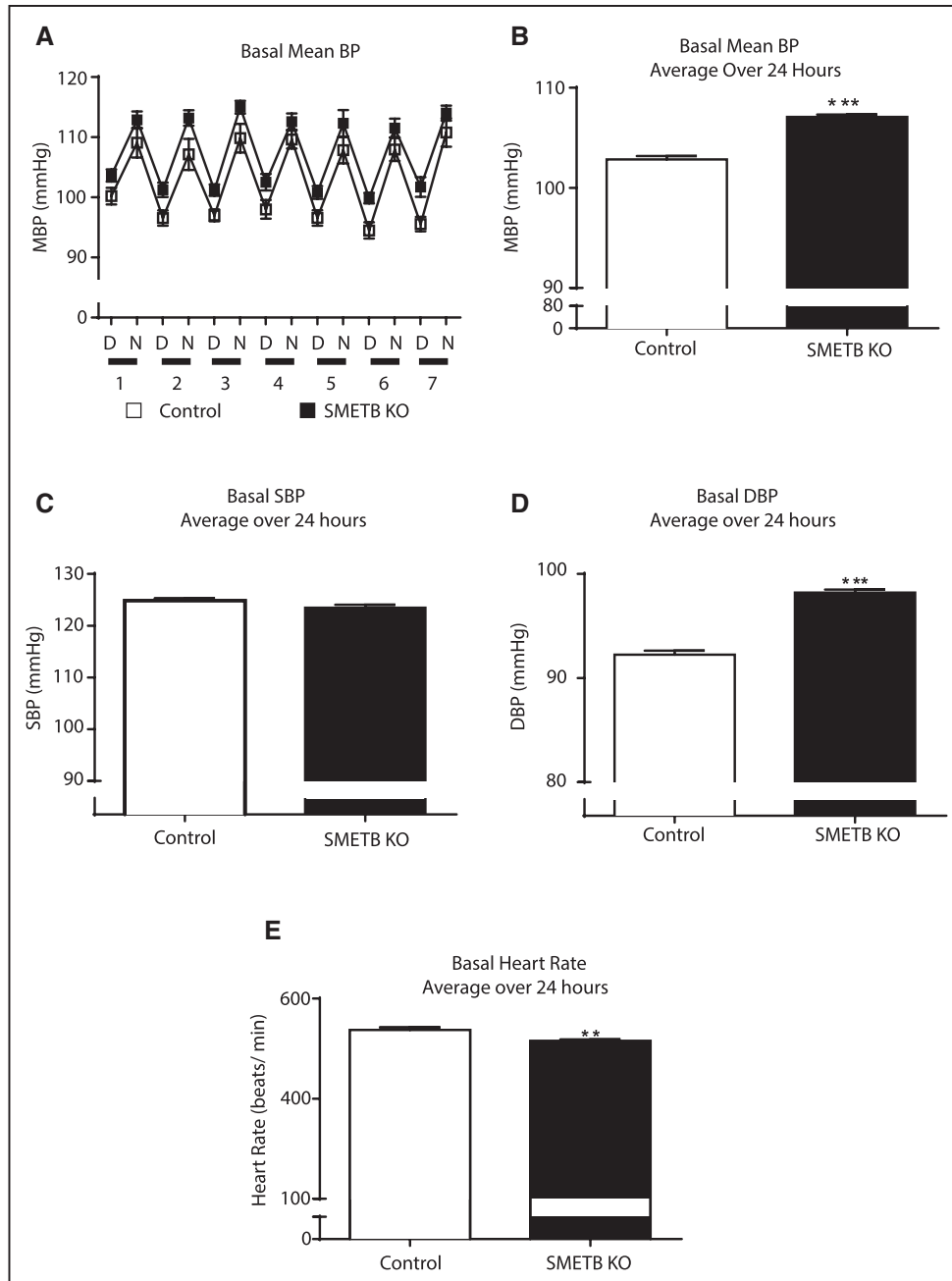
Incubation in culture medium (≤5 days) can induce ET<sub>B</sub>-mediated contraction in rat arteries.<sup>29</sup> Incubation of C57Bl/6J mesenteric veins in culture medium had no effect on S6c-mediated contraction (Figure 6A). In mesenteric arteries, incubation in culture medium selectively increased the contractile response to ET-1 (Table S5). Strikingly, S6c-mediated contraction was induced in isolated mesenteric arteries after incubation in culture medium (Figure 6B; Table S5), a response abolished by selective ET<sub>B</sub>, or mixed ET<sub>A/B</sub>, antagonism, but not by selective ET<sub>A</sub> antagonism (Figure 6C; Table S6). Incubation of mesenteric arteries from SMET<sub>B</sub> KO mice in culture medium did not induce S6c-mediated contraction (Figure 6D).

### No Induction of ET<sub>B</sub>-Mediated Contraction in Femoral Arteries

S6c-mediated contraction was variable in femoral arteries from WT C57Bl/6J mice: some contracted but others did not (Figure 6E). Neither incubation of femoral arteries in culture medium (24 hours; Figure 6F) nor lesion formation induced S6c-mediated contraction; femoral arteries isolated 28 days after ligation contracted in response to ET-1 (Figure 6G) but not to S6c (Figure 6H). Responses to acetylcholine, sodium



**Figure 2.** Functional consequences of selective endothelin<sub>B</sub> (ET<sub>B</sub>) deletion from smooth muscle (SM). **A**, Sarafotoxin S6c (S6c)-induced contraction of isolated trachea was abolished by ET<sub>B</sub> receptor antagonism (A192621; 100 nmol/L) but only reduced by selective smooth muscle ET<sub>B</sub> receptor (SMET<sub>B</sub>) deletion (residual contraction was blocked by A192621). Columns are mean±SEM (n=4). \*P<0.02, \*\*P<0.005. **B**, S6c-induced contraction in murine mesenteric veins was abolished by SMET<sub>B</sub> deletion. Symbols represent mean±SEM (n=4). \*P<0.05, \*\*P<0.01. KO indicates knockout; and KPSS, potassium physiological salt solution.



**Figure 3.** Selective deletion of endothelin<sub>B</sub> (ET<sub>B</sub>) receptors from smooth muscle increases baseline blood pressure (BP). **A**, BP, assessed in conscious, unrestrained male smooth muscle ET<sub>B</sub> receptor knockout (SMET<sub>B</sub> KO) mice and controls (n=8 per group) using radiotelemetry, demonstrated a clear diurnal rhythm. Mean blood pressure (MBP) in SMET<sub>B</sub> KO (filled symbols) mice was consistently higher than controls (open symbols). **B**, Data averaged over 24 h confirmed elevated MBP in SMET<sub>B</sub> KO, with no difference in **(C)** systolic blood pressure (SBP) but **(D)** elevated diastolic blood pressure (DBP). **E**, Increased MBP was accompanied by reduced heart rate. Data are mean±SEM (n=8 per group). \*\**P*<0.005, \*\*\**P*<0.0001. D indicates day; and N, night.

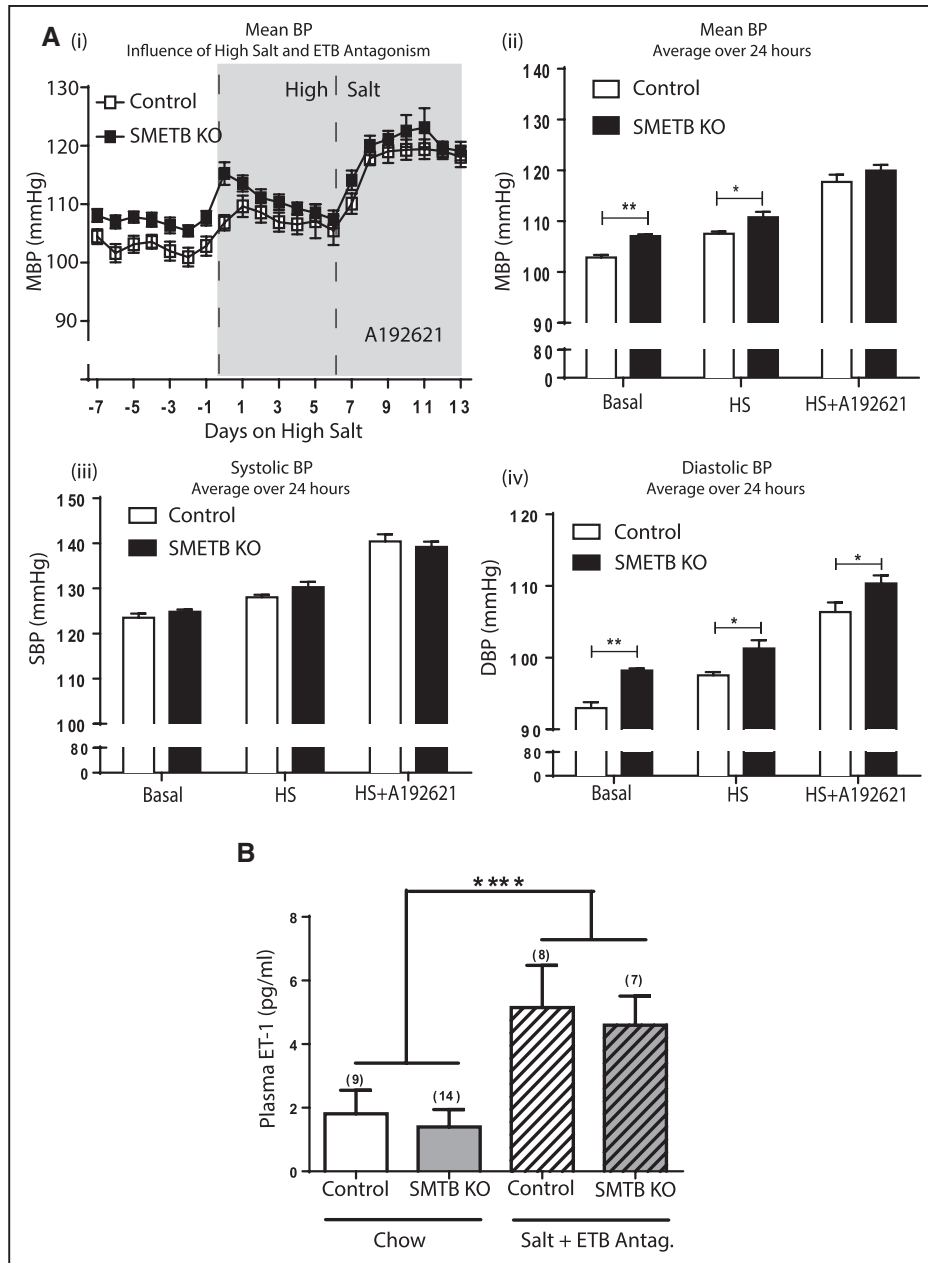
nitroprusside, and phenylephrine were unaltered by lesion formation (Figure S6).

### Discussion

Tissue-specific knockout mice were generated to address the hypothesis that selective deletion of ET<sub>B</sub> receptors from VSMC would impair arterial contraction, lower BP, and reduce neointimal lesion size. SMET<sub>B</sub> KO attenuated S6c-mediated vascular and tracheal contraction, without altering other functional responses, but produced a modest (≈4

mm Hg) increase in BP. ET<sub>B</sub>-mediated contraction was not induced in femoral arteries after ligation, although injury-induced intimal lesion formation was unaffected by SMET<sub>B</sub> KO. Key findings are summarized (Figure S7) and compared with the ECET<sub>B</sub> KO (Table S7).

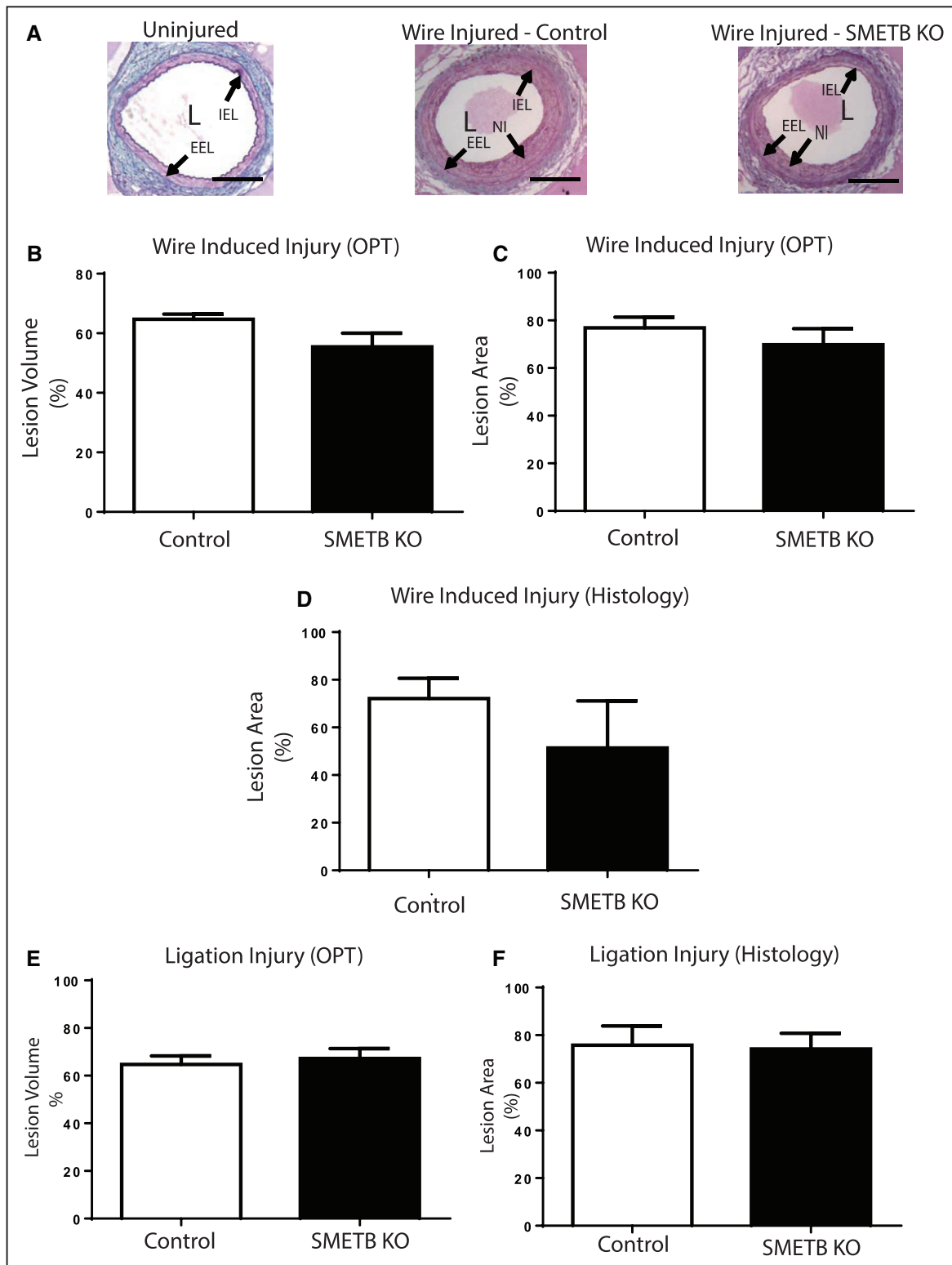
SMET<sub>B</sub> KO was based on our generation of ECET<sub>B</sub> KO,<sup>22</sup> crossing mice expressing Cre-recombinase controlled by the SM-specific SM22 promoter<sup>25</sup> with those bearing a floxed ET<sub>B</sub> gene.<sup>22</sup> This strategy was used to produce mice with SM-selective ET<sub>A</sub> deletion,<sup>4</sup> and renal collecting duct-selective



**Figure 4.** Selective deletion of endothelin<sub>B</sub> (ET<sub>B</sub>) receptors from smooth muscle does not alter blood pressure (BP) responses. **A**, BP, assessed in conscious, unrestrained male smooth muscle ET<sub>B</sub> receptor knockout (SMET<sub>B</sub> KO) mice and controls (n=8 per group) using radiotelemetry (i) was elevated by high salt diet (HS; 7 d) and by ET<sub>B</sub> antagonism (A192621; 30 mg<sup>-1</sup> kg<sup>-1</sup> d<sup>-1</sup>; 7 d) in both groups. (ii) Comparison of BP (averaged over 24 h) demonstrates the elevation in mean blood pressure (MBP) in response to high salt diet and high salt diet plus A192621. (iii) There was no difference in systolic blood pressure (SBP) in control compared with SMET<sub>B</sub> KO mice but (iv) diastolic blood pressure (DBP) was higher in SMET<sub>B</sub> KO for all treatment groups. **B**, Plasma endothelin-1 (ET-1) concentrations were similar in SMET<sub>B</sub> KO and controls and consistent with wild-type C57Bl/6J mice (1.14±0.08 pg/mL; n=6). ET-1 concentrations were elevated in control and SMET<sub>B</sub> KO mice after exposure to a high salt diet plus A192621. Data (mean±SEM) were analyzed using 2-way ANOVA with Tukey or Bonferroni post hoc test, as appropriate. **A**, \*P<0.05, \*\*P<0.01 compared with controls. **B**, \*\*\*\*P<0.00001 (effect of diet).

ET<sub>B</sub> deletion.<sup>21</sup> It has also been used within our group to produce mice with SM-selective deletion of glucocorticoid receptor<sup>31</sup> or 11 $\beta$ -hydroxysteroid dehydrogenase 1<sup>32</sup> (with LacZ staining in Rosa26 reporter mice showing SM22cre expression in the blood vessels and heart but not in the brain, kidney, or adrenal gland). As with ECET<sub>B</sub> KO,<sup>22</sup> SMET<sub>B</sub> KO mice were healthy. This contrasts with global ET<sub>B</sub> deletion, which causes coat spotting and death from megacolon,<sup>33</sup> requiring transgenic ET<sub>B</sub> rescue in the enteric nervous system.<sup>34</sup> Autoradiographic

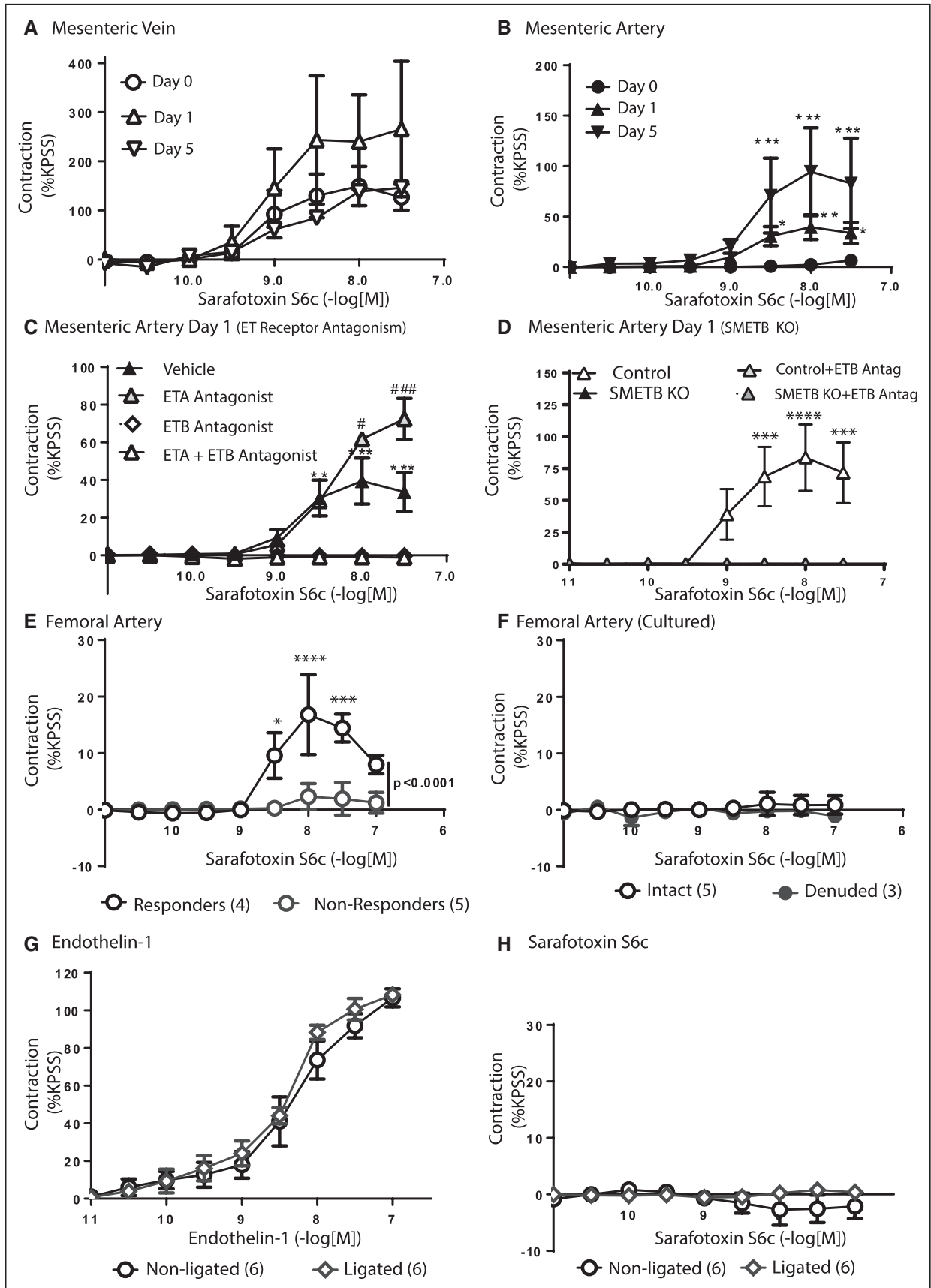
detection of ET<sub>B</sub> receptors in lungs of SMET<sub>B</sub> KO mice indicates maintained expression in EC (which was lost in ECET<sub>B</sub> KO).<sup>14</sup> This was supported by colocalization of immunoreactivity for ET<sub>B</sub> with an EC marker (von Willebrand factor) in coronary arteries; absence of medial ET<sub>B</sub> staining was consistent with deletion from SMCs. Polymerase chain reaction confirmed that ET<sub>B</sub> had been deleted from aortic smooth muscle but not from heart, colon, or skeletal muscle (although direct evidence of ET<sub>B</sub> deletion from tracheal, mesenteric vein,



**Figure 5.** Selective smooth muscle endothelin<sub>B</sub> (SMET<sub>B</sub>) deletion does not alter neointimal lesion formation in femoral arteries from control and SMET<sub>B</sub> knockout (KO) mice. Neointimal lesion volume (B) and maximal cross-sectional area (C) were similar in control and SMET<sub>B</sub> KO mice when measured by optical projection tomography. Similar results were obtained when maximal cross-sectional area was measured histologically (D). Volume (E) and maximal cross-sectional area (F) of lesions induced by ligation were similar in control and SMET<sub>B</sub> KO mice (optical projection tomography [OPT]). Data are mean±SEM (n=7). EEL indicates external elastic lamina; IEL, internal elastic lamina; L, lumen; and NI, neointima.

mesenteric, or femoral artery smooth muscle was not obtained using this technique). Functional investigations confirmed that SMET<sub>B</sub>-dependent responses were lost in the knockout,

with the abolition of S6c-mediated contraction in mesenteric veins. Furthermore, induction of S6c-mediated contraction in mesenteric arteries incubated in culture medium (as in rat



**Figure 6.** Impact of smooth muscle endothelin<sub>B</sub> (SMET<sub>B</sub>) receptors on vascular function. **A**, Sarafotoxin S6c (S6c)-induced contraction in mesenteric veins (n=6) was not increased by incubation for 1 (n=3) or 5 (n=1) d in culture. **B**, Freshly isolated mesenteric arteries (n=6) did not respond to S6c, but contractions were induced by incubation in culture medium for 1 (n=7) or 5 (n=3) \*P<0.05, (Continued)



**Figure 6 Continued.** \*\* $P < 0.01$ , \*\*\* $P < 0.005$  compared with d 0. **C**, S6c-mediated contraction of mesenteric arteries after 24 h in culture ( $n=7$ ) was abolished by ET<sub>B</sub> selective (A192621; 100 nmol/L;  $n=3$ ) or mixed ET<sub>A/B</sub> (BQ-123+A192621;  $n=3$ ) antagonism, but not by ET<sub>A</sub> receptor antagonism (BQ-123; 100 nmol/L;  $n=3$ ); \*\* $P < 0.01$ , \*\*\* $P < 0.005$  compared with ET<sub>B</sub> or ET<sub>A/B</sub> antagonism; # $P < 0.05$ , ### $P < 0.005$  compared with vehicle. **D**, In contrast to controls ( $n=4$ ), S6c-mediated, A192621 (100 nmol/L)-sensitive contraction was not induced in mesenteric arteries from SMET<sub>B</sub> knockout (KO) mice ( $n=4$ ) by incubation in culture medium (24 h); \*\*\* $P < 0.005$ , \*\*\*\* $P < 0.001$  compared with antagonists. **E**, Contractile responses to S6c were unreliable in femoral arteries—some failed to contract, whereas others produced small contractions. \* $P < 0.05$ , \*\*\* $P < 0.005$ , \*\*\*\* $P < 0.001$  compared with nonresponders. **F**, Incubation in culture did not induce S6c-mediated contraction in these arteries. Femoral arteries after ligation (28 d) contracted in response to endothelin-1 (**G**) but not to S6c (**H**). Data are mean $\pm$ SEM ( $n=3$  to 6). ET<sub>A</sub> indicates endothelin<sub>A</sub>; and KPSS, potassium physiological salt solution.

arteries<sup>35</sup>), was abolished by SMET<sub>B</sub> KO (although these functional changes do not necessarily confirm selective SMET<sub>B</sub> deletion). The failure to abolish S6c-induced contraction in trachea was unexpected and suggests either incomplete penetrance of SM22cre-mediated recombination or a role for ET<sub>B</sub> receptors in other cells (eg, epithelium) in mediating tracheal contraction. Detection of the delta band in some ear clip samples may suggest deletion of the floxed gene in germ cells, which is a possible limitation with these mice. However, our F<sup>+</sup>/Cre0 $\times$ F<sup>+</sup>/Cre0 crosses did not produce piebald mice (which inevitably would occur if germ-line recombination takes place). Therefore, the delta band during genotyping can only be explained by the presence of SMC in the ear clip preparations.

Selective deletion of ET<sub>B</sub> from EC increased plasma ET-1<sup>22</sup> because of impaired clearance.<sup>14</sup> In contrast, SMET<sub>B</sub> KO did not alter circulating ET-1, consistent with the proposal that ECET<sub>B</sub> predominantly mediate ET-1 clearance.

Transgenic and pharmacological approaches suggest ET<sub>B</sub> receptors regulate BP. Selective ET<sub>B</sub> receptor antagonism,<sup>20</sup> global ET<sub>B</sub> deletion,<sup>10</sup> and selective ET<sub>B</sub> deletion from the collecting duct<sup>21</sup> all increased ( $\approx 10$ – $13$  mm Hg) BP. Furthermore, ET<sub>B</sub> receptors in peripheral ganglia can influence BP,<sup>36</sup> suggesting that sympathetic activation accounts for ET<sub>B</sub>-induced hypertension.<sup>37</sup> In contrast, BP was not elevated by ECET<sub>B</sub> KO.<sup>22</sup> The small ( $\approx 4$  mm Hg) increase in BP, which persisted in SMET<sub>B</sub> KO mice despite reduced heart rate, suggests that loss of SMET<sub>B</sub> contributes to the increased BP induced by systemic ET<sub>B</sub> antagonism<sup>20</sup> or global ET<sub>B</sub> deletion.<sup>10</sup> However, it requires rejection of our hypothesis that ET<sub>B</sub>-mediated vascular contraction contributes to BP elevation. Indeed, our data support a role for extravascular ET<sub>B</sub> (eg, in the kidney or peripheral ganglia) in regulating BP. This is supported by the demonstration that, as in ECET<sub>B</sub> KO,<sup>22</sup> salt-induced and ET<sub>B</sub> antagonist-induced elevations of BP are unaltered by SMET<sub>B</sub> KO. The mechanism underlying increased BP after SMET<sub>B</sub> KO is not apparent but is unlikely to be a consequence of cre overexpression in SM because this did not alter baseline BP in SMET<sub>A</sub> KO mice.<sup>4</sup> Several possible explanations can be proposed. First, ET<sub>B</sub> in VSMC may contribute to the clearance of ET-1 from tissue where it is preferentially secreted by EC, and where it acts. Therefore, SMET<sub>B</sub> KO may cause ET-1 accumulation in the vascular wall, thus increasing ET-1-mediated vasoconstriction. Second, loss of SMET<sub>B</sub> may upregulate ET<sub>A</sub>-mediated contraction. Third, SMET<sub>B</sub> in the kidney may influence sodium homeostasis. Because SM22 may be expressed in perivascular fat precursors,<sup>36</sup> loss of ET<sub>B</sub> from perivascular fat may have caused developmental changes in vascular function that also contribute to elevated BP, but this has not been established. It is also not clear why basal diastolic blood

pressure is selectively increased in the SMET<sub>B</sub> KO, but this would be worthy of future investigation.

Increased BP in SMET<sub>B</sub> KO mice could not be attributed to vascular dysfunction as, with the exception of responses to S6c, we found no evidence of impaired arterial relaxation or contraction. Weak ET<sub>B</sub>-mediated contraction in arteries is consistent with studies in rats.<sup>35</sup> Preliminary investigations (unpublished data) indicated that S6c-induced contraction of freshly isolated murine arteries (femoral, mesenteric, and carotid) was not increased by NO synthase inhibition or by removal of the endothelium. These results indicate that we are not missing an ET<sub>B</sub>-mediated contraction that has been obscured by ET<sub>B</sub>-mediated relaxation. Induction of ET<sub>B</sub>-mediated contraction after incubation has been attributed to transcriptional regulation and MEK-ERK1/2 signaling.<sup>22,38</sup> Abolition of this response in mesenteric arteries from SMET<sub>B</sub> KO mice indicated that they lack both functional arterial ET<sub>B</sub> receptors and the means to generate new receptors in this tissue.

ET<sub>B</sub> upregulation in SMC, mediating vasoconstriction and proliferation in cardiovascular disease,<sup>18,19</sup> might explain studies reporting similar benefit from mixed ET<sub>A/B</sub> and selective ET<sub>A</sub> antagonism in reducing lesion formation<sup>23,39,40</sup> (despite the protective roles of ET<sub>B</sub> in several tissues, eg, EC and kidney). However, the effectiveness of mixed ET<sub>A/B</sub> and selective ET<sub>A</sub> antagonism is likely to depend on the balance of ET<sub>B</sub> receptor activity in EC and VSMC of an affected artery. Transient upregulation of ET<sub>A</sub> and ET<sub>B</sub> receptors has been demonstrated in arterial lesions.<sup>41</sup> If these ET<sub>B</sub> receptors contribute to lesion formation, then ET<sub>B</sub> antagonism would be desirable. There was, however, no evidence of induced ET<sub>B</sub>-mediated contraction in mouse femoral arteries after ligation. Similar investigations could not be performed after wire injury because these vessels fail to contract *ex vivo*. It remains possible that ET<sub>B</sub> upregulation occurs in other (eg, carotid) arteries.

Neointimal lesion formation is increased in rescued global ET<sub>B</sub> knockout mice<sup>10</sup> and in (spotted lethal) rats with global deletion of ET<sub>B</sub>,<sup>24</sup> consistent an antiproliferative role for ET<sub>B</sub> receptors. This is supported by demonstrations that ET<sub>B</sub> receptor antagonism increases lesion size,<sup>9,24</sup> with the suggestion that this is because of impaired ET<sub>B</sub>-mediated release of NO from EC. Indeed, increased lesion formation in mice with global ET<sub>B</sub> deletion was partly attributed to impaired EC-derived NO release.<sup>9</sup> In contrast, selective ECET<sub>B</sub> deletion inhibited ET<sub>B</sub>-mediated relaxation<sup>22</sup> but had no effect on arterial lesion formation.<sup>9</sup> These results suggest, therefore, that the protective role of ET<sub>B</sub> receptors is played by non-ECET<sub>B</sub> receptors. The demonstration here that deletion of ET<sub>B</sub> from the SMC does not alter lesion size indicates that, as with the receptors in EC,<sup>9</sup> ET<sub>B</sub> in SMC do not influence neointimal remodeling. This implicates nonvascular ET<sub>B</sub> receptors, for

example, in monocyte-derived macrophages, in the regulation of neointimal proliferation and atherosclerosis.<sup>42</sup>

In conclusion, we have demonstrated that selective ET<sub>B</sub> receptors in SMC may contribute modestly to regulation of BP but have little influence on vascular contraction or neointimal proliferation. These data suggest that any detrimental role of SMET<sub>B</sub> is minor (at least during normal physiology), and, therefore, that selective ET<sub>A</sub> receptor antagonists (which preserve protective EC/renal ET<sub>B</sub> signaling) should be preferred to mixed ET<sub>A/B</sub> antagonists for treatment of vascular disease.

### Perspectives

Generation of mice with selective deletion of ET<sub>B</sub> from SMC indicates that these receptors contribute to the increased BP induced by ET<sub>B</sub> receptor antagonism but do not regulate arterial function or the fibroproliferative response to acute arterial injury. It would be interesting to determine whether ET<sub>B</sub> in SMCs influence other cardiovascular diseases (eg, diabetic complications). Whether the data generated in these animals are replicated in mice with cardiovascular disease (eg, atherosclerosis), or in man, remains to be established. However, these results support the proposal that selective ET<sub>A</sub> receptor antagonists may have advantages over mixed ET<sub>A/B</sub> antagonists for combatting elevated BP or restenosis after revascularization.

### Acknowledgments

A192621 was a gift from AbbVie, United States.

### Sources of Funding

This work was funded by the British Heart Foundation (Project Grant PG/08/068/25461, P.W.F. Hadoke and D.J. Webb; Intermediate Clinical Research Fellowship FS/13/30/29994, N. Dhaun; and Centre of Research Excellence Award) and the Wellcome Trust (107715/Z/15/Z, A.P. Davenport and R.E. Kuc).

### Disclosures

D.J. Webb has provided advice to Abbott, AbbVie, AstraZeneca, Encysive, Pfizer, Retrophin, and Roche in relation to clinical development of endothelin receptor antagonists. K.M. Duthie received a Pfizer Young Investigator Award. N. Dhaun has received research grants from Pfizer.

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## Novelty and Significance

### What Is New?

- This study describes newly generated mice with selective endothelin<sub>B</sub> (ET<sub>B</sub>) receptor deletion from smooth muscle. This was used to clarify the influence of smooth muscle ET<sub>B</sub> receptors on (1) blood pressure, (2) arterial and venous contraction, and (3) arterial remodeling following injury.

### What Is Relevant?

- Generation of the knockout was necessary because ET<sub>B</sub> receptors in vascular endothelial and smooth muscle cells cannot be distinguished pharmacologically. This work shows that ET<sub>B</sub> receptors in smooth muscle have little influence on arterial function or neointimal remodeling but have a

small suppressive effect on diastolic blood pressure. This is consistent with the proposal that selective endothelin<sub>A</sub> (ET<sub>A</sub>) antagonism would be preferable to mixed ET<sub>A</sub>/ET<sub>B</sub> antagonism for inhibiting arterial remodeling.

### Summary

Selective smooth muscle ET<sub>B</sub> deletion indicated that these receptors play a minor role in regulation of BP but do not affect vascular function or remodeling. This suggests that, beyond endothelial cell ET<sub>B</sub>, ET<sub>B</sub>-dependent regulation of these processes is mediated by receptors in extravascular cells (eg, renal collecting ducts).