Serine Carboxypeptidase SCPEP1 and Cathepsin A Play Complementary Roles in Regulation of Vasoconstriction via Inactivation of Endothelin-1

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Abstract

The potent vasoconstrictor peptides, endothelin 1 (ET-1) and angiotensin II control adaptation of blood vessels to fluctuations of blood pressure. Previously we have shown that the circulating level of ET-1 is regulated through its proteolytic cleavage by secreted serine carboxypeptidase, cathepsin A (CathA). However, genetically-modified mouse expressing catalytically inactive CathA S190A mutant retained about 10–15% of the carboxypeptidase activity against ET-1 in its tissues suggesting a presence of parallel/redundant catabolic pathway(s). In the current work we provide direct evidence that the enzyme, which complements CathA action towards ET-1 is a retinoid-inducible lysosomal serine carboxypeptidase 1 (Scpep1), a CathA homolog with previously unknown biological function. We generated a mouse strain devoid of both CathA and Scpep1 activities (DD mice) and found that in response to high-salt diet and systemic injections of ET-1 these animals showed significantly increased blood pressure as compared to wild type mice or those with single deficiencies of CathA or Scpep1. We also found that the reactivity of mesenteric arteries from DD mice towards ET-1 was significantly higher than that for all other groups of mice. The DD mice had a reduced degradation rate of ET-1 in the blood whereas their cultured arterial vascular smooth muscle cells showed increased ET-1-dependent phosphorylation of myosin light chain 2. Together, our results define the biological role of mammalian serine carboxypeptidase Scpep1 and suggest that Scpep1 and CathA together participate in the control of ET-1 regulation of vascular tone and hemodynamics.

Introduction

Vascular resistance of the mammalian circulation system is tightly regulated by many endogenous agents that influence the blood volume, and diverse functions of endothelium, vascular smooth muscle and myocardium. When the balance of these agents is disturbed, persistent systemic hypertension develops. Short regulatory peptides, endothelin-1 (ET-1) and angiotensin II (AII) are recognized among the most potent vasoactive regulators. Their interaction with cell surface receptors both peptides can modulate blood pressure by contracting arteries, or by induction or suppression of vascular wall remodelling.

ET-1 also has mitogenic effects on vascular endothelium and smooth muscle [1], stimulates the secretion of atrial natriuretic peptide ANP and aldosterone and inhibits the release of renin to counteract its effects [2]. The elevated ET-1 values have been previously observed in human vascular and cardiovascular disorders such as acute myocardial infarction, congestive heart failure, ischemia, atherosclerosis, hypercholesteremia, systemic and pulmonary hypertension [3]. ET-1 deficient mice showed abnormal fetal development and haemodynamics [4], whereas the overexpression of human ET-1 in mice caused vascular remodelling and endothelial dysfunction [5,6].

AII is another potent blood pressure-inducing and mitogenic peptide that belongs to the renin-angiotensin system. It is derived from the precursor, angiotensin I (AI) by angiotensin converting enzymes (ACE or ACE2). Inhibitors of AII receptors, as well as ACE inhibitors normalize the high blood pressure and decrease inward remodelling of arteries [7].

The bioavailability and potency of AII and ET-1 can be regulated through many factors such as alteration of receptor density and affinity, up- and down-regulation of peptide synthesis or release, enzymatic activation (ACE and ACE2 for AII, ECE and MMP-2 for ET-1 [8]), or degradation (neutral endopeptidase NEP for ET-1 [9–11]). Previously we have shown that circulating ET-1 is inactivated by lysosomal carboxypeptidase, cathepsin A...
The amount *Scpep1* mRNA measured by RT-q-PCR in aorta, hear and kidney tissues of *Scpep1−/−* and *CathA+/−*/*Scpep1−/−* mice (Fig. S2) was below detection limit. Carboxypeptidase activity against ET-1 assayed in cultured AVSMC of *CathA+/−*/*Scpep1−/−* mice was reduced to ~10% of activity in WT mice whereas the activity in tissues of *Scpep1−/−* mice was reduced to ~70% (Fig. 1A). In the *CathA+/−*/*Scpep1−/−* mice carboxypeptidase activity was ~60% of WT and significantly lower than that in *CathA+/−*/*Scpep1−/−* mice, indicating that *Scpep1* partially contributes to ET-1 hydrolysis (Fig. 1A). The activity of *Scpep1* against ET-1 was further confirmed when the AVSMC of *CathA+/−*/*Scpep1−/−* mice were transiently transfected with *Scpep1*-expressing plasmid [19]. The level of carboxypeptidase activity measured with ET-1 in the transfected cells was significantly higher than in non-transfected cells or cells transfected with control plasmid, coding for green fluorescent protein (Fig. 1A) and similar to that in the cells of *CathA+/−*/*Scpep1−/−* mice, despite the modest transfection level of ~5% that could be achieved in the primary AVSMC cultures. In contrast when we transfected AVSMC from WT mice with a lentiviral vector expressing shRNA for *Scpep1* the carboxypeptidase activity against ET-1 in the cell homogenate in the transfected cells was reduced by ~50%, consistent with that in the AVSMC from *Scpep1−/−* mice. In the cells transfected with CathA shRNA-expressing vector the activity was decreased by ~90% and in the cells transfected with scrambled RNA constructs, not changed (Fig. 1B).

Finally, to test directly if *Scpep1* has carboxypeptidase activity against ET-1 we have expressed the protein, carrying a His6 tag at the C-terminus (*Scpep1-His6*) in HT1080 cells [19]. The secreted protein was purified until electrophoretic homogeneity by affinity chromatography on Ni-NTA resin followed by anion-exchange chromatography on Poros HQ resin (Fig. S3) and its carboxypeptidase activity was assayed as above with 50 µM ET-1 as a substrate. We found that at pH 5.5 purified *Scpep1-His6* was capable of cleaving the C-terminal Trp residue from ET-1 at a rate of 23.6 ± 2.6 µmol/h per mg of protein (Fig. 1C), i.e. close to that of purified CathA [20]. Lower activity was observed at higher pH of 6.5 and 7.5 (Fig. 1C).

**Scpep1 deficiency contributes to a further increase of blood pressure in CathA-deficient mice**

The heart rate (HR) and blood pressure (BP) in WT, *CathA+/−*, *Scpep1−/−* and double-mutant *CathA+/−*/*Scpep1−/−* male mice was measured by radiotelemetry over a 3-day period. Then, the mice were challenged by a high salt diet (8% NaCl for 2 weeks) with continuous measurement of BP and HR.

The day (Fig. 2A, C) and night (Fig. 2B, D) levels of systolic BP (SBP) were significantly increased in *CathA+/−*/*Scpep1−/−* animals as compared with WT both before and during a high-salt diet, whereas the BP levels in *Scpep1−/−* animals were similar to that of WT. Night SBP in *CathA+/−*/*Scpep1−/−* mice was significantly different from that of WT, *Scpep1-deficient* and CathA-deficient animals (Fig. 2 and Fig. S4). The HR values (Fig. S5) and the parameters characterizing kidney function (water intake, urine volume, urine sodium and urine creatinine levels, Fig. S6) were similar for all strains suggesting that the observed increase in SBP in DD mice relates to a vascular effect reflecting potential roles of *Scpep1* and CathA in conversion of vasoconstrictive peptides. This hypothesis was further tested by measuring changes in BP in response to ET-1 and AII, the precursor of vasoconstrictive peptide AII. The changes in the diastolic and systolic BP (ΔDBP and ΔSBP, respectively) were calculated as the differences between the measured BP values and the basal values measured for the 30 min interval preceding the injection. To reduce the impact of the stress on BP caused by animal handling/injection, animals were

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**Author Summary**

Arterial blood pressure is regulated by small peptide hormones (vasoactive peptides) that cause contraction or relaxation of the arterial wall. The blood and tissue levels of these peptides are controlled by two mechanisms: through their synthesis and through their inactivation by the enzymes that are capable of cleaving them. Our results demonstrate that vasoactive peptide endothelin-1, is inactivated by two homologous enzymes, lysosomal serine carboxypeptidase, cathepsin A and lysosomal serine carboxypeptidase 1. We have developed a mutant strain of mice that do not produce both enzymes and found that these mice rapidly develop high blood pressure and show a reduced degradation rate of endothelin-1. We also found that endothelin-1 causes higher contraction of arteries from mutant than from normal mice or mice that are deficient only in one of the two enzymes. Our mouse model provides insight into the functional engagement of lysosomal serine carboxypeptidases in pathophysiology of hypertension and may become a tool to explore whether induction of these enzymes would have any therapeutic value.
receiving daily saline injections for 3 days prior to the experiment. The data (Fig. 3) indicate that the BP response to the i.v. injections of ET-1 was significantly (p < 0.0001) dependent on animal genotype. The effect of ET-1 in Scpep-1-deficient mice was not significantly different from that in WT animals; there was no BP increase in response to the low (0.2 nmol/kg) dose of ET-1 and similar increase in response to the high (5 nmol/kg) dose. While CathA-deficient animals showed a higher response to ET-1 at the
Scpep1 deficiency increases the vasoconstrictive response of mesenteric arteries to ET-1 but not to AI

The vasoactivity of mesenteric arteries from the four groups of male mice was directly measured in ex vivo tests. Isolated arteries were exposed to ET-1 and the precursor of AII, AI as well as to known vasodilators (acetylcholine, ACh and sodium nitroprusside, SNP) and vasoconstrictors (norepinephrine, NE). We observed no differences in vessel reactivity in response to ACh, SNP or NE (Fig. 4A, B, C) as well as to AI (Fig. S8) between the four groups of mice. Reactivity to ET-1 was higher for CathA<sup>S190A</sup> and Scpep<sup>1</sup>/− mice than for WT mice (Fig. 4D). The reactivity of vessels from CathA<sup>S190A</sup>/Scpep<sup>1</sup>/− mice to ET-1 was significantly higher than that for all other groups of mice consistent with the in vivo data showing bigger increase of BP in CathA<sup>S190A</sup>/Scpep<sup>1</sup>/− mice in response to ET-1 (Fig. 4D).

Cultured AVSMC of CathA<sup>S190A</sup>/Scpep<sup>1</sup>/− mice show increased phosphorylation of myosin light chain

To verify at the molecular level if AVSMC from CathA<sup>S190A</sup>/Scpep<sup>1</sup>/− mice have increased reactivity to ET-1, we studied intracellular signalling events in these cells in response to ET-1. ET-1 interacts with G-protein-associated endothelin type A (ETR-A) and type B (ETR-B) receptors on the surface of AVSMC. Activation of the receptors induces phospholipase C and increases the intracellular Ca<sup>2+</sup> level leading to activation of myosin light chain kinase that phosphorylates myosin light chain (MLC) [21–24]. This causes contraction of myosin filaments and shrinkage of the cells. We therefore, compared the level of MLC phosphorylation in AVSMC before or after treatment with ET-1 for the 4 strains of mice. AVSMC cultured overnight in serum-free medium were treated with or without 100 nM ET-1, harvested and analyzed by Western blot using antibodies against MLC-2 phosphorylated at Thr<sup>18</sup> and Ser<sup>19</sup> residues or against total MLC protein. MLC-2 phosphorylation was blocked by pre-treatment of the cells with the low dose as compared to WT or Scpep1-deficient animals only, DD animals showed a higher response as compared to all other groups of mice (Fig. 3A, B). After injection of ET-1 at the high dose the BP in DD mice remained significantly elevated for at least 60 min, whereas in WT or single knockout mice it decreased already after 40 min (Fig. 3C, D). The effect of AI on BP was similar in all animal groups (Fig. S7).
known pharmacological antagonist of ETR-A, BQ610, and ETR-B antagonist, BQ788, suggesting that this effect is dependent on ET-1 action on its receptors (Fig. 5A). The cells from CathAS190A/Scpep1−/− mice had significantly higher level of pThr18/pSer19-MLC-2, 2 times higher than that in the control, CathA-deficient or Scpep1-deficient cells, and elevated basal levels of MLC-2 phosphorylation (Fig. 5B).

To determine the ET-1 degradation rate we injected mice in the tail vein with an ET-1 solution in saline at a dose of 0.1 nmol/kg BW. Fifteen minutes after injection mice were sacrificed and their lungs and aorta as well as blood were collected to measure the concentration of ET-1 by ELISA. Endogenous levels of ET-1 were

**Figure 3.** Mice with combined CathA/Scpep1 deficiency show higher increase in BP in response to systemic injection of ET-1. Sixteen week-old WT, Scpep1−/−, CathAS190A and DD male mice kept for two weeks on high-salt diet were intravenously injected with ET-1 solution in saline at a dose of 0.2 nmol/kg (A, B) and 5 nmol/kg (C, D). Changes in the systolic (ΔSBP) or diastolic (ΔDBP) blood pressure were calculated as differences between the means (±S.E) of the BP values recorded within 10 min intervals after the injections and means (±S.E) of the baseline BP values recorded within the 30 min interval before the injections. Two-way repeated measurements ANOVA was used to test differences between the mouse groups: significant differences between the mean BP values in Bonferroni post-test (* p<0.05, ** p<0.001, *** p<0.0001) are shown in the insert. N-value for each genotype is as follows: WT n = 5, DD n = 6, CathA190A n = 6, Scpep1−/− n = 7.

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CathAS190A/Scpep1−/− mice demonstrate elevated levels of plasma ET-1

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measured in the animals injected with saline. Our data show that, 15 min after the ET-1 injection, its concentration in lungs (Fig. 6A) and aorta (Fig. 6B) of CathA-deficient mice was higher than that in the WT or Scpep1-deficient animals thus confirming our previous findings about the involvement of this enzyme in the ET-1 degradation. In tissues or plasma of DD mice the concentration of ET-1 was significantly higher than that in WT, CathA-deficient or Scpep1-deficient mice suggesting that in CathAS190A/Scpep1<sup>−/−</sup> mice the degradation rate of ET-1 is considerably reduced. No differences in endogenous circulating levels of ET-1 (Fig. 6D) were recorded.

**Discussion**

Scpep1 was originally identified in rat aortic smooth muscle cells by screening for retinoid inducible genes [25]. Retinoids, natural and synthetic derivatives of vitamin A, block SMC proliferation and attenuate neointimal formation after vascular injury, presumably through retinoid receptor-mediated changes in gene expression. High transcript levels of Scpep1 were detected in kidney, lungs and heart. Scpep1 was localized to lysosomes by immunofluorescence, subcellular fractionation assays and mannose 6-phosphate receptor binding [19,26].
Scpep1 shows high similarity to other members of the serine carboxypeptidase family and, in particular, to CathA. Like CathA, Scpep1 has a cleavable signal peptide, N-linked glycans, the Ser-Asp-His catalytic triad and is proteolytically processed from a 55 kDa precursor into the 35 kDa and 18 kDa fragments [19]. Scpep1 gene-interrupted mice generated by us using a gene trap technology are fertile, have normal growth, normal clinical blood and urine parameters and did not have pathological changes in any tissue examined [19]. Later study by Lee at al. [27] reported that the Scpep1-null mice generated by replacing exons 1 and 2 of the Scpep1 gene with Neo cassette show a decrease in medial and intimal cell proliferation as well as in vessel remodelling following arterial injury. The same study also reported that a 50% knockdown of endogenous Scpep1 in mouse ASMC line showed dramatic decrease in serum-stimulated growth. This study did not identify a physiological substrate of Scpep1, but the authors concluded that Scpep1 and CathA have distinct functions and “non-overlapping pools of substrates that function in cardiovascular homeostasis”.

Our current data, however, provide evidence that both carboxypeptidases catabolize at least one common substrate, ET-1. Mice devoid of both CathA and Scpep1 activities show significantly higher BP on both normal and high-salt diet or in response to systemic injections of ET-1 as compared to WT mice or those with single deficiencies of CathA or Scpep1. ET-1 also causes higher constriction of mesenteric arteries from DD mice. Since the effects of other tested vasodilators and vasoconstrictors are similar, these results are consistent with increased sensitivity of arterial smooth muscle to ET-1. Indeed, in cultured AVSMC from DD mice ET-1 caused significantly increased phosphorylation of MLC-2 as compared with the control, CathA-deficient or Scpep1-deficient cells. Finally, the degradation rate of ET-1 in the blood plasma or aorta and lung tissues was significantly reduced in DD as compared to WT, CathA-deficient or Scpep1-deficient mice. This contradicts previously proposed role of CathA in the generation of AII from AI [15–17] and suggests that in general ET-1 and AII are controlled by different sets of proteases. We cannot exclude, however, that CathA still may participate in AII regulation in specific tissues, such as heart atrium, where the rate of AI conversion to A1–9 by CathA constitutes ~25% of that to AII by ACE [17].

Our data indicate that in mouse tissues CathA is sufficient for inactivation of ET-1, which justifies the apparent absence of

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**Figure 5. Cultured AVSMC from mice with combined CathA/Scpep1 deficiency show increased reactivity to ET-1.**

A. Pharmacological antagonists of ET receptors, BQ610 and BQ788, reduce MLC-2 phosphorylation in AVSMC treated with ET-1. Cultured AVSMC from WT mice were treated or not for 30 min with 2 μM BQ610 or BQ788 followed by 5 min induction with 100 nM ET-1 as indicated on the figure. Total protein extracts were analyzed by Western blotting using antibodies specific for phosphorylated (pThr18/Ser19-MLC) and total MLC-2 protein. Panel shows representative data of 3 independent experiments. Right panels show ratios (means and S.D.) of signal intensities for phosphorylated and total MLC-2 estimated with ImageQuant software. *p<0.05 in paired two-tailed t-test. B. Increased MLC-2 phosphorylation in AVSMC from double-deficient mice. Cultured AVSMC from WT, Scpep1−/−, CathA5190A and DD mice were treated for 5 min with 100 nM ET-1. Total protein extracts were analyzed by Western blotting using antibodies specific for phosphorylated and total MLC-2 protein. Panel shows representative data of 3 independent experiments. Graph below the panel shows ratios (mean values and S.D.) of signal intensities for phosphorylated and total MLC-2 protein. * p<0.05 in paired two-tailed t-test.

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phenotype in our line of Scpep1 KO mice. In contrast, Scpep1 activity is unable to fully compensate for the loss in CathA activity in the knock-in CathA-deficient mice that show elevated blood pressure [18]. In the absence of CathA, the Scpep1 activity becomes essential for degradation of ET-1 as demonstrated by induced BP and contractility of arteries in DD as compared to singe CathA KI mice. Importantly, CathA has also other functions non-overlapping with those of Scpep1, such as activation of sialidase Neu1 in the lysosome [12], regulation of elastogenesis through its function in elastin-binding protein complex [28,29] and inactivation of bradykinin [30]. Intravenous bolus injections of potent specific CathA inhibitors induced bradykinin-dependent diuresis [30], however in our experiments we did not see a difference in the urine volume between WT and CathA-deficient mice. One possible explanation is that CathA KI animals could adapt to deficiency of CathA by reducing bradykinin production or the number of bradykinin receptors.

The expression of Scpep1 in cardiovascular tissues can be effectively induced by retinoic acid, potentially providing a metabolic bypath to correct arterial hypertension attributed to a deficiency in ET-1 degradation in galactosialidosis patients with mutations in the CATHA gene [31,32]. Interestingly all-trans retinoic acid has been shown to inhibit pulmonary hypertension induced by monocrotaline in rats [33], whereas human patients with idiopathic pulmonary arterial hypertension were shown to have reduced retinoic acid levels [34]. The anti-hypertensive effect of retinoic acid treatment was attributed to its ability to elicit growth-inhibitory signals in pulmonary artery smooth muscle cells and influence pulmonary vascular remodelling [34–36], while our current data allow to propose that it may be also related to the induction of Scpep1 followed by increased degradation of ET-1. Together, our results define a biological role of Scpep1 protein, and suggest that Scpep1 and CathA participate together in the control of ET-1 regulation of vascular tone and hemodynamics.

Methods

Animals

Generation of mice containing Ser190Ala point mutation in the CathA active site (CathA<sup>190Ala</sup> strain) and those with the Scpep1 gene interrupted by gene-trap technology (Scpep1<sup>−/−</sup> strain) have been described [18,19]. In the Scpep1 gene-trap mouse β-galactosidase/neomycin phosphotransferase (geo) fusion gene was inserted into intron 7 of the Scpep1 gene resulting in deletion of downstream exons 8–13 encoding in particular the putative catalytic triad amino acids, Asp571 and His431 from the gene trap transcript.
The amount of Scpep1 mRNA and protein measured by Northern and Western blots in liver, kidney, heart, brain sphen and lung tissues of Scpep1+/− mice [19] as well as the amount Scpep1 mRNA measured by RT-q-PCR in aorta, heart and kidney tissues (Fig. S2) was reduced below detection threshold of the methods.

Both strains were back-crossed for at least 5 generations to C57BL/6Ncrl strain distributed by Charles River (QC, Canada). Homozygous animals from each genotype were cross-bred to obtain the Scpep1-deficient, CathA-deficient, double-mutant and wild type mice. Mice were housed in an enriched environment with continuous access to food and water, under constant temperature and humidity, on a 12 h light/dark cycle. Approval for the animal care and the use in the experiments was granted by the Animal Care and Use Committee of the St-Justine Hospital Research Center.

Genotyping of mice

50 µl of PCR mixture contained 100 pmol of each primer, 0.2 mM dNTPs, 1.5 U taq polymerase (Feldan, 9K-001-0002) and 50 mM KCl, and 1.5 mM MgCl2. Multiplex primers for detection of Scpep1 alleles were 5'-ATGCCTACAGATG-GAAAGCA (Scpep1-F), 5'-TATTGGCTGAGTGGAGAC (Scpep1-R) and 5'-CCGTCCTCCAGACAAATG (Scpep1-trap) and for detection of CathA alleles, 5'-GTTGGCGGAGAACAATTATG (Catha-F) and 5'-AACAGAAAGTGGCACCCCT-GAC (Catha-R). For Scpep1 allele genotyping, samples were denatured at 94°C for 2 min, followed by 35 cycles at 94°C for 15 s, 52°C for 15 s and 72°C for 1 min, with a final extension reaction at 72°C for 30 s. For CathA allele genotyping, samples were denatured at 92°C for 5 min, followed by 30 cycles at 92°C for 30 s, 56°C for 30 s and 72°C for 30 s, with a final extension reaction at 72°C for 5 min. Then the amplification product was digested with NdeI (Biolabs, R0111S) at 37°C overnight.

Quantitative RT-PCR

Total RNA was isolated from mouse tissues using the Trizol Reagent (Invitrogen 15596-026) according to the manufacturer’s protocol and reverse-transcribed using random primers and QuantiTect Reverse Transcription Kit (QIAGEN 205311). Quantification of mouse Scpep1 mRNA was performed using an SsoFast EvaGreen Supermix with Low ROX (BIO-RAD 172-5101) and the following set of primers: 5'-CATTAAGTGC-3' and 5'-GCTGAGTGGCCTCCTTGTAG (Scpep1-R), and 5'-GCTGAGTGGCCTCCTTGTAG (Scpep1-trap) for detection of Scpep1 alleles and 5'-GGTGGCGGAGAACAATTATG (Catha-F) and 5'-AACAGAAAGTGGCACCCCT-GAC (Catha-R) for Scpep1 allele genotyping. The transcript was normalized to the expression of RPL32 mRNA. All experiments were conducted in triplicate.

Blood pressure measurements by radiemetry

Male Cathk1/−/− mice and appropriate littermate controls were implanted with TA11PA-C10 radiemetry sensors (Data Sciences International) in the left carotid artery for direct measurement of arterial pressure and heart rate as described [37,38]. The transmitter was placed subcutaneously along the left flank. For basal measurements of mean day and night BP data were recorded continuously (sampling every hour for 20 sec) within 16 consecutive days and averaged for 12 h light and dark intervals. To measure changes in BP after ET-1 and AI injections data were recorded every 3 min for 2 h and averaged for 10-min consecutive intervals. At least 7 mice were studied for each genotype with the exception of WT mice for which only 5 mice were tested due to sudden death of 2 animals.

Vessel reactivity study

Vessel reactivity ex vivo was analyzed as described [39,40]. Briefly, male mice were sacrificed at five months and their mesenteric arteries were isolated and mounted onto glass capillaries in an arterograph chamber filled with cold oxygenated Krebs solution (118.6 mM NaCl, 4.7 mM KCl, 1.2 mM KH2PO4, 1.2 mM MgSO4, 25.1 mM NaHCO3, 26 µM EDTA, 0.18% glucose, 2.5 mM CaCl2). The arteries were constantly perfused intraluminaly with Krebs solution at 30 mmHg. After 45 minutes of equilibration vascular reactivity was measured in response to Norepinephrine (Sigma A-0937, 10−9–10−5 M), Acetylcholine (Sigma A-6623, 10−9–10−4 M), Sodium Nitroprusside (Calbiochem 56538, 10−9–10−4 M), Endothelin-1 (American Peptide Company 98-1-10, 10−11–10−6 M) and AI, (American Peptide Company 12-1-10, 10−8–10−4 M). Drugs were added extraluminally with a 30 min washout period in between each drug, during which the arteries were able to re-equilibrate to a baseline. To test vasodilatation arteries were pre-contracted with NE to 70% of their equilibration diameter. At least 3 concentration response curves were conducted for each vessel and at least 6 animals were studied for each genotype.

Isolation and culture of aortic vascular smooth muscle cells (AVSMC)

Combined tissues from 5–6 mouse aortas were minced in a DMEM containing collagenase type I (GIBCO, 17100-017, 5 mg/ml), trypsin (Sigma T-1426, 0.5 mg/ml), and DNase type I (Sigma D-4263, 20 µg/ml), incubated at 37°C for 2 h, and centrifuged for 5 min at 1000 g. The cells were resuspended in 10 ml of DMEM containing 10%FBS, 1% Antibiotic-Antimycotic (GIBCO 15240-062), 0.5% Fungizone (GIBCO 15290-018) and maintained in 5% CO2 incubator at 37°C. The medium was changed every three days. After 3 passages 100% of cell colonies were positive to VSMC marker, smooth muscle α-actin as assayed by FACS with A 2547 antibody (Sigma).

Purification of recombinant mouse Scpep1-His6 from HT1080 cells

HT1080 cells stably expressing Scpep1-His6 [19] were cultured in DMEM with 0.05% FBS. Medium was collected three times every 48 h and subjected to ammonium sulfate precipitation. After dialysis against PBS, the Scpep1-His6 was purified by metal affinity chromatography on Ni-NTA agarose (Qiagen) as recommended by the manufacturer. The eluate was dialyzed against PBS and subjected to HPLC anion exchange chromatography (BiocadVision, Applied Biosystems) by applying a step-wise gradient up to 500 mM NaCl in PBS. Purity of Scpep1-His6 was monitored by silver staining and Western blotting.

Carboxypeptidase activity assays

Carboxypeptidase activity in cultured AVSMC was measured against 50 µM ET-1 as previously described using the method measuring the liberation rate of the C-terminal amino acid of the peptide [18]. Subconfluent AVSMC were transiently transfected or not with Scpep1-RGS-His-Tag [19] and pEGFP-C1 (Clontech, Palo Alto, CA) plasmids, mouse CTSA shRNA (TF501716B/C) Scpep1 shRNA (TF505007A/B) or non-effective 29-mer scrambled shRNA (TR30015) cassette in pRFP-C-RS vector (Origene Technologies) using Effectene transfection reagent (Qiagen) at a ratio of 25 µl of Effectene to 1 µg of DNA. Forty eight hours after transfection (72 h for shRNA constructs) confluent cells were harvested, homogenized in water by sonication and 50 µl of cell homogenate was mixed with 100 µl of 0.1 mM ET-1 solution and measured by silver staining and Western blotting.
50 μl of 100 mM sodium acetate buffer, pH 5.4, and incubated for 30–180 min at 37°C. After addition of trichloroacetic acid (Sigma T0899, 3% final concentration) proteins were removed by 5 min centrifugation at 12,000 g. The 190 μl aliquot of supernatant was mixed with 3 ml of 50 mM sodium borate buffer, pH 9.5, containing 0.15 mg/ml of phthalic aldehyde and 1 mM of betamercaptoethanol (Sigma, M-6250) and incubated at room temperature for 30 min. The fluorescence was measured at 340 nm excitation and 495 emission wavelength and concentration of released amino acids determined using a calibration curve established with 1–100 μM leucine. Carboxypeptidase activity of recombinant Scpep1-His6 was measured by the same method using 0.4–0.8 μg of the purified enzyme.

Analysis of myosin light chain 2 phosphorylation by western blot

AVSMC cultured in 100 mm dishes to confluent layer were incubated overnight in a serum-free DMEM, and treated for 5 min with 100 nM ET-1. To test the pharmacological inhibition of the ET-1 receptors the cells were pre-treated for 30 min with 2 μM BQ610 (EMD 203715) or BQ788 (EMD 3223838) before stimulation with ET-1. The cells were washed with ice-cold PBS, and lysed in RIPA (RadioImmunoPrecipitation Assay) buffer containing 50 mM Tris HCl, pH 7.4, 150 mM NaCl, 1% NP-40, 0.25% sodium deoxycholate, 0.1% SDS, 2 mM EDTA, 1 mM PMSF, protease and phosphatase inhibitor cocktails (Roche 04939132001 and 0490637001). Cell lysates were analyzed by Western blot using anti-phospho-Thr18/Ser19 myosin light chain 2 antibody (Cell Signalling 3033S), and the enhanced chemiluminescence reagent (Thermo 32106).

Detection was performed with anti-rabbit IgG antibodies-HRP antibody (Cell Signalling 7074S), and the enhanced chemiluminescence reagent (Thermo 32106).

Measurement of ET-1 degradation rate in mouse blood and tissues

Three to four month old mice with 25–35 g body weight (BW) were anesthetised with urethane (1.5 g/kg BW) and injected into the tail vein with a solution of ET-1 in saline at a dose of 10 nmol/kg BW. Fifteen minutes post-injection, blood was collected in EDTA-coated tubes through cardiac puncture and immediately centrifuged to separate plasma. Aortas and lungs were dissected and rapidly frozen in liquid nitrogen.

For peptide extraction, tissues (200 mg) were homogenized in 1 ml/L CH3COOH/20 mM HCl. Plasma was supplemented with concentrated CH3COOH until the final concentration of 1 mol/L. Samples were boiled for 10 minutes and centrifuged at 20,000 g for 10 minutes. Supernatant was applied to a Strata C18-E column (Phenomenex, RK-Sepcol-1), washed with 3 volumes of 0.1% TFA in water, and peptides were eluted with 60% acetonitrile/0.1% TFA, lyophilized, and reconstituted in 0.1% TFA in DMSO. Quantitative assay of ET-1 was performed with an ELISA kit (Enzo Life Sciences ADI-900-020A) as described by the manufacturer.

Statistical analysis

Statistical analysis has been performed using two-tailed paired t-test (Fig. 1, and 4), Welch’s modification of two-tailed unpaired t-test (Fig. 5, S2 and S8) and two-way repeated measures ANOVA (Fig. 2, 3, and 4) tests using Prism Graphpad software. P-value of 0.05 or less was considered significant. Bonferroni post-hoc test was used to compare specific means, if significance was determined.

Statement of responsibility

The authors had full access to the data and take responsibility for its integrity. All authors have read and agreed to the manuscript as written.

Supporting Information

Figure S1 Genotyping of WT, Scpep1−/−, CathA1936A, and double-deficient (DD) mice by PCR analysis of tail genomic DNA. (A) Scpep1 allele-specific PCR amplifying a 200 bp fragment in wild type (WT) mice and 390 bp fragment in homozygous Scpep1-deficient animals (Scpep1−/−). (B) CathA allele-specific PCR followed by NdeI digestion produces a 350 bp fragment in wild type (WT) mice, and a 250 and 100 bp fragments in homozygous CathA-deficient animals (CathA1936A).

Figure S2 Scpep1 mRNA expression in mouse tissues. (A) Schematic representation of WT and Scpep1−/− mRNA showing the positions of primers for qPCR in Exxon 6 and Exxon 8. (B) Scpep1 relative mRNA expression in heart, kidney and aorta tissues. Total RNA was extracted from tissues of 16 week-old WT, Scpep1−/−, CathA1936A and double-deficient (DD) mice and analyzed for Scpep1 expression in different tissues by qPCR. The values were corrected for the level of control RPL23 mRNA.

Figure S3 Purification of recombinant mouse Scpep1-His6 from stably expressing HT1080 cells. Scpep1-His6 expressing HT1080 cells (Kollmann et al. 2009, FEBS Journal) were cultured in 0.05% FCS in DMEM. Medium was collected three times every 48 h and subjected to ammonium sulfate precipitation. After dialysis to PBS, the Scpep1-His6 was purified by Ni-NTA agarose (Qagen). The eluate was dialyzed to PBS and subjected to HPLC anion exchange chromatography (BiocadVision, Applied Biosystems) by applying a step-wise gradient up to 500 mM NaCl in PBS. Purification was monitored by silver staining and Western blotting. Fractions 9 and 10 were pooled and used for the assay of carboxypeptidase activity.

Figure S4 Mice with combined CathA/Scpep1 deficiency show significantly higher values of SBP. Diastolic (A) and systolic (B) blood pressure was recorded continuously (1 measurement per hour) during day and night 12-h periods in 16 week-old WT, Scpep1−/−, CathA1936A and DD male mice. Arrows indicate commencement of high salt diet. Two-way repeated measures ANOVA was used to test differences between the mouse groups: significant differences between the mean BP values in Bonferroni post-test (* p<0.05, ** p<0.001, *** p<0.0001) are shown in the insert. N-value for each genotype is as follows: WT n = 5, DD n = 6, CathA1936A n = 6, Scpep1−/− n = 7.

Figure S5 No significant differences were observed between heart rate in WT, Scpep1−/−, CathA1936A and double-deficient (DD) mice. Heart rate was recorded continuously (once each hour) during night (A) and day (B) 12-h periods in 16 week-old WT, Scpep1−/−, CathA1936A and double-deficient (DD) mice fed for three days with normal diet, followed by two weeks on high salt diet. Arrows indicate commencement of high salt diet. Two-way ANOVA was used to test differences between the mouse groups. N-value for each genotype is as follows: WT n = 5, DD n = 6, CathA1936A n = 6, Scpep1−/− n = 7.

Figure S6 No significant differences in kidney function were observed between WT, Scpep1−/−, CathA1936A and double-deficient
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WT n = 5, DD n = 6, between the mouse groups. N-value of each genotype is as follows: WT n = 5, DD n = 6, CathA190D n = 6, Scpep12/2 n = 7.

Figure S7 No significant difference was observed in the AI-induced constriction of mesenteric arteries between CathA190D, double deficient (DD) and WT mice. Mesenteric arteries isolated from sixteen week-old male WT, Scpep12/2, CathA190D and double deficient mice were mounted onto glass capillaries in an artereograph chamber filled with cold oxygenated Krebs solution and treated with increasing concentrations of AI. Two-way repeated measurements ANOVA was used to test differences between the mouse groups. N-value of each genotype is as follows: WT n = 8, DD n = 8, CathA190D n = 6, Scpep12/2 n = 6.

(PDF)

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Author Contributions

Conceived and designed the experiments: JT JLL AH TL AVP. Performed the experiments: XP LG VS JP KK. Analyzed the data: XP LG JP JLL TL AH AVP. Contributed reagents/materials/analysis tools: TL JT JLL AVP. Wrote the paper: XP LG AVP.

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