Endothelium-Derived C-Type Natriuretic Peptide Contributes to Blood Pressure Regulation by Maintaining Endothelial Integrity

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Abstract—We previously reported the secretion of C-type natriuretic peptide (CNP) from vascular endothelial cells and proposed the existence of a vascular natriuretic peptide system composed of endothelial CNP and smooth muscle guanylyl cyclase-B (GC-B), the CNP receptor, and involved in the regulation of vascular tone, remodeling, and regeneration. In this study, we assessed the functional significance of this system in the regulation of blood pressure in vivo using vascular endothelial cell–specific CNP knockout and vascular smooth muscle cell–specific GC-B knockout mice. These mice showed neither the skeletal abnormality nor the early mortality observed in systemic CNP or GC-B knockout mice. Endothelial cell–specific CNP knockout mice exhibited significantly increased blood pressures and an enhanced acute hypertensive response to nitric oxide synthase inhibition. Acetylcholine-induced, endothelium-dependent vasorelaxation was impaired in rings of mesenteric artery isolated from endothelial cell–specific CNP knockout mice. In addition, endothelin-1 gene expression was enhanced in pulmonary vascular endothelial cells from endothelial cell–specific CNP knockout mice, which also showed significantly higher plasma endothelin-1 concentrations and a greater reduction in blood pressure in response to an endothelin receptor antagonist than their control littermates. By contrast, vascular smooth muscle cell–specific GC-B knockout mice exhibited blood pressures similar to control mice, and acetylcholine-induced vasorelaxation was preserved in their isolated mesenteric arteries. Nonetheless, CNP-induced acute vasorelaxation was nearly completely abolished in mesenteric arteries from vascular smooth muscle cell–specific GC-B knockout mice. These results demonstrate that endothelium-derived CNP contributes to the chronic regulation of vascular tone and systemic blood pressure by maintaining endothelial function independently of vascular smooth muscle GC-B.

Key Words: blood pressure ■ C-type natriuretic peptide ■ endothelial function ■ endothelin ■ endothelium ■ guanylyl cyclase-B

The natriuretic peptide system is composed of 3 endogenous ligands, atrial natriuretic peptide (ANP), brain natriuretic peptide, and C-type natriuretic peptide (CNP), and 3 receptors, particulate guanylyl cyclase-A (GC-A, also called natriuretic peptide receptor [NPR]-A), particulate guanylyl cyclase-B (GC-B, also called NPR-B), and the clearance receptor (NPR-C).1 GC-A preferentially binds ANP and brain natriuretic peptide, whereas GC-B is selective for CNP.2 Both GC-A and GC-B catalyze the conversion of guanosine-5’-triphosphate to cyclic guanosine monophosphate. The clearance receptor, which lacks a guanylyl cyclase domain, has been implicated in the metabolic clearance of natriuretic peptides3 and is also reportedly coupled to Gi-dependent signaling in certain tissues.4,5 Based on its widespread distribution and relatively low plasma concentration, CNP is thought to function as a neurocrine/paracrine/autocrine regulator, especially in the brain,6 the growth plates of bones,7,8 and cardiovascular tissues.9-11 In earlier studies, we demonstrated that CNP is secreted from
vascular endothelial cells and proposed the existence of a vascular natriuretic peptide system consisting of endothelial CNP and smooth muscle GC-B, which could potentially contribute to the regulation of vascular tone, remodeling, and regeneration.3,10,12–14 To further clarify the physiological function of CNP in the vasculature in vivo, we previously generated mice lacking CNP; however, these CNP knockout mice presented marked skeletal dysplasia because of defective endochondral bone formation during development and a high perinatal mortality rate. These characteristics limited the usefulness of systemic CNP knockout mice for assessing the functional significance of CNP in the adult vasculature.15

For the present study, therefore, we used the Cre/loxP system to generate vascular endothelial cell–specific CNP knockout (CNP ecKO) mice and vascular smooth muscle cell (VSMC)–specific GC-B knockout (GC-B smcKO) mice. These conditional knockout mice showed neither the obvious skeletal abnormalities nor the perinatal mortality observed in systemic CNP or GC-B knockout mice. Using these animals, we were able to assess the function of endothelial cell–derived CNP and identify a novel role played by CNP in the chronic regulation of blood pressure in vivo. In addition, our findings raise the possibility that the long-term effects of CNP on blood pressure are mediated through modification of endothelial cell function and are independent of GC-B signaling in VSMCs.

Methods
A detailed description of the materials and methods is available in the online-only Data Supplement.

Animal Experiments
The protocol used to generate conditional knockout mice are presented below. The animal care and all experimental protocols were reviewed and approved by the Animal Research Committee at Kyoto University Graduate School of Medicine. Animal studies were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All mice used in this study had a C57BL/6J background. For the isolation and analysis of organs or blood samples, mice were anesthetized with 3.0% isoflurane and euthanized by cervical dislocation. Unless indicated otherwise, all data were obtained from female mice.

Generation of CNP ecKO and GC-B smcKO Mice
Details of the protocol used to generate Nppc-floxed heterozygous (Nppclox/+; Npr2lox/+; Tie2Cre) mice were described previously.3 Nppclox/+ and Npr2lox/+ mice were further bred to homozygosity. CNP ecKO mice were then obtained by breeding Nppclox/+ mice with heterozygous Tie2-cre Tg mice purchased from the Jackson Laboratory. GC-B smcKO mice were produced by breeding Npr2lox/+ mice with heterozygous sm22α-Cre knockin mice also purchased from the Jackson Laboratory.

Blood Pressure Measurement and Telemetry
Blood pressures were measured using blood pressure transmitters (Data Sciences International, TA11PA-C10) implanted in 13- to 16-week-old mice. After anesthetizing mice with 3.0% isoflurane, a radiotransmitter probe catheter was implanted in the aorta of each mouse via the left carotid artery. The mice were then housed individually in cages, where they were able to move about freely. The cages were situated above telemetric receivers with an output to a computer. The mice were maintained on a 12-hour light–dark cycle and were fed normal chow (0.19% sodium, F-2; Funabashi Farm Co. Ltd) and water. After allowing 1 week for the mice to recover from the implantation procedure, blood pressures were recorded for 24 hours.

Each point in the figures shows an average of all beats recorded over a 60-minute period (Dataquest LabPRO Acquisition System version 3.01; Data Sciences International).

Statistical Analysis
Data are presented as means±SEM. Unpaired t tests were used for comparisons between 2 groups. Blood pressure and dose–response curves were analyzed using 2-way analysis of variance for repeated measures followed by Bonferroni’s test. Values of P<0.05 were considered significant. All statistical analyses were performed using STATA 12.1 (Stata Corp, College Station, TX).

Results

Generation of Endothelial Cell–Specific CNP Knockout Mice
To elucidate the physiological function of endothelium–derived CNP in the vasculature, we generated CNP ecKO mice by crossing mice carrying floxed NPPC with transgenic mice expressing Cre recombinase under the control of the Tie2 promoter/enhancer (Tie2-Cre Tg mice).8,16,17 Using samples from the tails of CNP ecKO (Nppclox/flox; Tie2-Cre Tg) mice, we confirmed Cre-mediated recombination of the Nppc-floxed allele (Figure 1A). In CD31+ pulmonary microvascular endothelial cells collected from the lungs of CNP ecKO mice, we confirmed that CNP mRNA levels were reduced to <10% of that in cells from the control mouse (Nppclox/flox; Figure 1B).

Analysis of CNP mRNA levels in several organs revealed significant reductions in CNP mRNA levels in lung and heart tissues from CNP ecKO mice, as compared with tissues from control littermates (Figure 1C). The reduction of CNP mRNA expression in the lung and heart is consistent with the reduced endothelial expression of CNP, though a decrease in CNP expression in the endocardium or a small population of cardiomyocytes could also contribute to the reduction in CNP mRNA levels seen in the heart.18

Cardiac structure and function, plasma ANP levels, and renal function estimated based on creatinine clearance did not differ between 16-week-old control and CNP ecKO mice (Table S1 and Figure S1A in the online-only Data Supplement). Plasma CNP levels in CNP ecKO mice were significantly reduced to around 60% of the levels in control mice (Figure 1D). CNP ecKO mice did not show the dwarfism or early mortality characteristic of systemic CNP knockout mice and GC-B knockout mice,15,19 and their naso–anal length was similar to that of control mice (Nppclox/flox; Figure 1E). Whole body weight and the weights of several major organs did not differ significantly between 16-week-old control littermates and CNP ecKO mice (Table S1), nor did the histological findings in several major organs, including the heart, kidney, lung, liver, and brain (Figure S1A–S1C).

High Blood Pressure in CNP ecKO Mice
To clarify the role played by endothelial cell–derived CNP in the control of blood pressure in vivo, we used surgically implanted arterial catheters containing telemetry transmitters to measure blood pressures in conscious, unrestrained CNP ecKO mice and their control Nppclox/flox littermates.20

Examination of 24-hour telemetry data revealed a significant increase in systolic blood pressure (SBP) over time in both male and female CNP ecKO mice, as compared with their controls (Figure 2A through 2D). In both males and females,
SBP was consistently higher in CNP ecKO mice during both the light and dark portions of their day (Figure 2A and 2C; Figure S2A and S2B). On the contrary, heart rates did not differ between male and female CNP ecKO and control mice (Figure 2E and 2F; Figure S2C through S2F), nor did urinary noradrenaline levels (Figure S2G). Likewise, there were no differences in lumenal diameters, wall thicknesses, or the wall-to-lumen ratios in the aorta and mesenteric arteries between CNP ecKO and control mice (Table S2).

Acute Hypertensive Response to Inhibition of Nitric Oxide Synthesis Is Enhanced in CNP ecKO Mice

We next examined the acute hypertensive response evoked by intraperitoneal administration of the nitric oxide (NO) synthetase inhibitor N^G^-nitro-L-arginine methyl ester (L-NAME) to CNP ecKO and control mice. Administration of L-NAME increased SBP in CNP ecKO mice from 101.0±2.8 mm Hg to 156.0±6.0 mm Hg, while in control mice, SBP increased from 95.6±2.6 mm Hg to 140.3±4.2 mm Hg (Figure 3A). Consistent with the results summarized above, basal SBP before L-NAME administration was higher in CNP ecKO mice than in control mice. L-NAME evoked marked increases in SBP in both groups, but the enhancement was significantly larger in CNP ecKO mice than in control mice (Figure 3A and 3B). Apparently, the acute pressor response induced by NO synthetase inhibition is enhanced in CNP ecKO mice, as compared with the control mice (Figure 3B). On the other hand, heart rates after L-NAME administration did not significantly differ between CNP ecKO and control mice (Figure 3C and 3D).

Generation of Vascular Smooth Muscle Cell–Specific GC-B Knockout Mice

Based on results obtained using an in vitro model of vascular remodeling, we previously proposed the existence of a vascular CNP/GC-B system in which CNP secreted from endothelial cells acts via GC-B expressed on VSMCs.13,21 Therefore, to investigate the potential role of GC-B expressed on VSMCs in mediating blood pressure regulation by endothelium-derived CNP, we generated and analyzed GC-B smcKO mice.8,22,23 We confirmed deletion of the allele using genomic DNA from tail samples (Figure 4A) and found that expression of GC-B mRNA was, respectively, ≈90% and ≈80% lower in aortas and mesenteric arteries from GC-B smcKO mice than from their control Npr2^floX/floX littermates (Figure 4B and 4C). Whole body weights and the weights of the heart and kidneys did not differ between GC-B smcKO mice and their control littermates (Table S3). Likewise, plasma CNP levels also did not differ

Figure 1. Generation of endothelial cell–specific C-type natriuretic peptide (CNP) knockout mice. A, Detection of Cre-mediated recombination of DNA from mouse tail samples using polymerase chain reaction (PCR). Left. The presence of the cre gene; right, the deleted allele in the control Nppc^trans/ trans littermates (control) and endothelial cell–specific CNP knockout (CNP ecKO) mice (Nppc^floX/floX; Tie2-Cre Tg, shown as ecKO), as indicated. B, Quantification of CNP mRNA using real-time reverse transcriptase (RT)-PCR with mouse pulmonary microvascular endothelial cells from 16-week-old control and CNP ecKO mice (n=4 each). C, Quantification of CNP mRNA using real-time RT-PCR with the cerebrum (n=3 each), kidney (n=6 each), heart (n=6 each), and lung (n=6 control and 5 CNP ecKO mice) from 16-week-old control and CNP ecKO mice. The relative mRNA levels in control mice were assigned a value of 1.0. D, Plasma CNP levels in 16-week-old control and CNP ecKO mice (n=5 each). E, Nose–anal lengths in 16-week-old control and CNP ecKO mice (n=6 each). Data are presented as means±SEM. *P<0.05 vs control mice using Student’s t test. N.S. indicates not significant.
between the 2 groups (Figure S3A). In addition, the skeletal abnormalities characteristic of systemic GC-B KO mice were not detected in GC-B smcKO mice (Figure 4D).19

**SBP Is Normal in GC-B smcKO Mice**

We measured SBP in unrestrained GC-B smcKO mice and their control littermates using telemetry, as we did with the CNP ecKO mice. In both males and females, hourly average SBPs measured for 24 hours did not differ between GC-B smcKO and control mice (Figure 4E and 4F). Likewise, during both the light and dark portions of their day, average SBPs in GC-B smcKO and control mice were similar in both males and females (Figure S3B through S3E). Heart rates also did not differ between GC-B smcKO and control mice (Figure S3F through S3K).

**Acetylcholine-Induced, Endothelium-Dependent Vasorelaxation Is Impaired in Mesenteric Arteries From CNP ecKO Mice, but Is Preserved in Arteries From GC-B smcKO Mice**

To assess underlying mechanisms through which CNP participates in the regulation of blood pressure, we next investigated endothelium-dependent vasorelaxation using mesenteric arteries isolated from CNP ecKO and control mice. Initially, we confirmed that an NO donor, spermine NONOate, induced vasorelaxation and a thromboxane A2 agonist, U46619,
induced vasoconstriction that were comparable in mesenteric arteries isolated from CNP ecKO and control mice (Figure S4A and S4B). By contrast, acetylcholine (ACh)-induced, endothelium-dependent vasorelaxation was significantly impaired in mesenteric arteries from CNP ecKO mice, as compared with control mice (−log of half-maximal effective concentration [EC50]: CNP ecKO 7.0±0.2 versus control 7.5±0.1, n=8 in CNP ecKO and n=7 in control littermates; P<0.05; Figure 5A). Furthermore, the impairment of ACh-induced vasorelaxation was unaffected or enhanced in CNP ecKO arteries pretreated with the NO synthetase inhibitor L-NAME and the cyclooxygenase inhibitor indomethacin (−logEC50: CNP ecKO mice 6.6±0.2 versus control mice 7.1±0.2, n=9 in CNP ecKO and n=7 in control; P<0.05; Figure 5B). This suggests that the impairment of endothelium-dependent vasorelaxation in CNP ecKO mice involves, at least in part, NO- and prosta- glandin-independent pathways, possibly an endothelium-derived hyperpolarization factor (EDHF) system.

Examination of endothelium-dependent vasorelaxation of mesenteric arteries isolated from GC-B smcKO mice revealed that ACh-induced, endothelium-dependent vasorelaxation did not differ between arteries isolated from GC-B smcKO and control mice (−logEC50: CNP smcKO mice 7.4±0.3 versus control mice 7.5±0.2, n=5 each; Figure 5C), even when the arteries were pretreated with L-NAME and indomethacin (−logEC50: CNP smcKO mice 7.2±0.2 versus control mice 7.1±0.2, n=5 each; Figure S4C). This finding is consistent with the above results showing normal blood pressures in GC-B smcKO mice (Figure 4E and 4F). By contrast, CNP-induced vasorelaxation was greatly diminished or undetectable in mesenteric arteries from GC-B smcKO mice (Figure 5D). Moreover, in precontracted control arteries treated with L-NAME and indomethacin, CNP elicited a 50% reduction in tension, twice as large as in untreated vessels, but this response was almost completely abolished in arteries from GC-B smcKO mice (Figure 5E). CNP-induced vasorelaxation in mesenteric arteries did not differ between CNP ecKO and control mice (Figure S4D). It, thus, seems that CNP induces acute vasorelaxation by acting directly via GC-B on VSMCs in this ex vivo model. Consistent with these findings,
acute hypotensive effects induced by intravenous administration of CNP were markedly attenuated in GC-B smcKO mice (Figure 5F). Taken together, our findings clearly demonstrate that endothelium-derived CNP is involved in the physiological regulation of blood pressure, acting, at least in part, acutely via GC-B expressed on VSMCs and in the longer term via an alternative pathway involving endothelial function.

**Increased Levels of Preproendothelin-1 Gene Expression in Isolated Vascular Endothelial Cells and Plasma Endothelin-1 in CNP ecKO Mice**

To further characterize the functional alterations in vascular endothelial cells in CNP ecKO mice, we collected pulmonary microvascular endothelial cells from the lungs of CNP ecKO and control mice and examined the expression of endothelial genes potentially involved in the regulation of vascular tone. The genes of interest included those related to the natriuretic peptide system, NO system, renin–angiotensin system, and endothelin (ET) system. Among these genes, we found that the expression of preproET-1 gene encoding ET-1, a potent vasoconstrictor produced by vascular endothelial cells, and angiotensin-converting enzyme (ACE), which is highly concentrated in pulmonary endothelial cells and regulates blood pressure by converting inactive angiotensin I to the strongly vasoconstrictive angiotensin II, was significantly higher in pulmonary vascular endothelial cells from CNP ecKO mice than in those from control mice (Figure 6A and 6B). Expression of the other genes studied did not differ significantly between CNP ecKO and control mice (Figure S5A through S5D). We also found that plasma ET-1 concentrations were significantly elevated in CNP ecKO mice, as compared with control mice (Figure 6C). The difference in plasma angiotensin II levels between CNP ecKO and control mice did not reach the statistical significance (Figure S5E). In the treatment with the dual endothelin receptor antagonist bosentan, CNP ecKO mice showed significantly greater reduction in SBP than control mice, which is consistent with our idea that increased endothelial ET-1 production contributes to the increase in blood pressure seen in CNP ecKO mice (Figure 6D). Collectively, these results demonstrate that alterations in the characteristics of vascular endothelial cells, including a diminished ability to induce vasorelaxation and induction of vasoconstrictive ET-1 expression, at least in part, contribute to the chronic elevation of blood pressure seen in CNP ecKO mice (Figure 6E and 6F).

**Discussion**

Systemic CNP knockout mice present marked skeletal dysplasia (ie, dwarfism) because of defective endochondral bone formation and a high perinatal mortality rate of unknown cause. This makes systemic CNP knockout mice unsuitable for investigating the physiological role of CNP in the adult vasculature in vivo. To avoid these critical limitations, in the present study, we used the Cre/loxP system to generate CNP ecKO mice, which grew normally and survived to adulthood.
Using these CNP ecKO mice, we were able to demonstrate the essential role played by endothelial cell–derived CNP in the chronic regulation of blood pressure. CNP seems to act via smooth muscle GC-B, which has been shown to mediate endothelium-dependent vasorelaxation. When we selectively depleted GC-B from VSMCs by crossing floxed GC-B mice with sm22α-Cre knockin mice, the resultant GC-B smcKO mice appeared normal and, surprisingly, had blood pressures similar to those in control mice. In addition, ACh-induced vasorelaxation was preserved. These results raise the possibility that endothelium-derived CNP regulates blood pressure via pathways independent of VSMC-derived GC-B and that this receptor is not involved in ACh-induced mesenteric artery vasorelaxation. Notably, however, CNP-induced vasorelaxation was almost completely abolished in mesenteric arteries from GC-B smcKO mice. Moreover, the enhanced vasorelaxant response induced by CNP in precontracted mesenteric arteries treated with...
L-NAME and indomethacin was nearly completely abolished in arteries from GC-B smcKO mice, as was the acute hypotensive effect induced by intravenous administration of CNP to GC-B smcKO mice. These results suggest that the acute vasodilatory effect of CNP is mediated largely via GC-B expressed on VSMCs (Figure S5F). On the contrary, CNP contributes to the chronic regulation of blood pressure under normal physiological conditions via an alternative pathway that maintains endothelial function (Figure 6E and 6F). In addition, although rat endothelial CNP reportedly induces vasorelaxation via VSMC-expressed NPR-C acting as an EDHF, the mechanisms primarily involved may differ in mice because deletion of GC-B from VSMCs resulted in a nearly complete loss of the vasodilatory effect of CNP on mesenteric arteries.26,27 Consistent with that idea is the earlier report that EDHF activity is preserved in the mesenteric arteries of mice lacking NPR-C.25 However, because the mediators of EDHF potentially differ among different sized arteries and

Figure 6. Expression of preproET-1 mRNA in vascular endothelial cells is increased in endothelial cell–specific C-type natriuretic peptide knockout (CNP ecKO) mice. A and B, Relative levels of preproET-1 (A) and angiotensin-converting enzyme (ACE; B) mRNA determined using real-time polymerase chain reaction (PCR). The relative mRNA levels in endothelial cells from control mice were assigned a value of 1.0 (n=3 control littermates and 4 CNP ecKO mice). C, Plasma concentrations of endothelin-1 (ET-1) were determined using specific ELISAs (n=11 for each). *P<0.05. Data are presented as means±SEM. D, Changes in average systolic blood pressure (ΔSBP) measured 1 day before (pre) and 5 days after (post) initiation of bosentan treatment in control (n=5) and CNP ecKO mice (n=5). *P<0.05 using Student’s t test. E and F, Schematic drawing illustrating the role of endothelial CNP in chronic blood pressure regulation in wild-type (E) and CNP ecKO (F) mice. In acute responses, CNP derived from endothelial cells induces vasorelaxation via guanylyl cyclase-B (GC-B) expressed in VSMCs. This acute effect of CNP is abolished in VSMCs lacking GC-B as shown in Figure S5F. In chronic blood pressure regulation, alterations in the characteristics of vascular endothelial cells, including diminished ability to induce vasorelaxation and increased expression of the vasoconstrictor peptide ET-1, are caused by a deficit in cell autonomous effects exerted by vascular endothelial cell–derived CNP, but not by a deficit in GC-B signaling in VSMCs (E and F). Collectively, these endothelial alterations contribute to the elevated blood pressure seen in CNP ecKO mice (F). EC indicates vascular endothelial cell; and VSMC, vascular smooth muscle cell.
in different species, endothelial-derived CNP may act as an EDHF through NPR-C in some types of mouse arteries, arteries from different species, or arteries under different conditions. Indeed, although the acute hypotensive effect induced by intravenous administration of CNP was significantly attenuated in GC-B smcKO mice, CNP continued to elicit a partial response (Figure 5F). It is also noteworthy that chronic blood pressure regulation via GC-A, a common receptor for ANP and brain natriuretic peptide, is mediated by endothelial cell GC-A, not VMSC GC-A, though acute ANP-induced vasodilation is mediated by VSMC GC-A.33,29

CNP promotes endothelial cell proliferation and also reportedly stimulates NO synthetase activity in endothelial cells.14,30 Both of those effects may contribute to the chronic regulation of blood pressure by maintaining physiological endothelial function. This is consistent with the recent findings of Moyes et al, who reported that CNP derived from endothelial cells32 and suggest that for chronic regulation of pressure regulation by endothelium-derived CNP. In addition, our results demonstrating that endothelium-derived CNP maintains normal endothelial phenotypes that control blood pressure regulation via GC-A, a common receptor for ANP and brain natriuretic peptide, is mediated by endothelial cell GC-A, not VMSC GC-A, though acute ANP-induced vasodilation is mediated by VSMC GC-A.33,29

These results provide novel insight into the role of CNP/arterial cell–derived CNP signaling contributing to chronic blood pressure control. Alterations in the characteristics of vascular endothelial cells, including a diminished ability to induce vasorelaxation and increased expression of ET-1, could account for the elevation of blood pressure seen in CNP ecKO mice (Figure 6F). These results also provide additional insight into the antagonistic relation between the vasodilatory CNP-dependent pathway and the vasoconstrictive ET-1-dependent pathway in vascular endothelial cells and broaden our understanding of the vascular natriuretic peptide system.

**Perspectives**

We have shown that vascular endothelial cell–derived CNP plays a key role in the chronic regulation of blood pressure in vivo. Unexpectedly, although CNP-induced acute vasodilatation is predominantly mediated by GC-B on VSMCs, GC-B expressed on VSMC seems dispensable for endothelial cell–derived CNP signaling contributing to chronic blood pressure control. Alterations in the characteristics of vascular endothelial cells, including a diminished ability to induce vasorelaxation and increased expression of ET-1, underlie the blood pressure regulation by endothelial cell–derived CNP. These results provide novel insight into the role of CNP/
GC-B signaling in the vascular system and its underlying mechanisms.

Acknowledgments
We thank Akiko Abe, Mika Oya, and Aoi Fujishima for their excellent technical assistance and Yukari Kubo for her excellent secretarial work.

Sources of Funding
This research was supported by Grants-in-Aid for Scientific Research from the Japan Society for the Promotion of Science (to K. Kuwahara and Kazuwa Nakao); a grant from the Japanese Ministry of Health, Labour and Welfare (to Kazuwa Nakao); grants from the Japan Foundation for Applied Ergonomics, the Ministry of Health, Labour and Welfare, the Takeda Science Foundation, the Hoh-ansha Foundation, the SENSHE Medical Research Foundation (to K. Kuwahara). Kazuhiro Nakao conducted most of the experiments and contributed to data analysis. T. Tokudome helped isolate CD31

Nakao conducted most of the experiments and contributed to data analysis. C. Nagai-Okatani and N. Minamino measured plasma ANP, CNP, angiotensin II, and ET-1 levels. T. Nishikimi, Y.M. Nakao, K. Ueshima, M. Sone, and T. Kimura contributed to data analysis.

Disclosures
None.

References


What Is New?

- We show the contribution of vascular endothelial cell–derived C-type natriuretic peptide (CNP) to chronic blood pressure regulation, irrespective of sex, using endothelial cell–specific CNP knockout mice (CNP ecKO).
- Vascular smooth muscle cell–restricted knockout of guanylyl cyclase-B (GC-B), a functional receptor for CNP, had no effect on blood pressure.
- In CNP ecKO mice, endothelium-dependent vasorelaxation was impaired and endothelial expression of endothelin-1 was significantly increased.

What Is Relevant?

- The results of this study provide insight into the antagonistic relation between the vasodilatory CNP-dependent pathway and vasoconstrictive endothelin-1–dependent pathway in vascular endothelial cells and broaden our understanding of the vascular natriuretic peptide system.
- This study revealed the role of CNP/GC-B signaling in the normal physiological regulation of the vascular system and the potential significance of this system as a therapeutic target for the treatment of cardiovascular disease.

Novelty and Significance

Summary

CNP ecKO mice showed significantly elevated blood pressure and impaired endothelium-dependent vasorelaxation. By contrast, GC-B smcKO mice exhibited normal blood pressure and preserved endothelium-dependent vasorelaxation. Collectively, our findings suggest that the long-term effects of CNP derived from endothelial cells on blood pressure are mediated through modification of endothelial cell function and are independent of GC-B signaling in VSMCs. These results provide novel insight into the role of CNP/GC-B signaling in the vascular system and its underlying mechanisms.
Endothelium-Derived C-Type Natriuretic Peptide Contributes to Blood Pressure Regulation by Maintaining Endothelial Integrity

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_Hypertension_. 2017;69:286-296; originally published online January 3, 2017; doi: 10.1161/HYPERTENSIONAHA.116.08219

_Hypertension_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231

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Print ISSN: 0194-911X. Online ISSN: 1524-4563

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ENDOTHELIUM-DERIVED C-TYPE NATRIURETIC PEPTIDE CONTRIBUTES TO BLOOD PRESSURE REGULATION BY MAINTAINING ENDOTHELIAL INTEGRITY

Short title: C-type natriuretic peptide and blood pressure

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Supplemental Methods
Animal experiments
Details of the protocol used to generate conditional knockout mice are presented below. The animal care and all experimental protocols were reviewed and approved by the Animal Research Committee at Kyoto University Graduate School of Medicine, and conformed to the US National Institute of Health Guide for the Care and Use of Laboratory Animals. All mice used in this study had a C57BL/6J background. For the isolation and analysis of organs or blood samples, mice were anesthetized with 3.0% isoflurane and sacrificed by cervical dislocation.

Generation of CNP ecKO mice
The targeting vector used to generate CNP ecKO mice contained a loxP-flanked open reading frame (ORF) that included exons 1 and 2 with intron 1 and part of intron 2, as well as a FRT-flanked neomycin resistance cassette (Neo) and an additional 6.1 kbp containing the 5' sequence of the Nppc gene, the diphtheria toxin A gene (DTA) and a 2.8-kbp sequence spanning intron 2 and exon 3. The linearized targeting vector was electroporated into C57BL/6 embryonic stem (ES) cells, after which we screened for homologous recombination using PCR and confirmed recombination using Southern blot analysis. Mouse chimeras were generated by injecting the mutant ES cell clones into BALB/c host blastocysts, after which the chimeras were bred with Flp-expressing transgenic mice to remove Neo. Some of the offspring were bred with C57BL/6 mice to generate Nppc-floxed heterozygous (Nppcflox/+) mice, which were further bred to homozygosity. Endothelial cell-specific CNP conditional knockout mice were then obtained by breeding Nppcflox/flox mice with heterozygous Tie2-cretg mice, which were purchased from the Jackson Laboratory.

Generation of GC-B smcKO mice
The targeting vector used to generate GC-B smcKO mice contained loxP-flanked exons 3-7 (with introns), a FRT-flanked neomycin resistance cassette (Neo) and an additional 5.3 kbp sequence containing intron 2 of the Nppc gene, the diphtheria toxin A gene (DTA) and a 2.7-kbp sequence spanning exons 8-12 (with introns). The linearized targeting vector was electroporated into C57BL/6 embryonic stem (ES) cells, after which we screened for homologous recombination using PCR and confirmed recombination using Southern blot
Mouse chimeras were generated by injecting mutant ES cell clones into BALB/c host blastocysts, after which the chimeras were bred with Flp-expressing transgenic mice to remove Neo. Some of the offspring were then bred with C57BL/6 mice to generate Npr2-floxed heterozygous ($Npr2^{flox/+}$) mice, which were further bred to homozygosity. Vascular smooth muscle cell-specific GC-B conditional knockout mice were produced by breeding $Npr2^{flox/flox}$ mice with heterozygous sm22α-Cre knock in (KI) mice, which were purchased from the Jackson Laboratory (Bar Harbor, ME).

PCR genotyping for $Nppc$ and $Npr2$ genomic DNA
Genomic DNA was prepared from mouse tail samples. Genotyping was done using PCR. The following primer pairs were used for detection of floxed and deleted alleles in $Nppc$ and $Npr2$, respectively: for $Nppc$ floxed alleles, 5’-GTG TCC ACA GTG AGT TCT TTA CCA G-3’ (upstream) and 5’-GTA AAG TGT GTC TCT GCA CAC CTC GGT CCC ATC GGC A-3’ (downstream); for $Nppc$ deleted alleles, 5’-TCT GCA CAC CTC GGT CCC ATC GGC A-3’ (upstream) and 5’-GTA AAG TGT GTC TCT GCA CAC CTC GGT CCC ATC GGC A-3’ (downstream); for $Npr2$ floxed alleles, 5’-GTA ACC TGG GTA GAC TAG TTG TTG G-3’ (upstream) and 5’-ATG GTG GAG GAG GTC TTT AAT TCC-3’ (downstream); and for $Npr2$ deleted alleles, 5’-TTC TCA CAG GGT TTG AAA TCT AAT G-3’ (upstream) and 5’-ATG GTG GAG GAG GTC TTT AAT TCC-3’ (downstream).

Isolation of mouse pulmonary microvascular endothelial cells
Pulmonary microvascular endothelial cells were isolated from CNP ecKO mice and their control littermates as described previously with modification.1,2 In brief, lungs were removed from anesthetized, heparinized mice and minced in HBSS (Thermo Fisher Scientific, Waltham, MA). After incubation with collagenase A for 45 min at 37°C, the tissue was passed through a 40-µm filter to remove debris. The cells were then centrifuged (1000 g, 20 min, 21°C) in 40% Percoll solution, after which CD31+ pulmonary microvascular endothelial cells were purified from the middle layer using a magnetic cell sorting system (Miltenyi Biotec).

Blood pressure measurement and telemetry
Blood pressures in 16-week-old mice were measured using implantable blood pressure transmitters (Data Sciences International, TA11PA-C10). After anesthetizing mice with isoflurane, a radiotelemetry probe catheter was
implanted in the aorta of each mouse via the left carotid artery. The mice were then housed individually in cages where they were able to move about freely. The cages were situated above telemetric receivers with an output to a computer. The mice were maintained on a 12-h light-dark cycle and were fed normal chow (0.19% sodium, F-2, Funabashi Farm Co. Ltd) and water. After allowing 1 week for the mice to recover from the implantation procedure, blood pressures were recorded for 24 h. Each point in the figures shows the average of all beats over a 60-min period (Dataquest LabPRO Acquisition System version 3.01, Data Sciences International).

In another experiment, beginning 10 days after the implantation of blood pressure transmitters, CNP ecKO and control mice were administered the endothelin receptor antagonist bosentan (10 mg/kg/day, intragastric administration) daily for 1 week. Blood pressures recorded for 24 h immediately prior to the drug treatment and 5 days after initiating the treatment were then analyzed.

**Intraperitoneal injection of L-NAME**
After measuring the blood pressures of freely moving mice for 30 min, the NOS inhibitor L-NAME (50 mg/kg body weight, Dojin, Kumamoto, Japan) was administered intraperitoneally. Blood pressure measurements were then continued for an additional 2 h. Each point in the figures shows the SBP averaged over 5 min.

**Intravenous injection of CNP**
Mice were intubated and anesthetized with 0.5% to 1.0% isoflurane. After stabilization, 10 nmol/kg CNP (Peptide institute, Osaka, Japan) was intravenously administered via the tail vein to GC-B smcKO and control mice. Arterial blood pressures were monitored using a catheter inserted into a cervical artery. Monitoring began 2 min before injection and ended 10 min after injection.

**Wire myograph studies**
Female mice in estrous were used for these experiments. Vascular rings were isolated from second order mesenteric arteries and placed in cold physiological salt solution (PSS) containing (mM): 119 NaCl, 4.7 KCl, 2.5 CaCl$_2$, 1.2 MgSO$_4$, 25 NaHCO$_3$, 1.2 KH$_2$PO$_4$ and 5.5 glucose. The rings were then mounted in a tension myograph (Danish Myo Technology, Aarhus, Denmark) and bathed in
Krebs solution maintained at 37°C and gassed with 95% O₂, 5% O₂. After a wash period, the rings were stretched to the optimal pretension level (Danish Myo Technology Normalization Module) and were then repeatedly contracted using the thromboxane A2 analog U46619 (0.1 μM, Sigma-Aldrich, St. Louis, MO) until the responses were reproducible. If a vessel did not generate more than 1 mN of force, it was rejected. Concentration response curves were constructed for U46619 (0-100 nM). To assess vasorelaxation, the arteries were precontracted to 60% of maximum using U46619, after which the relaxant responses to ACh (0-10 μM, Sigma-Aldrich), CNP (0-1 μM, Peptide Institute) and the NO donor spermine NONOate (SpNO) (0-100 μM, CALBIOCHEM) were determined. In some instances, vessels were treated with L-NAME (300 μM, Dojin) and indomethacin (10 μM, Sigma-Aldrich) for 30 min before the dose-response curves were generated.

**Echocardiographic analysis**
Echocardiography was performed in 16-week-old mice as described previously using a Toshiba PowerVision 8000 (Toshiba Corp., Tokyo, Japan) equipped with a 12-MHz imaging transducer.³

**Histological Examination**
Organs taken from 16-week-old mice were fixed in 10% formalin and prepared for histological analysis as described previously.³

**Creatinine Clearance**
To measure creatinine clearance (mL/min), the amounts of creatinine in plasma samples and 24-h urine specimens from 16-week-old mice were determined enzymatically (SRL, Tokyo, Japan), as described previously.⁴

**Quantitative RT-PCR**
Total RNA was isolated from selected mouse tissues using TRIZOL following the manufacturer’s protocol. Real-time one-step RT-PCR was then carried out using 20 ng of total RNA with One-Step RT-PCR master mix reagent (Toyobo). All TaqMan primers and probes were purchased from Applied Biosystems.

In another experiment, total RNA was isolated from mouse CD31⁺ pulmonary microvascular endothelial cells collected from the lungs of CNP ecKO mice and their control littermates using a magnetic cell sorting system. Using 10 ng of total
RNA from the lung endothelial cells of control and CNP ecKO mice, mRNA levels in guanylyl cyclase A (GC-A), guanylyl cyclase B (GC-B), natriuretic peptide receptor C (NPR-C), endothelial nitric oxide synthase 3 (eNOS), small conductance calcium-activated potassium channel 3 (SKCa), intermediate conductance calcium-activated potassium channel 4 (IKCa), connexin37 (Cx37), connexin40 (Cx40), connexin43 (Cx43), angiotensin-converting enzyme (ACE), angiotensin II type1a receptor (AT1R), angiotensin II type2 receptor (AT2R), preproendothelin-1 (preproET-1), endothelin receptor type A (ETA), endothelin receptor type B (ETB) and endothelin converting enzyme 1 (ECE-1) were determined using One-Step RT-PCR master mix reagent (Toyobo, Osaka, Japan). The primers and probe sets were purchased from Applied Biosystems (Waltham, MA). Relative mRNA levels were determined by normalization to the level of GAPDH mRNA.

**Measurement of urinary noradrenalin excretion**
For analysis of urinary noradrenalin excretion, 16-week-old control and CNP ecKO mice and were placed in metabolic cages and given free access to food and water. Urinary samples were then collected for 1 day. Urinary noradrenalin excretion was assessed using reverse phase high-performance liquid chromatography.

**Morphological analysis of vessels**
After harvesting thoracic aortas (5 vessels from 5 mice) and mesenteric arteries (10 vessels from 5 mice) from 16-week-old mice, the samples were fixed in 4% PFA. Paraffin-embedded sections (4 μm) were then stained with hematoxylin-eosin, after which lumen diameter and wall thickness were measured (BZ-9000, KEYENCE Japan)

**Measurement of plasma ANP, CNP, ET-1 and angiotensin II concentrations**
Under anesthesia, blood was drawn from 16-week-old female mice by direct cardiac ventricle puncture and collected into tubes containing aprotinin and EDTA-2Na (final concentrations were 500 U/mL aprotinin and 3.3 mM EDTA-2Na). Plasma ANP concentrations were measured using a direct chemiluminescent enzyme immunoassay, as previously described5. Plasma CNP concentrations were measured using our newly developed sandwich chemiluminescent enzyme immunoassay, which has a detection limit down to 0.25pM and no cross-reactivity
with ANP, BNP and other related peptides (unpublished data). Plasma ET-1 and angiotensin II concentrations were measured using ELISAs according to manufacturers’ instructions (ET-1, R&D Systems, Minneapolis, MN; angiotensin II, Enzo Life Sciences, Farmingdale, NY).

**Statistical Analysis**

Data are presented as means ± SEM. Unpaired t-tests were used for comparisons between two groups. Blood pressure and dose-response curves were analyzed using two-way analysis of variance for repeated measures followed by Bonferroni’s test. Values of p<0.05 were considered significant. All statistical analyses were performed using STATA 12.1 (Stata Corp, College Station, TX).
References


Supplemental Table S1. Body and tissue weights, plasma ANP levels, renal function and echocardiographic parameters in 16-old–week \( Nppc^{flox/flox} \) and \( Nppc^{flox/flox}; \text{Tie2-Cre Tg} \) mice

<table>
<thead>
<tr>
<th>Variables</th>
<th>( NPPC^{flox/flox} )</th>
<th>( NPPC^{flox/flox}; \text{Tie2-Cre Tg} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body and tissue weights</td>
<td>n=6</td>
<td>n=6</td>
</tr>
<tr>
<td>BW (g)</td>
<td>23.7±0.4</td>
<td>22.8±0.4</td>
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<tr>
<td>Heart weight-to-BW ratio (mg/g)</td>
<td>4.7±0.1</td>
<td>4.7±0.1</td>
</tr>
<tr>
<td>Kidney weight-to-BW ratio (mg/g)</td>
<td>6.1±0.2</td>
<td>5.9±0.1</td>
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<tr>
<td>Liver weight-to-BW ratio (mg/g)</td>
<td>46.1±4.6</td>
<td>46.0±5.9</td>
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<tr>
<td>Lung weight-to-BW ratio (mg/g)</td>
<td>21.1±2.5</td>
<td>20.0±1.1</td>
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<tr>
<td>Plasma ANP concentration</td>
<td>n=10</td>
<td>n=9</td>
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<tr>
<td>Plasma ANP concentration (pg/ml)</td>
<td>96.9±22.2</td>
<td>140.0±41.2</td>
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<tr>
<td>Creatinine Clearance</td>
<td>n=7</td>
<td>n=7</td>
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<tr>
<td>Creatinine Clearance (ml/min)</td>
<td>0.21±0.03</td>
<td>0.29±0.02</td>
</tr>
<tr>
<td>Echocardiography parameters</td>
<td>n=10</td>
<td>n=11</td>
</tr>
<tr>
<td>LVDd (mm)</td>
<td>3.2±0.04</td>
<td>3.1±0.08</td>
</tr>
<tr>
<td>LVDs (mm)</td>
<td>1.6±0.06</td>
<td>1.6±0.08</td>
</tr>
<tr>
<td>IVST (mm)</td>
<td>0.71±0.02</td>
<td>0.71±0.02</td>
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<tr>
<td>PWT (mm)</td>
<td>0.71±0.01</td>
<td>0.71±0.02</td>
</tr>
<tr>
<td>FS (%)</td>
<td>49.2±1.9</td>
<td>48.8±1.9</td>
</tr>
<tr>
<td>EF (%)</td>
<td>81.1±1.7</td>
<td>80.7±1.8</td>
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</table>

Values are means ± SEM. BW, body weight; ANP, atrial natriuretic peptide; LVDd, left ventricular diastolic dimension; LVDs, left ventricular systolic dimension; FS, fractional shortening; IVST, intraventricular septum wall thickness; PWT, posterior wall thickness.
**Supplemental Table S2.** Morphological characteristics of vessels

<table>
<thead>
<tr>
<th>Variables</th>
<th>Nppc&lt;sup&gt;flox/flox&lt;/sup&gt;</th>
<th>Nppc&lt;sup&gt;flox/flox&lt;/sup&gt;;Tie2-Cre Tg</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Aorta</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lumen diameter (µm)</td>
<td>535.6±11.6</td>
<td>530.7±7.2</td>
</tr>
<tr>
<td>Wall thickness (µm)</td>
<td>48.7±1.5</td>
<td>49.0±0.8</td>
</tr>
<tr>
<td>Wall/Lumen ratio</td>
<td>0.090±0.002</td>
<td>0.092±0.002</td>
</tr>
<tr>
<td><strong>Mesenteric arteries</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lumen diameter (µm)</td>
<td>111.9±4.9</td>
<td>112.7±6.5</td>
</tr>
<tr>
<td>Wall thickness (µm)</td>
<td>16.9±0.4</td>
<td>17.6±0.6</td>
</tr>
<tr>
<td>Wall/Lumen ratio</td>
<td>0.151±0.007</td>
<td>0.156±0.008</td>
</tr>
</tbody>
</table>

Values are means±SEM, n=5 each in Aorta; n=10 each in Mesenteric arteries.
Supplemental Table S3. Body and tissue weights in 16-old–week Npr2\textsuperscript{flox/flox} and Npr2\textsuperscript{flox/flox}; sm22\textalpha-Cre KI mice

<table>
<thead>
<tr>
<th>Variables</th>
<th>Npr2\textsuperscript{flox/flox}</th>
<th>Npr2\textsuperscript{flox/flox};sm22\textalpha-Cre KI</th>
</tr>
</thead>
<tbody>
<tr>
<td>BW (g)</td>
<td>24.3±1.0</td>
<td>24.2±0.9</td>
</tr>
<tr>
<td>Heart weight-to-BW ratio (mg/g)</td>
<td>4.4±0.1</td>
<td>4.5±0.1</td>
</tr>
<tr>
<td>Kidney weight-to-BW ratio (mg/g)</td>
<td>5.0±0.1</td>
<td>4.9±0.1</td>
</tr>
</tbody>
</table>

Values are means ± SEM. BW, body weight. n=6 for Npr2\textsuperscript{flox/flox} and 5 for Npr2\textsuperscript{flox/flox};sm22\textalpha-Cre KI.
**Supplemental Figure S1.** Histology of major organs in CNP ecKO mice. **A**, **B** and **C**, Histological analysis of the heart (Sirius-red staining; **A**), kidney, lung, liver (Masson-trichrome staining; **B**) and Brain (hematoxylin-eosin staining; **C**) from control and CNP ecKO. H-E, hematoxylin-eosin. Scale bars, 100 µm. Samples were taken from 16-week-old mice (n=3 each).
**Supplemental Figure S2.** High blood pressure in CNP ecKO mice.  

**A,** Average SBPs measured over the 12-h light and dark periods in male mice. *p<0.05 using Student's *t*-test.  

**B,** Average SBPs measured over the 12-h light and dark periods in female mice. *p<0.05 using Student's *t*-test.  

**C,** Hourly average heart rates in male control (n=10) and CNP ecKO mice (n=10).  

**D,** Average heart rates measured over the 12-h light and dark periods in male mice.  

**E,** Hourly average heart rates in female control (n=6) and CNP ecKO mice (n=6).  

**F,** Average heart rates measured over the 12-h light and dark periods in female mice.  

**G,** Daily urinary norepinephrine (noradrenaline) excretion in control and CNP ecKO mice (control n=5, CNP ecKO n=7). Data are presented as means ± SEM. N.S.: not significant.
Supplemental Figure S3. Normal blood pressure in vascular smooth muscle cell-specific GC-B knockout mice. A, Plasma CNP levels in 16-week-old control (n=6) and GC-B smcKO mice (n=7). B, Average SBPs measured over the 12-h light and dark periods in male control (n=3) and GC-B smcKO (n=4) mice. C, Average SBPs measured over the entire 24-h day in male control (n=3) and GC-B smcKO (n=4) mice. D, Average SBPs measured over the 12-h light and dark periods in female control (n=3) and GC-B smcKO (n=3) mice. E, Average SBPs measured over the entire 24-h day in female control (n=3) and GC-B smcKO (n=3) mice. F, Hourly average heart rates in male control (n=3) and GC-B smcKO mice (n=4). G, Average heart rates measured over the 12-h light and dark periods in male mice. H, Average heart rates measured over the entire 24-h day in male mice. I, Hourly average heart rates in female control (n=3) and GC-B smcKO mice (n=3). J, Average heart rates measured over the 12-h light and dark periods in female mice. K, Average heart rates measured over the entire 24-h day in female mice. Data are presented as means ± SEM.
Supplemental Figure S4. NO donor spermine NONOate (SpNO)-induced vasorelaxation and U46619-induced vasoconstriction are comparable in mesenteric arteries from CNP ecKO mice and their control littermates. A, SpNO-induced vasorelaxation in mesenteric arteries from CNP ecKO mice and control littermates. B, U46619-induced vasoconstriction in mesenteric arteries from CNP ecKO mice and control littermates. C, ACh-induced relaxation of precontracted mesenteric arteries from female control and GC-B smcKO mice in the presence of L-NAME and indomethacin. Arteries were precontracted to 50-70% of maximum using U46619. Dose-response curves show ACh-induced relaxation in mesenteric arteries from control (n=5 each) and GC-B smcKO mice (n=5 each). D, CNP-induced relaxation of mesenteric arteries from control (n=5 each) and CNP ecKO mice (n=5 each). Data are presented as means ± SEM.
Supplemental Figure S5. Transcription of endothelial genes potentially associated with blood pressure regulation and endothelium dependent vasodilation. A, mRNA expression of genes related to natriuretic peptide system (GC-A, GC-B and NPR3) (control n=3, CNP ecKO n=4). B, Genes associated to NO and EDHF (Nos3, SK3, SK4, Cx37, Cx40 and Cx43) (control n=3, CNP ecKO n=4). C, Genes related to the renin-angiotensin system (AT1R and AT2R) (control n=3, CNP ecKO n=4). D, Genes related to endothelin system (ETA, ETB and ECE-1) (control n=3, CNP ecKO n=4). Relative mRNA levels were determined by normalization to the level of GAPDH mRNA. The relative mRNA levels in control mice were assigned a value of 1.0. n.d.: not detectable. E, Plasma concentrations of angiotensin II (AngII) were determined using specific ELISAs (n=4 CNP ecKO mice and 5 control littermates). N.S: not significant. Data are presented as means ± SEM. F, Schematic drawing illustrating the role of endothelial CNP in acute vasorelaxation. In acute responses, CNP derived from endothelial cells induces vasorelaxation via GC-B expressed in VSMCs. This acute effect of CNP is abolished in VSMCs lacking GC-B. VSMC: vascular smooth muscle cell.