Atrial natriuretic peptide prevents cancer metastasis through vascular endothelial cells

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Most patients suffering from cancer die of metastatic disease. Surgical removal of solid tumors is performed as an initial attempt to cure patients; however, surgery is often accompanied with trauma, which can promote early recurrence by provoking detachment of tumor cells into the blood stream or inducing systemic inflammation or both. We have previously reported that administration of atrial natriuretic peptide (ANP) during the perioperative period reduces inflammatory response and has a prophylactic effect on postoperative cardiopulmonary complications in lung cancer surgery. Here, we demonstrate that cancer recurrence after curative surgery was significantly lower in ANP-treated patients than in control patients (surgery alone). ANP is known to bind specifically to NPR1 [also called guanylyl cyclase-A (GC-A) receptor]. In mouse models, we found that metastasis of GC-A–nonexpressing tumor cells (i.e., B16 mouse melanoma cells) to the lung was increased in vascular endothelium-specific GC-A knockout mice and decreased in vascular endothelium-specific GC-A transgenic mice compared with control mice. We examined the effect of ANP on tumor metastasis in mice treated with lipopolysaccharide, which mimics systemic inflammation induced by surgical stress. ANP inhibited the adhesion of cancer cells to pulmonary arterial and micro-vascular endothelial cells by suppressing the E-selectin expression that is promoted by inflammation. These results suggest that ANP prevents cancer metastasis by inhibiting the adhesion of tumor cells to inflamed endothelial cells.

cardiac peptide | cancer metastasis | vascular endothelial cell | inflammation | surgery

The majority of cancer patients die from tumor metastasis. Despite substantial advances in our understanding of the mechanisms of tumor metastasis, effective prevention of metastasis has not been well established. Surgical removal of solid tumors is performed to cure patients if the primary tumor meets surgical indications; however, postoperative cancer recurrence is a major problem. Surgical trauma itself influences the development of early recurrence (1, 2). First, the procedure during tumor removal might provoke detachment of tumor cells; consistently, the number of circulating tumor cells is increased during primary tumor resection (3, 4). We previously reported that the presence of circulating tumor cells in pulmonary veins during lung cancer surgery could be a prognostic indicator for early cancer recurrence (4). Second, surgical trauma provokes a severe systemic inflammatory reaction. Emerging evidence suggests that systemic inflammation can accelerate the adhesion of circulating tumor cells to the vascular endothelium of distant organs, which is the first step of extravasation in hematogenous metastasis (5, 6). We identified human atrial natriuretic peptide (ANP) as a diuretic, natriuretic, and vasodilating hormone from the human heart in 1984 (7). ANP binds specifically to the guanylyl cyclase-A (GC-A) receptor to exhibit biological functions, including promotion of diuresis, antifibrinotic action, and inhibition of renin-angiotensin-aldosterone (8, 9). Thus, ANP has been used clinically for the treatment of heart failure since 1995 in Japan. We previously reported that administration of human ANP during the perioperative period reduces inflammatory responses and has a prophylactic effect on postoperative cardiopulmonary complications in lung cancer surgery (10–12). In those studies, organs, which is the first step of extravasation in hematogenous metastasis (5, 6).

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Significance

Postoperative cancer recurrence is a major problem following curative cancer surgery. Perioperative systemic inflammation induces the adhesion of circulating tumor cells released from the primary tumor to the vascular endothelium of distant organs, which is the first step in hematogenous metastasis. We have previously reported that administration of atrial natriuretic peptide (ANP) during the perioperative period reduces inflammatory response and has a prophylactic effect on postoperative cardiopulmonary complications in lung cancer surgery. Here, we demonstrate that cancer recurrence after lung cancer surgery was significantly lower in ANP-treated patients than in control patients (surgery alone). We show that ANP prevents cancer metastasis by suppressing the inflammatory reaction of endothelial cells, thereby inhibiting cancer cell adhesion to vascular endothelial cells.


Freely available online through the PNAS open access option.

Data deposition: The data reported in this paper have been deposited in the Gene Expression Omnibus (GEO) database, www.ncbi.nlm.nih.gov/geo (accession no. GSE56976).

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ANP was used to promote diuresis during perioperative right-side heart failure caused by lung damage. Here, we further analyzed the effect of ANP on prevention of cancer recurrence after surgery and found that ANP might have antitumor metastatic activity. We explored the antimitastatic action of ANP by using tissue-specific GC-A transgenic and knockout mice of tumor metastasis models. Our results suggest that ANP could be useful as an antimitastasis peptide to prevent cancer recurrence after surgery.

Results

Clinical Impacts of ANP Therapy on Cancer Recurrence After Lung Cancer Surgery. We performed a retrospective study of the incidence of cancer recurrence in lung cancer patients after curative surgery, comparing patients who underwent perioperative ANP treatment with those who were subjected to surgery alone (control patients). The 2-y relapse-free survival (RFS) after surgery was significantly greater in ANP-treated patients than in control patients (91% vs. 75%, P = 0.018) (Fig. 1A). To eliminate bias, we reanalyzed the data by using propensity score matching. The 2-y RFS in the propensity score-matched analysis was also significantly greater in ANP-treated patients than in control patients (91% vs. 67%, P = 0.0013) (Fig. 1B and SI Appendix, Table S1). We hypothesized from these retrospective observations that ANP may prevent recurrence of lung cancer.

Antimitastatic Effects of ANP in Hematogenous Pulmonary Metastatic Models. Vascular inflammation is considered to render the endothelium adhesive to circulating tumor cells, thereby allowing the metastasis of tumor cells (5, 6). We previously reported that postoperative complications induced by inflammation are reduced by ANP (10–12). Therefore, to investigate whether ANP inhibits the metastasis of cancer cells to inflamed organs, we examined the effect of ANP on tumor metastases in mice injected with LPS, which mimics systemic inflammation induced by surgical stress (6, 13). The LPS-treated mice showed numerous hematogenous pulmonary metastases of intravenously injected A549 lung cancer cells expressing EGFP (A549-EGFP) (Fig. 2A and B and SI Appendix, Fig. S1A). In contrast, the mice pretreated with ANP exhibited a large and significant reduction of LPS-induced pulmonary hematogenous metastasis of A549-EGFP cells (Fig. 2A). The ANP group and control group (surgery alone) in all patients (P = 0.018, log-rank test). (B) Kaplan–Meier curves of the above groups in propensity score-matched patients (P = 0.0013, log-rank test). RFS was measured from the day of surgery to cancer recurrence.

We hypothesized from these retrospective observations that ANP may prevent recurrence of lung cancer.

Mechanism of the Effect of ANP on Cancer Cell Adhesion to Vascular Endothelial Cells. We next attempted to uncover the molecular mechanism behind ANP-mediated inhibition of tumor metastasis through vascular endothelial cells. The attachment of A549-EGFP and H460 lung cancer cells expressing EGFP (H460-EGFP) cells to cultured human pulmonary artery endothelial cells (HPAECs) stimulated with LPS was dependent on the dose LPS-induced massive inflammation. More importantly, these data indicate that ANP acts through GC-A expressed in nontumor mouse cells.

To eliminate the direct effect of ANP on tumor cell proliferation, we first examined the direct effects of ANP on the growth of cancer cells and found that GC-A was expressed in A549 and H460 human lung cancer cells (SI Appendix, Fig. S1B). Even though GC-A was expressed on A549 and H460 cells, ANP did not inhibit the proliferation of these tumor cells (SI Appendix, Fig. S3 A–C). Natriuretic peptide receptor-C, which is also known as a receptor of ANP, was expressed in A549, H460, and B16/F10 cells (SI Appendix, Fig. S3 B–D). These results suggest that the inhibitory effect of ANP on tumor metastasis is dependent upon GC-A expressed on cells other than tumor cells.

We considered that GC-A expressed on endothelial cells might be responsible for the antimitastatic effect of ANP because cancer cell attachment to endothelial cells is the initial step in metastasis (5, 6). Vascular endothelial cells abundantly express GC-A, which exhibits a protective role in the cardiovascular system (8, 9). Therefore, to clearly show that the antimitastatic effect of ANP does not depend on GC-A expression in tumor cells, we examined the hematogenous pulmonary metastasis of B16/F10 cells in both endothelium-specific GC-A knockout mice (termed EC GC-A-KO mice) and GC-A transgenic mice (termed EC GC-A-Tg mice) (SI Appendix, Fig. S4). EC GC-A-KO mice exhibited a significant elevation of blood pressure and cardiac hypertrophy compared with GC-A flox/flox mice. These phenotypic data were consistent with the previous report (14). The number of pulmonary metastases was significantly higher in EC GC-A-KO mice than in GC-A flox/flox mice (Fig. 2E and F). Furthermore, cardiac metastases were found in one-third of EC GC-A-KO mice, whereas no cardiac metastasis was found in GC-A flox/flox mice (Fig. 2E). Overall survival was significantly shorter in EC GC-A-KO mice compared with GC-A flox/flox mice (Fig. 2G). In contrast, the number of pulmonary metastases was significantly lower in EC GC-A-Tg mice than in WT mice (Fig. 2H and I), and EC GC-A-Tg mice survived significantly longer than WT mice (Fig. 2J). Collectively, these data suggest that endothelial GC-A activated by ANP prevents hematogenous pulmonary metastasis of cancer cells in mice.

Fig. 1. Effect of ANP treatment on RFS in patients with surgically resected nonsmall cell lung cancer. (A) Kaplan–Meier curves of the ANP group and control group (surgery alone) in all patients (P = 0.018, log-rank test). (B) Kaplan–Meier curves of the above groups in propensity score-matched patients (P = 0.0013, log-rank test). RFS was measured from the day of surgery to cancer recurrence.
ANP was significantly inhibited by knockdown of E-selectin, but not by knockdown of VCAM-1 or ICAM-1 (Fig. 3 D and E and SI Appendix, Fig. S6A).

To search for genes that could be responsible for the ANP-mediated inhibition of tumor cell attachment to vascular endothelial cells, we performed microarray analyses of human umbilical vein endothelial cells (HUVECs) stimulated by ANP. E-selectin expression was markedly reduced in ANP-treated HUVECs compared with those treated with vehicle alone (SI Appendix, Table S2). Consistently, the expression of E-selectin induced by LPS in HPAECs was inhibited by ANP, whereas that of neither VCAM-1 nor ICAM-1 was affected (Fig. 4A). ANP also significantly inhibited the expression of E-selectin induced by LPS in HMVEC-L (SI Appendix, Fig. S5D). Furthermore, ANP-mediated suppression of LPS-induced E-selectin expression was not observed in HPAECs depleted of GC-A (Fig. 4B and SI Appendix, Fig. S6B). These data indicate that ANP suppresses E-selectin expression through GC-A. Because LPS induces inflammation by inducing nuclear translocation of NF-κB, which in turn promotes E-selectin expression (19), we examined the effect of ANP on nuclear translocation induced by LPS. ANP significantly inhibited the accumulation of NF-κB in the nucleus of LPS-induced HPAECs (Fig. 4 C and D).

Finally, we performed an in vivo study to evaluate the effects on LPS-induced E-selectin expression in the lung. Five hours after injection of LPS, E-selectin expression in the lung increased dose-dependently, whereas pretreatment with ANP attenuated this increase at both the gene and protein levels (Fig. 4E and F). Consistently, immunohistochemical analysis showed that pretreatment with ANP inhibited LPS-induced E-selectin expression in the CD31+ vascular endothelium (Fig. 4G). Furthermore, mice pretreated with E-selectin–neutralizing antibody exhibited a significant reduction of LPS-induced pulmonary metastasis of B16/F10 cells (SI Appendix, Fig. S7). Collectively, these data suggest that ANP inhibits E-selectin expression and reduces the E-selectin–mediated adhesion of tumor cells to vascular endothelium of the lung upon LPS-induced inflammation.

Fig. 2. ANP inhibits the LPS-augmented metastasis of A549-EGFP lung cancer cells and B16/F10 mice melanoma cells to the lung. (A) Representative EGFP images of the lungs of mice that were pretreated with or without LPS and then injected with A549-EGFP cells (1 × 10⁵ cells per mouse) and continuously treated with or without ANP for 4 wk. The mice were killed 6 wk after the injection of tumor cells. (B) Bar graph showing the number of nodules representing pulmonary metastasis of A549-EGFP cells in mice grouped as in A. Data are means ± SEM (n = 6, each group). **P < 0.01, unpaired two-tailed t test. (C) Representative images of the lungs of mice that were pretreated with or without LPS and then injected with B16/F10 cells (2 × 10⁵ cells per mouse) and continuously treated with or without ANP for 2 wk. The mice were killed 2 wk after the injection of the tumor cells. (D) Bar graph showing the number of nodules representing pulmonary metastasis of B16/F10 cells in mice grouped as in C. Data are means ± SEM (n = 6, each group). ***P < 0.001, unpaired two-tailed t test. (E) Representative images of the lungs and hearts (Top and Middle, respectively) and histological cross-sections of the hearts (Bottom) of the GC-A−/− mice and EC GC-A-KO mice after injection of B16/F10 cells (2 × 10⁵ cells per mouse). The mice were killed 2 wk after the injection of the tumor cells. (Scale bars, 500 μm.) Red arrows indicate metastasis in the heart. (F) Bar graph showing the number of nodules representing pulmonary metastasis of B16/F10 cells in mice grouped as in E. Data are means ± SEM (n = 10, each group). **P < 0.01, unpaired two-tailed t test. (G) Kaplan–Meier curves comparing survival times between WT and EC GC-A-KO mice after injection of B16/F10 cells (2 × 10⁵ cells per mouse). n = 15 (each group), **P < 0.05, log-rank test. (H) Representative images of the lungs of WT and EC GC-A-Tg mice after injection of B16/F10 cells (5 × 10⁴ cells per mouse). The mice were killed 2 wk after the injection of tumor cells. (I) Bar graph showing the number of nodules representing pulmonary metastasis of B16/F10 cells in mice grouped as in H. Data are means ± SEM (n = 10, each group). **P < 0.01, unpaired two-tailed t test. (J) Kaplan–Meier curves comparing survival times between WT and EC GC-A-Tg mice after injection of B16/F10 cells (5 × 10⁴ cells per mouse). n = 15 (each group), **P < 0.05, log-rank test.

Fig. 3. ANP inhibits LPS-regulated E-selectin–dependent adhesion of cancer cells to vascular endothelial cells. (A) Representative images of the adhesion of tumor cells (A549-EGFP, Upper; H460-EGFP, Lower) to monolayer-cultured HPAECs pretreated with or without ANP. (B and C) Bar graphs showing the number of A549-EGFP cells (B) or H460-EGFP cells (C) attached to monolayer-cultured HPAECs pretreated with or without ANP. Data are means ± SEM (n = 5, each group). *P < 0.05, **P < 0.01, ***P < 0.001, unpaired two-tailed t test. (D) Representative images of A549-EGFP cells attached to HPAECs depleted of the indicated molecules by siRNA treatment and treated with LPS. (E) Bar graph showing the number of A549-EGFP cells attached to HPAECs treated as in (D). Data are means ± SEM (n = 5, each group). *P < 0.05, one-way ANOVA. (Scale bars, 500 μm.)
Fig. 4. ANP–GC-A signaling attenuates LPS-induced E-selectin expression. (A) Immunoblot analysis of the lysates of HPAECs pretreated with or without ANP followed by LPS stimulation; antibodies used are indicated on the left. Each blot is representative of six independent experiments. (B) E-selectin expression assessed by immunoblot analysis of the lysates of HPAECs transfected with the indicated siRNAs and stimulated with LPS. The result shown is representative of six independent experiments. (C) Bright field images (Left) and NF-κB immunofluorescence images (Right) of HPAECs that were unstimulated (control, Top), stimulated with LPS alone (Middle), or pretreated with ANP followed by LPS stimulation (Bottom). Each image is representative of five independent experiments. (Scale bars, 100 μm.) (D) Quantitative analyses of C. Each column shows the percentage of HPAECs with nuclear NF-κB expression in the indicated group. Data are means ± SEM (n = 5, each group); **P < 0.01, unpaired two-tailed t test. (E) Quantitative reverse transcriptase PCR analysis of E-selectin mRNA levels in the lungs of mice pretreated with ANP or vehicle (control) and treated with LPS. Data are normalized to 36B4 mRNA levels. Data are means ± SEM (n = 6, each group); *P < 0.05, unpaired two-tailed t test. (F) Immunoblot analysis of E-selectin levels in lung lysates of mice pretreated with or without ANP followed by LPS stimulation (1.0 mg/kg) for 5 h. Each blot is representative of six independent experiments. (G) E-selectin images (Center), and merged images with DAPI staining (Right) of the lungs of mice pretreated with or without ANP followed by LPS stimulation (1.0 mg/kg) for 5 h. Each image is representative of six independent experiments. Nuclei are stained with DAPI (blue). (Scale bars, 100 μm.)

Discussion

Although many clinical trials aimed at preventing cancer recurrence during the perioperative period have been conducted, no prophylactic treatments have been established. The failure of these trials might be ascribed to the risk of surgery alone, and the side effects of the chemicals used in the trials (20, 21). We previously showed that ANP prevents the incidence of postoperative complications after lung cancer surgery (10–12). Here we demonstrate that cancer recurrence after curative surgery was significantly lower in ANP-treated patients than in control patients, suggesting that ANP could potentially be used to prevent cancer recurrence after surgery. We assumed two possibilities as to how ANP inhibited tumor metastases; one was that ANP directly inhibited the tumor cell proliferation and the other was that ANP indirectly inhibited tumor cell metastases by acting on nontumor cells. In previous studies of the direct effects of ANP on cancer cells, both inhibitory and stimulatory effects of ANP on the growth of cancer cells have been reported (22, 23); therefore, the direct effects of ANP on cancer cells remain controversial. In the present study, we focused on the possibility that ANP indirectly inhibits tumor cell metastases through effects on nontumor cells.

Our discovery that mice pretreated with ANP exhibited a dramatic reduction of LPS-induced pulmonary metastasis of introduced cancer cells provides direct evidence that ANP can prevent tumor metastasis in mice. This notion is supported by our finding that mice that specifically overexpress or lack expression of the receptor of ANP (i.e., GC-A) in the vascular endothelium have reduced or enhanced numbers of metastases, respectively, compared with the appropriate control mice. These results suggest that ANP prevents early relapse in patients at least in part by preventing metastasis through the vascular endothelium.

Surgical procedures induce postoperative complications and early recurrence after surgery by releasing inflammatory cytokines, such as IL-1β and TNF-α (1, 2). Recent studies indicate that postoperative complications, including severe inflammatory reaction and infection, after various types of cancer surgery are associated with poor cancer-specific survival (24–26). Endothelial cells that become inflamed during surgery are considered to be prone to adhering to circulating tumor cells, thereby allowing the initiation of metastasis (5, 6). Although most circulating tumor cells undergo rapid cell death by apoptosis (27, 28), it is possible that surgical inflammation promotes the adherence of residual cancer cells to inflamed endothelial cells (5, 6). ANP has anti-inflammatory and anti-infectious activity on endothelial cells. ANP pretreatment reduces serum TNF-α levels and NF-κB activation by inhibiting IκB-phosphorylation in mice injected with LPS (29) and has a protective role against LPS-induced lung injury and endothelial barrier dysfunction (30). Our finding that ANP has anti-inflammatory action (i.e., suppression of LPS-induced E-selectin) in vascular endothelial cells in mice is consistent with these studies. Taken together, our results suggest that ANP-mediated inhibition of metastasis occurs through inhibition of the inflammatory response.

Among the vascular adhesion molecules, E-selectin is essential for recruitment of inflammatory cells to damaged tissues (31), and it enables circulating tumor cells to roll and tether on the endothelium. Recent studies have shown that cross-talk between E-selectin and integrins could facilitate the movement of not only inflammatory cells but also tumor cells through the endothelium to inflammatory foci (16–18). In fact, tumor metastasis is increased in the lungs of E-selectin–overexpressing mice and reduced in E-selectin knockout mice (18). Therefore, E-selectin is considered to play a central role in hematogenous metastasis (16–18). In a clinical study, Gogali et al. reported that serum levels of soluble E-selectin in lung cancer patients were significantly elevated compared with those in control subjects (32). However, we assume that the antimetastasis activity of ANP does not solely depend upon the suppression of E-selectin, because extravasation of cancer cells in the metastatic process is regulated by many other steps. Recent experimental reports demonstrated that inflammatory chemokines including chemokine ligand (CCL) 2 and CCL5 contributed to not only leukocyte recruitment but also tumor cell homing to activated endothelial cells (33, 34). Because we focused on only E-selectin expressions in this study, further studies are necessary to elucidate the detailed mechanism and role of the ANP–GC-A system in cancer metastasis.

Because most current chemotherapeutic agents are cytotoxic and cause many side effects, chemotherapy cannot be used during surgical resection to prevent cancer recurrence. In contrast, ANP is an endogenous and physiological peptide and has been proved not to cause severe adverse effects when used in patients with heart failure (35). Because the target of ANP is considered to be vascular endothelium in all organs that express the GC-A receptor, including lung, liver, and brain, ANP might inhibit hematogenous cancer metastasis to all organs expressing GC-A receptor and could be used for all kinds of malignant tumors.

Materials and Methods

Clinical Study. We retrospectively evaluated 552 consecutive patients who underwent curative surgery for nonsmall cell lung cancer at Osaka University Hospital and National Hospital Organization Toneyama Hospital from August 2007 to December 2011. Patients with carcinoma in situ and those undergoing
a limited resection, including wedge resection, were excluded. Patients with incomplete postoperative follow up (n = 8) were also excluded. Segmentectomy for curative surgery were not excluded. Finally, 467 patients who underwent curative surgery were included in the present study.

RFS, defined as the time from the day of surgery to cancer recurrence, was compared between patients who received ANP during the perioperative period and those that received surgery only. In the ANP group, the subjects received ANP intravenously at 0.025 μg kg/min (Daichii-Sankyo Pharmaceutical) without a bolus for 3 d continuously, starting just before the induction of general anesthesia. We previously reported that ANP has a prophylactic effect against postoperative cardiopulmonary complications for patients with elevated preoperative brain natriuretic peptide levels (10, 12, 36).

Therefore, we performed a propensity score matched analysis to reduce the treatment selection bias for each group. The propensity score was estimated using a logistic regression model adjusted for age, sex, pathological staging (lung cancer tumor, node, metastases (TNM) staging seventh edition), cancer histology, and preoperative brain natriuretic peptide levels. These variables were chosen for potential associations with the outcome of interest. An independent statistician selected the patients by matching propensity scores without access to clinical outcome information. Patient characteristics for the full and propensity score-matched cohorts are listed in Table S4. ANP or vehicle infusion was continued until the mice were euthanized. Six or 8 wk (AS49-EGFP) or 2 wk (B16/F10) after tumor cell injection, the mice were killed for evaluation of pulmonary metastases. The number of nodules reflecting pulmonary metastasis of AS49-EGFP or B16/F10 cells was counted by using images obtained with a fluorescent microscope (OVI100, Olympus) or a camera (CX6, Ricol).

Adhesion Assay. To quantify tumor cell adhesion to HPAECs or HMVEC-L, a standardized cell adhesion assay was performed by using a modification of the method of van Rossen et al. (37). Briefly, HPAEC or HMVEC-L monolayers were established in 35-mm collagen-coated dishes (IWAKI). Before coculture with tumor cells, HPAEC or HMVEC-L were either pretreated with 0.1 μM ANP or vehicle for 3 d. ANP in 0.9% saline or vehicle was divided into two groups: vehicle and ANP groups with two kinds of cancer cells (either AS49-EGFP or B16/F10 cells). ANP or vehicle alone was implanted 1 d before cancer cells injection. On the next day, cancer cells (either AS49-EGFP or B16/F10 cells) were injected into the tail vein. To ascertain the efficiency of ANP administration, we confirmed that the blood levels of cGMP were elevated effectively when ANP (0.5 μg kg⁻¹ min⁻¹) was infused subcutaneously in the mice (Table S5). ANP or vehicle infusion was continued until the mice were euthanized. Six or 8 wk (AS49-EGFP) or 2 wk (B16/F10) after tumor cell injection, the mice were killed for evaluation of pulmonary metastases. The number of nodules reflecting pulmonary metastasis of AS49-EGFP or B16/F10 cells was counted by using images obtained with a fluorescent microscope (FSX100, Olympus) and a computer-aided manipulator program (Cell-sense, Olympus). In addition, the adhesion of the tumor cells to HPAECs depleted of either E-selectin, VCAM-1, or ICAM-1, and stimulated with LPS, was similarly analyzed.

Statistics. Data are presented as means ± SEM and were analyzed by using a two-tailed Student’s t-test for paired samples or one-way ANOVA for multiple groups. P values less than 0.05 were considered statistically significant.

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Supporting Information for

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SI Materials and Methods

Knockout and transgenic mice. To generate global and conditional depletion of the GC-A gene, TT2 embryonic stem (ES) cells derived from an F1 hybrid of C57BL/6 and CBA mice (1) were transfected with GC-A-targeting vector (SI Appendix, Fig. S2A), selected in the presence of G418 and screened for homologous recombination by PCR analysis and Southern blotting. Chimeric mice with a high ES cell contribution were bred with cytomegalovirus (CMV)-Cre mice (C57BL/6 strain background), which express Cre recombinase under the control of CMV promoter, to generate heterozygous GC-A+/− mice. GC-A+/− mice were intercrossed to obtain the chimeric mice (75% C57BL/6 and 25% CBA genetic background). To establish the conditional GC-A knockout mice, the chimeric mice were crossed with CMV-Flp mice (C57BL/6 strain background), which express Flp recombinase under the control of the CMV early enhancer and chicken β-actin promoter to remove the PKG-Neo-pA cassette. The resulting allele was designated as GC-A<sup>Δlox/Δlox</sup> (Accession. No. CDB0830K:

[http://www.cdb.riken.jp/arg/mutant%20mice%20list.html](http://www.cdb.riken.jp/arg/mutant%20mice%20list.html). To inactivate the GC-A gene
in vascular endothelial cells, the GC-A^{flox/flox} mice were bred with Tie2-Cre mice (C57BL/6 strain background) provided by T.N. Sato (Nara Institute of Science and Technology, Nara, Japan) and M. Yanagisawa (University of Texas Southwestern Medical Center, Dallas, TX) (2, 3). The genotype of the WT and GC-A^{flox/flox} mice was confirmed by means of PCR with the use of the F1-R1 and F2-R2 primer sets listed below (SI Appendix, Fig. S2B):

F1, 5’-GAGGGCGCAGTAGAACTAGG-3’;
R1, 5’-GTGAGCGACTCAACATCACAG-3’; and
F2, 5’-GCTATAGGAGTTTGGGAGGGTT-3’;
R2, 5’-TAATCCCATGGCGTTCCGT-3’.

We developed transgenic mice that express GC-A exclusively in the vascular endothelium. The transgene consists of a 2.1-kb fragment of the murine Tie2 promoter, a 10.0-kb fragment of the murine Tie2 enhancer, and exons 1–3 from the 5’ untranslated region of the murine Tie2 gene (provided by T.N. Sato) followed by GC-A cDNA and a human growth hormone polyadenylation site (SI Appendix, Fig. S2D). Linearized DNA containing the transgene without vector sequence was injected into the pronuclei of single-cell fertilized C57/BL6 embryos. The insertion of the transgene was confirmed by PCR by using one primer specific for the Tie2 promoter and another primer specific for GC-A. All mice were housed under specific pathogen-free conditions.

**Microarray analysis.** HUVECs were treated with 0.1 μM ANP (Peptide Institute, Inc., Osaka, Japan) or vehicle for 6 h, and then total RNA was isolated with the use of Trizol (Invitrogen, Carlsbad, CA). RNA was quantified by using an ND-1000 Spectrophotometer (NanoDrop Technologies, Wilminton, DE) and quality was confirmed by using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). Two hundred nanograms of total RNA was used to produce cyanine 3-CTP-labeled
cRNA by using an Agilent Low Input Quick Amp Labeling Kit. Following labeling and clean up, cRNA and dye incorporation were quantified with the use of an ND-1000 Spectrophotometer. After fragmentation, labeled cRNA samples were hybridized to Agilent whole human genome 4 x 44 K DNA microarrays. Following hybridization and washing, the microarrays were scanned with an Agilent DNA Microarray Scanner G2565BA and the intensities were extracted with Agilent Feature Extraction software ver.10.7.3.1. Data analyses were conducted with Agilent GeneSpring GX software ver.12.5. Microarray data were normalized with the 75th percentile shift method. Preprocessing of input data was concluded by baseline transformation to the mean of control samples. Differential gene expression was statistically determined by ANOVA. The resultant $P$-values were corrected for multiple testing according to the Benjamini–Hochberg method. A list was acquired of genes that were either induced or suppressed more than 1.5 fold (corrected $P$ value < 0.05) (SI Appendix, Table S2). The microarray data from HUVECs have been deposited in the NCBI Gene Expression Omnibus under accession number GSE56976.

**Immunofluorescence analyses in vivo.** To investigate the expression of E-selectin in the endothelium in vivo, lung samples of mice pre-treated with or without ANP (0.5 $\mu$g/kg/min) and stimulated with LPS (1.0 mg/kg) for 5 h were embedded in optimal cutting temperature compound (Sakura Finetek Japan Co, Ltd., Tokyo, Japan), snap-frozen in acetone–dry ice and sectioned to 6-µm thickness. The frozen tissue sections were fixed in acetone for 15 min at 4 °C and air-dried for 30 min. After each step, the tissue sections were rinsed twice in phosphate-buffered saline (PBS) for 5 min. The fixed-frozen tissue sections were then incubated with Protein Block (DakoCytomation, Glostrup, Denmark) for 15 min. The sections were immunostained with anti-mouse CD62E antibody (E-selectin; 1:500; 55029; BD Biosciences, Bedford, MA) and anti-
CD31 (PECAM1; 1:500; ab28364; Abcam, Cambridge, MA) and stained with DAPI (Vector Laboratories, Inc., Burlingame, CA). Immunofluorescence detection was performed by using species-matched secondary antibodies: Alexa Fluor 488 goat anti-rat IgG antibody (1:1000; A-11006; Molecular Probes, Eugene, OR) and Alexa Fluor 488 goat anti-rabbit IgG antibody (1:1000; A-11034; Molecular Probes). The immunofluorescence images were obtained by using a fluorescence microscope (FSX100). Five randomly selected fields per section were analyzed. NF-κB localization was examined by immunofluorescence analysis. HPAECs pre-treated with 0.1 μM ANP for 30 min and subsequently stimulated with 200 ng/ml LPS for 100 min were fixed with 4% paraformaldehyde and then membrane-permeabilized with TBS containing 0.01% Triton-X. The fixed cells were immunostained with anti-NF-κB (8242; Cell Signaling Technology, Danvers, MA), and the immunoreactivity was visualized with Alexa Fluor 488 goat anti-rabbit IgG antibody. Immunofluorescence images were obtained by using an epi-fluorescence microscope (IX-81, Olympus). The number of nuclear NF-κB signal-positive cells was counted in at least 5 images from each section.

**Measurements of blood pressure and heart rate.** Blood pressure and heart rate were measured in conscious mice by means of the tail-cuff method, as previously reported (4).

**Cell proliferation assay.** Proliferation of tumor cells treated with ANP was determined by assaying viable cell numbers with a Cell Counting Kit-8 (CCK-8) according to the manufacturer's protocol (Dojindo, Kumamoto, Japan). A549, H460, and B16/F10 cells were plated in 96-well plates at 2000–5000 cells/well in medium supplemented with 10% FCS. On the second day, the medium was changed to serum-free medium, and the cells were incubated for a further 24 h. Then, ANP at the concentrations indicated in the figures was applied to the cells. To count the number of cells, CCK-8 reagents were
added after 24 and 48 h, and the cells were incubated for an additional 1–1.5 h. The absorbance values were measured at 450 nm by using a microplate reader.

**Quantitative real-time polymerase chain reaction (qRT-PCR).** Total RNA was extracted from isolated HPAECs or lung tissues by using an RNeasy mini kit (Qiagen, Hilden, Germany) and then reverse-transcribed into cDNA by using a Quantitect Reverse Transcription kit (Qiagen). PCR amplification was performed by using SYBR Premix Ex Taq (Takara, Shiga, Japan) and a Light Cycler 480 System II (Roche Applied Science, Indianapolis, IN). For normalization, 36B4 was used as an internal control. PCR amplification was carried out with the following primer sets: 36B4 (Fwd, 5’-TCATTGTGGGAGCAGACAATGTGGG-3’, Rev, 5’-AGGTCCTCCTTTGGTAACACAAAGC-3’), human E-selectin (Fwd, 5’-CTCGGACATGTGGAGCCACAGGACA-3’, Rev, 5’-GGCTTTTGGAGCTGCTGGCAGGAAC-3’), mouse E-selectin (Fwd, 5’-GAGCTCAGAAAATCTACAGTGACTCCT-3’, Rev, 5’-GGATTTGTTGGTCTCAGCTGCTGACACAT-3’), human VCAM-1 (Fwd, 5’-GGAAAAAGTCTTTCTTTGGCCAGCTA-3’, Rev, 5’-GGAGCTGTAGACCCTCATTGGAAC-3’), human ICAM-1 (Fwd, 5’-GGCAAGAACCTTACCCTACGCTGCC-3’, Rev, 5’-GTTCAGTGCCAGCAGAAATTGGG-3’), mouse ICAM-1 (Fwd, 5’-GGCAAGAACCTTACCCTACGCTGCC-3’, Rev, 5’-GTTCAGTGCCAGCAGAAATTGGG-3’), human GC-A (Fwd, 5’-CAGCAACATCCTGGCACACCTTGTGCTGGC-3’, Rev, 5’-GAACTTTGCTTTGGCACCTTTCATTTTC-3’), human or mouse NPR-C (Fwd, 5’-ATTTGAAGGTATCGCCCAGGCAGGTG-3’, Rev, 5’-GGGCCCAAGGATATTGACATT-3’).
Short interfering RNAs (siRNAs). siRNAs and control siRNA were purchased from Ambion (Austin, TX) or Sigma-Aldrich. The sequences of the sense and antisense strands of the siRNAs were as follows: For E-selectin, 5'-CAACAAUAGGCAAAAGAUtt-3' and 5'-AUCUUUUGCCUAUUGUUGgg-3'; for VCAM-1, 5'-GGAGUUAUUUGAUUGGtt-3' and 5'-UCCCAAUCAAUACUCtt-3'; for ICAM-1, 5'-AGUCAACAGCUAAACCUCUt-3' and 5'-AACGUUUUAAGCUGCUGUUGACUgc-3'; and for GC-A, 5'-GGUACUCACUCACAAUGAtt-3' and 5'-UCAUUGGUGAGUGAGUACCgg-3', respectively. Reverse transfection of siRNA (5 nM final concentration) was carried out by using Lipofectamine RNAiMAX (Invitrogen). The efficiency of the knockdown of the molecules was examined by means of qRT-PCR and immunoblot analyses 48–72 h after transfection (SI Appendix, Fig. S3).

Western blot analysis. HPAECs, HMVEC-L, or lung tissues were lysed with RIPA buffer containing protease inhibitors. Total cell lysates (10 μg/lane) were separated on a 10%–20% gradient gel (Bio-Rad, Hercules, CA) and then transferred onto polyvinylidene difluoride membranes (Millipore, Bedford, MA). Anti–E-selectin (H-300), anti–VCAM-1 (C-19), anti–ICAM-1 (G-5), anti–GC-A (G-2), and anti-GAPDH (6C5) antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

Measurement of blood levels of cyclic GMP. To determine the effective dosage of ANP for continuous intravenous injection and subcutaneous injection, we measured the plasma level of cyclic GMP (cGMP) after intravenous infusion or subcutaneous administration of ANP. For intravenous ANP infusion, ANP dissolved in 0.9% saline was administered intravenously (0.025 or 0.1 μg/kg/min) with osmotic pumps (Alzet Model 1002) for 4 days. After mice were anesthetized with pentobarbital sodium, a
mouse jugular catheter (#7702, DURECT Corporation) attached to an osmotic pump was inserted into the left jugular vein. Then, the body of the osmotic pump was placed in the subcutaneous pocket created in the right flank. Mice that were treated with 0.9% saline alone in the same manner served as the vehicle control group. For subcutaneous ANP administration, ANP (0.5 µg/kg/min) dissolved in 0.9% saline was administered with an osmotic pump (Alzet Model 1002) for 4 days. Mice that were treated with 0.9% saline in the same manner served as the vehicle control group. The plasma concentration of cGMP was measured by radioimmunoassay with a cGMP assay kit (Yamasa Shoyu Co., Chiba, Japan) as previously described (5).

**E-selectin–neutralizing antibody for lung metastasis model.** To investigate whether E-selectin–neutralizing antibody exerts anti-tumor metastasis effects, we used the experimental lung metastasis model with LPS stimulation. Five hours before injection of tumor cells, C57BL/6 mice were intravenously injected with E-selectin–neutralizing antibody (#550290, 10E9.6, rat IgG2a; 30 µg per mouse; BD Biosciences) or the same amount of vehicle alone (#559073, ratIgG2a control; BD Biosciences), followed by injection or no injection of 1.0 mg/kg LPS. B16/F10 cells (3 × 10^5 cells/mouse) were then injected into the tail vein. Two weeks after tumor cell injection, the mice were euthanized for evaluation of pulmonary metastases.
References


Fig. S1. ANP inhibits the LPS-augmented metastasis of A549-EGFP lung cancer cells and B16/F10 mice melanoma cells to the lung. (A) All EGFP images of the lungs of mice that were pre-treated with or without LPS and then injected with A549-EGFP cells (1 × 10⁶ cells/mouse) and continuously treated with or without ANP for 4 weeks. The mice were sacrificed 6 weeks after the injection of the tumor cells. (B) Quantitative reverse transcriptase PCR analysis of GC-A mRNA levels in various cancer cell lines. Data are normalized relative to 36B4 mRNA levels and means ± SEM (n = 3). (C) All images of the lungs of mice that were pre-treated with or without LPS and then injected with B16/F10 (2 × 10⁵ cells/mouse) and continuously treated with or without ANP for 2 weeks. The mice were sacrificed 2 weeks after the injection of tumor cells.
**Fig. S2.** ANP inhibits pulmonary metastasis of A549-EGFP lung cancer cells and B16/F10 mice melanoma cells to the lung. (A) Representative EGFP images of the lungs of mice injected with A549-EGFP cells (1 × 10^6 cells/mouse) and continuously treated with or without ANP for 4 weeks. The mice were sacrificed 8 weeks after the injection of tumor cells. (B) Bar graph showing the number of nodules representing pulmonary metastasis of A549-EGFP cells in mice grouped as in (A). Data are means ± SEM. (n = 6, each group). **P < 0.01, unpaired two-tailed t-test. (C) Representative images of the lungs of mice injected with B16/F10 cells (5 × 10^5 cells/mouse) and continuously treated with or without ANP for 2 weeks. The mice were sacrificed 2 weeks after the injection of the tumor cells. (D) Bar graph showing the number of nodules representing pulmonary metastasis of B16/F10 cells in mice grouped as in (C). Data are means ± SEM (n = 6, each group). **P < 0.01, unpaired two-tailed t-test.
Fig. S3. Direct effects of ANP on cancer cell lines. (A) Representative images of the expression of the indicated genes in cancer cells including A549, H460, and B16/F10 cells. (B-D) Cell proliferation of A549 (B), H460 (C), and B16/F10 (D) cells with or without ANP treatment. Data are means ± SEM (n = 8–16, each group).
Fig. S4. Generation of GC-A<sup>flox/flox</sup> mice and EC GC-A-Tg mice. (A) Schematic representation of the WT allele, the targeted allele and the conditional allele. In the targeted allele, exon 1 of the GC-A gene (yellow box) is flanked by two lox P site (green arrowheads). Together with the first lox P site, a neomycin selection cassette, PKG-Neo-pA (gary box), flanked by two frt sites (red arrowheads) has been inserted. Mice carrying the targeted allele were crossed with transgenic mice expressing Flp recombinase under the control of CAG promoter (i.e., CMV early enhancer and chicken beta actin promoter) to remove the PKG-Neo-pA cassette, resulting in mice with a conditional allele (GC-A<sup>flox/flox</sup>). (B) PCR analysis of the genomic DNA from WT and GC-A<sup>flox/flox</sup> mice were performed by using two primer sets (F1-R1, F2-R2; indicated by red arrows) to confirm insertion of the lox P sites. In the WT allele, 356-bp (F1-R1) and 350-bp (F2-R2) fragments were amplified, whereas in the GC-A<sup>flox/flox</sup> allele, 570-bp (F1-R1) and 543-bp (F2-R2) fragments were amplified. (C) Representative images of immunoblot analysis of GC-A protein in lung endothelial cells of GC-A<sup>flox/flox</sup> mice and EC GC-A-KO mice, which have an inactivated GC-A gene in endothelial cells. EC GC-A-KO mice were produced by breeding GC-A floxed mice with Tie2-Cre mice, which carry the Cre recombinase driven by the Tie2 promoter. (D) Schematic diagram of the transgenic construct containing the Tie2 promoter and enhancer, which was used to produce the EC GC-A-Tg mice. (E) Representative images of immunoblot analysis of GC-A protein of lung endothelial cells of WT and EC GC-A-Tg mice.
Fig. S5. Effects of ANP on HMVEC-L with LPS stimulation. (A) Representative images of the adhesion of tumor cells (A549-EGFP, upper panels; H460-EGFP, lower panels) to monolayer-cultured HMVEC-L pre-treated with or without ANP. (B, C) Bar graphs showing the number of A549-EGFP (B) or H460-EGFP (C) cells attached to monolayer-cultured HMVEC-L pre-treated with or without ANP. Data are means ± SEM (n = 5, each group). *P < 0.05, **P < 0.01, unpaired two-tailed t-test. (D) Immunoblot analysis of the lysates of HMVEC-L pre-treated with or without ANP followed by LPS stimulation; antibodies used are indicated on the left. Each blot is representative of five independent experiments.
Fig. S6. Immunoblot analysis confirming the inhibition of E-selectin, VCAM-1, ICAM-1, and GC-A by siRNAs. (A) Immunoblot analysis confirming the inhibition of E-selectin, VCAM-1, and ICAM-1 in HPAECs treated with siRNAs specific for E-selectin, VCAM-1, or ICAM-1 followed by treated with LPS (50 ng/ml). (B) Immunoblot analysis confirming the inhibition of GC-A in HPAECs treated with siRNA specific for GC-A.
**Fig. S7.** E-selectin–neutralizing antibodies inhibit the LPS-augmented metastasis of B16/F10 mice melanoma cells to the lung. (A) All images of the lungs of mice that were pre-treated with E-selectin–neutralizing antibody or vehicle and then treated with or without LPS and finally injected with B16/F10 (3 × 10^5 cells/mouse). The mice were sacrificed 2 weeks after the injection of tumor cells. (B) Bar graph showing the number of nodules representing pulmonary metastasis of B16/F10 cells in mice grouped as in (A). Data are means ± SEM (n = 6, each group). **P < 0.01, ***P < 0.001, unpaired two-tailed t-test.
**Fig. S8.** Plasma levels of cGMP in mice injected with vehicle or ANP. Bar graph showing the plasma levels of cGMP in the indicated group. Data are means ± SEM (n = 6, each group). *P < 0.05, unpaired two-tailed t-test. iv., intravenous; sc., subcutaneous.
<table>
<thead>
<tr>
<th>Variables</th>
<th>Full cohort</th>
<th>Propensity score-matched cohort</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>ANP (n = 77)</td>
<td>Control (n = 390) S.D.</td>
</tr>
<tr>
<td>Age, years</td>
<td>68 (61–74)</td>
<td>74 (69–78) 0.61</td>
</tr>
<tr>
<td>Male</td>
<td>50 (65%)</td>
<td>268 (69%) 0.09</td>
</tr>
<tr>
<td>Stage</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1A</td>
<td>28 (36%)</td>
<td>165 (42%) 0.12</td>
</tr>
<tr>
<td>1B</td>
<td>28 (36%)</td>
<td>105 (27%) 0.20</td>
</tr>
<tr>
<td>2A</td>
<td>5 (6.5%)</td>
<td>31 (8.0%) 0.06</td>
</tr>
<tr>
<td>2B</td>
<td>9 (12%)</td>
<td>33 (8.5%) 0.11</td>
</tr>
<tr>
<td>3A</td>
<td>4 (5.2%)</td>
<td>45 (12%) 0.23</td>
</tr>
<tr>
<td>3B</td>
<td>2 (2.6%)</td>
<td>7 (1.8%) 0.05</td>
</tr>
<tr>
<td>4</td>
<td>1 (1.3%)</td>
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<tr>
<td>Histology</td>
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<tr>
<td>AD</td>
<td>39 (51%)</td>
<td>276 (71%) 0.40</td>
</tr>
<tr>
<td>SQ</td>
<td>30 (39%)</td>
<td>85 (22%) 0.37</td>
</tr>
<tr>
<td>Others</td>
<td>8 (10%)</td>
<td>29 (7.4%) 0.10</td>
</tr>
<tr>
<td>BNP*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1st</td>
<td>8 (10.4%)</td>
<td>110 (28.2%) 0.46</td>
</tr>
<tr>
<td>2nd</td>
<td>7 (9.1%)</td>
<td>111 (28.5%) 0.51</td>
</tr>
<tr>
<td>3rd</td>
<td>12 (15.6%)</td>
<td>102 (26.2%) 0.26</td>
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<tr>
<td>4th</td>
<td>50 (64.9%)</td>
<td>67 (17.2%) 1.11</td>
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</tbody>
</table>

AD, adenocarcinoma; BNP, brain natriuretic peptide; IQR, interquartile range; S.D., standardized difference; SQ, squamous cell carcinoma; *, preoperative BNP levels (pg/ml) are divided from 1st to 4th according to the IQR as follows: (min, 25%, 50%, 75%, max) = (2.0, 10.3, 18.4, 33.8, 307.1) in the full cohort and (2.9, 22.8, 43.1, 66.0, 307.1) in the propensity score-matched cohort.
Table S2. Microarray analysis of HUVECs stimulated with ANP

<table>
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<tr>
<th>Probe name</th>
<th>Gene symbol</th>
<th>Gene name</th>
<th>Fold change*</th>
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<tr>
<td>A_23_P97112</td>
<td>SELE</td>
<td>selectin E</td>
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<td>A_23_P17065</td>
<td>CCL20</td>
<td>chemokine (C-C motif) ligand 20</td>
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<td>A_23_P120227</td>
<td>LBH</td>
<td>limb bud and heart development homolog (mouse)</td>
<td>−1.94</td>
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<tr>
<td>A_23_P92499</td>
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<td>toll-like receptor 2</td>
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<tr>
<td>A_23_P37727</td>
<td>CX3CL1</td>
<td>chemokine (C-X3-C motif) ligand 1</td>
<td>−1.71</td>
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<tr>
<td>A_24_P151582</td>
<td>TEF</td>
<td>thyrotrrophic embryonic factor</td>
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<tr>
<td>A_23_P328740</td>
<td>NEURL3</td>
<td>neuralized homolog 3 (Drosophila) pseudogene</td>
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<tr>
<td>A_23_P53370</td>
<td>RND1</td>
<td>Rho family GTPase 1</td>
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<tr>
<td>A_32_P10133</td>
<td>PRINS</td>
<td>psoriasis associated RNA induced by stress (non-protein coding)</td>
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<tr>
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<td>A_32_P95852</td>
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<td>A_23_P152791</td>
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<tr>
<td></td>
<td></td>
<td>(monocarboxylic acid transporter 7)</td>
<td></td>
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<tr>
<td>A_23_P114983</td>
<td>TRIM63</td>
<td>tripartite motif containing 63</td>
<td>1.66</td>
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*Fold change in ANP-treated HUVECs compared with vehicle.
### Table S3. Hemodynamic parameters in mice with or without ANP

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Vehicle</th>
<th>ANP</th>
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<tbody>
<tr>
<td>Heart rate (bpm)</td>
<td>544±38</td>
<td>540±22</td>
</tr>
<tr>
<td>Systemic blood pressure (mmHg)</td>
<td>104±14</td>
<td>107±14</td>
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<tr>
<td>Diastolic blood pressure (mmHg)</td>
<td>57±8</td>
<td>60±7</td>
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Data are means ± SEM from 7-week–old mice treated with vehicle or ANP. (n = 6, per group).