ment. In terms of psychological tests, SDS and STAI showed no significant change during the study, and EDI did not show any increased fear of weight gain in these patients (data not shown).

Clinical course after discharge

All patients gained weight after discharge, as shown in Table 1. In case 3, menstruation resumed 6 months after discharge.

Discussion

The present study showed that ghrelin infusion (3 μg/kg twice a day) can decrease gastrointestinal symptoms and enhance hunger sensation and daily energy intake without serious adverse events in restricting-type AN patients. The major limitations of the present study relate to the lack of a randomized, placebo-controlled group and non-blindness of the investigators and the small number of patients recruited. A non-treated group is not possible due to ethical reasons. Although non-ghrelin infused subjects who receive intense counseling and supervision of dietitian might be considered as a control group, all subjects in the present study had already received those treatments as well as total parenteral nutrition during the previous admission but failed to increase body weight due to gastrointestinal symptoms. Since the daily energy intake of post-treatment period was still higher than that of pre-treatment period, we could not exclude a placebo effect of ghrelin. However, we insist that 4 patients who failed in gaining body weight for long periods but they could increase their food intake during and after ghrelin infusion. It is speculated as the patients told us that ghrelin triggered an improvement in gastrointestinal function, which ameliorated the fear of gastrointestinal discomfort after eating in these patients.

Ghrelin seems to improve gastrointestinal motility in AN patients in the present study. It is notable that borborygmi occurred immediately after ghrelin infusion and that abdominal fullness or constipation disappeared in all patients. Ghrelin plays a role in the regulation of gastrointestinal motility and acid secretion in rats [23-25] and increases the gastric emptying rate in normal-weight humans [26]. Although we did not investigate gastric emptying rate in AN patients after ghrelin injection, ghrelin improved epigastric discomfort. This was probably mediated partly through increased gastric peristalsis as shown in other diseases with gastrointestinal dysfunction [27-30].

Ghrelin infusion increased hunger scores evaluated by VAS questionnaires of AN patients in the present study. Although AN patients often report not to feel hunger or satiety sensation, hunger scores was higher just after ghrelin infusion than that before ghrelin infusion in 4 patients. Since the sensation of hunger is usually correlated with gastric emptying in humans [31], enhanced hunger sensation in AN patients may be caused in part by ghrelin-induced gastric motility. However, the stimulatory effects of ghrelin on hunger score did not last until the next meal. We considered that the short-term effect of ghrelin on hunger sensation is related to its rapid degradation. The plasma concentration of ghrelin reaches the peak at 15 min after injection and rapidly decreases [6]. Hunger scores before breakfast or dinner during ghrelin treatment were lower than those during both the pre- and post-treatment periods in case 1. It is likely that abdominal fullness induced by the increased amount of food eaten in the foregoing meal during ghrelin treatment probably disturbed the hunger sensation on the next meal.

In previous reports, continuous or repeated ghrelin infusion increased hunger sensation and food intake in healthy volunteers and various patients with appetite loss. Ghrelin infusion at a dose of 5 pmol/kg/min for 270 min increased food intake by 28 % in healthy young Caucasian volunteers [5] and by 31 % in middle-aged and elderly cancer patients [10]. Ghrelin infusion (2 μg/kg twice a day) for 3 weeks increased food intake and body weight by 0.8 kg in elderly patients with congestive heart failure [9], and by 1 kg in elderly patients with chronic obstructive pulmonary disease [8]. Moreover, in patients with functional dyspepsia, ghrelin infusion (3 μg/kg twice a day) for 2 weeks increased hunger sensation and food intake by 29 % without significant weight gain [11]. Since 1 kg weight gain requires 7000-8000 Kcal, the increase in energy intake achieved for 14 days in this study was not enough to lead to any considerable weight gain. Although case 4 gained 2.4 kg and showed remarkable improvement in nutritional parameters and malnutrition-related liver dysfunction, we believe that water retention during the refeeding period contributed to this weight gain [32]. A decrease in body weight of 2 patients (cases 1 and 2) during ghrelin study might be attributable to a decrease in malnutrition-induced fluid

retention or improvement in bowel movements.

There were two reports about the effects of ghrelin on appetite in AN patients. In one study, 5 pmol/kg/min ghrelin infusion for 300 min had little effect on appetite in severely emaciated as well as weight-recovered AN patients [33]. However, appetite was evaluated by VAS alone because the severely emaciated AN patients refused to eat in the study. Since it is well known that recognition of hunger and satiety in AN patients is generally impaired, appetite cannot be always analyzed correctly by VAS alone. Although 1 μg/kg ghrelin bolus infusion made AN patients feel hunger sensation in another study, their food intakes were not evaluated [34]. We therefore believe that studies aiming to investigate ghrelin as an appetite-stimulating substance should recruit only AN patients who are fully motivated to gain weight by psycho-educational therapy.

Adverse effects such as abdominal discomfort, diarrhea, transient flushing, truncal perspiration, and somnolence have been reported after ghrelin injection [6]. Two patients in the present study occasionally reported a warm sensation in the trunk and mild sweating. Since case 5 experienced mild abdominal pain and several episodes of loose stools per day, we reduced the dose of ghrelin to 1.5 μg/kg, which improved these symptoms. No other serious physical or biochemical deteriorations occurred. Moreover, malnutrition-related liver dysfunction and endocrinologic abnormalities in case 4 were improved after ghrelin treatment. Interestingly, ghrelin infusion increased somnolence in the study [33], however, none of the present 5 subjects reported increased sleepiness. We did not observe increased fear concerning weight gain, abnormal behavior, or unstable mental status owing to an increase in appetite during ghrelin treatment, and psychological tests did not demonstrate any significant change in mental state. The present patients who motivated to gain body weight felt happy to be able to eat after ghrelin infusion, and they were pleased to be free from uncomfortable gastrointestinal symptoms after this ghrelin study. It is notable that all patients gained weight after discharge.

In conclusion, we found that ghrelin decreases gastrointestinal symptoms and increases hunger sensation and daily energy intake without serious adverse events in AN patients. A double-blinded, randomized, and placebo-controlled study is indispensable for developing ghrelin as an effective appetite-stimulating therapy for AN patients. The present study would contribute to investigations for therapeutic potential of ghrelin in AN patients.

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Impaired Recovery of Blood Flow After Hind-Limb Ischemia in Mice Lacking Guanylyl Cyclase-A, a Receptor for Atrial and Brain Natriuretic Peptides

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Objective—Atrial and brain natriuretic peptides (ANP and BNP, respectively) function via guanylyl cyclase (GC)-A, resulting in diuresis, natriuresis, and blood vessel dilation. Here, we investigated the role of endogenous ANP/BNP-GC-A signaling on reparative vascular remodeling using a hind-limb ischemia model.

Methods and Results—In GC-A–deficient mice (GC-A-KO), hind-limb ischemia resulted in autoamputation or severe ulcers in 60% of mice (6/10) during the 28-day observation period. In wild-type (WT) mice, partial amputation or mild ulcers were detected in only 20% of mice (2/10). Laser Doppler perfusion imaging revealed that the recovery of blood flow in the ischemic limb was significantly inhibited in GC-A-KO mice compared with WT mice. Immunostainings with anti–PECAM-1 antibody demonstrated that, in GC-A-KO, the capillary density of the ischemic tissue was significantly diminished compared to WT. Furthermore, bone marrow transplantation showed the predominant role of GC-A on local ischemic tissue rather than on vascular progenitor cells mobilized from bone marrow during vascular remodeling. In cultured human endothelial cells, ANP treatment significantly stimulated mRNA expressions of vascular endothelial growth factor and endothelial nitric oxide synthase via Erk1/2-dependent mechanism.

Conclusion—These results suggest that endogenous ANP and BNP play important roles in reparative vascular remodeling in ischemic tissue. (***Arterioscler Thromb Vasc Biol.* 2009;29:1516-1521.)**

Key Words: atrial natriuretic peptide ■ brain natriuretic peptide ■ guanylyl cyclase-A ■ ischemia ■ mice

Vascular remodeling is critical for wound repair. In ischemic tissue, the presumed mechanism of hypoxia-induced angiogenesis involves the elevation of hypoxia-inducible factor-1α, resulting in increased expressions of growth factors, such as vascular endothelial growth factor (VEGF).[1] Angiogenic response to VEGF might involve the production of NO, as previously described in ischemic hind-limbs.[2]

The atrial and brain natriuretic peptides (ANP and BNP, respectively) are cardiac hormones that act via guanylyl cyclase (GC)-A to induce diuresis, natriuresis, and blood vessel dilation.[3] Yamahara et al found an activation of the natriuretic peptides/cyclic GMP/cyclic GMP-dependent protein kinase (PKG) pathway accelerated vascular regeneration and blood flow recovery in murine legs, for which ischemia had been induced by a femoral arterial ligation as a model for peripheral arterial diseases.[4] Recently, Park et al reported that intraperitoneal (i.p.) injection of carperitide, a recombinant human ANP, accelerated blood flow recovery with increasing capillary density in ischemic legs not only in nondiabetic mice but also in streptozotocin-induced diabetic mice, in which the blood flow recovery was significantly impaired compared with nondiabetic mice.[5] In the patients of peripheral arterial diseases, carperitide administration improved the ankle-brachial pressure index, intermittent claudication, rest pain, and ulcers.[5] These reports proved significance of the exogenous ANP and BNP in angiogenesis. However, the contributions of endogenous ANP and BNP on vascular remodeling are to be elucidated.

Here, we examined the roles of endogenous ANP and BNP in vascular remodeling using a hind-limb ischemia model.

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Methods

Animals

GC-A-knockout (GC-A-KO) mice were generated at the Howard Hughes Medical Institute (University of Texas Southwestern Medical Center).[6] Ten-week-old male GC-A-KO mice and wild-type (WT) littermates were used in the present study.

Ligation Model

After the mice were anesthetized with pentobarbital (80 mg/kg, i.p.), hind-limb ischemia was induced as reported previously.[7] After ligating the proximal end of the left femoral artery, the distal portion of the saphenous artery was ligated, and the artery and side branches were dissected. The femoral artery and attached side branches were then excised. Sham-operated mice underwent the identical surgical procedure as described above without the actual artery ligation and excision. The mice were observed for 4 weeks. All experimental procedures conformed to the guidelines for animal experimentation of the National Cardiovascular Center and were approved by the institutional review board for animal experimentation.

Assessment of Blood Perfusion

Hind-limb blood flow was assessed with a laser Doppler perfusion image (LDPI) analyzer (Moor Instrument) every week from the day of surgery to 4 weeks later. After blood flow was measured twice, the average flow values for the ischemic and nonischemic limbs were calculated using a computer. The LDPI index was determined as the ratio of ischemic to nonischemic hind-limb blood perfusion.

Immunohistochemistry

Ischemic hind-limb tissues 28 days after surgery were subjected to immunohistochemistry. After perfusion fixation with 4% paraformaldehyde, ischemic lower legs were embedded in OCT compound (Sakura Finetechnical) and frozen at −80°C. Cryostat sections (4 to 8 μm thick) of the tissues were stained with rat antimouse PECAM-1 antibody (Pharmingen) and alkaline phosphatase-conjugated anti-α-smooth muscle actin (α-SMA) monoclonal antibody (clone 1A4, SIGMA).

Analysis of Capillary Density

To measure capillary density, 4 random fields on 2 different sections from each mouse at ×200 magnification were photographed with a digital camera (Olympus). By computer-assisted analysis using WinRoof digital image analyzer (MITANI CORPORATION), capillary density was calculated as the mean percent positive stained area with PECAM-1 or α-SMA to the total field.

Radioimmunoassay for ANP

Radioimmunoassay (RIA) for ANP was performed as described previously.[8] Ventricle and femoral tissue were collected from mice 1 day and 7 days after operation. Tissues were diced and boiled for 10 minutes in 10 volumes of water. Glacial acetic acid was added (final concentration=1 mol/L) after cooling, and boiled tissues were homogenized. The supernatants obtained by centrifugation at 16 000*g* for 30 minutes were loaded onto Sep-pak C18 cartridges (Waters). After washing with 0.5 mol/L acetic acid and 0.1% trifluoroacetic acid (TFA), adsorbed materials were eluted with 60% CH_3CN containing 0.1% TFA. The eluted materials were lyophilized and submitted to RIA for ANP.

Bone Marrow Transplantation

Bone marrow transplantation (BMT) was performed as described previously.[9] Ten-week-old male WT mice and GC-A-KO mice were lethally X-irradiated with a total dose of 9 Gy (RX-650, Faxitron X-Ray Corporation). One day after, the recipient mice received unfractionated bone marrow cells (3×10^6) by cervical vein injection. Six weeks after the BMT, hind-limb ischemia was induced in the recipient mice. The reconstitution rate of the peripheral leukocytes was 80% to 85% as determined by flow cytometry.

Cell Culture, siRNA-Mediated Protein Knockdown

Human umbilical vein endothelial cells (HUVECs) and human aortic endothelial cells (HAECs) were purchased from KURABO. HUVECs were transfected 50 nmol/L siRNA duplexes using Lipofectamine RNAiMAX reagent (Invitrogen) according to the manufacturer's instructions. After 48 hours, the cells were used for the experiments.

Real-Time PCR

Real-Time PCR was performed as described previously.[10] Total RNA was extracted from mice femoral tissue and cultured HUVECs. First-strand cDNA was synthesized using SUPERSCRIPT II Reverse Transcriptase (Invitrogen) from 2 μg of total RNA. To quantitatively examine levels of GC-A endothelial nitric oxide synthase (eNOS), VEGF, and Angiopoietin2 (Ang2) mRNAs, real-time PCR amplification using a Light Cycler system (Roche Applied Science) was performed according to the manufacturer's instructions. Known concentrations of linearized plasmids containing mice GC-A, human eNOS, human VEGF, human Ang2 cDNA were used to generate standard curves. Gene expression was normalized to GAPDH. Primers were designed on Primer Express (PE Applied Biosystems Inc) coordinates as follows:

mice GC-A forward, (GCAACCAAGAGACCACTTTTCCA); mice GC-A reverse, (CGTTTTCCGGTTCACACGTTT); mice GAPDH forward, (TGCAGTGGCAAAGTGGAGATT); mice GAPDH reverse, (TCGCTCCTGGAAGATGGTGAT); human eNOS forward, (TCGTCCCTGTGGAAAGACAAG); human eNOS reverse, (TCTCGGAGCCATACAGGATTG); human VEGF forward, (CAGCTACTGCCATCCAATCGA); human VEGF reverse, (TTTGCCCCTTTCCCTTTCC); human Ang2 forward, (GACTGCCACGGTGAATAATTCA); human Ang2 reverse, (CGTGTAGATGCCATTCGTGGT); human GAPDH forward, (TGAAGGTCGGAGTCAACGGAT); human GAPDH reverse, (ACGGTGCCATGGAATTTGC).

Western Blot Analysis

The following antibodies were purchased from Cell Signaling Technology: phospho-Erk1/2 (Thr202/204) antibody, Erk1/2 antibody, phospho-p38 MAPK (Thr180/Tyr182) antibody, phospho-SAPK/JNK (Thr183/Tyr185) antibody, phospho-Akt (Thr308) antibody, phospho-Akt (Ser473) antibody, phospho-AMPKα (Thr172) antibody, phospho-AMPKα (Ser485) antibody, phospho-eNOS (Ser1177) antibody, phospho-VASP (Ser157) antibody, phospho-VASP (Ser239) antibody, VASP antibody, GAPDH antibody. Western blot analysis was performed as described previously.[11] HUVECs and HAECs were lysed with cell lysis buffer (20 mmol/L Tris-HCl, pH7.5, 150 mmol/L NaCl, 1mmol/L Na_2EDTA, 1 mmol/L EGTA, 1% Triton, 2.5 mmol/L Sodium pyrophosphate, 1 mmol/L β-glycerophosphate, 1 mmol/L Na_3VO_4, 1 μg/mL leupeptin; Cell Signaling Technology) with protease inhibitor cocktail (Roche). Lysates were cleared by centrifugation, and total protein concentrations were determined using Bio-Rad Protein Assay. Samples were electrophoresed through a reducing SDS-polyacrylamide gel and electroblotted onto a nitrocellulose membrane. The membrane was blocked with 5% nonfat dry milk and incubated with antibodies noted above. Protein expression was detected with a Phototope-HRP Western Blot Detection System (Cell Signaling Technology).

Results

Significance of Endogenous ANP and BNP in Postnatal Vascular Regeneration

As shown in Figure 1A, hind-limb ischemia in WT mice resulted in partial amputation of the limb and mild ulcers

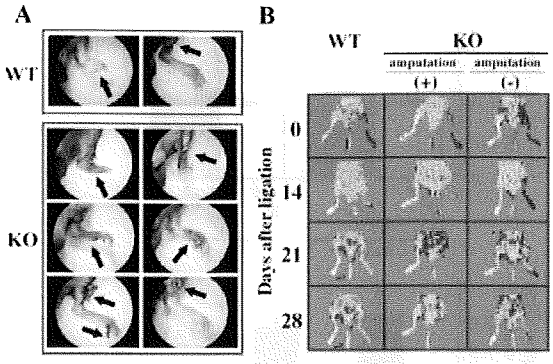

Figure 1. Contribution of GC-A–mediated signaling to vascular remodeling. A, Photographs obtained 28 days after operation in WT and GC-A-KO (KO) mice. Lesions are marked with arrows. B, Serial LDPI of hind-limb ischemia in WT and KO mice.

in 2 of 10 mice, whereas limb amputation and severe ulcers were observed in 6 of 10 GC-A-KO mice. Laser Doppler imaging revealed that, even though the rest of GC-A-KO mice did not show apparent limb lesions, blood flow recovery was significantly inhibited compared with WT mice (Figure 1B, amputation (−)). Figure 2A shows the time of course of blood flow recovery in GC-A-KO mice, which was reduced significantly compared with WT mice throughout the observation period.

Next, we evaluated capillary network formation and arteriole coverage in the ischemic tissue by immunostaining for PECAM-1 and α-SMA, respectively. As shown in Figure 2B, the PECAM-1–expressing area was significantly diminished in GC-A-KO mice compared with WT mice (3.5±0.2% versus 5.8±0.3%, $P<0.0001$). In the section, consecutive to that used in Figure 2B, the percentage of the sample area positive for α-SMA was also significantly smaller in GC-A-KO mice compared with WT mice (0.8±0.3% versus 3.9±0.7%, $P<0.01$; Figure 2C).

Importance of Local GC-A, Expressed in Ischemic Tissue in Vascular Remodeling

Recently, bone marrow (BM)-derived vascular progenitor cells (ie, EPC) have been reported to contribute to the reparative neovascularization.[12] Therefore, we next investigated the role of BM-derived cells in GC-A–mediated vascular remodeling with BMT experiments. As shown in Figure 3A, when GC-A-KO BM was transplanted to WT (BMT; KO to WT), blood flow recovery was significantly reduced compared with its control (BMT; WT to WT). Of note, inversely, when WT BM was transplanted to GC-A-KO (BMT; WT to KO), blood flow recovery was almost equal to its control (BMT; KO to KO). Next, we evaluated changes of GC-A expression level in ischemic limb tissue at day 1 and day 7 after operation using WT mice. As shown in Figure 3B, at day 1, GC-A expression level was comparable in femoral artery ligated and sham-operated group. However, at day 7, GC-A expression level was significantly increased in femoral artery ligated group (1.7-fold, $P<0.01$). These results indicate that local GC-A, expressed in ischemic limb tissue, and GC-A, expressed on BM-derived vascular progenitor cells, both play important roles in vascular remodeling.

Furthermore, we next examined whether ANP contents in the ventricle or in ischemic limb tissue were changed after ligation of femoral artery of the WT. As shown in supplemental Table I (available online at http://atvb.ahajournals.org), ANP contents in ventricle and ischemic limb tissue did not change significantly up to 7 days after ligation of the femoral artery.

ANP Stimulates eNOS and VEGF mRNA Expressions

To dissect the mechanism of ANP-mediated vascular repair promotion, we examined the changes of mRNA expression levels of eNOS, VEGF, and Ang2, all of which have been well known to contribute to angiogenesis, in cultured HUVECs after treatment of ANP. As shown in Figure 3C, ANP treatment significantly augmented eNOS (2.1-fold, $P<0.01$) and VEGF (2.0-fold, $P<0.001$) mRNA expression levels, whereas it did not change Ang2 mRNA expression level. Previous reports demonstrated that eNOS activity is regulated by phosphorylation at Ser1177.[13] Therefore, we performed Western blot analysis using a specific antibody against phospho-serine^{1177} of eNOS. As shown in Figure 3D,

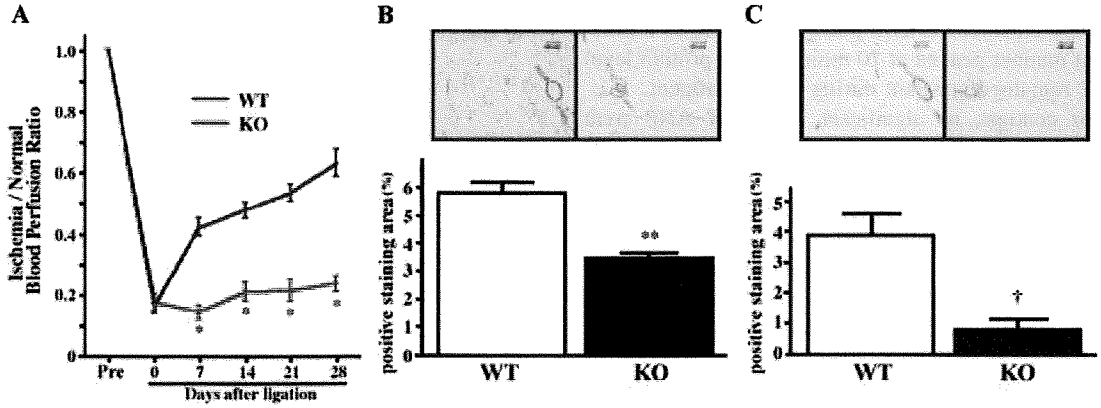

Figure 2. A, Serial quantitative analysis of the ischemic/normal hind-limb perfusion ratio in WT and GC-A-KO (KO) mice using LDPI. *$P<0.05$ vs WT. B and C, Immunostaining of ischemic hind-limbs using anti-PECAM-1 antibody (B) and anti-α-SMA antibody (C) on day 28. Values are expressed as the means±SEM. **$P<0.0001$ vs WT. †$P<0.01$ vs WT.

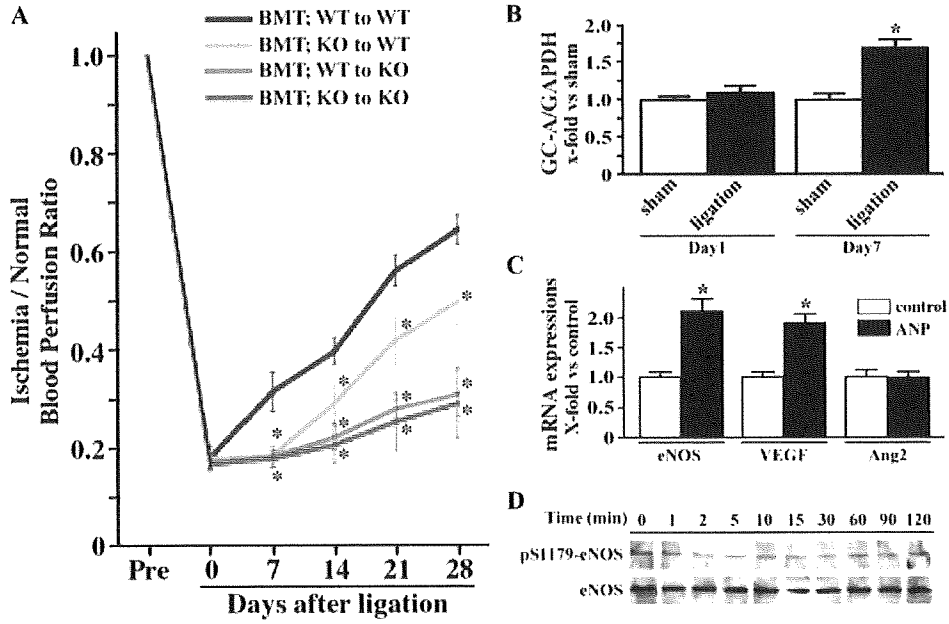

Figure 3. A, Serial quantitative analysis of ischemic/normal hind-limb perfusion ratio in WT and GC-A-KO (KO) mice using LDPI after BMT experiments. Values are expressed as the means±SEM. *$P<0.05$ vs BMT; WT to WT. B, Effect of femoral artery ligation and excision to GC-A mRNA expression at day 1 and day 7 after operation in the femoral tissue in WT mice. Values are expressed as the means±SEM. *$P<0.05$ vs sham-operation group. C, Effect of ANP (10^{-7} mol/L) treatment on eNOS, VEGF, and Angiopoietin2 (Ang2) mRNA expressions in HUVECs. Cells starved for 12 hours in medium containing 0.5% FCS were stimulated ANP for 8 hours. Results are means±SEM of 4 independent assays. *$P<0.05$ vs control. D, Effect of ANP (10^{-7} mol/L) treatment on eNOS phosphorylation in HUVECs. Cells starved for 12 hours in medium containing 0.5% FCS were stimulated with ANP (10^{-7} mol/L) for the time indicated at the top.

ANP treatment of HUVECs did not significantly change eNOS phosphorylation status on Ser1177. Therefore, it is suggested that the effect of ANP on eNOS activation could be mediated not by protein phosphorylation but by augmentation of gene expression.

Erk Mediates ANP-Induced eNOS and VEGF mRNA Expressions

Next, we investigated downstream signals of GC-A by Western blot analysis using phospho-specific antibodies of various kinase. We stimulated cultured HUVECs with ANP and assessed phosphorylation of Erk1/2 at different time points up to 120 minutes. As shown in Figure 4A, ANP greatly enhanced Erk1/2 phosphorylation, in a time-dependent manner peaked at 10 minutes. The protein level of Erk1/2 remained constant during ANP treatment.

In clear contrast, no significant effects of ANP were demonstrated on the phosphorylation status of the p38 MAPK (Thr180/Tyr182), SAPK/JNK (Thr183/Tyr185), Akt (Thr308), Akt (Ser473), AMPKα (Thr172), AMPKα (Ser485) in the same experimental condition describe above (data not shown). We also assessed whether Erk1/2 is phosphorylated by ANP in arterial endothelial cells. As shown in Figure 4B, ANP initiated Erk1/2 phosphorylation in cultured HAECs as well.

Vasodilator-stimulated phosphoprotein (VASP) is a member of the family of actin binding regulatory proteins, which induces stress fiber formation and membrane ruffling in vascular endothelial cells.[14] As shown in Figure 4C, ANP greatly enhanced VASP phosphorylation at Ser239, which reached maximum at 5 minutes and then remained relatively constant for up to 120 minutes, whereas the VASP protein expression remained constant during treatment.

Next, we sought to determine whether the ANP-induced Erk1/2 or VASP activation contributes to the upregulation of eNOS and VEGF genes, using PD98059, an Erk1/2 inhibitor, and VASP siRNA. The protein knockdown effects of VASP siRNAs were confirmed as shown in Figure 4D. Treatment of HUVECs with PD98059 significantly reduced ANP-mediated increases of eNOS and VEGF mRNA expressions (Figure 4E and 4F). In clear contrast, VASP protein knockdown by siRNA#1 had no effect on ANP-mediated increases of the gene expressions (Figure 4G and 4H).

Discussion

In the present study, we first demonstrated that, after ligation of the femoral artery, the blood flow recovery and capillary formation were reduced in the mice deficient for GC-A, a common receptor for ANP and BNP, compared with WT mice. Second, we proved that GC-A mRNA expression was upregulated in the ischemic hind-limb tissue at day 7 after operation. Third, we demonstrated that not only local GC-A in ischemic tissue but also GC-A on BM-derived cells contributes to the vascular regeneration. We also indicated that, in cultured endothelial cells, ANP treatment increased eNOS and VEGF mRNA expressions by Erk1/2-dependent mechanism.

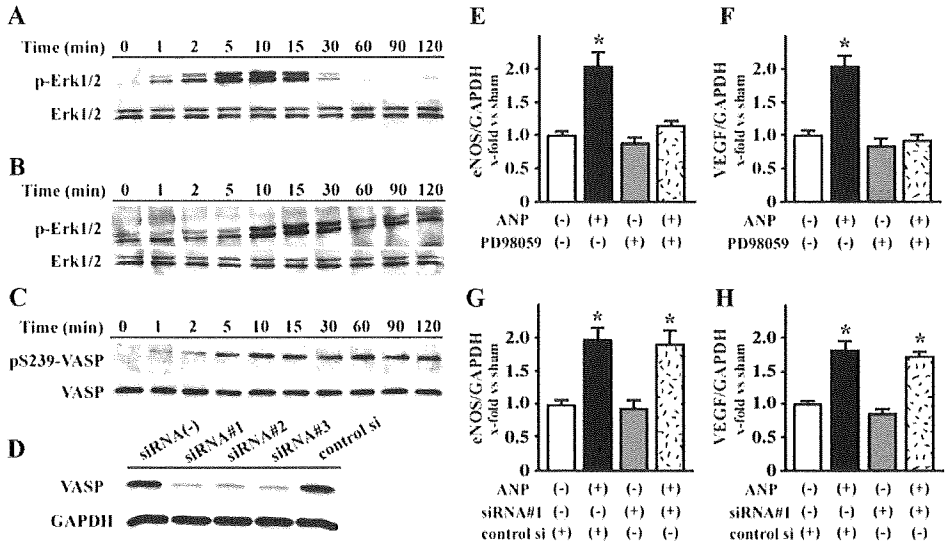

Figure 4. A–C, Effect of ANP (10^{-7} mol/L) treatment on Erk1/2 (A, B) and VASP (C) phosphorylation in HUVECs (A, C) and HAECs (B). Cells starved for 12 hours in medium containing 0.5% FCS were stimulated with ANP for the time indicated at the top. D, VASP protein knockdown effect of 3 types of siRNA in HUVECs. After siRNA transfection, cells were cultured in growth medium for 24 hours, and then starved for 12 hours in medium containing 0.5% FCS. Thereafter, cells were stimulated with ANP for 8 hours with or without siRNA. Control si means negative control siRNA. E and F, Effect of Erk1/2 inhibitor PD98059 (2×10^{-5} mol/L) on ANP (10^{-7} mol/L)-induced augmentation of eNOS (E) and VEGF (F) mRNA expressions in HUVECs. Cells starved for 12 hours in medium containing 0.5% FCS were stimulated by ANP with or without PD98059 for 8 hours. Results are means±SEM of 4 independent assays. *$P<0.05$ vs control. G and H, Effect of VASP siRNA on ANP (10^{-7} mol/L)-induced augmentation of eNOS (G) and VEGF (H) mRNA expressions in HUVECs. After siRNA transfection, cells were cultured in growth medium for 24 hours and then starved for 12 hours in medium containing 0.5% FCS. Thereafter, cells were stimulated with ANP for 8 hours. Results are means±SEM of 4 independent assays. *$P<0.05$ vs control.

Because GC-A is highly expressed in vascular endothelial cells,[15] we initially hypothesized that local GC-A on the endothelial cells of ischemic tissue could play an important role in the angiogenic effect of ANP. In fact, it has been reported that ANP induces an increase in the number of cultured endothelial cells and potentiates capillary network formation in vitro.[16] However, because recent evidences have indicated that BM-derived vascular progenitor cells contribute to neovascularization of ischemic lesions,[12] and because our preliminary experiment revealed that GC-A is expressed in these cells as well, sites of angiogenic action of ANP needed to be determined. We therefore designed BMT experiments that revealed that not only local GC-A but also GC-A on the BM-derived cells contributes to the postischemic vascular regeneration. Interestingly, in the GC-A-KO background, BMT of WT to KO had no significant change in postischemic blood flow recovery compared to BMT of KO to KO, implicating that GC-A in the local ischemic tissue is necessary for the development of ANP action on BM-derived cells.

In the present study, we have examined the effect of ANP on gene expressions of eNOS and VEGF, the key roles of which in vascular regeneration were clearly demonstrated by previous excellent studies.[1,2] Importantly, incubation of cultured endothelial cells with ANP for 8 hours significantly upregulated mRNA expressions of both eNOS and VEGF and the effects of ANP were efficiently blocked by the concomitant addition of Erk1/2 inhibitor. Taken together, it is suggested that ANP induces eNOS and VEGF mRNA expressions through Erk1/2 activation, which presumably plays a significant role in the ANP-induced vascular remodeling. In the present study, we also demonstrated that ANP treatment of HUVECs strongly phosphorylates VASP, which agrees with the recent report by Chen et al.[17] Because VASP has been known to have multiple effects on the physiological processes governed by cellular actin networks, such as cell motility, migrations, angiogenesis, and vascular permeability,[18] it is suggested that ANP-mediated VASP activation could promote actin stress fiber formation and endothelial tube formation.[17] However, whether inhibition of VASP may actually affect ANP-induced vascular remodeling in vivo awaits further studies.

Because ANP and BNP both bind to and activate GC-A, it would be worth speculating which ligand is pivotal for the angiogenic property of the endogenous natriuretic peptide system. Although the binding affinity of BNP to GC-A receptor is comparable to that of ANP,[19] plasma level of ANP is approximately 20-times higher than BNP concentration in humans and animals.[20] We therefore hypothesize that, rather than BNP, ANP may play a predominate role for the vascular remodeling in vivo. However, in heart failure, plasma level of BNP becomes comparable to or even higher than that of ANP.[21] Therefore, the role of BNP on peripheral vascular repair would become more important in subjects with cardiac diseases.

In conclusion, the present study strongly suggests that endogenous ANP and BNP play important roles in reparative vascular remodeling.

Acknowledgments

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Disclosures

None.

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