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Adrenomedullin provokes endothelial Akt activation and promotes vascular regeneration both in vitro and in vivo

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Abstract We previously reported that adrenomedullin (AM), a vasodilating hormone secreted from blood vessels, promotes proliferation and migration of human umbilical vein endothelial cells (HUVECs). In this study, we examined the ability of AM to promote vascular regeneration. AM increased the phosphorylation of Akt in HUVECs and the effect was inhibited by the AM antagonists and the inhibitors for protein kinase A (PKA) or phosphatidylinositol 3-kinase (PI3K). AM promoted re-endothelialization in vitro of wounded monolayer of HUVECs and neo-vascularization in vivo in murine gel plugs. These effects were also inhibited by the AM antagonists and the inhibitors for PKA or PI3K. The findings suggest that AM plays significant roles in vascular regeneration, associated with PKA- and PI3K-dependent activation of Akt in endothelial cells, and possesses therapeutic potential for vascular injury and tissue ischemia.

Key words: Adrenomedullin; cAMP; Akt; Angiogenesis; Re-endothelialization; Vascular regeneration

1. Introduction

Vascular regeneration is an essential event in recovery from endothelial injury or tissue ischemia. Thus, therapeutic strategies to promote re-endothelialization or neo-vascularization are now highlighted as promising treatment for atherosclerotic or ischemic diseases [1,2]. Many vasoactive substances secreted from endothelial cells (vascular hormones) have been reported to regulate not only vascular tone but also remodeling or regeneration. We revealed that C-type natriuretic peptide (CNP) is secreted from endothelial cells [3] and gene transfer of CNP promoted endothelial regeneration [4,5] and ischemia-induced angiogenesis [6] in vivo. We also reported that NPs directly promote endothelial regeneration in vitro [7]. In this way, NPs/cGMP/cGMP-dependent kinase (cGK) cascade is elucidated to be involved in the regulation of vascular regeneration.

Adrenomedullin (AM) is a potent vasorelaxant peptide that was originally isolated from human pheochromocytoma cells on the basis of its effect to elevate cAMP levels in rat platelets [8,9]. Recently, mice genetically engineered to overexpress or underexpress the AM gene were developed to determine the in vivo significance of AM [10–12]. Mice overexpressing the AM gene in their vasculature showed reduced blood pressure. On the other hand, mice lacking the AM gene did not survive the embryonic stage and showed abnormal vascular structure and subcutaneous hemorrhage. These observations suggest the significance of AM in vascular morphogenesis and regulation of vascular tone in vivo. AM has been shown to be present in atherosclerotic lesions and its secretion has been demonstrated to be augmented by inflammatory cytokines such as interleukin-1, TNF-α, and lipopolysaccharide [13]. Furthermore, hypoxia-responsive elements were identified in the AM gene and hypoxic conditions were reported to induce its expression and secretion from HUVECs [14]. These findings suggest the significance of AM for atherogenesis and angiogenesis.

Based on these findings, together with our recent report to show that AM enhanced proliferation and migration of cultured endothelial cells [15], we hypothesized that AM/cAMP/protein kinase A (PKA) cascade might have the potency to promote vascular regeneration. In this study, we tried to clarify whether AM has beneficial effects on vascular regeneration in the physiological in vitro model for endothelial regeneration and in vivo neo-vascularization in murine gel plugs.

2. Materials and methods

2.1. Materials and cell culture

All agents used were commercially available. Human AM, rat AM, proadrenomedullin N-terminal 20 peptide (PAMP), and the two AM antagonists, AM(22–52) and calcitonin gene-related peptide (8–37) (CGRP(8–37)) were purchased from the Peptide Institute (Osaka, Japan). The two PKA inhibitors, adenosine 3′,5′-cyclic monophosphorothioate Rp-isomer (Rp-cAMP) and myristoylated PKA inhibitor peptide sequence (14–22) cell-permeable (PKA Inh. Peptide), the two phosphatidylinositol 3-kinase (PI3K) inhibitors, LY294002 and wortmannin, and a cAMP analog, 8-Br-cAMP, were purchased from Calbiochem (San Diego, CA, USA). Vascular endothelial growth factor (VEGF) was purchased from Peprotech (London, UK).

HUVECs (Clonetics, Waltersville, MD, USA) were grown in the basic medium containing 2% fetal bovine serum (FBS) and growth supplements (EGM-2; Clonetics). Cell cultures between passages 4 and 6 were used for each experiment.

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Abbreviations: AM, adrenomedullin; PKA, protein kinase A; PI3K, phosphatidylinositol 3-kinase; HUVEC, human umbilical vein endothelial cell; NP, natriuretic peptide; PKA Inh. Peptide, myristoylated protein kinase A inhibitor peptide sequence (14–22) cell-permeable; FBS, fetal bovine serum

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2.2. Western blot analysis of phosphorylated Akt

HUVECs were treated with or without AM (10^{-8} mol/l) and they were harvested 30 min after the treatments otherwise indicated. Western blotting was performed according to a standard protocol, as we described previously [16]. Akt activity was evaluated by the ratio of phosphorylated Akt to total Akt detected by phospho-Akt (Ser473) and Akt antibody (Cell Signaling, Beverly, MA, USA), respectively. To evaluate Akt activation in endothelial injury, artificial wounds were made by a blue-tip at intervals of 5 mm on an over-confluent monolayer of HUVECs. Densitometric assays were done and the results were presented as fold increase compared to the control.

2.3. Wound healing assay in vitro

To examine whether AM promotes endothelial regeneration in vitro, wound healing assay was carried out as we described previously [7]. In the report, we confirmed that this assay could evaluate overall activity of endothelial proliferation and migration. Briefly, HUVECs were grown to over-confluent in six-well plates and a wound of approximately 2 mm width was made by a cell scraper. Cells were allowed to repair the wound for 40 h in the medium containing 0.5% FBS with or without experimental agents. The wounded monolayer was photographed before and after the incubation period and the re-endothelialized area was evaluated.

2.4. Gel plug assay in vivo

To examine the ability of AM to induce neo-vascularization in vivo, we used murine MATRIGEL plug assay, as described previously [17]. Mice were handled with care according to accepted ethical guidelines. Nude mice were anesthetized with pentobarbiturate (80 mg/kg) and 400 μl per plug of growth factor-reduced phenol red-free MATRIGEL (Becton Dickinson, Bedford, MA, USA) was injected into the abdomen of 6–8 week old male KSN-nude mice (Japan SLC; Hamamatsu, Japan) subcutaneously. A mouse was injected two gels symmetrically in the abdomen, gels with and without experimental agents.

On day 0, 4, 7, 14, and 21, the margins of the subcutaneous plugs were marked and the mean blood flow and the size of the plugs were estimated by a laser Doppler perfusion image analyzer (Moor instruments, Devon, UK). Blood flow in the plug was calculated by the formula: (blood flow) = (mean blood flow) × (plug size). The ratio of the blood flow of the two plugs in the same mouse, an agent-containing plug to its control plug, was considered to be the index of the angiogenic activity of the agent.

The gel plugs were resected from mice on day 21 and stained with rat anti-mouse PECAM-1 (Pharmingen, San Diego, CA, USA) and the number of PECAM-1 positive cells in the plug was estimated as capillary density. 1-mm thick slices were also processed and stained with rat anti-mouse PECAM-1 and a fluorescent agent, Alexa Flour 488 conjugated goat anti-rat IgG (Molecular Probes, Eugene, OR, USA) for the observation with a confocal microscope (LSM5 PASCAL; Carl Zeiss, Oberkochen, Germany) that can reconstruct the 3D structure of the plug from the obtained consecutive images. In this way, small capillaries in the plug were visualized stereoscopically.

2.5. Statistics

All data are expressed as the mean ± S.E.M. Statistical analysis was performed with ANOVA (analysis of variance) or Student's *t*-test. Values of $P < 0.05$ were considered to be statistically significant.

3. Results

3.1. AM activated Akt in HUVECs in a PKA- and PI3K-dependent manner

Fig. 1A demonstrates a time course of the phosphorylation of Akt at amino acid residue 473 (serine) after the treatment with AM (10^{-8} mol/l) on HUVECs. Akt phosphorylation was augmented within 15 min and peaked at 30 min.

To examine Akt activation in endothelial damage, we made artificial wounds in a confluent monolayer of HUVECs as described in Section 2. The endothelial injury itself increased Akt phosphorylation and the addition of AM to the injured endothelium further augmented the increase (Fig. 1B).

AM-induced phosphorylation of Akt was inhibited by the two AM antagonists, AM(22–52) (10^{-5} mol/l) and CGRP-(8–37) (10^{-5} mol/l). The PKA inhibitors, Rp-cAMP (10^{-5} mol/l) and PKA Inh. Peptide (5×10^{-7} mol/l), and the inhibitors for PI3K, LY294002 (10^{-5} mol/l) and wortmannin (10^{-7} mol/l), also suppressed Akt phosphorylation significantly (Fig. 1C).

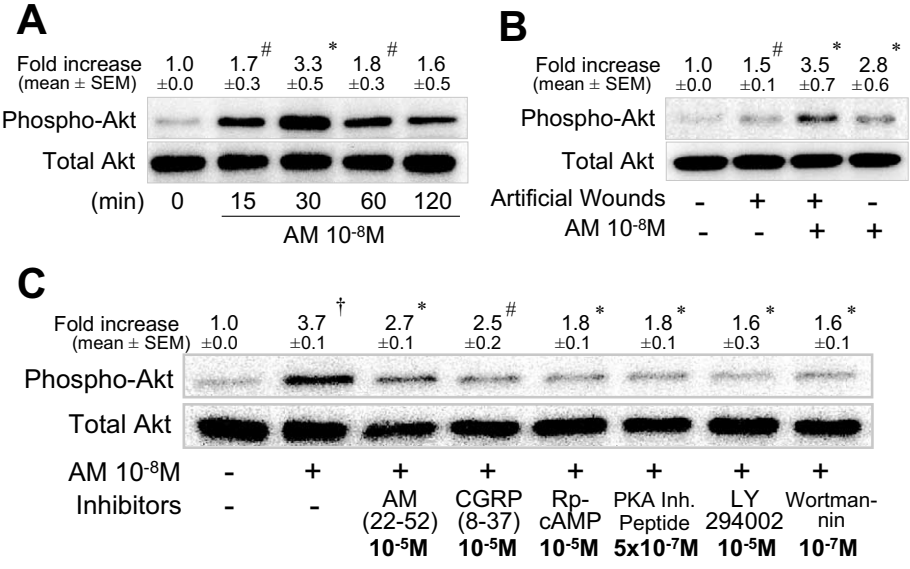

Fig. 1. Effect of AM on Akt activation in HUVECs. A: Time course of Akt phosphorylation in AM-treated HUVECs. Cells were treated with AM (10^{-8} mol/l) and harvested at the indicated times. B: Effect of artificial wounds and AM on Akt phosphorylation. Wounds were made at intervals of 5 mm on an over-confluent monolayer of HUVECs treated with or without AM. Cells were harvested 30 min after the treatments and phosphorylated Akt was detected. C: Effect of the AM antagonists and the inhibitors for PKA or PI3K on AM-induced Akt phosphorylation in HUVECs. Cells were pre-incubated with these inhibitors for 15 min before the administration of AM. They were harvested 30 min after the treatment and phosphorylated Akt was detected. Densitometric analyses were done and the ratio of phosphorylated Akt to total Akt is presented as fold increases compared to the control. #: $P < 0.05$; *: $P < 0.01$ versus the control (panels A and B) or the AM-treated group without inhibitors (panel C); †: $P < 0.01$ versus the control (panel C), $n = 3$.

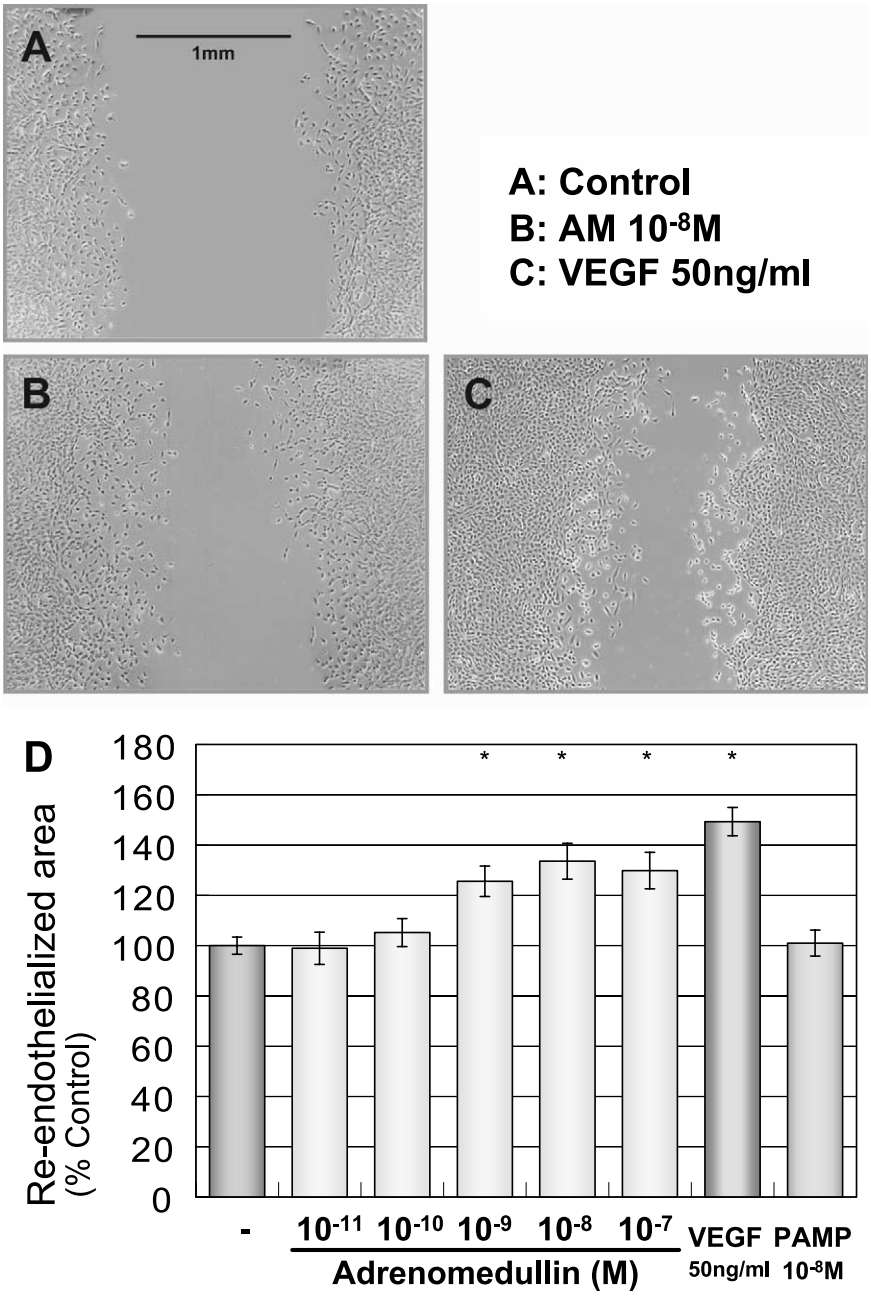

Fig. 2. Effect of AM on endothelial regeneration in wound healing assay in vitro. Wounded monolayer of HUVECs was incubated for 40 h. A–C: Representative photographs of re-endothelialized monolayer incubated for 40 h with the medium containing 0.5% FBS (control) (panel A), accompanied by AM (panel B), or VEGF (panel C). D: Dose-dependent effect of AM on endothelial regeneration in comparison to VEGF and PAMP. *: $P < 0.01$ versus the control, $n = 12$.

3.2. *AM promoted re-endothelialization of the wounded monolayer of HUVECs through a PKA- and PI3K-dependent pathway*

Fig. 2 shows the closure of wounded endothelium incubated with FBS 0.5% (control) (Fig. 2A), accompanied with AM (10^{-8} mol/l) (Fig. 2B), or VEGF (50 ng/ml) (Fig. 2C) for 40 h. Accelerated wound closure was observed in the group treated with AM, as well as that treated with VEGF. Fig. 2D shows the dose-dependent effect of AM on endothelial regeneration in comparison with VEGF and PAMP. AM promoted the wound closure significantly and dose-dependently. The increase of re-endothelialized area by 10^{-8} mol/l AM was 33.6% ± 7.1% over the control ($P < 0.01$, $n = 12$). On the other hand, PAMP, a hypotensive peptide that is synthesized from the same precursor of AM, had no significant effect on endothelial regeneration.

We next examined the effect of the same inhibitors at the same concentrations which were used in Akt phosphorylation assay and had a significant inhibitory effect on AM-induced Akt activation. The two AM antagonists and the inhibitors for PKA or PI3K at those concentrations suppressed AM (10^{-8} mol/l)-induced endothelial regeneration without affecting basal wound closure (Table 1).

In addition, 8-Br-cAMP (10^{-9} mol/l) mimicked the AM action to promote endothelial regeneration in wound healing assay (21.8% ± 5.0% over the control, $P < 0.01$, $n = 8$).

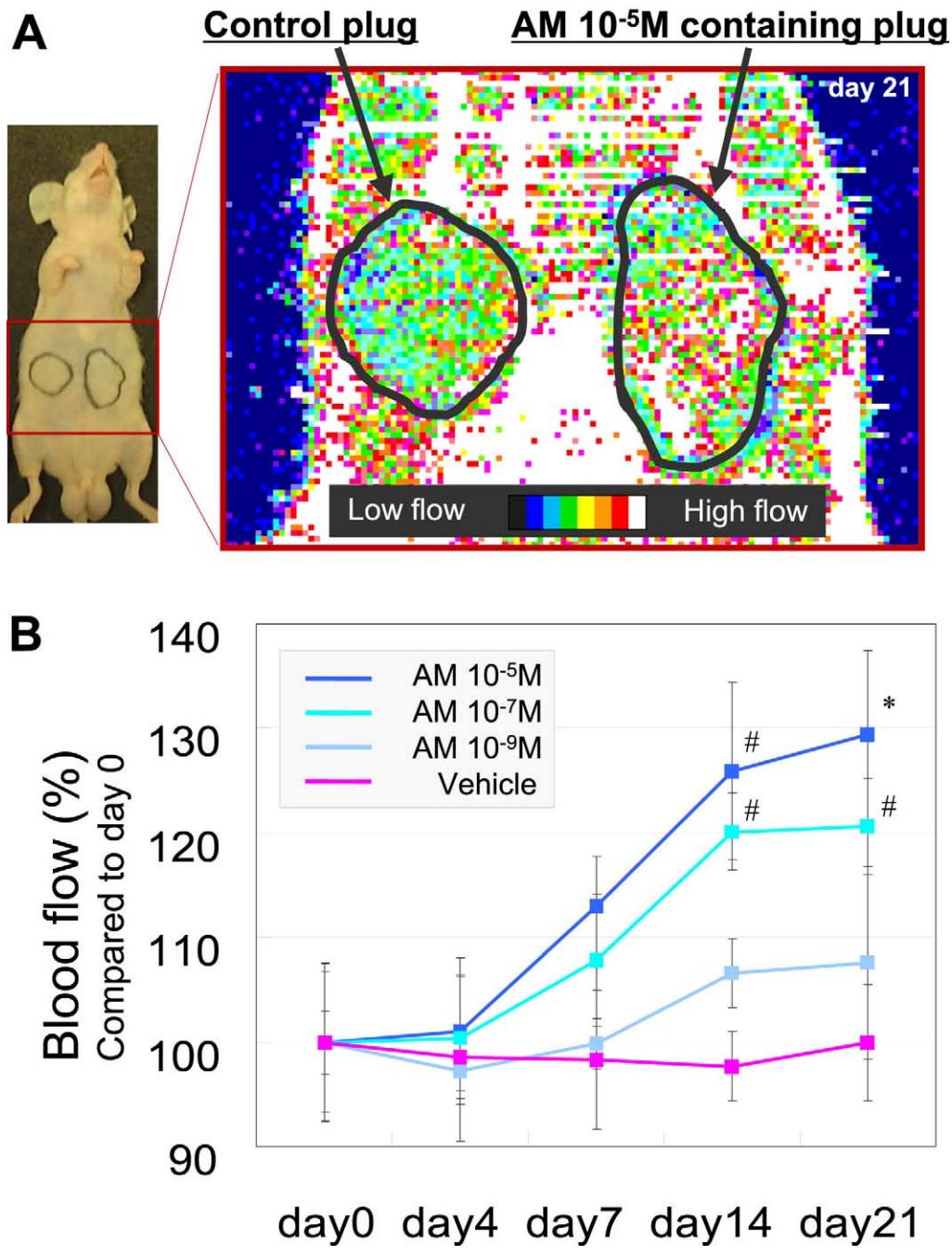

Fig. 3. Effect of AM on blood perfusion in murine gel plug assay. A: Blood flow measurement by a laser Doppler perfusion image analyzer. A representative view of a mouse injected with an AM-containing gel (10^{-5} mol/l) and a control gel is shown. The high-flow area is depicted in red to white and the low-flow in green to black. B: Time course of the effect of AM on blood flow in gel plugs. Blood flow on each day was measured by the laser Doppler perfusion image analyzer and compared to that on day 0. #: $P<0.05$; *: $P<0.01$ versus the blood flow on day 0. $n=12$ (AM 10^{-5} mol/l group), 8 (AM 10^{-7} mol/l and vehicle groups), and 4 (AM 10^{-9} mol/l group).

3.3. AM augmented blood flow in the plug through a PKA- and PI3K-dependent pathway

Fig. 3A demonstrates a representative image of the blood flow analysis of gel plugs on day 21. The AM-containing plug presented significantly higher blood perfusion compared to its control plug. The blood flow of the 10^{-5} mol/l AM-containing plug on day 21 was 29.4% ± 8.1% higher than that on day 0 ($P<0.01$, $n=12$; Fig. 3B).

The effects of the inhibitors on the AM-induced increase in blood flow were examined. Inclusion of the AM antagonists and the inhibitors for PKA or PI3K in AM-containing plugs significantly suppressed AM (10^{-7} mol/l)-induced augmentation of blood flow (Table 2).

The inhibitors at the doses used in the MATRIGEL plug assay had no significant effect on basal blood flow (Table 2) and the number of neo-vessels (data not shown) of gel plugs without AM. They did not have any significant toxic effects.

3.4. AM increased capillary density in gel plugs

Microscopic observation of whole and sliced plugs revealed

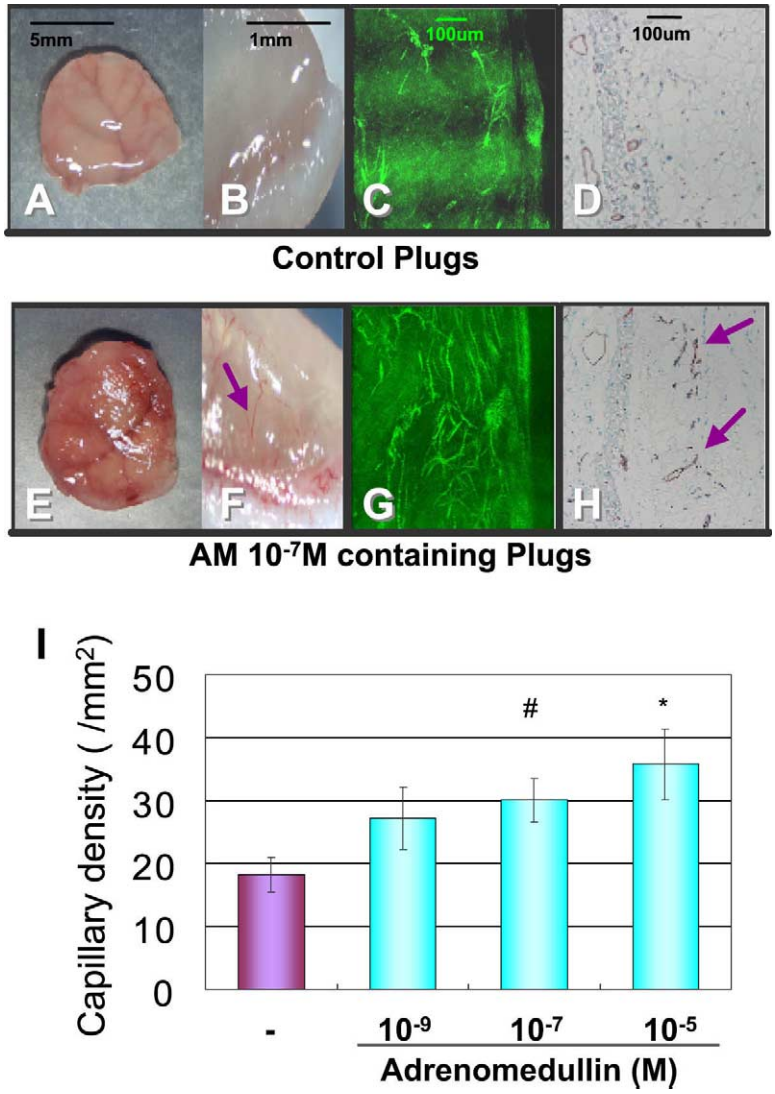

Fig. 4. Effect of AM on neo-vascularization in the gel plug. Gel plugs were harvested with underlying skin from mice on day 21 and angiogenic signs were microscopically examined. A–H: Representative images of a whole and sliced AM (10^{-7} mol/l)-containing plug (panels E and F) are shown, in comparison to the control plug (panels A and B). The plugs were stained with PECAM-1 to visualize neo-vessels. Immunochemically stained and fluorescent-labeled sections of an AM-containing plug (panels G and H) are shown, as compared to the control (panels C and D). The fluorescent-labeled sections were observed with a confocal microscope that can re-construct the 3D structure of small capillaries. I: Dose-dependent effect of AM on capillary density in the plugs determined by PECAM-1 immuno-reactivity on day 21. #: $P<0.05$; *: $P<0.01$ versus the control. $n=9$. Green: fluorescent-labeled PECAM-1 (panels C and G); brown: immunochemically stained PECAM-1 (panels D and H); arrows: neo-vessels in AM-containing plugs (panels F and H).

enhanced neo-vascularization in AM-containing plugs (Fig. 4E,F) compared to their control plugs (Fig. 4A,B). The capillary density estimated from PECAM-1 immuno-reactivity (Fig. 4D,H) was significantly and dose-dependently increased in AM-containing plugs (18.2 ± 2.8 per mm^2 in the control, 35.7 ± 5.7 per mm^2 in the 10^{-5} mol/l AM-containing plug on day 21; $P<0.01$ versus the control, $n=9$; Fig. 4I). We also confirmed increased capillary network in the AM-containing plug by observation with a confocal microscope that could visualize the 3D structure of capillaries of approximately 10 μm diameter (Fig. 4C,G).

4. Discussion

In this study, we showed that AM provokes endothelial Akt activation in a PKA- and PI3K-dependent manner and demonstrated that AM promotes endothelial regeneration in vitro and increases blood flow and capillary density in gel plugs in vivo through a PKA- and PI3K-dependent pathway.

AM-induced Akt activation in rat aortic tissues has been shown in a previous report [18]. In this study, we further confirmed that AM-induced Akt activation occurs in cultured endothelial cells. We also demonstrated that Akt is activated by the artificial wounds on HUVECs. Furthermore, we revealed that AM increases wound-induced Akt activation. Akt has been shown to regulate survival, migration, and nitric oxide production in endothelium [19]. In addition, constitutive Akt signaling in endothelium has been reported to be sufficient to promote angiogenesis in a rabbit hindlimb ischemia model [20]. Therefore, Akt activation in endothelium can be regarded as a key event in transduction of the angiogenic signal [21]. In the present study, AM-induced vascular regen-

Table 1
The effect of the AM antagonists and the inhibitors for PKA or PI3K on AM-induced endothelial regeneration

Treatment ($n=4$)	Re-endothelialized area (% control)	Treatment ($n=8$)	Re-endothelialized area (% control)
Control (FBS 0.5%)	100.0 ± 3.5	AM (10^{-8} mol/l)	132.6 ± 1.4[a]
+AM (22–52) (10^{-5} mol/l)	96.4 ± 5.4	+AM (22–52) (10^{-5} mol/l)	111.2 ± 7.4[b]
+CGRP (8–37) (10^{-5} mol/l)	103.3 ± 11.1	+CGRP (8–37) (10^{-5} mol/l)	119.8 ± 3.0[c]
+Rp-cAMP (10^{-5} mol/l)	104.0 ± 6.2	+Rp-cAMP (10^{-5} mol/l)	112.5 ± 11.7[b]
+PKA Inh. Peptide (5×10^{-7} mol/l)	101.7 ± 9.4	+PKA Inh. Peptide (5×10^{-7} mol/l)	113.0 ± 3.6[b]
+LY294002 (10^{-5} mol/l)	97.3 ± 14.7	+LY294002 (10^{-5} mol/l)	105.1 ± 6.6[b]
+Wortmannin (10^{-7} mol/l)	103.8 ± 13.1	+Wortmannin (10^{-7} mol/l)	103.0 ± 3.9[b]

Values are shown as mean ± S.E.M.
[a] $P<0.01$ versus the control.
[b] $P<0.01$ versus the AM (10^{-8} mol/l)-treated group without inhibitors.
[c] $P<0.05$ versus the AM (10^{-8} mol/l)-treated group without inhibitors.

Table 2
The effect of the AM antagonists and the inhibitors for PKA or PI3K on AM-induced augmentation of blood flow in gel plugs

Treatment ($n=4$)	Blood flow on day 21 (% control)	Treatment ($n=4$)	Blood flow on day 21 (% control)
Control	100.0 ± 0.0	AM (10^{-7} mol/l)	143.7 ± 5.5[a]
+AM (22–52) (10^{-4} mol/l)	101.1 ± 2.3	+AM (22–52) (10^{-4} mol/l)	104.1 ± 6.2[b]
+CGRP (8–37) (10^{-4} mol/l)	96.8 ± 10.1	+CGRP (8–37) (10^{-4} mol/l)	112.6 ± 3.7[b]
+Rp-cAMP (10^{-3} mol/l)	102.7 ± 11.7	+Rp-cAMP (10^{-3} mol/l)	113.2 ± 6.1[b]
+PKA Inh. Peptide (10^{-4} mol/l)	105.2 ± 6.1	+PKA Inh. Peptide (10^{-4} mol/l)	104.1 ± 9.8[b]
+LY294002 (10^{-3} mol/l)	100.1 ± 0.5	+LY294002 (10^{-3} mol/l)	109.3 ± 7.6[b]
+Wortmannin (10^{-5} mol/l)	101.2 ± 8.0	+Wortmannin (10^{-5} mol/l)	111.9 ± 7.4[b]

Values are shown as mean ± S.E.M.
[a] $P<0.01$ versus the control.
[b] $P<0.01$ versus AM (10^{-7} mol/l)-containing plugs without inhibitors.

eration both in vitro and in vivo was suppressed by PI3K inhibitors. Therefore, it is suggested that AM regulates neovascularization via enhancement of endothelial Akt activity following PI3K activation.

There are also several reports that imply the involvement of the cAMP/PKA cascade in vascular regeneration. However, whether PKA activation promotes or inhibits vascular regeneration is controversial [22,23]. In the present study, AM-induced vascular regeneration both in vitro and in vivo was significantly abrogated by the two PKA inhibitors, and 8-Br-cAMP simulated the effect of AM. Therefore, the cAMP/PKA cascade is supposed to have potency to promote vascular regeneration, at least in our experimental conditions. We also revealed that AM-induced Akt activation is suppressed by the inhibitors for not only PI3K but also for PKA. These results suggest the significance of the cAMP/PKA cascade on the regulation of Akt activity, which can induce vascular regeneration. The molecular mechanism in PKA-induced Akt activation is now under investigation.

In MATRIGEL plug assay, it has been reported that 10–100 times higher concentrations of substances than those used in cell culture experiments were required to exert enough influence in gel plugs [24,25]. Based on these previous reports, we planned to use higher concentrations of AM in MATRIGEL plug assay in vivo than in wound healing assay in vitro. As a result, the effective concentrations of AM were revealed to be 10^{-9}–10^{-7} mol/l in vitro and 10^{-7}–10^{-5} mol/l in vivo. The optimal concentrations of AM in vitro for cultured endothelial cells were similar to previous reports [26]. The difference between the effective concentrations in vitro and in vivo observed in our present study was also compatible with previous reports [24,25].

In conclusion, we demonstrated that AM provokes endothelial Akt activation and promotes vascular regeneration both in vitro and in vivo through a PKA- and PI3K-dependent pathway. These findings suggest the usefulness of AM as a new therapeutic agent for vascular injury, atherosclerotic diseases and tissue ischemia.

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