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Adrenomedullin augments collateral development in response to acute ischemia

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Abstract

Expression of adrenomedullin, discovered as a vasodilatory peptide, is markedly up-regulated under pathological conditions such as tissue ischemia and inflammation, which are associated with neovascularization. Here, we tested the hypothesis that overly expressed adrenomedullin may augment collateral flow to ischemic tissues. We induced hindlimb ischemia in wild-type mice and injected a naked plasmid expressing human adrenomedullin or an empty vector into the ischemic muscle, followed by in vivo electroporation. Adrenomedullin markedly enhanced blood flow recovery as determined by Laser Doppler imaging. The mice treated with an empty vector suffered frequent autoamputation of the ischemic toe, which was completely prevented by adrenomedullin. Anti-CD31 immunostaining revealed that adrenomedullin significantly increased capillary density. The angiogenic effect of adrenomedullin was abrogated in endothelial nitric oxide synthase (eNOS)-deficient mice. These results indicate that adrenomedullin may promote collateral growth in response to ischemia through activation of eNOS.

Keywords: Adrenomedullin; Angiogenesis; Nitric oxide; Vasodilatation; Hypoxia

Adrenomedullin is a vasodilatory peptide [1] and the major source of circulating adrenomedullin is the vascular wall [2,3]. The vascular action of adrenomedullin was at first considered to be solely due to elevated cAMP production, i.e., endothelium-independent vasodilation. However, we found that endothelial denudation substantially reduced the vasodilatory action of adrenomedullin in rodent aortic rings [4,5]. Furthermore, this adrenomedullin-induced endothelium-dependent vasorelaxation is exerted mostly through activation of the phosphatidylinositol 3-kinase (PI3-K)/Akt pathway [5]. It has been well established that this pathway is involved in various important actions such as anti-apoptosis and tissue protection as well as activation of endothelial nitric oxide synthase (eNOS).

Expression of the adrenomedullin gene is up-regulated by hypoxia [6,7] and inflammation [8], which are associated with neovascularization. Adrenomedullin abrogates ischemia/reperfusion renal injury. Studies using adrenomedullin gene knockout mice revealed that adrenomedullin plays an important role in vascular formation in embryos [9–11]. These findings suggest similarity of action of adrenomedullin to that of vascular endothelium growth factor (VEGF), a potent angiogenic factor. VEGF, either protein or gene, delivery to the ischemic limb resulted in collateral development even in patients with peripheral vascular diseases [12]. Thus, we hypothesized that overly

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expressed adrenomedullin may augment collateral flow to ischemic tissues.

Here, we studied the effect of adrenomedullin on collateral development in a murine ischemic hindlimb model. Adrenomedullin markedly augmented blood flow recovery in wild-type mice. The beneficial effects were abrogated in mice deficient for eNOS. Our findings suggest that adrenomedullin may promote angiogenesis in ischemic tissues through activation of eNOS.

Materials and methods

Animals. C3H/He mice and C57BL/6J mice were purchased from SLC Japan (Shizuoka, Japan). Endothelial nitric oxide synthase-deficient ($eNOS^{-/-}$) mice (C57BL/6J background) were purchased from Jackson Laboratory (Bar Harbor, ME) [13]. All protocols involving experimental animals were in accordance with the local institutional guidelines for animal care of the University of Tokyo and complied with the "Guide for the Care and Use of Laboratory Animals" (NIH publication No. 86-23, revised 1985).

Mouse ischemic hindlimb model. Unilateral hindlimb ischemia was induced in 30–35-week-old female mice [14]. The animals were anesthetized by intraperitoneal injection of pentobarbital (50 mg/kg). The proximal and distal portions of the right femoral artery and the distal portion of the saphenous artery were ligated. After that, the arteries and all side branches were dissected free and excised. The skin was closed with 5-0 surgical suture.

Gene transfer and measurement of plasma adrenomedullin concentration. Human adrenomedullin cDNA was subcloned into pcDNA3 at *Eco*RI and *Xba*I sites (hAMpcDNA3). We injected either hAMpcDNA3 or pcDNA3 (naked plasmid 50 μg/50 μl PBS) into the right thigh muscles followed by in vivo electroporation (100 V × 50 ms × 6 times) [15]. Gene delivery was repeated every week starting 1 week before surgery. The efficacy of adrenomedullin gene transfer was evaluated by measuring the concentration of plasma human adrenomedullin. At sacrifice, blood was drawn from the heart and transferred to chilled tubes containing 2 mM EDTA2Na and 500 IU/ml aprotinin. Plasma was separated by centrifuging at 4 °C and 3000 rpm for 15 min, and stored at −70 °C until the assay. The concentration of adrenomedullin in plasma was measured by radioimmunoassay as previously reported [16]. The sensitivity of this RIA system was 2 fmol/ml. The antiserum used crossreacts with mouse adrenomedullin by less than 2%.

Monitoring of hindlimb blood flow. Hindlimb blood perfusion was measured using a Laser Doppler perfusion imager (LDPI) system (Moor Instruments, Devon, UK) [14]. Excess hairs were removed from the limbs using depilatory cream before imaging and mice were placed on a heating plate at 40 °C. To avoid the influence of ambient light and temperature, the results are expressed as the ratio of perfusion in the right (ischemic) versus left (untreated) limb.

Measurement of capillary density in the ischemic leg. Five weeks after surgery, mice were sacrificed by intraperitoneal injection of an overdose of pentobarbital. The whole limbs were fixed in methanol overnight. The femora were carefully removed and the ischemic thigh muscles were embedded in paraffin. Sections (5 μm) were de-paraffinized and incubated with a rat-monoclonal antibody against murine CD31 (clone MEC13.1, BD PharMingen, San Diego, CA). Antibody distribution was visualized using the avidin–biotin-complex technique and Vector red chromogenic substrate (Vector Laboratories, Burlingame, CA), followed by counterstaining with hematoxylin. Capillaries were identified by positive staining for CD31 and their morphology. Ten different fields from each tissue preparation were randomly selected and capillaries were counted. Capillary density was expressed as the number of capillaries per square millimeter.

Aortic tension and ischemic hindlimb model in $eNOS^{-/-}$ mice. To explore the role of NO in AM-induced collateral formation, we examined the vascular effects of adrenomedullin in $eNOS^{-/-}$ mice. The thoracic aortas were carefully dissected from 15-week-old $eNOS^{-/-}$ mice and age-matched control mice ($n = 5$, each), as already described [5]. Moreover, we examined the effects of adrenomedullin on aortic tension in 30–35-week-old PI3-K knockout mice [17] and wild-type mice ($n = 5$, each). Aortic rings (5 mm in length) were mounted in organ chambers filled with 20 ml of an oxygenated Krebs–Ringer bicarbonate solution at 37 °C. Isometric tension was recorded using a force transducer (Oriental, Tokyo). The aortic rings were precontracted with 10^{-6} mol/L L-norepinephrine and responses to adrenomedullin were studied in the presence or absence of vascular endothelium. The endothelium of the aortic ring was removed by gentle rubbing with a twist of cotton. Relaxation in aortic rings was expressed as the percent decrease in tension. Collateral formation after hindlimb ischemia was also examined in 15-week-old $eNOS^{-/-}$ mice and age-matched control mice ($n = 5$, each).

Statistics. All data are expressed as mean values ± SEM. Statistical comparisons of mean values were performed by ANOVA followed by the Student–Neumann–Keuls test. A p value of <0.05 was considered as statistically significant.

Results

Blood flow recovery by adrenomedullin

To evaluate the angiogenic effect of adrenomedullin, we generated hindlimb ischemia in wild-type C57BL/6J mice, which were treated with pcDNA3 or hAMpcDNA3 ($n = 8$ for each group). The blood flow in the ischemic and non-ischemic legs was monitored weekly by Laser Doppler imaging (Fig. 1A). In the control mice, blood flow of the ischemic leg recovered gradually, reaching about 60% of the blood flow of the non-ischemic left leg by week 5 (Fig. 1B). Adrenomedullin dramatically enhanced blood flow recovery. In the mice treated with adrenomedullin, blood flow of the ischemic leg recovered to almost the same level observed in the non-ischemic limb in 2 weeks (Fig. 1B).

Collateral formation was evaluated based on the capillary density of the ischemic hindlimb muscle harvested 5 weeks after surgery. Consistent with the measurement by Laser Doppler imaging, anti-CD31 immunostaining revealed that adrenomedullin significantly increased the number of detectable capillaries in the ischemic leg (Figs. 2A and B).

Efficacy of adrenomedullin gene transfer

Plasma concentration of human adrenomedullin was detected only in mice treated with the adrenomedullin gene. As shown in Fig. 3, the plasma concentration of human adrenomedullin was significantly higher in mice transfected with the adrenomedullin gene than in control mice. The plasma adrenomedullin level in

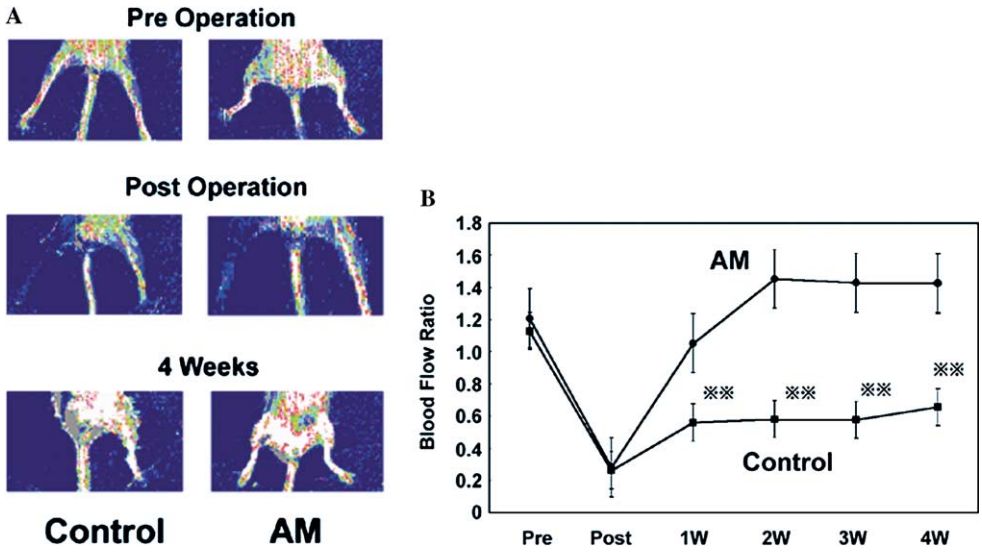

Fig. 1. Blood flow recovery by adrenomedullin. Unilateral hindlimb ischemia was induced in 30–35-week-old female wild-type C57BL/6J by resecting the right femoral artery. Hindlimb blood perfusion was measured using a Laser Doppler perfusion imager system (A). Either pc-DNA or hAMpcDNA3 (50 μg/week) was injected intramuscularly every week starting 1 week before surgery. The blood flow of the ischemic hindlimb was expressed as the ratio to that of the uninjured limb (B). $*p < 0.01$ vs AM.

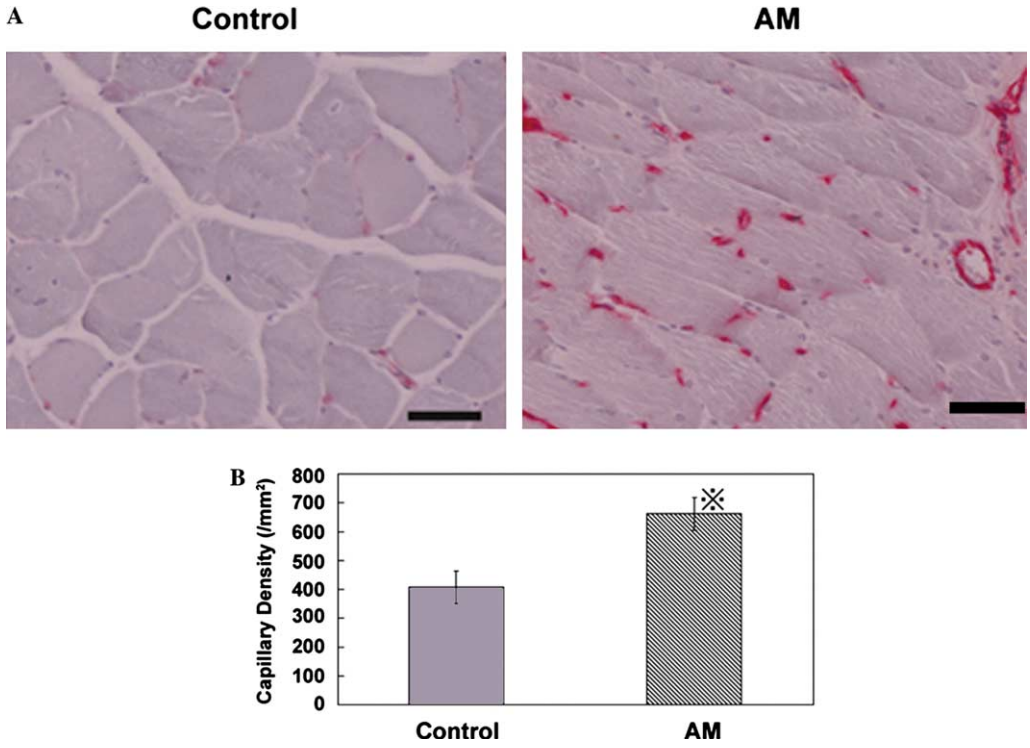

Fig. 2. Capillary density in the ischemic muscle 5 weeks after surgery. (A) Mice were sacrificed 5 weeks after surgery and capillaries in the ischemic muscles were visualized by anti-CD31 immunostaining. Bar, 50 μm. (B) The capillaries in 10 different fields were counted. Data are expressed as mean values ± SEM. $*p < 0.05$ vs Control.

adrenomedullin-treated mice exceeded that reported in healthy humans [18]. Immunoblotting using anti-human adrenomedullin antibody also showed a positive signal in thigh muscular tissues where the adrenomedullin gene was injected (data not shown).

Effects of adrenomedullin on blood flow recovery in eNOS-deficient mice

Accumulating evidence indicates that NO production by endothelial cells mediates the angiogenic effect

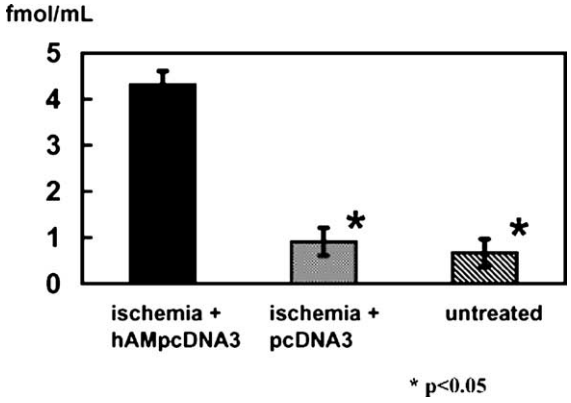

Fig. 3. Plasma adrenomedullin concentration in mice subjected to gene-transfer. We injected either hAMpcDNA3 or pcDNA3 (naked plasmid 50 μg/50 μl PBS/week) into the right thigh muscles followed by in vivo electroporation (100 V × 50 ms × 6 times). Plasma of mice was collected 5 weeks later ($n = 8$ for each group). The plasma concentration of adrenomedullin was higher in mice treated with adrenomedullin, demonstrating the efficacy of adrenomedullin gene transfer.

of many growth factors [19–22]. To investigate the role of eNOS in the enhancement of blood flow recovery by adrenomedullin, we evaluated the effects of adrenomedullin in eNOS-deficient mice. These mice lack the ability to dilate the capillaries via NO production in endothelial cells [23]. Surgical resection of the right femoral artery induced hindlimb ischemia in both eNOS$^{-/-}$ mice and wild-type mice to a similar extent (Fig. 4). However, blood flow recovery was severely impaired in eNOS$^{-/-}$ mice, as reported previously [19]. In contrast to the marked enhancement of collateral development observed in wild-type mice, adrenomedullin had no significant effects on blood flow recovery in eNOS$^{-/-}$ mice. Anti-CD31 immunostaining revealed that adrenomedullin treatment did not induce an increase in the number of visible capillaries in the ischemic leg.

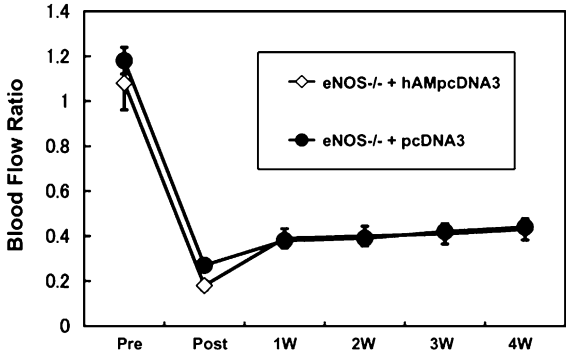

Fig. 4. Effects of adrenomedullin on blood flow recovery in eNOS-deficient mice. Hindlimb ischemia was induced in 15-week-old female eNOS$^{-/-}$ mice. The mice were treated with either pc-DNA or adrenomedullin (50 μg/week) ($n = 8$ for each group). Blood flow recovery was monitored weekly by a Laser Doppler imager.

Effects of adrenomedullin on tension of aortic rings

The effects of AM were examined in eNOS$^{-/-}$ mice. In control mice, adrenomedullin decreased aortic tension in a dose-dependent manner (Fig. 5). Although adrenomedullin reduced aortic tension, the extent was significantly smaller in eNOS$^{-/-}$ mice than in controls. Endothelial denudation did not affect aortic responses to AM in eNOS$^{-/-}$ mice. Furthermore, PI3-K$^{-/-}$ mice also showed an attenuated response to adrenomedullin, compared to wild-type mice. Adrenomedullin-induced vasorelaxation was again reduced in the aortas without endothelium and the extent of endothelium-independent vasorelaxation was similar between PI3-K$^{-/-}$ mice and wild-type mice (Fig. 5).

Discussion

Angiogenesis is a physiological response to ischemia and inflammation [24]. Recent progresses in this field have allowed the identification of many transcription factors, growth factors, and cytokines that are essential for spontaneous neovascularization [25]. It has been shown that administration of those factors to ischemic tissues effectively augments collateral development in animals [26] and humans [27]. In this study, we found that overly expressed adrenomedullin augmented collateral development in response to acute ischemia in murine hindlimbs. Endogenous adrenomedullin expression is markedly up-regulated during tissue ischemia and inflammation [6–8], which enhance capillary formation. Moreover, it was recently shown that targeting of the *adrenomedullin* gene leads to severe impairment of vascular formation [9–11]. Taken together, these findings suggest that adrenomedullin may play an important role in angiogenesis and that adrenomedullin may have a therapeutic utility by promoting collateral development in ischemic tissues.

The mechanism for adrenomedullin-induced angiogenesis was not elucidated by this study. However, adrenomedullin-induced development of collateral formation in the ischemic hindlimb was abolished in eNOS$^{-/-}$ mice. This suggests that the effects of adrenomedullin in the ischemic hindlimb were exerted through NO release by activation of eNOS. In fact, recent studies indicate that NO may promote angiogenesis because most of angiogenic factors, including VEGF, promote NO release from endothelial cells; these angiogenic effects are blocked by NOS inhibitors; angiogenesis is attenuated when NO bioactivity is reduced, e.g., hyperlipidemia [28,29]; furthermore, overly expressed eNOS increases the angiogenic response [30]. NO functions to promote migration [31] and survival of endothelial cells, although NO does stimulate their proliferation in vitro.

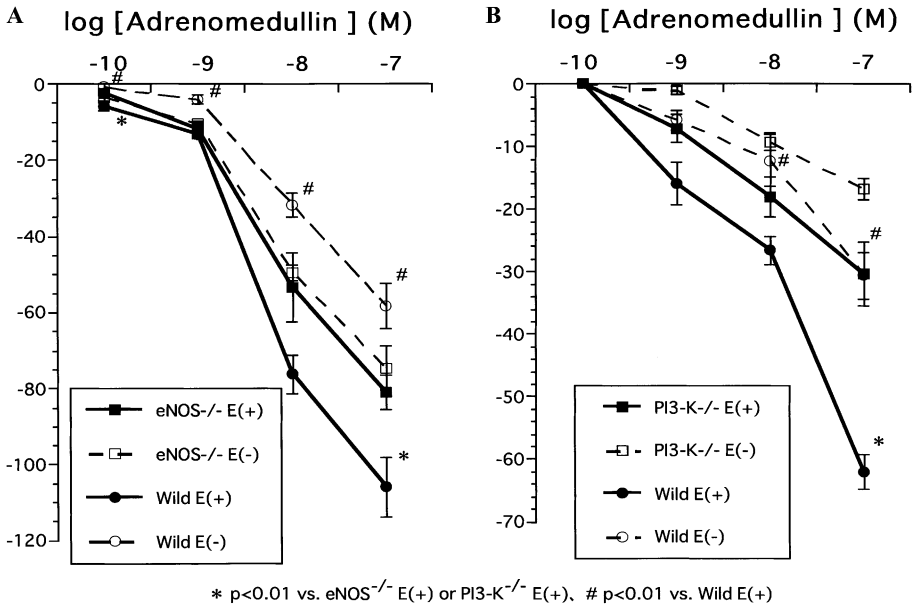

Fig. 5. Effects of adrenomedullin on aortic tension in eNOS-deficient mice (A) and in PI3-K-deficient mice (B). The thoracic aortas were dissected, mounted in a Krebs–Ringer bicarbonate solution, and precontracted by 10^{-6} mol/L L-norepinephrine ($n = 5$ for each group). The effects of endothelial denudation on the response to adrenomedullin were also examined. E(+), with endothelium; E(−), without endothelium.

In the present study, we transfected the adrenomedullin gene using electroporation. We have already confirmed the efficacy of the method with other genes. The expression of adrenomedullin peptide was still detected 1 week after plasmid injection. Adrenomedullin began to decrease aortic tension at around 10^{-10} mol/L; a 10-fold higher concentration than the circulating adrenomedullin attained by the present transfection method. However, it is possible that the locally expressed adrenomedullin level may be much higher, suggesting that adrenomedullin may exert some vascular action in the injected muscle.

In conclusion, we have demonstrated that adrenomedullin markedly enhances blood flow recovery after acute ischemia. Blood flow recovery correlated with the increase in the number of detectable capillaries. The plasma concentration of adrenomedullin was elevated in mice treated with adrenomedullin gene transfer. Adrenomedullin failed to promote blood flow recovery, when eNOS was genetically ablated. These results provide a mouse genetic evidence that eNOS is essential for adrenomedullin to promote collateral growth in response to tissue ischemia. Our findings suggest that adrenomedullin may potentially be used for therapeutic angiogenesis.

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