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Role of Na^+/Ca^{2+} Exchanger Type1 (NCX1) in the Angiogenesis Induced by Lipo-PGE_1 in Murine Hindlimb Ischemia Model

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Abstract：The Na^+/Ca^{2+} exchanger type-1 (NCX1) is considered to be involved in endothelial nitric oxide (NO) production. In this study, we examined the role of NCX1 in angiogenic response to acute hindlimb ischemia by using heterozygous NCX1 knockout ($NCX1^{+/-}$) mice. Furthermore, since Lipo-PGE_1 (prostaglandin E_1 encapsulated into lipid microsphere) is well known as a useful drug for peripheral arterial disease, we examined the effect of Lipo-PGE_1 in hindlimb ischemia-induced angiogenesis. We surgically induced unilateral hindlimb ischemia and monitored the blood flow recovery by Laser Doppler imaging for 4 weeks. Lipo-PGE_1 treatment enhanced the blood flow recovery and capillary density in wild-type mice. Western blotting at 28 day showed that Lipo-PGE_1 increased VEGF and phospho-Akt expression levels in the ischemic muscles. Interestingly, the blood flow recovery in $NCX^{+/-}$ mice was significantly augmented compared with that in wild-type mice, although it was similarly enhanced by Lipo-PGE_1 treatment. Moreover, to study possible involvement of endothelial NO synthase, we administered N^G-nitro-L-arginine methyl ester (L-NAME) to mice with hindlimb ischemia. L-NAME treatment eliminated the enhanced blood flow recovery observed in both $NCX1^{+/-}$ mice and Lipo-PGE_1-treated mice. These results suggest that NCX1, as well as Lipo-PGE_1, is involved in endothelial NO synthase-dependent angiogenesis.

Key words： **NCX, Lipo-PGE_1, Angiogenesis, eNOS, VEGF**

Introduction

The pathological role of endothelial cells is important, since locally synthesized substances from endothelial and vascular smooth muscle cells is considered to control vascular function.[1)] So far, many growth factors, such as vascular endothelial growth factor (VEGF) and transforming growth factor β (TGFβ), and their receptors have been identified as essential factors for angiogenesis.[2),3)] It has been shown that administration of VEGF and hepatocyte growth factor (HGF) to ischemic tissues effectively augments collateral development in animals and humans.[2),3)]

The regeneration therapy with angiogenic molecules has shown encouraging results for severe peripheral artery disease. Among them, prostaglandin E_1 (PGE_1) has proved to be beneficial for Peripheral arterial disease (PAD)[4)] as well as for end-stage heart failure in patients awaiting heart transplantation. However, it is known that PGE_1 is easily inactivated by hydrolysis in vivo. To dissolve this problem, Lipo-PGE_1 (prostaglandin E_1 incorporated in lipid microspheres), which is used as a potent vasodilator and platelet aggregation inhibitor, is well known as a useful drug for peripheral arterial disease.[5)] Lipo-PGE_1 is reported to increase blood flow in peripheral arteries by directly dilating vascular smooth muscle.[6)] However, the angiogenic effect of Lipo-PGE_1 is

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not well defined in animals and humans.

Sodium-calcium exchanger (NCX) is a plasma membrane transporter expressed in various cell types. The NCX family consists of three isoform, NCX1, 2 and 3. NCX1 is expressed in many tissues, whereas expression of NCX2 and NCX3 is restricted to brain and skeletal muscle. It is generally well known that NCX1 has the primary role in Ca^{2+} extrusion during excitation-contraction coupling. In endothelial cells, the expression of NCX1 protein has been demonstrated by immunoblotting and immunofluorescence.[7] Furthermore, cDNA coding for NCX1 has been detected in endothelial cells.[8] Recent reports suggest that endothelial NCX1 is involved in control of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$)[9),10] and nitric oxide (NO) production.[11),12] Thus, endothelial NO is an important regulator for endothelial cell growth, angiogenesis, and vascular tone. However, little is well known about the physiological and pathological roles of endothelial NCX1 in these processes.

Herein, we examined the role of NCX1 in angiogenesis in mice with hindlimb ischemia using heterozygous NCX1 knockout ($NCX1^{+/-}$) mice. Furthermore, we investigated the effect of Lipo-PGE_1 in ischemia-induced angiogenesis in wild-type and $NCX1^{+/-}$ mice. We showed that Lipo-PGE_1 accelerates angiogenesis in ischemic hindlimb. Effect of Lipo-PGE_1 was mediated by VEGF production, Akt phosphorylation, and NO production. NCX1 participated in the modulation of NO production. These observations revealed the possibility that NCX1 inhibitor would be useful as a therapeutic drug for PAD.

Materials and Methods

Animal model

Unilateral hindlimb ischemia was induced in wild-type and heterozygous NCX1 knockout mice ($NCX1^{+/-}$).[13] Animals were anesthetized by intraperitoneal injection of pentobarbital (50 mg/kg). The ligature was performed on the left femoral artery, 0.5cm proximal to the bifurcation of the saphenous and popliteal arteries. In some mice, intravenous injection of Lipo-PGE_1, a potent vasodilator and platelet aggregation inhibitor, were done before surgery and once a day after surgery for 7 days. To study possible involvement of endothelial NO synthase (eNOS), we treated mice with hindlimb ischemia with or without N^G-nitro-L-arginine methyl ester (L-NAME) (500 mg/l in the drinking water) for 28 days. We used all animals in accordance with the Guidelines for Animal Experiments in Fukuoka University.

Laser Doppler Perfusion Imaging

To provide functional evidence for ischemia-induced changes in collateral flow, Laser Doppler perfusion imaging (LDI, Moor Instruments) experiments were performed at post surgery and day 7, 14, 21, and 28. Mice were anesthetized with pentobarbital (40 mg/kg) and were placed on a heating plate at 37°C after excess hairs removal using depilatory cream from the hindlimb. Calculated perfusion was expressed as a ratio of ischemic to non-ischemic leg.

Western Blot Analysis

At day 28, gastrocnemius muscle from ischemic and non-ischemic legs were obtained, and homogenized in 1 ml of phosphate buffered saline (PBS) containing protease inhibitors. Protein content was then determined by BCA protein assay kit (Pierce). 100 μg of protein was separated on a polyacrylamide gel and electroblotted onto PVDF transfer membrane (Millipore). The membrane was blocked with 5% skim milk in PBS and then probed with antibodies against VEGF (1: 200, Sigma), phospho-Akt (1: 500, Sigma), Akt (1: 500, Sigma) and eNOS (1: 500, Sigma). We used the ECL system (GE Healthcare UK Ltd) for detection.

Arteriole Density

At day 28, gastrocnemius muscles from ischemic leg of wild-type mice with or without Lipo-PGE_1 were fixed with 10% formalin and embedded in paraffin. Sections at 5 μm thickness were stained with van Gieson. Arteriole densities in ischemic hindlimbs were analyzed by measuring the number of arterioles in light microscopic sections.

Statistical Analysis

All data are expressed as means ± SEM. Multiple comparisons were performed by one way analysis of variance (ANOVA) followed by the Fisher's test. The statistical difference between the two groups was examined using Student's t-test. A p value of <0.05 was considered as statistically significant.

Results

Effects of Lipo-PGE_1 treatment in mice with hindlimb ischemia

To examine the angiogenic effect of Lipo-PGE_1, we generated unilateral hindlimb ischemia in mice and treated them with Lipo-PGE_1 (3 and 10 μg/kg/day, i.v.) for 7 days. The blood flow in the ischemic and non-ischemic legs was monitored weekly by Laser Doppler imaging for 28 days (Fig.

1A). Immediately after ligature of left femoral artery and vein, a marked reduction of blood flow was observed in the left leg. In the control mice, the blood flow of ischemic leg recovered gradually and reached about 60% of the blood flow before ligature after 28 days (Fig. 1B). Lipo-PGE$_1$ treatment significantly enhanced the blood flow recovery. In the mice treated with Lipo-PGE$_1$, the blood flow of the ischemic leg recovered to 80% of pre-surgery in 28 days. Vehicle treatment did not affect blood flow of the ischemic leg. Collateral growth was evaluated by density of arteriole (>300 μm^2) of the ischemic hindlimb muscle at day 28. Consistent with the measurement by Laser Doppler imaging, the number of arterioles was increased in ischemic leg treated with Lipo-PGE$_1$.

Induction of VEGF expression and Akt phospholyration by Lipo-PGE$_1$ treatment

We next examined the protein levels of signaling factors in the ischemic and non-ischemic legs. The protein level of VEGF was about 1.7 fold increased in ischemic legs than non-ischemic legs in control mice. The increase was more prominent in Lipo-PGE$_1$ treated mice (Fig. 2). The protein level of phospho-Akt, but not Akt, was also increased in ischemic legs in Lipo-PGE$_1$-treated mice, suggesting that Lipo-PGE$_1$ enhances production of VEGF and activates Akt after ischemia.

Blood flow recovery in ischemic hindlimb of NCX1$^{+/-}$ mice

To investigate the role of NCX1 in blood flow recovery after hindlimb ischemia, unilateral hindlimb ischemia was induced surgically in wild-type and NCX1$^{+/-}$ mice. Systolic blood pressure and heart rate did not differ between wild-type and NCX1$^{+/-}$ mice (data not shown). In NCX1$^{+/-}$ mice, the blood flow recovery after ischemia was significantly augmented compared with wild-type mice (Fig. 3). Furthermore, Lipo-PGE$_1$ treatment similarly enhanced blood flow recovery both in wild-type and NCX1$^{+/-}$ mice.

Contribution of eNOS in the blood flow recovery

It is known that NO production by endothelial cells medi-

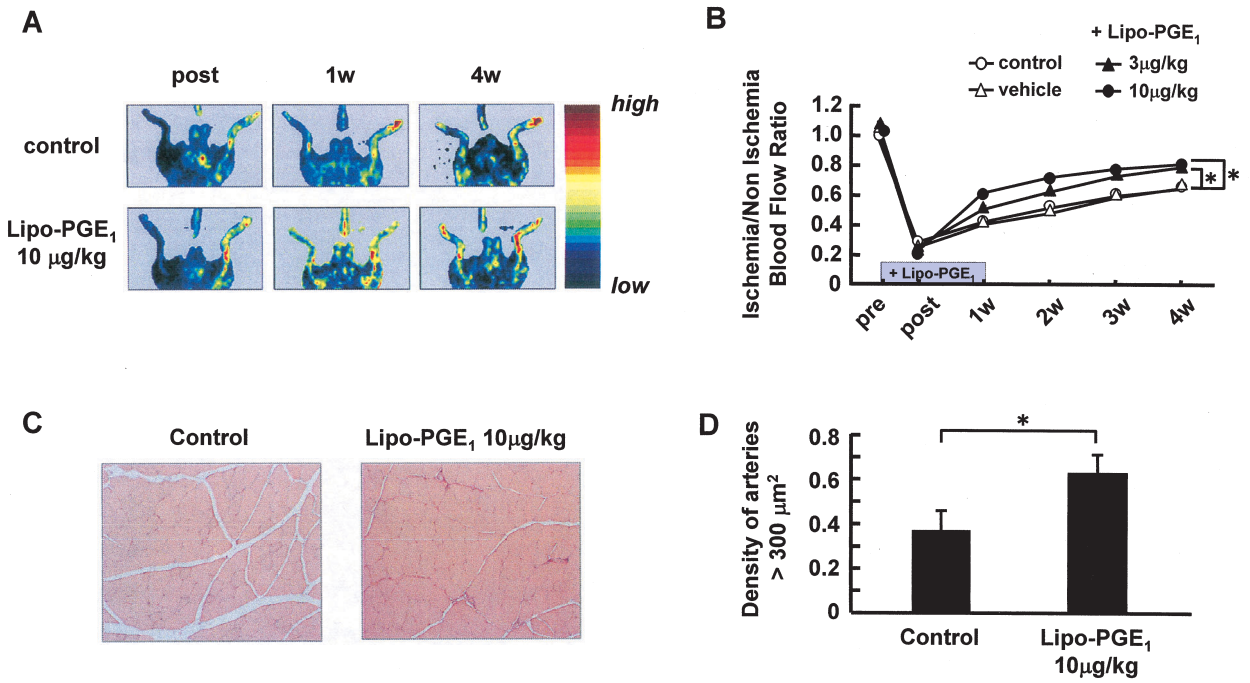

Fig. 1. Effect of Lipo-PGE$_1$ on blood flow recovery (A,B) and on capillarization after surgery (C,D). Unilateral hindlimb ischemia was induced in 10-week-old mice. Lipo-PGE$_1$ (3 and 10 μg/kg, iv) was injected before surgery and once a day after surgery for 7 days. Hindlimb blood perfusion was measured using Laser Doppler perfusion imaging system at post surgery and day 7 and 28. (A) Colors displayed in scale correspond to perfusion from 0% (dark blue) to 100% (red). (B) The ratio of blood flow on the ischemic (left) hindlimb to that in the non-ischemic (right) hindlimb (n=6–12). Lipo-PGE$_1$ treated mice (closed triangles and closed circles) showed accelerated perfusion recovery compared with vehicle-treated (open triangles) or control mice (open circles). (C) Arterioles in the ischemic muscle at 28 day after surgery were visualized by van Gieson staining. (D) The number of arterioles (> 300 μm^2) in ischemic muscle with or without Lipo-PGE$_1$ treatment was counted (n=4). The density of arterioles was increased by Lipo-PGE$_1$ (10 μg/kg, iv).

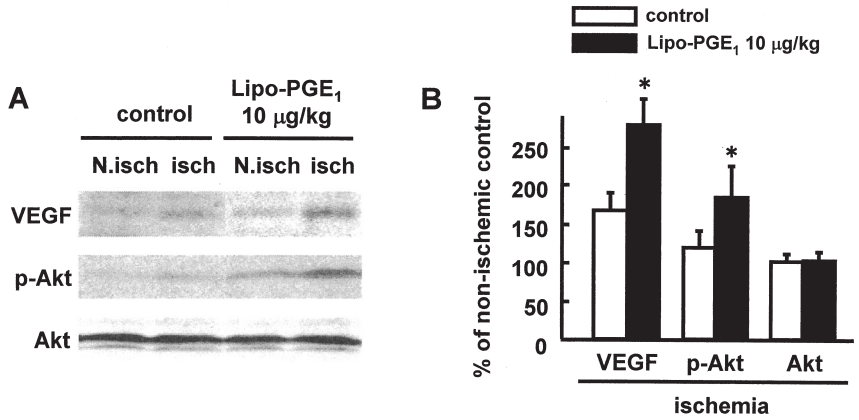

Fig. 2. Effect of Lipo-PGE$_1$ on VEGF expression and Akt activation. (A) Western blotting of VEGF, phospho-Akt and Akt proteins in the nonischemic and ischemic muscle at 28 day after surgery. (B) VEGF protein level of the ischemic leg was markedly upregulated by 250% in mice treated with Lipo-PGE$_1$ (10 mg/kg), and phospho-Akt content of the ischemic leg was upregulated by 180% (n=3–4).

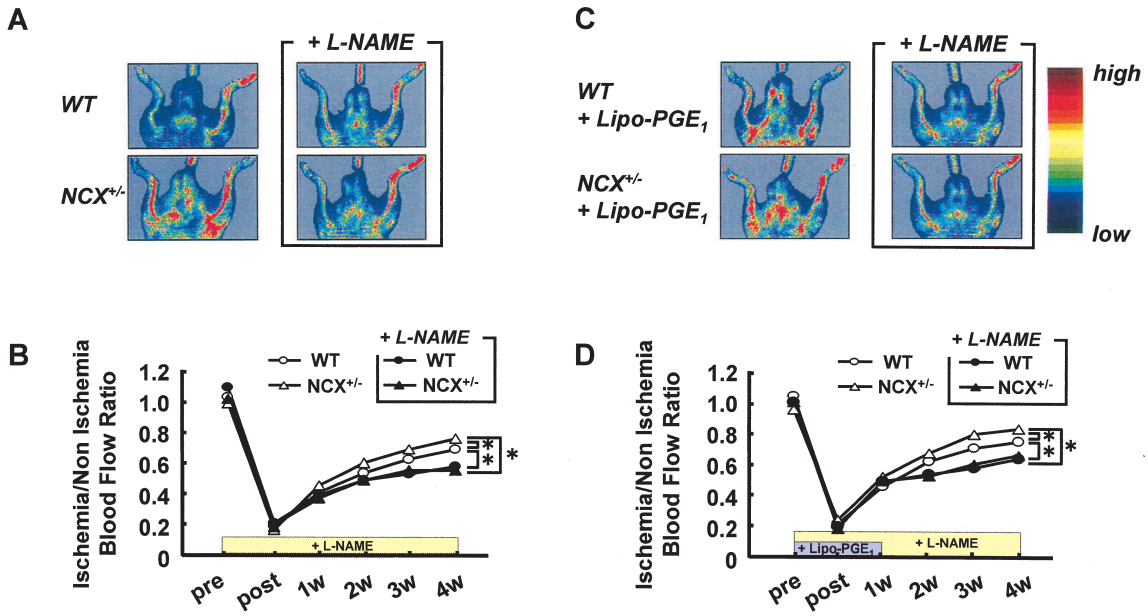

Fig. 3. Hindlimb blood flow in NCX1$^{+/-}$ and wild-type treated with L-NAME. Hindlimb blood flow of mice was monitored by Laser Doppler perfusion imaging. Lipo-PGE$_1$ treatment enhanced blood flow recovery in both mice (C, D). Blood flow recovery was enhanced in NCX1$^{+/-}$ mice. L-NAME treatment eliminated enhanced angiogenesis observed in both NCX1$^{+/-}$ mice and Lipo-PGE$_1$ treated mice (n=6–12).

ates the angiogenic effect of many growth factors. To evaluate the role of NO in enhanced blood flow recovery in mice treated with Lipo-PGE$_1$ and in NCX$^{+/-}$ mice, L-NAME, a NOS inhibitor, was administered to these mice. As shown in Fig. 3, L-NAME treatment eliminated enhanced angiogenesis observed in both Lipo-PGE$_1$ treated mice and NCX1$^{+/-}$ mice. We further examined the eNOS protein levels in ischemic hindlimb of Lipo-PGE$_1$-treated and NCX1$^{+/-}$ mice. The expression levels of eNOS were increased in these mice (Fig. 4).

Discussion

PAD is significant medical problems worldwide. In recent years, a variety of substances, including prostanoids have been identified as a vasodilator and/or angiogenic factor. Despite advance in medical treatment, limb salvage and relief of pain are still not satisfactory in patients with severe

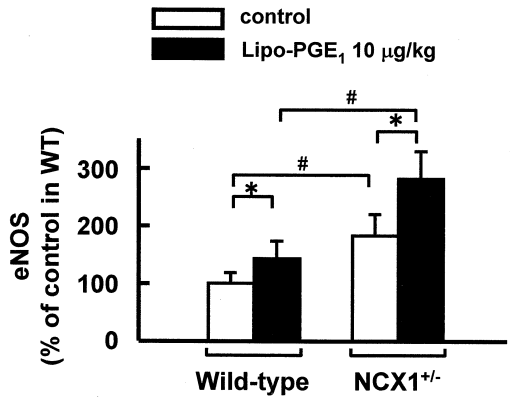

Fig. 4. Expression of eNOS protein in the ischemic and non-ishchemic hindlimb of NCX1$^{+/-}$ mice treated with Lipo-PGE$_1$. Quantitative evaluation of eNOS protein levels expressed as a percentage of the value in vehicle treated wild-type mice at 28 day after surgery. In the ischemic leg of Lipo-PGE$_1$ treated mice and NCX1$^{+/-}$ mice, eNOS expression levels were increased compared with wild-type mice (n=3-4).

disease. PGE$_1$ has various physiological actions such as vasodilation, reduction of blood viscosity, fibronolysis, and inhibition of platelet aggregation. PGE$_1$ also known to stimulate angiogenesis in several animal and clinical studies,[14),15)] it may serve as a useful drug for PAD. However, PGE$_1$ is easily inactivated by hydrolysis *in vivo*. A drug delivery system that enables the stabilization of PGE$_1$ is important. To dissolve this problem, several researcher use Lipo-PGE$_1$, PGE$_1$ incorporated in lipid microspheres. It is known that the liposomal formulation of some drugs enhances their efficacies. Recently, it is reported that Lipo-PGE$_1$ increases blood flow in peripheral arteries by directly dilating vascular smooth muscle.[5),6)] In this study, we examined the effect of Lipo-PGE$_1$ on hindlimb ischemia-induced angiogenesis in mice. We found that administration of Lipo-PGE$_1$ enhanced blood flow recovery and increased arteriole density in ischemic leg (Fig. 1A to 1D), suggesting that Lipo-PGE$_1$ may stimulate collateral development in ischemic hindlimb.

Recently, Gensch *et al.* have reported that PGE$_1$ increases the number of endothelial progenitor cell (EPC) in the blood and the bone marrow in mice.[16)] Kawabe *et al.* has also reported that the member of prostanoid, prostaglandin I$_2$ (PGI$_2$, prostacyclin), could recruit EPCs.[17)] He *et al.* have shown the role of PGI$_2$ in the proangiogenic function of EPCs.[18)] Furthermore, Dormond *et al.* reported that PGE$_2$ accelerates endothelial cell adhesion.[19)] These observations revealed that there are close relationship between angiogenesis and regulation of EPCs. At present, however, the relationship between NCX1 deficiency and EPC recruitment remains unclear, and further study will be needed.

It has been demonstrated that a number of growth factors, including VEGF, have an angiogenic potential. Among them, VEGF appears to be the most selective growth factor, insofar as it is specific for endothelial cells,[20)] whereas most of the other growth factors also stimulate growth of various types of cells. Previously, it was reported that prostaglandins may induce the expression of VEGF in various cells and tissues, including human monocytes [21)] and human synovial fibroblasts.[22)] Herein, we demonstrated that Lipo-PGE$_1$ treatment increased protein level of VEGF in ischemic hindlimb (Fig. 2). Phosphorylation of Akt also increased in ischemic leg, although the total level of Akt did not change (Fig. 2). These data suggest that the increased expression and function of VEGF with subsequent activation of Akt are involved in the mechanisms by which Lipo-PGE$_1$ stimulated ischemia-induced angiogenesis.

Akt is a serine/threonine protein kinase and is activated by phosphorylation from phosphoinositide-dependent kinases. Akt signaling regulates multiple steps in angiogenesis, including cell survival, migration, and capillary-like structure formation. Furthermore, this signaling pathway also regulates vessel integrity at least in part by controlling NO synthesis. It is generally accepted that NO is a critical angiogenic mediator. Previous studies have shown that the overexpression of eNOS in the vascular endothelium enhanced angiogenesis in response to hindlimb ischemia[23)] and the administration of NO donor stimulated proliferation of cultured rat endothelial cells.[24)] It has also been reported that NO is implicated in gene expression and regulation of gene products for angiogenic growth factors.[25)] Moreover, Haider *et al.* reported that PGE$_1$ analog induces eNOS expression in endothelial cells.[26)] From these observations, angiogenic effect of PGE$_1$ seems to be mediated by endothelial NO synthase. Therefore, to study possible involvement of eNOS in present model, we treated hindlimb ischemic mice with L-NAME for 4 weeks. L-NAME treatment eliminated Lipo-PGE$_1$-enhanced angiogenesis (Fig. 4). These results suggest that Lipo-PGE$_1$ may promote collateral growth in response to ischemia via VEGF/Akt/eNOS pathway.

We also studied the involvement of NCX1 in hindlimb ischemia-induced angiogenesis using NCX1$^{+/-}$ mice, in order to evaluate the role of $[Ca^{2+}]_i$ signaling in VEGF-mediated neovascularization. NCX1 can transport Ca^{2+} either out of cells (the forward mode) or into cells (the reverse mode) in exchange for $3Na^+$. NCX1 is driven by membrane potential as well as Na^+ and Ca^{2+} concentration gradients.[27)] In vascular endothelial cells, NCX1 has been reported to be involved

in endothelial NO production,[11),12)] and participate in endothelium-dependent control of vascular contraction and relaxation.[28),29)] Intriguingly, Schneider *et al.* suggested that NCX1 in the reverse mode may increase $[Ca^{2+}]_i$ and activate the Ca^{2+}/calmodulin complex, resulting in Ca^{2+}-dependent NO synthesis.[12)] In the present study, we found that ischemia-induced angiogenesis in $NCX^{+/-}$ mice was significantly augmented compared with that in wild-type mice (Fig. 4), suggesting that reduced function of NCX1 may promote collateral growth in response to ischemia. It seems that endothelial NCX1 primarily extrudes Ca^{2+} under physiological condition. Therefore, reduced NCX1 function in $NCX1^{+/-}$ mice may produce an increase in endothelial $[Ca^{2+}]_i$, resulting in the accelaration of Ca^{2+}-dependent NO synthesis. To further study possible involvement of eNOS, we administered L-NAME into $NCX1^{+/-}$ mice with hindlimb ischemia. L-NAME treatment eliminated enhanced ischemia-induced angiogenesis, which was observed in $NCX1^{+/-}$ mice as well as in Lipo-PGE_1 treated mice. These results suggest that NCX1 may be involved in eNOS-dependent angiogenesis probably through a change in $[Ca^{2+}]_i$ regulation.

In conclusion, Lipo-PGE_1 enhanced hindlimb ischemia-induced collateral development via increased expression of VEGF with subsequent activation of Akt and eNOS. On the other hand, the reduced function of NCX1 in endothelial cells may contribute to upregulation and activation of eNOS through an increase in $[Ca^{2+}]_i$ and subsequent neovascularization in ischemic hindlimb. Our findings suggest that NCX1 inhibitor, as well as Lipo-PGE_1, may potentially be used for therapeutic angiogenesis.

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