マウス下肢虚血モデルにおけるeNOS活性経路を介した メトフォルミンの血管新生促進作用に関する研究

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Metformin stimulates ischemia-induced revascularization through an eNOS dependent pathway in the ischemic hindlimb mice model

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Abstract

Objective: As first-line treatment for type2 diabetes, Metformin has gained a strong position. In addition, type2 diabetics benefit from the fact that Metformin is associated with a reduction in cardiovascular events. Nevertheless, there is a dearth of information concerning the functional role of metformin in regulating angiogenesis. Our present study explores whether metformin is involved in the modulation of the revascularization processes in vivo by employing a hindlimb mice model of ischemia-induced angiogenesis.

Methods: For comparative purposes, randomly selected Wild-type (WT) mice or eNOS deficient (eNOS-KO) were assigned to one of two groups. One group was orally administered a daily dose of metformin through a gastric tube while the other group served as a control with no metformin administered. Both groups were subjected to unilateral hind limb ischemia. Laser Doppler analysis coupled with capillary density staining with CD31was the method employed to determine Revascularization. AMPK and eNOS phosphorylation levels were assessed using Western blot analysis.

Results: Subsequent to hindlimb ischemic surgery, in comparison to the non-treated mice, metformin-treated WT mice showed accelerated limb perfusion, which was substantiated by laser Doppler blood-flow measurements and the presence of increased capillary density in the ischemic adductor muscle. Treatment with metformin significantly enhanced the increase in AMPK and eNOS phosphorylation levels of muscle tissues in WT mice induced by ischemia. In eNOS-KO mice, there was a significant increase in ischemic-tissue AMPK phosphorylation induced by metformin; however, blood flow recovery in ischemic limb after surgery was unaffected.

Conclusions: Metformin promoted revascularization in the presence of tissue ischemia through an AMPK/eNOS-related mechanism. Our study indicates that, in addition to its glucose-lowering effect, metformin fosters improved revascularization, which is responsible for its positive effect on patients with critical limb ischemia.

Abbreviations: AMPK: AMP-activated protein kinase; eNOS: endothelial nitric oxide synthase.

Introduction

As first-line treatment for type2 diabetes, Metformin (N',N'- dimethylbiguanide) has gained a strong position. Clinical trials in the UKPDS (United Kingdom Prospective Diabetes Study) demonstrated that treatment with metformin lessened the risk of macrovascular-related complications and all-cause mortality to a greater extent than is obtained through conventional

therapies associated with similar levels of lowered blood glucose[1]. Metformin has also displayed a correlation with decreased all-cause and cardiovascular risk of mortality[2-4]. Recently, it has been reported that monotherapy involving the most common insulin secretagogues appears to be linked with increased mortality as well as greater cardiovascular risk in comparison to treatment with metformin[5, 6] Furthermore, several authors have indicated the existence of a symptomatic benefit from metformin treatment in peripheral artery disease patients[7, 8]. Thus, clinical studies have led to the concept that metformin produces cardiovascular protective effects that are partly independent form its glycemic control function.

These clinical observations are in agreement with several experimental studies that have suggested that, besides its glucose-lowering effect, metformin positively affects vascular endothelial function and atherosclerosis. Metformin inhibits proinflammatory response and apoptosis in human vascular wall cells[9, 10]. Metformin treatment is also associated with a decrease in the activity of plasminogen activator inhibitor-1 (PAI-1) and tissue plasminogen antigen (tPA)[11, 12] and improvement of capillary flow[13]. Furthermore, metformin treatment attenuated vascular remodeling in monosodium glutamate-induced obese rats[14].

Numerous experimental studies indicate that activation of AMPK partly mediates the pleiotropic effects of metformin[15-17]. As a stress-activated protein kinase, AMPK plays a role in the regulation of energy and metabolic homeostasis[18-20]. Metformin activates AMPK in multiple cell types such as hepatocytes and endothelial cells[15-17]. It has also been reported that AMPK directly phosphorylates eNOS [21]. In addition, it has been shown that various AMPK activators, as a result of their ability to activate AMPK, stimulate phosphorylation of eNOS in endothelial cells and promote endothelial function[16, 22-24]. However, there is a dearth of information about the functional role of metformin, an AMPK activator, in regulating angiogenesis. In this study, we explored whether or not metformin modulates the revascularization processes in vivo by employing a hindlimb model of ischemia-induced angiogenesis.

Materials and Methods

Cell Signaling Technology (MA, USA) was the supplier for the following primary antibodies: phospho-eNOS antibody, eNOS antibody, phospho-AMPK (Thr 172) antibody, AMPK antibody and α-tubulin antibody. Dainipponn Sumitomo Pharmaceutical Co. (Tokyo, Japan) provided Metformin as a generous gift.

Animals and Experimental Protocol

Male wild-type mice (WT) and e-NOS deficient (eNOS-KO) mice in a C57BL/6J background between the ages of 8 to 10 weeks old served as the experimental subjects in this study[25, 26]. Western blot analysis was previously used to confirm that eNOS-KO mice are

deficient in eNOS protein[25]. The Institutional Animal Care and Use Committee of Nagoya University School of Medicine approved the study protocol. We employed a mouse model of neovascularization by surgically removing the entire left femoral artery and under anesthesia with sodium pentobarbital (50 mg/kg intraperitoneally) as previously described[23, 27, 28]. Mice were randomly assigned to one of three groups. Starting at 1day prior to surgery and continued for 4 weeks, one group received oral administration of metformin (150 or 300mg/kg/day, with 0.9% saline) by gastric tube and the control group received oral administration of saline (0.2ml/day) by gastric tube. We fixed the dosages at 150 or 300 mg/kg/day, since some groups of researchers had confirmed that these dosages were sufficient to investigate the effects of metformin in experimental mouse models[29, 30].

Laser Doppler Blood Flow Analysis and Clinical Score

Hindlimb blood flow was measured with a laser Dopplar blood flowmetry (LDBF; MoorLDI, Moor Instrument, Devon, UK), as previously described[23, 27, 28]. Before and on postoperative days 0, 3, 7,14,21 and 28, LDBF analysis on the legs and feet was performed. Depilatory cream was used to remove excess hairs from the limb before imaging, In order to minimize temperature variation, we placed the mice on a heating plate at 37°C. We analyzed the stored scanned images in order to quantify blood flow and calculate the mean LDBF values of the ischemic and non-ischemic limbs. In order to circumvent the possible occurrence of variations in data as a result from interference from ambient light and temperature, we expressed hindlimb blood flow as a ratio of the left (ischemic) to right (non-ischemic) hindlimb LDBF.

We designed a scoring system to more precisely evaluate the mobility of mice after limb ischemia: 0 = normal, 1 = pale foot or gait abnormalities, 2 = less than half of the foot is necrotic, 3 = more than half of the foot is necrotic without lower leg necrosis, 4 = more than half of the foot is necrotic with some lower leg necrosis, 5 = necrosis or auto amputation of entire lower limb. In order to obtain blood flow measurements, we carried out observation and recoding of the clinical scores for the mice at the same points in time[23].

Capillary and Arteriole Density Analysis

Analysis of the capillary density within thigh adductor muscle yielded specific evidence concerning microcirculation vascularity. On postoperative day 28, we gathered tissue samples from the ischemic thigh adductor skeletal muscles. From each sample, we removed 5um-thick tissue sections and froze them.

Tissue capillary and arteriole density were measured by histochemical staining with anti-mouse CD31 (BD Biosciences) and anti-mouse α-SMA (Sigma). In order to analyze capillary and

arteriole density we randomly chose fifteen microscopic fields from three different sections in each tissue block. Capillary and arteriole density were expressed as the quantity of CD31-positive cells and α-SMA positive cells per high power field (x 400) [23, 27]. A researcher blinded with respect to the samples preformed these quantifications.

Plasma Parameter Measurement

Enzymatic kits (Wako Chemicals, VA, USA) were used to measure plasma glucose and insulin levels. Blood was drawn from the mice by heart puncture on postoperative day 28.

Western Blot Analysis

Postoperative-day 7 tissue samples were homogenized in a lysis buffer prepared from 20 mm Tris-HCl (pH 8.0), 1% Nonidet P-40, 150 mM NaCl, 0.5% deoxycholic acid, 1 mM sodium orthovanadate, and protease inhibitor mixture (Roche). SDS-PAGE was employed to resolve cell lysates or culture media. Immunoblotting of the membranes with the indicated antibodies was carried out at a 1:1000 dilution in conjunction with subsequent secondary antibody conjugated with horseradish peroxidase at a 1:1000 dilution. Detection was carried out using ECL and ECL plus Western Blotting Detection kit (GE Healthcare, NJ, USA). Quantification of relative phosphorylation or protein levels was done using the Image J program. Immunoblots were normalized to α-tubulin.

Statistical Analysis

Data are presented as mean± S.E. as denoted in the figure legends. Statistical analysis was performed by ANOVA followed by Turkey's HSD test or Student's paired *t* test. A value of $P<0.05$ was accepted as statistically significant.

Results

Effect of Metformin on Ischemia-induced Revascularization

We subjected WT mice, treated with or without metformin, to unilateral hindlimb ischemia for the assessment of metformin's effect on the process of revascularization in response to ischemia. All mice survived surgery and remained healthy throughout the follow-up period. Metformin treatment (300mg/kg/day) had no effect on glucose in WT mice as previously described (metformin-treated WT mice: 100.2 ± 9.8 mg/dl and untreated mice: 101.6 ± 9.6 mg/dl)[31]. Representative LDBF images of hindlimb blood flow before surgery and at various postoperative points in time are presented in Figure 1A. There appeared to be accelerated blood flow recovery in the ischemic hindlimbs of the metformin (300mg/kg/day)-treated group of WT mice when compared to that of the group of untreated

mice. There was a significant increase in the ischemic muscle limb flow in WT mice treated with metformin (300mg/kg/day) on postoperative day 28 as demonstrated by quantitative analysis of hindlimb perfusion (Figure 1B).

Measuring capillary density and arteriole density in a histological section harvested from the ischemic muscle assessed the extent of revascularization at the microcirculatory level. Representative photomicrographs of endothelial cell marker CD31-stained muscle tissue are shown in Figure 2A. On postoperative day 28, metformin-treated WT mice had significantly greater capillary density in the ischemic hindlimb than non-treated WT mice, as demonstrated by quantitative analysis of CD31-positive cells (Figure 2B). Metformin-treated WT mice also demonstrated significantly greater arteriole density in the ischemic hindlimb than non-treated WT mice (Figure 2C and D). Thus, treatment with metformin promoted revascularization in ischemic tissue in WT mice.

Effect of Metformin on AMPK and eNOS Activation in Ischemic Muscle

eNOS plays an important role in regulating revascularization in response to tissue ischemia[26]. Western blot analysis was employed to assess the expression and phosphorylation of eNOS in ischemic adductor muscle at day 7 after surgery in order to explore the role of eNOS in metformin-mediated improvement of ischemia-induced revascularization. Although there was no difference between the two experimental groups of WT mice in relation to the total eNOS protein expression in ischemic muscles, there was significantly greater phosphorylation of eNOS in the ischemic muscle in the metformin-treated WT mice (Figure 3A and B).

The direct affect of AMPK on phosphorylate eNOS has been previously reported [21]. Thus, we assessed the expression and phosphorylation of AMPK in ischemic adductor muscle using Western blot analysis in order to clarify the role of AMPK in revascularization induced by metformin treatment. The results indicate that there was significant stimulation of phosphorylation of AMPK in WT mice ischemic muscle by metformin (Figure 3A and B).

eNOS Activation is Essential for Metformin-induced Revascularization

For additional analysis of the involvement of eNOS signaling in revascularization by metformin, we explored metformin's impact on blood flow in ischemic muscles of eNOS-KO mice. Upon LDBF analysis, no significant differences in limb perfusion were seen between metformin-treated and non-treated eNOS-KO mice on postoperative day 0, 3, 7 and 14 (Figure 4A). Because eNOS-KO mice exhibit severe ischemia-induced vascular insufficiency, which is accompanied by amputation, we assessed lower limb function and tissue salvage post-surgery using a clinical scoring system[32]. Similarly, there was no significant difference between the

index of severity of tissue ischemia after hindlimb surgery between metformin-treated and non-treated eNOS-KO mice (Figure 4B). In addition, according to immunohistochemical analysis, there was no significant difference in capillary and arteriole density between metformin-treated and non-treated eNOS-KO mice on postoperative day 28 (Figure 4C and D). Finally, we confirmed that there was an increase in the phosphorylation of AMPK in ischemic muscle of metformin-treated eNOS-KO mice in comparison to non-treated eNOS-KO mice (Figure 4E).

Discussion

We demonstrated that treatment with metformin promoted revascularization in response to ischemia, as seen in a state of vascular insufficiency created using a mouse model in the present study. Metformin treatment in WT mice stimulated recovery of limb perfusion and enhanced capillary density that was greater than seen in untreated mice, which was accompanied by eNOS activation. The beneficial actions of metformin on revascularization were nullified in eNOS-KO mice. Therefore, metformin has a beneficial effect on revascularization under ischemic conditions through eNOS signaling.

Type 2 diabetes increases the morbidity of coronary and peripheral artery diseases due to the occurrence of microvascular rarefaction and impaired collateral vessel growth, which are typical of ischemic conditions[33,34]. There is greater vulnerability to ischemic injury as well impairment of wound healing in the presence of these circulatory changes, ultimately yielding a more frequent occurrence of lower-limb amputation. In the present study, daily oral administration of metformin fostered revascularization in the presence of tissue ischemia. Thus, in addition to its glucose lowering effect, metformin could be beneficial for diabetes-related vascular complications.

A number of paper reported metformin improves tube formation via AMPK activation and increases eNOS activity in endothelial cells *in vitro* [16, 29, 35]. Previously, we have reported that AMPK activation in ischemic muscle enhances angiogenic repairs of ischemic muscle *in vivo*[23, 27, 36]. Metformin also improved cardiac function, an effect that was facilitated by the activation of AMPK and eNOS in a mouse model of myocardia infarction *in vivo*[17]. In the present study, despite the increased AMPK phosphorylation levels in ischemic muscle in eNOS-KO mice following metformin treatment, metformin did not affect perfusion recovery of ischemic limbs in eNOS-KO mice. Collectively, the ability of metformin to foster angiogenesis is likely a result of the its stimulation of the AMPK/eNOS signaling pathway within muscle.

The in vivo research carried out to illuminate the role of metformin in regulating angiogenesis has produced conflicting results. In agreement with our data, metformin treatment produces an increase in angiogenesis in endothelial cells[16, 35]. In contrast, others report that

metformin inhibits the growth of tumors in vivo by inhibition of neovascularization[37,38]. This discrepancy is possibly due to differences in the assay systems utilized for angiogenesis. There is also the possibility that metformin regulates pathological and physiological angiogenesis differentially as has been put forth to explain the effects of statins on vascularization[39]. Notably, it is recognized that activation of AMPK/eNOS signaling confers a pro-angiogenic phenotype in ischemic hindlimb[23, 26, 27]. Taking all these observations together suggests that the induction of AMPK/eNOS signaling by metformin treatment is able to facilitate revascularization in the presence of muscle ischemia.

The present study has several limitations. First, we employed a dosage of 300 mg/kg/ in order to activate AMPK/eNOS in mice. This dosage is higher than the usual dosage used in the case of humans. Nevertheless, therapeutic methods targeting the activation AMPK/eNOS could prove to be beneficial in the treatment of cardiovascular disease in humans. Second, the entire left femoral artery and vein were surgically removed in our mouse model of revascularization. This model may not replicate the human situation of arterial atherosclerotic occlusion. Therefore, it is necessary to carry out supplementary experimental research in order to obtain assessments using various models (chronic limb ischemia model, diabetic model and large animal model).

In patients suffering from peripheral arterial disease, the incidence of the diabetes mellitus is high[40]. Diabetes is one of the co-morbidities affecting wound healing. In Japan, in particular, the complication rates of diabetes mellitus and end-stage renal disease (ESRD) are high in patients with critical ischemic limbs. In addition to the glucose-lowering effect of metformin, the beneficial effects of metformin on cardiovascular disease have been demonstrated in various clinical studies[1-3, 5]. In the present study, metformin activated the AMPK-eNOS signaling pathway and thus promoted angiogenic repair in ischemic limbs. These results offer important fundamental data clarifying the efficacy of the clinical trials.

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Disclosures

None

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Figure legends

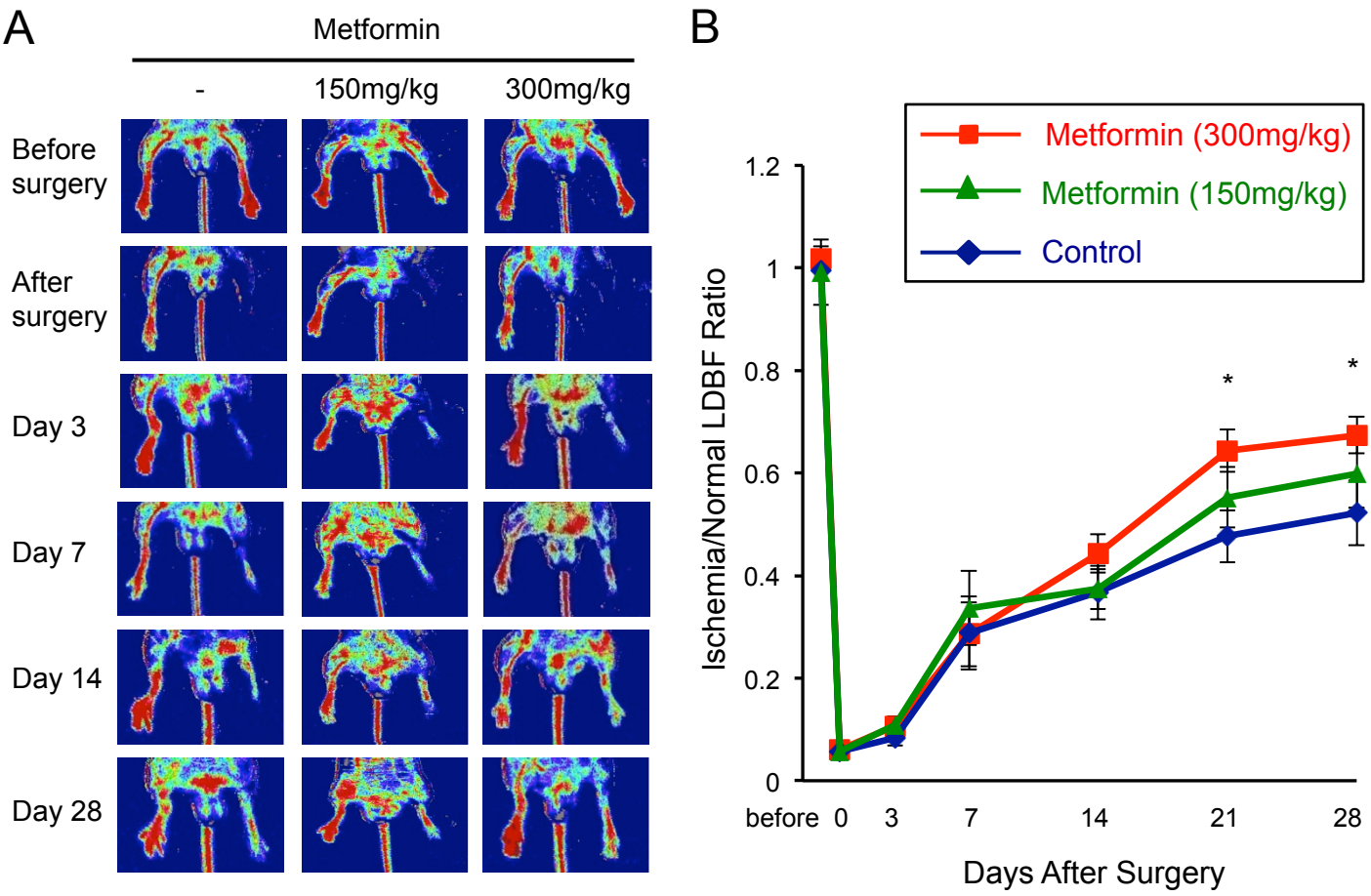
Figure 1: Metformin improves perfusion of ischemic limbs in WT mice. (A) Representative images of LDBF for WT mice treated with or without metformin before surgery and at different time points after surgery. Low perfusion signals (dark blue) were observed in the ischemic hindlimb of WT mice, whereas high perfusion signals (white to red) were detected in WT mice treated with metformin on post-operative day 3, 7, 14, 21 and 28. (B) Quantitative analysis of ischemic/normal LDBF ratio in WT mice (n=10 in each group) treated with or without metformin (*$p<0.05$ vs. control).

Figure 2: Increased capillary and arteriole density in ischemic metformin-treated WT mice. (A) Immunostaining of ischemic tissues with anti-CD31 monoclonal antibody (green) on post-operative day 28 (x400). (B) Quantitative analysis of capillary density in WT (n=10 in each group) treated with or without metformin (300mg/kg/day). (C) Immunostaining of ischemic tissues with anti-α-SMA antibody (red) on post-operative day 28 (x400). (D) Quantitative analysis of arteriole density in WT (n=10 in each group) treated with or without metformin (300mg/kg/day).

Figure 3: Effect of metformin on phosphoylation of AMPK and eNOS in ischemic muscle in WT mice. Western immunoblots with the indicated antibodies were performed on the ischemic adductor muscle of WT mice treated with or without metformin (300mg/kg/day) at 7 days after surgery. (A) The representative immunoblots and (B) quantitative analysis of relative changes in total and phosphorylated AMPK and eNOS. AMPK and eNOS were normalized to α-tubulin signal and expressed as percentage of the signal intensity of untreated WT mice (n=10).

Figure 4: AMPK/eNOS pathway is required for metformin-induced revascularization. (A) Quantitative analysis of ischemic/normal LDBF ratio in eNOS-KO mice (n=5 in each group) treated with or without metformin (300mg/kg/day). (B) Clinical Score in eNOS-KO mice treated with or without metformin (300mg/kg/day) as determined by an index of severity of limb ischemia. (C) Quantitative analysis of capillary density in eNOS-KO mice (n=5 in each group) treated with or without metformin (300mg/kg/day). (D) Quantitative analysis of arteriole density in eNOS-KO mice (n=5 in each group) treated with or without metformin (300mg/kg/day). (E) The representative immunoblots and quantitative analysis of relative changes in phosphorylated AMPK in eNOS-KO mice treated with or without metformin at 7 days after surgery. Western immunoblots with the indicated antibodies were performed on the ischemic adductor muscle of eNOS-KO mice treated with or without metformin at 7 days after surgery. Phosphorylation of AMPK was normalized to α-tubulin signal and expressed as a percentage of the signal intensity

of untreated eNOS-KO mice (n=5).


A
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150mg/kg
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Before surgery
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Control
Ischemia/Normal LDBF Ratio
1.2
1
0.8
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Days After Surgery
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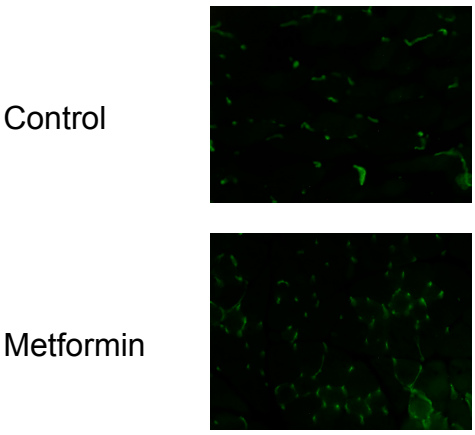
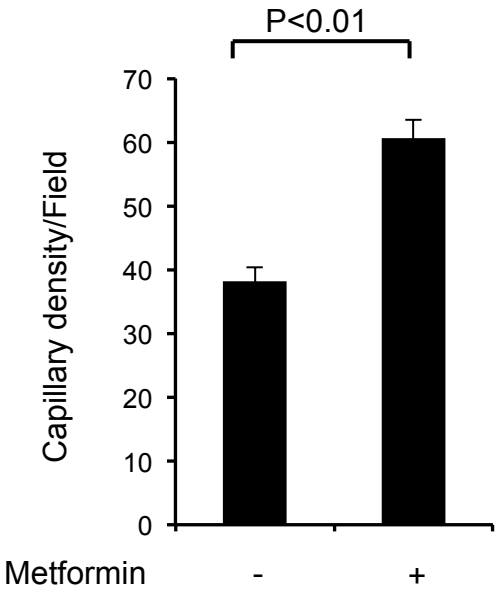
Figure 1

A



B



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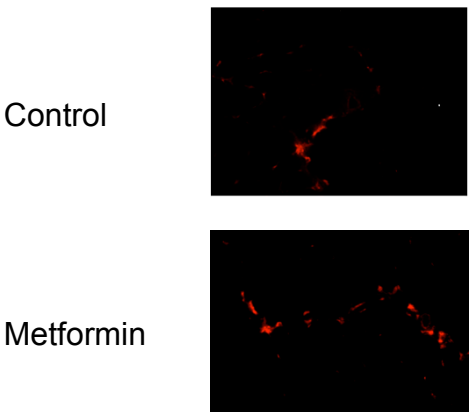

D

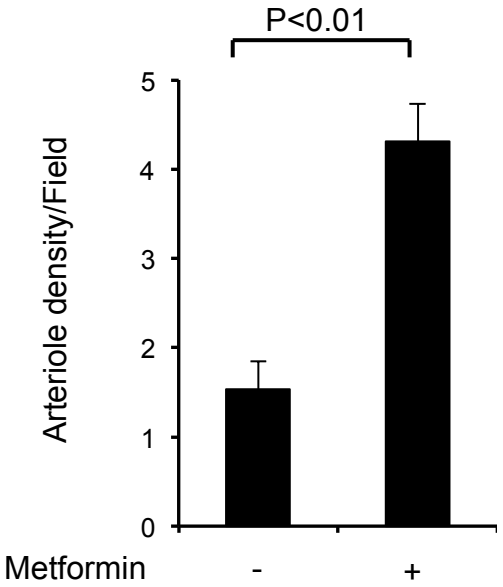

Figure 2

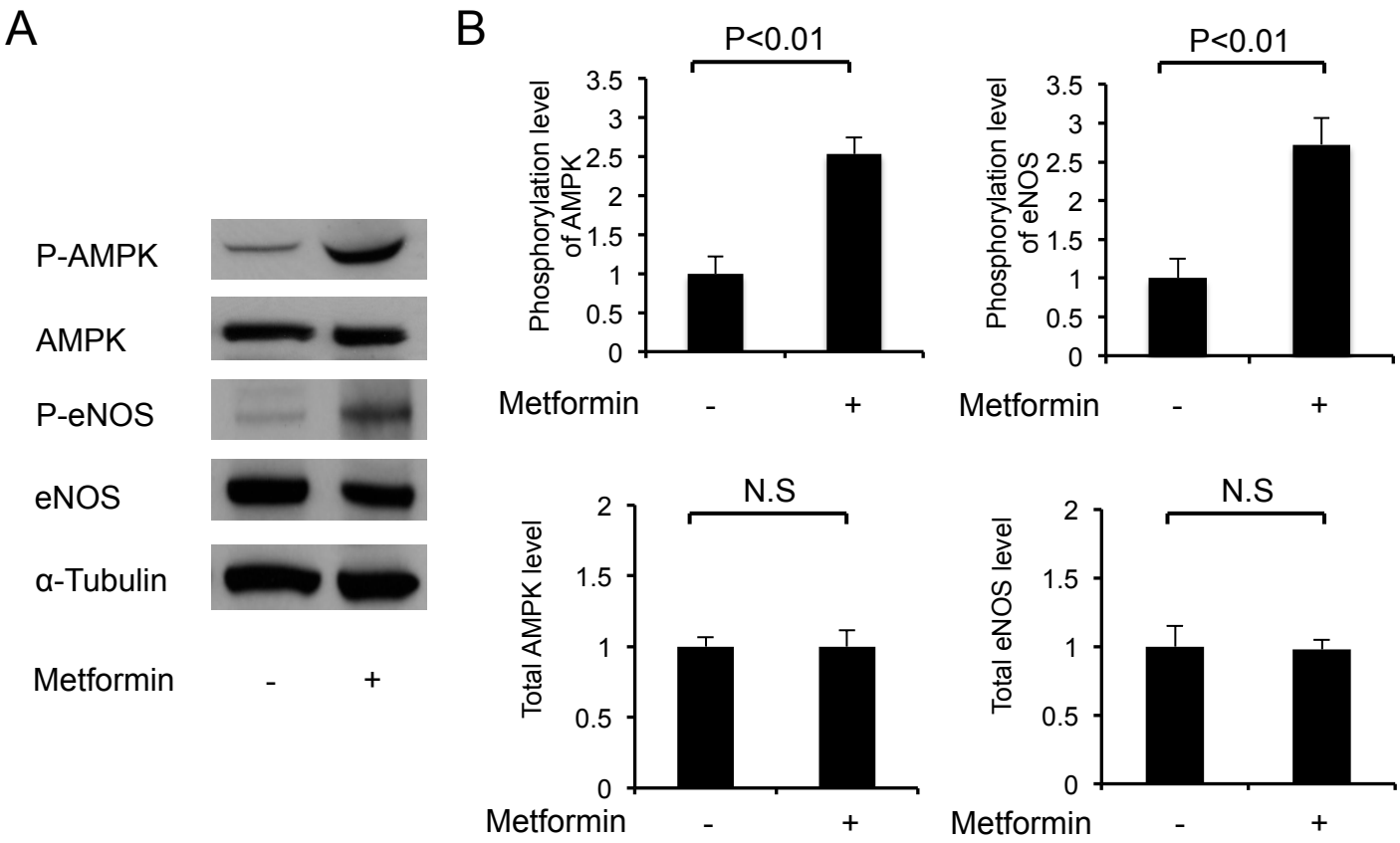
A
P-AMPK
AMPK
P-eNOS
eNOS
α-Tubulin
Metformin - +
B
P<0.01
Phosphorylation level of AMPK
3.5
3
2.5
2
1.5
1
0.5
0
Metformin - +
P<0.01
Phosphorylation level of eNOS
3.5
3
2.5
2
1.5
1
0.5
0
Metformin - +
N.S
Total AMPK level
2
1.5
1
0.5
0
Metformin - +
N.S
Total eNOS level
2
1.5
1
0.5
0
Metformin - +

Figure 3

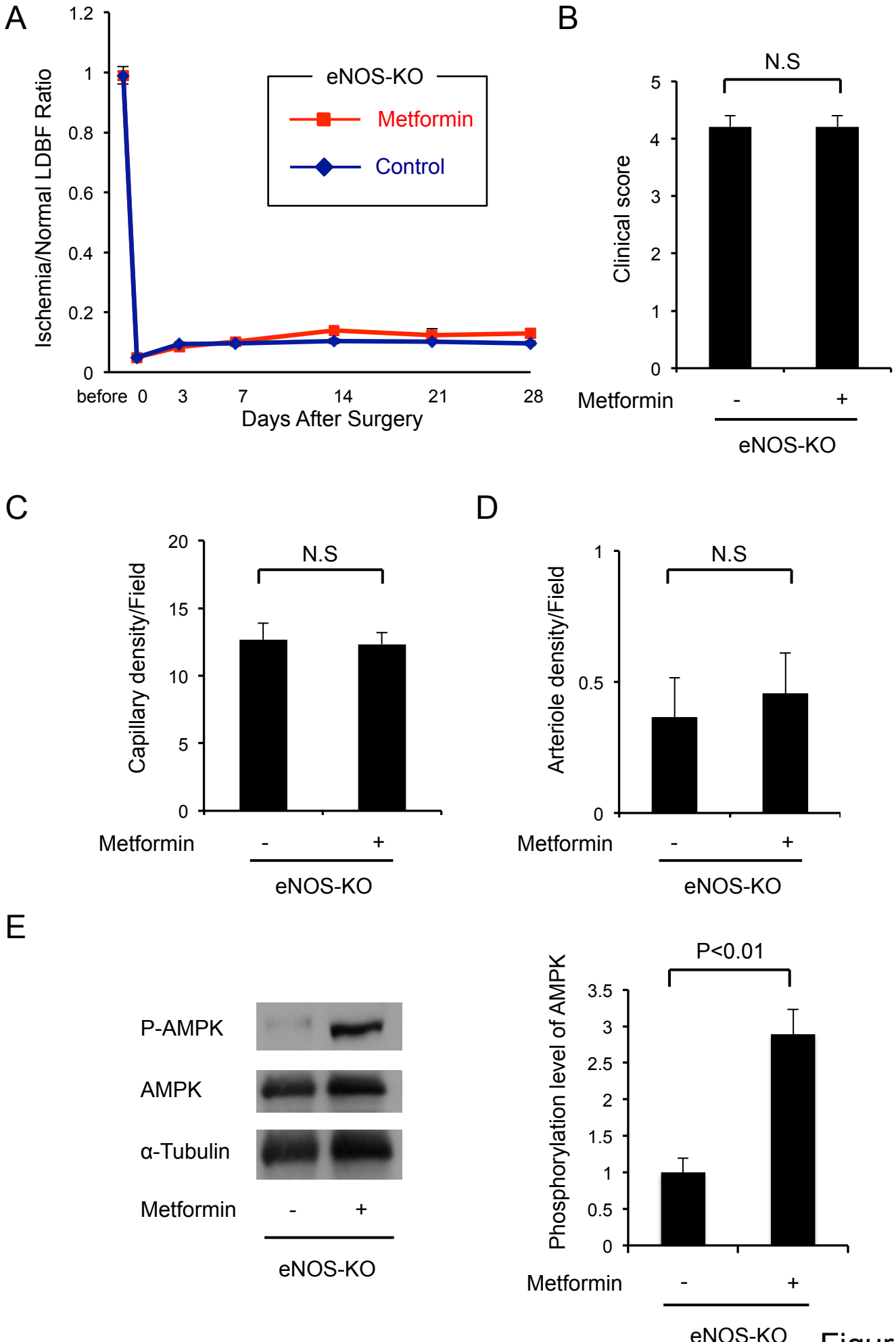
A
Ischemia/Normal LDBF Ratio
1.2
1
0.8
0.6
0.4
0.2
0
eNOS-KO
Metformin
Control
before
0
3
7
14
21
28
Days After Surgery
B
N.S
Clinical score
5
4
3
2
1
0
Metformin
-
+
eNOS-KO
C
Capillary density/Field
20
15
10
5
0
N.S
Metformin
-
+
eNOS-KO
D
Arteriole density/Field
1
0.5
0
N.S
Metformin
-
+
eNOS-KO
E
P-AMPK
AMPK
α-Tubulin
Metformin
-
+
eNOS-KO
P<0.01
Phosphorylation level of AMPK
3.5
3
2.5
2
1.5
1
0.5
0
Metformin
-
+
eNOS-KO

Figure 4

Figure legends

Figure 1: Metformin improves perfusion of ischemic limbs in WT mice. (A) Representative images of LDBF for WT mice treated with or without metformin before surgery and at different time points after surgery. Low perfusion signals (dark blue) were observed in the ischemic hindlimb of WT mice, whereas high perfusion signals (white to red) were detected in WT mice treated with metformin on post-operative day 3, 7, 14, 21 and 28. (B) Quantitative analysis of ischemic/normal LDBF ratio in WT mice (n=10 in each group) treated with or without metformin (*$p<0.05$ vs. control).

Figure 2: Increased capillary and arteriole density in ischemic metformin-treated WT mice. (A) Immunostaining of ischemic tissues with anti-CD31 monoclonal antibody (green) on post-operative day 28 (x400). (B) Quantitative analysis of capillary density in WT (n=10 in each group) treated with or without metformin (300mg/kg/day). (C) Immunostaining of ischemic tissues with anti-α-SMA antibody (red) on post-operative day 28 (x400). (D) Quantitative analysis of arteriole density in WT (n=10 in each group) treated with or without metformin (300mg/kg/day).

Figure 3: Effect of metformin on phosphoylation of AMPK and eNOS in ischemic muscle in WT mice. Western immunoblots with the indicated antibodies were performed on the ischemic adductor muscle of WT mice treated with or without metformin (300mg/kg/day) at 7 days after surgery. (A) The representative immunoblots and (B) quantitative analysis of relative changes in total and phosphorylated AMPK and eNOS. AMPK and eNOS were normalized to α-tubulin signal and expressed as percentage of the signal intensity of untreated WT mice (n=10).

Figure 4: AMPK/eNOS pathway is required for metformin-induced revascularization. (A) Quantitative analysis of ischemic/normal LDBF ratio in eNOS-KO mice (n=5 in each group) treated with or without metformin (300mg/kg/day). (B) Clinical Score in eNOS-KO mice treated with or without metformin (300mg/kg/day) as determined by an index of severity of limb ischemia. (C) Quantitative analysis of capillary density in eNOS-KO mice (n=5 in each group) treated with or without metformin (300mg/kg/day). (D) Quantitative analysis of arteriole density in eNOS-KO mice (n=5 in each group) treated with or without metformin (300mg/kg/day). (E) The representative immunoblots and quantitative analysis of relative changes in phosphorylated AMPK in eNOS-KO mice treated with or without metformin at 7 days after surgery. Western immunoblots with the indicated antibodies were performed on the ischemic adductor muscle of eNOS-KO mice treated with or without metformin at 7 days after surgery. Phosphorylation of AMPK was normalized to α-tubulin signal and expressed as a percentage of the signal intensity

of untreated eNOS-KO mice (n=5).