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Bovine milk-derived lactoferrin exerts proangiogenic effects in an Src-Akt-eNOS-dependent manner in response to ischemia

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Abstract

Lactoferrin (LF) exerts a variety of biological effects, including the promotion of angiogenesis by increasing the expression of angiogenesis-related genes and reducing blood pressure via a nitric oxide-dependent mechanism. In the present study, we investigated the effects of LF on angiogenesis using C57BL/6J mice that received daily unilateral treatment with or without bovine milk-derived LF (bLF) following unilateral hindlimb surgery. The analysis of laser speckle blood flow showed that bLF treatment promoted blood flow recovery in response to ischemic hindlimb. The capillary density of ischemic adductor muscles, as well as the phosphorylation of Src, Akt, and endothelial nitric oxide synthase (eNOS) was also significantly higher in bLF-treated mice than in vehicle-treated mice. Furthermore, bLF increased the phosphorylation levels of Src, Akt, and eNOS in *in vitro* experiments using human aortic endothelial cells (HAECs). The action of bLF on eNOS phosphorylation was abolished by both LY294002, a phosphatidylinositol 3-kinase inhibitor, and PP2, a Src inhibitor. Similarly, bLF-induced acceleration of tube formation, cell proliferation, and cell migration in HAECs were inhibited by LY294002 or PP2. Thus, bLF promotes vascular endothelial cell function via an Src-Akt-eNOS dependent pathway, thereby contributing to revascularization in response to ischemia.

Keywords: lactoferrin, angiogenesis, eNOS

Introduction

Lactoferrin (LF) is an 80-kD transferrin family, non-heme, iron-binding single-chain glycoprotein present in the secretory granules of neutrophils. LF is produced by mucosal epithelial cells and can be found in several biological exocrine fluids, including milk, and to a lesser extent, in bile, saliva, and tears (1). LF has various biological actions, including antibacterial and antiviral effects; antitumor activity; and functions involved in the regulation of the immune response, inflammation, and cellular growth, as well as in iron homeostasis (2). In addition, LF has been demonstrated to exert both angiogenic (3, 4) and antiangiogenic effects (5-7), making its involvement in angiogenesis controversial.

Angiogenesis plays a crucial role in the response to cardiovascular stress, including myocardial infarction (8), cardiac ischemia/reperfusion injury (9), cardiac hypertrophy (10), and peripheral circulating insufficiency (11). The enhancement of angiogenesis is an important therapeutic strategy for patients with the above diseases, and many studies investigating angiogenic mechanisms have identified factors or drugs that can be used to treat ischemic organs. Of these, endothelial nitric oxide synthase (eNOS) has been shown to be a main target for angiogenesis in response to ischemic stress in both *in vivo* (11-13) and *in vitro* conditions (14).

LF directly exerts its action on endothelial cells (ECs) by binding to specific sites on ECs through classical LF receptors such as a low-density lipoprotein-receptor related protein (15). In rats, bovine LF (bLF) is capable of inducing hypotension in a nitric oxide (NO)-dependent manner through the regulation of aortic relaxation (16). While this suggests that LF contributes to EC functions directly, the mechanism by which LF exerts effects on ECs has not yet been fully elucidated.

In the present study, we investigated the mechanisms of bLF action on ECs both *in vitro* and *in vivo*, and we showed that bLF promotes vascular endothelial function and angiogenesis in response to ischemia through a Src-Akt-eNOS-dependent mechanism.

Methods

Materials

Bovine lactoferrin (bLF) was purchased from Calbiochem (San Diego, CA, USA). The following commercially available antibodies were used in this study: anti-phospho-Akt (Ser473), anti-total Akt, anti-phospho-eNOS (Ser1177), anti-phospho-Src (Tyr416) from Cell Signaling Technology (Beverly, MA, USA); anti-total eNOS and anti-Src from Santa Cruz Biotechnology (Santa Cruz, CA USA); anti- α -tubulin, as a loading control, from Calbiochem (San Diego, CA, USA); and anti-CD31 (PECAM-1) from Becton, Dickinson and Company (BD; Tokyo, Japan). LY294002, a phosphatidylinositol 3-kinase (PI3-kinase) inhibitor, and 4-amino-5-(4-chlorophenyl)-7-(dimethylethyl)pyrazolo[3,4-*d*]pyrimidine (PP2), an Src inhibitor, were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Matrigel was obtained from BD Biosciences Japan (Tokyo, Japan). The CellTiter 96 AQueous nonradioactive cell proliferation assay kit ([3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] (MTS) reagent) was purchased from Promega KK (Tokyo, Japan).

Culture of vascular endothelial cells

Human aortic endothelial cells (HAECs) were purchased from Takara Bio Inc. (Otsu, Japan) and cultured in EGM-2 (Lonza, Tokyo, Japan), according to the

manufacturer's protocol. HAECs were passage from 5–8 times prior to use in each experiment. Cells were treated with bLF dissolved in sterilized water. In some experiments, cells were pretreated with LY294002 (2 μ M), PP2 (1 μ M), or vehicle alone for 30 min before bLF stimulation was initiated.

Tube formation assay

Tube formation assays were performed on growth factor-reduced Matrigel (BD Biosciences, San Jose, CA, USA), as described previously (17). In brief, 1×10^4 HAECs were cultured per well of Matrigel-coated plates in the presence of each concentration of bLF or vehicle, with or without LY294002 (2 μ M) or PP2 (1 μ M). Cells were then incubated at 37°C for 18 h. The formation of tube-like structures in the center field was observed using an inverted contrast microscope and captured with a CCD camera. Tube length was measured using the Image J 1.42 software.

Cell migration assay

Cell migration was evaluated using a modified Boyden chamber assay as described: 2.0×10^4 cells were added to the transwell insert (pore size, 3.0 μ m; BD Biosciences) and cultured for 6 h before cells that had migrated to the lower surface were fixed and stained with Giemsa stain solution. Five random microscopic fields per well were quantified.

Cell proliferation assay

Cells were seeded in 96-well plates at 1×10^4 cells/well for 24 h; subsequently, bLf was added for 8 h and then proliferation was assessed 1 h after the addition of MTS reagent by measuring absorbance at 490 nm with a plate reader.

Assay for cell death induction by serum starvation

HAECs were cultured in 96-well plates at 1×10^4 cells/well. Cells were incubated in EGM-2 as control or in serum- and growth factor-free EBM-2 with bLf or vehicle for 48 h. Serum deprivation-induced cell death was evaluated by an MTS-based assay.

Murine model of ischemic hindlimbs

All experimental procedures were performed in accordance with the guidelines of the Animal Research Committee, University of Tokushima Graduate School. Eight-week-old C57/BL6J mice were purchased from Nippon CLEA (Tokyo, Japan) and divided into 2 groups: a bLf ($50 \text{ mg}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$)-treated group and a vehicle-treated group. Mice were injected intraperitoneally with either bLf or vehicle 3 d before the surgery to induce ischemia in the hindlimbs; the procedure used has been previously described (17). In brief, mice were anesthetized through intraperitoneal injection of pentobarbital ($20 \text{ mg}\cdot\text{kg}^{-1}$) and subjected to unilateral hindlimb surgery. After ligation of the proximal and distal ends of the left femoral artery and vein, the entire artery and vein, with side branches, were resected.

Evaluation of peripheral blood flow

Hindlimb blood flow was measured before surgery and on postoperative days 0, 3, 7, 14, and 28 using a laser speckle blood flow (LSBF) analyzing system (Omega Zone,

Omega Wave Co., Tokyo, Japan), as described previously (17).

Capillary density in adductor muscles

Analysis of capillary density in adductor muscle was performed by CD31 immunohistochemistry (17). In brief, 28 d after surgery, adductor muscles were snap frozen in liquid nitrogen-cold isopentane containing OCT compound (Tissue-Tek, Sakura Finetek, Tokyo, Japan) and cryosectioned at 8 μm for immunohistochemistry and histological analyses. Capillary density was expressed as the number of CD31-positive cells corrected for the number of muscle fibers.

Protein extraction and western blot analysis

The methods used to extract protein from ECs and adductor muscles, as well as the western blotting protocol, have been previously described in detail (17). Immunoreactive bands were visualized using a chemiluminescence reagent (Forte Luminata Western HRP Substrates, Merck-Millipore, Billerica, MA), and membranes were exposed to Hyperfilm-ECL (GE Healthcare, Piscataway, NJ). Image J 1.42 software was used for semiquantitative analysis of densitometry.

Blood pressure measurement

Blood pressure was measured by the tail-cuff method, as previously described (17).

Statistical analysis

Data have been expressed as mean \pm standard error of mean (SEM) values. An unpaired 2-tailed Student's *t*-test was used to evaluate the differences between 2

groups. For comparisons among more than 2 groups, statistical significance was assessed using a one-way analysis of variance (ANOVA), and the significance of each difference was determined by post hoc testing using Tukey-Kramer's method. *P* values of <0.05 were considered to indicate statistical significance.

Results

Effects of bLF on eNOS activation and cell function in HAECs

It is widely recognized that eNOS activation is important for angiogenesis in ECs (11). First, we analyzed bLF action on eNOS phosphorylation in HAECs and found that bLF increased the phosphorylated levels of eNOS in a dose-dependent manner from 0.1 to 1.0 $\mu\text{g/mL}$ (Figure 1A). Our MTS-based assay demonstrated that treatment with 1.0 $\mu\text{g/mL}$ bLF significantly promoted cell proliferation (Figure 1B). To assess the effect of bLF on cell death, HAECs were treated with bLF or vehicle and incubated for 48 h in serum-free medium. We observed that bLF also reduced serum deprivation-induced cell death, as assessed by an MTS-based assay (Figure 1C). Moreover, quantitative analysis of tube-like formation in HAECs showed that bLF significantly stimulated tube formation relative to control cells treated with vehicle alone (Figure 1D). Interestingly, we found that higher concentrations of bLF (10 and 100 $\mu\text{g/mL}$) did not exert these proliferative effects, did not protect against serum starved-cell death, and did not promote tube formation in HAECs (Figure 1E-G).

bLF increases eNOS phosphorylation through the Src-Akt pathway

We found that eNOS activation and Src and Akt phosphorylation increased 1 h after bLF treatment (Figure 2). Because the PI3-kinase-Akt pathway is an important regulator of eNOS activation (18, 19) and Src is an upstream factor in PI3-kinase-Akt-eNOS signaling (20), we tested whether the Src-PI3-kinase-Akt pathway was involved in bLF-stimulated eNOS phosphorylation by using LY294002, a PI3-kinase inhibitor, and PP2, an Src kinase inhibitor. Pretreatment with either LY294002 or PP2 was found to markedly suppress bLF-stimulated eNOS activation (Figure 3A and E). Akt phosphorylation in response to bLF treatment was also diminished by PP2 (Figure 3E). Taken together, these findings suggest that bLF activates eNOS phosphorylation via an Src-PI3-kinase-Akt dependent mechanism.

Effects of bLF on tube formation, proliferation, and migration in HAECs

The effects of bLF on EC activation were assessed using assays for tube formation, cell proliferation, and migration. Quantitative analysis of tube length demonstrated that bLF increased tube formation in a concentration-dependent manner (Figure 1D), while MTS and Boyden chamber assays showed that bLF also promoted HAEC proliferation and migration (Figures 1B and C). We assessed the involvement of PI3-kinase, Akt, and Src in the pathway through which bLF activates cell tube formation, proliferation, and migration and found that bLF-activated EC functions were abolished by pretreatment with LY294002 or PP2 (Figure 3 A-D, Figure 3 E-H, respectively). Our results indicate that bLF promotes EC function through an Src-PI3-kinase-Akt mediated pathway.

bLF treatment ameliorated angiogenesis in response to ischemia in vivo

To assess the effects of bLF on angiogenesis *in vivo*, we used a mouse model of unilateral hindlimb ischemia induced via surgery. As shown in Table 1, there were no differences in body weight, systolic blood pressure, or pulse beat 1 week after the operation between mice with or without bLF treatment; however, consecutive LSBF analyses showed that the blood flow recovery after hindlimb ischemia was better in mice treated with bLF than in mice treated with the vehicle. The rate of LSBF in bLF-treated mice was also higher after 3 d and significantly increased at 7 d or later after ischemic hindlimb surgery, compared to that in control mice (Figure 4A).

Capillary density in the ischemic adductor muscle of bLF-treated mice

CD31-positive cells were measured in histological sections of ischemic adductor muscles to evaluate the angiogenic response following induction of hindlimb ischemia. Capillary density was found to be higher in bLF-treated mice than in vehicle-treated mice on day 28 after surgery (Figure 4B).

Action of bLF on eNOS, Akt, and Src phosphorylation in ischemic skeletal muscles

To identify the pathway through which bLF exerts its effects on angiogenesis, we assessed the phosphorylation levels of eNOS, Akt, and Src in ischemic skeletal muscle tissue. We found that eNOS phosphorylation, as well as Akt and Src phosphorylation, were significantly higher in the ischemic muscle tissues of mice treated with bLF than in mice treated with the vehicle on postoperative day 7 (Figure

4C). These results indicate that the effect of bLF on angiogenesis is exerted through Src-Akt-eNOS signaling, which is consistent with the findings for bLF action *in vitro*.

Discussion

In the present study, bLF was found to activate eNOS phosphorylation, tube formation, proliferation, and migration in ECs through an Src-Akt-dependent mechanism, leading to the promotion of angiogenesis. In an *in vivo* mouse model of hindlimb ischemia, bLF also augmented the phosphorylation of Src-Akt-eNOS and increased capillary number in ischemic adductor muscles, thereby contributing to the enhancement of blood flow recovery in response to ischemia.

LF is present in exocrine secretions from ECs such as milk and tears (1) and has a variety of biological functions, including antibacterial, antiviral, and anticarcinogenic activities, as well as functions in the regulation of the immune response, inflammation, cellular growth and differentiation, and iron homeostasis (2). LF has also been implicated in various pathophysiological conditions. For instance, LF increases bone formation (21), and the oral administration of LF has been shown to ameliorate bone density in ovariectomized mice (22). The action that LF exerts on bone occurs through stimulation of the proliferation and differentiation of osteoblasts, as well as inhibition of the differentiation of osteoclasts, and can contribute to the treatment of osteoporosis. In studies on cancer, LF has been found to inhibit tumor growth and metastasis through iron chelation (23, 24) and cause growth arrest through the inhibition of G1 cyclin dependent-kinase (25). Recently, several studies have shown that LF is also involved in the promotion of angiogenesis. In 1 study, human LF was found to promote vascular endothelial growth factor (VEGF)-mediated angiogenesis in ECs through the upregulation of KDR/Flt-1 expression (4). Similarly,

VEGF-mediated angiogenesis in ECs was enhanced by human apo-LF, but not human holo-LF (3). Finally, bLF has been shown to stimulate the expression of the angiogenic factors VEGF and fibroblast growth factor 2 via the extracellular regulated kinase 1/2 pathway in osteoblast cells (26), as well as to exert effects on hypotension and vascular relaxation through an NO-mediated pathway (16). Under ischemic conditions, increased LF concentration has been shown to be a predictor for the risk of fetal ischemic heart disease in the case of diabetes (27), suggesting compensatory elevation of LF against ischemia. Additionally, oral LF administration reduces intestinal ischemia-reperfusion injury through antioxidative, anti-inflammatory, and antiapoptotic actions in rats (28). Thus, LF has the potential to promote angiogenesis and is effective for revascularization in response to ischemia.

In the present study, we also found that bLF enhanced ischemia-induced angiogenesis *in vivo*, in addition to increasing EC function *in vitro* through an eNOS-dependent pathway. This bLF-induced eNOS activation was prevented by inhibitors of both PI3-kinase and Src, suggesting that bLF acts on eNOS in an Src-PI3-kinase-Akt-dependent manner. Indeed, eNOS is well known to be an important factor of angiogenesis activated by Akt-dependent signaling (18, 19). Similarly, bLF induced eNOS phosphorylation and EC functions in this study. These effects were suppressed by LY294002, a PI3-kinase inhibitor, indicating that LF activates EC function in an Akt-dependent manner. We also found that bLF increased Src phosphorylation and that the Src kinase inhibitor PP2 suppressed bLF-induced eNOS phosphorylation and EC activation. Src kinase has also been shown to be an upstream regulator of PI3-kinase-Akt-mediated eNOS activation by estrogen (20), a selective angiotensin II type 1 receptor (29), and grape juice (30). Taken together, our results demonstrate that bLF-induced eNOS activation is mediated through Src-PI3-kinase-Akt signaling.

In contrast to this study and other previous studies, several reports have shown LF to have an inhibitory effect on angiogenesis. For instance, the antitumor activity of bLF has been reported to be mediated by the inhibition of angiogenesis (6), and the oral administration of bLF has been found to inhibit VEGF-mediated angiogenesis in rats and ECs (5). Furthermore, bLF has been shown to suppress angiogenic activation in bovine aortic ECs (7). Thus, the effects of LF on angiogenesis remain controversial, although the results of the present study indicate that bLF activates endothelial function and promotes an ischemia induced-angiogenic response. While it is difficult to explain why our results are in opposition to those of previous studies, one plausible reason is that the doses of bLF used were different, ranging from 0.01 to 1 $\mu\text{g}/\text{mL}$ in our study versus 10 to greater than 100 $\mu\text{g}/\text{mL}$ in others. In support of this hypothesis, we found that a dose of 10 $\mu\text{g}/\text{mL}$ bLF had no effect on cell proliferation or tube formation in HAECs. Further investigations are necessary to address different effects of bLF on angiogenesis.

Conclusion

In this study, we have shown that bLF promotes revascularization in response to ischemic stress through an Src-Akt-eNOS-dependent pathway. Our observations indicate that bLF may have potential as a candidate agent that could be used to induce angiogenesis.

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Figure legends

Figure 1. Effects of bLF treatment in HAECs. (A) Dose-dependent effects of bLF on eNOS phosphorylation. Protein was extracted 1 h after bLF treatment. Left panel: Representative blots of phospho-eNOS, total eNOS, and tubulin. Right panel: Densitometric analysis of eNOS phosphorylation. Values are expressed in terms of

mean \pm SEM. * P < 0.05, ** P < 0.01, n = 6 in each group. (B) bLF action on endothelial cell proliferation. Cell proliferation was increased by bLF in a dose-dependent manner. Values are expressed in terms of mean \pm SEM. * P < 0.05, n = 6 in each group. (C) Effects of bLF on cell death induced by serum starvation. Cell death was inhibited by bLF in a dose-dependent manner. Values are expressed in terms of mean \pm SEM. * P < 0.05, n = 8 in each group. (D) Effects of bLF on tube formation. Endothelial cell tube formation was induced by bLF in a concentration-dependent manner. * P < 0.05, n = 4 in each group.

(E) Dose effect of 1–100 $\mu\text{g}/\text{mL}$ bLF on endothelial cell proliferation. Cell proliferation increased on treatment with 1 $\mu\text{g}/\text{mL}$ bLF, but not at 10 or 100 $\mu\text{g}/\text{mL}$ bLF. Values have been expressed in terms of mean \pm SEM. * P < 0.05, n = 8 in each group. (F) Effect of 1–100 $\mu\text{g}/\text{mL}$ bLF on cell death induced by serum starvation. Cell death was lower compared to that for the vehicle in the case of treatment with 1 $\mu\text{g}/\text{mL}$ bLF, but not in the case of 10 or 100 $\mu\text{g}/\text{mL}$ bLF. Values are expressed in terms of mean \pm SEM. * P < 0.05, n = 8 in each group. (G) Effect of 1–100 $\mu\text{g}/\text{mL}$ bLF on tube formation. Endothelial cell tube formation was induced by treatment with 1 $\mu\text{g}/\text{mL}$ bLF, but not in the case of 10 or 100 $\mu\text{g}/\text{mL}$ bLF. * P < 0.05, n = 4–5 in each group.

Figure 2. Time-dependent changes in eNOS, Akt, and Src phosphorylation in HAECs following bLF administration (1 $\mu\text{g}/\text{mL}$). Upper panel: Representative blots of phospho-eNOS, Akt, and Src, and total eNOS, Akt, Src, and tubulin. Lower panel: Densitometric analysis of eNOS, Akt, and Src phosphorylation. The values have been expressed in terms of mean \pm SEM. * P < 0.05, ** P < 0.01, n = 8 in each group.

Figure 3. Analysis of PI3-kinase-Akt or Src pathway involvement in bLF-induced

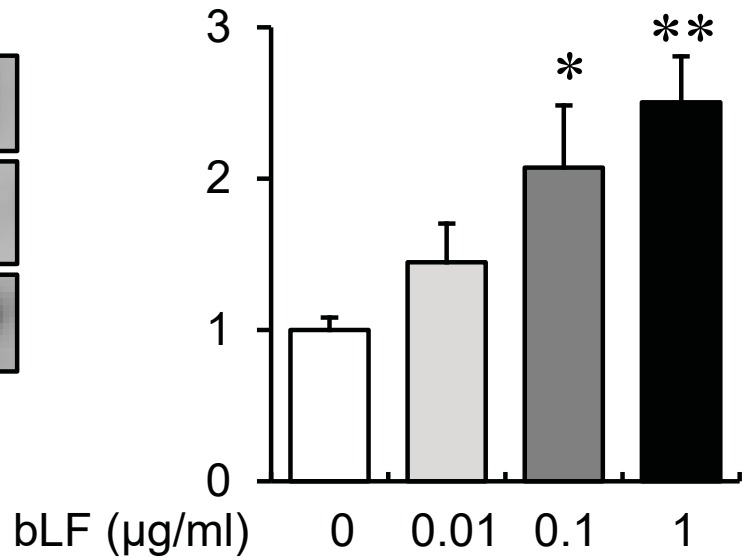
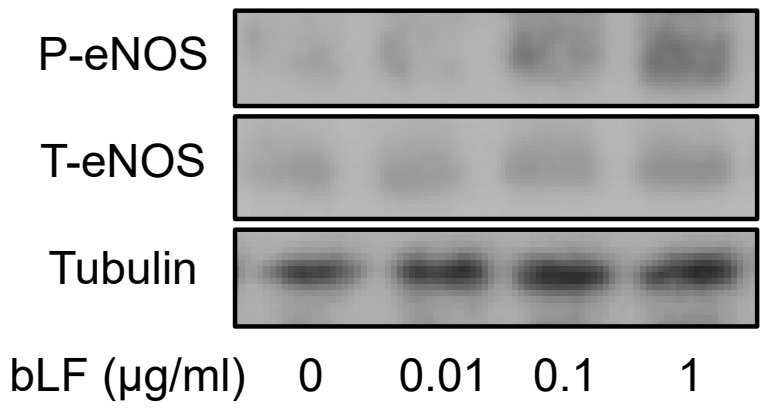
endothelial cell activation. (A) The induction of eNOS phosphorylation by bLF was abolished by LY294002. Protein was extracted 1 h after bLF treatment. Left panel: Representative blots of phospho-eNOS and Akt, and total eNOS, Akt, and tubulin. Right panel: Densitometric analysis of eNOS and Akt phosphorylation. The values have been expressed in terms of mean \pm SEM. * P < 0.05, ** P < 0.01, n = 8 in each group. The increases in (B) tube formation, (C) cell proliferation, and (D) cell migration elicited in HAECs by bLF were inhibited by LY294002. The values have been expressed in terms of mean \pm SEM. * P < 0.05, ** P < 0.01, n = 6–8 in each group. (E) bLF-induced eNOS phosphorylation was abolished by PP2. Protein was extracted 1 h after bLF treatment. Left panel: Representative blots of phospho-eNOS and Akt, and total eNOS, Akt, and tubulin. Right panel: Densitometric analysis of eNOS and Akt phosphorylation. The values have been expressed in terms of mean \pm SEM. * P < 0.05, ** P < 0.01, n = 8 in each group. The increases in (F) tube formation, (G) cell proliferation, and (H) cell migration elicited in HAECs by bLF were inhibited by PP2. The values have been expressed in terms of mean \pm SEM. * P < 0.05, ** P < 0.01, n = 6–8 in each group.

Figure 4. bLF treatment promoted revascularization in response to hindlimb ischemia in mice. (A) Left panel: Quantitative evaluation of ischemic to non-ischemic laser speckle blood flow (LSBF) ratio in mice treated with vehicle or bLF before surgery and on postoperative days 0, 3, 7, 14, and 28. Right panel: Representative LSBF images for hindlimb ischemia in vehicle or bLF-treated mice. * P < 0.05, ** P < 0.01 vs. mice treated with vehicle, n = 8 in each group. (B) Immunohistochemical analysis of capillaries in skeletal muscles. Left panel: Representative immunohistological staining of anti-CD31 in ischemic hindlimbs from mice treated with the vehicle or bLF. Right panel: Quantification of capillary density in mice treated with the vehicle

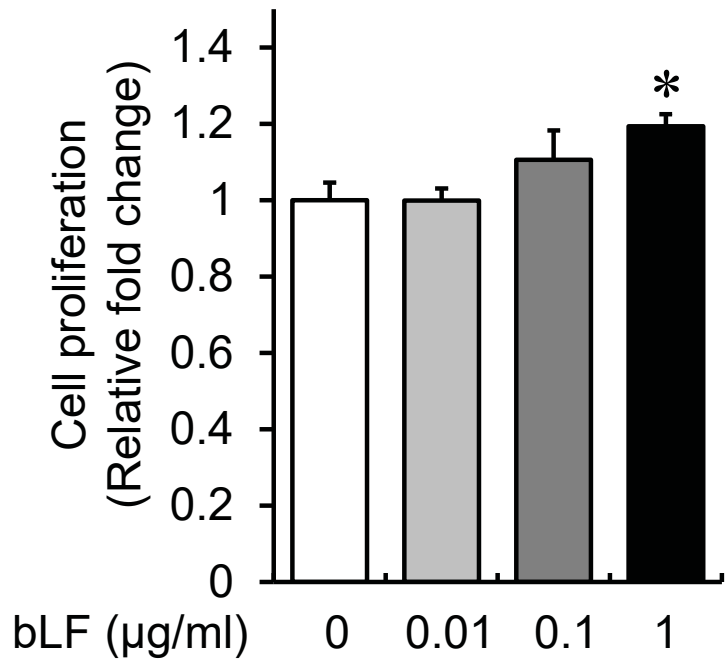
or bLF at 28 d after surgery. $*P < 0.05$ vs. mice treated with the vehicle, $n = 5-6$ in each group. (C) The levels of eNOS, Akt, and Src phosphorylation in the ischemic skeletal muscles of mice treated with the vehicle or bLF. Left panel: Representative blots of phospho- and total eNOS, Akt, and Src in the ischemic muscle tissue of mice treated with the vehicle or bLF at 7 d after surgery. Tubulin was used as a loading control. Right panel: Densitometric analysis of eNOS, Akt, and Src phosphorylation. The values have been expressed in terms of mean \pm SEM. $*P < 0.05$, $**P < 0.01$, $n = 6$ in each group.

Figure 1

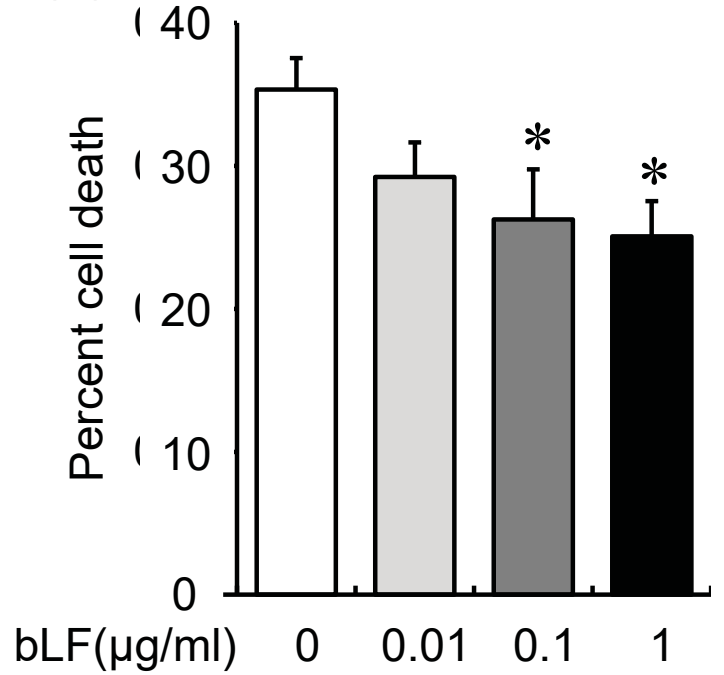
(A)



(B)



(C)



(D)

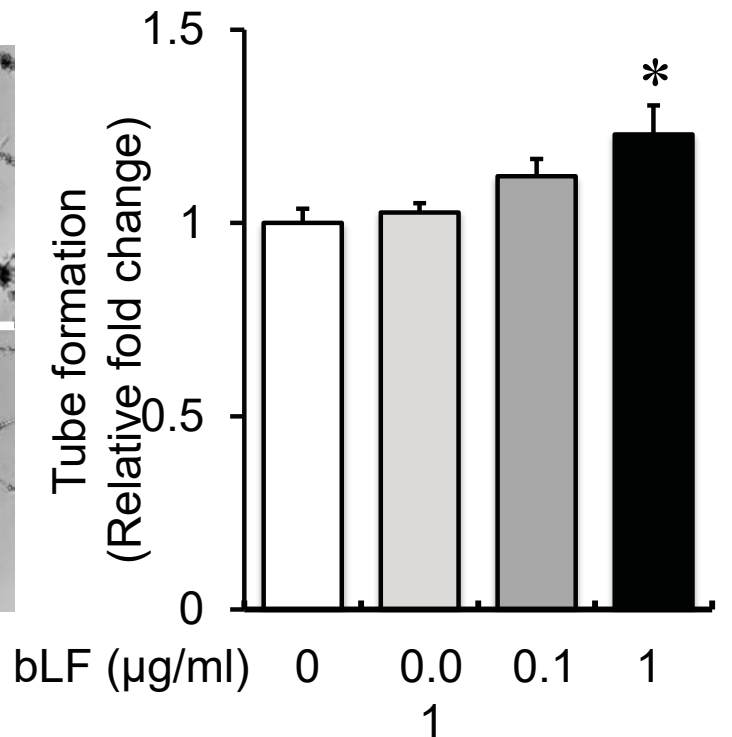
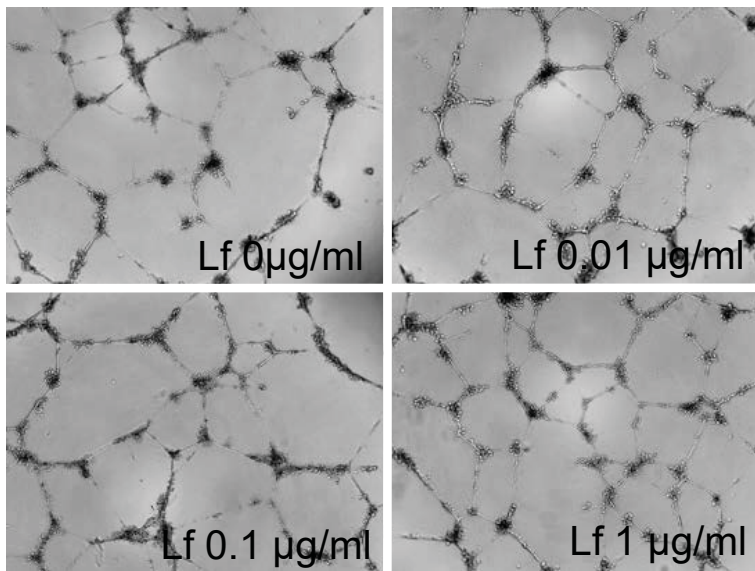
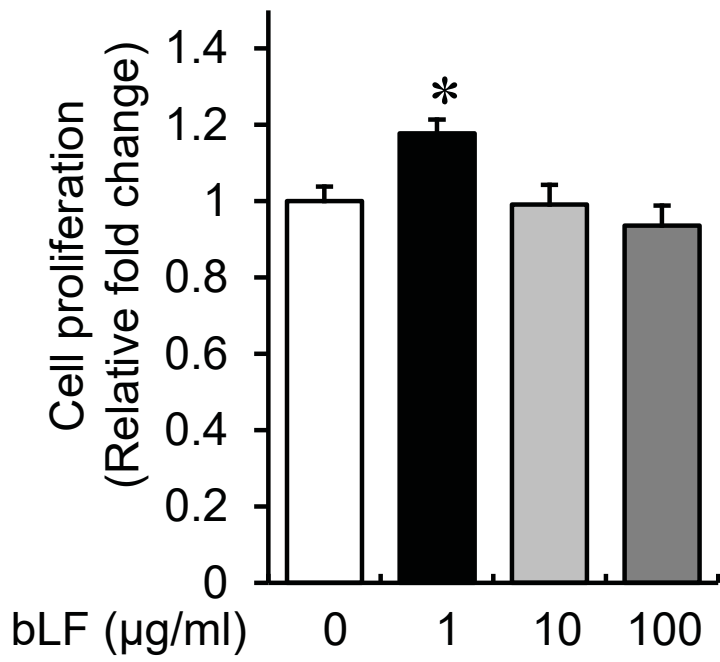
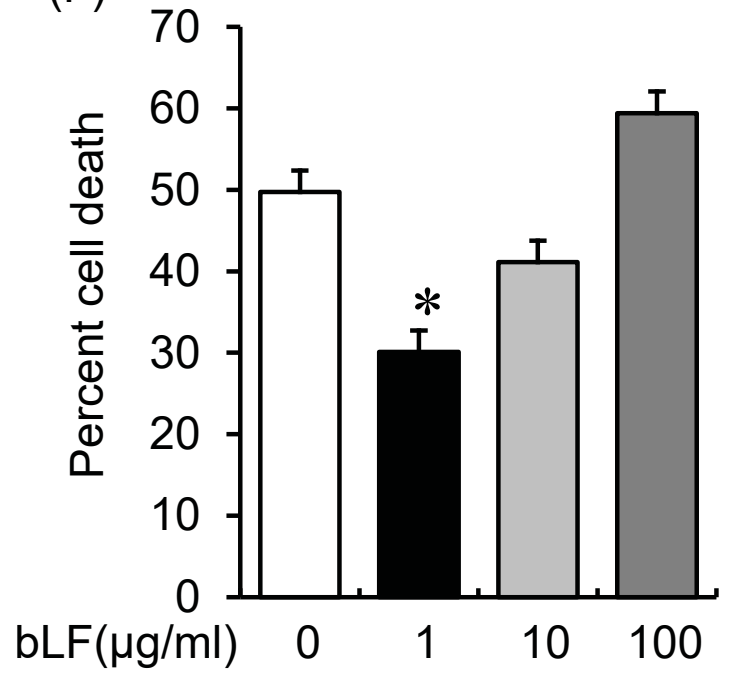


Figure 1continued

(E)



(F)



(G)

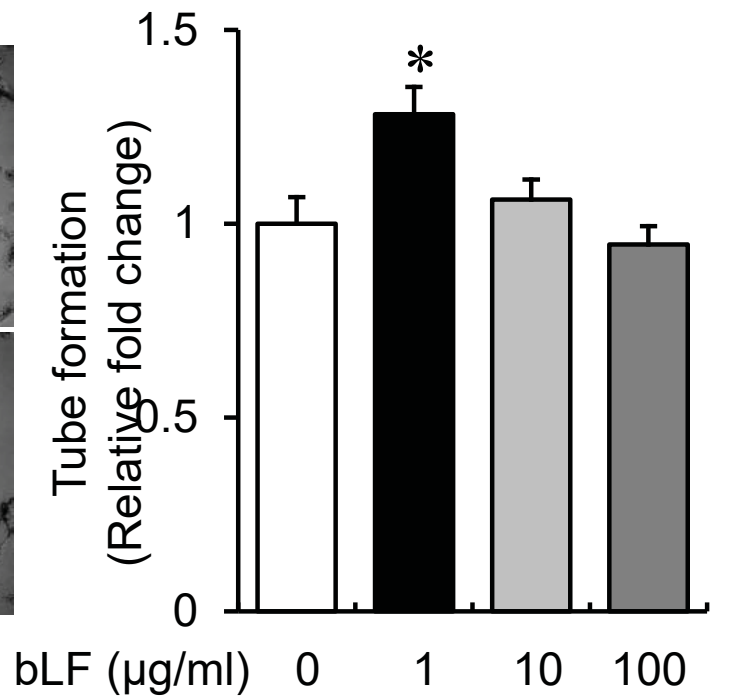
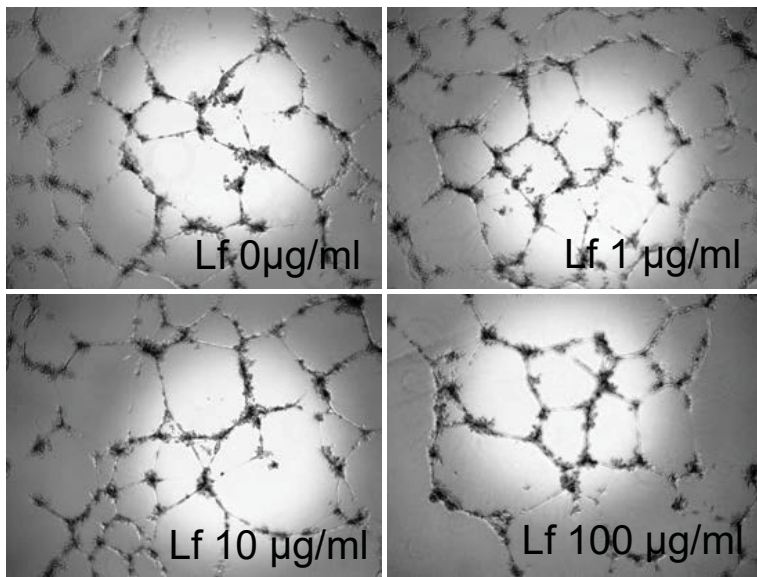


Figure 2

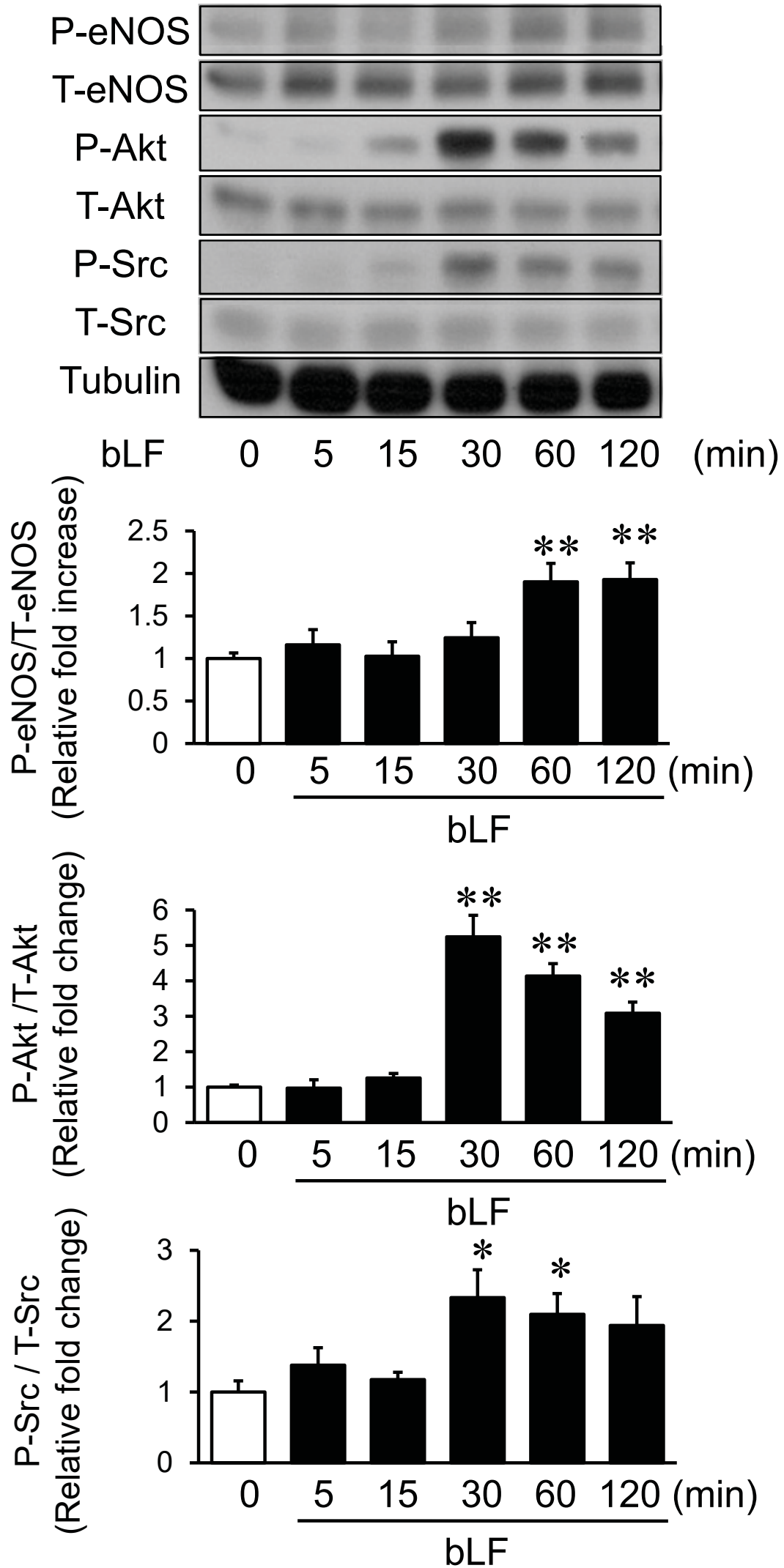


Figure 3

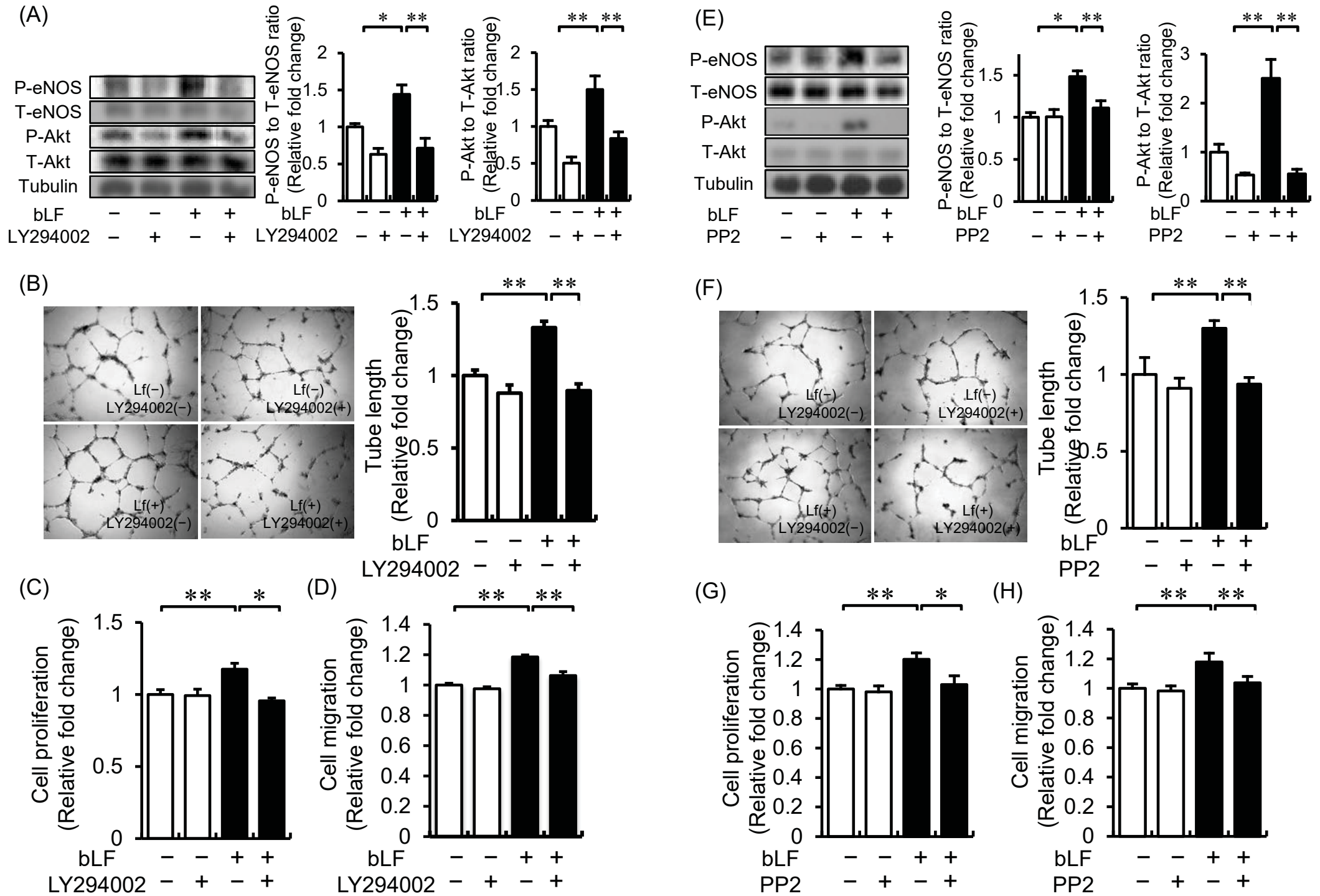
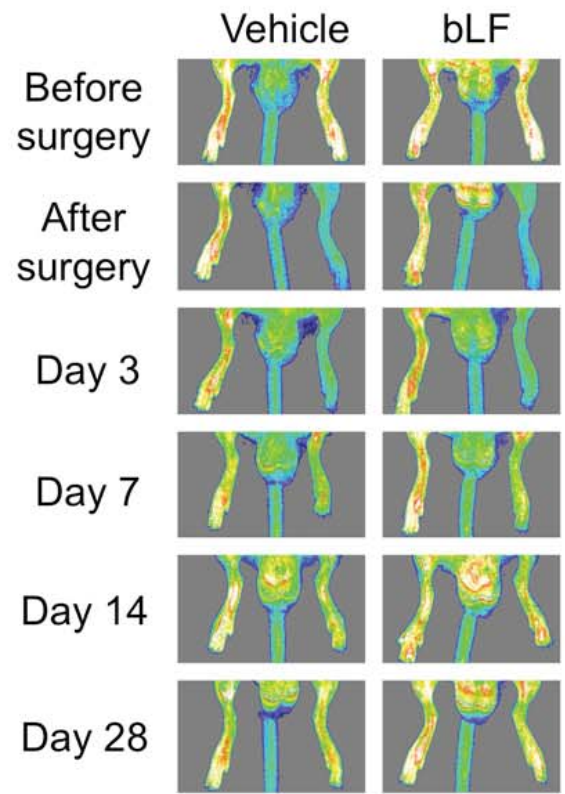
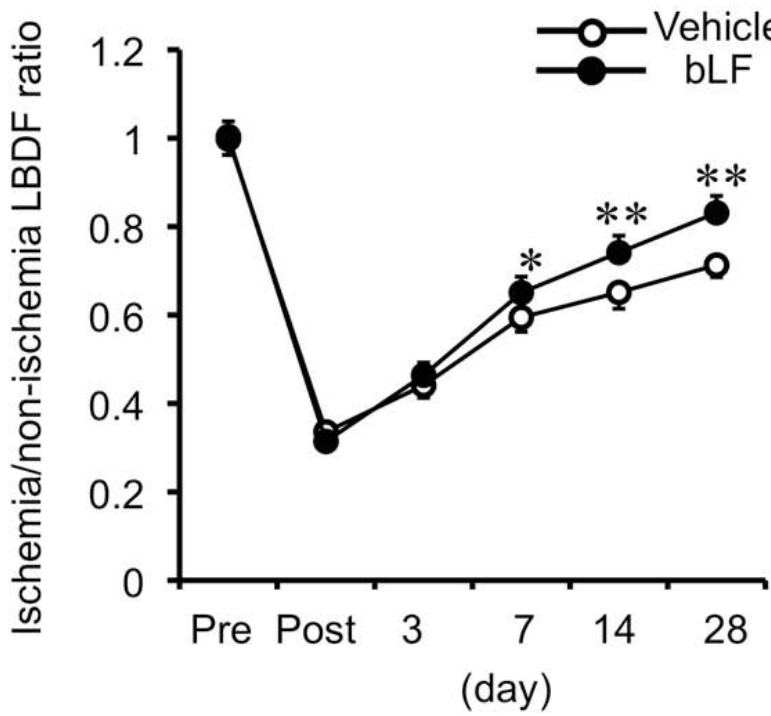
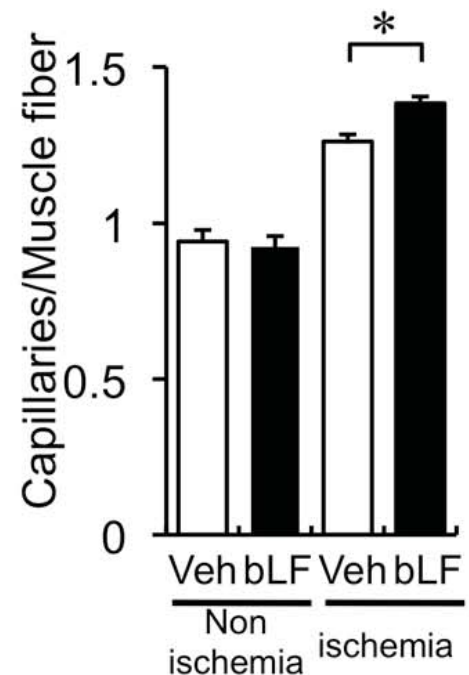
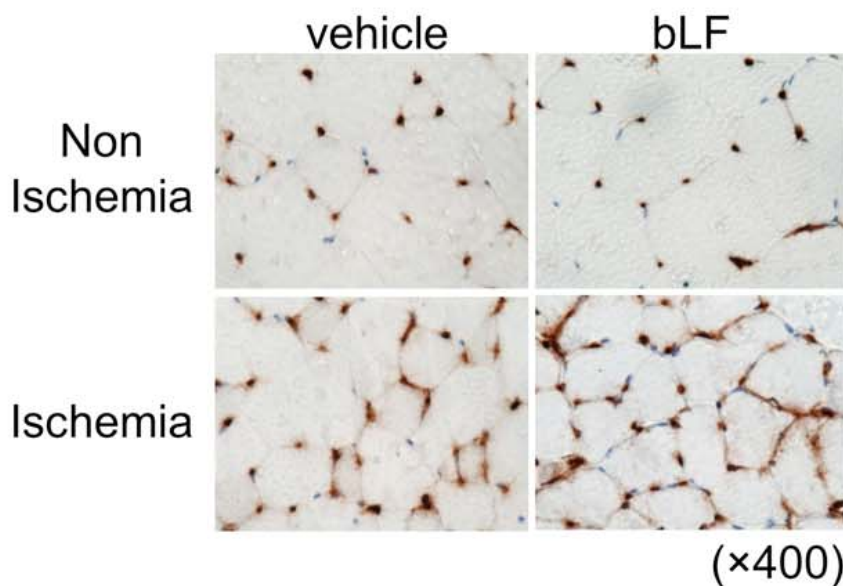


Figure 4

(A)



(B)



(C)

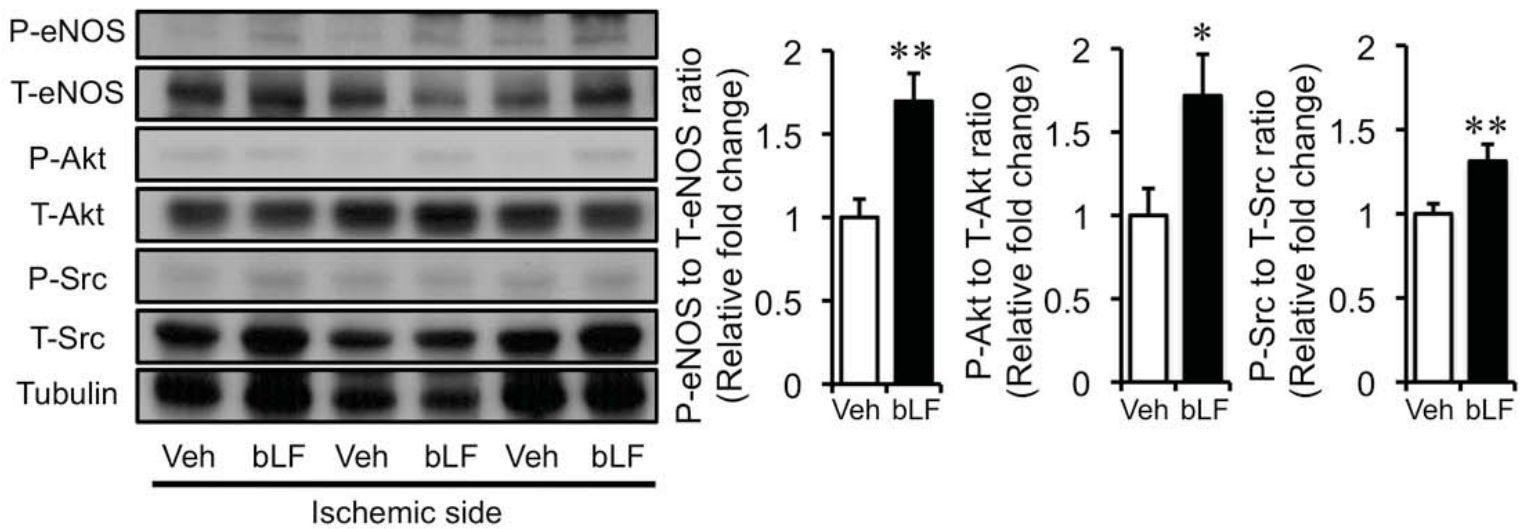


Table.1 Body weight, Blood pressure and pulse rate at day 7 after ischemic hindlimb surgery

	Post-surgery at day 7	
	vehicle	bLF
BW (g)	22.0 ± 0.3	22.1 ± 0.3
Syst. BP (mmHg)	99 ± 3	100 ± 2
Dias. BP (mmHg)	68 ± 3	73 ± 4
Pulse rate (beats/min)	663 ± 20	680 ± 8

Values are mean ± SEM. n=6, respectively.