Circ J 2006; **70:** 463–470

Repeated Thermal Therapy Up-Regulates Endothelial Nitric Oxide Synthase and Augments Angiogenesis in a Mouse Model of Hindlimb Ischemia

Yuichi Akasaki, MD; Masaaki Miyata, MD; Hideyuki Eto, MD; Takahiro Shirasawa, MD; Narisato Hamada, MD; Yoshiyuki Ikeda, MD; Sadatoshi Biro, MD; Yutaka Otsuji, MD; Chuwa Tei, MD

Background Nitric oxide (NO), constitutively produced by endothelial NO synthase (*e*NOS), plays roles in angiogenesis. Having reported that thermal therapy up-regulated the expression of arterial *e*NOS in hamsters, we investigated whether this therapy increased angiogenesis in mice with hindlimb ischemia.
Methods and Results Unilateral hindlimb ischemia was induced in apolipoprotein E-deficient mice, which were divided into control and thermal therapy groups. The latter mice were placed in a far-infrared dry sauna at 41°C for 15 min and then at 34°C for 20 min once daily for 5 weeks. Laser Doppler perfusion imaging demonstrated that the ischemic limb/normal side blood perfusion ratio in the thermal therapy group was significantly increased beyond that in controls (0.79±0.04 vs 0.54±0.08, $p<0.001$). Significantly greater capillary density was seen in thermal therapy group (757±123/mm^2 vs 416±20/mm^2, $p<0.01$). Western blotting showed thermal therapy markedly increased hindlimb *e*NOS expression. To study possible involvement of *e*NOS in thermally induced angiogenesis, thermal therapy was given to mice with hindlimb ischemia with or without N^G-nitro-L-arginine methyl ester (L-NAME) administration for 5 weeks. L-NAME treatment eliminated angiogenesis induced using thermal therapy. Thermal therapy did not increase angiogenesis in *e*NOS-deficient mice.
Conclusion Angiogenesis was induced via *e*NOS using thermal therapy in mice with hindlimb ischemia. (*Circ J* 2006; **70:** 463–470)

Key Words: Angiogenesis; Nitric oxide synthase; Thermal therapy

Nitric oxide (NO), constitutively produced by endothelial NO synthase (*e*NOS), plays important roles in vascular biology including regulation of vascular tone and blood pressure[1] as well as the regulation of angiogenesis.[2] Vascular endothelial growth factor (VEGF) augments the release of NO from cultured human umbilical venous endothelial cells and up-regulates expression of mRNA and protein expression for NOS.[3,4] Basic fibroblast growth factor (bFGF) and transforming growth factor-β (TGF-β) also increase the expression of *e*NOS and production of NO.[5,6] The release of NO in response to these growth factors is critical to their angiogenic actions. Direct in vitro evidence that NO may induce angiogenesis was demonstrated by Papapetropoulos et al,[7,8] while Ziche et al established the first line of evidence that NO can induce angiogenesis in vivo.[9,10] *e*NOS overexpressing or deficient mice showed differences in response to tissue ischemia, which demonstrates that *e*NOS modulates angiogenesis.[11,12]

We developed a form of thermal therapy that differed from the traditional sauna, finding that the repeated use of a dry sauna at 60°C improved hemodynamics, ameliorated symptoms and ventricular arrhythmias in patients with chronic heart failure.[13,14] In addition, we have found that repeated thermal therapy decreased the impairment of vascular endothelial function in patients with chronic heart failure[15] and in patients with coronary risk factors such as hypertension, hyperlipidemia, diabetes mellitus and smoking.[16]

Furthermore, we demonstrated that one of the molecular mechanisms by which repeated thermal therapy improved endothelial function involved an increase of mRNA and protein of *e*NOS.[17] We hypothesized that repeated thermal therapy can up-regulate *e*NOS and thereby augment ischemia-induced angiogenesis. The purpose of the present study was to determine whether repeated thermal therapy increased angiogenesis in a mouse model of hindlimb ischemia.

Methods

Animal Models

The investigation confirms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No.85-23, revised 1996). This study was carried out in accordance with the Guide for Animal Experimentation of the Faculty of Medicine at Kagoshima University. Unilateral hindlimb ischemia was induced by resecting the femoral artery and vein in 12-week-old apolipoprotein E (apoE)-deficient

(Received September 15, 2005; revised manuscript received January 6, 2006; accepted January 18, 2006)

Department of Cardiovascular, Respiratory and Metabolic Medicine, Graduate School of Medicine, Kagoshima University, Kagoshima, Japan

Part of this manuscript was presented at the scientific sessions 2004 of American Heart Association in New Orleans.

Mailing address: Chuwa Tei, MD, PhD, Department of Cardiovascular, Respiratory and Metabolic Medicine, Graduate School of Medicine, Kagoshima University, 8-35-1 Sakuragaoka, Kagoshima 890-8520, Japan. E-mail: tei@m.kufm.kagoshima-u.ac.jp

Table 1 Systolic Blood Pressure, Heart Rate and Bodyweight at Day 35

	Control (n=5)	Thermal therapy (n=5)	Thermal therapy plus L-NAME (n=5)
Systolic blood pressure (mmHg)	109.2±7.2	106.4±7.4	119.0±4.3*
Heart rate (beats/min)	638±35	636±18	635±23
Bodyweight (g)	21.4±0.8	21.5±0.7	21.0±0.6

*$p<0.01$ vs control and thermal therapy.
L-NAME, N^G-nitro-L-arginine methyl ester.

female mice (Jackson Laboratory). The procedure for creating the ischemic hindlimb has been described previously.[18] Briefly, after animals were anesthetized by intraperitoneal pentobarbital injection (50 mg/kg), the femoral artery was isolated and its proximal portion was ligated with 6-0 silk ligatures, followed by the distal portion of the saphenous artery. The remaining arteries and veins were all dissected free and then excised. The overlying skin was closed using 2 surgical staples.

Blood pressure and heart rate were measured in conscious mice using a computer-controlled tail-cuff system (MK-2000, Muromachi Kikai). Before recording measurements, mice were made accustomed to the apparatus for at least 3 days.

To chronically inhibit NO synthase, mice were given water containing 1 mg/ml N^G-nitro-L-arginine methyl ester (L-NAME) for 5 weeks. Furthermore, *e*NOS-knockout mice (*e*NOS-KO) were purchased from Jackson Laboratory. Unilateral hindlimb ischemia was induced by resecting the femoral artery and vein in 12-week-old *e*NOS-KO mice.

Thermal Therapy

The mice were divided into a control (n=7) and a thermal therapy group (n=7). Thermal therapy was carried out in an experimental far-infrared dry sauna system (Kyushu Olympia) at 41°C for 15 min and then at 34°C for 20 min. The thermal therapy group underwent daily thermal therapy 5 days a week for 5 weeks. We previously had established that this protocol elevates the rectal temperature by 1°C, with the elevation persisting for at least 20 min as shown in the clinical setting.[13] All animals were allowed food and water ad libitum and maintained under controlled environmental conditions (24°C, 12-h light/dark cycles).

Laser Doppler Perfusion Imaging (LDPI)

Serial assessment of hindlimb blood flow was performed with a Laser Doppler Imager (Moor Instruments) as previously described.[19] Excess hair was removed from the hindlimbs using a depilatory cream. To minimize variables including ambient light and temperature, calculated perfusion was expressed as a ratio of left (ischemic) to right (normal) hindlimb, and mice were placed on a heating plate at 37°C before initiating scanning. Perfusion analyses were performed sequentially under pentobarbital anesthesia before surgery, immediately after surgery and at 3, 7, 14, 21, 28 and 35 days after surgery.

Tissue Preparation

The animals were killed with an overdose of sodium pentobarbital at 35 days after surgery. For immunohistochemical analysis, intact ischemic and nonischemic limbs were immediately fixed in methanol overnight. Then, after removal of bones, 5-mm thick tissue sections were cut and embedded in paraffin. For Western blot analysis, isolated tissue samples were rinsed in phosphate buffered saline to remove excess blood, flash-frozen in liquid nitrogen and stored at −80°C until use.

Preparation of Protein Extracts

Proteins were extracted for Western blot analysis as reported previously.[20] Briefly, the hindlimb muscles (gastrocnemius muscle from ischemic and nonischemic hindlimbs) were ground to a fine powder under liquid nitrogen and incubated in ice-cold 0.1% Triton lysis solution (mmol/L: HEPES 10 (pH 7.4), sodium pyrophosphate 50, NaF 50, EDTA 5, EGTA 5, and NaCl 50; and 100 mmol/L Na_3VO_4, 0.1% Triton X-100, 500 mmol/L PMSF, and 10 mg/ml leupeptin) for 30 min. Insoluble matter was removed through centrifugation and the protein concentration was measured using a bicinchoninic acid assay (Pierce).

Western Blot Analysis

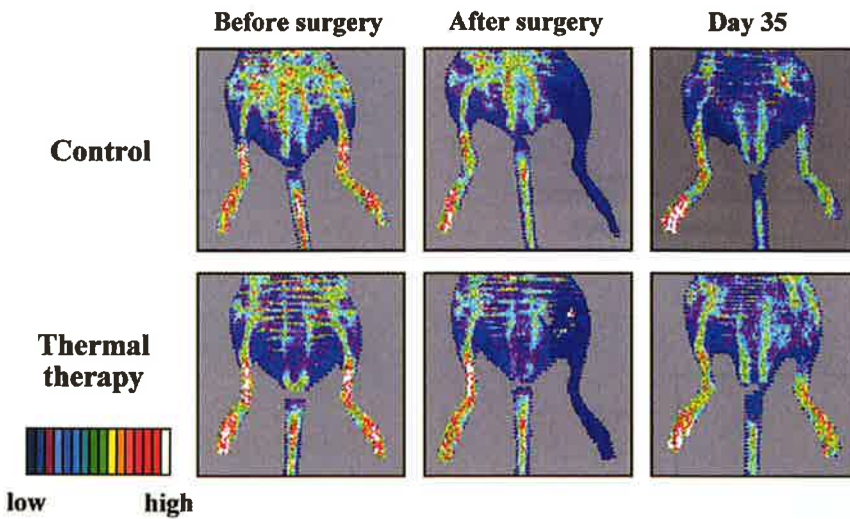
Western blotting was performed with a NuPAGE Electrophoresis System (Novex) as reported previously.[21] Briefly, 10-μg protein samples were resuspended in a reduced sample buffer, and then electrophoresed on a 4±12% Bis-Tris gel (Novex) with MOPS running buffer; blotted to nitrocellulose; and sequentially probed with polyclonal rabbit antisera raised against *e*NOS, VEGF or α-actin (Santa Cruz Biotechnology). A horseradish peroxidase-conjugated goat anti-rabbit antibody (Santa Cruz Biotechnology) was then added, and secondary antibodies were detected through autoradiography using enhanced chemiluminescence (ECL Plus, Amersham).

Measurement of Nitrate and Nitrite

Serum nitrate and nitrite (NOx) were measured using the Griess method, as previously described.[22] Serum samples were deproteinized using ultrafiltration through a 10 kDa micropore membrane (Millipore Corporation). Results of the assay were determined using a nitrate/nitrite colorimetric kit (Cayman Chemical).

Measurement of Capillary Density

Capillary densities in ischemic hindlimbs were analyzed for specific evidence of neovascularity. Endothelial cells were stained with a rat monoclonal antibody directed against mouse CD31 (PharMingen) and counted under a light microscope. Five different microscopic fields on 2 different sections from each of 3 animals at each time point were counted, and capillary density was expressed as number of capillaries/mm^2. The animals were coded in order to analyze without any knowledge of which treatment each individual animal had received. Intraobserver variability was determined from triplicate measurements performed by one observer for all sections. The mean ± SD difference among measurements made by the same observer was 2.0±0.4%. Interobserver variability was determined from



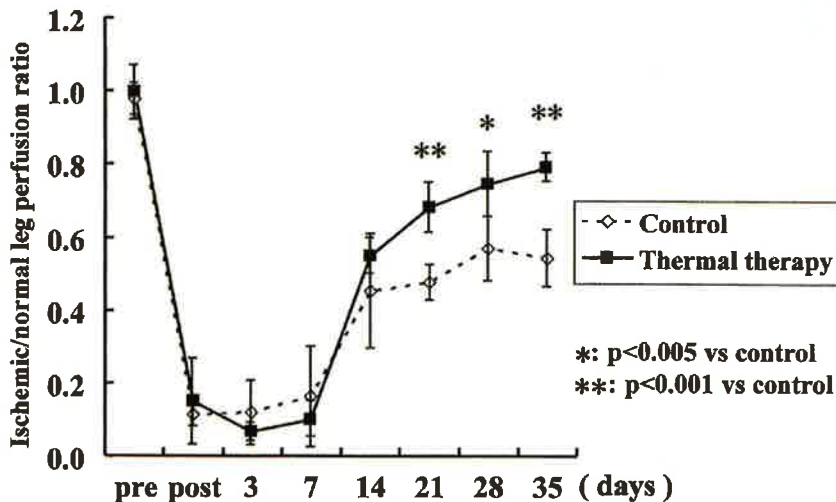


Fig 1. Hindlimb blood flow in apoE-deficient mice monitored by laser Doppler perfusion imaging. (A) Panels show color-coded images representing blood flow distribution. Low or absent perfusion is displayed as dark blue, while highest perfusion is displayed as white. (B) Time course of ischemic/normal leg blood perfusion ratio. Computer-assisted quantitative analysis demonstrated a significantly greater increase of ischemic/normal hindlimb perfusion ratio in the thermal therapy group (n=7) than in the control group (n=7). $^*p<0.005$, $^{**}p<0.001$ vs control group.

measurements of 10 randomly selected sections performed by a second observer in addition to the first. The numeric difference between the measurements made by the 2 observers was 2.3±0.9%.

Statistical Analysis

All values are expressed as the mean±SD. Statistical significance was evaluated using unpaired Student's t-tests for comparisons between 2 groups. A probability value of $p<0.05$ was considered to indicate statistical significance.

Results

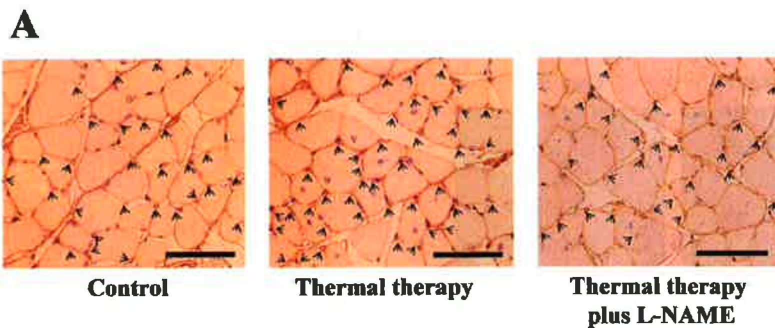
Repeated Thermal Therapy Increased Blood Flow in Ischemic Hindlimb

Unilateral hindlimb ischemia was induced surgically in apoE-deficient mice divided into control and thermal therapy groups. Systolic blood pressure, heart rate and body weight did not differ between control and thermal therapy groups at day 35 (Table 1).

Serial blood flow measurements were performed using a LDPI. Immediately after operative excision of left femoral artery and vein, a marked reduction of blood flow was observed in the left leg. Although flow in the ischemic leg had recovered slightly in the control group at day 35, thermal therapy increased the blood flow in the ischemic left leg at day 35 beyond that in the control (Fig 1A). Fig 1B shows the time course of computer-assisted determinations of LDPI. In the control group, blood flow remained impaired for 7 days and increased to 54% of flow in the nonischemic limb by day 35. A similar reduction of hindlimb blood flow occurred in the thermal therapy group, but in contrast to the control group, hindlimb blood flow then recovered to 79% (thermal therapy vs control, 0.79±0.04 vs 0.54±0.08, $p<0.001$).

Repeated Thermal Therapy Increased Capillary Density

We analyzed whether repeated thermal therapy induced angiogenesis. Capillary density was examined as an index of angiogenesis using immunohistochemistry with CD31 antibodies. A significant increase in capillary density at day 35 was found in the thermal therapy group as compared



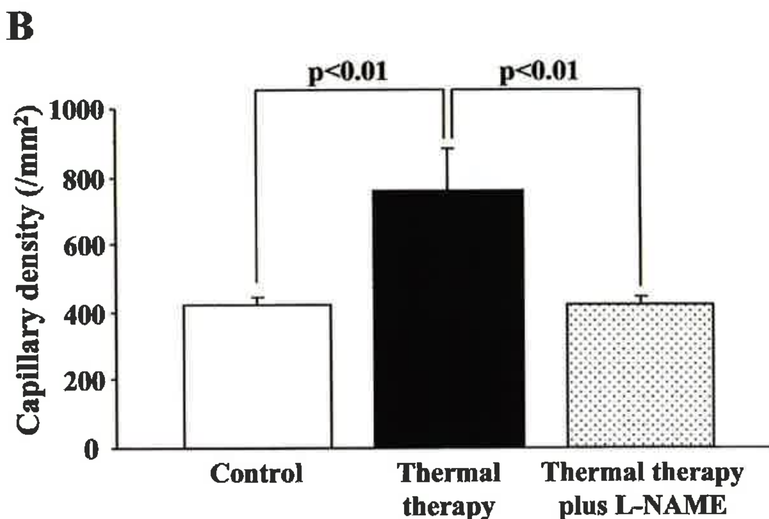


Fig 2. Capillary density in ischemic hindlimb. (A) Representative photomicrographs of immunohistochemical staining with CD31 of ischemic limb tissues at postoperative day 35. Repeated thermal therapy markedly increased the number of capillaries compared to the control, while thermal therapy accompanied by N^G-nitro-L-arginine methyl ester (L-NAME) treatment showed capillary formation only at the control level. Arrows indicate capillaries. Scale Bars= 50 μm. (B) Quantitative analyses disclosed that capillary density was significantly increased by repeated thermal therapy, but not when L-NAME was given additionally. In each group, n=3.

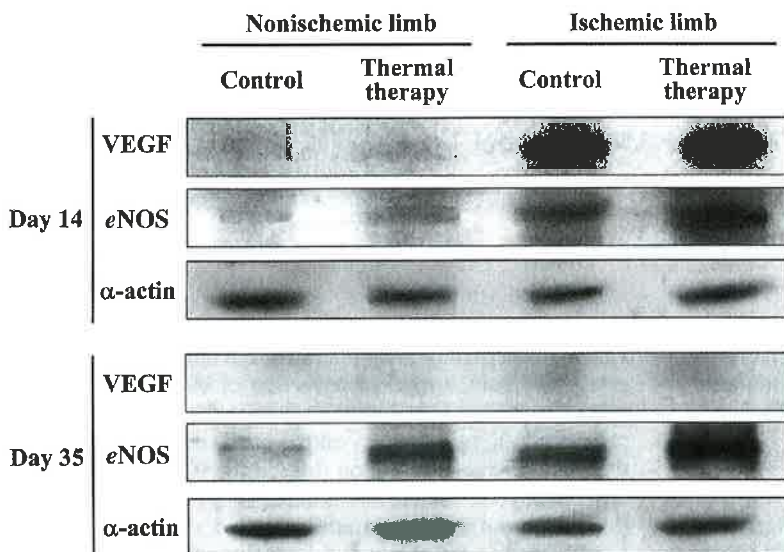


Fig 3. Western blotting of vascular endothelial growth factor (VEGF) and endothelial nitric oxide synthase (*e*NOS) protein in muscle of mouse hindlimb. VEGF expression in ischemic hindlimb did not differ between the control and thermal therapy groups at day 14. Thermal therapy markedly increased *e*NOS protein expression not only in the ischemic hindlimb but also in the nonischemic hindlimb.

with the control group (757±123/mm^2 vs 416±20/mm^2, $p<0.01$; Fig 2).

Repeated Thermal Therapy Increased eNOS and NOx

We examined the expression of angiogenic factors. Western blot analysis of VEGF demonstrated that VEGF protein expression level in the ischemic hindlimb did not differ between the control and thermal therapy groups at day 14, and it was not detected in either group at day 35. In contrast, Western blot analysis of *e*NOS at day 14 and 35 showed that thermal therapy markedly increased *e*NOS protein expression not only in the ischemic hindlimb but also in the nonischemic hindlimb (Fig 3). We measured serum NOx and confirmed that repeated thermal therapy increased NOx at day 35 (Fig 4).

L-NAME Treatment and eNOS Deficiency Abolished Angiogenesis Induced by Thermal Therapy

To determine the involvement of NO synthase in blood flow improvement induced by thermal therapy, we administered repeated thermal therapy in the mouse model of unilateral hindlimb ischemia with or without L-NAME

administration at 1 mg/ml for 5 weeks. By measuring serum NOx, we confirmed that L-NAME administration suppressed NO production induced by repeated thermal therapy (Fig 4). Although no significant difference in heart rate or body weight was seen between the thermal therapy group and the thermal therapy with L-NAME group, systolic blood pressure was significantly higher in the thermal therapy with L-NAME group than in the thermal therapy group (Table 1).

LDPI showed that L-NAME administration reduced the increase in blood flow induced by thermal therapy at day 35 (Fig 5A). Computer-assisted analysis of LDPI indicated that the increase of blood flow using thermal therapy was completely eliminated by L-NAME at day 35 (thermal therapy vs thermal therapy plus L-NAME, 0.80±0.06 vs 0.45±0.07, $p<0.001$; Fig 5B). L-NAME treatment with thermal therapy reduced an increase in capillary formation to the control level (thermal therapy vs thermal therapy plus L-NAME, 757±123/mm^2 vs 423±24/mm^2, $p<0.01$; Fig 2).

In addition, we performed the experiments using *e*NOS-KO mice to confirm the involvement of *e*NOS in blood flow improvement induced by thermal therapy. Unilateral

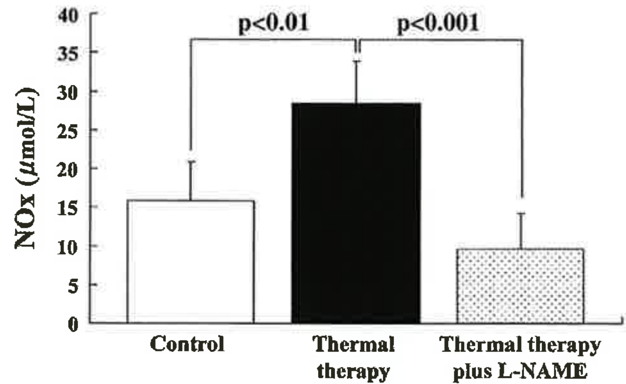
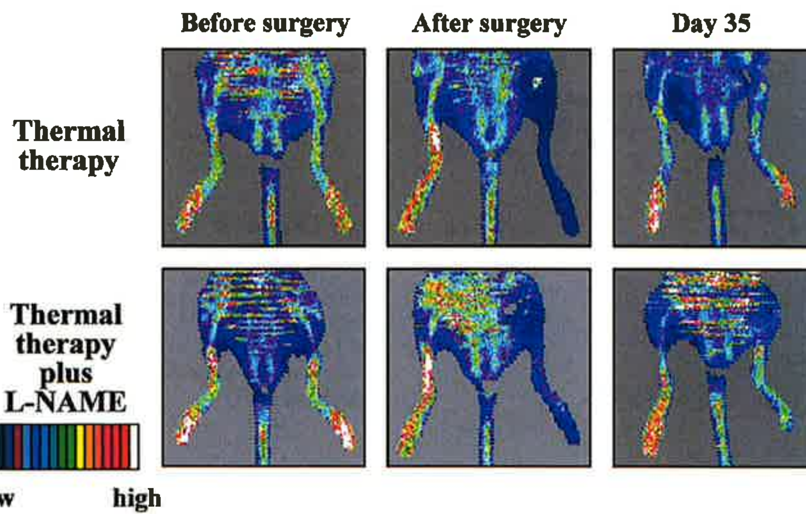


Fig 4. Serum concentration of nitrite plus nitrate (NOx) in mice at 35 days. Repeated thermal therapy increased NOx at day 35. N^G-nitro-L-arginine methyl ester (L-NAME) administration suppressed nitric oxide production induced by repeated thermal therapy. In each group, n=5.



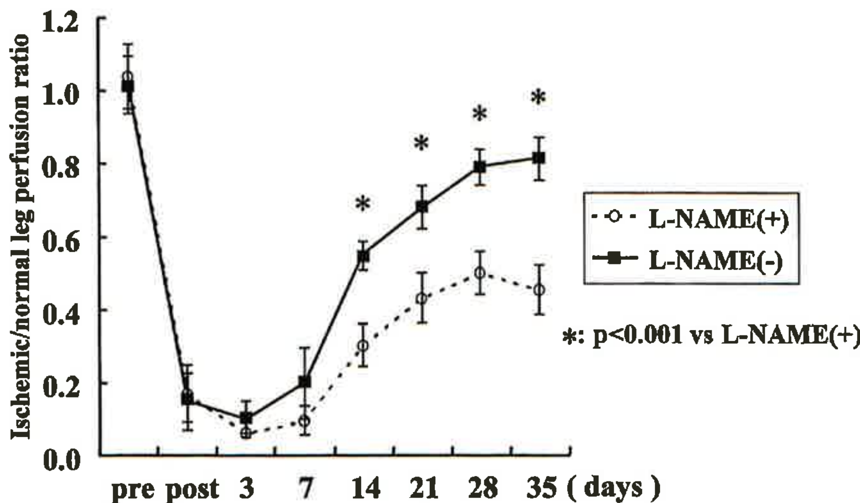
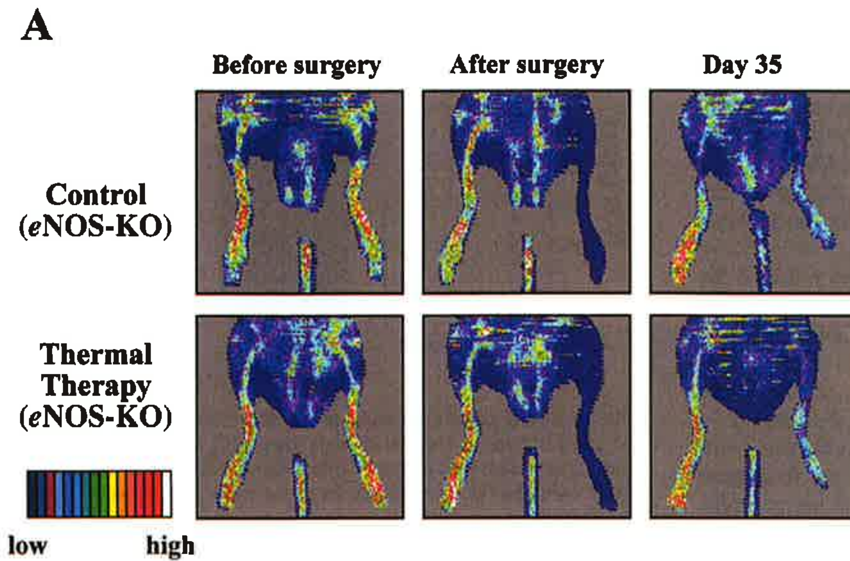


Fig 5. Results of laser Doppler perfusion imaging (LDPI). (A) Images show that N^G-nitro-L-arginine methyl ester (L-NAME) administration reduced induction of blood flow by thermal therapy at day 35. (B) Computer-assisted analysis of LDPI demonstrated that the increase in blood flow associated with thermal therapy was completely abolished by L-NAME treatment. In each group, n=7. *$p<0.001$ vs control group.



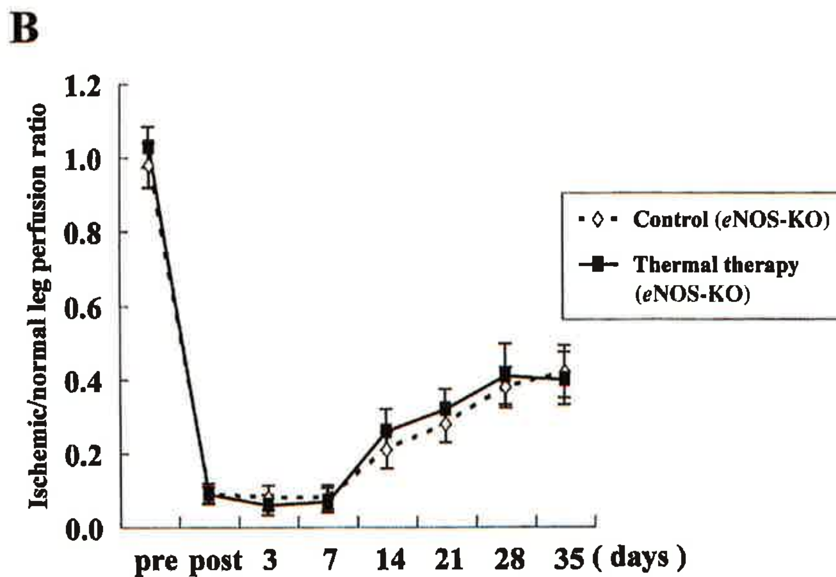


Fig 6. Results of laser Doppler perfusion imaging. (A) Images show that thermal therapy did not increase blood flow in endothelial nitric oxide synthase-knockout mice (*e*NOS-KO) at day 35. (B) Ischemic/normal leg blood perfusion ratio did not differ between control and thermal therapy groups. In each group, n=7.

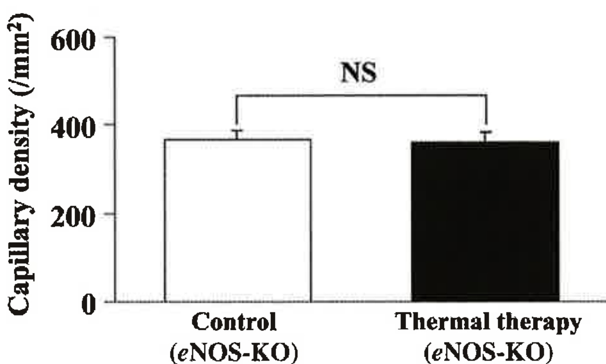


Fig 7. Capillary density in ischemic hindlimb of endothelial nitric oxide synthase-knockout mice (*e*NOS-KO). Capillary density in ischemic hindlimb of *e*NOS-KO did not differ between control and thermal therapy groups. In each group, n=3. NS, not significant.

hindlimb ischemia was surgically induced in *e*NOS-KO mice and they were divided into control (n=7) and thermal therapy groups (n=7). The thermal therapy groups underwent the thermal therapy for 5 weeks. The ischemic/normal leg blood perfusion ratio did not differ between the control and thermal therapy groups at day 35 (control vs thermal therapy: 0.40±0.08 vs 0.42±0.08; Fig 6). Capillary density in the ischemic hindlimb also did not differ between the control and thermal therapy groups (control vs thermal therapy: 357±20/mm^2 vs 350±20/mm^2; Fig 7). Thermal therapy did not increase angiogenesis in *e*NOS-KO mice with hindlimb ischemia. *e*NOS is suggested to be a critical regulator for angiogenesis using repeated thermal therapy.

Discussion

To better understand the effect of repeated thermal therapy on angiogenesis, we studied these effects in a mouse model of hindlimb ischemia. We demonstrated that repeated thermal therapy increased *e*NOS protein expression, blood flow and capillary density in the ischemic hindlimb. Furthermore, when we administered repeated thermal therapy

in mice with hindlimb ischemia also given L-NAME, interference with therapeutic benefits established that NO plays an important role in angiogenesis induced by thermal therapy. In addition, thermal therapy did not increase blood flow and capillary density in *e*NOS-KO mice, and *e*NOS is suggested to be a critical regulator for angiogenesis by thermal therapy.

NO is a mediator of angiogenesis, although in vitro results concerning this have not been uniform. NO inhibited migration of cultured endothelial cells[23] and NO donors inhibited angiogenesis in the chick chorioallantoic membrane and microvascular tube formation in the matrigel tube formation assay[24,25] In contrast, Guo et al reported that exogenous administration of an NO donor stimulated proliferation of cultured rat aortic endothelial cells[26] Ziche et al suggested that NO may be involved in angiogenesis elicited by VEGF but not bFGF[10] Finally, several in vivo studies demonstrated that NO modulates angiogenesis in response to tissue ischemia. Murohara et al showed that angiogenesis in the ischemic hindlimb was significantly impaired in *e*NOS-deficient mice compared with wild-type controls evaluated using either laser Doppler flow or capillary density measurement[11] Amano et al demonstrated that transgenic mice overexpressing *e*NOS in the endothelium increased new capillary formation in response to tissue ischemia[12] Namba et al reported that intramuscular injection of bovine *e*NOS plasmid induced therapeutic angiogenesis in a rat ischemic hindlimb model[19] Thus, NO is a critical regulatory molecule for angiogenesis in response to tissue ischemia. It is well established that endothelial progenitor cells or bone marrow-derived stem cells play a crucial role in angiogenesis in hindlimb ischemia and Aicher et al have demonstrated the essential role of *e*NOS for mobilization of stem and progenitor cells[27] As a next step, we are planning to analyze the effect of thermal therapy on the dynamics of endothelial progenitor cells and the involvement of bone marrow-derived cells.

NO is an angiogenic mediator acting downstream of VEGF; impaired angiogenesis in *e*NOS-deficient mice was not improved by the administration of VEGF[11] and transgenic mice overexpressing *e*NOS did not show a greater increase in VEGF expression in hindlimb ischemia[12] We believe that *e*NOS up-regulation induced by repeated thermal therapy is caused by an increase in cardiac output and blood flow, which in turn results in increased shear stress, although thermal stimulation might up-regulate arterial *e*NOS directly[13,17] Repeated thermal therapy increases cardiac output, shear stresses of the vessel wall and ultimately *e*NOS expression. Although repeated thermal therapy did not increase the expression of VEGF in the ischemic hindlimb, thermal therapy augmented angiogenesis by increasing *e*NOS expression in the course of ischemia-induced VEGF expression. Therefore, the presence of VEGF is necessary for angiogenesis induced by repeated thermal therapy, since repeated thermal therapy does not up-regulate VEGF expression itself.

Hypercholesterolemia adversely affects native collateral development. Collateral vessel development associated with hindlimb ischemia is severely attenuated in animal models of spontaneous hypercholesterolemia such as the Watanabe heritable hyperlipidemic rabbit (WHHL) and the apoE-deficient mouse[28,29] Administration of human VEGF to WHHL rabbits or adenoviral VEGF gene transfer to apoE-deficient mice resulted in marked augmentation of hindlimb blood flow and capillary density in hindlimb ischemia. Furthermore, Duan et al reported that collateral vessel formation and angiogenesis in response to hindlimb ischemia were significantly attenuated in rats with dietary hypercholesterolemia, and the mechanism may be related to reduced NO bioactivity in the ischemic tissue. They demonstrated that augmentation of the tissue NO activity by oral L-arginine supplementation restored impaired angiogenesis in hypercholesterolemia[30] We demonstrated in the present study that repeated thermal therapy up-regulated *e*NOS and augmented ischemia-induced angiogenesis in apoE-deficient mice.

Selective modulation of *e*NOS activity is a promising strategy for altering angiogenesis in vivo. A better strategy might be to manipulate endogenous generation of NO. Several previous studies have demonstrated angiogenesis induced by the *e*NOS gene by the use of adenoviral vectors in the rat hindlimb ischemia model[19,31,32] Compared to gene therapy with the *e*NOS gene, thermal therapy is safe and remarkably cost-effective. Modulation of *e*NOS activity by repeated thermal therapy is a promising strategy for altering angiogenesis in vivo. We now are applying thermal therapy to patients with peripheral arterial disease, analyzing its effect on angiogenesis in the ischemic limb.

Acknowledgments

This study was supported by grants-in-aid from the Japan Society for the Promotion of Science (#16300184).

References

1. Moncada S, Palmer RMJ, Higgs EA. Nitric oxide: Physiology, pathophysiology, and pharmacology. *Pharmacol Rev* 1991; **43:** 109–142.
2. Cooke JP, Losordo DW. Nitric oxide and angiogenesis. *Circulation* 2002; **105:** 2133–2135.
3. Hood JD, Meininger CJ, Ziche M, Harris JG. VEGF upregulates ecNOS message, protein, and NO production in human endothelial cells. *Am J Physiol* 1998; **274:** H1054–H1058.
4. van der Zee R, Murohara T, Luo Z, Zollman F, Passeri J, Lekutat C, et al. Vascular endothelial growth factor/vascular permeability factor augments nitric oxide release from quiescent rabbit and human vascular endothelium. *Circulation* 1997; **95:** 1030–1037.
5. Babaei S, Teichert-Kuliszewska K, Monge JC, Mohamed F, Bendeck MP, Stewart DJ. Role of nitric oxide in the angiogenic response in vitro to basic fibroblast growth factor. *Circ Res* 1998; **82:** 1007–1015.
6. Inoue N, Venema RC, Sayegh HS, Ohara Y, Murphy TJ, Harrison DG. Molecular regulation of the bovine endothelial cell nitric oxide synthase by transforming growth factor-beta 1. *Arterioscler Thromb Vasc Biol* 1995; **15:** 1255–1261.
7. Papapetropoulos A, Garcia-Cardena G, Madri JA, Sessa WC. Nitric oxide production contributes to the angiogenic properties of vascular endothelial growth factor in human endothelial cells. *J Clin Invest* 1997; **100:** 3131–3139.
8. Papapetropoulos A, Desai KM, Rudic RD, Mayer B, Zhang R, Ruiz-Torres MP, et al. Nitric oxide synthase inhibitors attenuate transforming-growth-factor-beta 1-stimulated capillary organization in vitro. *Am J Pathol* 1997; **150:** 1835–1844.
9. Ziche M, Morbidelli L, Masini E, Amerini S, Granger HJ, Maggi CA, et al. Nitric oxide mediates angiogenesis in vivo and endothelial cell growth and migration in vitro promoted by substance P. *J Clin Invest* 1994; **94:** 2036–2044.
10. Ziche M, Morbidelli L, Choudhuri R, Zhang H-T, Donnini S, Granger HJ, et al. Nitric oxide synthase lies downstream from vascular endothelial growth factor-induced but not basic fibroblast growth factor-induced angiogenesis. *J Clin Invest* 1997; **99:** 2625–2634.
11. Murohara T, Asahara T, Silver M, Bauters C, Masuda H, Kalka C, et al. Nitric oxide modulates angiogenesis in response to tissue ischemia. *J Clin Invest* 1998; **101:** 2567–2578.
12. Amano K, Matsubara H, Iba O, Okigaki M, Fujiyama S, Imada T, et al. Enhancement of ischemia-induced angiogenesis by *e*NOS overexpression. *Hypertension* 2003; **41:** 156–162.
13. Tei C, Horikiri Y, Park JC, Jeong JW, Chang KS, Toyama Y, et al. Acute hemodynamic improvement by thermal vasodilation in congestive heart failure. *Circulation* 1995; **91:** 2582–2590.

14. Kihara T, Biro S, Ikeda Y, Fukudome T, Shinsato T, Masuda A, et al. Effects of repeated sauna treatment on ventricular arrhythmias in patients with chronic heart failure. *Circ J* 2004; **68:** 1146–1151.
15. Kihara T, Biro S, Imamura M, Yoshifuku S, Takasaki K, Ikeda Y, et al. Repeated thermal therapy treatment improves vascular endothelial and cardiac function in patients with chronic heart failure. *J Am Coll Cardiol* 2002; **39:** 754–759.
16. Imamura M, Biro S, Kihara T, Yoshifuku S, Takasaki K, Otsuji Y, et al. Repeated thermal therapy improves impaired vascular endothelial function in patients with coronary risk factors. *J Am Coll Cardiol* 2001; **38:** 1083–1088.
17. Ikeda Y, Biro S, Kamogawa Y, Yoshifuku S, Eto H, Orihara K, et al. Repeated thermal therapy upregulates arterial endothelial nitric oxide synthase expression in Syrian golden hamsters. *Jpn Circ J* 2001; **65:** 434–438.
18. Couffinhal T, Silver M, Zheng LP, Kearney M, Witzenbichler B, Isner JM. A mouse model of angiogenesis. *Am J Pathol* 1998; **152:** 1667–1679.
19. Namba T, Koike H, Murakami K, Aoki M, Makino H, Hashiya N, et al. Angiogenesis induced by endothelial nitric oxide synthase gene through vascular endothelial growth factor expression in a rat hindlimb ischemia model. *Circulation* 2003; **108:** 2250–2257.
20. Orihara K, Biro S, Hamasaki S, Eto H, Miyata M, Ikeda Y, et al. Hyperthermia at 43°C for 2h inhibits the proliferation of vascular smooth muscle cells, but not endothelial cells. *J Mol Cell Cardiol* 2002; **34:** 1205–1215.
21. Miyata M, Biro S, Kaieda H, Eto H, Orihara K, Kihara T, et al. Apolipoprotein J/clusterin is induced in vascular smooth muscle cells after vascular injury. *Circulation* 2001; **104:** 1407–1412.
22. Ikeda Y, Biro S, Kamogawa Y, Yoshifuku S, Eto H, Orihara K, et al. Repeated sauna therapy increases arterial endothelial nitric oxide synthase expression and nitric oxide production in cardiomyopathic hamsters. *Circ J* 2005; **69:** 722–729.
23. Lau Y-T, Ma W-C. Nitric oxide inhibits migration of cultured endothelial cells. *Biochem Biophys Res Commun* 1996; **221:** 670–674.
24. Pipili-Synetos E, Sakkoula E, Maragoudakis ME. Nitric oxide is involved in the regulation of angiogenesis. *Br J Pharmacol* 1993; **108:** 855–857.
25. Pipili-Synetos E, Sakkoula E, Haralabopoulos G, Andriopoulou P, Peristeris P, Maragoudakis ME. Evidence that nitric oxide is an endogenous antiangiogenic mediator. *Br J Pharmacol* 1994; **111:** 894–902.
26. Guo JP, Panday MM, Consigny PM, Lefer AM. Mechanisms of vascular preservation by a novel NO donor following rat carotid artery intimal injury. *Am J Physiol* 1995; **269:** H1122–H1131.
27. Aicher A, Heeschen C, Mildner-Rihm C, Urbich C, Ihling C, Technau-Ihling K, et al. Essential role of endothelial nitric oxide synthase for mobilization of stem and progenitor cells. *Nat Med* 2003; **9:** 1370–1376.
28. van Belle E, Rivard A, Chen D, Silver M, Bunting S, Ferrara N, et al. Hypercholesterolemia attenuates angiogenesis but does not preclude augmentation by angiogenic cytokines. *Circulation* 1997; **96:** 2667–2674.
29. Couffinhal T, Silver M, Kearney M, Sullivan A, Witzenbichler B, Magner M, et al. Impaired collateral vessel development associated with reduced expression of vascular endothelial growth factor in apoE-/- mice. *Circulation* 1999; **99:** 3188–3198.
30. Duan J, Murohara T, Ikeda H, Katoh A, Shintani S, Sasaki K, et al. Hypercholesterolemia inhibits angiogenesis in response to hindlimb ischemia: Nitric oxide-dependent mechanism. *Circulation* 2000; **102:** III-370–III-376.
31. Brevetti LS, Sarkar R, Chang DS, Ma M, Paek R, Messina LM. Administration of adenoviral vectors induces gangrene in acutely ischemic rat hindlimbs: Role of capsid protein-induced inflammation. *J Vasc Surg* 2001; **34:** 489–496.
32. Smith RS Jr, Lin KF, Agata J, Chao L, Chao J. Human endothelial nitric oxide synthase gene delivery promotes angiogenesis in a rat model of hindlimb ischemia. *Arterioscler Thromb Vasc Biol* 2002; **22:** 1279–1285.