

Original Article

Percutaneous Carbon Dioxide Treatment using a Gas Mist Generator Enhances the Collateral Blood Flow in the Ischemic Hindlimb

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Aim: Highly concentrated carbon dioxide (CO₂) is thought to be useful for ischemic diseases. We investigated whether treatment with a few micrometers of CO₂ molecules atomized via two fluid-nozzles (CO₂ mist) exerts an angiogenic effect in a mouse ischemic hindlimb model.

Methods: Mice with unilateral hindlimb ischemia were divided into untreated (UT), 100% CO₂ gas alone-treated (CG), mixed air (O₂; 20%, N₂; 80%) mist-treated (AM) and 100% CO₂ mist-treated (CM) groups. The lower body of the mice was encased in a polyethylene bag filled with each gaseous agent using a gas mist generator for 10 minutes daily.

Results: According to a laser Doppler analysis, the ischemic hindlimb blood flow was persistently higher after the seventh day of induction of ischemia in the CM group than in the UT group. The capillary density was also greater in the CM group on day 28 compared with that observed in the UT group. In addition, the parameters in the AM and CG groups were similar to those obtained in the UT group. The observed effects were abolished by the administration of an inhibitor of nitric oxide synthase (NOS). The vascular endothelial growth factor mRNA expression and protein levels and the phosphorylated endothelial NOS level were increased in the CM group compared with that observed in the UT group. A proteomic analysis using liquid chromatography-tandem mass spectrometry identified novel protein candidates regulated by CO₂ mist.

Conclusion: Percutaneous CO₂ mist therapy may be useful for treating ischemia-induced angiogenesis.

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Key words: Angiogenesis, Carbon dioxide mist, Ischemia, Peripheral arterial disease, Mass spectrometry

Introduction

Patients with peripheral arterial disease (PAD) exhibit greater functional impairment, with a more

rapid functional decline and lower physical activity levels in daily life than those without PAD^{1, 2)}. The treatment of PAD has evolved over the past decade to include a broad approach, focusing on reducing the incidence of adverse cardiovascular events, improving the symptoms of claudication and preventing tissue loss under conditions of critical limb ischemia^{3, 4)}. Because these subjects have a severely limited quality of life, great emphasis is placed on ameliorating symptoms and reducing the risk of PAD progression. In

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spite of the development of various types of medicines⁴), the frequency of PAD has not yet decreased. Exercise rehabilitation is also recommended in patients with this disease⁵); however, supervised exercise is not covered by major medical insurance providers. Furthermore, it can be difficult to provide supervised exercise therapy for patients with PAD who are incapable of performing active exercise, such as those with neurodegenerative and rheumatologic diseases or age-related skeletal muscle wasting. Novel passive exercises, such as thermal therapy and whole-body periodic acceleration therapy, for PAD have been recently received much attention^{6,7}.

Balneotherapy using hot springs containing a high concentration of carbon dioxide (CO₂) has long been applied clinically to treat a variety of cardiovascular diseases, especially in European countries. The transfer of CO₂ across the skin is thought to have beneficial local vasomotor effects without inducing systemic hemodynamic modifications⁸). A recent study demonstrated that the immersion of ischemic mouse hindlimbs into baths of artificially created CO₂-enriched water results in a nitric oxide (NO)-dependent increase in collateral blood perfusion as well as the induction of regional vascular endothelial growth factor (VEGF) synthesis⁹). Hence, bathing in CO₂-enriched water may be a useful therapeutic approach in patients with PAD. Carbonate spring water is generally defined as water containing more than 1,000 parts per million (ppm) of CO₂, although the cost to set up such systems is expensive. Therefore, the development of more simple and affordable systems would be valuable.

Very recently, a new and simple system was developed in which CO₂ gas is dissolved in water containing a high concentration of CO₂ molecules using two fluid-nozzles, namely, CO₂ mist. We previously reported that the CO₂ mist produced by this device has a therapeutic benefit in the treatment of heart failure due to myocardial infarction¹⁰).

In the present study, we investigated whether treatment with CO₂ mist accelerates angiogenesis in a well-established mouse model of ischemia-induced angiogenesis. Consequently, we obtained evidence that CO₂ mist therapy stimulates ischemia-induced revascularization via upregulation of the angiogenic growth factor expression.

Materials and Methods

Animals and Experimental Protocol

All procedures involving animals were performed in compliance with the Osaka City University animal

care guidelines. The study protocol conformed with the Guidelines for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication No. 85-23, revised 1996). Male Wistar rats and male C57BL/6J (wild-type), inducible NO synthase deficient (iNOS^{-/-}) and endothelial NO synthase deficient (eNOS^{-/-}) mice 8-10 weeks of age were used in the present study. Unilateral hindlimb ischemia was induced under anesthesia with sodium pentobarbital (50 mg/kg intraperitoneally (i.p.)), as previously described^{7, 11-12}). Mixed air dry mist (AM) and CO₂ mist (CM) was generated using a dry mist production unit (Advance Biotron Co., Ltd., Tokyo, Japan)¹⁰). In brief, 100% concentrated CO₂ or mixed air (N₂: 80%; O₂: 20%) was compounded and compressed with water through double fluid nozzles at 4 atmospheres.

The mice were treated with 100% CO₂ gas (CG), AM or CM for 10 minutes once a day in a draft cabinet. Briefly, under anesthesia with sodium pentobarbital, the lower body of each mouse was encased in a sealed polyethylene bag on a heating plate at 37°C. The polyethylene bag was then filled with CG, AM or CM in the draft cabinet. The untreated (UT) group was kept on the heating plate at 37°C for 10 minutes once a day under anesthesia. The use of 100% CO₂ at 4 units of barometric pressure resulted in a CO₂ concentration in the bag of approximately 900,000 ppm¹⁰).

Effects of CO₂ Mist on the Vasodilation of Microvessels and Tissue Blood Flow

The first series of experiments was performed to compare the effects of CG, AM and CM on the vasodilation of microvessels. One week after the induction of hindlimb ischemia, the mouse femurs were slightly cut open, exposing the subcutaneous vessels, under anesthesia with sodium pentobarbital (50 mg/kg, i.p.). The incision site was then covered with a transparent seal. We then observed whether the CG or CM treatment dilated the microvessels using a digital microscope (VHX-1000, Keyence Co., Ltd., Osaka, Japan) equipped with a zoom lens.

Next, we investigated the effects of CG and CM on the tissue blood flow. At one week after the induction of hindlimb ischemia in the rats, we measured the blood flow in the shallow region of the tissue using laser tissue blood oxygenation monitors and near-infrared spectroscopy (BOM-L1TR SF, Omegawave Inc., Tokyo, Japan) during treatment with CG or CM. In brief, a saturation-monitoring sensor was attached to the rat toe. Changes in the tissue levels of oxygenated hemoglobin (oxy-Hb), deoxygenated hemoglobin

(deoxy-Hb), total hemoglobin (total-Hb) and tissue saturation (StO₂) compared with that observed at baseline were measured continuously during and after the 10-minute treatment period¹⁰.

Laser Doppler Blood Flow Analysis

In order to investigate the effects of a long duration of CG, AM or CM treatment on the hindlimb blood flow, mice with induced ischemia were randomly divided into four groups: the UT, CG, AM and CM groups. We performed the same experiment twice and obtained data for a total of 42 mice. The hindlimb blood flow was assessed under anesthesia with sodium pentobarbital (50 mg/kg, i.p.) using a laser Doppler blood flow (LDBF) analyzer (Moor LDI; Moor Instruments, Devon, UK) before and immediately after surgery and on postoperative days 4, 7, 14, 21 and 28, as previously described^{11, 12}. The LDBF analysis was performed before each treatment session on days 4, 7, 14 and 21 and 24 hours after the last treatment. After the scanning blood flow, the stored images were subjected to computer-assisted quantification, and the average flow in the ischemic and non-ischemic limbs was calculated. In order to prevent data variations resulting from the effects of ambient light and temperature, the hindlimb blood flow was expressed as the ratio of the left (ischemic) to the right (non-ischemic) LDBF values.

Capillary Density

At 28 days after surgery, the mice were euthanized under anesthesia with sodium pentobarbital (100 mg/kg, i.p.), and their hindlimbs were removed. The capillary density within the ischemic thigh adductor skeletal muscles was subsequently analyzed in order to obtain specific evidence of vascularity at the level of the microcirculation. Three sections of ischemic muscle were harvested from each animal, sliced and fixed in methanol. The tissues were then embedded in paraffin, and multiple tissue slices of 5 μ m in thickness were prepared. Capillary endothelial cells (ECs) were identified using immunohistochemical staining with a rat anti-mouse CD31 antibody (Ab) (PharMingen, CA). Fifteen random microscopic fields from three different sections in each tissue block were examined for the presence of capillary ECs, and the capillary density was expressed as the number of capillaries per high-power field ($\times 400$)^{11, 12}.

Measurement of the Nitrate (NO₃⁻) Levels in the Serum

At 28 days after surgery, the mouse serum nitrate (NO₃⁻) concentration was measured using high-per-

formance liquid chromatography, according to the manufacturer's instructions¹⁰.

Quantitative Real-Time Polymerase Chain Reaction

Total RNA was isolated using ISOGEN (Nippon Gene Co., Ltd., Toyama, Japan). In order to determine the transcript levels of vascular endothelial growth factor (VEGF) and fibroblast growth factor 2 (FGF-2), the RNA samples were subjected to quantitative real-time polymerase chain reaction (RT-PCR, 7500 Fast; Applied Biosystems, Carlsbad, CA). One-step quantitative RT-PCR reactions were performed using 100 ng of total RNA per reaction. The transcript levels were normalized to that of 18S for the analysis.

Effects of a NO Synthase Inhibitor on the Outcomes of CO₂ Dry Mist Therapy

In order to chronically inhibit NOS, wild-type mice with hindlimb ischemia were provided water containing 1 mg/mL of *N*^G-nitro- L-arginine methyl ester (L-NAME) for four weeks. Furthermore, iNOS^{-/-} and eNOS^{-/-} mice with hindlimb ischemia were treated with CO₂ mist for 28 days. The hindlimb blood flow was then measured, as described above.

Effects of a Low Concentration of CO₂ on the Tissue Blood Flow

In order to investigate whether blood perfusion is enhanced in a CO₂ concentration-dependent manner, we compared the effects of no treatment and treatment with 250,000 ppm (low CM) or 900,000 ppm (high CM) of CO₂ in the bags encasing the lower body of the mice. The high or low CM conditions were produced using 100% concentrated CO₂ or a mixture of 100% concentrated CO₂ and mixed air, respectively. The LDBF analysis was performed as described above.

Mass Analysis

At four days after ischemia, the mice were euthanized under anesthesia with sodium pentobarbital (100 mg/kg, i.p.) and their hindlimbs were removed. Digested proteins (150 μ g) from the muscles in the non-ischemic, UT and CM groups (pooling of each *n*=5) were reduced, alkylated, digested with trypsin and labeled with an isobaric tag for use with relative and absolute quantitation (iTRAQ) reagents (AB Sciex, Framingham, MA), according to the manufacturer's instructions, with the following minor modification.

After the labeling reaction (114, non-ischemic; 116, UT; 117, CM), the three samples were pooled

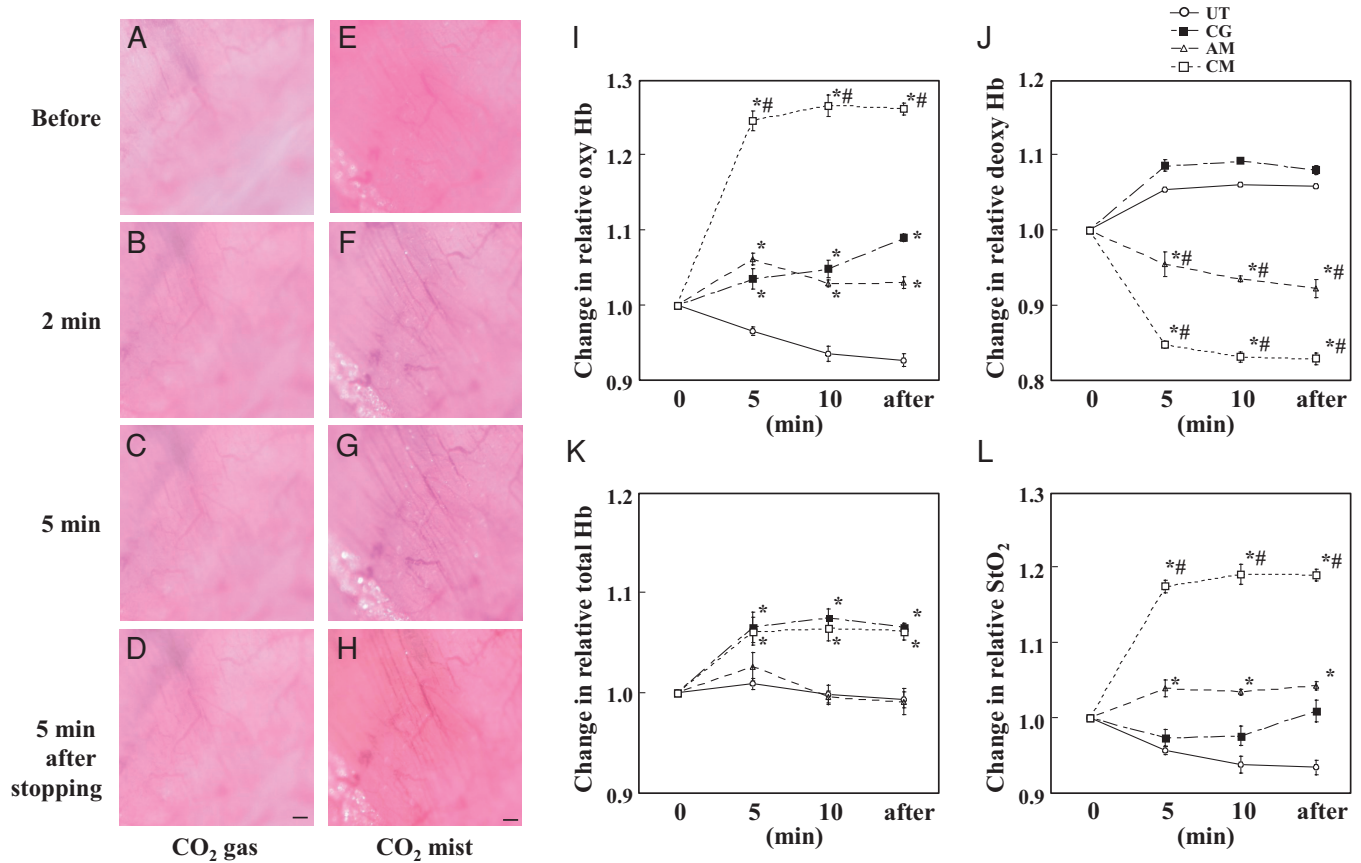


Fig. 1. Vasodilatory effects of the CO₂ mist and changes in the relative tissue blood flow. Photographs (A to H) taken with a digital microscope showing the vasodilatory effects of the CO₂ gas (A to D) and CO₂ mist (E to H) in the femoral subcutaneous tissue. After two minutes of spraying the CO₂ mist, microvessels gradually appeared. These microvessels were more prominent after five minutes of spraying the CO₂ mist (G) compared with that observed before treatment (E). Bar = 100 μ m. The CO₂ mist group had significantly increased oxygenated hemoglobin (Hb) levels (I) and decreased deoxygenated Hb levels (J) compared with the untreated, air mist and CO₂ gas groups. The total Hb levels were increased versus baseline in the CO₂ gas and CO₂ mist groups, but not the untreated and air mist groups (K). The tissue saturation levels were also significantly increased in the CO₂ mist (CM) group compared with those observed in the untreated (UT), air mist (AM) and CO₂ gas (CG) groups (L). oxy Hb, oxygenated hemoglobin; deoxy Hb, deoxygenated hemoglobin; total Hb, total hemoglobin; StO₂, tissue saturation. Each bar represents the mean \pm SEM. * p < 0.05 vs. UT; # p < 0.05, CO₂ mist vs. CO₂ gas.

and 10 μ L of 20% (v/v) trifluoroacetic acid was added to cleave RapiGest (Waters, Milford, MA). The samples were then vortexed, incubated at 37°C for one hour and centrifuged. The supernatant was subsequently purified using a cation exchange column (AB Sciex), according to standard procedures.

Next, a proteome analysis was performed using a DiNa-AI nano LC System (KYA Technologies, Tokyo, Japan) coupled with a QSTAR Elite hybrid mass spectrometer (AB Sciex) through a NanoSpray ion source (AB Sciex), as previously described¹³. Briefly, mobile phase A included 98% water (2% acetonitrile [ACN], 0.1% formic acid) and mobile phase B included 70% ACN (0.1% formic acid, 30% water). The column

effluent was introduced into the spray chamber through a tapered stainless steel emitter and directly electrosprayed into the QSTAR System ion trap mass spectrometer in the positive mode for the nanoESI-tandem mass spectrometry (MS/MS) analysis. One sample was run for 150 minutes. Protein identification was performed using the Analyst QS Software 2.0 program (AB Sciex) in the positive-ion mode. Both sets of data were processed using the ProteinPilot Software 2.0.1 package according to the Paragon™ search algorithm (AB Sciex), and the MS/MS data were searched against the NCBI database using a *Mus musculus* taxonomy filter. The minimum threshold for protein identification was set at a protein score of 1.3,

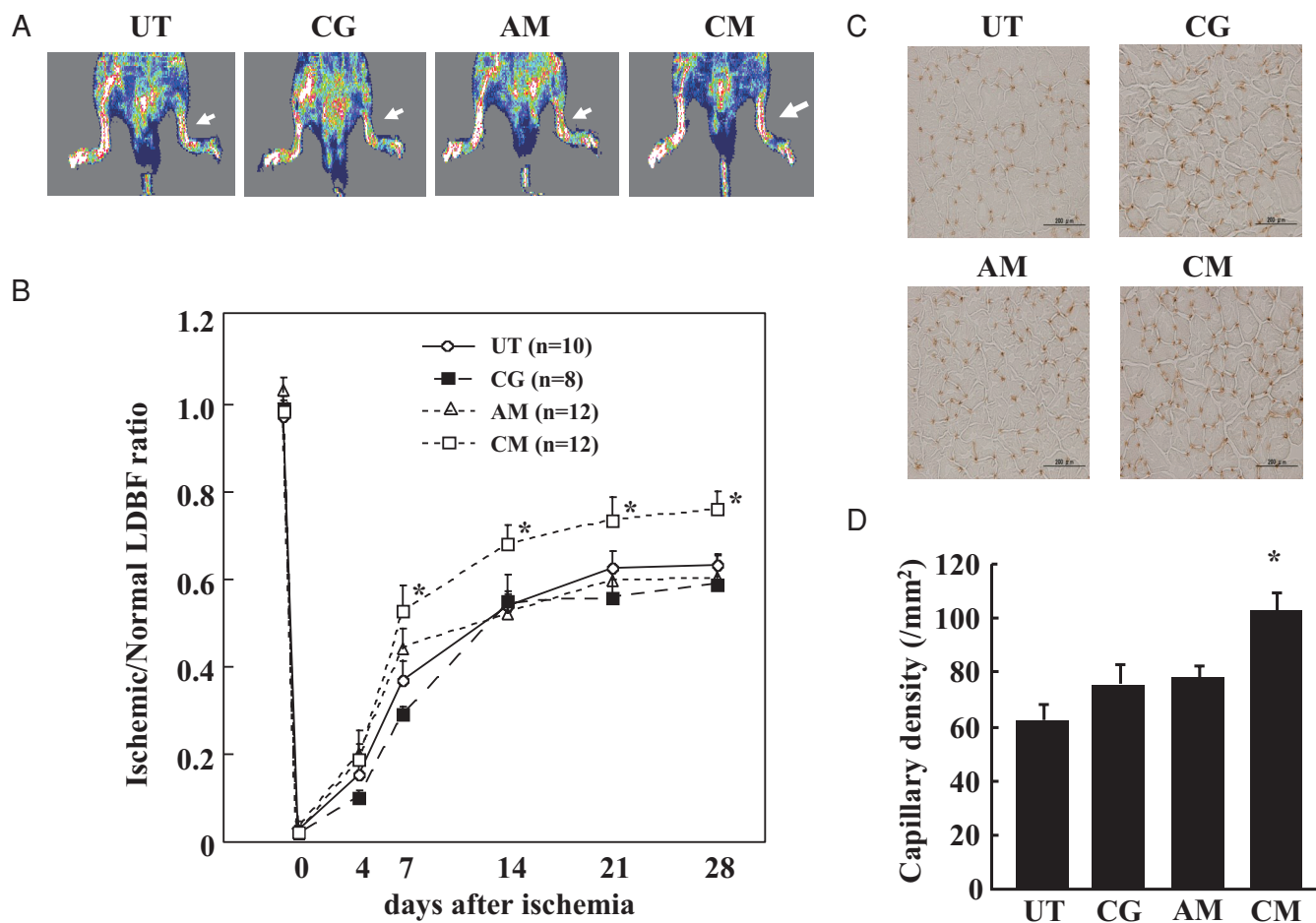


Fig. 2. Laser Doppler blood flow (LDBF) analysis and capillary density. (A) A representative LDBF image. A low-perfusion signal was observed in the ischemic hindlimbs of the untreated mice, whereas a high-perfusion pattern (white to red) was detected in the CO₂ mist-treated mice. The abbreviations are the same as in the Fig. 1 legend. (B) The computer-assisted quantitative analysis of hindlimb blood perfusion showed a significant improvement in the ischemic/normal hindlimb blood flow ratio in the CO₂ mist-treated mice compared with that seen in the untreated, CO₂ gas and air mist groups. Each bar represents the mean \pm SEM. * $p < 0.05$ vs. UT at each time point. (C) Representative angiograms and (D) capillary density assessed according to staining with anti-CD31 Abs on postoperative day 28. Each bar represents the mean \pm SEM. * $p < 0.05$ vs. UT.

which corresponded to a confidence level above 95% and a false discovery rate of 1%.

Western Blotting

Our detailed methods have been previously described^{11, 12}. Protein extracts were obtained from homogenized ischemic or non-ischemic skeletal muscles and electrophoretically transferred to polyvinylidene difluoride membranes (Immobilon-P, Millipore, Billerica, MA). The membranes were then probed with each primary antibody.

Antibodies were obtained from the following sources: anti-Ras-associated protein 1b (Rap1b), superoxide dismutase 2 (SOD2), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), VEGF, FGF-2

and actin antibodies from Santa Cruz Biotechnology (Dallas, TX); phosphor-ERK1/2 and anti-phospho-eNOS (p-Ser1177) antibodies from Cell Signaling Technology (Beverly, MA); anti-eNOS antibodies from BD Biosciences (San Jose, CA).

Statistical Analysis

All data are presented as the mean \pm standard error of the mean (SEM). Comparisons between two experimental groups were made to test for statistical significance using Student's *t*-test. Further comparisons between groups were made according to the Tukey-Kramer method using the StatView software program (SAS Institute, Inc., Cary, North Carolina, USA). Differences were considered to be statistically

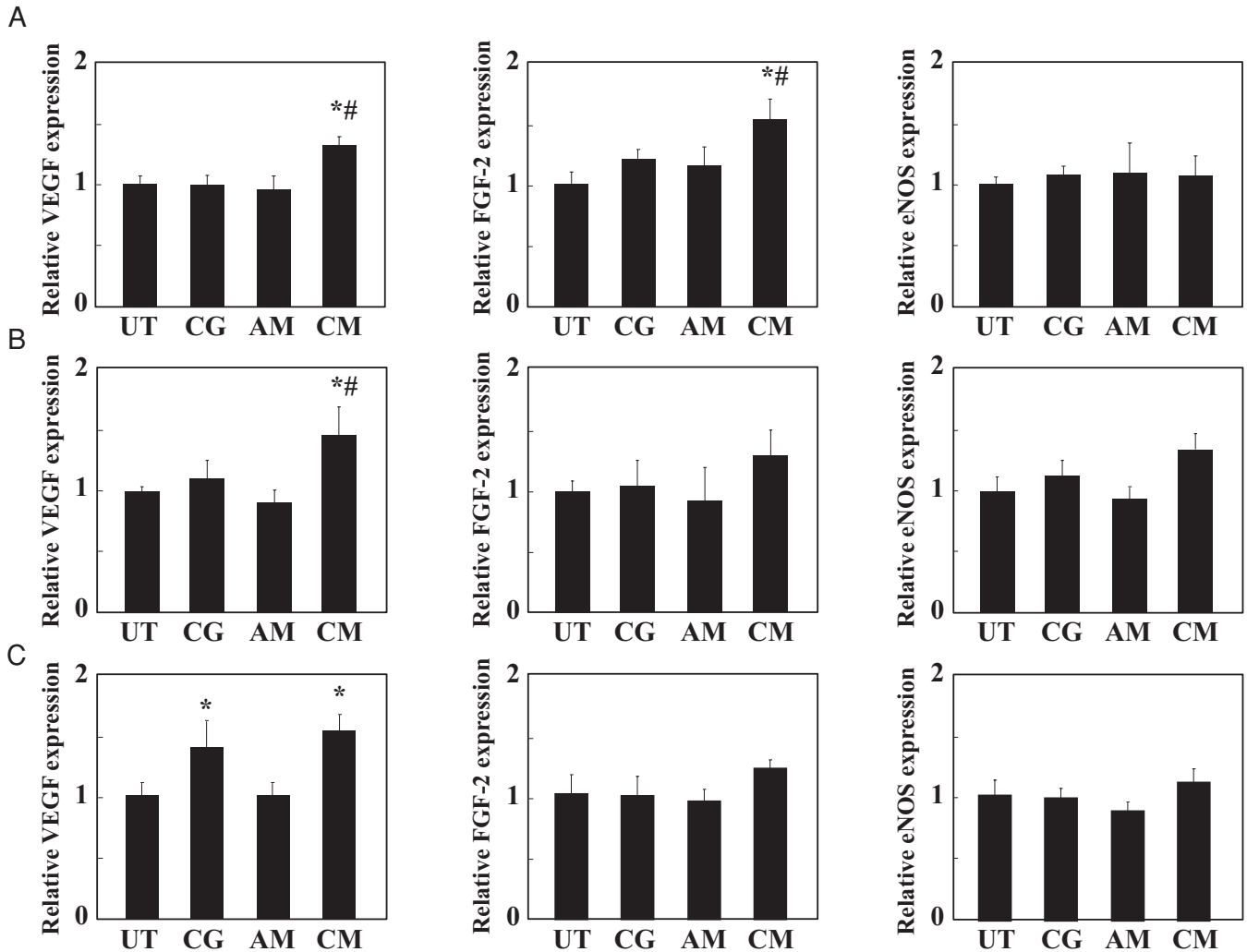


Fig. 3. Gene expression levels in the ischemic hindlimbs of the untreated, CO₂ gas, air mist-treated and CO₂ mist-treated mice. The bar graph shows each mRNA level corrected for the 18S rRNA level on postoperative day 4 (A), day 7 (B) and day 14 (C). The mean value in the untreated group was set at 1. The VEGF and FGF-2 expression levels were significantly increased by the CO₂ mist treatment. VEGF, vascular endothelial growth factor; FGF-2, fibroblast growth factor-2; eNOS, endothelial nitric oxide synthase. Other abbreviations are the same as in the Fig. 1 legend. The values are presented as the mean \pm SEM ($n=8$ to 12 (A) and (B), $n=4$ to 6 (C)). * $p < 0.05$ vs. UT; # $p < 0.05$, CO₂ mist vs. CO₂ gas.

significant at a value of $p < 0.05$.

Results

Effects of CO₂ Mist on Vasodilation and the Tissue Blood Flow

We compared the effects of treatment with CG, AM and CM on vasodilation. Wild-type mice in a transparent acrylic case were treated with CG, AM or CM. Treatment with CG did not change the detection of microvessels compared with that observed before treatment (**Fig. 1-A to D**), nor did AM treatment (data not shown). In contrast, a few microvessels were grad-

ually detected two to five minutes after treatment with the CO₂ mist compared with that noted before treatment (**Fig. 1-E to H**).

Next, we measured the changes in the relative tissue blood flow during CG, AM and CM treatment (**Fig. 1-I to L**). Rats were used in this experiment because the mice were too small to precisely measure the tissue blood flow. The CM group exhibited increased oxy-Hb levels (1.26-fold; **Fig. 1-I**), decreased deoxy-Hb levels (0.83-fold; **Fig. 1-J**) and increased StO₂ levels (1.19-fold; **Fig. 1-L**) compared with that observed at baseline. Importantly, the CM group exhibited significantly higher oxy-Hb levels than the

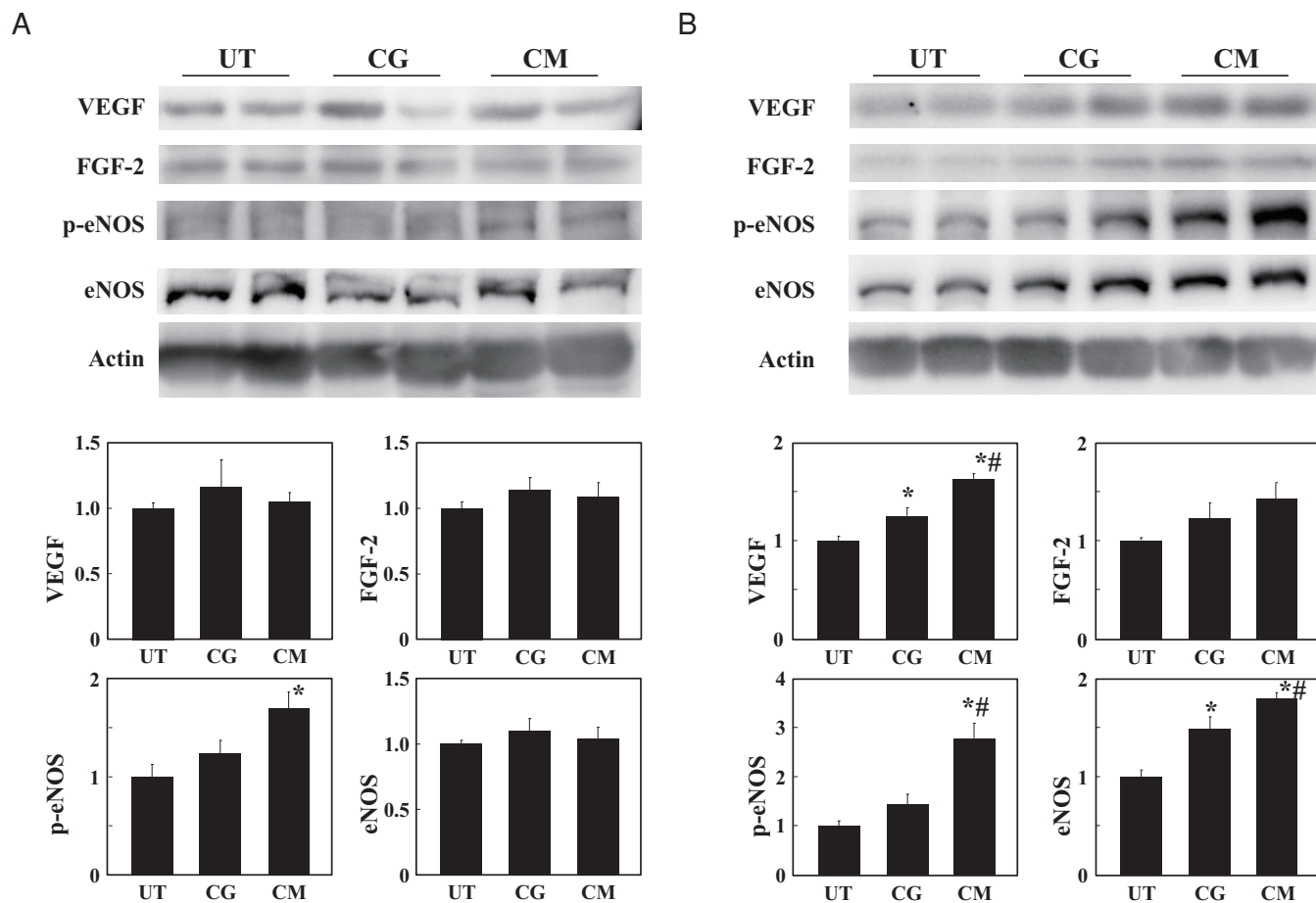


Fig. 4. Protein levels in the ischemic hindlimbs of the untreated and CO₂ gas- and CO₂ mist-treated mice. The upper panels show the findings of a representative Western blot analysis. The lower bar graphs show the level of each protein on postoperative day 4 (A) and day 14 (B) in the ischemic hindlimbs of the untreated and CO₂ gas- and CO₂ mist-treated mice. The mean value in the untreated group was set at 1. The VEGF and phosphorylated eNOS levels were significantly increased by the CO₂ mist treatment on day 14. p-eNOS, phosphorylated eNOS. Other abbreviations are the same as in the Fig. 3 legend. The values are presented as the mean \pm SEM ($n=4$ to 6). * $p<0.05$ vs. UT; # $p<0.05$, CO₂ mist vs. CO₂ gas.

UT group, as well as the CG and AM groups, after five minute of each treatment. Conversely, the CM group exhibited significantly lower deoxy-Hb levels than the UT, AM and CG groups after five minutes of each treatment. Interestingly, the StO₂ levels were also significantly higher in the CM group than in the CG group, although the total-Hb levels in the CM group were similar to those observed in the CG group (Fig. 1-L).

Laser Doppler Blood Perfusion and Tissue Capillary Density

We subsequently investigated whether treatment with CM accelerates blood flow recovery after ischemia. Immediately after the left femoral artery and vein were resected, the ratio of the ischemic (left) to

non-ischemic (right) hindlimb LDBF (the LDBF ratio) decreased by <0.05 in all groups. However, the CM-treated mice showed a significant blood flow recovery in the ischemic limb compared with the UT mice (day 14: 0.71 ± 0.04 vs 0.54 ± 0.03 , day 28: 0.78 ± 0.04 vs 0.63 ± 0.02 , $p<0.05$, respectively; Fig. 2-A and B), whereas the CG- and AM-treated mice demonstrated no acceleration in the recovery of the blood flow.

Next, we measured the capillary density in histological sections harvested from the ischemic tissues in order to investigate the extent of angiogenesis at the level of the microcirculation (Fig. 2-C). A quantitative analysis revealed that the capillary density was significantly greater on postoperative day 28 in the CM mice than in the UT mice (Fig. 2-D).

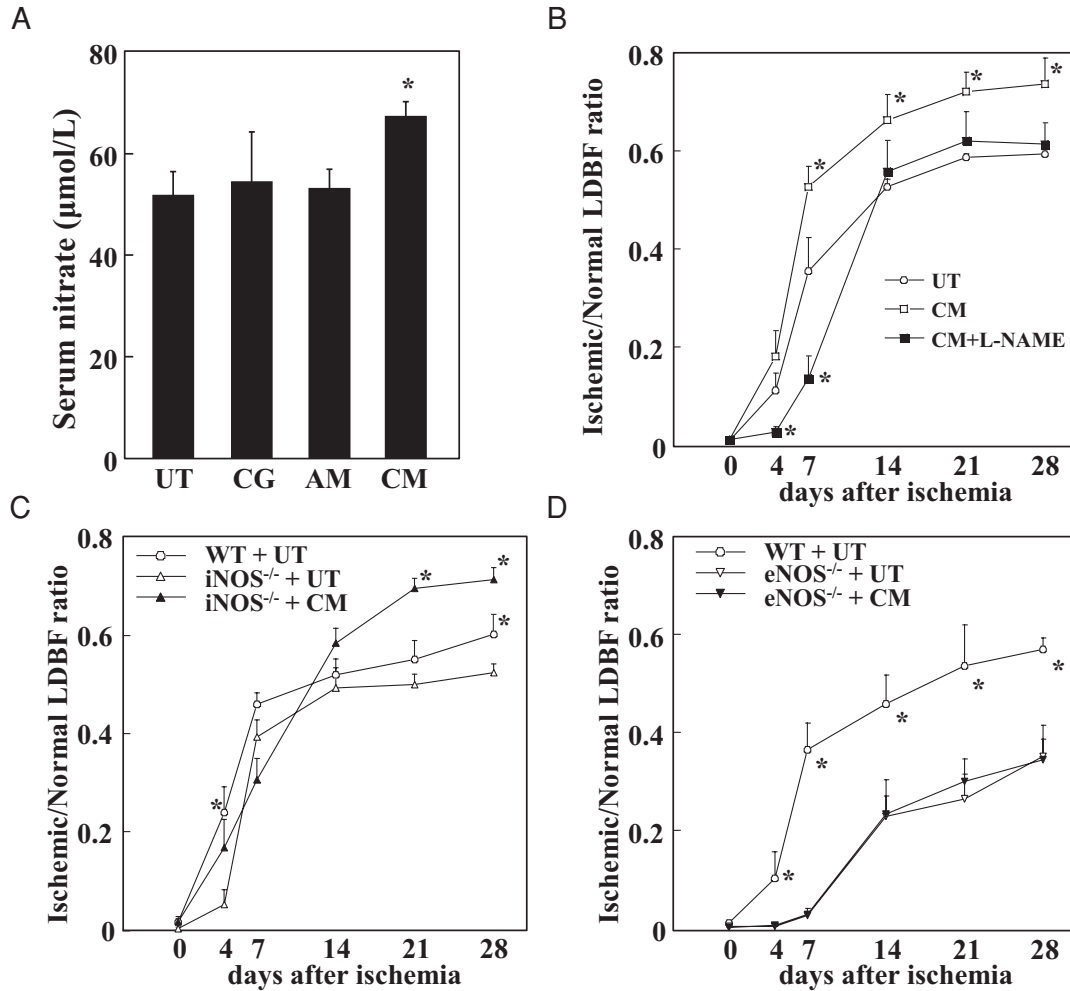


Fig. 5. Serum concentrations of nitrate and results of the LDBF analysis following NOS inhibition. (A) The serum concentrations of nitrate (NO_3^-) were significantly greater in the CM group than in the UT group on postoperative day 4. In contrast, the serum concentrations of NO_3^- in the CG and AM group did not differ significantly from the control values. The values are presented as the mean \pm SEM ($n=6$ to 8). * $p < 0.05$ vs. UT. (B) The administration of L-NAME (1 mg/mL in drinking water) reduced the increase in perfusion induced by the CO_2 mist to a normal level. The values are presented as the mean \pm SEM ($n=6$ to 8). * $p < 0.05$ vs. UT at each time point. (C) Treatment with CO_2 mist accelerated the recovery of the blood flow after ischemia in the $i\text{NOS}^{-/-}$ mice. The values are presented as the mean \pm SEM ($n=6$ to 8). * $p < 0.05$ vs. $i\text{NOS}^{-/-}$ + UT at each time point. In contrast, the CO_2 mist failed to increase the recovery of the blood flow in the $e\text{NOS}^{-/-}$ mice (D). The values are presented as the mean \pm SEM ($n=6$ to 8). * $p < 0.05$ vs. $e\text{NOS}^{-/-}$ + UT at each time point.

Expression of Angiogenic Growth Factors in the Ischemic Hindlimbs

VEGF and FGF-2 are the major cytokines responsible for ischemia-induced angiogenesis and arteriogenesis. Therefore, we examined the expression levels of VEGF and FGF-2 in the ischemic tissues of each group. Consequently, a quantitative RT-PCR analysis showed a significantly greater expression of VEGF on days 4, 7 and 14 and FGF2 on day 4 in the CM-treated mice compared with the UT mice (**Fig. 3-A, B**

and C). On the other hand, the eNOS expression was slightly but not significantly increased by CM treatment, whereas treatment with CG did not change the VEGF expression on days 4 and 7, but rather increased the expression on day 14.

We also examined the protein levels using a Western blot analysis. As shown in **Fig. 4**, the VEGF protein levels were increased in the CM group compared with those observed in the UT group. Meanwhile, the FGF-2 levels tended to be increased in the

CM group ($p=0.055$ vs. UT). In contrast to the mRNA expression, the level of phosphorylated eNOS was significantly increased in the CM group on both days 4 and 14. There were no differences in the actin levels between the groups.

Effects of CO₂ Mist Treatment on the NO and NOS Levels

The serum concentration of nitrate was measured at four days after ischemia, as shown in **Fig. 5-A**. The serum nitrate concentrations were significantly greater in the CM group than in the UT group ($p < 0.05$), whereas the nitrate levels in the CG and AM groups did not differ from the control values.

Next, the effects of the NO signaling pathway were investigated. Consequently, L-NAME completely inhibited the effects of the CM treatment (**Fig. 5-B**). Furthermore, the CM treatment significantly increased the LDBF ratio in the iNOS-deficient mice, but not in the eNOS-deficient mice (**Fig. 5-C and D**). These results suggest that the actions of CO₂ mist in stimulating revascularization *in vivo* are dependent, at least in part, on eNOS.

Effects of a Low Concentration of CO₂ Mist on the Tissue Blood Flow

We compared the differences in blood flow recovery in the ischemic hindlimbs between treatment with high and low CM concentrations. Similar to previous results, treatment with high CM significantly accelerated the blood flow recovery in the ischemic hindlimbs. On the other hand, treatment with low CM only slightly recovered the blood flow in the ischemic hindlimbs (**Fig. 6**), suggesting that recovery of the blood flow is enhanced in a CO₂ concentration-dependent manner.

Mass Analysis

In order to further investigate the detailed mechanisms of action of CM treatment, we performed a proteomic analysis of the hindlimb muscles at four days after ischemia. Proteins from the adductor muscle in the non-ischemic, UT and CM groups were labeled with 114, 116 and 117 tags, respectively, and 195 proteins were identified when the protein threshold was set at 1.3 to achieve a confidence level above 95%. We also included an additional 1.2-fold change cutoff for the iTRAQ ratio ($116/114 < 0.80$ or > 1.2) in order to reduce the rate of false-positive results for classifying proteins as upregulated or downregulated. Among the 195 proteins identified, 68 were upregulated and 62 were downregulated by ischemia. Furthermore, 18 of the 68 upregulated proteins were downregulated by

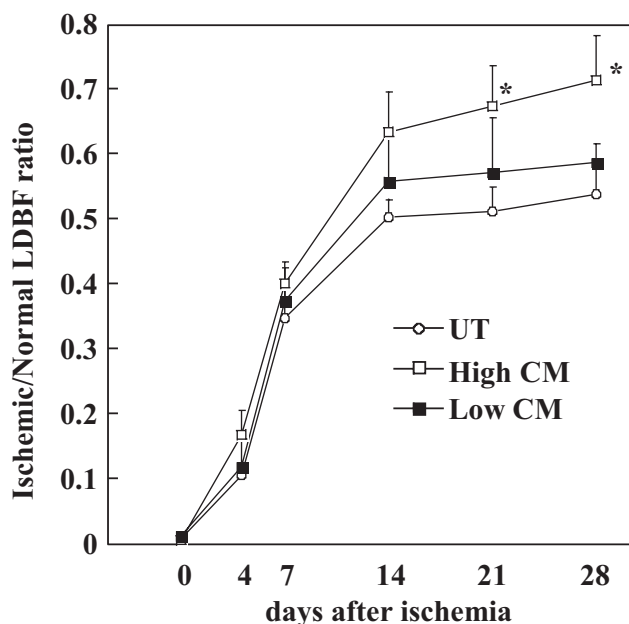


Fig. 6. Effects of high and low concentrations of CO₂ mist on ischemia-induced angiogenesis. Treatment with CO₂ mist at a high concentration of CO₂ (High CM) accelerated the blood flow recovery in the ischemic hindlimbs, whereas treatment with CO₂ mist at a low concentration of CO₂ (Low CM) only slightly recovered the blood flow. Each bar represents the mean \pm SEM (n =each 6). * $p < 0.05$ vs. UT at each time point.

CM treatment (ratio: $116/117 < 0.80$). As shown in **Table 1-A**, some of these proteins were related to signal transduction. In contrast, 21 of the 62 downregulated proteins were upregulated by CM treatment (ratio: $116/117 > 1.2$). As shown in **Table 1-B**, some of these proteins were related to metabolic enzymes.

In order to confirm the results of the mass analysis, we investigated the levels of several proteins using Western blotting (**Supplemental Fig. 1**). Consequently, the Rap1b level was remarkably increased by ischemia and subsequently decreased by CM treatment. In addition, the SOD-2 and GAPDH levels were decreased by ischemia and increased by CM treatment.

Discussion

The major findings of the present study are that angiogenesis and blood flow recovery in response to hindlimb ischemia were significantly improved by CO₂ mist treatment. Furthermore, we obtained the first evidence that CO₂ mist therapy affects both eNOS and VEGF, as well as the glycolytic system. Therefore, CO₂ mist treatment accelerates ischemia-induced

Table 1. Lists of the 18 proteins decreased by CO₂ mist among the 68 proteins upregulated by ischemia (A) and the 21 proteins increased by CO₂ mist among the 62 proteins downregulated by ischemia (B)

A			
Accession No.	Name	Ischemia/ non-ischemia	Ischemia + CO ₂ mist/Ischemia
gi 51172612	group specific component precursor	3.0807	0.6645
gi 12963497	kininogen 1 isoform 2	3.8908	0.6651
gi 160358829	hemopexin precursor	3.9433	0.7323
gi 6678768	myristoylated alanine rich protein kinase C substrate	6.2190	0.7396
gi 33563236	Rho, GDP dissociation inhibitor (GDI) beta	9.1627	0.7432
gi 149272386	PREDICTED: similar to thymosin beta-4	5.7131	0.7498
gi 33859753	RAS related protein 1b precursor	3.1921	0.7591
gi 124486712	ribosome binding protein 1 isoform a	3.3542	0.7599
gi 145301578	hemoglobin alpha 1 chain	2.7937	0.7624
gi 86476077	thymosin, beta 10	6.7223	0.7717
gi 21313162	RAB1B, member RAS oncogene family	1.9955	0.7815
gi 7110705	prothymosin alpha	4.1927	0.7853
gi 6681079	cathepsin B preproprotein	5.3743	0.7881
gi 6680836	calreticulin precursor	2.8233	0.7890
gi 18252782	serpin peptidase inhibitor, clade C, member 1 precursor	2.7453	0.7934
gi 6671549	peroxiredoxin	1.3707	0.7943
gi 12963729	INO80 complex subunit B	1.3092	0.7948
gi 6679439	peptidylprolyl isomerase A	2.9372	0.7985
B			
Accession No.	Name	Ischemia/ non-ischemia	Ischemia + CO ₂ mist/Ischemia
gi 130488506	four and a half LIM domains 3	0.6897	1.6215
gi 21313458	nucleolar protein 3	0.5381	1.6085
gi 149253821	PREDICTED: similar to Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) isoform 2	0.4492	1.5665
gi 114431240	adenosine monophosphate deaminase 1 (isoform M)	0.4481	1.5416
gi 33563266	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 4	0.4739	1.4803
gi 149266429	PREDICTED: similar to Glyceraldehyde-3-phosphate dehydrogenase isoform 2	0.5879	1.3685
gi 21313679	ATP synthase, H ⁺ transporting, mitochondrial F0 complex, subunit d	0.5153	1.3574
gi 257743039	lactate dehydrogenase A isoform 2	0.5176	1.3055
gi 149265502	PREDICTED: similar to Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	0.6516	1.2813
gi 33859811	mitochondrial trifunctional protein, alpha subunit precursor	0.7632	1.2807
gi 227430322	calsequestrin 1	0.7146	1.2730
gi 20452466	ferrochelatase	0.7089	1.2654
gi 6671762	muscle creatine kinase	0.5491	1.2503
gi 254553458	glucose phosphate isomerase 1	0.6630	1.2444
gi 31980762	superoxide dismutase 2, mitochondrial precursor	0.6287	1.2396
gi 40254393	hypothetical protein LOC66273	0.6046	1.2388
gi 6755256	muscle glycogen phosphorylase	0.5423	1.2289
gi 6671539	fructose-bisphosphate aldolase A	0.5888	1.2247
gi 6679651	enolase 3, beta muscle isoform 1	0.5824	1.2119
gi 6671519	adenylosuccinate synthase like 1	0.6999	1.2027
gi 6678391	troponin I, skeletal, fast 2	0.6375	1.2019

angiogenesis.

Carbonated springs have been used as spa therapy for many years to treat a variety of diseases, particularly in European countries. It is also well known that CO₂-enriched water induces peripheral vasodilation, which increases the cutaneous blood flow^{14, 15}. Furthermore, Toriyama *et al.* reported that the effects of CO₂-enriched water on the subcutaneous microcirculation are mediated by peripheral vasodilation resulting from increased parasympathetic and decreased sympathetic nerve activity¹⁶. Furthermore, a recent study showed that the immersion of ischemic hindlimbs into CO₂-enriched water results in a NO-dependent increase in collateral blood perfusion in mice in a unilateral hindlimb ischemia model⁹. Hence, highly concentrated CO₂ is thought to be useful for treating ischemic diseases. However, devising an artificial CO₂-rich bath system is difficult.

Although carbonated spring water has been found to contain more than 1,000 ppm of CO₂, it is difficult to constantly maintain the CO₂ concentration above 1,000 ppm for hours. In contrast, CO₂ dry mist production units can easily produce CO₂ concentrations of more than 900,000 ppm¹⁰. In the present study, we assessed whether treatment with CO₂ mist exerts an angiogenic effect in rodent ischemic hindlimbs and investigated the mechanisms responsible for the proangiogenic effects induced by CO₂ mist. The main finding of the present study is that percutaneously administered CO₂ mist accelerates angiogenesis, compared with no treatment or treatment with CO₂ gas or air mist, in mice with hindlimb ischemia.

There are several possible mechanisms for the effects of CO₂ mist. For example, the transfer of CO₂ across the skin results in beneficial local vasomotor effects without inducing systemic hemodynamic modifications⁸. In the present study, treatment with CO₂ mist, but not gas, had vasodilatory effects in rat femoral subcutaneous tissue (**Fig. 1-E to H**). These results suggest that CO₂ mist exerts a vasodepressor effect. Furthermore, the CO₂ mist significantly increased the oxy-Hb levels and significantly decreased the deoxy-Hb levels compared with the CO₂ gas (**Fig. 1-I and J**). Interestingly, the StO₂ levels were also significantly higher in the CO₂ mist group than in the CO₂ gas group (**Fig. 1-L**). These findings suggest that CO₂ mist increases the blood flow, consequently shortening the blood capillary transit time, thereby reducing the process of extraction of oxygen from the blood and releasing oxygen more easily.

A recent study demonstrated that the expression levels of potent angiogenic factors, such as VEGF or FGF-2, are increased and endothelial cell apoptosis is

inhibited in endothelial cells cultured in medium equilibrated with hypercapnia-associated acidosis¹⁷. It has also been established that VEGF promotes ischemia-induced angiogenesis¹⁸⁻²⁰. The present data further indicate that the VEGF and FGF-2 expression is induced in the hindlimb skeletal muscles by CO₂ mist treatment (**Fig. 3**), while the VEGF protein level is increased by CO₂ mist (**Fig. 4**). The increase in the VEGF expression caused by CO₂ mist treatment may, at least partially, contribute to angiogenesis after ischemia. Interestingly, treatment with CO₂ gas, as well as mist, significantly increased the VEGF expression on day 14 only in the present study, suggesting that CO₂ gas alone may increase the VEGF expression in the chronic phase, although the effect may not be intense.

Previous studies have reported that VEGF stimulates the release of NO from the arterial wall²¹ and promotes the recovery of a disturbed endothelium-dependent flow in the ischemic hindlimb. Moreover, the involvement of NO in the angiogenic effects of VEGF has been established in NO-deficient mice^{19, 22}. In the current analysis, CO₂ mist treatment significantly increased the eNOS mRNA activity, but not its expression (**Fig. 4**), as well as the serum NO₃⁻ concentration compared with that observed without treatment or treatment with CO₂ gas or air mist (**Fig. 5-A**). Furthermore, the inhibition of the NOS activity by L-NAME completely inhibited the recovery of the collateral blood flow induced by the CO₂ mist (**Fig. 5-B**). Furthermore, the CO₂ mist significantly increased the LDBF ratio in the iNOS^{-/-} mice, but not in the eNOS^{-/-} mice (**Fig. 5-C and D**). Taken together, these findings demonstrate that the proangiogenic effects of CO₂ mist are the product of the activation of eNOS-mediated signaling resulting from the downstream effects of VEGF. Very interestingly, treatment with CO₂ mist using highly concentrated CO₂ (100% CO₂ gas) significantly accelerated the blood flow recovery in the ischemic hindlimbs. In contrast, treatment with CO₂ mist using a low concentration of CO₂ only slightly recovered the blood flow in the ischemic hindlimbs (**Fig. 6**). These results suggest that blood flow recovery may be enhanced in a CO₂ concentration-dependent manner.

In order to further investigate the mechanisms underlying the effects of CO₂ mist, we performed a proteomic analysis of the hindlimb muscles four days after ischemia. Interestingly, the levels of 18 of 68 proteins upregulated by ischemia were decreased by CO₂ mist treatment. Rap1, a small GTPase, has been shown to regulate multiple basic cellular processes, and there is emerging evidence that Rap1 regulates basic endothelial responses to angiogenic stimulation^{23, 24}. In the

present study, the Rap1b level was remarkably increased by ischemia and subsequently decreased by treatment with CO₂ mist. Therefore, CO₂ mist may regulate excessive Rap1 and ERK activation in the hindlimb muscles, although there are currently no reports concerning the role of Rap1 in skeletal muscle.

On the other hand, we found that, of the 62 proteins downregulated by ischemia, the levels of 21 proteins were increased by CO₂ mist treatment. Some of these proteins are enzyme proteins involved in the glycolytic system, such as GAPDH, glucose phosphate isomerase 1, enolase and aldolase A. Therefore, CO₂ mist treatment may promote metabolism in ischemic muscles. Oxidative stress occurs when the production of reactive oxygen species (ROS) is enhanced and/or the antioxidant reserve is suppressed. Enhanced ROS production decreases the bioavailability of NO via an oxidative reaction that inactivates NO²⁵. Signal *et al.* provided evidence of a progressive decrease in the SOD and catalase activity in an infarcted rat heart²⁶. In addition, the present data show that the SOD2 level is decreased by ischemia and increased by CO₂ mist treatment. Therefore, CO₂ mist may have antioxidant actions.

Study Limitations

The proteomic analysis identified novel protein candidates regulated by the CO₂ mist. However, it could not be determined which molecules are directly regulated by CO₂ mist. Therefore, further studies are needed to explore the precise mechanisms underlying CO₂ mist-induced angiogenesis.

In conclusion, this study provides the first evidence that CO₂ mist promotes angiogenesis after ischemia, at least partially, by increasing the subcutaneous blood flow, as well as the VEGF and NO levels, in ischemic muscles. Hence, CO₂ mist therapy is potentially useful for treating patients with PAD.

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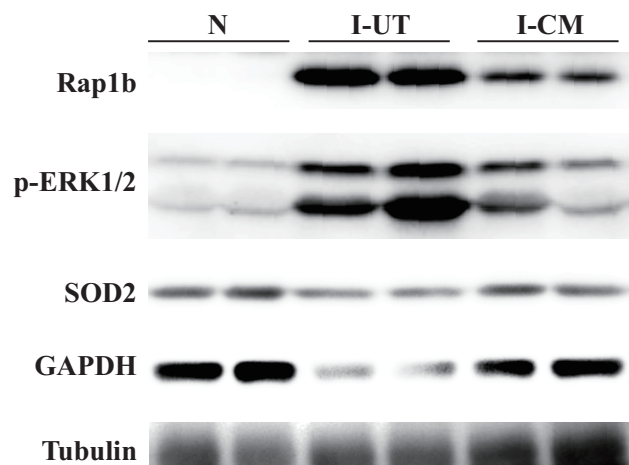
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The authors have no conflicts of interest to declare.

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Supplemental Fig. 1.

A representative Western blot analysis of Rap1b, p-ERK1/2, SOD-2 GAPDH and Tubulin in ischemic muscle tissue specimens at 4 days after ischemia. Rap1b, Ras-associated protein 1B; pERK1/2, phospho-ERK1/2; SOD2, superoxide dismutase 2; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; N, non-ischemic muscle; I-UT, ischemic muscle without treatment; I-CM, ischemic muscle with CO₂ mist treatment.