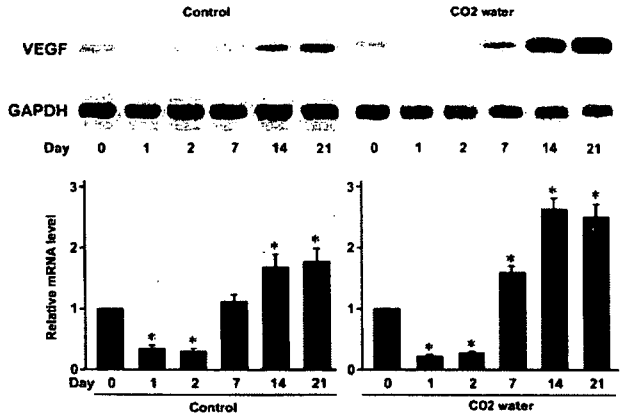

Figure 4. VEGF mRNA expression in ischemic hindlimb. Hindlimb skeletal muscles were dissected after ischemia, and RNA was extracted. Densities of VEGF mRNA signals were measured by densitometry and normalized relative to those of GAPDH mRNA signals. Results (mean±SEM, n=6) were arbitrarily indicated as values relative to VEGF mRNA levels at day 0. *$P<0.001$ vs day 0 preischemic levels.

ischemic mice did not change significantly after CO_2 bathing compared with the preischemic levels (Figure 5A). Although only induction of hindlimb ischemia did not affect plasma VEGF levels, VEGF levels in the CO_2-bathed ischemic mice were slightly but significantly elevated at day 14 (18%, $P<0.05$, n=6) compared with the water-immersed ischemic mice (n=6) (Figure 5B). Furthermore, we determined plasma pH levels to study whether CO_2 bathing–mediated effects are systemic. We found that CO_2 bathing of ischemic lower limbs did not significantly affect the pH levels in the peripheral blood (control, 7.2±0.04; CO_2 bathing, 7.2±0.03 at day 14; n=6 each). These findings demonstrate that VEGF synthesis by CO_2 bathing is induced only locally and that this increase in local VEGF synthesis leads to the elevation in plasma VEGF levels.

Skeletal muscles at day 14 (in which VEGF expression is maximally increased) were dissected, and eNOS phosphorylation and cGMP levels were examined. The eNOS phosphorylation levels at day 14 (normalized with expression levels of eNOS protein) were increased significantly in both control and CO_2-enriched water groups relative to the day 0 preischemic levels (1.6- and 2.4-fold, respectively) (Figure 6A). eNOS phosphorylation levels in the CO_2-enriched water group were significantly higher than those in the control group ($P<0.001$ versus the control group). Consistent with eNOS phosphorylation, cGMP levels in skeletal muscles at day 14 were also significantly higher (3.4-fold, $P<0.001$) in the CO_2-enriched water group compared with those in the control group (Figure 6B).

Effect of CO_2 Bathing on Circulating Endothelial-Lineage Progenitor Cells

CD34+/AC133+/Flk-1+ hematopoietic stem cells circulate in the peripheral blood of humans as an endothelial precursor cell and play a critical role in neovascularization in ischemic tissue.[16] Because AC133 marker is not available for mice, we isolated hematopoietic lineage-negative (Lin−) cells from the peripheral blood and then analyzed the CD34- and endothelial markers Flk-1– and endoglin-positive population to study whether endothelial-lineage precursor cells are mobilized by CO_2-enriched water bathing. FACS analysis indicated that Lin−/Flk-1+ cells are barely detected in the peripheral blood of normal mice (0.01±0.002% of total nuclear cells, n=12). Lin−/Flk-1+ cells were significantly increased after limb ischemia and showed a peak value at day 14 (≈7-fold versus the preischemic value) (from 0.01±0.002% to 0.073±0.002%, $P<0.001$, n=7). Interestingly, such a Lin−/Flk-1+ population was further increased by CO_2 bathing and showed a peak value at day 14 (≈24-fold increase, from 0.01±0.002% to 0.24±0.03%, $P<0.001$, n=7) (Figure 7A). The Lin−/Flk-1+ cells mobilized by CO_2 bathing were mostly positive for anti-endoglin antibody and in the CD34-negative fraction (Figure 7B). Considering that CD34−/Flk-1+ cells rather than CD34+/Flk-1+ are reported to be a real population of hematopoietic stem cells,[17] our present data

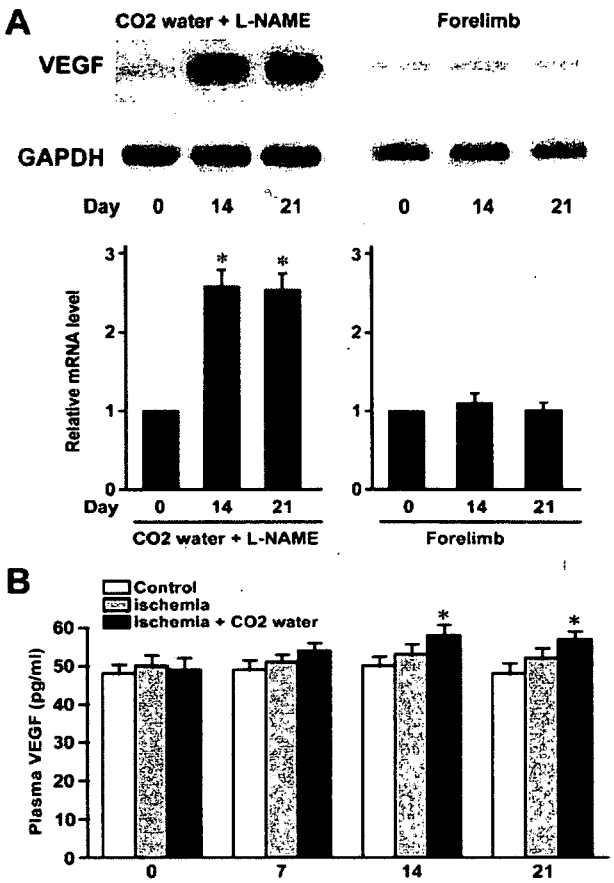

Figure 5. Effect of L-NAME on VEGF mRNA expression in hindlimb and forelimb skeletal muscles after hindlimb ischemia and plasma VEGF concentrations. A, Mice with hindlimb ischemia were provided water containing 1 mg/mL L-NAME, and time-dependent VEGF mRNA expression was measured as described in Figure 4. Forelimb skeletal muscles were dissected from hindlimb ischemia mice, and VEGF mRNA was analyzed. Results (mean±SEM, n=5) were arbitrarily indicated as values relative to VEGF mRNA levels at day 0. *$P<0.001$ vs day 0 preischemic levels, B, Plasma VEGF concentrations were measured by ELISA (control, CO_2 bathing of normal mice; ischemia, freshwater-bathed ischemic mice; ischemia+CO_2 water, CO_2-bathed ischemic mice, n=5 each). *$P<0.05$ vs day 0.

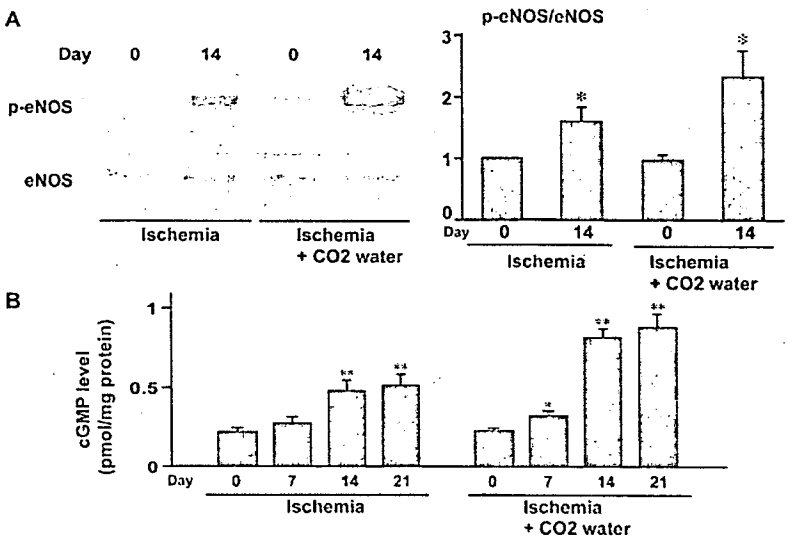

Figure 6. cGMP accumulation in ischemic limbs. A, Skeletal muscles were homogenized and immunoblotted with anti-phospho antibodies for eNOS. Phospho signals in filters were stripped and reprobed with anti-eNOS antibody. Phospho-eNOS densities were measured by densitometry and normalized relative to those of eNOS signals. Results are arbitrarily indicated as values relative to signal densities in day 0 preischemic control. Results shown are mean±SEM (n=6), and representative data are shown. *$P<0.001$ vs values in day 0 preischemic control. B, Skeletal muscles were dissected at days 7, 14, and 21 after hindlimb ischemia, and tissue cGMP levels were measured. Results shown are mean±SEM (n=6 each). *$P<0.05$, **$P<0.001$ vs day 0 preischemic control.

indicate that CO_2-enriched water bathing mobilizes very immature hematopoietic stem cells, including endothelial progenitor cells. To prove the specificity of anti-CD34 antibody, CD34+ cells were enriched by anti-mouse CD34 antibody from mouse bone marrow cells. As shown in Figure 7C, 83% purity of CD34-positive cells was detected by FACS, indicating that the staining for the CD34 antigen was properly performed.

We further examined whether Lin−/Flk-1+ cells express another endothelial marker, VE-cadherin, and also possess the characteristics of endothelial cells, such as acetylated LDL uptake. The Lin−/Flk-1+ cell population, mobilized by CO_2 bathing, was isolated and cultured with 100 nmol/L VEGF-supplemented medium for 4 days. Approximately 26±1.2% (n=12) of the Lin−/Flk-1+ cells adhered onto the fibronectin-coated plastic dishes. Approximately 74±2.3% (n=12) of the attaching cells showed the ability to incorporate the DiI-labeled acetylated LDL, and these cells expressed the VE-cadherin (Figure 7D).

Discussion

A number of reports about the physiological effects of CO_2-enriched water on subcutaneous microcirculation have been published. Savin et al[18] reported that transfer of CO_2 across the skin can have beneficial local vasomotor effects. Hartmann et al[19] demonstrated an increase in tissue oxygen brought about by the Bohr effect in addition to the vasodilation effect by CO_2 or vasodilation by decrease in plasma catecholamine levels. Toriyama et al[20] also reported that the effect of CO_2-enriched water on the subcutaneous microcirculation results from peripheral vasodilation resulting from increased parasympathetic and decreased sympathetic nerve activity. Findings in the intact coronary circulation[4] and in isolated aortic strips[5] have suggested that vasodilation in response to CO_2 may be mediated in part by NO. Consistent with these previous studies, our present study demonstrates that immersion of ischemic hindlimb into CO_2-enriched water bathing causes an NO-dependent increase in collateral blood perfusion, induction of regional VEGF synthesis, and mobilization of endothelial-lineage progenitor cells into the circulation.

What is the mechanism responsible for the proangiogenic effect by CO_2-enriched water bathing? In the endothelial cells cultured in the medium equilibrated with hypercapnia-associated acidosis, the expressions of potent angiogenic factors, such as VEGF or basic fibroblast growth factor, are increased and endothelial cell apoptosis is inhibited.[12] VEGF was well known to mobilize endothelial progenitor cells from bone marrow into the circulation.[21] Our present data clearly indicate that VEGF expression is markedly induced in hindlimb skeletal muscles after CO_2-enriched water bathing. A high concentration of CO_2 (1000 to 1200 mg/L) liberates free CO_2 in the freshwater (pH 7.0), resulting in a decrease in pH level (pH 5.0). We found that CO_2 bathing of ischemic lower limbs did not affect the pH levels in the peripheral blood and VEGF mRNA synthesis in the forelimb muscle. Considering that acidosis induces VEGF expression in the endothelial cells,[12] the local tissue acidosis by CO_2 bathing, rather than the CO_2 content of the water, may induce VEGF synthesis in the local skeletal muscles. Furthermore, calcium mobilization associated with local tissue pH changes may serve as alternate, or contributory, mechanisms for these observations.

Previous studies reported that VEGF stimulates the release of NO from the arterial wall[22,23] and promotes the recovery of disturbed endothelium-dependent flow in the ischemic hindlimb.[24] Involvement of NO in the angiogenic properties of VEGF has been established in the NO-deficient mice; Murohara et al[10] showed NO-mediated angiogenesis in the hindlimb ischemia model, and Aicher et al[25] reported that VEGF-mediated mobilization of endothelial progenitor cells is reduced in NO-deficient mice. The present study revealed that inhibition of NOS activity by L-NAME inhibited the recovery of collateral blood flow by CO_2 bathing without affecting local VEGF synthesis. Taken together, these findings demonstrate that the proangiogenic effect by CO_2 bathing is a result of activation of NO-mediated signaling and that this activation results from the downstream effects of VEGF. Considering that VEGF-mediated mobilization of endothelial

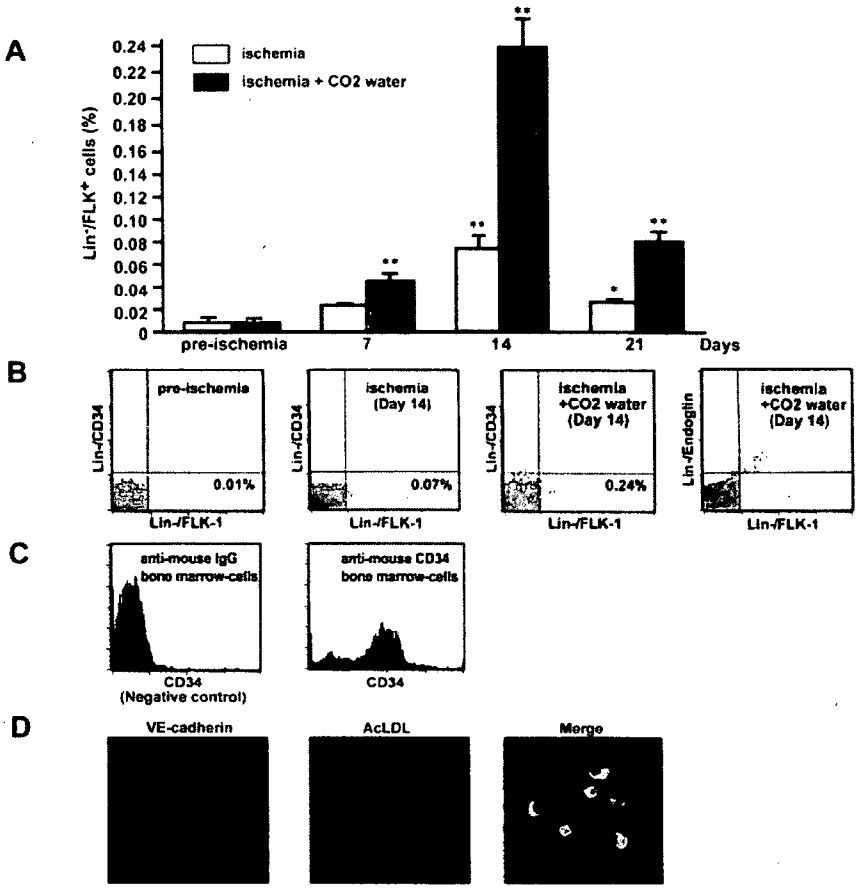

Figure 7. FACS analysis of circulating endothelial-lineage progenitor cells. A and B, Lin−/Flk+ cells in peripheral blood nuclear cells were isolated by PE-labeled lineage antigens, FITC-CD34 and biotin–Flk-1, and then analyzed. Lin−/Flk+/endoglin+ cells were isolated by FITC-labeled lineage antigens, PE-Flk-1 and biotin-endoglin. Cell number ratio of Lin−/Flk+ cells to total nuclear cells is shown (n=6 each). $^{*}P<0.05$, $^{**}P<0.001$ vs day 0 preischemic control. **C**, To prove specificity of anti-CD34 antibody, CD34+ cells in mouse bone marrow cells were enriched by biotin-labeled anti-mouse CD34 antibody and purified with streptavidin-magnet beads, followed by FACS analysis using streptavidin-PE. Rat anti-mouse IgG was used as a negative control. D, Lin−/Flk+ cells were cultured on fibronectin-coated plastic dishes in DMEM supplemented with 100 ng/mL VEGF and 10% FCS. After 4 days, DiI-labeled acetylated LDL was added into medium at 2 μg/mL for 6 hours, fixed with 4% paraformaldehyde, and stained with anti–VE-cadherin antibody and FITC-labeled anti-IgG antibody.

progenitor cells is NO-dependent,[25] our present study suggests that CO_2 bathing causes the induction of local VEGF synthesis, resulting in an NO-dependent neocapillary formation associated with mobilization of endothelial progenitor cells.

Hartman et al[19] reported that repeated CO_2-enriched water bathing increases arterial flow, transcutaneous oxygen tension, and pain-free walking distance in the clinical trial of peripheral arterial disease. Although they have not performed angiography, the enhancement of neovascularization may cause the increases in walking distance in addition to the improvement of blood vessel function. Toriyama et al[20] also showed that CO_2 foot bathing is clinically effective in the salvage of critical limb ischemia. In conclusion, our present study clearly demonstrates for the first time that CO_2-enriched water bathing causes the enhanced induction of local VEGF synthesis associated with activation of the NO-cGMP pathway and mobilization of endothelial progenitor cells, resulting in NO-dependent neocapillary formation that leads to an increase in collateral blood flow. Thus, these findings indicate that the CO_2-enriched water bathing therapy can be included in angiogenic therapies associated with neovascularization, such as the transplantation of bone marrow mononuclear cells[14] or VEGF gene therapy.[26]

Acknowledgments

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