

Tetrahydrobiopterin, L-Arginine and Vitamin C Act Synergistically to Decrease Oxidative Stress, Increase Nitric Oxide and Improve Blood Flow after Induction of Hindlimb Ischemia in the Rat

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Nitric oxide (NO) derived from endothelial nitric oxide synthase (eNOS) is a potent vasodilator and signaling molecule that plays an essential role in vascular remodeling of collateral arteries and perfusion recovery in response to hindlimb ischemia. In ischemic conditions, decreased NO bioavailability was observed because of increased oxidative stress, decreased L-arginine and tetrahydrobiopterin. This study tested the hypothesis that dietary cosupplementation with tetrahydrobiopterin (BH4), L-arginine, and vitamin C acts synergistically to decrease oxidative stress, increase nitric oxide and improve blood flow in response to acute hindlimb ischemia. Rats were fed normal chow, chow supplemented with BH4 or L-arginine (alone or in combination) or chow supplemented with BH4 + L-arginine + vitamin C for 1 wk before induction of unilateral hindlimb ischemia. Cosupplementation with BH4 + L-arginine resulted in greater eNOS expression, Ca^{2+} -dependent NOS activity and NO concentration in gastrocnemius from the ischemic hindlimb, as well as greater recovery of foot perfusion and more collateral artery enlargement than did rats receiving either agent separately. The addition of vitamin C to the BH4 + L-arginine regimen did further increase these dependent variables, although only the increase in eNOS expression reached statistical significances. In addition, rats given all three supplements demonstrated significantly less Ca^{2+} -independent activity, less nitrotyrosine accumulation, greater glutathione:glutathione disulfide (GSH:GSSG) ratio and less gastrocnemius muscle necrosis, on both macroscopic and microscopic levels. In conclusion, cosupplementation with BH4 + L-arginine + vitamin C significantly increased vascular perfusion after hindlimb ischemia by increasing eNOS activity and reducing oxidative stress and tissue necrosis. Oral cosupplementation of L-arginine, BH4 and vitamin C holds promise as a biological therapy to induce collateral artery enlargement.

Online address: <http://www.molmed.org>

doi: 10.2119/molmed.2011.00103

INTRODUCTION

Endothelial dysfunction is a hallmark of peripheral artery disease (PAD) (1). Central to the development of endothelial dysfunction, regardless of its cause, is a reduction in the bioavailability of nitric oxide (NO) derived from endothelial nitric oxide synthase (eNOS). Three fundamental mechanisms can compromise NO bioavailability: loss of eNOS expression,

loss of eNOS-derived NO production (that is, functional inactivation of eNOS) and inactivation of NO by a superoxide anion (O_2^-) to form peroxynitrite ($OONO^-$) (2,3). It is likely that all three mechanisms contribute to the endothelial dysfunction characteristic of PAD because increased oxidative stress is a common antecedent in the pathogenesis of this disease and can reduce NO bioavailability.

At least two characteristics of eNOS render it susceptible to oxidative stress. First, eNOS transcription, posttranslational modification and trafficking to the caveolae are attenuated by the accumulation of reactive oxygen species (ROS) within the endothelial cell (4). Second, the eNOS cofactor tetrahydrobiopterin (BH4) is highly susceptible to oxidation (5). BH4 maintains eNOS in its functional dimeric form; in the absence of BH4, eNOS becomes uncoupled so that the electron flux is diverted away from the L-arginine binding site and instead reduces molecular oxygen, generating O_2^- (6). This circumstance initiates a vicious cycle, wherein eNOS catalytic activity produces O_2^- , not NO, worsening existent oxidative stress.

These molecular characteristics of eNOS predict that several therapeutic options might prove effective for the

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Submitted March 22, 2011; Accepted for publication February 21, 2012; Epub www.molmed.org ahead of print February 24, 2012.

treatment of PAD, namely dietary supplementation with an antioxidant, or with the eNOS substrate L-arginine, or with the eNOS cofactor BH4. Vitamin C, or L-ascorbic acid, is a potent antioxidant and has been shown to preserve BH4 levels and enhance endothelial NO production *in vitro* (7). ONOO⁻ reacts with BH4 6–10 times faster when in the presence of ascorbate. The intermediate product of the reaction between ONOO⁻ and BH4 is the trihydrobiopterin radical (BH3^{*}), which is also reduced back to BH4 by ascorbate. Thus, ascorbate does not protect BH4 from oxidation but rather recycles the BH3 radical back to BH4 (8). Vitamin C levels are low in PAD patients (9), and acute (10) or short-term (11) vitamin C supplementation reduces PAD symptoms; however, cross-sectional epidemiological surveys have failed to find a clear link between long-term vitamin C intake and PAD symptoms or disease progression (12,13). L-arginine supplementation showed exciting promise in short-term studies of PAD (14,15), but this effect was not observed in a subsequent long-term study by the same group (16). BH4 improves eNOS-dependent vasodilation in long-term smokers and patients with type 2 diabetes, conditions associated with increased oxidative stress (17,18). To our knowledge, BH4 has not been specifically evaluated as a therapeutic modality in PAD.

An important gap in our understanding of BH4, L-arginine and L-ascorbic acid in the prevention and treatment of PAD is the potential synergistic effect of combined therapy, and there is convincing evidence to suggest that such an approach would prove successful. For example, supplementation with L-arginine alone might prove deleterious in the face of endothelial oxidative stress inasmuch as the resultant increase in eNOS catalytic activity might generate O₂⁻, not NO, if BH4 levels were reduced by oxidation. Thus, cosupplementation of L-arginine with L-ascorbic acid and BH4 might enhance the therapeutic outcome by reducing oxidant stress and preserving eNOS in its functional dimeric form,

respectively. This action would enhance eNOS-derived NO production and, by quenching existent O₂⁻, reduce NO inactivation by its reaction with O₂⁻.

In our previous work (19), we observed decreased eNOS expression, decreased bioavailable NO and increased oxidative stress in hindlimb ischemic rats, and oral supplementation of BH4 increased the beneficial effect of eNOS gene transfer. The goal of this study was to test the hypothesis that combined dietary supplementation with BH4, L-arginine and vitamin C will act synergistically to improve hindlimb perfusion recovery and preservation of tissue integrity in response to severe hindlimb ischemia. To this end, we generated severe hindlimb ischemia in the rat by means of femoral artery excision. Measured dependent variables included gastrocnemius muscle eNOS expression and activity, hindlimb laser Doppler perfusion and collateral artery enlargement, and gastrocnemius nitrotyrosine accumulation and tissue necrosis.

MATERIALS AND METHODS

Materials

Dietary supplements included the following: BH4 (10 mg/kg/d; Schircks Laboratories, Jonas, Switzerland); L-arginine, provided as L-arginine α -ketoglutarate (hereafter L-arginine; 88.5 mg/kg/d; Body Tech, North Bergen, NJ, USA); and L-ascorbic acid (that is, vitamin C; 88.5 mg/kg/d, Sigma-Aldrich, St. Louis, MO, USA). The dose of tetrahydrobiopterin was selected on the basis of a published report of its use in rats (35). And the dose of L-arginine and vitamin C was on the basis of their clinical dose in patients.

Animals

All protocols were approved by the Institutional Animal Care and Use Committee at the University of California, San Francisco, and University of Massachusetts Medical School. Adult male Sprague-Dawley rats weighing 265–285 g (Charles River Laboratories, Wilmington, MA, USA) were maintained in a clean housing facility on a 12-h light/dark cycle.

Preparation

Severe ischemia was induced in the left hindlimb. The femoral artery was ligated between the inguinal ligament and popliteal fossa, and the ligated section and its branches were excised. This procedure was carried out under anesthesia with 2% isoflurane. The untreated right hindlimb served as an internal control for each rat. An additional group of sham-operated rats were also used for selected assays. These rats underwent isolation of the femoral artery in the left hindlimb under 2% isoflurane anesthesia, but the artery was left intact.

Study Design

All rats were fed standard chow in powder form and water *ad libitum* (Deans Feeds, Redwood City, CA, USA). Animals were randomly selected to receive normal chow (control), chow with a single added supplement (BH4 or L-arginine), chow supplemented with BH4 + L-arginine or chow supplemented with BH4 + L-arginine + L-ascorbic acid. Dietary supplementation was commenced 7 d before the induction of hindlimb ischemia and was continued until sacrifice of the animal. Chow was replaced every 2 d. The time of sacrifice varied with the measured endpoint under consideration, as described below.

Western Blotting

Rats were sacrificed 14 d after induction of ischemia for measurement of gastrocnemius muscle eNOS, phosphorylated eNOS (p-eNOS) and nitrotyrosine expression. The timing of death was selected on the basis of previous work that demonstrated maximal postischemic change in these variables at this time (19). Samples were homogenized in liquid nitrogen and transferred to NP-40 lysis buffer, comprised of 50 mmol/L HEPES (pH 7.5), 150 mmol/L NaCl, 10% glycerol, 1.5 mmol/L MgCl₂, 1 mmol/L ethylenediaminetetraacetic acid (EDTA), 100 mmol/L NaF, 1% NP 40, 1 mmol/L phenylmethylsulfonyl fluoride (PMSF) and 1 g/mL aprotinin. The lysates were centrifuged, the supernatant recovered and the protein

concentration determined (Pierce Biotechnology, Rockford, IL, USA). A total of 100 μg protein per sample was separated on a 7.5% or 12% sodium dodecyl sulfate–polyacrylamide gel for determination of eNOS or nitrotyrosine, respectively, and then electroblotted on nitrocellulose membranes (Bio-Rad, Hercules, CA, USA). Membranes were incubated overnight at 4°C with mouse monoclonal anti-nitrotyrosine (Cayman Chemical, Ann Arbor, MI, USA; 1:1,000), mouse monoclonal anti-eNOS (BD Transduction Laboratories, San Diego, CA, USA; 1:1,000) or rabbit anti-p-eNOS (cell signaling) and then incubated for 2 h with horseradish peroxidase–conjugated anti-mouse or rabbit IgG antibody (Pierce Biotechnology; 1:5,000). Immunoreactive bands were visualized using the enhanced chemiluminescence system (Amersham, Arlington Heights, IL, USA). Band density was quantitated by standard densitometry, and the intensity of the band of interest was expressed as a function of the α -tubulin band.

eNOS Activity

Rats were sacrificed 14 d after induction of ischemia for determination of gastrocnemius muscle NOS activity. The timing of sacrifice was selected on the basis of previous work that demonstrated maximal postischemic change in this variable at this time (19). Muscles were homogenized in ice-cold buffer (250 mmol/L Tris-HCl, pH 7.4, 10 mmol/L EDTA, 10 mmol/L EGTA [ethylene glycol-bis(β -aminoethyl ether)- N,N,N',N' -tetraacetic acid]) and centrifuged, and the protein in the supernatant was adjusted to 5 $\mu\text{g}/\text{mL}$. Samples were incubated in 10 mmol/L NADPH, 1 $\mu\text{Ci}/\mu\text{L}$ ^{14}C -Arg, 6 mmol/L CaCl_2 , 50 mmol/L Tris-HCl (pH 7.4), 6 $\mu\text{mol}/\text{L}$ BH4, 2 $\mu\text{mol}/\text{L}$ flavin adenine dinucleotide (FAD) and 2 $\mu\text{mol}/\text{L}$ flavin mononucleotide (FMN) for 30 min at 37°C. The reaction was stopped with 400 μL of 50 mmol/L HEPES, pH 5.5, and 5 mmol/L EDTA. Identical samples were prepared without CaCl_2 , and all reactions were performed in duplicate. The radioactivity of the sample eluate was measured

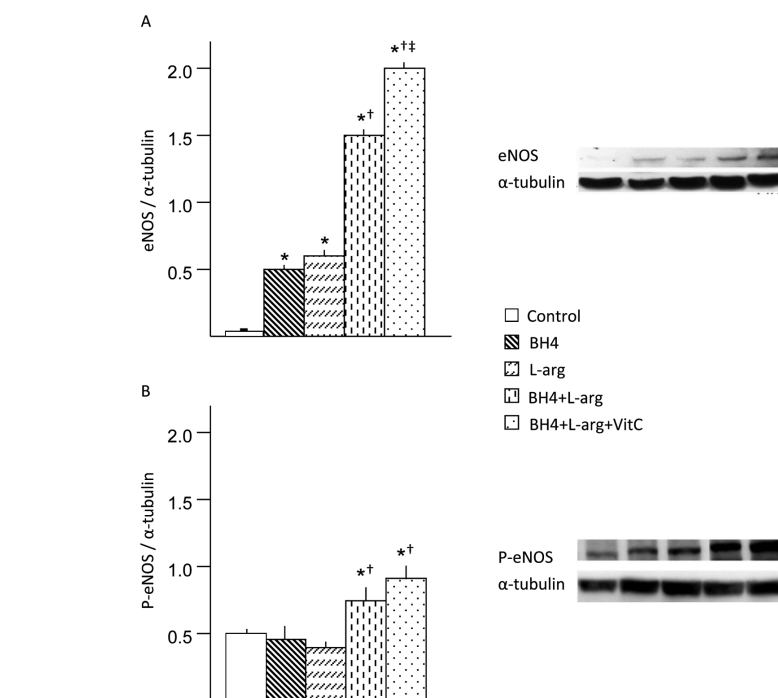


Figure 1. Effects of BH4, L-arginine (L-arg) and vitamin C (VitC) on eNOS and phosphorylated eNOS expression in the ischemic gastrocnemius muscle. Muscle was harvested 14 d after the induction of hindlimb ischemia, and supernatants from muscle homogenates were used in these assays. (A) Expression of eNOS was increased by all dietary additives, although the greatest increase was noted in rats that received BH4 + L-arginine + vitamin C. (B) Expression of p-eNOS was increased in rats fed BH4 + L-arginine or BH4 + L-arginine + vitamin C. Control rats were fed a standard diet, whereas the four treatment groups consumed diets supplemented with additives noted in the bar graph. Concentrations of these additives are noted in the text. Data are mean \pm standard deviation (sd); $n = 5$ –6. * $p < 0.05$ versus control; † $p < 0.05$ versus BH4 or L-arginine groups; ‡ $p < 0.05$ versus BH4 + L-arginine group.

and expressed as carboxypeptidase M (CPM)/ μg protein. The Ca^{2+} -dependent NOS activity, which corresponds to the sum of the endothelial and neural NOS isoforms, was calculated by subtracting the NOS activity measured in the absence of CaCl_2 from the NOS activity measured in the presence of CaCl_2 . The Ca^{2+} -independent NOS activity corresponds to inflammatory isoform of NOS, or iNOS.

NOx (Nitrite + Nitrate) Assay

The left gastrocnemius muscles after ischemic d 14 were collected and homogenized in phosphate-buffered saline (PBS). After centrifugation, ultrafilter tissue homogenates were passed through a 10-kDa molecular weight cutoff filter. Filtrate was used for nitrite and nitrate con-

centration measurement according to the protocol of the Nitrate/Nitrite fluorometric assay kit (Cayman Chemical).

Assay of GSH:GSSG Ratio

The left gastrocnemius muscles were homogenized and deproteinated. And then the supernatant was used for the measurement of glutathione disulfide (GSSG) and glutathione (GSH) according to the Glutathione assay kit (Cayman Chemical).

Hindlimb Perfusion

Hindlimb blood flow was determined by means of laser Doppler imaging (Moor Instruments, Devon, UK). Flow was measured preoperatively, immediately after arterial excision, and then 3, 7, 14, 21, 28, 35 and 42 d after induction of ischemia.

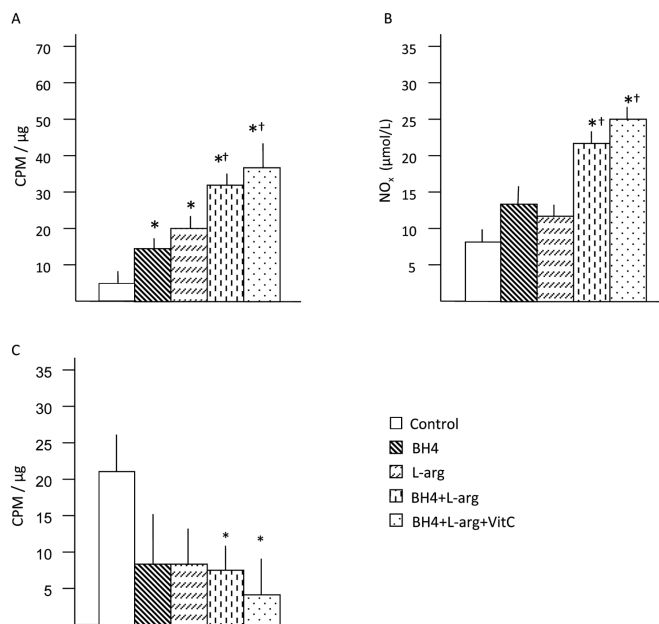


Figure 2. Effects of BH4, L-arginine (L-arg) and vitamin C (VitC) on NOS activity in the ischemic gastrocnemius muscle. Muscle was harvested 14 d after the induction of hindlimb ischemia, and supernatants from muscle homogenates were used in these assays. (A) Ca²⁺-dependent NOS activity was increased by all dietary additives, although the greatest increase was noted in mice receiving BH4 + L-arginine or BH4 + L-arginine + vitamin C. Data are mean \pm sd; n = 5–6. * p < 0.05 versus control; † p < 0.05 versus BH4 or L-arginine groups; ‡ p < 0.05 versus BH4 + L-arginine group. (B) NO_x (nitrite + nitrate) was increased in mice receiving BH4 + L-arginine or BH4 + L-arginine + vitamin C. Data are mean \pm sd; n = 5. * p < 0.05 versus control; † p < 0.05 versus BH4 or L-arginine groups. (C) Ca²⁺-independent NOS activity was less in mice receiving BH4 + L-arginine or BH4 + L-arginine + vitamin C than in control mice. Data are mean \pm sd; n = 5–6. * p < 0.05 versus control. In all graphs, control rats were fed normal chow and underwent hindlimb ischemia, whereas the four treatment groups consumed diets supplemented with additives noted in the bar graph. Concentrations of these additives are noted in the text.

Scans were obtained during inhalation of 1% isoflurane while core body temperature was maintained between 36.8 and 37.2°C. Scans were repeated three times, and the average for each rat was determined. Data were expressed as the ratio of ischemic to non-ischemic hindlimb.

Angiograms

Angiograms were performed 42 d after induction of ischemia. Barium sulfate (2.5 mL; EZPaq, Merry X-Ray, South San Francisco, CA, USA) was infused into the infrarenal aorta after ligation of the proximal aorta and inferior vena cava during inhalation of 2% isoflurane. A grid was superimposed over the film between the greater trochanter of the femur to the

patella. The number of intersections between contrast-filled vessels and gridlines was determined independently by three blinded observers. The angioscore was calculated as the average ratio of intersections to the total number of gridlines. Within the experimental setting of this study, the angioscore is a marker of collateral artery enlargement; thus, as collateral arteries dilate and remodel in response to femoral artery excision, their diameters increase, enhancing their visibility on the X-ray film, thus increasing the angioscore (19).

Nitroblue Tetrazolium Staining to Detect Muscle Necrosis

The left gastrocnemius muscle was removed 7 d after induction of ischemia.

The timing of sacrifice was selected on the basis of previous work that demonstrated maximal postischemic muscle necrosis at this time (19). The muscle was cut transversely into three 2-mm sections. Two sections were used for nitroblue tetrazolium (NBT) staining, while the third was frozen (–80°C) in optimal cutting temperature (OCT) embedding compound. Sections for NBT staining were incubated in PBS containing 0.033% NBT (Fisher Biotech, Austin, TX, USA) and 0.133% NADH (Roche Diagnostics, Indianapolis, IN, USA) at 21°C for 10 min. The samples were then fixed in 4% paraformaldehyde for 24 h. The areas of viable tissue, indicated by dark blue color, and nonviable tissue, indicated by white color, were measured by quantitative image analysis (ImagePro; Media Cybernetics, Bethesda, MD, USA). Data were expressed as the ratio of nonviable tissue to total tissue area. Measurements were made on the four exposed cut surfaces, and the average was taken and used as a single data point for each animal. The frozen section was used to prepare cryosections (10 μ m) for hematoxylin and eosin (H&E) staining to evaluate histological integrity of the muscle, as well as to detect the presence of an inflammatory infiltrate.

STATISTICAL ANALYSIS

Analyses were carried out by means of analysis of variance (ANOVA). *Post hoc* Student-Newman-Keuls tests were carried out if the ANOVA *F* statistic was significant to determine sites of difference within the ANOVA format. Probability values less <0.05 were accepted as significant for all statistical calculations.

RESULTS

Effects of Oral BH4, L-Arginine, and Vitamin C on Gastrocnemius eNOS and p-eNOS Expression

Dietary supplementation of BH4, L-arginine and vitamin C significantly affected eNOS and p-eNOS expression in the ischemic gastrocnemius muscle (Figure 1). Rats given single supplementation

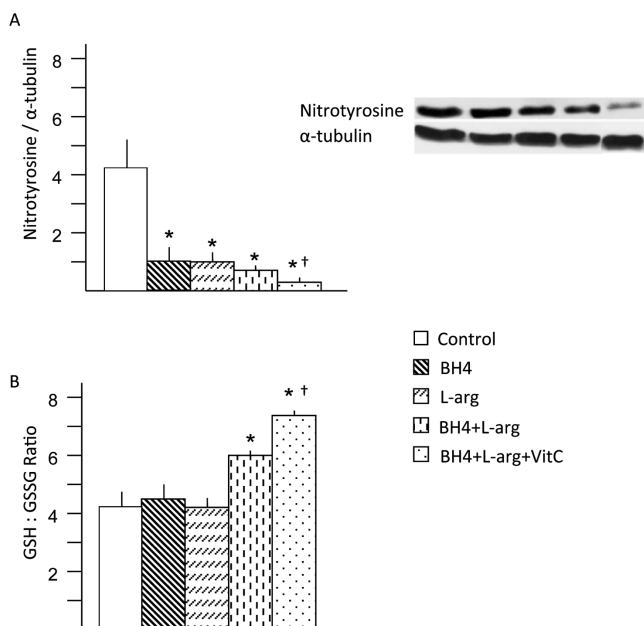


Figure 3. Effects of BH4, L-arginine (L-arg) and vitamin C (VitC) on oxidative stress in the ischemic gastrocnemius muscle. Muscle was harvested 14 d after the induction of hindlimb ischemia, and supernatants from muscle homogenates were used in these assays. (A) Nitrotyrosine expression was decreased by all dietary additives, although this decrease was greatest in rats receiving BH4 + L-arginine + vitamin C. (B) The GSH:GSSG ratio was increased in rats receiving BH4 + L-arginine; the addition of vitamin C to this regimen caused an additional increase in this ratio. Data are mean \pm sd; n = 5–6. **p* < 0.05 versus control, BH4 or L-arginine groups; †*p* < 0.05 versus BH4 + L-arginine groups.

with BH4 or L-arginine demonstrated similar levels of eNOS expression, and these levels were significantly greater than that of rats fed normal chow. Rats given two (BH4 + L-arginine) or three (BH4 + L-arginine + vitamin C) supplements also had greater levels of eNOS expression than rats given single supplements. Hence, the combination of BH4 and L-arginine had an additive effect on eNOS expression, and the addition of vitamin C provided an additional beneficial effect. However, increased p-eNOS expression was only observed in BH4 + L-arginine- and BH4 + L-arginine + vitamin C-fed rats.

Effects of Oral BH4, L-Arginine, and Vitamin C on Gastrocnemius Ca²⁺-Dependent NOS Activity

Dietary supplements affected Ca²⁺-dependent NOS activity in the ischemic gastrocnemius muscle (Figure 2A). Rats

given a single dietary supplement (BH4 or L-arginine) demonstrated Ca²⁺-dependent NOS activities that were similar to each other and greater than that of rats fed normal chow. Rats given two (BH4 + L-arginine) or three (BH4 + L-arginine + vitamin C) supplements displayed significantly greater Ca²⁺-dependent NOS activity than rats given a single dietary supplement. The combination of BH4 and L-arginine generated an additive effect. The addition of vitamin C further increased Ca²⁺-dependent NOS activity, although it did not reach statistical significance.

Effects of Oral BH4, L-Arginine and Vitamin C on Gastrocnemius NOx Levels

The final products of NO *in vivo* are nitrite (NO₂⁻) and nitrate (NO₃⁻). NOx is the sum of NO₂⁻ and NO₃⁻ and it is the best index of total NO production. The

NOx concentration in ischemic gastrocnemius was significantly increased from rats fed BH4 and L-arginine or BH4, L-arginine and vitamin C (Figure 2B). There were no changes in rats fed with either BH4 and L-arginine alone.

Effects of Oral BH4, L-Arginine and Vitamin C on Gastrocnemius Ca²⁺-Independent NOS Activity

Ca²⁺-independent NOS activity was greater in the ischemic gastrocnemius from rats fed normal chow than in the ischemic gastrocnemius of dietary fed rats (Figure 2C). Rats fed a single supplement (BH4 or L-arginine), or the combination of these two agents, demonstrated Ca²⁺-independent NOS activity statistically similar to the normal chow group (that is, there was no beneficial effect noted in these dietary intervention groups). However, rats provided with three dietary supplements (BH4 + L-arginine + vitamin C) demonstrated Ca²⁺-independent NOS activity levels in the ischemic gastrocnemius muscle that were lower than the other dietary intervention groups.

Effects of Oral BH4, L-Arginine and Vitamin C on Gastrocnemius Oxidative Stress

Dietary supplementation affected nitrotyrosine accumulation and the ratio of GSH versus GSSG in the ischemic gastrocnemius muscle (Figure 3). Rats given a single dietary supplement (BH4 or L-arginine), or the combination of these dietary agents, displayed similar nitrotyrosine levels. These levels were significantly less than the level noted in rats fed normal chow. Rats provided with all three dietary supplements (BH4 + L-arginine + vitamin C) exhibited a nitrotyrosine level significantly less than rats given a single supplement (BH4 or L-arginine) or the combination of these two agents. The ratio of reduced versus oxidized glutathione (GSH:GSSG) was also measured as another index of oxidative stress in the ischemic gastrocnemius muscle. GSH:GSSG ratio was increased in the ischemic gastrocnemius of

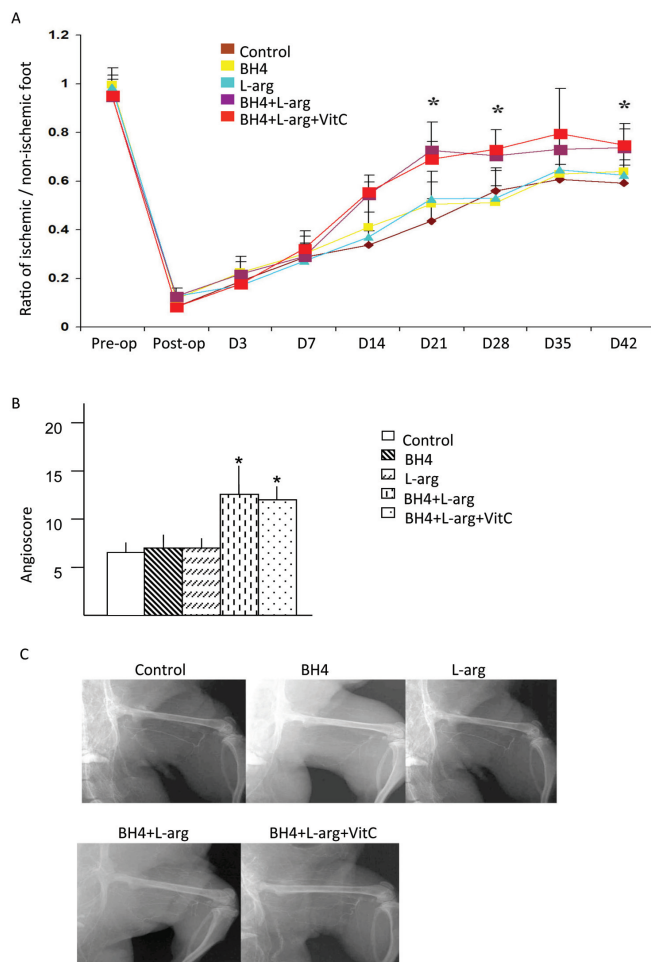


Figure 4. Effects of BH4, L-arginine and vitamin C on perfusion recovery after induction of hindlimb ischemia. (A) Laser Doppler perfusion imaging (LDPI) data, expressed as the ratio of blood flow from the ischemic to nonischemic hindlimbs, was determined before, immediately after and then serially over the ensuing 6 wks. Group identity is shown in the color key. Data are mean \pm sd; $n = 6$. * $p < 0.05$ for BH4 + L-arginine or BH4 + L-arginine + vitamin C groups versus all other groups. (B) The angioscore, determined 42 d after the induction of hindlimb ischemia and calculated as described in the text, was greater in rats receiving BH4 + L-arginine or BH4 + L-arginine + vitamin C than in all other groups. Data are mean \pm sd; $n = 6$. * $p < 0.05$ for BH4 + L-arginine or BH4 + L-arginine + vitamin C groups versus all other groups. (C) Representative angiogram from each study group.

rats fed the two dietary combination (BH4 + L-arginine) or the three dietary combination (BH4 + L-arginine + vitamin C). Addition of vitamin C had a beneficial effect in increasing GSH:GSSG ratio. Taken together, three dietary supplementation (BH4 + L-arginine + vitamin C) would be much more effective in decreasing ischemic muscles oxidative stress after induction of hindlimb ischemia in rats.

Effects of BH4, L-Arginine and Vitamin C on Hindlimb Perfusion

Dietary supplementation significantly increased the recovery of hindlimb perfusion after induction of severe ischemia, and this effect was regimen and time dependent (Figure 4A). Rats given a single supplement (BH4 or L-arginine) demonstrated similar degree of perfusion recovery in the foot, and this level was also similar to that noted in rats fed normal

chow. Rats provided with two (BH4 + L-arginine) or three (BH4 + L-arginine + vitamin C) supplements showed significantly greater recovery of foot perfusion than rats fed normal chow or rats given a single dietary supplement. This difference was evident at the later phase of recovery, on d 21, 28 or 42 after induction of ischemia, which was also the time of maximal collateral artery wall remodeling (20). A similar pattern was noted for collateral artery angioscores determined on d 42 after induction of ischemia. Hence, the angioscore was significantly greater in rats given two (BH4 + L-arginine) or three (BH4 + L-arginine + vitamin C) supplements than in rats fed normal chow or in rats given a single supplement (Figures 4B, C).

Effects of Oral BH4, L-Arginine and Vitamin C on Gastrocnemius Muscle Necrosis

The extent of gastrocnemius necrosis was affected by the provision of dietary supplements. Rats given a single supplement (BH4 or L-arginine) or the combination of these agents manifest a similar degree of gastrocnemius muscle necrosis. Moreover, the extent of necrosis noted in these dietary intervention groups was similar to that noted in rats fed normal chow (that is, these dietary regimens did not improve postischemic muscle integrity). However, rats provided with all three dietary supplements (BH4 + L-arginine + vitamin C) had significantly less gastrocnemius necrosis than rats fed normal chow, rats provided with a single dietary supplement or rats given the combination of BH4 + L-arginine. This difference was evident on macroscopic and microscopic levels. The percentage of the cut surface of the ischemic gastrocnemius muscle that was necrotic, determined by NBT staining, was significantly less in the BH4 + L-arginine + vitamin C group than in all other groups (Figures 5A, B). Groups fed normal chow, or supplemented with BH4, L-arginine, or both agents, demonstrated similar histological evidence of severe necrosis: muscle nuclei were nearly absent, intra-

myofiber vacuolization was substantial and the distance between myofibers was large (Figure 5C). A pronounced inflammatory infiltrate was also present in these groups. In contrast, the BH4 + L-arginine + vitamin C group demonstrated good preservation of muscle histology and only a limited inflammatory cell infiltrate.

DISCUSSION

The following study hypothesis was supported by our findings: dietary co-supplementation with tetrahydrobiopterin (BH4), L-arginine and vitamin C acts synergistically to decrease oxidative stress, increase nitric oxide and thereby improve perfusion and tissue recovery in response to acute hindlimb ischemia more than supplementation with a single supplement. Interestingly, two patterns of effect emerged. Cosupplementation with BH4 + L-arginine increased the dependent variables eNOS and p-eNOS expression, Ca²⁺-dependent NOS activity, foot perfusion and the collateral artery angioscore more than the addition of either component separately, whereas the addition of vitamin C provided a further beneficial effect on these variables, although only eNOS expression reached statistical significance. In addition, co-administration of all three dietary supplements had a significantly greater effect than BH4 or L-arginine, given individually or in combination, when the dependent variables of Ca²⁺-independent NOS activity, oxidative stress or muscle necrosis were measured.

eNOS and p-eNOS expression, Ca²⁺-dependent NOS activity, foot perfusion and the collateral artery angioscore are linked by established cause-and-effect relationships. eNOS-derived NO is a potent vasodilator (2); hence, the increased eNOS expression and activity present in the BH4 + L-arginine group should result in an NO-dependent increase in foot perfusion, and this expectation was realized by our findings. Moreover, eNOS-derived NO is a critical determinant in the response to hindlimb ischemia (21–23). This effect is direct, on the basis

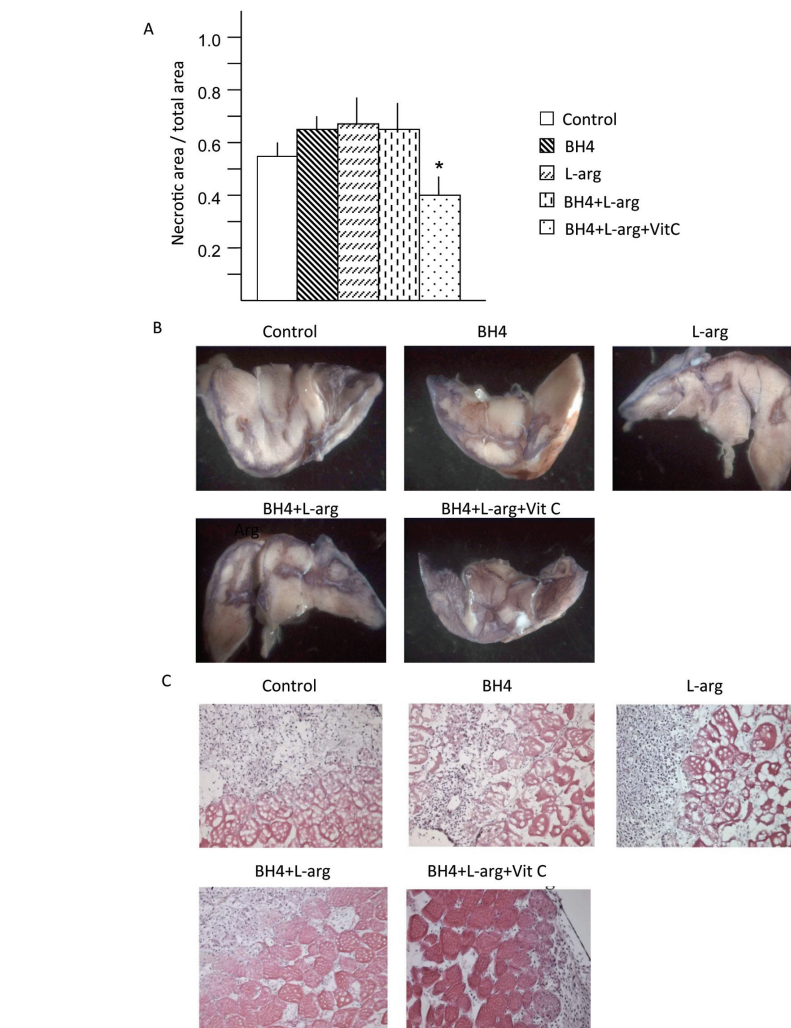


Figure 5. The effects of BH4, L-arginine (L-arg) and vitamin C (VitC) on necrosis in the ischemic gastrocnemius muscle. (A) Gastrocnemius muscle was removed 7 d after induction of hindlimb ischemia. Transverse sections of this muscle were stained with nitroblue tetrazolium to determine the ratio of necrotic versus viable surface area. This ratio was less in rats receiving BH4 + L-arginine + vitamin C than in all other groups. Data are mean ± sd; n = 6. *p < 0.05 for BH4 + L-arginine + vitamin C groups versus all other groups. (B) Representative images from each study group. (C) Histological sections of gastrocnemius muscle taken from the ischemic hindlimb 7 d after the induction of hindlimb ischemia were stained with H&E. Representative sections are shown. Note the loss of myofibers and the intense cellular infiltrate in the control, BH4 and L-arginine groups. Best preservation of muscle architecture was consistently observed in rats receiving BH4 + L-arginine + vitamin C.

of the vasodilator effect of NO (24), but also indirect, insofar as eNOS-derived NO is critical to collateral artery remodeling. These effects include mobilization of endothelial progenitor cells from bone marrow and their subsequent homing to the ischemic hindlimb (25). Once there, endothelial progenitor cells participate in

postischemic arteriogenesis, the process wherein existing collateral arteries undergo remodeling designed to restore vascular conductance (26). This process was evidenced by the increased angioscore, a marker of collateral artery enlargement, in rats provided with L-arginine + BH4 dietary supplements.

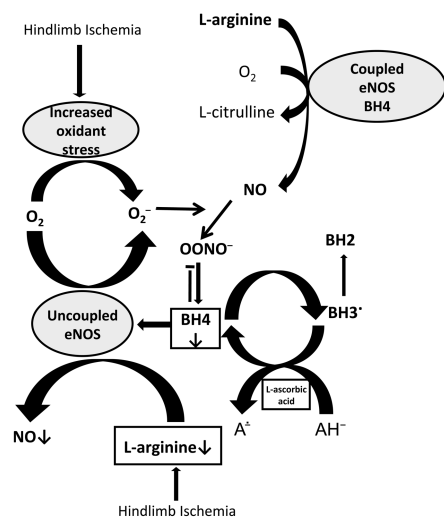


Figure 6. Schematic illustration of possible mechanism of three dietary combined regimen can increase NO bioavailability and decrease oxidative stress, accordingly increase blood flow recovery and reduce tissue necrosis. A[•], ascorbate; AH⁻, dehydroascorbate.

Vitamin C likely exerted its beneficial effects in this study through a variety of molecular mechanisms. In its capacity as an antioxidant, it enhances NO bioavailability by quenching O_2^- , thus limiting the inactivation of NO that occurs when O_2^- and NO combine to produce $OONO^-$ (3). Vitamin C also stabilizes existing BH4 (8) and increases endothelial BH4 synthesis (27), thus minimizing eNOS “uncoupling,” which in turn lessens generation of O_2^- by eNOS and reduces vascular oxidative stress (7). However, BH4 is itself a potent antioxidant (7), and administration of exogenous BH4 has been established to increase endothelial BH4 levels (28). Moreover, L-arginine directly stimulates eNOS expression (29), enhances eNOS activity by a receptor-dependent G protein-linked process (30) and limits the inhibitory effect of asymmetric dimethylarginine on eNOS-derived NO production (31). We propose that under the experimental conditions imposed by hindlimb ischemia, addition of vitamin C to BH4 + L-arginine significantly decreased oxidative stress and accordingly decreased tissue necrosis. It

may also restore BH4 or NO levels, since we observed an increase in eNOS activity and p-eNOS expression, although it did not reach statistical difference because of the number of samples analyzed, dosage of vitamin C or time of data collection. Cosupplementation with BH4 + L-arginine may have sufficiently restored endothelial BH4 levels and thereby intracellular redox balance. Hence, the addition of vitamin C did not further p-eNOS expression or activity, and hence NO bioavailability.

Ca^{2+} -independent NOS activity and tissue nitrotyrosine accumulation were significantly lower in rats receiving all three dietary supplements than in rats receiving BH4 or L-arginine, or the combination of the two. When measured by methods used herein, Ca^{2+} -independent NOS activity is an authentic reflection of iNOS activity, inasmuch as the assay was conducted *in vitro*, in the absence of shear stress, which can activate eNOS in the absence of Ca^{2+} via phosphorylation (32). The marked elevation of iNOS activity in rats fed normal chow indicates the presence of postischemia inflammation, which is also evidenced by the cellular inflammatory infiltrate in this group. Nitrotyrosine accumulation is indicative of $OONO^-$ -induced damage (33), and it is interesting that the group that exhibited the least amount of tissue damage (rats provided with all three supplements) also had the least nitrotyrosine accumulation. Moreover, rats in the triple therapy group demonstrated a virtual absence of nitrotyrosine. We interpret the present findings to indicate that muscle injury after ischemia is not entirely contingent upon loss of perfusion, inasmuch as cosupplementation with the antioxidant vitamin C clearly reduced tissue injury and eliminated nitrotyrosine accumulation, but did not affect perfusion or collateral artery enlargement. Instead, we speculate that vitamin C provided an antioxidant effect that limited tissue injury generated by inflammatory cells for which action depends in part on oxidant production (for example, neutrophils and macrophages). This effect could be direct, due to the antioxidant ac-

tivity of vitamin C, or indirect, due to the beneficial effect of vitamin C on BH4 levels (8,27), insofar as BH4 also exhibits potent antioxidant activity (7).

Although it is well established that endothelial dysfunction related to vascular oxidative stress is a critical factor in PAD pathogenesis, dietary supplementation with L-arginine or antioxidants, such as vitamin C, have had equivocal effects on long-term outcome (12,13,16). Dietary supplementation with L-arginine alone has a beneficial effect when given acutely (that is, via intravenous infusion [14] or for short duration [2 months] [15]), and these clinical results are consistent with the positive effects observed in rats provided with dietary L-arginine. However, long-term administration of L-arginine (6 months) not only failed to demonstrate a beneficial effect, but resulted in a degree of eNOS-dependent vascular reactivity significantly less than that of the placebo group (16). The present findings demonstrated increased iNOS activity after induction of ischemia. If a similar circumstance is present in PAD, then the singular dietary supplementation with L-arginine, the substrate for all NOS isoforms, might serve to worsen vascular inflammation, a critical participant in the pathogenesis of PAD (1). Vitamin C reduces vascular inflammation (34), improves redox balance (11) and eNOS-dependent vascular reactivity (10), but these effects have only been evaluated on a short-term basis, whereas retrospective cross-sectional studies have failed to confirm that dietary supplementation with antioxidants improves PAD outcome (12,13). We interpret the present findings to indicate that provision of BH4 + L-arginine + vitamin C acting synergistically might prove a useful therapeutic alternative in PAD treatment. To this end, the use of sapropterin dihydrochloride, a synthetic form of (6R)-L-erythro-5,6,7,8-tetrahydrobiopterin recently approved for the treatment of phenylalanine hydroxylase deficiency (35), might provide a practical means for the provision of BH4.

In conclusion, cosupplementation with BH4 + L-arginine + vitamin C resulted in

increased eNOS activity and NO concentration as well as greater foot blood flow recovery than rats receiving normal chow or either agent separately. The addition of vitamin C to the BH4 + L-arginine regimen further reduced oxidative stress and tissue injury in ischemic muscles (Figure 6). The clearly superior outcome of rats provided with BH4 + L-arginine + vitamin C warrants investigation of a cosupplementation strategy as a therapeutic alternative in PAD.

ACKNOWLEDGMENTS

This work was supported by National Institutes of Health, National Heart, Lung, and Blood Institute grant RO-1 HL-75353 (to LM Messina), as well as grants from the Pacific Vascular Research Foundation and the Wayne and Gladys Valley Foundation (to LM Messina).

DISCLOSURE

The authors declare that they have no competing interests as defined by *Molecular Medicine*, or other interests that might be perceived to influence the results and discussion reported in this paper.

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