

Interaction of 5-methyltetrahydrofolate and tetrahydrobiopterin on endothelial function

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Interaction of 5-methyltetrahydrofolate and tetrahydrobiopterin on endothelial function. *Am J Physiol Heart Circ Physiol* 282: H2167–H2172, 2002. First published February 28, 2002; 10.1152/ajpheart.00935.2001.—The present study was designed to investigate the interaction between 5-methyltetrahydrofolate and tetrahydrobiopterin in modulating endothelial function. Tetrahydrobiopterin is a critical cofactor for nitric oxide synthase and maintains this enzyme as a nitric oxide- versus superoxide-producing enzyme. The structure of 5-methyltetrahydrofolate is similar to tetrahydrobiopterin and both agents have been shown to improve endothelium-dependent vasodilatation. We hypothesized that 5-methyltetrahydrofolate interacts with nitric oxide synthase in a fashion analogous, yet independent, of tetrahydrobiopterin to improve endothelial function. We demonstrate that 5-methyltetrahydrofolate binds the active site of nitric oxide synthase and mimics the orientation of tetrahydrobiopterin. Furthermore, 5-methyltetrahydrofolate attenuates superoxide production (induced by inhibition of tetrahydrobiopterin synthesis) and improves endothelial function in aortae isolated from tetrahydrobiopterin-deficient rats. We suggest that 5-methyltetrahydrofolate directly interacts with nitric oxide synthase to promote nitric oxide (vs. superoxide) production and improve endothelial function. 5-Methyltetrahydrofolate may represent an important strategy for intervention aimed at improving tetrahydrobiopterin bioavailability.

nitric oxide; folic acid

NITRIC OXIDE (NO) can be produced by three homodimeric isozymes, termed NO synthases (NOS), including endothelial NOS (eNOS), neuronal NOS, and inducible NOS. Under normal conditions, eNOS efficiently catalyzes electron transfer from nicotinamide adenine dinucleotide phosphate (reduced)-flavin adenine dinucleotide-flavin mononucleotide (NADPH-FAD-FMN) to L-arginine generating citrulline and NO. This cascade of electron transfer to L-arginine is dependent on the presence of tetrahydrobiopterin (BH₄) bound within the active site. Conversely, eNOS devoid of BH₄ leads to the uncoupling of electron transfer

to L-arginine and facilitates the production of superoxide (4, 5, 28). Recent reports (10, 17, 22, 24, 25) indicate that supplementation with BH₄ improves endothelium-dependent vasodilatation in experimental diabetes, models of ischemia and reperfusion injury, hypercholesterolemia, and cigarette smoking.

The structure of 5-methyltetrahydrofolate (5-MTHF) is extremely similar to that of BH₄ with the exception of an extended tail attached to 5-MTHF. It has been proposed that the large size of 5-MTHF would exclude it from interacting with the active site of NOS (in a fashion similar to BH₄). However, clinical data (22, 27) contradicts this argument because direct infusion of 5-MTHF into the brachial artery of patients with hyperlipidemia mimics the effects of BH₄ on endothelial function. Stroes et al. (23) recently demonstrated that 5-MTHF augmented acetylcholine (ACh)-stimulated NO release in cultured endothelial cells. The authors further concluded that 5-MTHF was not acting as an antioxidant but directly interacting with eNOS.

The present study was designed to investigate the interaction among eNOS, BH₄, and 5-MTHF. Specifically, we examined 1) the effects of 5-MTHF on BH₄-inhibited superoxide and NO production, 2) the ability of 5-MTHF to directly interact with eNOS assessed by molecular computer modeling, and 3) the effect of 5-MTHF and BH₄ on endothelial function in a BH₄-deficient animal model. We herein report that 5-MTHF may interact with the active site of eNOS in a fashion analogous to BH₄. In addition, 5-MTHF restores BH₄-inhibited superoxide production. Finally, 5-MTHF exerts beneficial effects on endothelial function in a BH₄ deficient animal model to a similar degree as BH₄.

METHODS

Superoxide production. Superoxide produced by bovine recombinant eNOS (isolated from baculovirus overexpression in Sf9 cells) (Cayman Chemical; Ann Arbor, MI) was measured with 250 μmol bis-N-methylacridinium (lucigenin) chemiluminescence. Recombinant eNOS (7.5 μg) was incubated with 10 μM FAD, 1 mM CaCl₂, 1 mM NADPH, 300

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U/ml calmodulin, and either 100 or 500 μM of 5-MTHF or BH_4 . Total photons produced by the reaction between superoxide and lucigenin were measured in a lumometer for 300 s. All chemicals were prepared immediately before use. eNOS was purchased from Cayman Chemical; all other reagents were obtained from Sigma (St. Louis, MO).

Immunohistochemistry. Immortalized and bovine endothelial cells were both stained positively for eNOS protein. Cells were grown on German glass coverslips and fixed with 10% formalin for 15 min. Slides were rinsed with 100 μM Tris buffer (pH 7.6, 0.1% Triton X-100) and incubated with 1:250 primary polyclonal IgG rabbit anti-NOS antibodies in Tris buffer containing 0.005% bovine serum albumin for 24 h at 4°C ($n = 20$; Santa Cruz Biotechnology, Santa Cruz, CA). Slides were then rinsed and incubated with anti-rabbit IgG cyanogated conjugated in the aforementioned Tris buffer for 2 h at 4°C. Fluorescence was measured by an epifluorescence-equipped microscope.

Measurement of reactive oxygen species in cultured endothelial cells. Bovine aortic endothelial cells were isolated from aorta with 1 mg/ml collagenase in phosphate-buffered saline (PBS) and cultured in medium 199 containing 10% fetal calf serum (FCS) and 200 mM glutamine. Cells were then incubated in 5% FCS and treated for 24 h. All experiments were performed in cells between passages 2 and 6. After reaching confluence, the cells were loaded with 2 μM of the fluorescent probe dihydrorhodamine 123 for 30 min in FCS-free medium 199 and then stimulated with 10 μM ACh for an additional 30 min. Cells were rinsed, trypsinized, and resuspended in Hanks' balanced salt solution buffer (pH 7.2) and 5% FCS, and then centrifuged and resuspended in Hanks' buffer without FCS. Dihydrorhodamine 123 is irreversibly converted to the green fluorescent membrane impermeable compound rhodamine 123. Fluorescence-activated cell sorter procedure was carried out with a flow cytometer equipped with an ion argon laser emitting at 488 nm. Emission spectra were collected above 540 nm on 10,000 cells per sample in three separate experiments.

Measurement of superoxide production in cultured endothelial cells. Superoxide production was assessed using flow cytometry by comparing the mean dihydroethidine (DHE)-induced fluorescence of immortalized human EA.Hy926 endothelial line. Cells were cultured as previously mentioned and then incubated in serum-free medium 199 and 25 μM DHE and stimulated for another 30 min in 10 μM ACh. DHE reacts specifically with superoxide and is converted to ethidium. Cells were rinsed, trypsinized, resuspended in PBS buffer (pH 7.2) and 5% FCS, and then centrifuged and resuspended in PBS without FCS.

Computer modeling. Autodock version 3.0 was used to dock BH_4 and 5-MTHF. Partial charges for each atom were assigned to the ligand and enzyme with the use of SYBYL version 6.7 (Tripos; St. Louis, MO). Cubic affinity grid maps centered on the pterin site of bovine eNOS (dimensions $21 \times 21 \times 21 \text{ \AA}$, grid points spacing 0.35 \AA) were calculated for each pterin atom type and electrostatics using autogrid. Rotatable bonds for the pterins were specified with the use of the Autotors tool in Autodock. The pterins were individually docked to the eNOS pterin site using the Lamarckian genetic algorithm to search the precomputed affinity maps for low-energy binding orientations. Each docking trial was initiated with a randomly generated population of 50 binding orientations and completed after 1.5 million energy evaluations had been performed. Because of the stochastic nature of the docking process, 10 docking trials were performed. The resulting orientations were clustered with a 0.5 \AA tolerance and ranked according to binding energy predicted by docking.

Independent results of both BH_4 and 5-MTHF were compared with the crystal structure of eNOS containing bound BH_4 .

Experimental model of endothelial dysfunction and effects of 5-MTHF. Studies were conducted to assess the effect of BH_4 and 5-MTHF on endothelial function in fructose-fed rats. Fructose-fed rats have been demonstrated to have lower BH_4 levels and impaired endothelial function (20).

Male Sprague-Dawley rats ($n = 14$) were randomly assigned to control and fructose groups. The fructose group was administered a 60% fructose-enriched diet for a period of 6 wk, as described in Shinozaki et al. (20). After 6 wk, the rats were euthanized and the thoracic aortas removed for the isometric dose-response studies as described previously (1). After equilibration, the tissue was stimulated as follows: 1) cumulative dose-response curves (DRC) to phenylephrine (PE), 2) cumulative DRC to the endothelium-dependent vasodilator ACh in PE-precontracted arteries, and 3) cumulative DRC to the endothelial-independent vasodilator sodium nitroprusside in PE-precontracted arteries. These responses were repeated in the presence of BH_4 (100 μM) and 5-MTHF (100 μM). Percent maximum relaxation ($\%E_{\text{max}}$) was compared between groups by one-way ANOVA.

RESULTS

Immunocytochemistry. Endothelial cells isolated from the local abattoir and the immortalized human Ea.Hy96 cell line expressed eNOS. Cyclosporin in both the bovine and human endothelial cells lines induced eNOS expression in endothelial cells as previously demonstrated (not shown). Incubation with an inhibitory peptide specific for the antibody-binding site on eNOS markedly reduced fluorescence, confirming specificity of the primary and minimal nonspecific secondary antibody binding (not shown).

Attenuation of free radical production from eNOS by 5-MTHF and BH_4 . Figure 1 depicts the effects of 5-MTHF and BH_4 on superoxide production (by recombinant eNOS) assessed by lucigenin chemiluminescence. Native enzyme in the absence of cofactors produced large amounts of superoxide. This signal was completely abolished by superoxide dismutase. Importantly, both BH_4 and 5-MTHF caused a concentration-dependent attenuation in superoxide production.

Figure 2 depicts the effects of 5-MTHF and the BH_4 precursor (sepiapterin) on reactive oxygen species production in ACh-stimulated endothelial cells assessed by dihydrorhodamine 123 fluorescence. Dihydrorhodamine is an uncharged and nonfluorescent dye capable of diffusing across cell membranes. It reacts with hydrogen peroxide (14) and peroxyxynitrite (6, 16) to form the fluorescent and cell-impermeable rhodamine-123. As expected, cells exposed to the GTP-cyclohydrolase inhibitor 2,4-diamino-6-hydroxy-pyrimidine (DAHP, inhibitor of BH_4 synthesis) produced greater amounts of reactive oxygen species. Importantly, this effect was attenuated by the use of 5-MTHF and sepiapterin.

Figure 3 depicts the effects of DAHP on mean cellular fluorescence in cells incubated with the superoxide-specific dye DHE. DAHP increased superoxide product and this effect was attenuated by the use of 5-MTHF.

Computer modeling. Both BH_4 and 5-MTHF were docked in the eNOS active site and their positions were

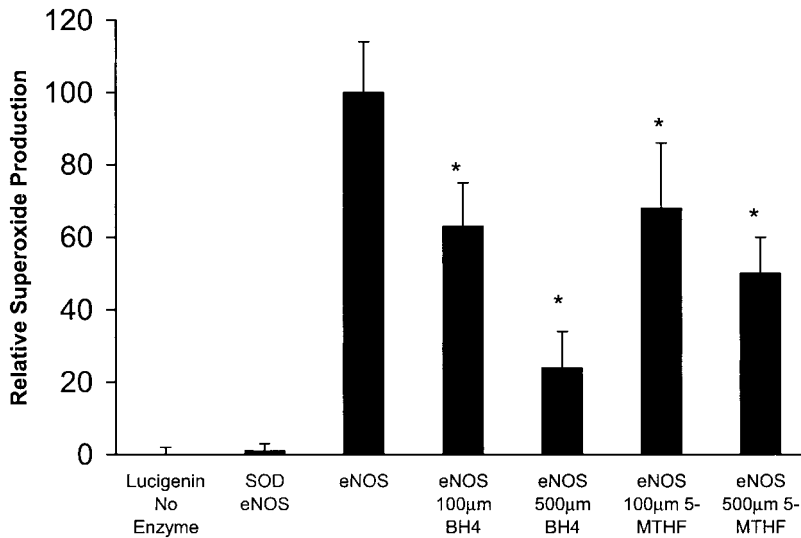


Fig. 1. Superoxide production measured by lucigenin (250 μM) enhanced chemiluminescence from recombinant endothelial nitric oxide synthase (eNOS). Total counts (300 s) expressed as percent reduction from control [eNOS without tetrahydrobiopterin (BH_4) or 5-methyltetrahydrofolate (5-MTHF)] over 300 s ($n = 4$ per treatment). Both BH_4 and 5-MTHF cause concentration-dependent attenuation of superoxide production. SOD, superoxide dismutase. * $P < 0.05$ vs. eNOS without 5-MTHF or BH_4 .

compared with that of the original BH_4 , as determined from the crystal structure (Fig. 4). The average atomic distance between the top-ranked BH_4 docking solutions was 1.26 and 1.01 \AA , respectively, accounting for 6 of 10 runs. Similarly, the docking solution for 5-MTHF was within 1.4 \AA (excluding the tail) of the actual pterin-binding site. Moreover, the 5-MTHF was π stacked with Trp-449, which is consistent with Fischmann et al. (7), who found a similar interaction between BH_4 and their analogous Trp-447. Furthermore, N3 and NA2 of the docked 5-MTHF were aligned identically to the corresponding BH_4 atoms and were 3.1 and 3.0 \AA from the propionate group of heme, again similar to what was reported by Fischmann et al. (7). Finally, the relative binding energies between BH_4 and 5-MTHF were -16.92 and -13.96 , respectively. The interaction between 5-MTHF and the eNOS active site

is illustrated in Fig. 4. The actual pterin-binding site is overlaid with 5-MTHF for reference.

Endothelial function assessment. Figures 5 and 6 depict the effects of acute BH_4 and 5-MTHF treatments on vascular responses in BH_4 -deficient fructose-fed rats. The fructose-fed rats exhibited endothelial dysfunction; the $\%E_{\text{max}}$ of ACh response was diminished in fructose-fed versus control rat aortas. Importantly, both BH_4 and 5-MTHF improved ACh-mediated vasorelaxation to a similar degree. Fructose-fed rats exhibited no changes in endothelium-independent responses (to sodium nitroprusside) in the presence or absence of BH_4 or 5-MTHF (Figs. 5 and 6).

DISCUSSION

This is the first study to provide evidence indicating that 5-MTHF is capable of binding the pterin site in eNOS. Furthermore, this direct binding to eNOS mimics the orientation and interactions of the natural cofactor BH_4 . Our in vitro data also indicate that

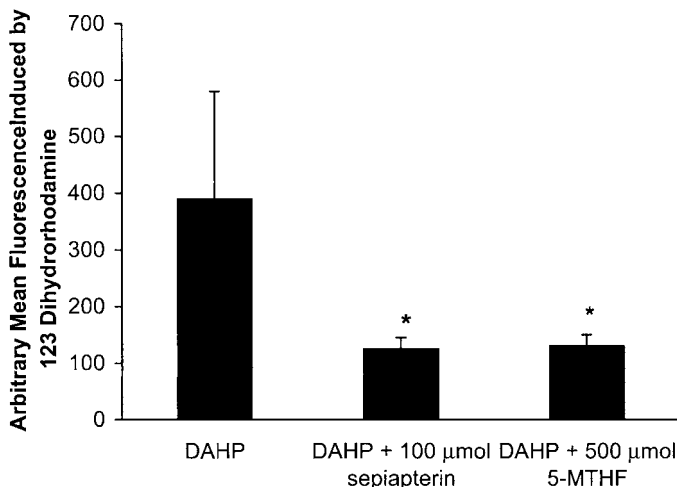


Fig. 2. Mean dihydrorhodamine 123-induced fluorescence of acetylcholine (ACh)-stimulated bovine aortic endothelial cells incubated with 2,4-diamino-6-hydroxy-pyrimidine (DAHP), DAHP + sepiapterin, or DAHP + 5-MTHF. Sepiapterin and 5-MTHF attenuate DAHP-induced superoxide production. * $P < 0.05$ vs. DAHP.

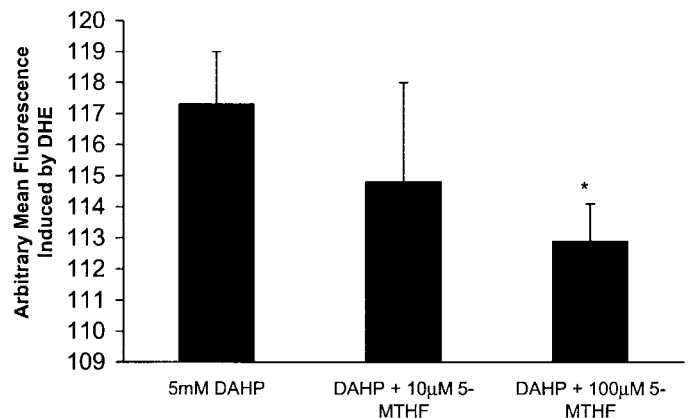


Fig. 3. Effects of DAHP on mean cellular fluorescence in cells incubated with the superoxide-specific dye dihydroethidine (DHE). DAHP increases superoxide production. * $P < 0.05$, this effect is attenuated by 100 μM 5-MTHF.

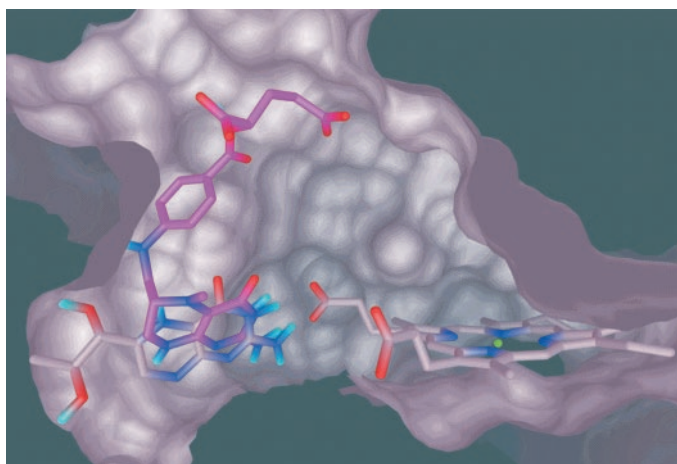


Fig. 4. Docking results of 5-MTHF in the active site of eNOS compared with the actual BH₄-binding site determined from the crystal structure. Heme iron is green with the propionyl group in red. 5-MTHF is capable of binding the pterin site in eNOS. The direct binding of 5-MTHF mimics the orientation and interactions of the natural cofactor BH₄.

5-MTHF prevents the exaggerated production of reactive oxygen species associated with a BH₄ deficiency in both recombinant enzyme and in cultured endothelial cells. Finally, we demonstrated that 5-MTHF supplementation restores NO-dependent endothelial function in BH₄-deficient fructose-fed rats.

It is well documented that BH₄ alters eNOS activity. However, the exact mechanism and the role of BH₄ in directing eNOS to produce NO instead of superoxide remain ill defined (2, 3, 13, 28). BH₄ has been proposed

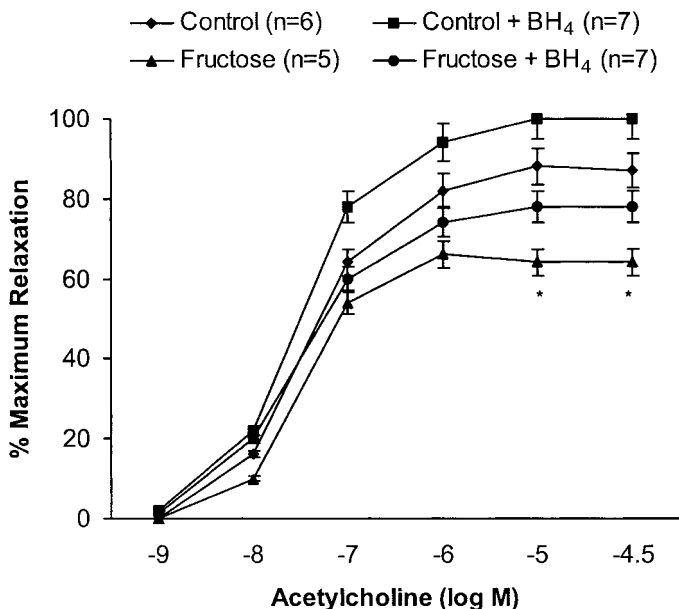


Fig. 5. Acute effects of BH₄ (100 μM, 3 h) on endothelium-dependent and -independent vascular relaxation in control vs. BH₄-deficient fructose-fed rat aortas. Fructose-fed animals have a significantly (**P* < 0.05) lower relaxation induced by ACh compared with all other groups. Acute treatment with BH₄ improved ACh-induced vasodilatation (**P* < 0.05). Endothelium-independent functional response to sodium nitroprusside (SNP) was preserved.

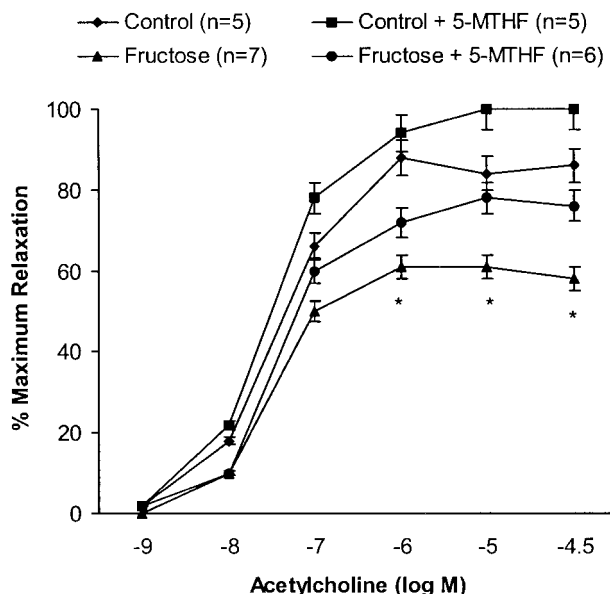


Fig. 6. Acute effects of 5-MTHF (100 μM, 3 h) on endothelium-dependent and independent vascular relaxation in control vs. BH₄-deficient fructose-fed aortas. Fructose-fed animals have a significantly (**P* < 0.05) lower relaxation induced by ACh compared with all other groups. Acute treatment with 5-MTHF improved ACh-induced vasodilatation in fructose-fed aortas (**P* < 0.05) while having no significant effect on the controls. There was no significant difference in dilatation between any of the groups induced by SNP.

to enhance the binding of L-arginine, activate heme into a high spin state, stabilize heme and donate electrons for the oxygenation reaction (2, 8). Various analogs of BH₄ have also been reported to catalyze NOS activity. Interestingly, only fully reduced tetrahydroanalogs are active and support NO generation whereas the dihydrobiopterins are inhibitory. One such compound reported to maintain NOS activity is 5-methyl-BH₄. Riethmuller et al. (19) recently demonstrated that 5-methyl-BH₄ exhibited a markedly reduced activity toward free oxygen while at the same time maintained catalytic activity, favoring NO production by neuronal NOS. The authors speculated that the methyl group helped stabilize the pterin ring (19). These results support the notion that the methyl group on 5-MTHF, analogous to the methyl group in 5-methyl-BH₄, will not interfere with interactions at the active site. The tetrahydro-reduced state of 5-MTHF also supports the observation that only the fully reduced analogs of BH₄ remain catalytically active. The docking of 5-MTHF to eNOS in this study demonstrates that 5-MTHF is capable of fitting into the active site and can interact in an almost identical manner as the natural cofactor BH₄.

Stroes et al. (23) recently demonstrated that 5-MTHF is unable to alter NOS activity in enzyme that is completely devoid of pterin. These observations do not support the concept that 5-MTHF can replace BH₄ as a cofactor for NOS. The authors speculated that 5-MTHF could facilitate the one-electron oxidation of BH₄, generating a pterin radical that may be necessary for the formation of NO (23). Such an interaction has been

shown to exist between ascorbic acid and BH₄ (12, 13). Although 5-MTHF can scavenge superoxide, its effects are several times lower than ascorbate. In addition, extracellular antioxidants such as superoxide dismutase are more relevant in vivo for scavenging superoxide compared with 5-MTHF (23).

The aforementioned paradigm may need to be reassessed in lieu of our data demonstrating that 5-MTHF is not only able to fit in the active site of eNOS but also mimic the orientation and interaction of BH₄ and eNOS. It is, however, possible that 5-MTHF does not bind eNOS unless BH₄ is already bound to the adjacent pterin-binding site. A similar relationship between BH₄ has been shown to exist by Gorren et al. (9), who determined that the binding of the first BH₄ has an anticooperative effect on the binding of the second BH₄ (9). Such an interaction would be in line with the observation that 5-MTHF has no effect on eNOS activity unless some pterin is present.

Previous clinical studies (26, 27) and evidence from this study highlight the importance of 5-MTHF on reactive oxygen species produced by NOS. Our studies, and those of Verhaar et al. (27) and Wever et al. (28), have shown that 5-MTHF can attenuate superoxide production in recombinant eNOS. This observation indicates that 5-MTHF can directly interact with eNOS rather than having an independent cellular action such as lowering homocysteine, which could then possibly lower cellular oxidative stress. In addition, we have demonstrated that coincubation with DAHP and 5-MTHF or the BH₄ precursor sepiapterin significantly lowers reactive oxygen species compared with cells incubated with DAHP alone (Fig. 2). Likewise, the superoxide-specific conversion of dihydroethidine to the fluorescent ethidium can be attenuated by coincubation with 5-MTHF.

The half-life of NO is dependent primarily on its reaction with superoxide or oxyhemoglobin (3). The reaction between NO and superoxide is extremely favorable, being three times more favorable than the reaction rate for superoxide and superoxide dismutase (3). Therefore, an eNOS enzyme partially deficient in BH₄ or 5-MTHF facilitates the generation of the potent oxidant peroxynitrite. Furthermore, excess superoxide production can lead to the production of other oxidants such as hydrogen peroxide and hydroxyl radicals. We have demonstrated that 5-MTHF is capable of inhibiting superoxide production by recombinant NOS and by endothelial cells deficient in BH₄. It can therefore be postulated that 5-MTHF binds and helps to convert NOS from a peroxynitrite synthase back to NOS.

Recent studies (11, 20) have indicated that BH₄ may be deficient in states of insulin resistance and diabetes whereas eNOS expression is increased by insulin. This combination of increased eNOS with the depletion of BH₄ would favor eNOS-mediated superoxide production rather than NO. Existing evidence suggests that the decreased NO production and increased superoxide generation can be linked to insulin resistance. Shinzaki et al. (21) have shown that endothelial BH₄ levels in fructose-fed rats were significantly decreased

and that exogenous BH₄ supplementation restored NO production while simultaneously decreasing superoxide levels. Using a different animal model, Pieper et al. (18) determined that 5-methyl-BH₄ could correct endothelial dysfunction in streptozotocin-induced diabetic rats. In the present study, we demonstrate 5-MTHF and BH₄ restore endothelial function in fructose-fed rats to an equal degree. Cellular changes in homocysteine are not likely to account for the improved endothelial function given that plasma homocysteine levels have been shown to be lower in diabetic rats (15). Similarly, treatment with BH₄ (10 mg·kg⁻¹·day⁻¹ for 8 wk) in fructose-fed rats has recently been demonstrated to improve endothelial function, insulin sensitivity and blood pressure (21). All of these effects were attributed to the restoration of eNOS function (21).

In summary, results from the present study demonstrate that 5-MTHF may interact with the active site of eNOS in a fashion analogous to BH₄. 5-MTHF prevents increases in superoxide production from recombinant eNOS and endothelial cells. Finally, 5-MTHF improves endothelial function in BH₄-deficient rat aortas. These data support the cellular and functional relationship between 5-MTHF, BH₄, and endothelial function. 5-MTHF may be a useful strategy for interventions targeting BH₄ bioavailability.

Study limitations. The results from our computer modeling suggest that 5-MTHF and BH₄ bind in a similar fashion within the active site of eNOS. In addition, both have similar effects ameliorating superoxide and promoting vasodilation further supporting our hypothesis. However, we acknowledge that the modeling is not definitive and further investigations such as crystallography or kinetic enzyme-binding studies are essential.

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