Critical Role for Tetrahydrobiopterin Recycling by Dihydrofolate Reductase in Regulation of Endothelial Nitric-oxide Synthase Coupling

RELATIVE IMPORTANCE OF THE DE NOVO BIOPTERIN SYNTHESIS VERSUS SALVAGE PATHWAYS

Tetrahydrobiopterin (BH4) is a required cofactor for the synthesis of nitric oxide by endothelial nitric-oxide synthase (eNOS), and BH4 bioavailability within the endothelium is a critical factor in regulating the balance between NO and superoxide production by eNOS (eNOS coupling). BH4 levels are determined by the activity of GTP cyclohydrolase I (GTPCH), the rate-limiting enzyme in de novo BH4 biosynthesis. However, BH4 levels may also be influenced by oxidation, forming 7,8-dihydrobiopterin (BH2), which promotes eNOS uncoupling. Conversely, dihydrofolate reductase (DHFR) can regenerate BH4 from BH2, but the functional importance of DHFR in maintaining eNOS coupling remains unclear. We investigated the role of DHFR in regulating BH4 versus BH2 levels in endothelial cells and in cell lines expressing eNOS combined with tet-regulated GTPCH expression in order to compare the effects of low or high levels of de novo BH4 biosynthesis. Pharmacological inhibition of DHFR activity by methotrexate or genetic knockdown of DHFR protein by RNA interference reduced BH4 from BH2, but the functional importance of DHFR in maintaining eNOS coupling remains unclear. We investigated the role of DHFR in regulating BH4 versus BH2 levels in endothelial cells and in cell lines expressing eNOS combined with tet-regulated GTPCH expression in order to compare the effects of low or high levels of de novo BH4 biosynthesis. Pharmacological inhibition of DHFR activity by methotrexate or genetic knockdown of DHFR protein by RNA interference reduced BH4 from BH2, but the functional importance of DHFR in maintaining eNOS coupling remains unclear.

In vascular disease states such as atherosclerosis and diabetes, endothelial nitric oxide (NO) bioactivity is reduced, and oxidative stress is increased, resulting in endothelial dysfunction. It has become apparent that enzymatic “coupling” of endothelial NO synthase by its cofactor tetrahydrobiopterin (BH4) plays a key role in maintaining endothelial function. Indeed, the balance between NO and superoxide production by eNOS appears to be determined by the availability of BH4 versus the abundance of 7,8-dihydrobiopterin (BH2), that is inactive for NOS cofactor function and may compete with BH4 for NOS binding (1). Intracellular biopterin levels are regulated principally by the activity of the de novo biosynthetic pathway (Fig. 1). Guanosine triphosphate cyclohydrolase I (GTPCH; EC 3.5.4.16) catalyzes the formation of dihydroprotoeptin triphosphate from GTP, and BH4 is generated by two further steps through 6-pyruvolytetrahydropterin synthase and sepiapterin reductase. GTPCH appears to be the rate-limiting enzyme in BH4 biosynthesis, and overexpression of GTPCH is sufficient to augment BH4 levels in cultured endothelial cells (2). Electron paramagnetic resonance spectroscopy studies have shown that BH4 both stabilizes and donates electrons to the ferrous-dioxygen complex in the oxygenase domain, as the initiating step of L-arginine oxidation (3–5). In this reaction BH4 forms the protonated trihydrobiopterin cation radical, which is subsequently reduced by electron transfer from NOS flavins. When BH4 availability is limiting, electron transfer from NOS flavins becomes uncoupled from L-arginine oxidation, eNOS generates superoxide rather than NO, BH4 becomes oxidized to catalytically incompetent BH2, and a futile feed-forward cascade of BH4 destruction proceeds (1). Recent studies reveal that BH4 and BH2 bind eNOS with equal affinity and that BH2 can efficiently replace eNOS-bound BH4, resulting in eNOS uncoupling (6). Indeed, we have previously shown that the relative abundance of eNOS versus BH4 together with the intracellular BH4:BH2 ratio, rather than absolute concentrations of BH4, are the key determinants of eNOS uncoupling (7), a hypothesis supported by a recent publication where BH2 levels are elevated after exposure of bovine aortic endothelial cells to DHFR-specific siRNA (8). Thus, mechanisms that regulate the BH4:BH2 ratio independently of overall biopterin levels may play an equally important role in regulating eNOS coupling as the well-established role of GTPCH, which regulates de novo BH4 bio-

References

1. The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. 1–3.

2. The abbreviations used are: BH4, tetrahydrobiopterin; BH2, 7,8-dihydrobiopterin; B, biopterin; DHFR, dihydrofolate reductase; NOS, nitric-oxide synthase; eNOS, endothelial NOS; GTPCH, guanosine triphosphate cyclohydrolase I; L-NAME, Nω-nitro-L-arginine methyl ester; SOD, superoxide dismutase; siRNA, small interfering RNA; GFP, green fluorescent protein; eGFP, enhanced GFP; HPLC, high performance liquid chromatography; MTX, methotrexate.

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Regulation of eNOS Coupling by DHFR

EXPERIMENTAL PROCEDURES

Generation of Cells with tet-regulatable GTPCH and eNOS Expression—We used NIH 3T3 murine fibroblasts stably transfected with a tet-off transactivator construct as previously described (7). In the presence of doxycycline, binding of the transactivator is blocked, and gene expression is prevented. These 3T3-tet-off cells, previously shown to express neither eNOS nor GTPCH (12) and also confirmed to be devoid of eNOS protein, were stably transfected with a plasmid encoding hemagglutinin antigen-tagged human GTPCH under the control of a tetracycline-responsive element. Individual colonies were isolated and analyzed for GTPCH expression, and a cell line termed “GCH cells” was established from expansion of a single colony. GCH/eNOS cells were produced by stable transfection of GCH cells with a plasmid encoding a human eNOS-eGFP fusion protein as described (13). All cell lines underwent at least three rounds of clonal selection.

Cell Culture—sEnd.1 murine endothelial cells were cultured in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with glutamine (2 mM), penicillin (100 units/mL), and streptomycin (0.1 mg/mL). Additionally, GCH cells were maintained using media containing the antibiotics hygromycin (200 μg/mL) and Geneticin (200 μg/mL), whereas eNOS/eGFP cell media also included puromycin (2 μg/mL). Where appropriate, doxycycline (1 μg/mL) was added to cell culture media to abolish transcription of GCH1 mRNA.

Biopterin Quantification by HPLC with Electrochemical Detection—BH4, BH2, and biopterin (B) levels in cell lysates were determined by HPLC followed by electrochemical and fluorescent detection, as previously described (14). Briefly, cells were grown to confluence and harvested by trypsinization. Cell pellets were resuspended in phosphate-buffered saline (50 mM), pH 7.4, containing diithioerythritol (1 mM) and EDTA (100 μM) and subjected to three freeze-thaw cycles. After centrifugation (15 min at 13,000 rpm and 4 °C), samples were transferred to new, cooled micro tubes and precipitated with cold phosphoric acid (1 M), trichloroacetic acid (2 M) and diithioerythritol (1 mM). Samples were vigorously mixed and then centrifuged for 15 min at 13,000 rpm and 4 °C. Samples were injected onto an isocratic column (Hichrom) and a mobile phase comprised of sodium acetate (50 mM), citric acid (5 mM), EDTA (48 μM), and dithioerythritol (160 μM) (pH 5.2) (all ultrapure electrochemical HPLC grade) at a flow rate of 1.3 ml/min. Background currents of +500 μA and −50 μA were used for the detection of BH4 on electrochemical cells E1 and E2, respectively. 7,8-BH2 and biopterin were measured using a Jasco FP2020 fluorescence detector. Quantification of BH4, BH2, and B was done by comparison with authentic external standards and normalized to sample protein content.

Western Blotting—Cells were suspended in radioimmune precipitation assay lysis buffer (20 mM Tris-HCl, 150 mM NaCl, 1 mM Na2EDTA, 1 mM EGTA, 1% Triton, 0.1% SDS, 0.1 sodium deoxycholate, pH 7.4) including a mixture of protease inhibitors (Roche Applied Science), and subjected to three freeze-
Regulation of eNOS Coupling by DHFR

thaw cycles in liquid nitrogen. Western blotting was carried out using standard techniques and anti-eNOS (BD Transduction Laboratories), anti-hemagglutinin, anti-DHFR, anti-SOD, and anti-GAPDH antibodies. The rabbit anti-rat GTPCH antibody was kindly provided by Prof. Steven S. Gross of Weill Medical College of Cornell University. Rabbit anti-human GTPCH was kindly provided by Gabriele Werner-Felmayer of the Institute for Medical Chemistry and Biochemistry, Innsbruck, Austria.

**Measurement of eNOS Protein Levels by eGFP Fluorescence**—Cell pellets were lysed in phosphate-buffered saline containing dithioerythritol (1 mM) and EDTA (100 μM) as for BH4 analysis. Sample fluorescence was quantified using a TEKAN fluorescence plate reader and a standard curve generated using recombinant eGFP. Because recombinant eNOS was expressed as an eNOS-eGFP fusion protein, eGFP fluorescence and eNOS levels were directly proportional. Importantly, determination of both eGFP fluorescence and BH4 levels was done on identical cell samples, allowing accurate determination of the BH4:eNOS ratio.

**Quantification of Superoxide Production by HPLC**—Measurement of 2-hydroxyethidium formation by HPLC was used to quantify superoxide production by methods adapted from those previously described (15, 16). Cells were washed 3 times in phosphate-buffered saline and incubated in Krebs-Hepes buffer in the presence or absence of L-NAME (100 μM). After 1 h, dihydroethidium (25 μM) was added, and cells were then incubated for an additional 20 min at 37 °C. Cells were then harvested by scraping, centrifuged, and lysed in ice-cold methanol. Hydrochloric acid (100 mM) was added (1:1 v/v) before loading into the autosampler for analysis. All samples were stored in darkened tubes and protected from light at all times. Separation of dihydroethidium, 2-hydroxyethidium, and ethidium was performed using a gradient HPLC system (Jasco) with an ODS3 reverse phase column (250 mm, 45 mm; Hichrom) and quantified using a fluorescence detector set at 510 nm (excitation) and 595 nm (emission). A linear gradient was applied from mobile phase A (0.1% trifluoroacetic acid) to mobile phase B (0.085% trifluoroacetic acid in acetonitrile) over 23 min (30% to 50% acetonitrile).

**Analysis of NO Synthesis by eNOS**—Cellular NO synthesis by eNOS was assessed by measuring the conversion of L-[14C]arginine to citrulline with HPLC detection in the presence and absence of L-NNAME (100 μM). After 30 min, samples were stored in darkened tubes and protected from light at all times. NO metabolites were separated on the gradient HPLC system (Jasco) and quantified using a fluorescence detector set at 450 nm (excitation) and 550 nm (emission). A linear gradient was applied from mobile phase A (0.1% trifluoroacetic acid) to mobile phase B (0.085% trifluoroacetic acid in acetonitrile) over 23 min (30% to 50% acetonitrile).

**DHFR and GCH Knockdown by RNA Interference**—DHFR and GCH-specific “ON-TARGETplus SMARTpool” siRNA was purchased from Dharmacon Thermo Scientific. The siRNAs were used as a pool of four specific siRNA duplexes with the following sequences: GCH duplex 1, GUAGAAGUGCUAAGAAGCU; GCH duplex 2, CGAAGAGUGUGCCUGUA; GCH duplex 3, GAAAGGGAGAUGCUCU; GCH duplex 4, AGUAGUAGAAAGCCGACA; DHFR duplex 1, AGUAGUAGAAGCUAGAGA; DHFR duplex 2, AGAAGACCACAGUGGGAU; DHFR duplex 3, GCGUGAAGGUUGUCUAGA; DHFR duplex 4, GCAAGUAAUGUGUGUAA.

sEnd.1 endothelial cells were grown in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with glutamine (2 mM), penicillin (100 units/ml), and streptomycin (0.1 mg/ml). 24 h before transfection, cells were seeded into 6-well plates. Cells were then transfected with GCH- or DHFR-specific siRNA (100 nm), and GAPDH-positive (100 nm) or nonspecific pooled duplex negative control siRNA (100 nm). Cells were cultured for 72 h, and gene silencing was detected by analysis of DHFR and GTPCH protein expression by Western blotting using DHFR and GTPCH-specific antibodies.

**Measurement of Cellular Antioxidant Capacity**—Cellular antioxidant status was assessed using an antioxidant detection kit that quantifies the combined antioxidant capacity of aqueous and lipid-soluble antioxidant including vitamins, proteins, lipids, glutathione, uric acid. Cells were harvested into assay buffer (50 mM phosphate buffered saline, pH 7.4, containing dithioerythritol (1 mM) and EDTA (100 μM) and subjected to three free-thaw cycles). Protocol was then followed as per the manufacturer’s instructions.

**Statistical Analysis**—Data are presented as the mean ± S.E. Data were subjected to the Kolmogorov-Smirnov test to determine distribution. Groups were compared using the Mann-Whitney U test for non-parametric data or Student’s t test for parametric data. When comparing multiple groups data were analyzed by analysis of variance with the Newman-Keuls post-test for parametric data or Kruskal-Wallis test with Dunns post-test for non-parametric data. Correlation testing was performed using the Pearson test. A value of p < 0.05 was considered statistically significant.

**RESULTS**

**Effects of DHFR Overexpression in Endothelial Cells**—We first investigated the effect of DHFR overexpression on biopterin levels and eNOS regulation in sEnd.1 murine endothelial cells. DHFR protein was increased 5-fold after transfection of sEnd.1 endothelial cells with DHFR cDNA, compared with untreated, GFP, or mock transfection controls (Fig. 2A). However, DHFR overexpression did not change the intracellular levels of either BH4 or total biopterins (Fig. 2, B and C). Similarly, neither endothelial-derived NO nor superoxide production was altered by DHFR overexpression, as evidenced by conversion of radiolabeled arginine to citrulline and by 2-hydroxyethidium accumulation, respectively (Fig. 2, D and E). These observations suggest that sEnd.1 endothelial cells contain sufficient DHFR to regulate steady-state BH4 levels and efficiently couple eNOS such that overexpression of DHFR has no further effects.

**Effects of DHFR Inhibition by Methotrexate (MTX) in Endothelial Cells**—We next sought to evaluate the effects of DHFR inhibition using MTX on steady-state biopterin levels to determine the requirement of DHFR for maintenance of BH4 homeostasis and eNOS coupling. We compared BH4, BH2, and biopterin levels in sEnd.1 endothelial cells after exposure to MTX (1 μM) for 16 h. Western blot analysis revealed that MTX did not alter protein levels of eNOS, DHFR, GCH, or GAPDH (Fig. 3A). However, intracellular BH4 levels were significantly decreased by exposure to MTX (from 308.0 ± 40.5 to 229.0 ± 36.3 pmol/mg protein) (Fig. 3B, p < 0.05), whereas the levels of BH2 and B were markedly elevated (Fig. 3, C and D, p < 0.001). Total biopterin levels (BH4 + BH2 + B) were unaltered by MTX, but there was a striking decrease in the ratio of reduced to oxidized biopterins (BH4:BH2 and B) (13.8 ± 2.3 versus 3.3 ± 3.3).
Furthermore, MTX induced eNOS uncoupling, demonstrated by the simultaneous decrease in NO synthesis \((p < 0.05; \text{Fig. 4A})\) and elevation in eNOS-dependent superoxide production \((p < 0.01; \text{Fig. 4B and C})\). Additional effects of MTX treatment on SOD protein levels and general cellular antioxidant capacity may explain changes in 2-hydroxyethidium accumulation observed in the presence of MTX. To eliminate these potentially confounding effects, we quantified CuZn-, Mn-, and endothelial cell SOD protein levels using Western blotting. No effect of MTX was observed on SOD levels \((p < 0.01)\). However, a significant reduction in cellular antioxidant capacity was detected after incubation of sEnd.1 endothelial cells, GCH, and GCH/eNOS cells with MTX. 

\[ \text{FIGURE 2. DHFR overexpression in sEnd.1 endothelial cells.} \] 

A, DHFR levels are increased by 5-fold after transfection of human DHFR cDNA in sEnd.1 endothelial cells as shown by Western blotting using a DHFR-specific antibody, as detailed under “Experimental Procedures.” Representative blots are shown; \(n = 3\). DHFR overexpression did not significantly affect intracellular BH4 levels \((B)\), total biopterin levels \((C)\), the production of nitric oxide as demonstrated by A23187-induced conversion of arginine to citrulline \((D)\), or the amount of eNOS-derived superoxide \((E)\); \(n = 3\) or 6 for each experiment.

\[ \text{FIGURE 3. Inhibition of endogenous DHFR activity by MTX:biopterin levels.} \] 

sEnd.1 cells were exposed to MTX \((1 \mu M)\) for 16 h at 37 °C, and intracellular biopterin levels were quantified by HPLC as detailed under “Experimental Procedures.” A, exposure to MTX did not affect intracellular eNOS, DHFR, or GTPCH \((GCH)\) protein levels. B, exposure of sEnd.1 cells to MTX significantly decreased BH4 levels \((*, p < 0.05)\), whereas a marked accumulation of BH2 \((**, p < 0.001)\) and biopterin \((*, p < 0.001)\) \((D)\) was observed. C, no significant change in total biopterin was demonstrated. D, BH4:BH2 ratio was significantly decreased after treatment of sEnd.1 cells with MTX \((*, p < 0.001, n = 3\) for all experiments).

\[ \text{FIGURE 4. Inhibition of endogenous DHFR activity by MTX:nitric oxide and superoxide production.} \] 

A23187-induced conversion of arginine to citrulline was used as a measure of eNOS activity. A, eNOS activity (as indicated by the inhibition of citrulline accumulation by \(N\)-methyl arginine; \(NMA)\) was attenuated in the presence of MTX in sEnd.1 cells \((*, p < 0.05)\). B, after exposure to MTX \((1 \mu M)\) for 16 h, cells were exposed to dihydroethidium \((25 \mu M)\) for 20 min, and accumulation of 2-hydroxyethidium was measured by HPLC with fluorescence detection. MTX exposure markedly elevated cellular superoxide production \((**, p < 0.01)\), which was significantly inhibited after pretreatment of cells with the NOS inhibitor, L-NAME \((C) (100 \mu M; *, p < 0.05)\). \(n = 6\) for each experiment.

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with MTX (*, p < 0.05, supplemental Fig. 2). No confounding effects of DHFR inhibition on cell growth and viability were observed (data not shown).

Effects of DHFR or GCH Knockdown by RNA Interference—We next investigated the effects of reduced DHFR expression by siRNA rather than pharmacologic DHFR inhibition and sought to compare with the effects of reduced GTPCH expression to determine the functional relationships between BH4 biosynthesis and BH4 recycling for eNOS coupling. After exposure of sEnd.1 endothelial cells to DHFR- or GTPCH-targeted siRNA for 72 h, DHFR or GTPCH protein was reduced by ~95 or 90%, respectively. No nonspecific effects of control GAPDH-targeted siRNA, non-targeted scrambled control siRNA, or transfection reagent alone (mock) were observed by Western blotting (Fig. 5A). Cells treated with DHFR, GTPCH, and control siRNA were simultaneously prepared for bioppterin analysis. As observed with DHFR inhibition using MTX, knockdown of DHFR resulted in a 40% decrease in intracellular BH4 (Fig. 5B) and a 2-fold elevation in BH2 levels (Fig. 5C), whereas total biopterins remained unchanged (Fig. 5D). Consequently, a striking reduction in the BH4:BH2 ratio was observed in the presence of DHFR knockdown but not GTPCH knockdown (4.8 ± 1.5 to 1.9 ± 0.8) (*, p < 0.05, Fig. 5E). eNOS uncoupling, evidenced by increased total and eNOS-dependent superoxide (**, p < 0.05, Fig. 5, G and H), as well as a reduced conversion of arginine to citrulline was significantly increased in the presence of both DHFR- and GCH-specific siRNAs (Fig. 5F). When combined, GTPCH- and DHFR-specific knockdown resulted in markedly more eNOS-derived superoxide (**, p < 0.05) and a diminished BH4:BH2 ratio (†, p < 0.05) when compared with either

FIGURE 5. Genetic knockdown of DHFR and GTPCH protein by siRNA. sEnd.1 cells were transfected with DHFR- and GCH-specific, control GAPDH, or scrambled nonspecific siRNA as described under "Experimental Procedures." A, DHFR and GTPCH-specific siRNAs alone or in combination knocked down protein levels by more than 90%. Importantly, GTPCH and GAPDH knockdown as well as scrambled control siRNAs did not have any effect on DHFR protein levels. B, Intracellular BH4 levels were decreased 40 and 95% by DHFR- and GTPCH-specific siRNAs, respectively (*, p < 0.05). C, a 2-fold increase in BH2 levels was induced by DHFR-specific siRNA (*, p < 0.05), and a 95% reduction was observed after exposure of sEnd.1 cells to GTPCH-specific siRNA (**, p < 0.001). Combined knockdown of GTPCH and DHFR resulted in a 4-fold increase in BH2 when compared with cells exposed to GTPCH-specific siRNA alone (*, p < 0.05). D, total biopterins were unchanged by DHFR-specific siRNA and reduced by ~95% after exposure of sEnd.1 endothelial cells to GTPCH-specific siRNA or when combined with DHFR-specific siRNA (**, p < 0.001). E, intracellular BH4:BH2 ratio was markedly decreased by DHFR-, but not GTPCH-specific siRNA exposure (*, p < 0.05). F, A23187-induced conversion of arginine to citrulline was diminished by DHFR (*, p < 0.05) and GTPCH-specific siRNAs (**, p < 0.001). G and H, eNOS-dependent superoxide production was induced by GTPCH- and DHFR-specific siRNA and when combined, led to a significantly increased production versus either GTPCH or DHFR siRNAs alone (**, p < 0.001). Blots shown are representative of three separate experiments. All biopterin and arginine to citrulline measurements were unaffected by GAPDH knockdown or scrambled control siRNAs (n = 6).
GTPCH or DHFR knockdown alone (Fig. 5). These data suggest that either major reductions in \textit{de novo} BH4 biosynthesis or an altered BH4:BH2 ratio through reduced BH4 recycling is sufficient to induce eNOS uncoupling. Furthermore, the exacerbation of both BH4:BH2 ratio and eNOS-dependent superoxide production by DHFR knockdown at low levels of total biopterin infers that DHFR activity is more significant at low, compared with high or saturating levels of BH4.

Quantitative Relationships between GTPCH Expression, DHFR, and BH4 Homeostasis in GCH Cells—We investigated the effects of DHFR inhibition using MTX in cells expressing hemagglutinin antigen-tagged human GTPCH under the control of a tetracycline-responsive element (GCH cells) (7). Titrating doxycycline concentration between 0.01 and 1 ng/ml to manipulate GTPCH expression, and therefore \textit{de novo} BH4 synthesis, resulted in the expected concentration-dependent reduction in steady-state intracellular BH4 levels. However, coinubcation with MTX significantly reduced BH4 levels across the whole range of GTPCH expression while markedly elevating intracellular BH2 levels (\(p < 0.05\); Fig. 6). Importantly, the relative magnitude of the effect of DHFR inhibition by MTX was significantly greater at low absolute biopterin levels (Fig. 6). Intracellular BH4 levels were reduced by 25% at 0.01 ng/ml doxycycline (i.e. high GTPCH and high total biopterins) versus 47% reduction at 1 ng/ml doxycycline (i.e. low GTPCH and low total biopterins).

\textbf{DHFR Protects against eNOS Uncoupling}—We reasoned that the potential importance of DHFR in BH4 recycling under low total biopterin conditions might be critical in eNOS uncoupling, where eNOS-dependent ROS production can itself lead to BH4 oxidation. To test this hypothesis, we compared the effects of DHFR inhibition by MTX in GCH cells versus GCH cells stably expressing an eNOS-GFP fusion protein (GCH/eNOS cells) at different levels of GTPCH expression as determined by doxycycline.

We confirmed by Western blotting that eNOS protein was abundant in GCH/eNOS cells but not GCH cells and that transgenic GTPCH protein was present equally in both GCH and GCH/eNOS cells but was undetectable after incubation with doxycycline for 7 days. Importantly, neither eNOS nor DHFR protein levels were altered by doxycycline or MTX exposure (Fig. 7A).
Incubation of both GCH and GCH/eNOS cells with doxycycline, MTX, or both in combination resulted in a marked suppression of intracellular BH4 levels (*, p < 0.05). However, BH4 levels in GCH/eNOS cells were significantly lower than those in GCH cells after doxycycline exposure, associated with increased accumulation of BH2 (Fig. 7, B and C). BH2 accumulation in GCH/eNOS cells was significantly higher in the presence of both MTX and DOX when compared with DOX alone (Fig. 7C; †, p < 0.05). The BH4:BH2 ratio, a key determinant of eNOS coupling, was not altered in GCH cells after doxycycline treatment in either the presence or absence of MTX. However, a striking doxycycline-induced decrease in the ratio of BH4:BH2 was observed in GCH/eNOS cells, which was further exacerbated in the presence of MTX (Fig. 7D). Similarly, the eNOS:BH4 ratio, determined by fluorescent quantification of eNOS-GFP fusion protein in cell lysates, was reduced by MTX (21.2 ± 0.50, p < 0.05), which was significantly inhibited by 1-NAME (100 μM) (†, p < 0.05, n = 6).

In this study we used both pharmacologic inhibition and genetic manipulation to determine the role of DHFR in the regulation of BH4 levels in sEnd.1 endothelial cells and in cell lines which express tetracycline-regulatable human GTPCH and an eNOS-GFP fusion construct, described previously (7). We reveal a key role for DHFR and the recycling pathway of BH4 biosynthesis in the maintenance of intracellular BH4 homeostasis. We also show that DHFR protein expression is an important factor in the preservation of eNOS coupling and subsequently the production of nitric oxide and superoxide. The major findings of this study are as follows. First, we demonstrate that endothelial cells contain substantial amounts of DHFR protein and that further augmentation of DHFR does not affect NO, BH4, or superoxide biosynthesis. Second, inhibition of DHFR activity or reduction of DHFR protein levels (after exposure to MTX- or DHFR-specific siRNA, respectively) results in BH4 oxidation to BH2, decreased production of NO, and elevation of eNOS-derived superoxide, indicative of eNOS uncoupling. Third, that this initiation of eNOS uncoupling by inhibition/knockdown of DHFR protein activity is exacerbated in conditions where BH4 is low or limiting. Finally, we reveal that DHFR is protective against eNOS uncoupling, demonstrated when cells expressing abundant eNOS are exposed to MTX, particularly at low levels of GTPCH expression, where reduced cellular BH4 increases the susceptibility to eNOS uncoupling, including "self-induced" eNOS uncoupling resulting from eNOS-dependent BH4 oxidation. Taken together, our findings provide clear mechanistic evidence to support a critical role of the recycling pathway in the regulation of cellular BH4 homeostasis and eNOS coupling, even in the absence of vascular disease or exogenous oxidative stress.

Biosynthesis of BH4, initially characterized by its cofactor role in reactions catalyzed by the aromatic amino acid hydroxylases, proceeds via the de novo pathway involving enzymes GTPCH, 6-pyruvoyl-tetrahydropterin synthase, and sepiapterin reductase (Fig. 1) (18). As the rate-limiting enzyme in BH4 synthesis, GTPCH regulation takes place at the transcriptional and post-translational levels, with activity and mRNA shown to be induced by mediators such as interferon-γ, tumor necrosis factor-α, and lipopolysaccharide (19–21). Indeed, steady-state BH4 levels in cells and tissues have previously been shown to directly correlate with GTPCH mRNA levels. BH4, also a cofactor for NO enzymes, is required for efficient oxidation of arginine and ultimately NO production. When BH4 availability is limiting, eNOS generates superoxide rather than NO, BH4 becomes oxidized to catalytically incompetent BH2, and a futile feed-forward cascade of BH4 destruction proceeds.

A BH2 reductase activity of DHFR was first observed in cell and tissue extracts. In a Chinese hamster ovary cell mutant lacking dihydrofolate reductase (DUKX-BII), endogenous formation of BH4 proceeds normally, but unlike the parent cells that express DHFR, extracts do not convert sepiapterin or BH2 to BH4 (22). These studies implicate the biopterin recycling pathway in the regulation of steady-state BH4 levels. Our data suggest that this BH2 reductase activity of DHFR is crucial in determining cellular BH4 homeostasis, NO bioavailability, and

| DOX | MTX | eNOS/BH4   | BH4/eNOS |
|-----|-----|------------|----------|
| –   | –   | 0.05 ± 0.01| 21.2 ± 4.63|
| +   | –   | 1.57 ± 0.94| 0.85 ± 0.58|
| –   | +   | 0.07 ± 0.00| 13.8 ± 0.90|
| +   | +   | 1.55 ± 0.50| 0.70 ± 0.24|

**DISCUSSION**

In this study we used both pharmacologic inhibition and genetic manipulation to determine the role of DHFR in the regulation of BH4 levels in sEnd.1 endothelial cells and in cell lines which express tetracycline-regulatable human GTPCH and an eNOS-GFP fusion construct, described previously (7). We reveal a key role for DHFR and the recycling pathway of BH4 biosynthesis in the maintenance of intracellular BH4 homeostasis. We also show that DHFR protein expression is an important factor in the preservation of eNOS coupling and subsequently the production of nitric oxide and superoxide. The major findings of this study are as follows. First, we demonstrate that endothelial cells contain substantial amounts of DHFR protein and that further augmentation of DHFR does not affect NO, BH4, or superoxide biosynthesis. Second, inhibition of DHFR activity or reduction of DHFR protein levels (after exposure to MTX- or DHFR-specific siRNA, respectively) results in BH4 oxidation to BH2, decreased production of NO, and elevation of eNOS-derived superoxide, indicative of eNOS uncoupling. Third, that this initiation of eNOS uncoupling by inhibition/knockdown of DHFR protein activity is exacerbated in conditions where BH4 is low or limiting. Finally, we reveal that DHFR is protective against eNOS uncoupling, demonstrated when cells expressing abundant eNOS are exposed to MTX, particularly at low levels of GTPCH expression, where reduced cellular BH4 increases the susceptibility to eNOS uncoupling, including “self-induced” eNOS uncoupling resulting from eNOS-dependent BH4 oxidation. Taken together, our findings provide clear mechanistic evidence to support a critical role of the recycling pathway in the regulation of cellular BH4 homeostasis and eNOS coupling, even in the absence of vascular disease or exogenous oxidative stress.

Biosynthesis of BH4, initially characterized by its cofactor role in reactions catalyzed by the aromatic amino acid hydroxylases, proceeds via the de novo pathway involving enzymes GTPCH, 6-pyruvoyl-tetrahydropterin synthase, and sepiapterin reductase (Fig. 1) (18). As the rate-limiting enzyme in BH4 synthesis, GTPCH regulation takes place at the transcriptional and post-translational levels, with activity and mRNA shown to be induced by mediators such as interferon-γ, tumor necrosis factor-α, and lipopolysaccharide (19–21). Indeed, steady-state BH4 levels in cells and tissues have previously been shown to directly correlate with GTPCH mRNA levels. BH4, also a cofactor for NO enzymes, is required for efficient oxidation of arginine and ultimately NO production. When BH4 availability is limiting, eNOS generates superoxide rather than NO, BH4 becomes oxidized to catalytically incompetent BH2, and a futile feed-forward cascade of BH4 destruction proceeds.

A BH2 reductase activity of DHFR was first observed in cell and tissue extracts. In a Chinese hamster ovary cell mutant lacking dihydrofolate reductase (DUKX-BII), endogenous formation of BH4 proceeds normally, but unlike the parent cells that express DHFR, extracts do not convert sepiapterin or BH2 to BH4 (22). These studies implicate the biopterin recycling pathway in the regulation of steady-state BH4 levels. Our data suggest that this BH2 reductase activity of DHFR is crucial in determining cellular BH4 homeostasis, NO bioavailability, and
ultimately eNOS coupling. The marked accumulation of BH2, resulting from DHFR inhibition or knockdown, would compete with BH4 for eNOS binding, diminish NO production, and elevate the synthesis of eNOS-derived superoxide. The lack of any change in total biopterin levels by DHFR inhibition or knockdown suggests that de novo synthesis of BH4 is unaffected. In contrast, GTPCH-specific siRNA diminished total intracellular biopterins by more than 90% with no change in BH4:BH2 ratio. Although elevated production of eNOS-derived superoxide was observed in GTPCH siRNA-treated cells, previous data from our group has shown this to be because of the reduced BH4:eNOS stoichiometry, not a decrease in BH4:BH2 ratio (7). Combined knockdown of GTPCH and DHFR in sEnd.1 endothelial cells provides insight into a differing significance or importance of DHFR activity at high versus low levels of total biopterin. When combined, DHFR- and GTPCH-specific knockdown resulted in significantly elevated eNOS-derived superoxide when compared with either treatment alone, possibly as a result of the increased accumulation of BH2 and diminished ratio of BH4:BH2.

To complement this finding we specifically tested the role of DHFR in eNOS coupling using cell lines with tetracycline-regulated expression of human GTPCH and an eNOS–GFP fusion protein (GCH and GCH/eNOS cells) (7) that are characterized by a doxycycline-induced decrease in BH4:BH2 ratio in the presence but not the absence of eNOS. Herein, we demonstrate that this eNOS uncoupling is further exacerbated in the absence of DHFR activity, as evidenced by MTX-induced superoxide production in GCH/eNOS cells that are inhibitable by l.-NAME. These data would suggest that GTPCH protein is the principal enzyme determining overall production of BH4, whereas DHFR maintains the BH4 pool in a reduced state, limiting the accumulation of oxidized biopterins. Because BH4 recycling by DHFR will simultaneously increase BH4 and reduce BH2, the impact of DHFR on BH4:BH2 ratio is substantial, conferring DHFR as having a potentially critical role in regulating eNOS coupling and ultimately eNOS function. Indeed, DHFR has recently been shown to have other regulatory effects on eNOS that may complement our observations. Michel and co-workers (8) have shown that perturbation of BH4 metabolism differentially affects BH4 levels and eNOS phosphorylation sites. Knockdown of DHFR by siRNA inhibits vascular endothelial growth factor-induced dephosphorylation of eNOS at Ser-116, an effect that is completely recovered by the addition of exogenous BH4. DHFR would have important consequences for the development of endothelial dysfunction in vascular disease states; our work supports and adds to the important observations of Chalupsky and Cai (2), who reported that oxidative stimuli such as angiotensin II can reduce DHFR activity in endothelial cells, leading to eNOS uncoupling.

Experiments using endothelium-targeted eNOS overexpressing mice have indicated that increased eNOS protein alone is not sufficient to maintain endothelial function in vascular disease. Kawashima and co-workers (23) reported that overexpression of eNOS in an apo-E KO background paradoxically resulted in increased vascular superoxide production, at least in part from uncoupled eNOS, which was prevented when supplementary BH4 was administered, or when these mice were further crossed with endothelium-targeted GTPCH transgenic mice (24). Although these data describe BH4 deficiency as a result of oxidation to BH2, evidence from this study and the observation that DHFR levels are diminished in mouse models of diabetes may suggest that insufficient recycling of BH2 to BH4 by DHFR is at least in part responsible, allowing the accumulation of BH2 and eNOS uncoupling to occur. Indeed, DHFR protein levels are significantly decreased in streptozotocin-induced diabetic mice, and diabetes-induced impairment of myocyte function is exacerbated after treatment of the mice with MTX (25). Furthermore, because of the lack of DHFR activity, adult cardiac myocytes have limited capacity to synthesize BH4 after cytochrome stimulation following treatment of rat cardiac allograft recipients with sepiapterin (26). An insufficiency of DHFR may also explain results obtained in hypercholesterolemic rabbits where prolonged exposure to sepiapterin impaired vasorelaxation despite reppletion of endogenous BH4 (27).

Pharmacological supplementation of BH4 improves endothelium-dependent relaxations and augments NO-mediated effects on forearm blood flow in smokers and those with diabetes and elevated cholesterol (28–30). However, inefficient utilization of the administered BH4 remains problematic (31, 32). Recent studies in cells and in vivo suggest that DHFR is required for biotoperin transport. BH4 accumulation in various tissues after supplementation with BH4, BH2, or sepiapterin can be inhibited by MTX; indeed after MTX treatment the increase in tissue biotoperin was almost exclusively BH2 (33). It was concluded that the elevation in BH4 by supplementation was mainly through a “salvage pathway” that included BH2 as the key intermediate in the production of BH4 through the action of dihydrofolate reductase. It is, therefore, likely that DHFR expression is critical to cells that do not contain the apparatus required for efficient synthesis of BH4. BH2 may be transported from cells such as endothelial cells, reduced back to BH4 by DHFR, and made available for the synthesis of NO or catecholamines.

NO homeostasis is tightly regulated, and sufficient NO production by the endothelium is required to maintain normal endothelial function. Because of the essential functions of DHFR and tetrahydrofolates, the DHFR inhibitor, MTX, has become the most widely used single therapeutic agent for cancer (1, 2) and the preferred therapeutic for rheumatoid arthritis (34, 35). Although little is known about the potential effects of MTX on the vasculature, one adverse effect of MTX treatment and DHFR inhibition may be because of the role of DHFR in regulating nitric oxide availability and biotoperin metabolism. Perhaps patients receiving MTX therapy should be administered BH4 to supplement possibly deficient levels and maintain vascular function. Accordingly, therapeutic strategies to augment endothelial BH4 levels and/or suppress intracellular BH2 accumulation are rational approaches to maintain eNOS coupling in vascular conditions.

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