Quantitative Regulation of Intracellular Endothelial Nitric-oxide Synthase (eNOS) Coupling by Both Tetrahydrobiopterin-eNOS Stoichiometry and Biopterin Redox Status

INSIGHTS FROM CELLS WITH TET-REGULATED GTP CYCLOHYDROLASE I EXPRESSION

Received for publication, July 16, 2008, and in revised form, November 14, 2008. Published, JBC Papers in Press, November 14, 2008, DOI 10.1074/jbc.M805403200

Mark J. Crabtree, Amy L. Tatham, Yasir Al-Wakeel, Nicholas Warrick, Ashley B. Hale, Shijie Cai, Keith M. Channon, and Nicholas J. Alp

From the Department of Cardiovascular Medicine, University of Oxford, John Radcliffe Hospital, Oxford OX3 9DU, United Kingdom

Tetrahydrobiopterin (BH4) is a critical determinant of endothelial nitric-oxide synthase (eNOS) activity. In the absence of BH4, eNOS becomes “uncoupled” and generates superoxide rather than NO. However, the stoichiometry of intracellular BH4/eNOS interactions is not well defined, and it is unclear whether intracellular BH4 deficiency alone is sufficient to induce eNOS uncoupling. To address these questions, we developed novel cell lines with tet-regulated expression of human GTP cyclohydrolase I (GTPCH), the rate-limiting enzyme in BH4 synthesis, to selectively induce intracellular BH4 deficiency by incubation with doxycycline. These cells were stably co-transfected to express a human eNOS-green fluorescent protein fusion protein, selecting clones expressing either low (GCH/eNOS-LOW) or high (GCH/eNOS-HIGH) levels. Doxycycline abolished GTPCH mRNA expression and GTPCH protein, leading to markedly diminished total biopterin levels and a decreased ratio of BH4 to oxidized biopterins in cells expressing eNOS. Intracellular BH4 deficiency induced superoxide generation from eNOS, as assessed by N-nitro-L-arginine methyl ester inhibitable 2-hydroxyethidium generation, and attenuation from eNOS, as assessed by N-nitro-L-arginine methyl ester inhibition of eNOS activity. In the absence of exogenous oxidative stress.

Nitric oxide, produced in the endothelium by endothelial nitric-oxide synthase (eNOS), is a critical regulator of vascular homeostasis (1, 2). NO has multiple antiatherogenic roles: inhibiting vascular smooth muscle cell proliferation, platelet aggregation, and leukocyte adhesion (1). Simultaneous loss of NO bioavailability and elevated production of superoxide is a hallmark of several vascular disease states including hypercholesterolemia, hypertension, diabetes, and throughout the progression of atherosclerosis (3–5).

The balance between NO and superoxide production by eNOS appears to be determined by the availability of its essential co-factor tetrahydrobiopterin (BH4) (6). Intracellular BH4 levels are regulated by the activity of the de novo biosynthetic pathway. Guanosine triphosphate cyclohydrolase I (GTPCH) catalyzes the formation of dihydrolipoic acid from GTP, and BH4 is generated by two further steps through 6-pyruvyltetrahydropterin 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 (7). 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 (8–10). In this reaction BH4 forms the protonated trihydrobiopterin cation radical, which is subsequently reduced by electron transfer from NOS flavins (9). When BH4 availability is limiting, electron transfer from NOS flavins becomes uncoupled from l-arginine oxidation, the ferrous-dioxygen complex dissociates, and superoxide is produced from the oxygenase domain (6, 11). In addition, BH4 also promotes eNOS dimer stabilization (12), which is required for enzymatic activity. In the apolipoprotein E knock-out mouse model of hypercholesterolemia, eNOS mRNA and protein levels remain unchanged or may even be increased compared with wild type litter mate controls. In these animals transgenic overexpression of eNOS paradoxically increases vascular superoxi-
ide production because of enzymatic uncoupling, which is reversed when mice are crossed with animals overexpressing GTPCH (13, 14). Recent focus has been toward the redox state of BH4 in disease models and the oxidation of BH4 to form 7,8-dihydrobiopterin (BH2) and biopterin, both incapable of NO-mediated effects on forearm blood flow in smokers and those with diabetes and elevated cholesterol (16–19). However, this may be due to nonspecific scavenging of superoxide by high dose BH4 treatment. The mechanisms that relate BH4 availability to eNOS activity in vivo are not fully understood, and approaches to develop BH4 as a therapeutic target remain speculative unless a better understanding of the BH4-eNOS stoichiometric relationship can be gained. Indeed, no study has demonstrated that diminished BH4 availability is solely sufficient to induce eNOS uncoupling in vivo. Accordingly, we sought to address these questions by developing novel stably transfected cell lines with both doxycycline-regulated expression of human GCH cDNA and expression of eNOS-GFP fusion protein. Using these cell lines we investigated the role of intracellular BH4 availability in eNOS coupling and report that both intracellular eNOS:BH4 reaction stoichiometry with the intracellular BH4:BH2 ratio are key determinants of eNOS uncoupling.

**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. In the presence of doxycycline, binding of the transactivator is blocked, and gene expression is prevented. These initial 3T3-tet-off cells, previously shown to express neither eNOS nor GTPCH (20) and also confirmed to be devoid of neuronal NOS, inducible NOS, and eNOS protein as tested by Western blotting (supplemental Fig. S1), were stably transfected with a plasmid encoding hemagglutinin (HA) antigen-tagged-human GCH1 under the control of a tetracycline-responsive element, as shown in supplemental Fig. S2. Individual colonies were isolated and analyzed for GCH1 expression and a cell line, termed “GCH cells,” was established from expansion of a single colony. GCH cells were stably transfected with a plasmid encoding a human eNOS-eGFP fusion protein (21). Clones were picked that expressed either low (GCH/eNOS-LOW) or high (GCH/eNOS-HIGH) levels. All of the cell lines underwent three rounds of clonal selection.

*Cell Culture*—The cells were cultured in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with glutamine (2 mmol/liter), 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 gentamicin (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 levels in cell lysates were determined by HPLC followed by electrochemical and fluorescent detection, as described previously (22). Briefly, the cells were grown to confluency and harvested by trypsinization. Sample pellets were resuspended in phosphate-buffered saline (50 mmol/liter), pH 7.4, containing dithioerythritol (1 mmol/liter) and EDTA (100 µmol/liter) and subjected to three free-thaw cycles. Following centrifugation (15 min at 13,000 rpm and 4 °C), the samples were transferred to new, cooled microtubes and precipitated with cold phosphoric acid (1 mol/liter), trichloroacetic acid (2 mol/liter), and dithioerythritol (1 mmol/liter). The samples were vigorously mixed and then centrifuged for 15 min at 13,000 rpm and 4 °C. The samples were injected onto an isotropic HPLC system and quantified using sequential electrochemical (Coulochem III, ESA Inc.) and fluorescence (Jasco) detection. HPLC separation was performed using a 250 mm, ACE C-18 column (Hichrom) and mobile phase comprising of sodium acetate (50 mmol/liter), citric acid (5 mmol/liter), EDTA (48 µmol/liter), and dithioerythritol (160 µmol/liter) (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 biopterin was done by comparison with authentic external standards and normalized to sample protein content.

**GTPCH Activity Assay**—GTPCH activity was assessed by HPLC, as described previously (23).

*Western Blotting*—Cells were suspended in radioimmune precipitation assay lysis buffer (20 mmol/liter Tris-HCl, 150 mmol/liter NaCl, 1 mmol/liter Na2EDTA, 1 mmol/liter 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-thaw cycles in liquid nitrogen. Western blotting was carried out using standard techniques and anti-eNOS, -inducible NOS, -neuronal NOS (BD Transduction Laboratories), -HA, -DHFR, -SOD, and -GAPDH antibodies. The rabbit anti-rat GTPCH antibody was kindly provided by Prof. Steven S. Gross of Weill Medical College of Cornell University.

**Quantitative Reverse Transcription-PCR**—Reverse transcription was carried out using SuperScript II (Invitrogen) on 1 µg of total cell RNA obtained by TRIzol extraction. Quantitative PCR was performed with an iCycler IQ real time detection system (Bio-Rad) with primers and probes from the gene expression assays system as supplied by Applied Biosystems.

**Measurement of eNOS Protein Levels by eGFP Fluorescence**—Cell pellets were lysed in phosphate-buffered saline containing dithioerythritol (1 mmol/liter) and EDTA (100 µmol/liter) 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.
Regulation of eNOS Coupling by BH4

Quantification of Superoxide Production by HPLC—Measurement of 2-hydroxyethidium formation by HPLC was used to quantify superoxide production, by methods adapted from those described previously (24, 25). The cells were washed three times in phosphate-buffered saline and incubated in Krebs-Hepes buffer in the presence or absence of L-NAME (100 μmol/liter). After 1 h, dihydroethidium (25 μmol/liter) was added, and the cells were then incubated for an additional 20 min at 37 °C. The cells were then harvested by scraping, centrifuged, and lysed in ice-cold methanol. Hydrochloric acid (100 mmol/liter) was added (1:1 v/v) prior to loading into the autosampler for analysis. All of the 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, 4.5 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 biopterin (0.085% trifluoroacetic acid in acetonitrile) over 23 min (30–50% acetonitrile).

Nitrite and Nitrate Determination—Following preincubation for 1 h in the presence or absence of L-monomethylarginine (100 μmol/liter) in Krebs-Henseleit buffer (consisting of 120 mmol/liter NaCl, 4.7 mmol/liter KCl, 1.2 mmol/liter MgSO4, 1.2 mmol/liter KH2PO4, 2.5 mmol/liter CaCl2, 25 mmol/liter NaHCO3, and 5.5 mmol/liter glucose), the cells were exposed to A23187 (1 μmol/liter) for 30 min. Total nitrite and nitrate accumulation was measured using the CLD88 NO analyzer (Ecophysics).

Analysis of NO Synthesis by eNOS—Cellular NO synthesis by eNOS was assessed by measuring the conversion of 14C L-arginine to citrulline with HPLC detection, in the presence and absence of N-monomethyl-L-arginine, as described previously (26).

Accumulation of 3-NT by Enzyme-linked Immunosorbent Assay—Intracellular accumulation of 3-NT as an indicator of peroxynitrite production was done using a specific 3-NT enzyme-linked immunosorbent assay kit (Millipore) as in the manufacturer’s instructions.

GCH Knockdown by RNA Interference—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: Duplex 1, GGUAGAAUGCUAAGACGUU; Duplex 2, CGAGAAGUGGCCUACGUA; Duplex 3, GAGAAGGGGAAUCCGCUUU; and Duplex 4, AGUAGUGAUAAGCGCACA. sEnd.1 endothelial cells were grown in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with glutamine (2 mmol/liter), penicillin (100 units/ml), and streptomycin (0.1 mg/ml). 24 h prior to transfection, the cells were seeded into 6-well plates. The cells were then transfected with GCH-specific siRNA (100 nmol/liter), GAPDH-positive (100 nmol/liter) or nonspecific pooled duplex negative control siRNA (100 nmol/liter). The cells were cultured for 72 h, and gene silencing was detected by analysis of GTPCH protein expression by Western blotting using GTPCH-specific antibodies.

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

RESULTS

Characterization of Doxycycline-induced BH4 Deficiency—We first investigated the response of the stably transfected cell lines to doxycycline exposure. Quantitative reverse transcription-PCR revealed maximal inhibition of GCH1 mRNA production following 3 h of doxycycline exposure (Fig. 1A) and confirmed that GCH1 transgene expression was equal in GCH and GCH/eNOS cells (Fig. 1B). Quantification of intracellular GTPCH activity by HPLC revealed greater than 98.5% inhibition following doxycycline exposure of GCH, GCH/eNOS-LOW, and GCH/eNOS-HIGH cells (Fig. 1C). Immunoblotting showed abundant eNOS protein in GCH/eNOS-HIGH cells, comparable with sEnd.1 endothelial cells and 5-fold greater than GCH/eNOS-LOW cells, whereas GCH cells expressed no detectable eNOS protein. Using anti-HA and anti-human GCH antibodies, transgenic GCH protein was undetectable following incubation with doxycycline for 10 days. Murine GCH protein was detected only in sEnd.1 murine endothelial cells, as expected (Fig. 1D). To determine whether doxycycline exposure induced compensatory or confounding alterations in anti-oxidant enzymes that might alter measurements of superoxide, we measured the protein levels of relevant enzymes by immunoblotting. Importantly, we observed that neither eNOS expression nor treatment of cells with doxycycline induced changes in the levels of antioxidant enzymes, including superoxide dismutase, catalase, and dihydrodololate reductase (Fig. 1E). Cellular levels of both GTPCH and eNOS protein in the presence and absence of doxycycline was confirmed by immunocytochemistry with fluorescence microscopy (Fig. 2). eNOS-eGFP fluorescence was observed in GCH/eNOS, but not in GCH cells. eNOS-eGFP fluorescence appeared to be localized to the plasma membrane and Golgi apparatus, and fluorescence intensity was markedly increased in GCH/eNOS-HIGH versus GCH/eNOS-LOW cells, consistent with the results from immunoblotting. Interestingly, eNOS-eGFP localization in GCH/eNOS-HIGH cells appeared to be localized in the cytoplasm as well as to the Golgi and plasma membrane. Similar anti-HA immunoreactivity, detecting GTPCH protein, was demonstrated in GCH, GCH/eNOS-LOW, and GCH/eNOS-HIGH cells, and this was no longer detectable following incubation with doxycycline. Doxycycline exposure had no effect on eNOS-GFP fluorescence among all cell types.

To evaluate the effects of reduced GTPCH activity on biopterin generation, we compared BH4 levels in GCH and GCH/eNOS cells with and without exposure to doxycycline. Intracellular BH4 levels were markedly suppressed following 3 days of treatment with doxycycline (p < 0.001) and remained suppressed thereafter (Fig. 3A). To assess the oxidative state of BH4-deficient cells, we quantified the BH4 oxidation product, BH2. A 3-fold increase in BH2 levels was observed in...
compared cell lines with four different eNOS:BH4 ratios, as shown in Table 1. Treatment of GCH/eNOS-LOW cells with doxycycline changed the eNOS:BH4 ratio from $0.17 \pm 0.02$ to $2.36 \pm 0.21$ ($p < 0.001$). In GCH/eNOS-HIGH cells, doxycycline altered the eNOS:BH4 ratio from $1.18 \pm 0.18$ to $7.10 \pm 2.17$ ($p < 0.01$). We now compared the eNOS:BH4 stoichiometry obtained in these conditions with eNOS enzymatic function by determination of NO versus superoxide generation.

**eNOS Enzymatic Activity: Nitric Oxide and Superoxide Generation**—To investigate whether the reduced BH4:eNOS ratio led to enzymatic uncoupling of eNOS, we measured superoxide production by the quantification of 2-hydroxyethidium fluorescence formed following exposure of cells to dihydroethidium. Low level superoxide production was detected in all cell types. Interestingly, a modest decrease in BH4 levels induced by doxyxycline was sufficient to increase superoxide production from GCH cells lacking eNOS (Fig. 4A). Importantly, this was not observed in control cells that do not contain tetracycline-regulated GCH expression, therefore suggesting that BH4 plays a general antioxidant role in our model. eNOS-dependent superoxide production, determined by co-incubation with the NOS inhibitor L-NAME, was markedly elevated in GCH/eNOS-LOW and was further exacerbated in GCH/eNOS-HIGH cells in the presence of doxycycline, suggesting eNOS uncoupling ($p < 0.01$). Moreover, L-NAME inhibitable 2-hydroxyethidium fluorescence (indicative of eNOS-derived superoxide production) positively correlated with increased ratios of eNOS:BH4 and the formation of BH2. By comparing NOS-dependent superoxide generation with the eNOS:BH4 stoichiometry, we observed that superoxide generation is initiated from eNOS when the eNOS:BH4 ratio exceeds one (Fig. 4C). 3-Nitrotyrosine accumulation, indicative of peroxynitrite formation, is shown to be significantly increased in GCH/eNOS-LOW and GCH/eNOS-HIGH cells in the presence of doxycycline, indicating increased oxidative stress in BH4-deficient cells consistent with eNOS uncoupling (Fig. 4D).
Basal NO generation by eNOS (as detected by both the conversion of arginine to citrulline and measurement of NOx accumulation in cell media) was undetectable across all cell types. Following activation in the presence of calcium ionophore (A23187), NO generation by eNOS was significantly elevated in GCH/eNOS-HIGH compared with GCH/eNOS-LOW cells (12.42 ± 0.65 versus 2.14 ± 0.14, p < 0.01), which was markedly attenuated in both cell lines following exposure to doxycycline. No eNOS activity or NOx accumulation was observed in GCH cells (Fig. 5, A and B). In support of these data, accumulation of nitrite (NO2−) and nitrate (NO3−) in cell culture medium revealed that levels of NOx found in media from GCH/eNOS-LOW and GCH/eNOS-HIGH cells treated with doxycycline (BH4-deficient) were substantially lower following exposure to calcium ionophore compared with their basal, nondoxycycline controls (Fig. 5, C and D). This attenuation of NO production by doxycycline treatment provides further evidence of eNOS uncoupling in this cell culture model of BH4 deficiency.

**Intracellular BH4: BH2 Ratio Determines eNOS Coupling**—Recent evidence suggests that intracellular accumulation of BH2, independent of absolute BH4 levels, can lead to eNOS uncoupling by competing with BH4 for binding to the active site of eNOS (8, 27). To determine the importance of intracellular BH2 versus BH4 levels in regulating eNOS coupling, we supplemented the levels of BH2 while “clamping” the absolute intracellular BH4 concentration. We exposed GCH/eNOS-LOW cells to BH2 (1 μmol/liter) either alone, or following pre-incubation with the DHFR inhibitor, methotrexate (MTX; 10 μmol/liter) at 37 °C for 4 h. Incubation of GCH/eNOS-LOW cells with BH2 alone was sufficient to raise intracellular levels of BH4, likely through the action of endogenous DHFR (data not shown). When exposed to BH2 after DHFR inhibition by MTX, BH4 levels remained unchanged, whereas intracellular BH2 levels were increased 25-fold (p < 0.05). Consequently, the ratio of BH4: BH2 was markedly decreased (p < 0.05). This change in BH4: BH2 ratio, in GCH/eNOS-LOW cells, was sufficient to cause an elevated production of eNOS-dependent superoxide (p < 0.05; Fig. 6). These data indicate that the ratio of BH4:BH2 can induce eNOS uncoupling, even in settings where eNOS:BH4 stoichiometry favors eNOS coupling.
Regulation of eNOS Coupling by BH4

GCH RNA Interference-induced eNOS Uncoupling in sEnd.1 Endothelial Cells—Having observed that reduction in GTPCH and BH4 levels was able to induce eNOS-derived superoxide production in our tetracycline-regulatable cell model, it was important to establish whether this phenomenon would also be observed in an endothelial cell line. Following exposure of sEnd.1 endothelial cells to GCH-targeted siRNA for 72 h, GTPCH protein was “knocked down” by ∼70% (Fig. 7A). Correspondingly, intracellular BH4 levels were decreased to a similar degree (275.52 ± 12.33 versus 88.44 ± 23.89 pmol/mg protein: p < 0.05). Importantly, GAPDH knockdown and nontargeted scrambled control siRNAs did not have any effect on either GTPCH protein (Fig. 7A) or BH4 levels (Fig. 7B), and there was no effect of siRNA transfection on eNOS protein levels (Fig. 7A). The effect of deficient BH4 levels on eNOS coupling was determined by measuring L-NAME-inhibitable 2-hydroxyethidium formation as an indicator of superoxide production (Fig. 7C). In accordance with the data from tet-regulatable cells, we observed that in sEnd.1 cells BH4 deficiency alone is sufficient to induce eNOS uncoupling in a mouse endothelial cell line.

DISCUSSION

In this study we describe novel cell lines in which a plasmid expressing tetracycline-regulatable human GCH1 was stably expressed with an eNOS-GFP fusion construct. We selected three experimental cell lines termed GCH, GCH/eNOS-LOW, and GCH/eNOS-HIGH, each with variable ratios of eNOS and BH4. We used this cellular model system to investigate the mechanisms by which BH4 deficiency may directly induce eNOS uncoupling and to determine whether attenuation of BH4 levels alone is sufficient to result in eNOS uncoupling in an intact cell, in the absence of pathological oxidative stress associated with eNOS uncoupling in vascular disease states.

The major findings of this study are as follows. First, we demonstrated that GCH1 mRNA expression was markedly attenuated following 3 h of doxycycline exposure. Second, this abrogation of GCH1 expression was sufficient to substantially decrease intracellular BH4 levels by 3 days. Third, doxycycline-induced BH4 deficiency resulted in attenuation of NO synthesis and induction of eNOS-dependent superoxide production. This ‘pro-oxidant’ switch in cellular redox status resulted in oxidation of BH4 to BH2 and a decrease in the BH4: BH2 ratio. Fourth, the ratio of BH4: BH2 in addition to the absolute molar concentration of BH4 is a key determinant of eNOS coupling in vivo. Finally, we reveal that BH4 deficiency alone, induced by genetic knockdown of GTPCH protein using GCH-specific siRNA, is sufficient to induce uncoupling of eNOS in endothelial cells. Taken together, our findings provide clear evidence that eNOS: BH4 stoichiometry and biopterin redox status are together responsible for determining the degree of eNOS coupling even in the absence of vascular disease or oxidative stress.

These findings provide important insights into the role of BH4 in regulating eNOS activity and eNOS coupling. Previous studies have reported BH4 deficiency in models of vascular disease such as hypercholesterolemia, hypertension, and diabetes (13, 14, 16–19, 28), but no studies have addressed the impact of selective BH4 deficiency on eNOS function or quantitative...
Regulation of eNOS Coupling by BH4

**FIGURE 5. Nitric oxide production from tet-regulatable cells.** A23187-induced conversion of arginine to citrulline and measurement of the accumulation of nitrate and nitrite (NOx) in cell culture medium was used as a measure of eNOS activity and NO production, respectively. A, doxycycline (DOX) had no effect on eNOS activity in GCH cells. In contrast, eNOS activity was attenuated in the presence of doxycycline in both GCH/eNOS-LOW and GCH/eNOS-HIGH cells (*, p < 0.05, n = 6). An example HPLC trace is shown in B. C, doxycycline had no effect on NOx accumulation in media from GCH cells. In contrast, NOx production from GCH/eNOS-LOW (30%) and GCH/eNOS-HIGH (35%) cells was attenuated in the presence of doxycycline (*, p < 0.05). An example trace from the NO analyzer is present in D (n = 6).

In eNOS-Tg mice where BH4 levels are limiting (eNOS-Tg/ApoE-KO), superoxide production is markedly elevated compared with wild type and eNOS-Tg littermates. However, eNOS overexpression in the presence of saturating levels of BH4 does not appear to elevate superoxide production (eNOS-Tg and eNOS/GCH-Tg) (32). In the present study we have specifically manipulated the relative expression of eNOS and GCH1, leading to a range of eNOS:BH4 ratios in a cell-based system. Our results confirm the previous findings in eNOS-Tg and eNOS/GCH-Tg mice. Indeed, the degree of BH4 oxidation, BH2 accumulation, and superoxide production directly correlated with the intracellular ratio of eNOS:BH4.

Our results from this reductionist cell culture model revealed that a moderate decrease in BH4 alone is sufficient to increase superoxide levels in GCH cells, without eNOS. This was not observed in control 3T3 cells lacking tet-regulatable GCH protein; therefore any effects of doxycycline on superoxide production in GCH cells must be dependent specifically on BH4 deficiency, but not on eNOS. Accordingly, we conclude that BH4 can have substantial effects on the levels of cellular reactive oxygen species production through mechanisms independent of eNOS, such as direct reactive oxygen species scavenging. However, superoxide production was further increased by doxycycline in GCH/eNOS-LOW and GCH/eNOS-HIGH cells, which was inhibitable with L-NAME. Taken together, this suggests that general antioxidant properties of BH4 are required for direct scavenging of superoxide and maintenance of intracellular redox balance. Moreover, these data demonstrate that eNOS-dependent superoxide production occurs in addition to basal superoxide levels and overwhelms cellular antioxidant defenses. This is surprising because superoxide reacts with BH4 in vitro at a rate constant >10,000-fold slower (3.9 × 10⁵ mol liter⁻¹ s⁻¹) than its near diffusion limited reaction with NO (6.7 × 10⁹ mol liter⁻¹ s⁻¹) (33, 34). Accordingly, NO should markedly outcompete BH4 for reaction with superoxide. Peroxynitrite formed by the NO/superoxide reaction could then oxidize BH4 as described previously (35) and thereby promote eNOS uncoupling. EPR has revealed that peroxynitrite oxidizes BH4 to BH2 via the nonprotonated BH3 radical with a rate constant estimated to be 6.0 × 10³ mol liter⁻¹ s⁻¹, severalfold higher than reactions between peroxynitrite, ascorbate, and intracellular thiols (36).

eNOS:BH4 reaction stoichiometry in the absence of oxidative stress. Other studies demonstrating the requirement for BH4 to maintain eNOS coupling have relied on purified recombinant proteins in reconstituted cell-free systems (6, 29–31). However, the quantitative relationship between eNOS coupling and BH4 has not been investigated in a living system in the absence of pathological oxidative stress. NO homeostasis is tightly regulated, and sufficient NO production by the endothelium is required to maintain normal endothelial function. 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 coworkers (13) reported that overexpression of eNOS in an ApoE-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 GCH-Tg mice (14). These data may be explained by the changing ratio of eNOS:BH4 across these animal models, but the precise quantitative stoichiometry could not be measured in these models.
Because the $K_m$ of BH4 and BH2 for eNOS are similar (~80 nM), previous studies have speculated that BH2 competes with BH4 for binding to eNOS, thus promoting eNOS uncoupling and superoxide production (8). Indeed, a recent study has demonstrated that BH2 is capable of displacing BH4 prebound to eNOS and that diminished BH4:BH2 ratio rather than a decrease in the absolute amount of BH4 determines NO and superoxide production by eNOS in endothelial cells following exposure to increased glucose concentrations (27). The present study now extends this hypothesis. In GCH/eNOS-LOW cells supplemented with BH2, intracellular BH4 levels were found to increase without any elevation in BH2 levels, possibly because of endogenous DHFR activity (data not shown). In the presence of the DHFR inhibitor MTX, we were able to manipulate the intracellular ratio of BH4 versus BH2 while effectively clamping BH4 concentrations. The resulting increase in BH2 and the subsequent 25-fold decrease in BH4:BH2 ratio resulted in a marked elevation in eNOS-derived superoxide production despite no change in BH4 levels (Fig. 6). Importantly, genetic knockdown of GTPCH protein expression was able to induce uncoupling of eNOS in endothelial cells as evidenced by the elevated production of superoxide that was inhibitable by L-NAME. This was striking because despite endothelial cells still containing substantial amounts of BH4 (88.44 ± 23.89 pmol/mg protein) following GCH1 knockdown, eNOS-derived superoxide production was increased as the ratio of eNOS:BH4 was decreased.

Taken together, these data provide clear evidence that eNOS coupling is determined by the eNOS:BH4 reaction stoichiometry and the ratio of BH4:BH2, even in the absence of pathological disease states.

Different therapeutic strategies have been studied to restore both reduced BH4 availability and impaired vascular function in the setting of oxidant stress. High dose BH4 supplementation augments NO-mediated effects on forearm blood flow in patients, but this may due, at least in part, to nonspecific anti-
Regulation of eNOS Coupling by BH4

oxidant nature of BH4 and direct scavenging of superoxide (37). In small scale clinical studies of high dose antioxidant treatment, vitamin C improves endothelial function in patients with hypercholesterolemia (38) and diabetes (39). However, larger scale randomized trials of antioxidants with several years of follow-up were unsuccessful and did not detect any significant benefit in terms of vascular end points (40). Accordingly, detailed understanding of the mechanisms of eNOS uncoupling are required to inform translational therapeutic approaches.

In the present study BH4 deficiency markedly elevated the production of eNOS-derived superoxide. Together with the attenuated production of NO by BH4-deficient cells, these data provide clear evidence for direct uncoupling of eNOS by BH4 deficiency in this cell-based model. Our data suggest a direct and pivotal role for BH4 availability in the regulation of eNOS function. Accordingly, therapeutic strategies to augment endothelial BH4 levels and/or suppress intracellular BH2 accumulation are rational approaches to maintain eNOS coupling in vascular disease.

Acknowledgments—We thank Dr. Joe de Bono for stimulating discussion of the project and for critical reading and assessment of this manuscript. We are grateful to Prof. Steven S. Gross of Weill Medical College of Cornell University for the kind gift of rabbit anti-rat GTPCH antibody.

REFERENCES

1. Ignarro, L. J. (2002) J. Physiol. Pharmacol. 53, 503–514
2. Furchgott, R. F., and Zawadzki, J. V. (1980) Nature 288, 373–376
3. Panza, J. A., Garcia, C. E., Kilcoyne, C. M., Quyyumi, A. A., and Cannon, R. O., III (1995) Circulation 91, 1732–1738
4. Ohara, Y., Peterson, T. E., and Harrison, D. G. (1993) J. Clin. Investig. 91, 2546–2551
5. White, C. R., Brock, T. A., Chang, L.-Y., Crapo, J., Briscoe, P., Ku, D., Bradley, W. A., Gianturco, S. H., Gore, J., Freeman, B. A., and Tarpey, M. M. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 1044–1048
6. Vasquez-Vivar, J., Kalyanaraman, B., Martasek, P., Hogg, N., Masters, B. S., Karoui, H., Tordo, P., and Pritchard, K. A., Jr. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 9220–9225
7. Cai, S., Khoo, J., and Channon, K. M. (2005) Cardiovasc. Res. 65, 823–831
8. Vasquez-Vivar, J., Martasek, P., Whitsett, J., Joseph, J., and Kalyanaraman, B. (2002) Biochem. J. 362, 733–739
9. Hurshman, A. R., Krebs, C., Edmondson, D. E., Huynh, B. H., and Marletta, M. A. (1999) Biochemistry 38, 15689–15696
10. Schmidt, P. P., Lange, R., Gorren, A. C., Werner, E. R., Mayer, B., and Andersons, K. K. (2001) J. Biol. Inorg. Chem. 6, 151–158
11. Stroes, E., Hjimering, M., van Zandvoort, M., Wever, R., Rabelink, T. J., and van Faassen, E. E. (1998) FEBS Lett. 438, 161–164
12. Cai, S., Khoo, J., Mussa, S., Alp, N. J., and Channon, K. M. (2005) Diabetes 48, 1933–1940
13. Ozaki, M., Kawashima, S., Yamashita, T., Hirase, T., Namiki, M., Inoue, N., Hirata, K.-i., Yasui, H., Sakurai, H., Yoshida, Y., Masada, M., and Yokoyama, M. (2002) J. Clin. Investig. 110, 331–340
14. Alp, N. J., McAteer, M. A., Khoo, J., Choudhury, R. P., and Channon, K. M. (2004) Arterioscler. Thromb. Vasc. Biol. 24, 445–450
15. Antoniades, C., Shirodaria, C., Warrick, N., Shijie, C., DeBono, J., Lee, I., Leeson, P., Neubauer, S., Ratnatunga, C., Pillar, R., Refsum, H., and Channon, K. (2006) Circulation 114, 1193–1201
16. Heitzer, T., Brockhoff, C., Mayer, B., Warnholtz, A., Mollnau, H., Henne, S., Meierertz, T., and Munzel, T. (2000) Circ. Res. 86, E36–E41
17. Stroes, E., Kastelein, J., Cosentino, F., Ekerlenkas, W., Wever, R., Koomans, H., Luscher, T., and Rabelink, T. (1997) J. Clin. Investig. 99, 41–46
18. Fukuda, Y., Teragawa, H., Matsuda, K., Yamagata, T., Matsuura, H., and Chayama, K. (2002) Heart 87, 264–269
19. Heitzer, T., Krohn, K., Albers, S., and Meierertz, T. (2000) Diabetologia 43, 1435–1438
20. Tseng, E., Billiar, T. R., Robbins, P. D., Lofus, M., and Stuehr, D. J. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 11711–11715
21. McDonald, D. M., Alp, N. J., and Channon, K. M. (2004) Pharmacogenetics 14, 831–839
22. Heales, S., and Hyland, K. (1989) J. Chromatogr. 494, 77–85
23. Werner-Felmayer, G., and Gross, S. S. (1996) in Methods in Nitric Oxide Research (Feilish, M., and Stamler, J. S., eds) pp. 271–299, John Wiley & Sons, Inc., New York
24. Zhao, H., Joseph, J., Fales, H. M., Sokoloski, E. A., Levine, R. L., Vasquez-Vivar, J., and Kalyanaraman, B. (2005) Proc. Natl. Acad. Sci. U. S. A 102, 5727–5732
25. Fink, B., Laude, K., McCann, L., Doughan, A., Harrison, D. G., and Dikalov, S. (2004) Am. J. Physiol. 287, C995–C902
26. de Bono, J., Warrick, N., Bendall, J., Channon, K. M., and Alp, N. (2007) Nitric Oxide 16, 1–9
27. Crabtree, M. J., Smith, C. L., Lam, G., Goligorsky, M. S., and Gross, S. S. (2008) Am. J. Physiol. 294, H1530–H1540
28. Landmesser, U., Dikalov, S., Price, S. R., McCann, L., Fukai, T., Holland, S. M., Mitch, W. E., and Harrison, D. G. (2003) J. Clin. Investig. 111, 1201–1209
29. Vasquez-Vivar, J., Martasek, P., and Kalyanaraman, B. (2004) in Biological Magnetic Resonance, pp. 75–91, Kluger Academic Publishers, Amsterdam
30. Vasquez-Vivar, J., Kalyanaraman, B., and Martasek, P. (2003) Free Radic. Res. 37, 121–127
31. Rodriguez-Crespo, I., Gerber, N. C., and Ortiz de Montellano, P. R. (1996) J. Biol. Chem. 271, 11462–11467
32. Bendall, J. K., Alp, N. J., Warrick, N., Cai, S., Adlam, D., Rockett, K., Yokoyama, M., Kawashima, S., and Channon, K. M. (2005) Circ. Res. 97, 864–871
33. Vasquez-Vivar, J., Whitsett, J., Martasek, P., Hogg, N., and Kalyanaraman, B. (2001) Free Radic. Biol. Med. 31, 975–985
34. Huie, R. E., and Padmaja, S. (1993) Free Radic. Res. Commun. 18, 195–199
35. Milstien, S., and Katusic, Z. (1999) Biochem. Biophys. Res. Commun. 263, 681–684
36. Kuzkaya, N., Weissmann, N., Harrison, D. G., and Dikalov, S. (2003) J. Biol. Chem. 278, 22546–22554
37. Kojima, S., Ona, S., lizuka, I., Arai, T., Mori, H., and Kubota, K. (1995) Free Radic. Res. 23, 419–430
38. Ting, H. H., Timimi, F. K., Haley, E. A., Roddy, M. A., Ganz, P., and Creager, M. A. (1997) Circulation 95, 2617–2622
39. Timimi, F. K., Ting, H. H., Haley, E. A., Roddy, M. A., Ganz, P., and Creager, M. A. (1998) J. Am. Coll Cardiol. 31, 552–557
40. Heart Protection Study Group (2002) Lancet 360, 23–33