Tetrahydrobiopterin Recycling, a Key Determinant of Endothelial Nitric-oxide Synthase-dependent Signaling Pathways in Cultured Vascular Endothelial Cells*[S]

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Tetrahydrobiopterin (BH4) is a key redox-active cofactor in endothelial isoform of NO synthase (eNOS) catalysis and is an important determinant of NO-dependent signaling pathways. BH4 oxidation is observed in vascular cells in the setting of the oxidative stress associated with diabetes. However, the relative roles of *de novo* BH4 synthesis and BH4 redox recycling in the regulation of eNOS bioactivity remain incompletely defined. We used small interference RNA (siRNA)-mediated "knockdown" GTP cyclohydrolase-1 (GTPCH1), the rate-limiting enzyme in BH4 biosynthesis, and dihydrofolate reductase (DHFR), an enzyme-recycling oxidized BH4 (7,8-dihydrobiopterin (BH2)), and studied the effects on eNOS regulation and biopterin metabolism in cultured aortic endothelial cells. Knockdown of either DHFR or GTPCH1 attenuated vascular endothelial growth factor (VEGF)-induced eNOS activity and NO production; these effects were recovered by supplementation with BH4. In contrast, supplementation with BH2 abolished VEGF-induced NO production. DHFR but not GTPCH1 knockdown increased reactive oxygen species (ROS) production. The increase in ROS production seen with siRNA-mediated DHFR knockdown was abolished either by simultaneous siRNA-mediated knockdown of eNOS or by supplementing with BH4. In contrast, addition of BH2 increased ROS production; this effect of BH2 was blocked by BH4 supplementation. DHFR but not GTPCH1 knockdown inhibited VEGF-induced dephosphorylation of eNOS at the inhibitory site serine 116; these effects were recovered by supplementation with BH4. These studies demonstrate a striking contrast in the pattern of eNOS regulation seen by the selective modulation of BH4 salvage/reduction *versus de novo* BH4 synthetic pathways. Our findings suggest that the depletion of BH4 is not sufficient to perturb NO signaling, but rather that concentration of intracellular BH2, as well as the relative concentrations of BH4 and BH2, together play a determining role in the redox regulation of eNOS-modulated endothelial responses.

Regulation of endothelial nitric oxide (NO)[2] production represents a critical mechanism for the modulation of vascular homeostasis. NO is released by endothelial cells in response to diverse humoral, neural, and mechanical stimuli (1–4). Endothelial cell-derived NO activates guanylate cyclase in vascular smooth muscle cells, leading to increased levels of cGMP and to smooth muscle relaxation. Blood platelets represent another key target for the actions of endothelium-derived NO (5): platelet aggregation is inhibited by NO-induced guanylate cyclase activation. Many other effects of NO have been identified in cultured vascular cells and in vascular tissues, including the regulation of apoptosis, cell adhesion, angiogenesis, thrombosis, vascular smooth muscle proliferation, and atherogenesis, among other cellular responses and (patho)physiological processes.

The endothelial isoform of NO synthase (eNOS) is a membrane-associated homodimeric 135-kDa protein that is robustly expressed in endothelial cells (2, 4, 6, 7). Similar to all the mammalian NOS isoforms, eNOS functions as an obligate homodimer that includes a cysteine-complex Zn^{2+} (zinc-tetrathiolate) at the dimer interface (8–10). eNOS is a Ca^{2+}/calmodulin-dependent enzyme that is activated in response to the stimulation of a variety of Ca^{2+}-mobilizing cell surface receptors in vascular endothelium and in cardiac myocytes. The activity of eNOS is also regulated by phosphorylation at multiple sites (11) that are differentially modulated following the activation of cell surface receptors by agonists such as insulin and vascular endothelial growth factor (VEGF) (12). The phosphorylation of eNOS at Ser-1179 activates eNOS, but phosphorylation at Thr-497 or Ser-116 is associated with inhibition of eNOS activity (13–17). eNOS is reversibly targeted to plasmalemmal caveolae as a consequence of the protein's *N*-myristoylation and thiopalmitoylation. The generation of NO by eNOS requires several redox-active cofactors, including nicotinamide adenine dinucleotide phosphate (NADPH), flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), calmodulin, and tetrahydrobiopterin (BH4), which have key roles in the electron flow required for eNOS catalysis. If the flow

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[2] The abbreviations used are: NO, nitric oxide; eNOS, endothelial isoform of nitric-oxide synthase; BH4, 5,6,7,8-tetrahydrobiopterin; BH2, 7,8-dihydrobiopterin; siRNA, small interfering RNA; GTPCH-1, guanosine triphosphate cyclohydrolase-1; DHFR, dihydrofolate reductase; ROS, reactive oxygen species; BAEC, bovine aortic endothelial cell; DAHP, 2,4-diamino-6-hydroxypyrimidine; VEGF, vascular endothelial growth factor; ANOVA, analysis of variance.

of electrons within eNOS is disrupted, the enzyme is uncoupled from NO production and other redox-active products are generated, including hydrogen peroxide and superoxide anion radical (18, 19).

In vascular disease states such as diabetes, endothelial dysfunction is characterized by a decrease in NO bioactivity and by a concomitant increase in superoxide formation, while eNOS mRNA and protein levels are maintained or even increased. "Uncoupled" eNOS generates reactive oxygen species (ROS), shifting the nitroso-redox balance and having adverse consequences in the vascular wall (20). Several enzymes expressed in vascular tissues contribute to the production and efficient degradation of ROS, and an enhanced activity of oxidant enzymes and/or reduced activity of antioxidant enzymes may cause oxidative stress. Various agonists, pathological conditions, and therapeutic interventions lead to modulated expression and function of oxidant and antioxidant enzymes. However, the intimate relationship between intracellular redox state, eNOS regulation, and NO bioavailability remains incompletely characterized.

BH4 is a key redox-active cofactor for activity of all NOS enzymes (21). The exact role of BH4 in NOS catalysis is not yet completely defined, but this cofactor appears to facilitate electron transfer from the eNOS reductase domain and maintains the heme prosthetic group of the enzyme in its redox-active form (18, 22, 23). Moreover, BH4 promotes formation of active NOS homodimers (24) and inhibits the formation of hydrogen peroxide or superoxide by uncoupled eNOS (18, 19). It has been reported that the endothelial dysfunction associated with diabetes is accompanied a decrease in the abundance of bioactive BH4. Supplementation with BH4 has been shown to improve endothelial function in the models of diabetes and hypertension (25, 26, 27). Moreover, BH4 oxidation is seen in vascular cells in the setting of oxidative stress associated with diabetes (28) and hypertension (29).

BH4 can be formed either by a *de novo* biosynthetic pathway or by a salvage pathway. Guanosine triphosphate cyclohydrolase-1 (GTPCH1) catalyzes the conversion of GTP to dihydroneopterin triphosphate. BH4 is generated by further steps catalyzed by 6-pyruvoyltetrahydropterin synthase and sepiapterin reductase (30). GTPCH1 appears to be the rate-limiting enzyme in BH4 biosynthesis; overexpression of GTPCH1 is sufficient to augment BH4 levels in cultured endothelial cells (31). On the other hand, dihydrofolate reductase (DHFR) catalyzes the regeneration of BH4 from its oxidized form, 7,8-dihydrobiopterin (BH2), in several cell types (30, 32). DHFR is mainly involved in folate metabolism and converts inactive BH2 back to BH4 and plays an important role in the metabolism of exogenously administered BH4. However, the relative contributions of endothelial GTPCH1 and DHFR to the modulation of eNOS-dependent pathways are incompletely understood.

In these studies, we have used siRNA-mediated "knockdown" of GTPCH1 and DHFR to explore the relative roles of BH4 synthesis and recycling in the modulation of eNOS bioactivity, as well as in the regulation of NO-dependent signaling pathways in endothelial cells.

EXPERIMENTAL PROCEDURES

Materials—Fetal bovine serum was from HyClone Laboratories (Logan, UT). Lipofectamine™ 2000, Amplex™ Red reagent, and most cell culture reagents were from Invitrogen. Polyclonal antibodies against phospho-Akt (Ser-473), Akt, and phospho-eNOS (Ser-1179) were from Cell Signaling Technology, Inc. (Danvers, MA). Polyclonal antibody against phospho-Ser-116-eNOS was from Upstate Biotechnology (Lake Placid, NY). Monoclonal antibodies against eNOS and DHFR were from BD Transduction Laboratories (Lexington, KY). Polyclonal antibody against GTPCH1 was from Novus Biologicals (Littleton, CO). The SuperSignal chemiluminescence detection reagents and secondary antibodies conjugated with horseradish peroxidase were from Pierce. Tetrahydro-L- biopterin (BH4), 7,8-dihydro-L-biopterin (BH2), and L-biopterin were from Cayman Chemical (Ann Arbor, MI). VEGF was from Calbiochem. Protein concentrations were determined using protein assay kits from Bio-Rad Laboratories (Philadelphia, PA). Determinations of protein abundance using immunoblot analyses were quantitated using a ChemiImager HD4000 (AlphaInnotech, San Leandro, CA). All other reagents were from Sigma.

Culture and Treatment of Cells—Bovine aortic endothelial cells (BAECs) were obtained from Cambrex (Walkersville, MD) and maintained in culture on gelatin-coated 100-mm culture dishes with Dulbecco's modified Eagle's medium supplemented with fetal bovine serum (10% v/v) as described previously (33). BAECs are used for experiments between passages 6 and 8, and serum-starved overnight before experiments. Treatments with VEGF and preparation of cell lysates were performed as described previously (34), with corresponding vehicle treatments as controls.

siRNA Preparation and Transfection—Consistent with previous work (35), we designed an siRNA duplex targeting bovine GTPCH1 mRNA (GTPCH1 siRNA, sequence 5′-CCGUGAC-GAGCACAAUGUU-3′, corresponding to bases 856–874 from the open reading frame of the bovine GTPCH1 mRNA; GenBank™ accession number XM_001251704.2). DHFR siRNA (sequence 5′-CCCAGAACAUGGGCAUCGGC-3′, corresponding to bases 527–546 from the open reading frame of the human DHFR mRNA; GenBank™ accession number NM_000791.3) was characterized in an earlier report (36). The siRNA duplex oligonucleotides were from Ambion, Inc. (Austin, TX). The nonspecific control siRNA 5′-AUUGUAUGC-GAUCGCAGAC-dTdT-3′ was from Dharmacon, Inc. (Lafayette, CO). We found that optimal conditions for siRNA knockdown involved transfecting BAECs when cells were at 50–70% confluence, and transfected cells were maintained in Dulbecco's modified Eagle's medium/10% fetal bovine serum; transfections with siRNA (30–45 nM) used Lipofectamine™ 2000 (0.15%, v/v) and followed protocols provided by the manufacturer (Invitrogen). Fresh medium was added 5 h after transfection, and experiments were typically conducted 48 h after transfection.

Measurement of Intracellular Levels of Biopterins—Oxidized and reduced forms of biopterins were analyzed by the differential oxidation method of Fukushima and Nixon (37). The whole procedure was performed in the dark. BAECs were washed and

suspended in cold extract buffer (0.1 M phosphoric acid, 5 mM dithiothreitol), and protein concentration was measured using the Bio-Rad protein assay. Proteins were removed by adding 35 μl of 2 M trichloroacetic acid to 300 μl of the extracts, followed by centrifugation. To determine total biopterins (BH4, BH2, and biopterin) by acid oxidation, 100 μl of cell extract was mixed with 15 μl of 0.2 M trichloroacetic acid and 15 μl of 1% iodine in 2% KI in 0.2 M trichloroacetic acid. To determine BH2 and biopterin by alkali oxidation, 15 μl of 1 M NaOH was added to 100 μl of extract, followed by addition of 15 μl of 1% iodine/2% KI in 3 M NaOH. After incubation at room temperature for 1 h in the dark, excess iodine was reduced by adding 25 μl of fresh ascorbic acid (20 mg/ml). After centrifugation, 10 μl of supernatant was injected into a high-performance liquid chromatography system (Agilent 1100 series, Agilent Technologies, Palo Alto, CA) equipped with a 150- × 0.32-mm ODS Hypersil column (Thermo Scientific, Waltham, MA), and coupled to a helium-cadmium laser-induced highly sensitive fluorescent detector (325 nm laser, series 56, Melles Griot, Carlsbad, CA; ZETALIF detector, Model LIF-SA-03, Picometrics, Ramonville, France), as described in Ref. 38. The mobile phase was methanol:doubly distilled H_2O (5:95, v/v) with a flow rate of 400 μl/min that was reduced to 4 μl/min with a precolumn flow splitter (100:1, series 620, Analytical Scientific Instruments, El Sobrante, CA) before laser-induced fluorescence detection. The criteria used for identification of biopterin were fluorescence, and retention time was compared with the standards. BH4 concentration, expressed as picomoles per milligram of protein, was calculated by subtracting BH2 plus biopterin from total biopterins.

eNOS Activity Assay— eNOS activity was quantified as the formation of L-[³H]citrulline from L-[³H]arginine as described before (16). Briefly, the reactions are initiated by adding L-[³H]arginine (10 μCi/ml, diluted with unlabeled L-arginine to give a final concentration of 10 μM) plus various drug treatments; each treatment was performed in triplicate cultures, which were analyzed in duplicate.

Measurement of NO$_2^-$—NO production from cells was measured by using an NO sensor (BioStat, ESA, Inc., Chelmsford, MA). Cell culture medium was replaced with Dulbecco's phosphate-buffered saline, and various drugs were added as indicated. After incubation for varying times, aliquots were removed and added to the reagent solution (100 mM H_2SO_4, 100 mM NaI) with the NO sensor. The amount of redox current generated is directly proportional to the NO_2^- concentration in the solution. The absolute NO_2^- concentrations were calculated against a standard curve prepared by using $NaNO_2$ solution. Cells were then harvested to determine protein concentration, permitting NO_2^- production to be reported as picomoles per mg of protein.

Measurement of H$_2$O$_2$—H_2O_2 production from cells was quantitated using the Amplex™ Red fluorescence assay using previously described methods (39). Cell culture medium was replaced with Amplex Red reaction mixture, which consists of 50 μM Amplex Red, 0.1 unit/ml horseradish peroxidase, and 10 units/ml superoxide dismutase diluted in Dulbecco's phosphate-buffered saline (10 units/ml superoxide dismutase is sufficient to convert all cell-derived superoxide to hydrogen per-
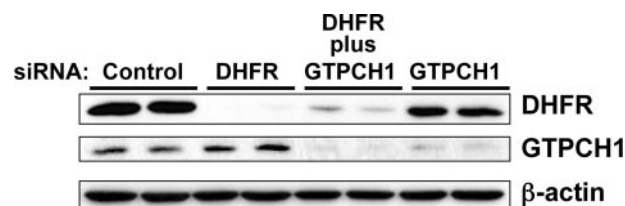

FIGURE 1. **siRNA-mediated knockdown of biopterin-metabolizing proteins in cultured endothelial cells.** Shown is a representative immunoblot of cell lysates prepared from BAECs transfected with duplex siRNA constructs targeted against DHFR (30 nM), GTPCH1 (45 nM), and/or a random sequence (*Control*). The total concentrations of siRNA for each transfection were brought up to 75 nM by adding control siRNA as needed. 48 h after transfection, cells were harvested, and protein expression was analyzed in immunoblots probed with antibodies as shown. This figure is representative of five similar experiments that yielded equivalent results.

oxide; data not shown). After incubation for varying times as indicated, aliquots were withdrawn and fluorescence was quantitated using a FluoroMax-2 spectrofluorometer (JY-Spex, Edison, NJ) at an excitation wavelength of 530 nm and an emission wavelength of 590 nm. The absolute H_2O_2 concentrations were calculated against a standard curve prepared by using H_2O_2 solution. Cells were then harvested to determine protein concentration, permitting H_2O_2 production to be reported as picomoles per mg of protein.

Immunoblot Analysis—BAEC lysates were prepared using a cell lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 0.025% sodium deoxycholate, 1 mM EDTA, 2 mM Na_3VO_4, 1 mM NaF, 2 μg/ml leupeptin, 2 μg/ml antipain, 2 μg/ml soybean trypsin inhibitor, and 2 μg/ml lima trypsin inhibitor). Immunoblot analyses of endothelial protein expression and phosphorylation were assessed as described previously in detail (34). Quantitative densitometric analyses of immunoblots were performed using a ChemImager HD4000 (AlphaInnotech).

Statistical Analysis—All experiments were performed at least three times. Mean values for individual experiments are presented as mean ± S.E. Statistical differences were assessed by ANOVA or t test when appropriate. A p value of less than 0.05 was considered significant.

RESULTS

siRNA-mediated Down-regulation of DHFR and GTPCH1 Expression in BAECs—To selectively knockdown the expression of DHFR and GTPCH1 protein in BAECs, we transfected siRNA duplex-targeting constructs, as described in detail under "Experimental Procedures." After validating the specificity of these DHFR and GTPCH1 siRNA constructs and optimizing experimental conditions for optimal protein knockdown, we determined the effects of "double knockdown" of these proteins transfecting both DHFR and GTPCH1 siRNA-targeting constructs in BAECs (Fig. 1). We analyzed immunoblots probed for DHFR, GTPCH1, or β-actin in cells transfected with DHFR siRNA and/or GTPCH1 siRNA constructs; the same quantity of siRNA was used in each transfection using nonspecific siRNA as indicated. DHFR protein expression was efficiently and specifically knocked down by transfection with DHFR siRNA, co-transfected either with control siRNA or GTPCH1-specific siRNA. Similarly, GTPCH1 expression was efficiently knocked down by transfection with GTPCH1 siRNA along with

TABLE 1

Intracellular levels of biopterins in siRNA-transfected endothelial cells supplemented with BH4 or BH2

This table shows the concentrations of total biopterins and BH4 in endothelial cells transfected with control, DHFR, or GTPCH1 siRNA and then supplemented with control vehicle or pterins as shown. 48 h following siRNA transfections, the cells were incubated with vehicle, BH4 (10 μM), or BH2 (10 μM) for 1 h, and intracellular levels of BH4 and total biopterins were assessed by differential oxidation method and HPLC analysis, as described in the text. Each datum represents the mean $\pm$ S.E. derived from three independent experiments, each performed in duplicate.

Supplement	Biopterins concentration					
	Control siRNA		DHFR siRNA		GTPCH1 siRNA	
	[Total biopterin]	[BH4]	[Total biopterin]	[BH4]	[Total biopterin]	[BH4]
	pmol/mg protein					
Vehicle	3.8 $\pm$ 0.5	3.0 $\pm$ 0.3	4.6 $\pm$ 1.3	1.3 $\pm$ 0.5[a]	0.7 $\pm$ 0.1[b]	0.5 $\pm$ 0.1[a]
BH4	22.5 $\pm$ 3.3[b]	18.7 $\pm$ 3.6[a]	20.4 $\pm$ 3.1[b]	14.4 $\pm$ 2.3[a]	23.4 $\pm$ 6.6[b]	20.2 $\pm$ 5.3[a]
BH2	17.7 $\pm$ 3.6[b]	6.6 $\pm$ 3.5	19.1 $\pm$ 2.8[b]	2.5 $\pm$ 2.0	20.1 $\pm$ 3.2[b]	5.1 $\pm$ 3.9

[a] $p < 0.01$ *versus* the BH4 concentration in control siRNA-transfected cells supplemented with vehicle (p values determined by ANOVA).
[b] $p < 0.01$ *versus* total biopterins concentration in control siRNA-transfected cells incubated with vehicle.
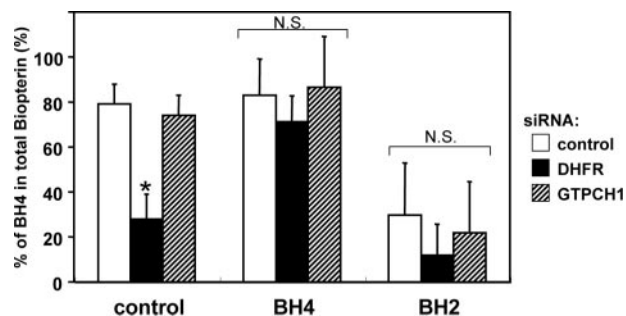

FIGURE 2. **Relative abundance of BH4 following pterin supplementation of siRNA-transfected endothelial cells.** BAECs were transfected with control, DHFR, or GTPCH1 siRNA, and intracellular levels of BH4 and total biopterins were determined by differential oxidation method and HPLC analysis, as detailed under "Experimental Procedures." This *bar graph* shows the percentage of total intracellular biopterins that are present as BH4 in siRNA-transfected BAECs following incubation with BH4 (10 μM) or BH2 (10 μM) for 1 h. Each data point represents the mean $\pm$ S.E. derived from three independent experiments, each performed in duplicate. The *asterisk* indicates $p < 0.01$ *versus* control (vehicle) treatment of siRNA-transfected cells (determined by ANOVA); following supplementation with BH4 and BH2, there were no longer any significant differences ("*N.S.*") in relative BH4 levels observed within groups following siRNA-mediated knockdowns. The absolute concentrations of total biopterins and BH4 are presented in detail in Table 1.

either control siRNA or DHFR-specific siRNA. Co-transfection of the DHFR siRNA along with GTPCH1 siRNA targeting constructs led to marked knockdown of both proteins. Under all experimental conditions, the levels of β-actin protein in cell lysates remained unchanged; as can be seen in this and subsequent figures and as noted in a prior report (36), expression of other endothelial proteins was not affected by transfection of DHFR and/or GTPCH1 siRNA-targeting constructs.

Intracellular Levels of BH4 and BH2 in siRNA-transfected BAECs—We next determined the intracellular concentrations of biopterins following siRNA-mediated knockdown of DHFR or GTPCH1 to determine the effects of these knockdowns on pterin metabolites (Table 1). As would be anticipated from its critical role in pterin synthesis, GTPCH1 knockdown markedly decreased the intracellular concentration of total biopterins, including BH4 and BH2 (82 $\pm$ 1% decrease compared with control siRNA-transfected cells, $n = 3$, $p < 0.01$), whereas the ratio of BH4 to BH2 was not affected (Fig. 2). On the other hand, DHFR knockdown significantly decreased the intracellular BH4 level (56 $\pm$ 10% decrease in BH4 concentration compared with control siRNA-transfected cells, $n = 3$, $p < 0.01$), with no significant change in the amount of total cellular biopterins (Table 1). As shown in Fig. 2, the percentage of BH4 relative to

total cellular biopterins was 79 $\pm$ 9% in control siRNA-transfected cells; 28 $\pm$ 11% in DHFR-knockdown cells ($p < 0.01$ compared with control cells); and 74 $\pm$ 9% in GTPCH1-knockdown cells (p not significantly different from control), respectively. These data indicate that DHFR siRNA-mediated suppression of the BH4 recycling pathway results in a relative increase in cellular BH2 levels. Because a number of studies have shown that administration of BH4 acutely improve endothelial function, we examined the effects of supplementation of BAECs with BH4 and BH2 on the intracellular levels of biopterins. As shown in Table 1, supplementation with BH4 (10 μM for 1 h) induced marked increases in total intracellular biopterins in treated cells. The decrease in BH4 levels seen with siRNA-mediated DHFR knockdown was recovered to the same levels as control cells following supplementation with BH4, as shown in Fig. 2 and Table 1. Supplementation with BH2 (10 μM, 1 h) also yielded marked increases in the levels of total biopterins (Table 1). However, as shown in Fig. 2, the proportion of BH4 in the BH2-treated cells was significantly and uniformly decreased compared with BH4-supplemented cells ($n = 3$, $p < 0.01$), independent of siRNA transfection (Table 1).

Effects of siRNA-mediated DHFR or GTPCH1 Knockdown or GTPCH1 Enzyme Inhibition on VEGF-induced NO Production in BAECs—To explore the effects of alterations of BH4 metabolism on eNOS bioactivity in endothelial cells, we measured eNOS enzyme activity (assaying the formation of [^{3}H]citrulline from L-[^{3}H]arginine) and NO production (using an electrochemical NO sensor) in endothelial cells transfected with DHFR or GTPCH1 siRNA. siRNA-mediated knockdown of either DHFR or GTPCH1 markedly suppressed VEGF-induced NO production by 87 $\pm$ 5% ($n = 8$, $p < 0.01$) or 95 $\pm$ 6% ($n = 8$, $p < 0.01$), respectively (Fig. 2A), and also blocked VEGF-induced eNOS enzyme activity (assayed by the formation of [^{3}H]citrulline from [^{3}H]arginine) by 85 $\pm$ 3% ($n = 4$, $p < 0.01$). Similarly, treatment of endothelial cells with GTPCH1 inhibitor 2,4-diamino-6-hydroxypyrimidine (10 mM) for 24 h markedly decreased VEGF-induced NO production by 91 $\pm$ 4% ($n = 4$, $p < 0.01$) (Fig. 3B) and also completely blocked eNOS enzymatic activity as assayed by the formation of [^{3}H]citrulline from [^{3}H]arginine ($n = 4$, $p < 0.01$). Supplementation of BAECs with BH4 (10 μM) completely restored the DHFR or GTPCH1 siRNA-mediated attenuation of NO production to the same level as control siRNA-transfected cells. In contrast, supplementation with the oxidized metabolite BH2 (10 μM) com-
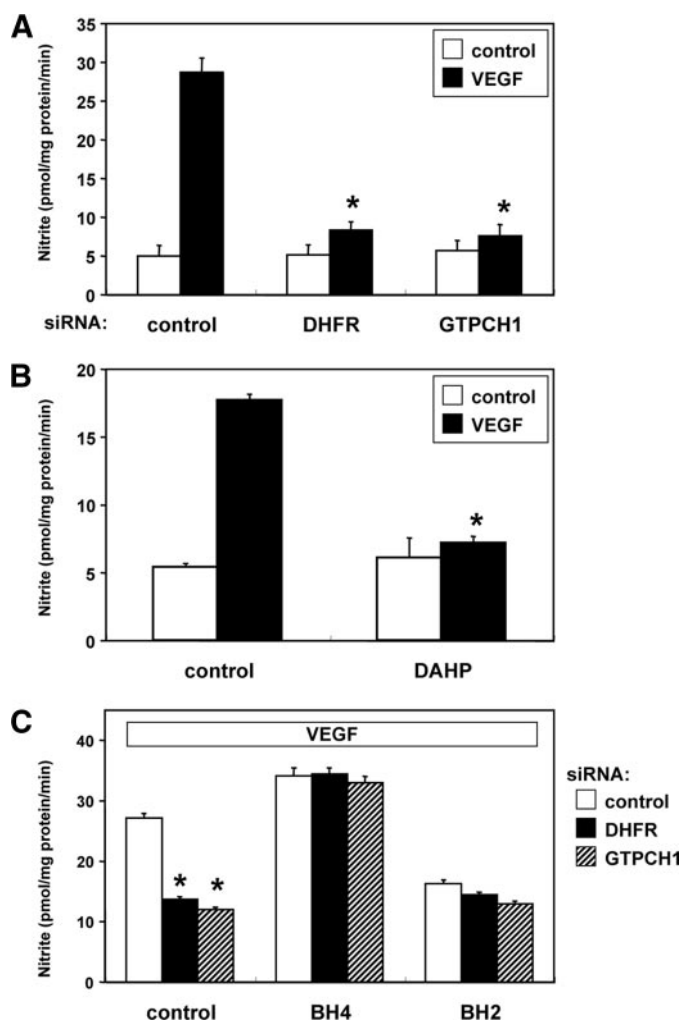
FIGURE 3. **Effects of siRNA-mediated DHFR or GTPCH1 knockdown or GTPCH1 enzyme inhibition on VEGF-induced NO production.** BAECs were incubated with VEGF (20 ng/ml) in Dulbecco's phosphate-buffered saline for 30 min, and NO_2^- accumulations in Dulbecco's phosphate-buffered saline were determined with an electrochemical NO sensor, as detailed under "Experimental Procedures." Each data point represents the mean ± S.E. derived from four to eight independent experiments. The addition of VEGF yielded a significant increase in NO production in control siRNA-transfected cells ($p < 0.01$) or cells treated with vehicle ($p < 0.01$). A, siRNA-transfected BAECs were assayed for NO production. *, $p < 0.01$ for VEGF *versus* control siRNA-transfected cells (ANOVA). B, BAECs pretreated with 2,4-diamino-6-hydroxypyrimidine (10 mM), an inhibitor of GTPCH1, for 24 h were assayed for NO production. *, $p < 0.01$ for VEGF *versus* cells treated with vehicle (control) (ANOVA). C, siRNA-transfected BAECs were assayed for NO production by incubating the cells with VEGF in the presence or absence of BH4 (10 μM) or BH2 (10 μM). *, $p < 0.01$ *versus* control siRNA-transfected cells (ANOVA).

pletely abolished VEGF-induced NO production (Fig. 3*C*). These data suggest that either an absolute decrease in BH4, or a relative decrease of BH4 concentration relative to BH2, can attenuate eNOS activity and NO production.

Effects of siRNA-mediated Down-regulation of BH4 Metabolic Proteins and Supplementation with BH4 or BH2 on ROS Production from BAECs—To investigate the effects of alterations of BH4 metabolism on eNOS-dependent generation of reactive oxygen species ("eNOS uncoupling"), we measured H_2O_2 production, including superoxide converted by superoxide dismutase from DHFR or GTPCH1 siRNA-transfected BAECs. It should be noted that the basal level of H_2O_2 produc-

tion in different independent experiments shows a ~2-fold variance. This variance in basal H_2O_2 production likely reflects the fact that all these experiments are performed in primary cells, and lot-to-lot variability is inevitably encountered. But within each series of experiments, which were performed independently, the variance was low, and the results shown are statistically significant. Still, caution must be exercised when extrapolating these results to other cellular systems. As can be seen in Fig. 4, siRNA-mediated DHFR but not GTPCH1 knockdown led to a marked increase in basal H_2O_2 production (77 ± 12% increase in H_2O_2 production compared with control siRNA-transfected cells, $n = 8$, $p < 0.01$). In contrast to the striking increase in NO production seen following VEGF treatment (Fig. 3), VEGF had no effect on H_2O_2 production in these cells (Fig. 4*A*). Moreover, the increase in H_2O_2 production seen following siRNA-mediated DHFR knockdown was unchanged by VEGF treatment (Fig. 4*A*). Because VEGF treatment had no effect on H_2O_2 production in these cells, we focused on exploring the basal production of H_2O_2 following perturbations in pterin metabolic pathways. The increase in basal H_2O_2 production seen with siRNA-mediated DHFR knockdown was abolished either by simultaneous siRNA-mediated knockdown of eNOS (Fig. 4*B*), or by supplementing the cells with BH4 (10 μM) (Fig. 4*C*). However, addition of BH2 (10 μM) markedly increased H_2O_2 production by 119 ± 6% ($n = 8$, $p < 0.01$) (Fig. 4*D*); this effect of BH2 on H_2O_2 production was dose-dependently inhibited by BH4 supplementation (Fig. 4*D*). These data suggested that eNOS-dependent ROS generation requires an increase in intracellular BH2 levels, not simply a depletion of BH4.

Differential Modulation of eNOS Signaling Pathways by BH4 Depletion Versus BH4 Oxidation—To determine whether perturbation of BH4 metabolism affects the status of eNOS phosphorylation and its signal transduction, we assessed VEGF-induced Akt phosphorylation and eNOS phosphorylation/dephosphorylation by immunoblotting using antibodies directed against phospho-eNOS at Ser-1179, phospho-eNOS at Ser-116, eNOS, phospho-Akt, and Akt. As shown in Fig. 4, DHFR knockdown completely blocked VEGF-induced eNOS dephosphorylation at the inhibitory phosphoserine residue Ser-116 ($n = 4$, $p < 0.01$) but had no effect on agonist-modulated eNOS phosphorylation at the enzyme-activating serine residue Ser-1179. GTPCH1 knockdown had no effect either on phosphorylation or dephosphorylation of eNOS at these residues. Phosphorylation of Akt was decreased by 85 ± 4% by DHFR knockdown ($p < 0.01$, $n = 4$), but Akt phosphorylation was unaffected by GTPCH1 knockdown (Fig. 5, *A* and *B*). Next, we tested the effects of double knockdown of DHFR and GTPCH1 to confirm that the inhibitory effects of DHFR knockdown on dephosphorylation of eNOS at Ser-116 and phosphorylation of Akt were induced by the accumulation of BH2. As shown in Fig. 6, siRNA-mediated double knockdown of both DHFR and GTPCH1 yielded no effect on VEGF-induced dephosphorylation of eNOS at Ser-116, whereas "single knockdown" of DHFR alone led to the suppression of VEGF-promoted dephosphorylation of eNOS at Ser-116. On the other hand, VEGF-induced phosphorylation of Akt was blocked by double knockdown of DHFR and GTPCH1 as well as by the
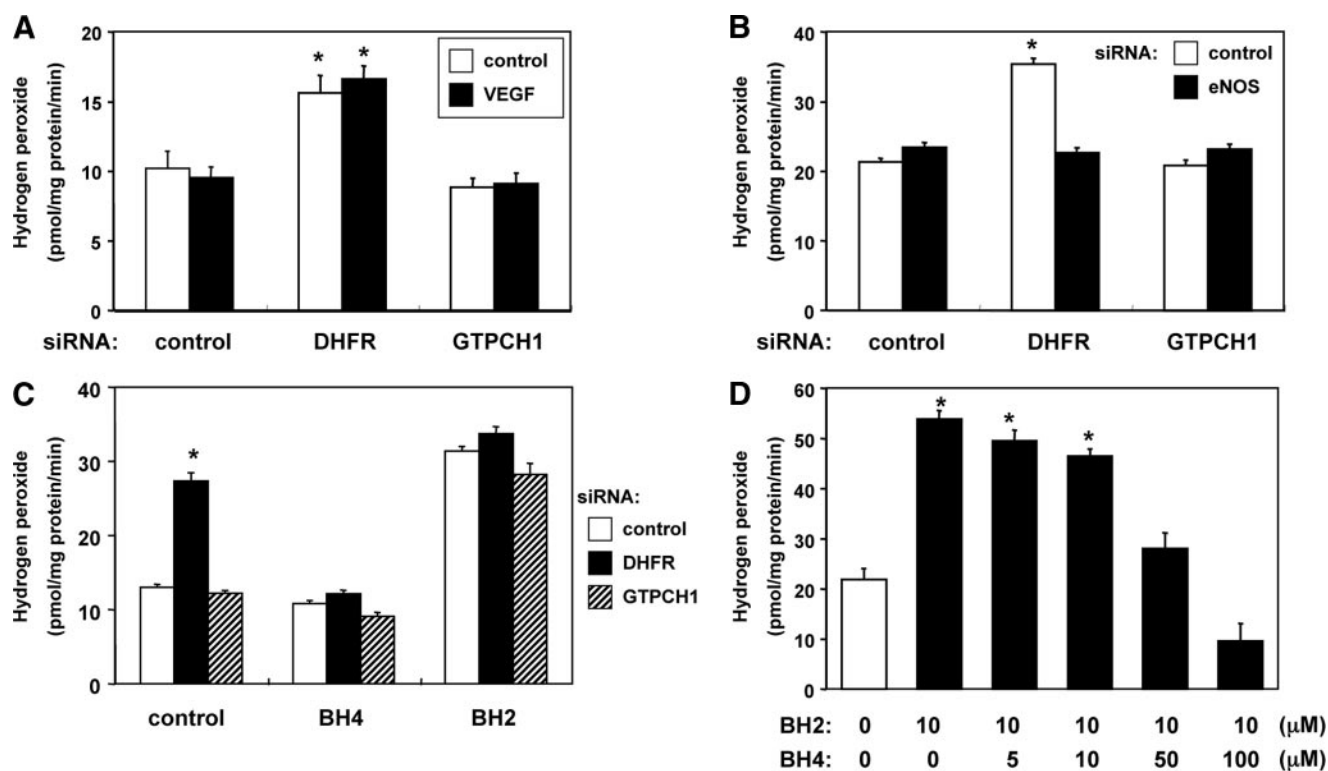
FIGURE 4. **Effects of siRNA-mediated down-regulation of BH4-metabolic proteins and supplementation with BH4 or BH2 on H_2O_2 production from cultured BAECs.** *A*, siRNA-transfected BAECs were assayed for H_2O_2 production in the presence or absence of VEGF (20 ng/ml) by incubating the cells with Amplex Red reaction mixture, and fluorescence was measured at an excitation wavelength of 530 nm and an emission wavelength of 590 nm, as detailed under "Experimental Procedures." *B*, BAECs were transfected with two different duplex siRNAs, targeted against DHFR (30 nM), GTPCH1 (45 nM), or control siRNA plus eNOS (30 nM) or nonspecific control siRNA. Total concentrations of siRNA for each well were aligned to 75 nM by adding control siRNA. 48 h after transfection, siRNA-transfected BAECs were assayed for H_2O_2 production. *C*, siRNA-transfected BAECs were assayed for H_2O_2 production in the presence or absence of BH4 (10 μM) or BH2 (10 μM). *D*, BAECs were assayed for H_2O_2 production in the presence of the indicated concentrations of BH4 and BH2. Each data point represents the mean $\pm$ S.E. derived from four to eight independent experiments. *, $p < 0.01$ *versus* control (ANOVA).

single knockdown of DHFR. These data suggest that the increase in intracellular BH2 levels caused by DHFR knockdown (Table 1) led to an inhibition of dephosphorylation of eNOS at Ser-116, associated with a decrease in eNOS activation (Fig. 3). However, Akt phosphorylation was inhibited by one or more other consequences of siRNA-mediated DHFR knockdown.

Effects of BH2 and BH4 on VEGF-promoted Akt and eNOS Phosphorylation and Dephosphorylation—We next assessed the effects of exogenous BH4 and BH2 on phosphorylation of eNOS and Akt. As shown in Fig. 7, exogenous BH4 completely recovers the VEGF-induced dephosphorylation response that is lost following siRNA-mediated DHFR knockdown (see also Figs. 5 and 6). In contrast, exogenous BH2 significantly inhibited VEGF-induced dephosphorylation in control siRNA-transfected cells: BH2 supplementation effectively replicates the effects of siRNA-mediated DHFR-knockdown (Fig. 7, *A* and *B*). On the other hand, VEGF-induced phosphorylation of Akt was not affected by cell treatments with either BH4 or BH2, and the suppression of VEGF-promoted Akt phosphorylation seen after siRNA-mediated DHFR knockdown was not rescued by BH4 supplementation (Fig. 7, *C* and *D*).

DISCUSSION

Each of the NOS isoforms not only produces NO but can also become uncoupled to produce superoxide and H_2O_2. In endo-

thelial cells, BH4 depletion has been shown to be associated with the production of superoxide by eNOS. However, the relative importance of BH4 synthetic *versus* salvage pathways in endothelial cells has not been established previously. The present study demonstrates a striking contrast in the consequences for eNOS regulation and oxidative stress from the selective modulation of BH4 salvage/reduction *versus* de novo BH4 synthetic pathways.

By using siRNA-mediated knockdown of key proteins involved in BH4 synthesis and recycling, we have been able to assess the differential effects of the simple depletion of cellular pterin levels consequent to GTPCH1 knockdown, *versus* the suppression of BH4 recycling as a consequence of DHFR knockdown. As shown in Fig. 1, transfection of specific duplex siRNAs led to the nearly complete knockdown of DHFR, GTPCH1, or both proteins by their cognate targeting constructs, either alone or in combination. As might be predicted based on the known roles of these proteins in biopterin metabolism, GTPCH1 knockdown led to a marked reduction in overall biopterin levels, whereas DHFR knockdown yielded a marked increase in the abundance of the oxidized product BH2, with no substantial effect on total biopterin levels (Table 1 and Fig. 2). In these experiments, siRNA-mediated GTPCH1 knockdown attenuated total cellular biopterin levels by an average of 82%, which is similar to the magnitude of biopterin sup-
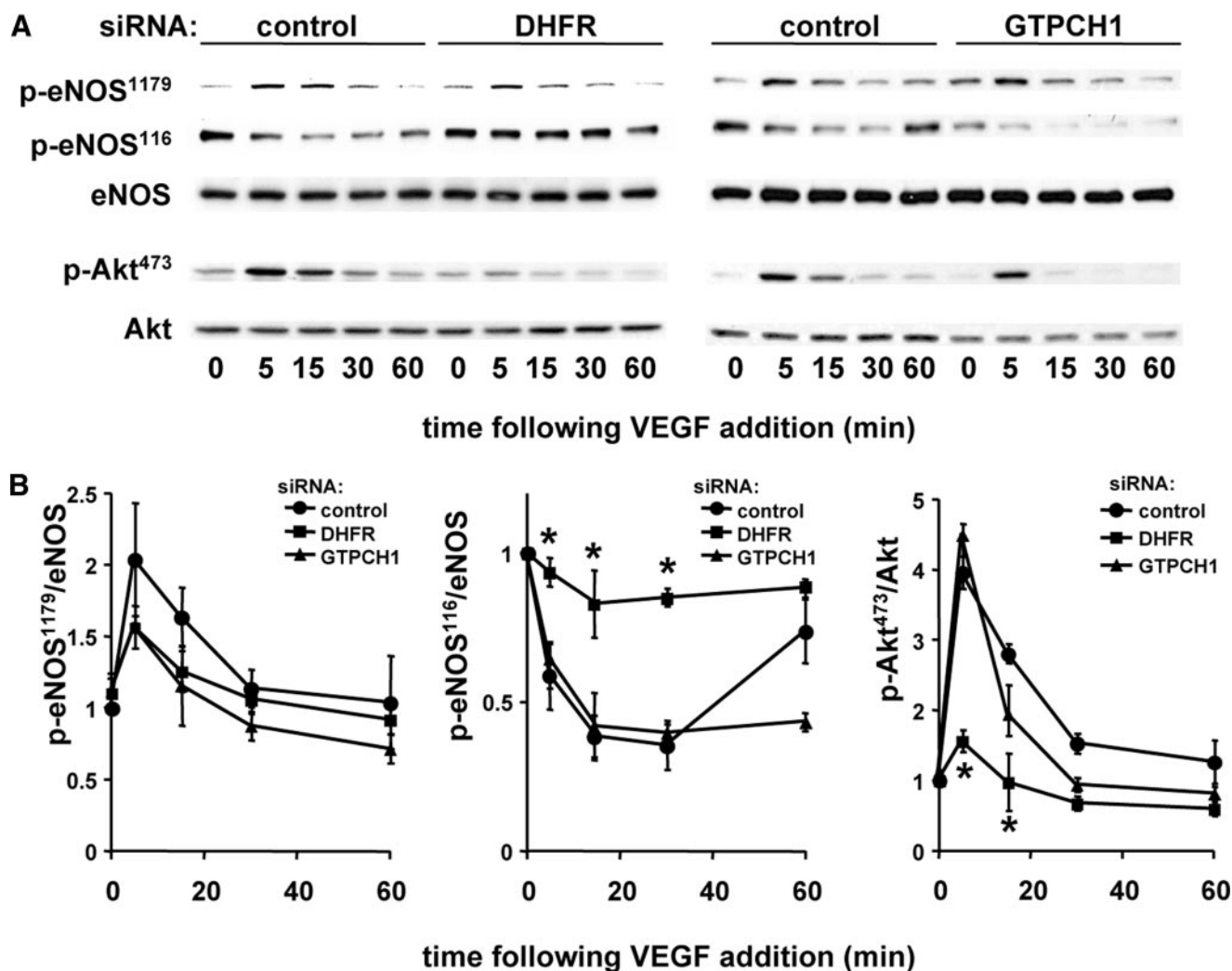
FIGURE 5. **Differential modulation of eNOS signaling pathways by siRNA-mediated DHFR *versus* GTPCH1 knockdown.** *A*, results of immunoblots analyzed in BAEC lysates prepared from cells transfected with control, DHFR, or GTPCH1 siRNA, and then treated with VEGF (20 ng/ml) for the indicated times. Cell lysates were resolved by SDS-PAGE and probed in immunoblots using antibodies directed against phospho-eNOS, total eNOS, phospho-Akt, and total Akt. Shown are the representative data of three independent experiments. *B*, results of densitometric analyses from pooled data, plotting the ratios of phosphorylated eNOS and Akt to total eNOS and Akt, respectively, relative to the signals present in the unstimulated cells. Each data point represents the mean ± S.E. derived from three independent experiments. *, $p < 0.05$ *versus* control siRNA-transfected cells (ANOVA).
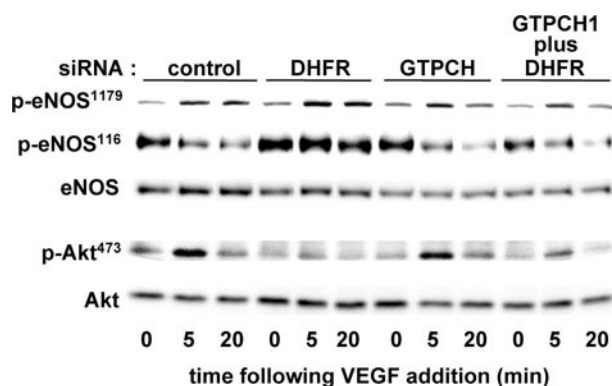

FIGURE 6. **Effects of siRNA-mediated double knockdown of DHFR and GTPCH1 on VEGF-modulated eNOS and Akt phosphorylation.** This figure presents the results of immunoblots analyzed in BAEC lysates prepared from cells transfected with siRNA targeting constructs as shown for control, DHFR, GTPCH1, or DHFR plus GTPCH1 siRNAs. 48 h after transfection, cells were treated with VEGF (20 ng/ml) for the indicated times. Cell lysates were resolved by SDS-PAGE and probed in immunoblots using antibodies directed against phospho-eNOS, eNOS, phospho-Akt, and Akt. The data shown are representative of three independent experiments that yielded equivalent results.

pression seen with the metabolic inhibitor 2,4-diamino-6-hydroxypyrimidine, which typically yields an 80–90% decrease in the level of intracellular biopterins (40–42). In contrast to some other cell types in which BH2 may be transported into cells more efficiently than BH4 (43), the cultured BAECs analyzed in the present study showed a marked increase in intracellular BH4 levels following supplementation with BH4. This increase in intracellular BH4 was seen even following siRNA-mediated DHFR knockdown, indicating that BH4 is likely to be transported as the reduced pterin and does not need to undergo redox cycling before being taking up by these cells.

siRNA-mediated knockdown of either DHFR or GTPCH1 significantly suppressed VEGF-stimulated NO synthesis (Fig. 3). Supplementation of transfected cells with BH4 but not BH2 led to the recovery of NO synthesis following either GTPCH1 or DHFR knockdown (Fig. 3). VEGF promoted a robust increase in NO synthesis (Fig. 3) but had no substantive effect on H_2O_2 production by these cells (Fig. 4*A*). The inhibition of BH4 recycling by DHFR knockdown, which led to the accumu-
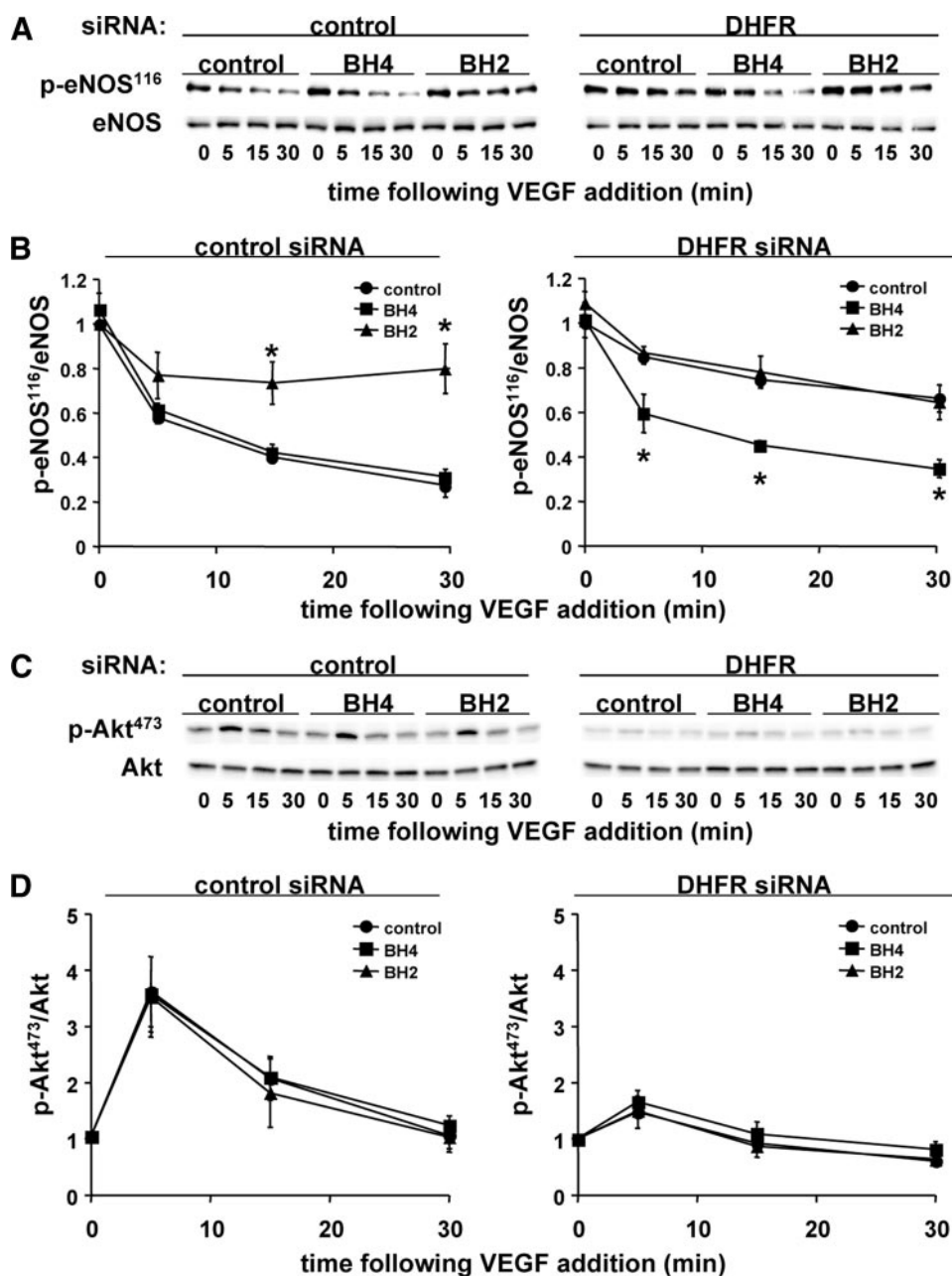
FIGURE 7. **Supplementation with BH4 or BH2: effects on eNOS and Akt phosphorylation responses.** BAECs were transfected with control or DHFR siRNA; 24 h after transfection, the cells were incubated with BH4 (10 μM) or BH2 (10 μM) for another 24 h, and then treated with VEGF (20 ng/ml) for the indicated times and analyzed in immunoblots probed with antibodies as shown. *A* and *C*, results from a representative experiment that was performed three times with equivalent results. *B* and *D*, results of densitometric analyses from pooled data, plotting the ratios of phosphorylated eNOS and Akt to total eNOS and Akt, respectively, relative to the signals present in the unstimulated cells. Each data point represents the mean $\pm$ S.E. derived from three independent experiments. *, $p < 0.05$ *versus* control siRNA-transfected cells (ANOVA).

to be required to see eNOS-dependent H_2O_2 synthesis. Addition of exogenous BH4 reversed the increase in ROS generation seen following siRNA-mediated DHFR knockdown (Fig. 4*C*). By contrast, the addition of exogenous BH2 effectively abolished NO production (Fig. 3*C*) and markedly increased ROS generation (Fig. 4*C*), yet once again these effects are reversed by the addition of BH4. Taken together, these data strongly indicated that BH2 plays a key role in generating ROS from eNOS in cultured endothelial cells, and that the ratio of BH2 to BH4 is a critical determinant of ROS synthesis. It should be noted that the measured concentrations of cell-derived nitrite (Fig. 3) and of cell-derived H_2O_2 (Fig. 4) do not necessarily indicate the actual stoichiometry of NO and H_2O_2 production, because there may be differential cellular metabolism of NO and H_2O_2 before these reactive species can be detected in the extracellular media.

VEGF-induced NO production is suppressed both by a decrease in total cellular biopterin following GTPCH1 knockdown, as well as by a relative decrease in BH4 following DHFR knockdown (Table 1). These findings are consistent with prior observations that BH4 is an essential cofactor for NO synthesis by eNOS (25). However, it should be noted that a marked depletion of cellular pterins is rarely found in pathophysiological states aside from genetic diseases such as phenylketonuria, and BH4 oxidation is more likely to be the underlying cause for decrease of BH4 bioavailability associated with endothelial dysfunction (44, 45). BH4 oxidation is observed in a number of vascular diseases, and although its oxidized product (BH2) cannot itself act as a NOS cofactor, BH2 can antagonize the effects of BH4 by competitive binding to NOS (44f). It has been recently shown that BH2 binds eNOS with an affinity equal to that of BH4 in murine endothelial cells (45).

A number of clinical studies have shown that supplementation with BH4 improves endothelial function in patients with hypercholesterolemia, hypertension, coronary artery disease, or diabetes (25–27). The present study has shown that supplementation of BH4 suppressed the increase in H_2O_2 production

lation of BH2 (Table 1) and a decrease in NO production (Fig. 3), also promoted a striking increase in eNOS-dependent ROS production (Fig. 4); again VEGF had no effect on H_2O_2 synthesis. By contrast, the depletion of total cellular biopterin by GTPCH1 knockdown (Table 1), which led to decrease NO production (Fig. 2), did not increase ROS production from endothelial cells (Fig. 4). These data suggested that decreased NO production and increased ROS production are not intrinsically linked when BH4 levels are depleted: even when BH4 levels are suppressed to the point of blocking NO synthesis, BH2 appears

seen with the increase in cellular BH2 levels following siRNA-mediated knockdown of DHFR (Fig. 4C). These results suggest that a relative increase in the BH2:BH4 ratio, rather than the absolute BH2 concentration, induces uncoupling of eNOS and ROS production. Our data are consistent with a recent study showing that superoxide production from endothelial cells in diabetic models is negatively correlated with intracellular ratio of BH4 to BH2 (45), as well as an *in vitro* study showing that eNOS uncoupling is critically controlled by the ratio between reduced and oxidized BH4 (46). This concept that the BH2:BH4 ratio is critically important for inducing eNOS uncoupling is also consistent with a resent study looking at pterin metabolism and eNOS coupling in a heterologous overexpression system (47). However, this recent study did find that knockdown of GTPCH1 also promoted production of ROS (47), in contrast to our findings in native endothelial cells, in which siRNA-mediated knockdown of DHFR but not GTPCH1 led to H$_2$O$_2$ production. Possibly these differences reflect the study of distinct cell types (native endothelial cells *versus* overexpressing cell lines) or other differences in experimental conditions.

The present study also showed that perturbation of BH4 metabolism differentially affects eNOS phosphorylation sites. eNOS is modulated by enzyme phosphorylation of multiple sites in the protein: phosphorylation at Ser-1179 activates eNOS, but phosphorylation at Thr497 or Ser-116 is associated with inhibition of eNOS activity (13–17). VEGF potently promotes eNOS activity not only by increasing intracellular Ca^{2+}, but also by stimulating kinase Akt and promoting eNOS phosphorylation at Ser-1179. VEGF stimulation of eNOS also involves the dephosphorylation of Ser-116 in a pathway that involves the Ca^{2+}/calmodulin-dependent phosphatase calcineurin (protein phosphatase 2B) (16). Our present studies show that siRNA-mediated DHFR knockdown inhibits VEGF-induced dephosphorylation of eNOS at Ser-116 (Fig. 5); this effect of DHFR knockdown is completely recovered by exogenous BH4. Because phosphorylation of eNOS at Ser-116 inhibits its enzymatic activity, and calcineurin-dependent pathways lead to eNOS dephosphorylation at Ser-116 (16), our current findings lead us to speculate that increases in the cellular BH2:BH4 ratio may modulate the activity of calcineurin pathways. These studies have also shown that siRNA-induced DHFR knockdown inhibits VEGF-induced Akt phosphorylation (Fig. 5). In contrast to the striking effects on VEGF-promoted eNOS Ser-116 dephosphorylation, neither double knockdown of DHFR and GTPCH1 nor supplementation with BH4 or BH2 have any effect on VEGF-stimulated Akt phosphorylation. These results suggest that the inhibitory effect of siRNA-mediated DHFR knockdown on Akt phosphorylation is not caused by an increase in intracellular BH2 levels. Further studies are required to investigate the relationship between BH4 metabolism and the activation of kinase Akt.

These studies also provide important evidence for the critical role of eNOS in the generation of reactive oxygen species from endothelial cells. Following siRNA-mediated knockdown of DHFR in endothelial cells, there was a marked increase in H$_2$O$_2$ production (Fig. 3), associated with an increase in the BH2:BH4 ratio (Table 1). However, this DHFR siRNA-dependent increase in endothelial cell-derived reactive oxygen species was completely blocked by simultaneously knocking down eNOS expression using its cognate siRNA (Fig. 3). These double knockdown experiments, in which eNOS expression can be suppressed along with other critical enzymes involved in eNOS catalysis or NO metabolism, are likely to be broadly informative in studies that examine other aspects of eNOS-dependent signaling pathways in cultured endothelial cells.

Taken together, these studies help to establish the central role of pterin redox recycling in the production of NO by eNOS and suggest that the simple depletion of BH4 is not sufficient to promote endothelial dysfunction. Our findings suggest instead that it is the concentration of intracellular BH2, as well as the relative concentrations of BH4 and BH2, that together play a determining role in the redox regulation of eNOS-modulated endothelial responses.

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