GTP cyclohydrolase I expression is regulated by nitric oxide: role of cyclic AMP

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Nitric oxide (NO) is a labile humoral factor synthesized from the oxidation of the guanidinon nitrogen moiety of L-arginine after activation of nitric oxide synthase (NOS) (42). There are three forms of NOS; constitutive forms are present in endothelial cells (eNOS) and neurons (nNOS), and a third, inducible isoform is present in macrophages (iNOS) (4, 32, 62). Pulmonary vascular endothelial cells synthesize NO after certain stimuli, such as shear stress and the receptor binding of specific vasodilators, activate eNOS (25, 47). Loss of NO production is the causative factor in the development of various pulmonary hypertensive disorders (6, 16). Inhaled NO is a selective, potent pulmonary vasodilator that is currently used as an adjunct therapy for these pulmonary vasculature diseases (36).

However, a number of studies have observed an acute and potentially life-threatening increase in pulmonary vascular resistance (PVR) upon acute withdrawal of inhaled NO. In children with congenital heart disease, this is manifested by an increase in PVR that may compromise cardiac output (2, 39). Nevertheless, in newborns with persistent pulmonary hypertension, there is a sudden decrease in systemic arterial oxygen saturation (26). This rebound pulmonary hypertension can occur after hours of therapy and is independent of the initial response; patients with no initial pulmonary vasodilatory response can have life-threatening pulmonary vasoconstriction upon withdrawal. These manifestations of acute NO withdrawal suggest that inhaled NO may alter endogenous endothelial function, although the mechanism is unclear. Both in vitro and in vivo studies have demonstrated that exogenous NO decreases endogenous eNOS activity and increase in the production of superoxide due to uncoupling of the enzyme, independently of changes in gene expression (3, 37, 50, 60). However, the mechanism for this uncoupling is still unresolved.

The redox sensitive NOS cofactor 5, 6, 7, 8-tetrahydrobiop- terin (BH4) is required for NO synthesis by all NOS isoforms (40). BH4 has profound effects on the structure of NOS, including the ability to shift the heme iron to a high spin state, increase arginine binding, and stabilize the active dimeric form of the enzyme (19, 58). Furthermore, BH4 deficiency correlates with diminished NO production and endothelial dysfunction in cardiovascular diseases such as hypertension and diabetes (33, 52), whereas, clinically, BH4 supplementation leads to an improvement in endothelial function in smokers (22), diabetics, hypertensive subjects (7), and patients with hypercholes- terolemia (55) or coronary artery disease (49).

Given the key role played by BH4 in regulating NO production, we were interested in testing the hypothesis that the uncoupling of eNOS induced by exogenous NO we have found both in vitro and in vivo could be due, at least in part, to a decrease in BH4 levels. However, contrary to our initial hypothesis, we found that exogenous NO caused an increase in BH4 levels both in vitro and in vivo. Furthermore, this was associated with an NO-mediated increase in GTP cyclohydro-lase I (GCH1) expression. We found that the increase was regulated at the levels of GCH1 transcription in a cAMP-CREB (cAMP response element binding protein)-dependent manner. Thus the NO-mediated increase in eNOS uncoupling...
does not appear to be mediated by a decrease in BH₄. Rather, there appears to be a coregulation between NO and BH₄.

MATERIALS AND METHODS

Lamb model of inhaled NO delivery. The inhaled NO lamb model has been reported previously (41, 46). In this lamb model, inhaled NO [40 parts per million (ppm)] was delivered in nitrogen in the inspiratory limb of the ventilator (Inovent; Ohmeda, Liberty, NJ) and continued for 24 h. The inspired concentrations of NO and nitrogen dioxide were continuously quantified by electrochemical methodology (Inovent). The hemodynamic variables were monitored continuously. Systemic arterial blood gases were determined intermittently, and ventilation was adjusted to achieve an arterial partial pressure of CO₂ (PaCO₂) between 35 and 45 Torr and an arterial partial pressure of O₂ (PaO₂) >50 Torr. Sodium bicarbonate was administered intermittently to maintain a pH > 7.30. Normal saline was administered intermittently to maintain stable atrial pressures throughout the study period. Peripheral lung wedge biopsies were performed, and blood was obtained after 24 h of therapy. At the end of the protocol, all lambs were killed with a lethal injection of pentobarbital sodium followed by bilateral thoracotomy, as described in the National Institutes of Health (NIH) Guidelines for the Care and Use of Laboratory Animals. All protocols and procedures were approved by the Committee on Animal Research of the University of California, San Francisco.

Cell culture. COS-7 cells (American Type Culture Collection) were cultured in Dulbecco’s modified Eagle’s-H16 medium (DME-H16) with 4.5-g glucose containing 10% fetal bovine serum and antibiotics (penicillin and streptomycin). Primary cultures of pulmonary arterial endothelial cells from late-gestation fetal lambs (PAECs) were isolated by the explant technique, as we have described previously (61). Endothelial cell identity was confirmed by the typical cobblestone appearance, contact inhibition, specific uptake of acetylated low-density lipoprotein labeled with 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine and positive staining for von Willebrand factor (Dako, Carpinteria, CA). PAECs were studied between passages 3 and 10. All cells were cultured at 37°C in 21% O₂-5% CO₂-balance N₂.

Quantification of BH₄ levels by high-performance liquid chromatography. BH₄ levels were measured by high-performance liquid chromatography (HPLC). Briefly, equal volumes of supernatant were separated into 1% iodine and 2% potassium iodide. The final ratio of chromatography (HPLC). Briefly, equal volumes of supernatant were reprobed with warere (Kodak 1D 3.6). To normalize for protein loading, all blots were reprobed with polyvinylidene difluoride membranes. Immunoblotting was carried out on 4–20% SDS-polyacrylamide gels and transferred to nitrocellulose membranes. Immunoblotting was carried out using an antibody raised against GCH1, as we have previously described (18), in Tris base-buffered saline with 0.1% Tween 20 and 5% BSA. After being washed, the membranes were probed with horseradish peroxidase-conjugated goat antiserum to rabbit or mouse. Reactive bands were visualized using chemiluminescence (SuperSignal West Femto; Pierce) on a Kodak 440CF image station (New Haven, CT). Bands were quantified using Kodak ImageStation software (Kodak ID 3.6). To normalize for protein loading, all blots were reprobed with β-actin.

Transient transfection and promoter activity analysis. The GCH1 promoter constructs 613 GTPCH and 313 GTPCH (a kind gift from Dr Gregory Kapatos, Wayne State University, School of Medicine, Detroit, MI) have been described previously (23). A 4-hp mutation was introduced into the CREB consensus sequence within 313 GTPCH construct. The sequence TGACCGGCA at –89 in the human GCH promoter (23) was changed to TGGaaGGA by site-directed mutagenesis, as described previously (61). Cells were cotransfected with 1.6 μg of test plasmid and 0.4 μg of β-gal plasmid (as an internal control to normalize for transfection efficiency) on a 10-cm² tissue culture plate at 90% confluency with Effectene transfection reagent (Qiagen, Valencia, CA) according to the manufacturer’s instructions. Next day, the cells were split onto six-well plates and allowed to adhere for 36 h. The culture medium was then replaced with fresh DMEM containing of spermine NONOate (SPNONO; 0–50 μM), forskolin (30 μM), the PKA inhibitor H-89 (10 μM), or another structurally unrelated PKA inhibitor, the Rp diastereomer of adenine 3′,5′-cyclic monophosphorothioate (Rp-cAMPS; 200 μM) for 16–18 h. The luciferase activity of 20 μl of protein extract was determined using the Luciferase assay system (Promega, Madison, WI), and β-galactosidase of 10 μl of protein extract was determined using β-galactosidase enzyme assay system (Promega) as a transfection efficiency control.

Nuclear extract preparation and electrophoretic mobility shift assays. Nuclear extracts were prepared using the NE-PER nuclear extraction kit (Pierce Biotechnology, Rockford, IL) as we have described previously (30, 31). Electrophoretic mobility shift assays (EMSA) were performed using biotinylated double-stranded oligonucleotides corresponding to the CREB site at –89 of the GCH promoter (23). The single-stranded oligonucleotides were biotinylated using the Biotin 3′ end DNA labeling kit (Pierce Biotechnology) to incorporate one to three biotinylated ribonucleotides (Biotin-11-UTP) onto the 3′ end of DNA strands using terminal deoxynucleotidyl transferase (TdT) and then annealed to make it double stranded. The sequence of the oligonucleotide is CREB, −102 5′-GGCGAGGCCGTGACCGGAAGGCCGCGGC 3′−73 (IDT technology, Coralville, IA). Binding reactions involved incubating 10 μg of nuclear extract with biotinylated oligonucleotide and 1 μg of poly(dI-dC) for 20 min at room temperature. The DNA-protein complexes were resolved on a 5% nondenaturing polyacrylamide gel in 1× Tris-borate-EDTA (TBE) buffer and then transferred to nylon membrane by 10.220.33.1 on June 14, 2017 http://ajplung.physiology.org/ Downloaded from
inhaled NO significantly increased plasma BH₄ levels (Fig. 1A), and this was associated with increased protein levels of GCH1 (Fig. 1, B and C). Similarly, we found that the NO donor SPNONO (0–100 μM, 4 h) induced a dose-dependent increase in GCH1 protein levels (Fig. 2, A and B) and an increase in BH₄ levels (Fig. 2C) in COS-7 cells.

Exogenous NO increases COS-7 cAMP and phosho-CREB levels. Previous studies have indicated that GCH1 transcription can be stimulated in certain cell types by the second messenger cAMP (23). Thus we next determined whether exogenous NO increased cAMP levels in COS-7 cells. Our data indicate that SPNONO (20–100 μM, 4 h) significantly increased cellular cAMP levels (Fig. 3A). Furthermore, this was associated with a dose-dependent increase in phosho-CREB levels (Fig. 3, B and C).

Effect of exogenous NO and cAMP on GCH1 promoter activity. We next examined the effect of SPNONO (0–50 μM) on GCH1 transcription using promoter constructs prepared from the human GCH1 gene fused upstream of a luciferase reporter (Fig. 4A). We found that SPNONO induced a significant increase in luciferase activity when COS-7 cells were transfected with construct containing −446 to +167 (613 GTPCH) of the human GCH1 promoter (Fig. 4B). Similar results were obtained with a truncated promoter construct containing −146 to +167 (313 GTPCH, Fig. 4B). Both these constructs contain a CREB response element (CRE) at −102 to −73 (Fig. 4A). To determine the role of CREB in the NO-mediated increase in GCH1 promoter activity, we mutated 4 bp of the CREB response element of 313 GTPCH from TGACGCGA to TGcaacGA to generate the construct 313mutGTPCH (Fig. 4A). We found that the mutated GCH1 promoter did not respond to SPNONO (Fig. 4C). Forskolin stimulates adenyl cyclase (AC) and increases cAMP levels in cells leading to CREB phosphorylation and activation (10, 23, 48). To detect the effect of PKA inhibition on SPNONO induced CREB phosphorylation, we pretreated COS-7 cells with H-89 (10 μM) or Rp-cAMPS (200 μM) for 30 min, after which the cells were exposed to SPNONO (50 μM, 24 h), and then whole cell extracts (10 μg) were subjected to Western blot analysis using an antibody raised against GCH1. GCH1 expression was also normalized for loading using β-actin. A representative blot is shown. B: the bar graph shows the relative change in GCH1 protein levels with SPNONO treatment. There was a dose-dependent increase in GCH1 protein levels. Values are means ± SE; n = 3. *P < 0.05 vs. untreated cells. †P < 0.05 vs. 20 μM SPNONO. C: COS-7 were treated with SPNONO (50 μM, 24 h), and then whole cell extracts (10 μg) prepared from peripheral lung tissue post-inhaled NO were analyzed using a specific antisera raised against GCH1. GCH1 expression was also normalized for loading using β-actin. A representative blot is shown. C: there is a significant increase in densitometric value for normalized GCH1 protein in peripheral lung tissue post-inhaled NO. Values are means ± SE with pre-inhaled NO values normalized to 1.0; n = 4. *P < 0.05 vs. pre-inhaled NO.

Fig. 1. Tetrahydrobiopterin (BH₄) and GTP cyclohydrolase I (GCH1) protein levels in lambs exposed to inhaled nitric oxide (NO). A: 4-wk-old lambs were exposed to inhaled NO (40 ppm, 0–24 h). Plasma BH₄ levels were measured before (Pre) and after (Post) NO inhalation using HPLC. There was an increase in plasma BH₄ levels post-inhaled NO. Values are means ± SE; n = 4. *P < 0.05 vs. pre-inhaled NO. B: protein extracts (50 μg) prepared from peripheral lung pre- and post-inhaled NO were analyzed using a specific antisera raised against GCH1. GCH1 expression was also normalized for loading using β-actin. A representative blot is shown. C: there is a significant increase in densitometric value for normalized GCH1 protein in peripheral lung tissue post-inhaled NO. Values are means ± SE with pre-inhaled NO values normalized to 1.0; n = 4. *P < 0.05 vs. pre-inhaled NO.

Fig. 2. Effect of exogenous NO on GCH1 protein and BH₄ levels in COS-7 cells. A: COS-7 cells were treated with increasing concentration of the NO donor spermine NONOate (SPNONO; 0–100 μM, 4 h), and then whole cell extracts (10 μg) were subjected to Western blot analysis using an antibody raised against GCH1. GCH1 expression was also normalized for loading using β-actin. A representative blot is shown. B: the bar graph shows the relative change in GCH1 protein levels with SPNONO treatment. There was a dose-dependent increase in GCH1 protein levels. Values are means ± SE; n = 3. *P < 0.05 vs. untreated cells. †P < 0.05 vs. 20 μM SPNONO. C: COS-7 were treated with SPNONO (50 μM, 24 h), and BH₄ levels were then measured using HPLC. There was a significant increase in BH₄ levels with exogenous NO treatment. Values are means ± SE; n = 6. *P < 0.05 vs. untreated cells.
Exogenous NO increases binding of CREB to the GCH1 promoter. We next examined the effect of exogenous NO on CREB binding at the GCH1 promoter sequence located at −89 (Fig. 6A). We performed EMSA using nuclear extracts prepared from COS-7 cells to evaluate the effect of SPNONO (0–50 μM, 4 h) on CREB DNA binding activity. Compared with untreated extracts, we observed a significant increase in the binding of CREB to the GCH1 DNA (Fig. 6A and B). The CREB complex was competed out with 50- and 100-fold excesses of unlabeled CREB oligonucleotide, demonstrating specificity of binding (Fig. 6C). The presence of CREB in the DNA binding complex was also confirmed using supershift analysis with antibodies against both CREB and phospho-CREB (Fig. 6D). The presence of a higher migrating supershifted band following incubation with anti-CREB and anti-phospho-CREB polyclonal antibodies provides evidence that increased signal in SPNONO-treated cells is due to CREB in the binding complex (Fig. 6D).

Effect of exogenous NO on GCH1 regulation in PAECs. Because our in vitro studies were carried out in COS-7 cells we wanted to verify that these same events occurred in a more physiologically relevant cell culture model. To accomplish this, we utilized PAECs isolated from the sheep. Initially, we examined the effect of SPNONO (50 μM, 4 h) on cellular cAMP levels. We found that SPNONO significantly increased cAMP levels in PAECs (Fig. 7A). Next, we transfected PAECs with the 313GTPCH and 313mutGTPCH promoter constructs and exposed them to SPNONO (0–50 μM, 16–18 h). Our data indicate that SPNONO significantly increased GCH1 promoter activity in the 313GTPCH construct (Fig. 7B), whereas the mutant GTPCH construct did not respond (Fig. 7C). Similar to findings in COS-7 cells, we found that SPNONO also dose-dependently increased GCH1 protein levels (Fig. 7, D and E) as well as cellular BH4 levels (Fig. 7E).

DISCUSSION

In infants and children with congenital heart disease and patients with acute lung injury, inhaled NO is used as an adjuvant therapy. It produces potent, selective pulmonary va-
sodilation that is independent of endothelial cell function. Although many patients benefit from inhaled NO, several problems associated with its use have emerged, including unpredictable or nonsustained responses to therapy and rapid, even life-threatening, increases in PVR following its acute withdrawal (2, 11). This “rebound pulmonary” hypertension is one of the most significant safety issues regarding inhaled NO therapy. Sudden increases in PVR on acute withdrawal of therapy have been described in patients with a variety of pulmonary vascular disorders (2, 8, 34, 39).

Our laboratory has previously demonstrated that exogenous NO can reduce eNOS activity in lamb and ovine PAECs, independently of eNOS protein levels (3, 50). Exogenously added NO or NO donor compounds inhibit the activity of nNOS in rat cerebellum, eNOS in BAECs, and iNOS in activated rat alveolar macrophages (5, 17, 44, 45). These reports have led to the concept of eNOS “uncoupling,” characterized by the discrepancy between eNOS protein levels and NO availability. Previously, we have reported in endothelial cells that exogenous addition of NO causes a decrease in eNOS

Fig. 6. Effect of exogenous NO on CREB binding to the CRE of the GCH1 promoter. A: COS-7 cells were treated with SPNONO (50 μM, 4 h) in the presence or absence of the PKA inhibitors H-89 (10 μM) or Rp-cAMPS (200 μM). Whole cell extracts (10 μg) were then subjected to SDS-PAGE, and immunoblots were performed using an antibody raised against pCREB. Loading was normalized by reprobing with an antibody specific to CREB. Values are means ± SE; n = 4. *P < 0.05 vs. untreated cells. B and C: COS-7 cells were transfected with the 313 GTPCH and 313mutGTPCH promoter constructs and treated with forskolin (30 μM, 16 h) in the presence or absence of the PKA inhibitors H-89 (10 μM) or Rp-cAMPS (200 μM). The luciferase activities were then determined. Forskolin significantly increased 313 GTPCH promoter activity, and this increase was attenuated in the presence of H-89 and Rp-cAMPS. Mutation of the CRE abolished the effect of both forskolin and PKA inhibition on GCH1 promoter activity. Values are means ± SE; n = 6. *P < 0.05 vs. untreated cells. †P < 0.05 vs. SPNONO alone.

sodilation that is independent of endothelial cell function. Although many patients benefit from inhaled NO, several problems associated with its use have emerged, including unpredictable or nonsustained responses to therapy and rapid, even life-threatening, increases in PVR following its acute
activity but an increase in superoxide levels (50). Similar
growth results were obtained in an in vivo study where lambs were
exposed to inhaled NO for 24 h, leading to an increase in
superoxide levels and decrease in eNOS activity without any
change in eNOS expression (3, 41). These results suggested
that there are posttranslational modifications of eNOS on
exposure to exogenous NO. Accumulating evidence points
toward a crucial role of the pterin cofactor BH4 in the regula-
tion of eNOS activity. BH4 is an essential cofactor for activity
of all NOS enzymes (3). The exact role of BH4 in NOS
catalysis remains incompletely defined, but it appears to facil-
itate electron transfer from the eNOS reductase domain, main-
tains the heme prosthetic group in its redox active form (56),
and enhances eNOS “coupling” (59).

Multiple studies have investigated the role BH4 levels and
eNOS function in various diseases. For example, it has been
shown that endothelial cells isolated from a BB rat (a strain
genetically predisposed to diabetes) have reduced BH4 levels
compared with cells from control stains, which cause reduced
NO production despite normal eNOS protein levels (38). Simi-
larly, insulin-resistant fructose-fed rats demonstrate increased
vascular superoxide production causing oxidative degradation
of BH4 in aorta, reduced eNOS activity, and impaired endo-
thelium-dependent vascular relaxations compared with control
(51, 53). Studies in a deoxycorticosterone acetate-salt (DOCA-
salt) model of hypertension have shown that higher amounts of
aortic superoxide leads to BH4 oxidation, as well as eNOS
uncoupling that can be prevented by oral BH4 supplementation
(33). Thus, in this study, we initially determined whether the
increased eNOS uncoupling we had observed both in vitro and
in vivo could be explained, at least in part, by a decrease in
BH4 levels. However, in our inhaled NO lamb model, we
observed an increase in BH4 level in plasma, despite a decrease
in eNOS activity (3). We also observed increased levels of BH4
in COS-7 cells and PAECs exposed to exogenous NO, indi-
cating that NO-mediated decreases in BH4 are unlikely to
underlie the increased eNOS uncoupling and that other mech-
anism must be involved. Although these are still unresolved,
we can speculate. We have previously found both in vitro and
in vivo that exogenous NO exposure causes an increase in
peroxynitrite production and that increased eNOS nitration is
involved in eNOS inhibition (60). Recently, using liquid chro-
matography/mass spectrometry, we elucidated the nitration
sites on eNOS (64). In this study, we have identified four
tyrosine residues that can be modified by nitration and are located in the region of eNOS responsible for the binding to heat shock protein 90 (HSP90). The interaction of HSP 90 with eNOS is important because it modulates the relative production of NO and superoxide (43); this interaction is inhibited in hypercholesterolemia and has been shown to cause eNOS uncoupling (43). In addition, limited availability of the substrate L-arginine, which may reduce NO synthesis and thereby increase superoxide production (54), could be involved. However, further studies are required to test these possibilities.

GCH1 is the first and the rate-limiting enzyme in BH4 biosynthesis. In inhaled NO lambs, we have observed an increase in GCH1 expression. Similar results were observed in COS-7 cells treated with NO donor SPNONOate. Previous studies have shown that GCH1 expression is regulated at the transcriptional level. Cytokines and inflammatory mediators such as interferon-γ, interleukin-1β, tumor necrosis factor (TNF), and lipopolysaccharide increase GCH1 transcription and enzymatic activity (9, 13, 15, 65). Studies of the rat and human GCH1 promoter have identified the 146 bp upstream from the transcription start site as the minimal sequence necessary for cell type-specific cAMP-dependent transcription (23, 27). In our study, we also observed an increase in activity of GCH1 promoter construct containing 146 bp upstream from the transcription start site with NO treatment. This sequence contains a GC-box, a CRE, and a CCAAT-box that are evolutionarily conserved. Both the CRE and the CCAAT-box are required for maximum basal and cAMP-dependent transcription (27, 28). Whereas the CRE sequence binds members of the basic leucine zipper family of transcription factors, including CREB, activating transcription factor-2 (ATF-2), C-Jun, and CAAT enhancer-binding protein β (C/EBPβ), the CCAAT-box binds the obligate heterotrimeric protein nuclear factor Y (NF-Y) (1, 23, 27, 28). In our study, mutating the CRE site in the −146 promoter led to a loss in response to NO. This indicates the important role of CREB in NO-mediated regulation of GCH1 promoter. It was reported previously that CREB binding to the GCH1 promoter can be enhanced with cAMP treatment (23). In this study, we also observed an increase in cAMP levels in both PAECs and COS-7 cells with SPNONO treatment. We also observed an increase in GCH1 promoter activity with forskolin (a rapid activator of AC) treatment and an attenuated response when we mutated the CREB binding site at −89, again supporting the conclusion that cAMP signaling plays a key role in regulating GCH1 expression.

It should be noted that our data do not identify the mechanism by which exogenous NO increases cAMP levels. However, there are prior studies that agree with our findings. For example, it has been reported in porcine PAECs exposed to sepiaptarin exhibited an increase in NO production and enhanced cAMP levels (20). In vivo increases in NO have been found to cause elevations in cAMP levels (20, 29), and increases in cAMP have been found to increase NO production (21, 63). However, in other studies, increases in NO have been shown to decrease cAMP levels. For example, in rat kidney cortical ducts, treatment with SPNONOate decreased cAMP levels, and similar results were observed in U937 cells treated with sodium nitroprusside (SNP) or S-nitroso-N-acetylpenicillamine (SNAP) (14, 35), whereas a study exposing rats to inhaled NO found no change in cAMP levels (24). Together, these reports point to conflicting results of NO on cAMP regulation. However, we speculate that the signaling molecule involved in increasing cAMP levels may be cGMP. NO stimulates cGMP synthesis from GTP by binding to the heme prosthetic group of soluble guanylate cyclase. Cyclic GMP (and cAMP) is degraded by the action of specific phosphodiesterases (PDEs). In the lung, PDE5 degrades cGMP, whereas cAMP can be degraded by a number of PDEs, including PDE2A, PDE3A, and PDE4 (12). Interestingly, in human umbilical vein endothelial cells, cGMP was found to inhibit PDE3A (57), suggesting that exogenous NO could increase cAMP levels via a cGMP-mediated inhibition of PDE3A. However, further studies are required to test this possibility.

In conclusion, our data indicate that NO-mediated decreases in BH4 levels do not play a major role in the increase in eNOS uncoupling induced by exogenous NO. Rather, NO enhances BH4 levels through the stimulation of GCH1 expression. This appears to involve an NO-mediated increase in cAMP levels that leads to the phosphorylation and activation of CREB. We believe this to be the first report elucidating the mechanism of BH4 regulation by exogenous NO. Furthermore, we speculate that further characterizing the molecular mechanisms by which GCH1 is regulated may identify potential therapies to stimulate BH4 levels and decrease the endothelial dysfunction associated with eNOS uncoupling that exists in a number of cardiovascular disease states.

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