GTP Cyclohydrolase I Phosphorylation and Interaction With GTP Cyclohydrolase Feedback Regulatory Protein Provide Novel Regulation of Endothelial Tetrahydrobiopterin and Nitric Oxide

Li Li, Amir Rezvan, John C. Salerno, Ahsan Husain, Kihwan Kwon, Hanjoong Jo, David G. Harrison, Wei Chen

Rationale: GTP cyclohydrolase I (GTPCH-1) is the rate-limiting enzyme involved in de novo biosynthesis of tetrahydrobiopterin (BH₄), an essential cofactor for NO synthases and aromatic amino acid hydroxylases. GTPCH-1 undergoes negative feedback regulation by its end-product BH₄ via interaction with the GTP cyclohydrolase feedback regulatory protein (GFRP). Such a negative feedback mechanism should maintain cellular BH₄ levels within a very narrow range; however, we recently identified a phosphorylation site (S81) on human GTPCH-1 that markedly increases BH₄ production in response to laminar shear.

Objective: We sought to define how S81 phosphorylation alters GTPCH-1 enzyme activity and how this is modulated by GFRP.

Methods and Results: Using prokaryotically expressed proteins, we found that the GTPCH-1 phospho-mimetic mutant (S81D) has increased enzyme activity, reduced binding to GFRP and resistance to inhibition by GFRP compared to wild-type GTPCH-1. Using small interfering RNA or overexpressing plasmids, GFRP was shown to modulate phosphorylation of GTPCH-1, BH₄ levels, and NO production in human endothelial cells. Laminar, but not oscillatory shear stress, caused dissociation of GTPCH-1 and GFRP, promoting GTPCH-1 phosphorylation. We also found that both GTPCH-1 phosphorylation and GFRP downregulation prevents endothelial NO synthase uncoupling in response to oscillatory shear. Finally oscillatory shear was associated with impaired GTPCH-1 phosphorylation and reduced BH₄ levels in vivo.

Conclusions: These studies provide a new mechanism for regulation of endothelial GTPCH-1 by its phosphorylation and interplay with GFRP. This mechanism allows for escape from GFRP negative feedback and permits large amounts of BH₄ to be produced in response to laminar shear stress. (Circ Res. 2010;106:328-336.)

Key Words: shear stress ■ eNOS uncoupling ■ coimmunoprecipitation ■ transfection ■ site-directed mutagenesis

Vascular endothelial cells, which line the inner surface of blood vessels, are directly and continuously exposed to fluid shear stress generated by blood flow. Shear forces vary throughout the circulation and affect vascular development, function and the progression of vascular diseases. At certain sites in the circulation, flow reverses during the cardiac cycle and these regions of oscillatory shear are prone to atherosclerotic lesion formation.

An important physiological effect of shear stress is modulation of NO production. Acutely, laminar shear stimulates NO production within seconds of onset, and over the long term, shear increases endothelial cell levels of NO synthase (eNOS), the enzyme responsible for NO production. Tetrahydrobiopterin (BH₄) is a critical cofactor for all three isoforms of NO synthase (NOS), and is involved in the sequential reduction of the heme iron of the enzyme to ultimately form an iron-oxy species that hydroxylates L-arginine. In the absence of this cofactor, NOS produces superoxide (O₂⁻) rather than NO, a situation referred to as NOS uncoupling. Experimental models such as hypercholesterolemia, diabetes, atherosclerosis, and hypertension have been associated with NOS uncoupling. Besides its critical function for NOS, BH₄ also serves as an essential cofactor for the aromatic amino acid hydroxylases, and therefore plays an important role in the synthesis of tyrosine, dopamine, and serotonin.

The rate-limiting enzyme for BH₄ synthesis is GTP cyclohydrolase I (GTPCH-1), which converts GTP to...
7,8-dihydroneopterin triphosphate. The GTP cyclohydrolase feedback regulatory protein (GFRP) is an important modulator of GTPCH-1 enzyme activity. Crystal structures of the GTPCH-1/GFRP complex have shown that BH₄ is bound at the interface of these 2 proteins, and enzymatic studies have shown that BH₄ promotes inhibition of GTPCH-1 by GFRP in a negative feedback fashion. Negative feedback is a ubiquitous mechanism that maintains the end-product of many enzymes within a very narrow range, and if fully functional in the case of GTPCH-1, should prevent large changes in intracellular BH₄. We have recently demonstrated that laminar shear stress increases GTPCH-1 activity and BH₄ levels by 30-fold in human aortic endothelial cells (HAECs) and that this is mediated by phosphorylation of GTPCH-1 at serine 81 (S81) by the α prime subunit of casein kinase 2 (CK2α'). These findings would suggest that laminar shear, and perhaps S81 phosphorylation of GTPCH-1, disrupts the negative feedback conferred by GFRP in endothelial cells. This study was therefore performed to determine how GTPCH-1 phosphorylation affects its enzyme activity and its association with GFRP and to determine whether this interaction is affected by shear stress. We also studied the role of GFRP in endothelial cells to modulate GTPCH-1 phosphorylation, BH₄ levels and NO production. We compared the effects of unidirectional laminar shear to oscillatory shear stress both in vitro and in vivo in attempt to understand differences of BH₄ regulation in responses to these 2 mechanical stimuli. Our findings illustrate a new, previously unknown mechanism for regulation of GTPCH-1 activity in endothelial cells, which involves its phosphorylation and interaction with GFRP.

Methods

An expanded Methods section is available in the Online Data Supplement at http://circres.ahajournals.org.
plasmids encoding either WT GTPCH-1 or GTPCH-1 in which S81 was replaced with an aspartate to mimic phosphorylation (S81D) or an alanine to block phosphorylation (S81A), respectively. Transfection with S81D caused a significantly higher increase in BH₄ levels and GTPCH-1 activity than transfection with either WT or S81A in both HUVECs (Figure 1A and 1B) and HAECs (Online Figure I). Transfection with empty vector had no effect on BH₄ levels. Exposure of cells to shear increased BH₄ levels and GTPCH-1 activity in cells transfected with the WT enzyme, but did not in cells transfected with either S81D or S81A mutant constructs (Figure 1B and Online Figure I), consistent with the concept that these mutants could not be phosphorylated in response to shear. Importantly, cells transfected with the S81D mutant produced significantly more NO than cells transfected with either WT or S81A GTPCH-1 (Figure 1C). Because GFRP is known to inhibit GTPCH-1 activity in the presence of BH₄ in vitro, we examined the effect of GFRP on activity of purified WT and S81D GTPCH-1 that had been expressed prokaryotically. The Vmax of purified S81D was increased by approximately 2-fold compared to the WT enzyme (Figure 1D and Online Table I). Surprisingly, whereas Vmax of the WT enzyme was halved by addition of an equimolar concentration of GFRP, the Vmax of S81D was resistant to inhibition by GFRP (Figure 1D and Online Table I). Moreover, GFRP binding increased the apparent Km of GTPCH-1, which also contributes to inhibition and increases the cooperativity of substrate binding. These effects were not seen in the S81D mutant. To summarize these results, GTPCH-1 phosphorylation at S81 increases its enzyme activity and its production of BH₄ and thus augments NO production in human endothelial cells. This is likely due, in part, to its resistance to feedback inhibition by GFRP.

**Effect of GTPCH-1 S81 Phosphorylation on Interaction With GFRP**

We next considered the hypothesis that phosphorylated GTPCH-1 has reduced binding to GFRP, which might explain its resistance to GFRP inhibition. To test this hypothesis, we used pull-down assays using prokaryotically expressed WT, S81D, and S81A GTPCH-1 fusion proteins containing a 6-His tag and a glutathione S-transferase–tagged GFRP fusion protein. These experiments demonstrated that S81D exhibited reduced interaction with GFRP compared to WT GTPCH-1, whereas S81A showed enhanced interaction both in the presence of BH₄ and GTP (Figure 2A and 2B) and in separate experiments, in the presence of phenylalanine

![Figure 1.](http://circres.ahajournals.org/)

**Figure 1.** Comparison of WT and S81 modified GTPCH-1 on endothelial BH₄ levels, GTPCH-1 activity and NO production. HUVECs after transfection were maintained in static conditions or exposed to unidirectional laminar shear for 14 hours. A, BH₄ and total biopterin levels were determined by HPLC (n=3). Bottom, Western blot illustrating similar expression of the HA-tagged proteins in these cells. B, GTPCH-1 activity as measured by HPLC (n=3). C, Nitric oxide production by HUVECs transfected with either WT, S81D, or S81A GTPCH-1 measured by ESR 48 hours after transfection (n=3). Right, Representative Fe²⁺ diethyldithiocarbamate ESR spectra for NO production. D, Saturation curves examining the rate of dihydro-neopterin trisphosphate (H₄NTP) from purified WT and S81D GTPCH-1 proteins expressed prokaryotically in the presence and absence of GFRP (n=3).

![Figure 2.](http://circres.ahajournals.org/)

**Figure 2.** Effect of GTPCH-1 S81 phosphorylation on its interaction with GFRP. A and B, Pull-down assays using glutathione S-transferase (GST)-tagged GFRP to immunoprecipitate His-tagged WT, S81D, and S81A GTPCH-1 (A) and His-tagged WT, S81D, and S81A GTPCH-1 to precipitate GST-GFRP (B). C, Analysis of GFRP and GTPCH-1 binding in intact cells by coimmunoprecipitation. cells were cotransfected with DsRed-GFRP and HA-GTPCH-1 constructs and subjected to immunoprecipitation using anti-DsRed (IP: DsRed) antibody. IgG was used as a control for coimmunoprecipitation. Input amounts of GFRP and HA-tagged GTPCH-1 are shown at the bottom. D, Densitometry values for GTPCH-1 divided by GFRP from coimmunoprecipitation (n=4 to 5).
by binding partners of the targets.18 In HAECs, GFRP small interfering (si)RNA decreased GFRP mRNA by 80% (Figure 3A), but did not change levels of GTPCH-1 protein, eNOS protein, or eNOS S1177 phosphorylation (Online Figure III). Importantly, siRNA inhibition of GFRP expression increased GTPCH-1 S81 phosphorylation in a manner similar to that induced by unidirectional laminar shear (Figure 3B and 3C). We also found that downregulation of GFRP by siRNA markedly increased HAEC BH4 levels and GTPCH-1 enzyme activity (Figure 3D and 3E). Of note, following downregulation of GFRP, shear no longer increased BH4 levels or GTPCH-1 activity. This finding suggests that GFRP is critical for the increase in endothelial BH4 and GTPCH-1 activity induced by shear. Finally, downregulation of GFRP by siRNA substantially increased endothelial cell production of NO (Figure 3F). In contrast to these effects of GFRP siRNA, nonsilencing RNA was without effect on GTPCH-1 activity, BH4 levels, or NO production. Thus, GFRP plays an inhibitory role in regulation of human endothelial cell GTPCH-1 enzyme activity, BH4 levels and NO production, in part, by mediating S81 phosphorylation.

To further examine the role of GFRP in modulation of endothelial cell GTPCH-1 phosphorylation and activity, we overexpressed GFRP in HAECs and exposed these cells to either static or shear conditions. Overexpression of GFRP alone almost completely abolished the increase in GTPCH-1 S81 phosphorylation caused by laminar shear (Figure 4A and 4B). Likewise, GFRP overexpression also suppressed the increase in BH4 levels, GTPCH-1 enzyme activity and NO production caused by laminar shear (Figure 4C through 4E). We also cotransfected HAECs with GFRP and WT GTPCH-1 (Online Figure IV). As expected, transfection of HAECs with GTPCH-1 alone significantly increased BH4 levels, whereas cotransfection of GTPCH-1 with GFRP prevented this effect (Online Figure IV). These findings further support the concept that GFRP regulates GTPCH-1 phosphorylation and BH4 levels at baseline and in response to laminar shear stress in human endothelial cells.

**Effects of GTPCH-1 S81 Phosphorylation and GFRP Downregulation on eNOS Uncoupling in Response of Oscillatory Shear Stress**

We have previously shown that in contrast to laminar shear, oscillatory shear induced a minimal increase in BH4 levels in HAECs.11 Such a paucity of BH4 in response to oscillatory shear might cause eNOS uncoupling. In keeping with this, oscillatory shear increased O2− production by ∼2-fold in control cells. In contrast, transfection of HAECs with GTPCH-1 WT, S81D, and S81A constructs all blocked the increase of O2− caused by oscillatory shear, but the greatest effect was observed with S81D (Figure 5A). Moreover, the NOS inhibitor L-NAME (N(G)-nitro-arginine methyl ester) blocked O2− production in untransfected cells, compatible with eNOS uncoupling, whereas transfection with any of the
GTPCH-1 constructs reversed this phenomenon such that L-NAME increased O$_2^\cdot$ production, suggesting that they were able to recouple eNOS in the setting of oscillatory shear stress (Figure 5A). All of the constructs prevented the decline of NO production in response to oscillatory shear stress, but S81D had the greatest effect on overall NO levels (Figure 5B). Downregulation of GFRP using siRNA also prevented eNOS uncoupling and reduction of NO in cells exposed to oscillatory shear (Figure 5C and 5D). These results indicate that GTPCH-1 levels, GTPCH-1 phosphorylation status and GFRP levels play critical roles in regulating eNOS uncoupling in response to oscillatory shear stress.

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**Figure 4.** Effect of GFRP overexpression on endothelial GTPCH-1 S81 phosphorylation and BH$_4$ levels. A. Representative Western blot for S81 phospho-GTPCH-1 in HAECs transfected with GFRP cDNA or a vector control cDNA and exposed to 14 hours of laminar shear stress or static conditions. B. Densitometry values for phospho/total GTPCH-1 (n=4). C. BH$_4$ levels in HAECs transfected with GFRP cDNA or a vector control cDNA and exposed to either laminar shear or static condition for 14 hours (n=4 to 5). D. GTPCH-1 activity measured by HPLC (n=5 to 6). E. NO production measured using ESR (n=5 to 6).

**Figure 5.** Effects of GTPCH-1 phosphorylation and GFRP downregulation on eNOS coupling in HAECs exposed to OSS. HAECs were either not transfected or transfected with plasmids encoding HA-tagged WT, S81D, or S81A GTPCH-1. Forty-eight hours later, cells were either left in static conditions or exposed to oscillatory shear for 14 hours. Comparison of WT and S81 modified GTPCH-1 on O$_2^\cdot$ production (n=5 to 6) (A) and NO production (n=5) (B). Effect of GFRP downregulation by siRNA on O$_2^\cdot$ production (n=4 to 5) (C) and NO production (n=5) (D).
Comparison of the Effects of Laminar Versus Oscillatory Shear Stress on CK2α Activity and Association of GFRP With GTPCH-1

We previously demonstrated that laminar, but not oscillatory, shear stress stimulated GTPCH-1 phosphorylation at S81 by CK2α; however, the mechanism by which shear induces S81 phosphorylation is unknown. In initial experiments, we found that CK2α activity was similar in HAECs exposed to no shear, laminar or oscillatory shear (Figure 6A). Thus, CK2α is not directly affected by either laminar or oscillatory shear stress. We next sought to determine whether GFRP, which, as shown above, prevents GTPCH-1 phosphorylation, is released from GTPCH-1 to allow phosphorylation in response to laminar shear. We used 2 different approaches to examine the interaction of GTPCH-1 and GFRP in static and sheared cells: coimmunoprecipitation and BRET. Coimmunoprecipitation of HA-GTPCH-1 and DsRed-GFRP from static and sheared HAECs showed that the amount of GTPCH-1 coprecipitated with GFRP decreased after cells were exposed to laminar but not oscillatory shear (Figure 6B and 6C). To analyze the interaction of these proteins using BRET, HAECs were cotransfected with plasmids encoding Renilla luciferase (Rluc8)-GTPCH-1 and Venus-GFRP fusion proteins and exposed to static and shear conditions. When Rluc8-GTPCH1 and Venus-GFRP were coexpressed and Rluc8 was excited with the substrate coelenterazine h, we detected a robust BRET signal with a value of 0.094±0.012 (Figure 6D). This BRET ratio was significantly lower in cells that were exposed to laminar but not oscillatory shear (Figure 6D). These results demonstrate that laminar shear causes the dissociation of GTPCH-1 and GFRP. Taken together with our experiments in which GFRP was either reduced by siRNA or overexpressed, this dissociation likely allows phosphorylation of GTPCH-1 at S81 and thus activates the enzyme.

In additional experiments, we sought to determine whether phosphorylation was either the cause or result of dissociation of GTPCH-1 and GFRP in the setting of laminar shear stress. Treatment of cells with 4,5,6,7-tetrabromobenzotriazole, a specific CK2 inhibitor that prevents GTPCH-1 phosphorylation, did not affect dissociation of GFRP and GTPCH-1, as detected by coimmunoprecipitation (Figure 6E and 6F). Similarly, shear also reduced the interaction of GFRP and the phosphorylation resistant S81A GTPCH-1 mutant (Figure 6G and 6H). These results indicate that S81 phosphorylation is likely the result of GFRP and GTPCH-1 dissociation and does not initiate dissociation of these proteins.

Effect of Oscillatory Flow on GTPCH-1 Phosphorylation and BH4 Levels In Vivo

To determine whether different shear profiles have different effects on GTPCH-1 phosphorylation and BH4 levels in vivo, we used an in vivo model of oscillatory shear stress by inducing partial carotid ligation. We ligated the external, internal and occipital branches of the LCA, while leaving the
superior thyroid artery intact in C57Bl/6 mice. This has been shown to result in low and oscillatory carotid shear stress in the LCA and accelerates atherosclerosis in ApoE KO mice.16 This procedure reduced GTPCH-1 S72-phosphorylation by 50% compared to right common carotid artery (RCA) in the same mouse as detected using a newly developed antibody to mouse GTPCH-1 phospho-S72, which is homologous to human phospho-S81 (Figure 7A and 7B). In accordance to impaired GTPCH-1 phosphorylation in LCA, partial ligation also decreased total biopterin production in LCA (Figure 7C). There was also evidence of BH4 oxidation in LCA, as indicated by decreased ratio of BH4 to BH2, likely attributable to increased reactive oxygen species production caused by oscillatory shear stress (Figure 7C). Together, these markedly decreased BH4 levels in regions of oscillatory shear in vivo. Moreover, these results show that shear forces modulate GTPCH-1 phosphorylation and BH4 production in intact vessels.

Discussion

Recently, our laboratory has demonstrated that unidirectional laminar shear stress stimulates endothelial GTPCH-1 phosphorylation by CK2α and that this is associated with a marked increase in GTPCH-1 catalytic activity and BH4 production.11 Our present study provides additional insight into mechanisms underlying GTPCH-1 phosphorylation and modulation of its activity by the regulatory protein, GFRP. A phospho-mimetic mutant of GTPCH-1, S81D, was shown to have increased activity compared to the WT enzyme and in contrast to the WT enzyme, was resistant to negative feedback inhibition by GFRP. We demonstrated that the phospho-mimetic mutant enzyme exhibited reduced binding to GFRP. Importantly, our data also indicate that GFRP regulates GTPCH-1 S81 phosphorylation. Reduction of GFRP levels using siRNA induced GTPCH-1 phosphorylation and dramatically increased endothelial cell GTPCH-1 activity, BH4 levels and NO production. In contrast, overexpression of GFRP inhibited GTPCH-1 phosphorylation and reduced endothelial cell levels of BH4 stimulated by laminar shear stress. Our data also showed that both transfection of S81D GTPCH-1 and downregulation of GFRP rescued eNOS uncoupling induced by oscillatory shear stress in HAECs. We also found that unidirectional laminar shear stress, which induces GTPCH-1 phosphorylation, decreased its association with GFRP. Our data also show that GTPCH-1 phosphorylation is likely a consequence, rather than a cause, of its dissociation from GFRP, because this phenomenon was not prevented by pharmacological inhibition of CK2 or by mutation of GTPCH-1 to prevent its phosphorylation. Finally, we demonstrated that oscillatory flow reduced GTPCH-1 phosphorylation, decreased total biopterin production, and augmented BH4 oxidation in vivo.

Taken together, our findings are compatible with the scheme illustrated in Figure 8. GTPCH-1 exists as a homodimer in complex with 2 homopentamers of GFRP. We propose that unidirectional laminar shear initially promotes dissociation of GFRP and GTPCH-1. This permits access of CK2α to GTPCH-1, allowing phosphorylation of one or more S81 residues. The phosphorylation of S81 reduces reassociation of GFRP and GTPCH-1, allowing further phosphorylation of other S81 residues. The fully phosphorylated GTPCH-1 exhibits markedly increased activity in part because it has reduced affinity for GFRP and is not inhibited by GFRP, which leads to increased BH4 levels and eNOS coupling. Our present in situ data do not allow discrimination between the direct effect of augmentation of GTPCH-1 activity by phosphorylation and the indirect effect of phosphorylation on altering association with GFRP; however our studies of purified WT and S81D GTPCH-1 would suggest that both are important.
Our present findings provide further insight into differences between the effects of unidirectional laminar and oscillatory shear on endothelial cell function (Figure 8). In humans and experimental animal models, areas of oscillatory shear, such as bifurcations and branching points of arterial trees, are associated with enhanced atherosclerotic lesion formation, whereas areas of well-developed laminar shear have reduced atherosclerosis. Over the long-term, laminar shear causes dissociation of GFRP and GTPCH-1 phosphorylation. The precise mechanism by which laminar shear stimulates GTPCH-1 phosphorylation and thus prevents dissociation of these 2 proteins in the setting of oscillatory shear is likely to lead to eNOS uncoupling and promote the development and/or progression of atherosclerosis. We previously demonstrated that GTPCH-1 was phosphorylated in response to unidirectional laminar shear, but not by oscillatory shear. In the present study, we confirmed this by using an in vivo model of oscillatory shear stress induced by partial carotid ligation. We showed that oscillatory flow reduced GTPCH-1 phosphorylation, decreased total biopterin production and promoted BH4 oxidation in vivo. A reduction in BH4 levels in regions of oscillatory shear is likely to lead to eNOS uncoupling and promote the development and/or progression of atherosclerosis. Our studies suggest that both GTPCH-1 phosphorylation and GFRP downregulation can rescue eNOS uncoupling in the setting of oscillatory shear.

To explore the mechanism by which laminar shear stimulates GTPCH-1 phosphorylation, whereas oscillatory shear does not, we used 2 separate methods to show that laminar shear dissociates GFRP and GTPCH-1, whereas oscillatory shear does not. Our data indicate that the continued association of these 2 proteins in the setting of oscillatory shear would inhibit GTPCH-1 phosphorylation and thus prevents activation of the enzyme. The precise mechanism by which laminar shear causes dissociation of GFRP and GTPCH-1 remains undefined. In preliminary studies, we found that the onset of GTPCH-1 phosphorylation begins approximately 4 to 5 hours after initiation of shear stress (data not shown). This temporally corresponds to the time that endothelial cell shape changes in response to shear. It is therefore interesting to speculate that endothelial cell cytoskeletal reorganization could alter the respective subcellular localizations of GTPCH-1 and GFRP and promote their dissociation. A very recent study showed that GTPCH-1 interacts with a Rho-GTPase, which is activated by shear is known to modulate cytoskeletal reorganization. Additional studies are needed to address this issue.

Comparison of results in endothelial cells with purified enzyme experiments is somewhat complicated by the contribution to GTPCH-1 regulation in cells of binding of ligands such as phenylalanine and BH4 to sites on both catalytic GTPCH-1 domains and regulatory GFRP domains. These effects are buffer-dependent and probably account for the greater effects of S81 phosphorylation observed in cells. Whereas GFRP is generally considered to inhibit GTPCH-1 in the presence of BH4, the ubiquitously present amino acid phenylalanine reverses the inhibition of GTPCH-1 without causing the dissociation of GFRP in vitro. Our experiments using siRNA show that in intact endothelial cells, the predominant role of GFRP is marked inhibition of GTPCH-1 activity. In keeping with an inhibitory role of GFRP, Nandi et al showed that its overexpression attenuated lipopolysaccharide- and cytokine-stimulated BH4 and NO production in an endothelial cell line.

Our results also reveal a novel mechanism for GFRP modulation of GTPCH-1 activity, by inhibiting its phosphorylation. Interestingly, in contrast to our conclusions, a very recent study showed no evidence for regulation of human GTPCH-1 by mouse GFRP when the former was expressed in murine fibroblasts. It is conceivable that mouse GFRP might not effectively interact with human GTPCH-1 and thus does not regulate its activity or that this interaction is unimportant in fibroblasts. Our present studies clearly indicate a role of GFRP in modulation of both basal and laminar shear stimulated GTPCH-1 activity in human endothelial cells.
Our previous data indicate that CK2α′ is responsible for GTPCH-1 phosphorylation in response to laminar shear. Pharmacological inhibition of CK2 and siRNA inhibition of CK2α′ eliminated S81 phosphorylation and the increase in GTPCH-1 activity caused by shear. Our present finding that GTPCH-1 is phosphorylated when it dissociates from GFRP is in accordance with the action of CK2 on other substrates. CK2 is considered constitutively expressed and active, and its ability to phosphorylate target substrates is often modulated by other proteins that bind to these targets. As an example, the nuclear factor κB subunit p65 is known to be phosphorylated by CK2 when it is released from the inhibitory subunit IκB. Our finding that shear does not modulate CK2α′ activity is in keeping with the concept that it simply gains access to GTPCH-1 on GFRP dissociation. In preliminary studies, we were unable to demonstrate binding of CK2α′ with GTPCH-1 (data not shown), perhaps because the transient nature of this association.

In summary, we find that phosphorylation of GTPCH-1 is critical in activation of this enzyme, because this not only enhances its intrinsic activity but also reduces the feedback inhibition by its regulatory protein, GFRP. Our results establish a pivotal role of GFRP in regulating GTPCH-1 S81 phosphorylation and activity, BH₄ levels, and NO production in human endothelial cells. These data also demonstrate that the constitutive interaction of GTPCH-1 and GFRP can be regulated by laminar shear stress, which dynamically modulates GTPCH-1 phosphorylation and enzyme activity. The interplay between GTPCH-1 and GFRP shown in our study defines a novel mechanism of GTPCH-1 control in the endothelium. Moreover, our findings suggest that the mechanism regulating shear-induced GTPCH-1 phosphorylation and BH₄ synthesis is important for the in vivo situation.

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Disclosures
None.

References
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GTP Cyclohydrolase I Phosphorylation and Interaction with GTP Cyclohydrolase Feedback Regulatory Protein Provide Novel Regulation of Endothelial Tetrahydrobiopterin and Nitric Oxide

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Supplemental Materials and Methods

Recombinant DNA Plasmids: Human GTPCH-1 cDNA was obtained form GeneCopoeia (Cat.No.EX-X0381-M07) and was cloned into the pCMV-HA vector (Clontech) and the pEGFP-C1 vector (Clontech) to create pCMV-HA/GTPCH-1 and pEGFP/GTPCH-1 constructs, respectively. Serine 81 in pCMV-HA/GTPCH-1 was changed to an alanine and an aspartate to produce the GTPCH-1 mutants S81A and S81D respectively using the QuikChange II Site-Directed Mutagenesis Kit (Stratagene). Prokaryotic expression of wild-type, S81D and S81A GTPCH-1 was accomplished by cloning these into the pET-30a(+) vector (EMDBiosciences, Madison, WI). The human GFRP cDNA was synthesized by Genscript (Piscataway, NJ) and cloned into the pDsRed-Monomer-C1 vector (Clontech), the pGEX-5X-3 vector (GE Healthcare) and the pET-15b vector (EMDBiosciences, Madison, WI) to create pDsRed/GFRP, pGEX-5X-3/GFRP and pET-15b/GFRP constructs, respectively. Rluc8 cDNA was amplified from the construct pcDNA3.1-Rluc8, which was kindly provided by Andreas Loening and Sanjiv Gambhir (Stanford University, Stanford, CA). PCR product for Rluc8 coding region was inserted in-frame
into pEGFP/GTPCH-1 replacing the EGFP cDNA to generate Rluc8/GTPCH-1. The Venus/GFRP construct was prepared from the construct pcS2-Venus kindly provided by Atsushi Miyawaki (RIKEN Brain Science Institute, Wako-city, Japan). A PCR product for the Venus coding sequence was substituted in-frame into pDsRed/GFRP replacing the DsRed cDNA. DNA sequencing was used to confirm construct sequences (Agencourt Biosciences, Beverly, MA).

**Cell Culture, Transfection Procedure and Shear Apparatus:** Human Aortic Endothelial Cells (HAEC) were purchased from Lonza (Walkersville, MD) and used between the 3rd and 6th passages. Human umbilical vein endothelial cells (HUVEC) were purchased from the Emory Skin Diseases Research Center and used between the 3rd and 6th passages. Plasmids were transfected in HAECs and HUVECs using PrimeFect II (Lonza) or Lipofectin (Invitrogen) respectively. HAECs were transfected with small interfering (si)RNA against GFRP (sense: GCCUUGGGAAACAACUUUtt) or transfected with a non-silencing control sequence (Applied Biosystems/Ambion, Austin, TX) as described previously 1. Laminar (15 dynes/cm²) and oscillatory shear (±15 dynes/cm²) were applied using a cone-in-plate viscometer with a 1° angle in an incubator at 37°C in 5% CO₂ for 14 hours 2.

**Antibodies and western blot analysis:** Antibodies against eNOS and P-S1177 eNOS were purchased from BD Biosciences (San Jose, CA). Antibodies against CK2α’, HA-tag and His-Tag were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and Cell Signaling Technology (Danvers, MA), respectively. An anti-Actin antibody (Sigma-Aldrich) was used for protein loading control. The Phospho-S81 GTPCH-1 antibody was described previously 1. An anti-GTPCH-1 rabbit polyclonal antibody was generated against a synthesized peptide, MEKPRGVRCTNGFPERE, which corresponds to amino acid residues 1-17 of rat GTPCH-1 (GenBank accession number NP 077332). An anti-GFRP rabbit polyclonal antibody was generated against a synthesized peptide, DPELMQHLGASKRRAL, which corresponds to amino
acid residues 25-40 of human GFRP (GenBank accession number NP 005249). Anti-GFRP IgG was purified from rabbit serum and tested using both purified GFRP and mouse liver lysates for western blotting. The anti-phospho-S72 GTPCH-1 antibody was generated against a synthesized peptide, SSILLBLGEDPQRC, which corresponds to amino acid residues 67-79 of mouse GTPCH-1 (GenBank accession number NP 032128). Cells were washed twice in cold PBS and scraped in 1X RIPA lysis buffer (Millipore, Billerica, MA) supplemented with Complete Protease Inhibitor Cocktail Tablet (Roche). Thirty μg of supernatant was separated by 12.5% SDS-PAGE and transferred to nitrocellulose membranes (Amersham Biosciences, UK). Membranes were probed using antibodies described above. Immunoreactive bands were visualized by enhanced chemiluminescence detection (GE Healthcare). Densitometry was performed with Quantity One software (Bio-Rad Laboratories).

**Real-time PCR analysis:** Endothelial cDNA was amplified using 7500 Fast Real Time PCR system (Applied Biosystems). Taqman primers for human GFRP were purchased from Applied Biosystems (Assay ID Hs00193360_m1). The resulting mRNA levels were normalized to the levels of human GAPDH mRNA.

**BH₄, GTPCH-1 activity, NO and O₂⁻ measurements:** Endothelial BH₄ levels and GTPCH-1 activity were measured by high-performance liquid chromatography (HPLC) as previously described ¹. For analysis of wild type GTPCH-1, S81D activity, a kinetic microplate assay was utilized as previously described ³ in the presence of GTP and BH₄. Endothelial cell NO production was measured by electron spin resonance (ESR) using the specific colloid probe Fe²⁺ diethyldithiocarbamate (Fe[DETC]₂) as described ⁴. O₂⁻ production in HAECs was measured by quantifying formation of 2-hydroxyethidium from dihydroethidium (10 μM) by HPLC. This product specifically reflects the reaction of O₂⁻ with dihydroethidium and has been validated previously ⁵.
GST and His Pull Down Assays: To detect association of GST-GFRP with His-tagged GTPCH-1, the ProFound Pull-Down GST Protein:Protein Interaction Kit (Pierce, Rockford, IL) was employed. *E. coli* (strain BL21) was transformed with pGEX-5X-3/GFRP and cultured overnight in LB medium. The culture was then diluted 1:100 with pre-warmed LB and incubated at 37°C with vigorous shaking. Recombinant GST-GFRP was obtained by isopropyl-β-d-thiogalactopyranoside (IPTG, 0.5 mmol/L) induction for 4 h at 30°C. Two mg of *E. Coli* lysate was incubated with 150 µg of purified His-GTPCH-1 recombinant protein in a buffer containing 10 µmol/L BH₄, 100 µmol/L GTP and 100 µmol/L ascorbic acid (Buffer A) or 1mmol/L L-phenylalanine (Buffer B). Protein complexes were eluted according to the manufacturer’s instructions and then separated on 12.5% gel by SDS–PAGE, followed by Western blot analysis using anti-His tag and anti-GFRP antibodies.

For His pull down assay, 50 µg of purified His-tagged GTPCH-1 recombinant protein was incubated with 50 µl of Ni-NTA (nickel-nitrilotriacetic acid) magnetic agarose beads (Qiagen, Valencia, CA) in protein binding buffer, according to the manufacturer's protocol. The beads and attached protein were separated from the buffer by using a magnetic separator, and then incubated with *E.Coli* lysate containing GST-GFRP fusion protein in buffer A. After washing once with the interaction buffer, the beads were heated in SDS-PAGE sample buffer and proteins analyzed by SDS-PAGE followed by western blotting using anti-His tag and anti-GFRP antibodies.

Immunoprecipitation and Immunoblotting: Cells were harvested in 1X RIPA lysis buffer (Millipore) supplemented with Complete Protease Inhibitor Cocktail Tablet (Roche). Supernatants (400 µg total protein) were pre-cleared with 20 µL of protein A/G Plus agarose beads (Santa Cruz Biotechnology) for 6 h at 4°C and incubated overnight at 4°C with 20 µL of protein A/G Plus agarose beads and 3 µL of anti-DsRed polyclonal antibody (Clontech), or an equal amount of non-immune rabbit IgG as a control. After four washes in phosphate-buffered...
saline buffer (PBS), the immunoprecipitated proteins were separated by SDS-PAGE, transferred to nitrocellulose membranes, and immunoblotted with anti-HA (Cell Signaling Technology) or our custom made anti-GFRP antibodies.

**CK2α’ Kinase Assay:** CK2α’ was immunoprecipitated from cell extracts by an anti-CK2α’ antibody (Santa Cruz Biotechnology) and enzyme activity was measured using a Casein Kinase 2 Assay Kit (Millipore) according to the manufacture’s instructions. Kinase activity was calculated by subtracting the mean of the background control samples without enzyme from the mean of samples with enzyme.

**BRET Assay:** HAECs were transfected with genes encoding Rluc8-GTPCH-1 and Venus-GFRP using the transfection method described above. Forty-eight hours after transfection, cells were detached with Trypsin/EDTA and washed once with PBS. BRET assays were performed in 96-well isoplates (Perkin–Elmer, Boston, MA) using approximately 100,000 cells resuspended in 180 µL of PBS. Coelenterazine h substrate (Invitrogen) was added at a final concentration of 5 µM, and luminescent readings at 460 and 528 nm wavelengths were collected immediately thereafter using a Synergy™ HT Multi-Mode Microplate Reader (Biotek, Winooski, VT). The BRET ratio was determined as described previously. In preliminary experiments, evidence for specific interaction was established by showing that transfection with increasing amounts of acceptor (Venus-GFRP) plasmid to a constant amount of donor (Rluc8-GTPCH-1) plasmid led to a linear increase in BRET ratio which saturated at a ratio of approximately 0.1 (Online Figure V). A donor:acceptor ratio of 1:4 was used as this represented the mid-point of the saturation curve. Co-expression of empty Rluc8 and Venus-GFRP led to a minimal BRET signal (Online Figure V).

**Animal studies with partial carotid ligation:** All experimental protocols were approved by the institutional Animal Care and Use Committee at Emory University. C57BL/6 mice were obtained...
from Jackson Laboratories. Partial ligation of left common carotid artery (LCA) was carried out as previously described to create low and oscillatory shear stress in LCA.

**Statistical Analysis:** All values are means ± SEM. The data were compared between groups by t test when comparison between two groups was performed and by ANOVA for multiple comparisons. When significance was indicated by ANOVA, the Bonferroni post hoc test was used to make selected comparisons and the Dunnett post hoc test was used when one group was served as a control. A value of P<0.05 was considered significant.
**Online Table I:** Kinetic properties of WT and S81D GTPCH-1 in the presence and absence of GFRP (n=3).

<table>
<thead>
<tr>
<th></th>
<th>Wild type</th>
<th>S81D</th>
<th>WT + GFRP</th>
<th>S81D + GFRP</th>
</tr>
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<tbody>
<tr>
<td><strong>Vmax</strong></td>
<td></td>
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<tr>
<td>nmol/min/mg protein</td>
<td>51.7 ± 2.4</td>
<td>84.3 ± 6.9*</td>
<td>32.3 ± 0.8†</td>
<td>85 ± 5.1</td>
</tr>
<tr>
<td><strong>Km (mmol/L)</strong></td>
<td>1.1 ± 0.2</td>
<td>1.1 ± 0.2</td>
<td>0.8 ± 0.2</td>
<td>1.1 ± 0.2</td>
</tr>
</tbody>
</table>

* p<0.001 vs. Wild type, †p<0.01 vs. Wild type
Online Figure I: Comparison of wild type and S81 modified GTPCH-1 on endothelial BH₄ levels in HAECs. HAECs were either not transfected or transfected with plasmids encoding HA-tagged WT, S81D or S81A GTPCH-1. Forty-eight hours later, cells were either left in static conditions or exposed to unidirectional laminar shear for 14 hours. BH₄ levels were determined by HPLC (n=3-4). The lower portion shows a western blot illustrating endogenous and overexpressed GTPCH-1 using an anti-GTPCH-1 antibody.
Online Figure II

**Online Figure II**: GST pull-down assay using GST-tagged GFRP to immunoprecipitate His-tagged WT, S81D and S81A GTPCH-1 in the presence of 1mmol/L L-phenylalanine.
Online Figure III: Protein levels determined by western blotting for GTPCH-1, phospho-S1177 eNOS and total eNOS in HAECs transfected with either a non-silencing siRNA or GFRP siRNA, or not transfected. Actin was used as a loading control.
Online Figure IV: A) Western blots for overexpressed GTPCH-1 and GFRP in HAECs co-transfected with HA-GTPCH and DsRed-GFRP. Western blot analysis was performed using antibodies against HA and DsRed, respectively. B) BH4 levels in HAECs co-transfected with HA-GTPCH and DsRed-GFRP (n=4-5).
Online Figure V: BRET saturation curve for constitutive interaction between GTPCH-1 and GFRP. Increasing amounts of Venus-GFRP was co-expressed with a constant amount of Rluc8-GTPCH-1 in HAECs. BRET ratios are plotted as a function of the ratio of Venus-GFRP over Rluc8-GTPCH-1 DNA. The Rluc8 empty vector was used as a negative control.
References


