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Abstract—An essential cofactor for the endothelial NO synthase is tetrahydrobiopterin (H_4B). In the present study, we show that in human endothelial cells, laminar shear stress dramatically increases H_4B levels and enzymatic activity of GTP cyclohydrolase (GTPCH)-1, the first step of H_4B biosynthesis. In contrast, protein levels of GTPCH-1 were not affected by shear. Shear did not change protein expression or activity of the downstream enzymes 6-pyruvoyl-tetrahydropterin synthase and sepiapterin reductase and decreased protein levels of the salvage enzyme dihydrofolate reductase. Oscillatory shear only modestly affected H_4B levels and GPTCH-1 activity. We also demonstrate that laminar, but not oscillatory shear stress, stimulates phosphorylation of GTPCH-1 on serine 81 and that this is mediated by the α prime (α') subunit of casein kinase 2. The increase in H_4B caused by shear is essential in allowing proper function of endothelial NO synthase because GPTCH-1 blockade with 2,4-diamino-6-hydroxypyrimidine during shear inhibited dimer formation of endothelial NO synthase, increased endothelial cell superoxide production, and prevented the increase in NO production caused by shear. Thus, shear stress not only increases endothelial NO synthase levels but also stimulates production of H_4B by markedly enhancing GTPCH-1 activity via casein kinase 2–dependent phosphorylation on serine 81. These findings illustrate a new function of casein kinase 2 in the endothelium and provide insight into regulation of GTPCH-1 activity. (***Circ Res*****. 2007;101:830-838.)**

Key Words: endothelial cell ■ endothelial NO synthase ■ GTP cyclohydrolase 1 ■ shear stress ■ tetrahydrobiopterin

The endothelial cell NO synthase (eNOS) is both acutely and chronically influenced by mechanical forces. Exposure of endothelial cells to unidirectional laminar shear acutely stimulates eNOS to produce NO within seconds.[1] Over the long term, laminar shear stimulates an increase in eNOS mRNA and protein expression. In vivo, exercise training increases eNOS expression, most likely by increasing cardiac output and endothelial shear.[2,3] These effects of laminar shear and exercise are thought to impart protection against atherosclerosis because in addition to its role as a vasodilator, NO prevents smooth muscle growth, platelet aggregation, and leukocyte adhesion and inhibits lipid oxidation and apoptosis in the vessel wall.[4] Unlike unidirectional laminar shear, oscillatory shear seems to predispose to atherosclerotic lesion formation.[5] Oscillatory shear stress stimulates production of reactive oxygen species and proinflammatory gene expression and promotes apoptosis.[6–8]

Tetrahydrobiopterin (H_4B) is an essential cofactor for eNOS to produce NO. H_4B is involved in the catalytic process of L-arginine oxidation and NO production. The oxygenase domain of each eNOS monomer binds 1 H_4B, so each functional dimer binds to 2 H_4Bs. In the initiating step of L-arginine oxidation, H_4B donates an electron to the ferrous–dioxygen complex in the oxygenase domain, leading to scission of the dioxygen and formation of an iron–oxy species that participates in L-arginine hydroxylation. The lack of sufficient amounts of H_4B impairs this process, such that superoxide (O_2^-) is released from the ferrous–dioxygen complex and NO is not formed.[9,10] This phenomenon has been referred to as eNOS uncoupling.

H_4B can be synthesized via a de novo pathway, which involves the sequential actions of 3 enzymes. The first enzyme, GTP cyclohydrolase (GTPCH)-1, cleaves GTP to 7,8-dihydroneopterin triphosphate, which is then converted by 6-pyruvoyl-tetrahydropterin synthase (PTPS) to 6-pyruvoyl-tetrahydropterin. Sepiapterin reductase (SR), in a final NADPH-dependent step, reduces 6-pyruvoyl-tetrahydropterin to H_4B.[11] In addition, H_4B can be formed by the action of a salvage pathway that converts 7,8-dihydrobiopterin (H_2B) and quinoid–dihydrobiopterin back

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to H_4B. It has recently been shown that endothelial cells predominantly convert H_2B back to H_4B by the enzyme dihydrofolate reductase (DHFR).[12]

Because eNOS protein is potently increased by shear and by exercise, it might also be necessary for the endothelial cell to increase H_4B levels to maintain proper function of the newly formed eNOS enzyme. In this study, we demonstrate that shear stress increases the H_4B levels in endothelial cells through a casein kinase 2 (CK2)-dependent phosphorylation of GTPCH-1 on serine 81. We further show that this increase in H_4B in response to shear is necessary to allow proper function of the endothelial NO synthase.

Materials and Methods

Materials

Chelerythrine and 4,5,6,7-tetrabromobenzotriazole (TBB) were from Calbiochem (San Diego, Calif). 1-Hydroxy-4-methoxy-2,2,6,6-tetramethylpiperidine was from Alexis Biochemicals (San Diego, Calif). All other biochemicals were purchased in the highest available grade from Sigma-Aldrich (St Louis, Mo).

Cell Culture

Human aortic endothelial cells (HAECs) (Cambrex, San Diego, Calif) were cultured in EBM2 Media (EBM2 Bullet Kit; Cambrex) on 10-cm cell culture dishes, as previously described.[13] Postconfluent HAECs between passages 4 to 8 were used for experiments. Cells were exposed to either unidirectional laminar shear (15 dynes) stress or oscillatory shear ($\pm$15 dynes) stress using a cone and plate viscometer.[14] Cells were pretreated with the indicated agent in media for 1 hour before shear.

Measurements of Biopterin

Measurements of biopterin content were performed using high-performance liquid chromatography (HPLC) analysis (System GOLD, Beckman; fluorescence detector FP2020, Jasoc Inc) and a differential oxidation method, as described previously.[15]

Enzyme Assays

The activity of GTPCH-1 was measured by monitoring the conversion of the substrate GTP under saturating conditions to dihydroneopterin trisphosphate, detected by HPLC as its oxidized and dephosphorylated product neopterin. PTPS activity was measured by monitoring the conversion of dihydroneopterin triphosphate to H_4B. Dihydroneopterin triphosphate was prepared enzymatically using recombinant GTPCH-1 protein (Abnova, Taipei, Taiwan). The activity of SR was assessed by monitoring the conversion of sepiapterin to H_4B under saturating conditions.[16]

Small Interfering RNA Transfection

Cells were transfected with small interfering (si)RNA against CK2α (AS-CK2α: GTCCATGAAATATTCCACCTG), CK2α' (AS-CK2α': GTTCTCCAGAATCTTAACCTC), and GTPCH-1 (AS-GTPCH-1: GATCGTTGGTACGATACGCTT) or transfected with a nonsilencing control sequence (Ambion). Transfection was performed as previously described.[8]

Real-Time PCR

Endothelial cDNA was amplified using a LightCycler real-time thermocycler (Roche Diagnostics, Indianapolis, Ind). Primers for CK2α and CK2α' were as follow: CK2α forward, 5-GAGGTCCCA-ACATCATCACAC-3; reverse, 5-TGACATTATGGGGCTTGA-CATCT-3; and CKα' forward, 5-AACCTTCGTGGTGGAACA-AAT-3; reverse, 5-CTTGCTGTGGCAGTAATCCAG-3. The resulting mRNA levels were expressed as a ratio to the level of 18S mRNA.

Western Blot and Detection of GTPCH-1 Phosphorylation

Using nonboiled lysates and low-temperature SDS-PAGE, eNOS dimer/monomer were immunoblotted (eNOS antibody 1:2500; BD-Transduction Laboratory) as described elsewhere.[17] DHFR antibody was purchased from BD-Transduction Laboratory. CK2α and CK2α' antibodies were from Santa Cruz Biotechnology. The GTPCH-1 and PTPS antibody are described elsewhere.[18,19] An alternate GTPCH-1 antibody was kindly provided by Irmgard Tegeder (University of Frankfurt, Germany). SR antibody was provided by Young Shik Park (Inje University, Kimhae, South Korea). To initially detect GTPCH-1 phosphorylation, the enzyme was immunoprecipitated with a monoclonal antibody (Abnova, Tapai, Taiwan) and A/G PLUS agarose beads (Santa Cruz Biotechnology). Phosphorylation was detected using ProQ Diamond staining (Invitrogen) according to the instructions of the manufacturer. Bands were visualized using a Kodak Image 2000MM station. To examine specific phosphorylation sites in GTPCH-1, rabbit polyclonal anti-phospho antibodies were raised against the phosphopeptides shown in Table I in the online data supplement at http://circres.ahajournals.org.

Measurement of NO and O_2^- Production

NO production was measured by electron spin resonance (ESR) using the specific colloid probe Fe^{2+} diethyldithiocarbamate ($Fe[DETC]_2$) as described.[20] For detection of O_2^- formation the spin probe 1-hydroxy-4-methoxy-2,2,6,6-tetramethylpiperidine (TMH) was used. Following either 14 hours of shear or static conditions, the medium was removed and a chelexed Krebs/HEPES containing TMH (0.5 mmol/L) was added to the cells for 60 minutes. The cells were then scraped from the dish, snap frozen, and placed in an ESR finger Dewer under liquid nitrogen. ESR settings were: field sweep, 80 G; microwave frequency, 9.39 GHz; microwave power, 2 mW; modulation amplitude, 5 G; conversion time, 327.68 ms; time constant, 5242.88 ms; 512 points resolution; and receiver gain, 1×10^4.

Statistical Analysis

All values are means$\pm$SEM. The data were compared between groups by *t* test when 1 comparison was performed and by ANOVA for multiple comparisons. A value of $P<0.05$ was considered significant. When significance was indicated by ANOVA, the Student–Newman–Keuls post hoc test was used when all groups were compared. The Bonferroni post hoc test was used to make selected comparisons and the Dunnett post hoc test was used when 1 group served as a control. In some cases, when the *F* test for comparison of variances was significant, groups were compared using the Mann–Whitney test and a Bonferroni correction.

Results

Effect of Shear Stress on H_4B Levels and Activity of Enzymes Involved in De Novo Biosynthesis

The influence of shear stress on H_4B levels was assessed using a cone and plate viscometer. HAECs were exposed to laminar shear (+15 dynes), oscillatory shear ($\pm$ 15 dynes), or static conditions. Fourteen hours of laminar shear markedly augmented H_4B levels compared with static conditions (Figure 1A). The levels of H_4B in cells exposed to oscillatory shear stress were significantly less than those in cells exposed to laminar shear (Figure 1A). The de novo synthesis of H_4B involves 3 enzymes, GTPCH-1, PTPS, and SR. Laminar shear caused a 30-fold increase in the activity of GTPCH-1 (Figure 1B and the Table). In keeping with the effect of laminar and oscillatory shear stress on H_4B levels, the increase in GTPCH-1 activity was much greater with laminar as compared with oscillatory shear. The activities of PTPS and SR were not regulated by shear stress (Table).

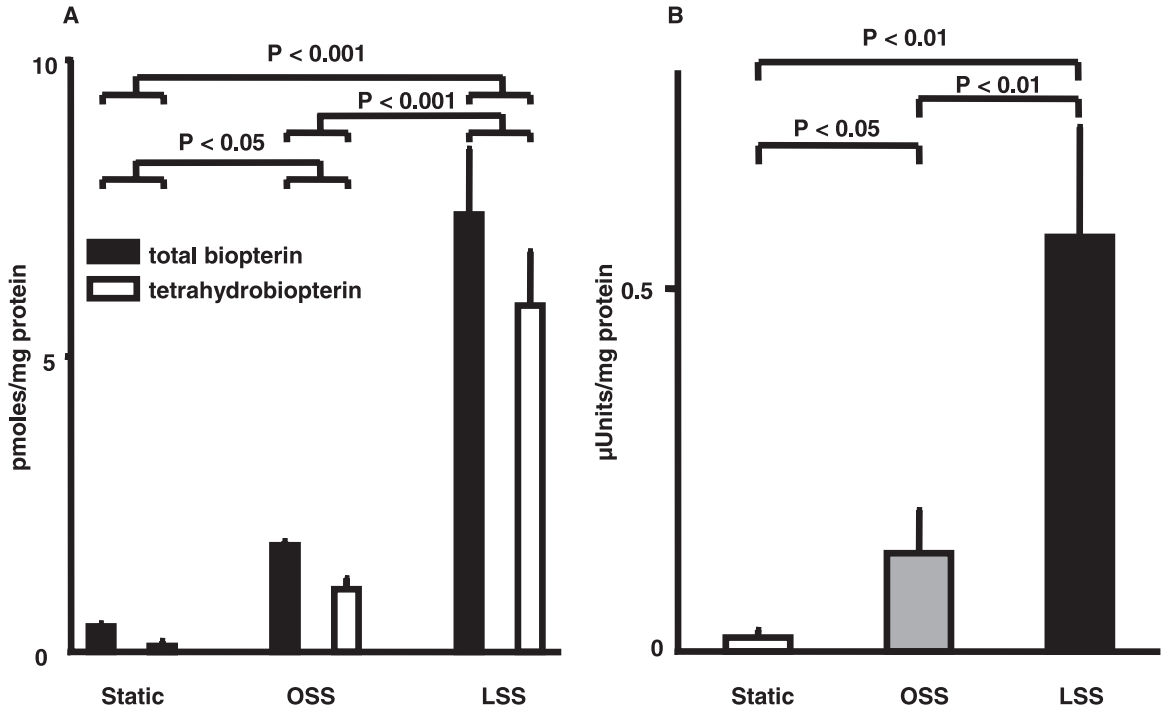

Figure 1. H_4B levels and activity of GTPCH-1 in response to shear. HAECs were exposed to 14 hours of either laminar shear stress (LSS) (+15 dynes) or oscillatory shear stress (OSS) (±15 dynes) or to static conditions. A, The levels of H_4B were determined using HPLC (n=7). B, GTPCH-1 activity was measured by monitoring the conversion of GTP to 7,8-dihydroneopterin trisphosphate, which was detected after oxidation and dephosphorylation to neopterin by HPLC (n=5 to 6).

Effect of Shear Stress on Expression of Enzymes Involved in the H_4B De Novo Biosynthesis

Other stimuli for H_4B de novo synthesis, such as cytokines, increase GTPCH-1 protein levels. We therefore investigated the effect of shear stress on protein levels of GTPCH-1, PTPS, and SR. Laminar shear did not appreciably change the protein levels of any of the enzymes involved in H_4B biosynthesis (Figure 2A). Western blots for GTPCH-1 demonstrate a predominant band at ≈30 kDa and inconsistently revealed a faint band at ≈35 kDa, which was inconsistently affected by oscillatory and laminar shear. Preliminary experiments using siRNA against GTPCH-1 indicated that both of these bands were specific for this enzyme. Western blots using a second GTPCH-1 antibody confirmed that neither oscillatory nor laminar shear increased GTPCH-1 protein levels (supplemental Figure I).[21]

Regulation of H_4B Levels Under Shear Stress by Casein Kinase 2

A common mechanism for enzyme activation is phosphorylation. We immunoprecipitated GTPCH-1 and examined its phosphorylation using ProQ Diamond staining. Laminar shear caused a 2-fold increase in GTPCH-1 phosphorylation (Figure 2B). GTPCH-1 contains 1 protein kinase C phosphorylation site and 5 CK2 phosphorylation sites and has been shown to be phosphorylated by protein kinase C and CK2 in vitro.[22,23] We therefore assessed the effects of protein kinase C or CK2 inhibition on H_4B levels under laminar shear stress. Protein kinase C inhibition with chelerythrine (3 μmol/L) had no effect on H_4B levels after 14 hours of laminar shear compared with untreated control cells. However, inhibition of CK2 with the specific inhibitor TBB dose-dependently blocked the increase in H_4B caused by laminar shear (Figure 2C). In addition, pretreatment of cells with TBB for 1 hour before shear also inhibited the increase in GTPCH-1 activity (Figure 2D).

CK2 is composed of a β regulatory subunit and either an α or α' catalytic subunit, the latter of which can function independently of the β subunit.[24] We therefore performed additional studies to confirm our results with TBB and to identify the catalytic subunit involved in regulation of GTPCH-1 activity. Using siRNA, we were able to selectively downregulate both protein and mRNA of either the α or α' subunits of CK2 (Figure 3A through 3C). Whereas downregulation of CK2α had no effect on the increase in H_4B levels caused by shear, downregulation of the α' subunit of CK2 markedly inhibited this increase in H_4B (Figure 3D). These changes in H_4B levels were paralleled by identical effects of siRNA against CK2 α and α' subunits on GTPCH-1 activity (Figure 3E). To clarify the role of CK2-mediated phosphorylation induced by shear, we raised polyclonal phosphospecifc antibodies against each of the 5 predicted CK2 phosphorylation sites. These revealed no evidence of

Table. Activity of Enzymes Involved in H_4B De Novo Synthesis

	GTPCH-1	PTPS	Sepiapterin Reductase
n	5–6	4	3–4
Static (μU/mg)	0.02±0.01	0.26±0.02	9.8±0.5
OSS (μU/mg)	0.13±0.06*	0.24±0.03	10.0±0.5
LSS (μU/mg)	0.57±0.15†‡	0.23±0.02	10.3±0.5

OSS indicates oscillatory shear stress; LSS, laminar shear stress. *$P<0.05$ vs static, †$P<0.01$ vs static, ‡$P<0.01$ vs oscillatory shear stress.

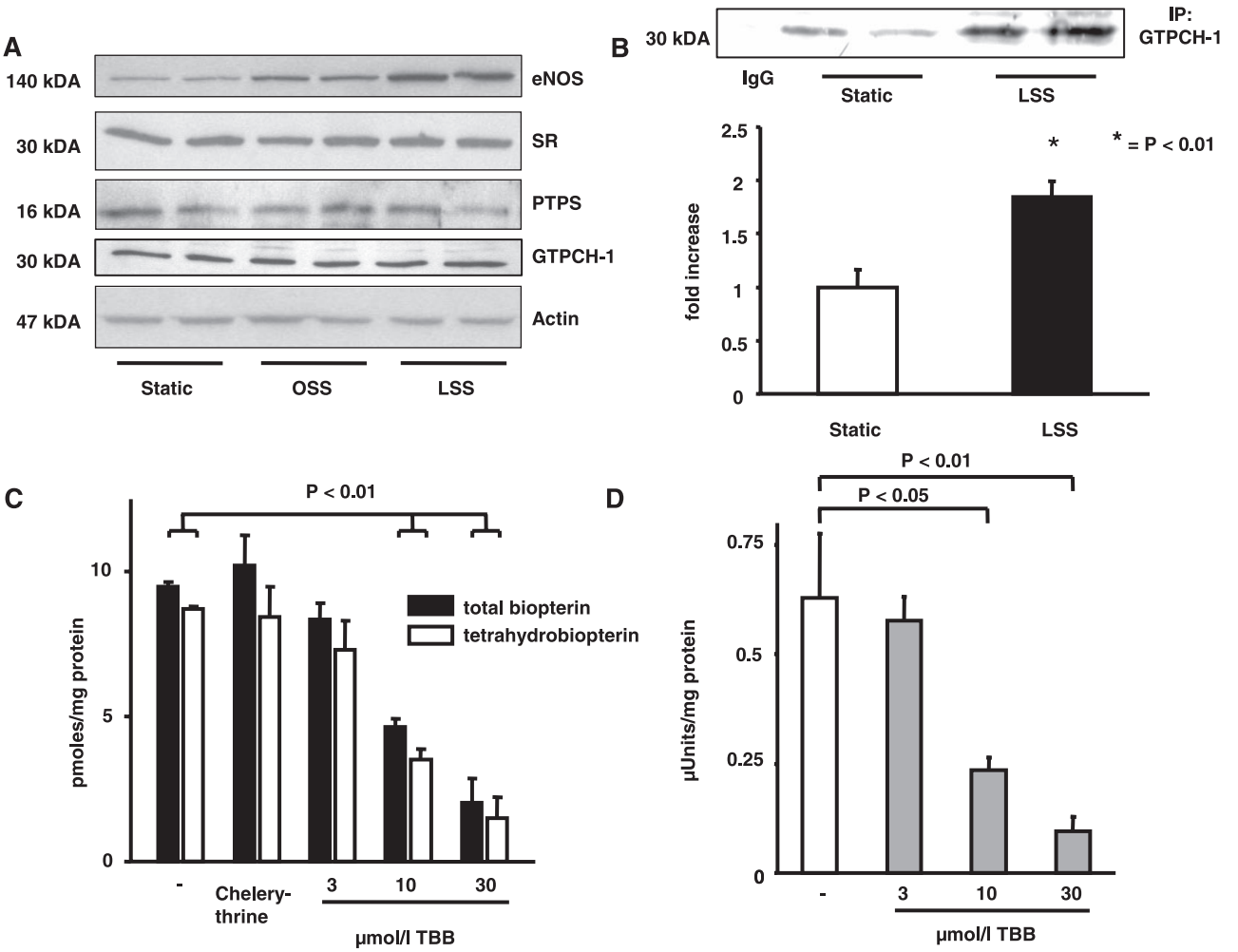

Figure 2. Mechanisms underlying increased H_4B de novo synthesis in response to shear. HAECs were exposed to 14 hours of either laminar shear stress (LSS) or oscillatory shear stress (OSS) or to static conditions. A, Representative Western blot showing protein expression of eNOS, GTPCH-1, PTPS, and SR. Actin was used as a loading control. B, Phosphorylation of GTPCH-1 in response to shear and role of casein kinase 2. HAECs were exposed to 14 hours of laminar shear stress or static conditions. GTPCH-1 was immunoprecipitated from cell lysates, run on SDS-PAGE, and transferred to a membrane that was stained with the ProQ diamond kit for phosphorylation. IgG was used as a control. The top portion shows an example blot; the bottom provides mean data for ProQ diamond staining (n=5 to 6). C, Cells were pretreated for 1 hour with either the CK2 inhibitor TBB (3 to 30 μmol/L) or the protein kinase inhibitor chelerythrine (3 μmol/L), or they were not pretreated, and exposed to 14 hours of laminar sheer stress. Levels of H_4B were measured using HPLC. D, Effect of TBB (3 to 30 μmol/L) on GTPCH-1 activity in sheared cells (n=3 to 6).

phosphorylation of serine 60, threonine 112, serine 139, or threonine 240 either at baseline or in response to shear. In contrast, serine 81 showed faint phosphorylation in unsheared cells and a striking increase in phosphorylation in response to laminar shear (Figure 4A and 4B). Of note, oscillatory shear did not stimulate phosphorylation of serine 81 (Figure 4B). The band identified by this phospho antibody corresponded in size to the major band identified in Figure 2A and supplemental Figure I and was reduced by siRNA against GTPCH-1 but not by nonsilencing siRNA (Figure 4C). Shear-induced GPTCH-1 phosphorylation on serine 81 was inhibited in endothelial cells transfected with siRNA against the α' subunit of CK2 (Figure 4D). These studies strongly support a role of CK2 in the activation of GTPCH-1 activity caused by shear and demonstrates that this is mediated by phosphorylation of this enzyme on serine 81. Shear stress did not change protein levels of either the α or α' subunits (Figure 4E).

Effect of Shear Stress on the Salvage Pathway

It has recently been shown that DHFR is a predominant enzyme of the salvage pathway for maintenance of H_4B in endothelial cells.[12] We considered the possibility that laminar shear stress might increase DHFR expression. Paradoxically, laminar shear downregulated DHFR protein levels in endothelial cells (Figure 5A and 5B). Moreover, the DHFR inhibitor methotrexate (1 μmol/L) had no effect on the increase in H_4B caused by shear and had no effect on the ratio of reduced to oxidized biopterin (Figure 5C). These data indicate that DHFR is likely not important for increasing H_4B in response to shear in human endothelial cells.

Role of H_4B Increase With Shear Stress for eNOS Coupling

The studies described above provide insight into how shear modulates H_4B and GTPCH-1 activity. It was unclear, how-

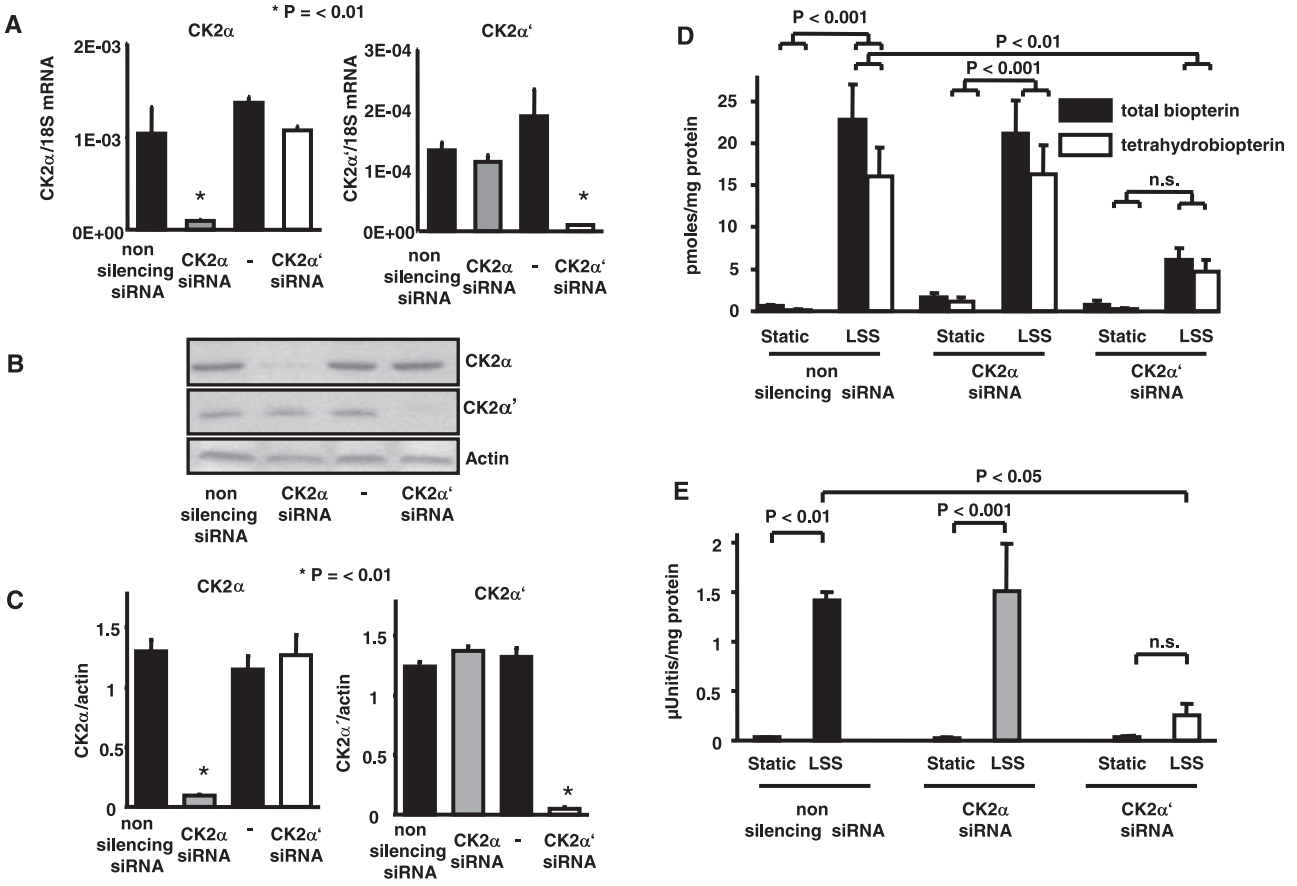

Figure 3. Role of CK2α and CK2α' in modulating H_4B levels and GTPCH-1 activity during shear. A through C, HAECs were transfected with siRNA against CK2α, CK2α', a nonsilencing control siRNA, or not transfected. A, mRNA was measured using real-time PCR (n=3). B, Representative Western blot of CK2 subunits protein expression. Actin was used as a loading control. C, Densitometry values for CK2 subunits protein expression normalized to actin (n=3). D and E, HAECs were transfected with siRNA against CK2α, CK2α', or a nonsilencing control siRNA and exposed to 14 hours of laminar shear stress (LSS) or static conditions. Thereafter, the levels of H_4B were assessed using HPLC (D), and GTPCH-1 activity (E) was measured (n=4 to 6).

ever, whether this increase in H_4B was essential for eNOS function or whether the small amount of H_4B present in human endothelial cells was sufficient to allow full eNOS catalytic function. To examine this question, we pretreated endothelial cells with the GTPCH-1 inhibitor 2,4-diamino-6-hydroxypyrimidine (DAHP) (1 mmol/L). This inhibited the shear-induced H_4B increase without changing the ratio of H_4B to oxidized pterins (Figure 6A). Basal NO production was then measured using ESR and the spin trap $Fe[DETC]_2$. In the absence of shear, cells produced minimal amounts of NO, and this was not changed by DAHP treatment (Figure 6B). Exposure of endothelial cells to 14 hours of shear strikingly increased endothelial cell NO production, and this was significantly reduced by DAHP (Figure 6B). In the absence of sufficient H_4B, eNOS not only fails to produce NO properly but also produces O_2^-. We therefore also measured O_2^- using ESR and the spin probe TMH. Under static conditions, the endothelial cell production of O_2^- was not affected by DAHP (Figure 6C). Laminar shear decreased O_2^- production in the absence of DAHP (Figure 6C). In the presence of DAHP, however, O_2^- production was significantly increased in sheared cells. This increase was inhibited by N^G-nitro-L-arginine methyl ester, suggesting that the source of O_2^- was NO synthase. In keeping with these results, low-temperature Western blots for eNOS revealed that when de novo generation of H_4B was prevented by DAHP, the formation of intact eNOS dimers caused by both oscillatory shear stress and laminar shear was blunted and monomer formation was increased (Figure 6D and 6E).

Discussion

In the present study, we demonstrate that laminar shear stress causes a marked increase in endothelial cell levels of the essential eNOS cofactor H_4B and provide insight into how this occurs. The activity of GTPCH-1, the first step in this pathway, was strikingly increased by ≈30-fold, whereas the activities of PTPS and SR were unchanged. Our data indicate that this increase in GTPCH-1 activity was modulated by its phosphorylation on serine 81 by CK2. In contrast to laminar shear, oscillatory shear did not stimulate GTPCH-1 phosphorylation and caused only a modest increase in H_4B and GTPCH-1 activity. The H_4B increase with shear was found to be essential for the newly synthesized eNOS to produce NO and to prevent O_2^- production by the enzyme.

In other cells, GTPCH-1 has been shown to be mediated by transcriptional mechanisms, posttranslationally by phosphorylation, and by the GTPCH-interacting protein GFRP, which modulates the activity of the enzyme. Cytokines lead to a

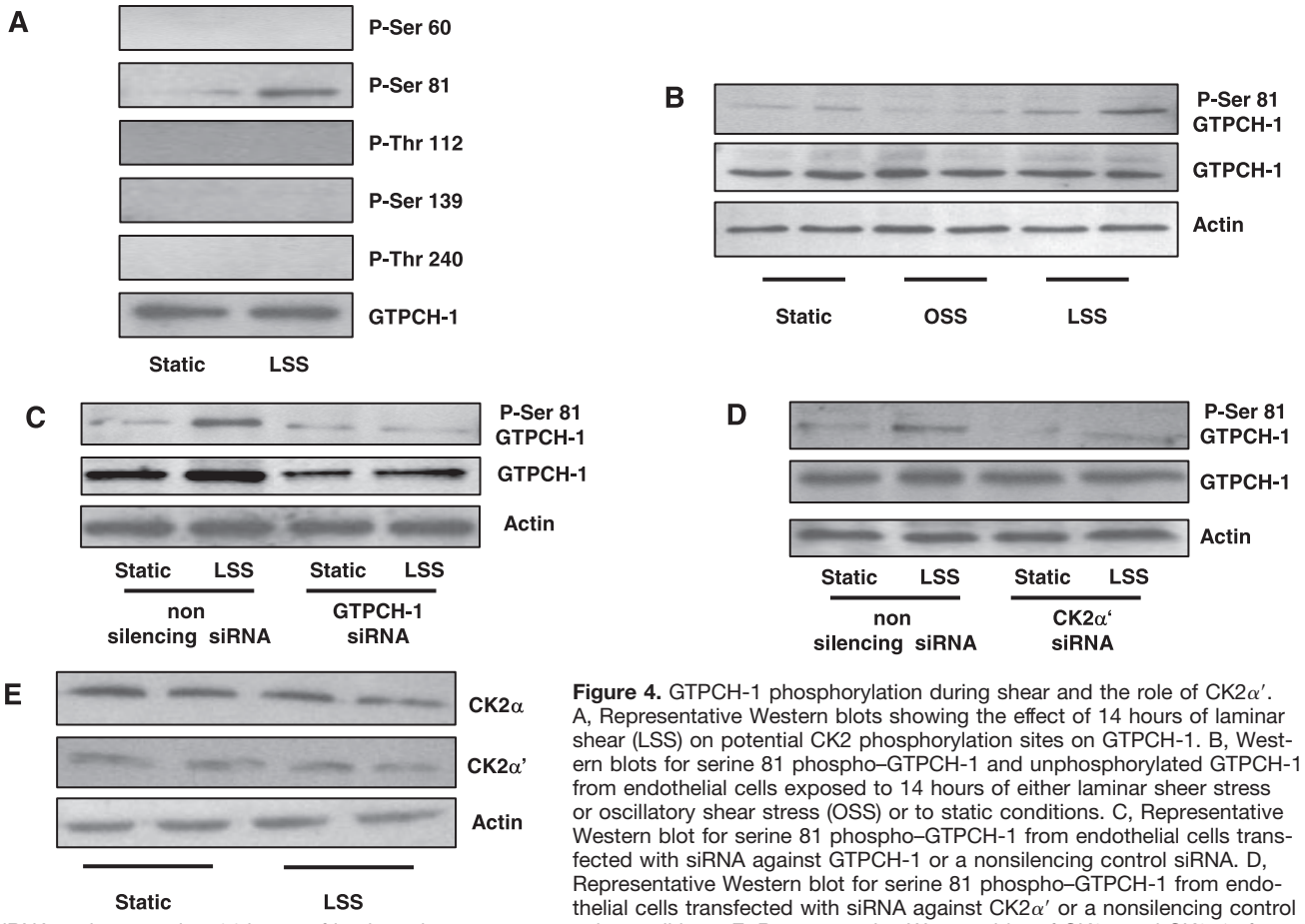

Figure 4. GTPCH-1 phosphorylation during shear and the role of CK2α'. A, Representative Western blots showing the effect of 14 hours of laminar shear (LSS) on potential CK2 phosphorylation sites on GTPCH-1. B, Western blots for serine 81 phospho–GTPCH-1 and unphosphorylated GTPCH-1 from endothelial cells exposed to 14 hours of either laminar sheer stress or oscillatory shear stress (OSS) or to static conditions. C, Representative Western blot for serine 81 phospho–GTPCH-1 from endothelial cells transfected with siRNA against GTPCH-1 or a nonsilencing control siRNA. D, Representative Western blot for serine 81 phospho–GTPCH-1 from endothelial cells transfected with siRNA against CK2α' or a nonsilencing control siRNA and exposed to 14 hours of laminar sheer stress or static conditions. E, Representative Western blot of CK2α and CK2α' after 14 hours of laminar sheer stress or static conditions.

marked upregulation of GTPCH-1 protein levels in several cells types.[18,25–27] Hydrogen peroxide has also been shown to stimulate GTPCH-1 mRNA levels in mouse brain microvascular endothelial cells and in bovine endothelial cells.[28] Unlike the cytokines and hydrogen peroxide, we found that laminar shear had no effect on GTPCH-1 protein expression but increased its activity by 30-fold. Of interest, the activity of PTPS and SR were unchanged by shear stress. Our data therefore indicate that the activity of GTPCH-1 is the regulating factor for H_4B levels with shear stress. If one compares the relative activities of GTPCH-1, PTPS, and SR at baseline, it is obvious that PTPS activity is ≈10-fold higher than GTPCH-1, whereas SR activity is almost 500-fold greater. Thus, at baseline, the rate limiting step in H_4B synthesis is GTPCH-1, the first step in the de novo pathway. Interestingly, in the setting of shear, the marked increase in GTPCH-1 activity exceeds that of PTPS activity, leading to a condition in which PTPS become rate limiting. The extraordinarily high level of SR activity is such that it is unlikely to ever become rate limiting in human endothelial cells. These findings are similar to the condition caused by interferon-γ in human umbilical vein endothelial cells, in which the marked increase in GTPCH-1 activity overcomes the activity of PTPS.[29] The very high levels of SR activity also explains why sepiapterin can markedly increase the endothelial cell level of H_4B.

Our data indicate that phosphorylation of GTPCH-1 on serine 81 is critical in activation of this enzyme by laminar shear. How phosphorylation at this site affects enzyme function remains unknown. An examination of the known crystal structure of human GTPCH-1 indicates that serine 81 resides in an extension of the enzyme, which could shield the GTP binding site. It is conceivable that phosphorylation alters GTP access to its binding site; however, additional studies are needed to address this.

Our data also indicate that the α' subunit of CK2 is responsible for GTPCH-1 Ser-81 phosphorylation. CK2 is composed of 3 subunits, including a regulatory β subunit and 2 catalytic subunits, α and α'. The α and α' subunits can function independently of the β subunit and also of each other.[24] Of interest, it has recently been shown that CK2 phosphorylates the angiotensin converting enzyme in endothelial cells, promoting its retention in the cell membrane.[30] CK2 has been demonstrated to phosphorylate calmodulin, which inhibits its binding to eNOS.[31] Relevant to the present study, laminar shear has been shown to cause CK2 phosphorylation of the transcription factor SP1.[32] The specific mechanisms underlying activation of CK2 by shear needs further investigation.

The level of H_4B is not only regulated by de novo synthesis but also by the salvage pathway, which converts H_2B and quinoid–H_2B to H_4B. Recently, it has been shown that DHFR

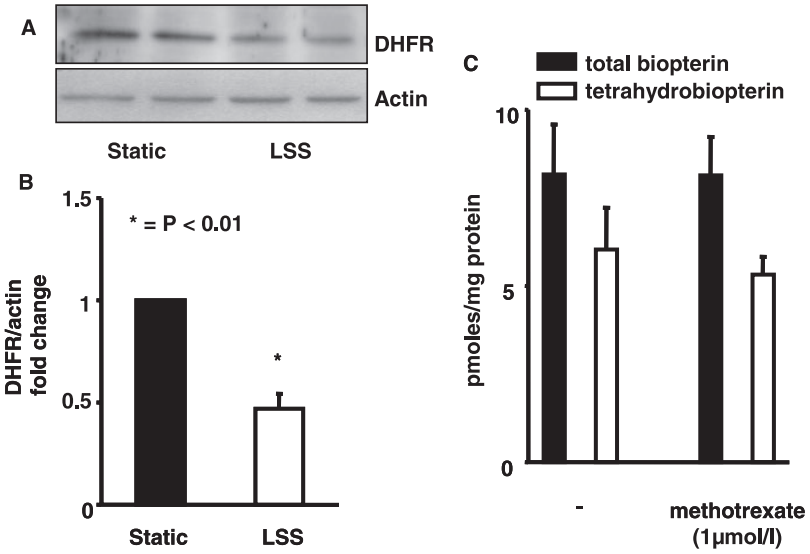

Figure 5. Expression and influence of blockade of the salvage enzyme DHFR. A, Representative Western blot of DHFR expression following static conditions or 14 hours of laminar shear stress (LSS). B, Densitometry values for DHFR expression normalized to actin with static condition set as 1 (n=5). C, H_4B levels in HAECs under laminar shear stress with or without blockade of the DHFR inhibitor methotrexate (1 μmol/L) (n=3).

plays a predominant role in the endothelial cell salvage pathway.[12] Our current study suggests that the increase in endothelial cell H_4B with shear is unlikely attributable to the salvage pathway because methotrexate, which inhibits DHFR, did not alter this response. Moreover, shear caused a decrease in endothelial DHFR protein levels. Taken together with the above findings, these data would suggest that the predominant effect of shear on H_4B synthesis is mediated by the de novo synthesis pathway and not via an alteration of the salvage pathway.

In contrast to laminar shear, oscillatory shear stress only minimally increased H_4B levels and GTPCH-1 activity. Our

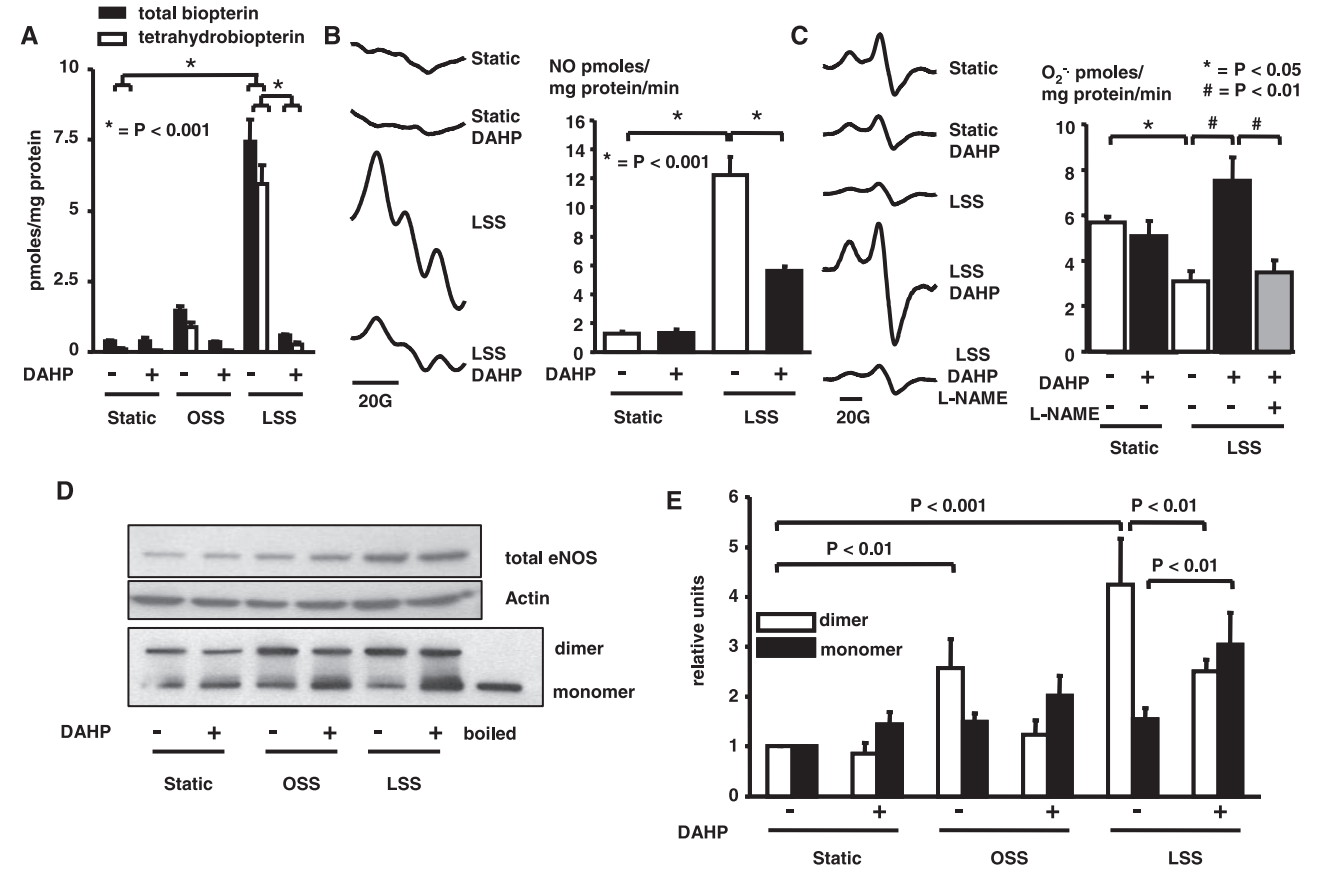

Figure 6. Effect of shear stress on H_4B levels and eNOS function during GTPCH-1 blockade. A, Effect of GTPCH-1 blockade with DAHP (1 mmol/L) on H_4B levels. B, Representative $Fe[DETC]_2$ ESR spectra for NO production (left) and mean data (right) (n=4 to 5). C, Representative ESR spectra for O_2^- production measured with the spin probe TMH (left) and mean data (right) (n=4 to 7). D, Representative Western blot for total eNOS and dimer/monomer using a low-temperature gel. E, Densitometry values for eNOS dimers and monomers. Values were normalized to static conditions, which were set as 1 (n=5).

data indicate that a major difference between laminar and oscillatory shear stress is that laminar shear stimulates serine 81 phosphorylation of GTPCH-1, whereas oscillatory shear stress does not. There are numerous prior examples of differing effects of laminar and oscillatory shear stress on endothelial cell function, with laminar shear having antiatherosclerotic effects, whereas oscillatory shear often promotes proinflammatory, proatherosclerotic events. Previously, we have found that oscillatory shear stress increases both NO production and eNOS expression, albeit to a lesser extent than that observed with laminar shear.[33] Taken together with the effect on H_4B levels, these results would help explain why endothelial cells in areas of disturbed flow have reduced levels of NO production.

Our present study and several others have indicated that an increase in H_4B is essential in conditions when eNOS protein levels are elevated. When we prevented the increase in H_4B during exposure to laminar shear, the cellular production of NO was only minimally increased and O_2^- production was increased. Moreover, eNOS dimer formation was blunted. In keeping with these data, Bendall et al recently showed that in transgenic mice overexpressing eNOS, a large portion of this enzyme was uncoupled, leading to excessive endothelial O_2^- production.[34] This situation was ameliorated when these mice were crossed with animals overexpressing GTPCH-1, such that vascular production of O_2^- was reduced and NO synthesis was increased. Likewise, Bevers et al have recently shown that overexpression of eNOS in microvascular endothelial cells led to enhanced production of both NO and reactive oxygen species.[35] When these cells were treated with H_4B, NO production dramatically increased and reactive oxygen species production declined.

In accord with our present study, Lam et al have recently shown that H_4B levels are increased in vivo by high shear caused by an aortocaval fistula.[36] These authors found that GTPCH-1 protein was increased in this model, which might involve stimuli other than shear, such as an inflammatory response. It is likely that GTPCH-1 is phosphorylated by high-flow states in vivo, such as the aortocaval fistula used by Lam et al.

In conclusion, we find that shear stress is a potent stimulus for H_4B de novo synthesis in human endothelial cells and that this is dependent on a marked GTPCH-1 activation by phosphorylation on serine 81. Our data indicate that this is likely mediated by CK2 and is necessary to maintain eNOS function during shear stress. These studies define a new role for CK2 in the endothelium and a novel mechanism of GTPCH-1 control.

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Disclosures

None.

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