Induction of KLF2 by Fluid Shear Stress Requires a Novel Promoter Element Activated by a Phosphatidylinositol 3-Kinase-dependent Chromatin-remodeling Pathway*

Justin P. Huddleson‡, Nisar Ahmad‡, Seetha Srinivasan, and Jerry B Lingrel§

From the Department of Molecular Genetics, Biochemistry, and Microbiology, University of Cincinnati College of Medicine, Cincinnati, Ohio 45267

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Fluid shear stress maintains vascular homeostasis by influencing endothelial gene expression. One mechanism by which shear stress achieves this is through the induction of transcription factors including Krüppel-like factor 2 (KLF2). We have previously reported that a 62-bp region of the KLF2 promoter is responsible for its shear stress-induced expression via the binding of nuclear factors. In this study, we find that the 62-bp shear stress response region contains a 30-bp tripartite palindrome motif. Electrophoretic mobility supershift and chromatin immunoprecipitation assays demonstrate that PCAF (P-300/cAMP-response element-binding protein-binding protein-associated factor) and heterogeneous nuclear ribonucleoprotein D bind this region as components of the shear stress regulatory complex. We have also characterized a PI3K-dependent/Akt-independent pathway responsible for shear stress-induced KLF2 nuclear binding, promoter activation, and mRNA expression. Furthermore, the shear stress response region of the KLF2 promoter was specifically immunoprecipitated by antibodies against acetylated histones H3 and H4 in shear-stressed but not static hemangioendothelioma cells. The acetylation of these histones was blocked by PI3K inhibition. Finally, we have found that KLF2 increases endothelial nitric-oxide synthase expression in murine endothelial cultures, an effect that is also blocked by PI3K inhibition. These results define the DNA regulatory element, signal transduction pathway, and molecular mechanism activating the flow-dependent expression of a vital endothelial transcription factor.

Mechanical forces influence endothelial phenotypes through signal transduction and gene activation. Hemodynamic shear stress, the frictional force that results from viscous blood flow (1), is of primary importance to the endothelium. It stimulates an adaptive response to generate antioxidant, anti-proliferative, anti-apoptotic, and anti-atherosclerotic patterns of gene expression (2, 3). One of the most important factors in this response is the level of nitric oxide (NO)\(^5\) production. In addition to being a potent vasodilator (4), NO inhibits inflammation (5), smooth muscle cell proliferation (6), platelet aggregation (7), and endothelial cell apoptosis (8). NO levels are increased, in part, through the activation of endothelial nitric-oxide synthase (eNOS) by phosphorylation on Ser\(^1177\) via the pro-survival phosphatidylinositol 3-kinase (PI3K)/Akt pathway, which plays an integral role in the response to fluid shear stress (9–11). It is also responsible for the induction of eNOS mRNA by shear stress (12). Although Akt is the canonical immediate target of PI3K activation, PI3K-dependent/Akt-independent shear stress signal transduction pathways have been reported (13, 14). However, little is known regarding the effectors of the PI3K mechanotransduction pathway or the molecular mechanisms underlying the signaling events from shear stress to gene expression in endothelial cells.

The application of shear stress up-regulates mRNA levels of vasoprotective genes by acetylating histones to facilitate DNA unwinding and increase the accessibility of chromatin to transcription factors (15, 16). Shear stress also increases the expression of transcription factors themselves, such as KLF2 (lung Krüppel-like transcription factor (LKLF)) (17, 18). KLF2 is found throughout the aorta with the exception of physiological levels of pulsatile and laminar shear stress (17). KLF2 is found throughout the aorta with the exception of branch points, which experience complex flow patterns and decreased shear stress. Because these sites are also more prone to atherosclerotic plaque formation, this finding suggests that KLF2 may have atheroprotective functions. Subsequent work (27, 28) has found that KLF2 overexpression up-regulates eNOS in human and bovine endothelial cells and is anti-inflammatory and anti-thrombotic. These findings suggest that nitric-oxide synthase; PI3K, phosphatidylinositol 3-kinase; Akt, cellular homolog of transforming oncogene of Akt-8 oncirus; KLF2, Krüppel-like factor 2; CREB, cAMP-response element-binding protein; CBP, CREB-binding protein; PCAF, P-300/CBP-associated factor; hnRNP D, heterogeneous nuclear ribonucleoprotein D; EOMA, hemangioendothelioma; FBS, fetal bovine serum; HUVEC, human umbilical vein endothelial cells; myr-Akt, constitutively active myristolylated Akt; EMSAs, electrophoretic mobility shifts assays; ChIP, chromatin immunoprecipitation; RT, reverse transcription; IP, immunoprecipitation; RNA Pol 2, RNA polymerase II; ERK, extracellular signal-regulated kinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; TFIIB, transcription factor II B.

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KLF2 gene expression is crucial for the prevention of vascular disease. However, the molecular mechanisms underlying its induction by shear stress have been largely unexplored.

Our laboratory has recently shown that a 62-bp region of the KLF2 promoter (from −157 to −95 nucleotides from the start site of transcription) mediates KLF2 flow induction via the binding of specific shear stress-induced nuclear factors in murine microvascular endothelial cultures (18). In this study, we have found that the 62-bp flow response region contains a 30-bp motif of three sequential palindromes. Our studies also indicate that P-300/CBP-associated factor (PCAF), and heterogenous nuclear ribonucleoprotein D (hnRNPD) bind this region as components of a shear stress-specific regulatory complex that functions in a PI3K-dependent pathway to acetylate histones, providing a molecular mechanism for the flow-dependent expression of KLF2.

MATERIALS AND METHODS

Cell Culture—EOMA cells (ATCC, Manassas, VA) were cultured in Dulbecco’s modified Eagle’s medium (Invitrogen) containing 15% fetal bovine serum (FBS). All of the HUVECs were used at less than the fifth passage. Cells (HUVEC, Cambrex, Walkersville, MD) were cultured in supplemented endothelial growth medium 2 media (Cambrex) containing 10% FBS. All of the HUVECs were used at less than the fifth passage.

Shear Stress Apparatus and Experiments—A Cellmax QUAD® artificial capillary cell culture system (Spectrum Laboratories, Rancho Dominguez, CA) was used as described previously (18) to shear cells at 19 dynes/cm² (30). For HUVEC experiments, the artificial capillaries were coated in 10 μg/ml human fibronectin (Sigma) prior to the addition of cells. For the signal transduction inhibitor experiments, pharmacological inhibitors (Calbiochem) for PI3K (LY294002), ERK1/2 (PD98059), or inactive analog control (SB202474) were dissolved in 0.25% Me2SO and then added to the media reservoir at the specified concentration, accounting for the total amount of liquid in the reservoir plus the capillary tubing. After the inhibitors were added, the medium was allowed to circulate over the cells at a minimal shear level of ≤1 dynes/cm² for 30 min before exposing the cells to a full shear force of 19 dynes/cm². During PD98058 treatment, the medium FBS concentration was reduced to 2%.

Transfection Protocol And Reporter Gene Assays—All of the transfections were carried out in 100-mm plates, except as otherwise noted. EOMA cells and HUVECs were grown to 70% confluence, washed with PBS, bathed in serum-free media, and transfected with 4 μg of DNA and 0.25 μg of β-galactosidase vector using FuGENE 6 (Roche Applied Science). FBS was added back to the plates 4 h after transfection. For deletion experiments, cells were trypsinized from the plates, allowed to settle in the capillary tubing for 12 h after transfection and seeded into the artificial capillary as described previously (18). The highest expressing construct (−924 to +14) was arbitrarily set at 100%, and all other expression systems are presented as a percentage of that value. For experiments testing transactivation of the KLF2 promoter, transfections were carried out in 6-well plates. 0.5 μg of a luciferase reporter plasmid containing the KLF2 −157 promoter was cotransfected into static EOMA cells along with 0.25 μg of β-galactosidase vector and 0.5 μg of expression plasmid for PCAF (a kind gift of Dr. Yoshio Nakatani, Dara Farber Cancer Institute, Boston, MA), hnRNPD (a kind gift of Dr. Arco Yukimoto, Jichi Medical School, Tochigi, Japan), or empty vector controls. Additional control experiments were done in identical fashion with a cyclic AMP-response element luciferase plasmid (Stratagene, La Jolla, CA) substituted for the KLF2 luciferase plasmid. The KLF2 expression vector was constructed by cloning the coding region of KLF2 cDNA into a pBK-cytoplasmavirus phagemid vector (Stratagene). The constitutively active Akt expression construct was a gift from Dr. Richard Roth (Stanford University School of Medicine, Stanford, CA) (31). The dominant-negative Akt expression vector (dominant-negative Akt (protein kinase B-CαAX)) was a gift from Dr. Beaudoin M. Th. Buringer (Utrecht University, Utrecht, The Netherlands) (32).

Electrophoretic Mobility Shift Assays (EMSA)—Nuclear extract, prepared as described previously (33), was isolated from EOMA cells subjected to 24 h of fluid shear stress. Protein concentration was determined by BCA assay. EMSAs were performed as described previously (18, 34). For experiments with the 62-bp promoter region, 2 μg of nuclear extract was used. In experiments with the 30-bp element, 20 μg of nuclear extract was used. For competition reactions, cold oligonucleotides were incubated with nuclear extract for 15 min prior to the addition of labeled oligonucleotide. For antibody supershifts, 5 μg of nuclear extract were used with either PCAF antibody (a gift from Dr. Yoshiro Nakatani, New York Medical School, and Santa Cruz Biotechnology) or hnRNPD antibody (a gift from Dr. Robert Schneider, New York, NY).

Chromatin Immunoprecipitation (ChIP) Assays—EOMA cells were exposed to shear stress for 12 h or kept in static conditions. ChIP assays were performed as described previously (34) using primer pairs to amplify a 139-bp segment encompassing the KLF2 promoter shear stress response region: forward 5′-GGCTTGGAGGCGCCGTC- CGGGCTCCCGGA-3′ and reverse 5′-CCGGGCTTAGGGGTCG- GACGAAACCCTG-3′. Anti-H3AcK14 and anti-H4AcK12 antibodies (Upstate Biotechnology), anti-RNA polymerase II antibody (Santa Cruz Biotechnology), and transcription factor II B (TFII B) antibody (Santa Cruz Biotechnology) were used to immunoprecipitate their corresponding proteins and bound DNA.

Immunoprecipitation and Immunoblotting—Static or sheared EOMA cells were lysed with non-ionic cell lysis buffer. Immunoprecipitations (IP) were performed essentially as described previously (35) with polyclonal rabbit anti-mouse hnRNPD antibody incubated with magnetic beads coated with sheep anti-rabbit antibody (Dynabeads M-280). Immunoblots were performed according to standard procedures with PCAF antibody (36). Rabbit primary antibodies against eNOS, Akt, and phospho-Akt (Ser473) were purchased from Cell Signaling Technology, Inc. (Beverly, MA). Mouse anti-GAPDH (Advanced ImmunoChemical, Long Beach, CA) and rabbit anti-hemagglutinin antibody (Y-11, Santa Cruz Biotechnology) antibodies were also used. After incubation with peroxidase-conjugated goat-anti-rabbit (Calbiochem) or goat anti-mouse (Jackson ImmunoResearch, West Grove, PA) IgG antibodies diluted in blocking buffer, immunoreactivity was visualized following treatment with ECL Western blotting detection reagent (Amersham Biosciences) and developed with Kodak Biomax MR x-ray film. Densitometric analyses were performed by ImageQuant 5.0, and results were normalized by arbitrarily setting the densitometry of static empty vector control cells to 1.0.

Semi-quantitative Real-time Reverse Transcription (RT)-PCR—Relative quantification of changes in KLF2 and eNOS expression following shear stress and transfection were determined by real-time RT-PCR, as described previously (18). Fold changes in mRNA expression were calculated using the ΔΔ Ct method (37). GAPDH levels, which are not influenced by shear stress (38), were used as an internal control. For the shear induction, a minimal shear promoter exists within the KLF2 promoter I–III regions (38). 18 S mRNA served as an internal control as follows: forward 5′-CGTCAAGGCACCCGGAGATT-3′ and reverse 5′-GACCCGACT- TACTGCGAATT-3′.

Summary—For comparisons between the means, independent experiments were performed and analyzed using Student’s t tests. p values < 0.05 were considered statistically significant.

RESULTS

The KLF2 Promoter Contains a 30-bp Minimal Shear Stress Response Element with a Palindrome Motif—We have previously reported that KLF2 is regulated by fluid flow through a 62-bp promoter region. Deletion analyses in HUVECs verified the importance of this region in primary human endothelial cells (Fig. 1A) and demonstrated its evolutionary conservation. In EOMA cells, we continued our deletion analysis through the KLF2 promoter and found that, although the whole 62-bp region from −157 to −95 nucleotides is required for maximal shear induction, a minimal shear promoter exists within the 62-bp region between −138 and −95 nucleotides that continues to generate a significant shear response compared with static controls (Fig. 1B).

This region contains a 30-bp element from −138 to −108 that is 100% conserved between mouse and human promoters and has been shown to regulate KLF2 transcriptional activity in LA-4 mouse lung cells (33, 39). The interesting feature of the 30-bp element is a motif of three palindromes occurring in tandem (Fig. 2A, I–III). Such palindrome motifs have been shown to be important for endothelial gene regulation but not in the context of shear stress induction (40, 41). The palin-
The KLF2 shear stress response element is conserved in HUVECs and contains a critical sequence between -157 and -95 bp. A, KLF2 promoter deletion luciferase constructs of indicated length from the start site of transcription (-924 nucleotides, -157, nucleotides, and so on) were transiently transfected into HUVECs along with a β-galactosidase plasmid prior to exposure to 24-h fluid shear stress (19 dyne/cm²) or static conditions. B, identical experiments were done in EOMA cells with finer deletion constructs. Schematics of the KLF2 promoter deletion constructs used in these experiments are shown on the left. Within the -924 construct (=100%), the 62-bp shear stress response region is shown in gray and the 30-bp minimal shear stress response element within is shown in white. The normalized luciferase activities in the cells are presented as percentage values compared with the value of shear stress-loaded cells transfected with the full KLF2 proximal promoter region from -924 to +14 bp. The results shown represent the mean ± S.E. of three to five independent experiments.

FIG. 2. The KLF2 shear stress response element is also noticeably AT-rich (100% AT in palindrome I; 80% AT in palindrome III) within a proximal promoter of ~70% GC content. An oligonucleotide spanning the -138 to -108 sequence was sufficient to bind additional shear stress nuclear factors above static base-line levels in electrophoretic mobility shift assays (Fig. 2B). The presence of two complexes is clearly visible under shear stress when using the 30-bp minimal promoter element (Fig. 2B, marked I and II) compared with our previous work that shows three shear stress-specific complexes binding the whole 62-bp region. No binding was evident when individual palindrome oligonucleotides were used in EMSAs (Fig. 2A, I alone and so on; data not shown). Interestingly, the protein binding within the 30-mer is best visualized at higher concentrations of nuclear extract (20 μg)
than required to see flow-specific binding using the full 62-bp region (2 μg). These findings suggest that the full length of the KLF2 shear stress response region is required for stability of the shear-induced activating complex, although the palindromic motif of the KLF2 promoter sufficiently displays dynamic flow-induced alterations in nuclear binding. In and of itself, however, the palindromic motif is insufficient to impart significant shear stress response compared with the −157 reporter plasmid in both EOMA cells and HUVECs (Fig. 2C). Therefore, transactivation of the KLF2 promoter by fluid flow depends significantly, but not exclusively, on the tripartite palindromic motif.

PCAF and hnRNP D Are Components of the Shear Stress Induction Complex on the KLF2 Promoter—Using DNA affinity chromatography and mass spectrometry, we have identified several proteins that regulate the −138 to −108 region of the KLF2 promoter in macrophages, including the transcriptional acetyltransferase PCAF and the RNA/DNA-processing factor hnRNP D (AUF1) (34). We reasoned that these proteins might be components of the endothelial shear stress KLF2 induction complex. Electromobility supershift assays were performed with oligonucleotide probes of the KLF2 palindromic motif and antibodies against PCAF and hnRNP D under static and flow conditions.

PCAF is a member of the GCN-5-related acetyltransferase family and has been proposed to play diverse roles in coactivation of gene expression (42, 43). In the present study, the addition of PCAF antibody resulted in no change in the pattern of static binding (Fig. 3A) but caused the immunodepletion of the shear-specific band (Fig. 2B, band I) (Fig. 3B). Therefore, PCAF binds the KLF2 promoter only under flow conditions. To confirm the results of the supershift assay in endothelial cells, we investigated the ability of PCAF to transactivate the KLF2 promoter through transient transfection of static cultured EOMA cells. As expected, ectopic PCAF expression resulted in an increase in KLF2 promoter activity (Fig. 3C). Transient transfection of PCAF did not influence the activity of empty pGL3 luciferase vector or a control cyclic AMP-response element luciferase plasmid, demonstrating the specificity of induction. Empty vector for PCAF also did not activate the KLF2 promoter. Therefore, our results indicate that PCAF is one component of the KLF2 shear stress induction complex able to activate the promoter in static cultures.

hnRNP D is a multi-functional protein that plays a role in mRNA turnover (44) but also functions as a transcription factor/coactivator (45, 46). Supershifts with antibody against hnRNP D produced results similar to the PCAF antibody. No change is noted in the static extract compared with nonspecific IgG control but caused the disappearance of the band in shear extract (B, marked by arrow). C, lack of trans-activation of the KLF2 promoter by hnRNP D (42 kDa) expression clone. Abbreviations: pGL3, empty luciferase vector; PCAF, PCAF expression vector; CRE, cyclic AMP luciferase reporter plasmid; 157, −157 reporter construct of KLF2 promoter; RLU, relative luciferase units; EV, empty vector. Mean ± S.E. from three separate experiments is shown. Asterisk indicates significant difference from luciferase values generated from empty vector transfections (*, p < 0.01).
interact under both static and shear conditions (Fig. 5A). However, ChIP analyses revealed that both PCAF and hnRNP D bind the KLF2 promoter only during shear stress (Fig. 5B). Because PCAF does not have a DNA binding domain and there is a loose hnRNP D DNA binding site (TTAGGC) (47) within the tripartite palindrome motif, this functional interaction suggests that it is targeted to the KLF2 promoter through its interaction with hnRNP D. Cotransfection of PCAF plus hnRNP D indicated that, in and of themselves, they are insufficient to cooperatively induce KLF2 in static cells (Fig. 5C). In fact, the presence of hnRNP D negated the induction of KLF2 mRNA by PCAF in static cultures. This finding may result from the ability of hnRNP D to degrade mRNA when overexpressed (44).

**Induction of KLF2 by Fluid Flow Is Regulated by a PI3K-dependent/Akt-independent Pathway**—To determine the signaling pathway responsible for KLF2 shear stress regulation, we tested the ability of pharmacological inhibitors of the ERK1/2 mitogen-activated protein kinase pathway and the PI3K pathway to block promoter activation and mRNA induction. ERK1/2 was chosen as a flow-activated mitogen-activated protein kinase pathway (48). Considering the anti-apoptotic properties of KLF2 (20, 23), PI3K was chosen for study because of its prosurvival functions. Interestingly, inhibition of ERK1/2 by PD98059 (50 μmol/liter) had no effect on KLF2 promoter activation (Fig. 6A). PI3K inhibition with LY294002, however, blocked KLF2 shear stress promoter activation in a dose-dependent manner (Fig. 6A). At 40 μmol/liter LY294002, promoter activation was reduced to 13.6% (± 7%) of cells treated with vehicle (Me2SO) alone. The effect of PI3K inhibition on flow-induced KLF2 mRNA levels was clearly visible by RT-PCR (Fig. 6B). Quantification of this effect by real-time RT-PCR revealed that PI3K inhibition completely blocked KLF2 mRNA fold induction by fluid shear stress (Fig. 6C). As expected, ERK1/2 inhibition had no effect on KLF2 mRNA levels. We next investigated the effect of PI3K inhibition on flow-induced nuclear binding within the 62-bp shear stress response region and the 30-bp minimal shear element. PI3K inhibition blocked shear-specific nuclear binding at both the 62- (Fig. 6D) and 30-bp levels (Fig. 6E). Finally, we verified that PI3K inhibition blocks flow-mediated KLF2 induction in HUVECs (Fig. 6F). These results demonstrate that a PI3K-dependent pathway regulates KLF2 flow induction.

Akt is the canonical downstream target of PI3K activation; however, PI3K-dependent/Akt-independent shear stress signal transduction pathways have been reported (13, 14). Therefore, we verified by immunoblot that Akt is activated (via phosphorylation) in EOMA cells exposed to flow and that PI3K inhibition blocks Akt activation (Fig. 7A). We subsequently investigated the involvement of Akt in the shear stress-PI3K-KLF2 pathway by using a constitutively active Akt construct. This Akt variant is rendered permanently active by replacement of the pleckstrin homology domain with a Src myristoylation signal (myr-Akt) that targets the protein to the membrane, even in the absence of stimulus. Transient transfection of myr-Akt into static EOMA cells for 24 h was sufficient to induce KLF2 only at an extremely high level of overexpression (25 μg/100-mm dish) (Fig. 7B). This level of induction was drastically lower than the induction of KLF2 by 24 h of shear stress (17.4-versus 3.0-fold change from static), as determined by semi-quantitative real-time RT-PCR. Lower doses of myr-Akt failed to elicit a response. Furthermore, the 25-μg level of myr-Akt transfection failed to transactivate a KLF2 reporter plasmid (Fig. 7C), suggesting that the slight level of mRNA induction at this level was an artifact of overexpression. Finally, dominant-negative Akt (protein kinase B-CAAAX) failed to block or even visibly reduce KLF2 induction by 12 h of fluid shear stress (Fig. 7D). Taken together, these studies suggest that fluid shear stress induces KLF2 independent of Akt.

**Shear Stress Mediates PI3K-dependent Chromatin Remodeling within the KLF2 Promoter**—Given the presence of the PCAF acetyltransferase within the KLF2 shear stress induction complex, we hypothesized that chromatin remodeling by histone acetylation may modulate the accessibility of the promoter to transcription factors. Both the H3 and H4 histones are recognized targets of PCAF acetyltransferase activity (49). We performed ChIP assays with primers to amplify the shear stress response region of the KLF2 promoter by polymerase chain reaction (Fig. 8A). This promoter region was immunoprecipitated by antibodies against acetylated histones H3 and H4 and RNA polymerase II (RNA Pol 2), specifically after exposure to shear stress. Using the same antibodies, no signal was detectable under static conditions. These data indicated that, in the absence of histone acetylation, no RNA Pol 2 binding and, therefore, no mRNA transcription can be generated from the KLF2 promoter. Furthermore, even during shear stress exposure, histone acetylation and RNA Pol 2 binding are blocked by PI3K inhibition via LY294002 treatment. No effect is noted by vehicle (Me2SO) treatment alone. Interestingly, LY294002 treatment also blocks PCAF, hnRNP D, and TFIIIB binding (Fig. 8B), although PI3K inhibition has no effect on the direct interaction of PCAF with hnRNP D (Fig. 8C). These
findings suggest that the recruitment of PCAF/hnRNP D to the KLF2 promoter via a PI3K pathway is responsible for histone acetylation and chromatin remodeling that allow the binding of the general transcription machinery.

**KLF2 Increases eNOS Expression in EOMA Mouse Microvascular Endothelial Cells**—Recent work (27) has demonstrated that eNOS is a direct downstream target of KLF2 in HUVECs and bovine aortic endothelial cells. We undertook an independent investigation of eNOS expression in response to forced KLF2 expression in static cultured microvascular EOMA cells. As expected, ectopic KLF2 expression increased eNOS mRNA levels 15.6-fold (±2.02) over empty vector controls in static cultures, a level comparable to the induction of KLF2 by 24 h of 19 dyne/cm² shear stress (Fig. 9A). The expression of KLF2 by the cytomegalovirus/KLF2 plasmid in static cultures was verified by RT-PCR (Fig. 9B). The ectopic expression of KLF2 in static cultures is 2.49-fold less (±0.20) than the induction of KLF2 by 24 h of 19 dyne/cm² shear stress. In this context, KLF2 was not overexpressed in these experiments because its induction by flow (a stimulus to which endothelial cells are continually exposed in vivo) is greater than its ectopic expression in static cultures.

We also found that ectopic KLF2 expression increased eNOS protein levels over empty vector controls in a manner similar to eNOS induction by fluid shear stress (Fig. 9, C and D). Ectopic KLF2 induced eNOS protein to a lesser extent than its induction of eNOS mRNA (±2-fold versus 15-fold). The KLF2-induced increase in protein level did correspond to the induction of eNOS mRNA by shear stress (Fig. 9E), suggesting that complex post-transcriptional and post-translation mechanisms regulate flow-dependent eNOS levels (50). The induction of eNOS by KLF2 in microvascular EOMA cells, in addition to large vessel HUVECs and bovine aortic endothelial cells, indicates that it is not a heterogenic phenomenon. Finally, we verified that, as reported previously (51), eNOS mRNA expression is up-regulated by shear stress (2.48-fold; ± 0.15). The increase in eNOS expression is blocked by LY294002 treatment at both the mRNA (Fig. 9E) and protein level (Fig. 9F), placing eNOS regulation into the shear stress-PI3K-KLF2 pathway.

**DISCUSSION**

We have previously reported that shear stress induces a sustained increase of KLF2 in microvascular EOMA cells. Our
present study has expanded on our prior work with the major findings that 1) the 62-bp shear stress response region is also important in HUVECs, 2) this region is largely comprised of a conserved tripartite palindrome motif, 3) PCAF and hnRNP D bind this region as components of the shear stress induction complex, and 4) there is a direct link among flow-induced PI3K activation, histone acetylation, KLF2 transcription, and eNOS induction.

Although palindrome motifs are a well known feature of both prokaryotic and eukaryotic promoters, to our knowledge, this is the first description of a palindromic shear stress response element. Palindrome motifs have been found in the regulatory
elements of the Tie2 gene (52), which is shear-responsive (53). The AT-rich nature of two of the three palindromes further suggests that they are an important feature of KLF2 transcriptional regulatory function. An AT-rich palindrome has been found to be important in transcriptional regulation of the endothelial von Willebrand factor gene (54). Interestingly, the von Willebrand factor AT-rich palindrome is at approximately the same location (∼135 to −126 bp) as the KLF2 AT-rich palindromes.

The connection of the PI3K pathway to KLF2 and eNOS induction is significant, because previous studies have shown that the PI3K pathway is activated by shear stress and that the anti-apoptotic effect of shear stress is mediated by PI3K- and NO-dependent mechanisms (55). The results of our present study establish the connection between flow, PI3K, KLF2, and eNOS. Therefore, they suggest a relationship between shear stress signal transduction, gene regulation, and the pro-survival functions of KLF2. Interestingly, the PI3K pathway has been shown to transcriptionally up-regulate cytochrome p450 in response to shear stress (56). This is the only other gene in addition to KLF2 that was found to be both highly responsive to prolonged shear stress and down-regulated by tumor necrosis factor α in the initial work describing the flow induction of KLF2 (17).

The involvement of PCAF corresponds to our recent work demonstrating its role in KLF2 gene regulation. This is the first report of PCAF in a shear stress induction complex, although it has been found to regulate the cyclooxygenase-2 promoter (57), which is shear-responsive (58). PCAF can acetylate both histone (42) and non-histone general transcription factors (59). Acetylation of other transcription factors may explain the ability of PCAF to transactivate the non-chromatin template KLF2 reporter plasmid. More importantly, acetylation of specific lysine residues within histone e-NH₂ tails plays a key role in opening the chromatin of transcriptionally active genes. PCAF-dependent histone acetylation is required for the recruitment of basal transcription factors and transcription from certain chromatin templates (43, 60), a model that is supported by our data. We have shown that PCAF binding, histone acetylation, and assembly of the general transcription machinery are flow-specific, which connects KLF2 induction to flow-dependent gene regulation through shear stress-mediated histone acetyltransferase-based chromatin remodeling (15).

The mechanism of PCAF recruitment to chromatin-remodeling complexes in response to shear stress is not understood and is currently under investigation. One possibility is that PCAF is a direct downstream target of PI3K. In response to nerve growth factor receptor signaling, the activation of PI3K has been found to be required for phosphorylation and nuclear translocation of PCAF, resulting in transcriptional activation.
of PC12 cells (61). A similar response may be generated by shear stress activation of endothelial mechanosensors, as suggested by the finding that PCAF is required for certain growth factor-induced signaling pathways (62). Considering that the vascular endothelial growth factor receptor 2 is activated in a ligand-independent manner by shear stress and associated with flow-dependent PI3K signaling (63), PCAF may provide a mechanism by which the physical force of shear stress acts through growth factor receptors to elicit specific biological responses on gene activation. In summary, our results support a model where, in response to flow activation, the PI3K signal transduction pathway converges on histones surrounding the KLF2 promoter to alter chromatin structure through the PCAF acetyltransferase.

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