Roles of KLF4 and AMPK in the inhibition of glycolysis by pulsatile shear stress in endothelial cells

Yue Han[a,b] , Ming He[b], Traci Marin[c], Hui Shen[b], Wei-Ting Wang[b], Tzong-Yi Lee[d], Hsiao-Chin Hong[d], Zong-Lai Jiang[a] , Theodore Garland Jr[e] , John Y.-J. Shyy[b,f,1] , Brendan Gongol[b,c,1], and Shu Chien[f,g,1]

[a]Institute of Mechanobiology and Medical Engineering, School of Life Sciences & Biotechnology, Shanghai Jiao Tong University, Shanghai, 200240, China; [b]Department of Medicine, University of California San Diego, La Jolla, CA 92093; [c]Department of Health Sciences, Victor Valley College, Victorville, CA 92395; [d]Warshel Institute for Computational Biology, School of Life and Health Sciences, The Chinese University of Hong Kong, Shenzhen, 999077, China; [e]Department of Evolution, Ecology, and Organismal Biology, University of California, Riverside, CA 92521; [f]Institute of Engineering in Medicine, University of California San Diego, La Jolla, CA 92093; and [g]Department of Bioengineering, University of California San Diego, La Jolla, CA 92093

Vascular endothelial cells (ECs) sense and respond to hemodynamic forces such as pulsatile shear stress (PS) and oscillatory shear stress (OS). Among the metabolic pathways, glycolysis is differentially regulated by atheroprone OS and atheroprotective PS. Studying the molecular mechanisms by which PS suppresses glycolytic flux at the epigenetic, transcriptomic, and kinomic levels, we have demonstrated that glucokinase regulatory protein (GCKR) was markedly induced by PS in vitro and in vivo, although PS down-regulates other glycolysis enzymes such as hexokinase (HK1). Using next-generation sequencing data, we identified the binding of PS-induced Krüppel-like factor 4 (KLF4), which functions as a pioneer transcription factor, binding to the GCKR promoter to change the chromatin structure for transactivation of GCKR. At the posttranslational level, PS-activated AMP-activated protein kinase (AMPK) phosphorylates GCKR at Ser-481, thereby enhancing the interaction between GCKR and HK1 in ECs. In vivo, the level of phosphorylated GCKR Ser-481 and the interaction between GCKR and HK1 were increased in the thoracic aorta of wild-type AMPKα2^{+/+} mice in comparison with littermates with EC ablation of AMPKα2 (AMPKα2^{−/−}). In addition, the level of GCKR was elevated in the aortas of mice with a high level of voluntary wheel running. The underlying mechanisms for the PS induction of GCKR involve regulation at the epigenetic level by KLF4 and at the posttranslational level by AMPK.

AMPK | KLF4 | epigenetics | GCKR | glycolysis

The endothelium lines the luminal surface of the arterial wall and is in direct contact with blood flow. The pulsatile shear stress (PS) at straight parts of arteries maintains endothelial homeostasis, whereas oscillatory shear stress (OS) at bifurcations and curvatures impairs endothelial function. Such OS-induced endothelial cell (EC) dysfunction is characterized by enhanced glycolysis, inflammation, proliferation, and production of reactive oxygen species (ROS) (1–5). Collectively, these EC phenotypic changes cause atherosclerosis (6).

As the sole pathway for glucose catabolism, glycolysis is a main energy source for the endothelium (7–9). Increased glycolysis in ECs meets the demand of glucose consumption required for EC migration and proliferation (3, 10). However, exaggerated glycolysis in endothelium is associated with disease states such as tumor angiogenesis, diabetic retinopathy, and atherosclerosis (8, 9). Mounting evidence indicates that shear stress regulates glycolysis in ECs as a function of the flow patterns. Doddaballapur et al. showed that the PS-induced Krüppel-like factor 2 (KLF2) reduces metabolic activity in ECs by repressing the expression of 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3 (PFKFB3), a key regulator of glycolysis (1). Analyzing RNA-sequencing (RNA-seq) data from ECs exposed to OS, Wu et al. concluded that OS increases endothelial glycolysis via stabilization of ROS-mediated hypoxia-inducible factor 1α (HIF-1α) (11). Using bulk assays, Feng et al. reported a similar result, namely, OS increased EC proliferation and inflammation via HIF-1α induction

of glycolysis enzymes (12). While these reports pointed out the increase in glycolysis under OS, a systemic study of the regulatory mechanisms of glycolysis in the endothelium in response to distinct flow patterns remains elusive.

Krüppel-like factor 4 (KLF4) and AMP-activated protein kinase (AMPK) are two principal molecules involved in the mechanotransduction mechanism in ECs. KLF4 is one of the Yamanaka factors that are necessary for embryonic cell pluripotency (13, 14). In ECs, KLF4 is a lineage-dependent transcription factor (TF) essential for endothelial lineage and a PS-induced signal-dependent TF (14). Under PS, KLF4 transcriptionally up-regulates many atheroprotective genes such as endothelial nitric oxide synthase (eNOS), thrombomodulin, and inositol 1,4,5-trisphosphate receptor, type 3 (ITPR3) (15, 16). Functioning as a pioneer TF, KLF4 binds to the promoter region of these PS-induced genes to interact with the basal transcriptional machinery and initiate epigenetic remodeling (16). As a metabolic gauge, AMPK globally regulates cellular metabolism by increasing catabolic pathways and decreasing anabolic pathways. AMPK activation decreases energy-consuming glycolysis while promoting mitochondrial oxidative metabolism to restore energy homeostasis (17). Upon activation, AMPK phosphorylates a number of target proteins in ECs that

contain a $\beta\theta\beta XXX(S/T)XXX\theta$ consensus sequence (β = basic amino acid, θ = hydrophobic amino acid, and X = any amino acid) (18). Many of these AMPK substrate proteins, such as eNOS and angiotensin converting enzyme 2, are critical for endothelial homeostasis (19, 20).
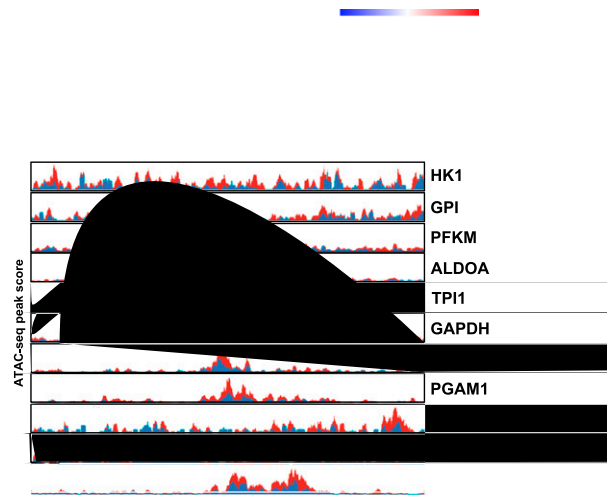
Hexokinase (HK1) catalyzes the first and also rate-limiting step of glycolysis. HK IV, also called glucokinase (GK), is expressed abundantly in ECs. Glycolysis is inhibited by the binding of glucokinase regulator protein (GCKR) to HK1, thereby sequestering HK1 in the nucleus (21). In homeostatic state, GCKR is usually present in molar excess of HK1 in the cell. However, the glycolytic rate is affected by the level of GCKR and the binding status of GCKR/HK1, which is dynamically and intricately modulated and can be rapidly changed by metabolic conditions (22). Thus, the expression of GCKR and its posttranslational modifications are essential regulatory mechanisms in glycolysis (23).

Given the lack of information on how shear stress regulates EC glycolysis via GCKR, we launched this study to investigate the roles of KLF4 and AMPK in regulating EC glycolysis via GCKR. Our results show that PS down-regulates glycolysis in ECs by 1) KLF4-mediated epigenetic and transcriptional up-regulation of GCKR expression and 2) AMPK phosphorylation of GCKR, with the ensuing increase in GCKR/HK1 interaction. This mechanotransduction mechanism is recapitulated in vascular health in mice with a high level of voluntary wheel running behavior.

Results

PS Down-Regulates Glycolysis in ECs at the Systems Level. We first assessed the dynamic changes in expression of genes involved in the glycolysis pathway in response to PS vs. OS by analyzing RNA-seq data from human umbilical vein ECs (HUVECs) subjected to PS and OS for 0 to 24 h (GSE103672) (16, 24). Pathway analysis demonstrated that the enzymes involved in glycolytic steps were generally down-regulated by PS starting from 16 h, as compared with OS (Fig. 1A). These results confirm that PS and OS regulate glycolysis in ECs in opposite directions. The RNA-seq findings that PS suppressed glycolysis enzymes were validated by qPCR (Fig. 1B). Because epigenetic regulations are integral parts of mechanotransduction in ECs in response to shear stress (16), we next investigated whether PS down-regulation of glycolysis genes involves chromatin remodeling. Thus, we determined the enrichment of transposase-accessible chromatin sequencing (ATAC-seq) peaks (measuring decondensed chromatin structure) in the regions flanking the transcription start site of the human glycolysis gene. The promoter regions of these glycolysis genes exhibited ATAC peak enrichment under OS vs. PS (Fig. 1C). Together, these results indicate that the PS inhibition of glycolysis genes occurs at epigenetic and transcriptional levels.

KLF4 Regulates GCKR Expression in Response to PS. In addition to testing PS down-regulated genes, we also examined PS up-regulated genes involved in glycolysis. PS markedly induced GCKR transcripts during 24 h (Fig. 2A), and this was validated by qPCR (Fig. 2B). We next examined the effect of PS on H3K27ac enrichment in the promoter region of GCKR. PS enriched H3K27ac signals (Fig. 2C), indicating that the GCKR promoter has a more decondensed chromatin structure under PS than OS. Given that KLF4 is a PS-induced pioneer TF transactivating a panel of genes essential for EC homeostasis (16), we next explored whether the PS-induced GCKR is mediated by KLF4 epigenetically and transcriptionally. Among the putative TFs that can bind to the GCKR promoter (determined by analyzing the binding sites for transcription factors [BSTF]), KLF4 mRNA was induced to the greatest extent by PS, according to the GSE103672 dataset (Fig. 2D). Consistent with the two putative KLF4 binding sites predicted in the GCKR promoter (Fig. 2C), GCKR was induced in ECs overexpressing KLF4, as indicated by the RNA-seq data (GSE90982, Fig. 2E). To validate the results in Fig. 2 A–E, we overexpressed KLF4 in ECs, mimicking the PS induction of KLF4. KLF4 overexpression increased KLF4 binding to the GCKR promoter and the expression of GCKR, as assessed by KLF4 chromatin immunoprecipitation (ChIP)-PCR, qPCR, and Western blot (Fig. 2 F–H). In reciprocal experiments with KLF4 knockdown in ECs, the PS induction of GCKR was attenuated at both mRNA and protein levels (Fig. 2 I and J). In line with these results, PS increased KLF4 binding to the GCKR promoter and augmented GCKR expression (Fig. 2 B, K, and L). The mouse thoracic aorta (TA) and aortic arch (AA) are under atheroprotective vs. atheroprone flow patterns. In vivo validation was provided by the finding that the GCKR mRNA level was higher in intima isolated from TA than AA (Fig. 2M). Taken together, the results in Fig. 2 demonstrate that the PS-induced GCKR depends on KLF4-mediated epigenetic and transcriptional regulations.

AMPK Phosphorylates and Regulates GCKR. The activities of several glycolysis enzymes (e.g., Phosphofructokinase 1 [PFK1]) are regulated by posttranslational modifications such as phosphorylation. To determine whether the PS-induced GCKR also involves protein phosphorylation, we used bioinformatics approaches to determine the phosphorylation sites and the corresponding kinases that phosphorylate GCKR. To accomplish this, kinase phosphorylation sites of GCKR were predicted first by evaluating the conservation of these sites among species using the Ensembl database (25). Then, kinases that putatively phosphorylate GCKR were ranked, based on the conservation of the cognate phosphorylation site. Among the predicted kinases, AMPK had the most preserved consensus sequences (Fig. 3A). To test whether AMPK can directly phosphorylate GCKR, we used in vitro kinase assays. Kinase reaction mixture containing recombinant AMPK caused an increase of the phosphorylation of GCKR (Fig. 3B). Among the seven putative AMPK phosphorylation sites, Ser-481 was the most homologous to the AMPK phosphorylation consensus sequence
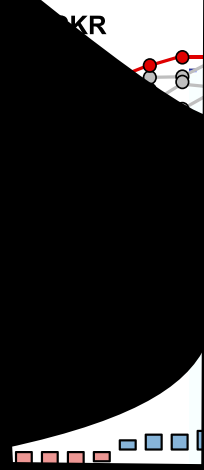
est whether GCKR Ser-481 was
 site, we mutated Ser-481 to
 in vitro kinase assays using the
eins. AMPK phosphorylation
s compared with the wild-type
 OS, PS increased the phos-
 mouse embryonic fibroblasts
-knockout (AMPK$^{-/-}$) MEFs
CKR Ser-481 (Fig. 3C). Next,
n of GCKR Ser-481 by AMPK
n that inhibits glycolysis (23, 26,
GCKR–HK1 interaction was
fected with GCKR S481A
ained in HUVECs transfected
netic) (Fig. 3D). Functionally,

HK1 activity was higher in HUVECs expressing GCKR S481A
than those expressing S481D (Fig. 3E). In vivo studies showed that
GCKR Ser-481 phosphorylation and GCKR–HK1 interaction
were higher in the TA from AMPKα2$^{+/+}$ than AMPKα2$^{-/-}$ mice
(Fig. 3 F and G). Overall, the results in Fig. 3 indicate that PS in-
creased AMPK phosphorylation of GCKR Ser-481, which in turn
enhanced GCKR–HK1 interaction to attenuate HK1 activity in ECs.

**KLF4-AMPK/GCKR Inhibition of Glycolysis in Mice with a High Level of
Voluntary Running.** We used adult males from a selectively bred
mouse model (generation 83) with a high level of voluntary wheel
running, namely, high-runner (HR) mice (28), to substantiate the
finding that KLF4-AMPK/GCKR inhibits glycolysis in the endo-
thelium in vivo. Presumably, PS is increased in the aorta of these
HR mice as a result of the increase in cardiac output (29), and we

Ultimately, such reduction in EC glycolysis by physiological activities may be important for the maintenance of vascular health.

Materials and Methods

Experimental methods are described in detail in *SI Appendix, SI Materials and Methods*. Cells for all experiments were cultured according to standard procedures and kept in a standard cell culture incubator held at 37 °C and 5% CO_2. Quantification of nucleic acids by qPCR was conducted with a Bio-Rad CFX96 real-time detection system using SYBR green. All primers used for ChIP or standard PCR are listed in *SI Appendix*, Table S1. Bioinformatic analyses were conducted in R programming language with support from Bioconductor or Comprehensive R Archive Network (CRAN) libraries.

Data Availability. All study data are included in the article and/or *SI Appendix*.

1. A. Doddaballapur *et al*., Laminar shear stress inhibits endothelial cell metabolism via KLF2-mediated repression of PFKFB3. *Arterioscler. Thromb. Vasc. Biol.* **35**, 137–145 (2015).
2. J. Wang, S. Zhang, Fluid shear stress modulates endothelial inflammation by targeting LIMS2. *Exp. Biol. Med. (Maywood)* **245**, 1656–1663 (2020).
3. S. Chien, Effects of disturbed flow on endothelial cells. *Ann. Biomed. Eng.* **36**, 554–562 (2008).
4. K. S. Heo, K. Fujiwara, J. Abe, Disturbed-flow-mediated vascular reactive oxygen species induce endothelial dysfunction. *Circ. J.* **75**, 2722–2730 (2011).
5. P. F. Davies, A. Remuzzi, E. J. Gordon, C. F. Dewey, Jr, M. A. Gimbrone, Jr, Turbulent fluid shear stress induces vascular endothelial cell turnover in vitro. *Proc. Natl. Acad. Sci. U.S.A.* **83**, 2114–2117 (1986).
6. J. J. Chiu, S. Chien, Effects of disturbed flow on vascular endothelium: Pathophysiological basis and clinical perspectives. *Physiol. Rev.* **91**, 327–387 (2011).
7. K. Theodorou, R. A. Boon, Endothelial cell metabolism in atherosclerosis. *Front. Cell Dev. Biol.* **6**, 82 (2018).
8. K. Rohlenova, K. Veys, I. Miranda-Santos, K. De Bock, P. Carmeliet, Endothelial cell metabolism in health and disease. *Trends Cell Biol.* **28**, 224–236 (2018).
9. G. Eelen, P. de Zeeuw, M. Simons, P. Carmeliet, Endothelial cell metabolism in normal and diseased vasculature. *Circ. Res.* **116**, 1231–1244 (2015).
10. K. De Bock, M. Georgiadou, P. Carmeliet, Role of endothelial cell metabolism in vessel sprouting. *Cell Metab.* **18**, 634–647 (2013).
11. D. Wu *et al*., HIF-1α is required for disturbed flow-induced metabolic reprogramming in human and porcine vascular endothelium. *eLife* **6**, e25217 (2017).
12. S. Feng *et al*., Mechanical activation of hypoxia-inducible factor 1α drives endothelial dysfunction at atheroprone sites. *Arterioscler. Thromb. Vasc. Biol.* **37**, 2087–2101 (2017).
13. A. Young *et al*., Flow activation of AMP-activated protein kinase in vascular endothelium leads to Krüppel-like factor 2 expression. *Arterioscler. Thromb. Vasc. Biol.* **29**, 1902–1908 (2009). Corrected in: *Arterioscler. Thromb. Vasc. Biol.* **30**, e325 (2010).
14. Y. Fan *et al*., Krüppel-like factors and vascular wall homeostasis. *J. Mol. Cell Biol.* **9**, 352–363 (2017).
15. G. Zhou *et al*., Endothelial Kruppel-like factor 4 protects against atherothrombosis in mice. *J. Clin. Invest.* **122**, 4727–4731 (2012).
16. M. He *et al*., Atheroprotective flow upregulates ITPR3 (Inositol 1,4,5-Trisphosphate Receptor 3) in vascular endothelium via KLF4 (Krüppel-Like Factor 4)-mediated histone modifications. *Arterioscler. Thromb. Vasc. Biol.* **39**, 902–914 (2019).
17. T. L. Marin *et al*., AMPK promotes mitochondrial biogenesis and function by phosphorylating the epigenetic factors DNMT1, RBBP7, and HAT1. *Sci. Signal.* **10**, eaaf7478 (2017).
18. T. L. Marin *et al*., Identification of AMP-activated protein kinase targets by a consensus sequence search of the proteome. *BMC Syst. Biol.* **9**, 13 (2015).
19. B. Thors, H. Halldórsson, G. Thorgeirsson, eNOS activation mediated by AMPK after stimulation of endothelial cells with histamine or thrombin is dependent on LKB1. *Biochim. Biophys. Acta* **1813**, 322–331 (2011).
20. J. Zhang *et al*., AMP-activated protein kinase phosphorylation of angiotensin-converting enzyme 2 in endothelium mitigates pulmonary hypertension. *Am. J. Respir. Crit. Care Med.* **198**, 509–520 (2018).
21. A. Raimondo, M. G. Rees, A. L. Gloyn, Glucokinase regulatory protein: Complexity at the crossroads of triglyceride and glucose metabolism. *Curr. Opin. Lipidol.* **26**, 88–95 (2015).
22. M. Salgado *et al*., When a little bit more makes the difference: Expression levels of GKRP determines the subcellular localization of GK in tanycytes. *Front. Neurosci.* **13**, 275 (2019).
23. B. Guigas *et al*., 5-Aminoimidazole-4-carboxamide-1-beta-D-ribofuranoside and metformin inhibit hepatic glucose phosphorylation by an AMP-activated protein kinase-independent effect on glucokinase translocation. *Diabetes* **55**, 865–874 (2006).
24. N. E. Ajami *et al*., Systems biology analysis of longitudinal functional response of endothelial cells to shear stress. *Proc. Natl. Acad. Sci. U.S.A.* **114**, 10990–10995 (2017).
25. D. R. Zerbino *et al*., Ensembl 2018. *Nucleic Acids Res.* **46**, D754–D761 (2018).
26. L. Agius, Hormonal and metabolite regulation of hepatic glucokinase. *Annu. Rev. Nutr.* **36**, 389–415 (2016).
27. A. Šmerc, E. Sodja, M. Legiša, Posttranslational modification of 6-phosphofructo-1-kinase as an important feature of cancer metabolism. *PLoS One* **6**, e19645 (2011).
28. Y. Zhang *et al*., AMP-activated protein kinase is involved in endothelial NO synthase activation in response to shear stress. *Arterioscler. Thromb. Vasc. Biol.* **26**, 1281–1287 (2006).
29. L. M. Vaanholt *et al*., Metabolic and behavioral responses to high-fat feeding in mice selectively bred for high wheel-running activity. *Int. J. Obes.* **32**, 1566–1575 (2008).
30. K. S. Zaret, Pioneer transcription factors initiating gene network changes. *Annu. Rev. Genet.* **54**, 367–385 (2020).
31. A. Moosavi, A. Motevalizadeh Ardekani, Role of epigenetics in biology and human diseases. *Iran. Biomed. J.* **20**, 246–258 (2016).
32. J. Grimsby *et al*., Characterization of glucokinase regulatory protein-deficient mice. *J. Biol. Chem.* **275**, 7826–7831 (2000).
33. B. Viollet *et al*., Physiological role of AMP-activated protein kinase (AMPK): Insights from knockout mouse models. *Biochem. Soc. Trans.* **31**, 216–219 (2003).
34. J. M. Park, T. H. Kim, S. H. Jo, M. Y. Kim, Y. H. Ahn, Acetylation of glucokinase regulatory protein decreases glucose metabolism by suppressing glucokinase activity. *Sci. Rep.* **5**, 17395 (2015).
35. Q. Yang *et al*., PRKAA1/AMPKα1-driven glycolysis in endothelial cells exposed to disturbed flow protects against atherosclerosis. *Nat. Commun.* **9**, 4667 (2018).
36. G. Soto-Heredero, M. M. Gómez de Las Heras, E. Gabandé-Rodríguez, J. Oller, M. Mittelbrunn, Glycolysis–A key player in the inflammatory response. *FEBS J.* **287**, 3350–3369 (2020).
37. C. M. Grant, Metabolic reconfiguration is a regulated response to oxidative stress. *J. Biol.* **7**, 1 (2008).

Han et al.
Roles of KLF4 and AMPK in the inhibition of glycolysis by pulsatile shear stress in endothelial cells