Response by Feng et al to Letter Regarding Article, “Mechanical Activation of Hypoxia-Inducible Factor 1α Drives Endothelial Dysfunction at Atheroprone Sites”

In Response:

We welcome the letter from Wu et al on our recent study, which showed that disturbed flow can activate hypoxia-inducible factor (HIF1α) leading to enhanced endothelial glycolysis, proliferation, and vascular inflammation. It is notable that Wu et al independently recapitulated our observation in a series of elegant experiments. In both studies, unbiased transcriptome-based methods identified enriched expression of HIF1α, and its target genes, in endothelial cells exposed to disturbed flow. It was also demonstrated, by both studies, that HIF1α-dependent glycolysis promotes vascular inflammation at atheroprone sites via induction of adhesion molecules and other inflammatory mediators. In contrast to Wu et al, our study revealed that HIF1α additionally induces excessive rates of endothelial cell proliferation—a phenotype that has been previously linked with vascular leakiness and atherogenesis. Taken together, the effects of HIF1α on inflammation and proliferation represent a plausible dual mechanism to explain its previously described proatherogenic effects.

We performed detailed biochemical studies to define the underlying molecular mechanism for HIF1α stabilization in the presence of oxygen in endothelial cells exposed to disturbed flow; dual regulation of HIF1α at both transcriptional and protein levels was identified. Evidence from multiple experiments led us to conclude that transcriptional upregulation of HIF1α mRNA in response to disturbed flow is under the control of NF-κB because (1) HIF1α expression was significantly reduced by overexpression of 1xIκB (an inhibitor of NF-κB), (2) silencing of RelA NF-κB subunits using siRNA reduced HIF1α expression, and (3) chromatin immunoprecipitation revealed that NF-κB interacts directly with HIF1α promoter sequences. Moreover, our results are in agreement with previous studies demonstrating that NF-κB can induce HIF1α in other contexts. Wu et al questioned our observation because they found that a peptide inhibitor of NF-κB did not alter HIF1α expression; however, we are uncertain of their conclusion because it seems to be based on data from a single Western blot that was not quantified. On the contrary, Wu et al show convincingly that HIF1α can activate NF-κB in endothelium exposed to disturbed flow. Considering the data from both studies, we posit that NF-κB is positioned both upstream and downstream from HIF1α in cells exposed to disturbed flow, thereby forming a positive feedback loop in HIF1α activation.

HIF1α is tightly regulated by enzymes that control the attachment of Lys48-linked polyubiquitin chains—a modification that targets proteins for degradation. In the presence of oxygen, HIF1α is modified with hydroxyl groups by prolyl hydroxylase domain (PHD) enzymes, and this modification is subsequently recognized by von Hippel-Lindau E3 ubiquitin ligase, which targets HIF1α for Lys48 polyubiquitination. However, in some situations, this process can be reversed by Cezanne—a deubiquitinating cysteine protease that can remove polyubiquitin chains from HIF1α and other proteins. Our study revealed that Cezanne was induced by disturbed flow, and loss-of-function approaches (siRNA, dominant-negative) revealed that it is essential for HIF1α protein expression under these conditions. Cezanne reduced the abundance of high molecular weight forms of HIF1α and coprecipitating polyubiquitin under conditions of disturbed flow suggesting that it stabilizes HIF1α via deubiquitination.

Wu et al have a different perspective on the mechanisms that stabilize HIF1α. They found that disturbed flow induces NOX4 (NADPH oxidase 4; 1 of 7 NADPH [nicotinamide adenine dinucleotide phosphate-oxidase] oxidases), which is required for HIF1α expression. This resonates with a previous observation that NOX4 can enhance HIF1α protein levels. The molecular mechanisms downstream from NOX4 were not identified by Wu et al; however, they speculate that reactive oxygen species (ROS) may stabilize HIF1α by inhibiting the activity of PHD and VHL (von Hippel–Lindau) enzymes. Although we observed that the expression of PHD and VHL was not reduced by disturbed flow, further work is necessary to investigate whether flow alters the activity of these enzymes. It should be noted that effects of flow on PHD, VHL, and Cezanne are not mutually exclusive; it is plausible that disturbed flow promotes HIF1α stability by simultaneously enhancing deubiquitination and reducing ubiquitination.

Wu et al comment on the hypothetical inhibitory effects of ROS on Cezanne enzymatic activity. However, although Cezanne can be inhibited by high levels of exogenous H2O2, it can function in conditions where endogenous ROS are abundant, for example, during ischemia/reperfusion. It is, therefore, not surprising that Cezanne can stabilize HIF1α in conditions of disturbed flow, associated with augmented ROS. More broadly, the influence of ROS on enzymatic activity is highly context specific and varies according to the particular species of reactive molecule, their levels, and intracellular localization. This is brought into sharp relief when considering the contrasting functions of NOX enzymes in the vasculature. Genetic deletion studies in mice revealed that although some NOX isoforms can promote vascular inflammation and injury, NOX4 (the molecule investigated by Wu et al) reduces inflammation and suppresses atherosclerosis. It is difficult to reconcile the anti-inflammatory and antiatherosclerotic effects of NOX4 with the observation from Wu et al that NOX4 drives HIF1α-dependent inflammation of arteries, leading to atherosclerosis. However, one explanation could come from a previous study showing that endothelial cells at atheroprone sites are simultaneously exposed to both proinflammatory and anti-inflammatory signals. Further work is necessary to define the ROS that are produced by NOX isoforms in response to disturbed flow and to identify their downstream molecular targets. It is conceivable that NOX4 promotes HIF1α stability.
by feeding into the NF-κB- and Cezanne-dependent mechanism that we described since crosstalk between NOX4 and NF-κB has been established in vascular endothelium.13

Overall, our findings1 and those of Wu et al12 reveal that HIF1α is activated at sites of disturbed flow in the presence of oxygen to promote pathophysiological changes in vascular endothelium by inducing glycolysis. These data collectively suggest that HIF1α-glycolysis pathways have the potential to be targeted therapeutically to enhance vascular function and treat atherosclerosis. Intervention upstream of HIF1α may include pharmacological modulation of the PHD or Cezanne enzymes.

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**References**


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