Glycocalyx heparan sulfate cleavage promotes endothelial cell angiopoietin-2 expression by impairing shear stress-related AMPK/FoxO1 signaling

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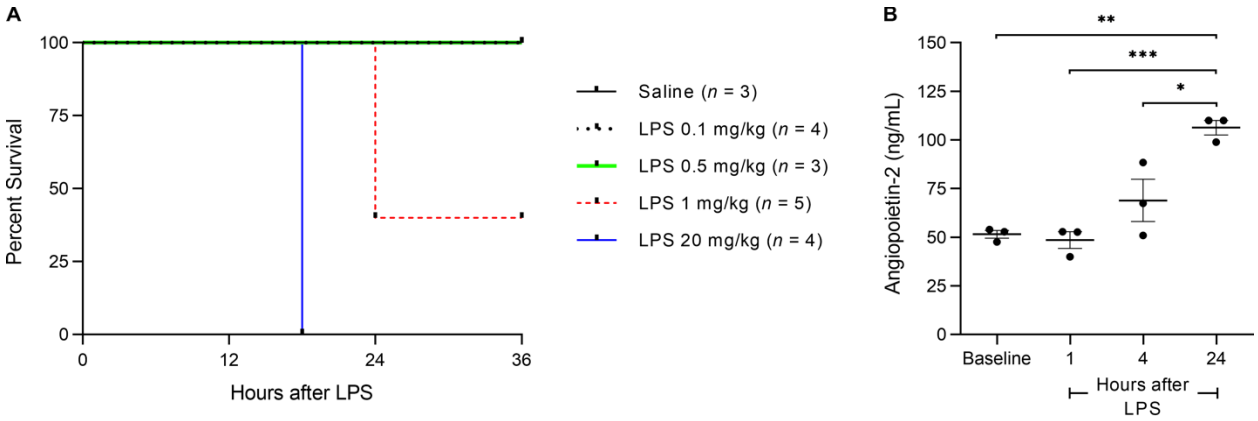
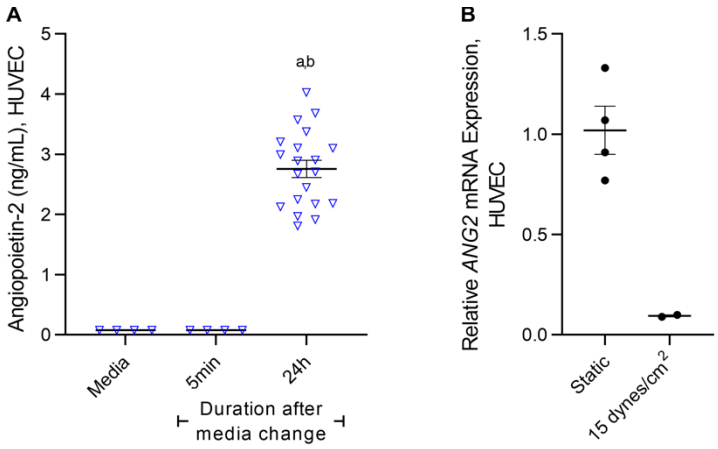
SUPPLEMENTAL MATERIAL

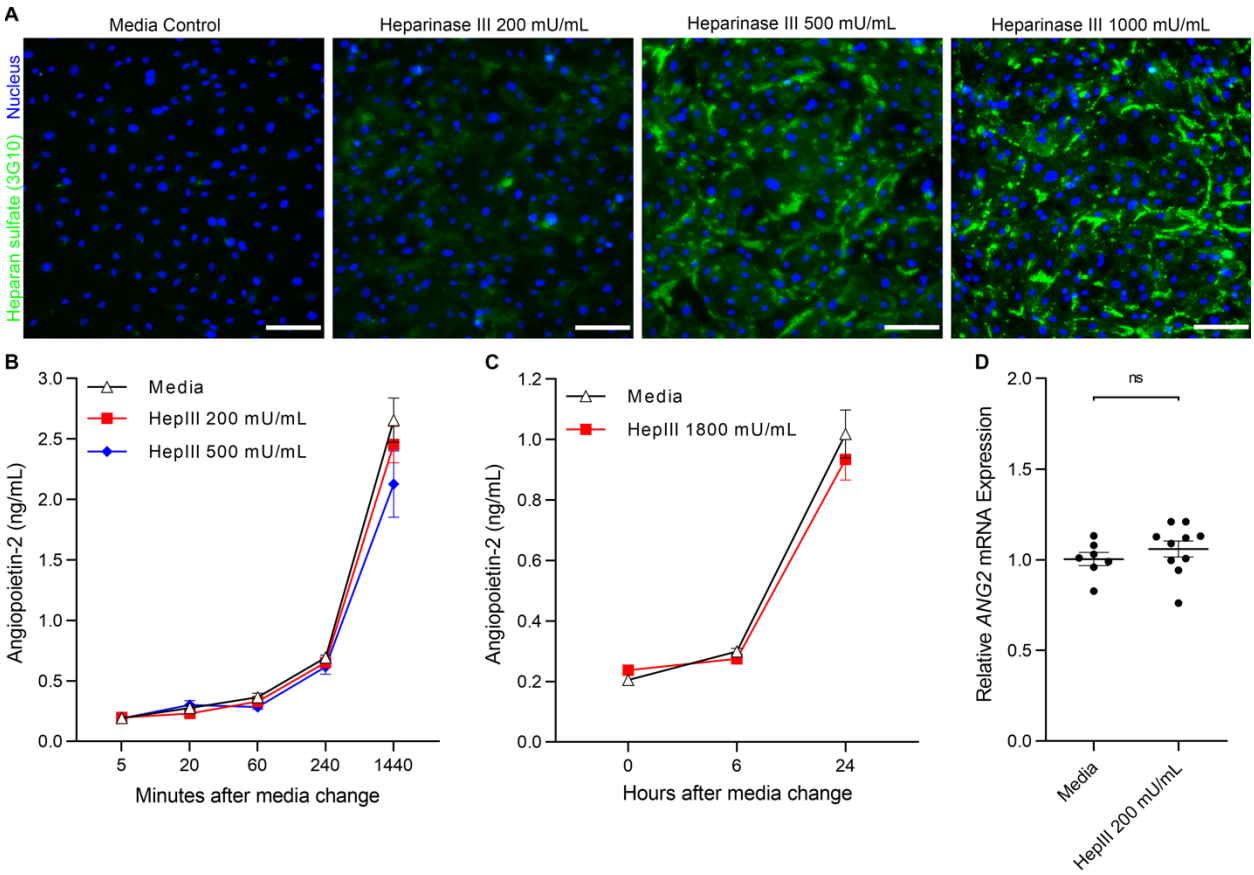
FIGURES



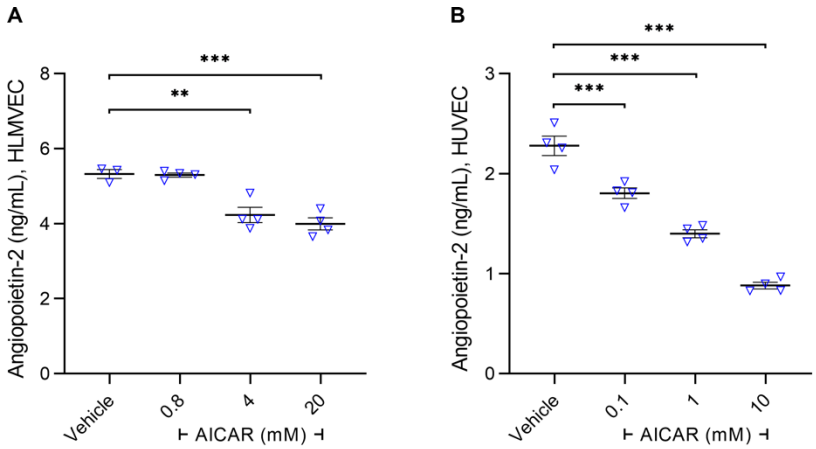
Supplemental Figure S1. Survival curve of increasing doses of lipopolysaccharide (LPS) administration and the time-dependent response of plasma angiopoietin-2 levels following LPS injection. (**A**) Kaplan-Meier survival curves of groups of C57BL/6J WT 12-14 week-old male mice who received retro-orbital injections with LPS 0.1, 0.5, 1, 20 mg/kg or vehicle (n = 3-5). We observed that LPS doses less than or equal to 0.5 mg/kg were sublethal, informing the LPS 0.1 mg/kg dose used to measure plasma levels of IL-6 and heparan sulfate depicted in Figure 2B and 2C. (**B**) Plasma angiopoietin-2 (Ang-2) levels in the three surviving mice following LPS 1 mg/kg injection shown in panel A. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ versus baseline after ordinary 1-way ANOVA corrected by Tukey's multiple comparisons test. Data are presented as mean ± SEM.



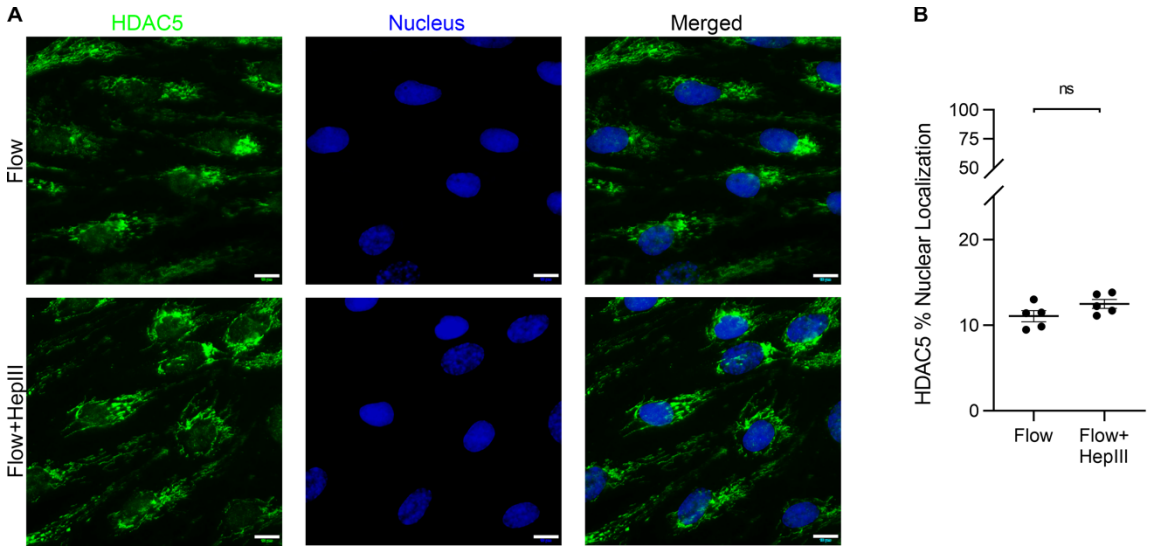
Supplemental Figure S2. Constitutive *ANG2* gene expression in human umbilical vein endothelial cells (HUVEC) is suppressed by shear stress application. (**A**) Ang-2 levels (ELISA) in HUVEC media (n = 4), HUVEC supernatant within 5min of media change (n = 4), and HUVEC supernatant after 24h of static culture (n = 20). [a]*P* < 0.001 versus media, [b]*P* < 0.001 versus 5min after ordinary 1-way ANOVA corrected by Tukey's multiple comparisons test. (**B**) Relative HUVEC *ANG2* gene expression after 24h of static culture (n = 4) or 48h of 15 dyn/cm^2 (n = 2). All data are presented as mean ± SEM.



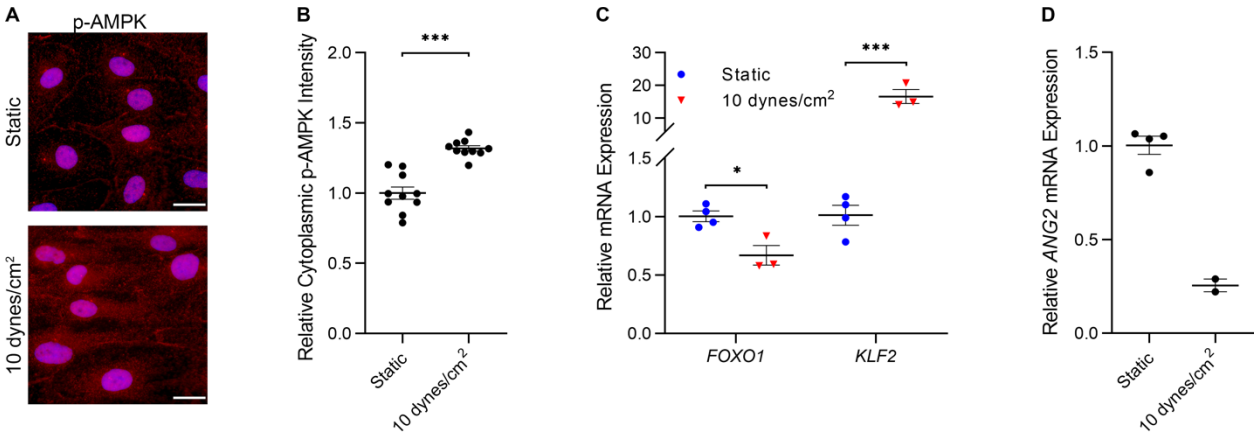
Supplemental Figure S3. Heparinase III (HepIII) treatment of statically cultured human lung microvascular endothelial cells (HLMVEC) results in a dose-dependent erosion of surface heparan sulfate but does not alter Ang-2 protein or *ANG2* gene expression. (**A**) Representative 20x epifluorescence images demonstrating staining for cleaved heparan sulfate (exposed 3G10 epitope) on HLMVECs treated with increasing doses of HepIII (2h treatment) (with DAPI overlay). Scale bar = 100 µm. Note the increased staining intensity for heparan sulfate 3G10 with increasing HepIII concentrations, becoming visually apparent by 200 mU/mL. (**B**) Supernatant Ang-2 levels (ELISA) from HLMVECs over the course of 24h after treatment with vehicle (media), HepIII 200 mU/mL, or HepIII 500 mU/mL (n = 4 per group per time point). There were no significant changes in Ang-2 secretion associated with HepIII dose after 2-way ANOVA corrected by Šídák's multiple comparisons test. (**C**) Supernatant Ang-2 levels (ELISA) from HLMVECs over the course of 24h after treatment with HepIII 1800 mU/mL or vehicle (media) (n = 4 per group per time point). There were no significant changes in Ang-2 levels associated with high-dose HepIII treatment after 2-way ANOVA corrected by Šídák's multiple comparisons test. (**D**) Relative *ANG2* gene expression from statically cultured HLMVECs in the presence (n = 10) or absence (n = 7) of HepIII 200 mU/mL (24h treatment). Ns represents non-significance after Student's *t*-test. All data are presented as mean ± SEM.



Supplemental Figure S4. Effect of AMPK activation on Ang-2 secretion from HLMVECs and HUVECs. (**A**) Supernatant Ang-2 levels (ELISA) from statically cultured HLMVECs 24h following treatment with increasing doses of AICAR or vehicle (n = 4 per group). ***P* < 0.01, ****P* < 0.001 after 1-way ANOVA corrected by Tukey's multiple comparisons test. (**B**) Supernatant Ang-2 levels (ELISA) from HUVECs 24h after treatment with increasing doses of AICAR or vehicle (n = 4 per group). ****P* < 0.001 after ordinary 1-way ANOVA corrected by Tukey's multiple comparisons test. All data are presented as mean ± SEM.



Supplemental Figure S5. Heparan sulfate cleavage from flow conditioned HLMVECs does not affect cellular localization of histone deacetylase 5 (HDAC5). (**A**) Representative 100x epifluorescence images demonstrating staining for HDAC5 in flow conditioned HLMVECs (15 dyn/cm^2, 48h) exposed to an additional 24h of 15 dyn/cm^2 in the absence (top row) or presence (bottom row) of HepIII 200 mU/mL (with DAPI overlay). Scale bar: 10 μm. (**B**) Relative staining intensity for HDAC5 within the nuclear fraction of flow conditioned HLMVECs (15 dyn/cm^2, 48h) exposed to an additional 24h of 15 dyn/cm^2 in the absence or presence of HepIII 200 mU/mL (from 5 representative 20x images, normalized to number of nuclei within field of view). Ns represents non-significance after Student's *t*-test. Data presented as mean ± SEM.



Supplemental Figure S6. Effect of lower shear stress on 5'-adenosine monophosphate-activated protein kinase (AMPK) phosphorylation and Forkhead Box O1 (*FOXO1*), Krüppel-like factor 2 (*KLF2*), and *ANG2* gene expression within HLMVECs. (**A**) Representative 100x epifluorescence images of HLMVECs stained for phosphorylated-AMPK (p-AMPK) following 48h of static culture (top) or 10 dyn/cm^2 (bottom) (with DAPI overlay). Scale bar = 20 μm. (**B**) Relative staining intensity for p-AMPK in the cytoplasm of HLMVECs following 48h of static culture or 10 dyn/cm^2 (from 10 representative 40x images, normalized to number of nuclei within field of view). ***P < 0.001 after Student's *t*-test. (**C**) Relative *FOXO1* and *KLF2* mRNA expression in HLMVECs following 48h of static culture (n = 4) or 10 dyn/cm^2 (n = 3). *P < 0.05, ***P < 0.001 after Student's *t*-test. (**D**) Relative *ANG2* mRNA expression in HLMVECs following 48h of static culture (n = 4) or 10 dyn/cm^2 (n = 2). All data are presented as mean ± SEM.