SUPPLEMENTAL MATERIAL

Interferon-gamma Impairs Human Coronary Artery Endothelial Glucose Metabolism via Tryptophan Catabolism and Activates Fatty Acid Oxidation

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Lee *et al. Interferon-gamma Impairs Endothelial Metabolism*

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Supplemental Methods

Real-time extracellular flux analyses

HCAEC were grown to full confluence and treated with IFN-γ (50 ng/mL) for 24 h prior to the assay. Real-time basal ECAR and OCR and subsequent OCR measurements following treatment with oligomycin and then the combination of rotenone and antimycin A were performed using an XFe24 Analyzer (Agilent) according to the manufacturer's protocol in the standard XF base medium (Agilent) containing 10 mM glucose, 1 mM pyruvate, and 2 mM glutamine. OCR and ECAR raw measurements were normalized by cell counts. For additional analysis of the different OCR components, the total basal OCR measurements were divided into mitochondrial respiration (defined as [basal OCR] - [OCR following rotenone + antimycin A injection]) and non-mitochondrial oxygen consumption (defined as minimal measurement value following rotenone + antimycin A injection). Additionally, mitochondrial respiration linked to ATP production was determined by [basal OCR] - [OCR following oligomycin injection]. Palmitate-BSA (100μM; Agilent) was supplemented to the above medium for the study of fatty acid oxidation with etomoxir or siCPT1A.

Intracellular metabolite extraction for LC-MS

Confluent HCAECs grown in a 6-well plate were treated with IFN-γ (50 ng/mL) for 1, 6, and 24 h in the above culture media. Cells were washed with cold PBS twice and were placed on a bed of dry ice. Intracellular metabolites were extracted with 1 ml 80% aqueous methanol pre-

cooled to –80°C. The internal standard *DL*-valine (D8) (Cambridge Isotope Laboratories) was added to each well simultaneously. After 10 min incubation at –80°C, cell debris was collected by scraping and transferred to clean Eppendorf tubes. After centrifugation, the supernatants were transferred to new tubes and evaporated to dryness using a SpeedVac Concentrator at 42 °C. The dry pellets were stored at –80°C and were resuspended in water prior to LC-MS analysis.

Targeted liquid chromatography mass spectrometry

Intracellular metabolites were analyzed using the Vanquish ultra-high-performance liquid chromatography system coupled to a Q Exactive mass spectrometer (Thermo Fisher) equipped with an Ion Max source and HESI II probe. Intracellular metabolite separation was performed with a ZIC-pHILIC stationary phase (150 mm × 2.1 mm, 3.5 μm; Merck) with a guard column. Mobile phase A was 0.1% ammonium hydroxide with 20 mM ammonium carbonate, and mobile phase B was acetonitrile. The gradient (%B) was 0 min, 80%; 20 min, 20%; 20.5 min, 80%; 28 min, 80%; and 42 min, stop. Additional parameters included an injection volume of 2.5 μl, a flow rate of 100 μL/min, a column compartment temperature of 25 °C, and an autosampler compartment temperature of 4 °C. The ionization source settings included sheath gas 40, auxiliary gas 15, sweep gas 1, spray voltage +3.0 or -3.1 kV, capillary temperature 275 °C, S-lens RF level 40, and probe temperature 350 °C. Additional MS parameters included a resolution of 70,000, an AGC target $1x10^6$ ions, and scan range 70-1000 m/z. TraceFinder software (Thermo Fisher) was used for data acquisition and analysis, with peak identifications based on m/*z* and retention time as compared to a custom library of 144 common metabolite standards. Data represent metabolite peak areas normalized to the internal standard peak area and cell

count. For further quantitative estimation of intracellular tryptophan and kynurenine, the peak area for each metabolite was interpolated from standard curves generated with serial dilutions of exogenous tryptophan and kynurenine (Sigma-Aldrich), respectively, added to the confluent HCAEC cell extracts and processed as described above.

Glucose uptake assay

HCAEC were grown to confluence and treated with IFN-γ (50 ng/mL) for 48 h in phenol red-free growth medium (Lonza) containing 5.5 mM glucose. Cells were washed twice with warm growth medium and were incubated with the 100 μM fluorescent glucose analogue 2-(*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-2-deoxyglucose (2-NBDG; Thermo Fisher Scientific) for 30 min at 37°C with 5 % CO_2. Cells were washed twice with warm phosphate buffered saline (PBS; Gibco) and gently detached with trypsin/EDTA (0.5%) (Gibco) before analysis by flow cytometry (Cytek Biosciences), according to the manufacturer's recommendation. Cell viability was assessed by propidium iodide (BioLegend), with no differences detected between the IFN-γ and untreated control groups. Analysis was performed using FlowJo™ Software.

Real time polymerase chain reaction

RNA was extracted from HCAEC following specified treatments and purified using the Qiagen RNeasy Mini Kit. cDNA synthesis was performed with 0.5 μg of RNA using the Advantage RT-for-PCR kit (Takara). Relative mRNA expressions were compared by quantitative PCR using TaqMan Gene Expression assays (Thermo Fisher) with the probes listed

in the **Table I in the Supplement**. All gene expressions were normalized to that of actin. Relative gene expressions are expressed as fold-change compared to untreated controls.

mRNA sequencing

Confluent HCAECs were treated with or without 50 ng/mL IFN-γ for 24 h in triplicates. RNA was extracted and purified using the RNeasy Mini Kit with DNase treatment (Qiagen). Libraries were prepared using Roche Kapa mRNA HyperPrep strand specific sample preparation kits from 200 ng of purified total RNA according to the manufacturer's protocol on a Beckman Coulter Biomek i7. The finished dsDNA libraries were quantified by Qubit fluorometry, Agilent TapeStation 2200, and RT-qPCR using the Kapa Biosystems library quantification kit according to the manufacturer's protocols. Uniquely dual indexed libraries were pooled in equimolar ratios and sequenced on an Illumina NovaSeq 6000 with paired-end 50 bp reads by the Dana-Farber Cancer Institute Molecular Biology Core Facility. Sequencing data were processed to remove any PCR primers, adaptors, and trim low-quality reads using FASTQC. These high quality, clean reads were aligned against the human reference genome hg19 using STAR [59]. The average rate of uniquely mapped reads is 88%. Gene expression levels were then measured by counting the unique reads in genomic features using HTSseq [60], with 20,114 genes in the raw count data. To increase the statistical power, we removed the genes that had 0 counts in all the samples, leaving 16,812 genes for further analysis. We next used edgeR to normalize the raw count data on the basis of library complexity and gene variation, and performed differential gene expression analysis [61]. In this method, an overdispersed Poisson model is used to account for both biological and technical variability, and empirical Bayes methods are used to moderate the degree of overdispersion across transcripts. The differentially expressed genes were identified on the basis of multiple-test

corrected P value (through the Benjamini-Hochberg procedure) and fold change. We used false discovery rate (FDR) < 0.05 as the significance threshold with p-values corrected by the Benjamini-Hochberg procedure for all genes sequenced.

Nuclear and cytoplasmic extraction

HCAECs grown and treated in a 100 mm culture dish were placed on ice in a 4°C cold room and washed twice with 3 volumes of cold PBS. One ml of cold Tris-EDTA saline buffer (40 mM Tris, 1 mM EDTA, 140 mM NaCl; pH 7.5) was added to each well before cells were scraped into 15 mL tubes. Cells were pelleted, washed with PBS, and processed for the sequential extraction of the cytoplasmic and nuclear compartments using the NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Fisher) according to the manufacturer's recommendations.

Co-immunoprecipitation of nuclear proteins

Endothelial nuclear lysates prepared as described above were pre-cleared using 100 µl SureBeads Protein A Magnetic Beads (Bio-Rad) with gentle rotation at 4°C for 30 min. One hundred twenty to two hundred micrograms of pre-cleared nuclear lysates were incubated with the magnetic beads for 5 min at room temperature, washed, and then incubated with rabbit anti-ARNT monoclonal antibody (C15A11; Cell Signaling) or rabbit anti-IgG monoclonal antibody (DA1E; Cell Signaling) for 1 h at room temperature with gentle rotation according to the manufacturer's recommendations. Antigen-antibody-bead complexes were washed, and antigen-

antibody complexes were eluted in Laemmli buffer according to the manufacturer's recommendations and analyzed by Western blot.

Plasmid cotransfection and luciferase assay

HCAECs were grown to 90 % confluence in a 6-well plate and were co-transfected with the reporter construct expressing the firefly luciferase gene *luc2P* under the control of hypoxia response element (HRE) (pGL4.42 luc2P/HRE/Hygro; Promega) and the control *Renilla* luciferase vector under the cytomegalovirus promotor (hRluc/CMV; Promega) at a 50:1 ratio using the Lipofectamin 3000 transfection system (Thermo Fisher) according to the manufacturer's instructions. After 24 h, confluent HCAECs were treated with 50 μM cobalt chloride with or without 2 ng/mL IFN-γ for 48 h before sequential measuring of the firefly and *Renilla* luciferase activities using the Dual-Luciferase Reporter Assay system and the GloMax plate reader (Promega). HRE firefly luciferase activity measured in each well was normalized to Renilla luciferase activity.

Western Blot

Nuclear and cytoplasmic lysates were prepared as detailed above. Whole cell lysates were prepared using the radioimmunoprecipitation assay lysis buffer (Cell Signaling Technology) mixed with phosphatase and protease inhibitors (Calbiochem). The lysates were heated in Laemmli buffer and were run on a sodium dodecyl sulfate–polyacrylamide gel electrophoresis system. The proteins were transferred to the polyvinylidene fluoride membranes and were probed with the following antibodies: primary antibodies; actin, LDHA, IDO1, TBP,

VHL, AMPK, phospho-AMPK (Thr 172), eNOS, phospho-eNOS (Ser 1177) (all Cell Signaling), AHR (Cell Signaling and Thermo Fisher), ARNT (Cell Signaling and Novus), CPT1A (Proteintech), HIF1α (BD); secondary antibodies: anti-rabbit or anti-mouse-horseradish peroxide (Cell Signaling). Further details of the antibodies are provided in **Table II in the Supplement**. Blots were developed with horseradish substrate WesternBright ECL (Advansta) and exposed on films or directly visualized using the ChemiDoc Touch Imaging System (Bio-Rad). Densitometric analysis was performed using the Image Lab Software (Bio-Rad).

Immunofluorescence analysis

HCAECs were grown to confluence on glass chamber slides and were treated with IFN-γ, 50 ng/mL, for 24 h. Cells were fixed and blocked with 10% goat albumin (Life Technologies) for 1 h at room temperature. Cells were probed using rabbit anti-AHR antibody (Abcam) and secondary goat anti-rabbit antibody conjugated with Alexa Fluor 488 (Abcam) and were placed on slides using VECTASHIELD antifading mounting medium containing DAPI (Vector Laboratories). Images were captured with the LSM 700 Confocal Microscope (ZEISS) and processed as described previously [62].

Gene silencing

HCAECs were transfected with small interfering RNA (siRNA) targeting *AHR, CPT1A* (Dharmacon), or *VHL* (50:50 mix of the two custom stealth siRNAs (Invitrogen) (see the **Table III in the Supplement** for the sequence details); or with non-targeting siRNAs using Lipofectamine 2000 and OptiMEM reduced serum media (Thermo Fisher), according to the

manufacturer's recommendations. 42-48 h following transfection, cells were treated with IFN-γ, 10 or 50 ng/mL, for 4 h (for siVHL), 12 h (for siAHR), or 24 h (siCPT1A) before proceeding with further analysis. Successful knockdown was confirmed by RT-qPCR and Western blot (see **Figure IV-V in the Supplement**).

NAD/NADH Assay

Quantification of intracellular NAD^+ and NADH was performed using the NAD^+/NADH-Glo bioluminescence assay (Promega) according to the manufacturer's recommendations.

High performance liquid chromatography

ATP and ADP content in the treated cells were measured by HPLC as described previously [63] with the following modifications. Approximately 2 million cells were washed twice with ice-cold PBS and harvested in 800 µl 0.6 M perchloric acid. The cells were then homogenized in grinding tubes (25 strokes). After neutralization and centrifugation, the supernatant was filtered through a 0.45-µm HV-Millipore filter. P^1,P^5-di(adenosine-5')-pentaphosphate (0.1 µM) was added to inhibit adenylyl kinase, and the supernatant was further filtered through a 0.2-µm HV-Millipore filter. One hundred microliters of the cell extract were injected into an HPLC system equipped with a Waters 1525 HPLC pump coupled to a Waters 2487 dual wavelength (UV/Vis) absorbance detector (Waters Co.). Separation was performed through a Kromasil C-18 reverse phase column (250 × 4.6 mm, 5 µm-particle-size) connected to its own guard column (3.9 × 20 mm, 5 µm-particle-size) with a gradient program from buffer A (10 mM tetrabutylammonium hydroxide, 10 mM KH_2PO_4, 0.125% methanol, pH 7.00) to buffer

B (2.8 mM tetrabutylammonium hydroxide, 100 mM KH_2PO_4, 30% methanol, pH 5.50), which was generated as follows: 10 min 100% buffer A, 3 min at up to 80% buffer A, 10 min at up to 70% buffer A, 12 min at up to 55% buffer A, 11 min at up to 40% buffer A, 9 min at up to 35% buffer A, 10 min at up to 25% buffer A, 15 min 0% buffer A, and 50 min 0% buffer A. A 0.8 ml/min pump flow rate and constant column temperature of 22 °C were used. The UV absorption at 257 nm of the column effluent was monitored, and compound peaks were identified by comparing retention times and absorption spectra with those of freshly prepared ultrapure standard mixtures. ATP and ADP were quantified by use of standard curves for each, and each was normalized to the protein content determined by the Lowry method.

Cyclic GMP Assay

Briefly, confluent HCAECs treated with IFN-γ for 25 h were treated with a phosphodiesterase inhibitor, 3-isobutyl-1-methylxanthine (0.5mM), in phenol red-free and serum-free basal medium prior to lysis in cold 6% trichloroacetic. Cells underwent three freeze and thaw cycles, and intracellular cyclic GMP was ether-extracted from the harvested supernatants and measured using the cGMP ELISA Kit (Cayman) as described previously [64]. The measured cGMP amounts were normalized to the total protein mass.

Endothelial monolayer wound healing assay

HCAECs were grown to confluence in a 6-well plate and were treated with 50 ng/mL of IFN-γ for 30 h. A thin vertical linear open wound was created using a small pipette tip scratching off the adherent cells across the well. Images were obtained using a Nikon E200POL polarized

light microscope at 0 and 15 h following the scratch injury. For assessing the effect of tryptophan depletion on endothelial migratory capacity, HCAECs were incubated with DMEM containing glucose (1 g/L) deficient in tryptophan (Thermo Fisher Scientific) with or without exogenous tryptophan supplementation (16.0 mg/L; 0.078 mM), equivalent to the concentration in the standard DMEM formulation, for 24 h before scratch injury was performed. Images were obtained at 0 and 48 h following the scratch injury. Images were analyzed using the Image J software where the open areas without adherent cells were quantified.

Supplemental Table I. RT-PCR TaqMan Assay probes used in the study

Gene names	Identifier
ADM	Hs00969450_g1
ADRA1B	Hs00171263_m1
AHR	Hs00169233_m1
AK3	Hs00927274_g1
ALDOA	Hs00605108_g1
ALDOA	Hs00605108_g1
ALDOC	Hs00902799_g1
CDKN1A	Hs00355782_m1
CITED2	Hs01897804_s1
CPT1A	Hs00912671_m1
CYP1B1	Hs00164383_m1
EDN1	Hs00174961_m1
ENO1	Hs00361415_m1
FLT1	Hs01052961_m1
HK1	Hs00175976_m1
HK2	Hs00606086_m1
HMOX1	Hs01110250_m1
ICAM	Hs01164932_m1
IDO1	Hs00158027_m1
IGF2	Hs01005963_m1
IGFBP1	Hs00236877_m1
IGFBP2	Hs01040719_m1
IGFBP3	Hs00181211_m1
LDHA	Hs00855332_g1
NOS3	Hs01574665_m1
P4HA2	Hs00990001_m1
PFKFB3	Hs00998698_m1
PFKL	Hs01036347_m1
PGK1	Hs00943178_g1
PKM	Hs00761782_s1
SERPINE1	Hs00167155_m1
SLC2A1	Hs00892681_m1
TFRC	Hs00951083_m1
TGFB3	Hs01086000_m1
TPI1	Hs03806547_s1
VCAM	Hs00365485_m1
VHL	Hs03046964_s1

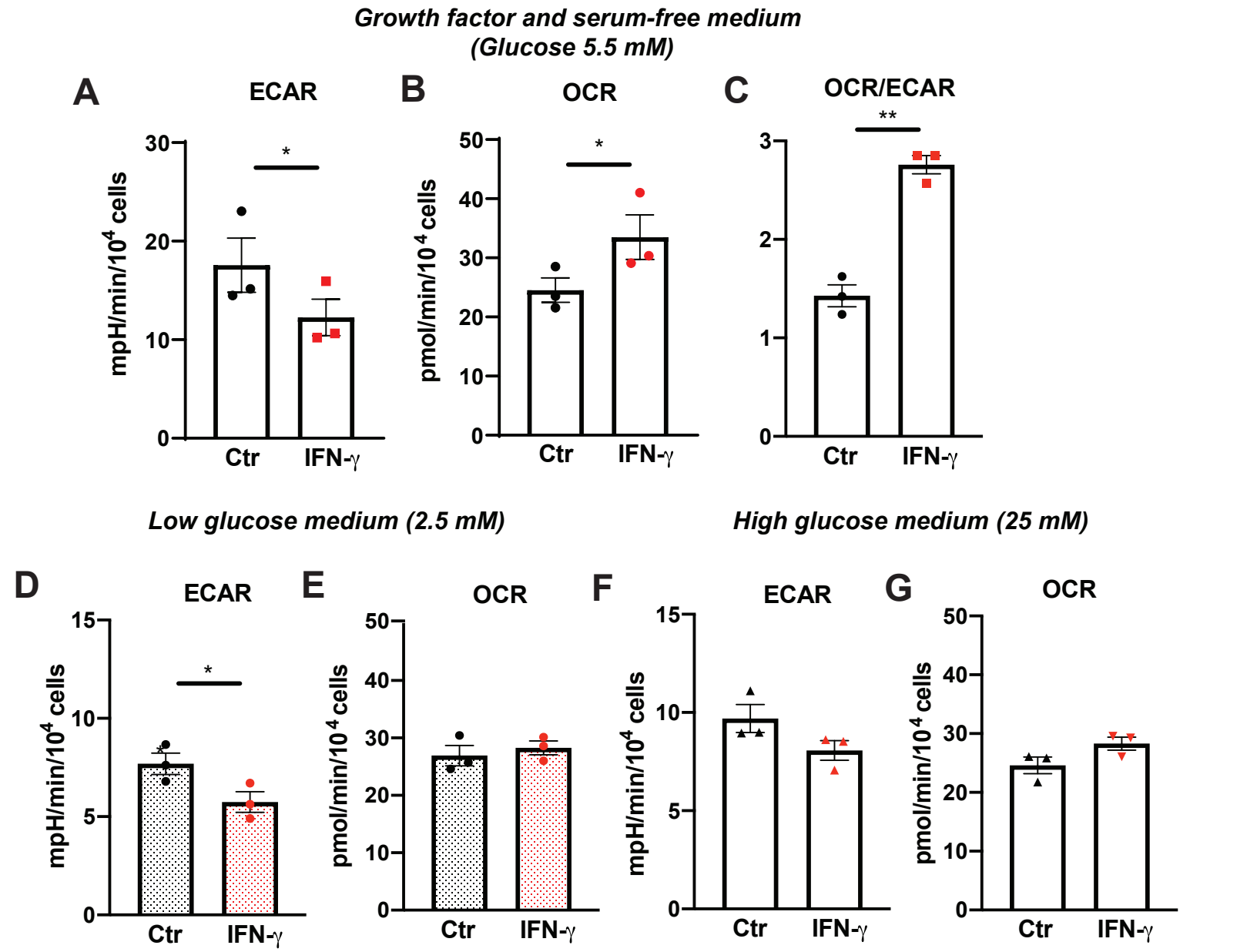
Supplemental Table II. Antibodies used in the study

Antibodies	Clone	Identifier	Sources
anti-AHR mouse Ab	RPT1	MA1-514	ThermoFisher
anti-AHR rabbit Ab	D5S6H	83200	Cell Signaling
anti-AHR polyclonal rabbit Ab		ab84833	Abcam
anti-AMPK rabbit Ab		2532	Cell Signaling
anti-phospho AMPK Thr172 rabbit Ab	40H9	2531S	Cell Signaling
anti-ARNT mouse Ab	H1beta234	NB100-124	Novus
anti-ARNT rabbit Ab	C15A11	3414S	Cell Signaling
anti-ARNT rabbit Ab	D28F3	5537S	Cell Signaling
anti-beta actin rabbit Ab		4970	Cell Signaling
anti-CPT1A rabbit Ab		15184-1-AP	Proteintech
Anti-eNOS mouse Ab	6H2	5880S	Cell Signaling
Anti-phospho-eNOS Ser1177 rabbit Ab	C9C3	9570S	Cell Signaling
anti-IDO1 rabbit Ab	D5J4E	86630S	Cell Signaling
anti-IgG rabbit Ab	DA1E	3900	Cell Signaling
anti-rabbit IgG Ab		7074S	Cell Signaling
anti-HIF1alpha mouse Ab	Clone 54	610958	BD
anti-LDHA rabbit Ab	2012	2012S	Cell Signaling
anti-TBP rabbit Ab	D5C9H	44059	Cell Signaling
Anti-VHL rabbit Ab		68547S	Cell Signaling

Supplemental Table III. siRNAs used in the study

Gene Target	Source	Identifier
AHR	Dharmacon	J-004990-08-0002
CPT1A	Dharmacon	L-009749-00-0005
Non-targeting	Dharmacon	D-001810-03-05 (Control for siAHR) D-001810-10-05 (Control for siVHL, siCPT1A)
VHL	Invitrogen*	siVHL #1: Sense: CCAGGUCAUCUUCUGCAAUCGCAGU Anti-sense: ACUGCGAUUGCAGAAGAUGACCUGG
		siVHL #2: Sense: CCAGGUCAUCUUCUGCAAUCGCAGU Anti-sense: ACUGCGAUUGCAGAAGAUGACCUGG

Supplemental Figure I



Supplemental Figure I. IFN-γ-mediated changes in endothelial glycolysis and oxidative phosphorylation in human coronary artery endothelial cells (HCAEC) in various culture medium conditions. (Related to **Figures 1** and **6**)

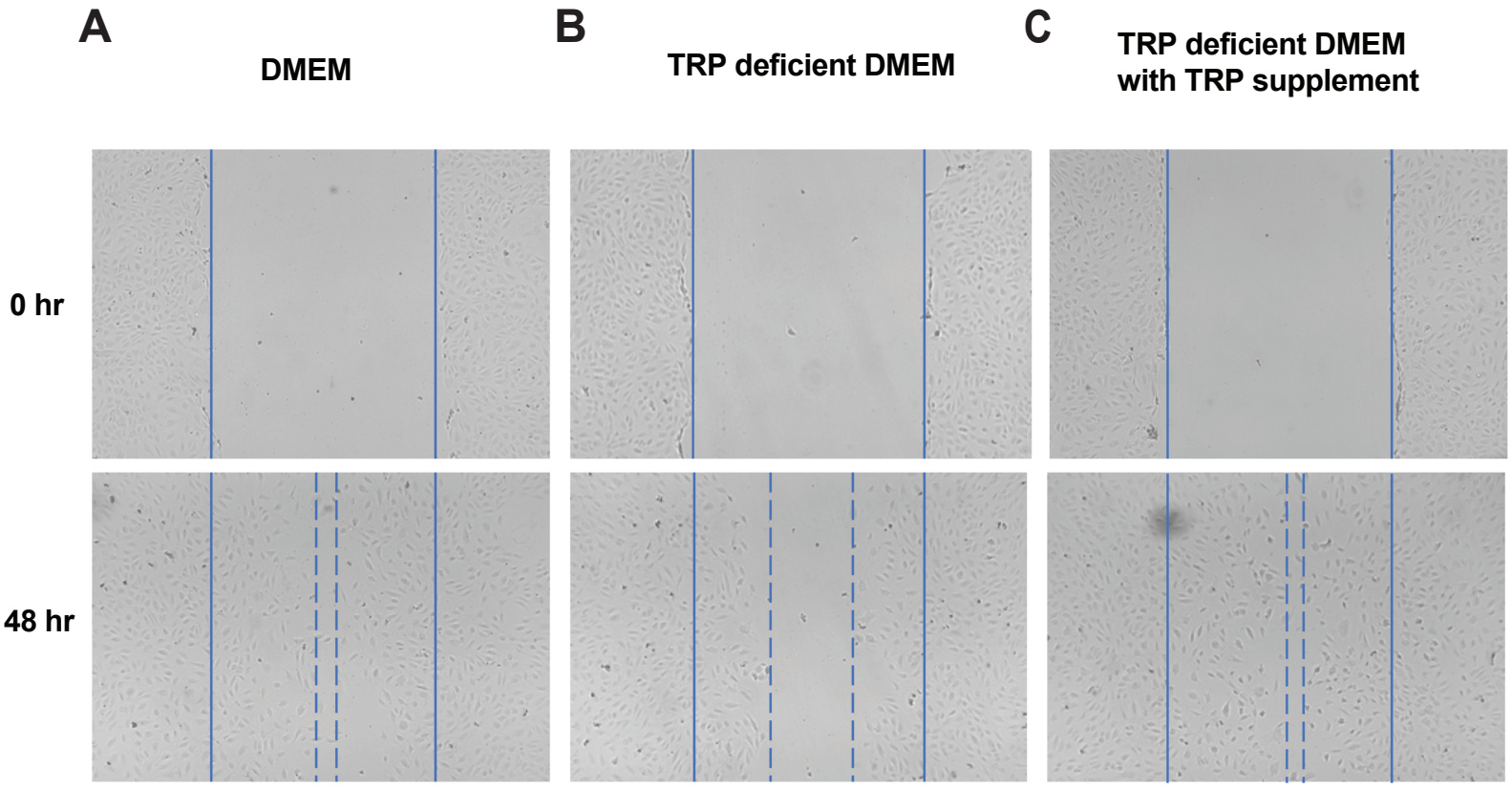
A-C, IFN-γ-mediated changes in endothelial extracellular acidification rate (ECAR) (**A**), oxygen consumption rate (OCR) (**B**) and ECAR/OCR (**C**) in HCAEC grown in the culture medium containing 5.5 mM glucose without serum or growth factors.

D-G, IFN-γ-mediated changes in endothelial ECAR (**D, F**) and OCR (**E, G**) in low glucose (2.5 mM) (**D-E**) and high glucose (25 mM) (**F-G**) media.

Assays were performed after 24 h of IFN-γ treatment at 50 ng/mL. Mean ± SEM from 3 independent experiments. Statistical significance was assessed using paired two-tailed Student's *t*-tests (**A-E, G**) or Wilcoxon matched-pairs signed rank test (**F**).

* $p < 0.05$, ** $p < 0.01$

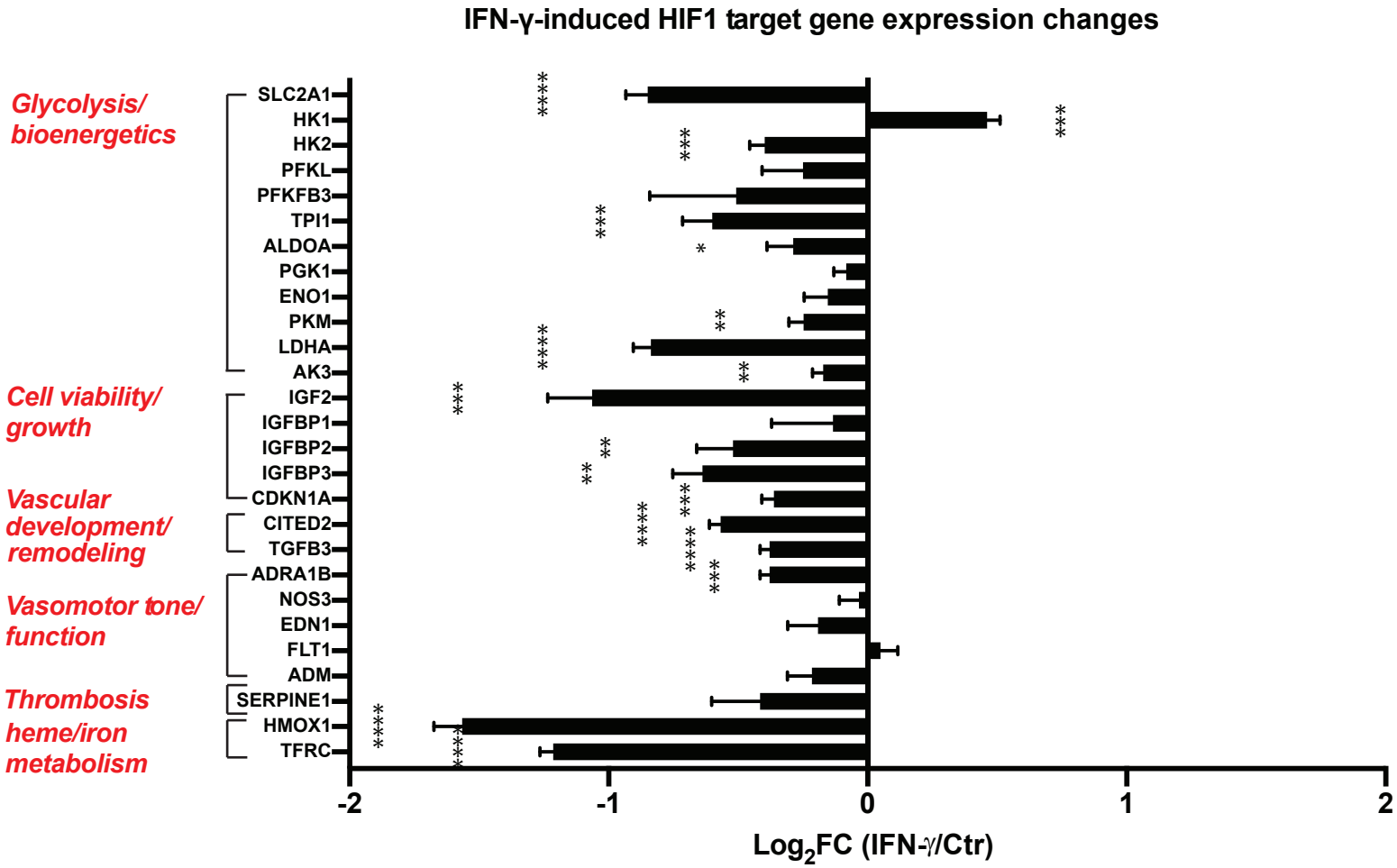
Supplemental Figure II



Supplemental Figure II. Tryptophan depletion impairs endothelial wound healing capacity. (Related to **Figure 2**)

A-C, The effect of tryptophan (TRP) deprivation in HCAEC migration capacities was assessed by scratch assay after exposure of the confluent HCAEC monolayer to a TRP deficient medium with (**C**) or without exogenous TRP supplementation (**B**) for 24 h before a mechanical scratch was made. Representative images are shown.

Supplemental Figure III



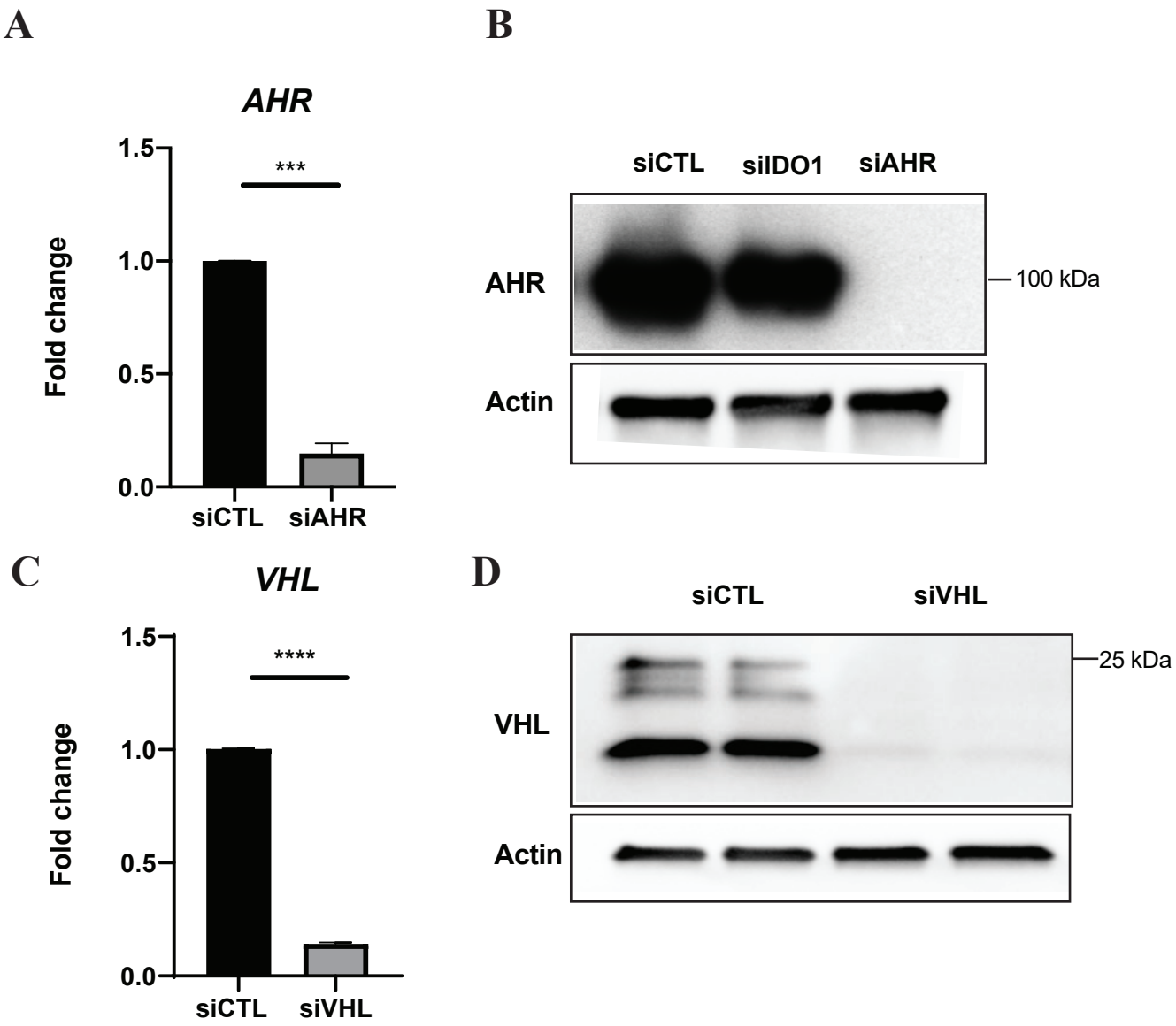
Supplemental Figure III. IFN-γ exposure results in global suppression of HIF1 target gene expressions in HCAEC in normoxia

Gene expression fold changes of known HIF1 target genes [65] in HCAEC were measured by RT-PCR following 24 h of IFN-γ treatment at 50 ng/mL. Mean ± SEM from 4 independent experiments. Statistical significance was assessed using two-tailed Student's *t*-tests. The significance levels were corrected for multiple testing using the Benjamini-Hochberg procedure.

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$

ADM: Adrenomedullin, ADRA1B: Adrenoceptor alpha 1B, AK3: Adenylate kinase 3, CDKN1A: Cyclin dependent kinase inhibitor 1A, CITED2: Cbp/P300-interacting transactivator 2, EDN: Endothelin, FLT: Fms-related receptor tyrosine kinase, HMOX: Heme oxygenase 1, IGF: Insulin-like growth factor, IGFBP: Insulin-like growth factor binding protein, NOS: Nitric oxide synthase, SERPINE1: Serpin family E member 1, TFRC: Transferrin receptor, and TGFB: Transforming growth factor beta

Supplemental Figure IV



Supplemental Figure IV. Gene silencing studies of AHR-HIF signaling pathways (Related to **Figure. 4**)

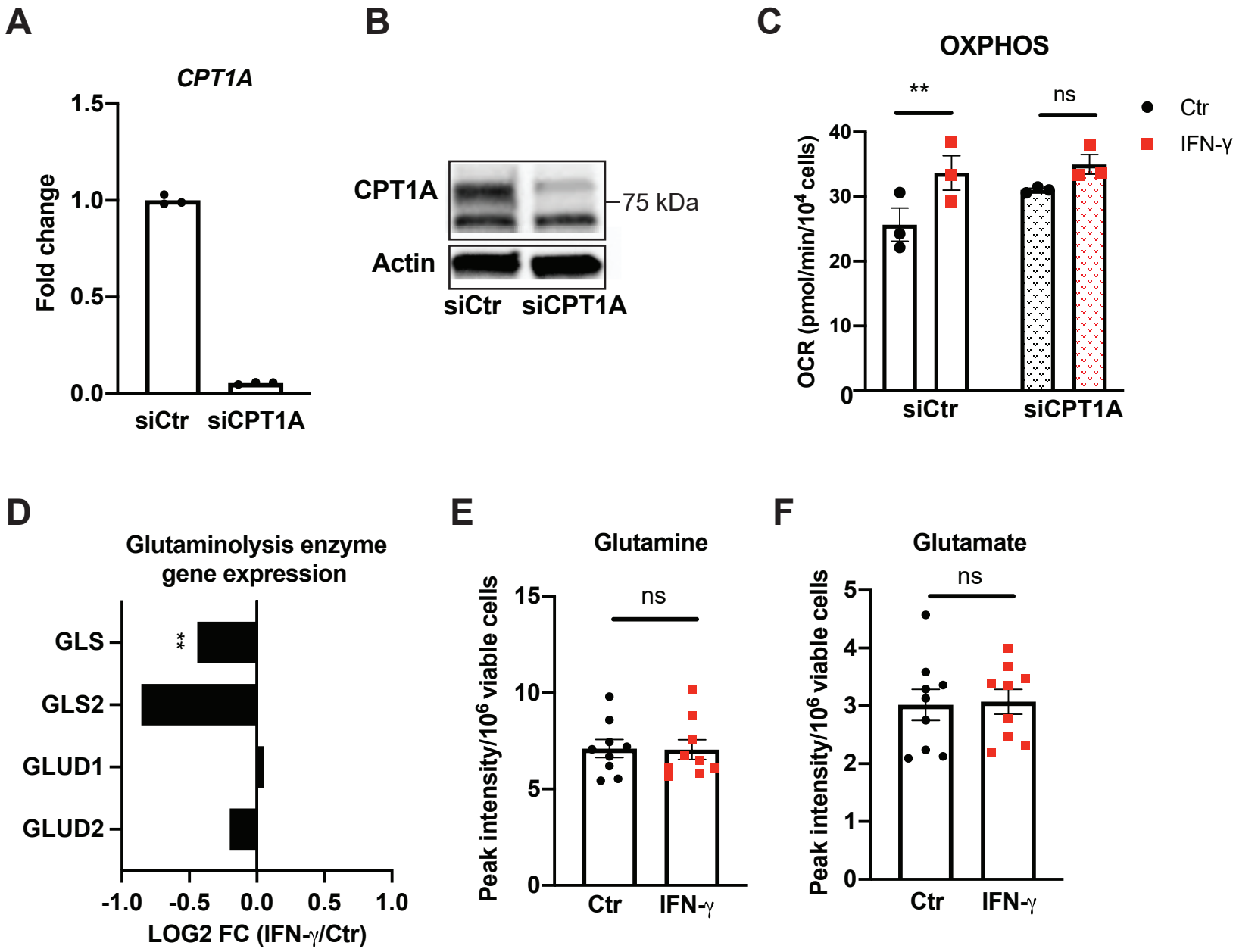
A-B, AHR silencing by siRNA treatment assessed at the mRNA (**A**) and protein levels (**B**).

C-D, VHL silencing by siRNA treatment assessed at the mRNA (**C**) and protein levels (**D**).

The mRNA expression levels assessed by RT-PCR represent mean ± SEM from 4 independent experiments. Protein expression levels assessed by Western blot are demonstrated with the representative images. Statistical significance was assessed using Welch's *t*-test (**A**) or two-tailed Student's *t*-test (**C**).

*** $p < 0.001$, **** $p < 0.0001$

Supplemental Figure V



Supplemental Figure V. IFN-γ augments endothelial fatty acid oxidation and preserves energy balance but does not promote glutaminolysis. (Related to **Figure 6**)

A-B, Carnitine palmitoyl transferase I (CPT1A) silencing by siRNA treatment assessed at the mRNA (**A**) and protein levels (**B**). Representative figures.

C, Real time extracellular flux analysis of basal oxidative phosphorylation (OXPHOS) of HCAEC following 24 h of IFN-γ treatment at 50 ng/ml following CPT1A gene silencing. Mean ± SEM from 3 independent experiments. Statistical significance was assessed by 2-way ANOVA with Sidak's multiple comparison test.

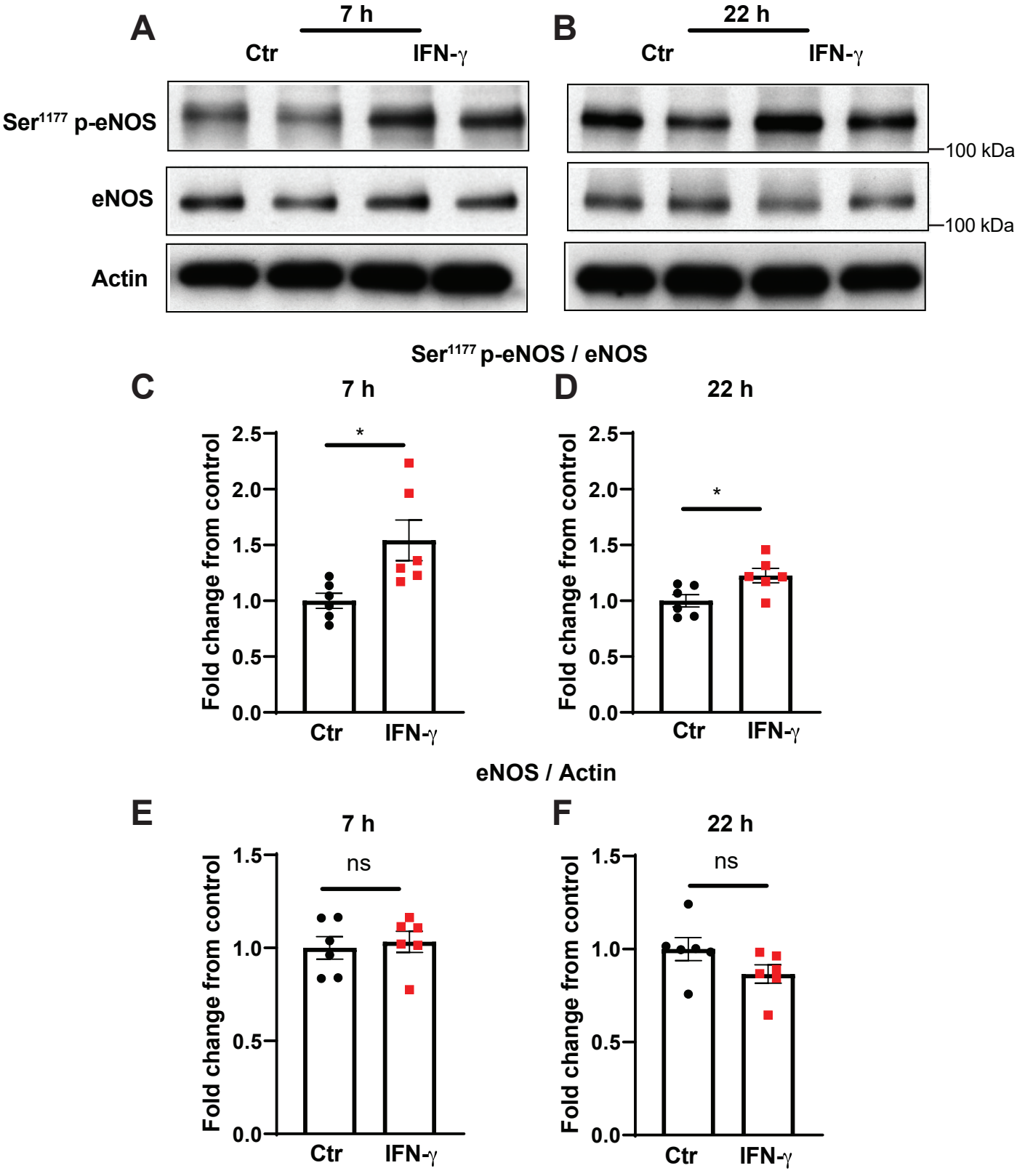
D, IFN-γ induced gene expression changes in the glutaminolysis enzymes by RNASeq analysis of HCAEC obtained in biological triplicates. Statistical significance was determined based on the *p*-values adjusted for multiple test hypotheses by Benjamini-Hochberg procedure.

E-F, Intracellular glutamine (**E**) and glutamate (**F**) in HCAEC measured by LC-MS following 24 h of IFN-γ treatment. Mean ± SEM from 9 biological replicates from 3 independent experiments. Statistical significance was assessed using two-tailed Student's *t*-tests.

GLS: Glutaminase, GLUD: Glutamate Dehydrogenase.

** $p < 0.01$

Supplemental Figure VI



Supplemental Figure VI. IFN-γ results in phosphorylation of endothelial nitric oxide synthase in HCAEC. (Related to **Figure 7**)

A-B, Representative images of Western blot analysis of Serine 1177 phosphorylated endothelial nitric oxide synthase (Ser^{1177} p-eNOS) and total eNOS following 7 h (**A**) and 22 h (**B**) of IFN-γ exposure at 50 ng/mL.

C-D, Densitometric quantification of Ser^{1177} p-eNOS from each sample was normalized to total eNOS before calculating the fold changes for IFN-γ treatment over untreated controls.

E-F, Densitometric quantification of total eNOS with and without IFN-γ treatment at 7 h (**E**) and 22 h (**F**).

Mean ± SEM of 6 biological replicates from 3 independent experiments. Statistical significance was assessed using two-tailed Student's *t*-test.

* $p < 0.05$