Online Supplementary Information

α-Ketoglutarate Inhibits Thrombosis and Inflammation by Prolyl Hydroxylase-2 Mediated Inactivation of Phospho-Akt.

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Supplementary Figures and Table

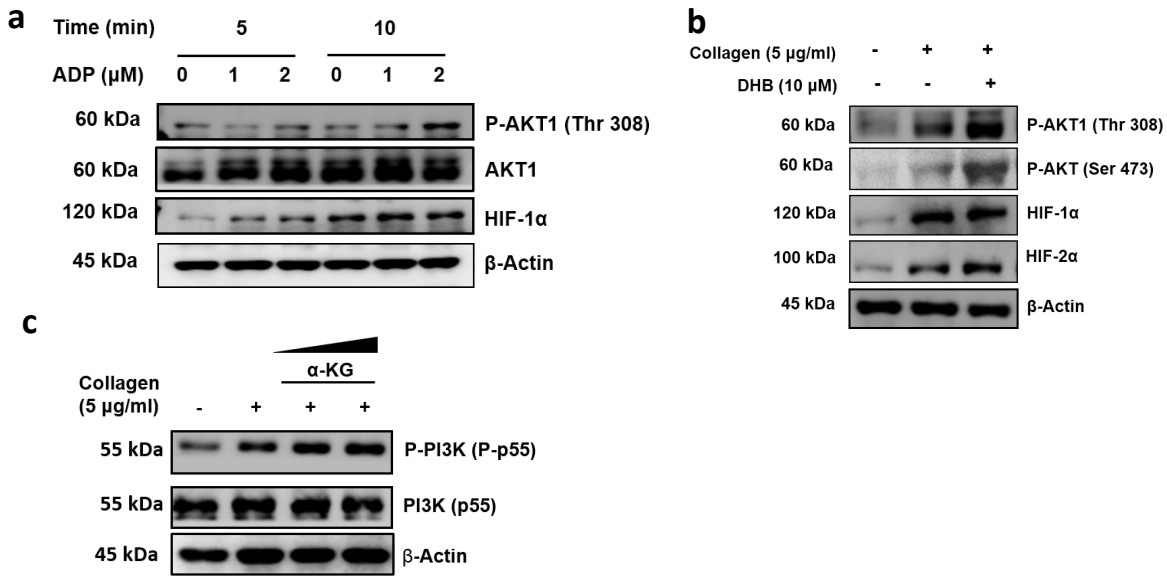


Fig. S1. Platelet activation to ADP and DHB. (a) Platelet-rich plasma (PRP) was incubated with ADP in a time- and concentration-dependent manner and expression of phosphorylated Akt1(Thr308), Akt1, and HIF-1α were measured in platelet pellet using WB assay. Densitometry analysis shows elevated expression of P-Akt1 and HIF-1α after ADP stimulation, densitometry data are mentioned in Fig. S14q-r. **(b)** PRP treated with collagen or DHB or both at mentioned concentration and platelet pellet was processed for WB. DHB found to increase pAkt1(Thr308), P-Akt (Ser473), HIF-1α and HIF-2α expression in platelets stimulated by collagen, densitometry data are mentioned in Fig. S14s-v. **(c)** PRP treated with collagen or αKG or both and platelet pellet was processed for WB of PI3K (p55). Densitometry data shows αKG do not affect collagen induced PI3K phosphorylation in platelets, densitometry data are mentioned in Fig. S14w.

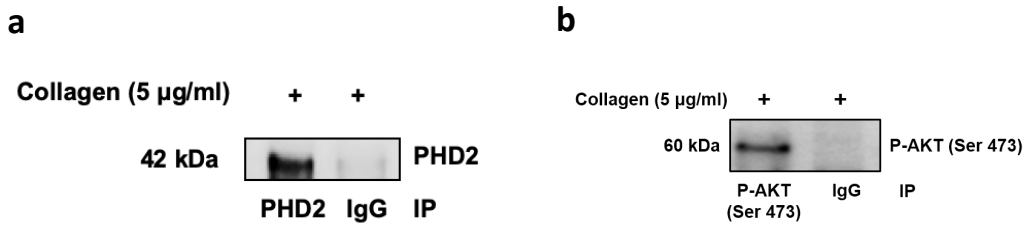


Fig. S2. Immunoprecipitation of PHD2 and pAkt from platelet lysate. Washed platelets were treated with collagen (5 µg/ml) and collected platelet pellet was lysed and immunoprecipitation was performed using protein A and protein G-Sepharose beads for PHD2 and pAkt(Ser473). Western blot images show immunoprecipitation of **(a)** PHD2 and **(b)** pAkt (Ser 473) with their respective IgG controls.

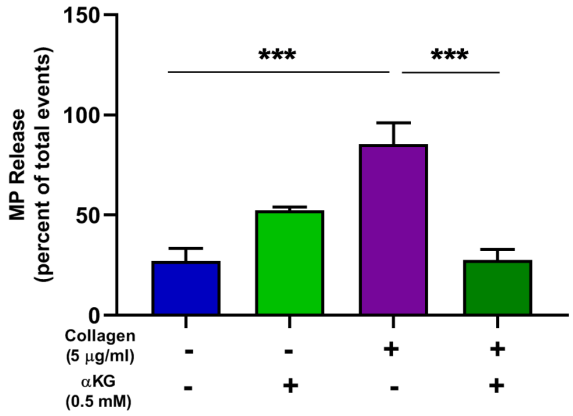
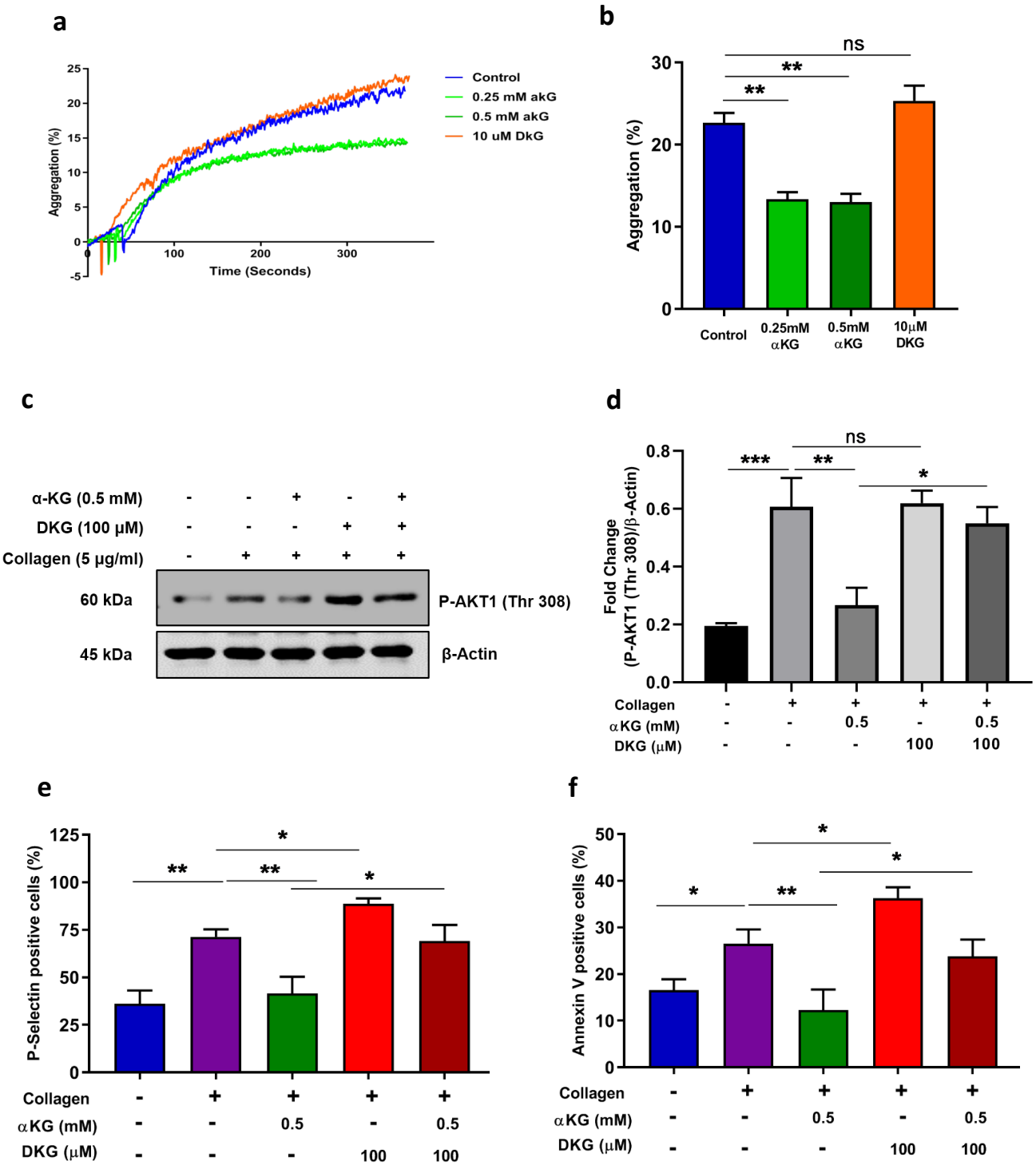


Fig. S3. Microparticles (MP) release from platelets after αKG treatment. Washed platelets were treated with collagen (5 µg/ml) or αKG (1 mM) or both, and collected supernatant processed for CD41a labelled microparticle analyses using flow cytometry. Bar diagram represents reduced microparticles release from α-KG treated platelets. Data are mean ± SEM from 3 independent experiments (one-way ANOVA, ***P<0.001).


a
Aggregation (%)
Time (Seconds)
Control
0.25 mM akG
0.5 mM akG
10 uM DkG
b
Aggregation (%)
ns
**
**
Control
0.25mM αKG
0.5mM αKG
10μM DKG
c
α-KG (0.5 mM)
DKG (100 μM)
Collagen (5 μg/ml)
60 kDa
45 kDa
P-AKT1 (Thr 308)
β-Actin
d
Fold Change (P-AKT1 (Thr 308)/β-Actin)
ns
***
**
*
Collagen
αKG (mM)
DKG (μM)
e
P-Selectin positive cells (%)
*
**
**
*
Collagen
αKG (mM)
DKG (μM)
f
Annexin V positive cells (%)
*
*
*
**
Collagen
αKG (mM)
DKG (μM)

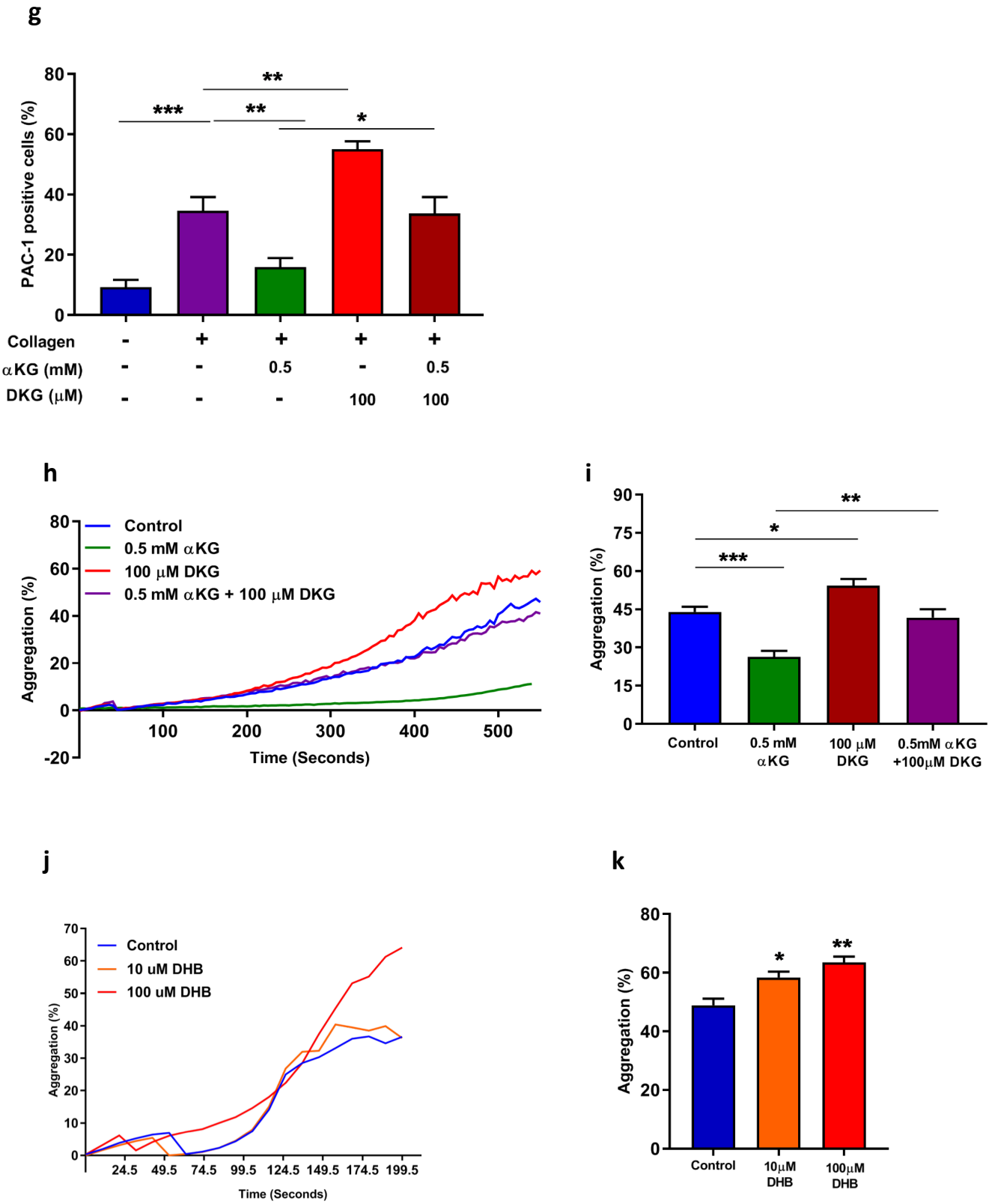

g
PAC-1 positive cells (%)
80
60
40
20
0
***
**
**
*
Collagen - + + + +
αKG (mM) - - 0.5 - 0.5
DKG (μM) - - - 100 100
h
Aggregation (%)
Control
0.5 mM αKG
100 μM DKG
0.5 mM αKG + 100 μM DKG
Time (Seconds)
i
Aggregation (%)
Control
0.5 mM αKG
100 μM DKG
0.5mM αKG +100μM DKG
j
Aggregation (%)
Control
10 uM DHB
100 uM DHB
Time (Seconds)
k
Aggregation (%)
Control
10μM DHB
100μM DHB

Fig. S4. Effect of αKG, DKG and DHB on platelet activation and aggregation. (a-b) Aggregation of platelets in response to ADP was reduced after αKG treatment, whereas, DKG increased it. **(a)** Percentage aggregation of representative assay. **(b)** Data from 3 independent assays. **(c-i) DKG rescues suppressed effect of αKG on platelet functions. (c)** pAkt1 (Thr 308) expression. **(d)** Densitometry of pAkt(Thr 308) from independent assays. **(e)** P-Selectin, **(f)** Annexin V binding to PS and **(g)** PAC-1 binding to platelets from above experiment. **(h)** Percentage of platelet aggregation of a representative assay. **(i)** Data from independent experiments. **(j-k) Effect of DHB on platelet aggregation. (j)** Percentage of platelet aggregation from a representative assay. **(k)** Data from independent experiments. Data are mean ± SEM from 3 independent experiments (one-way ANOVA, **P*<0.05, ***P*<0.01, ****P*<0.001 and ns=non-significant).

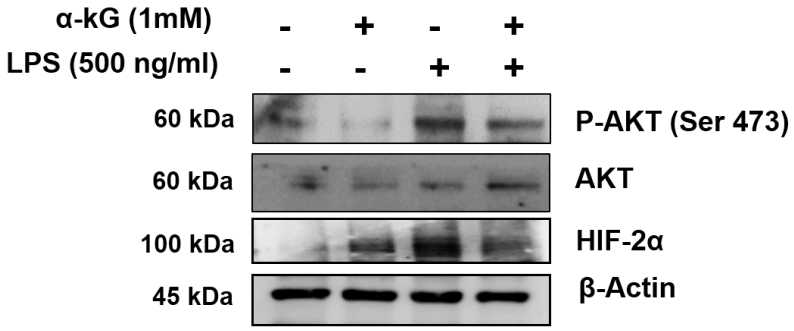


Fig. S5. Effect of αKG on LPS induced HIF-2α and pAkt (Ser 473) expression. Expression of pAkt (Ser 473) and HIF-2α are reduced in primary monocytes pre-treated with α-KG. Densitometry data in Fig. S14x-y.

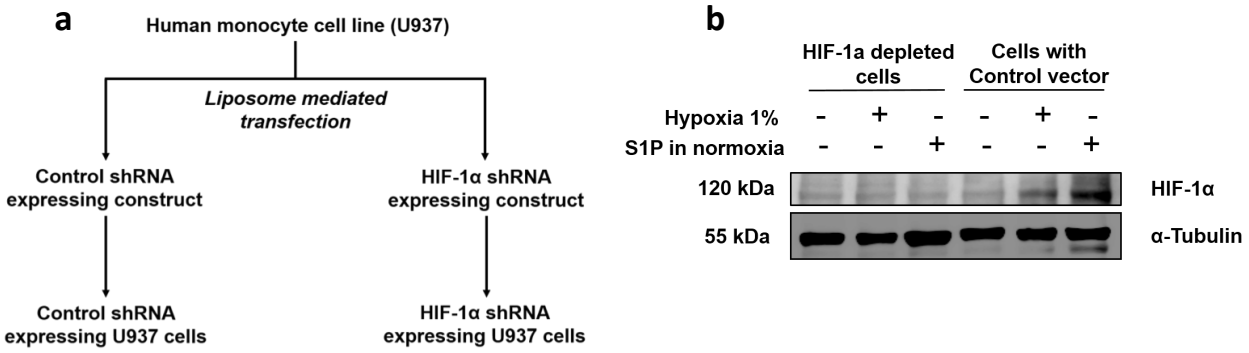


Fig. S6. Generation of HIF-1α depleted cells. (a) Schematic protocol for depletion of endogenous HIF-1α using shRNA in U937 cell line, using liposome-mediated delivery of construct. **(b)** HIF-1α protein expression in pLKO control shRNA and HIF-1α targeting constructs transfected cells with 24 hour 1% hypoxia or 1 μM S1P stimulation.

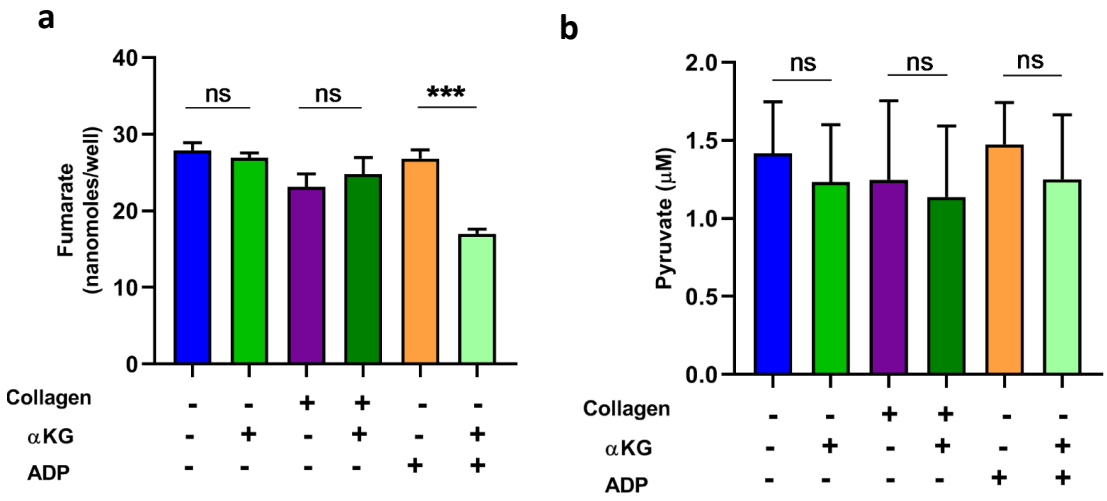


Fig. S7. Intracellular level of fumarate and pyruvate in activated platelets after αKG supplementation. Steady state level of **(a)** fumarate and **(b)** pyruvate were estimated from platelets lysate, pre-treated with α-KG and activated with collagen or ADP, as mentioned in Fig. 3. Data are mean ± SEM from 3 independent experiments (one-way ANOVA, ****P*<0.001, ns=non-significant).

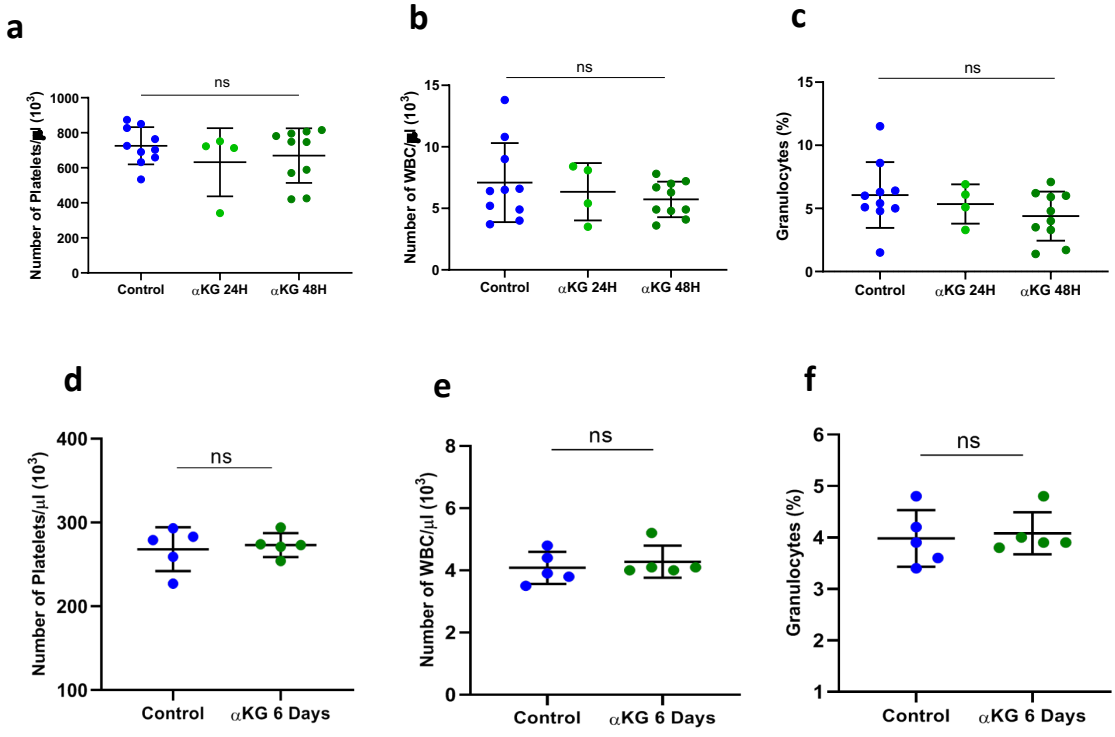


Fig. S8. Mice and Hamster blood counts. Count of **(a)** platelets, **(b)** WBC and **(c)** granulocytes determined from mice supplemented with dietary αKG for 24 and 48 hours, as mentioned in Figure 4a (Kruskal Wallis test, ns=non-significant). Count of **(d)** platelets, **(e)** WBC and **(f)** granulocytes determined from hamsters supplemented with dietary αKG for 6 days (as mentioned in Fig. 6), using Nihon Kohden's CelltacF haematology analyser (Mann Whitney test, ns=non-significant) Data are mean ± SEM.

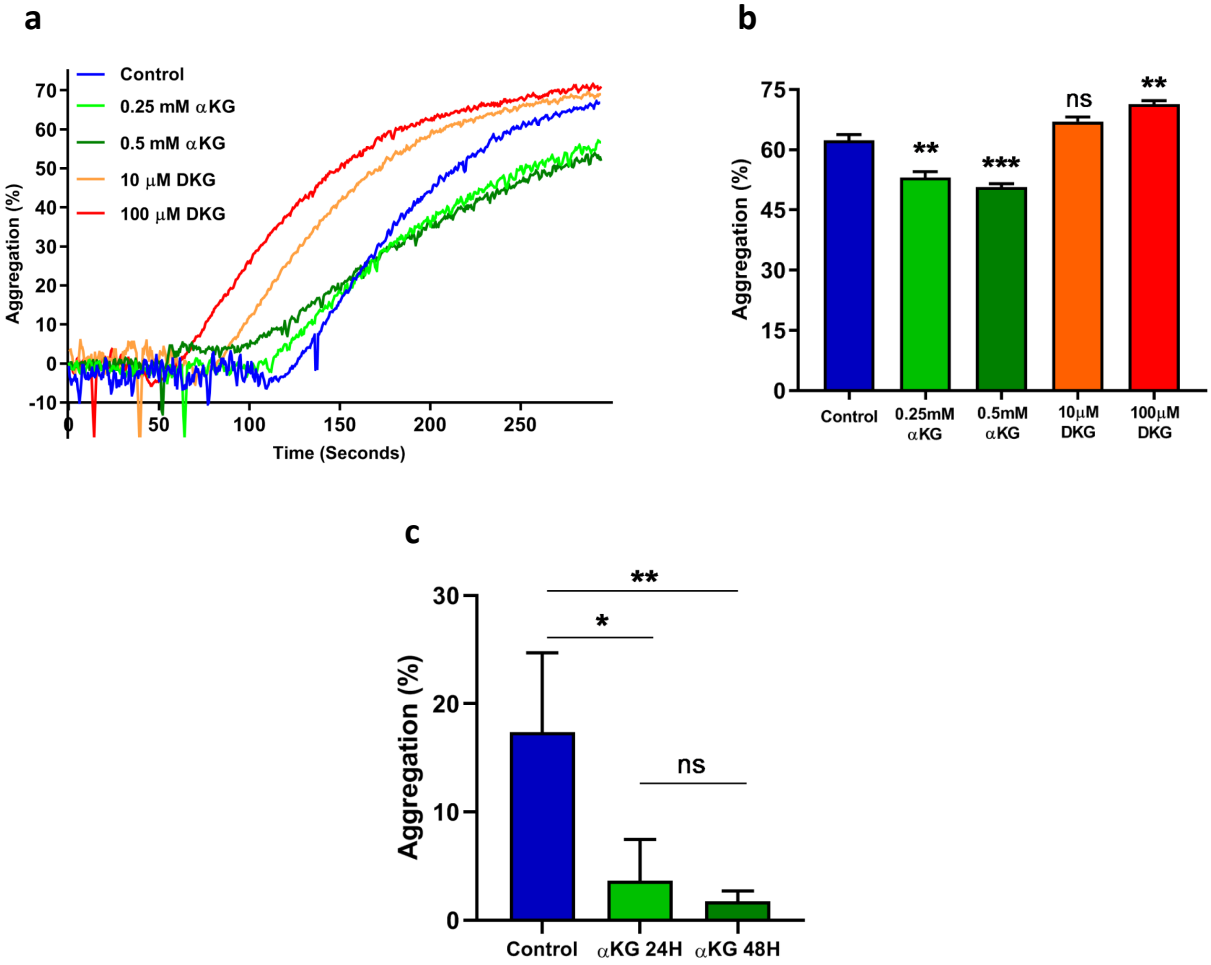


Fig. S9. Aggregation response of mice platelets with *in vitro* and *in vivo* treatment of αKG. **(a)** Aggregation response of mouse PRP to collagen with/without αKG or DKG treatment *in vitro*. **(b)** Percentage platelet aggregation. **(c)** Platelets from mice supplemented with dietary αKG for 24 and 48 hours were stimulated with ADP and aggregation response was quantified as percentage aggregation, as mentioned in Figure 4B. Data are mean ± SEM from 3 independent experiments (one-way ANOVA, **P*<0.05, ***P*<0.01, ****P*<0.001, ns=non-significant).

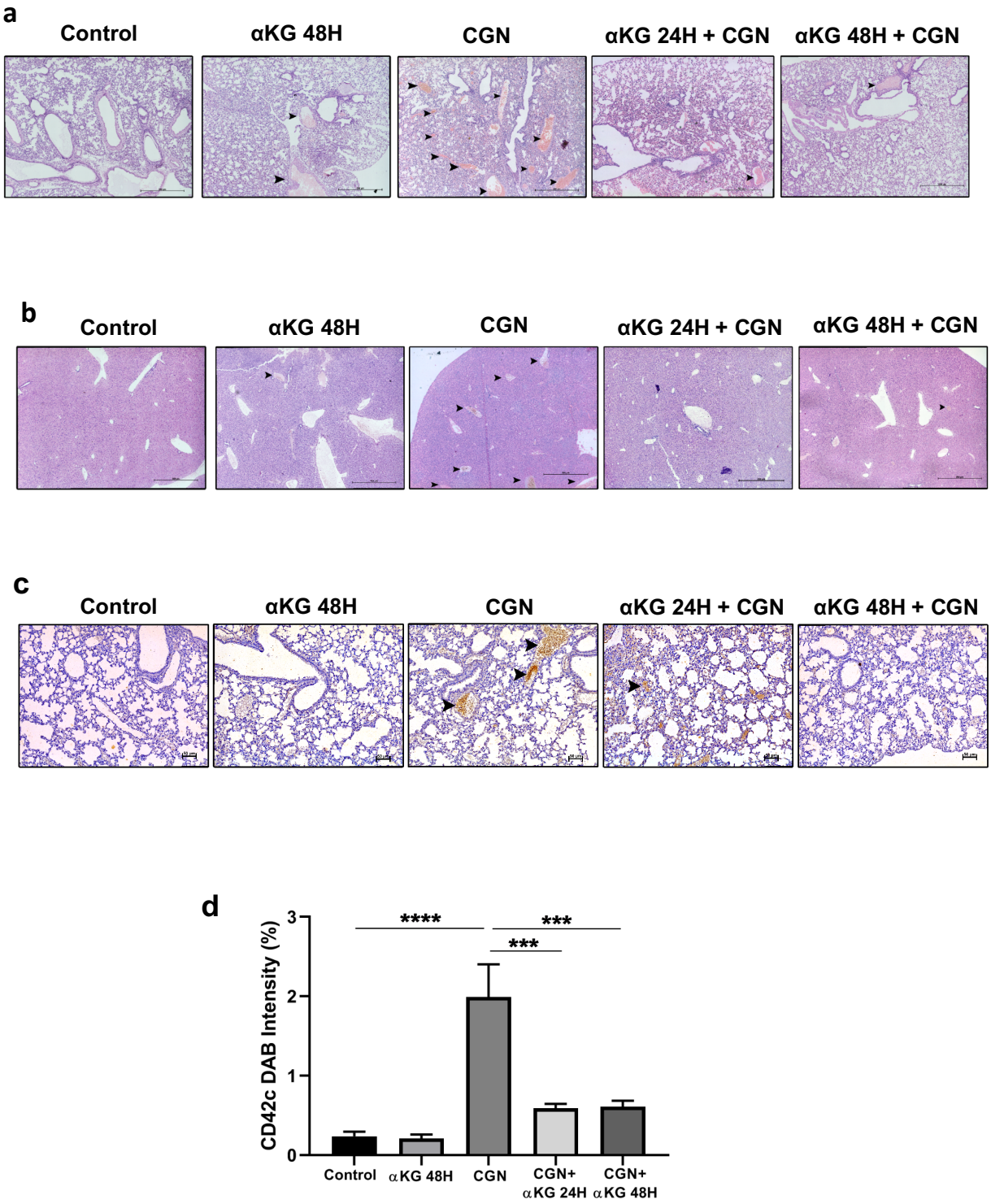


Fig. S10. Dietary αKG supplementation reduces lung and liver thrombosis in mice. H&E staining of mice **(a)** lung and **(b)** liver showing reduction of carrageenan induced thrombosis by dietary α-KG supplementation. Scale bar 500 µm. Similar MT staining data are mentioned in Fig. 5d. Treatment details are mentioned in Fig. 5a. **(c)** Mouse lung section from Fig. 5d were processed for IHC using CD42c antibody. Arrows indicate platelets in thrombus. Scale bar 50 µm. **(d)** Platelet accumulation in lung thrombus was measured by quantifying DAB intensity (n=8). Data are mean ± SEM (one-way ANOVA, ***P<0.001 and **** P<0.0001).

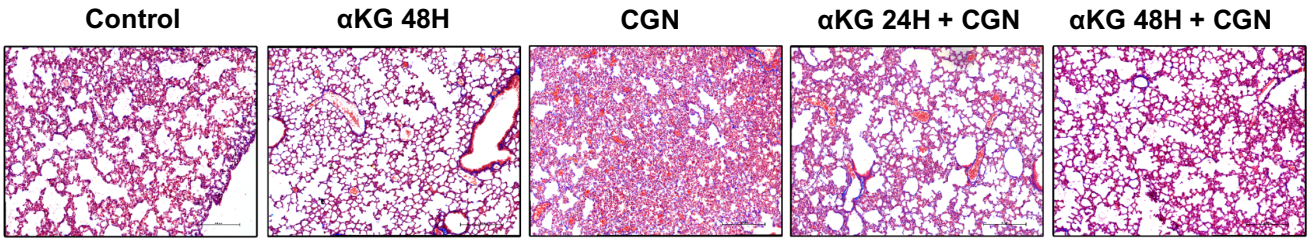


Fig. S11. Dietary αKG supplementation reduces inflammation in mice lung. Masson's trichrome (MT) staining of mice lung showing reduction of leukocyte infiltration, as the marker of inflammation, after dietary αKG supplementation in carrageenan-treated mice. Scale bar 500 μm. Similar H&E staining data are mentioned in Fig. 5h.

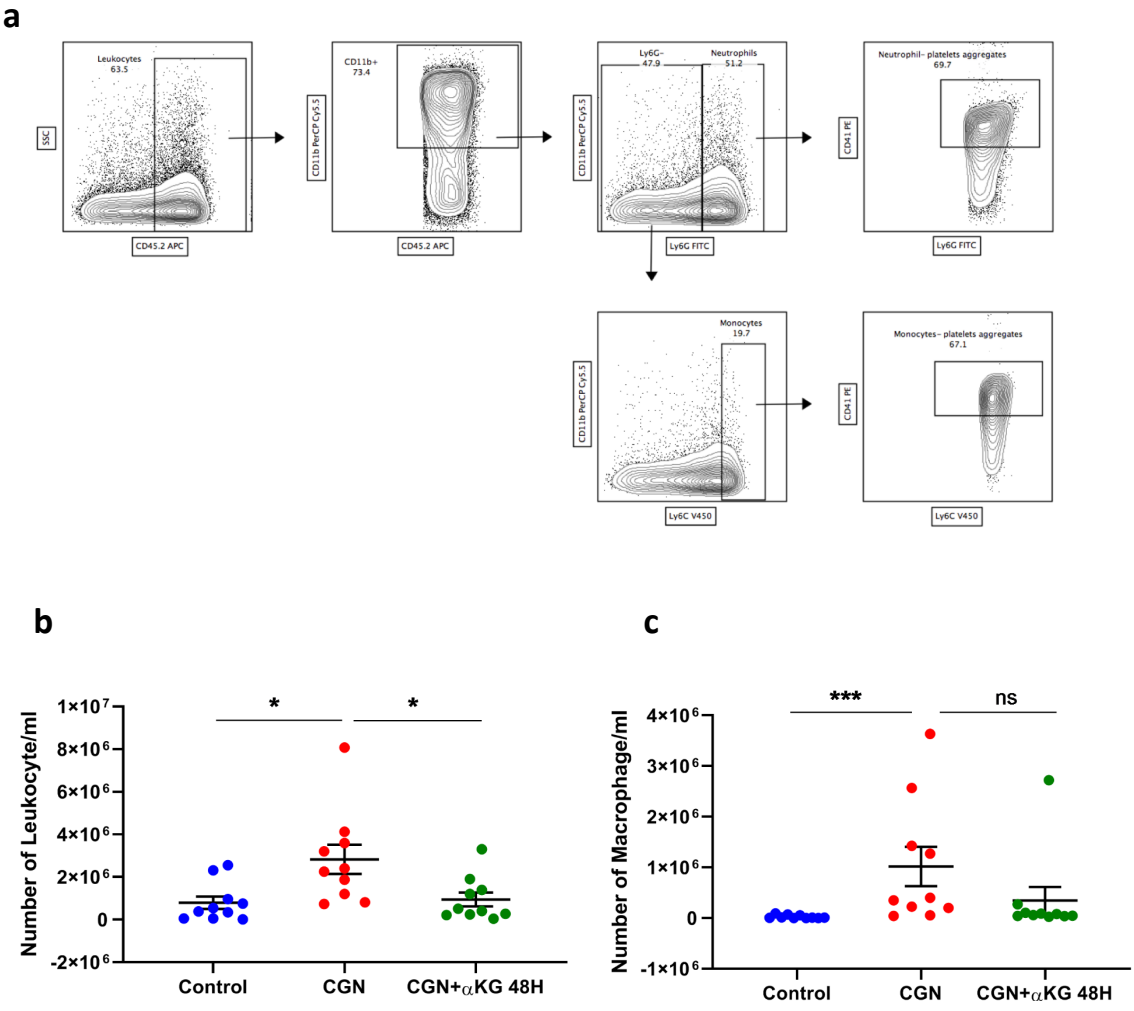


Fig. S12. Gating strategy for immune cells from mice peritoneal lavage fluid. Peritoneal lavage fluid collected from carrageenan treated mice was used to determine number of cells using flow cytometry. **(a)** Represents gating strategy used for determining different cell types. **(b)** Number of leukocytes and **(c)** macrophages in Peritoneal lavage fluid. Data are mean ± SEM. (Kruskal Wallis test, **P*<0.05, ****P*<0.001, ns=non-significant). Additional data for Fig. 5n-t.

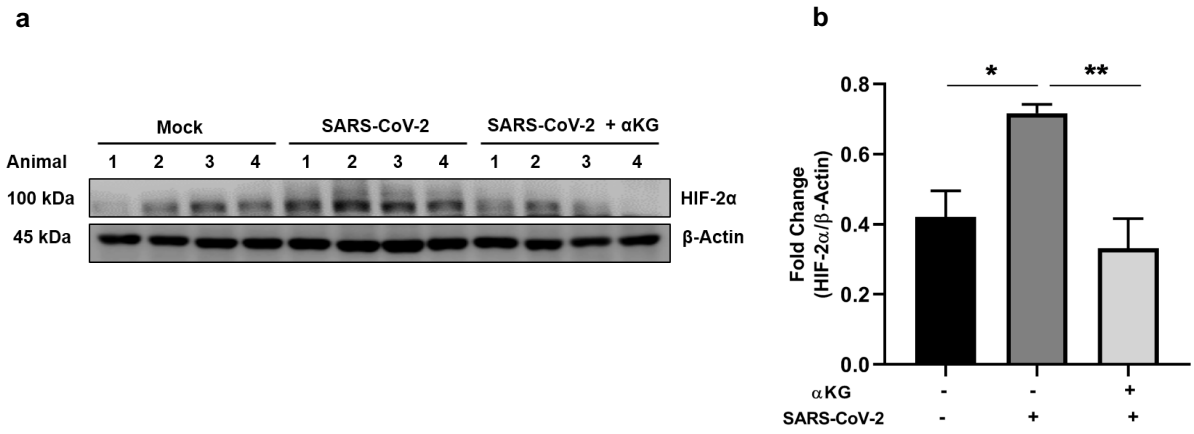
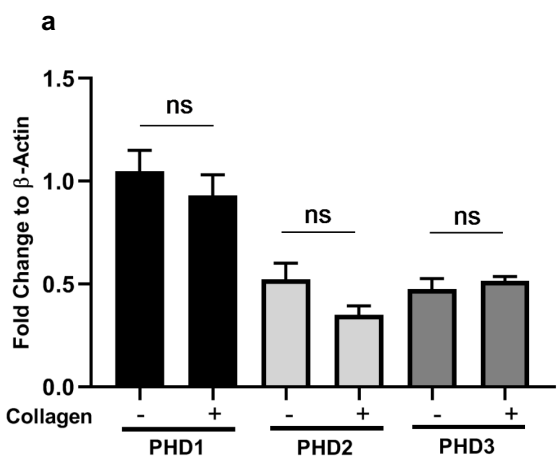
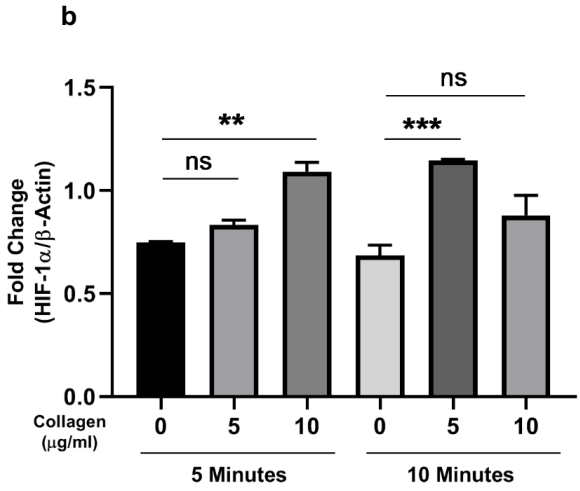
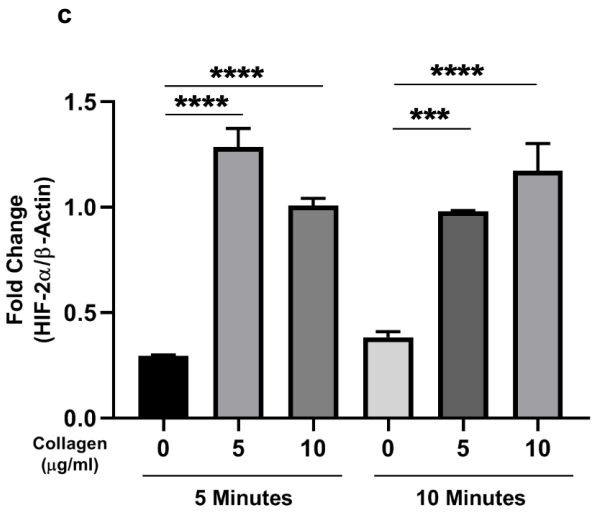
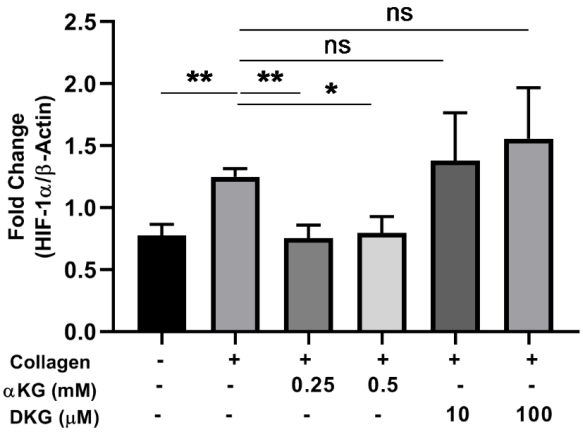


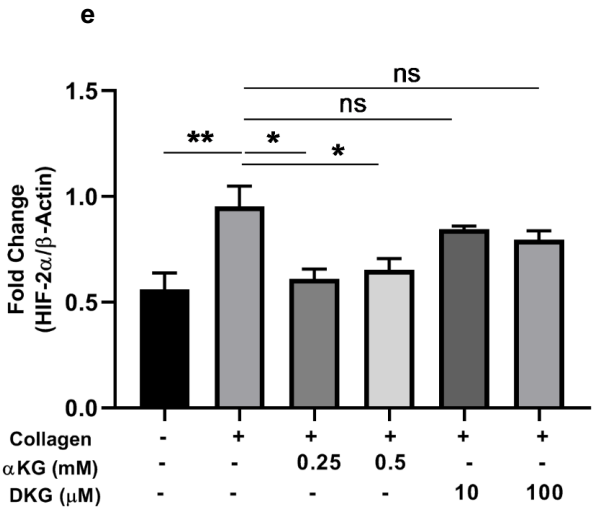
Fig. S13. HIF-2α expression in lung tissue of hamsters infected with SARS-CoV-2. (a) Immunoblots showing reduced expression of HIF2α in lung tissue lysates of hamsters treated with SARS-CoV-2 and supplemented with αKG compared to SARS-CoV-2 treated hamsters. **(b)** Densitometry data of immunoblots from above treatments (n=9 in each group). Data are mean ± SEM (one-way ANOVA, **P*<0.05, ***P*<0.01).

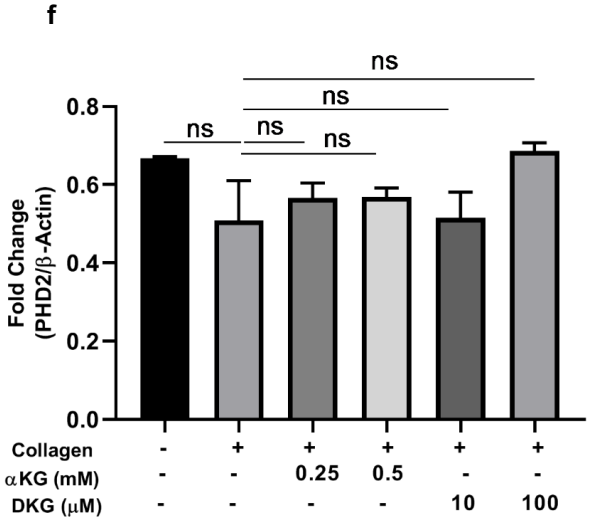

a
Fold Change to β-Actin
1.5
1.0
0.5
0.0
ns
ns
ns
Collagen
-
+
-
+
-
+
PHD1
PHD2
PHD3

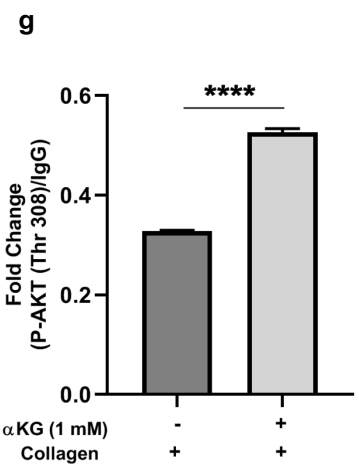

b
Fold Change
(HIF-1α/β-Actin)
1.5
1.0
0.5
0.0
ns
**
ns
***
Collagen
(μg/ml)
0
5
10
0
5
10
5 Minutes
10 Minutes

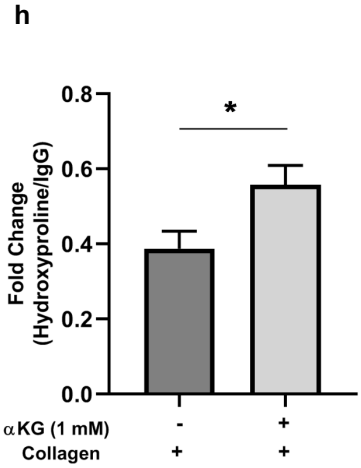

c
Fold Change
(HIF-2α/β-Actin)
1.5
1.0
0.5
0.0
****
****
****
***
Collagen
(μg/ml)
0
5
10
0
5
10
5 Minutes
10 Minutes

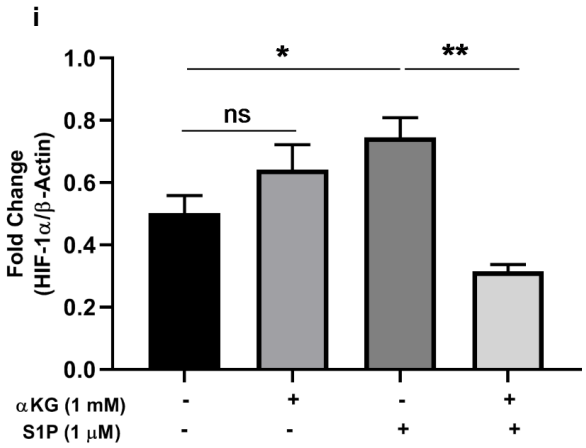

d
Fold Change
(HIF-1α/β-Actin)
2.5
2.0
1.5
1.0
0.5
0.0
ns
ns
**
**
*
Collagen
-
+
+
+
+
+
αKG (mM)
-
-
0.25
0.5
-
-
DKG (μM)
-
-
-
-
10
100

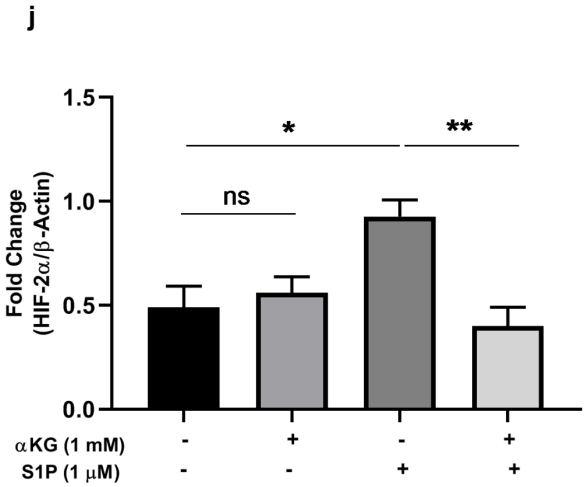

e
Fold Change
(HIF-2α/β-Actin)
1.5
1.0
0.5
0.0
ns
ns
**
*
*
Collagen
-
+
+
+
+
+
αKG (mM)
-
-
0.25
0.5
-
-
DKG (μM)
-
-
-
-
10
100

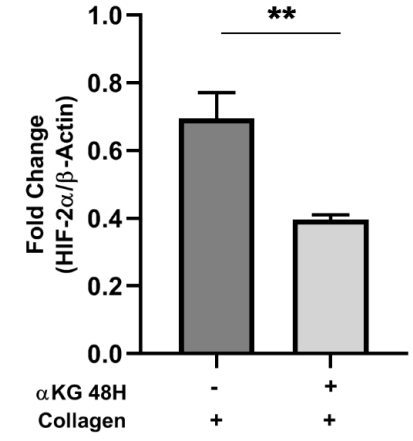

f
Fold Change
(PHD2/β-Actin)
0.8
0.6
0.4
0.2
0.0
ns
ns
ns
ns
ns
Collagen
-
+
+
+
+
+
αKG (mM)
-
-
0.25
0.5
-
-
DKG (μM)
-
-
-
-
10
100

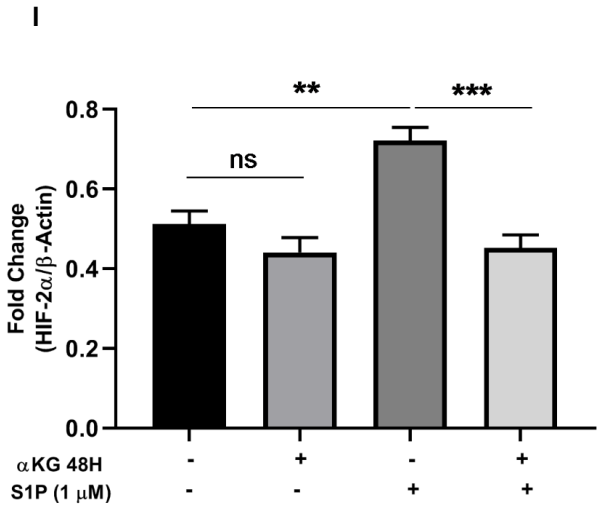

g
****
Fold Change (P-AKT (Thr 308)/IgG)
0.6
0.4
0.2
0.0
αKG (1 mM) - +
Collagen + +

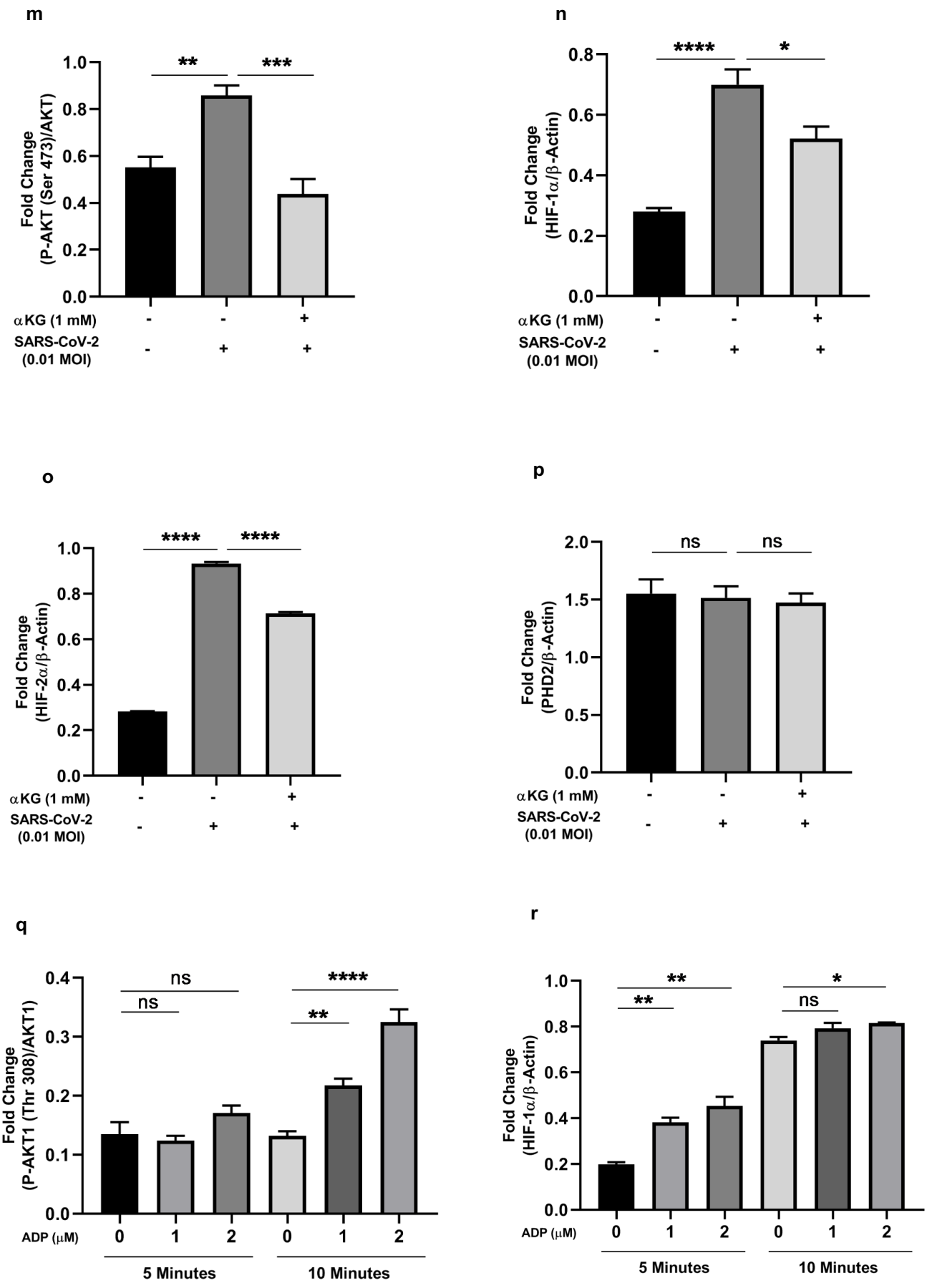

h
*
Fold Change (Hydroxyproline/IgG)
0.8
0.6
0.4
0.2
0.0
αKG (1 mM) - +
Collagen + +

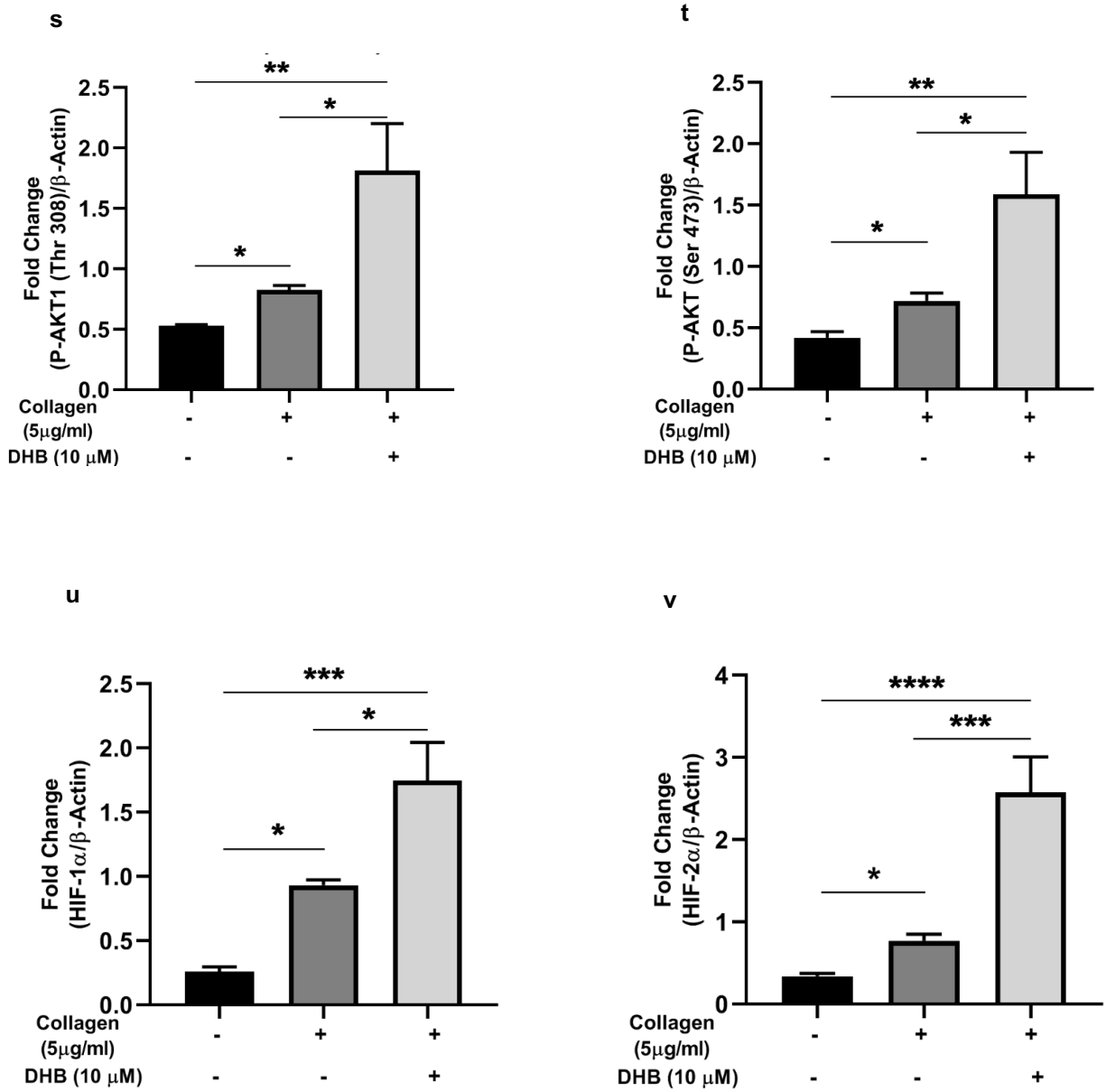

i
*
**
ns
Fold Change (HIF-1α/β-Actin)
1.0
0.8
0.6
0.4
0.2
0.0
αKG (1 mM) - + - +
S1P (1 μM) - - + +


j
*
**
ns
Fold Change (HIF-2α/β-Actin)
1.5
1.0
0.5
0.0
αKG (1 mM) - + - +
S1P (1 μM) - - + +


k
**
Fold Change (HIF-2α/β-Actin)
1.0
0.8
0.6
0.4
0.2
0.0
αKG 48H - +
Collagen + +


l
**
***
ns
Fold Change (HIF-2α/β-Actin)
0.8
0.6
0.4
0.2
0.0
αKG 48H - + - +
S1P (1 μM) - - + +


m
Fold Change (P-AKT (Ser 473)/AKT)
**
***
αKG (1 mM)
SARS-CoV-2 (0.01 MOI)
n
Fold Change (HIF-1α/β-Actin)
****
*
αKG (1 mM)
SARS-CoV-2 (0.01 MOI)
o
Fold Change (HIF-2α/β-Actin)
****
****
αKG (1 mM)
SARS-CoV-2 (0.01 MOI)
p
Fold Change (PHD2/β-Actin)
ns
ns
αKG (1 mM)
SARS-CoV-2 (0.01 MOI)
q
Fold Change (P-AKT1 (Thr 308)/AKT1)
ns
ns
****
**
ADP (μM)
5 Minutes
10 Minutes
r
Fold Change (HIF-1α/β-Actin)
**
**
*
ns
ADP (μM)
5 Minutes
10 Minutes


s
t
u
v
Fold Change (P-AKT1 (Thr 308)/β-Actin)
Fold Change (P-AKT (Ser 473)/β-Actin)
Fold Change (HIF-1α/β-Actin)
Fold Change (HIF-2α/β-Actin)
Collagen (5μg/ml)
DHB (10 μM)
**
*
***
****

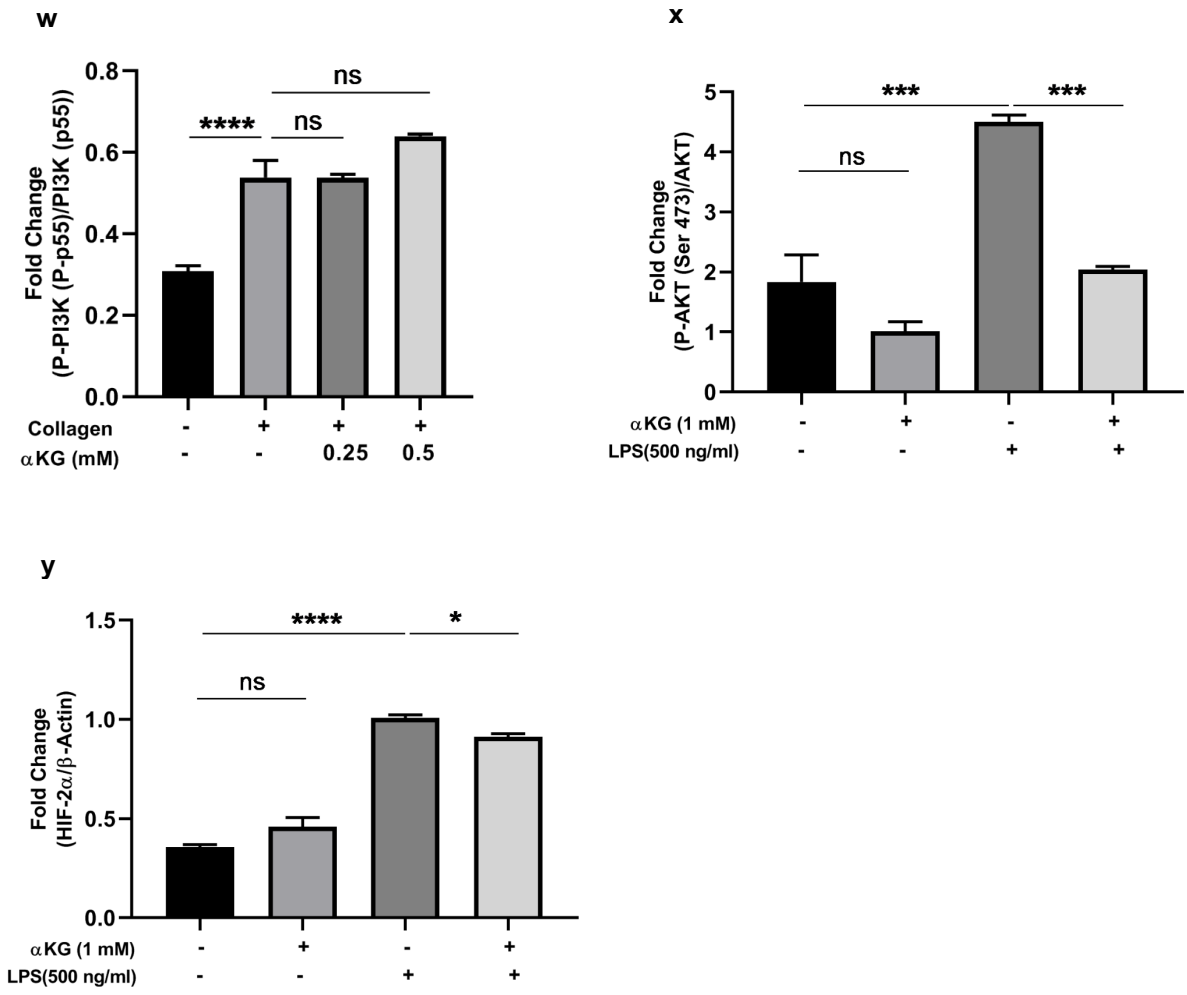


Fig. S14. Densitometry of western blots. Densitometric evaluation of the protein bands was performed using the Alpha imager software for western blots. Densitometry related to western depicted in main Fig. 1a-h (Fig. S14a-h), Fig. 2a (Fig. S14i-j), Fig. 4h (Fig. S14k), Fig. 4n (Fig. S14l), Fig. 6a (Fig. S14m-p). Densitometry related to western depicted in Fig. S1a (Fig. S14q-r), Fig. S1B (Fig. S14s-v), Fig. S1c (Fig. S14w), Fig. S5 (Fig. S14x-y). Bar represents mean ± SEM from triplicate blots. (Fig. S14 a, g, h and k, unpaired t-test, ns=non-significant) (One-way ANOVA, **P*<0.05, ***P*<0.01, ****P*<0.001, **** *P*<0.0001, ns=non-significant).

Supplementary Table S1: List of antibodies used in the study

Antibody	Manufacturer	Clone No.	Catalog No.
hu PAC-1 FITC	BD Biosciences	PAC-1	340507
hu P-Selectin PE Cy5	BD Biosciences	AK-4	551142
hu Annexin V FITC	BD Biosciences		556419
hu CD 41a PE	BD Biosciences	HIP8	555467
hu PHD1	Novus		NB100-310
hu PHD2	Novus		NB100-137
hu PHD3	Novus		NB100-139
hu PHD2	Cell Signalling Technology		3293S
hu PHD2	Cell Signalling Technology	D31E11	4835S
hu HIF1α	Cell Signalling Technology	D2U3T	14179S
hu HIF2α	Invitrogen	ep190b	MA1-16519
hu Phospho PI3K	Cell Signalling Technology		4228
hu PI3K	Cell Signalling Technology		4292
hu Phospho Akt1 (Thr 308)	Invitrogen	B18H12L21	701052
hu AKT1	Invitrogen	9Q7	AHO1112
hu Phospho Akt (Ser 473)	Cell Signalling Technology		9271
hu AKT	Cell Signalling Technology		9272
Anti-Hydroxyproline	Abcam		ab37067
hu α-tubulin	Thermo Fisher Scientific, USA	TU-01	13-8000
hu β-Actin	Cell Signalling Technology	1300000	4970
m CD42c	USBiological		363337
m CD45.2 APC	Biolegend	104	109814
m CD11b Percp 5.5	Invitrogen	M1/70	45-0112-82
m Ly6C V450	Invitrogen	HK1.4	48-5932-82
m Ly6G FITC	Biolegend	1A8	127606
m CD41a PE	Invitrogen	eBioMWReg30 (MWReg30)	12-0411-82
m CD11c APC Cy7	Biolegend	N418	117324
mF4/80 PE	Invitrogen	BM8	12-4801-82
m HIF1α	Cell Signalling Technology	D2U3T	14179S
m HIF2α	Invitrogen	ep190b	MA1-16519
m Phospho Akt1 (Thr 308)	Invitrogen	B18H12L21	701052
m AKT1	Invitrogen	9Q7	AHO1112
m Phospho Akt (Ser 473)	Cell Signalling Technology		9271
m AKT (PAN)	Cell Signalling Technology		9272
m α-tubulin	Thermo Fisher Scientific, USA	TU-01	13-8000
m β-Actin	Cell Signalling Technology	1300000	4970S
Secondary Goat Anti-Rabbit	Cell Signalling Technology		7074
Secondary Horse Anti-Mouse	Cell Signalling Technology		7076

Supplementary Table S2: List reagents used in the study

Reagent	Manufacturer	Catalog No.	Supplier
Octyl alpha ketoglutarate	Sigma Aldrich, USA	SML 2205	Sigma Aldrich, USA
ethyl-3-4-dihydroxybenzoic acid (DHB)	TCI America, Portland	D0571	Global Lab Solutions
Dimethyl 2-oxoglutarate	Sigma Aldrich, USA	349631	Sigma Aldrich, USA
Collagen	Bio/Data Corporation, USA	101562	Best Instuments
ADP	Bio/Data Corporation, USA	101312	Best Instuments
Sphingosine-1-Phosphate (S1P)	Sigma Aldrich, USA	S9666	Sigma Aldrich, USA
Lipopolysaccharide (LPS)	Sigma Aldrich, USA	L6386	Sigma Aldrich, USA
dietary α-ketoglutarate	SRL, Mumbai, India	28386	Linkbiotech
κ-carrageenan	Sigma Aldrich, USA	22048	Sigma Aldrich, USA
Trizol	Thermo Fisher Scientific	15596026	Nexgene lifescience
Superscript-III reverse-transcriptase	Invitrogen, USA	18080093	Nexgene lifescience
SYBR-green mix	Takara Bio, USA	RR390W	Clontech
protein G Sepharose beads	Sigma Aldrich, USA	17-0618-01	Sigma Aldrich, USA
protein A Sepharose beads	Sigma Aldrich, USA	17-5280-01	Sigma Aldrich, USA
RIPA lysis buffer	Sigma Aldrich, USA	R0278	Sigma Aldrich, USA
protease-phosphatase inhibitor	Thermo Fisher Scientific	78441	Nexgene lifescience
α-ketoglutarate estimation kit	Sigma Aldrich, USA	MAK054	Sigma Aldrich, USA
Succinate estimation kit	Sigma Aldrich, USA	MAK335	Sigma Aldrich, USA
Lactate estimation kit	Sigma Aldrich, USA	MAK064	Sigma Aldrich, USA
Fumarate estimation kit	Sigma Aldrich, USA	MAK060	Sigma Aldrich, USA
Pyruvate estimation kit	Sigma Aldrich, USA	MAK071	Sigma Aldrich, USA
Sphingosine-1-phosphate (S1P) estimation kit	Cloud clone corp.	CEG031Ge	Sunny corporation