Supplemental Material

Methods

Animals: All mouse experiments were approved by the NYU School of Medicine Institutional Animal Care Committee.

Materials: HDL_3 was purified from human plasma in an adaptation of a previously described ultracentrifugation method.[1] From HDL_3, apoA1 was isolated by delipidation. The apoA1 pellet was dissolved in 6 mol/L guanidine hydrochloride and dialyzed against Tris-buffered saline, pH 7.4.

Cell Culture: J774 or Raw 264.7 macrophages were cultured in 1g/L glucose (5.5 mM, "normal glucose") or 4.5 g/L (25 mM,"high glucose") containing DMEM supplemented with 15% FBS, 1% penicillin/streptozocin, 1% glutamine and 25 mmol/L HEPES buffer. Hypoxic incubations were carried out at 37°C in either a Plas-Labs chamber maintained at 1% oxygen (assessed by an Alpha Omega Oxygen Analyzer) or a Billups-Rothenberg modular incubator chamber flushed with 1% O_2, 5% CO_2 and 94% N_2. All assays on cells rendered hypoxic were performed either immediately upon removal from hypoxia or under hypoxic conditions in order to minimize re-oxygenation effects. For gene expression analysis, RNA was isolated using a PicoPure RNA Isolation Kit (Arcturus). RNA quality was verified with the Agilent2100 Bioanalyzer.

Primary bone marrow derived macrophage: (BMDM) were prepared from monocytes isolated from the tibia and femur of 6-8 week old apoE deficient mice as previously described.[2] Typically, cells were incubated for 7 days in DMEM, 10% FBS, and 10ng/mL Macrophage Colony-Stimulating Factor (M-CSF (PeproTech)) in normal D-glucose (100mg/dL).[3] Cells were then subjected to hypoxia as described above.

HIF-1α knockdown and overexpression studies: HIF-1α shRNA and scrambled lentiviruses were prepared as previously described [4], and after 3 serial infections J774 cells were selected with 2 ug/ml puromycin (Sigma). HIF-1α over-expression was achieved by infecting cells with a retrovirus expressing an epitope-tagged version of HIF 1α, (HA-HIF-1α P402A/P564A-pBabe-puro), which has been engineered to be stable in normoxic cells.[5-6]

Cellular protein, triglyceride and sterol determinations: Triglyceride content was determined by a standard colorimetric assay (Wako Chemical). Cholesterol levels were measured using an Amplex Red cholesterol kit. (Molecular Probes). Note that the cholesterol oxidase method used to assay cholesterol will also quantitatively measure demosterol.[7] All cellular protein quantifications were obtained using a standard colorimetric Lowry assay (Bio-Rad).

Immunostaining: HIF-1α staining was performed using a method modified from protocols previously described.[8-9] Cryosections were fixed in 4% paraformaldehyde and rinsed in TBS. Endogenous peroxidase activity was quenched with 0.3% H_20_2 in TBS for 5 minutes. Nonspecific sites were blocked with a commercial blocking buffer (Superblock, Pierce). Slides were incubated with a 1:50 dilution of polyclonal IgY anti-HIF-1α,[10-12] which recognizes the active form of HIF-1α, overnight at 4°C in 0.1% Tween 20, 10% FBS in TBS. After rinsing in TBS, slides were incubated with a peroxidase-conjugated rabbit anti-chicken IgY antibody (1:100, Pierce) in 10%FBS in TBS for 60 minutes at room temperature, then with a peroxidase conjugated goat anti-rabbit IgG (1:200, Dako) for sixty minutes. After TBS rinsing, antibody binding was localized using nickel-enhanced diaminobenzindine (Pierce). Sections were counterstained with hematoxylin and mounted.

For Glut-1 staining, cryosections were fixed in ice cold acetone and rinsed in PBS. Nonspecific sites were blocked with 4% goat serum in PBS for 90 minutes. Slides were incubated with a 1:200 dilution of polyclonal rabbit anti-mouse antibody (Alpha Diagnostics) in 4% goat serum, rinsed in PBS, and then incubated for 105 min with a 1:200 dilution of biotinylated goat anti-rabbit antibody in 4% goat serum. The slides were rinsed and incubated with alkaline phosphatase (Vectastain ABC Kit, Vector Labs) for 30 minutes. After rinsing in Tris buffer (pH 8.4), antibody binding was detected by Vector Red (Vector Labs) staining and counterstaining with hematoxylin.

VEGF staining was performed in a similar fashion, using a 1:400 dilution of polyclonal goat anti-mouse antibody (Santa Cruz Biotechnology) and 1:200 dilution of biotinylated horse anti-goat antibody. ABCA1 or CD31 staining was also performed in a similar fashion, using a 1:100 dilution of rabbit anti-mouse ABCA1 or CD31 antibody (Novus Biologicals), respectively, and a 1:200 biotinylated goat anti-rabbit antibody (Vector Laboratories). CD68 was performed using a 1:100 dilution of rat anti-CD68 antibody (Serotec) and a 1:200 dilution of biotinylated rabbit anti-rat antibody (Vector Labs).

For double immunofluorescence experiments, frozen aortic section from apoE-/- mice were incubated with the anti-HIF-1α antibody described above at 1:100 and with anti-CD68 antibody at 1:250. Following incubation and washes, the sections were incubated with secondary antibodies (1:200) anti-chicken alexa 488 (Sigma) and anti-rat biotinylated antibody, followed by streptavidin conjugated Texas Red (Sigma) 1:300. The cells were mounted in permount containing DAPI (Vector laboratories). As a control, primary antibody to HIF-1α, but not secondary antibody, was omitted; these controls demonstrated no detectable HIF-1α staining.

Cholesterol Loading: J774 or BMDM macrophages were plated onto 6 well plates under normoxic conditions at 60-80% confluence. After 24 h, the (1g/L glucose) media was changed to 0.2% BSA serum free medium with 15ug/ml cholesterol cyclodextrin complex and the cells were transferred to normoxic or hypoxic incubations for 24 additional hours. Lipids were extracted immediately on removal from culture, dried under nitrogen, resuspended in isopropanol and quantified.

Sterol Synthesis Assay: J774 cells were plated at 60-80% confluence on chamber slides and maintained under normoxia for 24 h. Then, the medium was changed to serum free (0.2% BSA) medium with 1μCi/ml [H^3]-acetate, and either rendered hypoxic or maintained as normoxic. The cells were then incubated for an additional 24 h and washed with PBS. The lipids were extracted with isopropanol, dried under nitrogen and resuspended in 1:1 methanol chloroform. The lipid suspension was spotted and separated by TLC using a petroleum ether/diethyl ether/acetic acid (90:5:4) solvent. After exposing the TLC plate to iodine, spots containing demosterol, the cholesterol-surrogate in J774 cells [13], were cut out and counted. Results are expressed as cpm H^3 incorporated into demosterol/mg cellular protein.

Cholesterol Efflux Assay: J774, RAW264 or BMDM cells were plated at 60-80% confluence onto 12 well plates under normoxic conditions. After 24 h, the (1g/L glucose) media was changed to 0.2% BSA with 15μg /ml cholesterol-cyclodextrin complex [1,2-^{3}H(N)]-cholesterol (0.5μCi/ml, Perkin-Elmer) and the cells were transferred to normoxic or hypoxic incubations for 16 h. After 16 h, the cells were washed with PBS x 2 and the media was changed to 0.2% BSA with 1μm of the ACAT inhibitor F1394 (to prevent re-esterification of cholesterol). The cells were allowed to equilibrate for 3 h, washed with PBS x2 and the medium was changed to either 0.2% BSA with 1μm F1394 alone, or with 50μg/ml HDL_3 or 50μg /ml apoAI. After 4 h, aliquots of media and cellular lipids were collected and cpm content measured by scintillation counting. Percent efflux was calculated as cpm in media/(cpm in media+cpm in cellular lipid).

Immunoprecipitation and Western blots: J774 or BMDM cells grown under normoxic or hypoxic conditions for 24 h were washed with PBS and lysed as previously described.[14] For determination of cellular ABCA1 content, 600 ug of cellular lysate protein was diluted in buffer containing 50 mmol/L Tris-HCl, pH 7.5, 150 mmol/L NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, and incubated with rabbit anti-ABCA1 (Novus Biologicals) at 4°C for 2 h followed by incubation with protein A-Sepharose (GE Healthcare) at 4°C for 2 h. The beads were washed three times with the same buffer, and immunoprecipitated material was released by heating at 100 °C in SDS-PAGE sample buffer. The resuspended immunoprecipitate was then resolved by 8% SDS-PAGE and transferred to a polyvinylidene difluoride membrane (PerkinElmer Life Sciences). The primary (monoclonal anti-mouse ABCA1, Novus Biologicals) and secondary antibodies (peroxidase-conjugated anti-mouse IgG, Calbiochem) were used at 1:1000 and 1:10,000 dilutions, respectively, with detection of the final signal by chemiluminescence (EC, Pierce). HMGCoA was detected using previously described methods.[15] The relative intensities of the bands were determined by densitometry (BioRad GS-800).

Cellular ABCA-1 staining: J774 or BMDM cells were plated at 60-80% confluence on chamber slides

and maintained in normoxic for 24 h. After 24 h, the media were changed and cells were rendered hypoxic or maintained as normoxic for an additional 24 h. After 24 h, the cells were washed with PBS x 2 and fixed in 3.3% paraformaldehyde for 30 minutes at room temperature. The cells were washed x 2 with PBS. Nonspecific sites were blocked by incubation in 2% horse serum in PBS for 45 minutes. The cells were incubated with a 1:500 dilution of polyclonal rabbit anti-ABCA1 (Novus Biologicals) at room temperature for 1 h, rinsed in PBS x2, and incubated with a 1:200 dilution of donkey anti-rabbit antibody coupled with Cy2 (Jackson) for 30 minutes at room temperature. Cells were rinsed with PBS, then mounted with a cover slide in Fluoromount (Southern Biotech). Cy2 fluorescence was detected using a Zeiss 510 Laser Scanning confocal microscope with an Argon laser (488 nm) and 505–530 nm bandpass emission filter.

Cell toxicity assays: BMDM were platted on 6 well plates and subject to 48h of 1% hypoxia or normoxia. After 48h, the media was collect and lactate dehydrogenase (LDH) leakage was measured following manufacture's protocol (Sigma). Another set of BMDM cells were tested for viability using 7-Amino-actinomycin D (Calbiochem) following manufactures protocol and were subjected to the C6 Flow Cytometer (Accuri).

Laser capture microdissection (LCM) of aortic plaque cells: To isolate cells from aortic plaques of apoE-deficient mice, LCM was performed with the PixCell IIe (Arcturus Bioscience, Mountain View, CA) as we have reported [16-17] with some modifications. Briefly, 6-µm frozen sections were dehydrated in ethanol and xylene and air dried. At 100-µm intervals, sections were immunostained for VEGF and GLUT1 to identify hypoxic areas. These were used as templates for the next five serial sections, and cells within and outside of the hypoxic area were selected.

RNA was isolated using the PicoPure RNA Isolation Kit (Arcturus, Mountain View, CA) and treated with DNase. The concentration of RNA was determined by the Ribogreen RNA Quantitation kit (Molecular Probes), and the RNA quality verified with the Agilent 2100 Bioanalyzer. RNA was not pooled; Expression of the genes noted in Results was analyzed by RT-PCR separately for each of the three mice used in this study. Approximately one third of the laser-captured cells were macrophages, based on co-localization studies with antibodies to VEGF and CD68. It was not possible to obtain a pure macrophage population from the hypoxic region in sufficient quantity for gene expression analysis.

Quantitative RT-PCR Analysis of mRNA Abundance: mRNA abundances were determined by qRT-PCR using 100 pg of total RNA and the i-Script One-Step RT-PCR kit (BioRad) according to the manufacturer's protocol. Primer sequences are given in Supplemental Table 1.

Statistical Analysis: Graph Pad software was used to perform unpaired two-tailed t-tests or ANOVA followed by Bonferroni's Multiple Comparison Test as appropriate. Results are displayed as mean+/-SEM and were considered statistically significant when * $p< 0.05$, ** $p<0.01$ and *** $p<0.001$.

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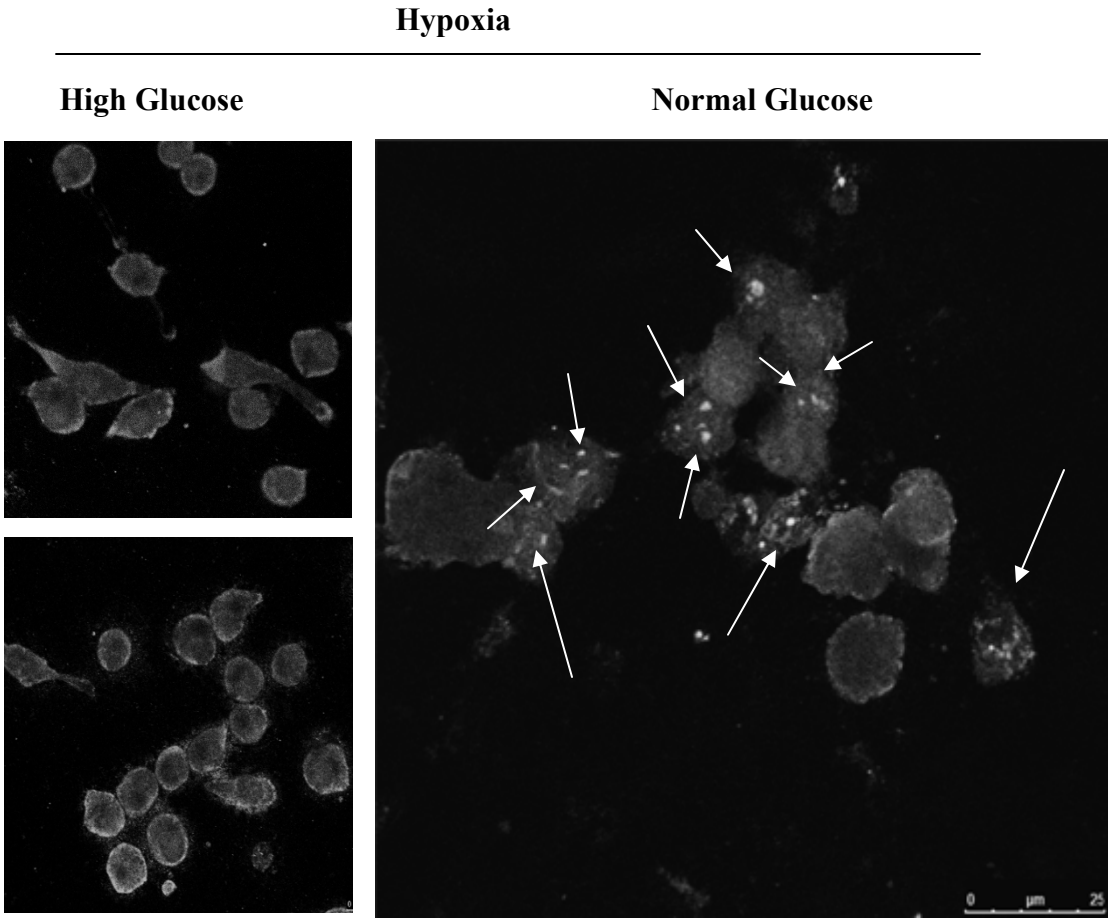
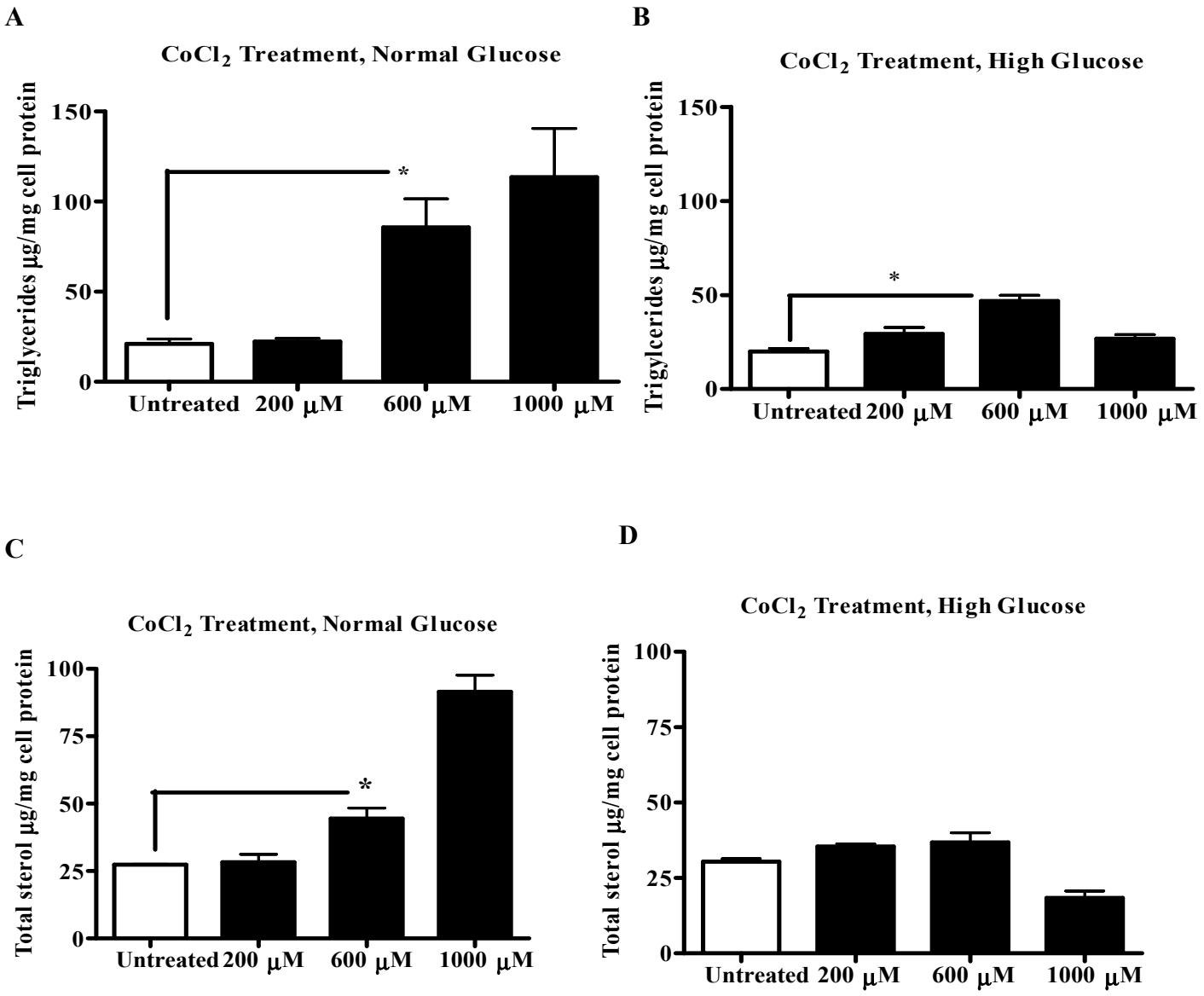
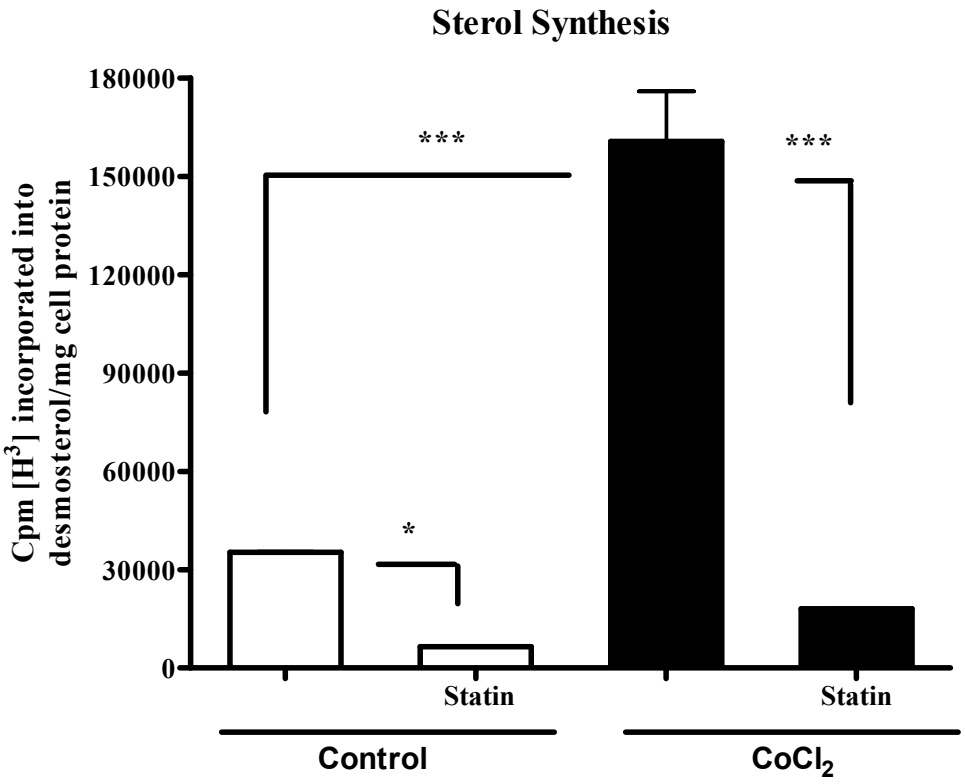


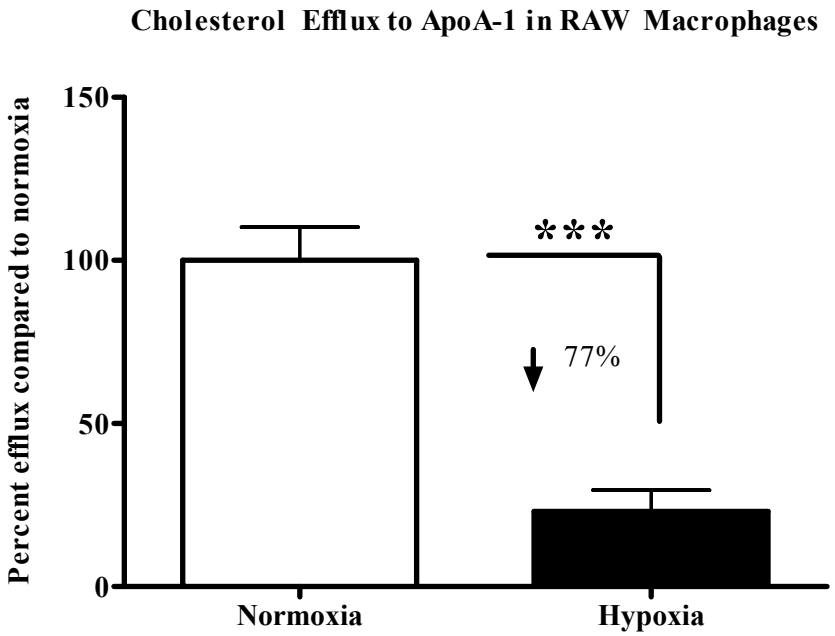
Online Figure I: Hyperglycemia blunts HIF-1α expression. J774 cells were plated on chamber slides with normal (5.5 mM) or high (25 mM) glucose. These cells were then incubated under hypoxic conditions for 24h and immunostained for HIF-1α. The images were taken using confocal microscopy through a 63X oil emersion lens. Arrows indicate high levels of HIF-1α staining.



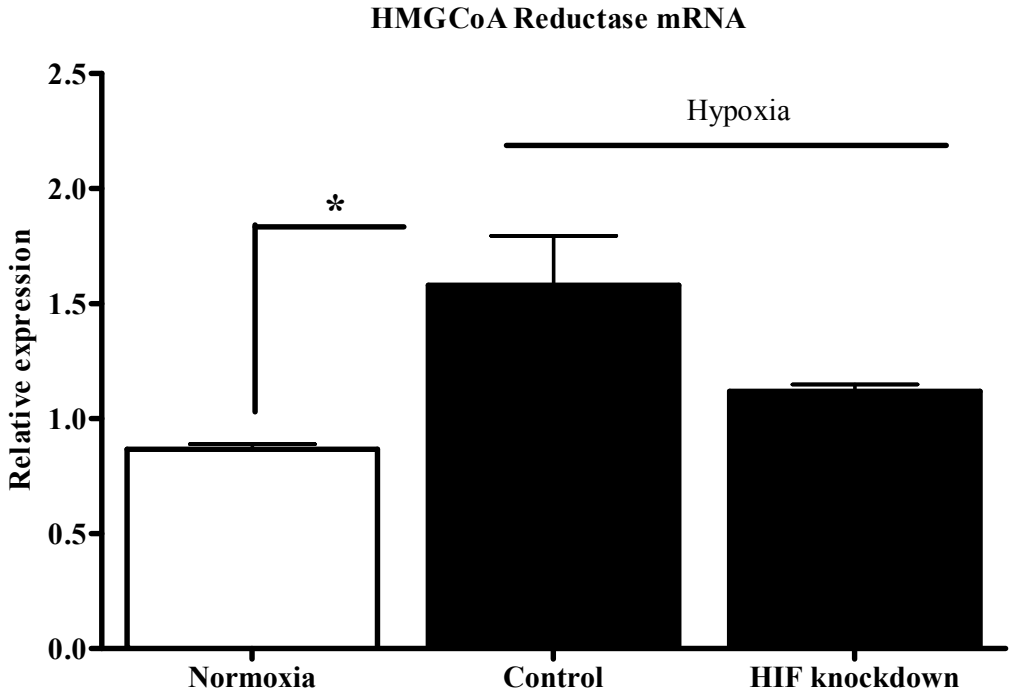
Online Figure II: Effect of $CoCl_2$ and hyperglycemia on macrophage lipid accumulation. **A,** In normal glucose conditions (5.5 mM), J774 macrophages incubated with increasing concentrations of the hypoxia mimic $CoCl_2$ showed significantly increased triglyceride content in untreated-vs-600 μ M treated cells. **B**, This effect was blunted by hyperglycemia (25 mM), however, at 600 μ M of $CoCl_2$ it was still statistically significant vs untreated. **C**, Similarly, increases in total cholesterol/desmosterol ("Total sterol") were observed under hypoxic, normal glucose conditions. **D**, In contrast, under hyperglycemia, no significant increase in total sterol content occurred with $CoCl_2$ treatment. These experiments were performed 3 times in triplicate. *$p<0.05$



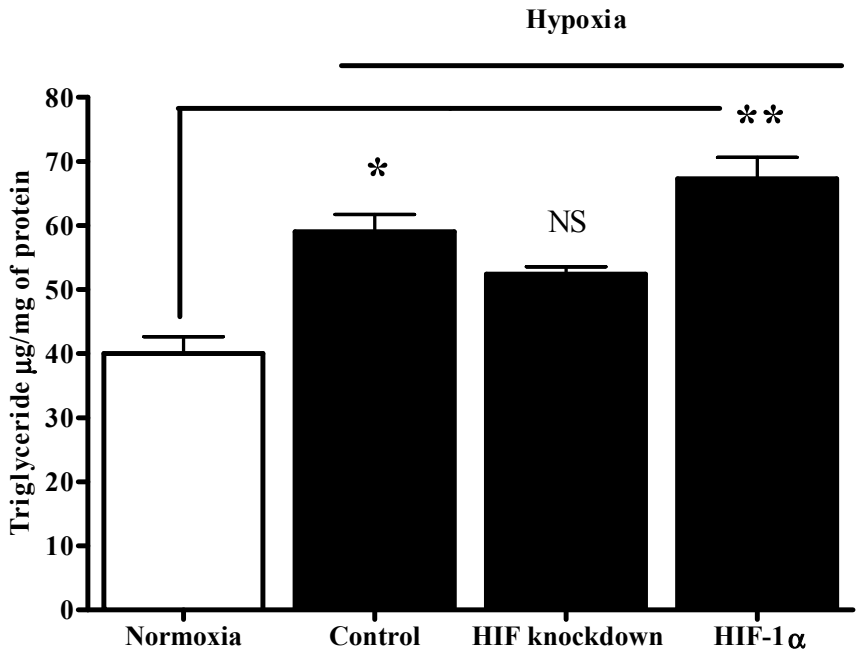
Online Figure III: Effects of $CoCl_2$ and the statin mevinolin on sterol synthesis. J774 macrophages were treated with or without 600 μ M $CoCl_2$ in serum-free conditions for 24h. **A**, Sterol (desmosterol) synthesis was measured at the end of 24h**.** Statins (5 μ M mevolin) were used to suppress sterol synthesis and showed 80% suppression of HMGCoA in cells treated with or without $CoCl_2$. These experiments were done twice in duplicate.



Online Figure IV: Cholesterol efflux in Raw264 macrophages. To confirm that the reduction in cholesterol efflux by hypoxia was not restricted to J774 cells, cells of another murine macrophage line, Raw264, were incubated for 24 h under normoxic and hypoxic conditions, and efflux of radiolabeled free cholesterol to lipid-poor apoAI (an ABCA-1 acceptor). As with J774 cells (Fig 4a.), cholesterol efflux was diminished by hypoxia, with ABCA-1 mediated efflux significantly decreased. These are the results of two experiments done in quadruplicates, *** $P<0.001$.



Online Figure V: HIF-1α regulates HMGCoA reductase expression. J774 cells exhibited increased HMGCoA reductase gene expression under hypoxic conditions for 24 h as before (Fig 3b). When cells stably expressing HIF-1α shRNA or scrambled shRNA ("Control") were subjected to hypoxic conditions, there was obvious inhibition of this increase. These experiments were performed twice in duplicate. * $P<0.05$



Online Figure IV: HIF-1α is a positive regulator of triglyceride content in macrophages . J774 cells were stably transfected either with control plasmid (pBabe-puro), scrambled shRNA ("Control") , HIF-1α shRNA ("HIF Knockdown") or a HIF-1α expression plasmid ("HIF-1α"). Cells are incubated in hypoxic conditions for 24 h and triglyceride levels were measured and compared to cells incubated in normoxia (control cells in normoxic conditions). These experiments were performed twice in duplicate; * $p<0.05$, ***$p<0.001$.

Online Table 1

Genes	Forward 5'-3´	Reverse5'-3´	Probe5'-3´
Cyclophilin A	GGCCGATGACGAGCCC	TGTCTTTGGAACTTTGTCTGCAA	TGGGCCGCGTCTCCTTCGA
VEGF	AGAAGTCCCATGAAGTGATCAAG	TGCACCCACGACAGAAGGA	GGCAGTAGCTTCGCTGGTAGAC
Glut-1	CAACTGTGCGGCCCCTACGTCTTC	GGGCATGTGCTTCCAGTATGT	ACGAGGAGCACCGTGAAGAT
CHOP	CTGGTCCACGTGCAGTCATGG	CCACCACACCTGAAAGCAGAA	AGGTGAAAGGCAGGGACTCA
ABCA-1	ATTGCCAGACGGAGCCG	TGCCAAAGGGTGGCACA	CCAGCTGTCTTTGTTTGCATTGCCC
ABCG-1	AAGGCCTACTACCTGGCAAAGA	GCAGTAGGCCACAGGGAACA	CATGGCCGATGTCCCCTTTCAGATC
SR-B1	GATGATGACCTTGGCGCTG	TCACCAACTGTGCGGTTCATA	TCACCATGGGCCAGCGTGCTT
HMGCoA Red	GGGAGCATAGGCGGCT	TGCGATGTAGATAGCAGTGACA	CAACGCCCACGCAGCAACA