Online Data Supplement:

LEUKOTRIENE B4 RECEPTOR-1 MEDIATES INTERMITTENT HYPOXIA-INDUCED ATHEROGENESIS

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Methods:

Cell Culture and IH Exposures. THP-1 cells were originally obtained from American Tissue Type Cell Collection (ATCC) and were cultured in Gibco RPMI 1640 medium (Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum (Hyclone), 50 U/ ml penicillin and 50 μg /ml streptomycin (Invitrogen) at 37 °C in 5% CO_2. THP-1Cells were passaged when they reached a density of $1*10^6$ cells/ml. THP-1 cells were used at passages <15 after reception from the ATCC. THP-1 monocytes were differentiated into macrophages with 100 nM phorbol 12-myristate 13-acetate (PMA) for 24 hrs. IH exposures were conducted using a custom-designed computer controlled incubator chamber attached to an external O_2/CO_2 computer-driven servocontroller (Biospherix, Lacona, NY). This system is able to continuously record the dissolved O_2 concentrations in the culture medium and thus allows for implementation of the desired oxygen concentration profile. The selected IH profile consisted of maintaining cells at 37°C in an hypoxic chamber in which gas concentrations were alternated between 0.1% O_2 and 30% O_2 every 10 min, in 5% CO_2, balanced with N_2. The dissolved O_2 concentration in culture medium was measured by a laser O_2 probe (Biospherix, Lacona, NY), which was alternated between 5% O_2 and 20% O_2 under the IH profile (see Figure E1). Control conditions corresponded to 21% O2 and 5% CO2 (room air; RA). Cells were exposed IH or RA for 3, 6, 24 and 48h.

Animals and IH Exposures: Animal care and experimental procedures were performed with approval from animal care committees of the University of Chicago and the University of Louisville. $ApoE^{-/-}$, $BLT1^{-/-}ApoE^{-/-}$ mice were fed with a high-cholesterol atherogenic western diet (TD88137, Harlan Laboratories, Indianapolis, IN). Animals

were housed in 4 identical commercially designed chambers (30"x20"x20") that can accommodate 24 mice each, and were operated under 12 hour light-dark cycle (Oxycycler model A44XO, Biospherix, Lacona, NY). Gas was circulated around each of the chambers, attached tubing and other units at 60 L.min-1. The O_2 concentration was continuously measured by an O_2 analyzer, and controlled by a computerized system regulating the gas valve outlets, such that the moment-to-moment desired oxygen concentration of the chamber was programmed, and adjusted automatically. This system permits delineation of custom oxygenation profiles that can mimic those of patients with OSA during sleep. Deviations from the desired concentration were operated by addition of N_2 or room air through solenoid valves. For the remaining 12 hours of nighttime (7 pm-7 am), oxygen concentrations were kept at 21%. The selected oxygen concentration profile for the 12 daylight hours has been extensively used in previous experiments in our laboratory (E1-E3), and consisted of 90 sec of 5.7% O_2 alternating with 90 sec room air for 12 hrs during the light phase. Ambient CO_2 in the chamber was periodically monitored and maintained at 0.03% by circulating the gas through soda lime. Ambient temperature was kept at 22-24^{o}C. Following a 10-week IH exposure, mice were anesthetized. The heart and aorta were dissected. Ascending aortas were embedded in OCT and cryosections were prepared for evaluating atherosclerotic lesions by Oil Red O staining.

Assessment of Atherosclerotic Lesions: Perfusion fixation, preparation of aortas and quantification of atherosclerotic lesions were performed as previously described. En face pinned-out aortas were stained with Sudan IV. The ascending aortas were embedded in OCT and snap-frozen in liquid nitrogen. Lesions in the ascending aorta from serial 7-μm-

thick cryosections were stained with Oil Red O and counterstained with haematoxylin. The images of the aortas were captured with a digital color camera mounted on a microscope (Nikon E300, Nikon USA, Melville, NY). Quantitative analysis of lipid-stained lesions was performed using SigmaImage software.

Intracellular Lipid Analysis In Macrophages: Differentiated macrophages were incubated for 24 hrs in RPMI 1640 supplemented with 5% LPDS and 50 μg ml-1 of acLDL (Biomedical Technologies, Stoughton, MA), followed by RPMI 1640 supplementation with 10% FBS. After 3 days, the macrophage foam cells were fixed in 10% formalin for 90 min, washed thoroughly with PBS, and incubated with a working solution of Oil Red O for 3 hrs. Intracellular lipids in macrophages were extracted with hexane/isopropanol (3:2), evaporated and dissolved in isopropanol containing 10% Triton X-100 for preparation of a sample solution. Free cholesterol and total cholesterol were determined by commercial assay systems (Amplex Red Cholesterol Assay kit, Invitrogen, Carlsbad, CA). Cholesterol ester was estimated by subtracting free cholesterol from total cholesterol. Data were normalized to cellular protein content.

Electrophoretic Mobility Shift Assay (EMSA): EMSA was performed using an EMSA kit from (AY1000, Panomics, Santa Clara, CA). In brief, cells were collected after treatment and frozen at -80°C. Nuclear extracts were prepared the using nuclear extraction kit (AY2002, Panomics, Santa Clara, CA) following the manufacturer's protocol. The nuclear extracts were then incubated with a biotin-tagged probe of NF-κB and HIF1-α for 30 min at 15°C, according to the manufacturer's protocol. The extracts were further electrophoresed using a 6% polyacrylamide gel at 4°C and transferred to S&S NYTRAN nylon membrane. The blots were developed using the detection kit

provided in the EMSA kit and visualized with an enhanced chemiluminescence detection system (Amersham Bioscience, Piscataway, NJ).

Quantitative Real-Time PCR. Total RNA was prepared from THP-1 and macrophages using the RNAesy kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. RNAs were quantified spectrophotometrically. 1µg total RNA from each experimental condition was reverse transcribed using oligo(dT)20 primers and Superscript II-Reverse Transcriptase (Invitrogen, Carlsbad California) following the manufacturer's protocol. cDNA equivalent to 20 ng of total RNA were subjected to subsequent real-time PCR (ABI 7500, Applied Biosystems, Foster City, CA) analysis following a standard protocol. Taqman probes and primers (5-LO, LTA4H, MCP-1, CCR2, CD14, CD68, CD36 SRA and ABCA1and ApoA1) were purchased from Applied Biosystems (Foster City, CA). Real-time PCR results were analyzed using ABI 7500 software (Applied Biosystems, Foster City, CA). Of note, the mRNA expression of 5-LO, LTA4H, BLT1 and BLT2 may not completely reflect their actual levels of protein expression due to potential post-translational modifications, such that protein expression studies will be required in future studies.

Assessment of TLB4 production in culture medium and plasma. Culture medium was collected after IH exposure. Plasma was obtained from blood drawn in an EDTA-pretreated tube after centrifugation. LTB4 was measured by ELISA kit (Cayman, Ann Arbor, Michigan) and its concentrations were determined according to the manufacturer's directions. The detection limits of the LTB4 assay is 13 pg/mL.

Reference List

E1. Kheirandish L, Row BW, Li RC, Brittian KR, Gozal D. Apolipoprotein E-deficient mice exhibit increased vulnerability to intermittent hypoxia-induced spatial learning deficits. Sleep 2005; 28:1412-1417

E2. Row BW, Kheirandish L, Li RC, Guo SZ, Brittian KR, Hardy M, Bazan NG, Gozal D. Platelet-activating factor receptor-deficient mice are protected from experimental sleep apnea-induced learning deficits. J.Neurochem. 2004; 89:189-196

E3. Xu W, Chi L, Row BW, Xu R, Ke Y, Xu B, Luo C, Kheirandish L, Gozal D, Liu R. Increased oxidative stress is associated with chronic intermittent hypoxia-mediated brain cortical neuronal cell apoptosis in a mouse model of sleep apnea. Neuroscience 2004; 126:313-323

Figure E1.

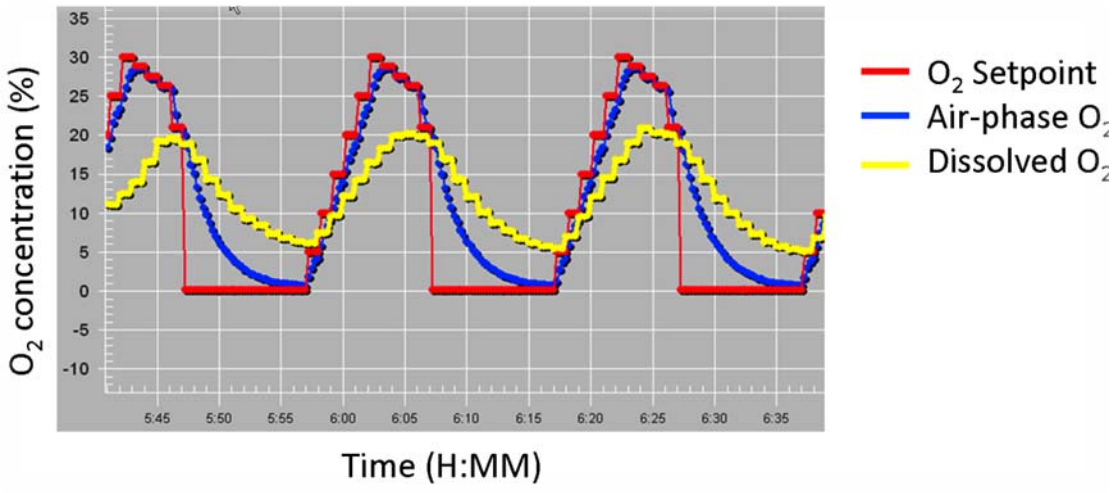

Figure E1. Hypoxia profile for IH cell model. As shown in the figure, the red line represents the theoretic setpoints of air phase O2 in hypoxia chamber. The blue line indicates the actual air-phase O2 concentration in the hypoxic chamber. The yellow line represents the dissolved O2 concentration in the cell culture medium.