Supporting Information to:

**Aescin Protection of Human Vascular Endothelial Cells Exposed to Cobalt Chloride Mimicked Hypoxia and Inflammatory Stimuli**

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**Reagents**

Pharmaceutical grade aescin (aescin 1a + aescin 1b) purified to 95 – 97%, was obtained from Madaus srl (Padova, Italy). Cobalt chloride, [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (MTT), isopronolol, sodium azide, Triton X-100, Mowiol, LPS were purchased from Sigma-Aldrich (St. Louis, MO, USA). Type 2 collagenase was obtained from Worthington (Lakewood, NJ, USA). EGM-bulletkit® medium, endothelium basal medium (EBM), foetal bovine serum (FBS), Pen-Strep (100 U/mL penicillin – 100 µg/mL streptomycin), 0.05% trypsin – 0.02% EDTA, PBS were supplied by Cambrex Bio Science (East Rutherford, NJ, USA); PE-conjugated anti-human CD31, PE-conjugated anti-human CD104 and isotypically matched PE-IgG1 murine antibody from BD Biosciences (Erembodegem, Belgium). Mouse anti-human CD31 was from Santa Cruz Biotechnology (St. Cruz, CA, USA); Alexa 633-conjugated phalloidin and anti-mouse Alexa488-conjugated from Molecular Probes (Eugene, OR, USA).

**Isolation and Culture of HUVEC**

Endothelial cells were freshly isolated from human umbilical cord veins (HUVEC) by digestion with 1% type 2 collagenase at 37 °C, according to Jaffe et al. (Jaffe EA, Hoger LW, Nachman RL, Becker CG, Minich CR. Culture of human endothelial cells derived from umbilical veins. Identification by morphologic and immunologic criteria. J Clin Invest 1973; 52: 2745-56), with minor modifications. Cells were initially cultured in EGM-bulletkit® medium, supplemented with 10% of heat inactivated FBS and 1% solution of Pen-Strep, maintained at 37 °C, 5% CO₂ and 100% humidity. At confluence, cells were harvested by treatment with 0.05% trypsin – 0.02% EDTA. For experiments, cells at 2 – 5 passages were seeded in multi-well plates or culture coverslips and incubated in EBM, 1% FBS and 1% Pen-Strep. 24 hours after seeding, the cells were incubated with CoCl₂ for 4 h or with aescin or with LPS for 24 hours. When HUVEC were treated with both aescin and cobalt chloride or LPS, cells were pre-incubated with aescin for 24 h, then the medium was replaced with fresh medium containing cobalt chloride or LPS and incubated for 4 or 24 hours further, respectively.
Cell Viability Assay

Cells were plated in 96 wells/plates. At the end of experimental protocol, 20 µL of a solution of MTT (5 mg/mL PBS) was added to each well and the plates were incubated for 4 h at 37 °C. The supernatant was discarded, 200 µL of isopropanol were added to dissolve the formazan crystals and the optical density was measured at 570 nm by an automated procedure (Victor², multilabel counter; Perkin-Elmer Wallac Instruments; Wellesley, MA, USA).

Flow Cytometry

HUVECs were grown on 24 well/plates and at the end of experimental protocol the cells were harvested using 0.05% trypsin – 0.02% EDTA, reacted for 30 min at 4 °C with PE-conjugated anti-human CD31 or with PE-conjugated anti-human CD104, diluted in PBS-2% BSA, then suspended in PBS, 2% FCS, 0.02% sodium azide. As an antibody control, the cells were reacted with the appropriate isotypically matched PE-IgG1 murine antibody. Cells were washed, suspended in cold PBS and fluorescence was measured on a FACScan instrument (Coulter Epics XL; Beckman; Fullerton, CA, USA).

Immunocytochemistry and Confocal Microscopy

HUVECs were grown on coverslips and, at the end of experimental protocols, fixed with 4% paraformaldehyde for 10 min, rinsed (PBS × 3) and permeabilized (0.1% Triton X-100). Cells were probed with mouse anti-human CD31, diluted in (PBS, 1% FCS) for 1 hour at 37 °C, rinsed (PBS × 3) and probed for 1 h with Alexa488-conjugated to detect CD31. To detect actin, the cells were probed with Alexa633-conjugated phalloidin. After washing (PBS × 3) the coverslips were mounted in Mowiol (Aldrich) and confocal images were obtained by laser scanning microscope (Nikon EZ C1; Nikon; Melville, NY, USA).

IL-6 Assay

HUVECs were incubated in 96 well-plates. At the end of the experimental protocol, the incubation medium was collected and IL-6 was assessed by kit enzyme-linked immunosorbent assay (Elisa Bender MedSystem; Vienna, Austria) and optical density
measured at 450 nm by an automated procedure (Victor², multilabel counter, Wallac Instruments).

**Statistical Analysis**

Results within the different experimental groups were analysed by using one-way ANOVA followed by Bonferroni’s ‘post hoc’ test for multiple comparisons and by Student’s t test for unpaired data, utilising GraphPad PRISM™ statistics software package. Values of p < 0.05 were considered statistically significant.