Effects of Chlorogenic Acid on Intracellular Calcium Regulation in Lysophosphatidylcholine-Treated Endothelial Cells

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Running Title: Role of chlorogenic acid in LPC-treated HUVECs

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SUPPLEMENTARY MATERIALS AND METHODS

Chemicals and reagents

Endothelial cell basal medium (EBM-2) for endothelial cell growth was purchased from Lonza (Walkersville, MD, USA). Trypsin EDTA was purchased from Gibco-Invitrogen (Grand Island, NY, USA). Intracellular Ca^{2+} was determined using the fura-2 acetoxymethyl (Fura-2/AM) ester from Molecular Probes (Eugene, OR, USA). Cell viability was determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) from Sigma (St. Louis, MO, USA). 1-palmitoyl-sn-glycero-3-phosphocholine, chlorogenic acid, gadolinium (Gd^{3+}), lanthanum (La^{3+}), and all other reagents were also purchased from Sigma.

Cell culture

HUVECs were cultivated in EBM-2 media supplemented with 2 % fetal bovine serum, 0.4 % recombinant human fibroblast growth factor-B, 0.1 % recombinant human epidermal growth factor, 0.1 % recombinant human vascular endothelial growth factor, 0.1 % ascorbic acid, 0.1 % recombinant long R insulin-like growth factor-1, 0.1 % heparin, 0.1 % gentamicin sulfate amphotericin-B, and 0.04% hydrocortisone at 37 °C and 5 % CO_2.

Assessment of Cell Survival

The MTT calorimetric assay determines the ability of viable cells to convert a soluble tetrazolium salt (MTT) into an insoluble formazan precipitate. MTT, a water-soluble yellow dye that is readily taken up by viable cells, is reduced by the action of mitochondrial dehydrogenases. The reduction product is blue, and the water-insoluble formazan crystal that can be dissolved in an organic solvent for spectrophotometric determination of its concentration. Cells were seeded at a density of 3×10^4 cells/well in a 96-well culture plate and treated with or without protective material prior to exposure to LPC (30 μg/ml). MTT was then added to each well and incubated for

an additional 3 h at 37 °C. Subsequently, 100 μl of dimethyl sulfoxide was added to dissolve the reduced MTT and the absorbance at 570 nm was read on a microplate reader (Molecular Devices, Sunnyvale, CA, USA). Data are expressed as a percentage of the control, which was considered 100 % viable.

Reverse Transcription (RT)-PCR Analysis

Total RNA was isolated from cultured cells using the Trizol reagent (Invitrogen, Carlsbad, CA, USA). Total RNA was reverse-transcribed into cDNA using a reverse transcription kit (Promega, Madison, WI, USA) and oligo dT, which was then used for semi-quantitative RT-PCR. The generated cDNA was amplified by Emerald Amp PCR (Takara, Shiga, Japan) using the following primers: TRPC1 forward 5'-GATTTTGGAAAATTTCTTGGGATGT-3', reverse 5'-TTTGTCTTCATGATTTGCTATCA-3' (369 bp); TRPC3 forward 5'-GAC ATA TTC AAG TTC ATG GTC CTC-3', reverse 5'-ACA TCA CTG TCA TCC TCA ATT TC-3'(223 bp); TRPC7 forward 5'-CAG AAG ATC GAG GAC ATC AGC-3', reverse 5'-GTG CCG GGC ATT CAC GTG GTA-3'(304 bp); glyceraldehyde-3-phosphate dehydrogenase (GAPDH) forward 5'-CGTCTTCACCACCATGCAGA-3', and reverse 5'-CGCCCATCACGCCACAGTTT-3' (300 bp). PCR products were electrophoresed on a 1.2 % agarose gel and images were captured with a photo-documentation imaging system.

Detection of Intracellular ROS

To measure ROS, HUVECs were grown in 8-well plates at 4×10^4 cells/well and incubated for 30 min in the dark with 10 μmol/L 2',7'-dichlorofluorescein diacetate (Invitrogen). After a gentle rinse with PBS, the cells were fixed in 3.7% formaldehyde for 10 min. The cells were then washed with PBS and mounted with slowfade gold antifade reagent (Invitrogen). The ROS signal was detected using a confocal microscope (Carl Zeiss, Dublin, CA, USA), set to 485 nm excitation

and 535 nm emission.

Measurement of intracellular calcium concentration

Fluorescence measurements of $[Ca^{2+}]_i$ were performed as previously described (36). Briefly, coverslips containing HUVECs were loaded with the acetoxymethyl ester of fura-2/AM. Fura-2/AM (3 μM) in 1.8 mM Ca^{2+}-containing physiological saline solution (PSS; 1.8 mM $CaCl_2$, 5 mM KCl, 10 mM HEPES [pH 7.4], 1.2 mM $MgCl_2$, 126 mM NaCl, 0.2 % BSA, and 10 mM glucose) was added to the HUVECs at room temperature for 30 min, and then washed three times with dye-free saline for 30 min. Fluorescence was monitored using a dual-wavelength system (Intracellular Imaging, Cincinnati, OH, USA). The cells were excited at 340 and 380 nm, and the emission intensity at 510 nm was recorded. The data were expressed as the ratio of 510 nm emissions of fura-2 fluorescence obtained from excitation at 340 and 380 nm, respectively. For the Ca^{2+}-free solution, Ca^{2+} was removed from the solution and substituted with 2 mM EGTA.

Immunoblotting.

Total proteins were prepared from HUVECs and analyzed following a standard protocol. Anti-TRPC1 (ab51255; Abcam, Cambridge, MA) and anti-GAPDH (2118s; Cell Signaling Technology, Danvers, MA) were used as specific antibody. The bands were developed using ECL immunoblotting detection kit (Amersham Bioscience, Piscataway, NJ) and detected using an imager (Fusion FX; Vilber, Lourmat, Australia).

Statistical analyses

All data are expressed as the mean ± S.E.M. Values were compared using Predictive Analytics Software Statistics 18 (PASW Statistics 18, IBM Corporation, NY, USA), followed by Student's t-test. $p < 0.05$ was regarded as statistically significant.