Abstract&Methods on «***Antioxidant properties of fullerene derivatives depend on their chemical structure: a study of two fullerene derivatives on HELFs***» researching

Abstract

Oxidative stress is a major issue in a wide number of pathologies (neurodegenerative, cardiovascular, immune diseases, cancer). Because of this, the search for new antioxidants is an important issue. One of the potential antioxidants that has been enthusiastically discussed in the past twenty years is fullerene and its derivatives. Although in aqueous solutions fullerene derivatives have shown to be antioxidants, their properties in this regard within the cells are controversially discussed. We have studied two different water-soluble fullerene C60 and C70 derivatives on HELFs in a wide range of concentrations. Both of them cause a decrease in cellular ROS at short times of incubation (1 hour). Their prolonged action, however, is fundamentally different: derivative G1-761 causes secondary oxidative stress whereas derivative VI-419-P3K keeps ROS levels under control values. What is the reason for this difference? To gain a better understanding we assessed factors that could play a role in response of cells to fullerene derivatives. Increased ROS production occurred due to NOX4 upregulation by GI-761. Derivative VI-419-P3K activated transcription of antioxidant master regulator NRF2 and caused its translocation to the nucleus. This data suggests that the antioxidant effect of fullerene derivatives depends on their chemical structure and not on the presence of fullerene cage.

Methods

Cell culture

HELFs (fourth passage) were obtained from the Research Centre for Medical Genetics (RCMG) collection. Approval was obtained from the Committee for Medical and Health Research Ethics of RCMG (approval #5). Cells were seeded at 1.7× 104 per mL in DMEM (PanEco, Moscow, Russia), 10% fetal calf serum (PAA, Vienna, Austria), 50 U/ml penicillin, 50 µg/ml streptomycin, 10 µg/ml gentamycin), and then incubated at 37°C for 2 or 24 h. Fullerene derivatives were added and the cells were cultured for periods ranging from 15 min to 48 h.

Antibodies

The following primary antibodies were used: FITC-γH2AX (pSer139) (Chemicon, Temecula, USA); NRF2, BRCA2 (Santa Cruz Biotechnology, Dallas, U.S.A.), pNRF2, NOX4, and FITC goat anti-rabbit IgG (Abcam, Cambridge, UK).

Flow cytometry (FCA)

HELFs were washed in Versene solution, treated with 0.25% trypsin, washed with culture medium, and suspended in PBS. Staining was performed with various antibodies. To fix the cells, paraformaldehyde (PFA, Sigma, Saint Louis, USA) was added (2%, 37°C, 10 min). Cells were washed three times with 0.5% BSA-PBS and permeabilized with 0.1% Triton X-100 (PBS, 15 min, 20°C) or with 90% methanol (4°C). Cells were washed three times with 0.5% BSA-PBS and stained with antibodies, 1 µg/mL, for 2 h (4°C), then again washed three times with 0.5% BSA-PBS. The cells were then incubated for 2 h (20°C) with FITC goat anti-rabbit IgG (1 µg/mL).

Fluorescence microscopy

Cell images were obtained using the AxioScope A1 microscope (Carl Zeiss) and confocal microscopy platform Leica TCS SP8 (Germany).

EGFP. Non – fixed cells were analyzed at 488 nm. To quantify the background fluorescence, the control cells were analyzed.

Immunocytochemistry

Cells were grown in slide flasks (25 cm3, Thermofisher scientific, Waltham, USA). Fibroblasts were fixed in 3% paraformaldehyde (4°C) for 20 min, washed with PBS and then permeabilized with 0.1% Triton X-100 in PBS for 15 min at room temperature, followed by blocking with 0.5% BSA in PBS for 1 h and incubation overnight at 4°C with the antibodies. After washing with 0.1% Triton X-100 in PBS, fibroblasts

were incubated for 2 h at room temperature with the FITC goat anti-mouse IgG, washed with PBS and then stained with DAPI.

ROS assay

Cells were grown in slide flasks and then washed in PBS. H2DCFH-DA (Molecular Probes/Invitrogen, Carlsbad, USA), 10 μM, was added to PBS for 20 min. Cells were washed three times with PBS and analyzed at 37°C using the total fluorescence assay at λex = 488nm, λem = 528 nm (EnSpire Equipment, Turku, Finland). The analysis was performed with three techniques: flow cytometry, fluorescent microscopy and total fluorescence assay in a 96-well plate. The reaction rate constant for formation of DCF (***k***) was calculated from the dependence of DCF signal intensity on time of cell incubation with H2DCFH-DA. The data are presented as ***ki/ko*** ratio***,*** where ***ki, ko*** are the rate constants in the exposed and unexposed cells, respectively. The average value of the DCF signal for 16 wells +/- standard deviation is reported.

Quantification of mRNA levels

Total mRNA was isolated using the RNeasy Mini kit (Qiagen, Hilden, Germany). After treatment with DNAse I, RNA samples were reverse transcribed by Reverse Transcriptase kit (Sileks, Moscow, Russia). The expression profiles were obtained using qRT-PCR with SYBRgreen PCR MasterMix (Applied Biosystems, Foster City, USA). The mRNA levels were analyzed using the StepOne Plus (Applied Biosystems); the technical error was approximately 2%. The following primers were used (Sintol, Moscow, Russia):

BRCA1 (F: TGTGAGGCACCTGTGGTGA, R: CAGCTCCTGGCACTGGTAGAG)
NRF2 *(NFE2L2*) (F: TCCAGTCAGAAACCAGTGGAT, R: GAATGTCTGCGCCAAAAGCTG);
NOX4 (F: TTGGGGCTAGGATTGTGTCTA; R: GAGTGTTCGGCACATGGGTA);
BRCA2 (F: CCTCTGCCCTTATCATCACTTT; R: CCAGATGATGTCTTCTCCATCC);
CCND1 (F: TTCGTGGCCTCTAAGATGAAGG; R: GAGCAGCTCCATTTGCAGC);
TBP (reference gene) (F: GCCCGAAACGCCGAATAT, R: CCGTGGTTCGTGGCTCTCT).

Comet assay

Cell suspension in low-melting-point agarose was dropped onto slides pre-coated with 1% normal-melting-point agarose. The slides were placed in a solution (10 mM Tris-HCl, pH 10, 2.5 M NaCl, 100 mM EDTA, 1% Triton X-100, 10% DMSO, 4°C, 1 h) and then in electrophoresis buffer (300 mM NaOH, 1 mM EDTA, pH>13). Electrophoresis was performed for 20 min at 1 V/cm, 300 mA. The slides were fixed in 70% ethanol and stained with SYBR Green I (Invitrogen, USA).
Images of comets were analyzed using CASP v.1.2.2 software.

Statistics

All reported results were repeated at least three times as independent biological replicates. In FCA, the medians of signal intensities were analyzed. The Figures show the mean and standard deviation (SD). The significance of the observed differences was analyzed with non-parametric Mann-Whitney U-tests. P-values < 0.05 were considered statistically significant and marked on Figures with (*). Data were analyzed with StatPlus2007 professional software (http://www.analystsoft.com).

Synthesis of the fullerene derivatives

Polycarboxylic water-soluble fullerene derivatives GI-761 and VI-419-P3K were synthetized in three steps from the readily available precursors $C_{60}Cl_6$ and $C_{70}Cl_8$ [11] following previously developed methodology [12,13].
Details of the synthesis of the compounds and their spectral characterization data are provided in supporting information. Both compounds showed high solubility in water and culture medium in the presence as well as in the absence of serum.