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Effects of mild ozonisation on gene expression and nuclear domains organization in vitro

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ABSTRACT

In the last two decades, the use of ozone (O_3) as a complementary medical approach has progressively been increasing; however, its application is still limited due to the numerous doubts about its possible toxicity, despite the low concentrations used in therapy. For an appropriate and safe clinical application of a potentially toxic agent such as O_3, it is crucial to elucidate the cellular response to its administration. Molecular analyses and transmission electron microscopy were here combined to investigate in vitro the effects of O_3 administration on transcriptional activity and nuclear domains organization of cultured SH-SY5Y neuronal cells; low O_3 concentrations were used as those currently administered in clinical practice. Mild ozonisation did not affect cell proliferation or death, while molecular analyses showed an O_3-induced modulation of some genes involved in the cell response to stress (*HMOX1*, *ERCC4*, *CDKN1A*) and in the transcription machinery (*CTDSP1*). Ultrastructural cytochemistry after experiments of bromouridine incorporation consistently demonstrated an increased transcriptional rate at both the nucleoplasmic (mRNA) and the nucleolar (rRNA) level. No ultrastructural alteration of nuclear domains was observed.

Our molecular, ultrastructural and cytochemical data demonstrate that a mild toxic stimulus such as mild ozonisation stimulate cell protective pathways and nuclear transcription, without altering cell viability. This could possibly account for the positive effects observed in ozone-treated patients.

1. Introduction

Ozone (O_3) is a highly unstable atmospheric gas that rapidly decomposes to normal oxygen (O_2). Although not being a radical molecule, O_3 is a very strong oxidant and, due to this highly toxic property, it has been widely used as a disinfectant agent, also for medical purposes (Travagli et al., 2010; Davies et al., 2011; Gupta and Mansi, 2012). In addition to its germicidal application, O_3 administration as O_2-O_3 gas mixture has proven to exert therapeutic effects in numerous diseases, including arthritis, heart and vascular diseases, asthma, emphysema, and multiple sclerosis (reviews in Re et al., 2008; Elvis and Ekta, 2011; Bocci, 2012). In the last two decades, the use of O_3 as a complementary medical approach has progressively been increasing all over the world; low O_3 concentrations are generally used in the medical practice but the application of O_3 therapy is still limited due to the numerous doubts about its possible toxicity. Knowledge of the cellular response to O_3 administration is therefore crucial for its appropriate and safe clinical application.

It has been hypothesized that exposure to mild O_3 concentrations may stimulate the cellular antioxidant defenses without inducing cell damage (Sagai and Bocci, 2011). Unfortunately, scientific data demonstrating activation of cytoprotective pathways are still scarce and inconclusive (Re et al., 2014; Güçlü et al., 2016).

To elucidate this point, we investigated the effects of low O_3

Abbreviations: SH-SY5Y, Human neuroblastoma cell line; *HMOX1*, Heme Oxygenase 1; *ERCC4*, Excision Repair Cross-Complementation Group 4; *CDKN1A*, Cyclin-Dependent Kinase Inhibitor 1A; *CTDSP1*, CTD Small Phosphatase 1

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concentrations currently used in clinical practice on the nuclear activity of cultured cells of neuronal origin. There is, in fact, a growing interest for therapeutic applications of O_3 to the nervous system based on experimental evidence that mild ozonisation induces metabolic stimulation and even regenerative effects in neurons (Molinari et al., 2014; Salem et al., 2016; Tural Emon et al., 2016; Ozbay et al., 2017). The use of an in vitro model ensured controlled experimental conditions, allowing analysis of the direct effects of mild ozonisation on nuclear function without the intermediation of blood/tissue factors as occurs in vivo. Moreover, the choice of a stabilised neuroblastoma cell line, characterized by negligible intersample variability compared to primary cell cultures, guaranteed the consistency of our experimental model with highly sensitive techniques such as genomic and ultrastructural analysis. The combination of molecular assays (microarray gene expression and Real Time qPCR) and microscopy techniques (transmission electron microscopy and ultrastructural immunocytochemistry) allowed investigation of the effects of O_3 exposure on gene expression and the organization of nuclear domains.

2. Materials and methods

2.1. Cell culture and ozone treatment

SH-SY5Y cells (a human neuroblastoma cell line) (5×10^5) were seeded on 75 cm^2 plastic flasks (Corning Inc., Corning, NY, USA) in a 1:1 mixture of EMEM (Eagle's Minimum Essential Medium) and F12 medium supplemented with 10% (v/v) fetal bovine serum, 1% (w/v) NEAA (Non-Essential Amino Acids Solution), 0.5% (w/v) glutamine, 100 U of penicillin and 100 μg/ml streptomycin (Gibco by Life Technologies), at 37 °C in a 5% CO_2 humidified atmosphere. When subconfluent, the cells were mildly trypsinized (0.25% trypsin containing 0.05% ethylene diamino tetraacetic acid (EDTA) in phosphate-buffered saline, PBS) and exposed in suspension to O_2-O_3 gas mixtures with two O_3 concentrations successfully used for therapeutic purposes (10 and 16 μg O_3/ml O_2). A high concentration (100 μg O_3/ml O_2) was also used as a control for a strong oxidative stress. The gas was produced by an OZO2 FUTURA apparatus (Alnitec s.r.l., Cremosano, CR, Italy), which generates O_3 from medical-grade O_2, and allows photometric real-time control of gas flow rate and O_3 concentration. The reliability of the experimental procedure was guaranteed by choosing a well-established technique for cell ozonisation (Larini et al., 2003), which ensures that a defined number of cells are exposed to an exact gas volume at a pressure corresponding to the atmospheric one, and that the cell sample reacts totally with the O_3 dose for a precise treatment time. Briefly, for each sample, 4×10^5 cells were suspended in 1 ml medium into a 10 ml syringe (Terumo Medical Corporation, Somerset, NJ, USA), an equal volume of gas was then collected in the syringe, and the sample was gently mixed with the gas for 10 min to allow cells to react with the O_3.

Cells exposed to pure O_2 under the same experimental conditions were used to discriminate the effect of O_3, while cells exposed to air served as control (CTR).

Three hours after the exposure to the different gases or air, the cells were processed for genomic or microscopy analyses, while other samples were allowed to grow for 24 h and 48 h, to estimate the effect of O_3 on cell viability and proliferation (see below). For Western blot analysis, the cells were processed 10 min after treatment.

2.2. Cell viability and proliferation

To determine the effect of gas exposure on cell survival and growth, 5×10^4 cells/well were seeded on 6 multiwell plastic microplates (Corning), and the fraction of dead cells was estimated 3 h after treatment, whereas the total cell number was assessed after 24 h and 48 h. The cells were detached by mild trypsinization as above, stained for 2 min with 0.1% trypan blue in the culture medium, and scored in a Burker hemocytometer using a Leica DM IL inverted microscope equipped with 10 × objective lens. Data were expressed as the mean of three independent experiments ± standard error of the mean (SE).

Results for each measured variable were pooled according to the experimental group and the mean ± SE value calculated. Statistical comparisons were performed by the one way-Anova test and post-hoc pairwise comparisons.

2.3. RNA isolation and microarray gene expression analyses

Three hours after gas exposure, RNA samples were extracted and purified from the cells by using the RNeasy Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's protocols. The concentration of total RNA was quantified using the Nanodrop 2000 (Nanodrop Technologies, Wilmington, DE, USA) by measuring the absorbance at 260 nm. Additionally, the OD260/230 and OD260/280 ratios were determined to assess RNA purity. RNA integrity was assessed with the 2100 Bioanalyzer (Agilent Technology, Santa Clara, CA, USA) using an RNA 6000 NanoChip and expressed as RNA Integrity Number (RIN), which was considered acceptable within the range of 7–10.

RNA samples from cells treated with O_2, 10 or 16 μg O_3/ml O_2 as well as the CTR were processed for transcriptomic analyses. Total RNA (250 ng) from each sample were reverse-transcribed with the Ambion WT Expression Kit (Invitrogen-Life Technologies, Carlsbad, CA, USA). Subsequently, 5.5 μg of sscDNA was fragmented and labelled with biotin. The labelled samples were hybridized onto Human Gene 1.1 ST Array Strips (Affymetrix, Inc., Santa Clara, CA, USA), comprising > 750,000 probes and representing > 28,000 genes mapped through UniGene or via RefSeq.

The reactions of hybridization, fluidics, and imaging were performed on the Affymetrix Gene Atlas instrument according to the manufacturer's instructions (http://www.affymetrix.com/support/technical/manuals.affx).

The descriptive features for each identified gene were obtained from http://www.genecards.org.

2.4. Microarray data analysis and pathway analysis

Affymetrix CEL files were imported into Partek Genomics Suite version 6.6 for data visualization and statistical testing. Quality control assessment was performed using Partek Genomic Suite 6.6. All the samples passed the quality criteria for hybridization controls, labelling controls and 3′/5′ Metrics. Background correction was conducted using Robust Multi-strip Average (RMA) (Irizarry et al., 2003) to remove noise from autofluorescence. After background correction, normalization was made using Quantiles Normalization (Bolstad et al., 2003) to normalize the distribution of probe intensities among different microarray chips. Subsequently, a summarization step was conducted using a linear median polish algorithm (Tukey, 1977) to integrate probe intensities in order to compute the expression levels for each gene transcript. Upon data upload, pre-processing of CEL data for the complete data set was performed using the Robust MultiChip Average ANOVA statistical test to assess treatment effects. Differential gene expression was assessed by applying a p-value filter (for attribute = -treatment) of $p < 0.05$ to the ANOVA results. Contrast analyses were then performed to get the following four comparisons: 10 μg O_3/ml vs CTR, 16 μg O_3/ml vs CTR, 10 μg O_3/ml vs O_2, 16 μg O_3/ml vs O_2. In this comparison, a maximum filter of $p < 0.05$ and a minimum absolute fold change cut-off of 1.2 were applied.

In order to identify common or specific genes modulated by each treatment condition, the gene lists were compared by an interactive tool Venny (Venny 2.0.2 http://bioinfogp.cnb.csic.es/tools/venny/index.html). DAVID software was used to identify molecular signalling pathways in each treatment conditions.

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