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Imidazole and beta-carotene photoprotection against photodynamic therapy evaluated by synchrotron infrared microscopy

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ABSTRACT

In order to better understand the role of β-carotene and imidazole on the Photodynamic Therapy (PDT) mechanism, synchrotron infrared microscopy was used to detect the associated intracellular biochemical modifications following the visible light irradiation of HeLa cells incubated with these compounds as typical hydrophobic and hydrophilic singlet oxygen quenchers, respectively. For this purpose, PDT was performed employing the hydrophilic sensitizer 5,10,15,20-Tetrakis (1-methyl-4-pyridinio) porphyrin tetra (p-toluenesulfonate), TMPyP, and the hydrophobic sensitizer 5-(4-Methoxycarboxyphenyl)-10,15,20-triphenyl-21H,23H–porphyrin. The single cell IR spectra of PDT-treated, PDT plus quencher-treated and control HeLa cells were recorded at the SOLEIL Synchrotron Infrared SMIS beamline targeting specifically the cell nucleus. Principal Component Analysis (PCA) was used to assess the IR spectral changes. PCA revealed that there is a frequency shift of the protein Amide I vibrational band for the assays with the TMPyP sensitizer, indicating changes in the protein secondary structures of the PDT-treated cancer cells compared to the controls. In addition, the scores in those cells treated with both quenchers appear to be similar to the controls indicating a photoprotective effect. Comparative experiments carried out with SKMEL-28 and HaCat cells showed non- significant photoprotective effects of β-carotene and imidazole.

1. Introduction

Singlet oxygen, $O_2(a^1\Delta_g)$, is the lowest excited state of molecular oxygen [1–3]. Production of sufficient quantities of singlet oxygen in a biological environment can perturb cellular processes and ultimately cause cell death via apoptosis or necrosis [4,5]. The cytotoxic effect of singlet oxygen is currently used in clinical practice in a treatment modality called photodynamic therapy (PDT), whereby the controlled production of singlet oxygen leads to the eradication of undesired tissue [6].

It was recently demonstrated using hydrophilic and hydrophobic sensitizers and HeLa cells, a cell type derived from human cervical cancer, that intracellular β-carotene does not quench $O_2(a^1\Delta_g)$ as revealed by time-resolved $O_2(a^1\Delta_g)$ phosphorescence experiments [7]. On the other hand, in the same paper the photoprotective effect of β-carotene was manifested in experiments also performed with the same sensitizers and cell lines. The photoprotective effect of the carotenoid was shown by different types of experiments, such as bright field microscope images for morphology changes (e.g., membrane located vacuole formation and chromatin condensation), the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay for enzymatic activity, the Trypan Blue assay for membrane permeability, among others [7]. From these results it was inferred that the principal mechanism of β-carotene photoprotection involves trapping of radicals that are also produced as a consequence of sensitizer irradiation with visible light and the subsequent reactions of $O_2(a^1\Delta_g)$.

Imidazole is a well-known $O_2(a^1\Delta_g)$ quencher [30], which was shown to inhibit the UV-A induced pattern of mitogen-activated protein kinase in human skin fibroblasts (MAPK) activation, a process mediated by $O_2(a^1\Delta_g)$, [8]. Imidazole derivatives also were demonstrated to inhibit MPAK in B16-F0 and HeLa cells [9].

On the basis of this background we were motivated to investigate whether: 1) even though β-carotene is unable to quench $O_2(a^1\Delta_g)$ in mammalian cells, its radical trapping effect has any consequence on the nuclear protein changes associated with early stages of apoptosis, 2) imidazole presents a photoprotective effect on cells exposed to PDT.

To this end, we employed here the Fourier transform infrared (FTIR) microspectroscopy, a vibrational spectroscopy technique, which is used as a diagnostic tool useful for probing molecular changes within cells or tissues submitted to different treatments. This technique was previously used to follow the intracellular changes of biological composition of cells such as proteins and DNA [10]. In particular, Srichan et al. employed synchrotron light to study the PDT on HeLa cells with the

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sensitizer hypocrillile [7]. For our experiments two different sensitizers were employed. One of them was the hydrophilic cationic porphyrin sensitizer 5,10,15,20-Tetrakis(1-methyl-4-pyridinio) porphyrin tetra (p-toluenesulfonate), TMPyP, which tends to accumulate in the cell nucleus, contrarily to what was observed with hypericine or hypocrelinne, but an appreciable amount can still be found in the cytoplasm [9,11]. The second photosensitizer employed here was 5-(4-Methoxycarboxyphenyl)-10,15,20-triphenyl-21H,23H–porphyrin, (TPPCOOMe). Because of its hydrophobic nature, this latter molecule localizes in a different domain of a living cell than does TMPyP.

The main goal of the present work is to probe molecular changes observed inside HeLa cells (i.e., proteins global changes) using the internal source of the continuum XL infrared microscope as a result of the localization of the sensitizers (TMPyP or TPPCOOMe) and β-carotene or imidazole as typical hydrophobic and hydrophilic potential photoprotective agents, respectively. For this purpose, experiments were performed with and without visible light irradiation at two different quenchers concentration. In particular, data were analyzed in the region of the Amide I band (1600–1700 cm^{-1}). Amide I and Amide II (1500–1560 cm^{-1}) bands arise primarily from the C═O and C—N stretching vibrations of the peptide backbone, respectively. FTIR has been shown to be particularly sensitive to protein secondary structure based on the vibrational frequency of the Amide I (C═O) band, which is affected by different hydrogen bonding environments for α-helix, β-sheet, and unordered structures. For most proteins, a mixture of secondary structures exists, and in this case, the Amide I band represents a combination of these components [11,12,10]. For comparison similar experiments were carried out with SKMEL-28 (human melanoma cells) and HaCat (human normal skin keratinocytes cells). HeLa and HaCat cell types are of epithelial origin and are considered to be an excellent comparative model between tumoral vs. non-tumoral studies in vitro [13]. SKMEL-28 cells derive from a metastatic melanoma, which is the most hardly treated skin cancer. Melanoma cells are resistant to traditional chemotherapy and radiation therapy, and thus photodynamic therapy can provide a promising treatment [14].

2. Materials and Methods

2.1. Chemicals

All trans-β-carotene (>97%, Sigma—Aldrich), TMPyP, (Sigma—Aldrich), 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide, MTT, (Sigma—Aldrich), and Imidazole (Sigma—Aldrich) were used as received. TTPCOOMe, was synthesized according to a published procedure [7].

2.2. PDT Experiments

Photodynamic therapy has been induced in cancer HeLa, SKMEL-28, and HaCat cell lines. The IR study aimed at identifying the change induced by various treatments, with or without visible light irradiation. According to previously published work from Dr. Dumas group [12], we concentrated on the biochemical changes that might have been induced in the nucleus of the cells.

2.3. Cell Culture

HeLa cells from the American Type Culture Collection (ATCC CCL-2) were cultured on low-e microscope slides in D-MEM containing 10% fetal bovine serum. SKMEL-28 cells from the American Type Culture Collection (ATCC HTB-72) were cultured on low-e microscope slides in MEM containing 10% fetal bovine serum. HaCaT cells, provided by IMBICE (Multidisciplinary Institute of Cellular Biology, Argentina), were cultured on low-e microscope slides in RPMI 1640 M (Micro-Vet SRL, C.A.B.A, Argentina). Then, the cells were incubated with 10 μM TMPyP or TPPMeCOO 24 h with or without the quenchers. After incubation cells were irradiated for 1 h with a 5 W fluorescent lamp (see spectral irradiance in the Supplementary Material, Fig. SM1) with a photon rate of 2.179×10^{-7} eins/Ls, as determined by ferrioxalate actinometry for λ ≤ 500 nm [15]. From Fig. SM1 the photon rate in the whole wavelength emission range was calculated to be $2{,}179 \times 10^{-8}$ eins/Ls. Then, cells were immediately fixed with 10% formalin in phosphate buffer saline (PBS) for 20 min at room temperature for conserving the cells [16]. The cells were washed first with PBS and then with water and dried.

2.4. MTT Assay

Cytotocixity in mammalian cells was estimated using metabolic competence by the colorimetric method of Mosma [17–19] as modified by Twentyman and Luscombe [20]. This assay measures the reduction of tetrazolium salt (3-(4,5-dethylthiazol-2-yl) 2,5 diphenyl tetrazolium bromide) to formazan by dehydrogenase enzymes of intact mitochondria in living cells.

For this analysis 1.5×10^3 cells/well were cultured in 96 multi-well plate and grown at 37 °C in 5% CO_2 humid atmosphere in complete culture medium for 24 h. This medium was then replaced with different extracts containing TMPyP or TPPCOOMe both in the presence and absence of β-carotene or imidazole. The samples were irradiated with visible light and then washed with PBS and fresh medium containing MTT reagent (1 mg/mL final concentration) (Sigma, St. Louis, MO, USA) was added. After 3 h incubation, cells were washed again with PBS. Color was developed by the addition of 100 μL dimethylsulfoxide (DMSO) (Merck, Química Argentina SAIC, Argentina) to each well for cells lysis and formazan crystals dissolution. The plates were shaken for 10 min and the absorbance was measured at 540 nm using an automatic ELISA Plate Solver reader (7530 Microplate Reader Cambridge Technology, Inc., St. Watertown, MA, USA). Ethanol (5%) was used as positive control. Each experiment was independently repeated three times.

Phototoxicity control experiments with HeLa, SKMEL-28, and HaCat cells were carried out with cells incubated in the presence of different amounts of β-carotene or imidazole solutions without the sensitizers. Results are shown in the Supplementary Material (Fig. SM2). No phototoxicity of the quenchers was observed in the concentration range employed in the PDT experiments.

2.5. FTIR Microspectroscopy

The synchrotron based Fourier transform infrared (SR-FTIR) microspectroscopy was used to particularly observe the changes of the chemical structure of the biological molecules in the nucleus of mammalian cells. The IR spectra of the untreated cells and sensitizers/quenchers treated cells were measured and compared. The IR spectral changes between the untreated cells and sensitizers/quenchers treated cells were analyzed by multivariate statistical analysis.

The experiments were performed at the infrared SMIS beamline, SOLEIL Synchrotron, L'Orme des Merisiers, Gif sur Yvette, France. The IR spectra collected in reflection mode from individual cells grown directly on low-e slides were recorded using a Continuum XL microscope coupled to a ThermoNicolet 5700 spectrometer (8 cm^{-1} spectral resolution, 256 scans per spectrum, aperture dimension 8×8 μm^2, magnification 32× NA 0.65 Schwarzschild objective, in reflection and confocale modes). The reference spectrum was recorded with the same parameters and conditions, but outside the cells.

2.6. Data Analysis

Cell spectra recorded one by one, with 50 to 100 individual cells per sample were analyzed. Principal Component Analysis (PCA) was used for outliers detection within samples and also for comparison between samples (see below). Several preprocessing techniques were tested including: Savitzky–Golay baseline correction [21], non-resonant Mie