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Spatial distribution of protein damage by singlet oxygen in keratinocytes

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Abstract

Singlet oxygen may be generated in cells by either endogenous or exogenous photosensitizers as a result of exposure to UV or visible irradiation. We have used immuno-spin trapping (*Free Rad. Biol. Med.* 36: 1214, 2004) to identify the subcellular targets of singlet oxygen generated by Rose Bengal (RB). Confocal fluorescence microscopy of HaCaT keratinocytes incubated with RB clearly showed that the dye entered the cells and was located mainly in the perinuclear region, probably associated with the Golgi apparatus and endoplasmic reticulum. Previous studies by Wright and coworkers (*Free Rad. Biol. Med.* 34, 637, 2003) have shown that long lived protein hydroperoxides (POOH) are present in cells exposed to singlet oxygen generating dyes. The addition of reducing metal ions such as Cu^+ to POOH results in the generation of protein derived radicals, $\text{POO}^\cdot$ and $\text{PO}^\cdot$, which react with the spin trap 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) to give relatively stable spin adducts. In order to determine the subcellular localization of the protein-DMPO adducts, we exposed keratinocytes to RB/light exposure and then incubated the cells with Cu^+ and DMPO. After staining with antibody against DMPO followed by a secondary Alexa Fluor 488 goat anti-rabbit IgG, the intracellular distribution of protein-DMPO adducts was determined by confocal microscopy. The subcellular localization of the protein DMPO adducts was coincident with that of Rose Bengal. This approach may provide information on the spatial distribution of singlet oxygen generated in cells.

Keywords

immuno-spin trapping; singlet oxygen; spatial distribution; Rose Bengal; HaCaT keratinocytes

Introduction

Singlet oxygen may be generated photochemically in cells by UV/visible irradiation of endogenous or exogenous photosensitizers (1) or metabolically *via* the decomposition of hydroperoxides by peroxidases (2). In a recent review Redmond and Kochevar (3) have pointed out that, because singlet oxygen is a highly reactive species with an inherent upper lifetime of 4 μs in water, its diffusion radius (~ 220 nm after 3 lifetimes) is much smaller than cellular dimensions (10–30 μm). Thus it is most likely that the primary reactions of singlet oxygen will occur close to its site of generation. Possible approaches to the spatial resolution of singlet oxygen in cells include the linkage of a photosensitizer to a target protein, genetically encoding a photosensitizer in a target protein and the use of organelle specific photosensitizers (see reference (3) for a description of these techniques). Another approach is the spectroscopic detection of singlet oxygen by monitoring its luminescence at 1268 nm (4,5). Skovsen and coworkers (4) have observed singlet oxygen luminescence generated by a DNA bound

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porphine dye, 5,10,15,20-tetrakis(*N*-methyl-4-pyridyl)-21*H*,23*H*-porphine (TMPyP), in a single nerve cell. They estimated that singlet oxygen would diffuse ~260 nm in 2 lifetimes.

Davies and coworkers (6) have shown that long lived protein hydroperoxides (POOH) are present in cells exposed to singlet oxygen generating dyes. The addition of reducing metal ions, e.g. Fe^{2+} , Cu^{+} , to POOH results in the generation of protein derived radicals (Equn. 1) which can react with the nitron spin trap 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) to give relatively long-lived nitroxide spin adducts (1),

Oxidation of the spin adduct generates a stable nitroxide (2) in which the radical moiety remains covalently attached.

Mason and coworkers (7,8) have developed an immunological technique, immuno-spin trapping, to locate the site(s) of free radical generation in cells. In this procedure DMPO reacts with free radicals generated on intracellular macromolecules and the resultant nitroxide products (2) are detected with the aid of a specific DMPO antibody. Previously we have successfully detected singlet oxygen generated by Rose Bengal (RB) stained HaCaT keratinocytes irradiated with visible light (9). However, we were unable to determine the precise location of singlet oxygen generation. Recently Bonini and coworkers have successfully employed immuno-spin trapping to image hypochlorite-induced, catalase-bound free radical formation in mouse hepatocytes (10). The aim of the present study was to determine whether a similar approach could be used to identify the subcellular targets of singlet oxygen generated by RB/light in HaCaT keratinocytes.

Materials and Methods

HaCaT keratinocytes, a transformed epidermal human cell line (11), were grown at 37°C in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal calf serum in an atmosphere of 95% air/5% CO_2 . RB and xylene orange were obtained from Sigma Chemical Co. (St. Louis, MO). 5,5-Dimethyl-1-pyrroline *N*-oxide (DMPO) (Aldrich Chemical Co., Milwaukee WI) was vacuum distilled and stored at -70°C until use. All other chemicals were reagent grade or better. Rabbit antiserum against DMPO (12) was kindly provided by Dr. R.P. Mason, NIEHS.

Light Treatment

Cells were incubated with RB (10 μM) in PBS at 37°C for 10 min. The solution was removed and cells were washed twice with sterile PBS-CMF (calcium/magnesium-free). For visible light treatment, cells were irradiated with cool white visible light (Phillips F40 AX50, 5000K Advantage) passed through a 30mm pathlength liquid filter (aqueous solution containing in g/l NaNO_2 48.4, Na_2CO_3 1, K_2CrO_4 0.2) to remove wavelengths below 400 nm. Fluence was measured using a YSI-Kettering Model 65A Radiometer (Yellow Springs Instrument Co., Yellow Springs, OH) and found to be 0.9 J cm^{-2} . Control samples were kept in the dark under the same conditions.

Confocal microscopy

HaCaT keratinocytes were seeded into 6-well plates containing glass coverslips (MatTek). Cells were treated with RB (10 μ M) with or without light and then exposed to DMPO/CuCl₂ (DMPO 100 mM, CuCl₂ 25 μ M). In order to carry out the reaction shown in Eqn. 1 Cu²⁺ must be reduced to Cu⁺. In HaCaT keratinocytes this is most likely achieved by intracellular glutathione (13) which is present at high concentrations in these cells (14). Cells were fixed and stained with anti-DMPO antibody followed by Alexa 488 secondary antibody and Hoechst 33342 to stain the nucleus. For RB localization, cells were seeded into 35-mm dishes containing a glass coverslip-covered 15-mm cutout (MatTek, Ashland, MA) for live cell microscopy measurement. The next day cells were incubated with RB (10 μ M) for 1h. Cell fluorescence was monitored using a Zeiss 510 Meta confocal microscope.

For localization of RB in the Golgi apparatus, HaCaT cells were transfected with Golgi-ECFP (Clontech, Mountain View, CA) with Nucleofector (Amaxa, Gaithersburg, MD) according to the instructions provided by the manufacturer. Briefly, one million HaCaT cells were electroporated in 100 μ l Nucleofector Solution V containing 2 μ g Golgi-ECFP plasmid using program U-20. After transfection, cells were seeded in 2 ml prewarmed medium in a MatTek dish. Using the kit and program specific for HaCaT cells, approximately 50% transfection efficiency was achieved. For confocal microscopy, excitation: 458 nm for ECFP and 543 nm for RB; emission: 475-525 nm for ECFP and LP560 for RB.

Pixel intensities in Figures 1 and 2 were determined using Matrox Inspector (Matrox Imaging, Toronto, Canada).

Protein peroxide assay

Keratinocytes were suspended in PBS and 2 ml aliquots containing 2×10^6 cells were placed in individual wells of a 6 well plate (Becton Dickinson, Franklin Lakes, NJ). After the addition of RB (10 μ M) the cells were incubated at 37°C for 10 min then exposed to visible light as described above. Control cells either contained no RB or were kept in the dark. Where indicated sodium azide (10 mM) was present during RB/light treatment. Intracellular protein peroxides were assayed using a modified FOX assay as described by Wright *et al.* (15). The absorbance was measured at 560 nm and compared to a standard curve prepared using H₂O₂.

Results

Confocal fluorescence microscopy of HaCaT keratinocytes incubated with RB clearly showed (Figure 1) that the dye entered the cells and was primarily located in the perinuclear region possibly associated with the Golgi apparatus. To confirm this we transfected the keratinocytes with Golgi-ECFP then examined the cells by confocal microscopy after the addition of RB. While RB was colocalized in the Golgi, most of the fluorescence was outside these structures possibly associated with the endoplasmic reticulum (Figure 2).

Wright *et al.* have detected singlet oxygen generated protein peroxides in THP-1 cells loaded with RB and exposed to visible light (15). We carried out similar experiments with HaCaT cells. Exposure of the cells to visible light alone generated 0.25 μ M peroxides/ 2×10^6 cells which increased to 4.2 μ M/ 2×10^6 cells in the presence of 10 μ M RB. When the singlet oxygen quencher sodium azide (10 mM) was present during RB irradiation the peroxide level decreased to 0.9 μ M/ 2×10^6 cells. These findings confirm that singlet oxygen generated protein peroxides are indeed present in HaCaT cells exposed to RB/light.

In order to determine the spatial distribution of RB generated singlet oxygen in keratinocytes we used confocal microscopy. For these experiments cells were exposed to RB/light then incubated with CuCl₂/DMPO (16). After fixation and permeabilization, cells were stained with

anti-DMPO antibody followed by Alexa 488 secondary antibody and Hoechst 33342 to stain the nucleus. Confocal microscopy of cells exposed to RB/light followed by CuCl_2 /DMPO showed extensive fluorescence in the perinuclear region (Figure 3). Cells kept in the dark or exposed to light in the absence of RB or DMPO showed no fluorescence (Figures 3 and 4). Sodium azide is a physical quencher of singlet oxygen (17) that can readily penetrate cell membranes (4). The addition of 10 mM sodium azide completely abolished the fluorescence (Figure 5).

Discussion

The subcellular localization of RB depends on the cell type and the dye concentration. In murine monocytes RB is located in the outer membrane (18) whereas in HeLa cells it is found in the Golgi apparatus and mitochondria (19-21). In this study confocal microscopy indicated that in HaCaT keratinocytes exposed to RB the dye was mostly confined to the perinuclear region (Figure 1). Confocal microscopy of HaCaT keratinocytes transfected with Golgi-ECFP and stained with RB showed colocalization (Figure 2) indicating that some of the dye was associated with the Golgi apparatus Soldani and coworkers have reported (21) that the Golgi apparatus is the main locus of damage in HeLa cells incubated with RB acetate and exposed to visible light. In our study the remainder of the RB exhibited a diffuse perinuclear pattern that was quite different from that of berberine stained mitochondria in HaCaT keratinocytes (22). We therefore presume that this RB is associated with the endoplasmic reticulum.

The diameter of the HaCaT keratinocyte is $\sim 20 \mu\text{m}$ (Figure 3). If singlet oxygen diffuses only $\sim 250 \text{ nm}$ before reacting (3,4) then it should be possible to map its intracellular spatial distribution by detecting the resultant products. Given that proteins make up the bulk of the cell contents and that singlet oxygen reacts rapidly with proteins (23) it seems reasonable to assume that protein hydroperoxides would be formed. By taking advantage of the conversion of hydroperoxides to peroxy and other radical species by reducing metals (Equation 1) then spin trapping the resultant radicals with DMPO should make it possible to locate damaged proteins by means of a DMPO specific antibody. A comparison of the pixel intensities across cells (Figures 1E and 3E) clearly shows that the fluorescence patterns are similar (i.e. high asymmetric fluorescence in the perinuclear region and no fluorescence associated with either the membranes, M, or the nuclei, N).

How certain can we be that we have achieved our goal of mapping the intracellular distribution of singlet oxygen? The addition of DMPO/ CuCl_2 after RB/light exposure should prevent the trapping of most of the radicals generated during irradiation. Even if protein radicals do persist after the light has been switched off and react with DMPO the corresponding DMPO/protein adducts would still be detected by the immunoassay. It is possible that superoxide generated in a Type I reaction may still be present after RB/light exposure. The generation of the DMPO/ $\text{O}_2^{\cdot -}$ adduct is not a confounding factor as it would not be detected. At micromolar concentrations the lifetime of superoxide is only a few microseconds, even less in the presence of superoxide dismutase or metal ions (24). While the resultant H_2O_2 may generate $\text{HO}^{\cdot}$ via the Fenton reaction this radical reacts rapidly with proteins. If the resultant protein radicals form adducts with DMPO then they would also be detected by the immunoassay. A reviewer has suggested that we may not be mapping intracellular singlet oxygen but the instead the distribution of the copper. While there are no studies on the intracellular distribution of copper in keratinocytes Palmer and coworkers have reported that in cardiomyocytes the metal is uniformly distributed throughout cytoplasm (25). Furthermore, copper binds readily to peptides and amino acids present in the cytoplasm so there is no reason to believe that it would be confined to a particular region of the cell.

In conclusion we believe the coincidence of the subcellular localization of RB with the damage detected by the immuno-spin assay technique (c.f. Figures 1A and 2A) strongly suggests that we have indeed successfully mapped the intracellular spatial localization of singlet oxygen.

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Figure 1. Confocal images of HaCaT cells incubated with Rose Bengal (RB 10 μ M) for 1h (A and B) or without RB (C and D). A and C, fluorescent images; B and D, transmission images showing the cells. Ex/Em: 543/LP560 nm. (E) Pixel intensity across a cell in A indicated by the yellow line; M, membrane, N, nucleus.

Figure 2.

Partial localization of Rose Bengal in Golgi apparatus. (A) HaCaT cells were transfected with Golgi-ECFP. 48 h after transfection, cells were incubated with Rose Bengal (10 μ M) and fluorescence was determined by confocal microscopy. Green is pseudocolor for Rose Bengal; and red is pseudocolor for Golgi-ECFP in order for better visibility of colocalization; grey is the transmission image showing the cells. (B) Part of a cell indicated by a red rectangle in the superimposed image was amplified. Yellow shows colocalization of Rose Bengal and Golgi apparatus.

Figure 3.

Confocal images of HaCaT cells stained with anti-DMPO antibody and Alexa 488 secondary antibody. Alexa 488 Ex/Em: 488/505-550nm. Cells were treated and then incubated with or without 100 mM DMPO and 25 μ M CuCl_2 in PBS for 10 min at room temperature. After fixation and permeabilization, cells were stained with anti-DMPO antibody followed by Alexa 488 secondary antibody. (A) Cells were exposed to light (wavelength > 400 nm, 0.9 J/cm^2) and then incubated without DMPO; left confocal fluorescence microscopy, right transmission microscopy (B) Same as in (A) except that cells were then incubated with DMPO; (C) Cells were incubated with Rose Bengal (RB, $10 \mu\text{M}$) for 1h, exposed to light as in (A) and then incubated without DMPO; (D) Same as in (C) except that cells were then incubated with DMPO. (E) Pixel intensity across a cell in D indicated by the yellow line; M, membrane, N, nucleus. Scale marker 20 microns.

Figure 4.

Confocal images of HaCaT cells stained with anti-DMPO antibody and Alexa 488 secondary antibody and Hoechst 33342 staining nucleus. Alexa 488 Ex/Em: 488/505-550nm. Hoechst Ex/Em: 364/385-470 nm. Cells were treated and then incubated with or without 100 mM DMPO and 25 μ M CuCl_2 in PBS for 10 min at room temperature. After fixation and permeabilization, cells were stained with anti-DMPO antibody followed by Alexa 488 secondary antibody (green) and Hoechst 33342 staining nucleus (blue); grey is the transmission image (A) Cells were kept in the dark and then incubated without DMPO; (B) Same as in (A) except that cells were then incubated with DMPO; (C) Cells were incubated with Rose Bengal (RB, 10 μ M) in the dark and then incubated without DMPO; (D) Same as in (C) except that cells were then incubated with DMPO

Figure 5.

Confocal images of HaCaT cells stained with anti-DMPO antibody and Alexa 488 secondary antibody (green) and Hoechst 33342 staining nucleus (blue). Alexa 488 Ex/Em: 488/505-550nm. Hoechst Ex/Em: 364/385-470 nm; grey is transmission image. Cells were treated and then incubated with or without 100 mM DMPO and 25 μ M CuCl₂ in PBS for 10 min at room temperature. After fixation and permeabilization, cells were stained with anti-DMPO antibody followed by Alexa 488 secondary antibody and Hoechst 33342 staining nucleus. (A) Cells were incubated with Rose Bengal (10 μ M), exposed to light (wavelength > 400 nm, 0.9 J/cm²) and then incubated with DMPO and CuCl₂; (B) Same as in (A) except that the cells were pretreated with NaN₃ (10 mM) for 10 min before exposure to light.