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Involvement of cathepsin B in mitochondrial apoptosis by *p*-phenylenediamine under ambient UV radiation

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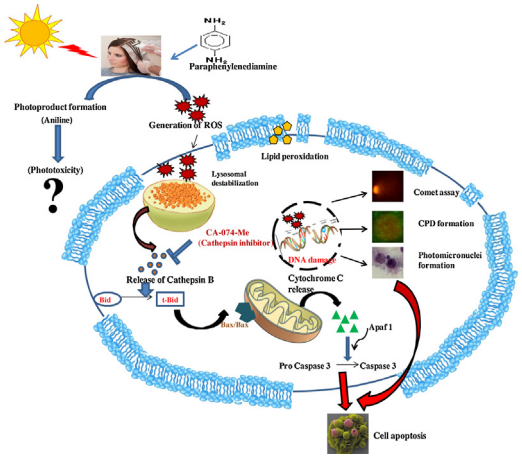
HIGHLIGHTS

- Photodegradation and formation of photoproduct.
- Involvement of ROS in PPD phototoxicity.
- Role of ROS in DNA damage, CPD and micronuclei formation.
- PPD induced lysosomal destabilization and release of cathepsin B.
- Cleavage of Bid and activation of mitochondrial apoptosis.

GRAPHICAL ABSTRACT



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ABSTRACT

Paraphenylenediamine (PPD), a derivative of paranitroaniline has been most commonly used as an ingredient of oxidative hair dye and permanent tattoos. We have studied the phototoxic potential of PPD under ambient ultraviolet radiation. PPD is photodegraded and form a novel photoproduct under UV A exposure. PPD shows a concentration dependent decrease in cell viability of human Keratinocyte cells (HaCaT) through MTT and NRU test. Significant intracellular ROS generation was measured by DCFDA assay. It caused an oxidative DNA damage via single stranded DNA breaks, micronuclei and CPD formation. Both lysosome and mitochondria is main target for PPD induced apoptosis which was proved through lysosomal destabilization and release of cathepsin B by immunofluorescence, real time PCR and western blot analysis. Cathepsin B process BID to active tBID which induces the release of cytochrome C from mitochondria. Mitochondrial depolarization was reported through transmission electron microscopy. The cathepsin inhibitor reduced the release of cytochrome C in PPD treated cells. Thus study suggests that PPD leads to apoptosis via the involvement of lysosome and mitochondria both under ambient UV radiation. Therefore, photosensitizing nature of hair dye ingredients should be tested before coming to market as a cosmetic product for the safety of human beings.

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1. Introduction

Paraphenylenediamine (PPD) is an intermediate oxidative dye used in hair dye formulation. When PPD is ingested, it absorbed through blood stream and redistributed to target to exert its effect [1]. PPD has been reported to increase the formation of liver tumors in mice [2]. Currently, PPD is present in more than 1000 hair dye formulations marketed all over the world [3]. The concentration of PPD used in hair dye is 2%. It has to be mixed with hydrogen peroxide in 1:1 prior to apply on hair.

The skin is more or less exposed to sunlight as part of daily life, in which the effects of ultraviolet radiation are not negligible. UVA constitutes >90% of the environmental solar UV radiation. It is generally accepted that UVA damages DNA, either through the generation of singlet oxygen ($^1O_2$) or by free radical resulting in oxidative base stress [4]. The rate of removal of UVA-generated cyclobutane pyrimidine dimmers was lower than those produced by UVB irradiation of skin. These observations emphasize the role of UV A induced DNA damage in skin carcinogenesis and should have consequences for photoprotection strategies [5].

Solar exposure and oxidative coloring are the most common and important factors that can lead to color fading and loss in hair manageability. UV induced damage changes the structure of keratin through the photooxidation of amino acids, and fatty acids, resulting in rupture of sulfur bridges, decomposition of lipids, melanin as well as numerous micro-molecular lesions [6,7]. Previous studies demonstrate the higher risk of leukemia, lymphoma and bladder cancer among user of hair dye containing PPD [8].

Recently, it has been demonstrated that the lysosomes are involved in apoptosis by the partial release of lysosomal protease enzyme such as cathepsins. Cathepsins (particularly cathepsin B) activate BID by its cleavage at Arg65 or Arg71, which was shown in tumor cells [9]. An important cytosolic Bcl2 family member BID was reported to be cleave by some cathepsins and translocated to the mitochondria following lysosomal destabilization [10]. Thus, a lysosomal pathway is connected with a mitochondrial pathway to initiate apoptosis through these Bcl2 family proteins. In this study, we examined that PPD target lysosomes of human keratinocyte cells and induce partial release of cathepsin B finally promotes mitochondrial apoptosis.

2. Materials and methods

2.1. Chemicals and culture wares

Paraphenylenediamine (PPD), superoxide dismutase (SOD), nitro-blue tetrazolium (NBT), mannitol, fetal bovine serum (FBS), Dulbecco's modified eagle's medium (DMEM F-12HAM), antibiotic and antimycotic solution, trypsin (0.25%), 3-(4,5-dimethylthiozolyl-2)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT), neutral red uptake (NRU), mannitol, Hank's Balanced Salt Solution (HBSS) were procured from Sigma Chemical Co. USA. Other reagents and chemicals were procured from Hi-Media and Merck India Ltd. All plastic wares including well plates and culture flasks (polystyrene coated) were purchased from Nunc (USA).

2.2. Cell culture

The HaCaT cell line was procured from National Centre for Cell Sciences Pune, India (passage number 46). It was sub cultured and maintained in our laboratory. The cell line was grown in DMEM culture medium supplemented with 10% FBS antibiotic-antimycotic solution (1.5%) at 5% CO_2 and 95% relative humidity (37 °C).

2.3. Source and radiation exposure

The UV-R system comprised an array of 1.2 m long UV-R emitting tubes manufactured by Vilber Lourmat (France). The intensity of emitted light was measured by a microprocessor-controlled RMX-3 W radiometer (Vilber Lourmat) equipped with calibrated UV A, UV B and UV C detecting probes. Intensities selected for irradiations were based on dosimetry carried out at our laboratory's roof top between 12.00 noon and 3.00 pm and were parallel to the ambient intensities of UV A and UV B in sunlight reaching at Lucknow (26°45′N latitude and 80°50′E longitude at 146 m above the mean sea level). The samples were put at a minimum distance of 22.0 cm from the source.

2.4. Analysis of PPD through LC/MS–MS

Stock solution of PPD (1 mg/mL) was prepared in double distilled water. Working solution 10 μg/mL was prepared in double distilled water. The solution was subjected to UV A irradiation for 0–4 h. Mass spectrometric detection was performed on an API 4000 mass spectrometer (Applied Biosystems, MDS Sciex Toronto, Canada) equipped with an API electro spray ionization (ESI) source. PPD was optimized by continuous infusion at 10 μL/min using syringe pump (Model 11, Harvard apparatus). Molecular weight of PPD is 108.14. The optimizes precursor (protonated form of analyte M^+H^+) was $m/z \rightarrow 109.0$. Zero air and nitrogen gas were used as source and curtain gas, respectively. The optimized declustering potential for PPD was 90 V. At these optimized conditions, Q1 scan for control and test sample was performed [11].

2.5. Photochemical assay

2.5.1. Determination of superoxide anion radical ($O_2^{\bullet -}$)

The generation of $O_2^{\bullet -}$ was monitored by recording the photosensitized reduction of nitroblue tetrazolium (NBT) to nitroblue diformazan (NBF) spectrophotometrically at 560 nm. Riboflavin (5 μg/mL) was used as a positive control [12].

2.5.2. Determination of hydroxyl radical (OH)

The OH generation was measured by ascorbic acid-iron-EDTA system and irradiated under UV A (2.88 J/cm^2), UV B (1.08 J/cm^2) and sunlight (30 min). Ascorbic acid (2 mM) was used as a positive control. The production of formaldehyde was monitored at 412 nm. [13].

2.6. Photocytotoxicity assay

2.6.1. MTT assay

Cells (2×10^4) were seeded per well in 96-well plates and kept in the CO_2 incubator for 48 h at 37 °C for 80–90% confluency. The medium was replaced by HBSS with PPD (5–100 μg/mL) for exposure. At the end of irradiation, HBSS was replaced by MTT (5 mg/mL) in 200 μL complete medium. After incubation, 200 μL of DMSO was added to each well by pipetting up and down to dissolve the content. The absorbance was recorded at 540 nm by using multiwell microplate reader (Fluostar Omega – BMG Labtech) [14].

2.6.2. Neutral red uptake (NRU) assay

Briefly, after irradiation, the cells containing PPD were washed with HBSS. The culture well plates were allowed to incubate for 3 h in complete medium (DMEM F-12HAM) containing neutral red (NR) dye (50 μg/mL). The absorbance was recorded at 540 nm [15].