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Journal of Photochemistry and Photobiology B: Biology 82 (2006) 224–235
Journal of Photochemistry and Photobiology B:Biology
www.elsevier.com/locate/jphotobiol

Synthetic melanin is a model for soluble natural eumelanin in UVA-photosensitised superoxide production

Rachel M. Haywood *, Martin Lee, Claire Linge

RAFT Institute of Plastic Surgery, Mount Vernon Hospital, Northwood, Middlesex, HA6 2RN, UK

Received 1 September 2005; received in revised form 2 December 2005; accepted 7 December 2005
Available online 30 January 2006

Abstract

Studies to UV-irradiate natural eumelanins in vitro have used insoluble pigment obtained by acid hydrolysis, which lacks melanoprotein. Eumelanin synthesised in the presence of a protein is not insoluble, and the insoluble form of melanin from acid hydrolysis may not have the same physicochemical properties as the natural pigment synthesised in vivo in the melanosome. Here we investigated radical production by three natural eumelanins exposed to solar levels of UVA; sepia melanin from *Sepia officinalis*, and eumelanins isolated from Oriental human and domestic cat hair. UVA irradiation of sepia melanin in solution at pH 4.5 in the presence of the spin trap 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) gave hydroperoxyl and hydroxyl radical-adducts, maximal at 0.6–2.5 mg/ml melanin concentrations. Hydroperoxyl radical production was relatively low in acetate buffer, but detected in aqueous suspensions of sepia melanin. Hair eumelanins were photoreactive with hydroperoxyl radical-adduct production at low concentrations (0.1–0.4 mg/ml melanin). Synthetic pigment after synthesis undergoes photo-oxidation (producing superoxide) at low concentrations ($\leqslant$0.3 mg/ml) and its oxidation increases the photoreactivity at higher melanin concentrations. These findings may be physiologically relevant to the properties and function of eumelanin in vivo when it is at low concentration (found in a small proportion of Caucasian melanocytes), and suggest that synthetic melanin has the potential for the basis of a model for natural eumelanin.
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Keywords: Electron spin resonance spectroscopy; Free radicals; Eumelanin; Spin trapping; Melanocyte; Photoprotection; UV radiation

1. Introduction

Synthetic dopa melanin is not the biological form of eumelanin, but several studies have suggested that it is a good model for melanin in melanosomes isolated from bovine eyes [1–5]. Synthetic dopa melanin synthesised in the absence of salts is a soluble form of eumelanin in vitro [1,6]. Studies of the natural pigment have been undertaken with hair melanin [7–10]; however, in order to remove melanoproteins, hair melanins have been subject to acid hydrolysis, which produces an insoluble pigment. In other studies solid hair samples [12] and sepia melanin, which exists predominantly as a suspension [11], have been used. The physical state of eumelanin in the melanosomes of viable melanocytes and keratinocytes, and in the non-viable keratinocytes of the stratum corneum, remains to be clarified. Eumelanin, however, is more likely to be insoluble in non-viable cells of the stratum corneum (forming a melanin dust upon terminal differentiation and degradation of keratinocytes) than in organelles of metabolically active viable cells. We have hypothesised that melanosomes may contain soluble pigment: eumelanin (which is typically associated with melanoprotein in vivo) is soluble when co-synthesised with protein in vitro [13]. Elleder and Borovansky demonstrated UV-induced autofluorescence of eumelanins in vitro, and also in histological sections of skin, which they attributed to the formation of oxidised eumelanin. This suggests that eumelanin in vivo may be in a soluble form, which can be oxidised [14].

Since the natural melanins used in previous studies [7–11] have lacked the protein moiety, we investigated the

* Corresponding author. Tel.: +44 1923 844350; fax: +44 1923 844031.
E-mail address: haywoodr@raft.ac.uk (R.M. Haywood).

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doi:10.1016/j.jphotobiol.2005.12.007

behaviour of soluble forms of natural eumelanin exposed to solar levels of UVA irradiation. In contrast with previous studies, which used acid reflux as part of the procedure to isolate melanin (to remove the melanoproteins), we isolated hair melanins without acid hydrolysis to yield a more soluble pigment. Natural eumelanin can be obtained as the melanoprotein sepia melanin (the ink produced by *Sepia officinalis*) and melanosomes from oriental human and animal hair. The natural eumelanins, in the presence of the spin trap DMPO at pH 4.5, were UVA-irradiated in situ in the cavity of an electron spin resonance spectrometer at constant fluence comparable to British summer direct sunlight. The irradiation of sepia melanin as a suspension was also investigated. UVA wavelengths were studied because of the link between UVA and skin aging and a possible association with melanoma [15–17]. Studies were carried out at pH 4.5 because it has been reported that the melanosome may be acidic [18], and detection of superoxide is facilitated at lower pH due to the greater stability of the protonated superoxide, or hydroperoxyl, radical-adduct [13,19,20]. Previously, we found using synthetic eumelanin synthesised from L-dopa autoxidation [13] that DMPO-$O_2H^{\cdot}$ was readily detected at pH 4.5, in approximate steady-state concentrations, at constant UVA fluence comparable to UK levels of sunlight. Detection of DMPO-$O_2H^{\cdot}$ at pH 7, in the presence of melanin, was complicated by metal-ion catalysed decay of DMPO-$O_2H^{\cdot}$ to a hydroxyl-type adduct and hydroxyl radical formation.

2. Materials and methods

Un-dyed human (oriental) and animal (black-haired domestic cat) hair samples were obtained, and heated in alkali (1 M NaOH) for 30 min (90–100 °C). The melanosomes were obtained by three different methods. *Method 1*: melanin was concentrated by gentle acid precipitation (2–3 drops of 3 M HCl) followed by centrifugation (4000*g*) to pellet the melanin, and repeated cycles of washing and precipitation to remove loosely bound protein and other non-melanin components of the hair. After washing, the melanin was resuspended in a small volume of 1 M NaOH to form a concentrated melanin solution (avoiding drying of the pigment at each stage). Melanin concentrations (of the concentrated pigment) were determined by spectrophotometric measurement of the absorbance at 490 nm and compared with a standard synthetic melanin (synthesised from tyrosine and hydrogen peroxide, Sigma) as described previously [13]. The synthetic standard was prepared at 10 mg/ml in pH 4.5 buffer and then both sample and standard diluted 10 fold in 1 M NaOH for spectrophotometry. Pigment concentrates in alkali were then either used immediately, or stored below 0 °C before use. For irradiation experiments the concentrated melanin was diluted in appropriate volumes of pH 4.5 buffer (at least 8 fold to reduce the pH to 4.5), and melanin solutions buffered at pH 4.5 were not used after 24 h at room temperature. *Method 2*: the melanin solution was dialysed for 24 h against PBS and then filtered through flanellette to remove undissolved hair and other debris; the filtered solution was diluted with distilled water and centrifuged 600 g to remove further debris and then ultracentrifuged (14,000*g*) for 10 mins and the resulting melanosome pellet (after removal of the supernatant) re-suspended in phosphate buffer pH 4.5 (NaH_2PO_4). *Method 3*: the melanosomes were isolated using a method based on that described by Liu et al. [21], which involved treatment of cut-up pieces of hair with dithiothreitol (10 mg/ml) and proteinase K (50 μg/ml) in phosphate buffer for 48 h. The dissolved hair was centrifuged as described for method 2 and the melanosome pellet resuspended in pH 4.5 buffer.

Sepia melanin (Sigma) was used as supplied. The powder, which was considerably less soluble in alkali than hair melanin, was solubilised by incubation with 1 M NaOH (at a concentration of 50 mg/ml) for a minimum of 24 h, and the saturated sepia solution then diluted 8 fold with pH 4.5 buffer. Sepia melanin was also prepared directly as a suspension in pH 4.5 buffer using either acetate (Sigma), or dH_2O to appropriate concentrations. Irradiations of sepia suspensions were carried out within 7 days of opening the commercial preparation, unless otherwise specified. The buffered solutions (at pH 4.5) were stored during this period at 4 °C in the dark.

5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) (Sigma) was used as a 0.9 M stock solution in phosphate-buffered-saline (PBS) (Gibco) or in distilled water (dH_2O) and purified before use by filtering through activated charcoal (1 g). In experiments involving DMSO, DMPO (0.9 M) was prepared in 50% aqueous DMSO solution (Sigma).

Synthetic melanin was synthesized from L-tyrosine (2.5 mg/ml) in phosphate buffer or pH 4.5 phosphate buffer (NaH_2PO_4) and mushroom tyrosinase (100 units/ml) in bovine serum albumin (BSA) solution. Synthetic melanin was also obtained commercially (Sigma); and synthesised from L-dopa autoxidation as described previously [13]. The A300/600 absorbance ratios of the different melanins were determined by measuring the UV–visible absorption spectrum at different dilutions (minimum 3), which met the criteria 2.5⟨A300⟩0.25. The absorption limits were defined to ensure all spectra gave measurable A300 and A600 values, and to reduce errors due to saturation of the instrument at high absorbance measurements (usually apparent > 2.5).

Electron-spin-resonance experiments were carried out using a Bruker EMX spectrometer (Rheinstetten/Karlsruhe, Germany) equipped with an ER 4103TM cavity and a Wilmad Glass Co. flat cell (Buena NJ). Typical ESR settings were 20 mW microwave power, 0.05 mT modulation amplitude, 2×10^5 receiver gain, sweep time 20 s with repeated scanning (5 scans) unless otherwise indicated. UV irradiation was carried out in situ in the spectrometer using a super high-pressure 100 W Nikon mercury lamp (model LH-M1100CB-1) focussed on the cavity transmission window. A 5 cm water filter was used to remove infra-red radiation together with two optical

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