Research Letter

Pigment Melanin Scavenges Nitric Oxide In Vitro: Possible Relevance to Keloid Formation

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Recently, nitric oxide (NO) has been implicated in the formation of keloids, preferentially formed in dark-skinned persons, and we suspected that pigment melanin itself may play a direct role by adsorbing NO. We tested the ability of cuttlefish sepia melanin to scavenge (adsorb) NO, generated in situ by 2-(N,N Diethylamino) diazeneolate-2-oxide (DEA/NO), through a dialysis membrane. NO was measured as NO_2^- and NO_3^- by the Griess method and as N_2O_3 by trapping experiments with the fluorogenic substrate 4,5-diaminofluorescein (DAF-2). Initial NO_2^- and NO_3^- concentrations were significantly lower in the test dialyzates than in controls. Scavenging of NO was rapid enough to compete with DAF adduct formation. Both analytical methods gave comparable results. Adsorbed NO and/or its oxidized products may undergo interactions with melanin, adsorbed O_2 , and/or dermal material that may lead to keloid formation.

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1. INTRODUCTION

Melanin pigments are responsible for epidermal skin coloring in humans. Melanin's broad optical absorption and stable free radical properties and binding capability make it an effective in vivo antioxidant [1, 2] photoprotective agent [1, 3], electron transfer agent [4–6], with semiconductor properties [6]. People of color are particularly susceptible to keloids, a recalcitrant consequence of aberrant wound healing, characterized by excessive collagen deposition that extends beyond the original wound [7–9]. Recently, nitric oxide (NO) has been implicated as a key player in the formation of keloids and hypertrophic scars [10] by stimulating over-expression of inducible nitric oxide synthase (iNOS) [11] which is in proximity to melanocytes and dermal collagen [11]. That NO can diffuse through biological membranes [12] suggests that these reactive species can reach the melanosomes within nearby melanocytes, so that NO adsorption to melanin and its sequelae may play a role in keloid formation, particularly in dark-skinned individuals.

2. MATERIALS AND METHODS

Sepia melanin, [13], (MelanInk[®]) was predialyzed through a Spectropore membrane (MW cutoff 6–8 kD) into 100 mL 0.1 M phosphate buffer, pH 7.4/0.1 M EDTA, followed by two changes of 0.1 M buffer alone. As a source of exogenous NO, we used DEA/NO (see Figure 1) (Sigma Chemical Co., Mo, USA). 200 mL of freshly made 0.9 mM DEA/NO stock solution (0.18 μmole) was placed into each of two stirred 25 mL graduated cylinders containing dialysis bags (Spectrum Laboratories, Inc., Calif, USA, MW cutoff 6–8 Kd) filled with (a) 3 mL of a 90 mg melanin buffer suspension (“melanin bag”) or (b) 3 mL buffer alone “control bag.” We measured DEA/NO generated—NO in 200 μL aliquots (1.44 nmole) of dialyzate test and control samples $t = 0$ –90 minutes as nitrite and nitrate by the Griess method with a SOFT maxPRO NO—measuring kit (Molecular Devices). Control experiments confirmed that no melanin escaped into the dialyzate, and there was no significant

FIGURE 1: Structure of DEA/NO. The sodium salt ($R = 1$) was used in the Griess analysis experiments. The diethylammonium salt ($R = 2$) was used for the trapping of NO with the fluorogenic DAF-2 to form the highly fluorescent DAF-2T.

FIGURE 2: Reaction of DAF with NO to form the highly fluorescent DAF-2T.

contamination by nitrite or nitrate prior to addition of DEA/NO.

NO was also detected by its ability of its oxidation product, N_2O_3 , to form highly fluorescent triazoles (DAF-2T) from 4, 5-diaminofluorescein (DAF-2) in the presence of molecular O_2 [14] (see Figure 2). Initially, 10.0 nmole of DAF-2 in buffer solution was mixed in disposable fluorescence cuvettes with the appropriate amount of added buffer to make a total volume of 2.0 mL. After further addition of 1.0 mL of DEA/NO (diethylammonium salt), the fluorescence intensity of melanin and control dialyzates was monitored as functions of time on a Perkin-Elmer 650–40 fluorescence spectrophotometer ($\lambda_{\text{ex}} = 495 \text{ nm}$, $\lambda_{\text{em}} = 515 \text{ nm}$). The *fluorescence scavenging ratio* was the ratio of fluorescence intensities of melanin and control bag dialyzates under steady-state conditions. In some experiments, a 20 mM melanin suspension in the absence of a dialysis membrane was analyzed as before. These latter results were corrected for the absorption and emission of melanin [15].

T-tests were conducted to determine the statistical significance of the results. The determined *P*-values assume normal distributions for each group.

3. RESULTS

3.1. NO measurement as nitrite and nitrate

Pigment melanin rapidly sequesters nitric oxide (see Figures 3(a) and 3(b)). At $t = 0$, the mean $\pm$ S.D. of 3 experiments afforded scavenging ratios of 0.503 ± 0.232 ($P < .03$) for NO_2^- and 0.323 ± 0.266 for NO_3^- ($P < .02$). Initial $[\text{NO}_3^-]/[\text{NO}_2^-]$ ratios were ~ 1.5 .

FIGURE 3: (a) Determination of NO as NO_2^- by Griess analysis in 0.1 M phosphate buffer, pH 7.4. Black circles: initial amount of NO = 1.44 nmole in “melanin bag” (see text). White circles: initial amount of 1.44 nmole in the absence of melanin (“blank bag”; see text). Mean $\pm$ S.D. of 3 determinations, $P < .03$. (b) Determination of NO as NO_3^- by Griess analysis in 0.1 M phosphate buffer, pH 7.4 under the same conditions as in Figure 3(a). Black circles: “melanin bag.” White circles: “blank bag”; see text. Mean $\pm$ S.D. of 3 determinations, $P < .02$.

3.2. Fluorescence measurements

The fluorescence intensities (i.e., DAF-2T formation) of both control and test samples increased to a steady-state value at $t \sim 20$ minutes (see Figure 4). Melanin competes successfully with DAF for NO as evidenced by a steady-state *fluorescence scavenging ratio* = 0.706 ± 0.103 ($n = 4$; $P < .0054$). These results were qualitatively the same whether dialysis systems or melanin suspensions were used.

FIGURE 4: Uptake of NO by sepia melanin as determined by trapping with DAF in 0.1 M phosphate buffer, pH 7.4 (see text). Black Circles: with added melanin (20 μ M in 3 mL). White circles: without added melanin Mean $\pm$ S.D. of 4 determinations, $P < .005$.

4. DISCUSSION

Melanin scavenging of NO takes place rapidly (see Figures 3(a) and 3(b)). The lower steady-state fluorescence intensity in the presence of melanin (see Figure 4) confirms that scavenging competes successfully with the relatively slow [14] triazole formation, and strongly suggests that NO itself is initially adsorbed to melanin. Adsorbed NO can form a variety of active species in oxygen-containing solution including NO₂, N₂O₃, O₂^{•-}, and ONOO⁻, and could change melanin's oxidation state [13, 16]. Any of these might stimulate keloid formation and might offer a basis for the observation that African-Americans, with higher and more robust concentrations of melanosomes, are more susceptible to keloids than are Caucasians.

Reaction of gaseous NO in solution with H₂O₂ and/or O₂^{•-} arising from melanin autooxidation [17] is unlikely to be significant. Since melanin acts as an efficient "pseudodismutase" [17], the steady-state [O₂^{•-}] is low outside the melanin "cage." Hydrogen peroxide does not react with NO [18].

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