The Role of Eumelanin in Generating Reactive Oxygen and Reactive Nitrogen in Solution: Possible Relevance to Keloid Formation

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ABSTRACT

Recently, nitric oxide (NO) has been implicated as an epigenetic factor in keloids, a scarring disease occurring primarily in dark skinned people who have relatively high amounts of pigment melanin. In this work, we tested whether a melanin-mediated redox reaction involving adsorbed NO and O₂ can couple NO oxidation with O₂ reduction to form reactive oxygen species (ROS) or reactive nitrogen species (RNS) in vitro at pH 7.4. We measured the formation of reactive species that oxidize dihydrorhodamine123 (DHR) to fluorescent rhodamine123 in the presence and absence of sepia melanin. In separate experiments, we monitored NO concentration with 4,5-diaminofluorescein (DAF) by measuring the highly fluorescent NO-adduct, DAF-2T. We attempted to detect peroxynitrite with 5 µM 3-methyl-1,2-cyclopentanedione (MCP), a selective scavenger of peroxynitrite (IC₅₀ = 3.6 µM for ONOO⁻ vs. 63.8 µM and >> 100 µM for NO and O₂⁻ respectively). However, MCP itself oxidized DHR. We found that in the absence of NO, melanin itself oxidizes DHR, with no loss of DAF-fluorescence (i.e. no net consumption of NO). In the presence of NO, there was a ~57% loss of DAF fluorescence, indicating that NOₓ is formed at the expense of NO. The data provided good fit (r² = 0.94) to a Langmuir adsorption isotherm, with pseudo first order rate k' = 8.2 × 10⁷ s⁻¹ and adsorption coefficient Kad = 4.04 M⁻¹. Both of these parameters are consistent with a facile chemisorption reaction between NO and O₂ on the melanin surface. Possible reactions are a) NO and O₂⁻ → ONOO⁻ and/or b) 2NO + O₂ → 2NO₂. The latter reaction is disfavored in solution but is significantly accelerated on the melanin surface via an entropy effect.

Keywords: Melanin; Nitric Oxide; NOₓ; Redox

1. Introduction

Pigment eumelanin binds a wide variety of compounds [1] and can act as a redox reagent [2,3]. Depending on its chemical environment, melanin can either donate or accept electrons from ambient molecules and/or couple the oxidation of one compound with the reduction of another [4-6]. Melanin is well known to undergo autoxidation as well as Fenton chemistry with bound or adventitious transition metals (usually iron or copper) to produce OH radicals. Depending on the relative metal and pigment concentrations, melanin may act as either an antioxidant or a pro-oxidant [7,8].

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 [9-11]. The etiology of keloids is complex, and both genetic [12] and epigenetic [13] factors are involved. Recent work [14-16] has indicated that nitric oxide (NO) enhances the synthesis of type I collagen by dermal fibroblasts and that the keloid lesion may arise from the NO/cGMP signaling pathway. The particular susceptibility of darkly-pigmented persons to keloids is intriguing, and it leads one to ask whether pigment itself may play an active role in keloid pathogenesis, given its ability to bind and mediate redox
reactions of bioactive molecules.

Recently, [4] we demonstrated that sepia melanin scavenged NO in solution through a cellulose dialysis membrane. Crippa et al. [17] demonstrated that molecular O₂ was adsorbed by melanin, and our observation of NO scavenging by melanin suggested a similar adsorption process for NO. We thought it quite conceivable that the scavenged nitric oxide could be oxidized to additional reactive nitrogen species (RNS; NOₓ) by direct redox reaction with melanin and/or with adsorbed O₂ or reactive oxygen species (ROS) that arose in the course of melanin autoxidation [2,3]. We used dihydrorhodamine 123 (DHR) oxidation to measure the formation of ROS and RNS in vitro at pH 7.4 in the presence and absence of added NO. We show that melanin can adsorb NO and couple the oxidation of NO with the reduction of O₂.

2. Methods

Buffer: All experiments were carried out in 0.1 M phosphate buffer (“buffer”) pH 7.4 made up in ultrapure deionized water. In several experiments, buffer was passed through a Chelex-100 resin column (8.0 × 1.0 cm) before use. This treatment had no effect on the reaction.

Melanin preparation: Impure sepia melanin was obtained as a kind gift from Dr. Miles Chedekel (Melan-Link®). Protein was removed by incubating this sample at 60°C for 30 min in 0.1 M HCl followed by predialysis 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.

Nitric oxide-generating compounds: We used freshly-prepared stock solutions of 0.1 mg/ml S-nitroso-N-acetyl-DL-penicillamine (SNAP; Sigma Chemical Co.) dissolved in 2.0 ml 95% ethanol and diluted to 10 ml through a Spectropore membrane (MW cutoff 6 - 8 kD) for 30 min in 0.1 M HCl followed by predialysis 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.

Detection of nitric oxide: Nitric oxide reacts with 4,5-diaminofluorescein (DAF) in the presence of molecular O₂ to form a highly fluorescent triazole (DAF-2T) [18]. Fluorometric measurements were made on 200 µl aliquots of control and test dialyzates, which were diluted to 1.0 ml in separate plastic cuvettes. At time t = 0, 1.0 ml of SNAP solution was added to each cuvette and the DAF-2T fluorescence intensity (excitation 495 nm, emission 515 nm) measured in a Perkin-Elmer 650 - 40 Fluorescence Spectrophotometer (Perkin Instruments, Norwalk, CT) as a function of time. The dialyzates of the control and test samples (200 µl) were analyzed for NO at times ranging from 0 to 120 minutes. Steady-state fluorescence levels were reached at 60 minutes under our experimental conditions. Data were expressed as the ratio sample/control (SNAP alone) at t = 100 minutes.

The Interaction of Sepia Melanin with nitric oxide: The reaction system consisted of 22 ml 0.1 M phosphate buffer (pH = 7.4) in a 25 ml graduated cylinder, surrounding dialysis bags that contained either a 5 ml suspension of 5 mg sepia melanin or 5 ml buffer alone (see below). Nitric oxide levels were measured from the test and control dialysis filtrates as previously described [4].

Detection of NOₓ: Oxidation of dihydrorhodamine 123 (DHR): Dialysis systems were prepared as before, with the addition of 1.0 ml of 2.0 mM dihydrorhodamine 123 (DHR; Life Sciences) in 25 ml of total solution. Control systems were a) SNAP (NO) alone (blank); b) SNAP (NO) + DHR (NO control); c) Melanin + DHR (melanin control). Test systems contained d) Melanin + DHR + SNAP (NO). (Oxidized) rhodamine fluorescence (excitation 475 nm; emission 540) was recorded for 0.5 ml aliquots of test and control systems as functions of time.

Estimation of the rate of NO oxidation: After correcting the DHR oxidation curves for melanin-produced OH, which oxidizes DHR [2,3,19], we used the initial part of the DHR oxidation curves which yielded reasonably straight lines and avoided build-up of secondary RNS products (e.g. NO₂) that could oxidize DHR [19]. Under these conditions, the rate of DHR oxidation is taken to be equal to the rate of disappearance of NO. For data analysis, the net oxidation of DHR by NOₓ was determined by subtracting the contribution of each control curve (see Figure 1, below). The corrected rate of DHR oxidation by NO is proportional to the melanin-mediated formation of NOₓ. The steady state concentration of NO (t) is proportional to added SNAP concentration at t = 0. We found the relationship between Rhodamine fluorescence and NOₓ concentration by means of a standard curve.*

*For further discussion, see Appendix.

Langmuir adsorption isotherm of melanin-coupled redox reaction(s) [4,20]: The Langmuir adsorption isotherm assumes that the reaction takes place with the reactants chemisorbed to the melanin surface. If θₜₙₒ and θₒ₂ are the fractions of NO and O₂ melanin binding sites bound by NO and O₂ respectively, we have:

\[ V = k\thetaₜₙₒ \thetaₒ₂ = k'\thetaₜₙₒ \]  \hspace{1cm} (1)

The Langmuir treatment yields [20]

\[ \thetaₜₙₒ = Kₐd [NO]/(1 + Kₐd [NO]) \]  \hspace{1cm} (2)

where [NO] is the solution concentration of NO in M, Kₐd = kₐ/kₜ is the adsorption coefficient, i.e. the ratio of ad-
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Figure 1. Oxidation of DHR by NO (white circles), Eumelanin (black triangles) and Eume lanin/NO system (white triangles) in 0.1 M phosphate buffer, pH 7.4 (see text). DHR is slowly autoxidized (black circles). In the absence of added melanin, NO slowly oxidizes DHR (white circles). Melanin itself oxidizes DHR at a significant rate with significant upward concavity (black triangles); the additional increases in DHR oxidation and concavity in the presence of NO suggests the generation of NOx (white triangles).

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sorption/dissociation constants of NO on the melanin backbone, having the units of M$^{-1}$. We assume that $k' = k \theta_{b_0}$ in O$_2$ saturated (aerated) solution, is equal to the pseudo first order rate constant for the bimolecular reaction between O$_2$ and NO on the melanin surface having the dimensions of s$^{-1}$ (see [17,20] for discussion). Substitution of (2) into (1) followed by inversion yields

$$\frac{1}{V} = \frac{1}{k'K_{ad}} [\text{NO}] + \frac{1}{k'}$$

Thus, if Langmuir mechanisms are operative, slopes of $1/V$ vs. $1/[\text{NO}]$ will yield a straight line with slope $1/k'K_{ad}$ and intercept $1/k'$ from which parameters $k'$ and $K_{ad}$ can be found.

The steady state concentration, [NO]$_{ss}$ was estimated from rate constants for formation and decline of NO in consecutive reactions. These constants were found from the approximate lifetimes of SNAP (~5 hr.) and NO decomposition in aqueous solution (~2 s). Under these conditions, NO is consumed virtually as fast as it is formed, and [NO] could be assumed to be invariant with time. Using these values, the integrated rate equation [20] ultimately yielded the value:

$$[\text{NO}]_{ss} = 1.12 \times 10^{-4} \left[ \text{SNAP}_{(0)} \right].$$

*For further discussion, see Appendix.

Statistical analysis: We used Student’s t test for small samples. Data were expressed as mean ± SD. Differences were considered significant for p < 0.05.

3. Results

DHR oxidation: Figure 1 shows the results of a representative experiment (n = 4) in aerated solution. Control groups were DHR alone (black dots); DHR + NO in the absence of melanin (white dots) and melanin + DHR (black triangles). DHR alone was slowly autoxidized to fluorescent rhodamine 123. Nitric oxide modestly increased the rate of DHR oxidation Melanin efficiently oxidized DHR with significant upward concavity. The test system, melanin + NO + DHR (white triangles), further increased the rate of growth and concavity. Qualitatively similar results were obtained for each experiment.

Attempts to Detect NO$_x$: Consumption of NO in solution: At 5 µM, 3-methyl-1,2-cyclopentanedione (MCP; Aldrich Chemical Co.) selectively scavenges ONOO$^-$ (IC$_{50}$ = 3.6 µM for ONOO$^-$ vs. 63.8 µM and >> 100 µM for NO and O$_2$ respectively [21]). Although we originally planned to use MCP to detect peroxynitrite, this did not prove successful, since MCP itself oxidized DHR, (not shown). However, we did test for NO consumption in the test system and control samples by using 4,5-diaminofluorescein (DAF-2; Sigma Chemical Co.) which forms a highly-fluorescent triazole adduct with NO (DAF-2T), [18] and which does not react with DHR. Figure 2 shows that 5 µM MCP scavenges NO at ~20% (Figure 2(b)). Nitric oxide consumption by melanin alone in the course of DHR oxidation does not occur (compare Figures 2(b) with 2(c)). However, the simultaneous presence of melanin and NO (Figure 2(d)) resulted in consumption of NO by an additional 57%, suggesting concomitant NO$_x$ formation at the expense of NO.

Figure 2. Disappearance of NO (SNAP) in the presence of sepia melanin in oxygen-saturated solution. DAF forms a highly fluorescent triazole DAF-2T in presence of NO. MCP (5 µM) scavenges virtually all ONOO$^-$ (IC$_{50}$ = 3.6 µM for ONOO$^-$ vs. 63.8 µM and >> 100 µM for NO and O$_2$ respectively [21]). Although we originally planned to use MCP to detect peroxynitrite, this did not prove successful, since MCP itself oxidized DHR, (not shown). However, we did test for NO consumption in the test system and control samples by using 4,5-diamino fluorescein (DAF-2; Sigma Chemical Co.) which forms a highly-fluorescent triazole adduct with NO (DAF-2T), [18] and which does not react with DHR. Figure 2 shows that 5 µM MCP scavenges NO at ~20% (Figure 2(b)). Nitric oxide consumption by melanin alone in the course of DHR oxidation does not occur (compare Figures 2(b) with 2(c)). However, the simultaneous presence of melanin and NO (Figure 2(d)) resulted in consumption of NO by an additional 57%, suggesting concomitant NO$_x$ formation at the expense of NO.

*For further discussion, see Appendix.

Statistical analysis: We used Student’s t test for small samples. Data were expressed as mean ± SD. Differences were considered significant for p < 0.05.
Kinetic Analysis of Melanin-Mediated NOx Formation: Previously, we and others have found that melanin can couple oxidation of one species with reduction of another. To test the hypothesis that melanin can mediate a redox reaction involving NO [4] and O2 [17] adsorbed on its surface, we analyzed our data according to a simple Langmuir adsorption isotherm [20].

Figure 3 shows a Langmuir plot of 1/V vs. 1/[NO]. This plot yielded a straight line, in good agreement with the Langmuir model ($\rho^2 = 0.94$). From the slope and intercept, we obtained a value of pseudo first order constant $k' = 8.2 \times 10^7$ s$^{-1}$ and adsorption coefficient $K_{ad} = 4.04$ M$^{-1}$.

4. Discussion

In the absence of sepi melanin, NO in aerated solution slowly oxidizes indicator DHR. Sepia melanin alone oxidizes DHR rapidly, but the addition of NO still further increases the rate of DHR oxidation. Figure 1 indicates that there is a synergistic effect in DHR oxidation when both melanin and NO are present.

Sepia melanin is known to consume O2 in aerated solution, producing H2O2 via a superoxide intermediate [2,3]. Neither superoxide H2O2 nor physiological NO concentrations will oxidize DHR, however, OH does oxidize DHR to fluorescent Rhodamine123 [19]. Hydroxyl radical could be generated from decomposition ONOOH at pH 7.4, [20] natural sepi melanin, [3] as well as adventitious and/or melanin-bound iron and copper ions. Bound transition metals are very difficult to remove, even after exhaustive dialysis against EDTA [1].

Figure 2 shows that under these experimental conditions, the NOx scavenger MCP does not scavenge ROS (compare Figures 2(b) and (c)). However, MCP will oxidize DHR (not shown). As indicated in Figure 2, 5.0 μM MCP scavenges ~20% of NO. There is an additional 57% NO consumption in the presence of added NO, presumably via formation of NOx. The results from Figure 2 confirm that the DHR-oxidizing species from melanin in the absence of NO is not NOx.

Crippa et al. [17] demonstrated that molecular O2 is adsorbed by melanin. We have observed melanin scavenging of NO that suggests a similar adsorption process [4]. The observed fit of our data to a simple Langmuir adsorption isotherm strongly suggests that the melanin-mediated reactions leading to increased DHR oxidation do involve adsorbed NO and O2 species. It seems reasonable that sepi melanin could couple NO oxidation with oxygen reduction to O2$. Super oxide and NO might then combine to form ONOO$ in a fast reaction

$$k_{NO2-O2} = 1.6 \times 10^{10} \text{ M}^{-1} \cdot \text{s}^{-1}$$

Alternatively, 2 adsorbed NO molecules in close proximity to molecular O2 could react to form NO2 and other NOx species. In the absence of melanin the reaction of 2 molecules of NO with one molecule of O2 to afford 2 molecules of NO2 is slow, (pseudo second order constant,

$$k_{NO-O2} = 4 \times 10^7 \text{ M}^{-1} \cdot \text{s}^{-1}$$

at NO saturation, (≈2 mM in aqueous solution), because it depends on a simultaneous attack of two molecules of NO on a molecule of O2 [23]. However, in adsorbing (“ordering”) NO and O2 on its surface, melanin might well facilitate formation of NO2, by significantly decreasing the entropy of the reaction. Our data does not presently allow distinction between these mechanisms.

Although comparison of our rate constant with solution data is not straightforward because of complications having to do with the melanin surface, our value of 8.2 × 10$^7$ s$^{-1}$ is in reasonable agreement with a value obtained from Crippa [17] who used a more rigorous treatment to obtain a value of 5.7 × 10$^6$ s$^{-1}$ for reduction of dioxygen by conduction band electrons of colloidal melanin (for discussion, see [17]).

Hsu et al. [16] found that keloid tissue expression showed significant increases in iNOS gene, NOx production, type I collagen mRNA, and type I collagen protein expression compared to normal fibroblasts. Our present work demonstrates that melanin adsorbs NO and mediates oxidation to NOx, with possible epigenetic cones-
quences. Although the consequences of these interactions are not well known with respect to keloids, one might gain insight by considering the closely-related case of systemic sclerosis, where imbalances in NO metabolism, type I collagen expression, and the occurrence of nitrated proteins have been known to occur for certain disease states [24-26]. Melanin sequestration of NO and its role in generating toxic NOx might have similar effects on normal fibroblasts that could possibly result in their transformation to keloid fibroblasts. This latter question is currently under investigation in our laboratory (Nok-kaew et al., in progress).

5. Conclusions

a) Sepia melanin accelerates (couples) the oxidation of NO to NO₂ and the reduction of molecular O₂ in buffered aqueous solution.

b) The reaction kinetics fit well to a simple Langmuir adsorption isotherm, and the reaction therefore appears to take place between adsorbed NO and O₂ via a chemisorption mechanism. We obtained a value of pseudo first order constant \( k' = 8.2 \times 10^{-4} \text{s}^{-1} \) and adsorption coefficient \( K_{ad} = 4.04 \text{M}^{-1} \).

c) Such a reaction occurring in situ in dermal tissue might have significant epigenetic consequences in keloid formation or related pathologies.

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REFERENCES


The concentration of NO is proportional to added SNAP. To make a standard curve, known concentrations of SNAP were treated with rhodamine B* and their rhodamine 123 fluorescence and [NO] were measured by means of a fluorimeter. In this case, NO decomposes as two consecutive reactions, of the form (2a):

\[
\frac{d[SNAP(t)]}{dt} = -k_2[SNAP(t)] + k_1[SNAP(o)] \exp(-k_1t)
\]

Substituting these values into the integrated equation (see 20) subsequently afforded the value of [NO] at steady state.

*Since the absorption/emission maxima of rhodamine B are red-shifted by ~1100 cm\(^{-1}\) with respect to rhodamine 123 and the quantum yields of fluorescence, \(\phi_f\) are similar, we shifted the excitation/emission measurement to 496 nm and 587 nm. The choice of excitation wavelength was chosen so that both samples absorbed the same amount of excitation radiation.

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**Appendix**

For analysis of the raw data from the Langmuir plot, the net oxidation of DHR by NO\(_x\) was determined by subtracting the contribution of each control curve (see Figure 1). The rate of DHR oxidation is proportional to the concentration of NO\(_x\); the concentration of steady state NO was estimated from the half-life of SNAP decomposition (~2 s). These allowed estimation of the respective rate constants for NO arising from decomposition of SNAP, (\(k_1 = 3.9 \times 10^{-5} \text{ s}^{-1}\) and \(k_2 = 0.347 \text{ s}^{-1}\) for decomposition of NO. Treating the process as two consecutive reactions, of the form (20) we get:

\[
\begin{align*}
\text{SNAP} \rightarrow& \ x \text{NO} \rightarrow \ x \text{NO}_x \\
\end{align*}
\]

In this case, \(k_2 \geq k_1\), and NO is consumed as fast as it is formed, leaving a steady-state NO concentration, \([\text{NO}_x]_s\). This treatment yields the differential equation for [NO]

\[
\frac{d[\text{SNAP}(t)]}{dt} = -k_2[\text{SNAP}(t)] + k_1[\text{SNAP}(o)] \exp(-k_1t)
\]