Open Journal of Physical Chemistry, 2013, 3, 157-162
Published Online November 2013 (http://www.scirp.org/journal/ojpc)
http://dx.doi.org/10.4236/ojpc.2013.34019

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

Julian M. Menter[1], Comnuan Nokkaew[2], Danita Eatman[3], Aquilla Sprewell[1], Natalia Silvestrov[3], Abrienne M. Patta[1], Sandra Harris-Hooker[2]

[1]Department of Microbiology, Biochemistry and Immunology, Morehouse School of Medicine, Atlanta, USA
[2]Office of Sponsored Research, Department of Pathology, Morehouse School of Medicine, Atlanta, USA
[3]Analytical Laboratory, Department of Pharmacology and Toxicology, Morehouse School of Medicine, Atlanta, USA
Email: jmenter@msm.edu

Received March 26, 2013; revised April 25, 2013; accepted May 1, 2013

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 O_2 can couple NO oxidation with O_2 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 O_2^- 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 NO_x is formed at the expense of NO. The data provided good fit (r^2 = 0.94) to a Langmuir adsorption isotherm, with pseudo first order rate $k' = 8.2 \times 10^7$) s^{-1} and adsorption coefficient K_{ad} = 4.04 M^{-1}. Both of these parameters are consistent with a facile chemisorption reaction between NO and O_2 on the melanin surface. Possible reactions are a) NO and $O_2^- \rightarrow ONOO^-$ and/or b) $2NO + O_2 \rightarrow 2NO_2$. The latter reaction is disfavored in solution but is significantly accelerated on the melanin surface via an entropy effect.

Keywords: Melanin; Nitric Oxide; NO_x; 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 O_2 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; NO_x) by direct redox reaction with melanin and/or with adsorbed O_2 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 O_2.

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 results.

Melanin preparation: Impure sepia melanin was obtained as a kind gift from Dr. Miles Chedekel (Melan-Ink@). 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-nitorso-N-acetyl-DL-penicillamine (SNAP; Sigma Chemical Co.) dissolved in 2.0 ml 95% ethanol and diluted to 10 ml with buffer (final concentration 4.5×10^{-5} M). Donors (250 µl of 0.02 mg/ml diluted stock) were added to test and control dialyzates in a total of 22 ml in a 25-ml graduated cylinder under mechanical stirring. All reactions involving use of SNAP were carried out in subdued light.

Detection of nitric oxide: Nitric oxide reacts with 4,5-diaminofluorescein (DAF) in the presence of molecular O_2 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 NO_x: 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 $^{\cdot}OH$, 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. NO_2) 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 NO_x 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 NO_x. The steady state concentration of NO (t) is proportional to added SNAP concentration at $t = 0$. We found the relationship between Rhodamine fluorescence and NO_x 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 θ_{NO} and θ_{O_2} are the fractions of NO and O_2 melanin binding sites bound by NO and O_2 respectively, we have:

$$V = k\theta_{NO}\theta_{O_2} = k'\theta_{NO} \quad (1)$$

The Langmuir treatment yields [20]

$$\theta_{NO} = K_{ad}[NO]/1 + K_{ad}[NO] \quad (2)$$

where [NO] is the solution concentration of NO in *M*, $K_{ad} = k_a/k_d$ is the adsorption coefficient, *i.e.* the ratio of ad-

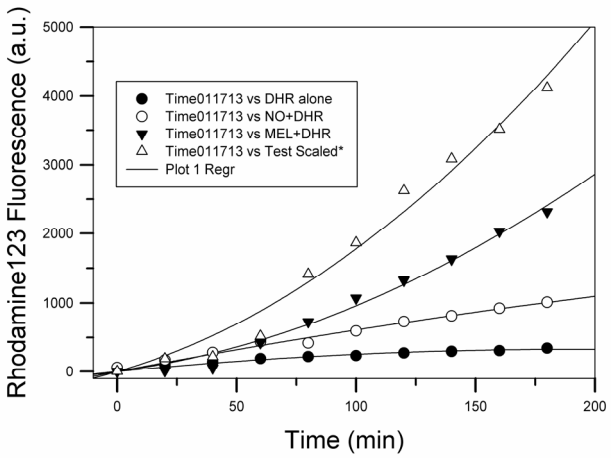

Figure 1. Oxidation of DHR by NO (white circles), Eumelanin (black triangles) and Eumelanin/NO system (white triangles) in 0.1 *M* phosphate buffer, pH 7.4 (see text). DHR is slowly autoxidized (black circles). In the absence of added melanin, NO slowly oxidizes DHR (white circles). Melanin itself oxidizes DHR at a significantly greater rate with significant upward concavity (black triangles); the additional increases in DHR oxidation and concavity in the presence of NO suggests the generation of NO_x (white triangles).

sorption/dissociation constants of NO on the melanin backbone, having the units of M^{-1}. We assume that $k' = k\theta_{O_2}$ 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

$$1/V = 1/k' K_{ad}[\mathrm{NO}] + 1/k' \quad (3)$$

Thus, if Langmuir mechanisms are operative, slopes of $1/V$ vs. 1/[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, $[\mathrm{NO}]_{ss}$ 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:

$$[\mathrm{NO}]_{ss} = 1.12\times10^{-4}\left[\mathrm{SNAP}_{(0)}\right]^{*}.$$

*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 $ONOO^-$ (IC50 = 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.

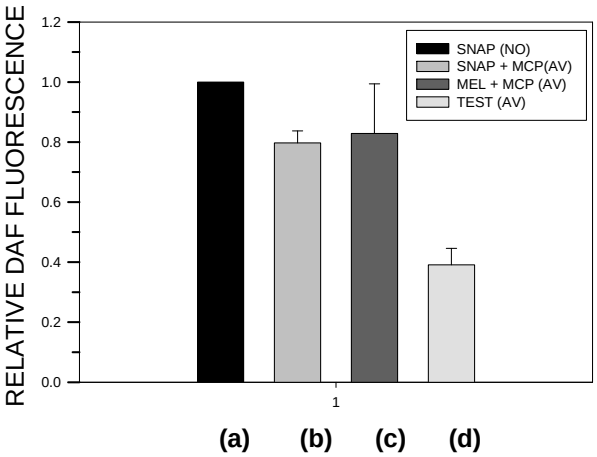

Figure 2. Disappearance of NO (SNAP) in the presence of sepia melanin in oxygen-saturated solution. DAF forms a highly fluorescent triazole DAF-2T in presence of NO. MCP (5 μM) scavenges virtually all ONOO (IC 50 = 3.6 μM), but only slightly scavenges NO (IC 50 = 63.3 μM), Taken together, Figures 1 and 2 suggest NO_x is formed at the expense of NO. (a) SNAP (NO) alone; (b) SNAP + MCP; (c) Melanin + MCP; (d) Melanin + MCP + SNAP. Data are expressed as *mean* ± *S.D*, *n* = 4.

Kinetic Analysis of Melanin-Mediated NO_x 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 O_2 [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 (r^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 sepia 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 O_2 in aerated solution, producing H_2O_2 via a superoxide intermediate [2,3]. Neither superoxide H_2O_2 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 sepia 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].

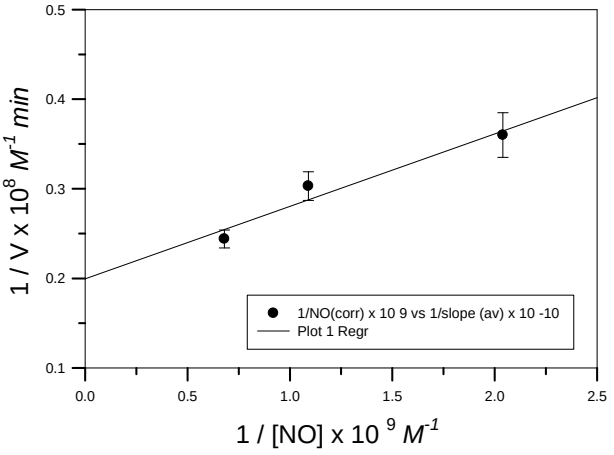

Figure 3. Langmuir Adsorption curve for NO + O_2 coupled redox reaction on sepia melanin surface. After correction for production of ROS by melanin, oxidation of DHR by NO in solution and spontaneous oxidation of DHR by ambient O_2 (see Figure 1), rates of production of NO_x were measured by rhodamine 123 fluorescence measurements (see *methods* section). Steady-state [NO] as a function of time was estimated from SNAP decomposition rates and from the lifetime of NO in aqueous solution (see text). Plots of $1/V$ vs. 1/[NO] afforded a straight line, (r^2 = 0.94) that intersected the 1/V axis above the origin, strongly indicating a chemisorption mechanism, with adsorption coefficient K_{ad} = 4.04 M^{-1} and k' = 4.98 × 10^9 min^{-1} = 8.2 × 10^7 s^{-1}.

Figure 2 shows that under these experimental conditions, the NO_x 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 NO_x. The results from **Figure 2** confirm that the DHR-oxidizing species from melanin in the absence of NO is not NO_x.

Crippa *et al.* [17] demonstrated that molecular O_2 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 O_2 species. It seems reasonable that sepia melanin could couple NO oxidation with oxygen reduction to O_2^-. Superoxide and NO might then combine to form $ONOO^-$ in a fast reaction $\left(k_{NO+O_2} = 1.6\times10^{10}\ M^{-1}\cdot s^{-1}\right)$ [22]. Alternatively, 2 adsorbed NO molecules in close proximity to molecular O_2 could react to form NO_2 and other NO_x species. In the absence of melanin the reaction of 2 molecules of NO with one molecule of O_2 to afford 2 molecules of NO_2 is slow, (pseudo second order constant, $k_{NO+O_2} = 4\times10^4\ M^{-1}\cdot s^{-1}$) at NO saturation, (~2 mM in aqueous solution), because it depends on a simultaneous attack of two molecules of NO on a molecule of O_2 [23]. However, in adsorbing ("ordering") NO and O_2 on its surface, melanin might well facilitate formation of NO_2, 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 straight-forward 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, NO_x 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 NO_x, 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 NO_x 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 (Nokkaew *et al.*, in progress).

5. Conclusions

a) Sepia melanin accelerates (couples) the oxidation of NO to NO_x and the reduction of molecular O_2 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 O_2 via a chemisorption mechanism. We obtained a value of pseudo first order constant $k' = 8.2 \times 10^7\ s^{-1}$ and adsorption coefficient $K_{ad} = 4.04\ M^{-1}$.

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

6. Acknowledgements

This work was funded in part by GRANTS: MBRS #GM08248, RCMI #8G12MD007602, and DOD # 911 NF-10-1 0448. There are no conflicts of interest.

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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 formation of NO_x; the concentration of steady state concentration of NO is proportional to added SNAP concentration at $t = 0$. We found the relationship between rhodamine 123 fluorescence and [NO] by means of a standard curve made by known concentrations of rhodamine B*.

The steady state [NO] was estimated from the half-lives of SNAP decomposition ($\tau \sim 5$ hr) and NO decomposition ($\tau \sim 2$ s). These allowed estimation of the respective rate constants for NO arising from decomposition of SNAP, ($k_1 = 3.9\times10^{-5}\ \mathrm{s}^{-1}$ and $k_2 = 0.347\ \mathrm{s}^{-1}$ for decomposition of NO. Treating the process as two consecutive reactions, of the form [20] we get:

$$[\mathrm{SNAP}] \xrightarrow{k_1} \mathrm{NO} \xrightarrow{k_2} \mathrm{NO_x} \qquad (1a)$$

In this case, $k_2 \geq k_1$, and NO is consumed as fast as it is formed, leaving a steady-state NO concentration, $[\mathrm{NO}]_{ss}$. This treatment yields the differential equation for [NO]

$$\mathrm{d}\left[\mathrm{SNAP}(t)\right]/\mathrm{d}t = -k_2\left[\mathrm{SNAP}(t)\right] + k_1\left[\mathrm{SNAP}(o)\right]\exp(-k_1 t) \qquad (2a)$$

Substituting these values into the integrated equation (see 20) subsequently afforded the value of

$$[\mathrm{NO}]_{ss} = 1.12\times10^{-4}\left[\mathrm{SNAP}(o)\right](\mathrm{M})$$

*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, φ_f very similar, we shifted the excitation/emission measurement to 496 nm and 587 nm. The choice of excitation wavelength was chosen so that the both samples absorbed the same amount of excitation radiation.