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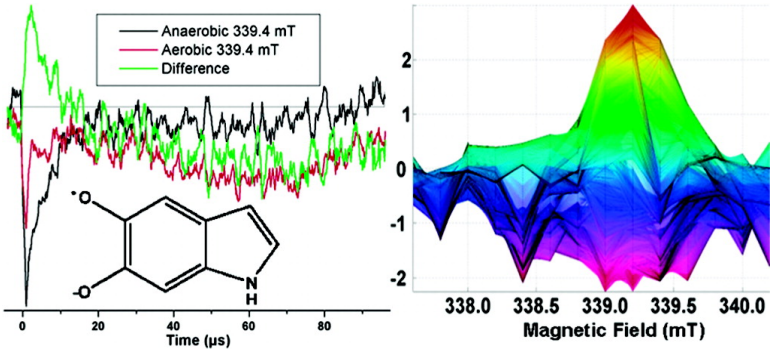

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Time-Resolved Detection of Melanin Free Radicals Quenching Reactive Oxygen Species

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Melanin is a heterogeneous biological polymer widespread in the biosphere and contains a population of *intrinsic*, quinone/semiquinone-like radicals.[1] Melanin, composed of many different monomers,[2] is the only known biopolymer containing *intrinsic* stationary free radicals.[1] Additional *extrinsic* semiquinone-like radicals are reversibly photogenerated in melanin under visible or UV irradiation.[2] Although different melanins are distinguishable by EPR,[3] the free radical chemistry of melanin is complex and not well characterized, especially the photochemistry of melanin in the presence of oxygen.

Melanin is found in the retinal pigment epithelium (RPE) of eyes. The RPE is a monolayer of cuboidal cells between the photoreceptors and choriocapillaris of the eye. The chemistry of the RPE is specialized to phagocytize and recycle the outer segment of photoreceptors and molecular retinaldehyde, the chromophore of rhodopsin.[4] RPE melanin, predominantly eumelanin, composed of monomers that are oxidation products of tyrosine, serves a photoprotective role by absorbing radiation and by scavenging reactive free radicals and reactive oxygen species (ROS).[1,5] However evidence also supports a phototoxic role of RPE melanin, especially in aged cells, including the increased photogeneration of ROS such as superoxide anions and hydroxyl radicals that are implicated in RPE cell death.[5,6] Melanin chemistry is of especial interest because RPE cell death is a major feature of the pathogenesis of age-related macular degeneration (AMD), the leading cause of blindness in the human population older than 60 years of age in the developed world.[7]

Since RPE cells, which must last a lifetime, are physiologically exposed to high oxidative stress and damaging irradiation,[7−9] the mechanism of survival is of general chemical interest. To determine directly if RPE melanin indeed scavenges photogenerated ROS, nanosecond time-resolved electron paramagnetic resonance (TREPR) spectroscopy was used to study melanin free radicals in human retinal pigment epithelium (RPE) cells under aerobic and anaerobic conditions.

RPE cells were obtained from human eyes[10] (Advanced Bioscience Resources Inc., Alameda, CA), cultured, and prepared for EPR experiments as described in the Supporting Information. Details of the EPR experimental design are also provided in the Supporting Information. TREPR experiments were conducted while the RPE cells flowed past the laser pulse under anaerobic or aerobic conditions sustained by slow, continuous bubbling of compressed O_2 or Ar through the cell volume. Typically, 15 TREPR time profiles were accumulated at individual field positions and used to produce the 3D spectra. The *g*-values (∼2.004) of the 3-D TREPR spectra as well as continuous wave (CW) EPR spectra of RPE melanin are compatible with assignment to *extrinsic* melanin free radicals.

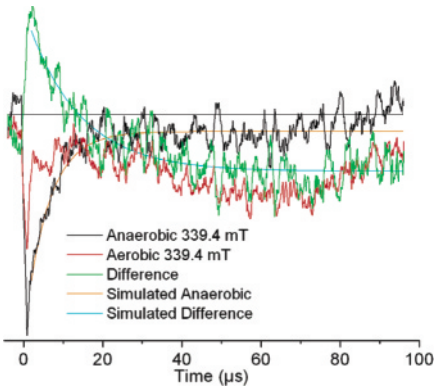

Figure 1. Representative TREPR time profiles of RPE melanin.

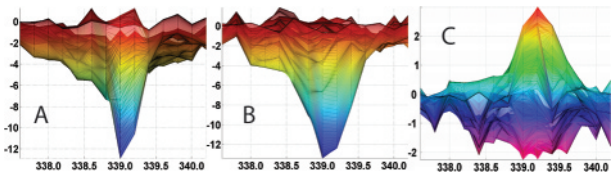

Figure 2. 3D TREPR spectra of RPE melanin: (A) aerobic, (B) anaerobic, and (C) difference, aerobic minus anaerobic.

Photochemistry resulting in TREPR signals was observed for several RPE samples (Figure 1). A large melanin *intrinsic* free radical CW EPR signal (see Supporting Information) is present in the dark. In the presence of visible or UV light, the CW EPR signal grows to a steady state, accompanied by the photoproduction of *extrinsic* free radicals and a shifting of the CW EPR *g*-value, and then reversibly decays back to the original dark *intrinsic* signal.

However, within 250 ns after the 15 ns laser pulse, at all resonant field values, the TREPR signal goes sharply negative (Figure 2). The negative amplitude of the TREPR signal represents microwave emission, indicating that melanin free radicals are generated from photoexcited triplet states.[11] In interpreting these data, it is useful to note that larger negative signals are indicative of increased free radical concentrations (see Supporting Information). Central to this work, a difference between aerobic and anaerobic conditions is readily observed, best documented by subtracting the anaerobic time profiles (Figure 1, black) from the aerobic TREPR time profiles (Figure 1, red). Under anaerobic conditions, the TREPR signal decays to the baseline with an exponential lifetime of approximately 13 μs (Figure 1, black (experimental) and orange (simulation)). However, the difference signal is initially positive and then decays to a negative baseline with an exponential decay lifetime of approximately 30 μs (Figure 1, green (experimental) and cyan

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(simulation)). The difference 3D spectrum (Figure 2C) is more symmetrical than the total spectrum (Figure 2B) and is positioned about 0.1 mT upfield from the total spectrum. This symmetrical difference signal indicates that, while at least two types of melanin radicals, M(1)$^\bullet$ and M(2)$^\bullet$, are present in the photoproduced *extrinsic* radical population, only one of the melanin radical species, M(2)$^\bullet$, is predominately involved in photochemistry with oxygen species. The species reacting with M(2) is not observed by TREPR, most likely because the associated EPR signal is too broad and weak for detection with the current signal-to-noise. Because the difference spectrum decays to negative baselines (Figures 1, green, and 2C), the difference spectrum establishes that, in the presence of oxygen, a chemical process occurs in which more melanin free radicals of a particular species are ultimately destroyed rather than created. In other words, some *intrinsic* free radicals, i.e., radicals present in the dark, are destroyed. The following scheme has been used to simulate the general features of the 3D spectra of Figure 2:

$$\mathrm{M(1)} + \mathrm{M(2)} + h\nu \rightarrow \mathrm{M(1)}^\bullet + \mathrm{M(2)}^\bullet \quad (1)$$

$$\mathrm{O_2} + h\nu \rightarrow \mathrm{ROS}^\bullet \quad (2)$$

$$\mathrm{ROS}^\bullet + \mathrm{M(2)}^\bullet \rightarrow \mathrm{M(2)} + \mathrm{ROS} \quad (3)$$

where all three reactions occur under aerobic conditions but only reaction 1 occurs under anaerobic conditions. Inside the cell, mitochondria are the primary source of ROS (superoxide anions, hydrogen peroxide, hydroxyl radicals, and peroxynitrite).[12] In melanin systems the ROS generation shown in reaction 2 arises from irradiating melanin under aerobic conditions, resulting in superoxide anion, $O_2^{\bullet-}$, hydroxyl radical, $^\bullet$OH, and hydrogen peroxide, H_2O_2.[1,5,13] While reaction 2 may lead to photodamage, reaction 3 scavenges reactive species. An imbalance between reactions 2 and 3 could explain the contradictory suggestions of both a phototoxic and a photoprotective role for melanin.

In this three-reaction scheme, more radicals, M(1)$^\bullet$ and M(2)$^\bullet$, are present after the laser pulse and eventually decay to the original *intrinsic* concentrations. The concentration ratio of M(1)$^\bullet$ to M(2)$^\bullet$ in the *intrinsic* radical population is different than that ratio in the *extrinsic* radical population. This ratio is also different in the presence of oxygen after a laser pulse because M(2)$^\bullet$ reacts almost exclusively with an oxygenic species. By experimental design, a reaction of oxygen with melanin radicals in the dark would not be observable in these experiments. Consequently, M(2)$^\bullet$ must be reacting with transient photoactive species synchronous with the laser pulse, species we have labeled ROS$^\bullet$. Because the difference signal (Figure 1, green) goes flat *below* the baseline, more M(2) is destroyed by ROS than is produced in reaction 1. Additionally, the *intrinsic* portion of M(2)$^\bullet$ that reacts in the presence of oxygen is eventually regenerated, presumably from the conversion of some photoproduced *extrinsic* radicals to new *intrinsic* radicals. This interpretation is collaborated by the well-established complete reversibility of RPE melanin CW EPR spectra (see Supporting Information). This study therefore supports the idea of melanin protecting biological systems as a free radical and reactive species buffer, and provides direct evidence that some of the *intrinsic* radicals partake in melanin photochemistry in the presence of oxygen.[14] Participation of *intrinsic* radicals in ROS scavenging allows melanin to protect the RPE against toxic species even in the dark.

The TREPR time profiles are the first direct EPR observations of melanin free radicals scavenging ROS. Direct observation of reaction 3 provides a new tool for understanding melanin photochemistry. Especially noteworthy is that at least two distinct melanin free radicals were present in irradiated RPE. At least four distinct synthetic melanin free radicals have been observed.[15] Remarkably, only one of these radicals is primarily involved in ROS scavenging.

Further TREPR experiments exploring the oxygen dependencies of melanin photochemistry in RPE cells and other melanin-containing systems are underway. By performing these experiments and correlating the results with RPE age and cell apoptosis, significant insight into melanin photoprotection and specific mechanistic details may be realized. Due to its potential therapeutic value, identifying the chemical structure of the ROS-reactive melanin species and developing a detailed mechanism of its reaction are our immediate goals. Certainly TREPR provides a direct means to explore melanin photochemistry in an unprecedented manner under experimentally variable oxidative and redox stresses.

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Supporting Information Available: Materials and Methods, CW EPR spectrum, and further discussion supporting our conclusions. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Sarna, T. *J. Photochem. Photobiol. B−Biol.* **1992**, *12* (3), 215−258.
(2) Sealy, R. C.; Felix, C. C.; Hyde, J. S.; Swartz, H. M. Structure and reactivity of melanins: influence of free radicals and metal ions. In *Free Radicals in Biology*; Pryor, W. A., Ed.; Academic Press: New York, 1980; Vol. 4, pp 209−259.
(3) (a) Sealy, R.; Hyde, J.; Felix, C.; Menon, I.; Prota, G. *Science* **1982**, *217* (4559), 545−547. (b) Sealy, R.; Hyde, J.; Felix, C.; Menon, I.; Prota, G.; Swartz, H.; Persad, S.; Haberman, H. *Proc. Natl. Acad. Sci. U.S.A.* **1982**, *79* (9), 2885−2889.
(4) Schraermeyer, U.; Heimann, K. *Pigm. Cell Res.* **1999**, *12* (4), 219−236.
(5) Boulton, M.; Rozanowska, M.; Rozanowski, B. *J. Photochem. Photobiol. B−Biol.* **2001**, *64*, 144−161.
(6) (a)Korytowski, W.; Sarna, T. *J. Biol. Chem.* **1990**, *265* (21), 12410−12416. (b) Kalyanaraman, B.; Korytowski, W.; Pilas, B.; Sarna, T.; Land, E. J.; Truscott, T. G. *Arch. Biochem. Biophys.* **1988**, *266* (1), 277−284. (c) Dayhaw-Barker, P.; Davies, S.; Shamsi, F.; Rozanowska, M.; Rozanowski, B.; Boulton, M. *Invest. Ophthalmol. Vis. Sci.* **2001**, *42* (4), S755−S755. (d) Rozanowska, M.; Korytowski, W.; Rozanowski, B.; Skumatz, C.; Boulton, M. E.; Burke, J. M.; Sarna, T. *Invest. Ophthalmol. Vis. Sci.* **2002**, *43* (7), 2088−2096. (e) Boulton, M.; Docchio, F.; Dayhawbarker, P.; Ramponi, R.; Cubeddu, R. *Vis. Res.* **1990**, *30* (9), 1291−1303. (f) Sarna, T.; Burke, J. M.; Korytowski, W.; Rozanowska, M.; Skumatz, C. M. B.; Zareba, A.; Zareba, M. *Exp. Eye Res.* **2003**, *76* (1), 89−98. (g) Sarna, T.; Swartz, H. M. Interactions of melanin with oxygen (and related species). In *Atmospheric Oxidation and Antioxidants*; Scott, G., Ed.; Elsevier: Amsterdam, 1993; Vol. 3, pp 129−169.
(7) Mainster, M. A. *Eye* **1987**, *1*, 304−310.
(8) Tanito, M.; Nishiyama, A.; Tanaka, T.; Masutani, H.; Nakamura, H.; Yodoi, J.; Ohira, A. *Invest. Ophthalmol. Vis. Sci.* **2002**, *43* (7), 2392−2400.
(9) VanBest, J. A.; Putting, B. J.; Oosterhuis, J. A.; Zweypfenning, R.; Vrensen, G. *Microsc. Res. Tech.* **1997**, *36* (2), 77−88.
(10) Rezai, K.; Semnani, R.; Patel, S.; Ernest, J.; vanSeventer, G. *Invest. Ophthalmol. Vis. Sci.* **1997**, *38* (12), 2662−2671.
(11) (a) Felix, C.; Hyde, J.; Sealy, R. *Biochem. Biophys. Res. Commun.* **1979**, *88*, 456−461. (b) Seagle, B. L.; Rezai, K. A.; Kobori, Y.; Gasyna, E. M.; Rezaei, K. A.; Norris, J. R. *Proc. Natl. Acad. Sci. U.S.A.* **2005**, *102* (25), 8978−8983.
(12) (a) Simonian, N. A.; Coyle, J. T. *Annu. Rev. Pharmacol. Toxicol.* **1996**, *36*, 83−106. (b) Wallace, D. C. *Science* **1999**, *283*, 1482−1488.
(13) (a) Haywood, R.; Linge, C. *J. Photochem. Photobiol. B−Biol.* **2004**, *76* (1−3), 19−32. (b) Sarna, T.; Burke, J.; Korytowski, W.; Rozanowska, M.; Skumatz, C.; Zareba, A.; Zareba, M., *Exp. Eye Res*. **2003**, *76* (1), 89−98. (c) Rozanowska, M.; Korytowski, W.; Rozanowski, B.; Skumatz, C.; Boulton, M.; Burke, J.; Sarna, T. *Invest. Ophthalmol. Vis. Sci.* **2002**, *43* (7), 2088−2096. (d) Dayhaw-Barker, P.; Davies, S.; Shamsi, F.; Rozanowska, M.; Rozanowski, B.; Boulton, M. *Invest. Ophthalmol. Vis. Sci.* **2001**, *42* (4), S755−S755. (e) Rozanowska, M.; Bober, A.; Burke, J.; Sarna, T. *Photochem. Photobiol.* **1997**, *65* (3), 472−479. (f) Koppenol, W. H.; Butler, J. *Adv. Free Radical Biol. Med.* **1985**, *1*, 91−131.
(14) Felix, C. C.; Hyde, J. S.; Sarna, T.; Sealy, R. C. *Biochem. Biophys. Res. Commun.* **1978**, *84*, 335−341. Cope, F. W.; Sever, R. J.; Polis, B. D. *Arch. Biochem. Biophys.* **1963**, *100*, 171. Van Woert, M. H. *Proc. Soc. Exp. Biol. Med.* **1968**, *129*, 161−165.
(15) Pasenkiewicz-Gierula, M.; Sealy, R. C. *Biochem. Biophy. Acta* **1986**, *884*, 510−516.

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