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ACTIVATED RATE PROCESSES AND A SPECIFIC BIOCHEMICAL MECHANISM FOR EXPLAINING DELAYED LASER INDUCED THERMAL DAMAGE TO THE RETINA

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ABSTRACT

Laser induced thermal damage to the retina is investigated. The one step Arrhenius type thermal damage integral of Henriques is analyzed for its strengths and weaknesses. The zero-order activated rate process is shown to well represent the data for pulse durations greater than 10 μs. A zero-order biochemical damage mechanism involving free radical formation and thermal disruption of the melanosome's protein coat is proposed as the initial molecular process that leads to cellular damage which appears after a delay. Data are presented that show the photoactivation of melanin granule oxidative reactivity. This *in vitro* data is evidence for an important step in our hypothesized damage pathway. © *1999 Society of Photo-Optical Instrumentation Engineers.* [S1083-3668(99)00403-7]

Keywords melanosome; free radical; threshold; damage; retinal pigment epithelium; melanin.

1 INTRODUCTION

As laser technology continues to advance, both the dangers and the benefits of laser usage increase. In order to better protect against damage, and to take additional advantage of surgical methods, it is important to understand the underlying biochemical and biophysical mechanisms of tissue interactions with laser energy. In this paper we investigate biochemical pathways that may be responsible for causing retinal damage at threshold laser fluences. We propose a photoactivated pathway that explains the delay of damage for minutes or hours after the laser insult. We also present experimental evidence for an important step in the pathway and discuss additional experiments that can be undertaken to test the remaining steps.

The effectiveness of lasers over conventional energy sources for surgery, and as a source of damage, is due to their high fluence (J/cm^2) and small spot size. A collimated laser beam that is millimeters in diameter on the cornea is focused by the optics of the eye to micrometer size on the retina resulting in retinal fluences that are 10^5 times[1] the corneal fluence. Data from experimental studies of the threshold corneal fluence for visible radiation required to cause retinal damage as a function of pulse duration are summarized in Figure 1. These data points can be found in Ref. 2 along with a mathematical treatment explaining the thermal mechanism responsible for threshold damage for durations longer than microseconds. The data points give the ED_{50} for each pulse duration, where ED_{50} represents the fluence for which there is a 50% chance of causing a minimum visible lesion (MVL). The data points[2] were obtained by different researchers using different types and wavelengths of lasers and on eyes from different species of animals so care must be used when interpreting the data. However, general trends are clearly discernible. These trends at different time scales may imply that different underlying physical mechanisms are responsible for biological damage at threshold fluences. These mechanisms are explained below. Briefly, in order of ascending duration, for subpicosecond pulses, intensities can be reached that allow for nonlinear propagation. Subnanosecond pulses deliver their energy in a short enough time to generate strong photoacoustic effects. Submicrosecond pulses generate high enough temperatures to produce vaporization, whereas pulse durations longer than a few microseconds produce lower temperature rises but which last longer and occur over a wider region in the retina. Finally, exposures that last for seconds or longer can produce photochemical effects.

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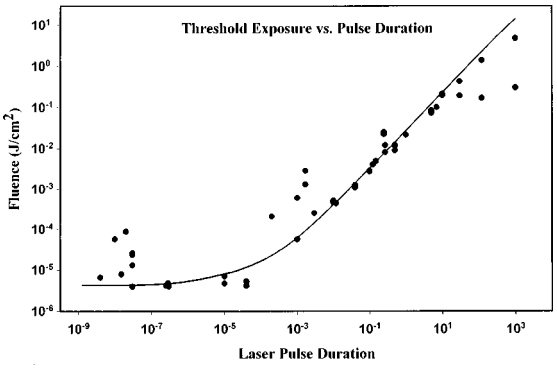

Fig. 1 Experimental ED_{50} threshold corneal fluence for retinal damage for visible wavelengths as a function of laser pulse duration. As a result of beam focusing, the fluence is increased by approximately 10^5 when it reaches the retina. The line is the fit to the data using a zero-order thermal model of Ref. 2.

The strongest absorbers in ocular tissues are melanosomes found in the retinal pigment epithelium (RPE). Melanosomes are micrometer size particles that have an absorption coefficient for light that is reported to be[3–5] in the range of 500–2000 cm^{-1}. For pulse lengths longer than 1 μs, MVL requires increasingly higher fluence as the pulse duration increases. This results from a mechanism in which temperature rises cause thermal damage. Since heat is conducted away from the absorbing melanosomes on microsecond time scales, a pulse longer than this will allow heat to conduct away during laser exposure, which will prevent the absorbing particles from reaching a high temperature unless the fluence is increased.

For pulse lengths between 1 μs and 1 ns the leveling off of the fluence required to cause damage is due to the duration of the pulse being shorter than heat conduction times away from the absorbing melanosomes. In this pulse length regime, all of the deposited energy remains localized in the absorbing melanosomes during the pulse duration and therefore the energy density in the melanosomes at the end of the pulse depends on the total energy absorbed but is independent of the pulse duration. In this pulse length range, studies have been performed[6,7] that show that bubble production in the RPE is the cause of threshold MVL damage. Damage resulting from the bubble production is primarily due to mechanical forces exerted in the cells. Above threshold levels, thermal damage may also occur. For pulse lengths shorter than 1 ns, the MVL threshold fluence appears to drop. The subnanosecond pulses are not only shorter than relevant thermal conduction times, they are also shorter than another critical relaxation time which is the time for sound to travel across the absorbing melanosomes. This pulse length regime is known as the ''stress confinement regime'' because pressure waves traveling at the speed of sound cannot escape during the duration of the pulse. This can lead to the buildup of extremely high pressure waves that are transmitted throughout the cell and cause damage, or the explosion of the melanosome within the cell. The high pressures generated by stress confinement could be the mechanism for threshold MVL damage and may explain why the threshold fluence drops for pulse lengths below 1 ns. For pulse lengths shorter than 1 ps, intensities ($J/cm^2 s$) can be achieved that are high enough for occurrence of nonlinear electromagnetic interactions between the beam and the transmitting material. This may lead to new damage mechanisms. Experimental data for the ultrashort, subpicosecond regime are only beginning to become available.[8,9]

We are interested in determining the specific molecular species that are affected by the laser energy and the biochemical reactions that lead to cellular damage. In order to understand the damage on a biochemical level, we have looked at the best characterized regime of pulse lengths which is for durations greater than 1 μs. In this regime, threshold level damage is almost certainly due to temperature rises of the cellular material. This allows us to use conventional chemical rate kinetics for analyzing data to ascertain possible reaction mechanisms.

We first briefly review Henriques'[10] original work on cellular damage caused by temperature rises and how it has been used by other workers. Then we propose a reaction pathway for causing damage that is consistent with the experimental observations, and present experimental evidence in support of one of the steps in the pathway.

2 METHODS

The Arrhenius Damage Integral, introduced in the context of thermal injury to biological tissue by Henriques,[10] assumes that the damage mechanism follows a zero-order reaction rate expression:

$$\frac{d\Omega}{dt} = Pe^{-\Delta E/R(T_0+\Delta T)}, \tag{1}$$

where $d\Omega/dt$ is the rate at which damage occurs. T_0 is the usual steady state temperature of the system (body temperature) and ΔT is the temperature rise, which can be due to any energy source such as laser exposure. ΔE is the activation energy for the damage process and P is the prefactor. Both ΔE and P are discussed in more detail after Eq. (5a). Equation (1) can be useful for determining the quantities ΔE and P, which are related to the thermodynamics of the underlying process. Equation (1) can be rewritten as

$$\Omega = \int Pe^{-\Delta E/R(T_0+\Delta T)}\,dt. \tag{2}$$

ΔE and P can be determined if the time dependence of the temperature rise ΔT is known for a laser pulse that is known to cause damage. Under these circumstances it is customary to set $\Omega = 1$, rep-

Table 1 Experimentally determined values of ΔE, P, and ΔS of Eqs. (3) and (4).

Reference	ΔE (Kcal/mole)	P (s^{-1})	ΔS (cal/mol K)
Henriques[a]	150	3.1×10^{98}	391
Welch[b]	150	1.3×10^{99}	394
Takata[c] $T\leqslant323$ K	99	4.3×10^{64}	240
$T>323$ K	158	9.4×10^{104}	420
Birngruber[d]	70	3.0×10^{44}	137

[a] See Ref. 10.
[b] See Ref. 11.
[c] See Ref. 12.
[d] See Ref. 13.

resenting that the system has gone from an undamaged state to a completely damaged state. As a simple example, if the laser pulse causes a temperature rise ΔT that remains constant for a time Δt that is long compared to its rise or fall times, then the integral is easily evaluated

$$\Omega=1=Pe^{-\Delta E/R(T_0+\Delta T)}\Delta t. \tag{3}$$

If this can be done with different pulses that result in different, but known ΔT and Δt, then ΔE and P can be determined.

As discussed by Henriques, using standard reaction rate theory ΔE is the activation energy of the process and the preexponential P is related to the entropy change ΔS

$$P=\frac{k_BT}{h}e^{\Delta S/R}. \tag{4}$$

ΔS can be obtained from P through

$$\Delta S=R\ln(P\times h/k_BT). \tag{5a}$$

Using $R=1.987$ cal/mol K, $h=6.6\times10^{-34}$ J s, $k_B=1.4\times10^{-23}$ J/K, and $T\approx350$ K, gives

$$\begin{aligned}\Delta S&=(1.987\text{ cal/mol K})\ln(P\times1.47\times10^{-13}\text{ s})\\&=(4.57\text{ cal/mol K})\log(P\times1.47\times10^{-13}\text{ s}).\end{aligned} \tag{5b}$$

This model has been used by a variety of researchers in analyzing thermal injuries. The results of some of these studies are listed in Table 1. Table 1 shows that there are differences in the values obtained for ΔE and ΔS, though they are all of the same order of magnitude.

The parameters ΔE and ΔS have precise meanings in chemical reactions in terms of the free energy for a reaction leading directly to the observed product. However, the usefulness of these parameters is related to the validity of the assumption of a well defined chemical reaction pathway and it is not clear that this is straightforwardly applicable to the complicated processes involved in laser induced retinal damage. In addition, Eqs. (1)–(3) imply that even at normal temperatures T_0, damage should be occurring, i.e., $d\Omega/dt>0$. Yet buildup of lesion type damage does not occur at normal temperatures. Thus, simple use of Eq. (1) ignores the kinetics of a repair process.

Given these problems, there are nevertheless reasons to maintain the Arrhenius damage integral approach in modeling the process. In this pulse length region, the underlying mechanism causing the damage is probably some type of thermally activated biological process and exponential dependencies on temperature can be expected, although possibly in a more complicated fashion than expressed in Eq. (1). Also, when ΔTs are calculated as a function of laser fluence using precise analytical and numerical models such as that found in Ref. 2, the data are consistent with the theoretical curve, as shown in Figure 1, in which the P and ΔE values of Welch and Polhamus[11] are used in Eq. (1). Furthermore, when an expression as simple as Eq. (1) is used to fit the data, the values of ΔE and $T\Delta S$ give ΔG that are in the same range as for protein denaturation, a very possible cause for the observed damage. For example, if we take the Welch and Polhamus[11] values of $\Delta E=150$ Kcal/mol and $\Delta S=394$ cal/mol K, then for $T=310$ K we have $\Delta G=\Delta E-T\Delta S=27$ Kcal/mol. This is quite similar to the free energy required to denature a protein[14] which is approximately 0.1 Kcal/mol/amino acid so that a typical 200 residue protein has a free energy stabilization of 20 Kcal/mol. Thus, it is worthwhile to determine if a biochemical mechanism can be found that is in accordance with both Eq. (1) and the energy parameters reported.

3 RESULTS AND DISCUSSION

3.1 MOLECULAR REACTION TIMES VS RETINAL DAMAGE TIME

In attempting to determine a molecular mechanism responsible for thermal damage to the retina, an explanation must be given for the difference in time scales between molecular processes compared to the time needed for observable retinal damage to occur. Heat conduction over cellular distances occurs on microsecond time scales and therefore heat can be dissipated in this time. Therefore, the time duration of elevated retinal temperatures for pulses longer than 1 μs is approximately the duration of the laser pulse. The time scale for large scale protein molecular rearrangement, such as folding or unfolding, is on the order of milliseconds to seconds and therefore can occur during the time that the temperature is elevated if the pulses are longer than 1 μs in duration. In contrast to subsecond duration laser exposures, elevated temperatures, and

biomolecular rearrangements, the time for appearance on the retina of ophthalmoscopically minimum visible lesions can take minutes or hours, which is long after the temperature has returned to normal. Therefore, in addition to the molecular damage caused during the temperature elevation there must be other steps leading from damaged molecules to observable cellular damage. There are two possible explanations:

1. A critical fraction of molecules or components of the cell is damaged and does not get repaired. These damaged molecules then continue to cause damage to other components of the cell and it is the secondary damage which slowly builds up and eventually results in MVL. This possibility can be distinguished by looking for experimental markers (e.g., circular dichroism markers of protein denaturation) which appear within 1 s or less after the end of the pulse, and do not disappear as MVL forms.

2. Alternatively, it is possible that most of the molecules that are directly damaged by the elevated temperature do get repaired within seconds, but during the few seconds that they are disabled they are biochemically active and damage other molecules in the cell, which in turn lead to MVL. In this case, the biochemical damage markers that appear immediately will return to normal within a short time (seconds) after the pulse even though MVL has not yet appeared.

3.2 ACTIVATION OF MELANOSOME DAMAGE MECHANISMS

Henriques' one step damage process of Eq. (1) is of the form of a zero-order reaction

$$\frac{dc}{dt} = k, \tag{6}$$

in which c represents the concentration of some species and k is the rate with which it changes with time. A reaction of this form has a rate that is independent of the concentration of reactants. This kind of reaction cannot be explained by a mechanism that involves a single molecule experiencing damage unless there is another process that introduces additional undamaged molecules at the same rate so that the concentration of undamaged molecules remains the same.

A zero-order reaction mechanism could conceivably explain thermal damage to retinal cells if the damage mechanism is catalyzed by an agent that is created by the temperature rise. The molecular species that causes the eventual observable MVL cellular damage need not be directly affected by the temperature rise but instead can have its concentration changed due to the action of the catalyzing agent which is activated directly by the temperature rise. A possible biochemical pathway of this type would be the disruption of the protein coat that encloses melanosomes.

Melanosomes contain melanin that is packaged with protein coats that restrict the direct interaction of the melanin with the surrounding cellular medium. Melanin, a pigment found in tissues such as hair, skin, and certain cell types of the eye and central nervous system, is generally thought to play a protective role against the deleterious effects of optical radiation by serving as a broadband light absorber.[15] Nevertheless, it has long been known that melanin, when irradiated with ultraviolet (UV) and visible-band light, is excited to a free radical,[16–20] and this radical is capable of supporting the generation of reactive free radicals which can adversely affect cellular components if the protein coat that encloses the melanosomes is disrupted. As shown in Eq. (7), the effect of visible light is to push the equilibrium between the quinone, hydroquinone, and semiquinone species of melanin to the right so that the occurrence of the radical semiquinone form is favored:[21–23]

$$\mathrm{MQ} + \mathrm{MQH_2} \overset{h\nu}{\rightleftarrows} 2\ \mathrm{MSQ}\bullet^{-}, \tag{7}$$

where we have used the following abbreviations: melanin with predominantly quinone forms (MQ); melanin with predominantly hydroquinone forms ($\mathrm{MQH_2}$); and melanin with predominantly semiquinone forms ($\mathrm{MSQ}\bullet^{-}$). This is presumably the only light-induced reaction. The semiquinones may then react in a type II mechanism directly with oxygen to produce the superoxide anion

$$\mathrm{MSQ}\bullet^{-} + \mathrm{O_2} \rightleftarrows \mathrm{MQ} + \mathrm{O_2}\bullet^{-}. \tag{8}$$

The formation of superoxide anion radicals within a cell may promote a variety of oxidative reactions. The occurrence of these reactions within a pigment-containing cell with inadequate antioxidant reserves would result in damage to the cellular membrane and intracellular organelles.

Although the precise interaction between excited melanin and cellular components within pigment cells is as yet unknown, photoactivated isolated melanin granules do support oxidative reactions with ascorbic acid,[21,24,25] fatty acids,[26] the nicotinamide cofactors 7-nicotinamide adenine dinucleotide, reduced form (NADH)[27,30] and β-nicotinamide adenine dinucleotide phosphate, reduced form (NADPH),[28] and retinal proteins.[29] Any of these reactions occurring *in situ* (i.e., in intact cells) would undoubtedly cause cellular damage. Because the rates of these photochemical reactions depend on the incident light flux, tissue damage induced by photoexcited melanin may take minutes or hours to appear given irradiation with low to moderate levels of ambient light.

The association of melanoproteins with melanin has been shown to inhibit photochemical interaction of excited melanin with various substrates including NADH.[30] Disturbances of the protein coat of melanosomes, for example by acid hydrolysis[30]

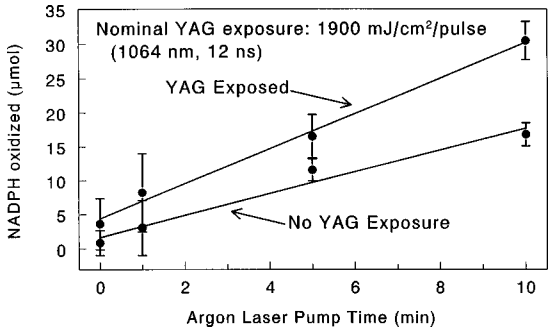

Fig. 2 Experimental data of NADPH photooxidation by isolated bovine RPE melanosomes showing enhanced photochemical activity of laser-disrupted melanosomes. Two samples of isolated bovine RPE melanosomes were exposed to the 488 nm output of a cw argon laser at ~2 w/cm^2 for the durations indicated. The sample that was first exposed to a train of 1800 pulses from a Nd:YAG laser (1064 nm, 10 ns pulse width, 10 pps) at an irradiance of 1900 mJ/cm^2/pulse shows statistically significantly more photoactivity as measured by the amount of NADPH oxidized. The increased photoactivity of the laser-disrupted melanosomes becomes more significant as the cw argon laser exposure time is increased.

or by photodisruption by pulsed laser exposure,[28] have been associated with increased photoreactivity. It is not yet known if physical disruption of the melanosome changes its effective action spectrum away from the blue–green region of the visible spectrum reported for intact melanosomes.[29] There is no reason, however, to predict that a spectral shift in the action spectrum would occur because of damage to the melanosome coats.

In Figure 2, we present experimental evidence showing that laser-disrupted melanosomes have enhanced photochemical reactivity compared to native melanosomes. The reactivity of melanosomes was assayed by their ability to oxidize the nicotinamide adenine dinucleotide cofactor, NADPH, during visible light exposure. Melanosomes were isolated from bovine RPE cells by the method of Glickman et al.,[25] and the melanin-NADPH assay, described in Glickman et al.[28,29] was used. Briefly, melanosomes were suspended in 80 mM *tris* buffer, *p*H 7.4, and mixed with NADPH at a concentration of 7 mM. This reaction mixture was divided into two aliquots, one of which was exposed to a train of 1800 pulses from a Nd:YAG laser (1064 nm, 10 ns pulse width, 10 pps) at an irradiance of 1900 mJ/cm^2/pulse, while the other aliquot was maintained in the dark. In order to demonstrate the difference in the reactivity of native and YAG-disrupted melanosomes, both samples were then exposed to the 488 nm output of a cw argon laser at ~2 W/cm^2 for the durations indicated. (Note that the photochemical reactivity of the melanosomes was negligible in the dark, but was excited by exposure to visible light. Therefore, the time lag between the YAG laser exposure and the argon laser exposure was not critical as long as the samples were kept in the dark or in low ambient light.) NADPH oxidation was estimated from the optical absorbance of the samples at 340 nm, measured after filtering the melanosomes from the samples. For both groups of melanosomes, the amount of NADPH oxidized was proportional to the duration of the argon laser pump period; however, the melanosomes preexposed to the Nd:YAG laser exposure were more photoactive than were the control melanosomes. Linear regression of NADPH oxidation against argon laser exposure duration, indicated that the average slope of the regression lines fit to YAG-exposed melanosome data was (mean$\pm$1 s.d.)2.72 ± 0.65($n=4$), while that of the regression lines fit to the control melanosome data was 1.55 ± 0.18($n=4$). These slopes were significantly different ($p\sim0.013$, 2-tailed *t*-test). Scanning electron microscopy revealed that the melanosomes exposed to the Nd:YAG laser were physically disrupted. These observations support the hypothesis that melanosomes, initially damaged by a laser exposure, may produce delayed injury in the RPE because of photochemical excitation by ambient visible light.

3.3 IMPLICATIONS FOR LASER INDUCED THERMAL DAMAGE

The possibility that thermal stress may damage the protein coats of melanosomes and increase their photoreactivity is a plausible hypothesis that may explain the delayed appearance of damage after some types of laser exposures to the eye. Although experimental evidence for other steps in the pathway is needed, the *in vitro* experimental evidence presented above supports the idea that reactions of melanin can produce oxidative stress in RPE cells. Cellular damage would occur as undamaged molecules in the cell interact with the products of the exposed melanin. The temperature dependence of the rate constant in the Henriques expression of Eq. (1) would be due to the temperature dependence of the integrity of the melanosome protein coat. Since the values for ΔE and ΔS of Table 1 are in the general range for protein denaturation, they are plausible values for disruption of the melanosome's protein coat and therefore may provide the mechanism that explains the ability of the simple zero-order expression to be used to model the process. Furthermore, this zero-order process allows the same exposed sites on melanosomes to cause damage to many molecules in the cell and fits within the general idea of ''biological amplification'' as a means for specific molecular reactions to result in larger scale cellular damage.

Isolated melanosomes, as well as intact RPE cells in culture, provide useful models to test the hypothesis. Melanosomes can be subjected to either specific laser exposures, or other forms of temperature stress, e.g., warming or boiling, to determine if the effect of thermal stress on their photoreactivity follows Eq. (1). Care must be taken to monitor the temperature–time history of the melanosomes, as

rapid heating will produce explosive vaporization of internal water which has already been shown to disrupt melanosomes.[28] Slower rates of warming may permit water vapor to dissipate without producing mechanical damage, but thermal denaturation of melanoproteins may still result, and could be associated with enhanced melanosome photoreactivity. The effects of thermal stress on isolated melanosomes may be straightforwardly tested by the use of a simple assay such as NADPH oxidation, as in the experiment shown in Figure 2. The effects of thermal damage in cultured RPE cells, however, will require other assays. One possibility is the use of an oxidation-sensitive fluorescent probe, e.g., 2′,7′-dichlorofluorescein, which can be taken up by RPE cells and oxidized by activated melanosomes. A preliminary report of the feasibility of this approach has been made.[31] The kinetics of an observed increase in the photochemical reactivity of melanosomes after thermal stress could then be modeled to determine consistency with the zero-order reaction mechanism described above.

Additionally, animal experiments might be useful for investigating the effects of melanosome damage. To determine the importance of light for photoexcitation of the damage process, laser lesions can be made in a group of animals. The animals would then be split into two groups; a group that is kept in the dark and another group that is exposed to ambient (or elevated) levels of light. Observation of a difference in the ultimate extent of delayed tissue damage would signify the importance of photoexcitation. A more complicated animal experiment would be to use an oxidation-sensitive fluorescent probe to assess the level of oxidative stress in the retina and RPE *in vivo* at various times following laser injury. Although these probes are commonly used with cultured cells and flow cytometry techniques, whether they could be used in the intact eye of an animal, in a way analogous to the clinical technique of fluorescein angiography, remains to be demonstrated.

4 CONCLUSION

Although cells carry out a variety of complex biochemical reactions, the Henriques zero-order damage integral does an excellent job of matching the experimental ED_{50}'s in the time range from 10^{-5}–1 s. A specific biochemical mechanism involving disruption of the melanosome's protein coat is proposed. Experimental evidence for an important step in the pathway is presented, and experimental protocols are described for testing other aspects. Since melanin becomes biochemically active at irradiation levels below the thermal damage threshold, disruption of the protein coat may be a biological endpoint which partly determines the thermal damage fluence level. If this is the underlying mechanism for threshold levels of thermal damage, it would be a direct example of biological amplification in which the creation of small scale changes on micron size melanosomes catalyze reactions that eventually lead to observable cellular death.

Acknowledgments

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