Hydroxyl radical formation and detection from multi-photon-ionization of tryptophan in the presence of hydrogen peroxide

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Introduction

One of the advantages claimed for the use of multiphoton excitation in microscopy is the reduction in photodamage to the sample because of the minimal absorption of near infrared photons in the areas outside of the focal region. Nevertheless, in the focal region the sample is exposed to very high photon fluxes to ensure multiphoton excitation and it is recognized that damage to biological samples may be significant unless efforts are made to limit the laser power at the sample[1]. The mechanisms of such photodamage are expected to include the excitation of endogenous photosensitizers (flavins etc.)[2] giving rise to damaging species such as oxygen-centered radicals (Type I mechanism) and singlet oxygen (Type II mechanism). At very high laser powers oxygen radicals may also be produced directly by breakdown in water.

We have previously demonstrated[3] that multiphoton excitation in aqueous solutions of the hydroxyl radical precursor 2-mercaptopyridine-N-oxide (MPNO) in the presence of tryptophan produces •OH within the focal volume as reported by the green fluorescence from resulting reaction to produce 5-hydroxytryptophan. We have now used a similar approach to demonstrate the multiphoton ionization of tryptophan using a focused femtosecond laser beam and suggest that such processes may contribute to damage in biological and cellular specimens during imaging. It is also suggested that this might be a suitable tool for the study of localized oxidative stress within live cells.

Results and discussion

In these experiments, solutions were exposed to a focused near infrared beam from a Ti-sapphire laser (180 fs pulses at 75 MHz and 750 nm) using the optics of a Nikon TE2000-U microscope to focus the beam to the required high power densities produced within the focal volume. Hydroxyl radicals were detected by formation of the characteristic luminescence of 5-hydroxytryptophan (5HTrp) induced by multiphoton excitation. For solutions containing tryptophan (Trp) and increasing concentrations of hydrogen peroxide, figure 1 shows a corresponding increase in fluorescence emission at 500 nm, characteristic of multiphoton excitation of 5hydroxytryptophan,[4,5] detected using a CCD camera and spectrograph attached to the microscope. This observation is explained by capture of the hydrated electron created by multi-photoionization of tryptophan (reaction [1]) by hydrogen peroxide (reaction [2]). This forms the reactive hydroxyl radical, which as in our previous experiments reacts further with tryptophan to give 5HTrp amongst other products (reaction [3]). These reactions are initiated within the femtolitre focal volume of the femtosecond laser beam and subsequently within this same volume 5HTrp is excited to produce characteristic green fluorescence. Overall this forms a basis for detection of hydroxyl radical generation within this system.

$$Trp + n\ hv \rightarrow Trp^{+\bullet} + e^-_{aq} \quad [1]$$

$$e^-_{aq} + H_2O_2 \rightarrow HO^\bullet + HO^- \quad [2]$$
$(k_2 = 1.2 \times 10^{10}\ dm^3\ mol^{-1}\ s^{-1})$

$$HO^\bullet + Trp \rightarrow \rightarrow 5HTrp \quad [3]$$

$$N_2O + e^-_{aq} \rightarrow N_2 + HO^\bullet \quad [4]$$
$(k_4 = 9.1 \times 10^9\ dm^3\ mol^{-1}\ s^{-1})$

$$HO^\bullet + CH_3CH_2OH \rightarrow H_2O + CH_3{}^\bullet CHOH \quad [5]$$
$(k_5 = 1.9 \times 10^9\ dm^3\ mol^{-1}\ s^{-1})$

The alternative explanation whereby hydrogen peroxide is directly photolysed to hydroxyl radical by 3-photon absorption at 750 nm appears very unlikely considering the low extinction coefficient at 250 nm (ca. 20 $dm^3\ mol^{-1}\ cm^{-1}$).

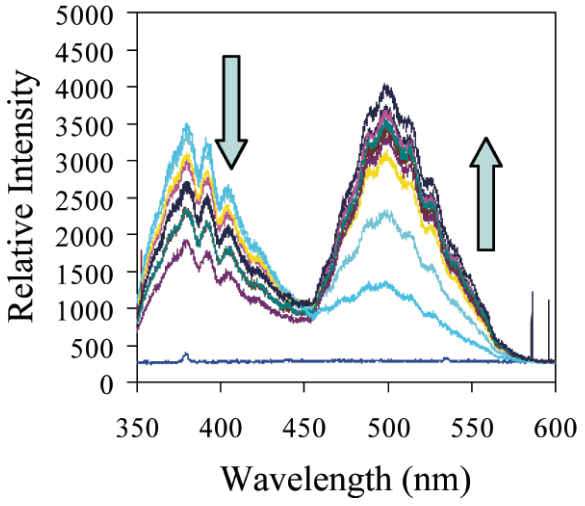

Figure 1. Emission spectra from solutions of Trp (2.4 mmol dm^{-3}) with H_2O_2 added from zero to 75 mmol dm^{-3} as indicted by the direction of the arrows.

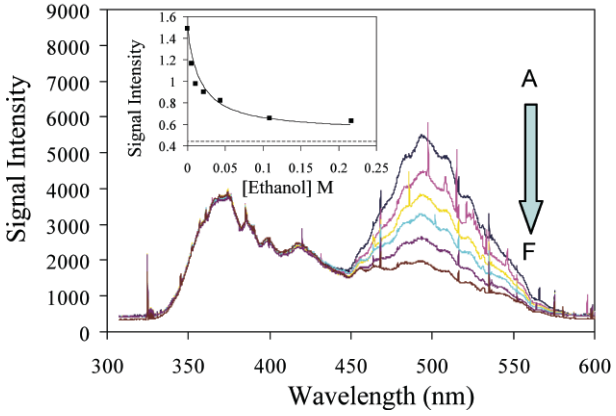

Figure 2. Emission spectra from solutions of Trp (2.4 mmol dm^{-3}) and H_2O_2 (14.4 mmol dm^{-3}) with added ethanol A) 0; B) 5; C) 11; D) 44; and E) 220 mmol dm^{-3}. Curve F is without H_2O_2.

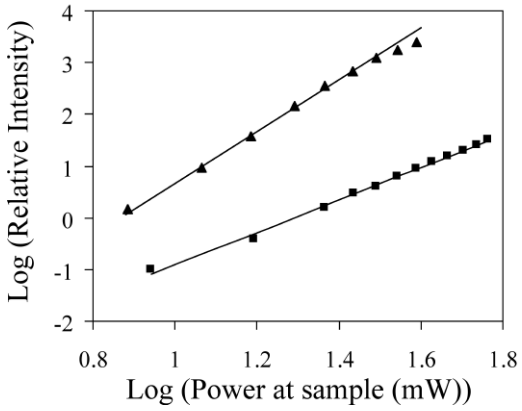

Figure 3. Power dependencies for emission at 500 nm from solutions of 5HTrp (▲) and Trp + H_2O_2 (■).

Saturation of a tryptophan solution with nitrous oxide was found to have the same effect as hydrogen peroxide. Since nitrous oxide converts the hydrated electron to hydroxyl radical (reaction [4]), this confirms the roles of these radicals in forming the 500 nm fluorescence. The direct involvement of hydroxyl radical was also demonstrated in measurements using both cystamine and ethanol as relatively specific scavengers for the hydrated electron and hydroxyl radicals respectively. The effect of ethanol as a hydroxyl radical scavenger (reaction [5]) is shown in figure 2 with the curve fitted using relevant rate constants for the reactions taking place in these solutions. The power dependences for luminescence at 500 nm in these experiments are shown in figure 3. A solution of 5HTrp shows the expected slope of 5 in the log-log plot[5], whereas for tryptophan in the presence of H_2O_2 the plot has a slope of 3, apparently corresponding to the 3-photon excitation of Trp at 750 nm and indicating that this is the process with limiting efficiency in the overall scheme.

Although multiphoton excitation of tryptophan fluorescence has been previously reported[6], this is the first report of multiphoton induced radical formation through photoionization. The one-photon excitation of tryptophan at 266 nm produces hydrated electrons though simultaneous one- and two-photon channels[7,8] with a quantum yield of 0.04 for the one-photon channel at room temperature. Further picosecond work would be required

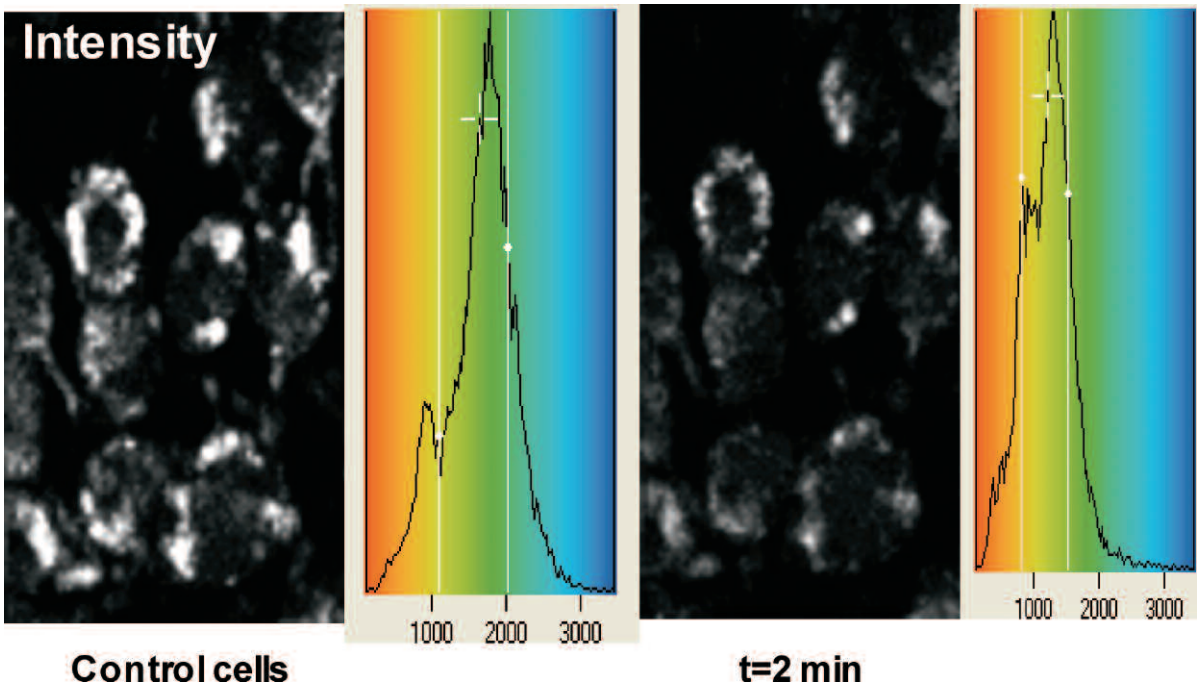

Figure 4. Fluorescence lifetime images of serotonin within RBL2 rat mast cells before (left) and 2 minutes after (right) addition of H_2O_2 (100 mmol dm^{-3}). The intensity images are shown together with fluorescence lifetime distributions.

to understand the processes involved in electron ejection from tryptophan. The results appear to indicate that photoionization of intracellular proteins may contribute to cellular damage during multiphoton imaging. Conversely the process might be used, when combined with either H_2O_2 or N_2O to scavenge the hydrated electrons, to produce a localised source of hydroxyl radical, defined by the focal volume of the laser beam, at an intracellular site for investigation of oxidative stress within cellular compartments. A preliminary observation of the diffusion of a high concentration of H_2O_2 into live cells is shown in figure 4. Solution experiments have shown that H_2O_2 is an efficient dynamic quencher of serotonin fluorescence with $k_q = 4 \times 10^9$ dm^3 mol^{-1} s^{-1}. This is very similar to the rate for quenching of Trp fluorescence by hydrogen peroxide [9]. The quenching effect enables observation of the intracellular diffusion of hydrogen peroxide in serotonin-loaded rat RBL-2H3 mast cells. Figure 4 shows that 2 minutes after H_2O_2 addition, the emission (UV fluorescence at 340 nm with two-photon excitation by 630 nm laser pulses) from serotonin within intracellular granules is quenched in intensity and the fluorescence lifetime distributions indicate that this is accompanied by quenching of the fluorescence lifetime.

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