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NOTE

Sodium fluorescein as a retinal pH indicator?

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Abstract

Retinal neovascularization is a symptom associated with various diseases revealing ocular fundus manifestation. Often, these neovascularizations originate from retinal hypoxia. A concomitant phenomenon of hypoxia is acidosis. To recognise this would permit the identification and treatment of hypoxic fundus areas long before first vascular modifications are seen. Thus, the goal of this investigation was to elucidate whether sodium fluorescein could be used as a retinal pH indicator. Sodium fluorescein solution was diluted in PBS (ratio: 1:150 000). The pH was varied from 6.5 to 8.6 by supplementation of HCl or NaOH, respectively. The fluorescence was excited by a pulsed diode laser (wavelength: 446 nm, pulse width: 100 ps) and detected by time-correlated single photon counting (TCSPC) technique. A least-squares fit of the measured fluorescence decay versus time by an exponential function results in the fluorescence lifetime. Ten measurements were taken at each pH for statistical analysis. The dependence of the fluorescence lifetime on the temperature and the concentration of sodium fluorescein was investigated in the same way. The fluorescence lifetime was found to rise from 3.775 ns to 4.11 ns with increasing pH (6.5 to 8.6). However, the gradient decreases with increasing pH. We found highly significant differences (Student's t-test, $P < 0.0005$) of the fluorescence lifetimes for pH values with a mean difference of 0.125 at pH < 7.65 whereas the differences were still significant ($P \leqslant 0.02$) at pH > 7.65 and mean pH differences of 0.2. The fluorescence lifetime was independent of the temperature (22 °C to 37 °C) and the concentration of sodium fluorescein (dilution 1:150 000 to 1:2000). The fluorescence lifetime of sodium fluorescein depends on the pH but not on temperature and concentration. Thus, the discrimination of areas with retinal acidosis should be possible by combination of the TCSPC technique with scanning laser ophthalmoscopy. Further investigations have to clarify whether the accuracy of the measurement at the fundus *in vivo* is sufficient.

Keywords: retina, hypoxia, acidosis, sodium fluorescein, fluorescence lifetime

Introduction

Vitreal, retinal or sub-retinal neovascularizations are severe complications in different sight-threatening diseases such as diabetic retinopathy, retinopathy of prematurity, as well as age-related macular degeneration. The outgrowth of new blood vessels from existing vessels is mediated by an imbalance of growth factors and signalling molecules (Agostini and Hansen 2003, Joussen *et al* 2003): the initial vasodilation is triggered by nitric oxide derived from the nitric oxide synthase (NOS). Up-regulated vascular endothelium growth factor (VEGF) results in the proliferation of endothelial cells and binding of Angiopoietin 2 (Ang-2) to its receptor Tie-2 enhances the break-up of the vessel wall (Maisonpierre *et al* 1997). NOS, VEGF and Ang-2 are up-regulated by the hypoxia inducible transcription factor HIF-1α (Semenza 2000). Thus, hypoxia is a major stimulus of neovascularization.

On the other hand, hypoxia has an impact on the retinal cellular metabolism: Müller glial cells, which have a high glycolytic capacity, release lactate which is metabolized in the respiratory chain of the photoreceptors (Poitry-Yamate *et al* 1995). Reduced pO_2 reduces the respiratory capacity of the receptors yielding an excess of lactate and, finally, retinal acidosis. This metabolic response to hypoxia is much faster than the inter-cellular signalling chain resulting in neovascularization which becomes visible clinically after months up to years. Thus, the possibility of detecting acidic fundus areas may be helpful in predicting the risk for neovascularization.

The fluorescence quantum efficacy of sodium fluorescein is known to depend on the pH. It decreases by about 60% if the pH is reduced from 7.4 to 6.0 (Wessing 1968). Though attempted for the evaluation of the temporal behaviour of the appearance of sodium fluorescein (Hipwell *et al* 1998), the absolute quantitation of the fluorescence intensity in fluorescein angiography is difficult since it may be influenced by the fluorescein concentration and the illumination conditions as well. However, if the reduction of fluorescence intensity in acidosis is caused by fluorescence quenching this should affect the fluorescence lifetime, which can be measured independently of the fluorescence intensity, too. Thus, the goal of this investigation was to elucidate the pH dependence of the fluorescence lifetime of sodium fluorescein.

Methods

Sodium fluorescein solution for injection (10%, Alcon Pharma GmbH, Freiburg, Germany) was diluted in phosphate buffer solution (PBS) in ratios of 1:17.5 up to 1:150 000. The pH was equilibrated by supplementation of HCl or NaOH, respectively, to 15 values between 6.5 and 8.6.

All measurements were carried out using a home-built fluorescence lifetime imager which is in use for patient investigations as well (Schweitzer *et al* 2000, 2004a, 2004b). The fluorescence was excited by a pulsed diode laser (wavelength: 446 nm, pulse width: 100 ps, Picoquant GmbH, Berlin, Germany) which was coupled to a confocal scanning laser ophthalmoscope (cLSO, Carl Zeiss, Oberkochen, Germany). The fluorescence light was separated from the reflected excitation light using a dichroic mirror (DT Blue, Linos Photonics GmbH, Göttingen, Germany) and a long-pass filter (OG 515, Andover Corp., Salem, USA). Light with wavelengths greater than 515 nm was detected by the time-correlated single photon counting (TCSPC) technique (SPC 536, Becker & Hickl GmbH, Berlin, Germany). The sodium fluorescein solution was contained in a cuvette with a thickness of 20 microns (Hellma GmbH, Müllheim, Germany) in the back focal plane of a model eye mounted in front of the cLSO. The fluorescence decay, measured in fluorescence intensity versus time, was approximated with an exponential function of time in a least-squares fit (software: fluofit,

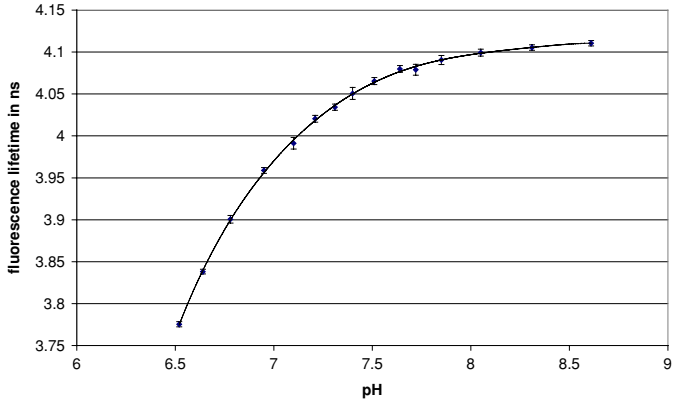

Figure 1. Fluorescence lifetime of sodium fluorescein (dilution: 1:150 000 in PBS) at 37 °C versus pH. Error bars show standard deviation of ten measurements at each pH value.

Picoquant GmbH, Berlin, Germany) providing the time constant of the decay, the so-called fluorescence lifetime, as a fit parameter. All measurements were repeated ten times. Besides the variation of the pH and the concentration, measurements were taken at 22 °C, 32 °C and 37 °C.

Results

The dependence of the fluorescence lifetime of sodium fluorescein is shown in figure 1. The sodium fluorescein was diluted in PBS in the ratio 1:150 000 and the measurements were taken at 37 °C. Though the changes in lifetime are small, the differences between measurements of two neighbouring pH values (mean step size: 0.125 for pH between 6.5 and 7.65 and 0.2 for pH between 7.65 and 8.6) were highly significant (Student's t-test, $P < 0.0005$) below pH 7.65 and still significant ($P \leqslant 0.02$) above. The fluorescence lifetime was independent of the temperature (22–37 °C). Furthermore, it was independent of the concentration up to a dilution of 1:2000. At higher concentrations, alterations due to the self-absorption of the fluorescence were observed.

Discussion

The fluorescence lifetime of sodium fluorescein depends on the pH but not on the temperature and concentration in a range covering the clinical values from vascular to tissue concentrations. Fluorescence lifetimes can be measured non-invasively at the patients fundus using scanning laser ophthalmoscopy combined with time-correlated single photon counting (Schweitzer *et al* 2000, 2004a, 2004b). Thus, the recording of fluorescence lifetime images in the late phase of a fluorescein angiogram, when the sodium fluorescein has diffused from the choriocapillris into the retina, could give information on the retinal pH and reveal local acidosis which may be a hint to hypoxic areas. This, however, needs a fluorescence signal with a sufficient signal-to-noise ratio to distinguish the alterations of the fluorescence decay time, which are very small over the expected pH range. Though the fluorescence quantum efficacy of sodium fluorescein is near 1 (Wessing 1968), clinical investigations will have to show whether the retinal concentration of sodium fluorescein in the late phase is high enough to produce such a strong signal. An optimization due to the time delay of the measurement after the injection

will be necessary in order to obtain maximal fluorescence from the retina and only minor contributions from the choriocapillaris and the vitreous. Furthermore, the fluorescence of the sodium fluorescein should be strong compared to that of intrinsic fluorophores such as lipofuscin (Delori *et al* 1995) and advanced glycation end products (Stitt 2001). This has to be scrutinized in investigations in elderly patients as well as in diabetics.

Lifetime imaging of the fluorescence of intra-retinal sodium fluorescein may be used for non-invasive retinal pH measurements *in vivo*. Further investigation, however, will have to clarify the conditions under which such measurements are possible.

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References

Agostini H T and Hansen L L 2003 *Ophthalmologe* **100** 371–7
Delori F C, Dorey K C, Staurenghi G, Arend O, Goger D C and Weiter J J 1995 *Invest. Ophthalmol.* **36** 718–29
Hipwell J, Manivannan A, Vieira P, Sharp P F and Forrester J V 1998 *Physiol. Meas.* **19** 165–80
Joussen A M, Kirchhof B and Gottstein C 2003 *Ophthalmologe* **100** 284–91
Maisonpierre P C *et al* 1997 *Science* **277** 55–60
Poitry-Yamate C L, Poitry S and Tsacopoulos M 1995 *J. Neurosci.* **15** 5179–91
Schweitzer D, Hammer M, Anders R, Doebbecke T and Schenke S 2004a *Ophthalmologe* **101** 66–72
Schweitzer D, Hammer M, Schweitzer F, R, Doebbecke T, Schenke S and Gaillard E R 2004b *J. Biomed. Opt.* **9** 1214–22
Schweitzer D, Kolb A, Hammer M and Thamm E 2000 *Proc. SPIE* **4164** 79–89
Semenza G L 2000 *Genes Deve.* **14** 1983–91
Stitt A W 2001 *Br. J. Ophthalmol.* **85** 746–53
Wessing A 1968 *Fluoreszenzangiographie der Reitna* (Stuttgart: Georg Thieme)