ALA UPTAKE AND LASER INDUCED ROS PRODUCTION IN KERATINOCYTES

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Abstract. Our study aims to assess the effect of photodynamic therapy (PDT) using ALA (5-aminolaevulinic acid, a precursor of intracellular porphyrins) and red LASER light on Human Caucasian dysplastic oral keratinocytes (DOK). As a first step we determined the best experimental conditions and the amount of reactive oxygen species (ROS) produced as a result of ALA-PDT. DOK cells were incubated with various concentrations of ALA, for different periods of time, in order to determine the optimal concentration of the photosensitizer and duration of incubation. The intracellular amount of ALA-induced protoporphyrin IX (PpIX) was estimated by its fluorescence emission spectra. The photolysis of PpIX in cells irradiated with a red LASER was measured by a decrease in the intensity of these spectra. Intracellular ROS were evaluated using a fluorescent nonselective marker, 2',7'–dichlorofluorescein diacetate (DCFDA). *Results*: The ALA concentration corresponding to optimal cell uptake and PpIX production was determined to be 1mM, over a 24h incubation period. The photolysis of PpIX in cells irradiated with red LASER light was proven by the decrease of PpIX emission peak. The rate of ROS concentration increase (as determined with DCFDA) was diminished by adding vitamin C in the cellular suspension.

Key words: photodynamic therapy, 5-aminolaevulinic acid, protoporphyrin IX, red LASER light, reactive oxygen species (ROS).

INTRODUCTION

Photodynamic therapy (PDT) is a treatment method currently accepted for some precancerous cutaneous lesions such as actinic keratosis or Bowen's disease and still under evaluation in nonmelanoma skin cancers and photorejuvenation.

Its effects are based on the generation of intracellular toxic compounds through photochemical reactions involving a photosensitizer. The subsequent chain of biochemical reactions leads to the formation of toxic intracellular compounds and ultimately to cell death through apoptosis.

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In the case of PDT using ALA, the photosensitizer is a substance naturally existing in living cells, protoporphyrin IX (PpIX), an intermediate in the biosynthesis of heme.

ALA is an important precursor for the *in vivo* production of porphyrins, including protoporphyrin IX. Since the synthesis of heme from PpIX is a rate limiting step, exogenous administration of ALA (topical or intra venous) leads to increased tissue levels of protoporphyrin [8]. It was found, however, that these increased levels can only be maintained for a short period in the body, the photosensitizing effects of PpIX lasting not much longer than 24 hours [11].

Reaching a high tissular level of ALA derived PpIX is further impeded by the fact that ALA crosses cellular membranes with difficulty [7]. Efforts were made to develop and test ALA-esters that would more easily penetrate the biological barriers and enter the cells, where they are hydrolyzed by unspecific esterases releasing ALA. Some of these esteric compounds showed improved bioavailability and/or phototoxicity [4].

The mechanisms by which ALA is taken into the cells seem to involve some type of transporter system. Studies on various types of cells pointed at different transporters, like the UGA4 transporter in the yeast *Saccharomyces cerevisiae* (the same transporting mechanism as for GABA) [13], or some type of β transporter in a murine mammary adenocarcinoma cell line [14]. The observed similarities between ALA and GABA transport are used, in current studies, in an effort to find ALA derivatives that could more easily penetrate into cells [5, 6].

Some studies indicate that ALA induced PpIX accumulation is higher in certain tumoral cells, compared to normal, surrounding tissue, due to abnormal enzyme activity, in particular the reduced activity of ferrochelatase and a relative enhancement of deaminase activity [3].

When exposed to light, PpIX is photodegraded. Analyses of emission spectra of the irradiated cells show both a decrease in the intensity of PpIX emission peak (known to be at 635 nm), and the formation, to some extent, of other intermediate compounds, such as photoprotoporphyrin (Ppp), with an excitation peak at 670 nm and an emission peak nearby, and with a shoulder of the emission spectrum beyond 720 nm, as reported by various authors [2, 12, 15]. The compound Ppp and other photointermediates are thought to have some photosensitizing activity themselves [9].

The process ultimately leads to the generation of cytotoxic reactive oxygen species (ROS) and free radicals and the destruction of the cells. Singlet oxygen is thought to play the main role in photodegradation [12].

The type of light used for irradiation, whether provided by LASER, LED or incoherent light lamps, seems to make no difference in PDT, as long as the necessary wavelength for the photodegradation of photosensitizer is provided [1].

Our study aims to assess the effect of photodynamic therapy PDT using ALA and red LASER light on Human Caucasian dysplastic oral keratinocytes (DOK). Since the mechanisms of ALA transportation may differ for different types of cells, we think that target cells for PDT should be investigated to find particularities in

their ALA uptake mechanisms. We tried to do the same for the cell line we used in our study, a keratinocyte cell line that had not been mentioned in literature as a subject for PDT studies before.

MATERIALS AND METHODS

CELL LINE AND CELL CULTURE

Human Caucasian dysplastic oral keratinocytes were cultured in DMEM supplemented with 2 mM glutamine, 5 μg/mL hydrocortisone and 10% Fetal Bovine Serum and incubated at 37 °C in an atmosphere containing 5% CO_2. The cells were seeded into 35 mm Petri dishes, 2.5×10^5 cells per dish, and incubated for 24 h.

CHEMICALS

ALA was dissolved in DMEM without phenol red and without supplements and used to replace the growth medium after the initial 24 h period.

DMEM and DMEM without phenol red and DCFDA were provided by Sigma-Aldrich Chemical Co., 0.5% trypsin without phenol red + 0.2% Na_2EDTA was provided by Biochrome. Other chemicals (PBS etc.) were prepared in our laboratory.

SAMPLE PREPARATION

After the ALA incubation period the cells were rinsed with PBS three times, (to remove the medium with ALA) and then twice with 1 mM Na_2EDTA in PBS (to facilitate the action of trypsin). Cells were then detached from the Petri dish's bottom using trypsin without phenol red, and resuspended in PBS. The samples thus obtained were placed in the fluorometer for irradiation and measurements.

SAMPLE IRRADIATION AND FLUOROMETRIC MEASUREMENTS

PpIX photolysis was induced by continuous irradiation with a He-Ne LASER (Melles Griot, 633 nm, 50 mW).

PpIX spectra were measured on a Perkin-Elmer fluorometer (MPF-44B). We used the excitation and emission wavelengths specific for PpIX, λ_{ex} = 407 nm and λ_{em} = 600–750 nm. Our equipment allowed irradiating the samples while they were placed inside the fluorometer:

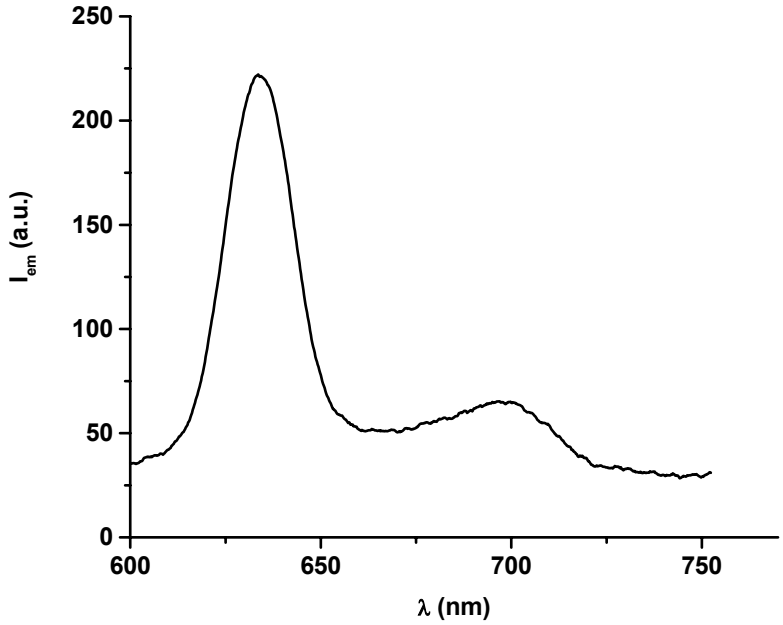

Fig. 1. PpIX emission spectra measured in DOK cells incubated with ALA for 24 h.

The fluorescent response of DCFDA was measured at λ_{ex} = 488 nm and λ_{em} = 523 nm.

ALA UPTAKE MEASUREMENTS

DOK cells were incubated in ALA / DMEM without phenol red, the following concentrations / periods of time:

- 1 mM / 2 h;
- 5 mM / 2 h;
- 10 mM / 3 h;
- 1 mM / 24 h;
- 1 mM / 26 h;
- 1 mM / 29 h.

ALA concentrations and periods of incubation were established starting from data found in literature for other types of cell cultures [6], and other keratinocyte cultures [1, 10]. As there are indications that PpIX has a very short half-life, we chose not to use incubation times much longer than 24 hours, as we expected the concentration to reach a plateau. The shorter incubation periods showed very little uptake of ALA and subsequent PpIX production, requiring a higher level of signal amplification upon reading, while incubation periods exceeding 24 h showed no important improvement in ALA uptake.

PPIX DEGRADATION DURING LASER IRRADIATION

After incubating DOK cells with 1 mM ALA for 24 h, the degradation of the photosensitizer was followed by monitoring the emission spectra of PpIX after different periods of LASER irradiation. Since the He-Ne LASER emission wavelength is very close to the PpIX emission peak, the irradiation was interrupted during fluorescence measurements. The interruptions lasted about 90 seconds and did not affect the fluorescence bleaching rates, since the photolysis of PpIX occurs only during irradiation and is not influenced by subsequent steps of the biochemical reaction chain.

A first batch of four samples (three samples incubated with ALA and a control without ALA) were irradiated for 30 minutes (real irradiation time that does not include the interruption for fluorescence measurements) and the PpIX emission spectra were taken every 5 minutes during the irradiation. Values obtained for the PpIX emission peak were then compared in order to assess the degradation rate of PpIX (Fig. 2).

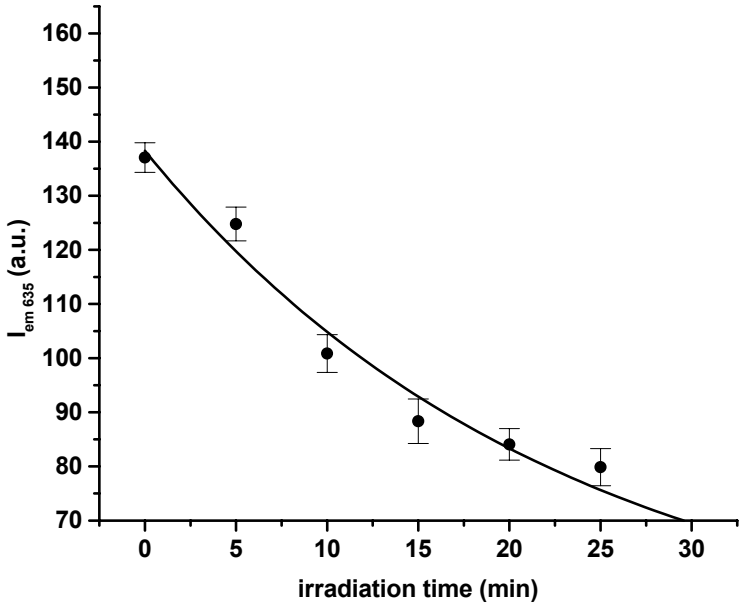

Fig. 2. PpIX photodegradation kinetics.

ROS INCREASE DURING LASER IRRADIATION

Measurements were performed on four batches of samples, prepared as described below.

DOK cells that had previously been incubated with 1 mM ALA for 24 h were rinsed twice with PBS and then incubated with 100 μM DCFDA in DMEM without phenol red for 30 minutes. After this procedure, the samples were handled using the protocol described in the sample preparation section.

The experimental protocol included three samples, two of which were irradiated and one control that was not exposed to LASER light. In one of the irradiated samples 10 mM vitamin C was added as an antioxidant in the final environment (PBS).

Since the LASER wavelength was different enough from the emission and excitation wavelength of DCFDA, the measurements could be accurately performed during irradiation. Emission intensity of DCFDA was recorded for 40 minutes.

The ROS production rate, represented by the increase in DCFDA emission intensity, is shown in Fig. 3.

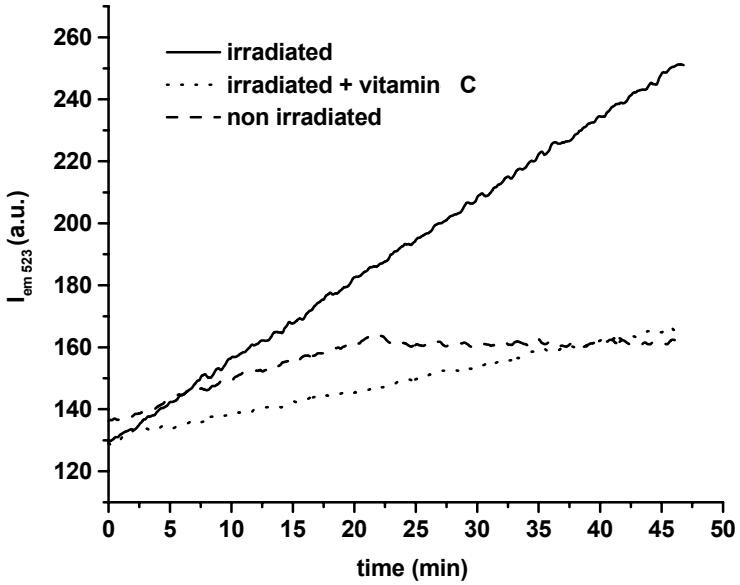

Fig. 3. DCFDA as function of the irradiation time.

RESULTS AND DISCUSSION

An important benefit of PDT at this time comes from its use in treating mostly oncological and non-oncological dermatological conditions. A lot of effort is, therefore, put in investigating the mechanisms and optimal treatment parameters of PDT using ALA and its derivatives (the main topical photosensitizers in use) on various keratinocyte cell lines.

In our study, optimal ALA uptake conditions were found to be: 1 mM ALA, incubated at 37 ºC, in 5% CO_2, over a period of 24 h. Production of PpIX was checked by measuring the emission spectra of the cellular suspension (Fig. 1).

Both the incubation period and the ALA concentration necessary for PpIX spectra measurement were higher for DOK cells than those found in literature referring to other cell lines (concentration range of μM, whereas our study required mM levels of ALA). We think this might be explained by the particularities in uptake mechanisms and enzyme activity within DOK cells. Further studies might clarify this and help improve ALA uptake for therapeutical purposes.

Comparing the values obtained for the PpIX emission peak at different irradiation times showed a clear decrease in emission intensity, due to the photolysis of PpIX. Under these experimental conditions, PpIX photolysis showed an apparent first order kinetics with a time constant of 4.4×10^{-2} min^{-1}.

Although our fluorescence emission spectra measurements included a wavelength interval large enough to encompass the emission peaks of the intermediate compound Ppp, we could not detect its increase in concentration during irradiation.

ROS production occurred with an average rate of 0.044 min^{-1} in ALA irradiated samples, while in samples incubated with vitamin C the rate of ROS production was only 0.013 min^{-1}. In control samples (with ALA but not irradiated) the ROS production rate was 0.008 min^{-1}.

For further information on the photodegradation kinetics of PpIX supplementary experiments are undergoing, using different PpIX concentrations and irradiation intensities. A better understanding of this mechanism can lead to a more rigorous optimization of therapeutic techniques.

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