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Influence of Physiological Parameters on the Production of Protoporphyrin IX in Human Skin by Topical Application of 5-Aminolevulinic Acid and its Hexylester

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Formation of protoporphyrin IX (PpIX) after topical application of 5-aminolevulinic acid (ALA) and its hexylester derivative (ALA-Hex) was studied on healthy human skin. Temperature, density of hair follicles, epidermal and skin thickness were measured on the application sites. The skin temperature was found to be the strongest determinant for PpIX formation. The PpIX fluorescence increase was about 25% per degree Celsius. Formation of PpIX was found to be independent of the density of hair follicles. A weak correlation was found between the PpIX fluorescence and the thickness of epidermis and skin. Sun exposure seems to reduce the production of PpIX slightly.

Key words: 5-aminolevulinic acid esters, epidermal thickness, hair follicles, fluorescence, skin physiology, skin thickness, spectroscopy, temperature

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INTRODUCTION

Photodynamic therapy (PDT) is now a standard treatment for several types of skin cancer (Fritsch *et al.*, 1998; Kalka *et al.*, 2000; Lang *et al.*, 2001; Morton *et al.*, 2002; Morton, 2002; Clark *et al.*, 2003). The treatment is based on selective retention of photosensitizers, usually porphyrins, in tumour tissue (Spikes, 1997; Dougherty *et al.*, 1998; Dolmans *et al.*, 2003). During light exposure, excited sensitizer molecules transfer their energy to neighboring oxygen molecules (Foote, 1991; Wilson *et al.*, 1997) and generate reactive oxygen species and/or other radicals, which in turn kill tumor cells (Ochsner, 1997; Konan *et al.*, 2002). Since most photosensitizers are fluorescent, they can also be used for photo diagnosis of cancer (Baumgartner *et al.*, 1996; Wang *et al.*, 1999; Leunig *et al.*, 2003).

In a recent PDT approach, 5-aminolevulinic acid (ALA), which is a natural intermediate in the heme biosynthesis, is applied. When administered exogenously, ALA or its derivative leads to accumulation of the photosensitizer protoporphyrin IX (PpIX) (Malik and Lugaci, 1987; Peng *et al.*, 1987; Kennedy *et al.*, 1990; Gardlo and Ruzicka, 2002). This approach can also be used for fluorescence diagnosis (Kennedy *et al.*, 1996; Lange *et al.*, 1999; Fischer *et al.*, 2001; Kelty *et al.*, 2002). The advantage of ALA-PDT is that endogenously formed PpIX does not give as prolonged cutaneous photosensitivity as the classical photosensitizers, such as Photofrin® or Foscan® (Dougherty *et al.*, 1998; Kelty *et al.*, 2002; Peng *et al.*, 2001). Moreover, in many cases, application of ALA or its derivatives leads to more selective tumour photosensitization than most of the other photosensitizers do (Kelty *et al.*, 2002; Peng *et al.*, 1997; Ludicke *et al.*, 2003).

An important reason for the tumour selectivity of PpIX synthesis seems to be a high activity of the rate limiting enzyme porphobilinogen deaminase (PBGD) in tumours (Gibson *et al.*, 1998; Krieg *et al.*, 2002). Other factors, such as temperature (Moan *et al.*, 1999; Juzeniene *et al.*, 2002), permeability and integrity of the skin overlaying tumours (Moan *et al.*, 2001; van den Akker *et al.*, 2003) and vehicle composition (De Rosa *et al.*, 2003; Merclin *et al.*, 2004; Winkler and Muller-Goymann, 2005; Bender *et al.*, 2005; McCarron *et al.*, 2005) also play crucial roles.

Large interlesion and interpatient variations make predictions of production of PpIX and photodynamic efficiency unreliable (Dougherty *et al.*, 1998; Kennedy and Pottier, 1992; Stefanidou *et al.*, 2000). The reasons for this are not completely understood. Further investigations and developments of instrumentation and methods,

allowing convenient monitoring of these parameters *in situ*, are needed. The present study we have investigated production of PpIX from ALA and its hexylester derivative with respect to different physiological parameters of human skin: Temperature, epidermal thickness, body site, number of hair follicles and sun exposure. A comparison of ALA and its hexylester (ALA-Hex) derivative is of interest, since both drugs are used clinically and since they are widely different in lipophilicity and pharmacological properties (Gaulhier *et al.*, 1997; Kloek *et al.*, 1998; Uehlinger *et al.*, 2000; Juzeniene *et al.*, 2002; Lopez *et al.*, 2004). Since fluorescence measurements are among the most sensitive analytical techniques available for complex biological systems and since PpIX has a strong characteristic fluorescence, *in situ* fluorescence measurements are well suited for monitoring changes in porphyrin levels in tissues (Pottier *et al.*, 1986; Webber *et al.*, 1997). Therefore, we have chosen non-invasive fluorescence spectroscopy to study the pharmacokinetics of PpIX in human skin in the present research.

MATERIALS AND METHODS

Human skin: Seven healthy male volunteers, age 25-55 years (mean 36.9 years), were included in this study. Non-invasive measurements were carried out on the investigators themselves under supervision of a licensed physician. None of the volunteers had a history of skin disease or excessive sun exposure. One had been significantly less exposed to the sun for cultural reasons. The following body sites were chosen: a lower arm, an upper arm, an upper part of a neck (above collar), a lower part of a neck (below collar), a back and a side of a trunk. For four subjects, spots were also selected on top of and behind an ear.

Topical application: 5-aminolevulinic acid (ALA) and its hexylester derivative (ALA-Hex) were kindly provided by PhotoCure ASA (Oslo, Norway). Different creams on a weight-to-weight basis (w/w) were prepared for topical application. The ALA cream was prepared by dissolving 10% ALA in an ointment (Unguentum Merck, Darmstadt, Germany), except for the sites on ears where 2% ALA concentration was used. The hexylester cream was prepared by dissolving 2% ALA-Hex in the ointment, which was used on the contra-lateral ear. Approximately 0.1 g of the freshly prepared cream was applied topically on the selected sites. The application site (patch area of the cream was about 1 cm²) was covered with a transparent dressing (OpSite Flexigrid, Smith and Nephew Medical Ltd., Hull, UK).

Fluorescence measurements: The amount of PpIX induced by ALA in the skin was monitored by measuring PpIX fluorescence as described earlier (Moan *et al.*, 1998). Briefly, a luminescence spectrometer (Perkin Elmer LS50B, Norwalk, CT) equipped with a photomultiplier (R928, Hamamatsu, Japan) and a non-invasive fibre-optic probe (Perkin Elmer accessory consisting of two bundles of silica fibres joined in parallel at a measuring tip along with an aluminium spacer of 10 mm length and 6.5 mm diameter) was used. The excitation wavelength was 407 nm and the fluorescence emission was registered at 637 nm, which correspond to the maxima of the PpIX excitation and emission spectra in the skin, respectively (Juzeniene *et al.*, 2002). The mean fluorescence was calculated for each experiment. In some cases, full fluorescence spectra were recorded to verify that the fluorescence originates mainly from PpIX.

Fluorescence was measured every hour up to 5 or 6 h after application of ALA or its hexylester. During all experiments, the cream and transparent dressings were continuously present, as they did not interfere with the fluorescence measurements.

Physiological parameters: The number of hair follicles was counted in a circular area of 0.8 cm² with the aid of a magnifying glass. The temperature was measured with a thin thermocouple (KM45 Kane-May Ltd., Welwyn Garden City, UK) immediately before the application of the creams. An ultrasound device (DermaScan C Ver. 3, Cortex Technology, Hadsuns, Denmark) with a 30 MHz transducer was used to measure epidermal and skin thickness.

RESULTS

Body sites with a history of frequent exposure to the sun (lower arm, top of ear, back of thorax) show lower PpIX formation than body sites that are usually less exposed to sunlight (upper arm, back of ear, side of thorax) (Fig. 1a). About twice as much PpIX was found behind the ear as on the top of the ear (Fig. 1b).

When the data from all subjects and body sites are pooled, temperature dependency of the PpIX production from ALA seems to be indicated (Fig. 2a). The temperature dependency is clearly seen for ALA and ALA-Hex applied on the ear (Fig. 2b).

There are large variations in PpIX production from subject to subject. However, the kinetics follows a similar pattern for all subjects: With one exception, the subjects produced more PpIX on the upper arm than on the lower arm (Fig. 3a). This also applies to the ear where, for all subjects, more PpIX is produced behind the ear than on

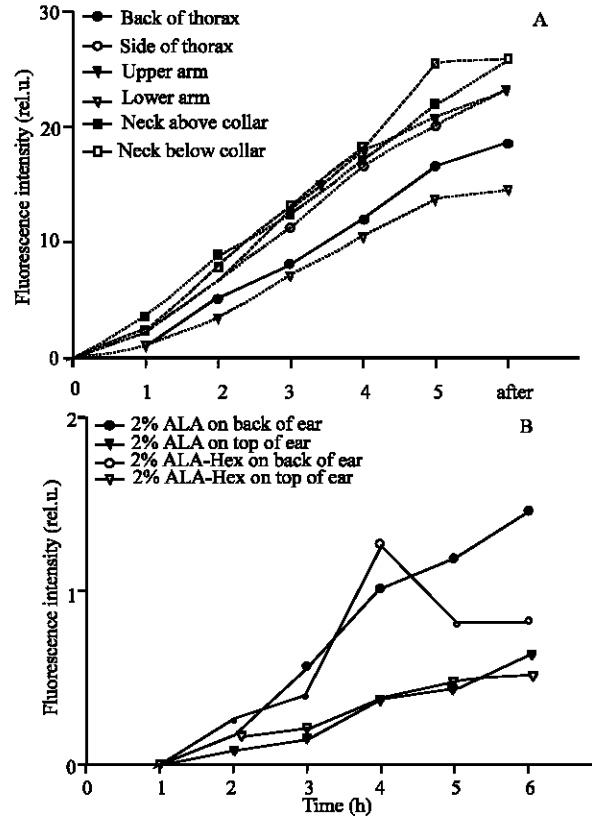


Fig. 1: Mean fluorescence of PpIX at different locations during continuous application of A) 10% (w/w) ALA and B) 2% (w/w) ALA or its hexylester

the top of it (Fig. 3c). After correction for the influence of the temperature, a similar pattern was obtained (Fig. 3b and d). The subject that did not follow the general pattern (subject 2) had a history of low sun exposure and also had visibly less pigmentation in sun exposed areas than the other subjects. There were only small differences in PpIX production that could be correlated to the sun exposure to be seen on the neck and when comparing back and side of the trunk.

There seems to be no correlation of the formation of PpIX and the density of hair follicles when the data of all four subjects are pooled (Fig. 4). There was a slight increase of PpIX formation with increasing the thickness of either epidermis or epidermis and dermis (skin) (Fig. 5 and 6).

DISCUSSION

The present study shows that there is a large variation of PpIX production in skin from subject to subject and from skin site to skin site. This is true for both ALA and its hexylester derivative and is in agreement

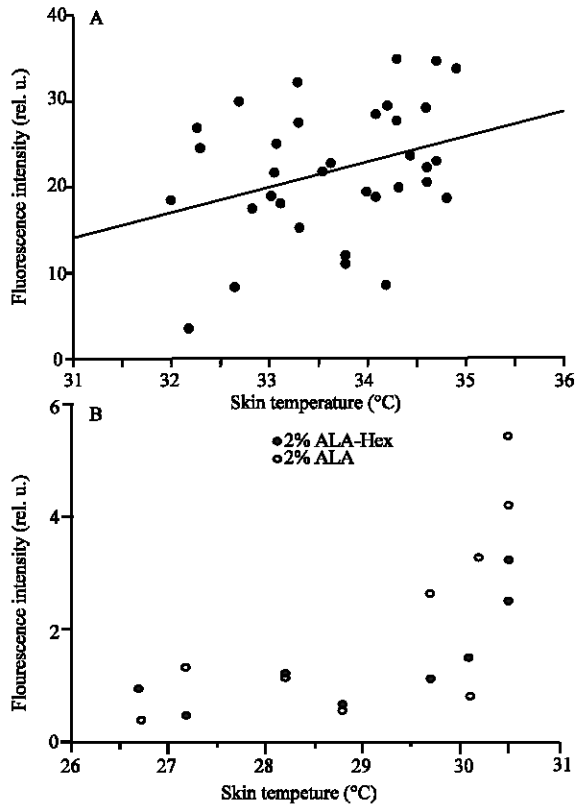


Fig. 2: Fluorescence of PpIX after 5 h application of A) 10% (w/w) ALA on upper arm, lower arm, neck above and below a collar, side and back of thorax and B) 2% (w/w) ALA or its hexylester on ear as a function of skin temperature measured

with the findings of other investigators. Skin temperature certainly varies from subject to subject and from one skin site to another (Liang and Norris, 1993). Temperature is an important determinant for PpIX formation. Even a small temperature increase may give rise to a significantly elevated production of PpIX (Moan *et al.*, 2001). Present data indicate that skin temperature is a major determinant for PpIX production in human skin. This is in agreement with our, as well as others, earlier work where the temperature of the skin of mice and humans was manipulated artificially (Moan *et al.*, 1999; van den Akker *et al.*, 2004). In the present study, the temperature dependency on PpIX formation is confirmed by natural variations of the skin temperature. An artificial cooling of mouse skin from around 36 to 30°C reduced the PpIX fluorescence by a factor of 2-3 (Juzeniene *et al.*, 2002). This is in good agreement with the present data obtained from human skin. An increase of 2°C on an ear roughly doubles the fluorescence both for ALA and ALA-Hex (Fig. 2b). On other body sites the average dependency is

less expressed, giving a rise of around 20% for 1°C (Fig. 2a).

Furthermore, skin thickness varies from subject to subject and from one body site to another. The impact of this variation is hard to foresee. On one hand, thick skin would retard the downward penetration of drug, but, on the other hand, thick skin contains more cells, also living ones, that may contribute to PpIX synthesis. Production of PpIX was found to be only slightly dependent on the skin thickness (Fig. 5 and 6). This statement is not strong because of the large scatter of the points, but, if true, it indicates a limited penetration downwards in the skin, even in the epidermis. It should be kept in mind that the excitation wavelength used for the fluorescence measurements was 407 nm and probes mainly superficial skin layers down to around 1 mm (Divaris *et al.*, 1990). This may partly explain why PpIX fluorescence is independent of the thickness of the whole skin (epidermis and dermis). The density of hair follicles also shows large individual variations. It has been shown that hair follicles in the skin of mice are preferentially damaged by ALA-PDT (Juzenas *et al.*, 2002). This may be due to the fact that after application of ALA, fluorescence of PpIX is mainly located in tumours, epidermis, hair follicles (van der Veen *et al.*, 1996) and sebaceous glands (Bissonnette *et al.*, 2000). Surprisingly, PpIX formation was found to be independent of the density of hair follicles (Fig. 4). This may indicate that, even though individual follicles exhibit a strong PpIX fluorescence after ALA application (Divaris *et al.*, 1990), the density of them is rather low so they do not contribute significantly to overall skin fluorescence. The discrepancy between our studies and microscopic studies is probably related to methodology. Fluorescence microscopy enables distinguishing of fluorescence pattern as a function of a fine skin structure. In our case the fibre-optic probe ensures an integrated record of fluorescence signal over a relatively large skin area (around 0.3 cm²). This may be an advantage whenever fast and reliable pharmacological *in situ* comparisons are needed, but the fine structure of the fluorescence pattern will be lost.

Sun exposure seems to play a role, since, in most cases, more PpIX was produced at skin sites shielded from the sun (upper arm) than on similar skin sites (of similar thickness and temperature) more regularly exposed to the sun (lower arm). Skin on the back of the ear produced twice as much PpIX as skin on the top of the ear (Fig. 3). This may be related to differences in both temperature and history of sun exposure (von Beckerath *et al.*, 2001). However, the results are surprising in view of animal experiments, which showed larger PpIX formation in UV-exposed mouse skin than in unexposed

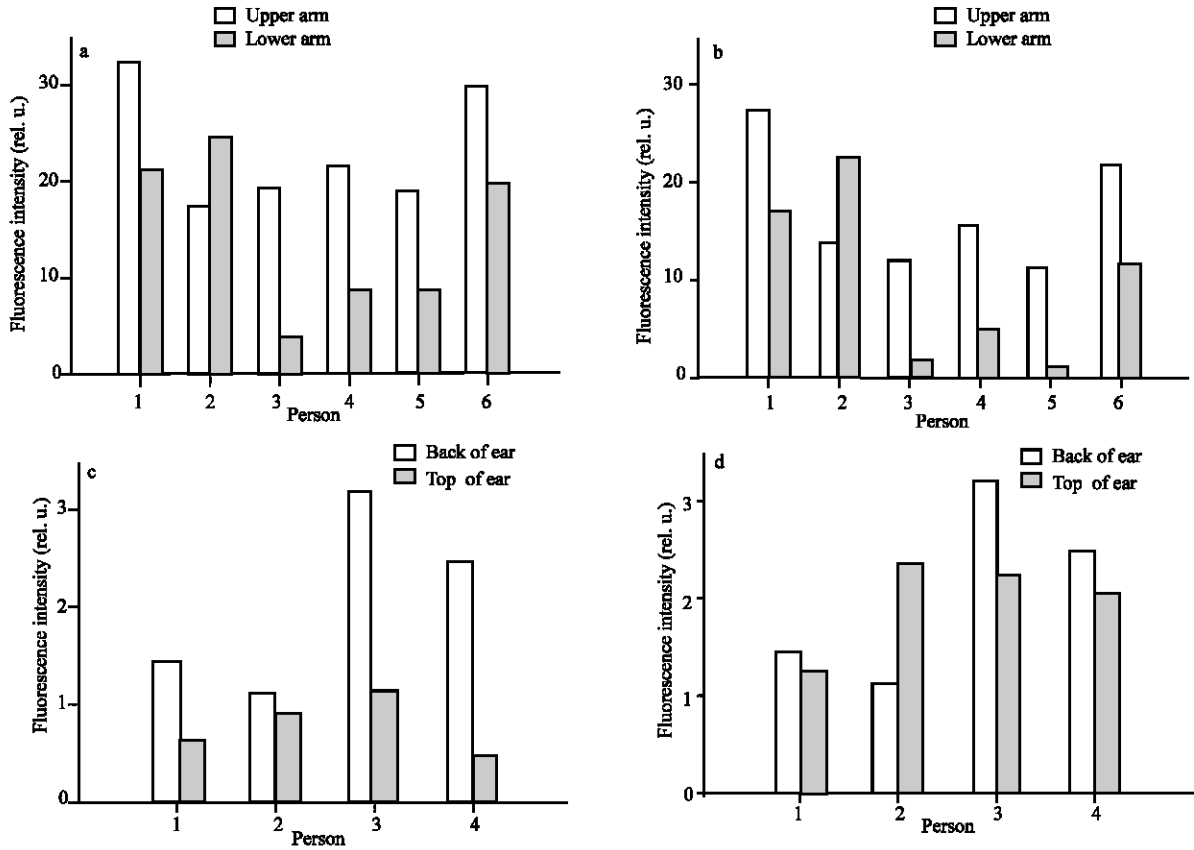


Fig. 3: Fluorescence of PpIX for different subjects after 5 h application of ALA. The data represent A) 10% ALA (w/w), B) 10% ALA (w/w) corrected for the temperature, C) 2% (w/w) ALA and D) 2% ALA (w/w) corrected for the temperature

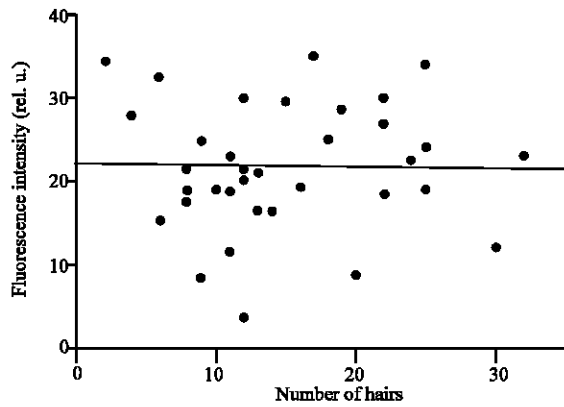


Fig. 4: Fluorescence of PpIX after 5 h application of 10% (w/w) ALA as a function of the number of hairs in an area of 0.8 cm²

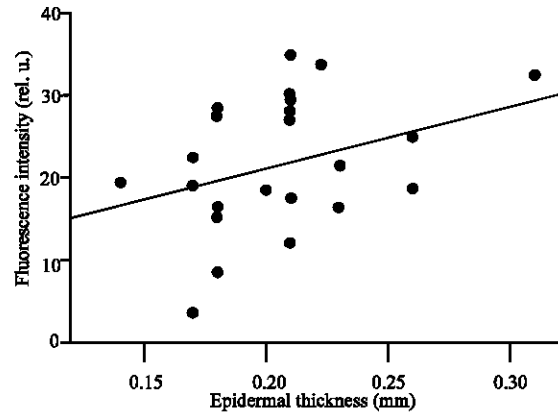


Fig. 5: Fluorescence of PpIX after 5 hours application of 10% (w/w) ALA as a function of the epidermal thickness

skin (van den Akker *et al.*, 2000; Sharfaei *et al.*, 2002). The reason for this discrepancy may be that in the animal experiments, we studied skin with acute UV-damage while in the present work, we consider body localizations with different long-time exposure to solar radiation.

In conclusion, the present results show large individual variations in PpIX production in skin. Increased PpIX production in skin with elevated temperature is indicated. Thus, heating may be a clinically relevant method of increasing the efficacy of ALA-PDT. There was

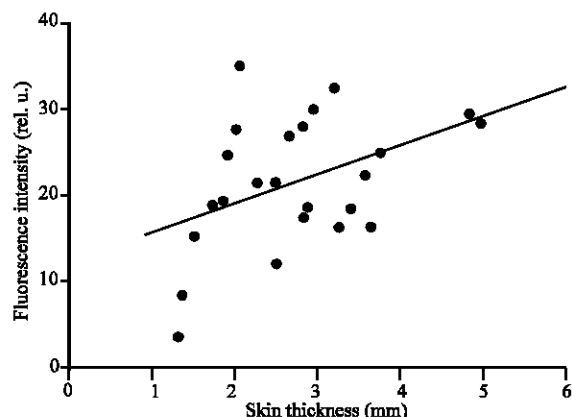


Fig. 6: Fluorescence of PpIX after 5 hours application of 10% (w/w) ALA as a function of the whole skin thickness.

only a weak dependency on PpIX production on skin thickness and no dependency on the density of hair follicles. However, the history of sun exposure, which is an important factor in dermatology, seems play a role, in that less PpIX formation was found in sun exposed than in unexposed skin.

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