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Effect of 5-aminolevulinic acid on kinetics of protoporphyrin IX production in CHO cells

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Abstract: 5-aminolevulinic acid (ALA) is utilized in a photodynamic therapy as a compound capable of augmenting intracellular pool of protoporphyrin IX (PpIX), which exhibits properties of a photosensitizer. The studies were aimed at monitoring accumulation of endogenous protoporphyrin IX in CHO cells under effect of various concentrations of ALA in culture medium and following removal of the compound from the culture medium. Cell content of PpIX was determined following incubation of the cells for 72 h in a culture medium containing different concentration of ALA. Moreover, the cells were preincubated for 2 h in ALA at various concentrations and separated from the compound by medium change and their PpIX content was monitored following incubation. PpIX content was defined by a fluorescent technique under the confocal microscope. In the course of continuous incubation of cells with ALA, biphasic alterations were noted in cellular PpIX concentration. Removal of ALA from the incubation medium resulted at first in a decrease in PpIX content in cells, which was followed by an evidently augmented accumulation of the compound in the cells. The results suggested that in the case of CHO cells, exogenous ALA was not an exclusive source of PpIX synthesis and that alterations in enzyme activities were responsible for production of PpIX.

Key words: Protoporphyrin IX - 5-aminolevulinic acid - CHO cells - Photodynamic therapy

Introduction

Photodynamic therapy (PDT) represents a therapeutic technique, in which cytotoxic effects in cells are induced by light. It requires that the cells contain a photosensitiser which induces the photochemical reactions leading to destruction of the cells [15]. Photosensitizers can be introduced to the cells exogenously, or from an endogenous source [12]. In the latter situation, protoporphyrin IX (PpIX) is used, which accumulates in cells under the effect of 5-aminolevulinic acid (ALA), particularly in neoplastic cells [2, 4, 7, 8]. ALA is a physiological precursor in the synthesis of heme, which is the porphyrin molecule (PpIX) with an iron atom in its ferrous state incorporated into its core. Free heme negatively regulates the activity of the enzyme ALA-synthase, which catalyses the initial metabolic step [1]. Addition of exogenous ALA circumvents this negative feedback control and induces an immediate increase in heme synthesizing activity, which results in intracellular accumulation of PpIX. Photodynamic therapy based on ALA application is at present increasingly widely applied in clinical practice [5].

Till now, particular attention has been paid to the way, in which PpIX is distributed and accumulated in cells under the effect of ALA, which undoubtedly has formed the basis for defining mechanisms linked to PDT effects [6, 10, 12]. For induction of a clinical effect it is important to recognise the kinetics of PpIX accumulation in cells, as influenced by the applied dose of ALA. Cellular content of the photosensitizer should be optimal for induction of the photodestructive effect, following light exposure of the treated neoplastic lesions. The kinetics of PpIX formation under the effect of exogenous ALA is thought to result from circumvented bottle-neck linked to synthesis of endogenous ALA, the level of which remains under control of free heme [1, 8, 11].

Considering that these problems may not only be of theoretical significance, but also have a practical value for establishing conditions of a photodynamic therapy, we decided to define kinetics of PpIX accumulation in CHO cells under the effect of various concentrations of ALA. Moreover, we decided to examine if application of extracellular ALA affects endogenous mechanisms of PpIX formation.

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Materials and methods

The studies were performed on tumour CHO (Chinese Hamster Ovary) cells, grown in RPMI 1640 supplemented with 100 g/ml streptomycin, 100 U/ml penicillin, 2 mmol/l L-glutamine and 5% FCS in a controlled CO_2 atmosphere (5%), at 37˚C. Effects of 5-aminolevulinic acid (ALA) concentration on accumulation of protoporphyrin IX in CHO cells were defined by continuous incubation of the cells in ALA concentrations of 1, 2.5 or 5 mmol/l culture medium for the period of 0, 1, 2, 4, 8, 18, 24, 48 or 72 h. Following that time, PpIX content in cells was evaluated using a confocal microscope (LSM 510, Zeiss). The estimations took advantage of PpIX-exciting laser beam of 458 nm wavelength (argon laser, HFT 458), while the emitted light was analysed using 585 nm filter (LP 585).

Patterns of the analysed cells were captured using 512×512 pixel measuring window (0.0530 mm^2) and the immersion fluorescence objective Plan-Neofluar 40×/1.3 Oil. Alterations in cellular PpIX content after removal of ALA from the incubation medium were defined in the following manner: CHO cells subjected to 2 h preincubation with ALA (the time was selected in a preceding experiment) at concentrations of 1, 2.5 or 5 mmol/l culture medium were washed thrice and placed in the same medium for another 0, 2, 4, 8, 18, 24, 48 or 72 h. After that time, PpIX content in cells was estimated by confocal microscopy in the above described procedure. In each experiment, cells of control group, incubated in the same way, but in the culture medium devoid of ALA were evaluated.

PpIX content was evaluated using the CytFlu 1.2 software and expressed in equipment units (e.u.), which reflected an average intensity of fluorescence per cross-section area of the analysed cells (1 mm^2). Each experiment was performed in 10 independent cultures. Statistical evaluation of the obtained results included the Kruskal-Wallis test (nonparametric ANOVA), performed using Statistica ver. 5 software. P value <0.05 was considered significant.

Results

At all tested ALA concentrations, continuous incubation of CHO cells resulted in a significant intensity increase of PpIX fluorescence. Results of PpIX-specific fluorescence estimation in a confocal microscope following continuous incubation with ALA are presented in Figure 1. At any of the applied ALA concentration, the relation between fluorescence in the examined cells and duration of incubation showed a biphasic character. Following 2 h incubation, augmented values of fluorescence were observed in cells: to 1262 e.u. for ALA concentration of 1 mmol/l, to 1350 e.u. for ALA concentration of 2.5 mmol/l and to 1504 e.u. for ALA concentration of 5 mmol/l. Intensities of the fluorescence differed only slightly between individual groups, despite clear differences in the applied ALA concentrations. In the subsequent 8 h of the experiment, intensity of the PpIX-specific fluorescence significantly decreased ($p<0.001$) and, then, slightly significantly increased ($p<0.05$) (particularly in cases of ALA concentrations of 1 and 2.5 mmol/l) to significantly decrease again ($p<0.05$). At ALA concentration of 5 mmol/l, the peak fluorescence (1956 e.u.) was noted in 48th h of the experiment (Fig. 2). PpIX-specific fluorescence in cells of the control group did not significantly change in the course of the entire experiment and never exceeded the value of 85 e.u.

A separate cycle of experiments was devoted to alterations in PpIX content in CHO cells, which were preincubated with ALA for 2 h and then transferred to the medium free of ALA. The obtained results are illustrated in Figure 2. Following incubation with 1 mmol/l ALA, the content of PpIX significantly decreased (from 1262 to 548 e.u., $p<0.01$) 2 h after removal of ALA. An augmented content of PpIX was observed after 18 h ($p<0.001$), with gradual significant decrease ($p<0.05$) in the subsequent hours of the experiment (Fig. 2). Likewise, in the case of 2.5 mmol/l ALA a significant decrease ($p<0.05$) in cellular PpIX content was noted 2 h following transfer of the cells to ALA-free medium. However, subsequent hours brought about a significant increase ($p<0.001$) in cellular PpIX content until 18 h of incubation, when the content reached 3547 e.u. This was followed by a logarithmic significant decrease ($p<0.001$) in cellular PpIX content. Two-hour incubation of the cells with 5 mmol/l ALA was followed by, on the average, a threefold significant decrease ($p<0.05$) in cellular PpIX content 2 h following transfer of the cells to the ALA-free medium. In the subsequent time period, cellular content of the compound gradually increased and between hours 8 and 24 of the incubation it persisted at the levels of 1082 to 1589 e.u. (Fig. 2).

Discussion

Properly conducted PDT initially requires an appropriate concentration of a photosensitizer and time which has to elapse between its application and light exposure. Accordingly, several studies have been focused on accumulation of photosensitizers in cells depending upon their physicochemical properties and their capacity to penetrate into the cells [4, 14]. The finding, that 5-aminolevulinic acid (ALA), which basically is not a photosensitizer itself, induces accumulation in cells (particularly tumour cells) of endogenous protoporphyrin IX has proved especially significant. Accumulation of PpIX in cells under the effect of ALA used to be evaluated by analysis of fluorescence intensity, employing either culture material or cryostat sections, which as a rule, was hampered by a significant measurement error. In our studies, the measurements of fluorescence intensity were performed using a confocal microscope, therefore the results reflect a thickness of the optical layer and not the total thickness of the examined material in optical axis of the microscope. This has allowed to obtain more reproducible results.

The results have demonstrated that incubation in media containing 1, 2.5 or 5 mmol/l ALA did not induce accumulation of PpIX in the cells, which would parallel the applied ALA concentrations. Moreover, application of extreme, five-fold higher ALA concentrations re-

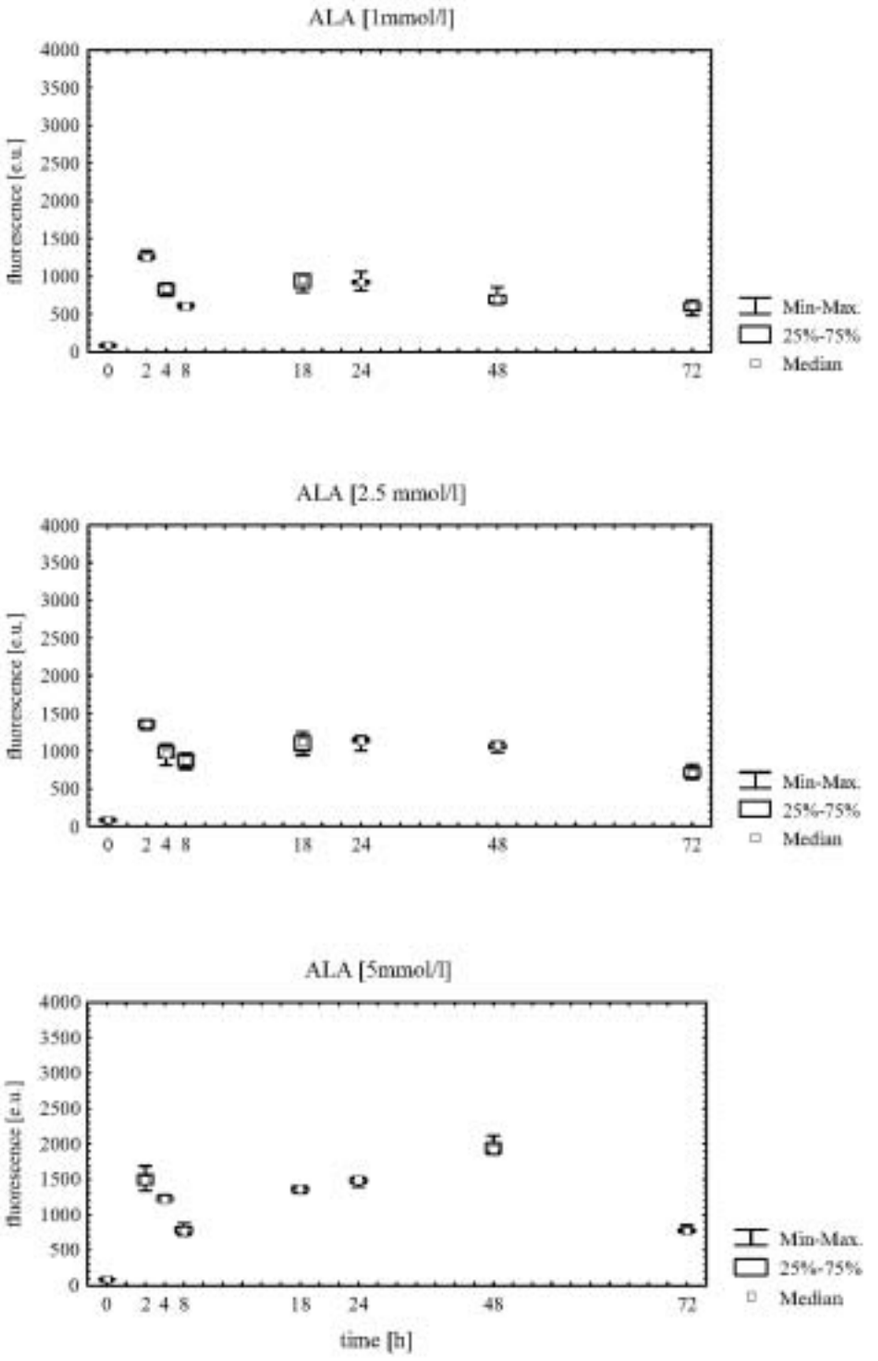

Fig. 1. Alterations in PpIX content in CHO cells during continuous incubation with ALA.

sulted in an increase in PpIX-related fluorescence only by 6-10%. Similar results were noted in *in vitro* studies on human cell lines, originating from lung and bladder cancers, in which application of higher than optimal ALA concentration resulted in a decrease in PpIX formation, paralleled by a decreased activity of mitochondria and lowered cell viability [14]. Continuous incubation of cells with ALA brought about biphasic changes in PpIX mean fluorescence related to duration of the incubation. The evident increase in the fluorescence intensity following 2 h incubation was followed by its decrease (after 8 h), increase and another decrease. The observed alterations should be interpreted as a result of equilibrium between synthesis of PpIX in the cells and removal of PpIX due to the binding of the compound to iron, its transformation to heme or its efflux out of the cells [2].

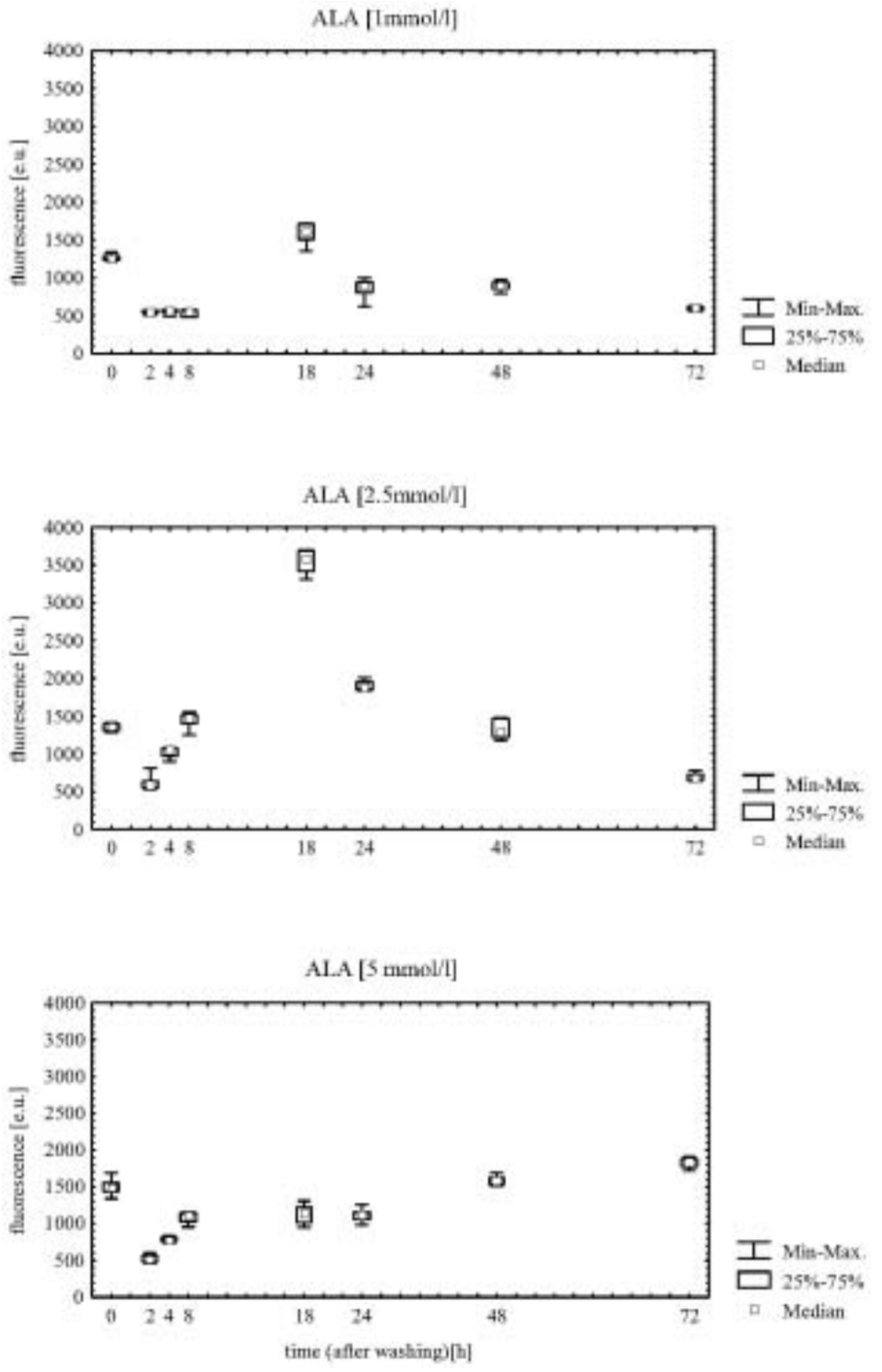

Fig. 2. Alterations in PpIX content in CHO cells, which were preincubated with ALA for 2 h and then transferred to the medium free of ALA.

The rate of PpIX formation is controlled by the slowest reactions of the biosynthetic pathway and may vary in different cells. At the preliminary stage of PpIX biosynthesis, 5-aminolevulinic acid is synthesized in the mitochondrial matrix from glycine and succinylCoA under the effect of ALA-synthase. Subsequently, ALA finds its way to the cytoplasm, in which in the presence of ALA-dehydratase (ALA-D) it becomes condensed to porphobilinogen. As the result of subsequent reactions (deamination, decarboxylation and oxidation), catalysed by appropriate enzymes (porphobilinogen deaminase, uroporphyrinogen decarboxylase, coproporphyrinogen oxidase and protoporphyrinogen oxidase) PpIX is formed, from which, following addition of iron (ferrochelatase - mitochondria), heme is produced [10]. In cases of augmented heme production, activity of ALA-

synthase is inhibited due to a negative feedback [3]. For that reason, the cells do not accumulate intermediate products of PpIX synthesis or heme.

Considering the above biosynthetic pathway of PpIX and heme, the ALA incubation-related augmented synthesis of PpIX in the cells could be interpreted as an effect of bypassing the exogenous ALA of the slowest reaction which controls the dynamics of PpIX formation [7]. In such a situation, one should expect that increased content of PpIX should develop as a second-order reaction and the maximum value should for certain time exhibit plateau, as long as the synthesis of PpIX will balance off its elimination. In cases of several cell lines, the change in PpIX content with duration of incubation with ALA corresponded in fact to the kinetics of second-order reactions, thus indicating that ALA evidently participated in PpIX synthesis [9, 13]. In contrast, in our studies changes in PpIX concentration in the first 8 hours of the experiment have corresponded to alterations, which used to be noted in sequential reactions, in which a product of one reaction serves as a substrate for the subsequent reaction. Similar results could be noted in studies of other authors [14]. The decrease in PpIX content in the studied CHO cells might have reflected efflux of the compound or increased activity of ferrochelatase, which catalyses binding of PpIX with iron.

In order to examine if exogenous ALA has been responsible for the observed effects, we performed the experiments, in which ALA was removed from the medium following 2 h exposure of the cells and subsequent alterations in PpIX content were recorded. As compared to continuous exposure of cells to the compound, removal of ALA from the medium was followed by evident decrease in the photosensitizer-specific fluorescence in the cells. This has confirmed our expectation that the augmented PpIX concentration in the first hours of the experiment has reflected action of exogenous ALA. Surprisingly, at later times following removal of ALA from incubation medium, augmented content of PpIX was detected; even higher levels were observed than in the medium containing ALA.

At the moment, the mechanism of this phenomenon remains difficult to be interpreted, but one should keep in mind the potential for induction of enzymes responsible for ALA synthesis and possibly lower efflux of the compound from the cells. It is certainly important for future investigations; it seems that in certain conditions the cell may preferentially switch to an endogenous mechanism, inducing a higher intracellular accumulation of the sensitizer.

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