Targeting of Sebocytes by Aminolevulinic Acid-dependent Photosensitization†

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Key Words: Aminolevulinic acid; sebaceous gland cells; photodynamic; differentiation.

Abbreviations: PDT, photodynamic therapy; ALA, aminolevulinic acid; PpIX, protoporphyrin IX

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Abstract

Photodynamic therapy using 5-aminolevulinic acid-induced protoporphyrin IX has been developed as a very useful therapeutic modality. Recently, several authors have reported on the efficacy of this procedure for acne. This approach is based on the fact that 5-aminolevulinic acid-induced protoporphyrin IX has strong selectivity for sebaceous glands. We used the immortalized human sebaceous gland cell line SZ95 to investigate cellular mechanisms of photodynamic therapy using 5-aminolevulinic acid-induced protoporphyrin IX. Quantification of induced protoporphyrin IX production showed dependence on the applied 5-aminolevulinic acid dose. When SZ95 sebocytes were differentiated by arachidonic acid treatment, there was no difference between them and the control cells with respect to both the amount of 5-aminolevulinic acid-induced protoporphyrin IX and the phototoxic effects. We altered protoporphyrin IX formation rates by growing cells scattered as single cells in the culture dishes. Single cells produced significantly lower protoporphyrin IX levels than those grown with intercellular contacts. Intracellular localization of protoporphyrin IX was imaged using confocal laser scanning microscopy. The differentiation-specific lipid droplets were virtually excluded from protoporphyrin IX fluorescence. In addition to weak mitochondrial and strong membrane fluorescence, distinctive spots with strong fluorescence were observed. These did not co-localize with fluorescent probes for mitochondria, lysosomes, or the Golgi apparati.

Introduction

Photodynamic therapy (PDT) is based on the tissue-selective accumulation of a photosensitizer that is generally a porphyrin derivative. Following illumination with light of an appropriate wavelength, and in the presence of oxygen, the photosensitizer generates active molecular species, such as free radicals and singlet oxygen that are toxic to cells and tissues. Since the initial study by Kennedy (1), it has been reported that PDT using 5-aminolevulinic acid (ALA)-induced protoporphyrin IX (PpIX), or ALA-PDT, is useful for treating non-melanoma skin cancers, actinic keratoses, and psoriasis, in addition to several other cutaneous indications (2). ALA is taken up by cutaneous cells and is metabolized via the porphyrin pathway to PpIX, the immediate precursor of heme.

Acne vulgaris is the most common skin disease, affecting nearly 80% of young adults (3) or almost 100% of male teenagers (4). Although the pathogenesis of acne has not been fully elucidated, the following factors are considered to be important in its pathogenesis: increased sebum excretion, hyperproliferation, and abnormal differentiation of follicular keratinocytes leading to comedogenesis. The proliferation of *Propionibacterium acnes (P. acnes)* plays an important role in inducing inflammation (5). Currently, a genuine inflammatory process added to the activity of excess tissue androgens and peroxisome proliferators-activated receptor ligands have been proposed as the initiators of the acne lesions (6). Standard treatments for acne include antibiotics, antibacterials, retinoids, anti-androgens (in females) and chemical peeling with α-hydroxy acids (e.g. glycolic acid) for comedonal acne. Although these therapies

contribute to improvement, all of them need prolonged and persistent use for optimal efficacy, which is a major challenge in the treatment of teenagers and young adults.

Several authors have reported that topical ALA-PDT was useful for the treatment of acne vulgaris (7, 8, 9, 10). This approach was based on the observation that porphyrins accumulate in the epidermis and hair follicles, and with some preference, in the sebaceous glands of mice receiving systemic ALA (11). Light exposure after ALA administration resulted in preferential photodamage to the sebaceous glands. This preference of porphyrin accumulation and PDT action for sebaceous glands was attributed to the lipophilicity of PpIX, the predominant ALA-induced metabolite. With experimental PDT of acne, a strong clinical response was also seen; this is associated with the pilosebaceous unit, supporting its preferential targeting by ALA-PpIX. At this time the critical target is not established. Besides its direct effects on sebocytes, ALA-PDT could potentially also affect *P. acnes* and target follicular obstruction.

In this study, we addressed ALA-dependent photosensitization of the human sebocyte, using the sebaceous gland cell line SZ95. SZ95 sebocytes retain the characteristics of normal human sebocytes, such as the ability to undergo cellular differentiation, which is associated with increase of cell volume, lipid synthesis, and apoptosis (12, 13). The expression of characteristic proteins of human sebaceous glands and the biological response to androgens and retinoids have been also preserved in this cell line. We therefore chose SZ95 sebocytes *in vitro* as a relevant cellular model to analyze ALA-induced PpIX formation, its distribution, and phototoxicity. We evaluated culture

condition-dependent variables of PpIX formation and explored differentiation-inducing pharmacological agents in their potential to enhance ALA-PDT.

Materials and Methods

Cell Line and Culture Conditions. Immortalized human SZ95 sebocytes (12) were maintained in Sebomed basal medium (Biochrom, Berlin, Germany) containing 10% (v/v) fetal bovine serum (FCS, Gibco, Invitrogen Corporation, Carlsbad, CA), 5 ng/ml human recombinant epidermal growth factor (Sigma, St Louis, MO), 50 IU/ml penicillin, and 50μg/ml streptomycin (Cellgro, Herndon, VA) in a humidified atmosphere containing 5% CO_2 at 37°C. Culture medium was changed every 2-3 d.

Chemicals. ALA, PpIX, arachidonic acid, spironolactone, methotrexate, Oil Red, and all-*trans* retinoic acid were obtained from Sigma (St. Louis, MO).

Treatment with Arachidonic Acid. Arachidonic acid (AA) was dissolved in 100% ethanol. The final concentration of ethanol in medium with and without AA did not exceed 0.1%. SZ95 sebocytes were treated with AA (10^{-4} M) for 48 h. Cells treated with 0.1% ethanol served as vehicle control.

Oil Red Staining. After treatment with AA, SZ95 sebocytes were washed with phosphate-buffered saline, fixed in Baker's formol for 5 min, washed twice in distilled water for 2 min each, incubated in 60% aqueous isopropanol for 5 min, stained in freshly

prepared 0.3% Oil Red solution in 60% isopropanol for 5 min, and washed in distilled water for 1 min. Nuclei were counterstained with hematoxyline for 20 seconds.

ALA Treatment and PpIX Quantification. In order to establish the dynamic range of ALA-dependent PpIX production in SZ95 sebocytes, cells were plated in 35 mm dishes. All manipulations of ALA-treated cells were performed under reduced light conditions. After 24 h the dishes were incubated in duplicate with 1 ml culture medium without FCS containing 0.2 to 1.0 mM ALA. After 4 h of treatment, samples were used for PpIX quantification as previously described (14). In brief, cells were solubilized in 1% SDS in 0.1 N NaOH and submitted to quantitative spectrofluorometry (excitation 405 nm, emission 580-720 nm).

Manipulation of Intercellular Proximity. In order to evaluate the effect of cell to cell proximity on PpIX production, we compared PpIX formation by exposing the same number of cells to the same amount of medium and ALA. We plated SZ95 sebocytes at $2x10^4$ in 35 mm plates. After 72 h incubation, we ascertained that epithelial cell nests had formed. These samples were denominated ‘nested sebocytes’. At that time, we counted the sebocytes and plated the same number of cells that were then incubated overnight. The next day, this set of dishes had approximately the same number of cells, but in a single cell distribution. Both sets of cells were exposed to the same concentrations of ALA, and PpIX was quantified after 4 h as described above.

Fluorescence Microscopy. For intracellular localization studies, cells were incubated with 5-ALA (4 h, 0.3 mM) or with exogenous PpIX (3 h, 300 nM). Co-staining was performed using standard markers of cellular organelles, such as for mitochondria (Mito Tracker Green FM, 4 nM), lysosomes (Lyso Tracker Green DND, 1 nM) and the Golgi apparatus (BODIPY FL ceramide, 25 nM) (all dyes from Molecular Probes, Eugene, OR). Cellular PpIX localization was imaged using a Leica confocal laser scanning microscope. Fluorescence was excited through a 63x water immersion objective using the 488 nm argon laser line. The fluorescence emission was separated into two bands using a 580 nm dichroic mirror; for the reflected portion, we used a 525 to 550 nm band-pass filter, and for the transmitted portion, a 590 nm long-pass filter. The images were displayed accordingly in green and red false color. Differential interference contrast images were obtained for matching transmission pictures.

Quantification of PpIX Phototoxicity. SZ95 sebocytes were cultured in 35 mm dishes and incubated with 0.2 to 1.0 mM ALA in medium without FCS. After 4 h, fresh medium without FCS was added, and the monolayers were immediately irradiated with 635 nm light from a diode laser (High Power Devices, Inc, North Brunswick, NJ, USA). The light intensity was measured with a Coherent Lasermate power meter (Coherent, Inc, Santa Clara, CA, USA) and was typically 0.06 W/cm^2. After PDT treatment, fresh medium with FCS was added, and cells were cultured under routine conditions. The 3-(4,5-Dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide (MTT) assay was performed at 24 h after light exposure as described in detail earlier (14). Briefly, MTT was added to a final concentration of 0.25 mg/mL in complete media. After 90 minutes, the conversion

product, formazan, was dissolved in DMSO and quantified spectrophotometrically. Quantification of cellular dehydrogenase activity provides a sensitive way of assessing survival after PDT and has been shown to correlate well with other established measures of cytotoxicity, such as colony formation (15).

Statistical Analysis. Mixed effects models were used to compare between experimental groups with each repetition treated as a random effect. Scheffe's adjustment was applied to avoid the inflation of type I error due to the multiple comparisons from between doses. All statistical analyses was done using SAS version 9.1 (Cary, NC).

Results

Small Dynamic Range of PpIX Formation in ALA-Treated SZ 95 Sebocytes.

First, we observed the PpIX amount in the cells and the supernatant when SZ95 sebocytes were incubated with ALA. PpIX released to the media were in range of 34% to 40% of the total amount of ALA at all concentrations (Fig. 1). When the cells were incubated with ALA up to 8 h, PpIX increased linearly in quantity at both low (0.3mM) and high concentrations (1.0mM) of ALA (data not shown), indicating that neither saturation nor induction of enzymatic functions occurred. When SZ95 sebocytes were exposed to a range of ALA concentrations, there was a clear dose dependence of PpIX formation (Fig. 2). This is consistent with the majority of other reports, but we found a relatively small range of dose response. While in LNCaP cells the PpIX values may vary more than 10

times from 0.2 to1.0 mM ALA (16), the range we found was only about 2-fold in SZ95 sebocytes.

AA Treatment Induces SZ95 Sebocyte Differentiation.

As previously reported (13), SZ95 sebocytes treated with 10^{-4} M AA for 48 h produced marked amounts of lipids, which could be visualized by Oil Red staining as cytoplasmic droplets. Most lipid droplets were seen in the paranuclear region. Vehicle-treated control cells showed occasional lipid droplets, but of greatly reduced numbers and sizes.

PpIX Production Depends on Cell Density.

Because cell density determines ALA-induced PpIX production in several cell lines, we compared SZ95sebocytes in two sets of plates, where the proximity of cells was a variable, while the amount of medium and ALA concentrations were kept constant. We found a strong reduction of PpIX production in those cells that were dispersed as single cells compared to those that were grouped in small monolayer aggregates. There appeared to be a significant difference at all concentrations between 0.2 and 1.0 mM ALA ($p<0.0001$) (Fig. 3).

Cellular PpIX Fluorescence Is Strongest in Cell Membranes.

We found PpIX to be mainly localized in the cell membranes. This was seen at the contact of two neighboring cells (Fig. 4). The higher intensity of cell membrane fluorescence was likely due to the vertical geometry of the membranes at the contact point. The nucleus was excluded from red PpIX fluorescence. In addition to a low level

diffuse red fluorescence, distinct spots of about 1-2 μm diameter were strongly red fluorescent. When probing for co-localization with green fluorescent dyes specific for mitochondria, lysosomes and Golgi apparati, no co-localization of these probes with the porphyrin fluorescence were seen (Fig. 5). The most striking finding, however, was the total lack of PpIX fluorescence in the lipid droplets of AA-treated SZ95 sebocytes. The only difference between undifferentiated cells and those treated with AA was that the latter had a reduced expression of the distinct round fluorescence spots (Fig. 4). When we applied exogenous PpIX, an identical pattern of PpIX accumulation was seen (data not shown).

AA Treatment neither Increases ALA-Induced PpIX Production nor ALA-PDT Efficacy.

When SZ95 sebocytes were pretreated with 10^{-4} M AA for 48 h, subsequent exposure to 0.2-1.0 mM ALA induced almost the same amount of PpIX accumulation in pretreated and in control cells (Fig. 2). Similarly, there was no difference in the phototoxic effects between these differently treated cells. Figure 6 shows an MTT assay of cells exposed to ALA and subsequently irradiated with 635nm light. Unirradiated cells and cells exposed to light only served as controls. Additional agents were used to explore their effects both on lipid formation and on ALA-PpIX. The amount of PpIX in retinoic acid treated cells (10 μM, 72 h), methotrexate treated cells (0.1 μg/ml, 72 h), vitamin D treated cells (10^{-3}-1 μM, 72 h), and control cells were almost the same (data not shown). These agents did not effect a change in the morphology of the cells or in the intracellular lipid formation.

Discussion

The motivation for this study lies in the rational development of an ALA-PDT regimen for acne. The objective of our study was to elucidate the kinetics of ALA-induced PpIX and the efficacy of ALA-PDT in human sebocytes. We also explored strategies that potentially may enhance ALA-induced PpIX formation and PDT.

In recent years, clinical ALA-PDT for acne treatment has been explored. Several clinical reports showed efficacy of ALA-PDT as acne therapy (7, 8, 9, 10). This approach to acne treatment was based on the fact that ALA-induced PpIX has strong selectivity for sebaceous glands in skin either by systemic (11) or topical (7) application of ALA.

We assumed that the strong lipophilicity of PpIX would cause accumulation in the lipid-rich environment of the sebaceous gland; data from mice and humans clearly supported this concept (7, 11). No critical evaluation of this issue at the cellular level has been performed. The exact mechanisms of accumulation of ALA-induced PpIX and its subcellular distribution have not been elucidated in sebocytes, which are the dominating cellular component of sebaceous glands. For a long time, investigations of the sebaceous gland were relatively difficult because studies had to be performed in primary cells or in an organ culture. The immortalized human facial sebaceous gland cell line SZ95 enabled us to observe ALA-PpIX kinetics and PpIX-induced phototoxicity in a clinically relevant *in vitro* model. In this study, we confirmed that SZ95 sebocytes accumulate ALA-

induced PpIX and undergo phototoxicity in a dose dependent manner. Our results show that the production of PpIX from exogenous ALA is cell-density dependent, as has been demonstrated in other cell lines, including several cancer cell lines, fibroblasts, and endothelial cells (17, 18, 19).

Measurement of PpIX content in the cells and the medium showed a constant time-dependent increase of porphyrin formation. PpIX showed a relatively high range of efflux to the medium. It has been proposed that PpIX is loosely attached to the plasma membrane and FCS components bind a certain fraction of membrane-bound porphyrin (20). The portion of PpIX that leaked into the medium from the seboctes was in a range of 34 to 40% when using 0.2 to 1.0 mM ALA and incubation times of 2 to 6 h (data shown for 4 h only). This relatively large fraction of PpIX in the medium is surprising, considering that the ALA-containing medium was without FCS, a protocol that enhances cellular retention of PpIX (14). Since lipoproteins are responsible for the majority of PpIX leakage into FCS-containing medium, we expected, in the absence of FCS, that lipophilic PpIX would be transferred from the cytoplasm to the intracellular lipid droplets.

This assumption, however, was not confirmed by our fluorescence imaging studies. These studies demonstrated a striking absence of fluorescence from intracellular lipid droplets. Intracellular localization of ALA-induced PpIX has a distinct cell-type-specific PpIX distribution that causes different phototoxic effects (21, 22). We found PpIX mainly localized to the cell membrane in SZ95 sebocytes, especially when the cells were close together. Furthermore, there were distinct dots in the cytoplasm of some cells.

These dots were not co-localized to the cell membrane, mitochondria, lysosomes, or the Golgi apparati, leaving these structures unidentified. Interestingly, exogenous PpIX induced the same pattern of PpIX distribution in SZ95 sebocytes, including distinct cytoplasmic dots and the sparing of lipid vacuoles. These results support an affinity of PpIX to the cell membrane and an unidentified preexisting structure in the cytoplasm. We noted a published report of a similar PpIX distribution pattern in normal urothelium cells (22).

Arachidonic acid treatment enhanced SZ95 sebocyte differentiation as reported previously (13). The treatment caused formation of a large number of lipid droplets in the cytoplasm of SZ95 sebocytes, as shown by Oil Red staining. In accordance with our keratinocyte data (14, 23), we expected to see increased PpIX formation and phototoxicity in AA-treated sebocytes. However, no increased accumulation of ALA-induced PpIX was seen. Furthermore, no alteration of the ALA-induced PpIX phototoxicity was seen in differentiated SZ95 sebocytes. This finding was similar to the data obtained with the promyelocytic leukemia cell line HL60 that did not show any increase of PpIX production in DMSO-treated, differentiated cells (24). On the other hand, it has been reported that cells such as erythroleukemia cells (25), primary mouse keratinocytes (14), lectin-stimulated lymphocytes (26), preadipocytes (24), and LNCaP cells (16) showed altered PpIX formation rates and cytotoxicity in differentiated versus undifferentiated cells.

Apparently, cell differentiation as a factor of enhanced accumulation of ALA-induced PpIX is cell-type specific. In addition to an apparent lack of increased PpIX forming capacity in differentiated SZ95 cells, we found a small range of dose response. In contrast, in other cell lines (e.g. LNCaP cells), a 5 to 10-fold PpIX production range can be found when cell lines are exposed to ALA concentrations between 0.2 and 1.0 mM (16). The range of ALA-induced PpIX accumulation is much smaller in SZ95 cells (Fig. 2) as well as in the rat keratinocyte cell line REK (23), while primary mouse keratinocytes had a large dynamic range of dose response (14). We suggest that SZ95 sebocytes are at their maximal PpIX production capacity even in their undifferentiated state. If these *in vitro* data are extrapolated to the clinical setting, we may draw two conclusions: first, that the concentration of ALA is not critical for effective PDT, and second, that pharmacological enhancement of ALA-induced PpIX accumulation in sebaceous glands is not a promising strategy to pursue.

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Figure Legends

Figure 1. PpIX is released into the media at all ALA concentrations. SZ95 sebocytes were incubated with 0.2 to 1.0 mM ALA for 4 h and intracellular PpIX and PpIX in the medium was quantified by spectrofluorometry. Experiments show mean±SD values of 4 samples. Closed diamonds, open circles, and open triangles indicate total PpIX, intracellular PpIX, and PpIX in the medium, respectively.

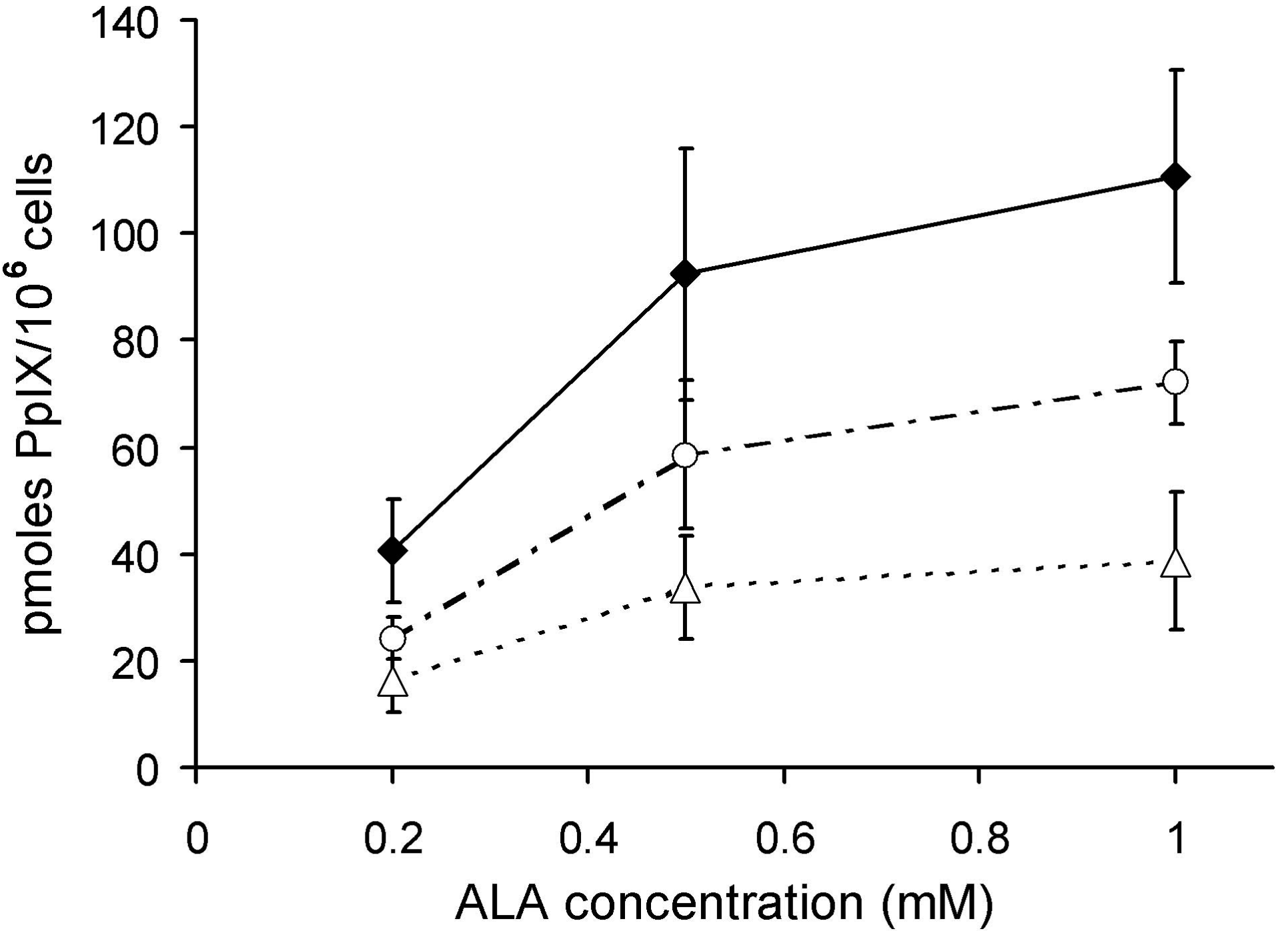
Figure 2. Limited dynamic range of ALA-induced PpIX production in SZ95 cells treated with and without arachidonic acid. SZ95 sebocytes were incubated with 0.2 to 1.0 mM ALA for 4 h and intracellular PpIX was quantified by spectrofluorometry. Cells were pretreated with 10^{-4} M arachidonic acid or with 0.1% ethanol for 48 h. Experiments show mean±SEM values of 6 samples. White bars indicate arachidonic acid treated cells and solid bars control cells.

Figure 3. Increased ALA-induced PpIX production in clustered SZ95 sebocytes. We plated two sets of dishes with the same number of cells, which were either grouped in clusters or in scattered single cell distributions. Both sets of cells were exposed to the same concentrations of ALA. PpIX was quantified after 4 h. Experiments show mean±SEM values of duplicate samples. Closed circles and open triangles indicate clustered cells and single cells, respectively.

Figure 4. Intracellular PpIX fluorescence distribution in SZ95 sebocytes treated with and without arachidonic acid. Transmission and fluorescence images of SZ95 sebocytes treated with vehicle (a,c) or with arachidonic acid (b,d) for 48 h. After incubation with 0.3 mM 5-ALA for 4 h, cellular PpIX fluorescence was imaged by confocal laser scanning microscopy.

Figure 5. Intracellular PpIX localization in SZ95 sebocytes. Red fluorescence, PpIX fluorescence after 4 h-incubation with 0.3 M ALA; green fluorescence from (a) co-staining for mitochondria (Mito Tracker green), (b) lysosomes (Lyso Tracker green), and (c) Golgi apparati (BODYPY FL ceramide).

Figure 6. Identical survival rates after ALA-PDT of undifferentiated SZ95 sebocytes and those pretreated with arachidonic acid. SZ95 sebocytes were pretreated with 10^{-4} M arachidonic acid or with vehicle for 48 h. Cells were incubated for 4 h with 0.3 mM ALA and irradiated. Survival was measured by MTT conversion 24 hours later. Experiments show mean±SEM values of six samples. Closed circles indicate arachidonic acid treated cells, and open triangles, control cells. Control sebocytes exposed to 8 J/cm^2 without ALA incubation showed 100% survival (data not shown).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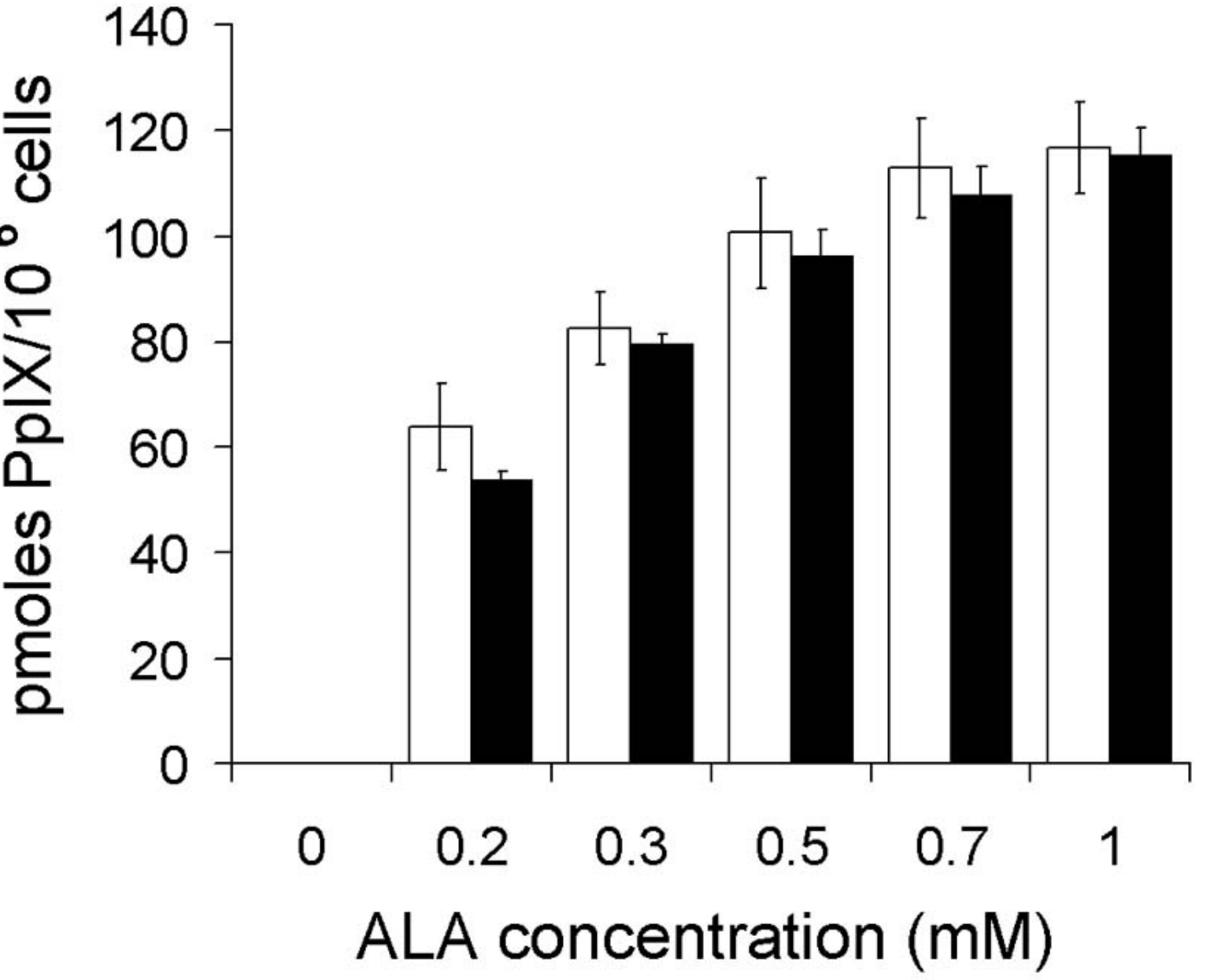
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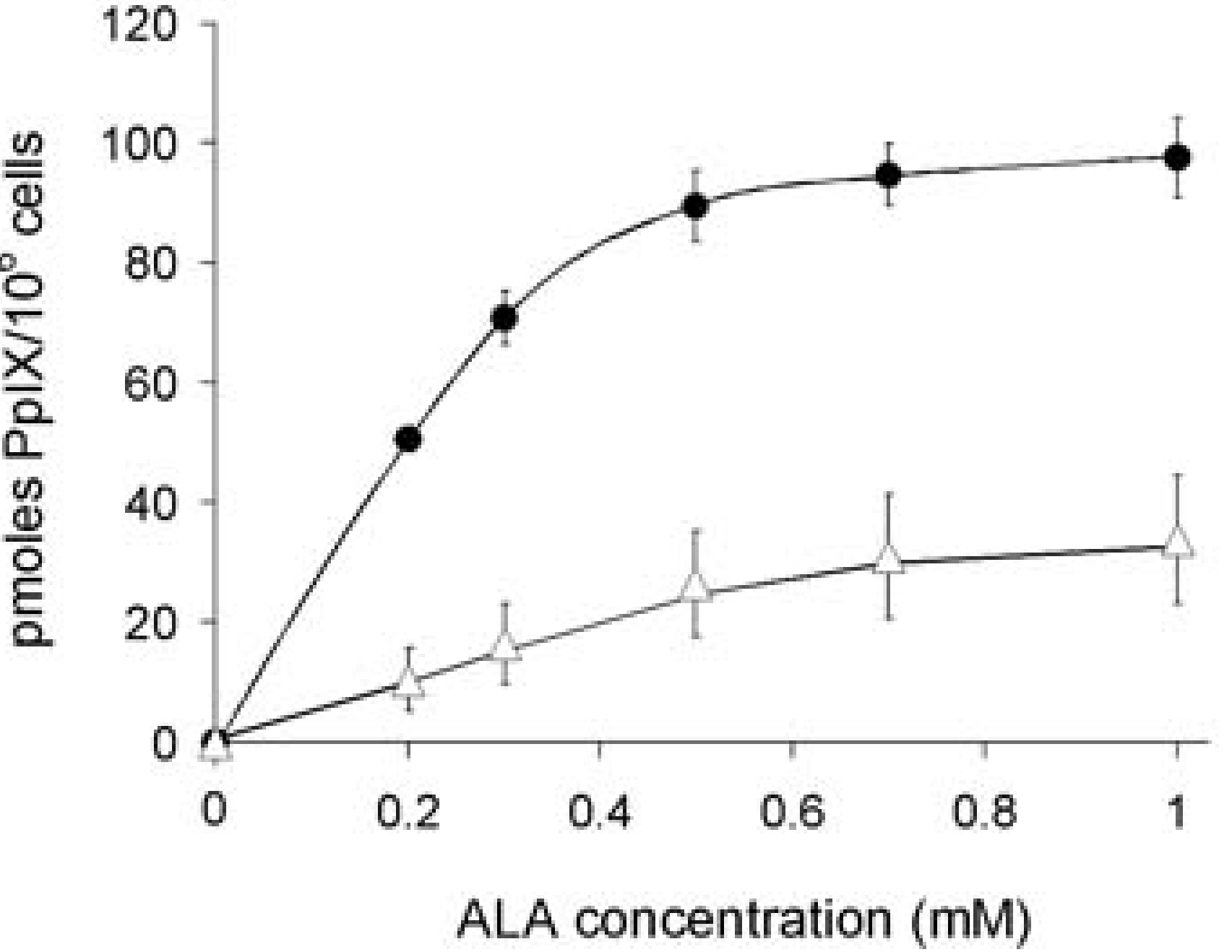
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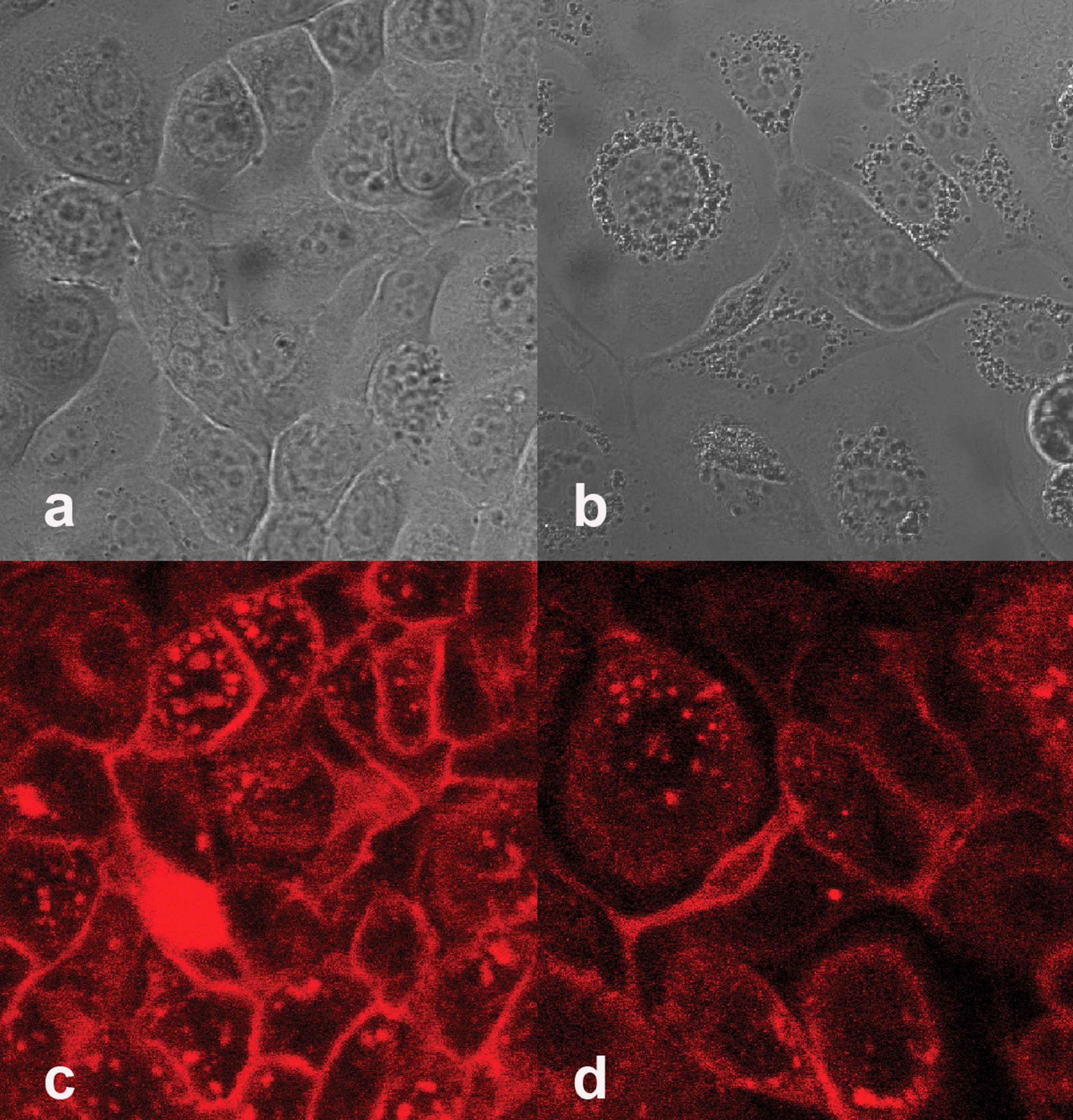
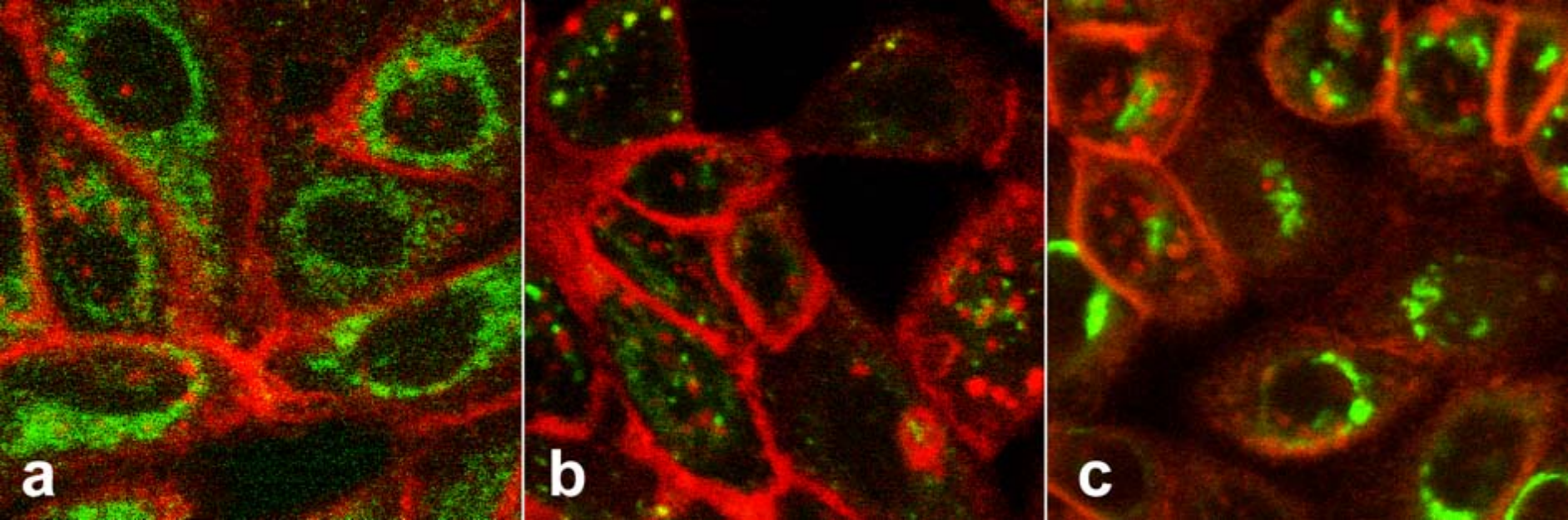
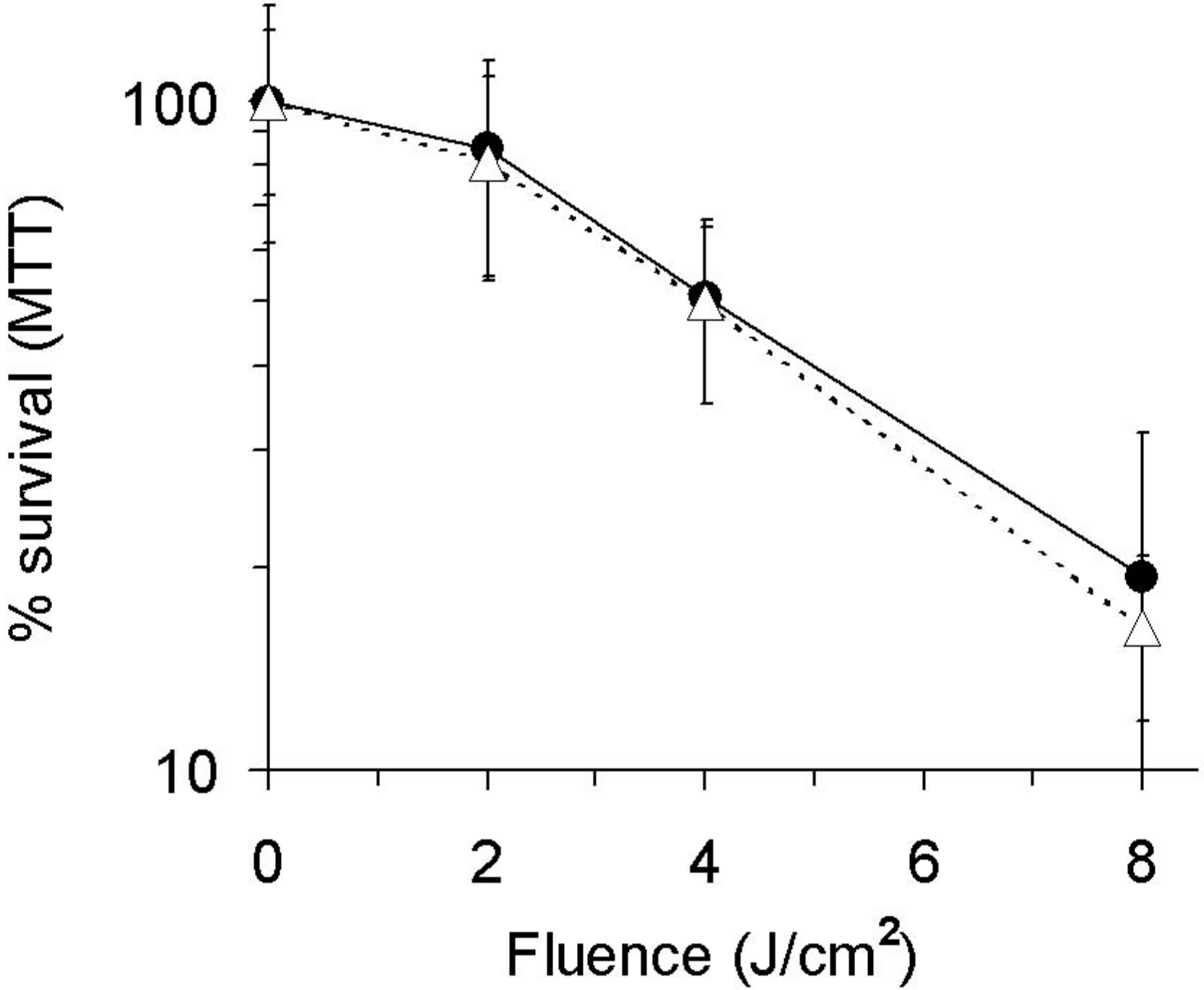
0.8
1
ALA concentration (mM)


a
b
c
d


a
b
c


100
10
% survival (MTT)
0
2
4
6
8
Fluence (J/cm²)