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Photodynamic action of the cationic dye tetrakis(N-ethylpyridinium-4-yl)porphyrin tetratosylate on *Escherichia coli* cell envelope

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We have analysed the photodynamic action of the cationic photosensitiser tetrakis(N-ethylpyridinium-4-yl)porphyrin tetratosylate (TN-Et-PyP) on the integrity and functions of *E. coli* AN 180 cell membranes. Photosensitisation of bacterial cells with photobactericidal concentrations of TN-Et-PyP resulted in abolition of plasma membrane (PM) voltage ($\Delta\Psi$), dissipation of transmembrane K^+ gradient and inhibition of the respiratory activity of bacteria contemporary with enhanced accumulation of the lipophilic anion phenyldicarbaundecaborane (PCB^-). Substantial impairment of cell PM functions suggests that the *E. coli* cell envelope, mainly at the level of PM, is a critical target of phototoxic action of TN-Et-PyP.

Key words: photosensitiser tetrakis(N-ethylpyridinium-4-yl)porphyrin tetratosylate (TN-Et-PyP), *Escherichia coli*, photodamage of cell membrane

INTRODUCTION

The rising prevalence of antibiotic-resistant bacteria stimulates the development of alternative antibacterial therapeutics [1]. Photodynamic antimicrobial chemotherapy (PACT) represents a promising alternative to the use of antibiotics and antiseptics for a selective combat against resistant bacteria, particularly for the treatment of localized infections [2–4]. PACT relies on a photosensitising compound, light and oxygen in order to generate phototoxic products, such as singlet oxygen and free radicals, which result in the eradication of target cells. The technique has been shown to be effective against multi-drug resistant strains and currently is gaining increased acceptance [3]. Reactive oxygen species react with almost every cellular component. The multiplicity of targets makes it difficult for the cells to develop resistance, and this is one of the advantages of PACT [2]. One of present directions in PACT development is a search for new photosensitisers with improved photobactericidal properties. There is a considerable current interest in the application of new generation photosensitisers based on various molecules containing a tetrapyrrole unit [5, 6]. Photosensitisation with different tetrapyrrole compounds has been shown to be efficient for the killing of cancerous and bacterial cells [5, 7].

We have previously reported that cationic photosensitiser tetrakis(N-ethylpyridinium-4-yl)porphyrin tetratosylate (TN-Et-PyP) expresses a strong photobactericidal action against certain Gram-positive and Gram-negative bacteria [8]. In the present study, in order to obtain information on the mechanism by which TN-Et-PyP induces photokilling of *E. coli*, we combined the estimations of cell viability with analysis of respiratory activity as well as potentiometric measurements of accumulation of K^+ and lipophilic ions (TPP^+ and PCB^-) by intact and TN-Et-PyP-treated cells to evaluate the primary target of the dye's photobactericidal action in the cell.

MATERIALS AND METHODS

Reagents. TN-Et-PyP was prepared by treating tetra(pyridin-4-yl)porphyrin with ethyl *p*-tosylate in dimethylformamide, using the general procedure of Pasternack and cowor-

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kers [9]. Stock solution was pepared in distilled water at a concentration of 1 mg/ml and stored at –20 °C in the dark. Potassium phenyldicarbaundecaborane was synthesized by Dr. A. Beganskienė (Faculty of Chemistry, Vilnius University). Tetraphenylphosphonium chloride was purchased from Aldrich. Other reagents were of analytical grade.

Bacterial growth. *Escherichia coli* K12 strain AN180 (F^-, arg, thi, xyl, str-r), kindly provided by Prof. F.Gibson (National University of Australia) was used in this study. The cells were grown in the Luria–Bertani [10] medium at 37 °C with aeration to mid-log phase (OD_{540} = 1.0; ~3×10^8 colony forming units (cfu)/ml), harvested by centrifugation (7500 g, 15 min) and resuspended to 1/80 of original volume in 0.1 M sodium phosphate buffer (pH 7.5). The suspension was used for photosensitisation experiments.

Photosensitisation experiments. Stock cell suspension was diluted with 0.1 M sodium phosphate buffer (pH 7.5) to ~1.5×10^8 cells/ml in 50 ml flat-bottomed flasks and treated with TN-Et-Pyp (2.5 μg/ml) (total volume of each sample 10 ml). The cells with and without photosensitiser were incubated at 37 °C in the dark for 30 min. Half of the samples were then exposed to light from the light-emitting diode array UNIMELA-509 (λ = 509 nm) for 30 min (the fluence rate 3 mW/cm^2), VU Laser Research Centre, Lithuania. After the treatment, cell suspensions from each sample were concentrated by centrifugation (12 000 g, 15 min) and subsequent resuspension in 100 μl of 0.1 M sodium phosphate buffer (pH 8.0). The cells were kept on ice until electrochemical measurements were performed.

Measurements of electrochemical parameters. Concentrations of TPP^+, PCB^- and K^+ in the media were measured as described earlier [11]. Briefly, measurements were performed by selective TPP^+ and PCB^- electrodes elaborated at the Laboratory of Membrane Biochemistry, Vilnius University, and a K^+-selective electrode (Orion model 970800) from Thermo Inc. The measurements were performed simultaneously in three reaction vessels. The calibration of the measuring system was performed at the beginning of each experiment by adding appropriate amounts of tetraphenylphosphonium chloride, phenyl-7,8-dicarbaundecaborane potassium salt or potassium chloride to 5 ml of 0.1 M Tris-HCl buffer (pH 8) containing 9 mM of glucose placed into a measurement vessel thermostatted at 37 °C. The concentrated cell suspension was added to the final concentration of ~3×10^8 cells/ml. The oxygen consumption by the cells (final concentration ~1.5×10^8 cells/ml) was carried on in the same medium in a 2 ml chamber with a Clark-type oxygen electrode of a digital oxygen measurement system (Rank Brothers Ltd, England) and Pico Recorder software. Sodium dithionite was used to define the zero level of oxygen in the incubation chamber. In the course of electrochemical experiments, cell suspensions were aerated by magnetic stirring. Experiments were repeated 3–5 times to ascertain reproducibility.

Estimation of the viability of bacterial cells. Bacterial viability was defined as the cell capability to form colonies on a solid nutrient broth. Photosensitiser- or/and light-treated cell suspensions were serially diluted in 0.9% NaCl, and 100 μl aliquots were plated on nutrient agar plates in duplicate. The colonies appearing on the plates were counted after overnight incubation at 37 °C in the dark. The data are presented as means ± standard error (SE) from 2–5 independent experiments. SigmaPlot 2001 for Windows version 7.101 software was used for the statistical analysis.

RESULTS AND DISCUSSION

A wide range of bacterial cell components, such as cell envelope, cytoplasmic enzymes or DNA could be the targets of photosensitisation-induced damage [2]. The interaction of the photosensitiser with bacterial cell occurs in several stages. The cell envelope is the first site of the photosensitiser–cell interaction and one of the main targets for a deleterious action of reactive oxygen species produced by the photodynamic process [4]. Both membrane lipids and proteins may be affected. Injurious effects of photodynamic action on membranes of microorganisms, including bacteria, were reported by several authors [12–15]. In the following set of experiments, changes in PM voltage (ΔΨ), transmembrane K^+ gradient, accumulation of the lipophilic ion PCB^- and respiratory activity of *E. coli* AN180 bacteria after dark treatment or photosensitisation with the cationic photosensitiser TN-Et-PyP were studied in order to elucidate the mode of action of the dye on the integrity and functions of *E. coli* cell membranes (Figure). The distribution of the lipophilic cation tetraphenylphosphonium (TPP^+) between the cells and the external medium was estimated to assay the ΔΨ (Figure, panel A). TPP^+ is widely used for estimating membrane voltages (ΔΨ, transmembrane differences of the electrical potential) in objects that are negatively charged inside, such as bacteria [16]. TPP^+ easily penetrates phospholipid bilayers. However, the outer membrane (OM) of Gram-negative bacteria is an asymmetric bilayer. The inner layer of the OM consists of phospholipids and the outer layer of lipopolysaccharide (LPS), and the latter forms an effective permeability barrier against various hydrophobic and lipophilic compounds, including TPP^+ and PCB^- [17]. Therefore, bacterial cells added to the medium containing TPP^+ bind only small amounts of this lipophilic cation (Figure, A). Addition of EDTA increases cell OM permeability to lipophilic ions. As has been shown previously [17], EDTA removes stabilizing divalent cations from their binding sites in LPS by chelation. This results in the release of a significant amount of LPS from the outer leaflet of the OM, which is changed by phospholipids. The formed phospholipid bilayer patches then act as channels through which TPP^+ can diffuse [17]. As a consequence, TPP^+ acquires the possibility to distribute between the cell cytoplasm and the external medium according to ΔΨ. The results of experiments, presented in Figure (panel A) show that both TN-Et-PyP-treated in the dark and untreated bacterial cells (Figure, A, curves 2 and 3) accumulated similar amounts of TPP^+, which corresponded to comparable magnitudes of their ΔΨ. The subsequent addition of polycationic antibiotic polymyxin B (PMB) was used to depolarize the cells completely in order to estimate the voltage-independent binding of TPP^+ by the cells. No uptake of TPP^+ by TN-Et-PyP–photosensitised cells was observed (Figure, A, curve 1), and this effect can be ascribed to the collapse of ΔΨ.

ΔΨ is stringently coupled with H^+ as well as with other ion gradients. The K^+ gradient plays one of the main roles in bacterial cells [18]. Therefore, in the parallel experiments, the metabolic competence of the cells was checked by measuring their

transmembrane K^+ gradient (Figure, panel B) and respiration rates (Figure, panel C). The results showed that the magnitude of K^+ gradient of the dark-treated cells remained unaffected, as revealed by addition of PMB, which dissipated the accumulated K^+ gradient and allowed estimating its original value (curves 2 and 3, Figure, B). However, Tn-Et-Pyp photosensitised cells did not show any significant amount of K^+ left in them after illumination, probably resulting from K^+ leakage into the media before placing bacteria into the measurement vessel (Figure, B, curve 1). The rate of oxygen consumption by the dark-treated cells only slightly decreased as compared with untreated ones (Figure, C, curves 2 and 3). However, the photodynamic treatment of the cells resulted in the abolishment of the K^+ gradient and a total inhibition of respiratory activity (curve 1, Figure C).

In the following set of experiments, the binding of the lipophilic anion PCB^- by intact, TN-Et-PyP dark-treated and TN-Et-PyP-sensitised cells was estimated (Figure, panel D). It had been shown previously [19, 20] that PCB^- binding depended on OM permeability as well as on the energy state of the cell. It is possible to distinguish the portion of metabolically active cells in a population, using the measurements of PCB^- binding [20]. Untreated cells were found to bind only negligible amounts of PCB^- (Figure, D, curve 3). A minor increase of PCB^- binding by cells treated with TN-Et-PyP in the dark (Figure, D, curve 2) could reflect both slightly enhanced cell OM permeability to lipophilic anions (though no increase of OM permeability to TPP^+ after the photosensitisation of cells was observed (see Figure panel A, curve 2) or some sublethal changes of PM properties, which are subsequently recovered, presumably after the plating of cells on a solid nutrient broth. Photosensitised cells bound a substantial amount of PCB^-, comparable to the amount bound by heat-inactivated cells (Figure, D, curves 1 and 4). PMB caused a further increase in PCB^- binding both to photosensitised and to heat-inactivated cells, probably by facilitating the partition of PCB^- into the interior of phospholipid bilayers (Figure, D, curves 1 and 4).

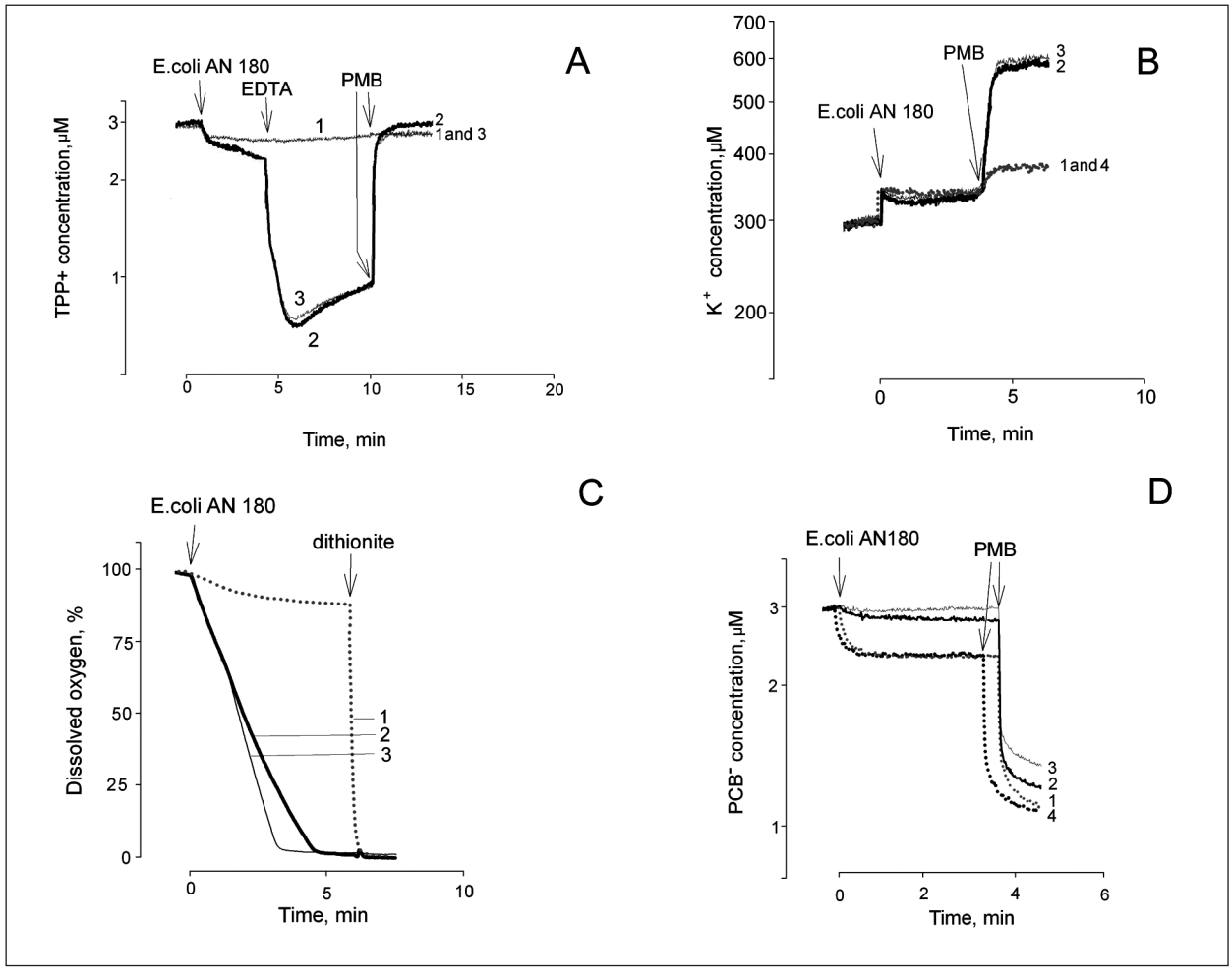

Figure. The accumulation of TPP^+ (A), K^+ (B), oxygen (C) and PCB^- (D) by intact and Tn-Et-Pyp treated *E. coli* AN180 cells. The measurements were performed at 37 °C in 0.1 Tris / HCl (pH 8.0) with 9 mM glucose, as described in "Materials and methods". Cells were added to the concentration of ~3×10^8 cells/ml (A, B and D) or 1.5×10^8 cells/ml (C). EDTA was added to the final concentration of 10 mM (A), PMB to the final concentration of 100 μg/ml (A, B and D). A grain of sodium dithionite was added to define the zero level of oxygen in the incubation chamber (C).

The grey dotted curve (1) represents Tn-Et-Pyp photosensitised cells, heavy solid curve (2) – Tn-Et-Pyp treated cells without illumination, solid thin curve (3) –untreated cells, black dotted curve (4) – cells treated with Tn-Et-Pyp and heated (10 min, 80 °C) after illumination (on B, curves 1 and 4 coincide)

Table. **Effects of TN-Et-PyP and light on *E. coli* AN180 cell viability, and optical density of the cell suspensions withdrawn from measurement vessels**

Treatment	Viable cells, % of control	OD of cell suspension (λ = 540 nm)
Illumination without Tn-Et-Pyp	100 ± 0.12	1.08 ± 0.25
Tn-Et-Pyp in the dark	94.42 ± 11.30	1.06 ± 0.23
Tn-Et-Pyp with illumination	0.84 ± 0.11	1.06 ± 0.27

The above results as well as data on the photobactericidal efficacy of TN-Et-PyP against *E. coli* cells, presented in Table, suggest that the OM of *E. coli* cells does not act as a permeability barrier preventing the access of cytotoxic reactive species (e. g., singlet oxygen) generated by TN-Et-PyP and light to the underlying sites, at least to PM. However, as shown in Table, the turbidity of cell cultures was decreased neither after the dark incubation nor photosensitisation with TN-Et-PyP, and there were no differences in the overall appearance (e. g., debris, visible change of viscosity, etc.) among TN-Et-PyP dark-treated, untreated and photosensitised samples. This observation tends to rule out the emergence of wide breaches in envelopes of photosensitised cells, however, existence of more subtle disturbances of envelope permeability is still to be checked.

Irradiation of the bacteria in the absence of a photosensitiser had no detectable effect either on bacterial viability or on membrane functions (data not shown).

The results reported here are consistent with the assumption that the *E. coli* cell envelope is the primary target of damage by TN-Et-PyP-photosensitisation. The molecular basis of photodamage could be a chemical modification of the proteins, such as components of respiratory chain and / or lipids, presumably via peroxidation. Further research is necessary to ascertain the molecular details of TN-Et-PyP-mediated membrane photodamage. Besides, it is not clear yet whether the sites of photodynamic damages are limited to the cell envelope. Our further work is intended for investigating these issues.

On the other hand, analysis of the respiratory activity or potentiometric measurements of TPP^+, PCB^- or K^+ accumulation by bacterial cells could be considered as a simple and rapid way to test the efficacy of photosensitisers exerting a membranotropic action.

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KATIJONINIO DAŽIKLIO TETRAKIS(N-ETILPIRIDIN-4-IL)PORFIRINO TETRATOSILATO FOTODINAMINIS POVEIKIS *ESCHERICHIA COLI* LĄSTELĖS APVALKALĖLIUI

Santrauka

Tirtas katijoninio dažiklio tetrakis(N-etilpiridin-4-il)porfirino tetratosilato (TN-Et-PyP) fotodinaminis poveikis *Escherichia coli* AN180 ląstelės membranų vientisumui ir funkcijoms. Nustatyta, kad ląstelių fotosensibilizacija, esant fotobaktericidinėms TN-Et-PyP koncentracijoms, depoliarizuoja ląsteles, naikina transmembraninį K^+ gradientą ir slopina ląstelių kvėpavimą. Tuo pat metu ląstelės įgyja gebėjimą surišti didelį kiekį lipofilinių anijonų (PCB^-). Esminių ląstelės plazminės membranos funkcijų slopinimas leidžia daryti prielaidą, kad ląstelės apvalkalėlis (daugiausia PM lygmeniu) yra pirminis TN-Et-PyP dažiklio fototoksinio poveikio taikinys.