Cytochrome c translocation in m-THPC photosensitised murine hepatoma MH22 cells *in vitro*

A. Sadauskaitė,
E. Pimpė,
J. Kadziauskas,
V. Kirvelienė,
B. Juodka

Department of Biochemistry and Biophysics, Vilnius University, M. K. Čiurlionio 21, LT-2009 Vilnius, Lithuania

We followed cytochrome c release to the cytosol of murine hepatoma MH22 cells photosensitised by m-THPC, which localizes to cell membranes including those of mitochondria. The effect of cyclosporin A, a specific inhibitor of mitochondrial permeability transition pore (PTP), on cytochrome c translocation and caspase activity was studied. Data presented here indicate that the passage of cytochrome c from mitochondria to the cytosol in photodamaged cells undergoing apoptosis induced by oxidative stress occuring directly in the cellular/mitochondrial membranes did not rely on PTP opening.

Key words: apoptosis, caspase, cytochrome c, mitochondria, m-THPC, photosensitisation

INTRODUCTION

The recent discovery of active involvement of mitochondria in apoptosis has made the field a topical one. Interest has centred around the opening of mitochondrial permeability transition pores (PTP) and the release of regulatory proteins of apoptosis [1]. In 1996 it was found that cytochrome c, a 12 kDa water-soluble mitochondrial haemoprotein, the only biological function of which was assumed to shuttle electrons between complexes III and IV of the electron transport chain, can activate cytosolic caspases [2]. The details of cytochrome c translocation from mitochondria to the cytosol are still unknown, and the following mechanisms were suggested: (1) direct induction of PTP opening leading to cytochrome c release [3]; (2) inhibition of mitochondrial respiration, resulting in oxidant production and decreased membrane potential, promoting PTP opening and cytochrome c release [4]; (3) oxidation and degradation of mitochondrial phospholipids leading to translocation of cytochrome c to the cytosol [5]. Besides, the formation of channels by pro-apoptotic members of Bcl-2 family was proposed [6]. It is possible that a universal mechanism for cytochrome c translocation does not exist, and either of the mechanisms presented above play a role in some settings.

Here, we followed cytochrome c release to the cytosol of murine hepatoma MH22 cells photosensitised by m-THPC, which localises to cell membranes including those of mitochondria [7]. Photosensitisation *per se* is a hot field due to its successful medical application for treatment of neo- and hyperplasias, and m-THPC is a promising photosensitiser of the so-called second generation [8]. It is generally accepted that reactive oxygen species (ROS) produced by photosensitisers exposed to light are the agents inducing further cellular damage that might lead up to cell death [9], and apoptosis and necrosis have been recorded in cells subjected to PDT [10]. Earlier we showed that apoptosis was the highly predominant mode of death of MH22 cells, which had exposed to light after accumulating m-THPC [11].

Data presented here indicate that the passage of cytochrome c from mitochondria to the cytosol in m-THPC photosensitised cells undergoing apoptosis induced by oxidative stress occuring directly in the cellular/mitochondrial membranes does not rely on PTP opening.

Corresponding author: V. Kirvelienė, Department of Biochemistry, Vilnius University, M. K. Čiurlionio 21, LT-2009 Vilnius Lithuania. E-mail: vida.kirveliene@gf.vu.lt

MATERIALS AND METHODS

Chemicals

Tissue culture products were obtained from Biochrom. Culture flasks and plates were from Becton-Dickinson. Meso-tetra(3-hydroxyphenyl)-chlorin (m-THPC) was kindly provided by R. Bonnett and B. A. Djelal (University of London, UK). Other chemicals were from Sigma, unless stated otherwise.

Cell culture

MH22 cells from murine hepatoma were cultivated in Dulbecco's modified Eagle's medium (DMEM) as described in Ref. 11 and underwent photosensitisation and light exposure as described in Ref. 12. The light source was LED array UNIMELA-660, emitting at 660 ± 5 nm (Vilnius University). Cyclosporin A was added 1 h before light exposure to a 3 μM final concentration. Cell viability was tested by MTT assay [13].

Western blot analysis of cell extracts

$7 \cdot 10^6$ cells were permeabilized for 30 min with 70 μl of PBS containing 50 μg/ml saponine. Opened cells were pelleted by centrifugation (15000 g for 10 min at 4 °C) and the supernatant was denaturated using Laemmli buffer [14]. Samples were separated by 15% SDS-PAGE, electrophoretically transferred to nitrocellulose membranes (Biorad) and incubated with antibodies according to Ref. 15. The membranes were incubated overnight with monoclonal antibody to cytochrome c (Biomol) and for 2 h with peroxidase-conjugated Anti-Mouse IgG (Jackson ImmunoResearch Laboratories, Inc.). Detection was carried out using an enhanced chemiluminescence detection system (Amersham Biosciences).

DEVD-specific caspase activity assay

The activity of DEVD-specific caspases was measured using the Caspase Fluorescent Substrate/Inhibitor QuantiPak (Biomol) based on the cleavage of 7-amino-4-trifluoromethyl coumarin dye from corresponding peptide derivative. The cleavage was followed by fluorescence measurements using an AscentFL microplate reader (Labsystems) at the exitation wavelength 390 nm and emission wavelength 510 nm. 10^5 cells were used for each measurement. The activity of the caspases was calculated as pmol substrate/min from a slope of the plot 'fluorescence *vs* time', using a conversion factor obtained from an appropriate calibration curve.

RESULTS AND DISCUSSION

In order to follow cytochrome c release in photosensitised cells undergoing apoptosis after exposure to light, permeability of plasma membrane was selectively increased by saponin under mild conditions without touching the mitochondria. When cells were exposed to light at a dose 1.8 kJ/m^2, after 24 h 11 ± 3% of cells were still viable (not shown). However, Western blot analysis indicated that nearly total cellular cytochrome c was present in the cytosol 2 h following light exposure (Figure, a).

The role of PTP in cytochrome c release was checked out by closing the pore with cyclosporin A, a specific inhibitor of cyclophilin D [16], which is a member of the multiprotein complex forming the pore. It was shown that during oxidant-induced apoptosis cyclosporin A significantly reduced cytochrome c loss from mitochondria [17]. However, cyclosporin A had a reverse effect on cytochrome c release in photodamaged cells. At a light dose 1.8 kJ/m^2, cyclosporin A exerted no effect on cytochrome c translocation to the cytosol, and at a light dose 0.6 kJ/m^2 (45 ± 6% viable cells after 24 h), disturbance of mitochondrial permeability transition by cyclosporin A stimulated cytochrome c release (Figure, b).

These unexpected results needed to be verified. Since cytochrome c in the cytosol activates caspases executing apoptotic transformations, the activity of DEVD-specific caspases (caspase-3) in photodamaged MH22 cells was checked. Again, no changes of caspase activity due to PTP closing by cyclosporin A were registered at a light dose 1.8 kJ/m^2, and a

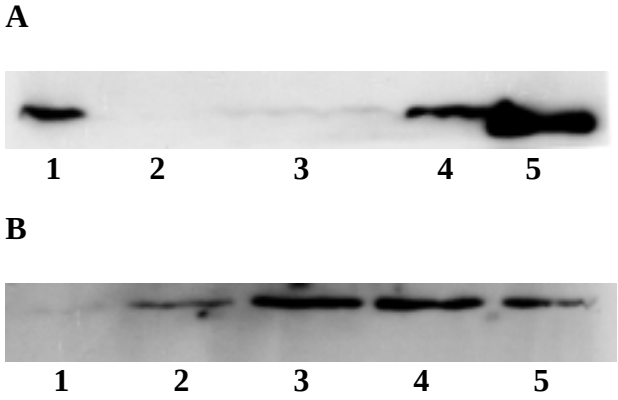

Figure. Translocation of cytochrome c to cytosol of m-THPC-photosensitised MH22 cells. Cells were incubated with 0.15 μg/ml m-THPC for 18 h.
A. Light dose 1.8 kJ/m^2; 1 – total cellular cytochrome c, 2 – untreated cells, 3 – 0.5 h after light exposure, 4–2 h after light exposure, 5 – cytochrome c, marker, 0.5 μg.
B. 2 h after light exposure; 1 – untreated cells, 2 – light dose 0.6 kJ/m^2, 3 – light dose 0.6 kJ/m^2 with cyclosporin A, 4 – light dose 1.8 kJ/m^2, 5 – light dose 1.8 kJ/m^2 with cyclosporin A.

Table. **DEVD-specific caspase activity in m-THPC-photosensitised MH22 cells**
The cells were incubated with 0.15 μg/ml m-THPC for 18 h. Cyclosporin A (CspA) was added, when indicated. Caspase activity was determined 2 h following light exposure

Conditions of cell treatment	Caspase activity, pmol/min
Control	0
Control with CspA	0
0.6 kJ/m^2	2.6
0.6 kJ/m^2 with CspA	3.3
1.8 kJ/m^2	1.8
1.8 kJ/m^2 with CspA	1.8

detectable increase of caspase activity at a light dose 0.6 kJ/m^2 was recorded (Table).

The observed effects of cyclosporin A on cytochrome c release and caspase activity do not support the suggested mechanisms of cytochrome c translocation relying on PTP opening. The hypothesis of degradation of mitochondrial phospholipids seems to be more acceptable in the case of cells undergoing apoptosis induced by membranously accumulated photosensitisers producing toxic ROS in close proximity of their location. An increased peroxidation of lipids in m-THPC-sensitised MH22 cells has already been registered [12]. Binding of cyclosporin A to a member of PTP could cause a hindrance to permanent translocation of proteins in the membrane. It could make phospholipids more susceptable to peroxidation by ROS, especially when the ROS flow is rather slow. However, this suggestion needs experimental verification.

ACKNOWLEDGEMENTS

We thank Vida Piskarskienė for the excellent care of the cells. This study was supported in part by Lithuanian State Foundation for Science and Studies through its programme 'Light in Biomedicine'.

References

1. Gotlieb RA. FEBS Lett 2000; 482: 6–12.
2. Liu X, Kim CN, Yong J. Cell 1996; 86: 147–57.
3. Hortelano S, Dallaporta B, Zamzami N et al. FEBS Lett 1997; 410: 373–7.
4. Brown GC. Biochim Biophys Acta 1999; 1411: 351–69.
5. Ushmorov A, Rattler F, Lehman V. Blood 1999; 93: 2342–52.
6. Shimizu S, Ide T, Yanagida T et al. J Biol Chem 2000; 275: 12321–5.
7. Boyle RW, Dolphin D. Photochem. Photobiol. 1996, 64(3) 469–85.
8. Ackroyd R, Kelty C, Brown N, Reed M. Photochem Photobiol 2001; 74: 656–69.
9. Waliszewski P, Skwarek R, Jeromin L et al. J. Photochem. Photobiol. B 1999, 52(1–3) 137–40.
10. Oleinick NL, Moris RL, Belichenko I. Photochem. Photobiol. Sci. 2002; 1: 1–12.
11. Kirvelienė V, Sadauskaitė A, Kadziauskas J et al. Biologija 2000; 145–8.
12. Kirveliene V, Prasmickaite L, Kadziauskas J et al. J Photochem Photobiol, B: Biol 1997, 41: 173–80.
13. Denizot F, Lang R. J Immunol Meth 1986; 89: 271–7.
14. Laemmli UK. Nature 1970; 227: 680–5.
15. Dunbar BS. Protein blotting: a practical approach. Oxford: Oxford University Press, 1996; 11–51.
16. Andreeva L, Tanveer A, Crompton M. Eur J Biochem 1995; 230: 1125–32.
17. Borutaite V, Morkuniene R, Brown GC. Biochim Biophys Acta 1999; 1453: 41–8.

A. Sadauskaitė, E. Pimpė, J. Kadziauskas, V. Kirvelienė, B. Juodka

CITOCHROMO C TRANSLOKACIJA M-THPC FOTOSENSIBILIZUOTOSE PELIŲ HEPATOMOS MH22 LĄSTELĖSE *IN VITRO*

S a n t r a u k a

Pelių hepatomos MH22 ląstelės *in* vitro buvo sensibilizuotos m-THPC ir stebėta citochromo c translokacija iš mitochondrijų į citozolį. Ištirtas ciklosporino A, laikino mitochondrijų pralaidumo inhibitoriaus, poveikis citochromo c translokacijai ir kaspazių aktyvumui. Rezultatai rodo, kad laikino pralaidumo porų atidarymas neturi lemiamos reikšmės citochromo c translokacijai iš mitochondrijų į citozolį ląstelėse, kuriose vyksta apotozė dėl ląstelės ir mitochondrijų membranose sukelto oksidacinio streso.