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Polyethyleneimine renders mitochondrial membranes permeable by interacting with negatively charged phospholipids in them

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ABSTRACT

Polyethyleneimines (PEIs) are used for transfection of cells with nucleic acids. Meanwhile, the interaction of PEI with mitochondria causes cytochrome *c* release prior to apoptosis; the mechanisms how PEI causes this permeabilization of mitochondrial membranes and the release of cytochrome *c* remain unclear. To clarify these mechanisms, we examined the effects of branched-type PEI and linear-type PEI, each of which was 25 kDa in size, on mitochondria. The permeabilization potency of mitochondrial membranes by branched PEI was stronger than that by linear PEI. The permeabilization by PEIs were insensitive to permeability-transition inhibitors, indicating that PEI-induced permeabilization was not attributed to permeability transition. Meanwhile, PEIs caused permeabilization of artificial lipid vesicles; again, the permeabilization potency of branched PEI was stronger than that of linear PEI. Such a difference in this potency was close to that in the case of isolated mitochondria, signifying that the PEI-induced permeabilization of mitochondrial membranes could be attributed to PEI's interaction with the phospholipid phase. Furthermore, this PEI-induced permeabilization of the lipid vesicles was observed only in the case of lipid vesicles including negatively charged phospholipids. These results indicate that PEIs interacted with negatively charged phospholipids in the mitochondrial membranes to directly lead to their permeabilization.

1. Introduction

Polyethyleneimines (PEIs) are commercially available polycationic reagents with wide molecular weights ranging from 200 Da to 1500 kDa, and they assume a variety of macromolecular structures, e.g., linear types and branched types. PEIs are well-studied polycations used in transfection protocols for intracellular delivery of nucleic acids both *in vitro* and *in vivo* [1–4]. Complexes of PEI and nucleic acids are generally believed to interact with plasma membranes and efficiently deliver the DNA into cells via 2 different mechanisms, i.e., endocytic and macropinocytic mechanisms, depending on the cell type; a part of the complexes are also considered to enter cells through cracks which are formed by PEIs [2]. On the other hand, many previous reports have indicated that PEI can also cause cytotoxicity [5–7]. Although earlier studies indicated that apoptosis, necrosis or autophagy was related to PEI-induced cell death [8–15], the induction mechanisms are still not fully understood. Some reports indicated that PEI interacts with mitochondria, with the result being cytotoxicity [8,9,12,14,15]. When 22-kDa linear PEI is added to HeLa cells, the PEI becomes localized in the mitochondria and causes their depolarization [12]. When 25-kDa branched PEI or 750-kDa linear PEI is added to mitochondria isolated from Jurkat cells or rat liver, cytochrome *c* release from the mitochondria is observed [8,9]. These findings indicate that the direct interaction of PEI with mitochondria causes the cytochrome *c* release, leading to apoptosis. However, the question as to how PEI causes the permeabilization of mitochondrial membranes and the subsequent release of cytochrome *c* still remains unanswered.

The mitochondrial inner membrane is highly resistant to the permeation of solutes and ions. However, under certain conditions, such as in the presence of Ca^{2+} and inorganic phosphate, the permeability of the inner membrane is known to become markedly increased. This phenomenon is referred to as the permeability transition (PT), and it is considered to result from the formation of a proteinaceous pore, the permeability transition pore (PTP), which makes the inner membrane permeable to various solutes and ions smaller than 1.5 kDa [16–18]. By this induction of PT, mitochondria become swollen, causing a decrease

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Abbreviations

CsA	cyclosporin A
CL	cardiolipin
DOPC	dioleoylphosphatidylcholine
DOPG	dioleoylphosphatidylglycerol
IMS	inter membrane space
PEI	polyethyleneimine
PG	phosphatidylglycerol
PT	permeability transition
PTP	permeability transition pore
TOCL	tetraoleoyl cardiolipin
VDAC	voltage-dependent anion channel

in membrane potential, acceleration of oxygen consumption, and rupture of the outer membrane and a part of the inner membrane [18–20]. From these swollen mitochondria, cytochrome *c* localized in the inter membrane space is released to trigger subsequent steps of cell death [21,22]. Thus, the PT is one of the major processes leading to the release of cytochrome *c* from mitochondria. PTP opening is caused by not only Ca^{2+} but also other inducers, e.g., atractyloside [23], phenylarsine oxide [24], and some kinds of cationic compounds [25–27]. On the other hand, mastoparan peptide at a high dose causes inner-membrane permealization and cytochrome *c* release by a mechanism independent of the PT; i.e., the peptide itself forms a pore to make the inner membrane permeable and to cause the subsequent release of cytochrome *c* [28].

Regarding PEI, whether it causes PTP opening remains an unanswered question. When Larsen et al. examined the effects of 25-kDa branched PEI on mitochondria isolated from rat liver, they showed that PEI causes mitochondrial swelling, a decrease in membrane potential, and acceleration of oxygen consumption, which are phenomena characteristically observed in PT-induced mitochondria [15]. However, in their study whether these phenomena observed in PEI-treated mitochondria were attributed to PTP opening was not practically investigated.

In the present study, to examine whether PEI induces the PT in mitochondria, we analyzed in detail the effects of PEI on mitochondria isolated from rat liver and on phospholipid vesicles.

2. Materials and methods

2.1. Materials

"Polyethylenimine (PEI), branched, Mw 25000," which was used as 25-kDa branched PEI in this study, was purchased from Sigma-Aldrich (St Louis, MO, USA). "PEI, linear, MW 25000" used as 25-kDa linear PEI in this study, was purchased from Polysciences (Warrington, PA, USA). Both of PEI products have 95–100% purities. The pH of each PEI solution was adjusted to 7.4. Cyclosporin A (CsA) was kindly provided by Novartis Pharma Inc. Dioleoylphosphatidylcholine (DOPC) and dioleoylphosphatidylglycerol (DOPG) were purchased from Nippon Fine Chemical (Osaka, Japan). 1,1′,2,2′-Tetraoleoyl Cardiolipin, Sodium Salt (TOCL) came from Avanti Polar Lipids (AL, USA). SF6847 was purchased from Wako Pure Chemical Industries Co. (Osaka Japan).

2.2. Preparation of mitochondria

Mitochondria were isolated from the livers of normal male Wistar rats according to the method described previously [20]. All animal experiments were performed according to the Guidelines for the Care and Use of Laboratory Animals of the University of Tokushima (approval number: T28-29). For determination of the protein concentration of mitochondrial suspensions, mitochondrial proteins were first solubilized with 1% SDS, and then subjected to Biuret analysis using bovine serum albumin as a standard.

2.3. Measurement of absorbance change in mitochondrial suspensions

The change in the absorbance of mitochondrial suspensions at 25 °C was examined essentially as described previously [28]. In brief, mitochondria were suspended in 2.2 ml of incubation medium (200 mM sucrose, 10 mM succinate, 0.68 μg/ml rotenone, 10 mM potassium phosphate; pH 7.4) to a final protein concentration of 0.7 mg protein/ml; succinate was added as an oxidative substrate of complex II of the respiratory chain: rotenone, an inhibitor of complex I of respiratory chain, was added for efficient energization of mitochondria. Time-dependent changes in the absorbance of mitochondrial suspensions were monitored at 540 nm with a Shimadzu spectrophotometer, model UV-1700. PEI-DNA complex was prepared using to an experimental protocol of "PEI MAX" (Polysciences, Inc), which is a transfection reagent using linear PEI; in this protocol, ref. 42 is cited as a reference. Briefly, 50 μM of PEI solution and 483 ng/μl of plasmid DNA (pcDNA3.1 hygro (−)) solution were prepared with 150 mM NaCl. PEI solution was mixed with plasmid DNA solution at the ratio of PEI nitrogen to DNA phosphorous (N/P) = 20. The mixture was incubated for 15 min at room temperature to allow DNA/PEI complexes to form. The mixture was added directly to the mitochondrial suspension.

2.4. Measurement of permeability of mitochondrial membrane to PEG

To examine the permeability of the mitochondrial membrane, we measured the effects of PEGs of various molecular sizes on the absorbance of mitochondrial suspensions, as described earlier [29]. Briefly, mitochondria were first treated with a reagent; and then, after complete induction of swelling, 1.1 ml of 300 mOsM PEG solution was added. Changes in the turbidity of the reaction mixture were then monitored at 540 nm.

2.5. Transmission electron microscopic analysis of mitochondrial configuration

The mitochondrial configuration was analyzed by transmission electron microscopy as described earlier, using a Hitachi electron microscope, model H-7650 [20].

2.6. Antibody preparation and measurement of protein release from mitochondria

Antibodies specific for cytochrome *c*, ornithine carbamoyltransferase, and voltage-dependent anion channel were raised by using synthetic peptides as immunogens, as described previously [20,30]. To examine the protein release from mitochondria after treatment under various conditions, we promptly centrifuged an aliquot (1 ml) of the mitochondrial suspension. The pelleted mitochondria were resuspended in 1 ml of the incubation medium, and 15 μl of this suspension and 15 μl of the supernatant were individually subjected to SDS-PAGE. Western blotting was carried out essentially as described earlier [30].

2.7. Preparation of calcein-loaded phospholipid vesicles and determination of calcein leakage from them

DOPC vesicles loaded with calcein and HEPES (K^+) were prepared as described previously [31]. Briefly, 20 mM DOPC in 100 μl of chloroform was dried under a nitrogen stream to produce a film on the