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Mitochondrial localization of P-glycoprotein and peptide transporters in corneal epithelial cells – Novel strategies for intracellular drug targeting

Megha Barot, Mitan R. Gokulgandhi, Dhananjay Pal, Ashim K. Mitra*

Division of Pharmaceutical Sciences, School of Pharmacy, University of Missouri – Kansas City, 2464 Charlotte Street, Kansas City, MO 64108, USA

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ABSTRACT

This study was designed to investigate functional localization of both efflux (P-glycoprotein, P-gp) and influx (peptide) transporters in the mitochondrial membrane of cultured rabbit primary corneal epithelial cells (rPCECs). Isolation and purification of mitochondria was performed by optimized cell fractionation method. Mitochondrial integrity was measured by JC-1 uptake experiment. The efflux activity of P-gp was assessed by performing *in vitro* uptake studies on isolated mitochondria with Rhodamine 123 (Rho-123) alone and in the presence of P-gp inhibitors (quinidine and cyclosporine A) using fluorimetry and flow cytometry analysis. Functional activity of peptide transporter was assessed by performing *in vitro* uptake studies of [3H] Gly-sar on isolated mitochondria in the presence or absence of peptide transporter substrate (Val–Val). Molecular characterization of P-gp and peptide transporter was assessed by western blot and confocal analysis. Enhanced JC-1 accumulation in the isolated fraction confirmed mitochondrial membrane integrity. Significantly higher uptake of Rho-123 on isolated mitochondria was observed in the presence of quinidine (75 and 100 μM) and cyclosporine A (10 μM). Significantly lower uptake of [3H] Gly-sar was observed in the presence of val–val due to competitive inhibition of peptide transporter on isolated mitochondria. Western blot and confocal analysis further confirmed the presence of P-gp and peptide transporter on the mitochondrial membrane of rPCECs. The present study demonstrates the functional and molecular characterization of P-gp and peptide transporters in the mitochondrial membranes of rPCECs. This knowledge of mitochondrial existence of P-gp and peptide transporter will aid in the development of subcellular ocular drug delivery strategies.

1. Introduction

Multidrug resistance (MDR) is the major complication of cancer chemotherapy. It is a situation in which cancer cells become simultaneously resistant to structurally unrelated drugs with different mechanisms of action (Gottesman, 2002). Alterations in common drug targets, increased drug detoxification, drug efflux, DNA repair, and apoptosis defects have all been implicated under MDR mechanisms (Gillet and Gottesman, 2010; Gottesman et al., 2002; Hendrich and Michalak, 2003; Lage and Dietel, 1999; Liscovitch and Lavie, 2000; Pommier et al., 2004). Besides cancer chemotherapy, MDR also represents a major barrier to the success of ocular drug delivery. Since most drug targets are located within specific intracellular compartments, drug accumulation into these sites is a critical determinant of therapeutic response (Duvvuri and Krise, 2005). Drug resistance phenotype showing altered intracellular distribution of drugs has been observed in MDR cancer cell lines relative to drug sensitive lines (Hindenburg et al., 1989; Slapak et al., 1992). Such intracellular redistribution proceedings may decrease the opportunity for a drug molecule to invade into a drug targeting compartment and thus limit its therapeutic response (Duvvuri and Krise, 2005).

MDR associated transporter proteins such as P-glycoprotein (P-gp), breast cancer resistance protein (BCRP) and multidrug resistance protein 1 (MRP1) are also expressed at corneal cell membrane level for reducing intracellular accumulation of toxins and drugs (Barot et al., 2011a,b; Karla et al., 2009, 2007). Nevertheless these proteins could be expressed in subcellular compartments and may actively sequester drugs and traffic away from their cellular targets (Duvvuri and Krise, 2005; Meschini et al., 2000; Munteanu et al., 2006; Rajagopal and Simon, 2003; Shapiro et al., 1998; Solazzo et al., 2009).

Mitochondria represent a vital intracellular organelle for ocular cell function and survival. It is an attractive target for drug delivery because there is growing confirmation to support an association between mitochondrial dysfunction and a number of ocular

* Corresponding author. Tel.: +1 816 235 1615; fax: +1 816 235 5779.
E-mail address: mitraa@umkc.edu (A.K. Mitra).

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diseases (such as age-related macular degeneration, diabetic retinopathy and glaucoma) (Barot et al., 2011a,b; Kowluru et al., 2006; Liang and Godley, 2003). Several reports have indicated the mitochondrial localization and activity of P-gp efflux protein. Munteanu et al. have shown presence and functional activity of mitochondrial P-gp in MDR resistant human myeloid leukemia cells (K562). Authors have observed reversed orientation of mitochondrial P-gp relative to its outward localization on cell surface. This finding suggested that inward mitochondrial P-gp can offer higher drug accumulation inside the organelle and extracellular antibodies cannot affect such accumulations since they cannot reach mitochondrial binding site. However, mitochondrial membrane permeable small molecule inhibitors can reduce such intra-organelle drug concentrations. Therefore, inward localization of mitochondrial P-gp in MDR cells can protect the nucleus and prevent the therapeutic drug entry in its nuclear targets (Munteanu et al., 2006). Another study reports functional localization of P-gp in the mitochondrial membrane of MDR resistant hepatocellular carcinoma (HCC) cells. This finding suggested that mitochondrial membrane localized P-gp can works like a pump to efflux cytotoxic agents from mitochondria to the cytosol. Therefore, outward orientation of mitochondrial P-gp is likely to protect mitochondrial DNA from cytotoxic damage (Solazzo et al., 2006). Furthermore, Ling et al. have shown that over-expression and localization of P-gp on mitochondrial membrane of mitochondrial DNA depleted human hepatoma cells (SK-Hep1) confers resistance to chemotoxic drug-induced apoptosis (Ling et al., 2012). Recently, another study has reported mitochondrial localization of P-gp only on doxorubicin-resistant human breast cancer cells (MCF-7) but not in parent cell line (Shen et al., 2012). Overall, above two findings have further supported the hypothesis that mitochondrial membrane localized P-gp posses efflux function and facilitates MDR at the intracellular site by pumping chemotoxic drug out from mitochondria to protect mitochondrial functioning.

Expression of P-gp efflux and peptide influx transporters have been previously identified by our laboratory on corneal epithelial cell surface (Dey et al., 2003; Janoria et al., 2010). Therefore, the aim of this study was to elucidate the expression, localization, and functional activity of P-gp efflux and peptide (PepT-1) influx transporters in the mitochondria of cultured rabbit primary corneal epithelial cells (rPCECs). In this study, *in vitro* efflux activity of P-gp was measured by a model fluorescent P-gp substrate rhodamine-123 (Rho-123) and two specific inhibitors of P-gp (quinidine and cyclosporine A, CsA). In addition, two peptide transporter substrates [3H] Glycylsarcosine (Gly-Sar) and val–val were selected to examine the *in vitro* function of PepT-1 transporter. All *in vitro* uptake experiments were performed in isolated mitochondria from rPCECs. Furthermore, localization and protein expressions of both the transporters were confirmed by confocal microscopy, and western blot analysis.

2. Materials and methods

2.1. Materials

Cell culture materials such as minimum essential medium (MEM), TripLE Express® solution and non-essential amino acids were obtained from Invitrogen (Carlsbad, CA). Fetal bovine serum (FBS) was procured from Atlanta biological (Lawrenceville, GA). Cell culture flasks (150 cm^2 area) were purchased from Fisher Scientific (Houston, TX). Rho-123, CsA and quinidine were procured from Sigma–Aldrich (St. Louis, MO). [3H] Gly-Sar (specific radioactivity, 4 Ci/mmol) was obtained from Moravek Biochemicals (Brea, CA, USA).

2.2. Cell culture

rPCECs were cultured according to our published procedure (Dey et al., 2003). Briefly, cells were grown with culture medium containing MEM, 10% FBS, HEPES, sodium bicarbonate, penicillin, streptomycin sulfate and 1% (v/v) non-essential amino acids, adjusted to pH 7.4. Cells were grown in 150 cm^2 culture flasks and maintained at 37 °C, in a humidified atmosphere of 5% CO_2 and 90% relative humidity. The culture medium was replaced every other day.

2.3. Mitochondria isolation

An isolation of mitochondria from the corneal cells was performed based on the principle of cell fractionation and differential centrifugation (Chaiyarit and Thongboonkerd, 2009; Munteanu et al., 2006; Bourgeron et al., 1992). Briefly, confluent rPCECs grown in 150 cm^2 flask were harvested by trypsinization, washed twice with ice-cold phosphate-buffered saline (PBS) and pelletized at 4 °C (1000 × *g*) for 10 min. Resulting pellet was re-suspended in 500 μL of ice-cold homogenization buffer (0.25 M sucrose, 1 mM EDTA, 10 mM HEPES; pH 7.4) and incubated on ice for 10 min. Following incubation, cells were homogenized with pre-chilled Dounce homogenizer (40–50 strokes) and cell lysis was ensured by LDH assay. The resulting homogenate was transferred into 10 mL centrifuge tube by making volume up to 5 mL with homogenization buffer and centrifuged at low speed (1000 × *g*, 10 min, 4 °C) to remove nuclei and unlysed cells. Resulting supernatant was again centrifuged at high speed (16,000 × *g*, 40 min, 4 °C) in order to remove lysosomal or peroxisomal contamination. The formed pellet ("crude mitochondria") was resuspended in homogenization buffer containing 0.25 M sucrose and centrifuged at 16,000 × *g* for 30 min at 4 °C. The resulting mitochondrial pellet was re-suspended in mitochondrial suspension buffer (pH 7.0) containing sucrose (250 mmol/L), tris (10 mmol/L) and protease inhibitors for further studies.

2.4. Mitochondrial membrane integrity evaluation by JC-1 uptake

Mitochondrial membrane integrity was assessed by measuring the potential gradient ($\Delta\psi$) across the membrane using the lipophilic, cationic JC-1 fluorescent dye as per the manufacturer's instructions (Sigma). Generally in healthy cells with high mitochondrial $\Delta\psi$m, JC-1 concentrates in the mitochondrial matrix and forms red fluorescent aggregates (J-aggregates). Any incident that disperses the mitochondrial membrane potential also averts accumulation of the JC-1 dye in the mitochondria. As an outcome the dye is dispersed all over the cytoplasm leading to a shift from red (J-aggregates) to green fluorescence (JC-1 monomers) (Reers et al., 1991). Valinomycin is an antibiotic agent permeabilizes the mitochondrial membrane and therefore, dissipates the mitochondrial potential gradient. In this experiment, valinomycin (1 μL) has been used as a control that prevents JC-1 aggregation. Fluorescence of JC-1 stained mitochondrial aggregates was measured by fluorimeter at 490 nm (excitation) and 590 nm (emission) wavelengths respectively.

2.5. Mitochondrial preparation for transmission electron microscopy (TEM)

For morphological characterization, 100 μL of mitochondrial suspension was centrifuged at 7000 × *g* for 10 min. The resulting pellet was fixed with glutaraldehyde (2.5%) in cacodylate buffer, post fixed with osmium tetroxide (2%) and dehydrated in ethanol.