Mitochondrial expression and activity of P-glycoprotein under oxidative stress in outer blood-retinal barrier

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Abstract

AIM: To investigate the role of oxidative stress in regulating the functional expression of P-glycoprotein (P-gp) in mitochondria of D407 cells.

METHODS: D407 cells were exposed to different ranges of concentrations of H₂O₂. The mitochondrial location of P-gp in the cells subjected to oxidative stress was detected by confocal analysis. Expression of P-gp in isolated mitochondria was assessed by Western blot. The pump activity of P-gp was evaluated by performing the efflux study on isolated mitochondria with Rhodamine 123 (Rho-123) alone and in the presence of P-gp inhibitor (Tariquidar) using flow cytometry analysis. The cells were pretreated with 10 mmol/L N-acetylcysteine (NAC) for 30min before exposing to H₂O₂, and analyzed the mitochondrial extracts by Western blot and flow cytometry.

RESULTS: P-gp was co-localized in the mitochondria by confocal laser scanning microscopy, and it was also detected in the mitochondria of D407 cells using Western blot. Exposure to increasing concentrations of H₂O₂ led to gradually increased expression and location of P-gp in the mitochondria of cells. Rho-123 efflux assay showed higher uptake of Rho-123 on isolated mitochondria in the presence of Tariquidar both in normal and oxidative stress state. H₂O₂ up-regulated P-gp in D407 cells, which could be reversed by NAC treatment.

CONCLUSION: H₂O₂ could up-regulate the functional expression of P-gp in mitochondria of D407 cells, while antioxidants might suppress oxidative-stress-induced over-expression of functional P-gp. It is indicative that limiting the mitochondrial P-gp transport in retinal pigment epithelium cells would be to improve the effect of mitochondria-targeted antioxidant therapy in age-related macular degeneration-like retinopathy.

KEYWORDS: P-glycoprotein; retinal pigment epithelium; oxidative stress; mitochondria

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INTRODUCTION

Oxidative stress in the mitochondria of retinal pigment epithelium (RPE) is thought to play a causative role in age-related macular degeneration (AMD). RPE is a part of the outer blood-retinal barrier (BRB). A primary function of RPE is to maintain the metabolic homeostasis of the retina by removing the waste products and providing nutrients for the neural retina. Dysfunction and degeneration of RPE by increased oxidative stress contributes to the irreversible loss of photoreceptors and central vision in AMD. One reason that RPE is sensitive to oxidative stress induced injury and dysfunction is the very high density of mitochondria in RPE. Mitochondrion is a rich source of reactive oxygen species (ROS) and vulnerable to oxidative stress, so RPE with abundant mitochondria is destined to be especially susceptible target of oxidative stress. It has been recognized that mitochondrial dysfunction due to oxidative stress aggregates in the AMD eyes and the anti-oxidative capacity of RPE from AMD patients decreases with age. There is a need for mitochondria-targeted antioxidant therapy in AMD-like retinopathy. Mitochondrial pharmaceutics, particularly those involving mitochondria-targeted antioxidants, execute therapeutic potential by maintaining the energy production and ameliorating apoptosis. However, some mitochondrial medicines may have fewer efficacies than expected due to their limited access.
P-glycoprotein in mitochondria
to the mitochondria, although they are accumulated several
hundred-fold into energized mitochondria. One potential
mechanism that the efficacy of these mitochondrial medicines
is limited may be the challenging of the drug efflux pump\textsuperscript{[18-19]}. If
these mitochondrial medicines are happened to be substrates
of some efflux pumps, such as P-glycoprotein (P-gp),
mitochondria-targeted therapies may be less efficient.
P-gp is the longest identified efflux pump, which is encoded
by the multidrug resistance (MDR) gene. Overexpression of P-gp and resistance to apoptosis are correlated well with
MDR, a very common observed molecular mechanism
in chemotherapeutic therapy. P-gp can pump anticancer
drugs and other compounds out of the cells, reducing the
intracellular concentration and chemotherapeutic efficacy
of these agents\textsuperscript{[20-21]}. P-gp has been also recognized to be
involved in other cell functions such as cellular metabolism
and cell survival\textsuperscript{[22-24]}. It is generally considered that P-gp
expresses on cell surface, but increasingly growing evidences
show the functional localization of P-gp in mitochondria\textsuperscript{[25-27]}.
In response to a broad range of endogenous and xenobiotic
stimuli, P-gp is proposed to either pump these substrates out of
mitochondria or pump them into mitochondria for sequestering,
both reducing the effect of substrates in mitochondria\textsuperscript{[25,28]}
Some novel mitochondria-targeting carrier has been designed
to transport drugs to mitochondria as well as limit the effects
of P-gp efflux pump\textsuperscript{[29]}. We propose that P-gp is present in the
mitochondria of RPE cells and may influence mitochondrial
medicines by changing transport across mitochondrial
membranes. We therefore built hydrogen peroxide (H$_2$O$_2$)-
induced oxidative stress in vitro model in D407 cells to mimic
RPE dysfunction in AMD-like retinopathy, and investigated
the functional expression of P-gp in mitochondria of D407
cells under oxidative stress conditions. This study will indicate
a possible role of P-gp activity regulation in the mitochondria
targeted therapeutic intervention in AMD.

**MATERIALS AND METHODS**

**Cell Culture and Treatment** The human RPE D407 cells
were from the Experimental Animal Center of Sun Yat-Sen
University, Guangzhou, China. Cells were routinely kept in
Dulbecco’s modified Eagle’s medium (DMEM) containing
10% fetal bovine serum and antibiotics (100 U/mL penicillin
and 0.1 mg/mL streptomycin) at 37°C in a 95% air and 5%
CO$_2$. All cell culture reagents were from Gibco BRL (Grand
Island, NY, USA). Treatment of D407 cells in this study
included the incubation with fresh medium containing different
concentrations of H$_2$O$_2$ (Sigma-Aldrich, Shanghai, China) or
antioxidant N-acetylcysteine (NAC, Sigma-Aldrich, Shanghai,
China) or P-gp pump inhibitor, Tariquidar (Selleckchem,
China).

**Measurement of Reactive Oxygen Species** D407 cells at a
density of 1×10$^6$ cells/mL were incubated in serum-free
medium containing 10 µmol/L dichloro-dihydro-fluorescein
diacetate (Beyotime Biotechnology, Jiangsu Province, China)
for 20min at 37°C after exposing to varying concentrations
of H$_2$O$_2$. Following washing, cells were collected by
tryptsinization. Intracellular ROS was analyzed from the
conversion of nonfluorescent dichloro-dihydro-fluorescein
diacetate to its fluorescent derivatives. The fluorescence
intensity was measured at 488 nm excitation and 525 nm
emission wavelengths by flow cytometry.

**Immunofluorescence Assay** Cells at a density of 2000 cells/
chamber were seeded onto polylysine-coated glass chamber
slides. Dilute 1 mmol/L MitoTracker Red CMXRos probe
(Life technology, USA), widely used to identify mitochondria,
to the final working concentration 100 nmol/L in DMEM.
Cells were reacted with MitoTracker probe for 20min, and
then fixed by ice-cold 4% Paraformaldehyde for 15min and
permeabilized for 10min in 1% Triton X-100. They were
immunolabeled with mouse monoclonal Mdr-1 antibody
(D-11, 1:200 dilution, Santa Cruz Biotechnology, USA) at
room temperature for 2h and were washed and incubated for
30min with anti-mouse IgG Alexa flour 488 (1:5000, Life
technology, USA), followed by the addition of 4', 6-diamidino-
2-phenylindole (ROCH, USA) to stain nucleus. The staining
pattern was visualized using a confocal microscope (Carl Zeiss
Jena GmbH, Jena, Germany).

**Preparation of Isolated Mitochondria from D407 Cells** The
preparation of isolated mitochondria was performed using the
mitochondria isolation kit for mammalian cells (Rockford,
IL USA). Cells at a density of 2×10$^6$ cells/mL following
H$_2$O$_2$ incubation were collected by centrifugation (850 g for
10min at 4°C) in a 2.0 mL microcentrifuge tube. Carefully
remove and discard the supernatant, and re-suspend cells in
10 packed cell volumes of buffer (1 mmol/L TrisHCl, pH 7.4,
0.13 mol/L NaCl, 5 mmol/L KCl, 7.5 mmol/L MgCl$_2$). Pellet
cells and decant supernatant, repeat this washing step 2 times.
Resuspend cells in 6 packed cell volumes of homogenization
buffer. Transfer cells to a glass homogenizer and incubate
for 10min on ice. Using a tight pestle, homogenize the cells.
Check under the microscope for cell breakage, the optimum is
around 60% which may require 35 strokes. Pour homogenate
into a conical centrifuge tube containing 1 packed cell volume
of 2 mol/L sucrose solution and mixed gently. Pellet unbroken
cells, nuclei, and large debris at 700 g for 10min at 4°C
and maintain the mitochondrial pellet on ice before downstream
processing.

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Western Blot The proteins of isolated mitochondria were extracted by resuspending the cells in RIPA buffer with protease inhibitor, and incubated on ice for 30 min. The samples were cleared by centrifuging at 12,000 g for 5 min at 4°C and supernatants were collected. The protein concentration was determined using a protein assay kit (Sangon Biotech, Shanghai, China). Equal amounts of protein were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred electrically onto polyvinylidene fluoride membranes (ROCH, USA) and blocked with 5% non-fat dry milk in Tris-buffered saline with 0.05% Tween-20. The membranes were probed with mouse monoclonal antibodies to P-gp (1:200 dilution, Santa Cruz Biotechnology, Santa Cruz, USA) or voltage-dependent anion channel 1 (VDAC1, 1:100 dilution, Santa Cruz Biotechnology) or glyceraldehyde-3-phosphate dehydrogenase (GAPDH, 1:3000, Santa Cruz Biotechnology) overnight at 4°C. The anti-mouse secondary antibodies (1:5000 dilution; Santa Cruz Biotechnology) were then rinsed twice in PBS and immediately analyzed by flow cytometry. The differences between data sets were analyzed by a one-way ANOVA and a P value less than 0.05 was considered to be statistically significant.

Measurement of Membrane Potential in Mitochondria The mitochondrial membrane potential (ΔΨm) was estimated in D407 cells using the dye JC-1 (5, 5′, 6, 6′-tetrachloro-1, 1′, 3, 3′-tetraethylbenzimidazole carbocyanine iodide) by flow cytometry. Cells with 80% confluence were incubated for 20 min at 37°C after the corresponding treatment in medium containing 10 μmol/L JC-1 (Beyotime Biotechnology, Jiangsu Province, China), a lipophilic fluorescent cation that exists as green fluorescent monomers at a low ΔΨm or as red fluorescent aggregates at a greater ΔΨm. The cells were then rinsed twice in PBS and immediately analyzed by flow cytometry. Data were collected at a 529 nm emission for green fluorescence and at 590 nm for red fluorescence. The green/red fluorescence intensity ratio is expressed as decreased ΔΨm, which represents the mitochondrial depolarization ratio.

Rhodamine 123 Accumulation Assay P-gp efflux activity was determined by flow cytometry with intracellular accumulation of rhodamine 123 (Rho-123, Sigma-Aldrich, Shanghai, China), which has been used as a typical probe to assess P-gp function in various cells and tissues. D407 cells were pretreated with 10 mmol/L NAC for 30 min or 1 μmol/L tariquidar for 1 h before exposing of different concentrations of H2O2 for 24 h. The optimal dose of tariquidar employed in this study was determined by the sub-toxic in D407 cells.

Figure 1 H2O2 increases ROS production in D407 cells in a dose-dependent manner. D407 cells subjected to varying concentrations of H2O2 for 24 h were loaded with DCFH-DA and the intracellular ROS level was measured by flow cytometry. Results shown are the mean±SE of three separate experiments performed in triplicate. Treatment with H2O2 for 24 h at a concentration range of 0 to 800 μmol/L caused a linear rise in ROS level and reached a maximum at 800 μmol/L in D407 cells. *P<0.05, **P<0.01 versus CTL for one-way ANOVA.

Statistical Analysis Data were presented as the mean±SE and analyzed using the SPSS package for Windows (version 17.0). The differences between data sets were analyzed by a one-way ANOVA and P value less than 0.05 was considered to be statistically significant.

RESULTS H2O2-induced Oxidative Stress in D407 Cells The oxidative stress state in D407 cells following exposure to different concentrations of H2O2 was determined by detecting the intracellular ROS level, indicated by DCF fluorescence using flow cytometry. As shown in Figure 1, the intracellular ROS level was increased in a concentration-dependent manner after
exposing D407 cells to varying concentrations of H₂O₂ for 24h.

Mitochondrial Location and Expression of P-glycoprotein in D407 Cells Subjected to Oxidative Stress

Figure 2A is the confocal microscopic picture presenting the mitochondrial location of P-gp in D407 cells subjected to oxidative stress. We found a visible but very faint overlap of P-gp and mitochondria fluorescence in untreated cells, indicating the colocalization of P-gp and mitochondria in D407 cells. A gradually increasing overlap of P-gp and mitochondria fluorescence was found in cells exposure to increased concentration of H₂O₂, indicating that P-gp mitochondrial expression increases with the increase of H₂O₂ exposure concentration. We next examined the P-gp expression in the mitochondria of D407 cells under oxidative stress state. The mitochondrial proteins of D407 cells were extracted after exposing to 0-800 µmol/L H₂O₂ and P-gp expression was analyzed by Western blot. Figure 2B shows that P-gp expression was observed in the mitochondrial extracts of untreated D407 cells and gradually increased expressions were observed in the increasing exposure concentrations of H₂O₂. No expression of GAPDH was observed in all samples.

Effect of Oxidative Stress on the Activity of P-glycoprotein in Mitochondria of D407 Cells

To excel the influence of mitochondrial integrity destruction induced by oxidative stress on P-gp activity detection, we first measured the alterations of ΔΨm using flow cytometry (Figure 3). When cells were exposed to 100 or 200 µmol/L H₂O₂, the ΔΨm had only a slight decrease (both P>0.05 when comparing to untreated cells) which indicates that the mitochondrial integrity has not been significantly damaged by exposure to H₂O₂ less than 200 µmol/L for 24h. When cells were exposed to 400 or 800 µmol/L H₂O₂ for 24h, the levels of ΔΨm were significantly decreased (both P<0.05 when comparing to untreated cells). To further exclude cytotoxic effects, the CCK-8 assay was performed. We found that the cell viability was 82.16%±5.03% when incubated with 200 µmol/L H₂O₂ for 24h. Pursuant to ISO 10993-5, percentages of cell viability above 80% are considered as non-cytotoxicity. We therefore chose cells exposure to 200 µmol/L H₂O₂ to investigate the effects of oxidative stress on P-gp activity in mitochondria of D407 cells. Figure 4 showed that in untreated cells, the intracellular Rho-123 fluorescence intensity was 22.31%±1.15% and it was increased to 33.98%±1.98% after P-gp activity was inhibited by 1 µmol/L tariquidar pretreatment for 1h (P<0.05). When cells were treated with 200 µmol/L H₂O₂ for 24h or combined pretreatment with 1 µmol/L tariquidar for 1h, the intracellular Rho-123 fluorescence intensity was 11.20%±1.01% and 18.10%±0.98%, respectively (P<0.05). These results indicate that the functional expression of P-gp be detected in the normal mitochondria of D407 cells and its functional expression was increased under oxidative stress state.
Involvement of Antioxidants in the Regulation of P-glycoprotein Functional Expression in Mitochondria of D407 Cells

To further determine whether antioxidants could change the functional expression of P-gp in mitochondria of D407 cells, we pretreated cells with 10 mmol/L NAC (a ROS scavenger) for 30 min before exposing D407 cells to H_2O_2, and analyzed the mitochondrial extracts by Western blot and flow cytometry. The pretreatment of NAC decreased the intracellular ROS level when comparing to cells exposure to H_2O_2 (data not shown). In Figure 5A, the Western blot result showed that P-gp expression was increased after 200 µmol/L H_2O_2 exposure while its expression was decreased after NAC pretreatment. Rho-123 accumulation assay further showed that H_2O_2 treatment induced a significant decrease in the fluorescence intensity of intracellular Rho-123, indicative of an increased P-gp function. As expected, the Rho-123 retention was increased with NAC pretreatment before exposure of 200 µmol/L H_2O_2 when compared with H_2O_2 alone, but did not fully return to the level of the control, untreated cultures (Figure 5B). These results indicate that the antioxidant may be involved in the regulation of P-gp functional expression in D407 cells.

DISCUSSION

Mitochondria is becoming an attractive target for drug-delivery in many oxidative stress-induced diseases. It remains elusive that whether the mitochondrial drug-delivery executes less therapeutic potential than expected due to the disturbance of drug efflux pump. We therefore conducted this study to investigate the functional expression of P-gp in mitochondria of human D407 RPE cells under normal and oxidative stress conditions and its influence to the drug-delivery across mitochondrial membranes. Here we demonstrated the mitochondrial presence of P-gp in the untreated D407 cells and increased mitochondrial expression under oxidative stress conditions. The efflux assay detected by quantifying the fluorescence intensity of intracellular Rho-123 shows that H_2O_2-exposed cells have increased transport of Rho-123 across mitochondrial membranes than untreated cells. In addition, administration of the antioxidant NAC led to the down-regulation of P-gp functional expression in mitochondria of H_2O_2-exposed D407 cells.
Age is the strongest risk factor for AMD and the oxidative stress is believed to be a primary contributor to the pathogenesis of AMD. Oxidative stress sensitizes RPE cells to injury and mediates the RPE cells dysfunction, so growing increasing studies on the AMD pathogenesis have been focused on defects in RPE function induced by oxidative stress. The sources of oxidative stress in RPE cells are diverse. High oxygen tension exposure from the choriocapillaris, long-term sunlight exposure and excess accumulation of lipofuscin are all significant source of oxidative stress to the RPE, which can lead to destruction of the RPE mitochondrial network and induce further pathological cascades of toxicity, inflammation and neurodegeneration process. Although some protective mechanisms work in RPE during the early phases of AMD, mitochondria are vulnerable to oxidative stress. We chose the point of mitochondrial network damage during oxidative stress in RPE cells because of the evidences that mitochondrial damage in AMD eyes parallels disease severity and eyes obtained postmortem from AMD patients have more obvious free radical damage to mitochondria of RPE cells than controls.

Expression of P-gp efflux transporter has been detected in different RPE models including primary RPE cells and secondary ARPE-19 and D407 RPE cell lines. It was indicated a different efflux transporter expression profile in the different RPE model. Constable et al. showed that P-gp was expressed in D407 cells but was not in ARPE-19 cells. Chen et al. found a significantly different efflux transporter expression profile in ARPE-19 cells when comparing other RPE models and suggested to use ARPE-19 with caution in the efflux transporter research. However, Mannermaa et al. thought that ARPE-19 and primary RPE cells had similar efflux protein profile. We think this discrepancy from different laboratory may be caused by the different process of cell culture and cell culture materials such as P-gp substrate and inhibitor. We previously identified expression of P-gp efflux transporter in our laboratory on human D407 RPE cell line. To see from these previously published literatures, we chose D407 cells to investigate the efflux pump in mitochondria of outer BRB in the present study. We demonstrated the mitochondrial localization and expression of P-gp in D407 cells by cellular staining assays and Western blot. In obtained confocal microscopic pictures, the exact overlapping P-gp (green) and mitochondria staining (red) indicates colocalization of P-gp and mitochondria in D407 cells. Purified mitochondria were isolated from D407 cells and the visible band of about 170 kDa following reacting with anti-P-gp antibody further indicated the presence of P-gp on the mitochondria of D407 cells. The functional expression of P-gp in mitochondria has been increasingly recognized. However, some researchers queried the location of P-gp in the mitochondrial membranes and they attributed the presence of P-gp in the mitochondrial fraction to the plasma membrane contamination. To expel the possibility of false positive induced by contaminated plasma membrane fractions, we chose both VDAC1 and GAPDH as the internal loading controls. VDAC1 was used as the internal loading control of mitochondrial proteins and GAPDH was used as a cytoplasm marker. Our findings showed...
the negative GAPDH expression in isolated mitochondria, which indicated that the isolated mitochondria had not been contaminated by plasma membrane fractions. We found that the intracellular ROS levels gradually increased in response to increased H$_2$O$_2$ concentration. Accordingly, P-gp expression in mitochondria also increased with the increased oxidative stress. P-gp expresses in a wide range of excretory and barrier tissues and it is proposed that P-gp serves a protective function in these tissues by limiting the absorption and distribution of harmful xenobiotics.[43] Induction of P-gp activity is becoming a new therapeutic focus to limit the toxicity caused by its substrates.[44] Alterations in P-gp functional expression following H$_2$O$_2$ exposure have been found in several tissues and cell lines and the potential role of P-gp under oxidative stress in these in vitro models of H$_2$O$_2$ exposure is also indicated. In most of the reports, P-gp expression was up-regulated after exposure of H$_2$O$_2$,[45-46] while only few reported the down-regulated expression.[47] It was proposed to be relative between the amount of ROS and P-gp expression following H$_2$O$_2$ exposure in these published literatures. Because RPE is functionally very similar to the blood-brain barrier (BBB), we focused on some investigations of P-gp in BBB and found that consistent with some of the reports, the finding that up-regulated P-gp expression parallels with the increased ROS generation in our study was also found in brain endothelial cells.[26,48]. In some published literatures, it is proposed that P-gp executes the protective effects from oxidative injury might be realized by activating the antioxidant defenses or up regulating some protective anti-oxidative signal pathways.[23-25] It needs further investigation whether P-gp has a specific role in regulating the caspase dependent apoptotic pathway or not in RPE cells. In our study, the fact that NAC attenuated P-gp expression induced by H$_2$O$_2$ indicates that the altered P-gp expression might affect the transport of P-gp substrates in RPE cells under conditions of oxidative stress. Another indication of our findings was that the oxidative stress in RPE may induce P-gp functional expression to an extent, but the excessive oxidative damage might increase difficulties in protection against toxicity caused by its substrates or alteration pharmacokinetics of therapeutic drugs targeted to the retinopathies. In summary, we investigated the location and expression of P-gp in mitochondria of D407 cells under oxidative stress conditions. One implication of our study is to propose a potential role of P-gp in the mitochondria targeted therapeutic intervention by changing drug transport across mitochondrial membranes. Another implication is to propose the necessity of concurrent application of antioxidant in limiting the mitochondrial P-gp transport and improving the effect of mitochondria-targeted therapy in AMD-like retinopathy.

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