Biochemical Pharmacology 78 (2009) 191–202

Contents lists available at ScienceDirect

Biochemical Pharmacology

journal homepage: www.elsevier.com/locate/biochempharm

Plasma membrane depolarization and Na,K-ATPase impairment induced by mitochondrial toxins augment leukemia cell apoptosis via a novel mitochondrial amplification mechanism

Wu Yin [a], Xiang Li [b], Su Feng [a], Wei Cheng [a], Bo Tang [a], Yi-Lin Shi [a], Zi-Chun Hua [a,*]

[a] The State Key Lab of Pharmaceutical Biotechnology, College of Life Science, Nanjing University, 22# HanKou Rd., Nanjing 210093, PR China
[b] Lab of Chemistry of Chinese Materia Medica, Nanjing University of Chinese Medicine, Nanjing 210046, PR China

ARTICLE INFO

Article history:
Received 15 January 2009
Accepted 26 March 2009

Keywords:
Mitochondria
Na,K-ATPase
Depolarization
Reactive oxygen species
Apoptosis

ABSTRACT

Na,K-ATPase is a ubiquitous transmembrane protein that regulates and maintains the intracellular Na^+ and K^+ gradient necessary for cell homeostasis. Recently, the importance of this pump in external stimuli-induced leukemia cell apoptosis has been increasingly appreciated, however, the exact role of Na,K-ATPase in mitochondrial apoptotic pathway still remains little understood. In this study, we found mitochondrial toxin rotenone caused a rapid mitochondrial membrane potential (MMP) collapse in Jurkat cells followed by plasma membrane depolarization (PMP). Similar results were also obtained in human U937 cells and non-cancerous mouse primary T cells. Rotenone-induced PMP depolarization occurred before apoptosis and well correlated with Na,K-ATPase impairment. To understand the mechanisms, Jurkat cells with mtDNA depletion and catalase overexpression were used. The results demonstrated that both PMP depolarization and Na,K-ATPase impairment induced by rotenone were regulated by mitochondrial H_2O_2 and Bcl-2. Finally, Na,K-ATPase suppression by ouabain greatly accelerated and enhanced mitochondrial toxins-induced cells apoptosis in Jurkat, U937 and primary T cells. In sum, by using leukemia cells and mouse primary T cells, we confirmed that mitochondria-to-Na,K-ATPase and PMP depolarization might represent a novel mechanism for mitochondria to amplify death signals in the initiation stage of cells apoptosis induced by mitochondrial toxins.

1. Introduction

Plasma membrane potential (PMP) depolarization has been recognized as a primary signal that conveys multiple biological messages within cells, such as gene transcription and expression [1], activation of Rho–Rho kinase (ROK) pathway to regulate paracellular transport in epithelial cells [2] or to regulate receptor stimulation-induced vascular smooth muscle contraction [3]. In cell death, PMP depolarization is an early event in Fas-triggered apoptosis [4]. Conversely, PMP depolarization could also protect cell from apoptosis [5]. Therefore, whether the PMP depolarization is just an epiphenomenon that occurs during apoptosis, or if there is a specific signaling role for PMP depolarization during apoptosis is currently unknown.

PMP depolarization is mainly mediated by ion fluxes, such as Na^+, Ca^{2+}, or Cl^- across plasma membrane. Impairment of ion channels or transporters responsible for these ion fluxes can thus disrupt the intracellular ion homeostasis and lead to PMP depolarization. Among ion transporters across plasma membrane, Na,K-ATPase stands up to be a key pump involved in PMP depolarization [6]. Report has revealed that Na,K-ATPase has been a specific target for inactivation in death receptors-mediated cell apoptosis. Furthermore, this Na,K-ATPase impairment leads to a significant increase of depolarized cells, the purpose of which is to enhance Fas ligand (Fas L)-induced human T-cell leukemia cells apoptosis [7]. These results suggest that PMP depolarization may play a role in predisposing leukemia cells to apoptotic stimuli. Besides Fas L, arsenic trioxide (As_2O_3) was also shown to be able to trigger PMP depolarization and enhance the activity of anti-cancer agents in leukemia cells [8,9]. Fas L and As_2O_3 are different substances that can kill leukemia cells, how these two agents are both able to depolarize leukemia cells appears to be intensively interesting. In principle, Fas L recruits intracellular FADD or caspase 8 to form death-inducing signaling complex (DISC) after binding with its receptor, leading to activation of caspase 8 and downstream caspase 3. Alternatively, the activated caspase 8 can also cleave and activate Bid, which then translocates to the mitochondria triggering the release of cytochrome *c* into cytosol. Once in cytosol, cytochrome *c* forms apoptosome to activate procaspase 9 and downstream caspase 3 [10]. Unlike that of Fas L,

* Corresponding author. Tel.: +86 25 83324605; fax: +86 25 83324605.
E-mail address: zchua@nju.edu.cn (Z.-C. Hua).

0006-2952/$ – see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.bcp.2009.03.025

As_2O_3 is suggested to directly target the mitochondria to induce leukemia cell apoptosis [11]. In whole cells, As_2O_3 can disrupt mitochondrial membrane potential ($\Delta\Phi$) and induce caspase activation [12], or trigger opening of permeability transition pore and release of soluble intermembrane proteins [13]. Apparently, mitochondrial impairment constitutes a shared mechanism for Fas L-mediated or As_2O_3-induced leukemia cells apoptosis. As such, we attempt to understand three important issues, (1) Whether mitochondrial impairment in human leukemia cells may have some intrinsic relationship with PMP depolarization, because TNF-α, another death family protein can also inhibit mitochondrial function at complex I [14] and depolarize human leukemia cells (Yin, W., personal communication). (2) What is the role of PMP depolarization in mitochondria-dependent leukemia cell apoptosis? (3) Whether mitochondrial impairment can also induce PMP depolarization in non-cancerous cells, such as primary T cells.

To address the above concern, rotenone was used in this study to cause mitochondrial damage and induce mitochondria-dependent apoptosis in leukemia cells. The results, as described in this study propose a novel mitochondria-involved apoptosis amplification mechanism mediated by plasma membrane Na,K-ATPase and PMP depolarization.

2. Materials and methods

2.1. Materials

Rotenone, diphenyleneiodonium (DPI), propidium iodide (PI), catalase, Mn(III) tetrakis (4-benzoic acid) porphyrin chloride (MnTBAP) and BAPTA/AM were purchased from Sigma Co. (St Louis, MO, USA). Squamocin was isolated and identified from chloroform part of Annona squamosa L. with purity over 99%. Various chromatographic and spectral techniques (^{1}H NMR, ^{13}C NMR and MS) were used to elucidate the structure. The general caspases inhibitor z-VAD.fmk was obtained from Kamiya Biomedical Co. (Seattle, WA, USA). Bis-(1,3-dibutylbarbiturate)-trimethine xonol ($DiBAC_4$), H_2DCFDA, dihydroethidium (DHE) and 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolcarbocyanine iodide (JC-1) were from Molecular Probes (Eugene, OR, USA). ^{86}RbCl was obtained from Amersham Pharmacia (Buckinghamshire, UK).

2.2. Cell culture

Jurkat A3 cells were generously gifted by Prof. Cohen [15]. U937 cells were purchased from American type culture collection (ATCC). Both Jurkat cells and U937 cells were maintained in RPMI 1640 medium containing 10% heat-inactivated fetal calf serum, 4 mM glutamine supplemented with 31 mg/l penicillin, and 50 mg/l streptomycin at 37 °C, 5% CO_2 atmosphere. U937 cells stably transfected with Bcl-2 and mock plasmid were kindly provided by Prof. Yang, R. from Nanjing University of Chinese Medicine (Nanjing, China).

2.3. Bcl-2 transgenic mice genotyping and isolation of mouse primary T cells

We used Bcl-2 transgenic mice in this study kindly provided by Astar Winoto [16]. Genotypes were determined by PCR amplification of genomic DNA obtained from mouse-tails. PCR primers used were Bcl-2 tg-F (5′-CATGTGTGTG GAGAG CGTCA AC-3′) and Bcl-2 tg-R (5′-TAGCCATTGCAGCTAGGTGAGC-3′). Based on previously described protocol [7], primary T cells suspension was prepared aseptically from lymph nodes of wild type or Bcl-2 transgenic mice. Mice were maintained in specific pathogen-free housing and cared for in accordance with "principles of laboratory animal care" approved by The Animal Care & Welfare Committee of China.

2.4. Generation of stable cell lines transfected with human catalase or MnSOD cDNA

The full-length cDNA of human catalase and MnSOD were amplified by using RT-PCR reaction and cloned in frame into bicistronic pIRES-EGFP eukaryotic expression vector containing the gene encoding GFP. These constructs (pIRES-EGFP-catalase, pIRES-EGFP-MnSOD) were stably transfected into Jurkat cells by electroporation using a Gene Pulser (Bio-Rad, Richmond, CA) at 250 V and 960 μF. Transfected clones, derived from single colonies were selected by limiting dilution in RMPI 1640 medium containing G418 (1.5 mg/ml) and screened for EGFP expression by flow cytometry, and further screened for catalase or MnSOD overexpression by western blot analysis. Control cell lines were generated by stably transfecting Jurkat cells with the pIRES-EGFP vector.

2.5. Generation of Jurkat ρ0 cells

Jurkat cells lacking mitochondrial DNA (ρ0) were generated by growing Jurkat cells in RPMI 1640 medium supplied with 10% fetal calf serum, 2 mM L-glutamine, 1 mM pyruvate, 50 μg/ml uridine, 25 mM glucose, and 50 ng/ml ethidium bromide (EB) for 5–6 weeks [17]. After selection, the cells were grown in the same medium without EB. Oxygen consumption was measured with a Clark-type oxygen electrode, and no oxygen uptake was observed for ρ0 Jurkat cells.

2.6. Assessment of plasma membrane or mitochondrial membrane depolarization

Acute changes in the plasma membrane potential were measured as previously described [7]. JC-1 was used to measure mitochondrial membrane depolarization [18,19]. In brief, cells after treatment were collected, stained with JC-1 (5 μg/ml) and analyzed with FACScan flow cytometer (Becton Dickinson, Oxford, USA). All flow cytometric analyses were accomplished using CellQuest software.

2.7. Ouabain sensitive $^{86}Rb^+$ uptake

$^{86}Rb^+$ was used as a cognate for measuring K^+ uptake in Jurkat cells [4,7].

2.8. DNA analysis by flow cytometry

Cells after treatment were fixed by the slow addition of cold 70% ethanol to a volume of 1 ml, stored at 4 °C overnight. For flow cytometry analysis, the fixed cells were pelleted, washed once in 1× phosphate-buffered saline (PBS), and stained in 1 ml of 20 μg/ml PI, 1 mg/ml RNase in 1× PBS for 20 min. 10,000 cells were examined by flow cytometry using a Becton Dickinson FASCalibur by gating on an area versus width dot plot to exclude cell debris and cell aggregates. The percentage of degraded DNA was determined by the number of cells with subdiploid DNA (sub G0) divided by the total number of cells examined under each experimental condition.

2.9. Measurement of intracellular H_2O_2 and O_2^-

Generation of intracellular H_2O_2 was measured using H_2DCFDA upon oxidation to the fluorescent derivative 2′-7′-dichlorofluorescin (DCF) by reactions with H_2O_2. Following treatments, cells were

collected and resuspended in 500 μl RPMI containing 2% FBS and 10 μM H_2DCFDA for 20 min at 37 °C. Subsequently, cells were washed with PBS and analyzed with flow cytometry (Becton Dickinson, Oxford, USA) with excitation set at 488 nm and emission at 530 nm. Immediately before flow cytometry analysis, PI (1 μg/ml) was added to exclude the non-viable cells. For measurement of intracellular O_2^-, cells after treatments were loaded with 5 μM DHE, analyzed with flow cytometry in FL3 channel.

2.10. Immunoblot analysis

The immunoblot analysis was performed as previously described [7]. Briefly, 50 μg or 100 μg protein was separated on 10% SDS-PAGE and electrophoretically blotted onto polyvinylidene fluoride (PVDF) membranes. Monoclonal antibody against human Na,K-ATPase α1 or β1 subunit was purchased from Affinity Bioreagents (Golden, CO, USA). α actin antibody was from Sigma Co. (St Louis, MO, USA). The immune blots were developed using enhanced chemiluminescence (ECL) system (Amersham Pharmacia Biotech, Piscataway, NJ).

2.11. Detection of plasma membrane Na,K-ATPase β1 subunit in Jurkat cells by flow cytometry

Following previously described protocol [7], Jurkat cells (2×10^6) were probed with primary antibody for 1 h, then FITC-conjugated secondary IgG for an additional 1 h. Finally, cells were resuspended in a 400 μl ice-cold PBS and analyzed with flow cytometry (Becton Dickinson, Oxford, USA). Immediately before flow cytometry analysis, PI (1 μg/ml) was added in each sample. PI impermeable cells were gated out as appropriate to measure plasma membrane Na,K-ATPase β1 subunit.

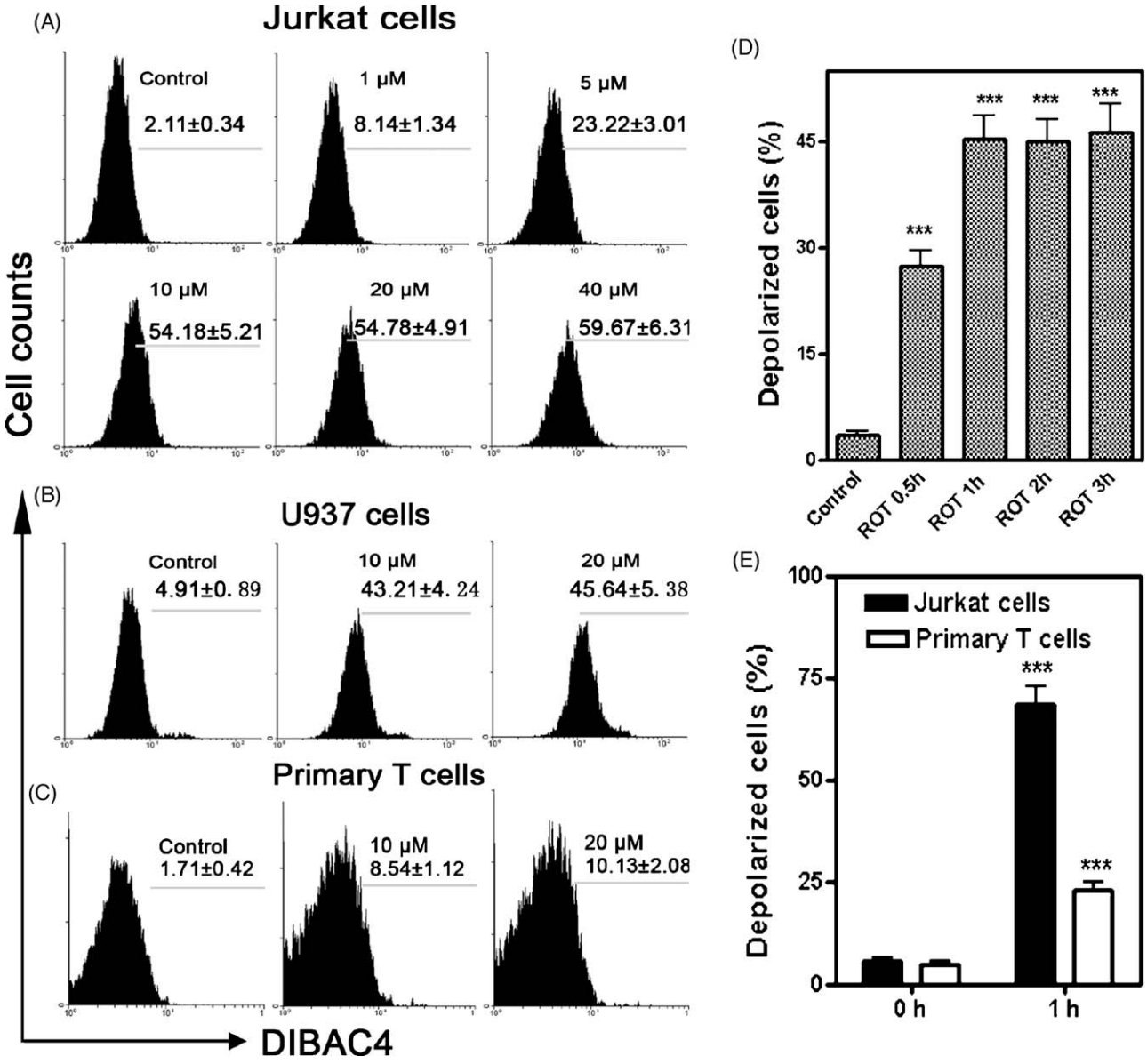

Fig. 1. Rotenone and squamocin trigger PMP depolarization in Jurkat cells or primary T cells. Dose–response effect of rotenone on PMP depolarization in Jurkat cells (A), U937 cells (B), or primary T cells isolated from mouse lymph nodes (C). Jurkat cells, U937 cells and primary T cells were treated with rotenone at different concentrations. (D) Time-course effect of rotenone on PMP depolarization in Jurkat cells, cells were treated with rotenone at 10 μM for different period of time. (E) Squamocin caused PMP depolarization in both Jurkat cells and primary T cells. Cells were treated with squamocin at 10 μM for 1 h. The percentage of cells stained with DiBAC4 is indicated (A–C). Data are presented as mean ± S.E.M. from three independent experiments, significant differences from the value obtained with cells incubated with medium alone are shown (D and E). ***$P < 0.001$. ROT: rotenone.

2.12. Detection of plasma membrane Na,K-ATPase α1 subunit by cell surface labeling

Based on previously described method and manufacturer's instruction with some modification [20], Jurkat cells were labeled using 0.5 mg/ml EZ-link NHS-SS-biotin (Pierce Chemical Co., Illinois, USA). The biotin-labeled membrane protein was pulled down by streptavidin beads (Pierce Chemical Co. Illinois, USA). The beads were thoroughly washed, boiled and proteins were analyzed by SDS-PAGE and western blot analysis using Na,K-ATPase α1 subunit antibody.

2.13. Measurement of caspases 3, 8 and 9 cleavage activities

The caspases 3, 8 and 9 activities in Jurkat cells were determined by using CaspGLOW Green Caspase-3, Caspase-8 and Caspase-9 Staining Kits (Biovision, Palo Alto, CA, USA) based on manufacturer's instructions.

2.14. Statistical analysis

The results were expressed as mean ± S.E.M. The statistical analysis involving two groups was performed by means of Student's *t*-test, whereas analysis of variance (ANOVA) followed by Dunnett's multiple comparison test was used in order to compare more than two groups. All data were processed with SPSS 10.0 software.

3. Results

3.1. Mitochondrial inhibitors induce PMP depolarization in human leukemia cells or primary T cells

In this study, we found rotenone triggered a dose-dependent PMP depolarization in Jurkat cells (Fig. 1A) and U937 cells (Fig. 1B). Rotenone also triggered PMP depolarization in Jurkat cells in a time-dependent manner with the least time was 0.5 h (Fig. 1D).

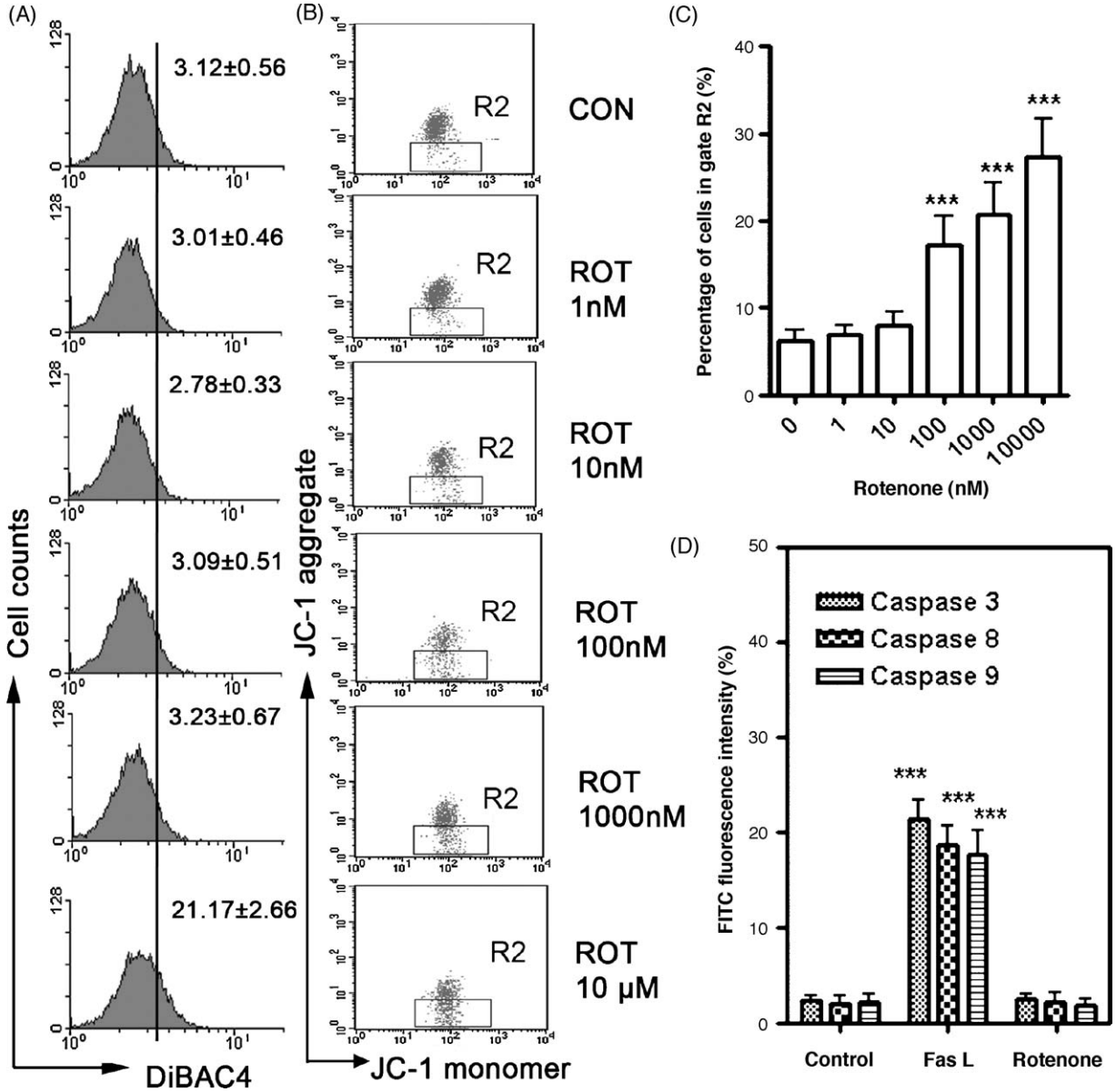

Fig. 2. Effect of rotenone on PMP depolarization (A), MMP depolarization (B and C), caspases activation (D) in Jurkat cells. Jurkat cells were treated with rotenone at different concentrations for 0.5 h (A and B). After treatment, cells were loaded with DiBAC4 for measurement of PMP depolarization (A), or loaded with JC-1 for measurement of MMP depolarization (B). The percentage of cells stained with DiBAC4 is indicated (A), cells with decreased JC-1 aggregates are gated as R2 (B), and the percentage of cells in gate R2 is plotted against rotenone concentration (C). Cells were treated with Fas L (50 ng/ml) or rotenone (10 μM) for 1 h, caspases 3, 8 and 9 activities in Jurkat cells were measured as described in Section 2 (D). Data are presented as mean ± S.E.M. from three independent experiments, significant differences from the value obtained with cells incubated with medium alone are shown (C and D). ***$P < 0.001$. ROT: rotenone.

Prolonged rotenone treatment, however, did not lead to further PMP depolarization, suggesting that rate-limiting steps might be involved in PMP depolarization. Squamocin is another potent mitochondrial inhibitor [21]. Like rotenone, squamocin also induced a remarkable PMP depolarization in Jurkat cells (Fig. 1E). Interestingly, both rotenone and squamocin were found to significantly depolarize primary T cells isolated from mouse lymph nodes (Fig. 1C and E), although their depolarizing effects on mouse primary T cells were less stronger than that on Jurkat cells or U937 cells.

3.2. Rotenone-induced PMP depolarization occurs after mitochondrial membrane potential (MMP) depolarization but before cell apoptosis in Jurkat cells

Because rotenone-induced PMP depolarization in Jurkat cells occurred so rapidly, we subsequently evaluated its relationship with other early events. First, we challenged Jurkat cells with a range of rotenone for 0.5 h to compare the temporal order of PMP and MMP depolarization. As shown in Fig. 2A, the minimal dosage required for rotenone to trigger PMP depolarization was 10 μM. However, rotenone used as little as 100 nM already caused a significant MMP depolarization as evidenced by the decreased JC-1 aggregates (Fig. 2B and C (percentage of cells in gate R2, $^{***}P < 0.001$)). Thus, PMP depolarization induced by rotenone was supposed to occur after MMP depolarization in Jurkat cells. To further confirm this observation, we did kinetic experiments by treatment of Jurkat cells with rotenone at 10 μM for 0.5, 1, 2, and 3 h. This approach, however, was finally proven to be unsuccessful in determining the temporal order of PMP and MMP depolarization because these two events almost occurred simultaneously in Jurkat cells, even when cells were subject to rotenone treatment for only 0.5 h (data not shown). To examine whether PMP depolarization induced by rotenone is a by-product of cell

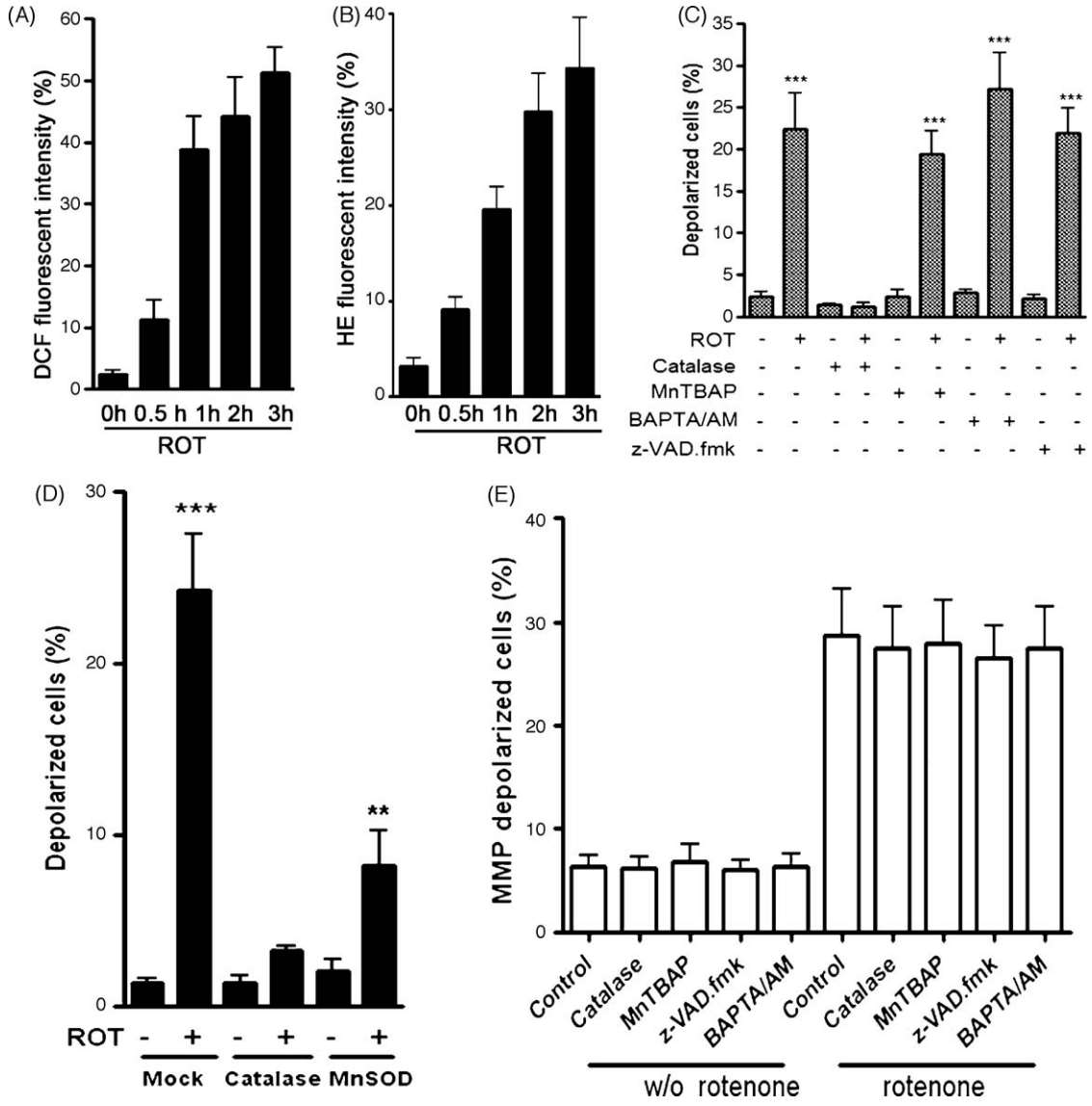

Fig. 3. ROS generation is involved in rotenone-induced PMP depolarization, but not MMP depolarization in Jurkat cells. Time-dependent increase of H_2O_2 (A) or O_2^- (B) generation in Jurkat cells after exposure to rotenone. Cells were treated with rotenone at 10 μM for different period of time. DCFH and DHE were used to measure intracellular H_2O_2 and O_2^-, respectively. (C) Exogenous catalase, but not MnTBAP, BAPTA/AM or z-VAD.fmk suppressed PMP depolarization in Jurkat cells induced by rotenone. (D) Rotenone-induced PMP depolarization was largely attenuated in catalase-transfected Jurkat cells, cells stably transfected with a vector coding for catalase, MnSOD or mock plasmid were treated with rotenone at 10 μM for 0.5 h. (E) Effects of H_2O_2, O_2^-, Ca^{2+}, caspases inhibitors on rotenone-induced MMP depolarization in Jurkat cells. Cells were preincubated with catalase (1000 U/ml), MnTBAP (10 mM), BAPTA/AM (10 μM) or z-VAD.fmk (4 μM) for 30 min, then challenged with rotenone at 10 μM for an additional 0.5 h (C and E). Data are presented as mean ± S.E.M. from three independent experiments, significant differences from the value obtained with cells incubated with medium alone are shown (C and D). $^{**}P < 0.01$; $^{***}P < 0.001$. ROT: rotenone.

apoptosis, caspases activation of Jurkat cells were measured. The results demonstrated that caspases 3, 8 and 9 cleavage activities remained inactivated in Jurkat cells after treatment with rotenone at 10 μM for 1 h, but were activated in Fas L-treated Jurkat cells as controls (Fig. 2D).

3.3. Mitochondrial H_2O_2 is involved in rotenone-induced PMP depolarization

It is generally acknowledged that lots of mitochondria couple electron transport to establish a proton gradient across mitochondrial inner membrane in the process of ATP production. However, the electron flow is not perfect, which often causes leakage of electrons from respiration complexes and generation of reactive oxygen species (ROS). Mitochondria-derived ROS have been implicated in a variety of biological processes [20,22,23]. To examine whether ROS might also be involved in PMP depolarization induced by rotenone, we first observed a time-dependent increase of cellular H_2O_2 (Fig. 3A) and O_2^- (Fig. 3B) in rotenone-treated Jurkat cells, furthermore, this ROS generation occurred as rapidly as 0.5 h and well correlated with PMP depolarization by rotenone (Fig. 1D). However, only H_2O_2 was mainly involved in rotenone-induced PMP depolarization which was completely suppressed in the presence of exogenous catalase, but not MnTBAP (Fig. 3C). The critical requirement of H_2O_2 was further confirmed in Jurkat cells stably transfected with catalase (Fig. 3D). In contrast, only partial protective effect was observed in cells stably transfected with MnSOD. Rotenone has been reported to increase cytosolic Ca^{2+} via suppression of mitochondrial Ca^{2+} uptake [24]. But surprisingly, Ca^{2+} chelator BAPTA/AM failed to suppress rotenone-induced PMP depolarization (Fig. 3C). Rotenone was unable to activate caspases 3, 8 and 9 activities when PMP depolarization was detected (Fig. 2D), thus it is not surprising to observe that PMP depolarization by rotenone was also not abrogated by caspases inhibitor z-VAD.fmk (Fig. 3C). Interestingly, none of the above-mentioned chemical inhibitors including catalase displayed protective effects on MMP depolarization induced by rotenone (Fig. 3E).

DPI is a potent NADP/NADPH oxidase inhibitor and was used in this study to examine whether PMP depolarization by rotenone is accounted by H_2O_2 from plasma membrane NADP/NADPH oxidase. Surprisingly, DPI did not prevent rotenone-induced PMP depolarization, but instead enhanced this effect. DPI alone even led to a significant increase of depolarized Jurkat cells as that of rotenone (Fig. 4A). To examine whether H_2O_2 from mitochondria is responsible for rotenone-induced PMP depolarization, we subsequently established Jurkat cells clone devoid of mtDNA (Jurkat ρ0 cells) by continuously incubating Jurkat cells in RMPI1640 culture medium supplemented with EB. As expected, both PMP depolarization (Fig. 4B) and cellular H_2O_2 generation (Fig. 4C) evoked by rotenone were largely attenuated in Jurkat ρ0 cells when compared with wild type Jurkat cells.

3.4. Impairment of Na,K-ATPase transport activity in rotenone-treated Jurkat cells

Na,K-ATPase is a critical ion pump involved in PMP depolarization. In this study, we observed a time-dependent decrease of Na,K-ATPase transport activity in Jurkat cells after treatment with rotenone at 10 μM (Fig. 5A). Furthermore, the decrease in Na,K-ATPase transport activity was well correlated with PMP depolarization (Fig. 1D) and ROS generation (Fig. 3A and B), suggesting that these events might be intrinsically linked. Rotenone-impaired Na,K-ATPase transport activity was not due to downregulation of Na,K-ATPase protein abundance, because neither Na,K-ATPase α1 nor β1 subunit expression was affected by rotenone (Fig. 5B-1 and B-2). To examine whether Na,K-ATPase subunits are internalized in rotenone-treated Jurkat cells, plasma membrane Na,K-ATPase α1 subunit were biotin labeled and subject to pull-down analysis. Consistent with previous observation [7], a time-dependent reduction of plasma membrane Na,K-ATPase α1 subunit was observed in Jurkat cells after exposure to Fas L (20 ng/ml, Fig. 5C-1), however, this effect was not mimicked by rotenone treatment since α1 subunit remained unchanged in rotenone-treated Jurkat cells (Fig. 5C-2). Like α1 subunit, plasma membrane Na,K-ATPase β1 subunit was also not internalized by rotenone in Jurkat cells as determined by flow cytometry analysis (Fig. 5D). As shown in this figure, Fas L-induced internalization of Na,K-ATPase β1 subunit was evidenced by the difference in mean FITC fluorescent intensity between Fas L-treated cells (D-3 and D-4) was much smaller than that of control cells (D-1 and D-2). In contrast, the difference in mean FITC fluorescent intensity between rotenone-treated cells (D-5 and D-6) was approximately the same as that of control cells (D-1 and D-2).

3.5. Mitochondria-derived H_2O_2 is involved in rotenone-impaired Na,K-ATPase in Jurkat cells

Since mitochondria-derived H_2O_2 was involved in PMP depolarization, we speculate that Na,K-ATPase impairment by

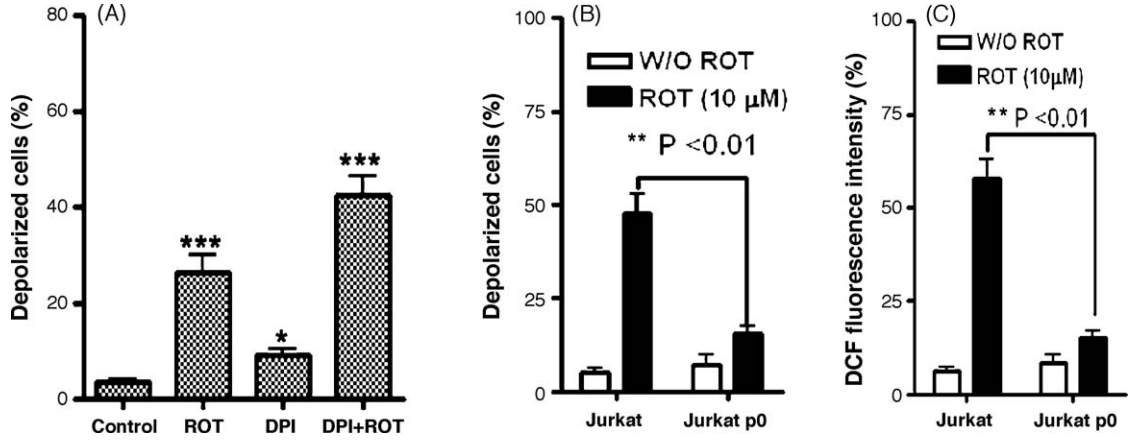

Fig. 4. Mitochondria-derived H_2O_2 is involved in rotenone-induced PMP depolarization. (A) Effect of DPI on rotenone-induced PMP depolarization in Jurkat cells. Cells were preincubated with DPI at 10 μM for 30 min, further challenged with rotenone at 10 μM for an additional 0.5 h. Rotenone-induced PMP depolarization (B) as well as H_2O_2 generation (C) was largely suppressed in Jurkat ρ0 cells. Control Jurkat cells or Jurkat ρ0 cells were challenged with rotenone at 10 μM for 1 h (B and C). Flow cytometric analysis was performed to measure cellular H_2O_2 and PMP depolarization. Data are presented as mean ± S.E.M. from three independent experiments, significant differences from the value obtained with cells incubated with medium alone are shown (A). $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. ROT, rotenone; DPI, diphenyleneiodonium.

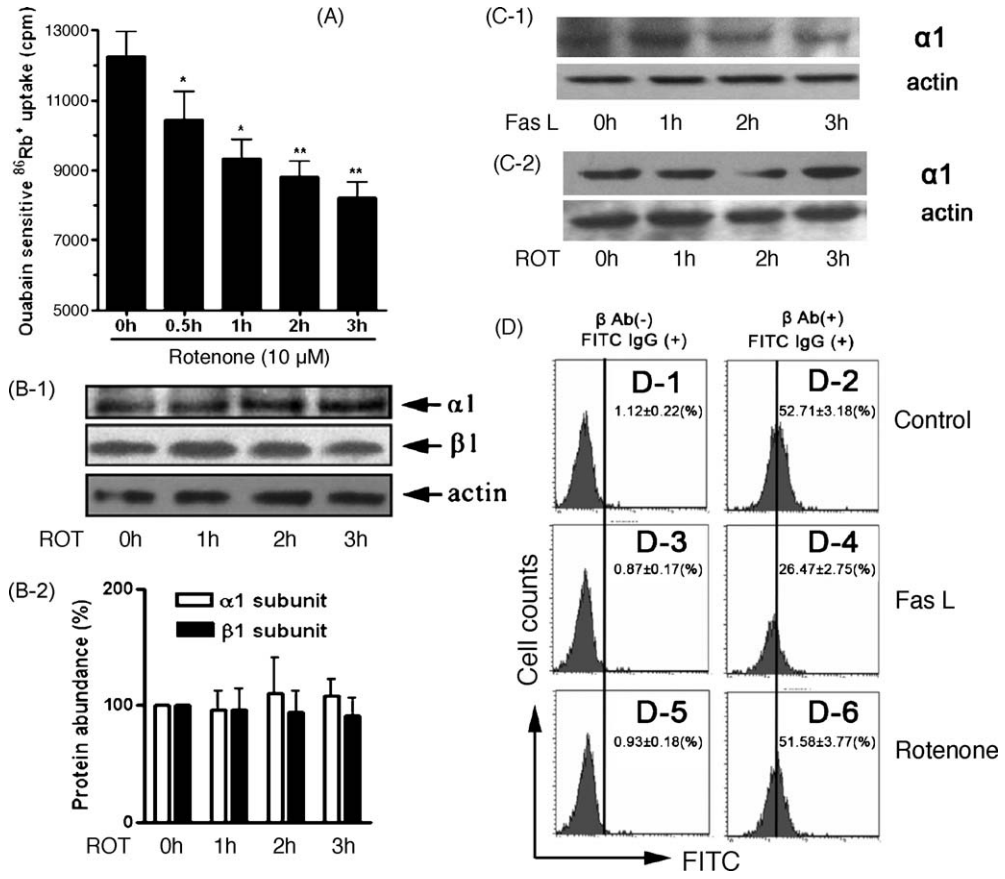

Fig. 5. Effect of rotenone on plasma membrane Na,K-ATPase transport activity in Jurkat cells. (A) Rotenone decreased plasma membrane Na,K-ATPase transport activity in Jurkat cells, cells were treated with rotenone at 10 μM for different period of time. Na,K-ATPase transport activity was measured as ouabain sensitive $^{86}Rb^+$ uptake. (B-1) Effect of rotenone on Na,K-ATPase α1 or β1 subunit expression in Jurkat cells. Cells were treated with rotenone at 10 μM for 1, 2 and 3 h, the western blot images of three independent experiments were scanned and quantified by using Gelpro software. Na,K-ATPase α1 or β1 subunit expression in control cells was arbitrarily set as 100% (B-2). Influence of Fas L (C-1) or rotenone (C-2) on plasma membrane Na,K-ATPase α1 subunit. After treatment with Fas L (20 ng/ml) or rotenone (10 μM) for 1, 2 and 3 h, the biotin-labeled cells were lysed and pulled down by streptavidin beads. Proteins bound to streptavidin beads were resolved by 10% SDS-PAGE. Western blot analysis was performed to measure Na,K-ATPase α1 subunit abundance. (D) Influence of Fas L or rotenone on plasma membrane Na,K-ATPase β1 subunit distribution. Detection of Na,K-ATPase β1 subunit was performed by flow cytometry analysis based on protocol as described in Section 2. The figures are representative of three independent experiments showing similar results. Significant difference from the value obtained with cells incubated with medium alone is shown (A). $^*P < 0.05$; $^{**}P < 0.01$.

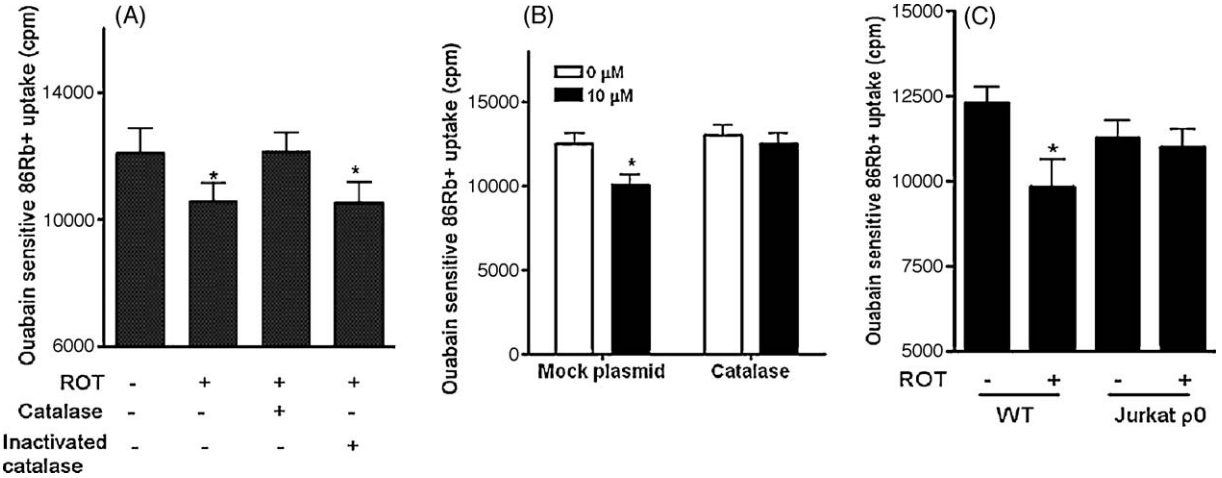

Fig. 6. Rotenone-impaired Na,K-ATPase in Jurkat cells is mediated by mitochondria-derived H_2O_2. (A) Effect of exogenous catalase or heat-inactivated catalase on rotenone-impaired Na,K-ATPase transport activity in Jurkat cells. Cells were preincubated with catalase (1000 U/ml) or inactivated catalase (1000 U/ml) for 30 min, further treated in the presence of rotenone (10 μM) for an additional 1 h. Na,K-ATPase transport activity was measured as ouabain sensitive $^{86}Rb^+$ uptake. (B) Rotenone-impaired Na,K-ATPase transport activity was largely attenuated in Jurkat cells stably transfected with catalase. Jurkat cells stably transfected with mock plasmid or catalase were treated with rotenone at 10 μM for 1 h. (C) Rotenone-impaired Na,K-ATPase transport activity was suppressed in Jurkat ρ0 cells. Control Jurkat cells or Jurkat ρ0 cells were treated in the presence of rotenone at 10 μM for 1 h. Data are presented as mean ± S.E.M. from three independent experiments, significant differences from the value obtained with cells incubated with medium alone are shown (A–C). $^*P < 0.05$.

rotenone is probably also relevant to H_2O_2 from mitochondria. As shown in Fig. 6A, Na,K-ATPase impairment by rotenone in Jurkat cells was abolished in the presence of exogenous catalase. Furthermore, this preventive effect elicited by catalase was specific because inactivated catalase after heat treatment failed to produce such an effect. Rotenone-impaired Na,K-ATPase activity was also significantly recovered in catalase transfectant as compared to mock transfectant (Fig. 6B). To determine if mitochondria-derived H_2O_2 is responsible for rotenone-impaired Na,K-ATPase, Jurkat cells devoid of mtDNA were used. The result, as shown in Fig. 6C demonstrated that Na,K-ATPase impairment was largely attenuated in Jurkat ρ0 cells as compared with the wild type Jurkat cells.

3.6. Suppression of rotenone and squamocin-induced cellular depolarization in leukemia cell lines or primary T cells after Bcl-2 overexpression

Bcl-2 is originally identified as an anti-apoptotic protein, but can also act as an antioxidant to suppress oxidative stress [25]. In this study, U937 cells stably expressing Bcl-2 and primary T cells isolated from Bcl-2 transgenic mouse were used to determine whether PMP depolarization by mitochondrial inhibitors is also related to Bcl-2. As shown in Fig. 7A and B, both PMP depolarization and Na,K-ATPase impairment by rotenone were largely attenuated in U937 cells with Bcl-2 overexpression, but not in the mock transfectant. Bcl-2 overexpression even resulted in a mild increase of the basal Na,K-ATPase transport activity (Fig. 7B). Similar results were also obtained in primary T cells isolated from Bcl-2 transgenic mouse. As shown in Fig. 7C, a left-shift of DiBAC4 fluorescence indicative of PMP hyperpolarization was observed in primary T cells from Bcl-2 transgenic mice (Fig. 7C). Secondly, both rotenone and squamocin triggered PMP depolarization in primary T cells isolated from wild type mice (Fig. 7D), however, their depolarizing effects were completely abolished in primary T cells isolated from Bcl-2 transgenic mice (Fig. 7D).

3.7. Inhibition of Na,K-ATPase activity enhances mitochondrial inhibitors-induced cell apoptosis in human leukemia cell lines and mouse primary T cells

We speculate that Na,K-ATPase impairment by mitochondrial inhibitors might be related to cell death enhancement, because in addition to ATP production, mitochondria also play critical roles in the initiation and amplification of programmed cell death. To test this hypothesis, we used ouabain to artificially suppress plasma membrane Na,K-ATPase activity and observed its influence on mitochondrial inhibitors-induced PMP depolarization and cell apoptosis. The results, as shown in Fig. 8A and B revealed that ouabain alone remarkably depolarized Jurkat cells, U937 cells as well as primary T cells. In addition, ouabain enhanced rotenone or squamocin-induced PMP depolarization in primary T cells, but not in

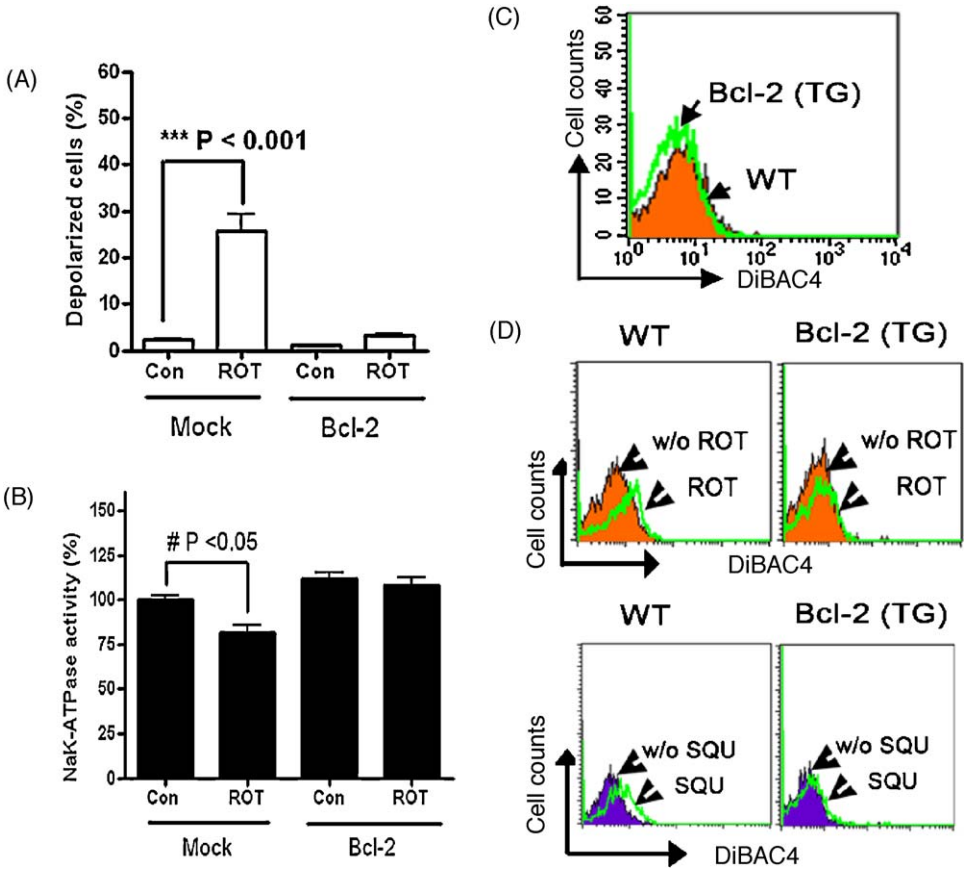

Fig. 7. Rotenone-induced PMP depolarization in U937 cells or primary T cells is suppressed in the presence of Bcl-2 overexpression. Inhibition of rotenone-induced PMP depolarization (A) or Na,K-ATPase impairment (B) in U937 cells with Bcl-2 overexpression. U937 cells stably transfected with mock plasmid or Bcl-2 were treated with rotenone at 10 μM for 1 h (A and B). Na,K-ATPase transport activity was measured as ouabain sensitive $^{86}Rb^+$ uptake, and Na,K-ATPase transport activity of Jurkat cells transfected with mock plasmid without rotenone treatment was arbitrarily set at 100% (B). (C) Hyperpolarization of primary T cells isolated from Bcl-2 transgenic mice. (D) Rotenone or squamocin-triggered PMP depolarization was suppressed in primary T cells isolated from Bcl-2 transgenic mice. Primary T cells were treated in the presence of rotenone (10 μM) or squamocin (10 μM) for 3 h, stained with $DiBAC_4$ and analyzed with flow cytometry for measurement of PMP depolarization. The figures are representative of three independent experiments showing similar results (C and D). Data are presented as mean ± S.E.M. from three independent experiments, significant differences from the value obtained with cells incubated with medium alone are shown (A and B), $^{\#}P < 0.05$; $^{***}P < 0.001$. ROT, rotenone; SQU, squamocin; TG, transgenic.

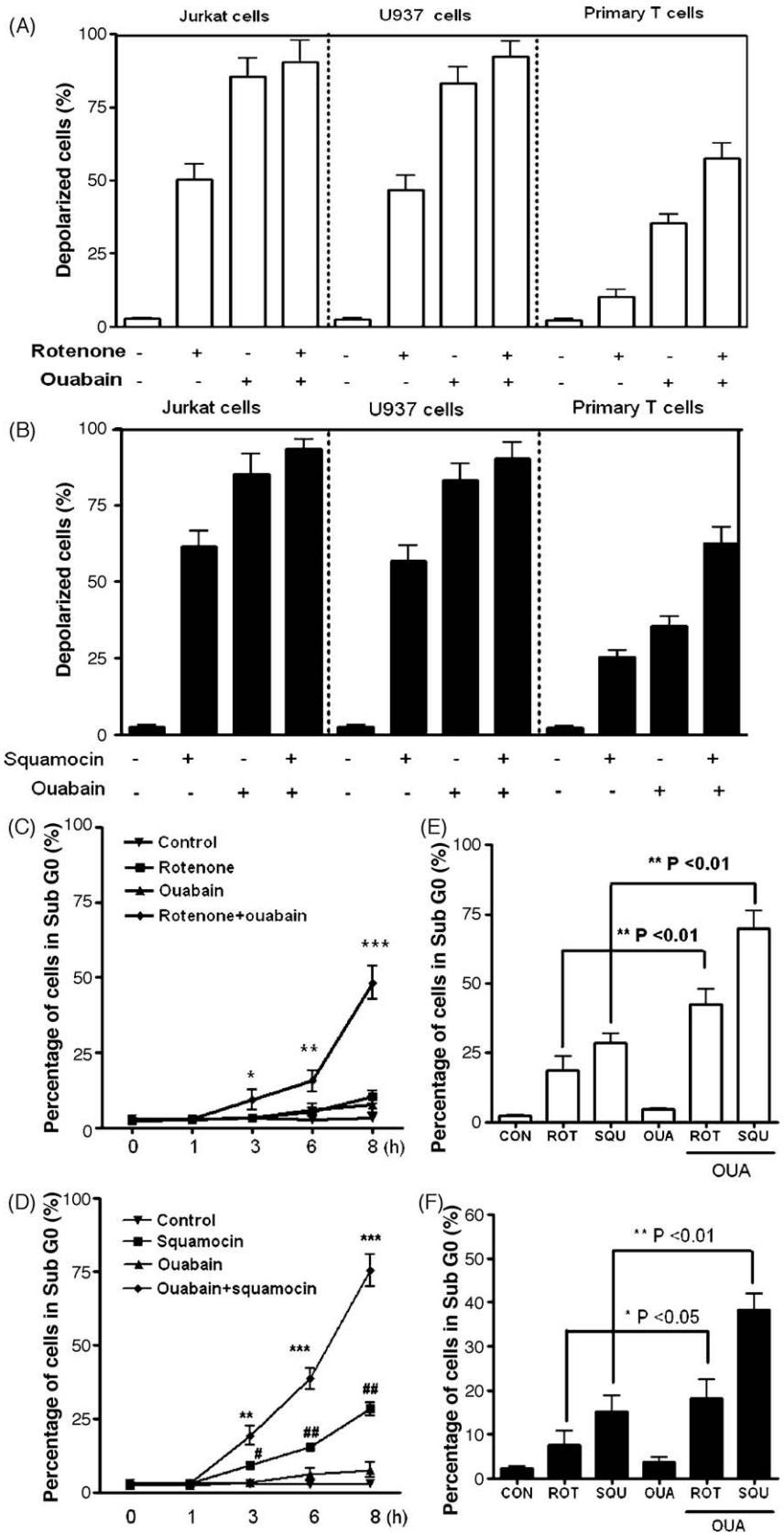

Fig. 8. Ouabain enhances PMP depolarization and cell apoptosis induced by rotenone or squamocin in Jurkat, U937 or mouse primary T cells. The PMP depolarization of Jurkat cells, U937 cells and mouse primary T cells induced by rotenone (A), or squamocin (B) in the presence or absence of ouabain. Cells were treated with these chemicals for 1 h (A and B). DiBAC4 was used to measure PMP depolarization. (C and D) Time-course effect of rotenone or squamocin on Jurkat cells death in the presence or absence of ouabain. Ouabain potentiated rotenone or squamocin-induced cell apoptosis in U937 cells (E) or in mouse primary T cells (F), cells were treated with these chemicals for 6 h (E and F). The concentration for rotenone used was 10 μM (A, C, E and F), for squamocin used was 10 μM (B, D, E and F), and for ouabain used was 100 μM (A–F). Data are presented as mean ± S.E.M. from three independent experiments (A–F), $^{\#}P < 0.05$; $^{\#\#}P < 0.01$; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ versus control. ROT, rotenone; SQU, squamocin; OUA, ouabain.

Jurkat or U937 cells, because ouabain alone already caused nearly 85% Jurkat or U937 cells to depolarize, but in primary T cells, only 33% cells were depolarized (Fig. 8A and B). Ouabain significantly accelerated and aggravated Jurkat cell apoptosis induced by rotenone (Fig. 8C) or squamocin (Fig. 8D), as quantified by sub G0 cells population (apoptotic DNA cleavage). In particular, consistent with other report [4], ouabain at 100 μM alone did not cause a significant sub G0 cells accumulation of Jurkat cells within the time examined (8 h). The cell apoptosis amplification by ouabain was also observed in U937 cells (Fig. 8E) or in primary T cells (Fig. 8F).

3.8. Suppression of mitochondrial inhibitors-induced leukemia cell apoptosis in the presence of catalase or Bcl-2 overexpression

Given the critical role of H_2O_2 and Bcl-2 in PMP depolarization and Na,K-ATPase impairment by rotenone, we subsequently wanted to know whether overexpression of catalase or Bcl-2 can rescue rotenone-induced cells apoptosis. The result demonstrated that rotenone-induced sub G0 cells accumulation was largely, but not completely attenuated in Jurkat cells overexpressing catalase (Fig. 9A). However, rotenone-induced sub G0 cells accumulation was completely suppressed in U937 cells overexpressing Bcl-2 (Fig. 9B). Similar results were also obtained in squamocin-treated Jurkat or U937 cells, or in mouse primary T cells challenged with rotenone or squamocin (data not shown).

4. Discussion

This paper described for the first time that Na,K-ATPase failure correlated with PMP depolarization induced by rotenone in leukemic cells. Furthermore, suppression of Na,K-ATPase activity accelerated and potentiated mitochondrial inhibitors-induced leukemia cells apoptosis. Researchers have found that Na,K-ATPase suppression can amplify cell death via loss of intracellular K^+ and caspase activation, or upregulation of death receptors in cancerous cells [26,27]. We did not observe the upregulation of death receptors such as Fas, DR4 or DR5 in Jurkat, U937 or primary T cells after treatment with mitochondrial toxins (data not shown), thus, Na,K-ATPase impairment most likely enhances cells apoptosis via disrupting intracellular ion homeostasis. Although this presumption needs more experiments to confirm, it seems reasonable to conclude that, at least in this study, mitochondria-to-Na,K-ATPase and PMP depolarization may represent a novel mechanism for mitochondria to amplify apoptosis signal and enhance rotenone-induced leukemia cell apoptosis.

Isolated from Derris genus, rotenone has been shown to block the electron flow and induce cellular ROS generation [22,28,29]. However, only H_2O_2 was found to be the major ROS involved in rotenone-induced PMP depolarization. Indeed, O_2^- and H_2O_2 often exert different biological functions despite the same origin, a good case in point is that in a recent study, rotenone-induced H_2O_2 was found to be specifically involved in the prevention of acute inflammatory processes in which activated neutrophils play a major role, such as acute lung injury [23]. Moreover, the regulatory mechanism of H_2O_2 on Na,K-ATPase varies depending on cell types and stimuli [7,20,30]. Rotenone-evoked H_2O_2 generation in Jurkat cells did not finally result in the internalization of Na,K-ATPase α1 and β1 subunits, we speculate that cellular ATP supply could be accounted because cellular ATP level is a critical determinant for membrane-bound protein internalization [31,32]. For instance, failure of chloride channel (CLC-2) internalization by cellular ATP depletion was observed in neurons [33]. As a result of electron transfer interruption, rotenone has been reported to dramatically decrease the ATP content in leukemia cells [29]. Rotenone at 10 μM was also found to cause nearly 20% ATP loss in Jurkat cells in 3 h (data not shown). In general, regulation of Na,K-ATPase occurs at several levels: (1) the long-term regulation which is relevant to the *de novo* synthesis of this pump; (2) the short-term regulation by recruitment/internalization of the active pumps to/from the plasma membrane; (3) the modulation of ATPase activity. Since rotenone failed to cause the suppression and internalization of Na,K-ATPase subunits, we speculate that rotenone probably inhibits Na,K-ATPase transport activity through direct modulation of its ATPase activity. Na,K-ATPase itself is an energy-consuming pump, its transport activity could be downregulated as long as cellular ATP supply is insufficient. As such, the relationship between ATP and H_2O_2 in rotenone-impaired Na,K-ATPase is an important issue needs to be addressed. Indeed, the interplays between ATP depletion and ROS in mitochondria-involved cellular effects are extremely complex. However, there is overwhelming evidence to suggest that ROS initiate the damage to lipids, protein and DNA and consequently result in mitochondrial dysfunction, ion balance deregulation and loss of membrane integrity [34]. As such, ATP depletion in rotenone-treated leukemia cells might be a by-product of H_2O_2 to cooperatively suppress the transport activity of Na,K-ATPase with H_2O_2.

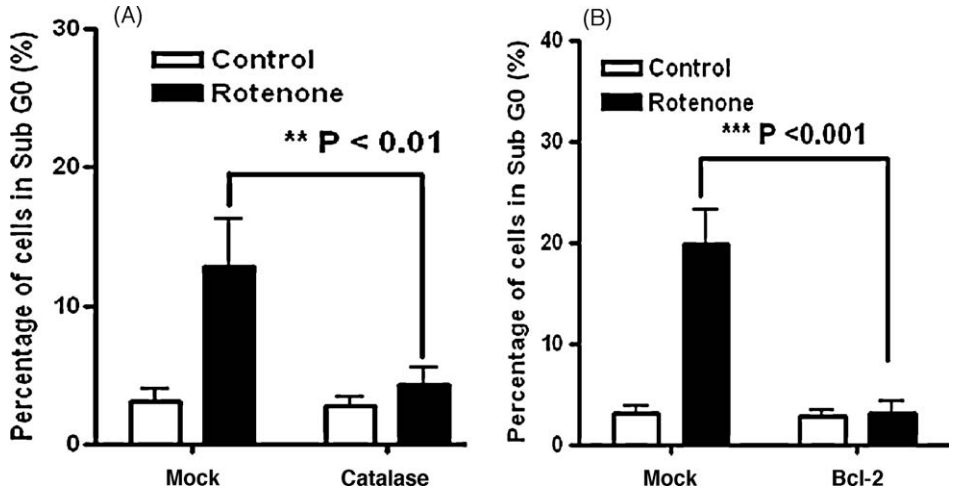

Fig. 9. Suppression of rotenone-induced cell apoptosis when catalase or Bcl-2 is overexpressed in Jurkat or U937 cells. (A) Effect of catalase overexpression on rotenone-induced Jurkat cells death. Jurkat cells stably transfected with mock plasmid or catalase were subject to rotenone treatment at 10 μM for 8 h. (B) Effect of Bcl-2 overexpression on rotenone-induced Jurkat cells death. U937 cells stably transfected with mock plasmid or Bcl-2 were subject to rotenone treatment at 10 μM for 6 h. Data are presented as mean ± S.E.M. from three independent experiments. $^{**}P < 0.01$; $^{***}P < 0.001$ versus control.

Until now, at least three functions are performed by Bcl-2 to exert protective effects. First, Bcl-2 can heterodimerize with and inhibit pro-apoptotic family members like Bax and BH3-only proteins [35]. Second, Bcl-2 regulates Ca^{2+} release from the endoplasmic reticulum by directly interacting with the inositol 1,4,5-trisphosphate receptor [36]. Third, Bcl-2 displays an antioxidant-like action, particularly at the level of the mitochondria [25,37]. Recent study even found that Bcl-2 can bind with GSH in mitochondria and exert antioxidant activity [38]. Based on these facts, Bcl-2 may act as an antioxidant to antagonize PMP depolarization and Na,K-ATPase impairment in rotenone-treated Jurkat cells. In addition to its possible antioxidant effect, Bcl-2 can also directly regulate Na,K-ATPase activity. For instance, Bcl-2 overexpression was found to increase the basal level of Na,K-ATPase transport activity in U937 cells (Fig. 7B), Bcl-2 overexpression even hyperpolarized mouse primary T cells (Fig. 7C), which is probably due to the upregulation of Na,K-ATPase activity because similar results were also obtained in PW cells [39]. In this study, it is noteworthy to mention that although mitochondrial inhibitors-induced cell apoptosis was also regulated by H_2O_2 and Bcl-2, PMP depolarization as well as Na,K-ATPase impairment could not be arbitrarily regarded as "by-products" of cell apoptosis, because these two cellular events were initiated before the occurrence of cell apoptosis. As such, the mitochondria-to-Na,K-ATPase amplification mechanism most likely forms in the initiation phase of rotenone-induced cells apoptosis.

We have to acknowledge that the concentration for rotenone used to trigger PMP depolarization in Jurkat cells was higher than its IC_{50} concentration on mitochondrial respiration, which suggests that the impaired mitochondrial respiration alone is probably not sufficient to trigger PMP depolarization. As such, mitochondrial H_2O_2 production constitutes the rate-limiting factor in rotenone-induced PMP depolarization as well as Na,K-ATPase impairment. In our opinion, this presumption may also help to understand why rotenone is usually used at different concentrations in different cells to exert ROS-relevant biological functions [9,22,23,29]. Likewise, the fact that leukemic cells presented greater depolarization than that of primary T cells in this study could also be accounted by H_2O_2 production, because cancer cells are in general metabolically active, produce high levels of H_2O_2, and are under intrinsic oxidative stress [40]. The malignant cells are more vulnerable to further oxidative stress by ROS-generating agents [9,40].

In sum, this study provides evidence that a novel mechanism exists for mitochondria to amplify death signals in the initiation stage of rotenone-induced leukemia cells apoptosis, which is mediated by PMP depolarization and Na,K-ATPase impairment. As a matter of fact, in addition to mitochondrial apoptotic pathway as presented in this study, Na,K-ATPase impairment and PMP depolarization were also involved in the apoptosis enhancement in death receptors-mediated cell apoptosis [4]. Therefore, Na,K-ATPase is probably a shared target for inactivation in either internal or external stimuli-induced apoptosis. On the other hand, the elevated Na,K-ATPase activity (PMP hyperpolarization) conferred cell resistance to radiation-induced programmed cell death [39]. Taken together, it seems reasonable to conclude that apart from its ion pump activity, Na,K-ATPase may also act as a sensor protein that facilitates cells to rapidly respond to apoptotic stimuli and controls the progress of apoptotic program.

Acknowledgements

This project was supported by Natural Science Fund of China to Dr. Zi-Chun Hua (30425009, 30330530) and to Dr. Yin Wu (30470644, 30600502), Natural Science Fund of Jiangsu Province to Dr. Yin Wu (BK2008272), Open Project of Jiangsu Key Lab of TCM Processing (ZYPZ001), the Ministry of Education of China (TRAPOY 199028418, 111 project).

References

[1] Sallmann S, Juttler E, Prinz S, Petersen N, Knopf U, Weiser T, et al. Induction of interleukin-6 by depolarization of neurons. J Neurosci 2000;20:8637–42.
[2] Szaszi K, Sirokmany G, Di Ciano-Oliveira C, Rotstein OD, Kapus A. Depolarization induces Rho–Rho kinase-mediated myosin light chain phosphorylation in kidney tubular cells. Am J Physiol Cell Physiol 2005;289:C673–85.
[3] Sakurada S, Takuwa N, Sugimoto N, Wang Y, Seto M, Sasaki Y, et al. Ca^{2+}-dependent activation of Rho and Rho kinase in membrane depolarization-induced and receptor stimulation-induced vascular smooth muscle contraction. Circ Res 2003;93:548–56.
[4] Bortner CD, Gomez-Angelats M, Cidlowski JA. Plasma membrane depolarization without repolarization is an early molecular event in anti-Fas-induced apoptosis. J Biol Chem 2001;276:4304–14.
[5] Ghoumari AM, Piochon C, Tomkiewicz C, Eychenne B, Levenes C, Dusart I, et al. Neuroprotective effect of mifepristone involves neuron depolarization. FASEB J 2006;20:1377–86.
[6] Franco R, Bortner CD, Cidlowski JA. Potential roles of electrogenic ion transport and plasma membrane depolarization in apoptosis. J Membr Biol 2006;209:43–58.
[7] Yin W, Cheng W, Shen W, Shu L, Zhao J, Zhang J, et al. Impairment of Na(+),K(+)-ATPase in CD95(APO-1)-induced human T-cell leukemia cell apoptosis mediated by glutathione depletion and generation of hydrogen peroxide. Leukemia 2007;21:1669–78.
[8] Nolte F, Friedrich O, Rojewski M, Fink RH, Schrezenmeier H, Korper S. Depolarisation of the plasma membrane in the arsenic trioxide (As_2O_3)-and anti-CD95-induced apoptosis in myeloid cells. FEBS Lett 2004;578:85–9.
[9] Pelicano H, Feng L, Zhou Y, Carew JS, Hileman EO, Plunkett W, et al. Inhibition of mitochondrial respiration: a novel strategy to enhance drug-induced apoptosis in human leukemia cells by a reactive oxygen species-mediated mechanism. J Biol Chem 2003;278:37832–9.
[10] Chinnaiyan AM, O'Rourke K, Tewari M, Dixit VM. FADD, a novel death domain-containing protein, interacts with the death domain of Fas and initiates apoptosis. Cell 1995;81:505–12.
[11] Kroemer G, de The H. Arsenic trioxide, a novel mitochondriotoxic anticancer agent? J Natl Cancer Inst 1999;91:743–5.
[12] Akao Y, Mizoguchi H, Kojima S, Naoe T, Ohishi N, Yagi K. Arsenic induces apoptosis in B-cell leukaemic cell lines in vitro: activation of caspases and down-regulation of Bcl-2 protein. Br J Haematol 1998;102:1055–60.
[13] Larochette N, Decaudin D, Jacotot E, Brenner C, Marzo I, Susin SA, et al. Arsenite induces apoptosis via a direct effect on the mitochondrial permeability transition pore. Exp Cell Res 1999;249:413–21.
[14] Higuchi M, Proske RJ, Yeh ET. Inhibition of mitochondrial respiratory chain complex I by TNF results in cytochrome *c* release, membrane permeability transition, and apoptosis. Oncogene 1998;17:2515–24.
[15] Harper N, Hughes M, MacFarlane M, Cohen GM. Fas-associated death domain protein and caspase-8 are not recruited to the tumor necrosis factor receptor 1 signaling complex during tumor necrosis factor-induced apoptosis. J Biol Chem 2003;278:25534–41.
[16] Rajpal A, Cho YA, Yelent B, Koza-Taylor PH, Li D, Chen E, et al. Transcriptional activation of known and novel apoptotic pathways by Nur77 orphan steroid receptor. EMBO J 2003;22:6526–36.
[17] King MP, Attardi G. Isolation of human cell lines lacking mitochondrial DNA. Methods Enzymol 1996;264:304–13.
[18] Salvioli S, Ardizzoni A, Franceschi C, Cossarizza A. JC-1, but not DiOC6(3) or rhodamine 123, is a reliable fluorescent probe to assess delta psi changes in intact cells: implications for studies on mitochondrial functionality during apoptosis. FEBS Lett 1997;411:77–82.
[19] Cossarizza A, Baccarani-Contri M, Kalashnikova G, Franceschi C. A new method for the cytofluorimetric analysis of mitochondrial membrane potential using the J-aggregate forming lipophilic cation 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolcarbocyanine iodide (JC-1). Biochem Biophys Res Commun 1993;197:40–5.
[20] Dada LA, Chandel NS, Ridge KM, Pedemonte C, Bertorello AM, Sznajder JI. Hypoxia-induced endocytosis of Na,K-ATPase in alveolar epithelial cells is mediated by mitochondrial reactive oxygen species and PKC-zeta. J Clin Invest 2003;111:1057–64.
[21] Degli Esposti M, Ghelli A, Ratta M, Cortes D, Estornell E. Natural substances (acetogenins) from the family Annonaceae are powerful inhibitors of mitochondrial NADH dehydrogenase (Complex I). Biochem J 1994;301(Pt 1):161–7.
[22] Chen Y, McMillan-Ward E, Kong J, Israels SJ, Gibson SB. Mitochondrial electron-transport-chain inhibitors of complexes I and II induce autophagic cell death mediated by reactive oxygen species. J Cell Sci 2007;120:4155–66.
[23] Zmijewski JW, Lorne E, Zhao X, Tsuruta Y, Sha Y, Liu G, et al. Mitochondrial respiratory complex I regulates neutrophil activation and severity of lung injury. Am J Respir Crit Care Med 2008;178:168–79.
[24] Murphy AN, Bredesen DE, Cortopassi G, Wang E, Fiskum G. Bcl-2 potentiates the maximal calcium uptake capacity of neural cell mitochondria. Proc Natl Acad Sci USA 1996;93:9893–8.
[25] Hockenbery DM, Oltvai ZN, Yin XM, Milliman CL, Korsmeyer SJ. Bcl-2 functions in an antioxidant pathway to prevent apoptosis. Cell 1993;75:241–51.

[26] Sreenivasan Y, Raghavendra PB, Manna SK. Oleandrin-mediated expression of Fas potentiates apoptosis in tumor cells. J Clin Immunol 2006;26:308–22.
[27] Frese S, Frese-Schaper M, Andres AC, Miescher D, Zumkehr B, Schmid RA. Cardiac glycosides initiate Apo2L/TRAIL-induced apoptosis in non-small cell lung cancer cells by up-regulation of death receptors 4 and 5. Cancer Res 2006;66:5867–74.
[28] Marella M, Seo BB, Matsuno-Yagi A, Yagi T. Mechanism of cell death caused by complex I defects in a rat dopaminergic cell line. J Biol Chem 2007;282:24146–5.
[29] Li N, Ragheb K, Lawler G, Sturgis J, Rajwa B, Melendez JA, et al. Mitochondrial complex I inhibitor rotenone induces apoptosis through enhancing mitochondrial reactive oxygen species production. J Biol Chem 2003;278:8516–25.
[30] Yin W, Yin FZ, Shen WX, Cai BC, Hua ZC. Requirement of hydrogen peroxide and Sp1 in the stimulation of Na,K-ATPase by low potassium in MDCK epithelial cells. Int J Biochem Cell Biol 2008;40:942–53.
[31] Hertel C, Coulter SJ, Perkins JP. The involvement of cellular ATP in receptor-mediated internalization of epidermal growth factor and hormone-induced internalization of beta-adrenergic receptors. J Biol Chem 1986;261:5974–80.
[32] Smith RM, Jarett L. Differences in adenosine triphosphate dependency of receptor-mediated endocytosis of alpha 2-macroglobulin and insulin correlate with separate routes of ligand-receptor complex internalization. Endocrinology 1990;126:1551–60.
[33] Dhani SU, Kim Chiaw P, Huan LJ, Bear CE. ATP depletion inhibits the endocytosis of ClC-2. J Cell Physiol 2008;214:273–80.
[34] Vanlangenakker N, Berghe TV, Krysko DV, Festjens N, Vandenabeele P. Molecular mechanisms and pathophysiology of necrotic cell death. Curr Mol Med 2008;8:207–20.
[35] Zha H, Aime-Sempe C, Sato T, Reed JC. Proapoptotic protein Bax heterodimerizes with Bcl-2 and homodimerizes with Bax via a novel domain (BH3) distinct from BH1 and BH2. J Biol Chem 1996;271:7440–4.
[36] Chen R, Valencia I, Zhong F, McColl KS, Roderick HL, Bootman MD, et al. Bcl-2 functionally interacts with inositol 1,4,5-trisphosphate receptors to regulate calcium release from the ER in response to inositol 1,4,5-trisphosphate. J Cell Biol 2004;166:193–203.
[37] Kane DJ, Sarafian TA, Anton R, Hahn H, Gralla EB, Valentine JS, et al. Bcl-2 inhibition of neural death: decreased generation of reactive oxygen species. Science 1993;262:1274–7.
[38] Zimmermann AK, Loucks FA, Schroeder EK, Bouchard RJ, Tyler KL, Linseman DA. Glutathione binding to the Bcl-2 homology-3 domain groove: a molecular basis for Bcl-2 antioxidant function at mitochondria. J Biol Chem 2007;282:29296–304.
[39] Gilbert M, Knox S. Influence of Bcl-2 overexpression on Na+/K(+)-ATPase pump activity: correlation with radiation-induced programmed cell death. J Cell Physiol 1997;171:299–304.
[40] Szatrowski TP, Nathan CF. Production of large amounts of hydrogen peroxide by human tumor cells. Cancer Res 1991;51:794–8.