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Role of mitochondria-associated hexokinase II in cancer cell death induced by 3-bromopyruvate

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ABSTRACT

It has long been observed that cancer cells rely more on glycolysis to generate ATP and actively use certain glycolytic metabolic intermediates for biosynthesis. Hexokinase II (HKII) is a key glycolytic enzyme that plays a role in the regulation of the mitochondria-initiated apoptotic cell death. As a potent inhibitor of hexokinase, 3-bromopyruvate (3-BrPA) is known to inhibit cancer cell energy metabolism and trigger cell death, supposedly through depletion of cellular ATP. The current study showed that 3-BrPA caused a covalent modification of HKII protein and directly triggered its dissociation from mitochondria, leading to a specific release of apoptosis-inducing factor (AIF) from the mitochondria to cytosol and eventual cell death. Co-immunoprecipitation revealed a physical interaction between HKII and AIF. Using a competitive peptide of HKII, we showed that the dissociation of hexokinase II from mitochondria alone could cause apoptotic cell death, especially in the mitochondria-deficient ρ^0 cells that highly express HKII. Interestingly, the dissociation of HKII itself did not directly affect the mitochondrial membrane potential, ROS generation, and oxidative phosphorylation. Our study suggests that the physical association between HKII and AIF is important for the normal localization of AIF in the mitochondria, and disruption of this protein complex by 3-BrPA leads to their release from the mitochondria and eventual cell death.

1. Introduction

In the 1920s Otto Warburg observed that liver tumor tissue exhibited an increase in glycolytic activity, even in the presence of oxygen. The phenomenon that tumor cells exhibit increased glycolysis and rely more on this metabolic pathway to generate ATP under aerobic conditions is known as the "Warburg effect" [1,2]. Such increased aerobic glycolysis has since been observed in a variety of cancer types. Although whether this metabolic switch in cancer cells is a causal event in cancer development or merely a downstream biochemical symptom of cancer is still a matter of debate, accumulating evidences seem to suggest that aerobic glycolysis is closely associated with tumorigenesis and plays important roles in maintaining the malignant behaviors of the cancer cells and promoting metastasis. High levels of glycolysis seem to confer malignant cell survival advantage in hypoxic or acidosis microenvironment during the process of cancer development. This unique metabolic profile of cancer cells provides a biochemical basis for developing new chemotherapeutic strategies targeting the glycolysis pathway in cancer cells [3].

Various inhibitors affecting the key enzymes of the glycolysis pathway have been identified. Among the glycolytic inhibitors currently under development for potential use in cancer treatment, 3-BrPA exhibits promising anticancer activity both in vitro and in vivo [4]. Although the details in the mechanisms of action responsible for the anticancer activity of 3-BrPA remain to be fully elucidated, this compound is believed to inhibit HKII and prevent glucose from entering the glycolytic pathway [4]. It should be noted, however, that 3-BrPA is a small molecule and chemically reactive. This compound may potentially affect multiple targets in the cells.

Hexokinase (ATP: D-hexose 6-phosphotransferase) is a key enzyme that catalyzes the first step in the glycolysis pathway. This enzyme transfers a phosphate group from ATP to glucose to form glucose-6-phosphate. In human cells, there are four isoforms of hexokinase (I–IV). Despite their overall structural similarity, these isoforms differ in their expression patterns, subcellular localizations, and catalytic/regulatory properties. In normal tissue, hexokinase I is mainly expressed in the brain and kidney, while hexokinase II is found mainly in the heart and skeletal muscle. The type III isozyme is found in virtually all tissues but is expressed at low levels. Hexokinase IV (glucokinase) is mainly expressed in liver and the islets of langerhans. Interestingly, hexokinase III exhibits perinuclear localization, but its biological function still remains unclear. All four isoforms are believed to play distinct roles in metabolism and affect cell fates [8,9]. Of note, hexokinase I and II are found to be able to bind to mitochondria through their interaction with VDAC (voltage dependent anion channel), and this association appears important for the homeostasis of mitochondria [10,11]. Additionally, such mitochondrial association has been reported to influence the enzyme kinetics of hexokinase [12].

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In cancer tissues, the high glycolytic activity requires an up-regulation of the key glycolytic enzymes including hexokinase. Interestingly, the percentage of hexokinase binding to the mitochondria is also significantly increased in cancer cells. For instance, in AS-20D liver tumor cells, the hexokinase protein level (mainly HKII) was found to be more than 500 times higher than in normal liver cells, which mainly express hexokinase IV instead. Furthermore, around 80% of hexokinase II is associated with mitochondria [13]. Due to the frequent up-regulation of HKII in cancer cells and its important role in glycolytic pathway, this enzyme seems to be an attractive target for anticancer drug development.

2. Materials and methods

2.1. Materials

3-BrPA, H_2O_2, clotrimazole (CTZ), 2-Deoxyglucose (2-DG), cyclosporin A (CsA), and rotenone were purchased from Sigma-Aldrich (St. Louis, MO). 3-BrPA was dissolved in water and neutralized with Na_2CO_3 immediately before use. Goat polyclonal anti-hexokinase II antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, California). Rabbit polyclonal anti-AIF was purchased from Chemicon (Temecula, CA). Cytochrome *c* monoclonal antibody (Clone/PAD: 7H8.2C12) was purchased from Invitrogen Corporation (Carlsbad, California). Immunoprecipitation Starter Pack was purchased from GE healthcare life sciences (Piscataway, NJ).

2.2. Cell culture

Human leukemia cells lines (HL-60) and lymphoma cell line (Raji) were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated FBS at 37 °C with 5% CO_2. Human colon cancer cells (HCT116 $p53^{+/+}$ and HCT116 $p53^{-/-}$) were cultured in McCoy's 5 A medium supplemented with 10% FBS. The mitochondrial defective clones (ρ^0 cells) HL-60/C6F, Raji/C2, Raji/C6 were maintained in RPMI 1640 supplemented with 10% fetal bovine serum, 1 mmol/L sodium pyruvate, 50 mmol/L uridine, and 2.7% glucose. The ρ^0 clone $HCT116^{+/+}$/c15 was maintained in McCoy's 5 A medium supplemented with 10% fetal bovine serum, 1 mmol/L sodium pyruvate, 50 mmol/L uridine, and 2.7% glucose.

2.3. Cytotoxicity assay

Apoptotic or necrotic cell death was determined by flow cytometry analysis of cells double-stained with annexin-V-FITC and propidium iodide (PI). After drug treatment, cells were collected and re-suspended in annexin-V binding buffer, then stained with annexin-V-FITC for 15 min at room temperature, and subsequently stained with PI. The samples were then analyzed using a FACS Calibur flow cytometer.

2.4. Measurements of mitochondria membrane potential and cellular ROS

Rhodamine-123 is a cell-permeable, cationic, green-fluorescent dye that can be readily sequestered by active mitochondria in proportion to the mitochondrial membrane potential. This compound has been commonly used for monitoring membrane potential and apoptosis. Rhodamine 123 (1 μM) was added into cell culture and incubated for 30 min before sample collection. After washing with PBS, the samples were analyzed by flow cytometry as described previously [5]. Cellular superoxide level was measured by hydroethidine by flow cytometry [5].

2.5. Measurement of mitochondria respiration activity

To determine cellular oxygen consumption, 5–10 million cells were suspended in 1 ml culture medium, which had been pre-saturated with oxygen (21%) by exposure to air in a 37 °C water bath. The cell suspension was sealed in a respiration chamber, and the oxygen concentration in the medium was monitored by a Clark-type oxygen electrode disc (Oxytherm, Hansatech Instrument, Cambridge, UK). The oxygen consumption rates were plotted on a graph and calculated as described previously [5].

2.6. Western blot analysis

Whole cells or mitochondrial fractions were lysed in 1 × SDS sample buffer and resolved by electrophoresis using SDS-PAGE and transferred to nitrocellulose membranes. The membranes were probed with primary antibodies overnight, and then incubated with appropriate horseradish peroxide-conjugated secondary antibodies for 3 h followed by detection with a SuperSignal Enhanced Chemiluminescence kit (Pierce, Rockford, IL). For sequential blotting, the membranes were stripped with Stripping Buffer (Pierce) and reprobed with proper antibodies.

2.7. Co-immunoprecipitation

Co-immunoprecipitation experiments were performed using the Immunoprecipitation Starter Pack according to the manufacture's protocol (GE healthcare life sciences). 1×10^7 cells were lysed in NP40 lysis buffer supplemented with PI cocktail, and co-immunoprecipitation was carried out using hexokinase II antibody. Primary antibody was incubated for 3 h at 4 °C. Protein-A beads were added and incubated for 1 h before the samples were collected and the washed. Proteins precipitated by protein-A beads were boiled and eluted in 1 × SDS-PAGE sample buffer for 5 min. Samples were analyzed by Western blotting using anti-hexokinase II and AIF antibodies.

2.8. Isolation of mitochondria and protein fractionation

Mitochondria of human leukemia cell line HL60 was isolated by conventional differential centrifugation as described previously [6]. Briefly, cells were harvested, washed once with cold PBS, and re-suspended in a buffer containing 10 mM Tris–HCl, pH 7.5, 10 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 70 mM sucrose, 210 mM mannitol, and protease inhibitors. After incubating in an ice-bath for 10 min, the cell suspension was homogenized with 15 strokes in a 2 ml glass homogenizer. The samples were centrifuged twice at 1500 × *g* at 4 °C for 5 min to remove nuclei and cell debris. The supernatants were centrifuged at 15,000 × *g* for 15 min to separate the mitochondrial fraction and cytosolic fraction. The mitochondria-enriched fractions were suspended in 50 μl KCl based respiratory buffer (150 mM KCl, 25 mM $NaHCO_3$, 1 mM $MgCl_2$, 3 mM KH_2PO_4, 20 mM HEPES, pH 7.4) [7]. After treatment with compounds such as 3-BrPA, CTZ and Glucose-6-P for 30 min at room temperature, the mitochondria fractions were then pelleted at 16,000 × *g* for 10 min. The pellet and resulting supernatant were separated on 10% SDS polyacrylamide gel and immunoblotted for Hexokinase II, AIF, cytochrome *c* and HSP 60 using appropriate antibodies.

3. Results and discussion

3.1. Upregulation of HK II in cancer cells with mitochondrial respiratory defect

To access the role of HKII in promoting the survival of cancer cells with mitochondrial respiratory defects, we first compared the expression levels of HKII protein in cells with competent mitochondrial function and cells deficient in respiration (ρ^0 cells) derived from the same tissue origins. Due to the inability to respire, the ρ^0 cells solely depend on the glycolytic pathway to generate ATP. As shown in Fig. 1, the ρ^0 cells derived from HCT116 ($p53^{+/+}$), HL-60, and Raji cells

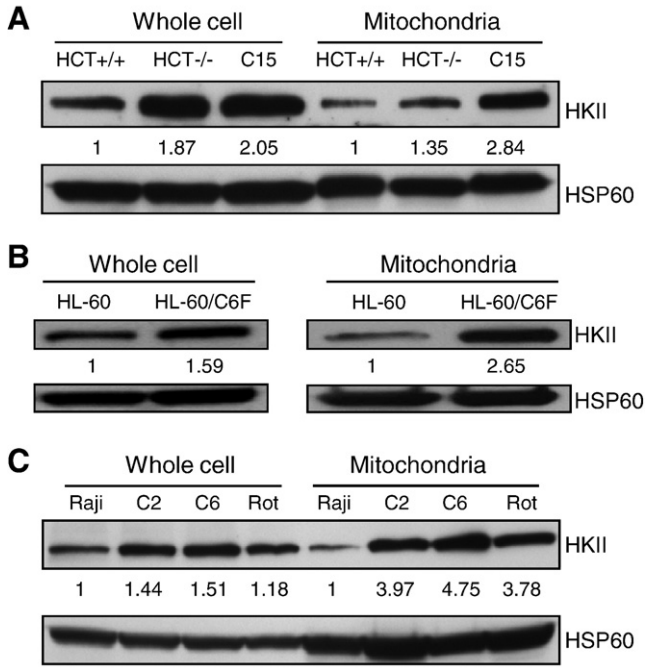

Fig. 1. Overexpression of HKII in ρ^0 cells and its localization to the mitochondria. (A) Comparison of HKII protein expression in HCT116 ($p53^{+/+}$) cells, their ρ^0 clone ($HCT116^{+/+}$ C15) and HCT116 ($p53^{-/-}$) cells. Approximately 10^6 cells were collected and lysed in 1 × SDS-PAGE sample buffer. Mitochondria from 5×10^6 cells of each cell line were isolated by differential centrifugation and lysed in 1 × SDS-PAGE sample buffer. Lysates were immunoblotted for HKII and HSP60. (B) Lysates of whole cell and mitochondria fractions of HL-60 cells and their ρ^0 derivatives HL-60/C6F cells were prepared, and immunoblotted for HKII and HSP60. (C) Lysates of whole cell and mitochondria fractions of Raji cell, their ρ^0 derivatives Raji C2, C6, and Raji cells treated with 1 nM rotenone (Rot) for 12 h were prepared as above, and then immunoblotted for HKII and HSP60. The level of hexokinase II was normalized to HSP60 using densitometry analysis.

exhibited higher expression levels of HKII protein compared to their respective parental cells. Interestingly, substantially more HKII proteins were located in the mitochondrial fraction of the ρ^0 cells (Fig. 1A, B). Chemical inhibition of mitochondrial respiration by the electron transport chain inhibitor rotenone also caused the translocation of HKII to the mitochondria (Fig. 1C). Hypoxia, which suppresses mitochondrial respiration, also has a similar effect on HKII (data not shown).

Emerging evidence suggests that the upregulation of HKII and its mitochondrial localization may not only make it easier for the cells to use ATP generated from mitochondria for phosphorylation of glucose, but may also have anti-apoptotic effects due to its interaction with the mitochondria. However, the detailed mechanisms still remain to be investigated. One possibility could be that HKII interacts with the Bcl-2 family members, for example through competing with Bax to bind to VDAC, to stabilize the MPTP (Mitochondrial permeability transition pore) and mitochondrial homeostasis [14]. Furthermore, because the product of hexokinase, glucose-6-phosphate is a common metabolic intermediate for both the glycolysis and the pentose phosphate pathways, inhibition of the hexokinase enzyme activity can have profound effect on cellular metabolism including mitochondria oxidative phosphorylation. Due to the important roles of HKII in both cancer cell metabolism and apoptosis, it is reasonable to speculate that this molecule may serve as a potential target for chemotherapy.

3.2. 3-Bromopyruvate covalently modifies HKII and causes its dissociation from mitochondria

Several chemicals including 2-deoxyglucose (2-DG), 3-bromopyruvate (3-BrPA), 5-thioglucose, clotrimazole, lonidamine, mannoheptulose and glufosfamide, have been shown to affect the activity of HKII [3]. 3-BrPA (3-bromopyruvatic acid, Br-CH_2-CO-COO-) is an analogue of pyruvate and an alkylating reagent that can react with the free SH groups of cysteine residues in certain proteins. This compound has been used for the treatment of the protozoan parasite *Trypanosoma brucei*, which generates ATP exclusively through glycolysis. According to early studies, the target of 3-BrPA is the trypanosomal glyceraldehyde-3-phosphate dehydrogenase [15]. Recent studies demonstrated that 3-BrPA is a potent inhibitor of hexokinase enzyme, and can inhibit both glycolysis and mitochondrial oxidative respiration in VX2 tumor model [4]. Importantly, 3-BrPA exhibited promising in vivo anticancer activity in the rabbit model of advanced hepatocellular carcinoma (AS-30D) without significant toxic side effect on normal liver tissue [16].

Previous studies showed that 3-BrPA can cause severe depletion of cellular ATP pool and induce cell death in various cancer cell lines [17]. Incubation of HL-60 cells with 100 μM 3-BrPA for 12 h caused up to 80% reduction of cellular ATP and significant cell death, which was associated with cytochrome *c* release and activation of caspase 3. Additionally, 3-BrPA was found to be more effective against cells under hypoxic conditions and ρ^0 cells, which are less sensitive to traditional chemotherapeutic agents such as doxorubicin, ara-C, and Taxol [17]. However, the detailed molecular mechanism by which 3-BrPA inhibits glycolysis and induces cell death still remains to be investigated.

Western blot analyses of protein extracts from cells treated with 3-BrPA revealed an interesting change in the electrophoresis mobility of HKII. As shown in Fig. 2, there was a time-dependent up-shifting of the HKII band after cells were incubated with 3-BrPA. This mobility alteration was not due to electrophoresis artifacts, since other proteins such the HSP60 and actin on the same gel did not exhibit a similar up-shift. Because 3-BrPA is an alkylating reagent capable of reacting with the -SH groups of the cysteine residues, it is likely that this up-shift reflects a covalent modification on cysteine residues in HKII protein.

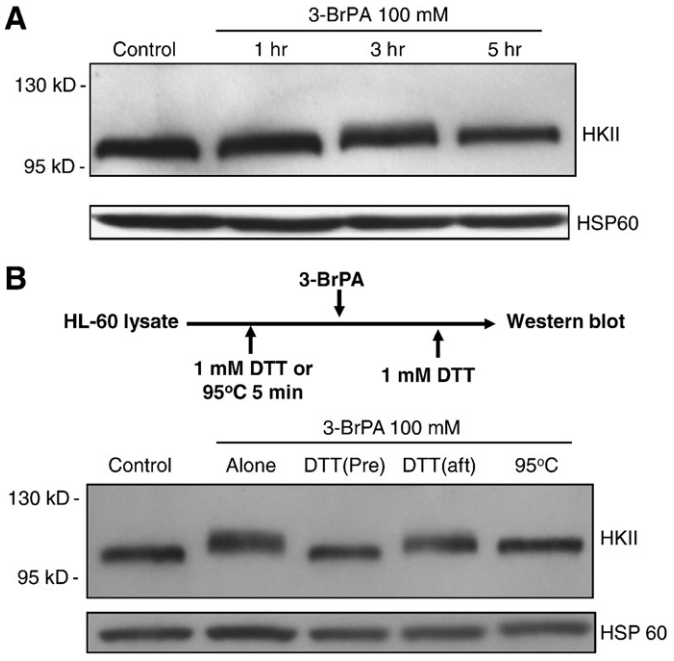

Fig. 2. Covalent modification of HKII protein induced by 3-BrPA. (A) HL-60 cells were treated with 100 μM 3-BrPA for 1, 3 and 5 h. Cells were collected and lysates were immunoblotted for HKII and HSP60. (B) HL-60 cell lysates were incubated with 100 μM 3-BrPA at room temperature for 10 min, two of the samples were mixed with 1 mM DTT 5 min before (Pre) or after (aft) addition of 3-BrPA. Another sample was boiled for 5 min at 95 °C to denature the proteins before treatment with 3-BrPA. Lysates were immunoblotted for HKII and HsP60.

To further test this possibility, another in vitro assay was performed using DTT to interfere with the reaction between 3-BrPA and -SH of cysteine. Cell lysates of HL-60 cells were incubated with 100 μM 3-BrPA at room temperature with 1 mM DTT being added before or after the addition of 3-BrPA. Another sample was heated at 95 °C for 5 min to denature the proteins before incubation with 3-BrPA. As shown in Fig. 2B, the sample treated 3-BrPA alone showed a significant up-shift compared with control sample, and pre-incubation with DTT or pre-denature by heating prevented 3-BrPA-induced band shift. This data suggests that 3-BrPA may indeed target the sulfhydryl group of cysteine residues in HKII. Because the estimation of molecular weight by electrophoresis in SDS-PAGE gel could not accurately determine the increments of band small shifting caused by different numbers of 3-BrPA molecules, the exact number of 3-BrPA molecules bound to each HKII protein is still unclear. The use of more accurate methods such as mass spectrometry analysis should provide useful information to calculate the number of 3-BrPA molecules bound to HKII. This chemical modification is likely redox sensitive and may depend on certain conformation of HKII. It would be interesting to further determine which specific cysteine residues in HKII are targets of 3-BrPA, and how this modification affects hexokinase II enzyme activity and functions. Experimental strategies based on site-specific mutation, immunoprecipitation, and mass spectrometry may help to answer this important question.

Previous studies showed that 3-BrPA can induce cell death through the mitochondria-mediated intrinsic apoptotic pathway, which is coupled with the release of cytochrome *c* from mitochondria to cytosol leading to activation of the caspase cascade. Also it is known that the mitochondria-associated HKII is involved in the regulation of apoptosis. However, the exact role of HKII in 3-BrPA-induced cell death remains unclear. To investigate the effect of 3-BrPA on HKII interaction with the mitochondria in whole cells, mitochondria-enriched fractions from 3-BrPA-treated cancer cells were isolated by differential centrifugation, and the release of mitochondrial proteins was analyzed by western blot analysis. As shown in Fig. 3A, mitochondria isolated from HL-60 cells treated with 100 μM 3-BrPA exhibited a significant loss of HK II with concurrent loss of Apoptosis inducing factor (AIF). Interestingly, there was no significant loss of cytochrome *c* from the mitochondria under the conditions (100 μM 3-BrPA, 3–6 h). Similar results were also observed in HCT116 and Raji cells (data not shown).

Although the release of HKII from the mitochondria could simply be a subsequent event of apoptosis induced by 3-BrPA, the rapid release of HKII and its up-shift suggests that 3-BrPA may have a direct effect on he mitochondrial-associated HKII. To test this possibility, an in vitro assay using isolated mitochondria from HL60 cells was performed. Mitochondria isolated from HL-60 cells were resuspended in a buffer containing KCl, and incubated with clotrimazole (CTZ), glucose-6-phosphate, or 3-BrPA to test the direct effect of these compounds. The rationale was that proteins released from the isolated mitochondria would be in the supernatant and could be separated from the mitochondrial-associated proteins by centrifugation. CTZ is known to dissociate HKII from mitochondria through targeting the interaction of HKII and VDAC, whereas glucose-6-phosphate is a known inhibitor of HK [18,19]. These two compounds were used as controls for comparison with 3-BrPA. The supernatant and mitochondria pellets were separated through centrifugation and immunoblotted for HKII and HSP60. As shown in Fig. 3B, 3-BrPA not only caused the up-shift of HKII, but also led to the release of HKII from the mitochondria. It should be noted that the amount of HKII released from the mitochondria seemed only moderate, probably due to the short-time (30 min) incubation of 3-BrPA with the isolated mitochondria. When whole cells were incubated with 3-BrPA for a longer time (6 h), there was a substantial amount of HKII release from the mitochondria (Fig. 3A, lane 3). Similar experiments were performed to test the release of AIF and cytochrome *c*. As shown in Fig. 3B, C,

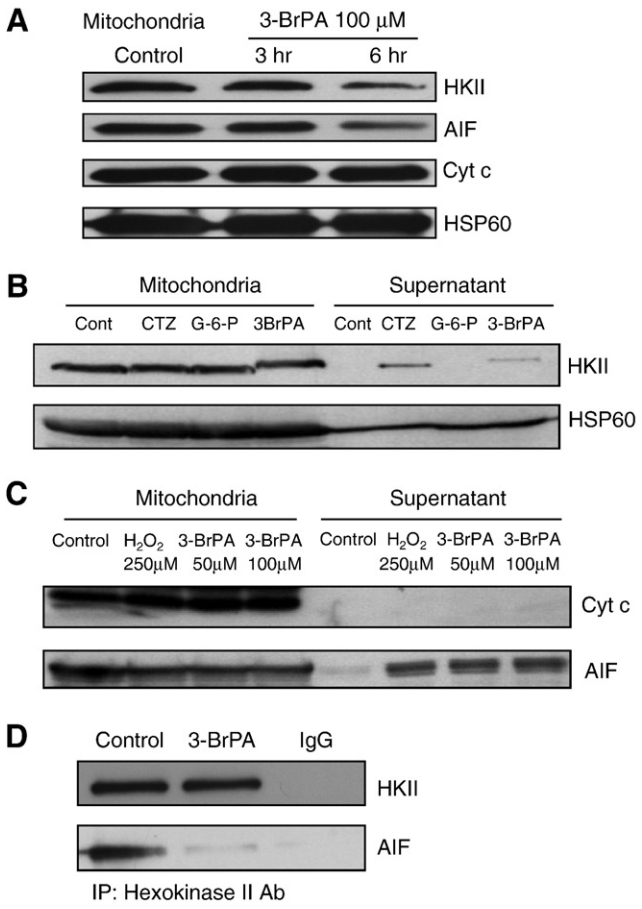

Fig. 3. Release of HKII and AIF from mitochondria induced by 3-BrPA. (A) 10^7 HL-60 cells were treated with 100 μM 3-BrPA for 3–6 h as indicated, mitochondria were isolated by differential centrifugation and lysates were immunoblotted for AIF, cytochrome *c* (Cyt c) and HSP60. (B) Mitochondria fractions were isolated from HL-60 cells and resuspended in 50 μl KCl-based respiratory buffer. Mitochondria were treated separately with 20 μM CTZ, 1 mM glucose-6-phosphate, 200 μM 3-BrPA for 30 min at room temperature. The supernatants and mitochondria pellets were separated by centrifugation at 16,000 ×*g* for 10 min. Lysates were immunoblotted for HKII and HSP60. (C) The mitochondrial fractions of HL-60 cells were incubated with H_2O_2 and 3-BrPA for 30 min at room temperature. The supernatants and mitochondria pellets were separated by centrifugation. Lysates were immunoblotted for cytochrome *c* and AIF. (D) HCT116 ($p53^{+/+}$) cells were treated with 100 μM 3-BrPA for 5 h, and cells were then lysed in NP-40 lysis buffer containing protease inhibitors cocktail. Co-immunoprecipitation was carried out using HKII antibody. The IP products were immunoblotted for HKII and AIF.

addition of 50 μM 3-BrPA caused the release of HKII and AIF from mitochondria, but the release of cytochrome *c* was again undetectable, consistent with the observation in Fig. 3A.

The preferential release of AIF, which is larger than cytochrome *c* in molecular weight, suggests that AIF may be physically associated with HKII, which can be disrupted by 3-BrPA. This possibility was tested by co-immunoprecipitation experiments. HCT116 colon cancer cells treated with 3-BrPA were lysed in NP-40 lysis buffer and co-immunoprecipitation was carried out using anti-HK antibody. The IP pulled-down samples were immunoblotted for both hexokinase II and AIF. As shown in Fig. 3D, there was a detectable physical interaction between HKII and AIF, and treatment of cells with 100 μM 3-BrPA significantly disrupted such interaction.

AIF is considered as a killer protein, which plays a major role in caspase-independent mitochondria-mediated cell death. Once released from the mitochondria, AIF may translocate to the nucleus and induce DNA condensation and fragmentation. AIF is also known as a redox molecule with enzyme similar to NADH oxidase, which generates ROS through oxidization of NADH. It has been reported to be

essential for maintaining mitochondria complex I activity, and *AIF*$^{+/-}$ cells seemed to have a lower level of ROS and were more sensitive to oxidant-induced apoptosis [20]. However, the biological function of AIF in the mitochondria and its regulation remain unclear. The co-immunoprecipitation experiments indicate that hexokinase II may form a complex with AIF and their interaction can be disrupted by 3-BrPA treatment. It would be interesting to test if HKII can regulate mitochondria function or redox level through its interaction with AIF.

3.3. Dissociation of HKII from the mitochondria triggers cancer cell death

The finding that 3-BrPA treatment caused a release of HKII and AIF from the mitochondria led us to hypothesize that the dissociation of HKII from mitochondria may play an important role in 3-BrPA-induced cell death. To test this possibility, a cell permeable N-terminal peptide of hexokinase II (N-HK II peptide) was synthesized based on the sequence of the N-terminal 15 amino acids of HKII, which is essential for association with mitochondria [18]. Once N-HKII is inside the cells, the peptide can cause dissociation of HKII from mitochondria through direct competition [18].

Fig. 4A illustrates the experimental procedures for testing the ability of N-HK II peptide to cause HKII dissociation from mitochondria and trigger cell death. In order to avoid the interference from FBS on peptide uptake, RPMI medium without FBS was use during the 1-h incubation with N-HK II peptide. After treatment with 10–30 μM N-HK II peptide, mitochondria were isolated from HL-60 cells and immunoblotted for HKII, AIF, and cytochrome *c* by western blotting. The data indicated that the N-HKII peptide treatment induced the release of HKII and AIF (Fig. 4B), similar to the action of 3-BrPA. The cytotoxicity of N-HK II peptide in HL-60 cells was analyzed by annexin-V/PI staining followed by flow cytometry analysis. A scrambled peptide was used as control. As shown in Fig. 4C, although the control scrambled peptide showed some cytotoxicity, the N-HK II peptide induced substantially more cytotoxic effect compared to the scrambled peptide at each concentration tested. This suggests that the dissociation of HKII from the mitochondria might play a role in promoting 3-BrPA-induced cell death. Interestingly, a 3-h incubation with 10 μM N-HK II peptide caused a substantial loss of HK II from the mitochondria without much release of AIF at this early time point, whereas a higher concentration of N-HK II peptide (30 μM) caused a substantial release of both HK II and AIF from the mitochondria (Fig. 4B). This data together with the dose-dependent cytotoxic action of N-HK II peptide in HL-60 cells (Fig. 4C) suggests that the release of AIF subsequent to HK II dissociation from the mitochondria may be an important event in the cell death process.

Previous studies showed that ρ^0 cells have higher levels of glycolysis and are more sensitive to glycolysis inhibitors [17]. Data shown in Fig. 1 also suggest that HKII were overexpressed in ρ^0 cells and largely localized to the mitochondria. This may in part explain the increase in glycolysis and resistance to chemotherapy in ρ^0 cells. Since ρ^0 cells have more HKII associated with mitochondria, we then tested whether ρ^0 cells are more sensitive to N-HK II peptide treatment. A shown in Fig. 4D, the HL-60 ρ^0 cells (C6F) with higher level of hexokinase II were likely to be more sensitive to N-HKII peptide treatment compared to the parental HL-60 cells.

3.4. Effect of 3-BrPA and N-HK II peptide on mitochondrial transmembrane potential

The loss of mitochondrial transmembrane potential is considered as a marker of mitochondria permeability transition and an early event of mitochondria-initiated cell death. Rhodamine-123 is a cell-permeable, cationic, green-fluorescent dye that can be readily sequestered by active mitochondria, and has been commonly used to monitor mitochondrial transmembrane potential and apoptosis. Since HKII can be associated with VDAC, a major component of the

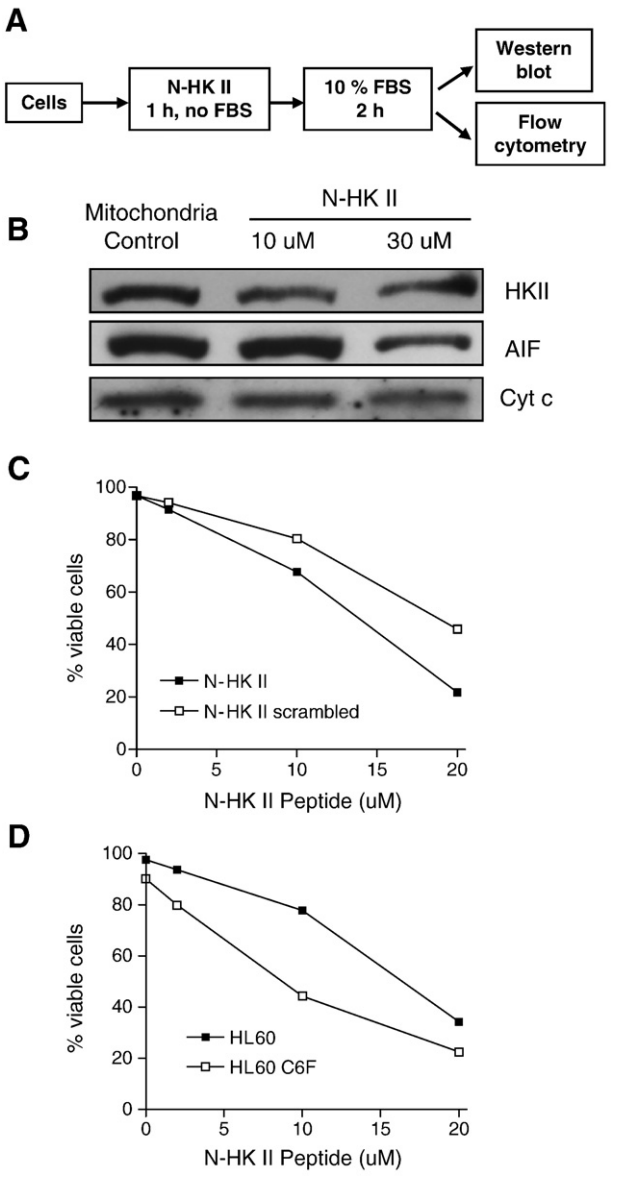

Fig. 4. Release of HKII and AIF from mitochondria and caused cell death induced by N-HK II peptide. (A) Experimental scheme. (B) HL-60 cells were treated with 10–30 μM N-HK II peptide. Culture medium was replaced with RPMI medium without serum for treatment with 10 or 30 μM N-HK II peptide for 1 h. Then the serum-free medium was replaced with RPMI medium with 10% FBS and incubated for additional 2 h. Mitochondria fractions were isolated and immunoblotted for HKII, AIF, and cytochrome *c*. (C) HL-60 cells were treated with 2, 10, and 20 μM N-HK II peptide and scrambled control peptide as described above. Cytotoxicity was analyzed by annexin-V/PI staining and flow cytometry analysis. (D) HL-60 cells and their ρ^0 clone HL-60/C6F were treated with 2, 10, and 20 μM N-HK II peptide as described above. Cells were collected and analyzed by annexin-V/PI staining to determine cytotoxicity.

MPTP, and 3-BrPA is able to cause cellular ATP depletion and the dissociation of HKII from the mitochondria, it is reasonable to speculate that 3-BrPA may affect mitochondrial transmembrane potential through its ability to cause dissociation of HKII from the organelle. To test if 3-BrPA can cause mitochondria permeability transition in living cells, HL-60 cells and its ρ^0 derivate HL-60/C6F cells were treated with 3-BrPA for various times and rhodamine-123 was used to monitor the change of membrane potential. 3-BrPA can cause a loss of membrane potential in a time-dependent manner in both HL-60 and their ρ^0 derivatives, with the ρ^0 cell being more sensitive to 3-BrPA (Fig. 5A, B). Cyclosporin A (CsA) is known to suppress the mitochondrial permeability transition through inter-

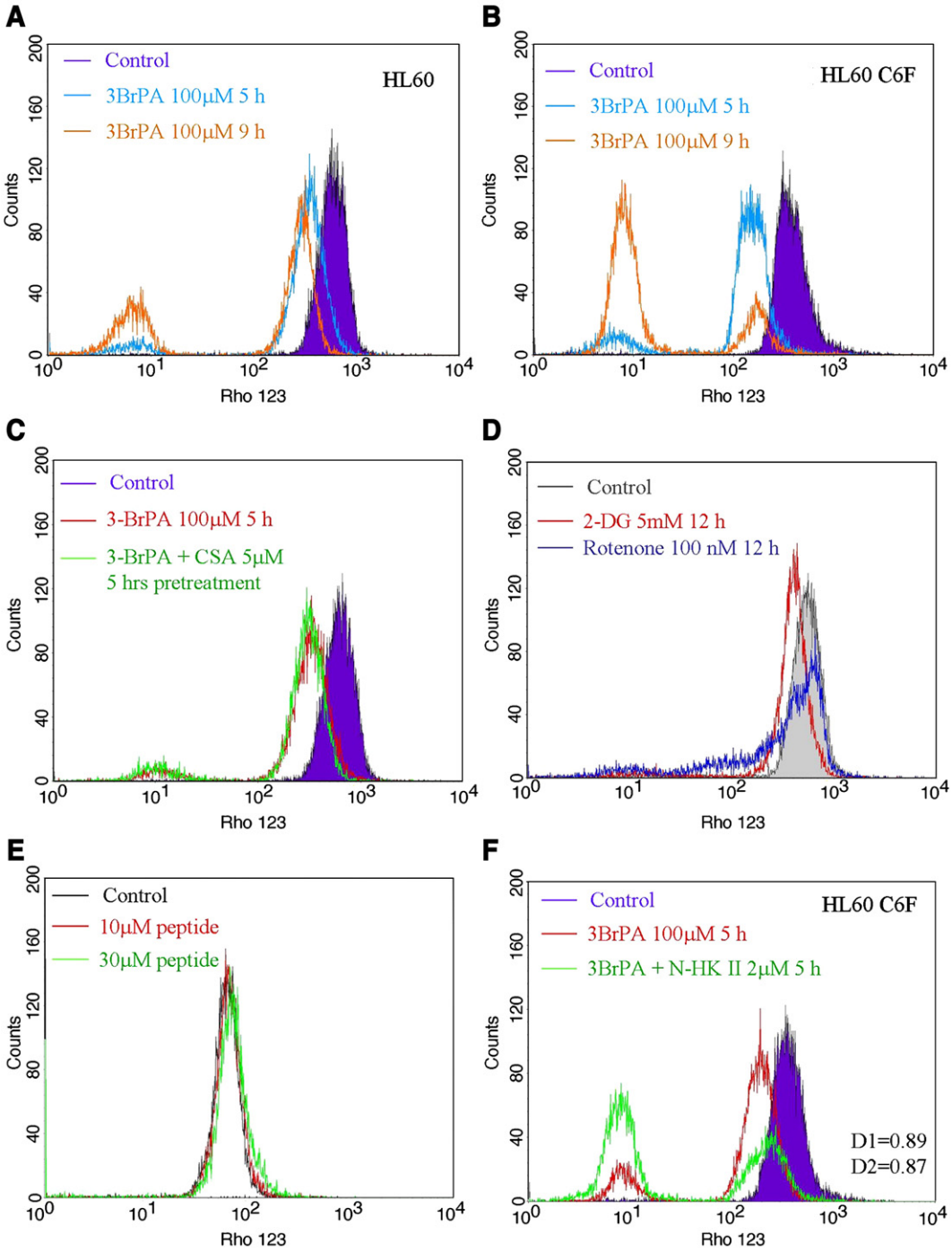

Fig. 5. Effect of 3-BrPA and N-HK II peptide on mitochondrial transmembrane potential. (A) HL-60 cells were treated with 100 μM 3-BrPA for 5–9 h, the mitochondrial membrane potential were analyzed by flow cytometry using rhodamine-123 as fluorescent dye (1 μM for 1 h). (B) HL-60 ρ^0 (C6F) cells were treated with 100 μM 3-BrPA for 5–9 h, the mitochondrial membrane potential were analyzed by flow cytometry using rhodamine-123 as fluorescent dye. (C) HL-60 cells were treated with 100 μM 3-BrPA for 5 h with or without pretreatment of 5 μM CsA (5 h). Mitochondrial transmembrane potential was analyzed by using rhodamine-123. (D) HL-60 cells were treated with 5 mM 2-DG or 100 nM rotenone for 12 h. Mitochondrial transmembrane potential was measured using rodamine-123. (E) HL-60 cells were incubated with 10 and 30 μM N-HK II peptides as described in Fig. 4. Mitochondrial membrane potential was measured using rodamine-123. (F) HL-60/C6F cells were treated with 100 μM 3-BrPA alone or in combination with 2 μM N-HK II peptide for 5 h. Rhodamine-123 was used to monitor the loss of mitochondrial transmembrane potential.

ference with the conformation change of cyclophilin D. To test whether the permeability transition caused by 3-BrPA can be prevented by CsA, HL-60 cells were pretreated with 5 μM CSA for 5 h, and then treated with 100 μM 3-BrPA. As shown in Fig. 5C, 3-BrPA caused a significant loss of membrane potential and CsA showed no protective effect. This indicates that 3-BrPA-induced mitochondria permeability transition may not involve cyclophilin D. The effects of mitochondrial respiration inhibitor rotenone and glycolysis inhibitor 2-DG were also tested (Fig. 5D). Both inhibitors also caused the loss of mitochondrial membrane potential, and the 2-DG inhibitory effect was similar to 3-BrPA.

Whether the dissociation of hexokinase II from the mitochondria leads to the loss of membrane potential still remained unclear. In order to address this question, the effect of N-HKII peptide on mitochondrial membrane potential was tested. HL-60 cells were treated with 10 and 30 μM peptides for 3 h using the same procedures

used above. Interestingly, although N-HK II peptide caused dissociation of hexokinase II from mitochondria and induce cell death, this peptide alone exhibited no effect on membrane potential (Fig. 5E). This was consistent with the observations by other research group [21]. Interestingly, when cells were treated with N-HK II peptide in combination with 3-BrPA, the depolarization was significantly enhanced. As shown in Fig. 5F, when HL-60/C6F cells were treated with 100 μM 3-BrPA in combination with 2 μM N-HKII peptide for 5 h, massive collapse of membrane potential was observed. These data suggested that N-HK II peptide caused dissociation of hexokinase from mitochondria will make the cells more vulnerable to the loss of membrane potential caused by 3-BrPA, but the HKII dissociation itself seems not affect mitochondria transmembrane potential. It is known that the association of HKII with mitochondrial is significantly affected by its phosphorylation by Akt [21]. However, it is unclear at the present time if 3-BrPA may cause a change in HKII phosphorylation status. When protein extracts were incubated with phosphatase in vitro, the up-shifted HKII band caused by 3-BrPA could not be shifted down by the phosphatase digestion (data not shown), suggesting that the band up-shifting was likely due to chemical modification by 3-BrPA, not by phosphorylation. Since the phosphatase incubation did not cause a down-shift of HKII band, it is possible that the HKII molecule from the 3-BrPA-treated cells was a in a dephosphorylated form (could not be further dephosphorylated by phasphatase), and thus was readily dissociated from mitochondria.

To further test if the dissociation of HKII from mitochondria may inhibit respiration, the effect of N-HK II peptide on mitochondrial oxidative phosphorylation and ROS generation was evaluated. Unlike 3-BrPA, N-HK II peptide alone had no effect on mitochondria respiratory activity, although it can induce significant cell death (Fig. 6). HL-60 cells were incubated with 3–30 μM peptides for 3 h, then oxygen consumption activity was measured using a Clark-type oxygen electrode. Even high concentration (30 μM) of N-HK II peptide did not show a significant inhibition on mitochondria respiration activity. It should be noted that apoptosis already occurred even at 10 μM N-HK II peptide, as measured by annexin-V/PI staining (Fig. 4).

The effect of N-HK II peptide on mitochondria ROS generation was also tested. Superoxide level was measured using hydroethidium (HEt) followed by flow cytometry analysis. HL-60 cells were incubated with various concentrations of peptide for 3 h, and superoxide level was not affected even at toxic concentrations of the peptide (Fig. 6B). Rotenone is a known inhibitor of mitochondrial respiratory chain complex I and, as expected, promoted ROS generation (Fig. 6C). Combination of rotenone with N-HK II peptide did not further enhance superoxide generation caused by rotenone. These data suggested that N-HK II peptide can cause dissociation of HKII from mitochondria and lead to induction of apoptosis, but this dissociation has no effect on mitochondria respiratory activity and ROS generation.

Although the exact molecular mechanisms of Warburg effect still remain to be elucidated, experimental evidences suggest that upregulation of glycolysis in cancer cells not only ensures the generation of ATP and other metabolic intermediate to meet the need of cancer cells, it also renders resistance to apoptosis and survival advantage compared to normal cells. These profound biochemical alterations in metabolic profile provide exciting opportunities for the development of therapeutic strategies to preferentially kill cancer cells.

As a potent inhibitor of hexokinase, 3-BrPA caused a mobility up-shift of HKII protein band, which indicates a covalent modification of HKII. Our data also indicates that 3-BrPA may target the sulfhydryl group of cysteine residues and this chemical modification is dependent on certain conformation of HKII. This covalent modification leads to the inhibition of hexokinase enzymatic activity and also triggers its dissociation from mitochondria, which also contributes to cell death. Experiment with isolated mitochondria or intact cells showed that the dissociation of HKII from the mitochondria induced by 3-BrPA was coupled with a preferential release of AIF instead of cytochrome *c*. The dissociation of HKII alone seems sufficient to trigger apoptosis. This is supported by the fact that N-HK II peptide induced HKII dissociation and cell death, but did not cause alteration in transmembrane potential and respiration.

Co-immunoprecipitation experiments suggest a possible existence of a cross-membrane protein complex between HKII and AIF, which can be disrupted by 3-BrPA. These findings provide new clues to understand the function of both HKII and AIF inside the mitochondria and how they may affect each other. Although incubation of cells with N-HK II peptide can directly cause cell death, the peptide did not show other effects of 3-BrPA such as inhibition of mitochondria respiration and decrease of mitochondria membrane potential. It seems that the enzymatic activity of HKII may be important for energy metabolism, while its mitochondria association may be important in regulation of apoptosis. Targeting HKII may be an effective mean to kill cancer cells and have potential clinical implications.

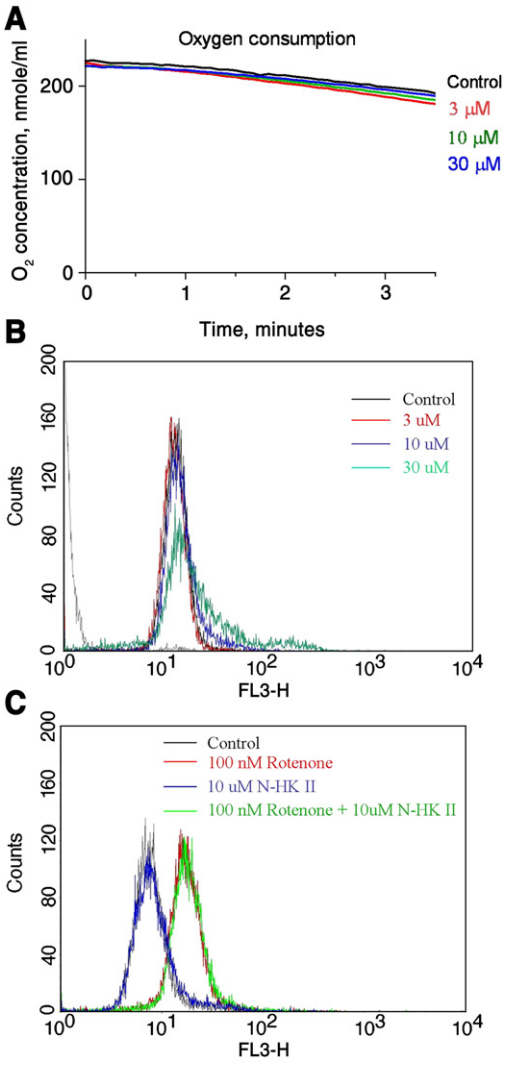

Fig. 6. Effect of N-HK II peptide and rotenone on mitochondrial respiration and ROS generation. (A) HL-60 cells were pre-treated with 3, 10 and 30 μM N-HK II peptide for a total of 3 h as described in Fig. 4A. Oxygen consumption of 10^7 cells was measured at 37 °C using a Clark-type oxygen electrode. (B) HL-60 cells were treated with 3, 10 and 30 μM N-HK II peptide and superoxide levels were measured by flow cytometry analysis using hydroethidine as a fluorescent dye. (C) HL-60 cells were pre-treated with 100 nM rotenone for 9 h, and then incubated with 10 μM N-HK II peptides for additional 3 h. Superoxide levels were measured by using hydroethidine.

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References

[1] O. Warburg, On the origin of cancer cells, Science 123 (1956) 309–314.
[2] O. Warburg, On respiratory impairment in cancer cells, Science 124 (1956) 269–270.
[3] Z. Chen, W. Lu, C. Garcia-Prieto, P. Huang, The Warburg effect and its cancer therapeutic implications, J. Bioenerg. Biomembr. 39 (2007) 267–274.
[4] Y.H. Ko, P.L. Pedersen, J.F. Geschwind, Glucose catabolism in the rabbit VX2 tumor model for liver cancer: characterization and targeting hexokinase, Cancer Lett. 173 (2001) 83–91.
[5] H. Pelicano, L. Feng, Y. Zhou, J.S. Carew, E.O. Hileman, W. Plunkett, M.J. Keating, P. Huang, 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. 278 (2003) 37832–37839.
[6] G. Achanta, R. Sasaki, L. Feng, J.S. Carew, W. Lu, H. Pelicano, M.J. Keating, P. Huang, Novel role of p53 in maintaining mitochondrial genetic stability through interaction with DNA Pol gamma, EMBO J. 24 (2005) 3482–3492.
[7] J.G. Pastorino, N. Shulga, J.B. Hoek, Mitochondrial binding of hexokinase II inhibits Bax-induced cytochrome *c* release and apoptosis, J. Biol. Chem. 277 (2002) 7610–7618.
[8] S. Sebastian, J.A. White, J.E. Wilson, Characterization of the rat type III hexokinase gene promoter. A functional octamer 1 motif is critical for basal promoter activity, J. Biol. Chem. 274 (1999) 31700–31706.
[9] J.E. Wilson, Isozymes of mammalian hexokinase: structure, subcellular localization and metabolic function, J. Exp. Biol. 206 (2003) 2049–2057.
[10] P.G. Polakis, J.E. Wilson, An intact hydrophobic N-terminal sequence is critical for binding of rat brain hexokinase to mitochondria, Arch. Biochem. Biophys. 236 (1985) 328–337.
[11] R.A. Nakashima, P.S. Mangan, M. Colombini, P.L. Pedersen, Hexokinase receptor complex in hepatoma mitochondria: evidence from N,N′-dicyclohexylcarbodiimide-labeling studies for the involvement of the pore-forming protein VDAC, Biochemistry 25 (1986) 1015–1021.
[12] W.Y. Li, R.L. Chieng, Studies on the physiological functions of the mitochondrial bound hexokinase of ascites tumor cells. Shi Yan Sheng Wu Xue Bao 10 (1965) 112–120.
[13] K.K. Arora, P.L. Pedersen, Functional significance of mitochondrial bound hexokinase in tumor cell metabolism. Evidence for preferential phosphorylation of glucose by intramitochondrially generated ATP, J. Biol. Chem. 263 (1988) 17422–17428.
[14] J.W. Kim, C.V. Dang, Multifaceted roles of glycolytic enzymes, Trends Biochem. Sci. 30 (2005) 142–150.
[15] J.P. Barnard, B. Reynafarje, P.L. Pedersen, Glucose catabolism in African trypanosomes. Evidence that the terminal step is catalyzed by a pyruvate transporter capable of facilitating uptake of toxic analogs, J. Biol. Chem. 268 (1993) 3654–3661.
[16] Y.H. Ko, B.L. Smith, Y. Wang, M.G. Pomper, D.A. Rini, M.S. Torbenson, J. Hullihen, P.L. Pedersen, Advanced cancers: eradication in all cases using 3-bromopyruvate therapy to deplete ATP, Biochem. Biophys. Res. Commun. 324 (2004) 269–275.
[17] R.H. Xu, H. Pelicano, Y. Zhou, J.S. Carew, L. Feng, K.N. Bhalla, M.J. Keating, P. Huang, Inhibition of glycolysis in cancer cells: a novel strategy to overcome drug resistance associated with mitochondrial respiratory defect and hypoxia, Cancer Res. 65 (2005) 613–621.
[18] N. Majewski, V. Nogueira, P. Bhaskar, P.E. Coy, J.E. Skeen, K. Gottlob, N.S. Chandel, C.B. Thompson, R.B. Robey, N. Hay, Hexokinase-mitochondria interaction mediated by Akt is required to inhibit apoptosis in the presence or absence of Bax and Bak, Mol. Cell 16 (2004) 819–830.
[19] P.A. Craven, R.E. Basford, Properties of the glucose 6-phosphate-solubilized brain hexokinase. Evidence for a lipoportein complex, Biochim. Biophys. Acta 255 (1972) 620–630.
[20] A. Urbano, U. Lakshmanan, P.H. Choo, J.C. Kwan, P.Y. Ng, K. Guo, S. Dhakshinamoorthy, A. Porter, AIF suppresses chemical stress-induced apoptosis and maintains the transformed state of tumor cells, EMBO J. 24 (2005) 2815–2826.
[21] S. Miyamoto, A.N. Murphy, J.H. Brown, Akt mediates mitochondrial protection in cardiomyocytes through phosphorylation of mitochondrial hexokinase-II, Cell Death Differ. 15 (2008) 521–529.