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ORIGINAL ARTICLE

Bo Yang · Lingling Fan · Liang Fang
Qiaojun He

Hypoxia-mediated fenretinide (4-HPR) resistance in childhood acute lymphoblastic leukemia cells

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Abstract *Purposes*: *N*-(4-Hydroxyphenyl)-retinamide (4-HPR, Fenretinide) is a synthetic retinoid with cytotoxicity in acute lymphoblastic leukemia (ALL) cell lines. Since ALL is a disease of the bone marrow, a hypoxic tissue compartment, and it has been reported that there is an antagonistic effect of hypoxia on many chemotherapeutic agents, our purpose was to observe whether hypoxia is able to inhibit the effect of 4-HPR for ALL cell lines and to investigate its mechanisms of antagonism to 4-HPR. *Methods*: Cytotoxicity was measured by MTT method, and apoptosis was measured by flow cytometry. Mitochondrial membrane potential ($\Delta\Psi_m$) was detected by JC1 staining and flow cytometry. Protein expression was analyzed by western blotting. *Results*: Hypoxia (2% O_2) induced 4-HPR resistance in the tested two ALL cell lines (Molt-4 and Molt-3), with at least a 2.8-fold increase in IC_{50} values ($P < 0.01$) compared with the IC_{50} values in normoxia (20% O_2). Apoptotic detection showed that 2% O_2 significantly suppressed 4-HPR-induced apoptosis and the percentages of 4-HPR-induced apoptotic cells at 12 and 24 h were 1.2 and 11.0%, respectively, compared with 12.6 and 76.3% in 20% O_2. In addition, in 20% O_2, but not in 2% O_2, 4-HPR obviously downregulated the protein expression of procaspase-3, ERK1/2 and XIAP, and increased the cleavage of PARP. Also, a significant $\Delta\Psi_m$ loss in response to 4-HPR was observed in normoxia, but not in hypoxia. *Conclusions*: Hypoxia is able to induce 4-HPR resistance in Molt-4 cells and the mechanism may be involved in the inhibition of 4HPR-induced $\Delta\Psi_m$ depolarization and regulation of mitochondrial pathway-related proteins associated in signaling apoptosis.

Keywords Hypoxia · Drug resistance · ALL · 4-HPR

Abbreviations 4-HPR: *N*-(4-Hydroxyphenyl)-retinamide or fenretinide · ALL: Acute lymphoblastic leukemia · MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide · $\Delta\Psi_m$: Mitochondrial membrane potential · PI: Propidium iodide · JC-1: 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolcarbocyanine iodide · PBS: Phosphate buffered saline · IAP: Inhibitor of apoptosis · XIAP: x-linked inhibitor of apoptosis protein · ERK: Extracellular signal-regulated kinase · PARP: poly(ADP-ribose) polymerase

Introduction

N-(4-Hydroxyphenyl)-retinamide (4-HPR, Fenretinide) is a synthetic retinoid with cytotoxic activity in a wide variety of cancer cells both in vitro and in vivo [1], including acute lymphoblastic leukemia (ALL) cell lines [2]. The mechanisms of 4-HPR-mediated apoptosis have not been fully elucidated. Previous reports have indicated that 4-HPR could induce apoptosis through RAR-dependent and/or RAR-independent pathways [3, 4]. The activation of the c-Jun N terminal kinase, the activation of the mitochondrial pathway via generation of reactive oxygen species (ROS) and/or the induction of increased ceramide (a sphingoid-based lipid second messenger molecule) production have all been implicated in 4-HPR-medaited apoptosis [5, 6]. It was also reported that 4-HPR enhanced the effect of chemotherapy, and the mechanism is related to mitochondrial dysfunction, which plays an important role in 4-HPR-induced apoptosis [7, 8].

ALL is a malignant (clonal) disease of the bone marrow in which early lymphoid precursors proliferate

B. Yang · L. Fan · L. Fang · Q. He
Institute of Pharmacology and Toxicology,
School of Pharmaceutical Sciences, Zhejiang University,
310031, Hangzhou, Zhejiang Province, China

B. Yang (✉) · Q. He (✉)
Department of Pharmacology, College of Pharmaceutical sciences,
Zhejiang University, Yan'an Rd. 353#,
310031, Hangzhou, China
E-mail: yang924@zju.edu.cn
E-mail: qiaojunhe@zju.edu.cn
Tel.: +86-571-87217206
Fax: +86-571-87217021

and replace the normal hematopoietic cells of the marrow. A poor prognosis of ALL patients is related to the recurrence in the bone marrow [9, 10], a hypoxic tissue compartment. It has been documented that the hypoxic tissue may serve as a "sanctuary" site for tumor cells, resulting in resistance to many common chemotherapeutic agents due to hypoxia [11–13]. Recently, a decrease in the cytotoxicity of 4-HPR by hypoxia was reported in Ewing's sarcoma and primitive neuroectodermal tumor cell lines [14]. Therefore, we were interested in identifying whether hypoxia could exert an inhibitive effect on the sensitivity of ALL cells to 4-HPR.

It has been demonstrated that a change in the mitochondrial membrane potential ($\Delta\Psi_m$) plays a pivotal role in transducing a variety of proapoptotic stimuli [15–17]. Opening of the permeability transition pores in the mitochondria induces the release of cell-death promoting factors, including cytochrome *c* and apoptosis-inducing factor (AIF) [18, 19]. Cytochrome *c* has been demonstrated to be involved in the activation of a caspase cascade [20, 21], while AIF has been shown to directly trigger apoptosis [22]. It was reported that hypoxia modulated $\Delta\Psi_m$ [14] and the expression of several proteins, such as ERK1/2 and XIAP, are involved in $\Delta\Psi_m$ stabilization and caspases activation [23–25]. Thus, we hypothesized that hypoxia might inhibit 4-HPR activity via the mitochondrial pathway.

Together, there are two specific aims in the current study: (1) to determine the in vitro cytotoxicity of 4-HPR against ALL cell lines in hypoxia; and (2) to explore the mechanisms of hypoxia-mediated 4-HPR resistance in ALL cell lines.

Materials and methods

Chemicals

4-HPR was kindly endowed by Dr. BJ Maurer (Childrens Hospital Los Angeles), dissolved in ethanol to make a 10-mM stock solution and stored at −20°C. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Sigma (St. Louis, MO, USA). MTT was dissolved in RPMI-1640 to make a 5 mg/ml solution. The mitochondrial fluorescent probe 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolcarbocyanine iodide (JC-1) was purchased from Molecular Probes (Eugene, OR, USA). Antibodies to procaspase-3, PARP, ERK1/2, XIAP, α-tublin and HRP-labeled secondary anti-mouse and anti-rabbit antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). ECL, Western Blotting detection reagents, was purchased from Amersham Biosciences (Piscataway, NJ, USA).

Cell culture

The human ALL cell lines (Molt-4 and Molt-3) were obtained from ATCC, and were maintained in RPMI-1640 medium (Gibco BRL, Grand Island, NY) with 10% heat-inactivated fetal bovine serum (FBS) (Gibco BRL, Grand Island, NY, USA) at 37°C in 5% CO_2 and 20% O_2 (normoxia). For the experiments, 2% O_2 was used as hypoxia, which is below the degree of hypoxia found in bone marrow [26].

Cytotoxicity assay

All cytotoxicity assays were performed in 96-well plates with MTT method. Each 100 μl suspended cells of Molt-4 and Molt-3 was plated at 5,000 cells per well. The cells were cultured in hypoxia (2% O_2) and were allowed to attach 1 day prior to the addition of 4-HPR (0–10 μM) in complete medium (to various final concentrations in 150 μl of complete medium) in replicates of 6 wells per condition. The plates were assayed at 72 h after initiation of drug exposure.

To measure cytotoxicity, MTT was added to the 96-well plate (final concentration = 0.25 mg/ml) and incubated for 4 h followed by 100 μl of DMSO. The OD value of each well was measured using a universal microplate reader (Bio-Tek Instruments, INC) at 570 nm and the results were expressed as the fractional survival of treated cells normalized to control cells. The concentration of drug inhibit for 50% of the cells (IC_{50}) was calculated using the software "Dose-Effect Analysis with Microcomputers" [27].

Apoptosis

To quantify apoptotic cells with sub-G0 DNA content, Molt-4 cells were cultured in complete medium in 25 cm^2 flasks, with or without 10 μM 4-HPR for 0–24 hours in 2% O_2 and 20% O_2, respectively. The cells were then harvested, washed in phosphate-buffered saline (PBS), centrifuged and resuspended in 1 ml of 0.1% sodium citrate containing 0.05 mg propidium iodide (PI) and 50 μg RNAse for 30 min at room temperature in the dark. RNAase was dissolved in 10 mmol/l Tris·Cl (pH 7.5) and 15 mmol/l NaCl to a concentration of 10 mg/ml, and boiled at 100°C for 15 min, then stored in −−20°C. Flow cytometric analysis of cell cycle distribution and apoptosis was performed with a BD FACSCalibur with a 488-nm (blue) argon laser (Becton Dickinson, San Jose, CA, USA). Data acquisition was performed with CellQuest 3.1 software and the data were analyzed with ModFit LT 3.0 software (Variety Software House, Topsham, ME, USA).

Assessment of mitochondrial potential transition

The $\Delta\Psi_m$ was determined [28] in the Molt-4 cell line after a 12 and 24-h treatment with 10 μM 4-HPR in 2% O_2 and 20% O_2. Afterward, the cells were collected and washed by PBS, and resuspended in PBS. The cells were

suspended in 1 ml of complete medium containing 10 μg/ml of JC-1 for 10 min at 37°C. JC-1 is a cationic dye that exhibits potential-dependent accumulation in mitochondria [29], indicated by a fluorescence emission shift from green to red. To assess the mitochondrial potential transition, 10,000 cells/sample stained by JC1 were observed by the fluorescent microscope (Olympus, Japan) and by flow cytometry. Mitochondria depolarization was specifically indicated by a decrease in the red to green fluorescence intensity.

Protein expression

Proteins of Molt-4 cells (incubated with 10 μM 4-HPR for 6, 12 and 24 h in 2% O_2 and 20% O_2) were extracted in lysis buffer (150 mM NaCl, 50 mM Tris, 2 mM EGTA, 2 mM EDTA, 25 mM NaF, 25 mM β-glycerophosphate, 0.2% Triton X-100, 0.3% NONIDET P-40, 0.1 mM PMSF) and 40 μg of total protein was loaded per lane. The proteins were fractionated on 10–12% Tris–Glycine pre-cast gels (Novex, San Diego, CA, USA), transferred to nitrocellulose membrane (Protran, Keene, NH, USA) and probed with primary antibodies and then HRP-labeled secondary antibodies. The proteins were visualized using ECL Western Blotting detection reagents.

Statistics

Significance of mean values was determined by unpaired two-tailed Student's t test. Significance of the dose–response curve was detected by ANOVA.

Results

Cytotoxicity assays of 4-HPR in normoxia and hypoxia

The dose–response curves of ALL cell lines to 4-HPR (0–10 μM), determined as single agent in 2% O_2 and 20% O_2, are shown in Fig. 1. IC_{50} values of 4-HPR for Molt-4 and Molt-3 cell lines in 20% O_2 were 2.6 ± 0.1 and 2.4 ± 0.1 μM, respectively. However, the IC_{50} values of 4-HPR in 2% O_2 increased to 9.1 ± 0.2 μM for Molt-4 cells and 6.7 ± 0.2 μM for Molt-3 cells, respectively. Hypoxia significantly ($P < 0.01$) inhibited, but not abrogated, the cell killing ability of 4-HPR.

4-HPR-induced apoptosis

The Molt-4 cells were treated with 10 μM 4-HPR for 6, 12 and 24 h and examined for apoptosis by PI staining and flow cytometry. Spontaneous apoptosis (1.1–1.4%) was observed in hypoxia and normoxia (Fig. 2a, e). In 20% O_2, 4-HPR induced apoptosis in 1.2, 12.6% and 76.3% at 6, 12 and 24 h in a time-dependent manner (Fig. 2b–d). In 2% O_2, apoptosis induced by 4-HPR declined to 1.4% (6 h), 1.2% (12 h) and 11.0% (24 h) at each time point, respectively (Fig. 2f–h).

Loss of mitochondrial membrane potential ($\Delta\Psi_m$)

We determined $\Delta\Psi_m$ in Molt-4 cells treated with 10 μM 4-HPR for 12 and 24 h in normoxia and hypoxia. A loss of $\Delta\Psi_m$ decline is specifically indicated by a switch from the red to green fluorescence intensity. Compared with the corresponding control (Fig. 3a, d), 4-HPR obviously decreased $\Delta\Psi_m$ in Molt-4 cells after 12 h (Fig. 3e) and 24 h of incubation (Fig. 3f) in 20% O_2, but slightly induce $\Delta\Psi_m$ decrease in 2% O_2 (Fig. 3b–c). The quantitated data from flow cytometry was represented as a histogram (Fig. 3g), which shows that the percentages of cells with low $\Delta\Psi_m$ show an eightfold increase in the cells exposed to 4-HPR for 24 hours in 20% oxygen compared with a twofold increase in the hypoxia.

Expression of procaspase-3, PARP, ERK and XIAP

The basal and 4-HPR-treated (10 μM for 6, 12 or 24 h) protein expression of procaspase-3, PARP, ERK and XIAP was measured by western blot. As shown in Fig. 4, 4-HPR (> 6-h treatment) obviously decreased the expression of procaspase-3, ERK and XIAP in Molt-4 cells, and increased the cleavage of PARP in a time-dependent manner in 20% O_2 (Fig. 4, left line). Whereas in 2% O_2, 4-HPR only slightly decreased procaspase-3 and increased the cleavage of PARP at 24 h. In addition, 4-HPR in 2% O_2 failed to decrease the expression of ERK and enhanced the expression of XIAP at 24 h (Fig. 4, right line).

Discussion

Retinoids have been shown to regulate vital cellular processes including cell proliferation, differentiation and apoptosis. *N*-(4-hydroxyphenyl)-all-trans-retinamide (fenretinide, 4-HPR) is a synthetic ATRA derivative with chemopreventive and cytotoxic activities against various cancer cell lines, including myeloid leukemia [30], and clinical trials of 4-HPR have been in progress in the United States. ALL is a malignant (clonal) disease of the bone marrow and a poor prognosis of ALL patients is related with the recurrence in the bone marrow [9, 10], a hypoxic tissue compartment. Since hypoxia reduced cytotoxicity of 4-HPR in neuroblastoma, Ewing's sarcoma and primitive neuroectodermal tumor cell lines [14], we investigated the 4-HPR activity for ALL cell lines in hypoxia, comparing with that in normoxia. Our results showed that, relative to the activity in 20% O_2, 2% O_2 inhibited the cell-killing ability of 4-HPR in ALL cells, with at least a 2.8-fold increase in IC_{50} values and more than a 6.9-fold decrease in apoptotic cells. These

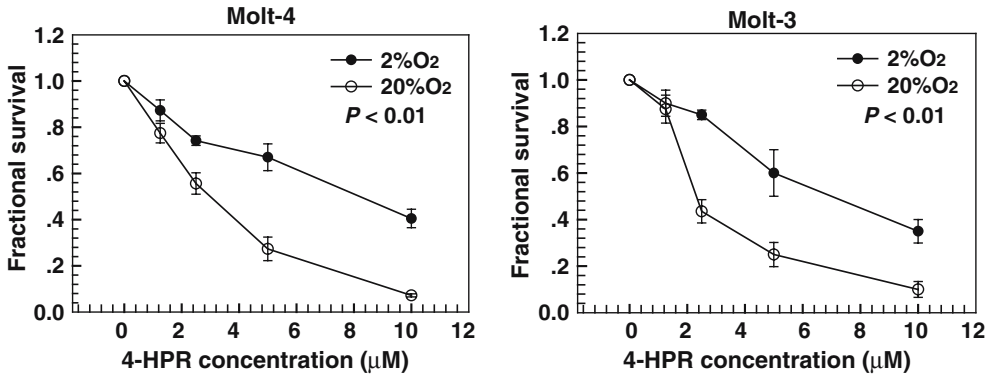

Fig. 1 Hypoxia (2% O_2)-induced 4-HPR resistance in Molt-4 cells. The cells were treated with 4-HPR (0–10.0 μM) for 72 h in 20% O_2 and 2% O_2, and cytotoxicity was analyzed with MTT method. 2% O_2 could significantly reduce the activity of 4-HPR. The points represent the mean fractional survival; error bars represent standard deviation. Values represent the means ± SD. for three separate experiments performed in triplicate. $P < 0.01$: statistical analysis of dose–response curves by ANOVA

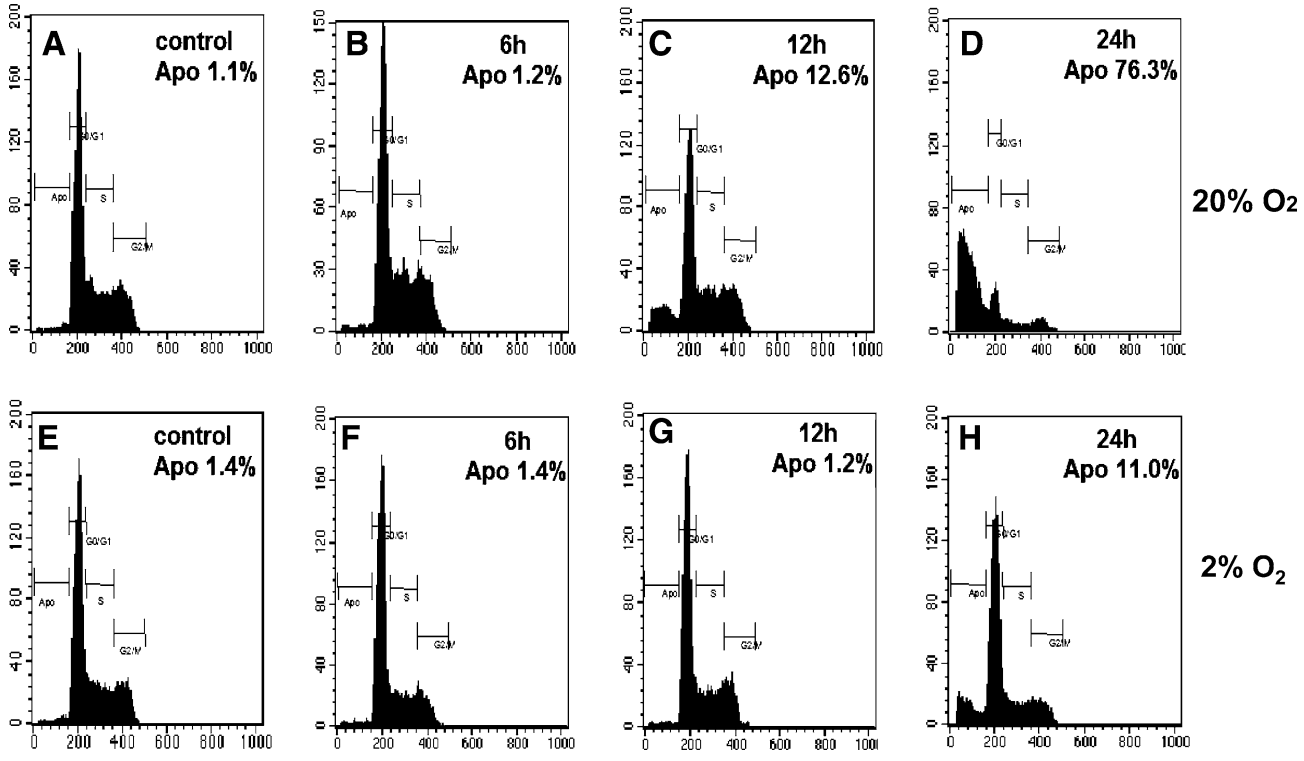

Fig. 2 Hypoxia inhibited apoptosis induced by 4-HPR in Molt-4 cells. The cells were treated with 10 μM 4-HPR for 0 to 24 h in 20% O_2 (*first line*) and 2% O_2 (*second line*), respectively. After treatment, apoptosis was assessed by propidium iodide (PI) staining of the lysed cell nuclei as described in Materials and methods. The DNA content of 15,000 events was analyzed by flow cytometry. *Apo* apoptotic percentage

data suggested that hypoxia exerted an antagonistic effect on the anti-leukemia activity of 4-HPR.

It has been demonstrated that the mitochondrial membrane potential ($\Delta\Psi_m$) plays a pivotal role in transducing a variety of proapoptotic stimuli [15–17]. The present study showed that 4-HPR in normoxia induced apoptosis and an obvious loss of $\Delta\Psi_m$ in Molt-4 cells at 12 and 24 h. However, 4-HPR in hypoxia only slightly induced $\Delta\Psi_m$ loss with less apoptosis, suggesting that hypoxia might exhibit inhibition on 4-HPR-induced apoptosis through stabilization of mitochondrial permeability and the release of mitochondrial apoptogenic factors in Molt-4 cells.

The inhibitors of the apoptosis (IAP) family, including cIAP-1, cIAP-2, XIAP, neuronal apoptosis inhibitory protein and survivin, were initially identified in baculovirus and highly conserved across species. These proteins act directly on caspases, distal to mitochondrial perturbation [24]. In addition, the IAP family is a likely target for ERK-mediated protection, which occurs

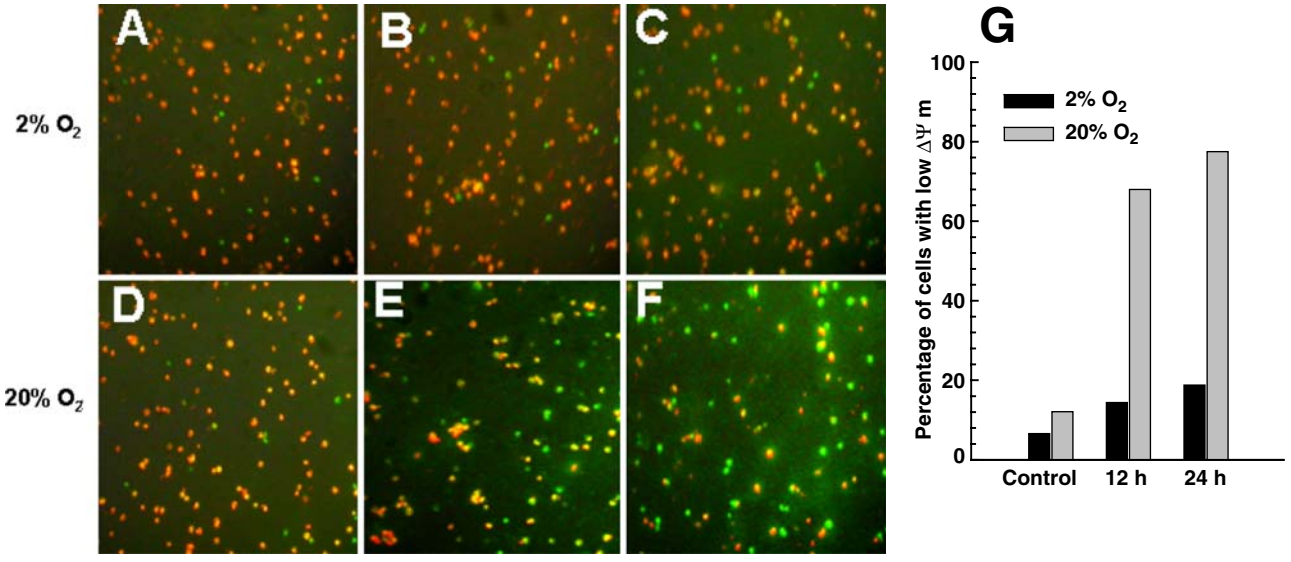

Fig. 3 Hypoxia inhibited mitochondrial membrane potential ($\Delta\Psi$m) decrease induced by 4-HPR in Molt-4 cells. The cells were treated with 4-HPR 10 μM for 12 and 24 h in 20% O_2 (**d**–**f**) and 2% O_2 (**a**–**c**), respectively. $\Delta\Psi$m was measured using the JC-1 dye as described in Materials and methods, in which mitochondria depolarization is indicated by a switch from the red to green fluorescence intensity. 2% O_2 could obviously inhibit $\Delta\Psi$m lose caused by 4-HPR. **a**, **d** Control groups, **e**, **d** cells exposed to 10 μM 4-HPR for 12 h, **c**, **f** cells exposed to 10 μM 4-HPR for 24 h. In addition, the cells with low $\Delta\Psi_m$ were quantitated by flow cytometry method after JC1 staining. The data was represented as a histogram (**g**)

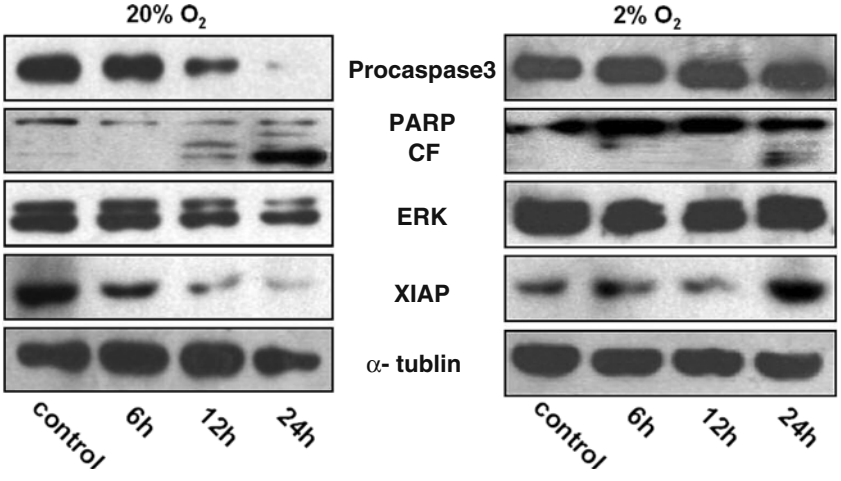

Fig. 4 Protein expressions of procaspase-3, cleavage of PARP, ERK and XIAP in Molt-4 treated with 4-HPR in 20% O_2 (*left group*) and 2% O_2 (*right group*). The cells were treated with 4-HPR 10 μM for 6, 12 and 24 h. After treatment, the cells were harvested, and protein expressions of procaspase-3, PARP, ERK and XIAP were detected by Western blotting. *Each lane* was loaded with 40 μg of protein. 2% O_2 could inhibit 4-HPR-induced down-regulation of procaspase-3, ERK, XIAP and activated PARP. α-tublin was shown as a loading control. *CF* cleavage fragment

downstream of mitochondrial alterations assessed as a decrease in membrane potential concomitant with enhanced cytochrome c release [25]. It was reported that the inhibition of apoptosis by enhanced XIAP transcription has been associated with ERK activation in acute myeloid leukemia cells and THP-1 cells [31, 32]. Since the activation of caspase-3 is inhibited by members of the IAP family [20, 23, 25] and ERK is able to maintain XIAP level to inhibit apoptosis at the downstream of the mitochondria-mediated pathway [25], we investigated the expression of procaspase-3, PARP (a substrate of caspase-3), ERK and XIAP to demonstrate whether $\Delta\Psi_m$-mediated apoptosis by 4-HPR is involved in caspase activation and affected by hypoxia. The time-course results showed that exposure to 4-HPR for 12 and 24 hours caused an obvious decrease in procaspase-3 expression and increase in cleavage of PARP in normoxia, but just slight changes in hypoxia. In the meantime, 4-HPR induced an obvious decrease in the expression of XIAP and ERK in normoxia, while in hypoxia, an elevated protein level of XIAP was observed in molt-4 cells treated with 4-HPR for 24 h. These

results suggest that ERK and XIAP are functionally involved in 4-HPR-induced apoptosis in ALL and the prevention of 4-HPR-induced inhibition of ERK and the enhancement of XIAP level in the cells exposed to hypoxia may play an important role in hypoxia-mediated antagonistic effect on 4-HPR. The slight cleavage of PARP at 24 h in the cells exposed to hypoxia suggested that 4-HPR-induced apoptosis could not be completely inhibited by hypoxia, which was consistent with the cytotoxicty results shown in Fig. 1.

In conclusion, the present study demonstrated that: (1) 4-HPR-induced apoptosis and cytotoxicity was reduced by hypoxia; (2) hypoxia antagonized 4-HPR-caused $\Delta\Psi_m$ loss and affected the expression of mitochondria pathway-related proteins, including ERK, XIAP, procaspase-3 and cleaved PARP; and (3) mitochondria pathway may be involved in the antagonistic effect of hypoxia on 4-HPR in ALL cells. Therefore, it is possible that a combination of 4-HPR with hypoxia-sensitive drugs, such as Tirapazamine, may result in a synergistic anti-cancer action.

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