Effect of radiation on glucose metabolism in breast cancer cells cultured under hypoxic condition

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BACKGROUND: Breast cancer is the most malignant disease in women, accounting for about 29% of all newly diagnosed cancer cases in women in 2014 [1]. Once it develops endocrine resistance, current treatments will provide limited clinical benefits and poor survival [2]. According to the findings of Lundgren et al., about 25%-40% invasive breast cancers contain the hypoxic regions [3]. Low oxygen of primary tumors has been linked with the increased risk of patients mortality and metastasis [4]. Additionally, the cancers with high hypoxic volumes usually have poor response to radiotherapy [5]. Thus, more efforts for the improvement of treatment effects are still needed in breast cancer.

Cellular metabolism within cancers is significantly different from that in normal tissues. The metabolism shift from mitochondrial respiration to glycolysis is reported to be associated with cancer malignancy [6]. Under normal supply of oxygen, cancer cells exhibit elevated glycolysis rates [7], and glycolysis composes approximately 50% energy sources [8]. HIF-1α stabilization is a key regulator of this process, which functions to activate the transcription of the genes encoding glucose transporters [9]. What is more, many genes regulated by HIF-1α have been

linked with glucose metabolism, including fructose biphoshatase 1 (FBP1), lactate dehydrogenase A (LDHA), hexokinase 2 (HK2), isocitrate dehydrogenase 2 (IDH2), and glucose transporter 1(GLUT1) [10, 11]. Hypoxia inducible factors (HIFs), the principal molecule responding to hypoxia [12], are essential for oxygen homeostasis in cancer cells, and when oxygen levels become too low for cells, they will help in the hypoxia adaption [13]. HIF-1 imposes a huge hurdle in radiotherapy due to its roles in cellular response to cancer hypoxia [14, 15]. The inhibition of HIF-1 makes it possible to reduce the metabolic flexibility of tumor cells, results in a high sensitivity to anticancer strategies [16, 17].

HYPOTHESIS: Oxygen is one of the most powerful radiosensitizes [18]. In this study, radiation treatment was conducted in hypoxic breast cancer cells to detect its influence on glucose metabolism. Glucose update and lactate release rate were recorded to observe the cell changes. Expression levels of HIF-1α and its several target genes, including FBP1, HK2, IDH2, GLUT1, and LDHA were also determined for a better elucidation.To investigate the influence of radiation hypoxia-treated breast cancers and the underlying mechanism, cell lines were conducted hypoxia and radiation treatments.

METHODS:*Cell Line and Culture Conditions*

A breast cancer cell line MCF-7 was maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (GBICO, MD, USA). Cells were cultivated in a 5% CO_2 incubator under 37°C with constant temperature and humidity.

Cells were placed in 96 well plates at the density of 5×10^4/well. For hypoxia treatment, $CoCl_2$ was added to a concentration of 200μmol/L for 24 hours. For radiation treatment, cells under aseptic conditions were performed 0 GY and 8GY radiation respectively, with a speed of

4GY/min. After the radiation, the cells were transferred into the incubator for 24 hours. Therefore, there were four groups of cells: normal group, hypoxia group (0 GY + $CoCl_2$), radiation group (8 GY), and hypoxia + radiation group (8 GY + $CoCl_2$).

To investigate the influence of HIF-1α inhibitor on these cells, another two groups were formed: inhibitor group (BAY), hypoxia + radiation + inhibitor group (8 GY + $CoCl_2$ + BAY) to compare with the normal and hypoxia + radiation groups. For the HIF-1α inhibitor treatment, the inhibitor BAY 87-2243 (selleck, YX, USA) was added into the cells to a concentration of 200μmol/L for 24 hours.

MTT Assay for Cell Proliferation

MTT assay was utilized to detect the proliferation rate of cells. Briefly, 10 μL MTT was added in to each well, followed by the incubation under 37°C for 4 hours. Then, the cells were centrifuged and washed for incubation with MTT-supplemented medium. After adding 100 μL DMSO into each well, the OD values were determined at 570nm.

Glucose Uptake and Lactate Release Determination

Supernatant incubation medium was collected for the evaluation of glucose absorption rate and lactate release rate, using Hexokinase kits and Lactate kits (Sigma, MO, USA). The uptake rate= (glucose content in control group-glucose content in treatment group)/glucose content in control group×100%. The release rate= (lactate content in treatment group-lactate content in control group)/glucose content in control group×100%.

Real-time PCR

For the expression determination of glucose metabolic pathway related genes in cell lines, RNA was DNase treated and reverse transcribed using TRIzol reagent (Invitrogen, Carlsbad, CA). Afterwards, an ultraviolet spectrophotometer (Merinton SMA4000, Beijing, China) was applied to test the RNA purity, and RNA with 2.0<A260/A280>2.3 was screened for further study. The experiments were performed on a ViiA7 Real-Time PCR System (Applied Biosystems) using a 5x primeScript RT Master MIX (perfect Real Time, Takara). The PCR reactions consisted of 3 min at 50 °C followed by 35 cycles of at 95°C for 3min, at 95°C for 10 s and at 60°C for 30 s. The primer sequences of all genes are listed in Table 1.

Western blot

Western blot analysis was conducted to detect the expression levels of FBP1 in hypoxia, radiation and HIF-1α inhibitor treated cells. Cell proteins were extracted and determined using BCA (bicinchinoninic acid) assay. Then, proteins were separated by polyacrylamide gel electrophoresis, transferred to PVDF membranes, and proved with anti-HIF-1α, anti-FBP1, anti-β-actin, and GAR-HRP antibodies (Proteintech, IL, USA). The dilutions of antibodies were recommended by the manufacturer. Finally, enhanced chemiluminescence regents were utilized for determination (Bio-Rad, CA, USA).

Statistical analysis

All experiments were performed in three reduplicate, and the differences between treated cells and control cells were compared by student's *t-test*. All data were expressed as mean ± SD of the three independent experiments and $P<0.05$ was set as the statistical significance level.

RESULTS:*MTT Assay for Cell Proliferation*

MTT assay showed that hypoxia and radiation treatment decreased the cell proliferation significantly comparing with untreated cells ($P<0.05$) (Fig.1 A). Specially, the radiation + hypoxia treatment exhibited most significant inhibition to cell proliferation.

Under normal cultivation condition, the addition of HIF-1α inhibitor could not inhibit cell proliferation, whereas the addition under radiation + hypoxia condition exhibited significant inhibiting effects ($P<0.05$) (Fig.1 B).

Glucose Uptake and Lactate Release Determination

The glucose uptake rate was higher in hypoxia and radiation cells than in untreated cells ($P<0.05$) (Fig.1 C). Lactate release rate was also remarkably increased in hypoxia and radiation cells ($P<0.05$) (Fig.1 E). When compared with hypoxia treatment, radiation treated cells displayed higher glucose uptake and lactate release rate, while the radiation + hypoxia had the most significant influence.

Under normal cultivation condition, the addition of HIF-1α inhibitor could not affect the glucose uptake rate, but it decreased the lactate release rate ($P<0.05$) (Fig.1 D). Under radiation + hypoxia condition, there were significant decrease effects ($P<0.05$) (Fig.1 F) for both glucose uptake and lactate release rate with the addition of HIF-1α inhibitor.

Expression levels of HIF-1α and FBPI

HIF-1α and FBPI levels were significantly increased in hypoxia, radiation, and radiation + hypoxia treated cells, at both mRNA and protein levels ($P<0.05$) (Fig. 2 A). What's more, their expression levels in radiation + hypoxia group were the highest. The addition of HIF-1α inhibitor decreased the expressions of HIF-1α comparing with the control group, and only in radiation +

hypoxia treated cells the addition showed significant decrease effects ($P<0.05$) (Fig. 2 A). Similar expression decreasing trends of FBP1 were observed, suggesting the inhibition of HIF-1α inhibitor on FBP1 (Fig. 2 B).

Expression levels of glucose metabolic pathway related genes

Comparing with the untreated cells, hypoxia, radiation and radiation + hypoxia treatments dramatically increased the mRNA levels of genes GLUT1 and LDHA ($P<0.05$) (Fig.3 A). Their expressions in radiation + hypoxia treated cells were the highest. Nevertheless, the expression increase of genes HK2 and IDH2 was not significant.

As shown in Fig.3 B, the addition of HIF-1α inhibitor in normal group significantly increased the mRNA levels of genes HK2, IDH2, and LDHA ($P<0.05$). The increasing effects in radiation + hypoxia groups were significantly decreased after the addition of HIF-1α inhibitor, with regard to genes IDH2, GLUT1, and LDHA ($P<0.05$).

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Figure legend

Figure 1 Cell viability (A), glucose uptake rate (B) and lactate release rate (C) of breast cancer cells cultured under different condition. $CoCl_2$ is used for the hypoxia treatment, 8GY is used for the radiation treatment, and BAY is the HIF-1α inhibitor.

Figure 2 mRNA and protein levels of HIF-1α and FBP1. $CoCl_2$ is used for the hypoxia treatment, 8GY is used for the radiation treatment, and BAY is the HIF-1α inhibitor.

Figure 3 Expressions of glucose metabolism related genes regulated by HIF-1α. A, $CoCl_2$ is used for the hypoxia treatment, 8GY is used for the radiation treatment, and BAY is the HIF-1α inhibitor.

Figure 1

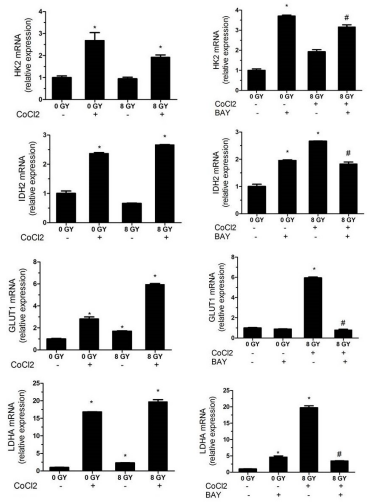


Figure 2

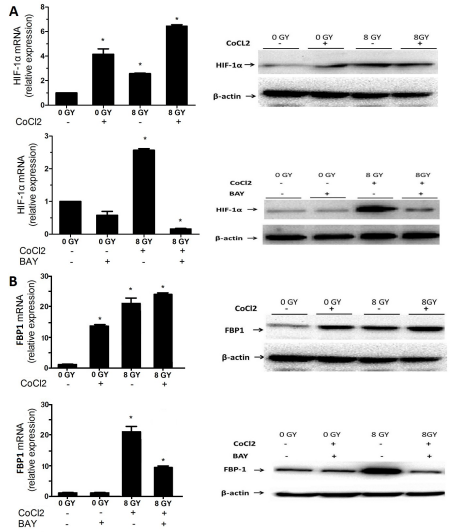
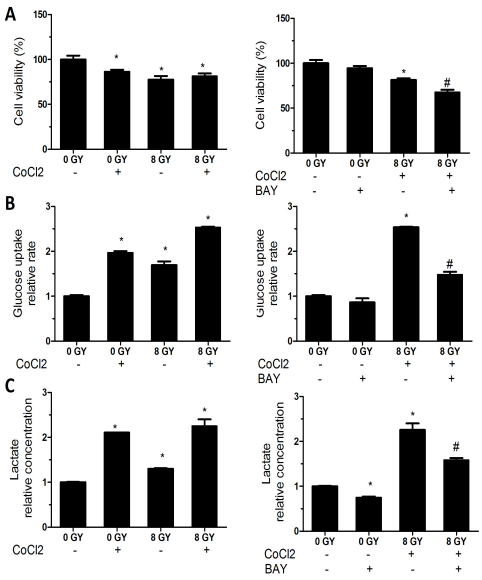


Figure 3


A
Cell viability (%)
150
100
50
0
0 GY
0 GY
8 GY
8 GY
CoCl2
-
+
-
+
BAY
*
#
B
Glucose uptake relative rate
3
2
1
C
Lactate relative concentration