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ORIGINAL ARTICLE

The Promoting Effect of Radiation on Glucose Metabolism in Breast Cancer Cells under the Treatment of Cobalt Chloride

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Abstract We aimed to investigate the influence of radiation on hypoxia-treated breast cancers cells and its underlying mechanism. We mimicked the hypoxic response in MCF-7 cells by the treatment of $CoCl_2$. Meanwhile, hypoxic MCF-7 cells induced by $CoCl_2$ or untreated MCF-7 cells were treated with or without radiation, and then treated with or without hypoxia inducible factors-1α (HIF-1α) inhibitor. Subsequently, glucose update and lactate release rate were determined by commercial kits, as well as the expressions of HIF-1α and the glucose metabolic pathway related genes, including fructose biphoshatase 1 (FBP1), glucose transporter 1 (GLUT1), lactate dehydrogenase A (LDHA), hexokinase 2 (HK2), and isocitrate dehydrogenase 2 (IDH20) were detected by western blotting and/or RT-PCR. The results showed that glucose uptake rate and lactate release rate were increased in cells under hypoxia and/or radiation condition compared with untreated cells ($p < 0.05$), while the addition of HIF-1α inhibitor decreased these rates in hypoxia + radiation treated cells ($p < 0.05$). In addition, compared with untreated cells, the mRNA and protein levels of HIF-1α were significantly increased under hypoxia and radiation condition ($p < 0.05$), while which decreased after the addition of HIF-1α inhibitor ($p < 0.05$). Similar content changing trends (all $p < 0.05$) were observed in FBP1, IDH2, GLUT1, and LDHA but not HK2. In conclusion, the combination of radiation and hypoxia could promote the glucose metabolism. Furthermore, HIF-1α might inhibit the promoting effect of radiation on glycolysis in hypoxic MCF-7 cells by regulating the glucose metabolic pathway.

Keywords Radiation; hypoxia · Glucose metabolism · Hypoxia inducible factors-1α · Cancer cell

Abbreviations

FBP1	Fructose biphoshatase 1
LDHA	Lactate dehydrogenase A
HK2	Hexokinase 2
IDH2	Isocitrate dehydrogenase 2
GLUT1	Glucose transporter 1
HIFs	Hypoxia inducible factors
BCA	Bicinchinoninic acid

Introduction

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 can provide limited clinical benefits and poor survival [2]. According to the findings of Lundgren et al. [3], about 25 %–40 % invasive breast cancers contain the hypoxic regions. Low oxygen of primary tumors has been considered to be linked with the increased risk of mortality and metastasis in patients [4]. Additionally, the cancers with high hypoxic volumes usually have poor response to radiotherapy [5]. Thus, more efforts for the improvement of therapeutic effects are still needed in breast cancer.

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Cellular metabolism in cancers is significantly different from that in normal tissues. The metabolism shifting from mitochondrial respiration to glycolysis is reported to be associated with cancer malignancy [6]. Under a normal supply of oxygen, cancer cells exhibit elevated glycolysis rates [7], and glycolysis composes approximately 50 % energy sources [8]. The stabilization of hypoxia-inducible factor-1α (HIF-1α), a key regulator of the glycolysis process, contributes to activate the transcription of the genes encoding glucose transporters (GLUTs) [9]. What's more, many genes regulated by HIF-1α are associated with glucose metabolism, including fructose biphoshatase 1 (FBP1), lactate dehydrogenase A (LDHA), hexokinase 2 (HK2), isocitrate dehydrogenase 2 (IDH2), and GLUT1 [10, 11]. HIFs, the principal molecules responding to hypoxia [12], are essential for oxygen homeostasis in cancer cells. When oxygen levels become too low for cells, HIFs will contribute to the hypoxia adaption [13]. Interestingly, 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]. Based on these results, we hypothesized that glucose metabolism was involved in the effect of radiation on hypoxia-treated breast cancers cells by the regulation of HIF-1α.

Oxygen is one of the most powerful radiosensitizers [18]. It is well known that cobalt chloride ($CoCl_2$) can mimic the hypoxic response through the up-regulations of HIF-1α, erythropoietin and glycolytic enzymes [19–21]. In this study, we mimicked the hypoxic response in breast cancer cells by the treatment of $CoCl_2$. Meanwhile, radiation treatment was conducted in hypoxic breast cancer cells induced by $CoCl_2$ to detect its influence on glucose metabolism. Glucose update and lactate release rate were measured to observe the cell glycolysis. Expression levels of HIF-1α and its several target genes, including *FBP1*, *HK2*, *IDH2*, *GLUT1*, and *LDHA* were also determined for a better elucidation. We aimed to investigate the influence of radiation on hypoxia-treated breast cancers cells and its underlying mechanism.

Materials and 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, cells were treated with $CoCl_2$ at a concentration of 200 μmol/L for 24 h [22]. For radiation treatment, cells under aseptic conditions were performed 0 Gy and 8 Gy radiation [23] respectively, with a speed of 4Gy/min. After the radiation, the cells were transferred into the incubator for 24 h. 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 with a concentration of 200 μmol/L for 24 h.

Glucose Uptake and Lactate Release Determination

Culture supernatant was collected for the evaluation of glucose uptake rate and lactate release rate, using Hexokinase kits and Lactate kits (Sigma, MO, USA), respectively. 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

To detect the expressions of glucose metabolic pathway related genes in cell lines, RNA was extracted using TRIzol reagent

Table 1 Primer sequence of glucose metabolic pathway related genes

Primer	Sequence (from 5'to3')	Primer	Sequence (from 5'to3')
FBP1-hf	TCACCCTAACCCGCTTCGTC	HK-2-hr	CAAGCCCTTTCTCCATCTCCT
FBP1-hr	ACTGCGGTGGAGATGGCTTT	GLUT1-hf	ACA GGC TCA AAG AGG TTA TG
LDHA-hf	GTCAGCAAGAGGGAGAAAGC	GLUT1-hr	TGG GTG GAG TTA ATG GAG TAG
LDHA-hr	TCCAAGCCACGTAGGTCAAG	HIF1α-hf	AGCCGAGGAAGAACTATGAA
IDH2-hf	TGGTGGAGATGGATGGTGAT	HIF1α-hr	TTCACAAATCAGCACCAAGC
IDH2-hr	TCAGTCTGGTCACGGTTTGG	GAPDH-hf	TGA CAA CTT TGG TAT CGT GGA AGG
HK-2-hf	CGCATCTGCTTGCCTACTTCT	GAPDH-hr	AGG CAG GGA TGA TGT TCT GGA GAG

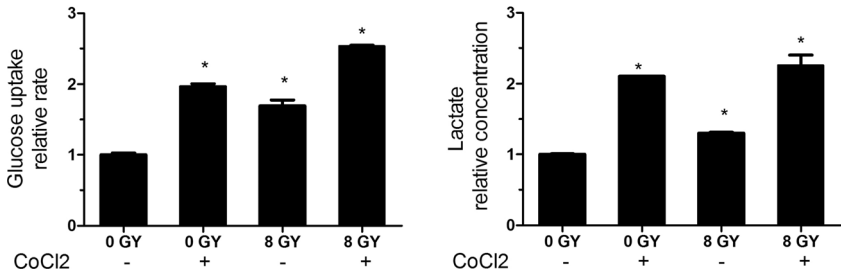

Fig. 1 Glucose uptake rate and lactate release rate of breast cancer cells cultured under hypoxia and/or radiation conditions. $CoCl_2$ is used for the hypoxia treatment, and 8 GY is used for the radiation treatment. *$p < 0.05$ versus 0 GY and no $CoCl_2$ group

(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 the further study. High-quality RNA was reversely transcribed into complementary DNA with the reverse transcription kit (Takara, Shiga, Japan). The primer sequences of FBP1, HK2, IDH2, GLUT1, LDHA, HIF-1α and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) are listed in Table 1. The experiments were performed on a ViiA7 Real-Time PCR System (Applied Biosystems) using a 5× 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 3 min, at 95 °C for 10 s and at 60 °C for 30 s. The comparative threshold (*C*t) cycle method ($2^{-\Delta\Delta}Ct$) was used to calculate relative quantification.

Western Blotting

Western blot analysis was conducted to detect the expression levels of FBP1 and HIF-1α. Proteins were extracted with RIPA lysis buffer (Shanghai Sangon Biotech Co., Ltd., China) and determined using bicinchoninic acid (BCA) assay (Shanghai Sangon Biotech Co., Ltd). Then, proteins were separated by polyacrylamide gel electrophoresis, transferred onto PVDF membranes, and then proved with anti-HIF-1α, anti-FBP1, and anti-β-actin antibodies (Proteintech, IL,

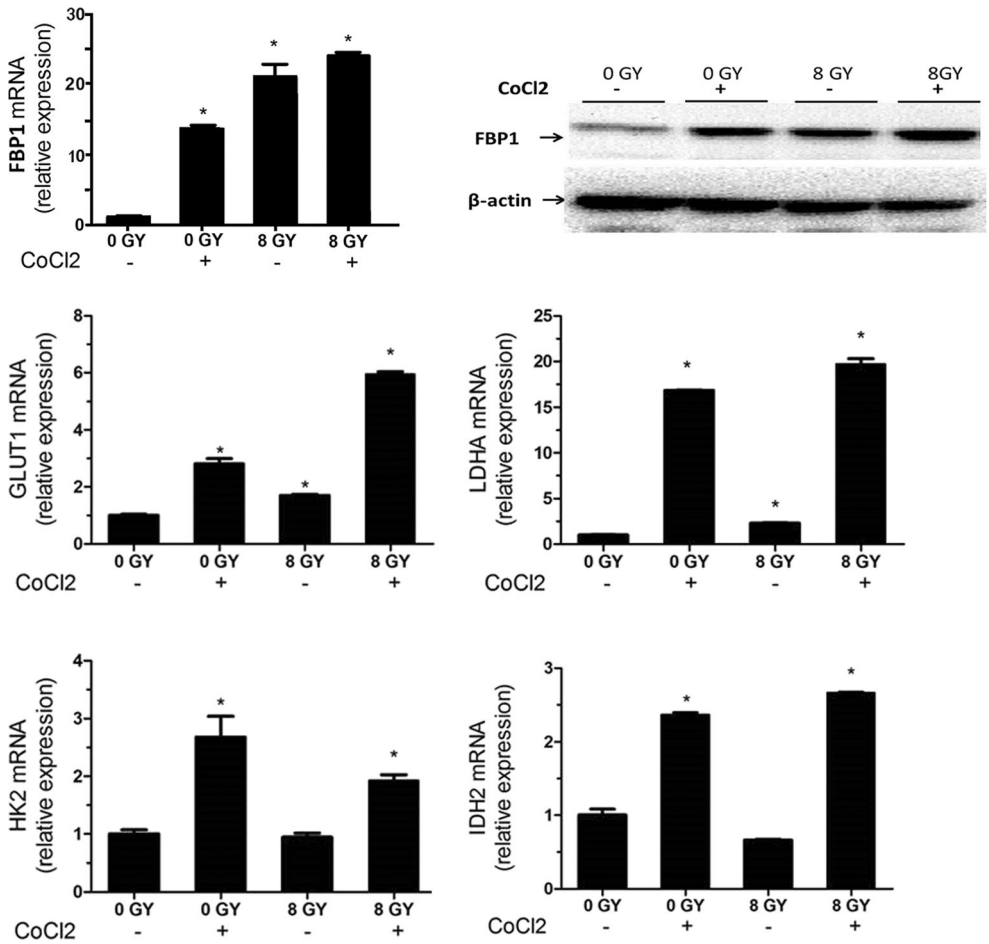

Fig. 2 Expressions of glucose metabolism related genes in breast cancer cells cultured under hypoxia and/or radiation conditions. $CoCl_2$ is used for the hypoxia treatment, and 8 GY is used for the radiation treatment. FBP1, fructose biphoshatase 1; LDHA, lactate dehydrogenase A; HK2, hexokinase 2; IDH2, isocitrate dehydrogenase 2; GLUT1, glucose transporter 1. *$p < 0.05$ versus 0 GY and no $CoCl_2$ group

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 and $p < 0.05$ was set as the statistical significance level.

Results

Effect of Radiation on Glycolysis in Hypoxic MCF-7 Cells

Compared with the normal group, the glucose uptake rate and lactate release rate were both remarkably increased in the hypoxia group ($p < 0.05$, Fig. 1). Similarly, the glucose uptake rate and lactate release rate in the radiation group were also higher than those in the normal group ($p < 0.05$, Fig. 1). Furthermore, the highest glucose uptake and lactate release rate were found in the radiation + hypoxia group compared with the normal group ($p < 0.05$, Fig. 1).

Effect of Radiation on the Glucose Metabolic Pathway Related Genes in Hypoxic MCF-7 Cells

The glucose metabolic pathway related genes, including *FBP1*, *GLUT1*, *LDHA*, *HK2* and *IDH2* were detected in this study. The results showed that FBP1 expression was significantly increased in hypoxia, radiation, and radiation + hypoxia treated cells in comparison with untreated cells, at both mRNA and protein levels ($p < 0.05$, Fig. 2). Compared with the normal group, the treatments of hypoxia, radiation and radiation + hypoxia, respectively, dramatically increased the mRNA levels of genes *GLUT1* and *LDHA* ($p < 0.05$, Fig. 2). Their expressions in radiation + hypoxia treated cells were the highest (Fig. 2). Nevertheless, hypoxia but not radiation could increase the mRNA levels of HK2 and IDH2 ($p < 0.05$, Fig. 2).

Effect of Radiation on Glycolysis in HIF-1α Inhibitor Treated Hypoxic MCF-7 Cells

This study found that the mRNA and protein expressions of HIF-1α were significantly increased in the hypoxia, radiation, and radiation + hypoxia groups compared with the normal group ($p < 0.05$, Fig. 3). However, the addition of HIF-1α

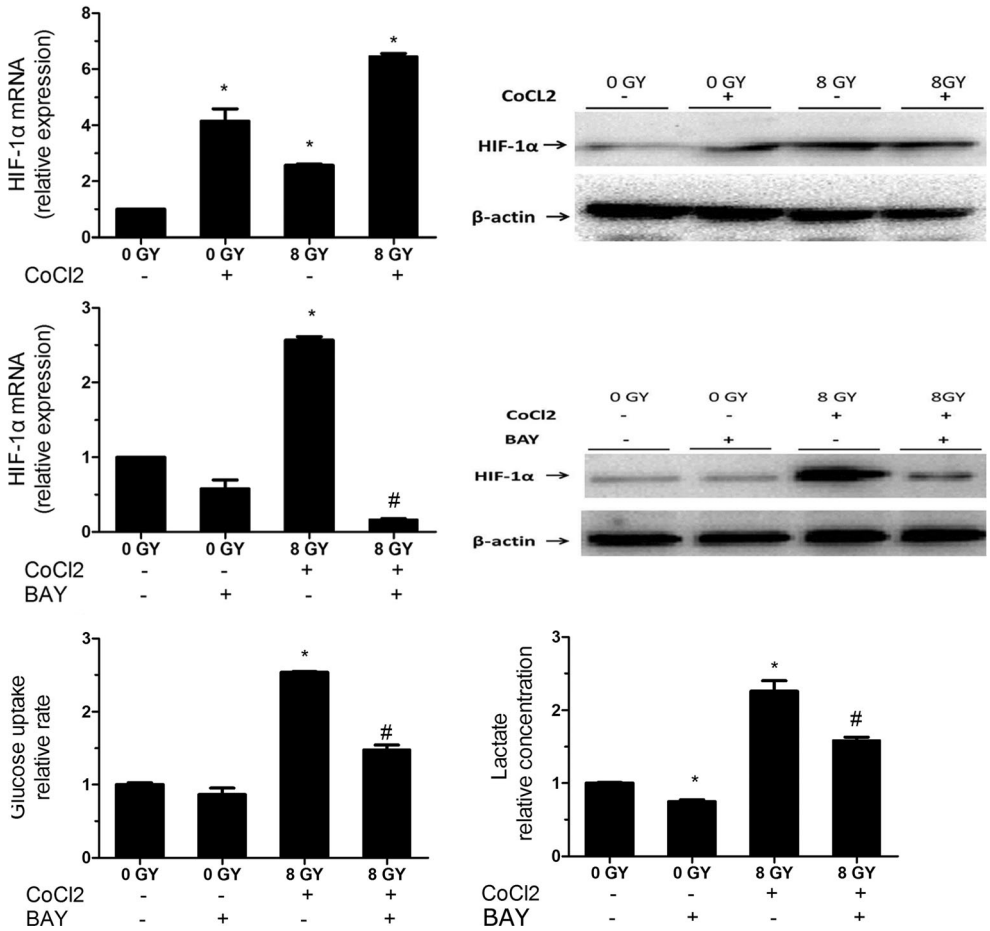

Fig. 3 Expressions of HIF-1α as well as glucose uptake rate and lactate release rate in breast cancer cells cultured under hypoxia and/or radiation conditions with or without the addition of HIF-1α inhibitor. $CoCl_2$ is used for the hypoxia treatment, 8 GY is used for the radiation treatment, and BAY is the HIF-1α inhibitor. HIF-1α, Hypoxia inducible factors-1α. $^{*}p < 0.05$ versus 0 GY and no $CoCl_2$ group, $^{\#}p < 0.05$ versus 8 GY, $CoCl_2$ and no BAY group

inhibitor significantly decreased the expression of HIF-1α compared with the radiation + hypoxia group ($p < 0.05$, Fig. 3). Furthermore, 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. 3). Under radiation + hypoxia condition, there were significant decreased effects ($p < 0.05$, Fig. 3) for both glucose uptake and lactate release rate with the addition of HIF-1α inhibitor.

Effect of Radiation on the Glucose Metabolic Pathway Related Genes in HIF-1α Inhibitor Treated Hypoxic MCF-7 Cells

As shown in Fig. 4, the addition of HIF-1α inhibitor in the normal group significantly increased the mRNA levels of genes *HK2*, *IDH2*, and *LDHA* ($p < 0.05$). The increasing effects in the radiation + hypoxia group were significantly inhibited after the addition of HIF-1α inhibitor, with regard to genes *FBP1*, *IDH2*, *GLUT1*, and *LDHA* ($p < 0.05$). However, the increased expression of HK2 was enhanced with the addition of HIF-1α inhibitor.

Discussion

To investigate the influence of radiation on glucose metabolism in hypoxic breast cancer cells, comprehensive examinations of the corresponding factors were performed. Glucose uptake rate and lactate release rate were increased under hypoxia and radiation condition, while the addition of HIF-1α inhibitor decreased these rates in hypoxia + radiation treated cells. In addition, the mRNA and protein levels of HIF-1α were significantly increased under hypoxia and radiation condition, while which decreased after the addition of HIF-1α inhibitor. Similar content changing trends were observed in FBP1, IDH2, GLUT1, and LDHA but not HK2.

It was well known that hypoxia was a pathophysiologic characteristic of solid malignancies [24], while radiotherapy had been considered as an important treatment modality in oncology through the induction of oxidative stress [25]. Under hypoxic conditions, tumor cells could contribute to anaerobic glycolysis, in which pyruvate, lactate, and hydrogen ions were produced [26, 27]. Similarly, our study showed that hypoxia could promote glycolysis. Previous study had shown that hypoxia might reduce tumor sensitivity to radiotherapy [28]. Interestingly, we also found that the combination of

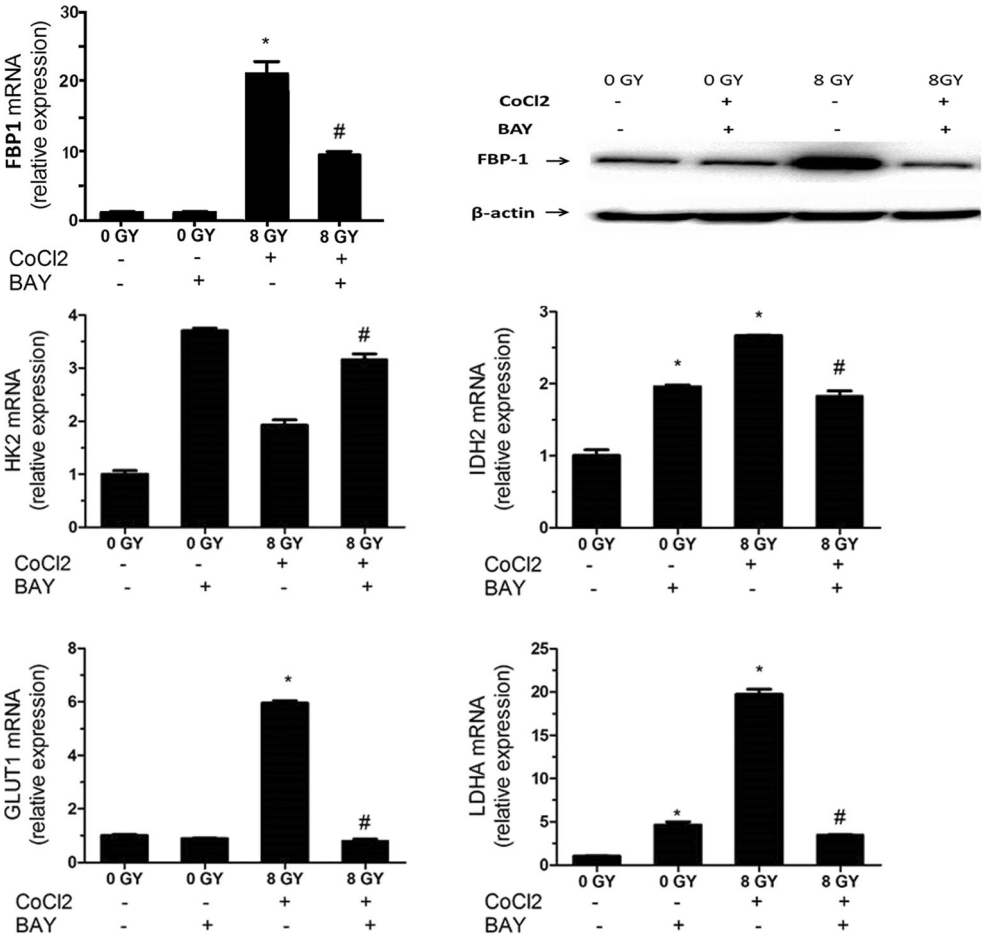

Fig. 4 Expressions of glucose metabolism related genes in breast cancer cells cultured under hypoxia and/or radiation conditions with or without the addition of HIF-1α inhibitor. $CoCl_2$ is used for the hypoxia treatment, 8 GY is used for the radiation treatment, and BAY is the HIF-1α inhibitor. FBP1, fructose biphoshatase 1; LDHA, lactate dehydrogenase A; HK2, hexokinase 2; IDH2, isocitrate dehydrogenase 2; GLUT1, glucose transporter 1. $^{*}p < 0.05$ versus 0 GY and no $CoCl_2$ group, $^{\#}p < 0.05$ versus 8 GY, $CoCl_2$ and no BAY group.

radiation and hypoxia had synergistic effect on glycolysis. This might be explained that hypoxic tumor cells could counter radiotherapy by the upregulation of glycolysis through accumulation of pyruvate and lactate [29].

In addition, we found that the glucose metabolic pathway related genes, including *FBP1*, *GLUT1*, *LDHA* and *IDH2*, significantly increased under the treatment of radiation and hypoxia in breast cancer cells. FBP1 was a rate limiting enzyme for gluconeogenesis [30]. Elevated FBP1 expression in cancer cells redirected cellular energy metabolism [31]. As one of the proteins responsible for increased glucose transport rate, GLUT1 expression was stimulated in cancer cells under hypoxia conditions, which was dually controlled by the low level of oxygen concentration as well as the inhibition of oxidative phosphorylation [32]. LDHA could convert pyruvate to lactate [33]. And the abundance of LDHA in tumors decreased as tumors regressed [34]. LHDA was believed to be involved in tumor maintenance, and its increased activity altered pyruvate rate in hypoxic cells [35]. In addition, IDH2 was associated with the metabolic transformation of cancer cells [36], and its expression was considerably elevated in cancer cells [37]. All these results indicated that radiation and hypoxia could promote the glucose metabolism through increasing the expression of these glucose metabolic pathway related genes.

HIF-1 was the regulatory center for the stable intracellular environment in hypoxia stress [11]. Its activation under hypoxic condition was believed to be closely related to a variety of oncogenic pathways and tumors [38], where HIF-1 functioned as a master regulator of several hypoxia-inducible genes. Our further results found that the mRNA and protein levels of HIF-1α were significantly increased under hypoxia and radiation condition in breast cancer cells. Similarly, the increased mRNA level of HIF-1α was proved in hypoxic adipocyte induced by $CoCl_2$ [39]. This might be explained that $CoCl_2$ could not only suppress HIF1α protein degradation, but also upregulate the mRNA levels of HIF1α; however, its mechanism still need to further investigated. In addition, we found that HIF-1α inhibitor BAY increased the expression of HIF-1α induced by $CoCl_2$. However, Ellinghaus et al. [40] showed that BAY inhibited the expression of HIF-1α protein under hypoxic conditions, but had no effect on HIF-1α protein levels induced by $CoCl_2$ in non-small cell lung cancer cells. This conflicting might be caused by the different cell lines and the treatment concentration of BAY. Furthermore, the HIF-1 pathway enabled tumor cells to survive by changing glucose metabolism toward a glycolytic phenotype. Tumor glucose metabolism could be targeted directly by inhibiting the related enzymes and transporters as well as indirectly through suppressing the expression of HIF-1 [41]. Previous study had proved that HIF-1 inhibition could result in glucose metabolic changes with a decreased rate of glucose uptake and lactate production in vitro [14]. Similarly, we found that the addition of HIF-1α inhibitor decreased glucose uptake and lactate release rates in hypoxia + radiation treated cells. Moreover, we found that HIF-1α inhibitor also reduced the expression of glucose metabolic pathway related genes. These results indicated that HIF-1α inhibitor could inhibit the promoting effect of radiation on glycolysis in hypoxic MCF-7 cells by regulating the glucose metabolic pathway.

Conclusions

In conclusion, the combination of radiation and hypoxia could promote the glucose metabolism. Furthermore, HIF-1α might inhibit the promoting effect of radiation on glycolysis in hypoxic MCF-7 cells by regulating the glucose metabolic pathway.

Compliance with Ethical Standards

Competing Interests The authors declare that they have no competing interests.

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