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Nicotinamide Inhibits Glycolysis of HL-60 Cells by Modulating Sirtuin 1 (SIRT1)/Peroxisome Proliferator-Activated Receptor γ Coactivator 1α (PGC-1α)/Hypoxia-Inducible Factor-2α (HIF2α) Signaling Pathway

Authors' Contribution:
Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
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Background: Nicotinamide can affect differentiation and proliferation of leukemia cells. This research aimed to explore the regulatory effect of nicotinamide on glycolysis metabolism of leukemia cells and to clarify the associated mechanisms.

Material/Methods: HL-60 cells were treated with nicotinamide and divided into 0.1, 1, and 10 μmol/l groups. HL-60 cells without any administration were assigned as negative control (CT group). Glucolytic activity was evaluated by detecting lactic acid production, and glucose level was measured using glucose consumption assay. Apoptosis of HL-60 was examined using flow cytometry assay, when cells were cultured for 24 h. Expressions of sirtuin 1 (SIRT1), peroxisome proliferator-activated receptor γ coactivator 1α (PGC-1α), and hypoxia-inducible factor-2α (HIF2α) were evaluated using a reverse transcription PCR assay and Western blotting assay, respectively.

Results: Nicotinamide remarkably decreased lactic acid production and glucose levels in leukemia cells compared with that of the CT group ($p<0.05$). Nicotinamide significantly induced the apoptosis of HL-60 cells compared to that of the negative control group ($p<0.05$). Nicotinamide significantly inhibited the SIRT1/PGC-1α/HIF2α signaling pathway mRNAs compared to that of the CT group ($p<0.05$). Nicotinamide remarkably reduced mitochondrial regulatory factors SIRT1/PGC-1α expression compared to that in the CT group ($p<0.05$). Nicotinamide obviously downregulated HIF2α compared with that of the CT group ($p<0.05$). Moreover, all of the above nicotinamide-induced effects, including glycolytic activity, apoptosis, and expression of SIRT1/PGC-1α/HIF2α, were changed in a dose-dependent manner.

Conclusions: Nicotinamide can inhibit glycolysis of HL-60 cells by inhibiting the mitochondrial regulatory factor SIRT1/PGC-1α and suppressing transcription factor HIF2α.

MeSH Keywords: Glycolysis • Leukemia • Nicorandil • Sirtuin 1

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Background

Leukemia is a group of highly heterogeneous malignant hematological diseases [1]. Leukemia is one of the 10 most common malignant tumors in China [2,3]. Leukemia demonstrates the highest morbidity and mortality in children and adolescents [4,5]. Although some patients with leukemia have specific chromosomal and genetic abnormalities, the pathogenesis has not been elucidated. A recent study [6] has shown that infinitely proliferating leukemia cells require vigorous glycometabolism to produce ATP to provide energy. Aerobic glycolysis can supply energy for cell growth and leads to high levels of glucose decomposition in many cancers [7]. Targeting glucose consumption or metabolism has been become an important strategy for eliminating cancer cells [8]. We speculated that there is an association between leukemia development or occurrence and glucose decomposition. Therefore, the relationship between leukemia cells and glycolysis and the specific mechanism involved need to be further studied.

Sirtuin 1 (SIRT1) is a member of the sirtuins deacetylase family that is involved in gene transcription, energy metabolism, and regulation of cell senescence [9,10]. Recent studies have shown that SIRT1 plays an important role in the occurrence and development of tumors [11,12]. Studies [13,14] have shown that SIRT1 is highly expressed in many kinds of cancer cells, and downregulation of SIRT1 can cause growth arrest and apoptosis of corresponding cancer cells. However, research on SIRT1 in leukemia is still preliminary. Abnormal energy metabolism is a distinct feature of tumors. SIRT1 is a key regulator of energy metabolism in mammals and can be used as a sensor and regulator of metabolic state *in vivo* [15]. Whether SIRT1 inhibitors can play an anti-tumor role by regulating the energy metabolism of leukemia cells is unclear. Moreover, peroxisome proliferator-activated receptor γ coactivator 1α (PGC-1α) coordinates many transcriptional processes that modulate glycolysis [16]. The hypoxia-inducible factor-2α (HIF2α) can modulate cell apoptosis, proliferation, and metabolism [17]. HIF2α is an important PGC-1α target in muscles that can be modulated by actions of SIRT1 and exercise [16]. Nicotinamide, as an amide derivative for VB3, plays crucial roles in many oxidation-reduction disorders by acting as a coenzyme [18]. Nicotinamide has been proven to protect against streptozotocin-caused diabetes, ischemia-reperfusion-induced acute lung injury, and cancers [19]. Previous studies also reported that nicotinamide can remarkably affect the differentiation of leukemia cells [20], and nicotinamide has lower toxicity *in vivo*, which could make it a candidate drug for treating diseases. However, the potential mechanism underlying the effects of nicotinamide on glycolysis metabolism of leukemia cells is unclear.

This study was designed to investigate the effects of nicotinamide, an inhibitor of SIRT1, on the glycolysis and metabolism pathway of human chronic myeloid leukemia cell line HL-60. We also attempted to clarify the role of the SIRT1/PGC-1α/HIF2α signaling pathway in the effects of nicotinamide, seeking to discover novel therapeutic targets for the treatment of refractory leukemia.

Material and Methods

Cell culture and trial grouping

Human chronic myeloid leukemia cell line HL-60 cells were purchased from the Basic College of Tongji Medical College, Huazhong University of Science and Technology (Wuhan, China). HL-60 cells were cultured in Roswell Park Memorial Institute 1640 (RPMI-1640) medium (Gibco BRL. Co., Grand Island, NY, USA), containing 10% FBS (Gibco BRL. Co.) supplemented with 100 U/ml penicillin/100 μg/ml streptomycin (Sigma-Aldrich, St. Louis, MO, USA) at 37° and 5% CO_2.

All experimental cells were taken at logarithmic growth phase, and the Trypan blue rejection rate was above 95%. The HL-60 cells were divided into 4 groups: a negative control group (CT group), a 0.1 μmol/l nicotinamide group, a 1 μmol/l nicotinamide group, and a 10 μmol/l nicotinamide group. The nicotinamide was purchased from Pfizer (Beijing, China), with purity greater than 99.5%.

Lactic acid production measurement

We evaluated lactic acid production (reflecting glucose consumption) in HL-60 cells. The HL-60 cells in each group were lysed using radioimmunoprecipitation assay (RIPA) lysis buffer. The supernatant of HL-60 cells after centrifugation was used for lactic acid detection. Lactic acid production was measured using the Lactic Acid Assay Kit (Cat. No. A019-2-1, Nanjing Jiangcheng Bioengineering Institute, Nanjing, China). Briefly, cell supernatant was incubated with the enzyme working fluid and chromogenic reagent at 37°C for 10 min. Then, the supernatants were terminated with the terminator. Finally, the absorbance of cells in each group was detected at a wavelength of 530 nm.

Evaluation of glucose levels in cells

Glucose levels in leukemia cells were detected. Briefly, the HL-60 cells in logarithmic growth phase were taken and cell concentration was adjusted to 1×10^6/ml. HL-60 cells were added into 96-well plates (Corning-Costar, Corning, NY, USA), adjusted amounts of 1×10^5/ml, and inoculated with 0.1 μg/ml, 1 μg/ml, and 10 μg/ml nicotinamide, culturing for 8 h, 16 h, and 24 h, respectively, at 37°C and 5% CO_2. The cultured cells were centrifuged at 2000 rpm for 5 min and the supernatant

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Table 1. Specific primers and amplification conditions for PCR assay.

Genes	Gene ID		Sequences	Annealing temperature (°C)	Cycle No. (n)	Length (bp)
SIRT1	23411	Forward	5'-ATCCCGTGGACCATACCT-3'	52	35	122
		Reverse	5'-AATGCGATTACGAAGCA-3'			
PGC-1α	10891	Forward	5'-CGAGTACCGGGAATTAT-3'	52	35	100
		Reverse	5'-AATGGCTGGAACCCGGA-3'			
HIF2α	3091	Forward	5'-GGACGCTATCGCGTGGA-3'	52	35	137
		Reverse	5'-GAGTTGAGAATGCAAGTT-3'			

was collected. The glucose levels were detected using the Glucose (HK) Assay Kit (Cat. No. GAHK20, Sigma-Aldrich, St. Louis, MO, USA) based on the protocol of the manufacturer. The absorbance of all groups was observed at 350 nm. The glucose content was calculated according to the following formula: glucose (mg/ml)=(sample A-reagent blank-sample A blank)×reaction volume (ml)×dilution multiple×0.029/sample volume (ml).

Flow cytometry assay

The apoptosis of HL-60 cells was evaluated using flow cytometry assay. The apoptosis was detected using the Annexin V-FITC/PI Apoptosis Detection Kit (Cat. No. CA1020, SolarBio. Sci. Tech. Co., Beijing, China), according to the manufacturer's protocol. In brief, HL-60 cells in logarithmic growth phase were incubated with 0.1 μg/ml, 1 μg/ml, and 10 μg/ml nicotinamide and then cultured at 37°C and 5% CO_2. Subsequently, the HL-60 cells in logarithmic phase were collected and inoculated into 96-well plates at a density of 1×10^5/ml for the flow cytometry assay.

Reverse transcription PCR assay

Gene expressions of SIRT1, PGC-1α, and HIF2α in HL-6 cells were detected using reverse transcriptional PCR assay. First, total RNAs in HL-60 cells were extracted with the One-step Total RNA Extractor (Trizol) regent (Cat. No. B511311, Sangon Biotech. Co., Shanghai, China). Then, the extracted total RNA was used to synthesize the complementary DNA (cDNA) using the High-capacity cDNA Reverse Transcription Kit (Cat. No. 4374967, Invitrogen/Life Technologies, Carlsbad, CA, USA). The specific PCR primers and reaction conditions are listed in Table 1. The PCR products were loaded onto 1.5% agarose gel for electrophoresis. The images of PCR products were scanned to obtain the grey value using Quantity-One Software (version: 4.6.9, Bio-Rad. Lab. Inc., Hercules, CA, USA). β-actin was used as an internal control for the mRNA expression. Each mRNA expression level was represented as the grey value and standardized to the housekeeping gene (β-actin).

Western blotting assay

The HL-60 cells in each group were homogenized in the presence of radioimmunoprecipitation assay (RIPA) lysis buffer (Cat. No. P0013C, Beyotime Biotech., Shanghai, China) containing protease inhibitors, and the protein content or concentration of supernatant was evaluated using the BCA Protein Assay Kit (Cat. No. 23225, Pierce, Rockford, IL, USA). The protein sample (about 20 μg) deriving from homogenates was loaded and separated using 15% sodium dodecyl sulphate-poly acrylamide gel electrophoresis (SDS-PAGE, Sangon Biotech.). Then, the proteins were electrotransferred onto polyvinylidene fluoride membranes (PVDF, Cat. No. FFP24, Beyotime Biotech., Shanghai, China). The PVDF membranes were blocked using 5% bovine serum albumin (BSA, Sangon Biotech.) in phosphate-buffered saline (PBS, Beyotime Biotech.) containing 0.05% Tween-20 (Sangon Biotech.) for 1 h at room temperature. Subsequently, PVDF membranes were incubated using mouse anti-human SIRT1 polyclonal antibody (Cat. No. #8469), rabbit anti-human PGC-1α polyclonal antibody (Cat. No. #2178), rabbit anti-human HIF2α polyclonal antibody (Cat. No. #59973), and mouse anti-human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) monoclonal antibody (Cat. No. #97166) at 4°C overnight. All primary antibodies were obtained from Cell Signaling Technology (Beverly, MA, USA). Then, the PVDF membranes were washed with PBS and continuously incubated using horseradish peroxidase (HRP)-labeled goat anti-rabbit IgG (Cat. No. ab205718, Abcam Biotech., Cambridge, MA, USA) and HRP-conjugated goat anti-mouse IgG (Cat. No. ab205719, Abcam Biotech., Cambridge, MA, USA) at 37°C for 2 h. The binding of the above antibodies in Western blotting assay was visualized with the Pierce Electrochemiluminescence (ECL) Western Blotting Kit (Cat. No. 32209, Thermo-Pierce, Rockford, IL, USA). Western blot bands (grey value) were analyzed using the Gel-Image Scanning System GDS8000 (UVP corporation, Sacramento, CA, USA). The protein expression was defined as the relative values of targeting protein grey value normalized to housekeeping protein (GAPDH) grey value.

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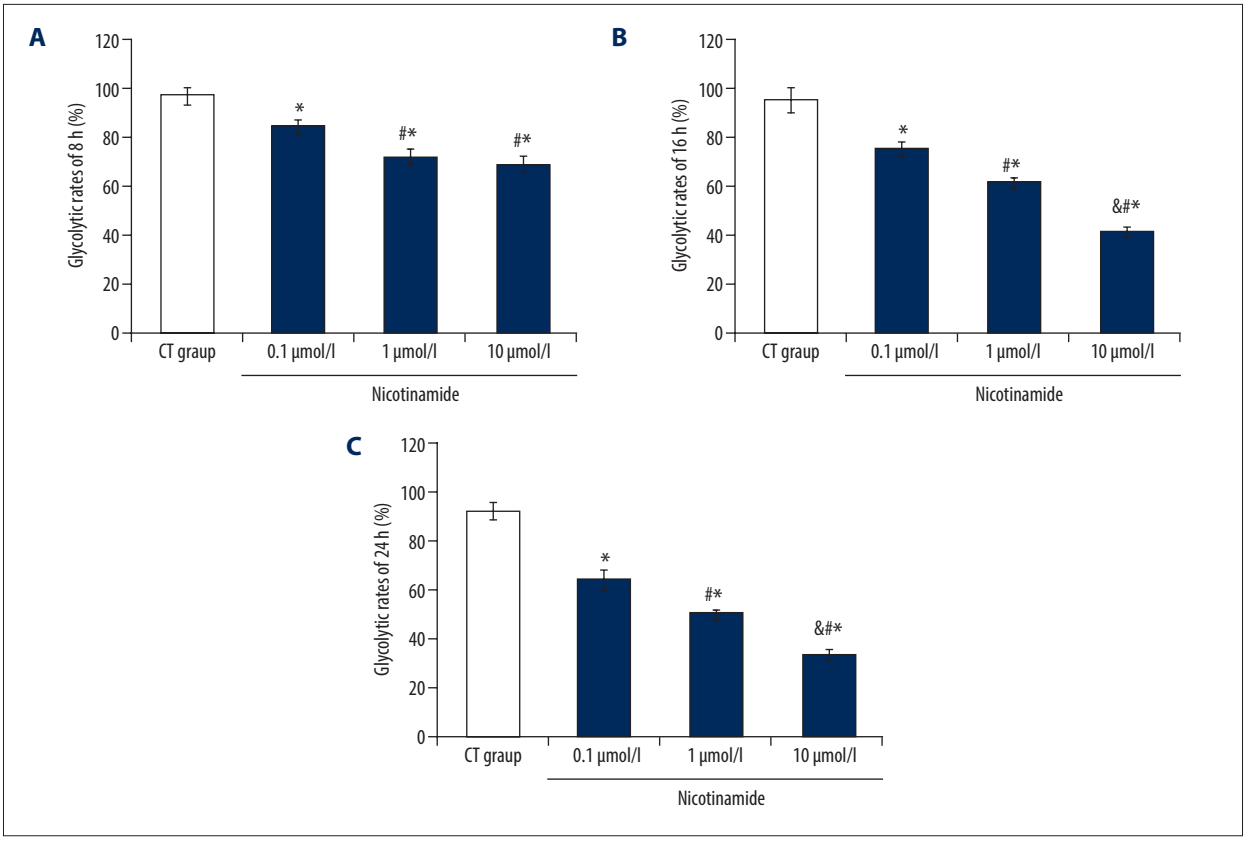

Figure 1. Nicotinamide reduced glucose levels in leukemia cells. The glucose levels in HL-60 cells cultured for 8 h (**A**), 16 h (**B**), and 24 h (**C**) were statistically analyzed. * $p<0.05$ *vs.* CT group. CT group: negative control group. # $p<0.05$ *vs.* 0.1 μg/ml nicotinamide group. & $p<0.05$ *vs.* 1 μg/ml nicotinamide group.

Statistical analysis

Data are represented as mean±standard deviation (SD) and were analyzed using professional SPSS software 20.0 (SPSS Inc., Chicago, IL, USA). Tukey's post hoc test-validated analysis of variance (ANOVA) was employed to compare differences in variables among multiple groups and the *t* test was used to compare difference in variables between 2 groups. All tests and experiments were performed for at least 6 replicates. $p<0.05$ was regarded as indicating a statistically significant difference.

Results

Nicotinamide decreased glucose levels in leukemia cells

The results showed that nicotinamide significantly inhibited the glucose levels of HL-60 cells, which was time-dependent and concentration-dependent (Figure 1). Compared with the control group, 0.1 μg/ml nicotinamide began to inhibit glucose levels in HL-60 cells after 8 h of intervention (Figure 1A, $p<0.05$). After the intervention with increased concentration of nicotinamide for 16 h, the glucose levels in HL-60 cells was remarkably decreased (Figure 1B, $p<0.05$). In addition, compared with the control group, 10 μg/ml nicotinamide had the highest inhibitory effect on glucose levels of HL-60 cells at 24 h after the intervention, and the relative glucose levels significantly decreased (Figure 1C, $p<0.05$).

Nicotinamide reduced lactic acid production in leukemia cells

The results of lactate testing showed that nicotinamide significantly inhibited the lactic acid production (glucolytic activity) of HL-60 cells, which was time-dependent and concentration-dependent (Figure 2). Compared with the CT group, 0.1 μmol/l nicotinamide began to decrease lactic acid production in HL-60 cells at 8 h after the intervention (Figure 2A, $p<0.05$). Nicotinamide also reduced the lactic acid production in HL-60 cells compared to that of CT group at 16 h after the intervention (Figure 2B, $p<0.05$). In addition, 10 μmol/l nicotinamide demonstrated the lowest lactic acid production and the highest inhibitory effect on lactic acid production in HL-60 cells at 24 h after the intervention (Figure 2C, $p<0.05$).

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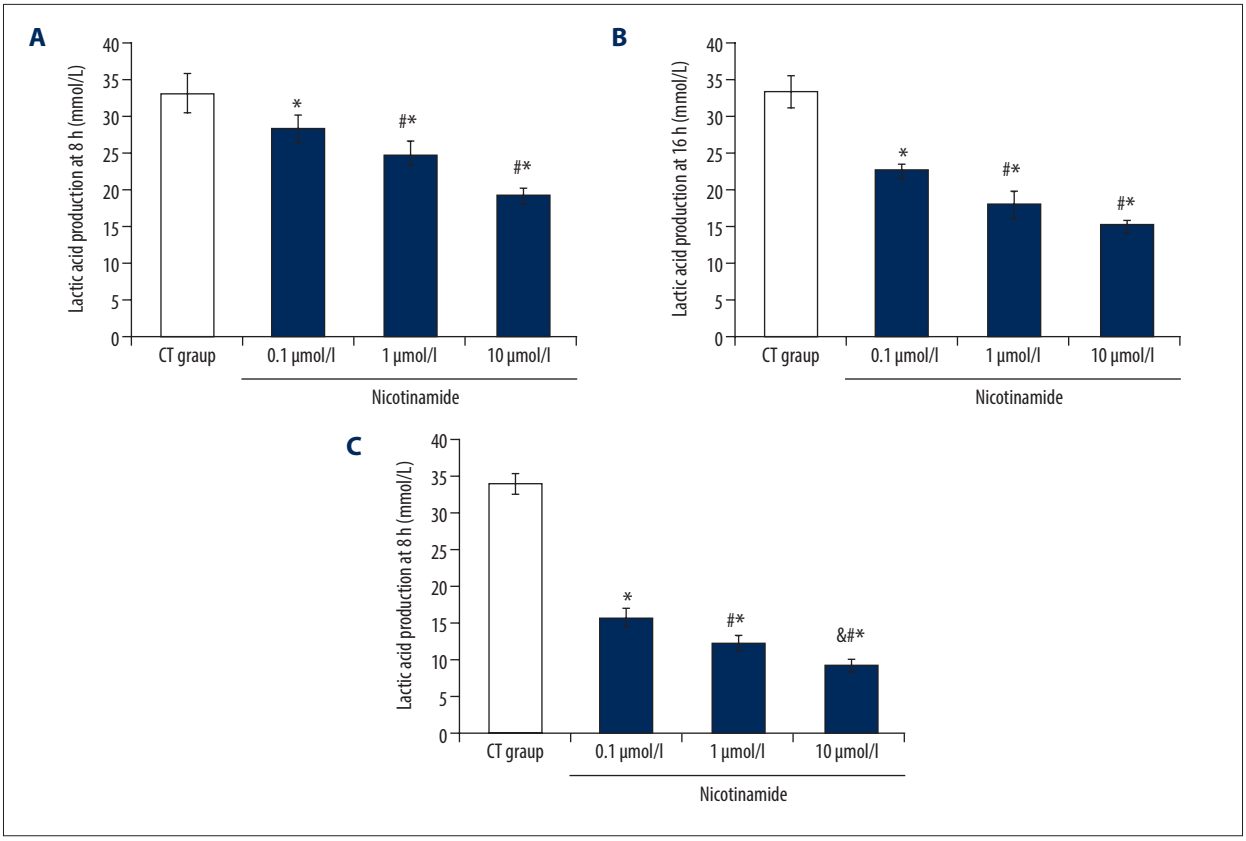

Figure 2. Nicotinamide inhibited lactic acid production in leukemia cells. Lactic acid production in HL-60 cells cultured for 8 h (**A**), 16 h (**B**), and 24 h (**C**) were statistically analyzed. * $p<0.05$ *vs.* CT group. CT group: negative control group. # $p<0.05$ *vs.* 0.1 μg/ml nicotinamide group. & $p<0.05$ *vs.* 1 μg/ml nicotinamide group.

Nicotinamide induced HL-60 cell apoptosis

The flow cytometry findings illustrated that nicotinamide could induce apoptosis of HL-60 cells in a concentration-dependent manner at 24 h after the intervention (Figure 3A). The effect of 0.1 μg/ml nicotinamide on inducing apoptosis began to appear after the intervention, and the difference was significant compared with that of the control group (Figure 3B, $p<0.05$). Meanwhile, with the increasing concentration of the nicotinamide, the apoptotic rate of HL-60 cells increased remarkably. The 10 μg/ml nicotinamide treatment demonstrated the strongest effects on HL-60 cells apoptosis, with higher apoptotic rate, exhibiting significant differences compared with the control group (Figure 3B, $p<0.05$).

Nicotinamide modulated SIRT1/PGC-1α signaling molecules

The reverse transcriptional PCR findings illustrated that the SIRT1 and PGC-1α were positively expressed in the CT group (Figure 4A). At 24 h after the nicotinamide intervention, compared with the CT group, expressions of SIRT1 and PGC-1α genes in HL-60 cells of the 3 treatment groups decreased in a concentration-dependent manner (Figure 4B, $p<0.05$). The SIRT1 and PGC-1α gene demonstrated the most significantly downregulating effects in 10 μg/ml nicotinamide-treated HL-60 cells, illustrating statistical differences when compared with the control group (Figure 4B, all $p<0.05$).

Expressions of SIRT1 and PGC-1α were also examined using Western blot assay (Figure 5A), showing that at 24 h after the HL-60 culture, SIRT1 and PGC-1α were positively expressed at higher levels (Figure 5B). The SIRT1 and PGC-1α expressions in the 0.1 μg/ml, 1 μg/ml, and 10 μg/ml nicotinamide groups were all significantly lower than in the control group (Figure 5B, all $p<0.05$), in a concentration-dependent manner. The 10 μg/ml nicotinamide treatment group demonstrated the lowest relative expressions of SIRT1 and PGC-1α comparing with that of the control group (both $p<0.05$).

Nicotinamide downregulated expression of transcription factor HIF2α

In this study, we also determined expression of transcription factor HIF2α using reverse transcriptional PCR (Figure 6A) and Western blotting assay (Figure 6B). The results showed

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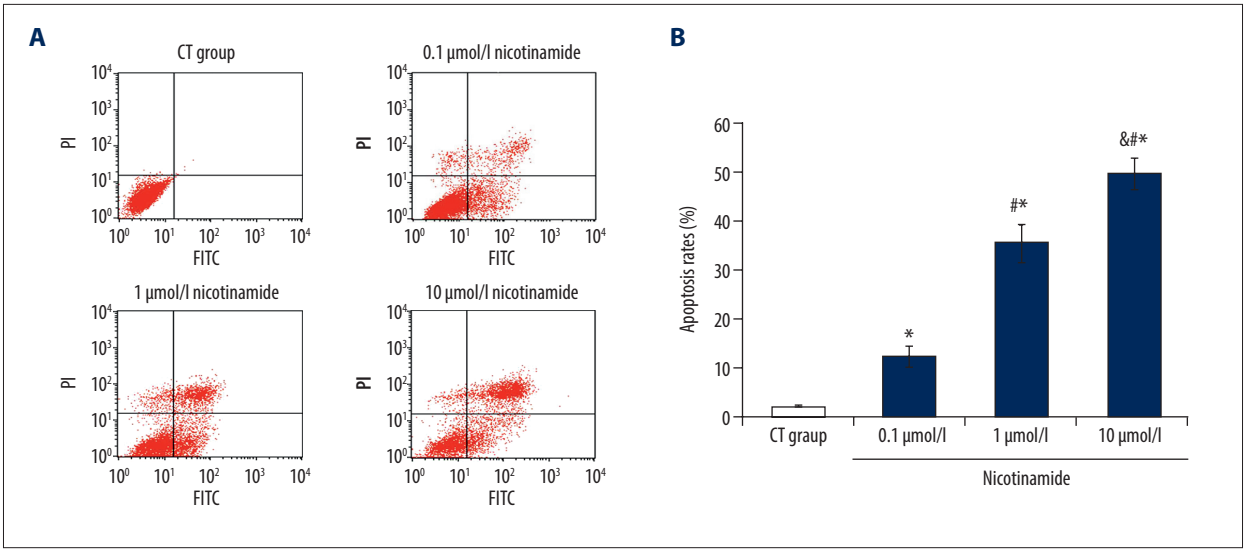

Figure 3. Nicotinamide treatment induced apoptosis of HL-60 cells. (**A**) Flow cytometry images for determined apoptosis of HL-60 cells. (**B**) Statistical analysis of the apoptosis rate of HL-60 cells in each group. * $p<0.05$ *vs.* CT group. CT group: negative control group. # $p<0.05$ *vs.* 0.1 μg/ml nicotinamide group. & $p<0.05$ *vs.* 1 μg/ml nicotinamide group.

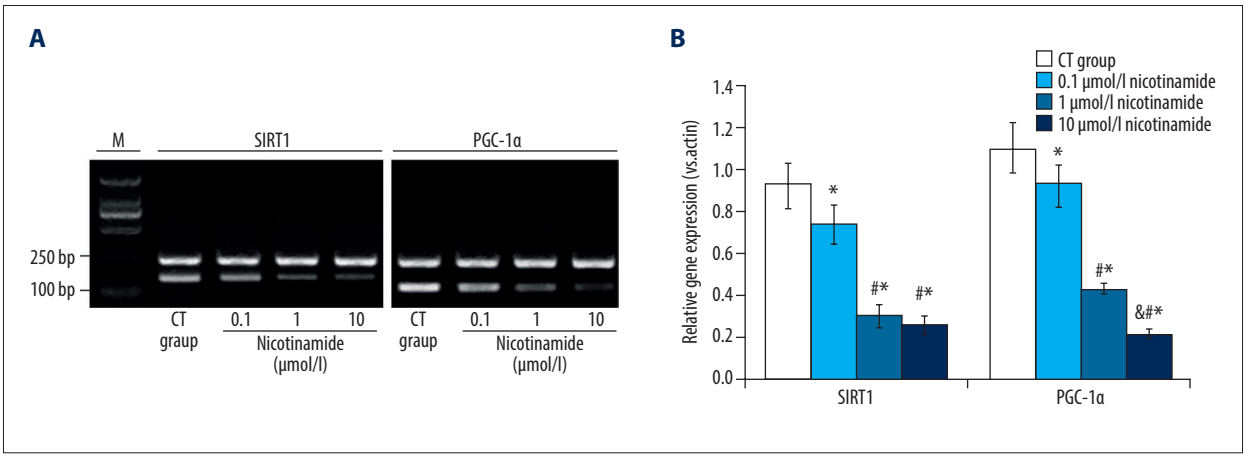

Figure 4. Nicotinamide regulated the SIRT1/PGC-1α mRNA expression in HL-60 cells. The changes of SIRT1/PGC-1α gene expression in HL-60 cells were determined with reverse transcriptional PCR assay. (**A**) Agarose gel electrophoresis images for the amplifying products of SIRT1/PGC-1α gene expression. (**B**) Statistical analysis for relative gene expression of SIRT1/PGC-1α molecules. * $p<0.05$ *vs.* CT group. CT group: negative control group. # $p<0.05$ *vs.* 0.1 μg/ml nicotinamide group. & $p<0.05$ *vs.* 1 μg/ml nicotinamide group.

that the nicotinamide treatments at different dosages all significantly inhibited expressions of HIF2α mRNA (Figure 6A) and protein (Figure 6B) in HL-60 cells after 24-h culture compared to that in the control group (all $p<0.05$). Especially in the 10 μg/ml nicotinamide administration, the HL-60 cells exhibited the most significant inhibition of HIF2α mRNA (Figure 6A) and protein (Figure 6B) expression compared with that in the control group (all $p<0.05$).

Discussion

Normal cells usually get energy through oxidative phosphorylation of the mitochondrial respiratory chain under aerobic conditions, while glycolysis is the most important route under hypoxic conditions. However, growth of cancer cells under aerobic conditions mainly uses glycolysis for energy. The most prominent characteristics of the metabolic reprogramming in cancer cells is the Warburg effect [21]. The pathogenesis of cancer involves reprogramming of cancer cell metabolism, such as avoidance of epidemic disease and tumor metastasis [22].

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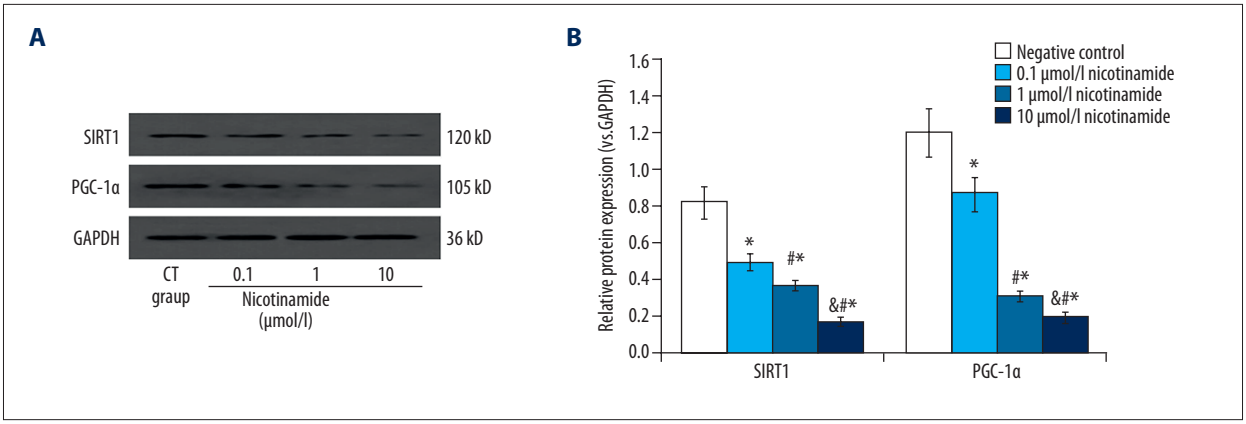

Figure 5. Nicotinamide modulated SIRT1/PGC-1α protein expression in HL-60 cells. (**A**) The protein expressions of SIRT1/PGC-1α in HL-60 cells were detected using Western blot assay. (**B**) Statistical analysis for the relative SIRT1 and PGC-1α expression. * $p<0.05$ *vs.* CT group. CT group: negative control group. # $p<0.05$ *vs.* 0.1 μg/ml nicotinamide group. & $p<0.05$ *vs.* 1 μg/ml nicotinamide group.

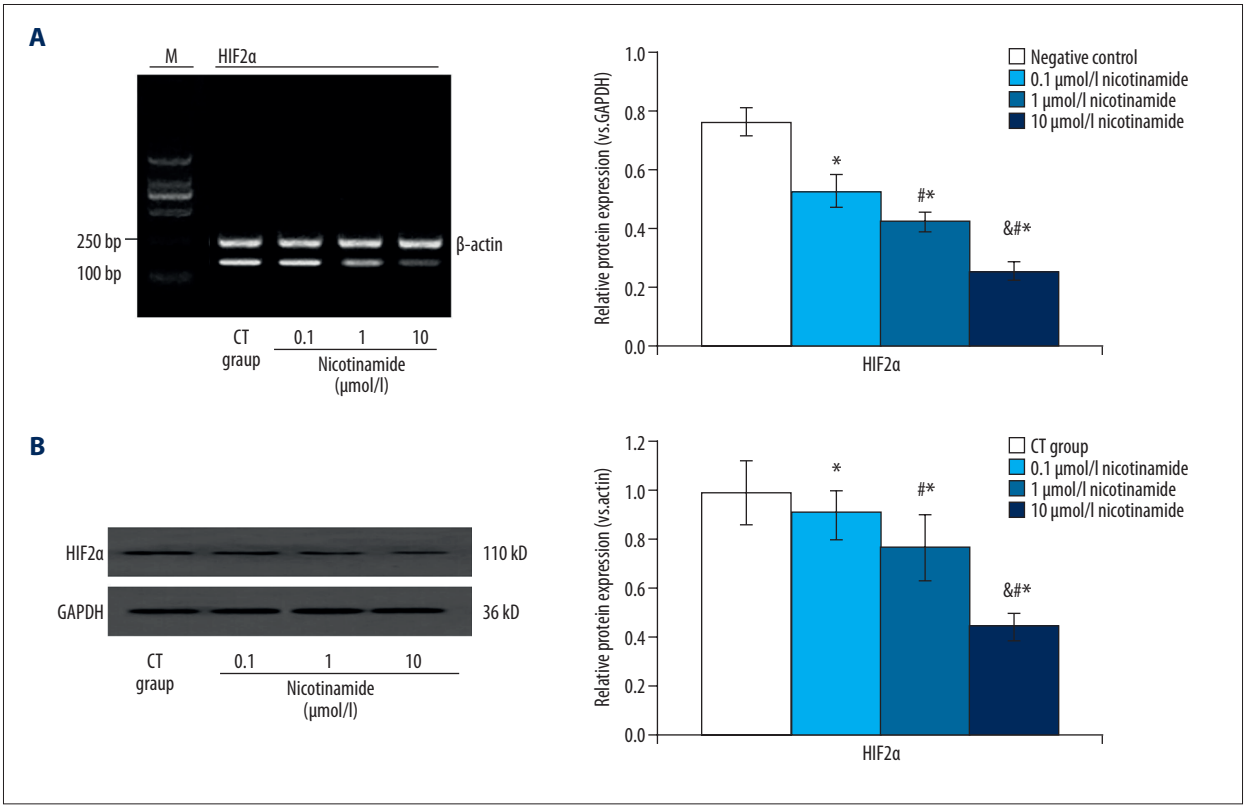

Figure 6. Nicotinamide decreased the expression of transcription factor HIF2α. (**A**) Evaluation of HIF2α gene expression in HL-60 cells using reverse transcriptional PCR assay. The statistical analysis results showed that nicotinamide downregulated HIF2α gene expression. (**B**) Determination of protein expression in HL-60 cells with Western blot assay. The statistical analysis results indicated that nicotinamide reduced HIF2α protein expression. * $p<0.05$ *vs.* CT group. CT group: negative control group. # $p<0.05$ *vs.* 0.1 μg/ml nicotinamide group. & $p<0.05$ *vs.* 1 μg/ml nicotinamide group.

In this study, lactic acid production (reflecting glucose consumption) was evaluated to reflect the glucolytic activity in HL-60 cells. Our results showed that nicotinamide remarkably decreased lactic acid production of HL-60 cells, which suggests that nicotinamide inhibits the growth of HL-60 cells by triggering the reduction of glycolytic activity. We also measured the glucose levels in leukemia cells treated with nicotinamide, showing that nicotinamide significantly reduced the

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glucose levels in HL-60 cells, which suggests that nicotinamide decreases glucose consumption. We also found that nicotinamide remarkably increased the apoptosis rates of HL-60 cells, which suggests that nicotinamide induced apoptosis. All of the above results demonstrate that glycolysis is associated with the growth of HL-60 cells. Then, we explored the mechanism by which nicotinamide triggers reduction of glycolytic activity.

SIRT1 participates in gene transcription, energy metabolism, and cell senescence, and regulates it [23,24]. Previous studies [25,26] have shown that SIRT1 plays a crucial role in the occurrence and development of tumors by regulating cell senescence and apoptosis. In addition, SIRT1, as an inductor and regulator of metabolic state *in vivo*, can also regulate energy metabolism in mammals. However, inhibitive effects of SIRT1 on energy metabolism in leukemia are unclear [27]. In the present study, nicotinamide, a specific inhibitor of SIRT1, was used to block the expression of SIRT1, and we also assessed the effects of nicotinamide on glycolysis and metabolism of leukemia cells. We found that nicotinamide induced apoptosis and inhibited glycolysis in HL-60 cells in a dose-dependent manner. Therefore, it is necessary to further investigate the expression of glycolysis-related factors in nicotinamide-treated HL-60 cells.

Because SIRT1 and PGC-1α can regulate the expression of coding genes in nuclei and mitochondria [28], SIRT1 and PGC-1α might regulate mitochondrial biosynthesis by influencing expression of mitochondrial transcription factor A and mediate cross-expression of nuclear and mitochondrial genes, finally affecting the energy metabolism of cells. PGC-1α can also interact with SIRTI and modulate glycolysis processes [16]. Therefore, this study explored the expression of SIRT1 and PGC-1α in HL-60 cells treated with nicotinamide. Our results showed that the expressions of SIRT1 and SIRT1 and PGC-1α in leukemia cells treated with nicotinamide were significantly downregulated in a dose-dependent manner.

Hypoxia-inducible factor (HIF) is one of the transcription factors and is a key speed-limiting enzyme in the aerobic glycolysis pathway of cancer cells [29]. HIF is also a cell receptor in response to hypoxia. Hypoxia in the tumor microenvironment can cause abnormal accumulation of HIF protein and significantly reduce the efficiency of oxidative phosphorylation, which prompts cancer cells to turn to glycolysis to obtain energy for survival [30]. In muscle tissues, PGC-1α targets HIF2α and interacts with SIRT1 [16]. SIRT1-activated HIF2α might be involved in the glycolysis pathway of tumors. Therefore, we speculated that HIF2 participates in the regulation of energy metabolism reprogramming in cancer cells. Our findings showed that expression of HIF2α in leukemia cells treated with nicotinamide was significantly inhibited in a dose-dependent manner. These results in SIRT1/PGC-1α and HIF2α suggest that nicotinamide downregulates the glycolytic activity of leukemic cells by inhibiting key speed-limiting enzymes of glycolysis. However, whether other signaling pathways play roles in glycolysis of HL-60 cells remains to be further clarified. We used reverse transcription PCR assay for quantification of gene expression, which is seldom used and less reliable. In further research, we plan to use real-time PCR analysis to evaluate gene expression.

Conclusions

This study demonstrated that nicotinamide remarkably decreased lactic acid production (glycolytic activity) and glucose levels, and induced the apoptosis of HL-60 cells. Nicotinamide significantly inhibited expressions of molecules in the SIRT1/PGC-1α/HIF2α signaling pathway. In summary, nicotinamide inhibits glycolytic activity and induces apoptosis of HL-60 cells through regulating the SIRT1/PGC-1α/HIF2α signaling pathway.

Conflict of interest

None.

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