Cobalt Chloride Induced Apoptosis by Inhibiting GPC3 Expression via the HIF-1α/c-Myc Axis in HepG2 Cells

Purpose: To investigate the role of glypican-3 (GPC3) in cobalt chloride (CoCl2)-induced cell apoptosis in hepatocellular carcinoma.

Methods: HepG2 cells were treated with CoCl2 in the absence or presence of GPC3 plasmid, and the growth and apoptosis were assessed using MTT assay and flow cytometry, respectively. The expression of GPC3, hypoxia-inducible factor 1α (HIF-1α), c-myc, sp1, poly-ADP-ribose polymerase (PARP) and caspase-3 was determined by real-time PCR, Western blotting, and immunofluorescence. The expression of GPC3 was decreased, and the expression of sp1 and c-myc were knocked down when HIF-1α was inhibited.

Results: CoCl2 significantly inhibited the proliferation of HepG2 cells and induced apoptosis. Additionally, the expression of GPC3 mRNA and protein was decreased, and the expression of GPC3 was attenuated. Further studies showed that CoCl2 increased the expression of HIF-1α while reducing the expression of sp1 and c-myc; knockdown of HIF-1α elevated the expression of GPC3, sp1, and c-myc.

Conclusion: CoCl2 inhibited the growth of HepG2 cells through downregulation of GPC3 expression via the HIF-1α/c-myc axis.

Keywords: cobalt chloride, c-myc, glypican-3, hepatocellular carcinoma, hypoxia-inducible factor 1α

Introduction

Hepatocellular carcinoma (HCC) is the most common malignancy, ranking third in morbidity and fifth in mortality among cancers worldwide. It is especially prevalent in Asia and sub-Saharan Africa. The complex mechanism underlying HCC carcinogenesis has yet to be elucidated for the development of effective targeted drugs. Therefore, the identification of the pathogenic mechanism of HCC is crucial for HCC therapy.

Tumour growth relies on the formation of new blood vessels to supply oxygen and nutrition. It has been demonstrated that oxygen deficiency modulates tumour growth, angiogenesis, vascular invasion, and metastasis by hypoxia-induced target genes, which is primarily mediated by hypoxia-inducible factor 1α (HIF-1α). When the tumour volume reaches 1–2 mm³, angiogenic factors, such as VEGF, are upregulated by HIF-1α and released to accelerate neovascularization. As a solid tumour, the hypoxic environment plays an important role in HCC tumour progression and metastasis.

Glypican-3 (GPC3) is a member of the heparan sulfate proteoglycan family, anchoring at the cell membrane by glycosylphosphatidylinositol. GPC3 is highly expressed in HCC tissues and has recently been identified as a novel potential HCC.
Several studies have shown that GPC3 plays a major role in HCC development and progression. However, the mechanism of hypoxia-mediated GPC3 regulation in HCC tissues is unknown. In the present study, CoCl$_2$ was used to mimic cell hypoxia in HCC cells and investigate the effect of hypoxia on GPC3 expression, as well as explore the role of GPC3 in hypoxia-induced cell apoptosis in HCC.

**Materials and Methods**

**Chemicals and Reagents**

CoCl$_2$ was purchased from Sigma-Aldrich (St. Louis, MO, USA). Primers for GAPDH, GPC3, and HIF-1$\alpha$ were synthesized by Sangon Biotech (Shanghai, China). The protease inhibitor was purchased from Roche (Mannheim, Germany). PowerUp™ SYBR™ Green Master Mix was purchased from Applied Biosystems (Foster City, CA, USA). Mouse anti-human monoclonal antibodies against $\beta$-actin and GPC3 were acquired from Santa Cruz Biotechnology (1:1000, Santa Cruz, CA, USA). Rabbit anti-human monoclonal antibodies against HIF-1$\alpha$, c-myc, spl, PARP and caspase-3 were obtained from Cell Signaling Technology (1:2000, Danvers, MA, USA). Anti-rabbit and anti-mouse IgG HRP-linked antibodies were procured from Cell Signaling Technology (1:1000, Danvers, MA, USA). Anti-human monoclonal antibodies against HIF-1$\alpha$, c-myc, spl, PARP and caspase-3 were obtained from Santa Cruz Biotechnology (1:1000, Santa Cruz, CA, USA). Rabbit anti-human monoclonal antibodies against HIF-1$\alpha$, c-myc, spl, PARP and caspase-3 were obtained from Cell Signaling Technology (1:2000, Danvers, MA, USA). RIPA lysis buffer was obtained from Beyotime Institute of Biotechnology (Shanghai, China).

**Cell Culture**

HepG2 cells were purchased from ATCC (Manassas, VA, USA) and maintained in DMEM medium (Gibco, Grand Island, NY, USA) with 10% foetal bovine serum (Gibco, Grand Island, NY, USA), 1% penicillin-streptomycin (10,000 U/mL penicillin and 10 mg/mL streptomycin) at 37 °C in a humidified atmosphere with 5% CO$_2$. The cells were passaged using 0.25% trypsin (Gibco, Grand Island, NY, USA).

**Cell Viability Assay**

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) (Beyotime Institute of Biotechnology, Shanghai, China) was used to assess cell viability according to the manufacturer’s instructions. Briefly, $2\times10^4$ HepG2 cells/well were seeded in 96-well plates and cultured for 24 h. The medium was replaced with 100 μL/well fresh medium containing various concentrations (0, 50, 100, and 200 μmol/L) of CoCl$_2$ for 24 h. Then, 20 μL of 5 mg/mL MTT was added to each well and incubated at 37 °C for 4 h. Subsequently, the reaction was quenched by adding 150 μL DMSO, and the absorbance was measured at 490 nm with a microplate reader (Foster City, CA, USA).

**Flow Cytometry**

To confirm the effects on cell apoptosis, annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) double staining was performed with an annexin V-FITC apoptosis detection kit (BD Biosciences, Bedford, MA, USA) as according to the manufacturer’s instructions. Briefly, the cells were harvested and resuspended in 1× annexin V binding buffer at a concentration of $1\times10^6$ cells/mL. Then, 100 μL of this suspension was incubated with 5 μL FITC annexin V and 5 μL PI for 15 min at room temperature. The stained cells were analysed by flow cytometry (Beckman Coulter, CA, USA) within 1 h.

**Real-Time PCR**

Real-time PCR was performed as described previously. Total RNA was extracted using TRIzol reagent. Approximately 1 μg of RNA from each sample was used to synthesize cDNA using the PrimeScript™ RT reagent kit with gDNA Eraser (TakaraBio, Inc., Otsu, Japan). PCR was performed using PowerUp™ SYBR™ Green Master Mix on a StepOne Plus instrument (Applied Biosystems, Foster City, CA, USA) according to the following programme: 30 s at 95 °C and 60 s at 60 °C for 40 cycles. The PCR primers were as follows: GAPDH-F: 5′-CTGGGCTACACTGAGCACC-3′; GAPDH-R: 5′-AAGTGTCGTTGAGGGCAATG-3′; GPC3-F: 5′-ATTGGCAAAGTATGTCCTAT-3′; GPC3-R: 5′-TCCGCTGGATAAGGTTTCTTC-3′; HIF-1$\alpha$-F: 5′-GAACGTCGTTGAGGGCAATG-3′; and HIF-1$\alpha$-R: 5′-CTGGATAAGGTTTCTTC-3′. GAPDH was used to normalize mRNA expression. Quantification of the real-time PCR results was performed with the 2$^{-}\Delta\Delta$CT method.

**Western Blot Analysis**

HepG2 cells were treated with CoCl$_2$ and harvested in RIPA lysis buffer (50 mM Tris-HCl pH 7.4, 0.5% sodium deoxycholate, 150 mM NaCl, 1% NP-40, and 0.1% sodium dodecyl sulfate) containing protease inhibitors. Then, the cells were lysed for 30 min on ice and subjected to centrifugation at 4 °C to collect the supernatant of the lysates. An equivalent amount of protein extracts was separated by SDS-PAGE and transferred to a nitrocellulose membrane. Subsequently, the membrane was blocked with 5% non-fat milk at room temperature and probed with primary antibodies overnight at 4 °C,
followed by incubation with HRP-conjugated secondary antibodies at room temperature for 2 h. The immunoreactive proteins were visualized using an ECL kit (Millipore, Billerica, MA, USA).

**Immunofluorescence and Laser Confocal Microscopy**

HepG2 cells were cultured on glass chamber slides in the presence or absence of 200 μmol/L CoCl₂ for 24 h, fixed with 4% paraformaldehyde, permeabilized with 0.3% Triton X-100, and blocked with 5% normal goat serum for 1 h at room temperature. Next, the cells were incubated with primary antibody (1:200) at 4 °C overnight, followed by probing with Alexa Fluor® 488-conjugated goat anti-rabbit or anti-mouse IgG (H+L) (1:100, ZSGB-BIO, Beijing, China). DAPI (Beyotime Institute of Biotechnology, Shanghai, China) was used for nuclei staining. The fluorescence intensity was analysed by confocal laser microscopy (Olympus Corporation, Japan).

**Vector Construction and siRNA Transfection**

The GPC3 plasmid was constructed in the pcDNA3.1(-) vector (Addgene, Watertown, MA, USA). The recombinant plasmid was verified by enzyme cleavage and sequencing analysis. The HIF-1α-targeting siRNA and control siRNA were synthesized by Guangzhou RiboBio (Guangzhou, China). HepG2 cells were seeded in 6-well plates and cultured overnight before plasmid or 20 nmol/L siRNA transfections were performed using Lipofectamine 3000™ (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s protocol.

**Luciferase Reporter Assay**

HepG2 cells were plated at a density of 2×10⁴ cells/well in 24-well plates. Then, the cells that had been transfected with the c-myc luciferase reporter plasmid (Genomeditech, Shanghai, China) were cultured in the presence or absence of 200 μmol/L CoCl₂ for 24 h. The cells were also co-transfected with pRL-TK (Renilla luciferase vector) for background normalization. The plasmid transfection was performed using Lipofectamine™ 3000 transfection reagent. After 24 h, the cells were lysed, and luciferase activity was detected using the Genecopoeia Luc-Pair Duo-Luciferase Assay Kit (Genecopoeia, Inc., Shanghai, China) according to the instructions recommended by the manufacturer.

**Statistical Analysis**

All experiments were repeated at least two times. Data are presented as the mean ± standard error. Student’s t-test was used for data analysis using SPSS 17.0 software. P<0.05 was considered to be statistically significant.

**Results**

CoCl₂ Induced Hypoxia Injury in HepG2 Cells by Inhibiting GPC3 Expression

It has been demonstrated that CoCl₂ induces apoptosis in several types of tumour cells. To explore the effect of CoCl₂ on HCC cells, HepG2 cells were treated with different concentrations of CoCl₂ for 24 h, and then cell viability and apoptosis were assessed by MTT assay and flow cytometry, respectively. As shown in Figure 1, CoCl₂ significantly reduced cell viability and induced cell apoptosis in a concentration-dependent manner, and cell apoptosis was further verified by the activation of caspase-3 and decreased expression of poly-ADP-ribose polymerase (PARP). The present study also confirmed that CoCl₂ successfully induced hypoxia in HepG2 cells, indicated by increased expression of HIF-1α protein (Figure 2). Interestingly, the HIF-1α mRNA level was downregulated, which might be a negative feedback mechanism to maintain homeostasis of the HIF-1α protein level. Moreover, the expression of GPC3 was detected at both the mRNA and protein levels. Compared to the levels in the control group, 50–200 μmol/L CoCl₂ treatment reduced the GPC3 mRNA level by more than 80%; accordingly, the protein level assessed by Western blotting and immunofluorescence was also significantly decreased in a concentration-dependent manner (Figure 2). Notably, immunofluorescence results suggested that CoCl₂ also induced the translocation of GPC3 from the cytoplasm to the membrane, but the underlying mechanism remains to be investigated.

CoCl₂ Downregulated GPC3 Expression via the HIF-1α/c-Myc Axis

Accumulating evidence has shown that GPC3 is transcriptionally regulated by c-myc, and overexpression of c-myc induces GPC3 promoter-dependent luciferase activity and elevates GPC3 expression at both the mRNA and protein levels. Since CoCl₂ decreased the expression of GPC3 mRNA, we hypothesized that CoCl₂ might suppress the transcriptional activity of c-myc. Strikingly, the luciferase reporter data demonstrated that CoCl₂ resulted in a 50% decline in the transcriptional activity of c-myc in HepG2 cells compared to that of the control group (Figure 3A).
Figure 1 CoCl₂ inhibited HepG2 cell viability and induced cell apoptosis. (A) HepG2 cells were treated with different concentrations of CoCl₂ for 24 h, and the cell viability was determined by MTT assay. (B) Cell apoptosis induced by CoCl₂ for 24 h was assessed by flow cytometry. (C) Apoptosis rate of HepG2 cells induced by different concentrations of CoCl₂. (D) Expression of PARP and caspase-3 induced by CoCl₂ for 24 h was determined by Western blotting. *p<0.05 vs 0 μM.

Figure 2 CoCl₂ inhibited the expression of GPC3 in HepG2 cells. (A, B) HepG2 cells were treated with 50~200 μM CoCl₂ for 24 h, and the mRNA levels of GPC3 and HIF-1α were evaluated by real-time PCR. (C) Protein expression of GPC3 and HIF-1α was determined by Western blotting. (D) Expression of GPC3 and HIF-1α in HepG2 cells was assessed by immunofluorescence, and the images were acquired by confocal laser microscopy. Scale bar=20μM. *p<0.05.
addition, the expression of c-myc was significantly reduced by CoCl$_2$ treatment in a concentration-dependent manner (Figure 3B). Additionally, the expression of sp1 was decreased by CoCl$_2$. Furthermore, the interaction between HIF-1$\alpha$ and c-myc has been shown to play a pivotal role in malignant progression. 16–18 Thus, to verify whether the HIF-1$\alpha$/c-myc axis mediates the CoCl$_2$-induced downregulation of GPC3, siRNA targeting HIF-1$\alpha$ was transfected into HepG2 cells, followed by 200$\mu$M CoCl$_2$ treatment. As shown in Figure 3C, knockdown of HIF-1$\alpha$ elevated the expression of GPC3, c-myc, and sp1, thereby indicating that the HIF-1$\alpha$/c-myc axis mediated the inhibitory effect of CoCl$_2$ on GPC3.

**Overexpression of GPC3 Attenuated CoCl$_2$-Induced Hypoxia Injury**

Our previous studies have demonstrated that GPC3 promotes HepG2 cell proliferation and inhibits cell apoptosis through the Wnt/β-catenin signalling pathway, 10 thereby suggesting a tumour-promoting effect of GPC3 on cell growth. To further explore the role of GPC3 in CoCl$_2$-induced hypoxia injury, a GPC3 overexpression plasmid was constructed and transfected into HepG2 cells with or without CoCl$_2$ treatment. As shown in Figure 4, GPC3 overexpression reversed cell proliferation and attenuated cell apoptosis induced by CoCl$_2$ compared to those of the CoCl$_2$ group.

**Discussion**

HCC is a common refractory tumour with high morbidity and mortality globally. Approximately 600,000 cases are diagnosed every year. 19 Although surgical resection is the primary choice for HCC therapy, the majority of patients are diagnosed at a late stage with distant metastasis due to the concealed symptoms of the cancer. Thus, the opportunity for surgery or transplantation is missed, and the five-year survival rate of 3–11% is due to the lack of effective treatments. 20

Although the pathogenesis of HCC is complicated, an array of critical genes or proteins have been identified as being involved in HCC tumour progression. Recent studies have shown that the level of GPC3 is drastically upregulated in HCC tissues, 6,21–23 while a low level has been observed or remained undetected in normal hepatic cells and benign liver tumours. The upregulated expression of GPC3 displays a positive association with tumour size, histopathological differentiation, tumour invasion, and metastasis 24 dysplasia in cirrhotic livers and could enrich the expression of HCC-related genes. 25 Accumulating evidence has shown that GPC3 promotes HCC tumour growth via the Wnt/β-catenin signalling pathway, 26,27 silences GPC3-induced cell apoptosis, and inhibits cell proliferation, 28–31 indicating a critical role for GPC3 in HCC tumorigenesis and development.

Hypoxia is a distinct hallmark of HCC. HIF-1$\alpha$ is a hypoxia-induced transcription factor and a critical regulator of genes that are activated in responses to an oxygen-deficient environment. Under normoxic conditions, HIF-1$\alpha$ is hydroxylated by prolyl hydroxylase (PHD) and undergoes proteasomal degradation, while during hypoxia, HIF-1$\alpha$ is stabilized and cannot be hydroxylated by PHD, thereby preventing it from undergoing proteasomal degradation. Then, the accumulated HIF-1$\alpha$ is translocated to the nucleus,
leading to the elevated expression of its target genes, which are involved in energy metabolism, cell proliferation, apoptosis, vascular remodelling, and erythropoiesis.\textsuperscript{32} Although cell survival is induced by the upregulation of HIF-1\textalpha{} target genes in the hypoxic microenvironment, rapid hypoxia could also induce irreversible cell damage.\textsuperscript{33} GPC3 is a critical oncoprotein in HCC; however, whether its expression is regulated by hypoxia remains unknown. Mimicking the oxygen-deficient environment in tumour cells by CoCl\textsubscript{2} treatment is the canonical in vitro model. It was confirmed that CoCl\textsubscript{2} blocks the oxygen signal and stabilizes the expression of HIF-1\textalpha{} by

Figure 4 Overexpression of GPC3 attenuated cell apoptosis induced by CoCl\textsubscript{2}. (A) HepG2 cells were transfected with the GPC3 plasmid or blank vector, and GPC3 expression was verified by Western blotting. (B) The transfected cells were treated with 200 \textmu{}M CoCl\textsubscript{2}, and cell viability was assessed by MTT assay. (C) The transfected cells were treated with 200 \textmu{}M CoCl\textsubscript{2}, and cell apoptosis was assessed. (D) Apoptosis rate of HepG2 cells in each group. (E) The transfected cells were treated with 200 \textmu{}M CoCl\textsubscript{2}, and the expression of PARP and caspase-3 was determined by Western blotting. *p<0.05.
displacing Fe$^{2+}$ in the proline hydroxylase cofactor, which led to a series of cell reactions induced by hypoxia, thereby making CoCl$_2$ useful in hypoxia studies. In the current study, it was found that CoCl$_2$ significantly reduced the expression of GPC3 at both the mRNA and protein levels in HepG2 cells and induced cell apoptosis in a dose-dependent manner. The overexpression of GPC3 attenuated CoCl$_2$-induced cell apoptosis, indicating that CoCl$_2$ induced cell apoptosis by inhibiting the expression of GPC3.

As expected, CoCl$_2$ significantly elevated HIF-1α protein levels. To verify whether HIF-1α mediated the inhibitory effects induced by CoCl$_2$, HIF-1α expression was silenced by siRNA. GPC3 expression was reversed by knockdown of HIF-1α in the presence of CoCl$_2$, suggesting that GPC3 expression may be negatively regulated by HIF-1α. Our present study also demonstrated that the HIF-1α/c-myc axis mediates this biological process.

Both HIF-1α, c-myc and sp1 are crucial transcription factors regulating proliferation and metabolism in cancer cells. HIF-1α and c-myc partially modulate the complex pathways by acting alone in response to low oxygen and also function in concert to reprogram cell metabolism. Reportedly, crosstalk between HIF-1α and the proto-oncogene c-myc during hypoxia plays a major role in controlling genetic instability and cancer progression. Although HIF-1α and c-myc share certain common target genes, they exert inverse effects on cell proliferation, mitochondrial biogenesis, and DNA repair. Under normal oxygen conditions, c-myc interacts with sp1 and Max to form a transcriptional complex which drives the transcription of c-myc target genes. During oxygen deficiency, c-myc could be replaced by HIF-1α due to the high affinity of sp1 to HIF-1α.

Conclusively, the present study revealed that CoCl$_2$ induced cell apoptosis via downregulated expression of GPC3. Further studies showed that the HIF-1α/c-myc axis mediates the inhibitory effects induced by CoCl$_2$ and that knockdown of HIF-1α upregulates the expression of GPC3. This was the first evidence reporting that GPC3 is reduced by a hypoxia mimicking reagent and that GPC3 is negatively regulated by HIF-1α. Notably, although CoCl$_2$ is commonly used to mimic hypoxia, cell metabolism is not consistent with oxygen deficit. Hypoxia is known to induce a more robust and extensive transcriptomic alteration than that of CoCl$_2$, while the latter induces a more marked upregulation of hypoxia-induced genes, thereby necessitating further hypoxia studies.

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**Disclosure**

The authors report no conflicts of interest in this work.

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