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HIF-1α-induced autophagy contributes to cisplatin resistance in ovarian cancer cells

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Received May 4, 2018, accepted June 1, 2018

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Pharmazie 73: 533-536 (2018) *doi: 10.1691/ph.2018.8514*

In this study, the viability and apoptosis of ovarian cancer cell line OVCAR-3 were assessed using MTT and flow cytometry analysis. GFP-tagged LC3 plasmid transfection was utilized to demonstrate the occurrence of autophagy. The expression of HIF-1α, Beclin1, LC3 and β-actin were determined with Western blot analysis. We found that hypoxia could inhibit cisplatin induced apoptosis in OVCAR-3 cells and enhance the chemoresistance to cisplatin. Furthermore, GFP-tagged LC3 plasmid transfection and western blot were used to demonstrate that hypoxia induced chemoresistance of OVCAR-3 cells to cisplatin is related to HIF-1α-induced autophagy. All these findings suggest that the cisplatin resistance of ovarian cancer cells is associated with HIF-1α-induced autophagy.

1. Introduction

Ovarian cancer is one of the common gynecological malignancies with highest mortality rate (Siegel et al. 2018). Co-treatment of cytoreductive surgery and platinum-based chemotherapy is currently standard utilized in the treatment of ovarian cancer (Conteduca et al. 2014). As the first-line treatment for ovarian cancer, cisplatin (DDP) is frequently used in the platinum-based chemotherapy (Khrunin et al. 2014). However, it was reported that many ovarian cancer patients are suffering from platinum-resistance including DDP, which could lead to metastasis and recurrence of ovarian cancer (Nawrocki et al. 2013). Thus it is important to investigate the underlying mechanisms of DDP resistance in ovarian cancer cells.

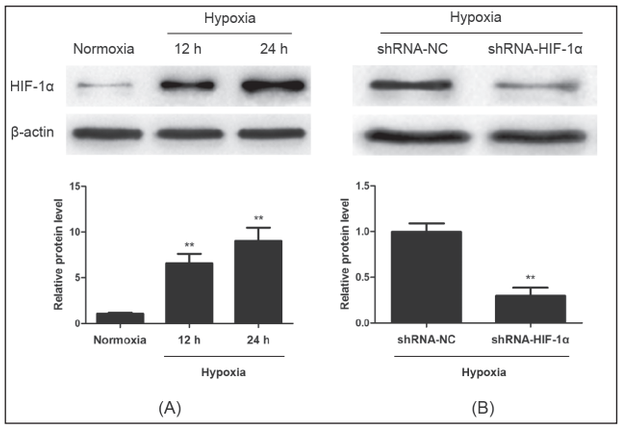

Fig. 1: Hypoxia induces HIF-1α expression in ovarian cancer cells. The effect of hypoxia on the HIF-1α expression and shRNA-HIF-1α vector knockdown effect in the OVCAR-3 cells were determined with Western blot. A: The effect of hypoxia on the HIF-1α expression. B: The knockdown effect of shRNA-HIF-1α vector on the HIF-1α expression under hypoxic condition. Data are presented as the mean±SEM (n=3). **p < 0.01, compared to Normoxia or shRNA-NC group.

DDP is a platinum compound, which could form DNA adducts and subsequently activate various signaling pathways that are related to DNA damage recognition, DNA repair and cell apoptosis. Previous research has revealed two main mechanisms contributing to platinum resistance of cancer cells: reduction of platinum-DNA adducts formation and the impairment of apoptotic response to adduct products in cancer cells (Kelland 2007; Shen et al. 2012). In view of the mechanisms of platinum resistance, a relationship between autophagy and cellular resistance to DDP was observed. Rationally, cells might be injured firstly in response to stress such as hypoxia or DDP (Degenhardt et al. 2006; Papandreou et al. 2008), and then the complicated regulated process 'autophagy' in cells occurrs to cope with the situation (Mizushima 2007; Terman et al. 2005). Autophagy allows energy supply during starvation, thus has been defined as a protective mechanism (Lum et al. 2005). It is suggested that hypoxia is able to upregulate autophagy, thereby increasing cell survival and chemoresistance (Del Bello et al. 2013; Liu et al. 2011; Wu et al. 2015), but the association between hypoxia induced autophagy and DDP resistance in ovarian cancer is not clear. The present study aimed to investigate how hypoxia and autophagy work together to mediate DDP resistance in ovarian cancer cells.

2. Investigations and results

2.1. Hypoxia induced expression of HIF-1α in ovarian cancer cells

As shown in Fig. 1, the results suggested that hypoxia could upregulate the expression of hypoxia-inducible factor-1α (HIF-1α) in OVCAR-3 cells. To further determine whether HIF-1α was involved in the DDP resistance of OVCAR-3 cells, we successfully downregulated HIF-1α in the cells by transfection of shRNA-HIF-1α vector, and the knockdown was subsequently confirmed with Western blot analysis.

2.2. Hypoxia enhances the DDP resistance of ovarian cancer cells

To evaluate the effects of hypoxia on chemoresistance of ovarian cancer cells, OVCAR-3 cells and OVCAR-3 cells that were stably transfected with shRNA-HIF-1α vector were incubated under normoxic or hypoxic conditions with DDP or 3-MA for 24 h. We tested

the percentage of cellular viability by MTT assay, and enhanced resistance to DDP were observed in OVCAR-3 cells exposed to hypoxia, while HIF-1α knockdown or inhibition of autophagy by 3-MA could prevent the enhanced resistance to DDP by hypoxic condition (Fig. 2).

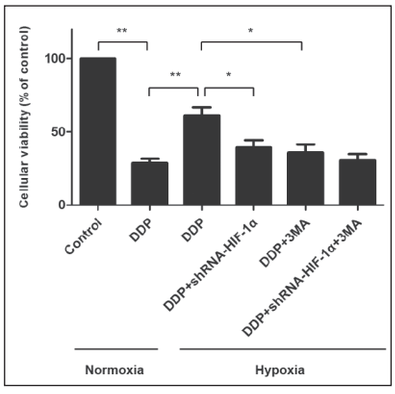

Fig. 2: Hypoxia enhances the chemoresistance of ovarian cancer cells to cisplatin. The effect of hypoxia on chemoresistance of ovarian cancer cells was measured by MTT assay. OVCAR-3 cells and OVCAR-3 cells that were stably transfected with shRNA-HIF-1α vector were cultured in complete medium under normoxic or hypoxic condition for 24 h in the presence or absence of DDP and 3-MA. Data are presented as the mean ± SEM (n=3). *$p < 0.05$ and **$p < 0.01$, compared to Normoxia+DDP or Hypoxia+DDP group.

2.3. Hypoxia inhibits cisplatin induced apoptosis in ovarian cancer cells

To evaluate whether hypoxia could inhibit DDP induced apoptosis, we conducted apoptosis detection by FITC-Annexin V/PI double staining in OVCAR-3 cells. As demonstrated in Fig. 3, OVCAR-3 cells exposed to hypoxia showed decreased early and late stages of apoptotic cells compared with that of the Normoxia+DDP group, but HIF-1α knockdown or inhibition of autophagy by 3-MA could prevent decreased early and late stages of apoptotic cells by hypoxic condition. These results demonstrated that hypoxia could inhibit DDP induced apoptosis in OVCAR-3 cells, while HIF-1α knockdown or inhibition of autophagy by 3-MA could prevent these effects by hypoxic condition.

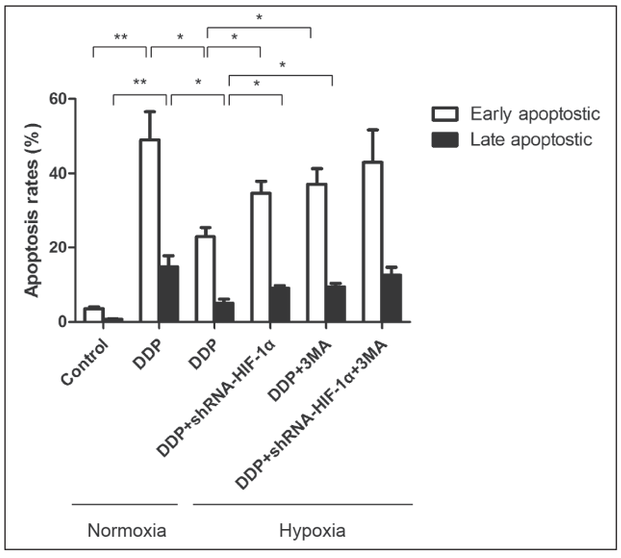

Fig. 3: Hypoxia inhibits cisplatin induced apoptosis in ovarian cancer cells. FACS analysis following Annexin V/PI staining was used to examine the percentage of early apoptotic cells (Ann+/PI–) and late apoptotic cells (Ann+/PI+) in OVCAR-3 cells or OVCAR-3 cells that were stably transfected with shRNA-HIF-1α vector. Data are presented as the mean ± SEM (n=3). *$p < 0.05$ and **$p < 0.01$, compared to Normoxia+DDP or Hypoxia+DDP group.

2.4. Hypoxia induced DDP resistance is associated with HIF-1α-induced autophagy

Previous results suggested that OVCAR-3 cells were protected by hypoxia from DDP treatment, and HIF-1α knockdown or inhibition of autophagy by 3-MA could prevent these protect effects. But how does it work? Is there any correlation between HIF-1α and autophagy in chemoresistance to DDP of OVCAR-3 cells by hypoxic condition? We further investigated the prevalence of autophagy under hypoxic conditions using GFP-LC3 vector, which could concentrate in autophagic vacuoles. OVCAR-3 cells and OVCAR-3 cells that were stably transfected with shRNA-HIF-1α vector were transiently transfected with GFP-LC3 plasmids, and the cells were cultured in the presence or absence of DDP under normoxic or hypoxic condition 24 h after transfection. As Fig. 4 suggests, the cells treated by DDP under hypoxic conditions exhibited a higher percentage of punctate GFP compared with the Normoxia+DDP group, while HIF-1α knockdown or inhibition of autophagy by 3-MA showed a lower percentage of punctate GFP compared with that of the Hypoxia+DDP group. To elucidate the role of autophagy by additional independent assays, we explored the LC3-II and Beclin1 protein level, which are hallmarks of autophagy. The results suggested that levels of endogenous LC3-II and Beclin1 were significantly increased in OVCAR-3 cells treated by DDP under hypoxic conditions as compared with normoxic conditions. However, HIF-1α knockdown or inhibition of autophagy by 3-MA could decrease these two protein levels compared to that of the Hypoxia+DDP group. These results demonstrated that DDP treatment under hypoxic conditions show high autophagy activity in OVCAR-3 cells, but HIF-1α knockdown and 3-MA treatment could decrease autophagy activity in OVCAR-3 cells.

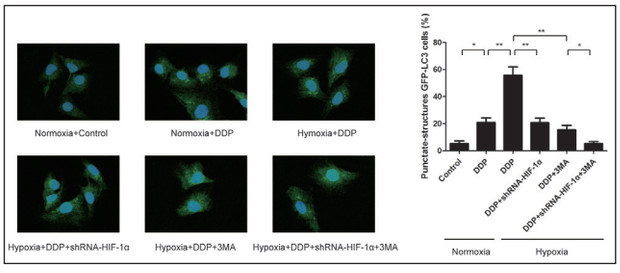

Fig. 4: Hypoxia promotes occurrence of autophagy in ovarian cancer cells. OVCAR-3 cells and OVCAR-3 cells that were stably transfected with shRNA-HIF-1α vector were transiently transfected with GFP-LC3 plasmid, 24 h post transfection, cells were incubated under normoxia or hypoxia with or without DDP treatment. At the end of treatment, cells were observed under a fluorescence microscope and the cells with diffused or punctuate GFP were counted. Data are presented as the mean ± SEM (n=3). *$p < 0.05$ and **$p < 0.01$, compared to Normoxia+DDP or Hypoxia+DDP group.

3. Discussion

As one of the most common features of tumor microenvironment, hypoxia plays an important role in the invasion and metastasis of tumors (Vaupel et al. 2014; Zhou et al. 2006). Previous studies suggested that a partial oxygen pressure (pO_2) of less than 10 mm was found in 60% of solid tumors, while pO_2 of 40-65 mm was found in the adjacent normal tissues. In addition, tumor cells were more prone to genetic and metabolic changes under hypoxic conditions, which contribute to the resistance of tumor cells to chemotherapy (Cosse et al. 2008; Höckel et al. 2001; Prabhakar et al. 2001; Zhou et al. 2006). Our results show that the percentage of death cell was lower for ovarian cancer cells cultured under hypoxic conditions than for those under normoxic conditions after DDP treatment, and HIF-1α had a key role in these hypoxia-mediated effects (Semenza 2010; Wohlkoenig et al. 2011). Furthermore, it was shown that HIF-1α is associated with hypoxia-induced chemoresistance (Brown et al. 2006; Hu et al. 2003; Hussein et al. 2006; Melillo 2006), but the underlying mechanisms remained unkown.

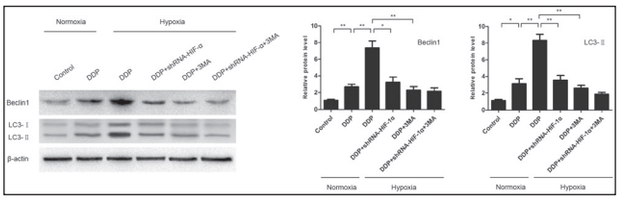

Fig. 5: Hypoxia induces autophagy activity in ovarian cancer cells. Levels of endogenous LC3-II and Beclin1 were measured by Western blot in OVCAR-3 cells and OVCAR-3 cells that were stably transfected with shRNA-HIF-1α vector treated by DDP under normoxic or hypoxic condition. Data are presented as the mean ± SEM (n=3). *$p < 0.05$ and **$p < 0.01$, compared to Normoxia+DDP or Hypoxia+DDP group.

Among all the underlying mechanisms that are responsible for hypoxia-induced DDP resistance, the decreased cellular apoptosis might be a vital one (Yu et al. 2012). In our study, DDP induced a significant increase of apoptosis in OVCAR-3 cells, but OVCAR-3 cells exposed to hypoxia showed decreased early and late stages of apoptotic cells compared with that of the Normoxia+DDP group, but HIF-1α knockdown could prevent decreased early and late stages of apoptotic cells. These results demonstrated that hypoxia could inhibit DDP induced apoptosis in ovarian cancer cells, but HIF-1α knockdown could prevent these effects by hypoxic conditions.

Furthermore, it has been reported that the upregulation of autophagy could enhance the chemoresistance of tumor cells to several anticancer agents including DDP (de Medina et al. 2009; Mizushima et al. 2009), and downregulation of autophagy in combination with other chemotherapeutic agents could increase cytotoxicity of tumor cells by triggering apoptosis (Herman-Antosiewicz et al. 2006; Nishikawa et al. 2010). Thus, the role of autophagy in the HIF-1α-induced DDP resistance deserved to be investigated. In our study, when treated by 3-MA under hypoxic conditions, the OVCAR-3 cells became sensitive to DDP, similar to the cells under normoxic conditions. Furthermore, inhibition of autophagy by 3-MA could prevent decreased early and late stages of apoptotic cells by hypoxic condition. Therefore, the effect of hypoxia and HIF-1α on autophagy in OVCAR-3 cells was studied by GFP-tagged LC3 plasmid transfection and Western blot assay. Autophagy occurred in cells could be measured by GFP-LC3, with the fluorescence pattern of GFP-LC3 concentrated in autophagic vacuoles changed from a diffuse to a punctate one. In our study, OVCAR-3 cells treated by DDP under hypoxic conditions exhibited a higher percentage of punctate GFP compared with that of Normoxia+DDP group, while HIF-1α knockdown or inhibition of autophagy by 3-MA showed a lower percentage of punctate GFP compared with the Hypoxia+DDP group. To further confirm the involvement of autophagy, we analyzed LC3-II and Beclin1 protein level, which are hallmarks of autophagy, by western blot. Levels of endogenous LC3-II and Beclin1 were markedly increased in OVCAR-3 cells treated by DDP under hypoxic conditions. But HIF-1α knockdown or inhibition of autophagy by 3-MA could decrease these two protein level compared to that of Hypoxia+DDP group. These results demonstrated that DDP treatment under hypoxic condition in OVCAR-3 cells show high autophagy activity, but HIF-1α knockdown and 3-MA treatment could decrease autophagy activity in OVCAR-3 cells.

In conclusion, our present study revealed that the induction of HIF-1α-induced autophagy could contribute to DDP resistance of ovarian cancer cells under hypoxic conditions. These results added further evidence of the molecular mechanisms of HIF-1α-induced autophagy in chemoresistance, and the agents targeting these molecules might be serving as novel chemotherapeutic agents for ovarian cancer therapy.

4. Experimental

4.1. Reagents

MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide), 3-methyladenine (3-MA) and cisplatin (DDP) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Primary antibodies against HIF-1α, beclin1 and β-actin were got from Cell Signaling Technology (Beverly, MA, USA). Primary antibody against LC3 was obtained from Sigma-Aldrich (St. Louis, MO, USA). Goat anti-rabbit and goat anti-mouse antibodies coupled to horseradish-peroxidase were from Boster (Wuhan, Hubei, China). Recombinant plasmid vectors harboring a shRNA sequence targeting HIF-1α (shRNA-HIF-1α) and its negative control vector shRNA-NC were from Santa Cruz (Dallas, TX, USA). GFP-tagged LC3 recombinant plasmid was obtained from Genomeditech (Shanghai, China). Lipofectamine 3000 was from Invitrogen (Carlsbad, CA, USA).

4.2. Cell culture

The ovarian cancer cell line OVCAR-3 was obtained from the American Type Culture Collection (ATCC, VA, USA). For normoxic conditions, the OVCAR-3 cells were maintained in RPMI 1640 (Gibco, USA) medium supplemented with 10% fetal bovine serum (HyClone, UT, USA) at 37 °C in the atmosphere consisting of 5% CO_2 and 95% air. For hypoxic conditions, the OVCAR-3 cells were kept in the atmosphere consisting of 1% O_2, 5% CO_2 and 94% N_2.

Cells were randomly allocated to six groups according to the different operation processes and drug treatment: Normoxia+Control group (cells were cultured under normoxia), Normoxia+DDP group (cells were cultured with DDP (20 μM) for 24 h under normoxic conditions), Hypoxia+DDP group (cells were cultured with DDP (20 μM) for 24 h under hypoxic conditions), Hypoxia+DDP+shRNA-HIF-1α group (cells that stably transfected by shRNA-HIF-1α were cultured with DDP (20 μM) for 24 h under hypoxic conditions), Hypoxia+DDP+3-MA group (cells were treated by DDP (20 μM) and 3-MA (10 μM) for 24 h under hypoxic conditions) and Hypoxia+DDP+shRNA-HIF-1α+3MA group (cells that were stably transfected by shRNA-HIF-1α were cultured in the presence of DDP (20 μM) and 3-MA (10 μM) for 24 h under hypoxic condition).

4.3. Cell viability assay

To evaluate the role of hypoxia in cisplatin resistance of ovarian cancer cells, OVCAR-3 cells or OVCAR-3 cells that were stably transfected by shRNA-HIF-1α were seeded in 96-well plates (1×10^4 per well) and kept in the medium containing DDP or 3-MA for 24 h under normoxic or hypoxic conditions. MTT assay was performed to detect the viability of cells. 10 μl of 5 mg/ml MTT solution was added to the wells, and incubated at 37 °C for 4 h. Then, the mixture solution containing MTT was discarded, and 150 μl DMSO was added to dissolve the formazan. The absorbance was determined at 570 nm wavelength, and the cell viability was calculated as (OD of experimental well/OD of control) × 100%.

4.4. Western blot analysis

Protein lysates of cells were isolated by the lysis buffer, and the concentrations were measured using bicinchoninic acid (BCA) (Beyotime, Jiangsu, China). Protein samples were then separated on 10% SDS-PAGE and transferred onto the PVDF membrane. After blocked with 5% BSA (Roche, Mannhein, Germany) for 1 h, the cropped membranes were incubated with primary antibodies against HIF-1α, Beclin1, LC3 or β-actin (1: 2000) at 4 °C overnight. Membranes were washed three times and incubated with horseradish peroxidase-conjugated secondary antibodies (Boster, Wuhan, China) for 1 h at 37 °C. Membranes were then washed again and detected with the ECL chemiluminescence kit (Bio-Rad, CA, USA). The band optical density was determined using Image Lab 5.0 software (Bio-Rad, CA, USA).

4.5. Apoptosis detection

FITC-Annexin V/PI double staining was conducted according to the instructions. In brief, the OVCAR-3 cells were treated with DDP or 3-MA for 24 h and washed twice with cold PBS, and then the double staining of the cells was performed by incubating with FITC-Annexin V/PI for 15 min at 25 °C. Furthermore, the fluorescence was analyzed by flow cytometry (BD Biosciences, NJ, USA) with proper machine settings, and the FITC signal detector (FL1) and PI signal detector (FL2) were utilized. The analysis of early-stage apoptotic cells (Ann+/PI-) and late-stage apoptotic cells (Ann+/PI+) was performed using Kaluza analysis software (Beckman Coulter, CA, USA).

4.6. Transient transfection and identification of autophagy

The GFP-tagged LC3 plasmid was commonly used to detect the occurrence of autophagy. OVCAR-3 cells or OVCAR-3 cells that were stably transfected with shRNA-HIF-1α were seeded in 96-well plates (1×10^4 cells/well), then the cells were transiently transfected with GFP-tagged LC3 plasmid using lipofectamine 3000 following the manufacturer's instructions. The cells expressed GFP-LC3 were cultured under normoxic or hypoxic condition in the medium with DDP or 3-MA for 24 h. After the treatment, the fluorescence images were acquired using an Olympus IX-70 inverted microscope (Olympus, NY, USA), and autophagy was measured by counting the cells with GFP-LC3-positive dots.

4.7. Statistical analysis

For continuous variables, the results are presented as the mean±S.E.M. (standard error of mean). The statistical analysis was performed by one-way analysis of variance (ANOVA) using SPSS 16.0 statistical software. P values less than 0.05 were considered statistically significant.

Acknowledgements: This work was funded by the National Natural Science Foundation of China (No. 81703922), Sichuan Science and Technology Program (2017JY0303), Basic Research Projects of Education Department of Sichuan Province (18ZB0164 and 18ZB0240) and Research Projects of Chengdu Medical College (CYZ17-24).

Conflicts of interest: None declared.

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