Research Paper

Hypoxia promotes drug resistance in osteosarcoma cells via activating AMP-activated protein kinase (AMPK) signaling

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ABSTRACT

Purpose: Drug resistance has been recognized to be a major obstacle to the chemotherapy for osteosarcoma. And the potential importance of hypoxia as a target to reverse drug resistance in osteosarcoma has been indicated, though the mechanism underlying such role is not clarified. The present study aims to investigate the role of hypoxia in the drug resistance in osteosarcoma cells via activating AMP-activated protein kinase (AMPK) signaling.

Experimental design: We investigated the promotion of the resistance to doxorubicin of osteosarcoma MG-63 and U2-os cells in vitro, and then determined the role of hypoxia-inducible factor-1 (HIF-1α) and HIF-1β, the activation and regulatory role of AMPK in the osteosarcoma U2-os cells which were treated with doxorubicin under hypoxia.

Results: It was demonstrated that hypoxia significantly reduced the sensitivity of MG-63 and U2-os cells to doxorubicin, indicating an inhibited viability reduction and a reduced apoptosis promotion. And such reduced sensitivity was not associated with HIF-1α, though it was promoted by hypoxia in U2-os cells. Interestingly, the AMPK signaling was significantly promoted by hypoxia in the doxorubicin-treated U2-os cells, with a marked upregulation of phosphorylated AMPK (Thr 172) and phosphorylated acetyl-CoA carboxylase (ACC) (Ser 79), which were sensitive to the AMPK activator, AICAR and the AMPK inhibitor, Compound C. Moreover, the promoted AMPK activity by AICAR or the downregulated AMPK activity by Compound C significantly reduced or promoted the sensitivity of U2-os cells to doxorubicin.

Conclusion: The present study confirmed the AMPK signaling activation in the doxorubicin-treated osteosarcoma cells, in response to hypoxia, and the chemical upregulation or downregulation of AMPK signaling reduced or increased the chemo-sensitivity of osteosarcoma U2-os cells in vitro. Our study implies that AMPK inhibition might be an effective strategy to sensitize osteosarcoma cells to chemotherapy.

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1. Introduction

Osteosarcoma is the most frequent primary bone malignancy, mainly attacks adolescents [1]. The combined chemotherapy with intensive dose has greatly improved the overall survival for osteosarcoma patients to over 70% [2]. However, the prognosis remains poor for those with metastasis, or for those who relapse, and survival rates only reach 20–30% [3,4]. Doxorubicin, methotrexate and Cisplatin are commonly used as anticancer drugs in osteosarcoma [5] for the last 20 years, and there has been no improvement in the survival of those osteosarcoma patients, who acquire the drug-resistant phenotype. Thus, it is urgent to recognize the drug-resistance mechanism of osteosarcoma and to provide novel therapeutic options for this disease.

Hypoxia-induced drug resistance has been confirmed for a variety of anti-tumor agents in various types of tumors [8,6,7], and even in osteosarcoma [9]. Such hypoxia markers as hypoxia-inducible factor-1 (HIF-1), vascular endothelial growth factor (VEGF) and carbonic anhydrase IX (CA IX) are detectable in osteosarcomas [10], and correlate with poor progress of osteosarcoma patients, suggesting the important role of hypoxia in the survival of osteosarcoma cells [11,12], and implying the potential importance of hypoxia as a target to antagonize drug resistance in osteosarcoma. However, it is indicated that the drug resistance in osteosarcoma is independent on the upregulated HIF-1α, suggesting other hypoxia-related signaling may be more relevant in the drug resistance to osteosarcoma.

Multiple other signaling pathways are deregulated in hypoxia and may exert regulatory roles in the hypoxia-induced drug resistance. Wild-type p53 is confirmed to be inactivated in some
tumor cells by hypoxia [14,13], c-jun, activator protein-1 (AP-1), Phosphoinositol-3-kinase (PI3K) pathway and nuclear factor kappa-B (NF-κB) have also been indicated to involve in the hypoxia-induced drug resistance, mainly by inhibiting the drug-induced apoptosis [16,15,17]. And the target inhibiting of these signaling pathways sensitizes cells to cytotoxic agents under the condition of hypoxia, implying these markers as possible targets to counteract the hypoxia-induced drug resistance.

AMP-activated protein kinase (AMPK) is the most important sensor of cellular energy [19,18], and is also activated by hypoxia as a compensatory response to the reduced mitochondrial respiration [20]. The heterotrimeric AMPK comprises of two regulatory subunits and one catalytic subunit, which is activated by the increased AMP/ATP ratio [21]. Then the activity was promoted of upstream kinases and phosphatases that control AMPK phosphorylation and dephosphorylation at the Thr-172 [22,23], particularly by the tumor suppressor LKB1, which phosphorylates the catalytic subunit of AMPK in an AMP-dependent manner [24,25]. AMPK is also activated by the Ca2⁺/calmodulin-dependent protein kinase (CaMK) [27,26], independently of cellular AMP levels. In addition, the activation of AMPK can also be induced in response to oxidant stress [28,29] with or without hypoxia. However, the role of the AMPK activation in hypoxia in osteosarcoma is still unclarified.

In the current study, we investigated the resistance of osteosarcoma cells to the widely utilized in clinic cytotoxic drug, doxorubicin under hypoxia, and then examined the association of such resistance with the activation of AMPK signaling. On the other side, we investigated the influence by the chemical inhibition of AMPK signaling on the hypoxia-induced resistance to doxorubicin. The present study indicated the key regulatory role of AMPK in the hypoxia-induced resistance to doxorubicin in osteosarcoma cells, suggesting a possible target of AMPK against the drug-resistance of osteosarcoma cells.

2. Materials and methods

2.1. Cell culture and treatment

Human osteosarcoma cell line MG-63 and U2-os were purchased from American Type Culture Collection (ATCC) (Rockville, MD, USA). MG-63 cells were cultured in Eagle’s minimal essential medium (EMEM), supplemented with 2 mM Glutamine, 1% Non Essential Amino Acids (NEAA) and 10% Fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA, USA), and were routinely incubated at 37 °C with 5% CO₂. U2-os cells were grown in RPMI-1640 medium (Ameresco, Framingham, MA, USA) supplemented with 10% FBS (Invitrogen, Carlsbad, CA, USA). For hypoxia culture, MG-63 or U2-os cells were incubated in a hypoxia incubator infused with a gas mixture of 5% CO₂, 2% oxygen and nitrogen, whereas cells were incubated in an ordinary incubator with 5% CO₂. For the doxorubicin treatment, MG-63 or U2-os cells with more than 85% confluence were incubated with medium containing 2% FBS and the doxorubicin with various concentrations. To induce or inhibit the AMPK activity, AICAR and Compound C (Sigma-Aldrich, St. Louis, MO, USA) were added to the medium with a concentration of 1 mM and 20 μM respectively.

2.2. MTT assay and apoptosis assay

The viability of MG-63 or U2-os cells which were seeded in 96-well plates with more than 85% confluence post various treatments were measured with MTT assay according to the standard protocol. The absorbance was measured at 570 nm with a reference wavelength of 750 nm using a spectrophotometer.

Apoptosis of U2-os cells was examined with an Annexin V-FITC apoptosis detection kit (Sigma-Aldrich, USA). Briefly, 6 × 10⁵ U2-os cells were stained with Annexin V-FITC and propidium iodide and then were detected by a FACSscan flow cytometer (Bio-Rad, Hercules, CA, USA). The results were presented as a percentage of apoptotic cells from total cells.

2.3. Western blot analysis

U2-os cells, post treatment, were harvested and were homogenized in an ice-cold Cell lysis buffer (Bio-Rad, Hercules, CA, USA). Cellular lysates was centrifuged with 12,000 × g for 30 min at 4 °C, and the supernatant was collected. Protein samples were separated with 8–12% SDS-PAGE gel and were transferred to a nitrocellulose membrane (Millipore, Bedford, MA, USA). The membrane was blocked for non-specific binding targets with 5% Skimmed milk powder overnight at 4 °C, and were incubated with the rabbit polyclone antibody (against caspase 3, Poly (ADP-ribose) polymerase (PARP), HIF-1α, HIF-1β, AMPKα with or without phosphorylated Thr 172, ACC with or without phosphorylated Ser 79, or β-actin) overnight at 4 °C, and then were incubated with HRP-linked secondary anti-rabbit antibody (New England Biolab, Ipswich, UK) for 1 h at room temperature. The specific binding band was scanned and quantified according to the band density by Image J software.

2.4. HIF-1α knockdown via RNA interference

The HIF-1α specific siRNA (siRNA- HIF-1α) (25 or 50 nM) or the scramble oligonucleotides as control (siRNA-Con) (25 or 50 nM) were purchased from Thermo Fisher (Waltham, MA, USA), and were transfected into U2-os cells with Opti-MEM containing Lipofectamine RNAMax (Invitrogen, Carlsbad, CA, USA). 6 h post transfection, cells were updated with fresh RPMI-1640 medium, which was supplemented with 2% FBS, and were subject to other treatment or were assayed for the knockdown efficiency post another inoculation of 24 h.

2.5. DCFH-DA assay ELISA for AMPK activity

Reactive oxygen species (ROS) was assessed using 2,7-di-chlorofluorescin diacetate (DCFH-DA). U2-os cells were added with BSS containing DCFH-DA (5 μM), and fluorescence signaling was collected at 535 nm using excitation at 484 nm. Cellular fluorescence density from individual cell well was collected and was averaged to provide an overall assessment for each group. The AMPK activity was examined with AMPK Kinase Assay Kit AMPK Kinase Assay Kit (CY-1182; CycLex, Nagano, Japan) according to the manufacturer’s manual. U2-os cells post various treatments were directly lysed with ice-cold lysis buffer; then the cellular lysates were serially diluted in Kinase buffer and supplemented with phosphorylation substrate, and the amount of phosphorylated substrate specifically bonded to an anti-phospho-mouse IgG, which then assayed with anti mouse IgG conjugated with horseradish peroxidase and its substrate, by absorbance 450 nm.

2.6. Statistical evaluations

Quantitative results are presented as mean ± SE. For the analysis between two groups, the Student’s t test was performed. A p value less than 0.05 was considered significant.
3. Results

3.1. Hypoxia reduces the sensitivity of osteosarcoma cells to doxorubicin in vitro

We firstly examined the viability of doxorubicin-treated osteosarcoma MG-63 cells under normoxia or hypoxia. It was shown in Fig. 1A, that the doxorubicin with 4, 8 or 16 μM significantly reduced the viability of MG-63 cells under normoxia (p < 0.01 or p < 0.001). Interestingly, the cellular viability reduction in the MG-63 cells under hypoxia was significantly less than in the cells under normoxia (p < 0.05 respectively for 4, 8 or 16 μM). Then we reevaluated such regulation by hypoxia in U2-os osteosarcoma cells. Fig. 1B also indicated that the doxorubicin treatment with 5, 10, 20 or 40 μM significantly reduced the cellular viability (p < 0.05, p < 0.01 or p < 0.001). And the hypoxia pre-treatment could significantly ameliorate such viability reduction in U2-os cells (p < 0.05 for 5 μM, p < 0.01 respectively for 10, 20 or 40 μM). Moreover, to reconfirm the influence by hypoxia on the doxorubicin sensitivity of osteosarcoma cells, we determined the apoptosis induction in U2-os cells treated with doxorubicin under normoxia or hypoxia. It was indicated that less apoptotic U2-os cells were induced by the doxorubicin with 10 or 20 μM in the hypoxia group (Fig. 1C; p < 0.05 respectively).

In addition, we analyzed the activation of caspase 3, which is the executive marker of apoptosis [30], in the doxorubicin-treated
U2-os cells under normoxia or hypoxia. As shown in Fig. 1D and E, the cleaved caspase 3 (activated form) was markedly lower in the U2-os cells under hypoxia (p < 0.01 respectively). And the substrate of caspase 3, Poly ADP ribose polymerase (PARP) was also markedly less lyzed in the U2-os cells under hypoxia (Fig. 1F; p < 0.05 respectively for 10, 20 or 40 μM). Therefore, hypoxia reduces the sensitivity of osteosarcoma cells to doxorubicin in either MG-63 or U2-os osteosarcoma cells.
3.2. Hypoxia-induced HIF-1α exerts no influence on the sensitivity of osteosarcoma cells to doxorubicin

HIF-1α is the best characterized marker for hypoxia and is sensitively accumulated under hypoxia [32,31]. To investigate a possible regulatory role of HIF-1α on the sensitivity of osteosarcoma cells to doxorubicin, we then quantified the level of HIF-1α and the constitutive HIF-1β in the doxorubicin-treated U2-os cells with or without hypoxia. The western blotting results demonstrated that the HIF-1α level was dramatically promoted by hypoxia in the doxorubicin-treated U2-os cells (Figs. 2A and 1B; \( p < 0.01 \) or \( p < 0.001 \)) at 6, 12 or 24 h post inoculation. However, there was no marked difference in the HIF-1β level between the hypoxia and normoxia groups (Fig. 2C). Then the siRNA targeting HIF-1α was used to knockdown the expression of HIF-1α and reevaluated the influence of hypoxia on the drug resistance. Fig. 2D and E confirmed the significant blockage of the HIF-1α induction by hypoxia in U2-os cells (\( p < 0.01 \) respectively). However, cleaved caspase 3 and lysed PARP were not significantly downregulated by the HIF-1α blockage (Fig. 2F); and the cellular viability (Fig. 2G) and the apoptosis (Fig. 2H) induction were also not regulated by the HIF-1α blockage. Thus, we deduced that the reduced drug sensitivity of U2-os cells under hypoxia was not associated with HIF-1α.
3.3. AMPK signaling was promoted by hypoxia in osteosarcoma cells in the presence of doxorubicin

It has been conceived that the oxygen deprivation under hypoxia upregulates the production of reactive oxygen species (ROS), which then triggers the AMPK activation, but independent of an increase in AMP/ATP ratio [34,33]. We then determined the activation of AMPK signaling under hypoxia in U2-os cells and examined the role of AMPK activation in drug sensitivity of U2-os osteosarcoma cells. It was indicated that the U2-os cells under hypoxia produced markedly increased ROS (Fig. 3A; \( p < 0.05, p < 0.01 \) or \( p < 0.001 \)) via the DCFH-DA fluorescence assay. And the western blot analysis demonstrated that the levels of phosphorylated AMPK and phosphorylated ACC, both of which are the active forms of AMPK signaling, were significantly promoted in the U2-os cells under hypoxia for 6 or 12 h (Fig. 3B–D; whichever in the presence of AMPK activator, AICAR, or AMPK inhibitor, Compound C; \( p < 0.05 \) or \( p < 0.01 \)). Moreover, the levels of phosphorylated AMPK and phosphorylated ACC was markedly up-regulated by the AMPK activator, AICAR, or downregulated by AMPK inhibitor, Compound C (\( p < 0.01 \) or \( p < 0.001 \)); and the AMPK activity assay also indicated the significant promotion of AMPK signaling by hypoxia, and the marked up- or down-regulation of AMPK signaling by AICAR or Compound C (Fig. 3E; \( p < 0.05, p < 0.01 \) or \( p < 0.001 \)).

3.4. Chemical manipulation of AMPK signaling regulates the sensitivity of osteosarcoma cells to doxorubicin

Then we examined the influence of AMPK activation on the U2-os cell sensitivity to doxorubicin. Fig. 4A indicated that the promotion by AICAR and the reduction by Compound C of the viability...
of U2-os cells were not significant. However, it was significant under hypoxia of the cellular viability promotion by AICAR and the reduction by Compound C (Fig. 4B; \( p < 0.05 \) respectively). And the apoptosis level was also significantly regulated by both chemical agents (reduced by AICAR, whereas promoted by Compound C; Fig. 4C; \( p < 0.05 \) or \( p < 0.01 \)) in the U2-os cells under hypoxia. In addition, levels of cleaved caspase 3 and the lysed PARP were also reduced by the treatment with AICAR, whereas were upregulated by Compound C (Fig. 4D–F; \( p < 0.05 \) or \( p < 0.01 \)). Thus, we confirmed that AMPK signaling was promoted by the hypoxia-mediated resistance of U2-os cells to doxorubicin.

4. Discussion

The role of hypoxia in the drug sensitivity has not been universally concluded, varying according to the type of tumor and the drug used [35,36]. It was observed to be resistant in rhabdomyosarcoma, Ewing's sarcoma and neuroblastoma [36,37]. Evidence exists of the importance of hypoxia in osteosarcoma [39,38]. In particular, the patients with a moderate or strong expression of HIF-1\( \alpha \), which is the best characterized marker for hypoxia, had a significantly shorter overall survival (OS) and disease-free survival (DFS), compared with HIF-1\( \alpha \) negative/weak expression [40]. And recently, a significant hypoxia-induced drug resistance in osteosarcoma cells has been confirmed in intro [9], highlighting the potential importance of hypoxia in the drug resistance in osteosarcoma. However, though the HIF-1\( \alpha \) was also found to be upregulated by hypoxia, it was irrelevant to such drug resistance.

In the present study, we firstly confirmed the resistance of osteosarcoma U2OS cells to doxorubicin under hypoxia via inhibiting the drug-induced apoptosis. The hypoxia pre-treatment could significantly ameliorate the viability reduction in U2-os cells which were treated with doxorubicin with 5, 10, 20 or 40 \( \mu \)M. And less apoptotic U2-os cells were induced, and less cleaved caspase (activated form) and less lysed PARP were promoted by the doxorubicin treatment in the hypoxia group. Therefore, hypoxia reduces the sensitivity of osteosarcoma cells to doxorubicin in either MG-63 or U2-os osteosarcoma cells. Then we investigated the role of HIF-1\( \alpha \), which is the best characterized marker for hypoxia [32,31], in the hypoxia-mediated resistance of U2-os cells to doxorubicin. Interestingly, HIF-1\( \alpha \) was significantly downregulated by hypoxia in U2-os cells which were treated with doxorubicin, whereas exerted no influence on the sensitivity of U2-os cells to doxorubicin.

Reactive oxygen species (ROS) has been conceived to be promoted under hypoxia and to trigger the AMPK activation, independent of an increase in AMP/ATP ratio [34,33]. In the present study, we also confirmed the activation of AMPK by hypoxia in U2-os cells. Markedly increased ROS was indicated in the U2-os cells under hypoxia. And significantly upregulated levels of phosphorylated AMPK and phosphorylated ACC, both of which are the active forms of AMPK signaling, were also promoted in the U2-os cells under hypoxia. And the levels of phosphorylated AMPK and phosphorylated ACC were significantly up-regulated by the AMPK activator, AICAR, or downregulated by AMPK inhibitor, Compound C in the doxorubicin-treated U2-os cells. Moreover, AMPK signaling has been recognized to involve in the hypoxia-mediated drug resistance in several types of cancers, such as non–small cell lung cancers to cisplatin and doxorubicin [41], prostate cancers [42]. However, there was a controversy about the activation of AMPK signaling in the drug resistance. AMPK is activated in the prostate cancer cells [42], while is inactivated in the lung cancer cells [41], during hypoxia leading to drug resistance, implying a cancer type-dependence. And the present study also confirmed the activation of AMPK signaling during the hypoxia-induced drug resistance in osteosarcoma cells. The chemical manipulation of AMPK signaling regulates the sensitivity of osteosarcoma cells to doxorubicin. The promotion by AICAR and the reduction by Compound C to the AMPK signaling significantly ameliorated or deteriorated the viability reduction or apoptosis induction of U2-os cells under hypoxia. In addition, the level of cleaved caspase 3 and the lysed PARP were also reduced by the treatment with AICAR, whereas were upregulated by Compound C. Thus, the AMPK signaling mediated the hypoxia-induced resistance of U2-os cells to doxorubicin.

Autophagy is a dynamic self-degradation process for cellular components by cellular lysosome under a stringent regulation [43,44], and has also been recognized during ischemic heart disease [45]. Autophagy normally maintains at a low level in heart, and is sharply promoted as a response to such environmental stress conditions as ATP depletion, excessive ROS and mitochondrial dysfunction [46,47]. Moreover, autophagy has been identified to mediate the hypoxia-induced resistance by regulating the angiogenesis in malignancies [48]. The present study has recognized the involvement of autophagy in the hypoxia-induced resistance in osteosarcoma cells. However, it is not clear about the orchestrated pathways about AMPK signaling and autophagy promotion during the hypoxia-induced resistance. The reduced adenosine-triphosphate (ATP) has been recognized to activate autophagy [49]. The increased AMP/ATP ratio activates AMP-activated protein kinase (AMPK) [50], which successively induces autophagy via inhibiting mammalian target of rapamycin (mTOR) [46]. The promoted reactive oxygen species (ROS) has also been recognized to induce autophagy in cardiocytes [51], in choriocarcinoma cells [52], and in breast cancer cells [53]. Therefore, the role of autophagy in the AMPK-mediated chemoresistance of osteosarcoma cells needs to be further clarified.

5. Conclusion

The present study confirmed the hypoxia induced resistance of osteosarcoma cells to doxorubicin, and such chemo-resistance was HIF-1\( \alpha \)-independent. Moreover, we recognized the involvement to AMPK signaling in such hypoxia-induced chemoresistance in U2-os osteosarcoma cells. Chemical manipulation of AMPK activity exerted an influence of such chemoresistance in U2-os osteosarcoma cells. Our study implies that AMPK inhibition might be a effective strategy to sensitize osteosarcoma cells to chemotherapy.

Conflict of interest

Authors declare no conflict of interests regarding the current study.

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