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Hypoxia Induces Mitochondrial Swelling and Invasive Potential of Cultured Cells

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ABSTRACT

Cells that are exposed to limiting oxygen environment (hypoxia) acquire resistance to many chemotherapeutic drugs. However the mechanism underlying in this phenomena is not fully understood. In this study we successfully standardized the induction of hypoxic condition in cultured cells like HEK293T and MRC5 cells. Upon CoCl2 treatment we noticed the induction of HIF1α in both the cell lines used in the study. Here we reveal that hypoxia condition induces the mitochondrial swelling without altering its protein levels. Also enhanced mitochondrial swelling increased the invasive potential of MRC5 cells which may result in neoplastic transformation. Together our result demonstrates that conditions to induce hypoxia and also its effect on cultured cells.

Keywords: Cultured Cells, Swelling, Tumurs, HIF-1.

INTRODUCTION

In solid tumours, when the oxygen levels were lower than 5% in the tumour microenvironment, the phenomenon is termed as "Hypoxia", the normal level of oxygen between 10-20% is coined as "Normoxia". When the oxygen levels were more than 21%, it is called as "Hyperoxia". Cancer cells are unresponsive to conventional therapies under chronic hypoxia. Due to hypoxia condition, there is an increased resistance to the chemotherapy and radiation treatment (Harris 2002, Shannon, Bouchier-Hayes *et al*. 2003). The solid tumour is not uniform and hence there will be difference in the concentration of the oxygen which leads to tumour heterogeneity (Pries, Cornelissen *et al*. 2009). Hypoxia-inducible factor 1 (HIF-1), which is a heterodimeric transcription factor composed of α and β subunit (HIF-1α and HIF-1β, respectively) and whose activity is mainly dependent on the expression levels of the former, is recognized as a key factor associated with chemo resistance of lung cancer (Semenza 2012), with HIF-1-induced glycolysis playing an important role in promoting this chemo resistance (Harris 2002). The influence of hypoxia in tumour biology is observed in the form of selection of genotypes favouring survival under hypoxia, augmented receptor tyrosine kinase (RTK) signalling, suppression of apoptosis, and alteration in central metabolism that favours the Warburg effect (Vaupel and Mayer 2007, Wilson and Hay 2011). Under hypoxia condition, there is an increase in the lactate production due to the secretion of increased levels of Glucose transporter-1 (GLUT), monocarboxylate transporter-1 (MCT) and Carbonic anhydrase IX (CAIX), which leads to the acidic microenvironment that facilitates metastasis (Subarsky and Hill 2003, Mayer, Vaupel *et al*. 2016). Hypoxia-inducible factor-1α (HIF-1α)

is one of the most important transcription factors mediating adaptation response to hypoxia and a regulator of gene products during hypoxia (Semenza 2000, Brahimi-Horn, Chiche *et al*. 2007). HIF-1α modulates the activation of Receptor Tyrosine Kinase cascade of signaling pathways including PI3K and Ras–Raf pathways. PI3K is hyper-activated under hypoxia (Kilic-Eren, Boylu *et al*. 2013).

MATERIAL AND METHODS

Cell line and reagents

MRC5 and HEK293T cells were cultured in DMEM media (Gibco) supplemented with 5% heat-inactivated fetal bovine serum (Gibco), penicillin (50 IU/ml), streptomycin (50 µg/ml) and 2 mM glutamine (all purchased from Sigma, UK). Cells were maintained at 37 °C in a humidified incubator containing 21% O_2, 5% CO_2 in air (referred to as normoxic conditions).

Matrigel-invasion assays

Matrigel-invasion assays were carried out in 6-well BD Biocoat Matrigel Invasion Chambers (BD Biosciences) according to the manufacturer's protocol. MRC5 cell line was grown in serum-free medium (in absence or presence of tetracycline) for 12 hours before being used for the assay. Post-invasion, the membranes were removed and observed at 40× magnification after staining with 1% Toluidiene Blue (Fluka) in 1% borax (Sigma) for 2 minutes. The entire experiment was repeated in parallel with control inserts. Percentage invasion was determined by the relative ratio of the mean number of cells invading through the matrigel insert membrane versus the number of cells invading through the control-insert membrane.. All experiments were set up at least in triplicates and repeated independently three times. The *P*-values were obtained by using two-tailed student's *t*-test, unpaired data with unequal variance. Values are mean ± s.d.

Isolation of mitochondria from HEK-293T cells

Mitochondria were isolated from HEK-293T cell lines. In brief, HEK-293T cells were grown as monolayers and harvested in mitochondria isolation buffer (20 mM Hepes, pH 7.5, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 210 mM sucrose and 70 mM mannitol). The cell suspension was subjected to homogenization using a Polytron 1600 homogenizer with two 5 s pulses at 15 rev./min. The lysate was subjected further to Dounce homogenization. The homogenate was centrifuged at 1000 ***g*** for 10 min at 4°C to separate the nucleus, and the supernatant was again centrifuged at 10000 ***g*** for 15 min at 4°C. The resultant mitochondrial pellet was washed twice and suspended in a buffer containing 250 mM sucrose, 5 mM magnesium acetate, 40 mM potassium acetate, 10 mM sodium succinate, 1 mM DTT and 20 mM Hepes/KOH, pH 7.4.

Ca^{2+}-induced mitochondrial swelling

For swelling assays, mitochondria (0.5 mg of protein/ml) were incubated in 96-well (0.3 ml/well) or 24-well (0.5 ml/well) microtitre plates in swelling buffer [200 mM sucrose, 20 *i*M EGTA, 5 mM succinate, 2 *i*M rotenone, 1 *i*g/ml oligomycin, 20 mM Tris, 20 mM Hepes and 1 mM KH_2PO_4 (pH 7.3 at 23°C)]. Swelling was assessed by decreased absorbance at 540 nm using a ThermoMax 96-well plate reader (Molecular Devices). Induction of the MPT was initiated with 200–300 *i*M $CaCl_2$. Swelling traces are representative of three or more experiments.

Hypoxic treatment

To prepare $CoCl_2$ stock solutions in DMEM tissue culture medium, usually as 10% w/v solutions, the chemicals were dissolved directly in culture medium. The stock solutions were filter-sterilized (0.22 µm). The resultant solutions were kept at 4 °C and used within 24 h for the assay. Nine separate serial $CoCl_2$ (Sigma, UK) concentrations in culture medium were prepared for addition to cell culture (1, 5, 10, 20, 25, 50, 100, 150 and 200 ìM). MRC5/HEK293T cell culture medium was removed from the flask and the cells were rinsed with Trypsin EDTA solution and Trypsin EDTA solution (sigma) were then added to the flask and incubated at 37 °C for 2 min. Cells were then resuspended with DMEM and cultured at a concentration of 5000 cells/well in tissue culture 96 wells plates. The cells were left 48 h to adhere at 37 °C in a humidified atmosphere tissue culture incubator containing 5% CO_2. After 48 h, cells were treated with serial $CoCl_2$ concentrations (1, 5, 10, 20, 25, 50, 100, 150 and 200 µM) with 10% O_2 at 37 °C for 16 h.

RESULTS

Standardization of Hypoxia condition in HEK293T cells and MRC5 cell line

Hypoxia is defined as the reduction or lack of oxygen in cells, organs and tissues. The decrease of oxygen causes oxygen tension might be due to reduced supply of oxygen or to an increased consumption of oxygen relative to the supply. To standardize the hypoxic induction, HEK293T and MRC5 cells were treated with 100μM CoCl2 with 20% O2 supply for 16 hours at 37oC. As shown in

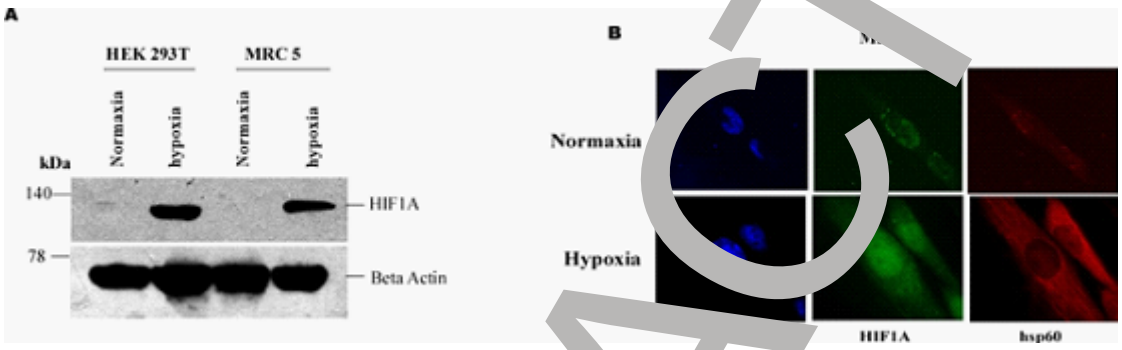

Fig. 1: Standardization of Hypoxia induction in cell culture (A) Asynchronous HEK293T cells and MRC5 cells were treated with or without 100μM CoCl2 for 16 hours at 37°C with the supply of 20% oxygen. Lysates were obtained from the treated cells and probed for HIF1α and β-actin using appropriate antibodies. (B) MRC5 cells treated with or without $CoCl_2$ were fixed using 4% Paraformaldehyde (PFA) and immunostained using anti-HIF1A and anti-hsp60 antibodies. DAPI was used as a nuclear staining

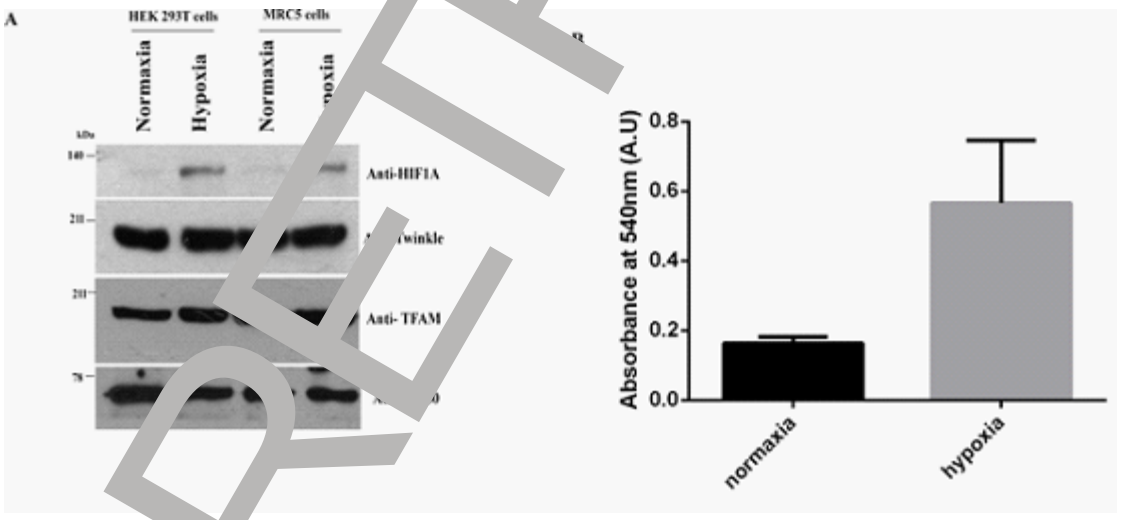

Fig. 2: Hypoxia condition causes mitochondrial swelling without affecting mitochondrial protein levels. (A) Cytoplasm was isolated from HEK293T and MRC5 cells under Normaxia and Hypoxia condition (as described in materials and methods). Mitochondrial extracts obtained from above mentioned cells under different conditions were probed for mitochondrial proteins such as hsp60, Twinkle and TFAM antibodies and also checked for HIF1α expression. (B) Isolated mitochondria from CoCl2 treated MRC5 cells were incubated in Mitochondria swelling buffer and swelling was assessed by decrease in the absorbance at 540nm. Experiment was carried out three times and graph was plotted.

Figure 1A, cells treated with CoCl2 significantly induced the Hypoxia inducible factor 1 (HIF1α). The same was not observed in normaxia condition. Microscopy analysis confirmed the induction of HIF1α under hypoxia condition (Figure 1 B), thereby indicating that above condition is suitable for Hypoxia studies.

Hypoxia condition causes mitochondrial swelling without affecting mitochondrial protein levels

Hypoxia is a nearly universal feature of tumor growth (Hockel and Vaupel 2001), conferring worse disease outcome via protection from apoptosis (Graeber, Osmanian *et al*. 1996), resistance to therapy. Mitochondria are the primary sites of hypoxia induced metabolic reprogramming in tumors (Denko 2008). This response involves HIF1-dependent transcription of mitochondrial pyruvate dehydrogenase kinase (PDK) (Kim, Rajagopal *et al*. 2006, Papandreou, Cairns *et al*. 2006), which in turn phosphorylates the pyruvate dehydrogenase complex (PDC) on three separate sites. Hence we hypothesized that Hypoxia condition may affect the mitochondrial integrity. To this end, Mitochondria swelling was measured and compared between isolated mitochondria isolated from Normaxia and Hypoxia condition. As shown in Figure 2 B, enhanced mitochondrial swelling was observed in Hypoxia condition as compared with Normaxia. This led us to find the possibility whether or not mitochondrial protein levels altered. For this purpose, cytoplasm was isolated from Normaxia and Hypoxia induced cells and checked for different mitochondrial protein levels. Interestingly, protein levels were unaltered between Normaxia and Hypoxia condition (Figure 2 A), suggesting that Hypoxia induces mitochondrial swelling without affecting its protein levels.

Hypoxia condition induces Metastatic potential of MRC5 cells

Hypoxia is a universal driver for aggressive tumor behavior, but the molecular mechanism behind is not well studied. Our previous experiments demonstrated that CoCl2 treatment induced HIF1α in both HEK293T and MRC5 cells. Hence, we hypothesized that Hypoxic condition might increase the metastatic potential of CoCl2 cells. For this purpose, we carried out in vitro invasion assay using MRC5 cells under two different conditions such as Normaxia and Hypoxia. As shown in Figure 3, MRC5 cells under Hypoxia condition exhibited enhanced metastatic potential as compared to Normaxia. Hence it can be surmised that Hyopxia potentiates invasive property of cells and drives them for neoplastic transformation.

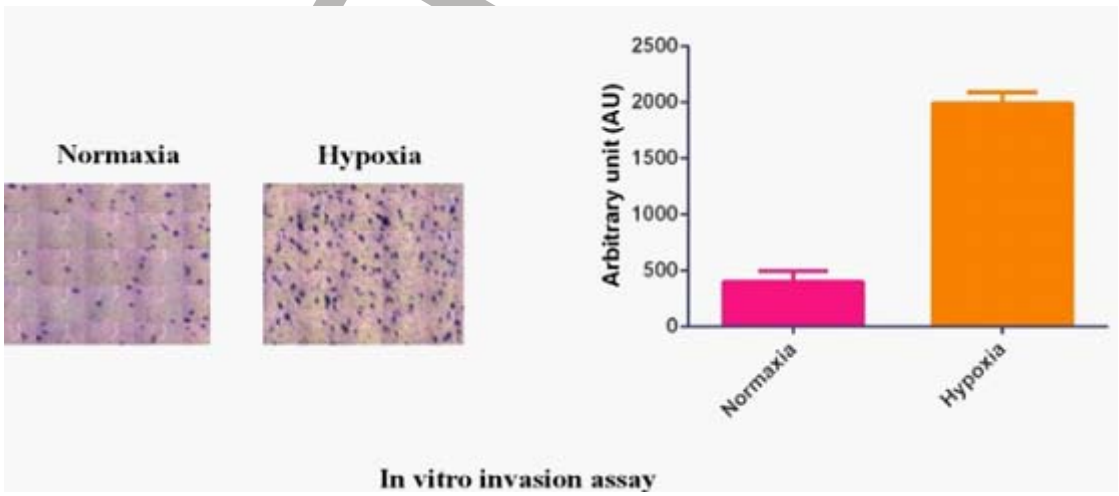

Fig. 3: Hypoxic condition enhances metastatic potential of MRC5 cells: To check the invasive property, MRC5 cells were treated either with or without CoCl2, followed by in vitro invasion assay as described in materials and methods. Invaded cells were stained using 1% Toluidiene Blue (Fluka) in 1% borax (Sigma) for 2 minutes and visualized under light microscope. Invaded cells were quantitated and graph was plotted with respect to arbitrary units

DISCUSSION

Defects in mitochondrial respiratory chain function underlie a spectrum of human diseases, ranging from impairment in cellular metabolisms and ageing (Semenza 2013). Hypoxia response is an endogenous program evolved to adapt the cellular milieu to limited oxygen condition (Kim, Rajagopal *et al*. 2006). Activation of Hypoxia response is protective mechanism against mitochondrial toxicity. In this study we have successfully standardized the best condition to induce hypoxia condition in cell culture systems which is 100µM CoCl2 at 10% O_2 (Figure 1). It is well known that cells in limited oxygen environment usually resistant to cancer chemotherapies. We are reporting here that under hypoxia condition, mitochondria are enlarged which in turn may block the pro-apoptotic stimuli. This blockade in the signal might protect the tumor cells from exogenous drugs like camptothecin and mitomycin which are widely used in chemotherapies (Fruman and Rommel 2014). It is important to note that hypoxia induces only the enlargement of mitochondria but keeping the mitochondrial protein levels intact. This signifies that under hypoxic condition mitochondria could still perform its native function but probably impaired in complex I activity of respiratory chain complex as increased ROS level has been reported in Hypoxia. HIF1α signaling is extensively studied in response to hypoxia induced tumor has been linked to metabolic re-programing, which suppresses the mitochondrial respiration in favor of glycolysis. Here our study shed lights on how Hypoxia condition increases the MRC5 cells metastatic potential which is probably due to the enlargement of mitochondria but not the protein levels.

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