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Cycling hypoxia up-regulates thioredoxin levels in human MDA-MB-231 breast cancer cells

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ABSTRACT

The thioredoxin system is a key cellular antioxidant system and is highly expressed in cancer cells, especially in more aggressive and therapeutic resistant tumors. We analysed the expression of the thioredoxin system in the MDA-MB-231 breast cancer cell line under conditions mimicking the tumor oxygen microenvironment. We grew breast cancer cells in either prolonged hypoxia or hypoxia followed by various lengths of reoxygenation and in each case cells were cultured with or without a hypoxic cycling preconditioning (PC) phase preceding the hypoxic growth. Flow cytometry-based assays were used to measure reactive oxygen species (ROS) levels. Cells grown in hypoxia showed a significant decrease in ROS levels compared to normoxic cells, while a significant increase in ROS levels over normoxic cells was observed after 4 h of reoxygenation. The PC pre-treatment did not have a significant effect on ROS levels. Thioredoxin levels were also highest after 4 h of reoxygenation, however cells subjected to PC pre-treatment displayed even higher thioredoxin levels. The high level of intracellular thioredoxin was also reflected on the cell surface. Reporter assays showed that activity of the thioredoxin and thioredoxin reductase gene promoters was also highest in the reoxygenation phase, although PC pre-treatment did not result in a significant increase over non-PC treated cells. The use of a dominant negative Nrf-2 negated the increased thioredoxin promoter activity during reoxygenation. This data suggests that the high levels of thioredoxin observed in tumors may arise due to cycling between hypoxia and reoxygenation.

1. Introduction

Thioredoxin (Trx), together with thioredoxin reductase (TrxR), are the major components of the Trx system [1], which is upregulated by oxidative stress [2,3] and functions to reduce key cellular proteins, including transcription factors, such as NF-κB, AP-1 and Ref-1 [4,5]. Trx is a 12 kDa redox protein that is over-expressed in cancers, particularly in the most metastatic and invasive cancers [6–8]. High levels of Trx protect cancer cells from apoptosis and also correlate with tumors resistant to chemotherapy [9,10]. Often the high levels of Trx present in cancers have been associated with the oxidative stress known to exist in tumors, since ROS induces Trx expression. In addition Trx is required for the activation of the hypoxic inducible factor-1 (HIF-1) transcription factor [11,12], which is responsible for up-regulating expression of many proteins required during hypoxic growth of cancer cells [13]. Therefore, Trx has potential functions in both the hypoxic regions and the oxidative stressed regions of tumors.

However the oxygenation status of tumors is quite complex and dynamic. Tumors often have a poorly developed vasculature resulting in an inefficient delivery of oxygen, which leads to 'cyclic hypoxia', with cycles of hypoxia followed by reoxygenation [14]. The cycling parameters can vary significantly. Fluctuations in red blood cell flux result in a frequency of a few cycles per hour [15], while vascular remodeling contributes to slower frequencies that vary from hours to days [16]. Studies have shown that cycling hypoxia conditions lead to an up-regulation of the HIF-1 transcription factor that supersedes the levels found in hypoxic growth alone [17]. Since cycling hypoxia involves several re-oxygenation phases it may also lead to increased levels of ROS and antioxidant enzymes.

A comparable situation occurs when hearts are subjected to cycles of ischemia/reperfusion, which preconditions (PC) for tolerance against a subsequent ischemic event. Rat hearts made tolerant to ischemia by 4 cycles of short-term ischemia followed by reperfusion also showed an upregulation of Trx expression [18]. The use of a Trx inhibitor demonstrated that Trx was required for the cardioprotective properties in the PC adapted heart. One of

Abbreviations: 7AAD, 7-aminoactinomycin D; DCF-DA, 2′,7′-dichlorofluorescein diacetate; HIF, Hypoxia-inducible-factor; Nrf-2, nuclear factor-erythroid-2 p45-related factor 2; PC, preconditioning; MMP, matrix metalloproteinases; Prx, peroxiredoxin; Trx, thioredoxin; TrxR, thioredoxin reductase.

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the consequences of ischemia is hypoxia. However as yet it has not been reported whether cycling hypoxia, as a pre-conditioning step, in cancer cells may similarly cause an upregulation of Trx expression. We therefore investigated Trx expression in breast cancer cells in response to several different oxygen growth conditions, including hypoxia, reoxygenation and in each case determining the effect of PC by subjecting the cells to short-term cycles of hypoxia and reoxygenation prior to subsequent hypoxia and reoxygenation. We also assessed if ROS levels increased during these same oxygen growth conditions.

2. Materials and methods

RPMI1640 and Lipofectamine 2000 were purchased from Life Technologies (Vic, Australia) and Fetal Bovine serum (FBS) from Quantum Scientific (Qld, Australia). The Trx antibody (5G8) is a mouse monoclonal IgG antibody generated against recombinant human thioredoxin; Ref-1 antibody (C-4) was purchased from Santa Cruz Biotechnology (CA, USA), HIF-1β (ARNT) and HIF-1α antibodies were purchased from BD (NSW, Australia); HRP conjugated secondary antibodies were purchased from Bio-Rad (NSW, Australia). Alexa Fluor 488 goat anti-mouse IgG was purchased from Life Technologies. Luciferase assay kits were purchased from Promega (Madison, WI, USA).

2.1. Cell culture and oxygenation conditions

The MDA-MB-231 breast cancer cell line was cultured in RPMI-1640 supplemented with 100 μg/ml penicillin and 100 μg/ml streptomycin and 10% FBS in a humidified atmosphere of 5% CO_2/95% air at 37 °C. For hypoxic growth, cells were cultured in 0.1% oxygen, 5% CO_2, 95% nitrogen in a hypoxic C-Chamber regulated by a ProOx C21 controller (Biospherix, NY, USA). Hypoxic samples were processed in a hypoxic C-Shuttle cell culture Glovebox (Biospherix). For cycling, cells were subjected to 4 cycles of 10 min hypoxia (as above) and 20 min reoxygenation by placing in 5% CO_2/95% air. This was followed by 16 h hypoxic growth and then culture in 5% CO_2/95% air for a reoxygenation stimulus (Fig. 1B).

2.2. ROS assays

Cells were grown in 25 cm^2 culture flasks under the oxygen growth conditions described above. The cells were then incubated in 10% FBS containing medium with 5 μM DCF-DA (Molecular probes, CA, USA) for 30 min at 37 °C. For hypoxic cells the addition of DCF-DA and incubation were also performed under hypoxic conditions. Cells were then detached using cell dissociation buffer (Life Technologies), counted and resuspended in phosphate buffered saline (PBS). The cells were centrifuged at 265×*g* for 5 min and resuspended in PBS or in PBS containing an appropriate dilution of 7AAD. The cells were left on ice until analysis using the BDAria FACS machine.

2.3. Western blotting

MDA-MB-231 cells were grown to confluency in a 9 cm petri dish, subjected to the various oxygen growth conditions and then lysed in NP-40 lysis buffer (150 mM NaCl, 50 mM Tris–HCl pH 8, 0.5% (v/v) Nonidet P-40, 0.5 mM EDTA, 2 mM PMSF, 1 μl/ml of protease inhibitor cocktail VI (Astral Scientific, NSW, Australia)) using a sonicator. The DC Protein assay kit (Bio-Rad) was used for protein estimation. Protein (50 μg) was run on a SDS–PAGE, transferred to a PVDF membrane (Bio-Rad) and probed with the applicable antibodies. HIF-1β (ARNT) was used as a loading control. Proteins were visualized using the Enhanced Chemiluminescence detection kit (GE Healthcare, NSW, Australia) and analysed with the Fujifilm Las-3000 machine.

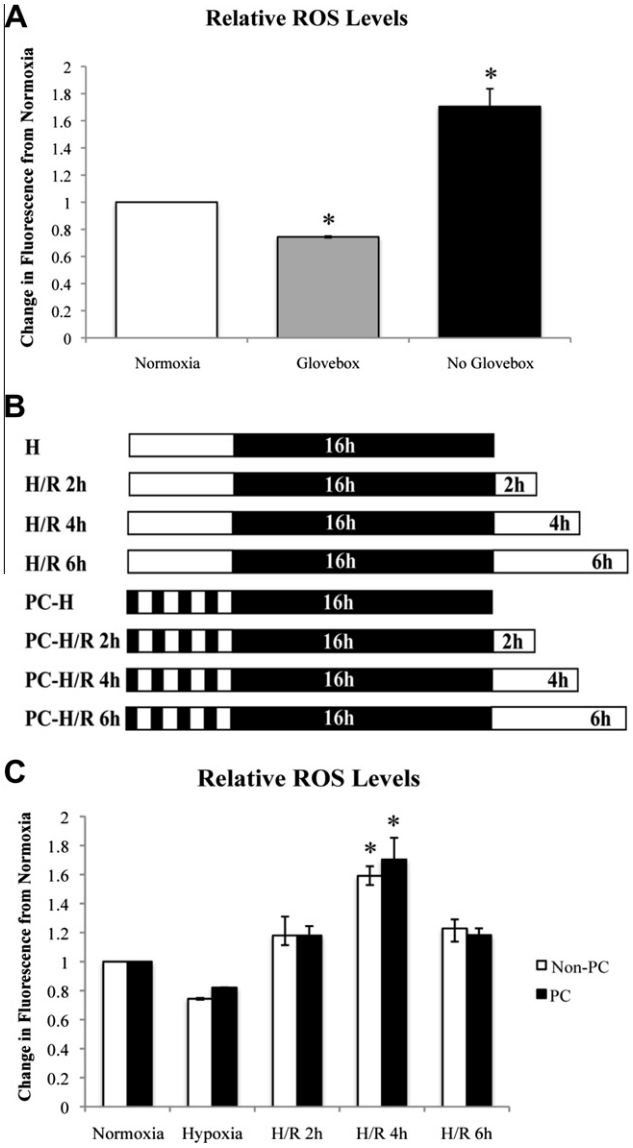

Fig. 1. Reactive oxygen species in MDA-MB-231 cells cultured in hypoxia and during reoxygenation (A) Relative ROS levels after processing hypoxic grown cells with or without a Glovebox. (B) Scheme of the hypoxic (H) and hypoxic followed by reoxygenation (H/R) culture conditions used for the cells with time of reoxygenation shown in hours. A pre-conditioning (PC) phase was also included for each H and H/R condition. Cells were grown under normoxia for equivalent lengths of growth. (C) ROS levels during the different oxygen conditions for cells cultured with (PC) or without (non-PC) preconditioning cyclic hypoxia. * = significant difference compared to normoxia with $p < 0.05$.

2.4. Immunocytochemistry

Cells were grown on collagen-coated coverslips in 4-well plates and washed with PBS. Blocking was achieved using 0.5 ml of 2 mg/ml BSA in PBS for 20 min at 4 °C. Cells were washed twice with PBS and then incubated with 20 μg/ml of the 5G8 anti-thioredoxin antibody in 0.1 mg/ml BSA for 30 min at 4 °C. Cells were washed 5 times with PBS and then incubated with secondary antibody diluted in 1 mg/ml BSA. Cells were washed 7 times in PBS and fixed using 3% formaldehyde and left at RT for 15 min. They were then washed twice with PBS and mounted onto glass microscope slides in VECTASHIELD mounting medium (containing DAPI (Vector