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

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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

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 hours of reoxygenation. The PC pre-treatment did not have a significant effect on ROS levels. Thioredoxin levels were also highest after 4 hours 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 12kDa redox protein that is over-expressed in cancers, particularly in the most metastatic and invasive cancers [6,7,8]. 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 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 hours hypoxic growth and then culture in 5% CO_2/95 % air for a reoxygenation stimulus (Figure 1B).

2.2 ROS assays

Cells were grown in 25cm^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.

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^{o}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^{o}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 laboratories, CA, USA). Cells were viewed with a Zeiss AxioImager Z1 microscope.

2.5 Cell surface FACS

Cells were resuspended in medium and left at room temperature for 40 min to block non-specific binding. The cells were centrifuged at 265×g for 5 min and resuspended in 1×PBS pH 7.4 to yield 1×10^6 cells/mL. 500μL of this suspension was centrifuged at 265×g for 5 min. Pellets were resuspended in 300μL of 2% BSA/PBS solution containing 20μg/ml of the 5G8 anti-thioredoxin antibody and incubated for 60 min at 4°C. Cells were washed with 2% BSA/PBS and resuspended in 300μL of 2% BSA/PBS solution containing the HRP conjugated anti-mouse secondary antibody and incubated for 30 min at 4°C. Cells were washed twice with 2% BSA/PBS and resuspended in PBS or PBS containing 7AAD (Life technologies) as required. The cells were left on ice until analysis using a BDAria FACS machine (BD).

2.6 Luciferase assays

Luciferase assays were performed as described previously [19,20]. Cells at 90 % confluency in 24-well plates were transfected with 500 ng of plasmid DNA using Lipofectamine 2000. For co-transfections an equal quantity of pcDNA3.1 (Life Technologies) or dnNrf-2 expressing plasmid [21] was added with the reporter construct. The Trx promoter constructs have been described previously [19,20] and the TrxR construct containing 830 bp of TrxR promoter sequence inserted into pGL3-Basic (Promega) was generated by PCR using the oligonucleotides 5'dCTGGAGTTAAAAGACTCT and 5'dCTGGGCTCGCGGCTTTGTCT with human genomic

DNA as template. The cells were grown under the oxygen growth conditions specified above. The reoxygenation phase was for either 4 or 24 hours.

2.7 Statistical Analysis

Data are presented as mean ±SEM from at least three independent experiments. A one-way analysis of variance (ANOVA) was performed to analyse the data sets. Once ANOVA analysis claimed the existence of significant difference in the data, Tukey's honest significance difference test was performed, post hoc. For testing of 2 parameters a two-way Anova was performed, followed by the Bonferroni test. Data were considered statistically significant when $p < 0.05$. All statistical analysis was performed using the Prism computer program (GraphPad Software, CA, USA).

3. Results

3.1 ROS levels

Intracellular ROS levels were measured using DCF-DA by FACS analysis. Our initial experiment was to test if it was necessary to process the hypoxic cells under hypoxic conditions. After growth in hypoxia cells exposed to air during processing of samples showed a statistically significant increase in ROS compared to cells grown in normoxia (Figure 1A), whereas if all processing was performed under consistent hypoxic conditions a significant decrease in ROS levels was obtained for cells grown in hypoxia. This result demonstrates that hypoxic cells exposed to even very short periods of normoxia can result in significantly higher levels of measured ROS. Thus all cells and samples representative of "hypoxia" were processed in a hypoxic Glovebox to avoid inadvertent reoxygenation.

MDA-MB-231 breast cancer cells were then grown under various hypoxia/reoxygenation conditions as shown in figure 1B. Cells were also grown with and without "pre-conditioning" (PC) through four cycles of short-term hypoxia/reoxygenation prior to the hypoxic growth phase. Significant differences in ROS levels were measured in the cells exposed to the different oxygen growth conditions (Figure 1C). Relative to normoxia, cells exposed to hypoxia exhibited a significant decrease in ROS levels while reoxygenation of the cells for either 2, 4 or 6 hours resulted in increase in ROS levels, with the highest level recorded after 4 hours. Cells subjected to PC before the hypoxic exposure yielded similar ROS levels to those obtained from the equivalent non-PC treated cells.

3.2 Intracellular Protein levels of Trx and Ref-1

Since Trx regulates the activity of Ref-1 [4] and Ref-1 in turn is required for HIF-1 transcriptional activity [22] we also sought to determine the protein expression levels of both Trx and Ref-1 in response to hypoxia, reoxygenation and cycling hypoxia. When cells were cultured in hypoxia for 16 hours there was an increase in Trx levels evident on Western blots (Figure 2A), which was confirmed by densitometry analysis (Figure 2C). With PC, an increase in Trx levels was also obtained after the subsequent hypoxic growth phase. However, these increased Trx levels were not shown to be statistically significant. There was no significant change in Ref-1 levels in cells grown in hypoxia, with or without PC, compared to cells grown in normoxia (Figure 2D).

The effect of PC for both Trx and Ref-1 expression was most pronounced during the reoxygenation phase. As shown in Figure 2C Trx levels were higher in cells that had undergone PC prior to the hypoxia and reoxygenation growth, compared to cells that did not undergo PC. The greatest difference was after 4 hours of reoxygenation, which also corresponded to the greatest increase of Trx levels compared to normoxic growth. A two-way Anova showed that both the length of reoxygenation time and whether the cells had undergone PC were statistically significant. For cells that had undergone PC Ref-1 levels were also highest after 4 hours of reoxygenation and a two-way Anova showed that PC treatment was also statistically significant for Ref-1, but the length of the reoxygenation phase was not statistically significant (Figure 2D).

3.3 Cell Surface Trx

To determine if the enhanced expression of intracellular Trx also influenced the levels of Trx present on the cell surface, both immunocytochemistry and FACS analysis were performed. Cells were grown under the condition that gave the highest intracellular Trx

levels (PC followed by hypoxia and 4 hours of reoxygenation) and visual analysis supported an increase in Trx expression on the cell surface (Figure 3C) compared to cells grown in either hypoxia (Figure 3B) or normoxia (Figure 3A). This was confirmed quantitatively using FACS and the increase in cell surface Trx was shown to be statistically significant (Figure 3D).

3.4 Trx system promoter activity during hypoxia and reoxygenation

To assess if the Trx promoter was activated during hypoxia or reoxygenation luciferase reporter assays were utilized. The TrxR promoter was also analysed to determine if both promoters were potentially co-regulated during the different oxygen growth phases. The effect of PC was also assessed. Two different lengths of the Trx promoter were utilized, a 1 kb full-length promoter [20] and a 547 bp fragment that does not contain the antioxidant response element (ARE). A construct containing a hypoxic response element (HRE) was also utilized as a positive control for a hypoxic response. During hypoxia both the Trx and TrxR promoter activities were decreased, although this decrease was not statistically significant (Figure 4A). During re-oxygenation both the Trx and TrxR promoters were activated and this was sustained for 24 hours of reoxygenation (Figure 4A). The increases during the reoxygenation phases were statistically significant. A prior PC step did not make any significant change to the promoter activities. The construct without the ARE element had a decreased basal level of expression (as previously reported [20]) and was not significantly induced during the reoxygenation phase (Figure 4A). PC cycling did not significantly affect the levels of promoter activity (Figure 4B). A further experiment confirmed that Nrf-2 is the transcription factor responsible for the increase during reoxygenation. Co-transfection

of the Trx promoter with a dnNrf-2 construct negated the increased Trx promoter activity observed during reoxygenation (Figure 4C).

4. Discussion

Trx is expressed at high levels in aggressive tumors [8], however the stimuli that give rise to these high levels are not fully defined. Trx is upregulated by oxidative stress [2,3] and Trx may also be induced during hypoxia [23] and both conditions are found within tumors. In reality the tumor oxygen environment is quite dynamic due to poorly formed vasculature leading to cycling between hypoxia and reoxygenation [15]. Our results show that Trx levels in the MDA-MB-231 breast cancer cell line are increased to maximum levels during 4 hours of reoxygenation following growth in hypoxia, while there was a much smaller (and not statistically significant) increase seen during culture in hypoxia. This result mimics the expression of peroxiredoxin I (Prx1), another redox protein and a substrate of Trx. Prx1 was upregulated in A549 lung cancer cells after 4 hours of reoxygenation following 4-16 hours of hypoxia [24] but no increase was observed during hypoxia. The increased levels of both Trx and Prx1 suggest that there may be a coordinated up-regulation of the antioxidant proteins in tumors during the reoxygenation phases. Both the Trx and TrxR gene promoters exhibited higher activity during the rexoygenation phase compared to the normoxic and hypoxic phases. The Prx1 gene promoter was also up-regulated by a reoxygenation phase following hypoxic growth and was shown to be activated by Nrf-2 [24]. Our studies have shown that the ARE element was required for the induction of the Trx promoter and that co-expression of a dominant negative Nrf-2 protein reduced Trx promoter activity. Therefore the co-induction of redox genes is likely to be facilitated through the action of Nrf-2.

Most significantly we show that cycles of hypoxia and reoxygenation preceding the hypoxic growth phase enhances the expression of both Trx and Ref-1 during the subsequent reoxygenation phase, compared to cells that had not undergone cycling. This is a new finding that provides further information on how the high levels of Trx observed in tumors may arise. Our result correlates with the increased Trx levels obtained when rat hearts were pre-conditioned with cycles of ischemia and reperfusion prior to an ischemic event [18]. However the Trx and TrxR promoters are not more active when cells are exposed to PC, indicating that the mechanism for inducing higher Trx protein levels is not at the transcriptional level.

The increase of intracellular Trx in cells subjected to PC cycling followed by hypoxia and reoxygenation was also mirrored on the cell surface. Cell surface Trx levels were significantly higher in cells subjected to cycling, hypoxia and reoxygenation compared to cells grown in normoxia or hypoxia. Recently Trx was shown to enhance both Matrix metalloproteinase (MMP) expression and extracellular activity in breast cancer cells [25]. Therefore Trx was suggested to have functional roles in cancer cells both intra- and extra-cellularly. The high levels of Trx seen inside and on the surface of cells grown in the cycling environment may therefore result in greater functionality of the MMP system, and thus cancer metastasis.

There are conflicting reports regarding whether ROS levels increase or decrease during hypoxic growth of cancer cells. Our data shows that in MDA-MB-231 breast cancer cells ROS levels are significantly decreased in hypoxia, while during the reoxygenation phase they are increased by more than 1.6-fold compared to normoxia. The increase in ROS occurred after 4 hours of reoxygenation, which mimics the increase observed for Trx. However unlike Trx protein levels a PC cycling hypoxia growth phase did not further enhance ROS levels. We also demonstrated that processing of cell samples grown in

hypoxia must also be performed in hypoxic conditions, as even a short exposure to an oxygenated environment leads to increases of ROS levels. Inconsistencies in processing of hypoxic samples may be part of the reason why there is such a difference in ROS levels reported in the literature.

In conclusion we have shown that higher Trx levels are observed in cancer cells subjected to cycles of hypoxia and reoxygenation. In addition the Trx gene promoter is activated in the reoxygenation phase through the action of Nrf-2. The contribution of redox systems in cancer cells is important, since Trx is used as a target for drug therapy. High levels of Trx in patient cancer cells are also correlated with a high likelihood of resistance to treatment [9]. Therefore, an understanding of how expression of the Trx system is regulated in response to the different oxygen conditions occurring in cancers may aid in elucidating the link between the Trx system and cancer progression. It is also necessary to assess the effectiveness of drugs targeting the Trx system in cancer cells cultured in these oxygen cycling conditions.

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Figure Legends

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 $p<0.05$.

Fig. 2. Intracellular expression of Trx and Ref-1 during hypoxia and reoxygenation

Representative Western blot showing Trx and Ref-1 expression levels in MDA-MB-231 cells grown under (A) non-PC or (B) PC conditions. HIF-1α was used as a positive control for hypoxia and the constitutively expressed HIF-1β as a loading control. Levels were quantitated using densitometry and Trx levels are shown in (C) and Ref-1 levels in (D) with respect to normoxic samples. A Δ indicates statistical significance between the non-PC and PC condition with $p<0.05$.

Fig 3. Cell Surface expression of Trx during hypoxia and reoxygenation. Using immunocytochemistry, Trx was detected on the cell surface of MDA-MB-231 cells. Representative images are shown for cells grown under (A) normoxia, (B) hypoxia and

(C) PC hypoxia followed by 4 hours reoxygenation. (D) Trx levels were quantitated by FACS; * = significant difference compared to hypoxia and normoxia, $p<0.05$.

Fig 4. Trx and TrxR promoter activity during hypoxia and reoxygenation. Luciferase assays were performed on lysates from MDA-MB-231 cells transfected with either a full length Trx promoter construct (Trx.1068), a shorter Trx promoter construct without the ARE element (Trx.547), a TrxR promoter construct (TrxR), a hypoxic control plasmid (HRE) or the pGL3.basic control plasmid (Basic). Cells were cultured in normoxia, hypoxia, and in hypoxia followed by reoxygenation for either 4 h (H/R 4h) or 24 hours (H/R 24h). (A) luciferase activity from non-PC treated cells; (B) luciferase activity from PC treated cells. (C) Trx promoter activity from cells co-transfected with the Trx.1068 promoter construct and either pcDNA3 or the dnNrf-2 plasmid. A * indicates significance in comparison with the respective normoxic sample. A # indicates significance difference in comparison with the respective hypoxic sample, $P< 0.01$.

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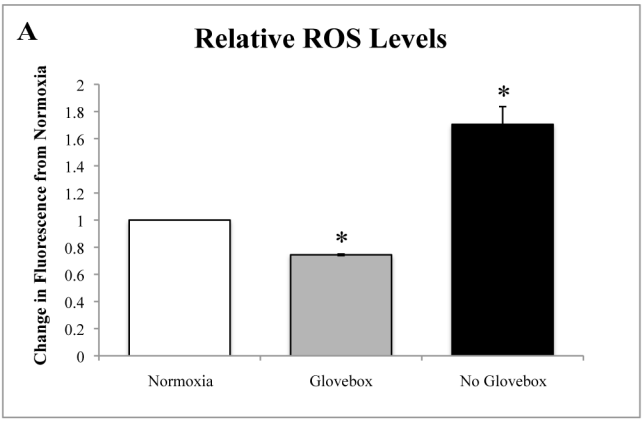
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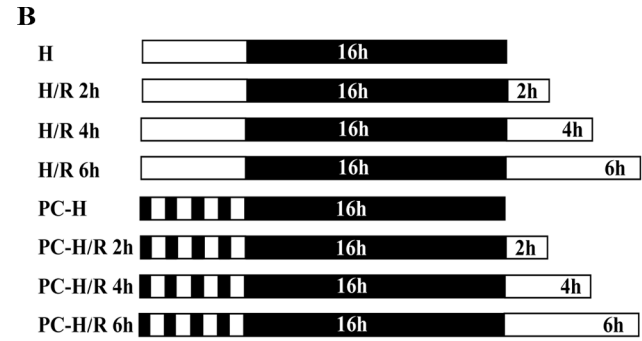
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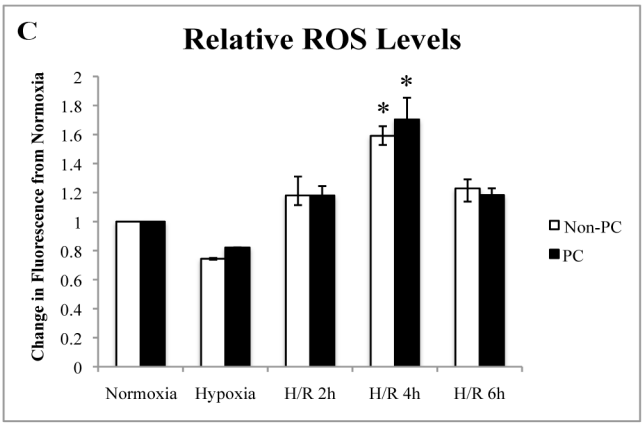
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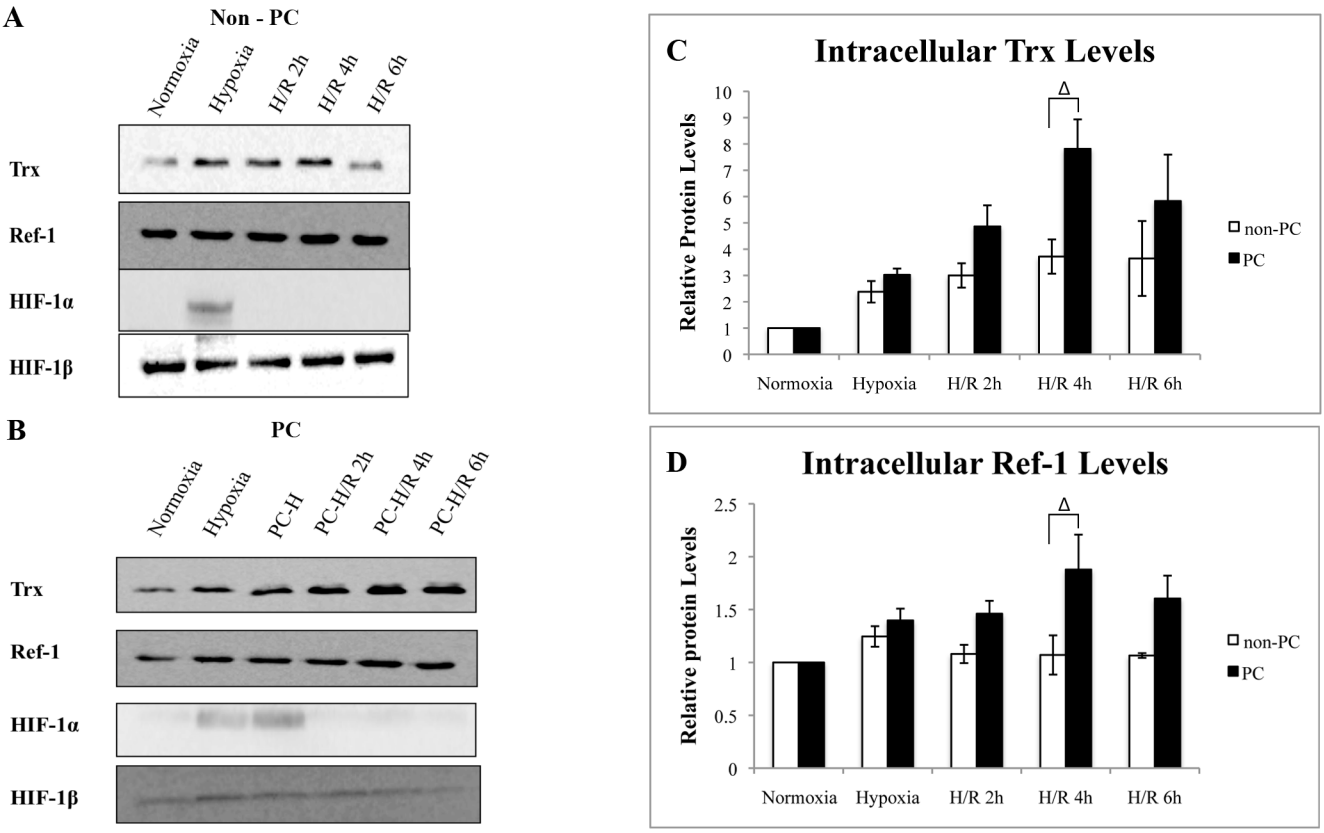
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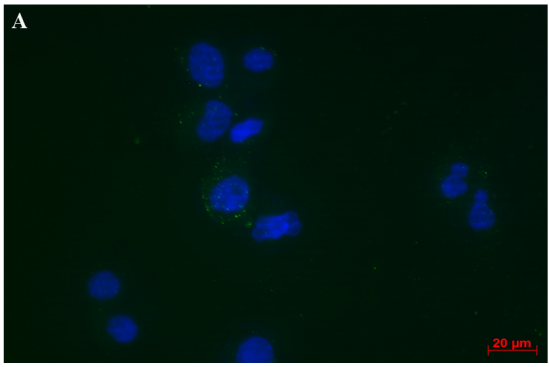
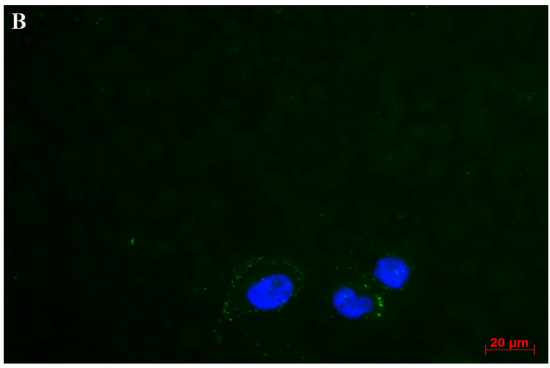
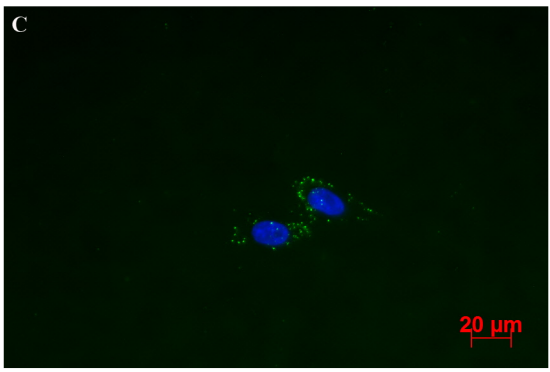
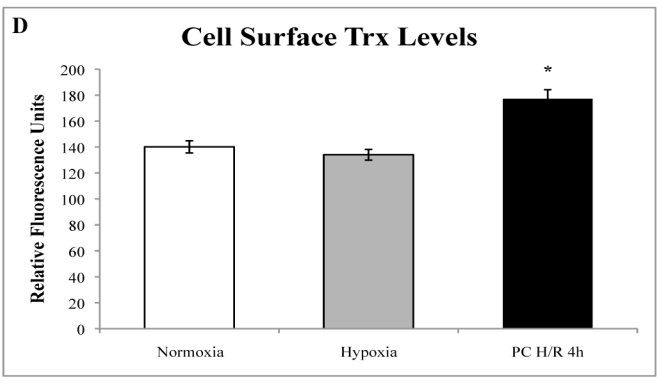
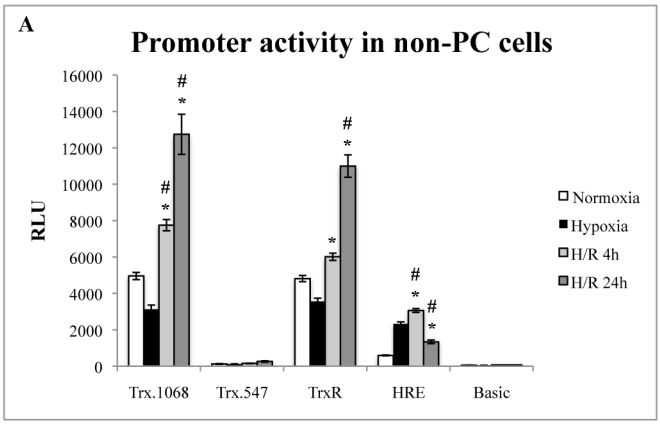
Normoxia
Hypoxia
H/R 2h
H/R 4h
H/R 6h
non-PC
PC
D
Intracellular Ref-1 Levels
Relative protein Levels
2.5
2
1.5
1
0.5
0
Δ
Normoxia
Hypoxia
H/R 2h
H/R 4h
H/R 6h
non-PC
PC

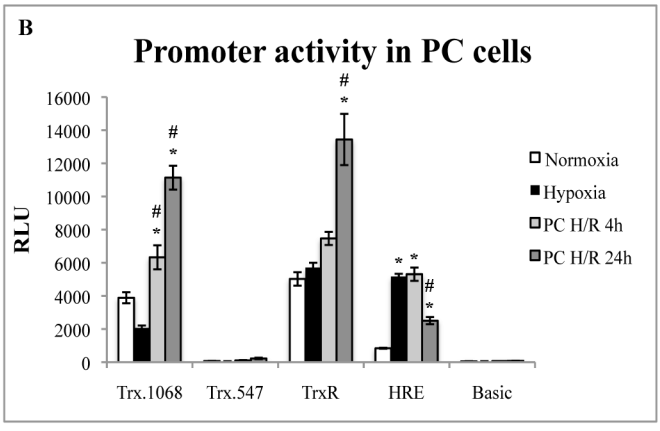

A
20 µm

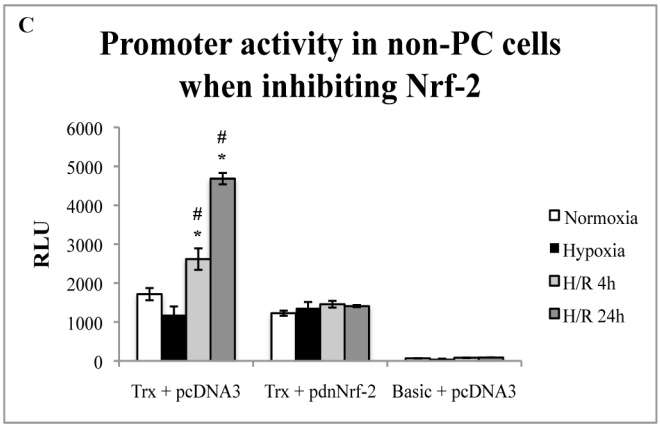

B
20 µm


C
20 µm


D
Cell Surface Trx Levels
Relative Fluorescence Units
200
180
160
140
120
100
80
60
40
20
0
*
Normoxia
Hypoxia
PC H/R 4h


A
Promoter activity in non-PC cells
RLU
16000
14000
12000
10000
8000
6000
4000
2000
0
Trx.1068
Trx.547
TrxR
HRE
Basic
Normoxia
Hypoxia
H/R 4h
H/R 24h


B
Promoter activity in PC cells
RLU
16000
14000
12000
10000
8000
6000
4000
2000
0
Trx.1068
Trx.547
TrxR
HRE
Basic
Normoxia
Hypoxia
PC H/R 4h
PC H/R 24h


C
Promoter activity in non-PC cells
when inhibiting Nrf-2
RLU
6000
5000
4000
3000
2000
1000
0
Trx + pcDNA3
Trx + pdnNrf-2
Basic + pcDNA3
Normoxia
Hypoxia
H/R 4h
H/R 24h