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The Role of Nitric Oxide in Cancer Cell DNA Repair, Hypoxia Adaptation and Drug Resistance

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Abstract

Nitric oxide (NO) has been widely characterized as an important signalling molecule in many biological systems. To study the role of NO in human breast cancer cells, we have developed tetracycline-inducible breast cancer cell MCF7 and co-culture systems in the breast cancer ZR75 cells. We have applied a functional genomic approach using micro arrays and antibody-based proteomic methods to study effects of NO in these cells. We found that many DNA-repair genes appear up-regulated including DNA-Pkcs, topoisomerase 2, and Rad 2. Some oncogenes and tumour suppressor genes also appear up regulated, such as Ret/Ptc2 and c-Yes-1. In accompanied with respiration inhibition, some of the hypoxia regulated genes; such HIF-α and GRP78 are also up-regulated. These different targets could contribute different effects in cancer cell DNA repair, hypoxia adaptation and drug resistance, leading us to develop the therapeutic intervention against cancer.

Keywords: Nitric oxide; cancer; DNA-PK; topoisomerase, rad 2; ret; c-yes-1; GRP78; MCF7; ZR75

Introduction

Nitric oxide (NO) has been widely characterized as an important signalling molecule in many biological systems [1]. NO is generated by a family of NO synthases (NOSs), of which there are three isoforms – endothelial (eNOS), neuronal (nNOS) and immunologically induced (iNOS). Various studies have shown that all three isoforms may be involved in promoting or inhibiting the aetiology of human cancer [2,3]. NO (and related reactive nitrogen species) may have both genotoxic and angiogenic properties. Increased NO generation in a cell may select mutant p53 cells and contribute to tumour angiogenesis by up-regulating VEGF and regulation of HIF-1α [4-7]. In addition, NO may modulate tumour DNA repair mechanisms by up-regulating p53, poly (ADP-ribose) polymerase (PARP) and the DNA-dependent protein kinase (DNA-PK) [7,8].

We were the first group to show that the expression of iNOS could lead to multiple-drug resistance in cancer cells [7,8]. The notion is supported by recent findings showing that depleting endogenous NO enhances cisplatin-induced apoptosis in melanoma cells [9]. Furthermore, iNOS expression in B-cell chronic lymphocytic leukaemia may confer apoptosis resistance by up-regulating DNA-PK activity [10]. However, high concentrations of NO may have the opposite effect. Adenoviral gene transfer of the iNOS gene enhances the radiation-response of human colorectal cancer treatment [11]. In fact, over the last decade, many reports have presented both positive and negative aspects of NO in tumour biology. Some reports found NO to be either cytostatic or cytotoxic [12]. For example some studies show that NO provides cytoprotection to tumour cells by increasing their angiogenic activity and hypoxia adaptation [13]. For instance, the key angiogenic factor, VEGF, has been found to be either up-regulated [14] or down-regulated by NO [15]. Jenkins et al [13] found that an iNOS cDNA expression cassette transfected into human colon cancer DLD-1 cells (which generate 20 pmol/min/mg NOS activity) promoted tumour growth [13]. However in DLD-1/recombinant iNOS-inducible cell culture, which generating 300 pmol/min/mg NOS activity showed a reduction in tumour growth [16] (Figure 1).We have proposed that there is a dose-dependent effect of NO on tumour growth [2]. In general, high levels of iNOS expression may be cytostatic or cytotoxic for tumour cells, whereas lower activity can have the opposite effect and promote tumour growth. Because of the complexity of NO reaction(s) in cancer, the traditional NOS inhibitors are difficulty to apply directly in cancer treatments. To study the role of NO in human breast cancer cells, we have developed tetracycline-inducible breast cancer cell MCF7 and co-culture systems in ZR75 cells. We have applied a functional

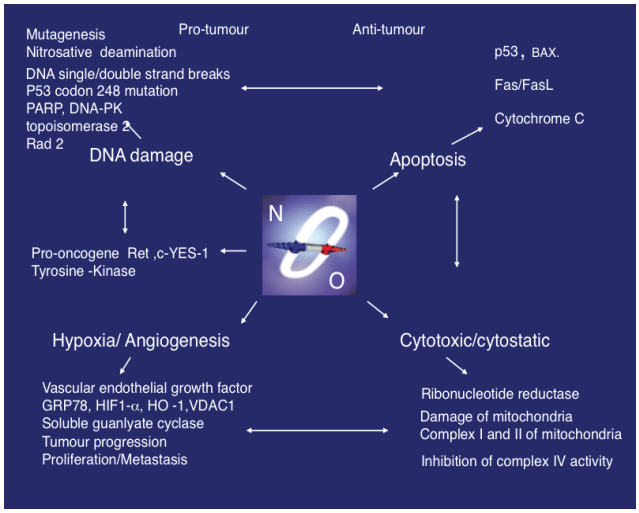

Figure 1: A schematic representation of the dual action of nitric oxide in tumour growth.

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genomic approach using micro arrays and antibody-based proteomic methods to study dose-dependent effects of NO in these cells.

Materials and Methods

Cell culture: MCF-7 and ZR75 cells were obtained from European collection of cell culture (Wiltshire, UK). The tetracyline-inducible cell line- Tex293 clone 22 carrying transfected human iNOS cDNA under the control of a tetracycline-inducible promoter was prepared as described previously [16]. Cell were grown in DMEM containing 25mM glucose and 10% fetal calf serum, as described [17].

DNA transfection and generation of tetracycline-inducible MCF7 cell line

The tetracycline-regulated mammalian expression vector pcDNA4/TO (Invitrogen, Carlsbad, CA) was used to generate a human iNOS cDNA expression construct. To develop tetracycline-inducible NO-generating cell lines, the pcDNA4/TO-iNOS and pcDNA6/TR (Invitrogen) constructs were co-transfected into MCF7 cells and dual selection using Zeocin for the human iNOS gene and blasticidin for the Tet repressor gene was carried out. A panel of 25 cell lines was isolated

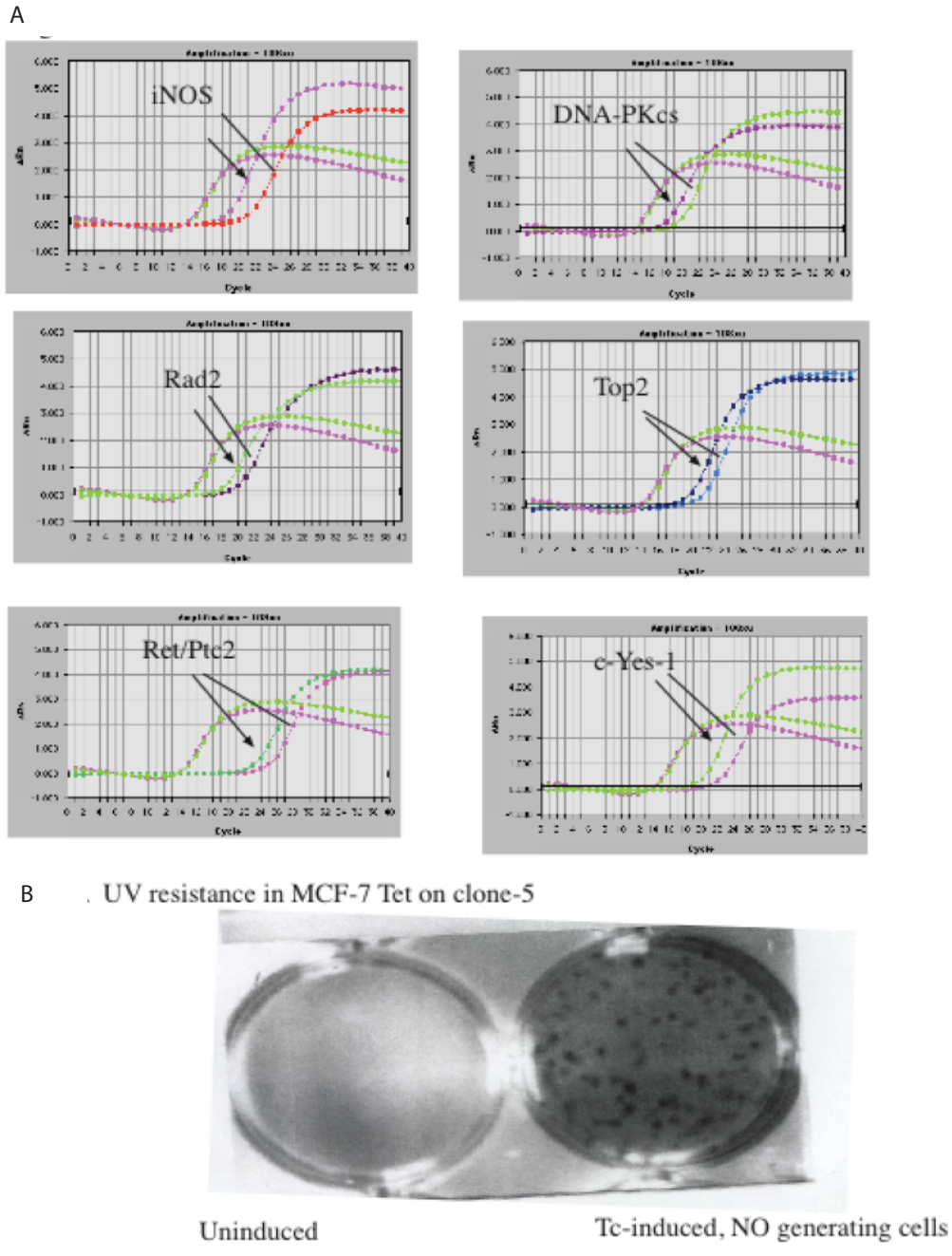

Figure 2: MCF-7 tet-on clone 5. A. Real-time RT PCR assay detecting iNOS, DNA-Pkcs, topoisomerase 2 (Top2) Rad 2, Ret and c-Yes-1 transcripts. All six genes are significantly up-regulated after induction (10 ng/ml tetracycline) for 16 h. B. Following exposure of cells to 120 mJ/cm^2 UV-C.m^2, the tetracycline-induced cells showed significant UV-c resistance with 10-fold more colonies surviving (stained with methyl green) than untreated cells after two weeks growth.

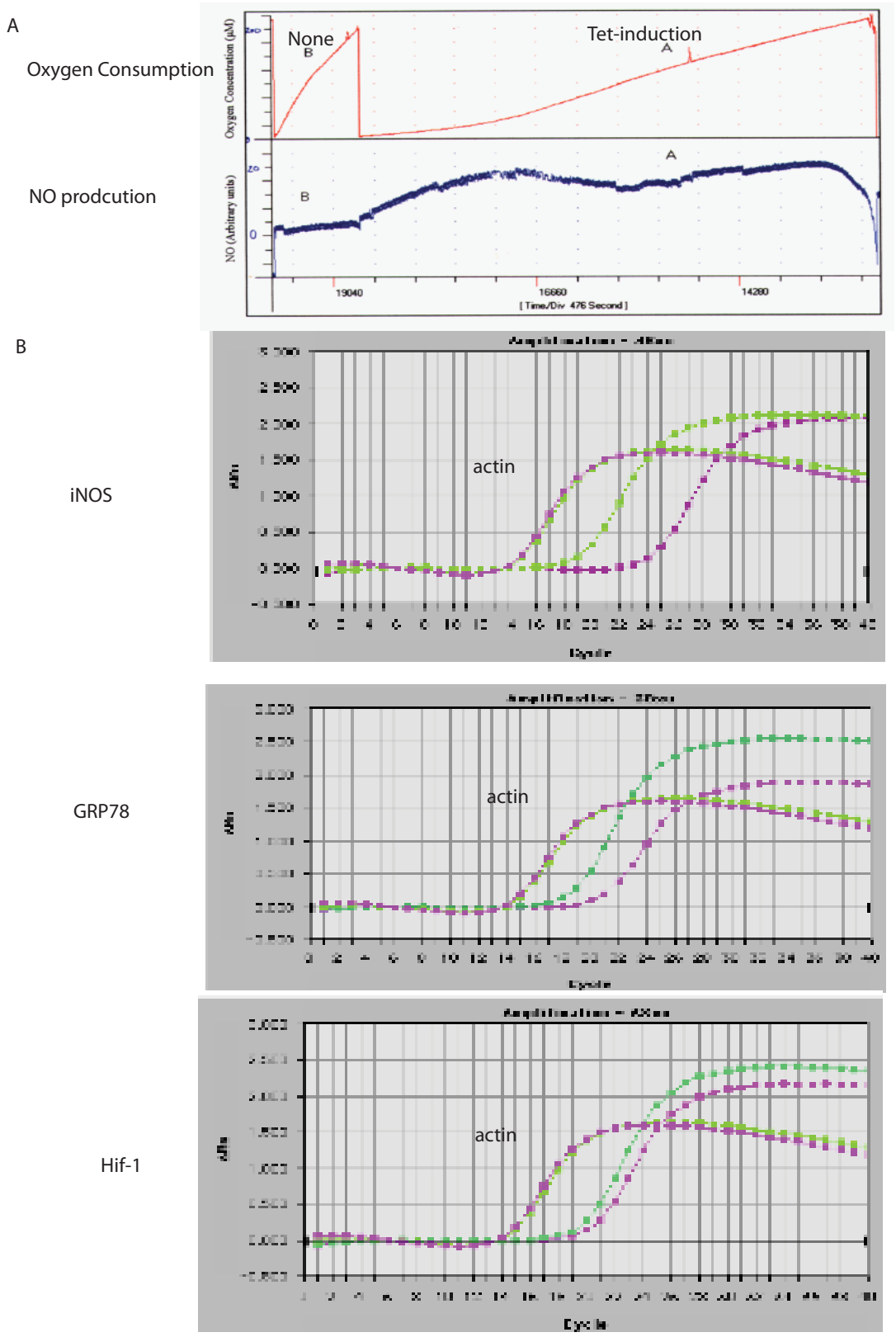

Figure 3: NO causes inhibition of respiration and alterations in patterns of gene expressing in a co-culture model using ZR75-1 breast cancer cells grown alongside NO-generating cells. A. Respiration rate is reduced significantly as NO concentration increases. B. Confirmation of the microarray data by real-time PCR for iNOS, Hinf-1a and Grp78 mRNA. Green lines indicate the NO-induced samples; Purple solid lines indicate the un-induced samples. Actin was used as a control, (show as the traces on the left of the graphs).

showing tetracycline-dependent (10 ng/ml) induction of NO. MCF7-5 was selected for further study.

Nitrite assay, nitric oxide and oxygen consumption measurement

Griess reaction (Promega, UK) which contains 1% sulfanilamide, 0.1% n-1-naphthylene diamine Dihydrochloride, was used to measure the nitrite concentration in the medium, which is used as a reflection of NO production. Absorbance at 540 nm against a reference 620 nm was measured with sodium nitrite as a standard. Nitric oxide and oxygen consumption measurements were carried out as described [17]

Breast cancer cell and NO-producing cell co-culture

To study the NO-mediated tumor cell-killing, we co-cultured human breast cancer cell line, ZR75-1with No-generating Tex293 clone 22 cells. We mixed together 10^6 DLD-1 cells and $2x10^6$ Tex293 clone 22 cells and seeded them overnight into 6-well plates.

RNA isolation, Microarrays and Real time RT-PCR analysis

Total RNAs were extracted from the NO-generating cells and control cells using Trizol reagent (Invitrogen, UK). Polyadenylated (poly A^+) RNA will be purified from total RNA and synthesis of cDNA involved use of T7-(dT)24 primers and SuperScript II according to manufacturer's instructions (Invitrogen, UK). *In vitro transcription* was performed to produce biotin labeled cRNA using *a bioarray high yield RNA transcription labeling kit* (Enzo Diagnostics, USA). cRNA was fragmented to about 200 bp (Affymetrix, USA) and hybridized with the HG-*U95Av2 array* represents approximately 10,000 full-length genes. The resulting expression data were analyzed by MAS 5.0 (Affymetrix) software for the data normalization, data clustering, expression profiling. The microarray results will be further confirmed by the Taq Man real time RT-PCR method. The relative amounts of all mRNAs were calculated using the relative cycle threshold method (Perkin-Elmer, Norwalk, CT).

Results and Discussion

Generation of the tetracycline-inducible NO-producing MCF7 cell line

We have carried out transfection experiment using our tetracycline constructs and successful isolated a panel of tetracycline-inducible NO-producing MCF7 cell lines. One of the clones, MCF7 Tet-on clone 5, can generate up to 20 ± 5 μM nitrite/10^6 cells (n=3) by 10 ng/ml tetracycline in 24 h. This induction can be completely inhibited by addition of the NOS inhibitor L-NIO (20 μM).

Gene Array Analysis of NO-producing MCF7 Tet-on clone 5 cell

We have also carried out Affrymetrix array analysis (U95 Av2) on the MCF7 Tet-on clone 5 and found that over 100 genes are up-regulated and 50 are down-regulated over 2 ± 0.5-fold gene expressing changes (Xu et al. unpublished data) using the 12,000-gene array format (using 10 ng/ml tetracycline induction). Many of the genes can be clustered into families, such as DNA-repair gene family including DNA-Pkcs, topoisomerase 2, and Rad 2. Some oncogenes and tumour suppressor genes appear regulated, such as Ret/Ptc2 and c-Yes-1. Interestingly, both of these genes are tyrosine kinases and are significantly up-regulated (over 3 fold). We have used real-time PCR to confirm some of the array data (Figure 2A).

NO-producing breast cancer cells are UV resistance

We have also carried out cytoprotection assays using 120 mJ/cm^2 UV-C to examine the MCF7 Tet-on clone 5 UV resistance. As shown on figure 1b, the tetracycline induced cells in MCF-7 tet-on clone 5 showed significant UV-C resistance after tetracycline induction (10 ng/ml), with 10-fold more colonies (stained with methyl green) surviving in NO-treated cells compared with untreated cells (Figure 2B).

NO regulates hypoxia regulated genes in breast cancer cell and NO-producing cell co-culture

Using NO-generating cells and breast cancer cell lines ZR75 co-culture system, we found that there were several hypoxia regulated genes, e.g. HIF1-α and GRP78 (Figure 3B) were up-regulated accompanied by nitric oxide increase and respiration rate decrease (Figure 3A).

Cancer had become one of the leading causes of death in the developed world. Multi-drug resistance and angiogenesis-related metastasis are the major problems facing cancer patients. We have shown here that a free radical gas, nitric oxide (NO) contributes to cancer cell DNA repair, hypoxia adaptation, drug resistance and apoptosis. Recently, our studies on NO-mediated cytoprotection showed an NO-mediated increase in expression of a hypoxia-regulated gene, glucose regulated protein 78 (Grp78) [17]. We have isolated specific anti-GRP78 scFV antibody [18] and conjugated it with quantum-dot and shows the conjugated scFV GRP78 antibody processes the antitumor activity *in vivo* and *in vitro* [19]. The antibody-mediated tumour killing with Fas:Fc antibody has previously successfully used to target cancer cell apoptosis [16]. Here we report developing further NO-inducible system in breast cancer cells. We found that hypoxia regulated gene HIF−α γενε expression was up-regulated (Figure 3B). We have also discovered several novel targets, which are NO-regulated, such as topoisomerase 2(TOP2), and Rad 2 genes (Figure 2A). TOP2 gene amplifications or deletions have been found to associated with an increase in responsiveness to anthracycline-containing chemotherapy regimens relative to non-anthracycline regimens that is similar to that seen in patients with *HER2* amplification [20]. Rad 2 gene is a single-stranded DNA endonuclease [21] being involved in excision repair of DNA damaged with UV light, bulky adducts, or cross-linking agents. Increasing its expression could lead UV-C resistance observed in NO-producing breast cancer cells (Figure 2B). Some oncogenes and tumour suppressor genes also appear regulated, such as Ret/Ptc2 and c-Yes-1. Ret mutations are involved in the hereditary Medullary Thyroid Carcinoma (MTC) [22] and tyrosine kinase inhibitors targeting activated RET are currently in clinical trials for the treatment of patients with MTC. C-Yes-1 is a non-receptor protein tyrosine kinase, involved in binding to many receptor tyrosine kinases including EGRF, PDGFR, CSF1R and FGFR [23]. Interesting, the Ret and c-YES-1 are both tyrosine-kinases, rich drugable targets for developing small molecules for cancer treatment. The newly discovered nitric oxide targets described here will lead us to develop more therapeutic agents against cancer.

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