Reactive oxygen species-linked regulation of the multidrug resistance transporter P-glycoprotein in Nox-1 overexpressing prostate tumor spheroids

Maria Wartenberg[a], Edda Hoffmann[b], Heinrich Schwindt[b], Frank Grünheck[b], John Petros[c,d], J. Rebecca S. Arnold[c,d], Jürgen Hescheler[b], Heinrich Sauer[e,*]

[a] *Department of Cell Biology, GKSS Research Center, Teltow, Germany*
[b] *Department of Neurophysiology, University of Cologne, Cologne, Germany*
[c] *Departments of Urology and Pathology and Hematology/Oncology, Emory University, Atlanta, GA, United States*
[d] *Atlanta VA Medical Center, Atlanta, GA, United States*
[e] *Department of Physiology, Justus-Liebig-University Giessen, Aulweg 129, 35392 Giessen, Germany*

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Abstract **Expression of the multidrug resistance (MDR) transporter P-glycoprotein (P-gp) has been demonstrated to be regulated by hypoxia-inducible factor-1α (HIF-1α) and inhibited by intracellular reactive oxygen species (ROS). Herein, P-gp and HIF-1α expression were investigated in multicellular prostate tumor spheroids overexpressing the ROS-generating enzyme Nox-1 in comparison to the mother cell line DU-145. In Nox-1-overexpressing tumor spheroids (DU-145Nox1) generation of ROS as well as expression of Nox-1 was significantly increased as compared to DU-145 tumor spheroids. ROS generation was significantly inhibited in the presence of the NADPH-oxidase antagonists diphenylen-iodonium chloride (DPI) and 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF). Albeit growth kinetic of DU-145Nox1 tumor spheroids was decreased as compared to DU-145 spheroids, elevated expression of Ki-67 was observed indicating increased cell cycle activity. In DU-145Nox1 tumor spheroids, expression of HIF-1α as well as P-gp was significantly decreased as compared to DU-145 spheroids, which resulted in an increased retention of the anticancer agent doxorubicin. Pretreatment with the free radical scavengers vitamin E and vitamin C increased the expression of P-gp as well as HIF-1α in Nox-1-overexpressing cells, whereas no effect of free radical scavengers was observed on *mdr-1* mRNA expression. In summary, the data of the present study demonstrate that the development of P-gp-mediated MDR is abolished under conditions of elevated ROS levels, suggesting that the MDR phenotype can be circumvented by modest increase of intracellular ROS generation.**

Keywords: P-glycoprotein; NADPH oxidase; Nox-1; Reactive oxygen species; Multicellular tumor spheroid; Hypoxia-inducible factor-1α

1. Introduction

Development of multidrug resistance (MDR) mainly through overexpression of MDR transporters belonging to the ABC cassette family of transporters, e.g. P-gp, MDR-associated protein (MRP), lung resistance protein, and breast cancer-related protein is currently the main cause for the failure of chemotherapeutic cancer treatment [3,15,16]. We have recently demonstrated that intrinsic expression of P-gp in three-dimensional tissues of multicellular tumor spheroids is correlated to hypoxia in central areas of the tumor tissue [28,31]. This may correspond to the well known chemoresistance of hypoxic tumors which results in a poorer prognosis of solid tumors with hypoxic regions than their well-oxygenated counterparts [12]. Consequently, we and others showed that P-glycoprotein (P-gp) expression is regulated by the transcription factor HIF-1α which is induced under conditions of hypoxia [8,31]. Under normoxia the HIF-1α subunit is subjected to oxygen-dependent ubiquitination and proteasomal degradation that is mediated by the von Hippel–Lindau protein [20]. HIF-1α ubiquitination and degradation might also be regulated by redox modifications of the protein. Iron regulatory protein 2 is targeted for ubiquitination and proteasomal degradation by oxidative modifications that occur in the presence of iron and oxygen [14].

Low levels of reactive oxygen species (ROS) have been implicated as intracellular signaling molecules in cellular processes such as proliferation, apoptosis, and senescence [19]. It is known for many years that cancer cells own the capacity to endogenously generate ROS to significant amounts which may be one or the only cause of their excessive growth [23]. We have previously shown that small exponentially growing tumor spheroids are active in ROS generation which is downregulated upon development of quiescent cell layers and initiation of MDR in the depth of large tumors [32]. Recently, it was demonstrated that the superoxide-generating oxidase Nox-1 is functionally required for ras oncogene transformation [17]. Furthermore, it was shown that NOX5 NADPH oxidase regulates growth and apoptosis in DU-145 prostate cancer cells [4]. The present study was undertaken to evaluate whether overexpression of Nox-1 with the consequence of elevated intracellular ROS levels would affect HIF-1α and P-gp levels as well as the development of a MDR phenotype. P-gp expression has been recently demonstrated by us and others to be regulated by exogenously added prooxidants [9,24,32]. Apparently, low levels of ROS downregulate P-gp expression whereas high concentrations of prooxidants which cause oxidative stress result in

*Corresponding author. Fax: +49 221 344527.
E-mail address: heinrich.sauer@physiologie.med.uni-giessen.de (H. Sauer).

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upregulation. The latter feature may be related to the anti-apoptotic properties of the P-gp transporter [5]. Our data show that overexpression of Nox-1 in DU-145 prostate cancer cells downregulated HIF-1α as well as P-gp expression, and prevented the development of a MDR phenotype. This effect is reversed in the presence of antioxidants which result in upregulation of HIF-1α as well as P-gp expression. The data of the present study shed new light on the ROS-linked regulation of P-gp, and should be considered in approaches that use anti-oxidants for cancer treatment in patients.

2. Materials and methods

2.1. Culture technique of multicellular tumor spheroids

The human prostate cancer cell line DU-145 as well as the DU-145Nox1 cell line overexpressing Nox-1 [1] were used throughout the whole study. The cell lines were grown routinely in 5% CO_2, humidified air at 37 °C with Ham's F-10 medium (Biochrom, Berlin, Germany) supplemented with 10% fetal calf serum (Sigma, Deisenhofen, Germany), 2 mM glutamine, 0.1 mM β-mercaptoethanol, 2 mM minimal essential medium, 100 IU/ml penicillin, and 100 μg/ml streptomycin (Invitrogen, Karlsruhe, Germany). Tumor spheroids were grown from single cells. Cell monolayers were enzymatically dissociated with 0.1% trypsin, 0.05% EDTA, and seeded in siliconized 250-ml spinner flasks (Integra Biosciences, Fernwald, Germany) with 250 ml of complete medium and agitated at 20 rotations per minute using a Cell-spin stirrer system (Integra Biosciences). Cell culture medium was partially (100 ml) changed every day.

2.2. Apoptosis and cell vitality assay

Apoptosis was assessed by annexin V staining using FITC-labeled annexin V (BD Pharmingen, San Diego, CA). Briefly, cells were incubated with 2 μl/ml annexin V solution for 15 min and fluorescence recorded at 488-nm excitation. For positive controls, cells were treated for 8 h with 2% dimethyl sulfoxide (DMSO). Cell lethality was assessed by the use of the lethal dye Sytox green (Molecular Probes, Eugene, OR) which labels the cell nuclei of dead cells with compromised cell membranes. For positive controls, cells were fixed with methanol prior to labeling with 100 nM Sytox green for 15 min. Sytox green fluorescence was excited at 488-nm.

2.3. Measurement of ROS generation

Intracellular ROS levels were measured using the fluorescent dye 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCF-DA) (Molecular Probes, Eugene, OR), which is a non-polar compound that is converted into a non-fluorescent polar derivative (H_2DCF) by cellular esterases after incorporation into cells. H_2DCF is membrane impermeable and is rapidly oxidized to the highly fluorescent 2′,7′-dichlorofluorescein (DCF) in the presence of intracellular ROS. For the experiments, multicellular tumor spheroids were incubated in E1 medium (containing in mM: NaCl 135, KCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 1, glucose 10, HEPES 10 (pH 7.4 at 23 °C)), and 20 μM H_2DCF-DA dissolved in DMSO was added. After 20 min, intracellular DCF fluorescence (corrected for background fluorescence) was evaluated in 3600 μm^2 regions of interest using an overlay mask unless otherwise indicated. For fluorescence excitation, the 488-nm band of the argon ion laser of a confocal laser scanning microscope (LSM410, Carl Zeiss, Jena, Germany) was used. Emission was recorded using a longpass LP 515-nm filter set.

2.4. Immunohistochemistry

Immunohistochemistry was performed with whole mount multicellular tumor spheroids. As primary antibodies the mouse polyclonal anti-mdr1 (clone Ab1) (Calbiochem, Bad Soden, Germany) (concentration of 2 μg/ml), the mouse monoclonal anti-HIF-1α antibody (BD Biosciences, Heidelberg, Germany) (dilution 1:200), the goat anti-human Nox1 (Santa Cruz Biotechnology, Santa Cruz, CA), and the mouse anti-human Ki-67 (Sigma) were used. Prior to incubation with primary antibodies tissues were fixed in 4% paraformaldehyde for 60 min at 4 °C and washed with PBS containing 1% Triton X-100 (PBST). Blocking against unspecific binding was performed for 60 min with 10% fat free milk powder (Heirler, Radolfzell, Germany) dissolved in PBST (0.01%). For P-gp staining, the tissues were subsequently incubated for 120 min at room temperature with primary antibodies dissolved in PBST (0.01%) supplemented with 10% milk powder. The tissues were thereafter washed three times with PBST (0.01% Triton) and reincubated with either a Cy3-conjugated goat anti-rabbit IgG (H + L) (P-gp), a Cy2-conjugated goat anti-mouse IgG (HIF-1α), a Cy5-conjugated donkey anti-goat (Nox-1) or a Cy5-conjugated goat anti-mouse IgG (Ki-67) (all from Dianova, Hamburg, Germany) at a concentration of 3.8 μg/ml in PBS containing 10% milk powder. After washing three times in PBST (0.01% Triton), the tissues were stored in PBST (0.01%) until inspection. For the excitation of the Cy2 fluorochrome, the 488-nm band of a helium/neon laser of the confocal setup was used. Emission was recorded using a 515-nm longpass filter set. The Cy3 fluorochrome was excited by the 543-nm band of a helium/neon laser and emission was recorded using a 570-nm longpass filter set. The Cy5 fluorochrome was excited with the 633-nm band of a helium/neon laser and emission recorded using a longpass 655-nm filter set.

2.5. Doxorubicin uptake experiments

Tumor spheroids were incubated in F10 cell culture medium supplemented with 10 μM doxorubicin for 4 h either in the presence of absence of the P-gp-reversing agent cyclosporin A (10 μM). Doxorubicin fluorescence was excited by the 543-nm line of a helium/neon laser of the confocal setup. Emission was recorded using a LP 570-nm filter set.

2.6. Quantitative RT-PCR

Total RNA from homogenized DU-145/DU-145Nox1 multicellular tumor spheroid samples was prepared using Trizol (Invitrogen) according to the manufacturer's recommendations followed by genomic DNA digestion using DNAse I/amp. grade (Invitrogen). Total RNA concentration was determined by $OD_{260\,nm}$ method. cDNA synthesis was carried out using SuperScript II RTase Kit (Invitrogen) and random hexamer primer according to the manufacturer's recommendations.

Primers were designed using the free online tool primer 3 (http://frodo.wi.mit.edu/cgibin/primer3/primer3_www.cgi) and analyzed using netprimer (Premier Biosoft; http://www.premierbiosoft.com/netprimer/netprlaunch/netprlaunch.html) and BLAST (http://www.ncbi.nlm.nih.gov/BLAST/). The forward sequence of *mdr-1* was 5′-AAGGAAGCCAATGCCTATGA-3′ and the reverse sequence 5′-ACCACTGCTTCGCTTTCTGT-3′.

PCR was performed in an MJResearch Opticon II in 96-well microtitre plates using QuantiTect SYBR Green PCR kit according to the manufacturer's recommendations. In brief, cDNA product of 25 ng total RNA was mixed with QuantiTect SYBR Green PCR mastermix. Primers were added to a final concentration of 0.3 μM and water was added to a final volume of 20 μl. Amplifications were performed starting with a 15-min template denaturation/hot start step at 95 °C, followed by 40 cycles of denaturation at 94 °C for 30 s, primer annealing for 40 s at 59 °C, and extension for 30 s at 75 °C. Fluorescence increase of SYBR Green was automatically measured after each extension step. The last cycle was followed by a melting curve analysis step from 55 to 95 °C.

Each test was carried out three times. C_T values were automatically obtained. PCR efficiency was determined using the LinReg applet (http://www.gene-quantification.de/ramakers-2003.pdf). Relative expression values were obtained by normalizing C_T values of the tested genes with C_T values of the housekeeping gene RNA-Polymerase II (hRPOLII) using the C_T Method [18].

2.7. Statistical analysis

Data are given as mean values ± S.E.M. with n denoting the number of experiments unless otherwise indicated. In each experiment, at least 30 tumor spheroids were analyzed. Student's t test for unpaired data were applied as appropriate. A value of $P < 0.05$ was considered significant.