Med Oncol (2015) 32:251
DOI 10.1007/s12032-015-0700-1

ORIGINAL PAPER

The effect of superoxide anion and hydrogen peroxide imbalance on prostate cancer: an integrative in vivo and in vitro analysis

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Received: 30 September 2015 / Accepted: 7 October 2015 / Published online: 14 October 2015

Abstract The epidemiological impact of SOD2 imbalance on prostate cancer (PC) risk associated with genetic variations has previously been studied. However, we found no previous studies clarifying the nature of SOD2 effects on prostate cancer. Here, we performed integrated in vivo and in vitro protocols that analyzed the association between Ala16Val-SOD2 polymorphism and prostate cancer aggressiveness at the time of diagnosis and evaluated the effect of the imbalance on PC proliferation using the DU-145 PC cell line treated with paraquat and porphyrin. In the pharmacological model, paraquat was used to increase superoxide anion levels and porphyrin was the SOD2 analog. The results confirmed the impact of superoxide–hydrogen peroxide imbalance on PC cell biology since porphyrin decreased cell proliferation and both treatments modulated antioxidant genes. Therefore, our results corroborate previous suggestions that alteration of redox status could be exploited therapeutically in the treatment of PC.

Keywords DU-145 · Genotypes · Paraquat · Porphyrin · Prostate cancer · Superoxide manganese dependent

Introduction

Prostate cancer development and aggression have been associated with an increase in oxidative stress due to an imbalance in reactive oxygen species (ROS) [1, 2]. The cellular control of ROS involves several mechanisms including an antioxidant system that initially dismutates superoxide anion into hydrogen peroxide via superoxide dismutase (SOD) and catalyzes hydrogen peroxidase into water via catalase (CAT) and glutathione peroxidase (GPX). Furthermore, superoxide dismutase enzyme manganese-dependent (MnSOD or SOD2) dysfunction was associated with risk of prostate cancer development as well as metastasis [3].

A nuclear gene produces this enzyme, which only acts in mitochondria [4]. Understanding the roles of SOD2 in cancer etiology has been approached by means of in vivo and in vitro cancer risk and cancer progression studies [5–7]. Although SOD2 has been considered a tumor suppressor protein that regulates cell death events under certain circumstances, a chronic increase in SOD2 levels can trigger DNA damage due to overproduction of hydrogen peroxide [8].

The epidemiological impact of SOD2 imbalance on cancer prostate risk associated with genetic variations has previously been studied. A single nucleotide polymorphism (SNP) resulting in a structural mutation that replaces a thymine (T) with a cytosine (C) is the most common genetic variation found in the SOD2 gene (rs4880). This nucleotide change results in the substitution of valine by alanine in codon 16, changing the valine amino acid (GTT)

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into alanine (GCT) [9, 10]. The polymorphism produces three possible genotypes: AA, VV and AV. The SOD2 protein produced by the V-allele has a β-sheet secondary structure, whereas the A-allele produces an α-helix protein structure. The A-SOD2 protein is able to quickly transverse mitochondrial membranes, whereas V-SOD2 is partially arrested in these membranes. An investigation performed by Sutton et al. [10] showed that A-SOD2 precursors generate 30–40 % more of the active, matricial, processed SOD2 homotetramer than V-SOD2 precursors.

Several studies have suggested that the A-allele or genotype represents an increased risk of prostate cancer development [11–14], whereas other studies found no association. The potential influence of the Ala16Val-SOD2 polymorphism on prostate cancer aggressiveness is also controversial. A review performed by Bresciani et al. [8] suggested that the conflicting data may be due to intervenient environmental or ethnic variables that influence the prostate risk of this polymorphism [15].

A recent investigation performed by Margalit et al. [16] suggested an association between three SOD2 polymorphisms (rs6917589; rs2758331 and rs4880) and death from prostate cancer or distant metastasis. However, other studies such as that performed by Duan et al. [17] suggest that SOD2 has an important tumor suppressor role in prostate cancer and could be used as genetic therapy in the treatment of human prostate cancer. Despite the above, there has been no integrated in vivo and in vitro study to clarify the effect of SOD2 on prostate cancer. Therefore, we performed an integrated investigation that initially analyzed the potential in vivo association between the Ala16Val-SOD2 polymorphism and prostate cancer aggressiveness at the time of diagnosis. A second in vitro study was performed to investigate the impact of SOD2 imbalance on prostate cancer proliferation using the DU-145 prostate cancer cell line, which was cultured under a superoxide anion–hydrogen peroxide imbalance in order to create some similarity potentially observed in AA and VV-SOD2 genotypes. This involved developing a pharmacological in vitro model using paraquat and porphyrin treatment. Paraquat is an organic compound that interferes with electron transfer by reduction of electron donors such as NADPH in the mitochondria, thus increasing superoxide anion levels [18], and porphyrin is a SOD2 mimic [19].

Materials and methods

In vivo analysis

Initially we conducted a case–control study that evaluated the association between Ala16Val-SOD2 polymorphism and prostate cancer. A total of 305 subjects (249 healthy controls and 56 patients) were selected from São Lucas Hospital, Porto Alegre, Brazil. All patients were ≥50 years old and Caucasians. The inclusion criteria included patients with a pathological diagnosis of prostatic adenocarcinoma. Other types of neoplasia were excluded, as well as dysfunction that could influence the results. Prostate cancer was classified by stage using the American Joint Committee on Cancer tumor–nodes–metastasis (TNM) staging manual [20] and by grade using Gleason score at diagnosis. 'Early stage' cases were those that were apparently organ confined with no involvement of the seminal vesicle (T1 or T2 or T3a and N0M0 stage), whereas 'advanced-stage' cases were those with local spread to the seminal vesicle and beyond (T3c or T4 and N0M0, any T and N (1–3) or any T and M1 stage). Tumors with Gleason score <7 were classified as 'well to moderately well differentiated,' whereas those with a Gleason score ≥7 were classified as 'poorly differentiated.' Finally, tumor aggressiveness was defined based on a combination of stage and Gleason grade: Apparently organ-confined tumors or those with a Gleason score <7 were defined as 'less aggressive'; tumors with advanced stage or Gleason score ≥7 were defined as 'more aggressive.' The Gleason stages were compared among subjects with different Ala16Val-SOD2 genotypes. The study protocol was approved by the Institutional Ethics Committees of all relevant institutions, and informed consent was obtained from all individuals whose information was collected prospectively.

Ala16Val-SOD2 polymorphism genotyping

Blood samples were collected from subjects by venipuncture, and Ala16Val-SOD2 genotyping was determined by polymerase chain reaction using a direct total blood cell sample and Tetra-Primer ARMS-PCR assay as described by Barbisan et al. [21] with slight modifications. Briefly, two primer pairs were used to amplify and determine the genotype of a DNA fragment containing the Ala16Val SNP in the human SOD2 sequence. The 3′-end of the allele-specific primers is underlined F1 (forward) 5′-CACCAGCACTAGCAGCATGT-3′; F2 (forward) 5′-GCAGGCAGCTGGCTaCGGT-3′; R1 (reverse) 5′-ACGCCTCCTGGTACTTCTCC-3′; R2 (reverse) 5′-CCTGGAGCCCAGATACCCtAAAG-3′. Underlined lowercase bases indicate the introduced mismatches. The PCR reaction was carried out in a total volume of 40 μL containing 20–40 ng of genomic DNA as the template, 0.5 μM of each primer, 100 μM of each dNTP, 1.25 mM of $MgCl_2$, PCR buffer (20 mM Tris–HCl (pH 8.4), 50 mM KCl), 5 % dimethyl sulfoxide (DMSO), and 1.25 units of DNA polymerase. The PCR amplification was carried out with an initial denaturation at 94 °C for 7 min, followed by 35 cycles of 60 s of denaturation at 94 °C, 20 s of annealing at 60 °C, and 30 s of extension at 72 °C, and an additional 7 min

of extension at 72 °C at the end of the final cycle. A 20-μL aliquot of the PCR product was mixed with 2 μL of loading buffer and resolved by electrophoresis in a 1.5 % agarose gel. This procedure resulted in three bands in heterozygotes (514, 366, and 189 bp) and two bands in homozygotes (Val/Val resulting in bands of 514 and 189 bp, and Ala/Ala resulting in bands of 514 and 366 bp).

In vitro protocol

Since the superoxide–hydrogen peroxide balance can be influenced by several environmental factors, an in vitro protocol was designed to test the effect of superoxide–hydrogen peroxide imbalance on prostate cancer cell proliferation. To perform this protocol, the DU-145 prostate cancer cell line was exposed to paraquat, which generates high levels of superoxide anion, and porphyrin, which mimics the SOD2 enzyme. As these molecules are cytotoxic depending on their concentrations, their effect on prostate cancer viability was initially determined using several concentrations of paraquat and porphyrin. Based on the results obtained, the concentrations of paraquat and porphyrin that positively modulated the cancer cells by increasing their viability when compared to the control group were used to test the effect on cell proliferation. To confirm that these concentrations caused oxidative imbalance in prostate cancer cells, we performed complementary assays that evaluated the levels of ROS, protein carbonylation, lipoperoxidation, and DNA damage.

Cell culture conditions

The DU 145 human prostate adenocarcinoma cell line was purchased from the American Type Culture Collection (Bethesda, MD). Cells were grown as monolayers in DMEM with 10 % fetal bovine serum and 1 % penicillin/streptomycin and amphotericin B. The cells were cultured at 37 °C with 5 % CO_2 and were expanded by obtaining the optimal amount for the experiments. Culture tubes for each subject were prepared at a final concentration of 1×10^5 cells/mL. After cell attachment, the cultures were treated with paraquat and porphyrin. The cells were then incubated at 37 °C in a humidified atmosphere with 5 % CO_2 for 6, 24 and 72 h for the assays.

Cell viability and proliferation assays

Cell viability after 24-h exposure to paraquat and porphyrin and cell proliferation after 72 h of exposure were analyzed by the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) reduction assay as described by Barbisan et al. [21]. Briefly, treated cells were incubated for 1 h with MTT reagent at 37 °C. After dissolving the formazan salt in DMSO (dimethyl sulfoxide), the absorbance was measured at 560 nm. The MTT assay was performed using a 96-well plate in three independent replications. One complementary analysis was performed to confirm the effect of paraquat and porphyrin on cell viability: quantification of free double-stranded DNA (dsDNA) picogreen as described by Cadoná et al. [23]. The results were expressed as a percentage of the untreated control values.

Oxidative assays

In order to confirm that porphyrin and paraquat at non-lethal concentrations cause changes in the oxidative and antioxidative status of cancer cells after 6-h exposure, we analyzed the followed variables: superoxide, nitric oxide, ROS and lipoperoxidation by spectrophotometry and fluorimetry; quantification of DNA damage by determination of 8-hydroxy-2′-deoxyguanosine (8-OHdG); and antioxidant enzyme levels (SOD1, SOD2, catalase (CAT) and glutathione peroxidase (GPX) using competitive enzyme-linked immunosorbent assay (ELISA) kits. Superoxide levels were measured according to Spitz and Oberley [24], and this methodology was used to evaluate the antioxidant activity of SOD and its isoforms. It is based on indirect measurement of the SOD activity involving competition between the enzyme and an indicator substance for superoxide radicals ($O_2^{-\cdot}$), NBT (nitro blue tetrazolium). This evaluation uses xanthine oxidase and a superoxide source, which reduces the NBT to formazan blue. Thus the higher the SOD activity in the tissue or sample analyzed, the lower the absorbance, i.e., less NBT is reduced by the superoxide formed. Nitric oxide levels were determined according to Choi et al. [25]. The ROS level was determined using the non-fluorescent cell permeating compound 2′-7′-dichlorofluorescein diacetate (DCFDA) assay. In this technique, the DCFDA is hydrolyzed by intracellular esterases to DCFH, which is trapped within the cell. This non-fluorescent molecule is then oxidized to fluorescent dichlorofluorescein (DCF) by cellular oxidants. After the designated treatment time, the cells were treated with DCFDA (10 μM) for 60 min at 37 °C. In the assay, 1×10^5 cells from each sample were used to measured ROS levels [15]. The fluorescence was measured at an excitation of 488 nm and an emission of 525 nm, and the results were expressed as picomoles/mL of 2′,7′-dichlorofluorescein (DCF) produced from 2′,7′-dichlorofluorescin in reaction with ROS molecules present in the samples. Lipoperoxidation was determined by quantification of thiobarbituric acid reactive substances (TBARS) according to the modified method of Jentzsch et al. [26]. The carbonylation of serum proteins was determined by the Levine et al. [27] method with modifications. Quantification of

8-oxo-dG was performed using an ELISA kit (Oxis Health Products Inc., Portland-OR, USA) following the manufacturer's instructions. Quantification was performed in a 96-well microplate, using a standard curve made by plotting absorbance versus log of concentration read at 450 nm. Antioxidant enzymes were determined by ELISA using kits produced by ABCAN (Cambridge, UK), following the manufacturer's instructions. Test sensitivity and absorbance readings were as follows: SOD1 = 0.1 ng/ml, 600 nm; SOD2 = 4 ng/ml, 600 nm; CAT = 4 ng/ml, 450 nm; and GPX = 0.5 mU/ml, 340 nm.

Cell cycle analysis by flow cytometry

To confirm the effect of paraquat and porphyrin on prostate cancer cell proliferation, an analysis of cell cycle after 72 h exposure was performed by flow cytometry using propidium iodide (PI) dye. The cell cycle analysis [28] was performed using flow cytometry after 72 h of DU145 treatment with paraquat and porphyrin. The PI reagent binds to DNA by intercalating between the bases with little or no sequence preference. PI also binds to RNA, necessitating treatment with nucleases to distinguish between RNA and DNA staining. Cells were seeded in 24-well plates at 1×10^5 cells per well in 2 mL of the different treatments in DMEM and incubated for 72 h. Following incubation, the cells were trypsinized, washed with PBS, and resuspended in 70 % ethanol (by vortexing) at −20 °C overnight. Prior to analysis, the cells were centrifuged and washed once with PBS, after which the cells were resuspended in 500 µl PI-solution in PBS (50 µg/ml PI from 50× stock solution (2.5 mg/ml), 0.1 mg/ml RNase A, 0.05 % Triton X-100), and incubated for 40 min at 37 °C. Finally, 3 ml of PBS was added for washing and the cells were then resuspended in 500 µl PBS for flow analysis.

mRNA expression analysis by quantitative QT-PCR assay of genes

The effect of paraquat and porphyrin on antioxidant enzyme genes (SOD1, SOD2, CAT and GPX) as well as four genes related to cell proliferative modulation (Bcl-2, BAX, Caspase 3, and Caspase 8) were analyzed by QT-PCR assay as previously described in Barbisan et al. [21]. Total RNA was isolated using TRIzol reagent. RNA yields were measured using a Nanodrop 2000 spectrophotometer. First-strand cDNA was synthesized from total RNA (2 µg) using a First-Strand cDNA Synthesis Kit and oligo dT primers. Q-PCR was performed in a 20-µl reaction that contained 1 µl of the cDNA and 12.5 µL of 2X QuantiFastSYBR Green PCR Master Mix, 3.9 µL of Rnase-free water, and 1 µM of the primers, and the following PCR parameters: PCR initial activation step 95 °C for 5 min followed by 40 cycles of 95 °C for 10 s, 60 °C for 30 s followed by a melt curve of 60–90 °C in 0.5 °C increments for 5 s. The expression level of beta-actin was used as an internal control. The relative expression was calculated using the comparative Ct and was expressed as the fold expression compared to the control. The specific primer pairs for the antioxidant enzyme genes used in this study were:

SOD1 = forward GCACACTGGTGGTCCATGAA and reverse ACACCACAAGCCAAACGACTT; SOD2 = forward GCCCTGGAACCTCACATCAA and reverse GGTA CTTCTCCTCGGTGACGTT; CAT = forward GATAGC CTTCGACCCAAGCA and reverse ATGGCGGTGAGTG TCAGGAT; GPX = forward GGTTTTCATCTATGAGG GTGTTTCC and reverse GCCTTGGTCTGGCAGAGAC T;BAX = forward CCCTTTTCTACTTTGCCAGCAA and reverse CCCGGAGGAAGTCCAATGT;Bcl-2 = forward GAGGATTGTGGCCTTCTTTGAGT; reverse AGTCATCCACAGGGCGATGT; CASP3 = forward TTTGAG CCTGAGCAGAGACATG and reverse TACCAGT GCGT ATGGAGAAATGG; CASP 8 = forward AGGAGCTGCT CTTCCGAATT and reverse CCCTGCCTGGTGTCTGAA GT.

Statistical analysis

Statistical software (SPSS 11.0, Chicago, USA) was used to analyze the in vivo data. Student's t test was used to compare quantitative baseline variables between case and control subjects. The allele frequencies were estimated by the gene-counting method. Chi-square ($\chi 2$) analysis was used to estimate the Hardy–Weinberg equilibrium. The allelic and genotype frequencies as well as the frequency of Gleason levels were compared among groups using Chi-square or the Fisher's exact test. In the in vitro protocols, the statistical analyses were carried out using Graph Pad Prism 5 software, and the results were expressed as the mean ± standard deviation (SD). Comparison of all groups was performed using two-way analysis of variance followed by post hoc Tukey's or Dunnet's tests. All p values were two-tailed. The alpha value was set to <0.05, indicating statistical relevance.

Results

Characteristic baselines and Ala16Val-SOD2 genotype frequencies are shown in Table 1. Mean age was similar in the control and prostate cancer groups, and as expected PSA values were higher in prostate cancer subjects. The genetic frequencies of Ala16Val-SOD2 polymorphism were in Hardy–Weinberg equilibrium. Allele frequencies were $A = 0.804$ and $V = 0.196$ in the control group and

Table 1 Baseline characteristics and Ala16Val-SOD2 genetic frequencies of controls and prostate cancer subjects

Variables	Controls	Prostate cancer	p*
Age (years, mean ± SD)	64.27 ± 7.79	66.47 ± 7.75	0.12
PSA levels (ng/dL, mean ± SD)	1.76 ± 1.34	21.54 ± 45.99	0.03
Genotype *n* (%)			
AA	18 (7.2)	13 (18.6)	0.01
VV	66 (26.8)	15 (21.4)	
AV	165 (66.0)	28 (60.0)	

* *SD* standard deviation; * statistical test: Chi square, as considered significant $p \leq 0.05$

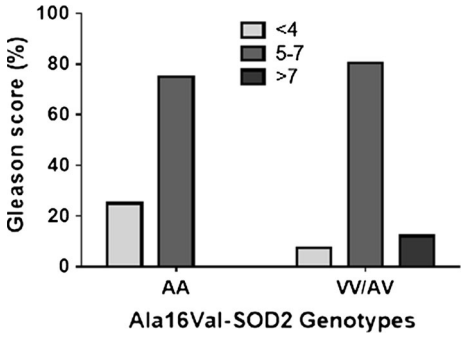

Fig. 1 Gleason score comparison between AA and V-carriers of Ala16Val-SOD2 polymorphism affected by prostate cancer

$A = 0.771$ and $V = 0.228$ in the prostate cancer group. Comparison between genotypes showed a higher frequency of the AA allele in prostate cancer subjects when compared to control subjects.

However, when tumoral aggressiveness at the time of diagnosis was evaluated, V-carriers presented higher Gleason scores than AA subjects (Fig. 1). Twelve percent of VV patients and 10 % of AV patients presented higher Gleason scores (>7), whereas no AA patients presented higher Gleason scores ($p = 0.02$).

A second approach described in the present investigation evaluated the effect of superoxide anion and hydrogen peroxide imbalance in the DU-145 prostate cancer cell line caused by exposure to a range of concentrations of paraquat and porphyrin (Fig. 2).

Lower paraquat doses significantly increased cell viability when compared to the control group, whereas doses $\geq$1 μM caused extensive mortality after 24 h exposure. In contrast, porphyrin was not toxic to prostate cancer cells, but increased cell viability when compared to the control group at 100 μM. For this reason, the effect of paraquat and porphyrin on cancer cell proliferation was tested at $\geq$0.1 and $\geq$100 μM, respectively, since these concentrations increased viability by around 1 % in relation to the control group.

The expression of four genes associated with the triggering of apoptosis was also analyzed, and the results are shown in Fig. 3. Porphyrin treatment upregulated all genes

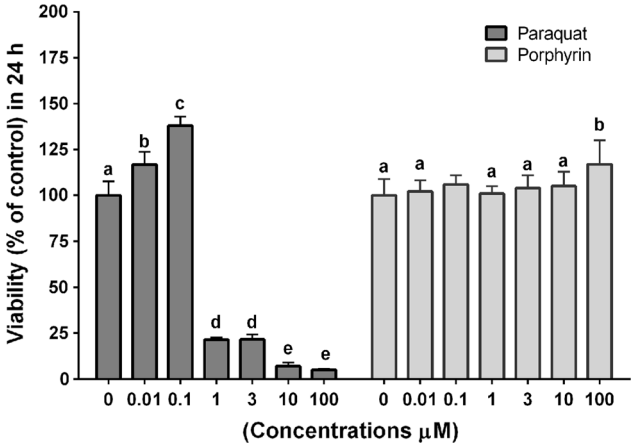

Fig. 2 Effect of paraquat and porphyrin on viability of DU-145 prostate cancer cells. The data are presented as % of untreated control group. Statistical comparison was performed by analysis of variance followed by Tukey's test. *Different letters* indicate statistical differences at $p \leq 0.05$

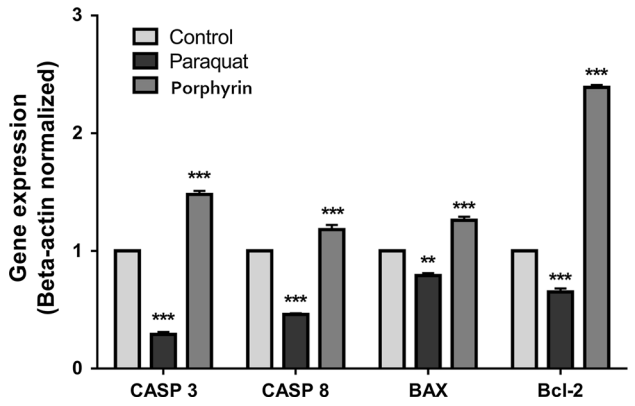

Fig. 3 Comparison of expression of four genes associated with apoptosis pathway among control and treated (paraquat, 0.1 μM; porphyrin, 100 μM) DU-145 prostate cancer cells after 6-h exposure. Statistical comparison was performed by analysis of variance followed by Dunnet's test. Statistical differences $^{**}p \leq 0.01$; $^{***}p \leq 0.001$

evaluated, including the anti-apoptotic Bcl-2 gene. The opposite was observed in cells exposed to paraquat, with the downregulation of all genes tested.

Oxidative variables evaluated in cells exposed to 0.1 μM paraquat and 100 μM porphyrin confirmed that these concentrations caused an imbalance in the oxidative status of prostate cancer cells (Fig. 4). Both treatments significantly increased superoxide levels when compared to the control group ($p = 0.01$), but only paraquat caused a decrease in nitric oxide levels ($p = 0.01$). ROS levels and dsDNA levels did not differ from the control group. However, protein carbonylation and DNA damage were higher in the paraquat-treated cells followed by porphyrin-treated cells when compared to levels found in untreated prostate cancer cells.

Treatments also significantly reduced the activity of antioxidant enzymes when compared to untreated prostate cancer cells. This effect was stronger in cells treated with porphyrin than with paraquat. A complementary analysis showed that both treatments trigger the modulation of gene expression of these enzymes (Fig. 5).

Both treatments caused differential antioxidant gene modulation. Porphyrin induced SOD1, SOD2, and CAT upregulation as well as downregulation of GPX. On the other hand, paraquat had no effect on SOD1 gene expression, but induced significant downregulation of other antioxidant enzyme genes.

Given these results, the effect of oxidative imbalance on prostate cancer cell proliferation was evaluated after 72 h exposure. Porphyrin at 100 μM significantly decreased cell proliferation ($p = 0.001$) when compared to the control group. However, this effect was not observed in cells treated with $\geq$0.1 μM paraquat (Fig. 6). The cell cycle was analyzed by flow cytometry, showing that porphyrin treatment caused a significant increase in cells in the S/M cycle phases when compared to the control group ($p = 0.02$). The number of cells marked by PI was lower in porphyrin-treated cells when compared to the control group. This effect was not observed in cells treated with paraquat.

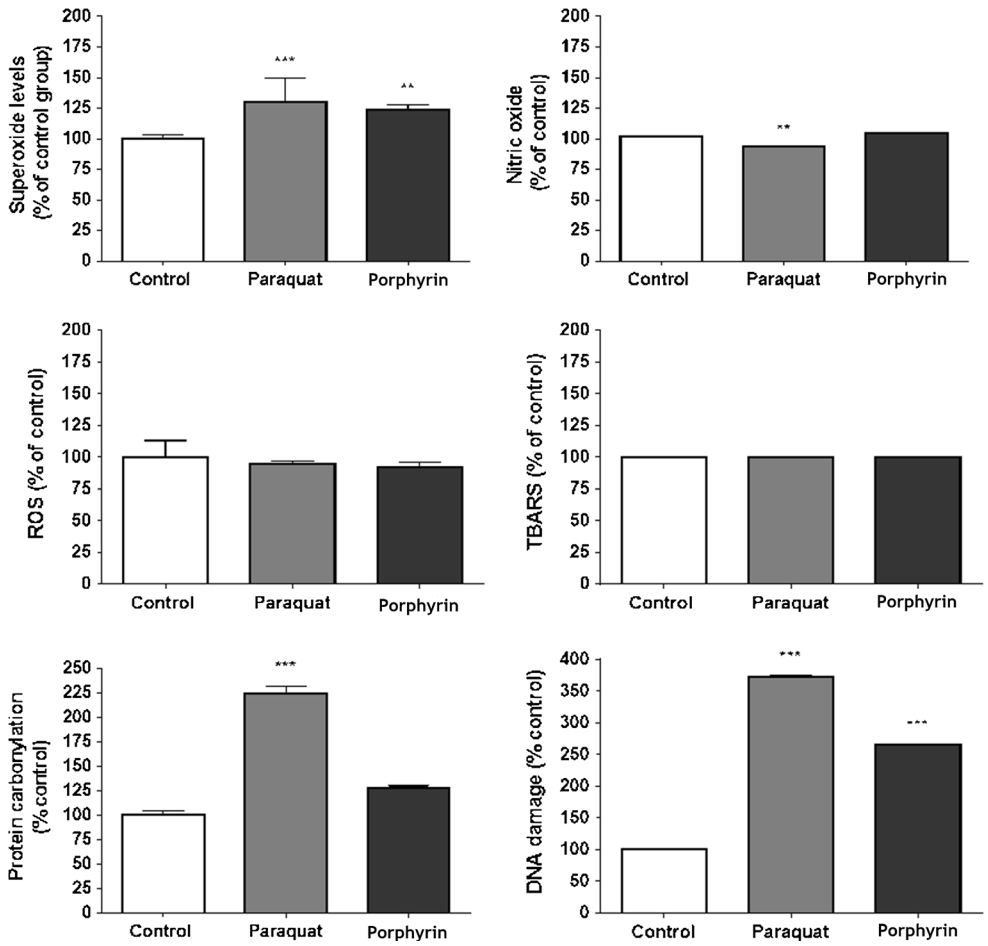

Fig. 4 Comparison of oxidative variables in control and treated (paraquat, 0.1 μM; porphyrin, 100 μM) DU-145 prostate cancer cells after 6-h exposure. The data are presented as % of untreated control group. Statistical comparison was performed by analysis of variance followed by Dunnet's test. Statistical differences $^{**}p \leq 0.01$; $^{***}p \leq 0.001$

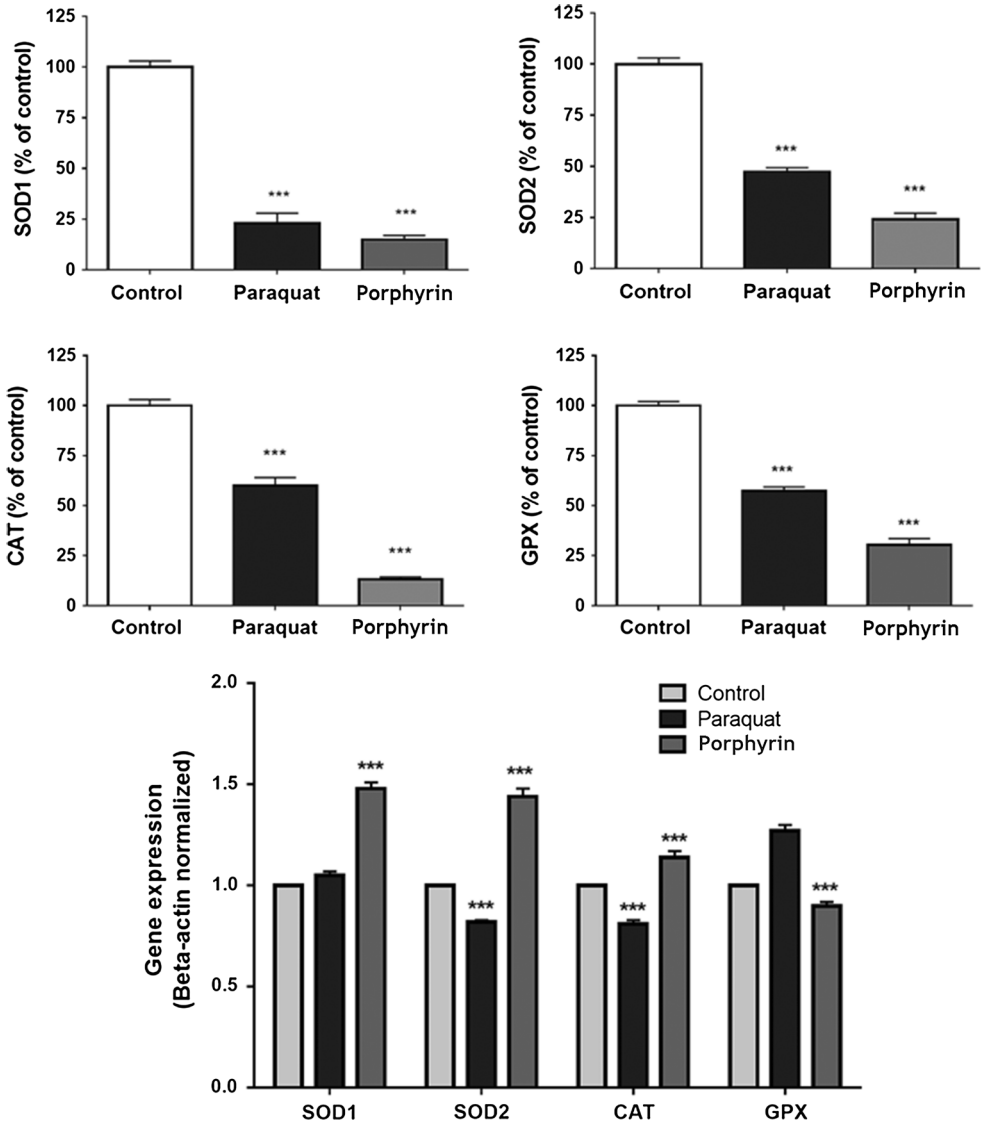

Fig. 5 Comparison of antioxidant enzyme activity and gene expression in control and treated (paraquat, 0.1 μM; porphyrin, 100 μM) DU-145 prostate cancer cells after 6-h exposure. The data are presented as % of untreated control group. Statistical comparison was performed by analysis of variance followed by Dunnet's test. Statistical differences $^{**}p \leq 0.01$; $^{***}p \leq 0.001$

Discussion

In the present investigation, we investigated whether the superoxide anion–hydrogen peroxide imbalance associated with Ala16Val-SOD2 SNP could be associated with prostate cancer using two integrated approaches. In the first analysis, we found an association between a higher risk of prostate cancer and the AA genotype. However, a second analysis showed an association with V-allele carriers and aggressive prostate cancer at the time of diagnosis. These two results suggest a dual role of SOD2 modulation in cancer biology, corroborating a previous investigation performed by Bica et al. [12] that described an association between AA and breast cancer risk and VV and cancer aggressiveness.

Some meta-analyses have suggested that the association between prostate cancer and Ala16Val-SOD2 is controversial: As some studies have found an association between prostate cancer and Ala16Val-SOD2 SNP [13–29], whereas others have not [30]. On the other hand, when we analyzed the aggressiveness of prostate cancer at diagnosis, we found that carriers of V-alleles (VV and AV genotypes) presented higher Gleason scores than AA patients. Ergen et al. [31] investigated Turkish patients and found an association between the AA genotype and risk of prostate cancer, but no association between aggressive prostate cancer determined by the Gleason score and Ala16Val-SOD2 SNP.

These contradictory data may be due to the strong influence of environmental factors such as nutrition and physical activity as well as ethnic origin, as described in a review performed by Bresciani et al. [8]. For example, the investigation performed by Mikhak et al. [32] found no general association between this polymorphism and aggressive prostate cancer risk. However, male carriers' of

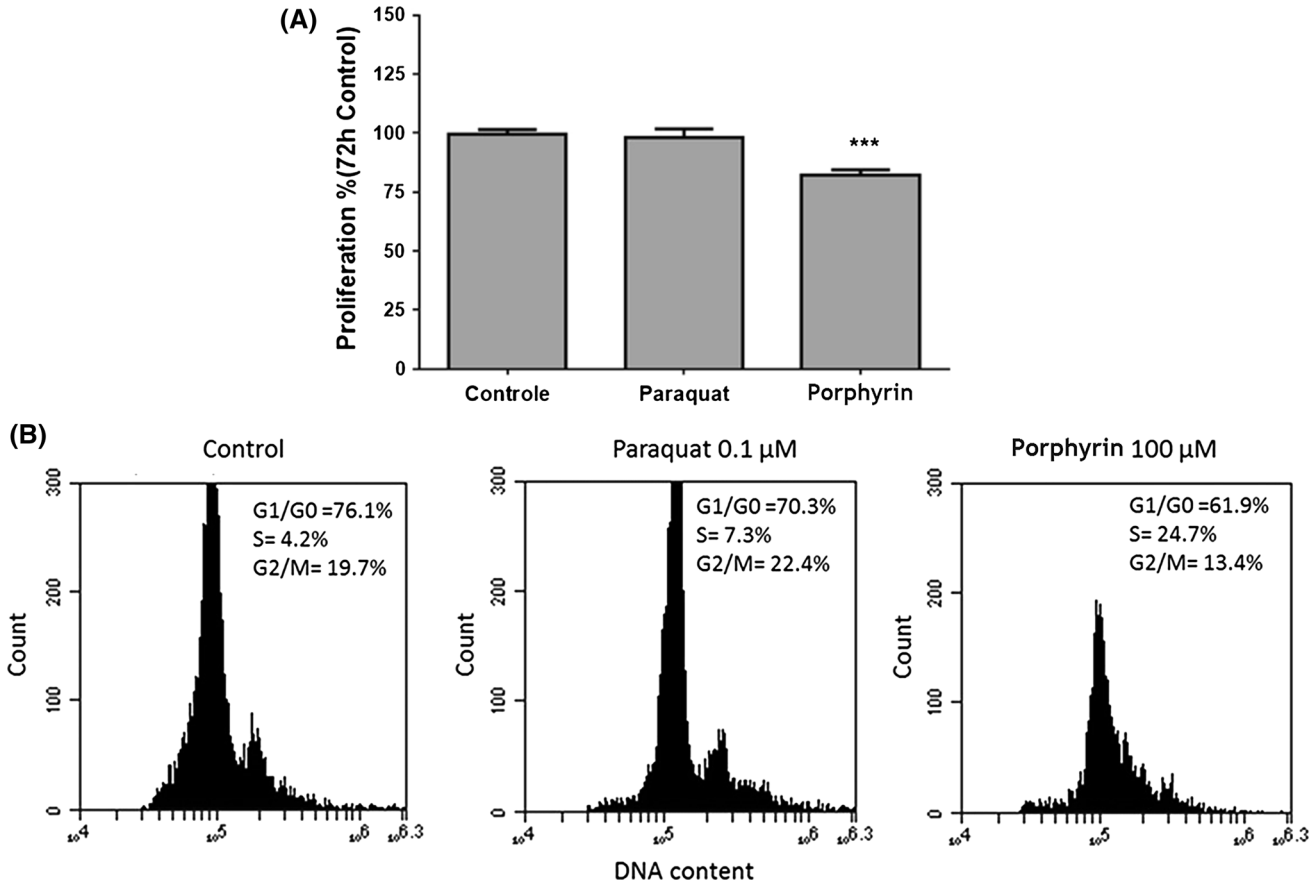

Fig. 6 Cellular proliferation (**a**) and representative results of cell cycle analyzed by flow cytometry (**b**) with propidium iodide after 72-h exposure to paraquat (0.1 µM) and porphyrin (100 µM). The median frequencies are presented for the main parts of the cell cycle

the AA genotype who had low long-term lycopene status presented higher aggressive prostate cancer risk than men who habitually consumed vegetables rich in antioxidant molecules.

Although we found no direct association between differential modulation of SOD2 related to genetic polymorphism and prostate cancer, we consider that investigating the role of SOD2 in prostate cancer is very important because it may be related to the prognosis and therapeutic management of this cancer. This premise is based on the well-known role of oxidative metabolism in carcinogenesis during tumor initiation, neoangiogenesis, and cell proliferation, which several studies have indicated involves alterations in SOD2 enzyme activity [5].

However, due to its high sensitivity to environmental and genetic variables, it is difficult to estimate the actual impact of SOD2 imbalance on prostate cancer cells. For this reason, we developed an in vitro experimental assay that investigated the role of SOD2 imbalance (pharmacologically caused by exposure to paraquat and porphyrin) in DU-145 cells, a prostate cancer cell line. Despite the methodological limitations potentially associated with the in vitro protocol and with the drugs used as the superoxide anion donor and to increase the concentration of SOD2 activity, our results suggested that SOD2 imbalance influences prostate cancer cell proliferation. The reduction in cancer cell proliferation caused by porphyrin exposure (which generated a cellular environment with higher SOD2 activity than in the control group) was more intense than that caused by paraquat. Cytometry flow analysis confirmed that porphyrin was able to inhibit the cell cycle mainly in the S phase, when compared to the control group.

Although previous studies have shown the role of SOD2 in cancer [5–7, 33], to the best of our knowledge, our investigation is the first to show the relevance of SOD2 imbalance in cancer risk and progression using concomitant in vivo and in vitro approaches.

The role of SOD2 in prostate cancer appears to be complex and is linked with other molecules related to cellular oxidative status. This premise is based on a recent investigation that showed discordant levels of antioxidant proteins and their enzymatic activities in prostate cancer tissues that probably contribute to the redox imbalance observed during prostate cancer progression [33]. In terms

of SOD2, the authors reported that whereas SOD2 enzyme activity was significantly increased in cancer prostate tissues with higher Gleason scores, the protein levels were lower. This discordance in the SOD2 protein/enzyme levels could be due to inactivation of SOD2 protein or post-translational modifications of tyrosine and lysine by nitration or by gene methylation. Among the proposed mechanisms for how alterations in intra- and extra-cellular redox states can induce prostate cancer progression, Chaiswing et al. [3] suggested that in high-grade cancer the SOD2 becomes non-functional and the increase in superoxide levels induces cancer progression. However, they did not explore the possible effect of superoxide imbalance on prostate cancer progression due to modulation of antioxidant gene expression. We analyzed this issue here, observing that when paraquat was added to prostate cancer cell cultures the SOD2 and CAT genes were downregulated, whereas the GPX gene was upregulated. In contrast, porphyrin upregulated all antioxidant genes with the exception of GPX. Despite this differential gene regulation, only porphyrin decreased prostate cancer cell proliferation, and paraquat did not alter this important parameter.

The combined results confirm the relevance of superoxide–hydrogen peroxide imbalance in prostate cancer cell biology and support previous suggestions that alteration of redox status could be exploited therapeutically in the treatment of prostate cancer. Nutritional supplementation during or after prostate cancer treatment cannot be ruled out and should be investigated in pre-clinical and clinical studies.

Acknowledgments The authors are grateful for the help of the Laboratory of Biogenômica—Federal University of Santa Maria, RS, Brazil. This study was funded by the following research grants and fellowships: CNPq and CAPES.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest including any financial, personal, or other relationships with other people or organizations that could inappropriately influence, or be perceived to influence, the present work.

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