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Modulation of redox signaling promotes apoptosis in epithelial ovarian cancer cells

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Abstract

Objective—Epithelial ovarian cancer (EOC) cells are known to be resistant to apoptosis through a mechanism that may involve alteration in their redox balance. NADPH oxidase is a major source of intracellular superoxide, which is converted to the less toxic product by superoxide dismutase (SOD). Superoxide contributes to hypoxia inducible factor (HIF)-1 α stabilization. We sought to determine the effects of inhibiting the generation of intracellular reactive oxygen species (ROS) on apoptosis of EOC cells.

Methods—Diphenyleneiodonium (DPI), an irreversible ROS inhibitor, was used to inhibit the generation of ROS in EOC cell lines, SKOV-3 and MDAH-2774, followed by assessment of apoptosis, NADPH oxidase, SOD3 and HIF-1 α expression. A combination of immunohistochemistry, immunoprecipitation/western blot, and real-time RT-PCR were utilized to evaluate the expression of these enzymes in EOC cells as well as normal ovarian tissue and ovarian cancer tissue specimens.

Results—DPI treatment significantly induced apoptosis in both EOC cell lines as evident by increased caspase-3 activity and TUNEL assay. Additionally, both EOC cell lines were found to express NADPH oxidase, HIF-1 α , and SOD3, which were highly sensitive to DPI treatment. DPI treatment resulted in reduced NADPH oxidase, SOD3 and HIF-1 α levels. Furthermore, ovarian

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Conflict of interest statement

Zhongliang Jiang – None

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cancer tissues were found to manifest higher NADPH oxidase levels as compared to normal ovarian tissues.

Conclusions—These data suggest that lowering oxidative stress, possibly through the inhibition of NADPH oxidase, induces apoptosis in ovarian cancer cells and may serve as a potential target for cancer therapy.

Keywords

NADPH oxidase; Diphenyleneiodonium; Epithelial ovarian cancer; Oxidative stress; Superoxide dismutase; Hypoxia inducible factor-1 alpha

Introduction

Ovarian cancer is the fifth leading cause of cancer death in women, the leading cause of death from gynecologic malignancies, and the second most commonly diagnosed gynecologic malignancy, however the underlying pathophysiology is not clearly understood [1]. Malignant cells are resistant to apoptosis through a mechanism that may involve alterations in their redox balance. Reactive oxygen species (ROS), generated in the mitochondria as a byproduct of oxidative phosphorylation, are continuously generated and eliminated in the biological system and play important roles in a variety of normal biochemical functions and abnormal pathological processes [2–5].

In addition to the ROS produced by the mitochondria, nicotin-amide adenine dinucleotide phosphate (NADPH)-oxidase, a flavoenzyme family member, generates a significant amount of endogenous ROS through the reduction of O_2 to superoxide ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), and other ROS [6–9]. The NADPH oxidase complex is composed of multiple subunits, including a glycoprotein gp91-phox, which is considered to be directly involved in the generation of $O_2^{\cdot-}$ [8]. Enzyme systems similar to the phagocyte NADPH oxidase are now known to exist in many other cell types, and are referred to as the NOX family of NADPH oxidases [8–13].

Several antioxidant enzymes, including superoxide dismutases (SOD), catalase, and various peroxidases are effective in removing destructive ROS [14,15]. Superoxide dismutases are key enzymes required for removal of $O_2^{\cdot-}$, thorough its conversion to H_2O_2 , which is further eliminated by both catalase and peroxidases [15,16]. Human extracellular Cu/Zn SOD (SOD3), is a unique SOD family member found in the extracellular matrix of tissues and is ideally situated to prevent cell and tissue damage, initiated by extracellularly produced ROS [17]. The loss of endogenous SOD3 activity can exacerbate oxidative stress and pathologic damage as it is a critical endogenous antioxidant enzyme involved in carcinogenesis, cancer proliferation and metastasis [18].

Cancer cells are under intrinsic oxidative stress and manifest significantly increased levels of ROS, and thus express higher levels of SOD, which has been reported to stabilize the HIF-1 α protein, enabling HIF-1 α to dimerize, forming an active transcription factor [11,19–21]. Since rapidly growing tumors become hypoxic, questions are raised as to what promotes an increase in SOD, and why HIF-1 α is stabilized and not degraded, with the

increase in ROS under the hypoxic environment in cancer cells [22,23]. Restoration of the ROS balance in cancer cells may provide a potential therapeutic intervention to selectively eliminate cancer cells via apoptosis. Inhibition of NADPH oxidase and other pro-oxidant enzymes has been reported to significantly induce apoptosis of cancer cells [24,25]. Several agents have been utilized to test this hypothesis, however, few have been tested in ovarian cancer [26–28].

In the present study, we investigated whether NADPH oxidase-mediated generation of intracellular reactive ROS lead to anti-apoptotic activity and thus a growth advantage to epithelial ovarian cancer (EOC) cells. Specifically, we have utilized DPI, an irreversible inhibitor of flavoproteins, including NADPH oxidase, to evaluate apoptosis of EOC cells. Additionally, we have evaluated other key players in the regulation of redox homeostasis and apoptosis, including SOD3 and HIF-1 α . Identification of targets to specifically induce apoptosis in EOC cells may provide a potential therapeutic target for selective elimination of cancer cells.

Materials and methods

Culture of EOC cell lines

The two human EOC cell lines, SKOV-3 and MDAH-2774, were obtained from ATCC (ATCC, Manassas, VA). Cells were cultured in 75cm² cell culture flasks (Corning Incorporated, Corning, NY) with McCoy's 5A medium (Invitrogen, Carlsbad, CA) supplemented with 100 U/mL penicillin and 100 μ g/mL streptomycin including 10% heat-inactivated FBS at 37 °C in 5% CO₂. Culture medium was replaced every two days.

Treatment of human EOC cells

Cells were plated (5 $\times$ 10⁶) in 100 cm² culture dishes and incubated for 24 hours. Cells were treated with 10 μ M DPI (Sigma-Aldrich, Saint Louis, MO) for 0, 0.5, 1, 3, 6, 12, and 24 hours. The dose was selected based on previous studies [26–28]. Cells were harvested at each time point. All experiments were performed in triplicate.

Measurement of caspase-3 activity in EOC cells

A Caspase-3 Colorimetric Activity Assay Kit (Chemicon, Billerica, MA) was utilized per the manufacturer's protocol. Cells (2 $\times$ 10⁶) were harvested and lysed in 300 μ l of lysis buffer, and concentrations were equalized for each sample set. Cell lysate (150 μ g) was combined with substrate reaction buffer containing 30 μ g of caspase-3 substrate, acetyl-DEVD-p-nitroaniline (Ac-DEVD-pNA). This mixture was incubated for 1 hr at 37 °C, and then absorbance was measured with a plate reader (Ultramark, BIO-RAD, Hercules, CA).

Detection of apoptosis in EOC cells

DNA fragmentation was assessed by the in situ Terminal Deoxynucleotidyl Transferase-mediated dUTP-biotin nick end labeling (TUNEL) technique per the DeadEnd Fluorometric TUNEL System (Promega, Madison, WI) manufacturer's protocol. Briefly, the cells were fixed with 4% paraformaldehyde in PBS for 20 min at 4 °C, and subjected to permeabilization for 5 min at room temperature with 0.2% Triton X-100. Next, cells were

labeled with the TUNEL reaction mixture for 60 min at 37 °C. The nuclei of these cells were also stained with 4',6'-diamino-2-phenylindole (DAPI). Fluorescein-labeled DNA, an indication of DNA fragmentation, was analyzed by using an Axiovert 40 CFL immuno-fluorescent microscope (Carl Zeiss Microimaging, Thornwood, NY) and recorded with a microscope-mounted camera (Carl Zeiss).

Real-time reverse transcription polymerase chain reaction (RT-PCR) for HIF-1 α , NADPH oxidase, and SOD3

RNA isolation—Total RNA was extracted from both SKOV-3 and MDAH-2774 cells using an RNeasy Mini Kit (QIAGEN, Valencia, CA) according to the protocol provided by the manufacturer.

Reverse transcription—A 20 μ L cDNA reaction volume was prepared with the use of QuantiTect Reverse Transcription Kit (QIAGEN) according to the protocol provided by the manufacturer.

Real-time RT-PCR—Real-time RT-PCR was performed with a QuantiTect SYBR Green RT-PCR kit (QIAGEN) and a Cepheid 1.2f Detection System (Cepheid, Sunnyvale, CA). Each 25- μ L reaction included 12.5 μ L of 2 $\times$ QuantiTect SYBR Green RT-PCR master mixes, 1 μ L of cDNA template, and 0.2 μ M each of target-specific primer were selected with the aid of the software program, Beacon Designer (Premier Biosoft, Palo Alto, CA). Human oligonucleotide primers (HIF-1 α , p22-phox, and SOD3) that amplify variable portions of the protein coding regions are listed in Table 1. Standards with known concentrations and lengths (base pairs (bp)) were designed specifically for HIF-1 α (100 bp), NADPH oxidase p22-phox subunit (82 bp), and SOD3 (99 bp) using the Beacon Designer software (Premier Biosoft), allowing for construction of a standard curve using a tenfold dilution series. A specific standard for each gene allows for absolute quantification of the gene in number of copies, which can then be expressed per μ g of RNA. The conditions for the three-step polymerase chain reaction protocol were as follows: an initial cycle at 95 °C for 900 s (HIF-1 α), 1000 s (p22-phox) and 850 s (SOD3) followed by 35 cycles of denaturation at 95 °C for 15 s, annealing at 60 °C (HIF-1 α and SOD3) and 54 °C (p22-phox) for 30 s, and extension at 72 °C for 30 s. Finally, a melting curve analysis was performed to demonstrate the specificity of the PCR product as a single peak. A control, which contained all the reaction components except for the template, was included in all experiments.

Measurement of SOD3 protein levels in EOC cells

Immunoprecipitation (IP)/Western blot was utilized as previously described with the following changes [29]. Cells were lysed with lysis buffer and cleared by centrifugation (10 minutes at 1,000 g, 4 °C). Protein concentration of cell lysates was measured with the Pierce BCA Protein Assay Kit (ThermoFisher Scientific, Rockford, IL) per the manufacturer's protocol. The same concentration of protein was utilized for each sample. Precleared cell lysates were incubated with anti-SOD3 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) for 1 hour at 4 °C, followed by precipitation with 20 μ L of Protein A/G PLUS-Agarose (Santa Cruz Biotechnology) and incubated at 4 °C overnight. Adherent proteins were eluted with 1 $\times$ protein loading buffer for 5 minutes at 80 °C and analyzed by a Western blot

detection kit (Visualizer, Millipore, Temecula, CA). SOD3 bands were scanned and analyzed by NIH Image J 3.0 (U. S. National Institutes of Health, Bethesda, Maryland).

Measurement of HIF-1 α levels in EOC cells

Protein concentration of cell lysates was measured as described above. Cell lysates were prepared from the various treatments of the EOC cell lines, SKOV-3 and MDAH-2774. HIF-1 α was measured with an enzyme-linked immunosorbent assay kit (HIF-1 α ELISA, R&D Systems, Minneapolis, MN), per the manufacturer's protocol.

Immunohistochemical staining of EOC tissue sections

Twenty benign ovarian tissues specimens, obtained from patients who underwent total abdominal hysterectomy-bilateral salpingo-oophorectomy for leiomyomas, and 20 invasive EOC cases (two sections per case) were retrieved from archival materials from the Detroit Medical Center/ Karmanos Cancer Center pathology department (Institutional review board number 072206MP2E). Histological diagnoses were as follows: 10 high grade and five low grade serous carcinomas, four grade 1 endometrioid and one grade 1 mucinous carcinomas. The mean age of the 20 patients was 56 (range 35–70). Sections were deparaffinized, hydrated with PBS (pH 7.4), and pretreated with H₂O₂ (3%) for 10 min to remove endogenous peroxidases and incubated in goat serum for 10 min. A primary antibody for NADPH oxidase (HPA015475, Sigma Aldrich) dilution (1:20) was applied to each section, followed by washing and incubation with the biotinylated secondary antibody for 10 min at room temperature. Detection was performed with AEC and counter-staining with Mayer's hematoxylin followed by mounting.

Ovaries had only surface epithelial inclusion cysts and epithelium was evaluated for expression of NADPH oxidase and was assessed based on the presence of cytoplasmic staining. Scoring was assigned based on the percentage of positive epithelial cells: a zero score assigned for cases with no cytoplasmic staining in any cells; score 1 with $\leq 5\%$ of cell staining positive; score 2 with 6–30% and score 3 with $\geq 30\%$ of cells staining positive. A gynecologic pathologist reviewed all slides, benign (2–5 slides) and malignant (5–15 slides). The most positive area in every tumor or benign cases was evaluated and only the percentage was evaluated because most of the cases had the same intensity. For statistical analysis, cases with score 0 or 1 were considered as being negative and cases with score 2 or 3 as positive.

Statistical Analysis

Data were analyzed using SPSS 19.0 for windows (SPSS for Windows, Chicago, IL). Data were analyzed using oneway ANOVA (analysis of variance) with Student Newman-Kuels post-hoc comparisons. Significance values of $p < 0.05$ were considered statistically significant for all analyses.

Results

DPI treated EOC cells exhibited increased caspase-3 activity and apoptosis

Caspase-3 activity significantly increased, in a time dependent fashion, in SKOV-3 cells, from 7.62 to 20.9, 20.3, 22.0, 24.5, 39.4, and 54.3 μM and in MDAH-2774 cells from 6.61 to 19.0, 20.4, 23.1, 25.6, 39.3, and 53.1 μM at the 0.5, 1, 3, 6, 12, and 24 hour time points, respectively ($p < 0.05$ as compared to control, Fig. 1A).

These results were confirmed by TUNEL staining, an indicator of the degree of DNA fragmentation, which is representative of apoptosis. Nuclei were stained with DAPI (blue) and apoptotic cells were visualized (60x) with fluorescein-12-dUTP (green). There was a significant increase in TUNEL staining (green) as compared to controls, in both EOC cell lines (Fig. 1B).

DPI treated EOC cells had reduced levels of NADPH oxidase mRNA

Real-time RT-PCR was utilized to determine the mRNA level of the NADPH oxidase p22-phox subunit, a representative O_2 sensing subunit of NADPH oxidase, in EOC cells treated with and without DPI for 0.5 hours. NADPH oxidase was significantly decreased by 51.6% in SKOV-3 cells, and by 40.1% in MDAH-2774 cells ($p < 0.05$ as compared to control, Fig. 2).

EOC tissues expressed higher levels of NADPH oxidase

NADPH oxidase expression was upregulated in 65% of EOC tissue sections, tested by immunohistochemistry as compared to 20% detectable expression for NADPH oxidase in normal ovarian epithelial tissue (surface epithelial inclusion cysts) (Fig. 3).

DPI treated EOC cells had reduced levels of HIF-1 α

DPI treatment significantly reduced HIF-1 α mRNA levels, in SKOV-3 cells, from 428.2 to 368.2, 318.6, 268.8, 261.3, and 206.8 pg/ μg RNA at the 0.5, 3, 6, 12, and 24 hour time points, and in MDAH-2774 cells from 421.3 to 356.9, 356.1, 327.4, 260.3, 240.0 and 223.1 pg/ μg RNA, at the 0.5, 1, 3, 6, 12, and 24 hour time points, respectively ($p < 0.05$ as compared to control, Fig. 4A). There was no statistical change in HIF-1 α mRNA levels at the 1 hour time point in SKOV-3 cells.

HIF-1 α protein levels, in SKOV-3 cells, were significantly reduced from 1699 to 828.0, 377.2, 311.0, 291.1, 224.6, and 44.27 pg/ml total protein and in MDAH-2774 cells from 1872 to 658.1, 332.4, 257.5, 111.8, 106.1, and 22.96 pg/ml total protein at the 0.5, 1, 3, 6, 12, and 24 hour time points, respectively ($p < 0.05$ as compared to control, Fig. 4B).

DPI treated EOC cells had reduced levels of SOD3

DPI treatment significantly reduced SOD3 mRNA levels, in a time dependent fashion, from 31.1 to 19.5, 24.6, 15.7, 9.23, 4.63, and 3.41 fg/ μg RNA at the 0.5, 1, 3, 6, 12, and 24 hour time points, respectively, in SKOV-3 cells ($p < 0.05$ as compared to control, Fig. 5A). Similarly, SOD3 mRNA levels were significantly reduced from 26.1 to 19.69, 16.0, 11.7, 8.63, and 6.68 fg/ μg RNA at the 0.5, 3, 6, 12, and 24 hour time points, respectively, in

MDAH-2774 cells ($p < 0.05$ as compared to control, Fig. 5A). There was no significant change in SOD3 mRNA levels at the 1 hour time point in MDAH-2774 cells.

SOD3 protein levels, in SKOV-3 cells, were reduced from 251.6 to 223.2, 215.7, 181.6, and 149.0 at the 3, 6, 12, and 24 hour time points, respectively ($p < 0.05$ as compared to control, Figs. 5B and C) and in MDAH-2774 cells from 254.4 to 237.9, 230.4, 208.5, 188.4, and 170.1 at the 1, 3, 6, 12, and 24 hour time points, respectively ($p < 0.05$ as compared to control, Figs. 5B and C). There was no significant change in SOD3 protein levels at the 0.5 hour time point in both SKOV-3 and MDAH-2774 cells and at the 1 hour time point in SKOV-3 cells. Results were expressed as relative levels as compared to their control.

Discussion

In this study we sought to determine the effects of inhibiting the generation of ROS by DPI, a well-characterized, potent inhibitor of flavoenzymes including NADPH oxidase, on apoptosis of EOC cells, and whether these effects are associated with SOD3 and HIF-1 α expression [30,31]. Diphenyleneiodonium has been used to inhibit ROS production mediated by NADPH oxidase in various cell types [21,31,32]. Our immunohistochemical results showed that NADPH oxidase is over-expressed in EOC tissues as compared to normal ovarian tissues (Fig. 3). Consistent with this observation, we demonstrated EOC cells to have elevated NADPH oxidase, which was reduced by DPI (Fig. 2). These findings are supported by the fact that increased NADPH oxidase levels promote the tumorigenic potential of NIH3T3 mouse fibroblasts as well as the DU-145 prostate epithelial cells [30].

Inhibition of NADPH oxidase has been reported to significantly limit the conversion of molecular O_2 to $O_2^{\cdot-}$, H_2O_2 and other ROS [24,25]. Growing evidence suggests that cancer cells exhibit increased intrinsic ROS stress, due in part to oncogenic stimulation, increased metabolic activity, and mitochondrial malfunction [2,3]. Further support for this increase in ROS is demonstrated by a cross-talk between mitochondria and the $O_2^{\cdot-}$ generating NADPH oxidase in ovarian tumors [33]. The mitochondria controls NADPH oxidase redox signaling, therefore loss of this control contributes to tumorigenesis [33]. In agreement with a previous study, we have shown that inhibition of NADPH oxidase-dependent ROS generation with DPI induced apoptosis in EOC cells, suggesting that the ROS produced by NADPH oxidase, at least in part, exert an anti-apoptotic function [34]. This anti-apoptotic mechanism involves induced inhibition of phosphorylation of AKT and subsequent suppression of AKT-mediated phosphorylation of ASK1 on Ser-83 [34–36]. Furthermore, the anticancer drug paclitaxel-induced apoptosis of ovarian cancer cells is mediated by negative regulation of AKT–ASK1 phosphorylation signaling whereas AKT activation by H_2O_2 confers protection against apoptosis [34–36].

In addition, we have shown that DPI treatment significantly reduced SOD3 and HIF-1 α mRNA levels as early as 30 minutes after treatment, with significant further reduction over the following 24 hours in EOC cells (Figs. 4 and 5). A parallel reduction in protein levels, although not with equivalent magnitude, was also observed for both SOD3 and HIF-1 α as determined by IP/Western blot and ELISA (Figs. 4 and 5). This may be a consequence of post-translation modifications, which may result in increased stability and/or lower

degradation of the proteins. These findings demonstrated that inhibition of NADPH oxidase attenuates the expression of both SOD3 and HIF-1 α , at the mRNA and protein levels. Moreover, our results showed that the inhibition of NADPH oxidase significantly induced apoptosis of EOC cells, as assessed by both caspase-3 activity and TUNEL assays (Fig. 1). Therefore, there appears to be a strong association between the inhibition of NADPH oxidase and the subsequent reduction in SOD3 and HIF-1 α levels and increase in apoptosis of EOC cells.

The correlation between HIF-1 α and cellular apoptosis has previously been demonstrated in lung and hepatoma cancer cells [25,37]. Apoptosis can regulate HIF-1 α through the modulation of the ratio of pro-apoptotic Bcl-2 and anti-apoptotic Bcl-2 family proteins [38]. Anti-apoptotic Bcl-2 and Bcl-xL levels were increased and proapoptotic BAX and BAK levels were decreased with the over-expression of HIF-1 α [38]. Also, it has been reported that inhibition of HIF-1 α by rapamycin resulted in an increase in apoptosis of cancer cells, and decrease in the expression of apoptosis inhibitor Bcl-2 in ovarian cancer xenografts and that rapamycin enhanced cell death through the inhibition of cell survival signals in a number of cell lines [39].

Most of the generated O₂⁻ undergoes a nonenzymatic or SOD-catalyzed reaction, generating H₂O₂ as an end product [40–42]. Hydrogen peroxide is freely diffusible through biological membranes, and its overproduction is extremely destructive to cells and tissues, yet it is physiologically important among ROS given its relative long half-life in the intracellular space, and that it is the precursor of the more toxic hydroxyl radicals [41–43]. It has been reported that increased SOD3 expression in ovarian cancer is a cellular response to intrinsic ROS stress [44]. However, the role of SOD3 in tumorigenesis is somewhat controversial. It has been recently demonstrated, in mice, that subcutaneous inoculation of the SOD3 gene significantly suppressed lung cancer metastasis and that over-expression of SOD3 resulted in *in vivo* inhibition of growth of B16-F1 melanoma tumors [45,46]. In contrast, inhibition of SOD has been shown to selectively induce apoptosis of leukemia and ovarian cancer cells, confirming our findings from the present study [5].

High expression levels of SOD3 was reported to significantly induce the expression of HIF-1 α in tumors, under hypoxic conditions, and thus demonstrates a relationship between SOD3 and HIF-1 α pathways [39]. The mechanism by which SOD3 upregulates HIF-1 α is not well understood, but there is substantial evidence to suggest that this mechanism is modulated, in part, by the steady-state level of O₂⁻ and the stabilization of HIF-1 α [47]. Therefore, reduction of O₂⁻ levels via inhibition of NADPH oxidase may result in lowering SOD3 levels, leading to the destabilization of HIF-1 α , subsequently increasing apoptosis.

Collectively, based on previously discussed published reports and the results from this study, we conclude that lowering oxidative stress, possibly through the inhibition of NADPH oxidase-generated O₂⁻, induces apoptosis in ovarian cancer cells and may serve as a potential target for cancer therapy.

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Fig. 1.

(A) Caspase-3 activity and (B) apoptosis in EOC cells. (A) Caspase-3 activity was measured in cell lysates from SKOV-3 and MDAH-2774 before and after DPI treatment (10 μ M) at various time points. (B) The amount of DNA fragmentation (apoptosis) was assessed by TUNEL assay in MDAH-2774 and SKOV-3, before and after DPI treatment (10 μ M, 12 and 24 hrs) as compared to control cells. Nuclei were stained with DAPI (blue) and apoptotic cells were visualized (60x) with fluorescein-12-dUTP (green). Results are representative of the mean of three independent experiments. (* All $p < 0.05$ as compared to their control).

Fig. 2.

Real-time RT-PCR for NADPH oxidase in EOC cells. Expression of the NADPH oxidase subunit p22-*phox* mRNA levels in SKOV-3 and MDAH-2774 before and after DPI treatment (10 μM, 0.5 hrs) was measured using real-time RT-PCR. Results are representative of the mean of three independent experiments. (* All $p < 0.05$ as compared to their control).

Fig. 3.

Immunohistochemical staining of EOC tissue sections. Normal and EOC tissue section were stained with a primary antibody for NADPH oxidase, followed by biotinylated secondary antibody. Detection was performed with AEC and counter-staining was done with Mayer's hematoxylin followed by mounting and imaging (20x). The expression of NADPH oxidase was assessed based on the presence of cytoplasmic staining.

Fig. 4.

HIF-1 α levels in EOC cells. (A) HIF-1 α mRNA levels in SKOV-3 and MDAH-2774 before and after DPI treatment (10 μ M), at various time points, were measured using real-time RT-PCR. (B) ELISA was performed at different time points for cell lysates from SKOV-3 and MDAH-2774 before and after DPI treatment (10 μ M) at various time points. Results are representative of the mean of three independent experiments. Results are representative of the mean of three independent experiments. (* All $p < 0.05$ as compared to their control).

Fig. 5.

SOD3 levels in EOC cells. (A) SOD3 mRNA levels in SKOV-3 and MDAH-2774 before and after DPI treatment (10 μ M), at various time points, were measured using real-time RT-PCR. (B) IP/Western blot was utilized to detect SOD3 protein levels in EOC cells. Cell lysates from SKOV-3 and MDAH-2774 before and after DPI treatment (10 μ M), at various time points, were precipitated with anti-SOD3 antibody and fractionated with SDS-PAGE. Membrane was probed with anti-SOD3 antibody. Immunoprecipitation of SOD3 exposed to SOD3 antibody are as follows: Lanes 1 & 8; control, Lanes 2 & 9; 0.5 hr, Lanes 3 & 10; 1 hr, Lanes 4 & 11; 3 hr, Lanes 5 & 12; 6 hr, Lanes 6 & 13; 12 hr, and Lanes 7 & 14; 24 hr. (C): IP/Western blot results were scanned and analyzed by NIH image J 3.0. Results are representative of the mean of three independent experiments. (* All $p < 0.05$ as compared to their control).

Table 1

Sequence of Human Oligonucleotide Primers.

Locus	Sense (5'-3')	Antisense (5'-3')	Product Length (bp)
HIF-1 α	AGCCGAGGAAGAACTATGAAC	ACTGAGGTTGGTTACTGTTGG	100
p22- <i>phox</i>	GTACTTTGGTGCTTACTC	GGAGCCCTTTTTCCTCTT	82
SOD3	GCCTCCATTTGTACCGAAAC	AGGGTCTGGGTGGAAAGG	78