Published in final edited form as:

Gynecol Oncol. 2010 February ; 116(2): 276–281. doi:10.1016/j.ygyno.2009.11.004.

Myeloperoxidase Serves as a Redox Switch that Regulates Apoptosis in Epithelial Ovarian Cancer

Ghassan M. Saed¹, Rouba Ali-Fehmi^{1,2}, Zhong L. Jiang¹, Nicole M. Fletcher¹, Michael P. Diamond¹, Husam M. Abu-Soud¹, and Adnan R. Munkarah^{1,3}

¹ Department of Obstetrics and Gynecology, The C.S. Mott Center for Human Growth and Development, Wayne State University School of Medicine, Detroit, Michigan

² Department of Pathology, Wayne State University

³ Department of Women's Health Services, Henry Ford Health System

Abstract

Objectives—Resistance to apoptosis is a key feature of cancer cells and is believed to be regulated by nitrosonium ion (NO⁺)-induced S-nitrosylation of key enzymes. Nitric oxide (NO), produced by inducible nitric oxide synthase (iNOS), is utilized by MPO to generate NO⁺. We sought to investigate the expression of myeloperoxidase (MPO) and iNOS in epithelial ovarian cancer (EOC) and determine their effect on S-nitrosylation of caspase-3 and its activity as well as apoptosis.

Methods—MPO and iNOS expression were determined using immunofluorescence in SKOV-3 and MDAH-2774 and EOC tissue sections. S-nitrosylation of caspase-3 and its activity, levels of MPO and iNOS, as well as apoptosis, were evaluated in the EOC cells before and after silencing MPO or iNOS genes with specific siRNA probes utilizing real-time RT-PCR, ELISA, and TUNEL assays.

Results—MPO and iNOS are expressed in EOC cell lines and in over 60% of invasive EOC cases with no expression in normal ovarian epithelium. Indeed, silencing of MPO or iNOS gene expression resulted in decreased S-nitrosylation of caspase-3, increased caspase-3 activity, and increased apoptosis but with a more significant effect when silencing MPO.

Conclusion—MPO and iNOS are co-localized to the same cells in EOC but not in the normal ovarian epithelium. Silencing of either MPO or iNOS significantly induced apoptosis, highlighting their role as a redox switch that regulates apoptosis in EOC. Understanding the mechanisms by which MPO functions as a redox switch in regulating apoptosis in EOC may lead to future diagnostic tools and therapeutic interventions.

Introduction

Apoptosis is a tightly regulated molecular process that removes excess or unwanted cells from organisms. Resistance to apoptosis is a key feature of cancer cells and is involved in the

Corresponding and Request: Ghassan M. Saed, PhD, Department of Obstetrics & Gynecology, C. S. Mott Center for Human Growth and Development, Wayne State University School of Medicine, 275 E. Hancock, Detroit, MI 48201, Phone: 313-577-5433; Fax: 313-577-4633; gsaed@med.wayne.edu.

Conflict of Interest Statement

The authors declare that there are no conflicts of interest.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

pathogenesis of cancer. We have previously reported that epithelial ovarian cancer (EOC) cells have significantly increased levels of nitric oxide (NO), which correlated with increased expression in inducible nitric oxide synthase (iNOS) [1]. We have also reported that EOC cells manifested lower apoptosis, which was markedly induced by inhibiting iNOS by L-NAME, indicating a strong link between apoptosis and NO/iNOS pathways in these cells [1]. Caspase-3 is known to play a critical role in controlling apoptosis, by participating in a cascade that is triggered in response to proapoptotic signals and culminates in cleavage of a set of proteins, resulting in disassembly of the cell [2–5]. Caspase-3 was found to be S-nitrosylated on the catalytic-site cysteine in unstimulated human lymphocyte cell lines and denitrosylated upon activation of the Fas apoptotic pathway [6]. Decreased caspase-3 S-nitrosylation was associated with an increase in intracellular caspase activity. Caspase-3 S-nitrosylation/denitrosylation is known to serve as an on/off switch regulating caspase activity during apoptosis in endothelial cells, lymphocytes and trophoblasts [7–10]. The mechanisms underlying S-nitrosothiol (SNO) formation *in vivo* are not well understood.

Myeloperoxidase (MPO) typically uses hydrogen peroxide (H_2O_2), in combination with chloride to generate hypochlorous acid [11–14]. We, and others, have demonstrated that MPO utilizes NO, produced by iNOS, as a one-electron substrate generating NO^+ , a labile nitrosating species that is rapidly hydrolyzed forming nitrite (NO_2^-) as an end product [15–18]. The ability of MPO to generate NO^+ , from NO, led us to believe that not only does MPO play a role in S-nitrosylation of caspase-3 in EOC cells, but also highlights a possible cross-talk between iNOS and MPO. In this study, we tested the hypothesis that MPO is responsible for the S-nitrosylation of caspase-3, which led to the inhibition of caspase-3 in EOC cells. Silencing MPO gene expression induced apoptosis in EOC cells through a mechanism that involved S-nitrosylation of caspase-3 by MPO. We also analyzed the expression of MPO in epithelial ovarian carcinoma specimens and compared it to its expression in normal ovarian epithelium.

Methods

Cell lines and cell culture

The human EOC cell lines, MDAH-2774 and SKOV-3, were obtained from American Type Culture Collection (ATCC, Manassas, VA). Cell lines 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 100µg/mL streptomycin including 10% heat-inactivated FBS at 37 °C in 5% CO₂. Culture medium was replaced every two days. For each experiment, cells were plated in 60 mm × 15mm cell culture dishes at a cell density of approximately 2×10^6 cells per dish. All experiments were performed in triplicate.

Transfection of siRNA for iNOS and MPO

Cells were maintained in McCoy's 5A medium supplemented with 100 U/mL penicillin and 100 µg/mL streptomycin including 10% heat-inactivated FBS at 37 °C in 5% CO₂. For siRNA transfection, cells were grown to a confluence of 30% to 40% in 12-well plates (BD Bioscience, Franklin Lakes, NJ) and transfected with the use of 3 µL NeoFX reagent (Ambion, Austin, TX), 2 µL of 20 µmol siRNA, and OptiMEM medium (Invitrogen) up to a final volume of 100 µL. Neo FX reagent and siRNA were incubated at room temperature for 10 minutes and then applied onto 1.0×10^5 cells per well. Transfection mixtures were incubated with cells for 24 hours before washing cells with medium and incubated for an additional 24 hours. Experiments were performed in triplicate for each of the 2 cell lines.

siRNA design, synthesis, and labeling

SiRNAs were designed after determination of target sequences by aligning iNOS and MPO sequences to an Ambion web-based algorithm. The 21-nucleotide duplex siRNA molecules

with 3-dTdT overhangs were re-suspended in nuclease-free water according to the instructions of the manufacturer (Ambion). To ensure stringent controls, both a 2A-based mutated control siRNA with 2 nucleotide mismatches (siRNA-2Amut) and a scrambled control sequence (siRNA-SCR) obtained from Ambion (Silencer Negative Control No. 1 siRNA) were used.

Real-time RT-PCR

Reverse transcription—A 20 μ L cDNA reaction volume was prepared using the QuantiTect Reverse Transcription Kit (Qiagen), as described by the manufacturer's protocol.

Real-time RT-PCR primer design and controls—Optimal oligonucleotide primer pairs for real-time RT-PCR amplification of reverse-transcribed cDNA were selected with the aid of the software program, Beacon Designer (Premier Biosoft Int., Palo Alto, CA). Human oligonucleotide primers, which amplify variable portions of the protein coding regions, were used. Sequences of the oligonucleotides used for amplification of iNOS, MPO, and β -actin mRNA are as described in Table S1.

Quantitative RT-PCR was performed using a QuantiTect SYBR Green RT-PCR kit (Qiagen, Valencia, CA) and Cepheid 1.2f Detection System. RT-PCR was performed in a 25 μ L total reaction volume including 12.5 μ L of 2 $\times$ QuantiTect SYBR Green RT-PCR master mix, 3 μ L of cDNA template, and 0.2 μ M each of target specific primers designed to amplify a part of each gene. To quantify each target transcript, a standard curve was constructed with serial dilutions of standard plasmid (Invitrogen). The PCR reaction was performed as follows: first at 95 $^{\circ}$ C for 10 minutes, and 40 cycles of 95 $^{\circ}$ C for 15 seconds and different annealing temperature. After RT-PCR, a melting curve analysis was performed to demonstrate the specificity of the PCR product as a single peak. A control, containing all the reaction components except for the template, was included in all experiments. The amount of mRNA was then normalized to the abundance of a housekeeping gene, β -actin. To evaluate the validity of using β -actin as an internal standard and changes in the amounts of β -actin, mRNA was tested as an external standard. Subsequently, the normalized values of the mRNA were divided by those in controls. Student's unpaired t test was used for group comparisons.

RT-PCR reaction conditions were programmed as follows: An initial cycle was performed at 94 $^{\circ}$ C for 5 minutes, followed by 35 cycles of denaturation at 94 $^{\circ}$ C for 1 minute, annealing at 56 $^{\circ}$ C for 1 minute (for iNOS), 60 $^{\circ}$ C for 1 minute (for MPO), and 58 $^{\circ}$ C for 1 minute (β -actin). This was followed by a final cycle at 72 $^{\circ}$ C for 7 minutes to allow completion of product synthesis.

Immunohistochemistry

Cells in Culture—Culture cells were grown on a Lab-Tek Chamber slide (Sigma Chemicals, St. Louis, MO) overnight at 37 $^{\circ}$ C. The cells were washed briefly with phosphate buffer saline solution (PBS) and fixed with 3% paraformaldehyde for 30 minutes followed by washing with PBS three times. Cells were blocked with 1% bovine serum albumin (BSA).

Slides were incubated with the FITC-conjugated iNOS and Texas Red-conjugated MPO antibodies (mouse anti-iNOS monoclonal antibody; BD Bioscience, rabbit anti-myeloperoxidase polyclonal antibody; Abcam, Cambridge, MA) diluted at 1:100 ratio for 1 hour at room temperature. The cover slips were mounted on the slide with a drop of mounting medium containing DAPI (Invitrogen), sealed with nail polish and stored in dark at 4 $^{\circ}$ C. Slides were examined with the Axiovert 25 inverted microscope (Zeiss, Thornwood, NY) using DAPI (blue), Texas red (red) and FITC (green), fluorescent filters with excitation and emission wavelengths of 365 and 445, 470 and 525, and 596 and 613 nm respectively. Images were taken using the Axiovision software (Zeiss) and a microscope-mounted camera.

Tissue sections—Twenty benign ovarian tissues specimens, obtained from cases who underwent salpingo-oophorectomy for benign uterine pathology, and 20 invasive epithelial ovarian cancer cases retrieved from archival materials from the Detroit Medical Center/ Karmanos Cancer Center pathology department. Case distribution by FIGO stage was as follows: 10 were stage I and 10 were advanced stage disease (III or IV) at diagnosis. H&E stained slides from each case were reviewed for validation/confirmation of diagnosis and histology by one of the authors (RAF). The histological diagnoses were as follows: 10 high grade serous carcinomas; 5 low grade serous carcinomas, 4 grade 1 endometrioid and one grade 1 mucinous carcinomas. The mean age of the 20 patients was 68 (range 38 to 89 years). After deparaffinizing and hydrating with phosphate-buffered saline (PBS, pH 7.4), the sections were pretreated with hydrogen peroxide (3%) for 10 minutes to remove endogenous peroxidases and incubated in goat serum for 10 minutes. A primary antibody for MPO (Dako, Denmark, A0398) and a primary antibody for iNOS (Santa Cruz, Santa Cruz, CA, sc7271) dilution (1:100) was then applied to each sections, followed by washing and incubation with the biotinylated secondary antibody for 10 minutes at room temperature. Detection was performed with AEC and counterstaining was done with Mayer's hematoxylin followed by mounting.

The expression of MPO and iNOS were assessed based on the presence of cytoplasmic staining. The 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 <5% of cell staining positive; score 2 with 6–30% and score 3 with >30% of cells staining positive. For statistical analysis, cases with score 0 or 1 were considered as being negative and cases with score 2 or 3 as positive.

Caspase-3 activity

Chemicon's Caspase-3 Colorimetric Activity Assay Kit (Chemicon, Temecula, CA) was used, which provides a simple and convenient means for assaying the activity of caspases that recognize the sequence DEVD. The assay is based on spectrophotometric detection of the chromophore p-nitroaniline (pNA) after cleavage from the labeled substrate DEVD-pNA. The free pNA can be quantified using a spectrophotometer or a microtiter plate reader at 405 nm. Comparison of the absorbance of pNA from an apoptotic sample with an uninduced control allows determination of the fold increase in caspase-3 activity (Chemicon). Cells (2×10^6) were harvested and lysed in 300 μ l of cell lysis buffer included with the kit, and concentrations were equalized for each sample set. Subsequently, 150 μ g of cell lysate 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 1h at 37 °C, and then absorbance was measured with a plate reader (Ultramark, BIO-RAD, Hercules, CA) by detection of the chromophore p-nitroaniline (pNA) after cleavage from the labeled substrate DEVD-pNA. Background reading from cell buffers and substrate were subtracted from the readings of samples before calculating increase in caspase-3 activity.

Detection of S-nitrosylation of caspase-3

Ovarian cancer cell lysates from the different treatments were immunoprecipitated with anti-caspase-3 polyclonal antibody conjugated with protein A/G plus agarose beads. Immunoprecipitated caspase-3 zymogen was released by boiling the beads at 95 °C for 5 minutes. Biotinylated proteins were separated by SDS-PAGE and detected using nitrosylation detection reagent I (HRP) according to the manufacturer's protocol.

Measurement of apoptosis by TUNEL assay

Apoptosis of treated and control EOC cells was assessed using the TUNEL technique as described by the Promega Apoptosis Detection System and as we have previously described [19,20]. Positive controls were performed by treating cells with DNase I (1mg/ml) in TdT

buffer for 10 minutes at room temperature before incubation with a biotinylated nucleotide. The TUNEL system detects the fragmented DNA of apoptotic cells by incorporating fluorescein-12-dUTP at the 3' –OH ends of the DNA using the enzyme Terminal deoxynucleotidyl Transferase, which form a polymeric tail using the principle of the TUNEL (TdT-mediated dUTP Nick End-Labeling) assay. Apoptotic cells were visualized using an Axiovert 25 inverted microscope (Zeiss) using DAPI (blue) and FITC (green), fluorescent filters with excitation and emission wavelengths of 365 and 445, 470 and 525 nm respectively. Images were taken using the Axiovision software (Zeiss) and a microscope-mounted camera.

Statistical analysis

Data were analyzed using SPSS 15.0 for Windows. A mixed model repeated measures ANOVA was used with treatment as the within factor and cell type as the between factor. Paired comparisons with a Bonferroni correction were used to compare pairs of treatments. Significant interactions between treatment and cell type were analyzed with independent sample t-tests by cell type on each treatment. Statistical significance of $P < 0.05$ is considered significant for all analyses.

Results

MPO expression in EOC cells and tissues

The ability of EOC cells and tissues to synthesize MPO and iNOS was investigated. The EOC cell lines, SKOV-3 and MDAH-2774, were dually stained with antibodies targeting iNOS (green) and MPO (red). Immunoreactivity showed co-localization of iNOS and MPO (yellow) in both ovarian cancer cell lines (Figure 1). Similar results were obtained for MDAH-2774 (results not shown). iNOS and MPO expression was upregulated in 70% and 65% of ovarian cancer tissue sections tested by immunohistochemistry, respectively (Figure 2). There was no detectable expression for either MPO or iNOS in any of the normal ovarian epithelial tissue. The immunoreactivity that was observed in normal ovarian tissue sections was localized to blood vessels.

Silencing iNOS or MPO gene expression increased caspase-3 activity and apoptosis in EOC cells

We have previously reported that EOC cell lines SKOV-3 and MDAH-2774 manifested a marked decrease in their rate of apoptosis and significantly higher rate of proliferation [1,21]. The cause for lower apoptosis is not yet known. Caspase-3 activity increased by 161 and 418% in SKOV-3 and 156 and 446% in MDAH-2774 when silencing iNOS or MPO gene expression utilizing specific siRNA for iNOS or MPO, respectively (Figure 3). TUNEL assay showed that these treatments were associated with increased apoptosis in both cell lines (Figure 4).

Silencing iNOS and MPO gene expression decreased S-nitrosylation of caspase-3 in EOC cells

Since the activity of caspase-3 depends on the level of its S-nitrosylation, we investigated the level of S-nitrosylation of caspase-3 in the two cell lines before and after silencing iNOS or MPO gene expression utilizing specific siRNA. The levels of S-nitrosylation of caspase-3 were markedly lower in response to silencing either iNOS or MPO, but to a greater extent when silencing MPO (Figure 5). There was no difference in the intensity between control and scrambled nonspecific siRNA (data not shown).

Cross-talk between MPO and iNOS gene expression in EOC cells

To determine the relationship between iNOS and MPO gene expression in EOC cells, we utilized the siRNA technology to silence iNOS gene expression and examined MPO

expression, and vice-versa. Our results clearly indicate that silencing iNOS gene expression resulted in a 47 and 36% reduction in MPO gene expression in MDAH-2774 and SKOV-3, respectively (Figure 6A). Similarly, silencing MPO gene expression also resulted in a 43 and 42% reduction in iNOS gene expression in MDAH-2774 and SKOV-3, respectively (Figure 6B). There was no difference in MPO and iNOS mRNA levels between control and scrambled nonspecific siRNA (data not shown).

Discussion

Molecular alterations that lead to apoptosis can be inhibited by S-nitrosylation of apoptotic proteins such as caspases. Thus, S-nitrosylation conveys a key influence of NO on apoptosis signaling and may act as a key regulator for apoptosis in cancer cells. It has been known that the effects of NO on apoptosis are not only stimulatory but also inhibitory as shown in this study. These paradoxical effects of NO on apoptosis seem to be influenced by a number of factors. It has been suggested that biological conditions, such as the redox state, concentration, exposure time and the combination with O₂, superoxide and other molecules, determine the net effects of NO on apoptosis [22]. Also, NO is implicated in both apoptotic and necrotic cell death depending on the NO chemistry and the cellular biological redox state [22]. We have previously demonstrated that the EOC cell lines, SKOV-3 and MDAH-2774, manifest lower apoptosis and have significantly high levels of NO due to the presence of high levels of iNOS [1,21]. Additionally, it has been shown that MPO can consume NO in the presence of the co-substrate H₂O₂ [16]. Based on these reports we hypothesized that MPO uses the existing cellular NO to produce NO⁺, which is the main source of protein S-nitrosylation, specifically caspase-3 in EOC cell lines.

In this study we detected significant levels of MPO, which was found to be co-localized with iNOS, expression in both EOC cell lines SKOV-3 and MDAH-2774. We have demonstrated that 65% of the invasive epithelial ovarian carcinoma specimens we tested express MPO in the neoplastic cells. The co-localization of MPO and iNOS has been demonstrated by immunohistochemical studies in cytokine-treated human neutrophils and primary granules of activated leukocytes [23]. The plasma levels and tissue expression of MPO in gynecologic malignancies were previously evaluated and it was found that gynecologic cancer patients had higher plasma MPO compared to control subjects [24]. Using immunostaining, it was also demonstrated that MPO expression was higher in cancer tissues compared to control [24].

MPO may function at sites with excessive NO levels leading to formation of NO⁺ and subsequent inactivation of caspase-3. In this study we found that MPO significantly increased S-nitrosylated caspase-3, which was accompanied by a parallel decrease in the level of apoptosis, suggesting a positive regulation of apoptosis through S-nitrosylation of caspase-3. Silencing MPO, by utilizing siRNA technology, resulted in a significant decrease in S-nitrosylation of caspase-3 and increase in apoptosis.

Since resistance to apoptosis is a hallmark of tumor growth, identifying mechanisms of this resistance such as S-nitrosylation may be a key in cancer progression. S-nitrosylation is reversible and seemingly specific post-translational modification that regulates the activity of several signaling proteins. S-nitrosylation of the catalytic site cysteine in caspases serves as an on/off switch regulating caspase activity during apoptosis in endothelial cells, lymphocytes, and trophoblasts [8–10]. Targeting MPO may be a potential therapeutic intervention to reverse the resistance to apoptosis in epithelial ovarian cancer cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

References

1. Malone JM, Saed GM, Diamond MP, Sokol RJ, Munkarah AR. The effects of the inhibition of inducible nitric oxide synthase on angiogenesis of epithelial ovarian cancer. *Am J Obstet Gynecol* 2006;194:1110–6. discussion 1116–8. [PubMed: 16580304]
2. Porter AG, Janicke RU. Emerging roles of caspase-3 in apoptosis. *Cell Death Differ* 1999;6:99–104. [PubMed: 10200555]
3. Liu L, Stamler JS. NO: an inhibitor of cell death. *Cell Death Differ* 1999;6:937–42. [PubMed: 10556969]
4. Dimmeler S, Haendeler J, Nehls M, Zeiher AM. Suppression of apoptosis by nitric oxide via inhibition of interleukin-1 β -converting enzyme (ICE)-like and cysteine protease protein (CPP)-32-like proteases. *J Exp Med* 1997;185:601–7. [PubMed: 9034139]
5. Thornberry NA, Lazebnik Y. Caspases: enemies within. *Science* 1998;281:1312–6. [PubMed: 9721091]
6. Mannick JB, Hausladen A, Liu L, Hess DT, Zeng M, Miao QX, Kane LS, Gow AJ, Stamler JS. Fas-induced caspase denitrosylation. *Science* 1999;284:651–4. [PubMed: 10213689]
7. Maejima Y, Adachi S, Morikawa K, Ito H, Isobe M. Nitric oxide inhibits myocardial apoptosis by preventing caspase-3 activity via S-nitrosylation. *J Mol Cell Cardiol* 2005;38:163–74. [PubMed: 15623433]
8. Rossig L, Fichtlscherer B, Breitschopf K, Haendeler J, Zeiher AM, Mulsch A, Dimmeler S. Nitric oxide inhibits caspase-3 by S-nitrosation in vivo. *J Biol Chem* 1999;274:6823–6. [PubMed: 10066732]
9. Dash PR, Cartwright JE, Baker PN, Johnstone AP, Whitley GS. Nitric oxide protects human extravillous trophoblast cells from apoptosis by a cyclic GMP-dependent mechanism and independently of caspase 3 nitrosylation. *Exp Cell Res* 2003;287:314–24. [PubMed: 12837287]
10. Mannick JB, Schonhoff C, Papeta N, Ghafourifar P, Szibor M, Fang K, Gaston B. S-Nitrosylation of mitochondrial caspases. *J Cell Biol* 2001;154:1111–6. [PubMed: 11551979]
11. Harrison JE, Schultz J. Studies on the chlorinating activity of myeloperoxidase. *J Biol Chem* 1976;251:1371–4. [PubMed: 176150]
12. Kettle AJ, van Dalen CJ, Winterbourn CC. Peroxynitrite and myeloperoxidase leave the same footprint in protein nitration. *Redox Rep* 1997;3:257–8. [PubMed: 9754322]
13. Weiss SJ, Klein R, Slivka A, Wei M. Chlorination of taurine by human neutrophils. Evidence for hypochlorous acid generation. *J Clin Invest* 1982;70:598–607. [PubMed: 6286728]
14. Ortiz de Montellano PR. Catalytic sites of hemoprotein peroxidases. *Annu Rev Pharmacol Toxicol* 1992;32:89–107. [PubMed: 1605582]
15. Stamler JS, Singel DJ, Loscalzo J. Biochemistry of nitric oxide and its redox-activated forms. *Science* 1992;258:1898–902. [PubMed: 1281928]
16. Abu-Soud HM, Hazen SL. Nitric oxide is a physiological substrate for mammalian peroxidases. *J Biol Chem* 2000;275:37524–32. [PubMed: 11090610]
17. Abu-Soud HM, Hazen SL. Nitric oxide modulates the catalytic activity of myeloperoxidase. *J Biol Chem* 2000;275:5425–30. [PubMed: 10681518]
18. Abu-Soud HM, Khassawneh MY, Sohn JT, Murray P, Haxhiu MA, Hazen SL. Peroxidases inhibit nitric oxide (NO) dependent bronchodilation: development of a model describing NO-peroxidase interactions. *Biochemistry* 2001;40:11866–75. [PubMed: 11570887]
19. Saed GM, Diamond MP. Apoptosis and proliferation of human peritoneal fibroblasts in response to hypoxia. *Fertil Steril* 2002;78:137–43. [PubMed: 12095503]
20. Saed GM, Abu-Soud HM, Diamond MP. Role of nitric oxide in apoptosis of human peritoneal and adhesion fibroblasts after hypoxia. *Fertil Steril* 2004;82(Suppl 3):1198–205. [PubMed: 15474096]
21. Munkarah AR, Morris R, Baumann P, Deppe G, Malone J, Diamond MP, Saed GM. Effects of prostaglandin E(2) on proliferation and apoptosis of epithelial ovarian cancer cells. *J Soc Gynecol Investig* 2002;9:168–73.
22. Nicotera P, Melino G. Regulation of the apoptosis-necrosis switch. *Oncogene* 2004;23:2757–65. [PubMed: 15077139]

23. Evans TJ, Buttery LD, Carpenter A, Springall DR, Polak JM, Cohen J. Cytokine-treated human neutrophils contain inducible nitric oxide synthase that produces nitration of ingested bacteria. *Proc Natl Acad Sci U S A* 1996;93:9553–8. [PubMed: 8790368]
24. Song M, Santanam N. Increased myeloperoxidase and lipid peroxide-modified protein in gynecological malignancies. *Antioxid Redox Signal* 2001;3:1139–46. [PubMed: 11813987]

Figure 1. Co-localization of MPO and iNOS to the same cells in EOC cells

MPO and iNOS staining in SKOV-3 ovarian cancer cells. A representative slide (100 x) was dually stained with antibody against MPO (red), iNOS (green), and nuclei (blue). Co-localization of MPO and iNOS is shown in yellow. Experiments were performed in triplicate.

Normal Ovary- MPO

Serous Carcinoma
MPO Serous Carcinoma
iNOS **Figure 2. Immunohistochemical staining of iNOS and MPO in normal and EOC tissues**

Normal ovarian epithelium showed no staining for either iNOS or MPO. Strong cytoplasmic staining for iNOS and MPO is shown in a case of high grade serous carcinoma. Experiments were performed in triplicate.

Figure 3. Caspase-3 activity in EOC cells

Caspase-3 Activity was measured in EOC cells, MDAH-2774 and SKOV-3, before and after silencing iNOS or MPO gene expression utilizing siRNA specific probes. A caspase-3 colorimetric activity assay kit was utilized as described in methods. There was no significant difference between controls and nonspecific scrambled siRNA (Data not shown). As can be seen from this figure, caspase-3 activity was significantly increased when silencing MPO or iNOS. Experiments were performed in triplicate.

Figure 4. Measurement of apoptosis

The amount of DNA fragmentation (apoptosis) was assessed by TUNEL assay for EOC cells, MDAH-2774 and SKOV-3, before and after silencing iNOS or MPO gene expression utilizing siRNA specific probes. Nuclei were stained with DAPI (blue) and apoptotic cells were visualized with fluorescein-12-dUTP (green). There was no significant difference between controls and nonspecific scrambled siRNA (Data not shown). Experiments were performed in triplicate.

Figure 5. Caspase-3 S-Nitrosylation in EOC Cells

S-nitrosylation of caspase-3 in EOC cells, SKOV-3 before (1) and after silencing MPO gene expression with specific siRNA (2) and MDAH-2774 before (3) and after silencing iNOS gene expression with specific siRNA probes (4). S-nitrosylated caspase-3 was detected with the S-nitrosylation protein detection assay kit as described in methods. There was no significant difference between controls and nonspecific scrambled siRNA (Data not shown). Experiments were performed in triplicate.

Figure 6. Real-time RT-PCR for MPO and iNOS in EOC cells

Total RNA isolated from EOC cells, MDAH-2774 and SKOV-3, before and after silencing MPO and iNOS gene expression using siRNA specific probes were analyzed utilizing real-time RT-PCR. There was no significant difference between controls and nonspecific scrambled siRNA (Data not shown). Experiments were performed in triplicate.