Nicotinamide Adenine Dinucleotide Phosphate Oxidase Expression Is Differentially Regulated to Favor a Pro-oxidant State That Contributes to Postoperative Adhesion Development

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Abstract

We have previously reported that superoxide ($O_2^{\bullet-}$) contributes to the development of postoperative adhesions. In this study, we determined whether $O_2^{\bullet-}$ generating nicotinamide adenine dinucleotide phosphate oxidase (NOX) is differentially expressed in normal peritoneal and adhesion fibroblasts and tissues. The NOX isoforms were measured utilizing Western blot, immunohistochemistry, high-performance liquid chromatography, and real-time reverse transcription polymerase chain reaction. Expression and activity of NOX were found to be significantly higher in adhesion tissues and cells than that in normal peritoneal tissues and cells ($P < .05$). Levels of NOX2, NOX4, NOX activating protein 1, DUOX1, p47^{phox}, and p22^{phox} messenger RNA increased in adhesion fibroblasts when compared to normal peritoneal and increased in response to hypoxia in normal peritoneal fibroblasts. Thus, adhesion fibroblasts are characterized by a unique NOX expression profile, which maintains a pro-oxidant state that may be responsible for the persistence of the adhesion phenotype. Decreasing the activity of NOX by targeting these isoforms may be beneficial for future therapeutic interventions of postoperative adhesions.

Keywords

NADPH oxidase, adhesions, oxidative stress, hypoxia, fibroblasts

Introduction

Postoperative adhesions are a common medical complication of gynecologic and other pelvic surgeries.¹ Pelvic adhesions have been reported to form in as many as 95% of patients within the first several weeks of major gynecologic surgery and are frequently associated with chronic or recurrent pelvic pain, intestinal obstruction and infertility.^{2,3} Although laparoscopic lysis of adhesions is considered a worthwhile treatment for adhesion-related chronic pelvic pain, adhesions have been shown to reform at one or more sites in almost all patients who undergo adhesiolysis and at two-thirds of the actual sites where adhesiolysis was performed.^{4,5} In addition, between 12% and 51% of patients also develop de novo adhesions following laparotomy or operative laparoscopy.^{3,4} The precise molecular mechanisms of postoperative adhesions remain unknown.

In an attempt to determine the underlying mechanisms of adhesion development, we have reported substantial phenotypic differences at the molecular level between fibroblasts isolated from normal peritoneum and adhesion

tissues of the same patients.⁶ These phenotypic differences have led to the characterization of the “adhesion phenotype” that includes an increase in fibronectin, type I collagen, vascular endothelial growth factor, transforming growth factor (TGF) β 1, α smooth muscle actin (α -SM A), cyclooxygenase 2, ratio of tissue plasminogen activator to plasminogen activator inhibitor, and ratio of matrix metalloproteinase 1 to tissue inhibitor of metalloproteinase 1.⁷ More importantly, we have shown that hypoxia can irreversibly induce the adhesion phenotype markers in normal

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peritoneal fibroblasts to levels observed in adhesion fibroblasts.⁸

Hypoxia triggers a number of critical adaptations that enable cell survival, including apoptosis suppression, altered glucose metabolism, and an angiogenic or profibrotic phenotype.⁹ Recent investigations suggest that O₂ depletion stimulates mitochondria to further increase reactive oxygen species (ROS), with subsequent activation of signaling pathways, such as hypoxia inducible factor 1 α , that promote cell survival and consequently fibrotic growth.⁹ Reactive oxygen species formed after exposure to oxidative stress in general have been implicated in the activation of signaling molecules.⁹⁻¹² Various enzyme systems produce ROS, including the mitochondrial electron transport chain, cytochrome P450, lipoxygenase, cyclooxygenase, xanthine oxidase, peroxisomes, and the nicotinamide adenine dinucleotide phosphate oxidase (NOX) complex, which is the focus of this study.^{9,10}

Nicotinamide adenine dinucleotide phosphate oxidases are the main source of superoxide (O₂^{•-}) in several cell types.¹³ The NOX consists of 7 isoforms: 5 NOXs, NOXs 1 to 5, and 2 NOX homologs, DUOX1 and DUOX2. The NOX enzymes are differentially activated by different binding molecules, including p22^{phox}, p40^{phox}, p47^{phox} or its homologue NOX-organizing protein 1 (NOXO1), p67^{phox} or its homologue NOX activating protein 1 (NOXA1), and Rac. Activation mechanisms and tissue distribution of the different members of the family are markedly different. Activation of NOX involves the translocation of regulatory elements from the cytoplasm to combine with catalytic subunits in the membrane.¹⁴ Increased NOX activity is known to contribute to a large number of pathologies, including cardiovascular diseases and neurodegeneration.¹⁵ The presence of multiple membrane-associated isoforms of NOX and their cytoplasmic subunits supported a major role of this enzyme complex in altering the oxidative stress balance.¹⁵ In this study, we compared the expression of the various NOX isoforms and their subunits in fibroblasts isolated from normal peritoneum and adhesions tissues as well as in tissues from normal peritoneum and adhesions.

Materials and Methods

Source and Culture of Human Fibroblasts

As described previously, normal parietal peritoneal tissue from the anterior abdominal wall lateral to midline incision and adhesion tissue were excised from patients undergoing laparotomy for pelvic pain at the initiation of the surgery following entry into the abdominal cavity.⁶ All patients gave informed written consent to tissue collection, which was conducted under a protocol approved by the Wayne State University Institutional Review Board. Fibroblast cell lines have been extensively characterized in previous studies and have been shown to be pure and solely fibroblast cells.¹⁶

Treatment of Fibroblasts

Cells (5 × 10⁶) were seeded in 100-mm dishes and were treated with or without hypoxia (2% O₂) for 24 hours followed by collection of cells for extraction of RNA and protein. Parallel cultures were placed in normoxia for 24 hours. Studies were conducted using passage 3 to 5 cells to maintain comparability. All hypoxic experiments were performed in an airtight modular incubator chamber (Billups-Rothenberg, Inc, Del Mar, California). The chamber was deoxygenated by a positive infusion of 2% O₂ in a CO₂-nitrogen gas mixture. Cultures were then placed in a standard humidified tissue incubator. There were no statistically significant differences in viability by crystal violet or trypan blue exclusion (data not shown).

RNA Isolation

Total RNA was extracted from human normal peritoneal fibroblasts and adhesion fibroblasts using the RNeasy Mini kit (Qiagen, Valencia, California) according to the protocol provided by the manufacturer.

Reverse Transcription

A 20 μ L complementary DNA (cDNA) reaction volume was prepared using 1 μ g RNA utilizing the QuantiTect reverse transcription kit (Qiagen) as described by the manufacturer's protocol.

Real-time reverse transcription polymerase chain reaction primer design and controls. Optimal oligonucleotide primer pairs for real-time reverse transcription polymerase chain reaction (RT-PCR) amplification of reverse-transcribed cDNA were selected with the aid of the software program, Beacon Designer (Premier Biosoft Int, Palo Alto, California). Human oligonucleotide primers, which amplify variable portions of the protein coding regions, are listed in Table 1. Standards with known concentrations were designed specifically for these primers using Beacon Designer software, allowing for construction of a standard curve using a 10-fold dilution series. A specific standard for each gene allows for absolute quantification of the gene in copy numbers, which can then be expressed as ng per μ g of RNA.

Real-time RT-PCR was performed with the QuantiTect SYBR Green RT-PCR kit (Qiagen) and a Cepheid 1.2f Detection System (Cepheid, Sunnyvale, California). Each reaction was 25 μ L consisting of 12.5 μ L of 2 × QuantiTect SYBR Green RT-PCR master mix, 1 μ L of cDNA template, and 0.2 μ mol/L each of target-specific primer that was designed to amplify a part of the gene of interest. To quantify each target transcript, a standard curve was constructed using a 10-fold dilution series of the standard for each specific gene investigated. The standard concentration is known and is expressed as copies per μ L and was utilized to convert copies per μ L of the target gene into fg of the gene of interest per μ g

Table 1. List of Oligonucleotide Primers Utilized in Real-Time RT-PCR Analysis of Normal and Adhesion Fibroblasts.

Accession #	Locus	Sense (5'-3')	Antisense (3'-5')	Amplicon Length	Initial PCR Cycle, Seconds	Annealing Temperature, °C
AF127763	NOX1	GCTAAATCCCATCCAGTC	GCTGAAGTTACCATGAGAA	100	1200	56
NM_000397	NOX2	GGAAACTACCTAAGATAGC	TAACATCACCACCTCATA	80	900	57
AF190122	NOX3	TTCTATTACAACAAGGAG	AATTATTATTCACCAGTTC	152	1100	49
AF261943	NOX4	GTAGGAGACTGGACAGAA	ATTGAATGAAGGGCAGAAT	82	1000	54
AF325189	NOX5	CCTTAGTCCTTCTAGTTG	CACCAATTCCAGATACAA	114	1000	53
NM_017434	DUOX1	TTCCACATCTTCTTCCTG	CTGATCTCCACCTTCTTC	83	1200	53
NM_014080	DUOX2	TTCCACATCTACTTCCTG	CTGATCTCCACCTTCTTC	83	1200	54
AY255769	NOXA1	TGGATGTTCTGTGTGAAG	GGGACTACAAAGCACTTG	88	1000	53
NM_144603	NOXO1	CATTCTTCTTCTTCCTCC	TCCTTTGCAGCTTTCTTG	104	900	55
NM_000101	p22 ^{phox}	GTACTTTGGTGCCTACTC	GGAGCCCTTCTTCCTCTT	82	1000	54
NM_000265	p47 ^{phox}	CAGCCAGCACTATGTGTA	ACTCGTAGATCTCGGTGAA	89	1200	54
NM_000433	p67 ^{phox}	TAATAACCAGACAACAGA	TTGATAACACCAGGATTA	125	1400	51

Abbreviations: PCR, polymerase chain reaction; RT-PCR, reverse transcription polymerase chain reaction.

RNA, as described previously.^{8,17} The PCR reaction conditions for the primers are summarized subsequently and in Table 1. An initial cycle was performed at 95°C as indicated in Table 1, followed by 35 cycles of denaturation at 95°C for 15 seconds, annealing for 30 seconds as described in Table 1, and a final cycle at 72°C for 30 seconds to allow completion of product synthesis. Following real-time 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.

Immunohistochemical Staining of Human Tissue Sections for NOX4

Normal peritoneal (n = 7) and adhesion tissues (n = 7) obtained from different women were deparaffinized and subjected to immunohistochemical staining, with standard streptavidin-biotin-peroxidase techniques, with diaminobenzidine as the chromogen. Tissue sections (4-5 µm) were prepared and incubated with primary antibody for NOX (represented by NOX4, HPA015475, Sigma Aldrich, St Louis, Missouri) as described previously.¹⁸ Secondary antibody was applied at 1:500 in phosphate-buffered saline (PBS) for 30 minutes at room temperature. After rinsing with PBS, the slides were incubated with streptavidin/peroxidase at 1:500 in PBS for 30 minutes at room temperature then rinsed with PBS and incubated for 15 minutes with 0.06% diaminobenzidine and counter-stained with Harris modified hematoxylin (Fisher Healthcare, Hanover Park, Illinois).

For the assessment of expression levels of NOX, the staining intensity in the cell membrane and cytoplasm and the percentage of stained cells were analyzed as follows: in Figure 1, staining intensity was scored as 0 (negative), 1+ (weak), 2+ (medium), or 3+ (strong). For the final score, low expression was defined as an intensity of 0, 1, 2, or 3 and <10% stained cells or an intensity of 0 or 1 and <50% stained cells. High expression was defined as an intensity of 2 or 3 and >10%

stained cells or an intensity of 1, 2, or 3 and >50% stained cells. Negative controls consisted of sections that underwent similar staining procedures without the addition of sera.

Immunofluorescence Staining of Human Normal Peritoneal and Adhesion Fibroblasts for NOX4

Normal peritoneal and adhesion fibroblast cells, from the same patient (n = 3), were grown on a Lab-Tek Chamber slides (Sigma Aldrich) overnight at 37°C. The cells were washed briefly with PBS and fixed with 3% paraformaldehyde for 30 minutes followed by washing with PBS 3 times. Cells were blocked with 1% bovine serum albumin. Slides were incubated with primary antibody, polyclonal rabbit anti-NOX4 (sc-30141, Santa Cruz Biotechnology, Santa Cruz, California), 1:100 ratio for 1 hour at room temperature followed by incubation with the secondary antibody, goat antirabbit immunoglobulin G-Texas Red (sc-2780, Santa Cruz), 1:200 for 1 hour at room temperature in the dark. The cover slips were mounted on the slide with a drop of mounting medium containing 4',6-diamidino-2-phenylindole (DAPI; Invitrogen, Carlsbad, California) and sealed with nail polish. Slides were examined with the Axiovert 25 inverted microscope (Zeiss, Thornwood, New York) using DAPI (blue) and Texas Red (red) fluorescent filters with excitation and emission wavelengths of 365 and 445 and 470 and 525 nm, respectively. Images were taken using the Axiovision software (Zeiss) and a microscope-mounted camera.

Western Blot Analysis

Protein extraction. Sections from normal peritoneal (n = 3) and adhesion (n = 3) tissues were cut from the paraffin-embedded blocks and were deparaffinized in xylene and rehydrated in graded ethanol as described previously.¹⁹ Normal peritoneal (n = 3) and adhesion (n = 3) cells and tissues were treated with extraction buffer (200 µL; 1 mol/L sodium dihydrogen phosphate, 10 mmol/L disodium hydrogen phosphate, 154 mmol/L

Figure 1. Quantitative real-time RT-PCR analysis of NADPH oxidase isoforms NOX2, NOX4, and p22^{phox}. Real-time RT-PCR was utilized to measure mRNA levels in NOX2 (A), NOX4 (B), and p22^{phox} (C) in normal peritoneal and adhesion fibroblasts treated with and without hypoxia (2% O₂, 24 hours). A representative figure using a single cell line is shown. Expression is depicted as the mean and with error bars representing standard deviation. *P < .05 as compared to untreated normal fibroblasts. RT-PCR indicates reverse transcription polymerase chain reaction; mRNA, messenger RNA; NADPH, nicotinamide adenine dinucleotide phosphate; NOX, NADPH oxidase.

sodium chloride, 1% Triton X-100, 12 mmol/L sodium deoxycholate, 0.2% sodium azide, 0.95 mmol/L fluoride, 2 mmol/L phenylmethylsulfonyl fluoride, 50 mg/mL aprotinin, 50 mmol/L leupeptin, pH 7.6) and incubated at 100°C for 20 minutes, followed by incubation at 60°C for 2 hours. After incubation, the lysates were centrifuged at 15 000g for 20 minutes at 4°C. Supernatants were collected and stored at -80°C until use for protein assay and Western blot analysis.

Protein assay. The protein concentration of the lysates was determined with the Bio-Rad Protein Assay per the manufacturer's protocol (Bio-Rad Hercules, California) at 750 nm. The protein concentration was determined based on the standard curve using BSA.

Western blot analysis. Lysates from cells or tissues were fractionated with sodium dodecyl sulfate polyacrylamide gel electrophoresis, 4% to 20% Tris-glycine gel (Invitrogen) at 130 V for 180 minutes against a molecular weight ladder. Proteins were transferred from the gel to a nitrocellulose membrane with the use of an electroblotting apparatus at 40 V for 2 hours. Primary antibodies diluted in 1% nonfat milk solution for polyclonal rabbit anti-NOX4 (sc-30141, Santa Cruz Biotechnology) NOX4 and rabbit anti-tubulin (Santa Cruz, sc-135659) primary

antibodies were incubated for 24 hours at 4°C. Membranes were washed and developed as described previously.²⁰ Protein bands were scanned and analyzed by NIH Image J 3.0 and normalized using a housekeeping gene, tubulin.

Nicotinamide Adenine Dinucleotide Phosphate Oxidases Activity Assay

Cell culture and treatment. Normal peritoneal (n = 3) and adhesion (n = 3) fibroblasts were seeded (5×10^6) in 150 mm diameter dish and allowed to grow overnight. Cells were washed with PBS/100 mmol/L diethylenetriaminepentaacetic acid (DTPA) 3 times. All subsequent steps were done under dim light. Hanks buffer of 10 mL (1.3 mmol/L CaCl₂, 0.8 mmol/L MgSO₄, 5.4 mmol/L KCl, 0.4 mmol/L KH₂PO₄, 4.3 mmol/L NaHCO₃, 137 mmol/L NaCl, 0.3 mmol/L Na₂HPO₄, and 5.6 mmol/L glucose, pH 7.4; 500 mL) containing DTPA (100 mmol/L) was added to each well, followed by dihydroethidium (DHE; 50 μmol/L)/dish. Plates were kept in an incubator (37°C, 5% CO₂) for 30 minutes. Thereafter, wells were washed 3 times with PBS/DTPA. Acetonitrile (10 mL) was then added, cells immediately harvested with a cell scraper, and lysate pipetted into an Eppendorf tube, kept on ice. Lysates were centrifuged (12 000g, 10 minutes, and 4°C),

supernatants were transferred to an Eppendorf tube, and the sample was dried under vacuum for 2 to 3 hours (Speed-Vac Plus SC-110A, Thermo Savant). For high-performance liquid chromatography (HPLC) analysis, samples were resuspended in 1 mL acetonitrile and a volume of 50 μ L was injected.

High-performance liquid chromatography. Dihydroethidium is converted to 2-hydroxyethidium (2-EOH) by NOX-produced $O_2^{\bullet -}$ and to a lesser extent peroxynitrite/ CO_2 and ethidium (Et) from H_2O_2 pathways involving metal proteins.²¹ We utilized the HPLC method that was validated by commercially available DHE and Et (Sigma Aldrich).²¹ The HPLC analysis was carried out using a Shimadzu HPLC system equipped with an SCL-10A system controller, with a binary pump solvent delivery (LC-10AD) module and an SIL-10AD autoinjector connected to an SPD-M10A diode array detector and an RF-10A XL fluorescence detector. An Alltech 5- μ m particle size, 4.6×150 -mm reverse-phase octadecylsilica (C18) HPLC column was used and kept at 27°C. The photodiode array detector was set at 245 nm, and the fluorescence detector was set at excitation of 510 nm and emission of 595 nm to monitor the chromatogram. The column was eluted at a flow rate of 1.0 mL/min with linear gradients of buffers A and B (A: water/10% acetonitrile/0.1% trifluoroacetic acid, v/v/v; and B: 100% acetonitrile). The solvent gradient was as follows: 0 to 10 minutes, 30% to 35% B; 10 to 11 minutes, 100% B; 11 to 16 minutes 100% B, then the buffer B composition dropped down to 30% within 16 to 17 minutes, the column equilibrated with 30% B for 13 minutes. Dihydroethidium was detected by the absorbance at 245 nm while 2-EOH and Et were detected at excitation 510 nm and emission 595 nm. According to the literature, the retention time of 2-EOH lies between that of DHE and Et. For normal peritoneal and adhesion fibroblast cell lysates, the products were identified by matching the retention times. 2-Hydroxyethidium was eluted at ~ 7 minutes as described previously.²¹

Statistical Analysis

Data were analyzed using SPSS 15.0 for Windows. Paired *t* tests were utilized for treatment within patient type while unpaired *t* tests were utilized for analysis between patient type. Statistical significance of $P < .05$ is considered significant for all analyses.

Results

Expression of NOX2

The NOX2 messenger RNA (mRNA) levels significantly increased (from 63.8 ± 7.3 to 91.4 ± 2.9 fg/ μ g RNA) in normal peritoneal as compared to adhesion fibroblasts ($P = .0097$, Figure 1A). Additionally, there was an increase, although not quite significant, in response to treatment with hypoxia (to

108.4 ± 1.2 fg/ μ g RNA, $P = .063$) in normal peritoneal fibroblasts while the increase in adhesion fibroblasts was not significant (Figure 1A).

Expression of NOX4

The NOX4 mRNA levels significantly increased (from 0.03 ± 0.02 to 0.2 ± 0.07 fg/ μ g RNA) in normal peritoneal as compared to adhesion fibroblasts ($P = .05$, Figure 1B). Additionally, there was a significant increase in response to treatment with hypoxia (to 0.07 ± 0.02 fg/ μ g RNA, $P = .0289$) in normal peritoneal fibroblasts while the observed increase in adhesion fibroblasts (to 0.4 ± 0.04 fg/ μ g RNA) was not quite significant (Figure 1B).

Expression of p22^{phox}

Levels of p22^{phox} mRNA significantly increased (from 942.3 ± 129.9 to 1633.9 ± 149.9 fg/ μ g RNA) in normal peritoneal as compared to adhesion fibroblasts ($P = .0388$, Figure 1C). Additionally, there was a significant increase in response to treatment with hypoxia (to 1447.0 ± 68.6 fg/ μ g RNA, $P = .0272$) in normal peritoneal fibroblasts while no change was observed in adhesion fibroblasts (Figure 1C).

Expression of p67^{phox} and NOXA1

The NOXA1 mRNA levels significantly increased (from 440.0 ± 16.5 to 612.8 ± 4.9 fg/ μ g RNA) in normal peritoneal as compared to adhesion fibroblasts ($P = .0045$, Figure 2A). Additionally, there was a significant increase in response to treatment with hypoxia (to 818.4 ± 37.3 fg/ μ g RNA, $P = .0248$) in normal peritoneal fibroblasts while the observed increase in adhesion fibroblasts (1061.9 ± 78.5 fg/ μ g RNA) was not quite significant ($P = .08$). For the homologue of NOXA1, p67^{phox}, there was no difference between normal peritoneal and adhesion mRNA levels (from 13.6 ± 0.3 to 14.2 ± 2.2 fg/ μ g RNA, $P = .7$) (Figure 2B). Hypoxia did not have an effect on normal peritoneal fibroblasts while a slight decrease, although not significant, was observed in adhesion fibroblasts (to 8.3 ± 0.07 fg/ μ g, $P = .06$) (Figure 2B).

Expression of p47^{phox} and NOXO1

Levels of p47^{phox} mRNA significantly increased (from 0.92 ± 0.13 to 1.62 ± 0.04 fg/ μ g RNA) in normal peritoneal as compared to adhesion fibroblasts ($P = .0177$, Figure 2C). Additionally, there was a significant increase in response to treatment with hypoxia (to 1.62 ± 0.16 and to 1.97 ± 0.03 fg/ μ g RNA) in both normal peritoneal and adhesion fibroblasts, respectively ($P = .0387$ and $P = .0144$, Figure 2C). There was no detectable mRNA level of NOXO1, a homolog of p47^{phox}, in either cell line.

Figure 2. Quantitative real-time RT-PCR analysis of NADPH oxidase subunits NOXA1, p67^{phox}, p47^{phox}, and DUOX1. Real-time RT-PCR was utilized to measure mRNA levels of NOXA1 (A) and its homolog, p67^{phox} (B), p47^{phox} (C), and DUOX1 (D) in normal peritoneal and adhesion fibroblasts treated with and without hypoxia (2% O₂, 24 hours). A representative figure using a single cell line is shown. Expression is depicted as the mean and with error bars representing standard deviation. *P < .05 as compared to untreated normal fibroblasts, **P < .05 as compared to untreated adhesion fibroblasts. RT-PCR indicates reverse transcription polymerase chain reaction; NADPH, nicotinamide adenine dinucleotide phosphate; NOXA1, NOX activating protein 1; NOX, NADPH oxidase.

Expression of DUOX1 and DUOX2

The DUOX1 mRNA levels significantly increased (from 76.7 ± 15.7 to 258.0 ± 1.9 fg/μg RNA) in normal peritoneal as compared to adhesion fibroblasts ($P = .0155$, Figure 2D). Additionally, there was a significant increase in response to treatment with hypoxia (to 209.3 ± 11.7 and 290.0 ± 1.7 fg/μg RNA) in both normal peritoneal and adhesion fibroblasts, respectively ($P = .0133$ and $P = .0493$). There was no detectable mRNA level of DUOX2 in either cell line.

Expression of NOX1, NOX3, and NOX5

There was no detectable mRNA level for NOX1, NOX3, or NOX5. The NOX is overexpressed in both human adhesion tissues and adhesion fibroblasts. Since NOX4 is the oxygen-sensing subunit controlling the overall activity of the enzyme, we sought to further confirm our real-time RT-PCR findings with protein levels and activity. We utilized immunohistochemistry using NOX4 antibody to demonstrate the expression of NOX in normal peritoneum and adhesion tissues ($n = 7$). There was high percentage of positive staining for NOX (as represented by NOX4) in adhesion tissues compared to normal peritoneal tissues (Figure 3A). The immunohistochemical

detection of the NOX4 subunit was further confirmed by Western blot analysis with adhesion tissue having a 4.3 fold higher NOX4 expression as compared to normal peritoneal tissue. (Figure 3B). Consistent with the observations in tissues, similar results were found in primary cultures of human fibroblasts ($n = 3$). The NOX4 expression was significantly higher in adhesion fibroblasts than that in normal peritoneal fibroblasts (Figure 3C) as indicated by an increase in red fluorescence. The immunofluorescence detection of the NOX4 subunit was further confirmed by Western blot analysis with adhesion fibroblasts having a 1.4 fold higher expression of NOX4 than normal peritoneal fibroblasts. (Figure 3D). There was no observed change in NOX4 protein levels in response to hypoxia (data not shown).

Adhesion Fibroblasts Manifested Higher NOX Activity

We have utilized an HPLC-based method to determine NOX activity in normal peritoneal and adhesion fibroblasts ($n = 3$). Oxidation of DHE by O₂^{•−} specifically generates 2-EOH while Et can represent the decay of 2-EOH and potentially other fluorescent intermediate products.²¹ The HPLC-based NOX4 assay was validated using a commercially available standard for DHE and Et (Figure 4A). Under our experimental conditions, DHE eluted at approximately 2.7 minutes and was

Figure 3. Immunohistochemistry and Western blot analysis of NADPH oxidase (represented by NOX4) in normal peritoneal and adhesion tissues and cells. A, A representative image of immunohistochemical staining for NADPH oxidase demonstrates high positivity in adhesions with no detectable reactivity in normal peritoneal tissue ($n = 3$). B, A representative Western blot analysis of NADPH oxidase demonstrates higher expression of NOX4 in adhesion tissue than in normal peritoneal tissue ($n = 7$). C, A representative image of immunofluorescence staining for NOX4 (red, indicated by arrows) and the nuclei (blue) in normal peritoneal and adhesion fibroblasts ($n = 3$). Staining for NOX4 demonstrates high positivity in adhesion fibroblasts with no detectable reactivity in normal peritoneal fibroblasts. Images were taken at $20\times$ using the Axiovert 25 inverted microscope. D, A representative Western blot analysis for NOX4 using cell lysates from human normal (lane 1) and adhesion fibroblasts (lane 2). There was no observed change in NOX4 levels in response to hypoxia (data not shown). Western blot results were scanned and analyzed by NIH image J 3.0 and demonstrated higher expression of NOX4 in adhesion fibroblasts than in normal peritoneal fibroblasts ($n = 3$). NIH indicates National Institutes of Health; NADPH, nicotinamide adenine dinucleotide phosphate; NOX, NADPH oxidase.

detected by absorbance at 245 nm (shown in black, Figure 4A) while Et eluted at approximately 8.8 minutes as detected by fluorescence (shown in red, Figure 4A). Under similar HPLC conditions, 2-EOH is expected to elute between DHE and Et.²² The relative amount of 2-EOH and Et in normal peritoneal and adhesion fibroblast cell lysate was determined by calculating the area under the curve for the respective peaks (Figure 4B). There was a distinct difference in the content of 2-EOH and Et in normal peritoneal and adhesion fibroblasts (Figure 4C). The amount of 2-EOH increased 1.9-fold while Et increased 2.4-fold in adhesion fibroblasts than that in normal peritoneal fibroblasts (Figure 4C).

Discussion

In this study, we have gathered further evidence to support a role for oxidative stress in the pathophysiology of postoperative adhesions. We have demonstrated that adhesion fibroblasts manifest a unique NOX isoform expression profile, which is responsible for an increased activity of this enzyme in adhesion tissues and fibroblast cells as evident by immunohistochemistry, immunofluorescence, and Western blot in both tissues and cells. Specifically, there was an increase in NOX2, NOX4, p22^{phox}, and

DUOX1 as well as in the cytosolic regulators p47^{phox} and NOXA1/p67^{phox} expression in adhesion fibroblasts as compared to normal peritoneal fibroblasts. These subunits were further increased in response to hypoxia in normal peritoneal fibroblasts.

Postoperative adhesions, like fibrosis, reflect a pathologic excess of biologic events involved in normal tissue repair.²³ Adhesion fibroblasts manifest a unique phenotype that resembles myofibroblasts, which we have termed as “adhesion phenotype.” Specifically, the adhesion phenotype is characterized by increased α -SMA, increased TGFs and cytokines and extracellular matrix production, decreased turnover via effects on matrix-degrading enzymes and their inhibitors as well as decreased apoptosis.^{7,8,16,25,26} Remarkably, hypoxia causes normal peritoneal fibroblasts to irreversibly acquire this phenotype.

Hypoxia, alone or through the activation of NOX, is known to acutely promote the generation of $O_2^{\bullet-}$, a potent ROS, from various intracellular enzyme systems. The generated $O_2^{\bullet-}$ has been previously demonstrated to be a key player in the formation of the adhesion phenotype and hypoxia induced an oxidative stress environment that favors the development of this phenotype.⁸ Adhesion fibroblasts manifest an altered redox balance that includes decreased myeloperoxidase, superoxide dismutase

Figure 4. A, Chromatogram showing the standard for DHE (50 $\mu\text{mol/L}$) in black and Et (10 $\mu\text{mol/L}$) in red. B, A representative HPLC chromatogram of cell lysate from normal peritoneal (dashed line) and adhesion (solid line) fibroblasts ($n = 3$). C, Relative amounts of 2-EOH and Et in normal peritoneal and adhesion fibroblast cell lysates. DHE indicates dihydroethidium; Et, ethidium; HPLC, high-performance liquid chromatography; EOH, 2-hydroxyethidium.

(SOD), caspase-3 activity, nitrate/nitrite, S-nitrosylation of caspase-3, and lipid peroxidation as compared to normal peritoneal fibroblasts.²⁶⁻²⁸ Indeed, scavenging $\text{O}_2^{\bullet-}$ by SOD, restores the adhesion phenotype markers, TGF- β 1 and type I collagen, levels in adhesion fibroblasts to the levels observed in normal peritoneal fibroblasts.⁸ Furthermore, during hypoxia exposure scavenging $\text{O}_2^{\bullet-}$ protected against the development of adhesion phenotype. Notably, normal peritoneal fibroblasts treated with SOD combined with hypoxia manifested a blockage to the development of the adhesion phenotype markers.⁸

A growing body of evidence, from clinical studies, support the notion that increases in NOX activity play a central role in the etiology of various diseases.^{29,30} Nicotinamide adenine dinucleotide phosphate oxidases can be activated by many mechanisms including tissue injury-induced hypoxia, mechanical forces, hormones, and cytokines, all of which can lead to an increase in expression of NOX subunits.^{31,32} All NOX family members are transmembrane proteins that transport electrons across biological membranes, producing $\text{O}_2^{\bullet-}$ from O_2 .¹⁵ The NOX complex includes a membrane-bound flavocytochrome containing 2 oxygen-sensing subunits, p22^{phox} and the glycoprotein gp91^{phox} (also known as NOX2), which are considered to be directly involved in the generation of $\text{O}_2^{\bullet-}$ and are both involved in initiation of cellular signaling in response to hypoxic conditions.³³ There are also the cytosolic proteins p47^{phox} and p67^{phox} and their respective homologous subunits, NOXO1 and NOXA1.^{15,33} During activation of NOX, p47^{phox}/NOXO1 and p67^{phox}/NOXA1

migrate to the plasma membrane where they associate with the flavocytochrome cytochrome b558 to form the active enzyme complex.¹⁵ Additionally, the NOX isozymes DUOX1 and DUOX2 are the sole proteins directly generating H_2O_2 outside the cell.³⁴ Collectively, NOX family members have been shown to be differentially expressed within the cells and tissues of the body. Specifically, gp91^{phox} is expressed in eosinophils, neutrophils, monocytes, and B lymphocytes whereas NOX1 is predominantly detected in the colon, uterus, and prostate.³⁵ Additionally, fibroblasts overexpressing NOX1 displayed increased levels of $\text{O}_2^{\bullet-}$ and exhibited a transformed phenotype in xenograft models.^{36,37} In pulmonary artery adventitial fibroblasts, NOX4 was also shown to maintain higher ROS levels under normoxic and hypoxic conditions resulting in enhanced proliferation and inhibited apoptosis.³⁸

To date, our overall understanding of the molecular mechanisms underlying peritoneal healing and specifically the development of postoperative adhesions, is extremely limited. Although, over the past decade, we have collected an immense amount of compelling evidence utilizing a clinically relevant rat cecal abrasion model and a unique cell culture system to support the hypothesis that hypoxia, as a result of tissue injury, is the initiating factor leading to postoperative adhesion development.^{8,24,25,39-46} A number of observations have been made using these models that illustrate the role of hypoxia and the subsequent oxidative stress, not only in the development but also in the persistent of the adhesion phenotype. In conclusion,

the adhesion phenotype is maintained under a continuous pro-oxidant state by the expression of a unique NOX isoform profile, which is responsible for the overall increased activity of this enzyme. Targeting these NOX family members may be beneficial for future therapeutic interventions.

Declaration of Conflicting Interests

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