



Original Contribution

Targeted subendothelial matrix oxidation by myeloperoxidase triggers myosin II-dependent de-adhesion and alters signaling in endothelial cells

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ABSTRACT

During inflammation, myeloperoxidase (MPO) released by circulating leukocytes accumulates within the subendothelial matrix by binding to and transcytosing the vascular endothelium. Oxidative reactions catalyzed by subendothelial-localized MPO are implicated as a cause of endothelial dysfunction in vascular disease. While the subendothelial matrix is a key target for MPO-derived oxidants during disease, the implications of this damage for endothelial morphology and signaling are largely unknown. We found that endothelial-transcytosed MPO produced hypochlorous acid (HOCl) that reacted locally with the subendothelial matrix and induced covalent cross-linking of the adhesive matrix protein fibronectin. Real-time biosensor and live cell imaging studies revealed that HOCl-mediated matrix oxidation triggered rapid membrane retraction from the substratum and adjacent cells (de-adhesion). De-adhesion was linked with the alteration of Tyr-118 phosphorylation of paxillin, a key adhesion-dependent signaling process, as well as Rho kinase-dependent myosin light chain-2 phosphorylation. De-adhesion dynamics were dependent on the contractile state of cells, with myosin II inhibition with blebbistatin attenuating the rate of membrane retraction. Rho kinase inhibition with Y-27632 also conferred protection, but not during the initial phase of membrane retraction, which was driven by pre-existing actomyosin tensile stress. Notably, diversion of MPO from HOCl production by thiocyanate or nitrite attenuated de-adhesion and associated signaling responses, despite the latter substrate supporting MPO-catalyzed fibronectin nitration. These data show that subendothelial-localized MPO employs a novel “outside-in” mode of redox signaling, involving HOCl-mediated matrix oxidation. These MPO-catalyzed oxidative events are likely to play a previously unrecognized role in altering endothelial integrity and signaling during inflammatory vascular disorders.

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Introduction

The vascular endothelium performs vital functions in cardiovascular homeostasis by regulating the initiation and resolution of

inflammatory responses and by controlling vascular tone. Central to the homeostatic properties of the endothelium is the production of nitric oxide (NO), which exerts anti-inflammatory actions by inhibiting leukocyte adhesion and platelet aggregation, and controlling vascular tone by signaling for the relaxation of adjacent vascular smooth muscle cells [1]. The homeostatic properties of the endothelium are also dependent on its function as a continuous, semipermeable barrier that tightly regulates the passage of nutrients, macromolecules, fluid, and cells between the blood and the interstitial space [2,3]. Inflammatory vascular diseases such as atherosclerosis are characterized by endothelial dysfunction manifested as a reduction in the bioactivity of endothelial-derived NO [1] and a loss of endothelial barrier integrity leading to endothelial hyperpermeability [4,5]. Enhanced vascular oxidative stress during inflammation is implicated as a principal cause of compromised endothelial NO signaling [1] and barrier function [2,3] and accordingly there is significant interest in understanding the oxidative reactions involved.

Considerable clinical and experimental evidence supports that myeloperoxidase (MPO), an oxidant-generating heme enzyme

Abbreviations: BAH, 4-aminobenzoic acid hydrazide; PP2, 4-amino-5-(4-chlorophenyl)-7-(dimethylethyl)pyrazolo[3,4-d]pyrimidine; ECs, bovine aortic endothelial cells; FAK, focal adhesion kinase; HBSS, Hank's balanced salt solution; HOCl, hypochlorous acid; HOSCN, hypothiocyanous acid; Met, methionine; MPO, myeloperoxidase; MLC-2, myosin light chain II; NO, nitric oxide; NO₂⁻, nitrite; •NO₂, nitrogen dioxide radical; PBS, phosphate-buffered saline; SCN⁻, thiocyanate; Y-27632, (+)-(R)-trans-4-(1-aminoethyl)-N-(4-pyridyl)cyclohexanecarboxamide dihydrochloride

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released by activated neutrophils and monocytes, plays a pathogenic role in impairing the signaling and vasodilatory function of endothelium-derived NO during inflammatory vascular disease. Thus, circulating MPO levels are elevated in patients with cardiovascular disease and the extent of this increase is an independent predictor of the incidence of endothelial dysfunction manifested as impaired endothelium-dependent vasorelaxation [6,7], as well as the prevalence and degree of coronary artery disease and clinical event risk in patients with acute coronary syndromes [8,9]. Inflammatory stimuli that increase circulating MPO levels promote endothelial dysfunction in healthy animals and humans, with a deficiency in functional MPO conferring protection [10,11]. Circulating MPO rapidly leaves the circulating compartment by binding to and transcytosing the endothelium [12–15], resulting in its accumulation within the subendothelial matrix, where it is anatomically positioned to impair the signaling and vasodilatory function of endothelium-derived NO by acting as a NO oxidase [10,13,16,17].

Subendothelial-localized MPO is not only positioned to interfere with NO signaling, but also to generate reactive oxidants capable of altering the structure and function of extracellular matrix proteins [13,18–20]. The potent chlorinating agent hypochlorous acid (HOCl) is the principal oxidant produced by MPO and is generated by oxidation of chloride ions (Cl^-) in the presence of the cosubstrate hydrogen peroxide (H_2O_2) [21,22]. Thiocyanate (SCN^-) and nitrite (NO_2^-) are also significant physiological substrates that divert the enzyme from HOCl production to the generation of the thiol-reactive oxidant hypothiocyanous acid (HOSCN) and the nitrating radical nitrogen dioxide ($\bullet\text{NO}_2$), respectively [22]. Previous studies identify that subendothelial matrix proteins are significant targets for modification by MPO-derived oxidants. Thus, MPO transcytosed by cultured endothelial cells binds to the adhesive matrix substrate fibronectin, conferring specificity of MPO-catalyzed tyrosine nitration to this protein [13]. In atherosclerotic coronary arteries and other inflamed vascular tissue from humans, MPO is characteristically found deposited within the subendothelium where it colocalizes with fibronectin-containing extracellular matrix and tyrosine-nitrated proteins [23]. Extracellular deposits of MPO and HOCl-modified proteins are similarly detected within the vascular endothelium of human atherosclerotic arteries [24–26].

While oxidative modification of subendothelial matrix proteins by MPO-derived oxidants is a characteristic event during inflammatory vascular disease, the implications of this process for endothelial cell morphology and signaling are largely unknown. In the current study, we show that endothelial-transcytosed MPO utilizes low micromolar levels of H_2O_2 to mediate HOCl-dependent subendothelial matrix oxidation, involving covalent cross-linking of fibronectin, and that this triggers cell–matrix de-adhesion driven by actomyosin-dependent tensile stress. We provide evidence that endothelial de-adhesion in response to HOCl-mediated matrix oxidation drives changes in key adhesion-dependent signaling processes, with this constituting a novel “outside-in” mode of redox signaling. Moreover, diversion of MPO from HOCl production by SCN^- or NO_2^- attenuates de-adhesion and associated signaling responses, despite the latter substrate supporting MPO-catalyzed matrix nitration. The ability of transcytosed MPO to disrupt endothelial integrity and alter signaling by targeted, HOCl-mediated subendothelial matrix oxidation may have important implications for the regulation of endothelial function during vascular inflammation.

Materials and methods

Cell culture

Bovine aortic endothelial cells (ECs, Lonza; passages 4–9) were cultured in EBM2 medium with all supplements added except

hydrocortisone (EGM medium; Lonza) and routinely passaged on gelatin-coated tissue culture flasks.

Cell experiments

For experiments with ECs containing transcytosed MPO, ECs were initially cultured to confluence in EGM medium on gelatin (Sigma; coated at 100 $\mu\text{g}/\text{ml}$ in PBS, 20 min, 22 °C). Confluent EC monolayers were then incubated for 2 h with 20 nM purified human neutrophil MPO (Merck Millipore) in Hanks balanced salt solution (HBSS) supplemented with 0.2% BSA, culture conditions that permit maximal endothelial transcytosis of MPO [13,14]. ECs containing transcytosed MPO were washed to remove unincorporated enzyme and then incubated in HBSS in the absence or presence of relevant reaction components including 1 mM methionine (Met), 100 μM 4-aminobenzoic acid hydrazide (ABAH), 100 μM SCN^- , and/or 100 μM NO_2^- for 15 min, or with cell signaling inhibitors (20 μM PP2, 40 μM blebbistatin, 10 μM Y-27632) for 30 min, prior to treatment with H_2O_2 (2–50 μM) for up to 1 h. In some experiments, cells were treated with reagent HOCl added as a bolus. H_2O_2 and HOCl solutions were prepared immediately before use and stock concentrations were determined spectrophotometrically (H_2O_2 , ϵ_{240} 43.6 $\text{M}^{-1}\text{cm}^{-1}$; HOCl, ϵ_{292} 350 $\text{M}^{-1}\text{cm}^{-1}$ at pH 12). For oxidant treatments, volumes were selected to ensure uniformity of oxidant exposure expressed relative to the adhesion area of cells (i.e., 1 ml for 12-well plates, 80 μl for 96-well plates).

For experiments with ECs adhered to fibronectin as the sole adhesive substrate, surfaces were first coated with bovine fibronectin (Sigma; 5 $\mu\text{g}/\text{ml}$, 22.7 nM in PBS, 2 h, 37 °C) and then blocked with BSA (0.2% in PBS, 0.5 h, 37 °C) prior to seeding of cells in serum-free media (medium-199 containing 1% BSA; 1 ml at 5×10^5 cells/ml in 12-well plates, 200 μl at 2.5×10^5 cells/ml in 96-well plates). These cell seeding densities yielded a near-confluent layer of adherent cells within 2 h. In experiments to examine a role for fibronectin-bound MPO on EC adhesion, MPO was first bound to fibronectin-coated surfaces (20 nM MPO in HBSS, 0.5 h, 37 °C, with subsequent washing) prior to the seeding of cells. For surface binding of proteins, volumes were selected to ensure uniformity of protein coating, expressed relative to the adhesion area of cells (i.e., μg protein/ mm^2 ; 1 ml for 12-well plates, 80 μl for 96-well plates). ECs adhered to fibronectin or MPO-bearing fibronectin-coated surfaces were then subjected to experimental treatments in the same manner as described above for ECs containing transcytosed MPO.

For experiments to measure the propensity of ECs to adhere to fibronectin previously treated with MPO-derived oxidants, cell-free surfaces with MPO-bearing fibronectin were first treated with H_2O_2 in the absence or presence of other reaction components in the same manner as MPO-containing ECs (see above). Surfaces were then incubated with Met (10 mM) for 10 min to quench residual reactive, surface-bound protein chloramines formed by HOCl, followed by washing, seeding of ECs, and measurement of cell adhesion by cell substrate impedance.

Experiments with purified fibronectin

Purified bovine fibronectin (100 nM, Sigma; twice desalted on PD MiniTrap G-25 columns, GE Healthcare) in HBSS was oxidized by pre-incubation with MPO (100 nM) for 15 min and exposure to H_2O_2 (2–50 μM) for 1 h in the absence and presence of 1 mM Met, 100 μM ABAH, 100 μM SCN^- , or 100 μM NO_2^- .

Real-time quantification of cell adhesion by cell–substrate impedance

Cell–substrate impedance at the surface of 96-well gold micro-electrode arrays (E-plate 96, Roche), expressed as “cell index”, a dimensionless value proportional to the area of cell–substrate contact [27], was measured continuously following seeding of ECs and subsequent treatments by an xCelligence system (Roche). During measurements, electrode arrays were housed in an incubator port maintained at 37 °C with 5% CO₂ saturation. The relative number of attached ECs on electrode surfaces was quantified by crystal violet staining, as previously detailed [20].

Live cell imaging

Differential interference contrast images of live cells on gelatin- or fibronectin-coated 35-mm glass-bottomed culture dishes (Fluorodish, World Precision Instruments) were recorded at 37 °C on a heated stage at 5-s intervals using a 63x water objective on a

Leica TCS SP5 microscope. The projected area of randomly selected cells was quantified by manually tracing the membrane edge and quantifying the number of enclosed pixels using ImageJ software (NIH). Change in area of individual cells over time was fitted to a single exponential decay using nonlinear regression (least squares fitting; Prism 5, GraphPad Software) to determine the half-life of cell contraction.

Measurement of H₂O₂

Cellular metabolism of exogenously added H₂O₂ was measured using an Amplex Red kit according to the manufacturer's instructions (Molecular Probes).

Western blotting

Following experiments, cell proteins and purified proteins were immediately harvested into reducing loading buffer (50 mM Tris-HCl,

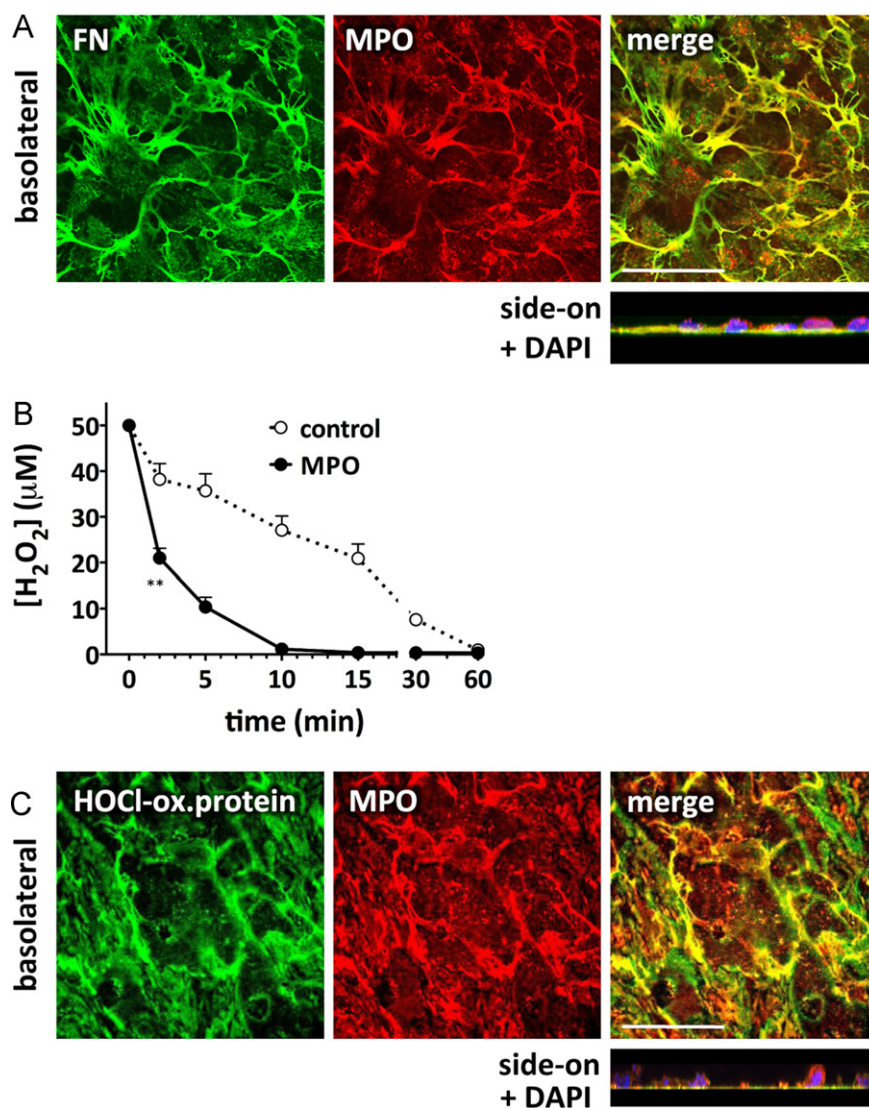


Fig. 1. Endothelial-transcytosed MPO colocalizes with matrix fibronectin and mediates HOCl-dependent protein oxidation. EC monolayers were incubated with MPO (20 nM) for 2 h and unincorporated MPO was removed by washing. MPO-containing ECs were then incubated with H₂O₂ (50 μM) for a further 1 h. (A) MPO-containing ECs prior to H₂O₂ treatment were fixed and imaged by laser confocal fluorescence microscopy using antibodies against fibronectin (FN; green) and MPO (red); control ECs not exposed to MPO displayed negligible immunofluorescence staining for MPO (data not shown). (B) H₂O₂ consumption by control and MPO-containing ECs over 1 h was measured by assaying cell supernatants by the Amplex Red assay at the indicated times. Data represent the mean ± SEM, *n* = 3 independent experiments, ***P* < 0.01 relative to control cells. (C) MPO-containing ECs treated with H₂O₂ were fixed and imaged by laser confocal fluorescence microscopy using antibodies against HOCl-oxidized protein (HOCl ox. protein; green) and MPO (red). In (A) and (C), images represent basolateral cell sections (upper panels) and merged side-on views showing DAPI staining of nuclei (blue); bars = 50 μm.

pH 6.8, 2% SDS, 200 mM dithiothreitol, 20% glycerol, and 0.2% bromophenol blue). Proteins were separated on 3–8% Tris/acetate NuPAGE gels or 10% Bis/Tris NuPAGE gels (Invitrogen), electroblotted to nitrocellulose (iBlot, Invitrogen), and then probed with mouse monoclonal antibodies against fibronectin (1:2000; clone E3E, Millipore), HOCl-oxidized protein (1:25; cell supernatant, clone 2D10G9, which does not cross-react with epitopes generated by oxidative reactions involving nitrating species, transition metals or lipid peroxidation reactions [28]), Src (1:1000; BD Biosciences), paxillin (1:1000; Cell Signaling), FAK (1:1000; Cell Signaling), and phospho-FAK Tyr-397 (1:1000; BD Biosciences) or with rabbit polyclonal antibodies against MPO (1:1000; Millipore), 3-nitrotyrosine (1:1000; Millipore), tubulin (1:1000; Sigma), phospho-paxillin Tyr-118 (1:1000; Cell Signaling), phospho-Src Tyr-416 (1:1000; Cell Signaling), phospho-Src Tyr-527 (1:1000; Cell Signaling), myosin light chain II (MLC-2) (1:1000; Cell Signaling), and diphospho-MLC-2 Thr-18/Ser-19 (1:1000; Cell Signaling). Proteins were visualized on membranes by horseradish peroxidase-conjugated secondary anti-mouse or anti-rabbit antibodies (Cell Signaling) and enhanced chemiluminescence detection (Amersham). Proteins were visualized in gels by SyproRuby staining (Invitrogen) and a Fuji FLA-5000 imaging system.

Immunofluorescence

Cells on glass coverslips were processed for immunostaining by fixing with 4% paraformaldehyde (22 °C, 20 min) and quenching free aldehydes with NH_4Cl (50 mM) for 5 min prior to washing

in PBS. Cells were immunostained using mouse monoclonal antibodies against fibronectin (1:100), HOCl-oxidized protein (clone 2D10G9) (1:50), and paxillin (1:1000) or a rabbit polyclonal antibody against MPO (1:300). Cyanine 2-conjugated goat anti-mouse (green) and cyanine 3-conjugated donkey anti-rabbit (red) antibodies were employed as secondary antibodies. Cell nuclei were stained with DAPI (1:2000) and F-actin was stained with Alexa 555-phalloidin (Invitrogen; 1:500). After washing with PBS, cells were mounted in Elvanol (Mowiol 4–88, Hoechst, Frankfurt, Germany). Laser confocal fluorescence images were recorded at room temperature using a 63x oil objective on a Leica TCS SP5 microscope. Reconstructions of confocal images were rendered using Imaris software.

Statistics

Statistical analyses were performed using one-way ANOVA with Newman Keul's post hoc testing with Prism 5 software. Statistical significance was accepted for P values < 0.05.

Results

Endothelial-transcytosed MPO oxidatively cross-links fibronectin in a HOCl-dependent manner

Incubation of confluent monolayers of ECs with MPO resulted in its uptake and accumulation within the subendothelial compartment, where it colocalized with matrix fibronectin (Fig. 1A),

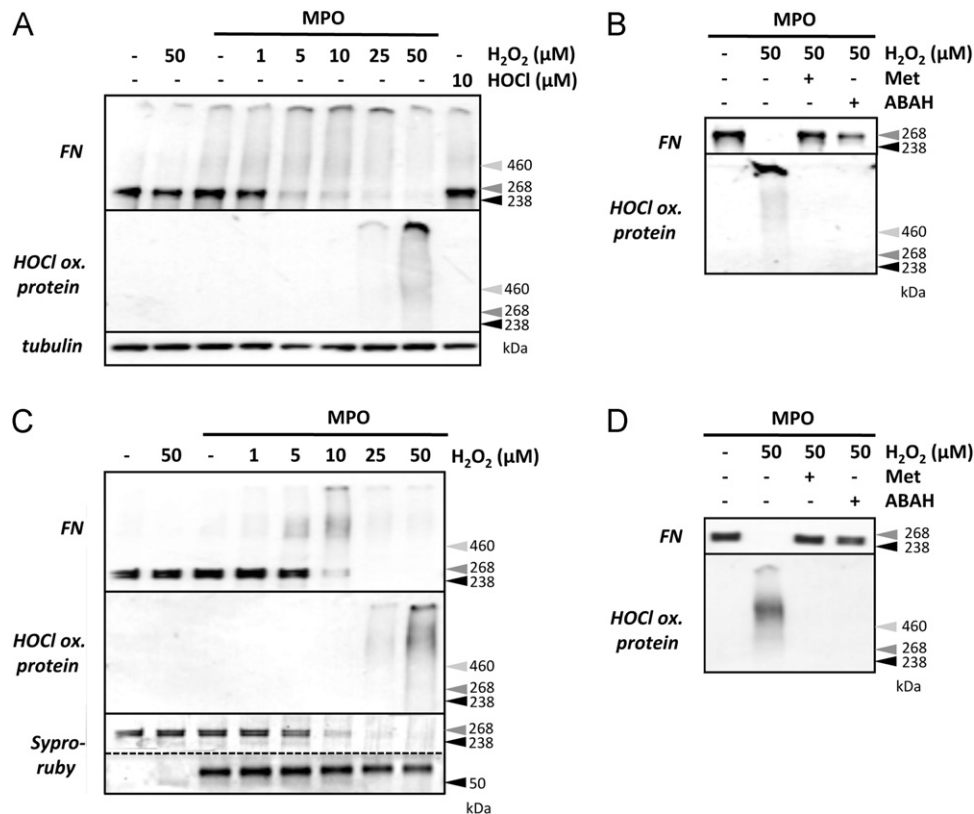


Fig. 2. Endothelial-transcytosed MPO oxidatively cross-links matrix fibronectin in a HOCl-dependent manner. (A, B) EC monolayers were incubated with MPO (20 nM) for 2 h and unincorporated MPO was removed by washing. Control (MPO-free) and MPO-containing ECs were treated with H_2O_2 (1–50 μM) for 1 h in the absence and presence of Met (1 mM) or ABAH (100 μM). In (A), control ECs were also treated with a bolus of reagent HOCl (10 μM) for 1 h. (C, D) Purified fibronectin (FN; 100 nM) was preincubated with MPO (100 nM) for 15 min prior to addition of H_2O_2 (0–50 μM) and incubation for a further 1 h in the absence and presence of Met (1 mM) or ABAH (100 μM). (A, B) Cell proteins and (C, D) purified proteins were analyzed by Western blotting (3–8% gels) under reducing conditions using antibodies against fibronectin (FN) or HOCl-oxidized protein (HOCl ox. protein). In (A), tubulin (55 kDa) was used as a protein loading control. In (C), protein bands of the purified proteins were visualized in the gels by SyproRuby staining (i.e., native FN monomer detected at 250 kDa and MPO α -chain monomer detected at ~60 kDa). Western blot/gel images are reduced by 50% on the vertical scale.

observations consistent with a previous study by Baldus et al. [13]. Control ECs not incubated with MPO displayed negligible immunofluorescence when probed with the antibody for MPO (data not shown). ECs containing transcytosed MPO consumed exogenously added H_2O_2 at significantly enhanced rates compared to control cells (Fig. 1B). Laser confocal microscopy and immunofluorescence showed that H_2O_2 consumption by MPO-containing ECs was accompanied by the formation of HOCl-oxidized protein, detected by the antibody clone 2D10G9 [28], which localized primarily within the subendothelium and displayed colocalization with MPO (Fig. 1C). Western blot analysis of protein extracts from ECs containing transcytosed MPO and exposed to low micromolar concentrations of H_2O_2 revealed extensive fibronectin cross-linking, reflected by loss of the parent fibronectin band and generation of non-reducible, high molecular weight (> 250 kDa) protein aggregates that were recognized by antibodies directed against fibronectin and HOCl-oxidized protein (Fig. 2A). The extent of fibronectin oxidation was dependent on H_2O_2 dose (Fig. 2A) and the concentration of MPO added (Supplementary Fig. 1A). HOCl-oxidized proteins and fibronectin oxidation/cross-linking were not detected in ECs treated with H_2O_2 alone or in MPO-containing ECs prior to H_2O_2 treatment (Fig. 2A, Supplementary Figs. 1B and C). Purified fibronectin was similarly converted into HOCl-oxidized, high

molecular weight aggregates by exposure to MPO in the presence of H_2O_2 (Fig. 2C), consistent with previous observations by Vissers and Winterbourn that purified fibronectin can be oxidatively cross-linked by MPO-derived HOCl [29]. Notably, extensive fibronectin cross-linking occurred when MPO-containing cells were exposed to as little as $5 \mu M$ H_2O_2 (Fig. 2A). Inclusion of the MPO inhibitor ABAH or the HOCl-scavenger methionine (Met) inhibited fibronectin cross-linking and generation of HOCl-oxidized protein within cells (Fig. 2B) and with purified fibronectin (Fig. 2D), observations that identify MPO-derived HOCl as the damaging oxidant. Notably, HOCl derived from low micromolar concentrations of H_2O_2 ($10 \mu M$) by endothelial-transcytosed MPO efficiently oxidized matrix fibronectin, as judged by the loss of the parent fibronectin band, while equivalent doses of reagent HOCl added as a bolus to the apical endothelial surface were ineffective (Fig. 2A). These data establish that transcytosed MPO mediates targeted matrix oxidation by producing HOCl focally within the subendothelial compartment.

Thiocyanate and nitrite suppress HOCl-mediated fibronectin oxidation by endothelial-transcytosed MPO

SCN^- and NO_2^- are significant physiological MPO substrates that divert the enzyme from HOCl production to the generation of

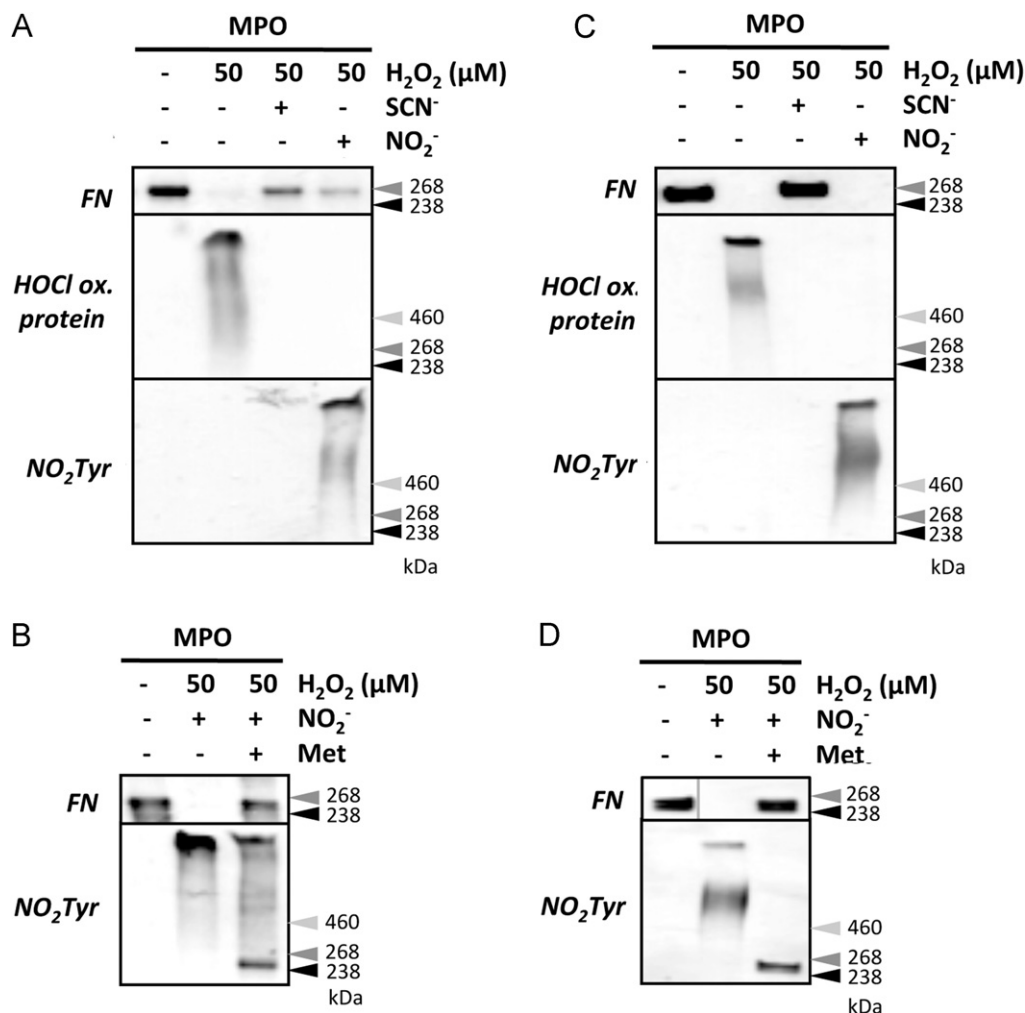


Fig. 3. Thiocyanate and nitrite suppress HOCl-mediated fibronectin oxidation by endothelial-transcytosed MPO. (A, B) EC monolayers were incubated with MPO (20 nM) for 2 h and unincorporated MPO was removed by washing. Control and MPO-containing ECs were then treated with H_2O_2 (50 μM) for 1 h in the absence and presence of SCN^- (100 μM), NO_2^- (100 μM), or Met (1 mM). (C, D) Purified fibronectin (FN; 100 nM) was incubated with MPO (100 nM) for 15 min and then treated with H_2O_2 (50 μM) for 1 h in the absence and presence of SCN^- (100 μM), NO_2^- (100 μM), or Met (1 mM). (A, B) Cell proteins and (C, D) purified proteins were analyzed by Western blotting (3–8% gels) using antibodies against FN, HOCl-oxidized protein (HOCl ox. protein), or 3-nitrotyrosine (NO_2Tyr). Western blot/gel images are reduced by 50% in the vertical scale.

HOSCN and $\bullet\text{NO}_2$, respectively. SCN^- is an excellent substrate for the halogenation activity of MPO and acts to stimulate MPO turnover [30]. While NO_2^- is also metabolized by the peroxidase activity of MPO it slows enzyme turnover by promoting MPO Compound II accumulation [31]. In line with their expected effects on MPO catalytic turnover, SCN^- accelerated and NO_2^- attenuated H_2O_2 consumption by ECs containing transcytosed MPO, with H_2O_2 completely consumed within 1 h after oxidant addition for all treatment conditions (Supplementary Fig. 2). After 1 h treatment, cell proteins were analyzed by Western blotting. SCN^- supplementation was found to inhibit MPO-catalyzed formation of HOCl-oxidized protein and fibronectin modification/cross-linking in ECs (Fig. 3A) and with purified fibronectin (Fig. 3C). NO_2^- also blocked the formation of HOCl-oxidized protein, attenuated fibronectin modification/cross-linking, and promoted the generation of 3-nitrotyrosine, detected on high molecular weight protein aggregates, both in ECs (Fig. 3A) and with purified fibronectin (Fig. 3C). To differentiate the potential roles of HOCl and $\bullet\text{NO}_2$ in mediating fibronectin cross-linking in the presence of NO_2^- , we examined the effect of including Met, which scavenges HOCl but not $\bullet\text{NO}_2$ [32]. Inclusion of Met strongly suppressed fibronectin cross-linking and caused a substantial proportion of 3-nitrotyrosine reactive protein to shift from high molecular weight aggregated material to a single band that comigrated with the parent fibronectin band, both in ECs (Fig. 3B) and with purified fibronectin (Fig. 3D). This reveals that endothelial-transcytosed MPO simultaneously promotes covalent cross-linking and nitration of matrix fibronectin in the presence of NO_2^- , and that HOCl is the oxidant primarily responsible for mediating fibronectin cross-linking.

MPO disrupts the cell-adhesive function of purified fibronectin in a HOCl-dependent manner

We next addressed the functional consequences of fibronectin oxidation by MPO, measured by its ability to support EC attachment and spreading. In these studies, MPO was first bound to fibronectin-coated surfaces and unbound MPO was removed by washing before initiating oxidant production by the fibronectin-bound MPO by exposure to H_2O_2 for 30 min. EC attachment and spreading on native and MPO-oxidized fibronectin was then measured in real time using a cell-substrate impedance electrode.

ECs seeded on native fibronectin in serum-free media were fully attached and spread within 2 h (Fig. 4A). Cell-substrate impedance values in fully adhered cells, quantified as “cell index”

(a value proportional to the area of cell-matrix contact [27]), correlated linearly with cell number and increased with the density of fibronectin coating ($\leq 20 \mu\text{g/ml}$) (Supplementary Fig. 3). Background EC adhesion levels on surfaces without fibronectin were negligible (Supplementary Fig. 3), confirming that adhesion under these conditions was dependent on this substrate.

Binding of MPO (20 nM) to approximately equimolar concentrations of fibronectin (coated at $5 \mu\text{g/ml}$ / 22 nM) prior to cell seeding did not alter the rate or extent of cell attachment and spreading, establishing that MPO binding does not mask fibronectin motifs that support EC adhesion (Fig. 4A). Pretreatment of the MPO-bearing fibronectin with low micromolar doses of H_2O_2 ($\leq 10 \mu\text{M}$) markedly impaired the ability of fibronectin to support subsequent EC attachment and spreading, with this loss of adhesive function abrogated by scavenging HOCl with Met and inhibiting MPO with ABAH (Fig. 4B). The MPO-dependent impairment of fibronectin's cell adhesive function was also blocked by inclusion of SCN^- or partially attenuated by NO_2^- (Fig. 4C). In reactions with NO_2^- , HOCl scavenging by Met completely preserved the cell adhesive function of fibronectin (Fig. 4C), despite the ability of MPO to catalyze tyrosine nitration of fibronectin under these reaction conditions (Figs. 3C and D). These findings establish that oxidative modification/cross-linking of fibronectin by MPO-derived HOCl disrupts its cell adhesive function and that suppression of HOCl-mediated oxidative modification by SCN^- and NO_2^- protects against this loss of function.

Subendothelial MPO induces EC de-adhesion in a HOCl-dependent manner

Having established that MPO-derived HOCl disrupts the cell adhesive function of fibronectin (Fig. 4) and that fibronectin is an important target for HOCl produced by subendothelial MPO (Figs. 1 and 2), we next examined the capacity of endothelial-transcytosed MPO to alter established EC cell-matrix adhesion. To more specifically address the potential role of fibronectin oxidation by subendothelial-localized MPO in altering EC cell-matrix adhesion, we performed equivalent studies with ECs adhered on fibronectin as the sole adhesive substrate. In these studies, MPO was first incorporated directly onto fibronectin-coated surfaces (as detailed in Fig. 4) and ECs were allowed to fully adhere for > 2 h (cf. Fig. 4A) before treatment with H_2O_2 to initiate subcellular oxidant production by the fibronectin-bound MPO. Changes in cell-matrix adhesion elicited by oxidant production within ECs by transcytosed

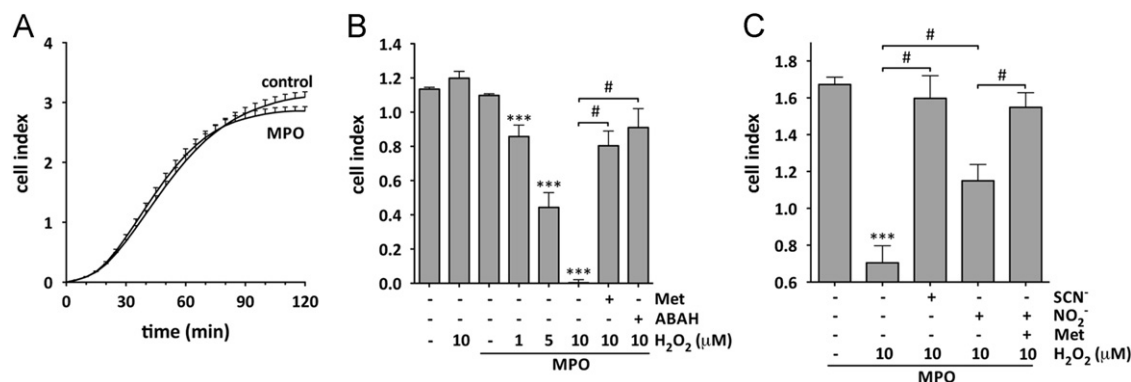


Fig. 4. MPO-derived HOCl impairs the cell-adhesive function of fibronectin. Fibronectin was coated at $5 \mu\text{g/ml}$ (ca. 22 nM) and incubated with MPO (20 nM) for 30 min and unbound MPO was removed by washing. Control (MPO-free) and MPO-bearing fibronectin surfaces were then treated with H_2O_2 (1–10 μM) for 1 h in the absence and presence of Met (1 mM), ABAH (100 μM), SCN^- (100 μM), or NO_2^- (100 μM). Surfaces were treated with Met (10 mM, 10 min) to quench residual protein-bound chloramines and surfaces washed before seeding ECs and measurement of EC adhesion and spreading by cell-substrate impedance. (A) EC adhesion (0–2 h post-seeding) on control or MPO-bearing fibronectin (“MPO”), without pre-treatment with H_2O_2 . (B) EC adhesion (2 h post-seeding) on control and MPO-bearing fibronectin pre-treated with H_2O_2 in the absence and presence of Met or ABAH. (C) EC adhesion (2 h post-seeding) on MPO-bearing fibronectin (“MPO”) pretreated with H_2O_2 in the absence and presence of SCN^- , NO_2^- and/or Met. Data represent the mean $\pm$ SEM, $n=3$. *** $P < 0.001$ relative to EC adhesion on MPO-bearing fibronectin without H_2O_2 treatment. # $P < 0.05$.

or fibronectin-bound MPO were quantified in real time using a cell–substrate impedance biosensor.

In ECs adhered on MPO-bearing fibronectin, H_2O_2 exposure provoked a rapid, time-dependent (Fig. 5A; $P < 0.001$ relative to control cells at 2 min after addition of $10 \mu M H_2O_2$) and dose-dependent (Fig. 5B) loss in cell–matrix contact. Quantification of ECs adhered to impedance electrodes by crystal violet staining revealed that this decrease in cell–matrix contact was independent of changes to the number of attached cells (Supplementary Fig. 4A). ECs not only maintained cell attachment but remained viable during oxidant treatment, revealed by their ability to reestablish adhesion to control levels following the addition of fresh media (Supplementary Fig. 4B). A similarly rapid loss of cell–matrix contact in response to H_2O_2 exposure was recapitulated in ECs containing transcytosed MPO (Fig. 6A). No loss of adhesion was detected in ECs exposed to equivalent doses of H_2O_2 in the absence of MPO (Figs. 5A and 6A). Live cell imaging of MPO-containing ECs exposed to H_2O_2 by differential interference contrast microscopy showed that the losses in cell–matrix contact were due to rapid membrane retraction and “rounding up” of cells (i.e., “de-adhesion”) and confirmed that all cells remained attached to the substratum (ECs adhered on MPO-

bearing fibronectin—Supplementary Movie 1; ECs containing transcytosed MPO—Supplementary Movie 2, with representative frames at 0 and 2 min after H_2O_2 exposure shown in Fig. 6C).

Supplementary material related to this article can be found online at <http://dx.doi.org/10.1016/j.freeradbiomed.2012.10.002>.

Supplementation of ECs adhered on MPO-bearing fibronectin with Met or ABAH (Fig. 5B) and SCN^- or NO_2^- (Fig. 5C) prior to H_2O_2 exposure attenuated MPO-mediated de-adhesion. The residual de-adhesion observed in the presence of SCN^- and NO_2^- was completely prevented by scavenging spurious amounts of HOCl with Met (Fig. 5C). Inclusion of Met and SCN^- alone or NO_2^- and Met in combination, also completely abrogated de-adhesion in ECs containing transcytosed MPO and treated with H_2O_2 (Fig. 6B).

Overall, these data establish that subendothelial-localized MPO triggers cell–matrix de-adhesion in a HOCl-dependent manner, and support that this involves disruption of the cell–adhesive function of fibronectin. Moreover, they identify that SCN^- and NO_2^- attenuate MPO-induced de-adhesion in accordance with their capacity to suppress HOCl-mediated fibronectin oxidation (Fig. 3).

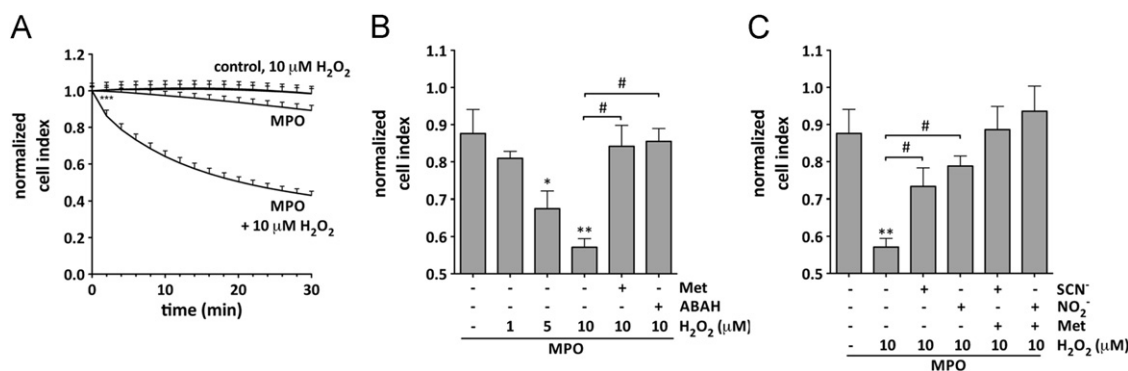


Fig. 5. Subendothelial MPO induces EC de-adhesion from fibronectin in a HOCl-dependent manner. Fibronectin was coated at $5 \mu g/ml$ (ca. $22 nM$) and incubated with MPO ($20 nM$) for 30 min and unbound MPO was removed by washing. ECs were seeded and allowed to fully adhere on control (MPO-free) and MPO-bearing fibronectin surfaces (2 h post-seeding; cf. Fig. 4A) before exposure to H_2O_2 (1 – $10 \mu M$) in the absence and presence of Met ($1 mM$), ABAH ($100 \mu M$), SCN^- ($100 \mu M$), or NO_2^- ($100 \mu M$). Changes in cell adhesion upon exposure to H_2O_2 were measured by cell–substrate impedance; cell index data are normalized to the cell index value at the time of H_2O_2 addition, which was given a value of 1. (A) Cell adhesion in ECs pre-adhered on control or MPO-bearing fibronectin (“MPO”) 0–30 min following exposure to H_2O_2 . (B) Cell adhesion of ECs pre-adhered on control or MPO-bearing fibronectin (“MPO”) 30 min following exposure to H_2O_2 in the absence or presence of Met or ABAH. (C) Cell adhesion in ECs pre-adhered on MPO-bearing fibronectin (“MPO”) 30 min following exposure to H_2O_2 in the absence or presence of SCN^- , NO_2^- , and NO_2^-/Met . Data represent the mean $\pm$ SEM, (A) $n = 6$ or (B, C) $n = 3$. $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ relative to cell adhesion changes of MPO-containing ECs without H_2O_2 treatment. $\# P < 0.05$.

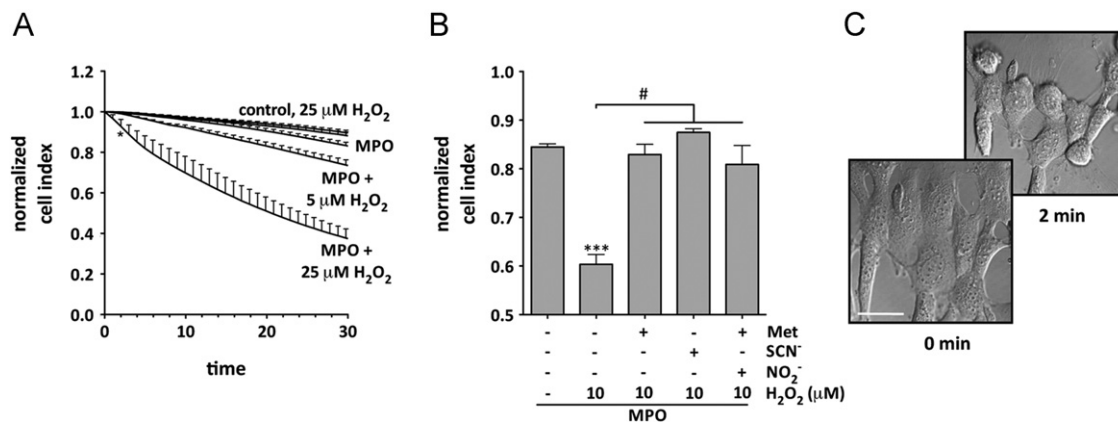


Fig. 6. Endothelial-transcytosed MPO induces EC de-adhesion in a HOCl-dependent manner. EC monolayers were incubated with MPO ($20 nM$) for 2 h and unincorporated MPO was removed by washing. Control (MPO-free) and MPO-containing ECs were then treated with H_2O_2 (5 or $25 \mu M$) in the absence and presence of Met ($1 mM$), SCN^- ($100 \mu M$), or NO_2^- ($100 \mu M$). Changes in cell adhesion upon exposure to H_2O_2 were measured by cell–substrate impedance; cell index data are normalized to the cell index value at the time of H_2O_2 addition, which was given a value of 1. (A) Cell adhesion of control or MPO-containing ECs 0–30 min following exposure to H_2O_2 . (B) Cell adhesion of MPO-containing ECs 30 min following treatment with H_2O_2 in the absence or presence of Met, SCN^- , NO_2^- , or Met/ NO_2^- . Data represent the mean $\pm$ SEM, $n = 6$. $^{***}P < 0.001$ relative to MPO-containing ECs without H_2O_2 exposure. $\# P < 0.05$. (C) Frames from a representative live cell differential interference contrast microscopy movie (Supplementary Movie 2) of MPO-containing ECs before (0 min) and after treatment with H_2O_2 ($10 \mu M$) for 2 min. Bar = $50 \mu m$.

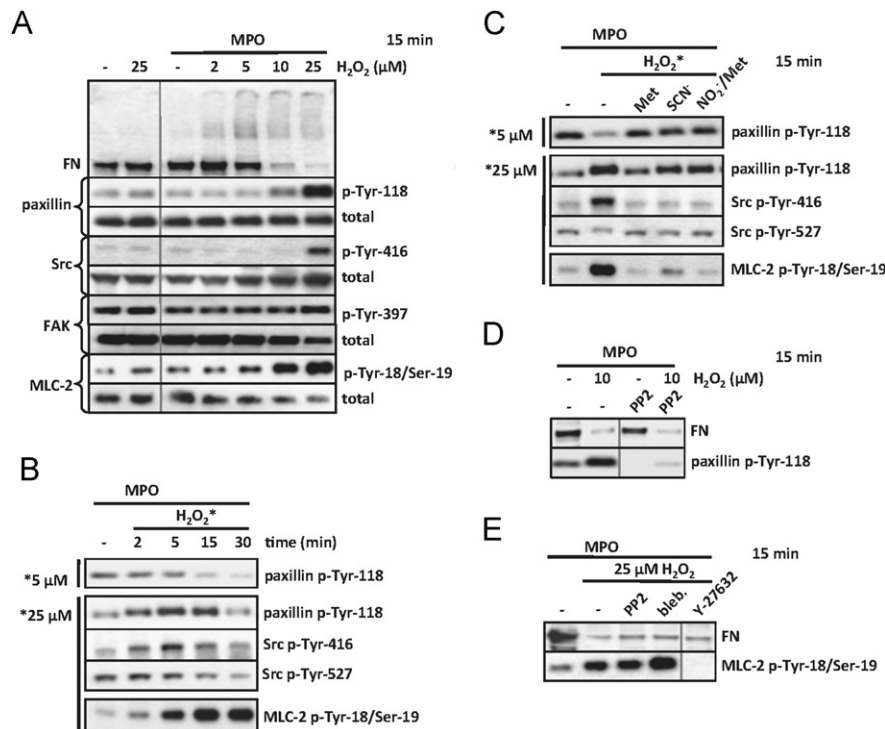


Fig. 7. Endothelial-transcytosed MPO alters the phosphorylation of paxillin, Src, and MLC-2 in a HOCl-dependent manner. (A–E) EC monolayers were incubated with MPO (20 nM) for 2 h and unincorporated MPO was removed by washing. Control (MPO-free) and MPO-containing ECs were then treated with H₂O₂ (2–25 μM) in the absence or presence of other reaction components (1 mM Met, 100 μM ABAH, 100 μM SCN⁻, or 100 μM NO₂⁻) or selective inhibitors of myosin II (blebbistatin, 40 μM), Rho kinase (Y-27632, 10 μM), or Src kinase (PP2, 20 μM). At the indicated times after the addition of H₂O₂, cellular protein extracts were prepared and analyzed by Western blotting for the levels of fibronectin (FN), paxillin Tyr-118 phosphorylation, paxillin, Src Tyr-416 or Tyr-527 phosphorylation, Src, FAK Tyr-397 phosphorylation, FAK, MLC-2 Tyr-18/Ser-19 diphosphorylation, and MLC-2.

EC de-adhesion induced by subendothelial MPO is linked with HOCl-dependent changes in focal adhesion and cytoskeletal signaling

Cell-matrix adhesion involves the assembly of membrane domains called focal adhesions that coordinate cellular responses to local changes in the chemical and physical state of the extracellular matrix by transducing “outside-in” signaling [33,34]. Phosphorylation of the focal adhesion protein paxillin at Tyr-118 has emerged as a major mechanobiological signaling “switch” that regulates responses to changes in cell-matrix adhesion, with loss of force transduction between the cell membrane and the underlying matrix up-regulating paxillin phosphorylation at this amino acid site [35]. Also, phosphorylation of myosin light chain II (MLC-2) at Thr-18 and Ser-19, which positively regulates actomyosin contractility, plays a central role in the remodeling of the cytoskeleton and adhesive contacts in response to external stimuli [35,36]. Therefore, we next investigated the extent to which matrix oxidation and disruption of cell-matrix adhesion by endothelial-localized MPO was linked with changes in the phosphorylation status of paxillin and MLC-2.

Exposure of MPO-containing ECs to H₂O₂ at ≥ 10 μM triggered a time-dependent increase in the phosphorylation of paxillin at Tyr-118 that was apparent after 2 min oxidant addition and maintained up until 15 min before returning to baseline levels at 30 min (Fig. 7A and B). Interestingly, exposure of MPO-containing ECs to lower H₂O₂ doses (5 μM), where EC adhesion was largely maintained (Fig. 6A), resulted in a time-dependent decrease in paxillin Tyr-118 phosphorylation (Fig. 7B). No alteration in paxillin phosphorylation was observed in control ECs exposed to equivalent doses of H₂O₂ in the absence of MPO (Fig. 7A). The dose-dependent modulation of paxillin phosphorylation by MPO (i.e., down-regulation at low oxidant dose or up-regulation at higher oxidant dose) was prevented by Met and suppressed in the presence of SCN⁻ and NO₂⁻ plus Met (Fig. 7C),

identifying HOCl as the primary signaling oxidant produced by EC-localized MPO.

Tyr-118 of paxillin is a phosphorylation target for FAK or Src kinase [37]. In MPO-containing ECs exposed to H₂O₂, while no marked change in Tyr-397 phosphorylation and hence activated FAK was apparent (Fig. 7A), exposure of these cells to 25 μM H₂O₂ resulted in the time-dependent increase in phosphorylation of Src at its activation/auto-phosphorylation site Tyr-416 that paralleled the time-dependent increase in paxillin Tyr-118 phosphorylation (Fig. 7A and B). Activation of Src by Tyr-416 phosphorylation can involve dephosphorylation of an inhibitory phosphorylation site (Tyr-527) [38]. Src Tyr-416 phosphorylation and paxillin Tyr-118 phosphorylation in MPO-containing ECs exposed to 25 μM H₂O₂ were not initially linked with Src Tyr-527 dephosphorylation; however, decreases in Tyr-527 phosphorylation were observed after 15–30 min (Fig. 7B). Src Tyr-416 phosphorylation and Tyr-527 dephosphorylation were both prevented by Met and suppressed in the presence of SCN⁻ or NO₂⁻ plus Met (Fig. 7C). A role of Src kinase for HOCl-dependent paxillin phosphorylation was indicated by the ability of the Src kinase inhibitor PP2 to block both basal and MPO-induced paxillin Tyr-118 phosphorylation (Fig. 7D).

With respect to myosin II signaling, exposure of MPO-containing ECs to H₂O₂ resulted in the dose- (Fig. 7A) and time-dependent (Fig. 7B) increase in the cellular levels of diphosphorylated MLC-2 (Tyr-18/Ser-19). MPO-induced MLC-2 phosphorylation was inhibited by addition of Met, SCN⁻, or NO₂⁻ plus Met (Fig. 7C), confirming its dependence on HOCl, and was prevented by inhibiting Rho kinase with Y-27632 (Fig. 7E). Y-27632 did not suppress HOCl-mediated fibronectin oxidation, confirming that its activity reflected its action as a Rho kinase inhibitor and not by suppressing production of or damage by HOCl (Fig. 7E).

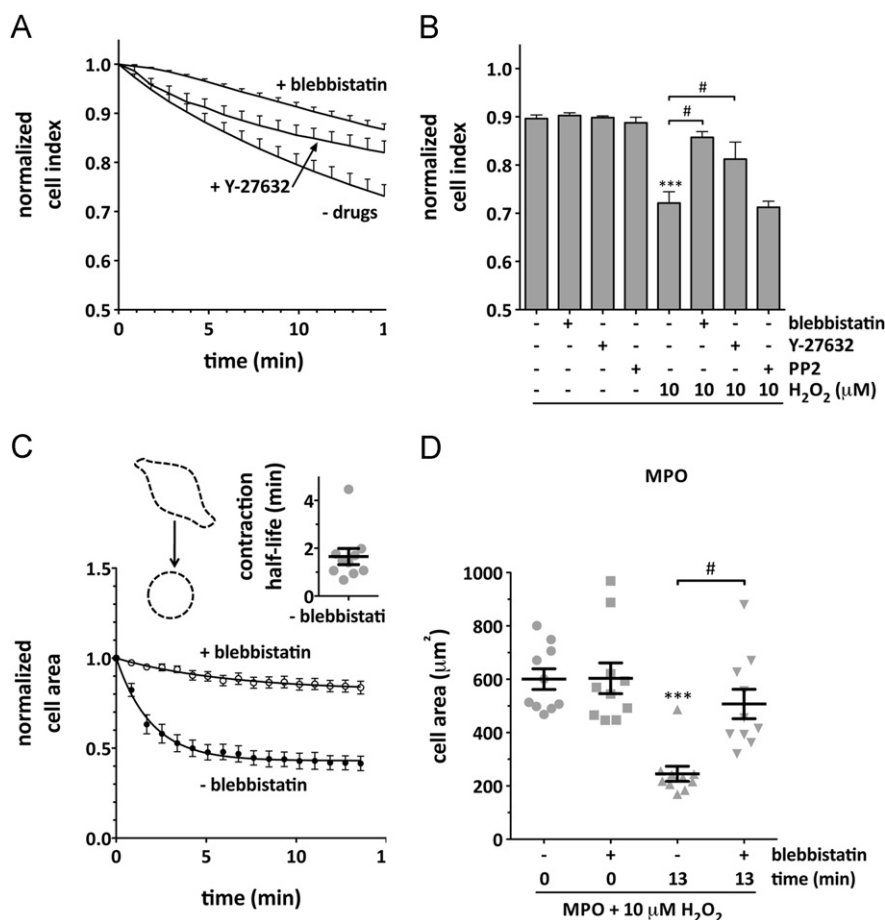


Fig. 8. EC de-adhesion induced by endothelial-transcytosed MPO involves myosin II-mediated contractility. EC monolayers were incubated with MPO (20 nM) for 2 h and unincorporated MPO was removed by washing. MPO-containing ECs were then treated with H₂O₂ (10 μM) for 15 min in the absence or presence of inhibitors of myosin II (blebbistatin, 40 μM), Rho kinase (Y-27632, 10 μM), or Src kinase (PP2, 20 μM). Changes in cell adhesion upon exposure to H₂O₂ were measured by cell-substrate impedance and changes in projected cell area ("cell area") were measured by image analysis of time-lapse differential interference contrast microscopy movies. (A, B) Cell adhesion of MPO-containing ECs 0–15 min (15 min in panel B) following exposure to H₂O₂ in the absence or presence of blebbistatin, Y-27632, or PP2. Data represent the mean ± SEM, *n* = 6. Cell index data are normalized to the cell index value at the time of H₂O₂ addition, which was given a value of 1. (C, D) Cell area of MPO-containing ECs 0–13 min following exposure to H₂O₂ in the absence or presence of blebbistatin (*n* = 10 cells, 2 replicate movies, 5 randomly selected cells per movie; cf. Supplementary Movies 2 and 4); cell area data are normalized to the cell area value at the time of H₂O₂ addition, which was given a value of 1. (C, inset) Cell contraction half-life as determined by fitting the decrease in the area of individual cells to a single exponential decay. (D) Cell area before (0 min) and 13 min following exposure to H₂O₂. ****P* < 0.001 relative to MPO-containing ECs without exposure to H₂O₂. # *P* < 0.05.

Actomyosin contractility promotes EC de-adhesion induced by subendothelial MPO

The preceding studies identify that HOCl produced by subendothelial-localized MPO modulates Src-dependent paxillin phosphorylation and elicits Rho kinase-dependent MLC-2 phosphorylation, which signals for increased myosin II-dependent contractility. We next examined the potential functional role of these signaling events in MPO-induced de-adhesion by selectively inhibiting myosin II signaling and Src-dependent paxillin phosphorylation. Changes in EC adhesion were quantified over time by cell-substrate impedance and/or by measuring decreases in the projected area of individual cells by image analysis of time-lapse differential interference contrast movies.

Inhibition of Src with PP2 had no material impact on MPO-induced MLC2 phosphorylation (Fig. 7E) and de-adhesion (Fig. 8B), indicating that while Src-dependent paxillin phosphorylation is a signaling response linked with endothelial de-adhesion, it does not directly contribute to this process. In contrast, inhibition of myosin II motor function with blebbistatin markedly abrogated MPO-induced membrane retraction from the substratum and from adjacent cells in ECs containing transcytosed MPO (Fig. 8A–D) and in ECs adhered on MPO-bearing

fibronectin (Supplementary Figs. 5A–D) (see also Supplementary Movies 1–4 for representative time-lapse differential interference contrast movies used for image analysis). Blebbistatin prevented EC de-adhesion without inhibiting HOCl-mediated fibronectin oxidation or MLC-2 phosphorylation (Fig. 7E), confirming that its inhibitory activity reflected its action as an inhibitor of myosin II motor function.

The rates of membrane retraction in individual cells during MPO-induced de-adhesion (measured as decreases in the projected cell area over time) were quite uniform, with losses in cell area plateauing at ca. 50% of initial values and cells exhibiting average contraction half-lives of ca. 1–2 min both in ECs containing transcytosed MPO (Fig. 8C and D) and in ECs adhered on MPO-bearing fibronectin (Supplementary Figs. 5C and D). Of note, robust increases in Rho kinase-dependent MLC-2 phosphorylation occurred after 5 min, when membrane retraction was already extensive (Fig. 7B). Furthermore, while Rho kinase inhibition with Y-27632 did not suppress the initial rapid phase of MPO-induced de-adhesion (i.e., <5 min following H₂O₂ exposure) it was inhibitory during the subsequent phase of de-adhesion (i.e., >5 min following H₂O₂ exposure) (Fig. 8A and B), a time period when Rho kinase-dependent MLC-2 phosphorylation was markedly increased (Fig. 7B).

Staining of MPO-containing ECs for F-actin prior to H₂O₂ exposure (Supplementary Fig. 6A) and 10 min following H₂O₂ exposure (Supplementary Fig. 6B) showed that de-adhesion involved extensive inward remodeling of the actin cytoskeleton and accumulation of F-actin at the membrane periphery, which was abrogated by scavenging HOCl with Met (Supplementary Fig. 6C). Notably, paxillin also accumulated at the membrane periphery during HOCl-mediated de-adhesion in association with F-actin (Supplementary Figs. 6A–C). Although Rho kinase-dependent MLC-2 phosphorylation peaked within 5–15 min following H₂O₂ exposure (Fig. 7B), this was not accompanied by an increase in long actin stress fibers and instead de-adhesion was characterized by a loss of preexisting stress fibers (Supplementary Figs. 6A and B).

Overall, these findings support that while up-regulation of Rho kinase-dependent contractility contributes to the overall extent of MPO-induced de-adhesion, the initial rapid phase of membrane retraction represents a response to oxidative disruption of cell–matrix contacts by MPO-derived HOCl, and is primarily driven by preexisting stress in the actin cytoskeleton.

Discussion

During vascular inflammation, MPO is released from circulating leukocytes and accumulates within the subendothelial extracellular matrix by binding to and transcytosing the endothelium [13,23]. Compelling clinical and experimental evidence has identified endothelial-transcytosed MPO as a key mediator of impaired NO signaling in inflammatory vascular disorders and in human cardiovascular disease, with this thought to reflect its ability to efficiently intercept and oxidatively consume endothelial-derived NO within the subendothelial space [7,10,11]. However, subendothelial-localized MPO is also anatomically positioned to impact on endothelial morphology and signaling by oxidatively modifying the structure and function of adhesive extracellular matrix proteins [13,19,20].

This study has shown for the first time that MPO transcytosed by ECs utilizes low micromolar concentrations of H₂O₂ to mediate targeted, HOCl-dependent oxidation of the subendothelial matrix, and that this involves covalent modification/cross-linking of fibronectin. Employing real-time cell–substrate impedance biosensor measurements and quantitative live cell image analysis, we showed that HOCl-mediated oxidation of the subendothelial matrix triggers rapid retraction of the cell membrane from the substratum and from adjacent ECs (de-adhesion). EC de-adhesion was linked with the activation of Src and modulation of Tyr-118 phosphorylation of paxillin, a key focal adhesion-dependent signaling process, as well as Rho kinase-dependent MLC-2 phosphorylation. Notably, de-adhesion dynamics were dependent on the contractile state of cells, with inhibition of myosin II and Rho kinase-mediated MLC-2 phosphorylation significantly retarding the rate of membrane retraction.

Importantly, these rapid de-adhesion and signaling responses were absent in ECs treated with H₂O₂ alone in the absence of MPO. While other studies identify that H₂O₂ can alter cell–cell/cell–matrix adhesion and focal adhesion/cytoskeletal signaling (e.g., paxillin phosphorylation, MLC-2 phosphorylation) in ECs, significantly higher oxidant doses ($\geq 100 \mu\text{M}$ H₂O₂) are typically required to elicit robust morphological and signaling responses [39,40]. Moreover, instead of eliciting de-adhesion as we observed in MPO-containing ECs, treatment with H₂O₂ alone *enhances* cell–matrix adhesion in ECs in a concentration-dependent manner [39]. Our data therefore highlight that EC-transcytosed MPO significantly potentiates, and alters, the signaling actions of H₂O₂ by converting it to HOCl.

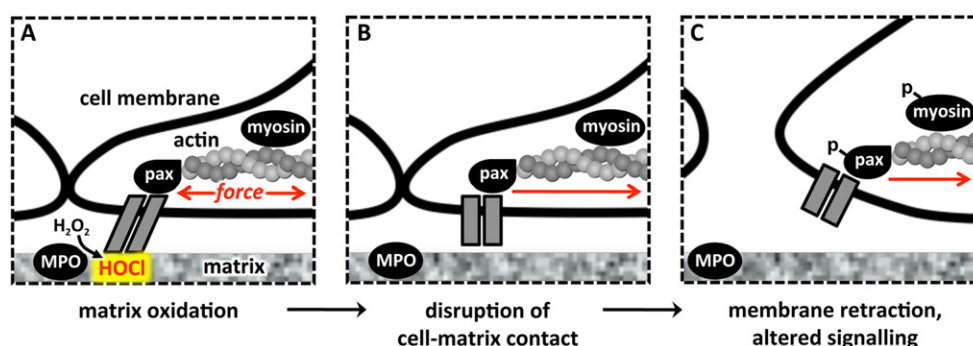
Previous studies have shown that transcytosed MPO associates basolaterally with fibronectin and locally catabolizes NO₂[−] to the nitrating radical •NO₂, conferring specificity of MPO-mediated protein tyrosine nitration to fibronectin [13]. Immunohistochemical studies support that fibronectin is an important target for MPO-derived •NO₂ in inflammatory vascular disorders, with MPO, fibronectin, and tyrosine-nitrated proteins colocalizing within diseased human vessels [23]. However, the functional implications of •NO₂ production and resultant protein nitration of fibronectin within the subendothelium by MPO are largely unknown. The current study reveals that in the presence of NO₂[−] subendothelial MPO concomitantly catalyzes HOCl-dependent cross-linking and tyrosine nitration of matrix fibronectin. However, HOCl-mediated reactions were shown to be primarily responsible for triggering EC de-adhesion and associated cell signaling events; selective scavenging of HOCl with Met markedly attenuated fibronectin cross-linking, de-adhesion, and cell signaling responses, but did not prevent fibronectin nitration.

Another biologically important MPO substrate is SCN[−], which is derived primarily from dietary sources (e.g., broccoli) and is elevated in smokers. Currently, there is considerable interest in whether diversion of MPO from HOCl production by SCN[−] to instead produce the thiol-reactive oxidant HOSCN is protective or deleterious in the context of inflammatory vascular conditions [41,42]. SCN[−] strongly suppressed HOCl production by EC-localized MPO, reflecting its action as a competitive inhibitor of HOCl production (indicated by its ability to stimulate H₂O₂ metabolism) and also likely reflecting its ability to rapidly scavenge HOCl, with both of these processes yielding HOSCN [30,43]. We found that SCN[−] protected against MPO-induced EC de-adhesion and associated signaling responses, indicating that these processes are insensitive to HOSCN production by EC-localized MPO. These findings are in line with other studies that identify a protective, antioxidant function for SCN[−] against MPO-mediated cellular injury [41].

Model studies employing purified fibronectin as the sole adhesive substrate provided strong evidence that induction of EC de-adhesion by MPO-derived HOCl involves disruption of the cell-adhesive function of fibronectin. Thus, prior oxidation of fibronectin by MPO-derived HOCl readily impaired its ability to support EC attachment and spreading, and this loss in function correlated with the capacity of HOCl, produced at the basolateral surface of ECs by fibronectin-bound MPO, to disrupt established EC adhesion on fibronectin. In accordance with their ability to inhibit HOCl-mediated fibronectin modification/cross-linking, SCN[−] and NO₂[−] suppressed MPO-induced impairment of attachment and spreading of ECs on, and de-adhesion of ECs from, fibronectin.

While our data identify a causal role for HOCl-mediated matrix oxidation in triggering EC de-adhesion, we show that actomyosin tensile forces significantly accelerate this process. Thus, the rate of de-adhesion triggered by H₂O₂ exposure in MPO-containing ECs was markedly attenuated by the inhibition of myosin II motor function with blebbistatin. Up-regulation of Rho kinase-dependent contractility contributed to the MPO-induced EC de-adhesion, but not during the initial rapid phase of membrane retraction, which preceded robust increases in Rho kinase-dependent MLC-2 phosphorylation and was not attenuated by Rho kinase inhibition.

Our data support a model where subendothelial-localized MPO catalyzes targeted, HOCl-mediated matrix oxidation that disrupts adhesive forces at the cell–matrix interface to trigger membrane retraction driven by pre-existing tensile force within the actin cytoskeleton (Scheme 1A and B). Notably, recent studies identify that targeted disruption of cell–matrix contacts by



Scheme 1. Proposed model of MPO-induced EC de-adhesion and “outside-in” redox signaling. (A) MPO binds to the subendothelial matrix and mediates targeted HOCl-dependent matrix oxidation that (B) disrupts cell–matrix contact, leading to (C) membrane retraction driven by unopposed tension in the actin cytoskeleton and alteration of adhesion-dependent signaling. pax, paxillin.

electrochemical means likewise provokes rapid cell contraction driven by preexisting actomyosin tensile stress [44].

As well as mechanically coupling the actin cytoskeleton to the subendothelial matrix, adhesive contacts are enriched in cytoplasmic signaling proteins and rapidly transduce mechanical signals provided by changes in cell–matrix adhesion into intracellular phosphorylation events. This “outside-in” signaling, in which paxillin phosphorylation plays a central regulatory role, allows cells to respond rapidly and sensitively to chemical and physical changes in the extracellular matrix and engages signaling networks that control cell shape, motility, proliferation, and survival [33–36].

We propose that the local disruption of cell–matrix contacts by HOCl in the subendothelium drives signaling changes by altering cell–matrix mechanotransduction (Scheme 1C). Paxillin Tyr-118 phosphorylation is negatively regulated by force transduction between the actin cytoskeleton and the underlying matrix at focal adhesions (i.e., cell–matrix traction) [35]. We show that F-actin and paxillin are extensively remodeled during HOCl-induced de-adhesion and the increase in paxillin Tyr-118 phosphorylation observed at higher oxidant doses (i.e., $\geq 10 \mu\text{M}$) is consistent with a global loss in cell–matrix traction. Phosphorylation of paxillin at Tyr-118 generates docking sites for SH2 domain containing proteins [34] and engages signaling networks that regulate the assembly of new adhesive contacts [35]. Notably, at lower oxidant doses, where matrix oxidation and de-adhesion were less extensive, the levels of paxillin phosphorylation were initially maintained and subsequently down-regulated, consistent with a compensatory increase in cell–matrix traction at pre-existing and/or newly formed adhesive contacts. Importantly, very low oxidant doses ($5 \mu\text{M}$) were required to induce extensive fibronectin cross-linking and changes in paxillin phosphorylation (Fig. 2A, Fig. 7C).

Another important novel aspect of this work is the finding that HOCl produced subendothelially by MPO induces Rho kinase-dependent MLC-2 diphosphorylation at Tyr-18 and Ser-19 and Src activation, as indexed by Tyr-416 phosphorylation and Tyr-527 dephosphorylation. Together with alterations in paxillin phosphorylation, MLC-2 phosphorylation and Src activation represent key signaling processes involved in adhesion responses [35,36]. The specificity of these responses to the action of MPO-derived HOCl indicates that they may be functionally linked to changes in cell–matrix mechanotransduction (see discussion below). Notably, previous studies identify that loss of cell adhesion alone can trigger increases in Src activation and paxillin phosphorylation [45,46].

Significantly, no previous study has addressed the possibility that oxidants might drive changes in cell signaling by altering cell–matrix mechanotransduction. Our proposed model of “outside-in” redox signaling (Scheme 1) differs from the conventional model of redox signaling in that it involves oxidants

modifying extracellular proteins and generating *mechanical signals* to propagate intracellular phosphorylation events instead of directly modifying and altering the activity of intracellular signaling proteins.

We do not exclude that “conventional” redox signaling mechanisms contribute to the signaling responses elicited by subendothelial-localized MPO. HOCl could contribute to the observed signaling responses by directly oxidizing critical cysteine thiol residues present on RhoA (which activates Rho kinase) and Src, leading to their activation [38,47,48], or by oxidizing active site cysteine thiols in regulatory protein phosphatases [1]. However, if the observed signaling responses were primarily driven by oxidation of intracellular protein thiols, the diversion of MPO from HOCl production by SCN^- would also be expected to be stimulatory, not inhibitory as observed, given that HOSCN is a more thiol-selective oxidant than HOCl [22]. Furthermore, kinetic considerations of oxidant reactivity support that HOCl exerts its biological actions by oxidizing targets within a very short distance of its site of production [49]. In line with this prediction, our current data identify that HOCl produced extracellularly by endothelial-transcytosed MPO mediates focal oxidation of matrix proteins within the subendothelial compartment to alter cellular adhesion and adhesion-dependent signaling. We propose that this involves HOCl-mediated impairment of the cell-adhesion (integrin- and/or heparin-binding) motifs of fibronectin. The lack of cysteine and tyrosine residues in these adhesion motifs, and hence their resistance to functional damage by HOSCN and $\cdot\text{NO}_2$, could explain why diversion of MPO from HOCl production by SCN^- and NO_2^- protects against MPO-induced de-adhesion and associated signaling responses.

Physiological redox signaling processes are increasingly recognized to be highly regulated, involving targeted, oxidative modification of specific proteins, with signaling specificity achieved through spatial association of oxidant-generating enzymes and target proteins within specific cellular compartments [1,50]. Interestingly, the alteration of endothelial signaling by subendothelial matrix-bound MPO fits within this paradigm. It is therefore tempting to speculate that the delivery of MPO to the subendothelial compartment by endothelial-transcytosis to promote HOCl-mediated matrix oxidation might serve an unforeseen regulatory function during inflammatory vascular responses. However, its potential pathophysiological significance is clear: our present findings identify that matrix oxidation resulting from the production of low, physiological fluxes of HOCl by subendothelial-localized MPO triggers a rapid loss in endothelial cell–matrix adhesion and promotes membrane retraction, events that will significantly perturb the integrity of the endothelium. These processes could play a key pathological role in inflammatory vascular conditions by promoting tissue edema and

compromising the anti-inflammatory/antithrombotic function of the endothelium by exposing intimal proteins.

Acknowledgments

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.freeradbiomed.2012.10.002>.

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