Free Radical Biology and Medicine 53 (2012) 2344–2356

Contents lists available at SciVerse ScienceDirect

Free Radical Biology and Medicine

journal homepage: www.elsevier.com/locate/freeradbiomed

Original Contribution

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

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ARTICLE INFO

Article history:
Received 21 July 2012
Received in revised form
27 September 2012
Accepted 1 October 2012
Available online 9 October 2012

Keywords:
Myeloperoxidase
Extracellular matrix
Endothelial dysfunction
Redox signaling
Free radicals

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.

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; NO_2^-, nitrite; $^{\bullet}NO_2$, 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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http://dx.doi.org/10.1016/j.freeradbiomed.2012.10.002

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 ($^{\bullet}NO_2$), 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 μg/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 M^{-1} cm^{-1}; HOCl, ε_{292} 350 M^{-1} 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 μg/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^-.