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Endothelial-Transcytosed Myeloperoxidase Activates Endothelial Nitric Oxide Synthase via a Phospholipase C-Dependent Calcium Signaling Pathway

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ABSTRACT

During vascular inflammation, the leukocyte-derived enzyme myeloperoxidase (MPO) is transcytosed across the endothelium and into the sub-endothelial extracellular matrix, where it promotes endothelial dysfunction by catalytically consuming nitric oxide (NO) produced by endothelial NO synthase (eNOS). In the presence of chloride ions and hydrogen peroxide (H_2O_2), MPO forms the oxidant hypochlorous acid (HOCl). Here we examined the short-term implications of HOCl produced by endothelial-transcytosed MPO for eNOS activity. Incubation of MPO with cultured aortic endothelial cells (ECs) resulted in its transport into the sub-endothelium. Exposure of MPO-containing ECs to low micromolar concentrations of H_2O_2 yielded enhanced rates of H_2O_2 consumption that correlated with HOCl formation and increased eNOS enzyme activity. The MPO-dependent activation of eNOS occurred despite reduced cellular uptake of the eNOS substrate L-arginine, which involved a decrease in the maximal activity (V_{max}), but not substrate affinity (K_m), of the major endothelial L-arginine transporter, cationic amino acid transporter-1. Activation of eNOS in MPO-containing ECs exposed to H_2O_2 involved a rapid elevation in cytosolic calcium and increased eNOS phosphorylation at Ser-1179 and de-phosphorylation at Thr-497. These signaling events were attenuated by intracellular calcium chelation, removal of extracellular calcium and inhibition of phospholipase C. This study shows that stimulation of endothelial-transcytosed MPO activates eNOS by promoting phospholipase C-dependent calcium signaling and altered eNOS phosphorylation at Ser-1179 and Thr-497. This may constitute a compensatory signaling response of ECs aimed at maintaining eNOS activity and NO production in the face of MPO-catalyzed oxidative stress.

Key Words: Endothelial dysfunction; endothelial nitric oxide synthase; hydrogen peroxide; hypochlorous acid; L-arginine; myeloperoxidase; nitric oxide; oxidative stress; reactive oxygen species; redox signaling.

Abbreviations: BAPTA-AM, 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid, (acetoxymethyl)ester; CAT1, cationic amino acid transporter-1; ECs, endothelial cells; eNOS, endothelial nitric oxide synthase; H_2O_2 , hydrogen peroxide; HOCl, hypochlorous acid; L-Arg, L-arginine; MPO, myeloperoxidase; NO, nitric oxide; $O_2^{\bullet-}$, superoxide anion radical; PLC, phospholipase C; PSS, physiological salt solution.

HIGHLIGHTS

- Endothelial-transcytosed MPO generates HOCl in the sub-endothelium
- MPO-derived HOCl activates eNOS despite impairing endothelial L-arginine uptake
- Activation involves PLC/Ca²⁺-induced changes in eNOS Ser¹¹⁷⁹/Thr⁴⁹⁷ phosphorylation
- This pathway preserves eNOS activity in the face of MPO-mediated oxidative stress

INTRODUCTION

The vascular endothelium plays an essential role in maintaining cardiovascular homeostasis. Key to this process is the synthesis of nitric oxide (NO) by the endothelial isoform of NO synthase (eNOS) [1, 2]. In addition to its role in regulating arterial pressure and vascular tone, eNOS-derived NO retards endothelial-leukocyte interactions, platelet activation and smooth muscle cell proliferation, thereby exhibiting anti-inflammatory and anti-atherogenic actions [2]. In endothelial cells (ECs), eNOS is a homodimer comprised of a C-terminal reductase domain linked by a calmodulin-binding site to an N-terminal oxygenase domain [2, 3]. Enzyme activation requires the flavin-mediated transport of NADPH-derived electrons through the reductase domain to the N-terminal active-site heme that catalyzes incorporation of molecular oxygen into the substrate L-arginine (L-Arg), generating L-citrulline and NO [2-5]. The activity of eNOS is controlled through various post-translational mechanisms. For example, enzyme activity is dependent on the rate of intracellular L-Arg transport, principally controlled by the cationic amino acid transporter-1 (CAT1) that resides in the plasma membrane in close proximity to eNOS [6]. Enzyme activation also occurs in response to rapid increases in intracellular calcium that promotes binding of the calcium-calmodulin complex and disengagement of an auto-inhibitory loop, thereby accelerating enzyme electron flow [7, 8]. Post-translational eNOS control also involves numerous regulatory protein-protein interactions and temporal changes to the phosphorylation status of particular amino acid residues [4, 9]. Activation of eNOS in response to a variety of stimuli commonly involves a coordinated increase in phosphorylation at Ser-1177 (human sequence) or Ser-1179 (bovine sequence) and de-phosphorylation at Thr-495 (human sequence) or Thr-497 (bovine sequence) [10-14].

Inflammatory vascular disorders including atherosclerosis, diabetes and hypertension are characterized by endothelial dysfunction that manifests as an impaired bioactivity of NO [2], with the extent of impairment an independent predictor of future clinical cardiovascular events [15-18]. Convincing evidence points to oxidative stress as an underlying cause and significant interest surrounds understanding the oxidative mechanisms at play [2, 19, 20]. Diseased blood vessels produce elevated levels of reactive oxygen species including the superoxide anion radical ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2). The rapid reaction of $O_2^{\bullet-}$ with NO to form peroxynitrite is a significant mechanism by which oxidative stress impairs NO bioactivity to cause endothelial dysfunction [2, 20]. H_2O_2 also has important implications for NO bioactivity through its ability to stimulate redox signaling pathways that modulate eNOS activity, in part, by increasing phosphorylation of the enzyme at Ser-1177/Ser-1179 and de-phosphorylation at Thr-495/Thr-497 [21, 22]. H_2O_2 also influences endothelial dysfunction by promoting oxidative reactions that can compromise NO bioactivity including elevating endothelial levels of $O_2^{\bullet-}$ and labile iron [23] or

through its role as a co-substrate for the oxidant-generating heme enzyme myeloperoxidase (MPO) [24].

MPO is primarily expressed in circulating neutrophils and monocytes, and in sub-sets of tissue macrophages [24]. Upon leukocyte activation, MPO is secreted into the phagolysosome and extracellular milieu where it uses H_2O_2 and chloride ions to catalyze the formation of hypochlorous acid (HOCl), a potent oxidant that plays a physiological role in the innate immune host defence [25]. However, a growing body of clinical and experimental evidence indicates that MPO mediates oxidative mechanisms modulating vascular NO signaling during cardiovascular disorders. For example, Vita and colleagues reported that elevated levels of circulating MPO correlated with the extent of impairment of endothelial function in coronary artery disease patients and Rudolph et al., demonstrated that MPO gene-deficiency protected healthy subjects from endothelial dysfunction induced by the intravascular administration of nicotine [26, 27]. In animal models of vascular inflammation or atherosclerosis, MPO inhibitor drugs or MPO gene-deficiency protect against impairment of NO bioactivity and resultant endothelial dysfunction [28-30].

Key to MPO promoting endothelial dysfunction is its binding to the luminal surface of the arterial endothelium and subsequent transcytosis and accumulation in the sub-endothelial extracellular matrix [28, 31-35]. At this site, MPO is positioned to catalyze focal oxidative reactions capable of altering endothelial function. Accordingly, MPO and evidence of oxidative tissue damage mediated by MPO-derived oxidants, including HOCl, is detected within the endothelium and sub-endothelium of diseased human arteries [32, 33, 36-39]. Eiserich et al. reported that exposure of endothelial-transcytosed MPO to H_2O_2 promoted the oxidative consumption of NO via the enzyme's NO oxidase function, causing impaired NO bioactivity and endothelial dysfunction [28]. Sub-endothelial MPO also uses H_2O_2 to generate HOCl within the sub-endothelium [34]. Here, we investigated the implications of endothelial-transcytosed MPO for eNOS activity and the signaling events involved.

MATERIALS & METHODS

Cell culture and treatment protocols— Bovine aortic ECs (Lonza) were cultured in flasks coated with gelatin (Sigma; 0.1% (w/v) in phosphate buffer saline [PBS]) in EBM-2 medium plus supplements as outlined by the manufacturer (Lonza). Confluent cells between passages 3-9 were used for experiments. For experiments, ECs were incubated in physiologic salt solution (PSS) (119 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 0.15 mM Na_2HPO_4 , 0.4 mM KH_2PO_4 , 5 mM $NaHCO_3$, 5.6 mM glucose, 1.5 mM $CaCl_2$, 22 mM HEPES, pH 7.4). For MPO loading, ECs in PSS containing

0.2% bovine serum albumin (BSA) were incubated with purified human neutrophil MPO (20 nM; Merck Millipore) for up to 2 h, then washed twice in PBS to remove any residual unbound MPO and incubated in PSS. Control or MPO-containing ECs were then exposed to H₂O₂ (Merck Millipore) at increasing concentrations (0-100 μ M) and incubated for 0-30 min. In some experiments, cells were pre-incubated in PSS supplemented with the pharmacological inhibitors BAPTA-AM or U73122 (Merck Millipore), or incubated in calcium-free PSS prior to H₂O₂ addition.

Western Blotting– At the end of experiments, PBS-washed ECs were lysed with SDS-loading buffer (62.5 mM Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, 50 mM dithiothreitol and 0.1% bromophenol blue). Lysates were boiled (5 min, 100°C), centrifuged (5 min, 12,000 rpm) and cellular proteins resolved by SDS-PAGE using 7% Tris-Acetate NuPAGE gels (Invitrogen). Resolved proteins were transferred onto nitrocellulose membranes using the iBlot Gel Transfer System (Invitrogen). Membranes were blocked with 5% non-fat milk in Tris-buffered saline (TBS)-Tween buffer (20 mM Tris, 137 mM NaCl, pH 7.6 and 0.1% Tween) for 30 min and probed with the relevant primary antibody (1:1000 dilution) in 5% non-fat dry milk in TBS-Tween buffer overnight at 4°C. Primary antibodies included mouse monoclonal antibodies against eNOS (BD Biosciences) and HOCl-oxidized protein (Clone 2D10G9; that does not cross-react with epitopes generated by oxidative reactions involving nitrating species, transition metals or lipid peroxidation reactions [40]) or rabbit polyclonal antibodies against MPO (Merck Millipore), tubulin (Sigma), phospho-eNOS (Ser-1177/Ser-1179) (Merck Millipore) and phospho-eNOS (Thr-495/Thr-497) (Merck Millipore). TBS-Tween washed membranes were incubated with the relevant anti-mouse or anti-rabbit horseradish peroxidase-conjugated secondary antibody (1:5000 dilution; Cell Signaling Technology) in 5% non-fat dry milk in TBS-Tween buffer for 1 h at room temperature. Membranes were washed in TBS-Tween buffer and protein bands detected using ECL Western Blotting detection reagents (Amersham Biosciences). Densitometric analysis of protein bands was performed using ImageJ software (National Institute of Health).

Immunocytochemistry– Cells cultured on glass coverslips were fixed with 4% para-formaldehyde (22 °C, 20 min), free aldehydes quenched with NH₄Cl (50 mM) for 5 min and then washed with PBS. Fixed cells were probed with a rabbit polyclonal antibody against MPO (1:300; Merck Millipore) or a mouse monoclonal antibody against fibronectin (1:100; Clone E3E, Merck Millipore), followed by the relevant labelled secondary antibody; i.e., cyanine 2-conjugated goat anti-mouse (green) or cyanine 3-conjugated donkey anti-rabbit (red) antibodies. Cell nuclei were stained with DAPI (1:2000). After washing with PBS, cells were mounted in Elvanol (Mowiol 4–88, Hoechst). Confocal fluorescent images were recorded at room temperature using a 63x oil

objective on a Leica TCS SP5 microscope. Reconstructions of confocal images were performed using Imaris software.

Measurement of H₂O₂– Cellular metabolism of exogenous H₂O₂ added to ECs was measured using the Amplex Red H₂O₂/peroxidase assay kit (Invitrogen). Briefly, following H₂O₂ addition to confluent ECs in PSS, 50 µL aliquots of buffer were collected at the indicated times and transferred into a 96-well plate containing 50 µL of Amplex Red reaction mix prepared according to the manufacturer's instructions. The concentrations of H₂O₂ were then quantified using a fluorescence microplate reader with 544 nm excitation and 590 nm emission.

Measurement of cellular L-Arg uptake and eNOS activity– The enzyme activity of eNOS was quantified by measuring the conversion of L-[³H]-Arg into L-[³H]-citrulline in ECs as previously described [21]. Briefly, confluent control or MPO-containing ECs in 6-well plates were exposed to 10 µM L-Arg and 3.3 µCi L-[³H]-Arg (Perkin Elmer) that was immediately followed by the addition of H₂O₂ and incubation of the cells for a further 10 min. ECs were then washed 3x with PBS, lysed with 200 µL of 100% ethanol and then mixed with 2 mL of ice-cold stop buffer (20 mM sodium acetate, pH 5.5, 1 mM L-citrulline, 2 mM EDTA, 2 mM EGTA). The cellular uptake of [³H]-L-Arg was measured by collecting 20 µL of the lysate followed by liquid scintillation counting. The remaining lysate was subjected to anion exchange chromatography using Dowex AG50W-X8 resin columns (Bio-Rad) for the determination of L-[³H]-citrulline as previously described [21]. To study L-Arg uptake kinetics, confluent control or MPO-containing ECs in 12-well plates that were non-treated or H₂O₂-treated for 30 mins were washed and incubated in PSS containing 2.5 µCi/mL [³H]-L-Arg (Perkin-Elmer) and varying concentrations of cold L-Arg (0 – 200 µM) for 5 min before washing and measurement of [³H]-L-Arg in EC lysates by liquid scintillation counting. L-Arg kinetic analyses were performed as previously described [41].

Measurement of cellular calcium– To measure intracellular calcium transients, ECs were grown on 0.1% gelatin-coated 24-well glass-bottom culture plates (MatTek Corporation) until confluency. After MPO loading, confluent ECs were incubated in PSS containing the calcium-sensitive dye Fura-2-AM (5 µM; Thermo Fischer Scientific) and Pluronic F-127 (5 mg/mL; Sigma Aldrich) for 30 min. Cells were washed with warm PBS and replaced with fresh PSS, then left in the microscope chamber (maintained at 37 °C, 5% CO₂) for 90 s to equilibrate before the addition of H₂O₂. Fluorescent images were taken every 5 s at 510 nm emission with alternating 340 nm and 380 nm excitation using a Zeiss SD/TIRF microscope (Zeiss Axio Observer, 63x/1.3 Water/Glycerol objective, and 21 HE FURA filter). Intracellular calcium was determined by the ratiometric analysis of F340/F380 images of 3-5 individual cells on a pixel-by-pixel basis using ImageJ software.

Statistics— All numerical data are expressed as the mean $\pm$ SEM of three or more independent experiments. Western blots shown are representative of three or more independent experiments. Statistical differences in column graphs were assessed using Student t-test for the comparison of 2 different treatments or one-way ANOVA with the Bonferroni's *post-hoc* test for more than 2 treatments. A two-way ANOVA was performed for statistical analysis of 2 different curves or for grouped treatments. Statistical analyses were performed using Prism 6 software. A *p*-value <0.05 was considered statistically significant.

RESULTS

Catalytically active MPO accumulates within the sub-endothelial space of confluent arterial ECs— Incubation of confluent ECs with purified human neutrophil MPO resulted in a time-dependent accumulation of the enzyme, as judged by Western blotting (Fig. 1A). Cellular incorporation of MPO was detected within 5 min and maximal after 2 h (Fig. 1A). Immunofluorescence confocal microscopy showed that after 2 h incubation, the majority of MPO was located within the basolateral or sub-endothelial compartment with some immunoreactivity observed intracellularly but none apparent at the apical surface (Fig. 1B). Within the basolateral compartment, MPO co-localized with the sub-endothelial matrix protein fibronectin (Fig. 1B & C). MPO metabolizes H_2O_2 to form compound I, which oxidizes Cl^- ions to generate HOCl [42]. Endothelial-transcytosed MPO was active and capable of producing HOCl. Thus, MPO-containing ECs exhibited a >3 -fold enhanced rate of H_2O_2 consumption relative to control ECs (Fig. 2A). The enhanced H_2O_2 consumption in MPO-containing ECs coincided with HOCl production, measured as the increased formation of high-molecular weight immuno-reactive material detected by a monoclonal antibody specific for HOCl-oxidized proteins [37, 40, 43] (Fig. 2B).

H_2O_2 treatment activates eNOS in MPO-containing ECs despite impairing L-Arg uptake— We next examined the implications of endothelial-transcytosed MPO for eNOS activity in the absence or presence of H_2O_2 . Addition of 50 μM H_2O_2 to MPO-containing ECs induced an approximately 2-fold increase in eNOS activity compared to control ECs, ECs treated with H_2O_2 alone or ECs containing MPO alone (Fig. 3A). The magnitude of the MPO/ H_2O_2 -induced increase in eNOS activity was similar to that obtained by EC-treatment with the calcium ionophore A23187 (Fig. 3A), which is commonly employed to elicit robust eNOS activation in ECs. Enzyme activity of eNOS was significantly elevated in MPO-containing ECs exposed to H_2O_2 despite an approximately 50% decrease in EC uptake of L-Arg, a change that was not apparent in cells incubated with MPO or H_2O_2 alone (Fig. 3B). When EC levels of L-citrulline produced were normalized to the amount of EC L-Arg uptake, eNOS activation afforded by the exposure of MPO-containing ECs to H_2O_2 was

approximately 3.5-fold greater than that exhibited by control ECs (Fig. 3C). These findings show that despite inhibiting the intracellular transport of L-Arg, activation of endothelial-transcytosed MPO, by H₂O₂ addition, stimulates eNOS activity in ECs.

Sub-endothelial MPO impairs the rate of EC L-Arg transport– As MPO in the presence of H₂O₂ impaired EC L-Arg uptake, we next analyzed the amino acids transport kinetics. In ECs, CAT1 is the principal membrane-bound transporter controlling EC L-Arg uptake and eNOS activity [6, 44]. Consistent with this, EC L-Arg uptake was strongly inhibited by L-lysine (Fig. 4A), a competitive CAT-1 inhibitor [41, 45]. Analysis of the L-Arg transport kinetics showed that the presence of MPO significantly inhibited the maximal L-Arg transport rate in ECs exposed to H₂O₂ (Fig. 4B). A corresponding Eadie-Hofstee plot analysis (Fig. 4C & D) showed that in the presence of MPO/H₂O₂ the *V*_{max} value was significantly reduced by >50% versus ECs treated with H₂O₂ alone (Fig. 4E). No significant change was noted for the affinity of L-Arg for its transporter (*K*_m value) (Fig. 4F).

H₂O₂ induces rapid eNOS phosphorylation changes in MPO-containing ECs– We and others have previously reported that activation of eNOS by H₂O₂ at concentrations ≥100 μM involved an increase in phosphorylation at Ser-1177/Ser-1179 with a concomitant de-phosphorylation of Thr-495/Thr-497 [21-23, 46]. We next examined the implications of endothelial-transcytosed MPO for eNOS phosphorylation at these two key regulatory amino acid sites. While treatment of ECs with 50 μM H₂O₂ alone caused minor changes to the phosphorylation status of eNOS at Ser-1179 (bovine) and Thr-497 (bovine), addition of H₂O₂ to MPO-containing ECs induced a time-dependent increase in eNOS phosphorylation at Ser-1179 and de-phosphorylation at Thr-497 (Fig. 5). Increases in eNOS Ser-1177 phosphorylation or Thr-495 de-phosphorylation in MPO-containing ECs were rapid and maximal over 2-5 min after H₂O₂ addition and returned to baseline over 10-30 min (Fig. 5). Significantly increased eNOS Ser-1179 phosphorylation and Thr-495 de-phosphorylation was apparent in MPO-containing ECs exposed to 10-50 μM of H₂O₂ versus ECs treated with H₂O₂ alone (Fig. 6). This data shows that sub-endothelial MPO augments the ability of low micromolar levels of H₂O₂ to alter eNOS phosphorylation.

Treatment of MPO-containing ECs with H₂O₂ increases intracellular calcium– Increases in cytosolic calcium is a key signaling event necessary for maximal eNOS activation by stimulating the binding of calmodulin to the enzyme [4, 7]. We therefore next measured the implications of MPO for intracellular calcium transients. In MPO-containing ECs, H₂O₂ addition rapidly increased

cytosolic calcium levels (Fig. 7A-C). In contrast, no detectable changes were evident in ECs treated with H₂O₂ alone. Quantification of the peak intracellular calcium response indicated that exposure of MPO-containing ECs to 50 μ M H₂O₂ promoted an approximately 10-fold increase in intracellular calcium compared with H₂O₂ treatment alone (Fig. 7C). The ability of H₂O₂ to elevate cytosolic calcium levels in MPO-containing ECs (Fig. 7D) was inhibited by the removal of extracellular calcium (Fig. 7E). In MPO-containing ECs initially treated with H₂O₂ in the absence of extracellular calcium, which evoked a rapid and small transient increase in cytosolic calcium (Fig. 7E & F), subsequent addition of extracellular calcium generated an immediate and larger secondary increase in intracellular calcium levels (Fig. 7F). Together, this data indicates that H₂O₂ treatment of MPO-containing ECs rapidly increases intracellular calcium levels via stimulation of capacitative calcium entry from the extracellular environment [47].

MPO-induced changes to eNOS phosphorylation require phospholipase C (PLC)-dependent calcium signaling— Induction of eNOS phosphorylation at Ser-1177/Ser-1179 by several stimuli depends on calcium signaling [13, 14, 21]. Chelation of intracellular calcium with BAPTA-AM (Fig. 8A & B) or removal of extracellular calcium (Fig. 8C & D) both inhibited the MPO/H₂O₂-dependent increase in eNOS phosphorylation at Ser-1179. A principal regulator of calcium signaling is PLC, which converts phosphatidylinositol 4,5-bisphosphate (PIP₂) to inositol 1,4,5-trisphosphate (IP₃) that promotes intracellular calcium transients [48]. Treatment of MPO-containing ECs with the selective PLC inhibitor U73122 abrogated the increase in cytosolic calcium induced by H₂O₂ addition (Fig. 9A & B). PLC inhibition with U73122 also significantly inhibited the MPO/H₂O₂-dependent increases in eNOS phosphorylation at Ser-1179 and de-phosphorylation at Thr-497 (Fig. 9C-E). These results support a role for PLC-dependent calcium signaling in the H₂O₂-induced changes in eNOS phosphorylation that occur in MPO-containing ECs.

DISCUSSION

Inflammatory vascular disease is characterized by the accumulation of leukocyte-derived MPO within the sub-endothelium where it promotes endothelial dysfunction by catalyzing local oxidative reactions [28, 32-36]. However, the implications of the oxidative reactions catalyzed by sub-endothelial MPO for EC signaling and function remain largely unexplored. A major finding of this study is that the addition of H₂O₂ to confluent aortic ECs containing sub-endothelial MPO elicits HOCl formation and the rapid stimulation of eNOS enzyme activity, despite inhibiting EC uptake of the eNOS substrate L-Arg. The MPO-dependent activation of eNOS involved the PLC-

dependent increase in intracellular calcium levels that signaled for the coordinated changes in eNOS phosphorylation status at Ser-1179 and Thr-497. Together, this study establishes that sub-endothelial MPO acutely activates eNOS by stimulating PLC-dependent calcium signaling, which may represent a compensatory redox cell signaling pathway aimed at preserving NO bioactivity in the face of MPO-catalyzed oxidative stress (Fig. 10).

To study the implications of sub-endothelial MPO for eNOS we incubated confluent cultures of arterial ECs with low nanomolar concentrations of purified human MPO and in agreement with prior work [33, 34], demonstrated that the enzyme is rapidly transported into the basolateral or sub-endothelial space. The sub-endothelial enzyme deposits remained catalytically active as MPO-containing ECs more avidly consumed H_2O_2 concomitant with the generation of HOCl, as indicated by the formation of high molecular weight HOCl-oxidized proteins. Our recent work [34] identified that this high molecular weight material recognized by a well-characterized monoclonal antibody specific for HOCl-oxidized proteins [37, 40, 43] constitutes cross-linked aggregates of sub-endothelial matrix proteins, including fibronectin. The present study links the stimulation of HOCl generation by sub-endothelial MPO with activation of PLC-dependent calcium signaling and resultant activation of eNOS.

Activation of eNOS after stimulation of sub-endothelial MPO by H_2O_2 addition involved a coordinated increase in eNOS phosphorylation at Ser-1179 and de-phosphorylation at Thr-497 in a H_2O_2 time- and dose-dependent manner. These coordinated changes in eNOS phosphorylation promote enzyme electron flow and activation [9] and have been observed for ECs exposed to various stimuli including bradykinin, VEGF, shear stress and H_2O_2 [12-14, 21]. For H_2O_2 to promote robust changes in eNOS phosphorylation at these amino acid sites requires exposure of ECs to concentrations $>100 \mu\text{M}$, with the changes maximal over 15-45 mins [21, 23]. Here we demonstrate that in the presence of endothelial-localized MPO, as little as $10 \mu\text{M}$ H_2O_2 was sufficient to rapidly alter eNOS phosphorylation that was maximal at 2-5 mins after oxidant addition. Therefore, the catalytic conversion of low concentrations of H_2O_2 into HOCl by transcytosed-MPO significantly augments the capacity of H_2O_2 to coordinate changes in eNOS phosphorylation at Ser-1179 and Thr-497 and stimulate enzyme activity.

Rapid increases in intracellular calcium also contribute to eNOS activation by stimulating the binding of the calcium-calmodulin complex to the enzyme's calmodulin-binding domain causing disruption of an auto-inhibitory loop to facilitate electron transport [7, 8, 49]. In the presence of transcytosed-MPO, addition of H_2O_2 rapidly increased intracellular calcium flux that was not apparent with ECs exposed to H_2O_2 alone. This rapid MPO-dependent increase in cytosolic calcium was dependent on PLC activity and the intracellular influx of calcium from the extracellular medium as it was abrogated by PLC inhibition with U73122 or removal of calcium from the

medium. In the absence of extracellular calcium, a small and transient increase in cytosolic calcium was apparent in MPO-containing ECs upon H₂O₂ addition, which likely constitutes the PLC-dependent transient release of calcium from internal endoplasmic reticulum stores. Notably, re-addition of calcium to the extracellular medium generated a larger sustained elevation in intracellular calcium. Together, these findings are consistent with MPO-dependent activation of capacitative calcium entry that involves an initial PLC-dependent release of calcium from endoplasmic reticulum stores, which triggers the subsequent influx of extracellular calcium across the plasma membrane into the cytosol [47]. Earlier reports demonstrated that exposure of ECs to H₂O₂ increased intracellular calcium levels by activating PLC-dependent signaling and the resultant influx of extracellular calcium [50-52]. This study therefore supports that the conversion of H₂O₂ into HOCl by endothelial-transcytosed MPO significantly augments PLC-dependent capacitative calcium signaling responses induced by addition of H₂O₂. The detailed molecular mechanisms by which endothelial-transcytosed MPO triggers endothelial calcium signaling requires more detailed investigations.

Our findings that HOCl produced by endothelial-transcytosed MPO promotes PLC-dependent elevation in intracellular calcium and acute activation of eNOS differs from prior studies reporting that exogenous exposure of ECs to reagent HOCl increases intracellular calcium levels independent of extracellular calcium by oxidatively inactivating the sarco/endoplasmic reticulum Ca²⁺-ATPase activity [53] or promotes eNOS uncoupling by oxidizing a zinc-thiolate cluster in the enzyme [54, 55]. Reasons for these differences are unknown but may reflect differences in the cellular sites with which HOCl reacts; *i.e.*, due to its high reactivity [56] HOCl added exogenously as a bolus of reagent oxidant would be expected to principally react at the apical plasma membrane surface of ECs. In contrast, HOCl produced by endothelial-transcytosed MPO reacts primarily within the sub-endothelial matrix where MPO accumulates [34].

Endothelial calcium signaling is reported to serve as an upstream signal for the phosphorylation of eNOS at Ser-1177/Ser-1179 in response to several stimuli including bradykinin, VEGF, polyphenols and H₂O₂ [12, 13, 21, 23, 57, 58]. Our data show that PLC-dependent calcium signaling is important for the H₂O₂/MPO-dependent increase in eNOS Ser-1179 phosphorylation as chelation of intracellular calcium with BAPTA-AM, incubation of ECs in the absence of extracellular calcium or PLC inhibition with U73122, all inhibited eNOS Ser-1179 phosphorylation. An involvement of PLC-dependent calcium signaling was also apparent for the MPO-dependent de-phosphorylation of eNOS at Thr-497 as U73122 also significantly inhibited the decreased Thr-497 phosphorylation in MPO-containing ECs exposed to H₂O₂. Interestingly, PLC inhibition with U73122 also elevated basal eNOS phosphorylation at Thr-497, findings consistent with a prior

report of an involvement of the calcium-dependent phosphatase calcineurin in the dephosphorylation of eNOS at this amino acid under basal conditions in bovine aortic ECs [12].

While our data identify PLC-dependent calcium signaling as an upstream event necessary for the MPO-mediated changes in eNOS phosphorylation status, the identity of the PLC isoforms involved remain unknown. The phosphoinositide-specific PLC enzyme family consists of 13 isoforms that are categorized into 6 families; i.e., β , γ , δ , ϵ , ζ and η [59, 60]. Depending on the vascular bed, ECs express 8 or more PLC isoforms [61, 62] and further studies are required to identify the respective PLC isoform(s) involved in MPO-dependent signaling in ECs. Similarly, the downstream protein kinase(s) or phosphatase(s) responsible for the MPO-induced changes in eNOS phosphorylation at Ser-1179 and Thr-497 are unknown. Our prior work showed that exposure of ECs to H_2O_2 at concentrations $>100\ \mu M$ alone increased eNOS phosphorylation at Ser-1177 and dephosphorylation at Thr-495 in a PI3K- and calcium-dependent manner, with the phosphorylation at Ser-1177 mediated by Akt [21]. However, our preliminary studies testing selective inhibitors of the PI3K/Akt pathway and other putative kinases (AMPK, CaMKII, PKC) or phosphatases (PP1, PP2A or calcineurin) [4] do not support their role in the MPO-induced changes in eNOS phosphorylation status at Ser-1179 or Thr-497. This indicates that the redox signaling pathways responsible for eNOS activation by sub-endothelial MPO differs from those elicited by H_2O_2 alone. As such, more detailed studies are necessary to identify the kinases and phosphatases primarily responsible for the MPO-dependent changes in eNOS phosphorylation.

While our data show that oxidative reactions catalyzed by transcytosed MPO promote redox cell signaling events that modulate eNOS activity it is also plausible that MPO could impact on eNOS in other ways. For example, albumin-bound MPO is transcytosed via caveolae-mediated endocytosis [63], an active transport mechanism shown to acutely control eNOS activity [64]. Therefore, it is plausible that the process of MPO transcytosis itself may modulate eNOS activity in real-time. As endothelial cavolin-1/caveolae levels are reduced during vascular inflammation [65], it is possible that this also alters MPO transcytosis and its implications for eNOS and NO bioactivity.

A significant finding was that exposure of MPO-containing ECs to low micromolar concentrations of H_2O_2 elevated eNOS activity despite a significant reduction in endothelial L-Arg uptake. Our kinetic analysis support that HOCl produced by endothelial-transcytosed MPO directly impairs L-Arg uptake principally by reducing the rate of CAT1-mediated transport (V_{max}), but not the affinity of L-Arg (K_m) for CAT1. This finding is similar to Venardos *et al.* reporting that peroxynitrite attenuated endothelial L-Arg uptake by reducing the V_{max} , but not the K_m , of the CAT1 transporter [41]. The molecular and oxidative mechanisms by which MPO-derived HOCl (the current study) or peroxynitrite [41] inhibits CAT1 warrant further investigations.

Impairment of CAT1-mediated L-Arg transport contributes to endothelial dysfunction during cardiovascular disease [66-68]. Our data therefore support the concept that the generation of HOCl and inhibition of CAT-1-mediated L-Arg transport represents another mechanism by which sub-endothelial MPO can mediate endothelial dysfunction. Prior studies have linked MPO to the impairment of L-Arg bioavailability in other ways. For example, Zhang et al. showed that HOCl chlorinates L-Arg to form competitive eNOS inhibitors [69]. In further work, von Leitner and colleagues reported that MPO-derived HOCl also inhibits the activity of dimethylarginine dimethylaminohydrolase (DDAH-1) causing a build-up of asymmetric dimethylarginine (ADMA) [70], a competitive substrate with L-Arg for binding to CAT-1 and endogenous inhibitor of NOS enzymes [71, 72]. Together with the results presented here it is clear that MPO-derived HOCl can adversely impact on L-Arg bioavailability, and hence eNOS activity, through its actions on L-Arg uptake, L-Arg oxidation and inhibition of DDAH-1.

Collectively, we demonstrate for the first time that despite impairing EC L-Arg uptake, activation of endothelial-transcytosed MPO acutely stimulates eNOS activity by signaling for PLC-dependent increases in intracellular calcium and the resultant reciprocal changes in the phosphorylation status of eNOS at Ser-1179 and Thr-497. We envisage that this redox signaling pathway represents an initial compensatory response of the endothelium to bolster or preserve NO production in the face of MPO-catalyzed oxidative stress. This protective response is likely, however, to be futile if MPO continues to catalyze oxidative reactions for prolonged periods within the sub-endothelium, including MPO's NO oxidase function that can directly scavenge endothelial-derived NO [28, 73] and the ongoing formation of HOCl, which can inhibit EC L-Arg uptake, and at higher concentrations, promote endothelial dysfunction by inducing eNOS uncoupling [54, 55] or oxidizing soluble guanylate cyclase [30].

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FIGURE LEGENDS

Figure 1. MPO is taken up by ECs in a time-dependent manner and accumulates within the sub-endothelium. (A) Confluent ECs were incubated with MPO (20 nM) for the indicated times (0-120 min) and cellular levels of eNOS and MPO assessed in cell lysates by Western Blotting. (B, C) Confluent ECs on coverslips were incubated with MPO (20 nM) for 2 h, washed and then fixed with paraformaldehyde and immunostained for MPO (red) or fibronectin (green) by laser confocal fluorescence microscopy. Serial images were taken along the z-plane using the z-stack feature included in the microscopy software. Images were processed using ImageJ, where the apical, intracellular and basolateral planes were determined by referencing the DAPI-stained cell nuclei (blue).

Figure 2. Endothelial-transcytosed MPO enhances H₂O₂ metabolism and HOCl production.

(A) ECs were incubated in the absence (Control) or presence of MPO (20 nM) for 2 h, washed and then exposed to H₂O₂ (50 µM). At the indicated times, H₂O₂ concentration in supernatants was

quantified by the Amplex Red assay. Data represent the mean $\pm$ SEM of $n=4$ independent experiments; **** $p<0.0001$ comparing curves using a two-way ANOVA. (B) MPO-containing ECs were non-treated or treated with H_2O_2 (50 or 100 μM) for 30 min. Cell lysates were analyzed for HOCl-oxidized protein or tubulin by Western Blotting, using a monoclonal antibody specific for HOCl-oxidized proteins (clone 2D10G9) or an anti-tubulin antibody, respectively.

Figure 3. H_2O_2 activates eNOS in MPO-containing ECs, despite impairing L-Arg uptake. ECs were incubated in the absence (control, CTL) or presence of MPO (20 nM) for 2 h, washed and then non-treated or treated with H_2O_2 (50 μM) for 10 min in buffer containing 10 μM L-Arg and 3.3 $\mu Ci/mL$ [3H]-L-Arg. ECs were also stimulated with A23187 (1 μM) for 10 min. (A) eNOS catalytic activity was indexed as the conversion of [3H]-L-Arg into [3H]-L-Citrulline by ECs and expressed as % control values. (B) L-Arg uptake was indexed as the total radiolabel in EC lysates and expressed as % control. (C) EC L-Citrulline levels in (A) were normalized to the corresponding L-Arg uptake levels in (B) and expressed as % control. Data are the mean $\pm$ SEM, $n=3$ independent experiments; * $p<0.05$, ** $p<0.01$ vs. MPO/ H_2O_2 (one-way ANOVA with Bonferroni's post-hoc test), ns (non-significant).

Figure 4. MPO impairs the rate of EC L-Arg transport. (A) ECs were incubated in the absence (control, CTL) or presence of L-lysine (Lysine, 1 mM) prior to addition of a cocktail of 10 μM L-Arg and 2.5 $\mu Ci/mL$ [3H]-L-Arg and incubation for the indicated times. ECs were washed, harvested and the levels of intracellular [3H]-L-Arg quantified. Data are the mean DPM $\pm$ SEM, $n=3$, * $p<0.05$ comparing curves with a two-way ANOVA. (B-F) ECs were incubated in the absence or presence of MPO (20 nM) for 2 h, washed and then treated with H_2O_2 (50 μM) for 30 min. ECs were then washed and incubated in buffer containing 2.5 $\mu Ci/mL$ [3H]-L-Arg (or 50 nM) and varying concentrations of cold L-Arg (0-200 μM) for 5 min. (C & D) Eadie-Hofstee plots were generated using linear regression transformation. (E & F) V_{max} and K_m were determined using the Michaelis-Menten equation. Data are the mean $\pm$ SEM, $n=3$, ** $p<0.01$ comparing curves using a two-way ANOVA or (B) comparing columns using a student t-test (E & F).

Figure 5. H_2O_2 induces time-dependent changes in eNOS phosphorylation in MPO-containing ECs. ECs were incubated in the absence or presence of MPO (20 nM) for 2 h, washed and treated with H_2O_2 (50 μM) for the indicated times. Cell lysates were analyzed for eNOS phosphorylation status at Ser-1179 or Thr-497 and total eNOS by Western Blotting. Note: the immunoblots for p-eNOS (Ser-1179) and p-eNOS (Thr-497) are derived from the same gel and nitrocellulose membrane, initially probed for p-eNOS (Ser-1179), stripped, and then re-probed for p-eNOS (Thr-

497). The eNOS blot shown represents the identical test samples run at the same time on a separate gel and nitrocellulose membrane, which was probed for total eNOS. The band intensities of eNOS phosphorylation at (B) Ser-1179 and (C) Thr-497 were quantified by densitometry using ImageJ and expressed as a % of untreated control ECs or ECs containing MPO alone. Data are mean $\pm$ SEM, n=3 independent experiments; *** p <0.001, **** p <0.0001 comparing curves using a two-way ANOVA.

Figure 6. H₂O₂ dose-dependently alters eNOS phosphorylation in MPO-containing ECs. (A) ECs were incubated in the absence or presence of MPO (20 nM) for 2 h, washed and then exposed to varying H₂O₂ concentrations (0-50 μ M) for 5 min. Cell lysates were analyzed for eNOS phosphorylation status at Ser-1179 and Thr-497 or total eNOS by Western Blotting. Note: the immunoblots for p-eNOS (Thr-497) and eNOS are from the same gel and nitrocellulose membrane, initially probed for p-eNOS Thr-497, stripped, and probed for total eNOS. The p-eNOS (Ser-1179) blot shown represents the identical test samples run at the same time on a separate gel and nitrocellulose membrane, which was probed for p-eNOS (Ser-1179). The band intensities of eNOS phosphorylation at (B) Ser-1179 or (C) Thr-497 were quantified by densitometry using ImageJ and expressed as a % of untreated control ECs or ECs containing MPO alone. Data are the mean $\pm$ SEM from immunoblots of n=3 independent experiments, * p <0.05, **** p <0.0001 by two-way ANOVA.

Figure 7. Treatment of MPO-containing ECs with H₂O₂ increases intracellular calcium transients. Confluent ECs were incubated in the absence or presence of MPO (20 nM) for 2 h, washed and loaded with Fura2-AM (5 μ M) for 30 min. Basal EC calcium levels were initially recorded for 90 s before addition of H₂O₂ (50 μ M). Fluorescent images were taken every 5 s at 510 nm emission with alternating 340 nm and 380 nm excitation using a Zeiss SD/TIRF microscope. Intracellular calcium was quantified by the ratio-metric analysis of F340/F380 images. (A) Fluorescent images of Fura2-loaded control or MPO-containing ECs before (baseline) or after H₂O₂ treatment (H₂O₂). (B) Time-dependent change in Fura2 fluorescence in control or MPO-containing ECs before or after H₂O₂ treatment, which was added at 1.5 mins. Data is representative of n=4 independent experiments. (C) The peak increase in F340/F380 values above basal levels was calculated in ECs treated with H₂O₂ alone (H₂O₂) and MPO-containing ECs treated with H₂O₂ (MPO+H₂O₂). Data are the mean $\pm$ SEM, n=4 independent experiments; ** p <0.01 Student t-test. (D-F) Fura-2-loaded MPO-containing ECs incubated in calcium-free buffer were: (D) stimulated with H₂O₂ (50 μ M) 1 min after the re-addition of calcium (Ca²⁺, 1.5 mM), (E) treated with H₂O₂ in the absence of extracellular calcium, or (F) treated with H₂O₂ in the absence of extracellular

calcium for 3 min before the re-addition of calcium (Ca^{2+} , 1.5 mM) and incubation for a further 2 min. Data shown is representative of n=3 independent experiments.

Figure 8. MPO-induced eNOS phosphorylation at Ser-1179 is calcium-dependent. (A) ECs were incubated with MPO (20 nM) for 2 h, washed and pre-treated with or without the intracellular calcium-chelator BAPTA-AM (10 μM) for 30 min. Cells were then treated with H_2O_2 (50 μM) for the indicated times. Cell lysates were assessed for eNOS Ser-1179 phosphorylation or total eNOS by Western Blot. (C) ECs were incubated with MPO (20 nM) for 2 h, washed and replaced with buffer lacking ($-\text{Ca}^{2+}$) or containing ($+\text{Ca}^{2+}$) 1.5 mM calcium prior to the treatment with H_2O_2 (50 μM) for 2 or 5 mins. Cell lysates were assessed for eNOS Ser-1177 phosphorylation or total eNOS by Western Blot. Note: immunoblots in (A) or (C) of p-eNOS (Ser-1179) and eNOS are from the same gel and nitrocellulose membrane, initially probed for p-eNOS (Ser-1179), stripped, and re-probed for eNOS. (B & D) The band intensities of eNOS phosphorylation at Ser-1179 were quantified by densitometry using ImageJ and expressed as % untreated control ECs. Data are the mean $\pm$ SEM from n=3 independent experiments; * $p<0.05$, ** $p<0.01$ using a two-way ANOVA.

Figure 9. PLC inhibition attenuates MPO-dependent calcium signaling and alterations in eNOS phosphorylation. (A, B) ECs were incubated with 20 nM MPO for 2 h, washed and loaded with 5 μM Fura-2-AM for 30 min. Cells were incubated in the absence (Control, CTL) or presence of U73122 (2.5 μM) for 5 min prior to addition of 50 μM H_2O_2 and calcium imaging. Fluorescent images were taken every 5 s at 510 nm emission with alternating 340/380 nm excitation. Intracellular calcium was quantified by the ratio-metric analysis of F340/F380 images. Data in (A) are representative of n=3 independent experiments and in (B) the mean $\pm$ SEM of n=3 independent experiments; * $p<0.05$. (C-E) ECs were incubated with 20 nM MPO for 2 h, washed and pre-treated with or without U73122 (1 μM) for 5 min before H_2O_2 (50 μM) treatment for the indicated times. Cell lysates were assessed for eNOS phosphorylation at Ser-1179 or Thr-497 and total eNOS by Western Blotting. Note the immunoblots for p-eNOS (Ser-1179) and eNOS are from the same gel and nitrocellulose membrane that was initially probed for p-eNOS (Ser-1179), stripped, and re-probed for total eNOS. The p-eNOS (Thr-497) blot shown is the identical test samples run at the same time on a separate gel and the nitrocellulose membrane probed for p-eNOS (Thr-497). (D, E) The band intensities of eNOS phosphorylation at Ser-1179 or Thr-497 were quantified by densitometry using ImageJ and expressed as % untreated control ECs or ECs containing MPO alone. Data are the mean $\pm$ SEM from immunoblots of n=3 independent experiments; * $p<0.05$, ** $p<0.01$, **** $p<0.0001$ using a two-way ANOVA.

Figure 10. Implications of endothelial-transcytosed MPO for eNOS. (A) During heightened vascular inflammation increased circulating levels of leukocyte derived MPO are transcytosed through endothelial cells and into the sub-endothelial extracellular matrix (ECM). (B) In the presence of H_2O_2 , endothelial-localized MPO catalyzes the formation of HOCl that (C) impairs the cellular uptake of the eNOS substrate, L-arginine (L-Arg), via the cationic amino acid transporter-1 (CAT-1). In parallel, MPO-derived HOCl also (D) activates phospholipase C (PLC)-dependent increases in intracellular calcium, sourced from (E) the extracellular environment. (F) This increased intracellular calcium activates yet-to-be defined protein kinases/phosphatases that afford increases in eNOS phosphorylation at Ser-1179 and de-phosphorylation at Thr-497, (G) resulting in an increase in eNOS activity and NO production. (H) The MPO-dependent increase in NO may, however, not translate into increased NO bioactivity as MPO in the presence of H_2O_2 can catalytically consume NO via its NO oxidase activity. Note: this summary scheme does not accurately illustrate the specific sub-cellular locations at which the different oxidative and cell signaling events occur, the nature of which require further investigation.

HIGHLIGHTS

- Endothelial-transcytosed MPO generates HOCl in the sub-endothelium
- MPO-derived HOCl activates eNOS despite impairing endothelial L-arginine uptake
- Activation involves PLC/Ca²⁺-induced changes in eNOS Ser¹¹⁷⁹/Thr⁴⁹⁷ phosphorylation
- This pathway preserves eNOS activity in the face of MPO-mediated oxidative stress

GRAPHICAL ABSTRACT

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