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Myeloperoxidase promotes tube formation, triggers ERK1/2 and Akt pathways and is expressed endogenously in endothelial cells

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ABSTRACT

Myeloperoxidase is a member of the mammalian peroxidase family, mainly expressed in the myeloblastic cell lineage. It is considered a major bactericidal agent as it is released in the phagosome where it catalyzes the formation of reactive oxygen species. It is also released in the extracellular spaces including blood where it is absorbed on (lipo)proteins and endothelial cell surface, interfering with endothelial function. We performed RNA sequencing on MPO-treated endothelial cells, analyzed their transcriptome and validated the profile of gene expression by individual qRT-PCR. Some of the induced genes could be grouped in several functional networks, including tubulogenesis, angiogenesis, and blood vessel morphogenesis and development as well as signal transduction pathways associated to these mechanisms. MPO treatment mimicked the effects of VEGF on several signal transduction pathways, such as Akt, ERK or FAK involved in angiogenesis. Accordingly MPO, independently of its enzymatic activity, stimulated tube formation by endothelial cells. RNA interference also pointed at a role of endogenous MPO in tubulogenesis and endothelium wound repair *in vitro*. These data suggest that MPO, whether from endogenous or exogenous sources, could play a role in angiogenesis and vascular repair *in vivo*.

1. Introduction

Myeloperoxidase is a member of the mammalian heme peroxidase family. It catalyzes the oxidation of halides (bromide, iodide, and chloride) and the pseudohalide, thiocyanate using as a main substrate hydrogen peroxide (H_2O_2) to generate their hypohalous acids [1].

It shows a cell specific expression as it is mainly expressed in cells of the myeloid lineage, namely monocytes, macrophages and neutrophils where it is stored in azurophilic granules. Through its enzymatic action on chloride, it is believed to be the major antimicrobial actor when bacterias are ingested into phagosomes where the content of granules is released [1]. There, HOCl is able to oxidize a variety of biological molecules including proteins, nucleic acids or lipids. In pro-inflammatory conditions, MPO can be released in the extracellular medium where it can illegitimately modify encountered molecules [2]. For example, concentrations as high as hundreds of ng/ml can be found in the serum of septic patients which correlates with the presence of MPO catalyzed post translational modifications [3].

Non canonical expression has also been occasionally reported in non myeloid cell types such as neurons, some tissues macrophages and prostatic epithelial cells [4–6].

The endothelium is an epithelium that constitutes the innermost layer of blood vessels. As part of the blood vessel wall, its most evident function is to form an active semi permeable barrier involved in both partition and exchange of fluids, ions, biological molecules and cells between blood and other tissues. Endothelial cells are therefore actors

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in the process of angiogenesis, the creation of new blood vessels from preexisting ones. Endothelial cells also play a regulatory role in other functions including hemostasis, vascular tone or interactions with blood cells. These controls involve the expression of molecules involved in the fine tuning of the balance between coagulation and fibrinolysis, vasoconstriction and vasodilation, and blood cells repulsion and recruitment respectively. Endothelial dysfunction is the perturbation of this balance and is involved in the development of various pathologies such as cardiovascular diseases, tumor development and metastasis [7]. Endothelial cells are at the front row to interact with blood molecules.

We report the presence of MPO in endothelial cells that can be of exogenous or endogenous origin. We show that MPO is involved in endothelial cell behaviors associated with angiogenesis, that it stimulates angiogenic signal transduction pathways and expression of genes involved in this process.

2. Material and methods

2.1. Cell culture

Human Aortic endothelial cells immortalized with human telomerase catalytic subunit hTERT (TeloHAEC) were purchased from ATCC and cultured in Vascular Cell Basal Medium (ATCC® PCS100030 ™) supplemented with 2% fetal bovine serum, human epidermal growth factor (5 ng/ml), basic fibroblast growth factor (5 ng/ml), vascular endothelial growth factor (VEGF) (5 ng/ml), Insulin-like growth factor (15 ng/ml), glutamine (10 mM), Heparin Sulfate (0.75U/ml), hydrocortisone (1 μg/ml) and ascorbic acid (50 μg/ml) at 37 °C and 5% CO2 in a humidified air incubator.

HUVEC (Human umbilical vein endothelial cells) were cultured in M200 growth medium with growth supplements LSGS (S-003-10) (thermofisher). HMEC-1 (human microvascular endothelial cells) obtained from the Center for Disease Control (Atlanta, Georgia), were cultured in MCDB-131 media (Gibco) supplemented with 10% fetal bovine serum (FBS), L-glutamine (2 mmol/L), human epidermal growth factor (10 ng/ml), penicillin/streptomycin (50 μg/ml) and hydrocortisone (1 μg/ml; Sigma).

EA.hy926 were cultured in DMEM media supplemented with 10% fetal bovine serum (FBS), L-glutamine (2 mmol/L) and penicillin/streptomycin (50 μg/ml). HL-60 (Human promyelocytic leukemia cells), THP1 (Human monocytic leukemia cells) and Jurkat cells (Human lymphoid leukemia cells) were cultured in RPMI supplemented with 10% fetal bovine serum (FBS), L-glutamine (2 mmol/L) and penicillin/streptomycin (50 μg/ml).

2.2. Recombinant MPO preparation

Recombinant MPO was prepared as described previously [8]. Briefly, the pNIV2703 plasmid was constructed that codes for prepromyeloperoxidase. It contains an MPO ORF coding for amino acid 11 in the putative signal sequence to amino acid 696. The pNIV2703 expression vector was transfected into CHO cells by electroporation. Cell supernatants were recovered to assay the production level and the enzymatic activity of secreted molecules. Each batch solution was characterized for its activity (U/ml), protein concentration (mg/ml) and specific activity. Peroxidase activity was determined using *o*-dianiside as a substrate. Protein concentration was measured using the Lowry assay, with ovalbumin as a standard. Each batch was checked for endotoxin using the Lonza Endotoxin Detection Kit QCL-1000 (Catalog Number: 50–647U). Enzymatically inactive human recombinant myeloperoxidase (iMPO) was generously supplied by Dr. Christian Obinger from BOKU-University, Vienna, Austria.

This mutant has been generated by site directed mutagenesis. It is a Q91T mutant devoid of enzymatic activity.

2.3. Transcriptome analysis by RNA-Seq

Total RNA samples extracted by Trizol from cells upon MPO treatment, were phenol/chloroform extracted and 5 μg of total RNA were treated by 1MBU of DNAse (BaseLine-Zero™ DNAse, Epicentre, USA) for 20 min at 37 °C to remove residual genomic DNA contamination. RNA quality was verified by PicoRNA chip on Bioanalyzer 2100 (Agilent, USA) to ensure RIN (RNA integrity number) > 8.0.

PolyA + fraction was isolated from 4.5 μg of DNAse-treated total RNA using NEBNext Oligo d(T)25 Magnetic beads kit (NEB, USA), according to manufacturer's recommendations. PolyA + enrichment and the absence of residual rRNA contamination were verified using PicoRNA chip on Bioanalyzer 2100 (Agilent, USA).

PolyA + fraction (1 ng for each sample) was used for whole-transcriptome library preparation using ScriptSeqv2 RNA-Seq kit (Illumina, USA). Libraries amplified in 14 PCR cycles were purified using Agencourt AMPure XP beads (Beckman-Coulter, USA), at a ratio 0.9x to remove adapter dimer contamination. Quality of the libraries was verified by HS DNA Chip on Bioanalyzer 2100 (Agilent, USA) and quantification done by Qubit 2.0 with appropriate RNA quantification kit.

Sequencing was performed on HiSeq1000 (Illumina, USA) in single-read SR50 mode. About 50 mln or raw sequencing reads were obtained for each sample (See Table S1). Adapter contamination was removed by Trimmomatic v0.32 [9] and the resulting sequencing reads aligned in sensitive-local mode by Bowtie 2 v2.2.4 [10] to hg19 build of human genome. Differential expression was analyzed using *.bam files in DESeq2 package [11] under R environment. Analysis of KEGG and Gene Ontology pathways for differentially expressed genes was done under R environment.

2.4. Cell treatment and transfection

TeloHAEC cells were treated with TNF-α (10 ng/ml), LPS (1 μg/ml), MPO or iMPO (200 ng/ml), H_2O_2 (10 μM), IFN-γ (50 ng/ml) or VEGF (100 ng/ml) for 24 h or 1 h depending on the experiment. In experiments using catalase TeloHAEC cells were pre-treated with 300 nM catalase for 2 h then stimulated with 200 ng/ml active (MPO) or inactive (iMPO) MPO for 1 h.

For cell transfection, TeloHAEC were grown to 70% confluency, and transiently transfected with universal negative control siRNA or siRNA targeting human MPO (Sigma), using GeneXPlus transfection reagent (ATCC). Cells were incubated at 37 °C, 5% CO_2 for 48 h in culture medium devoid of heparin sulfate and antibiotics.

2.5. Matrigel tube formation assay

The μ-slide Angiogenesis System (ibidi) was used to assess the formation of tube-like structures according to the manufacturer's instructions. Briefly, μ-slide wells were coated with 10 μl of growth factor reduced matrigel (ThermoFisher), then allowed to polymerize for 30 min at 37 °C. 10^4 cells in 50 μl culture medium (which were either supplemented or not with MPO, iMPO (200 ng/ml) or VEGF (100 ng/ml)) were seeded per well, incubated at 37 °C, 5% CO_2 and inspected for tubule formation after 6 h. Pictures were taken from different fields with the 10× objective using phase contrast microscopy (Nikon, Eclipse Ti). Vessel morphometric parameters including vessel length and junction density were measured using AngioTool software. Experiments were reproduced 3 times independently.

2.6. Scratch assay

EC migration was monitored using the wound-healing (or scratch) assay. Briefly, 3.10^4 cells were seeded into 2 well silicone insert defining a cell-free gap (ibidi) and incubated overnight in growth medium at 37 °C and 5% CO_2 to allow the cells to attach and to form a confluent