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RESEARCH

Role of Myeloperoxidase in ROS Generation and Inflammation Response on Prostate Epithelial Cells

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Abstract—Myeloperoxidase (MPO) has been reported in prostate tissue, and considering its pro-oxidant properties, this location might be linked to prostate pathology. The possibility that the glandular prostatic tissue might be the source of MPO and its potential inflammatory effects must be tested. Human prostate material was obtained from prostate biopsies and radical prostatectomies. Immunohistochemistry was performed using MPO-specific human antibody. *In situ* hybridization using MPO-specific probes and laser-assisted microdissection for quantitative real-time RT-PCR were performed to observe whether MPO is being produced in prostate tissue. Mass spectrometry on prostate biopsies was used to detect products of MPO activity in nucleic acids (DNA/RNA). MPO contribution to intracellular accumulation of ROS and interleukin-8 in prostatic epithelial cells was monitored *in vitro*. Immunohistochemistry confirmed cellular localization of MPO in epithelial cells of the prostate. The staining varied from light to high intensity. *In situ* hybridization did not address the presence of mRNA coding for MPO. No MPO-specific modifications on nucleic acids were detected. Mox-LDL was a major factor inducing ROS and cytokines production in prostatic epithelial cells. We did not demonstrate that MPO was synthetized by prostatic epithelial cells. However, *in vitro* experiments showed the ability of MPO to potentiate the ROS production and inflammation on prostate epithelial cells. Results do not allow us to demonstrate a role of MPO in prostate to date but further studies are mandatory to focus on the potential impact of MPO in the development of prostatic diseases.

KEY WORDS: prostate; inflammation; oxidative stress; MPO; *in situ* synthesis

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INTRODUCTION

Chronic inflammation is associated with prostate diseases. Several parameters including androgens pathway, inflammatory mediators, and oxidative stress have been considered to play a role, but there is no consensus as to which is the primary one. Most of the prostate pathological hypertrophy specimens contain inflammatory infiltrates [1]. Causes of intraprostatic inflammation remain unclear, and it has been suggested that systemic inflammation could contribute to the progression of inflammation within the prostate [2]. However, the significance of inflammation on the development and severity of lower urinary tract syndrome (LUTS) due to benign prostatic hyperplasia (BPH) or prostate cancer has not been established yet [3]. Oxidative stress (OS) is associated with age-related degenerative diseases such as BPH. *In vivo*, OS might be modulated by several enzymes or proteins such as myeloperoxidase (MPO) and angiotensin II (Ang II).

MPO is a lysosomal enzyme located in azurophilic granules of neutrophils and monocytes to provide microbicidal activity [4]. However, in some conditions such as oxidative stress or chronic inflammation diseases, MPO can be released in the extracellular fluid in response to a high level of pro-inflammatory cytokines, and MPO can promote oxidative damages to surrounding tissues [4]. Ang II is the main effector peptide of the renin angiotensin system (RAS) and exerts a variety of biological actions, including NADPH oxidase activation, stimulation of cell growth, and migration as well as promotion of inflammation of smooth muscle cells and fibroblasts [5, 6]. By facilitating of sympathetic activity, Ang II could interfere within the pathophysiology of BPH [7]. In this context, a higher Ang II-specific activity was reported in patients suffering of BPH compared to healthy patients [8]. Moreover, MPO and Ang II interplay in the bloodstream to produce Mox-LDLs (oxidation of low-density lipoproteins (LDLs) by MPO) which have a pro-inflammatory action as they promote the release of cytokines such as IL-8 and TNF-α by endothelial cells and monocytes, respectively [9, 10]. It is worth stressing that MPO has been observed in prostate tissue, and in view of the pro-oxidant properties of this enzyme, this location might be linked to prostate pathology [11]. But its origin remained unknown and questions that arise are the potential *in situ* synthesis of the MPO by prostate cells and its potential effect on inflammation and reactive oxygen species (ROS) production.

MATERIAL AND METHODS

This study was approved by the Ethics Committee of Erasme Hospital (Brussels University Clinics, hôpital Erasme-ULB), Brussels, Belgium, and was conducted in accordance with the ethical standards as laid down in the 1964 Declaration of Helsinki. All patients signed an informed consent before enrollment. Patients underwent 12 2D TRUS-guided biopsies including transition zone biopsies. All the samples (prostate biopsies and radical prostatectomy specimens) were reviewed by the same pathologist for cancer diagnosis and Gleason score assessment (International Society of Pathology, ISUP 2005), according to the International Union for Cancer control (IUCC 2009) classification.

MPO and Immunohistochemistry

Immunohistochemistry was performed on 5-µm paraffin-embedded, 10% formalin-fixed prostatic tissue sections. Tissue sections were deparaffinized, and heat-induced epitope retrieval was carried out in EnVisionTM Flex target retrieval solution, high pH (Dako K8004, DAKO, 6392 Via Real Carpinteria, CA 93013, USA) for 10 min at 97 °C using the Dako PTLink apparatus (Dako, Code PT100/PT101), followed by a 20-min cool down and rinse in tris-buffer-saline (TBS). All subsequent steps were performed using the EnVision Flex-HRP kit (Dako, code K8000) on a Dako autostainer link 48 according to the standard Dako protocol. Polyclonal rabbit anti-human MPO ready-to-use (prediluted primary antibody, Dako code IR511) was used as the primary antibody and incubated for 20 min on the tissue slides. Omission of the primary antibody was used as control.

MPO immunostaining validation has been previously reported [11]. Some immunostaining was also performed using the same polyclonal rabbit anti-human MPO (Dako, IG fraction, code A0398), but after immune-purification on a MPO column. The results obtained with this affinity purified MPO antibody were identical to those obtained with the standard antibody (Dako code IR511). We, therefore, routinely used the standard prediluted Dako antibody without immunopurification.

In situ Hybridization (ISH)

With the aim to report the potential synthesis of mRNA encoding for MPO protein, we used ISH and gene

expression profiling analysis. We used frozen human tissues and followed a laser-assisted microdissection approach to obtain RNA for reverse transcriptase-polymerase chain reaction (RT-PCR). MPO mRNA detection was performed on formalin-fixed, paraffin-embedded tissues using the RNAscope® 2.0 HD detection kit (BROWN) (Advanced Cell Diagnostics, USA, 7707 Gateway Blvd. Newark, CA, Inc., Cat No 320497) and a MPO probe (Advanced Cell Diagnostics, Inc., Cat No.603091) according to the manufacturer's instructions. The housekeeping gene polymerase (RNA) II (POLR2A) was used as a positive control for RNA quality, and the bacterial gene DapB was used as a negative control. As positive control, we used embryonic liver with extramedullary hematopoiesis in human fetus autopsies well known to produce MPO mRNA.

Tissue Samples, Initial Handling

The human prostate tissue samples were obtained immediately after radical prostatectomies and submerged in RNA later (Quiagen), an aqueous non-toxic tissue storage reagent that rapidly permeates tissues to stabilize and protect cellular RNA. After 24 h at 4 °C, the tissue sample was removed from RNA later and kept frozen at – 80 °C until microdissection.

Tissue Preparation and Microdissection of Frozen Specimens [12]

On the day before microdissection, the frozen samples were rinsed twice for 15 min in phosphate-buffered saline and then refrozen at – 30 °C in Tissue-tek OCT compound (Bayer, Sakura Findtech Europe B.V.). The first, middle, and last sections were cut at 5-µm thickness and stained with hematoxylin and eosin (H&E, Sigma-Aldrich) to identify the components they contained and to identify areas to capture or avoid. The remaining sections for LCM were cut at 10 µm, prepared on glass slides coated with a special membrane that facilitates the catapulting of large tissue areas and entirely preserves the morphology of the microdissected specimens, and colored with hematoxylin and eosin (H&E, Sigma-Aldrich) by immersing them sequentially in the following: 70% ethanol and then water (3 min each), Meyer's hematoxylin (Sigma-Aldrich; 2 min), water (1 min), 1% eosin (Sigma-Aldrich; 10 s), and then for 30 s each in water, 70% ethanol, 96% ethanol, and 100% ethanol. Microdissection was performed in the 24 h after the slide preparation using a PALM system (Microbeam, Zeiss, Germany). The microdissection makes it possible to isolate the glandular cells of the inflammatory cells. The selected cells were excised from the surrounding structures by the highly focused ultraviolet laser beam and were subsequently catapulted in groups of 10–100 cells (depending on the tissue) into the sterile cap of a microtube placed above the section. Depending on the tissue microdissected, 50–200 zones were collected per sample.

RNA Extraction from Microdissected Tissues

For the total RNA isolation from the samples obtained by microdissection, RNA easy mini kit (Qiagen, Westburg) was used. The RNA extractions were carried out according to the manufacturer's instructions. To ensure the integrity of RNA, dissection was performed within 30 min, and the cells on the cap transferred to a lysis buffer (350 µl buffer RLT with 10 µl β-mercaptoethanol) and stored at – 80 °C after centrifugation (2 min at 13,400 rcf) until use. After thawing and centrifugation, the samples containing the RNA were mixed with 350 µl of 70% ethanol, applied to the column and centrifuged. After washing, the RNA was treated with DNase I (RNase-free DNase Set Protocol, Qiagen). The column was washed twice with 500 µl buffer RPE, and the RNA was finally eluted from the column with 30 µl water and stored at – 80 °C.

Complementary DNA (cDNA) Synthesis and Quantitative Real-Time PCR (q RT PCR)

For each sample, 250 ng RNA was used to generate cDNA by reverse transcription (transcriptor high fidelity cDNA synthesis kit®, Roche Diagnostics GmbH, Germany) using random hexamers, according to manufacturer's instructions. Quantitative real-time PCR was performed on a Light Cycler® 480 II apparatus (Roche Diagnostics GmbH, Germany) using SYBR Green I (Roche Diagnostics GmbH, Germany). Primers for human MPO were manually designed and checked for secondary structures and specificity. The following sequences were used: forward: 5′-CCACACCCTCAT CCAACCCT-3′; reverse: 5′ CGCTCCCGGATCTCATCC AC-3′. Final primer concentrations used for qPCR was 500 nM. The following conditions for qPCR were used: denaturation step at 95 °C (5 min), followed by 45 amplification cycles (15 s at 95 °C, 30 s at 58 °C (annealing), and 30 s at 72 °C (elongation)).

Extraction of Cytoplasmic Pool, RNA, and DNA

Briefly, tissues were crushed in liquid nitrogen before DNA and RNA extractions using, respectively, DNeasy and RNeasy kits (Advanced Cell Diagnostics, USA. 7707 Gateway Blvd. Newark, CA). The extractions were carried out according to the manufacturer's protocols.

Enzymatic Hydrolysis Nucleoside Analysis by LC/MSMS

Enzymatic hydrolysis was performed for extracted DNA and RNA, as described by Noyon et al. [13]. Briefly, DNA and RNA were digested into nucleosides in the presence of internal standards (labeled 15N5-dGua and 5-fluorocytidine) using nuclease P1, PDE II, PDE I, alkaline phosphatase, and appropriated buffers. Thereafter, all samples were dried by vacuum centrifuge, dissolved in 50 µl aqueous mobile phase, and 10 µl was injected into LC/MSMS (in dynamic MRM positive mode) for the analysis of chloro(deoxy)cytidine (Cl-(d) Cyt) and 8-oxo(deoxy)guanosine (oxo-(d)Gua). Briefly, the analyses were performed using a LC/MS system from Agilent Technologies (Santa Clara, CA, USA): Agilent 1290 Infinity Binary – UHPLC system fitted to a mass spectrometer Agilent Jet Stream electrospray ionization source (AJS) – Triple Quadrupole (QqQ) 6490 series. Nucleoside separation was performed at 4 °C on Poroshell 120, EC-C18, 2.1 × 100 mm, 2.7-µm column, preceded by a Poroshell 120, EC-C18, 2.1 × 5 mm, 2.7-µm guard column, using an ammonium acetate 10 mM in water pH 5/methanol gradient. All these LC and MS parameters were detailed and validated in a previous article [13]. The results are expressed as the ratio Cl-dCyt/dCyt, Cl-Cyt/Cyt, oxo-dGua/dGua, and oxo-Gua/Gua.

Prostatic Epithelial Cell Culture

To assess the MPO dependent oxidation on prostate cells *in vitro*, normal human prostate epithelial cells (PrEC) were purchased from Lonza (Braine-l′Alleud, Belgium) and cultured in PrEBMTM (prostate epithelial cell basal medium, Lonza) supplemented with PrEGMTM Single QuotsTM (bovine pituitary extract, hydrocortisone, hEGF, epinephrine, transferrin, insulin, retinoic acid, triiodothyronine, and gentamicin; Lonza). Cells were maintained in accordance with manufacturer's instructions (CloneticsTM prostate epithelial cell system).

Mox-LDL Preparation [14]

Native LDLs (LDLs) were isolated from plasma of healthy donors by ultracentrifugation using sequential density gradients at the Laboratory of Experimental Medicine (A. Vésale Hospital, Charleroi, Belgium). The concentrations of lipoprotein solution were adjusted to 1 mg/ml in PBS (150 mM Cl^- and 10 mM PO_4^{3-} pH 7.4). Mox-LDLs were produced by mixing 1.6 mg LDLs in PBS with 8 µl of 1 M HCl, 45 µl of MPO (final concentration 250 nM), and 40 µl of 50 mM H_2O_2 and incubated for 4 h at 37 °C. PBS containing 1 g/l EDTA was added to bring the volume to 2 ml. LDLs and Mox-LDLs were desalted using RPMI-1640 without glutamine (Lonza, Belgium) by PD-10 desalting columns (GE Healthcare, Little Chalfont, Buckinghamshire, UK). All LDL solutions were stored in the dark at 4 °C after sterilization using sterile filters (0.2 µm) and were used within 4 days to avoid any further oxidation. The concentration of LDLs was calculated and adjusted to 1000 µg/ml using the Lowry method.

ROS Production in Prostate Epithelial Cells

Carboxy-H2DCFDA dye (Life technologies) was used to study the intracellular ROS generation in the PrE cells. The study was performed in 12-well plates, wherein the cells were seeded at a density of 2500 cells/cm^2 in prostate epithelial cell growth medium. At 80–90% confluency, the cells were washed twice with PBS, carboxy-DCFDA dye was added at a concentration of 10 µM, and the plates were kept in a 37 °C, 5% CO2 incubator for 30 min. Following incubation, the dye solution was removed; the cells were washed twice with PBS, and to assess the impact of those markers on prostate cells *in vitro*, normal human prostate epithelial cells were then treated for 24, 48, and 64 h, respectively, with native LDLs (200 µg/ml), MPO (200 ng/ml), MPO oxidized LDLs (Mox-LDL, 200 µg/ml), angiotensin II (AngII, 100 nM), and/or glucose (GLU, 0.3%). After various incubation times at 37 °C in the dark, the fluorescence of the oxidized DCFDA dye was measured at the respective excitation and emission wave lengths of 485 nm and 520 nm in a Berthold (Tristar) plate reader. The protein concentration was measured by the Bradford method after lysis in 1 N NaOH, and the fluorescence was expressed as relative fluorescence in AU per mg protein for normalization. The intracellular accumulation of ROS was monitored.

Cytokine (IL-8) Production Quantification in Prostate Epithelial Cells

Interleukin-8 measurements in prostatic cells supernatants were measured at 24 h using highly sensitive commercially available enzyme-linked immunosorbent assay with Il-8 ELISA kits according to the manufacturer's protocols (Roche, Mannheim, Germany) and performed by the Laboratory of Experimental Medicine, 222 unit-ULB at the CHU de Charleroi (site Vésale, Belgium).

Statistics

Statistical analyses were performed using GraphPad Prism Version 5.02. Data were evaluated using Student's *t*-test and one-way ANOVA. Results are presented as mean ± SEM from at least three independent experiments in triplicates. Differences were considered significant at the values of $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***), respectively.

RESULTS

Detection of MPO Protein and mRNA in Glandular Prostatic Cells

As previously described [11], immunostaining (IHC) showed cellular localization of MPO protein in secretory epithelial cells of the prostate (Fig. 1A–C). The staining varied from light to high intensity. Some glands were found heavily stained coexisting with some completely unstained neighboring glands.

Staining of basal as well as of luminal glandular cells was present. The fibromuscular stroma was unstained or displayed faint background staining. To explore the endogenous production of MPO by prostatic cells, *in situ* hybridization (ISH) was performed on prostatic tissues obtained from radical prostatectomies (Fig. 1D–F). Negative control experiment performed with DapB probe did not generate any staining (Fig. 1I), while the positive control, using a housekeeping gene polymerase2 (POLR2A), stained most of the cells (Fig. 1G, H). ISH and IHC were performed on serial sections. *In situ* hybridization appears as brown spots inside the cells. About 1 point can be observed for 8 to 10 cells. This result can be considered as no signal. We therefore view this as a negative result although we cannot completely rule out being below background noise.

Another positive control was obtained by using embryonic liver with extramedullary hematopoiesis in human fetus autopsies (Fig. 2A–D).

Laser-Assisted Microdissection

With the aim to report the synthesis of mRNA coding for MPO protein, we used a second method. After laser microdissection, mRNA was isolated and a q RT PCR was performed. The real-time PCR amplification curves after laser microdissection of prostatic epithelial cells highlight the presence of RNA encoding for the myeloperoxidase (MPO). The amplification threshold is crossed between 30 and 34 cycle timers (Fig. 3A, B). As negative control, we used stroma region, and the amplification threshold was over 38.

Detection of MPO Products Activity on Prostate Biopsies

In our previous work, we demonstrated that modified MPO nucleosides could be incorporated into RNA of epithelial prostatic cells *in vitro* [15]. We tried to detect products of MPO activity by mass spectrometry on prostate biopsies. We analyzed specific markers of the MPO activity in nucleic acid (DNA/RNA)-chloro(deoxy)cytidine (Cl-(d)Cyt) compared to non-specific 8-oxo(deoxy) guanosine (oxo-(d)gua). The results showed no detectable chloro nucleobase such as Cl-dCyt and Cl-Cyt. Moreover, no statistical differences for the ratio's oxo-dGua/dGua in DNA between patients with positive (whatever the Gleason score) or negative biopsies (Table 1). The same results were obtained for RNA when analyzing oxo-Gua/Gua (Table 2).

Those results do not allow us to demonstrate a role of MPO in carcinogenesis as far as no nucleic acid-specific modifications have been observed.

Reactive Oxygen Species (ROS) Production and Interleukin-8 Secretion in Prostatic Epithelial Cells

In this part of the present work, we exposed *in vitro* prostatic epithelial cells to factors identified to induce oxidative stress (OS) such as angiotensin II (Ang II), glucose, LDL, MPO, and Mox- LDL. Figure 4 shows

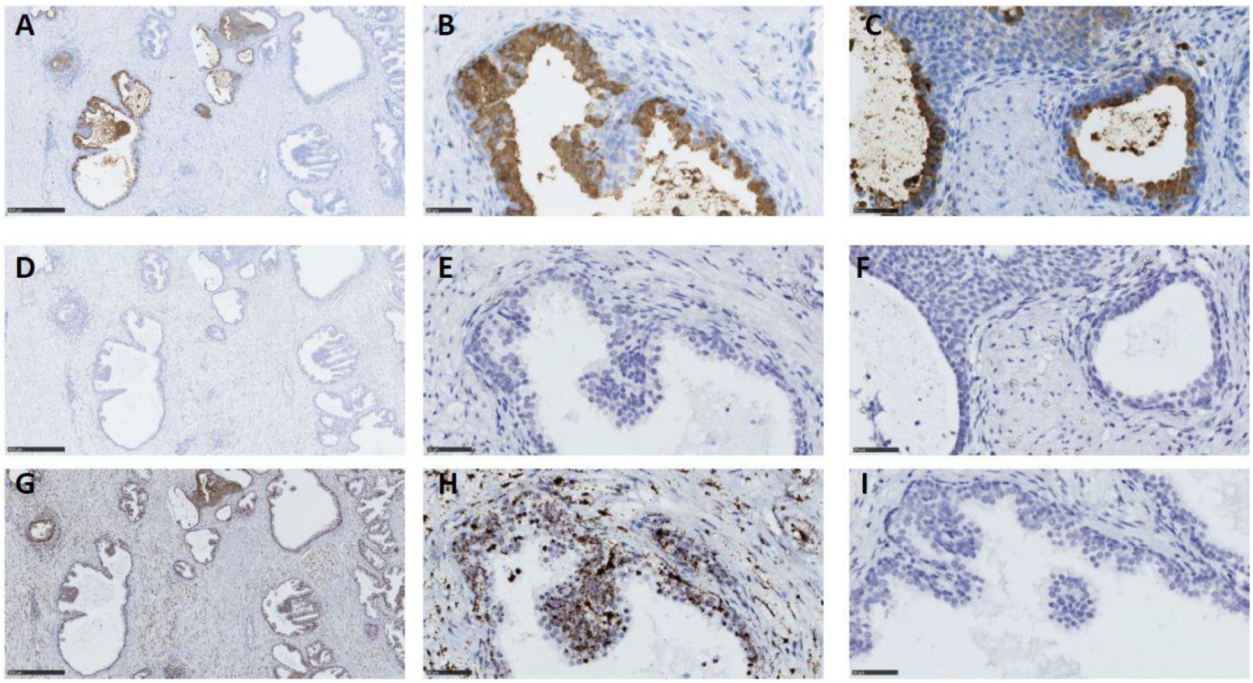

Fig. 1 MPO stained on prostate epithelial cells. **A** Detection of MPO by IHC, 5×. **B** and **C** 40×. **D** Detection of MPO by ISH, 5×. **E** and **F** 40×. **G** Positive control 5×(probe POLR2A). **H** Positive control 40×. **I** Negative control 40×(probe DapB) (scale bars: 50 µm).

that native LDLs, MPO, glucose, and Ang II alone did not induce OS after 24 and 48 h, respectively. OS was increased only after 64 h of incubation with Ang II, whereas an accumulation of ROS was observed in the presence of Mox-LDLs after 24 h and is stable over time. The oxidative stress produced by the Mox LDLs increased significantly in the presence of Ang II and in the presence of both Ang II and glucose. In general, the

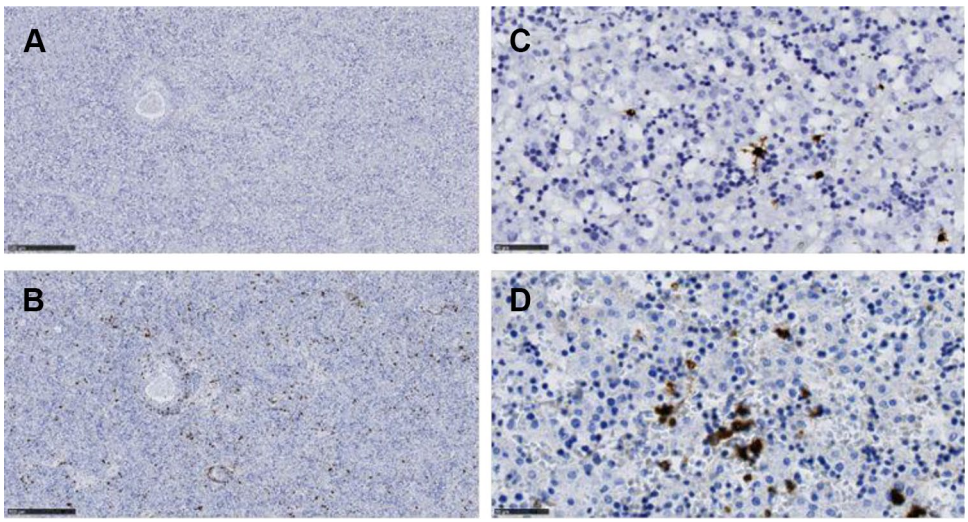

Fig. 2 Positive control obtained from embryonic liver with extramedullary hematopoiesis in human fetus autopsies. **A** Detection of MPO by ISH, 5×. **B** 40×. **C** Detection of MPO by IHC, 5×. **D** 40×(scale bars: 50 µm).

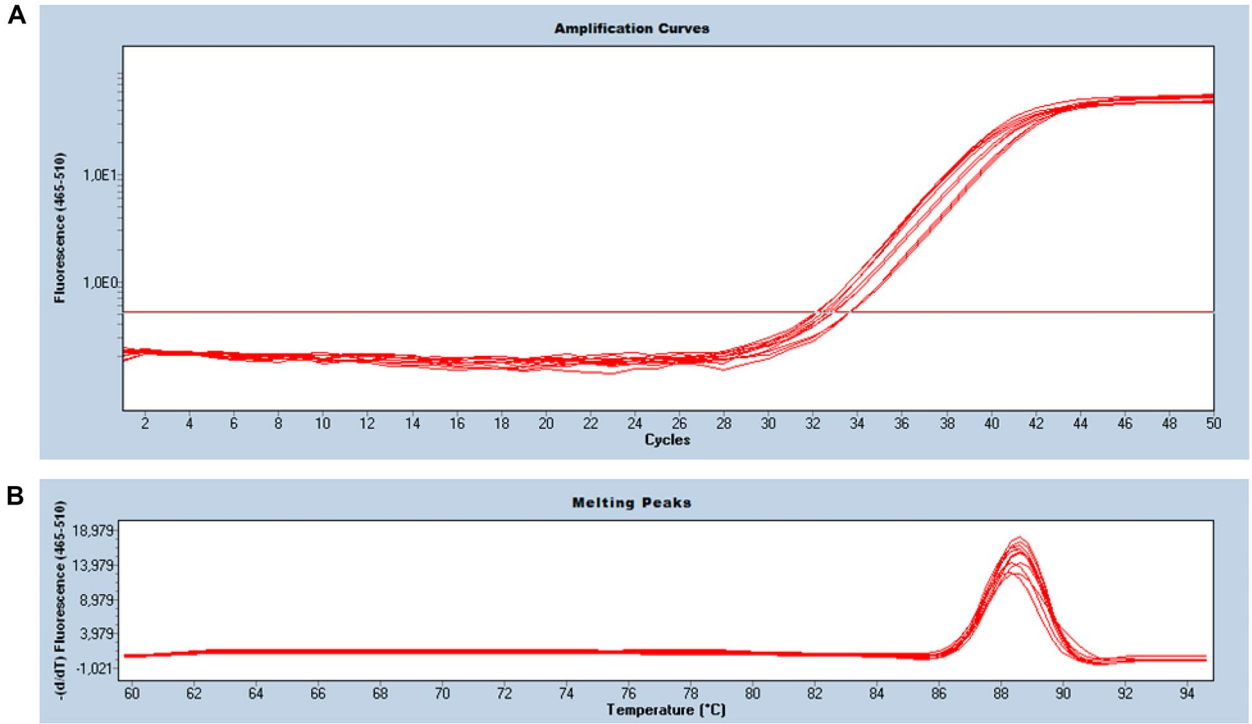

Fig. 3 Detection of MPO cDNA in samples obtained by microdissection using qRT-PCR: **A** Real-time PCR amplification curves for MPO cDNA in samples obtained by microdissection. The CT in all samples are between 32 and 34 cycles. **B** Melting curve analysis of cDNA samples amplified with primers specifically targeting MPO. A single peak was seen at 89 °C.

addition of Mox-LDL, MPO, and Ang II produced more ROS over time.

We observed a significant effect of LDL and Mox-LDL on IL-8 production (Fig. 5).

No one effect was observed with the ANG II or MPO alone.

DISCUSSION

Chronic inflammation has been documented for years in prostatic diseases, being associated to either disease initiation or progression [16]. The number of cytokines and growth factors are associated to immune dysregulation and chronic inflammation in BPH, including those responsible for the permanent attraction of leukocytes and those that promote the growth of prostate cells [17]. Inflammation influences the tissue microenvironment through the production of ROS, cyclooxygenase activity, and nitric oxide synthesis that are all linked to the deleterious effects of inflammation on prostate tissue [2]. We previously identified the presence of MPO protein in some prostatic glandular cells [11]. This observation raises the point of the (exogen or endogen) origin of the prostatic MPO, and this is one of the questions we addressed in this report. In other words, is the MPO present in some prostatic glandular cells the result of endocytosis of extracellular MPO produced by leukocytes or the result of a true prostatic epithelial synthesis? We confirmed our previous results with new positive immunostainings for MPO. We aimed to evaluate a local synthesis for MPO in identifying the presence of mRNA coding for MPO, but our results are not in accordance with this hypothesis. Indeed, by using *in situ* hybridization method, we did not confirm the presence of mRNA coding for MPO in epithelial cells of the prostate. This may support the hypothesis in favor of an external capture for MPO rather than a local synthesis. Even if it is a result that seems “negative”, it is still the conclusion of a methodical work that deserves to be communicated. This

Table 1 Detection of 8-oxodGua in Prostate Biopsies: the Results Are Expressed as Mean oxo-dGua/dGua Ratios +/− SD (expressed in %) in Biopsies DNA with the Corresponding Anapathological Results

	Ratio 8-oxodGua/dGua (%)		Anapathological results
	Mean	SD	
Patient 1	0,2	0,1	Negative
Patient 2	0,16	0,05	Negative
Patient 3	0,29	0,06	Negative
Patient 4	0,31	0,09	1 positive biopsies/10—Gleason score 7 (3 + 4)
Patient 5	0,31	0,06	Negative
Patient 6	0,14	0,06	Negative
Patient 7	0,19	0,06	3 positive biopsies/12—Gleason score 6 (3 + 3)
Patient 8	0,20	0,04	1 biopsy/10 with prostatitis
Patient 9	0,15	0,05	1 positive biopsy/12—Gleason score 6 (3 + 3)
Patient 10	0,16	0,06	1 positive biopsy/12
Patient 11	0,08	0,02	1 positive biopsy/10—Gleason score 6 (3 + 3)
Patient 12	0,24	0,07	1 biopsy/10 with acute prostatitis
Patient 13	0,26	0,06	3 positive biopsies/10—Gleason score 7 (3 + 4)
Patient 14	0,12	0,03	Negative
Patient 15	0,18	0,04	Negative
Patient 16	0,17	0,06	3 positive biopsies/12—Gleason score 6 (3 + 3)
Patient 17	0,12	0,06	Negative
Patient 18	0,09	0,08	Negative
Patient 19	0,14	0,06	2 positive biopsies/12 positive—Gleason score 9 (4 + 5) 2 biopsies/12 with prostatitis
Patient 20	0,20	0,06	2 positive biopsies/12—Gleason score 6 (3 + 3)
Patient 21	0,21	0,07	1 positive biopsy/12—Gleason score 6 (3 + 3)

means that we have to look elsewhere and make other assumptions.

In contrast, the extraction of mRNA by laser microdissection of epithelial cells is demonstrated by q RT-PCR and amplification of mRNA coding for MPO. Q RT-PCR test is a highly efficient and reliable sensitive detection assay with the advantage of a much smaller tumor content requirement than FISH [18]. How to interpret this

Table 2 Detection of 8-oxoGua in Prostate Biopsies: the Results Are Expressed as Mean oxo-Gua/Gua Ratios +/− SD (Expressed in ppm) in Biopsies mRNA with the Corresponding Anapathological Results

	Ratio 8-oxoGua/Gua (ppm)		Anapathological results
	Mean	SD	
Patient 22	56	57	3 positive biopsies/10—Gleason score 6 (3 + 3) 1 biopsy/10 with prostatitis
Patient 23	115	79	1 biopsy/12 with high-grade intra-epithelial dysplasia
Patient 24	74	36	4 positive biopsies/9—2 with Gleason score 6 (3 + 3) 2 with Gleason score 7 (4 + 3 and 3 + 4)
Patient 25	149	67	Negative
Patient 26	153	157	6 positive biopsies/12—Gleason score 6 (3 + 3)
Patient 27	152	118	2 positive biopsies/12—Gleason score 6 (3 + 3)
Patient 28	349	131	1 biopsy/12 with inflammation

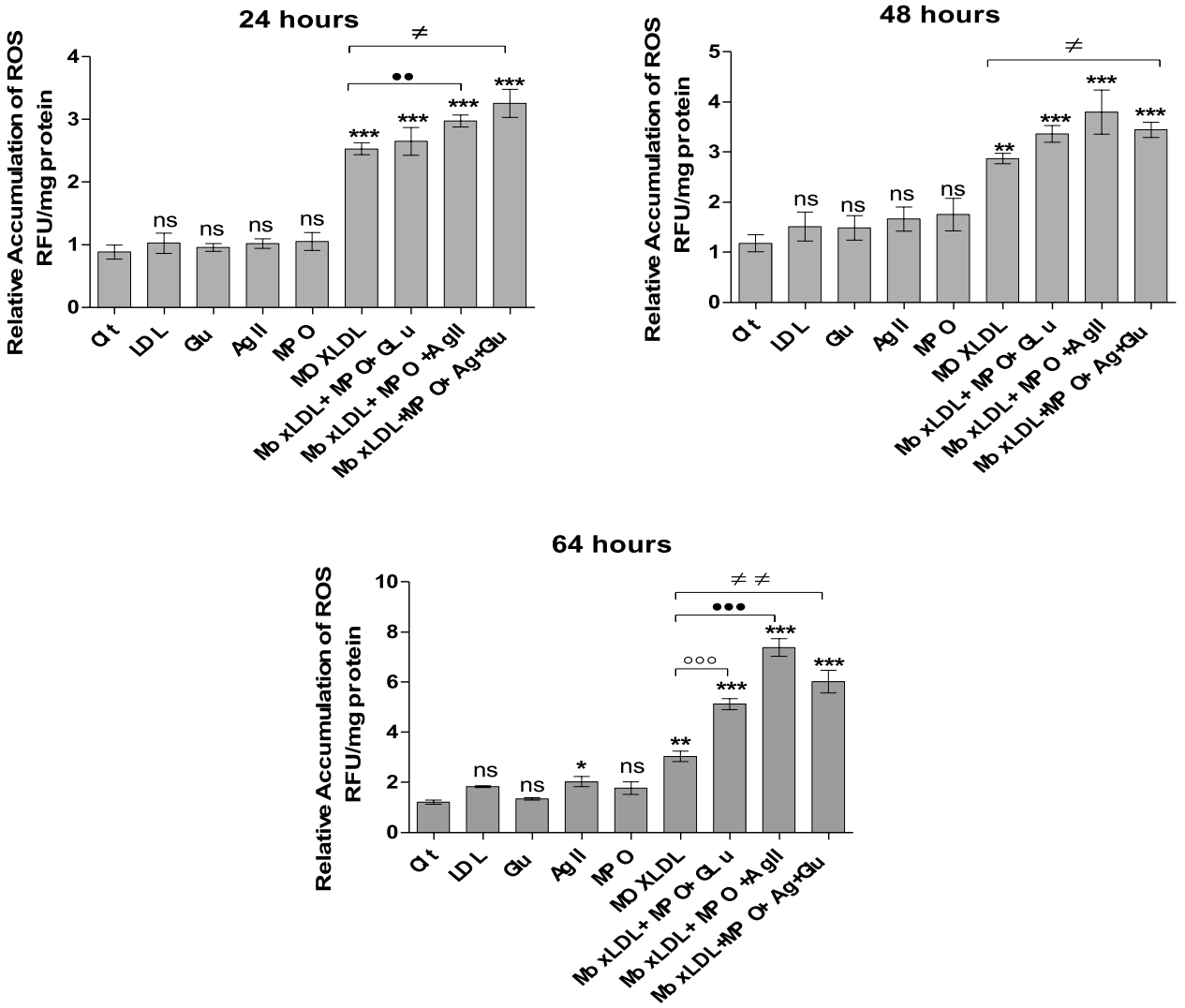

Fig. 4 Intracellular accumulation of ROS. PrEC loaded with the H2DCF-DA probe were stimulated with native LDL (200 µg/ml), MPO-oxidized LDLs (200 µg/ml), MPO (200 ng/ml) glucose (0.3%), or/and angiotensin (100 µM) for 24,48, and 64 h. The fluorescence of oxidized DCF was normalized by the protein quantity. The graphs are the means of three independent experiments. $^{***}p<0.001$, $^{**}p<0.005$, $^{*}p<0.05$ vs. control. $^{\neq\neq}p<0.005$, $^{\neq}p<0.05$ MoxLDL vs MoxLDL + Mpo + Ag + Glu. $^{\bullet\bullet\bullet}p<0.001$, $^{\bullet\bullet}p<0.001$, MoxLDL vs MoxLDL + Mpo + Ag. $^{\circ\circ\circ}p<0.001$ MoxLDL vs MoxLDL + Mpo + Glu.

discrepancy? We find that hybridization worked very well on positive controls (Fig. 3). We believe that the observed amplification in q RT-PCR is probably related to the presence of macrophages present in the prostate glands. The presence of neutrophils and/or macrophages comes quite quickly in the reasoning for interpretation as leukocytes in the prostate represent +/− 2% of the total cells [19]. We showed that there was RNA coding for MPO. As CT > 30, this means few mRNA and few MPO. We cannot exclude if it is not coming from other cells. A larger study should be able to analyze the relationship between the presence of leukocytes and the immunolabeling obtained for MPO. In the difficulties, it will be necessary to identify the neutrophils as well as the monocytes/macrophages. To achieve this, it will be necessary to highlight elastase, CD14 and iNOS. iNOS has been detected in both basal epithelial cells and secretory cells of the glandular epithelium of prostate [20]. As we confirmed the presence

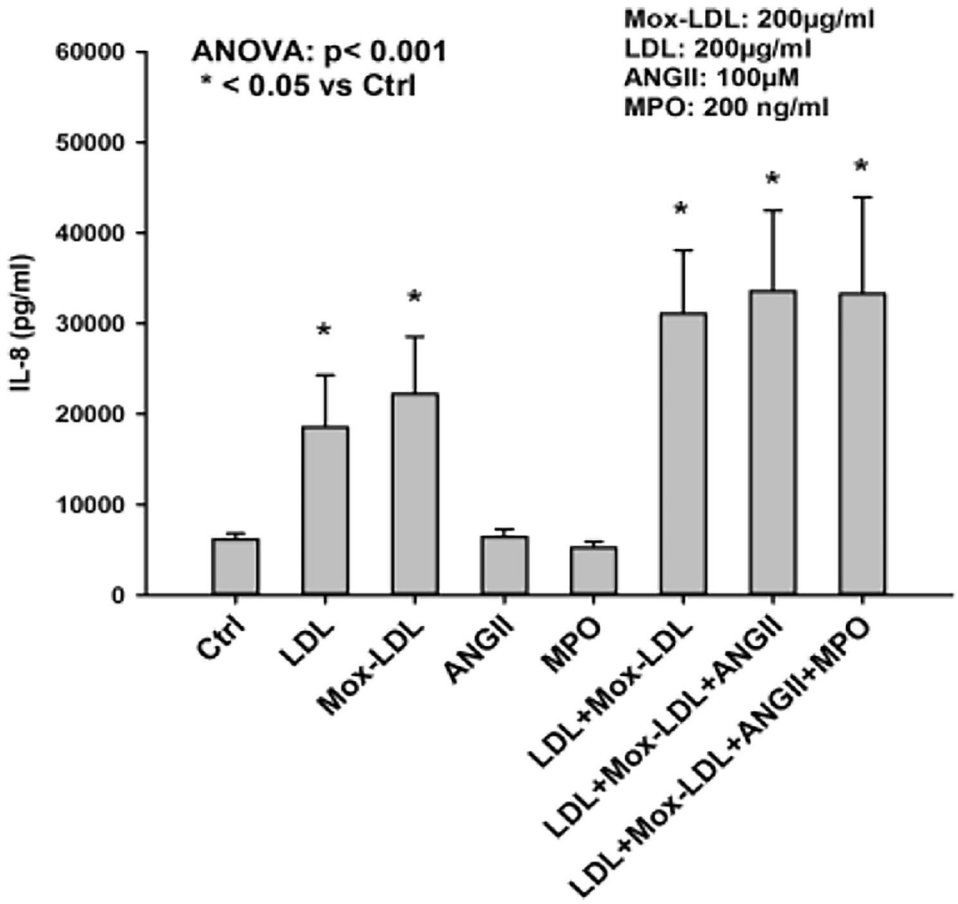

Fig. 5 Interleukin-8 production by epithelial prostate cells after 24 h of incubation with native LDL, Mox-LDL, ANG II, MPO, and different combinations (in triplicate).

of MPO protein, we aimed at detecting MPO products of activity. In previous work, we observed that chloro(deoxy) cytidine are specific markers of MPO oxidation *in vitro* [13]. Consequently, we measured those nucleotides, and it was observed that chlorocytidine (Cl-Cyt) was present in plasma of healthy volunteers and specifically incorporated in mRNA [15]. In the present study, we failed to detect MPO-specific modifications on nucleic acids by monitoring Cl(d)Cyt in positive or negative biopsies for prostate cancer. These results are corroborated by previous results wherein we demonstrated that ex vivo cell incubation with MPO does not produce nucleic acids modifications [15]. Looking at non-specific markers such as oxo(deoxy)guanidine, no significant oxidative damage was observed. This confirmed that the presence of MPO in epithelial cells of prostate is not related to oxidative damage in nucleic acid. Therefore, the analysis of chloronucleobase in the extracellular media was not expected. This could be related to our analysis, not sensitive enough, as performed on the whole biopsies (the only possible analysis with mass spectrometry to date) and not in whole prostate specimen. The negative results may be related to the fact that much background noise is generated by the ratio epithelial cells/whole biopsy tissue. This could preclude detection as the detection threshold is too high. Analysis with the positive tissue or at a single cell level would be more appropriate for the detection of MPO products of activity. Looking at non-specific markers such as oxo(deoxy) guanidine, no significant oxidative damage was observed. However, even if MPO in epithelial cells of prostate is not related to oxidative damage in nucleic acid, the presence of this enzyme with pro-oxidant properties emphasizes

the potential involvement of MPO in the development of prostatic disease, but physiological process inducing the mechanism of MPO synthesis is always needed. A criticism of our work is based on the observation that cellular test results stressed on the noxious role of the MPO and its products of activity, while we failed to detect any modification on RNA/DNA. The presence of the MPO in prostatic epithelial cells could then be physiological. The presence of MPO in the prostate glandular cells could be of the "exocrine" type with an antimicrobial purpose which does not attack the DNA of the producing cell. We could postulate for an antiseptic role of MPO in seminal fluid produced by the prostate and not only by the infiltration of neutrophils during an infection, like reported for the lactoperoxidase in saliva and breast milk [21]. Ang II is secreted in part by the prostate basal epithelial cells *in vitro*, and angiotensin receptors are expressed in the prostate glandular epithelium [22]. A causal link between increased Ang II plasma levels and Ang II prostate tissue concentrations and prostatic cells growth in BPH remain to be established. In looking at non-specific markers such as oxo(deoxy)guanidine, no significant oxidative damage were observed. Indeed, Ang II plasma level and Ang II prostatic level regulations are suggested to be independent. In this context, the incubation of prostatic epithelial cells with Ang II demonstrates its contribution to the intracellular production of ROS and it could contribute to cell growth [5]. In oxidative stress conditions or chronic inflammation diseases, MPO can promote oxidative damages and enhance slightly ROS production [4]. We have recently emphasized the predominant contribution of both MPO and Ang II for production of Mox-LDL in bloodstream [10]. Our experiments on prostate epithelial cells show that MPO alone does not contribute directly to the ROS production; however, its product of activity, namely, Mox-LDL, is one of the major factors that induce ROS production in prostatic epithelial cells and trigger the inflammatory response. The question raises about the role of MPO in prostate cancer by the production of reactive oxygen species.

CONCLUSION

Although having confirmed the presence of MPO in the prostate, we did not demonstrate that MPO was synthetized by prostatic epithelial cells. However, *in vitro* experiments showed that MPO is able to potentiate the effect of ANGII, glucose, and Mox-LDL on ROS production and interleukin-8 by prostate epithelial cells. Understanding the role of the presence of MPO in epithelial cells of the prostate, whether pathological or physiological, will be the subject of our further works.

AUTHOR CONTRIBUTION

T Roumeguère and K Zouaoui Boudjeltia had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. Conceptualization: T Roumeguère, P Van Antwerpen and K Zouaoui Boudjeltia. Acquisition, analysis, or interpretation of data: All authors. Drafting of the manuscript: T Roumeguère, P Van Antwerpen, I Bar, and K Zouaoui Boudjeltia. Critical revision of the manuscript for important intellectual content: All authors. Study supervision: P Van Antwerpen, P Delree, and K Zouaoui Boudjeltia. All authors read and approved the final manuscript.

AVAILABILITY OF DATA AND MATERIALS

Data could be available on request.

DECLARATIONS

Ethics Approval This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the Ethics Committee of Erasme Hospital, Université Libre de Bruxelles, Brussels, Belgium.

Consent to Participate Informed consent was obtained from all individual participants included in the study.

Consent for Publication Not applicable.

Competing Interests The authors declare no competing interests.

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