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ScienceDirect
The Veterinary Journal 178 (2008) 62–69
The Veterinary Journal
www.elsevier.com/locate/tvjl

Effects of unfractionated and fractionated heparins on myeloperoxidase activity and interactions with endothelial cells: Possible effects on the pathophysiology of equine laminitis

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Accepted 29 August 2007

Abstract

As heparins are sometimes used to prevent equine laminitis, the interactions between equine neutrophil myeloperoxidase (MPO), unfractionated (UFH) and fractionated low molecular weight (LMWH) heparins and digital endothelium have been investigated. The effects of the heparins on purified equine MPO activity were tested by immunocapture followed by enzymatic detection. Endothelium–MPO interactions were assessed by measuring total and active MPO uptake by arterial and venous digital endothelial cells in culture with or without the addition of heparins. A dose-dependent MPO inhibition by UFH and LMWH was seen, with the greatest reduction in MPO activity noted with the highest concentration of LMWH. The MPO capture was greater in arterial cells, but heparins better inhibited MPO capture in venous cells. The activity of cell-bound MPO was almost completely suppressed by the heparins, and no differences were observed between UFH and LMWH. The results confirm the anti-inflammatory properties of heparins and allow a better understanding of the potential role of MPO in laminitis.

Keywords: Myeloperoxidase; Heparin; Endothelial cell culture; Equine laminitis; Inflammation

Introduction

Laminitis is a painful systemic disease of complex aetiology and is one of the major complications of many surgical or medical diseases in horses, especially gastrointestinal diseases (Eades et al., 2002). Its prevalence reaches 7.1% in UK (Hinckley and Henderson, 1996), and in the USA 13% of horse establishments (excluding racetracks) reported problems with laminitis with a mortality of 4.7% of affected horses (Anon., 2000). Its principal manifestation is a diffuse aseptic pododermatitis leading to the dermo-epidermic separation of laminar tissues of the foot (Pollitt, 1996). However, the pathophysiology and the pathway of prevention and treatment have not been completely elucidated.

Laminitis may be explained either by haemodynamic modifications leading to local ischemia, or by an increased activity of the matrix metalloproteinases from the laminar endothelial cells and keratinocytes, or by a combination of both of these (Bailey, 2004). Studies using laminitis models have reported morphological modifications of the laminar tissues such as cellular oedema, degeneration and necrosis of the basal epithelium accompanied by a disintegration of the basement membrane (Pollitt, 1996; Morgan et al., 2003; French and Pollitt, 2004). Similar histological lesions have been described in the vascular endothelium (Hood et al., 1993). These findings support the occurrence of an

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doi:10.1016/j.tvjl.2007.08.033

ischemia–reperfusion process and therefore a vascular hypothesis for the initiation of the disease.

The role of platelet activation also appears significant, as evidenced by both the release of the vasoconstrictor molecules serotonin and thromboxane (TX) A_2 and by the formation of microthrombi. Microthrombi are found in the early stages of laminitis (Weiss et al., 1994) and their incidence is promoted by damage to the vascular endothelium (Vanhoutte, 1991; Hood et al., 1993). By virtue of their anti-coagulant properties heparins have been proposed as substances that may be of use in preventing laminitis (Cohen et al., 1994; Moore and Hinchcliff, 1994).

Recently, modifications to the circulating leucocytes, characterised by a decrease in the total white blood cell count and changes in leucocyte size and granularity indicating cell activation and recruitment, have been described in the prodromal stage of induced laminitis (Hurley et al., 2006), and the perivascular presence of monocytes and polymorphonuclear neutrophils (PMNs) in laminar tissues was reported (Black et al., 2006). These observations were confirmed in a further study in which myeloperoxidase (MPO) was found in the skin and laminar tissues in a model of induced laminitis (Riggs et al., 2007). MPO is considered to be a specific marker of the activation and degranulation of PMNs (Biasucci et al., 1996; Wu, 2005), demonstrating their presence despite their fast degeneration.

In the inflammatory process, activated PMNs produce reactive oxygen species (ROS), and MPO is involved in this through the synthesis of hypochlorous acid (HOCl), one of the most potent oxidising agents, that acts on neighbouring cells and tissues (Deby-Dupont et al., 1999; Klebanoff, 2005). The cytotoxicity of stimulated equine PMNs on equine endothelial cells in culture was found to correlate with the release of MPO in the medium (Benbarek et al., 2000). Interactions between endothelial cells and activated PMNs have been implicated in disorders such as septic shock (Turek et al., 1985) and intestinal ischemia, conditions that are associated with a high risk of developing laminitis. In small or large intestine strangulation, a significant local activity of MPO, and an increase in MPO concentration in the peritoneal fluid and plasma have been reported (Grulke et al., 1999, in press). Limitation of these interactions could therefore be therapeutically advantageous.

As heparin has been used to prevent laminitis and also possesses anti-inflammatory effects on the endothelium (Baldus et al., 2001; Manduteanu et al., 2003; Ranjbaran et al., 2006), the objective of this study was to examine the effects of fractionated heparin (low molecular weight heparin, LMWH) and unfractionated heparin (UFH) on the MPO oxidant activity and on the MPO capture by distal limb venous and arterial endothelial cells.

Materials and methods

Reagents

Sodium chloride (NaCl), $NaHCO_3$, Na_2HPO_4, KCl, KH_2PO_4, $MgSO_4$, $MgCl_2$, $CaCl_2$, *N*-cetyl-*N*,*N*,*N*-trimethyl-ammoniumbromide (CETAB), dimethylsulfoxide (DMSO), trypan blue, and H_2O_2 were from Merck. Bicinchoninic acid kit, glucose, Percoll, cytochalazine B, *N*-formyl-methionyl-leucyl-phenylalanine (fMLP), $NaNO_2$, unfractionated heparin (UFH), low molecular weight heparin (LMWH, 3000 Da), collagenase, gelatine (from porcine skin, type A), antibiotics, amphotericin B, anti-von Willebrand factor and anti-endothelin-1 antibodies came from Sigma–Aldrich. Dulbecco's Modified Eagle Medium (DMEM), trypsin-EDTA, fetal bovine serum (FBS) and horse serum were acquired from Gibco. Endothelial cell growth factor (ECGF) and Hepes were from Roche. Amplex red came from molecular probes Europe BV. LSAB2 System-HRP, haematoxylin and aqueous permanent mounting medium were from DakoCytomation. Phosphate-buffered saline (PBS) comprised 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4 and 1.5 mM KH_2PO_4 (pH 7.4). Hepes buffer solution (HBS) was made up of 137 mM NaCl, 4 mM KCl and 5.55 mM glucose (pH 7.4). Hank's balanced salt solution (HBSS) was 137 mM NaCl, 5.4 mM KCl, 4.2 mM $NaHCO_3$, 0.34 mM Na_2HPO_4, 0.44 mM KH_2PO_4, 0.4 mM $MgSO_4 \cdot 7H_2O$, 0.5 mM $MgCl_2 \cdot 6H_2O$, 1.2 mM $CaCl_2 \cdot 2H_2O$ and 5.55 mM glucose (pH 7.4).

Isolation of equine neutrophils

Neutrophils were isolated from EDTA (1.6 mg/mL blood) anticoagulated-blood drawn from the jugular vein of healthy horses, fed and bred in identical conditions and not under medical treatment (Faculty of Veterinary Medicine, University of Liège, Belgium). Each batch of neutrophils was obtained from 150 mL blood drawn from one horse. The neutrophils were isolated at room temperature (18–22 °C) by centrifugation (400 *g*, 30 min, 20 °C) on a discontinuous Percoll density gradient (Pycock et al., 1987). The PMN fraction was gently collected and washed in two volumes of physiological saline solution and the cells were resuspended in PBS. With this technique, the cell preparation was $\geqslant$95% neutrophils with a viability of 95% as measured by the trypan blue exclusion test.

Preparation of the supernatant of activated PMNs

Each millilitre of the PMN suspension (about 270×10^6 cells) was incubated with 5 μL cytochalazine B (1 mg/mL in DMSO) for 10 min at 37 °C. Then, 10 μL of fMLP (10^{-4} M in 10% DMSO solution) were added to the suspension and incubated for 30 min at 37 °C. Total MPO in the supernatant was measured by an original ELISA designed for equine MPO as previously described (Franck et al., 2005). After the measurement of MPO, the supernatant was diluted with HBSS to attain a final MPO concentration of 13 nM for use in the MPO capture assays (see below).

Effect of unfractionated heparin and low molecular weight heparin on the activity of equine myeloperoxidase

The intrinsic inhibition capacity of UFH and LMWH on the activity of MPO was determined by SIEFED (''Specific Immunological Extraction Followed by Enzymatic Detection''), an original method developed for the specific detection of active MPO from horse's neutrophils (Franck et al., 2006). The method consists of capturing MPO from a solution or a biological sample by specific anti-MPO antibodies immobilised onto multi-well plates (ThermoLabSystems). The capture step is followed by washing to eliminate all the compounds of the sample which are not bound to the antibodies (proteins or potential modulating or interfering substances), and by an in situ detection of MPO activity with a reaction mixture containing H_2O_2, a fluorogenic substrate (Amplex Red), and nitrite as enhancer of the reaction.

Stock solutions of 10^{-2} M UFH and LMWH were prepared in distilled water and diluted with a 10 mM PBS buffer at pH 7.4 to be used at the final concentrations of 10^{-4}, 10^{-5} or 10^{-6} M. UFH and LMWH were incubated for 15 min with purified equine MPO (20 ng/mL). The vehicle solution of UFH and LMWH was used as control. After incubation, the mixture was loaded on the SIEFED microplate and incubated (2 h, 37 °C) to allow the specific binding of MPO to the antibodies. After washing to

eliminate the incubation medium containing UFH or LMWH, the activity of MPO was measured. The activity of MPO alone was taken as 100%.

Endothelial cells culture

Digital veins and arteries from two horses were taken after euthanasia for clinical reasons (lethal injection of T61, Intervet) and conveyed to the laboratory in 100 mM sterile HBS. The vessels were blotted and their lumen rinsed several times with HBS to eliminate residual blood clots. The ends of the vessels were closed after intraluminal injection of a 0.1% solution of collagenase in HBS for 10 min at 37 °C. The digestion mixtures were individually collected in sterile tubes (Falcon, BD Biosciences) containing a 25% solution of FBS in PBS.

The vessels were rinsed several times with FBS solution and then longitudinally opened. The intima was gently scraped and the scrapings added to the tubes containing the respective digestion mixture. These were then centrifuged for 10 min at 180 *g*. Pellets were suspended in fresh culture medium (DMEM with L-glutamine, pyruvate, and 5.55 mM of glucose, penicillin [100 UI/mL], streptomycin [100 μg/mL], gentamycin [100 μg/mL], amphotericin B [1.25 μg/mL], ECGF [20 μg/mL], heparin [50 μg/mL]) and placed in culture dishes (9.6 cm^2) (Nunc A/S) coated with a 0.2% gelatine solution.

Cells were incubated at 38 °C with 5% CO_2, and the medium was changed three times per week. At confluence, cells were detached by incubation at 37 °C with a 0.5% trypsin-EDTA solution, collected and centrifuged (10 min, 180 *g*). The cell pellet was suspended in the culture medium and distributed in gelatin-coated culture dishes for further growth. Cells were characterised by the immunofluorescence detection of von Willebrand factor and endothelin–1 with their respective antibodies. Endothelial cells were used at passage 3.

MPO uptake by endothelial cells and effects of UFH and LMWH

Cells (400 000 cells/well) were seeded onto six-well plates (6×9.6 cm^2) (Nunc A/S) coated with a 0.2% gelatine solution. They were cultured for 48 h (until confluence) in the complete medium without heparin under a controlled atmosphere (5% CO_2) at 38 °C. The PMN supernatant with an MPO concentration of 13 nM (4 μg) was added to the wells. For the assays with the heparins, the diluted supernatant was preincubated for 20 min with UHF or LMWH at the final concentration of 6.7×10^{-7} M before the addition to the cells. For the control wells, HBSS alone was added.

After 2 h incubation at 37 °C, the cell supernatants were recovered and the wells washed three times with HBSS. Cells were then scraped and recovered in 1 mL of a lytic solution (HBSS + 0.1% of CETAB) before freezing at −80 °C. Cells from some wells under each experimental condition were fixed with 100% ethanol, and used for immunocytochemistry.

Determination of total and active MPO taken up by endothelial cells

After thawing, the solutions of lysed endothelial cells were centrifuged at 180 *g* for 3 min to obtain a homogeneous supernatant and protein quantities were determined by the bicinchoninic acid method. Total MPO associated with the cells was measured by ELISA (Franck et al., 2005) whereas active MPO was determined in cellular extract by a direct enzymatic reaction, using the same technique as applied in SIEFED (with H_2O_2, nitrite and the Amplex Red fluoregenic substrate), but without a preliminary extraction by specific antibodies. The increase in fluorescence due to MPO activity was monitored for 30 min after the addition of the substrate with the Fluoroscan Ascent (Thermo Electron Corporation) at the excitation and emission wavelengths of 544 and 590 nm, respectively. Results were expressed as the variation of fluorescence (ΔFU) per mg of proteins per min.

Immunostaining for MPO

After fixing in 100% ethanol, cells were dried and preserved at room temperature until use. Immunostaining procedures were carried out with the reagents of the Dakocytomation kit. Briefly, cells were covered with 500 μL of diluted anti-equine MPO antibodies (6 μg/mL) produced in rabbits, and incubated overnight in a wet chamber before a new incubation for 1 h with 500 μL of secondary biotinylated anti-rabbit antibodies diluted twice in PBS. Then, a horse radish peroxidase-conjugated streptavidin solution diluted twice in PBS was added and after an incubation of 60 min in the wet chamber, the chromatogen diaminobenzidine (DAB) was added. Between each step, cells were rinsed three times with PBS. Haematoxylin solution was dropped on the cells for 30 s before washing with tap water for 3 min. The brown colour was allowed to develop for 10 min and then stopped by three washings with distilled water. Some drops of ultramount aqueous permanent mounting medium were finally applied on the cells before morphological observations with a microscope (Axioskop, Zeiss). Pictures were taken using a Nikon D-70 camera and qualitatively compared for brownish colouration.

Statistical analysis

Data are given as means ± SD. Statistical analyses were performed with the GraphPad Instat 3.0 software. All data series were tested by the Kolmogorov and Smirnov method to assume a Gaussian bell-shaped distribution. ANOVA with Tukey's post test were used to compare UFH and LMWH data with control values, differences in a same group according to UFH or LMWH concentrations and differences between UFH and LMWH groups at each concentration. Results were considered significant at $P < 0.05$.

Results

Effect of unfractionated heparin and low molecular weight heparin on MPO activity

The in vitro studies using the SIEFED technique showed that the heparins reduced the MPO activity. UFH had a significant effect at all concentrations (Fig. 1). The inhibition rates were 21.42 ± 6.49 ($P < 0.01$), 34.11 ± 12.99 ($P < 0.001$), and $63.91 \pm 14.87\%$ ($P < 0.001$) for the UFH concentrations of 10^{-6}, 10^{-5} and 10^{-4} M, respectively, compared to control values. At 10^{-4} M, UFH showed

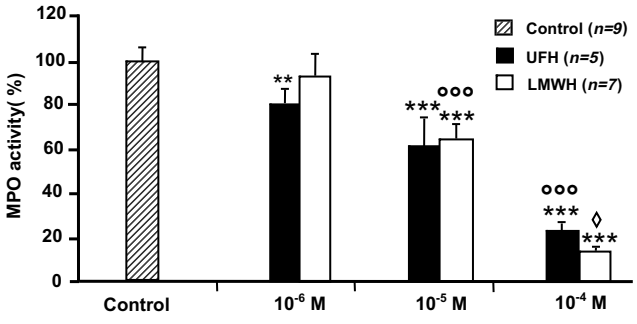

Fig. 1. Effect of unfractionated (UFH) and fractionated low molecular weight (LMWH) heparins on neutrophil myeloperoxidase (MPO) activity measured by specific immunological extraction followed by enzymatic detection (SIEFED). Purified equine MPO (20 ng/mL) was incubated with heparins for 20 min before immunocapture. The percentages of inhibition were calculated, and the MPO activity of the control without heparin determined as the 100% control value. Data are given as means ± standard deviation (SD). $^{**}P < 0.01$ and $^{***}P < 0.001$ for UFH and LMWH vs. control. $^{\diamond}P < 0.05$ between UFH and LMWH at 10^{-4} M. $^{ooo}P < 0.001$ for UFH vs. 10^{-5} M and for LMWH vs. 10^{-4} and 10^{-6} M.

significantly more inhibition ($P < 0.001$) than at other concentrations.

LMWH also produced a significant and dose related inhibition on MPO activity: the percentage inhibitions were 5.98 ± 11.20, 37.99 ± 6.95 ($P < 0.001$) and $86.45 \pm 3.86\%$ ($P < 0.001$) at 10^{-6}, 10^{-5} and 10^{-4} M, respectively. In the LMWH group the inhibition of the MPO activity was not significant for the lowest concentration, and at 10^{-5} M the inhibition was significantly different ($P < 0.001$) from that at the two other concentrations. At 10^{-4} M LMWH caused greater inhibition of MPO activity than UFH ($P < 0.01$) (Fig. 1).

Determination of MPO uptake by endothelial cells

Three techniques were applied to measure the amount of enzyme taken up by the endothelial cells: an ELISA technique to quantify the total amount of MPO, an enzymatic method to quantify the active fraction of the enzyme, and an in situ detection by immunostaining of the endothelial cells.

Using the ELISA, we demonstrated that MPO was taken up by endothelial cells, and that UFH and LMWH both inhibited the uptake by arterial and venous cells (Fig. 2). The uptake reductions were expressed as percentages by taking the control value as 100% for arterial and venous cells, respectively. In arterial endothelial cells, percentage inhibitions were 62.44 ± 5.42 ($P < 0.001$) and $58.86 \pm 12.55\%$ ($P < 0.001$) for UFH and LMWH, respectively. In the venous cells, the percentage uptake reductions for UFH and LMWH were $87.32 \pm 3.62\%$ ($P < 0.001$) and $90.01 \pm 3.05\%$ ($P < 0.001$), respectively. Neither venous nor arterial cells showed a different response to UFH or LMWH. However, in each group (without heparins, with UFH, with LMWH), there were significant differences between arteries and veins ($P < 0.01$ for UFH; $P < 0.001$ for control and LMWH).

The activity measurements indicated that the enzyme that was taken up by the endothelial cells conserved its enzymatic activity, at least partially. They also indicated

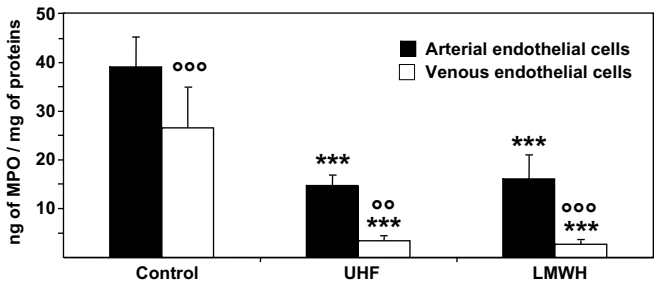

Fig. 2. Effect of UFH and LMWH (6.7×10^{-7} M) on total MPO uptake by venous ($n = 9$) and arterial ($n = 6$) confluent endothelial cells. Total MPO content was measured by a specific ELISA test. Controls were carried out without heparins. Data are given as means ± SD. $^{***}P < 0.001$ for UHF or LMWH vs. their respective control cells. $^{\circ\circ}P < 0.01$ and $^{\circ\circ\circ}P < 0.001$ between arteries and veins in the same group.

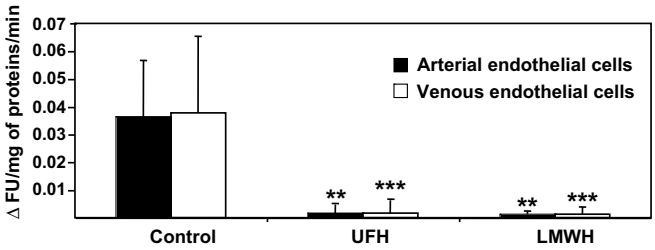

Fig. 3. Effect of UFH and LMWH (6.7×10^{-7} M) on the activity of MPO taken up by confluent venous ($n = 9$) and arterial ($n = 6$) endothelial cells. Activity is expressed as the variation of fluorescence units (ΔFU) per min of reaction. Controls were carried out without heparins. Data are given as means ± SD. $^{**}P < 0.01$ and $^{***}P < 0.001$ for UFH or LMWH vs. their respective control cells.

for arterial cells a loss of cell-associated MPO activity of 95.61 ± 9.79 ($P < 0.01$) and $96.62 \pm 3.38\%$ ($P < 0.01$), respectively, for the UFH and LMWH groups vs. controls (Fig. 3). The inhibition rate was similar for venous cell-associated MPO activity: 95.69 ± 12.29 ($P < 0.001$) and $96.47 \pm 6.80\%$ ($P < 0.001$) for UFH and LMWH, respectively (Fig. 3). No significant differences were found between arteries and veins in each group.

The immunostaining of endothelial cells after incubation with 13 nM (4 μg) of equine MPO solution in HBSS with or without the addition of UFH/LMWH, confirmed qualitatively the enzyme capture by the cells and the inhibiting effect of the heparins (Fig. 4). A brownish pigmentation was observed in cells incubated with MPO (Fig. 4b), which was greatly decreased in cells treated with UFH (Fig. 4c) or LMWH (Fig. 4d), and absent in control cells (no incubation with MPO) (Fig. 4a).

Discussion

In this study, we first compared the inhibitory effects of UFH and LMWH on the activity of equine MPO. We demonstrated that both types of heparins significantly reduced MPO activity, except at the lowest concentration of LMWH (10^{-6} M), and found that UFH was a better inhibitor than LMWH except at the highest concentration.

In previous studies, it was reported that heparins had potential protective effects against the activation of PMNs by decreasing the superoxide anion production and MPO release by PMNs (Leculier et al., 1993). The data from these studies indicated that heparins acted indirectly on MPO by inhibiting the degranulation of PMNs.

The capacity of heparins to inhibit the activity of MPO has never been assessed. The original SIEFED technique, recently developed by Franck et al. (2006), appeared to be an ideal method to study the potential pharmacological effects of the heparin-derived compounds on the activity of this enzyme because by using this method the heparins were washed before the enzymatic revelation of MPO bound to its antibodies. The inhibitory response can be attributed to an interaction with heparins, which either

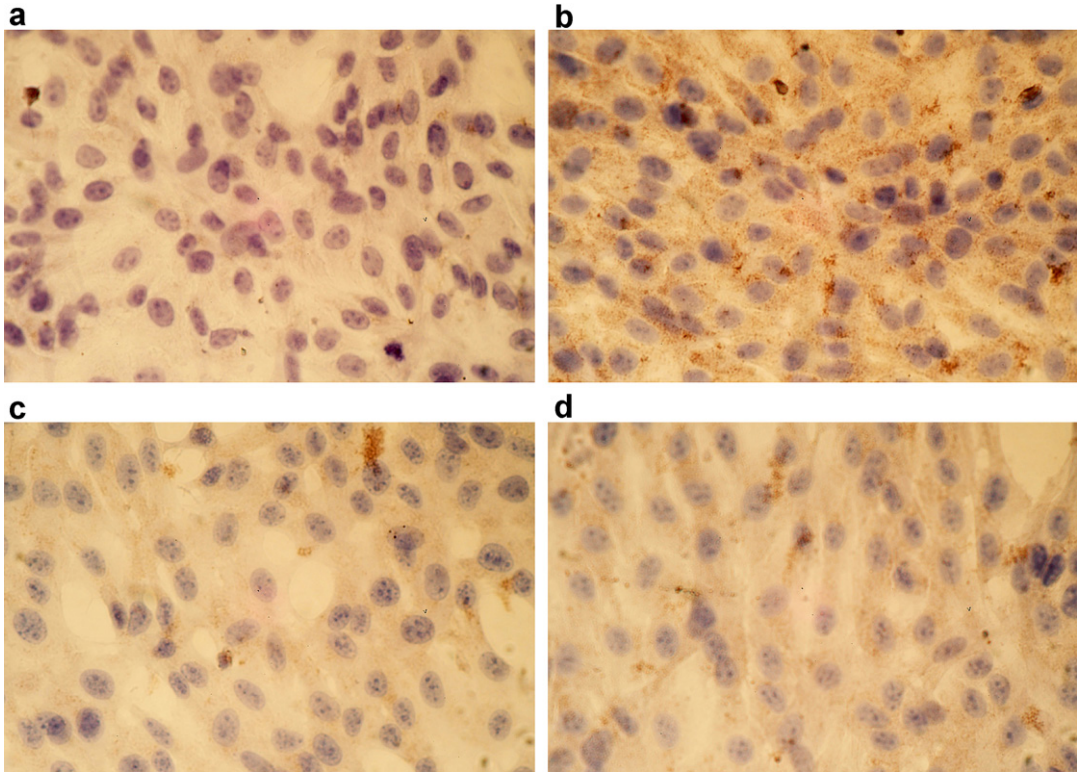

Fig. 4. Immunodetection of MPO in arterial endothelial cells by specific rabbit anti-MPO antibodies (×40). (a) no incubation with MPO (control); (b) incubation (2 h) with 4 μg equine MPO; (c) same as B + 6.7×10^{-7} M UHF and (d) same as B + 6.7×10^{-7} M LMWH.

modify the enzyme structure or block access to the active site, leading to inactivation of MPO or a reduction in the capture of MPO by the specific antibodies. Decrease in MPO activity cannot be attributed to protein degradation since heparins have no enzymatic activity and even possess anti-protease activity (Sissi et al., 2006; Spencer et al., 2006) able to protect MPO against proteases from the PMN.

MPO is a cationic enzyme able to interact strongly with polyanionic polysaccharide molecules like heparins (Daphna et al., 1998). Previous studies have reported a hyperbolic and competitive inhibitory mechanism of heparins on elastase or cathepsin G, two serine proteases present in PMNs (Sissi et al., 2006; Spencer et al., 2006). As with these two enzymes, we may hypothesise that the inhibitory effects of heparins on MPO are not specific but linked to electrostatic interactions and dependent on a similar competitive inhibitory mechanism: at a high concentration, the inhibitory capacity of LMWH would be greater than UFH because the access of UFH to MPO would be limited by steric effects.

Following the demonstration of an in vitro inhibitory effect of UFH and LMWH on MPO activity, we were interested to verify the existence of interactions between MPO and arterial or venous equine endothelial cells in culture models and to assess the influence of heparins on these interactions. We found that arterial and venous endothelial cells were able to bind MPO (Figs. 2 and 4) and that some differences existed between arterial and venous cells. The uptake of MPO by arterial endothelial cells was significantly more effective than that observed for venous arterial cells. Heparins inhibited MPO binding to arterial cells more efficiently than to venous cells suggesting a better affinity of MPO for the former.

The binding of MPO to cultured endothelial cells (Savage et al., 1993; Daphna et al., 1998; Baldus et al., 2001) was considered to play a role in vascular inflammatory disease, but these studies used either human umbilical vein endothelial cells (HUVEC) or bovine aortic endothelial cells (BAEC). HUVEC are embryonic cells which possess unique properties compared to adult and mature endothelial cells. In BAEC models, MPO obtained from human PMNs was generally used without considering species differences. Moreover previous studies have shown differences in the distribution of von Willebrand factor or Weibel–Palade bodies between endothelial cells from arteries and veins (Gebrane-Younes et al., 1991; Smith et al., 1996). Our microscopic observations revealed different morphological features, with venous cells being bigger and flatter and less compact than arterial cells, with a shadow aspect (data not shown). Thus, the originality of our model is that we used both mature digital arterial and venous endothelial cells and MPO from horses to study interactions between them. Furthermore, MPO was obtained by collecting the supernatant of stimulated PMNs and after measurement of its concentration by ELISA, used without further purification to mimic as much as

possible the in vivo interaction between endothelial cells, MPO and other compounds released from activated PMNs, such as proteases or interleukins.

Interestingly, when MPO was bound or picked up by the cells in the absence of heparins, it remained active. Under these circumstances, MPO can produce oxidant species in the cells or on the cell surface. However, to exert its activity, the enzyme must find H_2O_2 as cofactor and substrate. H_2O_2 is produced by many cells in inflammation or following hypoxic conditions. In the presence of heparins, the activity of cell-bound MPO was strongly reduced. There were no differences in the activity of MPO bound to arterial or venous cells, although the total MPO concentrations were different. Further investigations are necessary to explain this phenomenon.

MPO-endothelium interactions have been attributed to electrostatic attractions (Johnson et al., 1987), and more recent studies using heparinase, heparitinase or chondroitinase demonstrated that the cell-MPO interactions were effectively dependent on the glycosaminoglycans (GAGs) of the cell glycocalyx (Daphna et al., 1998; Baldus et al., 2001). GAGs are associated with most of the body cells, either on the surface or stored in intracellular granules (Kjellen and Lindahl, 1991). They contain chondroitin sulfate, dermatan sulfate, keratin sulfate, heparin sulfate and heparin, which are polyanionic compounds with increasing charge density (Christer Busch, 1984). The differentiated distribution pattern of binding sites for cationic ferritin that was observed in capillaries (Simionescu et al., 1981) supports a different distribution of GAGs. Studies by Baldus et al. (2001) showed that xanthine oxidase and MPO both fastened to the endothelial cells, but without being in competition since the binding of xanthine oxidase depended on the presence of chondroitin sulfate, whereas the binding of MPO depended on heparin sulfate and heparin. We therefore hypothesise that the differences observed between venous and arterial cells in the binding of MPO were linked to a differentiated distribution pattern of GAGs between these two endothelial cell lines.

Specific immunostaining of endothelial cells confirmed the ELISA results and the inhibiting effects of heparins on MPO binding. The distribution of the brownish colour (in the immunostaining assay without UFH or LMWH) was not homogenous, but indicated scattered or clustered spots with a granular aspect. These observations suggest that MPO was internalised, maybe by transcytosis as has been previously described for bovine arterial cells (Baldus et al., 2001). The possibility of a transcytosis pathway for MPO internalisation is also supported by studies reporting a similar process for other proteins such as interleukins or lipoprotein lipase (Middleton et al., 1997; Obunike et al., 2001).

The demonstration of MPO uptake by endothelial cells in horses seems particularly interesting, as it may improve our understanding of the neutrophil-endothelium interaction in the pathophysiology of laminitis, well-recognised as a vascular inflammatory disease with endothelial dysfunction (Eades et al., 2007). In carbohydrate-induced laminitis and endotoxaemia models, the endothelium relaxation induced by acetylcholine is impaired, very likely due to endothelial damage or dysfunction (Baxter, 1995; Schneider et al., 1999). The vasodilation response to acetylcholine, bradykinin or substance P is dependent on nitric oxide (NO) release by the endothelium (Cogswell et al., 1995; Elliott et al., 1994; Katz et al., 2003; Berhane et al., 2006). Consequently, it could be that the impairment of the vasodilation response in laminitis pathophysiology is linked to NO depletion. Recent studies in human cardiology have demonstrated that myeloperoxidase enhances NO catabolism in myocardial ischaemia and reperfusion (Baldus et al., 2004). In laminitis, the binding or the internalisation of MPO by digital endothelial cells could lead to a similar process, leading to an NO depletion and altering the balance in favour of vasoconstriction. Moreover, damage induced by the activity of cell bound MPO is perhaps also responsible for an activation of the endothelium leading to the release of other active mediators such as endothelin-1, the most potent endogenous vasoconstrictor (Katwa et al., 1999). Endothelial activation is also responsible for the development of a predominant procoagulant activity in inflammatory processes occurring in laminitis (Johnson et al., 2004; Eades et al., 2007), favouring microthrombosis in laminar tissues.

Our data indicate that the infiltration of laminar tissues by MPO as reported by Riggs et al. (2007) may be due not only to a PMN infiltration and degranulation but also to an endothelial transcytosis of MPO from blood, occurring after the PMN degranulation induced by proinflammatory substances such as endotoxins.

Finally, recent studies in man and rats have indicated that heparins attenuate inflammatory responses and ischemia/reperfusion injuries (Dotan et al., 2001; Derhaschnig et al., 2003; Harada et al., 2006). Furthermore, in humans heparins have been found to increase endothelial NO bioavailability by discharging vessel-immobilised MPO and restoring vasodilator function at the clinical concentration of 70 U/kg BID (Baldus et al., 2006), a dose that is used clinically in horses. Therefore, both our results and the literature data indicate preventive and curative anti-inflammatory properties of UFH and LMWH either by inhibiting the binding of MPO or by releasing cell-bound MPO.

Conclusions

Our results may help to understand the role of MPO in vascular inflammatory disease in horses, especially in laminitis in which active MPO uptake by endothelial cells in the laminar tissues could participate in the ischemic process. MPO-endothelial cell interactions could favour either microthrombi formation after cell injuries or vasoconstriction induced by NO depletion. In such diseases, heparin may not only act as an anticoagulant but also as an anti-inflammatory drug with protective effects for the endothe-

lium against the deleterious activity of MPO. The findings could open the field for future investigations in the development of specific modulators of MPO activity for use in the treatment of inflammatory disease.

Acknowledgements

This work was supported by a FNRS (National Fund for Scientific Research, Belgium) Grant. G. de la Rebière is recipient of a FRIA (Fund for the Research in Industry and Agriculture) fellowship.

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