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BASIC SCIENCE

Myeloperoxidase deficiency preserves vasomotor function in humans

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Aims Observational studies have suggested a mechanistic link between the leucocyte-derived enzyme myeloperoxidase (MPO) and vasomotor function. Here, we tested whether MPO is systemically affecting vascular tone in humans.

Methods and results A total of 12 135 patients were screened for leucocyte peroxidase activity. We identified 15 individuals with low MPO expression and activity (MPO^{low}), who were matched with 30 participants exhibiting normal MPO protein content and activity (control). Nicotine-dependent activation of leucocytes caused attenuation of endothelial nitric oxide (NO) bioavailability in the control group ($P < 0.01$), but not in MPO^{low} individuals ($P = 0.12$); here the MPO burden of leucocytes correlated with the degree of vasomotor dysfunction ($P = 0.008$). To directly test the vasoactive properties of free circulating MPO, the enzyme was injected into the left atrium of anaesthetized, open-chest pigs. Myeloperoxidase plasma levels peaked within minutes and rapidly declined thereafter, reflecting vascular binding of MPO. Blood flow in the left anterior descending artery and the internal mammary artery (IMA) as well as myocardial perfusion decreased following MPO injection when compared with albumin-treated animals ($P < 0.001$). Isolated IMA-rings from animals subjected to MPO revealed markedly diminished relaxation in response to acetylcholine ($P < 0.01$) and nitroglycerine as opposed to controls ($P < 0.001$).

Conclusion Myeloperoxidase elicits profound effects on vascular tone of conductance and resistance vessels *in vivo*. These findings not only call for revisiting the biological functions of leucocytes as systemic and mobile effectors of vascular tone, but also identify MPO as a critical systemic regulator of vasomotion in humans and thus a potential therapeutic target.

Keywords Leucocyte • Endothelium • Myeloperoxidase • Vascular tone • Nitric oxide

Introduction

Myeloperoxidase (MPO), a haeme enzyme stored in and released by activated polymorphonuclear neutrophils (PMN), monocytes, and macrophages, has long been considered to be an enzyme solely involved in host defence: in the presence of its principal substrate hydrogen peroxide (H_2O_2), MPO generates a myriad of reactive oxygen species with documented cytotoxic properties,

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such as hypochlorous acid (HOCl) and nitrogen dioxide.[1] However, over the last years, MPO has gained increasing attention as a critical mechanistic contributor to vascular inflammatory disease, in particular in atherosclerosis: Circulating MPO plasma and serum levels not only predict adverse outcome in patients with acute coronary syndromes and heart failure, MPO is also localized to atherosclerotic plaques[2,3] and the subendothelial matrix.[4,5] Here, MPO is capable of oxidizing and carbamylating lipoproteins thereby facilitating its uptake by macrophages and propagating foam cell formation.[6,7] Also, MPO revealed to activate matrix metalloproteinases (MMPs), redox- and in particular HOCl-sensitive matrix degrading enzyme systems, critically affecting the stability of the plaque's fibrous cap and remodelling cardiac tissue.[8,9]

Importantly, MPO not only proved to affect the structural homogeneity of the vessel wall, but was also suggested to critically modulate signalling cascades. One of the most intensively characterized signalling pathways in the vessel wall centres around nitric oxide (NO), which is synthesized in the endothelium and passes the subendothelial space to reach one of its effectors, the vascular smooth muscle cell.[10,11] Given the strategic accumulation of MPO at the interface between endothelium and media,[5] MPO was put forward to affect vascular tone by depleting endothelium-derived NO.[12,13]

So far *ex vivo* assays measuring MPO-dependent NO consumption and isometric tension studies assessing NO bioavailability in explanted aortic rings from rodents suggested that MPO might indeed adversely affect vasorelaxation.[12] However, direct evidence for MPO to systemically affect vascular tone *in vivo* has not yet been provided.

Here, we report vasoactive effects of MPO in humans and in a large animal model. Individuals with inherited low MPO activity were protected from leucocyte activation-induced deterioration of vascular function. Direct MPO administration in anaesthetized pigs increased the tone of conductance and resistance vessels and adversely affected myocardial blood flow (MBF), thereby strengthening the concept that MPO indeed acts as a modulator of vascular tone *in vivo*.

Methods

Human studies

A total of 12 135 consecutive patients undergoing assessment of the white blood cell count at the University Hospital Hamburg-Eppendorf were screened for neutrophil peroxidase activity using a Technicon H1 Hematology Analyzer (Bayer). Whereas no reports exist to use this screening method for identifying patients with high PMN-derived peroxidase activity, previous studies established a peroxidase index below -15 as indicative of enzyme deficiency[14]; accordingly subjects exhibiting a peroxidase index below -15 were considered eligible for this study. Patients had to be above age 18, healthy, and free of overt infections (as evidenced clinically and by a C-reactive protein level <5 mg/L at the day of the vascular function tests). Given the overall low incidence of MPO deficiency and the fact that there is no substantial evidence that leucocyte activation by nicotine differs between smokers or non-smokers current smoking was not an exclusion criterion. However, the subjects were advised not to smoke 12 h prior to vascular function testing and blood sampling. The primary endpoint was the between-group difference in flow-mediated dilation (FMD) during the study. The study was approved by the local Ethics Committee of the Board of Physicians of Hamburg and was conducted in accordance with the Declaration of Helsinki. All enrolled and screened subjects gave written informed consent.

All subjects were treated with a 4 mg nicotine containing gum, which was chewed for 30 min, a manoeuver reported to activate leucocytes.[15] Prior to and 2 h after nicotine application blood was drawn and forearm FMD according to the principles set by the international brachial artery reactivity task force[16] was assessed. Plasma was obtained and stored immediately at -80°C. Plasma levels of MPO, MMP-9, nitrite,[17] and nicotine were determined by ELISA and by gas chromatography-mass spectrometry (GC-MS), respectively. Polymorphonuclear neutrophils were isolated from whole blood anticoagulated with EDTA.[18] Myeloperoxidase protein expression in PMN was assessed by western blotting using a polyclonal antibody to MPO (Calbiochem). Myeloperoxidase activity in PMN was measured by determination of oxidation of tetramethylbenzidine as described previously.[5] Details are provided in the Supplementary material online.

Nitric oxide consumption was determined *ex vivo* in PMN lysates from MPO^{low} and control subjects using an NO electrode (amiNO-700).

To determine endothelium-dependent and independent relaxation isolated murine aortic rings were incubated with nicotine (10 μM), nicotine-activated leucocytes (1 h pre-incubation of WT-derived leucocytes with nicotine (10 μM), MPO (20 μg/mL), or control in combination with 10 μM H_2O_2 as previously.[12]

Animal studies

Experimental protocol

The experiments were performed in 16 domestic male pigs (56 ± 6 kg) at the Animal Laboratory, University Heart Center Hamburg, Germany. The study was performed in accordance with the 'Position of the American Heart Association on Research Animal Use' (Circulation, April 1985) and approved by the government animal care committee and the institutional review board for the care of animal subjects. Human MPO (18 μg/kg, Planta, Vienna, Austria) or human serum albumin (HSA) (18 μg/kg) was injected into the left atrium (infused volume: 3 ± 0.4 mL/animal) in a blinded, randomized manner. For quantitative determination of MPO plasma levels blood samples were retrieved at baseline and 1, 5, 15, 30, 60, and 90 min after MPO or HSA application and stored at -80°C until further analysis.

Myocardial blood flow measurements as well left artery descends (LAD) and IMA flow measurement were repeated at 30, 60, and 90 min. All haemodynamic parameters were continuously monitored, and recorded during each intervention.

Vessel preparation and organ chamber studies

Internal mammary artery (IMA) segments were dissected at the end of the experiment and endothelium-dependent and independent relaxation was determined in response to increasing doses of acetylcholine (Ach, 10^{-9}–10^{-6} mol/L) and nitroglycerine (NTG) as previously.[12]

Myocardial blood flow measurements using fluorescent microspheres

Approximately 4×10^6 FMs, 15 μm in diameter (Molecular Probes, Eugene, Oregon), were injected into the left atrium during withdrawal of a reference blood sample via the abdominal aorta as previously described.[19] Hearts were excised and fixed in 10% formaldehyde for 6–8 days. The anterior wall of the left ventricle was sliced into 20 wedge-shaped transmural tissue pieces. Tissue samples and arterial blood

reference were processed for determination of MBF by spectrofluorometry according to the standard method described by Glenny *et al.*[20]

Myocardial perfusion measurement using fluorescent cardiac imaging

Myocardial perfusion was assessed using a fluorescent cardiac imaging (FCI) system (LLS GmbH, Ulm, Germany) as described previously.[19] Following intravenous application of indocyanine green (ICG; 0.01 mg/kgBW) FCI sequences were recorded for 60 s (25 frames/s). To assess myocardial perfusion by FCI the slope of fluorescent intensity (SFI) derived from the time dependent fluorescence signal was calculated and correlated to fluorescent microspheres (FM) data in the corresponding area, as previously.[19]

Biochemical analyses and immunofluorescence

Myeloperoxidase plasma levels were assessed by ELISA (PrognostiX). For detection of vascular deposition of MPO, excised IMA segments (60 mm) from animals treated with MPO ($n = 4$) or with HSA ($n = 3$) were cannulated and perfused using phosphate buffered saline to remove residual blood. Then the segments were incubated for 15 min with phosphate-buffered saline containing unfractionated heparin (150 mg/mL) and the eluate was collected and analysed for MPO by ELISA. In addition vessel-adherent MPO was detected in IMA segments by immunofluorescence using a specific antibody (Calbiochem).

Further details on the animal experiments are provided in the Supplementary material online.

Statistical analysis

For the human studies we aimed to detect a minimal difference in the primary target variable, mean change in flow-mediated dilation of 2% in absolute terms, between the two groups, with a two-sided type alpha level of 0.05 and a power of 0.80. Based on previous trials and pilot data, we estimated the standard deviation of the difference between the two values for the same patient to range from 20 to 50% (in relative terms), which gave formal sample size requirements of 40 individuals. Secondary endpoints were the between-group differences in the levels markers of neutrophil activation and nitrite. Categorical data are presented as frequencies and percentages and were compared by χ^2 test or Fisher's exact test as appropriate. Continuous variables were tested for normal distribution using the Kolmogorov–Smirnov test. Normally distributed variables are presented as mean $\pm$ standard deviation; for non-normally distributed data the median and inter-quartile range are given. To consider the subject-level match, a variance analysis was applied including the factors: group (MPO^{low} or control) and an indicator variable for the matched triple.

For comparison between groups Student's unpaired *t*-test was used in case of normal distribution, in case of non-normal distribution Mann–Whitney U test was employed. For comparison within the groups, Student's paired *t*-test or Wilcoxon test was applied. For the assessment of the relationship, Spearman's rank correlation or Pearson's product-moment correlation was employed as appropriate.

For animal studies random group means and their standard errors at different time points were calculated using an adjusted two-way analysis of variance. Time trends were tested based on random coefficient models with time as regressor and group as independent factor. Within groups, trends were tested vs. no trend (slope of zero), between-groups trends were tested on equality. For the analysis of relaxation studies, a general linear model for repeated measures was applied.

Results

Human studies

Within the study period of 12 months, 30 out of the 12 135 examined subjects were detected to display markedly reduced or no detectable neutrophil peroxidase activity. Of these 30 subjects, 17 met all eligibility criteria but 2 declined to participate. Accordingly, 15 subjects—who were not related to each other—with low or no expression of the active enzyme (MPO^{low}) were included. The MPO^{low} group was matched in a 2:1 fashion with a control group characterized by non-reduced MPO expression and/or activity as assessed by MPO protein content and activity in isolated PMN ($n = 30$; control).

As displayed in *Table 1*, the baseline characteristics were similar in both groups. To exclude a heparin-induced modulation of vascular function, none of the subjects was on heparin at least 12 h prior to vascular function testing. Subjects were also advised not to smoke 12 h prior to blood sampling and vascular function testing.

To confirm that the differences in leucocyte peroxidase activity indeed reflects differences in MPO protein expression and/or activity, PMN from all participants were isolated. Polymorphonuclear neutrophils lysates from individuals in the MPO^{low} group revealed significantly reduced immunoreactivity for MPO when compared with participants in the control group (*Figure 1A*). Since the active form of MPO is processed by cleavage of precursors and deficiency in the active enzyme does not exclude the presence of these MPO precursors, lysates were also analysed for MPO activity, which was reduced in the MPO^{low} group and thus recapitulated the results obtained for total peroxidase activity in whole blood (*Figure 1B*).

Upon nicotine exposure, plasma levels of nicotine were comparable between both groups (MPO^{low}: 6.05 $\pm$ 7.1 ng/mL vs. control: 5.23 $\pm$ 3.0 ng/mL; $P = 0.59$) indicating a not significantly different systemic nicotine bioavailability between both groups.

Markers of leucocyte activation

In order to test whether nicotine indeed provoked leucocyte activation, plasma levels of MMP-9 and MPO, which are released upon activation of neutrophils, were determined. The increase in plasma levels of leucocyte-derived MMP-9 was similar in both groups following nicotine application (*Figure 2A*, mean increase MPO^{low}: 12.9 $\pm$ 6.6 ng/mL, control: 31.8 $\pm$ 14.9 ng/mL, $P = 0.39$) reflecting a comparable degree of leucocyte activation in both groups. As opposed to MPO^{low} subjects, participants in the control group exhibited a significant rise in MPO plasma levels following administration of nicotine (*Figure 2B*).

Vascular function tests

To further test whether nicotine-induced leucocyte activation indeed translates into a decreased bioavailability of endothelium-derived NO, vascular function tests were performed prior to and following nicotine exposure. Whereas baseline FMD was similar in both groups, nicotine administration significantly deteriorated FMD in subjects with normal neutrophil peroxidase activity. In contrast, nicotine did not lead to any change of FMD in MPO^{low}

Table 1 Baseline clinical characteristics

	MPO^{low} (n = 15)	Control (n = 30)	P-value
Age, years	56 ± 14.7	56 ± 15.8	0.887
Gender (male), %	40.0	40.0	0.629
BMI, kg/m^2	24.7 ± 4.2	23.5 ± 3.4	0.284
Hypertension, %	46.7	46.7	0.623
Hyperlipoproteinaemia, %	26.7	26.7	0.632
Diabetes mellitus, %	20.0	20.0	0.644
Current smoking, %	20.0	20.0	0.644
ASA, %	20.0	26.7	0.460
Clopidogrel, %	6.7	6.7	0.714
Beta-blockers, %	46.7	33.3	0.292
ACE-Inhibitors, %	20.0	26.7	0.460
CSE inhibitor,%	26.7	23.3	0.540
Haemoglobin, g/dL	13.0 ± 1.1	13.6 ± 1.4	0.226
Leucocytes, 10e^6/mL	7.48 ± 4.65	6.55 ± 2.25	0.375
Creatinine, mg/dL	1.06 ± 0.44	1.04 ± 0.64	0.914
C-reactive protein, mg/dL	0.87 ± 2.29	1.11 ± 2.68	0.762
Total cholesterol, mg/dL	171.4 ± 42.0	186.7 ± 48.1	0.302
LDL, mg/dL	86.7 ± 27.2	102.0 ± 34.6	0.144
HDL, mg/dL	63.7 ± 13.9	62.3 ± 19.1	0.811
Triglycerides, mg/dL	134.3 ± 78.7	111.7 ± 71.4	0.338
RRsys, mmHg	130 ± 21	140 ± 30	0.371
RRdia, mmHg	77 ± 15	77 ± 12	0.900
Heart rate, b.p.m.	68 ± 11	69 ± 10	0.830

BMI, body mass index; ASA, acetyl salicylic acid; ACE, angiotensin-converting enzyme, CSE, cholesterol synthase; MPO, myeloperoxidase. Mean ± SD.

subjects (*Figure 3A*). Flow ratios were not different between the two groups and remained unchanged following nicotine exposure (MPO^{low}: 6.3 ± 2.4 vs. 6.6 ± 2.8, $P = 0.75$; control: 8.7 ± 4.9 vs. 7.2 ± 3.3, $P = 0.76$; between groups: pre-treatment $P = 0.59$, post-treatment $P = 0.15$). Nitroglycerine-mediated dilation (NMD) was significantly greater in MPO^{low} subjects when compared with the control group at baseline and following nicotine administration, with nicotine not significantly altering NMD within the groups (*Figure 3B*). Correlation analysis revealed a significant inverse correlation between FMD and MPO plasma levels prior to and following nicotine application ($r = -0.33$, $P = 0.03$; $r = -0.30$, $P = 0.04$). Also, FMD upon nicotine application was inversely related to MPO activity in PMN ($r = -0.48$, $P = 0.003$, *Figure 3C*). *Ex vivo* NO consumption by cell lysates from isolated PMN was significantly reduced in MPO^{low} subjects when compared with controls. If PMN lysates from MPO^{low} subjects were fortified with exogenous MPO NO consumption was reconstituted (*Figure 3D*).

Ex vivo endothelium-dependent and independent function testing underscored the leucocyte activating effects of nicotine and reinforced the tenet, that nicotine-induced liberation of MPO from leucocytes significantly contributes to endothelial dysfunction (see Supplementary material online, *Figure S1*).

MPO^{low} subjects also displayed significantly higher baseline plasma levels of nitrite, a surrogate of NO availability, when compared with controls (2.98 ± 1.11 vs. 1.5 ± 0.75 mM, $P < 0.001$, *Figure 3E*) with baseline nitrite plasma levels being inversely correlated to circulating MPO ($r = -0.40$, $P = 0.034$). Nicotine treatment did not significantly impact on nitrite plasma levels. (3.41 ± 1.05 vs. 1.8 ± 1.23 mM, $P = 0.001$ between groups; $P = 0.31$ and $P = 0.32$ vs. baseline). There was no correlation between baseline vessel diameter and nitrite.

Animal studies

In order to explore whether *free* MPO is capable of systemically regulating vascular tone independent of the activation state of leucocytes, MPO was given to anaesthetized open-chest pigs. The study cohort consisted of 10 animals, which received MPO and 6 animals being challenged with HSA. One pig in the MPO group was lost during the experiment due to sustained ventricular arrhythmias, leaving nine pigs in the MPO group for final assessment.

Biochemical analysis

At baseline, MPO plasma levels were undetectable in both groups, indicating that intrinsic, pig-derived MPO was not recognized by the MPO-antibody used for the ELISA. Plasma levels of the administered MPO peaked immediately after injection and rapidly declined thereafter (*Figure 4*), suggesting a redistribution of the enzyme with binding of MPO to extraplasmatic compartments. In the control group no significant MPO levels could be detected (*Figure 4*).

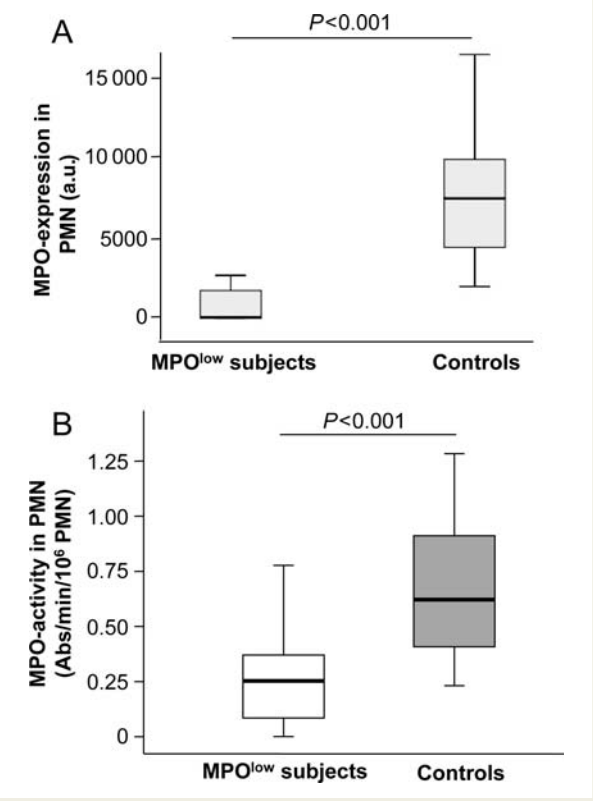


Figure 1 Assessment of myeloperoxidase protein expression and enzyme activity in human neutrophils. (*A*) Densitometric analysis of myeloperoxidase protein expression of all participants (MPO^{low}: $n = 15$; control $n = 30$). (*B*) Quantification of peroxidase activity in isolated polymorphonuclear neutrophils from all participants.

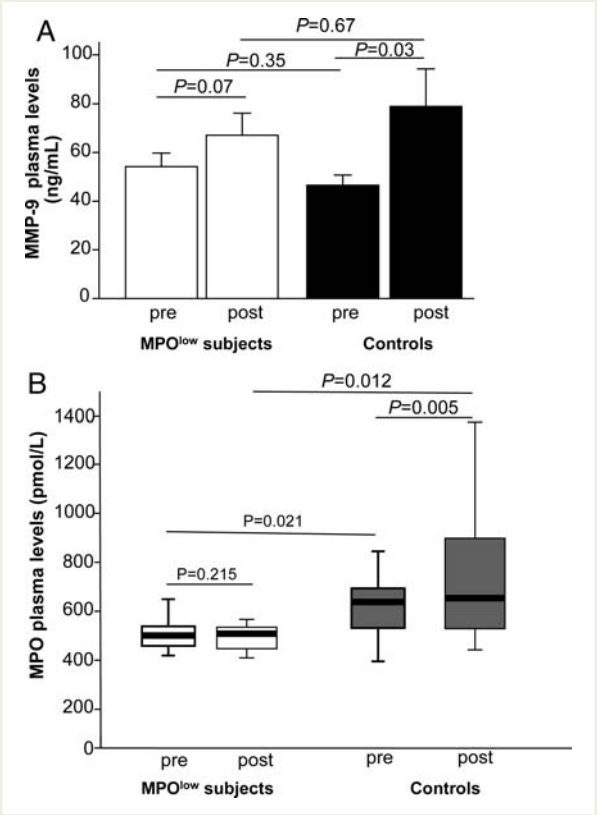


Figure 2 Leucocyte activation at baseline and following nicotine administration in humans with normal and reduced myeloperoxidase activity. (*A*) Matrix metalloproteinase-9 plasma levels before and after stimulation with nicotine. (*B*) Myeloperoxidase plasma levels before and following nicotine administration are shown. Data are displayed for the 15 subjects with reduced myeloperoxidase expression and/or activity (MPO^{low}) subjects and for the 30 matched controls.

Effect of myeloperoxidase on blood flow in internal mammary artery and left artery descends

In the control group, IMA and LAD flow remained unchanged throughout the entire study ($P = 0.563$ and $P = 0.706$), whereas IMA and LAD flow decreased significantly in the MPO group [mean decrease per 30 min: 30.2 (95% CI: 22.5–37.9) mL/min and 7.5 (95% CI: 4.1–10.9) mL/min, $P < 0.001$, *Figure 5A*].

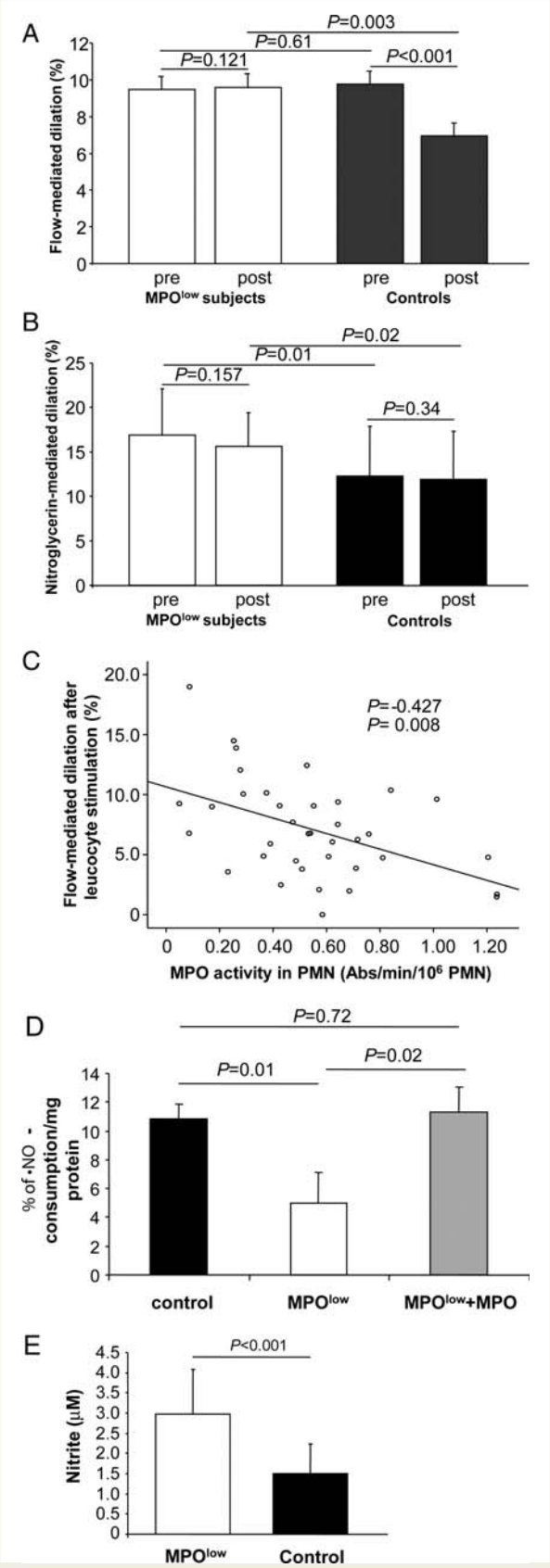
Myocardial blood flow and perfusion

In order to test whether MPO also impacts on vascular tone of resistance vessels, MBF was assessed using FM. *Figure 5B* depicts MBF assessed at baseline and 30, 60, and 90 min after MPO injection in the LAD area. In the HSA group, MBF increased throughout the experiment ($P = 0.026$ for trend). In contrast, animals subjected to MPO revealed a markedly reduced MBF during the course of the experiment ($P = 0.009$ for trend). The difference between the two treatment groups was highly significant ($P = 0.006$).

Myocardial perfusion (SFI) assessed by FCI revealed no changes over time in the HSA-treated animals ($P = 0.890$ for trend), whereas SFI decreased significantly in the MPO-treated animals ($P < 0.001$ for trend, *Figure 5C*). Slope of fluorescent intensity was significantly different between the two groups ($P = 0.002$).

Haemodynamic measurements

To assess whether systemic application of MPO finally affects haemodynamics, systemic vascular and pulmonary vascular resistance (PVR) was assessed (*Table 2*). Given that open-chest animal experiments lead to significant loss of fluid the central venous pressure (CVP) was assessed throughout the experiment. In order to reliably assess the fluid status of the animals, the heart rate was kept at 100 b.p.m. using a pacemaker. To keep the CVP constant, the infused volume of sodium chloride was lower in the MPO group when compared with the HSA group infused volume of sodium chloride (1.0 ± 0.2 l vs. 1.8 ± 0.3 l). Despite reduced volume administration in the MPO group, pulmonary artery pressure (PAP) as well as PVR increased significantly in MPO-treated animals ($P = 0.028$ and $P = 0.003$) when compared with the HSA group (*Table 2*). The systemic vascular resistance (SVR) did not change significantly in both treatment groups (*Table 2*). Of importance, all animals were normoxemic throughout the experiments.



Ex vivo endothelial function tests and assessment of vessel adherent myeloperoxidase

In order to explore whether alterations in vascular tone are a reflection of NO oxidation by MPO, IMA-segments from HSA- and MPO-treated animals were explanted and vessel relaxation in response to Ach was tested: vessel segments from MPO-treated animals revealed markedly reduced relaxation in response to Ach (89.4 ± 4% in rings from HSA-treated animals vs. 56.9 ± 3% in MPO-treated pigs, $P < 0.01$, *Figure 6A*). Exogenous addition of the MPO substrate H_2O_2 prior to Ach challenge further attenuated NO-dependent relaxation in vessels from MPO-treated animals (56.9 ± 3 and 45.4 ± 4% after addition of H_2O_2, $P = 0.021$), whereas vessels from HSA-treated pigs revealed no further impairment of vessel relaxation upon H_2O_2 (89.5 ± 5% before and 89.4 ± 4% after addition of H_2O_2, $P = 0.381$, *Figure 6A*). In addition, non-endothelial-derived relaxation in response to NTG was also attenuated in vessels rings from MPO-treated animals ($P < 0.001$; *Figure 6B*).

In order to test, whether decreased NO bioavailability and concomitant attenuated relaxation in rings from animals exposed to MPO was indeed reflected by association of MPO with the vessel wall, explanted IMA segments were perfused with heparin containing buffer. Perfusion partially recovered MPO, reflecting release of vessel-adherent MPO by heparin ($P = 0.03$ vs. HSA-treated vessel explants; *Figure 6C*). Immunohistochemistry re-confirmed vascular deposition of the enzyme in explanted IMA segments (*Figure 6D*).

Discussion

The principal findings of the current study are that MPO profoundly affects vascular tone of resistance and conductance vessels, which directly impacts on forearm blood flow in humans

Figure 3 Vascular function in humans with low and normal myeloperoxidase activity in response to nicotine. (*A*) Flow-mediated and (*B*) nitroglycerine-mediated dilation before and after nicotine stimulation. Data are displayed for all 15 subjects with diminished myeloperoxidase expression and/or activity (MPO^{low}) subjects and for the 30 matched controls. (*C*) Spearman's correlation between flow-mediated dilation following leucocyte activation and MPO activity. Myeloperoxidase-dependent consumption of nitric oxide. (*D*) Polymorphonuclear neutrophils derived from MPO^{low} subjects exhibited significantly less nitric oxide consumption when compared with controls. Exogenous addition of MPO (344 pmol/L) to myeloperoxidase-deficient polymorphonuclear neutrophils restored nitric oxide consumption. (*E*) MPO^{low} subjects also displayed significantly higher baseline plasma levels of nitrite when compared with controls with baseline nitrite plasma levels being inversely correlated to circulating myeloperoxidase ($r = -0.40$, $P = 0.034$). Nicotine treatment did not significantly impact on nitrite plasma levels.

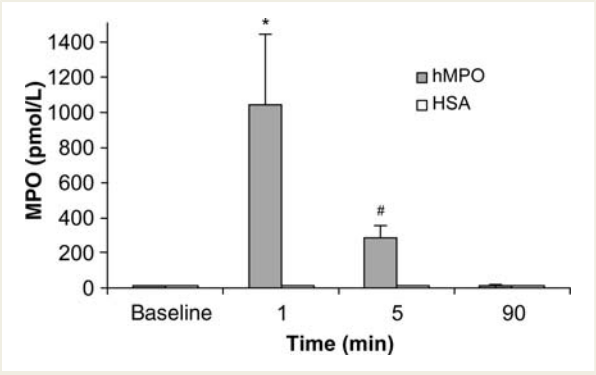


Figure 4 Myeloperoxidase plasma levels following injection of human myeloperoxidase vs. human serum albumin in an open-chest pig model. Injection of human myeloperoxidase resulted in a rapid increase in MPO levels in pig plasma ($n = 9$, as assessed by ELISA), and decreased thereafter when compared with HSA-treated ($n = 6$) animals ($^{*}P < 0.01$ vs. baseline and $^{\#}P < 0.01$ vs. 1 min).

and similarly affected epicardial, microvascular, and extramyocardial blood flow in anaesthetized pigs. Assessment of NO-dependent and independent relaxation in humans and *ex vivo* testing of NO-dependent vasorelaxation in vessels from the treated animals reinforced the tenet that MPO-driven oxidation of endothelium-derived NO in addition is one pivotal underlying mechanism for the effect of MPO on vascular tone.

Although numerous studies have advocated for MPO-driven consumption of endothelial NO, no study to date has directly assessed the haemodynamic consequences of systemic MPO release or administration under *in vivo* conditions. Whereas investigations in $MPO^{-/-}$ mice assessed vascular function and endothelial NO bioavailability in explanted rings of conductance vessels following LPS challenge,[12] studies in patients with vascular inflammatory disease only provided an association between MPO and vascular dysfunction.[3,21,22]

The current study now advances the concept of MPO-driven changes in vasomotion and reveals that liberation of MPO from leucocytes as well as administration of free MPO suffices to critically influence vascular reactivity: Following nicotine exposure, a tool to activate PMN and liberate MPO, humans with normal MPO expression/activity displayed significantly impaired FMD, whereas subjects with low MPO expression/activity were protected against the nicotine-induced deterioration of FMD. This tenet was reinforced by the observation that NO consumption was significantly reduced in PMN isolated from subjects with low MPO expression/activity. The correlation between MPO and nitrite plasma levels on the one hand and MPO levels and endothelial function on the other hand further affirms that MPO indeed impacts on vascular reactivity. Interestingly, individuals with low MPO expression/activity not only displayed improved NO-dependent vasorelaxation, baseline assessment of NO-independent relaxation was also augmented in the MPO^{low} group. This suggests that MPO under chronic conditions, e.g. by increased matrix turnover, post-translational oxidation of matrix proteins or oxidation of lipoproteins, also affects the structural integrity of the vessel wall.

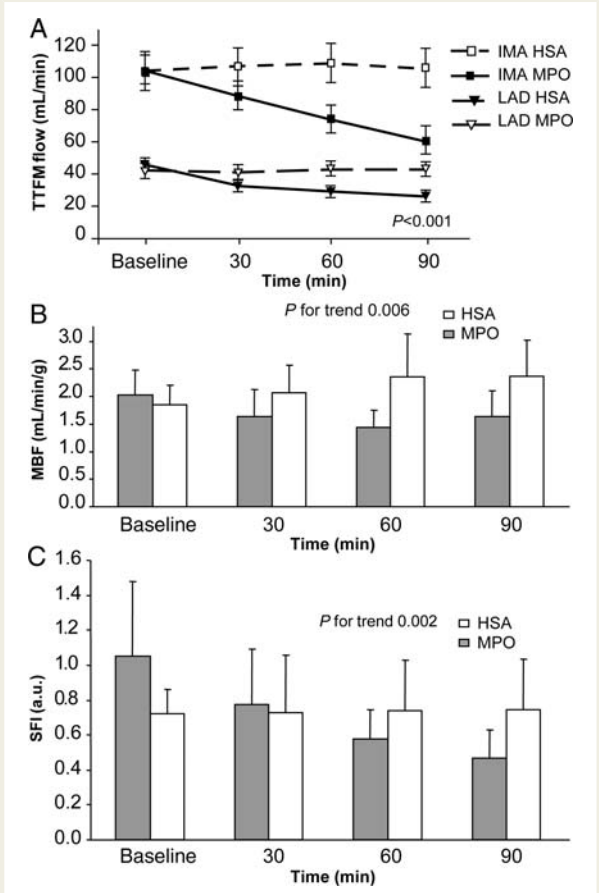


Figure 5 Effect of myeloperoxidase on blood flow of conductance vessels and myocardial blood flow in anaesthetized pigs. (*A*) Mean internal mammary artery and left artery descends flow in the myeloperoxidase group decreased significantly during the course of the experiment ($P < 0.001$) and when compared with human serum albumin-treated animals ($P < 0.001$). (*B*) Myocardial blood flow and perfusion as measured by fluorescent microspheres and fluorescent imaging in myeloperoxidase and human serum albumin-treated animals. Mean blood flow decreased significantly in the myeloperoxidase group (*P* for trend 0.009) as opposed to human serum albumin-treated animals (*P* for trend 0.026). The difference between the groups was also statistically significant ($P = 0.006$). (*C*) In accordance, myocardial perfusion (slope of fluorescent intensity) decreased in myeloperoxidase-treated animals (*P* for trend <0.001) and remained unchanged in human serum albumin-treated animals (*P* for trend 0.890). Slope of fluorescent intensity was significantly different between the groups ($P = 0.002$).

Of note, the experiments performed in pigs revisited the adverse effects of MPO on vascular function and revealed attenuated vasodilation in conductance (IMA and LAD) and resistance vessels (myocardial microvasculature). Importantly, the MPO plasma concentrations needed to elicit these effects on vascular tone in humans and in the animals did not exceed plasma levels

Table 2 Haemodynamic parameters in anaesthetized pigs

Parameter	Treatment	Baseline	30 min	60 min	90 min	*P* (trend)	*P* (trend MPO vs. trend HSA)
CO (l/min)	MPO	5.74 ± 1.51	5.60 ± 1.03	5.47 ± 0.94	5.19 ± 1.05	0.0298	$P = 0.129$
	HSA	5.19 ± 1.05	6.10 ± 1.72	5.55 ± 1.32	5.62 ± 1.34	0.116	
MAP (mmHg)	MPO	79.9 ± 15.9	80.4 ± 20.5	77.4 ± 19.2	72.3 ± 13.5	0.012	$P = 0.028$
	HSA	72.3 ± 13.5	72.0 ± 6.6	72.7 ± 10.2	76.3 ± 6.7	0.346	
PAP mean (mmHg)	MPO	31.3 ± 11.5	36.2 ± 15.5	37.6 ± 12.8	40.5 ± 15.6	0.028	$P = 0.078$
	HSA	29.3 ± 15.6	29.4 ± 14.0	29.2 ± 10.5	28.2 ± 7.6	0.830	
LAP mean (mmHg)	MPO	8.4 ± 2.4	9.0 ± 3.6	8.8 ± 3.6	8.0 ± 2.3	0.615	$P = 0.669$
	HSA	8.0 ± 2.3	8.5 ± 2.9	9.3 ± 3.4	9.2 ± 4.1	0.467	
CVP (mmHg)	MPO	5.9 ± 2.0	6.0 ± 2.2	6.2 ± 1.64	6.2 ± 1.6	0.440	$P = 0.047$
	HSA	6.2 ± 1.6	7.2 ± 2.6	8.2 ± 3.2	8.3 ± 3.3	0.019	
SVR (Picco) (dynes*s*cm^{-5})	MPO	1088 ± 425	1106 ± 381	1099 ± 345	1014 ± 334	0.492	$P = 0.455$
	HSA	1014 ± 334	934 ± 413	964 ± 295	1056 ± 350	0.306	
SVR (calculated) (dynes*s*cm^{-5})	MPO	1142 ± 536	1099 ± 345	1077 ± 341	1083 ± 423	0.492	$P = 0.535$
	HSA	1083 ± 423	933 ± 392	979 ± 335	1026 ± 349	0.384	
PVR (dynes*s*cm^{-5})	MPO	279 ± 127	366 ± 136	397 ± 142	464 ± 229	0.001	$P = 0.003$
	HSA	222 ± 145	239 ± 148	248 ± 173	249 ± 118	0.54	

CO, cardiac output; MAP, mean blood pressure; PAP, pulmonary artery pressure; LAP, left atrial pressure; CVP, central venous pressure; SVR, systemic vascular resistance and PVR, pulmonary vascular resistance. Mean ± SD.

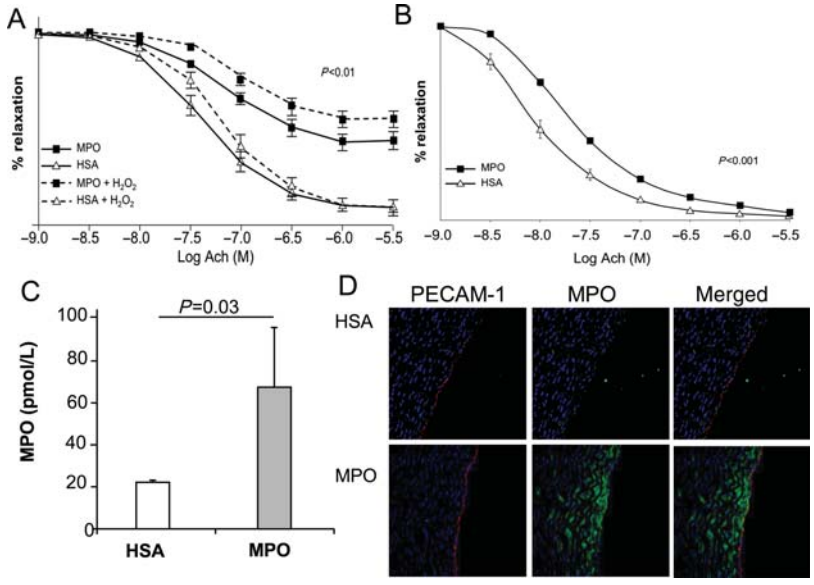


Figure 6 Nitric oxide-dependent relaxation of explanted pig vessel segments and vascular myeloperoxidase deposition. (*A*) Relaxation of explanted segments of the internal mammary artery in response to acetylcholine. There was a significant attenuation of acetylcholine-dependent relaxation in vessel segments from animals treated with myeloperoxidase (56.9 ± 3% in myeloperoxidase-treated pigs vs. 89.4 ± 4% in rings from human serum albumin-treated animals, $P < 0.01$). Addition of H_2O_2 further decreased acetylcholine-dependent vasorelaxation in rings explanted from myeloperoxidase-treated animals (45.4 ± 4%, $^*P = 0.021$), whereas vessel segments from human serum albumin-treated animals showed no H_2O_2-dependent impairment of vessel relaxation (89.5 ± 5%). (*B*) Nitroglycerine-induced relaxation was significantly reduced in explanted segments of the internal mammary artery derived from myeloperoxidase-treated animals ($P < 0.001$). (*C*) Heparin-dependent liberation of myeloperoxidase after *ex vivo* perfusion of explanted pig internal mammary artery. Vessels from myeloperoxidase-treated animals revealed increased levels of myeloperoxidase in the eluate when compared with human serum albumin-treated pigs. (*D*) Immunofluorescent imaging displayed vascular myeloperoxidase deposition in explanted internal mammary artery segments of myeloperoxidase-treated pigs when compared with human serum albumin-treated animals (red, PECAM; green, MPO; blue, DAPI; magnification ×200).

of MPO previously reported for patients with acute coronary disease, heart failure, or sepsis[23–25] and thus have to be considered pathophysiologically relevant. On the other hand, the highly dynamic change in MPO plasma levels upon exogenous MPO administration elucidates the fate of MPO in the vasculature: myeloperoxidase plasma levels peaked within minutes after injection and rapidly declined thereafter. The fact that MPO leaves the circulatory compartment not only is an important observation reflecting the avidity of this enzyme to bind to the vessel wall, but it also demonstrates that assessment of circulating MPO levels in plasma or serum poorly reflects the non-PMN associated, 'free' fraction of this enzyme. In other words, circulating MPO levels as determined in patients with acute coronary syndromes, heart failure, or acute septic disease might significantly underestimate the extent of vascular-immobilized MPO. Heparins have been shown to compete with extracellular matrix proteins for binding of MPO with the extent of MPO liberation being an indicator of vascular NO bioavailability.[22] In agreement with these findings, MPO was not only localized to the vessel wall by means of immunohistochemistry, vessel-immobilized MPO could also been retrieved upon heparinization. Importantly, MPO containing vessel rings were indicative of substantially attenuated vasorelaxation: Organ chamber isometric tension studies revealed significantly attenuated acetylcholine-dependent and thus NO-induced vasorelaxation in vessel segments from MPO-treated animals. These studies also demonstrate that MPO positive vessel rings even in the absence of exogenously added H_2O_2—the substrate of MPO—were indicative of reduced vasorelaxation. This implies that endogenous sources of H_2O_2 in the vessel wall provide sufficient substrate for MPO *in vivo*, which further supports the notion that MPO is capable of affecting vascular NO bioavailability remote of its degranulating host cell. The fact that NTG-induced relaxation was also attenuated in vessels from MPO-treated animals revisits the observations made in humans, and suggests that MPO also impacts on the structural integrity of the vessel wall.

Interestingly, the vasoactive properties of MPO were not only restricted to conductance vessels like the brachial artery in humans and the internal mammary and the left coronary artery in pigs, MPO also affected resistance vessels: Myocardial blood flow and perfusion as assessed by FM and FCI was significantly diminished in the animal model. Myeloperoxidase-dependent modulation of vascular tone was not restricted to the myocardial circulation but rather holds true for the pulmonary vascular system also: In pigs exposed to MPO, PAP and PVR increased. Given that MPO-treated animals were volume-restricted to keep the CVP equal between groups, the effect of MPO on PAP and resistance may even be underestimated. The fact that mean PAP and PVR increased upon MPO administration underscores the haemodynamic significance of the observed MPO effects since pulmonary vasomotion is vastly independent of circulatory reflexes.[26–28] Taken together, the MPO-induced increase in PVR on the one hand and the decrease in myocardial perfusion on the other hand accounted for the decrease in cardiac output, which drove the decrease in blood pressure in the MPO group.

Lower LDL levels as confounders for attenuated nicotine-induced endothelial dysfunction in MPO-deficient individuals were excluded by multivariate regression analysis. Certainly, the current study bears several limitations: (i) Using the Technicon H1 Hematology Analyzer is not an established tool to assess MPO deficiency, however it is a valid screening method before low MPO expression/activity was validated in every single subject by means of western blotting and activity assays. (ii) Since nicotine has vasoactive properties, it cannot be excluded that nicotine exposure *per se* altered vascular function—even if we did not observe significant effects of nicotine on vascular relaxation in explanted murine aorta. (iii) Furthermore, the current data cannot exclude that MPO elicits effects on endothelial NO bioavailability apart from NO oxidation; e.g. MPO-dependent inactivation of endothelial NO synthase or HOCl-mediated NO consumption, respectively.[29,30] Also, MPO suggests to modulate the structural integrity of the vessel wall, in light of the differences in endothelial-independent vasorelaxation between MPO competent individuals and those with low expression/activity of the enzyme.

However, given the profound differences in NO-dependent vasomotor function between the two groups of individuals, the significant correlation between MPO activity and flow-mediated dilation following leucocyte activation and the systemic effects of MPO administration on different vascular territories in the animals investigated the current study provides compelling evidence for MPO acting as a leucocyte-derived mediator affecting vascular NO bioavailability *in vivo*. This effect of leucocytes on vascular function may be physiological under states of acute infection and sepsis, in which the secreted MPO provides a sink for the excess synthesis of NO provided but may be detrimental under conditions of chronic vascular disease such as atherosclerosis, in which diffuse MPO sequestration into the vessel lumen may lead to oxidation of endothelium-derived NO thereby pathologically increasing vascular tone. Thus the current studies reinforces observational studies in humans with MPO deficiency indeed being characterized by a reduced occurrence of cardiovascular disease,[31] whereas increased levels of MPO were indicative of cardiovascular disease in asymptomatic individuals.[32,33]

Taken together, the current results not only call for appreciation of alternative, vasoactive properties of leucocytes, but—with respect to MPO—may point towards a novel, clinically applicable target for modulating vascular NO bioavailability under inflammatory conditions.

Supplementary material

Supplementary material is available at *European Heart Journal* online.

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