Myeloperoxidase evokes substantial vasomotor responses in isolated skeletal muscle arterioles of the rat

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Abstract

Aims: Myeloperoxidase (MPO) catalyses the formation of a wide variety of oxidants, including hypochlorous acid (HOCl), and contributes to cardiovascular disease progression. We hypothesized that during its action MPO evokes substantial vasomotor responses.

Methods: Following exposure to MPO (1.92 mU mL⁻¹) in the presence of increasing concentrations of hydrogen peroxide (H₂O₂), changes in arteriolar diameter of isolated gracilis skeletal muscle arterioles (SMAs) and coronary arterioles (CAs) and in the isometric force in basilar arteries (BAs) of the rat were monitored.

Results: Myeloperoxidase increased vascular tone to different degrees in CAs, SMAs and BAs. The mechanism of increased vasoconstriction was studied in detail in SMAs. MPO-evoked vasoconstrictions were prevented by the MPO inhibitor 4-aminobenzhydrazide (50 μM), by endothelium removal in the SMAs. Surprisingly, the HOCl scavenger L-methionine (100 μM), the thromboxane A₂ (TXA₂) antagonist SQ-29548 (1 μM) or the non-specific cyclooxygenase (COX) antagonist indomethacin (1 μM) converted the MPO-evoked vasoconstrictions to pronounced vasodilations in SMAs, not seen in the presence of H₂O₂. In contrast to noradrenaline-induced vasoconstrictions, the MPO-evoked vasoconstrictions were not accompanied by significant increases in arteriolar [Ca²⁺] levels in SMAs.

Conclusion: These data showed that H₂O₂-derived HOCl to be a potent vasoconstrictor upon MPO application. HOCl activated the COX pathway, causing the synthesis and release of a TXA₂-like substance to increase the Ca²⁺ sensitivity of the contractile apparatus in vascular smooth muscle cells and thereby to augment H₂O₂-evoked vasoconstrictions. Nevertheless, inhibition of the HOCl–COX–TXA₂ pathway unmasked the effects of additional MPO-derived radicals with a marked vasodilatory potential in SMAs.

Keywords: hydrogen peroxide, myeloperoxidase, smooth muscle calcium, thromboxane A₂, vasoconstrictions.

The effector enzyme myeloperoxidase (MPO) has a protective role in inflammatory processes. However, the activation of MPO may become deleterious and can also contribute to the development of cardiovascular diseases (Nicholls & Hazen 2005, Podrez et al. 2000, Klebanoff 2005). Accordingly, excessive levels of MPO in the plasma may be accompanied by an increased risk of subsequent cardiovascular events.
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(Baldus et al. 2003, Zhang et al. 2001c, Vita et al. 2004, Brennan et al. 2003, Karakas & Koenig 2012, Kataoka et al. 2014), whereas individuals with an inherited MPO deficiency are at a reduced cardiovascular risk (Nikpoor et al. 2001, Hoy et al. 2001). There is currently no clear explanation of this situation.

Myeloperoxidase, a haem-containing, intensely green protein, was originally isolated from canine pus and from purulent fluids from patients with tuberculosis (Klebanoff 2005, Malle et al. 2007). The synthesis of MPO is initiated in the bone marrow during myeloid differentiation and is completed in the granulocytes (Lau & Baldus 2006, Hansson et al. 2006). MPO is stored primarily in the azurophil granules of the polymorphonuclear neutrophils and monocytes, but it has also been found in tissue macrophages (Daugherty et al. 1994, Lau & Baldus 2006, Hampton et al. 1998, Klebanoff 2005). To exert its antimicrobial effects, MPO primarily catalyses the reaction of hydrogen peroxide (H₂O₂) with chloride (Hampton et al. 1998), to form hypochlorous acid (HOCl) (Malle et al. 2007, Cook et al. 2012). The activation of MPO additionally gives rise to a number of other pro-oxidative radicals through its peroxidase activity. The biological effects of the MPO (e.g. vasomotor activity, permeability, apoptotic effect) system depend on the local concentration of H₂O₂ (Golubinskaya et al. 2014) of other substrates and/or antioxidant molecules (e.g. methionine (Met) (Podrez et al. 2000, Porszasz et al. 2002). Taken together, the involvement of MPO has been implicated in vascular inflammation in association with infection, diabetes and atherosclerosis (Malle et al. 2007, Cook et al. 2012, Zhang et al. 2004, Kataoka et al. 2014, Sugiyama et al. 2001, Sipal 2009, Woods et al. 2003, Ford 2010).

It is not known at present how the persistent generation of MPO-derived oxidants evokes adverse effects in vascular tissues. MPO and its oxidative products are highly abundant in human atherosclerotic lesions (Daugherty et al. 1994, Hazen & Heinecke 1997, Hazen et al. 2000, Hazell et al. 1996). MPO is presumed to be involved in the oxidative modification of low-density lipoprotein, thereby converting it into a high uptake form and hence promoting foamy cell formation (Podrez et al. 1999, Savenkova et al. 1994). Through its catalytic activity, MPO can consume nitrogen monoxide (NO), thereby limiting its bioavailability (Eiserich et al. 2002, Abu-Soud & Hazen 2000). MPO-derived HOCl reacts with L-arginine and produces NO synthesis inhibitors (Zhang et al. 2001b, a), and HOCl can impair endothelial NO bioactivity in a superoxide-dependent manner (Stocker et al. 2004). Furthermore, MPO and HOCl can activate matrix metalloproteinases and deactivate matrix metalloproteinase inhibitors, leading to weakening of the fibrous cap and the development of destabilized atherosclerotic plaque (Karakas & Koenig 2012, Fu et al. 2001). From a functional aspect, MPO treatment led to a decrease in myocardial perfusion in pigs and inhibited the acetylcholine-evoked relaxation in the internal mammary arteries (Rudolph et al. 2012). Vasorelaxation in response to acetylcholine was also found to be impaired in mice at relatively high plasma MPO levels (Zhang et al. 2013). Nevertheless, the mechanisms through which MPO modulates the vascular responses are not well understood. In this study, we made an effort to investigate the effects of MPO activation in vascular preparations in vitro. Moreover, we tried to characterize the possible mechanism of the vasomotor action of MPO in SMAs.

As the MPO substrate H₂O₂ was earlier identified as an important regulator of vascular diameter under both normal and pathological conditions, the vasoactive effects of MPO were contrasted to those of H₂O₂. H₂O₂ evokes a concentration-dependent biphasic effect in the skeletal muscle arterioles (SMAs) and mesenteric arteries in the rat, causing vasoconstriction at lower concentrations and vasodilatation at higher concentrations (Gao et al. 2003, Cseko et al. 2004, Csató et al. 2014), whereas, H₂O₂ induces only vasodilatation in the rat coronaries (Csató et al. 2014).

In this study, we investigated (i) the acute effects of MPO on the H₂O₂-evoked changes in diameter in isolated SMAs and coronary arterioles (CAs) and on the contractile force in the basilar arteries (BAs) of the rat, and (ii) the signal transduction pathways mediating the vascular effects of MPO-derived oxidative radicals.

Materials and methods

Animals, anaesthesia and tissue dissection

Male Wistar rats (weighing 250–350 g, 6–9 weeks old) obtained from Toxic-Coop Toxicological Research Center, Dunakeszi, Hungary, were fed a standard chow and drank tap water ad libitum. Anaesthesia was performed with an intraperitoneal injection of sodium pentobarbital (150 mg kg⁻¹), and all efforts were made to minimize suffering of animals. The gracilis muscle, the heart and the brain were removed and placed into silicone-coated petri dishes containing 0–4 °C Krebs solution (composition in mM: 110 NaCl, 5.0 KCl, 2.5 CaCl₂, 1.0 MgSO₄, 1.0 KH₂PO₄, 5.0 glucose and 24.0 NaHCO₃, obtained from Sigma-Aldrich, St. Louis, MO, USA) equilibrated with a gaseous mixture of 5% CO₂, 10% O₂ and 85% N₂ at pH 7.4. All animal procedures used in this study were in full accordance with the rules of the Ethical
Committee of the University of Debrecen and approved by the appropriate governmental body Directive 2010/63/EU of the European Parliament. The study conforms with Persson PB. Good Publication Practice in Physiology 2013 Guidelines for Acta Physiol (Oxf) (Persson 2013).

Materials and drugs
The TXA2 inhibitor SQ-29548 was purchased from BioMarker Kft. (Gödöllő, Hungary). MPO protein, MPO inhibitor and COX antibodies were obtained from Abcam (Cambridge, UK). Secondary antibodies were from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA, USA). All other chemicals were from Sigma-Aldrich and were kept under the conditions recommended by the manufacturer. All reported concentrations are cumulative concentrations in the organ chamber.

Measurement of arteriolar diameter
The rat SMAs and CAs were isolated and the changes in their diameters were measured as described earlier (Csato et al. 2014). Briefly, the isolated arterioles were transferred into an organ organ and then were cannulated. The intraluminal pressure was set at 80 mmHg (pressure servo control system, Living Systems Instrumentation, St. Albans, VT, USA). The temperature was maintained at 37 °C by the built-in temperature controller in the tissue chamber (Living Systems Instrumentation). Changes in arteriolar diameter were recorded by a video microscope system (microscope: Nikon, Eclipse 80i; CCD camera: Topica Systems Instrumentation). Successful endothelium denudation was verified by the loss of dilation in response to acetylcholine (10 μM, 96 ± 4% dilation before and −6 ± 4% after endothelium removal, n = 5), while a maintained smooth muscle function was confirmed with noradrenaline (71 ± 1% constriction before and 64 ± 2% after endothelium removal). The effects of MPO and H2O2 were also measured in the presence of an MPO inhibitor (50 μM 4-aminobenzhydrazide), a TXA2 receptor inhibitor (1 μM SQ-29548) and a COX antagonist (10 μM indomethacin) in the SMAs. The effects of MPO were tested after incubation of the vessels with the HOCl scavenger L-Met (20, 40 and 100 μM) in all three vessel types. At the end of the experiments, the maximal (passive) arteriolar diameter was determined in the absence of extracellular Ca2+.

Measurement of arteriolar contractions under isometric conditions
Basilar arteries were prepared from rat brains with microsurgical tools, and approx. 4-mm-long rings were then mounted in an isometric contraction measurement system (DMT-510, Danish Myotechnology, Aarhus, Denmark). Before exposure to test solutions, vessel tone was normalized. To this end, preparations were stretched at a force by increasing 1.5 mN every 15 s until the calculated intraluminal pressure reached 13.4 kPa. The experiments were then performed at this stretch level (isometric contractions).

Experimental protocols
The endothelial function was tested with acetylcholine (1 nm–10 μM) and the smooth muscle function with noradrenaline (1 nm–10 μM, in SMAs), serotonin (1 nm–10 μM in CAs) or potassium chloride (10–60 mM, in BAs).

Myeloperoxidase activity was measured via detection of the chemiluminescence produced upon the oxidation of luminol. H2O2 working solutions were prepared from the stabilized 30% stock solution (Sigma-Aldrich) immediately before the experiments and were stored on ice. The arterioles were first treated with MPO (1.92 μM mL−1, 300 s treatment duration, diameter measured every 10 s) to record the effects of MPO alone. This was followed by the addition of H2O2 (1 μM–10 mM) and the responses to MPO+H2O2 were then determined. In the BAs, the effects of MPO and H2O2 were tested after pre-constrictions were evoked with 60 mM potassium chloride.

The mechanism of MPO-evoked vasomotor responses was explored in detail in SMAs. In some experiments, the endothelium was removed by the perfusion of air bubbles through the arterioles (denudation). Successful endothelium denudation was verified by the loss of dilation in response to acetylcholine (10 μM, 96 ± 4% dilation before and −6 ± 4% after endothelium removal, n = 5), while a maintained smooth muscle function was confirmed with noradrenaline (71 ± 1% constriction before and 64 ± 2% after endothelium removal). The effects of MPO and H2O2 were also measured in the presence of an MPO inhibitor (50 μM 4-aminobenzhydrazide), a TXA2 receptor inhibitor (1 μM SQ-29548) and a COX antagonist (10 μM indomethacin) in the SMAs. The effects of MPO were tested after incubation of the vessels with the HOCl scavenger L-Met (20, 40 and 100 μM) in all three vessel types. At the end of the experiments, the maximal (passive) arteriolar diameter was determined in the absence of extracellular Ca2+.

Simultaneous measurement of vascular diameter and intracellular Ca2+ concentrations
Simultaneous measurements of intracellular Ca2+ and arteriolar diameter were performed as described previously (Csato et al. 2014, Czikora et al. 2012, Kandasamy et al. 2013). Briefly, SMAs were isolated and cannulated as mentioned above, except that the tissue bath was supplemented with 1% bovine serum albumin (Sigma-Aldrich) and 5 μM Fura-2AM a ratiometric fluorescent Ca2+ indicator dye (Molecular Probes, Eugene, OR, USA) until a spontaneous myogenic tone
developed. Intracellular \( Ca^{2+} \) concentrations were measured with an Incyte IM system (Intracellular Imaging Inc, Cincinnati, OH, USA). Fura-2 fluorescence (recorded every 2–5 s) was excited alternately by 340- and 380-nm light, and the emitted fluorescence was detected above 510 nm. The outer arteriolar diameter was determined in each recorded image. Arteriolar \( Ca^{2+} \) concentration was determined as the difference from the initial force in the case of isometric measurements. Statistical analyses were performed with Microsoft Office Excel software by the Student’s \( t \)-test. \( P<0.05 \) was considered statistically significant.

**Results**

**MPO promotes \( H_2O_2 \)-evoked vasoconstriction**

Myeloperoxidase (1.92 mU mL\(^{-1}\)) increased the vascular tone and promoted the development of vasoconstriction in the presence of \( H_2O_2 \) in vascular beds of different origin. In the SMAs, a robust MPO-dependent vasoconstrictive effect was observed, that is from a 50 ± 21% level of vasodilation (at 1 mM \( H_2O_2 \)) to 47 ± 11% vasoconstriction following the addition of MPO (\( P = 0.004 \); Fig. 1a). In the CAs, where \( H_2O_2 \) evoked only vasodilation, MPO administration resulted in significant vasoconstriction in a wide range of \( H_2O_2 \) concentrations, for example 13 ± 4% dilation at 100 \( \mu M \) \( H_2O_2 \), but 6 ± 3% constriction following the addition of MPO (\( P = 0.006 \); Fig. 1b). In the BAs, the MPO-dependent vasoconstriction was relatively less pronounced, for example 1.1 ± 0.5 mN dilation at 100 \( \mu M \) \( H_2O_2 \) and 1.6 ± 0.7 mN constriction following the addition of MPO (\( P < 0.05 \); Fig. 1c). Vascular diameters measured under various test conditions are to be seen in Tables 1 and 2.

Myeloperoxidase alone (without the addition of its substrate \( H_2O_2 \)) did not affect the diameters of the SMAs or the CAs or the contractile force in the BAs (data not shown).

**HOCl mediates the MPO-induced vasoconstriction in the SMAs**

The mechanical effects of the chlorinating activity of MPO were assessed comparing the vascular responses in the presence of the HOCl scavenger L-Met (100 \( \mu M \)) with those in the presence of the MPO-specific inhibitor 4-aminobenzhydrazide (50 \( \mu M \)) (Fig. 2a and b). The extracellular concentration of \( H_2O_2 \) can reach as high as 300 \( \mu M \) \textit{in vivo}, and our studies were...
therefore highlighted at this H2O2 concentration. The MPO-specific inhibitor prevented the development of MPO-dependent vasoconstriction (maximal vasoconstriction at 300 μM H2O2-MPO: 47 ± 7% vs. 16 ± 6% vasoconstriction, P < 0.0001) as expected. In the presence of L-Met, however, the MPO-induced vasoconstrictions were converted to robust vasodilations (e.g. 73 ± 11% dilation at 300 μM H2O2, P < 0.0001 vs. MPO+H2O2) suggesting an MPO-evoked, but HOCl-independent vasodilation mechanism. L-Met (100 μM) alone did not affect the H2O2-evoked vasoconstriction in the absence of MPO (Fig. 2c). In a parallel in vitro enzyme assay, 100 μM L-Met fully opposed the chlorinating activity of MPO (Fig. 2d).

Divergent effects of L-Met treatments on MPO-evoked vasodilations in different vessel types

The MPO-stimulated HOCl-independent vasodilating mechanism was screened in different vascular beds (Fig. 3). In the SMAs, the above mechanism exhibited an apparent L-Met concentration dependence (maximal vasoconstriction at 300 μM H2O2 47 ± 7% vs. vasodilations of 8 ± 19, 35 ± 23 and 73 ± 11% in the presence of 20, 40 and 100 μM L-Met respectively; Fig. 3a and b). In the CA, the maximal L-Met concentration (100 μM) also provoked vasodilation at a high (1 mM) H2O2 concentration, whereas at 300 μM H2O2, L-Met did not modulate the vascular tone (i.e. 3 ± 9 vs. 13 ± 7% vasodilation; P = 0.44, Fig. 3c and d). Finally, 100 μM L-Met treatment did not significantly influence the MPO-evoked vascular responses in the BAs (e.g. 3.3 ± 0.1 mN vasoconstriction at 300 μM H2O2 vs. 4.0 ± 1 mN vasoconstriction, P = 0.61; Fig. 3e and f).

The signalling mechanism of MPO-evoked vasoconstriction in the SMAs

Endothelium removal inhibited the MPO-evoked vasoconstriction in the SMAs (e.g. 47 ± 7% vasoconstr-
Table 1 Effects of different inhibitors and endothelium removal on the MPO- and H$_2$O$_2$-induced arteriolar responses

| Type of arteriole | Coronary arterioles | Skeletal muscle arterioles |
|------------------|---------------------|---------------------------|
| Treatment        | None/Control MPO+ L-Met | None/Control MPO+ SQ-29548 MPO+ endothelium denudation MPO+ indomethacin MPO+ 100 µM MPO+ 100 µM MPO+ 40 µM MPO+ 20 µM MPO+ 4-aminobenzhydrazide |
| No. of experiments | 5 4 | 5 5 5 5 5 5 4 6 5 |
| Initial diameter | 180 ± 17 85 ± 15 182 ± 12 136 ± 15 171 ± 7 178 ± 8 115 ± 23 123 ± 8 151 ± 9 183 ± 25 187 ± 7 |
| Diameter after inhibitor | – 76 ± 12 – 141 ± 14 – 166 ± 7 112 ± 20 – 143 ± 12 176 ± 25 – |
| Diameter after MPO | 190 ± 16 73 ± 9 182 ± 12 142 ± 13 172 ± 7 168 ± 8 115 ± 19 120 ± 14 143 ± 13 175 ± 24 181 ± 8 |
| Diameter after 1 mM H$_2$O$_2$ | 191 ± 12 105 ± 15 93 ± 17 171 ± 19 179 ± 6 193 ± 8 175 ± 22 168 ± 13 184 ± 18 191 ± 26 143 ± 28 |
| Passive diameter | 234 ± 12 123 ± 10 233 ± 11 182 ± 13 190 ± 4 199 ± 8 179 ± 18 184 ± 6 193 ± 15 208 ± 26 225 ± 3 |

Tissue sources of arteriolar beds are indicated (CAs or SMAs). Diameters are shown as means ± SEM in absolute values (µm). The number of experiments performed is also indicated. Arteriolar diameters are given at the beginning of the experiments (initial diameter) and after treatment with 100 µM (the maximum constrictor dose in the control) or 10 mM (the maximum dilator dose in the control) H$_2$O$_2$. The effects of pre-incubations with inhibitors (diameter after the inhibitor) and the maximum diameter of the vessels (the passive diameter) are also indicated.
Vascular expression of COXs in the SMAs

The expression of COX isoenzymes in SMAs was tested by immunohistochemistry. Both the vascular smooth muscle layer and the endothelial cells were stained positively with the anti-COX-1 antibody, whereas the anti-COX-2 antibody did not produce a COX-specific staining pattern (Fig. 5).

Table 2 Effects of different treatments on the MPO- and H2O2-induced changes in isometric contractile force in the BAs

| Treatment               | None/Control | MPO+100 μM L-Met |
|-------------------------|--------------|------------------|
| No. of experiments      | 5            | 5                |
| Initial force           | 5.5 ± 1.70   | 0.55 ± 0.65      |
| Force after 10 mM KCl   | 1 ± 0.47     | 0.52 ± 0.41      |
| Force after 60 mM KCl   | 9.97 ± 1.41  | 7.16 ± 1.41      |
| Force after MPO         | 9.97 ± 1.41  | 8.02 ± 1.39      |
| Force after 1 mM H2O2   | 2.77 ± 0.46  | 2.35 ± 0.80      |

Force values are given as means ± SEM in absolute values (mN). The number of experiments performed is also indicated. Contractile forces refer to the beginnings of the experiments (initial force), after pre-contraction with KCl (10 mM or 60 mM) and after treatment with MPO and 1 mM H2O2.

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Figure 2 HOCl mediates the vasoconstriction evoked by MPO in the SMAs. MPO-induced vasoconstriction was inhibited with the MPO inhibitor 4-aminobenzhydrazide (50 μM) (id: 182 ± 8 μm, n = 5 arterioles from four different animals; closed triangles); however, significant vasoconstriction was still observed at 100 μM and 300 μM (P < 0.05) compared to the baseline, panel a. 100 μM L-Met converted the MPO-induced vasoconstriction to vasodilation (id: 115 ± 19 μm, n = 5 arterioles from five different animals; closed squares). Open circles represent the effects of H2O2 alone, while closed circles illustrate the effects of H2O2 in the presence of MPO. Asterisks denote significant differences from the MPO, and # denote significant differences between MPO+MPO inhibitor and MPO+L-Met. The effects of MPO alone and in combination with the MPO inhibitor or L-Met in the presence of 300 μM H2O2 (control) on the vascular diameter in the SMAs (panel b). The H2O2-induced biphasic response did not change in the presence of 100 μM L-Met (id: 120 ± 14 μm, n = 5 arterioles from five different animals; closed squares, but it caused significant vasoconstriction relative to the zero line at 30 μM and 300 μM H2O2; panel c). Increasing concentrations of L-Met inhibited the chlorinating activity of MPO in a concentration-dependent manner (100%: maximal activity without L-Met, panel d).
MPO-induced vasoconstriction develops in the absence of significant intracellular Ca\(^{2+}\) concentration changes

Measurements of the intracellular Ca\(^{2+}\) concentration and the arteriolar diameter changes were performed in parallel in the SMAs. MPO-evoked vasoconstriction (29 ± 3% vasoconstriction at 1 mM H\(_2\)O\(_2\); \(P = 0.04\) vs. the baseline) developed without significant changes in the F\(_{340/380}\) ratio signal in the range of H\(_2\)O\(_2\) concentrations between 1 \(\mu\)M and 1 mM (Fig. 6a). In contrast, the noradrenaline-evoked (1 nM–10 \(\mu\)M) vasoconstrictions with comparable magnitudes (44 ± 4% constriction at 10 \(\mu\)M noradrenaline; \(P = 0.0005\) vs. the baseline) were accompanied by
significant increases in the F 340/380 ratio (from 0.85 /C6 0.03 to 1.15 /C6 0.09; Fig. 6b). MPO alone did not have any effect on the arteriolar diameter or on the F340/380 signal (not shown).

Discussion

Vascular inflammation during endothelial dysfunction (Zhang et al. 2001a), atherosclerosis (Sugiyama et al. 2001, Sirpal 2009, Woods et al. 2003, Ford 2010), diabetes mellitus (Zhang et al. 2004, Kataoka et al. 2014) and coronary artery disease (Cavusoglu et al. 2007, Mayyas et al. 2014) is characterized by increased levels of production and local release of both H2O2 and MPO. Moreover, the increased generation of MPO was observed in neurodegenerative disorders (Reynolds et al. 1999, Pennathur et al. 1999), arthritis (Bender et al. 1986) and some cancers (Reynolds et al. 1997). We hypothesized that MPO evokes substantial vasomotor responses in the presence of H2O2. This process may have immediate (acute) effects on the vascular diameter, which was tested here under in vitro conditions. The details of intracellular mechanisms responsible for the MPO elicited vasomotor responses were studied in SMAs. The most important findings of this study are that (1) MPO has the potential to promote vasoconstriction in H2O2-treated SMAs, CAs or BAs of the rat; (2) in the SMAs, MPO facilitates the H2O2-dependent activation of COX-1 and the TXA2 receptors, resulting in an increase in the Ca2+ sensitivity of force production in the smooth muscle cells; and (3) L-Met inhibits the chlorinating activity of MPO and converts MPO-evoked vasoconstrictions to vasodilations in the SMAs.

The question arises as to whether the observed decreased vasodilation in the presence of MPO originates from H2O2 consumption by MPO, thereby requiring a higher nominal H2O2 concentration to produce comparable vasodilations. At lower concentrations of H2O2, the level of vasoconstriction was similar in the absence and in the presence of MPO, while at higher concentrations of H2O2, MPO led to higher maximal vasoconstriction levels, thereby suggesting that MPO did not simply shift the apparent H2O2 concentration dependences of the vascular responses. We therefore postulate alternative mechanisms for the explanation of the MPO-dependent vascular effects.

One of the major products of the MPO-mediated conversion of H2O2 is HOCl. Our in vitro vascular measurements were performed in Ca2+ containing Krebs solution which provided the chloride ions for the MPO to generate HOCl. The mechanisms through which HOCl can affect vascular tissues have been examined by a number of research groups. HOCl initiates the halogenation, nitration and oxidative cross-linking of amino acids, lipids and nucleotides (Pruzt 1996, Albrich et al. 1981). Less is known about the molecular pathways involved in the HOCl-evoked changes in vascular dynamics. One such possibility
relates to a decrease in NO bioavailability, as suggested by observations on HOCl-dependent impairments in endothelial function (Yang et al. 2006, Stocker et al. 2004, Xu et al. 2006). Similarly to our findings, HOCl was found to cause vasoconstriction in bovine pulmonary arteries, but the exact mechanism of this effect remained unclear (Turan et al. 2000). The present investigation revealed increases in vasoconstriction in the SMAs, CAs and BAs, thereby extending the range of vascular beds affected in this way by MPO. We additionally made an effort to identify the molecular mechanisms contributing to these vasoconstrictive effects, besides to the decreased NO bioavailability reported earlier. One of the major observations was that the widely accepted HOCl scavenger L-Met (Okabe et al. 1993, Zhang et al. 2003, 2004) not only inhibited the vasoconstriction evoked by MPO, but also unmasked a robust vasodilatory effect in the SMAs. The employed MPO-specific inhibitor, 4-aminobenzhydrazide, blocked both the chlorinating and the peroxidase activities of the MPO (Malle et al. 2007, Kettle et al. 1995, 1997) and prevented the vasoconstriction evoked by MPO. In the presence of 4-aminobenzhydrazide and MPO, however, the vascular responses to H2O2 did not differ significantly from those in the absence of MPO. Collectively, the above data suggested that MPO-mediated chlorination has a major role in the activation of a signalling pathway leading to vasoconstriction. L-Met not only antagonized this effect, but revealed an additional MPO-dependent mechanism leading to vasodilation. This latter effect was probably related to the peroxidase activity of MPO that was not inhibited by L-Met. It is worthy of consideration that in the CAs and BAs, where MPO-evoked vasoconstrictions were less pronounced than those in the SMAs, L-Met did not result in significant vasodilations, which is suggestive of differential expressions of the MPO-responsive vasodilatory pathways in the different vascular beds.

Effector structures responding to MPO-derived radicals were first tested by removal of the endothelium in SMAs, which eliminated the endothelium-derived effects, including decreased NO bioavailability reported earlier (Stocker et al. 2004, Xu et al. 2006, Turan et al. 2000). Importantly, H2O2-evoked vasoconstrictions were found in a previous study to be completely endothelium dependent (Csató et al. 2014). However, the vasoconstriction evoked by H2O2 in the presence of MPO was only partially opposed by endothelium removal (Fig. 4a), suggesting that the MPO-evoked vasoconstriction was only partially endothelium dependent. These observations, together with those in the presence of the COX inhibitor indomethacin and the TXA2 inhibitor SQ-29548, implicate that MPO causes the generation of a vasoconstrictive prostanoid derivate (potentially TXA2) not only in the endothelial cells, but also in the vascular smooth muscle cells, through the activation of COXs. To confirm this possibility, the expression of COXs enzymes was
explored by means of immunohistochemistry, and COX-1-specific staining was indeed confirmed both in the endothelial layer and in the smooth muscle cells of the SMAs. Interestingly, not only was the MPO-mediated vasoconstriction prevented by either TXA2 receptor inhibition or COX inhibition, but similarly as when L-Met was applied, it was converted to vasodilation. A role for TXA2 was implicated by its pharmacological inhibitor; nevertheless, we did not examine TXA2 production upon MPO exposures. Taken together, we postulate that the MPO-evoked vasoconstriction is mediated by a vasoconstrictive prostanoïd derivative through TXA2 receptor activation. Hence, the above findings point to a HOCl–COX1–TXA2 pathway as being decisive in the prevention of MPO-dependent vasodilation in the SMAs (Fig. 7).

Numerous previous studies have furnished evidence that H2O2 is an important regulator of the vascular diameter (Matoba et al. 2003, Koller & Bagi 2004, Miura et al. 2003, Gao & Lee 2005, Gao et al. 2003, Gao & Lee 2001). It is difficult to specify the physiologic concentration of H2O2 in vascular tissues in vivo. Nevertheless, it has been found that under pathological conditions, it may increase up to 0.3 mM. In our study, H2O2 was used in a wide concentration range (1 μM–10 mM), thus covering also pharmacological levels. This approach allowed us to reveal the mechanisms of MPO-derived vascular effects developing on top of the biphasic H2O2-dependent responses (Liu & Zweier 2001, Root & Metcalf 1977, Cseko et al. 2004). In higher concentrations, H2O2 may cause vasodilation. The possible mechanism of the H2O2-evoked vasodilation has been investigated by a number of groups in different vessel types (Iida & Katusic 2000, Thengchaisri & Kuo 2003, Zhang et al. 2012). Our previous results implicated the involvement of the NO/cyclic guanosine monophosphate pathway and the activation of K⁺ channels in SMAs (Cseko et al. 2004).

Under pathological conditions associated with inflammation, such as acute infections (Hampton et al. 1998, Pullar et al. 2000, Hirche et al. 2005), diabetes (Zhang et al. 2004, Kataoka et al. 2014), atherosclerosis (Sugiyama et al. 2001, Sirpal 2009, Woods et al. 2003, Ford 2010), arthritis (Bender et al. 1986), Alzheimer disease (Reynolds et al. 1999) and
Parkinson’s disease (Pennathur et al. 1999), MPO is released together with H2O2. In vivo conditions, MPO is released together with H2O2. Under these circumstances, L-Met may prevent H2O2-evoked vasoconstriction or even convert it into vasodilation, because L-Met in its presumed physiological concentration range (i.e. 20–40 μM) (Mayo Medical Laboratories 2015) also largely prevents the vasoconstrictions evoked by MPO in the SMAs. Hence, the ultimate effect on the vascular tone and thereby on local microcirculation will be a function of the availability of a range of local regulators (e.g. H2O2, MPO, L-Met) which are of high potency in vasoregulation (Cséko et al. 2004).

The MPO-induced vasoconstrictions were not accompanied by significant increases in the intracellular Ca2+ concentration in the H2O2 concentration range of between 100 μM and 1 mM. In contrast, noradrenaline treatment evoked vasoconstrictions to similar degrees, together with significant increases in the intracellular Ca2+ concentration, suggesting that MPO (similarly to the thromboxane A2 receptor agonist U46619) activated a Ca2+-sensitizing mechanism, causing vasoconstriction rather than increasing the intracellular Ca2+ concentration (Csáto et al. 2014). The mechanism of MPO-mediated vasodilation was beyond the scope of this study.

Overall, our present results suggest that MPO-derived HOCl can enhance the production of a TXA2-like vasoconstrictive molecule both in the endothelium and in the vascular smooth muscle cells of SMAs, thereby increasing the sensitivity of the contractile protein machinery in the vascular smooth muscle cells to produce vasoconstriction. Nevertheless, in the absence of a functional HOCl–COX1–TXA2 pathway, an MPO-dependent vasodilatory mechanism may prevail in the SMAs of the rat during tissue inflammation associated with neutrophil degranulation.

**Study limitations**

In this study, we aimed to explore the effects of MPO and H2O2 in vascular preparations with different origins. Due to differences in vascular diameters for SMAs, CAs and BAs (i.e. approx. 160 μm, approx. 180 μm and approx. 250 μm respectively), the same experimental set-up could not be employed for all vascular beds. Prior to test incubations, spontaneous myogenic tone developed in isotonic preparations (SMAs and CAs), while during isometric measurements (BAs), agonist-induced constrictions were applied. Consequently, the extent of the observed vascular responses may reflect differences in experimental arrangements. Nevertheless, the direction of vascular responses (vasodilation vs. vasoconstriction) could be determined convincingly because results were contrasted to controls under the same experimental conditions.

**Significance**

Cardiovascular diseases are associated with inflammation and increased oxidative stress. An understanding of the physiological responses as concerns pro-oxidant mechanisms may contribute to the development of new and more effective drugs in the fight against cardiovascular diseases. The most important message of this paper is that L-Met not only has the potential to prevent the vasoconstrictive responses due to activation of the HOCl–COX1–TXA2 pathway, but can evoke pronounced vasodilations in the presence of the proinflammatory enzyme MPO.

**Acknowledgments**

The work was funded by grants from the Hungarian Scientific Research Fund (OTKA): K 84300 (to AT) and K 109083 (to ZP), and the Hungarian Social Renewal Operational Program TÁMOP-4.2.A-11/1KONV-2012-0045.

**Conflicts of interest**

None declared.

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