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

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Myeloperoxidase-mediated vasoregulation

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Abstract

Aims: Myeloperoxidase (MPO) catalyzes 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 substantional vasomotor responses.

Methods: Following exposure to MPO (1.92 mU ml^{-1}) in the presence of increasing concentrations of hydrogen peroxide (H_2O_2) 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: MPO 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 nonspecific cyclooxygenase (COX) antagonist indomethacin (1 µM) converted the MPO-evoked vasoconstrictions to pronounced vasodilations in SMAs; not seen in the presence of H_2O_2. In contrast to norepinephrine-induced vasoconstrictions, the MPO-evoked vasoconstrictions were not accompanied by significant increases in arteriolar [Ca^{2+}] levels in SMAs.

Conclusion: These data showed, H_2O_2-derived HOCl to be a potent vasoconstrictor upon MPO application. HOCl activated the COX pathway, causing the synthesis and release of TXA2-like substance to increase the Ca^{2+} sensitivity of the contractile apparatus in vascular smooth muscle cells and thereby to augment H_2O_2-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.

Key words: hydrogen peroxide, myeloperoxidase, smooth muscle calcium, thromboxane A2, vasoconstrictions

Introduction

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 and 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 (Baldus et al., 2003, Zhang et al., 2001c, Vita et al., 2004, Brennan et al., 2003, Karakas and 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.

MPO, a heme-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 and 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 and Baldus, 2006, Hampton et al., 1998, Klebanoff, 2005). To exert its antimicrobial effects, MPO primarily catalyzes the reaction of hydrogen peroxide (H_2O_2) 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 H_2O_2 (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, Sirpal, 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 and 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 and Hazen, 2000). MPO-derived HOCl reacts with L-arginine and produces NO-synthesis inhibitors (Zhang et al., 2001b, Zhang et al., 2001a), 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 and 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 the present 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.

Since the MPO substrate H_2O_2 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 H_2O_2. H_2O_2 evokes a concentration-dependent biphasic effect in the skeletal muscle arterioles (SMAs) and mesenteric arteries in the rat, causing vasoconstriction at lower concentrations, and vasodilation at higher concentrations (Gao et al., 2003, Cseko et al., 2004, Csato et al., 2014), whereas, H_2O_2 induces only vasodilation in the rat coronaries (Csato et al., 2014).

In the present study, we investigated (i) the acute effects of MPO on the H_2O_2-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, anesthesia and tissue dissection

Male Wistar rats (weighing 250-350 g, 6-9 weeks old) obtained from Toxi-Coop Toxicological Research Center, Dunakeszi, Hungary) were fed a standard chow and drank tap water *ad libitum*. Anesthesia was performed with an intraperitoneal injection of sodium pentobarbital (150 mg kg^{-1}) 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 $CaCl_2$, 1.0 $MgSO_4$, 1.0 KH_2PO_4, 5.0 glucose and 24.0 $NaHCO_3$, obtained from Sigma-Aldrich, St. Louis, MO, USA) equilibrated with a gaseous mixture of 5% CO_2, 10% O_2 and 85% N_2 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 is conforming 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 (St. Louis, MO, USA) 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 mesaured as described earlier (Csato et al., 2014). Briefly, the isolated arterioles were transferred into an organ chamber 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, St. Albans, VT, USA). Changes in arteriolar diameter were recorded by a video microscope system (microscope: Nikon, Eclipse 80i; CCD camera: Topica Technology Co Ltd, Taipei, Taiwan; video digitalizer: National Institutes, Bethesda, USA). The isolated SMAs and CAs spontaneously developed a substantial myogenic tone (a decrease in diameter from 196±6 μm to 160±6 μm, n=45, and from 234±14 μm to 178±14 μm, n=9, respectively) in response to an intraluminal pressure of 80 mmHg.

Measurement of arteriolar contractions under isometric conditions

BAs were prepared from rat brains with microsurgical tools, and ~ 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 norepinephrine (1 nM-10 μM, in SMAs), serotonin (1 nM-10 μM, in CAs) or potassium chloride (10-60 mM, in BAs).

MPO activity was measured via detection of the chemiluminescence produced upon the oxidation of luminol. H_2O_2 working solutions were prepared from the

stabilized 30% stock solution (Sigma Aldrich, St. Louis, MO, USA) immediately before the experiments and were stored on ice. The arterioles were first treated with MPO (1.92 mU 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 H_2O_2 (1 μM-10 mM) and the responses to MPO+H_2O_2 were then determined. In the BAs, the effects of MPO and H_2O_2 were tested after precontractions 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 norepinephrine (71±1% constriction before and 64±2% after endothelium removal). The effects of MPO and H_2O_2 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 (1 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 Ca^{2+}.

Simultaneous measurement of vascular diameter and intracellular Ca^{2+} concentrations

Simultaneous measurements of intracellular Ca^{2+} 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, St. Louis, MO, USA) and 5 μM Fura-2AM, a ratiometric fluorescent Ca^{2+} 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 Fura-2 fluorescence ratio ($F_{340/380}$).

Immunohistochemistry

The gracilis muscle was removed from the rat and embedded in Tissue-Tek O.C.T compound (Electron Microscopy Sciences; Hatfield, PA, USA). Cryostat sections (10-μm-thick, Electron Microscopy Sciences; Hatfield, PA, USA) were prepared, fixed in acetone for 5-10 min and blocked with normal goat sera for 20 min (1.5% in PBS, Sigma-Aldrich; St. Louis, MO, USA). COX enzymes were stained with COX-1 (Rabbit anti COX1: ab109025, dilution: 1:50) and COX-2-specific antibodies (Rabbit anti-COX2: ab15191, dilution: 1:50). Antibodies were visualized through the use of fluorescent secondary antibodies (Goat anti-rabbit biotin, dilution: 1:100; goat anti-mouse FITZ, dilution: 1:300). Gracilis muscle was co-stained with anti-smooth muscle actin (NCL-SMA, dilution, 1:20; Novocastra Laboratories, Newcastle, UK) and DAPI

(Vector Laboratories, Burlingame, California, USA). Pictures were processed by ImageJ software (NIH, Bethesda, MD, USA).

Measurement of inhibitory effect of L-Met on the chlorinating activity of MPO

MPO-evoked chlorinating activity was measured with a commercial assay kit (Cayman Chemical Company, Ann Arbor, MI, USA) in accordance with the manufacturer's instructions. The measurement is based on the cleavage of nonfluorescent 2-[6-(4-aminophenoxy)-3-oxo-3H-xanthen-9-yl] benzoic acid (APF) to fluorescein by MPO-generated hypochlorite (-OCl). The reaction mixtures contained 45 µM APF, 30 µM H_2O_2, 3 U l^{-1} MPO and 200-0.39 mM L-Met (serially diluted). The measurements were performed in phosphate-buffered saline (PBS, pH=7.4) independently from the *in vitro* vascular experiments. Changes in fluorescence intensity (λ_{ex}=485 nm, λ_{em}=520 nm) were measured at 30-s intervals for 5 min with a plate reader (NovoStar plate reader, BMG Labtech). Fluorescence intensities values were plotted as a function of time and fitted by linear regression (before saturation). The slope of this relation was used to calculate MPO activities.

Data analysis and statistical procedures

The internal diameters of arterioles are shown as means±SEM. Arteriolar constriction was expressed as the change in diameter as a percentage of the initial diameter (before addition of the vasoactive agents) measured at an intraluminal pressure of 80 mmHg. Arteriolar dilation was calculated as the percentage of the maximal (passive) diameter determined in the absence of extracellular Ca^{2+} at the end of the experiments. The contractile force was indicated in absolute values, as the difference from the initial force in the case of isometric measurements. Statistical analyses were

249 performed with Microsoft Office Excel software by the Student's *t*-test. $P<0.05$ was
250 considered statistically significant.
251
252 **Results**
253 **MPO promotes H_2O_2-evoked vasoconstriction**
254 MPO (1.92 mU ml^{-1}) increased the vascular tone, and promoted the development of
255 vasoconstriction in the presence of H_2O_2 in vascular beds of different origin. In the
256 SMAs, a robust MPO-dependent vasoconstrictive effect was observed, *i.e.* from a
257 50±21% level of vasodilation (at 1 mM H_2O_2), to 47±11% vasoconstriction following
258 the addition of MPO (P=0.004; Fig. 1A). In the CAs, where H_2O_2 evoked only
259 vasodilation, MPO administration resulted in significant vasoconstriction in a wide
260 range of H_2O_2 concentrations, *e.g.* 13±4% dilation at 100 μM H_2O_2, but 6±3%
261 constriction following the addition of MPO (P=0.006; Fig. 1B). In the BAs, the MPO-
262 dependent vasoconstriction was relatively less pronounced *e.g.* 1.1±0.5 mN dilation
263 at 100 μM H_2O_2 and 1.6±0.7 mN constriction following the addition of MPO ($P<0.05$;
264 Fig. 1C). Vascular diameters measured under various test conditions are to be seen
265 in Tables 1 and 2.
266 MPO alone (without the addition of its substrate H_2O_2) did not affect the
267 diameters of the SMAs or the CAs or the contractile force in the BAs (data not
268 shown).
269
270 **HOCl mediates the MPO-induced vasoconstriction in the SMAs**
271 The mechanical effects of the chlorinating activity of MPO were assessed comparing
272 the vascular responses in the presence of the HOCl scavenger L-Met (100 μM) with
273 those in the presence of the MPO-specific inhibitor 4-aminobenzhydrazide (50 μM)

(Fig. 2A and 2B). The extracellular concentration of H_2O_2 can reach as high as 300 μM *in vivo*, and our studies were therefore highlighted at this H_2O_2 concentration. The MPO-specific inhibitor prevented the development of MPO-dependent vasoconstriction (maximal vasoconstriction at 300 μM H_2O_2+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.* to 73±11% dilation at 300 μM H_2O_2, $P<0.0001$ *vs.* MPO+H_2O_2) suggesting an MPO-evoked, but HOCl-independent vasodilation mechanism. L-Met (100 μM) alone did not affect the H_2O_2-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 H_2O_2 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 3B). In the CA, the maximal L-Met concentration (100 μM) also provoked vasodilation at a high (1 mM) H_2O_2 concentration, whereas at 300 μM H_2O_2 L-Met did not modulate the vascular tone (*i.e.* 3±9% *vs.* 13±7% vasodilation; $P=0.44$, Fig. 3C and 3D). Finally, 100 μM L-Met treatment did not significantly influence the MPO-evoked vascular responses in the BAs (*e.g.* 3.3±1 mN vasoconstriction at 300 μM H_2O_2 *vs.* 4.0±1 mN vasoconstriction, $P=0.61$; Fig. 3E and 3F).

The signaling mechanism of MPO-evoked vasoconstriction in the SMAs

Endothelium removal inhibited the MPO-evoked vasoconstriction in the SMAs (*e.g.* 47±7% vasoconstriction at 300 μM H_2O_2+MPO with intact endothelium, *vs.* 13±15% vasoconstriction + MPO without endothelium, *P*=0.07; Fig. 4A).

Next, the involvement of the TXA2 receptors in the MPO-evoked vasoconstrictive effects was tested. Inhibition of the TXA2 receptors by 1 μM SQ-29548 converted the MPO-evoked vasoconstrictions to vasodilations (*e.g.* 47±7% vasoconstriction at 300 μM H_2O_2+MPO *vs.* 30±17% dilation at 300 μM H_2O_2+MPO+TXA2 receptor inhibitor; P=0.002, Fig. 4B).

The role of COXs in the MPO-evoked vascular responses was also examined by using the nonspecific COX inhibitor indomethacin (1 μM); similarly to TXA2 inhibition, this not only prevented the MPO-evoked vasoconstriction, but converted it that to vasodilation (47±7% vasoconstriction at 300 μM H_2O_2 *vs.* 69±16% vasodilation; *P*=0.002; Fig. 4C).

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).

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_2O_2; *P*=0.04 *vs.* the baseline) developed without significant changes in the $F_{340/380}$ ratio signal in the range of H_2O_2 concentrations between 1 µM and 1 mM (Fig. 6A). In contrast, the norepinephrine-evoked (1 nM-10 µM) vasoconstrictions with comparable magnitudes (44±4% constriction at 10 µM norepinephrine; *P*=0.0005 *vs.* the baseline) were accompanied by significant increases in the $F_{340/380}$ ratio (from 0.85±0.03 to 1.15±0.09; Fig. 6B). MPO alone did not have any effect on the arteriolar diameter or on the $F_{340/380}$ 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), coronary artery disease (Cavusoglu et al., 2007, Mayyas et al., 2014) is characterized by increased levels of production and local release of both H_2O_2 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 H_2O_2. 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 H_2O_2-treated SMAs, CAs or BAs of the rat; (2) in the SMAs, MPO facilitates the H_2O_2-dependent activation of COX-1 and the TXA2 receptors, resulting in an increase in the Ca^{2+} sensitivity of force production in

348 the smooth muscle cells; and (3) L-Met inhibits the chlorinating activity of MPO, and
349 converts MPO-evoked vasoconstrictions to vasodilations in the SMAs.

350 The question arises as to whether the observed decreased vasodilation in the
351 presence of MPO originates from H_2O_2 consumption by MPO, thereby requiring a
352 higher nominal H_2O_2 concentration to produce comparable vasodilations. At lower
353 concentrations of H_2O_2, the level of vasoconstriction was similar in the absence and
354 in the presence of MPO, while at higher concentrations of H_2O_2 MPO led to higher
355 maximal vasoconstriction levels, thereby suggesting that MPO did not simply shift the
356 apparent H_2O_2 concentration dependences of the vascular responses. We therefore
357 postulate alternative mechanisms for the explanation of the MPO-dependent vascular
358 effects.

359 One of the major products of the MPO-mediated conversion of H_2O_2 is HOCl.
360 Our *in vitro* vascular measurements were performed in Ca^{2+} containing Krebs
361 solution which provided the chloride ions for the MPO to generate HOCl. The
362 mechanisms through which HOCl can affect vascular tissues have been examined by
363 a number of research groups. HOCl initiates the halogenation, nitration and oxidative-
364 crosslinking of amino acids, lipids and nucleotides (Prutz, 1996, Albrich et al., 1981).
365 Less is known about the molecular pathways involved in the HOCl-evoked changes
366 in vascular dynamics. One such possibility relates to a decrease in NO bioavailability,
367 as suggested by observations on HOCl-dependent impairments in endothelial
368 function (Yang et al., 2006, Stocker et al., 2004, Xu et al., 2006). Similarly to our
369 findings, HOCl was found to cause vasoconstriction in bovine pulmonary arteries, but
370 the exact mechanism of this effect remained unclear (Turan et al., 2000). The present
371 investigation revealed increases in vasoconstriction in the SMAs, CAs and BAs,
372 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, Zhang et al., 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, Kettle et al., 1997) and prevented the vasoconstriction evoked by MPO. In the presence of 4-aminobenzhydrazide and MPO however, the vascular responses to H_2O_2 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 signaling 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 (Stocker et al., 2004, Xu et al., 2006, Turan et al., 2000). Importantly, H_2O_2-evoked vasoconstrictions were found in a previous study to be completely endothelium-dependent (Csato et al., 2014). However, the vasoconstriction evoked by H_2O_2 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 indomethachin and the TXA2 inhibitor SQ-29548, implicate that MPO causes the generation of a vasoconstrictive prostanoide 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 prostanoid 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 H_2O_2 is an important regulator of the vascular diameter (Matoba et al., 2000, Yada et al., 2003, Matoba et al., 2003, Koller and Bagi, 2004, Miura et al., 2003, Gao and Lee, 2005, Gao et al., 2003, Gao and Lee, 2001). It is difficult to specify the physiologic concentration of H_2O_2 in vascular tissues *in vivo*. Nevertheless, it has been found that under pathologic conditions it may increase up to 0.3 mM. In our study, H_2O_2 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 H_2O_2 dependent responses (Liu and Zweier, 2001, Root and Metcalf, 1977, Cseko et al., 2004). In higher concentrations H_2O_2 may cause vasodilation. The possible mechanism of the H_2O_2-evoked vasodilation has been investigated by a number of groups in different vessel types (Iida and Katusic, 2000, Thengchaisri and 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 disease (Pennathur et al., 1999) MPO is released together with H_2O_2. In vivo conditions, MPO is released together with H_2O_2. Under these circumstances L-Met may prevent H_2O_2-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.* H_2O_2, MPO, L-Met, *etc.*) which are of high potency in vasoregulation (Cseko et al., 2004).

The MPO-induced vasoconstrictions were not accompanied by significant increases in the intracellular Ca^{2+} concentration in the H_2O_2 concentration range of between 100 μM and 1 mM. In contrast, norepinephrine treatment evoked vasoconstrictions to similar degrees, together with significant increases in the intracellular Ca^{2+} concentration, suggesting that MPO (similarly to the thromboxane

A2 receptor agonist U46619) activated a Ca^{2+}-sensitizing mechanism, causing vasoconstriction rather than increasing the intracellular Ca^{2+} concentration (Csato 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.

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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. ~160 μm, ~180 μm, ~250 μm, respectively) the same experimental set-up could not be employed for all vascular beds. Prior 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.

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676

677 **Significance**

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

685 **Tables**

686 **Table 1. Effects of different inhibitors and endothelium removal on the MPO-**
687 **and H_2O_2-induced arteriolar responses**

688 Tissue sources of arteriolar beds are indicated (CAs or SMAs). Diameters are shown
689 as means±S.E.M. in absolute values (μm). The number of experiments performed is
690 also indicated. Arteriolar diameters are given at the beginning of the experiments
691 (initial diameter) and after treatment with 100 μM (the maximum constrictor dose in
692 the control) or 10 mM (the maximum dilator dose in the control) H_2O_2. The effects of
693 preincubations with inhibitors (diameter after the inhibitor) and the maximum diameter
694 of the vessels (the passive diameter) are also indicated.

695 **Table 2. Effects of different treatments on the MPO-and H_2O_2-induced changes**
696 **in isometric contractile force in the BAs**

Force values are given as means±S.E.M. in absolute values (mN). The number of experiments performed is also indicated. Contractile forces refer to the beginnings of the experiments (initial force), after precontraction with KCl (10 mM or 60 mM), and after treatment with MPO and 1 mM H_2O_2.

Table 1.

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 L-Met	100 µM L-Met	MPO+ 40 µM L-met	MPO+ 20 µM L-Met	MPO+4-amino-benzhydrazide
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	188±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_2O_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

Table 2.

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±1.59
Force after 1 mM H_2O_2	2.77±0.46	2.35±0.80

Figure legends

Figure 1. MPO promotes H_2O_2-evoked vasoconstriction in different vascular beds

After preincubation with MPO (activity: 1.92 mU ml^{-1}, 600 s), isolated, cannulated SMAs (initial diameter (id): 182±12 µm, n=5 arterioles from 4 different animals; panel **A**) or CAs (id: 180±17 µm, n=5 arterioles from 5 different animals; panel **B**) with intact endothelium were treated with increasing concentrations (1 µM-10 mM) of H_2O_2. In SMAs H_2O_2 alone (10 µM, 30 µM and 100 µM) evoked significant vasoconstriction compared to the zero line (P<0.02). In the presence of MPO, H_2O_2 caused significant vasoconstriction from 10 µM-1 mM H_2O_2 compared to the control and the zero line (P<0.05, panel **A**) In CAs H_2O_2 (30 µM and 100 µM) and MPO evoked significant vasoconstriction comparing to the control (P<0.05) which was not significant compared to the baseline (panel **B**) The arteriolar diameter was recorded and cumulative concentration-response relationships were determined. Changes in relative arteriolar diameter are shown. Values during vasodilations are expressed as percentages of the difference between the maximal passive diameter (maximal dilation (100%) in the absence of extracellular Ca^{2+}) and the initial diameter, while constriction is expressed as a percentage of the initial diameter (illustrated at 0% on the y scale). Similarly, isolated BAs (n=5 arterioles from 5 different animals) precontracted with KCl were incubated in the presence of MPO (activity: 1.92 mU ml^{-1}, 600 s). Arteries were exposed to the increasing concentrations of H_2O_2 (1 µM-3 mM, panel **C**). H_2O_2 evoked vasoconstriction was significant at 30 µM, whereas in the presence of MPO the vasoconstriction was significant at 10 µM, 30 µM and 100 µM H_2O_2 compared to the baseline. MPO and H_2O_2 caused significant vasoconstriction compared to the control (10, 30 and 100 µM H_2O_2 panel **C).** The contractile forces

are indicated in absolute values, as differences from the initial baseline force. Asterisks denote significant differences from the control (H_2O_2 without MPO).

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 4 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 5 different animals; closed squares). Open circles represent the effects of H_2O_2 alone, while closed circles illustrate the effects of H_2O_2 in the presence of MPO. Asterisks denote significant differences from the MPO, and crosses 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 H_2O_2 (control) on the vascular diameter in the SMAs (Panel **B**). The H_2O_2-induced biphasic response did not change in the presence of 100 μM L-Met (id: 120±14 μm, n=5 arterioles from 5 different animals; closed squares, but it caused significant vasoconstriction relative to the zero line at 10 μM and 30 μM H_2O_2; 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**).

Figure 3. Effects of L-Met on the MPO-mediated vascular effects in different arteriolar beds

Increasing concentrations of L-Met (20, 40 or 100 μM) inhibited the MPO-mediated vasoconstriction in the SMAs in concentration-dependent manner (Id: 175±24 μm, n=6 arterioles from 4 different animals, with 20 μM L-Met, (closed triangles); id:

143±13 µm, n=4 arterioles from 4 different animals, with 40 µM L-methionine, (open triangles), id: 115±19 µm, n=5 arterioles from 5 different animals, with 100 µM L-Met (open squares). MPO and 20 µM L-methionin evoked significant vasoconstriction at 10 µM H_2O_2 compared to the baseline; Panel **A**). The effects of MPO alone and in combination with increasing L-Met concentrations in the presence of 300 µM H_2O_2 (control) on the vascular diameter in the SMAs (Panel **B**). In the CAs L-Met (100 µM; open squares) inhibited the MPO-evoked vasoconstriction only at a higher concentration of H_2O_2 (id: 73±10 µm n=4 arterioles from 4 different animals). Asterisks denote significant differences from MPO (Panel **C**). The effects of MPO alone and in combination with 100 µM L-Met in the presence of 300 µM H_2O_2 (control) on the vascular diameter in the CAs (Panel **D**). L-Met (100 µM; open squares) did not significantly influence the MPO-evoked changes in the isometric force in the BAs compared to the control (n=6 arterioles from 3 different animals, Panel **E**), but comparing to the zero line MPO together with L-met caused significant vasoconstriction at 30 µM and 100 µM H_2O_2 (P=<0.05). The effects of MPO alone and in combination with 100 µM L-Met in the presence of 300 µM H_2O_2 (control) on the vascular diameter in the BAs (Panel **F**).

Figure 4. The mechanism of MPO-induced vasoconstriction in the SMAs

H_2O_2-evoked vasoconstriction (open circles; control) was abolished after endothelium denudation (id: 138±10 µm, n=4 arterioles from 4 different animals; closed diamonds, Panel **A**). However, in the presence of MPO, and at relatively low H_2O_2 concentrations, vasoconstrictions (significant vasoconstriction at 10 µM -100 µM H_2O_2 compared to the baseline; P<0.05). were still observed in the absence of endothelium (id: 172±7 µm, n=5 arterioles from 4 different arterioles; open triangles). Closed circles illustrate the effects of MPO. Asterisks denote significant differences

from the action of MPO in the presence and absence of endothelium, and crosses indicate significant differences between the endothelium removal and the control. The MPO and H_2O_2-induced vasoconstriction was tested in the presence of the TXA2 receptor antagonist (id: 142±13 μm, n=5 arterioles from 4 different animals; closed triangles, Panel **B**) and in the presence of the COX inhibitor (id: 168±8 μm, n=5 arterioles from 3 different animals; open triangles, Panel **C**). Asterisks denote significant differences from MPO.

Figure 5. COX-1 isoenzyme is present in the vascular endothelial and smooth muscle cells in the SMAs

The presence of COX-1 isoenzyme in the vascular smooth muscle cells and in the vascular endothelium was confirmed by immunohistochemistry. Smooth muscle actin is labeled in green, COX in red, and nuclei in blue (from top to bottom). Control images (without primary antibodies) are indicated in the right-hand column.

Figure 6. MPO increases the Ca^{2+} sensitivity of force production in the vascular smooth muscle cells

The changes in intracellular Ca^{2+} levels ($F_{340/380}$ signals) and external arteriolar diameters were studied in SMAs under control conditions (id: 297±9 μm, n=7 arterioles from 6 different animals; panel **A**), or after treatment with norepinephrine (id: 314±16 μm, n=7 arterioles from 6 different animals; panel **B**). Asterisks denote significant differences from the initial values.

Figure 7. A proposed mechanism for the vascular effects of MPO in the SMA

During its anti-inflammatory activity, MPO modulates the vascular action of H_2O_2. The release of MPO causes the production of hypochlorous acid (HOCl), which increases

the generation of thromboxane A2 (TXA2) both in endothelial cells and in vascular smooth muscle cells, leading to vascoconstriction through a Ca^{2+}-sensitizing mechanism in vascular smooth muscle cells. An MPO inhibitor prevents both the peroxidation and the chlorinating activity, while L-Met inhibits only the chlorinating activity of the enzyme. In the presence of L-Met, the peroxidation pathway is still functional and vasodilation is observed, probably due to the generation of a vasodilatative peroxidation product (marked by a question mark).