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

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Short title:

Myeloperoxidase-mediated vasoregulation

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25

26 **Abstract**

27 **Aims:** Myeloperoxidase (MPO) catalyzes the formation of a wide variety of oxidants,
28 including hypochlorous acid (HOCl), and contributes to cardiovascular disease
29 progression. We hypothesized that during its action MPO evokes substantional
30 vasomotor responses.

31 **Methods:** Following exposure to MPO (1.92 mU ml^{-1}) in the presence of increasing
32 concentrations of hydrogen peroxide (H_2O_2) changes in arteriolar diameter of isolated
33 gracilis skeletal muscle arterioles (SMAs), and coronary arterioles (CAs) and in the
34 isometric force in basilar arteries (BAs) of the rat were monitored.

35 Results: MPO increased vascular tone to different degrees in CAs, SMAs and BAs.
36 The mechanism of increased vasoconstriction was studied in detail in SMAs. MPO-
37 evoked vasoconstrictions were prevented by the MPO inhibitor 4-
38 aminobenzhydrazide (50 μM), by endothelium removal in the SMAs. Surprisingly, the
39 HOCl scavenger L-methionine (100 μM), the thromboxane A2 (TXA2) antagonist SQ-
40 29548 (1 μM) or the nonspecific cyclooxygenase (COX) antagonist indomethacin (1
41 μM) converted the MPO-evoked vasoconstrictions to pronounced vasodilations in
42 SMAs; not seen in the presence of H_2O_2. In contrast to norepinephrine-induced
43 vasoconstrictions, the MPO-evoked vasoconstrictions were not accompanied by
44 significant increases in arteriolar [Ca^{2+}] levels in SMAs.

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

50 unmasked the effects of additional MPO-derived radicals with a marked vasodilatory
51 potential in SMAs.

52 **Key words:** hydrogen peroxide, myeloperoxidase, smooth muscle calcium,
53 thromboxane A2, vasoconstrictions
54

55 Introduction

56 The effector enzyme myeloperoxidase (MPO) has a protective role in inflammatory
57 processes. However, the activation of MPO may become deleterious and can also
58 contribute to the development of cardiovascular diseases (Nicholls and Hazen, 2005,
59 Podrez et al., 2000, Klebanoff, 2005). Accordingly, excessive levels of MPO in the
60 plasma may be accompanied by an increased risk of subsequent cardiovascular
61 events (Baldus et al., 2003, Zhang et al., 2001c, Vita et al., 2004, Brennan et al.,
62 2003, Karakas and Koenig, 2012, Kataoka et al., 2014), whereas individuals with an
63 inherited MPO deficiency are at a reduced cardiovascular risk (Nikpoor et al., 2001,
64 Hoy et al., 2001). There is currently no clear explanation of this situation.

65 MPO, a heme-containing, intensely green protein, was originally isolated from
66 canine pus and from purulent fluids from patients with tuberculosis (Klebanoff, 2005,
67 Malle et al., 2007). The synthesis of MPO is initiated in the bone marrow during
68 myeloid differentiation and is completed in the granulocytes (Lau and Baldus, 2006,
69 Hansson et al., 2006). MPO is stored primarily in the azurophil granules of the
70 polymorphonuclear neutrophils and monocytes, but it has also been found in tissue
71 macrophages (Daugherty et al., 1994, Lau and Baldus, 2006, Hampton et al., 1998,
72 Klebanoff, 2005). To exert its antimicrobial effects, MPO primarily catalyzes the
73 reaction of hydrogen peroxide (H_2O_2) with chloride (Hampton et al., 1998), to form
74 hypochlorous acid (HOCl) (Malle et al., 2007, Cook et al., 2012). The activation of
75 MPO additionally gives rise to a number of other pro-oxidative radicals through its

76 peroxidase activity. The biological effects of the MPO (*e.g.* vasomotor activity,
77 permeability, apoptotic effect) system depend on the local concentration of H_2O_2
78 (Golubinskaya et al., 2014) of other substrates and/or antioxidant molecules (*e.g.*
79 methionine (Met) (Podrez et al., 2000, Porszasz et al., 2002). Taken together, the
80 involvement of MPO has been implicated in vascular inflammation in association with
81 infection, diabetes and atherosclerosis (Malle et al., 2007, Cook et al., 2012, Zhang
82 et al., 2004, Kataoka et al., 2014, Sugiyama et al., 2001, Sirpal, 2009, Woods et al.,
83 2003, Ford, 2010).

84 It is not known at present how the persistent generation of MPO-derived
85 oxidants evokes adverse effects in vascular tissues. MPO and its oxidative products
86 are highly abundant in human atherosclerotic lesions (Daugherty et al., 1994, Hazen
87 and Heinecke, 1997, Hazen et al., 2000, Hazell et al., 1996). MPO is presumed to be
88 involved in the oxidative modification of low-density lipoprotein, thereby converting it
89 into a high-uptake form and hence promoting foamy cell formation. (Podrez et al.,
90 1999, Savenkova et al., 1994) Through its catalytic activity, MPO can consume
91 nitrogen monoxide (NO), thereby limiting its bioavailability (Eiserich et al., 2002, Abu-
92 Soud and Hazen, 2000). MPO-derived HOCl reacts with L-arginine and produces
93 NO-synthesis inhibitors (Zhang et al., 2001b, Zhang et al., 2001a), and HOCl can
94 impair endothelial NO bioactivity in a superoxide-dependent manner (Stocker et al.,
95 2004). Furthermore, MPO and HOCl can activate matrix metalloproteinases and
96 deactivate matrix metalloproteinase inhibitors, leading to weakening of the fibrous
97 cap and the development of destabilized atherosclerotic plaque (Karakas and
98 Koenig, 2012, Fu et al., 2001). From a functional aspect, MPO treatment led to a
99 decrease in myocardial perfusion in pigs and inhibited the acetylcholine-evoked
100 relaxation in the internal mammary arteries (Rudolph et al., 2012). Vasorelaxation in

101 response to acetylcholine was also found to be impaired in mice at relatively high
102 plasma MPO levels (Zhang et al., 2013). Nevertheless, the mechanisms through,
103 which MPO modulates the vascular responses are not well understood. In the
104 present study, we made an effort to investigate the effects of MPO activation in
105 vascular preparations *in vitro*. Moreover, we tried to characterize the possible
106 mechanism of the vasomotor action of MPO in SMAs.

107 Since the MPO substrate H_2O_2 was earlier identified as an important regulator
108 of vascular diameter under both normal and pathological conditions, the vasoactive
109 effects of MPO were contrasted to those of H_2O_2. H_2O_2 evokes a concentration-
110 dependent biphasic effect in the skeletal muscle arterioles (SMAs) and mesenteric
111 arteries in the rat, causing vasoconstriction at lower concentrations, and vasodilation
112 at higher concentrations (Gao et al., 2003, Cseko et al., 2004, Csato et al., 2014),
113 whereas, H_2O_2 induces only vasodilation in the rat coronaries (Csato et al., 2014).

114 In the present study, we investigated (i) the acute effects of MPO on the H_2O_2-
115 evoked changes in diameter in isolated SMAs and coronary arterioles (CAs) and on
116 the contractile force in the basilar arteries (BAs) of the rat, and (ii) the signal
117 transduction pathways mediating the vascular effects of MPO derived-oxidative
118 radicals.

119

120 **Materials and Methods**

121 **Animals, anesthesia and tissue dissection**

122 Male Wistar rats (weighing 250-350 g, 6-9 weeks old) obtained from Toxi-Coop
123 Toxicological Research Center, Dunakeszi, Hungary) were fed a standard chow and
124 drank tap water *ad libitum*. Anesthesia was performed with an intraperitoneal
125 injection of sodium pentobarbital (150 mg kg^{-1}) all efforts were made to minimize

126 suffering of animals. The gracilis muscle, the heart and the brain were removed and
127 placed into silicone-coated Petri dishes containing 0-4 ˚C Krebs solution (composition
128 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
129 $NaHCO_3$, obtained from Sigma-Aldrich, St. Louis, MO, USA) equilibrated with a
130 gaseous mixture of 5% CO_2, 10% O_2 and 85% N_2 at pH 7.4. All animal procedures
131 used in this study were in full accordance with the rules of the Ethical Committee of
132 the University of Debrecen and approved by the appropriate governmental body
133 Directive 2010/63/EU of the European Parliament. The study is conforming with:
134 Persson PB. Good Publication Practice in Physiology 2013 Guidelines for Acta
135 Physiol (Oxf) (Persson, 2013).
136
137 **Materials and drugs**
138 The TXA2 inhibitor SQ-29548 was purchased from BioMarker Kft. (Gödöllő,
139 Hungary). MPO protein, MPO inhibitor and COX antibodies were obtained from
140 Abcam (Cambridge, UK). Secondary antibodies were from Jackson Immunoresearch
141 Laboratories, Inc. (West Grove, PA, USA). All other chemicals were from Sigma-
142 Aldrich (St. Louis, MO, USA) and were kept under the conditions recommended by
143 the manufacturer. All reported concentrations are cumulative concentrations in the
144 organ chamber.
145
146 **Measurement of arteriolar diameter**
147 The rat SMAs and CAs were isolated and the changes in their diameters were
148 mesaured as described earlier (Csato et al., 2014). Briefly, the isolated arterioles
149 were transferred into an organ chamber and then were cannulated. The intraluminal
150 pressure was set at 80 mmHg (pressure servo control system, Living Systems

151 Instrumentation, St. Albans, VT, USA). The temperature was maintained at 37 ˚C by
152 the built in temperature controller in the tissue chamber (Living Systems
153 Instrumentation, St. Albans, VT, USA). Changes in arteriolar diameter were recorded
154 by a video microscope system (microscope: Nikon, Eclipse 80i; CCD camera: Topica
155 Technology Co Ltd, Taipei, Taiwan; video digitalizer: National Institutes, Bethesda,
156 USA). The isolated SMAs and CAs spontaneously developed a substantial myogenic
157 tone (a decrease in diameter from 196±6 μm to 160±6 μm, n=45, and from 234±14
158 μm to 178±14 μm, n=9, respectively) in response to an intraluminal pressure of 80
159 mmHg.
160

161 **Measurement of arteriolar contractions under isometric conditions**

162 BAs were prepared from rat brains with microsurgical tools, and ~ 4-mm-long rings
163 were then mounted in an isometric contraction measurement system (DMT-510,
164 Danish Myotechnology, Aarhus, Denmark). Before exposure to test solutions, vessel
165 tone was normalized. To this end, preparations were stretched at a force by
166 increasing 1.5 mN every 15 s until the calculated intraluminal pressure reached 13.4
167 kPa. The experiments were then performed at this stretch level (isometric
168 contractions).
169

170 **Experimental protocols**

171 The endothelial function was tested with acetylcholine (1 nM-10 μM), and the smooth
172 muscle function with norepinephrine (1 nM-10 μM, in SMAs), serotonin (1 nM-10 μM,
173 in CAs) or potassium chloride (10-60 mM, in BAs).

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

176 stabilized 30% stock solution (Sigma Aldrich, St. Louis, MO, USA) immediately
177 before the experiments and were stored on ice. The arterioles were first treated with
178 MPO (1.92 mU ml^{-1}, 300 s treatment duration, diameter measured every 10 s) to
179 record the effects of MPO alone. This was followed by the addition of H_2O_2 (1 μM-10
180 mM) and the responses to MPO+H_2O_2 were then determined. In the BAs, the effects
181 of MPO and H_2O_2 were tested after precontractions were evoked with 60 mM
182 potassium chloride.

183 The mechanism of MPO-evoked vasomotor responses was explored in detail
184 in SMAs. In some experiments, the endothelium was removed by the perfusion of air
185 bubbles through the arterioles (denudation). Successful endothelium denudation was
186 verified by the loss of dilation in response to acetylcholine (10 μM, 96±4% dilation
187 before and -6±4% after endothelium removal, n=5), while a maintained smooth
188 muscle function was confirmed with norepinephrine (71±1% constriction before and
189 64±2% after endothelium removal). The effects of MPO and H_2O_2 were also
190 measured in the presence of an MPO inhibitor (50 μM 4-aminobenzhydrazide), a
191 TXA2 receptor inhibitor (1 μM SQ-29548) and a COX antagonist (1 μM indomethacin)
192 in the SMAs. The effects of MPO were tested after incubation of the vessels with the
193 HOCl scavenger L-Met (20, 40 and 100 μM) in all three vessel types. At the end of
194 the experiments, the maximal (passive) arteriolar diameter was determined in the
195 absence of extracellular Ca^{2+}.

196

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

199

200 Simultaneous measurements of intracellular Ca^{2+} and arteriolar diameter were
201 performed as described previously (Csato et al., 2014, Czikora et al., 2012,
202 Kandasamy et al., 2013). Briefly, SMAs were isolated and cannulated as mentioned
203 above, except that the tissue bath was supplemented with 1% bovine serum albumin
204 (Sigma Aldrich, St. Louis, MO, USA) and 5 μM Fura-2AM, a ratiometric fluorescent
205 Ca^{2+} indicator dye (Molecular Probes, Eugene, OR, USA) until a spontaneous
206 myogenic tone developed. Intracellular Ca^{2+} concentrations were measured with an
207 Incyte IM system (Intracellular Imaging Inc, Cincinnati, OH, USA). Fura-2
208 fluorescence (recorded every 2-5 s) was excited alternately by 340 and 380-nm light,
209 and the emitted fluorescence was detected above 510 nm. The outer arteriolar
210 diameter was determined in each recorded image. Arteriolar Ca^{2+} concentration was
211 determined as the Fura-2 fluorescence ratio ($F_{340/380}$).
212

213 **Immunohistochemistry**

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

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

226

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

228 MPO-evoked chlorinating activity was measured with a commercial assay kit
229 (Cayman Chemical Company, Ann Arbor, MI, USA) in accordance with the
230 manufacturer's instructions. The measurement is based on the cleavage of
231 nonfluorescent 2-[6-(4-aminophenoxy)-3-oxo-3H-xanthen-9-yl] benzoic acid (APF) to
232 fluorescein by MPO-generated hypochlorite (-OCl). The reaction mixtures contained
233 45 μM APF, 30 μM H_2O_2, 3 U l^{-1} MPO and 200-0.39 mM L-Met (serially diluted). The
234 measurements were performed in phosphate-buffered saline (PBS, pH=7.4)
235 independently from the *in vitro* vascular experiments. Changes in fluorescence
236 intensity (λ_{ex}=485 nm, λ_{em}=520 nm) were measured at 30-s intervals for 5 min with a
237 plate reader (NovoStar plate reader, BMG Labtech). Fluorescence intensities values
238 were plotted as a function of time and fitted by linear regression (before saturation).
239 The slope of this relation was used to calculate MPO activities.

240

241 **Data analysis and statistical procedures**

242 The internal diameters of arterioles are shown as means±SEM. Arteriolar constriction
243 was expressed as the change in diameter as a percentage of the initial diameter
244 (before addition of the vasoactive agents) measured at an intraluminal pressure of 80
245 mmHg. Arteriolar dilation was calculated as the percentage of the maximal (passive)
246 diameter determined in the absence of extracellular Ca^{2+} at the end of the
247 experiments. The contractile force was indicated in absolute values, as the difference
248 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).

299 **The signaling mechanism of MPO-evoked vasoconstriction in the SMAs**

300 Endothelium removal inhibited the MPO-evoked vasoconstriction in the SMAs (*e.g.*
301 47±7% vasoconstriction at 300 μM H_2O_2+MPO with intact endothelium, *vs.* 13±15%
302 vasoconstriction + MPO without endothelium, *P*=0.07; Fig. 4A).

303 Next, the involvement of the TXA2 receptors in the MPO-evoked
304 vasoconstrictive effects was tested. Inhibition of the TXA2 receptors by 1 μM SQ-
305 29548 converted the MPO-evoked vasoconstrictions to vasodilations (*e.g.* 47±7%
306 vasoconstriction at 300 μM H_2O_2+MPO *vs.* 30±17% dilation at 300 μM
307 H_2O_2+MPO+TXA2 receptor inhibitor; P=0.002, Fig. 4B).

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

313

314 **Vascular expression of COXs in the SMAs**

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

319

320 **MPO-induced vasoconstriction develops in the absence of significant**
321 **intracellular Ca^{2+} concentration changes**

322 Measurements of the intracellular Ca^{2+} concentration and the arteriolar diameter
323 changes were performed in parallel in the SMAs. MPO-evoked vasoconstriction

324 (29±3% vasoconstriction at 1 mM H_2O_2; *P*=0.04 *vs.* the baseline) developed without
325 significant changes in the $F_{340/380}$ ratio signal in the range of H_2O_2 concentrations
326 between 1 μM and 1 mM (Fig. 6A). In contrast, the norepinephrine-evoked (1 nM-10
327 μM) vasoconstrictions with comparable magnitudes (44±4% constriction at 10 μM
328 norepinephrine; *P*=0.0005 *vs.* the baseline) were accompanied by significant
329 increases in the $F_{340/380}$ ratio (from 0.85±0.03 to 1.15±0.09; Fig. 6B). MPO alone did
330 not have any effect on the arteriolar diameter or on the $F_{340/380}$ signal (not shown).
331

332 **Discussion**

333 Vascular inflammation during endothelial dysfunction (Zhang et al., 2001a),
334 atherosclerosis (Sugiyama et al., 2001, Sirpal, 2009, Woods et al., 2003, Ford, 2010)
335 diabetes mellitus (Zhang et al., 2004, Kataoka et al., 2014), coronary artery disease
336 (Cavusoglu et al., 2007, Mayyas et al., 2014) is characterized by increased levels of
337 production and local release of both H_2O_2 and MPO. Moreover the increased
338 generation of MPO was observed in neurodegenerative disorders (Reynolds et al.,
339 1999, Pennathur et al., 1999), arthritis (Bender et al., 1986) and some cancers
340 (Reynolds et al., 1997). We hypothesized that MPO evokes substantial vasomotor
341 responses in the presence of H_2O_2. This process may have immediate (acute) effects
342 on the vascular diameter, which was tested here under *in vitro* conditions. The details
343 of intracellular mechanisms responsible for the MPO elicited vasomotor responses
344 were studied in SMAs. The most important findings of this study are that (1) MPO has
345 the potential to promote vasoconstriction in H_2O_2-treated SMAs, CAs or BAs of the
346 rat; (2) in the SMAs, MPO facilitates the H_2O_2-dependent activation of COX-1 and the
347 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

373 additionally made an effort to identify the molecular mechanisms contributing to these
374 vasoconstrictive effects, besides to the decreased NO bioavailability reported earlier.
375 One of the major observations was that the widely accepted HOCl scavenger L-Met
376 (Okabe et al., 1993, Zhang et al., 2003, Zhang et al., 2004) not only inhibited the
377 vasoconstriction evoked by MPO, but also unmasked a robust vasodilatory effect in
378 the SMAs. The employed MPO-specific inhibitor, 4-aminobenzhydrazide blocked
379 both the chlorinating and the peroxidase activities of the MPO (Malle et al., 2007,
380 Kettle et al., 1995, Kettle et al., 1997) and prevented the vasoconstriction evoked by
381 MPO. In the presence of 4-aminobenzhydrazide and MPO however, the vascular
382 responses to H_2O_2 did not differ significantly from those in the absence of MPO.
383 Collectively, the above data suggested that MPO-mediated chlorination has a major
384 role in the activation of a signaling pathway leading to vasoconstriction. L-Met not
385 only antagonized this effect, but revealed an additional MPO-dependent mechanism
386 leading to vasodilation. This latter effect was probably related to the peroxidase
387 activity of MPO that was not inhibited by L-Met. It is worthy of consideration that in
388 the CAs and BAs, where MPO evoked vasoconstrictions were less pronounced than
389 those in the SMAs, L-Met did not result in significant vasodilations, which is
390 suggestive of differential expressions of the MPO-responsive vasodilatory pathways
391 in the different vascular beds.

392 Effector structures responding to MPO-derived radicals were first tested by
393 removal of the endothelium in SMAs, which eliminated the endothelium-derived
394 effects, including decreased NO bioavailability (Stocker et al., 2004, Xu et al., 2006,
395 Turan et al., 2000). Importantly, H_2O_2-evoked vasoconstrictions were found in a
396 previous study to be completely endothelium-dependent (Csato et al., 2014).
397 However, the vasoconstriction evoked by H_2O_2 in the presence of MPO was only

398 partially opposed by endothelium removal (Fig. 4A), suggesting that the MPO-evoked
399 vasoconstriction was only partially endothelium-dependent. These observations,
400 together with those in the presence of the COX inhibitor indomethachin and the TXA2
401 inhibitor SQ-29548, implicate that MPO causes the generation of a vasoconstrictive
402 prostanoide derivate (potentially TXA2) not only in the endothelial cells, but also in
403 the vascular smooth muscle cells, through the activation of COXs. To confirm this
404 possibility, the expression of COXs enzymes was explored by means of
405 immunohistochemistry, and COX-1-specific staining was indeed confirmed both in
406 the endothelial layer and in the smooth muscle cells of the SMAs. Interestingly, not
407 only was the MPO-mediated vasoconstriction prevented by either TXA2 receptor
408 inhibition or COX inhibition, but similarly as when L-Met was applied it was converted
409 to vasodilation. A role for TXA2 was implicated by its pharmacological inhibitor,
410 nevertheless we did not examine TXA2 production upon MPO exposures. Taken
411 together, we postulate that the MPO-evoked vasoconstriction is mediated by a
412 vasoconstrictive prostanoid derivative through TXA2 receptor activation. Hence, the
413 above findings point to a HOCl – COX1 – TXA2 pathway as being decisive in the
414 prevention of MPO-dependent vasodilation in the SMAs (Fig. 7).

415 Numerous previous studies have furnished evidence that H_2O_2 is an important
416 regulator of the vascular diameter (Matoba et al., 2000, Yada et al., 2003, Matoba et
417 al., 2003, Koller and Bagi, 2004, Miura et al., 2003, Gao and Lee, 2005, Gao et al.,
418 2003, Gao and Lee, 2001). It is difficult to specify the physiologic concentration of
419 H_2O_2 in vascular tissues *in vivo*. Nevertheless, it has been found that under
420 pathologic conditions it may increase up to 0.3 mM. In our study, H_2O_2 was used in a
421 wide concentration range (1 μM-10 mM), thus covering also pharmacological levels.
422 This approach allowed us to reveal the mechanisms of MPO derived vascular effects

423 developing on top of the biphasic H_2O_2 dependent responses (Liu and Zweier, 2001,
424 Root and Metcalf, 1977, Cseko et al., 2004). In higher concentrations H_2O_2 may
425 cause vasodilation. The possible mechanism of the H_2O_2-evoked vasodilation has
426 been investigated by a number of groups in different vessel types (Iida and Katusic,
427 2000, Thengchaisri and Kuo, 2003, Zhang et al., 2012). Our previous results
428 implicated the involvement of the NO/cyclic guanosine monophosphate pathway and
429 the activation of K^+ channels in SMAs (Cseko et al., 2004).

430 Under pathological conditions associated with inflammation, such as acute
431 infections (Hampton et al., 1998, Pullar et al., 2000, Hirche et al., 2005), diabetes
432 (Zhang et al., 2004, Kataoka et al., 2014), atherosclerosis (Sugiyama et al., 2001,
433 Sirpal, 2009, Woods et al., 2003, Ford, 2010), arthritis (Bender et al., 1986),
434 Alzheimer disease (Reynolds et al., 1999), and Parkinson disease (Pennathur et al.,
435 1999) MPO is released together with H_2O_2. In vivo conditions, MPO is released
436 together with H_2O_2. Under these circumstances L-Met may prevent H_2O_2-evoked
437 vasoconstriction or even convert it into vasodilation, because L-Met in its presumed
438 physiological concentration range (i.e. 20-40 μM) (Mayo Medical Laboratories, 2015)
439 also largely prevents the vasoconstrictions evoked by MPO in the SMAs. Hence, the
440 ultimate effect on the vascular tone and thereby on local microcirculation will be a
441 function of the availability of a range of local regulators (*e.g.* H_2O_2, MPO, L-Met, *etc.*)
442 which are of high potency in vasoregulation (Cseko et al., 2004).

443 The MPO-induced vasoconstrictions were not accompanied by significant
444 increases in the intracellular Ca^{2+} concentration in the H_2O_2 concentration range of
445 between 100 μM and 1 mM. In contrast, norepinephrine treatment evoked
446 vasoconstrictions to similar degrees, together with significant increases in the
447 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

473 vascular responses (vasodilation vs. vasoconstriction) could be determined
474 convincingly because results were contrasted to controls under the same
475 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**

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

701

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).