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

**V. Csató, A. Pető, G. Á. Fülöp, I. Rutkai, E. T. Pásztor, M. Fagyas, J. Kalász, I. Édes, A. Tóth and Z. Papp**

*Division of Clinical Physiology, Institute of Cardiology, Research Center for Molecular Medicine, Faculty of Medicine, University of Debrecen, Debrecen, Hungary*

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Correspondence: Zoltán Papp, Division of Clinical Physiology, Institute of Cardiology, Research Center for Molecular Medicine, Faculty of Medicine, University of Debrecen, Móricz Zsigmond krt. 22., H-4032 Debrecen, Hungary. E-mail: pappz@med.unideb.hu

## 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 ($H_2O_2$), 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:** 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 $\mu$M), by endothelium removal in the SMAs. Surprisingly, the HOCl scavenger L-methionine (100 $\mu$M), the thromboxane A2 (TXA2) antagonist SQ-29548 (1 $\mu$M) or the non-specific cyclooxygenase (COX) antagonist indomethacin (1 $\mu$M) converted the MPO-evoked vasoconstrictions to pronounced vasodilations in SMAs, not seen in the presence of $H_2O_2$. In contrast to noradrenaline-induced vasoconstrictions, the MPO-evoked vasoconstrictions were not accompanied by significant increases in arteriolar [$Ca^{2+}$] levels in SMAs.

**Conclusion:** These data showed that $H_2O_2$-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 $Ca^{2+}$ 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.

*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

 

(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 ($H_2O_2$) 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 & 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 $H_2O_2$ 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 this 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, anaesthesia 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*. 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 $CaCl_2$, 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

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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 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). Changes in arteriolar diameter were recorded by a video microscope system (microscope: Nikon, Eclipse 80i; CCD camera: Topica Technology Co. Ltd., Taipei, Taiwan; video digitizer: National Institutes, Bethesda, USA). The isolated SMAs and CAs spontaneously developed a substantial myogenic tone (a decrease in diameter from 196 ± 6 $\mu$m to 160 ± 6 $\mu$m, $n = 45$, and from 234 ± 14 $\mu$m to 178 ± 14 $\mu$m, $n = 9$ respectively) in response to an intraluminal pressure of 80 mmHg.

### *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 $\mu$M) and the smooth muscle function with noradrenaline (1 nM–10 $\mu$M, in SMAs), serotonin (1 nM–10 $\mu$M 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. $H_2O_2$ 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 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 $\mu$M–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 pre-contractions 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 $\mu$M, 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 $H_2O_2$ were also measured in the presence of an MPO inhibitor (50 $\mu$M 4-aminobenzhydrazide), a TXA2 receptor inhibitor (1 $\mu$M SQ-29548) and a COX antagonist (10 $\mu$M 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 $\mu$M) 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) and 5 $\mu$M 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 OCT compound (Electron Microscopy Sciences; Hatfield, PA, USA). Cryostat sections (10 $\mu$m 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). COX enzymes were stained with COX-1 (rabbit anti-COX-1: ab109025, dilution: 1:50) and COX-2-specific antibodies (rabbit anti-COX-2: 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*

Myeloperoxidase-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 non-fluorescent 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 $\mu$M APF, 30 $\mu$M $H_2O_2$, 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 ($\lambda_{ex}$ = 485 nm and $\lambda_{em}$ = 520 nm) were measured at 30-s intervals for 5 min with a plate reader (NovoStar plate reader, BMG Labtech). Fluorescence intensity 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 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 *in vivo*, and our studies were

 

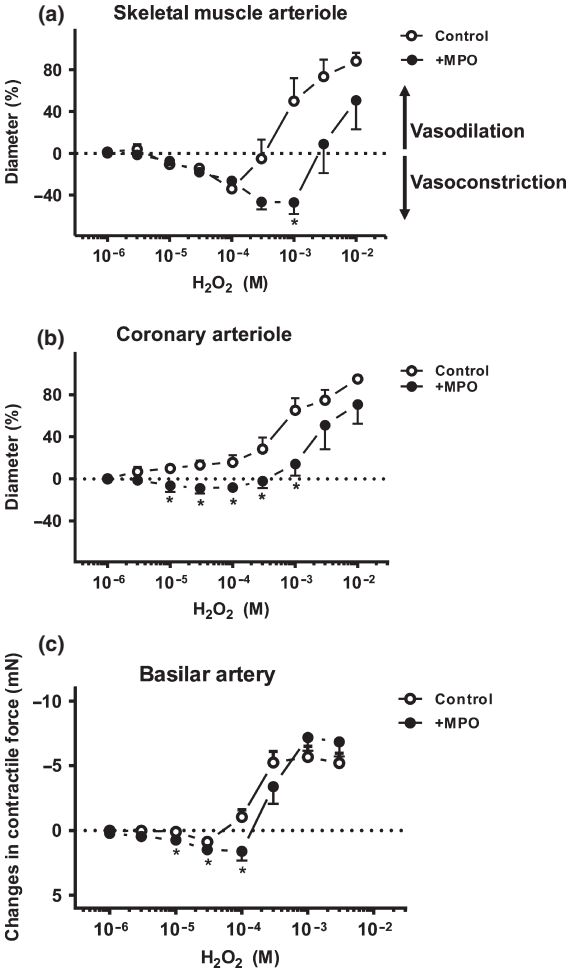


**Figure 1** MPO promotes $H_2O_2$-evoked vasoconstriction in different vascular beds. After pre-incubation with MPO (activity: 1.92 mU $mL^{-1}$, 600 s), isolated, cannulated SMAs (initial diameter (id): 182 ± 12 $\mu$m, $n$ = 5 arterioles from four different animals; panel (a) or CAs (id: 180 ± 17 $\mu$m, $n$ = 5 arterioles from five different animals; panel (b) with intact endothelium were treated with increasing concentrations (1 $\mu$M–10 mM) of $H_2O_2$. In SMAs, $H_2O_2$ alone (10 $\mu$M, 30 $\mu$M and 100 $\mu$M) evoked significant vasoconstriction compared to the zero line ($P < 0.02$). In the presence of MPO, $H_2O_2$ caused significant vasoconstriction from 10 $\mu$M–1 mM $H_2O_2$ compared to the control and the zero line ($P < 0.05$, panel a). In CAs, $H_2O_2$ (30 $\mu$M and 100 $\mu$M) 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 five different animals) pre-contracted 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 $\mu$M–3 mM, panel c). $H_2O_2$-evoked vasoconstriction was significant at 30 $\mu$M, whereas in the presence of MPO, the vasoconstriction was significant at 10 $\mu$M, 30 $\mu$M and 100 $\mu$M $H_2O_2$ compared to the baseline. MPO and $H_2O_2$ caused significant vasoconstriction compared to the control (10, 30 and 100 $\mu$M $H_2O_2$, 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).

therefore highlighted at this $H_2O_2$ concentration. The MPO-specific inhibitor prevented the development of MPO-dependent vasoconstriction (maximal vasoconstriction at 300 $\mu$M $H_2O_2$+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. 73 ± 11% dilation at 300 $\mu$M $H_2O_2$, $P < 0.0001$ vs. MPO+$H_2O_2$) suggesting an MPO-evoked, but HOCl-independent vasodilation mechanism. L-Met (100 $\mu$M) 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 $\mu$M 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 $\mu$M $H_2O_2$ 47 ± 7% vs. vasodilations of 8 ± 19, 35 ± 23 and 73 ± 11% in the presence of 20, 40 and 100 $\mu$M L-Met respectively; Fig. 3a and b). In the CA, the maximal L-Met concentration (100 $\mu$M) also provoked vasodilation at a high (1 mM) $H_2O_2$ concentration, whereas at 300 $\mu$M $H_2O_2$, L-Met did not modulate the vascular tone (i.e. 3 ± 9 vs. 13 ± 7% vasodilation; $P = 0.44$, Fig. 3c and d). Finally, 100 $\mu$M L-Met treatment did not significantly influence the MPO-evoked vascular responses in the BAs (e.g. 3.3 ± 1 mN vasoconstriction at 300 $\mu$M $H_2O_2$ 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 vasoconstr-

 

**Table 1** Effects of different inhibitors and endothelium removal on the MPO- and $H_2O_2$-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 L-Met | 100 μM L-Met | MPO+ 40 μM L-met | MPO+ 20 μM L-Met | 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 | 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 |

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) $H_2O_2$. The effects of pre-incubations with inhibitors (diameter after the inhibitor) and the maximum diameter of the vessels (the passive diameter) are also indicated.

**Table 2** Effects of different treatments on the MPO- and $H_2O_2$-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 ± 1.59 |
| Force after 1 mM $H_2O_2$ | 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 $H_2O_2$.

iction 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 using the non-specific COX inhibitor indomethacin (1 μM); similarly to TXA2 inhibition, this not only prevented the MPO-evoked vasoconstriction, but converted it 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).

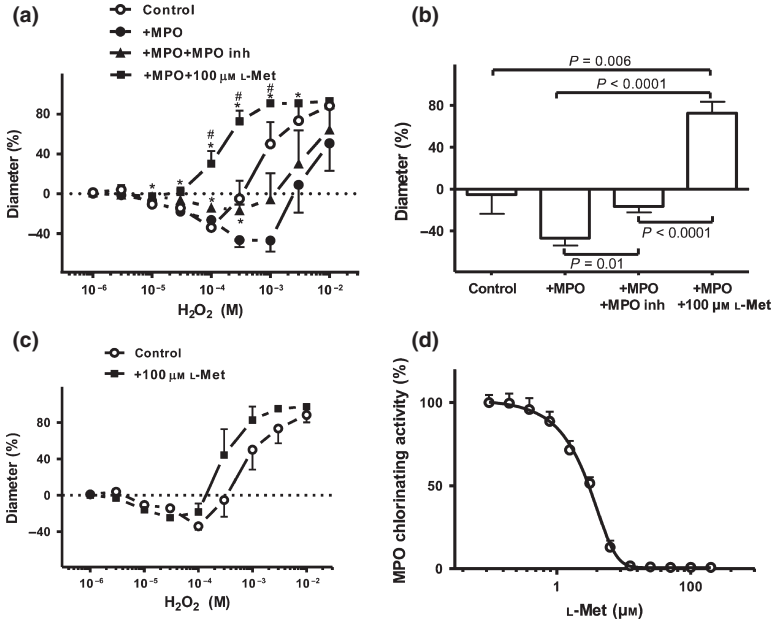


**Figure 2** HOCl mediates the vasoconstriction evoked by MPO in the SMAs. MPO-induced vasoconstriction was inhibited with the MPO inhibitor 4-aminobenzhydrazide (50 μM) (id: 182 ± 8 μm, $n = 5$ arterioles from four different animals; closed triangles); however, significant vasoconstriction was still 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 $H_2O_2$ alone, while closed circles illustrate the effects of $H_2O_2$ in the presence of MPO. Asterisks denote significant differences from the MPO, and # 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 $H_2O_2$ (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 five 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).

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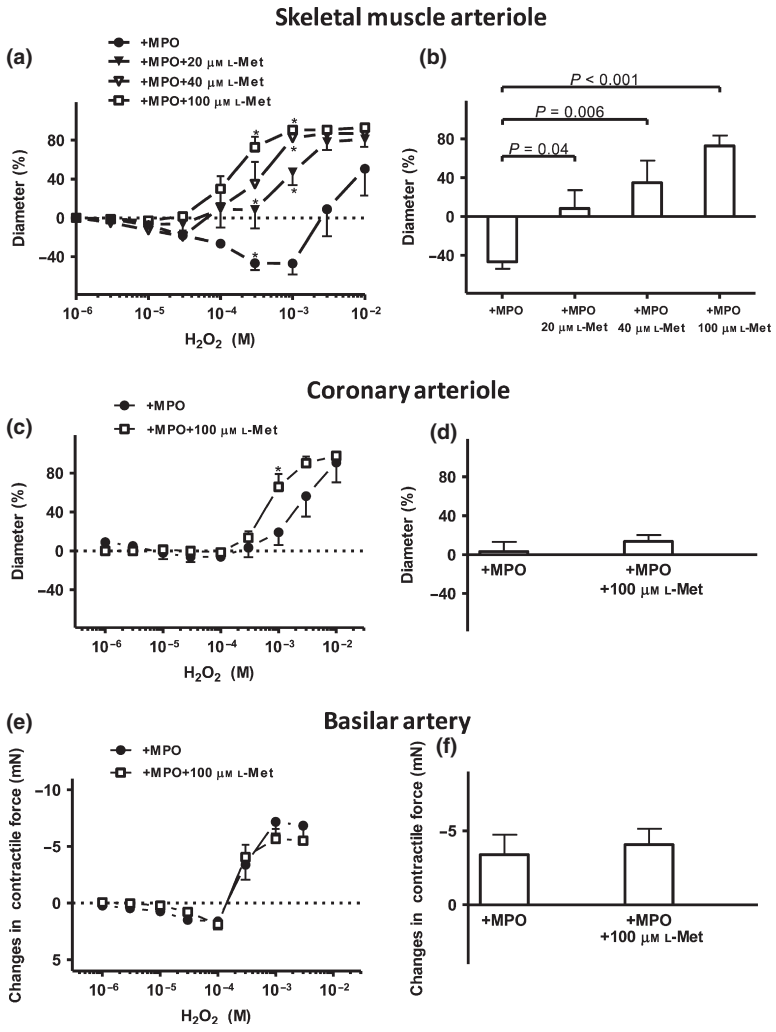


**Figure 3** Effects of L-Met on the MPO-mediated vascular effects in different arteriolar beds. Increasing concentrations of L-Met (20, 40 or 100 $\mu$M) inhibited the MPO-mediated vasoconstriction in the SMAs in concentration-dependent manner (Id: 175 ± 24 $\mu$m, $n$ = 6 arterioles from four different animals, with 20 $\mu$M L-Met, (closed triangles); id: 143 ± 13 $\mu$m, $n$ = 4 arterioles from four different animals, with 40 $\mu$M L-methionine, (open triangles), id: 115 ± 19 $\mu$m, $n$ = 5 arterioles from five different animals, with 100 $\mu$M L-Met (open squares). MPO and 20 $\mu$M L-methionine evoked significant vasoconstriction at 10 $\mu$M $H_2O_2$ compared to the baseline; panel a). The effects of MPO alone and in combination with increasing L-Met concentrations in the presence of 300 $\mu$M $H_2O_2$ (control) on the vascular diameter in the SMAs (panel b). In the CAs, L-Met (100 $\mu$M; open squares) inhibited the MPO-evoked vasoconstriction only at a higher concentration of $H_2O_2$ (id: 73 ± 10 $\mu$m, $n$ = 4 arterioles from four different animals). Asterisks denote significant differences from MPO (panel c). The effects of MPO alone and in combination with 100 $\mu$M L-Met in the presence of 300 $\mu$M $H_2O_2$ (control) on the vascular diameter in the CAs (panel d). L-Met (100 $\mu$M; 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 three different animals, panel e), but comparing to the zero line, MPO together with L-met caused significant vasoconstriction at 30 $\mu$M and 100 $\mu$M $H_2O_2$ ($P \leq 0.05$). The effects of MPO alone and in combination with 100 $\mu$M L-Met in the presence of 300 $\mu$M $H_2O_2$ (control) on the vascular diameter in the BAs (panel f).

### *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 $\mu$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

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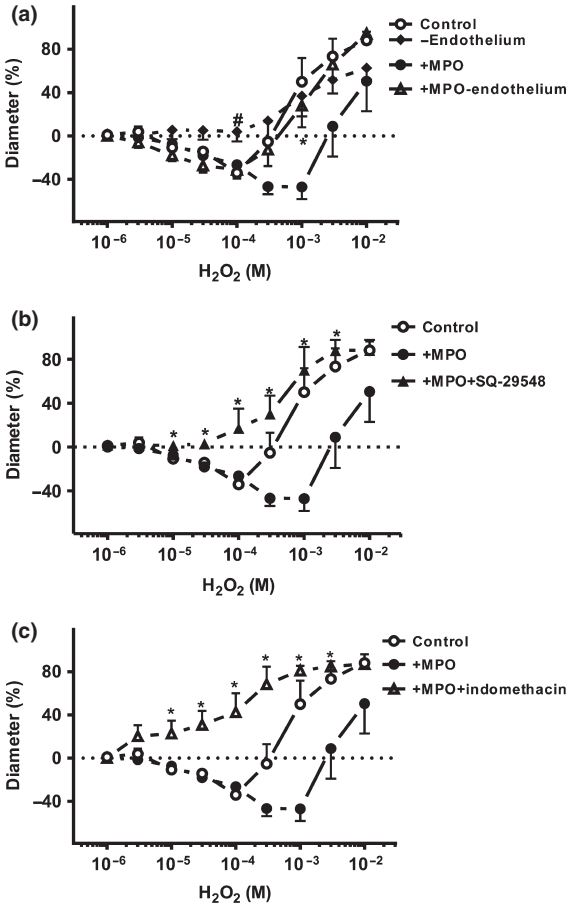


**Figure 4** The mechanism of MPO-induced vasoconstriction in the SMAs. $H_2O_2$-evoked vasoconstriction (open circles, control) was abolished after endothelium denudation (id: 138 ± 10 $\mu$m, $n$ = 4 arterioles from four different animals; closed diamonds, panel a). However, in the presence of MPO, and at relatively low $H_2O_2$ concentrations, vasoconstrictions (significant vasoconstriction at 10 $\mu$M–100 $\mu$M $H_2O_2$ compared to the baseline; $P < 0.05$) were still observed in the absence of endothelium (id: 172 ± 7 $\mu$m, $n$ = 5 arterioles from four 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 # 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 $\mu$m, $n$ = 5 arterioles from four different animals; closed triangles, panel b) and in the presence of the COX inhibitor (id: 168 ± 8 $\mu$m, $n$ = 5 arterioles from three different animals; open triangles, panel c). Asterisks denote significant differences from MPO.

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) and 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 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 $H_2O_2$ consumption by MPO, thereby requiring a higher nominal $H_2O_2$ concentration to produce comparable vasodilations. At lower concentrations of $H_2O_2$, the level of vasoconstriction was similar in the absence and in the presence of MPO, while at higher concentrations of $H_2O_2$, MPO led to higher maximal vasoconstriction levels, thereby suggesting that MPO did not simply shift the apparent $H_2O_2$ 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 $H_2O_2$ is HOCl. Our *in vitro* vascular measurements were performed in $Ca^{2+}$ 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 (Prutz 1996, Albrich *et al.* 1981). Less is known about the molecular pathways involved in the HOCl-evoked changes in vascular dynamics. One such possibility

 

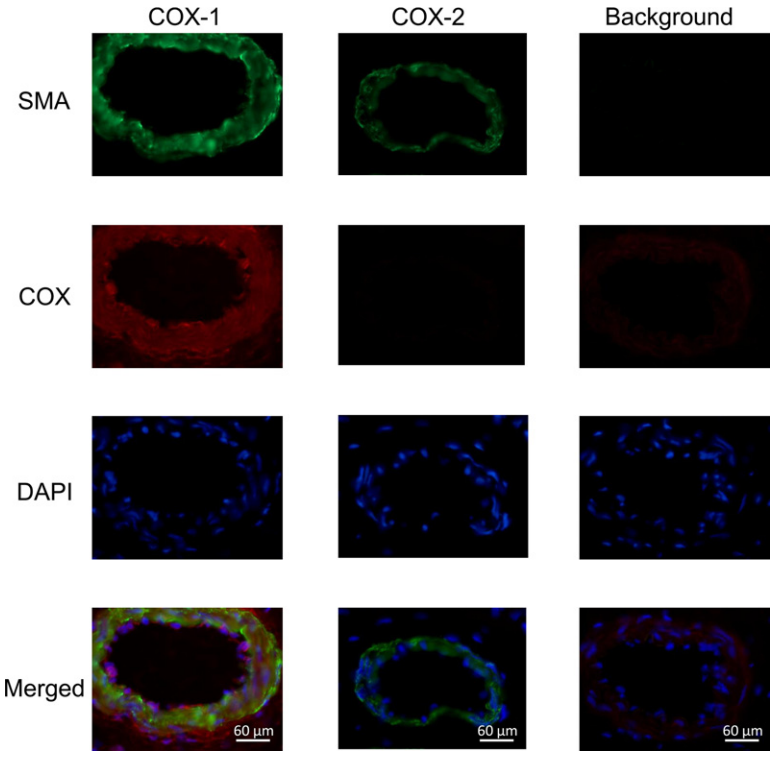


**Figure 5** COX-1 isoenzyme is present in the vascular endothelial and smooth muscle cells in the SMAs. The presence of COX-1 isoenzyme in the vascular smooth muscle cells and in the vascular endothelium was confirmed by immunohistochemistry. Smooth muscle actin is labelled in green, COX in red, and nuclei in blue (from top to bottom). Control images (without primary antibodies) are indicated in the right-hand column.

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 $H_2O_2$ 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 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 (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 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

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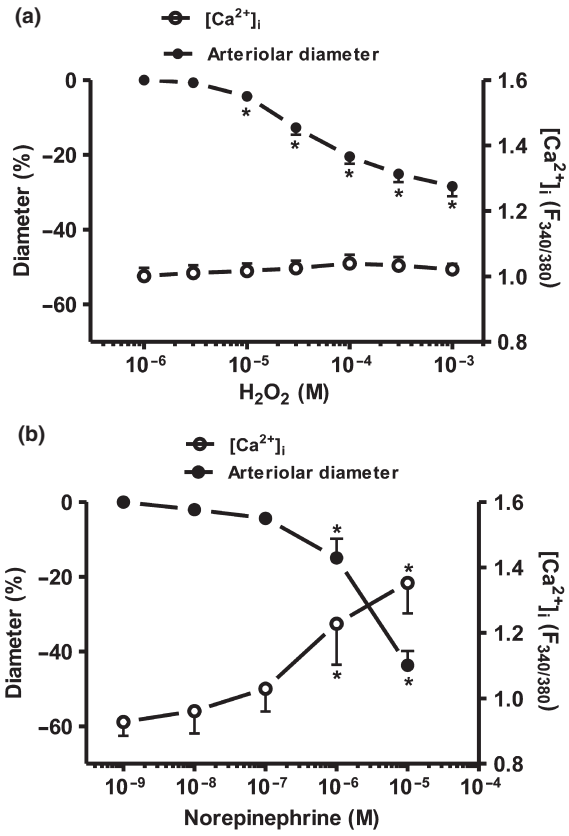


**Figure 6** MPO increases the $Ca^{2+}$ sensitivity of force production in the vascular smooth muscle cells. The changes in intracellular $Ca^{2+}$ levels ($F_{340/380}$ signals) and external arteriolar diameters were studied in SMAs under control conditions (id: 297 ± 9 $\mu$m, $n$ = 7 arterioles from six different animals; panel a), or after treatment with noradrenaline (id: 314 ± 16 $\mu$m, $n$ = 7 arterioles from six different animals; panel b). Asterisks denote significant differences from the initial values.

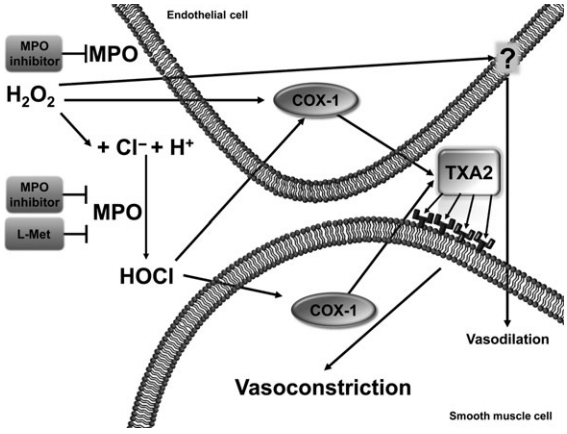


**Figure 7** A proposed mechanism for the vascular effects of MPO in the SMA. During its anti-inflammatory activity, MPO modulates the vascular action of $H_2O_2$. The release of MPO causes the production of hypochlorous acid (HOCl), which increases the generation of thromboxane A2 (TXA2) both in endothelial cells and in vascular smooth muscle cells, leading to vasoconstriction 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).

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 & 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 $H_2O_2$ 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, $H_2O_2$ was used in a wide concentration range (1 $\mu$M–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 & Zweier 2001, Root & 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 & 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 $H_2O_2$. *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 $\mu$M) (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) 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 $\mu$M and 1 mM. In contrast, noradrenaline 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.

## Study limitations

In this study, we aimed to explore the effects of MPO and $H_2O_2$ in vascular preparations with different origins. Due to differences in vascular diameters for SMAs, CAs and BAs (i.e. approx. 160 $\mu$m, approx. 180 $\mu$m and approx. 250 $\mu$m 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.

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## Conflicts of interest

None declared.

## References

Abu-Soud, H.M. & Hazen, S.L. 2000. Nitric oxide is a physiological substrate for mammalian peroxidases. *J Biol Chem* **275**, 37524–37532.

Albrich, J.M., McCarthy, C.A. & Hurst, J.K. 1981. Biological reactivity of hypochlorous acid: implications for microbicidal mechanisms of leukocyte myeloperoxidase. *Proc Natl Acad Sci USA* **78**, 210–214.

Baldus, S., Heeschen, C., Meinertz, T., Zeiher, A.M., Eiserich, J.P., Munzel, T., Simoons, M.L. & Hamm, C.W. 2003. Myeloperoxidase serum levels predict risk in patients with acute coronary syndromes. *Circulation* **108**, 1440–1445.

Bender, J.G., Van Epps, D.E., Searles, R. & Williams, R.C. Jr 1986. Altered function of synovial fluid granulocytes in patients with acute inflammatory arthritis: evidence for activation of neutrophils and its mediation by a factor present in synovial fluid. *Inflammation* **10**, 443–453.

Brennan, M.L., Penn, M.S., Van Lente, F., Nambi, V., Shishehbor, M.H., Aviles, R.J., Goormastic, M., Pepoy, M.L., McErlean, E.S., Topol, E.J., Nissen, S.E. & Hazen, S.L. 2003. Prognostic value of myeloperoxidase in patients with chest pain. *N Engl J Med* **349**, 1595–1604.

Cavusoglu, E., Ruwende, C., Eng, C., Chopra, V., Yanamadala, S., Clark, L.T., Pinsky, D.J. & Marmur, J.D. 2007. Usefulness of baseline plasma myeloperoxidase levels as an independent predictor of myocardial infarction at two years in patients presenting with acute coronary syndrome. *Am J Cardiol* **99**, 1364–1368.

  doi: 10.1111/apha.12488

Cook, N.L., Viola, H.M., Sharov, V.S., Hool, L.C., Schoneich, C. & Davies, M.J. 2012. Myeloperoxidase-derived oxidants inhibit sarco/endoplasmic reticulum $Ca^{2+}$-ATPase activity and perturb $Ca^{2+}$ homeostasis in human coronary artery endothelial cells. *Free Radic Biol Med* **52**, 951–961.

Csato, V., Peto, A., Koller, A., Edes, I., Toth, A. & Papp, Z. 2014. Hydrogen peroxide elicits constriction of skeletal muscle arterioles by activating the arachidonic acid pathway. *PLoS ONE* **9**, e103858.

Cseko, C., Bagi, Z. & Koller, A. 2004. Biphasic effect of hydrogen peroxide on skeletal muscle arteriolar tone via activation of endothelial and smooth muscle signaling pathways. *J Appl Physiol (1985)* **97**, 1130–1137.

Czikora, A., Lizanecz, E., Bako, P., Rutkai, I., Ruzsnavszky, F., Magyar, J., Porszasz, R., Kark, T., Facsko, A., Papp, Z., Edes, I. & Toth, A. 2012. Structure-activity relationships of vanilloid receptor agonists for arteriolar TRPV1. *Br J Pharmacol* **165**, 1801–1812.

Daugherty, A., Dunn, J.L., Rateri, D.L. & Heinecke, J.W. 1994. Myeloperoxidase, a catalyst for lipoprotein oxidation, is expressed in human atherosclerotic lesions. *J Clin Invest* **94**, 437–444.

Eiserich, J.P., Baldus, S., Brennan, M.L., Ma, W., Zhang, C., Tousson, A., Castro, L., Lusis, A.J., Nauseef, W.M., White, C.R. & Freeman, B.A. 2002. Myeloperoxidase, a leukocyte-derived vascular NO oxidase. *Science* **296**, 2391–2394.

Ford, D.A. 2010. Lipid oxidation by hypochlorous acid: chlorinated lipids in atherosclerosis and myocardial ischemia. *Clin Lipidol* **5**, 835–852.

Fu, X., Kassim, S.Y., Parks, W.C. & Heinecke, J.W. 2001. Hypochlorous acid oxygenates the cysteine switch domain of pro-matrilysin (MMP-7). A mechanism for matrix metalloproteinase activation and atherosclerotic plaque rupture by myeloperoxidase. *J Biol Chem* **276**, 41279–41287.

Gao, Y.J. & Lee, R.M. 2001. Hydrogen peroxide induces a greater contraction in mesenteric arteries of spontaneously hypertensive rats through thromboxane A(2) production. *Br J Pharmacol* **134**, 1639–1646.

Gao, Y.J. & Lee, R.M. 2005. Hydrogen peroxide is an endothelium-dependent contracting factor in rat renal artery. *Br J Pharmacol* **146**, 1061–1068.

Gao, Y.J., Hirota, S., Zhang, D.W., Janssen, L.J. & Lee, R.M. 2003. Mechanisms of hydrogen-peroxide-induced biphasic response in rat mesenteric artery. *Br J Pharmacol* **138**, 1085–1092.

Golubinskaya, V., Brandt-Eliasson, U., Gan, L.M., Kjerrulf, M. & Nilsson, H. 2014. Endothelial function in a mouse model of myeloperoxidase deficiency. *Biomed Res Int* **2014**, 128046.

Hampton, M.B., Kettle, A.J. & Winterbourn, C.C. 1998. Inside the neutrophil phagosome: oxidants, myeloperoxidase, and bacterial killing. *Blood* **92**, 3007–3017.

Hansson, M., Olsson, I. & Nauseef, W.M. 2006. Biosynthesis, processing, and sorting of human myeloperoxidase. *Arch Biochem Biophys* **445**, 214–224.

Hazell, L.J., Arnold, L., Flowers, D., Waeg, G., Malle, E. & Stocker, R. 1996. Presence of hypochlorite-modified proteins in human atherosclerotic lesions. *J Clin Invest* **97**, 1535–1544.

Hazen, S.L. & Heinecke, J.W. 1997. 3-Chlorotyrosine, a specific marker of myeloperoxidase-catalyzed oxidation, is markedly elevated in low density lipoprotein isolated from human atherosclerotic intima. *J Clin Invest* **99**, 2075–2081.

Hazen, S.L., Gaut, J.P., Crowley, J.R., Hsu, F.F. & Heinecke, J.W. 2000. Elevated levels of protein-bound p-hydroxyphenylacetaldehyde, an amino-acid-derived aldehyde generated by myeloperoxidase, are present in human fatty streaks, intermediate lesions and advanced atherosclerotic lesions. *Biochem J* **352**(Pt 3), 693–699.

Hirche, T.O., Gaut, J.P., Heinecke, J.W. & Belaaouaj, A. 2005. Myeloperoxidase plays critical roles in killing Klebsiella pneumoniae and inactivating neutrophil elastase: effects on host defense. *J Immunol* **174**, 1557–1565.

Hoy, A., Tregouet, D., Leininger-Muller, B., Poirier, O., Maurice, M., Sass, C., Siest, G., Tiret, L. & Visvikis, S. 2001. Serum myeloperoxidase concentration in a healthy population: biological variations, familial resemblance and new genetic polymorphisms. *Eur J Hum Genet* **9**, 780–786.

Iida, Y. & Katusic, Z.S. 2000. Mechanisms of cerebral arterial relaxations to hydrogen peroxide. *Stroke* **31**, 2224–2230.

Kandasamy, K., Bezavada, L., Escue, R.B. & Parthasarathi, K. 2013. Lipopolysaccharide induces endoplasmic store $Ca^{2+}$-dependent inflammatory responses in lung microvessels. *PLoS ONE* **8**, e63465.

Karakas, M. & Koenig, W. 2012. Myeloperoxidase production by macrophage and risk of atherosclerosis. *Curr Atheroscler Rep* **14**, 277–283.

Kataoka, Y., Shao, M., Wolski, K., Uno, K., Puri, R., Murat Tuzcu, E., Hazen, S.L., Nissen, S.E. & Nicholls, S.J. 2014. Myeloperoxidase levels predict accelerated progression of coronary atherosclerosis in diabetic patients: insights from intravascular ultrasound. *Atherosclerosis* **232**, 377–383.

Kettle, A.J., Gedye, C.A., Hampton, M.B. & Winterbourn, C.C. 1995. Inhibition of myeloperoxidase by benzoic acid hydrazides. *Biochem J* **308**(Pt 2), 559–563.

Kettle, A.J., Gedye, C.A. & Winterbourn, C.C. 1997. Mechanism of inactivation of myeloperoxidase by 4-aminobenzoic acid hydrazide. *Biochem J* **321**(Pt 2), 503–508.

Klebanoff, S.J. 2005. Myeloperoxidase: friend and foe. *J Leukoc Biol* **77**, 598–625.

Koller, A. & Bagi, Z. 2004. Nitric oxide and $H_2O_2$ contribute to reactive dilation of isolated coronary arterioles. *Am J Physiol Heart Circ Physiol* **287**, H2461–H2467.

Lau, D. & Baldus, S. 2006. Myeloperoxidase and its contributory role in inflammatory vascular disease. *Pharmacol Ther* **111**, 16–26.

Liu, X. & Zweier, J.L. 2001. A real-time electrochemical technique for measurement of cellular hydrogen peroxide generation and consumption: evaluation in human polymorphonuclear leukocytes. *Free Radic Biol Med* **31**, 894–901.

Malle, E., Furtmuller, P.G., Sattler, W. & Obinger, C. 2007. Myeloperoxidase: a target for new drug development? *Br J Pharmacol* **152**, 838–854.

 

Matoba, T., Shimokawa, H., Nakashima, M., Hirakawa, Y., Mukai, Y., Hirano, K., Kanaide, H. & Takeshita, A. 2000. Hydrogen peroxide is an endothelium-derived hyperpolarizing factor in mice. *J Clin Invest* **106**, 1521–1530.

Matoba, T., Shimokawa, H., Morikawa, K., Kubota, H., Kunihiro, I., Urakami-Harasawa, L., Mukai, Y., Hirakawa, Y., Akaike, T. & Takeshita, A. 2003. Electron spin resonance detection of hydrogen peroxide as an endothelium-derived hyperpolarizing factor in porcine coronary microvessels. *Arterioscler Thromb Vasc Biol* **23**, 1224–1230.

Mayo Medical Laboratories 2015. Test ID: AAQP Amino Acids, Quantitative, Plasma (www document) http://www.mayomedicallaboratories.com/test-catalog/Clinical+and+Interpretive/9265

Mayyas, F.A., Al-Jarrah, M.I., Ibrahim, K.S. & Alzoubi, K.H. 2014. Level and significance of plasma myeloperoxidase and the neutrophil to lymphocyte ratio in patients with coronary artery disease. *Exp Ther Med* **8**, 1951–1957.

Miura, H., Bosnjak, J.J., Ning, G., Saito, T., Miura, M. & Gutterman, D.D. 2003. Role for hydrogen peroxide in flow-induced dilation of human coronary arterioles. *Circ Res* **92**, e31–e40.

Nicholls, S.J. & Hazen, S.L. 2005. Myeloperoxidase and cardiovascular disease. *Arterioscler Thromb Vasc Biol* **25**, 1102–1111.

Nikpoor, B., Turecki, G., Fournier, C., Theroux, P. & Rouleau, G.A. 2001. A functional myeloperoxidase polymorphic variant is associated with coronary artery disease in French-Canadians. *Am Heart J* **142**, 336–339.

Okabe, E., Takahashi, S., Norisue, M., Manson, N.H., Kukreja, R.C., Hess, M.L. & Ito, H. 1993. The effect of hypochlorous acid and hydrogen peroxide on coronary flow and arrhythmogenesis in myocardial ischemia and reperfusion. *Eur J Pharmacol* **248**, 33–39.

Pennathur, S., Jackson-Lewis, V., Przedborski, S. & Heinecke, J.W. 1999. Mass spectrometric quantification of 3-nitrotyrosine, ortho-tyrosine, and o, o'-dityrosine in brain tissue of 1-methyl-4-phenyl-1,2,3, 6-tetrahydropyridine-treated mice, a model of oxidative stress in Parkinson's disease. *J Biol Chem* **274**, 34621–34628.

Persson, P.B. 2013. Good publication practice in physiology: guidelines for Acta Physiol. *Acta Physiol* **209**, 250–253.

Podrez, E.A., Schmitt, D., Hoff, H.F. & Hazen, S.L. 1999. Myeloperoxidase-generated reactive nitrogen species convert LDL into an atherogenic form in vitro. *J Clin Invest* **103**, 1547–1560.

Podrez, E.A., Abu-Soud, H.M. & Hazen, S.L. 2000. Myeloperoxidase-generated oxidants and atherosclerosis. *Free Radic Biol Med* **28**, 1717–1725.

Porszasz, R., Porkolab, A., Ferencz, A., Pataki, T., Szilvassy, Z. & Szolcsanyi, J. 2002. Capsaicin-induced nonneural vasoconstriction in canine mesenteric arteries. *Eur J Pharmacol* **441**, 173–175.

Prutz, W.A. 1996. Hypochlorous acid interactions with thiols, nucleotides, DNA, and other biological substrates. *Arch Biochem Biophys* **332**, 110–120.

Pullar, J.M., Vissers, M.C. & Winterbourn, C.C. 2000. Living with a killer: the effects of hypochlorous acid on mammalian cells. *IUBMB Life* **50**, 259–266.

Reynolds, W.F., Chang, E., Douer, D., Ball, E.D. & Kanda, V. 1997. An allelic association implicates myeloperoxidase in the etiology of acute promyelocytic leukemia. *Blood* **90**, 2730–2737.

Reynolds, W.F., Rhees, J., Maciejewski, D., Paladino, T., Sieburg, H., Maki, R.A. & Masliah, E. 1999. Myeloperoxidase polymorphism is associated with gender specific risk for Alzheimer's disease. *Exp Neurol* **155**, 31–41.

Root, R.K. & Metcalf, J.A. 1977. $H_2O_2$ release from human granulocytes during phagocytosis. Relationship to superoxide anion formation and cellular catabolism of $H_2O_2$: studies with normal and cytochalasin B-treated cells. *J Clin Invest* **60**, 1266–1279.

Rudolph, T.K., Wipper, S., Reiter, B., Rudolph, V., Coym, A., Detter, C., Lau, D., Klinke, A., Friedrichs, K., Rau, T. *et al.* , *et al.* 2012. Myeloperoxidase deficiency preserves vasomotor function in humans. *Eur Heart J* **33**, 1625–1634.

Savenkova, M.L., Mueller, D.M. & Heinecke, J.W. 1994. Tyrosyl radical generated by myeloperoxidase is a physiological catalyst for the initiation of lipid peroxidation in low density lipoprotein. *J Biol Chem* **269**, 20394–20400.

Sirpal, S. 2009. Myeloperoxidase-mediated lipoprotein carbamylation as a mechanistic pathway for atherosclerotic vascular disease. *Clin Sci (Lond)* **116**, 681–695.

Stocker, R., Huang, A., Jeranian, E., Hou, J.Y., Wu, T.T., Thomas, S.R. & Keaney, J.F. Jr 2004. Hypochlorous acid impairs endothelium-derived nitric oxide bioactivity through a superoxide-dependent mechanism. *Arterioscler Thromb Vasc Biol* **24**, 2028–2033.

Sugiyama, S., Okada, Y., Sukhova, G.K., Virmani, R., Heinecke, J.W. & Libby, P. 2001. Macrophage myeloperoxidase regulation by granulocyte macrophage colony-stimulating factor in human atherosclerosis and implications in acute coronary syndromes. *Am J Pathol* **158**, 879–891.

Thengchaisri, N. & Kuo, L. 2003. Hydrogen peroxide induces endothelium-dependent and -independent coronary arteriolar dilation: role of cyclooxygenase and potassium channels. *Am J Physiol Heart Circ Physiol* **285**, H2255–H2263.

Turan, N.N., Demiryurek, A.T. & Kanzik, I. 2000. Hypochlorous acid-induced responses in sheep isolated pulmonary artery rings. *Pharmacol Res* **41**, 589–596.

Vita, J.A., Brennan, M.L., Gokce, N., Mann, S.A., Goormastic, M., Shishehbor, M.H., Penn, M.S., Keaney, J.F. Jr & Hazen, S.L. 2004. Serum myeloperoxidase levels independently predict endothelial dysfunction in humans. *Circulation* **110**, 1134–1139.

Woods, A.A., Linton, S.M. & Davies, M.J. 2003. Detection of HOCl-mediated protein oxidation products in the extracellular matrix of human atherosclerotic plaques. *Biochem J* **370**, 729–735.

Xu, J., Xie, Z., Reece, R., Pimental, D. & Zou, M.H. 2006. Uncoupling of endothelial nitric oxidase synthase by hypochlorous acid: role of NAD(P)H oxidase-derived superoxide and peroxynitrite. *Arterioscler Thromb Vasc Biol* **26**, 2688–2695.

Yada, T., Shimokawa, H., Hiramatsu, O., Kajita, T., Shigeto, F., Goto, M., Ogasawara, Y. & Kajiya, F. 2003. Hydrogen

  doi: 10.1111/apha.12488

peroxide, an endogenous endothelium-derived hyperpolarizing factor, plays an important role in coronary autoregulation in vivo. *Circulation* **107**, 1040–1045.

Yang, J., Ji, R., Cheng, Y., Sun, J.Z., Jennings, L.K. & Zhang, C. 2006. L-arginine chlorination results in the formation of a nonselective nitric-oxide synthase inhibitor. *J Pharmacol Exp Ther* **318**, 1044–1049.

Zhang, C., Patel, R., Eiserich, J.P., Zhou, F., Kelpke, S., Ma, W., Parks, D.A., Darley-Usmar, V. & White, C.R. 2001a. Endothelial dysfunction is induced by proinflammatory oxidant hypochlorous acid. *Am J Physiol Heart Circ Physiol* **281**, H1469–H1475.

Zhang, C., Reiter, C., Eiserich, J.P., Boersma, B., Parks, D.A., Beckman, J.S., Barnes, S., Kirk, M., Baldus, S., Darley-Usmar, V.M. & White, C.R. 2001b. L-arginine chlorination products inhibit endothelial nitric oxide production. *J Biol Chem* **276**, 27159–27165.

Zhang, R., Brennan, M.L., Fu, X., Aviles, R.J., Pearce, G.L., Penn, M.S., Topol, E.J., Sprecher, D.L. & Hazen, S.L. 2001c. Association between myeloperoxidase levels and risk of coronary artery disease. *JAMA* **286**, 2136–2142.

Zhang, C., Yang, J., Jacobs, J.D. & Jennings, L.K. 2003. Interaction of myeloperoxidase with vascular NAD(P)H oxidase-derived reactive oxygen species in vasculature: implications for vascular diseases. *Am J Physiol Heart Circ Physiol* **285**, H2563–H2572.

Zhang, C., Yang, J. & Jennings, L.K. 2004. Leukocyte-derived myeloperoxidase amplifies high-glucose–induced endothelial dysfunction through interaction with high-glucose–stimulated, vascular non–leukocyte-derived reactive oxygen species. *Diabetes* **53**, 2950–2959.

Zhang, D.X., Borbouse, L., Gebremedhin, D., Mendoza, S.A., Zinkevich, N.S., Li, R. & Gutterman, D.D. 2012. H2O2-induced dilation in human coronary arterioles: role of protein kinase G dimerization and large-conductance $Ca^{2+}$-activated $K^+$ channel activation. *Circ Res* **110**, 471–480.

Zhang, H., Xu, H., Weihrauch, D., Jones, D.W., Jing, X., Shi, Y., Gourlay, D., Oldham, K.T., Hillery, C.A. & Pritchard, K.A. Jr 2013. Inhibition of myeloperoxidase decreases vascular oxidative stress and increases vasodilatation in sickle cell disease mice. *J Lipid Res* **54**, 3009–3015.

 