SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

The vasomotor effects of inflammatory mediators

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The Examination takes place at the Department of Pediatrics, Faculty of Medicine, University of Debrecen at 11 am, on May 11, 2015.

Head of the **Defense Committee**:	Prof. György Balla, MD, PhD, DSc, MHAS
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The PhD Defense takes place at the Lecture Hall of Department of Pediatrics, Faculty of Medicine, University of Debrecen at 1 pm, on May 11, 2015.

1. Introduction

Cardiovascular disorders (such as hypertension, ischaemic heart disease, stroke or peripherial vasoconstriction) are often related to the inflammatory processes of the blood vessels. Lot of inflammatory mediators have vasomotor effects thereby contribute to the regulation of the microcirculation. Inflammation is an important defense mechanism; also its features were already described. However, the exact mechanism and all of the molecules contributing to the vascular diameter under inflammatory processes have not investigated yet. We suppose that the inflammatory enzyme, myeloperoxidase (MPO) has vasomotor effects.

1.1. Hydrogen-peroxide, as a reactive oxygen derivative agent

H_2O_2 can be produced by endothelial cells, smooth muscle cells and fibroblasts, under both physiological and pathological conditions. Moreover, significant amounts of H_2O_2 are released by activated leukocytes under inflammatory conditions. Numerous enzyme systems, including NAD(P)H oxidase the mitochondrial respiratory chain, xanthine oxidase, uncoupled endothelial nitric oxide (NO) synthase, cytochrome P-450 enzymes, lipoxygenase and the cyclooxygenases (COX), can generate the superoxide anion ($O_2^{\cdot -}$), which is then reduced to H_2O_2. There can be a great variation in the extracellular concentration of H_2O_2, but it can probably reach 0.3 mM.

1.2. Hydrogen-peroxide, as a vasoactive agent

H_2O_2 has been shown to act as an endothelium-derived hyperpolarizing factor (EDHF) in several vascular beds, including porcine coronary arterioles, mouse mesenteric arterioles, rat ophthalmic arteries and rat coronary arterioles. It has been proposed that, as an EDHF, H_2O_2 contributes to the development of functional hyperaemia in human coronary and mesenteric arterioles. Another important role ascribed to H_2O_2 is the mediation of flow-induced dilation in human coronary arterioles and as such it may provide an important back-up dilator mechanism when levels of NO are reduced. In contrast, H_2O_2 results in vasoconstriction in the rat aorta and renal artery, the rabbit pulmonary artery and the canine basilar arterioles. Surprisingly, H_2O_2 has also been shown to exert a concentration-dependent biphasic effect (*i.e.* vasoconstriction followed by vasodilation) in the skeletal muscle arterioles and mesenteric arterioles of the rat.

Previous studies have revealed certain fragments of the signalling cascades responsible for the H_2O_2-evoked vascular constrictions and dilations in various species and preparations. Thus, H_2O_2 has been shown to evoke vasodilation by activation of arachidonic acid (AA) metabolism and subsequent cyclic adenosine monophosphate production in canine cerebral arteries. Moreover, H_2O_2 has been claimed to activate the NO/cyclic guanosine monophosphate pathway in rat skeletal muscle arterioles and in the rabbit aorta. Increased cGMP levels lead to the release of endothelium-derived dilator prostaglandins in porcine coronary arterioles, whereas the endothelium-independent relaxation to H_2O_2 in porcine coronary arterioles involves the activation of K^+ channels. Similarly to the above

vasodilator mechanisms, it is hypothesized that in different vessel types/species several distinct signalling molecules can contribute to the H_2O_2-evoked constrictor effects, including COX products, tyrosine kinases and mitogen-activated protein kinase.

1.3. Myeloperoxidase: structure and function

MPO, a heme-containing, intensely green protein, was originally isolated from canine pus and from purulent fluids from patients with tuberculosis. The synthesis of MPO is initiated in the bone marrow during myeloid differentiation and is completed in the granulocytes. MPO is stored primarily in the azurophil granules of the polymorphonuclear neutrophils and monocytes, but it has also been found in tissue macrophages. To exert its antimicrobial effects, MPO primarily catalyzes the reaction of H_2O_2 with chloride, to form hypochlorous acid (HOCl). 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 system depend on the local concentration of H_2O_2 and on the availability of other substrates and/or antioxidant molecules (*e.g.* methionine (Met).

1.4. Myeloperoxidase and hydrogen-peroxide in cardiovascular disorders

H_2O_2 is involved as a signalling molecule in the physiological regulation of the vascular diameter however; during inflammation the capacity of the antioxidant system is not enough to eliminate the oxidative agents. Therefore, H_2O_2 can

contribute to the development of the vascular dysfunction in hypertension, diabetes and atherosclerosis.

The activation of MPO may also become deleterious and can contribute to the development of cardiovascular diseases Excessive levels of MPO in the plasma may be accompanied by an increased risk of subsequent cardiovascular events, whereas individuals with an inherited MPO deficiency are at a reduced cardiovascular risk. There is currently no clear explanation of this situation.

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. 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. Through its catalytic activity, MPO can consume nitrogen monoxide (NO), thereby limiting its bioavailability. MPO-derived HOCl reacts with L-arginine and produces NO-synthesis inhibitors, and HOCl can impair endothelial NO bioactivity in a superoxide-dependent manner. 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. From a functional aspect, MPO treatment let to a decrease in myocardial perfusion in pigs and inhibited the acetylcholine-evoked relaxation in the internal mammary arteries. Vasorelaxation in response to acetylcholine was also found to be impaired in mice at relatively high plasma MPO levels.

Since the MPO substrate H_2O_2 was earlier identified as an important regulator of vascular diameter under both normal and pathological conditions, the vasoactive effects of MPO were contrasted to those of H_2O_2.

2. Aims

Our aims were:

1. to investigate the mechanism of the H_2O_2-evoked vasoconstriction in skeletal muscle arterioles
2. to examine the acute vasomotor effects of MPO

3. Materials and methods

All procedures employed in this work conformed to strictly Directive 2010/63/EU of the European Parliament and were approved by the Ethical Committee of the University of Debrecen. Experiments were performed on male Wistar rats; animals were anaesthetized with an intraperitoneal injection of sodium pentobarbital (150 mg/kg).

3.1. Measurement of arteriolar diameter

The isolated arterioles were transferred into an organ chamber containing two glass micropipettes filled with Krebs solution. The arterioles were cannulated at both ends and the micropipettes were connected via silicone tubing to a pressure servo control system. The intraluminal pressure was set at 80 mmHg, the temperature was maintained at 37 ˚C by the built in temperature controller in the

tissue chamber. Changes in arteriolar diameter were recorded by a video microscope system. The isolated skeletal muscle arterioles and coronary arterioles spontaneously developed a substantial myogenic tone (a decrease in diameter from 202±0.3 μm to 156±0.3 μm ±6 μm, n=118, and from 180±11 μm to 115±6 μm, n=33, respectively) in response to an intraluminal pressure of 80 mmHg.

3.2. Measurement of arteriolar contractions under isometric conditions

Basilar arteries were prepared from rat brains with microsurgical tools, and ~ 4-mm-long rings were then mounted in an isometric contraction measurement system. Before exposure to test solutions, vessel tones were 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).

3.3. Experimental protocols

H_2O_2 solutions were prepared immediately before the experiments and were stored on ice. In the first series of experiments, cumulative concentrations of H_2O_2 (1 μM-10 mM) were added to the skeletal muscle arterioles or coronary arterioles and the responses to the H_2O_2 were determined. The diameters were recorded 60 s after the application of each H_2O_2 concentration. To study the kinetics of diameter changes, various concentrations of H_2O_2 (10, 30, 100, 300 μM and 3 mM) were used (600 s treatment duration, diameter measured every 10 s). In some groups of experiments, the endothelium was removed by air perfusion of the arterioles The

effects of H_2O_2 on the diameter of the arterioles were also measured in the presence (15-30-min preincubation) of a PKC inhibitor, a PLC inhibitor, a PLA inhibitor, a Src kinase inhibitor, a non-selective COX-1 and COX-2 inhibitor, a COX-1or COX-2-selective inhibitor and a TXA2 receptor inhibitor.

MPO activity was measured via detection of the chemiluminescence produced upon the oxidation of luminol. The arterioles were treated with MPO (1.92 mU ml^{-1}, 300 s treatment duration, diameter measured every 10 s) to record the effects of MPO alone. This was followed by the addition of H_2O_2 (1 μM-10 mM) and the responses to MPO+H_2O_2 were then determined. In some experiments, the endothelium was removed by the perfusion of air bubbles through the arterioles. The effects of MPO and H_2O_2 were also measured in the presence of an MPO inhibitor, a TXA2 receptor inhibitor and a COX antagonist in the skeletal muscle arterioles. The effects of MPO were tested after incubation of the vessels with the HOCl scavenger L-Met in all three vascular preparations. At the end of the experiments, the maximum (passive) arteriolar diameter was determined in the absence of extracellular Ca^{2+} at an intraluminal pressure of 80 mmHg.

3.4. Parallel measurement of vascular diameter and intracellular Ca^{2+} concentrations

Isolated and cannulated arterioles were incubated for 60 min in the presence of physiological buffer solution containing 1% bovine serum albumin and 5 μM Fura-2AM fluorescent Ca^{2+} indicator dye. Intracellular Ca^{2+} concentrations were measured with an Incyte IM system. Fura-2 fluorescence (recorded every 2-5 s)

was excited alternately by 340 and 380 nm light, while the emitted fluorescence was detected at 510 nm by selecting at least 1000 pixels within the arteriolar wall. Arteriolar Ca^{2+} concentrations were assessed via the Fura-2 fluorescence ratio ($F_{340/380}$), and in these assays the outer arteriolar diameters were determined for each recorded image.

3.5. Immunohistochemistry

The gracilis muscle was removed from the rat and embedded in Tissue-Tek O.C.T compound. Cryostat sections (10 μm thick) were prepared, fixed in acetone for 5-10 min and blocked with normal goat sera for 20 min. COX enzymes were stained with COX-1 and COX-2-specific antibodies. Gracilis muscle was co-stained with anti-smooth muscle actin and DAPI.

3.6. Measurement of the inhibitory effect of L-Methionine on the chlorinating activity of myeloperoxidase

MPO-evoked chlorinating activity was measured with a commercial assay kit in accordance with the manufacturer's instructions. The measurement is based on the cleavage of nonfluorescent 2-[6-(4-aminophenoxy)-3-oxo-3H-xanthen-9-yl] benzoic acid (APF) to fluorescein by MPO-generated hypochlorite (-OCl). The reaction mixtures contained 45 μM APF, 30 μM H_2O_2, 3 U l^{-1} MPO and 200-0.39 mM L-Met (serially diluted). The measurements were performed in phosphate-buffered saline (PBS, pH=7.4) independently from the *in vitro* vascular experiments. Changes in fluorescence intensity (λ_{ex}=485 nm, λ_{em}=520 nm) were

measured at 30-s intervals for 5 min with a plate reader. Fluorescence intensities values were plotted as a function of time and fitted by linear regression (before saturation). The slope of this relation was used to calculate MPO activities.

3.7. 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 GraphPad Prism 5.0 software by the Student t-test and ANOVA (Dunnett's *post hoc* test). $P<0.05$ was considered statistically significant.

4. Results

4.1. Hydrogen-peroxide induced arteriolar responses

Increasing concentrations of H_2O_2 evoked a concentration-dependent biphasic effect in the skeletal muscle arterioles: lower concentrations (10-100 μM) of H_2O_2 produced vasoconstriction (maximum at 100 μM, 34±3% constriction, $P < 0.001$ *vs.* id,), whereas higher concentrations (3-10 mM) of H_2O_2 resulted in

vasodilation (maximum at 10 mM, 80±11% dilation, $P < 0.001$ *vs.* id). In contrast, H_2O_2 evoked only vasodilation in the coronary arterioles (maximum at 10 mM, 96±3% dilation, $P = 0.01$). The kinetics of the H_2O_2-evoked changes in the diameter of the skeletal muscle arterioles was also tested. Although the H_2O_2-evoked vasoconstrictions were mostly transient, vasoconstrictions at lower H_2O_2 concentrations (10 μM and 30 μM) were not followed by significant vasodilations. In contrast, 100 μM or 300 μM H_2O_2 caused time-dependent biphasic changes: after the initial vasoconstriction, a substantial vasodilation developed. Application of 3 mM H_2O_2 resulted in substantial vasodilation without initial vasoconstriction.

4.2. Role of the endothelium in hydrogen-peroxide induced vasoconstriction

The H_2O_2-induced constriction was abolished in the endothelium-denuded skeletal muscle arterioles (0±8% constriction at 100 μM H_2O_2, $P = 0.03$ *vs.* control), but the dilations were not affected (69±10% dilation at 10 mM H_2O_2).

4.3. Hydrogen-peroxide stimulated endothelial signalling processes, leading to the activation of cyclooxygenase

The H_2O_2-evoked vasoconstriction (measured at 100 μM H_2O_2) was inhibited by the application of the PLA antagonist (7±2% constriction, $P < 0.005$ *vs.* control), the PKC antagonist (9±4% constriction at 100 μM H_2O_2, $P < 0.005$ *vs.* control), the PLC inhibitor (15±18% dilation, $P < 0.05$ *vs.* control) or the Src kinase antagonist (8±3% vasoconstriction, $P < 0.005$ *vs.* control).

4.4. Effects of non-selective and selective cyclooxygenase inhibition on hydrogen-peroxide induced arteriolar responses

The H_2O_2-induced constrictions were converted to dilations in the presence of a non-selective COX inhibitor (41±17% dilation at 100 μM H_2O_2, $P < 0.005$ *vs.* control). In separate experiments, we investigated the specific roles of COX-1 and COX-2 in the mediation of the H_2O_2-evoked vascular responses. It emerged that the selective COX-1 inhibitor abolished the constriction induced by H_2O_2 (23±9% dilation at 100 μM H_2O_2, $P < 0.05$ *vs.* control) and converted it to dilation, whereas the inhibitory effect of the COX-2 antagonist was not significant (13±4% constriction at 100 μM H_2O_2, $P > 0.05$ *vs.* control).

4.5. Hydrogen-peroxide evoked effector mechanisms leading to vasconstrictive responses

The H_2O_2-evoked vasoconstriction in the skeletal muscle arterioles was abolished and converted to dilation (36±11% dilation at 100 μM H_2O_2, $P < 0.005$ *vs.* control) by TXA2 receptor inhibition. In contrast, the same treatment did not affect the H_2O_2-evoked dilation in the coronary arterioles (96±2% dilation at 10 mM H_2O_2). Activation of the TXA2 receptors with the stable analogue of TXA2, resulted in constriction of both the skeletal muscle arterioles (69±2%, n=5, $P < 0.002$ *vs*. id) and the coronary arterioles (42±6%, $P = 0.002$ *vs*. id).

4.6. Characterization of hydrogen-peroxide evoked changes in intracellular Ca^{2+} concentrations of vascular smooth muscle cells

The H_2O_2-evoked vasoconstriction was not accompanied by significant changes in the $F_{340/380}$ ratio signal in the range of H_2O_2 concentrations between 1 μM and 100 μM). However the noradrenaline (10 μM)-induced vasoconstriction was accompanied by a significant increase in $F_{340/380}$ (from 0.96±0.04 to 1.36±0.07, P = 0.001). Moreover, the TXA2 agonist-evoked peak in $F_{340/380}$ was significantly smaller than that evoked by noradrenaline (from 0,87±0,04 to 0,93±0,04 *vs.* from 0,92±0,04 to 1,36±0,07, respectively, $P < 0.05$) despite their largely comparable vasoconstrictive responses (to 44±5% *vs.* 57±6%, respectively, $P > 0.05$;).

4.7. Myeloperoxidase promotes hydrogen-peroxide evoked vasoconstriction

MPO (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 skeletal muscle arterioles, a robust MPO-dependent vasoconstrictive effect was observed, *i.e.* 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). In the coronary arterioles, where H_2O_2 evoked only vasodilation, MPO administration resulted in significant vasoconstriction in a wide range of H_2O_2 concentrations, *e.g.* 13±4% dilation at 100 μM H_2O_2, but 6±3% constriction following the addition of MPO (P=0.006). In the basilar arteries, the MPO-dependent vasoconstriction was relatively less pronounced *e.g.* 1.1±0.5 mN dilation at 100 μM H_2O_2 and 1.6±0.7 mN constriction following the addition of MPO (P<0.05).

MPO alone (without the addition of its substrate H_2O_2) did not affect the diameters of the skeletal muscle arterioles or the coronary arterioles or the contractile force in the basilar arteries (data not shown).

4.8. Hypochlorous acid mediates the myeloperoxidase induced vasoconstriction in the skeletal muscle arterioles

The mechanical effects of the chlorinating activity of MPO were assessed comparing the vascular responses in the presence of the HOCl scavenger L-Met with those in the presence of the MPO-specific inhibitor 4-aminobenzhydrazide. 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.* 5±18% vasoconstriction, P=0.067) 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 alone did not affect the H_2O_2-evoked vasoconstriction in the absence of MPO. In a parallel *in vitro* enzyme assay, L-Met fully opposed the chlorinating activity of MPO.

4.9. Divergent effects of L-Methionine treatments on myeloperoxidase evoked vasodilations in different vessel types

The MPO-stimulated HOCl-independent vasodilating mechanism was screened in different vascular beds. In the skeletal muscle arterioles, 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). In the coronary arterioles, the maximal L-Met concentration 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). Finally, L-Met treatment did not significantly influence the MPO-evoked vascular responses in the basilar arteries (*e.g.* 3.3±1 mN vasoconstriction at 300 μM H_2O_2 *vs.* 4.0±1 mN vasoconstriction, *P*=0.61).

4.10. The signalling mechanism of myeloperoxidase evoked vasoconstriction in the skeletal muscle arterioles

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

Next, the involvement of the TXA2 receptors in the MPO-evoked vasoconstrictive effects was tested. Inhibition of the TXA2 receptors 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).

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

4.11. Vascular expression of cyclooxygenase in the skeletal muscle arterioles

The expression of COX isoenzymes in skeletal muscle arterioles 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.

4.12. Myeloperoxidase 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 skeletal muscle arterioles. MPO-evoked vasoconstriction (29±3% vasoconstriction at 1 mM H_2O_2; P=0.04 *vs.* the baseline) developed without significant changes in the $F_{340/380}$ ratio signal in the range of H_2O_2 concentrations between 1 μM and 1 mM. In contrast, the noradrenaline-evoked (1 nM-10 μM) vasoconstrictions with comparable magnitudes (44±4% constriction at 10 μM noradrenaline; P=0.0005 *vs.* the baseline) were accompanied by significant increases in the $F_{340/380}$ ratio (from

0.85±0.03 to 1.15±0.09). MPO alone did not have any effect on the arteriolar diameter or on the $F_{340/380}$ signal (not shown).

5. Discussion

A number of attempts have been made to investigate the mechanism of H_2O_2-evoked vasodilation but much less is known as regards the mechanism of H_2O_2-evoked vasoconstriction. H_2O_2 can modulate the vascular diameter in the rat renal artery, the canine basilar artery, the porcine coronary arterioles and the rabbit aorta in an endothelium-dependent manner. It may also display endothelium-independent effects in human coronary arterioles, canine coronary arterioles and the rat aorta. In the present study, H_2O_2-induced vasoconstriction was completely inhibited by endothelium denudation or by inhibition of the TXA2 receptor. Our observations suggest that H_2O_2 causes the generation of TXA2 in the endothelium, leading to vasoconstriction, and also that H_2O_2 may elicit endothelium-dependent dilation in skeletal muscle arterioles when the TXA2-mediated vasoconstriction is blocked. In contrast, H_2O_2-evoked vasodilation in the coronary arterioles was not influenced by a TXA2 inhibitor, although the activation of TXA2 receptors with U46619 resulted in vasoconstriction in both the coronary and the skeletal muscle arterioles. These results suggest that TXA2 receptors are present in both types of vessel, but H_2O_2 activates different signalling pathways. It evokes TXA2 synthesis and release from endothelial cells in the skeletal muscle arterioles, but has no such effect in the coronary arterioles.

PLA is responsible for the generation of AA (the substrate of COX) in various vascular preparations. In our study, H_2O_2-evoked vasoconstriction was inhibited in the presence of the PLA antagonist, suggesting a role for PLA in the H_2O_2-induced vasomotor response. The activation of PLA can be a consequence of PKC-mediated phosphorylation. Indeed, preincubation of skeletal muscle arterioles with the PKC antagonist resulted in a significantly reduced H_2O_2-evoked constriction. PKC can be activated by the diacylglycerols released by PLC, and inhibition of PLC resulted in a significantly decreased H_2O_2-mediated vasoconstriction. It might be argued that inhibition of the PKC pathway (*e.g.* PLC and PKC inhibition) can affect TXA2 receptor stimulation-evoked constrictions independently of the endothelial effects of H_2O_2. However, PLC inhibition was without effects on the constrictions evoked by the TXA2 receptor agonist U46619, suggesting an upstream (endothelial) target in H_2O_2-mediated constriction.

The H_2O_2-evoked activation of PLC was earlier shown to be mediated by Src kinase in mouse embryonic fibroblasts. Indeed, the constrictor effects of H_2O_2 in skeletal muscle arterioles were inhibited in the presence of a Src kinase antagonist. Moreover, H_2O_2-evoked vasoconstriction was completely inhibited by the non-specific COX antagonist indomethacin. Furthermore, the H_2O_2-induced vasoconstriction was also fully inhibited in the presence of a specific COX-1 antagonist, while it was not influenced significantly by a specific COX-2 antagonist, suggesting a prominent role of COX-1 in H_2O_2-evoked vasoconstriction.

Taken together, the H_2O_2-induced constriction component was largely abolished by inhibitors of PLA, PKC, PLC and Src kinases, indicating a complex network of intracellular signalling in the H_2O_2 response. Interestingly, H_2O_2-evoked vasoconstriction was also prevented in the absence of endothelium. These findings, implicate a sequence of signalling events in the endothelial layer during H_2O_2-evoked vasoconstrictions. Nevertheless, alternative mechanisms cannot be excluded.

TXA2 receptors are expressed in numerous cell types, including vascular smooth muscle cells. TXA2 receptors can couple with G_q protein, thereby activating the PLC pathway, giving rise to Ca^{2+} release and PKC activation (a Ca^{2+}-dependent pathway). However, TXA2 also binds to G_{12} proteins, leading to the activation of Rho-kinase-mediated signalling (a Ca^{2+}-independent pathway), and hence to Ca^{2+} sensitization of the contractile protein machinery. Nevertheless, G_{12} proteins may also evoke vasoconstriction by promoting Ca^{2+} entry through another Ca^{2+}-dependent mechanism, as has been demonstrated in the rat caudal arterial smooth muscle. Our experimental results indicated that H_2O_2-evoked vasoconstrictions were not accompanied by significant increases in intracellular Ca^{2+} concentration. In contrast, the treatment with noradrenaline increased the intracellular Ca^{2+} concentration in parallel with a significant decrease in arteriolar diameter. In comparison, the TXA2 receptor agonist U46619-evoked vasoconstriction was accompanied by a significantly lower increase in intracellular Ca^{2+} concentration than that evoked by noradrenaline, supporting our hypothesis

that H_2O_2 increases the Ca^{2+} sensitivity of the vascular smooth muscle, rather than stimulating Ca^{2+} entry into smooth muscle cells. Although the explanation of the apparent increase in vascular Ca^{2+} sensitivity is beyond the scope of this study, we speculate that the potential mechanism may involve the inhibition of myosin light chain phosphatase via Rho-associated kinase (ROCK) or PKC, leading to increased phosphorylation of LC20 (myosin regulatory light chain). Alternatively, vascular Ca^{2+} sensitization of constriction could be elicited by dynamic regulation of the actin cytoskeleton by PKC and ROCK.

It is rather difficult to estimate the real concentration of H_2O_2 in vascular beds *in vivo*. Nevertheless, it has been shown that in certain pathological conditions it may reach relatively high levels (up to about 0.3 mM). In this study, the use of even higher concentrations of H_2O_2 (up to 10 mM) allowed us to characterize the biphasic vascular effects of H_2O_2. Lower concentrations of H_2O_2 evoked vasodilation in coronary arterioles, but elicited the constriction of skeletal muscle arterioles. This is consistent with the previous finding an important regulatory role of H_2O_2 as an EDHF in the coronary microcirculation, and the conclusion that, H_2O_2 cannot be regarded as an EDHF in skeletal muscle arterioles under physiological conditions. It is unclear whether H_2O_2 concentrations reach levels high enough to evoke vasodilation and hence to increase the skeletal muscle blood flow under pathological conditions (*e.g.* inflammation).

Vascular inflammation is characterized by increased levels of production and local release of both H_2O_2 and MPO. We hypothesized that MPO can modulate

the vascular responses to H_2O_2. This process may have immediate (acute) effects on the vascular diameter, which was tested here under *in vitro* conditions.

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. 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-crosslinking of amino acids, lipids and nucleotides. Less is known about the molecular pathways involved in the HOCl-evoked changes in vascular dynamics. One such possibility relates to a decrease in NO bioavailability, as suggested by observations on HOCl-dependent impairments in endothelial function. Similarly to our findings, HOCl was found to cause vasoconstriction in bovine pulmonary arteries, but the exact mechanism of this effect remained unclear. The present investigation revealed increases in vasoconstriction in the skeletal muscle arterioles, coronary arterioles and basilar arteries, 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 not only inhibited the vasoconstriction evoked by MPO, but also unmasked a robust vasodilatory effect in the skeletal muscle arterioles. The employed MPO-specific inhibitor, 4-aminobenzhydrazide blocked both the chlorinating and the peroxidase activities of the MPO 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 coronary arterioles and basilar arteries, where MPO evoked vasoconstrictions were less pronounced than those in the skeletal muscle arterioles, 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, which eliminated the endothelium-derived effects,

including decreased NO bioavailability. Importantly, H_2O_2-evoked vasoconstrictions were found in a previous study to be completely endothelium-dependent. However, the vasoconstriction evoked by H_2O_2 in the presence of MPO was only partially opposed by endothelium removal, 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 TXA2 not only in the endothelial cells, but also in the vascular smooth muscle cells, through the activation of COXs. To confirm this possibility, the expression of COXs enzymes was explored by means of immunohistochemistry, and COX-1-specific staining was indeed confirmed both in the endothelial layer and in the smooth muscle cells of the skeletal muscle arterioles. 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. Hence, the above findings point to a HOCl – COX1 – TXA2 pathway as being decisive in the prevention of MPO-dependent vasodilation in the skeletal muscle arterioles.

Numerous previous studies have furnished evidence that H_2O_2 is an important regulator of the vascular diameter. Under pathological conditions associated with inflammation, MPO is released together with H_2O_2. Under these circumstances L-Met may prevent H_2O_2-evoked vasoconstriction or even convert it into vasodilation, because L-Met in its presumed physiological concentration range

(i.e. 20-40 μM) also largely prevents the vasoconstrictions evoked by MPO in the skeletal muscle arterioles. Hence, the ultimate effect on the vascular tone and thereby on local the microcirculation will be a function of the availability of a range of local regulators (*e.g.* H_2O_2, MPO, L-Met, *etc.*) which are of high potency in vasoregulation.

The MPO-induced vasoconstrictions were not accompanied by significant increases in the intracellular Ca^{2+} concentration in the H_2O_2 concentration range of between 100 μM and 1 mM. In contrast, noradrenaline treatment evoked vasoconstrictions to similar degrees, together with significant increases in the intracellular Ca^{2+} concentration, suggesting that MPO activated a Ca^{2+}-sensitizing mechanism, causing vasoconstriction rather than increasing the intracellular Ca^{2+} concentration.

Overall, our present results suggest that MPO-derived HOCl can enhance the production of TXA2 both in the endothelium and in the vascular smooth muscle cells, 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 vasodilator mechanism may prevail in the skeletal muscle arterioles of the rat during tissue inflammation associated with neutrophil degranulation.

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List of publications related to the dissertation

1. **Csató, V.**, Pető, A., Fülöp, G.Á., Rutkai, I., Pásztorné T., E., Fagyas, M., Kalász, J., Édes, I., Tóth, A., Papp, Z.: Myeloperoxidase evokes substantial vasomotor responses in isolated skeletal muscle arterioles of the rat.
   Acta Physiol. "accepted by publisher" (2015)
   IF:4.251 (2013)

2. **Csató, V.**, Pető, A., Koller, Á., Édes, I., Tóth, A., Papp, Z.: Hydrogen peroxide elicits constriction of skeletal muscle arterioles by activating the arachidonic acid pathway.
   PLoS One. 9 (8), e103858-, 2014.
   DOI: http://dx.doi.org/10.1371/journal.pone.0103858
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List of other publications

3. Kalász, J., Pásztorné Tóth, E., Fagyas, M., Balogh, Á., Tóth, A., **Csató, V.**, Édes, I., Papp, Z., Borbély, A.: Myeloperoxidase impairs the contractile function in isolated human cardiomyocytes.
   Free Radic. Biol. Med. "accepted by publisher" (2015)
   IF:5.71 (2013)

4. Fagyas, M., Úri, K., Siket, M.I., Fülöp, G.Á., **Csató, V.**, Daragó, A., Boczán, J., Bányai, E., Szentkirályi, I.E., Maros, T.M., Szerafin, T., Édes, I., Papp, Z., Tóth, A.: New perspectives in the renin-angiotensin-aldosterone system (RAAS) II: Albumin suppresses angiotensin converting enzyme (ACE) activity in human.
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5. Tóth, A., Czikora, Á., Pásztor, T.E., Dienes, B., Bai, P., Csernoch, L., Rutkai, I., **Csató, V.**, Mányiné, I.S., Pórszász, R., Édes, I., Papp, Z., Boczán, J.: Vanilloid receptor-1 (TRPV1) expression and function in the vasculature of the rat.
   J. Histochem. Cytochem. 62 (2), 2014.
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