



Hydrogen Peroxide Elicits Constriction of Skeletal Muscle Arterioles by Activating the Arachidonic Acid Pathway

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Abstract

Aims: The molecular mechanisms of the vasoconstrictor responses evoked by hydrogen peroxide (H₂O₂) have not been clearly elucidated in skeletal muscle arterioles.

Methods and Results: Changes in diameter of isolated, cannulated and pressurized gracilis muscle arterioles (GAs) of Wistar-Kyoto rats were determined under various test conditions. H₂O₂ (10–100 μM) evoked concentration-dependent constrictions in the GAs, which were inhibited by endothelium removal, or by antagonists of phospholipase A (PLA; 100 μM 7,7-dimethyl-(5Z,8Z)-eicosadienoic acid), protein kinase C (PKC; 10 μM chelerythrine), phospholipase C (PLC; 10 μM U-73122), or Src family tyrosine kinase (Src kinase; 1 μM Src Inhibitor-1). Antagonists of thromboxane A₂ (TXA₂; 1 μM SQ-29548) or the non-specific cyclooxygenase (COX) inhibitor indomethacin (10 μM) converted constrictions to dilations. The COX-1 inhibitor (SC-560, 1 μM) demonstrated a greater reduction in constriction and conversion to dilation than that of COX-2 (celecoxib, 3 μM). H₂O₂ did not elicit significant changes in arteriolar Ca²⁺ levels measured with Fura-2.

Conclusions: These data suggest that H₂O₂ activates the endothelial Src kinase/PLC/PKC/PLA pathway, ultimately leading to the synthesis and release of TXA₂ by COX-1, thereby increasing the Ca²⁺ sensitivity of the vascular smooth muscle cells and eliciting constriction in rat skeletal muscle arterioles.

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Introduction

Among its many important roles, H₂O₂ is involved as a signalling molecule in the physiological regulation of the vascular diameter. Moreover, H₂O₂ can contribute to the development of a vascular dysfunction in hypertension [1,2], diabetes [3,4] and atherosclerosis [5]. Nevertheless, the vascular signalling pathways mobilized by H₂O₂ have not been fully elucidated.

H₂O₂ can be produced by endothelial cells, smooth muscle cells and fibroblasts [6,7], under both physiological and pathological conditions. Moreover, significant amounts of H₂O₂ are released by activated leukocytes under inflammatory conditions [8]. Numerous enzyme systems, including NAD(P)H oxidase [9,10], the mitochondrial respiratory chain, xanthine oxidase, uncoupled endothelial nitric oxide (NO) synthase, cytochrome P-450 enzymes, lipoxygenase and the cyclooxygenases [11–16], can generate the superoxide anion (O₂^{•−}), which is then reduced to H₂O₂. There can be a great variation in the extracellular concentration of H₂O₂, but it can probably reach 0.3 mM [8,17,18].

H₂O₂ 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 [19–23]. It has been proposed that, as an EDHF, H₂O₂ contributes to the development of functional hyperaemia in human coronary and mesenteric arterioles [24,25]. Another important role ascribed to H₂O₂ is the mediation of flow-induced dilation in human coronary arterioles [26,27] and as such it may provide an important back-up dilator mechanism when levels of NO are reduced [28]. In contrast, H₂O₂ results in vasoconstriction in the rat aorta [29,30] and renal artery [31], the rabbit pulmonary artery [32] and the canine basilar arterioles [33,34]. Surprisingly, H₂O₂ has also been shown to exert a concentration-dependent biphasic effect (*i.e.* vasoconstriction followed by vasodilation) in the skeletal muscle and mesenteric arterioles of the rat [8,35].

Previous studies have revealed certain fragments of the signalling cascades responsible for the H₂O₂-evoked vascular constrictions and dilations in various species and preparations.

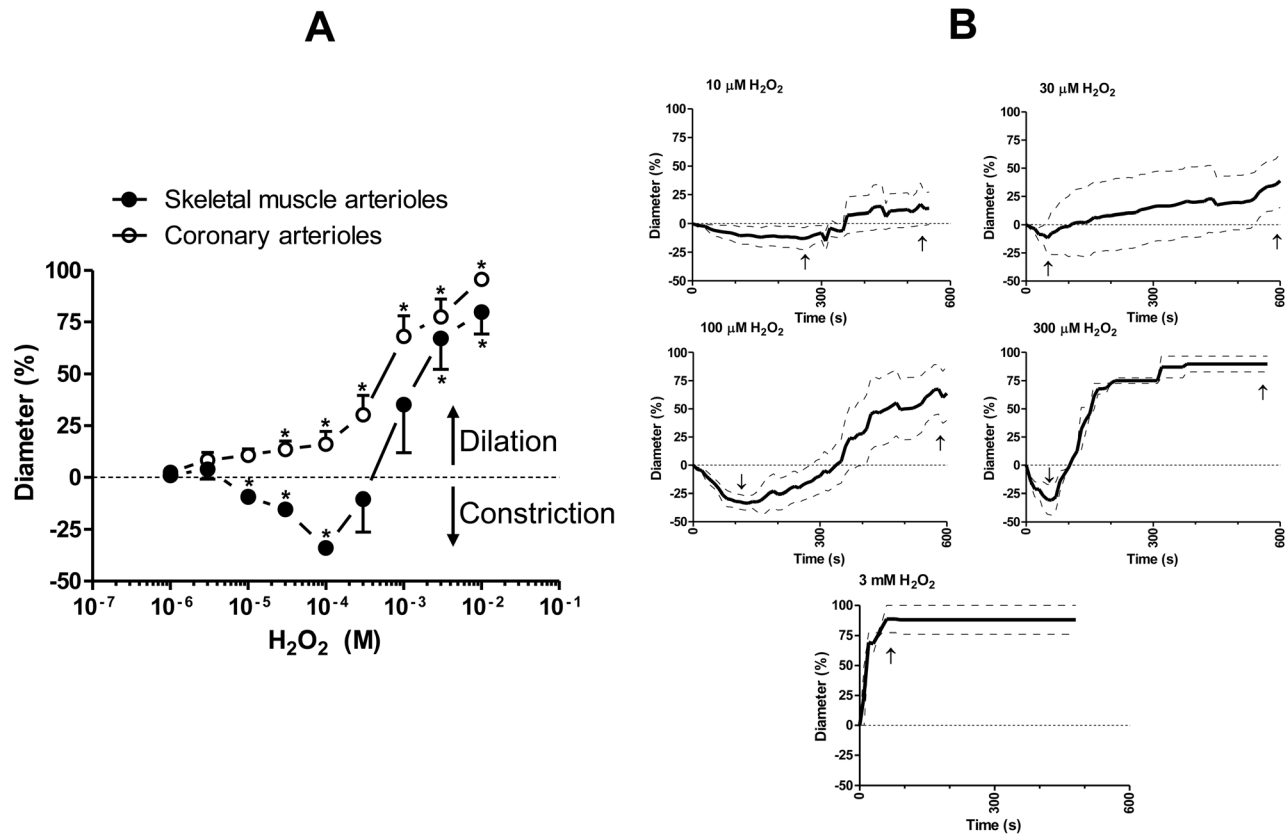


Figure 1. Effects of H₂O₂ on arterioles isolated from skeletal muscle and heart. H₂O₂ (1 μM–10 mM) was added to isolated, cannulated, skeletal muscle (initial diameter (id): 191 ± 17 μm, n = 6 arterioles from 6 different animals) or coronary arterioles (id: 110 ± 18 μm, n = 7 arterioles from 7 different animals) with intact endothelium. The arteriolar diameter was recorded and concentration-response (cumulative application) relationships were determined (panel A). Changes in relative arteriolar diameter are shown. Relative diameter changes during vasodilations were expressed as percentages of the difference between the maximum passive diameter (maximum dilation: 100%, determined in the absence of extracellular Ca²⁺) and initial diameter with positive values, while during constrictions they were expressed relative to the initial diameter (illustrated at 0% on the y axis) with negative values. Asterisks denote significant differences from the initial values. The kinetics of H₂O₂-evoked responses was studied in isolated skeletal muscle arterioles (panel B; means ± SEM with solid and dashed lines, respectively). The effects of the indicated concentrations of H₂O₂ were recorded for 600 s in the continuous presence of H₂O₂ (n = 3–5 arterioles at each concentration from 11 different animals). The positions of maximum constrictions and dilations are illustrated by arrows. doi:10.1371/journal.pone.0103858.g001

Thus, H₂O₂ has been shown to evoke vasodilation by activation of arachidonic acid (AA) metabolism and subsequent cyclic adenosine monophosphate production in canine cerebral arteries [36]. Moreover, H₂O₂ has been claimed to activate the NO/cyclic guanosine monophosphate pathway in rat skeletal muscle arterioles and in the rabbit aorta [8,37]. Increased cGMP levels lead to the release of endothelium-derived dilator prostaglandins in porcine coronary arterioles [38], whereas the endothelium-independent relaxation to H₂O₂ in porcine coronary arterioles involves the activation of K⁺ channels [39–42]. Similarly to the above vasodilatory mechanisms, it is hypothesized that in different vessel types/species several distinct signalling molecules can contribute to the H₂O₂-evoked constrictor effects, including COX products [8,29,30,43], tyrosine kinases [29,34] and mitogen-activated protein kinase [34,44,45]. Moreover, these pathways may mobilize intracellular Ca²⁺-dependent mechanisms in vascular smooth muscle cells to evoke vasoconstriction [29,34], although the activation of Ca²⁺-independent alternative pathways cannot be excluded [46].

Taken together, H₂O₂ apparently activates complex second messenger systems in the vascular endothelium and smooth muscle cells to evoke vasoconstriction, although the exact signalling

pathway and its ability to change intracellular Ca²⁺ concentrations are not well understood. In the present study, therefore, we investigated the acute effects of H₂O₂ on the diameter of arterioles isolated from rat skeletal muscle and rat coronaries, the signal transduction pathway initiating H₂O₂-evoked vasoconstriction, and the changes in vascular smooth muscle intracellular Ca²⁺ concentrations induced by H₂O₂.

Methods

Ethical statement

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.

Animals, anaesthesia and tissue dissection

Experiments were performed on male Wistar rats (approximately 10 weeks of age, weighing 250–350 g, obtained from Toxi-Coop Toxicological Research Centre, Dunakeszi, Hungary). The animals were fed a standard chow and drank tap water *ad libitum*. For the study, animals were anaesthetized with an intraperitoneal injection of sodium pentobarbital (150 mg/kg). All efforts were

Table 1. Effects of different inhibitors and endothelium removal on the H₂O₂-induced responses.

Type of arteriole	Rat skeletal muscle arterioles					Rat coronary arterioles							
	Control	None/Control	SQ-29548	Indomethacin	7,7-Dimethyl-(5Z,8Z) eicosadienoic acid	Chelerythrine	U-73122	Src inhibitor-1	Celecoxib	SC-560	NS-398	Control	SQ-29548
No. of experiments	6	7	5	5	5	5	4	5	4	5	3	7	5
Initial diameter	191±17	110±18	109±12	111±2	130±11	121±12	133±3	138±11	148±13	122±9	156±8	110±18	109±12
Diameter after inhibitor	-	-	108±12	111±3	130±11	164±11	126±10	143±12	146±13	113±14	155±8	-	108±12
Diameter after 100 μM H ₂ O ₂	128±15	128±20	117±18	130±4	120±11	157±12 *	132±18	133±14 *	135±16	131±17 *	142±9 *	128±20	117±18
Diameter after 10 mM H ₂ O ₂	248±7	200±25	142±13	151±3 *	175±8 *	179±5 *	175±12 *	187±5 *	180±11 *	191±7 *	215±13 *	200±25	142±13
Passive diameter	261±8	205±27	143±12	156±5 *	176±8 *	185±4 *	179±12 *	190±4 *	185±11 *	200±4 *	218±13 *	205±27	143±12

Effects of various treatments on the diameter of isolated, cannulated, pressurized (80 mmHg) arterioles of the rat. The tissue sources of the arteriolar beds are indicated (coronary arterioles or skeletal muscle arterioles). Diameters are shown as means±S.E.M. in absolute values (μm). The number of experiments performed is also indicated. Arteriolar diameters are shown at the beginning of the experiments (initial diameter) and after treatment with 100 μM (maximum constrictor dose in the control) or 10 mM (maximum dilator dose in the control) H₂O₂. The effects of preincubations with the inhibitors (diameter after the inhibitor) and the maximum diameter of the vessels (passive diameter) are also shown. Significant effects of the treatments on the arteriolar diameters are indicated by asterisks (paired t-test relative to the initial diameter). doi:10.1371/journal.pone.0103858.t001

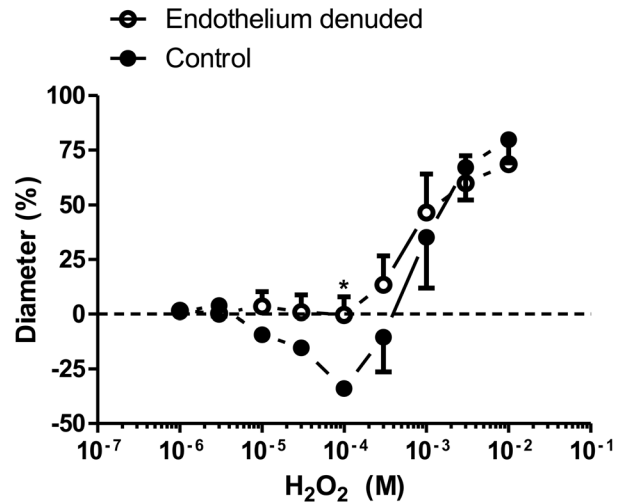


Figure 2. H₂O₂-induced vasoconstrictions are mediated by the endothelium in skeletal muscle arterioles. H₂O₂ concentration-response relationships were determined (as given in Fig. 1A) in intact (control, closed symbols, n = 6 from 6 different animals) and endothelium-denuded arterioles (id: 131±10 μm, open symbols, n = 5 arterioles from 5 different animals). The asterisk denotes a significant difference from the control. doi:10.1371/journal.pone.0103858.g002

made to minimize the suffering of the animals. The gracilis muscle and the heart were removed and placed in silicone-coated petri dishes containing cold (0–4°C) Krebs solution (in mM: 110 NaCl, 5.0 KCl, 2.5 CaCl₂, 1.0 MgSO₄, 1.0 KH₂PO₄, 5.0 glucose and 24.0 NaHCO₃) equilibrated with a gaseous mixture of 5% CO₂, 10% O₂ and 85% N₂ at pH 7.4.

Materials and drugs

The TXA₂ agonist (U46619) was obtained from Calbiochem (Billerica, MA, USA), and the TXA₂ inhibitor (SQ-29548) from BioMarker Kft. (Gödöllő, Hungary). All other chemicals were from Sigma-Aldrich (St. Louis, MO, USA) and were kept under the conditions prescribed by the manufacturer. All reported concentrations are the final concentrations in the organ chamber.

Isolation of arterioles and measurement of vascular diameter

Arterioles were isolated and cannulated as described previously [47]. Briefly, gracilis muscle arterioles and the second branch of the septal coronary artery (both ~1.5 mm long) running intramuscularly were isolated through the use of microsurgical instruments and an operating microscope and 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 (Living Systems Instrumentation, St. Albans, VT, USA) to set the intraluminal pressure at 80 mmHg. The temperature was maintained at 37°C by a temperature controller. Changes in internal arteriolar diameter were recorded continuously with a video microscope system (Topica CCD camera).

Experimental protocols

In response to the intraluminal pressure of 80 mmHg the isolated arterioles spontaneously developed a substantial myogenic tone without the use of any exogenous constrictor agents (a decrease from an initial diameter of 205±5 μm to 149±5 μm

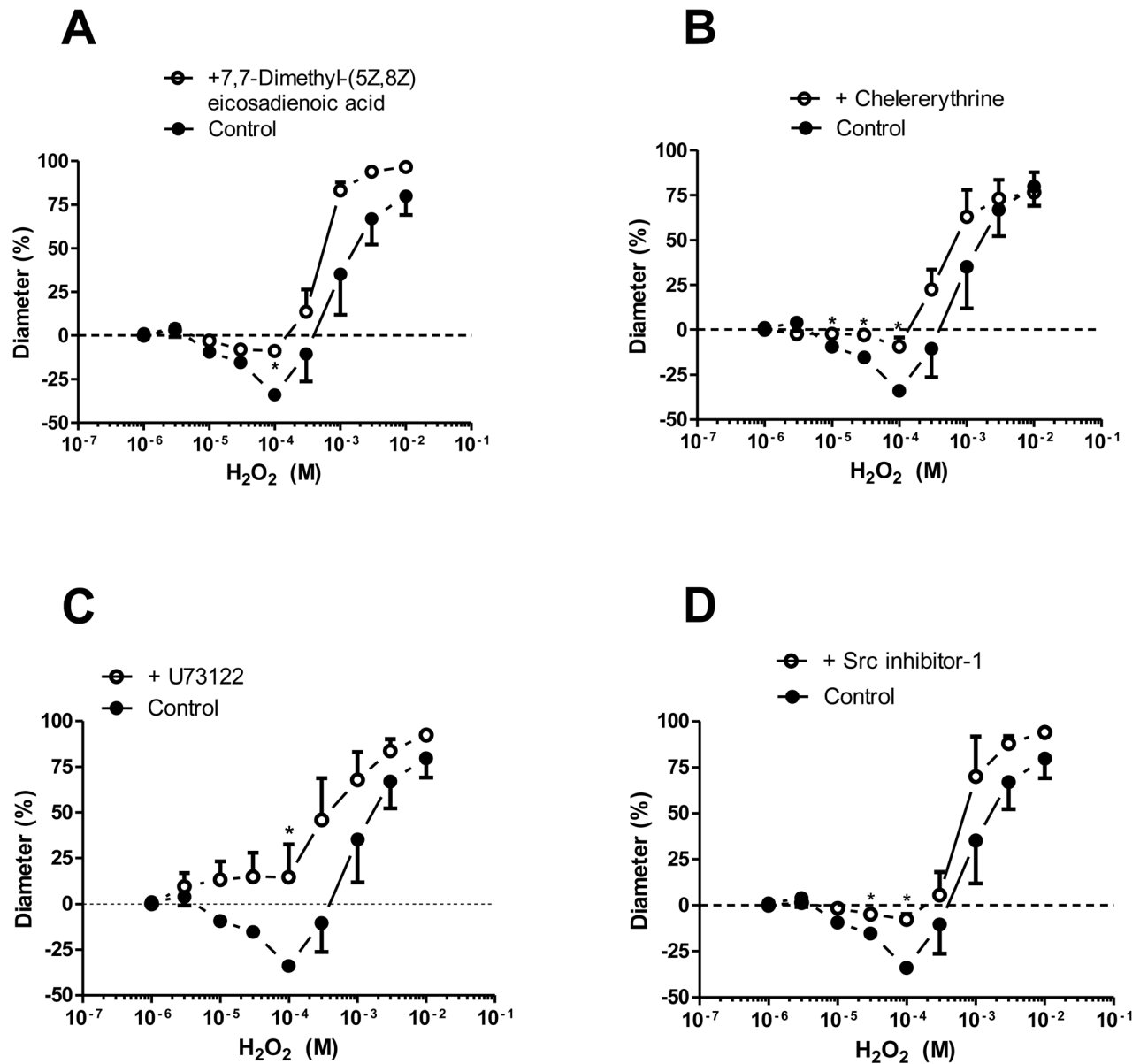


Figure 3. Endothelial mechanisms of H₂O₂-evoked vasoconstriction of skeletal muscle arterioles. Arteriolar diameter was recorded in response to H₂O₂ without pretreatment (control, as given in Fig. 1A, closed symbols) or after test incubations (open symbols) for at least 15 min in the presence of PLA inhibitor 7,7-dimethyl-(5Z,8Z)-eicosadienoic acid (100 μ M, n = 5 arterioles from 5 different animals, id:130 \pm 11 μ m; panel A), or in the presence of PKC inhibitor chelerythrine (10 μ M, n = 5 arterioles from 5 different animals, id: 164 \pm 11 μ m; panel B), or in the presence of PLC inhibitor U-73122 (10 μ M, n = 4 arterioles from 4 different animals, id: 126 \pm 10 μ m; panel C), or in the presence of Src kinase inhibitor Src inhibitor-1 (5 μ M, n = 5 arterioles from 5 different animals, id: 143 \pm 12 μ m; panel D). Asterisks denote significant differences from the control. doi:10.1371/journal.pone.0103858.g003

(n = 99 arterioles from 82 different animals) and from 170 \pm 14 μ m to 107 \pm 7 μ m (n = 17 arterioles from 17 different animals) in the skeletal and coronary arterioles of the rat, respectively).

Cumulative concentrations of acetylcholine (1 nM–10 μ M) were used to test the vasomotor function of the endothelium. The smooth muscle function was tested with norepinephrine (skeletal muscle artery) or serotonin (coronary artery, 1 nM–10 μ M). H₂O₂ solutions were prepared immediately before the experiments and were stored on ice. In the first series of experiments, cumulative concentrations of H₂O₂ (1 μ M–10 mM) were added to the skeletal muscle arterioles (n = 6 arterioles from 6 different animals) or coronary arterioles (n = 7 arterioles from 7 different animals) and

the responses to the H₂O₂ were determined and diameters were recorded 60 s after the application of each H₂O₂ concentration. During measurements, the changes in the pH of the chamber containing H₂O₂ were also checked. The pH of the control solutions did not vary significantly with the final concentration of H₂O₂ (pH 7.52 \pm 0.03 in the absence of H₂O₂, pH 7.58 \pm 0.03 in the presence of 10 mM H₂O₂, n = 3). To study the kinetics of diameter changes, various concentrations of H₂O₂ (10, 30, 100 and 300 μ M) were used (600 s treatment duration, diameter measured every 10 s, n = 3–5 arterioles from 11 different animals at each concentration). In some groups of experiments, the endothelium was removed by air perfusion of the arterioles

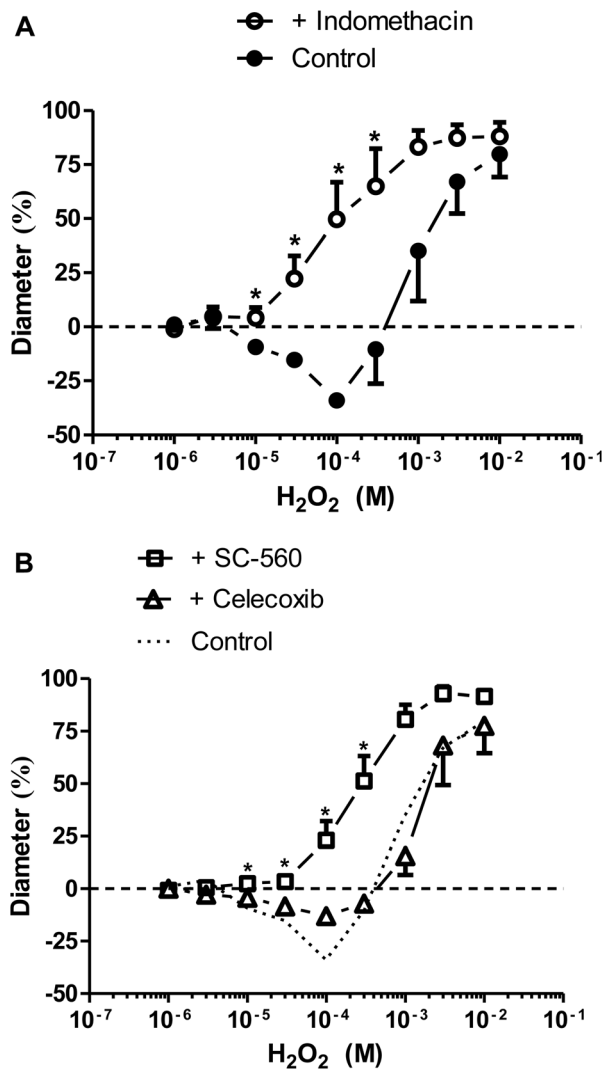


Figure 4. H₂O₂-induced vasoconstriction is mediated by COX-1. Arteriolar constrictions (control, as given in Fig. 1A, closed symbols) were prevented in the presence of the non-specific COX inhibitor indomethacin (10 μM, n=5 arterioles from 4 different animals, preincubation for 30 min, id: 111±3 μm, open symbols; panel A). Panel B: The roles of COX isoforms in H₂O₂-evoked responses were studied by comparing vascular diameters in the absence of COX inhibitors (dotted line) with those in the presence of COX-1 inhibitor SC-560 (1 μM, n=5 arterioles from 3 different animals, id: 113±14 μm; open squares) or with COX-2 inhibitor celecoxib (3 μM, n=4 arterioles from 4 different animals, id: 146±13 μm; open triangles). Asterisks denote significant differences from the control. doi:10.1371/journal.pone.0103858.g004

(denudation, n=6 arterioles from 6 different animals). Successful endothelium denudation was verified by the loss of dilation in response to acetylcholine (96±5% dilation before and 0.3±0.2% after endothelium removal), whereas a maintained smooth muscle function was confirmed through the use of norepinephrine (62±6% constriction before and 55±6% after endothelium removal).

The effects of H₂O₂ on the diameter of the arterioles were also measured in the presence (15–30-min preincubation) of a PKC inhibitor (chelerythrine, 10 μM, n=5 arterioles from 5 different animals), a PLC inhibitor (U73122, 10 μM, n=4 arterioles from 4 different animals), a PLA inhibitor (7,7-dimethyl-(5Z,8Z)-eicos-

dienoic acid, 100 μM, n=5 arterioles from 5 different animals), a Src kinase inhibitor (Src inhibitor-1, 5 μM, n=5 arterioles from 5 different animals), a COX-1 and COX-2 inhibitor (indomethacin, 10 μM, n=5 arterioles from 4 different animals), a COX-1-selective inhibitor (SC-560, 1 μM, n=5 arterioles from 3 different animals), a COX-2-selective inhibitor (celecoxib, 3 μM, n=4 arterioles from 4 different animals), another COX-2-selective inhibitor (NS-398, 10 μM, n=3 arterioles from 3 different animals) and a TXA₂ receptor inhibitor (SQ-29548, 1 μM, n=10 arterioles from 10 different animals). The inhibitors were dissolved in dimethyl sulphoxide (DMSO), ethanol or in water. The maximum concentration of non-aqueous solvent (DMSO or ethanol) in the organ chamber was 0.1%. The solvents alone had no vascular effects.

At the end of the experiments, the maximum (passive) arteriolar diameter was determined in the absence of extracellular Ca²⁺ at an intraluminal pressure of 80 mmHg.

Parallel measurement of vascular diameter and intracellular Ca²⁺ concentrations

Simultaneous measurements of intracellular Ca²⁺ and arteriolar diameter were performed as described previously [48,49]. Briefly, isolated and cannulated arterioles (n=9 arterioles from 6 animals) were incubated for 60 min in the presence of physiological buffer solution containing 1% bovine serum albumin and 5 μM Fura-2AM fluorescent Ca²⁺ indicator dye. Intracellular Ca²⁺ 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, while the emitted fluorescence was detected at 510 nm by selecting at least 1000 pixels within the arteriolar wall. Arteriolar Ca²⁺ 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. The exact dimensions of the sampling region depended on the ongoing treatment: and it was variable in different vessels. The average dimensions of the sampling region were 285±15 μm×105±6 μm.

Data analysis and statistical procedures

The diameters of arterioles are shown as means±SEM. Arteriolar constriction was expressed as the change in the baseline initial diameter (id, immediately before the addition of H₂O₂) as a percentage of the baseline diameter measured at an intraluminal pressure of 80 mmHg. Arteriolar dilation was calculated as the percentage change from the baseline id (immediately before the addition of H₂O₂) to the “passive” diameter in the absence of extracellular Ca²⁺. Statistical analyses were performed with GraphPad Prism 5.0 Software (La Jolla, CA, USA) by the Student's *t*-test and by ANOVA (Dunnnett's *post hoc* test). *P*<0.05 was considered statistically significant.

Results

H₂O₂-induced arteriolar responses

Increasing concentrations of H₂O₂ evoked a concentration-dependent biphasic effect in the skeletal muscle arterioles: lower concentrations (10–100 μM) of H₂O₂ produced vasoconstriction (maximum at 100 μM, 34±3% constriction, *P*<0.001 *vs.* id, Fig. 1A, Table 1), whereas higher concentrations (3–10 mM) of H₂O₂ resulted in vasodilation (maximum at 10 mM, 80±11% dilation, *P*<0.001 *vs.* id). In contrast, H₂O₂ evoked only vasodilation in the coronary arterioles (maximum at 10 mM, 96±3% dilation, *P*=0.01). The kinetics of the H₂O₂-evoked changes in the diameter of the skeletal muscle arterioles was also

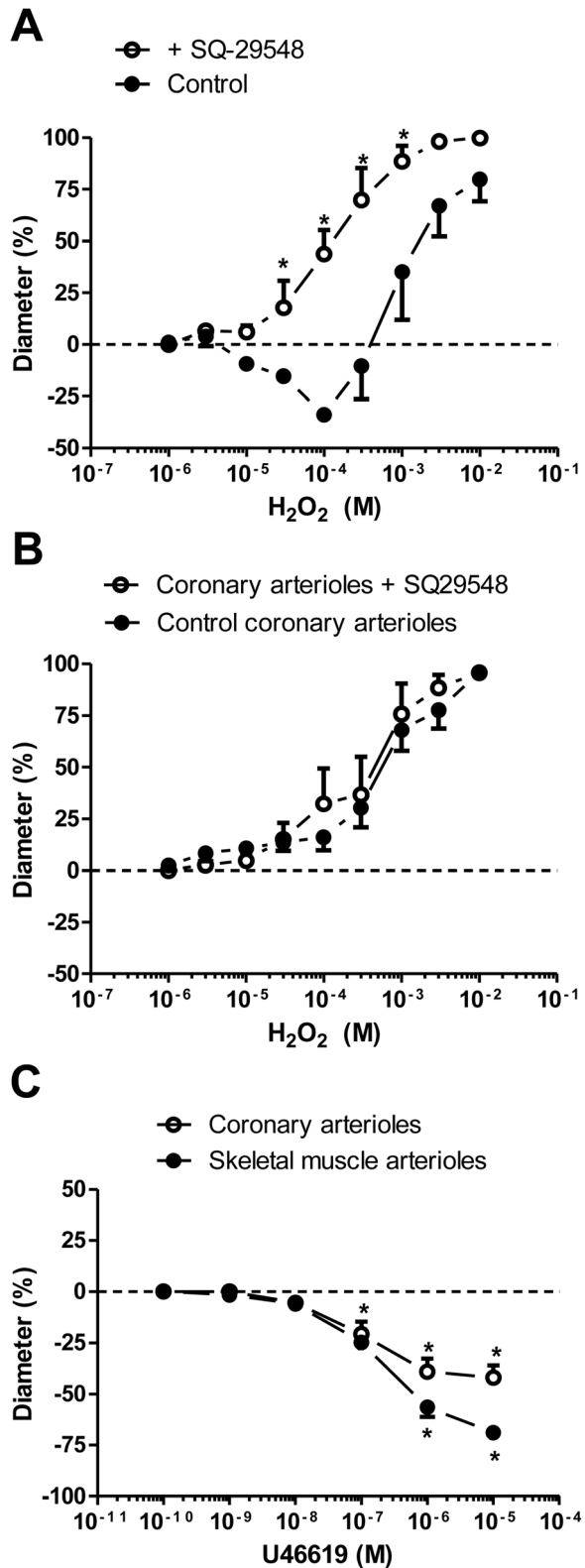


Figure 5. H₂O₂-induced vasoconstriction is mediated by TXA₂. The role of TXA₂ receptors was tested by comparing H₂O₂-induced vascular responses under control conditions (closed symbols) with those in the presence of TXA₂ receptor antagonist SQ-29548 (1 μM, n=10 arterioles from 10 different animals, 15-min preincubation) in skeletal muscle arterioles (panel A, open symbols; id: 133±7 μm, asterisks denote significant differences from the control) and in

coronary arterioles (panel B, open symbols; id: 108±12 μm). Panel C: The presence of functional TXA₂ receptors was verified by the application of TXA₂ receptor agonist U46619 (0.1 nM–10 μM) in skeletal muscle (closed symbols; id: 189±7 μm, n=5 arterioles from 5 different animals) and coronary arterioles (open symbols; id: 119±12 μm, n=5 arterioles from 5 different animals). Asterisks denote significant differences from the initial diameter. doi:10.1371/journal.pone.0103858.g005

tested. Although the H₂O₂-evoked vasoconstrictions were mostly transient, vasoconstrictions at lower H₂O₂ concentrations (10 μM and 30 μM) were not followed by significant vasodilations (Fig. 1B). In contrast, 100 μM or 300 μM H₂O₂ caused time-dependent biphasic changes: after the initial vasoconstriction, a substantial vasodilation developed. Application of 3 mM H₂O₂ resulted in substantial vasodilation without initial vasoconstriction.

Role of the endothelium in H₂O₂-induced vasoconstriction

The H₂O₂-induced constriction was abolished in the endothelium-denuded skeletal muscle arterioles (0±8% constriction at 100 μM H₂O₂, *P*=0.03 *vs.* control; Fig. 2A), but the dilations were not affected (69±10% dilation at 10 mM H₂O₂).

H₂O₂ stimulated endothelial signalling processes, leading to the activation of COX

The H₂O₂-evoked vasoconstriction was inhibited by the application of the PLA antagonist (7,7-dimethyl-(5*Z*,8*Z*)-eicosadienoic acid, 7±2% constriction, *P*<0.005 *vs.* control; Fig. 3A), the PKC antagonist (chelerythrine, 9±4% constriction at 100 μM H₂O₂, *P*<0.005 *vs.* control; Fig. 3B), the PLC inhibitor (U-73122, 15±18% dilation, *P*<0.05 *vs.* control, Fig. 3C) or the Src kinase antagonist (Src inhibitor-1, 8±3% vasoconstriction, *P*<0.005 *vs.* control; Fig. 3D).

Effects of non-selective and selective COX inhibition on H₂O₂-induced arteriolar responses

The H₂O₂-induced constrictions were converted to dilations in the presence of a non-selective COX inhibitor (indomethacin, 41±17% dilation at 100 μM H₂O₂, *P*<0.005 *vs.* control; Fig. 4A). In separate experiments, we investigated the specific roles of COX-1 and COX-2 in the mediation of the H₂O₂-evoked vascular responses. It emerged that the selective COX-1 inhibitor SC-560 abolished the constriction induced by H₂O₂ (23±9% dilation at 100 μM H₂O₂, *P*<0.05 *vs.* control; Fig. 4B) and converted it to dilation, whereas the inhibitory effect of the COX-2 antagonist celecoxib was not significant (13±4% constriction at 100 μM H₂O₂, *P*>0.05 *vs.* control). Moreover, another COX-2 specific antagonist, NS-398 (10 μM, n=3 arterioles from 3 different animals), did not prevent the H₂O₂-evoked vasoconstrictions either (8±1% constriction at 100 μM H₂O₂, *P*>0.05 *vs.* control; Figure S1).

H₂O₂-evoked effector mechanisms leading to vasoconstrictive responses

The H₂O₂-evoked vasoconstriction in the skeletal muscle arterioles was abolished and converted to dilation (36±11% dilation at 100 μM H₂O₂, *P*<0.005 *vs.* control; Fig. 5A) by TXA₂ receptor inhibition (SQ-29548). In contrast, the same treatment did not affect the H₂O₂-evoked dilation in the coronary arterioles (96±2% dilation at 10 mM H₂O₂; Fig. 5B). Activation of the TXA₂ receptors with the stable analogue of TXA₂, U46619, resulted in constriction of both the skeletal muscle (69±2%, n=5,

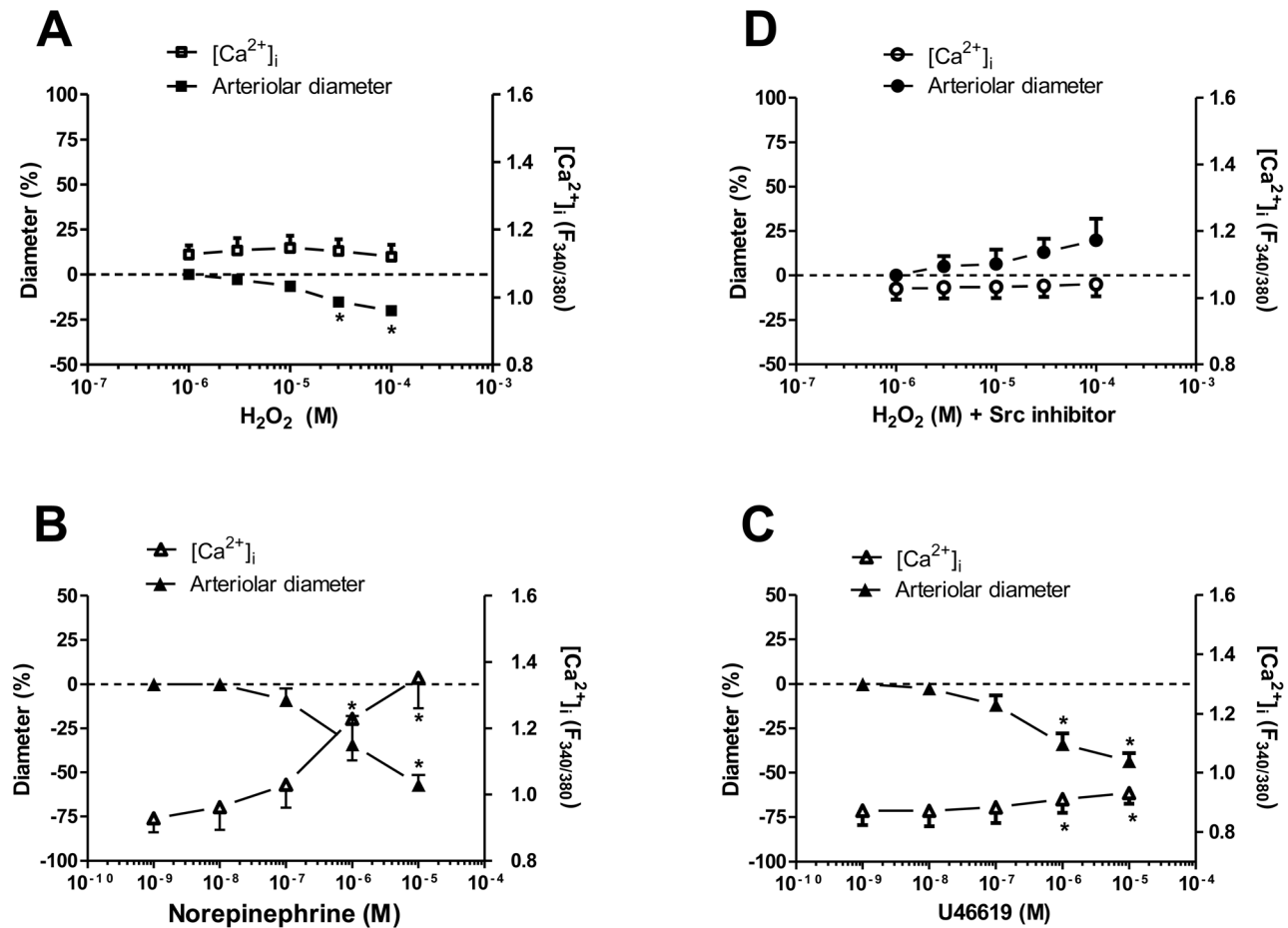


Figure 6. H₂O₂ increases the Ca²⁺ sensitivity of force production in vascular smooth muscle cells. The changes in intracellular Ca²⁺ levels and arteriolar diameters were studied in skeletal muscle arterioles under control conditions (panel A; n = 5 arterioles from 3 different animals), or after treatment with norepinephrine (panel B; n = 5 arterioles from 3 different animals), or by addition of the TXA2 receptor agonist U46619 (0.1 nM–10 μM; panel C; n = 5 arterioles from 4 different animals). Experiments were also performed in the presence of H₂O₂ together with Src kinase inhibitor, (Src inhibitor-1, 5 μM n = 4 arterioles from 3 different animals, 20-min preincubation; panel D). Asterisks denote significant differences from the initial values.

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$P < 0.002$ vs. id; Fig. 5C) and the coronary arterioles ($42 \pm 6\%$, $P = 0.002$ vs. id; Fig. 5C).

Characterization of H₂O₂-evoked changes in intracellular Ca²⁺ concentrations of vascular smooth muscle cells

The H₂O₂-evoked vasoconstriction was not accompanied by significant changes in the F_{340/380} ratio signal in the range of H₂O₂ concentrations between 1 μM and 100 μM (Fig. 6A). However the norepinephrine (10 μM)-induced vasoconstriction was accompanied by a significant increase in F_{340/380} (from 0.96 ± 0.04 to 1.36 ± 0.07 , $P = 0.001$; Fig. 6B). Moreover, the U46619-evoked peak in F_{340/380} was significantly smaller than that evoked by norepinephrine (0.93 ± 0.04 vs. 1.36 ± 0.07 , respectively, $P < 0.05$) despite their largely comparable vasoconstrictive responses (to $44 \pm 5\%$ vs. $57 \pm 6\%$, respectively, $P > 0.05$; Fig. 6C). In another set of experiments, the H₂O₂-evoked changes in vascular diameter and Ca²⁺ concentration were measured in the presence of an Src kinase inhibitor (Src inhibitor-1), where vasoconstriction was inhibited by this inhibitor, and F_{340/380} did not change (Fig. 6D). In arterioles with intact endothelium the acetylcholine-induced vasodilation was accompanied by a significant decrease in F_{340/380} (from 1.05 ± 0.05 to 0.89 ± 0.04 , $P < 0.05$, n = 5).

Discussion

As far as we are aware this is the first study that has revealed the signalling mechanisms of H₂O₂-induced vasoconstriction in the skeletal muscle arterioles of the rat. Besides confirming some steps identified earlier in different vascular preparations, we have now supplemented the signalling cascade with additional molecular interactions. Thus, we have shown that H₂O₂ promotes endothelial Src activation and that it leads ultimately to an increased Ca²⁺ sensitivity of force production in vascular smooth muscle cells.

A number of attempts have been made to investigate the mechanism of H₂O₂-evoked vasodilation [8,36,39,40], but much less is known as regards the mechanism of H₂O₂-evoked vasoconstriction. H₂O₂ can modulate the vascular diameter in the rat renal artery [31], the canine basilar artery [50], the porcine coronary arterioles [38] and the rabbit aorta [37] in an endothelium-dependent manner. It may also display endothelium-independent effects in human coronary arterioles [26], canine coronary arterioles [51] and the rat aorta [29]. In the present study, H₂O₂-induced vasoconstriction was completely inhibited by endothelium denudation or by inhibition of the TXA2 receptor. Our observations suggest that H₂O₂ causes the generation of TXA2 in the endothelium, leading to vasoconstriction [31–33],

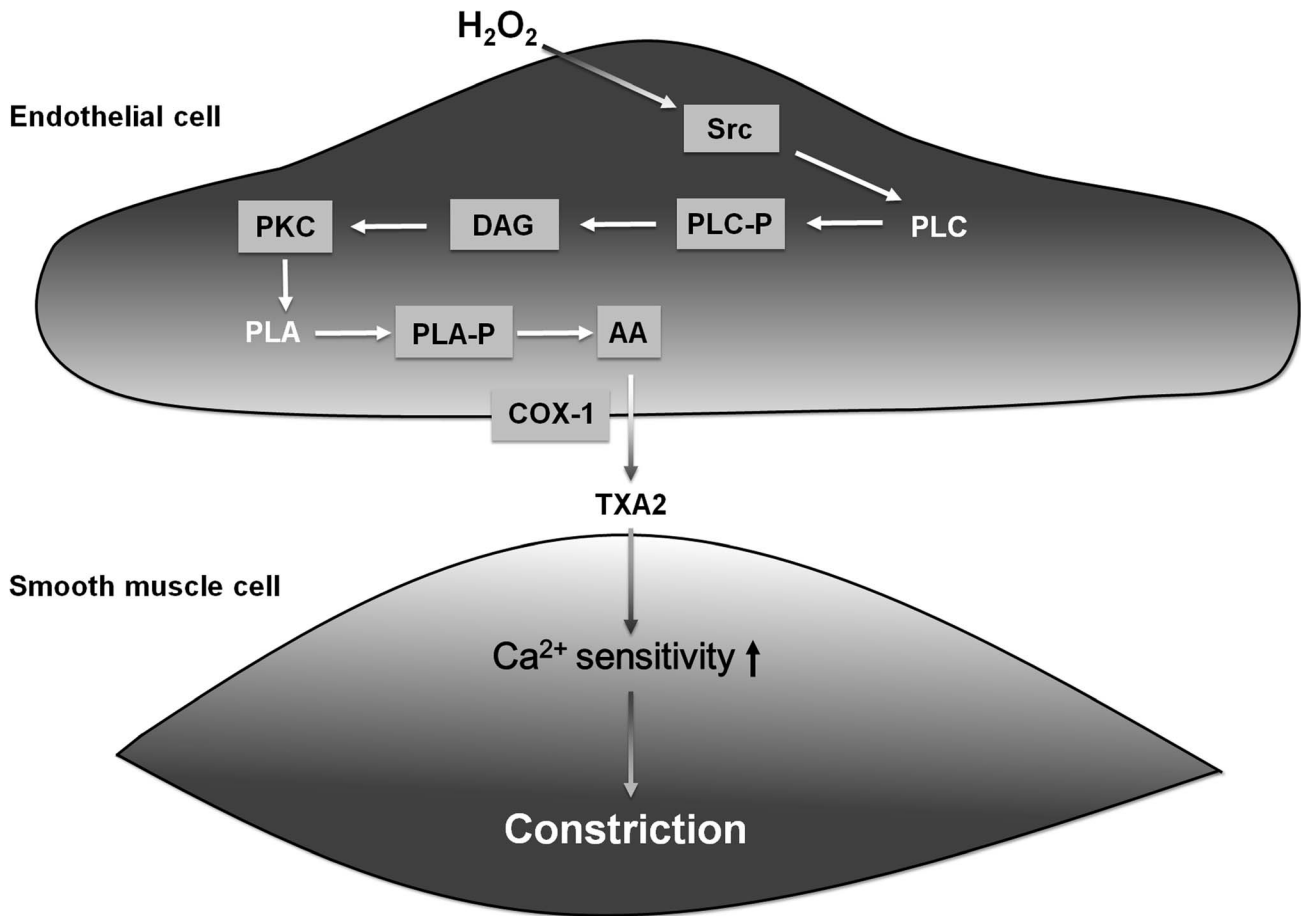


Figure 7. Proposed molecular mechanisms of H₂O₂-evoked vasoconstriction, based on the present study. H₂O₂ may induce both vasodilation and vasoconstriction, depending on the applied H₂O₂ concentration, vessel type, species and experimental protocol (e.g. exposure time). Our data imply that H₂O₂ elicits vasoconstriction by activating Src kinase, which activates the phospholipase C (PLC), protein kinase (PKC), phospholipase A (PLA) and cyclooxygenase (COX) pathway, leading to the production of thromboxane A2 (TXA2), which increases the Ca²⁺ sensitivity of the vascular smooth muscle in skeletal muscle arterioles of the rat (DAG: diacylglycerol).
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and also that H₂O₂ may elicit endothelium-dependent dilation in skeletal muscle arterioles when the TXA₂-mediated vasoconstriction is blocked. In contrast, H₂O₂-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 H₂O₂ 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 [52]. In our study, H₂O₂-evoked vasoconstriction was inhibited in the presence of the PLA antagonist (7,7-dimethyl-(5Z,8Z)-eicosadienoic acid, 100 μM), suggesting a role for PLA in the H₂O₂-induced vasomotor response. This observation is in accordance with the findings reported by Gao *et al.* on rat mesenteric arterioles [35]. The activation of PLA can be a consequence of PKC-mediated phosphorylation [53]. Indeed, preincubation of skeletal muscle arterioles with the PKC antagonist chelerythrine (10 μM) resulted in a significantly reduced H₂O₂-evoked constriction. PKC can be activated by the diacylglycerols released by PLC [54], and

inhibition of PLC by U73122 (10 μM) resulted in a significantly decreased H₂O₂-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 H₂O₂. However, PLC inhibition was without effects on the constrictions evoked by the TXA₂ receptor agonist U46619 (Figure S2), suggesting an upstream (endothelial) target in H₂O₂-mediated constriction.

The H₂O₂-evoked activation of PLC was earlier shown to be mediated by Src kinase in mouse embryonic fibroblasts [55]. Indeed, the constrictor effects of H₂O₂ in skeletal muscle arterioles were inhibited in the presence of an Src kinase antagonist. Moreover, H₂O₂-evoked vasoconstriction was completely inhibited by the non-specific COX antagonist indomethacin. These results are in line with previous findings [8,29,31,35,56,57]. Furthermore, the H₂O₂-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 H₂O₂-evoked vasoconstriction.

Taken together, the H₂O₂-induced constriction component was largely abolished by inhibitors of PLA, PKC, PLC and Src kinases, indicating a complex network of intracellular signalling in the

H₂O₂ response. Interestingly, H₂O₂-evoked vasoconstriction was also prevented in the absence of endothelium. These findings, together with concordant previous observations by others [31–33], implicate a sequence of signalling events in the endothelial layer during H₂O₂-evoked vasoconstrictions. Nevertheless, alternative mechanisms cannot be excluded.

TXA₂ receptors are expressed in numerous cell types, including vascular smooth muscle cells [58]. TXA₂ receptors can couple with G_q protein, thereby activating the PLC pathway, giving rise to Ca²⁺ release and PKC activation (a Ca²⁺-dependent pathway) [59,60]. However, TXA₂ also binds to G₁₂ proteins [60], leading to the activation of Rho-kinase-mediated signalling (a Ca²⁺-independent pathway), and hence to Ca²⁺ sensitization of the contractile protein machinery [59]. Nevertheless, G₁₂ proteins may also evoke vasoconstriction by promoting Ca²⁺ entry through another Ca²⁺-dependent mechanism, as has been demonstrated in the rat caudal arterial smooth muscle [61]. Our experimental results indicated that H₂O₂-evoked vasoconstrictions were not accompanied by significant increases in intracellular Ca²⁺ concentration. In contrast, the treatment with norepinephrine increased the intracellular Ca²⁺ 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 Ca²⁺ concentration than that evoked by norepinephrine, supporting our hypothesis that H₂O₂ increases the Ca²⁺ sensitivity of the vascular smooth muscle, rather than stimulating Ca²⁺ entry into smooth muscle cells. Similar conclusions were reached in previous studies, where the H₂O₂-induced constriction of isolated rabbit [32,46] or porcine (36) pulmonary arterioles was not influenced by extracellular Ca²⁺ removal. Although the explanation of the apparent increase in vascular Ca²⁺ 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) [62]. Alternatively, vascular Ca²⁺ sensitization of constriction could be elicited by dynamic regulation of the actin cytoskeleton by PKC and ROCK [63].

It is rather difficult to estimate the real concentration of H₂O₂ 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) [8,17,18]. In this study, the use of even higher concentrations of H₂O₂ (up to 10 mM) allowed us to characterize the biphasic vascular effects of H₂O₂. Lower concentrations of H₂O₂ 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 H₂O₂ as an EDHF in the coronary microcirculation [20,21,26],

and the conclusion that, H₂O₂ cannot be regarded as an EDHF in skeletal muscle arterioles under physiological conditions [8]. It is unclear whether H₂O₂ concentrations reach levels high enough to evoke vasodilation and hence to increase the skeletal muscle blood flow under pathological conditions (*e.g.* inflammation).

The findings of the present study suggest that H₂O₂ activates an endothelial signalling pathway, leading to the synthesis of TXA₂, which then activates its receptors of smooth muscle cells, leading to an increase in the Ca²⁺ sensitivity of their contractile protein machinery. Figure 7 summarizes the detailed mechanisms identified or confirmed in the present study that lead to H₂O₂-evoked constrictions of the skeletal muscle arterioles. Elucidation of these details of this H₂O₂-induced signalling not only adds to our knowledge of H₂O₂-induced vasomotor responses, but may also furnish novel molecular targets for the treatment of H₂O₂-driven vascular dysfunctions.

Supporting Information

Figure S1 Effects of different COX-2 specific inhibitors on H₂O₂-induced vasoconstriction. The lack of the effects of COX-2 in the vasoconstriction evoked by H₂O₂ was confirmed by using another COX-2-specific inhibitor, NS-398 (10 μM, n = 3 arterioles from 3 different animals, id: 155 ± 8 μm; closed triangles). The effects of celecoxib are indicated by open triangles (3 μM celecoxib, n = 4 arterioles from 4 different animals, id: 146 ± 13 μm); the dotted line denotes the control. (TIF)

Figure S2 PLC inhibition had no effects on the constrictions evoked by the TXA₂ receptor agonist. PLC inhibition (10 μM U73122) significantly decreased the constriction evoked by norepinephrine (n = 4 arterioles from 2 different animals, id: 170 ± 10 μm and 154 ± 8 μm; panel A), but did not influence the constrictions evoked by increasing concentrations of the TXA₂ receptor agonist U46619 in skeletal muscle arterioles (n = 5 arterioles from 4 different animals, id: 171 ± 10 μm and 154 ± 8 μm; panel B). Means ± SEM are plotted. Asterisks denote significant differences from the control. (TIF)

File S1 Data in supporting information file. (PDF)

Author Contributions

Conceived and designed the experiments: VC AP ÁK IÉ AT ZP. Performed the experiments: VC AP AT. Analyzed the data: VC AP AT ÁK. Contributed reagents/materials/analysis tools: VC AT ÁK. Wrote the paper: VC AT ÁK IÉ ZP.

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