Hydrogen peroxide increases nerve-evoked contractions in mouse tail artery by an endothelium-dependent mechanism

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Abstract

Reactive oxygen species contribute to regulating the excitability of vascular smooth muscle. This study investigated the actions of the relatively stable reactive oxygen species, H$_2$O$_2$, on nerve-evoked contractions of mouse distal tail artery. H$_2$O$_2$ (10-100 μM) increased nerve-evoked contractions of isometrically mounted segments of tail artery. Endothelium denudation increased nerve-evoked contractions and abolished the facilitatory effect of H$_2$O$_2$. Inhibition of nitric oxide synthase with L-nitroarginine methyl ester (0.1 mM) also increased nerve-evoked contractions and reduced the late phase of H$_2$O$_2$-induced facilitation. H$_2$O$_2$-induced facilitation of nerve-evoked contractions depended, in part, on synthesis of prostanoids and was reduced by the cyclooxygenase inhibitor indomethacin (1 μM) and the thromboxane A$_2$ receptor antagonist SQ 29,548 (1 μM). H$_2$O$_2$ increased sensitivity of nerve-evoked contractions to the α$_2$-adrenoceptor antagonist idazoxan (0.1 μM) but not to the α$_1$-adrenoceptor antagonist prazosin (10 nM). Idazoxan and the α$_2C$-adrenoceptor antagonist JP 1302 (0.5-1 μM) reduced H$_2$O$_2$-induced facilitation. H$_2$O$_2$ induced facilitation of nerve-evoked contractions was abolished by the non-selective cation channel blocker SKF-96365 (10 μM), suggesting it depends on Ca$^{2+}$ influx. In conclusion, H$_2$O$_2$-induced increases in nerve-evoked contractions depended on an intact endothelium and were mediated by activating thromboxane A$_2$ receptors and by increasing the contribution of α$_2$-adrenoceptors to these responses.

Keywords: Mouse tail artery, Sympathetic neurovascular transmission, Hydrogen peroxide, Endothelium,
1. Introduction

Reactive oxygen species are produced in both vascular smooth muscle and endothelial cells, and are generated by NADPH oxides, xanthine oxidases, mitochondrial respiration and uncoupled nitric oxide synthases (Ardanaz and Pagano, 2006). The reactive oxygen species produced include unstable free radicals such as superoxide (O$_2^•$–), and longer-lived non-free radical oxidants, such as hydrogen peroxide (H$_2$O$_2$). Because it is relatively stable, H$_2$O$_2$ has been considered to function as both an intracellular second messenger and a paracrine factor that modifies vascular contractions (Ardanaz and Pagano, 2006).

This study investigated the actions of H$_2$O$_2$ on nerve-evoked constrictions of the mouse tail artery. No studies have reported effects of H$_2$O$_2$ on neurovascular transmission, but H$_2$O$_2$ elicits vasoconstriction that depends on activity of cyclooxygenases and the generation of constrictor prostanoids that activate vascular muscle via thromboxane A$_2$/prostaglandin H$_2$ receptors (Tang and Vanhoutte, 2009). This action of H$_2$O$_2$ has been reported to be mediated either via a direct action on vascular muscle (Gao and Lee, 2005) or by triggering release of constrictor prostanoids from the endothelium (Katusic et al., 1993). Importantly, low concentrations of the thromboxane A$_2$/prostaglandin H$_2$ receptor agonist U-46619 that do not produce a contraction, increase neurovascular transmission (Vila et al., 2001). It therefore seems possible that H$_2$O$_2$ will increase nerve-evoked contractions of mouse tail artery. In addition to prostanoid-mediated contraction, H$_2$O$_2$ can contract vascular muscle by releasing Ca$^{2+}$ from intracellular stores (Pourmahram et al., 2008) and by increasing extracellular Ca$^{2+}$ influx (Lin et al., 2007; Shen et al., 2000).

The mouse tail artery is a thermoregulatory vessel and cooling increases its sensitivity to α$_2$-adrenoceptor agonists (Chotani et al., 2000). This effect of cooling on reactivity to α$_2$-adrenoceptor agonists depends on reactive oxygen species signalling (Bailey et al., 2005). This conclusion is based on
observations that cooling stimulates mitochondrial production of reactive oxygen species and that the cold-induced increase in reactivity to the \( \alpha_2 \)-adrenoceptor agonist UK 14,304 was prevented by inhibiting mitochondrial generation of reactive oxygen species (Bailey et al., 2005). This increased sensitivity to \( \alpha_2 \)-adrenoceptor agonists is suggested to contribute to cold-induced vasoconstriction by amplifying nerve-evoked contractions (Chotani et al., 2000).

The cold-induced increase in sensitivity to \( \alpha_2 \)-adrenoceptor agonists in mouse tail artery depends on activity of Rho kinase and recruitment of previously “silent” \( \alpha_2\text{C} \)-adrenoceptors (Bailey et al., 2004; Jeyaraj et al., 2012). Rho kinase inhibition reduces \( \text{H}_2\text{O}_2 \)-induced contractions of tracheal smooth muscle (Kojima et al., 2007) and pulmonary arteries (Pourmahram et al., 2008). Therefore activation of Rho kinase by \( \text{H}_2\text{O}_2 \) may contribute to modifying nerve-evoked contractions. In pulmonary arteries, \( \text{H}_2\text{O}_2 \)-induced contractions were also reduced by inhibition of protein kinase C (Pourmahram et al., 2008).

The present study tested the hypothesis that \( \text{H}_2\text{O}_2 \) increases nerve-evoked contractions of mouse tail artery and demonstrated that this was the case. Further studies investigated involvement of the endothelium in this facilitatory effect of \( \text{H}_2\text{O}_2 \) and whether it involved the production of prostanoids. In addition, we investigated whether \( \text{H}_2\text{O}_2 \) increased the contribution of \( \alpha_2 \)-adrenoceptors to neural activation of vascular muscle and whether the augmentation of nerve-evoked contractions depends on \( \text{Ca}^{2+} \) influx and on the activity of Rho kinase or protein kinase C.

2. Materials and Methods

2.1 Animals and tissue preparation
All procedures conformed to the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes and were approved by the animal ethics committee at the University of Melbourne. Male C57Bl/6 mice aged 9 - 13 weeks were obtained from the Animal Resource Centre (Perth, Australia). Mice were deeply anaesthetised with isoflurane and then killed by cervical dislocation. Segments of ventral tail artery ~1.75 mm in length were dissected from 60 - 80 mm along the tail. Vessels were maintained in physiological saline solution containing (mM): NaCl, 133; KCl, 4.7; CaCl₂, 2.0; MgCl₂, 1.2; NaH₂PO₄.H₂O, 1.3; NaHCO₃, 16.3; glucose, 7.8; ethylenediamine tetraacetic acid, 0.02. This solution was bubbled with 95% O₂ / 5% CO₂ and heated to ~36.5°C.

2.2 Drugs

Phenylephrine HCl, carbachol (carbamoylcholine chloride), prazosin HCl, nifedipine, L-NAME (L-nitroarginine methyl ester), indomethacin, sodium nitroprusside and polyethylene glycol-catalase were obtained from Sigma-Aldrich Chemical Company (Castle Hill, NSW), UK 14,304 (5-Bromo-6-(2-imidazolin-2-ylamino)quinoxaline),Y-27632 (trans-4-[(1R)-1-aminoethyl]-N-4-pyridinylcyclohexanecarboxamide dihydrochloride), JP 1302 (N-[4-(4-methyl-1-piperaziny1) phenyl]-9-acridinamine dihydrochloride) and NF449 (4,4',4'',4''''-{Carbonylbis(imino-5,1,3-benzenetriyl-bis(carbonylimino))}tetrakis-1,3-benzenedisulfonic acid, octasodium salt) were obtained from Tocris Bioscience (Bristol, UK), SQ 29,548 ([1S-[1α,2α(Z),3α,4α]]-7-[3-[2-[[phenylamino]carbonyl]hydrazino]methyl]-7-oxabicyclo[2.2.1]hept-2-yl]-5-heptenoic acid) and U-46619 (9,11-dideoxy-9α,11α-methanoeoxy-prosta-5Z,13E-dien-1-oic acid) were obtained from Cayman Chemical Company (Ann Arbor, USA), H₂O₂ was obtained from Merck Pty Ltd (Kilsyth, Vic, Australia), and tetrodotoxin was obtained from Alamone (Jerusalem, Israel). Prazosin was prepared as a 1 mM stock in 10% (v/v) dimethylsulphoxide (Chem-Supply Pty Ltd, Port Adelaide, SA, Australia) in water. Nifedipine, indomethacin and UK 14,304 were prepared as 10 mM stock solutions in
ethanol (final working concentration of ethanol ≤ 0.1% (v/v)). SQ 29,548 was prepared as a 10 mM stock in dimethylsulphoxide (final working concentration of dimethylsulphoxide was 0.01% (v/v)). U-46619 was purchased as a 28.5 mM stock solution in methyl acetate (final working concentration of methyl acetate was 0.003 % (v/v)). All other drugs were made up as ≥1 mM stock solutions in water.

2.3 Mechanical responses

Artery segments were mounted isometrically between two stainless-steel wires (40 μm diameter) in a four-chamber myograph (Multi Myograph Model 610M, Danish Myo Technology, Aarhus, Denmark). To normalize the basal conditions, Laplace’s equation was used to convert the measured force to the effective transmural pressure exerted on the vessel wall (Mulvany and Halpern, 1977). Initially the arteries were stretched in small steps until the calculated transmural pressure was 13.3 kN/m² (100 mmHg), and then lumen circumference measured at this pressure was reduced by 10% (Mulvany and Halpern, 1977). Under these conditions the lumen circumference was 0.74 ± 0.01 mm. The arteries were allowed to equilibrate for 40 mins at which time the effective transmural pressure was ~4.6 kN/m² (~35 mmHg). All vessels were then stimulated with 2 applications of phenylephrine (2 μM) and when the second contraction had plateaued, carbachol was applied (1 μM) to determine if the endothelium was intact (defined as relaxation to carbachol >70%). In some experiments, the endothelium was removed by rubbing the lumen surface of the vessels with a human hair and the success of this procedure was defined as relaxation to carbachol <10%. In all experiments, test and control assessments were made in parallel using tissues obtained from the same animal.

2.4 Electrical stimulation
Electrical stimuli were applied through platinum plate electrodes mounted on either side of the artery along its length. The stimulus pulse width was 0.2 ms and the voltage was set at 120% of the minimum voltage required for a maximal contraction to 50 pulses at 3 Hz (typically 12V). At the end of all nerve stimulation experiments it was confirmed that α-adrenoceptor blockade (with 0.01 μM prazosin + 0.1 μM idazoxan) or tetrodotoxin (0.5 μM) completely abolished electrically evoked contractions (establishing that the electrical stimuli did not directly activate the muscle). In rats, postganglionic sympathetic neurons supplying the tail artery typically discharge action potentials at <1 Hz, with the level of activity increasing maximally up to about 2 Hz when the body core temperature is lowered (Ootsuka et al., 2004). For this reason we chose to study contractions evoked by trains of stimuli at 1 Hz.

In the experiments investigating the effects of H$_2$O$_2$ and other drugs on nerve-evoked contractions, the arteries were stimulated with trains of 50 stimuli at 1 Hz delivered at 8 min intervals. The first series of experiments assessed the concentration dependence of the H$_2$O$_2$-induced increase in nerve-evoked contractions. After 2 control responses, H$_2$O$_2$ was added cumulatively at increasing concentrations (10, 30 and 100 μM), with each concentration present for 4 contractions. Because the rates of membrane permeation are comparable to the rates at which H$_2$O$_2$ is degraded by peroxidases and catalases, the intracellular concentration would be expected to be maximally about 10% of that applied (Antunes and Cadenas, 2000). In all subsequent experiments, 100 μM H$_2$O$_2$ was used because this concentration robustly increased nerve-evoked contractions. Unless otherwise stated, the experiments investigating the effects of various drugs on the H$_2$O$_2$-induced increase in nerve-evoked contractions consisted of a series of 8 contractions; with 2 under control conditions followed by 2 in the absence (control) or in the presence of a drug and then 4 in the presence of H$_2$O$_2$. The effect of the drug on nerve-evoked contraction was determined at the second response in its presence (i.e. after ~16 mins application) and is expressed as a percentage of the contraction immediately prior to the application of
the drug. Comparisons were made with measures made in the time matched control tissue in which no
drug was added. The facilitatory effect of H$_2$O$_2$ on nerve-evoked contractions was expressed as a % of
the response immediately prior to its addition.

2.5 Alpha-adrenoceptor agonist stimulation

In the experiments with the $\alpha_1$-adrenoceptor agonist phenylephrine (0.03 - 30 $\mu$M) or the $\alpha_2$-
adrenoceptor agonist UK 14,304 (0.001 - 1 $\mu$M), 2 cumulative concentration response curves were
constructed with the concentration increased by half log increments at 4 min intervals. The first
concentration response curve was done in the absence of H$_2$O$_2$ and the second 30 min after the addition
of H$_2$O$_2$. In addition, a control tissue was treated in a similar manner but had no H$_2$O$_2$ added.

2.6 Assessment of nitric oxide signalling

To assess whether H$_2$O$_2$ changed the vasodilator effect of activating endothelium with carbachol and of
exogenously delivered nitric oxide, vessels were constricted with phenylephrine (2 $\mu$M) and cumulative
concentration response curves were constructed for carbachol (0.01 - 1 $\mu$M) or the nitric oxide-donor
sodium nitroprusside (0.001 - 0.1 $\mu$M) in control tissues and in tissues pre-treated with H$_2$O$_2$ for 30 mins.
In these experiments, the concentration of carbachol or sodium nitroprusside was increased by log
increments at 2 min intervals.

2.7 Data analysis
The output from the myograph was recorded and analysed using a PowerLab data acquisition system and the program Chart (ADInstruments, Bella Vista, NSW, Australia). For nerve-evoked contractions their peak amplitude was measured. The α-adrenoceptor agonist-evoked contractions often peaked and then declined slightly to a stable level and in these cases the amplitude of the stable response was measured. In some experiments, the α-adrenoceptor agonists induced small amplitude vasomotions and in these cases the amplitude of the contractions was measured at the lowest point of the phasic contractions. The EC₅₀s for phenylephrine concentration response curves were estimated by fitting the data to the Hill equation using nonlinear regression analysis (Prism 4; GraphPad Software, San Diego, CA). All values are expressed as the mean ± S.E.M.. The effects of the various drugs on the facilitation of nerve-evoked contractions produced by H₂O₂ were assessed by repeated measures analysis of variance. All pair-wise comparisons were made with paired t-tests and when multiple comparisons were made, the P values were adjusted using the false discovery rate procedure (Curran-Everett, 2000). Results were considered significant at P < 0.05. Presented P values were determined using paired t-tests unless otherwise indicated. In all cases, n refers to the number of animals used.

3. Results

3.1 H₂O₂ increases the amplitude of nerve-evoked contractions

All concentrations of H₂O₂ tested (10 - 100 μM) increased the amplitude of nerve-evoked contractions (Fig. 1A). For all the following experiments investigating the mechanisms underlying the facilitatory effect of H₂O₂ on nerve-evoked contractions 100 μM was used. Fig. 1B shows a representative trace of nerve-evoked contractions in a tissue treated with 100 μM H₂O₂ after the second contraction. A small
increase in resting tension was also observed following \( \text{H}_2\text{O}_2 \) application (0.04 ± 0.01 mN/mm, \( P < 0.05; \ n = 9 \)).

The cell permeable polyethylene glycol-catalase (333 U/ml), which degrades \( \text{H}_2\text{O}_2 \), had no effect on nerve-evoked contractions (polyethylene glycol-catalase, 116 ± 15%; time control, 94 ± 11%; \( P = 0.35, \ n = 5 \)). Fig. 1C shows the amplitude of successive nerve-evoked contractions measured as a percentage of the first contraction in control, \( \text{H}_2\text{O}_2 \) and \( \text{H}_2\text{O}_2 + \text{polyethylene glycol-catalase} \) treated arteries. Polyethylene glycol-catalase abolished the \( \text{H}_2\text{O}_2 \)-induced facilitation of nerve-evoked contractions, confirming this effect was due to this reactive oxygen species.

3.2 \( \text{H}_2\text{O}_2 \)-induced facilitation of nerve-evoked contractions is endothelium-dependent

In arteries with an intact endothelium, stimulation of the endothelium with carbachol (1 μM) reduced contractions to phenylephrine (2 μM) by ~90% (87 ± 2%, \( n = 37 \)). In arteries that had their endothelium denuded, the carbachol-induced relaxation was almost abolished (6 ± 1%, \( n = 5 \)). In these vessels, removal of the endothelium approximately doubled the amplitude of nerve-evoked contractions (no endothelium, 0.40 ± 0.09 mN/mm; control, 0.16 ± 0.04 mN/mm; \( P < 0.05 \)) and virtually abolished the \( \text{H}_2\text{O}_2 \)-induced facilitation of nerve-evoked contractions (Fig. 1D).

In endothelium-intact tissues, blockade of nitric oxide synthesis with L-NAME (100 μM) approximately doubled the amplitude of nerve-evoked contractions (L-Name, 208 ± 24%; time control, 89 ± 4%; \( P < 0.05, \ n = 7 \)) and reduced the late phase of the \( \text{H}_2\text{O}_2 \)-induced facilitation of these responses (Fig. 2A). In the absence of \( \text{H}_2\text{O}_2 \), nitric oxide appears to be the primary, but not the only, endothelium derived vasodilator substance, as inhibition of nitric oxide-synthase with L-NAME abolished the large relaxation to 0.1 μM carbachol, and greatly reduced the almost complete relaxation to 1 μM carbachol.
(Fig. 2B). In arteries pre-contracted with phenylephrine (2 μM), concentration response curves for carbachol (0.01 - 1 μM) did not differ in the absence or in the presence of H₂O₂ (Fig. 2C). H₂O₂ also did not change concentration response curves for the nitric oxide-donor, sodium nitroprusside (0.001 - 0.1 μM; Fig. 2D).

### 3.3 Activity of cyclooxygenase contributes to H₂O₂-induced facilitation of nerve-evoked contractions

To assess the possible involvement of cyclooxygenases and vasoactive prostanoids in the facilitatory actions of H₂O₂, the effects of inhibiting cyclooxygenases (with indomethacin, 1 μM) and antagonising thromboxane A₂/prostaglandin H₂ receptors (with SQ 29,548, 1 μM) were investigated. Neither indomethacin (indomethacin, 103 ± 8%; time control, 93 ± 4%; \( P = 0.33, n = 6 \)) nor SQ 29,548 (SQ 29,548, 114 ± 11%; time control, 99 ± 5%; \( P = 0.24, n = 9 \)) on their own changed the amplitude of nerve-evoked contractions, but both these agents attenuated the H₂O₂-induced facilitation of nerve-evoked contractions (Fig. 3A and B). At the concentration used, SQ 29,548 fully blocked the contraction and the increase in nerve-evoked contraction produced by the thromboxane A₂/prostaglandin H₂ receptor agonist U-46619 (10 nM; results not shown). These findings suggest that activity of cyclooxygenases and constrictor prostanoids contribute to H₂O₂-induced facilitation of nerve-evoked contraction.

### 3.4 H₂O₂-induced facilitation of nerve-evoked contractions is dependent on activation of SKF-96365 sensitive cation channels

To evaluate the potential role of Ca²⁺ entry in mediating the augmentation of nerve-evoked contractions produced by H₂O₂, the effects of SKF-96365 (10 μM) which blocks both store-operated and voltage-activated Ca²⁺ influx, and the L-type Ca²⁺ channel blocker nifedipine (1 μM), were assessed. In
comparison to pre-treatment responses, neither SKF-96365 (SKF-96365, 87 ± 24%; time control, 93 ± 4%; P = 0.82, n = 5) nor nifedipine (nifedipine, 84 ± 8%; time control, 88 ± 5%; P = 0.73, n = 4) on their own changed nerve-evoked contractions. However, SKF-96365 (Fig. 3C), but not nifedipine (repeated measures analysis of variance P = 0.72), strongly attenuated the H$_2$O$_2$-induced facilitation. These findings suggest that H$_2$O$_2$-induced facilitation of nerve-evoked contractions depend on Ca$^{2+}$ entry through an SKF-96365-sensitive Ca$^{2+}$ channel that is not an L-type Ca$^{2+}$ channel.

3.5 The role of Rho kinase and protein kinase C in H$_2$O$_2$-induced facilitation of nerve-evoked contractions

To determine if increased activity of Rho kinase or protein kinase C contributes to the facilitation of nerve-evoked contraction produced by H$_2$O$_2$, the effects of blocking their activity respectively with Y-27632 (1 μM) and RO31-8220 (1 μM) were assessed. Both Y-27632 (Y-27632, 52 ± 6%; time control, 90 ± 4%; P < 0.01, n = 4) and RO31-822 (RO31-822, 72 ± 5%; time control, 94 ± 3%; P < 0.01, n = 4) reduced nerve-evoked contractions, but neither of these agents significantly attenuated the facilitation of nerve-evoked contraction produced by H$_2$O$_2$ when the data were compared using repeated measures analysis of variance (P > 0.15 for both comparisons). However, while RO31-8220 did not reduce the early phase of the facilitation produced by H$_2$O$_2$ (Fig. 3D), it did reduce that measured at the 8th stimulus (P <0.05).

3.6 H$_2$O$_2$ increases the contribution of α$_2$-adrenoceptors to nerve-evoked contractions

To investigate the possibility that H$_2$O$_2$ changes the contribution of α$_1$- or α$_2$-adrenoceptors to nerve-evoked contractions, the effects of blocking these receptors respectively with prazosin (0.01 μM) or idazoxan (0.1 μM) were assessed. When applied immediately following the fourth stimulation in the
presence of H$_2$O$_2$, the % blockade of nerve-evoked contractions produced by prazosin was similar to that in time-matched control tissues (Fig. 4A). By contrast, H$_2$O$_2$ markedly increased the % blockade of nerve-evoked contractions produced by idazoxan (Fig. 4A). We also investigated the effects of idazoxan (0.1 μM) and the α$_2$C-adrenoceptor selective antagonist JP 1302 (0.5 - 1 μM) added prior to the addition of H$_2$O$_2$. Both idazoxan (idazoxan, 34 ± 2%; time control, 99 ± 4%; $P < 0.05$, $n = 5$) and JP 1302 (JP 1302, 64 ± 6%; time control, 98 ± 3%; $P < 0.05$, $n = 8$) reduced nerve-evoked contractions and attenuated the H$_2$O$_2$-induced facilitation of the contractions (Fig. 4B and C). These data suggest that H$_2$O$_2$ increases the contribution of α$_2$-adrenoceptors to nerve-evoked contractions.

To assess the possibility that H$_2$O$_2$ increases the contribution of α$_2$-adrenoceptors to nerve-evoked contractions via the release of a vasoactive prostanoid (see Bhattacharya and Roberts, 2003), we assessed if blockade of α$_2$-adrenoceptors reduced the facilitatory effect of the thromboxane A$_2$/prostaglandin H$_2$ receptor agonist U-46619 on nerve-evoked contractions. However, as U-46619 (1 nM) increased the amplitude of nerve-evoked contractions to a similar extent in the absence (277 ± 68 %, $n = 4$) and in the presence (244 ± 40 %, $n = 4$) of idazoxan (0.1 μM, $P = 0.73$), it appears that the augmentation of the α$_2$-adrenoceptor-mediated component of nerve-evoked contraction produced by H$_2$O$_2$ is not mediated via activation of thromboxane A$_2$/prostaglandin H$_2$ receptors.

The effects of blocking P2X1-purinoceptors with NF449 (10 μM) prior to the addition of H$_2$O$_2$ were also assessed to determine if purinergic transmission played a role in effects of H$_2$O$_2$. NF449 alone reduced nerve-evoked contractions (NF449, 50 ± 5 %; time control, 98 ± 4 %; $P <0.001; n = 5$), but did not reduce the facilitation produced by H$_2$O$_2$ (repeated measures analysis of variance $P = 0.33$).

3.7 H$_2$O$_2$ revealed α$_2$-adrenoceptor agonist-evoked contractions
The effects of H₂O₂ on concentration response curves for phenylephrine (α₁-adrenoceptor selective agonist) and UK 14,304 (α₂-adrenoceptor selective agonist) were assessed. H₂O₂ produced a small but significant increase in sensitivity to phenylephrine (pEC₅₀; control, 6.03 ± 0.03; H₂O₂, 6.16 ± 0.05; P <0.05; n = 9), but reduced the maximum contraction to this agent (Fig. 5A). In the absence of H₂O₂, the highest concentration of UK 14,304 tested (1 μM) produced a very small increase in wall tension (Fig. 5B). By contrast, when tested 30 mins after applying H₂O₂, UK 14,304 produced a concentration dependent increase in wall tension that plateaued at 0.01-0.03 μM. In time-matched control tissues treated with H₂O₂ alone, wall tension did not increase during the time period that UK 14,304 was applied (n = 3, data not shown). These data confirm that H₂O₂ increases the responsiveness of the tail artery to α₂-adrenoceptor agonists.

4. Discussion

This study demonstrates that H₂O₂ produces a marked enhancement of nerve-evoked contractions in mouse tail artery that is dependent on the endothelium. Stimulation of the endothelium with H₂O₂ appears to increase the excitability of the vascular muscle via a mechanism that is dependent on Ca²⁺ influx through SKF-96365-sensitive channels and is mediated, at least in part, by activation of a cyclooxygenase and production of constrictor prostanoids.

The facilitatory effect of H₂O₂ on nerve-evoked contraction was almost abolished in tissues that had their endothelium denuded. As endothelium denudation by itself increased nerve-evoked contractions, the simplest explanation is that H₂O₂ inhibits a tonic inhibitory influence of the endothelium. In mouse tail artery, nitric oxide appears to be the primary, but not the only, endothelium-derived vasodilator substance. However, while L-NAME increased nerve-evoked
contractions, confirming that the endothelium has a tonic inhibitory effect on the vascular muscle, this agent only reduced the late phase of the enhancement of nerve-evoked contractions produced by H$_2$O$_2$. Our findings indicate that H$_2$O$_2$ does not reduce carbachol-evoked release of nitric oxide or the sensitivity of the muscle to nitric oxide. The reduction in H$_2$O$_2$-induced facilitation of nerve-evoked contractions produced by L-NAME may result from a reduction in the basal release of nitric oxide from the endothelium, perhaps by uncoupling nitric oxide synthase (see Karaa et al., 2005). Alternatively, as the facilitation produced by the sequential addition of L-NAME (~200%) and H$_2$O$_2$ (~200%) is similar to that of H$_2$O$_2$ alone (~400%), the nerve-evoked contractions in the presence of these agents may have reached a maximum level. While we cannot exclude the possibility that H$_2$O$_2$ reduces the tonic inhibitory influence of the endothelium, the finding that this agent markedly increased nerve-evoked contractions in the presence of L-NAME suggests that it triggers the release of factors from the endothelium that increase nerve-evoked responses.

Endothelial denudation, inhibition of cyclooxygenases or blockade of thromboxane A$_2$/prostaglandin H$_2$ receptors reduced the H$_2$O$_2$-induced contractions in both the rat skeletal arterioles (Cseko et al., 2004) and canine basilar artery (Katusic et al., 1993) and reduced H$_2$O$_2$-induced facilitation of nerve-evoked contractions in the present study. These findings suggest that H$_2$O$_2$ stimulates endothelial production of constrictor prostanoids (e.g. thromboxane A2; Wong and Vanhoutte, 2010) and that this contributes to increasing nerve-evoked constrictions. In accord with this suggestion, a low concentration of the thromboxane A$_2$/prostaglandin H$_2$ receptor agonist U-46619 (1 nM) increased nerve-evoked contractions. In the endothelium, an increase in cytosolic Ca$^{2+}$ can stimulate the synthesis of prostanoids by cyclooxygenase (Wong and Vanhoutte, 2010). As H$_2$O$_2$ increases cytosolic Ca$^{2+}$ in endothelial cells by releasing Ca$^{2+}$ from intracellular stores and by increasing Ca$^{2+}$ entry through store-operated Ca$^{2+}$ channels (Doan et al., 1994; Volk et al., 1997), these mechanisms could explain how H$_2$O$_2$ stimulates the production of constrictor prostanoids. An alternative explanation is that H$_2$O$_2$ triggers
Ca\(^{2+}\) entry through transient receptor potential melastatin 2 channels, which are known to be expressed in human endothelial cells (Hecquet et al., 2008). As SKF-96365 blocks both store-operated Ca\(^{2+}\) channels and transient receptor potential melastatin 2 channels, our finding that this agent abolished the facilitatory effect of H\(_{2}\)O\(_{2}\) does not discriminate between these two possible explanations.

H\(_{2}\)O\(_{2}\) selectively increased the contribution to \(\alpha_2\)-adrenoceptors to neural activation of the mouse distal tail artery and increased the responsiveness of this vessel to the \(\alpha_2\)-adrenoceptor agonist UK 14,304. Cooling has been shown to increase the reactivity of this vessel to UK 14,304 by a reactive oxygen species and Rho-kinase dependent mechanism (see introduction). The cooling induced increase in reactivity to UK 14,304 is explained by translocation of \(\alpha_{2C}\)-adrenoceptors to the cell surface (Bailey et al., 2004; Jeyaraj et al., 2012) As the \(\alpha_{2C}\)-adrenoceptor antagonist JP1302 reduced the facilitatory effect of H\(_{2}\)O\(_{2}\) on nerve-evoked contractions, perhaps this also involves translocation of these receptors. However, the Rho kinase inhibitor Y-27632 did not reduce the facilitatory effect of H\(_{2}\)O\(_{2}\), indicating that \(\alpha_{2C}\)-adrenoceptor signalling can be increased by a mechanism that does not involve Rho kinase.

As contractions to UK 14,304 are enhanced by the thromboxane A\(_2\)/prostaglandin H\(_2\) receptor agonist U-46619 in another thermo-regulatory vessel, the porcine ear artery (Bhattacharya and Roberts, 2003), it is possible that the inhibitory effects of thromboxane A\(_2\)/prostaglandin H\(_2\) receptor and \(\alpha_2\)-adrenoceptor antagonists on the actions of H\(_{2}\)O\(_{2}\) are similarly linked. However, the facilitatory effect of U-46619 on nerve-evoked contractions was not changed by idazoxan, indicating that stimulation of thromboxane A\(_2\)/prostaglandin H\(_2\) receptors does not increase the contribution of \(\alpha_2\)-adrenoceptors to neural activation of the vascular muscle. These findings suggest that thromboxane A\(_2\)/prostaglandin H\(_2\) receptors and \(\alpha_2\)-adrenoceptors contribute to separate components of the enhancement of nerve-evoked contractions produced by H\(_{2}\)O\(_{2}\).
In conclusion, in the mouse tail artery H$_2$O$_2$ produced an endothelium-dependent increase in nerve-evoked contractions. This effect was observed with exogenous application of 10-100 μM H$_2$O$_2$, which would be expected to increase intracellular concentration maximally in the range 1-10 μM (see Antunes and Cadenas, 2000) and this is at the upper end of the physiological range of concentrations (Schröder and Eaton, 2008). Indeed in vascular tissues where there is evidence that H$_2$O$_2$ acts as a paracrine messenger it is necessary to use 10-100 μM H$_2$O$_2$ to mimic the effects attributed to endogenously generated H$_2$O$_2$ (e.g; Capettini et al., 2008; Gao and Lee, 2005; Matoba et al., 2003). Therefore we believe it possible that the effects we observed for H$_2$O$_2$ could contribute to normal vascular function. In mouse tail artery, it has been suggested that mitochondria are a cold sensor, signalling via the generation of reactive oxygen species to increase reactivity to nerve-released NA (Bailey et al., 2005). Our findings are in accord with this idea, but suggest that Rho kinase mediated recruitment of ‘silent’ α$_{2C}$-adrenoceptors is not the only mechanism whereby reactive oxygen species can increase reactivity of vascular smooth muscle. High levels of H$_2$O$_2$ are generated in inflamed tissues and during ischemia-reperfusion (Li and Shah, 2004), so it is also possible that H$_2$O$_2$-induced changes in neurovascular transmission contribute to pathophysiology.

**Acknowledgements:** The work was supported by a grant from the National Health and Medical Research Council of Australia (grant number 568850).
References


Figure legends

**Fig. 1.** H$_2$O$_2$ increased the amplitude of nerve-evoked contractions in an endothelial dependent manner. (A) The peak amplitude of contractions to 50 pulses at 1 Hz before and during the sequential addition of increasing concentrations of H$_2$O$_2$ (10-100 μM; n = 5) in artery segments; * indicate differences between contractions before and during application of H$_2$O$_2$ (P <0.01). (B) Original trace showing the effect of 100 μM H$_2$O$_2$ on both resting force and nerve-evoked contractions. (C) The peak amplitude of nerve-evoked contractions produced by H$_2$O$_2$ in the absence ( ) and in the presence ( ) of polyethylene glycol-catalase (n = 5) expressed as a percentage of the contraction immediately prior the addition of H$_2$O$_2$ (stimulus number 4). This graph also displays data for time-matched control tissues that did not receive H$_2$O$_2$ or polyethylene glycol-catalase (Δ). (D) The change in the peak amplitude of nerve-evoked contractions produced by H$_2$O$_2$ in the absence ( ) and in the presence ( ) of an intact endothelium (n = 5) expressed as a percentage of the contraction immediately prior the addition of H$_2$O$_2$ (stimulus number 4). Comparisons between the effects of H$_2$O$_2$ in the absence or in the presence of polyethylene glycol-catalase or an intact endothelium were made with repeated measures analysis of variance (P values indicated in C and D). Data are presented as mean and S.E.M.. Artery segments used in A, B and C had an intact endothelium.

**Fig. 2.** L-NAME reduced the facilitatory effect of H$_2$O$_2$ on nerve-evoked contractions

(A) The change in the peak amplitude of nerve-evoked contractions produced by H$_2$O$_2$ in the absence ( ) and in the presence ( ) of L-NAME (100 μM, n = 7) expressed as a percentage of the contraction immediately prior the addition of H$_2$O$_2$ (stimulus number 4). Comparison between the effects of H$_2$O$_2$ in the absence or in the presence of L-NAME was made with repeated measures analysis of variance (P
value is indicated in A). (B) The % relaxation of phenylephrine constricted non-H₂O₂ treated tissues produced by the muscarinic agonist carbachol (0.01 - 1 μM) in the absence (hatched bars) or in the presence (white bars) of L-NAME (n = 4); * indicates that L-NAME reduced relaxations to 0.1 and 1 μM carbachol (P <0.001). (C and D) The % relaxation of phenylephrine constricted tissues produced by (C) carbachol or by (D) the nitric oxide donor sodium nitroprusside in the absence (stippled bars) or in the presence (white bars) of H₂O₂ (n = 4). H₂O₂ did not change the relaxation produced by carbachol or sodium nitroprusside. Data are presented as mean and S.E.M.. All artery segments had an intact endothelium.

**Fig. 3.** Indomethacin, SQ 29,548 and SKF-96365, reduced the facilitatory effect of H₂O₂ on nerve-evoked contractions. The change in the amplitude of contractions to 50 pulses at 1 Hz produced by H₂O₂ in the absence ( ) and in the presence ( ) of (A) the non-selective cyclooxygenase inhibitor indomethacin (1 μM, n = 6), (B) the thromboxane A₂/prostaglandin H₂ receptor antagonist SQ 29,548 (1 μM, n = 9), (C) the non-specific Ca²⁺ channel blocker SKF-96365 (10 μM, n = 5) or (D) the protein kinase C selective inhibitor RO31-8220 (RO, 1 μM, n = 5) expressed as a percentage of the contraction immediately prior the addition of H₂O₂ (stimulus number 4). Comparisons between the effects of H₂O₂ in the absence or in the presence of these drugs were made with repeated measures analysis of variance (P values indicated in A, B, C and D). Data are presented as mean and S.E.M.. All artery segments had an intact endothelium.

**Fig. 4.** H₂O₂ selectively increased the contribution of α₂-adrenoceptors to nerve-evoked contractions. (A) Effect of the α₁-adrenoceptor antagonist prazosin (0.01 μM, n = 3) or the α₂-adrenoceptor antagonist idazoxan (0.1 μM, n = 5) on the peak amplitude of contractions to 50
pulses at 1 Hz in the absence (stippled bars) and in the presence (white bars) of H$_2$O$_2$ expressed as a percentage of the contraction immediately prior to adding the antagonist; * indicates that H$_2$O$_2$ significantly increased the blockade produced by idazoxan ($P<0.01$). (B and C) The change in the peak amplitude of nerve-evoked contractions produced by H$_2$O$_2$ in the absence (●) and in the presence (○) of (B) idazoxan (n = 5) or (C) JP 1302 (n = 8) expressed as a percentage of the contraction immediately prior the addition of H$_2$O$_2$ (stimulus number 4). Comparisons between the effects of H$_2$O$_2$ in the absence or in the presence of idazoxan or JP 1302 were made with repeated measures analysis of variance ($P$ values indicated in B and C). Data are presented as mean and S.E.M.. All artery segments had an intact endothelium.

**Fig. 5.** H$_2$O$_2$ reduced the maximum contractions to phenylephrine and revealed contractions to UK 14,304. (A and B) Concentration response curves for (A) the $\alpha_1$-adrenoceptor agonist phenylephrine (n = 9) and (B) the $\alpha_2$-adrenoceptor agonist UK 14,304 (n = 7) in the absence ( ) or in the presence ( ) of H$_2$O$_2$. Comparisons between concentration response curves in the absence and presence of H$_2$O$_2$ were made with repeated measures analysis of variance ($P$ values are indicated in A and B). In A, * indicates a difference between the maximum contraction to phenylephrine in the absence or in the presence of H$_2$O$_2$ ($P<0.05$). Data are presented as mean and S.E.M.. All artery segments had an intact endothelium.
Figures 1 to 5

Fig. 1.

A

\[
\text{Wall tension (mN/mm)}
\]

B

\[
\text{1 mN}
\]

C

D

\[
\text{% of pre-treatment}
\]

\[
\text{Stimulus number}
\]

\[
P < 0.05
\]

\[
P < 0.001
\]
Fig. 2.
Fig. 3.

![Graphs showing changes in stimulus number and percentage of pre-treatment for different conditions.](image)

- **Fig. 3A**: Stimulus number vs. percentage of pre-treatment with a significant difference ($P < 0.05$).
- **Fig. 3B**: Stimulus number vs. percentage of pre-treatment with a significant difference ($P < 0.01$).
- **Fig. 3C**: Stimulus number vs. percentage of pre-treatment with a significant difference ($P < 0.05$).
- **Fig. 3D**: Stimulus number vs. percentage of pre-treatment with a significant difference ($P = 0.16$).
Fig. 4.

A

B

C

P < 0.05

% of pre-treatment

Stimulus number

H$_2$O$_2$

Idazoxan

Prasosin

% of pre-treatment
Fig. 5.

A

B

Phenylephrine (µM)

[UK 14,304j (µM)

∆ Wall tension (mN/mm)

P < 0.05

P < 0.01

0.001 0.01 0.1

0.01 1 10

0.01 0.1 1

0.01 1 100

0.01 0.1 1

0.01 1 100
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Title:
Hydrogen peroxide increases nerve-evoked contractions in mouse tail artery by an endothelium-dependent mechanism

Date:
2013-01-05

Citation:
Reardon, TF; Brock, JA, Hydrogen peroxide increases nerve-evoked contractions in mouse tail artery by an endothelium-dependent mechanism, EUROPEAN JOURNAL OF PHARMACOLOGY, 2013, 698 (1-3), pp. 362 - 369

Publication Status:
Accepted manuscript

Persistent Link:
http://hdl.handle.net/11343/43915