Catalase has negligible inhibitory effects on endothelium-dependent relaxations in mouse isolated aorta and small mesenteric artery

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1 The current study examined the hypothesis that endothelial production of hydrogen peroxide (H_2O_2) mediates relaxations to acetylcholine (ACh) in aorta and small mesenteric arteries (SMA) from mice.

2 Relaxations to ACh (0.01–10 μM) and H_2O_2 (0.1–1000 μM) were produced in aorta and SMA isolated from wild-type C57BL/6 mice and type II diabetic mice (db/db). In SMA, relaxations to ACh were produced in the presence of N^{ω}-nitro-L-arginine methyl ester (100 μM) and indomethacin (Indo, 10 μM).

3 1-H[1,2,4]oxadiazolo[4,3-]quinoxalin-1-one (10 μM) significantly reduced ACh-induced relaxations in SMA, abolished responses in aorta, but had no effect on relaxations induced by H_2O_2. Catalase (2500 U ml^{-1}) abolished responses to H_2O_2, but did not alter relaxations to ACh in the SMA and only caused a small rightward shift in responses to ACh in the aorta.

4 ACh-, but not H_2O_2-, mediated relaxations were significantly reduced by tetraethylammonium (10 mM), the combination of apamin (1 μM) and charybdotoxin (100 nM), and 25 mM potassium chloride (KCl). Higher KCl (60 mM) abolished relaxations to both ACh and H_2O_2. Polyethylene glycolated superoxide dismutase (100 U ml^{-1}), the catalase inhibitor 3-amino-1,2,4-triazole (3-AT, 50 mM) and treatment with the copper chelator diethyldithiolcarbamate (3 mM) did not affect relaxations to ACh.

5 H_2O_2-induced relaxations were endothelium-independent and were not affected by ethylene diamine tetraacetic acid (EDTA 0.067 mM), 4-aminopyridine (1 mM), ouabain (100 μM) and barium (30 μM), 3-AT or Indo.

6 Although the data from this study show that H_2O_2 dilates vessels, they do not support the notion that H_2O_2 mediates endothelium-dependent relaxations to ACh in either aorta or SMA from mice.
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Abbreviations: ACh, acetylcholine; Ap, apamin; 4-AP, 4-aminopyridine; 3-AT, 3-amino-1,2,4-triazole; carboxy-PTIO, 2-[4-carboxyphenyl]-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide; ChTx, charybdotoxin; DETCA, diethyldithiolcarbamate; EDHF, endothelium-derived hyperpolarizing factor; EDTA, ethylene diamine tetraacetic acid; H_2O_2, hydrogen peroxide; Indo, indomethacin; KCl, potassium chloride; L-NAME, N^{ω}-nitro-L-arginine methyl ester; NO, nitric oxide; NOS, nitric oxide synthase; ODQ, 1-H[1,2,4]oxadiazolo[4,3-]quinoxalin-1-one; PEG-SOD, polyethylene glycolated superoxide dismutase; Phe, phenylephrine; PSS, physiological saline solution; ROS, reactive oxygen species; SMA, small mesenteric arteries; TEA, tetraethylammonium

Introduction

The release of endothelium-derived substances, such as nitric oxide (NO) and prostacyclin, is important in mediating vasodilatation and maintaining blood pressure homeostasis. The reactive oxygen species (ROS) superoxide and hydrogen peroxide (H_2O_2) are also increasingly gaining acceptance as endogenously derived modulators of vascular tone. While the NO scavenging properties of superoxide are well known, it appears that H_2O_2 exerts a direct modulatory effect on vasomotor tone to mediate relaxation. As H_2O_2 relaxes smooth muscle (Burke & Wolin, 1987; Wei & Kontos, 1990; Barlow & White, 1998; Hayabuchi et al., 1998; Iida & Katusic,

2000) and endothelial cells can generate significant amounts of ROS (Arroyo et al., 1990; Holland et al., 1998; Stroes et al., 1998; Zhang et al., 1999), it is conceivable that endogenously produced H_2O_2 might also regulate vascular tone. Indeed, several studies support this concept and have demonstrated a catalase-sensitive and, therefore, an assumed H_2O_2-mediated component to endothelium-dependent relaxation in some vascular beds (Kontos et al., 1984; Rubanyi & Vanhoutte, 1986; Yang et al., 1991; Sobey et al., 1997; Cosentino et al., 2001; Rabelo et al., 2003). Since the relaxant effects of H_2O_2 have been associated with an action on potassium channels (Barlow & White, 1998; Hayabuchi et al., 1998; Bychkov et al., 1999), it was proposed that H_2O_2 mediated the relaxations attributed to endothelium-derived hyperpolarizing factor (EDHF) in mouse mesenteric arteries (Matoba et al., 2000).

EDHF-mediated relaxations are characteristically resistant to inhibitors of nitric oxide synthase (NOS), cyclooxygenase or soluble guanylate cyclase, and are attributed to an endothelial-derived hyperpolarization that is conducted to adjacent vascular smooth muscle cells to cause relaxation (Triggle *et al.*, 1999; McGuire *et al.*, 2001; Feletou & Vanhoutte, 2003). Depolarizing stimuli such as high extracellular concentrations of potassium, or a combination of the calcium-activated potassium channel blockers apamin (Ap) and charybdotoxin (ChTx), can inhibit EDHF relaxations (Edwards *et al.*, 1998; Busse *et al.*, 2002). Matoba *et al.* (2000) drew comparisons between relaxations resistant to inhibitors of NOS and cyclooxygenase and those to H_2O_2 in mouse mesenteric arteries because both were similarly sensitive to the nonselective calcium-activated potassium channel inhibitor tetrabutylammonium (TBA). Furthermore, both the endothelium-dependent hyperpolarizations and relaxations in these vessels were inhibited by catalase. It was also recently proposed that endothelium-dependent relaxations in the mouse aorta were mediated by H_2O_2 and that impaired endothelium-dependent dilatation in hypercholesterolemic mice resulted from a reduced output of H_2O_2 (Rabelo *et al.*, 2003). In the current study, we sought to further investigate the prospect that endothelium-dependent relaxations in mouse vessels are mediated at least in part by the ROS H_2O_2. The approach we took was to compare endothelium-dependent relaxations induced by acetylcholine (ACh) to those induced by H_2O_2 in the presence of agents that modify either the production or site of action of these dilators.

Methods

Animals

A total of 6- to 8-week-old male mice (C57BL/6, Charles River Laboratories, Montreal, PQ, Canada; or type II diabetic db/db and their respective wild-type controls db $+/?$, Jackson Laboratories, Bar Harbour, ME, U.S.A.) were killed by cervical dislocation. The mesenteric bed was isolated and placed in physiological saline solution (PSS) for dissection. First-order small mesenteric arteries (SMA) were cleared of any adherent connective tissue and fat, and cut into 2 mm segments. These were then mounted onto a wire myograph chamber, set at a resting tension of 1 mN and allowed to equilibrate for 60 min with intermittent washes every 15 min. The thoracic aorta was isolated and cleared of connective tissue and fat, and cut into 2 mm segments. Rings were placed onto tissue hooks in a wire myograph chamber and set at a resting tension of 4.5 mN. The experimental use of animals in this study conformed to guidelines as set out by the Health Sciences Animal Care Committee at the University of Calgary.

Experimental protocol

At the end of the equilibration period, the vessels were tested for responsiveness with a bolus concentration of potassium chloride (KCl, 120 mM) and promptly washed with PSS. To assess the integrity of the endothelium, tissues were contracted with a submaximal concentration of prostaglandin $F_{2\alpha}$ (PGF$_{2\alpha}$, $0.01-0.1\,\mu$M) in SMA and phenylephrine (Phe, $0.1-0.3\,\mu$M) in aorta, and if the relaxation to ACh ($10\,\mu$M) was greater than

70% of the contraction they were considered to be endothelium-intact. In some vessels, the endothelium was removed by passing a wire through the lumen several times. If vessels failed to relax to ACh, they were considered to be devoid of endothelial cells.

Endothelium-dependent relaxations

Aortic rings were constricted with submaximal concentrations of Phe ($0.1-0.3\,\mu$M), and control concentration–response curves to ACh ($0.01-10\,\mu$M) were produced and compared to those produced in rings treated with catalase (2500 U ml^{-1}; activity: 19,990 U mg^{-1} solid; catalog number: C40, Sigma) or 1-H[1,2,4]oxadiazolo[4,3-]quinoxalin-1-one (ODQ, $10\,\mu$M). SMA were pretreated with N^{ω}-nitro-L-arginine methyl ester (L-NAME, $100\,\mu$M) and indomethacin (Indo, $10\,\mu$M) (incubated for 15 min), and control concentration–response curves to ACh ($0.01-10\,\mu$M) were produced during a submaximal contraction to PGF$_{2\alpha}$ ($0.01-0.3\,\mu$M). These responses were then repeated in the presence of catalase, high KCl (25 and 60 mM), tetraethylammonium (TEA, 10 mM), ODQ, a combination of Ap ($1\,\mu$M) and ChTx (100 nM), polyethylene-glycolated superoxide dismutase (PEG-SOD, 100 U ml^{-1}) and in tissues pretreated with the copper chelator diethyldithiocarbamate (DETCA, 3 mM, 30 min incubation followed by wash) or the catalase inhibitor 3-amino-1,2,4-triazole (3-AT, 50 mM, 1 h incubation followed by wash). In some experiments, SMA were treated with the combination of L-NAME, Indo and ODQ or L-NAME, Indo and 2-[4-carboxyphenyl]-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide (carboxy-PTIO, $300\,\mu$M) to assess any further effects that catalase might have on responses to ACh under these conditions. The effects of the different agents on relaxations to ACh or H_2O_2 were tested following a 30 min incubation period unless otherwise stated.

H_2O_2-induced relaxations

Endothelium-intact or denuded mouse SMA and aorta were constricted with PGF$_{2\alpha}$ or Phe to observe concentration–response curves to H_2O_2 ($1-1000\,\mu$M). Tissues that were exposed to an initial concentration–response curve to H_2O_2 did not contract as effectively with PGF$_{2\alpha}$ or Phe following washout; therefore, each treatment was performed in a separate vessel preparation. Relaxations to H_2O_2 were produced in the presence of high KCl (25 and 60 mM), Indo, ODQ, catalase, TEA (10 mM), Ap/ChTx ($1\,\mu$M and 100 nM, respectively), ouabain and BaCl$_2$ (100 and 30 μM, respectively), 4-aminopyridine (4-AP, 1 mM) or ethylene diamine tetraacetic acid (EDTA, 0.067 mM).

Drugs and materials

The composition of the PSS was as follows (mM): NaCl (118.0), KCl (4.7), KH$_2$PO$_4$ (0.8), MgSO$_4$ (1.2), NaHCO$_3$ (24.9), dextrose (11.1) and CaCl$_2$ (2.5). The PSS was maintained at a temperature of 37°C and bubbled with carbogen (95% O_2, 5% CO_2). All drugs and reagents, except TEA, were purchased from Sigma-Aldrich (St Louis, MO, U.S.A.), while TEA was obtained from BDH Chemicals Ltd (Poole, U.K.). Drugs were prepared as stock solutions, which were then diluted to appropriate concentrations in PSS or distilled water. H_2O_2 was purchased as a 30% w w^{-1}

concentrated solution and was subsequently diluted in distilled water. All drugs except prostaglandin $F_{2\alpha}$, Indo and ODQ were dissolved in water. Indo and prostaglandin $F_{2\alpha}$ were dissolved in ethanol, and ODQ was prepared in DMSO.

Statistical analysis

Data are expressed as a percentage of $PGF_{2\alpha}$-, Phe- or KCl-induced contractions. Symbols represent means and T-bars are standard error of means (s.e.m.). Some data are also displayed as pEC_{50} values, which are derived according to the equation $pEC_{50} = -\log(EC_{50})$. Comparison of the effects of different treatments on responses to ACh and H_2O_2 was determined by one-way analysis of variance followed by Dunnett's multiple comparison test (Prism, Graph Pad). Asterisks represent *P*-values less than 0.05 and indicate statistical significance. The *n* values in parentheses indicate the number of animals used for an experiment.

Results

Endothelium-dependent relaxations

In the presence of L-NAME (100 μM) and Indo (10 μM), ACh (0. 01–10 μM) relaxed C57BL/6 mouse SMA to a maximum of $62.6 \pm 3.6\%$ ($n = 32$) of the $PGF_{2\alpha}$-induced contraction. The addition of the soluble guanylate cyclase inhibitor ODQ (10 μM) significantly reduced the relaxations to $25.9 \pm 6.2\%$ ($n = 9$) (Figure 1a). In the aorta, ACh (0.001–10 μM) produced relaxations that reached a maximum of $84.2 \pm 3.0\%$ ($n = 8$) of the Phe-induced contraction. Relaxations were abolished in rings treated with ODQ (Figure 1b).

L-NAME- and Indo-resistant relaxations to ACh in SMA were not affected by catalase (2500 U ml^{-1}) in either SMA from C57BL/6 or db/db mice (Figure 2a and c). In aorta, catalase (2500 U ml^{-1}) caused a significant rightward shift of the concentration–response curve to ACh (pEC_{50} control: 7.38 ± 0.1, catalase: 6.98 ± 0.1, $n = 5$, $P < 0.05$, paired *t*-test) but caused no significant change to maximal relaxation (Figure 2b).

Both ODQ and the NO scavenger carboxy-PTIO (300 μM) further reduced the L-NAME- and Indo-resistant relaxations to ACh in SMA; however, catalase did not have any additional effects on the residual relaxations that remained in the presence of these agents (Figure 3). Relaxations to ACh were significantly reduced by 25 mM KCl and were abolished by 60 mM KCl (Figure 4a). Relaxations were also significantly reduced by the potassium channel inhibitors TEA (10 mM) and by the combination of Ap/ChTx (Figure 4b). Treatment of SMA with DETCA (3 mM) to inhibit the production of H_2O_2 by endogenous Cu/Zn SOD, with 3-AT (50 mM), to inhibit endogenous catalases, or the addition of cell-permeable PEG-SOD (100 U ml^{-1}) to augment endogenous formation of H_2O_2, all had no effect on ACh-induced relaxations (Figure 5).

Hydrogen peroxide-induced relaxations

H_2O_2 (1–1000 μM) produced concentration-dependent relaxations, which fully relaxed SMA. In aortic rings, H_2O_2 induced relaxations that reached a maximum of $57.6 \pm 7.1\%$ of the Phe-induced contraction, which were abolished by catalase but were not affected by ODQ (Figures 1b and 2b, $n = 4$–6).
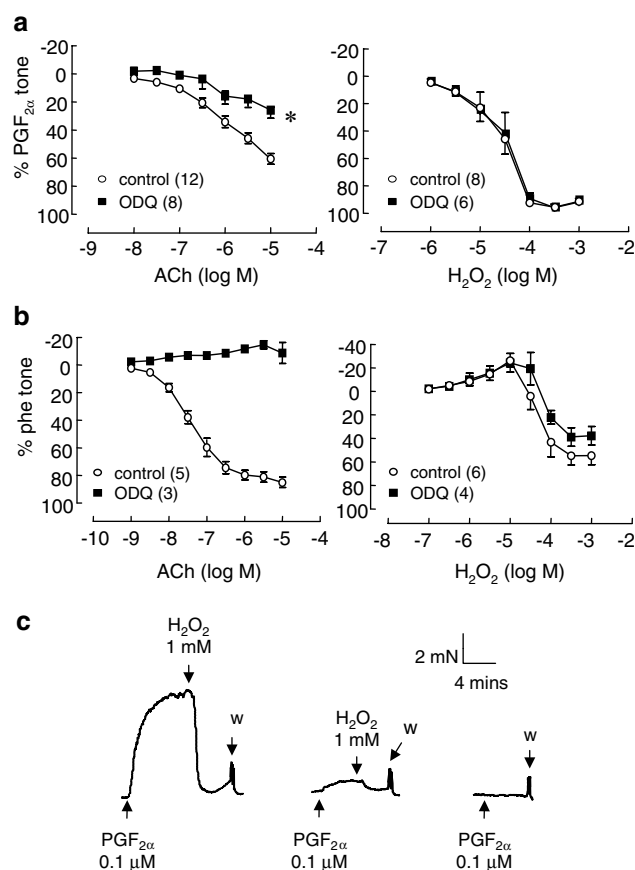

Figure 1 Relaxations produced to ACh (0.01–10 μM) or H_2O_2 (0.1–1000 μM) in (a) SMA and (b) aorta, in the absence and presence of ODQ (10 μM). Relaxations to ACh in SMA were performed in the presence of L-NAME (100 μM) and Indo (10 μM). (c) Trace depicting the effect of repeated exposures of 1 mM H_2O_2 on contractile tone in SMA. Symbols represent means and t-bars are standard error of means (s.e.m.). *$P < 0.05$ when compared to control (one-way analysis of variance). Numbers in parentheses refer to number of experiments performed.

Following washout, H_2O_2 impaired the ability of the aorta and SMA to contract again to their respective contractile agonists (Figure 1c). Relaxations were not dependent on the presence of endothelial cells since there was no difference in the concentration–response curve to H_2O_2 between endothelium-intact or denuded SMA (Table 1). Relaxations were abolished by catalase (2500 U ml^{-1}) and 60 mM KCl; however, lower KCl (25 mM) did not significantly reduce responses to H_2O_2 (Figure 4). Furthermore, Indo, ODQ, TEA, Ap/ChTx, 4-AP, ouabain/BaCl$_2$, pretreatment of vessels with 3-AT and the inclusion of EDTA in the PSS all failed to alter H_2O_2-induced relaxations in SMA (Table 1).

Discussion

The findings of the current study do not support the contention that endothelium-dependent relaxations to ACh in either aorta or SMA from mouse are mediated by H_2O_2. Catalase, which metabolizes H_2O_2 into water and oxygen, did not inhibit relaxations to ACh in SMA and only slightly reduced responses in aorta. The irreversible nature of the relaxations to H_2O_2 coupled with the fact that very high
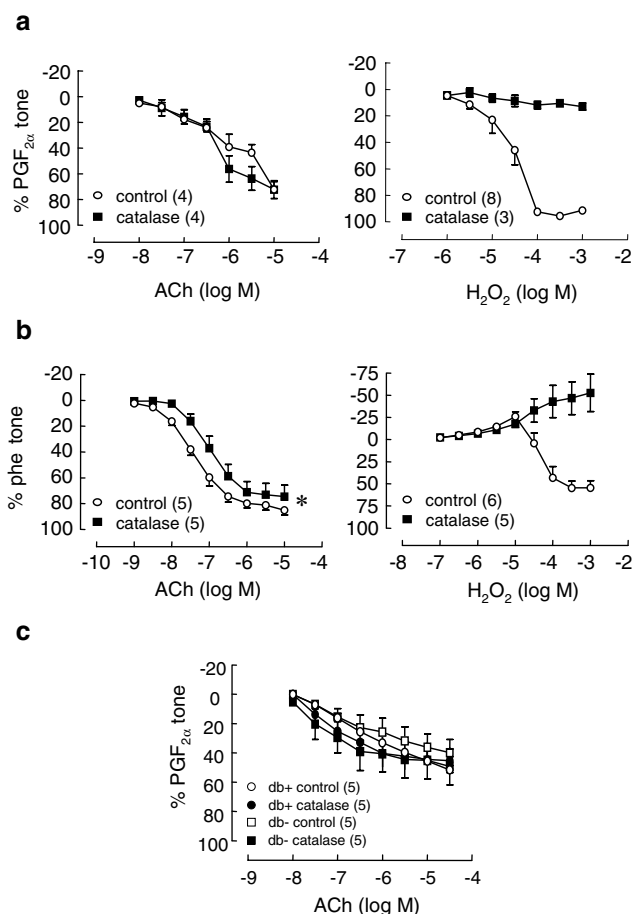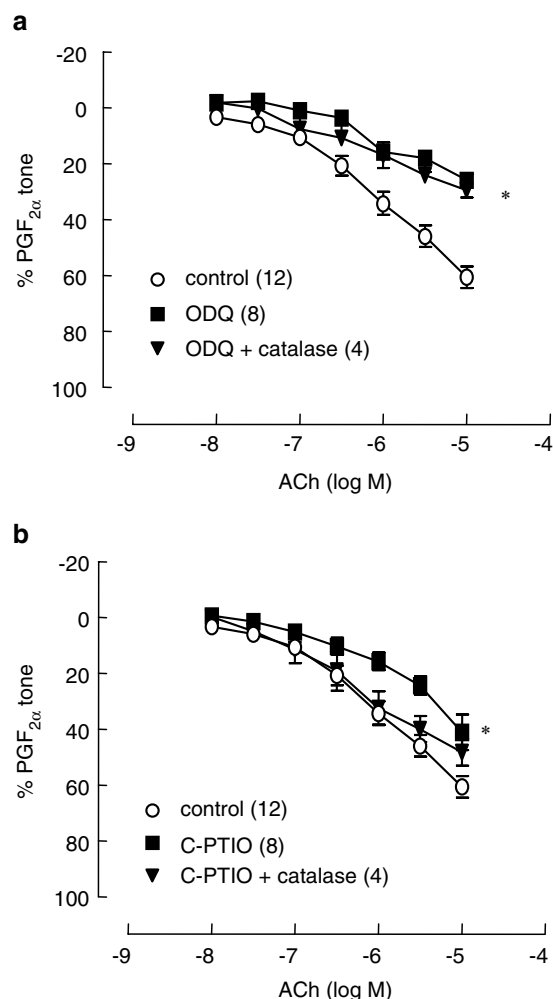
Figure 2 Effect of catalase (2500 U ml^{-1}) on relaxations to ACh (0.01–10 μM) in (a) SMA and (b) aorta from C57BL/6 mice, and (c) SMA from type II diabetic mice db/db (and respective wild-type controls db +/?; $n = 5$). Relaxations to H$_2$O$_2$ (1–1000 μM) were abolished by catalase in both aorta and SMA. Symbols represent means and t-bars are standard error of means (s.e.m.). *$P < 0.05$ when compared to control (comparison of pEC$_{50}$ values, paired t-test). Numbers in parentheses refer to number of experiments performed.

concentrations were required to induce a response, indicate that H$_2$O$_2$ is unlikely to function as a physiological endothelium-derived dilator in mouse vessels.

Endothelium-dependent relaxation

In the aorta, ACh produced relaxations that were abolished by the soluble guanylate cyclase (sGC) inhibitor ODQ. In SMA, relaxations to ACh persisted in the presence of L-NAME and Indo. ODQ further reduced responses, indicating that ACh relaxations in these vessels are mostly mediated by the activation of sGC by a residual source of NO, whose synthesis cannot be fully inhibited by L-NAME alone. The possibility that the endothelium-dependent relaxations in these vessels were mediated by H$_2$O$_2$ was discounted in this study because catalase (2500 U ml^{-1}) did not inhibit relaxations in SMA and only caused a small rightward shift in response curves to ACh in aorta. In SMA, both ODQ and the NO scavenger carboxy-PTIO further reduced the relaxations to ACh, and catalase did not have any additional effect on the residual relaxations seen under these conditions.
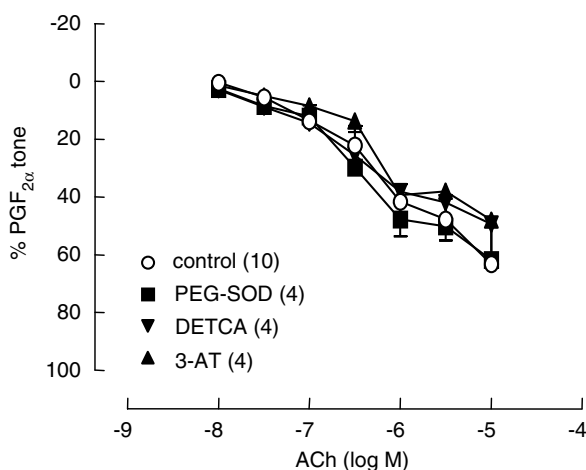

Figure 3 L-NAME/Indo-resistant relaxations to ACh (0.01–10 μM) in C57BL/6 mouse SMA were further reduced by (a) ODQ (10 μM) and (b) carboxy-PTIO (C-PTIO, 300 μM). However, the subsequent addition of catalase (2500 U ml^{-1}) did not have an additional effect on residual relaxation that persisted in the presence of these agents. Symbols represent means and t-bars are standard error of means (s.e.m.). *$P < 0.05$ when compared to control (one-way analysis of variance). Numbers in parentheses refer to number of experiments performed.

In order to determine whether the synthesis of H$_2$O$_2$ by Cu/Zn SOD could contribute to endothelium-dependent relaxation, SMA were also pretreated with the copper chelator diethyldithiocarbamate (DETCA, 3 mM), commonly used to inhibit the Cu/Zn form of superoxide dismutase (Cu/Zn SOD) (Omar *et al.*, 1991; Martin *et al.*, 1994). DETCA treatment of vessels did not reduce ACh-mediated relaxations, indicating that endogenous Cu/Zn SOD is unlikely to synthesize sufficient amounts of H$_2$O$_2$ to relax vessels. Nonetheless, non-copper-containing forms of SOD are also expressed by endothelial cells that could also produce H$_2$O$_2$, thus the lack of effect by DETCA does not entirely exclude a role for H$_2$O$_2$. On the other hand, if endogenously synthesized H$_2$O$_2$ contributes to ACh-mediated relaxation, supplementing vessels with SOD would be expected to augment the production of H$_2$O$_2$; however, the addition of PEG-SOD did not enhance ACh-mediated relaxations in SMA. The possibility that endogenous catalases were contributing to the destruction of endothelial
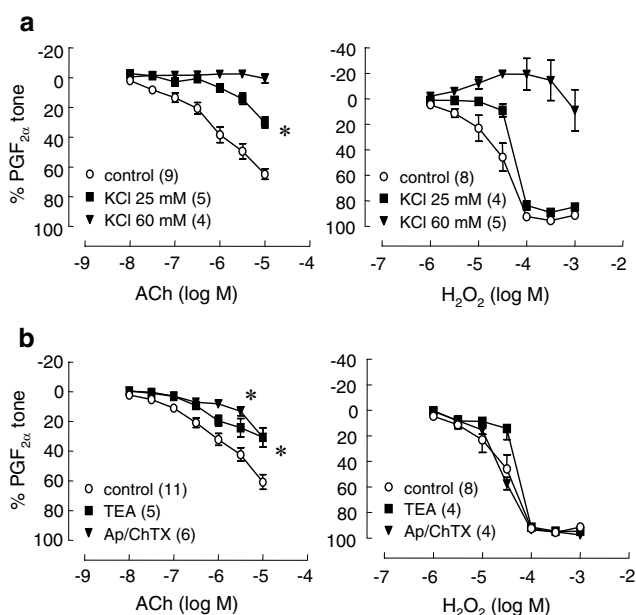
Figure 4 Comparative effects of (a) varied concentrations of KCl (25 and 60 mM), or (b) the potassium channel inhibitors TEA (10 mM) and Ap (1 μM) and ChTX (100 nM), on L-NAME/Indo-resistant relaxations to ACh (0.01 – 10 μM) and those induced by H_2O_2 (1 – 1000 μM) in C57BL/6 mouse SMA. Symbols represent means and t-bars are standard error of means (s.e.m.). *$P < 0.05$ when compared to control (one-way analysis of variance). Numbers in parentheses refer to number of experiments performed.

Figure 5 L-NAME/Indo-resistant relaxations to ACh (0.01 – 10 μM) in C57BL/6 mouse SMA were not affected by DETCA pretreatment (3 mM, 30 min incubation), the addition of PEG-SOD (100 U ml^{-1}) or the treatment of vessels with 3-amino-1,2,4-triazole (3-AT, 50 mM, 1 h incubation). Symbols represent means and t-bars are standard error of means (s.e.m.). Numbers in parentheses refer to number of experiments performed.

Table 1 Summary of the effects of different agents or treatments on pEC_{50} values and maximal relaxations to H_2O_2 in $PGF_{2\alpha}$ contracted mouse SMA

Treatment	n	pEC_{50}	Maximal relaxation[a] (%)
Control (EC+)	8	4.54 ± 0.13	94.4 ± 1
Control (EC−)	5	4.6 ± 0.2	95.1 ± 1
Indomethacin 10 μM	4	4.57 ± 0.14	93.7 ± 1
ODQ 10 μM	6	4.51 ± 0.18	94.8 ± 1
EDTA 0.067 mM	4	4.37 ± 0.04	96.6 ± 1
3-AT 50 mM	4	4.58 ± 0.03	93.2 ± 1
KCl 25 mM	4	4.31 ± 0.06	87.1 ± 1[b]
TEA 10 mM	4	4.27 ± 0.06	94.8 ± 1
Ap 1 μM/ChTx 100 nM	4	4.57 ± 0.04	97.6 ± 1
4-AP 1 mM	4	4.38 ± 0.11	93.8 ± 1
TEA/4-AP	4	4.79 ± 0.05	91.2 ± 2
Oua 100 μM/ Ba^{2+} 30 μM	4	4.52 ± 0.1	90.3 ± 5

[a]Maximal relaxations are expressed as percentages of the contraction to $PGF_{2\alpha}$ (0.01 – 0.1 μM).
[b]$P < 0.05$ when compared to control (EC+), ANOVA, followed by Dunnett's multiple comparison test.
3-AT: 3-amino-1,2,4-triazole; 4-AP: 4-aminopyridine; Ap: apamin; Ba^{2+}: barium chloride; ChTx: charybdotoxin; EC+: endothelium-intact; EC−: endothelium-denuded; EDTA: ethylene diamine tetraacetic acid; H_2O_2: hydrogen peroxide; KCl: potassium chloride; ODQ: 1-H[1,2,4]oxadia-zolo[4,3-]quinoxalin-1-one; oua: ouabain; pEC_{50}: −log[EC_{50}]; TEA: tetraethylammonium.

H_2O_2, thus explaining the lack of effect of exogenously applied catalase, is unlikely because the catalase inhibitor 3-AT did not significantly alter relaxations to ACh.

Oxidative stress is a common characteristic of diabetes (De Vriese *et al.*, 2000), and studies from our laboratory have demonstrated that elevated production of ROS contributes to endothelial dysfunction in the leptin-receptor-deficient, type II diabetic mouse db/db (Pannirselvam *et al.*, 2002). Thus, the prospect of H_2O_2 being the mediator of EDHF highlighted the possibility that its role in vasodilatory tone might be more pronounced in conditions characterized by oxidative stress. Given the smooth muscle relaxant effects of H_2O_2, we wondered whether increased endothelial production of H_2O_2 in blood vessels from diabetic animals might compensate for impaired endothelium-dependent dilatation that results from the reduced bioavailability of NO. Thus, we initially set out to investigate the contribution of H_2O_2 to endothelium-dependent vasodilation in db/db mice. However, catalase did not inhibit endothelium-dependent relaxations in SMA from these mice. As, Matoba *et al.* (2000) showed that L-NAME/Indo-resistant relaxations in wild-type C57BL/6 mice were sensitive to catalase, our next step was to see if we could at least observe catalase sensitivity in SMA from these mice; however, catalase again did not affect relaxations. Overall, even in conditions where increased production of ROS is expected to occur, H_2O_2 does not appear to mediate relaxant tone in the mesenteric vasculature.

Hydrogen peroxide-induced relaxations

H_2O_2 produced concentration-dependent relaxations in both aorta and SMA and these were abolished by catalase. In order to induce a relaxation to H_2O_2, it was necessary to exceed concentrations of 100 μM, which may have irreversibly damaged smooth muscle cells, as vessels did not contract effectively on subsequent challenges with $PGF_{2\alpha}$ or Phe (see Figure 1c). H_2O_2 has been reported to damage the calcium ATPase of the sarcoplasmic reticulum (SR) in smooth muscle

(Grover *et al.*, 1992), reducing the ability of cells to replenish the SR with calcium and thus affecting their ability to respond to a contractile agonist (Grover *et al.*, 1995). In the present study, relaxations to H_2O_2 were prevented by high extracellular concentrations of KCl (60 mM), suggesting that relaxations could be mediated by the activation of potassium channels. However, when a lower concentration of KCl (25 mM) was applied, relaxation to H_2O_2 remained largely unchanged whereas relaxations to ACh were substantially reduced. Also, incubation with TEA, ouabain and barium, apamin and ChTx, or 4-AP all failed to inhibit relaxations to H_2O_2, suggesting the lack of involvement of K_{Ca}, K_V, K_{IR} and the $Na^+/K^+/ATPase$ in mediating responses to H_2O_2. Some studies have suggested that the effects of H_2O_2 may be cyclic GMP (cGMP)-mediated (Burke & Wolin, 1987; Burke-Wolin *et al.*, 1991; Fujimoto *et al.*, 2002) or endothelium-dependent (Zembowicz *et al.*, 1993); however, in the present study ODQ did not inhibit relaxations to H_2O_2, and removal of endothelium did not significantly alter relaxations to H_2O_2 in SMA.

EDTA did not significantly affect responses to H_2O_2, indicating that these responses were unlikely to be due to the metal-catalyzed transformation of H_2O_2 into other ROS (e.g. hydroxyl radicals) that might mediate the relaxation. Some of the vasoactive effects of H_2O_2 have also been associated with arachidonic acid-dependent pathways, resulting in elevated formation of prostanoids (Clyman *et al.*, 1989; Iida & Katusic, 2000). However, the conditions required to observe EDHF relaxations (i.e. the presence of L-NAME and Indo) would exclude the possibility that prostanoids contribute to the relaxant effects of endogenous H_2O_2 because any formation of prostanoids would be inhibited by Indo. Indo itself did not prevent relaxations to H_2O_2 in the present study; therefore, cyclooxygenase-derived metabolites are unlikely to be involved in these responses.

The findings of the present study suggest that relaxations mediated by H_2O_2 result from nonspecific effects on vascular smooth muscle tone. The fact that, aside from catalase, only depolarizing concentrations of KCl (60 mM) were able to prevent the 'relaxations' to H_2O_2 indicates that the effects might be associated with alterations in intracellular calcium signaling. High KCl (60 mM) produces contractions by depolarizing smooth muscle cells, which activates a voltage-dependent calcium influx. Contractions to $PGF_{2\alpha}$ are mediated by the activation of thromboxane receptors, which, depending on the tissue, raise tone either by stimulating an influx of calcium from extracellular sources (Godfraind & Miller, 1982) or by calcium sensitization (Nobe & Paul, 2001). Although we did not further investigate the possible mechanisms that might account for the decrease in tone by H_2O_2, recent evidence suggests that it may relax smooth muscle by interfering with calcium sensitization processes. For example, H_2O_2 increased cytosolic calcium in canine tracheal smooth muscle, but paradoxically also caused a decrease in the amount of myosin phosphorylated in response to ACh, causing a loss in tone (Lorenz *et al.*, 1999). Fujimoto *et al.* (2002) reported that H_2O_2 reduced the amount of myosin phosphorylated by noradrenaline in guinea pig aorta partly through a cGMP-mediated mechanism. It has also been reported that H_2O_2 irreversibly inhibited myosin ATPase activity to prevent cross-bridge formation during contractions (Perkins *et al.*, 2003); thus it is possible that H_2O_2 elicits relaxation, or a loss in tone, by indiscriminately impairing smooth muscle contractile processes.

Discrepancy with other studies

In contrast to some recent reports (Matoba *et al.*, 2000; Rabelo *et al.*, 2003), we were unable to establish the existence of catalase-sensitive, and therefore H_2O_2-mediated, endothelium-dependent relaxations in the SMA and aorta from mouse. Other studies have also reported negligible inhibitory effects by catalase on endothelium-dependent relaxations (Beny & von der Weid, 1991; Fulton *et al.*, 1997; Pomposiello *et al.*, 1999; Hamilton *et al.*, 2001; Itoh *et al.*, 2003). Both Beny & von der Weid (1991) and Pomposiello *et al.* (1999) found that catalase did not affect L-NAME- and Indo-resistant relaxations to bradykinin in porcine coronary arteries, indicating that H_2O_2 is not an endogenous vasodilator in this preparation. Subsequent studies by another group proposed that an epoxyeicosatrienoic acid was the mediator of EDHF-type relaxations in these vessels (Fisslthaler *et al.*, 1999), and although the enzyme that synthesizes this substance, CYP-2C, was also reported to generate significant amounts of ROS in response to bradykinin, these ROS do not appear to mediate vasorelaxation (Fleming *et al.*, 2001). Itoh *et al.* (2003) reported that even though SOD enhanced ACh-induced relaxations, neither SOD nor catalase significantly affected ACh-induced hyperpolarizations in rabbit SMA, ruling out the likelihood that H_2O_2 is an EDHF in this vessel preparation. We also found that in the rat SMA, catalase has no effect on L-NAME/Indo-resistant relaxations to ACh (data not shown). On the other hand, Matoba *et al.* (2000) reported that catalase effectively inhibited both ACh-induced relaxations and hyperpolarizations in both wild-type and eNOS-deficient mouse SMA. The reason for the discrepancy between the data from Matoba *et al.* (2000) and those from our laboratory is unclear. Conceivably, differences in the activity of the catalase used in the studies might contribute to the discrepancies or, alternatively, variations in the experimental procedure might suitably explain these differences. The form of catalase used in our studies had a higher activity than the one used by Matoba and others (Sigma catalog number C40 – 19,900 U mg^{-1} solid cf. Sigma catalog number C9322 – 2390 U mg^{-1} solid). Thus, one point to consider is whether the lower activity catalase has contaminants that themselves might have an inhibitory effect on relaxations (Halliwell & Gutteridge, 1999). However, when we performed experiments with this other form of catalase, we found that other than causing excessive foaming when in the myograph chamber, it did not significantly affect ACh-induced relaxations (data not shown). Indirect approaches that were used (e.g. inhibition of synthesis and metabolism of H_2O_2 and supplementation with enzymatic sources of H_2O_2) also failed to confirm the involvement of H_2O_2 as an endothelium-derived dilator in SMA. Although our study does not support the idea of H_2O_2 as an endothelium-derived dilator in the mesenteric vasculature, it may be worthwhile to consider if the contribution of H_2O_2 is greater in vascular beds that are noted for their sensitivity to changes in oxygen tension and redox conditions such as the cerebral or pulmonary vasculature (Burke & Wolin, 1987; Yang *et al.*, 1991).

Our main reservation against the suggestion that H_2O_2 is a physiological vasodilator is based on our observation that

high, and likely supraphysiological, concentrations of H_2O_2 were required to induce dilatations, which progressively impaired contractile tone. In contrast, although high concentrations of exogenous NO (10- to 100-fold higher than those generated physiologically) are routinely used in studies to compare with endogenous NO processes, under these conditions the responses to NO (either as NO donors or NO solutions) are reversible and do not usually impair contractile function.

In summary, the data from the current study demonstrate that H_2O_2 relaxes aorta and SMA from mouse possibly through nonspecific alterations to smooth muscle function. We also conclude that H_2O_2 is unlikely to mediate endothelium-dependent relaxations in these vessels.

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