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Depletion of intracellular Ca^{2+} stores enhances flow-induced vascular dilatation in rat small mesenteric artery

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1 The effect of depleting intracellular Ca^{2+} stores on flow-induced vascular dilatation and the mechanism responsible for the vasodilatation were examined in rat isolated small mesenteric arteries.

2 The arteries were pressurized to 50 mmHg and preconstricted with phenylephrine. Intraluminal flow reversed the effect of phenylephrine, resulting in vasodilatation. Flow dilatation consisted of an initial transient peak followed by a sustained plateau phase. The magnitude of dilatation was markedly reduced by removing Ca^{2+} from the intraluminal flow medium.

3 Depletion of intracellular Ca^{2+} stores with either cyclopiazonic acid (CPA, 2 μM) or 1,4-dihydroxy-2,5-di-tert-butylbenzene (BHQ, 10 μM) significantly augmented the magnitude of flow dilatation. Flow-induced endothelial cell Ca^{2+} influx was also markedly enhanced in arteries pretreated with CPA or BHQ.

4 Flow-induced dilatation was insensitive to N^{w}-nitro-L-arginine methyl ester (100 μM) plus indomethacin (3 μM) or to oxyhemoglobin (3 μM), but was markedly reduced by 30 mM extracellular K^+ or 2 mM tetrabutylammonium (TBA), suggesting an involvement of EDHF.

5 Catalase at 1200 U ml^{-1} abolished the flow-induced dilatation, while the application of exogenous H_2O_2 (90–220 μM) induced relaxation in phenylephrine-preconstricted arteries. Relaxation to exogenous H_2O_2 was blocked in the presence of 30 mM extracellular K^+, and H_2O_2 (90 μM) hyperpolarized the smooth muscle cells, indicating that H_2O_2 can act as an EDHF.

6 In conclusion, flow-induced dilatation in rat mesenteric arteries can be markedly enhanced by prior depletion of intracellular Ca^{2+} stores. Furthermore, these data are consistent with a role for H_2O_2 as the vasodilator involved.

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Abbreviations: EDHF, endothelium-derived hyperpolarizing factors; H_2O_2, hydrogen peroxide

Introduction

Hemodynamic shear stress generated by blood flow exerts an important control on vascular tone in resistance arteries. Shear stress acts on the endothelium to stimulate the release of vasodilators such as nitric oxide (NO), prostacyclin (PGI_2) and endothelium-derived hyperpolarizing factor (EDHF), causing endothelium-dependent vascular relaxation (Busse & Fleming, 2003).

There is considerable controversy as to whether flow dilatation is Ca^{2+}-dependent. Some studies report flow dilatation independent of a $[Ca^{2+}]_i$ rise in endothelial cells (Muller *et al*., 1999; Marchenko & Sage, 2000; Ungvari *et al*., 2001), where the underlying mechanism might be related to flow-induced eNOS activation *via* Akt or PKA pathways (Busse & Fleming, 2003). Other studies, however, show Ca^{2+}-dependent flow-induced dilatation. For example, Cooke *et al*. (1991) reported that flow-induced dilatation of rabbit iliac arteries required Ca^{2+} influx. Falcone *et al*. (1993) also demonstrated a close association between flow-induced endothelial cell $[Ca^{2+}]_i$ rise and the vasodilatation of rat cremaster muscle arterioles. Taken together, it appears that flow may elicit vasodilatation either *via* a $[Ca^{2+}]_i$-dependent mechanism or a $[Ca^{2+}]_i$-independent mechanism. However, at least in some vascular beds, flow-induced endothelial Ca^{2+} influx plays an important role in flow dilatation.

Endothelial cell Ca^{2+} influx can be stimulated either by mechanical shear stress or chemical agonists (Nilius & Droogmans, 2001). Flow-induced shear stress induces Ca^{2+} influx through a number of different mechanisms, including activation of mechanosensitive cation channels (Yao *et al*., 2000), stimulation of $P2X_4$ purinoceptor (Yamamoto *et al*., 2000) or modulation of cytoskeleton-mediated signal transduction pathways (Busse & Fleming, 2003). On the other hand, Ca^{2+}-mobilizing agonists such as ATP, bradykinin and histamine elicit Ca^{2+} influx mainly *via* the store-operated mechanism (Schilling *et al*., 1992; Parekh & Putney, 2005).

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These agonists trigger $InsP_3$ receptor-mediated Ca^{2+} release from intracellular Ca^{2+} stores. The consequent depletion of Ca^{2+} stores then stimulates Ca^{2+} influx across the plasma lemma (Parekh & Putney, 2005). Our previous data demonstrated an interaction between flow-induced Ca^{2+} influx and store-operated Ca^{2+} release. Depletion of intracellular Ca^{2+} stores increased the sensitivity of endothelial cell Ca^{2+} influx to shear stress (Kwan *et al*., 2003). Based on the assumption that an increased endothelial cell Ca^{2+} influx may enhance the release of vasodilators, in the present study we hypothesized that depletion of intracellular Ca^{2+} stores might enhance the flow-induced vasodilatation. If so, this may represent an important physiological control mechanism.

To examine this hypothesis, we used cyclopiazonic acid (CPA) and 1,4-dihydroxy-2,5-di-tert-butylbenzene (BHQ), each of which induce Ca^{2+} store depletion by specifically inhibiting endoplasmic reticulum Ca^{2+}-ATPase (SERCA), and then compared flow-induced dilatation between arteries with or without exposure to these SERCA inhibitors. Our results showed that store depletion enhanced the flow-induced dilatation in rat mesenteric arteries, and also suggested that H_2O_2 was the major vasodilator involved in the flow dilatation.

Methods

Pressure myography

All animal experiments were conducted in accordance with the regulations of the U.S. National Institutes of Health (NIH Publication No. 8523, revised 1996) and U.K. Animals (Scientific Procedures) Act 1986. Sprague–Dawley rats were placed in a chamber and were killed by carbon dioxide. Pressure myography studies were performed as described elsewhere (Liu *et al*., 2004). Briefly, a third- or fourth-order mesenteric artery (about 2–3 mm long) was dissected and transferred to a pressure myograph (Danish Myotechnology) filled with oxygenated Tyrode's solution at 37°C. The external diameter of the artery was recorded continuously with a CCD (video camera module) camera using software MyoView (Photonics Engineering). The artery was cannulated at both ends with glass micropipettes. Phenylephrine (concentration varied to achieve similar constriction in different arteries, 0.1–4 μM) was used to preconstrict the artery to 55–70% of its initial vessel diameter. Flow was initiated by creating the pressure difference of 5 mmHg between inflow and outflow. The mean intraluminal pressure was maintained at 50 mmHg throughout the flow protocol. Shear stress was calculated by the equation $\tau = 4\mu f\pi^{-1}r^{-3}$ (Ando & Kamiya, 1993). The initial shear force was near 10 dynes cm^{-2}. Flow dilatation reduced the shear stress to $\sim$3.5 dynes cm^{-2}. At the end of each experiment, acetylcholine (1 μM) was used to assess the viability of the endothelium. CPA, BHQ, N^{w}-nitro-L-arginine methyl ester (L-NAME), indomethacin, charybdotoxin plus apamin, tetrabutylammonium (TBA), miconazole, $BaCl_2$ and ouabain, glybenclamide, 4-aminopyridine, carbenoxolene, 18α-glycyrrhetinic acid and catalase were added both extra- and intraluminally. Phenylephrine, acetylcholine, dimethyl sulfoxide (DMSO) and H_2O_2 were only added into extraluminal solution. ATP (1 μM) was included in all experiments both extra- and intraluminally for the purpose of producing consistent flow dilatation (Liu *et al*., 2004). Abluminal drug delivery was achieved by adding the drugs to the side of the chamber (10 ml) and gently mixing by pipetting up and down several times. Intraluminal drug administration was achieved *via* intraluminal flow perfusion, followed by a stabilization period of 30 min. Denudation of endothelial cells was achieved by inserting a nylon suture into the vessel lumen, which was then gently rolled back and forth. The success of denudation was confirmed by an absence of relaxation to acetylcholine (1 μM).

Measurement of $[Ca^{2+}]_i$ and store Ca^{2+} content in endothelial cells

An artery was placed in a specially designed flow chamber, which was mounted on an inverted microscope equipped with a $\times$20 Olympus water immersion objective. The artery was pressurized to 50 mmHg and stabilized for 30 min in Tyrode's solution. The endothelial cell layer was then loaded with Ca^{2+}-sensitive fluorescence dyes by pumping 20 μM fluo-4/AM (for monitoring cytosolic Ca^{2+} level) or 10 μM mag-fluo-4/AM (for monitoring store Ca^{2+} content) and 0.02% pluronic F127 in Tyrode's solution into the artery lumen at a pressure $<$10 mmHg and incubated for 1 h in dark at room temperature. After the dye loading, the pressure was re-raised to 50 mmHg. To study the flow effect on cytosolic Ca^{2+}, the artery was treated with 2 μM CPA or 10 μM BHQ for 20 min to deplete intracellular Ca^{2+} stores before the flow. ATP (1 μM) was included in all experiments both extra- and intraluminally. In addition, 30 μM xestospongin C (XeC) was also added before the flow to eliminate possible flow-induced Ca^{2+} release from $InsP_3$-sensitive intracellular Ca^{2+} stores. Fluo-4 fluorescence in endothelial cells, which was visualized as the innermost cell layer of the vessel and aligned longitudinally, was measured before and during the flow with a FV500 Fluoview confocal system (Olympus, Japan). The Ca^{2+} responses to flow were displayed as the ratio of fluorescence relative to the intensity before flow (F1/F0). For store Ca^{2+} monitoring, CPA or BHQ was applied to extraluminal solution and mag-fluo-4 fluorescence in endothelial cells was monitored. In each experiment, fluorescent intensity was measured in 15–20 individual endothelial cells and then the average was obtained. For Ca^{2+}-free experiments, the arteries were kept both extra- and intraluminally in a Ca^{2+}-free Tyrode's solution containing 2 mM EGTA before and during the flow. Fluo-4 fluorescence could be ablated by physical removal of the endothelium. Our dye-loading protocol did not label the smooth muscle cells, because no visible fluorescence could be observed in smooth muscle cells after endothelium denudation.

Membrane potential measurement

Isolated small mesenteric arteries were bathed in Tyrode's solution. A conventional intracellular glass microelectrode filled with 3 M KCl (tip resistance, 40–80 MΩ) was used to impale smooth muscle cells (He *et al*., 1996). Membrane potential was recorded using PICOLOG (Pico Technology) Software. The following criteria were used to assess the validity of a successful impalement: a sudden negative shift in voltage followed by (i) a stable negative voltage for $>$2 min and (ii) an instantaneous return to the previous voltage level on dislodgement of the microelectrode. Each artery was impaled $\geqslant$4 times and the results were then averaged to

obtain membrane potential. After a stable membrane potential was recorded for $\geqslant 2$ min, H_2O_2 (90 μM) was applied.

Chemicals and solutions

Phenylephrine hydrochloride, L-NAME and indomethacin were obtained from RBI (Natick, MA, U.S.A.). Fluo-4/AM, mag-fluo-4/AM and pluronic F127 were from Molecular Probe (Eugene, OR, U.S.A.). BHQ was from Tocris (Ellisville, MI, U.S.A.). ATP, acetylcholine, charybdotoxin, apamin, CPA, DMSO, TBA, miconazole, $BaCl_2$, catalase, ouabain and BSA were purchased from Sigma (St Louis, MO, U.S.A.). Indomethacin, CPA, BHQ and XeC were dissolved in DMSO, and the others were dissolved in H_2O. Heat inactivation of catalase was achieved by a 5-min incubation at 100°C.

Tyrode's solution contained (in mM): 117 NaCl, 1 $MgCl_2$, 4.7 KCl, 1.2 KH_2PO_4, 1.6 $CaCl_2$, 10 HEPES, 30 D-mannitol, 11 D-glucose and 1% BSA, pH 7.4. Ca^{2+}-free Tyrode's solution contained (in mM): 117 NaCl, 1 $MgCl_2$, 4.7 KCl, 1.2 KH_2PO_4, 10 HEPES, 30 D-mannitol, 11 D-glucose, 2 EGTA and 1% BSA, pH 7.4. K^+ Tyrode's solution (30 mM) contained in mM: 92.9 NaCl, 1 $MgCl_2$, 28.8 KCl, 1.2 KH_2PO_4, 1.6 $CaCl_2$, 10 HEPES, 30 D-mannitol and 11 D-glucose, pH 7.4.

Data analysis

Vasodilatation to flow was calculated as the percentage of phenylephrine-induced constriction by the following equation:

$$\%\ \text{vasodilatation} = 100\times(D_f - D_{phe}/D_i - D_{phe})$$

where D represents the external diameter of vessels; D_f is the vessel diameter after flow; D_{phe} is the diameter after phenylephrine constriction and before flow; D_i is the initial diameter without any treatment. In all experiments, n equals the number of mesenteric arteries taken from different rats.

Results

Effect of extracellular Ca^{2+} on flow-induced vascular dilatation

Small mesenteric arteries pressurized to 50 mmHg had an external diameter of 300–450 μm ($n=104$). Each vessel was first preconstricted to a similar level (55–70% of its initial diameter) by application of phenylephrine. In some vessels, phenylephrine application evoked one or two oscillations before artery diameter stabilized. Preliminary experiments indicated that stable contraction to phenylephrine was achieved within 1–2 min and lasted for more than 30 min ($n=6$). After stabilization, intraluminal flow was applied to evoke dilatation. Flow-induced dilatation consisted of an initial transient peak followed by a sustained plateau phase (>10 min) (Figure 1a). The magnitude of peak flow dilatation was calculated to be $82\pm8\%$ ($n=19$) (Figure 1b), whereas that of sustained dilatation was $37\pm4\%$ ($n=19$). The vessels rapidly contracted again as soon as the flow was stopped (Figure 1a). The diameter of vessels after stopping the flow was $65.6\pm1.5\%$ ($n=14$) of the initial diameter (before phenylephrine preconstriction), and was comparable to that before the initiation of flow ($66.7\pm1.3\%$ of the initial diameter, $n=14$). Flow-induced dilatation was entirely endothelium-dependent, as it was abolished by removal of the endothelium (Figure 1b).

In order to test the role of Ca^{2+} influx in flow-induced dilatation, intraluminal flow was carried out using a Ca^{2+}-free medium. The results showed that the removal of Ca^{2+} from the intraluminal flow medium reduced both the peak and sustained flow-induced dilatations by $50\pm8\%$ ($n=7$) and $51\pm10\%$ ($n=7$), respectively (Figure 2a and b). Note that in our experimental setup, although the intraluminal flow media could be Ca^{2+}-free, extraluminal solution was normal Tyrode's solution with 1.6 mM Ca^{2+} to enable phenylephrine-induced preconstriction. Therefore, as the mesenteric artery wall was only $\sim$30–35 μm thick, which would enable Ca^{2+} diffusion across the wall, and because of a large extra- compared to intraluminal volume (10 ml vs $\sim$0.2 μl), the solution surrounding the endothelial cells was not likely to be completely Ca^{2+}-free even though 2 mM EGTA was present intraluminally to chelate Ca^{2+}.

We also tested whether flow could enhance endothelial cell Ca^{2+} influx in intact small mesenteric arteries. Figure 2c–e shows that the flow evoked a rise in endothelial cell $[Ca^{2+}]_i$. This $[Ca^{2+}]_i$ rise was greatly reduced if intraluminal solution is Ca^{2+}-free and was abolished if both intra- and extraluminal solutions were Ca^{2+}-free (Figure 2c–e).

Effect of depleting intracellular Ca^{2+} stores on flow-induced dilatation

The magnitude of flow-induced vasodilatation with or without either CPA or BHQ was measured in each case and in the same artery. The results indicated that store depletion with either CPA (2 μM) or BHQ (10 μM) for 15–20 min markedly increased the magnitude of subsequent flow-induced dilatation (Figure 3a and b). Vehicle (1.4 μM DMSO) alone had no effect (Figure 3c). Figure 3d summarizes these data, and shows that, in the presence of CPA and BHQ, both peak and sustained flow dilatations were significantly increased compared to the controls. The initial peak dilatation is a full dilatation. In some arteries, this full dilatation was maintained (Figure 3a and b); therefore, initial phase could not be distinguished from sustained phase. However, for the purpose of data comparison

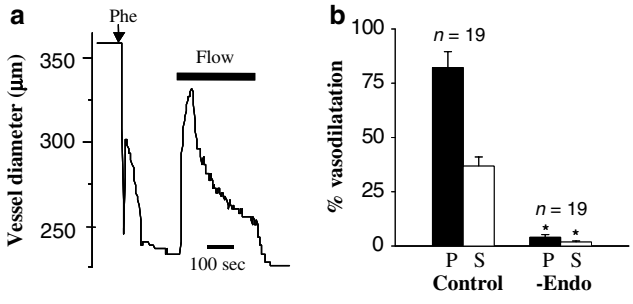

Figure 1 Flow-induced dilatory responses in rat small mesenteric arteries. (a) A representative trace showing dilatory response to flow challenge. The vessel was preconstricted with phenylephrine. Solid bar on the top of the trace indicates the time period when intraluminal flow (Tyrode's solution with 1% BSA) was applied. (b) Comparison of the peak (P) and sustained (S) flow dilatations between normal and endothelium-denuded vessels. Values are means$\pm$s.e.m. ($n=19$). *$P<0.05$ compared to control.

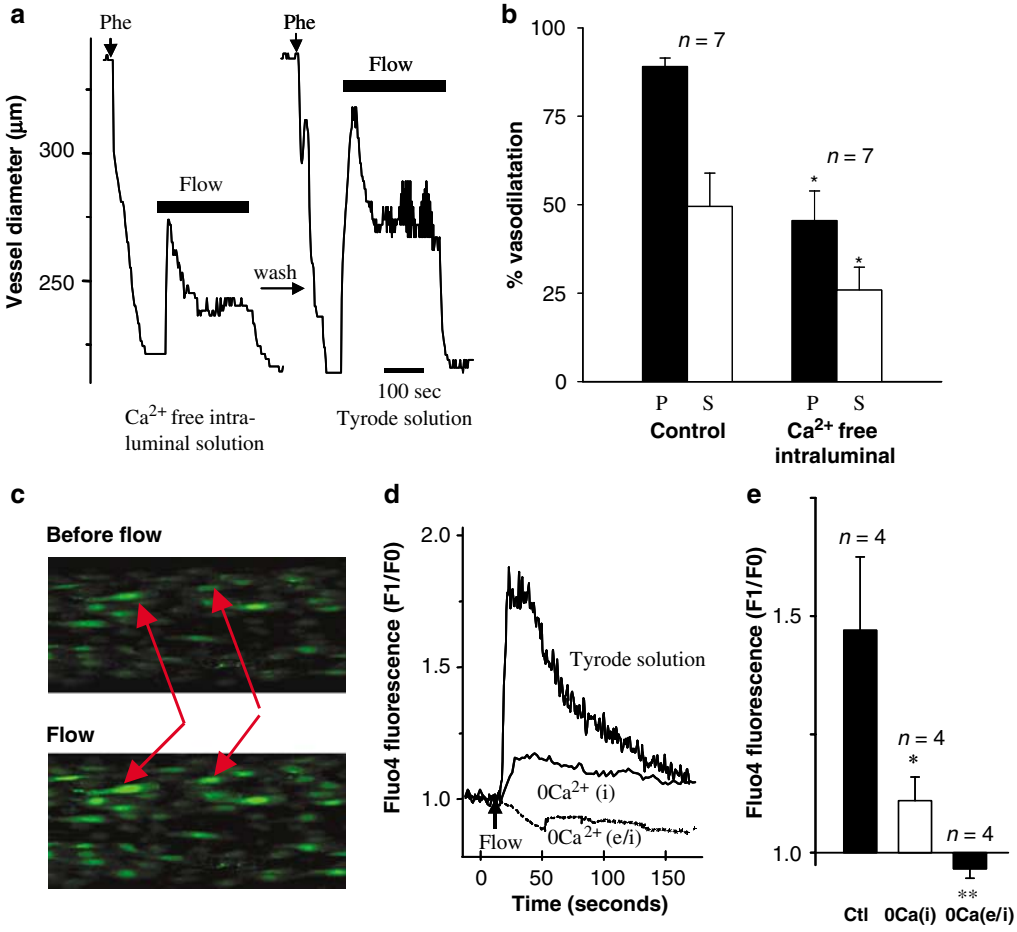

Figure 2 Effect of extracellular Ca^{2+} on flow-induced vasodilatation and flow-induced endothelial cell $[Ca^{2+}]_i$ rise in isolated small mesenteric arteries. (a) Representative traces of flow dilatation. The first flow dilatation was initiated by a Ca^{2+}-free intraluminal Tyrode's solution. The vessel was then washed by and maintained in normal Tyrode's solution for 1 h, followed by the second intraluminal flow experiment with the normal Ca^{2+}-containing Tyrode's solution. (b) Summary showing the effect of intraluminal Ca^{2+} removal on the peak (P) and sustained (S) flow dilatations. (c) Representative endothelial cell fluo-4 images of an artery in Tyrode's solution before and during flow. Each pair of arrows point to one particular cell before and during flow. (d) Representative traces of flow-induced endothelial $[Ca^{2+}]_i$ rise in the presence (control) or absence of Ca^{2+} intraluminally ($0Ca^{2+}$(i)) or absence of Ca^{2+} both intra- and extraluminally ($0Ca^{2+}$(e/i)). Each trace represents the average fluorescence of 10–15 cells. The arrow indicates the time point when an intraluminal flow was initiated. (e) Summary of the peak $[Ca^{2+}]_i$ rise as in (d). Values are means ± s.e.m. ($n = 4$–7). *$P < 0.05$ compared to respective controls; **$P < 0.05$ compared to $0Ca^{2+}$(i).

in Figure 3d, we still arbitrarily separate this dilatation into initial and sustained phases, with both counted as full dilatation. In preliminary experiments, we also tested another SERCA inhibitor thapsigargin. However, thapsigargin was unsuitable as it evoked repetitive oscillations in contraction, and it was not possible to obtain stable responses.

It is known that SERCA inhibitors may modulate vascular tone, which could indirectly influence flow responses. At low concentration (0.3–1 μM CPA or <10 μM BHQ), these inhibitors may cause vascular relaxation due to the release of endothelial cell vasodilators (Fusi *et al*., 1999; Okon *et al*., 2002), while at high concentrations (>10 μM CPA or >10 μM BHQ), they induce contraction due to an enhanced store-operated Ca^{2+} influx in vascular smooth muscle (Fusi *et al*., 1998; Ng & Gurney, 2001) and/or increased release of vasocontractile prostaglandins from the endothelium (Okon *et al*., 2002). In the present study, we used an intermediate concentration of 2 μM CPA or 10 μM BHQ, neither of which caused significant change in stable vascular tone (Figure 3a and b). The treatment did cause short-term rhythmic changes in vessel diameter (Figure 3a and b), which were absent in endothelium denuded arteries ($n = 6$), indicating endothelium-dependency. Rhythmic contractions of this form are likely due to SERCA inhibitor-induced endothelial cell Ca^{2+} oscillations (Li *et al*., 1995), which lead to rhythmic changes in the release of endothelial vasodilators.

Effect of Ca^{2+} store depletion on flow-induced Ca^{2+} influx

As expected, treatment of the arteries with CPA (2 μM) or BHQ (10 μM) caused a decrease in store Ca^{2+} content in endothelial cells of isolated small mesenteric arteries (Figure 4a and b), as determined by changes in mag-fluo-4 fluorescence (Shmigol *et al*., 2001). Consistent with other reports (Morgan & Jacob, 1998; Kwan *et al*., 2003), CPA/BHQ treatment also

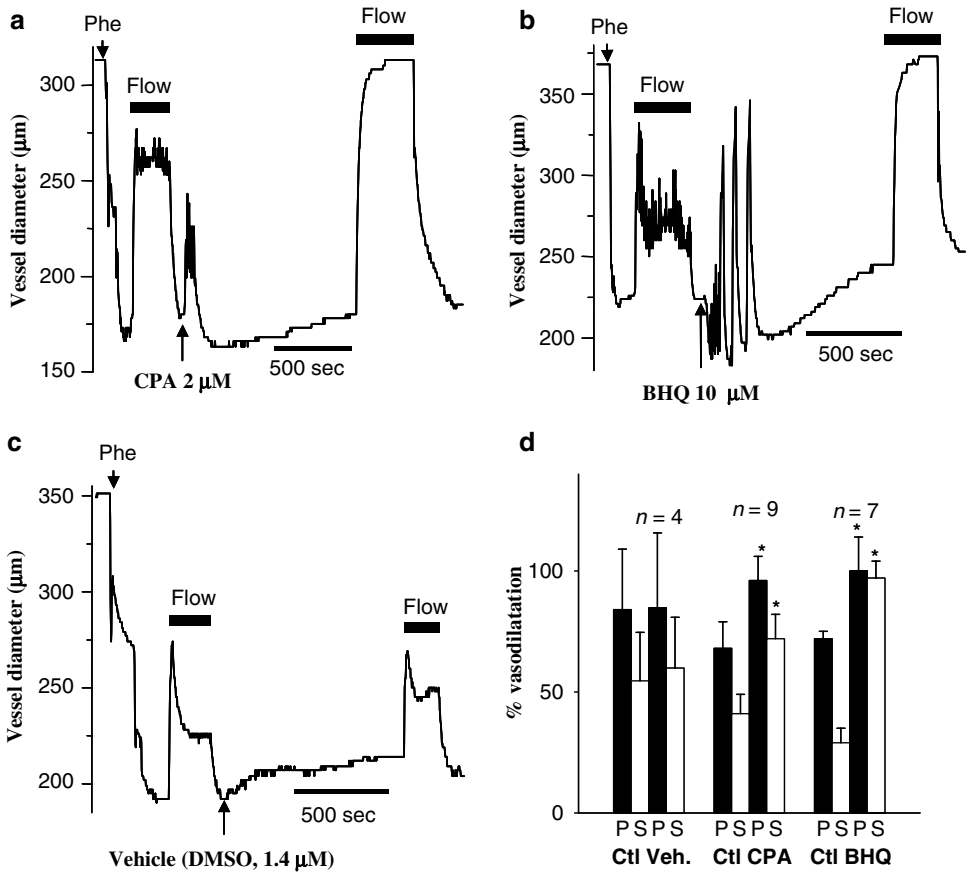

Figure 3 Effect of SERCA inhibitors on flow vasodilatation. (a–c) Representative traces showing the effect of CPA (2 μM) (a) or BHQ (10 μM) (b) or vehicle (DMSO, 1.4 μM) (c) on flow dilatation. (d) Summary of responses to CPA, BHQ and vehicle (DMSO). The control values (Ctl) for peak (P) and sustained (S) flow dilatations were obtained from the first flow dilatation in the same vessels before respective treatments. Values are means $\pm$ s.e.m. ($n = 4$–9). *$P < 0.05$ compared to control.

induced a transient rise in endothelial cell $[Ca^{2+}]_i$, which returned to basal levels within 10–15 min (data not shown). Figure 4c and d show that flow evoked a rise in endothelial cell $[Ca^{2+}]_i$ and that the magnitude of $[Ca^{2+}]_i$ rise was much larger in arteries that were pretreated with CPA or BHQ for 20 min, compared to control (vehicle-treated) arteries. The rise in $[Ca^{2+}]_i$ was apparently due to Ca^{2+} influx, because it was absent if intra- and extraluminal solutions were Ca^{2+}-free (Figure 2d and e). Note that in this series of experiments (Figure 4c and d), an $InsP_3$ receptor inhibitor XeC (30 μM) was added through the intraluminal solution before flow, in order to prevent possible residual flow-induced Ca^{2+} release from $InsP_3$-sensitive Ca^{2+} stores.

H_2O_2 may be the vasodilator involved in flow-induced dilatation

Flow-induced relaxation was not significantly affected by L-NAME (100 μM) plus indomethacin (3 μM) or by oxyhemoglobin (3 μM) (Figure 5a), indicating a lack of involvement by NO and PGI_2. However, the relaxation to flow was blocked by 30 mM K^+ (Figure 5b and d) and the peak flow dilatation was greatly reduced by a nonselective K^+ channel blocker TBA (2 mM) (Figure 5c and d). These data suggested an important role for EDHF and K^+ channels in flow dilatation. However, note that TBA only affected the peak but not the sustained flow dilatations, suggesting that two components may occur *via* distinct mechanisms.

Pharmacological inhibitors were then used in an attempt to identify the specific EDHF involved. The results showed that the flow dilatation was insensitive to miconazole (3 μM) (peak and sustained dilatations of 76 $\pm$ 7 and 37 $\pm$ 12% vs the control values of 81 $\pm$ 7 and 40 $\pm$ 10% ($n = 6$)), charybdotoxin (100 nM) plus apamin (100 nM) (peak and sustained dilatations of 66 $\pm$ 17 and 39 $\pm$ 10% vs the control values of 68 $\pm$ 14 and 23 $\pm$ 6% ($n = 5$)), Ba^{2+} (30 μM) plus ouabain (100 μM) (peak and sustained dilatations of 75 $\pm$ 10 and 34 $\pm$ 10% vs the control values of 67 $\pm$ 8 and 31 $\pm$ 8% ($n = 5$)), glybenclamide (100 nM) (peak and sustained dilatations of 80 $\pm$ 7 and 58 $\pm$ 11% vs the control values of 88 $\pm$ 6 and 57 $\pm$ 11% ($n = 6$)), 4-aminopyridine (5 mM) (peak and sustained dilatations of 84 $\pm$ 4 and 66 $\pm$ 5% vs the control values of 72 $\pm$ 7 and 54 $\pm$ 10% ($n = 5$)), carbenoxolene (100 μM) (peak and sustained dilatations of 88 $\pm$ 7 and 62 $\pm$ 9% vs the control values of 79 $\pm$ 9 and 55 $\pm$ 14% ($n = 5$)) or 18α-glycyrrhetinic acid (100 μM) (peak and sustained dilatations of 62 $\pm$ 8 and 47 $\pm$ 15% vs the control values of 76 $\pm$ 7 and 41 $\pm$ 15% ($n = 4$)), suggesting that metabolites of cytochrome P_{450}, K_{Ca} channels, K_{ATP} channels, K_v channels, K_{ir} channels, Na^+–K^+–ATPase, or gap junctions do not play an important role. On the other hand, flow-

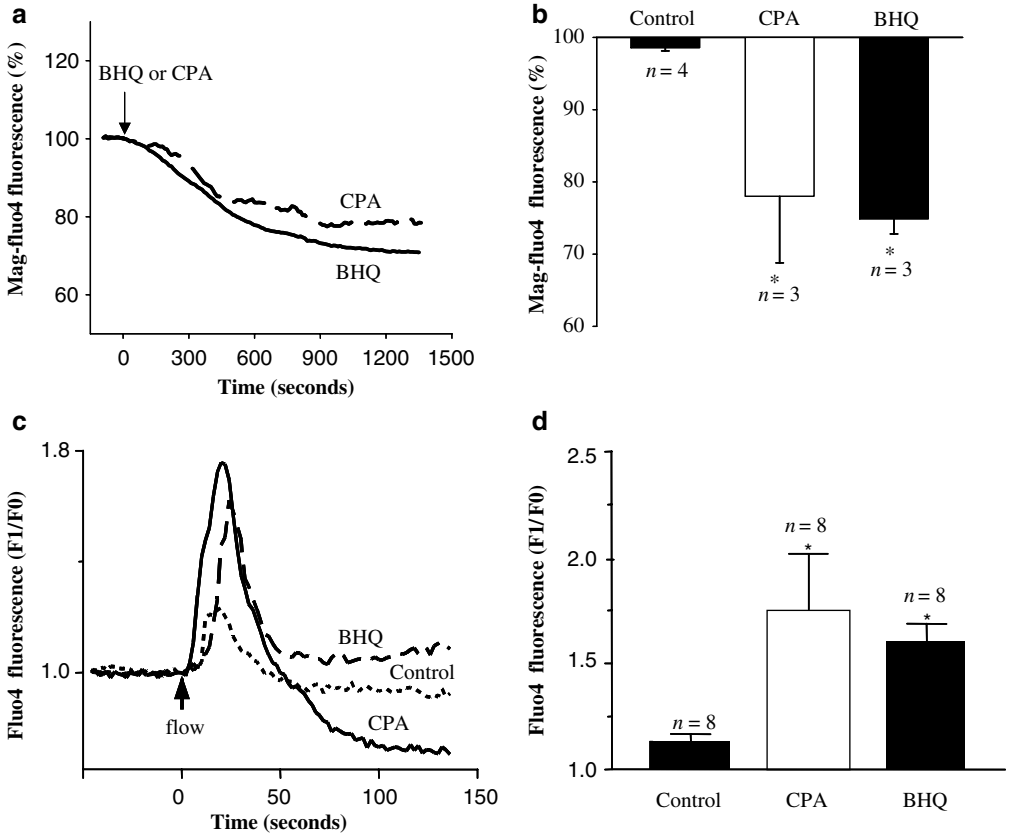

Figure 4 Effect of SERCA inhibitors on store Ca^{2+} content and flow-induced Ca^{2+} influx. (a) Representative traces showing the changes in mag-fluo-4 fluorescence in endothelial cells in response to CPA (2 μM, long dash) or BHQ (10 μM, solid line). (b) Summary of the changes in mag-fluo-4 fluorescence as in (a). (c) Representative traces showing the effect of CPA (2 μM, solid line) or BHQ (10 μM, long dash) treatment on flow-induced endothelial $[Ca^{2+}]_i$ rise. XeC (30 μM) was included in intraluminal solutions before flow. (d) Summary of the peak $[Ca^{2+}]_i$ rise as in (c). Each trace represents the average fluorescence of 10–15 cells. The arrows indicate the time point when an intraluminal flow (c) or a chemical (a) was applied. Control had no CPA or BHQ treatment. Values are means$\pm$s.e.m. ($n=3$–8). *$P<0.05$ compared to control.

induced dilatation was markedly reduced by catalase, an enzyme that catalyzes the conversion of H_2O_2 to water and oxygen. In the presence of 600 U ml^{-1} catalase, the peak and sustained flow dilatations were reduced to $29\pm11\%$ ($n=6$) and $17\pm7\%$ ($n=6$), respectively, from the control values of $81\pm4\%$ ($n=6$) and $39\pm6\%$ ($n=6$) (Figure 6a and b). Catalase at a concentration of 1200 U ml^{-1} further reduced the peak and sustained flow dilatations to $6\pm4\%$ ($n=6$) and $4\pm3\%$, respectively ($n=6$) (Figure 6a and b). The effect of catalase was reversed after washout of the enzyme (Figure 6a). In the presence of a catalase inhibitor, aminotriazole (AT) (50 mM) (Figure 6b), or after heat inactivation (data not shown), catalase had no influence on flow-induced dilatation. These results suggest that the flow-induced dilatation was mediated by H_2O_2.

Application of exogenous H_2O_2 completely reversed phenylephrine-induced arterial constriction ($\sim$100% relaxation) in both intact ($n=5$) (data not shown) and endothelium-denuded arteries ($n=5$) (Figure 6c). In the presence of 30 mM K^+, to prevent membrane hyperpolarization, H_2O_2 (90 μM and up to 440 μM) failed to dilate the arteries (Figure 6d). Instead, H_2O_2 caused further contraction by $12\pm1\%$ ($n=5$), possibly reflecting a direct contractile action on the smooth muscle cells independent of a role as EDHF (Figure 6d). The membrane potential of vascular smooth muscle cells in the endothelium-denuded arteries was -60 ± 3 mV ($n=4$). H_2O_2 (90 μM) hyperpolarized the membrane potential by -16 ± 1 mV ($n=4$) (Figure 6e).

Discussion

In the present study, we show that flow-induced dilatation in small mesenteric arteries was largely dependent upon endothelial cell Ca^{2+} influx. The vasodilatation was accompanied by a parallel rise in endothelial cell Ca^{2+} influx, and furthermore removal of Ca^{2+} from the intraluminal solution greatly reduced the magnitude of flow-induced dilatation. The dilatation was endothelium-dependent and mediated by EDHF, but not NO or PGI_2. Furthermore, our data show for the first time that depletion of intracellular Ca^{2+} stores markedly augments the magnitude of flow-induced dilatation. This finding potentially has profound physiological implications. Vascular endothelial cells are exposed to circulating blood that contains numerous Ca^{2+}-mobilizing agents, including metabolites, local paracrine agents, growth factors and

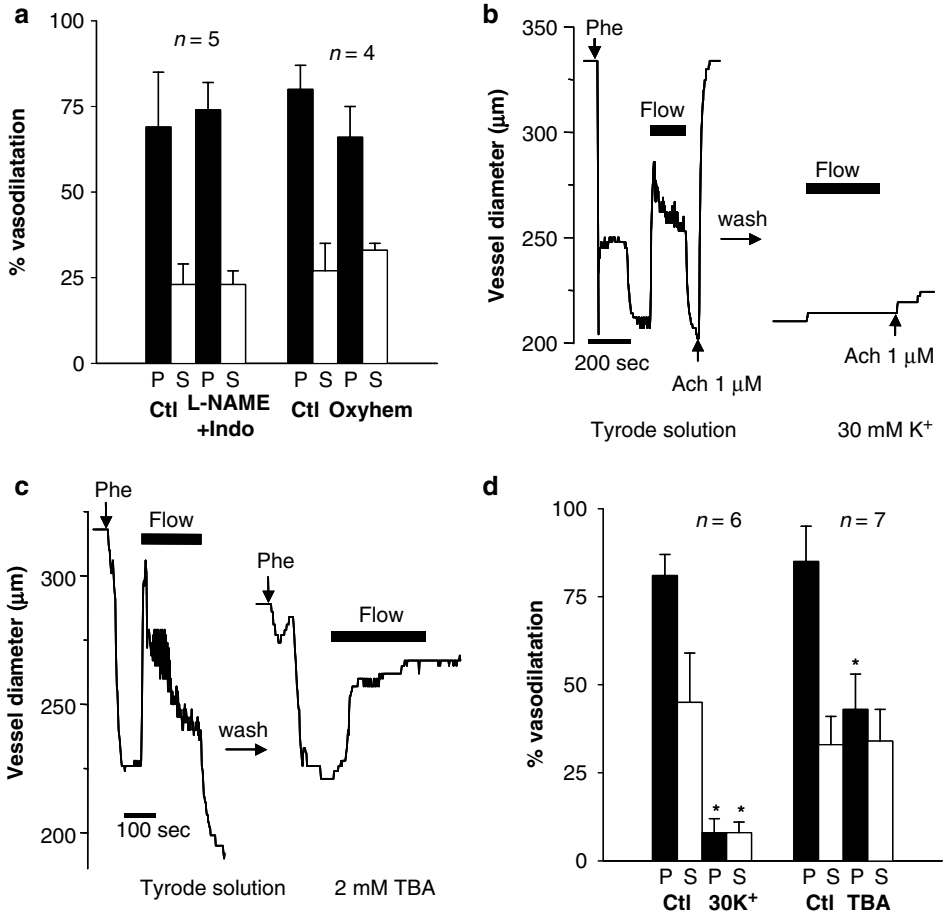

Figure 5 Effect of L-NAME plus indomethacin, oxyhemoglobin, 30 mM K^+, or TBA on flow-induced vasodilatation. (a and d) Summary of the peak (P) and sustained (S) flow dilatations in the absence (control, Ctl) and presence of L-NAME (100 μM) plus indomethacin (3 μM) (a) or oxyhemoglobin (3 μM) (a) or 30 mM K^+ (d) or TBA (2 mM) (d). Data for treatments and their respective controls were generated from the same arteries. (b and c) Representative traces showing the effect of 30 mM K^+ (b) or 2 mM TBA (c) on the flow dilatation. Values are means $\pm$ s.e.m. ($n = 4$–7). *$P < 0.05$ compared to control.

cytokines. Our data suggest that these endogenous substances, by stimulating Ca^{2+} store depletion in the endothelium, may significantly potentiate EDHF-mediated flow dilatation in resistance arteries. Our hypothesis provides a mechanistic explanation for several previous reports from us and others, in which the Ca^{2+}-mobilizing agonists ATP, endothelin-1 and angiotension II each potentiate flow-induced vasodilatation (Henrion *et al*., 1999; Matrougui *et al*., 1999; Liu *et al*., 2004). A likely explanation is that store depletion may enhance flow-induced dilatation through enhanced Ca^{2+} influx into endothelial cells.

In our flow experiments, the arteries were preconstricted with phenylephrine and continuously bathed in 1 μM ATP. Therefore, one concern is whether these agonists may affect flow-induced dilatation. Phenylephrine and ATP can cause vascular contraction in part by inducing intracellular Ca^{2+} release and facilitating Ca^{2+} influx into smooth muscle cells (Nilsson *et al*., 1998; Parekh & Putney, 2005). However, it is not likely that this effect of phenylephrine or ATP on the smooth muscle will influence flow-induced dilatation, because the flow acts on endothelial cells, not on smooth muscle cells. Further, in all experiments artery diameter was titrated to a similar level before the start of flow and therefore would be unlikely to affect our comparison of the subsequent flow-induced dilatation. In addition to an action on the smooth muscle cells, ATP may also induce intracellular Ca^{2+} release in endothelial cells, thereby enhancing the flow dilatation. But 1 μM ATP was included in all vessels, including controls, and those exposed to CPA/BHQ treatment. Thus, any effect should be comparable in control and treated vessels. Finally, we have previously shown that phenylephrine up to 10 μM does not stimulate intracellular Ca^{2+} release in endothelial cells of rat small mesenteric arteries (Dora *et al*., 2000).

The term EDHF describes a diverse array of chemicals with different structures such as epoxyeicosatrienoic acids generated from cytochrome P_{450}, K^+ ions and H_2O_2 (Busse *et al*., 2002; Busse & Fleming, 2003). Among these, cytochrome P_{450} metabolites and activation of K_{Ca} channels have been shown to be important in flow-induced dilatation in human coronary arterioles (Miura *et al*., 2001; 2003), rat mesenteric arteries (Takamura *et al*., 1999) and mouse skeletal muscle arterioles (Huang *et al*., 2001). More recently, it was suggested that H_2O_2 may act as an EDHF and contribute to the flow-induced dilatation in human coronary arterioles (Liu *et al*., 2003;

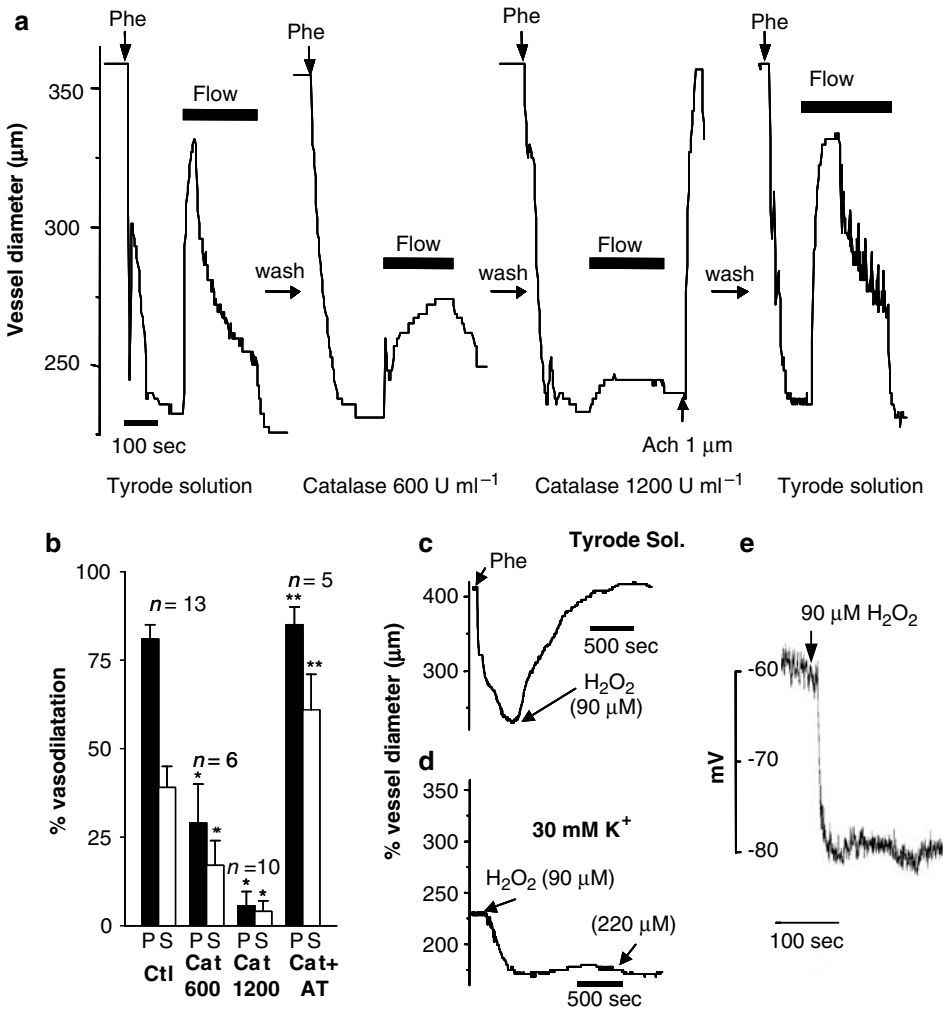

Figure 6 Role of H_2O_2 in flow-induced vasodilatation. (a) Representative traces showing the effect of catalase (600 and 1200 U ml^{-1}) on flow dilatation. The vessel was subjected to multiple intraluminal flow challenges, first with the normal Tyrode's solution, then in the presence of 600 and 1200 U ml^{-1} catalase both intra- and extraluminally, and at last after washout of catalase. Between two consecutive flow challenges, vessel was washed by and maintained in Tyrode's solution for 1 h. (b) Comparison of the peak (P) and sustained (S) flow dilatations as in (a). Also shown were the flow dilatations in the presence of 1200 U ml^{-1} catalase, plus 50 mM AT. Values are means ± s.e.m. ($n = 6$–13). *$P < 0.05$ compared to control. **$P < 0.05$ compared to 1200 U ml^{-1} catalase. (c–e) Representative traces showing relaxant (c, artery bathed in Tyrode's solution; d, in the presence of 30 mM K^+) and hyperpolarizing responses (e) to exogenous H_2O_2 in endothelium-denuded small mesenteric arteries.

Miura *et al*., 2003). In the present study, we found that flow-induced dilatation in rat small mesenteric arteries was not modified by inhibitors of K_{Ca}, K_v, K_{ATP}, K_{ir}, Na^+-K^+-ATPase, gap junctions and cytochrome P_{450}. In contrast, the flow-induced dilatation was abolished in the presence of catalase. Furthermore, exogenous H_2O_2 was able to hyperpolarize the smooth muscle cells and induce vascular relaxation. These data strongly suggest that H_2O_2 can act as an EDHF under these conditions, released in response to flow in rat small mesenteric arteries. H_2O_2 is produced from superoxide anion (O_2^-) and potential sources of endothelial O_2^- generation include mitochondria, xanthine oxidase, uncoupled eNOS, cytochrome P_{450} enzymes, NADPH oxidases, phospholipase A_2, cyclooxygenase and lipoxygenases (Li & Shah, 2004). The activity of many of these enzymes/pathways is Ca^{2+}-sensitive (Gordeeva *et al*., 2003). It is likely therefore that flow-induced rise in endothelial cell $[Ca^{2+}]_i$ may stimulate the activity of such enzymes, leading to the production of H_2O_2 and subsequent vascular dilatation.

We and others have previously shown that vasoactive agonists such as acetylcholine and ATP can stimulate a rise in endothelial cell $[Ca^{2+}]_i$ and elicit vasodilatation in the same mesenteric artery preparation (Dora *et al*., 2001; Malmsjo *et al*., 2002). In this case, the agonist-induced vasodilatation reflects the activation of K_{Ca} channels (Dora *et al*., 2001; Malmsjo *et al*., 2002). H_2O_2 is apparently not involved, because the dilatation was completely abolished by the combined presence of L-NAME, indomethacin and charybdotoxin plus apamin (Dora *et al*., 2001; Malmsjo *et al*., 2002). Taken together with the present data, these observations suggest that flow and agonists both initiate a similar rise in endothelial cell $[Ca^{2+}]_i$ level, but that different mechanisms then lead to dilatation. It appears that a rise in endothelial cell $[Ca^{2+}]_i$ level *per se* may not be sufficient for H_2O_2 production;

other flow-induced signal(s) may also be needed to enable H_2O_2 production.

The differences in participating vasodilators could also be one of the reasons of why, in the present study, store depletion was found to enhance flow-induced dilatation in the rat small mesenteric arteries, whereas other reports in rat and rabbit aorta (Hutcheson & Griffith, 1997; Huang *et al*., 2000) and our results in rat small mesenteric arteries (data not shown) both indicated that store depletion decreased agonist-induced dilatation. We have shown here that flow dilatation of rat small mesenteric arteries is mediated by H_2O_2. On the other hand, the agonist-induced dilatations in rat and rabbit aortas as well as in rat mesenteric arteries are mediated by NO (Hutcheson & Griffith, 1997; Huang *et al*., 2000) or K_{Ca} channels (Dora *et al*., 2001; Malmsjo *et al*., 2002). As the underlying mechanism of flow- and agonist-induced vasodilatation is very different, it is not surprising that store depletion has different effects on these two routes to vasodilatation. Note also that it was previously reported that store depletion could reduce the flow dilatation in rabbit aorta (Hutcheson & Griffith, 1997), but again the dilatation was mediated by a different vasodilator, NO.

In conclusion, flow-induced vascular dilatation is greatly influenced by the status of the intracellular Ca^{2+} stores. Depletion of intracellular Ca^{2+} stores in the endothelium dramatically potentiates flow-induced vasodilatation in rat small mesenteric arteries. The dilatation appeared to be predominately mediated by the generation of H_2O_2.

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