Flow Dilation in Rat Small Mesenteric Arteries is Mediated by Hydrogen Peroxide Generated from CYP Epoxygenases and Xanthine Oxidase

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Abstract: We have previously demonstrated that hydrogen peroxide (H_2O_2) mediates flow-induced vascular dilation in rat small mesenteric arteries. In the present study, the source of this flow-induced H_2O_2 was explored. The arteries were pressurized to 50 mm Hg and preconstricted with phenylephrine. Intraluminal flow reversed the effect of phenylephrine, resulting in vascular dilation. Cytochrome P450 (CYP) inhibitors N-methylsulfonyl-6-(2-proparglyoxyphenyl) hexanoic acid (MS-PPOH, 10 μM) and miconazole (30 μM) reduced the magnitude of peak flow dilation by ~20%-30%, and reduced the duration of dilatory response by ~70-80%. Nevertheless, sulphaphenazole (10 μM), a selective inhibitor of CYP 2C9, had no effect neither on the peak flow dilation nor the duration of dilatory response. Oxypurinol (100 μM), an inhibitor of xanthine oxidase, attenuated the duration of dilatory response by ~60% but exerted no effect on the magnitude of peak flow dilation. Cyclosporin A (2 μM), an inhibitor for mitochondrial permeability transition pore, MitoQ (300 nM), a mitochondria-targeted antioxidant, and apocynin (1 mM), a NADPH oxidase inhibitor, had no effect neither on the magnitude of peak flow dilation nor the duration of dilatory response. To further confirm the role of CYP in flow-induced H_2O_2 production, a fluorescent probe CM-H_2DCFDA was used to monitor the production of H_2O_2 in the primary endothelial cells isolated from rat small mesenteric arteries. The results showed that flow-induced H_2O_2 production was markedly reduced in MS-PPOH and miconazole pretreated endothelial cells. Taken together, our results suggest that, during flow dilation, H_2O_2 is generated from CYP epoxygenases and xanthine oxidase.

INTRODUCTION

Blood flow through an artery generates hemodynamic shear stress which plays an important role in the control of vascular tone. *In vivo* studies showed that shear stress causes dilation in a variety of vessels from different species including humans [1, 2]. The mechanism of flow-induced vascular dilation has been intensively studied. It is found that flow dilation is endothelium-dependent, although the endothelial factor varies, depending on species, vascular bed, and vessel size [3]. Three principal dilators are released from endothelial cells upon stimulation by shear stress. These include nitric oxide (NO) and/or prostacyclin and/or endothelium-derived hyperpolarizing factor (EDHF) [3].

Rat small mesenteric arteries are one of favorite models for studying the regulation of vascular tone in resistance arteries. However, there is controversy as to which vasodilator is responsible for flow dilation in rat small mesenteric arteries. Published results suggest that either NO [4-6] or EDHF [7, 8] could be the main vasodilator responsible for flow dilation in rat small mesenteric arteries. This discrepancy may be due to the differences in experimental conditions used in different laboratories. Note that different groups used different systems, pressure perfusion system [7, 8] *vs* peristaltic pump [6], to generate intraluminar flow. Furthermore, in some studies, animals under specific conditions, such as pregnant [4] or with chronic L-NAME treatment [5], were used for the experiments. Recently, we reported that flow dilation in rat small mesenteric arteries was mediated by EDHF, but not NO nor prostacyclin [8]. The dilation was completely abolished by catalase, suggesting an involvement of H_2O_2. Furthermore, exogenous application of H_2O_2 induced vascular dilation in these arteries and it also caused hyperpolarization of smooth muscle cells in the same arteries [8]. These results suggest that endothelial H_2O_2, as an EDHF, is the main vasodilator responsible for flow dilation in rat small mesenteric arteries. Note that H_2O_2 has previously been suggested to be the main vasodilator involved in flow dilation in other vascular beds [9, 10].

H_2O_2 is produced from superoxide anion (O_2^-) by superoxide dismutase (SOD). However, endothelial O_2^- could result from activity of multiple enzymes/organelles including NAD(P)H oxidase, cytochrome P450 (CYP), mitochondrial respiration, and xanthine oxidase [11]. Several studies have explored the source of H_2O_2 in flow dilation. Liu *et al.* show that inhibition of mitochondrial respiration reduced the flow-induced H_2O_2 production and abolished the flow dilation in human coronary arterioles from the patients with coronary artery disease, suggesting an involvement of mitochondria-generated H_2O_2 [12]. In contrast, two other studies [13, 14] demonstrated that NAD(P)H oxidase may be essential for shear stress-induced O_2^- generation in human umbilical vein endothelial cells.

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Our previous studies showed that flow dilation in rat small mesenteric arteries is mediated by H_2O_2. In the current study, we investigated the source of H_2O_2 for this flow dilation. Pharmacological inhibitors were employed to differentiate the enzymes/organelles that are responsible for H_2O_2 production during flow dilation in rat small mesenteric arteries. The results demonstrated that, during flow dilation, H_2O_2 is generated from CYP epoxygenases and xanthine oxidase.

MATERIALS AND METHODOLOGY

Pressure Myography

We followed the *Guide for the Care and Use of Laboratory Animals* published by US Institute for Laboratory Animal Research, National Research Council in 1996. Male Sprague-Dawley rats (260-280 g) were placed in a chamber and were killed by carbon dioxide. Pressure myography studies were performed as described elsewhere with slight modification [8, 15]. 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 Krebs-Henseleit solution at 37°C. Krebs-Henseleit solution contained in mM: 119 NaCl, 25 $NaHCO_3$, 1 $MgCl_2$, 4.7 KCl, 2.5 $CaCl_2$, 1.2 KH_2PO_4 and 11 D-glucose. The artery was cannulated at both ends with glass micropipettes that were connected to independent reservoirs set at the same height and solution level to ensure no flow. Both reservoirs were filled with Krebs-Henseleit solution containing 1% BSA. The intraluminal pressure was then set to 50 mmHg by raising both reservoirs at the same time under no-flow conditions, and the artery was equilibrated for 30 min at 37°C. Phenylephrine (concentration varied to achieve similar constriction in different arteries, 0.1-4 μM) was used to preconstrict the artery to 60% - 80% of its initial vessel diameter. Flow was initiated by creating the pressure difference of 5 mm Hg between inflow and outflow flow (5-6 mmHg), by moving the two reservoirs an equal distance but in opposite vertical directions at the same time. The mean intraluminal pressure was maintained at 50 mmHg throughout the flow protocol. Shear stress was calculated by equation $\tau = 4\ \mu f \pi^{-1} r^{-3}$ [16]. The initial shear force was near 10 dynes cm^{-2}. Flow dilation reduced the shear stress to ~3.5 dynes cm^{-2}. At the end of each experiment, acetylcholine (1 μM) was used to assess the viability of the endothelium. ATP (1 μM) was included in all experiments both extra- and intraluminally for the purpose of producing consistent flow dilation [15]. The external diameter of the artery was recorded continuously with a CCD (video camera module) camera using software MyoView (Photonics Engineering).

After the first dilatory response to flow, which acted as the control, the same arteries were washed then treated with a panel of different pharmacological agents both extra- and intraluminally for 30 min, followed by a second flow dilation. Dilatory response to the second flow was measured and compared to the control. The pharmacological agents included: MS-PPOH (10 μM), miconazole (30 μM), sulfaphenazole (10 μM), oxypurinol (100 μM), cyclosporin A (2 μM), MitoQ (300 nM), DecylTPP (300 nM), apocynin 1 mM). Solvent (vehicle) controls were also performed, in which the arteries were treated with dimethyl sulfoxide (DMSO, 0.01%) or ethanol (0.01%)

Isolation of Primary Microvascular Endothelial Cells (MVECs) from Rat Mesentery

Primary cultured microvascular endothelial cells were isolated from male Sprague-Dawley rat mesentery as described elsewhere [17]. Briefly, vessels of mesenteric bed were dissected and digested with 0.02% collagenase in phenol red-fee EBM basal medium for 45 min at 37°C. Dispersed cells were pelleted and then cultured in EGM medium supplemented with 1% bovine brain extract and 1% penicillin-streptomycin. The identity of endothelial cells was verified by immunostaining with an antibody against von Willebrand Factor.

Detection of H_2O_2 Production in Endothelial Cells

CM-H_2DCFDA, a fluorescent dye sensitive to H_2O_2 but not to O_2^- [18], was used to evaluate the production of H_2O_2 in MVECs upon shear stress. Briefly, the cells were grown on cover slips (Menzel-Glaser, Germany) to reach 70-80% confluence, and were loaded with 5 μM CM-H_2DCFDA for 40 minutes in dark at room temperature. Loaded cells were washed two times with normal physiological saline solution (NPSS) and incubated for additional 30 minutes with or without MS-PPOH (10 μM) or miconazole (30 μM) in NPSS containing 1% BSA and 1 μM ATP. Flow was initiated by pumping normal physiological saline solution (NPSS) containing 1% BSA and 1 μM ATP to a specially-designed parallel plate flow chamber, in which the cells were adhered to the bottom [19]. The flow shear stress was ~20 dyne/cm^2. NPSS contained in mM: 140 NaCl, 5 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 glucose, 5 Hepes, pH 7.4. Fluorescence signals before and 1 hour after flow challenge was recorded by an Olympus FV1000 Fluoview confocal laser scanning system. An excitation wavelength of 488 nm was provided by Multiline-Argon laser and the fluorescence signal was collected using a 515 nm-long pass emission filter. Changes of CM-H_2DCFDA fluorescence in response to flow were displayed as a ratio of the fluorescence after flow relative to the fluorescence before flow (F1/F0).

Chemicals

Phenylephrine hydrochloride was obtained from RBI (Natick, USA). ATP, acetylcholine, DMSO, BSA, miconazole, oxypuinol, sulphaphenazole were purchased from Sigma-Aldrich. Apocynin and cyclosporin A were from Calbiochem. CM-H_2DCFDA [5-(and-6)-chloromethyl-2'7'-dichlorodihydrofluorescein diacetate acetyl ester] was from Molecular Probes. Miconazole was dissolved in DMSO, MS-PPOH was dissolved in either ethanol or DMSO, MitoQ and Decyl TPP Bromide were dissolved in ethanol, oxypurinol was dissolved in 1N sodium hydroxide, and the others were dissolved in water. EBM basal medium and EGM complete medium were purchased from Lonza (Switzerland). Penicillin-streptomycin was obtained from Gibco-BRL.

Data Analysis

The peak magnitude of flow dilation was calculated as the percentage of phenylephrine-induced constriction by the following equation:

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

where D represents the external diameter of vessels; D_f is the maximum 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 most cases, flow dilation is transient with a rapid rise phase followed by a relatively slow falling phase (Fig. **1**). During the falling phase of flow transient, vessel diameter decreases exponentially. Because the decrease in diameter became very slow when flow transient approached its end, it was nearly impossible to accurately determine the "real time duration of flow dilation", which should be "from the start of flow dilation till its end". To overcome this problem and to reduce the error in estimation, the "duration of flow dilation" was arbitrarily set as the time difference between the start of flow dilation till the time when diameter of the vessel reduced to 50% of its maximal flow dilation (Fig. **1**). This arbitrary parameter of "duration of dilatory response" was used to indicate the sustainability of flow dilation. Vessels that showed less than 10% reduction in diameter were considered to demonstrate sustained dilatory response and were excluded from the calculation.

Statistical Analysis

Data are given as mean±s.e.m. The Student's two-sample t-test was used to compare the treatment groups with relevant controls. *P*-values <0.05 were taken to show significant differences between means. In all of the pressure myograph experiments, n is the number of mesenteric arteries from different rats.

RESULTS

Effect of CYP Epoxygenase Inhibitors on Flow Dilation

Isolated small mesenteric arteries with a vessel external diameter between 250-400 μm at 50 mmHg were preconstricted with phenylephrine to a similar level (60% - 80% of its initial diameter), and intraluminal flow was applied to induce dilation (Fig. **2**). After stopping the flow and testing the viability of the vessels with acetylcholine (1 μM), the vessels were washed with and stabilized in Krebs-Henseleit solution for 30 min to 1 hr and then reconstricted with phenylephrine. Flow was able to elicit dilation on the

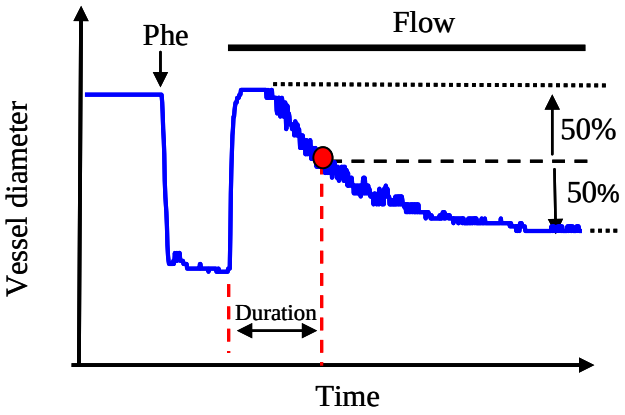

Fig. (1). Determination of the duration of dilatory response to flow. The duration of dilatory response is defined as the time elapsed from the start of flow dilation till the time when the magnitude of dilation reduced to 50% of its maximal value.

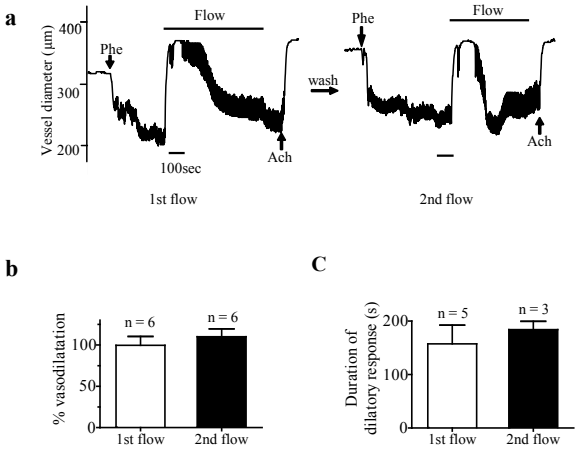

Fig. (2). Flow-induced dilatory responses in rat small mesenteric arteries. (**a**) A representative trace showing the dilatory response to flow challenge. The vessel was preconstricted with phenylephrine. Solid bar on the top of the trace indicates the time period when intraluminal flow (Krebs-Henseleit solution with 1% BSA) was applied. After the first flow-induced dilatory response, the vessel was then washed by and maintained in Krebs-Henseleit solution for 30 minutes to 1 hour before the second flow challenge. (**b**) Comparison of the peak magnitude of dilation in response to the first and the second flow. (**c**) Comparison of the duration of dilatory response. Values are means ± s.e.m. (n = 3-6).

vessels again (Fig. **2**). There is no difference in dilatory responses, either in the peak magnitude of flow dilation or in the duration of dilatory response, from the first flow and to the second flow (Fig. **2**). In other words, the dilatory responses to flow can be repeated in the same arteries without any significant reduction in flow dilatory response.

Pharmacological inhibitors were employed to examine the possibility of CYP epoxygenase involvement in flow dilation. Previous results showed that 3 μM miconazole, a nonselective CYP inhibitor [20], failed to inhibit flow-induced dilation [8]. In the present study, we found the same agent (miconazole) at a significant higher concentration (30 μM) was able to reduce the peak magnitude of flow dilation as well as the duration of dilatory responses (Fig. **3**) while the vehicle control (0.01% DMSO) did not, suggesting an involvement of CYP in flow dilation. To confirm the involvement of CYP, we used a structurally distinct and highly selective epoxygenase inhibitor MS-PPOH [21]. The results showed that MS-PPOH (10 μM) also decreased the peak magnitude and the duration of dilatory response while the vehicle control (0.01% ethanol) failed to do so, confirming the involvement of CYP epoxygenases in flow dilation. Sulphaphenazole (10 μM), a selective inhibitor of CYP 2C9 which is an isozyme of CYP epoxygenases [22], had no effect neither on the peak magnitude nor on the duration of dilatory response. These results suggest that CYP epoxygenases, but not the isoform CYP 2C9, were involved in flow dilation.

Effect of Xanthine Oxidase Inhibitor on Flow Dilation

Oxypurinol, a selective xanthine oxidase inhibitor [23], was used to examine the involvement of xanthine oxidase in

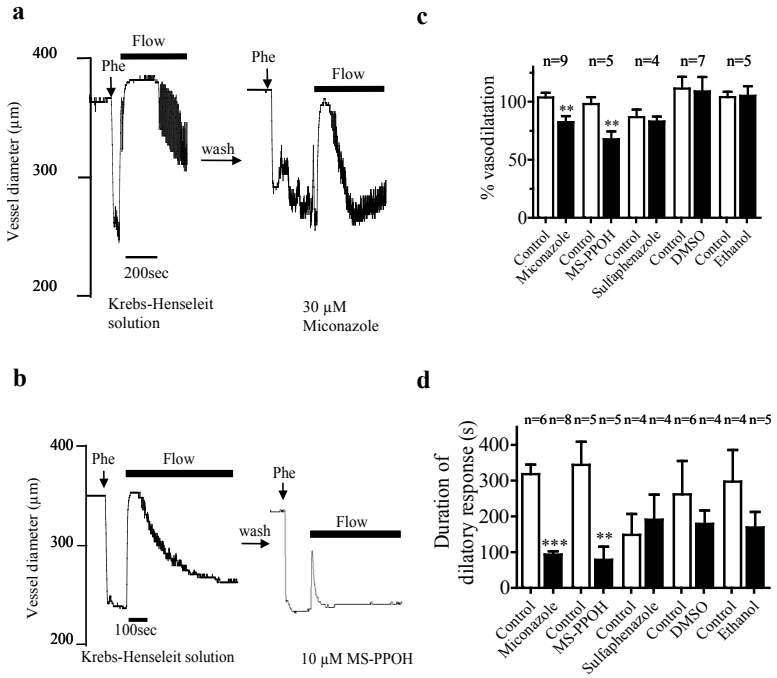

Fig. (3). Effect of CYP inhibitor on flow-induced vascular dilation. (**a**) A representative trace showing the effect of miconazole on flow dilation. After the 1st flow-induced dilatory response, the vessels were washed and incubated in miconazole (30 μM) for 30 minutes before the 2nd flow challenge. (**b**) A representative trace showing the effect of MS-PPOH (10 μM) on flow dilation. (**c**) Summary of data showing the peak flow dilation in the absence (control) and presence of miconazole (30 μM), MS-PPOH (10 μM), sulfaphenazole (10 μM) or vehicle (0.01% DMSO or 0.01% ethanol). (**d**) Summary of data showing the duration of dilatory response in the absence (control) and presence of respective agent. Values are means ± s.e.m. (n = 4-9). $^{**}P < 0.01$; $^{***}P<0.001$.

flow dilation. Oxypurinol (100 μM) reduced the duration of dilatory response but had no effect on the peak magnitude of flow dilation (Fig. **4**). These results suggest an involvement of xanthine oxidase in flow dilation.

Effect of Mitochondria-Generated H_2O_2 on Flow Dilation

Cyclosporin A and MitoQ were used to examine the involvement of mitochondrial reactive oxygen species (ROS) in flow dilation. Cyclosporin A inhibits the opening of the permeability transition pore in the inner mitochondrial membrane and thus prevents the production of O_2^- [24]. MitoQ is an elegantly-designed mitochondria-targeted antioxidant, which can selectively scavenge mitochondria-generated H_2O_2 and other ROS [25]. Decyltriphenylphosphonium bromide (decylTPP bromide) is the triphenylphosphonium moiety that is responsible for targeting MitoQ to

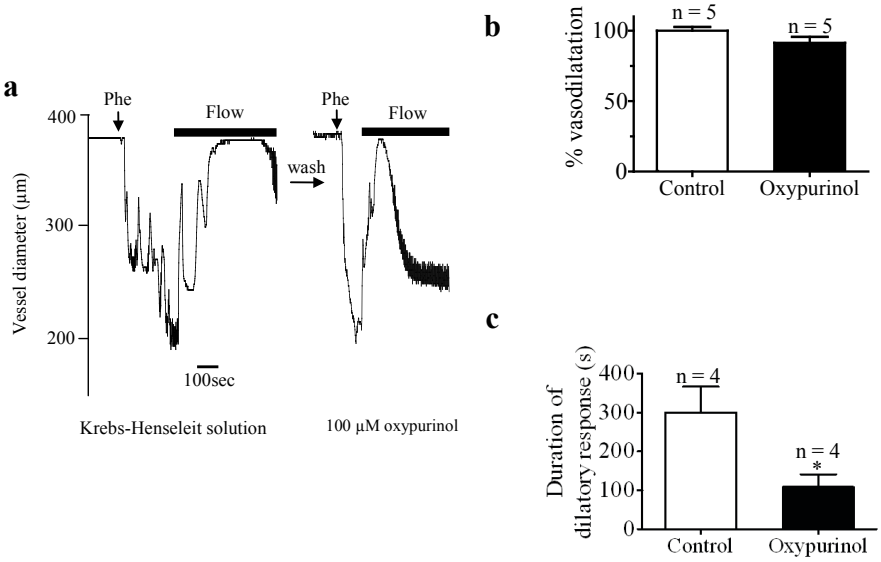

Fig. (4). Effect of xanthine oxidase inhibitor on flow dilation. (**a**) A representative trace showing the effect of oxpurinol (100 μM) on flow dilation. (**b**) Summary of data showing the peak flow dilation in the absence (control) and presence of oxypurinol. (**c**) Summary of data showing the duration of dilatory response. Values are means ± s.e.m. (n = 4-5). $^{*}P < 0.05$.

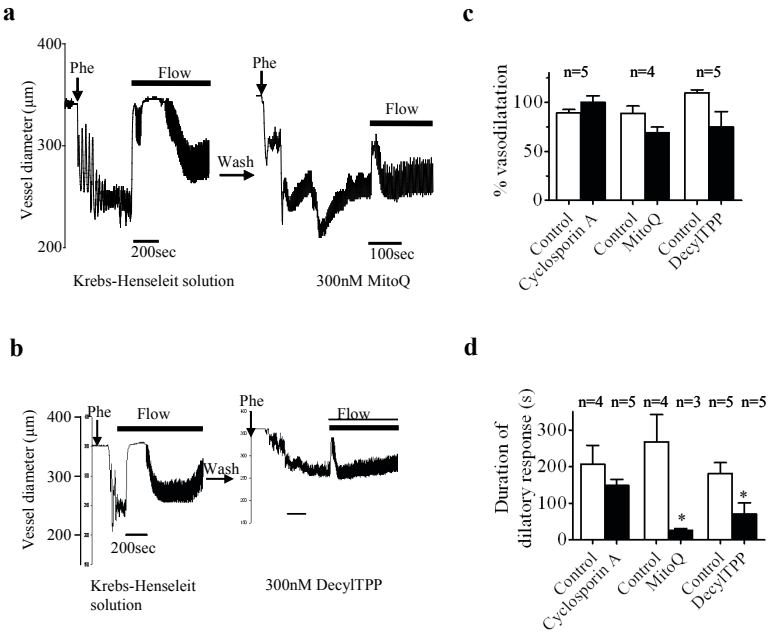

Fig. (5). Effect of inhibiting/scavenging mitochondrial H_2O_2 on flow dilation. (**a**) A representative trace showing the effect of MitoQ (300 nM) on flow dilation. (**b**) A representative trace showing the effect of decylTPP (300 nM) on flow dilation. (**c**) Summary of data showing the peak flow dilation in the absence (control) and presence of MitoQ, decylTPP, or cyclosporin A (100 μM). (**d**) Summary of data showing the duration of dilatory response. Values are means ± s.e.m. (n = 3-5). *$P < 0.05$.

the mitochondria, but it is inactive against mitochondria-generated H_2O_2 [25, 26]. It serves as a negative control for MitoQ to account for any non-specific effect of lipophilic cations [25, 26]. In experiments, cyclosporin A (2 μM) had no effect either on the peak magnitude of flow dilation or on the duration of dilatory response whereas MitoQ (300 nM) reduced the duration of dilatory response but did not affect the peak magnitude (Fig. **5**). However, decylTPP bromide (300 nM) exerted the same effect as MitoQ (Fig. **5**). No difference in flow dilation was found between MitoQ- and decylTPP bromide-treated arteries (Fig. **5**). Therefore, the effect of MitoQ on the duration of dilatory response to flow was due to the non-specific effect of lipophilic cations. These data suggest that mitochondria-generated H_2O_2 did not contribute to the flow dilation.

Effect of NADPH Oxidase Inhibitor on Flow Dilation

Apocynin is a selective inhibitor of NADPH oxidase that acts by inhibiting incorporation of the $p47^{phox}$ subunit of NADPH oxidase into the membrane unit [27]. Apocynin (1 mM) exerted no effect neither on the peak magnitude of flow dilation nor the duration of dilatory response. Hence, our data suggest that NADPH oxidase was not involved in flow dilation in rat small mesenteric arteries.

Effect of MS-PPOH and Miconazole on H_2O_2 Production in MVECs

To further confirm the role of CYP epoxygenases in flow-induced H_2O_2 production, we monitored the change of CM-H_2DCFDA fluorescence in the primary cultured mesenteric endothelial cells in response to flow challenge. CM-H_2DCFDA is a fluorescent dye widely used to detect the change in cytosolic H_2O_2 level [12, 18]. In experiments, exposure of cultured endothelial cells to flow resulted in a rise in CM-H_2DCFDA fluorescence (Fig. **6a**, left column; and **6b**), indicating a flow-induced production of H_2O_2. This fluorescence increase was completely abolished in those cells that were treated with MS-PPOH (10 μM) (Fig. **6a**, middle column; and **6b**) or miconazole (30 μM) (Fig. **6a**, right column; and **6b**). As another control, vehicle (0.01% DMSO) treatment had no effect on flow-induced rise in CM-H_2DCFDA fluorescence (Fig. **6b**). These data are consistent with those from flow dilation study, supporting an important role of CYP epoxygenases in flow-induced H_2O_2 production.

DISCUSSION

Our previous studies [8] have showed that the flow-induced vascular dilation in rat mesenteric arteries is mainly mediated by H_2O_2, because 1) the dilation was completely abolished by catalase, and the inhibition can be reversed by washing away the catalase; 2) in the presence of a catalase inhibitor aminotriazole, catalase loses its inhibitory effect; 3) exogenous application of H_2O_2 induces relaxation in phenylephrine–preconstricted arteries and hyperpolarized the smooth muscle cells. In the present study, we explored the source of H_2O_2 during flow-induced vascular dilation in rat small mesenteric arteries. The results demonstrated that, during flow dilation, H_2O_2 is generated from CYP epoxygenases and xanthine oxidase. Mitochondria-generated H_2O_2 and NADPH oxidase are not involved in the process. Within the two sources that contribute to the H_2O_2 generation, CYP epoxygenases appear to play a more important role as the inhibition of CYP epoxygenases reduced both the peak magnitude and the duration of dilatory response, and it also suppressed the H_2O_2 production in MVECs. However, note that inhibition of CYP epoxygenases or xanthine oxidase alone failed to completely

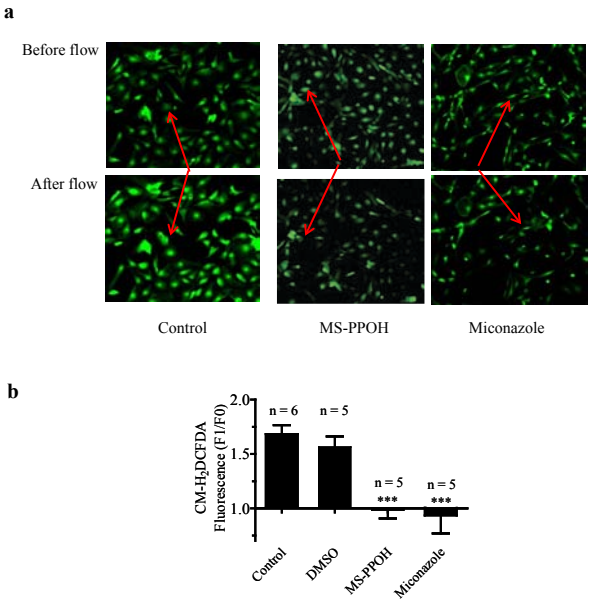

Fig. (6). Effect of MS-PPOH and miconazole on flow-induced H_2O_2 production. (**a**) Representative vascular endothelial cell CM-H_2DCFDA images before and after flow. Each pair of arrows point to one particular cell before and after flow. (**b**) Summary of data showing the change in CM-H_2DCFDA fluorescence as in (a). Miconazole, 30 μM; MS-PPOH, 10 μM; DMSO, 0.01%.Values are means ± s.e.m. (n = 5-6). ***P < 0.001.

abolish the flow dilation, suggesting an involvement of multiple sources for H_2O_2 generation during flow dilation.

CYP epoxygenases metabolize arachidonic acid to four regioisomeric epoxyeicosatrienoic acids (EETs) (5, 6-, 8, 9-, 11,12-, and 14,15-EET) [28]. The EETs have been identified as EDHFs in several vascular beds including coronary and renal arteries [28, 29]. Based on the results that flow dilation can be markedly inhibited by miconazole and 17-octadecynoic acid, Miura *et al*. suggested that EETs are the vasodilator responsible for flow dilation in human coronary arterioles in the patients with coronary artery disease [30]. However, note that catalytic cycle of CYP epoxygenases can also produce ROS, including $O_2^{\cdot-}$, H_2O_2, and hydroxyl radicals [22, 31, 32]. H_2O_2 thus produced may act as an EDHF, contributing to flow dilation. In fact, later studies from the same group have concluded that H_2O_2 is the major vasodilator responsible for flow dilation in human coronary arterioles [10, 12], although they argued that, during flow dilation, H_2O_2 is generated from mitochondria. In the present study, we demonstrated an important role of CYP epoxygenases in flow dilation. As mentioned, activity of CYP epoxygenases may produce EET and H_2O_2, both of which may act as EDHF to cause vascular dilation. Although we cannot exclude the possibility that EET thus produced may also contribute to the flow dilation, the main vasodilator appears to be H_2O_2 instead of EET because our previous studies have already demonstrated that H_2O_2 scavenging could completely abolish the flow dilation [8]. Fleming suggested that, among numerous CYP isoforms, CYP 2C9 is a physiologically relevant source of ROS in porcine coronary endothelial cells [22]. Recent studies showed that CYP 2C9 is involved in flow dilation in human peripheral conduit arteries in healthy subjects and in patients with heart diseases [33]. Thus we tested the possible involvement of CYP 2C9 in flow dilation in rat small mesenteric arteries. Our data showed that sulphaphenazole, a specific inhibitor for CYP 2C9, did not have any effect on flow dilation, suggesting that CYP 2C9 is not involved in the flow dilation of rat small mesenteric arteries.

Xanthine oxidase catalyzes the oxidation of xanthine or hypoxanthine during purine metabolism, leading to the formation of $O_2^{\cdot-}$ and H_2O_2. It has been shown that xanthine oxidase is a source for $O_2^{\cdot-}$ production in human internal mammary arteries and saphenous veins [34]. Xanthine oxidase also contributes to an increased ROS levels in spontaneous hypertensive rats [35, 36]. We used oxypurinol, a selective inhibitor of xanthine oxidase, to examine the possible involvement of xanthine oxidase in flow dilation. The results showed that oxypurinol reduced the duration of dilatory response to flow, suggesting an involvement of xanthine oxidase in flow dilation in rat small mesenteric arteries.

The electron transport chain within mitochondria can also generate $O_2^{\cdot-}$ and H_2O_2 [37]. One previous study from Liu *et al*. suggests that, during flow dilation in human coronary arterioles, H_2O_2 is generated from mitochondria respiration [12]. Their conclusion was based on 1) flow-induced H_2O_2 production, as determined by H_2DCFDA fluorescence and electron spin resonance, is reduced by rotenone, which is mitochondrial complex I inhibitor; 2) the flow dilation of human coronary resistance arteries is almost completely abolished by rotenone, and furthermore the dilation was partially inhibited by myxothiazol, a mitochondrial complex III inhibitor. These results are contradictory to the findings from the present study. In the present study, we found that scavenging mitochondria-generated H_2O_2 with MitoQ or inhibiting mitochondrial ROS production with cyclosporin A had no effect on flow dilation, both arguing against an involvement of mitochondria-generated H_2O_2 in flow dilation. The reason for this discrepancy is unclear. However, compared to the study from Liu *et al.* [12], we used different animal species and different arteries. Furthermore, the study from Liu *et al*. used rotenone to inhibit mitochondria $O_2^{\cdot-}$ generation [12]. There is a controversy on whether rotenone can indeed inhibit mitochondria $O_2^{\cdot-}$ generation because rotenone was found to stimulate mitochondria $O_2^{\cdot-}$ generation in some cases [38, 39]. In the present study, we used a much superior agent MitoQ that can selectively scavenge mitochondria-generated H_2O_2 and other ROS. These differences in experimental designs could contribute to the conflicting results about the role of mitochondria-generated H_2O_2 in flow dilation.

NADPH oxidase is another putative source of $O_2^{\cdot-}$ in vascular endothelial cells. It is a membrane-bound flavocytochrome and uses both flavin and heme groups to transfer electrons from NADPH to oxygen, yielding $O_2^{\cdot-}$. Previous reports showed that laminar shear stress may generate $O_2^{\cdot-}$ via NADPH oxidase in cultured human umbilical vein endothelial cells [13, 14]. In our experiments, apocynin, a selective inhibitor for NADPH oxidase, did not have effect on flow dilation, suggesting that NADPH oxidase is not involved in the process. However, there is a major difference in experimental designs between ours and others': we used isolated artery segments as experimental

materials while the others used cultured human umbilical vein endothelial cells. The findings obtained from culture cells may not readily apply to *in vivo* situation. These could contribute to the discrepancy in results.

CONCLUSION

In summary, during flow dilation in rat small mesenteric arteries, H_2O_2 as the dilator is generated from CYP epoxygenases and xanthine oxidase.

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