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H$_2$O$_2$-induced microvessel barrier dysfunction: the interplay between reactive oxygen species, nitric oxide, and peroxynitrite

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Keywords
Endothelial [Ca$^{2+}$], H$_2$O$_2$, nitric oxide, microvessel permeability, peroxynitrite.

Abstract
Elevated H$_2$O$_2$ is implicated in many cardiovascular diseases. We previously demonstrated that H$_2$O$_2$-induced endothelial nitric oxide synthase (eNOS) activation and excessive NO production contribute to vascular cell injury and increases in microvessel permeability. However, the mechanisms of excessive NO-mediated vascular injury and hyperpermeability remain unknown. This study aims to examine the functional role of NO-derived peroxynitrite (ONOO$^{-}$) in H$_2$O$_2$-induced vascular barrier dysfunction by elucidating the interrelationships between H$_2$O$_2$-induced NO, superoxide, ONOO$^{-}$, and changes in endothelial [Ca$^{2+}$]$$_i$ and microvessel permeability. Experiments were conducted on intact rat mesenteric venules. Microvessel permeability was determined by measuring hydraulic conductivity (Lp). Endothelial [Ca$^{2+}$]$$_i$, NO, and O$_2$/O$_2$ were assessed with fluorescence imaging. Perfusion of vessels with H$_2$O$_2$ (10 µmol/L) induced marked productions of NO and O$_2$/O$_2$, resulting in extensive protein tyrosine nitration, a biomarker of ONOO$^{-}$. The formation of ONOO$^{-}$ was abolished by inhibition of NOS with N$^G$-Methyl-L-arginine. Blocking NO production or scavenging ONOO$^{-}$ by uric acid prevented H$_2$O$_2$-induced increases in endothelial [Ca$^{2+}$]$$_i$ and Lp. Additionally, the application of exogenous ONOO$^{-}$ to microvessels induced delayed and progressive increases in endothelial [Ca$^{2+}$]$$_i$ and microvessel Lp, a pattern similar to that observed in H$_2$O$_2$-perfused vessels. Importantly, ONOO$^{-}$ caused further activation of eNOS with amplified NO production. We conclude that the augmentation of NO-derived ONOO$^{-}$ is essential for H$_2$O$_2$-induced endothelial Ca$^{2+}$ overload and progressively increased microvessel permeability, which is achieved by self-promoted amplifications of NO-dependent signaling cascades. This novel mechanism provides new insight into the reactive oxygen and/or reactive nitrogen species-mediated vascular dysfunction in cardiovascular diseases.

Introduction
Increased production of reactive oxygen species (ROS) has been demonstrated to cause impaired vascular barrier function, resulting in tissue damage and organ dysfunction (Del Maestro et al., 1981; Cai and Harrison, 2000; Zhu and He, 2006; Zhou et al., 2009; Di et al., 2016; Mundi et al., 2017). Our previous studies demonstrated that hydrogen peroxide (H$_2$O$_2$) at a pathophysiologically relevant concentration (10 µmol/L) induced delayed and progressive increases in endothelial calcium concentration (EC [Ca$^{2+}$]$$_i$) and microvessel permeability that was attributed to H$_2$O$_2$-induced excessive nitric oxide (NO) production through endothelial nitric oxide synthase (eNOS) activation (Zhou et al., 2013).

While physiological levels of NO have been recognized to be essential for the maintenance of many vascular functions, excessive NO has been demonstrated to play important roles in mediating inflammation-induced vascular leakage (Yuan et al., 1993; He et al., 1997; Zhu and...
Role of ROS and RNS in microvessel barrier dysfunction

Jiang et al., 2008; Zhou and He, 2011), large amounts of $H_2O_2$-induced NO production resulted in caspase activation and impairment of $Ca^{2+}$ homeostasis, leading to vascular cell apoptosis, and delayed and progressive increases in microvessel permeability (Zhou et al., 2013).

Exogenously supplied NO has been reported to have potential cytotoxic effects in cultured endothelial cells (Shen et al., 1998; Shimizu et al., 1998). However, the mechanisms of NO-mediated vascular cell injury, particularly in $H_2O_2$-stimulated intact vessels remain unknown. Accumulating evidence suggest that the cytotoxic effects of NO are most likely mediated by its oxidation product, particularly peroxynitrite (ONOO$^-$), rather than NO itself (Xia et al., 1996; Ribo et al., 2001; Pacher et al., 2007). NO reacts rapidly with superoxide (O$_2^-$) at a diffusion-controlled rate ($\sim 1 \times 10^{10} \text{M}^{-1} \text{s}^{-1}$) to form ONOO$^-$, a strong oxidant capable of oxidizing and nitrating lipid, protein, and DNA, resulting in cell death and tissue injury (Radi et al., 1991; Pacher et al., 2007; Szabo et al., 2007). Recently, $H_2O_2$ has been shown to induce further production of O$_2^-$ and $H_2O_2$ mainly through activation of NAD (P)H oxidase (Li et al., 2001; Cai, 2005), which is considered as an important mechanism for ROS-mediated pathogenesis of cardiovascular diseases. However, whether the interplay between reactive oxygen species, nitric oxide, and peroxynitrite is accountable for $H_2O_2$-induced microvessel barrier dysfunction remains unknown.

Currently, the role of ONOO$^-$ in the regulation of eNOS activity and NO production, particularly under in vivo conditions, has not been well defined. While some early studies reported that ONOO$^-$ induced vasodilatation through increased NO production (Liu et al., 1994; Villa et al., 1994), others on cultured bovine endothelial cells suggested that, although ONOO$^-$ increased eNOS activity through enhanced phosphorylation at Ser$^{1179}$, it increased O$_2^-$ production, instead of NO (Zou et al., 2002; Zou et al., 2002). The interrelationship between ONOO$^-$ and eNOS activity and their roles in the regulation of vascular barrier function in intact vessels has not been well explored.

This study is designed to examine the functional roles of ONOO$^-$ in $H_2O_2$-induced microvessel barrier dysfunction by elucidating the interrelationships between $H_2O_2$, eNOS-derived NO, $O_2^-$, ONOO$^-$, and the changes in EC $[Ca^{2+}]_i$ and microvessel permeability. Experiments were conducted on individually perfused mesenteric venules with intact surrounding circulation. EC $[Ca^{2+}]_i$ and NO were measured in Fura-2 and DAF-2-loaded vessels, respectively. Microvessel permeability was determined by measuring hydraulic conductivity (Lp). We first measured $H_2O_2$-induced changes in EC $[Ca^{2+}]_i$, NO production, and microvessel Lp. Under the same experimental conditions, $H_2O_2$-induced $O_2^-$ production was assessed using dihydroethidium. The formation of ONOO$^-$ was examined by fluorescent immunostaining of nitrotyrosine, a biomarker of ONOO$^-$, in $H_2O_2$-perfused vessels. The functional relationship between ONOO$^-$, and changes in EC $[Ca^{2+}]_i$, and microvessel permeability was further investigated using uric acid, an endogenous ONOO$^-$ scavenger. The direct effects of exogenously applied ONOO$^-$ on EC $[Ca^{2+}]_i$, NO production, as well as microvessel Lp, were also examined.

Materials and Methods

Animal preparation

All animal experiments were conducted on female Sprague–Dawley rats (2–3 months old, 220–250 g; Hilltop Laboratory Animal, Scottsdale, PA). All procedures and animal use were approved by the Animal Care and Use Committee at West Virginia University. Rats were anesthetized with pentobarbital sodium (65 mg/kg body wt) administered subcutaneously. A midline surgical incision (1.5–2 cm) was made in the abdominal wall and the mesentery was gently taken out from the abdominal cavity and spread over a glass coverslip attached to an animal tray for studies. The upper surface of the mesentery was continuously superfused with mammalian Ringer’s solution at 37°C. Each experiment was performed on one microvessel per animal.

Measurement of Lp in individually perfused rat mesenteric microvessels

Microvessel permeability was assessed by measuring hydraulic conductivity, Lp, using modified Landis technique, which measures the volume flux of water across the microvessel wall. Details have been described previously (Curry and Sarelius, 1983; Kendall and Michel, 1995; He et al., 1996). Briefly, a single venular microvessel with diameters ranging between 35 and 50 µm was cannulated with a micropipette and perfused with albumin-Ringer solution (control) containing 1% (vol/vol) hamster red blood cells as markers. A known hydrostatic pressure (40–60 cmH$_2$O), controlled by a water manometer, is applied through the micropipette to the vessel lumen, which allows the perfusate to continuously flow through the vessel. For each measurement, the perfused vessel was occluded briefly downstream with a glass rod. The initial
water flux/unit area of microvessel wall (Jv/A) was calculated from the velocity of the marker cell after vessel occlusion, the vessel radius, and the distance between the marker cell and the occlusion site. Lp was calculated as the slope of the relationship between Jv/A and the pressure difference across the vessel wall. In each experiment, the baseline Lp and the Lp after the application of testing solutions were measured in the same vessel, and the changes in Lp were expressed as the ratio of \( \frac{\text{Lp}_{\text{test}}}{\text{Lp}_{\text{control}}} \). All testing agents were added to the perfusate and delivered into the vessel lumen through the cannulation pipette. To prevent the marker red blood cells from interacting with \( \text{H}_2\text{O}_2 \) and ONOO\(^{-} \) in the perfusate, marker cells were absent during the perfusion period and added back for Lp measurements after designated duration of perfusion.

**Measurements of EC \([\text{Ca}^{2+}]_i\)**

EC \([\text{Ca}^{2+}]_i\) was measured in individually perfused microvessels using the fluorescent \( \text{Ca}^{2+} \) indicator fura 2-AM. Experiments were performed on a Nikon Diaphod 300 microscope equipped with a Nikon photometry system. In each experiment, a venular microvessel was cannulated and perfused first with albumin-Ringer solution that contained 10 \( \mu \text{mol/L} \) of fura 2-AM for 45 min. The vessel was then recannulated and perfused with albumin-Ringer solution for 10 min to remove fura 2-AM from the vessel lumen. A segment of fura 2-AM-loaded vessel at least 100 \( \mu \text{m} \) away from the cannulation site was then positioned within the measuring window which covered ~50 ECs forming the vessel wall. The excitation wavelengths for fura 2-AM were selected by two narrow-band interference filters (340 \( \pm 5 \) and 380 \( \pm 5 \) nm; Oriel), and the emission was separated with a dichroic mirror (DM400) and a wide-band interference filter (500 \( \pm 35 \) nm; Oriel). The corresponding FI values (FI\(_{340}\) and FI\(_{380}\) respectively) were collected with a 0.25-sec exposure at each wavelength. Baseline EC \([\text{Ca}^{2+}]_i\) was measured after fura 2-AM was removed from the vessel lumen with albumin-Ringer perfusion for 10 min. The same vessel was recannulated with pipette containing test reagents (e.g., \( \text{H}_2\text{O}_2 \), ONOO\(^{-} \), L-NMMA, uric acid, \( \text{LaCl}_3 \), etc.) and the changes in EC \([\text{Ca}^{2+}]_i\) were measured immediately after the recannulation. At the end of the experiment, the microvessel was superfused with a modified Ringer solution (5 \( \text{mmol/L} \) of \( \text{Mn}^{2+} \) without \( \text{Ca}^{2+} \)) and perfused with the same solution that contained ionomycin (10 \( \mu \text{mol/L} \) to bleach the \( \text{Ca}^{2+} \)-sensitive form of fura 2. The background FI due to unconverted fura 2-AM and other \( \text{Ca}^{2+} \)-insensitive forms of fura 2 were subtracted from FI\(_{340}\) and FI\(_{380}\) values. The ratios of the two FI values were converted to \( \text{Ca}^{2+} \) concentrations using an in vitro calibration curve (He et al., 1996).

**Measurement of endothelial NO production**

Levels of endothelial NO were quantified at cellular level in individually perfused microvessels using a fluorescence imaging system and 4,5-diaminofluorescein diacetate (DAF-2 DA). The experimental rigs were the same as that used for \( \text{Ca}^{2+} \) measurements, except that a 12-bit digital, cooled, charge-coupled device camera (ORCA; Hamamatsu) was used for image acquisition. The excitation wavelength for DAF-2 DA was selected by an interference filter (480/40 nm), and emission was separated by a dichroic mirror (305 nm) and a band-pass barrier (535/50 nm). To minimize photo bleaching, a neutral density filter (0.5 N) was positioned in front of the interference filter and the exposure time was minimized to 0.12 sec at 1-min intervals. All the images were acquired and analyzed using Metafluor software (Universal Imaging). During the experiment, each vessel was continuously perfused with albumin-Ringer solution containing DAF-2 DA (5 \( \mu \text{mol/L} \)). NO production was measured after the DAF-2 loading reached the steady state (Zhou and He, 2011). Basal NO was measured for 10 min before perfusion with test reagents. All images were collected from a group of ECs located at the same focal plane of the vessel wall. At the end of the experiment, the NO donor, sodium nitroprusside (SNP, 50 \( \mu \text{mol/L} \)) was applied to the superfusate to ensure sufficient intracellular DAF-2 DA to react with NO. Quantitative analysis was conducted at the individual EC level using manually selected regions of interest (ROIs) along the vessel wall. Each ROI covers the area of one individual EC as indicated by the fluorescence outline. The tissue autofluorescence was subtracted from all the measured FIs. The changes in FI\(_{\text{DAF}}\) upon addition of test reagents were expressed as the percentage of FI\(_{\text{DAF}}\) over the baseline FI\(_0\) (FI/FI\(_0\) *100). The rate of FI\(_{\text{DAF}}\) change was derived by the first differential conversion of cumulative FI\(_{\text{DAF}}\) over time. DAF-2 specificity for NO detection was examined by mixing DAF-2 DA (10 \( \mu \text{mol/L} \)) with albumin-Ringer solution, \( \text{H}_2\text{O}_2 \) (5, 50 and 500 \( \mu \text{mol/L} \)), SNP (20 and 200 \( \mu \text{mol/L} \)), hypoxanthine/xanthine oxidase (0.05 \( \mu \text{mol/L} /1 \mu \text{U} \), 0.5 \( \mu \text{mol/L} /10 \mu \text{U} \), and 5 \( \mu \text{mol/L} /100 \mu \text{U} \), or peroxynitrite (5, 50, and 500 \( \mu \text{mol/L} \)) using the same experimental settings as those used for NO measurement on intact vessels. No significant increase in FI\(_{\text{DAF}}\) was observed for all the test agents except SNP at 200 \( \mu \text{mol/L} \) that caused an accumulative increase in FI\(_{\text{DAF}}\), indicating the specificity of DAF-2 as a NO fluorescent indicator. Peroxynitrite at 500 \( \mu \text{mol/L} \) caused a small increase in FI\(_{\text{DAF}}\) but that concentration is beyond the range we used in the experiments. The representative measurements with each agent are shown in Fig 1.
Confocal images of fluorescent immunostaining for nitrotyrosine and superoxide detection

Leica confocal system (SP2) was used to detect fluorescent immunostaining for nitrotyrosine and superoxide production with dihydroethidium (DHE) in H\textsubscript{2}O\textsubscript{2}-perfused vessels. Images were acquired using Leica objective ×63 (HCX PL APO, NA 1.2) with ×1.5 electronic zoom, and the vertical step was 0.3 μm. The mean FI of each stack of ROIs that cover the vessel wall was quantified using Leica confocal software. The averaged FI of the perfused vessels was calculated. The ratio of the FI over control value was used to present the changes in the nitrated tyrosine content. The absolute mean FI in arbitrary unit from each vessel was calculated for DHE images to semi-quantify the magnitude of superoxide production. Nitrotyrosine immunostaining was conducted in fixed vessels. The mesentery bearing each perfused vessel was fixed with paraformaldehyde, followed by 10-min permeabilization with 0.1% Triton X-100 and 1 h blocking with 5% bovine serum before overnight exposure to the mouse Anti-Nitro tyrosine antibody (ab7048, abcam, USA) at 4°C on an automatic laboratory shaker. The tissue was then washed with BSA-Ringer solution for three times before incubation with Alexa 488-conjugated goat anti-mouse secondary antibody (ab150113, abcam, USA) at room temperature for 3 h. For detection of superoxide, each vessel was loaded with DHE (10 µmol/L) for 30 min followed by perfusion with either BSA-Ringer alone or containing H\textsubscript{2}O\textsubscript{2} (10 µmol/L) for 30 min. The images were acquired with excitation at 488 nm and emission at 590–700 nm. To determine the source of superoxide, diphenyle ylide iodonium (DPI, an NADPH oxidase inhibitor, 20 µmol/L) was applied to the vessel for 20 min after DHE loading, followed by perfusion with H\textsubscript{2}O\textsubscript{2} (10 µmol/L) in the presence of DPI.

Solutions and reagents

Mammalian Ringer solution (He et al., 1996) was used for dissecting mesenteries, superfusing tissues, and preparing perfusion solutions. The composition of the mammalian Ringer solution was (in mmol/L): 132 NaCl, 4.6 KCl, 2 CaCl\textsubscript{2}, 1.2 MgSO\textsubscript{4}, 5.5 glucose, 5.0 NaHCO\textsubscript{3}, 20 N-2-hydroxyethylpiperazine–N’–2-ethanesulfonic acid (HEPES), and Na-HEPES. All perfusates used for control and test perfusion contained BSA (10 mg/ml). Fura-2 AM was purchased from Invitrogen. H\textsubscript{2}O\textsubscript{2} (30%), DAF-2, DAF-2 DA, LaCl\textsubscript{3}, L-NMMA, DPI, and DHE were purchased from Sigma. All the fluorescent dyes except for Alexa488-conjugated secondary antibody (in BSA-Ringer solution) were prepared in DMSO for stock solution, and at least 1:1000 dilution was made for the final working solutions. ONOO\textsuperscript− and degraded ONOO\textsuperscript− were purchased from EMD Millipore and were diluted in the BSA-Ringer solution into final concentrations of 10 or 50 µmol/L. All perfusates containing the test reagents were freshly prepared before each cannulation.

Data analysis and statistics

All values are presented as means ± SD. Each “n” represents the number of vessels (one vessel per rat). Paired
The application of L-NMMA nearly abolished H$_2$O$_2$ the same experimental conditions that NO was measured. FIDAF increase indicates a higher NO production rate and (irreversible chemical conversion), the greater slope of the 41 min of H$_2$O$_2$ perfusion with a total of 830 lower sustained level. A second increase in FIDAF was 425/C$_6$H$_2$O$_2$ (10$^{-10}$)/C$_6$ since the FIDAF increase in reaction to NO is cumulative an additional increase in FIDAF, and reached a plateau level at 655/C$_6$H$_2$O$_2$ (10$^{-10}$)/C$_6$ when H$_2$O$_2$ perfusion (2 h) reduced the FI to 1.3 0.27 times that of the control (4, n = 5, P < 0.0001). Then Lp was measured under nitrated tyrosine formation. Importantly, L-NMMA (2 mmol/L), without affecting the baseline EC [Ca$^{2+}$]$_i$ (67 ± 7.8 vs. 75 ± 10.8 nmol/L), completely abolished H$_2$O$_2$-induced increase in EC [Ca$^{2+}$], (Fig. 2C), indicating a causal relationship between H$_2$O$_2$-induced excessive NO and the subsequent Ca$^{2+}$ influx and eventually Ca$^{2+}$ overload in ECs lining microvessel wall.

### Results

**H$_2$O$_2$ induced NO-dependent increases in microvessel Lp and EC [Ca$^{2+}$]$_i$**

We previously demonstrated that H$_2$O$_2$ at pathologically relevant concentrations induced delayed and progressive increases in microvessel Lp, which was initiated by the activation of eNOS and excessive NO production (Zhou et al., 2009; Zhou et al., 2013). Fig 2A shows the summary results of H$_2$O$_2$-induced time- and dose-dependent increases in Lp. The H$_2$O$_2$-induced endothelial NO production was measured in DAF-2 DA-loaded venules. Fig 2B shows the time course of H$_2$O$_2$-induced NO production (presented as the mean changes in FIDAF-2 pooled from four vessels). Since the FIDAF increase in reaction to NO is cumulative (irreversible chemical conversion), the greater slope of the FIDAF increase indicates a higher NO production rate and the plateau indicates no further increase in NO. When H$_2$O$_2$ (10 µmol/L) was perfused into the microvessel, there was an immediate increase in FIDAF that reached 425 ± 18.1% of the baseline level in 5 min, followed by a lower sustained level. A second increase in FIDAF was observed after 20 min of H$_2$O$_2$ perfusion with 280 ± 41% additional increase in FIDAF, and reached a plateau level at 41 min of H$_2$O$_2$ perfusion with a total of 830 ± 85% increase in FIDAF. In another four DAF-2 DA-loaded vessels, perfusion of albumin-Ringer solution as a control showed approximately 1% increase per minute in FIDAF, representing basal NO production. Preperfusion of vessels with NOS inhibitor, L-NMMA (2 mmol/L), for 30 min followed by the administration of H$_2$O$_2$ in the presence of L-NMMA completely abolished H$_2$O$_2$-induced NO production (n = 4, P < 0.0001). Then Lp was measured under the same experimental conditions that NO was measured. The application of L-NMMA nearly abolished H$_2$O$_2$ (10 µmol/L)-induced increases in microvessel Lp with decrease in mean Lp from 9.3 ± 1.3 to 1.3 ± 0.16 times of the control after 2-h perfusion of H$_2$O$_2$ (Lp$_{test}$/Lp$_{contro}$ n = 5 per group, P < 0.0001, Fig. 2D).

The effect of H$_2$O$_2$ on EC [Ca$^{2+}$], was examined in fura-2 loaded microvessels (n = 5). The mean baseline EC [Ca$^{2+}$]$_i$ was 80 ± 10.5 nmol/L. Perfusion of venules with H$_2$O$_2$ (10 µmol/L) did not cause immediate increase in EC [Ca$^{2+}$]$_i$. A slow but progressive increase in EC [Ca$^{2+}$]$_i$ occurred after 15 min of H$_2$O$_2$ perfusion and reached a significant increase (207 ± 47.1 nmol/L) from control levels at 36 min of perfusion (p = 0.004), and plateaued at 436 ± 52.7 nmol/L with H$_2$O$_2$ perfusion for ~84 min (P < 0.0001 vs. vehicle control, Fig. 2C). The application of LaCl$_3$, a nonselective divalent cation blocker (50 µmol/L, added 20 min before H$_2$O$_2$ administration and present throughout the study), prevented H$_2$O$_2$-induced increase in EC [Ca$^{2+}$],. The mean EC [Ca$^{2+}$], before and after 2-hour perfusion of H$_2$O$_2$ in the presence of LaCl$_3$ were 80 ± 10.5 nmol/L and 96 ± 7.2 nmol/L, respectively (n = 4, P < 0.0001 vs. H$_2$O$_2$ perfusion alone, Fig. 2C), indicating Ca$^{2+}$ influx to be the main source for H$_2$O$_2$-induced increases in EC [Ca$^{2+}$]$_i$. Blocking Ca$^{2+}$ influx by LaCl$_3$ also prevented H$_2$O$_2$ (10 µmol/L)-induced delayed increases in Lp (n = 4 & 5 per group, P < 0.0001, Fig. 2D), indicating the increased EC [Ca$^{2+}$], after reaching certain threshold levels (>300 nmol/L, i.e., at least after 1h of H$_2$O$_2$ perfusion) to be responsible for increased Lp.

Since H$_2$O$_2$-induced NO production is immediate and EC [Ca$^{2+}$], did not increase until 15 min later, we predict that, if they are related, the delayed increase in EC [Ca$^{2+}$], could be the result of the initial large amount of NO production in H$_2$O$_2$-perfused vessels. To test this relationship, we measured EC [Ca$^{2+}$]$_i$ after blocking NO production using L-NMMA in four vessels. L-NMMA (2 mmol/L), without affecting the baseline EC [Ca$^{2+}$]$_i$ (67 ± 7.8 vs. 75 ± 10.8 nmol/L), completely abolished H$_2$O$_2$-induced increase in EC [Ca$^{2+}$], (Fig. 2C), indicating a causal relationship between H$_2$O$_2$-induced excessive NO and the subsequent Ca$^{2+}$ influx and eventually Ca$^{2+}$ overload in ECs lining microvessel wall.

**H$_2$O$_2$ induced NO-dependent tyrosine nitration in intact venules**

To examine whether ONOO$^-$ plays a role in H$_2$O$_2$-induced increases in microvessel Lp, we examined nitrotyrosine, a biomarker of ONOO$^-$, in H$_2$O$_2$-perfused venules. In contrast to the low fluorescence signal in albumin-Ringer-perfused vessels (Fig. 3A left panel), H$_2$O$_2$ perfusion (10 µmol/L for 2 h) induced an extensive tyrosine nitration in the cytosol and cell membrane (Fig. 3A middle panel). Arrows indicate the nucleus of ECs without nitrated tyrosine formation. Importantly, L-NMMA (2 mmol/L) significantly attenuated the nitrated tyrosine signal (Fig. 3A right panel). The fluorescence intensity (FI) was quantified in each vessel and summarized in Fig 3B. The mean FI in vessels with 2-h H$_2$O$_2$ perfusion was 4.8 ± 0.27 times that of the control (n = 4, P < 0.0001), and the application of L-NMMA before and during H$_2$O$_2$ perfusion (2 h) reduced the FI to 1.3 ± 0.1 times to that...
of the control \((n = 3, P < 0.0001)\), indicating H2O2-induced ONOO\(^{-}\) formation is NO dependent.

**H2O2 induced superoxide production in intact venules**

NO reacts quickly with superoxide to form ONOO\(^{-}\). To examine the role of H2O2-induced NO in the formation of ONOO\(^{-}\), we examined the intracellular superoxide levels in H2O2-perfused vessels with DHE staining. As shown in Fig. 4A, a marked increase in the DHE FI was observed in both endothelium and pericytes after 30-min perfusion of H2O2 (10 \(\mu\)mol/L), which was abolished by pretreatment of the vessels with an inhibitor of NADPH oxidase, diphenylene iodonium (DPI, 20 \(\mu\)mol/L). The mean FIs of DHE were 48.6, 141.15.6, and 59.0.5 AU in BSA-Ringer (control, \(n = 6\)), H2O2 (\(n = 5\)), and H2O2 plus DPI-perfused vessels (\(n = 6\)), respectively (Fig. 4B, \(P < 0.0001, \text{H2O2 group vs. control and H2O2 plus DPI group, respectively}\).
Role of ONOO\textsuperscript{−} in H\textsubscript{2}O\textsubscript{2}-induced increases in microvessel Lp and endothelial [Ca\textsuperscript{2+}]

To examine whether NO-derived ONOO\textsuperscript{−} accounts for the H\textsubscript{2}O\textsubscript{2}-induced increases in EC [Ca\textsuperscript{2+}], and microvessel Lp, the effects of H\textsubscript{2}O\textsubscript{2} on EC [Ca\textsuperscript{2+}], and microvessel Lp were investigated with the application of uric acid, a ONOO\textsuperscript{−} scavenger. Uric acid (50 µmol/L) perfusion for 20 min did not alter the baseline EC [Ca\textsuperscript{2+}] (69 ± 4.7 vs. 85 ± 4.1 nmol/L) and Lp (2.3 ± 0.34 vs. 2.5 ± 0.19 × 10\textsuperscript{-7} cm\textsuperscript{-1} cmH\textsubscript{2}O\textsuperscript{−1}, P > 0.8, n = 4 for each group), but significantly reduced H\textsubscript{2}O\textsubscript{2}-induced increase in EC [Ca\textsuperscript{2+}], from a mean peak value of 403 ± 43.0 (n = 5) to 139 ± 30.6 nmol/L (n = 4, P = 0.002), and completely prevented the H\textsubscript{2}O\textsubscript{2}-induced increase in Lp (L\textsubscript{p_test}/L\textsubscript{p_control}) from 9.3 ± 1.34 (n = 5) to 1.0 ± 0.08 times the control level (n = 4, P = 0.003). All values are compared after 2-h perfusion of H\textsubscript{2}O\textsubscript{2} (10 µmol/L). Fig. 5A,B show the representative experiments and Fig. 5C shows the results summary.
measured the changes in Lp. The mean baseline Lp was $2.1 \pm 0.16 \times 10^{-7} \text{ cm s}^{-1} \text{ cmH}_2\text{O}^{-1}$ ($n = 25$). No changes in Lp were observed with ONOO$^-$ perfusion for 30 min at 10 and 50 µmol/L ($L_{\text{test}}/L_{\text{control}}$ was $1.0 \pm 0.02$ and $1.0 \pm 0.03$, respectively, $n = 3$ per group, Fig. 6A,D). However, extending the perfusion duration to 1 and 2 h resulted in time- and dose-dependent Lp increases similar to that observed in H$_2$O$_2$-perfused vessels (Fig 2A). Lp increased significantly to $2.8 \pm 0.45$ ($P = 0.03$) and $5.9 \pm 0.44$ ($P = 0.003$) times the control at 10 µmol/L, and to $6.0 \pm 0.57$ and $12.0 \pm 0.96$ times the control at 50 µmol/L ($P < 0.001$) after 1 and 2 h of ONOO$^-$ perfusion, respectively (Fig. 6B,D, $n = 4-6$ per group). We also examined the effect of degraded peroxynitrite on microvessel Lp in two vessels. No changes in Lp were observed after 1-h perfusion of degraded peroxynitrite (50 µmol/L). Fig 6C represents the contrast of the Lp changes in peroxynitrite- and degraded peroxynitrite-perfused vessels.

### Peroxynitrite-induced progressive increases in EC [Ca$^{2+}$]$\text{i}$ contributed to the increases in microvessel Lp

The causal relationship between ONOO$^-$ and EC [Ca$^{2+}$]$\text{i}$, was further examined in seven vessels loaded with fura-2. Baseline EC [Ca$^{2+}$]$\text{i}$ was $82 \pm 12.0$ nmol/L. A significant increase in EC [Ca$^{2+}$]$\text{i}$, occurred at $20 \pm 4.0$ min of ONOO$^-$ perfusion (50 µmol/L), and progressively reached to a sustained level of $375 \pm 30.0$ nmol/L at $78 \pm 2.3$ min of ONOO$^-$ perfusion ($n = 4$, Fig. 7A). No changes in EC [Ca$^{2+}$]$\text{i}$ were observed on vessels perfused with albumin-Ringer for 1 h ($n = 3$, data not shown). Preperfusion of vessels with LaCl$_3$ (50 µmol/L) for 20 min did not affect baseline EC [Ca$^{2+}$]$\text{i}$, but prevented ONOO$^-$-induced increases in EC [Ca$^{2+}$]$\text{i}$ (the mean EC [Ca$^{2+}$]$\text{i}$, was $98 \pm 15.2$ nmol/L at 80 min of ONOO$^-$ perfusion ($n = 3$, $P = 0.0027$, Fig. 7A), indicating the role of Ca$^{2+}$ influx in ONOO$^-$-induced increase in EC [Ca$^{2+}$]$\text{i}$).

The role of Ca$^{2+}$ influx in ONOO$^-$-induced Lp increase was then examined. The application of LaCl$_3$ prevented the increased Lp in ONOO$^-$ (50 µmol/L, 1 h)-perfused vessels. The $L_{\text{test}}/L_{\text{control}}$ reduced from $2.1 \pm 0.44$ ($n = 4$, $P = 0.0027$, Fig. 7B), indicating that the sustained increase in EC [Ca$^{2+}$]$\text{i}$, to be responsible for ONOO$^-$-induced Lp increase.

### Peroxynitrite induced eNOS activation and NO production and Ca$^{2+}$ influx dependency

To evaluate the relationship between ONOO$^-$ and eNOS activity, NO was measured in ONOO$^-$ (50 µmol/L)-
perfused vessels \( (n = 4) \). Perfusion of vessels with ONOO\(^{-}\) induced an immediate increase in NO production with an initial peak rate at 31.9 ± 6.43% min\(^{-1}\) for 2 min followed by a rate of 6.5 ± 2.30% min\(^{-1}\) till 10 ± 1.4 min (Fig. 8C). This initial increase in FI\(_{\text{DAF}}\) (ΔFI%) was 332 ± 25% of baseline control. With continuous ONOO\(^{-}\) perfusion, a second peak of NO production occurred at ~18 min with a rate of 16.6 ± 4.50% min\(^{-1}\) and fell to 1.9 ± 0.10% min\(^{-1}\) at ~62 min (Fig. 8C). This second phase of FI increase (ΔFI%) was 222.0 ± 18.50% of the control (Fig. 8A,B). Control experiments in vessels perfused with albumin-Ringer solution \( (n = 3) \) only showed basal NO production at a rate of 0.1%/min (Fig. 8A,B).

By comparing ONOO\(^{-}\)-induced NO production with the time course of the increases in EC \([\text{Ca}\(^{2+}\)]\), we found that the initial increase in NO production occurred in the absence of increased EC \([\text{Ca}\(^{2+}\)]\), whereas the second phase of NO production occurred concomitantly with increases in EC \([\text{Ca}\(^{2+}\)]\) (Fig. 8C). To examine the role of \( \text{Ca}^{2+}\) influx in ONOO\(^{-}\)-induced NO production, NO was measured on four vessels pretreated with LaCl\(_3\) (50 µmol/L). As described above (Fig. 7A), LaCl\(_3\) prevented ONOO\(^{-}\)-induced increases in EC \([\text{Ca}^{2+}\]) Under the same experimental conditions as those EC \([\text{Ca}^{2+}\] were measured, NO measurements showed that inhibition of \( \text{Ca}^{2+}\) influx by \( \lambda\) had no effect on the ONOO\(^{-}\)-induced initial NO production, but attenuated the second phase of NO production (ΔFI% reduced from 222.0 ± 18.50% to 15.0 ± 3.25% of control, Fig. 7B,C). These data suggest that ONOO\(^{-}\)-induced initial NO does not require increased \( \text{Ca}^{2+}\) influx, but the second phase of NO production was initiated by the increased EC \([\text{Ca}^{2+}\]).
We also examined whether ONOO\(^{-}\)-induced NO production was a result of NOS activation. Treatment of vessels with NOS inhibitor, L-NMMA (2 mmol/L), 20 min before and during ONOO\(^{-}\) perfusion significantly decreased ONOO\(^{-}\)-induced NO production. \(F_{DAF}\) reduced from 570 ± 141.0% to 154 ± 50.0% of control, \((n = 4, \text{Fig. 8B})\).

**Discussion**

Our study, using quantitative measurement of microvessel permeability in combination with fluorescence and confocal microscopy, identified a novel mechanism by which \(H_2O_2\), a commonly known reactive oxygen species essential for the pathogeneses of cardiovascular diseases, interplays with superoxide, eNOS-derived NO, and ONOO\(^{-}\), leading to EC Ca\(^{2+}\) overload, vascular cell damage, and barrier dysfunction in intact vessels. \(H_2O_2\) at a pathophysiological relevant concentration (10 \(\mu\)mol/L) (Lacy et al., 2000; Banerjee et al., 2003) induced eNOS activation with a large amount of NO production. Instead of causing an immediate increase in microvessel permeability that occurred in PAF or other inflammatory mediator-stimulated microvessels (He et al., 1996; Zhu and He, 2005; Zhou and He, 2010), this large amount of NO (~10 times the amount of NO detected in PAF-perfused microvessels) was shown to be responsible for intracellular Ca\(^{2+}\) accumulation, subsequent cell apoptosis, and the delayed and progressive increases in microvessel permeability (Zhou et al., 2013). Concomitant with the excessive NO production, a marked production of superoxide, as well as extensive protein tyrosine nitration, a biomarker for ONOO\(^{-}\), were detected in \(H_2O_2\)-perfused vessels, indicating \(H_2O_2\)-induced ONOO\(^{-}\) formation in intact microvessels. Functionally, \(H_2O_2\)-induced increases in microvessel Lp was prevented by pretreatment of the vessels with uric acid, a strong ONOO\(^{-}\) scavenger, suggesting a causal relationship between ONOO\(^{-}\) and \(H_2O_2\)-induced microvessel barrier dysfunction. The direct evidence of the role of ONOO\(^{-}\) in \(H_2O_2\)-induced vascular barrier dysfunction was demonstrated by exogenously applied peroxynitrite to the microvessels, which showed a dose- and time-dependent increase in microvessel Lp, a pattern similar to that observed in \(H_2O_2\)-perfused vessels. Importantly, ONOO\(^{-}\), a downstream product derived from NO, is demonstrated to cause further eNOS activation and NO production. This reciprocal interaction between \(H_2O_2\), ONOO\(^{-}\), and eNOS constitues a self-promoted amplification of ONOO\(^{-}\). Taken together, this study provides the first in vivo evidence that ONOO\(^{-}\), derived from \(H_2O_2\)-induced NO and superoxide, further activates eNOS, leading to a self-promoted amplification of signaling cascades, resulting in \(H_2O_2\)-induced ONOO\(^{-}\)-mediated cell damage and microvessel barrier dysfunction. Details are illustrated in Fig. 9.

**Role of eNOS-derived NO production in the regulation of \(H_2O_2\)-induced microvessel barrier dysfunction**

Previous studies from our laboratory and others have demonstrated that eNOS-derived excessive NO plays an essential role in inflammatory mediators-induced vascular leakage (Mayhan, 1992; Yuan et al., 1993; He et al., 1997; Hatakeyama et al., 2006; Zhou and He, 2010; Zhou and
He, 2011). A typical inflammatory mediator-induced increase in microvessel permeability usually is immediate and transient, which is associated with the transient formation of endothelial gaps between adjacent ECs (Majno and Palade, 1961; Wu and Baldwin, 1992; Baluk et al., 1997; Jiang et al., 2008; Zhou and He, 2011). In contrast to the inflammatory mediator-induced immediate and transient increase in microvessel permeability, H₂O₂ caused a delayed and progressive increase in microvessel Lp in a dose- and time-dependent manner (Zhou et al., 2009; Zhou et al., 2013), indicating the involvement of different mechanisms. Our study demonstrated that H₂O₂ at pathophysiologically relevant concentrations (10 µmol/L) induce both Ca²⁺-independent and -dependent eNOS activation and the excessively produced NO triggers caspase activation and the impairment of intracellular Ca²⁺ homeostasis, leading to vascular cell apoptosis and consequently delayed and progressive increases in microvessel permeability (Zhou et al., 2013). In this case, the H₂O₂-induced initial NO production in the absence of increased

**Figure 8.** ONOO⁻ induced Ca²⁺ influx-independent and -dependent eNOS activation and NO production in perfused venules. (A) Representative DAF-2 fluorescent images in one individual vessel showing the changes in FIDAF-2 at selected time points upon ONOO⁻ (50 µmol/L) perfusion. Sodium nitroprusside (SNP, 50 mmol/L) was applied to the superfusate at the end of the experiment to ensure sufficiently loaded DAF-2 to react with NO. Scale bar indicates the FIDAF-2 in arbitrary unit. (B) Pooled cumulative FIDAF-2 curve in ONOO⁻-perfused vessels in the absence (n = 4) and presence of L-NMMA (2 mmol/L, n = 4) or LaCl₃ (50 µmol/L, n = 4). Ringer solution containing 1% BSA was applied as vehicle control (n = 3). C ONOO⁻-induced changes in NO production rate (df/dt, left Y axis) superimposed with the changes in EC [Ca²⁺] (right Y axis). *P = 0.015 (Two-way ANOVA).
EC \([\text{Ca}^{2+}]\), although being five times more than that observed in PAF-perfused vessels, did not cause immediate increase in microvessel permeability (Zhu and He, 2005; Zhou and He, 2010). These findings suggest that the excessive NO-mediated vascular barrier dysfunction may depend on the activation of other concurrent signaling pathways, such as increased EC \([\text{Ca}^{2+}]\), or the location of NO production (Sanchez et al., 2006), thus the pattern of NO-mediated increase in microvessel permeability is stimulus specific.

Our previous study illustrated that the \(\text{H}_2\text{O}_2\)-induced initial phase of eNOS activation is predominantly through increased phosphorylation at Ser\(^{1177}\) independent of elevated EC \([\text{Ca}^{2+}]\), whereas the second phase of eNOS activation is mainly triggered by increased EC \([\text{Ca}^{2+}]\), and \(\text{Ca}^{2+}/\text{CaM}\) binding (inhibited by caveolin-1, CAV-1), coordinated with decreased phosphorylation at Thr\(^{495}\) (Zhou et al., 2013). Thus, not only does the delayed increases in EC \([\text{Ca}^{2+}]\), contribute to EC \(\text{Ca}^{2+}\) overload, it also triggers a second phase of \(\text{Ca}^{2+}\)-dependent NO production, further amplifying the NO-dependent signaling cascade. This notion is supported by the fact that blocking NOS activation by L-NMMA prevented the delayed and progressive increases in EC \([\text{Ca}^{2+}]\) (Fig. 2C). The sustained increase in EC \([\text{Ca}^{2+}]\), an indication of impaired \(\text{Ca}^{2+}\) homeostasis, appears to be essential for vascular cell apoptosis and subsequently increased microvessel permeability.

**Role of NO-derived ONOO\(^-\) in \(\text{H}_2\text{O}_2\)-induced increases in microvessel \(\text{Lp}\)**

Although NO has been reported to have potential cytotoxic effects, many of them are attributed to its oxidation product, particularly ONOO\(^-\) rather than NO itself (Xia et al., 1996; Riobo et al., 2001; Pacher et al., 2007). NO reacts rapidly with superoxide to form ONOO\(^-\), a strong oxidant, capable of oxidizing and nitrating lipid, protein, and DNA, resulting in cell death and tissue injury (Radi et al., 1991; Pacher et al., 2007; Szabo et al., 2007). Consistent with this concept, our results support that \(\text{H}_2\text{O}_2\)-induced excessive NO-derived ONOO\(^-\) is the key contributing to increased microvessel permeability in intact rat venules. This conclusion is supported by the following evidence: (1) \(\text{H}_2\text{O}_2\) induced concomitant NO and superoxide production, the two elements required to form ONOO\(^-\), (2) \(\text{H}_2\text{O}_2\) induced NO-dependent extensive protein tyrosine nitration (a commonly recognized biomarker for ONOO\(^-\)) in the vascular wall, (3) uric acid, an endogenous ONOO\(^-\) scavenger (Ames et al., 1981; Hoover et al., 1998; Tsukada et al., 2000; Walford et al., 2004; Kuzkaya et al., 2005), prevented \(\text{H}_2\text{O}_2\)-induced impairment of \(\text{Ca}^{2+}\) homeostasis and increases in microvessel permeability; and (4) exogenously applied ONOO\(^-\) induced a delayed and progressive increases in EC \([\text{Ca}^{2+}]\) and microvessel \(\text{Lp}\) in a dose- and time-dependent manner, a pattern similar to that observed in \(\text{H}_2\text{O}_2\)-perfused vessels.

The \(\text{H}_2\text{O}_2\)-induced superoxide production has been considered as an important feed-forward mechanism for ROS-mediated pathogenesis of cardiovascular diseases (Cai, 2005). In vitro studies showed that inhibition of p22\(^{\text{phox}}\) by siRNA and knockout of gp91\(^{\text{phox}}\) (the two transmembrane subunits of NAD(P)H oxidase) prevented the \(\text{H}_2\text{O}_2\)-induced increases in superoxide production in vascular endothelial cells (Li et al., 2001; Djordjevic et al., 2005). Using confocal microscopy, we detected a threefold increase in DHE FI in the nuclei of both ECs and pericytes as early as 30 min of \(\text{H}_2\text{O}_2\) perfusion, indicating a large amount of superoxide production (Fig. 4). Within the same period, NO production increased eightfold...
(indicated by the increased FlDAF-2, Fig. 2B). When both NO and superoxide are produced in close proximity, a spontaneous ONOO⁻ formation is expected in the H₂O₂ perfused vessels. Our results that DPI, a commonly used NAD(P)H oxidase inhibitor, prevented the increases in superoxide production in both endothelium and pericytes in H₂O₂-perfused intact vessels (Fig. 4) support NAD(P) H oxidase as the primary source for the H₂O₂-induced superoxide production.

More direct evidence of H₂O₂-induced ONOO⁻ formation is the extensive NO-dependent tyrosine nitration detected after 2-h H₂O₂ perfusion (Fig. 3). Nitrotyrosine has been recognized as an important biomarker for ONOO⁻ and an indication of systemic nitrooxidative stress (Prutz et al., 1985; Reiter et al., 2000; Radi, 2004; Radi, 2013).

The application of uric acid, an endogenous ONOO⁻ scavenger (Hooper et al., 1998; Tsukada et al., 2000; Walford et al., 2004; Kuzkaya et al., 2005), that abolished the H₂O₂-induced increases in EC [Ca²⁺]i (Fig. 5B,C) and microvessel Lp (Fig. 5A,C) further support a role of ONOO⁻ in H₂O₂-mediated changes in microvessels. Furthermore, the direct perfusion of ONOO⁻ in microvessels showed delayed and progressive increases in both EC [Ca²⁺]i and microvessel Lp in a dose- and time-dependent manner (Fig. 6), a similar pattern to that observed in H₂O₂-perfused vessels (Zhou et al., 2009; Zhou et al., 2013), and inhibition of Ca²⁺ influx with LaCl₃ prevented Ca²⁺ accumulation in ECs and the increased Lp in peroxynitrite-perfused vessels (Fig. 7). These results demonstrate a direct role of ONOO⁻ in the impairment of Ca²⁺ homeostasis and vascular barrier dysfunction.

**Interaction between ONOO⁻ and eNOS-derived NO production: a self-promoted amplification, contributing to ONOO⁻-mediated vascular damage and barrier dysfunction**

Currently, the role of ONOO⁻ in eNOS activity, particularly under in vivo conditions, remains unknown. While some early studies reported that ONOO⁻ induced coronary vasodilation in isolated rat hearts and coronary arterial rings of dogs through increased NO production (Liu et al., 1994; Villa et al., 1994), other studies on cultured bovine ECs indicated that ONOO⁻-induced eNOS phosphorylation at Ser1179 (corresponding to Ser1176 in human and rats) increased O₂⁻ production, rather than that of NO (Zou et al., 2002; Zou et al., 2002). Using quantitative fluorescent imaging in intact venules, our study demonstrated that ONOO⁻ induced a large amount of NO that was abolished by L-NMMA, indicating eNOS-derived NO production (Fig. 7). Similar to what have been observed in H₂O₂-perfused vessels (Shen et al., 1998), ONOO⁻ induced two phases of eNOS-derived NO production. As shown in Fig. 8C, the initial NO production with a higher peak rate occurred in the absence of increases in EC [Ca²⁺]i, whereas the second phase NO production is slow and concomitant with progressively increased EC [Ca²⁺]i. Blockade of Ca²⁺ influx by LaCl₃ did not affect the initial NO production, but abolished the NO production in the second phase (Fig 8B,C), suggesting that peroxynitrite-induced initial eNOS activation is independent on Ca²⁺ increase, whereas the second phase of eNOS activation is Ca²⁺ dependent. This interesting finding suggests that ONOO⁻, a downstream product formed by NO and superoxide, further amplified NO production, resulting in an amplification of NO-dependent signaling and augmented ONOO⁻-mediated vascular cell damage and barrier dysfunction.

**Methodology assessment**

Vascular barrier function is usually referred to vascular permeability to both fluid and solutes. In this study, microvascular permeability is determined by measuring hydraulic conductivity, Lp, one of the three permeability coefficients that assesses the transport of fluid. Although microvessel permeability to different sizes of solute was not measured, experimental evidence showed that Lp well correlates with the transports of small- to intermediate-sized molecules (Michel and Curry, 1999). Importantly, most of the increases in solute flux (including large molecules) are coupled to water flows. When permeability increases, the magnitude changes in fluid or small molecules could be different from macromolecules, depending on transport pathways involved, they usually followed the same time course as the changes in Lp (Rutledge et al., 1990; Rutledge et al., 1995; Michel and Curry, 1999). The permeability coefficients are more quantitative and accurate measures of vascular wall properties than the commonly used flux or leaky sites, since they are not affected by unknown variations of hemodynamic factors and surface area for transport (Michel and Curry, 1999). Importantly, all the studies presented here were conducted in individually perfused intact microvessels under well-controlled conditions, and the changes in NO, superoxide, ONOO⁻, and EC [Ca²⁺]i were all measured under the same experimental conditions similar to those where Lp was measured, which enabled us to directly correlate the changes in signaling pathways with microvessel permeability.

Another advantage of using individually perfused vessel to investigate the effects of reactive reagents is that the study containing H₂O₂ or ONOO⁻ in the perfusion pipette constantly refreshes the perfusate in the vessel lumen, which provides a relatively constant concentration.
of H$_2$O$_2$ or ONOO$^-$ to the cells lining vascular walls. This constantly refreshed perfusate makes a fundamental difference from the one time addition of reactive reagents to the tissue or cell culture medium that results in an immediate reaction of the reactive agents with the cells and other components in the medium and lost its activity quickly. In those studies, usually hundreds of µmol/L of mmol/L concentrations of peroxide (far more than the pathological concentrations detected in the plasma) were used in order to show some of the responses (Alexander et al., 2000; Kevil et al., 2000; Lee et al., 2006; Jin et al., 2012; Suresh et al., 2015). We also knew that H$_2$O$_2$ or ONOO$^-$ even reacts with marker cells (about 1% RBCs) in the perfusate and affects the effectiveness of the treatment. That is why we perfuse the vessel without marker cells for a certain time period and only add the marker cells during Lp measurements. This also explains why we can get the responses with pathological range of plasma H$_2$O$_2$ concentrations (10 µmol/L). Based on our current (Figs 2, 6, 7) and previous studies (He et al., 1996; Zhou et al., 2013), the cell marker-free perfusate in the presence of 1% BSA is able to well maintain the baseline Lp, EC and basal NO up to 2-h perfusion period which we have tested.

Compared to normal blood, our perfused vessels lack blood cells, plasma SOD, catalase, and substances released from blood cells, which could affect the production of ROS, reactive nitrogen species (RNS), and NO. Under diseased conditions, measured plasma peroxide levels are close to 10 µmol/L (Lacy et al., 2000). We consider that the detected plasma H$_2$O$_2$ level is the effective level of peroxide that affects endothelial cells lining the vascular walls and is the net balance of the constant production of ROS and its reaction with plasma enzymes such as SOD and catalase in the vasculature. Our experimental conditions bypassed the constant ROS production and catalytic processes when blood is circulating in the vasculature, and applied this pathologically relevant net plasma H$_2$O$_2$ concentration to the vessel lumen, which allows us to investigate its direct effect on vascular walls and avoid other confounding factors.

The use of DHE to detect intracellular formation of superoxide has been assessed and characterized by many studies. The formation of 2-hydroxyethidium is considered as the specific reaction of DHE with superoxide and not with other biologically relevant oxidants, which can be separated from its parent DHE and ethidium (Zielonka et al., 2008), including vessel segment studies and confirmed with high performance liquid chromatography (HPLC, Dikalov et al., 2007). The advantage of using the DHE fluorescence imaging in our perfused intact vessels is its ability to provide spatial detection of superoxide production at vascular walls using the same experimental approach where other signaling changes and microvessel permeability are measured, which could not be achieved with other methods. The pharmacological inhibitors used in this study such as DPI and uric acid showed consistent results as NADPH oxidase inhibitor and ONOO$^-$ scavenger (Hooper et al., 1998; Kean et al., 2000; Kim et al., 2018; Pietruczuk et al., 2019), respectively, even though they may have nonspecific effects. These additional supporting evidence, together with the results of DHE staining and the nitrotyrosine immunostaining provided multiple lines of evidence to support the H$_2$O$_2$-induced superoxide production and subsequent ONOO$^-$ formation.

One potential concern is the specificity of using DAF-2 to measure NO in the presence of the reactive oxidants (Jourd’heuil, 2002). DAF-2 DA has been used to measure intracellular NO in a wide range of biological systems. However, since the interaction of DAF-2 with NO to form DAF-2 triazole form (DAF-2T, high fluorescence product) requires the presence of O$_2$, it raises concerns about the potential effects of oxidants on DAF-2T conversion (Jourd’heuil, 2002; Wardman, 2007; Hall and Garthwaite, 2009). To address this issue, we have carefully characterized this NO probe in individually perfused vessels and demonstrated the cautious procedures to avoid non-NO-related DAF-2 FI (Zhou and He, 2011). We also assess the specificity of DAF-2 by measuring its direct reaction with H$_2$O$_2$, superoxide (generated by HX/XO), and ONOO$^-$ in solution using the same imaging system when NO was measured in intact microvessels. The results shown in Fig 1 demonstrated that DAF-2 (10 µmol/L, closer to intracellular DAF-2 concentration when 5 µmol/L DAF-2 DA was used to load vessels) does not directly interact with ROS and ONOO$^-$ in the absence of NO within the reagents’ concentrations we used in the experiments, although at this point we may not exclude the possibility that the presence of oxidants may facilitate the DAF-2T formation with increased flux of NO and enhance DAF-2 FI. Our results show that the use of NOS inhibitor completely blocked the H$_2$O$_2$- or ONOO$^-$-induced increases in DAF-2 FI indicating a strong association between DAF-2T and increased NO production. We consider that DAF-2 is the most reliable specific NO indicator comparing with other up to date methods. Since the H$_2$O$_2$- and ONOO$^-$-induced DAF-2 FI changes are in large magnitude, some contributions from oxidant effect will not change the nature of our findings. Especially, our previous studies have provided the validation of the NO measurements with DAF-2 by conducting parallel studies with immunostaining of eNOS activation (Zhou et al., 2013).

In conclusion, our study uncovered a novel mechanism of elevated H$_2$O$_2$-mediated microvascular dysfunction.
that commonly occurs in many cardiovascular diseases. H$_2$O$_2$ at pathophysiological relevant concentrations, induces a large amount of eNOS-derived NO concomitant with increased superoxide production, and subsequent formation of ONOO$^-$, a more potent reactive nitrogen species (RNS). The formed ONOO$^-$ is able to further activate eNOS and augment NO production, resulting in a self-promoted augmentation of nitrooxidative stress and subsequent vascular cell damage and vascular barrier dysfunction. The signaling cascades and sequential functional changes illustrated in Fig. 9 offer novel insight into ROS and/or RNS-mediated pathogenesis of cardiovascular diseases.

**Conflict of Interest**

The authors declare no competing financial interests.

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