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**H₂O₂-induced microvessel barrier dysfunction: the interplay between reactive oxygen species, nitric oxide, and peroxynitrite**

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**Abstract**

Elevated H₂O₂ is implicated in many cardiovascular diseases. We previously demonstrated that H₂O₂-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 H₂O₂-induced vascular barrier dysfunction by elucidating the interrelationships between H₂O₂-induced NO, superoxide, ONOO⁻, and changes in endothelial [Ca²⁺]i and microvessel permeability. Experiments were conducted on intact rat mesenteric venules. Microvessel permeability was determined by measuring hydraulic conductivity (Lp). Endothelial [Ca²⁺]i, NO, and O₂⁻/O₂ were assessed with fluorescence imaging. Perfusion of vessels with H₂O₂ (10 µmol/L) induced marked productions of NO and O₂⁻, resulting in extensive protein tyrosine nitration, a biomarker of ONOO⁻. The formation of ONOO⁻ was abolished by inhibition of NOS with NG-Methyl-L-arginine. Blocking NO production or scavenging ONOO⁻ by uric acid prevented H₂O₂-induced increases in endothelial [Ca²⁺]i and Lp. Additionally, the application of exogenous ONOO⁻ to microvessels induced delayed and progressive increases in endothelial [Ca²⁺]i and microvessel Lp, a pattern similar to that observed in H₂O₂-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 H₂O₂-induced endothelial Ca²⁺ 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 (H₂O₂) at a pathophysiologically relevant concentration (10 µmol/L) induced delayed and progressive increases in endothelial calcium concentration ([Ca²⁺]i) and microvessel permeability that was attributed to H₂O₂-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

Zhou et al.

He, 2005; Hatakeyama et al., 2006; Zhou and He, 2010; Zhou and He, 2011; Mundi et al., 2017. Our previous studies demonstrated that, instead of causing immediate and transient increases in microvessel permeability as observed in inflammatory mediator-stimulated vessels (He et al., 1996; Baluk et al., 1997; Zhu and He, 2005; Jiang et al., 2008; Zhou and He, 2011), large amounts of H₂O₂-induced NO production resulted in caspase activation and impairment of Ca²⁺ 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 H₂O₂-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; Riobo et al., 2001; Pacher et al., 2007). NO reacts rapidly with superoxide (O₂⁻) at a diffusion-controlled rate (−1 × 10¹⁰ M⁻¹ 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, H₂O₂ has been shown to induce further production of O₂⁻ and H₂O₂ 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 H₂O₂-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 vasodilation 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 Ser¹¹⁷⁹, it increased O₂⁻ 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 H₂O₂-induced microvessel barrier dysfunction by elucidating the interrelationships between H₂O₂, eNOS-derived NO, O₂⁻, ONOO⁻, and the changes in EC [Ca²⁺]i and microvessel permeability. Experiments were conducted on individually perfused mesenteric venules with intact surrounding circulation. EC [Ca²⁺]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 H₂O₂-induced changes in EC [Ca²⁺]i, NO production, and microvessel Lp. Under the same experimental conditions, H₂O₂-induced O₂⁻ production was assessed using dihydroethidium. The formation of ONOO⁻ was examined by fluorescent immunostaining of nitrotyrosine, a biomarker of ONOO⁻, in H₂O₂-perfused vessels. The functional relationship between ONOO⁻ and changes in EC [Ca²⁺]i, and microvessel permeability was further investigated using uric acid, an endogenous ONOO⁻ scavenger. The direct effects of exogenously applied ONOO⁻ on EC [Ca²⁺]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 cmH₂O), 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 Lp{\text{test}}/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 H2O2 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 [Ca^{2+}]i**

EC [Ca^{2+}]i was measured in individually perfused microvessels using the fluorescent 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 μmol/L of fura 2-AM for 45 min. The vessel was then cannulates 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 μm 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 ± 5 and 380 ± 5 nm; Oriel), and the emission was separated with a dichroic mirror (DM400) and a wide-band interference filter (505/50 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 μmol/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 mmol/L) was applied to the superfusate to ensure sufficient intracellular DAF-2 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 auto-fluorescence 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 FI0 (FI/FI0*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 (10 μmol/L) with albumin-Ringer solution, H2O2 (5, 50 and 500 μmol/L), SNP (20 and 200 μmol/L), hypoxanthine/xanthine oxidase (0.05 mmol/L /1 mU, 0.5 mmol/L /10 mU, and 5 mmol/L /100 mU), or peroxynitrite (5, 50, and 500 μmol/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 μmol/L that caused an accumulative increase in FI{\text{DAF}}, indicating the specificity of DAF-2 as a NO fluorescent indicator. Peroxynitrite at 500 μmol/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.

**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 rig were the same as that used for 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 (505 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 μmol/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 mmol/L) was applied to the superfusate to ensure sufficient intracellular DAF-2 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 auto-fluorescence 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 FI0 (FI/FI0*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 (10 μmol/L) with albumin-Ringer solution, H2O2 (5, 50 and 500 μmol/L), SNP (20 and 200 μmol/L), hypoxanthine/xanthine oxidase (0.05 mmol/L /1 mU, 0.5 mmol/L /10 mU, and 5 mmol/L /100 mU), or peroxynitrite (5, 50, and 500 μmol/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 μmol/L that caused an accumulative increase in FI{\text{DAF}}, indicating the specificity of DAF-2 as a NO fluorescent indicator. Peroxynitrite at 500 μmol/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_2O_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_2O_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, diphenylethene iodinum (DPI, an NADPH oxidase inhibitor, 20 μmol/L) was applied to the vessel for 20 min after DHE loading, followed by perfusion with H_2O_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_2, 1.2 MgSO_4, 5.5 glucose, 5.0 NaHCO_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_2O_2 (30%), DAF-2, DAF-2 DA, LaCl_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^- and degraded ONOO^- 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
t-test was used for paired data analysis, that is, control and treatment were conducted in the same vessel. Unpaired t-test with Welch’s correction was used for data comparison between two groups, and one-way or two-way ANOVA with Tukey post hoc test was applied for multiple group comparison. A probability value of $P < 0.05$ was considered as statistically significant, and the exact or the range of $p$ values is presented in figure legends.

**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 FI$_{DAF}$-2 pooled from four vessels). Since the FI$_{DAF}$ increase in reaction to NO is cumulative (irreversible chemical conversion), the greater slope of the FI$_{DAF}$ 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 FI$_{DAF}$ that reached 425 ± 18.1% of the baseline level in 5 min, followed by a lower sustained level. A second increase in FI$_{DAF}$ was observed after 20 min of H$_2$O$_2$ perfusion with 280 ± 41% additional increase in FI$_{DAF}$, and reached a plateau level at 41 min of H$_2$O$_2$ perfusion with a total of 830 ± 85% increase in FI$_{DAF}$. In another four DAF-2 DA-loaded vessels, perfusion of albumin-Ringer solution as a control showed approximately 1% increase per minute in FI$_{DAF}$, 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$^{2+}$]$_i$ 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 $(207 \pm 47.1$ 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$^{2+}$]$_i$. The mean EC [Ca$^{2+}$]$_i$ 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$^{2+}$]$_i$, 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$^{2+}$]$_i$ did not increase until 15 min later, we predict that, if they are related, the delayed increase in EC [Ca$^{2+}$]$_i$ 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$^{2+}$]$_i$ (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 lining microvessels with 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 µmol/L), which was abolished by pretreatment of the vessels with an inhibitor of NADPH oxidase, diphenylene iodonium (DPI, 20 µmol/L). The mean FIs of DHE were 48 ± 2.5, 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, \(P < 0.0001\), H₂O₂ group vs. control and H₂O₂ plus DPI group, respectively).
Role of ONOO\(^{-}\) in \(\text{H}_2\text{O}_2\)-induced increases in microvessel Lp and endothelial [Ca\(^{2+}\)]

To examine whether NO-derived ONOO\(^{-}\) accounts for the \(\text{H}_2\text{O}_2\)-induced increases in EC [Ca\(^{2+}\)], and microvessel Lp, the effects of \(\text{H}_2\text{O}_2\) on EC [Ca\(^{2+}\)], and microvessel Lp were investigated with the application of uric acid, a ONOO\(^{-}\) scavenger. Uric acid (50 \(\mu\text{mol/L}\)) perfusion for 20 min did not alter the baseline EC [Ca\(^{2+}\)] (69 \(\pm\) 4.7 vs. 85 \(\pm\) 4.1 nmol/L) and Lp (2.3 \(\pm\) 0.34 vs. 2.5 \(\pm\) 0.19 \(\times\) 10\(^{-7}\) cm\(^{-1}\) cm\(H_2O_2\)^{-1}, \(P > 0.8\), \(n = 4\) for each group), but significantly reduced \(\text{H}_2\text{O}_2\)-induced increase in EC [Ca\(^{2+}\)] from a mean peak value of 403 \(\pm\) 43.0 (\(n = 5\)) to 139 \(\pm\) 30.6 nmol/L (\(n = 4\), \(P = 0.002\)), and completely prevented the \(\text{H}_2\text{O}_2\)-induced increase in Lp (Lp\(_{\text{test}}\)/Lp\(_{\text{control}}\)) from 9.3 \(\pm\) 1.34 (\(n = 5\)) to 1.0 \(\pm\) 0.08 times the control level (\(n = 4\), \(P = 0.003\)). All values are compared after 2-h perfusion of \(\text{H}_2\text{O}_2\) (10 \(\mu\text{mol/L}\)). Fig. 5A,B show the representative experiments and Fig. 5C shows the results summary.
Peroxynitrite induced delayed and progressive increases in microvessel Lp in a dose- and time-dependent manner, a pattern similar to that observed in H₂O₂-
perfused vessels

To investigate the direct effect of ONOO⁻ on microvessel permeability, we perfused ONOO⁻ to microvessels and measured the changes in Lp. The mean baseline Lp was 2.1 ± 0.16 × 10⁻⁷ cm s⁻¹ cmH₂O⁻¹ (n = 25). No changes in Lp were observed with ONOO⁻ perfusion for 30 min at 10 and 50 µmol/L (Lp_test/Lp_control was 1.0 ± 0.02 and 1.0 ± 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 H₂O₂-perfused vessels (Fig 2A). Lp increased significantly to 2.8 ± 0.45 (P = 0.03) and 5.9 ± 0.44 (P = 0.003) times the control at 10 µmol/L, and to 6.0 ± 0.57 and 12.0 ± 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 [Ca²⁺]i contributed to the increases in microvessel Lp

The causal relationship between ONOO⁻ and EC [Ca²⁺]i, was further examined in seven vessels loaded with fura-2. Baseline EC [Ca²⁺]i was 82 ± 12.0 nmol/L. A significant increase in EC [Ca²⁺]i, occurred at 20 ± 4.0 min of ONOO⁻ perfusion (50 µmol/L), and progressively reached to a sustained level of 375 ± 30.0 nmol/L at 78 ± 2.3 min of ONOO⁻ perfusion (n = 6, Fig. 7A). No changes in EC [Ca²⁺]i were observed on vessels perfused with albumin-Ringer for 1 h (n = 3, data not shown). Preperfusion of vessels with LaCl₃ (50 µmol/L) for 20 min did not affect baseline EC [Ca²⁺]i, but prevented ONOO⁻-induced increases in EC [Ca²⁺]i (the mean EC [Ca²⁺]i, was 98 ± 15.2 nmol/L at 80 min of ONOO⁻ perfusion (n = 3, P = 0.0027, Fig. 7A), indicating the role of Ca²⁺ influx in ONOO⁻-induced increase in EC [Ca²⁺]i).

The role of Ca²⁺ influx in ONOO⁻-induced Lp increase was then examined. The application of LaCl₃, prevented the increased Lp in ONOO⁻ (50 µmol/L, 1 h)-perfused vessels. The Lp_test/Lp_control reduced from 6.0 ± 0.57 (n = 6) to 1.1 ± 0.18 times the control (n = 4, P = 0.0002, Fig. 7B), indicating that the sustained increase in EC [Ca²⁺]i, to be responsible for ONOO⁻-induced Lp increase.

Peroxynitrite induced eNOS activation and NO production and Ca²⁺ 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\(_{DAF}\) \((\DeltaFI\%)\) 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 \((\DeltaFI\%)\) 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 \([Ca^{2+}]_{i}\) \(, \) we found that the initial increase in NO production occurred in the absence of increased EC \([Ca^{2+}]_{i}\), whereas the second phase of NO production occurred concomitantly with increases in EC \([Ca^{2+}]_{i}\) \(, \) (Fig. 8C). To examine the role of Ca\(^{2+}\) influx in ONOO\(^-\)-induced NO production, NO was measured on four vessels pretreated with LaCl\(_3\) \( (50 \mu mol/L) \). As described above (Fig. 7A), LaCl\(_3\) prevented ONOO\(^-\)-induced increases in EC \([Ca^{2+}]_{i}\). Under the same experimental conditions as those EC \([Ca^{2+}]_{i}\) were measured, NO measurements showed that inhibition of Ca\(^{2+}\) influx by \( \) had no effect on the ONOO\(^-\)-induced initial NO production, but attenuated the second phase of NO production \((\DeltaFI\% \) 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 Ca\(^{2+}\) influx, but the second phase of NO production was initiated by the increased EC \([Ca^{2+}]_{i}\).
Role of ROS and RNS in microvessel barrier dysfunction

Zhou et al.

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. \(F_{\text{DAF}}\) 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 pathogenesis 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 constitutes 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, H$_2$O$_2$ 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 H$_2$O$_2$ at pathophysiologically relevant concentrations (10 µmol/L) induce both Ca$^{2+}$-independent and -dependent eNOS activation and the excessively produced NO triggers caspase activation and the impairment of intracellular Ca$^{2+}$ homeostasis, leading to vascular cell apoptosis and consequently delayed and progressive increases in microvessel permeability (Zhou et al., 2013). In this case, the H$_2$O$_2$-induced initial NO production in the absence of increased 

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**Figure 8.** ONOO$^-$/CO induced Ca$^{2+}$ 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 $F_{DAF-2}$ at selected time points upon ONOO$^-$/CO (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 $F_{DAF-2}$ in arbitrary unit. (B) Pooled cumulative $F_{DAF-2}$ curve in ONOO$^-$/CO-perfused vessels in the absence ($n=4$) and presence of L-NMMA (2 mmol/L, $n=4$) or LaCl$_3$ (50 µmol/L, $n=4$). Ringer solution containing 1% BSA was applied as vehicle control ($n=3$). C) ONOO$^-$/CO-induced changes in NO production rate (df/dt, left Y axis) superimposed with the changes in EC [Ca$^{2+}$], (right Y axis). *$P=0.015$ (Two-way ANOVA)
EC [Ca\textsuperscript{2+}]i, 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 [Ca\textsuperscript{2+}]i, 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 H\textsubscript{2}O\textsubscript{2}-induced initial phase of eNOS activation is predominantly through increased phosphorylation at Ser\textsuperscript{1177} independent of elevated EC [Ca\textsuperscript{2+}]i, whereas the second phase of eNOS activation is mainly triggered by increased EC [Ca\textsuperscript{2+}]i and Ca\textsuperscript{2+}/CaM binding (inhibited by caveolin-1, CAV-1), coordinated with decreased phosphorylation at Thr\textsuperscript{495} (Zhou et al., 2013). Thus, not only does the delayed increases in EC [Ca\textsuperscript{2+}]i contribute to EC Ca\textsuperscript{2+} overload, it also triggers a second phase of Ca\textsuperscript{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 [Ca\textsuperscript{2+}]i, (Fig. 2C). The sustained increase in EC [Ca\textsuperscript{2+}]i, an indication of impaired Ca\textsuperscript{2+} homeostasis, appears to be essential for vascular cell apoptosis and subsequently increased microvessel permeability.

**Role of NO-derived ONOO\textsuperscript{-} in H\textsubscript{2}O\textsubscript{2}-induced increases in microvessel Lp**

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

The H\textsubscript{2}O\textsubscript{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\textsuperscript{phox} by siRNA and knockout of gp91\textsuperscript{phox} (the two transmembrane subunits of NAD(P)H oxidase) prevented the H\textsubscript{2}O\textsubscript{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 H\textsubscript{2}O\textsubscript{2} perfusion, indicating a large amount of superoxide production (Fig. 4). Within the same period, NO production increased ~eightfold.

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**Figure 9.** Schematic diagram illustrating the mechanisms of H\textsubscript{2}O\textsubscript{2}-induced microvessel barrier dysfunction. H\textsubscript{2}O\textsubscript{2} at pathophysiological levels activate eNOS and NADPH oxidase in the endothelium and pericytes, leading to increased formation of NO and O\textsubscript{2}\textsuperscript{-}. The temporal and spatial proximity of largely produced NO and O\textsubscript{2}\textsuperscript{-} promote the formation of ONOO\textsuperscript{-}, a more potent oxidant, leading to ONOO\textsuperscript{-}-mediated lipid peroxidation, protein tyrosine nitration, subsequent impairment of intracellular Ca\textsuperscript{2+} homeostasis, and vascular cell apoptosis. Both NO-derived ONOO\textsuperscript{-} and the delayed increases in EC [Ca\textsuperscript{2+}]i, are able to further activate eNOS, resulting in amplified NO production and NO-dependent downstream cascade. These positive feedback loops, indicated by red dotted lines, play an important role in sustained production of ONOO\textsuperscript{-}, and eventually ONOO\textsuperscript{-}-mediated endothelial cell Ca\textsuperscript{2+} overload, vascular cell apoptosis, and progressively increased microvessel permeability. The application of inhibitors (shaded boxes) blocks the downstream events. This diagram include both previous (Zhou et al., 2009; Zhou et al., 2013) and current studies. DPI (diphenylene iodonium, NADPH oxidase inhibitor); eNOS: endothelial nitric oxide synthase; CAV-1: Caveolin-1 (eNOS inhibitor); z-VAD-FMK: caspase inhibitor.
(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 H\(_2\)O\(_2\)-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 H\(_2\)O\(_2\)-perfused intact vessels (Fig. 4) support NAD(P)H oxidase as the primary source for the H\(_2\)O\(_2\)-induced superoxide production.

More direct evidence of H\(_2\)O\(_2\)-induced ONOO\(^{-}\) formation is the extensive NO-dependent tyrosine nitration detected after 2-h H\(_2\)O\(_2\) perfusion (Fig. 3). Nitrotyrosine has been recognized as an important biomarker for ONOO\(^{-}\) and an indication of systemic nitrosative 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 H\(_2\)O\(_2\)-induced increases in EC [Ca\(^{2+}\)]\(_i\) (Fig. 5B,C) and microvessel Lp (Fig. 5A,C) further support a role of ONOO\(^{-}\) in H\(_2\)O\(_2\)-mediated changes in microvessels. Furthermore, the direct perfusion of ONOO\(^{-}\) in microvessels showed delayed and progressive increases in both EC [Ca\(^{2+}\)]\(_i\), and microvessel Lp in a dose- and time-dependent manner (Fig. 6), a similar pattern to that observed in H\(_2\)O\(_2\)-perfused vessels (Zhou et al., 2009; Zhou et al., 2013), and inhibition of Ca\(^{2+}\) influx with LaCl\(_3\) prevented Ca\(^{2+}\) 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 Ca\(^{2+}\)-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 Ser\(^{1179}\) (corresponding to Ser\(^{1176}\) in human and rats) increased O\(_2^*\) 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 H\(_2\)O\(_2\)-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 [Ca\(^{2+}\)]\(_i\), whereas the second phase NO production is slow and concomitant with progressively increased EC [Ca\(^{2+}\)]. Blockade of Ca\(^{2+}\) influx by LaCl\(_3\) 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 Ca\(^{2+}\) increase, whereas the second phase of eNOS activation is Ca\(^{2+}\) 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 [Ca\(^{2+}\)]\(_i\) 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 solution containing H\(_2\)O\(_2\) or ONOO\(^{-}\) in the perfusion pipette constantly refreshes the perfusate in the vessel lumen, which provides a relatively constant concentration
of \( \text{H}_2\text{O}_2 \) or \( \text{ONOO}^- \) 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 \( \mu\text{mol/L} \) or \( \text{mmol/L} \) 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 \( \text{H}_2\text{O}_2 \) or \( \text{ONOO}^- \) 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 \( \text{H}_2\text{O}_2 \) concentrations (10 \( \mu\text{mol/L} \)). 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 previous studies (He et al., 1996; Zhou et al., 2000; Kevil et al., 2000; Lee et al., 2006; Jin 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 \( \text{H}_2\text{O}_2 \)-induced superoxide production and subsequent \( \text{ONOO}^- \) 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 fluorescence. We also assess the specificity of DAF-2 by measuring its direct reaction with \( \text{H}_2\text{O}_2 \), 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 \( \mu\text{mol/L} \), closer to intracellular DAF-2 concentration when 5 \( \mu\text{mol/L} \) 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 fluorescence. Our results show that the use of NOS inhibitor completely blocked the \( \text{H}_2\text{O}_2 \) - or ONOO\(^-\) -induced increases in DAF-2 fluorescence 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 \( \text{H}_2\text{O}_2 \) - and ONOO\(^-\) -induced DAF-2 fluorescence 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 \( \text{H}_2\text{O}_2 \)-mediated microvascular dysfunction
that commonly occurs in many cardiovascular diseases. H$_2$O$_2$ at pathophysiologically 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.

**References**


