Reactive oxygen species cause endothelial dysfunction in chronic flow overload

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FLOW OVERLOAD (FO) occurs when the blood flow rate in blood vessels is elevated over its basal physiological level. FO may occur physiologically during exercise or pregnancy and pathophysiological in arteriovenous fistula, contralateral stenosis, or cardiac hypertrophy (1, 6, 8, 15, 19, 48). Since flow-induced shear stress on the endothelium regulates vascular function and dictates structural homeostasis of the blood vessel wall, the blood flow rate is closely regulated. FO in an artery elicits an acute endothelium-derived vasodilatation mediated by endothelial nitric oxide (NO), prostacyclin (PGI2), endothelium-derived hyperpolarizing factor (EDHF) (3, 9, 37, 42), and other signaling molecules to mediate specific cellular responses in the vasculature, including activating matrix metalloproteinases (MMPs), vascular remodeling, vascular smooth muscle cell (VSMC) hypertrophy, and cellular apoptosis (6, 28, 34, 43, 47, 51). Furthermore, O2− production may lead to hydrogen peroxide (H2O2) formation, which is thought to regulate potassium channels (23). In the vasculature, there may be several sources of O2− such as NADPH oxidase, xanthine oxidase, mitochondria, and endothelial NO synthase (eNOS) when uncoupled (30).

The increase in ROS generation in hypertension, diabetes, hypercholesterolemia, atherosclerosis, and ischemia is considered deleterious to the vasculature (2, 5, 11, 12, 20, 25, 27, 33, 44). NADPH oxidase has been identified as a major source of ROS in blood vessels in response to chemical (e.g., hypercholesterolemia and diabetes) or physical (e.g., hypertension and oscillatory shear stress) stimuli (5, 7, 17, 20, 28, 44). The increase in ROS production from NADPH oxidase, as occurs in hypertension, atherosclerosis, and diabetes, attenuates endothelium-dependent vasodilatation and causes endothelial dysfunction (5, 20, 32, 41, 49, 53).

Although the increase in ROS production in CFO has been established in both conduit artery and various small arteries (6, 22, 23, 47) and endothelial dysfunction in CFO has also been found in mesenteric artery of obese rats (4), the effect of CFO on endothelial function of elastic (carotid) artery of large animal is unknown. Hence, we hypothesize that 1) the ROS increase compromises endothelial function in CFO and 2) the source of ROS is, in part, due to NADPH oxidase. To test these hypotheses, an intact porcine carotid artery was exposed to CFO for 1 wk by a contralateral ligation. ROS was detected with the spin trap N-tert-butyl-α-phenylnitrone (PBN) by electron paramagnetic resonance (EPR) spectroscopy. The production of ROS was also assayed with ethidium fluorescence analysis and luminol-enhanced chemiluminescence. The expressions of eNOS and NADPH oxidase were measured. Endothelial function was examined through endothelium-dependent relaxation in response to acetylcholine (ACh). Uncoupled eNOS in CFO was found with acute administration of eNOS cofactor tetrahydrobiopterin (BH4). In one additional group of swine, the animals with CFO carotid arteries were orally fed apocynin (4-hydroxy-3-methoxy-acetophenone) to determine the effect of NADPH oxidase in the vessel wall. The results support our hypotheses and demonstrate that CFO causes endothelial dysfunction in conduit vessels.

MATERIALS AND METHODS

Twelve male Duroc swine weighing 34 ± 4 kg (range 30–39 kg) were randomly divided into two groups. In group I, the right carotid artery was exposed to CFO for 1 wk by ligation of the contralateral
carotid artery. In group II, the right carotid artery was exposed to CFO
for 1 wk while animals were orally fed apocynin at the dose of 60
mg·kg$^{-1}$·day$^{-1}$ from the postoperative first day to the termination
(CFO+$\alpha$). The left carotid artery served as a control for each group
and was harvested at the time of ligation. All experiments were
performed in accordance with national and local ethical guidelines,
including the Institute of Laboratory Animal Research (ILAR) Guide,
Public Health Service policies, Animal Welfare Act, and an approved
Indiana University School of Medicine IACUC protocol.

Animal Preparation

Surgical anesthesia was induced with ketamine (20 mg/kg im) and
atropine (0.04 mg/kg im) and maintained with isoflurane (1–2%).
Blood gas values were measured, and ventilation was adjusted to
maintain normal values of $P_{O_2}$ and $P_{CO_2}$. In preliminary experiments,
we measured the flow rates and external diameters to confirm that the
left and right common carotid arteries are equivalent to ensure that
one can serve as control for the other. Furthermore, the flow rates and
diameter of the right carotid artery were immediately mea-
sured after the left carotid artery was excised to quantify the imme-
diate FO.

Subsequent to a left cervicotomy, the left common carotid artery
was exposed gently to avoid vasomotion by dissection, and the in vivo
external diameter was measured with the aid of a stereo microscope.
The vessel was further dissected to place a flow probe (TS420
Transonic System). After data collection, the artery was ligated by suture and excised (length of $\approx$3 cm); the right carotid artery was not
exposed to any surgical trauma. This measurement was taken on day
0 to avoid intervention on the experimental vessel. The incision was
closed, and the animal was recovered. The animals in group II were
fed apocynin (60 mg·kg$^{-1}$·day$^{-1}$) orally mixed in food. After 1 wk
and following a right cervicotomy, the in vivo external diameter and
flow rate of right carotid artery were measured (data points for week
4), and the vessel segment was excised. The excised vessels from
control and experimental groups were immediately stored in 4°C
HEPES physiological salt solution (HEPES-PSS, pH 7.4, mM: 142
NaCl, 4.7 KCl, 2.7 sodium HEPES, 1.17 MgSO$_4$, 2.79
CaCl$_2$, 5.5 glucose) and divided into segments for various measure-
ments after dissection of adjacent tissue. HEPES and HEPES salt
were purchased from Sigma, whereas other chemicals were purchased from
Fisher Scientific.

Vasoactivity

An isovolumic myograph, which retains the physiological loading
of a pressure myograph with sensitivity of a wire myograph, was used
to evaluate the vasoactivity of the artery (24). Briefly, the carotid
vessel was cannulated on the connectors, which were fixed in a bath
containing HEPES-PSS. The vessel was incubated in the bath at 37°C
for 40 min. The vessel was stretched to in situ length with the aid of
a digital caliper (resolution of 0.1 mm) and preloaded at a physiolog-
nical pressure of 80 mmHg. Contraction or relaxation was pharmaco-
logically induced with both ends closed. The contraction or relaxation
was normalized to dry tissue weight.

ROS Detection

We detected ROS with paramagnetic resonance spectroscopy,
ethidium fluorescence assay, and luminol derivative (L-012) enhanced
chemiluminescence analysis as described below.

Electron paramagnetic resonance spectroscopy. Immediately after
the vessel was divided, the vessel ring for electron paramagnetic
resonance (EPR) was videotaped from the side (5 mm in length) and
cross-views under stereo microscope. The volume of the segment was
calculated based on the product of cross-sectional area and axial
length. After measurement by EPR as described below, ROS genera-
tion was expressed as mole per unit of volume.

ROS concentration in tissue samples was determined from the EPR
spectra obtained by incubating the tissue samples with the spin
trapping agent N-tert-butyl-$\alpha$-phenylnitrone (PBN; Sigma) at 190
mM in HEPES-PSS for 30 min at 37°C in the dark. A ring incubated
with 4-Hydroxy-TEMPO (a superoxide dismutase mimic) served as
the control for ROS measurement. The tissue was subsequently
inserted into a syringe along with the supernatant, immediately frozen
in liquid nitrogen, and stored at $\approx$80°C until EPR analysis was
performed. To avoid ROS produced during freezing and thawing of
samples, the sample was quickly removed while in its frozen state
from the syringe and placed in a Dewar containing liquid nitrogen.
The Dewar was then inserted into the microwave cavity of the EPR
spectrometer. The sample remained at liquid nitrogen temperature
throughout the EPR analysis (1, 21).

The EPR equipment and settings were as follows. A Bruker ESP
X-band spectrometer equipped with a TE102 cavity was utilized to
detect signals. Parameters for the spectra were 9.4-GHz microwave
frequency, 25.2-mW microwave power, 4.0-G modulation amplitude,
and 100-G magnetic field sweep width. All experiments were
completed at liquid nitrogen temperature.

Four EPR scans were taken per tissue sample and analyzed with
Bruker WINEPR software (version 2.11) based on the spectral intensity
and line width. ROS concentrations were determined with 2,2,6,6-
tetramethylpiperidined 1-oxyl, TEMPO, solution (0.1 mM, Sigma) used
as a concentration standard. All EPR parameters and conditions were
applied to both standard and experimental samples.

Chemiluminescence. A highly sensitive chemiluminescence probe,
luminol derivative L-012 (Wako Chemicals) was used to detect ROS
in the vessel tissue. Three arterial segments, 2–3 mm in length, were
incubated in 96-well plates with HEPES-PSS at 37°C for 1 h. NADPH
(0.3 M) and L-012 (500 μM) were administered in wells (36). Some
of the segments were incubated with 1 mM oxyphenol (a xanthine
oxidase inhibitor), 50 μM rotenone (a mitochondria inhibitor), or 1
μM t-NAMe (N4-nitro-L-arginine methyl ester; an eNOS inhibitor)
to verify the involvement of xanthine oxidase, mitochondria, and
eNOS. Light emission was detected by ultra-sensitive photon counter
(Wallac EnVision, 2104 Multilabel Reader, PerkinElmer). Counts
were obtained at 1-min intervals for 40 min. ROS levels were reported
as relative light units after subtracting background luminescence and
were normalized to dry tissue weight.

Ethidium fluorescent assay. A 5-mm ring was cut from the segment
of carotid artery and incubated with nitrogen-bubbled HEPES-PSS (to
remove possible ROS) containing dihydroethidium (DHE, Sigma) at

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optimal concentration (determined in pilot experiments) of 0.2 μM for 30 min at 37°C in the dark (47). Another ring that served as a control for ROS measurement was first incubated with 4-hydroxy-TEMPO (Tempol, Sigma), an O₂ scavenger, for 30 min at 37°C, and then with DHE for 30 min at 37°C. An additional ring without DHE incubation was used as the control for autofluorescence of the vessel tissue. A 4′-6-diamidino-2-phenylindole (DAPI) staining was performed to determine nuclear area, which was a reference for analysis of ethidium fluorescence. After being washed five times with saline, the rings were sliced at 20-μm-thick transverse sections by a cryostat (Leica CM 1850). Confocal microscopy (LSM 510 META, Zeiss) was used to visualize the fluorescence (excitation/emission: 518/605 nm, 63× oil objective). A gray scale analysis was carried out to determine the fluorescence area fraction. The autofluorescence of vessel tissue was subtracted to remove the background.

Protein Expression

The segment for Western blotting was homogenized in a lysis buffer and then incubated on ice for 1 h. The sample was centrifuged at 1,000 g for 15 min at 1°C, and the supernatant was drawn off. The total value of protein was measured by a BCA kit (Bio-Rad). Equal amounts of protein (25 μg) were loaded, electrophoresed in 10% SDS-PAGE gel, and transferred onto a polyvinylidene difluoride membrane. After blocking the sample for 2 h in 8% dried milk in TBS-Tween buffer, the membrane was incubated overnight at 4°C with specific primary antibody with either anti-eNOS (1:1,000 dilution in blocking buffer, BD transduction laboratory), anti-p22phox (1:1,000, Santa Cruz Biotech), anti-p47phox (1:1,000, Santa Cruz Biotech), anti-NOX2 (1:250, Santa Cruz Biotech), or anti-NOX4 (1:250, Santa Cruz Biotech). The membrane was then rinsed and incubated with horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotech), to correct for sample loading.

Statistical Analysis

All data given in the text and figures are expressed as means ± SD. Student’s t-test (two-tailed distribution, two-sample unequal variance) and Duncan’s test following ANOVA were used to detect differences between groups. For all analyses, P < 0.05 was used to indicate statistical significance.

RESULTS

The blood flow in carotid arteries was significantly increased in CFO and CFO + A after contralateral ligation (189.2 ± 25.3, 369.6 ± 61.9, and 382.3 ± 62.5 ml/min in the control, CFO, and CFO + A groups, respectively; P < 0.001). The flow rate in the apocynin treatment group was not significantly different than the untreated group. Figure 1 represents a typical tracing of a control and a carotid artery exposed to CPO. The mean and peak of the blood flow doubled in CFO (Fig. 1A). After normalization of pulsatile blood flow with mean flow rate, the two curves with respect to their mean values largely overlapped (Fig. 1B), which suggests that the oscillatory components of pulsatile blood flow were not significantly changed in the CFO model.

The outer diameter of the vessel significantly increased by 8.6% (4.60 ± 0.41 to 4.98 ± 0.46 mm) after 1 wk of exposure to CFO (P < 0.05) and did not change significantly after treatment with apocynin (4.98 ± 0.46 to 5.06 ± 0.48 mm). We did not observe a significant change of arterial wall thickness between either the CFO or the CFO + A group compared with the control group (data not shown). The systemic blood pressure measured at the femoral artery did not change after either surgical ligation of the carotid artery or after treatment with apocynin compared with the pressure before ligation (88 ± 12 vs. 86 ± 11 mmHg). The wall shear stress (WSS; WSS ∼ QD⁻³, where Q and D represent flow rate and inner diameter, respectively) was found to remain elevated by ~50% after 1 wk of the CFO and CFO + A groups.

Endothelial Function

The endothelial function was quantified by the % decrease in tension in response to ACh (Fig. 2). The dose curves indicated that endothelial function was compromised in the CFO group (P < 0.05) but was preserved with apocynin treatment. This result implied that endothelial dysfunction in CFO was related to ROS upregulation. Furthermore, we found that the endothelial dysfunction of the vessels in CFO were completely reversed after acute incubation with BH₄ and L-arginine, which suggests that eNOS uncoupling may play a role in CFO. The endothelium-independent vasorelaxation in response to SNP did not show differences in groups, which implied that the VSMC did not develop resistance to nitric oxide (103.5 ± 15.5, 102.6 ± 17.2, and 103.6 ± 21.1% for control, CFO, and CFO + A, respectively). The potassium-induced contraction, which is not receptor-dependent, did not reveal differences of VSMC contractility (tension) in the three groups (49.6 ± 6.98, 51.3 ± 7.66, and 51.1 ± 7.96 mN/mm for control, CFO, and CFO + A, respectively).

Fig. 1. A typical waveform of blood flow in the carotid artery at baseline and flow overload (CFO). A: the real time recordings. B: the normalized tracing by the mean flow rate. The normalized flow tracing at chronic flow overload (CFO) approximately overlaps the normalized baseline.

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The EPR measurements are presented in Fig. 3. ROS generation significantly increased in the CFO group. Apocynin treatment restored ROS generation to the control value. Since PBN is lipophilic and forms stable lipid-derived, spin adducts (radical bound to spin trap), it provides reliable overall concentration measurements of ROS in biological tissue under physiological conditions without lipid radical adduct extraction that may involve artifacts such as failure to retrieve all of the adducts from the biological sample and additional ROS generation during centrifugation. Since PBN is indeed able to trap NO in vascular tissue, we verified that NO was not significantly produced in our experimental preparation with DAF-2DA (NO probe) and l-NAME (an eNOS inhibitor).

Luminol derivative L-012 is highly sensitive at physiological range of pH (7.5) and reacts with various types of ROS generated from various biological tissues. L-012 enhanced chemiluminescence increased in the vessel tissue in CFO after addition of NADPH (Fig. 4). A 1-wk treatment of apocynin decreased the level of ROS. The inhibitors of xanthine oxidase, mitochondria, and eNOS did not cause any change of chemiluminescence (data not shown), which indicates that those ROS sources were not involved in ROS production in CFO.

Confocal microscopic images of ethidium fluorescence showed that ROS increased in all three wall layers in CFO group (Fig. 5A). The fraction area of fluorescent assay in the carotid vessels exposed to CFO was more than three times higher than that of control segments (Fig. 5B). With the treatment of apocynin, the production of ROS was significantly lower than that in the CFO group (Fig. 5A). When the tissue from either the control or the CFO group was first incubated with Tempol, the DHE assay was almost completely eliminated by Tempol (Fig. 5A).

Fig. 2. Endothelium-dependent vasorelaxation of carotid arteries. The arteries were precontracted to approximate tension with phenylephrine (PE), and dose-responsive vasorelaxation was induced with acetylcholine (ACh). C, control group; CFO, chronic flow overload group; CFO+A, chronic flow overload group treated with apocynin; CFO+BH4, vessel segment in CFO acute incubation with tetrahydrobiopterin (BH4). *Significant difference between groups (P < 0.05; ANOVA followed by Duncan’s test for multiple groups).

Fig. 3. A: representative samples of the EPR spectra. The concentration of ROS is proportional to the signal intensity. B: ROS concentration by EPR spectroscopy using the PBN as a spin trap in HEPE-PSS. *Significant difference compared with control (P < 0.05). #Significant difference compared with CFO (P < 0.05).

Fig. 4. ROS production determined by chemiluminescence analysis in arterial segments. A luminol derivative L-012 was used to enhance chemiluminescence by ROS in arterial segments. A: representative samples of L-012 enhanced-chemiluminescence. B: ROS production measured by chemiluminescence analysis. *Significant difference compared with control (P < 0.05). #Significant difference compared with CFO (P < 0.05).
Expression of eNOS and NADPH Oxidase

The expression of eNOS was significantly upregulated in the CFO group and unaffected by apocynin treatment (Fig. 6). The subunits p22phox and p47phox are well known to be expressed ubiquitously in endothelial and VSMC. The protein expression of cytosolic assembling subunits of NADPH oxidase (p22phox and p47phox) were significantly elevated in carotid segments exposed to CFO but not in CFO/H11001A (Fig. 6). NOX2 and NOX4 (NADPH oxidase 2 and 4) were found to be upregulated in CFO and CFO/H11001A.

DISCUSSION

The major findings are that chronic shear stress elevation of ~50% increases ROS production mediated by NADPH oxidase and induces endothelial dysfunction in swine carotid artery. Recent observations suggest that NADPH oxidase is directly involved in $O_2^-$ production in mouse carotid arteries where the flow was increased by a factor of three to five by construction of an arteriovenous fistula (6). The present study demonstrates a similar finding in conduit artery of the porcine model in response to a more modest increase in WSS. Moreover, we show for the first time endothelial dysfunction as a consequence of increased oxidative stress in conduit artery where eNOS uncoupling may play a role during CFO.

The porcine carotid artery was exposed to approximately twice the physiological flow rate for 1 wk in this study. Although the change of flow rate was relatively small compared with a typical arteriovenous fistula (over fivefold increase in flow rate) (18), the objective was to study a model with minimal disturbance since surgery elicits inflammatory response and consequent oxidative stress. The diameter enlargement of the artery was accordingly small (8.6%). Such a change in flow and diameter in a conduit artery may occur under physiological conditions, such as in exercise (15, 50). Interestingly, ROS generation increased by onefold in response...
Apocynin is an inhibitor of NADPH oxidase under in vivo conditions where \( \text{H}_2\text{O}_2 \) and myeloperoxidase is present and is suggested to inhibit the translocation of cytoplasmic subunits (45). It is also possible that treatment of apocynin in this study shifted the balance of oxidative stress in vascular tissue through nonspecific antioxidant effects (13, 36). Since the expression of NOX isoforms may be located in endothelial and VSMC, we cannot separate the effect of WSS on endothelial and circumferential stretch acting throughout the vessel wall on NOX isoforms. The elucidation of the role of NOX in endothelial and VSMC during CFO requires further study.

Ethidium fluorescence visualized by confocal microscopy indicates that ROS increases not only in endothelium but also in the media and in the adventitia. This is interesting because WSS acts directly on the luminal surface of endothelium; i.e., WSS is not likely to directly stimulate the media and the adventitia. A fundamental question involves the mechanism by which elevated WSS sensed by the endothelium transmits its effect on more remote regions of the wall. A possible mechanism may be similar to hypertension via the NADPH oxidase pathway (44, 45, 49). The common factor in CFO and hypertension is the increase in circumferential stretch and stress. Hypertension increases circumferential stress and strain by an increase in blood pressure, whereas flow-induced vasodilation increases vessel stretch similarly through an increase in diameter. The circumferential stress or strain, mediated by mechanotransduction in CMO, may activate NADPH oxidase and elicit ROS generation.

In summary, ROS production increases in porcine carotid arteries in response to a onefold increase in the blood flow rate, which leads to endothelial dysfunction. NOX2 and NOX4 oxidase and p22phox and p47phox are upregulated in CMO, and NADPH oxidase is likely involved in the increase in oxidative stress. The chronic use of apocynin prevents the elevation of ROS levels, even though NOX2 and NOX4 are upregulated, and preserves endothelial function. The mechanisms by which apocynin prevents the upregulation of p22phox and p47phox but not NOX2 and 4 remain unclear. Although the process of CFO-induced remodeling to restore WSS has previously been thought of as a physiological response, the present data suggest that CFO mediated by ROS causes endothelial dysfunction, which may result from eNOS uncoupling in the first week of outward vascular remodeling.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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