NAD(P)H oxidase-derived peroxide mediates elevated basal and impaired flow-induced NO production in SHR mesenteric arteries in vivo

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Submitted 4 February 2008; accepted in final form 1 July 2008

Zhou X, Bohlen HG, Miller SJ, Unthank JL. NAD(P)H oxidase-derived peroxide mediates elevated basal and impaired flow-induced NO production in SHR mesenteric arteries in vivo. Am J Physiol Heart Circ Physiol 295: H1008–H1016, 2008. First published July 3, 2008; doi:10.1152/ajpheart.00114.2008.—Nitric oxide (NO) and reactive oxygen species (ROS) have fundamentally important roles in the regulation of vascular tone and remodeling. Although arterial disease and endothelial dysfunction alter NO and ROS levels to impact vasodilation and vascular structure, direct measurements of these reactive species under in vivo conditions with flow alterations are unavailable. In this study, in vivo measurements of NO and \( \text{H}_2\text{O}_2 \) were made on mesenteric arteries to determine whether antioxidant therapies could restore normal NO production in spontaneously hypertensive rats (SHR). Flow was altered from ~50–200% of control in anesthetized Wistar-Kyoto rats (WKY) and SHR by selective placement of microvascular clamps on adjacent arteries while NO and \( \text{H}_2\text{O}_2 \) were directly measured with microelectrodes. Relative to WKY, SHR had significantly increased baseline NO and \( \text{H}_2\text{O}_2 \) concentrations (2.572 ± 241 vs. 1.059 ± 160 nM, \( P < 0.01 \), and 26 ± 7 vs. 7 ± 1 \( \mu \)M, \( P < 0.05 \), respectively). With flow elevation, \( \text{H}_2\text{O}_2 \) but not NO increased in SHR; NO but not \( \text{H}_2\text{O}_2 \) was elevated in WKY. Apocynin and polyethylene-glycolated catalase decreased baseline SHR NO and \( \text{H}_2\text{O}_2 \) to WKY levels and restored flow-mediated NO production. Suppression of NAD(P)H oxidase with gp91ds-tat decreased SHR \( \text{H}_2\text{O}_2 \) levels. Addition of topical \( \text{H}_2\text{O}_2 \) to increase peroxide to the basal concentration measured in SHR elevated WKY NO to levels observed in SHR. The results support the hypothesis that increased vascular peroxide in SHR is primarily derived from NAD(P)H oxidase and increases NO concentration to levels that cannot be further elevated with increased flow. Short-term and even acute administration of antioxidants are able to restore normal flow-mediated NO signaling in young SHR.

Hypertension; oxidative stress; flow mediated; NAD(P)H oxidase inhibition; spontaneously hypertensive rats

NITRIC OXIDE (NO) and reactive oxygen species (ROS) have fundamentally important roles in the regulation of vascular tone and remodeling. The presence of arterial disease and endothelial dysfunction are believed to alter NO and ROS levels to impact vasodilation and vascular structure. Although studies have demonstrated that flow elevation increases both NO (5, 9, 31) and ROS (37, 43, 51) production in arteries and arterioles, there is a lack of direct measurements of these reactive species under in vivo conditions, especially with flow alterations in the presence of arterial disease. Recent in vitro studies with arterioles from patients with arterial disease (43, 51) have shown that \( \text{H}_2\text{O}_2 \) increases with flow and mediates flow-dependent dilation. It is not known whether similar abnormalities in flow-modulated NO and \( \text{H}_2\text{O}_2 \) production occur in animal models of arterial disease or how elevated peroxide affects NO production in vivo.

We conducted this study to provide direct, in vivo measurements of NO and \( \text{H}_2\text{O}_2 \) and to test the hypothesis that in the presence of endothelial dysfunction, the perivascular NO concentration of resistance arteries is suppressed and the \( \text{H}_2\text{O}_2 \) concentration enhanced during both basal conditions and with flow elevation. In addition, we proposed and tested that this imbalance is reversed by antioxidant therapy. We utilized spontaneously hypertensive rats (SHR), because the presence of endothelial dysfunction (55, 69, 72) and impaired collateral artery growth (23, 42, 66) are well documented in this strain. In addition, we have previously shown that the SHR collateral growth impairment is reversed by antioxidant therapy (42). For the current study, we focused on basal levels of NO and \( \text{H}_2\text{O}_2 \), and the presence of endothelial dysfunction, the perivascular NO concentration, but not the NO concentration, was modulated by flow in SHR mesenteric arteries. However, basal concentrations of both NO and \( \text{H}_2\text{O}_2 \) were elevated in SHR relative to those in Wistar-Kyoto rats (WKY). Treatment of SHR with the antioxidant apocynin, as well as the NAD(P)H oxidase inhibitor gp91ds-tat, restored both basal concentrations of NO and \( \text{H}_2\text{O}_2 \) and flow-mediated NO production. The results suggest that in SHR, oxidative stress is primarily responsible for the elevated \( \text{H}_2\text{O}_2 \) concentrations and abnormal NO production. The data also imply that the collateral growth impairment in the young SHR is not the result of reduced NO bioavailability.

METHODS

Animals. Male SHR and WKY rats were obtained from Harlan (Indianapolis, IN) and studied at ~10 wk of age. The first experiments investigated the effect of short-term apocynin treatment on in vivo mesenteric artery NO and \( \text{H}_2\text{O}_2 \) concentrations. For these experiments, apocynin (acetovanillone; Fisher Scientific, Hampton, NH) was given in drinking water (3 mM) for 1 wk at a dose previously shown to reduce oxidative stress (49) and restore collateral growth capacity (42) in SHR. Subsequent studies utilized acute polyethylene-glycolated catalase (PEG-catalase; Sigma Chemical, St Louis, MO; topical concentration 250 U/ml, 20-min incubation) and, also in acute treatment, the specific NAD(P)H oxidase inhibitor gp91ds-tat (EZBiocarb, Westfield, IN; topical concentration 1.0 \( \mu \)M, 40-min incubation) (50) to clarify mechanisms. All procedures were approved by the Indiana University School of Medicine Institutional Animal Care and Use Committee.

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The rats were anesthetized with 250 mg·ml⁻¹·kg⁻¹ thiopental sodium given subcutaneously. Using this mode of anesthesia injection avoided any possibility of the anesthetic directly interacting with the intestinal vasculature, as would be the case with intraperitoneal injection. Furthermore, although thiopental sodium is a long-acting anesthetic in rats, the subcutaneous injection extended the time of anesthesia such that supplemental dosage was rarely needed. Depth of anesthesia was monitored by pinching a forelimb toe and observing any trend for reduction in arterial pressure, which generally predicted an impending need for additional anesthetic injection (subcutaneously). Once animals reached insensate conditions, the trachea was cannulated, and the animals were immediately mechanically ventilated at 70 breaths/min (the typical breathing rate of conscious adult rats) and at sufficient tidal volume to generate oxygen percent saturation of hemoglobin in the 92–95% range in the ear vasculature. The right femoral artery was cannulated to monitor the arterial blood pressure and infuse lactated Ringer solution (0.5 ml·h⁻¹·100 g⁻¹) to maintain a stable arterial blood pressure. The animals were maintained at body temperature as soon as they became immobile and thereafter by placing a 35–37°C water jacket beneath the body.

**Model preparation.** The small intestine and mesentery were prepared for in vivo observation with a standardized technique that maintained innervation and all arterial and venous connections (5, 8). The antimesenteric edges of the bowel wall were used to apply small sutures by pinching a small amount of the gut wall. The sutures were used to stabilize the bowel and gently spread the mesentery. The mesenteric arteries were bathed in a solution of bicarbonate-buffered (pH 7.35–7.45) physiological saline, equilibrated with 5% O₂, 5% CO₂, and 90% N₂ (7, 9). To minimize bowel motility, both isoproterenol and norepinephrine at concentrations below 10⁻⁶ were added to the medium to suppress visceral smooth muscle spontaneous contractions. Prior studies have shown this approach has a minimal effect on both normal and hypertensive vasculatures (4, 26).

**Arterial occlusion and flow and diameter measurement.** A 3-mm length of mesenteric artery ~2 cm from the bowel was isolated from its companion vein for placement of a precalibrated 0.5-mm blood flow probe (0.5PSB nanoprobe; Transonic Systems, Ithaca, NY) (Fig. 1). This location was selected to be ≥10 mm from the site of NO and H₂O₂ measurements to prevent electrical interference. The zero-flow value was set with the flow probe in the bath above the vessel. To assess the modulation of NO and H₂O₂ by flow, we placed three microvascular clamps (S&T B-1; Fine Science Tools, Foster City, CA) sequentially at different locations on adjacent mesenteric arteries as indicated in Fig. 1. After baseline blood flow was determined, blood flow was increased first with a single clamp at position 1 and then with double clamps at positions 1 and 2. After measurements of flow and NO or H₂O₂ were obtained for ~10 min, clamps were removed from positions 1 and 2 and the return to baseline was verified. In the experiments measuring NO concentration, a single clamp was also placed at position 3 to decrease flow. This was done to determine whether the abnormal flow-modulated NO production in SHR occurred only with flow elevation. Videotape recordings of mesenteric arteries were acquired at ×30 magnification with a dissecting microscope (Olympus SZH) and camera (model C2400-50 charge-coupled device; Hamamatsu) for offline measurement of diameter with image analysis software (Image-1/AT).

**NO measurement.** NO was measured with the use of NO-selective microelectrodes based on well-established techniques (6, 9, 10, 74). The microelectrode was sensitive to NO only at the open glass envelope tip, which was sharpened to an outer diameter of 10–15 μm. The microelectrodes were calibrated against known NO gas concentrations (Matheson, Joliet, IL) with the highest concentration being equivalent to 1.200 nM. If the micro electrode did not have a stable current during NO exposure or did not fully recover to the expected baseline current during 100% nitrogen, the micro electrode was not used. NO microelectrodes were polarized relative to a silver-silver chloride electrode to the voltage (0.7 or 0.9 V) most sensitive to NO concentration as indicated by a Keithley model 6517A electrometer (Cleveland, OH). Electronic drift of the electrode was compensated mathematically using a virtual baseline adjusted for time and the linear rate of drift (2). “Zero” NO concentration was obtained by simply raising the electrode tip ~200 μm above the tissue. Initial NO measurements were made 0.5 cm from the vessel wall in an avascular region of the mesentery to represent a mesenteric tissue background NO concentration. For periarterial NO measurement, the microelectrode tip penetrated the mesentery beside the mesenteric artery and the tip was advanced into the outer connective tissue over the vessel wall. When microvascular clamps were placed on or removed from adjacent arteries, it was necessary to briefly remove the micro electrode from the perivascular region to prevent tip breakage. However, the microelectrode was returned to the exact same vascular site using the previous penetration track and vessel/tissue landmarks. To verify electrode specificity to NO, measurements were made at the end of some of the experiments after 1 mM N⁵-nitro-L-arginine methyl ester (L-NAME; Sigma Chemical) was added to the bathing solution for 20–30 min to suppress all forms of nitric oxide synthase (NOS). At the prevailing in vivo conditions, the NO electrode current was not affected by H₂O₂, because the electrodes were unresponsive to topical H₂O₂ (≤100 μM) added to the bath (data not shown). NO measurement. The perivascular H₂O₂ concentration was measured with a World Precision Instruments (Sarasota, Florida) Apollo 4000 system using their ISO-HPO-100 sensor (100 μm diameter, 2 mm length), a carbon fiber-based microelectrode coated with proprietary materials to increase selectivity for H₂O₂. This system has been utilized by other groups for in vitro and in vivo studies (14, 54). The electrode was calibrated against freshly prepared H₂O₂ solutions in the range of 0–100 μM in Ringer solution at 37.5°C. The electrodes were insensitive to NO gas (1,200 nM) in nitrogen-equilibrated Ringer solution. To measure the H₂O₂ beside an artery, the exposed carbon fiber sensor approached the vessel wall at a shallow angle to ensure the entire sensor tip touched the outside wall of the vessel. Very similarly to the NO measurement, the H₂O₂ electrode was briefly moved away from the arteries when microvascular clamps were placed on or removed from adjacent arteries. The microelectrode was returned to the exact same vascular site using vessel/tissue landmarks. To confirm the H₂O₂ electrode specificity, we added 250 U/ml...
PEG-catalase to the bathing solution at the end of the experiments for 20–30 min to scavenge the H$_2$O$_2$ in the bath, and then the H$_2$O$_2$ concentration measurement was repeated. Additional experiments were performed to evaluate the impact of background H$_2$O$_2$ signal from plasma on the perivascular H$_2$O$_2$ measurement. Rat plasma (3–4 ml) was collected from four WKY and four SHR and H$_2$O$_2$ measured using the electrode directly.

**Statistical analyses.** Data analyses were performed with SigmaStat 3.0 (Systat Software, San Jose, CA). One- or two-way ANOVA was used with or without repeated measures, as indicated. When the ANOVA indicated statistical differences existed between groups, multiple pairwise comparisons were performed with the Student-Neman-Keuls test. The correlations between changes in mesenteric artery blood flow and arterial NO and H$_2$O$_2$ concentrations were evaluated with linear regression analysis. Data are presented as group averages ± SE. Criterion for significance was $P < 0.05$.

**RESULTS**

**Body weight and arterial pressures.** Among all groups of animals, the body weight range was similar (225–250 g). The young SHR at ~10 wk showed significantly elevated mean arterial blood pressure compared with WKY (135 ± 3 vs. 92 ± 3 mmHg, $P < 0.01$). Apocynin treatment for 1 wk significantly lowered the blood pressure of SHR (110 ± 3 mmHg, $P < 0.01$ compared with untreated SHR), but it remained elevated relative to WKY ($P = 0.05$). There was no significant blood pressure difference between WKY and apocynin-treated WKY (98 ± 9 mmHg). Topical PEG-catalase did not change the mean arterial pressure in the SHR (135 ± 6 vs. 137 ± 5 mmHg). Similarly, acute treatment with gp91ds-tat did not alter mean arterial pressure in the SHR (138 ± 3 vs. 141 ± 4 mmHg).

**Basal NO concentration measurement.** Our initial experiments revealed the unexpected observation that, as shown by in vivo direct measurement, the baseline periarterial NO concentration was significantly greater (143%, $P < 0.01$, Fig. 2A) in young SHR than in normotensive WKY. With the addition of L-NAME, the perivascular NO concentration was substantially decreased from 1,059 ± 160 to 275 ± 62 nM in WKY and from 2,572 ± 241 to 770 ± 265 nM in SHR. The percent reduction in NO concentration was similar between SHR and WKY, as shown in Fig. 2B. Pretreatment of SHR with apocynin (3 mM for 7 days) reduced the baseline perivascular NO concentration to 778 ± 101 nM, a concentration similar to the range in WKY (Fig. 2A). In contrast, apocynin pretreatment did not change the baseline NO concentration in WKY (1,020 ± 197 nM).

**Modulation of perivascular NO concentration by flow.** The baseline arterial blood flow and arterial diameters were similar among WKY, apocynin-pretreated WKY, SHR, and apocynin-pretreated SHR (0.19 ± 0.03, 0.21 ± 0.03, 0.22 ± 0.02, and 0.16 ± 0.02 ml/min and 256 ± 17, 240 ± 5, 224 ± 11, and 227 ± 7 µm, respectively). Two-way repeated-measures ANOVA indicated significant differences in flow for the clamping status within an animal group but no differences among groups and no interaction between group and clamping status. Changes in blood flow with each clamp state are shown in Fig. 3A. The changes in flow were similar among all groups and experimental conditions. The correlation between the percent changes in NO concentration and flow are shown in Fig. 3B. In WKY and apocynin-treated WKY, perivascular NO concentration was directly correlated with changes in flow.

However, in SHR, there was no correlation between changes in NO and flow. Analysis of group means demonstrated an actual decrease in NO from baseline to the highest flow condition (Fig. 3C). These data demonstrate a profound impairment in flow-modulated NO production in the SHR. However, apocynin pretreatment completely restored the correlation between changes in flow and NO concentration. The actual NO concentrations in the apocynin-pretreated SHR were similar to those in WKY for all flow conditions (Fig. 3C).

**Basal and flow-modulated perivascular H$_2$O$_2$ and plasma H$_2$O$_2$ concentration.** Measurements of in vivo perivascular H$_2$O$_2$ concentration were obtained with a peroxide-sensitive electrode under basal conditions and with flow elevation similar to that for the NO measurements and are reported in Fig. 4.
In Fig. 4A, a typical calibration of the peroxide electrode is shown and demonstrates both linearity and sensitivity over the range of the lowest perivascular measurements. In our experience, the linear response extended to 100 μM H₂O₂. Figure 4B presents baseline peroxide levels in untreated and apocynin-treated WKY and SHR and also demonstrates specificity of the peroxide electrode. Basal H₂O₂ concentration in SHR was increased 266% compared with that in WKY. Pretreatment with apocynin decreased H₂O₂ concentration in SHR to a range similar to that in WKY. Electrode specificity in the in vivo setting was confirmed by topical application of PEG-catalase (250 U/ml), a dose that has been shown to efficiently scavenge H₂O₂ in vitro (48). With the topical use of PEG-catalase, H₂O₂ concentration was substantially decreased 70 ± 2.6% in WKY, 75 ± 8.7% in apocynin-treated WKY, 78 ± 10.5% in SHR, and 70 ± 8.1% in apocynin-treated SHR. The percent reduction in H₂O₂ concentration was similar in all groups. Although the basal H₂O₂ concentration after topical PEG-catalase was similar between all groups, direct H₂O₂ measurements were also made on plasma collected from WKY and SHR to further confirm that differences were specific for H₂O₂ and not due to blood-borne factors. The electrode measurements of plasma H₂O₂ concentrations were 0.4 ± 0.3 and 0.95 ± 0.2 μM in WKY and SHR, respectively. These levels are too low in plasma to significantly influence the in vivo perivascular H₂O₂ measurement. The relationships between percent changes in flow and H₂O₂ concentration are presented in Fig. 4C for untreated and apocynin-treated WKY and SHR. Regression analysis indicated that a correlation existed only in the untreated SHR such that H₂O₂ concentration increased with flow (P = 0.012, r² = 0.372). This positive correlation between flow-mediated H₂O₂ production was abolished by pretreatment with apocynin (P = 0.80, r² = 0.000) in the SHR. Although there is significant scatter in the individual data points of Fig. 4C, the group means presented in Fig. 4D clearly show the increase in H₂O₂ concentration with flow in SHR unlike what is observed in the other groups.

Effect of H₂O₂ on in vivo NO production. Because existing studies of the peroxide effect on NO levels are contradictory (13, 59), we performed additional experiments to determine whether the elevated H₂O₂ concentration in SHR could account for the abnormal NO concentrations. Figure 5A shows the results of increasing bath peroxide levels. Topical H₂O₂ at concentrations of 20 and 100 μM increased NO concentration in WKY (2 clamps vs. baseline, P = 0.010) but actually decreased in SHR (2 clamps vs. baseline, P = 0.011). With apocynin pretreatment, NO was statistically increased from baseline at both clamp conditions in both WKY (P = 0.001) and SHR (P = 0.029 for 1 clamp and 0.009 for 2 clamps). n = 6, 3, 5, and 6 for WKY, WKY + Apo, SHR, and SHR + Apo, respectively.
the basal periarterial concentration measured in SHR (20 μM), the NO concentration measured in WKY was within the same range observed in the SHR. Next, experiments were performed with acute incubation of PEG-catalase to determine whether abnormal NO production in SHR was a result of the elevated H₂O₂ concentration. The results in Fig. 5B show that topical PEG-catalase not only decreased basal NO concentration but also restored flow-mediated NO production in SHR.

**Effect of gp91ds-tat on basal and flow-mediated H₂O₂ production.** Because the specificity of apocynin as an NAD(P)H oxidase inhibitor has recently been questioned (30), additional experiments were performed with addition of gp91ds-tat (topical 1 μM for 40 min) to the bath. As shown in Fig. 6, this specific NAD(P)H oxidase inhibitor significantly decreased SHR basal H₂O₂ concentration and blocked its flow-mediated H₂O₂ production. Gp91ds-tat had no effect on H₂O₂ concentration in WKY at baseline or during increased blood flow.

**DISCUSSION**

To our knowledge, this is the first study to investigate in vivo perivascular NO and H₂O₂ concentrations in resistance arteries from an animal model of arterial disease under conditions of altered flow. In SHR mesenteric arteries, basal NO and H₂O₂ concentrations were elevated relative to those in WKY. In response to flow elevation, H₂O₂ but not NO was increased in SHR; the opposite was observed in WKY. Exogenous peroxide elevated NO concentration in WKY but not SHR, whereas suppression of peroxide lowered NO concentration in SHR and restored flow-mediated NO production. Experiments with apocynin and gp91ds-tat indicated the primary source of the elevated H₂O₂ in SHR mesenteric arteries was NAD(P)H oxidase.

**Basal elevation of H₂O₂.**

The basal elevation of periarterial H₂O₂ concentration in SHR relative to WKY (Fig. 4) is consistent with the direct measurement by peroxide electrode by Cosentino et al. (16) in isolated aortic rings, although higher in absolute magnitude. The peroxide electrode used for the in vivo periarterial measurements was demonstrated to be linear, even at the lowest range of measurements (Fig. 4A). The use of topical PEG-catalase (Fig. 4B) demonstrated the majority of the signal measured was specific for peroxide. In fact, much of the remaining signal may be due to incomplete degradation of H₂O₂ by topical PEG-catalase, because plasma peroxide concentrations were much lower than periarterial levels. The concentration of the basal periarterial H₂O₂ measured in SHR (20 μM)

**Fig. 4. Periarterial [H₂O₂] measurements.**

A: linearity of the WPI ISO-HPO-100 electrode is shown in a typical calibration plot (electrode voltage vs. [H₂O₂], r² = 0.99). The electrode has excellent sensitivity and linearity for the lowest levels of perivascular peroxide measured. B: specificity of the electrode for H₂O₂ is demonstrated by measurements made before and after topical polyethylene-glycolated catalase (PEG-catalase) in control and apocynin-treated WKY and SHR. In rats not pretreated with apocynin, SHR had significantly higher basal [H₂O₂] than WKY rats. With apocynin treatment, the baseline in WKY was not altered but was decreased in SHR to a level similar to that in WKY. Topical PEG-catalase greatly suppressed the peroxide signal in all groups. Two-way ANOVA was used for statistical analyses; n = 4, 3, 4, and 4 for WKY, WKY + Apo, SHR, and SHR + Apo, respectively. C: line plots demonstrate the relationship between percent changes in [H₂O₂] and arterial blood flow in control (untreated) and apocynin-treated WKY and SHR. As shown by regression analysis, there was a significant correlation between [H₂O₂] and flow only in untreated SHR (γ = 1.65 + 0.42x, r² = 0.42, P = 0.012); n = 5, 3, 5, and 5 for WKY, WKY + Apo, SHR, and SHR + Apo, respectively. D: bar graphs of group means for [H₂O₂] at baseline and during flow elevation. [H₂O₂] increased from baseline with flow only in untreated SHR (P = 0.028 and 0.002 for 1 and 2 clamps, respectively). Two-way repeated-measures ANOVA was used for statistical analyses; n = 4, 3, 4, and 4 for WKY, WKY + Apo, SHR, and SHR + Apo, respectively.
and SHR. This percent decrease is similar to the l-NAME-induced reduction in nitrite observed by Yan et al. (70) in isolated mesenteric arteries. Our expectation of reduced basal NO was based on previous direct measurement by NO electrodes in isolated SHR aorta (16) and mesenteric arteries (63) and measurement of SHR endothelial NOS activity (eNOS) (55, 72). However, there are numerous studies that have reported increased eNOS activity and/or elevated NO production in SHR (11, 34, 40, 46, 47, 52). Most relevant to this study are the reports by Nava et al. (46), showing an approximately twofold increase in SHR mesenteric artery eNOS activity, and Maffei et al. (40), who reported elevated NO levels, as determined by DAF-2 fluorescence, to be approximately twofold greater in SHR than in WKY mesenteric arteries. Chu and Bohlen (15) have reported good correlation in relative changes in ex vivo NO measured using NO electrodes and DAF-2 fluorescence. Thus considerable evidence exists to support our measurements of elevated basal NO concentration in SHR mesenteric arteries.

Flow modulation of NO production. Our results demonstrated an increase in NO with elevated flow in WKY but not in SHR (Fig. 3). This observation is consistent with earlier in vivo findings in healthy animals (6, 31) and with the suppression of flow-mediated NO production observed by Bohlen in obese rats (6). The impairment of SHR flow-mediated NO production is also consistent with previous studies that have demonstrated a suppressed NO component of flow-induced dilation in SHR (33, 36, 52) and in humans with hypertension (25) and coronary artery disease (51). In other experimental models, increased superoxide production and NO scavenging (39, 57), eNOS uncoupling (3, 56), and reduced eNOS activation (57) have all been shown to be involved in reduced NO concentration or release in response to elevated flow. However, as discussed below, our results suggest that elevated peroxide in the SHR mesenteric arteries stimulate NO production to a level that cannot be further increased by flow elevation.

Flow modulation of peroxide production. The flow-mediated elevation of peroxide in SHR but not WKY (Figs. 4 and 6) is

Fig. 5. Role of peroxide in NO regulation. A: exogenous H2O2 effects on NO. Periarterial [NO] was increased in the WKY but not SHR by addition of peroxide to the suffusion solution to yield final bath concentrations of 20 or 100 μM H2O2. Note that with 20 μM H2O2, [NO] in WKY was increased to the baseline SHR level. B: effect of PEG-catalase on SHR NO production. Acute PEG-catalase treatment (250 U/ml topically) lowered basal periarterial [NO] and restored the flow-mediated NO production in SHR. Two-way ANOVA was used for statistical analyses; n = 4 in each group in A and B.

Fig. 6. Effect of exogenous gp91ds-tat on periarterial [H2O2]. Topically applied gp91ds-tat (1 μM) had no effect on WKY [H2O2], but in SHR the basal level was reduced to the WKY level and flow-related H2O2 production was abolished. Two-way ANOVA was used for statistical analyses; n = 3 WKY and 4 SHR.
consistent with observations by Gutterman and colleagues (43, 51) with vessels from patients with and without arterial disease. In our study the flow-induced peroxide elevation was abolished by short-term treatment with apocynin and acute administration of gp91ds-tat, implying that the enzymatic source of the elevated peroxide in SHR mesenteric arteries is NAD(P)H oxidase, as discussed below. This is consistent with the increases in NAD(P)H oxidase-derived superoxide in cultured human endothelial cells induced by laminar shear (18, 22). However, Gutterman’s group (38) has shown that in coronary arteries obtained from patients undergoing cardiopulmonary bypass surgery, the primary source of flow-induced peroxide production is not NAD(P)H oxidase but mitochondrial complexes I and III. Results from a recent study suggest that potential pathways exist for interactions between oxidants produced by mitochondria and NAD(P)H oxidase (20).

Effect of peroxide on in vivo NO production. In vitro studies have shown that peroxide can increase eNOS expression and activation and, thus, NO production (13, 21, 61). Our data demonstrate that peroxide is a powerful activator of eNOS in vivo and is responsible for both the elevated basal NO and impaired flow-mediated NO production in the young SHR (Fig. 5). Topical application of peroxide increased WKY NO concentration to the same range observed in SHR. Unlike in WKY, topical addition of peroxide in SHR had no effect. However, reduction of peroxide level in the SHR by PEG-catalase lowered basal NO and restored flow-dependent NO production. These results indicate that the impaired flow-mediated NO production in mesenteric arteries of young SHR occurred because locally produced peroxide elevates baseline NO concentration to a level that cannot be raised further in response to increased flow.

Enzymatic source of elevated peroxide in SHR. NAD(P)H oxidase is upregulated in vascular tissue of SHR (19, 55, 69, 73). Our results demonstrate that both apocynin and gp91ds-tat decrease basal peroxide and prevent flow-mediated peroxide production in SHR (Figs. 4 and 6). Although the ability of apocynin to inhibit NAD(P)H oxidase in vascular cells has been challenged recently, the experiments were performed with modified, immortalized HEK-293 cells (30), which do not contain peroxidase to convert apocynin to its active form (62). Abundant evidence exists that in vascular tissues in vitro and in animals in vivo, apocynin does indeed inhibit NAD(P)H oxidase (62). Regardless, gp91ds-tat is a competitive NAD(P)H oxidase inhibitor (53). Thus available data together with our results provide strong evidence that the enzymatic source of the increased peroxide in SHR is NAD(P)H oxidase.

Study limitations. We did not attempt to estimate shear stress, the presumed stimulus for flow-mediated NO production, because accurate measurements of diameter changes could not be made due to the adipose tissue overlying the mesenteric arteries. We could not displace the adipocytes with a glass plate as in our collateral growth studies (42, 67), because this would have precluded the electrode measurements. Adipocytes could have been removed from the artery, but this might have impacted the levels of paracrine factors and altered in vivo NO and H2O2 production. However, since others have shown flow-mediated dilation to be impaired in the SHR mesentery (33, 52) and because the changes in flow were similar between WKY and SHR (Fig. 3), the stimulus for NO production would be expected to be greater in SHR than WKY. Thus we do not believe the differences in flow-mediated NO production were the result of a decreased stimulus level in SHR.

In the initial experiments, we chose to use short-term rather than acute apocynin treatment. This was done because, as we have shown, this antioxidant protocol promotes collateral artery growth in SHR (42). This therapy reduced arterial pressure in the SHR and also may have influenced expression levels of eNOS and its cofactors as well as oxidant-forming and scavenging molecules. Although such potential mechanisms may be involved in alteration of NO and peroxide concentrations observed with chronic apocynin therapy, acute treatment with PEG-catalase and gp91ds-tat produced similar effects on NO and H2O2 concentrations (Figs. 4 and 6) without altering arterial pressures and before significant changes in protein expression could have occurred. In addition, although many antioxidants have antihypertensive effects, they also provide benefits that are independent of decreased pressure (42, 45).

It is important to note that our study was performed in relatively young animals with acute flow elevation. The results could be different in older animals or in animals with longer term hypertension or different vascular disease. Longer elevation of flow, NO, or ROS may induce changes in the expression and activity of molecules involved in the production of both of these species, including eNOS, NAD(P)H oxidase, superoxide dismutase, catalase, and arginase, among others (57, 65). There also may be differences between vascular beds; however, elevated flow-induced peroxide production has been observed in human arterioles from heart, intestinal, and adipose tissues (27, 43, 51). Other investigators have shown that peroxide elevation can result in elevated as well as reduced NO production (12, 13, 59). The differences could be related to the peroxide concentration and/or duration of exposure.

Clinical implications. Flow-mediated changes in NO and H2O2 are important in regulating both arterial tone and wall remodeling, such as that occurring during collateral growth. Substantial evidence indicates that collateral growth is impaired in the presence of arterial disease and its risk factors, including aging (44, 66), hypertension (29, 42, 67), hypercholesterolemia (68), metabolic syndrome (28, 64, 71), and diabetes (1, 35). All of these risk factors are characterized by endothelial dysfunction and elevated oxidative stress. Significant effort is being made to develop cellular and molecular therapies to promote collateral growth. We believe such therapies will only achieve their maximal effectiveness after abnormalities in redox balance are corrected. For example, as previously reviewed (58), ROS can influence many processes involved in physiological or pathological arterial remodeling, including cell proliferation, hypertrophy and migration, matrix metabolism, and cytokine production, and recent studies have shown that peroxide impairs the function of stem/progenitor cells (24, 32). The similarity of acute effects of catalase and gp91ds-tat and the short-term effects of apocynin on peroxide and NO concentrations suggest that appropriate antioxidant therapies could provide vascular benefit when administered chronically in the clinic.

In summary, this study demonstrates that peroxide and NO concentrations in mesenteric arteries of young SHR are abnormal under both basal conditions and during acute flow elevation. Acute and short-term treatment with antioxidants restores concentrations of these reactive species to normal values.
Future studies are warranted to determine whether similar results are obtained in other models of endothelial dysfunction, including older SHR with more established disease.

REFERENCES


