Hypoxic relaxation of penile arteries: involvement of endothelial nitric oxide and modulation by reactive oxygen species

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Penile erection occurs when nitric oxide (NO) released from nerve terminals upon sexual stimulation decreases vascular resistance and increases blood flow through cavernous and helicine arteries, thus stimulating further NO release from penile endothelium, relaxation of corporal smooth muscle, and sustained erection (40, 41). The enzyme responsible for NO generation NO synthase (NOS) uses l-arginine as a substrate and promotes its oxidation with NADPH and O2 consumption to yield citrulline and NO (33). Since molecular O2 is a substrate for the synthesis of both neural and endothelial NO by NOS, partial O2 pressure (Po2) in the blood of the corpus cavernosum plays a key role in the regulation of penile hemodynamics. During the flaccid state, Po2 is similar to that of venous blood and it rises to 90–100 mmHg during erection as a result of the increased arterial inflow to the sinuses (2). Early studies by Kim et al. (23) demonstrated that Po2 modulates penile erection by regulating NO synthesis in corpus cavernosum tissue and the ability of cavernosal smooth muscle to relax in response to electrical stimulation of the nerves. In addition, responses to endothelium-dependent vasodilators are progressively inhibited as a function of decreasing Po2 levels. Thus increased blood flow and Po2 to the penis after arterial dilatation would trigger NO synthesis, relaxation of trabecular smooth muscle, and erection (23).

The effects of chronic hypoxia on erectile function have been reported in both clinical and experimental studies. Erectile dysfunction (ED) is commonly found in male patients with several pathological conditions related with chronic hypoxia such as aging, heart and respiratory failure, sleep apneas, chronic obstructive respiratory disease, diabetes, hypertension, and arteriosclerosis (5, 43). In a rabbit model of atherosclerosis-induced chronic cavernosal ischemia, both neuronal NOS and endothelial NOS (eNOS) proteins dramatically decrease in erectile tissue, which suggests that arterial insufficiency and subsequent exposure of erectile tissue to hypoxia impair constitutive NOS expression and thus NO synthesis and relaxation (3, 4). This probably contributes to the arteriogenic ED induced by atherosclerosis and other arterial occlusive diseases.

ED is currently considered as an early sign of subclinical vascular disease and frequently coexists with vascular diseases such as hypertension, atherosclerosis, and diabetes mellitus (34). Endothelial dysfunction, increased oxidative stress, and reduced NO bioavailability endanger tissue blood flow under these pathological conditions. Studies in prediabetic animal models have demonstrated that hypoxic dilatation of systemic small arteries is impaired because of the scavenging of endothelial mediators by the enhanced generation of reactive oxygen species (ROS) in the arterial wall (6, 7, 10, 11). It is well established that the dysregulation of ROS production is involved in the pathogenesis of vascular diseases such as hypertension, hypercholesterolemia, and diabetes (37, 48). However, changes in ROS production and in redox status have been hypothesized also to play a role in the O2 sensing mechanisms of vascular smooth muscle cells (48).

Although the association of chronic hypoxia and ED is well documented, the mechanisms underlying the acute effects of hypoxia on the integrated function of erectile tissue are not well understood (22). Most systemic blood vessels relax to hypoxia as a part of local autoregulatory mechanisms that match blood perfusion to the metabolic demands of the tissue. The aim of the present study was to assess the responses of penile arteries to acute hypoxia and the mechanisms responsible for the hypoxic vascular responses with special regard to
the involvement of the endothelium and ROS production in the vascular wall. Since obesity and insulin resistance increase the risk of cardiovascular disease and the prevalence of ED (8, 12), we hypothesize that penile hypoxic vasodilator responses might be impaired under these conditions of cardiovascular risk. The effects of obesity were hence assessed on the penile vascular responses to hypoxia in a rat model of high-fat diet (HFD)-induced obesity.

MATERIALS AND METHODS

Animals. Male Wistar rats (n = 60) were maintained in the animal care facility at the New York Medical College on standard rat chow (n = 50) or on a HFD (n = 10; 60% of saturated fat; 58Y1, TestDiet) for 10 wk and were given free access to food and water (7, 9). Experiments were performed on 14–16-wk-old rats. Animals were treated in a manner approved by the Institutional Animal Care and Use Committee of New York Medical College, and the protocols followed the current guidelines of the National Institutes of Health and American Physiological Society for the use and care of laboratory animals.

Dissection, mounting, and force measurement. Adult male Wistar rats were given an intraperitoneal injection of pentobarbital sodium (50 mg/kg body wt) and euthanized by the removal of the heart and lungs. The penile arteries, first- or second-order branches of the rat dorsal penile artery, were carefully dissected as previously described (45) and mounted in microvascular myographs (DMT, Denmark) as ring preparations about 2 mm long by inserting two 40-μm tungsten wires into the vessel lumen. The arteries were equilibrated for 30 min in Krebs solution at 37°C and continuously gassed with 21% O2-5% CO2-74% N2, and the relationship between passive wall tension and second exposure after 1 h, the effects of endothelial removal and of peroxide including ebselen (5 M Phe. The effects of hypoxia elicited by the gas changes used in functional studies on superoxide levels detected by chemiluminescence from arteries placed in a 1-cm² cuvette-type thermostated tissue bath inside a light-tight box containing Krebs buffer with 5 μM lucigenin was determined as described previously (32). After 1 h equilibration, the arteries were exposed to hypoxia (20 min), followed by reoxygenation (10 min).

Data presentation and statistical analysis. In the functional experiments, the mechanical responses of the arteries were measured as force and expressed as active wall tension, ΔT, which is the increase in force, ΔF, divided by twice the segment length. The results are expressed as either newtons per meter of tension or as a percentage of the response to either high-K+ physiological salt solution or the precontractor drug in each artery. Sensitivity to the agonists is expressed in terms of pD2 values = −log(EC50), EC50 being the molar concentration of the agonist producing 50% of the maximum response. EC50 was calculated by nonlinear curve fitting of the concentration-response curves for the inhibitor to the classical Hill Equation by using the computer program GraphPad Prism 5.0 (GraphPad Software, San Diego, CA). The EC50 value for each individual curve was first obtained, and thereafter the average value for a given set of experiments was calculated. The statistical differences between means were analyzed by paired or unpaired Student’s t-test where appropriate. P < 0.05 was considered statistically significant.

RESULTS

The penile arteries used in the present study had an effective internal lumen diameter (l) of 216 ± 4 μm (n = 53) and responded to a high-K+ solution with a contraction of 2.26 ± 0.08 N/m (n = 53). Phe (1 μM) induced a prompt and stable contraction of 2.90 ± 0.12 N/m. ACh evoked concentration-dependent relaxations, pD2 and maximal responses being 6.08 ± 0.06 and 80 ± 2% of the Phe-induced preconstriction arteries (n = 50).

Effects of acute hypoxia on rat penile arteries. In arteries precontracted with Phe, acute hypoxia produced a relaxation of 52 ± 2% (n = 52) of the Phe-induced tone over the 20-min period examined, reaching a maximum at 15 min. A small transient contraction within the first 5 min of exposure to hypoxia could often be observed (Fig. 1A). Following hypoxia,
reoxygenation with 21% O2 for 10 min induced a recovery of 95 ± 1% (n = 52) of the initial tone induced by Phe (Fig. 1A).

Effect of endothelial removal, inhibition of NOS and cyclooxygenase on relaxation to hypoxia and ACh. To assess the role of the vascular endothelium in penile hypoxic relaxations, the effects of endothelial cell removal and inhibitors of endothelial mediators such as NO and prostanoids were tested. The mechanical removal of the endothelium significantly reduced the relaxant response of penile arteries to hypoxia (Fig. 1C) and blunted the relaxations elicited by 10 μM ACh (Fig. 1, B and D). The blockade of NOS with L-NNA markedly inhibited the relaxations obtained after 10 min of hypoxia without affecting reoxygenation (Fig. 2A) and abolished the ACh relaxant responses in the same arteries (Fig. 2B). The blockade of cyclooxygenase with indomethacin caused a modest but significant inhibition of the relaxation elicited by hypoxia after 10 min and by 0.1 μM ACh (Fig. 2, C and D). These data suggest an involvement of endothelial-derived NO and relaxant prostanoids in the hypoxic vasodilator responses of penile arteries.

Scavenging superoxide with the SOD mimetic Tempol and inhibition of Nox activation enhance relaxation to both hypoxia and ACh. A possible influence of ROS on the relaxation of penile arteries to hypoxia was further assessed. Figures 3 and 4 show the effect of superoxide scavengers and inhibitors of Nox oxidases on the relaxation of penile arteries to hypoxia and ACh. Treatment with the SOD mimetic Tempol (30 μM) significantly enhanced relaxation to hypoxia (Fig. 3A) and also the relaxant responses elicited by ACh (Fig. 3B). The inhibition of nonselective Nox activation with apocynin (30 μM) and of specific Nox2 activation with gp91ds-tat (1 μM) also caused a significant increase in relaxation to both hypoxia and ACh without altering Phe-induced tone (Fig. 4), suggesting that Nox2-derived superoxide is interfering with relaxant responses of penile arteries to both hypoxia and ACh.

Basal superoxide production in penile arteries: influence of endogenous NO, Nox inhibition, and hypoxia. Figure 5A shows the quantification of basal superoxide release in intact penile arteries measured by lucigenin chemiluminescence. Since protein kinase C (PKC) has been shown to activate Nox2 in bovine coronary arteries and mouse aorta (16), superoxide levels obtained after the activation of PKC with phorbol 12,13-dibutyrate (PDBu, 10 μM) were used as a positive control of superoxide production in penile arteries. The preincubation with Tempol (30 μM) reduced chemiluminescence by almost 70%, confirming specificity for superoxide, whereas the activation of PKC with PDBu increased by more than twofold basal superoxide production (Fig. 5A). An investigation of the potential sources of superoxide generation showed that the inhibition of Nox activation with 1 μM gp91ds-tat partially inhibited both basal and PDBu-induced superoxide release (Fig. 5A). On the other hand, the inhibition of NOS with L-NNA (100 μM) increased by 30% basal superoxide, suggesting that basal superoxide levels had a scavenging effect of arterial NO production (Fig. 5A).

Whereas Phe (10 μM) had a small effect, which was not statistically significant, on increasing basal superoxide levels (4 ± 2%, n = 6), exposure to acute hypoxia reduced super-
Oxidation production in penile arteries by 46%, whereas reoxygenation induced a recovery of 97% of the basal superoxide levels. The reduced chemiluminescence over a 20-min period and the recovery after 10 min reoxygenation are normalized to the basal chemiluminescence at time 0.

Effect of scavenging peroxide with catalase or ebselen on hypoxia- and ACh-induced relaxation. To assess an influence of endogenous peroxide on the hypoxic relaxant responses of penile arteries, the effects of catalase and ebselen were tested. Scavenging peroxide with catalase (200 U/ml) markedly enhanced hypoxia- and ACh-induced relaxations (Fig. 6, A and B). Ebselen, a glutathione peroxidase mimetic that can decrease intracellular peroxide (13), induced an even greater enhancement of the hypoxic and ACh relaxant responses in penile arteries (Fig. 6, C and D). This agent also reduced the contractile response upon reoxygenation from 97 ± 1 to 53 ± 15 of the Phe-induced contraction (n = 6, P < 0.05) (Fig. 6C).

Effect of exogenous H2O2 on penile arteries. An exogenous addition of H2O2 (1–100 μM) caused a concentration-dependent relaxation of Phe-precontracted penile arteries, with the...
maximum response at 100 μM being 85 ± 3% (n = 16). A biphasic response consisting of a transient contraction followed by a profound and persistent relaxation was often observed at the highest concentrations of H₂O₂ (Fig. 7A). Scavenging peroxide with catalase (200 U/ml) blunted relaxation to exogenous H₂O₂ (Fig. 7B). The removal of the endothelium reduced the contractile component of the response to H₂O₂ at the highest concentration used (100 μM) to 54 ± 9% (P < 0.01 vs. control, n = 6) (Fig. 7C). The inhibition of cyclooxygenase with indomethacin (1 μM) blunted the relaxations elicited by low concentrations of H₂O₂ (Fig. 7D). Since the vascular effects of peroxide can be mediated through the activation of both the ERK MAPK (13) and the p38 MAPK (1), the effects of selective inhibitors were assessed on the exogenous peroxide-elicited relaxations. PD-98050 (10 μM) inhibited the relaxation responses elicited by low concentrations of H₂O₂ (Fig. 7E), suggesting that ERK MAPK influenced the observed relaxation responses. In contrast, SB-203580 (10 μM) enhanced the H₂O₂-elicited relaxation (Fig. 7F), suggesting that p38 MAPK has a negative modulatory effect upon peroxide-induced relaxation in penile arteries.

Effect of HFD-induced obesity on penile hypoxic vasodilator responses. Similar to our previous observations (7, 9, 21), rats fed a HFD developed obesity, which is characterized by
increased body weight (418 ± 15 and 504 ± 18 g, n = 10, P < 0.001, in control and HFD-fed rats, respectively), plasma insulin, and cholesterol levels. Arterial blood pressure was significantly increased in anesthetized HFD rats (7). The penile arteries from HFD-fed rats had normalized lumen diameters of 222 ± 16 (n = 12), which were not different from those in lean control rats. Neither contractile responses induced by either high K+ (2.42 ± 0.26 N/m, n = 12) or 1 μM Phe (2.92 ± 0.31 N/m, n = 12) nor endothelium-dependent relaxations elicited by ACh (pD2, 5.91 ± 0.07; and Emax, 73 ± 3%, n = 12) of penile arteries from HFD-fed animals were significantly different from those in arteries of control rats.

Figure 8 shows the effects of dietary fat-induced obesity on the relaxant responses to hypoxia and on the basal superoxide production of penile arteries. The relaxation induced by acute hypoxia was significantly impaired in arteries from HFD-fed animals, whereas the contractile responses upon reoxygenation were unchanged compared with control arteries (Fig. 8A). A quantification of superoxide production in intact arteries by lucigenin-enhanced chemiluminescence demonstrated that basal superoxide production was significantly enhanced by about twofold in arteries of HFD rats (Fig. 8C). In contrast to that observed in arteries from control animals, the treatment with L-NNA did not further reduce hypoxic relaxant responses in penile arteries of HFD-fed rats (Fig. 8C). In contrast to the marked inhibition of relaxation to ACh by NOS blockade seen in arteries from control rats (Fig. 2B), penile arteries from HFD rats showed substantially less inhibition of relaxation to ACh when NOS activity was inhibited (Fig. 8D).

**DISCUSSION**

The present study demonstrates that acute hypoxia relaxes penile arteries through a mechanism involving the release of NO from the endothelium and that its interaction with the basal ROS production in the arterial wall is a major factor in limiting the response that is observed. In animals with HFD-induced obesity, the relaxant responses to hypoxia are impaired and associated with increased superoxide levels and a blunting of the NO component of the hypoxic relaxation. This suggests that under pathophysiological conditions, enhanced oxidative stress and consequently reduced NO bioavailability seem to contribute to endothelial/vascular dysfunction in penile arteries in a manner that impairs hypoxic relaxant responses.

Penile arteries responded to acute hypoxia with a significant relaxation similar to that found in other systemic arteries and also in penile corpus cavernosum, where hypoxia induced relaxation and the accumulation of intracellular Ca2+ (22). Although a significant fraction of the penile hypoxic relaxant response originates from vascular smooth muscle, the present results demonstrate that the endothelium modulates this response, and endothelium-derived mediators including both NO and relaxant prostanoids appear to be involved in the relaxation to acute hypoxia that is observed. These data are consistent with earlier observations in other systemic arteries such as...
skeletal muscle and coronary small arteries from the rat (10, 26). However, the mechanisms for relaxation to hypoxia in penile arteries seem to differ from those in penile corpus cavernosum, because the response in this tissue was found to be independent of the endothelium, suggesting differences in the signaling pathways that control relaxation of penile erectile tissues under hypoxic conditions (22). Acute hypoxia has been shown to upregulate eNOS expression in vascular endothelial cells, and hypoxic eNOS-derived NO release has been proposed as a protective mechanism to counteract the negative effects of acute episodic hypoxia near the endothelium (20, 30, 36). The blockade of NOS with L-NNA mimicked the effect of endothelial removal and caused a significant inhibition of penile relaxation to hypoxia, suggesting the release of endothelial-derived NO. This is consistent with earlier in vitro (10, 26) and in vivo (27, 38) studies supporting the involvement of NO in the systemic vasodilatation to acute hypoxia. On the other hand, the inhibitory effect of indomethacin on the relaxations observed after 10 min of acute hypoxia in penile arteries further suggests a release of relaxant prostanoids, which is in agreement with that reported for human arteries where the endothelium-dependent relaxation to hypoxia is in part mediated by prostacyclin release (39).

The participation of ROS in the vascular O2 sensing mechanisms remains a matter of debate (48), and both ROS generated by either Nox or mitochondria (29, 36, 46) and ROS-independent pathways (15, 32, 49) have been suggested to play a role in the arterial responses to hypoxia. In the present study, the enhancing effect of the SOD mimic Tempol and of the Nox inhibitors apocynin and gp91ds-tat suggests that ROS are interfering with the hypoxic relaxations of penile arteries under normal conditions. An increased formation of superoxide along with an upregulation of gp91phox and enhanced Nox activity in response to hypoxia has been reported in pulmonary endothelial and smooth muscle cells (36). However, consistent with findings in both pulmonary and coronary arteries (17, 32, 48), an exposure to acute hypoxia significantly decreased basal superoxide levels measured by lucigenin chemiluminescence in intact penile arteries, which recovered to control levels upon reoxygenation. The enhancing effect of ROS scavengers on the relaxation to hypoxia despite the inhibitory effect of hypoxia on superoxide production could be explained on the basis of the interactions between basally released superoxide and NO released by hypoxia. Thus a significant basal production of superoxide increased by PKC activation (16) and blunted by Tempol was found in penile arteries. Vascular smooth muscle from resistance arteries contains Nox2, a Nox also known as gp91phox. The modest inhibitory effect of Nox2 inhibition on basal superoxide production suggests that other sources also contribute to ROS production in penile arteries. This is consistent with findings in systemic arteries from Nox2-deficient mice where superoxide production was unaltered by Nox2 deletion (31). Also, in human vessels, superoxide production by the Nox system was greater in veins than arteries, where
other sources such as xanthine oxidase substantially contribute to superoxide production (18, 19).

In the present study, the blockade of NOS increased basal superoxide production in penile arteries, suggesting that the release of ROS is modulated by its interaction with endogenous endothelial-derived NO, probably by producing peroxynitrite that reduces the bioavailability of both radicals. The interaction between basal NO and superoxide has earlier been shown in human arteries, where there is a greater superoxide production than in veins, which is balanced by NO, resulting in a higher arterial peroxynitrite formation (18, 19). The present results also suggest that NO released by either acute hypoxia or by the endothelial agonist ACh could be scavenging basal superoxide, and this in turn may account for the enhancing effect of superoxide scavengers and Nox inhibitors on the hypoxia- and ACh-induced relaxations of penile arteries. Accordingly, acute hypoxia has been shown to enhance eNOS expression, to reduce nitrate content (index of NO formation), and to increase nitrotyrosine (a potential indicator of peroxynitrite formation) in intact pulmonary arteries (18, 19). Thus ROS can modulate hypoxia-induced vasodilatation by decreasing NO bioavailability through quenching by superoxide in a manner that is associated with the formation of peroxynitrite.

Hypoxia and the inhibition of the electron transport chain increases mitochondrial ROS generation in arterial smooth muscle (13, 29), and the subsequent dismutation of superoxide to peroxide makes ROS readily diffusible across mitochondrial membranes. In the present study, the enhancing effect found for peroxide scavengers such as catalase and the glutathione peroxidase mimetic ebselen on the relaxant responses induced by hypoxia suggests an interaction of peroxide-mediated contractile effects with the hypoxia relaxation of penile arteries. Ebselen has been shown to efficiently scavenge peroxynitrite (24, 28), and this could account in part for the enhancing effect of this agent on the hypoxia-induced relaxations. However, recent studies demonstrate that elevated peroxide levels can impair the relaxation induced by acute hypoxia in coronary arteries through the activation of ERK MAPK (13). The predominant vasoactive effect found for exogenous peroxide on penile arteries was a relaxation partially mediated by the endothelium, the release of relaxant prostanoids, and ERK MAPK. However, the vasoconstrictor effects of peroxide could also be observed and unmasked by the inhibition of the p38 MAPK, which is consistent with the dual vasodilator and p38 MAPK-mediated vasoconstrictor effects reported for peroxide in mouse mesenteric resistance arteries (25). On the other hand, peroxide derived from p47phox Nox2 activation by PKC is a major contributor to vasoconstrictor (thromboxane A2)-elicited contraction in coronary arteries (16). In the present study we confirm that the activation of PKC markedly increases superoxide generation through a mechanism involving Nox2 activation, as depicted by the inhibition caused by gp91ds-tat on the PDBu-induced enhancement of lucigenin chemiluminescence. However, while the data in Fig. 5B suggest that the activation of α1-adrenoceptors with Phe may have had a small effect on increasing superoxide, this effect did not reach statistical significance. Thus the processes contributing to the basal Nox-
associated vasoactive levels of superoxide generation in penile arteries remain to be defined.

Several earlier studies have shown impaired hypoxic vasoconstriction responses in arteries from animal models of cardiovascular disease (10, 11, 14). Obesity, along with a constellation of metabolic and vascular abnormalities including insulin resistance, dyslipidemia and hypertension, jointly referred to as metabolic syndrome, markedly increases the risk for cardiovascular disease and diabetes (42). These metabolic alterations are associated with endothelial dysfunction, and they act as independent risk factors for ED (8, 12). In the obese Zucker rat, an experimental model of genetic obesity and metabolic syndrome-associated ED (47), endothelial dysfunction correlates with abnormal structure in penile arteries, and both NO- and prostanooid-mediated endothelium-dependent relaxations are impaired (44). The data provided in the present study further extend the concept that HFD-induced obesity is associated with penile vascular dysfunction and demonstrate that HFD-induced obesity causes endothelial dysfunction depicted by the impaired endothelial NO-mediated component of the hypoxic relaxations of penile arteries. Impaired vasodilator responses to reduced Po2 have earlier been demonstrated in systemic microvessels from the obese Zucker rat model of genetic obesity (10, 11, 14). Both increased vascular thromboxane A2 production (14) and reduced NO-mediated responses (10) have been shown to be involved in the blunted hypoxic relaxations of arteries from obese animals. In the present study, the lack of an inhibitory effect of NOS blockade on the hypoxic responses of penile arteries from HFD-induced obese rats suggests that hypoxia-induced release of NO is impaired. Moreover, the reduced inhibitory effect of L-NNA on the endothelium-dependent relaxations to ACh further supports an impaired NO release/production in these arteries, although the whole ACh relaxant response was preserved. Several mechanisms have been suggested to compensate the impaired NO-mediated component of the arterial endothelial-dependent relaxations in obesity. Thus, in the obese Zucker rat, protanoids maintain the hypoxic vasodilation of skeletal muscle microvessels (10), whereas a major contribution of the endothelium-derived hyperpolarizing factor preserves the ACh arteriolar coronary relaxation in HFD-induced obese rats (9).

The expression of eNOS has been demonstrated to be either unaltered or upregulated in arteries from both genetic (44) and dietary fat-induced (21) models of obesity. Therefore, changes in eNOS protein content are not likely to be involved in the reduced NO-mediated responses of the hypoxic relaxations in penile arteries from HFD-fed rats. However, increased oxidative stress was found in these arteries, which confirms that observed in skeletal muscle small arteries from the same model of obesity, where an enhanced xanthine oxidase-derived superoxide production has been demonstrated (7). The present findings therefore suggest that enhanced ROS production is probably interfering with the NO signaling of penile arteries, thus impairing NO-mediated hypoxic relaxant responses. Although erectile function was not assessed in the present study, the endothelial dysfunction demonstrated for penile arteries is likely to be a cause of ED in this model of dietary fat-induced obesity, as demonstrated in rodent models of genetic obesity (44, 47) and in clinical studies showing the association between obesity and ED (8, 12).

In summary, the present study provides evidence for the involvement of endothelial-derived NO and its modulation by ROS in the hypoxic relaxation of penile arteries. Decreased NO bioavailability and enhanced ROS production impair penile hypoxic responses and contribute to the endothelial/vascular dysfunction in dietary fat-induced obesity. However, further studies are needed to document the origins and biological importance of these mechanisms in metabolic disease-associated ED.

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

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