Differential structural and functional changes in penile and coronary arteries from obese Zucker rats

Nuria Villalba,1 Pilar Martínez,2 Ana María Briones,3 Ana Sánchez,1 Mercedes Saláces,3 Albino García-Sacristán,1 Medardo Hernández,1 Sara Benedeto,1 and Dolores Prieto1

1Department of Physiology, Faculty of Pharmacy, and 2Department of Anatomy, Faculty of Veterinary Sciences, Universidad Complutense, Madrid; and 3Department of Pharmacology, Faculty of Medicine, Universidad Autónoma, Madrid, Spain

Submitted 18 December 2008; accepted in final form 11 June 2009

Villalba N, Martínez P, Briones AM, Sánchez A, Saláces M, García-Sacristán A, Hernández M, Benedeto S, Prieto D. Differential structural and functional changes in penile and coronary arteries from obese Zucker rats. Am J Physiol Heart Circ Physiol 297: H696–H707, 2009. First published June 19, 2009; doi:10.1152/ajpheart.01308.2008.—Erectile dysfunction frequently coexists with coronary artery disease and has been proposed as a potential marker for silent coronary artery disease in type 2 diabetes. In the present study, we comparatively assessed the structural and functional changes of both penile arteries (PAs) and coronary arteries (CAs) from a prediabetic animal model. PAs and CAs from 17- to 18-week-old obese Zucker rats (OZRs) and from their control counterparts [lean Zucker rats (LZRs)] were mounted in microvascular myographs to evaluate vascular function, and stained arteries were subjected to morphometric analysis. Endothelial nitric oxide (NO) synthase (eNOS) protein expression was also assessed. The internal diameter was reduced and the wall-to-lumen ratio was increased in PAs from OZRs, but structure was preserved in CAs. Ach-elicited relaxations were severely impaired in PAs but not in CAs from OZRs, although eNOS expression was unaltered. Contractions to norepinephrine and 5-HT were significantly enhanced in both PAs and CAs, respectively, from OZRs. Blockade of NOS abolished endothelium-dependent relaxations in PAs and CAs and potentiated norepinephrine and 5-HT contractions in arteries from LZRs but not from OZRs. The vasodilator response to the phosphodiesterase 5 inhibitor sildenafil was reduced in both PAs and CAs from OZRs. Pretreatment with SOD reduced the enhanced vasoconstriction in both PAs and CAs from OZRs but did not restore Ach-induced relaxations in PAs. In conclusion, the present results demonstrate vascular inward remodeling in PAs and a differential impairment of endothelial relaxant responses in PAs and CAs from insulin-resistant OZRs. Enhanced superoxide production and reduced basal NO activity seem to underlie the augmented vasoconstriction in both PAs and CAs. The severity of the structural and functional abnormalities in PAs might anticipate the vascular dysfunction of the more preserved coronary vascular bed.

vascular remodeling; endothelial dysfunction; coronary artery; penile artery; erectile dysfunction

ERECTILE DYSFUNCTION (ED) is currently considered an early clinical manifestation of a more generalized vascular disease due to its high prevalence in patients with cardiovascular risk factors including diabetes, hypertension, hyperlipidemia, and tobacco abuse (32, 46). ED is a common complication and an important cause of decreased quality of life in men with diabetes, and its prevalence is three times higher in type 1 and type 2 diabetic patients than in the general population (18, 48).

Growing epidemiological evidence associates the subsequent risk of ED with the presence of risk factors for coronary artery disease (CAD) such as obesity, hypertension, and dyslipidemia (32, 34). On the other hand, the rate of ED in patients with CAD is as high as 42–57%, and the incidence of ED in diabetic patients with silent ischemia is 34.8% versus 4.7% in those without silent ischemia (16, 32, 34). This has recently led to the suggestion that ED could be a potential marker for silent CAD in type 2 diabetes mellitus patients (16, 34).

Normal erectile function is primarily a vascular event that relies on vasodilatation, which is largely due to both nerve- and endothelium-derived nitric oxide (NO). Thus, neural NO-mediated arterial dilatation and increased blood inflow to the corpora cavernosa produced by parasympathetic activation at the initiation of the erection leads to shear stress-mediated stimulation of the endothelial lining in penile arteries (PAs), which, in turn, releases NO from the endothelium and produces further vasodilatation and sustained erection (23, 40). Previous studies (1, 27, 44) have shown that both endothelium and nerve-derived NO-mediated relaxant responses are impaired in penile erectile tissues from diabetic patients and most type 1 diabetic animal models. However, endothelial dysfunction is a key factor in the development of vascular complications in insulin resistance and diabetes and is thought to be one of the links between ED and CAD (6, 46).

The purpose of the present study was to further investigate the association between ED and CAD by evaluating the functional response of PAs and coronary arteries (CA) from the obese Zucker rat (OZR), a well-established genetic model of insulin resistance caused by a dysfunctional gene of the leptin receptor. These animals are used as a model of type 2 diabetes because of impaired glucose tolerance associated with inherited obesity gene mutation, and they progressively develop obesity, type 2 diabetes, and hypertension (17). The adverse effects of insulin resistance on the peripheral and coronary circulations are well documented, and endothelial dysfunction emerges as major factor in the development of hypertension and CAD (2, 7, 26, 35). However, the effects of insulin resistance are virtually unexplored in the PA circulation. Both corporal venuoocclusive dysfunction (28) and reduced erectile responses to stimulation of the cavernosal nerves (50) have been demonstrated in the OZR, thus confirming the development of ED in this prediabetic animal model. Therefore, we assessed the effects of insulin resistance on the vascular structure and function of PAs and CAs with special regard to the role of endothelium-derived NO in the genesis of vascular dysfunction.

METHODS

Animal model. All the experiments were approved by the Animal Experimentation Ethics Committee of Complutense University and conformed to National Institutes of Health Guidelines for the Care of Reprint requests and other correspondence: D. Prieto, Dept. of Physiology, Faculty of Pharmacy, Universidad Complutense de Madrid, Madrid 28040, Spain (e-mail: d prieto@farm.ucm.es).
and the Use of Laboratory Animals: OZR (fafa, n = 43) and their control counterparts [lean Zucker rats (LZRs); fat-, n = 44] were purchased from Charles River Laboratories (Barcelona, Spain) at 8–10 wk of age. Animals were housed at the Pharmacy School animal care facility and maintained on standard chow and water ad libitum until they were used for study. Experiments were performed on 17- to 18-wk-old rats. On the day of the experiment, rats were weighed, blood samples were collected, and plasma was frozen for the determination of nonfasting glucose, total triglyceride, and total cholesterol levels using commercially available kits. Plasma insulin levels were measured by a specific ELISA. Systolic blood pressures and heart rates were measured weekly by tail-cuff plethysmography. Rats were killed by cervical dislocation and exanguination, and the heart and penile tissues were quickly removed and placed in cold physiological saline solution (PSS) maintained at 4°C of the following composition (in mM): 119 NaCl, 4.7 KCl, 1.18 KH2PO4, 1.17 MgSO4, 1.5 CaCl2, 24.9 NaHCO3, 0.027 EDTA, and 11 glucose (pH 7.4).

Preparation of isolated small arteries. First- and second-order branches of the rat dorsal PA, which run in parallel along the dorsal surface of the penis just above the penile dorsal vein, and second-order branches of the left descending CA were carefully dissected by removing the connective and fat tissue and adhering myocardium, respectively, as previously described (41, 49). Arterial segments from OZRs and LZRs were mounted in parallel in double microvascular myographs (Danish Myotechnology) and equilibrated for 30 min in saline solution (PSS) at 37°C gassed with 95% O2-5% CO2, and the relationship between passive wall tension and internal circumference was then determined for each individual artery. From this, the internal circumference corresponding to a transmural pressure of 100 mmHg for a relaxed vessel in situ (L100) was calculated. Arteries were set to an internal circumference equal to 0.9 × L100 (l), at which tension development is maximal in these arteries (41, 49).

Experimental procedures for functional experiments. At the beginning of the experiment, arteries were challenged twice with 124 mM high-K+ PSS (KPSS) to test vessel viability. Endothelial function and vasoconstrictor responses were evaluated in both PAs and CAs from LZRs and OZRs by constructing concentration-dependent response curves (CRCs) to ACh and norepinephrine or 5-HT, respectively, in the absence and presence of the NO synthase (NOS) inhibitor Nω-nitro-l-arginine (L-NNa; 100 μM), which was applied directly into the bath 30 min before the second CRC to ACh or norepinephrine was constructed. The effects of the phosphodiesterase 5 (PDE5) inhibitor sildenafil and the NO donor SNAP-S-nitroso-N-acetylpenicillamine (SNAP) were also evaluated in arteries precontracted with either 1 μM phenylephrine or 20 μM 5-HT. To evaluate superoxide production in the vessel wall, arteries were incubated for 30 min with 150 U/ml SOD (8) before the construction of a CRC to either norepinephrine, 5-HT, or ACh. Since agonist-elicted responses were reproducible in two consecutive CRCs, the effect of treatment was assessed in the same artery, with the first CRC being used as the control and the second CRC being constructed after treatment.

Vessel morphometry and immunohistochemistry. Tissue samples from the myocardium and penis containing the left descending CA and dorsal PA, respectively, were immersion fixed in 4% paraformaldehyde in 0.1 M sodium phosphate buffer (PB), cryoprotected in 30% sucrose in PB, snap frozen in liquid nitrogen, and stored at −80°C. Transversal sections of 10 μm were obtained by means of a cryostat and stained with hematoxylin and eosin for the morphometrical experiments. Morphometric determination of the lumen, media, and vessel areas was performed using Metamorph 4.0 software. Briefly, all microscopic images of the sections were traced for the calculations of the areas. To determine the luminal area, the cross-sectional area enclosed by the internal elastic lamina was corrected to a circle by applying the form factor F/4π to the measurement of the internal elastic lamina, where l is the length of the lamina. The vessel area was determined by the cross-sectional area enclosed by the external elastic lamina corrected to a circle, by applying the same form factor (F/4π) to the measurement of the external elastic lamina. This method avoids miscalculations of areas caused by the eventual collapse of the immersion-fixed arteries. Internal and external diameters were calculated from luminal and vessel areas, respectively. Mean values were obtained from three different sections from the same artery from LZRs (n = 4) and OZRs (n = 4) using one CA and two PAs from each animal. For immunocytochemistry, tissue sections were processed following the avidin-biotin-peroxidase complex method (22).

Sections were first immersed in a mixture of 1% H2O2 and 90% methanol in distilled water for 30 min, washed in PB, and then preincubated in 10% normal goat serum in PB containing 0.3% Triton X-100 for 2–3 h. Sections were incubated with mouse anti-endothelial NOS (eNOS) antibody (Chemicon) diluted 1:500 for 48 h, washed, and then reacted with biotinylated goat anti-mouse secondary serum (Chemicon) diluted 1:400 for 2 h at room temperature, followed by an incubation with avidin-biotin complex (Vector) diluted 1:100 for 90 min at room temperature. The immunocomplex was visualized with 0.05% 3,3-diaminobenzidine and 0.001% H2O2 in PB. No immunoreactivity could be detected in sections incubated in the absence of the primary or secondary antibody.

Western blot analysis. To determine the expression of total eNOS, dorsal PAs and left descending CAs from LZRs (n = 10 and 7, respectively) and OZRs (n = 10 and 7, respectively) were homogenized in 100 μl of lysis buffer containing 10 mM Tris+HCl (pH 7.4), 1% SDS, and 1 mM sodium vanadate. Equal amounts of proteins (15 μg) were loaded in wells and subjected to electrophoresis on a 10% polyacrylamide-SDS gel followed by blotting to a polyvinylidene difluoride membrane and blocked for 1 h with agitation. For the analysis of eNOS, blots were incubated for 1 h at room temperature in the presence of specific primary antibodies against eNOS (BD Transduction Laboratories) and α-actin (Sigma) at 1:200 dilution and 1:125,000 dilution, respectively, followed by horseradish peroxidase (HRP)-conjugated secondary antibody [goat anti-mouse IgG-HRP, StressGen, for eNOS and goat anti-rabbit IgG (H+L)-HRP conjugate, Bio-Rad, for α-actin] at 1:2,500 dilution for 1 h at room temperature with agitation. Blots were detected with the ECL kit (Amersham Life Sciences, Barcelona, Spain). To normalize the protein levels, membranes were stripped and reprobed with anti-α-actin antibodies (Sigma). The expression level of each protein was presented as a ratio of density of the eNOS band versus that of the α-actin from the same sample. One artery (~1 cm in length) from each animal was used to determine the total expression of eNOS.

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 (AT), which is the increase in force (ΔF) divided by twice the segment length. The results are expressed as either Nm−1 of tension, kPa of transmural pressure (Apresure = 2000 × ΔT/0.9 × l100), or as a percentage of the response to either KPSS or the preconstrictor drug in each artery. Sensitivity to the agonists was expressed in terms of pD2 values [equal to −log(EC50), where EC50 is the concentration of the agonist required to give half-maximal relaxation]. The concentration of the agonists producing 50% of the maximal effect (EC50) of the responses elicited by either phenylephrine or KPSS was calculated by nonlinear curve fitting of the CRCs for the inhibitor to the classical Hill equation using the GraphPad Prism 5.0 (GraphPad Software, San Diego, CA) computer program. The EC50 value for each individual curve was first obtained, and, thereafter, the average value for a given set of experiments was calculated.

Data are expressed as means ± SE; n represents the number of arteries, one or two arteries from each animal in the functional and morphometrical experiments and one artery from each animal in the Western blot analysis. The differences between means were analyzed using one-way ANOVA or paired or unpaired Student’s t-tests when appropriate. P values of <0.05 were considered significant. All calculations were made using a standard software package (Prism 4.0, GraphPad Software).
RESULTS

Metabolic parameters. At 17–18 wk of age, OZRs were significantly heavier and had elevated total cholesterol and triglycerides levels compared with controls. Insulin levels were about threefold higher and nonfasting glucose levels were also found to be elevated in OZRs versus LZRs (Table 1). However, there were no differences in either systolic blood pressures or heart rates between the two strains. Heart weights were similar in OZRs and LZRs, whereas penile weights for OZRs were less than those of LZR (not shown). The left ventricular ratio was higher in LZRs than in OZRs (Table 1).

Vascular structure is altered in PAs but not CAs from OZRs. Representative photomicrographs of hematoxylin-eosin-stained cross sections of CAs and PAs from LZRs and OZRs are shown and the morphometry data are summarized in Fig. 1. The morphometric analysis demonstrated that external and internal diameters were reduced and the wall-to-lumen ratio was increased in PAs from OZRs (Fig. 1B). The media-to-lumen ratio of PAs in OZRs was about fourfold higher than that in LZRs (Fig. 1C). No changes were observed in either external or internal diameters, media thickness, or media-to-lumen ratio in CAs from OZRs compared with controls (Fig. 1, B and C). In accordance with the morphometry data, normalized internal lumen diameters (li) were found to be significantly smaller in PAs from OZRs compared with controls (132 ± 4 and 160 ± 6 μm, n = 23, P < 0.001, in OZRs and LZRs, respectively), whereas li values were comparable in CAs of LZRs and OZRs [289 ± 10 and 273 ± 11 μm, n = 26, not significant (NS), respectively).

Impaired vasoconstrictor responses in both PAs and CAs from OZRs. Stimulation of the arteries with KPSS evoked contractions significantly smaller in PAs from OZRs compared with LZRs (equivalent to 21.1 ± 1.7 vs. 30.8 ± 1.3 kPa, n = 26, P < 0.001, respectively; Fig. 2A). Contractions to KPSS were similar in CAs from LZRs and OZRs (6.0 ± 0.7 kPa, n = 28, and 5.6 ± 0.5 kPa, n = 26, NS, respectively; Fig. 2B). The comparison of the contractile response to agonists revealed that vasoconstriction was enhanced in both PAs and CAs of OZRs compared with LZRs (Fig. 2, C–F). Although maximal contractile elicited by norepinephrine was significantly smaller in PAs of OZRs compared with LZRs (33.7 ± 2.3 vs. 44.5 ± 1.8 kPa, n = 26, P < 0.001; Fig. 2C), the sensitivity to the adrenergic agonist was significantly enhanced in PAs of OZRs (pD2 values of 6.51 ± 0.06 vs. 6.71 ± 0.09 in LZRs and OZRs, respectively, n = 27, P < 0.05). Vasoconstrictor responses to 5-HT were found to be markedly enhanced in CAs of OZRs compared with LZRs, both in terms of maximal contraction (8.4 ± 0.8 vs. 6.6 ± 0.7 kPa, P < 0.05, n = 26; Fig. 2, D and F) and sensitivity (pD2 values of 6.14 ± 0.07 vs. 5.66 ± 0.11, P < 0.001, n = 26, respectively).

Impaired endothelium-dependent vasodilator responses in PAs from OZRs. To explore endothelium-dependent vasodilation, ACh was cumulatively added to arteries precontracted with either 1 μM phenylephrine (PAs) or 20 μM 5-HT (CAs). ACh elicited concentration-dependent relaxations in arteries from all groups, which were severely impaired in PAs of OZRs compared with LZRs (pD2 values of 5.79 ± 0.06 vs. 5.51 ± 0.09, n = 23, P < 0.01, in LZRs and OZRs, respectively; Fig. 3, A and C). ACh-induced relaxations were similar in CAs of LZRs and OZRs (pD2 values of 6.90 ± 0.06 and 6.74 ± 0.07, n = 27, NS, in LZRs vs. OZRs, respectively; Fig. 3, B and D). In contrast to ACh, the relaxations elicited by the NO donor SNAP were comparable in both PAs and CAs of either LZRs or OZRs. Thus, pD2 values were 6.36 ± 0.17 and 6.39 ± 0.04 and maximal responses were 94.4 ± 1.5% and 93.3 ± 2.2% (n = 9, NS) in PAs of LZRs and OZRs, respectively, and pD2 values were 6.22 ± 0.14 and 6.37 ± 0.23 and maximal responses were 94.4 ± 4.7% and 87.8 ± 3% (n = 8, NS) in CAs of LZRs and OZRs, respectively.

Role of NO in the abnormal vascular reactivity of PAs and CAs of OZRs. To investigate whether alterations in endogenous NO production may play a role in the impaired endothelium-dependent responses of PAs and in the enhanced vasoconstriction of both PAs and CAs of OZRs, the responses to ACh, to the vasoconstrictor agonists norepinephrine and 5-HT, and to the PDE5 inhibitor sildenafil were evaluated after blockade of NOS in arteries from LZRs and OZRs. Treatment with L-NNA (100 μM) largely reduced the relaxations induced by ACh in both PAs and CAs from LZRs (Fig. 4, A and B, and Table 2), suggesting that these responses are mostly a result of NO production. Treatment with L-NNA also reduced ACh relaxant responses in PAs (Fig. 4A and Table 2) and in CA (Fig. 4B and Table 2) from OZRs.

Figure 4, C and D, shows that inhibition of endogenous NO synthesis enhanced the vasoconstrictor responses to norepinephrine in PAs and to 5-HT in CAs from LZRs, respectively, with this enhancement being especially marked in CA. In contrast to LZRs, NOS blockade did not significantly increase the contractile responses to either norepinephrine (Fig. 4C and Table 2) or 5-HT (Fig. 4D and Table 2) in PAs and CAs from

Table 1. Hemodynamic and metabolic parameters of LZRs and OZRs

<table>
<thead>
<tr>
<th></th>
<th>LZRs</th>
<th>n</th>
<th>OZRs</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight, g</td>
<td>368 ± 9</td>
<td>22</td>
<td>483 ± 8†</td>
<td>22</td>
</tr>
<tr>
<td>Heart weight, g</td>
<td>1.95 ± 0.12</td>
<td>8</td>
<td>1.91 ± 0.10</td>
<td>8</td>
</tr>
<tr>
<td>Systolic blood pressure, mmHg</td>
<td>122 ± 1.9</td>
<td>6</td>
<td>128 ± 5</td>
<td>6</td>
</tr>
<tr>
<td>Heart rate, beats/min</td>
<td>344 ± 8</td>
<td>6</td>
<td>341 ± 7</td>
<td>6</td>
</tr>
<tr>
<td>LV ratio</td>
<td>0.37 ± 0.02</td>
<td>8</td>
<td>0.28 ± 0.01†</td>
<td>8</td>
</tr>
<tr>
<td>Blood glucose concentration, mg/dl</td>
<td>106 ± 8</td>
<td>15</td>
<td>181 ± 22*</td>
<td>16</td>
</tr>
<tr>
<td>Plasma insulin concentration, ng/ml</td>
<td>1.27 ± 0.17</td>
<td>10</td>
<td>3.78 ± 0.34†</td>
<td>10</td>
</tr>
<tr>
<td>Plasma total cholesterol concentration, mg/dl</td>
<td>92 ± 4</td>
<td>15</td>
<td>188 ± 11‡</td>
<td>16</td>
</tr>
<tr>
<td>Plasma triglyceride concentration, mg/dl</td>
<td>85 ± 6</td>
<td>15</td>
<td>322 ± 28‡</td>
<td>16</td>
</tr>
</tbody>
</table>

Data are means ± SE; n, number of animals. Lean Zucker rats (LZRs) and obese Zucker rats of 17–18 wk of age were studied. The left ventricular (LV) ratio was calculated as grams of LV weight/100 g body wt. Significant differences were analyzed by an unpaired Student’s t-test. *P < 0.05, †P < 0.001, and ‡P < 0.0001 vs. LZRs.
OZR. These results demonstrate that there is a significant endogenous NO production that counterbalances vasoconstriction in arteries from LZR. This basal NO production and/or effects are impaired in OZR.

The selective PDE5 inhibitor sildenafil produced concentration-dependent relaxations in both PAs and CAs from all groups, which were significantly reduced in the OZR group compared with controls (Fig. 5). The vasodilator responses induced by sildenafil in PAs and CAs were blunted by NO synthesis blockade with L-NNA in both LZR and OZR (Fig. 5 and Table 2). This demonstrates that the sildenafil relaxant effect is due to the amplification of endogenous NO production, which is impaired in both PAs and CAs from OZR compared with controls.

Fig. 1. There was a fourfold increase in the media-to-lumen ratio in penile arteries (PAs) from obese Zucker rats (OZR), but structure was preserved in coronary arteries (CAs). A: representative sections of PAs (left) and CAs (right) from OZR and lean Zucker rats (LZR) stained with hematoxylin and eosin. B and C: histograms showing the average measurements of the morphometric analysis (vessel diameters (B) and media-to-lumen ratio (C)). ID, internal diameter; ED, external diameter; MT, media thickness. Data are shown as means ± SE of 8 arteries for PAs and 4 arteries for CAs (2 arteries/animal for PAs and 1 artery/animal for CAs). *P < 0.05, **P < 0.01, and ***P < 0.001 vs. LZR.
**eNOS protein expression.** Staining of arterial cross sections with a monoclonal antibody against eNOS revealed no differences in the presence and distribution of this constitutive NOS isoform in the endothelium of either PAs or CAs from OZRs compared with LZRs (Fig. 6, A and B). Western blot analysis of eNOS protein from PAs and CAs confirmed no significant differences in the levels of expression between the LZR and OZR groups (Fig. 6, C and D). The expression of \( \beta \)-actin was not significantly different between LZRs and OZRs.

**Role of superoxide production in the abnormal vascular reactivity of PAs and CAs of OZRs.** The incidence of superoxide anion production in the impaired vasodilator and vasoconstrictor responses of PAs and CAs from OZRs was assessed in arteries pretreated with 150 U/ml SOD. Under these conditions, the enhanced contractions elicited by norepinephrine (PAs) and 5-HT (CAs) were both significantly reduced in OZRs, although they were not restored to the control levels of arteries from the LZR group (Fig. 7, A and C, and Table 3). In contrast, ACh relaxant responses in PAs from OZRs were unchanged by the acute antioxidant treatment (Fig. 7B and Table 3).

**DISCUSSION**

The present study comparatively assessed the structural and functional characteristics of PAs and CAs from an animal model of insulin resistance, based on previous clinical studies showing that ED is often a sentinel sign of vascular disease and has been proposed as a potential marker of silent CAD in diabetic men (16). The hypothesis of the study was that at a given stage in the course of diabetes, there must be marked differences in vascular function between the PA and CA beds since ED is clinically evident when CAD is still absent or silent in diabetic patients. We used the OZR, a well-established...
model of insulin resistance and metabolic syndrome, which are conditions that usually anticipate type 2 diabetes. Our results demonstrated profound alterations in the structure, abnormal vascular reactivity, and endothelial dysfunction of PAs from 17- to 18-wk-old OZR, whereas in CAs from the same animals, structure was preserved and there was augmented vasoconstriction likely due to emerging endothelial dysfunction.

Remodeling is considered a hallmark of the vascular disorders associated with metabolic syndrome and diabetes mellitus. The present study demonstrates structural abnormalities in PAs from a prediabetic animal model, as indicated by the increased media-to-lumen ratio found in PAs from OZRs. This is due to the narrowing of the internal lumen and to the increase of the media wall thickness, suggesting inward vascular remodeling. In contrast, no significant changes were observed in CA branches of similar order from OZRs, demonstrating a preserved vascular structure. Vascular remodeling has recently been reported in asymptomatic middle-aged women with metabolic syndrome, where carotid artery measurements by noninvasive B-mode ultrasound technique showed a preserved luminal diameter and blood flow in response to arterial wall thickening (24). On the other hand, skeletal muscle resistance arterioles of OZRs have consistently been demonstrated to remodel, resulting in a reduced passive diameter and distensibility but also thinner walls, indicative of atrophic vascular remodeling (11, 45). Our results in the small PAs of OZRs would be in agreement with those reported in subcutaneous resistance arteries from type 2 diabetic patients, which displayed inward hypertrophic remod-

Fig. 3. Endothelium-dependent relaxations to ACh were severely impaired in PAs but not CAs from OZRs compared with LZRs. A and B: representative traces showing ACh-induced relaxations in PAs (A) and CAs (B) from LZRs and OZRs. Phe, phenylephrine. C and D: average concentration-response curves for relaxation to ACh in PAs (C) and CAs (D) from LZRs and OZRs. Data are shown as means ± SE of 23 arteries (C) and 27 arteries (1–2 arteries/animal; D). ***P < 0.001 vs. LZRs.
eling with reduced lumen diameters and an increased media-to-lumen ratio (43). Diabetic vascular complications and organ damage are associated with remodeling of the vessel wall in the retinal, renal, and mesenteric circulations. Structural changes in the PAs from OZRs may therefore be related to the ED reported in this animal model (28, 50) and to the high incidence of ED in metabolic syndrome and diabetic patients (10, 18, 48).

Prepenile pudendal arteries, a major site of resistance in the penile vasculature, and intrapenile structures have been demonstrated to remodel in hypertension, and antihypertensive therapy has been proposed for the long-term correction of ED and risk reduction (20, 47). Interestingly, in the OZR model, corporal venoocclusive dysfunction is associated with a decreased smooth muscle cell-to-collagen ratio and fibrosis of the erectile tissue (28). This reduction of the smooth muscle content would be consistent with the reduced contractility found in the PAs of OZRs in the present study. On the other hand, a recent study (30) in the type 2 diabetic db/db mouse also reported matrix alterations in the corpora cavernosa consisting of dysregulation of the collagen and elastin content and associated with impaired venoocclusion and reduced erectile responses. Therefore, remodeling of penile tissues, both arterial and corporal, correlates with ED in metabolic syndrome and type 2 diabetes (28, 30).

Metabolic abnormalities, such as hyperglycemia and hyperinsulinemia, have been suggested to play a role in the genesis of the structural abnormalities observed in diabetes mellitus. Thus, insulin promotes vascular cell growth, and a correlation between plasma insulin and the media-to-lumen ratio of small arterioles from type 2 diabetic patients has been reported (43).

Increased production of local vasoactive factors such as endothelin-1 can also contribute to the vascular remodeling of diabetic arteries (21). The OZR used in the present study displayed mild hyperglycemia and hyperinsulinemia, along with an abnormal lipid profile. However, under these metabolic conditions, structure in CAs was preserved, suggesting that factors other than the metabolic ones may additionally be involved in the genesis of the vascular remodeling observed in PAs. Studies in OZRs have suggested that remodeling of the microcirculation reflects chronic reductions in blood flow secondary to abnormal vascular reactivity (11, 45). Decreased NO bioavailability and reduced endothelium-dependent relaxations have recently been associated with the vascular remodeling in resistance arteries from OZRs (4). Accordingly, in the present study, structural abnormalities were associated with severe endothelial dysfunction in PAs, whereas structure was preserved in CAs, which displayed a moderate endothelial dysfunction in the same OZRs. Interestingly, PA vascular reactivity and structure were impaired before the onset of hypertension and in contrast to that recently reported for cerebral arteries in this animal model, where structural remodeling, enhanced myogenic tone, and increased stroke risk and injury correlate with the development of hypertension (37). Our findings in PAs of OZRs therefore reinforce the view of ED as an early sign of subclinical vascular disease (46).

Abnormal vasoconstriction due to either augmented smooth muscle contraction or endothelial dysfunction has previously been shown in arteries from diabetic type 2 and metabolic syndrome animal models and patients (12, 35, 50). Our data demonstrate an enhanced vasoconstriction upon stimulation in
response given as either a percentage of the high-K⁺ responses of ACh and sildenafil. pD₂ is equal to percentage of the precontraction induced by NE or 5-HT for the vasodilator solution (KPSS)-induced contraction for norepinephrine (NE) and 5-HT or a concentration of agonist giving the half-maximal response. L-NNA, constrictor responses to both nerve-released norepinephrine (5, 6) and 5-HT and the sensitivity to norepinephrine was augmented. Enhanced vasoconstrictor responses to NE and 5-HT and the endothelial dysfunction rather than to enhanced contractility of smooth muscle. The reduced maximal sensitivity to norepinephrine was augmented.

### Table 2. Effect of nitric oxide synthase blockade on the vasomotor responses to NE and 5-HT and the vasodilator responses to ACh and sildenafil in penile and coronary arteries from LZRs and OZRs

<table>
<thead>
<tr>
<th></th>
<th>Penile arteries</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pD₂</td>
<td>E₉₀, %</td>
<td>pD₂</td>
<td>E₉₀, %</td>
<td>n</td>
<td>pD₂</td>
<td>E₉₀, %</td>
<td>E₉₀, %</td>
<td>n</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>NE</td>
<td>6.53 ± 0.09</td>
<td>154 ± 6</td>
<td>6.87 ± 0.12</td>
<td>180 ± 22</td>
<td>7</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>+L-NNA</td>
<td>7.28 ± 0.38</td>
<td>150 ± 6</td>
<td>6.94 ± 0.11</td>
<td>169 ± 19</td>
<td>7</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>ACh</td>
<td>5.90 ± 0.16</td>
<td>78 ± 7</td>
<td>5.13 ± 0.18</td>
<td>29 ± 8</td>
<td>7</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>+L-NNA</td>
<td>2 ± 4*</td>
<td></td>
<td>1 ± 4*</td>
<td></td>
<td>7</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Sildenafil</td>
<td>6.29 ± 0.10</td>
<td>64 ± 4</td>
<td>5.75 ± 0.12</td>
<td>42 ± 6*</td>
<td>7</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>+L-NNA</td>
<td>7 ± 5*</td>
<td></td>
<td>5 ± 6*</td>
<td></td>
<td>7</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

|                | 5-HT           | 6.57 ± 0.15 | 122 ± 12 | 6.35 ± 0.11 | 174 ± 15* | 8        |
|                | +L-NNA         | 6.31 ± 0.18 | 175 ± 19 | 6.61 ± 0.12 | 177 ± 19 | 8        |
|                | ACh            | 6.90 ± 0.11 | 92 ± 2   | 6.71 ± 0.16 | 82 ± 7 | 9        |
|                | +L-NNA         | 2 ± 8*    |          | 19 ± 7*    |        | 9        |
|                | Sildenafil     | 7.93 ± 0.16 | 64 ± 4   | 8.00 ± 0.17 | 48 ± 2* | 6        |
|                | +L-NNA         | 8 ± 7*    |          | 12 ± 8*    |        | 6        |

**Penile arteries**

Values are means ± SE; n, number of individual arteries. E₉₀, maximal response given as either a percentage of the high-K⁺ physiological saline solution (KPSS)-induced contraction for norepinephrine (NE) and 5-HT or a percentage of the precontraction induced by NE or 5-HT for the vasodilator responses of ACh and sildenafil. pD₂ is equal to −log EC₅₀, where EC₅₀ is the concentration of agonist giving the half-maximal response. L-NNA, N-nitro-L-arginine. Significant differences from controls were analyzed by paired or unpaired Student t-tests. *P < 0.05, **P < 0.01, and ***P < 0.001 vs. the control value within that group (by paired Student’s t-tests); †P < 0.05, ‡P < 0.01, and §P < 0.001 vs. LZRs (by unpaired Student’s t-test).

Both PAs and CAs from OZRs, which seems to be due to endothelial dysfunction rather than to enhanced contractility of smooth muscle cells. Thus, contractions elicited by KPSS were either decreased (PAs) or unaltered (CAs) in OZRs, thus ruling out hyperreactivity of smooth muscle. The reduced maximal contractile responses elicited by both KPSS and norepinephrine in PAs from OZRs probably reflect the smaller size of these arteries due to the structural remodeling, although the sensitivity to norepinephrine was augmented. Enhanced vasoconstrictor responses to both nerve-released norepinephrine (5, 30) and α₁-adrenergic agonists (30, 50) have been demonstrated in the corpus cavernosum of OZRs and of type 2 diabetic mice, and they have been ascribed to both prejunc- tional and postjunctional mechanisms. In the present study, basal levels of NO appear to be insufficient to counterbalance vasoconstriction in both PAs and CAs from OZRs, since blockade of NOS failed to enhance contractions induced by either norepinephrine or 5-HT, respectively, in contrast to that observed in control arteries. This is in accordance with recent observations in arteries from type 2 diabetic patients, where impaired basal NO activity augments α-adrenergic vasoconstriction (35), and also in skeletal muscle arteries from prediabetic and short-term diabetic Zucker rats, where a reduction in NO signaling enhances reactivity to both norepinephrine and endothelin-1 and precedes the development of overt diabetes and the onset of hypertension (29). The lesser inhibitory effect of NOS blockade on PAs from LZRs compared with CAs is likely related to the differential regulation of PA tone and CA tone by endothelial vasoactive factors and, subsequently, to the corresponding adaptive changes after NOS inhibition.

In the present study, the impairment of basal NO activity in arteries from OZRs is further supported by the reduced vaso- dilatation elicited by the selective PDE5 inhibitor sildenafil in both PAs and CAs. Sildenafil has been reported to dilate human epicardial arteries and to improve endothelial dysfunc- tion in patients with CAD (19). Our results showing the lesser relaxant responses to sildenafil in PAs from OZRs are consistent with the inability reported for this PDE5 inhibitor to normalize the endothelium-dependent responses to ACh in human penile resistance arteries from diabetic patients (1). This was initially ascribed to the involvement of pathways different from NO and not affected by PDE5 inhibition, i.e., EDHF, in penile endothelium-dependent arterial vasodilatations. However, in the present study, we assessed the direct relaxant effect of sildenafil, which is due the enhancement of cGMP accumulation and thus to the potentiation of the relaxant effects of basal-released endothelial NO (42). The involvement of NO is confirmed in the present study by the marked inhibitory effect of NOS blockade on the sildenafil relaxant responses of both PAs and CAs. Therefore, our results showing reduced sildenafil-elicited vasodilations support impaired basal NO activity in arteries from OZRs and, in the case of PAs,
would be consistent with clinical data showing a lower efficacy of PDE5 inhibitors to treat ED in some diabetic patients (48).

Several studies have investigated endothelium-dependent relaxations in various vascular beds of insulin resistance and type 2 diabetic animals and humans, and reduced NO-mediated responses have been reported in the majority of these studies (1, 2, 4, 14, 35, 43). In the present study, we demonstrate a differential impairment of endothelial function of PAs and CAs from the same OZRs. Thus, whereas basal NO activity seemed to be similarly impaired in both PAs and CAs, agonist-stimulated endothelium-dependent vasodilatation was markedly reduced in PAs but preserved in CAs from OZRs. Our observations in CAs are in agreement with data from a recent longitudinal study (36) showing that ACh relaxant responses in CAs from OZRs are unaffected until the age of 24 wk but blunted in 28- to 36-wk-old animals. However, ACh dilator responses have been reported to be markedly attenuated in CAs from the type 2 diabetic db/db mouse (31). These differences can be ascribed to the more deleterious effect of the elevated plasma glucose levels in established diabetes compared with the mild hyperglycemia found in the prediabetic OZR. On the other hand, dilatations to ACh were more severely impaired in PAs from OZRs than in the corpus cavernosum from db/db mice (30), which is in agreement with that reported for human PAs and the corpus cavernosum from diabetic patients (1). The differential impairment of endothelial function in PAs and the corpus cavernosum may be due to the different contribution of the various endothelial factors, i.e., NO, prostanoids, and EDHF, to the relaxations of penile vascular tissues (1, 40). In the present study, the experiments with L-NNA demonstrate that the failure to dilate in PAs might be due in part to abnormalities in the NO-mediated component of the endothelium-dependent relaxations.

Fig. 6. Endothelial NOS (eNOS) expression was unchanged in arteries from OZRs compared with LZR. A and B: immunohistochemical demonstration of eNOS in the endothelial cell layer of PAs (A) and CAs (B) from LZR (left) and OZR (right). C and D: Western blot analysis of eNOS expression in PAs (C) and CAs (D) from LZR and OZRs. Results were quantified by densitometry. Data are shown as means ± SE of 10 animals in A and 7 animals in B.
Increased oxidative stress is a well-established pathogenic factor for vascular dysfunction in insulin resistance and diabetes, and an excessive production of ROS has been associated with the reduced NO bioavailability and endothelial dysfunction in cerebral arteries (8), CAs (2, 25, 39), and various peripheral arteries (4, 13) from OZRs and type 2 diabetic animals. In CAs from OZRs, increased oxidative stress impairs insulin-dependent vasodilatation but not endothelium-dependent responses to ACh (25), as in the present study. Our experiments further show that treatment with SOD partially reduced the enhanced contractile responses to both norepinephrine and 5-HT in PAs and CAs from OZRs, respectively, which suggests that increased superoxide production may contribute to the reduced NO bioavailability and to the enhanced vasoconstriction found in arteries from OZRs. However, acute antioxidant treatment did not completely normalize contractile responses, nor could it restore the endothelium-dependent relaxations to ACh in PAs from OZRs. These results suggest that factors other than oxidative stress may contribute to the blunted dilator reactivity of PAs. The long-term effects of oxidative stress as well as the role of changes in the posttrans-
lational regulation of eNOS and prostanoid and endothelin metabolism in the pathogenesis of the endothelial dysfunction of PAs warrant further investigation.

In conclusion, the present results demonstrate inward vascular remodeling in PAs and a differential impairment of the vasodilator endothelial responses in both PAs and CAs of a prediabetic animal model. Whereas basal NO activity was similarly altered in both arteries, the endothelium-dependent relaxations to ACh were severely impaired in PAs but not CAs despite unchanged eNOS levels. The structural abnormalities and severity of the altered endothelial relaxant responses in PAs from OZRs might explain the ED reported in these animals (28, 50). Although the mechanisms underlying the altered vasodilator reactivity require further elucidation, augmented vasoconstriction emerges as a common consequence of the endothelial dysfunction observed in both PAs and CAs. Impaired endothelial function is considered as an early sign of proatherogenic vascular environment, since the upregulated contractile state of blood vessels contributes to hypertension and reduces blood flow, thus accelerating the development of atherosclerosis associated with diabetes. The pathological implication of the present findings is that the severity of the altered structure and function in PAs might anticipate the functional impairment of the more preserved coronary vascular bed. This is consistent with the “artery size hypothesis” recently proposed as a pathophysiological mechanism to address the link between ED and CAD (33), where atherosclerosis would affect PAs earlier than CAs due to their smaller diameters. Thus, PAs will be severely narrowed, eliciting sexual symptoms that precede the clinical manifestation of CAD, but ED and CAD could be considered as two different aspects of the same disease.

ACKNOWLEDGMENTS

The authors thank Fernanda Roque for the measurements of systolic blood pressure and heart rate and Francisco Puente and Manuel Perales for expert technical assistance.

GRANTS

This work was supported by Spanish Ministry of Science and Education Grants SAF2006-09191 and SAF 2006-02376 and Universidad Complutense de Madrid Grant PR1/106-14441-A. N. Villalba is a research fellow of Spanish Grants SAF2006-09191 and SAF 2006-02376 and Universidad Computense de GRANTS technical assistance. Pressure and heart rate and Francisco Puente and Manuel Perales for expert assistance.

REFERENCES