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Vascular endothelial function masks increased sympathetic vasopressor activity in rats with metabolic syndrome

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hypertension; metabolic syndrome; endothelium; sympathetic activation; nitric oxide

—Sympathetic hyperactivation, a common feature of obesity and metabolic syndrome, is a key trigger of hypertension. However, some obese subjects with autonomic imbalance present a dissociation between sympathetic activity-mediated vasoconstriction and increased blood pressure. Here, we aimed to determine in a rat model of metabolic syndrome whether the endothelium endothelial nitric oxide (NO) synthase (eNOS)-NO pathway contributes to counteract the vasopressor effect of the sympathetic system. Rats were fed a high-fat and high-sucrose (HFS) diet for 15 wk. Sympathovagal balance was evaluated by spectral analysis of heart rate variability and plasmatic catecholamine measurements. Blood pressure was measured in the presence or absence of *N*-nitro-*L*-arginine methyl ester (*L*-NAME) to inhibit the contribution of eNOS. Vascular reactivity was assessed on isolated aortic rings in response to α_1 -adrenergic agonist. The HFS diet increased sympathetic tone, which is characterized by a higher low on the high-frequency spectral power ratio and a higher plasmatic concentration of epinephrine. Despite this, no change in blood pressure was observed. Interestingly, HFS rats exhibited vascular hyporeactivity (-23.6%) to α_1 -adrenergic receptor stimulation that was abolished by endothelial removal or eNOS inhibition (*L*-NAME). In addition, eNOS phosphorylation (Ser¹¹⁷⁷) was increased in response to phenylephrine in HFS rats only. Accordingly, eNOS inhibition *in vivo* revealed higher blood pressure in HFS rats compared with control rats (147 vs. 126 mmHg for mean blood pressure, respectively). Restrain of adrenergic vasopressor action by endothelium eNOS is increased in HFS rats and contributes to maintained blood pressure in the physiological range.

NEW & NOTEWORTHY Despite the fact that prohypertensive sympathetic nervous system activity is markedly increased in rats with early metabolic syndrome, they present with normal blood pressure. These observations appear to be explained by increased endothelial nitric oxide synthase response to adrenergic stimulation, which results in vascular hyporeactivity to α -adrenergic stimulation, and therefore blood pressure is preserved in the physiological range.

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INTRODUCTION

Metabolic syndrome (MetS) is a cluster of physiological dysregulations that includes visceral obesity, hyperglycemia, insulin resistance, and dyslipidemia. All abnormalities are well-known risk factors for the development of cardiovascular disease (CVD) and type 2 diabetes. Moreover, patients with MetS commonly develop hypertension, a major comorbidity factor for CVD. Several components of MetS, such as hyperinsulinemia (14, 16, 25), visceral obesity (2, 21, 27), and others, are positive modulators of the sympathetic nervous system (8, 20, 27). The sympathetic nervous system is a major vasoconstrictor system, which may contribute to the hypertension observed in patients with MetS. Surprisingly, some patients who are obese and have MetS present with normal arterial blood pressure (ABP) and vascular tone despite obvious sympathetic activation (1, 27). These observations suggest that 1) compensatory mechanisms counterbalance sympathetic action and therefore dissociate sympathetic activity from its hypertensive effect and 2) vascular tone control might be involved in such a phenomenon.

A healthy endothelium preserves the balance between vasodilatation and vasoconstriction, which determine the arterial diameter and consequently blood pressure. Notably, endothelium liberates the vasorelaxant gasotransmitter nitric oxide (NO) produced by endothelial NO synthase (eNOS) (43), thereby assuming a suitable adaptation and control of blood pressure (43). Indeed, various stimuli can activate eNOS, such as bradykinin, hypoxia, or shear stress to adapt blood flow and satisfy metabolic demand. eNOS is also activated in vascular smooth muscle during α_1 -adrenergic receptor (α_1 -AR) stimulation to limit potential side effects of massive vasoconstriction during adrenergic stress (11, 47). Interestingly, endothelial function and the eNOS pathway are altered in various pathological states. Metabolic diseases are classically associated with endothelial dysfunction; however, the ability of the endo-

thelium to limit arterial vasoconstriction can also be exacerbated. For example, this is the case during adrenergic stress in hepatic and renal diseases (3, 45), but similar observations have been made in an obese animal model (30). Until now, the role of endothelium in preventing elevated vascular tone and ABP during sympathetic outflow in some obese subjects has never been studied.

The aim of this work was to evaluate the role of endothelium and the eNOS-NO pathway in vascular α_1 -adrenergic hyporesponsiveness in a population with MetS. For this purpose, we investigated sympathetic activation, the impact of α_1 -AR stimulation on arterial vasomotricity, and how the endothelium and especially eNOS activation state could modulate this response in a rat model of high-fat and high-sucrose (HFS) diet-induced metabolic disorders.

METHODS

Experimental protocol. All investigations conformed with European Parliament Directive 2010/63/EU (no. CEEA-00322.03) and were approved by the local research ethics committee (experimentation no. 84.004). Seven-week-old male Wistar rats, obtained from Janvier, were randomly assigned into either the control (Ctrl) group fed with a standard diet (A04, with a caloric value of 3.339 Kcal/kg, SAFE) or to the HFS group. The HFS diet is a high-fat diet (230 HF, containing 60% kcal as fat with a caloric value of 5.317 kcal/kg, SAFE) completed with 10% sucrose in drinking water for 15 wk to induce MetS. At the end of the 15-wk diet period, cardiac function was explored in vivo (ECG and blood pressure). Rats were then euthanized, blood was collected for biochemistry analysis, abdominal pelvic fat and epididymal fat were removed and weighed as index of visceral obesity, and the aorta was dissected as follows: the upper part of the descending aorta was immediately flash frozen in liquid nitrogen for Western blot analysis (basal condition) and the lower part was directly cut into several rings that were either used directly for vascular reactivity assessment or incubation with phenylephrine (PE) and then flash frozen for Western blot analysis.

ECG recording and heart rate variability analysis. ECG recordings were obtained through implantable CA-F40 telemetric ECG transmitters (DSI) and using a signal RCP-1 receiver connected to a data acquisition system (Ponemah Physiology Platform, DSI). Rats were instrumented under general anesthesia (inhaled aerae: 2.5% in 95% O₂) and allowed to recover from surgery for 8 days. Autonomic nervous system activity on cardiac function was assessed by studying beat-to-beat heart rate variability (HRV) as previously described (57, 58). Twenty segments of 3 min (5 segments/h) were analyzed using Kubios HRV software (version 2.2) to assess frequency-domain HRV (53). First, all RR intervals were measured, and the mean RR interval was then calculated. After application of a cubic spline interpolation (20 Hz), the power spectrum was estimated with Welch's periodogram modeling, i.e., the RR series was divided into overlapping segments (50%), each segment was windowed to decrease the leakage effect (1,024 s), and the spectrum estimate was obtained by averaging the fast Fourier transformation spectra of all windowed segments. Thus, the fast Fourier transformation [low-frequency (LF) band: 0.4–1.5 Hz and high-frequency (HF) band: 1.5–5 Hz] was exclusively applied to sinus RR intervals. As commonly published, the LF band power reflects sympathetic autonomic nervous system and baroreflex activities on the heart (26, 51). The HF band power reflects parasympathetic activity (45). Thus, the LF-to-HF ratio could be used to estimate sympathovagal activity on cardiac rhythm (5).

Blood pressure measurements. Mean blood pressure (MBP), systolic blood pressure (SBP), and diastolic blood pressure (DBP) were assessed in conscious rats by the tail-cuff method using the CODA

tail-cuff system (Kent Scientific) at the end of the study. Animals were trained to the measurement procedure for 8 days before the beginning of the experiment. A single experimenter was designated to conduct all measurements between 9 AM and 12 PM. Obtained results were the mean of at least 7 valid measurements on 15 reads in a recording session. To confirm that this system was sensitive enough to detect an adrenergic hypertensive effect, blood pressure was measured in some rats before and after an intraperitoneal injection of norepinephrine (1 mg/kg), a catecholamine involved in sympathetic nervous activity (SBP was 132.5 ± 4.1 vs. 167.4 ± 9.0 mmHg, respectively). Finally, to assess the contribution of NO in the regulation of blood pressure, blood pressure was measured in some rats before and 15 min after an intraperitoneal injection of the NO synthase inhibitor *N*-nitro-L-arginine methyl ester (L-NAME; 20 mg/kg). The method was adapted from Huerper et al. (26).

Isolated aortic rings. Under anesthesia [pentobarbital sodium (100 mg/kg ip)], the thoracic aorta was quickly removed and placed in cold Krebs-Henseleit bicarbonate buffer [composed of (in mM) 118.00 NaCl, 25.00 NaHCO₃, 4.80 KCl, 1.20 KH₂PO₄, 1.25 CaCl₂, and 11.00 glucose]. After removal of adherent tissue, the aorta was cut in small segments 2 mm long. Aortic rings were mounted onto stainless steel supports and suspended in a tissue bath containing Krebs-Henseleit buffer at 37°C continuously bubbled with a 95% O₂-5% CO₂ gas mixture. Rings were connected to an isometric force transducer (EMKA Technologies) linked to an amplifier (EMKA Technologies) and a computerized acquisition system to record changes in isometric force. Resting tension was adjusted to 2.0 g, and aortic rings were allowed to stabilize for 60 min. From there, KCl solution (60 mM) was applied to obtain a reference contraction, which was used to normalize subsequent contractile responses. Endothelial integrity was then tested with a single dose of PE (1 μ M) followed by a vasorelaxing dose of acetylcholine (ACh; 10 μ M). To assess vasocontractile capacity to the α_1 -adrenergic agonist, cumulative doses of PE were added to the bath (0.1 nM–10.0 μ M). To evaluate the implication of endothelial activity on α -adrenergic vasoconstriction, the inner surface of some rings was gently rubbed to remove endothelium before the rings were mounted. Endothelium removal efficacy was confirmed when no vasorelaxation was observed in response to ACh injection. To test the capacity of the endothelium to inhibit agonist- and nonagonist-induced vasoconstriction, the aortic ring response to a single dose of PE (1 μ M) and a single dose of KCl (60 mM) was measured. In the same way, the effect of eNOS activity was evaluated by treating aortic rings with L-NAME (0.3 mM) 30 min before the vasoconstriction protocol. Responses were characterized by E_{max} values corresponding to the maximal contractile effect of the drug and EC₅₀ values, which represent the concentration of drug that induces a contraction equal to 50% of its own maximal effect.

Fasting blood glucose and glucose tolerance tests. Intraperitoneal glucose tolerance tests were performed at the end of the 13th week of the protocol. First, blood was obtained via tail clip to assess fasting blood glucose (Caresens N, DinnoSante). Rats then received an intraperitoneal injection of a glucose solution (2 g/kg), and blood glucose was measured at 10, 20, 30, 60, and 120 min after the glucose injection.

Blood analysis. Blood samples were stored at -20°C until analysis. Total cholesterol, HDL-cholesterol, LDL-cholesterol, and triglycerides were determined in plasma by automated enzymatic kits on a Cobas 6000 autoanalyzer. Serum insulin was determined using a commercial rat insulin ELISA kit (10-1250-01, Mercodia). Plasmatic norepinephrine and epinephrine were determined by HPLC analysis (column: Vydac 218TP54, 5 μ m, inner diameter: 4.6×250 mm, Waters Separations Module 2695, multiwavelength fluorescence detector, Waters 2475).

Western blot analysis. Western blots were performed as previously described (4). Briefly, proteins from aortic homogenates were separated onto SDS-PAGE gels and transferred onto PVDF membranes.

Membranes were incubated with primary antibodies at 4°C in 1% milk (α_{1A} -, α_{1B} -, and α_{1D} -ARs: 1:500, Santa Cruz Biotechnology; eNOS: 1:1,000, BD Transduction; and GAPDH: 1:5,000, Santa Cruz Biotechnology) or 0.3% BSA (eNOS-phospho-Ser¹¹⁷⁷: 1:500, BD Transduction) in Tris-buffered saline containing 0.05% Tween 20 overnight. Immunodetection was carried out using enhanced chemiluminescence (ECL) or ECL Plus system (SuperSignal West Pico Chemiluminescence substrate, Thermo Scientific; Luminata Forte Western HRP substrate, Millipore, respectively), and membranes were then exposed to X-ray films for revelation. eNOS protein content was expressed relative to GAPDH content. eNOS-phospho-Ser¹¹⁷⁷ protein content was expressed relative to eNOS content. To evaluate the impact of PE on eNOS activation, the phosphorylation level at Ser¹¹⁷⁷ of eNOS was measured from aorta rings mounted in an organ bath at a resting tension of 2 g and then incubated with or without PE (10 μ M) for 15 min.

Statistical analysis. Data are expressed as means \pm SE. Unpaired *t*-tests were used for single comparisons between groups. For experiments involving multiple measures on the same animal over time as well as for dose-response curves, comparisons between groups were made using two-way ANOVA followed by a Sidak post hoc test where appropriate. Values of *P* < 0.05 were considered statistically significant. Statistical analysis was done using GraphPad Prism software.

RESULTS

HFS diet induces MetS in Wistar rats. After 13 wk, enriched fat and sucrose diet feeding significantly increased the body mass of HFS rats compared with their littermates assigned to the standard chow diet-fed group (Fig. 1A). HFS rats had higher epididymal and abdominopelvic fat mass in than Ctrl rats (Fig. 1B). HFS rats also presented elevated fasting blood glucose (Fig. 1C), elevated fasting serum insulin (Fig. 1D), and enhanced elevation of blood glucose during glucose tolerance tests (Fig. 1E). Blood analysis revealed that HFS rats had higher LDL and triglyceride levels with unchanged total cholesterol and HDL levels (Fig. 1F). Altogether, these results showed that the HFS diet induced central obesity (increased body mass and visceral fat accretion), alterations of glucose and insulin metabolism, and dyslipidemia, which fit the definition of MetS in humans (22).

Increased sympathetic nervous outflows in HFS rats do not translate into higher blood pressure. Obesity and MetS are classically associated with increased sympathetic nervous outflows (2, 21, 27). We monitored cardiac electrical activity (ECG) using a telemetric system over 24 h in Ctrl and HFS rats

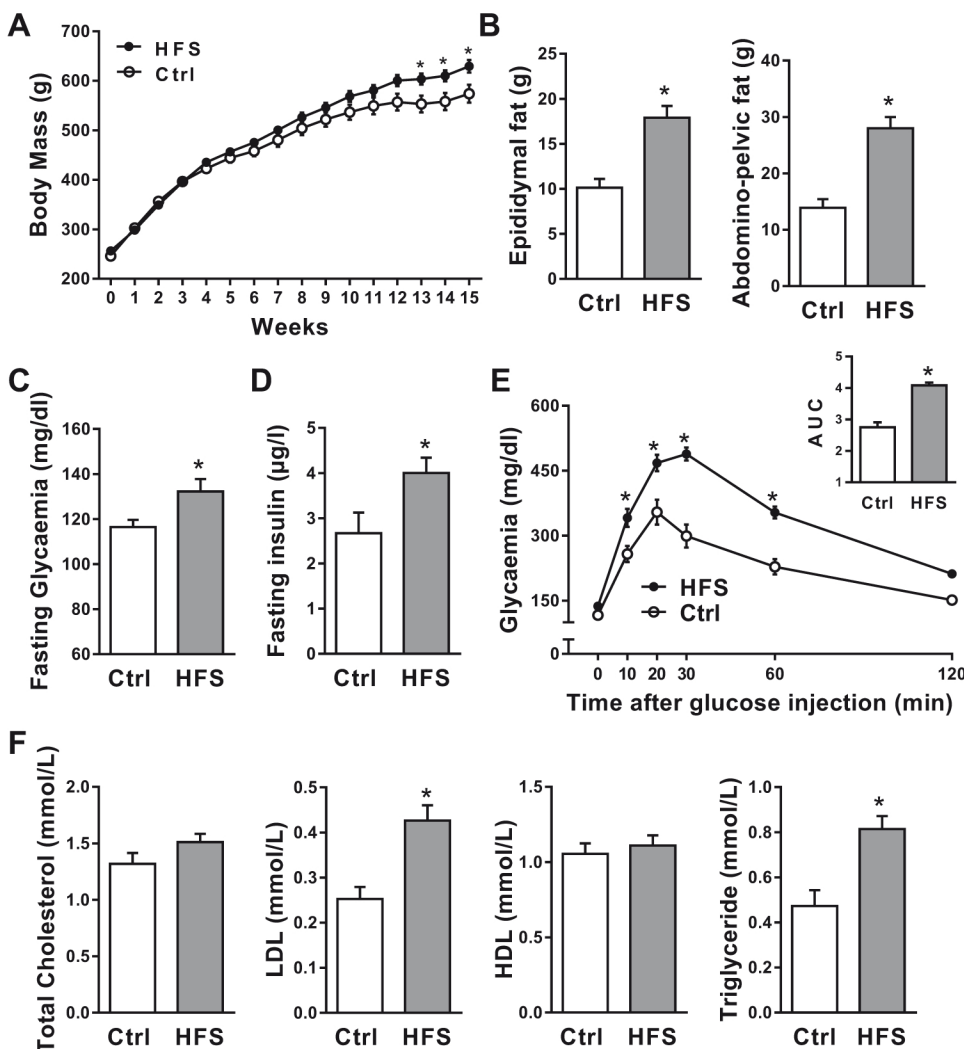


Fig. 1. High-fat and high-sucrose (HFS) diet induces metabolic syndrome in Wistar rats. **A:** followup of body mass during the 15 wk of HFS or standard [control (Ctrl)] diet ($n = 14-15$ rats/group). **P* < 0.05 compared with the Ctrl group by two-way ANOVA followed by the Sidak post hoc test. **B:** epididymal and abdominopelvic fat mass at the end of the protocol ($n = 14-15$ rats/group). **P* < 0.05 compared with the Ctrl group by unpaired *t*-test. **C:** fasting blood glucose measured at weeks 13 and 14 of the protocol ($n = 10-11$ rats/group). **P* < 0.05 compared with the Ctrl group by unpaired *t*-test. **D:** fasting blood insulin concentration measured at week 12 of the protocol ($n = 10-11$ rats/group). **P* < 0.05 compared with the Ctrl group by unpaired *t*-test. **E:** blood glucose concentration measured during an intraperitoneal glucose tolerance test performed in fasted rats during the 13th week of the protocol and the corresponding area under the curve (AUC; inset) ($n = 10-11$ rats/group). **P* < 0.05 compared with the Ctrl group by two-way ANOVA followed by the Sidak post hoc test. **F:** plasmatic total cholesterol, low-density lipoprotein (LDL), high-density lipoprotein (HDL), and triglycerides measured at the end of the protocol ($n = 12$ rats/groups). **P* < 0.05 compared with the Ctrl group by unpaired *t*-test. All values are means \pm SE.

and then analyzed the activity of the autonomic nervous system through beat-to-beat HRV analysis in the frequency domain (Fig. 2, *A* and *B*). The LF band reflects mostly the influences of the sympathetic system on heart rhythm, and oscillations in HF bands reflect exclusively vagal activity. Thus, the LF-to-HF ratio reflects sympathovagal balance (58). HFS rats had a drastic increase in LF bands (Fig. 2, *A*–*C*) associated with a smaller increase in HF bands (Fig. 2, *A*, *B*, and *D*). Consequently, HFS rats exhibited a higher LF-to-HF ratio compared with Ctrl rats (Fig. 2*E*). This result indicates that alteration of sympathovagal balance in HFS rats is characterized by higher sympathetic tone. Classically, increased peripheral sympathetic outflow is associated with a high level of circulating

catecholamines (19, 61). We thus evaluated plasmatic levels of epinephrine and norepinephrine by HPLC. Consistent with the higher LF-to-HF ratio in HFS rats, the plasmatic level of epinephrine was higher in HFS rats compared with Ctrl rats (Fig. 2*F*). A similar tendency was observed with norepinephrine levels, although it did not reach significance (Fig. 2*G*). Because sympathetic tone is known to affect vascular tone and to increase blood pressure, we measured ABP in conscious rats by the tail-cuff method. First, we evaluated the ABP response to norepinephrine administration (1 mg/kg) in Ctrl animals and observed that this adrenergic stress produced a large increase in ABP in Ctrl animals (132.5 ± 4.1 vs. 167.4 ± 9.0 mmHg). In contrast, although our ABP measurement device effectively

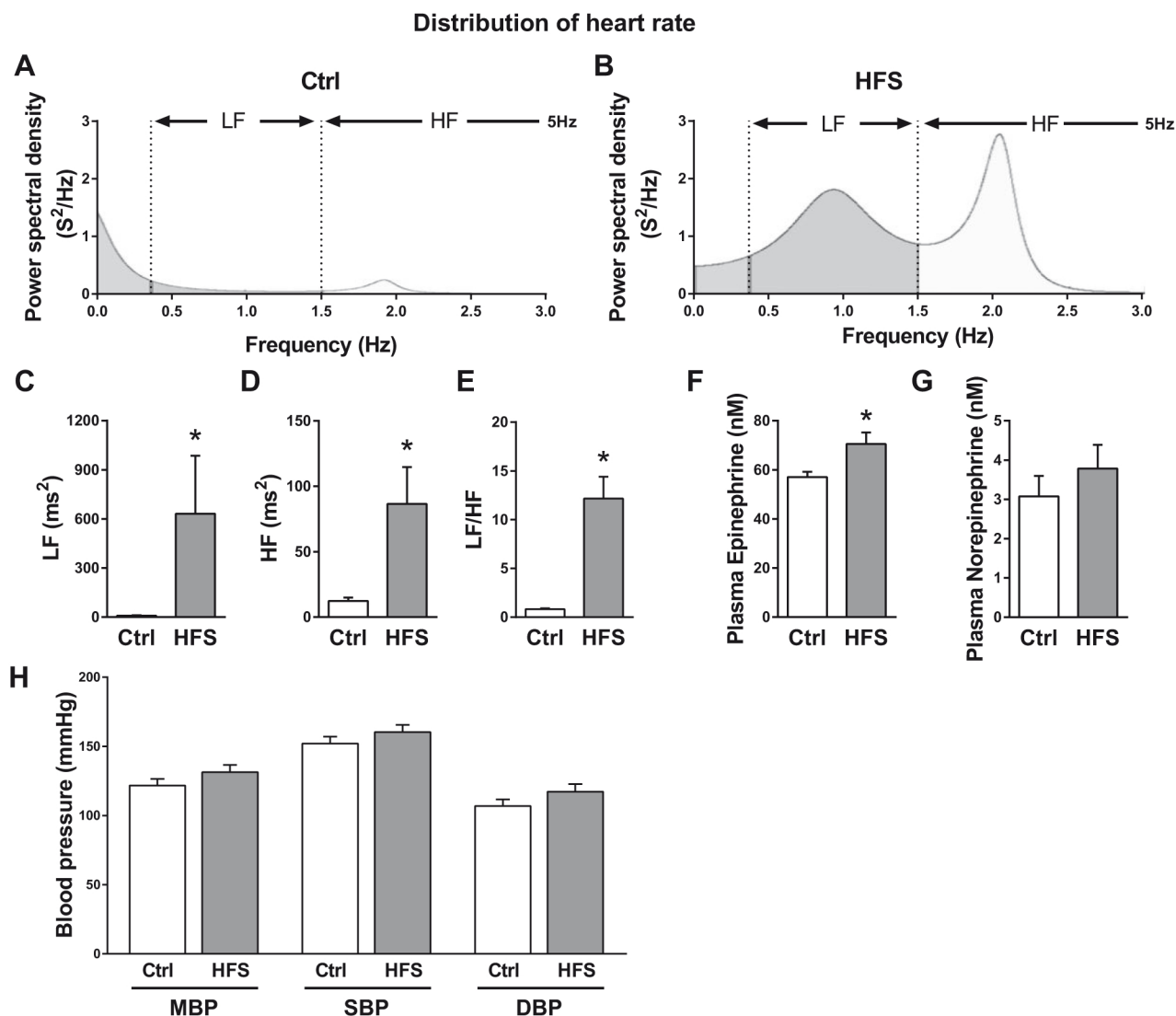


Fig. 2. Rats fed a high-fat and high-sucrose (HFS) diet present sympathetic hyperactivation with unchanged blood pressure. *A*: representative fast Fourier transformation spectrum of RR interval variability obtained in the control (Ctrl) population. HF, high frequency; LF, low frequency. *B*: representative fast Fourier transformation spectrum of RR interval variability obtained in the HFS population. *C*: LF spectral power measured by heart rate variability analysis in the frequency and time domain at week 14 of the protocol ($n = 4-9$ rats/group). * $P < 0.05$ compared with the Ctrl group by unpaired *t*-test. *D*: HF spectral power measured by heart rate variability analysis in the frequency and time domain at week 14 of the protocol ($n = 4-9$ rats/group). * $P < 0.05$ compared with the Ctrl group by unpaired *t*-test. *E*: LF-to-HF ratio (LF/HF; $n = 4-9$ rats/group). * $P < 0.05$ compared with the Ctrl group by unpaired *t*-test. *F*: plasmatic epinephrine measured at the end of the protocol ($n = 12$ rats/group). *G*: plasmatic norepinephrine measured at the end of the protocol ($n = 12-13$ rats/group). Data are compared between groups by unpaired *t*-test. *H*: mean blood pressure (MBP), systolic blood pressure (SBP), and diastolic blood pressure (DBP) measured by the tail-cuff method at week 14 of the protocol ($n = 12$ rats/group). Data are compared between groups by unpaired *t*-tests. All values are means \pm SE.

detected ABP variation in response to adrenergic receptor stress, we observed no difference of the ABP indexes between Ctrl and HFS animals in the steady state (Fig. 2H). Altogether, these results clearly supported sympathetic hyperactivity in HFS rats without any observable consequence on ABP.

Endothelium-dependent vascular hyporeactivity to vasoconstrictive agent masks sympathetic-dependent hypertension in HFS rats. Vascular tone is the consequence of the balance between vasoconstrictive and vasorelaxant factors. We first examined the vasocontractile function known to depend on the sympathetic activity through the adrenergic signaling pathway. To decipher why ABP was unchanged in HFS rats despite increased sympathetic outflow, we evaluated whether HFS rats could present downregulation of α_1 -AR. Western blot examination of α_{1A} -AR and α_{1D} -AR expression levels showed no difference between groups, but the HFS diet tended to reduce

α_{1B} -ARs ($P = 0.0695$). We next assessed the impact of the HFS diet on the arterial contractile response to the specific α_1 -AR agonist PE. The dose response to PE obtained in isolated aortic rings of HFS rats was reduced compared with Ctrl rats (Fig. 3B). This alteration was characterized by a reduced maximal response (E_{max} ; Fig. 3C) and sensitivity (pD_2 ; Fig. 3D) to PE in HFS aortic rings compared with Ctrl aortic rings. Altogether, these results show that the HFS diet alters α_1 -AR vascular signaling.

During α_1 -AR stimulation, the vasocontractile response is determined by both the smooth muscle response and capacity of the endothelium to release some vasorelaxant factors that restrain the contractile response (31, 54, 59). Therefore, the involvement of the endothelium in the modifications of the vascular responses in HFS rats was tested by mechanically removing the endothelium in the aorta (Fig. 4). In this condi-

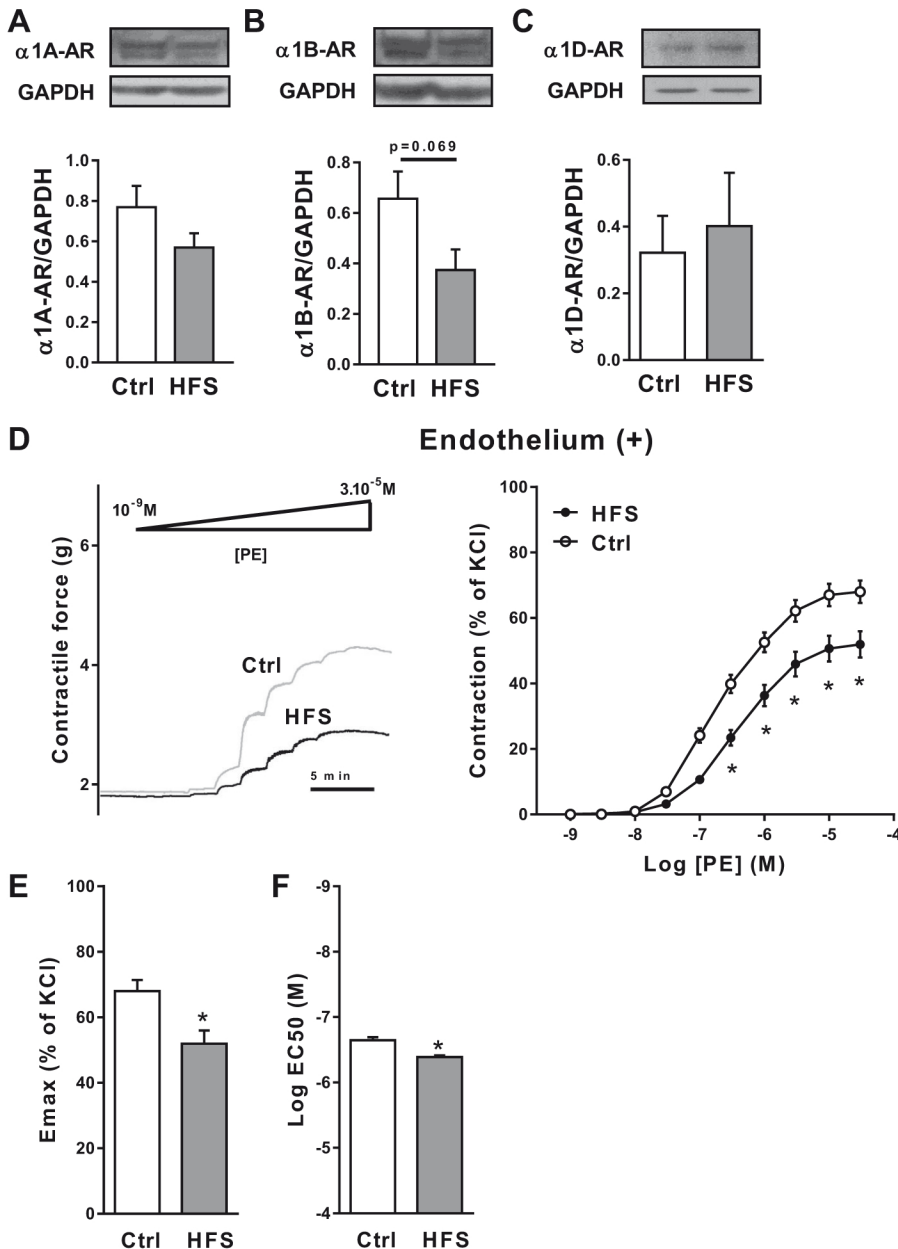


Fig. 3. Rats fed a high-fat and high-sucrose (HFS) diet exhibit vascular hyporeactivity to α_1 -adrenergic agonist. **A:** α_{1A} -adrenergic receptor (AR) expression measured in aortic tissue lysates by Western immunoblot analysis ($n = 4-5$ rats/group). Data were compared between groups by an unpaired t -test. **B:** α_{1B} -AR expression measured in aortic tissue lysates by Western immunoblot analysis ($n = 4-5$ rats/group). Data were compared between groups by an unpaired t -test. **C:** α_{1D} -AR expression measured in aortic tissue lysates by Western immunoblot analysis ($n = 7-9$ rats/group). Data were compared between groups by an unpaired t -test. **D, left:** representative recordings demonstrating the responses evoked by phenylephrine (PE) in aortic rings of control (Ctrl) and HFS rats. **Right:** dose-dependent response to cumulative doses of PE on aortic rings ($n = 4$ rats/group and $n = 23-24$ aortic rings/group). * $P < 0.05$ compared with the Ctrl group by two-way ANOVA followed by the Sidak post hoc test. **E:** maximal contraction of aortic rings to PE. Data are expressed in percent contractions relative to maximal contraction (E_{max}) obtained with 60 mM KCl ($n = 4$ rats/group and $n = 23-24$ aortic rings/group). * $P < 0.05$ compared with the Ctrl group by an unpaired t -test. **F:** logarithm of PE concentration inducing 50% of the maximal response (EC_{50}) to PE on aortic rings ($n = 4$ rats/group and $n = 23-24$ aortic rings/group). * $P < 0.05$ compared with the Ctrl group by an unpaired t -test. All values are means \pm SE.

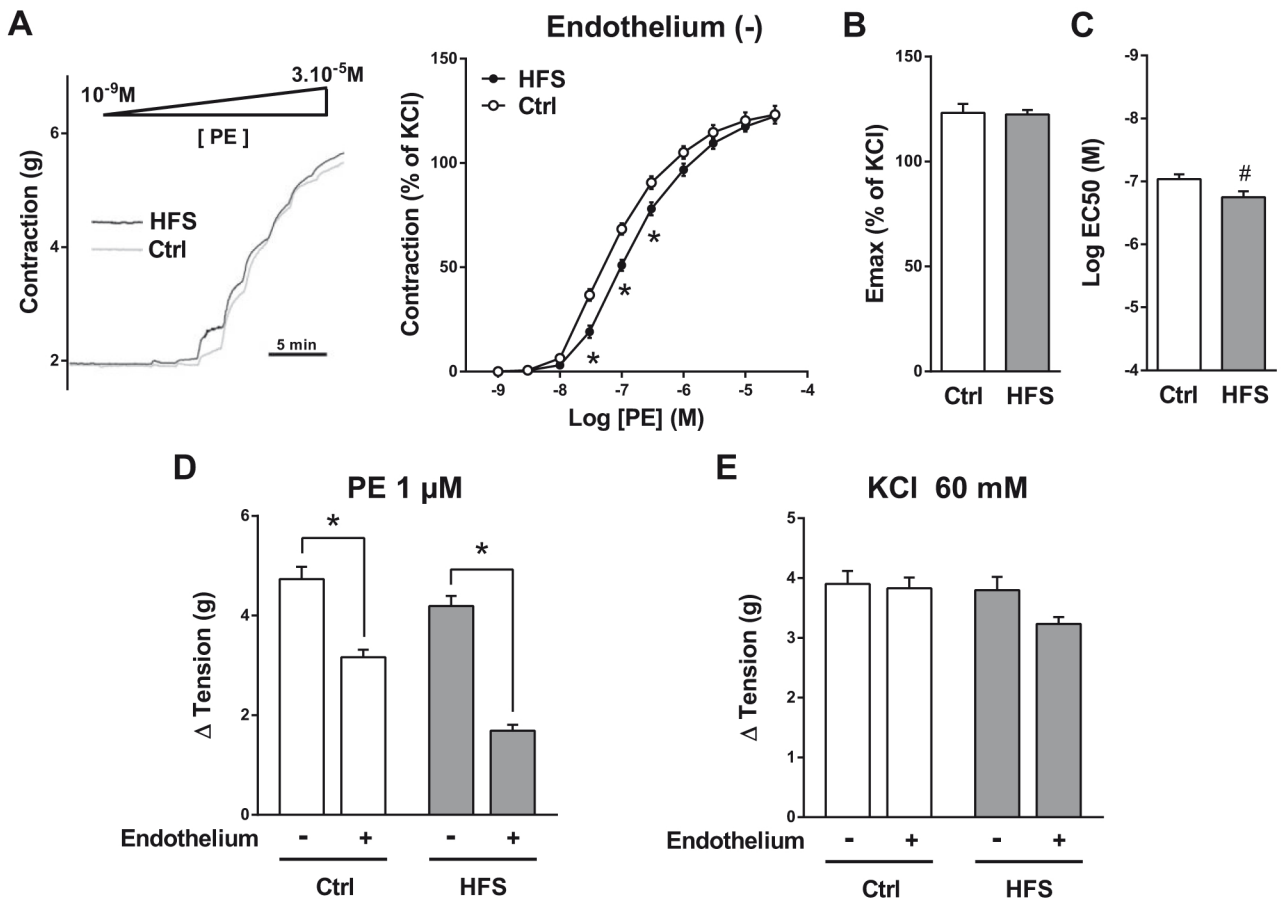


Fig. 4. Endothelium mediates vascular α_1 -adrenergic hyporeactivity in high-fat and high-sucrose (HFS) diet-fed rats. *A, left*: representative recordings demonstrating the responses evoked by phenylephrine (PE) in aortic rings of control (Ctrl) and HFS rats on which the endothelium was removed. *Right*, dose-dependent response to cumulative doses of PE on aortic rings on which endothelium was removed expressed relative to the amplitude of contraction with KCl ($n = 4$ rats/group and $n = 7$ aortic rings/group). $*P < 0.05$ compared with the Ctrl group by two-way ANOVA followed by the Sidak post hoc test. *B*: maximal contraction of aortic rings to PE. Data are expressed as percent contraction relative to maximal contraction obtained with 60 mM KCl ($n = 4$ rats/group and $n = 7$ aortic rings/group). Data were compared between groups by an unpaired *t*-test. *C*: logarithm of PE concentration inducing 50% of the maximal response (EC_{50}) to PE on aortic rings on which endothelium was removed ($n = 4$ rats/group and $n = 7$ aortic rings/group). Data were compared between groups by an unpaired *t*-test. $\#P < 0.05$ compared with the Ctrl group by an unpaired *t*-test. *D*: developed tension by aortic rings with or without endothelium in response to 1 μ M PE ($n = 4$ rats/group and $n = 10$ –28 aortic rings/group). $*P < 0.05$ compared with aortic rings from the same group without endothelium by two-way ANOVA followed by the Sidak post hoc test. *E*: developed tension by aortic rings with or without endothelium in response to 60 mM KCl ($n = 4$ rats/group and $n = 10$ –28 aortic rings/group). Data were compared by two-way ANOVA followed by the Sidak post hoc test. All values are means \pm SE.

tion, the Ctrl aorta contractile response to PE was strengthened, increasing maximal responses from 68% to 123% of KCl contraction (Fig. 4B). This result shows that the endothelium limits the vasoconstrictive effect of PE in healthy conditions. Interestingly, this increase in the contractile response to PE in endothelium-free aortic rings was higher in HFS rats than in Ctrl rats (here, the maximal response increased from 52% to 122% of the KCl contraction between the respective endothelium-intact and endothelium-free aortas; Fig. 4B). It is important to note that, in the absence of endothelium, HFS and Ctrl aortas reached a similar level of contraction (Fig. 4, A and B). The aortic sensitivity to PE ($-\log EC_{50}$) was increased by endothelium removal, and the Ctrl aorta remained slightly more sensitive to PE than the HFS aorta (Fig. 4C). Taken together, these data show that HFS rats demonstrate reduced adrenergic vasocontractile capacity, with no alteration at the muscular level, attributable to an enhancement of the endothelial anticontractile effect.

Endothelial cells maintain vascular tone by the elaboration of relaxing factors in response to various molecular and physical factors. To examine whether the endothelium inhibitory effect on vasoconstriction and its amplification in HFS rats was specific to α_1 -AR stimulation or relied on common features of vasoconstriction, we evaluated Ctrl and HFS aortic ring responses to a high single dose of PE (1 μ M) and to a high single dose of KCl (60 mM), a nonreceptor-dependent vasoconstrictor. As expected, we observed that, in both populations, developed contractions to PE were significantly attenuated when the endothelium was preserved. Once again, this phenomenon was amplified in the HFS aorta (developed contraction reduced from 4.7 to 3.2 g in the Ctrl aorta and from 4.2 to 1.6 g in the HFS aorta; Fig. 4D). Interestingly, when aortas were stimulated with KCl, we found no difference between developed contraction obtained in aortas without and with endothelium in Ctrl aortic rings. Only a slight decrease was observed between these two conditions in HFS aortic rings; however, this was not statistically

significant (Fig. 4E). These results definitively confirm that the endothelium preferentially antagonizes α_1 -AR-induced vasoconstriction and that this effect is potentiated in HFS rats.

Endothelium-derived NO is known to modulate the contractile response during α_1 -AR-mediated vasoconstriction (45, 47). Therefore, the implication of the eNOS-NO pathway in the modification of α_1 -AR vascular responses was tested by treating intact aortas with an eNOS inhibitor, L-NAME. Inhibition of eNOS increased the constrictive response to PE to a similar value in both Ctrl and HFS aortas. Indeed, between conditions without and with L-NAME pretreatment, the maximal response to PE increased from 68% to 118% of the KCl contraction in Ctrl rats and from 52% to 120% of the KCl contraction in HFS rats. As a consequence, differences observed between Ctrl and HFS were totally abolished in the presence of L-NAME (Fig. 5, A–C). The production of NO by eNOS depends on both the level of eNOS expression and level of activation by its phosphorylation on its activation site (Ser¹¹⁷⁷). Under basal conditions, both the levels of eNOS expression and the eNOS phospho-Ser¹¹⁷⁷-to-eNOS ratio were similar between Ctrl and HFS aortas (Fig. 5, D and E), which cannot explain the lower response to PE in the HFS aorta. We next evaluated the level of eNOS activation by PE in Ctrl and HFS aortas. Interestingly, PE reduced the level of eNOS phospho-Ser¹¹⁷⁷ in Ctrl aortic rings by 13% and increased it in HFS aortas by 31% (Fig. 5F). Finally, to confirm endothelium and eNOS contributions in the blunting of the sympathetic activation on blood pressure, we measured *in vivo* blood pressure in Ctrl and HFS rats before and after an intraperitoneal injection of L-NAME (20 mg/kg). In Ctrl rats, L-NAME had modest and not significant effects on SBP (+6%), DBP (+5%), and MBP (+5%) (Fig. 5G). In HFS rats, the same injection of L-NAME produced a significant increase of SBP (+14%), DBP (+17%), and MBP (+17%) (Fig. 5G). Thus, in the presence of L-NAME, SBP, DBP, and MBP were higher in HFS rats compared with Ctrl rats (Fig. 5G). Altogether, these results show that the HFS diet was associated with increased sympathetic vasopressor activity that was counterbalanced by an eNOS-dependent mechanism in the endothelium.

DISCUSSION

The main findings of the present study are that 1) HFS rats exhibited increased peripheral sympathetic outflow without an effect on blood pressure and 2) the increased eNOS response to α_1 -AR stimulation contributes to mask such vasopressor impact of the sympathetic outflow.

Hemodynamic consequences of the HFS diet. Obesity and MetS are important risk factors for the establishment of hypertension. The underlying physiological mechanisms involved are complex and have not been fully elucidated. To clarify the link between the development of obesity/MetS and the increase in blood pressure, numerous animal models have been used and assigned to different obesogenic diets. Those models display several features of obesity/MetS but, as observed in humans, do not systematically report increased blood pressure (8). This could be explained by discrepancies regarding the age of the rodents at the beginning of the diet, the duration and the composition of the diet, and the use of different strains of mice or rats (6, 8). For example, fructose consumption induces hyperuricemia, which is known to mediate endothelial dys-

function and to trigger the development of renal diseases (38, 42). Variation in these parameters will have different consequences on potent regulators of blood pressure, such as the renin-angiotensin-aldosterone system, renal damages, vascular endothelial function, and sympathetic nervous system activity. However, despite strong evidence that obesity and metabolic dysregulation are key triggers of increased blood pressure, in humans and in rodents some unknown factors could also contribute to explain uncoupling between MetS and hypertension.

Hemodynamic consequences of sympathetic activation. Some changes in the vascular response to sympathetic activation may significantly account for this complex association between MetS and hypertension. Indeed, sympathetic activation, a common feature of obesity and metabolic disorders (33), is known as a major trigger of hypertension. There is accumulating evidence that hyperinsulinemia, obesity, and possibly other components of MetS exert this sympathoexcitatory effect (44). Body fat products such as leptin and nonesterified fatty acids have also been associated with whole body norepinephrine spillover (49) and α_1 -AR vasopressor activity (23). Consistently, in our rat model of diet-induced MetS with abdominal obesity and hyperinsulinemia, both HRV analysis and plasma catecholamine levels evidenced high sympathetic activity in HFS rats. Using sympatholytic agents (9) or α_1 -AR blockers (17), several groups have highlighted the implication of sympathetic activity in higher vasopressor activity and hypertension observed in obesity and MetS. This contrasts with other studies (1, 8, 21, 27) and the present study in which, despite obvious sympathetic activation, no apparent change in ABP was observed. In line with our observation, it is interesting to note that, in hypertensive obese/MetS subjects, sympathetic overactivation was associated with increased vascular α -AR-dependent tone (12), whereas, in normotensive subjects, this coupling is lost (1). This suggests that, when MetS is not associated with hypertension, some vascular mechanisms may counteract the expected increase in arterial pressure.

Reduced adrenergic vasocontractile capacity in obesity. In several studies, the increased sympathetic activation associated with the development of MetS resulted in a lower adrenergic vasoconstrictive response of the vascular bed (1, 7, 39). This mechanism may represent a compensatory mechanism that counteracts the blood pressure elevation observed in response to adrenergic stress. The underlying mechanisms remain to be elucidated. It has been shown that leptin, an adipokine that increases sympathetic tone, induces a lower vascular contractile response to epinephrine associated with lower α_{1D} -AR gene expression (4). This was confirmed in obese mice; however, the vascular consequences and underlying mechanisms remain unclear (8). In the present study, α_{1A} -AR and α_{1D} -AR levels, measured by Western blot analysis, were not altered, whereas the α_{1B} -AR level tended to be lower in the aorta of HFS rats ($P = 0.069$). This could account for the reduced contractile response observed in the HFS aorta compared with the Ctrl aorta. However, at the level of the aorta, the vasoconstrictive response to PE differed between groups only when the endothelium was intact. Indeed, in endothelium-free aortic rings, the altered response to PE between Ctrl and HFS groups was abolished. Altogether, these data suggest that, despite the fact that the level of α_1 -AR could partly account for the altered

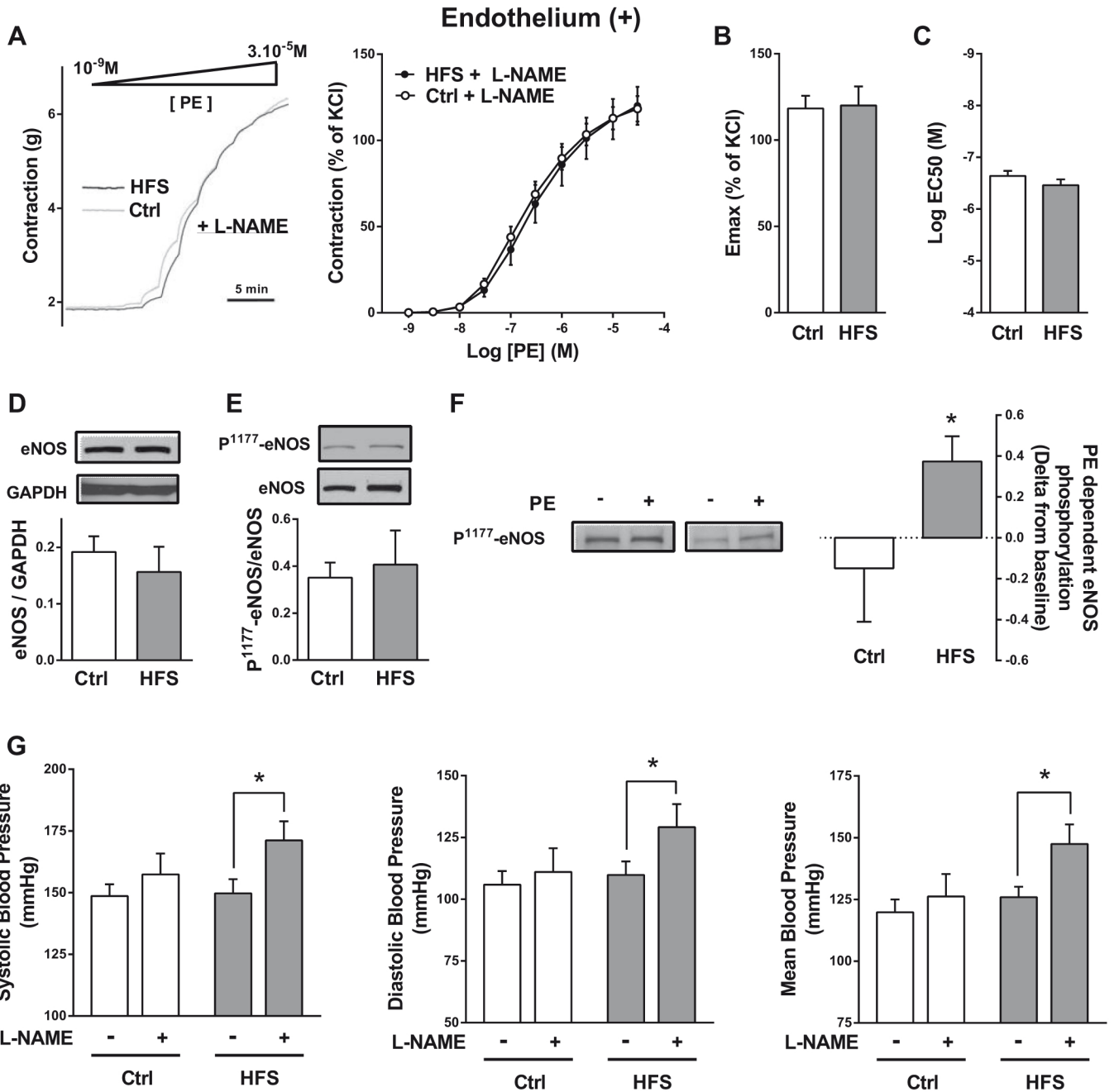


Fig. 5. Endothelial nitric oxide synthase (eNOS) depresses vascular α_1 -adrenergic receptor (α_1 -AR)-dependent vasopressor activity in high-fat and high-sucrose (HFS) diet-fed rats. *A, left:* representative recordings demonstrating the responses evoked by phenylephrine (PE) in aortic rings of control (Ctrl) and HFS rats pretreated with *N*-nitro-L-arginine methyl ester (L-NAME; 0.3 mM). *Right,* dose-dependent response to cumulative doses of PE on aortic rings pretreated with L-NAME (0.3 mM, $n = 4$ rats/group and $n = 7$ aortic rings/group). *B:* maximal contraction of aortic rings to PE. Data are expressed as percent contraction relative to maximal contraction obtained with 80 mM KCl ($n = 4$ rats/group and $n = 7$ aortic rings/group). *C:* logarithm of PE concentration inducing 50% of the maximal response (EC₅₀) to PE on aortic rings pretreated with L-NAME (0.3 mM, $n = 4$ rats/group and $n = 7$ aortic rings/group). *D:* eNOS expression measured in aortic tissue lysates by Western immunoblot analysis ($n = 5$ rats/group). Data were compared between groups by an unpaired *t*-test. *E:* eNOS phosphorylation at Ser¹¹⁷⁷ (P¹¹⁷⁷-eNOS) measured in aortic tissue lysates by Western immunoblot analysis ($n = 5$ rats/group). Data were compared between groups by an unpaired *t*-test. *F:* PE-dependent eNOS phosphorylation at Ser¹¹⁷⁷ measured by Western immunoblot analysis in tissue lysates of the aorta preincubated at 37°C with or without PE (10 μ M) for 15 min ($n = 3-6$ rats/group). * $P < 0.05$ compared with the Ctrl group by an unpaired *t*-test. Values correspond to the difference between eNOS phosphorylation measurements obtained in samples of the same aorta that were previously incubated in Krebs-HEPES solution with or without PE (10 μ M). *G:* mean, systolic, and diastolic blood pressures measured before and after an intraperitoneal injection of L-NAME (20 mg/kg, $n = 6-8$ rats/group). * $P < 0.05$ compared with data of the same group not treated with L-NAME by two-way ANOVA followed by the Sidak post hoc test. All values are means \pm SE.

vasocontractile response to epinephrine in HFS vessels, the role of the endothelium seems to be critical in the phenomenon.

Mechanisms of the reduced adrenergic response in HFS rats. In our obese/MetS rats, L-NAME increased blood pressure and unmasked a negative regulatory role of eNOS even under the basal condition. Considering that a sympathetic-dependent increase in ABP was obvious in HFS rats only in the presence of L-NAME, it seems that, at this stage of obesity/MetS, some compensatory mechanisms, triggered by the eNOS-NO pathway, contribute to maintain normal blood pressure in HFS rats by counteracting the impact of the sympathetic tone.

There are at least two mechanisms by which NO can lower blood pressure. NO produced in vascular endothelial cells acts as a potent vasodilator and thereby reduces vascular peripheral resistance (24). NO is also able to modulate autonomic nervous system activity and thus the impact of sympathetic tone on blood pressure (43, 62). In our work, the vascular contribution of eNOS on arterial pressure was obvious because the aortic hyporeactivity to α_1 -AR agonist in HFS aortic rings was blunted either by endothelial removal or by eNOS inhibition with L-NAME. This clearly highlights the key role of the endothelium and eNOS-NO-dependent vasodilation in maintaining blood pressure of HFS rats at the level seen in Ctrl rats.

Hyperinsulinemia, classically reported in obese/MetS subjects (15) and in our model, could be one of the triggers of this phenomenon. Indeed, insulin is known to activate eNOS by a phosphoinositide 3-kinase-Akt pathway. However, in conditions of metabolic disorders, insulin resistance compromises its ability to activate eNOS and also increases an insulin-MAPK-endothelin-1 pathway. Both may contribute to switching insulin from a vasorelaxant agent to a vasocontractile agent. In our study, both the glucose tolerance test and fasting hyperinsulinemia (Fig. 1) strongly suggest that HFS rats also developed insulin resistance. This is in line with the lack of difference between groups regarding basal eNOS phosphorylation (Ser¹¹⁷⁷) in the aorta. We thus can assume that insulin resistance was also present at the vascular level in our model. Altogether, these data do not support a role of hyperinsulinemia in the blunted vasomotor response to high sympathetic tone and α_1 -AR stimulation.

To understand this phenomenon, we also considered the possibility that our experimental model may have an increase in eNOS expression, but we found no difference between groups on these parameters.

Previous studies that have evaluated intercellular signaling between vascular smooth muscle cells and endothelial cells indicated that eNOS is activated in response to α_1 -AR agonist (34, 46). In our study, eNOS phosphorylation on its activation site in response to α_1 -AR agonist was highly evident in HFS rats but not in Ctrl rats. This phenomenon has also been reported in the rat model of renovascular hypertension, known as the two-kidney, one-clip model, and explained by activation of the phosphoinositide 3-kinase-Akt pathway. Indeed, in this model, increased phospho-eNOS- in response to PE was abolished by the use of wortmannin (45). Thus, despite the fact that the level of eNOS phosphorylation was not markedly altered in the HFS aorta, its higher ability to be activated during the stimulation of α_1 -AR stimulation constitutes a key element in maintaining blood pressure at the Ctrl level in HFS rats.

Transition from normal blood pressure to hypertension. As mentioned above, patients in the MetS condition may segregate into normotensive and hypertensive patients according to the fact that sympathetic activation translates or not into increased vascular tone. In light of our results, we suggest that such differences between those two subpopulations may reflect a rupture in the ability of the vascular endothelium to compensate the increase in adrenergic stimulation of the vascular smooth muscle cells. As a consequence, it allows hypertension to develop in those specific conditions. This is supported by the lost ability of the endothelium to counteract the vasocontractile response to epinephrine observed in aging and hypertensive models (28). At the cellular level, it is well known that depletion of the eNOS cofactor tetrahydrobiopterin, eNOS uncoupling, or diminished NO bioavailability by NADPH oxidase-dependent production of superoxide anion are mechanisms associated both with diabetes and hypertension that could be responsible for this rupture (36, 40). It seems then that all factors that could contribute to reduce the eNOS-NO response to adrenergic stress could constitute key triggers in the development of hypertension when subjects suffered for altered sympathovagal balance.

To conclude, we emphasize here the key role of the endothelium-dependent eNOS-NO pathway to limit or prevent the development of hypertension in subjects in the MetS condition (see schematic illustration of the proposed mechanism in Fig. 6). Indeed, in such a pathological state associated with impaired sympathovagal balance, the ability of the endothelium

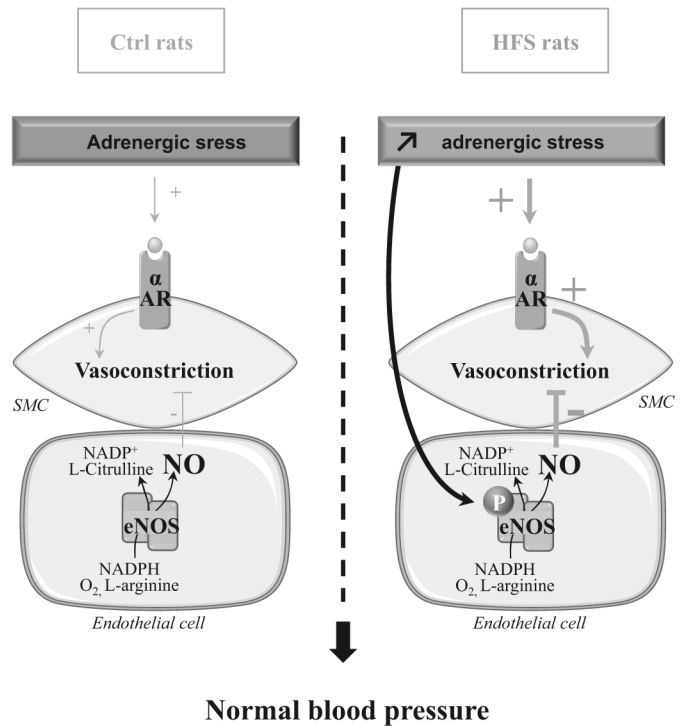


Fig. 6. The endothelium masks increased sympathetic vasopressor activity in rats with metabolic syndrome. In the control (Ctrl) condition, adrenergic vasoconstriction is blunted by endothelial nitric oxide (NO) synthase-NO anticontractile activity. In high-fat and high-sucrose (HFS) diet-fed rats, the vasocontractile adrenergic influence is increased but does not translate into elevated blood pressure because of a greater eNOS-NO anticontractile activity. AR, adrenergic receptor, SMC, smooth muscle cells.

to modulate adrenergic-dependent vasoconstriction seems to constitute the last defense against hypertension, which constitutes one of the worst cardiovascular risk factors. Thus, in MetS, all strategies that contribute to protect or improve endothelial function, such as exercise training or an appropriate diet, have to be considered to reduce the proliferation of cardiovascular risk factors.

Limitations of the study. In our study, the ex vivo experiments were performed on aortic isolated rings, which is considered a good and reliable model to study the interaction between the endothelium and vascular smooth muscle cells (60). However, the study of resistance vessels rather than conductance vessels would be physiologically more relevant to decipher mechanisms related to blood pressure regulation. Indeed, the functional impairments and timing of the alterations induced by MetS may be different in resistance vessels than in conductance vessels. Further studies will be needed to evaluate whether such compensatory mechanisms also exist at the level of resistance vessels. Another potential limitation of this work was that blood pressure was measured using the tail-cuff method, which may be considered poorly reliable (29). Doubts about this technic are partly due to the difficulty to use it properly. Indeed, to obtain reliable results, as recommended by the American Heart Association (32), animals need to be exposed to the procedure for at least 7 days before the beginning of the experiment to avoid animal stress. Results should also be obtained from an average of at least 3–10 measurements. Here, data were acquired by rigorously using American Heart Association recommendations (refer to METHODS). In addition, the accuracy of indirect blood pressure measurement may vary widely according the technology used in the tail-cuff method. Here, we used a volume-pressure-based tail-cuff method that has been proven accurate by comparison to simultaneous telemetry measurements (13). Finally, the ability of our method to accurately detect blood pressure variation was validated by measuring a substantial blood pressure increase in response to an intraperitoneal injection of norepinephrine.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

S.B., G.M., and C.R. conceived and designed research; S.B., C.M., A.R.N., L.B., S.G., C.L., F.D.N., J.D., O.C., J.T., and G.M. performed experiments; S.B., A.R.N., L.B., C.L., F.D.N., J.D., J.T., G.M., and C.R. analyzed data; S.B., C.M., A.R.N., G.M., and C.R. interpreted results of experiments; S.B. and C.R. drafted manuscript; S.B., O.C., J.T., G.M., and C.R. edited and revised manuscript; S.B. and C.R. approved final version of manuscript.

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