# ETUDE N°2

## La fonction endothéliale vasculaire masque l'effet vasopresseur du système nerveux sympathique chez le rat syndrome métabolique.

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

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## RÉSUMÉ D'ARTICLE

### Contexte scientifique

Le syndrome métabolique est caractérisé par différents facteurs de risques interdépendants, dont l'obésité viscérale et une intolérance au glucose associée à une dérégulation de l'insulinémie. Ces facteurs peuvent être responsables du déclenchement d'une hypertension artérielle, notamment en favorisant l'hyper-activation du système nerveux sympathique (SNS). Ce dernier est responsable d'une augmentation du tonus vasculaire et des résistances vasculaires périphériques. Cependant, malgré une augmentation de l'activité sympathique chez certains sujets obèses ou souffrant de syndrome métabolique, le tonus vasculaire et la pression artérielle ne sont pas toujours altérés (Huggett et al. 2004; Agapitov et al. 2008).

Il est bien décrit à ce jour que la régulation du tonus vasculaire est localement assurée par les agents vasoactifs produits par les cellules endothéliales, et notamment par le monoxyde d'azote (NO). Cet agent vasodilatateur joue un rôle important dans le contrôle du tonus vasculaire et donc de la pression artérielle. Ce gazotransmetteur pourrait être responsable d'une hypo-réactivité vasculaire face à une stimulation adrénergique, et ainsi limiter les effets néfastes induits par une sur-activation du SNS. **Ainsi, l'hypothèse de cette étude n°2 était que les effets vasoconstricteurs et hypertenseurs de l'hyperactivité du SNS, chez un modèle rat syndrome métabolique normotendu, pourraient être compensés par une biodisponibilité supérieure du NO au niveau vasculaire.**

### Méthodologie

Des rats Wistars ont été soumis à un régime enrichi en lipide et en sucre (HFS : *high fat high sucrose*) pendant 15 semaines, permettant d'induire un syndrome métabolique.

L'activité du SNS a été évaluée grâce à une analyse des domaines fréquentiels de la variabilité de la fréquence cardiaque, et par un bilan des catécholamines circulantes (noradrénaline et adrénaline). La perfusion sanguine a été évaluée par une technique de laser doppler au niveau de la microcirculation cutanée, et la pression artérielle a été mesurée par la technique dite de « tailcuff » utilisant un brassard caudal. L'implication du NO dans la régulation du tonus vasculaire a été évaluée en mesurant la pression artérielle d'animaux préalablement traités à la L-NAME (20mg/kg).

Une exploration ex vivo de la réactivité vasculaire aortique, a permis de mettre en évidence le rôle de l'endothélium et du NO dans la régulation de la vasomotricité au cours d'un stress adrénergique. Enfin, l'expression et la phosphorylation de la eNOS (Ser1177) suite à un stress α-adrénergique ont été mesurées par western blot.

**Résultats majeurs**

Les animaux nourris avec un régime HFS présentaient une obésité viscérale et une hyperinsulinémie, associées à une hyperactivité du SNS. Néanmoins, ces animaux ne présentaient pas d'hypertension artérielle. De façon intéressante, nous avons pu mesurer une hyporeactivité vasculaire en réponse à un stress α1-adrénergique (phényléphrine) sans changement apparent du niveau de l'expression des récepteurs α1-adrénergiques. Cette hyporéactivité semble être liée à un phénomène endothélium- et eNOS-dépendant, impliquant l'hyperactivation de la eNOS au cours d'un stress adrénergique. **Ainsi, ces travaux démontrent l'implication de l'endothélium et plus particulièrement de la voie eNOS/NO dans le maintien d'une pression artérielle normale malgré une hyperactivation sympathique caractéristique d'un syndrome métabolique.**

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

**Running Head:** Endothelial control of blood pressure.

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**Abstract**

**Objective**: Sympathetic hyperactivation, a common feature of obesity and metabolic syndrome, is a key trigger of hypertension. However, some obese subjects with autonomic unbalance present a dissociation between sympathetic nerve activity and sympathetic mediated vascular tone and thus normal blood pressure. Here, we aimed to determine in a rat model of metabolic syndrome, whether the endothelium and eNOS-NO pathway could counteract the vasopressor effect of the sympathetic system.

**Methods:** Rats were fed a high fat and sucrose diet for 15 weeks (HFS). Blood glucose homeostasis was evaluated at 13-14 weeks. Sympatho-vagal balance was evaluated by spectral analysis of heart rate variability and plasmatic catecholamines measurements. Blood pressure was measured at rest and during eNOS inhibition (L-NAME). Adrenergic vascular reactivity was assessed isometrically in response to α1-adrenergic agonist, phenylephrine.

**Results:** HFS diet increased fat accretion (+77% epididymal, +101% dorsal), fasting insulinemia (+49.8%), fasting (+13.6%) and postprandial glycemia (+51%). HFS rats presented higher low on high-frequency spectral power ratio (x13), higher plasmatic epinephrine (+23%) but unchanged blood pressure. Interestingly, HFS rats exhibited vascular hyporeactivity (-23.6%) to α1-adrenergic receptors stimulation that was abolished by endothelial removing or eNOS inhibition (L-NAME). In addition, eNOS phosphorylation (ser1177) was increased (+31%) by phenylephrine in HFS rats only. Accordingly, eNOS inhibition in-vivo revealed higher blood pressure in HFS compared to control rats (Respectively 147 vs 126 mmHg for mean blood pressure).

**Conclusion:** Restrain of adrenergic vasopressor action by the endothelium is increased in HFS rats and contributes to maintain blood pressure in physiological range.

**Condensed abstract:**

Despite sympathetic activation, some MetS and obese subjects present normal blood pressure and dissociation between sympathetic nerve activity and increased vascular tone. This suggest compensatory mechanisms occurring at vascular level in MetS/obese subjects that oppose sympathetic vasoconstrictor and hypertensive effect. Using a high fat, high sucrose diet, this study shows that in the time where rats develop metabolic syndrome and an autonomic unbalance, they also develop un increased eNOS-NO sensitivity to adrenergic stimulation resulting in vascular hypoadrenergic reactivity and therefore maintenance of physiological blood pressure.

**Keywords:** hypertension, metabolic syndrome, endothelium, sympathetic activation, nitric oxide.

**Introduction**

Metabolic syndrome (MetS) is a cluster of physiological dysregulations that include 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 develop commonly hypertension, a major comorbidity factor for CVD. Several components of MetS such as hyperinsulinemia [1–3], visceral obesity [4–6] and others are positive modulators of the sympathetic nervous system [4,7]. The sympathetic nervous system is a major vasoconstrictor system, which may contribute to the hypertension observed in patients with MetS. Surprisingly, some obese and MetS patients present normal arterial blood pressure (ABP) and vascular tone despite obvious sympathetic activation [4,8]. These observations suggest that (1) compensatory mechanisms counterbalance sympathetic action and therefore dissociate sympathetic activity from its hypertensive effect and that (2) vascular tone control might be involved in such 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 gazotransmitter nitric oxide (NO) produced by the endothelial NO synthase (eNOS) [9], thereby assuming a suitable adaptation and control of blood pressure [9]. Indeed, various stimuli can activate eNOS such as bradykinine, hypoxia or shear stress in order to adapt blood flow and satisfy metabolic demand. eNOS is also activated in vascular smooth muscle during α1-adrenergic receptors (α1-AR) –stimulation to limit potential side effects of massive vasoconstriction during adrenergic stress [10,11]. Interestingly, endothelial function and eNOS pathway are altered in various pathological states. Metabolic diseases are classically associated with endothelial dysfunction; however, endothelium ability to limit arterial vasoconstriction can also be exacerbated. As exemple, this is the case during adrenergic stress in hepatic and renal diseases [12,13] but similar observations have been made in obese animal model [14]. 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 α1-adrenergic hyporesponsiveness in population with MetS. For this purpose, we investigated the sympathetic activation, the impact of α1-adrenergic receptors stimulation on arterial vasomotricity and how the endothelium and especially the eNOS activation state

could modulate this response in a rat model of high fat and high sucrose diet induced metabolic disorders.

## Methods

### Experimental protocol

All investigations conformed to European Parliament Directive 2010/63/EU (N° CEEA-00322.03) and were approved by the local research ethics committee (experimentation n°: 84.004). Male Wistar rats were randomly assigned into either the control group (Ctrl) fed with a standard diet (A04, SAFE, France) or to the high fat high sucrose group (HFS). The HFS diet is a high fat diet (230 HF containing 60% kcal as fat with a caloric value of 5.317 kcal/g; SAFE, France), completed with 10% of sucrose in drinking water during 15 weeks to induce MetS. At the end of the 15 weeks diet period, cardiac function was explored *in vivo* (ECG, blood pressure). Then rats were sacrificed, blood was collected for biochemistry analysis, total visceral and epididymal fat were removed and weighed as index of visceral obesity, and aorta was dissected for ex-vivo analysis of vascular function.

### ECG recording and heart rate variability analysis.

ECG recordings were obtained through implantable CA-F40 telemetric ECG transmitters (DSI, St. Paul, MN) and by using a signal RCP-1 receiver connected to a data acquisition system (Ponemah Physiology Platform, DSI). Rats were instrumented under general anesthesia and allowed to recover from surgery for 8 days. The autonomic nervous system activity on cardiac function was assessed by studying beat-to-beat Heart Rate Variabilty (HRV) as described previously [15,16]. Twenty segments of 3-minutes (5 segments/hour) were analyzed using Kubios HRV software v2.2 to assess frequency domain - HRV [17]. First all RR intervals were measured and then mean RR interval was calculated. Then, after application of a cubic spline interpolation (20 Hz), the power spectrum is estimated with Welch's periodogram modelling i.e. the RR series is divided into overlapping segments (50%), each segment is windowed to decrease the leakage effect (1024s), and the spectrum estimate is obtained by averaging the FFT spectra of all windowed segments. Thus, the Fast Fourier Transform (Low frequency band, LF: 0.4-1.5Hz; high frequency band, HF: 1.5-5Hz) was applied to exclusively sinus RR intervals. As commonly published, the LF band power reflects sympathetic autonomic nervous system and baroreflex activities on heart (26, 51). The HF band power reflects parasympathetic activity (45). Thus, the LF/HF ratio could be used to estimate the sympatho-vagal activity on cardiac rhythm (5).

**Blood pressure measurements.**

Mean (MBP), systolic (SBP) and diastolic (DBP) blood pressures were assessed in conscious rats by tail-cuff method using the CODA tail-cuff system (Kent Scientific) at the end of the protocol. To confirm that this system was sensitive enough to detect 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. Finally, to assess NO contribution in the regulation of blood pressure, blood pressure was measured in some rats before and 15 minutes after intraperitoneal injection of a NOS inhibitor, L-NAME (20mg/kg) to assess NO contribution in the regulation of blood pressure.

**Isolated aortic rings**

Under anesthesia (sodium pentobarbital, 100 mg/kg, i.p.), thoracic aorta was quickly removed and placed in cold Krebs–Henseleit bicarbonate buffer (composition in mM: NaCl 118, NaHCO3 25, KCl 4.8, KH2PO4 1.2, CaCl2 1.25, Glucose 11). After removal of adherent tissue, the aorta was cut in small segments of 2mm long. The aortic rings were mounted onto stainless steel supports and suspended in the tissue bath containing Krebs–Henseleit buffer at 37°C continuously bubbled with $O_2$–$CO_2$ (95%–5%) gas mixture. The rings were connected to an isometric force transducer (EMKA technologies, EMKA Paris, France), linked to an amplifier (EMKA technologies, EMKA Paris, France) and a computerized acquisition system, to record changes in isometric force. The 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 in order to obtain a reference contraction, which was used to normalize subsequent contractile responses. Endothelial integrity was then tested with a single dose of phenylephrine (PE, 1 µM) followed by a vasorelaxing dose of acetylcholine (ACh, 10 µM). To assess vasocontractile capacity to α1 adrenergic agonist, cumulative doses of phenylephrine were added in the bath (0.1 nM to 10 µM). To evaluate the implication of endothelial activity on alpha adrenergic vasoconstriction, the inner surface of some rings was gently rubbed to remove endothelium before the rings were mounted. To test endothelium capacity to inhibit agonist and non-agonist induced vasoconstriction, aortic rings response to respectively a single dose of PE (1 µM) and a single dose of KCl (60 mM). In the same way, the effect of eNOS activity was evaluated by treating aortic rings with L-NAME (0.3 mM) 30min prior to the vasoconstriction protocol. The responses were characterized by Emax 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.**

Intra-Peritoneal Glucose Tolerance Tests (IPGTT) were performed at the end of the 13$^{th}$ week of the protocol. First, blood was obtained via tail clip to assess fasting blood glucose (Caresens® N, DinnoSanteTM). Then, rats received an intraperitoneal injection of a glucose solution (2g/kg), and blood glucose was measured at 10, 20, 30, 60 and 120 minutes after the glucose injection.

**Blood analysis**

Blood samples were stored at -20°C until analysis. Total cholesterol (TC), high-density lipoprotein cholesterol (HDL), low lipoprotein cholesterol (LDL) and triglycerides (TG) were determined in plasma by automated enzymatic kits on 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 high performance liquid chromatography analysis (Column: Vydac 218TP54, 5 µm, 4.6 mm i.d. x 250 mm; Waters Separations Module 2695; multi-wavelength fluorescence detector Waters 2475).

**Western blot analysis**

Western blots were performed as previously described [25]. Briefly, proteins from aorta homogenates were separated onto sodium dodecyl sulfate-polyacrylamide gels and transferred onto polyvinylidene difluoride membranes. Membranes were incubated with primary antibodies at 4°C in 1 % milk (α1D adrenergic receptor, 1:500 Santa Cruz; eNOS, 1:1000 BD Transduction and GAPDH, 1:5000; Santa Cruz), or 0.3 % bovine serum albumin (eNOS-PSer1177 1:500; BD Transduction) in Tris-buffered saline containing 0.05 % Tween-20 overnight. Immunodetection was carried out using ECL or ECL Plus system (SuperSignal® West Pico Chemiluminescence Substrate, Thermo Scientific; Luminata™ Forte Western HRP substrate, Millipore Corporation, respectively) and membranes were then exposed to X-ray films for revelation. eNOS protein content was expressed relative to GAPDH content. eNOS-PSer1177 protein content was expressed relative to eNOS content. To evaluate PE impact on eNOS activation, the phosphorylation level at Ser1177 of eNOS was measured from aorta segments previously incubated with or without PE (10 µM) for 15 minutes.

**Statistical analysis**

Data were expressed as the mean ± SEM. Unpaired t-tests were used for single comparison between groups. For studies involving multiple measures on the same animal over time as well as for dose-response curves, comparison between groups were made using two-way ANOVA followed by a Sidak post hoc test where appropriate. A value of $p<0.05$ was considered statistically significant. Statistical analysis was done using GraphPad Prism software.

**Results**

**High fat and high sucrose diet induces MetS in Wistar rats**

After thirteen weeks, enriched fat and sucrose diet feeding significantly increased body mass of HFS rats compared to their littermate assigned to standard chow diet (Fig 1A). HFS rats had higher epididymal and dorsal 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 test (Fig 1E). Blood analysis revealed that HFS rats had higher LDL cholesterol and triglyceride levels with unchanged total cholesterol and HDL levels (Fig 1 F). All together these results showed that HFS diet induced central obesity (increased body mass and visceral fat accretion), alteration of glucose and insulin metabolisms and dyslipidemia, which fit the definition of MetS in Human [26].

**Increased sympathetic nervous outflows in HFS rats does not translate into higher blood pressure**

Obesity and MetS are classically associated with increased sympathetic nervous outflows [4–6]. We monitored cardiac electrical activity (ECG) using telemetric system over 24 hours in Ctrl and HFS rats and then analyzed the activity of autonomic nervous system through beat-to-beat HRV analysis in the frequency domain (Figure 2A & 2B). The ‘low’ frequency band (LF) reflects mostly the influences of the sympathetic system on heart rhythm and oscillations in ‘high‘ frequency bands (HF) reflect exclusively vagal activity. Thus LF/HF reflects sympatho-vagal balance [16]. HFS rats had a drastic increase in LF bands (Fig 2A, 2B & 2C) associated with a smaller increase in HF bands (Fig 2A, B and D). Consequently, HFS rats exhibited higher LF/HF ratio when compared to Ctrl rats (Fig 2E). This result indicates that alteration of sympatho-vagal balance in HFS rats is characterized by higher sympathetic tone. Classically, increased peripheral sympathetic outflow is associated with high level of circulating catecholamines [27,28]. We thus evaluated plasmatic levels of epinephrine and

norepinephrine by high performance liquid chromatography. Consistent with the higher LF/HF ratio in HFS rats, plasmatic level of epinephrine was higher in HFS when compared to Ctrl ones (Fig 2F). Similar tendency was observed with norepinephrine levels, although it did not reach significance (Fig 2G). Since sympathetic tone is known to affect vascular tone and to increase blood pressure, we measured ABP in conscious rats by tail-cuff method. Firstly, we evaluated ABP response to NE administration (1 mg/kg) in Ctrl animals and observed that this adrenergic stress produced a large increase in ABP in Ctrl animals (Fig1 suppl data). By contrast, although our ABP measurement device effectively detected ABP variation in response to AR-stress, we observed no difference of the ABP indexes between Ctrl and HFS animals in steady state (Fig 2I). Altogether, these results supported clearly 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**

The 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 adrenergic signaling pathway. To decipher why the arterial blood pressure was unchanged in HFS rats despite increased sympathetic outflow, we evaluated HFS rats could present down regulation of $\alpha_{1D}$-AR, which appears to be highly implicated in this pathway [29–31], but we found no difference in its expression level between groups as confirmed by western blot (Fig 3A). Next we assessed the impact of HFS diet on arterial contractile response to the specific $\alpha_1$-AR agonist PE. The dose-response to PE obtained in isolated aortic rings of HFS rats was reduced when compared to Ctrl ones (Fig 3B). This alteration was characterized by reduced maximal response ($E_{max}$, Fig 3C) and sensitivity (pD2, Fig 3D) to PE in HFS aortic rings compared to Ctrl ones. Altogether, those results clearly showed that HFS diet does not alter $\alpha_{1D}$-AR expression level but alters the vasoconstrictive response mediated by the $\alpha_1$-AR stimulation.

During vasoconstriction, the endothelium also releases some vasorelaxant factors in order to restrain the contractile response [32–34]. 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 condition, 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 than in Ctrl rats (Here, the maximal response increased from to 52% to 122% of KCl contraction between the respective endothelium-intact and endothelium-free aorta) (Fig 4B). It is important to note that in the absence of endothelium, HFS and Ctrl aorta reached the similar level of contraction (Fig 4A, B). The aorta sensitivity to PE (-Log EC50) was increased by the endothelium removal and Ctrl aorta remained slightly more sensitive to PE than HFS one (Fig 4C). Taken together these data show that HFS rats exert reduced adrenergic vasocontractile capacity, with no alteration at the muscular level, but due to an enhancement of endothelial anticontractile effect.

The endothelial cells maintain vascular tone by the elaboration of relaxing factors in response to various molecular and physical factors. To examine whether endothelium inhibitory effect on vasoconstriction and its amplification in HFS rats was specific to $\alpha_1$-AR stimulation or relied on common features of vasoconstriction, we evaluated Ctrl and HFS aortic rings responses to a high single dose of PE (1 µM) and to a high single dose of KCl (60 mM), a non-receptor dependent vasoconstrictor. As expected, we observed that in both populations, developed contraction to PE were significantly attenuated when endothelium was preserved. Once again, this phenomenon was amplified in HFS aorta (Developed contraction reduced from 4.7 g to 3.2 g in Ctrl aorta and from 4.2 g to 1.6 g in HFS ones) (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 endothelium preferentially antagonizes $\alpha_1$-AR induced vasoconstriction and that this effect is potentiated in HFS rats.

The endothelium-derived NO is known to modulate the contractile response during α1-AR mediated vasoconstriction [11,13]. Therefore, eNOS-NO pathway implication in the modification of α1-AR vascular responses was tested by treating intact aorta with an eNOS inhibitor, L-NAME. The inhibition of eNOS increased the constrictive response to PE to a similar value in both Ctrl and HFS aorta. Indeed, between conditions without and with L-NAME pre-treatment, maximal response to PE increased from 68% to 118% of KCl contraction in Ctrl rats and from 52 to 120 % of KCl contraction in HFS rats. As a

consequence, differences observed between Ctrl and HFS were totally abolished in presence of L-NAME, (Fig 5A, B & C). The production of NO by eNOS depends on both the level of eNOS expression and the level of activation by its phosphorylation on its activation site (ser1177). In basal conditions, both the levels of eNOS expression and eNOS-Pser1177/eNOS ratio were similar between Ctrl and HFS aorta (Fig 5D and E), which cannot explain the lower response to PE in HFS aorta. Next we evaluated the level of eNOS activation by PE in Ctrl and HFS aorta. Interestingly, PE reduced the level of eNOS-Pser1177 in Ctrl aortic rings by 13% and increased it in HFS aortas by 31% (Fig 5F). Finally, to confirm the 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 intraperitoneal injection of L-NAME (20mg/kg). In Ctrl rats, L-NAME had modest and not significant effects on systolic (+6%), diastolic (+5%), and mean (+5%) blood pressures (Fig 5G). In HFS rats, the same injection of L-NAME produced a significant increase of systolic (+14%), diastolic (+17%) and mean (+17%) blood pressures (Fig 5G). Thus, in presence of L-NAME, systolic, diastolic and mean blood pressures were higher in HFS rats when compared to Ctrl ones (Fig 5G). Altogether these results showed that 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 effect on blood pressure, (2) increased eNOS response to α1-AR stimulation contributes to mask such vasopressor impact of the sympathetic outflow.

Sympathetic activation is a common feature of obesity and metabolic disorders [35]. There is accumulating evidence that hyperinsulinemia, obesity and possibly other components of MetS exert this sympatho-excitatory effect. For example insulin infusion under euglycemic conditions has been demonstrated to increase muscle sympathetic nerve activity [36]. Body fat products such as leptin and non-esterified fatty acids have also been associated with whole-body norepinephrine spillover [37] and α1-AR vasopressor activity [38]. 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 [39] or α1-AR blockers [40], 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 [4,8] and the

present one in which despite obvious sympathetic activation no apparent change in arterial blood pressure was observed. Thus, some unknown mechanisms counteract the subsequent increase in arterial pressure expected with sympathetic activation.

Nevertheless, in our MetS rats, L-NAME increased blood pressure and unmasked a negative regulatory role of eNOS even in basal condition. Considering that sympathetic-dependent increase in arterial blood pressure was obvious in HFS rats only in presence of L-NAME, it seems that at this stage of the MetS pathology, some compensatory mechanisms, triggered by eNOS-NO pathway, contribute to maintain a 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 [41]. NO is also able to modulate the autonomic nervous system activity and thus the impact of sympathetic tone on blood pressure [9,42]. In our work, the vascular contribution of eNOS on arterial pressure was obvious since aortic hyporeactivity to α1-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 level seen in Ctrl rats.

To understand this phenomenon, we first considered the possibility that our experimental model may have an increase in eNOS expression and/or its phosphorylation on its main activation site (Ser1177), but we found no difference between groups on these parameters.

However, previous studies evaluating intercellular signaling between vascular smooth muscle cell and endothelial cell indicate that eNOS is activated in response to α1-AR agonist [43,44]. In our study, eNOS phosphorylation on its activation site in response to α1-AR agonist was highly evident in HFS rats but not in Ctrl ones. This phenomenon has been also reported in the rat model of renovascular hypertension known as 2 kidneys 1 clip and explained by the activation of the Pi3K-Akt pathway. Indeed, in this model increased eNOS-P in response to PE was abolished by the use of wortmannin [13]. Thus, despite the level of eNOS phosphorylation was not markedly altered in HFS aorta, its higher ability to be activated during the stimulation of α1-AR stimulation constitutes a key element in maintaining blood pressure at Ctrl level in HFS rats.

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

with impaired sympatho-vagal balance, the endothelium ability 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 strategy that contribute to protect or improve endothelial function, such as exercise training or an appropriate diet have to be considered in order to reduce the proliferation of cardiovascular risk factors.

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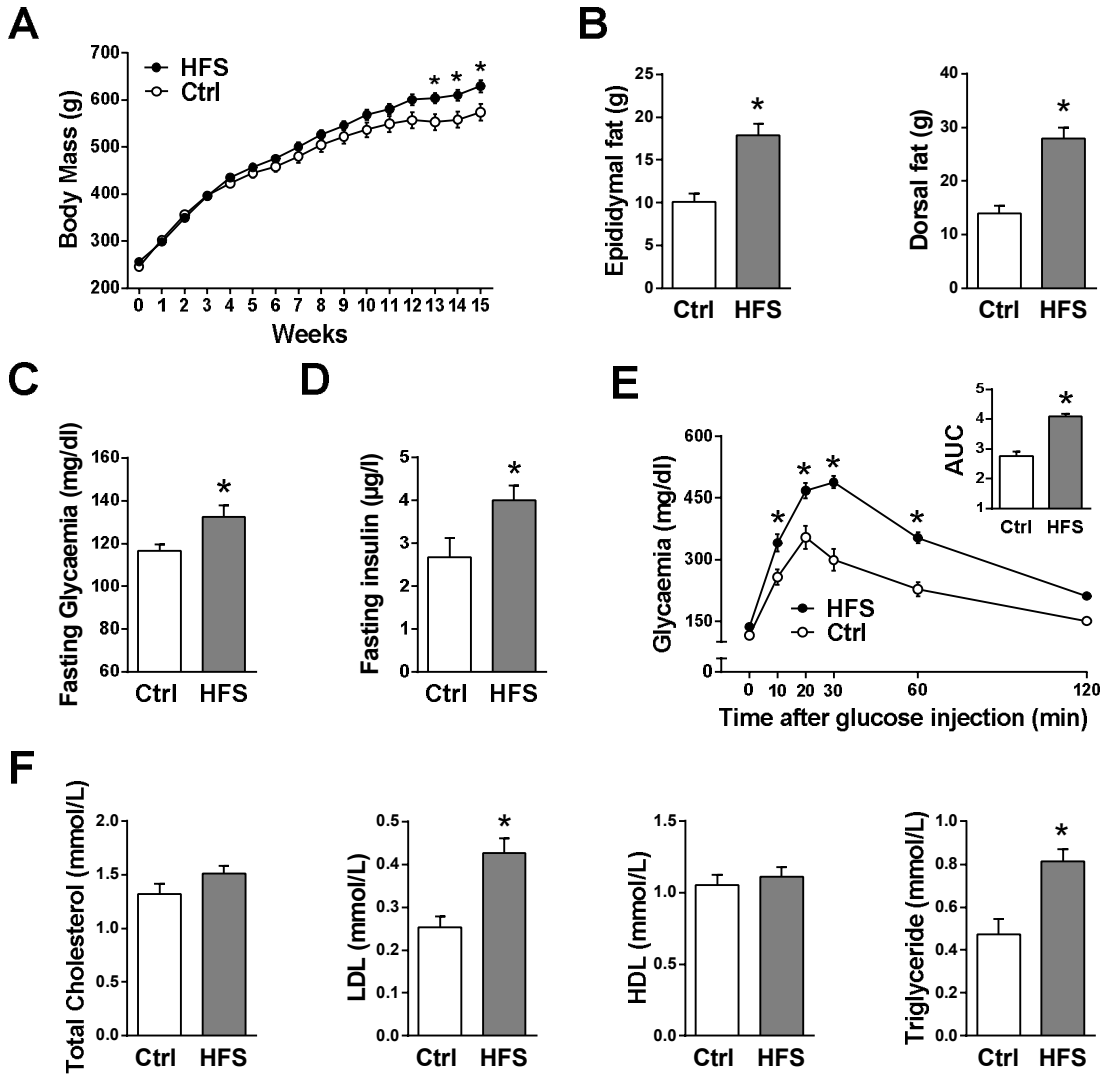


**Figure. 1. High fat and high sucrose diet induces MetS in Wistar rats.** A: Follow up of body mass during the 15 weeks of high fat/high sucrose diet (HFS) or standard diet (Ctrl). B: Epididymal and dorsal fat mass at the end of the protocol. C: Fasting blood glucose measured at week 13 and 14 of the protocol. D: Fasting blood insulin concentration measured at week 12 of the protocol. E: Blood glucose concentration measured during a glucose tolerance test (IPGTT) performed in fasted rats during the 13$^{th}$ week of the protocol and corresponding area under the curve (AUC)(inner graph). G: Plasmatic total cholesterol, Low Density Lipoprotein (LDL), High Density Lipoprotein (HDL) and triglyceride measured at the end of the protocol. Values are the mean ± SEM. *P<0.05 vs. Ctrl group.

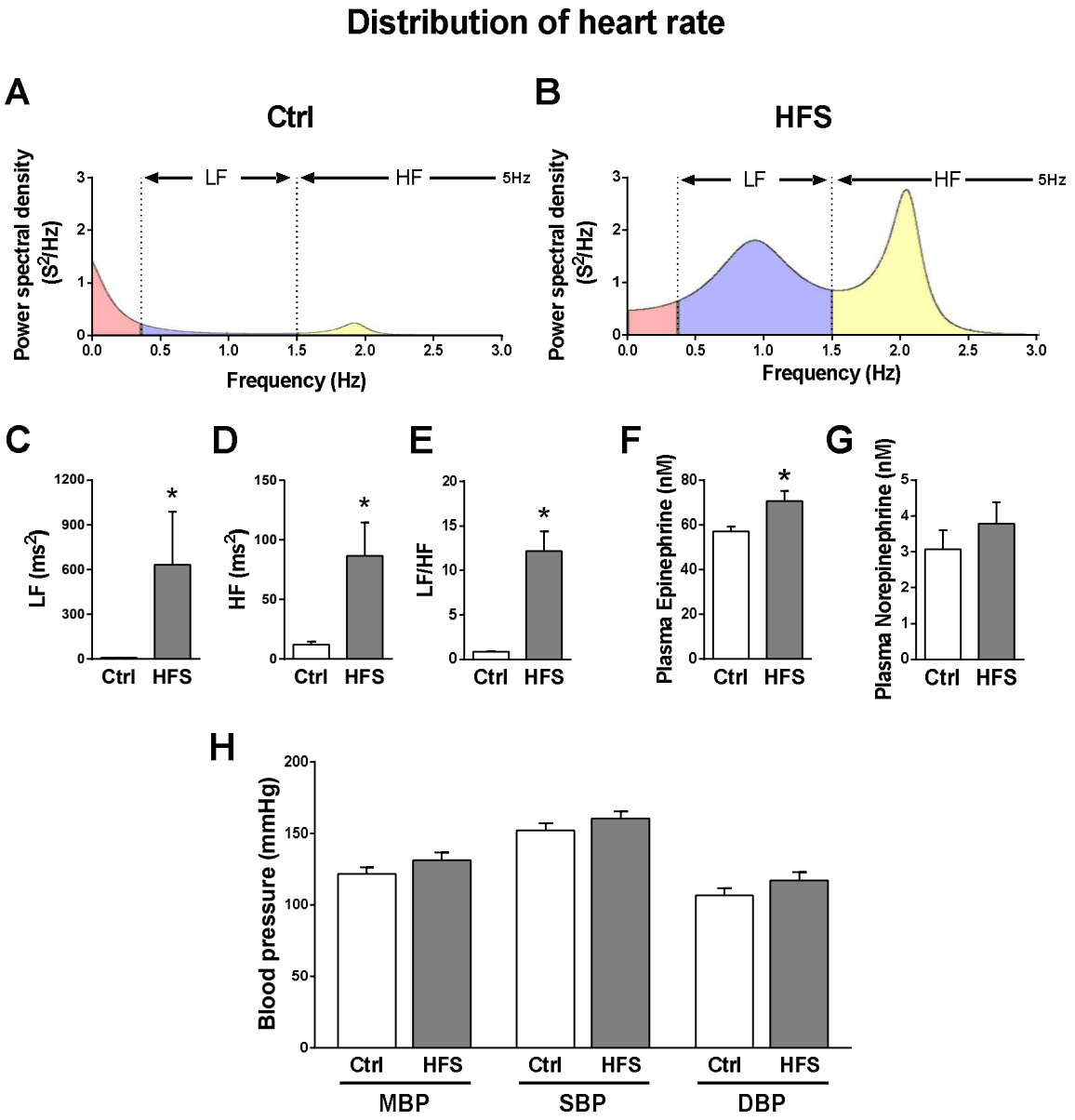


**Figure. 2. HFS rats present sympathetic hyperactivation with unchanged blood pressure.** A: Representative Fast Fourier Transform spectrum of RR intervals variability obtained in Ctrl population. B: Representative Fast Fourier Transform spectrum of RR intervals variability obtained in HFS population. C: Low frequencies (LF) spectral power measured by heart rate variability analysis in the frequency and time-domain at week 14 of the protocol. D: High frequencies spectral power measured by heart rate variability analysis in the frequency- and time-domain at week 14 of the protocol. E: LF to HF ratio. F: Plasmatic epinephrine measured at the end of the protocol. G: Plasmatic norepinephrine measured at the end of the protocol. H: Mean (MBP), systolic (SBP) and diastolic (DBP) blood pressure

measured by tailcuff method at week 14 of the protocol. Values are the mean ± SEM. *P<0.05 vs. Ctrl group.

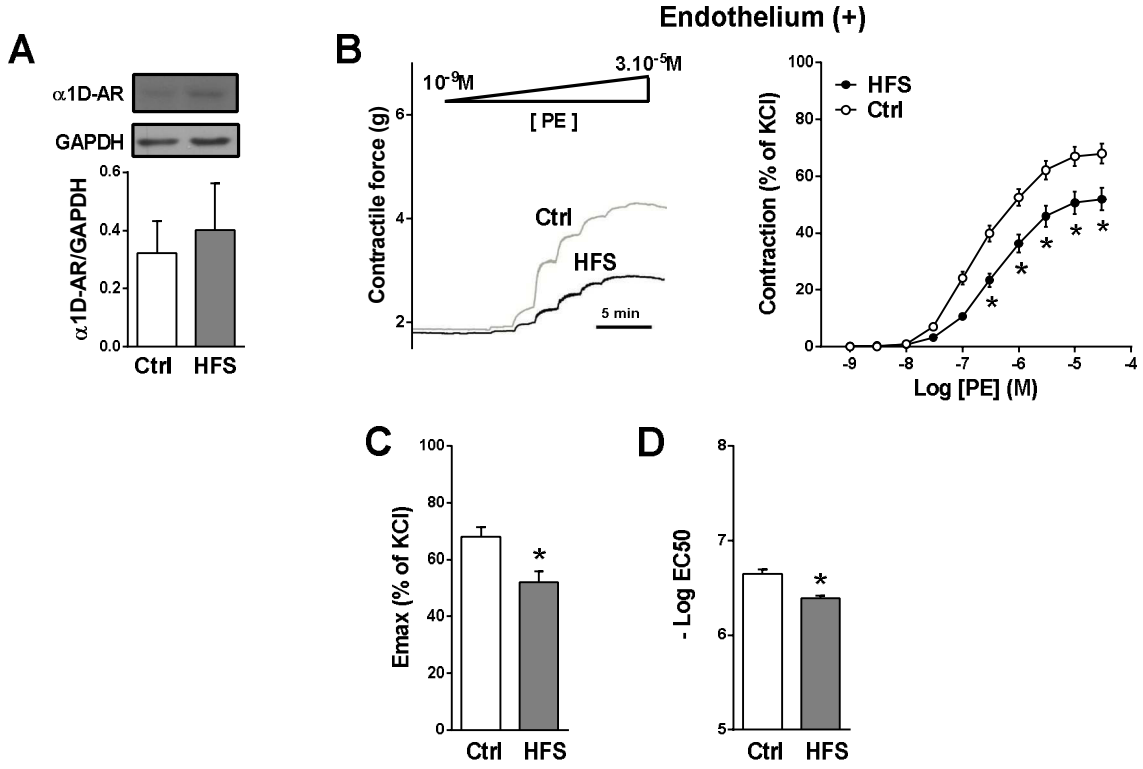


**Figure. 3. HFS rats exhibit vascular hyporeactivity to α1-adrenergic agonist.**

A: α1D-AR expression measured in aortic tissue lysates by Western immunoblotting. B: Left: Representative recordings demonstrating the responses evoked by PE in aortic rings of Ctrl and HFS rats. Right: Dose-dependent response to cumulative dose of PE on aortic rings. C: Maximal contraction of aortic rings to PE. Data are expressed in percent contraction relative to maximal contraction obtained with 60 mM KCl. D: Logarithm of PE concentration inducing 50% of maximal response (EC50) to PE on aortic rings. Values are the mean ± SEM. *P<0.05 vs. Ctrl group.

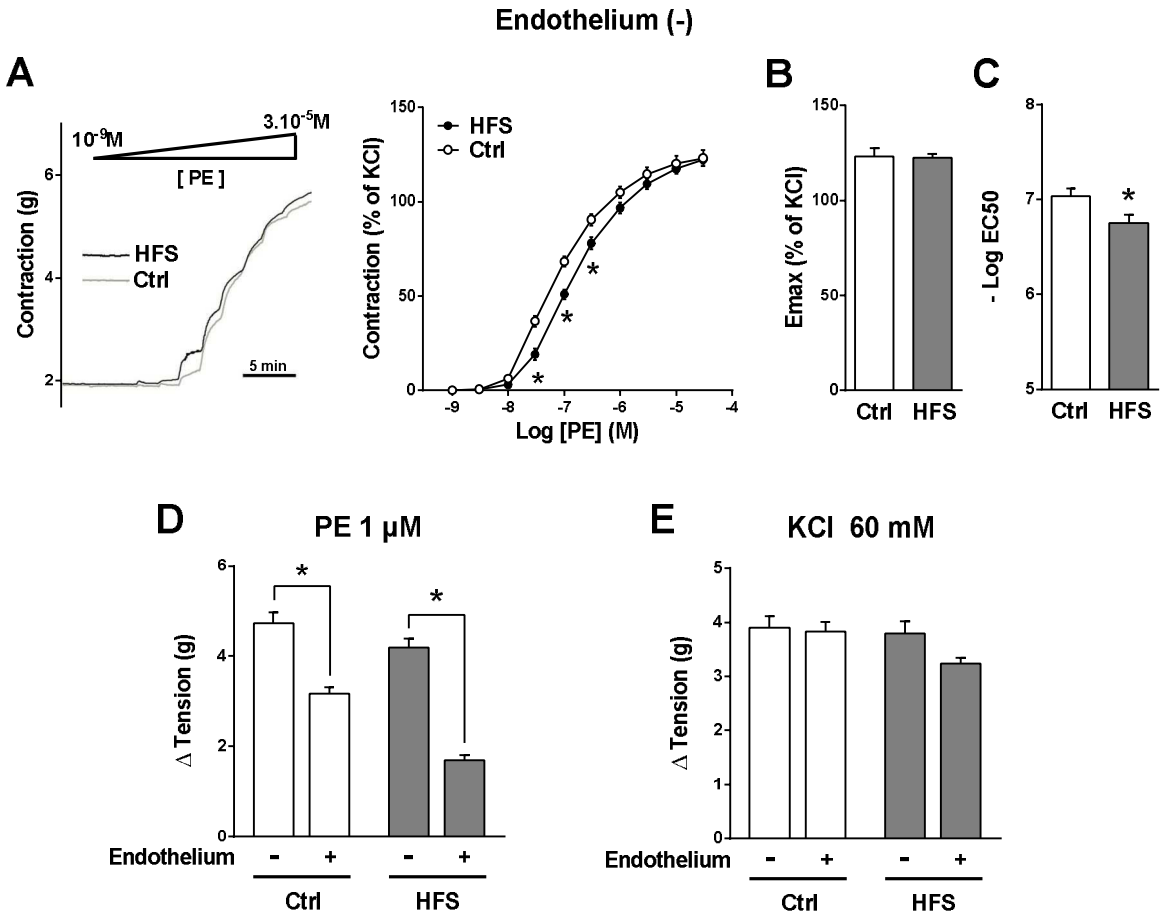


**Figure. 4. Endothelium mediates vascular α1-adrenergic hyporeactivity in HFS rats.** A: Left: Representative recordings demonstrating the responses evoked by PE in aortic rings of Ctrl and HFS rats on which endothelium was removed. Right: Dose-dependent response to cumulative dose of PE on aortic rings on which endothelium was removed expressed relative to the amplitude of contraction with KCl. B: Maximal contraction of aortic rings to PE. Data are expressed in percent contraction relative to maximal contraction obtained with 60 mM KCl. C: Logarithm of PE concentration inducing 50% of maximal response (EC50) to PE on aortic rings on which endothelium was removed. D: Developed tension by aortic rings with or without endothelium in response to 1 µM of PE. E: Developed tension by aortic rings with or without endothelium in response to 60 mM of KCl. Values are the mean ± SEM. *P<0.05 vs. Ctrl group.

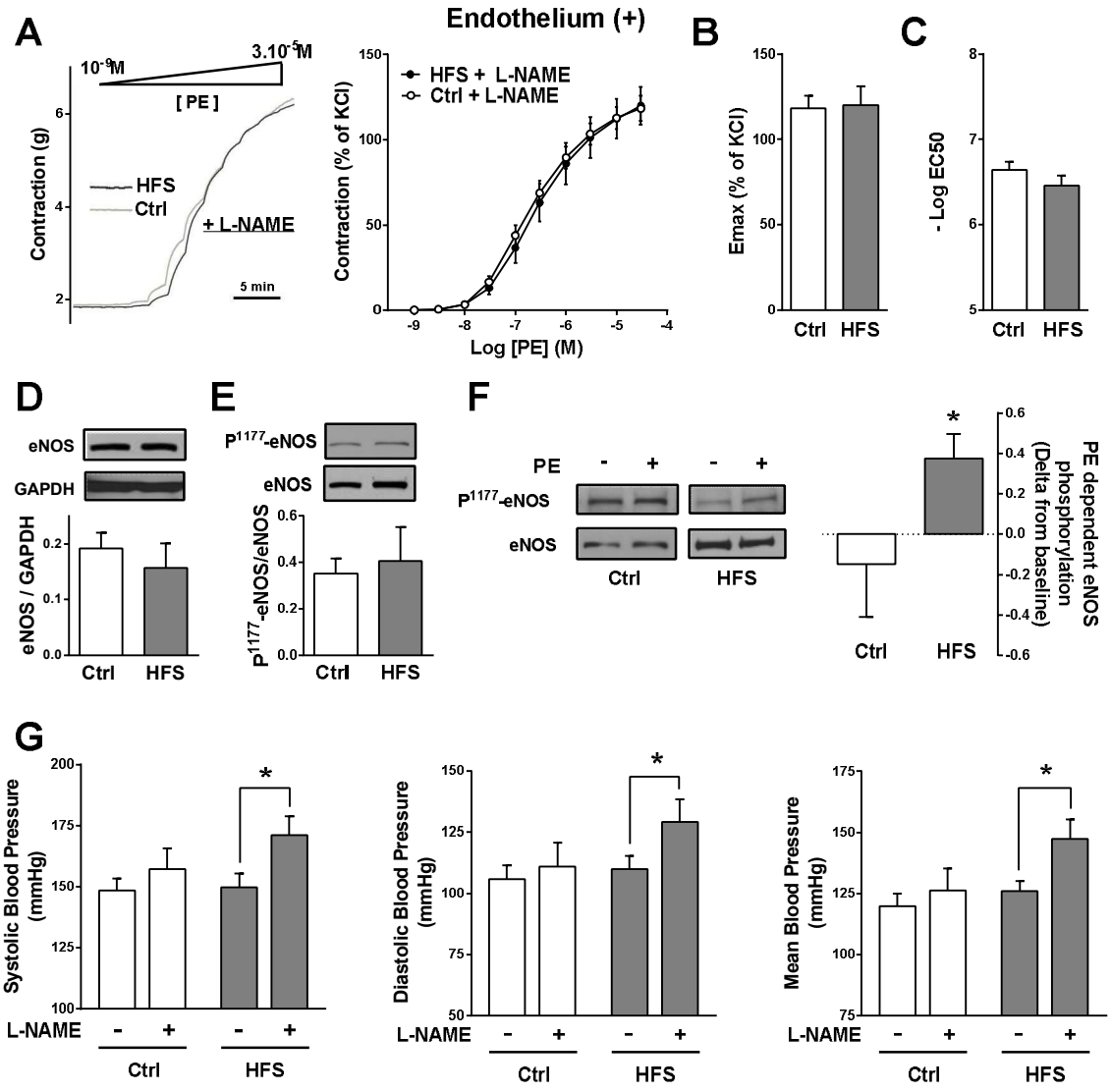


**Figure. 5. Endothelial nitric oxide synthase depresses vascular α1-AR dependent vasopressor activity in HFS rats.** A: Left: Representative recordings demonstrating the responses evoked by PE in aortic rings of Ctrl and HFS rats that were pre-treated with L-NAME (0.3 mM). Right: Dose-dependent response to cumulative dose of PE on aortic rings pre-treated with L-NAME (0.3 mM). B: Maximal contraction of aortic rings to PE. Data are expressed in percent contraction relative to maximal contraction obtained with 80 mM KCl. C: Logarithm of PE concentration inducing 50% of maximal response (EC50) to PE on aortic rings that were pre-treated with L-NAME (0.3 mM). D: eNOS expression measured in aortic tissue lysates by Western immunoblotting. E: eNOS phosphorylation at Ser 1177 (P1177-eNOS) measured in aortic tissue lysates by Western immunoblotting. F: PE dependent eNOS phosphorylation at Ser 1177 (P1177-eNOS) measured by Western immunoblotting in tissue lysates of aorta pre-incubated à 37°C with or without PE (10 µM) for 15 min. The values

correspond to the difference between eNOS phosphorylation measurement obtained in samples of a same aorta that were previously incubated in Krebs-HEPES solution with or without PE (10 µM). G: Mean, systolic and diastolic blood pressure measured before and after intraperitoneal injection of L-NAME (20mg/kg). Values are the mean ± SEM. *P<0.05.

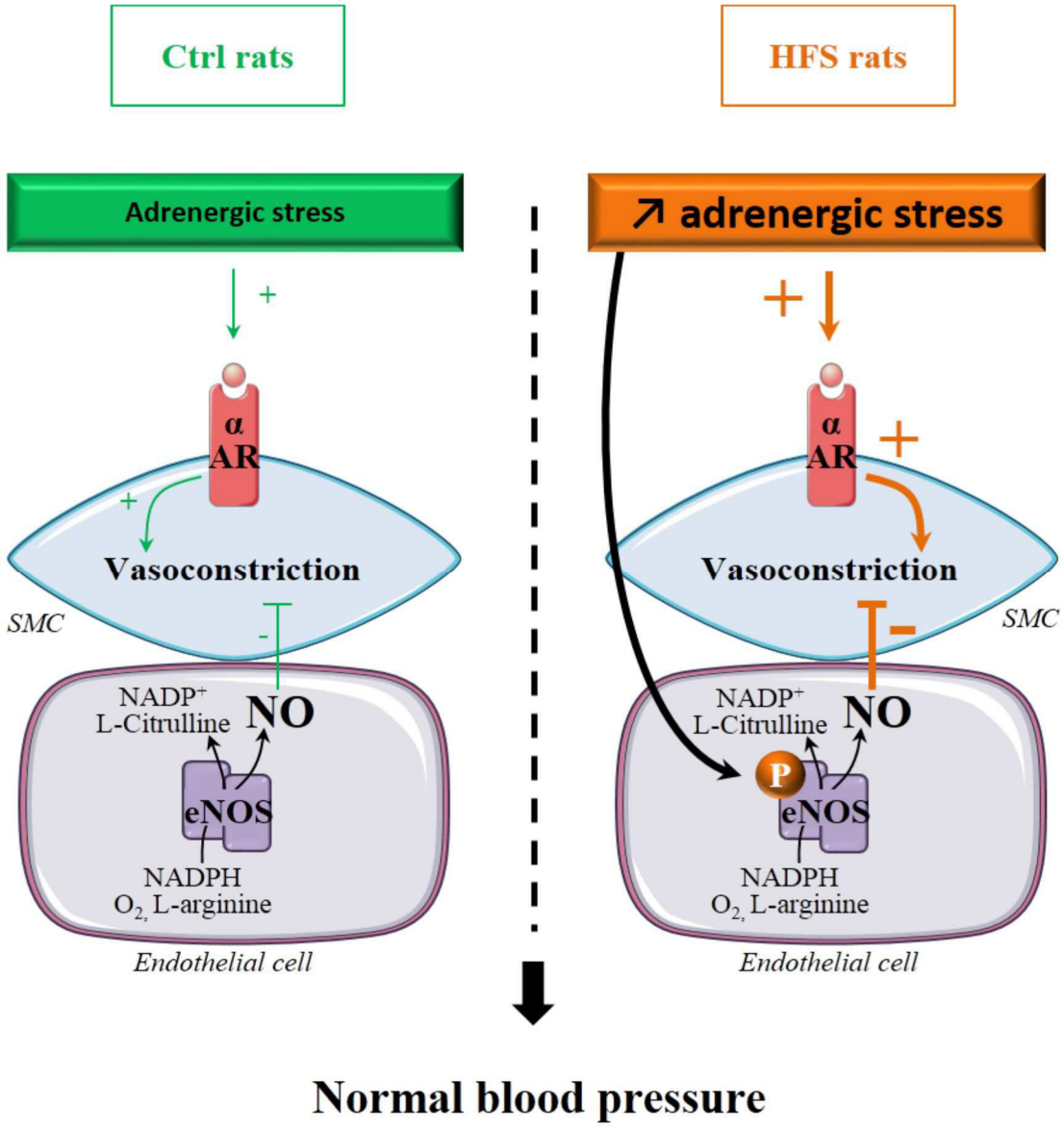


**Figure. 6. Endothelium masks increased sympathetic vasopressor activity in rats with metabolic syndrome.** In control condition, adrenergic vasoconstriction is blunted by eNOS-NO anticontractile activity. In HFS rats, vasoconstritive adrenergic influence is increased but does not translate into elevated blood pressure because of a greater eNOS-NO anticontractile activity.

## Résultats additionnels

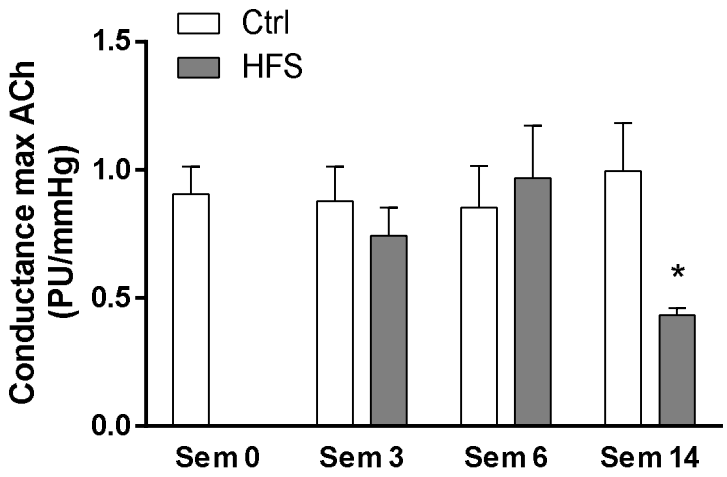


**Figure 36: Suivi longitudinal du flux sanguin cutanée mesuré par Laser Doppler, en réponse à une iontophorèse d'acétylcholine (ACh) permettant d'évaluer la fonction de relaxation vasculaire endothélium-dépendante au niveau de la microcirculation cutanée de rats sains (Ctrl) et SMet (HFS).** Les résultats sont exprimés en conductance (PU/mmHg)* :$p<0.05$ vs Ctrl. Les valeurs ont été exprimées en moyenne ± SEM.