Exercise training restores eNOS activation in the perivascular adipose tissue of obese rats: Impact on vascular function

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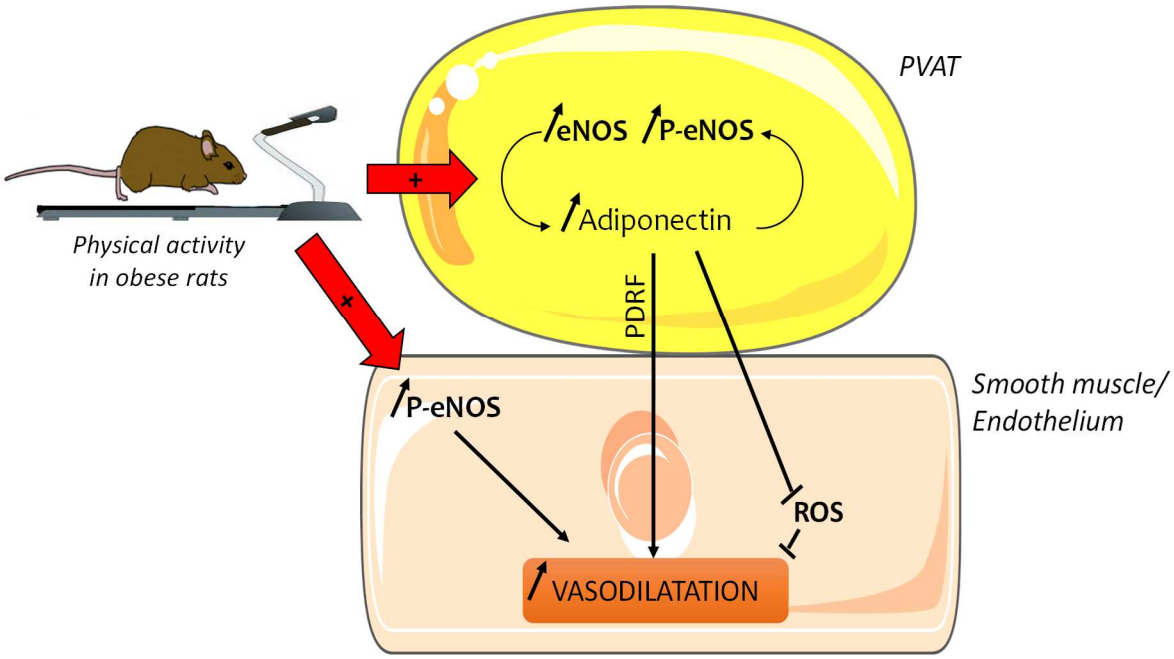
ABSTRACT

Objective: This study evaluated in obese rats the effect of exercise training on eNOS expressed in perivascular adipose tissue (PVAT) and its consequences on vascular function. **Methods:** Wistar rats were divided in 3 groups: control (standard diet), obese (high fat/high sucrose diet, HFS for 15 weeks), and exercised obese (HFS diet and exercise from week 6 to week 15, HFS-Ex) rats. The eNOS-adiponectin pathway and reactive oxygen species (ROS) were evaluated. Vascular reactivity was assessed on isolated aortic rings with or without PVAT and/or endothelium and exposed or not to the conditioned media of PVAT. **Results**: Obesity reduced eNOS level and phosphorylation on its activation site in the PVAT and had no impact on the vascular wall. Exercise training was able to increase eNOS and P-eNOS both in the vascular wall and in the PVAT. Interestingly, this was associated with increased level of adiponectin in the PVAT and to lower ROS in the vascular wall. Finally, PVAT of HFS-Ex aorta has eNOS-dependent anticontractile effects on endothelium denuded aortic rings and has beneficial effects on the endothelium-dependent vasorelaxation to ACh. **Conclusion:** Exercise training in obese rats is able to impact PVAT eNOS with subsequent beneficial impact on vascular function.

KEYWORDS: eNOS, exercise, obesity, oxidative stress, perivascular adipose tissue.

GRAPHICAL ABSTRACT



HIGHLIGHTS

- Exercise training in obese rats impacts eNOS both in the perivascular adipose tissue and in the endothelium

- Beneficial effects of exercise training on the perivascular adipose tissue is also obvious regarding adiponectin level and ROS production in the aortic wall

- Perivascular adipose tissue contributes to the beneficial effects of exercise training on vascular function

1. INTRODUCTION

Obesity constitutes a global healthcare problem and has many adverse effects on the cardiovascular function[1,2]. Recently, the perivascular adipose tissue (PVAT) has emerged as a relevant fat depot for cardiovascular risk and as a potential trigger in vascular dysfunction. Indeed, PVAT known as an endocrine and paracrine organ[3], due to its localization close to the smooth muscle layer of most systemic blood vessels is considered as a regulator of vascular function. PVAT can secrete some relaxing factors, called PVAT-derived relaxing factors (PDRF), that modulate the vasoconstrictive response to adrenergic stress[4], via endothelium-dependent and -independent mechanisms[5,6]. Among PDRF, adiponectin is known to increase eNOS phosphorylation[7] and reduce ROS production[8] in the vascular wall. Interestingly, the anti-contractile effect of PVAT is lost in patients with metabolic disorders[9] and in rodent models of metabolic syndrome[10]. In pathological conditions, PVAT also contributes to increase the production of reactive oxygen species (ROS) in the aortic wall[8]. Alterations of the eNOS[11] and adiponectin-dependent pathways[8] appear to be involved.

Among strategies that can influence both the adipose tissue and the cardiovascular system, exercise training is highly recommended in obese patients[12]. Exercise training improves the vascular endothelial function in healthy subjects[13] and in obese patients[14]. This is mainly mediated by the beneficial effects of exercise on eNOS[15]. To the best of our knowledge, despite eNOS is expressed in the PVAT[16] and exercise is known to activate this enzyme in the cardiovascular system[15], the effects of exercise training in obese rats on eNOS specifically expressed in the PVAT have never been challenged. In this study, we determined, using a rat model of obesity, the impact of exercise training on eNOS expressed in the PVAT and its consequences on vascular function.

2. MATERIALS AND METHODS

2.1. Animal model

All animal experiments were performed according to the European Parliament Directive 2010/63/EU (N° CEEA-00322.03) and approved by the local research ethics committee (n°3487). Male Wistar rats (200-225g) were randomly assigned into three groups: controls (Ctrl; N=24) fed with standard diet (A04, SAFE, France), rats fed with high fat/high sucrose diet (230 HF, SAFE, France, completed with 10% sucrose in drinking water) for 15 weeks, exercised (HFS-Ex; N=22) or not (HFS; N=24) from the 6th week to the end of the experiments at 50-60% of their expected maximal aerobic velocity (1 hour once per day, four times a week) using an incremental protocol previously described by Ko et al.[17]. At the end of the study, the efficiency of exercise training in the HFS-Ex group was confirmed by the higher maximal aerobic velocity, measured as described[18], in HFS-Ex rats compared to sedentary (HFS: 25.20±1.46 m.min^{-1}; HFS-Ex: 39.50±0.29 m.min^{-1}; $p<0.05$).

2.2. Isolated thoracic aortic rings

Isolated aortic rings procedure was performed as previously described (51). Briefly, aortic rings with PVAT or endothelium removed or not, were mounted on stainless steel connected to an isometric force transducer (EMKA Technologies, EMKA Paris, France). Endothelium integrity was confirmed by adding acetylcholine (ACh, 10µM) to phenylephrine (PE, 1µM) pre-contracted rings. One dose of potassium chloride (KCl, 60mM) was added to determine maximal contraction. The vascular function was evaluated in different conditions: **1/** the vascular response to norepinephrine (1nM to 10µM) on endothelium denuded aortic rings with intact PVAT was evaluated in presence or not of the NOS inhibitor L-NAME (100µM);

2/ we evaluated in PE (1µM) pre-contracted vessels the effect of ACh (1nM to 100µM) and sodium nitroprusside (SNP, 1nM to 100µM) on aortic rings with or without PVAT; **3/** we evaluated the impact of PVAT conditioned media from the three different groups on aortic rings of Ctrl animals. The PVAT conditioned media was obtained as previously described[19] and transferred (1.5ml) in a 5ml organ bath containing Ctrl aortic rings without PVAT. The endothelium-dependent and -independent vasorelaxation was then evaluated as described above.

2.3. Western blotting

Immunoblotting was performed using standard techniques as previously described[20]. Briefly, proteins from aorta or PVAT homogenates were separated on polyacrylamide-SDS gels and transferred onto PVDF membranes. Membranes were blocked and next incubated at 4°C with primary antibodies against eNOS-$P^{Ser1177}$ (1:500; BD Transduction), eNOS (1:1000 BD Transduction), GAPDH (1:5000 Santa Cruz), adiponectin (1:1000 Cell Signaling), tubulin (1:1000 BD Bioscience). Immunodetection was carried out using ECL Plus system (Luminata™ Forte Western HRP substrate, Millipore Corporation).

2.4. ROS measurement with dihydroethidium (DHE).

Aortic segments with PVAT were embedded in Optimal Cutting Temperature (OCT from Tissue-Tek) and flash-frozen in liquid nitrogen. Frozen sections were covered with 10µM DHE and incubated in a light-protected humidified chamber at 37°C for 5mn. Images were obtained with a fluorescence microscope (OLYMPUS BX60, Excitation: 488nm; emission: 610nm) at the Imaging facility 3A INRA/University of Avignon. TEMPOL (10mM) was used as negative control to confirm that the signals resulted from ROS production.

2.4. Statistical analyses

Data were expressed as the mean ± SEM. For comparison of experimental conditions, the Student's *t* test, analysis of variance (ANOVA) or repeated measures ANOVA followed by the Tukey's adjusted test were used. A value of $p<0.05$ was considered as statistically significant. Statistical analyses were done with the GraphPad Prism software.

3. RESULTS

3.1. Exercise training impact eNOS expression and activation state in the PVAT of obese rats.

Using a rat model of obesity (Table 1) previously characterized by our team[20], we assessed the impact of exercise training on the PVAT. Obesogenic diet increased the mass of aortic PVAT (Figure 1A). This was prevented by exercise training (Figure 1A). We next evaluated the impact of our experimental conditions on eNOS in both the aorta and the PVAT. In aortic wall, HFS diet did not have any significant effect on eNOS level and phosphorylation (Figure 1B). In line with previous work[21], exercise training increased both eNOS level and phosphorylation (Figure 1B). In PVAT, eNOS and P-eNOS were both reduced in HFS rats. Interestingly, exercise training increased these levels in the PVAT of HFS-Ex rats compared to HFS rats (Figure 1B). The vascular effect of NO synthetized by the PVAT is mainly explained by a NO-PKG-adiponectin pathway[19]. Thus, we next evaluated the impact of HFS diet with or without exercise on PVAT adiponectin level. HFS diet reduced the level of adiponectin in the PVAT compared with Ctrl animals. Yet, exercise increased adiponectin level in the PVAT of HFS-Ex compared to HFS rats (Figure 1C). Considering that in obesogenic environment, ROS production in the aortic wall is closely related to adiponectin level and to NO bioavailability[22], we evaluated the impact of HFS diet with or without exercise training on ROS production in the aortic wall. Quantification of ROS production

using the superoxide indicator DHE in aorta with PVAT showed that ROS production in HFS-Ex aorta samples was significantly reduced compared with HFS (Figure 1D). Altogether, those results show that exercise training is able to impact the eNOS both in the arterial wall and in the PVAT. Such effects could probably have vascular functional consequences.

3.2. Vascular functional effects of PVAT: impact of exercise training in obese rats.

The anticontractile effect of PVAT in response to various vasoconstrictor agonists[5] is mainly dependent on eNOS activation[16]. Thus, we first evaluated how the effect of exercise on PVAT eNOS was able to modulate the vasoconstrictive response of the vascular wall to norepinephrine. We used endothelium-denuded aortic rings with PVAT to test the contractile response to norepinephrine in presence or not of the eNOS inhibitor L-NAME. L-NAME did not influence the aortic contractile response in Ctrl and HFS groups (Figure 2A and B). The maximal response to norepinephrine was reduced in HFS-Ex compared with HFS (HFS: 106.0±4.6% vs HFS-Ex: 93.7±5.5%; $p<0.05$). Moreover, in HFS-Ex L-NAME increased the response to norepinephrine by 23% (Figure 2C) and abolished the difference between HFS and HFS-Ex (HFS-L-NAME: 105.6±3.7 vs HFS-Ex-L-NAME: 115.7±6.8, NS). These results suggest a pivotal role for eNOS in the PVAT anticontractile impact of exercise training in HFS rats. Thus, we next evaluated how the presence of PVAT left intact around aortic rings affect the endothelium-dependent vasodilation to ACh as PVDR can also impact the endothelial function[23]. PVAT has no effect on the maximal response to ACh in Ctrl and HFS aorta (Figure 2D and 2E). Conversely, in HFS-Ex rats, the presence of PVAT increased the maximal response to ACh by 26% (Figure 2F). The maximal response to SNP was increased in presence of PVAT but to the same extent into the 3 groups (Figure2D, E and F). Thus, PVAT is able to modulate both vascular smooth muscle and endothelial reactivity but the beneficial effects of exercise appeared obvious only on the endothelial function. We next

tested the endothelium-dependent vasodilation of Ctrl aortic rings incubated with the conditioned media obtained from the PVAT of the 3 groups (Figure 2G) to distinguish the effects of exercise on the PVAT from those on the arterial wall. The PVAT conditioned media of HFS rats reduced the vascular response to ACh compared to the conditioned media of Ctrl animals. This deleterious effect was lost with the conditioned media of exercised HFS rats (Figure 2H). Altogether, our data strongly support that exercise training altered the eNOS-NO pathway in the PVAT of obese rats with subsequent impact on functional vascular parameters.

4. DISCUSSION

The aim of this work was to evaluate the impact of exercise training on eNOS in the PVAT of obese rats and its consequence on the vascular function. We observed that exercise training was able to modulate eNOS both in the aortic wall and in the PVAT, leading to an improvement of the vascular function, partially explained by the effect of exercise training on PVAT.

PVAT is a key trigger of vascular dysfunction in obese mice[11] and in patients with type 2 diabetes[12]. We reported here that exercise training is able to reduce PVAT mass as it does on other adipose tissue. More interestingly is the impact of exercise training on the functional state of eNOS in the PVAT. Indeed, NO derived from eNOS in the PVAT is a strong candidate for PDRF[16]. The impact of exercise training on the eNOS-NO pathway in the vascular endothelium is mainly dependent of shear stress. In the PVAT, how exercise is able to impact the expression and the functional state of this enzyme is less evident. Several studies have demonstrated a link between β_3-adrenoreceptor-mediated signalling and eNOS activation in the adipocytes[24] and more particularly in the PVAT[15]. In the heart, eNOS activation by exercise is dependent of β_3-adrenorecepetor stimulation by catecholamines[25]. In

addition, exercise training increases the level of β_3-adrenoreceptor[25], which contributes to potentiate NO synthesis in response to adrenergic stress. Conversely, obesity or type2 diabetes is known to reduce the level of β_3-adrenoreceptor[2,26]. The β_3-adrenorecepetors appear then as potential candidates to explain the opposite effect of HFS diet and exercise on eNOS in the PVAT. Further studies will be needed to evaluate this hypothesis and to decipher the underlying mechanisms.

Among the candidate for PDRF, adiponectin is also under the spotlight[23]. Indeed, in the adipocytes surrounding the vasculature, eNOS-PKG dependent synthesis of adiponectin is known to activate eNOS in the vascular wall[23] and to modulate ROS production[27]. In our model exercise training increases both eNOS and adiponectin levels in the PVAT, and reduces ROS production in the vascular wall. In addition, the anticontractile effect of PVAT, obvious only in HFS-Ex aorta in absence of the endothelium, appears to be dependent of eNOS expressed in the PVAT. This suggests that an interaction between eNOS and adiponectin in the PVAT could explain the beneficial effects of exercise on the vascular wall in HFS rats. Our work suggests then that exercise is able to impact eNOS expression and phosphorylation at the level of PVAT. However, the potential role of weight loss cannot be excluded since exercise training in our model of obese rats is associated with reduced body mass (HFS: 720±42g; HFS-Ex: 585±15g, $p<0.05$) Indeed, it has been previously reported that weight loss in obese rats normalized the level of eNOS in the PVAT[16], that could contribute to the restoration of PVAT anticontractile capacity. Further studies will be needed to decipher how exercise training is able to directly impact eNOS in the PVAT and to identify the signalling pathway involved.

To conclude, we demonstrate that PVAT can be considered as a pivotal player in the beneficial effects of exercise on the vascular function in obese animals. In addition to its

effect on PVAT mass, exercise training also modulates the adiponectin-eNOS pathway and ROS production in the aortic wall. Altogether, these elements could contribute to the beneficial effects of exercise training on vascular function.

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TABLE 1

	Ctrl rats	HFS rats	HFS Ex rats
Body mass (g)	628.1±15.9	720.2±42.5*	585.8±15.5#
Food intake (g/rat/day)	24.2±0.7	16.8±0.7*	15.1±1.0*
Fluid intake (ml/rat/day)	33.2±1.7	66.9±4.6*	73.9±5.4*
Visceral fat (g)	24.9±2.4	62.2±4.8*	39.9±4.0#
Fasting glycemia (mg/dl)	116.5±3.2	141.1±6.2*	119.8±5.1#
Fasting insulinemia (µg/l)	2.67±0.46	4.00±0.34*	3.45±0.43
AUC-IPGTT	28165±1618	40825±929*	34566±1866#
HOMA-IR	12.89±2.42	22.83±1.44*	19.01±2.74
Triglycerides (mmol/l)	0.41±0.03	0.81±0.06*	0.56±0.05#

Table1: Impact of HFS diet with or without exercise training on obesity, food and fluid intakes and metabolic disorders in rats. AUC-IPGTT: area under curve of intraperitoneal glucose tolerance test; HOMA-IR: Homeostasis model assessment estimated insulin-resistance**.**

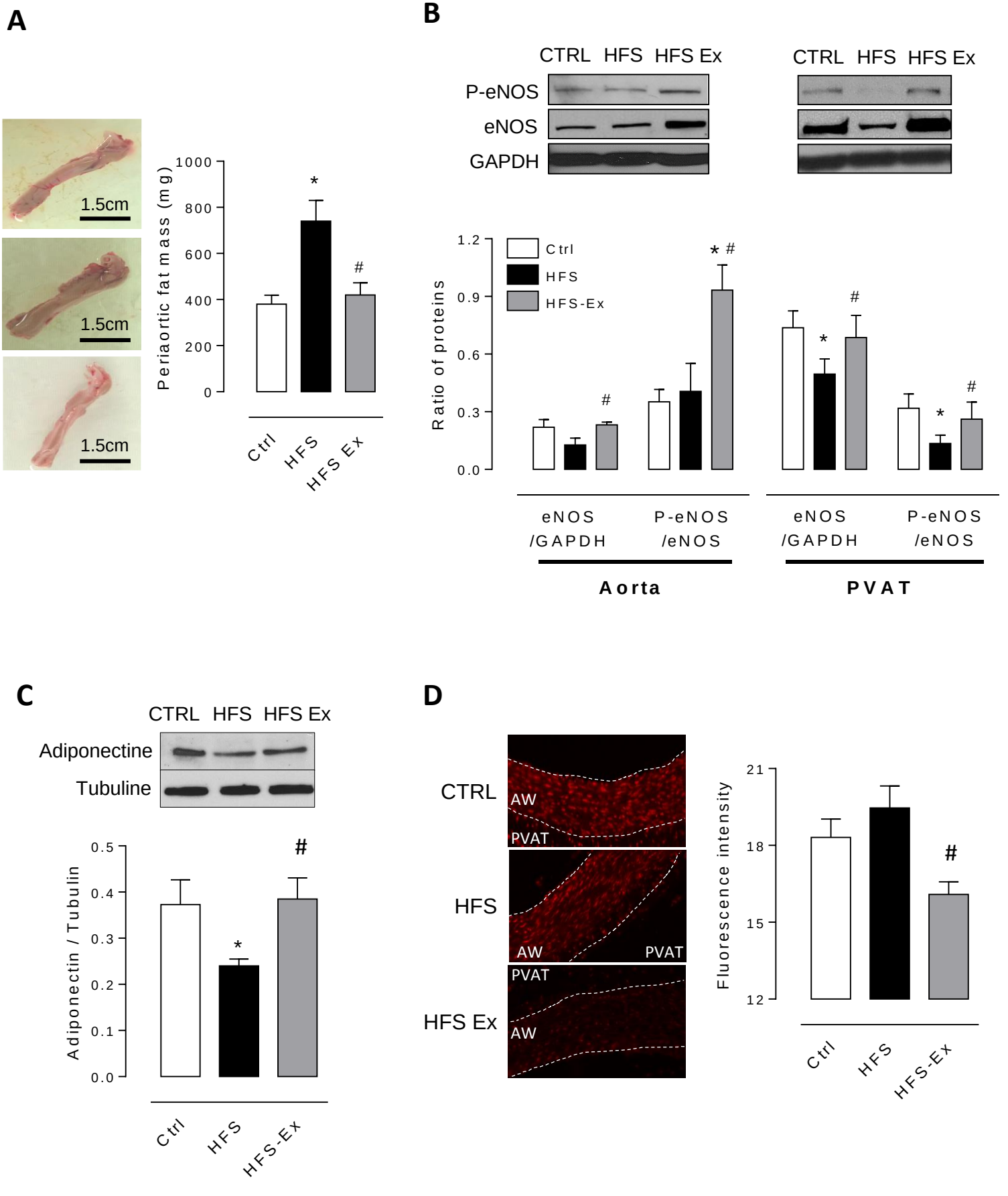
All data are the mean ± SEM. Control (Ctrl, n=12), high fat/high sucrose diet without exercise (HFS,,n=12), high fat/high sucrose diet with exercise training for 9 weeks (HFS-Ex, n=12); *, $p<0.05$ vs control group, #, $p<0.05$ vs HFS group (ANOVA followed by the Tukey's multiple comparison post-hoc test).

FIGURES LEGENDS

Figure 1: Beneficial impact of exercise training on eNOS level and phosphorylation in the PVAT is associated with increased adiponectin level in the PVAT and reduced reactive oxygen species in the vascular wall. (A) Left panels: Representative images of thoracic aorta samples with perivascular adipose tissue (PVAT) in Ctrl, HFS and HFS-Ex rats. Right panels: Periaortic fat mass at the end of the study in Ctrl, HFS and HFS-Ex rats (N=6-8 rats/group). (**B**) eNOS expression (eNOS) and phosphorylation at Ser 1177 (P-eNOS) measured in aorta (left panel) or PVAT (right panel) by western blotting (N=9-10 rats/group). eNOS is expressed relative to GAPDH content and P-eNOS relative to eNOS content. (**C**) Adiponectin expression measured in PVAT by western blotting (N=5-6 rats/group). Adiponectin is expressed relative to tubulin expression. (**D**) Reactive oxygen species production in aortic wall with PVAT was assessed by dihydroethidium fluorescence (N=22-34 sections/group). Dashed lines delineate the aortic aortic wall (AW). *, $p<0.05$ vs CTRL group, #, $p<0.05$ vs HFS group (two-way ANOVA followed by Tukey's multiple comparison post-hoc correction). All data are the mean ± SEM.

Figure 2. PVAT contribute to the beneficial effects of exercise training on vascular function. Dose-response curves to norepinephrine (Nor) in endothelium denuded aortic rings with intact surrounding PVAT, treated or not with N(ω)-nitro-L-arginine methyl ester (L-NAME), obtained in **(A)** Ctrl (N=7 aortic rings), **(B)** high fat/high sucrose (HFS) (N=9 aortic rings) and **(C)** HFS-exercised (HFS-Ex) (N=9 aortic rings) rats. Dose-response curves to acetylcholine (ACh) and maximal response to SNP in aortic rings from (**D)** Ctrl, (**E**) HFS or (**F**) HFS-Ex rats, with intact surrounding PVAT ((+)PVAT) or not (N=8 aortic rings/group/condition). (**G)** Schematic illustration of conditioned media obtained from the

PVAT of Ctrl, HFS and HFS-Ex rats and transfer in organ baths containing a CTRL aortic rings. **(H)** Dose-response curves to ACh in control aortic rings exposed to the conditioned media of Ctrl, HFS or HFS-Ex PVAT. For each graph, N=8 Ctrl aortic rings/condition. Maximal response to SNP obtained in the same conditions are presented respectively on each dose-response figure. *, $p<0.05$ vs Ctrl group, #, $p<0.05$ vs HFS group (two-way ANOVA followed by the Tukey's multiple comparison post-hoc test). All values are the mean ± SEM.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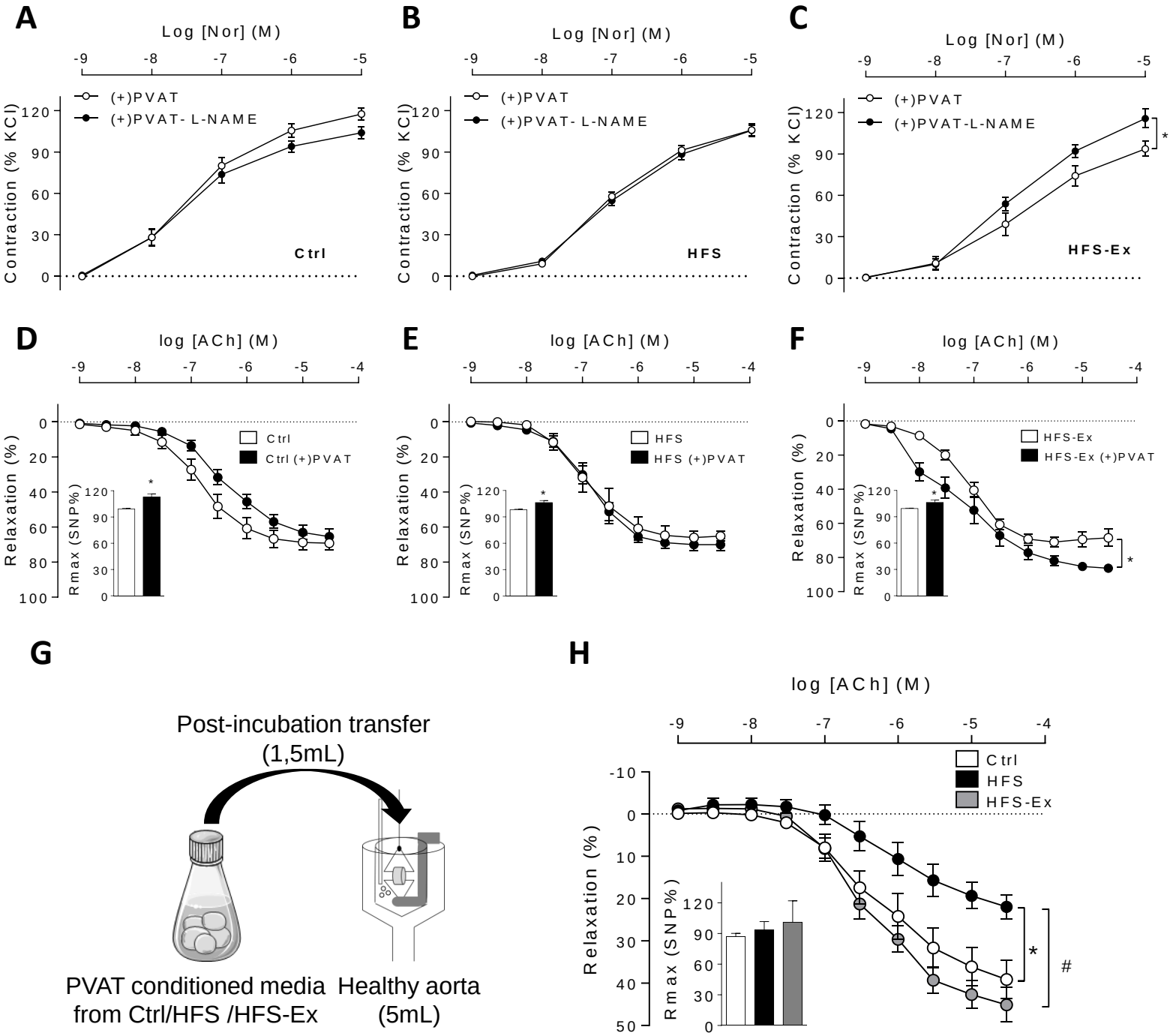
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