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Effets bénéfiques de l'exercice physique sur la fonction vasoactive du PVAT de rats obèses.

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Exercise training impacts perivascular adipose tissue in obese rats: beneficial consequences on vascular function.

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Article en préparation

RÉSUMÉ D'ARTICLE

Contexte scientifique

Depuis quelques années, un nouvel acteur impliqué dans la régulation de la fonction vasculaire est apparu : le tissu adipeux périvasculaire (PVAT). En condition physiologique, celui-ci semble participer au maintien de l'homéostasie vasculaire grâce notamment à des échanges de molécules vasoactives avec les cellules de l'endothélium et du muscle lisse vasculaire (Lohn et al., 2002 ; Wojcicka et al., 2010). Cependant, en situation pathologique, comme dans le cadre de l'obésité ou du syndrome métabolique, ce tissu adipeux semble participer à l'installation de dysfonctions vasculaires (Ketonen et al., 2010 ; Marchesi et al., 2009). L'exercice physique est connu pour être une stratégie préventive et thérapeutique intéressante dans le traitement des pathologies cardiométaboliques (Roque et al., 2013). En effet il permet, en augmentant la biodisponibilité du NO, de prévenir ou d'améliorer la fonction vasculaire chez des sujets sains comme chez des sujets souffrants de désordres métaboliques (Maiorana et al., 2003). Plus récemment, des travaux ont démontré que l'exercice physique est capable de modifier le phénotype et le métabolisme du tissu adipeux, réduisant ainsi ses effets délétères sur la physiologie cardiovasculaire (Thompson et al., 2012). Néanmoins, aucune étude à ce jour ne s'est intéressée aux effets de l'exercice physique sur le PVAT dans le cadre de pathologies métaboliques. **Ainsi, au cours de cette étude, nous avons évalué, chez un modèle de rats syndromes métaboliques, les effets de l'exercice physique sur la biocommunication entre le PVAT et la fonction vasculaire. Une attention toute particulière a été portée sur le couple stress oxydant / eNOS..**

Méthodologie

Des rats males Wistar ont été soumis à un régime riche en lipides et en sucre (HFS : *high fat high sucrose*) pendant 15 semaines. Après 6 semaines, les rats ont été assignés aléatoirement au groupe de rat sédentaire (HFS) ou entraînés (HFS-Ex). Des tests de vasoréactivité de l'aorte ont été réalisés sur ces animaux au terme du régime, en présence ou non de PVAT autour du vaisseau. Dans un deuxième temps, nous avons évalué l'influence du secretome des PVAT de rats contrôles (Ctrl), HFS et HFS-Ex, sur des anneaux d'aortes saines. Afin de caractériser ce PVAT, des coupes histologiques ont été réalisées permettant de visualiser le phénotype du PVAT, et d'évaluer la production d'espèces oxygénées réactives

par fluorescence. Enfin, l'expression protéique de la thermogénine (UCP-1), de l'adiponectine, de la eNOS ainsi que sa phosphorylation (Ser 1177), a été mesurée au sein du PVAT et du secretome. L'expression et la phosphorylation de la eNOS ont été quantifiées au niveau du tissu artériel.

Résultats majeurs

Au terme du régime, la masse et la « brunisation » du PVAT des rats HFS avait tendance à être augmentée par rapport au groupe Ctrl. De manière intéressante, l'exercice a permis de normaliser la masse du PVAT et de potentialiser le phénomène de « brunisation » du tissu. Nous avons observé qu'en l'absence de PVAT, le régime HFS avait tendance à altérer la relaxation liée à l'endothélium, alors que lorsque le PVAT était présent autour de l'artère HFS, sa sensibilité à l'ACh était augmentée. L'exercice physique a permis de potentialiser la relaxation endothélium-dépendante et la sensibilité du muscle lisse vasculaire au monoxyde d'azote, et ceci était encore plus marqué en présence du PVAT. De manière intéressante, le sécrétome du PVAT d'animaux HFS sédentaire est à l'origine d'une dysfonction endothéliale marquée alors que ce phénomène n'est pas observé chez le sécrétome du PVAT d'animaux HFS-Ex. Il est de plus intéressant de noter que l'exercice physique permet de prévenir les effets délétères du régime HFS sur la voie adiponectine/eNOS et de limiter la production d'ERO dans la paroi vasculaire. **Cette 3ème étude de mon travail de doctorat, a permis de montrer que l'exercice physique permet de prévenir les modifications des propriétés vaso-actives du PVAT liées à un régime HFS. Ce phénomène pourrait être expliqué par une amélioration du statut oxydant de la paroi artérielle, et à une potentialisation de la voie adiponectine/eNOS par l'exercice physique.**

Exercise training impacts perivascular adipose tissue in obese rats: beneficial consequences on vascular function.

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Running Head: Exercise training alters perivascular adipose tissue effect in obesity.

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Abstract

Background: The perivascular adipose tissue (PVAT), a new key regulator of vascular function, seems beneficial in healthy arterial tissue but deleterious in subjects with metabolic disorders. Exercise training is widely known to modulate vascular tone, and has also been demonstrated to impact adipose tissue biology. Here we evaluated for the first time the impact of exercise training on PVAT of obese rats and its potential consequences on vascular function.

Methods: Male Wistar rats were fed with high fat high sucrose diet (HFS) for 15 weeks. After 6 weeks rats were randomly assigned into two groups: sedentary or exercised group (HFS ex).

Results: We reported increased PVAT mass in HFS rats associated with a tendency to increase both white to brown adipocytes ratio and uncoupling protein 1 level. Interestingly, exercise training normalized PVAT mass and markedly increased this browning process. In HFS rats, no deleterious effect of PVAT was observed on ACh response of aortic rings. However, when the secretome of the PVAT of HFS animals was tested on healthy aortic rings strong deleterious effects appeared. In exercised HFS animals this phenomenon was totally blunted. Interestingly, the improvement of the aortic response to ACh, obvious in free PVAT aortic rings, was potentiated in aorta with PVAT, suggesting a role of PVAT in the beneficial effect of exercise. Finally, we observed that HFS diet resulted altered adiponectin-eNOS pathway in PVAT, whereas exercise training normalized this pathway at the level seen in Ctrl animals and reduced ROS production in the aortic wall.

Conclusion: To conclude, exercise training is able, in obese animals, to restore the adiponectine-eNOS pathway in the PVAT which could contribute to the reduction of ROS production in the aortic wall and to the subsequent increase in vascular endothelial function.

INTRODUCTION

Obesity and its closely associated complications, known as metabolic syndrome, are associated with poor cardiovascular prognosis and constitute then a global healthcare phenomenon. Indeed, obesity has numerous adverse effects on vascular (Steinberg et al., 1996; Walther et al., 2015) and cardiac function (Iacobellis et al., 2002). Especially, vascular dysfunction appears as an early trigger of cardiovascular outcomes (Cai and Harrison, 2000). Despite the underlying biological mechanisms are not fully understood, endothelial dysfunction appears as a key element (Hadi et al., 2005). Indeed, increased inflammatory processes as well as nitro-oxidative stress observed in those populations, negatively impact the endothelial function (Heitzer et al., 2001; Hamilton et al., 2013) and especially the ability of the endothelium to synthetize nitric oxide (NO) by the endothelial NO synthase (eNOS) (Forstermann et al., 2006).

Recently, a new potential player of the vascular dysfunction is under the spotlight, the perivascular adipose tissue (PVAT). PVAT has been recently recognized as an active endocrine and paracrine organ (Lee et al., 2011; Weston et al., 2013) and emerges as a potential key regulator of vascular function. Its localization, close to the smooth muscles layer of most of systemic blood vessels made him a potential potent regulator of vascular function, like the endothelium in its own time. Indeed, PVAT is able to secrete some relaxing factor, named PVAT-derived relaxing factors (PVRF), that modulate the vasoconstrictive response to adrenergic stress (Dubrovska et al., 2004; Takemori et al., 2006), by both endothelium-dependent and independent mechanisms (Wojcicka et al., 2011; Gao et al., 2007). Interestingly, this anti-contractile effect is lost in subjects with metabolic disorders. This is obvious in humans (Greenstein et al., 2009) as well as in rodents (Marchesi et al., 2009). In such pathological states, PVAT even contributes to increased ROS production in the aortic wall (Antonopoulos et al., 2015; 2015; Xia et al., 2016) and to reduce vascular function.

Dysfunctional state of eNOS (Xia et al., 2016) and alteration of adiponectine-dependent pathways (Antonopoulos et al., 2015) appears implied.

Thus PVAT could be considered as an interesting target to prevent or correct vascular dysfunctions in such pathological populations. Among strategies well described to impact both adipose tissue and the cardiovascular system, exercise training is recognized as efficient and is highly recommended in patients with metabolic disorders for the prevention and rehabilitation of cardiovascular diseases (Thijssen et al., 2010). Indeed, exercise training is well known to improve vascular endothelial function in healthy subjects (Green et al., 2011) as well as in some pathologies (Pedersen et al., 2006). This seems to be mainly due to the beneficial effects of exercise on eNOS level and activation state (Zhang et al., 2009; Farah et al., 2013). Exercise also impacts the adipose tissue. Indeed, its effects on lipid metabolism (Berg et al., 1994; Teixera Lemos et al., 2009), adipocytes size and lipid content reduction (Gollisch et al., 2009; Thompson et al., 2012) are well described. In addition, exercise training is also associated with a browning of white subcutaneous adipose tissue, and thus with increased of its metabolic rate (Sutherland et al., 2009; Stanford et al., 2015; Vernochet et al., 2012). To the best of our knowledge, only one study was interested by the effect of exercise on PVAT and reported in healthy rats that exercise training impacted the phenotype of PVAT but has no alteration on its vascular effects (Araujo et al., 2015). The impact of exercise training on the PVAT of obese animals and its consequence on vascular function has never been challenged.

In this study we aim to investigate, using a model of high fat high sucrose diet in rats, whether exercise training is able to modulate the phenotype of PVAT and its impact on the control of vascular function.

MATERIALS AND METHODS

Animal model

All investigations conformed to the European Parliament Directive 2010/63/EU (N° CEEA-00322.03) and were approved by the local research ethics committee (n°84.004).

Male Wistar rats (200-225g) from Janvier (France) were used for this study, housed four by cage under controlled conditions of temperature (21°C ± 1), hygrometry (60% ± 10) and lightening (12 hours a day), with free access to tap water and standard food. After one acclimation week, rats were randomly assigned into three different group: control rats (Ctrl rats; N=55) fed with standard diet (A04, SAFE, France), rats fed with high fat and high sucrose diet (HFS diet, 230 HF, SAFE, France completed with 10% sucrose in drinking water; HFS rats, N=37) for 15 weeks , and HFS exercised rats fed with HFS diet and exercised from the 6^{th} week of the diet to the end of the experiments (HFS-Ex; N=32). The exercise program consisted in treadmill running once per day, four times a week for 9 weeks at 60% of their maximal aerobic velocity (MAV) for one hour.

Preparation of isolated thoracic aortic rings

After the diet ended, rats were anesthetized (sodium pentobarbital, 120 $mg.kg^{-1}$, i.p.) and thoracic aorta was quickly removed and placed in cold Krebs-Henseleit bicarbonate buffer (composition in mM: NaCl 118; $NaHCO_3$ 25; Glucose 11; KCl 4.8; KH_2PO_4 1.2; $MgSO_47H_2O$ 1.1; $CaCl_2$ 1.25). In some experiments, PVAT surrounded was excised ((-) PVAT) or not ((+) PVAT), and aorta was cut into 2-3mm thicks The aortic rings were mounted into stainless steels supports and suspended in the tissue bath containing Kreb-Henseleit buffer at 37°C continuously bubbled with O_2-CO_2 gas mixture (95%-5%). The rings were connected to an isometric force transducer (EMKA technologies, EMKA Paris, France), and linked to an amplifier (EMKA technologies, EMKA Paris, France), and a

computer acquisition system to record changes in isometric force. After equilibration for 45mn, the intactness of endothelium was confirmed by acetylcholine (ACh, 10µM) in phenylephrine (PE, 1µM) pre-contracted vessels. More than 70% vasorelaxation induced by ACh was considered as no injury of the endothelium. Data acquisition was performed using IOX (EMKA technologies, EMKA Paris, France).

Vascular reactivity

In the first series of bioassay experiments (protocol 1), PVAT was left intact around aortas, and endothelium was removed mechanically by inserting a roughened stainless-steel wire into the lumen. Following the integrity test, one dose of potassium chloride (KCl, 60mM) was applied to determine maximal smooth muscle contraction. Then, vascular contraction was assessed by the contractile response to norepinephrine (Nor) (1nM to 10µM), on aortic rings incubated with NO synthase inhibitor Nω-nitro-L-arginine methylester hydrochloride (L-NAME, 100µM) ((+) L-NAME) or not ((-) L-NAME). Vascular tension was expressed as a percentage of the steady-state tension (100%) induced by KCl.

In the second series of bioassay experiments (protocol 2), endothelium-dependant and independent relaxation in PE (1µM) pre-contracted vessels were assessed by measuring the dilatory effect of ACh (1nM to 100µM) and sodium nitroprusside (SNP, 1nM to 100µM) respectively, on aortic rings (+) or (-) PVAT. The vascular dilatation was expressed as a percentage of maximum response to PE.

In the third series of bioassay experiments (protocol 3), we replaced 1.5mL of the three different PVAT-incubated solution (secretome) in a donor bath chamber to a healthy (Ctrl, (-) PVAT) aorta in an acceptor chamber (Fig 3A). Then we performed a cumulative concentration-dependent response to ACh (1nM to 100µM) in PE (1µM) pre-contracted aortic

rings. To investigate the part of oxidative stress induced by PVAT secretions, we incubated aortic rings with N-acetylcysteine (NAC, 10µM) (secretome-NAC) or not.

In the fourth series of bioassay experiments (protocol 4), we replaces 1.5mL of each secretome (Ctrl/HFS/HFSEx) in a donor bath chamber to respective aorta ((-) PVAT) in an acceptor chamber. Next, we performed a cumulative concentration-dependent response to ACh (1nM to 100µM) in PE (1µM) pre-contracted aortic rings.

Biochemical analyses

Histological sections

Formalin-fixed PVAT samples were processed through parafin embedment, sectioned at five microns, stained with hematoxylin and eosin for morphometric determinations. Sections were examined with a TM300 microscope associated with a system of digitization of image (Nikon camera, DXM1200) at X10 magnification.

Western Blot

Immunoblotting of total and phosphorylated eNOS (Ser 1177), Adiponectine, uncoupling protein 1 (UCP-1), was performed using standard techniques. Briefly, proteins from aorta and PVAT homogenates were separated on polyacrylamide-SDS gels and transferred onto PVDF membranes at 100V for 1h30. Membranes were blocked for 1h with 10% milk or 3% bovine serum albumine in Tris-buffered saline containing 0.05 % Tween-20 overnight. Then membranes were incubated with primary antibodies at 4°C (eNOS-PSer 1177 1:500; BD transduction; eNOS, 1:1000 BD Transduction, GAPDH, 1:5000 Santa Cruz; Adiponectin, 1:1000 Cell Signaling; Tubulin, 1:1000 BD Bioscience; UCP1, 1:300 Santa Cruz). 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.

Proteomic profiler evaluation

Capture and control antibodies have been spotted in duplicate on nitrocellulose membranes. PVAT lysates are diluted, mixed with a cocktail of biotinylated detection antibodies, and incubated overnight with the Proteome Profiler Rat Adipokine Array®. After washes, Streptavidin-HRP and chemiluminescent detection reagents are applied, and a signal is produced at each capture spot corresponding to the amount of protein bound. Membranes were exposed to X-ray films for revelation.

ROS measurements with fluorescent dye dihydroethidium (DHE).

Aortic segments with PVAT left intact surround were embedded in Optimal Cutting Temperature compound (OCT from Tissue-Tek) and flash frozen in liquid nitrogen. Frozen sections (14 µm thick) was covered with 10µM DHE on top, incubated in a light-protected humidified chamber at 37°C for 5mn, and a cover slip was applied. Slides were viewed with a fluorescence microscope (OLYMPUS BX60, Excitation: 488nm; emission: 610nm). Images were analysed using the software Image J.

Statistical analyses

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

RESULTS

Exercise training reduces HFS diet induced obesity and metabolic disorders.

At the end of the 15 weeks of HFS diet visceral fat was increased (2.5 times) in HFS rats compared with Ctrl, whereas no changes in total body weight has been observed (Table 1). Blood glucose as well as blood insulin measured after overnight fasting was markedly higher in HFS than Ctrl rats (Table 1). In addition, blood glucose remained higher throughout the 2-hours IPGTT, as indicated by the higher area under the curve (Table 1). However, the Homeostatic Model Assessment of Insulin Resistance score (HOMA-IR) only tended to be higher in HFS than in Ctrl animals without reaching significance (Table 1). HFS diet resulted also in a 2 times increase in blood triglycerides (Table 1). The efficiency of exercise training in the HFS-Ex group was first confirmed by the higher maximal aerobic velocity in HFS-Ex rats than in sedentary HFS animals (HFS: 25.20±1.46 m.min-1; HFS-Ex: 39.50±0.29 m.min-1; $p<0.0001$). Exercise training reduced body weight (table 1), that was explained by the lower visceral fat accumulation in HFS-Ex rats (Table 1). Exercise training also reduced the difference between Ctrl and HFS rats concerning fasting blood glucose and insulin but the difference between HFS and HFS-Ex rats did not reach significance (Table 1). However, the glycemic response to IPGTT in HFS rats was significantly reduced by exercise training (Table 1). Finally, exercise training markedly reduced blood triglycerides (Table 1). Altogether, these results indicate that 1) HFS rats are a reliable model of visceral obesity associated with its body of metabolic disorders and that 2) exercise training performed in HFS rats was able to prevent or reduce many features of obesity.

Exercise reduced PVAT mass and increased its brownisation..

In line with results obtained on visceral adipose tissue, HFS diet increased PVAT mass (Figure 1A and B). This was associated with a tendency to increase brown adipose tissue, as

highlighted by the higher number of multilocular adipocytes in HFS PVAT compared to Ctrl ones (Figure 1C). In accordance with this, the level of uncoupling protein 1 (UCP1), used as a marker of thermogenic respiration, tended to be increased in the PVAT of HFS compared to Ctrl ones but did not reach significance (Figure 1D). Exercise in HFS rats (HFS-Ex group) normalized PVAT mass at level seen in Ctrl (Fig 1A and B). In addition, the slight increase in brown adipose tissue seen in PVAT of HFS rats was exacerbated in HFS-Ex as shown by the significant increase of small multilocular adipocytes (Figure 1C) and confirmed by the significant increase of UCP1 level in PVAT of HFS rats (Figure 1D). Those results contribute to show that exercise training is able to impact PVAT as it does on other adipose tissue (Thompson et al., 2012).

Exercise training altered the role of PVAT on vascular function.

It has been previously reported that obesity in mice resulted in a PVAT-dependent alteration of endothelium response to ACh (Xia et al., 2016). Thus, we challenged whether our experimental conditions and their effects on PVAT could affect the endothelium-dependent and independent relaxation of aortic rings. In PVAT free aortic rings, the vasorelaxant response to ACh tended to be reduced in HFS compared to Ctrl ones (Figure 2A). This was obvious regarding maximal response to ACh (Rmax Ctrl: 69.14±4.00%; HFS: 55.21±6.89%; p=0.07). However, no effect was observed when we tested the endothelium-independent vascular smooth muscle cells sensitivity to NO with increasing dose of SNP (Figure 2B). In line with previous work (Lozano et al., 2016; Jenkins et al., 2016), these results strongly supported that HFS diet was associated with endothelium-dependent vascular dysfunction (Figure 2A and B). Next we performed the same experiments but in aorta with PVAT. It has been previously reported that PVAT exacerbates the vascular dysfunction observed in obese mice (Xia et al., 2016). Unexpectedly, in our model the dose-response curve to ACh was

shifted leftward in HFS aorta when compared to Ctrl rats (Figure 2C), meaning of higher sensitivity of aortic rings to ACh (EC_{50} Ctrl: $7.12e^{-07}$±$1.69e^{-07}$M; HFS: $1.67e^{-07}$±$3.26e^{-08}$M, $p<0.05$). The same results were obtained regarding the endothelium-independent response to SNP (Figure 2D), which strongly supports that in presence of PVAT vascular smooth muscle cells sensitivity to NO was improved. In this work, we especially aimed to study whether exercise training is able to modulate the impact of PVAT on vascular function. In line with the literature (Touati et al., 2011), endothelium-dependent relaxation was improved in HFS exercised rats when compared to sedentary HFS ones. Indeed, despite no effect of exercise was observed on the response to SNP (Figure 2B), endothelium-dependent response to ACh was tended to be increased (Figure 2A). This was obvious regarding maximal response (HFS: 55.21±6.89%, HFS Ex: 68.42±5.06%, p=0.07) as well as EC50 (HFS: $1.97e^{-7}$±$5.98e^{-8}$M, HFS-Ex: $9.60e^{-8}$±$2.08e^{-8}$M, p=0.06). When we performed the same experiments but in aorta with PVAT, we reported that the beneficial effect of exercise on response to ACh was exacerbated (Figure 2C). This phenomenon was not observed when we tested the vascular response to SNP (Figure 2D). Our results can be explained by the effects of our experimental conditions on both the PVAT, the aortic wall and their interactions. Thus, to discriminate the impact on PVAT from those on aortic wall, we next evaluated the impact of PVAT secretion, named secretome, from each experimental populations on healthy aortic rings without PVAT (Figure 3A). We first observed, in line with previous work (Lee et al., 2014), that ACh-vasorelaxation was lowered when performed in presence of the secretome of healthy PVAT (Rmax without Secretome: 69.14±4.00% vs Rmax with Secretome: 39.13±4.48%, P=0.0004). Since, the response to SNP remained preserved (Rmax without PVAT: 84.85±2.88% vs Rmax with PVAT-Secretome: 86.99±3.17%,P=0.6), we can conclude that the effects of the secretome were mainly due to endothelial dysfunction. Interestingly, these effects were strongly exacerbated when healthy aortic rings were incubated with the secretome of HFS

PVAT. Indeed, ACh response of healthy aortic rings incubated with the secretome of HFS rats was markedly lower than those obtained with the secretome of Ctrl rats (Figure 3B). Finally, the effect of exercise training on the PVAT of HFS rats was able to prevent this phenomenon, since in presence of secretome of HFS-Ex rats, the aortic response to ACh was normalized at level seen in Ctrl conditions (Figure 3B). Because no effect of the secretome was observed on the response to SNP whatever the experimental conditions (Figure 3C), we can firmly stipulate that such effects were endothelium-dependent. Altogether those results shown that despite higher sensitivity of vascular smooth muscles to NO in HFS aorta with PVAT, in this group of rats this specific adipose tissue released some deleterious compounds that could have negative effects on healthy arteries. This strongly support the idea of compensatory mechanisms at the level of the aorta in HFS rats. Interestingly exercise training prevented the deleterious effects of PVAT secretion on arterial function and potentiate the vascular response to ACh in aorta with PVAT.

Exercise training impact the adiponectin-eNOS pathway and ROS production in PVAT. In arterial tissue, vasodilatation is mainly mediated by eNOS-NO dependent vascular smooth muscle relaxation (Ignarro et al., 1999). The eNOS is largely express in the vascular endothelium, but recently has also been observed in PVAT (Xia et al. 2016; Gil-Ortega et al., 2010). Next, we measured the impact of our experimental conditions on eNOS level and phosphorylation on its main activation site serine^{1177} (eNOS-P). In PVAT free aorta, HFS diet only tended to reduce eNOS level (P=0.12; Suppl Figure 1) and has no effect on eNOS-P. In line with previous work (Davis et al., 2003; Dimmeler et al., 1999), exercise training increased both eNOS level and eNOS-Pser1177 (Suppl Figure 1).We next focused our biochemical assay on PVAT. Interestingly, as evidenced by western blot analyses, HFS diet reduced both eNOS level and eNOS-P in PVAT (Figure 4A). Exercise training in HFS rats

totally blunted this phenomenon (Figure 4A). The role of eNOS of the endothelium in the regulation of vascular tone is largely described in the literature (Ignarro et al., 1999; Zhao et al., 2015), however, whether eNOS expressed in the PVAT could impact the vascular function has never been clearly identified. To test this, we challenged in endothelium denuded aortic rings with PVAT the contractile response to norepinephrine in presence or not of L-NAME to inhibit eNOS of the PVAT. No specific effect of L-NAME was reported in Ctrl and HFS aorta, suggesting that eNOS-NO pathway from the PVAT did not contribute directly to modulate aortic response to norepinephrine (Figure 4B and C). In contrast, in HFS-Ex aortic rings, the maximal response to norepinephrine was reduced when compared to HFS (Rmax HFS: 106.00±4.66 %, HFS-Ex: 93.70±5.52 %; $P<0.05$). Moreover, L-NAME increased the response to norepinephrine (Figure 4D). In line with the effects of exercise training on eNOS in the PVAT, these results strongly suggest that eNOS from the PVAT of HFS-Ex animals contributed to modulate vascular smooth muscle cells contractile response to adrenergic agonists. Altogether those results contribute to show that eNOS was clearly impacted by our exercise training not only in the endothelium but also in the PVAT. Subsequently, eNOS from the PVAT became efficient to modulate vascular tone.

Despite we reported here some effects of PVAT on vascular smooth muscle cells function, we have shown above that the deleterious effects of the PVAT secretome was obvious only when we challenged the endothelium-dependent vasorelaxation (Figure 3B). When we performed a proteome profile of adipokines secreted by the PVAT we observed that HFS diet have no strong impact on the level of different adipokines (Suppl Figure 2), and that exercise tended to reduce the production of almost all adipokines (Suppl Figure 2). Among the adipokine known to interact with eNOS, adiponectine has been largely studied and shown to activate the eNOS-NO pathway in the cardiovascular system (Hattori et al., 2003; Chen et al., 2003). In addition, it has been reported in type 2 diabetic human that reduced adiponectine

level in PVAT increased ROS production in the arterial wall and thus contribute to the development of vascular dysfunction (Antonopoulos et al., 2015). Interestingly, in our model, when we measured the level of adiponectine by western blot in the PVAT, we reported exactly the same pattern than those observed on eNOS level and phosphorylation. Indeed, in line with the results of Antonopoulos et al. (Antonopoulos et al., 2015), adiponectine level was reduced in HFS PVAT when compared to Ctrl PVAT (Figure 4E). A key result of this work was that exercise training was able to normalize the level of adiponectine in HFS PVAT at level seen in Ctrl PVAT (Figure 4E). Those results were also obvious when biochemical assay were performed on PVAT secretome (Suppl Figure 3). Considering the close relationship between adiponectine availability and ROS production in the arterial wall (Nour-Eldine et al., 2016), we evaluated the level of ROS production by DHE staining in aorta with PVAT. HFS diet tended to increase ROS production, however differences did not reach significance (Figure 4F). Exercise training markedly reduced ROS production in the aortic wall (Figure 4F). Altogether those results reported that exercise training is able, in obese animals, to restore the adiponectine-eNOS pathway in the PVAT which could contribute to the reduction of ROS production in the aortic wall and then to increased vascular endothelial function.

DISCUSSION

The aim of this work was to evaluate the impact of exercise training on PVAT of obese rats and its potential consequences on vascular function. We observed here using a model of HFS diet in rats that exercise training was able to normalize the adiponectine-eNOS pathway in aorta of obese rats, and thus have beneficial effects on vascular function.

PVAT has been previously shown as a key trigger of vascular dysfunction in obese mice (Xia et al., 2016) and type 2 diabetics subjects (Antonopoulos et al., 2015). In our model of high fat and high sucrose diet in rats, we observed yet an improvement of vascular response to ACh in aorta with PVAT, which is explained by higher sensitivity of the VSM to NO in presence of the PVAT. Thus, this result appears contradictory with the scientific literature. However, when we tested the impact of PVAT secretome of HFS animals on healthy aortic rings, clear deleterious effects appeared. Thus, it seems likely that in our model of HFS rats compensatory mechanisms, involving a non-explored communication between smooth muscle cells layer and PVAT, contribute to normalize vascular function during the development of the metabolic pathology. Such discrepancies between our work and the literature could be explained by differences regarding the development of the metabolic diseases. Indeed, our model of HFS diet in rats is surely associated with visceral obesity, however metabolic disorders were not at a late stage of the disease. For example, body mass was not significantly different between HFS and Ctrl rats and no insulin-resistance was obvious. In addition, in our model, PVAT remodelling is characterized by some features of browning adipose tissue, whereas with more prolonged diet and a later stage of the metabolic diseases, a marked whitening of the PVAT has been reported with deleterious effect on vascular function (Ketonen et al., 2010). This reinforce the idea of an early stage of the pathology in our model. Indeed, excessive calories intake results first in an adaptive process named diet-induced thermogenesis (Rothwell et al., 1979). This process contributes to increase energy expenditure and is explained by increased UCP-1 level in the adipose tissue. Such a tendency was also observed in the PVAT of our model, since we observed that HFS diet tended to increase the level of mutlilocular adipocytes and of UCP1 in the PVAT. It seems then that the whitening of the PVAT tissue is observed only when the excess in calories intake is more prolonged. Thus, it is of main interest to note that, at an early stage of the pathology, deleterious vascular

effects of PVAT secretion were counteracted by compensatory high VSM sensitivity to NO. This suggest that PVAT was impacted by metabolic pathology earlier than the vascular function, and constitute then an early trigger of future vascular disorders. However, further studies are needed to confirm this hypothesis.

Considering this potential forerunner role of PVAT in the development of vascular dysfunction in metabolic diseases, it could constitute an interesting therapeutic target. Among the strategy well known to impact the adipose tissue, exercise training is known as really efficient (Boulé et al., 2001; Mourrier et al., 1997). Indeed, exercise training results in an increased level of beige adipocytes and then of the metabolic rate of white adipose tissue. Beige cells differed from white adipocytes because of their higher metabolic rate and UCP 1 protein level (Enerback et al., 2009; Petrovic et al., 2010). We reported here, in line with previous studies, that exercise training has comparable effect on the PVAT, characterized by reduced adiposity, browning and increased UCP1 level. However, whether the effects of exercise on PVAT have an impact on vascular function remains not clear. In pigs fed with high fat diet, exercise increases anticontractile effect of PVAT but has no effect on relaxing function (Reifenberg et al., 2007). Araujo et al. reported in healthy rats that exercise training impacted the phenotype of PVAT but with no consequences on vascular function (Araujo et al., 2015). In our work, we show for the first time that, in HFS rats, PVAT contributes to the beneficial effects of exercise training on vascular function. Indeed, the improvement of the endothelium-dependent vasodilatation was more obvious in presence of PVAT in HFS-Ex aorta. We also reported that eNOS from the PVAT contribute to blunt the contractile response to NE, independently of the endothelium. Finally, we observed that exercise modulates the release of deleterious vasoactive compounds as observed with the PVAT of HFS rats. PVAT exhibit a heightened proinflammatory state and reduced adipocytic differentiation under basal

conditions. It seems to be highly sensitive to the effects of high-fat feeding, which causes further reductions in adipocytes-associated gene expression. This profil let thinking that PVAT could be play a major role in development of vascular inflammation, which could contribute to atherosclerotic lesion development (Chatterjee et al., 2009).. In our model, except for adiponectin, no major effects of the diet was observed on other adipokines. However, exercise training reduced the level of all adipokines and increased the level of adiponectin. The effects of exercise on adiponectin in the adipose tissue is relatively well described (Bruun et al., 2006; Berggren et al., 2005). Indeed, in obese subjects exercise increases plasma adiponectin and its release by the adipose tissue (Wang et al., 2015). However, to the best of our knowledge, this is the first time that this phenomenon is observed in the PVAT. This is of importance since, reduced adiponectin release by the PVAT has been previously shown as a key trigger of vascular dysfunction in type 2 diabetic subjects (Antonopoulos et al., 2015). The role of adiponectin on the vascular tissue is classically explained by its ability to activate the eNOS-NO pathway (Hattori et al., 2003; Xi et al., 2005) and to reduce ROS production (Motoshima et al., 2004). Interestingly, in our model, exercise training is able to prevent the reduction of eNOS and eNOS-P in PVAT of HFS rats and also to reduce ROS production in the wall of aorta with PVAT. Altogether, those results help us to understand why the PVAT secretome of HFS-Ex was less deleterious for the endothelial function that the one of sedentary HFS animals. However, the role of the eNOS expressed in the PVAT on the control of vascular function is not clear. In obese mice, PVAT eNOS is reported to contribute to global arterial oxidative stress, mainly due to its uncoupling state (Xia et al., 2016). However, in our model no difference in ROS production between Ctrl and HFS aorta was observed. This could be due to the early stage of the metabolic disease in our model, as discuss above. Thus, whether eNOS in the PVAT contribute or not to modulate the vascular function in Ctrl and HFS animals remains not clear. However, when the HFS animals

are exercised, the inhibition of eNOS expressed in the PVAT increased the contractile response to NE in endothelium-denuded aortic rings with PVAT, which clearly emphasis the role of PVAT eNOS on the regulation of vascular tone. These results associated with the beneficial effects of exercise training on adiponectin-eNOS pathway contribute certainly to explain why the beneficial effects of exercise training were more obvious when the PVAT surrounded the aorta.

To conclude, we reported here that PVAT can be considered as an effective player of the beneficial effects of exercise on vascular function in obese animals. Indeed, in addition to its effect on the phenotype of the PVAT, exercise training also impact the adiponectin-eNOS pathway and also the ROS production in the aortic wall. Altogether, those elements could contribute to the beneficial effects of exercise training on vascular function. This work highlight the PVAT as a potential early trigger of vascular dysfunction in metabolic diseases and that exercise training constitute an efficient strategy to impact both vascular and PVAT eNOS-dependent signalling.

Disclosures

No authors reports conflict of interest

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	Control rats	HFS rats	HFS Ex rats
Body mass (g)	639.0±12.3	640.6±20.5	554.6±17.0 #
Visceral fat (g)	24.9±2.4	62.2±4.8 *	39.9±4.0 #
Fasting glycemia (mg/dl)	116,5±3,2	137,1±6,9 *	119,8±5,1
Fasting insulinemia (µg/l)	2.67±0.46	3.66±0.46 *	3.45±0.43
AUC-IPGTT	28165±1618	40825±929 *	34566±1866 #
HOMA-IR	14.72±2.85	20.72±2.47	19.01±2.74
Triglycerides (mmol/l)	0.41±0.03	0.81±0.06 *	0.56±0.05 #

Table: Exercise enhances endothelium-dependant relaxation of aorta. AUC-IPGTT: area under curve intraperitoneal glucose tolerance test; HOMA-IR: Homeostasis model assessment estimated insulino-resistance. Data are presented as Mean ± SEM, *, $p<0.05$ vs control group, #, $p<0.05$ vs HFS group.

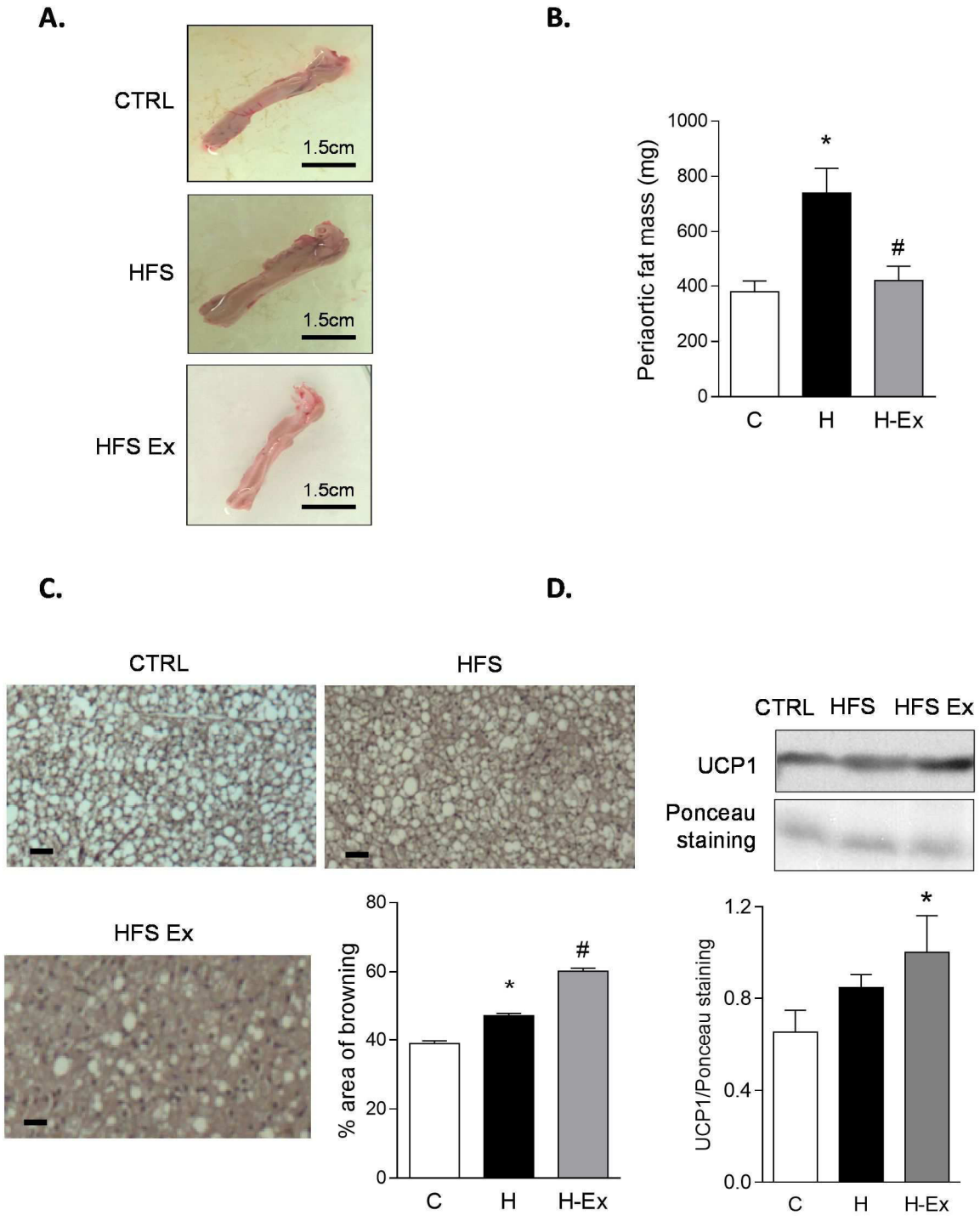


Figure 1. Exercise reduced PVAT mass and increased browning. (A) Representative pictures of aorta with PVAT from Ctrl, HFS and HFS-Ex rats. (B) Periaortic fat mass at the end of the protocol. (C) Light microscopic appearance of perivascular adipose tissue. Tissues were harvested from Ctrl, HFS and HFS-Ex rats, stained with haematoxylin-eosin, sectioned and photographed (magnification X10). Graph represent the percentage of browning area. (D) UCP1 expression measured in PVAT lysates by Western immunoblotting. Scale: 100µm. Data are presented as Mean ± SEM, *, $p<0.05$ vs control group, #, $p<0.05$ vs HFS group.

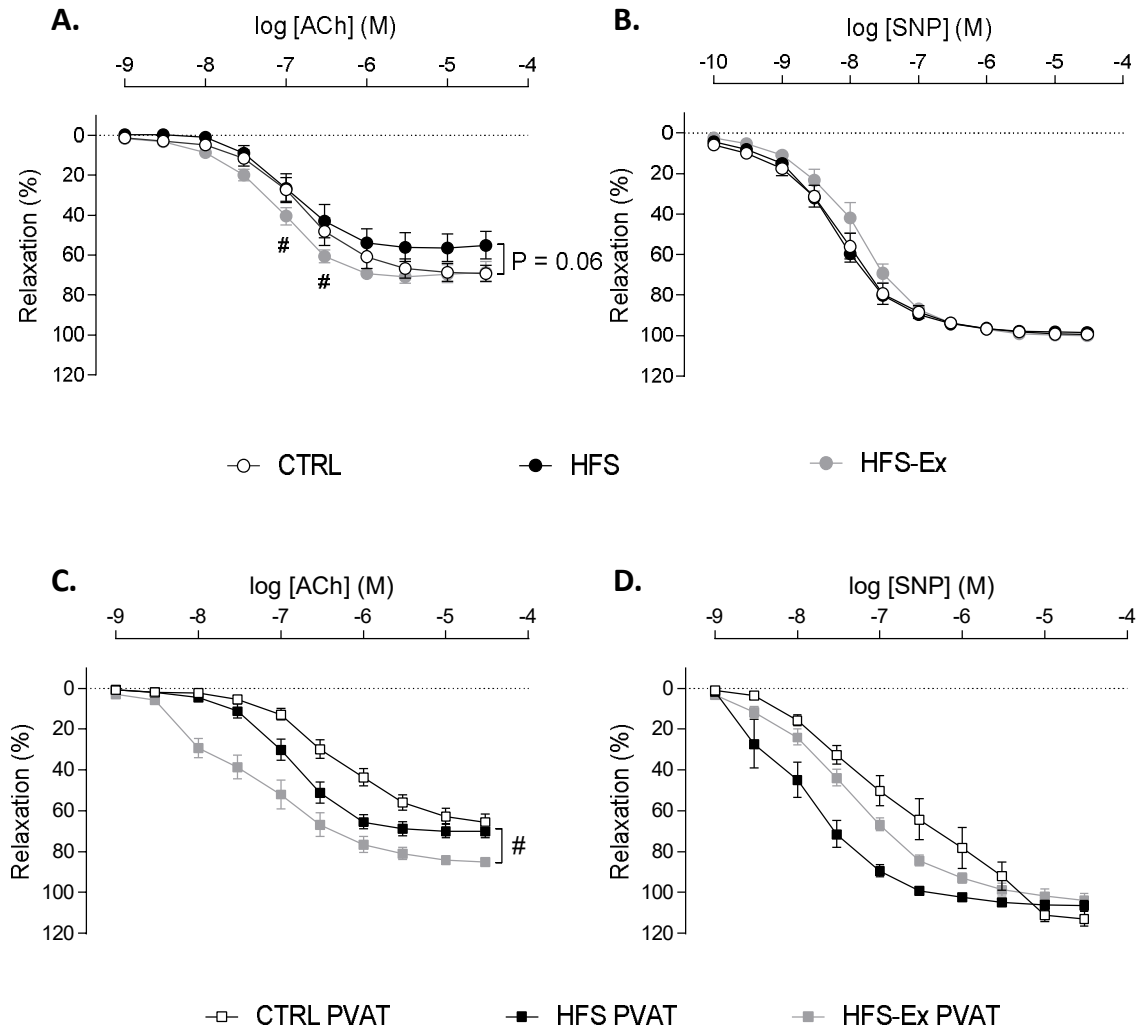


Figure 2. Exercise training altered the role of PVAT on vascular function. Dose-response curves to acetylcholine (A and C), and to sodium nitroprusside (B and D), in aortic ring preparations without PVAT (A and B), and intact aortic ring preparations (C and D). Data are presented as Mean ± SEM, #, $p<0.05$.

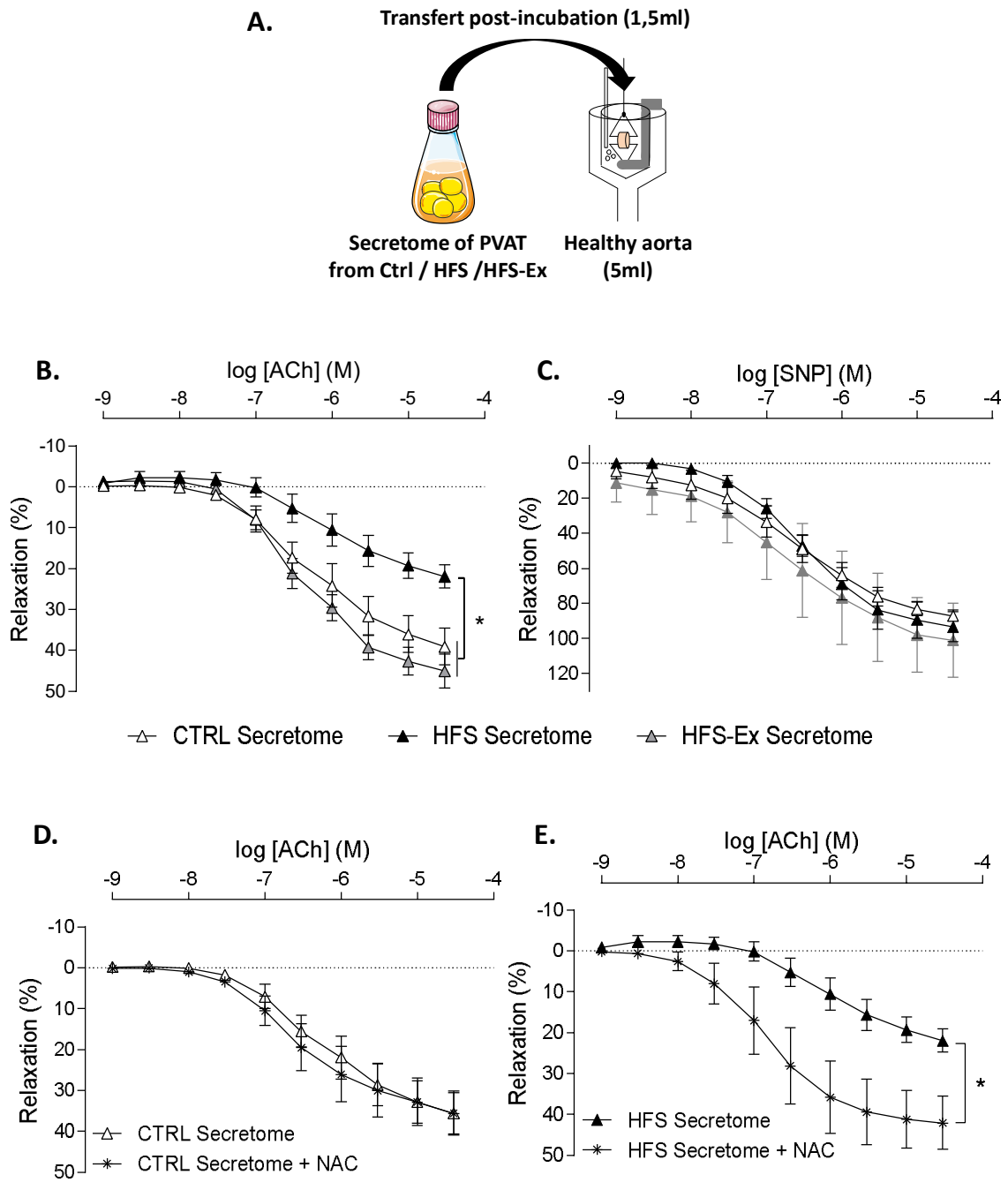
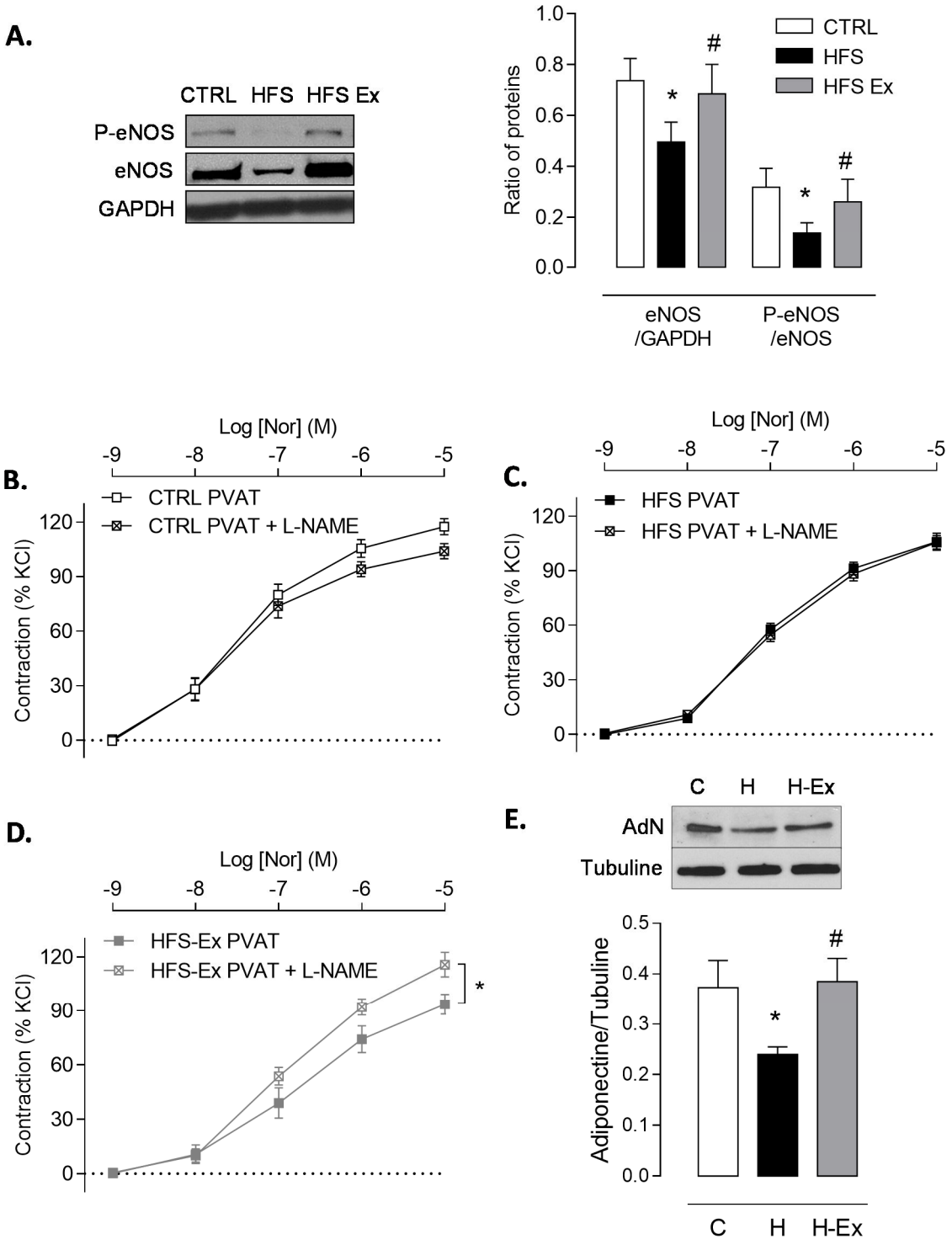


Figure 3. PVAT from exercise rats have enhanced antioxidant properties. (A) Schematic illustration of the bioassay experiments. Secretome (1.5mL) of different PVAT incubated for 30 minutes at 37°C were transferred to healthy aortic preparations without PVAT in a closed acceptor bath chamber. Dose response to acetylcholine (B) and to sodium nitroprusside (C) in healthy aortic rings without PVAT, with effect of Ctrl, HFS or HFS-Ex secretome. Dose response to acetylcholine on healthy aortic rings without PVAT, incubated with Ctrl-secretome (D) or HFS-secretome (E), pre-treated with N-acetylcystein or not. Data are presented as Mean ± SEM, *, $p<0.05$.


A.
CTRL HFS HFS Ex
P-eNOS
eNOS
GAPDH
Ratio of proteins
CTRL
HFS
HFS Ex
eNOS /GAPDH
P-eNOS /eNOS
B.
Log [Nor] (M)
CTRL PVAT
CTRL PVAT + L-NAME
Contraction (% KCl)
C.
HFS PVAT
HFS PVAT + L-NAME
D.
HFS-Ex PVAT
HFS-Ex PVAT + L-NAME
E.
C H H-Ex
AdN
Tubuline
Adiponectine/Tubuline

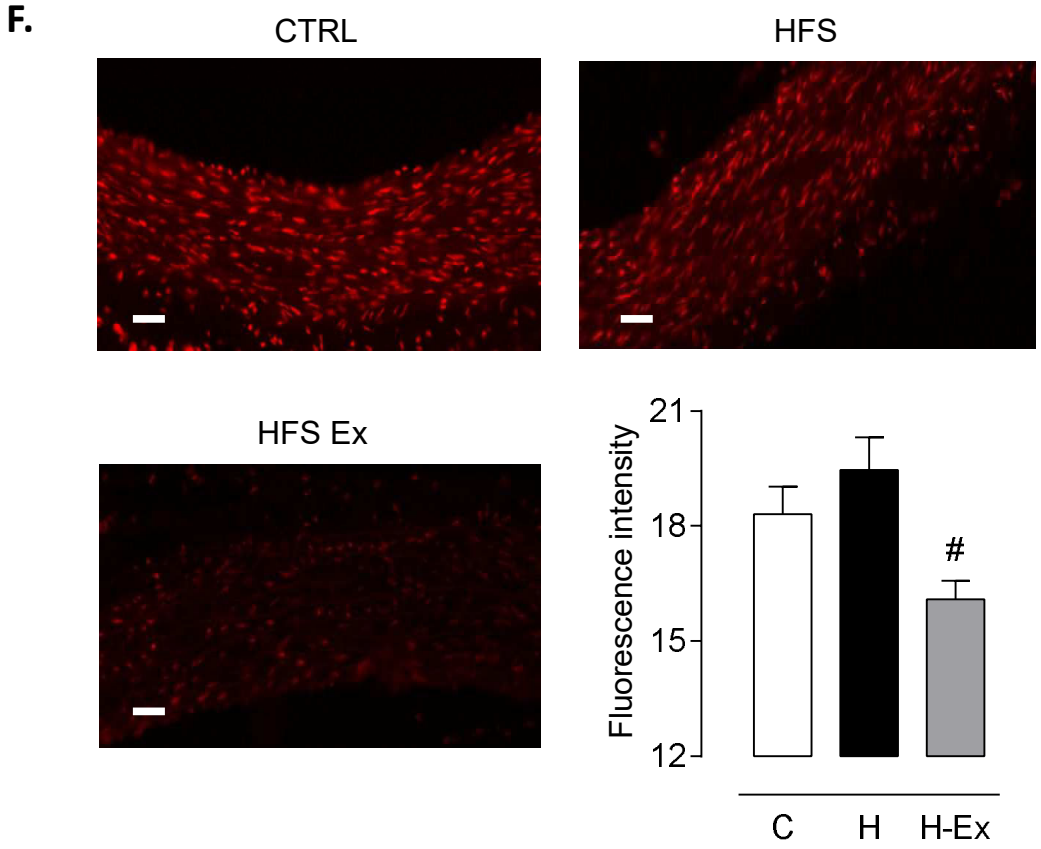
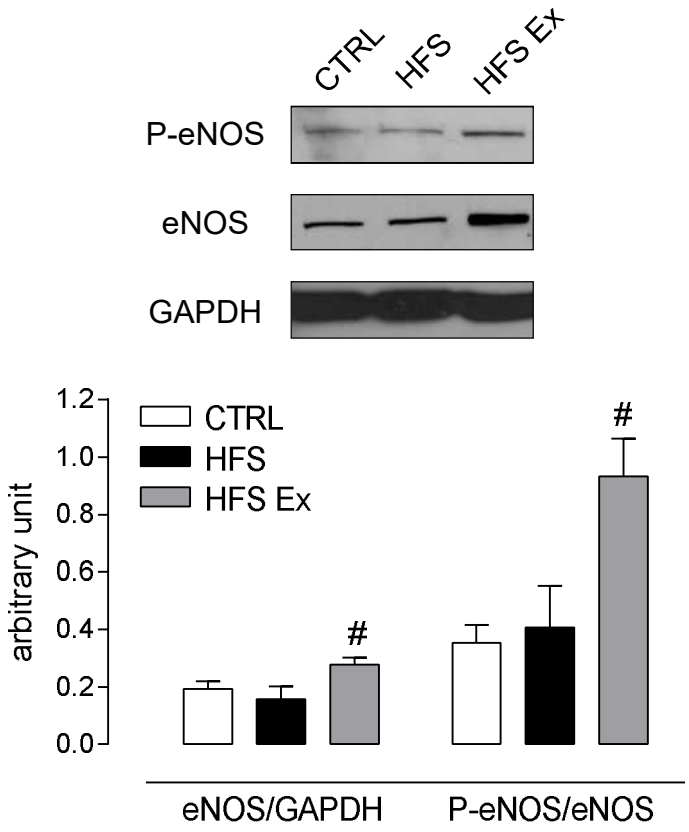
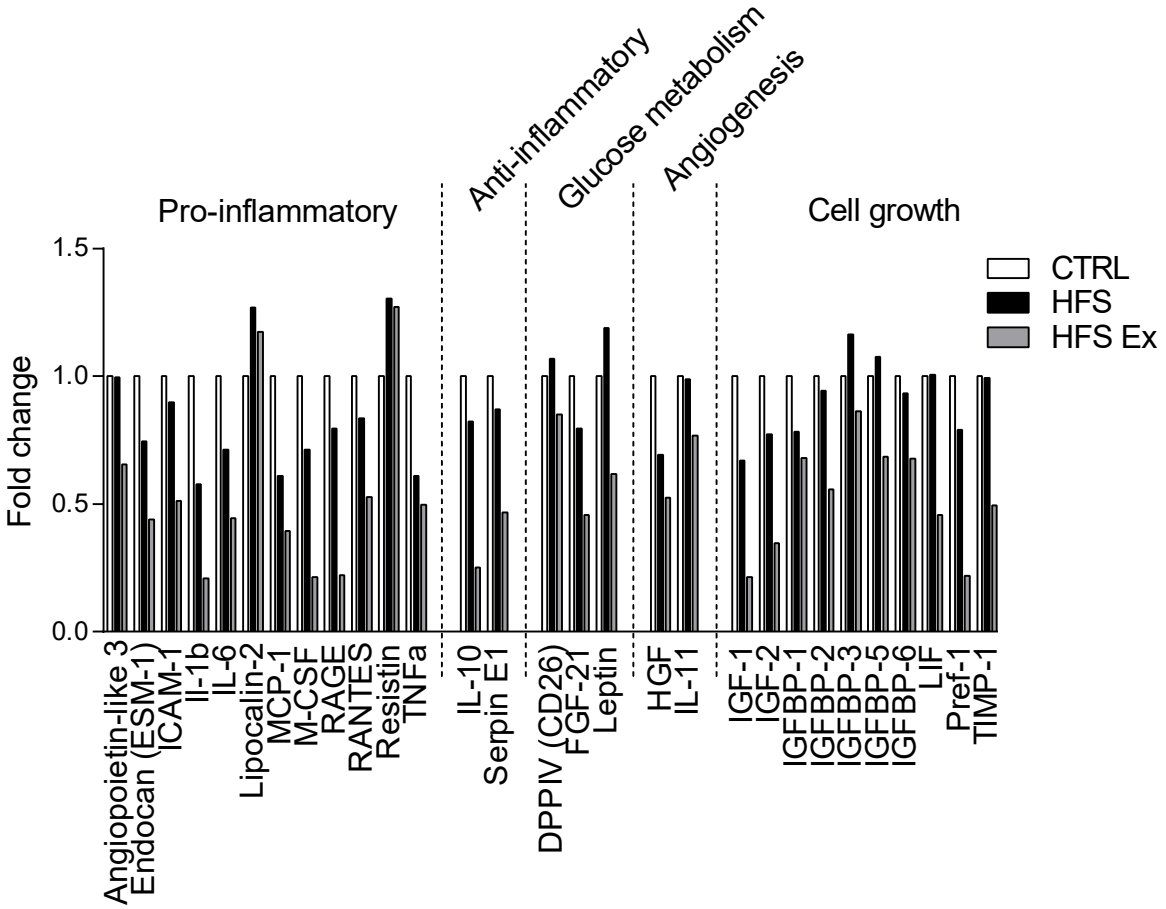


Figure 4: Exercise training impact the adiponectin-eNOS pathway. A. eNOS expression and eNOS phosphorylation at Ser 1177 (P-eNOS) measured in PVAT tissue lysates by Western immunoblotting. Dose response to noradrenaline on Ctrl (B), HFS (C), and HFS-Ex (D) aortic rings with PVAT surrounded, pre-treated or not with L-NAME. (E) Adiponectin expression measured in PVAT tissue lysates by Western immunoblotting. (F) ROS production in aortic wall with PVAT surrounded, assessed by DHE staining in Ctrl, HFS and HFS-Ex rats. Scale: 100µm. Data are presented as Mean ± SEM, *, $p<0.05$ vs control group, #, $p<0.05$ vs HFS group.

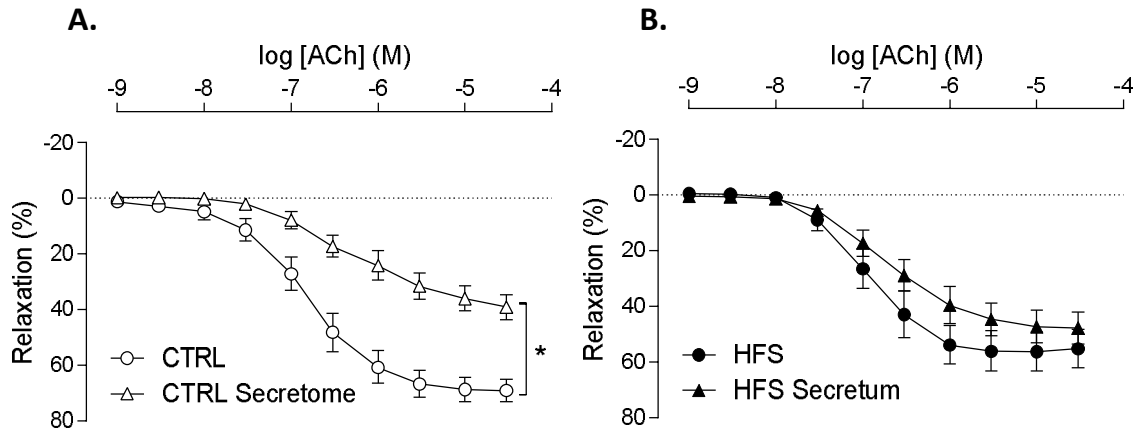
Supplemental Data



Supplemental figure 1. Exercise improves eNOS expression and activation in aorta. eNOS expression and eNOS phosphorylation at Ser 1177 (P-eNOS) measured in aortic tissue lysates by Western immunoblotting. Data are presented as Mean ± SEM, #, $p<0.05$ vs HFS group.



Supplemental figure 2. Proteomic profile of PVAT. Adipokines profile from different PVAT lysates were measured with Rat Adipokine Array Kit®. Data are presented as fold change to Ctrl group.



Supplemental figure 3. Vascular consequences of PVAT remodelling. Dose response to acetylcholine on Ctrl (A) and HFS (B) aortic rings without PVAT surrounded, incubated or not with their corresponding PVAT-secretome. Data are presented as Mean ± SEM, *: $p<0.05$.