Etude n°II : La dysfonction endothéliale chez les sujets obèses : implication des microparticules circulantes ?

Role of eNOS and NOX containing microparticles in the endothelial dysfunction in patients with obesity

Saloua DIMASSI, Karim CHAHED, Soumaya BOUMIZA, Matthias CANAULT, Zouhair TABKA, Pascal LAURANT, Catherine RIVA.

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But de l'étude

Le but de cette étude était, dans un premier temps, d'étudier l'intégrité de la fonction endothéliale chez des sujets obèses par exploration fonctionnelle de l'endothélium et par mesure de différents marqueurs vasculaires et notamment les MPs circulantes. Dans un deuxième temps, nous nous sommes intéressés au rôle de ces MPs sur la fonction endothéliale et à leur contenu, et plus particulièrement en protéines eNOS et NADPH oxydase, afin d'essayer de comprendre leur implication dans la fonction endothéliale au niveau vasculaire dans un contexte d'obésité.

Principaux résultats

Nos principaux résultats nous ont permis d'observer que la population de sujets obèses présentait une augmentation des concentrations plasmatiques des différents marqueurs lipidiques, inflammatoires et du stress oxydant par rapport aux sujets normo-pondérés. De plus, l'exploration de la fonction endothéliale chez ces sujets obèses a

révélé une altération de la réponse endothélium-dépendante. Ces résultats suggèrent une dysfonction endothéliale au sein de la population d'obèses. Toutefois, le taux de nitrites plasmatiques, l'un des principaux métabolites du NO, était élevé suggérant que la biodisponibilité du NO n'était pas altérée au niveau vasculaire.

Par la suite, nous nous sommes intéressés aux MPs circulantes chez les sujets obèses et normo-pondérés. Leur quantification au niveau plasmatique a tout d'abord révélé une augmentation de leurs concentrations plasmatiques chez les sujets obèses. L'étude fonctionnelle de ces MPs nous a permis d'observer que ces MPs n'altéraient pas la fonction endothéliale *ex vivo*. De plus, l'analyse de leur contenu en eNOS a montré que l'activité de cette protéine était augmentée chez les sujets obèses. L'expression de la NOX2, principale protéine impliquée dans le stress oxydant était par contre diminuée et sa fraction cytosolique p47, essentielle à son activité, n'a pas pu être détectée dans les MPs.

Conclusion

D'après nos résultats, nous avons pu observer que notre population de sujets obèses présentait une altération de la fonction endothéliale. L'étude des MPs circulantes a révélé que, même si leurs concentrations plasmatiques étaient élevées chez les sujets obèses. Toutefois, d'après l'étude fonctionnelle *ex vivo* et leur contenu en protéines eNOS et NADPH oxydase, nous avons pu constater que ces MPs n'étaient pas impliquées dans la dysfonction endothéliale observée chez les sujets obèses. De plus, nos résultats sur la biodisponibilité du NO et sur les MPs circulantes suggèreraient que ces MPs sont le reflet d'un mécanisme compensatoire au niveau vasculaire qui tend à

contrebalancer les effets néfastes du stress oxydant et de la dysfonction endothéliale chez ces sujets obèses.

Article

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ROLE OF ENOS- AND NOX-CONTAINING MICROPARTICLES IN ENDOTHELIAL DYSFUNCTION IN PATIENTS WITH OBESITY

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What is already known about this subject? Elevated circulating levels of microparticles have been associated with several diseases and cardiovascular risk factors, such as obesity.

What did the study add? Microparticles do not contribute directly to the endothelial dysfunction associated with obesity. On the contrary, eNOS- and NOX-containing microparticles could play a role in the compensatory mechanism observed in vascular endothelial cells to counteract the pathologic mechanisms underlying obesity-induced endothelial dysfunction.

Abstract:

Objective: To explore the pathophysiological profile of patients with obesity and to investigate the potential role of circulating microparticles (MPs) in endothelial dysfunction in patients with obesity.

Methods: The inflammatory and oxidative status and the cutaneous microvascular blood flow were characterized in 69 patients with android obesity and 46 subjects with normal weight (controls) by using laser Doppler flowmetry. Circulating MP levels were measured by flow cytometry and endothelial nitric oxide synthase (eNOS) and NADPH oxidase (NOX) expression in MPs were investigated by western blotting. MP effect on vascular reactivity was assessed in rat aorta rings.

Results: Patients with obesity showed endothelial dysfunction, hyperglycemia, inflammation and oxidative stress. In controls, low MP levels were positively correlated with normal microvascular function Western blot analysis revealed reduced eNOS and increased NOX4D expression in MPs from subjects with obesity compared with controls. However, this was not correlated with endothelial dysfunction parameters and did not impair *ex vivo* endothelium-dependent vasodilation.

Conclusions: These results suggest that MPs do not contribute directly to endothelial dysfunction associated with obesity. Conversely, eNOS- and NOX- containing MPs could be involved in the compensatory mechanism of vascular endothelial cells to counteract the pathologic mechanisms underlying endothelial dysfunction.

Abbreviation list:

ACh: Acetylcholine

AOPP: Advanced Oxidation Protein Products

ApoA: Apolipoprotein A

ApoB: Apolipoprotein B

BF: Blood flow

BMI: Body mass index

CBF: Cutaneous blood flow

CVC: Cutaneous vascular conductance

CVD: Cardiovascular disease

DBP: Diastolic blood pressure

DTNB: 5'-dithiobis (2- nitrobenzoic acid)

eNOS: Endothelial nitric oxide synthase

FMD: Flow-mediated dilation

GSH: reduced Glutathione

HDLc: High density Lipoprotein cholesterol

HsCRP: high sensitive C-reactive protein

IL-6: Interleukin-6

LDF: Laser Doppler Flowmetry

LDLc: Low density Lipoprotein cholesterol

LSH: Local skin heating

MPs: microparticles

NO: Nitric oxide

NOX: NADPH oxidase

PFP: Platelet-free plasma

PRP: Platelet-rich plasma

ROS: Reactive oxygen species

SBP: Systolic blood pressure

TBARS: Thiobarbituric Acid Reactive Substances

WHR: Waist-to-hip Ratio

Disclosure:

The authors have no potential conflicts of interest

Author Contributions:

S.D. researched data/wrote the manuscript, K.C. reviewed the manuscript, S.B. researched data, M.C. reviewed the manuscript, Z.T. reviewed the manuscript, P.L. reviewed the manuscript, C.R. researched data/wrote/edited the manuscript and is the guarantor of this work and, as such, had full access to all study data and takes responsibility for the data integrity and the data analysis accuracy.

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Introduction:

Obesity is a health problem worldwide. It also is a major risk factor for cardiovascular diseases by predisposing to endothelial alterations (1). Endothelial dysfunction is generally associated with decreased nitric oxide (NO) bioavailability. NO production is catalyzed by endothelial nitric oxide synthase (eNOS), while reactive oxygen species (ROS) negatively regulate NO bioavailability (2). Several enzymes are involved in ROS generation and, among them, the NADPH oxidase represents a major source of vascular superoxide anion (O_2^-) that generates oxidative stress as observed in patients with obesity and type 2 diabetes (3).

Microparticles (MPs) are membranous vesicles released from many types of vascular cells, such as endothelial cells, platelets and leukocytes, in response to cell stimulation, stress or apoptosis (4). Circulating MP composition and function are directly dependent on the characteristics of the cells from which they originate (5). Moreover, MPs could contribute to intercellular communication and may works as vectors of biological messages leading to endothelial and vascular dysfunctions, or platelet activation (6). Elevated circulating MP level has been associated with several diseases and cardiovascular risk factors, such as obesity (7, 8). However, depending on the pathological context and the mechanism of MP formation, MPs could have favorable or deleterious effects on vascular homeostasis (9).

The aim of this study was to investigate MP potential role in the pathophysiology of endothelial function alterations in obesity. First, clinical parameters, oxidative stress and inflammatory status as well as endothelial function (cutaneous microvascular blood flow) were evaluated in young subjects with obesity and in controls with normal weight. Then, the number of circulating MPs and eNOS and NADPH oxidase (NOX) content (two proteins implicated in the development of

endothelial dysfunction and oxidative stress associated with obesity) in MPs from the two groups were determined. Finally, MP influence on the vasoreactivity of rat aortic rings was assessed.

Methods

Subjects

The study, approved by the Farhat Hached Hospital Ethics Committee in Tunisia, included 115 healthy Caucasian subjects (45 males, 70 females) who signed an informed consent before inclusion. Based on their body mass index (BMI), they were divided in subjects with obesity (BMI $\geq$ 30kg/m², n=69) and controls with normal weight (BMI < 25kg/m², n=46). Medical history and physical examination data were collected for all subjects to exclude co-morbid factors. Exclusion criteria were: 1) history of hypertension, antihypertensive medication or elevated blood pressure in sitting position (systolic blood pressure (SBP) $\geq$ 140 mmHg and/or diastolic blood pressure (DBP) $\geq$ 90 mmHg); 2) history of diabetes mellitus or fasting glucose > 7mmol.l^{-1}; 3) history of cardiovascular disease or current cardiovascular procedures; 4) hyperlipidemia (total cholesterol > 6.7 mmol.l^{-1} and/or triglyceride level > 4.5 mmol.l^{-1}); 5) past or present history of smoking; 6) consumption of vasoactive medications or antioxidant supplementation in the past six months. Anthropometric (height, weight, waist and bi-iliac circumference) measurements and the mean arterial pressure (MAP= 1/3 (SBP) + 2/3 (DBP)) were recorded. Fasting glucose, lipids and inflammatory markers (high-sensitivity C-reactive protein, hsCRP, and sedimentation rate) were evaluated in all subjects.

Oxidative stress measurement

Blood samples (n=115) were collected in EDTA tubes after overnight fasting and were centrifuged at 4,000 g for 10 min. Plasma aliquots were immediately removed and stored at -80°C until analysis. Nitrite content was assessed using the colorimetric-based Griess method. Oxidative damage (lipid peroxidation level) was estimated by measuring the plasma levels of ThioBarbituric Acid Reactive Substances (TBARS) and Advanced Oxidation Protein Products (AOPP). The plasma concentration of reduced Glutathione (GSH) was determined with a 5'-dithiobis (2-nitrobenzoic acid) (DTNB)-based enzymatic method.

Endothelial function assessment

Endothelial function was explored in all subjects (n=115) by assessing the forearm microvascular cutaneous vasoreactivity using Laser Doppler Flowmetry coupled with iontophoresis (Periflux System 5000, Perimed, Jarfalla Sweden). Endothelium-dependent vasodilation was evaluated by stimulation with 2% acetylcholine chloride (ACh) (Sigma Aldrich, Switzerland) and endothelium-independent vasodilation, after local skin heating (LSH) (10). Briefly, cutaneous blood flow (CBF) was recorded at rest for 2min and during the functional exploration. Three doses of ACh were delivered using an anodal current (0.1mA for 10s) at 2-min intervals. Finally, the local skin temperature, which was initially maintained at 32°C, was increased to 44°C for 5min (LSH) (Figure 1) (11). Data were expressed as cutaneous vascular conductance (CVC), which represents the ratio between the CBF and MAP values, to take into account variations in blood pressure between subjects (12). The endothelium-dependent response was calculated as the difference between the peak CVC upon ACh stimulation (i.e., the CVC after the third

dose of ACh) and the baseline CVC (ΔACh-CVC). The endothelium-independent response was calculated as the difference between the peak CVC following LSH-induced vasodilation and the baseline CVC (ΔLSH-CVC).

MP preparation and flow cytometry analysis

MP preparation

Citrated blood samples were collected from 46 subjects (14 controls and 32 subjects with obesity) and processed within 2h. Platelet-rich plasma (PRP) was collected after centrifugation at 1,500g for 15min at room temperature (RT). Platelet-free plasma (PFP) was obtained by further centrifugation at 13,000g at RT for 2min, and the MP pellet after ultracentrifugation of the PFP samples at 20,000g, 4°C for 90 min. MP and PFP aliquots were stored at -80°C until analysis (13).

MP quantification by flow cytometry

MP samples were analyzed using an Accuri C6 flow cytometer and software (Accuri Cytometers, Ann Arbor, MI) to define the MP gate and to quantify the absolute numbers of MPs per µl of plasma. Regions corresponding to MPs were identified in forward and side-angle light scatter intensity dot plot representation set at logarithmic gain, based on the diameter using standard microbeads (0.1 and 1.1µm) (14).

Vascular reactivity measurement

Rat aorta rings from 3-month-old Wistar rats were incubated for 5h in Dulbecco's modified eagle medium/Krebs-HEPES solution (117mM NaCl, 74.5mM KCl, 84mM $NaHCO_3$, 1.5mM KH_2PO_4, 1.7mM $MgCl_2$, 21mM HEPES, 11.1mM glucose) in the absence (n=8) or presence of MPs from controls (n=6) or subjects

with obesity (n=7). The final MP concentration corresponded to the MP circulating plasma level. Aorta rings were then mounted in organ chambers filled with Krebs solution warmed at 37°C and continuously aerated with a 95%O_2-5%CO_2 gas mixture. Aorta rings were connected to an isometric force transducer (EMKA Technologies, EMKA Paris, France), linked to an amplifier (EMKA Technologies, EMKA Paris, France) and changes in isometric force were recorded on a computer equipped with a data acquisition system. Resting tension was adjusted to the maximal length tension of 2g and aorta rings were allowed to equilibrate for 1h. Contractions were produced by addition of 10-4 M phenylephrine and the endothelium-dependent response was studied by addition of ACh at different concentrations (5.10-9 to 10-4 M). Finally, 10-5 M sodium nitroprusside (SNP) was used to reach the maximum response and to test the endothelium-independent response (16). MP effect on the ACh-sensitivity of aorta rings was evaluated by calculating the ACh concentration inducing the half-maximal response (EC_{50}). Care and use of laboratory animals were according to the European Community standards.

Western blot analysis

MP pellets from controls (n=6) and subjects with obesity (n=6) were suspended in lysis buffer (25mM Tris pH=7.6, 150mM NaCl, 1% Triton X100) containing phosphatase inhibitor (Fisher Scientific) and protease inhibitor cocktails (Sigma Aldrich). Proteins were transferred to PVDF membranes and probed with mouse monoclonal anti-eNOS (1:300; BD Biosciences) and anti-eNOS-$P^{Ser1177}$ (1:200; BD Biosciences) and goat polyclonal anti-gp91-phox (1:200; Santa Cruz Biotechnology), anti-p47-phox (1:200; Santa Cruz Biotechnology), anti-NOX4 (1:200; Santa Cruz Biotechnology) and rabbit polyclonal anti-αβ tubulin (1:3000; BD Biosciences) antibodies. Immunodetection was carried out using the ECL System

(Super Signal West Pico Chemiluminescence Substrate, Thermo Scientific) followed by exposure to X-ray films for revelation.

Statistical analysis

Data analysis was performed using the SPSS 17.0 software (SPSS Inc, Chicago, IL, USA). Results are expressed as the mean ± SEM. The data normal distribution was tested using the Kolmogorov-Smirnov test. The independent-samples t-test was used after investigation of variances equality by using the Levene's test. Vasoreactivity data were analyzed using a two-way ANOVA and Dunnett's multiple comparisons test. Correlations were obtained using the Pearson correlation test. Statistical significance was inferred when the two-tailed p-value was lower than 0.05.

Results

Population characteristics

This study included 69 subjects with obesity (BMI ≥ 30kg/m²) and 46 controls with normal weight (BMI < 25kg/m²). Anthropometric and biochemical data are presented in Table 1. As age and gender distribution were not significantly different between groups, the results were presented independently of gender.

The waist circumference and waist to hip ratio (WHR) indicated that the subjects with obesity had android obesity. Moreover, the mean fasting glucose concentration exceeded 5.6 mmol/L in subjects with obesity, suggesting a hyperglycemic state (15). Plasma triglycerides and the mean fasting glucose levels as well as SBP, DBP and MAP were higher in subjects with obesity than in controls.

The elevated hsCRP and sedimentation rate values (compared with controls) suggested the presence of chronic inflammation in the subjects with obesity.

Oxidative stress analysis

Plasma TBARS, AOPP and nitrite levels were significantly higher in the group with obesity than in controls (Table 1). Conversely, plasma GSH level (a marker of the anti-oxidative defense status) was significantly lower in subjects with obesity than in controls (Table 1).

Assessment of endothelial function by Laser Doppler Flowmetry

Basal CVC values and after stimulation with ACh (peak ACh-CVC, ΔACh-CVC and AUC) were significantly lower in the group with obesity than in controls (Table 2). Conversely, peak LSH-CVC and ΔLSH-CVC were similar between groups (Table 2). These results indicate that endothelium-dependent, but not endothelium-independent vasodilation is impaired in subjects with obesity.

Relationships between circulating MP levels and endothelial function parameters

Circulating MP number was significantly higher in subjects with obesity (n=32) than in controls (n=14) (Figure 2A). Correlations between circulating MP levels, endothelial function parameters were assessed separately in each group, to highlight the possible role of "good" MPs in controls (normal weight) and of "modified" MPs in subjects with obesity (Table 3). In controls, circulating MP number was significantly and positively correlated with endothelium-dependent response parameters (AUC, peak ACh-CVC and ΔACh-CVC). Conversely, no correlation was found in the population with obesity (Table 3).

Effect of MPs on the *ex vivo* endothelium-dependent response

ACh-induced relaxation in rat aortic rings was not modified by incubation with MPs (used at their mean plasma concentration) from controls (n=6) or from subjects with obesity (n=7) compared with buffer alone (n=8) (Figure 2B). Similarly, MPs (from controls or subjects with obesity) did not influence ACh sensitivity (EC_{50}) and maximum relaxation upon ACh stimulation (Figure 2C). Moreover, MPs did not affect significantly the response to SNP.

Nitric Oxide Synthase and NADPH Oxidase levels in circulating MPs

The levels of eNOS and eNOS phosphorylated on Ser1177 ($P^{Ser1177}$eNOS, activating phosphorylation) were assessed by western blot analysis of crude MP lysates (n=6/each group). Total eNOS expression was significantly lower in MPs from subjects with obesity than in controls (Figure 3A). Conversely, the $P^{Ser1177}$-eNOS to total eNOS ratio was significantly higher in MPs from subjects with obesity than in controls (Figure 3B).

Western blot analysis of the expression of NADPH oxidase isoforms in crude MP lysates showed that the catalytic trans-membrane NADPH oxidase type 2 (NOX2) could be detected as two characteristic bands (a 65 kDa band representing the non-glycosylated form and a broad band with an average size of 110 kDa corresponding to the heavily glycosylated mature form). Total NOX2 expression (65 kDa plus 110 kDa bands) was significantly decreased in MPs from subjects with obesity compared with controls (Figure 3C). The cytosolic p47phox NADPH oxidase subunit could not be detected in any MP sample (Figure 3D). The level of NADPH oxidase type 4 (NOX4) (specific 67 kDa band) was not significantly different between groups (Figure 3E). Finally, expression of the NOX4D variant (32 kDa band) was

significantly higher in MPs from subjects with obesity than from controls (Figure 3F).

Discussion

The main findings of this study are: 1/ endothelial function is impaired and inflammatory markers are elevated in subjects with obesity and hyperglycemia compared with controls (BMI < 25kg/m²); 2/ the number of circulating MP is higher in subjects with obesity, but this does not correlate with *in vivo* endothelial dysfunction parameters; moreover, MPs from subjects with obesity do not influence *ex vivo* endothelial-dependent vasorelaxation; 3/ eNOS and NOX2 levels are reduced, whereas $P^{Ser1177}$-eNOS and NOX4D levels are increased in MPs from subjects with obesity compared with controls.

Our results show that endothelium-dependent vasodilation is altered in young subjects with obesity compared with age-matched controls, suggesting endothelial dysfunction. The pathophysiological mechanisms underlying the link between obesity and microvascular dysfunction are various and complex (16–18). Endothelial dysfunction in resistance vessels evolves progressively, together with the increase in body fat (19). The hyperglycemia we observed in the group with obesity could also be involved in endothelial dysfunction development, as demonstrated by Kawano et al. (20). Moreover, the high hsCRP levels (an inflammatory marker) in the group with obesity suggest a systemic inflammatory state that could further impair endothelial function through inhibition of eNOS gene expression in endothelial cells (21). Many reports suggest that oxidative stress is involved in the pathophysiology of cardiovascular diseases by reducing NO bioavailability and consequently NO

levels (2). In our population with obesity, reduced antioxidant defenses (i.e., GSH) were associated with increased levels of lipid peroxidation (TBARS) and protein oxidation (AOPP) markers. Oxidative damage has been linked to enhanced ROS production in patients with obesity (22). High NO and ROS levels could lead to the production of peroxynitrite, resulting in microvascular function impairment (3). It has been proposed that nitrite anion (NO_2^-), which is abundant in blood and tissues, represents the largest intravascular and tissue storage form of NO. Approximately 80-90% of basal plasma nitrites are derived from eNOS-produced NO, suggesting that nitrites could be used as a endothelial function biomarker (23). In agreement, in our study NO bioavailability (nitrites) was significantly increased in the group with obesity. Our results are consistent with those by Ghasemi et al. (24), who showed increased nitrite/nitrate levels in women with obesity. Fujita et al. (25) also reported higher circulating nitrite/nitrate levels in subjects with obesity due to NO production by visceral fat. Moreover, NO synthetized within a specific location could be transported in blood as nitrites to distant organs, providing protection against ischemic injury (26). Therefore, one can hypothesize that increased levels of NO metabolites in obesity may be a compensatory mechanism against obesity-related changes.

Our study shows that circulating MP levels are significantly higher in subjects with obesity than in controls. However, MP levels were positively correlated with the microvascular response (AUC and ΔACh-CVC) only in the control group. This is different from previous works showing that high MP levels correlate with impaired flow-mediated dilation of the coronary (27) or brachial artery (7). Moreover, we found that MPs from subjects with obesity and from controls did not influence *ex vivo* ACh-induced relaxation, whereas other studies observed an effect using MPs

from patients with myocardial infarction (28). It is generally acknowledged that MP levels are increased in pathological contexts, but their functional characteristics are closely dependent on their biochemical composition. For example, Mostefai et al. (30) observed that MP levels were increased in patients with septic shock compared with healthy controls, but such MPs did not trigger vascular inflammation. On the contrary, they exerted a vascular protective role (29). The absence of effect of MPs from subjects with obesity suggests that such MPs do not carry a "pathological" content. As circulating MPs from subjects with vascular dysfunction originate largely from the endothelium (30), we analyzed eNOS expression and activity in circulating MPs, as a marker of the vessel endothelial cell content. Total eNOS level was reduced in circulating MPs from subjects with obesity compared with controls. Conversely, the eNOS-$P^{Ser1177}$/eNOS ratio was increased in MPs from subjects with obesity, suggesting higher eNOS activation in these MPs than in MPs from controls. Horn et al., (31) also reported the presence of functional eNOS in circulating MPs with a significant decrease of its level in patients with endothelial dysfunction. However, they did not analyze eNOS activation level (i.e., $P^{Ser1177}$eNOS). Oxidative stress or hyperglycemia could modulate eNOS expression. Indeed, exposure of cultured human coronary endothelial cells to high glucose leads to eNOS expression and NO production downregulation (32). Peng et al (33) also observed eNOS (mRNA and protein) downregulation in cardiomyocytes incubated with high glucose and insulin concentrations. Reduced eNOS expression was also reported in MPs from patients with advanced atherosclerosis (32). On the other hand, eNOS activity could be increased even if its expression level does not change. Silver et al. observed that although eNOS expression in endothelial cells was not increased in overweight adults and subjects with obesity compared with normal-weight controls, $P^{Ser1177}$eNOS level

was higher (34). Increased eNOS activation, and therefore enhanced NO production, could be the result of oxidative stress, and more specifically of ROS production (35). Indeed, H_2O_2 can activate eNOS by stimulating phosphorylation at Ser1177 (36). Based on these data, we suggest that the increased $P^{Ser1177}$eNOS level in MPs and in endothelial cells and the high plasma nitrite levels in subjects with obesity indicate the presence of a vascular compensatory mechanism to increase NO production in order to maintain its bioavailability in the presence of oxidative stress.

As NADPH oxidase is considered the main source of ROS production within the vasculature, we first investigated whether NOX2 was expressed in MPs. In agreement with a previous work showing the presence of NOX2 in circulating MPs from patients with hypercholesterolemia (37), we found that NOX2 was expressed in MPs, but its level was reduced in MPs from patients with obesity. NOX2 activation requires the translocation of its cytosolic subunit p47 to the cytoplasmic membrane (34). We could not detect this subunit in circulating MPs from both groups. This suggests that p47 was not localized at the membrane surface or in proximity of the MP blubbing site in endothelial cells and that it could not contribute to NOX2 activation (38). Therefore, we hypothesize that these MPs cannot produce ROS through NOX2 activity. NOX2 contributes to endothelial dysfunction and vascular oxidative stress, whereas NOX4 has a vasoprotective role by increasing NO bioavailability (39). Indeed, NOX2 produces O_2^- and NOX4 catalyzes production of H_2O_2 that could activate eNOS by enhancing its phosphorylation at Ser1177 (36). In our study, total NOX4 expression was comparable in MPs from controls and subjects with obesity. Conversely, expression of NOX4D, a NOX4 variant that produces H_2O_2 *in vitro* at similar levels as NOX4, was higher in MPs from subjects with obesity than in controls (40).

To conclude, although circulating MP levels were higher in subjects with obesity and endothelial dysfunction, they did not affect endothelial function *ex vivo* and were not correlated with impaired endothelial function parameters *in vivo*. Moreover, the higher $P^{Ser1177}$eNOS level in MPs from subjects with obesity than in controls suggests increased eNOS activity. Expression of the NOX2 isoform, which promotes endothelial dysfunction, was lower, while NOX4D, which has vasoprotective effects, was higher in MPs from subjects with obesity than in controls. Taken together, these results suggest that MPs from subjects with obesity do not contribute directly to endothelial dysfunction. As the MP phenotype reflects the phenotype of the cells from which they originate (endothelial cells) and the vascular state, we hypothesize that in subjects with obesity, MPs could participate in a compensatory mechanism to counterbalance the pathologic effects of obesity-related endothelial dysfunction.

Legends to figures

Figure 1 - Typical Laser Doppler recordings of a normal response at the forearm of a healthy subject. Vertical lines separate the different recording phases: resting CBF, the three successive administrations of 2% ACh (delivered using 0.1mA current for 10s) and local skin heating (LSH). The third dose of ACh was used to determine the maximum ACh response (i.e., the peak ACh-CBF). Temperature was maintained at 33°C during baseline recording and ACh administration and increased to 44°C for LSH. PU: perfusion unit.

Figure 2 – MP quantification and MP influence on *ex vivo* vasoreactivity. A/ Quantification of circulating MP levels in controls (n=14) and subjects with obesity (n=32); B and C/ Effect of MPs on the endothelium-dependent response of rat aorta

rings incubated with vehicle (n=8), MPs from controls (n=6) or MPs from subjects with obesity (n=7) for 5 hours. B/ Relaxation upon exposure to ACh; and C/ ACh EC_{50}. Values are the mean ± SEM. *p<0.05 versus control group.

Figure 3 – Western blot analysis using MPs from subjects with obesity (n=6) and controls (n=6) to determine: A/ total eNOS expression; B/ the phosphorylated eNOS to total eNOS ratio (eNOS-$P^{Ser1177}$/total eNOS); C/ total NOX2 expression (glycosylated and non-glycosylated NOX2); D/ p47 expression; E/ NOX4 expression; F/ NOX4D variant expression. Bands were quantified using tubulin. Values are the mean ± SEM. *p<0.05 versus control group.

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Table 1- Clinical and inflammatory characteristics of control group and obesity group

Characteristics	Control (n=46)	Obesity (n=69)	*P-value*
Demographic parameters			
Age (years)	33.11 ± 1.46	36.46 ± 1.36	*p= 0.106*
Gender (males/females)	18/28	27/42	*p= 0.578*
Clinical parameters			
BMI ($kg.m^{-2}$)	22.73 ± 0.29	37.27 ± 0.99	*p< 0.0001****
Waist circumference (cm)			
Waist circumference (males)	80.23 ± 3.04	118.63 ± 3.80	*p< 0.0001****
Waist circumference (females)	80.15 ± 2.67	111.97 ± 2.73	*p< 0.0001****
WHR			
WHR (males)	0.82 ± 0.02	0.99 ± 0.01	*p< 0.0001****
WHR (females)	0.81 ± 0.02	0.90 ± 0.01	*p< 0.0001****
Systolic blood pressure (mmHg)	113.00 ± 1.25	118.40 ± 1.92	*p= 0.021**
Diastolic blood pressure (mmHg)	74.50 ± 1.07	78.00 ± 0.90	*p= 0.014**
Mean arterial pressure (mmHg)	87.33 ± 1.03	91.46 ± 1.06	*p= 0.008***
Fasting glucose ($mmol.l^{-1}$)	5.03 ±0.71	5.68 ± 0.20	*p= 0.015**
Total cholesterol ($mmol.l^{-1}$)	4.42 ± 0.13	4.77 ± 0.10	*p= 0.048**
Triglycerides ($mmol.l^{-1}$)	0.89 ± 0.05	1.54 ± 0.13	*p< 0.0001****
HDL cholesterol ($mmol.l^{-1}$)	1.21 ± 0.48	1.14 ± 0.04	*p= 0.268*
LDL cholesterol ($mmol.l^{-1}$)	2.76 ± 0.12	2.96 ± 0.10	*p= 0.225*
ApoA ($g.l^{-1}$)	1.62 ±0.04	1.61 ± 0.03	*p= 0.919*
ApoB ($g.l^{-1}$)	0.82 ±0.02	0.87 ± 0.03	*p= 0.317*
Inflammatory blood markers			
hsCRP ($mg.l^{-1}$)	1.39 ±0.35	4.86 ± 0.54	*p< 0.0001****
Sedimentation rate (mm/h)	9.09 ± 1.85	15.68 ± 2.09	*p= 0.030**
Oxidative stress markers			
Nitrites (µmol/l)	0.26 ± 0.02	0.39 ± 0.02	*p= 0.003***
TBARS (µmol/l)	3.75 ± 0.39	4.95 ± 0.39	*p= 0.048**
AOPP (mmol/l)	14.30 ± 1.01	16.67 ± 0.55	*p= 0.028**
GSH (mmol/l)	17.61 ± 1.84	12.77 ± 1.14	*p= 0.020**

Abbreviations: BMI, Body Mass Index; WHR, Waist-to-Hip Ratio; HDL cholesterol, High Density Lipoprotein cholesterol; LDL cholesterol, low Density Lipoprotein cholesterol; ApoA, ApolipoproteinA; ApoB, ApolipoproteinB; hsCRP, high sensitive C- Reactive Protein; TBARS: ThioBarbituric Acid Reactive Species; AOPP: Advanced Oxidized Protein Products; GSH : reduced Glutathione. Data are expressed as mean ± SEM; * :p<0.05 ; ** :p<0.001 ; *** :p<0.0001

Table 2- Microvascular function of control group and group with obesity

	Control (n=46)	Obesity (n=69)	*P-value*
AUC (PU*sec)	8168.59 ± 833.26	5712.77 ± 553.56	0.012*
Basal CVC (PU/mmHg)	0.075 ± 0.006	0.053 ± 0.004	0.007 **
Peak ACh-CVC (PU/mmHg)	0.47 ± 0.05	0.32 ± 0.03	0.014 *
Peak LSH-CVC (PU/mmHg)	0.73 ± 0.06	0.63 ± 0.05	0.196
Δ ACh-CVC (PU/mmHg)	0.39 ± 0.04	0.27 ± 0.03	0.033*
Δ LSH-CVC (PU/mmHg)	0.66 ± 0.06	0.57 ± 0.05	0.295

Abbreviations: PU: Perfusion Unit; ACh : Acetylcholine; LSH : Local Skin Heat; AUC: Area Under Curve; CVC: cutaneous vascular conductance; Δ ACh CVC : peak ACh CVC minus baseline; Δ LSH CVC: peak LSH CVC minus baseline. Data are expressed as mean ± SEM.*:p<0.05 ; **p<0.01.

Table 3- Relationships between circulating MPs levels and parameters of endothelial function

Parameters	MPs levels (MPs/µl plasma)			
	Control (n=14)		Obesity (n=32)	
	r value	P-value	r value	P-value
AUC (PU*sec)	0.639*	0.014	-0.194	0.288
Peak ACh-CVC (PU/mmHg)	0.658*	0.015	-0.076	0.731
Δ ACh-CVC (PU/mmHg)	0.659*	0.014	-0.066	0.764

Abbreviations: MPs : Microparticles; AUC: Area Under Curve; PU : Perfusion Unit; ACh : Acetylcholine; CVC: Conductance Vascular Conductance; : Δ ACh CVC : peak ACh CVC minus baseline; * :p<0.05.

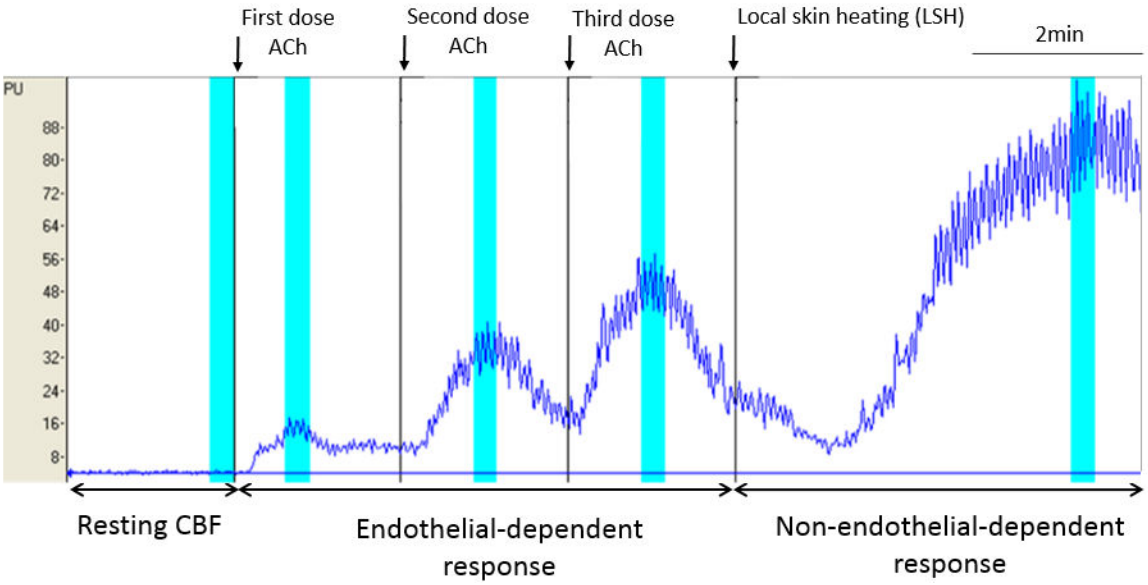

Figure 1 - Typical Laser Doppler recordings of a normal response at the forearm of a healthy subject. Vertical lines separate the different recording phases: resting CBF, the three successive administrations of 2% ACh (delivered using 0.1mA current for 10s) and local skin heating (LSH). The third dose of ACh was used to determine the maximum ACh response (i.e., the peak ACh-CBF). Temperature was maintained at 33°C during baseline recording and ACh administration and increased to 44°C for LSH. PU: perfusion unit.

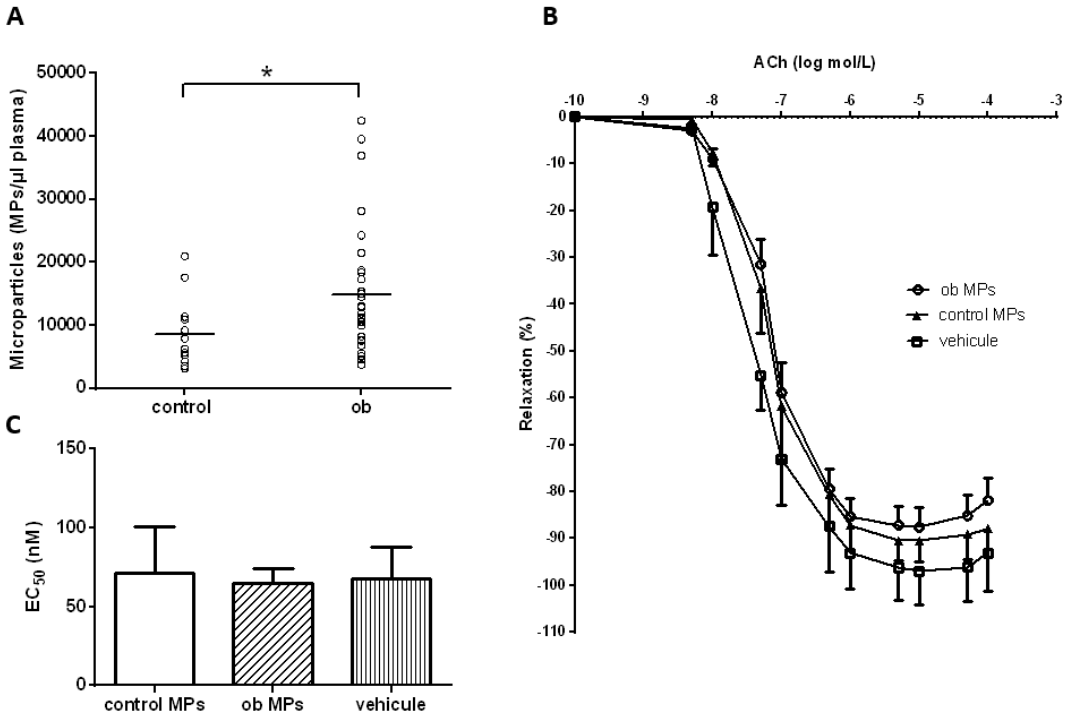

Figure 2 – MP quantification and MP influence on *ex vivo* vasoreactivity. A/ Quantification of circulating MP levels in controls (n=14) and subjects with obesity (n=32); B and C/ Effect of MPs on the endothelium-dependent response of rat aorta rings incubated with vehicle (n=8), MPs from controls (n=6) or MPs from subjects with obesity (n=7) for 5 hours. B/ Relaxation upon exposure to ACh; and C/ ACh EC_{50}. Values are the mean ± SEM. *$p<0.05$ versus control group.

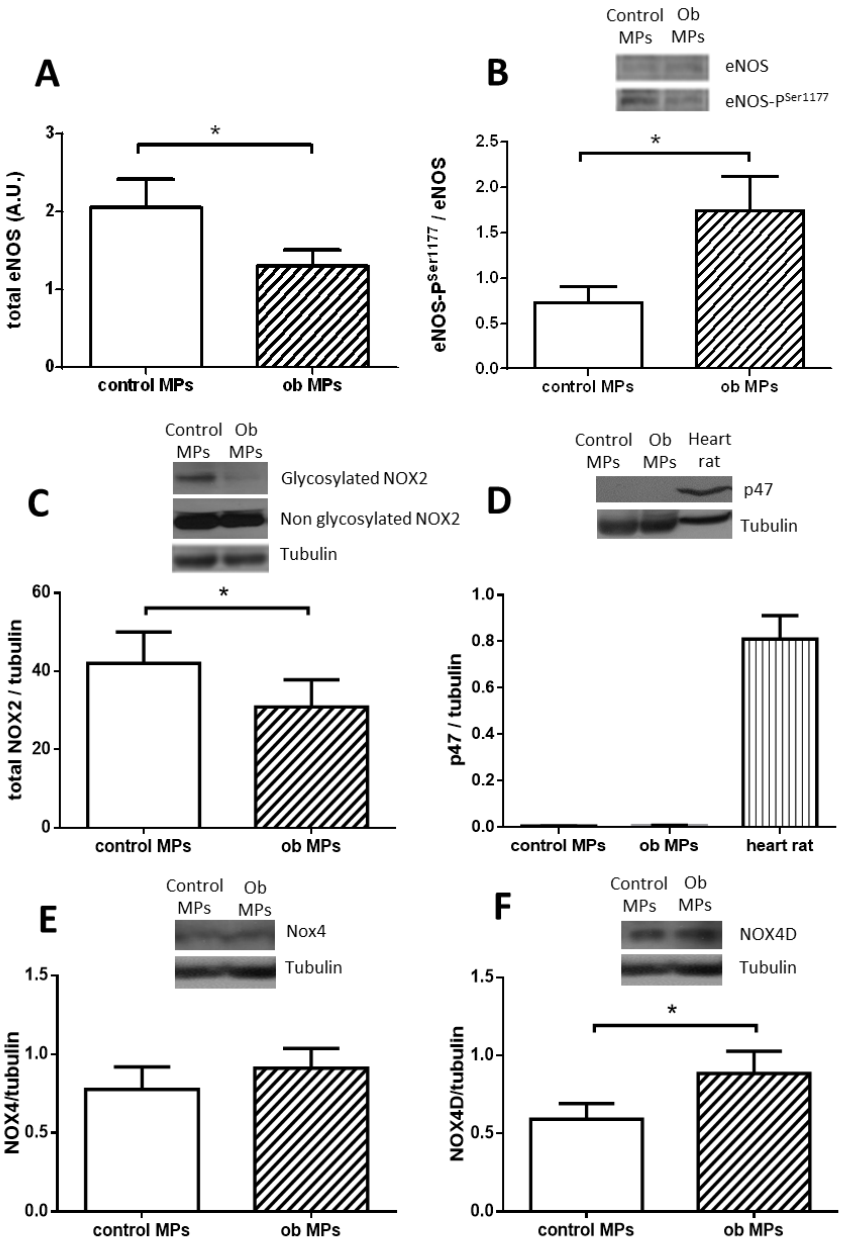

Figure 3 – Western blot analysis using MPs from subjects with obesity (n=6) and controls (n=6) to determine: A/ total eNOS expression; B/ the phosphorylated eNOS to total eNOS ratio (eNOS-$P^{Ser1177}$/total eNOS); C/ total NOX2 expression (glycosylated and non-glycosylated NOX2); D/ p47 expression; E/ NOX4 expression; F/ NOX4D variant expression. Bands were quantified using tubulin. Values are the mean ± SEM. *p<0.05 versus control group.