Vascular Biology, Atherosclerosis, and Endothelium Biology

Circulating Microparticles from Patients with Obstructive Sleep Apnea Enhance Vascular Contraction

Mandatory Role of the Endothelium

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Obstructive sleep apnea (OSA) is characterized by repetitive apnea-hypopnea cycles during sleep associated with oxygen desaturation and sleep disruption. We evaluated the role of circulating microparticles (MPs) from patients with OSA in the regulation of vascular function. MPs from whole blood from patients with OSA or control subjects were injected i.v. into mice. Injection of MPs from patients with OSA induced *ex vivo* vascular hyperreactivity in aortas with functional endothelium but, in contrast, hyporeactivity in vessels without functional endothelium. Vascular hyperreactivity was blunted in the presence of a nitric oxide synthase inhibitor alone or combined with the cyclooxygenase inhibitor indomethacin. MPs from patients with OSA reduced endothelial nitric oxide synthase activity and nitric oxide production, increased aortic cyclooxygenase-1 and cyclooxygenase-2 expression, and increased thromboxane A₂ and prostacyclin production. Blockade of thromboxane A₂ receptor did not affect the serotonin response in arteries from OSA MP-treated mice. A superoxide dismutase mimetic reduced the vascular hyperreactivity induced by MPs from patients with OSA but had no effect on contraction in vessels from control and non-OSA

MP-treated mice. These data provide evidence that circulating MPs from patients with OSA induce *ex vivo* vascular hyperreactivity with the obligatory role of the endothelium and subtle interactions between the nitric oxide and cyclooxygenase pathways and metabolites. These results highlight the participation of MPs in vascular dysfunction associated with OSA. (Am J Pathol 2012, 181: 1473–1482; <http://dx.doi.org/10.1016/j.ajpath.2012.06.020>)

Obstructive sleep apnea (OSA) is a highly prevalent disease characterized by recurrent episodes of partial or complete obstruction of the upper airways during sleep, leading to repeated falls in oxygen saturation. OSA is associated with hypertension, coronary artery disease, stroke, and premature death, and considerable evidence is now available in support of an independent association between OSA and cardiovascular diseases.¹ Various pathophysiologic mechanisms have been proposed to contribute to the pathogenesis of vascular dysfunction in patients with OSA.² In this view, circulating microparticles (MPs) have been considered as biomarkers of vascular injury and inflammation in several cardiovascular abnormalities.^{3–5} Indeed, altered levels of MPs have been detected and frequently correlate with the severity of the pathologic condition. MPs are vesicles with procoagulant and proinflammatory properties shed from the plasma membrane of various cell types, such as circulating cells and cells from the vascular wall, during activation or apoptosis.^{6,7} Recently, MPs have also been considered

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as effectors to vehicle biological messages on the target cells. However, few studies set out to demonstrate the importance of MPs in OSA, and they mainly evoke an alteration of the origin of MP levels.^{8–11} We provided evidence that patients with OSA display the same levels of circulating MPs as healthy individuals but that levels of MPs from activated leukocytes (CD62L⁺ MPs) are higher in patients with OSA.¹² There is a positive correlation between circulating levels of CD62L⁺ MPs and nocturnal desaturations severity.^{12,13} The evening-to-morning change in activated leukocyte-derived MPs in patients with OSA provided evidence in support of a direct link between sleep-disordered breathing and nocturnal MP release.¹³ *In vitro* and *ex vivo* studies have also demonstrated that MPs from patients with OSA can induce endothelial dysfunction, reduce endothelial nitric oxide (NO) release, and increase endothelial adhesion molecule expression.¹² These recent data suggest that MPs could play a role in the pathogenesis of endothelial dysfunction that may initiate the atherogenic process in OSA. However, to our knowledge, the role played by MPs in the regulation of vascular contractility regarding exaggerated vasoconstriction that occurs in patients with OSA¹⁴ has never been assessed. Therefore, the present study was designed to investigate the effects of MPs from patients with OSA on vascular reactivity in response to vasoconstrictor agonists. The mechanisms by which OSA MPs affect vascular contractility were also investigated. MPs were injected *i.v.* into mice to test their pathophysiologic relevance *in vivo*.

Materials and Methods

Patients

Consecutive male patients (19 to 70 years old) investigated by polysomnography or overnight respiratory recording in the Sleep Unit of the Department of Respiratory Medicine of Angers University, Angers, France, for suspected OSA were screened for the study. Exclusion criteria were previous treatment for OSA, body mass index (calculated as weight in kilograms divided by height in meters squared) ≥ 35 , history of coronary artery disease, heart failure, stroke, hypertension, diabetes mellitus, dyslipidemia, and treatment with any drug known to affect endothelial function. Patients with an apnea-hypopnea index (AHI) of ≥ 5 events per hour were included in the OSA group. Patients with an AHI < 5 were included in the non-OSA (N-OSA) group. All the patients underwent evaluation of clinical profile and daytime sleepiness using the Epworth Sleepiness Scale.¹⁵ Standard in-laboratory overnight polysomnography was performed as previously described¹⁶ using a computerized recording system (CID 102; Cidelec, Angers, France) with the following channels: electroencephalogram, electrooculogram, chin electromyogram, arterial oxygen saturation (finger oximetry), nasal-oral airflow (pressure cannula), tracheal sound (suprasternal microphone), electrocardiogram, chest and abdominal wall motion (piezo electrodes), bilateral tibialis electromyogram, and body position. Overnight respiratory recording (CID 102 L; Cidelec) was performed as previously de-

scribed¹⁷ with the same channels except for electroencephalogram, electrooculogram, chin electromyogram, and tibialis electromyogram. Respiratory events were scored manually using recommended criteria.¹⁸ Hypopneas had to be associated with $\geq 4\%$ oxygen desaturation. The University of Angers ethics committee approved the study, and patients gave their informed consent.

MP Isolation and Characterization

MP characterization was performed in the morning after sleep recording, at approximately 7 or 8 AM, before breakfast. Routine laboratory tests, including glucose, glycated hemoglobin, triglycerides, total cholesterol, high-density lipoprotein cholesterol, low-density lipoprotein cholesterol, and blood cell count, were also performed using a morning blood sample. For MP isolation, blood samples were collected in EDTA tubes (Vacutainer; Becton Dickinson, Le Pont de Claix, France) from a peripheral vein using a 21-gauge needle to minimize platelet activation and were processed for assay within 2 hours. Samples were centrifuged for 20 minutes at $270 \times g$, and plasma was then harvested and centrifuged for 20 minutes at $1500 \times g$ to obtain platelet-free plasma (PFP). Two hundred microliters of PFP was frozen and stored at -80°C until use. As previously described,^{4,12} the remaining PFP was subjected to two series of centrifugation at $21,000 \times g$ for 45 minutes to eliminate plasma and to pellet MPs for studies, and supernatant was replaced by 0.9% NaCl saline solution. Finally, MP pellets were suspended in 150 μL of 0.9% saline salt solution and were stored at 4°C until subsequent use.

MP subpopulations were discriminated into PFP according to the expression of membrane-specific antigens by flow cytometry. MPs derived from platelets, lymphocytes, and endothelial cells were identified using anti-CD41, anti-CD45, and anti-CD146 antibodies, respectively. Anti-CD62L antibody was used to identify MPs derived from activated L-selectin⁺ leukocytes. Irrelevant human IgG was used as an isotype-matched negative control for each sample. Five microliters of PFP was incubated with 5 μL of specific antibody (Beckman Coulter, Villepinte, France), and after 45 minutes of incubation, samples were diluted in 300 μL of 0.9% NaCl. Annexin V-FITC (BioVision Research Products, Mountain View, CA) binding was used to count phosphatidylserine-expressing MPs. To determine the MP concentration, equal volumes of sample and FlowCount beads were then added to calculate the MP concentration, and samples were analyzed using a 500 MPL system flow cytometer (Beckman Coulter). Regions corresponding to MPs were identified in forward and side-angle light scatter intensity dot plot representation set at logarithmic gain, depending on their diameter (0.1 to 1.0 μm). Sample analysis was stopped after counting 10,000 events.

Vascular Reactivity

All the animal studies were performed using approved institutional protocols and conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health. Male Swiss mice (8 to 10

weeks old) were treated by i.v. injection of MPs into the tail vein at the circulating levels detected in the blood of each patient (OSA group: range, 1276 to 52,581 MPs per microliter of plasma; N-OSA group: range, 2887 to 32,703 MPs per microliter of plasma) or 0.9% NaCl saline solution (CTL), as previously performed in other studies.^{4,12} Thus, several patients in the N-OSA group had circulating levels of MPs greater than several patients in the OSA group. In addition, for all the experiments, the OSA and N-OSA groups were matched for age, body mass index, and biological data.

Aortic rings were isolated from mice treated with MPs from patients or healthy individuals during 24 hours. The mouse aorta was removed and carefully cleaned of adhering fat and connective tissue and then was cut into rings with or without endothelium (1.5 to 2 mm long) that were mounted on a wire myograph filled with physiologic saline solution as previously described.^{5,19} Concentration-response curves were constructed by cumulative application of serotonin (5-HT, 1 nmol/L to 10 μ mol/L; Sigma-Aldrich, St. Quentin, France) to vessels with functional endothelium in the absence or presence of the given inhibitor preincubated for 30 minutes: the NO synthase (NOS) inhibitor N^G-nitro-L-arginine (L-NA, 100 μ mol/L; Sigma-Aldrich), the selective cyclooxygenase (COX)-2 inhibitor *N*-(2-cyclohexyloxy-4-nitrophenyl) methanesulfonamide (NS-39810 μ mol/L; Sigma-Aldrich), the nonselective COX inhibitor indomethacin (100 μ mol/L; Sigma-Aldrich), the selective thromboxane A₂ receptor antagonist SQ-29548 (5 μ mol/L; Sigma-Aldrich), L-NA plus indomethacin, the superoxide dismutase mimetic manganese (III) tetrakis-(1-methyl-4-pyridyl)-porphyrin pentachloride (MnTMPyP, 100 μ mol/L; Calbiochem, Darmstadt, Germany), or tetrahydrobiopterin (BH4, 10 μ mol/L; Sigma-Aldrich). All the inhibitors were used at the maximal active concentrations at which they inhibit the release of NO from all isoforms of NOSs, metabolites from COX-2 isoform, or metabolites from COX in blood vessels, as reported in many previous studies.^{4,20} Higher concentrations of L-NA, NS-398, SQ-29548, or indomethacin did not induce further inhibition. Also, the contractile response to a cumulative concentration-response to the thromboxane A₂ agonist (9,11-dideoxy-11a,9a-epoxymethanoprostaglandin F₂) (U-46619; Cayman Chemicals, Ann Arbor, MI) or to the combination of U-46619 (100 nmol/L) and KCl (80 mmol/L) was assessed in mouse aortas. Finally, a concentration-response curve to 5-HT was performed in aortas without functional endothelium. The endothelium was removed by rubbing the intimal surface of the rings with a rough needle. Aortas were considered denuded of their endothelium when acetylcholine (1 μ mol/L) did not produce any relaxation.

NO Determination by Electron Paramagnetic Resonance

Detection of NO production was performed using a technique with Fe²⁺ diethyldithiocarbamate (Sigma-Aldrich) as spin trap as previously described.²¹ Briefly, 24 hours after administration of MPs, the animals were sacrificed.

The aortas were dissected and incubated for NO production in 250 μ L of Krebs-HEPES buffer solution, treated with 250 μ L of colloid Fe(diethyldithiocarbamate)₂, and incubated for 45 minutes at 37°C. NO detection was measured *in situ* by electron paramagnetic resonance, and values are expressed as amplitude signal per milligram tissue (dry weight).

Western Blot Analysis

Aortas were dissected, homogenized, and lysed. Proteins (40 μ g) were separated on NuPAGE gels (Invitrogen, Carlsbad, CA). Blots were probed with anti-endothelial NOS (eNOS) (BD Biosciences, San Jose, CA), anti-phospho-eNOS Thr 495, anti-phospho-eNOS Ser 1117 (Cell Signaling Technology Inc., Beverly, MA), and anti-COX-1 and 2 (Santa Cruz Biotechnology, Santa Cruz, CA) antibodies. A monoclonal mouse β -actin antibody (Sigma-Aldrich) was used at 1:1000 dilution to visualize protein gel loading. The membranes were then washed at least three times in Tris buffer solution containing 0.05% Tween and were incubated for 1 hour at room temperature with the appropriate horseradish peroxidase-conjugated secondary antibody. The protein antibody complexes were detected by ECL Plus reagent (Amersham Biosciences, Piscataway, NJ) according to the manufacturer's instructions.

Determination of Prostanoid Production

After sacrificing the mice pretreated with MPs, their aortas were dissected and treated with 5-HT (1 μ mol/L, 37°C, 30 minutes). After collection of the medium, thromboxane A₂, prostaglandin E metabolite, prostacyclin, and total 8-isoprostane concentrations were measured by enzyme immunoassay kits (Cayman Chemicals). Prostanoid concentrations are expressed as pictograms per milliliter per milligram tissue (dry weight).

Data Analysis

Data were analyzed using GraphPad Prism software (GraphPad Software Inc., San Diego, CA). Data are expressed as mean $\pm$ SEM, and *n* represents the number of patients or experiments performed with mice. $pD_2 = -\log EC_{50}$, EC_{50} being the molar concentration of the agonist that produces 50% of the maximal effect; EC_{50} values were calculated by logit-log regression. For all the experiments, statistical analyses were performed using nonparametric *U*-tests or two-way analysis of variance for repeated measures and subsequent Bonferroni post hoc tests. *P* < 0.05 was considered statistically significant.

Results

Baseline Clinical and Biological Characteristics

Thirty-four patients without any cardiovascular history of risk factor were included in this study. Patients were split into N-OSA (*n* = 20) and OSA (*n* = 22) groups according to AHI.

Table 1. Characterization of the Study Population

Characteristic	N-OSA group (n = 20)	OSA group (n = 22)	P value
Age (years)	42.1 ± 3	53.8 ± 1.9	0.0143
Body mass index*	25.8 ± 1	26.7 ± 0.5	NS
Weight (kg)	82.4 ± 4	82.8 ± 1.8	NS
Systolic BP (mm Hg)	124.1 ± 4.1	137.7 ± 4.9	NS
Diastolic BP (mm Hg)	72.9 ± 2	80 ± 3.1	NS
Smokers (No.)	5	3	NS
Plasma glucose (mmol/L)	5.3 ± 0.2	5.7 ± 0.3	NS
Glycated hemoglobin (%)	5.7 ± 0.1	5.7 ± 0.1	NS
Total cholesterol (mmol/L)	5.5 ± 0.3	5.8 ± 0.2	NS
Triglycerides (g/L)	1.3 ± 0.1	1.5 ± 0.2	NS
HDL cholesterol (mmol/L)	1.3 ± 0.1	1.3 ± 0.1	NS
LDL cholesterol (mmol/L)	3.7 ± 0.3	3.9 ± 0.2	NS
Epworth Sleepiness Scale score	9.3 ± 1.5	9.9 ± 0.8	NS
AHI (events/hour)	1.3 ± 0.3	21.6 ± 3.2	<0.0001
Mean SaO ₂ (%)	93.7 ± 0.4	93.2 ± 0.3	NS
4% ODI (events/hour)	1.5 ± 0.5	15.4 ± 2	<0.0001

All values are expressed as mean ± SEM except where indicated otherwise.

*Calculated as weight in kilograms divided by height in meters squared.

BP, blood pressure; HDL, high-density lipoprotein; LDL, low-density lipoprotein; ODI, oxygen desaturation index; NS, not significant; SaO₂, oxyhemoglobin saturation.

The two groups differed by age but did not differ by weight, body mass index, blood pressure, daytime sleepiness as assessed by the Epworth Sleepiness Scale,¹⁵ lipid metabolism, glucose metabolism, or smoking rate (Table 1).

Phenotype of Circulating MPs

As previously described,¹² the total number of circulating MPs was not significantly different between the N-OSA and OSA groups. Phenotypical characterization of MPs did not show significant differences between the N-OSA and OSA groups (Table 2). However, a positive correlation was found between CD62L⁺ MPs and the AHI (Figure 1).

Table 2. Circulating MP Levels

MPs (events/ μ L)	N-OSA group (n = 20)	OSA group (n = 22)
Total circulating MPs	13,125 ± 2058 (2887–32,703)	20,957 ± 3617 (1276–52,581)
CD41 ⁺ MPs	10,647 ± 2016	15,964 ± 3059
Annexin V ⁺ MPs	4283 ± 842	5135 ± 1913
CD45 ⁺ MPs	356 ± 124	244 ± 79
CD66b ⁺ MPs	174 ± 40	145 ± 21
CD62L ⁺ MPs	193 ± 38	260 ± 42
CD62P ⁺ MPs	257 ± 48	273 ± 202
CD146 ⁺ MPs	306 ± 64	363 ± 84
CD235a ⁺ MPs	818 ± 161	502 ± 71

Values are expressed as mean ± SEM, and none are statistically significant. CD41⁺ MPs: platelet-derived MPs; Annexin V⁺ MPs: procoagulant MPs; CD45⁺ MPs: leukocyte-derived MPs; CD66b⁺ MPs: granulocyte-derived MPs; CD62L⁺ MPs: L-selectin⁺ MPs; CD62P⁺ MPs: P-selectin⁺ MPs; CD146⁺ MPs: endothelial-derived MPs; CD235a⁺ MPs: erythrocyte-derived MPs.

Figure 1. Positive correlation between CD62L⁺ MPs and the severity of OSA according to the AHI.

OSA MPs Increase Responsiveness of Mouse Aortic Rings to Vasoconstrictor Agents

5-HT produced a concentration-dependent increase in tension in mouse aortic rings with functional endothelium in the CTL group and in those treated with circulating levels of either N-OSA or OSA MPs. Maximal response but not sensitivity to 5-HT was markedly increased in mice treated with OSA MPs (mean ± SEM maximal contractions, 3.1 ± 0.2 mN/mm) compared with those treated with either vehicle or N-OSA MPs (mean ± SEM maximal contractions, 2.3 ± 0.2 and 2.1 ± 0.2 mN/mm, respectively) (Figure 2A). Because concentrations of MPs used from the OSA and N-OSA groups were not significantly different and only OSA MPs induced vascular hyperreactivity, the effects observed cannot be related to the different concentrations of circulating MPs in both groups but rather to the composition of MPs.

As for 5-HT, aortic rings taken from mice treated with OSA MPs displayed increased maximal contraction in response to the stable analog of thromboxane A₂, U-46619, compared with those from vehicle- and N-OSA MP-treated animals (Figure 2B). Finally, aortas from OSA MP-treated mice exhibited an increased contractile response to the concomitant application of KCl (100 mmol/L) and a single concentration of U-46619 (100 nmol/L) compared with those from vehicle- and N-OSA MP-treated animals (Figure 2C).

Altogether, these data suggest that the OSA MP-induced vascular hyperreactivity is independent of the agonist or vasoconstrictor used. The OSA MP-induced vascular hyperreactivity may result from the release of vasoconstrictor products from different cellular origins (eg, endothelial cells, smooth muscle cells, or fibroblasts).

Involvement of the NO Pathway in OSA MP-Induced Vascular Hyperreactivity

To investigate the role of NO, we studied the effect of the NOS inhibitor L-NA on the response to 5-HT. L-NA alone increased mean ± SEM basal tone in aortas from control (0.286 ± 0.04 mN/mm) and N-OSA MP-treated (0.576 ± 0.14 mN/mm) mice but not in those taken from mice treated with OSA MPs. Moreover, L-NA enhanced maximal response to 5-HT in control and N-OSA MP-treated vessels. In aortas from mice treated with OSA MPs, L-NA

Figure 2. OSA MPs increase the responsiveness of mouse aortic rings to vasoconstrictor agents. Concentration-response curves to 5-HT ($n = 8$) (A) and U-46619 ($n = 5$) (B) in mouse aortic rings from vehicle- (CTL), N-OSA MP-, and OSA MP-treated mice. C: Contractile response to the concomitant application of KCl (80 mmol/L) and a single concentration of U-46619 (1 μmol/L) ($n = 6$) in aortic rings from mice treated with vehicle, N-OSA MPs, or OSA MPs. Data are given as mean $\pm$ SEM. * $P < 0.001$, ** $P < 0.01$, and *** $P < 0.05$.

did not affect maximal response to 5-HT. Moreover, it induced a rightward shift of the concentration-response curve to the agonist (mean $\pm$ SEM pD₂ being 5.7 ± 0.2 , 5.5 ± 0.3 , and 4.7 ± 0.2 for CTL, N-OSA, and OSA, respectively) (Figure 3). Thus, L-NA inhibited rather than potentiated the response to 5-HT in aortas from mice treated with OSA MPs (Figure 3D). NO production in aortas taken from OSA MP-treated mice was significantly lower than that measured in vessels from vehicle- or N-OSA MP-treated mice (Figure 4A). To identify the molecular mechanism implicated in the decrease in NO production, we analyzed the levels of expression and phosphorylation of aortic eNOS by Western blot analysis. As shown in Figure 4B, eNOS expression was not significantly modified by MP treatment, although a trend toward an increase in eNOS expression was observed after treatment with MPs. However, treatment with OSA MPs, but not N-OSA MPs, induced a significant decrease in eNOS phosphorylation on the activator (Ser1177) and

inhibitor (Thr495) sites (Figure 4, C and D). After normalization of the amount of phosphorylated eNOS to the total amount of enzyme, the ratio of phosphorylated eNOS at the inhibitor and activation sites was greater in aortas from OSA MP-treated mice compared with those treated with N-OSA MPs or untreated (Figure 4E). These results indicate a down-regulation of eNOS activity caused by OSA MPs.

A Superoxide Dismutase Mimetic-Sensitive Mechanism Is Involved in OSA MP-Induced Vascular Hyperreactivity

Because superoxide anion production by uncoupled eNOS may account for the vascular hyperreactivity induced by OSA MPs, the effects of either MnTMPyP, a superoxide dismutase mimetic, or BH4 were analyzed in the contractile responses in aortas treated with MPs from patients in the N-OSA and OSA groups. Pretreatment with MnTMPyP of aortas from control or N-OSA MP-treated mice did not modify significantly the maximal contraction induced by 5-HT. The mean $\pm$ SEM values of the maximal contraction were 2.3 ± 0.2 versus 2.05 ± 0.1 for vessels from control mice in the absence and presence of MnTMPyP, respectively. In vessels from N-OSA MP-treated mice, the mean $\pm$ SEM values were 2.1 ± 0.2 versus 2.03 ± 0.09 . In contrast, MnTMPyP induced an $\sim 33\%$ reduction in the mean $\pm$ SEM maximal contraction induced by 5-HT of vessels from OSA MP-treated mice (3.1 ± 0.2 versus 2.08 ± 0.2 in the absence and presence of MnTMPyP, respectively) without significant modification of the mean $\pm$ SEM pD₂ values (6.04 ± 0.02 versus 5.9 ± 0.10 in the absence and presence of MnTMPyP, respectively). Furthermore, BH4 induced an increase in mean $\pm$ SEM basal tone (0.23 ± 0.05 mN/mm) but was unable to modify contraction to 5-HT of aortas from mice treated with MPs from patients with OSA. Indeed, maximal contraction to 5-HT in the presence of BH4 was 11% higher (not significant) than in the absence of BH4. Also, pD₂ values were not statistically significantly different between both conditions, the mean $\pm$ SEM values being 6.04 ± 0.02 versus 5.96 ± 0.06 in the absence and presence of BH4, respectively.

Figure 3. Involvement of the NO pathway in OSA MP-induced vascular hyperreactivity. Concentration-response curves to 5-HT in mouse aortic rings from vehicle-treated mice (CTL; $n = 5$) (A), N-OSA MP-treated mice ($n = 5$) (B), and OSA MP-treated mice ($n = 5$) (C) with or without the presence of NOS inhibitor (L-NA, 100 μmol/L). D: Concentration-response curves to 5-HT of aortic rings from mice treated with vehicle (CTL), N-OSA MPs-, or OSA- MPs in the presence of L-NA ($n = 5$). Data are given as mean $\pm$ SEM. * $P < 0.001$.

Figure 4. Down-regulation of eNOS activity by OSA MPs. **A:** Quantification of the amplitude of NO-Fe(diethyldithiocarbamate)₂ signal in aortas from mice treated with vehicle (CTL), N-OSA MPs, and OSA MPs. Values are expressed as amplitude per milligram (A/mg) weight of dried aorta ($n = 6$). Western blots show eNOS expression (**B**), phospho-eNOS Ser 1177 (**C**), and phospho-eNOS Thr 495 (**D**), and histograms show the ratio of phosphorylation of eNOS Ser 1177 (**C**) or eNOS Thr 495 (**D**) versus total eNOS. Immunoblots were quantified by densitometric analysis. Data are representative of five separate blots, and the densitometry values are expressed in arbitrary units (AU). **E:** The ratio of phosphorylation of eNOS at the inhibitory Thr 495 and activation (Ser 1177) sites is expressed in arbitrary units (AU). Data are given as mean $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$.

Involvement of COX Metabolites in OSA MP-Induced Vascular Hyperreactivity

To investigate the role of COX metabolites in 5-HT-induced vascular reactivity, the effects of a nonselective inhibitor of COX (indomethacin) and a selective inhibitor of COX-2 (NS-398) were examined. In the presence of indomethacin, the contractile response to 5-HT was reduced in aortas from all groups of mice; the decrease was even greater in the OSA group (60%) compared with the N-OSA and vehicle groups (47% and 48%, respectively) (Table 3). When COX-2 was specifically silenced using NS-398, the response to 5-HT was impaired in vessels from controls, N-OSA MPs, and OSA MPs, with a stronger inhibition for aortas treated by OSA MPs (Table 3). These results suggest that OSA MP treatment leads to an increase in the release of vasoconstrictor metabolites derived from COX, including COX-2.

OSA MPs were able to enhance the expression levels of COX-1 and COX-2 compared with N-OSA MPs and vehicle (Figure 5, A and B). To test the hypothesis that COX activity could be affected by this treatment, we measured the production of different COX metabolites. Aortas treated with OSA-MPs displayed increased throm-

boxane A₂ and prostacyclin production compared with N-OSA MP-treated and untreated aortas. In contrast, release of prostaglandin E₂ or 8-isoprostane was not significantly different among the three groups (Figure 5, C–F). The hyperreactivity observed might be due to the increase in thromboxane A₂ production. To test this hypothesis, we performed a concentration-response curve to 5-HT in the presence of SQ-29548, a selective thromboxane A₂ receptor antagonist. SQ-29548 significantly reduced the response to 5-HT in vessels from vehicle- and N-OSA MP-treated mice but not in those from animals injected with OSA MPs (Table 3). These results suggest that the SQ-29548-sensitive pathway is involved in the response to 5-HT in vessels from vehicle- and N-OSA MP-treated mice. In contrast, in those from OSA MP-treated mice, the SQ-29548-sensitive pathway might not be implicated in the response, or, alternatively, vasodilator metabolites might mask the effect of thromboxane A₂.

Interaction between the NO and COX Pathways

To study the eventual interaction between the NO and COX pathways, we used inhibitors, L-NA and indomethacin, in combination. On the one hand, blockade of the two pathways

Table 3. pD₂ Values and Maximal Contractions (Emax) of Serotonin Obtained in Aorta from Mice Treated with Vehicle (CTL), N-OSA MPs, or OSA MPs in the Absence or Presence of COX Inhibitor (Indomethacin, 10 μ mol/L), in the Presence of COX-2 Inhibitor (NS-398, 1 μ mol/L), or in the Presence of Thromboxane A₂ Antagonist (SQ-29548, 5 μ mol/L)

Treatment	pD ₂	Emax (mN/mm)
CTL		
Without inhibitors	5.4 $\pm$ 0.2	2.3 $\pm$ 0.2
With indomethacin	6.3 $\pm$ 0.2*	1.1 $\pm$ 0.1*
With NS-398	5.8 $\pm$ 0.2	1.3 $\pm$ 0.1*
With SQ-29548	6 $\pm$ 0.2	1.2 $\pm$ 0.1*
N-OSA MPs		
Without inhibitors	5.8 $\pm$ 0.2	2.1 $\pm$ 0.2
With indomethacin	5.9 $\pm$ 0.2	1.1 $\pm$ 0.1**
With NS-398	5.5 $\pm$ 0.2	1.1 $\pm$ 0.1***
With SQ-29548	5.9 $\pm$ 0.1	1.6 $\pm$ 0.1*
OSA MPs		
Without inhibitors	6.1 $\pm$ 0.1	3.1 $\pm$ 0.2
With indomethacin	5.6 $\pm$ 0.3	1.3 $\pm$ 0.2**
With NS-398	5.1 $\pm$ 0.2	2.2 $\pm$ 0.4***
With SQ-29548	6 $\pm$ 0.2	3.3 $\pm$ 0.3

All values are expressed as mean $\pm$ SEM.

* P < 0.05, ** P < 0.001, *** P < 0.01 versus without inhibitors.

enhanced the response to 5-HT in aortas from control and N-OSA MP-treated mice. On the other hand, the presence of the two blockers induced a rightward shift of the response to 5-HT in vessels from mice treated with OSA MPs (Figure 6).

Role of the Endothelium

In vessels without functional endothelium, contraction to 5-HT was significantly reduced in vessels taken from mice treated with OSA MPs compared with those taken from control or N-OSA MP-treated mice (Figure 7). These results strongly suggest the obligatory role of the endothelium in the hyperreactivity of the aorta to 5-HT after treatment of mice with OSA MPs.

Discussion

In the present study, we showed that i.v. injection of OSA MPs into mice promotes vascular hyperreactivity in response to vasoconstrictor agents, with the obligatory role of the endothelium. This effect was associated with the ability of OSA MPs to reduce the activity of eNOS concomitant with a decrease in NO production. In addition, OSA MPs increased aortic COX-1 and COX-2 expression associated with enhanced production of thromboxane A₂ and prostacyclin, and indomethacin or the selective COX-2 inhibitor NS-398 reduced hyperreactivity in response to 5-HT. The SQ-29548-sensitive pathway might not be implicated in vascular hyperreactivity of OSA MPs, or, alternatively, vasodilator metabolites, including prostacyclin, might mask the effect of thromboxane A₂. Moreover, a mechanism sensitive to MnTMPyP is implicated in the vascular hyperreactivity induced by OSA MPs. These

Figure 5. OSA MPs and the COX pathway. Western blots reveal expression of COX-1 (A) and COX-2 (B) in aortas from mice treated with vehicle (CTL), N-OSA MPs, and OSA MPs. Data are representative of six separate blots. Concentrations of the COX derivatives thromboxane A₂ (C), 8-isoprostane (D), prostacyclin (E), and prostaglandin E₂ (F) in the supernatants of mouse aortas pretreated with vehicle (CTL), N-OSA MPs, and OSA MPs and stimulated with 5-HT (n = 5). Data are given as mean $\pm$ SEM. * P < 0.01, ** P < 0.05.

Figure 6. Interaction between the NO and COX pathways. Concentration-response curves to 5-HT in mouse aortic rings from vehicle-treated mice (CTL; $n = 5$) (A), N-OSA MP-treated mice ($n = 5$) (B), and OSA MP-treated mice ($n = 5$) (C) in the absence or presence of L-NA plus indomethacin. D: Concentration-response curves to 5-HT of aortic rings from mice treated with vehicle (CTL), N-OSA MPs, or OSA MPs in the presence of L-NA plus indomethacin ($n = 5$). Data are given as mean $\pm$ SEM. * $P < 0.001$.

data provide evidence of the pathophysiologic relevance of MPs as vectors of transcellular exchange of messages in promoting endothelial¹² and vascular (the present study) hyperreactivity in patients with OSA.

It is well accepted that MPs contribute, at least in part, to the alteration of vascular function in several cardiovascular diseases.^{6,7} Evidence of the pathophysiologic relevance of MPs in OSA was recently provided *in vitro* and *ex vivo* by their ability to impair endothelium-dependent relaxation in response to acetylcholine and to flow when injected into mice.¹² In line with a previous study,¹² circulating levels of MPs were not different between patients in the OSA and N-OSA groups, but levels of CD62L⁺ MPs were positively correlated with the severity of sleep-disordered breathing. We observed a significant difference in age between the N-OSA and OSA populations. Although we cannot exclude that age might affect the level of circulating MPs, it has been shown that MP levels from platelets, red blood cells, and those expressing phosphatidylserine in young patients are not different from those in elderly patients,^{22,23} suggesting that age does not affect MP generation or their procoagulant activity.

We provide further evidence that OSA MPs promote vascular hyperreactivity in response to vasoconstrictor agents in mouse aortas. These findings are in line with previous studies in which MPs from patients with septic shock enhance contraction in response to 5-HT.⁴ Septic MPs may, rather, be protective in counteracting the decrease in peripheral resistance and progressive hypotension during severe sepsis and explain the fact that increased levels of MPs may predict a more favorable outcome in severe sepsis in terms of mortality rate and organ dysfunction.²⁴ In contrast to patients with septic shock, increased vascular reactivity in patients with OSA is deleterious and is probably involved in vascular re-

modeling, leading to the exacerbation of vasoconstriction reported in this disease. Indeed, exaggerated vasoconstriction occurs in the forearms of patients with OSA in response to angiotensin II.¹⁴ In a rat model of intermittent hypoxia, enhanced responses to endothelin-1 have also been reported in several vascular beds.^{25,26} Furthermore, increased contraction to norepinephrine in mouse hindquarters has also been observed.²⁷ In contrast, contractile responses to potassium, endothelin-1, 5-HT, and thromboxane A₂ mimetic were not affected in rat aortas after 35 days of intermittent hypoxia.²⁶ Nevertheless, we provide evidence that MPs from OSA induced vascular hyperreactivity independently of the vasoconstrictor under the experimental conditions used and, therefore, participate in the regulation of vascular tone in this disease.

OSA MPs induced vascular hyperreactivity only in the presence of functional endothelium, suggesting the obligatory role of this tunica. In contrast, OSA MPs elicited vascular hyporeactivity in the absence of functional endothelium, suggesting that they can also act directly on smooth muscle cells. The pathways involved in this vascular hyporeactivity were not assessed since, *in vivo*, MPs interact with intact blood vessels in which the endothelium is present.

We previously reported decreased endothelial NO production in response to OSA MPs.¹² These data corroborate studies showing a decreased plasma concentration of NO metabolites in OSA, which is correlated with OSA severity.²⁸ In a previous work,¹² MPs from patients with OSA reduced NO production from endothelial cells by negative regulation of eNOS activity. We reinforce this hypothesis inasmuch as i.v. injection of mice with OSA MPs decreased NO production in the aorta by a mechanism associated with reduced eNOS activity without alteration of its expression. Moreover, blockade of the NO pathway with L-NA alone increased basal tone in aortas from control and N-OSA MP-treated mice but not in those from mice treated with OSA MPs, suggesting that OSA MPs reduced basal NO production. Decrease of the basal level of NO has been reported in a rat model of intermittent hypoxia.²⁹ Furthermore, L-NA enhanced maximal response to 5-HT in control and N-OSA MP-treated vessels. In aortas from mice treated with OSA

Figure 7. Role of the endothelium in the increased responsiveness to 5-HT induced by OSA MPs. Concentration-response curves to 5-HT without functional endothelium ($n = 5$) in mouse aortic rings from vehicle (CTL), N-OSA MP-, and OSA MP-treated mice. Data are given as mean $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$.

MPs, L-NA did not affect maximal response to 5-HT. Altogether, these data demonstrate that decreased endothelial NO participates in vascular hyperreactivity elicited by OSA MPs. It is expected that the concentration-response curve to 5-HT would remain unchanged in the presence of L-NA owing to reduced endothelial NO production in vessels from mice treated with OSA MPs. L-NA instead induced a rightward shift of the concentration-response curve to the agonist. One can advance the hypothesis that inhibition of NOS unmasks the existence of vasoconstrictor factor(s) released on OSA MP treatment. These factors might participate in the vascular hyperreactivity induced by OSA-MPs. Indeed, a plausible explanation may be the superoxide production via the uncoupled eNOS. Although the superoxide dismutase mimetic MnTMPyP decreased contraction to 5-HT in vessels from mice treated with OSA MPs, incubation with BH₄, an essential cofactor required for the activity of eNOS, had no effect. These results indicate that vascular hyperreactivity induced by OSA MPs may be due, at least in part, to increased reactive oxygen species production. The characterization of the source of reactive oxygen species remains to be evaluated.

The contraction in response to 5-HT involved the participation of COX vasoconstrictor metabolites in the aorta independent of the treatment (vehicle, N-OSA MPs, or OSA MPs). Blockade of either the nonselective COX inhibitor indomethacin or the COX-2 inhibitor resulted in a greater reduction in the 5-HT response in vessels from mice treated with OSA MPs, suggesting that COX metabolites participate, in part, in the increased response. Indeed, COX-1 and COX-2 expression was increased in aortas taken from OSA MP-treated mice. Up-regulation of COX-2 has been reported in endothelial cells treated with MPs from OSA¹² and in venous endothelial cells harvested from patients with untreated OSA.³⁰ The nature of the COX metabolites involved was found to be increased thromboxane A₂ and prostacyclin. Concerning thromboxane A₂, blockade of thromboxane receptor by SQ-29548 significantly reduced the response to 5-HT in vessels from vehicle- and N-OSA MP-treated mice but not in those from animals injected with OSA MPs. Thus, the SQ-29548-sensitive pathway is involved in the response to 5-HT in vessels from vehicle- and N-OSA MP-treated mice. In contrast to OSA MP-treated mice, the SQ-29548-sensitive pathway might not be implicated in the response, or, alternatively, vasodilator metabolites, probably prostacyclin, might mask the effect of thromboxane A₂. Moreover, another vasoconstrictor factor specific to COX metabolites might participate in OSA MP-induced vascular hyperreactivity. Indeed, concomitant blockade of the NO and COX pathways induced a rightward shift of the response to 5-HT in OSA MP-treated aortas. The nature of this vasoconstrictor factor remains to be identified.

In conclusion, we provide evidence that OSA MPs induce *in vivo* vascular hyperreactivity to vasoconstrictor agents in the aorta with the obligatory role of the endothelium, probably via increased production of reactive oxygen species. This effect is associated with a combination of reduced eNOS activity and NO production and a subtle interaction with the COX pathway and metabo-

lites. The critical role of MPs as a vector of biological messages leading to vascular dysfunction in OSA is, therefore, underlined.

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