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Shed Membrane Particles from Preeclamptic Women Generate Vascular Wall Inflammation and Blunt Vascular Contractility

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We investigated the role of microparticles in vascular dysfunction of the multisystemic disorder of preeclampsia in women's omental arteries or mouse arteries. Preeclamptic women displayed increased circulating levels of leukocyte- and platelet-derived microparticles compared with healthy pregnant individuals. Microparticles from preeclamptic, but not healthy, pregnant women induced *ex vivo* vascular hyporeactivity to serotonin in human omental arteries and mouse aortas. Hyporeactivity was reversed by a nitric-oxide (NO) synthase inhibitor and associated with increased NO production. In the presence of a cyclooxygenase (COX)-2 inhibitor, serotonin-mediated contraction was partially reduced in arteries treated with healthy microparticles but was abolished after treatment with preeclamptic microparticles. This was associated with increased 8-isoprostane production. Preeclamptic microparticles induced up-regulation of inducible nitric-oxide synthase and COX-2 expression, evoked nuclear factor- κ B activation, and enhanced oxidative and nitrosative stress. Interestingly, the microparticles originating most probably from leukocytes were responsible for the COX-2 vasoconstrictor component of preeclamptic microparticles, whereas those of platelet origin were mainly involved in NO release. Moreover, vascular hyporeactivity was observed in arteries taken from mice treated *in vivo* with preeclamptic microparticles. This study demonstrates pathophysiological relevance and

provides a paradoxical effect of preeclamptic microparticles associated with proinflammatory properties on vessels, leading to enhanced NO and superoxide anion levels and counteraction of increased COX-2 metabolites. (Am J Pathol 2006, 169:1473-1483; DOI: 10.2353/ajpath.2006.051304)

Approximately 10% of pregnancies are associated with hypertension, 75% of them being related to preeclampsia.¹ This condition is a multiorgan disorder associated with generalized endothelial dysfunction, resulting in hypertension, proteinuria, and fetal growth delay. Despite extensive research, the mechanisms involved in the vascular dysfunction are still not well understood. The current hypotheses are as follows: 1) an endothelial dysfunction occurs as a consequence of placental ischemia; 2) the invading cytotrophoblasts cause shallow invasion of spiral arteries and systemic inflammation, leading to an immune disorder between mother and fetuses; and 3) genetic imprinting may be the cause of the whole problem. Animal models for such vascular dysfunction are not available at present, which makes it difficult to study the vascular component of the disease. What is clearly established is that endothelial cells overexpress procoagulant factors and that endothelium-dependent regulation of vasomotricity is impaired and even abolished in preeclampsia.^{2,3} Nevertheless, it is still unclear whether the altered levels of vascular tone observed in preeclampsia are merely a result of changes in circulating vasoactive substances^{4,5} and/or alterations of the vascular smooth muscle signaling and contraction systems.

Recently, several groups have reported elevated plasma concentration of shed membrane microparticles (MPs) during preeclampsia and have subsequently sug-

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gested their involvement in the unrelenting hypertension associated with this disease. MPs are fragments released from the plasma membrane of stimulated or apoptotic cells.^{6,7} Although the total number of circulating MPs was unaltered in preeclampsia, the proportion of T-lymphocyte and granulocyte MPs was increased.^{8,9} Circulating MPs from these patients abolished the endothelium-dependent relaxation in contrast to MPs from healthy pregnant women.¹⁰ However, the mechanisms triggering the modifications of the vessel contraction/relaxation balance in this disease are not fully elucidated inasmuch as an alteration of responsiveness of vascular smooth muscle to vasoconstrictor stimuli by MPs from preeclamptic patients have not yet been studied.

The current study was therefore designed to investigate the effect of MPs, harvested from preeclamptic women, on vascular wall structure and reactivity to vasoactive drugs. Such an understanding could help to establish whether these MPs are bad or good for the patients and whether pharmacological manipulation of their metabolism would modify this vascular issue.

Materials and Methods

Patients

This study was approved by the Ethics committee of the Hospital of Strasbourg, Strasbourg, France. After written consent, women with ($n = 21$) or without ($n = 17$) preeclampsia were included to provide MPs or omental vessels. Preeclampsia was defined according to standard criteria.¹¹

Preeclampsia is defined by the *de novo* appearance of hypertension (systolic blood pressure ≥ 140 mmHg or diastolic blood pressure ≥ 90 mmHg) associated with new onset of proteinuria, defined as $\geq 2+$ on dipstick per 24 hours detected for the first time after 20 weeks of gestation. The majority of the patients enrolled in the present study showed persistent headaches (75%), abdominal pain (62%), and severe systolic blood pressure of 164 ± 20 mmHg and diastolic blood pressure of 111 ± 10 mmHg. They exhibited new onset of proteinuria of $3+$ on dipstick associated with a clinical edema and a subsequent preterm birth and intrauterine growth restriction. Although the patients had the severe form, they did not display characteristics of central nervous system, renal, or respiratory failures. All patients were admitted to the intensive care unit for monitoring and control of their blood pressure. Patients with preexistent hypertension, gestational diabetes mellitus, coagulation abnormalities, and previous renal or hepatic disease were not included. Women with normal pregnancy without preeclampsia were considered as controls.

Circulating MPs were harvested after a two-step centrifugation ($1500 \times g$ for 15 minutes; $14,000 \times g$ for 2 minutes) of plasma samples pelleted and recovered in 1 ml of RPMI medium (Cambrex, Verviers, Belgium). A further 45 minutes of $13,000 \times g$ centrifugation was then performed, and supernatants corresponding to the last MP washing medium were used as control. Amounts of

MPs were expressed as nmol/L phosphatidylserine equivalents (nmol/L PS Eq), and their phenotyping was performed with specific monoclonal antibodies (anti-GPIb α , anti-CD11a, and anti-CD31) as described elsewhere.¹²

For each patient, circulating MP levels were determined. Vessels were then incubated with circulating levels of MPs detected in the blood of each patient to better mimic their *in vivo* effect on the vascular wall. These values ranged from 6 to 22 nmol/L Eq PS for preeclamptic patients to 3 to 17 nmol/L Eq PS for nonpreeclamptic patients. Preliminary experiments showed that 24-hour treatment of vessels with MPs leads to maximal effect. Thus, all MP experiments were performed under these conditions. Levels of endotoxin were assessed in all MP preparations with the Limulus amoebocyte lysate kit QCL-1000 (Cambrex) and were found to be below the lower detection limit of the kit (<0.1 endotoxin U/ml).

During cesareans, the specimens of omentum were immediately collected into ice-cold physiological salt solution to harvest small omental arteries (200 to 400 μ m in diameter, 2.5 to 3 mm in length). Vessels were quickly dissected of fat within 2 hours in ice-cold physiological salt solution of the following composition in mmol/L: NaCl, 119; KCl, 4.7; NaHCO₃, 14.9; MgSO₄·7H₂O, 1.2; CaCl₂, 2.5; KH₂PO₄, 1.18; and glucose, 5.5, to test vascular reactivity in a wire myograph system. The viability of omental arteries was tested by their capacity to produce reproducible contractions in response to the combination of depolarizing solution and maximally active concentration of the thromboxane agonist U46619 (10 μ mol/L; Calbiochem, London, UK).

Vascular Reactivity

Aortic rings and second generation of mesenteric arteries were obtained from female C57BL/6 mice (8 to 10 weeks of age). After 24 hours of incubation with various circulating levels of MPs from normal women (CMPs: range, 3 to 17 nmol/L Eq PS) or preeclamptic women (PrMPs: range, 6 to 22 nmol/L Eq PS), or from the last washing medium of MPs as control, human omental arteries or mouse aortas were mounted on a wire myograph. The functionality of the endothelium was assessed by the ability of acetylcholine to induce relaxation.

Concentration-response curves were constructed by cumulative application of serotonin (5-HT, 3 nmol/L to 10 μ mol/L; Sigma Aldrich, St. Quentin, Fallavier, France) to vessels with functional endothelium in the absence or presence of the given inhibitor: the nitric-oxide (NO) synthase 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-398, 10 μ mol/L; Sigma Aldrich), or L-NA plus NS-398. Both inhibitors were used at maximal active concentrations at which they inhibit the release of either NO from all isoforms of NO synthases or metabolites from COX-2 isoforms in blood vessels, as reported in many of our previous studies.^{13,14} Higher concentrations of either L-NA or NS-398 did not induce further inhibition.

Mouse mesenteric arteries (100 to 130 μm in diameter) were isolated and incubated *ex vivo* for 24 hours with the different circulating levels of PrMPs (range, 6 to 22 nmol/L Eq PS) or CMPs (range, 3 to 17 nmol/L Eq PS). They were then mounted in a video-monitored perfusion system (Living Systems Instrumentation, Burlington, VT) to study the physiological endothelial dilatation in response to shear stress. Diameter changes were measured by increasing flow rate (0 to 92 $\mu\text{l}/\text{minute}$) under a constant intraluminal pressure of 75 mmHg.

To determine which component(s) of MPs are responsible for the vascular effects, GPIIb α ⁺ MPs were isolated from plasma using the magnetic anti-biotin microbeads MidiMACS isolation kit according to the instructions of the manufacturer. Briefly, MPs were incubated with biotinylated antibody to GPIIb α -biotin (gift of Dr. François Lanza, U. 311 INSERM, Strasbourg, France) for 30 minutes at 4°C, and then 20 μl of anti-biotin microbeads were added for 15 minutes at 4°C. MP suspension was applied onto the MS column, and GPIIb α ⁺ MPs were recovered. GPIIb α ⁺ MPs were obtained after removing the column from the magnetic separator. Anti-biotin microbeads alone were without effect on the vasomotricity measurements. Ninety-nine percent of circulating platelet-derived MPs (GPIIb α ⁺ MPs) were removed. For each sample 10,000 events were analyzed by flow cytometry.

In another set of experiments, mice were treated *in vivo* by intravenous injection into the tail vein of MPs ($n = 5$) isolated from either healthy pregnant or preeclamptic women. We injected the mice with the equivalent of the circulating level of MPs detected *in vivo* for each preeclamptic or nonpreeclamptic patient. After 24 hours, aortas were isolated to study vascular reactivity.

NO Spin Trapping and Electronic Paramagnetic Resonance Studies

The detection of NO production was performed using the technique with Fe²⁺ diethyldithiocarbamate (DETC; Sigma Aldrich) as spin trap.¹⁵ After administration of 5-HT, vessels that had been preincubated with MPs were placed in 24-well clusters filled with 250 μl of Krebs solution and then treated with 250 μl of colloid Fe(DETC)₂ and incubated at 37°C for 1 hour. These studies were performed on a tabletop x-band spectrometer miniscope (Magnetech, Berlin, Germany). Recordings were made at 77°K using a Dewar flask. Instrument settings were 10 mW of microwave power, 1 mT of amplitude modulation, 100 kHz of modulation frequency, 60 seconds of sweep time, and 10 scans.

Determination of Prostanoid Production

After incubation with MPs, vessels were treated with 5-HT (10 $\mu\text{mol}/\text{L}$, 37°C, 20 minutes). After collection of the medium, thromboxane B₂, prostaglandin E metabolites, and total 8-isoprostanes were measured by enzyme immunoassays kits (Cayman Chemicals, Montluçon, France). The concentration of prostanoids was expressed as pg/mg tissue (dry weight).

Staining and Imaging by Confocal Microscopy

Vessels were frozen and cut into 10- μm sections. Fixed sections were incubated (2 hours at room temperature) in a blocking buffer (5% nonfat dry milk in phosphate-buffered saline). Tissue sections were then incubated overnight (4°C) with monoclonal murine anti-iNOS (1:50; Transduction Laboratories, Heidelberg, Germany) or anti-COX-2 (1:100; Transduction Laboratories) antibodies. Polyclonal nuclear factor (NF)- κB p65 antibody (1:100; Abcam, Cambridge, UK) or a mouse monoclonal anti-nitrotyrosine (clone 1A6) antibody (1:100; Upstate Cell Signaling Solutions, Hampshire, UK) were used for the NF- κB p65 or for nitrotyrosine immunostaining, respectively. Three washes were followed by incubation (1 hour, 37°C) with secondary murine or rabbit fluorescent Alexa fluor-488-labeled antibody (1:100; Invitrogen Molecular Probes, Leiden, The Netherlands). In another set of experiments, *in situ* production of superoxide was evaluated with the oxidative fluorescent dye dihydroethidine (Sigma Aldrich) according to Miller and colleagues.¹⁴ After washing, vessel sections were mounted on glass slides. MRC-1024ES confocal equipment mounted on a Nikon Eclipse TE 300 inverted microscope was used for the optical sectioning of the tissue. Digital image recording was performed using the LaseSharp Software.

Data Analysis

Data are presented as mean $\pm$ SEM, and n represents the number of animal or human samples. Statistical analyses were performed by a one-way analysis of variance, Kruskal-Wallis and Mann-Whitney *U*-tests, or two-way analysis of variance for repeated measurements, and subsequent Tukey post hoc tests were performed with the Statview version 5.0 software (SAS Institute, Cary, NC). $P < 0.05$ was considered to be statistically significant.

Results

Circulating Level of MPs Is Increased in Preeclamptic Patients Compared with Healthy Pregnant Women

The total number of circulating MPs was significantly increased in preeclampsics (Table 1). Phenotypic characterization of cellular origin of MPs showed increased leukocyte- and platelet-derived MPs in the bloodstream of preeclampsics.

MPs from Preeclampsics Did Not Impair Endothelial Responses in Either Aorta or Mesenteric Arteries of the Mice

The relaxation to acetylcholine was not significantly different between control aortas, CMP- or PrMP-treated vessels being, respectively, $85 \pm 6\%$ ($n = 6$), $86 \pm 7\%$ ($n = 9$), and $86.9 \pm 3\%$ ($n = 9$) of the level of the same precontraction. Likewise, CMP or PrMP treatment did not

Table 1. Clinical Characteristics and MP Phenotyping of Normal Pregnant Women (CMPs) or Women with Preeclampsia (PrMPs)

	CMPs (normal pregnancy) (n = 17)	PrMPs (preeclampsia) (n = 21)
Age (years)	28 ± 5	28 ± 6
Gestational age (weeks)	35 ± 4	32 ± 4
Parity	0.64 ± 0.78	0.18 ± 0.54
Systolic blood pressure (mmHg)	114 ± 10	164 ± 20 [†]
Diastolic blood pressure (mmHg)	71 ± 7	111 ± 10*
Proteinuria	0	+++
Delivery gestational age (weeks)	39 ± 1	32 ± 4*
Baby birth weight (g)	3229 ± 438	1517 ± 676*
MPs (nmol/L Eq PS)	7.5 ± 1.35	11.68 ± 1.09*
Lymphocytes MPs (CD11a) (nmol/L Eq PS)	2.9 ± 0.65	5.85 ± 0.61 [†]
Platelet MPs (GP1b) (nmol/L Eq PS)	4.2 ± 0.94	5.83 ± 0.65*
Endothelial MPs (CD31) (nmol/L Eq PS)	Nonidentified	Nonidentified

*P < 0.05.

[†]P < 0.01.

affect flow-induced endothelial vasodilatation in mesenteric arteries being $15.1 \pm 1.81 \mu\text{m}$ (n = 5) and $14.97 \pm 2.29 \mu\text{m}$ (n = 5), respectively.

MPs from Preeclamptics Decreased the Responsiveness to 5-HT of Both Human Resistance Vessels and Mouse Aortic Rings

5-HT produced a concentration-dependent increase in tension in human vessels and mouse aortic rings with functional endothelium. Incubation for 24 hours of either human omental arteries (Figure 1A) or mouse aortic rings (Figure 1B) with circulating levels of CMPs did not significantly affect contractile response to 5-HT compared with control vessels not treated with MPs (Figure 1, A and B). In contrast, PrMPs decreased vascular reactivity to this agonist in the two groups of vessels studied. It should be noted that whatever the circulating level of MPs in non-preeclamptic patients was, they were not able to induce vascular hyporeactivity. On the other hand, whatever the circulating level of MPs from preeclamptic patients was,

they were able to reduce contraction to 5-HT under these experimental conditions.

To investigate the mechanisms involved in vascular hyporeactivity induced by PrMPs, the role of NOS and COX-2 metabolites was evaluated by studying the effect of L-NA alone, NS-398 alone, or in combination, in mouse aortas in response to 5-HT. L-NA did not significantly modify the contractile responses to 5-HT in mouse aortas incubated with CMPs (Figure 2A). However, L-NA significantly enhanced the response to the same agonist in aortas treated with PrMPs compared with those treated with CMPs (Figure 2B). Both aortic rings with functional endothelium treated with either CMPs or PrMPs, preincubated with Fe(DETC)₂, exhibited an electronic paramagnetic resonance feature of signals derived from NO-Fe-(DETC)₂ (not shown). As shown in Figure 2C, incubation of aortas for 24 hours with PrMPs elicited an ~3.5-fold increase in the electronic paramagnetic resonance signal when compared with the signal obtained in aortas incubated with CMPs. Immunohistochemical detection of iNOS using confocal microscopy was conducted.

Figure 1. PrMPs decrease a 5-HT contraction elicited on the human and mouse vessels. **A:** Concentration-effect curves of human omental arteries (n = 5) exposed to increasing concentrations of 5-HT after incubation for 24 hours with either PrMPs (range, 6 to 22 nmol/L Eq PS; ■) or CMPs (range, 3 to 17 nmol/L Eq PS; □) or medium containing the last washing medium of MPs used as control (△). **B:** Concentration-effect curves to increasing concentrations 5-HT in mouse aortic rings (n = 9) after incubation for 24 hours with either PrMPs (range, 6 to 22 nmol/L Eq PS; ■) or CMPs (range, 3 to 17 nmol/L Eq PS; □) or medium without MPs (△). PrMPs decreased significantly the maximal contraction induced by 5-HT in human and mouse vessels. Please note that there was no decrease in contraction to the same agonist between vessels exposed to CMPs and the control obtained after incubation in medium without MPs.

Figure 2. PrMPs induce NO overproduction and iNOS expression in vessels. **A:** Concentration-effect curves of 5-HT in the presence (○) and in the absence (□) of L-NA (100 μ mol/L) in mouse aortas incubated with CMPs for 24 hours (range, 3 to 17 nmol/L Eq PS; $n = 9$). **B:** Concentration-effect curves of 5-HT after 24 hours of preincubation with PrMP mouse aortas (range, 6 to 22 nmol/L Eq PS; $n = 9$) in the presence (●) and in the absence (■) of L-NA (100 μ mol/L). **C:** Quantification of the amplitude of NO-Fe(DETC)₂ signal in unit/weight (mg of the dried sample A/W_(ds), $n = 8$) in mouse aortas after incubation with either CMPs or PrMPs and exposed to 5-HT (10 μ mol/L). **D–F:** Immunohistochemical staining of inducible NO synthase for mouse aorta after incubation with either medium without MPs, medium containing CMPs, or medium containing PrMPs, respectively.

Whereas weak or no staining of iNOS was found in the vessel wall of control (2.43 ± 0.13 arbitrary units) or CMP-treated aortas (2.80 ± 0.31 arbitrary units; Figure 2, D and E), marked iNOS labeling was observed in the medial layer of aortas treated with PrMPs [44.40 ± 8.13 arbitrary units (Figure 2F), $*P < 0.05$ significantly different to control or CMP-treated aortas]. The negative control obtained by incubation with the secondary murine fluorescence-labeled antibodies did not display any staining (not shown).

A selective inhibitor of COX-2, NS-398, significantly reduced the contractile response to 5-HT in vessels treated with CMPs (Figure 3A). Surprisingly, NS-398 abolished the contraction to the same agonist in mouse arteries treated with PrMPs (Figure 3B). These results suggest that both CMPs and PrMPs stimulate the release of COX-2-vasoconstrictor products. No or weak staining of COX-2 was found in the vessel wall of control (2.11 ± 1.08 arbitrary units) or CMP-treated aortas [5.71 ± 0.85 arbitrary units (Figure 3, C and D, respectively), $*P < 0.05$ significantly different to control vessels]. However, marked COX-2 labeling was observed in the medial layer of aortas treated with PrMPs [32.53 ± 0.67 arbitrary units (Figure 3E), $*P < 0.05$ significantly different to control or CMP-treated vessels]. Finally, the combination of NO and COX-2 inhibitors strongly enhanced the contraction in response to serotonin administration in mouse aortas treated with PrMPs (Figure 3B) but not in CMP-treated aortas (Figure 3A), suggesting a role for

relaxant factors in vascular hyporeactivity of PrMP-treated vessels.

MPs Stimulate the Release of Vasoconstrictor Prostanoids

Assaying for thromboxane B₂, the stable metabolite of thromboxane A₂, showed a reduced production of thromboxane A₂ in mouse aortas treated with PrMPs compared with those incubated with CMPs (Figure 4A). The release of PGE₂ was not different between CMP- and PrMP-treated arteries (Figure 4B). Interestingly, measurement of 8-isoprostane production showed an increased synthesis of this compound in aortas treated with PrMPs when compared with vessels treated with CMPs (Figure 4C).

PrMPs Activate NF- κ B Detected by p65/RelA Staining and Induce Oxidative and Nitrosative Stresses in the Vascular Wall of Mouse Aortas

Because enhanced expression of proinflammatory enzymes such as iNOS and COX-2 is under the control of the nuclear factor (NF)- κ B/RelA family of transcription factors, we assessed their activation. NF- κ B family members are heterodimers of p65/RelA and p50/NF- κ B, but only the p65 subunit has transactivation do-

Figure 3. PrMPs induce COX-2 expression. **A:** Concentration-effect curves of 5-HT in the presence (◊) and in the absence (□) of NS-398 (10 μmol/L) or in the presence of L-NA plus NS-398 (Δ) in mouse aortas incubated with CMPs for 24 hours (range, 3 to 17 nmol/L Eq PS; n = 9). ***P* < 0.01, significantly different from CMP-incubated vessels without inhibitors. **B:** Concentration-effect curves of 5-HT after 24 hours of incubation with PrMP mouse aortas (range, 6 to 22 nmol/L Eq PS; n = 9) in the presence (◆) and in the absence (■) of NS-398 (10 μmol/L) or in the presence of L-NA plus NS-398 (▲). After incubation for 24 hours with PrMPs, the association of L-NA and NS-398 enhanced the contraction to 5-HT in aortas preincubated with PrMPs but not with CMPs. ****P* < 0.001, significantly different from PrMP-incubated vessels without inhibitors. **C–E:** Immunohistochemical staining of inducible COX-2 for mouse aortas after incubation with medium without MPs, medium containing CMPs, or medium containing PrMPs, respectively. Background of secondary Alexa 488-conjugated anti-mouse antibody (not shown).

mains capable of initiating transcription. The NF-κB heterodimer co-localizes in the cytoplasm with the inhibitory protein IκB. On cell stimulation, IκB is phosphorylated, removed, and degraded, allowing free NF-κB to induce transcription. Activation can be evidenced by staining the p65 subunit. No specific stain-

ing was found in control aortas (6.24 ± 0.05 arbitrary units), whereas weak staining was found in aortas incubated with CMPs [12.96 ± 0.33 arbitrary units (Figure 5, A and B, respectively), **P* < 0.05 significantly different from control vessels]. By contrast, immunohistochemical studies showed marked aortic staining of

Figure 4. PrMPs and prostaglandin production. Concentration of the COX derivatives in the supernatants of mouse aortas exposed to CMPs (range, 3 to 17 nmol/L Eq PS; white) or to PrMPs (range, 6 to 22 nmol/L Eq PS; black) for 24 hours and stimulated with 5-HT (10 μmol/L). **A:** Thromboxane B₂ was decreased in medium deriving from vessels preincubated with PrMPs (n = 3). **B:** Concentration of PGE₂ was not different in the two groups of medium from CMP- or PrMP-treated vessels (n = 3). **C:** 8-Isoprostane was strongly enhanced in the medium from aortas preincubated with PrMPs compared with CMP-treated vessels (n = 3). **P* < 0.05, ****P* < 0.001, significantly different from medium of CMP-incubated vessels.

Figure 5. PrMPs induce the activation of NF- κ B p65 and oxidative stress. **A–C:** Immunohistochemical staining for NF- κ B p65 of mouse aortas after incubation with medium without MPs or containing either CMPs or PrMPs, respectively ($n = 3$). Green fluorescence was linked to secondary Alexa 488-conjugated anti-rabbit antibody. **D–F:** Immunohistochemical staining for nitrotyrosine of mouse aortas after incubation with either medium without or containing CMPs or PrMPs, respectively ($n = 3$). Green fluorescence was linked to secondary Alexa 488-conjugated anti-mouse antibody. **G–I:** *In situ* detection of superoxide in mouse aorta sections after incubation with either medium with or without CMPs or PrMPs, respectively ($n = 3$). Sections of aortas were labeled with the oxidative dye hydroethidine (red fluorescence when oxidized to EtBr by superoxide).

p65/RelA subunit of NF- κ B in the vascular wall of aortas incubated with PrMPs [29.45 ± 4.14 arbitrary units (Figure 5C), $*P < 0.05$ significantly different to control or CMP-treated aortas]. Negative controls obtained by incubation with the secondary rabbit fluorescence-labeled antibody do not display any staining (not shown).

To investigate the possible involvement of MPs in oxidative stress of the vascular wall, we evaluated the *in situ* production and the topographical distribution of reactive oxygen species (ROS). Tissue sections from vessels treated with PrMPs displayed a marked increase in ethidium bromide fluorescence [12.21 ± 1.15 arbitrary units (Figure 5I), $*P < 0.05$], reflecting an increase in the vascular wall

oxidative stress compared with control (2.80 ± 0.34 arbitrary units) and CMP-treated aortas (4.61 ± 0.65 arbitrary units) (Figure 5, G and H, respectively).

NO reacts with O_2^- , and this reaction leads to the production of the powerful oxidant peroxynitrite. Peroxynitrite modifies tyrosine in proteins, resulting in nitrotyrosine residues. The sections from vessels treated with PrMPs showed a marked increase in nitrotyrosine staining (15.74 ± 2.70 arbitrary units, $*P < 0.05$; Figure 5F), reflecting an increase degree of nitrotyrosilation in the vascular wall compared with control (1.61 ± 0.23 arbitrary units) and CMP-treated aortas (2.74 ± 0.74 arbitrary units) (Figure 5, D and E, respectively).

Figure 6. Effect of platelet or leukocyte MPs from preeclampsics in 5-HT-induced contraction in mice aortic rings. **A:** Flow cytometry histograms showing the surface expression levels of GPIIb/alpha in total PrMP samples and in fractions obtained after separation of MPs as described in Materials and Methods. Fluorescence (FL2, red fluorescence channel intensity, log scale) is expressed in arbitrary units (a.u.). **B:** Concentration-effect curves to 5-HT in mouse aortic rings (n = 5) after incubation for 24 hours with either platelet MPs (■) or leukocyte MPs (●) from preeclampsics or medium without MPs (□). **C:** Concentration-effect curves to 5-HT in the absence (□) and in the presence of the NO synthase inhibitor (○) L-NA (100 μmol/L) or of NS-398 (10 μmol/L) (◇) or of L-NA plus NS-398 (Δ) in mouse aortic rings incubated with platelet MPs from preeclampsics for 24 hours (n = 5). **D:** Concentration-effect curves to 5-HT in the absence (■) and in the presence of the NO synthase inhibitor (●) L-NA (100 μmol/L) or of NS-398 (10 μmol/L) (◆) or of L-NA plus NS-398 (▲) in mouse aortic rings incubated with leukocyte MPs from preeclampsics for 24 hours (n = 5). **P < 0.01, ***P < 0.001, significantly different data obtained in the absence of inhibitors.

Vascular Effects of the Differential Component(s) of Circulating MPs

After separation of MP samples, two fractions were obtained (Figure 6A): GPIIb/alpha⁺ MPs, which were selected from the platelet fraction, and GPIIb/alpha⁻ MPs of nonplatelet origin. Incubation for 24 hours of mouse aortic rings (Figure 6B) with circulating levels of GPIIb/alpha⁺ or GPIIb/alpha⁻ MPs from preeclampsics significantly decreased vascular contraction to 5-HT. To investigate the mechanisms involved in vascular hyporeactivity induced by the two fractions of MPs (GPIIb/alpha⁺ and GPIIb/alpha⁻ MPs), the role of NOS and COX-2 metabolites was evaluated by studying the effect of L-NA and NS-398 either alone or in combination. The inhibition of NOS with L-NA significantly enhanced the response to 5-HT in vessels treated with either GPIIb/alpha⁺ or GPIIb/alpha⁻ MPs (Figure 6, C and D). The selective inhibitor of COX-2 NS-398 did not modify the

contraction to 5-HT in vessels treated with GPIIb/alpha⁺ MPs (Figure 6C), but, surprisingly, it abolished the contraction to the same agonist in mouse arteries treated with GPIIb/alpha⁻ MPs (Figure 6D). These results suggest that only GPIIb/alpha⁻ MPs stimulate the release of COX-2 vasoconstrictor products. It is interesting to note that MPs of endothelial origin were not detected under the experimental conditions used. Thus, it is likely that GPIIb/alpha⁻ MPs are mainly derived from leukocyte origin.

Finally, the combination of NO and COX-2 inhibitors enhanced the contraction in response to 5-HT administration in aortas treated with GPIIb/alpha⁺ MPs (Figure 6C) but not in GPIIb/alpha⁻ MP-treated aortas (Figure 6D). Altogether, these results support the relevance of relaxant factors in vascular hyporeactivity of GPIIb/alpha⁺ MPs from platelet origin-treated vessels on the one hand and, on the other, the important role played by COX-2-dependent vasoconstrictor factors in GPIIb/alpha⁻ MP-treated vessels.

Figure 7. *In vivo* treatment of mice with PrMPs decreases 5-HT-induced contraction in aortic rings. Concentration-effect curves to 5-HT in mouse aortic rings ($n = 5$) after *in vivo* treatment for 24 hours with either PrMPs (range, 6 to 22 nmol/L Eq PS; ■) or CMPs (range, 3 to 17 nmol/L Eq PS; □).

In Vivo Treatment of Mice with PrMPs Induces Vascular Hyporeactivity

To evaluate the *in vivo* significance of PrMPs in the vascular reactivity, circulating levels of PrMPs or CMPs were injected in the tail vein of mice. Twenty-four hours after the injection, vessels were isolated from mice to study the vasomotricity. In intact endothelium preparations, aortas taken from PrMP-treated mice displayed lower contractile response to 5-HT than vessels from mice treated with CMPs (Figure 7). Furthermore, PrMP-induced vascular hyporeactivity to 5-HT was reversed by the combination of L-NA and NS-398 (not shown).

Discussion

In the present study, we provide evidence that PrMPs induced vascular hyporesponsiveness to a vasoconstrictor agent in both human omental arteries and mouse aortic rings without changes in endothelial function. These effects were observed at circulating levels of PrMPs, and they were associated with an up-regulation of proinflammatory protein expression, namely iNOS and COX-2, through the activation of the transcription factor RelA/NF- κ B in the vessel wall. Thus, PrMPs stimulated the release of NO and COX-2 metabolites. The proinflammatory property of PrMPs leads to the observation of surrogate signs of oxidative and nitrosative stresses in the vascular wall. Interestingly, GPIb α ⁺ MPs, most probably of leukocyte origin, induced both the release of NO and COX-2 vasoconstrictor products of PrMPs, whereas those from platelet origin are able to stimulate the release of NO only. In this context, the balance of the release of these products is shifted toward an enhanced participation of NO and thus vascular hyporeactivity. All these findings suggest that PrMPs of platelet origin could play a protective role in preeclampsia because NO blunts the increased production of vasoconstrictor substances from COX-2 induced by PrMPs from leukocytes. Finally, we provide evidence of the *in vivo* effect of PrMPs and

thus their role in preeclampsia in terms of vascular hyporeactivity.

The pathophysiology of preeclampsia is associated with systemic vascular inflammation with neutrophil activation¹⁶ and oxidative stress.^{17–19} However, the mechanisms of vascular alteration during preeclampsia are not well understood with regard to the role of MPs. Previous studies concerning MPs in normal pregnancy and preeclampsia showed that the total number of circulating MPs was not significantly altered despite an increase in the number of T-cell and granulocyte MPs.¹⁰ In another study,⁹ a close relationship between endothelial dysfunction and circulating levels of endothelial MPs was reported in preeclamptic patients. The level of platelet MPs was either reduced¹⁰ or unchanged⁹ between preeclamptic and nonpreeclamptic patients. In the present work, women with preeclampsia displayed elevated levels of MPs derived from lymphomonocytes and platelets in their bloodstream compared with normal pregnancy. The levels of the former are more elevated than in those of the latter. We could not detect endothelial MPs in the plasma from normal or preeclamptic women, possibly because the levels of these MPs were too low to be detected by the sensitivity of the method used.

In previous studies,^{2,20} the involvement of MPs in the vascular dysfunction of preeclampsia has been suggested, in line with the idea that these vesicles are a sign of severity including the impairment of endothelial-dependent relaxation. In the present study, PrMP-treated vessels did not display endothelial dysfunction. The discrepancy with the previous study of VanWijk and co-workers²⁰ could be attributable to the different experimental conditions (method used to harvest PrMPs, MP concentration, time, and means used to incubate vessels). The experimental conditions used in the present work were conducted using circulating level of MPs from each patient to better mimic the *in vivo* conditions. We showed that MPs might be a means to blunt the hyperreactivity of vessels to vasoconstriction, which is a classic feature of preeclampsia-induced hypertension. Thus, our data shed new light on the significance of enhanced MP levels during pregnancy. According to recent reviews^{21,22} and our previous study,¹³ in which MPs participate in inflammation and vascular (dys)function, we found that PrMPs are effective in reducing the 5-HT-elicited vascular contraction *ex vivo*. The composition and constituents of PrMPs probably drive the vascular effects of such MPs.

The mechanisms involved in vascular effects of MPs were studied in the aortas of mice. In the present study, PrMPs compared with CMPs were able to produce both oxidative and nitrosative stresses in the vessel wall as shown by the increase of dihydroethidine staining for O₂^{•−} and protein nitrotyrosine staining for peroxynitrite formation. Reactivity for both occurs throughout the vessel wall, mainly the medial and adventitial layers. The synthesis of acute phase inflammatory mediators including NO through iNOS and COX-2 metabolites is regulated by the activation of NF- κ B. We showed an up-regulation of NF- κ B associated with a significant up-regulation of iNOS

in the vessel wall on PrMP exposure. Such an effect might lead to intense vasodilatation, and subsequent potential hypotension such as that observed in severe sepsis²³ or acute liver failure.¹⁴ The discrepancy between up-regulation of iNOS releasing huge amounts of NO and hypertension is striking in light of a simultaneous up-regulation of COX-2,^{24,25} which has been reported in pregnant animals²⁶ and in preeclamptic women.²⁷ Many studies have established that MPs up-regulate COX-2^{6,28}; moreover, preeclamptic women have increased plasma concentrations of substances such as vasoconstrictor eicosanoids.²⁹ Here we found both up-regulation of COX-2 and higher release of the vasoconstrictor 8-isoprostane on PrMP stimulation compared with CMPs. This suggests that PrMPs directly or indirectly modify the activity of 5-HT with a shift toward substantial vasoconstriction through isoprostanes.^{30,31} In line with these results, women with preeclampsia have increased participation of the COX-2 pathway, which decreases only after delivery.^{32,33} An increase in 8-isoprostane may lead to a progressive increase in vascular resistance and vasoconstriction and counterbalance the effects of NO itself. Indeed, during preeclampsia, 8-isoprostane is present and active as reported by Rajmakers and colleagues,¹⁸ and its inhibition can prevent potential hypertension.³⁴ It should be noted that the net effect of PrMPs on vascular hyporeactivity in response to 5-HT despite increased 8-isoprostane is probably attributable to their capacity in releasing a huge amount of NO. At the present, further studies are needed to sort out this paradox.

The components of PrMPs responsible for vascular reactivity have been investigated, and they can be considered to be of both platelet and leukocyte origins because MPs were GPIb α^+ and GPIb α^- , respectively. Under the experimental conditions used, we were not able to detect MPs from endothelial origin. Interestingly, we found opposite effects of MPs depending on their origin. Whereas platelet PrMPs induced production of NOS-related vasodilator products, aortas treated by PrMPs from leukocytes released, in addition to NO, COX-2-dependent vasoconstrictor metabolites. This could be related to the different MP compositions as functions of their origin, as shown in the proteomic analyses from platelet and leukocytic MPs.^{35,36} PrMPs from platelets induced vasodilator products, and this property might protect against vasoconstrictor factors generated by leukocyte PrMPs.

Finally, we show that *in vivo* treatment of mice with PrMPs induced similar results to those obtained in *ex vivo* incubation of mouse vessels with PrMPs. Together, these results strongly suggest that circulating MPs from preeclamptic patients are able to induce *in vivo* vascular hyporeactivity and demonstrate, for the first time, the *in vivo* effect of PrMPs, and thus their pathophysiological relevance, in vascular reactivity.

In conclusion, we report that PrMPs but not CMPs elicit an intense vascular wall inflammation in vessels from different species. These particles have a double-sided action: they blunt the vasoconstrictive effect of 5-HT by up-regulating iNOS with subsequent NO overproduction, and this vasodilator effect seems to be induced by the

PrMPs from both platelet and leukocyte origins. Furthermore, PrMPs can also be considered as protective against a potential increase in vascular tone by 8-isoprostane, which may occur from COX-2 likely induced by leukocyte-derived PrMPs. Because we demonstrate that PrMPs are effective *in vivo* in terms of vascular reactivity, one can advance the hypothesis that PrMPs from platelets may serve as a protective mechanism against hypertension during preeclampsia.

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