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Vascular Biology, Atherosclerosis and Endothelium Biology

Endothelial Dysfunction Caused by Circulating Microparticles from Patients with Metabolic Syndrome

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Microparticles are membrane vesicles that are released during cell activation and apoptosis. Elevated levels of microparticles occur in many cardiovascular diseases; therefore, we characterized circulating microparticles from both metabolic syndrome (MS) patients and healthy patients. We evaluated microparticle effects on endothelial function; however, links between circulating microparticles and endothelial dysfunction have not yet been demonstrated. Circulating microparticles and their cellular origins were examined by flow cytometry of blood samples from patients and healthy subjects. Microparticles were used either to treat human endothelial cells *in vitro* or to assess endothelium function in mice after intravenous injection. MS patients had increased circulating levels of microparticles compared with healthy patients, including microparticles from platelet, endothelial, erythrocyte, and procoagulant origins. *In vitro* treatment of endothelial cells with microparticles from MS patients reduced both nitric oxide (NO) and superoxide anion production, resulting in protein tyrosine nitration. These effects were associated with enhanced phosphorylation of endothelial NO synthase at the site of inhibition. The reduction of O_2^- was linked to both reduced expression of $p47^{phox}$ of NADPH oxidase and overexpression of extracellular superoxide dismutase. The decrease in NO production was triggered by nonplatelet-derived microparticles. *In vivo* injection of MS microparticles into mice impaired endothelium-dependent relaxation and decreased endothelial NO synthase expression. These data provide evidence that circulating microparticles from MS patients influence endothelial dysfunction. *(Am J Pathol 2008, 173:1210–1219; DOI: 10.2353/ajpath.2008.080228)*

The metabolic syndrome (MS) is a cluster of metabolic abnormalities including visceral obesity, high blood pressure, hyperglycemia, and dyslipidemia,[1–4] which are associated with increased cardiovascular risk and diabetes of type 2.[5,6] The MS is becoming a public health concern because its prevalence is rapidly increasing in the world because of an increased incidence of overweight or obesity and physical inactivity.[7,8] In this context, it is now well established that MS is associated with cardiovascular morbidity and mortality.

The pathophysiology seems to be primarily attributable to insulin resistance with excessive flux of fatty acids implicated.[2,9] A proinflammatory state linked to the production of cytokines from a variety of cells including adipocytes and macrophages contributes to the syndrome.[2–5] Thus, the structural and cardiovascular alterations linked to MS are associated with both increased inflammatory factors and reactive oxygen species. In the context of MS, a variety of defective mechanisms occurs including increased reactive oxygen species from the hyperreactivity of the neuro-humoral systems, endothelial dysfunction and alterations of vascular reactivity, and inflammation that take place in the chronic macro- and microcirculation perturbations.[2–5] Inflammation is the key pathogenic component of atherosclerosis, promotes thrombosis, a process that underlies acute coronary event and stroke. Inflammation is orchestrated by the

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interactions between inflammatory cells, such as leukocytes and vascular cells, endothelial and smooth muscle cells, which under activation or apoptosis, for example, lead to the release of circulating microparticles (MPs).[10–12]

MPs are membrane vesicles with procoagulant and proinflammatory properties.[10–12] MPs are present in blood from healthy individuals and elevated in patients under pathological states, such as sepsis,[13] preeclampsia,[14] Crohn's disease,[15] and also in various clinical situations associated with thrombosis[16] and in diabetic patients,[17] strengthening the notion that MPs may play a role in these diseases. Indeed, MPs can be considered as vectors of biological messages, such as induction of endothelial and vascular dysfunctions or platelet activation.[10–12]

With regard to MS, tissue factor-positive MPs are associated with components of the MS but not with coagulation in asymptomatic patients with well-regulated type 2 diabetes.[18] Thus, tissue factor on MPs may be involved in processes other than coagulation, including transcellular signaling or angiogenesis. Recently, Arteaga and colleagues[19] reported endothelial cell MP release, platelet and leukocyte activation, and increased binding of MPs from endothelial cells and platelets to leukocytes in patients with the MS. Whether endothelial MPs are purely a marker of endothelial activation in the MS or play a role in promoting coagulation and atherogenesis *in vivo* is unknown. Finally, Chironi and colleagues[20] showed in patients with MS, leukocyte-derived MP level is higher than in those free of such syndrome and in the overall study population, leukocyte-derived MP level increased gradually in parallel with the number of components of MS.

To the best of our knowledge, none of the above studies have made a direct correlation between circulating MPs and endothelial dysfunction inasmuch the later is the primary target leading to atherothrombotic diseases. Thus, the aim of this work was firstly to characterize circulating MPs from healthy patients and patients with MS according to their cellular origins and procoagulant properties, and secondly to determine *in vitro* effects of MPs from MS on endothelial cells with respect to the molecular pathways implicated in NO and superoxide anion O_2^- productions and to the release of cytokines involved in inflammation. Finally, MPs were injected intravenously into mice to test their pathophysiological relevance *in vivo*. For the later, endothelial function was investigated by assessing the endothelium-dependent relaxation in response to acetylcholine in aorta of the mice.

Materials and Methods

Patients

This study was approved by the ethics committee of the University Hospital of Angers (France). We included 54 patients with MS from the Department of Endocrinology and Nutrition of the University Hospital of Angers. Patients were eligible for inclusion, according to the National Cholesterol Education Program-Adult Treatment Panel III, when they had at least three criteria of the five following: 1) waist circumference >102 or 88 cm for men and women, respectively; 2) high systolic and diastolic pressures >130/85 mmHg; 3) fasting glycemia >5.5 mmol/L; 4) triglycerides >1.65 mmol/L; and 5) high-density lipoprotein <1 mmol/L in men or <1.3 mmol/L in women. Patients with a history of cardiovascular diseases, preexistent chronic inflammatory disease, and cancer were not included. Normal controls consisted of 43 patients who met less than two of the MS criteria (40% without any component of MS). All donors accepted to participate to the follow-up study.

MP Isolation

Peripheral blood (20 ml) from healthy donors and patients was collected in ethylenediaminetetraacetic acid tubes (Vacutainers; 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. After a 20-minute centrifugation at $270 \times g$, platelet-rich plasma was separated from whole blood. Then, platelet-rich plasma was centrifuged 20 minutes at $1500 \times g$ to obtain platelet-free plasma (PFP). Two hundred μl of PFP were frozen and stored at −80°C until subsequent use. Remaining PFP was subjected to three series of centrifugations at $21{,}000 \times g$ for 45 minutes to pellet MPs for *in vitro* studies, and the supernatant was replaced by 200 μl of 0.9% saline salt solution and stored at 4°C until subsequent use.

Characterization of MP Phenotype

Membrane MP subpopulations were discriminated in PFP according the expression of membrane-specific antigens. Numeration of endothelial MPs was performed using anti-CD146 labeling; numeration of platelet, erythrocyte, and leukocyte MPs was performed using anti-CD41, anti-CD235a, and anti-CD45 labeling, respectively. Anti-CD62L and anti-CD62P were used to numerate P-selectin$^+$ and L-selectin$^+$ MPs, respectively. Irrelevant human IgG was used as an isotype-matched negative control for each sample. For numeration studies, 10 μl of PFP were incubated with either 5 μl of specific antibody (Beckman Coulter, Villepinte, France). Annexin V binding was used to numerate phosphatidylserine-expressing circulating MPs (2 μl of annexin V/5 μl PFP). After 30 minutes of incubation at room temperature, samples were diluted in 300 μl of 0.9% saline salt solution or annexin-V labeling buffer, respectively. Then, an equal volume of sample and Flowcount beads were added and samples were analyzed in a flow cytometer 500 MPL system (Beckman Coulter).

In another set of experiments, to determine which component(s) of MPs are responsible for the cellular effects, platelet MPs were isolated from plasma using the magnetic anti-CD61 microbeads MidiMACS isolation kit (Bergisch Gladbach, Germany) according to the instructions of the manufacturer. Briefly, MPs were incubated with

20 μl of human anti-CD61 microbeads for 15 minutes at 4°C. MP suspension was applied onto the MS column, and nonplatelet MPs were recovered. Platelet MPs were obtained after removing the column from the magnetic separator. Ninety-nine percent of circulating platelet-derived MPs was removed.

Cell Culture

The Eahy 926 endothelial cell line was maintained in culture in medium as previously described.[21] Cells were treated for 24 hours by MPs from patients or healthy subjects at the circulating levels of MPs detected in the blood of each patient or healthy subject, as previously performed in other pathologies.[22,23] In another set of experiments, cells were treated with vehicle (anti-CD61 microbeads alone), platelet- or nonplatelet-derived MPs from MS patients before NO production being assessed.

NO Spin Trapping and Electronic Paramagnetic Resonance (EPR) Studies

The detection of NO production was performed using the technique with Fe^{2+} diethyldithiocarbamate (DETC; Sigma-Aldrich, St. Quentin, Fallavier, France) as spin trap as described previously.[21,24] Briefly, cells were treated with 250 μl of colloid $Fe(DETC)_2$ and incubated at 37°C for 45 minutes. NO measurement was performed on a tabletop x-band spectrometer miniscope (MS200; Magnettech, Berlin, Germany).

Superoxide Anion (O_2^-) Determination by EPR

Cells were allowed to equilibrate in deferoxamine-chelated Krebs-Hepes solution containing 1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidin (CMH; Noxygen, Mainz, Germany) (500 μmol/L), deferoxamine (25 μmol/L), and DETC (5 μmol/L) under constant temperature (37°C) for 20 minutes. Cells were then scrapped and frozen in plastic tubes and analyzed in a Dewar flask by EPR spectroscopy. For EPR study, values are expressed as amplitude of signal per protein concentration.

Western Blotting

After treatments, cells were homogenized and lysed. Proteins (20 μg) were separated on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Blots were probed with anti-endothelial NOS (eNOS), caveolin-1, NOX-2, $p47^{phox}$, and $p67^{phox}$ (BD Biosciences, San Jose, CA), phospho-caveolin-1 Tyr 14, phospho-eNOS Ser 1177, phospho-eNOS Thr 495, phospho-p38 (Cell Signaling, Beverly, MA), extracellular superoxide dismutase (SOD), Mn SOD, Cu/Zn SOD (Stressgen Biotechnologies Corporation, Victoria, Canada), nitrotyrosine (US Biological, Swampscott, MA), β-actin (Sigma-Aldrich), NOX-1, and NOX-4 antibodies. Also, aortas from mice injected with vehicle or MPs from either MS patients or healthy patients were removed, dissected, and proteins analyzed by Western blotting. Blots were probed with anti-eNOS and anti-caveolin-1 antibodies (BD Biosciences).

Peroxynitrite ($ONOO^-$) Detection

The fluorescent probe 3′-(*p*-aminophenyl) fluorescein (APF) probe (Molecular Probes, Eugene, OR) was used according to the manufacturer protocol in the aim to detect alternatively the $ONOO^-$ level. Briefly, cells were treated with vehicle or MPs from either MS patients or healthy patients for 24 hours. Then cells were incubated for 1 hour with APF (10 μmol/L). Fluorescence excitation and emission maxima were 490 and 515 nm, respectively.

Quantitative Real-Time Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Analysis

Cells were treated 24 hours with MPs from MS patients or healthy patients and then frozen in liquid N_2 and used to investigate the expression of mRNA for interleukin (IL)-6, IL-8, IL-1α, IL-1β, tumor necrosis factor-α, monocyte chemotactic protein-1, transforming growth factor (TGF)-β1, TGF-β2, and TGF-β3 by RT-PCR. RT-PCR analyses were performed using a Chromo 4[tm] (Bio-Rad, Marnesla-Coquete, France) and SYBR Green detection. Quantifications were realized according to the ΔCt method as previously described.[25]

Vascular Reactivity

All animal studies were performed using approved institutional protocols. Male Swiss mice (8 to 10 weeks old) were treated *in vivo* by intravenous injection into the tail vein of MPs at the circulating levels of MPs detected in the blood of each patient or healthy subject as previously performed.[21,24] After 24 hours, aortic rings were obtained from animals and mounted on a wire myograph as previously described.[21,24] Endothelium-dependent vasodilatation was studied by cumulative application of acetylcholine (1 nmol/L to 10 μmol/L, Sigma-Aldrich) in aortas with functional endothelium precontracted with U46619 (Sigma-Aldrich).

Data Analysis

Data are represented as mean $\pm$ SEM, *n* represents the number of experiences. Statistical analyses were performed by a one-way analysis of variance and Mann-Whitney *U* or analysis of variance for repeated measures and subsequent Bonferroni post hoc test. $P < 0.05$ was considered to be statistically significant.

Results

As shown in Table 1, there were no significant differences between MS patients and control patients with respect to

Table 1. Baseline Characteristics of MS and Healthy Patients

	Healthy patients	MS patients
Number	37	43
Mean age (years)	52.7 ± 1.3	52 ± 1.7
Sex ratio (male:female)	21:16	32:11
BMI (kg/m^2)	28.1 ± 1.6	34.9 ± 1.2†
Waist circumference (cm)	95 ± 5	110 ± 2†
Systolic blood pressure (mmHg)	125 ± 3	138 ± 3†
Diastolic blood pressure (mmHg)	75 ± 2	81 ± 2*
Glycemia (mmol/L)	5 ± 0.06	6.8 ± 0.27†
Insulinemia (μg/L)	0.5 ± 0.04	1.15 ± 0.13†
HbA1c (%)	5.5 ± 0.07	6.3 ± 0.13†
HOMA	2.8 ± 0.4	7.7 ± 1.4†
Total cholesterol (mmol/L)	5.5 ± 0.19	5 ± 0.14
HDL cholesterol (mmol/L)	1.8 ± 0.09	1.5 ± 0.07
LDL cholesterol (mmol/L)	3.17 ± 0.16	2.6 ± 0.14
Triglycerides (mmol/L)	1.1 ± 0.06	2 ± 0.21†
Creatininemia (mmol/L)	77.4 ± 2.4	77.2 ± 3.6
Treatments (%)	0	40
Oral antidiabetic	0	19
Insulin	24	71
Antihypertensive	23	57
Hypolipidemic		

Baseline characteristics of MS patients (n = 43) compared to healthy patients (n = 37). Patients were fasted before blood collection. All values are expressed in Système International (SI) units. The homeostatic model assessment (HOMA) is a method used to quantify insulin resistance and beta cell function (HOMA = glycemia × insulinemia/22.5). Glycosylated hemoglobin A1c (HbA1c) is expressed as percentage from total hemoglobin.

$^*P < 0.01$; $^\dagger P < 0.001$.

age. As expected MS patients showed greater visceral obesity as given by waist circumference, enhanced triglyceridemia, and increased blood pressure. Insulinemia, fasting glycemia, and calculated homeostatic model assessment are increased arguing for insulin resistance in MS patients. They also displayed higher glycosylated level of hemoglobin A1c (HbA1c), a test that gives a good estimate of how well diabetes is being managed throughout the last 2 or 3 months, because of the presence of type 2 diabetes in a third of the 43 MS patients. But, none of the diabetic patients had individual HbA1c more than 7.5% and none of them were under oral antidiabetic medication at the time of exploration.

Circulating MPs and Their Cellular Origins

The total number of circulating MPs was significantly increased in patients with MS compared to healthy patients (Figure 1A). Phenotypical characterization of cellular origin of MPs showed an increase of ~5-fold (annexin V$^+$), ~2.6-fold (CD41$^+$), and 1.7-fold (CD146$^+$) of the circulating level of procoagulant-, platelet-, and endothelial-derived MPs in MS patients compared to healthy patients respectively (Figure 1, B–D). Also, a greater erythrocyte-derived MPs (CD235a$^+$) was observed in these patients compared to healthy patients (Figure 1E). MPs of other cellular origins were not significantly different between the two groups including those from leukocytes (CD45$^+$), granulocytes (CD66b$^+$), monocytes, and macrophages (CD11b$^+$) (not shown). Moreover, there was no difference in circulating levels of MPs labeled for P-selectin (CD62P$^+$) or L-selectin expression (CD62L$^+$) between MS patients and healthy patients (data not shown).

MS MPs Reduce NO in Endothelial Cells by Reducing eNOS Activity

Control and cells treated with MPs from either MS or healthy patients exhibited an EPR feature of signals derived from NO-Fe(DETC)$_2$. As shown in Figure 2A, MPs from MS patients, but not from healthy patients, reduced significantly NO release in endothelial cells by ~50%. In the aim to dissect the molecular changes governing the

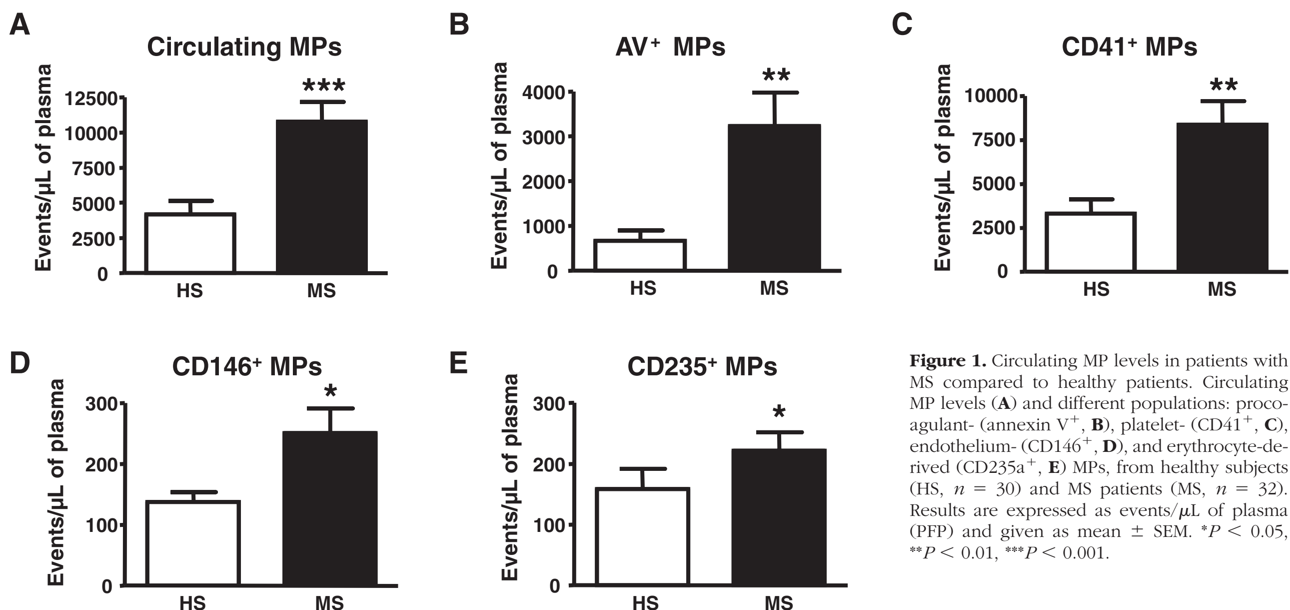

Figure 1. Circulating MP levels in patients with MS compared to healthy patients. Circulating MP levels (**A**) and different populations: procoagulant- (annexin V$^+$, **B**), platelet- (CD41$^+$, **C**), endothelium- (CD146$^+$, **D**), and erythrocyte-derived (CD235a$^+$, **E**) MPs, from healthy subjects (HS, n = 30) and MS patients (MS, n = 32). Results are expressed as events/μL of plasma (PFP) and given as mean ± SEM. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

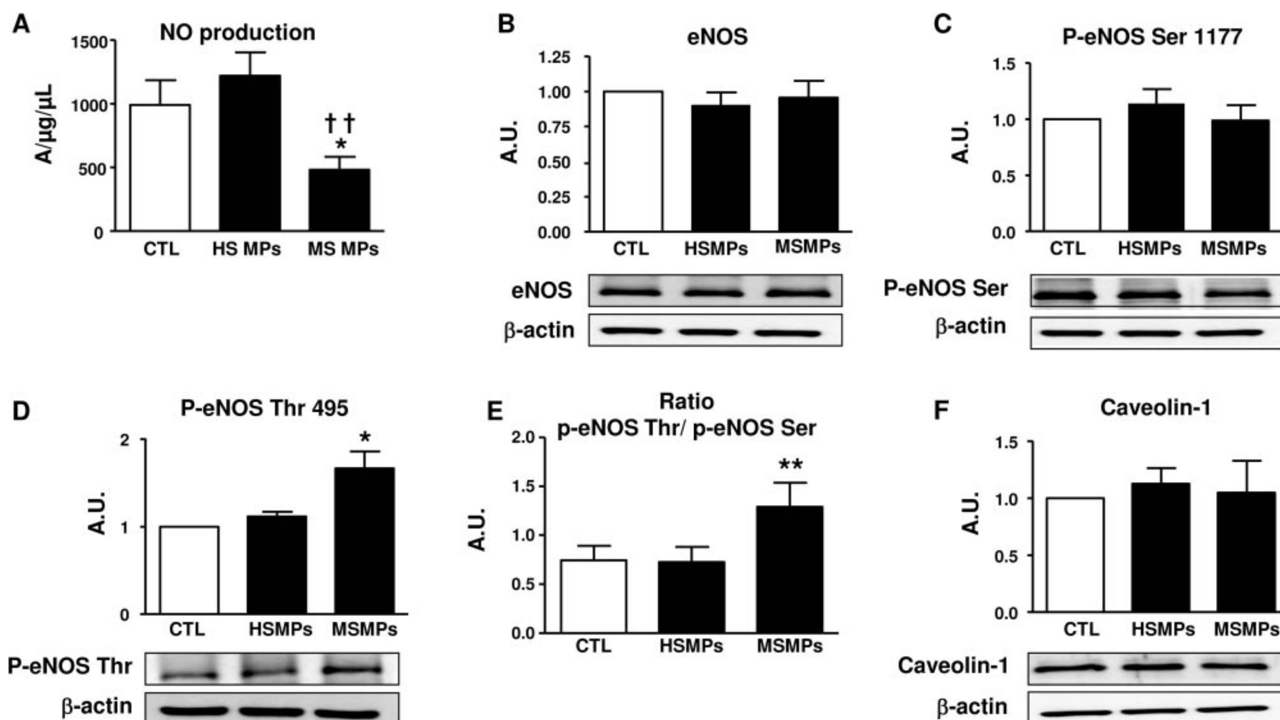

Figure 2. MPs from MS patients decrease NO production in endothelial cells via inhibition of NOS activity. Cells were incubated for 24 hours in the presence of vehicle (CTL), MPs from healthy subjects (HSMPs), or in the presence of MPs from MS patients (MSMPs). **A:** Quantification of the amplitude of the NO-Fe(DETC)$_2$ complex signal in human endothelial Eahy 926 cells. Values are expressed in units of amplitude/µg/µl of proteins of samples ($n = 6$). **B–F:** Western blotting using antibodies raised against eNOS (**B**), phospho-eNOS Ser 1177 (**C**), phospho-eNOS Thr 495 (**D**), or caveolin-1 (**F**). Immunoblots were quantified by densitometric analysis. Data are representative of four separate blots, and the densitometry values are expressed in arbitrary units (A.U.) as mean ± SEM. **E:** The ratio of phosphorylation of eNOS at the inhibitor site (Thr 495) and activation site (Ser 1177) is expressed in arbitrary units (A.U.) as mean ± SEM. $^{*}P < 0.05$ versus CTL, $^{**}P < 0.01$ versus CTL and HSMPs for (E) and $^{††}P < 0.01$ versus HSMPs.

reduction of NO release induced by MPs from patients in endothelial cells, we analyzed by Western blotting expression and activation of enzymes linked to NO pathway. MPs from either healthy subjects or patients did not modify expression of eNOS (Figure 2B). MPs from MS patients did not alter eNOS phosphorylation on its activator (Ser 1177) site but they greatly increased its phosphorylation at the inhibitor (Thr 495) site (Figure 2, C and D) when compared either to nontreated or those treated with MPs from healthy patients. In addition, after normalization of the amount of phosphorylated eNOS to total amount of the enzyme, the ratio of phosphorylated eNOS at the inhibitor and activation sites was calculated. Interestingly, the ratio of phosphorylation was greater in cells treated with MPs from MS patients compared to those from healthy patients (Figure 2F). We examined the possible regulation by MPs of the caveolin-1 expression. Figure 2E shows that neither MPs from patients nor healthy patients were able to modify caveolin-1 expression.

MS MPs Reduce O_2^- Release in Endothelial Cells

EPR measurement of O_2^- production showed that MPs from MS patients but not those from healthy patients significantly reduced O_2^- production compared to nontreated endothelial cells (Figure 3A). Similar results were obtained by flow cytometer analysis using the fluorescent dye dihydroethidine as a marker of reactive oxygen species production (data not shown). We evaluated expression of membrane (NOX-1, NOX-2, and NOX-4) and cytosolic subunits ($p47^{phox}$ and $p67^{phox}$) of NADPH oxidase, a major source of cellular O_2^-. Treatment of cells with MPs from both healthy patients and MS patients reduced expression NOX-1 subunit (Figure 3B). Both types of MPs did not change expression of the NOX-2 subunit (Figure 3C). MPs from healthy patients enhanced expression of NOX-4 membrane subunit (Figure 3D). Interestingly, MPs from MS patients reduced expression of $p47^{phox}$ cytosolic subunit without affecting that of $p67^{phox}$ (Figure 3, E and F). To evaluate the cellular capacity of endothelial cells to reduce O_2^- in the presence of MPs, we examined expression of different isoforms of SOD. MPs from both healthy patients and MS patients did not significantly modify Mn-SOD and Cu/Zn-SOD expressions (Figure 3, G and H). Interestingly, MPs from MS patients but not those from healthy patients significantly enhanced extracellular SOD expression (Figure 3I).

MS MPs Increase Nitration of Proteins in Endothelial Cells

The reaction between NO and O_2^- generates $ONOO^-$, a strong oxidant, which could react directly with electron-

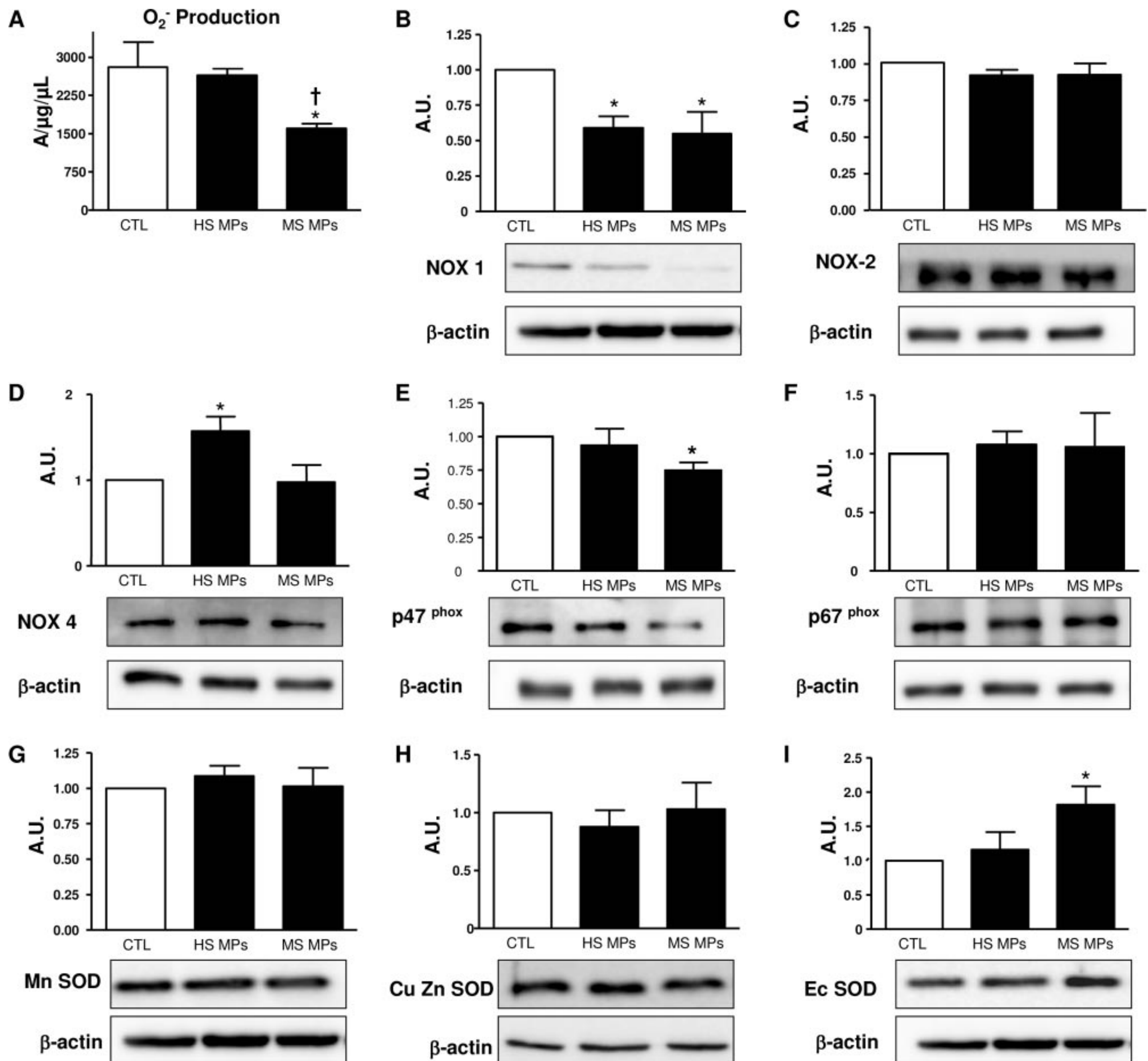

Figure 3. MPs from MS patients reduce O_2^- release in endothelial cells. Cells were incubated for 24 hours in the presence of either vehicle (CTL), MPs from healthy subjects (HSMPs), or in the presence of MPs from MS patients (MSMPs). **A:** Quantification of the amplitude of the O_2^--CMH complex signal in human endothelial Eahy 926 cells expressed in units of amplitude/μg/μl of proteins of samples as mean ± SEM ($n = 6$). **B--I:** Western blotting using antibodies raised against: NOX-1 (**B**), NOX-2 (**C**), NOX-4 (**D**), p47^{phox} (**E**), p67^{phox} (**F**), Mn SOD (**G**), Cu/Zn SOD (**H**), extracellular SOD (**I**). Immunoblots were quantified by densitometric analysis. Data are representative of five separate blots, and the densitometry values are expressed in arbitrary units (A.U.) as mean ± SEM. $*P < 0.05$ versus CTL and $^{\dagger}P <$ 0.05 versus HSMPs.

rich groups. Then, we evaluated its formation in endothelial cells by studying nitration of proteins, a subsequent step to $ONOO^-$ formation, using specific antibodies raised against nitrotyrosine. Interestingly, MPs from MS patients, but not from healthy patients, markedly enhanced nitration of proteins in endothelial cells (Figure 4, A and B).

Another technique using APF fluorescent probe was conducted to further detect $ONOO^-$ formation. We observed a higher fluorescence from cells treated with MPs from both MS patients and healthy patients compared to vehicle [delta of fluorescence from basal condition (arbitrary units): 35.5 ± 4.8 ($n = 7$), 31.6 ± 4.6 ($n = 10$), 20.7 ± 2.7 ($n = 12$) in MS MP- ($P < 0.01$ versus control), healthy patients MP- ($P < 0.05$ versus control), and vehicle-treated cells, respectively]. This result together with the previous finding concerning nitration of proteins indicate an increase of $ONOO^-$ production in cells treated with MS MPs compared to vehicle and healthy patients' MPs. The increase of fluorescence observed with healthy patients' MPs probably results in an increase of hydroxyl radical and the hypochlorite anion in addition to $ONOO^-$ level. Furthermore, it should be noted that the increase in protein nitration induced by MS MPs was analyzed by taking into account total proteins. However as shown on Figure 4A, only some but not all proteins are increased on MS MP treatment. Such subtle changes cannot be detected using the fluorescence technique.

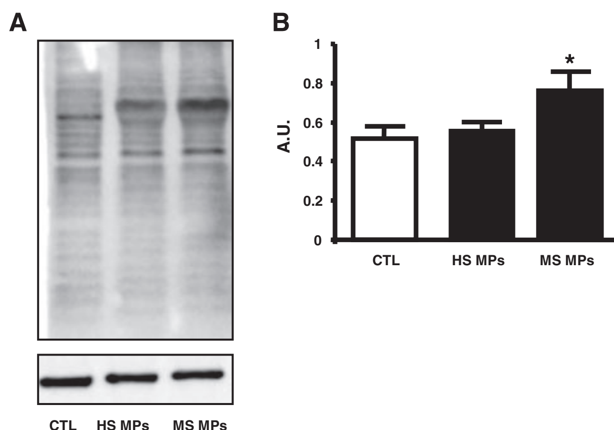

Figure 4. MPs from MS patients increase nitration of proteins in endothelial cells. Cells were incubated for 24 hours in the presence of vehicle (CTL), MPs from healthy subjects (HSMPs), or in the presence of MPs from MS patients (MSMPs). **A:** The protein nitration was evaluated by Western blotting using antibody raised against nitrotyrosine-modified proteins. **B:** Immunoblots were quantified by densitometric analysis. Data are representative of four separate blots, and the densitometry values are expressed in arbitrary units (A.U.) as mean ± SEM. *$P < 0.05$ versus CTL.

MPs from Either Healthy Patients or MS Patients Did Not Modify Pro-Inflammatory Cytokine Release in Endothelial Cells

The possible regulation of cellular expression and release of cytokines by MPs was further evaluated. Both MPs from healthy patients and MS patients did not modify expression of IL-6 and IL-8 in endothelial cells as assessed by RT-PCR (Table 2). Moreover, the release of IL-6 and IL-8 was not modified in MP-treated cells compared to controls as measured by enzyme-linked immunosorbent assay (data not shown). Besides, tumor necrosis factor-α mRNA expression and that of other pro-inflammatory cytokines including monocyte chemotactic protein-1, IL-1-α, IL-1-β, TGF-β1, TGF-β2, and TGF-β3 were not affected by the treatment of endothelial cells with both MS and healthy patient MPs (Table 2).

Table 2. Cytokines mRNA Expression

	mRNA normalized relative quantity ($\times 10^3$)		
	CTL	HSMPs	MSMPs
IL-6	5 ± 0.5	5.9 ± 0.2	5.6 ± 0.7
TNF-α	0.03 ± 0.003	0.03 ± 0.005	0.06 ± 0.04
MCP-1	17.9 ± 1.1	14.5 ± 1.07	17.9 ± 2.4
IL-1α	3.74 ± 0.3	4.2 ± 0.7	3 ± 0.6
IL-1β	0.11 ± 0.02	0.06 ± 0.01	0.08 ± 0.02
IL-8	28.3 ± 4.5	21.5 ± 0.7	23.3 ± 1.2
TGF-β2	8.9 ± 1.2	8.2 ± 0.2	8.3 ± 0.8
TGF-β1	975 ± 80	1139 ± 117	944 ± 82
TGF-β3	5.5 ± 1.2	4.2 ± 0.8	1.9 ± 1.5

Quantitative real-time RT-PCR analysis of normalized relative quantity of IL-6, IL-8, IL-1α, IL-1β, TNF-α, MCP-1, TGF-β1, TGF-β2, and TGF-β3 mRNA expressions in human endothelial cells, Eahy 926, after 24 hours of incubation with either vehicle (CTL, $n = 6$), MPs from healthy patients (HSMPs, $n = 5$), or MPs from MS patients (MSMPs, $n = 5$).

MS MPs Impair Endothelium-Dependent Relaxation in Vivo *in Aorta of the Mice*

The relaxation to acetylcholine was significantly impaired in aortas taken from mice subjected to intravenous injection of MPs from MS patients compared to those from mice injected with either vehicle or MPs from healthy patients ($E_{max} = 50 \pm 4\%$, $61 \pm 3\%$, and $60 \pm 4\%$, respectively; $P < 0.01$) (Figure 5A). These results suggest MPs from MS patients induce *in vivo* endothelial dysfunction in mice aortas. Interestingly, MS MPs markedly reduced eNOS expression in mice aortas (Figure 5B) without affecting that of caveolin-1 (Figure 5C) compared to vehicle or MPs from healthy patients.

Nonplatelet-Derived MS MPs Decrease NO Production in Endothelial Cells

As shown in Figure 6 nonplatelet- but not platelet-derived MS MPs reduced NO production in endothelial cells, suggesting that the effects of MS MPs are probably supported by endothelial- and/or erythrocyte-derived MS MPs because the level of these subtypes of MPs is increased in patients with MS compared to healthy donors.

Discussion

In the present study, we show that circulating levels of MPs are elevated in patients with MS compared to healthy patients. Of particular interest was the increase in endothelial-, erythrocyte-, and platelet-derived MPs in addition to procoagulant (annexin V^+) MPs in MS patients. Besides, we demonstrate *in vitro* that MPs from MS patients decreased both NO production and O_2^- in cultured endothelial cells. These effects were associated with a reduction of eNOS activity via increased phosphorylation of eNOS at the inhibitory site in one hand and at least in part a decreased expression of some NADPH oxidase subunits and increased of extracellular SOD. Surprisingly, MPs from MS increased the nitration of proteins. Moreover, MS MPs did not change release of inflammatory cytokines in endothelial cells. These data underline dissociations between MPs from MS patients especially those from nonplatelet origin in decreasing NO availability and inflammation in endothelial cells accounting for their capacity to promote endothelial dysfunction. Evidence of the pathophysiological relevance of MS MPs was provided *in vivo*, by their capacity in impairing endothelium-dependent relaxation in response to acetylcholine and by reducing eNOS expression in the aorta. Thus, these results demonstrate an association between increase of circulating levels of MPs including those with procoagulant properties and their involvement in promoting endothelial dysfunction leading to atherogenesis *in vivo*.

MS is a cluster of several atherosclerotic risk factors that includes commonly type 2 mellitus diabetes and obesity.[1–5] Circulating procoagulant MPs have been described in various clinical situations associated with

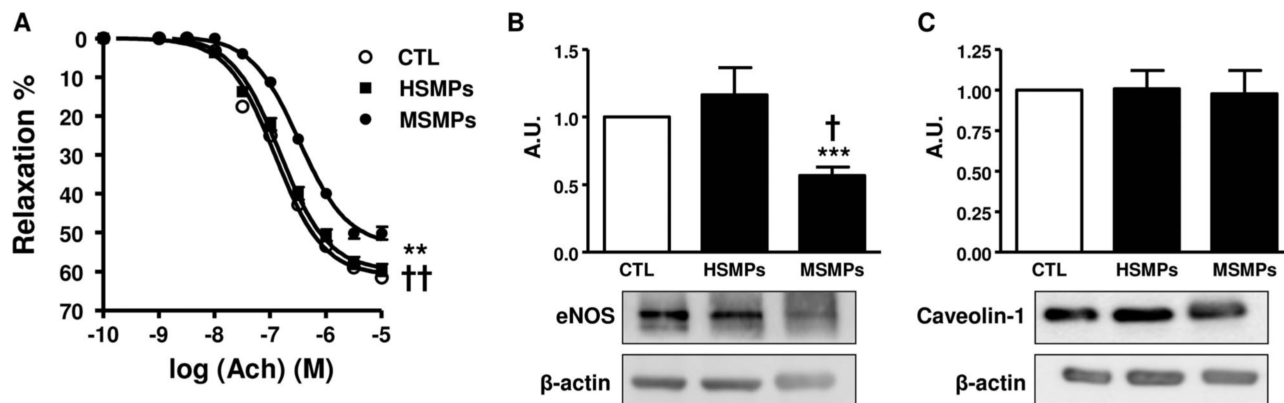

Figure 5. MPs from MS patients impair endothelium-dependent relaxation in mouse aorta. **A:** Acetylcholine (Ach)-induced relaxation in vehicle- (CTL), healthy subjects MP- (HSMPs), and MS patients MP (MSMPs)-treated mouse aorta (n = 5 to 8). Results are expressed as a percentage of relaxation of U46619-induced precontraction. Western blotting of mouse aorta using antibodies raised against: eNOS (**B**), caveolin-1 (**C**). Immunoblots were quantified by densitometric analysis. Data are representative of four separate blots, and the densitometry values are expressed in arbitrary units (A.U.) as mean ± SEM. ***$P < 0.001$, **$P < 0.01$ versus CTL; †$P < 0.05$, ††$P < 0.01$ versus HSMPs.

thrombosis and in diabetic patients.[16,17] Tissue factor-positive MPs are associated with components of the MS but not with coagulation in asymptomatic patients with well-regulated type 2 diabetes. Thus, tissue factor on MPs may be involved in processes other than coagulation, including transcellular signaling or angiogenesis. The origin of tissue factor-positive MPs occurs mainly from platelets, T-helper cells, and granulocytes.[18] In obese patients, an increase in circulating MPs including procoagulant MP levels might account for the increased risk of thrombotic complications in obesity.[26] Recently, Arteaga and colleagues[19] reported endothelial cell MP release, platelet and leukocyte activation, and increased binding of MPs from endothelial cells and platelets to leukocytes in patients with the MS. Finally, Chironi and colleagues[20] showed in patients with MS, leukocyte-derived MP level is higher than in those free of such syndrome and in the overall study population, leukocyte-derived MP level increased gradually in parallel with the number of components of MS. Altogether, the above reports underline the difficulty in attributing a role for MPs in MS patients although they are probably involved in promoting coagulation and in some extent atherogenesis *in vivo*. Most importantly, whether MPs are purely a marker or activator of endothelial activation is not known. In the present study, we report for the first time an association with increased circulated MPs in MS patients that displayed at least four of five of the international criteria of the syndrome and their properties in inducing endothelial dysfunction both *in vitro* and *in vivo*.

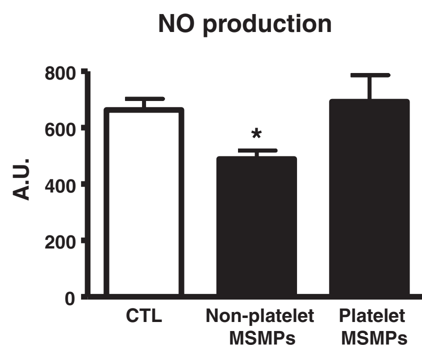

Figure 6. Nonplatelet-derived MS MPs decrease NO production in endothelial cells. Endothelial cells were incubated 24 hours with vehicle (anti-CD61 microbeads alone), platelet- or nonplatelet-derived MSMPs. Quantification of the amplitude of the NO-Fe(DETC)$_2$ complex signal in cells is expressed in units of amplitude/μg/μl of proteins of samples as mean ± SEM (n = 5). *$P < 0.05$.

Interestingly, procoagulant (annexin V$^+$) MPs are increased in MS patients compared to controls. Because of their procoagulant properties, this subtype of MPs may participate to fibrinolysis impairment and thrombinogenesis elevation as observed for patients with type 2 diabetes and higher markers of abdominal obesity, a major component of MS, compared to those with lower abdominal obesity markers.[17] The cellular origin of procoagulant MPs may be diverse and includes those from platelets, leukocytes, erythrocytes, and endothelial cells.[10–12] According to Chironi and colleagues,[20] the main origin of procoagulant MPs in MS patients derived from leukocytes based on the method used that detects only MPs that expose functional phosphatidylserine on their surface therefore cannot exclude the existence of annexin V$^-$ MPs. This is particularly important inasmuch as only 45% of MPs detected in our present study was annexin V$^+$ using flow cytometry analysis in MS patients. The difference in the method used also probably accounts for the fact that we did not detect an increase in total leukocyte MPs in MS patients.

In addition to procoagulant MPs, we showed an increase in platelet, erythrocyte, and endothelial MPs in MS patients. With regard to platelet MPs, Arteaga and colleagues[19] did not report a significant change in platelet MPs although they show increased platelet activation in MS patients. The difference probably relies on the markers used for determination of platelet MPs (CD31$^+$/CD42b$^+$ from Arteaga and colleagues,[19] versus CD41$^+$ in the present study). From these data, one can advance the hypothesis that MPs (annexin V$^+$ and platelet MPs) might play a role in an increase in thrombotic risk in this pathology. Increased endothelial MPs have been reported in multiple proinflammatory and prothrombotic states, including trauma-induced systemic inflammatory-response syndrome,[27] venous thromboembolism,[28] antiphospholipid antibody syndrome,[29] acute

coronary syndromes,[16] and end-stage renal failure patients.[23] According to Arteaga and colleagues,[19] the increase in endothelial MPs results from endothelial cell apoptosis but not activation among patients with MS. Endothelial MPs correlate with endothelial dysfunction appreciated by loss of endothelium-dependent flow-mediated vasodilatation in obese women but the mechanism involved has not been analyzed yet.[30]

Endothelial dysfunction is the primary event leading to the failure of vasoactive, anti-coagulant, and anti-inflammatory effects of healthy endothelium. The most important mechanism for endothelial dysfunction is the decrease in NO availability. Endothelial production of NO was initially considered to be dependent on increases in intracellular calcium and binding of calcium/calmodulin to eNOS. However, eNOS regulation is a process determined by a cascade of events, including changes in eNOS mRNA and protein levels,[31] and phosphorylation of Ser, Thr, and tyrosine residues.[32] Here, we show that MPs from MS patients reduced NO release in endothelial cells without affecting expression of eNOS or its phosphorylation on Ser 1177. However, MS MPs increased markedly its phosphorylation on the inhibitor site (Thr 495). In conjunction with our findings, Densmore and colleagues[33] showed that co-incubation of endothelial MPs with endothelial cells yields a reduction of NO release that is associated with a decrease in eNOS phosphorylation at Ser1179. Hence, MPs from MS patients reduced NO production in endothelial cells by regulating negatively eNOS activity.

The bioavailability of NO depends not only on its generation but also on reactive oxygen species production. Here, we evaluated the effect of MPs from both healthy patients and MS patients on O_2^- release. Surprisingly, MPs from patients reduced the O_2^- release in endothelial cells. In contrast, Brodsky and colleagues[34] showed that endothelium-derived MP treatment induces O_2^- production in rat renal microvascular endothelial cells. The differential effects on oxidative stress may be attributable to the origin of MPs (total MPs in the present study versus endothelial MPs) and/or to the endothelial cell used (Eahy 926 endothelial cell line, the present study versus renal microvascular endothelial cells). We cannot distinguish among these possibilities. Also, it cannot be excluded that the affinity of NO issued by eNOS is greater in acting with O_2^- within the endothelial cell compared to its affinity with the spin trap used. Thus, in the present study, NO may blunt the O_2^- release by forming $ONOO^-$ as shown by the increase of protein nitration induced by MS MPs. $ONOO^-$ has been shown to trigger apoptosis in cardiomyocytes as well as in endothelial and vascular smooth muscle cells, induces the up-regulation of adhesion molecules in endothelial cells, the disruption of endothelial glycocalyx, and may enhance the adhesion of neutrophils to the endothelium.[35] This effect of MS MPs on endothelial cells may explain the dissociation between reduced NO and O_2^- formation in cultured endothelial cells, and may account for endothelial dysfunction.

We focused the study on NADPH oxidase among xanthine oxidase or complex I in mitochondria as a source of O_2^- production in endothelial cells. MPs from both healthy and MS patients exerted subtle effects on different subunit of NADPH oxidase including the different subunits of NOX, $p47^{phox}$, $p67^{phox}$. Even though MPs from healthy MPs reduced NOX-1 expression, they increased that of NOX-4 without reducing $p47^{phox}$ expression. Turning to MPs from MS, one can advance the hypothesis that the reduced O_2^- production by these MPs in culture endothelial cells may partly involve both the decrease in NOX-1 and $p47^{phox}$ expressions. Moreover, MPs from MS patients enhanced the capacity of endothelial cells to reduce the O_2^- by increasing expression of extracellular SOD. This event also contributes to the decrease of O_2^- occurring in cells treated with MS MPs.

Finally, we show that *in vivo* treatment of mice with MS MPs but not those from healthy subjects reduced the ability of acetylcholine to promote endothelium-dependent relaxation in aorta accompanied with a decrease of eNOS expression in association with their capacity to reduce NO formation and increase of $ONOO^-$ in cultured endothelial cells *in vitro*. Together, these results strongly suggest that circulating MPs from MS patients are able to induce *in vivo* endothelial dysfunction and demonstrate, for the first time, their pathophysiological relevance, in this syndrome.

All of the effects observed with MPs were obtained by treating cells or animals with total circulating MPs from patients or healthy subjects. We show that platelet-derived MPs represent the main proportion of circulating MS MPs. Interestingly; we demonstrate here that nonplatelet-derived MS MPs reduced NO production in endothelial cells suggesting that the effects of MS MPs on endothelial function are probably supported by endothelial- and/or erythrocyte-derived MS MPs because the level of these subtypes of MPs is increased in patients compared to healthy donors. This new finding underscores that the biological message carried by MPs does not depend on quantity, but may be associated to the quality of antigens harbored by them.

The MS and metabolic pathologies are characterized by a chronic inflammatory state.[2–5] In the present work we evaluated the effect of MPs from healthy patients and MS patients on the release of different inflammatory cytokines from endothelial cells. Interestingly, MPs from either healthy subjects or patients did not modify the release cytokines from endothelial cells. Previous works showed that MPs could directly activate and stimulate cells to produce inflammatory mediators such as cytokines.[36–39] We report an uncoupling between inflammation and endothelial dysfunction by MPs from MS patients.

In summary, we demonstrate that patients with MS have increased levels of circulating MPs, especially procoagulant, platelet-, endothelial-, and erythrocyte-derived MPs. MPs from MS patients induce *in vivo* endothelial dysfunction in mice aortas. *In vitro* MPs from MS patients reduce endothelial NO bioavailability by regulating negatively eNOS activity independently of oxidative stress. Nonplatelet-derived MS MPs are incriminated in these effects on endothelial function. From these results, one can advance the hypothesis of a potential association between increased circulating procoagulant, platelet-, endothelial-, and erythrocyte-derived MPs with endothelial dysfunction in patients with MS.

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