

# Endothelium-derived microparticles impair endothelial function in vitro

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**Brodsky, Sergey V., Fan Zhang, Alberto Nasjletti, and Michael S. Goligorsky.** Endothelium-derived microparticles impair endothelial function in vitro. *Am J Physiol Heart Circ Physiol* 286: H1910–H1915, 2004; 10.1152/ajpheart.01172.2003.—Endothelial cell dysfunction (ECD) is emerging as the common denominator for diverse and highly prevalent cardiovascular diseases. Recently, an increased number of procoagulant circulating endothelial microparticles (EMPs) has been identified in patients with acute myocardial ischemia, preeclampsia, and diabetes, which suggests that these particles represent a surrogate marker of ECD. Our previous studies showed procoagulant potential of endothelial microparticles and mobilization of microparticles by PAI-1. The aim of this study was to test the effects of isolated EMPs on the vascular endothelium. EMPs impaired ACh-induced vasorelaxation and nitric oxide production by aortic rings obtained from Sprague-Dawley rats in a concentration-dependent manner. This effect was accompanied by increased superoxide production by aortic rings and cultured endothelial cells that were coincubated with EMPs and was inhibited by a SOD mimetic and blunted by an endothelial nitric oxide synthase inhibitor. Superoxide was also produced by isolated EMP. In addition, p22(phox) subunit of NADPH-oxidase was detected in EMP. Our data strongly suggest that circulating EMPs directly affect the endothelium and thus not only act as a marker for ECD but also aggravate preexisting ECD. superoxide; nitric oxide; cell dysfunction; cardiovascular disease

ENDOTHELIAL CELL DYSFUNCTION (ECD) is a precursor and common denominator of cardiovascular diseases including atherosclerosis, diabetic vasculopathy, hypertension, and progressive cardiomyopathy. It is well documented that altered function of endothelial nitric oxide (NO) synthase (eNOS) and/or decreased bioavailability of NO are fundamental abnormalities that lead to the pathophysiological manifestations of ECD (9, 11, 22). In fact, accumulated data suggest that many clinical manifestations of ECD are intimately linked to the expression and function of eNOS. Endothelium-dependent relaxation of vascular smooth muscle is in part governed by the integrity of the L-arginine-eNOS-NO system (35). Moreover, NO is responsible for the regulation of diverse functions of endothelial cells and their interactions (both inflammatory and thrombogenic) with circulating formed elements and vascular smooth muscle cells (for review, see Ref. 9).

Since the first description of “platelet dust” more than 35 years ago (36), the ability of eukaryotic cells to shed components of their plasma membrane into the extracellular space has been established for many cell types (1, 3, 5, 17, 23, 38). Such sealed fragments, which are known as microparticles, typically range in size from 0.1 to 2  $\mu\text{m}$ . Microparticles contain cell surface proteins and cytoplasmic components of the cells of origin (10, 39). The release of microparticles was first described (14) from platelets after activation by different stimuli

such as thrombin, collagen, and shear stress. Microparticles shed by different cells express a subset of cell surface proteins derived from the plasma membrane of the cells of origin. For example, microparticles shed by polymorphonuclear neutrophils express selectins and integrins, complement regulators, HLA-1, and other markers of neutrophils (13), whereas microparticles derived from endothelial cells express CD31, CD54, CD62E,  $\alpha_v\beta_3$ -integrins, etc. (10, 18, 27). The shedding of membrane-associated endothelial cell surface elements that contain  $\beta_1$ -integrins and different matrix metalloproteinases (MMPs) such as MMP-2, MMP-9, and membrane type-1 MMP (MT1-MMP) has been observed in cultured cells (28).

Previous studies (2) suggest that some microparticles activate platelets and endothelial cells via the transcellular delivery of arachidonic acid and thereby increase the binding of monocytes to endothelial cells. Increased numbers of circulating microparticles have been reported in patients during cardiopulmonary bypass, unstable angina, and lacunar infarcts and with diabetes mellitus (25, 27, 33). Increased microparticle formation by endothelial cells has been documented in patients with lupus and acute coronary syndromes (5, 9, 17, 23, 38, 39). Significantly elevated numbers of circulating endothelium- and platelet-derived microparticles, which correlated with blood pressure, were found in patients with severe hypertension (26). Elevated plasminogen activator inhibitor-1 (PAI-1), which is one of the hallmarks of ECD, increases endothelial microparticle (EMP) formation (7), which further confirms the link between dysfunctional endothelial cells and EMPs. Although a consensus is building regarding the potential role of circulating EMPs as a hallmark of ECD, it is not known whether EMPs per se could aggravate ECD. Importantly, circulating microparticles isolated from the blood of preeclamptic women (but not from healthy pregnant women) were suggested to impair endothelial functions as inferred from observations that exposure to microparticles diminishes endothelium-dependent relaxation of isolated myometrial arteries (32). Similar impairment of endothelium-dependent relaxation of rat aortic rings by circulating microparticles obtained from patients with myocardial infarction has been observed (4).

The aim of this study was to investigate the direct effects of EMPs on vascular endothelium.

## MATERIALS AND METHODS

**Cell culture.** Rat renal microvascular endothelial cells (RMVECs) were previously established and characterized by our laboratory (29). Cells were grown and maintained in endothelial basal medium-2 (EBM-2; Clonetics) supplemented with 2% FBS and growth factors at 37°C in an atmosphere that contained 5% CO<sub>2</sub>.

**Chemicals.** Dihydroethidium (DHE) was purchased from Molecular Probes (Eugene, OR); manganese(III)tetrakis(4-benzoic acid)por-

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phyrin (MnTBAP) was from Alexis Biochemical (San Diego, CA); and calcium ionophore A-23187,  $N^G$ -nitro-L-arginine methyl ester (L-NAME), and 2-( $N,N$ -diethylamino)-diazololate-2-oxide (NONOate) were from Sigma (St. Louis, MO).

**Isolation of microparticles from cultured endothelial cells.** Microparticles were isolated from RMVECs cultured on 150-mm dishes ( $\sim 10^8$  cells). Confluent cells were incubated for 2 h in serum-free EBM-2 as previously detailed (7). Culture medium was collected and cleared from cells and cell debris by centrifugation at 5,000 g for 10 min. The supernatant was subjected to ultracentrifugation at 100,000 g for 2 h (7, 21), and sedimented EMPs were used immediately in the experiments. The number of resulting microparticles was calculated using fluorescence-activated cell sorting analysis as previously described (7). The high-speed supernatant was used as a vehicle control.

**Assessment of agonist-induced vasorelaxation in aortic rings.** The animal-study protocol was approved by the Institutional Animal Care and Use Committee. Thoracic aortas were obtained from male Sprague-Dawley rats (body wt, 180–200 g) that were anesthetized with ketamine and xylazine. Aortas were cleared of periadventitial tissue and cut transversely into 1.5–2.0-mm rings. The aortic rings were incubated with different concentrations of EMPs in serum-free EBM-2 or with high-speed supernatant from microparticles for 3 h at 37°C in a 5% CO<sub>2</sub> atmosphere in a cell culture incubator. Vascular rings, which were handled carefully to avoid damage to the inner surface, were mounted on wires in the chambers of a multivessel myograph (J. P. Trading and Danish Myo Technology; Aarhus) bathed in Krebs buffer. The medium was gassed with 95% O<sub>2</sub>-5% CO<sub>2</sub> and maintained at 37°C (pH 7.4). After equilibration (30 min), the rings were set to an internal circumference equivalent to 90% of the relaxed circumference under a transmural pressure of 100 mmHg and were allowed to stabilize for 20–30 min. The rings were then depolarized with KCl (60 mM) to evaluate maximal contraction. After the vascular preparations were washed with Krebs buffer, they were contracted with phenylephrine ( $10^{-6}$  M). When the contractile response was stabilized (steady-state phase, 12–15 min), vasorelaxation responses to cumulative increments in the ACh or NONOate concentrations were examined (24).

**Measurement of NO production by aortic rings.** Aortic rings were treated with EMPs or supernatant as described above. NO concentration was measured using a porphyrin-electroplated, Nafion-coated, carbon-fiber electrode as previously described (16). Calibration of the electrode was performed before each experiment using dilutions of freshly prepared NO-saturated Krebs-Ringer solution. Aortic rings were placed into 100  $\mu$ l of Krebs buffer, and after a stable baseline was obtained, 5  $\mu$ g/ml of calcium ionophore A-23187 was pipetted into the buffer while the electrochemical current was continuously recorded.

**Detection of superoxide production from aortic rings.** Intravital fluorescence microscopy of aortic rings loaded with DHE (10  $\mu$ M) for 30 min was performed using a Nikon Diaphot epifluorescence inverted microscope equipped with a silicon-intensified target camera (Hamamatsu) and a temperature-controlled chamber at 37°C. Rings were illuminated for 30 ms at a 510-nm wavelength in 120-s intervals using an automatic shutter (Lambda 10-2; Sutter Instruments) interfaced to Image-1-Fluor software (Universal Imaging). Images were collected at a 620-nm wavelength ( $\times 200$  magnification) using an appropriate dichroic mirror and were stored and analyzed using Image-1 software. A bright-field image of aortic rings was collected at the end of each experiment to localize the endothelium-derived fluorescence. The fluorescence intensity was measured from 6–8 different endothelial cells before and after the aortic rings were treated with EMPs ( $10^6$  EMPs/ml).

**Fluorescence plate-reader assays.** Fluorescence plate-reader assays were performed in 96-well black microtiter plates (Corning; Corning, NY) using an FLx800 fluorescence plate reader (Bio-Tek Instruments; Winooski, VT). To detect superoxide, RMVECs or isolated microparticles were loaded with 10  $\mu$ M DHE in a Krebs-HEPES buffer for 30 min; L-NAME and MnTBAP were added after DHE loading at concentrations specified (see RESULTS). After cells were washed, EMPs were added and cells were incubated at 37°C for 1 h. Plates were read in the plate reader using 510- and 620-nm wavelength excitation and emission filters, respectively (6, 37). The background fluorescence value (obtained during cell-free incubation) was subtracted from the recorded data.

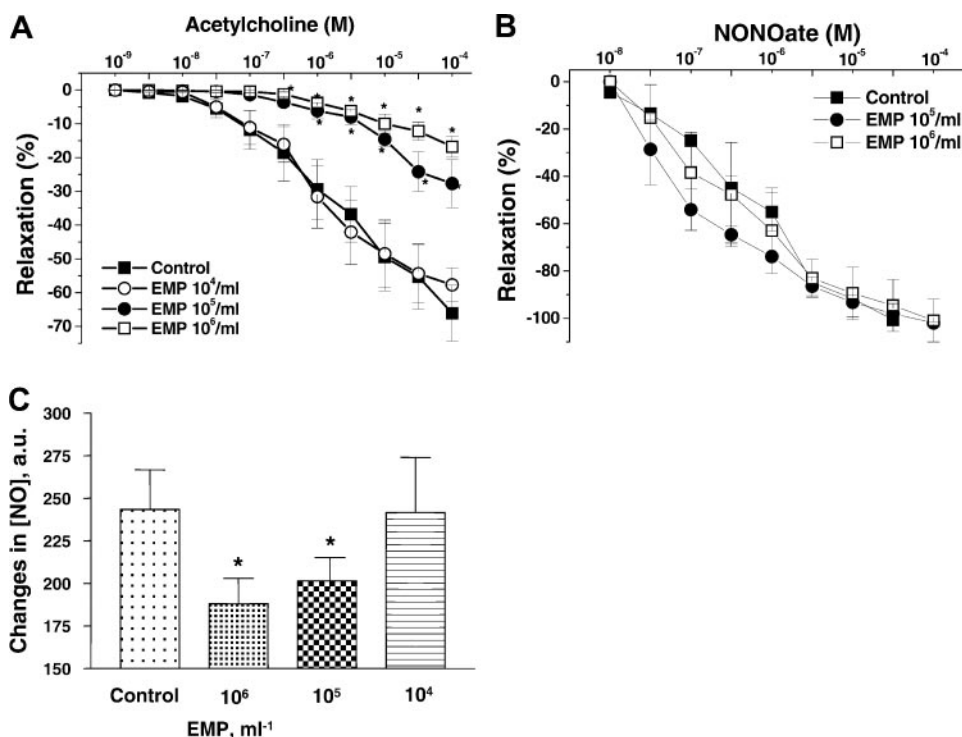


Fig. 1. Effects of microparticles on endothelial function. **A:** ACh-induced vasorelaxation of aortic rings ( $n = 6$ ) incubated for 3 h with endothelial microparticles (EMPs). **B:** vasorelaxation of aortic rings ( $n = 6$ ) induced by incubation of rings with 2-( $N,N$ -diethylamino)-diazololate-2-oxide (NONOate) for 3 h with EMPs. **C:** nitric oxide (NO) production in response to A-23187 (5  $\mu$ g/ml) by aortic rings ( $n = 6$ ) incubated with EMPs for 3 h is represented as NO concentration ([NO]); au, arbitrary units. Data are means  $\pm$  SE; \* $P < 0.05$  compared with control.

**Statistical analysis.** Paired or unpaired *t*-tests and/or ANOVA with subsequent Tukey's post-test were used.  $P < 0.05$  was considered statistically significant. All values are presented as means  $\pm$  SE. All experiments were repeated at least three times.

## RESULTS

**EMPs impair endothelium-dependent vasorelaxation and reduce NO levels.** Aortic rings were incubated for 3 h with and without increasing concentrations of EMPs to study the effects on ACh- and NONOate-induced vasorelaxation. The basal tone of the aortic rings showed no significant change in all experimental groups. The relaxation response to ACh but not to NONOate diminished greatly in aortic rings preincubated with  $10^5$ – $10^6$  EMPs/ml (Fig. 1, A and B). This implies that EMPs selectively affect the endothelium, as relaxation responses to ACh and NONOate are endothelium dependent and independent, respectively. The impaired vasorelaxation in response to ACh in aortic rings treated with EMPs could not be attributed to an impaired responsiveness of vascular smooth muscle cells to NO, because vessels from all experimental groups were equally responsive to the NO donor NONOate.

To study NO production by aortic rings, changes in NO concentration in a response to A-23187 (5  $\mu$ g/ml) were tested using an NO-selective electrochemical electrode. Calcium ionophore-induced NO release was diminished by 35% in aortic rings pretreated with  $10^5$  and  $10^6$  EMPs/ml but not with  $10^4$  EMPs/ml or the corresponding EMP-free supernatant (Fig. 1C).

**EMPs dose-dependently increase superoxide levels in cultured endothelial cells.** To test whether EMPs decrease NO production or bioavailability as a result of oxidative stress, the level of superoxide was assessed in cultured RMVECs. Cells were loaded with DHE (10  $\mu$ M) and treated with EMPs for 1 h, after which the level of superoxide was measured using a fluorescence plate reader. Treatment with EMPs (but not with an EMP-free supernatant) resulted in dose-dependent increases of superoxide levels in cultured RMVECs (Fig. 2A). This increase in superoxide levels was completely prevented by pretreatment of RMVECs with the SOD mimetic MnTBAP (40  $\mu$ M) and was reduced 60% by pretreatment with the eNOS inhibitor L-NAME (1 mM; Fig. 2B).

**EMPs increase superoxide levels in aortic ring endothelium.** Intravital microscopy of aortic rings pretreated ex vivo with EMPs was used to assess superoxide levels. Aortic rings were loaded with 10  $\mu$ M DHE for 30 min, washed, and subsequently incubated in Krebs buffer. After a 20-min baseline recording, EMPs were added to the chamber and changes in endothelium-derived fluorescence were recorded. Addition of EMPs but not

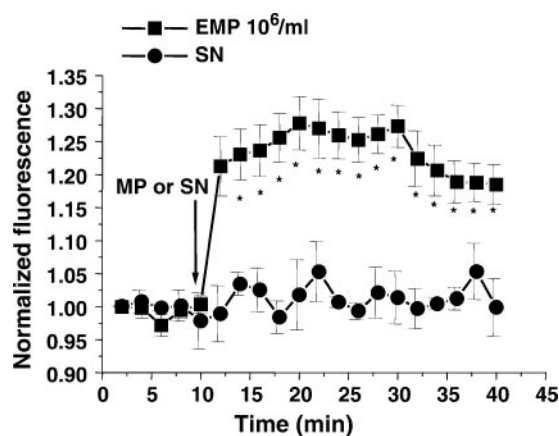


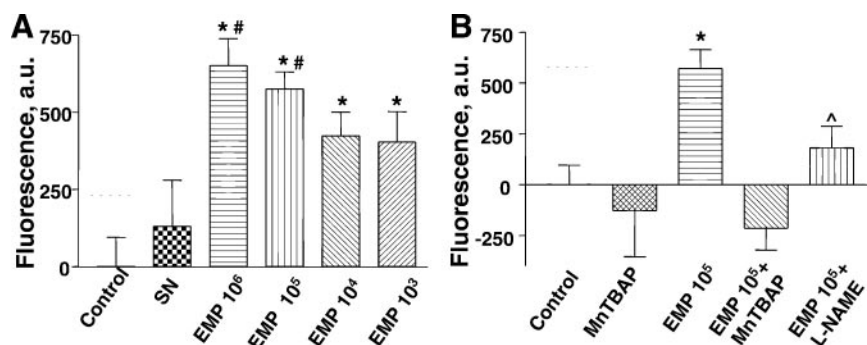
Fig. 3. EMPs induce superoxide production by aortic rings. Superoxide production was detected by fluorescence of DHE (10 mg/ml) by aortic rings treated with  $10^6$  EMPs/ml or high-speed supernatant. \* $P < 0.05$  compared with control.

EMP-free supernatant elicited a sustained increase in superoxide levels in the endothelium of intact aortic rings (Fig. 3).

**EMPs per se produce superoxide and express NAD(P)H oxidase subunit p22<sup>phox</sup>.** To test whether EMPs per se produce superoxide and thus contribute to superoxide production by cultured endothelial cells, EMPs were incubated with DHE, and the fluorescence was measured using the fluorescence plate reader. As shown in Fig. 4, EMPs produced superoxide, whereas EMP-free supernatant had no detectable changes in fluorescence. Moreover, immunoblot analysis revealed that the p22<sup>phox</sup> subunit of NAD(P)H oxidase was expressed in RMVECs and microparticles but not in the high-speed supernatant (Fig. 4).

**Treatment with SOD mimetic restores endothelium-dependent vasorelaxation and NO levels impaired by isolated EMPs.** The possibility to preserve endothelium-dependent vasorelaxation after EMP treatment by scavenging superoxide was tested using the SOD mimetic MnTBAP. ACh-induced relaxation of aortic rings was compared with NO levels in preparations preincubated for 3 h in buffer only and in preparations preincubated with buffer containing EMPs alone or both EMPs and 40  $\mu$ M MnTBAP. ACh-induced vasorelaxation as well as NO concentrations were completely restored in aortic rings treated with both EMPs and MnTBAP (Fig. 5). MnTBAP had no effects on endothelium-dependent vasorelaxation or NO levels in control aortic rings (data not shown).

Fig. 2. EMPs induce superoxide production by cultured endothelial cells. A: superoxide production by rat renal microvascular endothelial cells (RMVECs) treated with EMPs or supernatant (SN) for 1 h ( $n = 12$ ) detected by fluorescence of dihydroethidium (DHE, 10  $\mu$ M). B: superoxide production by RMVECs ( $n = 8$ ) treated with  $10^5$  EMPs/ml and manganese(III)tetrakis(4-benzoic acid)porphyrin (MnTBAP, 40  $\mu$ M) or *N*<sup>G</sup>-nitro-L-arginine methyl ester (L-NAME, 1 mM). Data are means  $\pm$  SE; \* $P < 0.05$  compared with control; # $P < 0.05$  compared to supernatant.





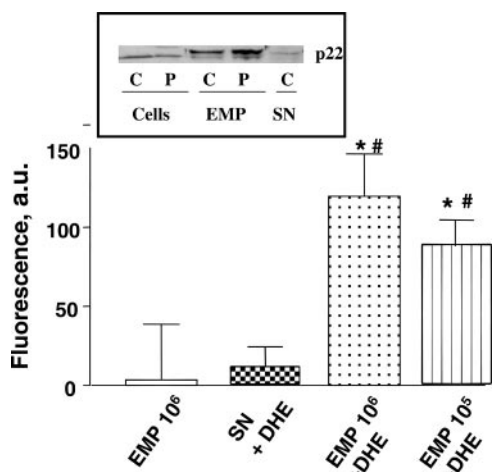


Fig. 4. EMPs per se produce superoxide. Superoxide production by EMPs or high-speed supernatant was detected by DHE (10 mM;  $n = 8$ ). Inset: p22<sup>phox</sup> subunit of NAD(P)H oxidase was detected by immunoblotting in isolated EMPs. C, control; P, PAI-1 (10 ng/ml, 3 h)-treated cells. Data are means  $\pm$  SE; \* $P < 0.05$  compared with control; # $P < 0.05$  compared with supernatant.

## DISCUSSION

One of the underlying pathophysiological mechanisms of endothelial cell dysfunction is an impaired balance between NO and reactive oxygen species including superoxide. Increased oxidative stress and diminished bioavailability of NO are key determinants of impaired endothelial function. Increased production of superoxide anion has been identified with many cardiovascular diseases including hypertension, atherosclerosis, and dyslipidemia (8, 15, 16). Formation of microparticles is a common mechanism of membrane shedding by many cell types including endothelial cells (5, 17, 23, 28). Increased numbers of circulating EMPs have been documented in patients with lupus anticoagulant, acute coronary syndromes, and diabetes mellitus (33).

This study provides evidence that EMPs directly affect endothelium-dependent vasorelaxation via a mechanism that involves diminished production and/or bioavailability of NO. This effect was accompanied by increased superoxide levels in aortic rings and cultured endothelial cells treated with microparticles and was inhibited by a SOD mimetic and partially inhibited by an eNOS inhibitor. Additionally, EMPs per se produced detectable amounts of superoxide and contained an NAD(P)H oxidase.

Although the increased numbers of circulating microparticles have been implicated in many cardiovascular diseases (33), the pathophysiological role of EMPs has not been well investigated. Thus the number of circulating platelet- and endothelium-derived microparticles is elevated in women with normal pregnancy compared with nonpregnant healthy women, whereas the presence of preeclampsia is associated with an increased procoagulant potential of circulating microparticles (5, 30) without a change in the total number of circulating microparticles (31). In addition, circulating microparticles isolated from the blood of preeclamptic women (but not from healthy pregnant women) directly affected endothelium by impairing endothelium-dependent relaxation in vitro in isolated myometrial arteries (32). Also, it has been demonstrated that rat aortic rings exposed for 24 h to circulating microparticles obtained from patients with acute myocardial infarction show significant impairment in endothelium-dependent relaxation, whereas microparticles obtained from patients with nonischemic diseases had no effects on endothelium-dependent relaxation (4). However, the role of EMPs in this process has not been determined. The pool of circulating microparticles contains microparticles shed by different cell types including platelets, leukocytes, and endothelial cells; EMPs represent  $\sim 10$ –15% of the total microparticle population (7, 32). In healthy humans, circulating microparticles of endothelial origin are present within the concentration range of  $(1 \text{ to } 70) \times 10^3$  EMPs/ml (3, 5, 17, 21). In our studies, we used a pure population of EMPs and demonstrated that EMPs obtained from cultured cells impair endothelium-dependent relaxation responses to ACh at concentrations similar to those observed in patients with diverse cardiovascular diseases (2, 7, 27, 28). Thus in our experiments, ex vivo 3-h incubation of aortic rings with  $10^5$ – $10^6$  EMPs/ml led to a significant impairment of ACh-induced relaxation, which suggests decreased production or bioavailability of NO. Indeed, direct measurement of the NO released by these aortic rings showed a decreased response to calcium ionophore after treatment with  $10^5$  and  $10^6$  EMPs/ml. It is important to note that incubation of aortic rings with physiological ( $10^3$ – $10^4$  EMPs/ml) concentrations of EMPs or EMP-free supernatant affected neither ACh-dependent vasorelaxation or NO production. One of the pathogenetic mechanisms of impaired endothelial function is an increase in superoxide production by the vascular endothelium, which was demonstrated by intravital microscopy of aortic rings treated with EMPs.

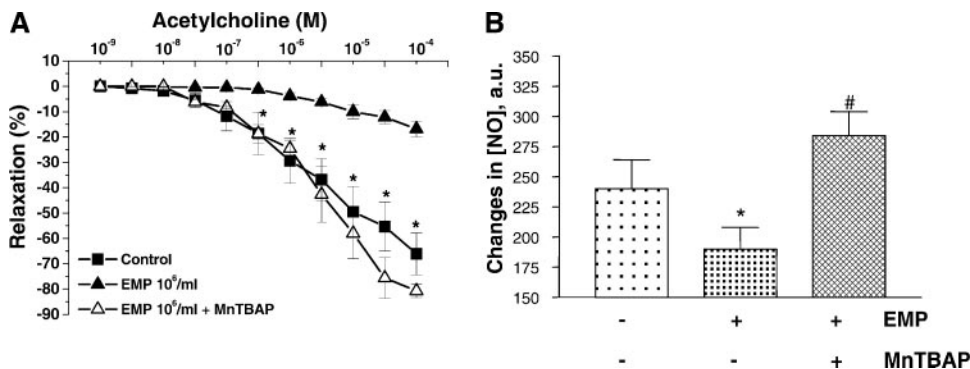


Fig. 5. Treatment with SOD mimetic restores endothelium-dependent vasorelaxation and NO production. A: endothelium-dependent vasorelaxation induced with ACh by aortic rings treated both with EMPs ( $10^6$  EMPs/ml) and SOD mimetic MnTBAP (40  $\mu$ M) was restored to the level of intact vessels. B: NO production by aortic rings treated with both  $10^6$  EMPs/ml and MnTBAP (40  $\mu$ M) was improved compared with the rings treated with  $10^6$  EMPs/ml alone. Data are presented as means  $\pm$  SE; \* $P < 0.05$  compared with  $10^6$  EMPs/ml.

To elucidate the mechanism(s) that leads to a decrease in NO production and/or bioavailability, we demonstrated that EMPs induce superoxide production in cultured endothelial cells as well as by the endothelium of aortic rings. The mechanisms of the increased production of superoxide by microparticles require additional investigation, but one of the hypothetical mechanisms may be an uncoupling of eNOS, which leads to a switch from the NO-producing enzyme to superoxide production (8, 10, 15, 18, 34). In fact, inhibition of eNOS by L-NAME partially inhibited the microparticle-induced release of superoxide, which suggests an important role of this enzyme in the production of superoxide by these cells. An initiator mechanism for eNOS uncoupling may be represented by superoxide ions produced by EMPs themselves (as shown in Fig. 4). Another possible source of superoxide in isolated EMPs may be represented by NAD(P)H oxidase. Indeed, we demonstrated that the NAD(P)H oxidase subunit p22<sup>phox</sup> is present in EMPs. This observation opens the door for additional investigations of the role of NAD(P)H oxidase, delivered by microparticles directly to the endothelium, in the pathogenesis of endothelial cell dysfunction.

The increase in superoxide production that is induced by EMPs plays an important role in impaired endothelium-dependent relaxation. Treatment with the SOD mimetic MnTBAP restored not only vascular responses to ACh by aortic rings incubated with EMPs but NO production as well. Regardless of the source of the increased superoxide levels in the endothelium, these observations suggest an important role of oxidative stress in the pathogenesis of abnormal vascular function.

The data presented demonstrate that EMPs are an important pathogenetic factor in the development of ECD and strongly suggest the possibility of a vicious cycle: the elevated number of microparticles is a marker of ECD and, in turn, elevated levels of microparticles thus formed could aggravate ECD.

#### GRANTS

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