Upregulation of Proinflammatory Proteins Through NF-κB Pathway by Shed Membrane Microparticles Results in Vascular Hyporeactivity

Angela Tresse, M. Carmen Martínez, Bénédicte Hugel, Karel Chalupsky, Christian D. Muller, Ferhat Meziani, Delia Mitolo-Chieppa, Jean-Marie Freyssinet, Ramaroson Andriantsitohaina

Objective—Microparticles are membrane vesicles with procoagulant and proinflammatory properties released during cell activation, including apoptosis. The present study was designed in dissecting the effects evoked by microparticles on vascular reactivity.

Methods and Results—Microparticles from either apoptotic T lymphocytic cells or from plasma of diabetic patients with vascular complications induced vascular hyporeactivity in response to vasoconstrictor agents in mouse aorta. Hyporeactivity was reversed by nitric oxide (NO) synthase plus cyclooxygenase-2 inhibitors, and associated with an increased production of vasodilatory products such as NO and prostacyclin. Microparticles induced an upregulation of proinflammatory protein expressions, inducible NO-synthase and cyclooxygenase-2, mainly in the medial layer of the vessels as evidenced by immunochemical staining. In addition, microparticles evoke NF-κB activation probably through the interaction with the Fas/Fas Ligand pathway. Finally, in vivo treatment of mice with lymphocyte-derived MPs induces vascular hyporeactivity, which was reversed by the combination of NO and cyclooxygenase-2 inhibitors.

Conclusion—These data provide a rationale to explain the paracrine role of microparticles as vectors of transcellular exchange of message in promoting vascular dysfunction during inflammatory diseases. (Arterioscler Thromb Vasc Biol. 2005;25:2522-2527.)

Key Words: Fas/Fas Ligand ■ microvesicles ■ proinflammatory proteins ■ vascular dysfunction ■ vasoactivity
T cells on vascular smooth muscle reactivity. It is interesting to note that under different pathological conditions, circulating levels of MPs from lymphocytes are enhanced. In diabetic patients the amount of MPs from leukocyte origin is 3-fold higher than in healthy donors. In addition, HIV-infected patients show elevated levels of MPs bearing CD4 antigens. In preeclampsia, elevated levels of MPs from granulocytes and lymphocytes have been reported. We have previously observed that MPs captured from blood samples from HIV-infected individuals have properties comparable to those of their counterparts from cultured apoptotic T CEM cells and from circulating plasma of diabetic patients. Hence, the use of MPs from T cells appears relevant in exploring their effects on vasoactivity.

In the present study, we provide evidence that MPs from either apoptotic T lymphocytes or from diabetic patients with vascular complications promote vascular hyporeactivity by inducing an increased production of vasodilatory products such as NO and prostacyclin. These MPs also induced upregulation of proinflammatory protein expression, the inducible NO-synthase (iNOS) and COX-2, mainly in the medial layer of vessels through NF-κB–dependent transcription via Fas/FasL pathway. Interestingly, in vivo treatment of mice with lymphocyte-derived MPs induces vascular hyporeactivity, suggesting their pathophysiological relevance during inflammatory diseases.

**Methods**

For a detailed Methods section, please see http://atvb.ahajournals.org.

**Results**

**MPs From T Cell Line Induce Vascular Hyporeactivity to Vasoconstrictor Agonists**

5-HT produced a concentration-dependent increase in tension in aortic rings with or without functional endothelium. Incubation of mice aortic rings with 30 nmol/L PEq MPs for 24-hour decreased vascular reactivity to the agonist both in endothelium-intact and endothelium-denuded vessels (Figure 1A and 1B). MP treatment-induced vascular hyporeactivity may result from the release of vasodilatory products from different cellular origin (ie, endothelial, smooth muscle, or fibroblast). However, the fact that 5-HT–induced contractions were also reduced in vessels without functional endothelium, strongly suggests that vasodilatory products from non-endothelial origin are implicated in the hyporeactivity produced by MP treatment. Therefore, the following experiments were performed in vessels without functional endothelium.

To investigate the role of NO and COX metabolites, the effect of the NO-synthase inhibitor, L-NA, the selective inhibitor of COX-2, NS-398, alone or in combination, were studied on the response to 5-HT. L-NA, NS-398, nor L-NA plus NS-398 had a significant effect on contractile responses to 5-HT in control arteries (Figure 1C and 1E). In aorta incubated with MPs, L-NA alone or NS-398 alone increased the contractile response to the agonist (Figure 1D). In vessels treated with MPs, the combination of the 2 inhibitors produced further potentiation of contraction when compared with that observed in the presence of either L-NA or NS-398 alone (Figure 1F) and restored the contractile response to 5-HT toward that of aortic rings not exposed to MPs. Similar data were obtained with the nonselective COX inhibitor, indomethacin (not shown). Also, MPs induced hyporeactivity in response to another agonist phenylephrine that was reversed in the combination of L-NA and NS-398 (not shown).

**MPs Generated From T Cell Line Stimulate NO and 6-keto-PGF₁α Production**

Direct in situ measurements of NO production were performed by EPR spectroscopy using Fe(DETC)₂ as a spin trap. Both control and MP-treated aortae with functional endothelium, preincubated with Fe(DETC)₂, exhibited an EPR feature of signals derived from NO-Fe(DETC)₂. The quantitative measurement of the NO-Fe(DETC)₂ signal amplitude was reported to the relative units for weight in mg of the dried sample (Wₐ). The NO-Fe(DETC)₂ EPR signal was greater in aortae treated with MPs for 24 hours (429±80 A/Wₐ) compared with nontreated vessels (179±18 A/Wₐ), (**P<0.01, n=8). Assay of 6-keto-PGF₁α, the stable product of PGI₂, showed increased production in aortae treated with MPs for 24 hours (3907±625 pmol/mg Wₐ) compared with nontreated vessels (1875±390 pmol/mg Wₐ), (**P<0.01, n=6).
**MPs From T Cell Line Induce iNOS and COX-2 Expression**

Immunohistochemical detection of iNOS and COX-2 was conducted by confocal microscopy. Whereas weak or no staining of either iNOS or COX-2 was found in the vessel wall of control aorta (Figure 2A and 2H), marked iNOS and COX-2 labeling were observed in the medial layer of aorta treated with MPs (Figure 2B and 2I). The negative control obtained by incubation with the secondary murine fluorescence-labeled antibody did not display any staining (Figure 2C and 2J). Western blot analysis showed an increase of both iNOS and COX-2 expression in aorta treated with MPs compared with control vessels (Figure 2G and 2N). The ability of MPs from T CEM cells to interact with human smooth muscle cells (HSMCs) has been studied by their capacity in promoting upregulation of iNOS and COX-2 expression. As shown in Figure 2O to 2R, HSMCs treated with T cell MPs exhibit a marked labeling of iNOS and COX-2 when compared with controls (Figure 2P and 2R versus 2O and 2Q, respectively).

**Sprad MPS From T Cell Line Activate NF-κB Detected by p65/Rel A and Phosphorylated 1kB-Alpha Staining**

Because enhanced expression of proinflammatory enzymes such as iNOS and COX-2 is under the control of the NF-κB/Rel family of transcription factors, the activation of the latter was assessed. NF-κB family members are heterodimers consisting of p65/RelA and p50/NF-κB1,14 but only the p65 subunit has transactivation domains capable of initiating transcription. Immunohistochemical studies showed marked aortic staining of p65/RelA subunit of NF-κB in the medial layer and weaker in the adventitial layer of aorta incubated with MPs (Figure 3B). No specific staining was found in control aorta (Figure 3A). Negative controls obtained by incubation with the secondary rabbit fluorescence-labeled antibody did not display any staining (Figure 3C).

As illustrated in Figure 3G to 3H, T cells MPs were able to activate NF-κB p65/Rel family in HSMC. Indeed, p65 translocates to the nucleus of cultured smooth muscle cells as shown by the merged picture after MPs stimulation (Figure 3H). These data were confirmed by the phosphorylation of 1kB-alpha at Ser32, which is essential for release of active NF-κB p65/Rel A. Whereas weak or no staining was found in control cells, a marked labeling of phosphorylated 1kB-alpha was found in HSMC treated with MPs (Figure 3I and 3J).

**Fas/FasL Signaling Accounts for Vascular Hyporeactivity Induced by MPs From T Cell Line**

The possibility that a direct interaction of membrane MPs with vascular cell (ie, smooth muscle) surface may account for activation of intracellular pathway was assessed. For this, the interaction between FasL, harbored by lymphocytic MPs, and Fas receptor expressed by vascular cells was considered using Fas receptor labeling by immunohistochemical studies. The vessel wall of control aorta that has not been in contact with MPs was considered the negative control. Immunohistochemical staining of either iNOS (O, P) or COX-2 (Q, R) from cultured human smooth muscle cells after incubation with either RPMI 1640/M199 medium (O, Q, n=5) or 30 nmol/L PS eq MPs (P, R, n=3). Propidium iodide in red, iNOS and COX-2 in green.

**Figure 2.** Enhanced iNOS and COX-2 expression evoked by MPs from T cell line. Immunohistochemical staining of iNOS from mouse aorta after incubation with either RPMI 1640/M199 medium (A, D, control n=5) or 30 nmol/L PS eq MPs (B, E, n=5) Immunohistochemical staining of COX-2 from mouse aorta after incubation with either RPMI 1640/M199 medium (H, K, control n=5) or 30 nmol/L PS eq MPs (I, L, n=5). (C, F, J, and M) Background of secondary Alexa 488-anti-mouse conjugated antibody. D to F and K to M, Corresponding phase-contrast images. Bars=50 μm. Western blot revealing expression of iNOS (G) and COX-2 (N) in mouse aorta exposed to 30 nmol/L PS eq MPs. Histograms show densitometric analysis of iNOS and COX-2 expression of 3 separate experiments. **P<0.01, significantly different from control. Immunohistochemical staining of either iNOS (O, P) or COX-2 (Q, R) from cultured human smooth muscle cells after incubation with either RPMI 1640/M199 medium (O, Q, n=5) or 30 nmol/L PS eq MPs (P, R, n=3). Propidium iodide in red, iNOS and COX-2 in green.
Isolated from mice to study vascular reactivity. In endotheli-

um intact preparations, the contractile response to 5-HT was

lower in aorta taken from mice treated with MPs compared

with the response obtained in vessels from mice treated with

vehicle (Figure 4A). Furthermore MP-induced vascular hy-

poreactivity to 5-HT was reversed by the combination of

L-NA and NS-398 (Figure 4A). Moreover, increased CD4

labeling was found in the media layer of aortas from

MP-treated mice, whereas weak CD4 staining was detected in

control vessels (Figure 4B through 4G).

**In Vivo Circulating MPs and Lymphocyte-Derived

MPs from Diabetic Patients Induce

Vascular Hyporeactivity**

To assess the pathophysiologic relevance of the present study,

the effect of circulating MPs from diabetic patients that had

typical symptoms, such as microvascular complications, was

examined. As shown in Figure 5A, treatment of mouse aorta

with circulating MPs from 5 diabetic patients significantly

reduced the contractile response to phenylephrine, and reac-
tivity was restored in the presence of anti-FasL antibody.

Furthermore, MPs shed by the actinomycin D-challenged

lymphocytes of the same diabetic patients were also able to

induce vascular hyporeactivity (Figure 5B). These data con-

firm that MPs play a significant role in the development of

vascular dysfunction under several pathophysiological situa-
tions such as cardiovascular disorders (ie, diabetes).

**Discussion**

In the present study, MPs stemming from apoptotic T

lymphocytes or circulating in diabetic patients with vascular

complications promote vascular hyporeactivity by inducing

the production of vasodilatory mediators such as NO and

prostacyclin. This effect resulted in an upregulation of iNOS

and COX-2 through NF-κB–dependent transcription via the

Fas/FasL pathway. These data provide valuable information
to explain the paracrine role of MPs as vectors of transcellular
exchange of message in promoting vascular dysfunction
during inflammatory diseases.

In general, studies of the impact of MPs on cell activation
or function have been performed using MPs from stimulated

platelets, because they represent the main source of circulat-
ing procoagulant MPs and play a role in hematopoiesis and

cell activation. Elevated levels of circulating MPs of
different cell types, including T cells. Here, we have used 30

nmol/L PS eq MPs because this concentration is frequently

observed in several pathologies like paroxysmal nocturnal

hemoglobinuria, diabetes, or unstable angina. However, MPs

are not only released from platelets or endothelial cells, but also

from various cell types, including T cells. Here, we have used 30

nmol/L PS eq MPs because this concentration is frequently

observed in several pathologies like paroxysmal nocturnal

hemoglobinuria, diabetes, or unstable angina. Thus, this

concentration of MPs may well be reached in vivo, in the

plaque vicinity, for instance.

Several pathologies are associated with vascular dysfunc-
tion including attenuation of endothelium-dependent vasodi-
latation, alteration of responsiveness of vascular smooth

muscle to vasoconstrictor stimuli, or both. For instance, in

inflammatory disorders such as cirrhosis, portal hyperten-
sion, or sepsis both vascular hypo-responsiveness to


Figure 3. NF-κB p65 activation induced by MPs from T cell line. Immunohistochemical staining for NF-κB p65 of mouse aorta after incubation with either RPMI 1640/M199 medium (A, D, control, n=3) or 30 nmol/L PS eq MPs (B, E, n=3). C and F, Background of secondary Alexa 488-anti-rabbit conjugated antibody. D to F, Corresponding phase-contrast images. Immunohistochemical staining for NF-κB p65 (G, H) and phosphorylated IκB-alpha (I, J) of cultured human smooth muscle cells after incubation with either RPMI 1640/M199 medium (G, I, control, n=3) or 30 nmol/L PS eq MPs (H, J, n=3). Propidium iodide in red, NF-κB p65 and phosphorylated IκB-alpha in green.
vasoconstrictors and reduced endothelium-dependent relaxation have been reported. We have previously shown that the same type of MPs used in the present study is able to induce endothelial dysfunction.11

Here, MPs from apoptotic T cells and from plasma of diabetic patients impaired the contraction induced by agonists, and this hyporeactivity was observed even in vessels without functional endothelium. These results and those obtained on cultured HUVECs indicate that MPs are able to act on the smooth muscle cells and induce the release of vasodilatory factors, or alternatively, MPs can evoke an alteration of the balance between relaxant and constrictrion factors in smooth muscle cells.

With regard to the vasodilatory factors, the inhibition of proinflammatory enzymes, iNOS or COX-2, reduced the hyporeactivity in MP-treated vessels. In addition, the combined inhibition of both enzymes restored the contractile response. Moreover, MPs stimulated the production and release of NO and prostacyclin, account for the hyporeactivity induced by MPs. This is corroborated by the expression of iNOS and COX-2 in the vessel wall. Interestingly, the observations by confocal microscopy show that the increases of iNOS and COX-2 expressions were mainly localized in the medial layer of aorta treated with MPs. This is partially in accordance with the upregulation reported by Barry16,21 in HUVECs, platelets, or monocytic cells subjected to platelet MPs, but in their study arachidonic acid was the key mediator of these effects. The difference of the vasodilatory product released by COX-2 might be related to the cell origin of MPs, the stimuli responsible for MP release or target cells. Concerning the effect of MPs on iNOS, to the best of our knowledge the present study shows for the first time upregulation of iNOS induced by MP treatment. Our previous results on endothelial dysfunction through alteration of eNOS11 and those obtained in the present study show that MPs from T cells can affect the expression of different types of NO-synthases to alter vascular function and this includes iNOS for its activating effect on the release of vasodilatory products from smooth muscle origin.

NF-κB activation is upstream of the synthesis of acute phase inflammatory mediators. Among the genes known to be positively regulated by NF-κB are iNOS and COX-2. One possible explanation is that both promoter regions of iNOS and COX-2 genes have NF-κB binding sequences, and, thus, the same pathophysiologic stimuli (MPs in this case) may turn on the expression of both genes simultaneously.17 Alternatively, NO produced through NF-κB–induced iNOS expression may affect COX-2 expression and/or activity as shown in other models. Nevertheless, here MPs from T cells were able to induce NF-κB activation in the vessel wall and in HSMC, as revealed staining of its p65 subunit and phosphorylation of IkB-alpha.

In vascular smooth muscle cells it has been shown that the activation of the Fas/FasL pathway results in the increased expression of a specific program of inflammatory genes.23,24 We have previously shown that MPs used in the present study expressed FasL.11 Here, MP-treated vessels displayed a lack of Fas labeling. It cannot be excluded that MPs induced downregulation of Fas expression at the surface of smooth muscle cells. However, the fact that after incubation of MPs with an anti-FasL antibody, the vessel reactivity was restored is in favor of an interaction of FasL from MPs with Fas from the vessel wall, leading to intracellular signaling. Concerning the possible pathway linked to MPs, it has been shown that MP-borne FasL, but not the soluble form, is cytotoxic through activation of NF-κB.25,26 These works are in accordance with the
present study where MPs from apoptotic T cells activated NF-κB through the Fas/FasL pathway independently of its ability to induce smooth muscle cell apoptosis.

Finally, we show that in vivo treatment of mice with MPs generated from T cells induces similar results than those obtained in ex vivo incubation of mice vessels with MPs. In addition, CD4 staining was observed in vessels from in vivo-treated mice. Altogether, these results strongly suggest that circulating MPs are able to induce in vivo vascular hyporeactivity that is associated with an increase of CD4 expression in the media layer of the vessel wall. Although CD4 labeling could be related to an infiltration of in vivo monocytes-macrophages into the media layer of the vessel wall, one can advanced the hypothesis that MPs reach smooth muscle and mediate their effect through Fas/FasL interaction. The mechanism by which MPs cross the endothelial barrier and affect vascular smooth muscle function remains to be determined. Nevertheless, the present data demonstrate for the first time to our knowledge the in vivo effect of MPs and thus their pathophysiological relevance in terms of vascular hyporeactivity.

In summary, we propose that MPs interacting with smooth muscle cells through the Fas/FasL pathway evoke NF-κB activation, which in turn upregulates iNOS and COX-2 expression leading to the production and release of the vasodilatory factors NO and prostacyclin. Both NO and prostacyclin account for the MP-induced hyporeactivity. Hatano et al.17 have shown that NO from endogenous iNOS, which is activated by NF-κB, provides partial protection from Fas-mediated inflammation. In this respect, vasodilatory factors released from smooth muscle cells may counteract the cytotoxic effects of Fas/FasL interaction. Our results indicate that MPs released from T cells can act as true mediators of an inflammatory signal. The hyporeactivity to vasoconstrictr agents observed on vessel treatment with MPs from diabetic patients emphasizes the role of MPs as vectors of transcellular exchange of message in promoting vascular dysfunction accompanying inflammatory diseases.

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References

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Methods

MPs production

The human lymphoid CEM T-cell line (ATCC number CRL-2265) was cultured in free-serum X-vivo15 medium. MP production was induced by treatment of $2 \times 10^6$ cells with actinomycin D (0.5 µg/mL, 18 h, 37°C). The cell supernatant was obtained after two centrifugation steps (750 g for 15 min; 200 g for 5 min). The supernatant was centrifuged for 45 min at 14,000 g. The pelleted MPs were suspended in Hank’s buffer and centrifuged for 45 min at 14,000 g. The pellet was recovered in 1 mL RPMI 1640/M199 medium. MPs isolated from at least five independent preparations were analyzed for each experimental condition.

To prepare circulating MP samples, peripheral blood was collected from five informed and consenting diabetic individuals with typical symptoms, such as microvascular complications. Circulating MPs were obtained after a two step centrifugation of plasma samples (1,500 g for 15 min; 13,000 g for 2 min), and pelleted by centrifugation at 13,000 g for 45 min. The pellet was recovered in 1 mL.

Lymphocytes were also isolated by differential centrifugation on Ficoll. Isolated lymphocytes were treated with actinomycin D and generated MPs were obtained as described above.

MP amounts and their phenotypic characterization were determined by measurement of their procoagulant phosphatidylserine content in a prothrombinase assay described elsewhere, and were expressed as nmol/L phosphatidylserine equivalent (nmol/L PS eq) by reference to a calibration curve. As previously described, MPs from plasma of diabetic patients and from actinomycin D-treated lymphocytes from the same patients expressed surface markers characteristic of T CEM cells.

Levels of endotoxin were assessed with the Limulus Amebocyte Lysate kit QCL-1000 (Cambrex) and found to be below the lower detection limit of the kit (<0.1 endotoxin units/ml).
**Measurement of vascular reactivity**

Male Swiss mice (6-8 weeks old) were anesthetized and sacrificed by decapitation. Aortic rings were mounted on a wire myograph filled with PBS (37°C, 95% O₂-5% CO₂). Mechanical activity was recorded isometrically by a force transducer (Kistler-Morse, DSG BE4). The endothelium functionality was assessed by the acetylcholine ability to induce relaxation.

In preliminary experiments, treatment of vessels with 30 nmol/L PS eq MPs for 24 h corresponded to the concentration and time required to obtain maximal endothelial dysfunction. Thus, all experiments MPs were performed under these conditions, which could be relevant with respect to pathophysiologic situations. Supernatants corresponding to the last MPs washing medium have been used as control and it did not display any modification of vascular reactivity. Thus, it is unlikely that the effects of MPs on vascular reactivity result from a probable contamination by endotoxin that would induce hyporesponsiveness to vasoconstrictor agents (see above). In another set of experiments, male Swiss mice were treated in vivo by i.v. injection of either 30 nmol/L PS eq. MPs generated from T cell line or supernatant corresponding to the last MPs washing medium in the tail vein of the animals. After 24 h vessels were isolated from mice to study vascular reactivity.

Concentration-response curves were constructed by cumulative application of either 5-HT or phenylephrine to vessels with or without functional endothelium after 30 min pre-incubation with either the NO-synthase inhibitor, N⁶-nitro-L-arginine (L-NA), the COX inhibitor, indomethacin, the selective COX-2 inhibitor, N-(2-cyclohexyloxy-4-nitrophenyl) methanesulfonamide (NS-398) or L-NA plus indomethacin or plus NS-398. The anti-human FasL antibody (dilution 1:10) was incubated with MPs (30 min, 4°C) to allow neutralization of MP FasL. Vessels were then treated with this mixture before contraction experiments.

**Human smooth muscle cell (HSMC) culture**
Human umbilical artery smooth muscle cells from PromoCell bioscience alive (PromoCell GmbH) were cultured with Dubbelco Eagle's medium/Ham's F12 (3:1), 10% bovine serum, 100 U/mL penicillin and 25 g/mL gentamicin. Cells showed a constant phenotype during subculturing and were used on passage 4.

**NO spin trapping and EPR studies**

Detection of NO production was performed using a previously described technique with Fe$^{2+}$ diethylldithiocarbamate (DETC) as spin trap.$^{13}$ The phenylephrine-treated vessels were placed in 24-well clusters filled with 250µL of Krebs solution, and then treated with 250µL of colloid Fe(DETC)$_2$ and incubated (37°C, 1 h). These studies were performed on a table-top x-band spectrometer Miniscope (Magnettech). Recordings were made at 77°K, using a Dewar flask. Instrument settings were 10mW of microwave power, 1mT of amplitude modulation, 100 kHz of modulation frequency, 60 s of sweep time and 10 scans.

**Determination of prostanoid production**

Vessels with endothelium were treated with 5-HT (10 µmol/L, 20 min). The medium was next collected. Then, 6-keto-PGF$_{1a}$ was measured by enzyme immunoassay kits. The concentration of 6-keto-PGF$_{1a}$ was expressed as pmol/mg tissue (dry weight).

**Staining and imaging by confocal microscopy**

Vessels with endothelium were frozen and cut in 10 µm sections. Fixed sections were incubated (2 h) in blocking buffer (5% non-fat dry milk in PBS). After three washes, tissue sections were incubated overnight with monoclonal murine anti-iNOS (1:50), anti-COX-2 (1:100) or phosphorylated IκB-alpha (1:100) antibodies. For NF-κB p65 immunostaining we used a polyclonal NF-κB p65 antibody (1:100). Three washes were followed by incubation (1 h) with murine Alexa fluor-488-labeled antibody (1:100). Biotin-conjugated mouse anti-human CD95 monoclonal antibody (1:50) was used for Fas receptor immunostaining. For
CD4 immunostaining, we used biotin-conjugated anti-human CD4 monoclonal antibody (1:10). After three washes were followed by incubation (1h) with streptavidin-phycoerythrin (1:100). After washes, vessel sections were mounted on glass slides.

After fixation, HSMC were incubated with anti-iNOS, anti-COX-2, phosphorylated IκB-alpha or NF-κB p65 antibodies associated with propidium iodide, to stain the nucleus, for 10 minutes at room temperature. Three washes were followed by incubation (15 min) with the secondary murine or rabbit Alexa fluor-488-labeled antibody. After washes, cells were mounted on glass slides.

The 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 LaseSharp Software. Confocal Assistant was used for image analysis (TC Brelje).

**Western Blot analysis**

Crushed tissue were homogenized and lysed. Seventy-five μg proteins were separated on 7% or 12% SDS-PAGE. Blots were probed with anti-iNOS and COX-2 followed by the anti-mouse horseradish peroxidase-conjugated antibody.

**Statistical Analysis**

Data are represented as mean ± SEM. n represents to the number of animals studied. Statistical analyses were performed by Student t-test or analysis of variance. Differences were considered significant at a value of $P<0.05$. 
Figure I. Involvement of Fas/FasLigand pathway in the hyporeactivity induced by MPs from T cell line. Immunohistochemical staining for Fas receptor CD95 of mouse aortic vessels after incubation with either RPMI 1640/M199 medium (A, D, control, n=5) or 30 nmol/L PS eq MPs (B, E, n=5). (C, F) Background of streptavidin signal. (D-F) show the corresponding phase-contrast images. Bars = 100 µm. Immunohistochemical staining for iNOS (G-I) or COX-2 (J-L) of cultured human smooth muscle cells after incubation with either RPMI 1640/M199 medium (G, J, control, n=3) or 30 nmol/L PS eq MPs in the absence (H, K, n=3) or in the presence of anti-FasL antibody (I, L, n=3). Propidium iodide in red, iNOS or COX-2 in green. (M) Concentration-response curves to phenylephrine of mouse aortic rings without functional endothelium after incubation with either RPMI 1640/M199 medium (open circles, control, n=6) or 30 nmol/L PS eq MPs in the absence (filled circles, n=6) or in the presence of anti-FasL antibody (open squares, n=6). ***P<0.001 significantly different from control.
Figure I