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Macrophages Derived From Septic Mice Modulate Nitric Oxide Synthase and Angiogenic Mediators in the Heart

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Macrophages (Mps) can exert the defense against invading pathogens. During sepsis, bacterial lipopolisaccharide (LPS) activates the production of inflammatory mediators by Mps. Nitric oxide synthase (NOS) derived-nitric oxide (NO) is one of them. Besides, Mps may produce pro-angiogenic molecules such as vascular endothelial growth factor-A (VEGF-A) and metalloproteinases (MMPs). The mechanisms involved in the cardiac neovascular response by Mps during sepsis are not completely known. We investigated the ability of LPS-treated Mps from septic mice to modulate the behavior of cardiac cells as producers of NO and angiogenic molecules. In vivo LPS treatment (0.1 mg/mouse) increased NO production more than fourfold and induced de novo NOS2 expression in Mps. Immunoblotting assays also showed an induction in VEGF-A and MMP-9 expression in lysates obtained from LPS-treated Mps, and MMP-9 activity was detected by zymography in cell supernatants. LPS-activated Mps co-cultured with normal heart induced the expression of CD31 and VEGF-A in heart homogenates and increased MMP-9 activity in the supernatants. By immunohistochemistry, we detected new blood vessel formation in hearts cultured with LPS treated Mps. When LPS-stimulated Mps were co-cultured with isolated cardiomyocytes in a transwell assay, the expression of NOS2, VEGF-A and MMP-9 was induced in cardiac cells. In addition, MMP-9 activity was up-regulated in the supernatant of cardiomyocytes. The latter was due to NOS2 induction in Mps from in vivo LPS-treated mice. In conclusion LPS-treated Mps are inducers of inflammatory/angiogenic mediators in cardiac cells, which could be triggering neovascularization, as an attempt to improve cardiac performance in sepsis.

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Macrophages (Mps) are innate immune cells that form the first line of defense against invading pathogens. They are released from the bone marrow and after circulating in the blood stream, they migrate into tissues to undergo final differentiation into resident Mps (Valledor et al., 2010). These cells perform many functions that are essential for tissue remodeling, inflammation and immunity, including phagocytosis, cytotoxicity and secretion of a wide array of cytokines, growth factors, lysozymes, proteases, complement components, clotting factors and prostaglandins (Ren et al., 2008). One of the most potent stimuli for Mps is bacterial lipopolysaccharide (LPS), a constituent of the cell wall of Gram negative bacteria, and a major causative agent of septic shock. This condition affects different organs, among others the myocardium and the blood vascular system. In experimental models, LPS challenge leads to pathophysiological changes similar to the human septic shock syndrome. During sepsis, LPS starts a complex cascade of events in monocytes, Mps and neutrophils that leads to the production of endogenous mediators, preceding multiple organ dysfunction syndrome (Ren et al., 2008). One of these mediators is nitric oxide (NO) derived from calcium-dependent isoforms of nitric oxide synthase (NOS) 1 and 3 and/or from the calcium-independent NOS2 isoform. LPS is a potent inducer of NOS2 leading to high production of NO (Moncada and Higgs, 2006). NO is one of the vasoactive substances released from a variety of cells under conditions of endotoxemia and sepsis, and it is now becoming clear that NO plays a pivotal role in the regulation of gene expression (Connelly et al., 2001).

For a decade the function of vascular endothelial growth factor (VEGF) (also referred to as VEGF-A) in the regulation of angiogenesis has been extensively reviewed (Ferrara et al., 2003). VEGF-A belongs to a gene family that includes VEGF-B, VEGF-C and VEGF-D. Alternative splicing of VEGF-A yields in the generation of different isoforms ($VEGF_{121}$, $VEGF_{165}$, $VEGF_{189}$, $VEGF_{206}$). Less frequent splice variants have also been reported, such as $VEGF_{145}$ and $VEGF_{185}$. Native VEGF is a heparin-binding homodimeric glycoprotein of 45 kDa and its properties closely correspond to those of $VEGF_{165}$. VEGF-A is a potent angiogenic and vascular permeability factor (Ferrara et al., 2003). It has been shown that VEGF levels are regulated in

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several cell types, for example, Mps and smooth muscle cells after LPS stimulation, suggesting that it is important in the initiation and development of sepsis (Kim et al., 2008). Other factors mainly involved in angiogenesis are metalloproteases (MMPs). A variety of cell types such as Mps, lymphocytes and tumor cells, have been shown to produce MMPs (Sato et al., 1994; Goetzl et al., 1996; Malik et al., 1996). Cytokines as well as other factors such as lectins, hormones, viruses, bacteria, and LPS, have been shown to play a crucial role in the regulation of MMPs expression in vivo and in vitro (Paemen et al., 1997). Significantly increased MMPs activity has been observed in a variety of inflammatory disorders such as rheumatoid arthritis, multiple sclerosis and in bacterial sepsis, implicating a role for the MMPs in the tissue injury that accompanies these diseases (Gijbels et al., 1992; Pagenstecher et al., 2000; Takaishi et al., 2008).

Because the mechanisms by which Mps are involved in the modulation of cardiac angiogenesis during septic shock are not completely known, we here investigated the ability of Mps to produce NOS derived NO and angiogenic mediators by itself and to induce those molecules in the heart in an experimental model of sepsis induced by LPS.

Materials and Methods

Animals

Female BALB/c mice (12 weeks old) from our Animal Care Division were used. Mice were maintained on a 12 h day/night cycle and fed ad libitum with a standard diet. Euthanasia was performed by inhalation of CO_2. Animals were maintained in accordance with the guidelines of the NIH (Guide for the Care and Use of Laboratory Animals, 1996) and protocols were approved by the Internal Committee for the Care and Use of Laboratory Animals (CICUAL) from the School of Medicine, University of Buenos Aires.

Purification of peritoneal macrophages

Mps were obtained by washing the peritoneal cavity of BALB/c mice with 5 ml of Dulbeccós Modified Eagle Medium (D-MEM; Invitrogen Life Technologies, Grand Island, NY) supplemented with 10% of heat-inactivated fetal bovine serum (FBS) (PAA, Greiner Bio-One, Frickenhausen, Germany). Cells were left to adhere to plastic for 2 h at 37°C. After washing twice with phosphate buffer saline (PBS), adherent cells were scraped and suspended in culture medium. Cell viability (>95%) was assessed by Trypan blue exclusion test. For in vitro assays, Mps were treated with 10 μg/ml of LPS from *Eschericchia coli* (*E. coli*; L2654 Sigma–Aldrich, St. Louis, MO) for 24 h. The in vivo model of septic shock was obtained by inoculating mice with 0.1 mg/mouse of LPS in 0.1 ml PBS intraperitoneally (i.p.) (Ren et al., 2008). To prevent NOS2 induction, animals were treated i.p. with aminoguanidine (150 mg/kg) 1 h before LPS treatment. Animals were sacrificed 6 or 24 h post-inoculation and peritoneal Mps were purified. After 24 h, culture supernatants were collected and after washing with PBS, Mps were lysed in buffer: 100 mM NaCl; 10 mM EGTA; 10 mM EDTA; 50 mM Tris–HCl pH 8; 1% Triton X-100 and protease inhibitors. Lysates were centrifuged at 8,000*g* for 10 min at 4°C. Samples were stored at −80°C and protein concentration was measured by the method of Bradford (1976).

Determination of nitric oxide

Levels of NO released into the cell culture were determined by Griess reaction (Green et al., 1982). Briefly, Mps were seeded in 6-well plates, at 10^6 cells/well in 1 ml D-MEM:F12 with 5% FBS. After 24 h, 100 μl of the supernatants were added to an equal volume of Griess reagent (1% sulphanyl amine in 30% acetic acid with 0.1% N-1-naphtyl ethylenediamine dihydrochloride in 60% acetic acid). Absorbance was measured at 540 nm with an ELISA Reader (Biotek, Winooski, VT). Results were expressed as nitrite (NO_2^-) production in micromolar concentration per million cells ($\mu M/10^6$ cells).

Western blot assays

Heart tissue homogenates or cell lysates were prepared according previously described methods and were subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE), seeding 20–50 μg/lane (Davel et al., 2002; Penas et al., 2013). Proteins were transferred to nitrocellulose strips and incubated with the following polyclonal antibodies: rabbit anti-NOS1 or anti-NOS3 (sc-1025 or sc-654 Santa Cruz Biotechnology, Santa Cruz CA), goat anti-NOS-2 (sc-8310 Santa Cruz Biotechnology), goat anti-PECAM-1 (CD31; sc-1506 Santa Cruz Biotechnology), goat anti-VEGF-A (sc-1836 Santa Cruz Biotechnology) or rabbit anti-MMP-9 (#3852 Cell Signaling Technology, Danvers, MA). Then, the corresponding second antibody: anti-rabbit IgG conjugated with peroxidase, diluted 1:10,000 or anti-goat IgG conjugated with peroxidase, diluted 1:20,000 (A5420 or A0545 Sigma–Aldrich) was added. Bands were visualized by chemiluminiscence. Optical density (O.D.) of the bands was quantified by Image J software (NIH) and expressed in O.D. units relativized to that obtained for actin or glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression (detected with polyclonal antibodies: rabbit anti-β actin or rabbit anti-GAPDH (sc-130656 or sc-25778 Santa Cruz Biotechnology), which were used as loading controls.

Zymography assays

Culture supernatants were subjected to 10% SDS–PAGE gel electrophoresis in the absence of reducing agents. Gels were copolymerized with gelatin from porcine skin as enzyme substrate. After electrophoresis, gels were incubated in 5 mM $CaCl_2$ and 50 mM Tris–HCl buffer, pH 7.4, at 37°C for 24 h. Then, the zymograms were stained with Coomassie Brilliant Blue R-250 and destained with 30% methanol and 10% acetic acid in distilled water. Bands of gelatine degradation could be seen as transparent areas against a blue background. O.D. of the bands was quantified by Image J software and values were relativized to control (cells or tissue without treatment) considered as 1.

Co-culture of macrophages with normal hearts

Mps (3×10^6) from BALB/c mice were obtained 6 h after treatment (0.1 mg/mouse LPS administered i.p.) and cultured with hearts slices (100 mg/sample) from normal BALB/c mice in 4 ml of D-MEM medium. After 24 or 48 h, the culture supernatants were collected and hearts were homogenized following procedures previously described (Davel et al., 2002).

Immunohistochemistry

Heart tissue obtained from co-culture experiments mentioned above were fixed by immersion in formalin solution and then dehydrated, cleared, embedded in paraffin wax and cut (7-μm thickness) on a rotating microtome. The slides were deparaffinized and rehydrated through xylene and descending ethanol series. The quenching of endogenous peroxidase activity was made by incubation in 3% H_2O_2 in methanol for 30 min. After several washes in PBS, the slides were heated in microwave oven in buffer citrate pH 6 for 1 min three times for antigen retrieval. After cooling at room temperature and rinsing with PBS, the sections were incubated with blocking solution (PBS with 5% skimmed milk) for 1 h at room temperature. Then, after PBS washing, the tissue were bordered with pap-pen and incubated with a goat polyclonal antibody against mouse PECAM-1 (CD31) molecule (sc-1506 Santa Cruz Biotechnology) diluted 1:100 in blocking solution overnight at 4°C in a humidified chamber. After washes, sections were incubated 30 min at room temperature with diluted biotinylated antibody solution provided by Vectastain Elite ABC system kit (Vector, Burlingame, CA) and then with ABC reagent from the same kit during 30 min more. The immune reaction was detected

using H_2O_2 with 3, 3′-diaminobenzidine tetrahydrochloride (D9015 Sigma Chemical Co.) within 10 min, when desired stain intensity was achieved. Control sections were prepared by omitting the primary antibody. Controls did not show immunolabelling. An Arcano ZOU T biological photomicroscope equipped with a digital camera (Arkano, China) was used for the observation of sections. The images obtained were analyzed and stored using a TSView Image software.

Primary cardiomyocytes culture

Hearts from neonatal BALB/c mice were removed and kept in Hanks' balanced salt solution (HBSS) without Ca^{2+} and Mg^{2+} The cells were dissociated with trypsin (Sigma–Aldrich; 0.25% wt/vol in HBSS) at 37°C. After successive digestions cells were added to an equal volume of cold HBSS with Ca^{2+} and Mg^{2+}. The cell suspension was centrifuged at 200*g* for 8 min and the cells were suspended in D-MEM:M199 medium (Gibco Life Technologies, Grand Island, NY; 4:1, vol/vol) supplemented with 10% FBS and antibiotics. The suspended cells were then collected and plated at a density of 10^5 cells/cm^2 in D-MEM:M199 with 10% FBS and antibiotics. It was confirmed that more than 90% of cells were cardiomyocytes (Hovsepian et al., 2011). For co-culture experiments, cardiomyocytes (2×10^6) were plated in 24-well plates, were washed extensively with PBS, and transwells with 0.4-μm porosity polyester membrane filters were placed above them. LPS-activated peritoneal Mps (2.5×10^6; obtained from animals treated i.p. with 0.1 mg/mouse LPS for 6 h) were added to the upper chamber. After 24 or 48 h, the supernatants from cardiomyocytes were collected and after washing with PBS cells were lysed in buffer: 100 mM NaCl; 10 mM EGTA; 10 mM EDTA; 50 mM Tris–HCl pH 8; 1% Triton X-100 supplemented with protease inhibitors. Lysates were centrifuged at 8,000*g* for 10 min at 4°C. Samples were stored at −80°C and protein concentration was measured by the method of Bradford (1976).

Statistics

Data were expressed as mean ± SEM of at least three experiments. A GraphPad Prism computer program one-way ANOVA analysis for paired samples was used to determine the significance of differences between mean values in all control and test samples. The analysis was complemented by using a Tukey test to compare among mean values. Differences between means were considered significant if $P < 0.05$.

Results

Effect of LPS added in vitro to macrophages

We tested the action of 10 μg/ml LPS added in vitro during 24 h to normal peritoneal Mps by quantifying NO production, one of the main modulators of inflammation, accumulated in culture supernatants. Figure 1A shows that the production of NO from LPS-treated Mps increased by 100 ± 9%. Concomitantly, LPS induced de novo expression of NOS2 protein, while it decreased the levels of calcium-dependent isoforms, NOS1 and NOS3 (Fig. 1B). In addition, LPS significantly increased MMP-9 enzyme expression by 49 ± 5% and activity by 130 ± 12% respect to control (untreated Mps; Fig. 2).

Effect of LPS administered in vivo on macrophages

When Mps were purified from animals treated in vivo with LPS, the maximal effect on nitrite accumulation in culture supernatants was observed 6 h post-inoculation (27 ± 2 μM/10^6 cells) in comparison to untreated Mps cultured for the same period of time (6 ± 1 μM/10^6 cells; $P < 0.001$; Fig. 3A). NO production was still increased in Mps after 24 h treatment with LPS in vivo (13 ± 2 μM/10^6 cells) in comparison to control (7 ± 1 μM/10^6 cells; Fig. 3A). These results were paralleled to a maximal induction in the expression of NOS2 observed in Mps at 6 h, which decreased 24 h post-inoculation of LPS (Fig. 3 B). As it was observed in vitro, NOS1 expression was also down-regulated in Mps.

In vivo activation of Mps by LPS induced de novo expression of VEGF-A in cell lysates, being greater at 24 h than at 6 h ($P < 0.01$ vs. control: untreated Mps; Fig. 4A). In a similar manner than Mps treated in vitro with LPS, in vivo treatment increased MMP-9 expression in cell lysates and raised MMP-9

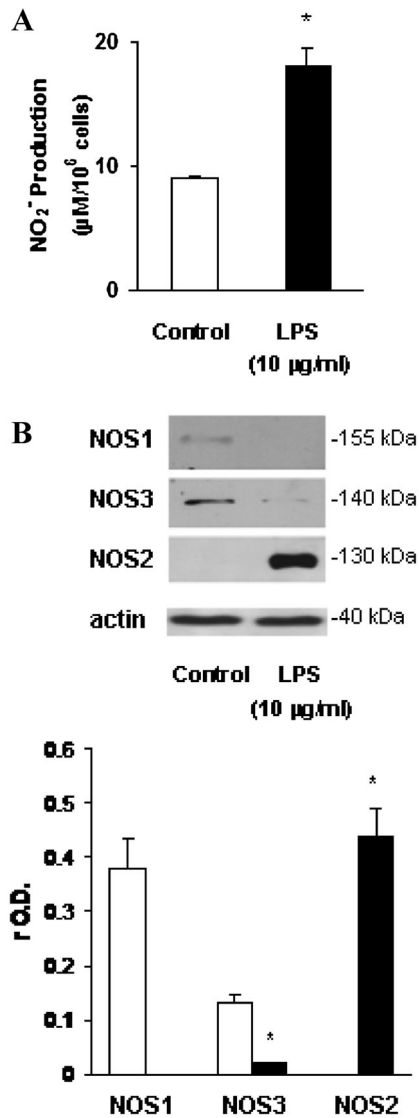

Fig. 1. Nitric oxide synthase (NOS) derived nitric oxide (NO) production by macrophages treated in vitro with LPS. A: Peritoneal macrophages from normal BALB/c mice were stimulated with 10 μg/ml lipopolisaccharide (LPS) as it was stated in Materials and Methods. NOS activity was measured as nitrite (NO_2^-) production in micromolar concentration per million cells (μM/10^6 cells). Values are means ± SEM of six experiments. *$P < 0.001$ versus control (untreated macrophages). B: Western blot assays to detect the expression of NOS1, 2, and 3 (upper part). One representative experiment of 3 is shown. Densitometric analysis of the bands was expressed as optical density units relative (rO.D.) to the expression of actin used as loading control (lower part). White bars: control. Black bars: LPS treatment. Values represent mean ± SEM of three independent experiments. *$P < 0.05$ versus control.

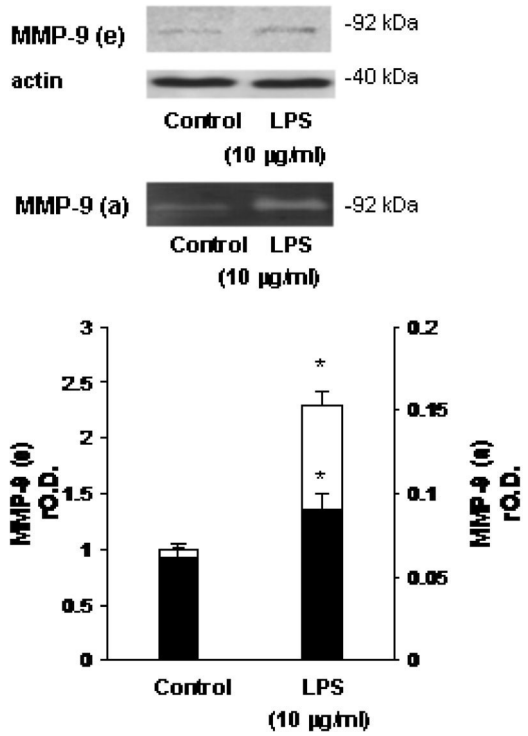

Fig. 2. Metalloproteinase-9 (MMP-9) expression (e) and activity (a) in macrophages treated in vitro with lipopolisaccharide (LPS). Densitometric analysis of the bands was expressed in optical density units relative (rO.D.) to control (untreated macrophages) considered as 1. White bars: MMP-9 (e) Black bars: MMP-9 (a). Values represent means ± SEM of three experiments. *$P < 0.05$ versus control. Molecular weights of proteins are indicated on the right.

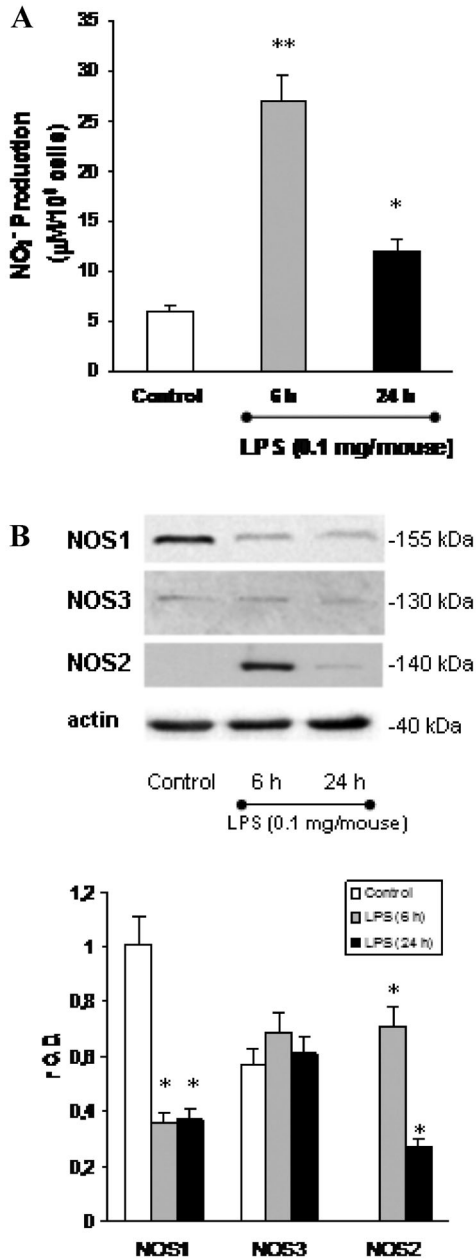

Fig. 3. Nitric oxide synthase (NOS) derived nitric oxide (NO) production by macrophages obtained from animals treated in vivo with (LPS). BALB/c mice were inoculated with 0.1 mg/mouse LPS as it is stated in Materials and Methods. A: NOS activity was measured as nitrite (NO_2^-) production in micromolar concentration per million cells (μM/10^6 cells). Values represent means ± SEM of four experiments. **$P < 0.001$; *$P < 0.01$ versus control (untreated macrophages). B: Western blot assays to detect the expression of NOS1, 2, and 3 in macrophage lysates (upper part). One representative experiment of three is shown. Densitometric analysis of the bands was expressed in optical density units (rO.D.) relative to the expression of actin used as loading control (lower part). Values represent means ± SEM of three independent experiments. *$P < 0.05$ versus control.

activity by around 60 ± 7% in the supernatants of Mps, obtained after 6 h of LPS inoculation (Fig. 4B). Both, the expression and the activity of MMP-9 were higher at 24 h than at 6 h. The activity was tested either in the supernatant of Mps or in the sera of LPS-treated animals ($P < 0.05$; Fig. 4B).

Effect of macrophages activated in vivo with LPS on heart tissue

Also, Mps from animals treated in vivo with LPS up-regulated NO production in the supernatants of the co-culture with normal heart (Fig. 5A) and slightly induced NOS2 expression in heart tissue after 48 h in culture ($P < 0.001$ vs. control; Fig. 5B). In addition, when hearts were obtained from animals treated with LPS (0.1 mg/mouse) during 6 h and were co-cultured with Mps derived from the same animals, a significant increase in the production of NO was observed in the supernatants after 24 h (25.2 ± 2.3 μM) or after 48 h (33.5 ± 3.1 μM) ($P < 0.001$ vs. control) of co-culturing in comparison to control (co-culture of untreated heart plus untreated Mps: after 24 h: 7.4 ± 0.9 μM or after 48 h: 14.9 ± 1.9 μM).

It has been proposed that during sepsis immune cells are recruited to different organs, like heart, regulating the production of inflammatory/angiogenic mediators. To test this hypothesis, we co-cultured Mps from mice treated i.p. with LPS for 6 h with normal heart slices for 24 h or 48 h. Figure 6A shows, that the expression of CD31, a marker of new blood vessels formation, as well as 40 and 33 kDa VEGF-A expression were induced in heart homogenates after 24 h of culture ($P < 0.05$ vs. control: untreated heart). When Mps were in contact with heart for 48 h, an increment in VEGF-A

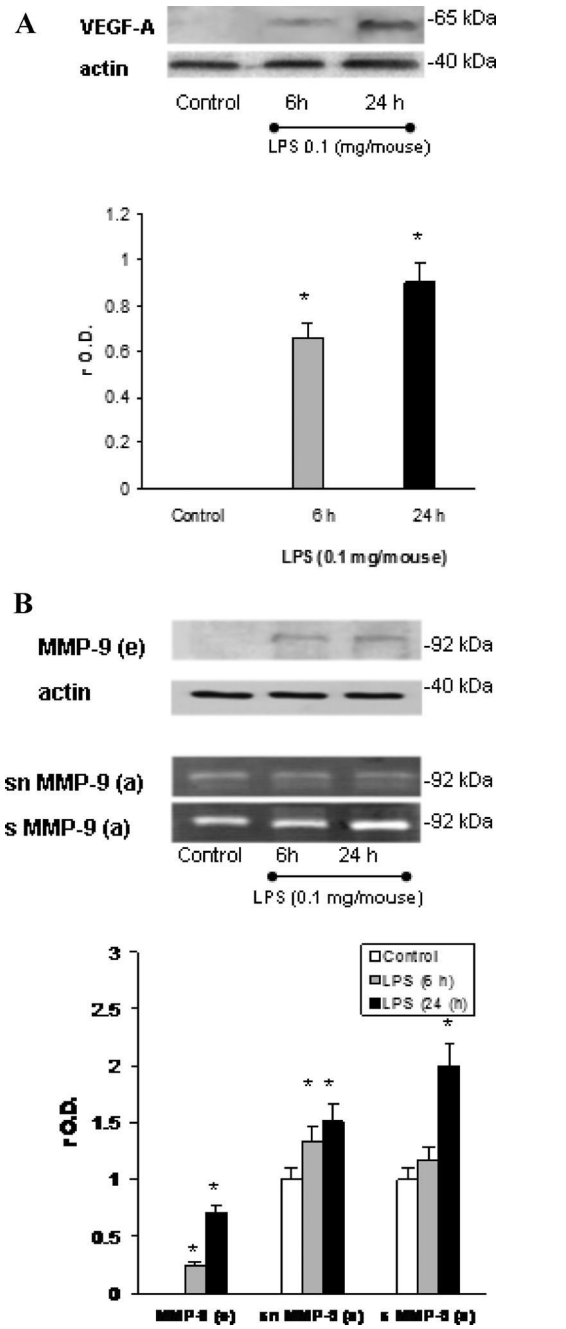

Fig. 4. Expression of angiogenic proteins in macrophages obtained from animals treated in vivo with lipopolisaccharide (LPS). A: Western blot assays to detect the expression of vascular endothelial growth factor-A (VEGF-A; upper part) in macrophage lysates. One representative experiment of 3 is shown. Densitometric analysis of the bands was expressed in optical density units relative (rO.D.) to the expression of actin used as loading control (lower part). $^{*}P < 0.05$ versus control. B: metalloproteinase-9 (MMP-9) expression (e) in macrophages lysates. Densitometric analysis of the bands was expressed in optical density units relative (rO.D.) to the expression of actin used as loading control (lower part). MMP-9 activity (a) in macrophage supernatants (sn; upper part) and in the sera (s) of LPS-treated mice (middle part). Densitometric analysis of the bands was expressed as optical density units relative (rO.D.) to control (untreated macrophages) considered as 1. Values represent means ± SEM of three independent experiments. $^{*}P < 0.05$ versus control (untreated macrophages). Molecular weights of proteins are on the right.

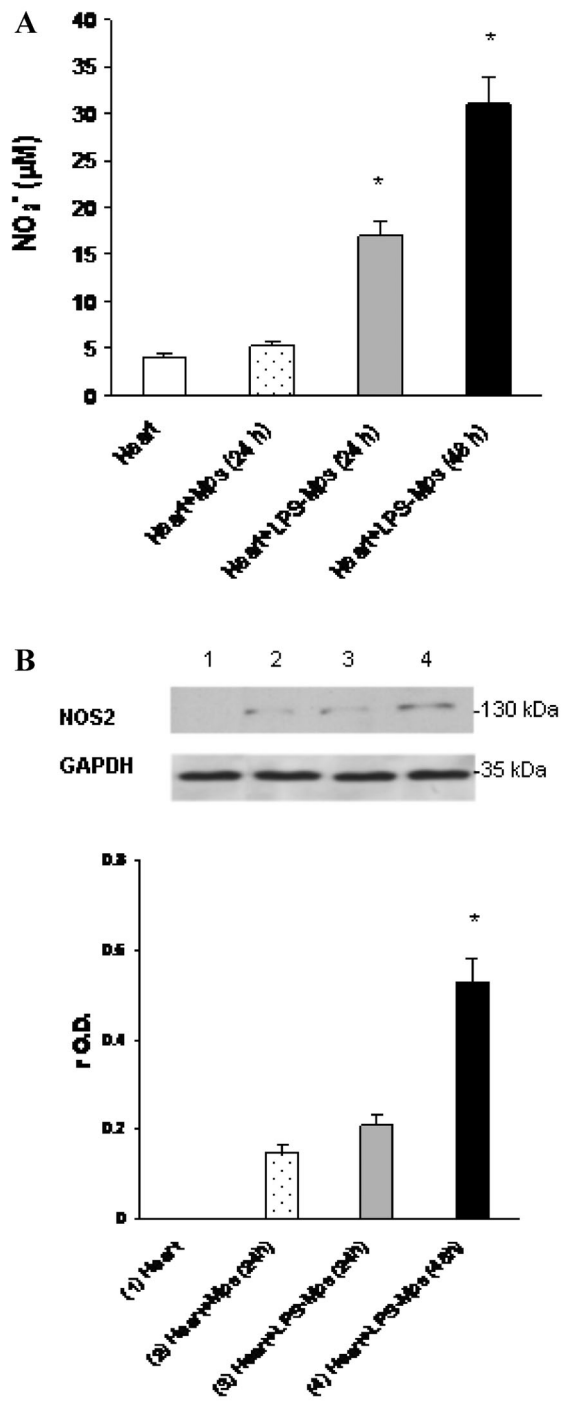

Fig. 5. Effect of in vivo lipopolisaccharide (LPS) treated macrophages (Mps) on nitric oxide (NO) production and nitric oxide synthase (NOS) expression in normal hearts. A: NOS activity was measured as nitrite (NO_2^-) production in micromolar concentration (μM) in the supernatants. Values represent means ± SEM of three independent experiments. $^{*}P < 0.01$ versus control (normal heart). B: Western blot assays to detect the expression of NOS2 in heart homogenates (upper part). One representative experiment of 3 is shown. Densitometric analysis of the bands was expressed as optical density units relative (rO.D.) to the expression of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) used as loading control (lower part). Values represent means ± SEM of three independent experiments. $^{*}P < 0.05$ versus control. Molecular weights of proteins are indicated on the right.

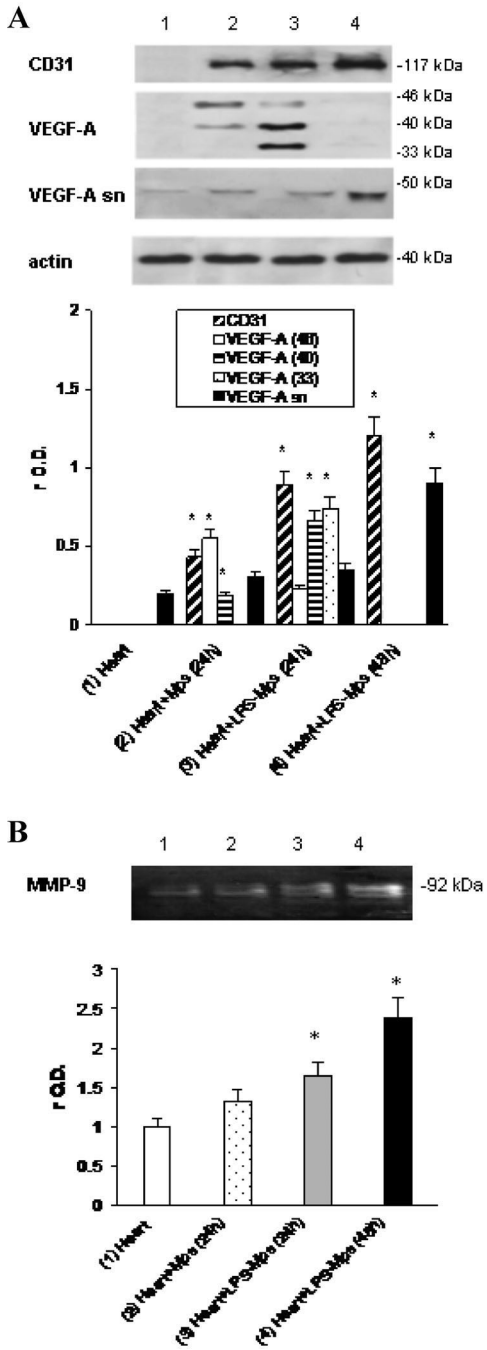

Fig. 6. Effect of in vivo lipopolisaccharide (LPS) treated macrophages (Mps) on angiogenic proteins in normal hearts. Heart slices were co-cultured with LPS-Mps (3×10^6) for 24 h or 48 h as it was stated in Materials and Methods Section. A: Western blot assays to detect the expression of CD31 and vascular endothelial growth factor-A (VEGF-A) in heart homogenates or in culture supernatants (sn; upper part). One representative experiment of 3 is shown. Densitometric analysis of the bands was expressed as optical density units relative (rO.D.) to the expression of actin used as loading control (lower part). Values represent means ± SEM of three independent experiments. *$P < 0.05$ versus control (normal heart). B: Zymography assays to detect metalloproteinase-9 (MMP-9) activity in supernatants. One representative experiment of 3 is shown (upper part). Densitometric analysis of the bands was expressed as optical density units relative (rO.D.) to control (normal heart) considered as 1 (lower part). Values represent means ± SEM of three independent experiments. *$P < 0.05$ versus control.

expression in the supernatants was observed ($P < 0.05$; Fig. 6A). In addition, a raised in MMP-9 activity in the supernatants of the co-culture (heart plus LPS-activated Mps) after 24 and 48 h was detected ($P < 0.05$; Fig. 6B).

In addition, positive immunostaining for CD31 molecule was observed in some blood vessels of heart slices obtained from the co-culture with LPS-treated Mps during 48 h revealing the formation of new blood vessels in hearts from this experimental group (Fig. 7A). No positive staining was observed in heart slices obtained from 24 h of co-culture with LPS-activated Mps or normal Mps or untreated tissue (Fig. 7B–D).

Since heart is composed by cardiomyocytes, vascular endothelial cells and connective tissue, we analyzed the action of peritoneal Mps obtained 6 h after the inoculation of 0.1 mg/mouse LPS (i.p.) on isolated cardiomyocytes in a transwell assay. This kind of assay let us elucidate if soluble mediators derived from Mps were responsible for the regulation of angiogenic molecules in purified cardiomyocytes. LPS-treated Mps increased the production of nitrites by 100 ± 6% at 24 h and fivefold after 48 h of co-culturing (Fig. 8A). Activated Mps also induced de novo expression of NOS2 in cardiomyocytes, being this effect more potent after 48 h than after 24 h of culture ($P < 0.05$; Fig. 8B).

Also VEGF-A expression was up-regulated in cardiomyocytes by LPS-stimulated Mps (Fig. 9A); this effect was more potent at 48 h than at 24 h of co-culturing ($P < 0.05$). Similar results were observed in relation to MMP-9 activity in the supernatant of cardiomyocytes (Fig. 9B).

We confirmed that aminoguanidine treatment of LPS-activated Mps reduced nitrite production in the supernatant of cardiomyocytes as well as NOS2 expression in the same cells (Fig. 10A,B). In order to analyze whether NOS2-derived NO from Mps was involved in the regulation of angiogenic molecules expressed in cardiomyocytes, we tested the expression of VEGF-A in these cells after culturing them with aminoguanidine-LPS-treated Mps. Figure 11A shows that the inhibition of NOS2 in LPS-treated Mps did not modify VEGF-A expression in cardiomyocytes. On the other hand, the inhibition of NOS2 by aminoguanidine in LPS-treated Mps dramatically reduced MMP-9 expression and activity in cardiomyocytes (Fig. 11B).

Discussion

It is known that LPS activates monocytes/Mps to produce pro-inflammatory cytokines such as tumor necrosis factor alpha and interleukins (Cohen, 2002). Mps also secrete, in response to LPS, a wide variety of other biological mediators including platelet-activating factor, prostaglandins and free radicals, such as NO (Cohen, 2002; Connelly et al., 2003). Production of these inflammatory cytokines and mediators by monocytes/Mps may exert a dual role: on the one hand, they may contribute to the efficient control of growth and dissemination of invading pathogens, and on the other to exacerbate inflammatory/infection diseases like sepsis (Cohen, 2002). As a consequence of Mps stimulation either in vitro or in vivo with this endotoxin, NOS2 enzyme is expressed de novo. Concomitantly, we observed a decreased in calcium-dependent NOS isoforms expression, mainly in NOS1 protein. Colasanti et al. (1999) reported that in astroglial cells treated with LPS plus interferon gamma, expressing both constitutive and inducible NOS, the induction of the latter is an event that takes place when NOS1 is preventively and quickly down-regulated. This phenomenon was seen in our experimental model either in vitro or in vivo in LPS-activated Mps.

MMPs play an important role in acute and chronic inflammation by promoting the turnover of extracellular matrix (ECM), and by modulating the action of inflammatory mediators. MMPs aid efficient invasion of inflammatory cells to

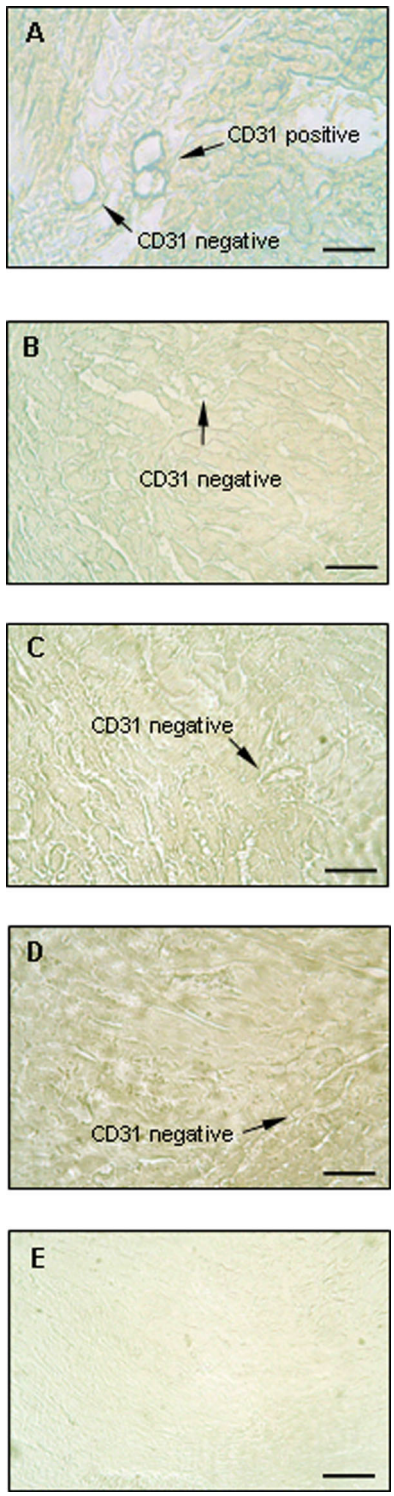

Fig. 7. CD31 expression in murine heart co-cultured with macrophages (Mps). Immunohistochemistry using a specific antibody against CD31 (PECAM1) in heart slices. Positive immunostaining is seen in the endothelium of new blood vessels in (A) heart from the co-culture with LPS activated-Mps during 48 h. Scale bar = 50 μm. Heart slices from a co-culture with (B) LPS activated-Mps during 24 h, (C) normal Mps, or (D) untreated heart did not show positive immunostaining. E: negative control, omitting the primary antibody. Scale bar = 50 μm.

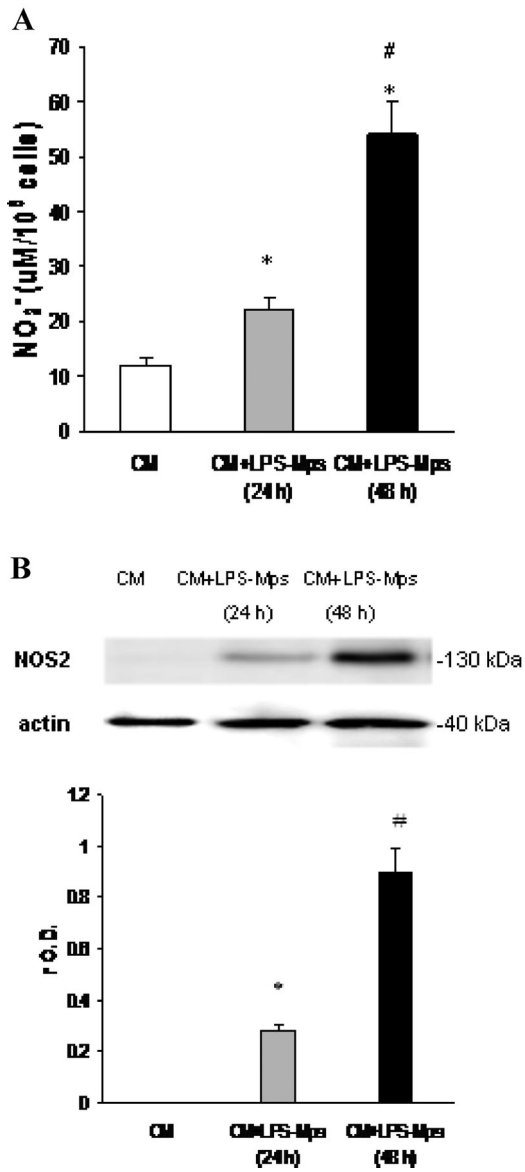

Fig. 8. Effect of in vivo lipopolisaccharide (LPS) treated macrophages (Mps) on nitric oxide (NO) production and nitric oxide synthase (NOS) expression in normal cardiomyocytes (CM). A: NOS activity was measured as nitrite (NO_2^-) production in micromolar concentration per million cells (μM/10^6 cells) in the supernatants of CM. Values represent means ± SEM of three independent experiments. $^*P < 0.05$ versus CM; $^\#P < 0.05$ versus CM + LPS-Mps (co-cultured during 48 h). B: Western blot assays to detect the expression of NOS2 in CM lysates (upper part). One representative experiment of 3 is shown. Densitometric analysis of the bands was expressed as optical density units relative (rO.D.) to the expression of actin used as loading control (lower part). Values represent means ± SEM of three independent experiments. $^*P < 0.05$ versus CM. Molecular weights of proteins are indicated on the right.

sites of damage or infection, allow angiogenesis and prepare the tissue for deposition of new ECM (Parks et al., 2004). Reel et al. (2011) found that LPS-treatment of monocytes selectively increased the steady-state mRNA levels of MMP-1, MMP-7, MMP-9, MMP-10, and MMP-14 to a greater degree than TIMPs, implying a shift in the protease/anti-protease balance in favor of

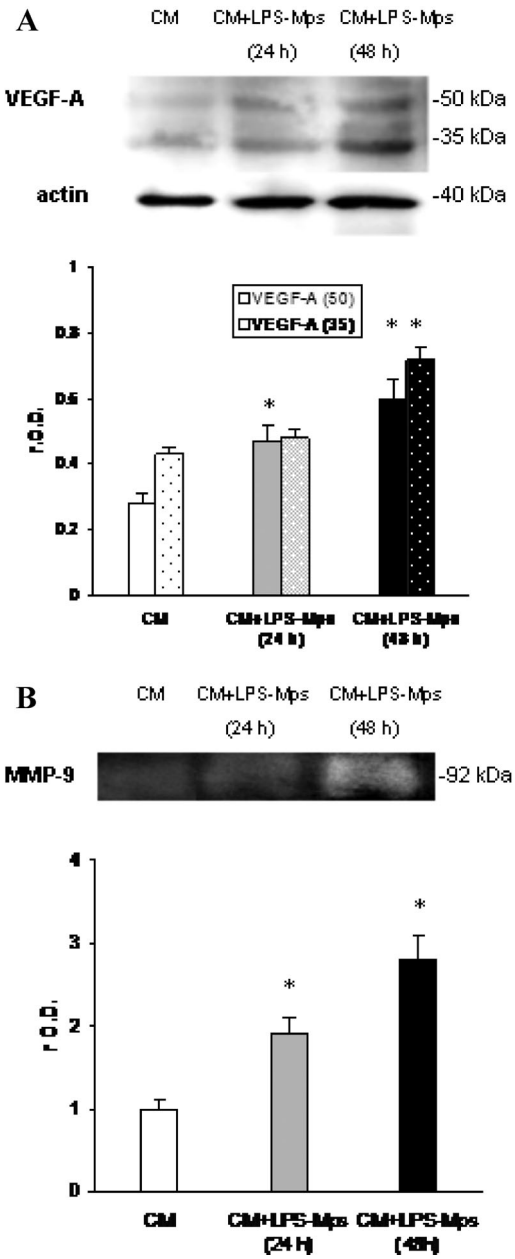

Fig. 9. Effect of in vivo lipopolisaccharide (LPS) treated macrophages (Mps) on angiogenic proteins in normal cardiomyocytes (CM). LPS-Mps (2.5×10^6) were co-cultivated with CM (2×10^6) in a transwell assay for 24 h or 48 h as it was stated in Materials and Methods Section. A: Western blot assays to detect the expression of vascular endothelial growth factor-A (VEGF-A) in CM lysates (upper part). One representative experiment of 3 is shown. Densitometric analysis of the bands was expressed as optical density units relative (rO.D.) to the expression of actin used as loading control (lower part). Values represent means ± SEM of three independent experiments. *$P < 0.05$ versus CM. B: Zymography assays to detect metalloproteinase-9 (MMP-9) activity in the supernatants of CM. One representative experiment of 3 is shown (upper part). Densitometric analysis of the bands was expressed as optical density units relative (rO.D.) to control (normal heart) considered as 1 (lower part). Values represent means ± SEM of three experiments. *$P < 0.05$ versus CM.

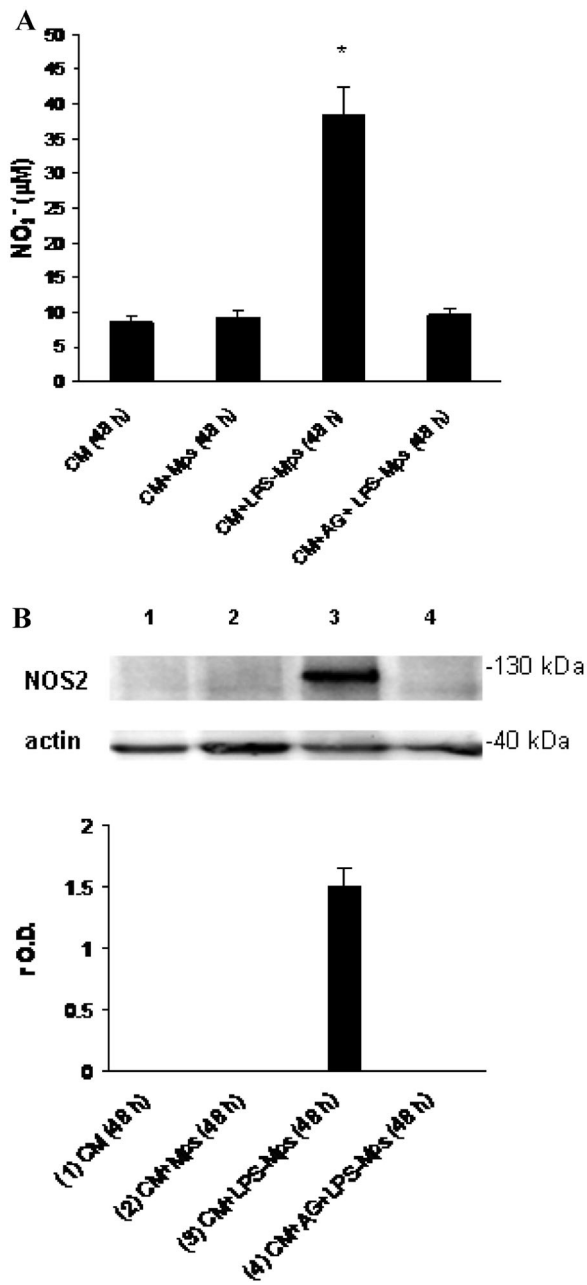

Fig. 10. Role of nitric oxide synthase 2-derived nitric oxide from in vivo lipopolisaccharide (LPS) treated macrophages (Mps) on the expression of angiogenic proteins in normal cardiomyocytes (CM). Mps (2.5×10^6) were obtained from mice treated with aminoguanidine (AG) and LPS and were co-cultivated with CM (2×10^6) in a transwell assay for 48 h as it was stated in Materials and Methods Section. A: Western blot assays to detect the expression of vascular endothelial growth factor-A (VEGF-A) in CM lysates (upper part). One representative experiment of 3 is shown. Densitometric analysis of the bands was expressed as optical density units relative (rO.D.) to the expression of actin used as loading control (lower part). Values represent means ± SEM of three independent experiments. *$P < 0.05$; **$P < 0.001$ versus CM. B: Western blot and zymography assays to detect metalloproteinase-9 (MMP-9) expression and activity in lysates and supernatants of CM respectively. One representative experiment of 3 is shown (upper part). Densitometric analysis of the bands was expressed as optical density units relative (rO.D.) to control (CM; lower part). White bars MMP-9 expression; black bars MMP-9 activity. Values represent means ± SEM of three experiments. *$P < 0.05$; **$P < 0.001$ versus CM.

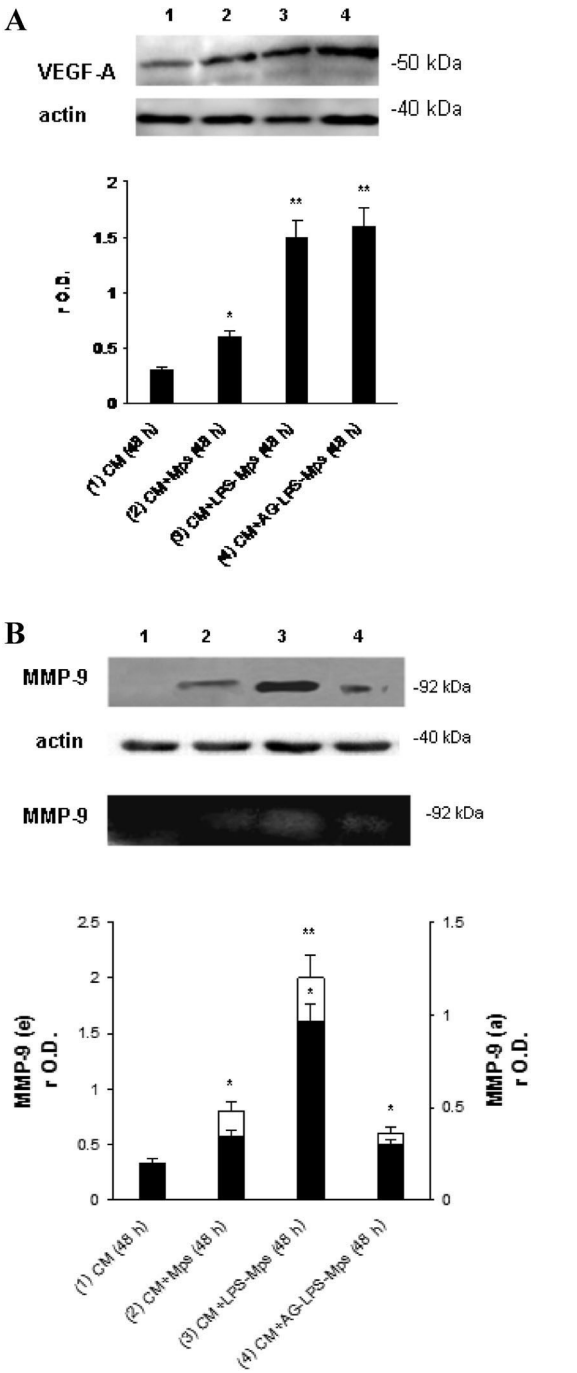

Fig. 11. Inhibition of nitric oxide synthase 2 (NOS2) activity and expression in normal cardiomyocytes (CM) co-cultured with in vivo lipopolisaccharide (LPS) activated macrophages (Mps). Mps (2.5×10^6) were obtained from mice treated with aminoguanidine (AG) and LPS and were co-cultivated with CM (2×10^6) in a transwell assay for 48 h as it was stated in Materials and Methods Section. A: NOS activity was measured as nitrite (NO_2^-) production in micromolar concentration (μM) in the supernatants. Values represent means ± SEM of three independent experiments. *P < 0.01 versus CM. B: Western blot assays to detect the expression of NOS2 in CM lysates (upper part). One representative experiment of 3 is shown. Densitometric analysis of the bands was expressed as optical density units relative (rO.D.) to the expression of actin used as loading control (lower part). Values represent means ± SEM of three independent experiments. Molecular weights of proteins are indicated on the right.

matrix degradation. In line with these results, we demonstrated that LPS stimulates the expression and activity of MMP-9 in peritoneal Mps. Also, we found an increase in MMP-9 activity in the sera of animals treated with LPS. Other authors indicated that the migration of immune cells from the bloodstream to sites of inflammation requires MMPs-mediated proteolysis of the basement membrane, as it was reported in vitro and in animal models (Leppert et al., 1995; Faveeuw et al., 2001; Cuenca et al., 2006).

When analyzing the action of LPS administered in vivo on peritoneal Mps, a maximum in the expression and activity of NOS2 at 6 h post-inoculation was observed. Regarding angiogenic proteins, VEGF-A and MMP-9, were induced by LPS in a delayed manner in comparison to NOS2. Wells et al. (2003) described abundant differences between the genes expressed early (within the first 6 h) or late (12–24 h or later) after LPS stimulation in different mouse strains. They mentioned that NOS protein is derived from an early gene induced, while angiogenic factors could be considered as products of late induced genes. Besides the differences observed in the kinetics of protein induction in LPS-treated Mps, they can display a broad spectrum of phenotypes which allows them to be equally critical in both the initiation and the resolution of inflammation (Mantovani et al., 2007). The two major Mps phenotypes reported are the classic pro-inflammatory M1 and the alternative anti-inflammatory M2 phenotypes (Mantovani et al., 2007; Martinez et al., 2009). Mps activated with Th1 cytokines (interferon gamma) and/or bacterial endotoxins polarize to M1 phenotype. In contrast, Mps activated with Th2 cytokines (IL-4, IL-13), IL-10, or glucocorticoid hormones polarize to M2 profile.

The formation of new blood vessels requires the sprouting of pre-existing blood vessels and their subsequent fusion with other blood vessels (Carmeliet, 2005). It has been suggested that Mps could participate in this process, but little is known about the mechanisms involved in it (Nucera et al., 2011). We had previously reported that peritoneal Mps from tumor-bearing mice are able to induce a strong neovascular response in the skin of singeneic mice (de la Torre et al., 2005). Similar results accompanied by the increment of VEGF-A and MMP-9 are observed when LPS-treated Mps are inoculated in the skin of singeneic mice (data not shown). Cursiefen et al. (2004) demonstrated in a model of inflammatory corneal neovascularization that VEGF-A is essential for recruitment of monocytes/Mps and that these cells play a crucial role in inducing inflammatory neovascularization by supplying/amplifying signals essential for pathological angiogenesis and lymphangiogenesis, since Mps depletion inhibited these processes.

It is known that an excessive and uncontrolled production of inflammatory cytokines and mediators in response to LPS may lead to serious systemic complications such as microcirculatory dysfunction, tissue damage, and septic shock with a high mortality (Morrison and Ryan, 1987). One of the most affected organs during sepsis is the heart. When we investigated the effect of LPS-activated Mps on the heart, we observed an induction in the expression of CD31, a marker of new blood vessels and confirmed the formation of new blood vessels in the heart from the co-cultures with LPS-treated Mps for 48 h by immunohistochemistry. Also the expression of VEGF-A, more specifically of the low molecular weight fragments were detected by Western blot after 24 h of co-culture either in heart homogenates or in cardiomyocyte lysates. These results were in parallel with an increment in MMP-9 activity in the supernatants. Previous reports indicated that tumor associated-Mps release MMPs that contribute to tumor dissemination by degrading ECM and/or by processing VEGF-A to low molecular weight fragments that could in turn activate in a more potent manner VEGF receptors (Allavena et al., 2008).

We also showed a progressive increment in the expression of VEGF-A and NOS2, and in the activity of MMP-9 when the supernatant of LPS-activated Mps reaches cardiomyocytes in the transwell assay. This reveals that Mps produce soluble mediators which induce cardiomyocytes to produce angiogenic factors.

Previous reports indicated interactions and possible cross talk between iNOS and MMP-9 in rat aortic vascular smooth muscle cells stimulated with LPS, interferon-gamma, and phorbol 12-myristate13-acetate. The NOS inhibitor N omega-nitro-L-arginine methyl ester exerted an inhibitory effect on COX-2 and MMP-9 mRNA synthesis (Marcet-Palacios et al., 2003). Similarly, our results indicate that NOS2 induction by LPS in Mps is involved in the up-regulation of MMP-9 expression and activity in cardiomyocytes derived from co-culture experiments.

Conclusions

Other authors have mentioned that interstitial inflammatory immuno-infiltrates are present in the ischemic heart disease and in myocardium of LPS-induced sepsis (Cuenca et al., 2006; Lorier et al., 2011). This study suggests that Mps obtained from in vivo LPS-treated mice are able to up-regulate its own production of NO as well as VEGF-A levels, MMP-9 expression and activity and new blood vessel formation in heart tissue. LPS-activated Mps are also inducers of VEGF-A and MMP-9 expression and activity in cardiomyocytes being the latter dependent on NOS2 induction in activated Mps, supporting the hypothesis that during sepsis neovascularization occurs in the myocardium as an attempt to improve cardiac function.

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Literature Cited

Allavena P, Sica A, Garlanda C, Mantovani A. 2008. The Yin-Yang of tumor-associated macrophages in neoplastic progression and immune surveillance. Immunol Rev 222:155–161.

Bradford MM. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254.

Carmeliet P. 2005. Angiogenesis in life, health and medicine. Nature 438:932–936.

Cohen J. 2002. The immunopathogenesis of sepsis. Nature 420:885–891.

Colasanti M, Persichini T, Cavallieri E, Fabrizi C, Mariotto S, Menegazzi M, Lauro GM, Suzuli H. 1999. Rapid inactivation of NOS-I by lipopolisaccharide plus interferon-gamma-induced tyrosine phosphorilation. J Biol Chem 274:9915–9917.

Connelly L, Palacios-Callender M, Ameixa C, Moncada S, Hobbs AJ. 2001. Biphasic regulation of NF-kappa B activity underlies the pro- and anti-inflammatory actions of nitric oxide. J Immunol 166:3873–3881.

Connelly L, Jacobs AT, Palacios-Callender M, Moncada S, Hobbs AJ. 2003. Macrophage endothelial nitric-oxide synthase autoregulates cellular activation and pro-inflammatory protein expression. J Biol Chem 278:26480–26487.

Cuenca J, Martín-Sanz P, Alvarez-Barrientos AM, Boscá L, Goren N. 2006. Infiltration of inflammatory cells plays an important role in matrix metalloproteinase expression and activation in the heart during sepsis. Am J Pathol 169:1567–1576.

Cursiefen C, Chen L, Borges LP, Jackson D, Cao J, Radziejewski C, D'Amore PA, Dana MR, Wiegand SJ, Streilein JW. 2004. VEGF-A stimulates lymphangiogenesis and hemangiogenesis in inflammatory neovascularization via macrophage recruitment. J Clin Invest 113:1040–1050.

Davel LE, Jasnis MA, de la Torre E, Gotoh T, Diament M, Sacerdote de Lustig E, Sales ME. 2002. Arginine metabolic pathways involved in the modulation of tumor induced angiogenesis by macrophages. FEBS Let 532:216–220.

de la Torre E, Davel L, Jasnis MA, Gotoh T, Sacerdote de Lustig E, Sales ME. 2005. Muscarinic receptors participation in angiogenic response induced by macrophages from mammary adenocarcinoma-bearing mice. Breast Cancer Res 7:R345–R352.

Faveeuw C, Preece G, Ager A. 2001. Transendothelial migration of lymphocytes across high endothelial venules into lymph nodes is affected by metalloproteinases. Blood 98:688–695.

Ferrara N, Gerber HP, LeCouter J. 2003. The biology of VEGF and its receptors. Nature Med 9:669–676.

Gijbels KSM, Carton H, Opdenakker G. 1992. Gelatinase in the cerebrospinal fluid of patients with multiple sclerosis and other inflammatory neurological disorders. J Neuroimmunol 41:29–34.

Goetzl EJ, Banda MJ, Leppert D. 1996. Matrix metalloproteinases in immunity. J Immunol 156:1–4.

Green LC, Wagner DA, Glowoski J, Skipper PL, Wishnok JS, Tannembaum SR. 1982. Analysis of nitrate, nitrite and (^{15}N) in biological fluids. Anal Biochem 126:131–138.

Hovsepian E, Mirkin GA, Penas F, Manzano A, Bartrons R, Goren NB. 2011. Modulation of inflammatory response and parasitism by 15-Deoxy-Δ(12,14) prostaglandin J(2) in Trypanosoma cruzi-infected cardiomyocytes. Int J Parasitol 41:553–562.

Kim CO, Huh AJ, Kim MS, Chin BS, Han SH, Choi SH, Jeong SJ, Choi HK, Choi JY, Song YG, Kim JM. 2008. LPS-induced vascular endothelial growth factor expression in rat lung pericytes. Shock 30:92–97.

Leppert D, Waubant E, Galardy R, Bunnett NW, Hauser SL. 1995. T cell gelatinases mediate basement membrane transmigration in vitro. J Immunol 154:4379–4389.

Lorier G, Touriño C, Kalil RAK. 2011. Coronary angiogenesis as an endogenous response to myocardial ischemia in adults. Arq Bras Cardiol 97:e140–e148.

Malik N, Greenfield BW, Wahl AF, Kiener PA. 1996. Activation of human monocytes through CD40 induces matrix metalloproteinases. J Immunol 156:3952–3960.

Mantovani A, Sica A, Locati M. 2007. New vistas on macrophage differentiation and activation. Eur J Immunol 37:14–16.

Marcet-Palacios M, Graham K, Cass C, Befus AD, Mayers I, Radomski MW. 2003. Nitric oxide and cyclic GMP increase the expression of matrix metalloproteinase-9 in vascular smooth muscle. J Pharmacol Exp Ther 307:429–436.

Martinez FO, Helming L, Gordon S. 2009. Alternative activation of macrophages: An immunologic functional perspective. Annu Rev Immunol 27:451–483.

Moncada S, Higgs EA. 2006. The discovery of nitric oxide and its role in vascular biology. Br J Pharmacol 147:193–201.

Morrison DC, Ryan JL. 1987. Endotoxins and disease mechanisms. Annu Rev Med 38:417–432.

Nucera S, Biziato D, de Palma M. 2011. The interplay between macrophages and angiogenesis in development, tissue injury and regeneration. Int J Dev Biol 55:495–503.

Paemen L, Jansen PM, Proost P, Van DJ, Opdenakker G, Hack E, Taylor FB. 1997. Induction of gelatinase B and MCP-2 in baboons during sublethal and lethal bacteraemia. Cytokine 9:412–415.

Pagenstecher A, Stalder AK, Kincaid CL, Volk B, Campbell IL. 2000. Regulation of matrix metalloproteinases and their inhibitor genes in lipopolysaccharide-induced endotoxemia in mice. Am J Pathol 157:197–210.

Parks WC, Wilson CL, Lopez-Boado YS. 2004. Matrix metalloproteinases as modulators of inflammation and innate immunity. Nat Rev Immunol 4:617–629.

Penas F, Mirkin GA, Hovsepian E, Cevey A, Caccuri R, Sales ME, Goren NB. 2013. PPARγ ligand treatment inhibits cardiac inflammatory mediators induced by infection with different lethality strains of Trypanosoma cruzi. Biochim Biophys Acta 1832:239–248.

Reel B, Sala-Newby GB, Huang WC, Newby AC. 2011. Diverse patterns of cyclooxygenase-independent metalloproteinase gene regulation in human monocytes. Br J Pharmacol 163:1679–1690.

Ren Y, Xie Y, Jiang G, Fan J, Yeung J, Li W, Tam PK, Savill J. 2008. Apoptotic cells protect mice against lipopolysaccharide-induced shock. J Immunol 180:4978–4985.

Sato H, Takino T, Okada Y, Cao J, Shinagawa A, Yamamoto E, Seiki M. 1994. A matrix metalloproteinase expressed on the surface of invasive tumour cells. Nature 370:61–65.

Takaishi H, Kimura T, Dalal S, Okada Y, D'Armiento J. 2008. Joint diseases and metalloproteinases: A role for MMP-13. Curr Pharm Biotecnol 9:47–54.

Valledor AF, Comalada M, Santamaría-Babi LF, Lloberas J, Celada A. 2010. Macrophage proinflammatory activation and deactivation: A question of balance. Adv Immunol 108:1–20.

Wells CA, Ravasi T, Faulkner GJ, Carninci P, Okazaki Y, Hayashizaki Y, Sweet M, Wainwright BJ, Hume DA. 2003. Genetic control of the innate immune response. BMC Immunol 4: 5–23.