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Infiltration of Inflammatory Cells Plays an Important Role in Matrix Metalloproteinase Expression and Activation in the Heart during Sepsis

Jimena Cuenca,* Paloma Martín-Sanz,*[†]
Alberto M. Álvarez-Barrientos,* Lisardo Boscá,*[†]
and Nora Goren*

From the Centro Nacional de Investigaciones Cardiovasculares*
and the Consejo Superior de Investigaciones Científicas,[†]
Madrid, Spain

Septicemia is an emerging pathological condition involving, among other effects, refractory hypotension and heart dysfunction. Here we have investigated the contribution of resident nonmyocytic cells to heart alterations after lipopolysaccharide administration. These cells contributed to the rapid infiltration of additional inflammatory cells that enhance the onset of heart disease through the release of inflammatory mediators. Early activation of resident monocytic cells played a relevant role on the infiltration process, mainly of major histocompatibility complex class II- and CD11b-positive cells. This infiltration was significantly impaired in animals lacking the nitric-oxide synthase-2 (NOS-2) gene or after pharmacological inhibition of NOS-2 or cyclooxygenase-2, suggesting a significant contribution of nitric oxide and prostanoids to the infiltration process. Under these conditions, the expression of NOS-2 and cyclooxygenase-2 in the whole organ was attenuated because cardiomyocytes failed to express these enzymes. However, cardiomyocytes expressed and activated matrix metalloproteinase-9 through mechanisms regulated, at least in part, by nitric oxide and prostaglandins in an additive way. These results directly link the inflammatory response in the heart and extracellular matrix remodeling by the matrix metalloproteinases released by the cardiomyocytes, suggesting that activation and recruitment of inflammatory cells to the heart is a major early event in cardiac dysfunction promoted by septicemia. (Am J Pathol 2006, 169:1567–1576; DOI: 10.2353/ajpath.2006.060109)

Sepsis is still associated with a high mortality rate and is a prevalent disease in developed countries. Septic shock is

caused by massive systemic microbial infection and is characterized by refractory hypotension and severe cardiac dysfunction. Although the pathogenesis of this cardiac dysfunction remains poorly understood, the contribution of proinflammatory mediators, such as nitric oxide (NO) and prostaglandins (PGs), has been recognized,^{1–5} and the condition is characterized by the persistent presence of macrophages and other mononuclear cells, tissue destruction, fibroblast proliferation, and the deposition of extracellular matrix (ECM).^{6,7} Indeed, ECM remodeling is considered one of the ways to arrive at the failing heart situation.^{8,9} Matrix metalloproteinases (MMPs), a large family of zinc-dependent endopeptidases, are able to degrade components of the ECM, and increased MMP activity is associated with several cardiovascular pathologies including heart failure, arteriosclerosis, and tissue damage during inflammatory onset.^{10,11}

In addition to sepsis, several studies have demonstrated the expression of cyclooxygenase-2 (COX-2) in the myocardium of failing human hearts,¹² and inhibition of COX-2 is known to improve cardiac function after myocardial infarction¹³ and to decrease fibroblast proliferation.^{14,15} The relationship between NO, PGs, and MMP activation has been clearly demonstrated in vascular smooth muscle, where NO and cyclic GMP up-regulate the expression of MMP-9.¹⁶ Moreover, in atherome plaques the PGs produced by macrophage-expressed COX-2 enhance MMP-9 synthesis and activation.¹⁷ However, confusion exists regarding the contribution of cardiomyocytes to the expression and activity of NOS-2, COX-2, and MMPs because most of the studies have been performed using whole organ extracts or isolated neonatal cardiomyocytes.^{18–21} Using isolated fetal, neonatal, and adult cultured cardiomyocytes, we showed

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Address reprint requests to Lisardo Boscá, CNIC, Melchor Fernández Almagro 3, 28029 Madrid, Spain. E-mail: lbosca@cnic.es.

that only the fetal and neonatal cells express NOS-2 and have a restricted expression of genes depending on nuclear factor- κ B activation,²² despite exhibiting a response via TLR-4 signaling.²³

In the present study, we have chosen a mouse model of endotoxic shock after systemic lipopolysaccharide (LPS) administration because these conditions contribute to decreased contractile efficiency and left ventricular enlargement and heart dysfunction.²⁴ Moreover, in this model we have investigated how the inflammatory response after LPS challenge contributes in the mouse heart to the infiltration of circulating cells and to ECM remodeling by secreted MMPs. Our data show that the rapid expression of NOS-2 and COX-2 after LPS administration is attributable to the activation of resident and infiltrating cells, mostly monocytes/macrophages, with a minor (COX-2) or negligible (NOS-2) contribution by cardiac cells. Moreover, using NOS-2 KO mice or NOS-2 and COX-2 pharmacological inhibitors, we observed an impaired LPS-dependent infiltration in the heart. The release of NO and PGs by these cells plays an important role in MMP-9 accumulation and activation not only in infiltrating cells but also in cardiomyocytes, contributing to the well-known organ dysfunction and tissue damage characteristic of septic shock.

Materials and Methods

Animals and Induction of Endotoxic Shock

C57BL/6J0004 (wild type) and NOS-2 KO mice (B61292P2) were obtained from Jackson Laboratories (Bar Harbor, ME). Genotyping was performed by tail DNA polymerase chain reaction (PCR) as recommended by the supplier. Mice, 2 months of age (20 to 25 g), were supplied with food and water *ad libitum* and exposed to a 12-hour light-dark cycle. Animals were intraperitoneally administered with a single dose of LPS from *Salmonella typhimurium* (Sigma Chemical Co., St. Louis, MO) at 2 mg/kg body weight. Untreated (control) animals received 0.5 ml of 0.9% NaCl. In some experiments, animals were treated before LPS injection with 20 mg/kg 1400W (a selective inhibitor of NOS-2) and 5 mg/kg DFU [5,5-dimethyl-3(3-fluorophenyl)-4-(4-methylsulphonyl)phenyl-2(5H)-furanone; a selective COX-2 inhibitor kindly donated by MSD, Rahway, NJ]. Animals were sacrificed at the indicated times (up to 96 hours), and hearts were used either to isolate cardiomyocytes (*vide infra*), to prepare heart extracts, or to perform immunohistochemistry on fixed sections of the tissue. All animal experiments conformed to the Guide for the Care and Use of Laboratory Animals (National Institutes of Health publication 85-23, revised 1996).

Functional Measurements

Echocardiography was performed using a 13-MHz ultrasound probe (Sequoia Acuson) after sedation of the mice with 50 mg/kg body weight of ketamine. The ejection time, LV-end diastolic diameter, LV end-systolic diame-

ter, and the heart rate were measured on M-mode echocardiograms.

Isolation and Culture of Adult Cardiomyocytes

When adult cardiomyocytes were prepared, the heart was perfused and digested with collagenase as described.^{25,26} After disaggregation of the tissue, isolated cells were processed as described previously.²² In brief, after incubation for 1 hour, the cell suspension was sedimented by centrifugation at low speed ($80 \times g$) and seeded for 30 minutes on plastic dishes to favor macrophage adhesion, and the remaining cell suspension was distributed in plates pre-coated with a 2% solution of gelatin and cultured with Dulbecco's modified Eagle's medium supplemented with 10 μ g/ml transferrin, 10 μ g/ml insulin, vitamin mix, antibiotics, and 10% fetal bovine serum. After overnight incubation to favor cardiomyocyte adhesion, the dishes were washed with phosphate-buffered saline (PBS), and the medium was replaced. Usually, cells were plated in 6-cm culture dishes (1 to 2×10^6 cells) or 24-multiwell tissue culture plates (2×10^5 cells per well). Cell viability was assessed by microscopic observation of Trypan blue exclusion and was ~80%.

Cardiomyocyte Cell Lines and Co-Culture with Macrophage Raw 264.7 Cells

The cell line H9c2 was purchased from the American Type Culture Collection, Rockville, MD [CRL 1446, H9c2(2-1)]. These cells are spontaneously immortalized rat embryo ventricular myoblasts that preserve many electrical and biochemical characteristics of adult cardiomyocytes. The HL-1 adult mouse cardiac muscle cell line was obtained from Dr. W.C. Claycomb²⁵ and was maintained in culture as described.²² For co-culture experiments, LPS/interferon (IFN)- γ -activated Raw 264.7 cells (8 hours with 200 ng/ml LPS; 50 U/ml IFN- γ) in 24-well plates were washed extensively with PBS, and transwells with 0.4- μ m porosity polyester membrane filters (Costar Corp., Cambridge, MA) were placed above them. Cardiomyocyte cell lines were added to the upper chamber in 600 μ l of medium containing 0.5% FCS. Co-cultures were maintained for 24 hours. After co-culture, the filters were fixed with 70% ethanol at -20°C , washed with PBS, and blocked with 3% bovine serum albumin for 1 hour at room temperature. Filters were then incubated overnight with anti-MMP-9 antibody in PBS, 1% bovine serum albumin at 4°C . The filters were incubated with fluorescent secondary antibody (IgG-Cy3) and treated with Hoechst 33258 for 30 minutes at room temperature. Fluorescence was visualized on a MRC 1024 microscope (Bio-Rad, Hercules, CA) with Laser-sharp software.²⁷

Quantitative Real-Time Reverse Transcriptase-Polymerase Chain Reaction (Q-RT-PCR)

Total RNA was extracted from frozen mouse hearts by using Trizol reagent (Life Technologies, Inc., Grand

Island, NY). Total RNA (1 μ g per sample) was reverse-transcribed, with oligo(dT) as primer, using Expand Reverse Transcriptase (Roche) according to the manufacturer's protocol. Q-RT-PCR was performed with the SYBR Green PCR kit (PE Applied Biosystems) in an Applied Biosystems 7700 sequence detector. Primer sequences were as follows: MMP-9 forward 5'-CAGACCAAGGGTACAGCCTGTT-3', MMP-9 reverse 5'-AGTGCATGGCCGAAGCTC-3'; NOS-2 forward, 5'-CAG CTGGGCTGTACAAACCTT-3'; NOS-2 reverse, 5'-CATTGGAAGTGAAGCGTTTCG-3'; COX-2 forward, 5'-GCTGTACAAGCAGTGGCAAAG-3'; and COX-2 reverse, 5'-GCGTTTGGCGTACTCATTGAGA-3'. For normalization, all samples were analyzed in the same run for GAPDH expression. The GAPDH primers were forward, 5'-GAAGGTGGTGAAGCAGGCAT-3'; and reverse, 5'-TCGAAGGTGGAAGAGTGGGA-3'. PCR parameters were 50°C for 2 minutes, 95°C for 10 minutes, and 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. Quantitative expression values were extrapolated from separate standard curves for GAPDH or the indicated gene, generated with 10-fold dilutions of cDNA (in duplicate).

Immunohistochemistry

Hearts were extracted in 30% sucrose in PBS and frozen in liquid N₂ and serial 7- μ m-thick sections were cut with a Leitz sledge microtome onto gelatinized glass coverslips. The preparations were fixed in a 4% paraformaldehyde solution in PBS, pH 7.4, for 45 minutes at room temperature, washed with PBS, and permeabilized with ice-cold methanol for 15 minutes at room temperature. After blocking with 3% bovine serum albumin for 1 hour at room temperature, the sections were incubated overnight with the indicated antibodies in PBS, 1% bovine serum albumin at 4°C. The antibodies were against the major histocompatibility complex class II [fluorescein isothiocyanate-anti-mouse MHC class II (I-A/I-E), catalog no. 11-5321; Bioscience] that identified noncardiomyocytic but immunocompetent cells; Cy5-anti-mouse CD11b (catalog no. 19-0112; Bioscience), and polyclonal antibodies against NOS-2 and COX-2 (Santa Cruz Biotechnology, Santa Cruz, CA). The sections were incubated with fluorescent secondary antibodies (IgG-Cy3) and treated with Hoechst 33258 for 30 minutes at room temperature. Fluorescence was visualized on a MRC 1024 microscope (Bio-Rad) with Lasersharp software.²⁷ In some experiments (see Figure 1C), anesthetized mice (2.5 ml/kg Equithesin; Janssen) were perfused *in vivo* through the left ventricle with 50 ml of PBS and fixed with 500 ml of 4% paraformaldehyde in PBS. The hearts were removed, cut in blocks, and postfixed for 4 hours. Sections of tissue were used for immunohistochemistry by light microscopy.

Preparation of Total Tissue Extracts

Tissue samples were homogenized in 3 vol of 10 mmol/L Tris-HCl, pH 7.5, containing 1 mmol/L MgCl₂, 1 mmol/L

EGTA, 10% glycerol, 0.5% 3-((3-cholamidopropyl)-dimethylammonio)-1-propanesulfonate (CHAPS), 1 mmol/L β -mercaptoethanol, and 0.1 mmol/L phenylmethyl sulfonyl fluoride. Extracts were vortexed for 30 minutes at 4°C, and after centrifuging for 20 minutes at 13,000 $\times$ g, the supernatants were stored at -20°C. For Western blot analysis, the protein concentration was determined with Bradford reagent,²⁸ total protein extracts were boiled in Laemmli sample buffer, and equal amounts of protein (20 to 30 μ g) were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Determination of MMP-9 Activity

MMP-9 activity was measured with a specific Matrix Metalloproteinase-9 Biotrak, Activity Assay System (Amersham Biosciences) in accordance with the manufacturer's protocol.²⁹ The assay uses the QuickZyme detection enzyme, in its proform, which can be activated by captured active MMP-9 or by pro-MMP-9 activated with *p*-aminophenylmercuric acetate (APMA). This assay is specific and quantitative.

Determination of Tumor Necrosis Factor- α in Serum

The serum levels of tumor necrosis factor- α were measured in duplicate with a commercial kit (Biotrak, Amersham), following the instructions of the supplier.

Western Blotting Analysis

The amounts of COX-2, NOS-2, and MMP-9 in total tissue extracts were determined by immunoblotting with antibodies from Santa Cruz (COX-2 and NOS-2) and Torrey Pines Biolabs, Houston, TX (MMP-9). Proteins were transferred from 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis to polyvinylidene difluoride membranes (Amersham). After blocking with 5% nonfat dry milk in Tris-buffered saline containing 1% Tween 20 (TBST), membranes were incubated for 1 hour at room temperature with the corresponding antibodies. Membranes were washed with TBST and incubated with horseradish peroxidase-conjugated secondary antibody, and after further washes bound antibody was revealed with the ECL detection system (Amersham). Band intensities were measured on a densitometric scanner (Amersham) and are expressed in arbitrary units.

Data Analysis

The number of experiments is indicated in each figure legend. The results of different groups are given as means $\pm$ SD. The statistical significance within each group was calculated by Student's *t*-test for paired data (parametric test) and by the Wilcoxon test (nonparametric values). The statistical significance of differences between groups for each condition was evaluated by the Mann-Whitney *U*-test. Differences between NOS-2^{+/+}

Table 1. Characterization of the Mouse Endotoxemic Model

	NOS-2 ^{+/+}		NOS-2 ^{-/-}	
	Saline	LPS	Saline	LPS
Ejection time (ms)	53 ± 4	82 ± 5*	52 ± 5	91 ± 7*†
LV end-diastolic diameter (mm)	3.2 ± 0.1	3.5 ± 0.2	3.1 ± 0.2	3.4 ± 0.2
Heart rate (bpm)	441 ± 12	302 ± 10*	444 ± 14	359 ± 10*†
TNF-α (ng/ml serum, 2 hours)	<0.01	1.2 ± 0.3	<0.01	1.4 ± 0.3

Mice were intraperitoneally injected with 2 mg/kg body wt of LPS, and echocardiographic measurements were performed at 18 hours in anesthetized animals. Serum levels of TNF-α were determined in samples of serum obtained at 2 hours after LPS administration. Results show the mean ± SD. bpm, beats per minute.

**P* < 0.05 versus animals receiving saline; ANOVA result versus WT mice, †*P* < 0.05.

and NOS-2^{-/-} were determined by analysis of variance. All tests have been calculated by two-tail, and the significance has been considered at *P* < 0.05.

Results

Resident and Infiltrating Inflammatory Cells, but Not Cardiomyocytes, Express COX-2 and NOS-2 in Hearts of Animals Treated with LPS

In the present study we used a mouse model of endotoxic shock after LPS administration that has been character-

ized in terms of the rise in tumor necrosis factor-α serum levels and echocardiographic parameters (Table 1). Intraperitoneal injection of adult mice with LPS induced expression of NOS-2 and COX-2 protein in heart extracts at 24 hours, with a much lower COX-2 expression in NOS-2 KO mice (Figure 1A) or when animals were treated with the selective NOS-2 inhibitor 1400W (not shown). These data suggest that NO is influencing the expression of COX-2 in the heart tissue (see below). The NOS-2 and COX-2 protein levels were in agreement with the mRNA amounts measured by Q-RT-PCR (Figure 1B). However, immunohistochemical staining of heart sections showed that most of the NOS-2- and COX-2-positive

Figure 1. The early expression of NOS-2 and COX-2 in heart is attributable to inflammatory cells. **A:** Wild-type or NOS-2 KO mice were intraperitoneally injected with LPS (2 mg/kg body wt) and sacrificed after 24 hours. The expression levels of NOS-2 and COX-2 were determined by Western blot in heart extracts. α-Actin was used to normalize the blots. The quantitative expression levels of COX-2 are indicated. **B:** NOS-2 and COX-2 mRNA levels were determined by Q-RT-PCR in heart extracts prepared at the times indicated after injection of LPS or saline. Expression of mRNA in arbitrary units (a.u.) is presented as the ratio of the amount of target gene to GAPDH. **C:** The distribution of NOS-2 and COX-2 in heart was assessed by confocal microscopy in mounted tissue sections. Target protein (secondary antibody) is in green, and nuclear staining (Hoechst) is blue. **D:** Representative immunohistochemical staining of NOS-2 expression in heart sections from wild-type mice 24 hours after intraperitoneal injection with LPS. White arrows show the positive cells that exhibited a noncardiac morphology. Results show a representative experiment of four, and the mean ± SD. **P* < 0.05; ***P* < 0.001 versus time 0 hours.

Figure 2. Absence of NOS-2 expression and minor COX-2 levels in isolated cardiomyocyte cultures. **A:** Wild-type or NOS-2 KO mice were intraperitoneally injected with LPS (2 mg/kg body wt) for 24 hours, and cardiomyocytes were prepared and cultured for an additional period of 24 hours in the absence or presence of 200 ng/ml LPS, 20 ng/ml interleukin-1 β , and 10 ng/ml murine IFN- γ . The expression levels of NOS-2 and COX-2 were determined by Western blot in cell extracts, and the accumulation of nitrites and nitrates and PGE₂ was determined in the culture medium. **B:** The same experiment was performed after isolation of cardiomyocytes from control mice and treatment for 24 hours with the indicated stimuli. **C:** NOS-2 and COX-2 mRNA levels were determined by Q-RT-PCR in cell extracts prepared after 6 hours of cell culture. Results show a representative experiment of four (**A**, **B**), and the means $\pm$ SD ($n > 4$); * $P < 0.05$, ** $P < 0.01$ versus the corresponding condition in the absence of proinflammatory challenge (mRNA levels at 6 hours; **C**).

cells exhibited morphology distinct from that of cardiomyocytes (Figure 1, C and D) and these cells were also negative for selective cardiomyocyte markers such as α -actin and troponin T-C (not shown). To better characterize the NOS-2 and COX-2 expression in the heart, isolated cardiomyocytes were prepared. NOS-2 expression was undetectable in cardiomyocytes from animals treated *in vivo* for 24 hours with LPS and maintained in culture overnight to favor adhesion (Figure 2A), or when cultured cardiomyocytes from untreated mice were challenged for 24 hours with LPS and proinflammatory cytokines (Figure 2B). Regarding COX-2, moderate expression was observed in cardiomyocytes from animals treated *in vivo* with LPS (note that the presence of a band in lanes 1 and 3 was attributable to the 24 hours of *in vivo* treatment with LPS). In agreement with these data, the accumulation of nitrites plus nitrates in the medium (Figure 2, A and B) and the NOS-2 mRNA levels measured after 6 hours of isolation (Figure 2C, left) or LPS challenge (Figure 2C, right) did not respond to proinflammatory stimulation. The levels of PGE₂ and COX-2 mRNA exhibited a moderate increase in these cultured cells (Figure 2, A–C). This suggested that cardiac expression of these enzymes was attributable to other cell types, probably infiltrating cells, and that NO was involved in the infiltration process. Indeed, previous work showed that fetal or neonatal cardiomyocytes, but not the adult cells, express

genes involved in the inflammatory response, such as NOS-2 and COX-2.²²

Further immunohistochemical studies were performed to identify the cells expressing NOS-2 and COX-2 in the heart. These cells stained positive for the expression of CD11b, a monocyte/macrophage marker, and MHC class II, confirming them as resident or infiltrated inflammatory cells but not cardiomyocytes. Representative sections are shown in Figure 3A. The time course of colocalization of CD11b and MHC class II with COX-2 and NOS-2 after LPS treatment was determined (Figure 3, B and C) as well as that of α -actin and NOS-2 and COX-2 (Figure 3D). Noteworthy, the presence of MHC II-COX-2 or CD11b-COX-2-positive cells in NOS-2 KO mice was significantly impaired (Figure 3, B and C). It is important to note that NO down-regulates MHC class II levels and therefore, CD11b-positive cells better reflect the infiltration level in the presence of NOS-2 (see Discussion). These data confirmed that the cells expressing NOS-2 were not cardiac myocytes, but activated resident and infiltrated monocytes.

MMPs Are Expressed in Cardiomyocytes under Proinflammatory Conditions

Infiltration of inflammatory cells requires changes in the ECM, and because alterations to the myocardial ECM are pathognomonic of the failing heart,³⁰ we decided to investigate the role of MMPs in tissue damage during the inflammatory response. Wild-type mice were intraperitoneally injected with LPS as before, and the expression levels of MMP-9 mRNA and protein were determined in whole heart extracts. Increased MMP-9 mRNA and protein expression was detected in the hearts of LPS-injected animals 6 hours after injection and was further increased after 24 hours (Figure 4, A and B). The increased protein expression included that of the active 82-kD form (Figure 4B), and MMP-9 activity in heart extracts prepared 24 hours after injection was markedly increased (Figure 4C). Immunohistochemical examination confirmed a significantly increased expression of active MMP-9 (Figure 4D) and showed that this included expression by cardiomyocytes (Figure 4D, bottom).

To investigate whether NOS-2 and COX-2 expression in infiltrating inflammatory cells is causally linked to the whole-organ induction of MMP-9, we injected wild-type mice with LPS and selective inhibitors of COX-2 (DFU) and/or NOS-2 (1400W). Figure 5A shows the immunohistochemical staining corresponding to the expression of MMP-9 after LPS treatment. Inhibition of COX-2 with DFU or of NOS-2 with 1400W decreased the levels of MMP-9 significantly, and simultaneous inhibition of both COX-2 and NOS-2 activities decreased MMP-9 levels near to the control values. Good correlation was observed between the MMP-9 protein levels detected by immunofluorescence in Figure 5A and MMP-9 enzyme activity (Figure 5B). Moreover, a reduced activity of MMP-9 was observed in heart extracts from NOS-2 KO mice, data that

Figure 3. Characterization of cells expressing NOS-2 and COX-2 in heart sections from wild-type and NOS-2 KO mice treated with LPS. Tissue sections from the hearts of animals treated for 24 hours with LPS were fixed and immunostained with antibodies for NOS-2 and COX-2 and for specific cell-type markers. **A:** Representative sections of co-localization of MHC class II and CD11b with NOS-2 and COX-2. **B:** Time-dependent distribution of MHC class II-positive cells also expressing NOS-2 and COX-2 in wild-type or NOS-2 KO mice, respectively. **C:** Time-dependent distribution of CD11b-positive cells also expressing NOS-2 and COX-2 in wild-type or NOS-2 KO mice, respectively. **D:** Time-dependent distribution of α -actin, NOS-2, and COX-2 in heart sections. Data show the means $\pm$ SD ($n > 15$); * $P < 0.01$ versus time 0 hours. Immunohistograms are representative of at least 20 examined.

were in agreement with pharmacological NOS-2 inhibition with 1400W (Figure 5C).

Cardiomyocytic Cell Lines Express MMPs in Response to Proinflammatory Stimulation

To gain further insight into the contribution of infiltrating immune cells to cardiomyocyte MMP-9 expression, we examined MMP-9 expression in two cardiac cell lines, H9c2 (rat) and HL-1 (mice). Although these cell lines activate nuclear factor- κ B in response to LPS, they fail to express other inflammatory molecules, such as NOS-2 and COX-2 (not shown). They do, however, express MMP-9 and MMP-2. As Figure 6A shows, both cell lines expressed MMP-9 in response to LPS challenge, but a greater response was observed to combined treatment with the NO donor DETA-NO and PGE₂. Moreover, the effect of the exogenous addition of NO and PGE₂ was mimicked in co-cultures of H9c2 or HL-1 cells with LPS/IFN- γ -activated Raw 264.7 macrophages, supporting the idea that these molecules are key regulators in the ex-

pression and activation of MMP-9 in cardiac cells (Figure 6B). Inhibition of NOS-2 and COX-2 activities with 1400W and DFU, respectively, in activated macrophages decreased MMP-9 levels significantly.

Discussion

In this study we have investigated the ability of LPS to activate resident inflammatory cells present in the heart and the contribution of these nonmyocytic cells to the infiltration process and to the cardiac activation of MMPs. Although few studies have been devoted to evaluate the role of infiltration, it has been described that in animals lacking TLR4 the recruitment of inflammatory cells in the heart and the characteristic organ dysfunction are impaired under endotoxemic conditions.^{7,18} Our previous data showed that isolated adult cardiomyocytes, maintained in culture for short periods, lack the ability to express typical enzymes mediating inflammation, such as NOS-2 or COX-2, when stimulated with LPS and com-

Figure 4. MMP-9 expression and activation in heart after *in vivo* LPS administration. **A:** Wild-type mice were intraperitoneally injected with LPS or saline, and the levels of MMP-9 mRNA were determined by Q-RT-PCR in hearts extracted at the indicated times. **B:** Protein levels of MMP-9 and α -actin in heart extracts from LPS-treated mice. The immunograph shows typical results of a Western blot, and the plot shows the densitometric intensities for each condition from four independent determinations, normalized against α -actin expression and presented in arbitrary units. **C:** MMP-9 enzyme activity in whole heart extracts from LPS-treated mice. One unit equals 1 ng of peptide substrate hydrolyzed per minute per mg of protein. **D:** Immunohistochemical detection of active MMP-9 expression in heart tissue of mice 24 hours after injection with LPS; bottom: magnification of tissue sections showing MMP-9 fluorescence in cardiomyocytes. Data are the means $\pm$ SD of four experiments. * P < 0.05 versus saline or time 0 hours.

binations of proinflammatory cytokines (interleukin-1 β , tumor necrosis factor- α , and IFN- γ), due to the attenuation of I κ B kinase activation.²² Moreover, array studies revealed that the ability of neonatal cardiomyocytes to respond to LPS did not become fully functional until after more than 5 days in culture. Indeed, this type of refractory response is observed in various cardiac pathologies such as myocarditis, septic shock, and myocardial infarction and seems to correlate with contractile dysfunction, local inflammation, and promotion of cardiomyocyte death.^{6,23} The present study shows that early NOS-2 and COX-2 expression in the heart occurs mainly in resident and infiltrated immune cells and that these events play an important role in the progression of heart dysfunction, being MMP-2 and -9 overexpression and activation one of the targets. Our data well support this conclusion: 1) NOS-2 was undetectable, and COX-2 was barely expressed in cardiomyocytes prepared from adult animals treated *in vivo* with LPS and maintained in culture; 2)

COX-2 and NOS-2 were present in cells also expressing markers of inflammatory cells (for example CD11b or MHC class II) but not in cells expressing cardiomyocyte markers; 3) when NOS-2 and COX-2 activities were inhibited in LPS-treated animals (or when NOS-2 KO mice were intraperitoneally given LPS), the infiltration of inflammatory cells and the whole-organ expression of NOS-2, COX-2, and MMP-9 were all decreased, suggesting that these enzymes were expressed by resident/infiltrating cells; and 4) the morphology of the cells expressing NOS-2 and COX-2 differed from cardiomyocytes. Indeed, extensive analysis of published data on the expression of NOS-2 in cardiomyocytes shows that this has been described in neonatal cells, and only a few studies report the expression in cultured myocytes from adult animals, in which the potential contribution of activated noncardiac cells or regional differences in cardiomyocytic populations cannot be excluded.¹⁸ Attempts to identify a rapid and transient expression of NOS-2 in cardiomyo-

Figure 5. Regulation of MMP-9 expression in heart. Wild-type and NOS-2 KO mice were intraperitoneally injected with 1400W, DFU, and LPS, as indicated. **A:** Immunofluorescence determination of MMP-9 expression in wild-type mice. The immunohistographs show typical staining with anti-MMP-9 antibody in heart tissue sections taken 24 hours after injection with the agents indicated underneath. Average fluorescence intensities for each condition are indicated above each image. **B:** MMP-9 enzyme activity in whole-heart extracts from wild-type mice taken 24 hours after injection. **C:** MMP-9 enzyme activity in whole-heart extracts from NOS-2 KO mice taken 24 hours after injection. Data are the means $\pm$ SD of four experiments. * $P < 0.01$ versus the control. Analysis of variance results * $P < 0.01$ with respect to NOS-2^{+/+} mice.

cytes (up to 6 to 8 hours) after LPS challenge or inhibition of a potential desensitization of TLR-4 by contaminating LPS during the cell isolation procedure (using polymixin B), failed to show the presence of this enzyme in the cardiomyocytes (not shown).

The identity of the infiltrating cells is an important question. In several cardiac diseases such as myocarditis induced by myosin injection or infection by specific viruses, the inflammatory response is mediated by neutrophils and bone marrow-derived macrophage infiltration, which then triggers the activation of other mononuclear cells. Moreover, in addition to the recruitment of macrophages, lymphocytes, and neutrophils, the inflammatory response is accompanied by myocardial necrosis.³¹ Previous studies also reported that innate and specific immune cells participate in inflammatory infiltration and that a large number of leukocytes are recruited to the site of infection.³² In viral myocarditis, tissue injury becomes progressively evident by days 3 and 4, after which the process is complicated by an inflammatory infiltration.³³ The onset of viral myocarditis depends on the pathogen, and, for instance, viral infection by CVB3 promotes a

rapid release of chemokines that stimulate an early infiltration of mononuclear cells in the heart.³¹ In agreement with this suggestion, we observed in our model a time-dependent increase of MHC class II- and CD11b-positive cells after LPS treatment, followed by a decrease in the case of MHC class II cells attributable to the expression of NOS-2 and the release of NO, a well-known repressor of MHC class II expression.³⁴ We have also examined the ability of the heart to modulate ECM in the course of inflammation. Whereas adult cardiomyocytes fail to express NOS-2 or COX-2, they express MMPs. Interestingly, COX-2 metabolites released by the activated inflammatory cells exert profound effects on MMP expression and processing by the cardiomyocytes. Indeed, evidence that PGE₂ regulates ECM degradation and tissue remodeling has been well documented in several models of cardiac dysfunction.³⁵ The pathophysiological effects resulting from the activities of secreted MMPs, in particular 2 and 9, have been reported in various models of heart dysfunction.^{10,36–38} These MMPs contribute to tissue repair after ischemic or infarction damage, but the observation that gene transfer of the

Figure 6. MMP-9 immunofluorescence in H9c2 and HL-1 cardiomyocytes. **A:** Cells were maintained in culture and exposed for 24 hours to LPS (200 ng/ml), 1400W (50 μ mol/L), DFU (500 nmol/L), DETA-NO (100 μ mol/L), and PGE₂ (1 μ mol/L) in the combinations indicated. **B:** Cardiomyocyte cell lines were co-cultured with Raw 264.7 macrophages that had been previously activated with LPS/IFN- γ , and MMP-9 expression was determined in the upper compartment, containing the cell lines. Results show the means $\pm$ SD of four experiments. * P < 0.01, ** P < 0.005 versus the corresponding untreated control cells (**A**) or cultures in the absence of LPS/IFN- γ (**B**).

MMP inhibitor TIMP-1 has a cardioprotective effect shows that sustained MMP activation can be detrimental.³⁷ Furthermore, *in vivo* mouse models in which MMP activities can be monitored by noninvasive techniques (near-infrared spectroscopy of fluorescent substrates), enable observation of MMP-2 and MMP-9 co-localized with neutrophils in the infarct zone.^{36,39} Interestingly, in our inflammatory model, we found by immunocytochemistry that cardiomyocytes are able to express and activate MMP-9 (this response was observed both in adult cultured cardiomyocytes and in cardiomyocyte cell lines). This interplay between COX-2 expression, release of

PGs, and the regulation of MMP activity has been recognized in vascular pathologies in which macrophage proteinase expression contributes to the rupture of atherosclerotic plaques, thrombosis, myocardial infarction, and stroke. Indeed, treatment with selective COX-2 inhibitors leads to reduced tissue damage and MMP expression and activation.^{40,41} In addition to COX-2 metabolites there is a contribution of NO to the process of MMP secretion and activity. In this regard, endogenous NO from the constitutive NOS-3 enzyme (ie, physiological, but low levels) can enhance MMP expression.^{42,43} However, expression of NOS-2 by nonmyocytic cells, with its associated high NO output, exerts a dual role: on the one hand it promotes MMP expression, but on the other hand high concentrations of NO reversibly inhibit MMPs through nitrosylation reactions on specific cysteine residues.^{44,45} Unraveling the specific contribution of these nonmyocytic cells to the alteration in the functional response of the cardiomyocyte might help to develop additional strategies for the pharmacological protection of heart function in the course of septicemia and the later evolution to septic shock.

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