Selective Impairment of Nuclear Factor-κB-Dependent Gene Transcription in Adult Cardiomyocytes

Relevance for the Regulation of the Inflammatory Response in the Heart

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The ability of neonatal and adult cardiomyocytes to activate the nuclear factor (NF)-κB pathway in response to lipopolysaccharide and interleukin-1β challenge has been investigated and compared with that of peritoneal macrophages. The activation of the IκB kinase and the phosphorylation and degradation of IκBa and IκBβ was much lower in adult cardiomyocytes than in the neonatal counterparts and macrophages. This restricted activation of the NF-κB pathway resulted in a significant reduction in the time of nuclear activation of NF-κB, as deduced by electrophoretic mobility shift assays and in the transcription of target genes, such as IκBa, cyclooxygenase-2 (COX-2) and nitric-oxide synthase-2 (NOS-2). Studies on chromatin immunoprecipitation showed binding of NF-κB proteins to the regulatory κB sites identified in the promoters of the IκBa, COX-2, and NOS-2 genes in macrophages and, to a lower extent, in neonatal cardiomyocytes. The binding to these κB sites in adult cardiomyocytes was observed only in the IκBa promoter and was minimal or absent in the COX-2 and NOS-2 promoters, respectively, suggesting a restricted activation of NF-κB-regulated genes in these cells. These data indicate that the function of the NF-κB pathway in adult cardiomyocytes is limited in time, which results in the expression of a reduced number of genes and provides a functional explanation for the absence of NOS-2 inducibility in these cells under proinflammatory conditions. (Am J Pathol 2007, 171:820–828; DOI: 10.2353/ajpath.2007.061076)

The ability of the heart to respond to proinflammatory cytokines and pathogen-associated molecular patterns has been reported by several authors.1–3 However, in isolated cardiac cells from neonatal or adult animals there are discrepancies regarding the conditions required to observe the expression of genes involved in the inflammatory response such as nitric-oxide synthase-2 (NOS-2), cyclooxygenase-2 (COX-2), and matrix metalloproteinases.4–6

The nuclear factor (NF)-κB family of transcription factors plays an essential role in regulating the induction of genes involved in inflammation, and it has been shown that they are important mediators of the inflammatory response in the heart. The presence of p65, p50, IκBα, and IκBβ in the myocardium has been described in postnatal and adult heart, but less is known regarding the role of c-Rel that mediates late events in the transcriptional activity of the NF-κB/c-Rel complex, preferentially in immune cells.7 The p65 protein is a key transcriptionally active component of NF-κB, but the activation of this factor mainly depends on the phosphorylation of inhibitory molecules including IκBα. However, optimal induction of NF-κB target genes also requires phosphorylation of NF-κB proteins such as p65.10

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We have shown that recently isolated neonatal and adult cardiomyocytes are unable to express most of the NF-κB-regulated enzymes related to inflammation, except after a prolonged period of culture in the case of neonatal cells. This effect is attributable, at least in part, to a reduced activation of the IκB kinase/NF-κB pathway observed in cardiomyocytes but exerts differential responses depending on the genes considered; in particular, the expression of NOS-2 is significantly impaired. To gain insight on the mechanisms that regulate the expression of NF-κB-dependent genes in the cardiomyocyte, we used the in situ method of chromatin immunoprecipitation (ChIP) for the study of the transcription of IκBα, COX-2, and NOS-2, as three genes related to NF-κB activation. Our data show that after lipopolysaccharide (LPS) and interleukin (IL)-1β challenge, the activation of the IκB kinase (IKK) exhibits marked differences among cells, with adult cardiomyocytes being the less activated. Neonatal and adult cardiomyocytes expressed IκB proteins and COX-2. However, adult cardiomyocytes failed to express NOS-2 indicating an inefficient capacity of the NF-κB complex to accomplish the transcription of this gene. This behavior was compared with that of macrophages, which exhibited a robust activation of the NF-κB pathway, resulting in the expression of higher levels of COX-2 and NOS-2.

Materials and Methods

Chemicals

LPS from Salmonella typhimurium and all other reagents not specified were from Sigma Chemical Co. (St. Louis, MO). Cytokines were from Roche (Mannheim, Germany). Collagenase type II was from Worthington (Lakewood, NJ). Antibodies were obtained from Santa Cruz (Dallas, CA) or Calbiochem (San Jose, CA). Tissue culture dishes were from Falcon (Lincoln Park, NJ), and culture media were from BioWhittaker (Walkersville, MD).

Isolation and Culture of Neonatal Cardiomyocytes

Neonatal cardiomyocytes (NeoC) were isolated from 1-day-old BALB/c mice as follows. The hearts were pooled, minced, disaggregated mechanically, and digested for 5 minutes at 37°C with 0.8% collagenase (type II, Worthington Biochemical Corp.) in phosphate-buffered saline (PBS), according to a previous protocol of successive digestions. The cell pellets were resuspended in Dulbecco's modified Eagle's medium/F-12/M-199 medium [4:1 (v/v)], supplemented with 10% fetal bovine serum (FBS) and antibiotics (penicillin, 100 U/ml; streptomycin, 100 μg/ml). The isolated cells were incubated for 1 hour in an uncoated flask, allowing the non-myocytic population to differentially attach to the flask. Then, the cell suspension was sedimented by centrifugation at low speed (80 × g), and cells were distributed in plates precoated with a 2% solution of gelatin. After overnight incubation, the dishes were washed with PBS, and the medium was replaced. Usually, cells were plated at 4 × 10⁴ cells/cm² and used after 3 days in culture.

Isolation of Adult Cardiomyocytes

Ventricular myocytes (AdtC) were isolated as previously described. In brief, 8-week-old male mice were anesthetized (92.7 mg/kg ketamine/xylazine) and sacrificed by cervical dislocation. Hearts were rapidly removed and placed into Tyrode buffer (140 mmol/L NaCl, 5.4 mmol/L KCl, 1 mmol/L Na₂HPO₄, 5 mmol/L HEPES, 10 mmol/L glucose, and 1 mmol/L MgCl₂, pH 7.4), containing 1 mmol/L CaCl₂ at 4°C and then cannulated via the aorta for retrograde perfusion of the coronary arteries. Hearts were perfused with Tyrode buffer containing 1 mmol/L CaCl₂ at 2 ml/minute for 5 minutes at 37°C and then Tyrode buffer without CaCl₂ at 2 ml/minute for 5 minutes. Final perfusion continued at 2 ml/minute for 8 to 10 minutes with Tyrode buffer containing 40 μmol/L CaCl₂, 20 μg/ml collagenase (Yakult Pharmaceuticals Co., Seoul, Korea), and 4 μg/ml protease (Sigma). Ven- tricles were then minced in Tyrode buffer containing 1 mmol/L CaCl₂, 500 μg/ml collagenase, 100 μg/ml protease, and 2.5% bovine serum albumin (Sigma). Ventricular tissue segments were put into a shaking water bath for 10 to 20 minutes at 37°C to complete dispersion of myocytes. Myocytes were cultured at a density of 1 to 2 × 10⁴ cells/cm² on laminin-coated tissue cultureware. The cardiomyocytes were cultured in Dulbecco’s modified Eagle’s medium with 10% FBS, 10 μg of transferrin, 10 μg/ml insulin, minimal essential medium with vitamin mix and nonessential amino acids, and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin), and used within the next 24 hours. For both NeoC and AdtC cultures, the viability of the cells was determined by propidium iodide exclusion (>90%), and the purity by immunocytochemistry for α-actin and cardiac actinin (>85% positive).

Cardiomyocyte Cell Line

HL-1 cells are cardiomyocytes derived from a mouse atrial tumor and were kindly provided by Dr. W.C. Claycomb, Louisiana State University Health Science Center, New Orleans, LA, and cultured in Claycomb medium (JRH Biosciences, Lenexa, KS) containing 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 0.1 mmol/L nor- epinephrine, and 2 mmol/L L-glutamine. Cells were cultivated at 37°C under 5% CO₂ on fibronectin-coated flasks. HL-1 is a cardiac muscle cell line that contracts and retains phenotypic characteristics of the adult cardiomyocyte.

Preparation of Macrophages

Elicited peritoneal macrophages were prepared from male mice 4 days after intraperitoneal administration of 1 ml of 10% thioglycollate broth. Cells were seeded at 2 × 10⁶ in 6-cm plates or 5 × 10⁵ in 24-multiwell plates and cultured with RPMI 1640 medium supplemented with...
10% heat-inactivated FBS and antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin) at 37°C in an humidified atmosphere with 5% CO₂. After incubation for 4 hours, nonadherent cells were removed, and remnant cells were cultured for the indicated times.16

**Transfection of Macrophages and Cardiomyocytes**

Cells were transfected per triplicate using JetPel transfection reagent (PolyPlus Transfection, San Marcos, CA) with a JetPel/DNA ratio of 3:1 and in the presence of 2% FBS. Twenty-four hours after transfection, the culture medium was changed, and cells were activated with the indicated stimuli. The vectors used were p-Luc, (κB)₂-, Luc, p-p65, c-Rel, and the respective dominant-negative forms p-DN-p65 and p-DN-c-Rel, respectively. These vectors were provided generously by Dr. M. Fresno, Universidad Autónoma de Madrid, Madrid, Spain, and have been described previously.17,18 Reporter activities were assayed using the dual luciferase assay system per the manufacturer’s instructions (Promega, Madison, WI). For normalization of transfection efficiency, cells were co-transfected with the reporter plasmid pTk Renilla (Promega), and luminescence data were expressed as the relative light ratio between both enzymes. A vector encoding the GFP gene was used for the evaluation of the transfection efficiency.

**Measurement of Metabolites**

The amounts of nitrate and nitrate that accumulate in the culture medium were determined after reduction of nitrates to nitrates followed by the determination of NO₂⁻ levels with Griess reagent.11 The levels of PGE₂ were determined using a Biotrak kit from Amersham, Arlington Heights, IL, following the instructions of the supplier.

**Determination of TLR2/4 Expression Levels by Flow Cytometry**

Cells were resuspended in PBS 1× and incubated 15 minutes with a fluorescein isothiocyanate/phycocerythrin-conjugated antibody (Ab) against the indicated TLR and incubated with the indicated antibodies against NF-κB proteins (p50, p65, and c-Rel) before the addition of the probe.

**Preparation of Cytosolic and Nuclear Extracts**

Cultured cells were washed with PBS, scraped off the dishes in ice-cold PBS, and centrifuged. Cell pellets were homogenized in 200 µl of buffer A (10 mmol/L HEPES, pH 7.9, 1 mmol/L ethylenediamine tetraacetic acid (EDTA), 1 mmol/L ethylene glycol bis(β-aminoethyl ether)-N,N',N″,N‴-tetraacetic acid, 10 mmol/L KCl, 1 mmol/L dithiothreitol, 0.5 mmol/L phenylmethyl sulfonyl fluoride, 40 µg/ml leupeptin, 2 µg/ml tosyl-lysyl-chloromethane, 5 mmol/L NaF, 1 mmol/L NaVO₄, and 10 mmol/L Na₂MoO₄), and Nonidet P-40 was added to reach 0.5% (v/v). After 15 minutes at 4°C, the tubes were gently vortexed for 15 seconds, and nuclei were collected by centrifugation at 8000 × g for 15 minutes. The supernatants were stored at −80°C (cytotoxic extracts), and the pellets were resuspended in 50 µl of buffer A supplemented with 20% (v/v) glycerol and 0.4 mol/L KCl, and mixed for 30 minutes at 4°C. Nuclear proteins were obtained by centrifugation at 13,000 × g for 15 minutes, and aliquots of the supernatant (nuclear extracts) were stored at −80°C. For Western blot analysis, cytosolic and nuclear proteins were boiled in Laemmli sample buffer, and equal amounts of protein (30 to 15 µg) were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

**Measurement of IKK Activity**

Macrophages and NeoC and AdtC cells (5 to 6 × 10⁶) were stimulated, and at the indicated times the cells were homogenized in 1 ml of buffer A and centrifuged for 10 minutes in a microcentrifuge. The IKK from equal amounts of protein from these supernatants was immunoprecipitated with 1 µg of anti-IKK2 Ab. After washing with 4 ml of buffer A, the pellet was resuspended in kinase buffer (20 mmol/L HEPES, pH 7.4, 0.1 mmol/L EDTA, 100 mmol/L NaCl, 1 mmol/L dithiothreitol, 0.5 mmol/L phenylmethyl sulfonyl fluoride, 2 µg/ml aprotinin, 10 µg/ml leupeptin, 2 µg/ml TLCK, 5 mmol/L NaF, 1 mmol/L NaVO₄, 10 mmol/L Na₂MoO₄, and 10 mmol/L okadaic acid). Kinase activity was assayed in 100 µl of kinase buffer containing 50 ng of precipitated protein. Myelin basic protein was used as substrate in the presence of 50 µmol/L [γ-³²P]ATP (0.5 µCi). Reactions were stopped with 1 ml of ice-cold buffer A supplemented with 5 mmol/L EDTA, and after retention onto a P81 Whatman filter and counted.

**Electrophoretic Mobility Shift Assays**

The oligonucleotide sequence corresponding to the NF-κB site of the rat COX-2 promoter (5′-GGCAAGGG-GATTCCCTTAGTT-3′)11 was annealed with the complementary sequence by incubation for 5 minutes at 85°C in 10 mmol/L Tris-HCl, pH 8.0, 50 mmol/L NaCl, 10 mmol/L MgCl₂, 1 mmol/L dithiothreitol. Aliquots (100 ng) were end-labeled with Klenow enzyme fragment in the presence of 50 µCi of [α-³²P]dCTP and the other unlabeled dNTPs in a final volume of 50 µl. DNA probe (5 × 10⁴ dpm) was used for each binding assay: 5 µg of nuclear protein were incubated for 15 minutes at 4°C with the probe and with 1 µg of poly (dl-dC), 5% glycerol, 1 mmol/L EDTA, 10 mmol/L KCl, 5 mmol/L MgCl₂, 1 mmol/L dithiothreitol, and 10 mmol/L Tris-HCl (pH 7.8) in a final volume of 20 µl. The DNA-protein complexes were separated on native 6% polyacrylamide gels in 0.5% Tris-borate-EDTA buffer. Supershift assays were performed by incubating the nuclear extracts (3 µg of protein) with the indicated antibodies against NF-κB proteins (p50, p65, and c-Rel) before the addition of the probe.
Western Blot Analysis

The protein levels of IκBα, IκBβ, p65, c-Rel, and α/β-actin were determined in cytosolic or nuclear extracts as indicated. Equal amounts of protein (15 to 30 μg) were size-fractionated in a 10% acrylamide gel, transferred to a Hybond P membrane (Amersham), and after blocking with 5% nonfat dry milk, were incubated with the corresponding antibodies and visualized by enhanced chemiluminescence. Different exposure times were performed with each blot to ensure the linearity of the band intensities. Band intensities were measured on a densitometric scanner (Amersham) and expressed in arbitrary units.

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

Total RNA was extracted from frozen cells by using TRIzol reagent (Life Technologies, Inc., Grand Island, NY). Total RNA (1 μg per sample) was reverse-transcribed with oligo(dT) as primer using Expand Reverse Transcriptase (Roche, Basel, Switzerland) according to the manufacturer’s protocol. Q-RT-PCR was performed using SYBR Green PCR kit (PE Applied Biosystems, Foster City, CA) in an Applied Biosystems 7700 sequence detector. Primer sequences were: NOS-2 (mouse) forward, 5'-CAGCTGGGCTGTACAAACCTT-3', reverse 5'-CATTGGAAGTGAAGCGTTCTTCG-3'; COX-2 (mouse) forward, 5'-GCTGTAACAAGCA-GTGCA AAAG-3', reverse 5'-GGGATCTAAGGTCCTAACTTGACA-3'; IκBα (mouse) forward, 5'-GCTCCGAGACTTTCGAGGAA-3', reverse 5'-TTGTAGTTGGCCTGCA-3'. All samples were analyzed in the same run for 18S expression for normalization: forward, 5'-AACACGGGAAA-CCTCACCC-3' and reverse, 5'-CCACCAACTAAAGCG-GCCA-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. The quantitative expression values were extrapolated from standard curves for 18S mRNA. The replicates were then averaged, and fold induction was determined, considering the value at zero time as 1.

ChIP Assay

ChIP assay was performed according to Saccani and colleagues. In brief, after stimulation, cells were fixed by adding directly to the medium formaldehyde solution to reach a final concentration of 1%. After incubation for 15 minutes, the cells were washed extensively with PBS, scraped off the dishes, and centrifuged. Cells were resuspended in ice-cold buffer I (10 mmol/L HEPES, pH 7.9, 10 mmol/L EDTA, 0.5 mmol/L ethylene glycol bis(β-aminoethyl ether)-N,N′,N′,N′′-tetraacetic acid, and 0.25% Triton X-100), centrifuged, and the pellet resuspended in buffer II (10 mmol/L HEPES, pH 7.9, 1 mol/L EDTA, 0.5 mmol/L ethylene glycol bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid, and 200 mmol/L NaCl). After centrifugation, nuclei were lysed with ice-cold lysis buffer (50 mmol/L Tris-HCl, pH 8.0, 10 mmol/L EDTA, 1% SDS, and protease inhibitors). Chromatin was sheared by sonication (5 × 18 seconds each at 10% power), centrifuged, and diluted five times with the IP dilution buffer (20 mmol/L Tris-HCl, pH 8.0; 2 mmol/L EDTA, 1% Triton X-100, 150 mmol/L NaCl, and protease inhibitors). The extracts were preclotted for 30 minutes with a 50% suspension of salmon sperm-saturated protein A/G agarose. Immunoprecipitations with anti-p65 or anti-c-Rel Abs (Santa Cruz Biotechnologies) or with no Ab, were performed overnight at 4°C. Immune complexes were collected with protein A/G agarose and washed sequentially for 5 minutes each in buffer W150 (20 mmol/L Tris-HCl, pH 8.0, 2 mmol/L EDTA, 1% Triton X-100, 0.1% SDS, and 150 mmol/L NaCl), buffer W600 (20 mmol/L Tris-HCl, pH 8.0, 2 mmol/L EDTA, 1% Triton X-100, 0.1% SDS, and 500 mmol/L NaCl), and buffer W100 (10 mmol/L Tris-HCl, pH 8.0, 1 mmol/L EDTA, 0.25 mol/L LiCl, 1% Nonidet P-40, and 1% deoxycholate). Precipitates were then washed twice with TE (10 mmol/L Tris-HCl, pH 8.0, and 1 mmol/L EDTA), extracted with TES (25 mmol/L Tris-HCl, pH 7.5, 10 mmol/L EDTA, and 0.5% SDS) and heated at 65°C to elute the chromatin. After proteinase K digestion, chromatin DNA was extracted with phenol-chloroform followed by ethanol precipitation. PCR reactions were performed with promoter-specific primers and normalized with the input controls. Primer sequences are shown in Table 1. After amplification, the PCR products were electrophoresed in a 2% agarose gel and visualized by ethidium bromide staining.

Statistical Analysis

Unless otherwise stated, data are expressed as mean ± SD. To compare means between two independent experiments the Mann-Whitney rank sum test was used. Statistical analysis of mRNA expression data was performed by using the two-tailed homoscedastic t-test. The results were considered significant at P < 0.05. Data were analyzed by SPSS for Windows statistical package version 9.0.1 (SPSS Inc., Chicago, IL).

Results

Differential Degradation of IκB Proteins after Proinflammatory Challenge

The response of primary cultures of neonatal (NeoC) and adult (AdC) cardiomyocytes to tumor necrosis factor (TNF)-α, IL-1β, and LPS in terms of NOS-2 and COX-2 expression (two well-characterized NF-κB-dependent

### Table 1. Primers for ChIP Assays

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primers</th>
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<tbody>
<tr>
<td>COX-2</td>
<td>Forward 5'-TTAACCGGCTAGCTGTGTGCCTGTGCTG-3' Reverse 5'-CCTGGGATCTAAGGTCCTAACT-3'</td>
</tr>
<tr>
<td>NOS-2</td>
<td>Forward 5'-GACCATGCGAAGATGAGTGGGA-3' Reverse 5'-TGAAATGCAATTTGACAGTGTTCGACCCG-3'</td>
</tr>
<tr>
<td>IκBα (1)</td>
<td>Forward 5'-GACCTTTCCAGCCACTACGG-3' Reverse 5'-CCTAACCACGGCCCGG-3'</td>
</tr>
<tr>
<td>IκBα (2)</td>
<td>Forward 5'-TACTAGTGCAGCAGGTCAG-3' Reverse 5'-GGTCATGCAGAGGACCTT-3'</td>
</tr>
<tr>
<td>IκBα (3)</td>
<td>Forward 5'-ACCCAGGGGAAAGAAGGTT-3' Reverse 5'-CTGGAAGTGCTTCACGG-3'</td>
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mined in these cells, the activation in AdtC represented only a minor content of NOS-2 genes) and nitrite and PGE$_2$ synthesis was analyzed and compared with that of peritoneal macrophages treated under identical conditions. Figure 1A shows the response of these cells to combinations of these proinflammatory stimuli, and maximal accumulation of NOx and/or PGE$_2$ in the culture medium was obtained when LPS and IL-1β were combined. TNF-α promoted a significant apoptosis in cardiomyocytes after 24 hours of culture and for this reason it was omitted in the proinflammatory mixture. As Figure 1, A and B, shows, AdtC failed to express NOS-2, and only moderate levels of COX-2 were detected, whereas NeoC and macrophages exhibited a robust expression of both NOS-2 and COX-2. Agreement was observed between the levels of NOx and PGE$_2$ and the corresponding protein as substrate. Results show the mean ± SD of the metabolites or fluorescence measurements, the relative levels of TLR4 and TLR2 in the cell extracts and cell membrane were determined in intact cultured cells. Macrophages exhibited approximately twofold and fourfold higher levels than those measured in NeoC and AdtC, respectively (Figure 1C); however, the levels of IKK2, p65, and IκBα were quite similar in the three cell types, and only a minor content of IκBα was observed in AdtC (Figure 1D).

In view of these data, we compared the ability of these cells to activate the NF-κB pathway after proinflammatory challenge. As Figure 2A shows, on normalization to the basal levels, macrophages and NeoC exhibited a rapid degradation of IκBα and IκBβ, as a result of the activation of IKK and phosphorylation of IκBα in S32 in response to LPS and IL-1β (Figure 2B). However, AdtC exhibited a moderate phosphorylation of IκBα in S32 and a decrease of both IκBα proteins, minimal in the case of IκBβ, followed by a rapid resynthesis of IκBα. When the activation of IKK was determined in these cells, the activation in AdtC represented only 21% and 32% of the activity in macrophages and NeoC, respectively, after 15 minutes of stimulation (Figure 2C). These differences in IKK activity and IκB degradation and recovery resulted in different responses in the mRNA levels of the genes studied: IκBα, NOS-2, and COX-2, with a significantly attenuated response in the case of the NOS-2 gene in AdtC (Figure 3; to note the difference in scales).

To gain insight on the nuclear translocation of NF-κB in these cells after LPS/IL-1β challenge, serial electrophoretic mobility shift assays were performed. As Figure 4 shows, the binding of the κB complexes was most restricted in time in AdtC than in macrophages or NeoC. Moreover, supershift studies showed that the complexes at early times after activation involved p50 and p65 proteins, whereas at 24 hours the presence of c-Rel was detected in nuclear extracts from activated macrophages and to a lesser extent in NeoC. In agreement with these data, the presence of p65 in the nucleus was higher in macrophages and NeoC than in AdtC, with a minimal contribution of c-Rel in the case of NeoC.

Analysis of NF-κB Complexes in the Promoter Region of Selected NF-κB-Dependent Genes

Further to determine the binding of NF-κB proteins to previously recognized regulatory sequences in the pro-
motors of iκBα, NOS-2, and COX-2 ChIP assays were performed in samples obtained after 2 hours and 24 hours of activation with LPS/IL-1β. As Figure 5 shows, binding of p65 complexes was observed in the three motifs investigated of the iκBα promoter, regardless of the nature of the cells. When the binding to the distal iκB site from the murine NOS-2 promoter was investigated, a significant and reproducible presence of p65 was observed in macrophages and NeoC; however, AdtC completely failed to show such binding, and only a modest recognition was present in the case of the iκB site from the COX-2 promoter. ChIP analysis was also performed on the NOS-2 and COX-2 promoters after 24 hours of stimulation, including anti-c-Rel Abs in the immunoprecipitation reaction. As Figure 5 (bottom) shows, c-Rel was absent in AdtC, and only a minor binding in the case of the COX-2 promoter was observed in NeoC. Opposite to this behavior, macrophages exhibited a clear presence of c-Rel at 24 hours in both promoters.

To investigate the possibility of a restoration of the NF-κB response via the expression of p65 and c-Rel proteins, macrophages, NeoC, AdtC, and the adult cardiomyocytic cell line HL-1 were transfected with a p65 reporter vector or with the 1.1-kb NOS-2 promoter linked to a luciferase gene and various proteins of the p65 and c-Rel family. After transfection and stimulation with LPS/IL-1β, the reporter activity was measured. The efficiency for the transfection of AdtC was quite low (1 to 4%), and therefore, the experiments were performed with the HL-1 cell line (see Discussion). As Figure 6 shows, the activity of the NF-κB reporter increased in the three types of cells after LPS/IL-1β challenge, although with significant quan-
the control condition; cyttes, a situation that is dependent on the age of the sized, which implies a differential regulation of NF-
only in macrophages was it fully degraded and resynthe-
expressed. Of note, although I to macrophages. Moreover, in AdtC only COX-2 was
expressed in the three types of cells studied, COX-2 and

Discussion
In this work we have studied quantitative aspects of NF-κB activation in cardiomyocytes using as reference peritoneal macrophages that exhibit a robust response of this pathway after proinflammatory stimulation. Our data show a differential occupancy and transcriptional activity of various NF-κB-regulated genes in cardiomyocytes, a situation that is dependent on the age of the animals: whereas IκBα was rapidly degraded and re-expressed in the three types of cells studied, COX-2 and NOS-2 expression was attenuated in NeoC with respect to macrophages. Moreover, in AdtC only COX-2 was expressed. Of note, although IκBα was processed in all these cells, IκBβ remained virtually stable in AdtC, and only in macrophages was it fully degraded and resynthe-
sized, which implies a differential regulation of NF-κB activity among these cells. In agreement with these data, previous work showed that AdtC exhibit a moderate re-
response in the expression of genes depending on NF-κB activity, despite containing similar levels of most of the proteins of the pathway, such as IKK, IκBα, and p65. To explain this loss of expression of κB-dependent genes in AdtC, we investigated the contribution of early signal-
leading to NF-κB activation and the existence of potential transcriptional silencing mechanisms once the complex is located in the nucleus (ie, cooperative actions of histone deacetylases, HDACs).

Regarding the proinflammatory stimulation, we investi-
igated the effect of LPS and IL-1β that provided the most potent response in terms of IκBα degradation, almost identical to that observed when TNF-α was included as stimulus. However, TNF-α was excluded in this study because this cytokine promoted apoptotic death of AdtC and to a lesser extent in NeoC. Co-treatment with IFN-γ did not influence NF-κB activation or the expression of NOS-2 in AdtC (not shown). The profile of genes altered in the heart after systemic administration of LPS is known, and a reversible cardiac dysfunction starting 24 hours after endotoxin administration has been reported. These data show a spatial and temporal heterogeneity in the changes of the expression of genes that are important to understand the complexity of the sepsis in the whole organ, including the infiltration of inflammatory cells, but most of these effects are lost when the organ is digested to prepare cardiomyocytic cultures, suggesting a major contribution of infiltrated nonmyocytic cells to the inflammatory response in the heart.

The role of TLR2 and TLR4 in the expression of inflam-
mary mediators in the cardiomyocyte is well documented. Our data show that TLR2 and TLR4 are present in the cell membrane of NeoC and AdtC, although at concentrations that are less than twofold and fourfold those present in the macrophages, respectively. It can be suggested accordingly that the initial downstream signaling is attenuated because the activation of IKK and accu-
cumulation of phosphorylated IκBα in AdtC treated for 20 minutes with LPS/IL-1β represents only 20 and 27%, respectively, of the levels reached in macrophages. Moreover, these data suggest that the rapid resetting of IκBα protein after stimulation of AdtC is responsible, at least in part, for the decreased activation of the NF-κB pathway as deduced from the results obtained in cells transfectected with a p65 expression vector. In addition to this, it has been reported that the levels of TLR2 and TLR4 can be up-regulated and expressed in the cell mem-
brane on proinflammatory challenge, and in this way a second round of stimulation on cells expressing higher levels of TLRs can result in a more effective response in the case of the AdtC. Indeed, the mRNA levels of TLR4 increased three times after 24 hours of LPS admin-
istration (results not shown), and transfection with consti-
tuitively active forms of TLR4 induced NF-κB activity but not the transcription of some promoters, such as NOS-
2. The transcription of NOS-2 in rodent cells is strictly dependent on NF-κB activity, and the distal κB site, which has been used in our ChIP study, confers induc-
ability in response to interferon (IFN)-γ. According to our data, the NF-κB proteins remained unbound to this

Figure 6. Effect of p65 and c-Rel transfection on the NOS-2 promoter activity. Primary cultures of macrophages (Mø), neonatal (NeoC), and the adult cardiomyocytic cell line HL-1 were cotransfected with the indicated vectors, and the luciferase activity was determined after 24 hours of culture. Results are expressed as the luminescence ratio between the light corresponding to the reporter activity versus the light of Renilla luciferase used as normaliza-
tion vector. Data show the mean ± SD of four experiments. *P < 0.01 versus the control condition; **P < 0.01 versus the condition in the absence of p65 and c-Rel proteins.
κB site required for the expression of NOS-2, even in the presence of IFN-γ during the stimulation (not shown), at the time that equivalent sites, in particular those found in the IκBα promoter, were occupied and transcriptionally active in view of the rapid up-regulation and accumulation of IκBα mRNA and proteins after LPS/IL-1β challenge. The question remains if prolonged exposure to proinflammatory stimuli, a condition that enhances the expression of TLRs, would be sufficient to improve NF-κB responsiveness leading to a behavior similar to that observed in NeoC. In this regard, we described in a previous work that prolonged culture of cardiomyocytes enhanced notably the expression of genes related to inflammation, specifically the expression of NOS-2 in NeoC in response to LPS/IL-1β challenge.11

We also investigated the contribution of p65 and c-Rel proteins in the particular case of NOS-2 transcription. c-Rel has been detected in macrophages and other immune cells where it mediates late and sustained responses in the activation of specific promoters.32,33 As expected, macrophages expressed c-Rel, and only minor levels were detected at 24 hours in NeoC, but not in AdtC. However, transfection with c-Rel of macrophages, NeoC, and the HL-1 cell line, which behaves very closely to AdtC, resulted in a clear inhibitory effect in the promoter activity of NOS-2 in the three cell systems assayed, precluding a potential inducing effect of this transcription factor. This was not the case of p65 that, as expected, restored a notable activation of the NOS-2 promoter in the three cell types analyzed.

The attenuated response of the NF-κB pathway in cardiomyocytes seems to have important physiopathological consequences. On the one hand, our data suggest that the activation of the NF-κB pathway only induces a moderate expression of some of the genes involved in the release of potent effector molecules, such as NO and prostaglandins that have been described to contribute to heart dysfunction.34 On the other hand, data from various groups have stressed the relevance of this activation of the NF-κB pathway to mediate cell protection and function under pathological circumstances; for example, in animals infected with Trypanosoma cruzi, there is a selective impairment of the apoptotic response to TNF-α in cardiomyocytes and neurons via the activation of the NF-κB pathway, and this response seems to be specific because T cells undergo apoptosis after infection.35,36 In agreement with this view, hypertrophic heart can be considered as a condition in which NF-κB is activated and cells are protected against apoptotic death.37 This situation can be pharmacologically prevented with salicylate analogues, such as triflusal, that are potent inhibitors of NF-κB pathway and, therefore, efficient in preventing cardiac hypertrophy.38 The mechanisms by which the activation of NF-κB protects cardiomyocytes from apoptosis seems to include, in addition to the expression of common anti-apoptotic targets,22,39–41 the specific inhibition in the heart of the hypoxia-inducible death factor BNIP3.42 Moreover, initial experiments to evaluate the contribution of HDAC activity on the p65-dependent transactivation revealed that the association between HDAC1 and p65 is minimal in AdtC, and inhibitors of HDAC, such as trichostatin A, failed to enhance the expression of NOS-2 and COX-2 in AdtC or to increase the binding of NF-κB to the sites studied in the ChIP experiments (not shown).

Finally, it should be mentioned that in the myocytic cell line C2C12, derived from skeletal muscle, LPS is able to induce a significant expression of NOS-2 and other genes involved in the inflammatory response; however, in the cardiomyocytic cell line HL-1 as well as in AdtC we failed to observe such an effect, reinforcing the view that these cells have an attenuated response in the NF-κB pathway.43 In summary, our data suggest that adult cardiomyocytes display a moderate capacity to activate the NF-κB pathway, which might be important for the pathophysiology of the organ. Indeed, various studies suggest that the major input of proinflammatory stimuli and effector molecules, such as TNF-α, NO, and prostaglandins that are present in the heart under infectious or inflammatory conditions are released by infiltrating macrophages and neutrophils.25,26 supporting the view that NF-κB activation in adult cardiac cells is involved in other cell functions, distinct from the acute inflammatory response. Ancillary to this conclusion, pharmacological achievements to regulate heart dysfunction under sepsis should take into account the relative contribution of the distinct types of cells present in the heart, as well as those infiltrating from the circulatory system, to the whole inflammatory response in the organ.

References

40. Frost RA, Nystrom GJ, Lang CH: Lipopolysaccharide stimulates nitric oxide delivery delays nitric oxide-dependent apoptosis in cardio