Induction of Nitric Oxide Synthase Gene by Interleukin-1β in Cultured Rat Cardiocytes

Motoryoshi Tsujino, MD; Yukio Hirata, MD; Taihei Imai, MD; Kazuo Kanno, MD; Satoru Eguchi, MD; Hiroshi Ito, MD; Fumiaki Marumo, MD

Background. Impaired myocardial contractility in septic shock is protracting, which may be caused by cytokine-induced nitric oxide (NO) synthesis in the heart. However, the cellular mechanism by which cytokines induce nitric oxide synthase (NOS) in cardiocytes remains obscure.

Methods and Results. We studied the effect of human recombinant interleukin-1β (IL-1β) on synthesis of NO\textsubscript{2}-/NO\textsubscript{3}\ (-\ NO\textsubscript{3}) and the expression of NOS mRNA and protein in cultured neonatal rat cardiocytes. IL-1β dose-dependently (0.1 to 10 ng/mL) stimulated NO\textsubscript{3} production as a function of time (6 to 48 hours). Northern blot analysis using complementary DNAs for rat brain constitutive (c) NOS and mouse macrophage-type inducible (i) NOS as probes showed that IL-1β induced expression of mRNA for iNOS but not for cNOS, starting after 6 hours and reaching a maximum after 48 hours in cardiocytes. IL-1β similarly induced iNOS mRNA expression in cultured adult rat cardiocytes in a time-dependent manner. Western blot analysis using specific antibody against the N-terminal fragment of mouse iNOS revealed the expression of 130-kD iNOS-like protein in IL-1β-treated cardiocytes. Northern blotting and immunocytochemical study revealed that IL-1β-induced iNOS mRNA and iNOS-like immunoreactivity were exclusively localized to cardiac myocytes but also to nonmyocytes, to a lesser extent. N\textsuperscript{G}O\textsubscript{2}-monomethyl-L-arginine, an NOS inhibitor, completely blocked the IL-1β-induced NO\textsubscript{3} production, whose effect was reversed by L-arginine but not by d-arginine. Dexamethasone inhibited the IL-1β-induced NO\textsubscript{3} production as well as iNOS mRNA expression. Cycloheximide and actinomycin D completely inhibited the IL-1β-induced NO\textsubscript{3} production and iNOS mRNA expression. Neither a calmodulin inhibitor (W-7), a protein kinase C inhibitor (calphostin C), nor a Ca\textsuperscript{2+} channel antagonist (nicardipine) showed any effect on the IL-1β-induced NO\textsubscript{3} production.

Conclusions. These data demonstrate that IL-1β induces macrophage-type iNOS mRNA expression mainly by cardiac myocytes but also by nonmyocytes to a lesser extent, and subsequent de novo protein synthesis of iNOS leads to excessive local production of NO by cardiocytes. (Circulation. 1994;90:375-383.)

Key Words. • nitric oxide • myocytes • interleukin

Nitric oxide (NO), which is generated from L-arginine by nitric oxide synthase(s) (NOS) in a wide variety of tissues, appears to play diverse physiological roles, including regulation of vascular tone, neurotransmission, and mediation of immune responses. At least two different isoenzymes of NOS exist; one is Ca\textsuperscript{2+}/calmodulin-dependent and constitutive (c), and the other is Ca\textsuperscript{2+}-independent and cytokine-inducible (i). cNOS is present mainly in brain and endothelium, can be activated by Ca\textsuperscript{2+}-mobilizing agonists within a few minutes. Conversely, iNOS, which exists in a number of cells and tissues, such as macrophages, vascular endothelial cells, smooth muscle cells, hepatocytes, fibroblasts, and myocardium, can be induced by bacterial lipopolysaccharides (LPS) and cytokines over several hours. Recently, two isoforms of cNOS from rat cerebellum and bovine endothelium have been cloned and sequenced; the overall amino acid sequence identity of both isoforms is 60%, and they have binding sites for NADPH, FAD, FMN, and calmodulin. More recently, iNOS from an activated mouse macrophage cell line has been cloned and sequenced; mouse macrophage iNOS has about 50% amino acid sequence similarities to rat brain cNOS and bovine endothelial cNOS and has binding sites for NADPH, flavin coenzymes, and calmodulin as well.

In septic shock, sustained depression of myocardial contractility and protracted hypotension are determinant pathophysiological events in the outcome of this condition. Recently, it has been reported that several cytokines, such as tumor necrosis factor (TNF)-α, interleukin (IL)-6, and IL-2, but not IL-1, inhibited contractility of isolated hamster papillary muscles via generation of NO in the myocardium, because an NOS inhibitor, N\textsuperscript{G}O\textsubscript{2}-monomethyl-L-arginine (L-NMMA), blocked negative inotropic effect by cytokines. However, it remains unknown whether cytokines directly stimulate cNOS and/or iNOS in cardiocytes. Therefore, we investigated whether IL-1β stimulates NO synthesis by induction of iNOS and/or cNOS in cultured neonatal rat cardiocytes and whether IL-1β-induced iNOS mRNA is expressed by cardiac myocytes and/or nonmyocytes, by measurement of NO production, Northern blot analysis using mouse iNOS cDNA as a probe, and immunocytochemical study using a specific antibody for mouse iNOS. To confirm the potential physiological relevance, we studied whether IL-1β induces iNOS mRNA in cultured adult rat myocytes as well.
Methods

Materials
Recombinant IL-1β (lymphocyte-activating factor activity, 2 x 10^6 half-maximal U/mg protein) was a generous gift from Otsuka Pharmaceutical Co. Nicardipine was a gift from Yanamouchi Pharmaceutical Co. Dexamethasone, I- and D-arginine, N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W-7), and cytosine arabinoside (Ara-C) were obtained from Sigma Chemical Co, calphostin C from Kyowa Medex Co, L-NMMA from Calbiochem Co, cycloheximide and actinomycin D from Wako Pure Chemical, and deoxyctydine 5'-[32P]triphosphate from DuPont NEN Research Products. cDNA for human GAPDH was generously provided by Dr K. Webster (SRI International).

Culture of Neonatal and Adult Rat Cardiocytes
Primary culture of rat neonatal cardiocytes was prepared as previously described.16 Briefly, cardiac ventricles from 1- or 2-day-old Wistar rats (Japan Laboratory Animals) were minced and dissociated with 0.1% collagenase (Worthington Biochemical Corp). After dispersed cells were incubated on 100-mm culture dishes (Falcon) for 60 minutes at 37°C, nonattached cardiocytes were collected and seeded into 60-mm dishes (2 x 10^6 cells per dish) for total RNA extraction or 12-well plates (4 x 10^5 cells per well) for measurement of NO_2⁻/NO_3⁻ (NOx) production. Cardiocytes were incubated in Dulbecco's modified Eagle's medium (DMEM; Flow Laboratories) supplemented with 5% fetal calf serum (FCS) for 24 hours, then treated with Ara-C (10^-5 mol/L) for 48 hours to inhibit proliferation of nonmyocytes, and finally incubated in serum-free DMEM for 24 hours. At the end of Ara-C treatment, the percentage of nonmyocyte contamination in neonatal rat cardiocyte primary cultures was approximately 10% of total cell population. Cardiac myocytes could be distinguished from nonmyocytes by immunocytochemical localization of myosin heavy chain as reported.18 Myocytes were morphologically distinguished by a coarse, granular cytoplasm with small, round, and dense nuclei, whereas nonmyocytes were readily distinguished by morphological features, such as polyhedral cells with phase-lucent cytoplasm and larger, less dense, and more oval nuclei than those of myocytes.19 In some experiments, primary cultures of both cardiomyocytes and nonmyocytes were prepared to compare the differences of NOX production and the expression of iNOS mRNA and protein between two cultures.

Ventricular myocytes of adult male Wistar rats (250 to 300 g) were prepared by 0.1% collagenase as described by Claycomb and Palazzo.20 Isolated rod-shaped myocytes in DMEM with 10% FCS were plated in 60-mm dishes (3 x 10^5 cells per dish). Cells were then treated with or without IL-1B (10 ng/mL) for 12 and 24 hours for RNA extraction. Cell viability after experiments was ~60%.

Determination of NOX
NOX concentrations in culture media were determined in essentially the same manner as recently reported.21 In brief, the samples were applied to a copperized cadmium reduction column to reduce NOX⁻ to NOX⁺, which reacts with Griess reagent in an autoanalyzer (TCI-NOXI1000, Tokyo Kasei Kogyo); the absorbance at 540 nm was measured. NOX⁺ was used as a reference standard.

Preparation of Probes by Polymerase Chain Reaction
The subcloned cDNA for rat brain cNOS, whose nucleotide sequence coincides with that of rat brain cNOS, was prepared as recently reported.22 Polymerase chain reaction (PCR) primers for mouse macrophage iNOS were synthesized according to the oligonucleotide sequences GATCAGGAACTTGAAAGCCCC and CCTATTGGCCAAAAGGGGC corre-

sponding to those of the amino acid residues (942-948) and (1127-1134), respectively.11 The PCR products were amplified from LPS-induced mouse liver RNA by 40 cycles of denaturing at 94°C for 1 minute, annealing at 60°C for 1.5 minutes, and extension at 72°C for 2 minutes. No amplified products were observed in the reverse transcriptase negative control. The PCR product of expected size (577 bp) was ligated into the pGEM plasmid vector (Promega) by the T/A cloning method. The nucleotide sequence of the subcloned iNOS cDNA was determined by the dideoxy chain termination method and coincided with that of mouse macrophage iNOS as recently reported.22 BamHI-EcoRI restriction fragments of the subcloned cDNAs for rat brain cNOS and mouse liver iNOS were used as probes for Northern blot analysis.

Northern Blot Analysis
Northern blot analysis was performed essentially as described.22 Total RNAs from cultured neonatal rat cardiac myocytes and nonmyocytes were prepared by the acid guanidinium thiocyanate–phenol–chloroform method.24 Total RNA (20 µg) was separated by formaldehyde/1% agarose gel electrophoresis and transferred to a Magnagraph nylon membrane (Micron Separations Inc). The subcloned cDNAs for mouse liver iNOS, rat brain iNOS, and mouse cDNAs were labeled with deoxyctydine 5'-[32P]triphosphate by the random-prime labeling method. After ultraviolet wave cross-linking, RNA immobilized on the membrane was hybridized with the cDNA probes for mouse liver iNOS, rat brain iNOS, and human GAPDH, respectively, at 42°C in the presence of 50% formamide. The membrane was washed five times with 0.1 x SSPE (15 mmol/L NaCl, 1 mmol/L NaHPO_4, and 0.1 mmol/L EDTA)/0.5% SDS at 37°C for 15 minutes and autoradiographed with an intensifying screen for 24 hours at ~80°C.

Preparation of Antibody for Mouse iNOS
N-terminal fragment (1-14) of mouse iNOS (Met-Ala-Cys-Pro-Trp-lys-Phe-Leu-Phe-Lys-Val-Lys-Ser-Tyr)11 was synthesized by the solid-phase method and purified by high-performance liquid chromatography (Dr. K. Nakajima and S. Sakakibara, Peptide Institute Inc, Osaka, Japan). The synthetic N-terminal fragment (10 mg) was conjugated to bovine thyroglobulin (50 mg) by carbodiimide (Peptide Institute Inc) as a coupling agent. The conjugate (100 µg) emulsified with complete Freund's adjuvant (Difco Laboratories) was injected intradermally biweekly at multiple sites on the back of Japanese White rabbits. After repeated immunization, the antisera suitable for immunocytochemistry and Western blotting were obtained. The antibody used showed full cross-reactivity with N-terminal fragment (1-14) and a 130-kD iNOS-like protein of liver extracts from LPS-treated mice by gel filtration chromatography.

Western Blot Analysis
After treatment with or without IL-1B (10 ng/mL) for 48 hours, cardiocytes were washed with phosphate-buffered saline (pH 7.4), scraped, and centrifuged. Cell pellets were resuspended in 0.1 mL lysis buffer (100 mmol/L phosphate, 25 mmol/L NaCl, 5 mg/mL pepstatin-A, 1 mg/mL chymostatin, 5 mg/mL aprotinin, and 0.1 mmol/L PMSF). After sonication, cell lysate was subjected to SDS–polyacrylamide gel electrophoresis (7.5% acrylamide, 1.5-mm-thick slab gels). Proteins were then transferred to nitrocellulose membrane (Hybond-C super, 0.45-mm pore size; Amersham) by “semi-dry” electroblotting. The membrane was blocked for 1 hour at room temperature with 5% skim milk in TBS (25 mmol/L Tris, 150 mmol/L NaCl, and 0.02% NaN_3) containing 0.2% Tween-20 and incubated with rabbit polyclonal iNOS antibody (1:1000 dilution) for 2 hours. After washing, the membrane was incubated for 1 hour with donkey anti-rabbit IgG conjugated to horseradish peroxidase; peroxidase activity was visualized.
with an enhanced chemiluminescence Western blotting detection system (Amersham).

**Immunocytochemical Study**

Cardiac myocytes and nonmyocytes with or without IL-1β (10 ng/mL) for 48 hours were cultured in a Labtek chamber (Nunc Inc), after which cells were fixed for 15 minutes in ice-cold 70% acetone. The cells were immunostained with an avidin-biotin-peroxidase (ABC) kit (Vector Laboratories). In brief, the cells were sequentially exposed to normal goat serum (1:60 dilution) for 20 minutes, rabbit iNOS antiserum (1:400 to 1:800 dilution) or nonimmunized rabbit serum at the same dilution for 24 hours, and biotinylated goat anti-rabbit IgG (1:400 dilution) for 30 minutes. The cells were then incubated for 60 minutes in ABC solution prepared by adding avidin DH and biotinylated peroxidase to dilution buffer. Peroxidase activity was visualized by incubation with 3,3′-diaminobenzidine tetrahydrochloride and 0.03% H2O2 in 0.1 mol/L Tris-HCl (pH 7.6).

**Measurements of Cell Number and Viability and Lactate Dehydrogenase Activity**

Cell number was determined by a Coulter counter (Coulter Electronics, Inc), and cell viability was assayed by trypan blue dye exclusion. Lactate dehydrogenase (LDH) activity in culture media was measured by an autoanalyzer.

**Statistics**

Results are expressed as mean±SEM. Statistical analysis was performed by the Wilcoxon rank-sum test. A value of P<.05 was considered statistically significant.

**Results**

As shown in Fig 1A, IL-1β (10 ng/mL) stimulated NO production by rat neonatal cardiocytes in a time-dependent manner (6 to 48 hours); about 2.5-fold (6 hours), 4-fold (12 to 24 hours), and 10-fold (48 hours) increases above control levels were observed. Northern blot analysis using subcloned cDNA for mouse liver iNOS as a probe revealed a single band corresponding to the size of mouse macrophage iNOS mRNA (4.4 kb) after stimulation with IL-1β, whereas no band was detected in the absence of IL-1β (Fig 1B). IL-1β time-dependently (6 to 48 hours) increased steady-state levels of iNOS mRNA. However, Northern blotting using cDNA for rat brain cNOS as a probe did not show any significant signals in either the absence or presence of IL-1β during the incubation periods (Fig 1B).

Northern blot analysis of total RNA from cultured adult cardiomyocytes with mouse iNOS cDNA as a probe also revealed a single hybridization band with a size (4.4 kb) similar to that of neonatal cardiomyocytes after stimulation with IL-1β in a time-dependent manner (12 to 24 hours); no band was detected in the absence of IL-1β (Fig 2).

IL-1β dose-dependently (0.1 to 10 ng/mL) increased NO production, reaching a maximum (about 10-fold increase over control) by 10 ng/mL (Fig 3A); the approximate dose for half-maximal stimulation was 0.7 ng/mL. Likewise, IL-1β dose-dependently induced iNOS mRNA expression, causing a maximal signal by 10 ng/mL but decreasing the signal by 100 ng/mL (Fig 3B).

Western blot analysis using a specific antibody against the N-terminal fragment of mouse iNOS showed that a 130-kD band corresponding to the size of mouse iNOS protein appeared in lysates of cardiocytes treated with IL-1β, whereas such a distinct band was not detectable in lysates of nontreated cardiocytes (Fig 4).

To characterize cellular localization of iNOS-like protein by cardiocytes, we performed immunocytochemical study by the ABC method using polyclonal rabbit antibody for mouse iNOS. Abundant iNOS-like
immunoreactivities were exclusively localized in cytoplasm of cardiac myocytes but also of nonmyocytes to a lesser extent only after treatment with IL-1β (10 ng/ml) (Fig 5); no iNOS-like immunoreactivity was noted in either cardiac myocytes or nonmyocytes in the absence of IL-1β. Neither nonimmunized rabbit serum nor rabbit antibody preabsorbed with excess (10 μg/mL) N-terminal fragment (1-14) of mouse iNOS showed any immunostaining (data not shown).

To determine whether cardiac myocytes and/or nonmyocytes express iNOS in response to IL-1β, we studied NOx production and iNOS mRNA expression in both cells. IL-1β (10 ng/mL) caused about a sevenfold increase in NOx production above control level in cardiac myocytes and a sixfold increase above control level in nonmyocytes as well (Fig 6A). Northern blot analysis also revealed that IL-1β induced iNOS mRNA in both cardiac myocytes and nonmyocytes, although the expression of IL-1β-induced iNOS mRNA in cardiac myocytes was about threefold greater than that in nonmyocytes (Fig 6B).

L-NMMA (10⁻⁵ mol/L) completely blocked the IL-1β-induced NOx production in cardiocytes, whose effect was reversed by a 10-fold excess of l-arginine but not of d-arginine (Fig 7A). However, the IL-1β-induced iNOS mRNA signals were slightly reduced by L-NMMA with or without l- and d-arginine (Fig 7B).

Both a protein synthesis inhibitor, cycloheximide (1 μg/mL), and an RNA synthesis inhibitor, actinomycin D (1 μg/mL), completely blocked the IL-1β-induced NOx production in cardiocytes, whereas both compounds failed to affect basal NOx production (Fig 8A). The IL-1β-induced iNOS mRNA expression was abolished either by cycloheximide or actinomycin D. Cycloheximide had no effect on GAPDH mRNA expression under basal as well as IL-1β-treated conditions; however, actinomycin D reduced GAPDH mRNA expression under either condition (Fig 8B). Cycloheximide did not affect cell number (treated cells, 7.8±0.4×10⁵ versus nontreated cells, 7.6±0.3×10⁵), cell viability (>95% in either cell), or LDH activity in cultured media (treated cells, 71.8±4.2 mU/mL versus nontreated cells, 69.8±3.8 U/mL).

Dexamethasone (10⁻⁶ mol/L) significantly (P<.001) inhibited the IL-1β–induced NOx production, whereas a calmodulin inhibitor, W-7(10⁻⁶ mol/L), had no effect (Fig 9A). Likewise, dexamethasone but not W-7 blocked the IL-1β–induced iNOS mRNA expression (Fig 9B). Neither a protein kinase C (PKC) inhibitor, calphostin C (10⁻⁷ mol/L), nor a voltage-dependent Ca⁺⁺ channel antagonist, nicardipine (10⁻⁷ mol/L), exerted any effect on basal or IL-1β–induced NOx production (Table).

**Discussion**

Proinflammatory cytokines are synthesized and released locally from macrophages, leukocytes, and vascular endothelium in response to ischemia–reperfusion myocardium, which may be responsible for a transient depression of myocardial contractility. A sustained depression of myocardial contractility occurs in septic shock associated with the release of several cytokines and during cytokine antitumor therapy. Recently, Finkel et al reported that the direct negative inotropic effect of several cytokines, such as TNF-α, IL-6, and IL-2, is mediated through myocardial NO synthesis. The rapid onset and reversibility of the cytokine-induced negative inotropic effects in their
study argue against iNOS activation, which requires several hours for gene induction. Therefore, the sustained depression of myocardial contractility by cytokines may result from enhanced activity of iNOS in the myocardium.

The present study clearly demonstrated that IL-1β increased NO production by cultured rat cardiocytes in a time- and dose-dependent manner. A stereospecific NOS inhibitor (L-NMMA) inhibited the IL-1β-induced NO, production; its effect was reversed by an excess substrate of L-arginine but not of D-arginine. These findings indicate that IL-1β directly stimulates NOS, which catalyzes L-arginine as a substrate to generation of NO by cardiocytes. A lag time of at least several hours for NO, production by IL-1β in cardiocytes suggests de novo protein synthesis of iNOS rather than cNOS responsible for NO, production. In fact, Western blot analysis using a specific antibody against synthetic N-terminal fragment (1-14) of mouse iNOS revealed the expression of 130-kD protein corresponding to the molecular weight of mouse iNOS in IL-1β-treated cardiocytes but not in nontreated cells.

Recently, cDNA cloning for cNOS from rat cerebellum revealed recognition sites for NADPH, FAD, FMN, and calmodulin. More recently, cDNA cloning for iNOS from an activated mouse macrophage cell line (RAW 264.7) has also revealed that mouse iNOS is 51% identical to that of rat brain cNOS and shares binding sites for NADPH, flavin cofactors, and calmodulin. Two recent studies showed that IL-1β stimulates NO production by inducing iNOS in cultured rat cardiocytes. Roberts et al demonstrated that IL-1β induced 130-kD iNOS-like protein in cultured neonatal rat cardiomyocytes by Western blot analysis using mouse iNOS antibody. Schulz et al showed that combination of IL-1β and TNF-α induced Ca2+-independent but not Ca2+-dependent NOS activities in adult rat cardiomyocytes as measured by enzymatic assay. However, regulation of iNOS gene expression by cardiocytes was not examined in either study. The present Northern blot study revealed for the first time the expression of macrophage-type iNOS mRNA but not brain-type cNOS mRNA after stimulation with IL-1β in neonatal as well as adult rat cardiocytes. Time and dose dependence of IL-1β on the expression of iNOS mRNA in neonatal rat cardiomyocytes paralleled that of NO, production by the cells. Although rat cardiac iNOS has not yet been cloned, it has recently been reported that iNOS cloned from rat hepatocytes induced by LPS and cytokine treatment is 93% identical to that of mouse macrophage iNOS. Thus, rat cardiac iNOS is very homologous, if not identical, to mouse liver iNOS. Our results indicate that IL-1β induces macrophage-type iNOS but not brain-type cNOS to generate NO in rat cardiocytes. A slow-onset and long-lasting iNOS mRNA expression and NO, production in response to IL-1β in this study may account for the prolonged depression of myocardial contractility in septic shock associated with cytokine
studied whether we for treatment and our primary cytos. Given cardiac Northern blot analysis, cytos represent mainly of three dishes; bar indicates SEM. A, Northern blots of iNOS mRNA and GAPDH mRNA in the same manner as Fig 1B.

overproduction and during antitumor therapy with cytokines.

Our primary cultures of rat neonatal cardiocytes consist mainly of cardiac myocytes, although nonmyocytes represent ~10% of the cell population. Therefore, we studied whether the IL-1β-induced NOx production and iNOS mRNA expression are attributable to cardiac myocytes and/or nonmyocytes by measurement of NOx, Northern blot analysis, and immunocytochemical study. Our results show that IL-1β stimulates NOx production and iNOS mRNA expression in both cardiac myocytes and nonmyocytes. However, the stimulatory effects by IL-1β on NOx production and iNOS mRNA expression in cardiac myocytes are greater than those in nonmyocytes. Given only 1/10 contamination by nonmyocytes in our primary culture, this would strengthen the contention that cardiac myocytes are the major source of NOx and iNOS mRNA. Immunocytochemical study using specific antibody for the N-terminal fragment of mouse iNOS also shows that iNOS-like immunoreactivity is exclusively localized in cardiac myocytes, and to a lesser extent in nonmyocytes as well, only after IL-1β treatment. Our data are consistent with those of a recent study showing that IL-1β stimulates NOx production and iNOS-like protein as determined by Western immunoblotting in cultured neonatal rat cardiac myocytes prepared by Percoll gradients.29 Furthermore, cultured adult rat ventricular myocytes with rod-shaped and contractile properties have been shown to possess cytokine-inducible Ca2+-independent NOS activities as well as iNOS mRNA expression as demonstrated in this study. These data lend substantial support to the assertion that cardiomyocytes express iNOS mRNA and protein predominantly in response to IL-1β. Cardiac nonmyocytes consist of fibroblasts, vascular smooth muscle cells, and endothelial cells, all of which have been shown to have cytokine-inducible NOS activity.5,6,8

Taken together, it is suggested that, although both cardiac myocytes and nonmyocytes have an IL-1β-inducible NOS gene, NOx production and the expression of iNOS mRNA and protein by our rat cardiac primary cultures are attributable largely to cardiac myocytes.

The present study has further shown that an RNA synthesis inhibitor (actinomycin D) completely blocked the IL-1β-induced iNOS mRNA expression as well as
Fig 8. Effects of cycloheximide (CHX) and actinomycin D (AMD) on interleukin-1β (IL-1β)-induced NOx-/NO3- (NOx) production and inducible nitric oxide synthase (iNOS) mRNA and GAPDH mRNA expression by rat neonatal cardiocytes. A, Bar graph showing measurement of cells incubated with (hatched columns) or without (open columns) IL-1β (10 ng/mL) in the absence and presence of CHX (1 μg/mL) or AMD (1 μg/mL) for 48 hours (n=3). Data are plotted in the same manner as in Fig 6A. B, Northern blots of iNOS and GAPDH mRNA in the same manner as in Fig 1B. Lane 1, control; 2, IL-1β; 3, CHX; 4, IL-1β plus CHX; 5, AMD; and 6, IL-1β plus AMD.

NOx production by rat cardiocytes, suggesting the transcriptional regulation of iNOS gene and subsequent de novo synthesis of iNOS. However, the present study has revealed that a protein synthesis inhibitor (cycloheximide) also blocked the IL-1β-induced iNOS mRNA expression and NOx production. Thus, our data suggest that an intermediate labile protein(s) stimulated by IL-1β induces iNOS mRNA expression as a transcriptional factor. Our data are consistent with those of a recent report showing that cytokine-induced iNOS mRNA in rat hepatocytes is inhibited by actinomycin D and cycloheximide.31 It seems unlikely that the inhibition by cycloheximide is due to its nonspecific cytotoxicity because cell number and viability, LDH activity in culture media, and GAPDH mRNA expression were unchanged after cycloheximide treatment.

 Unexpectedly, the iNOS mRNA signal appears to decline at the highest concentration (100 ng/mL) of IL-1β compared with 10 ng/mL, although no apparent cytotoxic effect by IL-1β was observed. IL-1β in higher doses may induce other cytokines (IL-4, IL-10) that inhibit iNOS mRNA expression.32,33 The IL-1β-induced iNOS mRNA was apparently reduced by L-NMMA with or without excess L- and D-arginine.

L-NMMA may somehow decrease stability of IL-1β and/or IL-1β-induced intermediate protein(s), if any. However, the precise mechanisms responsible for these effects by L-NMMA and higher doses of IL-1β need to be determined.

Effects of Nicardipine and Calphostin C on Interleukin-1β-Induced NOx-/NO3- Production by Cultured Rat Neonatal Cardiocytes

<table>
<thead>
<tr>
<th>Drugs</th>
<th>NOx Produced, nmol per 48 h per 10⁶ cells</th>
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<tbody>
<tr>
<td>None</td>
<td>16.3±0.6</td>
</tr>
<tr>
<td>IL-1β, 10 ng/mL</td>
<td>158.3±2.3*</td>
</tr>
<tr>
<td>Nicardipine, 10⁻⁷ mol/L</td>
<td>15.8±0.5</td>
</tr>
<tr>
<td>IL-1β+nicardipine</td>
<td>161.5±2.9*</td>
</tr>
<tr>
<td>Calphostin C, 10⁻⁷ mol/L</td>
<td>15.9±0.7</td>
</tr>
<tr>
<td>IL-1β+calphostin C</td>
<td>155.1±3.0*</td>
</tr>
</tbody>
</table>

NOx indicates NOx-/NO3-. IL, interleukin. Cardiocytes were incubated with or without IL-1β (10 ng/mL) in the presence and absence of nicardipine (10⁻⁷ mol/L) and calphostin C (10⁻⁷ mol/L) for 48 hours; NOx production was measured. Each value is the mean (n=3)±SEM.

*P<.05 vs control.
In the present study, neither PKC inhibitor (calphostin C), calmodulin inhibitor (W-7), nor Ca\(^{2+}\) channel antagonist (nicardipine) affected the IL-1\(\beta\)-induced NO\(_3\) production. These results are in good agreement with the previous observations that enzymatic activity of iNOS does not require Ca\(^{2+}\)/calmodulin or PKC.\(^{34}\) Despite the presence of a presumptive calmodulin binding site in mouse macrophage iNOS molecule,\(^{11,12}\) the reason why iNOS is apparently independent of Ca\(^{2+}\)/calmodulin remains unknown. Recent reports have shown that Ca\(^{2+}\)/calmodulin-independent iNOS from rat liver and mouse macrophages is a calmodulin-bound form of calmodulin-dependent iNOS.\(^{35}\) Thus, Ca\(^{2+}\)/calmodulin-independent NO\(_3\) activities versus Ca\(^{2+}\)/calmodulin-dependent NO\(_3\) activities as measured by enzymatic assay could not accurately distinguish iNOS from cNOS. Therefore, molecular interaction of Ca\(^{2+}\)/calmodulin with its binding site in iNOS needs to be clarified.

Glucocorticoids have been shown to have a protective effect against endotoxin shock.\(^{36}\) However, it remains unknown whether such beneficial effects of glucocorticoids are mediated via direct inhibition on enzyme activity and/or synthesis of iNOS. It has been reported that glucocorticoids inhibit enzyme activity of iNOS but not of cNOS in porcine endothelial cells.\(^{37}\) We recently demonstrated that dexamethasone blocked the IL-1\(\beta\)-induced NO\(_3\) production by direct inhibition of iNOS gene expression in rat vascular smooth muscle cells.\(^{38}\) In neonatal rat cardiocytes, dexamethasone similarly inhibited IL-1\(\beta\)-induced NO\(_3\) production by blocking iNOS mRNA expression. Taken together, it is strongly suggested that glucocorticoids directly inhibit iNOS mRNA expression in the cardiovascular system. However, it is unclear whether glucocorticoids inhibit iNOS gene either at the transcriptional or at the posttranscriptional level.

It remains unknown how enhanced NO generation by cytokines leads to sustained reduced myocardial contractility associated with endotoxin shock and cytokine therapy. It is well known that NO activates soluble guanylate cyclase to generate cGMP.\(^{39}\) cGMP has been shown to decrease myocardial contractility by decreasing cytoplasmic Ca\(^{2+}\) concentration.\(^{40,41}\) Thus, it is possible that cGMP is the second messenger for the negative inotropic action by cytokines. Alternatively, NO may inhibit Krebs’ cycle enzyme cis-aconitase activity,\(^{42,43}\) thereby decreasing mitochondrial respiration in the myocardium. It should be noted that Ca\(^{2+}\)-independent NO\(_3\) activity but not Ca\(^{2+}\)-dependent NO\(_3\) activity is increased in the myocardium from patients with dilated cardiomyopathy.\(^{44}\) Taking into account the present observations that cytokine can induce iNOS expression in myocardial tissues from neonatal and adult rats, the possible involvement of local production of NO by cytokine-inducible iNOS in the pathogenesis of idiopathic cardiomyopathy and acute myocarditis warrants further investigation.

In conclusion, IL-1\(\beta\) increases NO production in rat cardiocytes by induction of iNOS gene expression, possibly via a cycloheximide-sensitive intermediate protein(s). Thus, the sustained depression of myocardial contractility induced by cytokines may be partly mediated by local NO production generated by enhanced iNOS activity in cardiac myocytes and nonmyocytes in the same fashion as in macrophages and endothelial cells.

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