Cytokine-inducible Nitric Oxide Synthase (iNOS) Expression in Cardiac Myocytes

CHARACTERIZATION AND REGULATION OF iNOS EXPRESSION AND DETECTION OF iNOS ACTIVITY IN SINGLE CARDIAC MYOCYTES IN VITRO

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Cellular constituents of heart muscle contain both constitutive and inducible nitric oxide (NO) signaling pathways that modulate the contractile properties of cardiac myocytes. The identities of the inducible NO synthase (iNOS) isoform(s) expressed in cardiac muscle, and of the specific cell types expressing iNOS activity, remain poorly characterized. We amplified a 217-base pair cDNA by reverse transcriptase-polymerase chain reaction from primary cultures of inflammatory cytokine-pretreated adult rat ventricular myocytes (ARVM) that was nearly identical to other iNOS cDNA sequences. Using this 217-base pair cDNA as a probe in Northern blots, we found no evidence of iNOS mRNA in control myocytes, but both interleukin-1β and interferon-γ individually increased iNOS mRNA abundance in primary cultures of ARVM, with maximal expression at 12 h. The half-life of iNOS mRNA in actinomycin C1-treated cells was 4 h. Both dexamethasone and transforming growth factor-β attenuated the induction of iNOS mRNA abundance and enzyme activity by IL-1β and INFγ. Pretreatment with dexamethasone also abolished the induction of iNOS mRNA, but not the increase in GTP cyclohydrolase mRNA in purified cardiac myocytes from lipopolysaccharide-injected rats. In order to further characterize the specific cell type producing NO, we used a NO-specific porphyrinic/Nafion-coated microsensor to record NO release from a single, isolated ARVM pretreated with IL-1β and INFγ. NO release could be detected following microinjection of L-arginine in the vicinity of the cell juxtaposed to the NO microsensor, but not following microinjection of D-arginine, and not from ARVM pretreated with L-N-nitroarginine. Cytokine-pretreated ARVM that had been maintained in L-arginine-depleted medium also exhibited a depressed contractile response to proterenol after addition of L-arginine, but not D-arginine. These results indicate that altered contractile function of cardiac myocytes following exposure to specific inflammatory cytokines is due to induction of myocyte iNOS.

Recent evidence indicates that nitric oxide (NO) signaling pathways play a direct role in regulating the contractile properties of cardiac muscle in vitro and in vivo. We have previously established that both neonatal and adult cardiac myocytes contain a constitutive NO synthase (cNOS) activity that mediates the responsiveness of these two myocyte phenotypes to muscarinic and β-adrenergic agonists, respectively (1). Han et al. (2) also have shown that NO plays an essential role in mediating the effects of muscarinic agonists on the rate of depolarization of sinoatrial nodal cells. In addition to the modulation by NO of cardiac myocyte function under normal physiologic conditions, there is also accumulating evidence that specific soluble inflammatory mediators may cause a delayed but sustained increase in inducible NO synthase (iNOS) activity within cellular constituents of cardiac muscle (3–5). In vitro, addition of IL-1β for at least 24 h to neonatal rat myocytes has been shown to decrease the spontaneous beating rate of these cells, a change that can be reversed by addition of the L-arginine analogue L-NMMA, a NO synthase antagonist. This effect of IL-1β was associated with the expression of iNOS protein in these primary isolates, and was antagonized by co-treatment with TGFB (6, 7). Primary isolates of adult rat ventricular myocytes (ARVM) exposed to medium conditioned by LPS-activated rat macrophages become less responsive to the positive inotropic effect of β-adrenergic agonists, coincident with a sustained rise in nitrite release into the myocyte-conditioned medium, that can be completely reversed by L-NMMA (3).

While these and other reports in the literature provide compelling evidence for the existence of cNOS and iNOS activities within cellular components of cardiac muscle, the relative contributions of NO synthesized within cardiac myocytes themselves and NO released from neighboring cells to produce the functional effect mentioned above remain unclear. Also, the
molecular identity of the isofos of NOS responsible for the sustained rise in NO production following exposure to cytokines in adult ventricular myocytes is poorly characterized: the previous report of a NO-mediated negative inotropic effect of cytokines on adult guinea pig hearts within minutes (8) was in apparent contradiction with the classical time dependence (in hours) for iNOS expression in neonatal preparations (6), but instead pointed to the possible activation of a constitutively kines in adult ventricular myocytes is poorly characterized: the hours) for iNOS expression in neonatal preparations

mM L-N-monomethyl arginine (L-NMMA), an inhibitor of NO synthase, normalized per mg of protein, of ARVM cultured in the absence of added

cytokines in vascular smooth muscle and aortic endothelial cells (10, 11). However, the relevance of the co-induction of GTP cyclohydrolase I and NOS seen in vitro to pathophysiological situations in vivo has been less clearly established (12).

In this report, we describe the isolation and sequencing of a partial rat cDNA from cytokine-treated, primary NRVM isolates that is identical to the other iNOS isoforms cloned from other cell types, as well as the regulation of iNOS mRNA and protein content and enzyme activity by specific cytokines and glucocorticoids. To determine whether cardiac myocytes were a source of NO in these primary cultures, we employed an NO-selective microsensor (13, 14) and experimental conditions that permitted quantitative detection of NO release from individual cytokine-treated adult ventricular myocytes in vitro. Finally, using a model of LPS injection in rats, we verified that the transcripts for iNOS and GTP cyclohydrolase are both induced in adult cardiac myocytes in vivo, but are differentially regulated by glucocorticoids.

**EXPERIMENTAL PROCEDURES**

Isolation and Preparation of ARVM—Calcium-tolerant ventricular myocytes were isolated from adult male Sprague-Dawley rats (225-275 g) as described previously (15), with modifications to limit the number of non-myocyte cells contaminating primary isolates. These included two density gradient sedimentation steps through a 6% bovine serum albumin (Sigma) cushion followed by centrifugation of the resulting myocyte fraction through a Percoll suspension (Pharmacia Biotech Inc.) and differential attachment to laminin-coated tissue culture dishes, as described previously (16). This method typically limits the amount of non-myocyte cell contamination to 2-5%. All myocytes were cultured in a defined medium modified from that originally described by Voiz et al. (17) consisting of Dulbecco's modified Eagle's medium with phosphate-buffered saline, including 25 mM HEPES and NaHCO3 with L-glutamine (Life Technologies, Inc.), supplemented with 2 mg/ml bovine serum albumin, 2 mM L-carnitine, 5 mM creatine, and 5 mM taurine, with 100 IU/ml penicillin, and 100 g/ml streptomycin (Life Technologies, Inc.). This medium, which we termed ACCT (15), was used for all primary cultures except for the NO microsensor and contractility experiments, for which ACCT was supplemented with 0.1 mM insulin and 10-7 M T3 (referred to as ACCTT).

Since previous reports have shown that LPS contamination of culture media and reagents can profoundly influence the expression of iNOS in neonatal cardiac myocyte primary isolates (6), we measured the LPS levels in all the solutions used for the isolation and culture of our adult cardiac myocytes preparations using a Chromogenic Limulus Amebocyte Lysate assay kit (Whitaker Biochemicals, Walkersville, MD): all the physiologic buffers (70 mM), Percoll gradients (stock solution, up to 1 ng/ml) and enzyme mixtures (stock solution, up to 2 ng/ml) contained significant amounts of LPS, from which, after repetitious 40,000 g supranatant of 24-h cultured myocytes in ACCTT (80 pg to 0.4 ng/ml); we then measured the cyclic GMP content (using a RIA kit, Biomedical Technologies Inc, Stoughton, MA), normalized per mg of protein, of ARVM cultured in the absence of added cytokines for 24 h in ACCTT containing either 0.6 mM l-arginine or 1 mM L-normonethyl arginine (L-NMA). L-NMMA, an inhibitor of NO synthase, and found no significant difference (10.6 ± 0.57 and 10.3 ± 0.41 pmol/mg protein, respectively, for 12 determinations in each group from two different cultures) that would suggest a NOS-dependent increase in cyclic GMP by contaminating LPS in culture.

For experiments in which cardiac myocytes were isolated from animals pretreated with an intraperitoneal injection (4 mg/kg) of a lipopoly saccharide (LPS) component of Salmonella typhimurium (87F402; Sigma Chemical Co.). The animals were allowed to recover with saline, and were sacrificed by intracardial perfusion with 4% paraformaldehyde, were perfused 6-7 h after injection. A subgroup of rats were pretreated with intraperitoneal injection of dexmethasone (1.2 mg/kg) 45 min before the injection of LPS. ARVM were isolated as described above, including perfusion through a Percoll sedimentation gradient, and total RNA was extracted from fresh isolates.

**Regulation of NO Synthase (NOS) Activity—**Activity MYOCT NOS was quantified by measuring the conversion of L-[3H]arginine to L-[3H]citrulline in the presence of saturating concentrations of the enzyme's cofactors. Total cellular homogenates were prepared from approximately 7 x 10^6 ARVM that had been washed three times in warm Hank's balanced salt solution (Life Technologies, Inc.) and suspended by gentle trypsinization in Hank's balanced salt solution containing 0.25% trypsin and 1 mM EDTA. The cells were centrifuged at 100 x g at 4 °C, washed twice in ice-cold phosphate-buffered saline, and resuspended in 200 µl of a lysis buffer with added 0.5 mM EDTA, 0.5 mM EGTA, 1 mM dithiobitol, 1 µM tetrahydrobiopterin, 1 µM leupeptin, 0.2 mM phenyl methylsulfonyl fluoride, and 20 mM Tris-HCl (pH 7.4 at 4 °C). Cells were incubated on ice for 15 min, and then sonicated on ice for 30 s (Branson Sonifier 450; Danbury, CT). The homogenates were centrifuged at 1,500 x g for 15 min at 4 °C and protein content was determined using the Bradford technique (Bio-Rad) with albumin as a standard. Twenty-five µg of supernatant (approximately 50 µg of protein) were added to 125 µl of buffer containing 50 mM HEPES (pH 7.4, 37 °C), 1.25 mM CaCl2, 1 mM EDTA, 0.5 mM NADPH, 10 mM FAD, 5 µm FMN, 10 µM tetrahydrobiopterin, 10 µg/ml calmodulin, 1 mM dithiobitol, and 0.2 mM L-[3H]arginine (3.2 x 10^6 cpm/ml; Amersham) for 1 h at 37 °C. The reaction was stopped by the addition of 2 ml of ice-cold 20 mM HEPES (pH 7.5) and 5 mM EDTA, and the total volume was applied to a Dowex 50WX8 column that had been pre-equilibrated with 20 mM HEPES (pH 5.5). L-[3H]citrulline was eluted with 2 ml of deionized water, and radioactivity was quantified by scintillation counting. The results are expressed as counts (cpm)/mg protein.

Control and Experimental Groups—Myocyte contractility studies were performed exactly as described previously (3). Briefly, ARVM plated on laminin-coated plastic coverslips following pretreatment with control or experimental (i.e. cytokine-containing) media were placed in a superfusion chamber on the heated stage of an inverted phase-contrast microscope. Cell motion was detected by an optical-video system and digitized for later computer analysis. Cells were stimulated at 2 Hz at 37 °C and superfused with a HEPES-buffered physiologic salt solution with or without isoproterenol (final concentration, 2 nm). One cell per coverslip was examined.

**PCR Cloning and Sequencing of iNOS mRNA from ARVM**—In order to identify the iNOS expressed in isolated ARVM, the following PCR and sequencing strategies were used. Total RNA was isolated from both cytokine-pretreated and control ARVM using the method of Chomczynski and Sacchi (18), and stored in diethylylcarboxamide (Sigma)-treated water at -70 °C. Reverse transcription of ARVM RNA was accomplished using standard protocols. Briefly, 10 µg of total RNA were treated with 100 units of RNase inhibitor (Promega), and denatured at 65 °C for 10 min. A reverse transcription mixture containing deoxynucleotides (dNTPs; 1 mM), random hexamers (20 µM), and 1,000 units of reverse transcriptase (Superscript; Life Technologies, Inc.) was then added in buffer containing 50 mM Tris-HCl (pH 8.3), 40 mM KCl, and 2.5 mM MgCl2. For control experiments, primers were prepared in the same way, except that the reverse transcriptase enzyme was omitted. All samples were incubated at 26 °C for 10 min and at 42 °C for 35 min and the reaction was stopped by heating at 100 °C for 5 min.

Amplification of reverse transcriptase (RT) products was accomplished by subjecting 5-µl aliquots to 35 cycles of PCR (94 °C for 1 min; 55 °C for 1 min; 72 °C for 2 min) in the presence of dNTPs (0.125 mM) and 2.5 units of Taq polymerase (Promega) in a standard buffer containing 1.5 mM MgCl2. The sense oligonucleotide 5'-AGAATGGAGATAGGACGT-3' is complementary to base pairs 1541-1558 and lies in the 5'-half of the cDNA sequence of the rat NOS3 isozyme (19). For each experiment, control samples were always run in the absence of cDNA template, and amplified with RNA samples processed in parallel without reverse transcriptase.

The single PCR amplified product of the expected size (217 bp) was...
purified by agarose gel electrophoresis, cloned into pBluescript (Stratagene, La Jolla, CA), and used to transfect DH5α Escherichia coli. Two positive clones were selected for further characterization. Following plasmid purification, two sequences were segmented in both directions by the dideoxy chain termination technique using a Sequenase kit (U. S. Biochemical Corp.).

**Northern Blots**—After electrophoresis of 15 μg of total RNA through a 1.5% formaldehyde-agarose gel, and transfer onto a nylon membrane using a vacuum blotter (Bio-Rad model 785), Northern blots were hybridized with the 32P-radiolabeled 217-bp cDNA obtained by RT-PCR or the full-length cDNA of the rat GTP cyclohydrolase I (kindly provided by Dr. Hatakeyama under standard conditions, optimized), and then washed with 2 x SSC, 0.1% SDS for 30 min at room temperature, followed by 1 x SSC, 0.1% SDS at 37 °C, and 0.2 x SSC, 0.1% SDS at 65 °C. The blots were prepared for autoradiography at -70 °C with intensifier screens for at least 6 h, or as noted.

**Western Blot of Inducible NOS Synthase (iNOS) Protein**—ARVM were lysed directly in each well by application of a buffer containing 0.062 M Tris-HCl (pH 8.8), 10% glycerol, 2% SDS (w/v), 5% (v/v) β-mercaptoethanol, and the mixture boiled for 5 min. Equal amounts (90 μg) of the denatured proteins per lane were loaded and separated on a 12% SDS-polyacrylamide gel (Mini Protean II, Bio-Rad) and transferred to a nitrocellulose membrane that was reversibly stained with Ponceau red to verify equal loading and/or transfer between lanes (Millipore, HATF 20200 membrane). The membrane was blocked with 1% bovine serum albumin in Tris-buffered saline with 0.06% (v/v) Tween 20 (TBST; Sigma). Membranes were incubated with rabbit polyclonal anti-iNOS primary antibody that had undergone affinity purification on a synthetic peptide composed of a unique sequence (residues 1-20 of the N terminus of the murine macrophage iNOS (21)) for 3 h in TBST. After three washes (10 min each), the membranes were incubated for 1 h at room temperature with a 125I-coupled goat anti-rabbit IgG secondary antibody at a 1:2000 dilution in TBST with 1% bovine serum albumin. After three additional washes in TBST, the membranes were rinsed twice in TBS and autoradiographed for 48-72 h.

**Detection of NO by Porphyrinic Microsensor**—Porcine-purified ARVM treated in vitro with cytokines were cultured on 13-mm diameter plastic coverslips (Thermanox; Nunc, Naperville, IL), and then transferred to a temperature-controlled stage of a Zeiss Axiovert epifluorescence microscope (Inovision IC-300), attached to a Zeiss Axiosvert epifluorescence microscope. A mixture containing both parameters were validated in separate experiments in which the change in intracellular Ca2+ activity was observed in ARVM loaded with the Ca2+-sensitive probe fura-2 (Molecular Probes, Eugene, OR) previously described (22). Following exposure by this technique to the β-adrenergic agonist isoproterenol, the few nucleotide differences we observed compared to the human macrophage iNOS (19), 27582 Regulation of iNOS mRNA by Northern Analysis—In order to explore further the abundance and regulation of transcripts corresponding to this PCR product, total RNA from ARVM induced by inflammatory cytokines in vitro was hybridized in Northern blot experiments with the 32P-labeled insert described above. As shown in Fig. 2A, a transcript of approximately 4.6 kb was identified in ARVM pretreated in vitro with TNF-α, IFN-γ, and NO synthase inhibitor, or removal of NAPDH from the enzyme assay buffer, decreased NO synthase activity to control levels. As shown in Fig. 2B, co-illumination of rhTGFβ1 (100 ng/ml) with rhIL-1β (2 ng/ml) and rmIFNγ (500 units/ml) reduced NOS activation by about 50%, as does 3 μM dexamethasone added to primary myocyte cultures 45 min before the cytokines. Reverse Transcriptase PCR Cloning and Sequence of an iNOS cDNA from ARVM—To determine if an iNOS transcript could be amplified in cytokine-pretreated myocytes, PCR was performed on reverse-transcribed total RNA obtained from ARVM that had been isolated using sequential density gradient sedimentations and a Percoll centrifugation procedure, followed by differential attachment to laminin (16). Primary cultures of ARVM isolated in this fashion were used for all the experiments reported in this article.

Using amplifiers derived from the nucleotide sequence of the iNOS cDNA from rat vascular smooth muscle (19), we amplified a 217-bp cDNA fragment from reverse transcribed RNA isolated from ARVM pretreated with cytokines for 24 h. No RT-PCR product could be identified in control myocytes not exposed to cytokines. Importantly, neither ARVM RNA that had not been reverse transcribed, nor samples from which the cDNA template had been deleted, generated any positive PCR products when otherwise handled identically. The 217-bp PCR product was cloned in pBluescript (Stratagene) and the nucleotide sequence of two independent clones was determined in both directions and yielded identical 217-bp sequences. This clone (GenBank accession number L36063) was nearly identical to the published murine macrophage (5 mismatches) and rat vascular smooth muscle iNOS (2 mismatches) sequences (19, 24). The few nucleotide differences we observed were present in the sequence of both strands in two independent clones.

**Detection of iNOS mRNA by Northern Analysis**—In order to explore further the abundance and regulation of transcripts corresponding to this PCR product, total RNA from ARVM induced by inflammatory cytokines in vitro was hybridized in Northern blot experiments with the 32P-labeled insert described above. As shown in Fig. 2A, a transcript of approximately 4.6 kb was identified in ARVM pretreated in vitro with TNF-α, IFN-γ, and NO synthase inhibitor, or removal of NAPDH from the enzyme assay buffer, decreased NO synthase activity to control levels. As shown in Fig. 2B, co-illumination of rhTGFβ1 (100 ng/ml) with rhIL-1β (2 ng/ml) and rmIFNγ (500 units/ml) reduced NOS activation by about 50%, as does 3 μM dexamethasone added to primary myocyte cultures 45 min before the cytokines.

**Reverse Transcriptase PCR Cloning and Sequence of an iNOS cDNA from ARVM**—To determine if an iNOS transcript could be amplified in cytokine-pretreated myocytes, PCR was performed on reverse-transcribed total RNA obtained from ARVM that had been isolated using sequential density gradient sedimentations and a Percoll centrifugation procedure, followed by differential attachment to laminin (16). Primary cultures of ARVM isolated in this fashion were used for all the experiments reported in this article.
The half-life of iNOS mRNA was examined using actinomycin D, which effectively prevented the appearance of iNOS transcripts when given concurrently with recombinant cytokines (data not shown). When actinomycin C1 (10 μg/ml) was added at 18 h following ARVM exposure to cytokines, iNOS transcript levels decreased to the detection limit by Northern analysis at 10 h, and completely disappeared at 24 h. As a control for the possible spontaneous decline of iNOS transcript, RNA was isolated at each time point from ARVM treated in parallel with cytokines under the same conditions, but in the absence of actinomycin; in the absence of actinomycin, the iNOS transcript declined over time with a calculated half-life of 4 h (Fig. 3B).

Regulation of iNOS mRNA Abundance and Protein Content—Both IL-1β and IFNγ alone were sufficient at 18 h to increase iNOS mRNA abundance in primary isolates of ARVM...
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Figure 3. iNOS mRNA stability in cytokine-treated ARVM. A, effect of actinomycin C1 on iNOS mRNA abundance: Northern blot analysis of iNOS mRNA in ARVM maintained in ACCT and exposed to the combination of IL-1α, TNFα, and IFNγ as described in the legend to Fig. 2. iNOS mRNA stability was assessed by addition of 10 μg/ml actinomycin C1 after 18 h of cytokine exposure and then by harvesting cells immediately and after 3, 6, and 10 h of actinomycin C1 exposure. The hybridization signal for iNOS transcript was analyzed as described in the legend to Fig. 2, and compared with that of cytokine-treated ARVM not exposed to actinomycin, harvested at the same time points. The blot was rehybridized with a 32P-labeled cDNA probe for 18 S rRNA. B, calculated iNOS mRNA half-life: the signal density of iNOS RNA was normalized for 18 S rRNA abundance, plotted against time, and the half-life of the iNOS transcript, which was 4 h. In the absence of actinomycin, the iNOS signal intensity remained unchanged over the indicated time course.

In defined medium (i.e. ACCT), as shown in Fig. 4B. Both dexamethasone (3 μM) and rhTGFβ (100 ng/ml) decreased iNOS mRNA abundance when added to ACCT medium containing rhIL-1α and rmIFNγ (Fig. 4A), consistent with the decrease in enzyme activity in myocyte cellular homogenates as measured by the conversion of L-[3H]arginine to L-[3H]citrulline, shown in Fig. 1. Additional experiments (repeated three times) showed that the antagonistic effect of TGFβ on iNOS mRNA abundance was bimodal with almost complete inhibition of the iNOS signal at 1 ng/ml TGFβ and a gradual decrease of the inhibitory effect at 5 and 10 ng/ml (data not shown).

To determine whether the changes in iNOS mRNA abundance with dexamethasone were paralleled by similar relative changes in iNOS protein content by Western analysis, we used an iNOS-specific polyclonal antibody raised against epitopes on the murine macrophage iNOS isoform (21). Primary ARVM isolates were exposed to a combination of IL-1α and IFNγ with and without 3 μM dexamethasone. As shown in Fig. 4C, while no protein can be detected in ARVM exposed only to control medium for 24 h, there is a detectable protein band in IL-1α and IFNγ-treated cells at the size expected for the iNOS isoform. iNOS protein content in dexamethasone- and cytokine-treated cells was markedly decreased compared to myocytes treated with cytokines alone, consistent with the decline in iNOS mRNA abundance in Fig. 4A and enzyme activity in Fig. 1B.

Regulation of iNOS and GTP Cyclohydrolase mRNA Abundance by Dexamethasone in Vivo—iNOS transcripts were detectable in purified ARVM from LPS-injected rats at 6–7 h, but not in ARVM from control, saline-injected rats (Fig. 5). Separate experiments showed that iNOS mRNA abundance continued to increase at 12 h postinjection (not shown). In LPS-injected animals, transcripts for GTP cyclohydrolase I also increased at 6–7 h in purified ARVM. Pretreatment with dexamethasone nearly abolished the increase in iNOS mRNA in ARVM, but had little effect on the transcript for GTP cyclohydrolase (Fig. 5).

NO Release by Single Cytokine-exposed ARVM Detected by a NO-selective Porphyrinic Microsensor—In order to further explore the cell of origin producing NO in the ARVM preparations, we took advantage of the sensitivity and selectivity for NO of a porphyrinic/Nafion-coated microsensor. In preliminary
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Figure 5. Regulation of iNOS and GTP cyclohydrolase (GTP ch) mRNA by dexamethasone in ARVM freshly isolated from hearts of LPS-injected rats. Total RNA from ventricular myocytes freshly isolated from hearts of adult rats injected 6-7 h previously with saline (control, lane 1), 4 mg/kg LPS alone (lane 2), or preceded with 1.2 mg/kg dexamethasone 45 min before (lane 3) was hybridized with the 217-bp iNOS 32P-labeled cloned cDNA fragment described under "Results." After autoradiography for 36 h (iNOS), a band of the expected size, at 4.6 kb, was detected in RNA from myocytes from LPS-injected rats but not from control rats; the iNOS-hybridizable mRNA was much less in ARVM from dexamethasone-treated rats injected with LPS (lane 3). The same blot was later rehybridized with the full-length cDNA of GTP cyclohydrolase 1 and autoradiographed for 3 days. Two transcripts, at 1.1 and 3.5 kb, were seen in ARVM from LPS-injected rats, with the same abundance regardless of dexamethasone treatment (lanes 2 and 3). The same blot was later rehybridized to a 32P-labeled cDNA probe for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) RNA to reflect the amount of RNA loaded per lane (15 μg). This experiment was repeated three times with similar results.

In the experiments described here we have determined that specific recombinant cytokines added to primary cultures of adult ventricular myocytes leads to an increase in iNOS mRNA abundance and NO synthase activity that is decreased by dexamethasone and TGFβ. The induction of iNOS in ARVM by recombinant cytokines had a magnitude and time course that was consistent with the iNOS induction we observed in ARVM incubated in conditioned medium from LPS-activated rat alveolar macrophages (3). Sequence analysis of a partial cDNA showed that the iNOS that is expressed in these cells is highly similar to the iNOS described in other tissues following activation by LPS or cytokines. Cytokine-pretreated cardiac myocytes were clearly identified as a source of NO release in ARVM primary cultures, confirming that these cells contain iNOS activity. We verified that purified adult myocytes expressed iNOS mRNA after injection of LPS in vivo and that this stimulus coincided with an increase in GTP cyclohydrolase mRNA abundance; pretreatment with dexamethasone in vivo decreased iNOS mRNA, while having little effect on GTP cyclohydrolase mRNA.

In contrast with the two constitutive isoforms of NOS (eNOS) which show considerable sequence differences and are encoded by two different genes (26, 27), the cDNAs encoding the inducible isoforms of NOS (iNOS) show a high degree of identity across tissues (19, 24, 25). The partial cDNA reported here is almost identical to previously published sequences and the deduced amino acid sequence, using the same reading frame as for the rat vascular smooth muscle iNOS, is completely identical to the equivalent portion of the originally identified iNOS sequence from murine macrophages (24), as well as other rat iNOS sequences identified in other cells and tissues (19, 28). It is also similar (i.e. 81% identity in amino acid sequence) to human iNOS sequences published to date (25, 29, 30). Using the same amplimers, we identified a similar 217-bp cDNA fragment following RT-PCR amplification of RNA from cytokine-pretreated primary cultures of microvascular endothelial cells isolated from adult rat ventricular muscle (31). However, subsequent Northern analysis showed that iNOS is differentially regulated in ARVM and microvascular endothelial cells (e.g. interferon-γ alone induces iNOS in ARVM (see Fig. 4B) but not in cardiac microvascular endothelial cells) (31). Although we described above, after microinjection of D-arginine (Fig. 6B, b) or L-arginine following a 4-h incubation with the L-arginine analog L-NMMA (Fig. 6B, a), both conditions in which no signal could be recorded.

In order to verify that the experimental conditions in which NO release was detected from single cardiac myocytes with the NO microsensor also would result in decreased ARVM contractile responsiveness to β-adrenergic agonists, as expected from our previous studies (1, 3), both control and cytokine-pretreated myocytes were incubated in L-arginine-depleted medium for 4 h, and then either D-arginine or L-arginine was added in the superfusion buffer and myocyte contractile responses at baseline and in response to isoproterenol were recorded. The contractile responses to isoproterenol were not different between the two control, non-cytokine-treated groups of cells exposed to either L- or D-arginine (2.8 ± 0.45 and 2.1 ± 0.26 x the baseline value, respectively, p > 0.05; Fig. 6C, bars 1 and 3). There was no decreased contractile response to isoproterenol in cytokine-pretreated ARVM superfused with D-arginine (Fig. 6C, bar 2), but cytokine-pretreated ARVM demonstrated a decline in their inotropic response to isoproterenol in L-arginine-containing buffer compared to uninduced cells, consistent with the data shown above indicating induction of iNOS and NO release from these cells.

Discussion

In the experiments described here we have determined that specific recombinant cytokines added to primary cultures of adult ventricular myocytes leads to an increase in iNOS mRNA abundance and NO synthase activity that is decreased by dexamethasone and TGFβ. The induction of iNOS in ARVM by recombinant cytokines had a magnitude and time course that was consistent with the iNOS induction we observed in ARVM incubated in conditioned medium from LPS-activated rat alveolar macrophages (3). Sequence analysis of a partial cDNA showed that the iNOS that is expressed in these cells is highly similar to the iNOS described in other tissues following activation by LPS or cytokines. Cytokine-pretreated cardiac myocytes were clearly identified as a source of NO release in ARVM primary cultures, confirming that these cells contain iNOS activity. We verified that purified adult myocytes expressed iNOS mRNA after injection of LPS in vivo and that this stimulus coincided with an increase in GTP cyclohydrolase mRNA abundance; pretreatment with dexamethasone in vivo decreased iNOS mRNA, while having little effect on GTP cyclohydrolase mRNA.

In contrast with the two constitutive isoforms of NOS (eNOS) which show considerable sequence differences and are encoded by two different genes (26, 27), the cDNAs encoding the inducible isoforms of NOS (iNOS) show a high degree of identity across tissues (19, 24, 25). The partial cDNA reported here is almost identical to previously published sequences and the deduced amino acid sequence, using the same reading frame as for the rat vascular smooth muscle iNOS, is completely identical to the equivalent portion of the originally identified iNOS sequence from murine macrophages (24), as well as other rat iNOS sequences identified in other cells and tissues (19, 28). It is also similar (i.e. 81% identity in amino acid sequence) to human iNOS sequences published to date (25, 29, 30). Using the same amplimers, we identified a similar 217-bp cDNA fragment following RT-PCR amplification of RNA from cytokine-pretreated primary cultures of microvascular endothelial cells isolated from adult rat ventricular muscle (31). However, subsequent Northern analysis showed that iNOS is differentially regulated in ARVM and microvascular endothelial cells (e.g. interferon-γ alone induces iNOS in ARVM (see Fig. 4B) but not in cardiac microvascular endothelial cells) (31). Although we
did not clone the entire cDNA, our data, taken together with previous reports, support the likelihood that iNOS expressed in these different tissues represent the product of a single gene.

The RT-PCR approach we used clearly does not exclude the possibility that we amplified a cDNA product from non-myocyte cells in these primary cultures, despite careful attempts at obtaining homogenous preparations of cardiac myocytes. However, subsequent Northern analysis of total RNA from the same cultures using our PCR product as a probe revealed an abundant distinctive transcript, supporting the view that cardiac myocytes themselves express an isofrom of iNOS close to, or identical to other iNOS. The time course of the increase in iNOS mRNA abundance is consistent with that reported for iNOS induction in hepatocytes (32) and murine macrophages (24, 33) in response to LPS and cytokines. As shown in Fig. 3, iNOS mRNA abundance is sustained over 24 h in the continuous presence of IL-1β, IFNγ, and TNFα, with a calculated half-life for iNOS mRNA of 4 h. This is consistent with the data reported by Vodovotz et al. (34) in murine macrophages 18 h following treatment with IFNγ, although these authors did observe a markedly decreased iNOS mRNA half-life after shorter periods of exposure to IFNγ.

Although we (as others (Ref. 6)) measured detectable levels of LPS in our culture reagents, we never found detectable levels of iNOS mRNA in ARVM cultured in the absence of added cytokines either by Northern analysis or RT-PCR, which argues against a significant iNOS induction by traces of LPS alone in our cultured adult myocytes; this is consistent with the absence of the characteristic iNOS-mediated attenuation of the contractile response to isoproterenol when ARVM are cultured in the presence of added LPS alone (3). Of note, in our serum-free defined medium, the ARVM are never exposed to serum that could provide exogenous lipopolysaccharide-binding protein and/or CD 14 molecules, known to enhance endotoxin bioactivity (35); this factor (or developmental differences between neonatal (6) and adult cardiocytes) may provide an explanation for the absence of any effect at relatively low LPS concentrations (35). We cannot exclude, however, that traces of LPS may have potentiated, or even attenuated the induction of iNOS with added cytokines, as shown with other cell types (21, 36).

By contrast with its effects in vitro, LPS injected in vivo caused an increase in iNOS mRNA in freshly isolated ARVM.
that both iNOS translation and protein half-life appeared to be breakdown, or acting by additional post-translational mechanisms. Heterotypic co-cultures of ARVM and cardiac microvascular endothelial cells acting to reduce iNOS transcription or increase iNOS mRNA in ventricular muscle, in which dexamethasone caused only a range of activity that we found to be endogenously released in (37), including heart (4); importantly, this model of induction differs by the fact that, in addition to LPS, it involves the secondary release of endogenous cytokines, among which TNF-α and IL-1β seem to play an important role for iNOS expression (37). LPS in vivo induced the co-expression of GTP cyclohydrolase I mRNA in freshly isolated ARVM, with the appearance of two transcripts (1.1 and 3.5 kb) consistent with previous descriptions from other cell types in culture (38, 39) (Fig. 5); although we did not specifically measure the functional impact of GTP cyclohydrolase I expression on iNOS activity in vivo, this observation nevertheless lends credence to the view that the co-regulation of both enzymes may be of physiological importance upon immune stimulation and does not only represent the consequence of a culture-induced artifact (9, 12). Werner-Felmayer et al. (40) had also observed variable increases in GTP cyclohydrolase I activity in different organ tissues upon LPS challenge in vivo, although heart tissue was not examined at that time.

Dexamethasone largely prevented the increase in iNOS mRNA abundance and protein content in cytokine-pretreated ARVM (Fig. 4), as well as maximal iNOS activity in these cells in culture. This is consistent with reports of dexamethasone reducing iNOS mRNA abundance in cytokine-pretreated hepatocytes (32). This report and our data on dexamethasone’s effects in ARVM contrast with our observations in cytokine-pretreated microvascular endothelial cells isolated from adult rat ventricular muscle, in which dexamethasone caused only a small (15%) decline in iNOS mRNA abundance, protein content, or maximal iNOS activity in cell lysates (31). Importantly, we verified that dexamethasone produced the same effect on iNOS mRNA from ARVM in vivo; however, dexamethasone had little (if any) effect on GTP cyclohydrolase I mRNA (Fig. 5). Similarly, the effects of dexamethasone were shown to differ on the production of NO and tetrahydrobiopterin in macrophages (41). The mechanism of iNOS mRNA suppression by dexamethasone is not completely understood (9); we have observed a parallel suppression of IRF-1 mRNA, a critical transcription factor for iNOS expression (42), by glucocorticoids in cytokine-pretreated ARVM.3 The elucidation of GTP cyclohydrolase I gene regulation will await full characterization of its promoter.

Roberts et al. (6) reported that TGFβ suppressed the induction of iNOS protein by LPS and IL-1β in primary cultures of neonatal rat cardiac myocytes. The increased and continuous rate of NO release following iNOS induction by IL-1β caused a marked sustained decrease in beating rate at 20 h that could largely be prevented by TGFβ, L-NMMA, or methylene blue. TGFβ also decreased iNOS activity (Fig. 1) and iNOS mRNA abundance (Fig. 5) in IL-1β and IFNγ-pretreated ARVM. The magnitude of the decrease is similar to that which we observed in IL-1β and IFNγ-pretreated cardiac microvascular endothelial cells (31). Concentration-response experiments showed a maximal inhibitory effect at 1 ng/ml TGFβ consistent with a bimodal effect; interestingly, this concentration is also in the range of activity that we found to be endogenously released in heterotypic co-cultures of ARVM and cardiac microvascular endothelial cells (43). We cannot comment on whether TGFβ is acting to reduce iNOS transcription or increase iNOS mRNA breakdown, or acting by additional post-translational mechanisms. Vodovotz et al. (34) noted that TGFβ suppressed murine macrophage iNOS mRNA abundance by reducing iNOS mRNA stability and not by inhibiting transcription. They also noted that both iNOS translation and protein half-life appeared to be reduced by TGFβ.

3 J.-L. Balligand, unpublished observations.

Finally, a NO-selective microsensor was used in order to verify that NO release, detected at the cell membrane, was present in ventricular myocytes in vitro. The NO microsensor technology has been described and validated in both tissue and cell culture systems, with a detection limit of approximately 10 nm (i.e. approximately 10⁻¹⁰ mol of NO in the volume of the average cell). The amperometric mode used in our experiments has been successfully used to measure the kinetics of NO release from endothelial cells in response to eNOS activation by calcium-raising agonists. In contrast with cNOS, the activity of the largely calcium-insensitive iNOS is not rapidly regulated by changes in intracellular calcium, but is usually sustained for several hours after it has been induced. In order to take advantage of the sensitivity and time resolution of the electrode, we deprived the cells of L-arginine, the substrate for NOS, in order to reduce their production of NO and then abruptly re-introduced the amino acid to observe an outburst of NO release with an acceptable signal-to-noise ratio. Microinjection parameters and micropipette placement were designed to minimize the likelihood of significant NO release from neighboring cells. The detection of NO release following the injection of L-arginine into the medium immediately adjacent to a cytokine-pretreated myocyte, as shown in Fig. 6, demonstrates that these cells contain iNOS activity. That the amperometric signal recorded by the microsensor was specific for NO released by myocytes following L-arginine is supported by the failure of the sensor to detect NO release following either β-arginine or L-NMMA. Under identical conditions, myocytes pretreated with cytokines for 24 h in defined medium, and then deprived of L-arginine for 4 h, exhibited the typical decline in inotropic responsiveness to isoproterenol following readuction of L-arginine, but not β-arginine, that we had originally observed in ARVM exposed to LPS-activated macrophage-conditioned medium (3).

While the role of the constitutive NO signaling pathway in cardiac myocytes is becoming clarified, it remains unclear what role the much higher levels of NO released from cytokine-exposed cardiac myocytes and other cellular constituents of cardiac muscle play in the normal physiologic response to injury or stress or in the pathogenesis of disease. In addition to its cytotoxic and antiviral effects, NO has been shown to either directly interact with the cGMP cyclic nucleotide complex directly or to facilitate post-translational modification of a number of cellular and extracellular proteins that could affect myocyte phenotype or function (23, 45–47). These effects may be relevant to the pathophysiology of certain cardiac diseases.

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