Induction of NO synthase in rat cardiac microvascular endothelial cells by IL-1β and IFN-γ

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Cardiovascular Division, Department of Medicine, Brigham and Women's Hospital and Harvard Medical School; Respiratory Physiology Program, Harvard School of Public Health; and Department of Pathology, Brigham and Women's Hospital, Boston, Massachusetts 02115; and Department of Medicine, Johns Hopkins University, Baltimore, Maryland 21205

Balligand, Jean-Luc, Dan Ungureanu-Longrois, William W. Simmons, Lester Kobzik, Charles J. Lowenstein, Santiago Lamas, Ralph A. Kelly, Thomas W. Smith, and Thomas Michel. Induction of NO synthase in rat cardiac microvascular endothelial cells by IL-1β and IFN-γ. *Am. J. Physiol.* 268 (*Heart Circ. Physiol.* 37): H1293–H1303, 1995.—There are important phenotypic differences between endothelial cells of large vessels and the microvasculature and among microvascular endothelial cells isolated from different tissues and organs. In contrast to most macrovascular endothelial cells, we demonstrate that cultured cardiac microvascular endothelial cells (CMEC) have no detectable constitutive NO synthase (NOS) activity but have a robust increase in NOS activity in response to specific inflammatory cytokines. To determine the identity of the inducible NOS (iNOS) isoform(s) induced by cytokines, we used reverse-transcription polymerase chain reaction techniques to clone and sequence a 217-bp cDNA fragment from CMEC cultures pretreated with interleukin-1β (IL-1β) and interferon-γ (IFN-γ) that was identical to the corresponding portion of the murine macrophage iNOS cDNA. By use of this CMEC iNOS cDNA as a probe in Northern analyses, IL-1β, but not IFN-γ, increased iNOS mRNA content in CMEC, although IFN-γ markedly potentiated iNOS induction in these cells. In IL-1β- and IFN-γ-pretreated CMEC, dexamethasone only minimally suppressed the rise in iNOS mRNA, protein abundance, or maximal iNOS enzyme activity in whole cell lysates but suppressed nitrite production by 60% in intact CMEC. Dual labeling of cytokine-pretreated CMEC in primary culture with an anti-iNOS antiserum and a fluorescein-labeled lectin specific for the microvascular endothelium of rat heart (GS-1) confirmed the presence of iNOS expression in these cells. iNOS was also detected in microvascular endothelium in situ in ventricular muscle from lipopolysaccharide-, but not sham-injected, rat hearts. The induction of iNOS in the endothelium of the cardiac microvasculature may have important implications for understanding the pathophysiology of some forms of inflammatory cardiomyopathies.

cytokine; glucocorticoid; lipopolysaccharide; endothelium; cardiac myocytes

THE IDENTIFICATION of NO synthase (NOS) isoforms in endothelial cells and the mechanisms regulating their activation has been the focus of much recent research. The constitutive NOS (cNOS) in large-vessel endothelium has been cloned and characterized by us and by others in aortic and umbilical vein endothelial cells (2, 11, 17, 21, 22, 32). Indeed, most of our knowledge about the cellular biology and physiology of the endothelium, including endothelial cell activation by inflammatory mediators, is based on studies in these large-vessel endothelial cells (9). However, there are important differences in phenotype between most large-vessel endothelium and microvascular endothelium, as well as considerable phenotypic heterogeneity among microvascular endothelial cells isolated from different tissues and organs (9, 15, 18). It remains controversial, for example, whether bovine aortic or human umbilical vein endothelial cells (HUVEC) express an inducible NOS (iNOS) isoform in response to lipopolysaccharide (LPS) or inflammatory cytokines. In contrast, Kleeman et al. (13) observed iNOS expression in pancreatic islets of prediabetic rats, including endothelial cells, and we reported a large increase in the release of nitrite, an oxidation product of NO, in primary cultures of cardiac microvascular endothelial cells (CMEC) after a 24-h incubation in medium conditioned by LPS-activated rat alveolar macrophages (1). This increase could theoretically be explained by increased expression of cNOS or by increased activity of cNOS through some posttranslational modification, as we have described for the endothelial cNOS (ecNOS) (22). Alternatively, cytokines may promote the expression, in endothelial cells, of a separate isoform that could be similar or identical to the iNOS originally cloned from macrophages (35) and subsequently identified in a number of other cell types (3, 7, 25, 33), including cardiac myocytes (1a).

In this report, we show that the increased production of NO by microvascular endothelial cells isolated from adult rat ventricular muscle in response to cytokines is due to the expression of an iNOS isoform. In contrast to large-vessel endothelium, cNOS activity in these cultured cells was almost undetectable. Using a reverse-transcription polymerase chain reaction (RT-PCR) approach, we characterized a partial iNOS cDNA from cytokine-pretreated endothelial cells that was essentially identical to the iNOS isoform sequence originally identified in activated murine macrophages (35). However, the regulation of iNOS expression in microvascular endothelial cells in response to specific cytokines and drugs differs from that of other cellular constituents of ventricular muscle. Finally, iNOS was identified in the microvascular endothelium in hearts of LPS-injected rats and in cytokine-pretreated CMEC in vitro by immunostaining with an antimurine iNOS-specific antiserum.

METHODS

Isolation of CMEC. The methods for the isolation and characterization of CMEC from adult rat ventricular tissue have been described in detail elsewhere (24). Briefly, the atria, valvular tissue, and right ventricle were removed from hearts obtained from ether-anesthetized male Sprague-Dawley rats (175–200 g) after a short (5-min) retrograde perfusion of the heart through the ascending aorta with a Krebs-Henseleit bicarbonate (KHB) buffer containing (in mM) 118 NaCl, 4.7 KCl, 1.25 $CaCl_2$, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 25 Na HCO_3, and 11 dextrose, saturated with 95% O_2-5% CO_2 at pH 7.4, at 37°C. The remainder of the left ventricle was briefly immersed (30 s) in 70% ethanol to devitalize epicardial mesothelial and endocardial endothelial cells. The ventricular tissue was washed, and the outer one-fourth to one-third of the epicardial surface of the left ventricle was dissected away and discarded. The remaining ventricular tissue was minced finely and treated with collagenase and trypsin in nominally Ca^{2+}-free KHB buffer. Dissociated cells were washed and resuspended in Dulbecco's modified Eagle's medium (DMEM; GIBCO-BRL, Gaithersburg, MD) with 20% fetal calf serum (FCS; GIBCO) and penicillin-streptomycin and plated on laminin (1 μg/cm^2; GIBCO)-coated culture dishes at a density of 2,500 cells/cm^2. The medium was removed, and the cells were washed once at 1 h to remove loosely adherent cells. These primary isolates have been documented to contain >90% endothelial cells, with a phenotype, at low passage number, consistent with their microvascular origin, as previously described (24). The consistency of this isolation procedure is periodically checked by submitting resuspended CMEC primary isolates to fluorescence-activated cell sorting (FACS) analysis after overnight loading with DiI-Ac-LDL (acetylated low-density lipoprotein labeled with 1,1′-dioctadecyl-1-3,3,3′,3′-tetramethyl-indocarbocyanine perchlorate; Biomedical Technologies, Stoughton, MA), as well as by other criteria, including selective staining with fluorescein isothiocyanate (FITC)-linked *Griffonia (Bandeiraea) simplicifolia* I (GS-1) lectin (Sigma Chemical, St. Louis, MO), as reported previously (24). Additional immunohistochemical analysis was performed on primary cultures using a specific antibody for OX-1 (leukocyte common antigen, Serotech, Indianapolis, IN), a marker for mononuclear cells, and consistently revealed ≤5% positively staining cells (4 different preparations). Vascular smooth muscle cells from rat pulmonary arteries (*passages 13–16*; kindly provided by Drs. N. Izzo and W. S. Colucci, Brigham and Women's Hospital, Boston, MA) were cultured in medium 199 (Sigma Chemical) with 20% fetal calf serum (FCS, GIBCO) and penicillin-streptomycin until confluent; cells were serum starved for 12 h in medium 199 and then treated with recombinant cytokines for 16–18 h before they were harvested.

PCR cloning and sequencing of iNOS mRNA in CMEC. Total RNA was isolated from cytokine-pretreated and control confluent CMEC primary cultures by the method of Chomczynski and Sacchi (5) and stored in diethylpyrocarbonate (DEPC; Sigma Chemical)-treated water at −70°C. Reverse transcription (RT) of CMEC RNA was accomplished using standard protocols (30). Briefly, 10 μg of total RNA were treated with 100 U of RNase inhibitor (Promega, Madison, WI) and denatured at 65°C for 10 min. An RT mix containing 1 mM deoxynucleotide trisphosphates (dNTPs), 20 μM random hexamers, and 1,000 U of reverse transcriptase (Superscript, GIBCO) was added in buffer containing 50 mM tris(hydroxymethyl)aminomethane (Tris)-HCl, 40 mM KCl, and 2.5 mM $MgCl_2$. For each experiment, parallel control samples were prepared in which reverse transcriptase 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 RT products was accomplished by subjecting 5-μl aliquots to 35 cycles of polymerase chain reaction (PCR; 1 min each at 94°C denaturing and 55°C annealing and 2 min at 72°C extension) in the presence of 0.125 mM dNTPs and 2.5 U of Taq polymerase (Promega) in a standard buffer containing 1.5 mM $MgCl_2$. The sense oligonucleotide 5′-(GAGATCAATGCAGCTGTG)-3′ corresponded to *base pair 1342–1359* and the antisense oligonucleotide 5′-(AGAATGGAGATAGGACGT)-3′ was complementary to *base pair 1541–1558* of the cDNA sequence of the rat vascular smooth muscle cell iNOS isoform (25). For each experiment, control samples were run in the absence of cDNA template, and RNA samples were processed in parallel without reverse transcriptase. Products of PCR amplification were resolved by agarose gel electrophoresis, and the expected 217-bp amplification product obtained from RNA of cytokine-pretreated CMEC was purified and cloned into the expression vector pBluescript (Stratagene, La Jolla, CA). After ligation, the plasmid was transfected into competent DH5α *Escherichia coli*, and two positive clones were further characterized. The nucleotide sequence of the inserts from these positive plasmids was verified on both strands by the dideoxy chain termination technique with use of Sequenase 2.0 (US Biochemical).

Northern blots. Northern blot analysis was performed by electrophoresing 15 μg of total RNA through a 1.5% formaldehyde-agarose gel and blotting onto nylon membranes by overnight capillary transfer or by vacuum blotting (model 785, BioRad). The 217-bp cDNA insert obtained from RT-PCR (see above) was radiolabeled using deoxycytidine-[^{32}P]triphosphate by random primer labeling. After 4 h of prehybridization at 42°C, the blots were hybridized overnight at 42°C by standard techniques (30) and then washed with 2× saline-sodium citrate buffer (SSC) and 0.1% sodium dodecyl sulfate (SDS) for 30 min at room temperature followed by 1× SSC and 0.1% SDS at 37°C and 0.2× SSC and 0.1% SDS at 65°C and autoradiographed at −70°C with intensifying screens for 6 h, or as otherwise stated.

Measurement of NOS activity in CMEC. Endothelial cell NOS activity was quantified by measuring the conversion of L-[^{3}H]arginine to L-[^{3}H]citrulline in the presence of saturating concentrations of cofactors. Total cellular homogenates were prepared from one 100-mm culture dish of confluent endothelial cells that had been washed three times in warm Hanks' balanced salt solution (HBSS, without $MgCl_2$, $CaCl_2$, or $MgSO_4$; GIBCO-BRL) and suspended by gentle trypsinization in HBSS containing 0.25% trypsin and 1 mM EDTA. Cells were centrifuged at 100 *g* at 4°C and washed twice in ice-cold phosphate-buffered saline (PBS), and the pellet was resuspended in 300 μl of a lysis buffer containing 20 mM Tris-HCl (pH 7.4 at 4°C), 0.5 mM EDTA, 0.5 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid, 1 mM dithiothreitol, 1 μM tetrahydrobiopterin (THB_4; Dr. B. Schircks Laboratories, Jona, Switzerland), 1 μM leupeptin, and 0.2 mM phenylmethylsulfonyl fluoride. Cells were lysed by three cycles of freeze-thawing, and the homogenates were centrifuged at 1,500 *g* for 15 min at 4°C. The protein content was determined using the Bradford technique (Bio-Rad, Melville, NY) with albumin as a standard. Twenty-five microliters of supernatant (~50 μg of protein) were added to 125 μl of buffer containing 50 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES, pH 7.4, 37°C), 1.25 mM $CaCl_2$, 1 mM EDTA, 0.5 mM NADPH, 10 mM flavin adenine dinucleotide, 5 μM flavin mononucleotide, 10 μM THB_4, 10 μg/ml calmodulin, and 0.2 nM L-[^{3}H]arginine (3.2×10^6 cpm/ml; Amersham, Arlington Heights, IL) for 1 h at 37°C. The reaction was stopped by the addition of 2 ml of

ice-cold 20 mM HEPES (pH 5.5) and 5 mM EDTA, and the total volume was applied to a Dowex-50W X8 column that had been preequilibrated with 20 mM HEPES (pH 5.5). L-[^{3}H]citrulline was eluted with 2 ml of deionized water, and radioactivity was quantified by liquid scintillation counting. The results are expressed as counts per minute per milligram of protein.

Nitrite release in CMEC-conditioned medium. The nitrite content of endothelial cell-conditioned medium was determined using standard techniques. Microvascular endothelial cells were cultured in 12-well tissue culture plates (Costar, Cambridge, MA) in DMEM with 20% FCS until confluent and then for 24 h in 400 μl of phenol red-free DMEM (GIBCO-BRL). After a 24-h incubation, the medium was collected and centrifuged once at 1,500 *g* for 15 min at 4°C to remove cellular debris, and 150 μl of this supernatant was added to a 1:1 (vol/vol) mixture of Griess reagent [0.75% sulfanilamide (final concn) in 0.5 N HCl-0.075% naphthylethylenediamine; Sigma Chemical], and absorbance was determined at 543 nm spectrophotometrically. A standard curve was constructed using known concentrations of sodium nitrite over the linear range of the assay (0.1–50 μM nitrite).

Western blot of iNOS protein. CMEC extracts were prepared, and the total protein concentration in the extracts was determined as described above. Protein extracts were reconstituted in sample buffer containing 0.062 M Tris-HCl, 2% SDS, 10% glycerol, and 5% (vol/vol) β-mercaptoethanol, and the mixture was boiled for 5 min. Equal amounts (60 μg) of the denatured proteins per lane were loaded and separated on 12% SDS-polyacrylamide gels (Mini Protean II, BioRad, Hercules, CA) and transferred to nitrocellulose membranes (HATF 20200 membrane, Millipore, Bedford, MA). The membrane was blocked with 1% bovine serum albumin (BSA) in Tris-buffered saline with 0.05% (vol/vol) Tween 20 (Sigma Chemical; TBST). Membranes were incubated with rabbit polyclonal anti-mouse iNOS primary antibody that had undergone affinity purification on a synthetic peptide composed of a unique sequence (*residues 1–20* of the NH_2-terminus of the murine macrophage iNOS) (20) for 3 h in TBST with 1% BSA. After three washes (10 min each), the membranes were incubated for 1 h at room temperature with a horseradish peroxidase-labeled goat anti-rabbit immunoglobulin G (IgG) secondary antibody (Pierce, Rockford, IL) at a 1:10,000 dilution in TBST with 1% BSA. After three additional washes in TBST, the membranes were rinsed twice in TBST, exposed to a chemiluminescent reagent (Renaissance, NEN Dupont, Boston, MA), and autoradiographed for 20–30 s.

Immunolocalization of iNOS in situ in ventricular muscle and in vitro in cytokine-treated CMEC. Affinity-purified polyclonal rabbit antibodies to iNOS (see above) were used for immunolocalization of iNOS in sections of rat ventricular muscle and in primary CMEC cultures in vitro. Cryostat sections of snap-frozen ventricular muscle from adult rats that had been injected intraperitoneally with LPS or saline 14 h before the animals were killed were fixed in fresh 2% buffered paraformaldehyde for 10 min and rinsed in PBS. After exogenous biotin sites were blocked by sequential incubation with avidin and biotin (biotin blocking kit, Vector Laboratories, Burlingame, CA), the sections were subjected to an overnight incubation with primary specific anti-NOS antibody or control solutions. Controls included the use of buffer alone or dilutions of nonspecific purified rabbit IgG in the primary layer. Specific binding was detected using a biotin-conjugated goat anti-rabbit IgG, avidin-biotin peroxidase complex (Vector Laboratories), and a substitute solution of H_2O_2 (0.03%) and diaminobenzidine (2 mg/ml) in 0.5 M Tris-saline (pH 7.6) with 1 M imidazole and 0.3% azide (to block mammalian peroxidase). The slides were then counterstained with hematoxylin, dehydrated through graded alcohols, mounted, and coverslipped.

For the immunofluorescence localization of iNOS in primary CMEC cultures, microvascular endothelial cells were prepared in the standard manner and plated on laminin-coated tissue culture chamber slides (Nunc, Naperville, IL) and allowed to grow to confluency before the medium was switched to DMEM for 24 h followed by addition of cytokines in DMEM, or DMEM alone, for another 24 h. The cells were then air-dried and fixed with fresh 2% paraformaldehyde for 10 min and rinsed with PBS. Cells were labeled first with FITC-labeled GS-1 (Sigma Chemical), as previously described (24), in PBS for 30 min followed by several washes in PBS. The cells were then labeled with the affinity-purified rabbit anti-iNOS antiserum (see above) followed by a goat anti-rabbit IgG-Texas Red-linked secondary antibody. Controls included omission of FITC-labeled GS-1, omission of the anti-iNOS antibody or use of normal rabbit IgG to control for specificity, and omission of the Texas Red-linked secondary antibody. Slides were mounted in glycerol and examined using appropriate filter sets on a Nikon Diaphot epifluorescence microscope.

RESULTS

iNOS activity in induced CMEC. After 24 h of exposure to interleukin-1β (IL-1β) and interferon-γ (IFN-γ), individually or in combination, the production of nitrite was measured from confluent serum-starved CMEC (Fig. 1*A*). We found that these cells produce very little nitrite in the absence of cytokine stimulation. IL-1β alone produced an increase in nitrite production that was clearly potentiated by the addition of IFN-γ to IL-1β; however, IFN-γ alone failed to produce any detectable nitrite release.

To characterize further this NOS enzyme activity, protein extracts from CMEC were assayed for the conversion of L-[^{3}H]arginine to L-[^{3}H]citrulline. As shown in Fig. 1*B*, there was an ~10-fold increase in activity in cytokine-treated cells compared with control untreated cells, which, as in Fig. 1*A*, had undetectable levels of constitutive activity. The specificity for NOS activity was verified by addition of the L-arginine analogue and NOS inhibitor *N*-monomethyl-L-arginine (1 mM) to the protein extract and by removal of NADPH, a necessary cofactor for NOS, both of which resulted in a nearly complete inhibition of assayable activity (Fig. 1*B*).

In contrast to cNOS isoforms, removal of calcium from the enzyme reaction buffer had no effect on NOS activity in cytokine-treated endothelial cell extracts (Fig. 1*B*).

PCR cloning and sequence of an iNOS cDNA from CMEC. To determine whether the increase in NOS activity after cytokine exposure, as shown above, was the result of increased expression of one of the two cNOS isoforms, the iNOS isoform cloned and sequenced from activated macrophages, or possibly of a novel iNOS isoform, we used an RT-PCR strategy to amplify putative iNOS transcript(s) expressed in IL-1β- and IFN-γ-treated CMEC primary cultures. Oligonucleotides were synthesized that encode a portion of the 5′ end of the iNOS cDNA sequence recently identified in rat vascular smooth muscle (25). These oligonucleotides were used in RT-PCR reactions with RNA obtained from IL-1β- and IFN-γ-pretreated and control CMEC. As shown in Fig.

2*A*, no RT-PCR products were identified from control endothelial cells (*lane 5*), nor were any PCR products obtained with RNA samples in the absence of reverse transcriptase or when cDNA was omitted from the PCR reaction (*lanes 3–4*). A single 217-bp product was generated, however, from total RNA from cytokine-pretreated cells (Fig. 2*A*, *lane 2*). After cloning into the vector pBluescript, numerous positive clones were isolated and two clones were selected for analysis. The sequences of these two clones were identical, revealing a single 217-bp cDNA shown in Fig. 2*B*. There is a 77% identity at the nucleotide level with the bovine aortic endothelial cell cNOS sequence (17) and a 99% identity with the rat vascular smooth muscle iNOS (25). The two nucleotide differences we observed, compared with the equivalent portion of the cDNA sequence of the rat vascular smooth muscle iNOS, were present in the sequence of both strands in two independent clones. Comparison of the deduced amino acid sequences according to the reading frame of the rat vascular smooth muscle iNOS cDNA shows that the two peptides are identical. In contrast with macrovascular endothelial cells, Northern analyses of total RNA from cytokine-pretreated and control CMEC with use of a full-length bovine endothelial cNOS cDNA (17) or a PCR-amplified partial rat cNOS cDNA (J.-L. Balligand, unpublished results), under a number of hybridization conditions, failed to reveal detectable transcripts (data not shown).

To quantitate the abundance of the iNOS transcript, this 217-bp cDNA sequence was used as a probe in Northern analyses of total RNA from CMEC. As shown in Fig. 3, a 4.6-kb transcript, consistent with the size of the murine macrophage iNOS mRNA (35), was detected in CMEC pretreated with IL-1β and IFN-γ for 6 h (Fig. 3, *top*). iNOS mRNA abundance peaked at 18 h and began to decline at 24 h. In control cells untreated with cytokines, no iNOS mRNA signal could be detected.

Differential regulation of iNOS induction in CMEC and vascular smooth muscle. We previously observed that tumor necrosis factor-α (TNF-α) and IFN-γ, in addition to IL-1β, induce iNOS activity in adult rat ventricular myocytes (1a; D. Ungureanu-Longrois, J.-L. Balligand, K. Okada, W. W. Simmons, L. Kobzik, C. J. Lowenstein, S. Kunkel, T. Michel, R. A. Kelly, and T. W. Smith, unpublished results). As shown in Fig. 4*B*, confluent serum-starved vascular smooth muscle cells in culture also respond to IFN-γ alone, as well as IL-1β and endotoxin (LPS), with an increase in iNOS mRNA abundance. The same experiment was repeated with

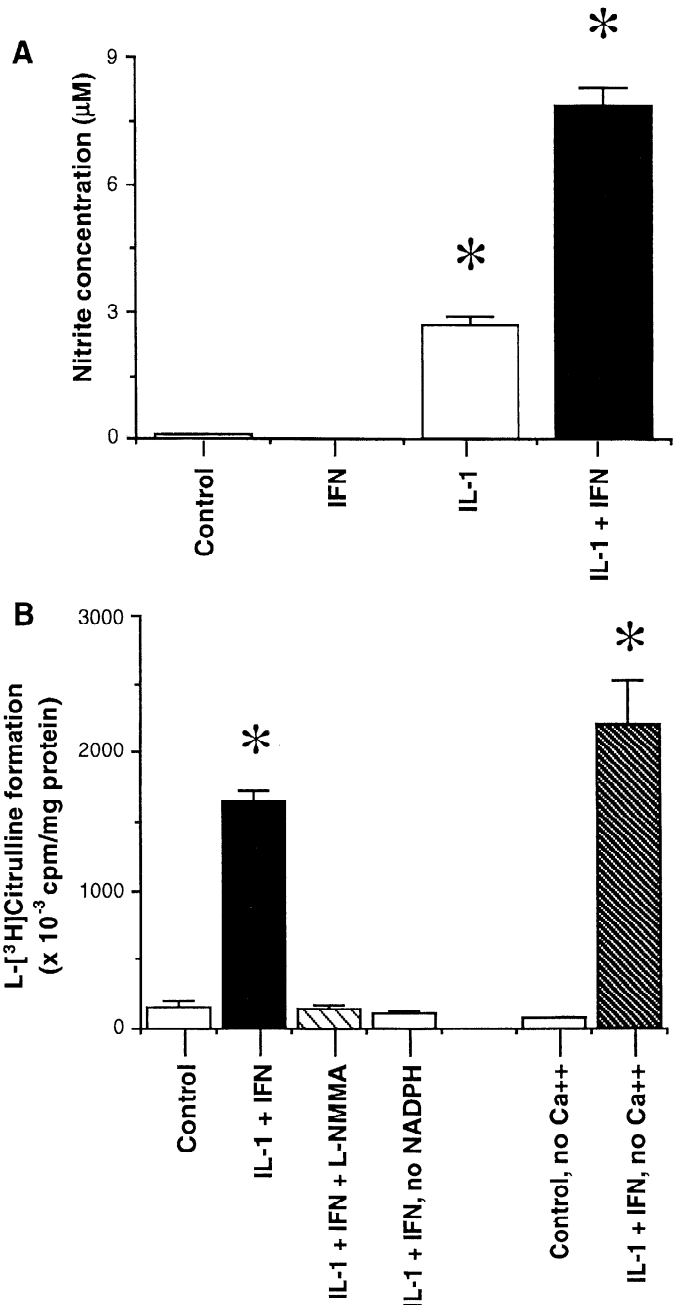


Fig. 1. Induction of inducible nitric oxide synthase (iNOS) activity in cardiac microvascular endothelial cells (CMEC) by interleukin-1β (IL-1β) and interferon-γ (IFN-γ). *A*: nitrite production by cytokine-treated CMEC. NOS activity was detected as nitrite (see METHODS) released into supernatant of confluent primary cultures of CMEC that were serum starved for 24 h and incubated in 12-well plates for a further 24 h in phenol red-free DMEM alone (control, *bar 1*) or with recombinant mouse IFN-γ (rmIFN-γ, 500 U/ml, *bar 2*), recombinant human IL-1β (rhIL-1β, 2 ng/ml, *bar 3*), or rmIFN-γ (500 U/ml) + rhIL-1β (2 ng/ml; *bar 4*). Note absence of detectable production of nitrite from cells treated with rmIFN-γ alone. Data are means ± SE of triplicate samples from a representative experiment (repeated 3 times with similar results). $^*P < 0.01$ vs. control. *B*: [^{3}H]citrulline formation by cytokine-treated CMEC extracts. NOS activity was detected by measuring rate of conversion of L-[^{3}H]arginine to L-[^{3}H]citrulline in cell lysates by quantitative cation-exchange chromatography (see METHODS). Confluent primary cultures of CMEC, serum starved for 24 h, were incubated in regular culture medium (i.e., DMEM; control, *bar 1*) or rhIL-1β (2 ng/ml) + rmIFN-γ (500 U/ml; *bar 2*). Total cell extracts were incubated at 37°C in a reaction mixture that contained sufficient cofactors to permit maximal enzyme activity (see METHODS). After a 24-h exposure to IL-1β and IFN-γ, there was a marked induction of iNOS activity (*bar 2*); in parallel experiments, the same cytokine-treated cell extracts were incubated in a reaction mixture in which 1 mM *N*-monomethyl-L-arginine (L-NMMA) was added (*bar 3*) or NADPH was omitted (*bar 4*). The same cell extracts were also incubated in a separate buffer with the same components as for *bars 1–4*, except Ca^{2+} was omitted and 3 mM EDTA and 3 mM EGTA were added (*bars 5* and *6*). iNOS activity was unaffected by removal of Ca^{2+} from reaction mixture. Data are means ± SE from 3 expts (each in triplicate). $^*P < 0.01$ vs. respective control.

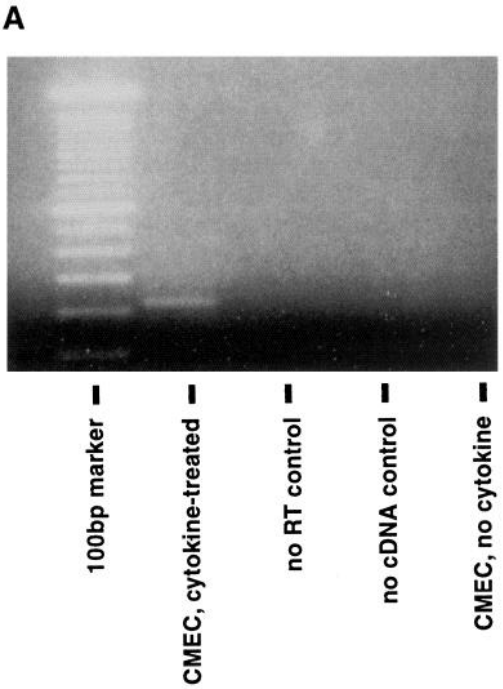


Fig. 2. Sequence of iNOS cDNA fragment cloned by reverse-transcription polymerase chain reaction (RT-PCR) from cytokine-pretreated CMEC. *A*: ethidium-stained agarose electrophoresis gel showing PCR-amplified iNOS cDNA from CMEC. Total RNA (10 μg) from confluent low-passage CMEC treated with rhIL-1β (2 ng/ml) and rmIFN-γ (500 U/ml) was reverse transcribed using standard protocols (see METHODS). By use of primers from the rat vascular smooth muscle iNOS cDNA (25), 5 μl of cDNA were amplified by PCR, and product was electrophoresed through a 1.5% agarose gel, along with a 100-bp molecular marker (*lane 1*). Parallel reaction products were run from cDNA of unstimulated endothelial cells (*lane 5*), RNA samples from stimulated endothelial cells processed identically in the absence of reverse transcriptase (*lane 3*), and samples without cDNA template (*lane 4*). No detectable band was visible from these controls (*lanes 3–5*). An RT-PCR product of ~0.2 kb is visible only from cytokine-stimulated CMEC (*lane 2*). *B*: sequence of 217-bp PCR-amplified iNOS cDNA from CMEC. Two independent PCR-generated clones were sequenced in both directions with use of dideoxy method (Sequenase, US Biochemical). Sequences of forward (*base pair 1342–1359*) and reverse (*base pair 1541–1558*) primers, derived from the vascular smooth muscle iNOS cDNA sequence (25), are underlined. Sequence is 98% identical to sequence between *base pairs 1360* and *1540* reported for rat vascular smooth muscle iNOS cDNA (25) (2 mismatches, bold characters) and 97% identical to sequence between *base pairs 1429* and *1609* of murine macrophage iNOS cDNA (35). It is also identical to iNOS cDNA we have cloned from adult rat ventricular myocytes (1a).

confluent low-passage rat aortic smooth muscle cells, in which an iNOS transcript was also detected after treatment with IFN alone (data not shown). In contrast to rat vascular smooth muscle cells and our recent observations in cardiac myocytes in adult rat hearts (1a), there was no detectable iNOS hybridization signal in CMEC pretreated with IFN-γ alone (Fig. 4*A*).

Effect of dexamethasone on iNOS induction in CMEC after IL-1β and IFN-γ. We have shown that dexamethasone given 45 min before exposure of ventricular myocytes to IL-1β and IFN-γ diminishes iNOS mRNA abundance and enzyme activity in adult rat ventricular myocytes (1a). To determine the effect of this corticosteroid on iNOS induction in CMEC, confluent serum-starved microvascular endothelial cell primary cultures were exposed for 45 min to 3 μM dexamethasone before recombinant human IL-1β (2 ng/ml) and recombinant mouse IFN-γ (500 U/ml) were added, and the cells or conditioned medium were harvested 24 h later. As shown in Fig. 5*A*, 3 μM dexamethasone decreased iNOS mRNA abundance by only 10–15%, in marked contrast to our results in cardiac myocytes. Similarly, iNOS protein content on Western analysis and enzyme activity in cytokine-pretreated endothelial cell lysates were only slightly decreased by dexamethasone, as shown in Fig. 5, *B* and *C*. However, 3 μM dexamethasone significantly reduced nitrite accumulation in medium conditioned by cytokine-pretreated CMEC by ~60% (Fig. 5*D*).

Immunohistochemical detection of iNOS in CMEC in vivo and in vitro. Using an affinity-purified rabbit polyclonal antiserum raised against a murine macrophage iNOS peptide, we determined whether iNOS could be detected in microvascular endothelium of ven-

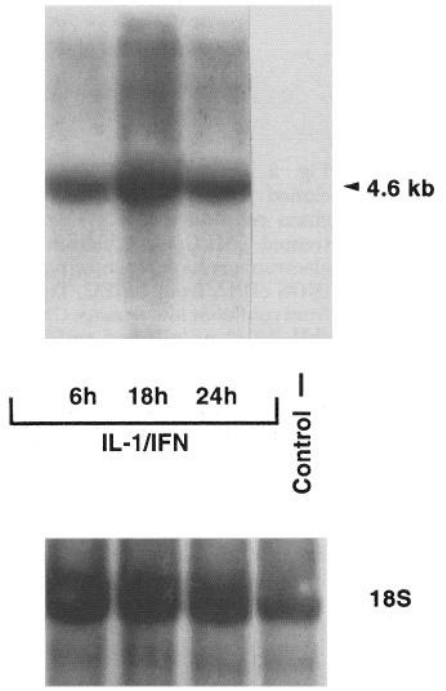


Fig. 3. *Top*: time course of induction of iNOS transcript by IL-1β and IFN-γ in CMEC. Time course of induction of iNOS mRNA by rhIL-1β (2 ng/ml) and rmIFN-γ (500 U/ml) in CMEC is shown on this Northern analysis using 217-bp iNOS [^{32}P]cDNA probe described in Fig. 2. Data are shown for iNOS mRNA abundance at *time 0* (i.e., control CMEC) and at 6, 18, and 24 h after exposure to cytokines. *Bottom*: blot was rehybridized to a ^{32}P-labeled probe for 18S rRNA to correct for loading differences.

tricular muscle obtained from rats that had been killed 14 h after injection with intraperitoneal LPS or in cytokine-pretreated CMEC in primary culture (Fig. 6). In hearts of sham-injected rats, background was minimal, and there was no staining of endothelium. In contrast, in hearts from LPS-injected animals, most fields showed ~20% of microvessels with positive labeling with anti-iNOS. As shown in Fig. 6, *A–C*, the vessel staining appeared to be limited to the endothelium. The endocardial endothelium also exhibited iNOS staining in LPS-injected rat hearts (data not shown). Evaluation of subtle increases in iNOS expression in ventricular myocytes from LPS-injected hearts is precluded by background staining in these cells, even in the absence of the primary antimurine macrophage iNOS antiserum. In contrast, staining of CMEC was never observed if the primary antibody was omitted or when irrelevant control rabbit IgG was used (Fig. 6).

In vitro, primary cultures of CMEC that had reached confluence and were then serum starved in DMEM for ≥ 24 h before exposure to the combination of IL-1β and IFN-γ for a further 24 h were examined using double labeling with the fluorescein-labeled lectin GS-1 and the murine macrophage iNOS antiserum with a secondary antibody linked to Texas Red. GS-1 has been shown specifically to label endothelium of the entire microvasculature of the rat heart (29) and has been used by us to characterize these endothelial cells in primary culture (24). Although these cells exhibit a typical cobblestone appearance at confluency when plated on laminin, with each cell exhibiting a flattened polygonal shape, many CMEC underwent a dramatic change in phenotype after 24 h in DMEM containing IL-1β and IFN-γ, contracting and assuming a spindle shape (Fig. 6, *F* and *G*). These cells stained intensely for GS-1 and iNOS, although fainter GS-1 and iNOS staining could also be observed in cells that remained flattened and polygonal.

DISCUSSION

In this report, we have established that CMEC express an iNOS that is identical to the iNOS identified in cytokine-activated murine macrophages, vascular smooth muscle, and other cell types. Unlike vascular smooth muscle cells and cardiac myocytes, iNOS in CMEC is not induced by IFN-γ, although IFN-γ mark-

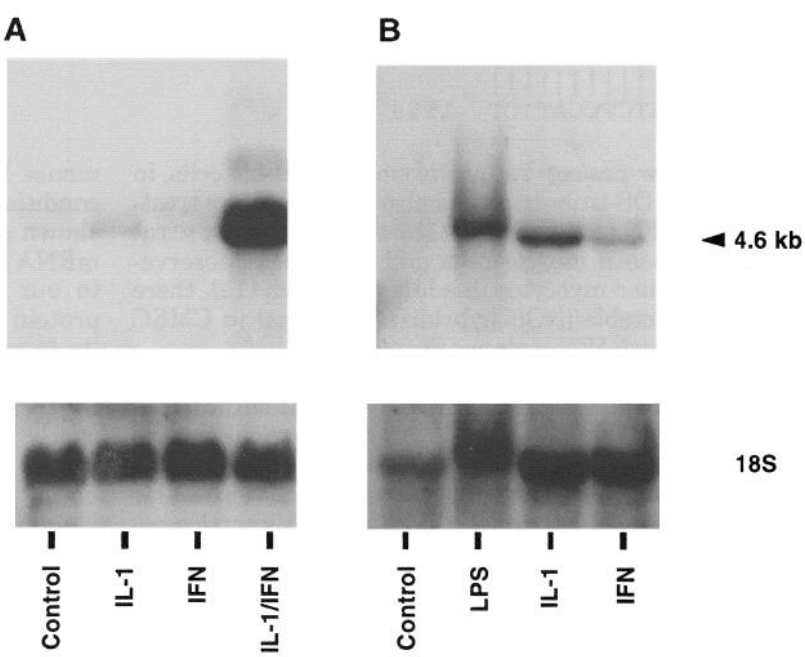


Fig. 4. Differential regulation of iNOS mRNA abundance in response to IL-1β and IFN-γ in CMEC and vascular smooth muscle cells. *A*: Northern blot analysis of iNOS transcript induction in CMEC in response to IL-1β (2 ng/ml), IFN-γ (500 U/ml), or both for 18 h. Culture conditions and Northern blot hybridization were the same as in Fig. 3, except blots were autoradiographed for 14 h. Note absence of detectable iNOS transcript from CMEC treated with IFN-γ alone. *B*: Northern blot analysis of iNOS transcript induction in rat pulmonary arterial smooth muscle cells (PASM) in response to IL-1β (2 ng/ml), IFN-γ (500 U/ml), or lipopolysaccharide (LPS, from *Salmonella typhimurium*, 10 μg/ml) for 18 h. PASM (*passages 13–16*) were cultured in medium 199 (GIBCO) with 20% fetal bovine serum (Sigma Chemical) until confluent and then serum starved in medium 199 for 24 h before treatment with cytokines or LPS, as indicated. Northern blot hybridization and autoradiography were performed as described above. Note clear induction of iNOS transcript with IFN-γ alone in contrast with CMEC. *A* and *B* are representative of ≥ 3 expts with similar results.

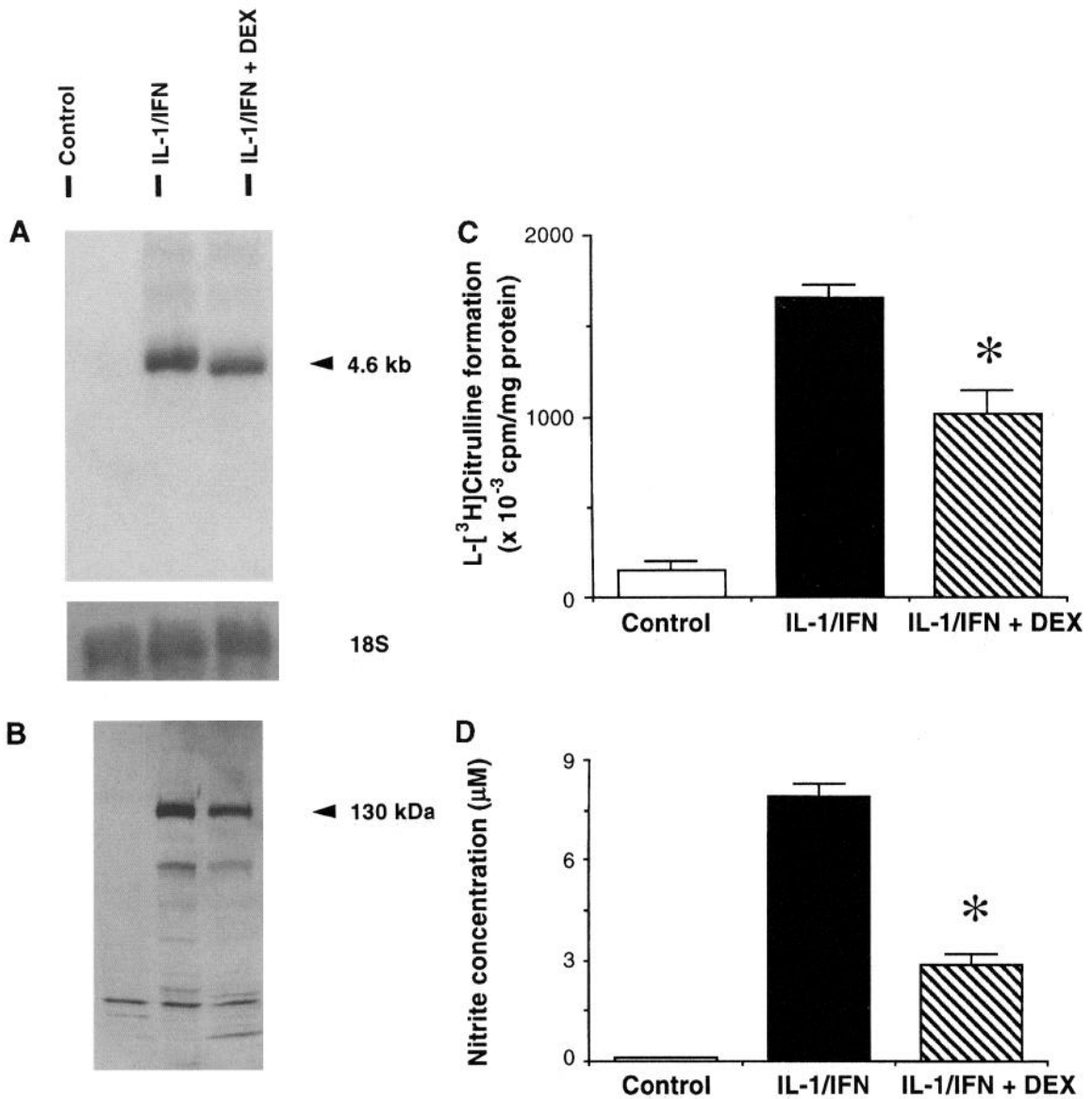


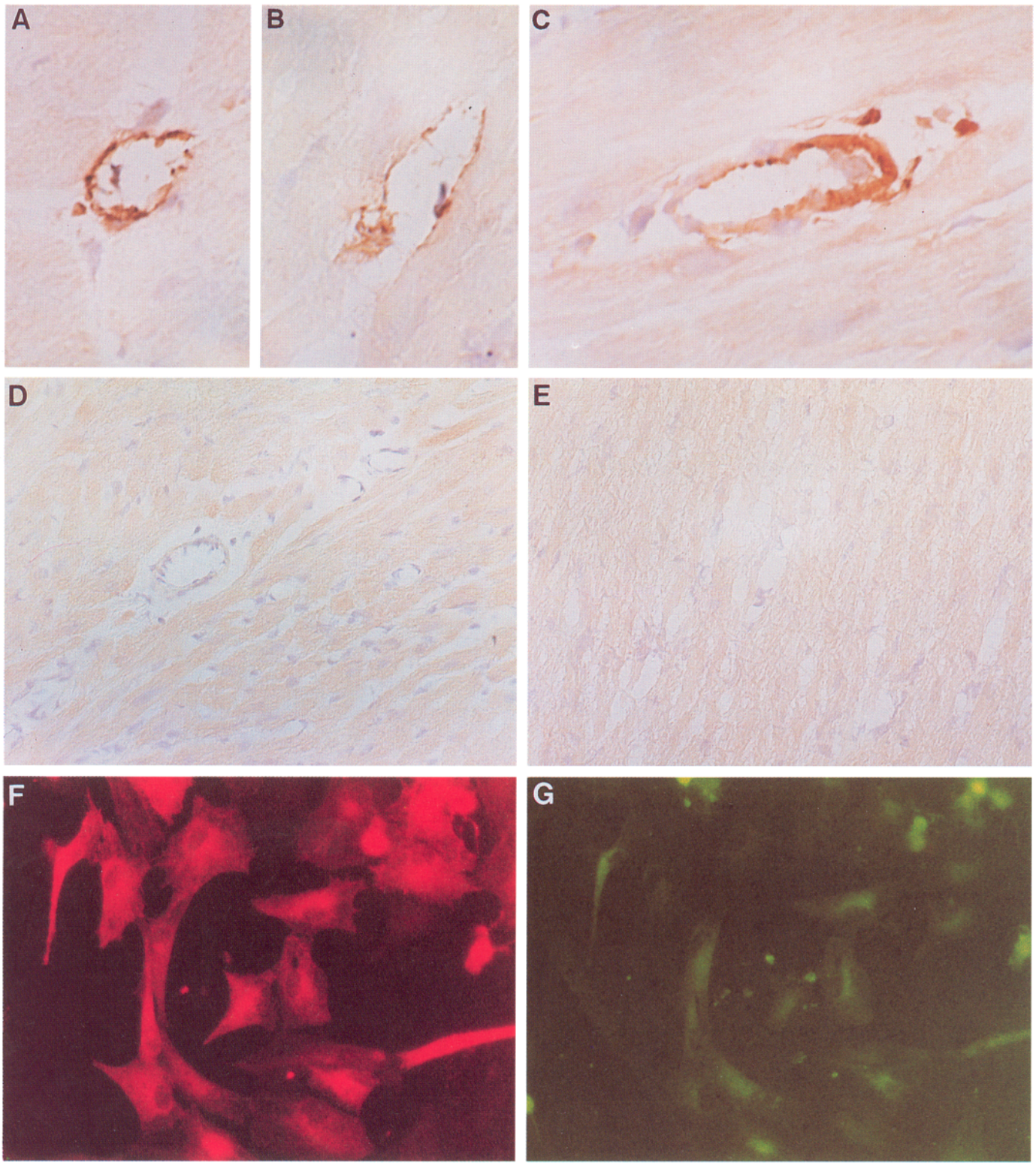
Fig. 5. Effect of dexamethasone (Dex) on iNOS induction in response to IL-1β and IFN-γ. Effect of 3 μM Dex added to confluent serum-starved CMEC primary cultures in DMEM 45 min before addition of rhIL-1β (2 ng/ml) and rmIFN-γ (500 U/ml) was examined on iNOS mRNA abundance (*A*), iNOS protein content (*B*), iNOS activity (*C*), and nitrite accumulation (*D*) at 24 h. iNOS mRNA abundance was examined by Northern analysis in *A* with use of ^{32}P-labeled 217-bp iNOS cDNA probe identified by RT-PCR for cytokine-pretreated CMEC (Fig. 2) and a ^{32}P-labeled 18S rRNA probe to correct for amount of RNA loaded per lane. iNOS protein was detected by Western analysis in *B* with use of an affinity-purified polyclonal antibody raised against murine macrophage iNOS. In *C*, iNOS activity was detected in CMEC lysates by measuring rate of conversion of L-[^{3}H]arginine to L-[^{3}H]citrulline (see Fig. 1*B*). In *D*, nitrite accumulation was measured spectrophotometrically with use of Griess reaction (see METHODS). Experiments in *A–D* were repeated ≥ 3 times with similar results. Data are means ± SE.

edly enhanced mRNA abundance and activity in response to IL-1β. Unlike most large-vessel endothelial cells, CMEC, in addition to expressing iNOS in response to specific inflammatory cytokines, also have little or no cNOS activity. iNOS could be localized immunohistochemically to the microvascular endothelium in situ in hearts of rats after intraperitoneal injection of LPS, but not control animals, and in CMEC that had been activated in vitro by inflammatory cytokines.

Two different assays, the measurement of nitrite production by intact cells and conversion of L-[^{3}H]arginine to L-[^{3}H]citrulline in CMEC cell lysates, were used to document the increase in NOS activity by cytokines in CMEC, and both techniques documented a large increase in NOS activity after treatment with IL-1β and IFN-γ. In agreement with previous reports on the lack of sensitivity of iNOS to calcium (4, 23), the cytokine-induced NOS activity of CMEC was unaffected by calcium chelation in the assay buffer, supporting the possibility that CMEC expressed an iNOS isoform. Moreover we could not identify endothelial cNOS transcripts in this cell type using our bovine ecNOS cDNA (17) or a partial cNOS cDNA from rat (J.-L. Balligand, unpublished observations) as a probe. The very low (if

any) constitutive production of NO by CMEC in primary culture is in keeping with this interpretation. In intact organs or tissues, we (T. Michel and J. Polak, unpublished observations) and others (28) documented patchy and less robust ecNOS expression in a variety of microvessels relative to the staining seen in large vessels with use of immunohistochemical techniques.

To identify a putative iNOS from cytokine-treated CMEC, we used an RT-PCR approach that employed amplimers, the design of which was guided by the recognition that the putative iNOS in CMEC would be identical to or different from the other iNOS isoforms cloned to date. A cDNA fragment of the expected size (217 bp) was identified that was virtually identical to the



reported vascular smooth muscle (25) and murine macrophage (35) iNOS sequences. It is also identical to the cDNA we have identified in cytokine-treated rat cardiac myocytes (1a). In comparison with the rat vascular smooth muscle and murine macrophage iNOS cDNA sequences, the few base pair mismatches do not alter the deduced amino acid sequence (Fig. 2*B*) and probably represent silent polymorphisms in the same gene. Together with the high degree of similarity at the nucleotide level with sequences reported for the human hepatocytes (7), human chondrocytes (3), and a human colorectal adenocarcinoma cell line (33), these data add to a growing consensus that there may be one mammalian iNOS isoform being expressed in a number of cell types.

The RT-PCR technique employed here clearly does not exclude the possibility that the iNOS cDNA fragment we identified could have been amplified from mRNA from a small fraction of contaminating nonendothelial cells in our cultures. We believe that this is unlikely, though, on the basis of the strong iNOS hybridization signal in Northern analysis using this cloned radiolabeled amplification product in our cytokine-treated CMEC cultures. These primary CMEC cultures have been extensively characterized and routinely contain >95% endothelial cells, as verified by FACS analysis of DiI-Ac-LDL-loaded cells (24). Also the observation of a positive immunohistochemical staining for iNOS in microvascular endothelium in vivo and in cytokine-pretreated CMEC in vitro argues that CMEC express the iNOS we identified or one that is highly homologous.

Further evidence that microvascular endothelial cells in these CMEC primary cultures are responsible for the molecular and biochemical evidence of iNOS induction is supported by the observation that the regulation of the enzyme in these cells is different from that in vascular smooth muscle cells or cardiac myocytes. IFN-γ alone produced no detectable increase in iNOS mRNA levels (Fig. 4), protein content, or NOS activity in cell lysates (data not shown) or nitrite release (Fig. 1*A*) by CMEC. The observation that murine IFN-γ potentiates the expression of iNOS mRNA (Fig. 4) and activity (Fig. 1*A*) induced by IL-1 rules out the possibility that species differences in the activity of the recombinant peptide or the absence of receptors for IFN-γ accounts for the lack of an effect of this cytokine alone in rat CMEC. This is in agreement with previous observations in rat aortic endothelial cells (34) but contrasts with the effect of IFN-γ on adult rat vascular smooth muscle cells and ventricular myocytes, both of which show a clear expression of iNOS transcript (1a; Fig. 4), an observation that also argues against any significant contamination of our CMEC cultures by either of these latter two cell types.

The response of cytokine-pretreated CMEC to dexamethasone does identify another difference between these cells and adult rat ventricular myocytes (D. Ungureanu-Longrois, J.-L. Balligand, K. Okada, W. W. Simmons, L. Kobzik, C. J. Lowenstein, S. Kunkel, T. Michel, R. A. Kelly, and T. W. Smith, unpublished results; 31) and rat hepatocytes (8) with respect to dexamethasone-regulated iNOS induction. In contrast to these other cell types, in which dexamethasone decreases iNOS mRNA abundance ~10-fold, dexamethasone only slightly affected the extent of the increase in iNOS mRNA or protein content, or maximal iNOS activity, in cell lysates of cytokine-pretreated CMEC (Fig. 5). However, iNOS activity, in intact cells, as indicated by the rate of release of nitrite into CMEC-conditioned medium, was significantly decreased. This decrease in nitrite release that was disproportionate to the small change in iNOS protein abundance may reflect the influence of dexamethasone on other cellular pathways, perhaps related to L-arginine transport or cofactor availability or to posttranslational modifications of the enzyme. These hypotheses are the subject of ongoing investigation in the laboratory.

We identified iNOS protein using an affinity-purified murine macrophage iNOS-specific antiserum in microvascular endothelium in situ in ventricular muscle from LPS-injected rats and in cytokine-pretreated CMEC in vitro that had been dual labeled with the lectin GS-1 (Fig. 6). The differences in the intensity of iNOS staining, as well as that of GS-1 lectin, as shown in Fig. 6, *F* and *G*, are attributable in part to differences in the shape of cytokine-treated cells at 24 h, with many intensely stained cells exhibiting a retracted spindle shape that has been noted in cytokine-treated HUVEC. Staining for iNOS in situ in immunohistochemical

Fig. 6. Immunohistochemical detection of iNOS in hearts from LPS-injected rats and in IL-1β- and IFN-γ-pretreated CMEC in vitro. iNOS was identified in sections of adult rat ventricular muscle obtained from hearts of adult rats 14 h after an intraperitoneal injection of LPS, and sections of muscle were isolated from control sham-injected rats with use of an affinity-purified antibody raised against murine macrophage iNOS (20) and a secondary antibody linked to a peroxidase reaction (see METHODS). iNOS staining could also be identified in intravascular or infiltrating leukocytes in LPS-injected hearts, but these cells contributed less than one-half of total cells staining positively for iNOS; positive cells were only rarely seen in sections from sham-injected animals (data not shown). In hearts from LPS-injected animals, specific iNOS peroxidase staining could be observed in all fields in microvasculature. *A* and *B*: capillary or venular staining (×1,000); *C*: endothelial cell staining in a small arteriole (×1,000). In marked contrast, there was little or no iNOS-specific staining in hearts from sham-injected rats (*D*, ×400). There was no iNOS-specific staining in LPS-injected hearts if a nonspecific IgG control antiserum was used as primary antibody (*E*, ×400). Also, no iNOS-specific peroxidase staining was observed when primary antibody was omitted, although cardiac myocytes exhibited a persistent background staining, as is evident in these photomicrographs. For in vitro staining of CMEC exposed to IL-1β (2 ng/ml) and IFN-γ (500 U/ml) for 24 h, cells were dual labeled with fluorescein-conjugated lectin GS-1 (*G*, ×600) and antimurine macrophage iNOS antiserum and a secondary antibody linked to Texas Red (*F*, ×600); the same field is illustrated in *F* and *G*. GS-1 lectin is relatively specific for endothelium of microvasculature in rat heart (29) and is particularly evident when condensed into elongated spindle-shaped phenotype of some CMEC after a 24-h exposure to IL-1β and IFN-γ (*G*). These cells are positive for iNOS, as indicated by positive Texas Red fluorescence of field in *F*.

sections of hearts from animals previously treated with LPS did not uniformly stain positively for iNOS in the endothelium of the microvasculature but was present in ~20% of the microvessels in each field, as well as the endocardial endothelium. It is unclear whether this represents selective activation of iNOS (e.g., in a venular endothelium) or incomplete iNOS activation in this particular experimental model. Pober and Cotran (27), using a dermal organ culture model, also emphasized that endothelial cells from distinct anatomic levels of the microvasculature respond differently to specific inflammatory cytokines, at least in the skin.

Most of our information about the cell and molecular biology of endothelial cell function and responsiveness is based on primary cultures of aortic endothelium from several species and on studies in HUVEC, cells that are relatively easy to isolate and maintain in primary culture (9). Aortic endothelial cells and HUVEC have been used extensively in studies of endothelial cell activation by inflammatory mediators (9, 27). However, Gerritsen et al. (10) noted important differences in the responses of human microvascular endothelium from lung and rheumatoid synovium to TNF-α, IL-1β, or IFN-γ and the responses of HUVEC in primary culture. The physiological relevance of iNOS induction in the microvasculature may be related to the cytotoxic and antiviral actions of NO (12), its effects on microvascular permeability, and the regulation of neutrophil recruitment from the vasculature (9, 14, 16). We have shown that iNOS induction in CMEC by IL-1β, a cytokine that alone does not affect the function of cardiac muscle cells, leads to a marked decrease in cardiac myocyte contractile responsiveness to β-adrenergic agonists in coculture with CMEC (D. Ungureanu-Longrois, J.-L. Balligand, K. Okada, W. W. Simmons, L. Kobzik, C. J. Lowenstein, S. Kunkel, T. Michel, R. A. Kelly, and T. W. Smith, unpublished results). Endothelial cells of the microvasculature may act to transduce or amplify an immunologic response by releasing NO as well as other autacoids and inflammatory mediators that act to regulate heart cell function and may also affect myocyte survival (6, 19).

We thank Darlene Toupin for expert technical work.

This work was supported by National Heart, Lung, and Blood Institute Grants R37-HL-36141 (to T. W. Smith) and R29-HL-46457 (to T. Michel). J.-L. Balligand and D. Ungureanu-Longrois were recipients of fellowship awards from the American Heart Association, Massachusetts Affiliate. W. W. Simmons is the recipient of a Medical Research Council of Canada fellowship award. T. Michel is an Established Investigator of the American Heart Association.

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Received 2 June 1994; accepted in final form 28 September 1994.

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