Purification and cDNA Sequence of an Inducible Nitric Oxide Synthase from a Human Tumor Cell Line†

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ABSTRACT: A combination of cytokines induced the expression of nitric oxide synthase (NOS) in a human colorectal adenocarcinoma cell line, DLD-1. We have purified the enzyme and examined some of its biochemical properties. An antiserum to an inducible NOS from murine macrophages cross-reacted with the DLD-1 NOS. The purified human and murine enzymes displayed a similar lack of dependence on exogenous calcium and calmodulin for activity, which contrasts with the requirement for calcium and calmodulin of purified brain and endothelial isoforms of NOS. We have also isolated a cDNA for a cytokine-induced NOS from DLD-1 cells. Sequence analysis of this cDNA and NOS cDNAs from human liver, smooth muscle, and macrophages suggests that, at the genetic level, there is a single isoform of human-inducible NOS.

Nitric oxide synthase (NOS)[1] converts L-arginine to L-citrulline and nitric oxide (NO). NO is thought to play a role in a wide variety of physiological processes ranging from regulation of vascular tone to neurotransmission [for a recent review, see Nathan (1992)]. Three distinct isoforms of nitric oxide synthase have been well described both biochemically and genetically. These include constitutively expressed brain (Bredt & Snyder, 1990; Bredt et al., 1991; Schmidt et al., 1991) and endothelial (Pollack et al., 1991; Lamas et al., 1992; Sessa et al., 1992) isoforms and a cytokine-induced isoform from murine macrophages (Stuehr et al., 1991; Hevel et al., 1991; Lyons et al., 1992; Xie et al., 1992; Lowenstein et al., 1992). In humans, brain and endothelial isoforms of NOS are fairly well documented (Schmidt & Murad, 1991; Nakane et al., 1993; Janssens et al., 1992). However, it has been more difficult to demonstrate the existence of an inducible NOS in any human tissue. Recently, the induction of NOS activity has been described in primary cultures of human macrophages (Denis, 1991), hepatocytes (Nussler et al., 1992), vascular smooth muscle (Scott-Burden et al., 1992), and chondrocytes (Charles et al., 1993). There is only one report of inducible NOS in a human cell line, and the level of enzyme activity described in this study was low (Lelchuk et al., 1992). There has been insufficient biochemical and/or genetic data to determine if there are tissue-specific variants of the inducible enzyme.

In the present study, we demonstrate the existence of a cytokine-induced NOS in a human tumor cell line. We have purified the protein and examined some of its biochemical properties. We have also isolated a cDNA for an inducible NOS from the same tumor cell line. Sequence analysis of this cDNA and NOS cDNAs from several other human tissues suggests that, at the genetic level, there is a single isoform of inducible NOS.

† The nucleic acid sequence in this paper has been submitted to GenBank.

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[1] Abbreviations: NOS, nitric oxide synthase; NO, nitric oxide; IFN-γ, interferon-γ; LPS, lipopolysaccharide; IL-1β, interleukin-1β; TNF α, tumor necrosis factor α; IL-6, interleukin-6.

EXPERIMENTAL PROCEDURES

Cell Culture. DLD-1 (ATCC no. CCL 221) is a human colorectal adenocarcinoma cell line. RAW 264.7 (ATCC no. TIB 71) is a murine macrophage cell line. Cells were grown at 37 °C, 5% CO_2 in RPMI 1640 medium (Gibco BRL) supplemented with L-glutamine, penicillin, streptomycin, and 10% heat-inactivated fetal bovine serum (Hyclone). Human liver cell lines were provided by B. Huber (Burroughs Wellcome Co.). All other cell lines were obtained from ATCC.

Induction Studies. Cells were grown to confluence in 12-well plates. Cytokines or other agents were added to fresh media (1 mL/well). At 18–24-h postinduction, culture supernatants were harvested and stored at −20 °C for nitrite analysis.

Nitrite Analysis. Nitrite was measured spectrophotometrically using the Griess reagent (Green et al., 1982). Four hundred microliters of Griess reagent (1% sulfanilamide and 0.1% naphthylethylenediamine in 2% phosphoric acid) was added to 400 μL of culture supernatant. After 15–30 min at room temperature, the absorbance at 540 nm was measured. Nitrite concentrations were determined by comparison with standard solutions of sodium nitrite prepared in water.

Enzyme Purification. Cells were plated into 10 150 cm^2 flasks (20 mL of medium/flask), grown to confluence, and treated with 100 units/mL interferon-γ (IFN-γ), 200 units/mL interleukin-6 (IL-6), 10 ng/mL tumor necrosis factor α (TNF α), and 0.5 ng/mL interleukin-β (IL-1β). IFN-γ, IL-6, and TNF α were from Boehringer Mannheim; IL-1β was from Genzyme. At 18–24-h postinduction, cells were harvested by scraping and washed with phosphate-buffered saline. Pelleted cells were stored at −70 °C. All further steps in the purification were performed at 4 °C. Cell extract was prepared by resuspending the cell pellet in 5 mL of 20 mM Tris-HCl, pH 7.5, containing 1 mM dithiothreitol and 2 μM tetrahydrobiopterin, lysing by three cycles of rapid freeze–thawing, and centrifuging the lysate for 1 h at 100000*g*. Enzyme activity in the extract was stable when stored at −70 °C. The extract (15–25 mg of protein) was applied by gravity flow to a column containing 200 μL of 2′,5′-ADP–Sepharose (Pharmacia LKB) that had been equilibrated with 20 mM Tris-HCl, pH 7.5,

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containing 10% glycerol, 1 mM dithiothreitol, and 2 μM tetrahydrobiopterin (EB). The column was then washed sequentially with 3 mL each of EB, 0.5 M NaCl in EB, 3 mM malic acid plus 0.15 mM $NADP^+$ in EB, 1 mM 2′-AMP in EB, 1 mM NADH in EB, and EB. NOS was eluted with 3 mL of 3 mM NADPH in EB. Enzyme activity in the eluate was stabilized by adding bovine serum albumin (BSA) to a concentration of 1 mg/mL. The eluate from the ADP–Sepharose column was applied (either directly or after storage at −70 °C) at a flow rate of 0.2 mL/min to a column containing 1 mL of DEAE Bio-Gel A–agarose (Bio-Rad) that had been equilibrated with EB. The column was washed with 5 mL each of EB and 0.05 M NaCl in EB. NOS was eluted with a linear gradient (10 mL, 0.05–0.5 M) of NaCl in EB. When protein concentrations were determined in the column fractions, dithiothreitol was omitted from the buffers due to its interference with the colloidal gold protein assay. The purified enzyme was stable when stored at −70 °C in the presence of 1 mg/mL BSA.

NOS was purified from a murine macrophage cell line (RAW 264.7) by the same procedure and from bovine brain as described (Furfine et al., 1993).

Enzyme Assay. NOS activity was measured by a radiochemical assay that monitors the conversion of labeled L-arginine to L-citrulline (Bredt & Snyder, 1989). Reactions contained 50 mM Hepes, pH 7.5, 200 μM NADPH, 1 mM dithiothreitol, 10 μM FAD, 100 μM tetrahydrobiopterin, 10 μM L-arginine, and 2.9 μM L-[U-^{14}C]arginine (New England Nuclear, NEC-267E) in a total volume of 50 μL. Typically, reactions were started by adding 10 μL of protein sample and run for 10 min at 37 °C. Reactions were terminated by adding 0.5 mL of cold stop buffer containing 0.1 M Hepes, pH 6, 1 mM citrulline, and 5 mM EDTA. The samples were applied to disposable columns containing 0.5 mL of AG 50W-X8 cation-exchange resin (Na form, Bio-Rad). The reaction product was eluted with two 0.75-mL portions of water, and 12 mL of Ecolume (ICN) scintillation fluid was added to the pooled effluents from each column.

Protein Analysis and Antibody Production. Protein concentrations were determined by the colloidal gold method (Ciesiolka & Gabius, 1988) using the Quantigold reagent (Diversified Biotech, Boston, MA) and BSA as a standard. A rabbit polyclonal antiserum to murine macrophage NOS was produced by immunization with an NOS fusion protein that was purified from an *Escherichia coli* expression system. cDNA encoding amino acids 1–224 of murine macrophage-inducible NOS (Lyons et al., 1992; Xie et al., 1992; Lowenstein et al., 1992) was amplified by RNA-PCR as described (Wood et al., 1993) and inserted into the pGEX-2T protein expression vector (Pharmacia LKB). This expression system produced a fusion protein consisting of a 26-kDa fragment from the carboxyl terminus of glutathione *S*-transferase fused to the amino terminus of the murine macrophage NOS fragment. The fusion protein was purified by affinity chromatography on glutathione–Sepharose (Pharmacia LKB) followed by preparative SDS/polyacrylamide gel electrophoresis. Western blot analysis was performed by standard procedures using the rabbit antiserum to the NOS fusion protein and an alkaline phosphatase-conjugated secondary antibody (Protoblot system, Promega).

Amplification of NOS cDNA from DLD-1 Cells by RNA-PCR. NOS was induced in DLD-1 cells as described above. The cells from one confluent 75 cm^2 flask were harvested by scraping and washed with phosphate-buffered saline. Poly-(A^+) RNA was isolated from the cells using the Micro-Fast Track procedure (Invitrogen). RNA-PCR was performed using the GeneAmp RNA-PCR kit (Perkin Elmer). After an initial denaturation step at 95 °C, 35 PCR cycles were performed as follows: 1 min at 95 °C, 2 min at 55 °C, 3 min at 72 °C. The following oligonucleotide primers based on the human chondrocyte NOS cDNA sequence (Charles et al., 1993) were used to amplify the DLD-1 NOS cDNA: fragment 1 forward, 5′-AAGCCCCACAGTGAAGAACAT-3′; fragment 1 reverse, 5′-ATGTACCAGCCATTGAAGGGG-3′; fragment 2 forward, 5′-ATCGACCTGGGCTGGAAGCCCAAG-3′; fragment 2 reverse, 5′-GCTGCCTTGAAGGTTTGCACGGCC-3′; fragment 3 forward, 5′-CTCAACAACAAATTCAGGTAC-3′; fragment 3 reverse, 5′-AGCCGCTGGCATTCCGCACAA-3′; fragment 4 forward, 5′-CTCCCCATTCTGAAGCCCAGG-3′; fragment 4 reverse, 5′-AGAGCTGGCTCCATCCTTAAG-3′. Oligonucleotides were synthesized and purified by Oligos, Etc. (Wilsonville, OR), except for control primers for human β-actin which were obtained from Stratagene.

Cloning and Sequencing of NOS cDNA from DLD-1 Cells. RNA-PCR reaction products were analyzed by agarose gel electrophoresis. Products of the appropriate size were excised from the gels and inserted into the pCR II TA cloning vector (Invitrogen). DNA sequences of the cloned PCR products were determined from plasmid templates using the dideoxy chain termination method with Sequenase Version 2 T7 DNA polymerase (United States Biochemical). Sequence analysis was performed with Wisconsin Genetics Computer Group sequence analysis software.

Table I: Nitrite Levels in Culture Supernatants of DLD-1 Cells 24-h Postinduction[a]

treatment	[nitrite], μM	treatment	[nitrite], μM
none	1	IFN-γ + IL-6	3
IFN-γ	2	IL-1β + TNFα	1
LPS	2	IL-1β + IL-6	2
IL-1β	2	IL-6 + TNFα	2
TNFα	2	IFN-γ + IL-1β + LPS	26
IL-6	1	IFN-γ + IL-1β + TNFα	38
IFN-γ + LPS	2	IFN-γ + IL-1β + IL-6	39
IFN-γ + IL-1β	25	IFN-γ + IL-1β + TNFα + IL-6	44
IFN-γ + TNFα	4		

[a] Results are reported as the averages of assays run on duplicate or triplicate wells. Well-to-well variation was less than 10%.

RESULTS

Induction of NOS in DLD-1 Cells. Human cell lines were screened for the presence of an inducible NOS by measuring the production of nitrite, a stable oxidation product of NO, in cell culture supernatants. Accumulation of detectable nitrite in culture supernatants within 24 h implies the existence of a high level of NOS activity, since the spectrophotometric assay for nitrite is relatively insensitive. Such a high level of inducible NOS activity is easily observed in murine macrophage cell lines that have been exposed to IFN-γ and LPS (Marletta et al., 1988). A variety of cytokines that have been reported to induce NOS in a variety of cell types [reviewed in Nathan (1992)] were tested for their ability to induce NOS in human cell lines. Induction of NOS was not detected in the following cell lines: liver (Hep G2, Hep 3B, and five immortalized liver cell lines), monocyte (U-937, THP-1, K-562), colon (SW 480), and cervix (ME 180). The results of induction experiments with the human colorectal adenocarcinoma cell line DLD-1 are shown in Table I. None of the cytokines or LPS used alone resulted in detectable nitrite in

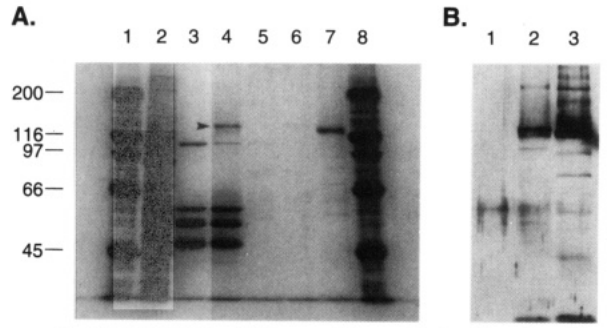

FIGURE 1: SDS/PAGE and Western blot analysis of fractions from purification of DLD-1 NOS. (A) The polyacrylamide gel (7.5%) was stained with Coomassie Brilliant Blue R-250. Lanes 1 and 8, molecular mass markers (kDa). Lane 2, cell extract, 6 μg of protein. Lane 3, peak fraction from ADP–Sepharose column, uninduced DLD-1 cells, 1 μg of protein. Lane 4, same as lane 3 but induced DLD-1 cells. Lane 5, peak fraction from DEAE Bio-Gel A column, uninduced DLD-1 cells, 0.1 μg of protein. Lane 6, same as lane 5 but induced DLD-1 cells. Lane 7, peak fraction from DEAE Bio-Gel A column, induced RAW 264.7 cells. The arrow indicates putative DLD-1 NOS. (B) Proteins were detected using an antiserum to the murine macrophage NOS. Lane 1, peak fraction from DEAE Bio-Gel A column, uninduced DLD-1 cells, 0.1 μg of protein. Lane 2, same as lane 1 but induced DLD-1 cells. Lane 3, peak fraction from DEAE Bio-Gel A column, induced RAW 264.7 cells.

Table II: Purification of Cytokine-Induced NOS from DLD-1 Cells

fraction	sp. act.[a]	purification factor	total act., pmol/min	yield, %
cell extract	236	1	3350	100
ADP–Sepharose	27037	115	1370	41
DEAE Bio-Gel A	287879	1220	504	15

[a] Picomoles of citrulline per minute per milligram of protein.

the culture supernatants. The combination of IFN-γ and LPS, which induced a very high level of NOS activity in the murine macrophage cell line RAW 267.4, did not induce a measurable amount of NOS in DLD-1 cells. All of the cytokines were tested in pairs: the combination of IFN-γ and IL-1β resulted in a marked increase in the amount of nitrite produced. This induction was further enhanced by the addition of either TNF α or IL-6. The greatest level of induction was observed when all four cytokines were used together.

Purification and Properties of Cytokine-Induced NOS from DLD-1 Cells. Cytokine-induced NOS from DLD-1 cells was purified in two chromatographic steps by modifications of protocols published for the purification of NOS from murine macrophages (Stuehr et al., 1991; Hevel et al., 1991). The results of a typical purification are shown in Table II. A series of selective washes removed several proteins that copurified with NOS on ADP–Sepharose. Most of the additional contaminating proteins were removed by anion-exchange chromatography. The peak of NOS activity eluted from the DEAE Bio-Gel A column at approximately 0.2 M NaCl. The NOS-specific activity of extracts from DLD-1 cells was about 20 times lower than the specific activity of extracts from RAW 264.7 cells; 1–2 μg of purified NOS was obtained from 15–20 mg of extracted protein.

NOS purified from DLD-1 cells was stabilized by the addition of BSA and storage at −70 °C. Compared to storage at −70 °C in the presence of 1 mg/mL BSA, enzyme activity declined by 30% after overnight storage at −70 °C in the absence of BSA or at 4 °C in the presence of BSA, and declined by 60% after overnight storage at 4 °C in the absence of BSA.

SDS/PAGE analysis of the NOS peak fraction from the ADP–Sepharose column revealed a protein with a molecular

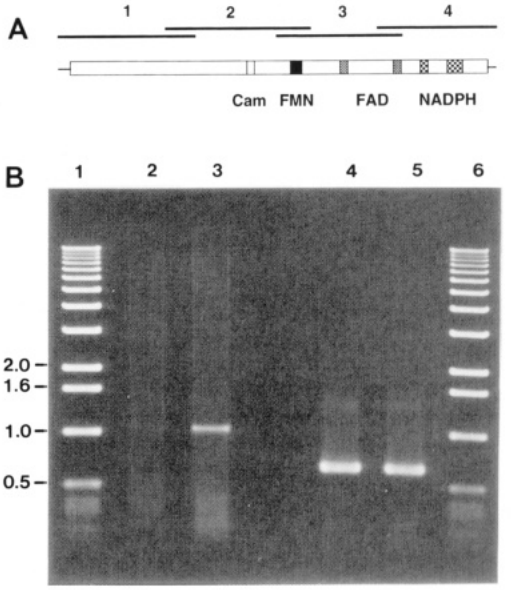

FIGURE 2: Amplification of NOS cDNA from DLD-1 cells by RNA-PCR. (A) Schematic of NOS cDNA. The coding sequence is depicted as a boxed region; the noncoding sequence is depicted as a line. Predicted amplification products are indicated above the cDNA. (B) Products of RNA-PCR reactions were analyzed by agarose gel electrophoresis. Lanes 1 and 6, molecular mass markers (kb). Lanes 2 and 4, uninduced DLD-1 cells. Lanes 3 and 5, induced DLD-1 cells. The primer pair for fragment 3 of the DLD-1 cDNA was used in lanes 2 and 3; a primer pair for human β-actin was used in lanes 4 and 5.

Table III: Calcium/Calmodulin Dependence of NOS Purified from DLD-1 Cells, RAW 264.7 Cells, and Bovine Brain

conditions	rel act. DLD-1	rel act. RAW	rel act. bovine brain
no additions	100	100	0
+1 mM EGTA	40	74	
+1 mM Ca^{2+}	100	108	
+10 μg/mL calmodulin	97	101	
+Ca^{2+}, +10 μg/mL calmodulin	101	103	100[a]
+50 μM trifluoperazine	96	96	0[a]
+50 μM R24571	96	8	0[a]

[a] 1 mM Ca^{2+}, 1 μg/mL calmodulin.

mass of ~130 kDa (Figure 1A, lane 4). This protein migrated with a slightly lower mobility than NOS purified by the same method from RAW 264.7 cells (Figure 1A, lane 7). When the purification was performed with an equivalent amount of starting material from DLD-1 cells that were not treated with cytokines, the 130-kDa protein was not detected (Figure 1A, compare lanes 3 and 4). Therefore, the 130-kDa protein was specifically induced in DLD-1 cells by cytokine treatment. The identity of the 130-kDa protein was further confirmed as NOS by Western blot analysis. An antiserum to a murine macrophage NOS fusion protein reacted with both the putative NOS from DLD-1 cells and the NOS purified from murine macrophages (Figure 1B, lanes 2 and 3).

Constitutive isoforms of NOS, including the enzymes from brain and vascular endothelium, require the addition of calcium and calmodulin for activity after purification and are inhibited by EGTA and a variety of calmodulin antagonist (Bredt & Snyder, 1990; Schmidt et al., 1991; Pollack et al., 1991). In contrast, the cytokine-induced NOS purified from murine macrophages does not require added calcium or calmodulin (Stuehr et al., 1991; Hevel et al., 1991). The calcium/calmodulin dependence of inducible NOS purified from DLD-1 cells compared with enzymes purified from murine

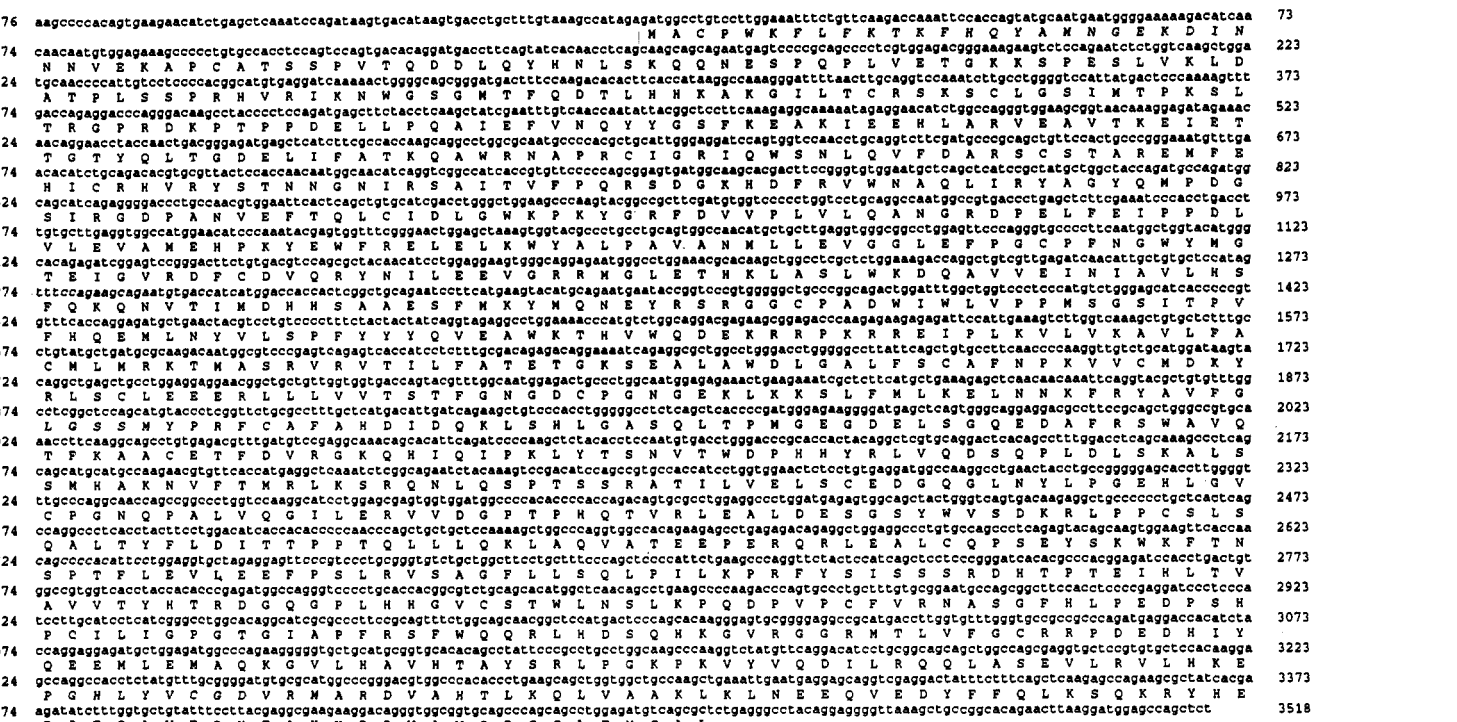

FIGURE 3: Nucleic acid and deduced amino acid sequences of DLD-1 NOS cDNA. Sequence numbering begins at the first nucleotide of the initiation codon.

macrophages and bovine brain is shown in Table III. Both the DLD-1 and murine enzymes were significantly inhibited by 1 mM EGTA. The inhibition of DLD-1 NOS by 1 mM EGTA could be overcome by adding an excess of either calcium or magnesium (data not shown). Neither inducible NOS was stimulated by the addition of calcium and/or calmodulin, whereas purified brain NOS was completely dependent on added calcium and calmodulin for activity. Trifluoperazine, a calmodulin antagonist, had no effect on the activity of either inducible isoform at a concentration that completely inhibited the brain enzyme. A second calmodulin antagonist, R24571, completely inhibited the brain enzyme, greatly inhibited the murine macrophage enzyme, and had no effect on the DLD-1 enzyme.

The apparent K_m of the purified DLD-1 enzyme for L-arginine was 3 μM.

Isolation and Characterization of a cDNA for Human Inducible NOS. RNA-PCR on poly(A^+) RNA obtained from cytokine-treated DLD-1 cells was used to isolate overlapping cDNA clones that encode a human inducible NOS. The human chondrocyte NOS cDNA sequence (Charles et al., 1993) was used to design four sets of oligonucleotide primers that encompass the complete coding region of the cDNA plus a small amount of 5′ and 3′ untranslated sequence (Figure 2A). The results obtained with the primers for fragment 3 are shown in Figure 2B. When RNA from untreated DLD-1 cells was used as the template for RNA-PCR, no amplification product of the expected size was observed (Figure 2B, lane 2). The PCR products were inserted into a plasmid vector, and the sequence of both strands of the cloned cDNA was determined.

A contiguous DLD-1 NOS cDNA sequence of 3595 bp was compiled from sequences of the overlapping PCR products. This sequence contains an open reading frame encoding a 1153 amino acid protein with a calculated molecular mass of 131 116 Da (Figure 3). This is slightly larger than the calculated molecular mass of the murine macrophage NOS (130 566 Da). The NOS from DLD-1 cells is very similar to the NOS from murine macrophages; the deduced amino acid sequences are 81% identical. DLD-1 NOS is 58% identical to human brain NOS (Nakane et al., 1993) and 52% identical to human endothelial NOS (Janssens et al., 1992).

The nucleic acid sequence that we obtained for DLD-1 NOS cDNA is identical to a recently obtained cDNA sequence for human chondrocyte NOS (Charles et al., 1993), except for two single-base substitutions that do not result in a difference in the deduced amino acid sequences for the proteins. We also compared the DLD-1 NOS cDNA sequence to a recently published cDNA sequence for inducible NOS from human hepatocytes (Geller et al., 1993). The DLD-1 sequence differs from the hepatocyte sequence at 10 scattered nucleotides, resulting in 7 amino acid differences (the deduced amino acid sequences are 99% identical). The DLD-1 cDNA sequence is identical to the hepatocyte sequence at the two nucleotides that differ from the chondrocyte cDNA sequence.

DISCUSSION

An inducible isoform of NOS that causes prolonged release of large quantities of NO is easily observed in a variety of tissues from rodents. The biological role of the macrophage NOS is most likely immune defense. The functions of NOS isoforms induced in liver and vascular smooth muscle are less clear. Induction of NOS in vascular smooth muscle may play a role in septic hypotension; induction of NOS in other tissues may likewise contribute to other pathological states.

It has been difficult to demonstrate induction of NOS in human tissues to the same extent as has been observed in rodents. This has slowed the progress of research directed toward fully characterizing the human inducible enzyme and understanding its role in pathological conditions such as septic shock. Recently, some success has been reported in attempts to induce NOS in primary cultures of human macrophages (Denis, 1991), hepatocytes (Nussler et al., 1992), and smooth muscle (Scott-Burden et al., 1992). Induction appears to occur most reproducibly and to the highest level in hepatocytes.

In an effort to obtain an easily accessible supply of human tissue for purification of inducible NOS, we screened a number of human cell lines. NOS activity was detected by a relatively insensitive but very simple assay for the production of nitrite in cell culture supernatants. We did not detect induction of NOS activity in any of the human macrophage or liver cell lines that were tested. We were able to induce NOS activity in a human colorectal adenocarcinoma cell line, DLD-1. Although the quantity of NOS induced in DLD-1 cells was considerably lower than what we had been able to achieve in a murine macrophage cell line, sufficient activity was present to attempt purification. A purification of more than a thousandfold was achieved in two chromatographic steps. A rabbit antiserum to the murine macrophage NOS cross-reacted with the DLD-1 NOS, and the two purified enzymes displayed a similar lack of dependence on exogenous calcium and calmodulin for activity. It was recently shown that purified murine macrophage NOS contains tightly bound calmodulin (Cho et al., 1992); we are currently investigating whether the same is true for the DLD-1 enzyme. As has been reported for an inducible rat liver NOS (Iida et al., 1992), it may be possible to remove at least a portion of this tightly bound calmodulin from the enzyme during purification by including metal chelators in the column buffers.

There are some apparent discrepancies in the literature regarding the calcium and calmodulin dependence of inducible NOS. Enzymes from different tissues would probably best be compared after purification by identical methods. It is generally agreed that the interaction between calmodulin and inducible NOS is different from that between calmodulin and constitutive NOS from brain and vascular endothelium. A lower concentration of calcium is required for the interaction of calmodulin with inducible NOS (Cho et al., 1992). A variety of calmodulin antagonists, including trifluoperazine, do not inhibit inducible NOS at a concentration that abolishes the activity of brain NOS. The differential inhibition of inducible enzymes from RAW 264.7 and DLD-1 cells that we observed with the calmodulin antagonist R24571 may be due to a species difference in the calmodulin binding site. The metal-chelating agents EDTA and EGTA partially inhibit inducible NOS at concentrations that totally inhibit the constitutive isoforms—the mechanism for this inhibition is not clear. The RAW 264.7 and rat liver enzymes appear to be less sensitive to metal chelators than the DLD-1 and human liver enzymes (our data; Geller et al., 1993; Iida et al., 1992; Evans et al., 1992), another possible indication of a species difference in the calmodulin binding site.

There are at least two distinct types of constitutive NOS, the brain and the endothelial forms. When we began this work, it was not known if there were multiple, tissue-specific forms of inducible NOS. We recently reported that the murine macrophage and the rat liver enzymes are essentially identical at the genetic level (Wood et al., 1993). Furthermore, the cDNA for inducible NOS from rat vascular smooth muscle (Nunokawa et al., 1993) appears to be identical to the cDNA for inducible NOS from rat liver. We have isolated and characterized a cDNA for an inducible NOS from DLD-1 cells. Coincident with our studies, cDNAs for inducible NOS from primary human chondrocytes (Charles et al., 1993) and primary human hepatocytes (Geller et al., 1993) were cloned and sequenced. We have also completely sequenced an inducible NOS cDNA from primary human hepatocytes, and partially sequenced NOS cDNAs from primary human smooth muscle and macrophages (unpublished data). The accumulated sequence data suggest that, at the genetic level, there is only one form of inducible NOS. Although the identity at the genetic level does not preclude the possibility of tissue-specific posttranslational variations in the inducible isoform, it should simplify the path toward more fully understanding the biological roles of inducible NOS.

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