Proc. Natl. Acad. Sci. USA
Vol. 90, pp. 11419–11423, December 1993
Biochemistry

Cloning, characterization, and expression of a cDNA encoding an inducible nitric oxide synthase from the human chondrocyte

IAN G. CHARLES*†, RICHARD M. J. PALMER*, MARK S. HICKERY‡, MICHAEL T. BAYLISS‡, ANN P. CHUBB*, VALERIE S. HALL*, DAVID W. MOSS*, AND SALVADOR MONCADA*

*Wellcome Research Laboratories, Langley Court, Beckenham, Kent BR3 3BS, United Kingdom; and ‡Kennedy Institute of Rheumatology, Hammersmith, London W6 7DW, United Kingdom

Communicated by Gertrude B. Elion, July 22, 1993 (received for review March 5, 1993)

ABSTRACT **Incubation of human articular chondrocytes with interleukin 1β results in the time-dependent expression of nitric oxide (NO) synthase. We report here the isolation of a cDNA clone which encodes a protein of 1153 amino acids with a molecular mass of 131,213 Da and a calculated isoelectric point of 7.9. CHO cells transfected with a plasmid harboring this cDNA clone expressed NO synthase activity that was inhibited by some L-arginine analogues. The deduced amino acid sequence of the human chondrocyte inducible NO synthase shows 51% identity and 68% similarity with the endothelial NO synthase and 54% identity and 70% similarity with the neuronal NO synthase. The similarity (88%) between the human chondrocyte NO synthase cDNA sequence and that reported for the murine macrophage suggests that the inducible class of enzyme is conserved between different cell types and across species.**

The synthesis of nitric oxide (NO) from L-arginine is now recognized as an important pathway for regulating the function of a wide variety of cells and tissues. NO exerts many of these effects through activation of the soluble guanylate cyclase (1). In the vessel wall, NO is synthesized by the vascular endothelium, to regulate smooth muscle tone and thus blood pressure (2–7). NO synthase is also present in the central nervous system, where NO is a neurotransmitter/neuromodulator mediating the action of glutamate on *N*-methyl-D-aspartate receptors (8–11) and mediating/modulating transmission in nerves previously recognized as nonadrenergic and noncholinergic (12). NO can also act as an autocrine regulator of some cells, including platelets, where it modulates their activation (13).

NO generated by activated macrophages is also an important effector molecule in host defense, through a mechanism involving its interaction with iron-sulfur-centered enzymes (14) and/or superoxide anions (15). In this role, NO has been shown to possess antitumor (16) and antimicrobial activity against various parasites *in vitro* (17) and *in vivo* (18).

NO is synthesized from L-arginine by the action of NO synthase(s), generating citrulline as a co-product. These enzymes are all NADPH-, FAD-, FMN-, and tetrahydrobiopterin-dependent (19). Both the neuronal and inducible NO synthases are P450-type heme proteins (20). In addition to control at the enzyme level, NO synthesis can also be regulated through the expression of different enzymes in various cell types. In endothelial cells (21, 22) and neuronal (9, 10) and other (13, 23, 24) tissues, NO synthase activity is constitutively expressed and has a requirement for Ca^{2+} and calmodulin (22). In contrast, NO synthase is synthesized *de novo* in macrophages, hepatocytes, Kupffer cells, vascular smooth muscle, and vascular endothelium following activation with endotoxin and/or cytokines (1, 19). The inducible NO synthase in these cells does not require exogenous Ca^{2+} or calmodulin for activity, and the induction is inhibited by glucocorticoids (25, 26). Interestingly, the enzyme in murine macrophages has been shown to have calmodulin tightly bound to the protein (27).

Recently, NO synthase has been shown to be induced in rabbit articular chondrocytes (28, 29), where the enzyme is Ca^{2+}-dependent and not affected by calmodulin inhibitors or glucocorticoids (29). We have recently extended this observation to human chondrocytes, where the enzyme is induced by interleukin 1β (IL-1β) but, unlike that from the rabbit chondrocyte, is Ca^{2+}-independent and marginally affected by glucocorticoids (30). The induction of NO synthase in human cells has previously only been reliably shown in hepatocytes (31).

The NO synthases comprise a family that can be distinguished on the basis of comparative DNA sequence analysis. Sequences have been reported for the cDNAs encoding the constitutive neuronal NO synthases from rat and man (32, 33), the constitutive endothelial NO synthases from bovine and human tissue (34–37), and the inducible NO synthase from a rodent macrophage line (38–40). A comparison of the deduced protein sequences derived from the three classes of cDNAs shows, overall, 50–60% similarity and a high degree of similarity with the enzyme cytochrome P450 reductase.

Although two distinct forms of the constitutive enzyme have been described at the molecular level, with high identity between species for both neuronal forms (33) and endothelial forms (37), only the murine macrophage form of the inducible NO synthase (38–40) has been similarly characterized. In the present study we have characterized the inducible form of NO synthase from human articular chondrocytes activated with IL-1β, by cloning and expressing the full-length cDNA from these cells.§

MATERIALS AND METHODS

Cell Culture and Isolation of mRNA. Human chondrocytes were isolated and cultured as described (30). Cells were incubated with or without IL-1β (1 ng/ml) for 24 hr, harvested with trypsin, washed, and frozen at −70°C. Poly(A)$^+$ mRNA (1–2 μg) was extracted with a Micro-FastTrack kit (Invitrogen) from 1–2 × 10^6 cells. The murine macrophage cell line J774 was cultured and induced to express NO synthase as described previously (41), and poly(A)$^+$ mRNA was extracted as described above. Dihydrofolate reductase-negative (DHFR$^-$) CHO cells were maintained in 75-cm^2 flasks in Dulbecco's modified Eagle's medium (GIBCO) plus 10% fetal bovine serum, 1 mM L-glutamine, nonessential

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: IL-1, interleukin 1; DHFR, dihydrofolate reductase; L-NIO, N^{δ}-iminoethyl-L-ornithine; L-NMMA, N^{ω}-monomethyl-L-arginine.

†To whom reprint requests should be addressed.

§The sequence reported in this paper has been deposited in the GenBank database (accession no. X73029).

amino acids, antibiotics, 100 μM hypoxanthine, and 16 μM thymidine. pSVL-transfected cells were cultured in the absence of hypoxanthine and thymidine but in the presence of dialyzed fetal bovine serum and 100 μM methotrexate. Samples (100 μl) were removed from triplicate cultures at 24-hr intervals and stored at 4°C before determination of NO_2^- by chemiluminescence (3).

Identification of Human Chondrocyte Inducible NO Synthase by Reverse Transcription–Polymerase Chain Reaction (RT–PCR) and Construction of a cDNA Library. We have previously used the degenerate oligonucleotides BB3 (5′-CGGGATCCGGNACNGGNATHGCNCCNTT-3′) and BB4 (5′-GCGAATTCNCCRCANACRTADATRTG-3′) (35) to clone a fragment of the rabbit chondrocyte inducible NO synthase cDNA (30). The same primers (50 ng) were used to amplify random-primed cDNA generated from human induced and uninduced chondrocyte poly(A)$^+$ mRNA (50 ng) by using a GeneAmp RT–PCR kit (Perkin–Elmer/Cetus). The primers, AL14 (5′-ACGGAGAAGCTTAGATCTG-GAGCAGAAGTG-3′) and AL15 (5′-CTGCAGGTTGGAC-CACTGGATCCTGCCGAT-3′) were derived from the RAW 264.7 sequence (38–40) and generated a 630-bp band corresponding to the 5′ end of the inducible NO synthase from J774 cells. This fragment was used as a probe to screen the λZAPII (Stratagene) human chondrocyte cDNA library. The following conditions were used for 30 cycles: denaturation at 96°C, 35 sec; anneal at 55°C, 2 min; and extension at 72°C, 3 min. PCR products from BB3 and BB4 were digested with *Eco*RI/*Bam*HI and those from AL14 and AL15 with *Hin*dIII/*Bam*HI, before being cloned into pBluescript SKII(+) (Stratagene) by standard methods (42).

Poly(A)$^+$ mRNA (1.5 μg) isolated from chondrocytes activated with IL-1β (1 ng/ml) for 24 hr was used to generate a cDNA library in the bacteriophage λZAPII. Both random and oligo(dT) primers were used in the cDNA synthesis, and 5×10^5 independent recombinant phages were generated. Phages were amplified once, and 10^6 plaques were plated out and screened (in duplicate) by standard techniques (42) with the 630-bp fragment from the murine inducible cDNA clone labeled with α-^{32}P.

Hybridization and DNA Sequencing. Blot and plaque hybridizations were carried out on GeneScreenPlus hybridization membranes (DuPont). Northern blot analysis was carried out using digoxigenin-labeled probes (Boehringer Mannheim) after electrophoresis and transfer of mRNA from denaturing formaldehyde/agarose gels (42). The signal was developed by using digoxigenin antibody coupled to alkaline phosphatase and the chemiluminescent substrate 3-(2′ spirodamantane)-4-methoxy-4-(3″-phosphoryloxy)phenyl-1,2-dioxetane, disodium salt. The filter was washed, exposed to film for 10 min, and then developed.

Recombinant DNA was sequenced by using double-stranded DNA as template (43). An overlapping series of deletions was made in template DNA (44) with the exonuclease III kit (Pharmacia). Sequencing was carried out using universal primer, [α-^{35}S]dATP, wedge gels (45), and modified T7 DNA polymerase (46). Compression artifacts (47) were resolved using 7-deaza-dGTP (Pharmacia). Gaps in the sequence were filled in by using synthetic oligonucleotides (48).

Generation of a Stable CHO Cell Line Expressing NO Synthase. DHFR$^-$ CHO cells were cotransfected with 10 μg of the full-length cDNA for the human inducible NO synthase, pSVL-NO, cloned as an *Xba* I fragment into the vector pSVL (Pharmacia), and with 1 μg of the DHFR-encoding plasmid pRDN2. NO synthesis by individual clones was assayed as NO_2^- concentration in growth medium. One cell line, CHO-INOS-20, was selected for further study.

Preparation and Assay of NO Synthase in CHO-INOS Cytosol. NO synthase was prepared from confluent cultures (29). Parental CHO and transfected-control cytosols were prepared similarly and protein concentration was estimated with a Coomassie blue dye binding kit (Pierce). NO synthase activity was measured spectrophotometrically (29). Reactions were initiated by the addition of L-arginine (30 μM). In some experiments the effects of calmodulin, EGTA, calmidazolium (R24571), and the NO synthase inhibitors N^{δ}-iminoethyl-L-ornithine (L-NIO) and N^{ω}-monomethyl-L-arginine (L-NMMA) (1) were investigated.

RESULTS

Cloning of an Inducible Human NO Synthase cDNA. The strategy used to clone the human chondrocyte inducible NO synthase cDNA relied on the finding that significant levels of NO synthase activity could be induced in these cells by IL-1β (30). Northern blotting showed the presence of a 4.4-kb NO synthase-specific band in mRNA extracted from induced cells that was absent in uninduced cells (Fig. 1). In preliminary experiments using RT–PCR and degenerate oligonucleotide primers (BB3 and BB4), a 350-bp fragment of the rabbit chondrocyte inducible NO synthase was cloned and sequenced which had >90% identity with the murine inducible NO synthase cDNA sequence over this region (data not shown). When RT–PCR was carried out on human chondrocyte mRNA with the same primer set a similar 350-bp band was purified, cloned, and sequenced. Analysis of the sequence demonstrated that the human chondrocyte inducible NO synthase fragment had >80% identity with the murine inducible NO synthase cDNA sequence over this region.

A cDNA library was constructed in λZAPII by using oligo(dT) and random primed poly(A)$^+$ mRNA isolated from induced human cells. To obtain a full-length clone, an α-^{32}P-labeled probe was prepared from a 630-bp 5′ fragment of the murine inducible NO synthase cDNA cloned from mRNA isolated from the cell line J774, whose cDNA sequence is identical to that described for the RAW 264.7 cell line (unpublished observations).

Several clones were identified, one of which (pBS HSI-NOS) contained the full-length cDNA for inducible NO synthase. DNA sequence analysis of the 4164-bp cDNA clone showed the presence of an open reading frame capable of encoding a protein of 1153 amino acids with a calculated molecular mass of 131,213 Da (Fig. 2). The putative ATG initiation codon lies in a Kozak consensus sequence (TAGAGATGG; ref. 49). The sequence of the human inducible NO synthase is highly related to that of the murine enzyme, which comprises 1144 amino acids with a calculated molecular mass of 130,556 Da. Overall the two proteins have

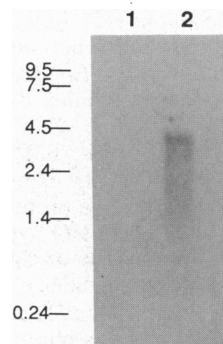

FIG. 1. Northern blot analysis of inducible NO synthase-specific mRNA from human chondrocytes. Poly(A)$^+$ mRNA (0.25 μg per lane) was electrophoresed and the blot was hybridized with a full-length cDNA probe. A positively hybridizing band at 4.4 kb is apparent only in lane 2 (induced cells). Lane 1 was loaded with the same amount of poly(A)$^+$ mRNA from uninduced cells and shows no positively hybridizing band. The positions of molecular size (kb) markers are indicated.

```
NNOS  MEDHMFGVQQ IQPNVISVRL FKRKVGGLGF LVKERVSKPP VIISDLIRGG   50
NNOS  AAEQSGLIQA GDIILAVNGR PLVDLSYDSA LEVLRGIASE THVVLILRGP  100
NNOS  EGFTTHLETT FTGDGTPKTI RVTQPLGPPT KAVDLSHQPP AGKEQPLAVD  150
NNOS  GASGPGNGPQ HAYDDGQEAG SLPHANGWPQ APRQDPAKKA TRVSLQGRGE  200

ENOS  .......... ........MG NLKSVAQEPG PPCGLGLGLG LGLCGKQGPA   32
NNOS  NNELLKEIEP VLSLLTSGSR GVKGGAPAKA EMKDMGIQVD RDLDGKSHKP  250
INOS  .......... .MACPWKFLF KTKFHQYAMN GEKDINNNVE KAPCATSSPV   39

ENOS  TPAP...... .....EPSRA PASLLPPAPE HSPPSSPLTQ .......PPE   64
NNOS  LPLGVENDRV FNDLWGKGNV PVVLNNPYSE KEQPPTSGKQ SPTKNGSPSK  300
INOS  TQDDLQYHNL SKQQNESPQP LVE.....TG KKSPESLVKL DAT....PLS   80

ENOS  GPKFPRVKNW EVGSITYDTL SAQAQQDGPC TPRRCLGSLV FPRKLQGRPS  114
NNOS  CPRFLKVKNW ETEVVLTDTL HLKSTLETGC TEYICMGSIM HPSQ.HARRP  349
INOS  SPRHVRIKNW GSGMTFQDTL HHKAKGILTC RSKSCLGSIM TPKSLTRGPR  130

ENOS  PGPPAPEQLL SQARDFINQY YSSIKRSGSQ AHEQRLQEVE AEVAATGTYQ  164
NNOS  EDVRTKGQLF PLAKEFIDQY YSSIKRFGSK AHMERLEEVN KEIDTTSTYQ  399
INOS  DKPTPPDELL PQAIEFVNQY YGSFKEAKIE EHLARVEAVT KEIETTGTYQ  180

ENOS  LRESELVFGA KQAWRNAPRC VGRIQWGKLQ VFDARDCRSA QEMFTYICNH  214
NNOS  LKDTELIYGA KHAWRNASRC VGRIQWSKLQ VFDARDCTTA HGMFNYICNH  449
INOS  LTGDELIFAT KQAWRNAPRC IGRIQWSNLQ VFDARSCSTA REMFEHICRH  230

ENOS  IKYATNRGNL RSAITVFPQR CPGRGDFRIW NSQLVRYAGY RQQDGSVRGD  264
NNOS  VKYATNKGNL RSAITIFPQR TDGKHDFRVW NSQLIRYAGY KHRDGSTLGD  499
INOS  VRYSTNNGNI RSAITVFPQR SDGKHDFRVW NAQLIRYAGY QMPDGSIRGD  280

ENOS  PANVEITELC IQHGWTPGNG RFDVLPLLLQ APDEPPELFL LPPELVLEVP  314
NNOS  PANVQFTEIC IQQGWKPPRG RFDVLPLLLQ ANGNDPELFQ IPPELVLELP  549
INOS  PANVEFTQLC IDLGWKPKYG RFDVVPLVLQ ANGRDPELFE IPPDLVLEVA  330

ENOS  LEHPTLEWFA ALGLRWYALP AVSNMLLEIG GLEFPAAPFS GWYMSTEIGT  364
NNOS  IRHPKFEWFK DLALKWYGLP AVSNMLLEIG GLEFSACPFS GWYMGTEIGV  599
INOS  MEHPKYEWFR ELELKWYALP AVANMLLEVG GLEFPGCPFN GWYMGTEIGV  380

ENOS  RNLCDPHRYN ILEDVAVCMD LDTRTTSSLW KDKAAVEINV AVLHSYQLAK  414
NNOS  RDYCDNSRYN ILEEVAKKMN LDMRKTSSLW KDQALVEINI AVLYSFQSDK  649
INOS  RDFCDVQRYN ILEEVGRRMG LETHKLASLW KDQAVVEINI AVLHSFQKQN  430

ENOS  VTIVDHHAAT ASFMKHLENE QKARGGCPAD WAWIVPPISG SLTPVFHQEM  464
NNOS  VTIVDHHSAT ESFIKHMENE YRCRGGCPAD WVWIVPPMSG SITPVFHQEM  698
INOS  VTIMDHHSAA ESFMKYMQNE YRSRGGCPAD WIWLVPPMSG SITPVFHQEM  480

                                 __________CaM__________
ENOS  VNYFLSPAFR YQPDPWKGSA AKGTGITRKK ....TFKEVA NAVKISASLM  510
NNOS  LNYRLTPSFE YQPDPWNTHV WKGTNGTPTK RRAIGFKKLA EAVKFSAKLM  749
INOS  LNYVLSPFYY YQVEAWKTHV WQD.EKRRPK RREIPLKVLV KAVLFACMLM  529

      ________
ENOS  GTVMAKRVKA TILYGSETGR AQSYAQQLGR LFRKAFDPRV LCMDEYDVVS  560
NNOS  GQAMAKRVKA TILYATETGK SQAYAKTLCE IFKHAFDAKV MSMEEYDIVH  799
INOS  RKTMASRVRV TILFATETGK SEALAWDLGA LFSCAFNPKV VCMDKYRLSC  579

ENOS  LEHETLWLVV TSTFGNGDPP ENGESFAAAL MEMSGPYNSS PRPEQHKSYK  610
NNOS  LEHETLVLVV TSTFGNGDPP ENGEKFGCAL MEMRHP...N SVQEERKSYK  846
INOS  LEEERLLLVV TSTFGNGDCP GNGEKLKKSL FMLKELNN.. ..........  617

                                           ________________
ENOS  IRFNSISCSD PLVSSWRRKR KESSNTDSAG ALGTLRFCVF GLGSRAYPHF  660
NNOS  VRFNSVSSYS DSQKSSGDGP DLRDNFESAG PLANVRFSVF GLGSRAYPHF  896
INOS  .......... .......... .......... ...KFRYAVF GLGSSMYPRF  634

      ____FMN_________
ENOS  CAFARAVDTR LEELGGERLL QLGQGDELCG QEEAFRGWAQ AAFQAACETF  710
NNOS  CAFGHAVDTL LEELGGERIL KMREGDELCG QEEAFRTWAK KVFKAACDVF  946
INOS  CAFAHDIDQK LSHLGASQLT PMGEGDELSG QEDAFRSWAV QTFKAACETF  684

ENOS  CVGEDAK..A AARDIFSPKR SWKRQRYRLS AQAEGLQLLP GLIHVHRRKM  758
NNOS  CVGDDVNIEK ANNSLISNDR SWKRNKFRLT FVAEAPELTQ GLSNVHKKRV  996
INOS  DVRGKQHIQI P..KLYTSNV TWDPHHYRLV QDSQPLDLSK ALSSMHAKNV  732

                                  _____FAD-PPi____
ENOS  FQATIRSVEN LQSSKSTRAT ILVRLDTGGQ EGLQYQPGDH IGVCPPNRPG  808
NNOS  SAARLLSRQN LQSPKSSRST IFVRLHTNGS QELQYQPGDH LGVFPGNHED 1046
INOS  FTMRLKSRQN LQSPTSSRAT ILVELSCEDG QGLNYLPGEH LGVCPGNQPA  782

ENOS  LVEALLSRVE DPPAPTEPVA VEQL.EKGSP GGPPPGWVRD PRLPPCTVRQ  857
NNOS  LVNALIERLE DAPPVNQMVK VELLEERNTA LGVISNWTDE LRLPPCTIFQ 1096
INOS  LVQGILERVV DGPTPHQTVR LEALDESG.. ....SYWVSD KRLPPCSLSQ  826

ENOS  ALTFFLDITS PPSPQLLRLL STLAEEPREQ QELEALSQDP RRYEEWKWFR  907
NNOS  AFKYYLDITT PPTPLQLQQF ASLATSEKEK QRLLVLSKGL QEYEEWKWGK 1146
INOS  ALTYFLDITT PPTQLLLQKL AQVATEEPER QRLEALCQ.P SEYSKWKFTN  875

                                 ____FAD-ISO____
ENOS  CPTLLEVLEQ FPSVALPAPL LLTQLPLLQP RYYSVSSAPS THPGEIHLTV  957
NNOS  NPTIVEVLEE FPSIQMPATL LLTQLSLLQP RYYSISSSPD MYPDEVHLTV 1196
INOS  SPTFLEVLEE FPSLRVSAGF LLSQLPILKP RFYSISSSRD HTPTEIHLTV  925

ENOS  AVLAYRTQDG LGPLHYGVCS TWLSQLKPGD PVPCFIRGAP SFRLPPDPSL 1007
NNOS  AIVSYRTRDG EGPIHHGVCS SWLNRIQADE LVPCFVRGAP SFHLPRNPQV 1246
INOS  AVVTYHTRDG QGPLHHGVCS TWLNSLKPQD PVPCFVRNAS GFHLPEDPSH  975

        __NADPH-Ribose__________
ENOS  PCILVGPGTG IAPFRGFWQE RLHDIESKGL QPTPMTLVFG CRCSQLDHLY 1057
NNOS  PCILVGPGTG IAPFRSFWQQ RQFDIQHKGM NPCPMVLVFG CRQSKIDHIY 1296
INOS  PCILIGPGTG IAPFRSFWQQ RLHDSQHKGV RGGRMTLVFG CRRPDEDHIY 1025

ENOS  RDEVQNAQQR GVFGRVLTAF SREPDNPKTY VQDILRTELA AEVHRVLCLE 1107
NNOS  REETLQAKNK GVFRELYTAY SREPDKPKKY VQDILQEQLA ESVYRALKEQ 1346
INOS  QEEMLEMAQK GVLHAVHTAY SRLPGKPKVY VQDILRQQLA SEVLRVLHKE 1075

      ___NADPH-Ade_______
ENOS  RGHMFVCGDV TMATNVLQTV QRILATEGDM ELDEAGDVIG VLRDQQRYHE 1157
NNOS  GGHIYVCGDV TMAADVLKAI QRIMTQQGKL SAEDAGVFIS RMRDDNRYHE 1396
INOS  PGHLYVCGDV RMARDVAHTL KQLVAAKLKL NEEQVEDYFF QLKSQKRYHE 1125

ENOS  DIFGLTLRTQ EVTSRIRTQS FSLQERQLRG AVPWAFDPPG SDTNSP*... 1203
NNOS  DIFGVTLRTI EVTNRLRSES IAFIEESKKD TDEVFSS*.. .......... 1433
INOS  DIFGAVFPYE AKKDRVAVQP SSLEMSAL*. .......... .......... 1153
```

FIG. 2. Alignment of deduced sequences from human neuronal (NNOS; ref. 33), endothelial (ENOS; refs. 35 and 37), and chondrocyte (INOS) NO synthase sequences. Gaps introduced in the sequences to optimize alignments are shown as dots. Conserved residues found in all three sequences are shown in bold, and regions are marked depicting consensus motifs: CaM (putative calmodulin binding site), FMN (flavin mononucleotide binding), FAD-PPi (flavin adenine dinucleotide pyrophosphate), FAD-ISO (FAD isoalloxazine), NADPH-Ribose (nicotinamide adenine dinucleotide phosphate ribose), and NADPH-Ade (NADPH-adenine).

81% identity and 88% similarity, as determined by the GAPALIGN program (Wisconsin Genetics Computer Group). Both molecules have consensus recognition sites for the cofactors FAD, FMN, and NADPH and a calmodulin recognition motif, although both enzymes are Ca^{2+}-independent (30, 38).

Comparison of the deduced sequence of the human inducible NO synthase with those of the two other human NO synthases (Fig. 2) shows 51% and 54% identity and 68% and 70% similarity with the endothelial and neuronal NO synthases, respectively. The human inducible enzyme is the shortest member of the family, lacking a 45-amino acid region from the middle of the molecule that is present in both the other enzymes. All three enzymes showed similarity in those regions representing the consensus motifs for binding of calmodulin, FAD, FMN, and NADPH.

Transfection of the pSVL-NO construct into CHO cells led to the isolation of a stable cell line expressing human inducible NO synthase under the control of a heterologous constitutive promoter (Fig. 3). The expressed NO synthase activity was inhibited by L-NIO (100 μM) and by L-NMMA (100 μM).

NO synthase activity in CHO-INOS cytosol was substantially increased following the addition of 30 μM L-arginine (Fig. 4). Neither EGTA (1 mM) nor the calmodulin antagonist calmidazolium (R24571; 10 and 50 μM) had any significant effect on NO synthase activity ($P < 0.05$, Student's t test). The synthesis of NO in the presence of 30 μM L-arginine was inhibited by 1 mM L-NMMA. NO synthase activity was not detected in the cytosols of the untransfected parental or the negative control transfectant cell lines (data not shown).

DISCUSSION

Stimulation of human chondrocytes with 1L-1β results in the expression of NO synthase activity which correlates with the presence of a 4.4-kb mRNA band in Northern blots. It is unlikely that this is due to the presence of a contaminating cell type, since cartilage is known to contain only chondrocytes, which synthesize as much NO per cell (29, 30) as any other cell type so far reported (1). The cDNA for human inducible NO synthase reveals that the gene is capable of encoding a protein with high homology (81% identity and 88% similarity) to the murine inducible NO synthase. A comparison of human chondrocyte inducible NO synthase with human

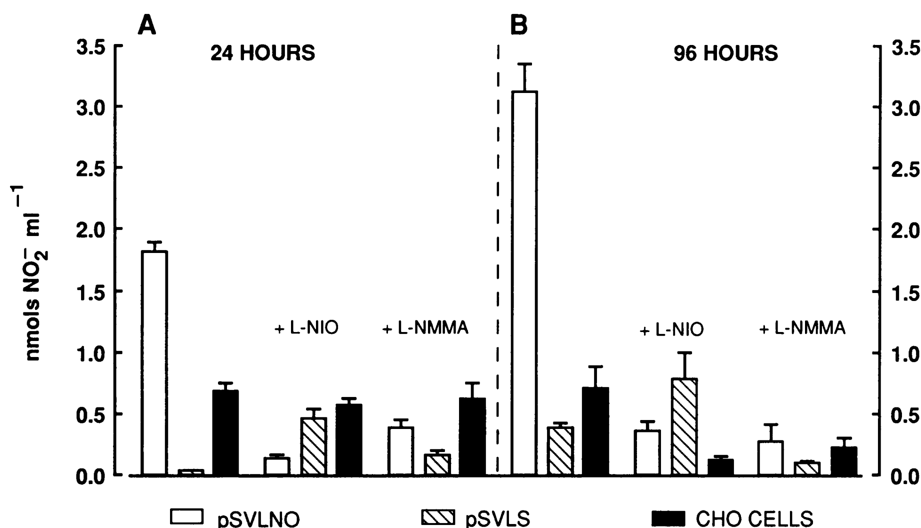

FIG. 3. Expression of recombinant human chondrocyte inducible NO synthase in CHO cells. CHO cells were transfected with pSVL-NO containing the full-length cDNA for human inducible NO synthase, and NO synthase activity was assayed as NO_2^- in the culture supernatant after 24 hr (*A*) or 96 hr (*B*). Controls were parent cells alone (i.e., untransfected; filled bars) and an unrelated CHO-recombinant (pSVLS; hatched bars). Only the pSVL-NO recombinant CHO cells produced significant NO_2^- in the medium (open bars) and this was inhibited by L-NIO (100 μM) and L-NMMA (100 μM).

neuronal and endothelial NO synthases shows the overall identity to be between 50% and 55%. The neuronal sequence is longer than those reported for the endothelial and inducible forms of the enzyme by some 200 amino acids at the N terminus. All three enzymes contain recognition motifs for FAD, FMN, and NADPH cofactor binding. The potential myristoylation site (37) found at the N terminus of the endothelial enzyme is absent from both neuronal and inducible sequences.

Some forms of the inducible enzyme appear to be Ca^{2+}/calmodulin-independent (14, 19, 30), whereas others do not (19, 29). However, the apparent Ca^{2+} dependence of NO synthases may be misleading as a criterion for classifying these enzymes, since each of the three members of the NO synthase family contains a consensus motif for calmodulin binding, suggesting the ability to bind Ca^{2+}/calmodulin, albeit with different affinities. Phosphorylation sites are also

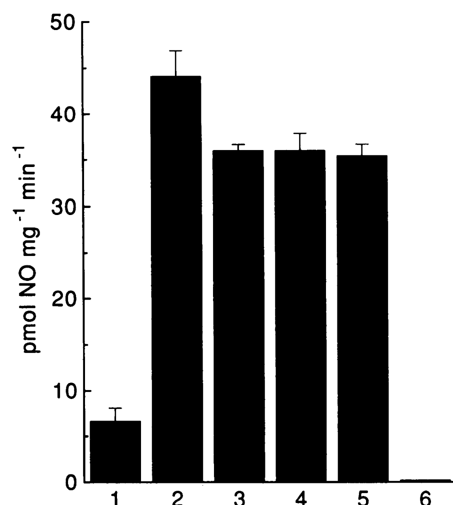

FIG. 4. NO synthesis by CHO-INOS cytosol. Cytosol from CHO cells transfected with pSVL-NO was prepared and assayed as described in *Materials and Methods*. NO synthesis (bar 1) was enhanced by L-arginine (30 μM; bar 2) and was not significantly affected by EGTA (1 mM; bar 3) or calmidazolium (R24571, 10 and 50 μM; bars 4 and 5, respectively). NO synthesis was significantly inhibited by L-NMMA (1 mM; bars 6). For each condition, n = 3.

present in both the murine macrophage (38–40) and human chondrocyte inducible NO synthase sequences, although their importance has yet to be determined.

Two different forms of the RAW 264.7 murine macrophage inducible NO synthase have been characterized by Xie *et al.* (39) by DNA sequence analysis. We looked for similar variations in the human chondrocyte inducible NO synthase by partial sequencing of the 3′ end of a further six cDNA clones and found none (data not shown). If other forms of human inducible NO synthase do exist, it is not known whether they result from differential gene expression, differential splicing, or posttranslational modification.

Transfection of CHO cells with a plasmid carrying the human chondrocyte inducible NO synthase cDNA resulted in the expression of inducible NO synthase activity which was inhibited by L-NMMA and L-NIO. NO synthesis by cytosol from recombinant CHO-INOS was dependent on L-arginine and this activity was abolished by L-NMMA. Removal of free Ca^{2+} by EGTA or addition of the calmodulin antagonist calmidazolium (R24571) had no significant effect on enzyme activity, suggesting that the recombinant inducible NO synthase, in common with NO synthase from human chondrocytes (30), was independent of exogenous Ca^{2+} and calmodulin. The preparation of a stable cell line producing recombinant human inducible NO synthase should allow the isolation and purification of larger amounts of enzyme, so that its properties can be fully evaluated.

Inducible forms of NO synthase are widespread in mammalian tissues (1, 50); however, the role of NO synthase induction in chondrocytes is not understood. IL-1β concentrations are increased in inflamed joints (51, 52), and under these conditions this enzyme is also likely to be induced. The resulting NO may affect cell function, either through the elevation of cGMP levels or through the interaction with iron-containing enzymes involved in respiration and in nucleotide synthesis (1).

Note. After this paper was submitted for review, Geller *et al.* (53) reported the cDNA cloning of a similar inducible NO synthase from human hepatocytes. This sequence differs from the human chondrocyte sequence at seven amino acid positions, and heterologous expression produces an NO synthase that is partially dependent on Ca^{2+} and calmodulin.

We thank Hugh Spence for synthesizing the oligonucleotides used

in this study, Dr. Nigel Sharp for the kind gift of the DHFR plasmid pRDN2, Sally Baylis for computing advice, Neil Foxwell for technical support, Piers Emson for useful discussions, and Maria Parkinson for typing the manuscript.

1. Moncada, S., Palmer, R. M. J. & Higgs, E. A. (1991) *Pharmacol. Rev.* **43**, 109–142.
2. Furchgott, R. W. & Zawadzki, J. (1980) *Nature (London)* **288**, 373–376.
3. Palmer, R. M. J., Ferrige, A. G. & Moncada, S. (1987) *Nature (London)* **327**, 524–526.
4. Ignarro, L. J., Buga, G. M., Wood, K. S., Byrns, R. E. & Chaudhuri, G. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 9265–9269.
5. Palmer, R. M. J., Ashton, D. S. & Moncada, S. (1988) *Nature (London)* **333**, 664–666.
6. Rees, D. D., Palmer, R. M. J. & Moncada, S. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 3375–3378.
7. Aisaka, K., Gross, S. S., Griffith, O. W. & Levi, R. (1989) *Biochem. Biophys. Res. Commun.* **160**, 881–886.
8. Garthwaite, J., Charles, S. L. & Chess-Williams, R. (1988) *Nature (London)* **336**, 385–388.
9. Knowles, R. G., Palacios, M., Palmer, R. M. J. & Moncada, S. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 5159–5162.
10. Bredt, D. S. & Snyder, S. H. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 9030–9033.
11. Garthwaite, J. (1991) *Trends Neurosci.* **14**, 60–67.
12. Gillespie, J. S., Liu, X. & Martin, W. (1989) *Br. J. Pharmacol.* **98**, 1080–1082.
13. Radomski, M. W., Palmer, R. M. J. & Moncada, S. (1990) *Proc. Natl. Acad. Sci. USA* **87**, 5193–5197.
14. Hibbs, J. B., Jr., Taintor, R. R., Vavrin, Z., Granger, D. L., Drapier, J.-C., Amber, I. J. & Lancaster, J. R., Jr. (1990) in *Nitric Oxide from L-Arginine: A Bioregulatory System*, eds. Moncada, S. & Higgs, E. A. (Elsevier, Amsterdam), pp. 189–223.
15. Beckman, J. S., Beckman, T. W., Chen, J., Marshall, P. & Freeman, B. A. (1990) *Proc. Natl. Acad. Sci. USA* **87**, 1620–1624.
16. Hibbs, J. B., Jr., Vavrin, Z. & Taintor, R. R. (1987) *J. Immunol.* **138**, 550–556.
17. James, S. L. & Hibbs, J. B., Jr. (1990) *Parasitol. Today* **6**, 303–305.
18. Liew, F. Y., Millot, S., Parkinson, C., Palmer, R. M. J. & Moncada, S. (1990) *J. Immunol.* **144**, 4794–4797.
19. Stuehr, D. J. & Griffith, O. W. (1992) *Adv. Enzymol.* **65**, 287–346.
20. Stuehr, D. J. & Ikeda-Saito, M. (1992) *J. Biol. Chem.* **267**, 20547–20550.
21. Palmer, R. M. J. & Moncada, S. (1989) *Biochem. Biophys. Res. Commun.* **158**, 348–352.
22. Busse, R. & Mulsch, A. (1990) *FEBS Lett.* **265**, 133–136.
23. Palacios, M., Knowles, R. G., Palmer, R. M. J. & Moncada, S. (1989) *Biochem. Biophys. Res. Commun.* **158**, 348–352.
24. Schulz, R., Smith, J. A., Lewis, M. J. & Moncada, S. (1991) *Br. J. Pharmacol.* **104**, 21–24.
25. Di Rosa, M., Radomski, M., Carnuccio, R. & Moncada, S. (1990) *Biochem. Biophys. Res. Commun.* **172**, 1246–1252.
26. Radomski, M. W., Palmer, R. M. J. & Moncada, S. (1990) *Proc. Natl. Acad. Sci. USA* **87**, 10043–10047.
27. Cho, H. J., Xie, Q. W., Calaycay, J., Mumford, R. A., Swiderek, K. M., Lee, T. D. & Nathan, C. (1992) *J. Exp. Med.* **176**, 599–604.
28. Stadler, J., Stefanovic-Racic, M., Billiar, J. R., Curran, R. D., McIntyre, L. A., Georgescu, H. I., Simmons, R. L. & Evans, C. H. (1991) *J. Immunol.* **147**, 3915–3920.
29. Palmer, R. M. J., Andrews, T., Foxwell, N. A. & Moncada, S. (1992) *Biochem. Biophys. Res. Commun.* **188**, 209–215.
30. Palmer, R. M. J., Hickery, M. S., Charles, I. G., Moncada, S. & Bayliss, M. T. (1993) *Biochem. Biophys. Res. Commun.* **193**, 398–405.
31. Nussler, A. K., Di Silvio, M., Billiar, T. R., Hoffman, R. A., Geller, D. A., Selby, R., Madriaga, J. & Simmons, R. L. (1992) *J. Exp. Med.* **176**, 261–264.
32. Bredt, D. S., Hwang, P. H., Glatt, C. E., Lowenstein, C., Reed, R. R. & Snyder, S. H. (1991) *Nature (London)* **351**, 714–718.
33. Nakane, M., Schmidt, H. H. H. W., Pollock, J. S., Forsterman, U. & Murad, F. (1993) *FEBS Lett.* **316**, 175–180.
34. Lamas, S., Marsden, P. A., Li, G. K., Tempst, P. & Michel, T. (1992) *Proc. Natl. Acad. Sci. USA* **89**, 6348–6352.
35. Janssens, S. P., Shimouchi, A., Quertermous, T., Bloch, D. B. & Bloch, K. D. (1992) *J. Biol. Chem.* **267**, 14519–14522.
36. Sessa, W. C., Harrison, J. K., Barber, C. M., Zeng, D., Durieux, M. E., D'Angelo, D. D., Lynch, K. R. & Peach, M. J. (1992) *J. Biol. Chem.* **267**, 15274–15276.
37. Marsden, P. A., Schappert, K. T., Chen, H. S., Flowers, M., Sundell, C. L., Wilcox, J. N., Lamas, S. & Michel, T. (1992) *FEBS Lett.* **307**, 287–293.
38. Lyons, C. R., Orloff, G. J. & Cunningham, J. M. (1992) *J. Biol. Chem.* **267**, 6370–6374.
39. Xie, Q. W., Cho, H. J., Calaycay, J., Mumford, R. A., Swiderek, K. M., Lee, T. D., Ding, A., Troso, T. & Nathan, C. (1992) *Science* **256**, 225–228.
40. Lowenstein, C. J., Glatt, C. S., Bredt, D. S. & Snyder, S. M. (1992) *Proc. Natl. Acad. Sci. USA* **89**, 6711–6715.
41. Cunha, F. Q., Weiser, W. Y., David, J. R., Moss, D. W., Moncada, S. & Liew, F. Y. (1993) *J. Immunol.* **150**, 1–6.
42. Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab. Press, Plainview, NY), 2nd Ed.
43. Stephen, D., Jones, C. & Schofield, J. P. (1991) *Nucleic Acids Res.* **24**, 7463–7464.
44. Henikoff, S. (1984) *Gene* **28**, 351–359.
45. Sanger, F., Nicklen, S. & Coulson, A. R. (1983) *Proc. Natl. Acad. Sci. USA* **74**, 5463–5467.
46. Tabor, S. & Richardson, C. C. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 4767–4771.
47. Mizusawa, S., Nishimura, S. & Seela, F. (1986) *Nucleic Acids Res.* **14**, 1319–1324.
48. Charles, I. G., Keyte, J., Brammar, W. J., Smith, M. & Hawkins, A. R. (1986) *Nucleic Acids Res.* **14**, 2201–2213.
49. Kozak, M. (1991) *J. Cell Biol.* **115**, 887–903.
50. Salter, M., Knowles, R. G. & Moncada, S. (1991) *FEBS Lett.* **291**, 145–149.
51. Arend, W. P. & Dayer, J. M. (1990) *Arthritis Rheum.* **33**, 305–315.
52. Nguyen, Q., Mort, J. S. & Roughley, T. J. (1992) *J. Clin. Invest.* **89**, 1189–1197.
53. Geller, D. A., Lowenstein, C. J., Shapiro, R. A., Nussler, A. K., Di Silvio, M., Wang, S. C., Nakayama, D. K., Simmons, R. L., Snyder, S. H. & Billiar, T. R. (1993) *Proc. Natl. Acad. Sci. USA* **90**, 3491–3495.