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Stable Expression of Human Inducible Nitric Oxide Synthase in V79 Chinese Hamster Cells

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ABSTRACT. A recombinant expression vector containing the full-length cDNA for human inducible nitric oxide (NO) synthase was constructed for constitutive expression in V79 Chinese hamster cells. Expression was followed by Western analyses using three different NO synthase antisera. Activity remained stable during 4 months of continued cultivation. Activities were 25 pmol min^{-1} mg^{-1} cytosolic protein with L-arginine and 47 pmol min^{-1} mg^{-1} cytosolic protein with N^G-hydroxy-L-arginine as substrates. Activity was concentration-dependently inhibited by inhibitors such as N^G-methyl-L-arginine, N^G-nitro-L-arginine, N^G-nitro-L-arginine methyl ester, aminoguanidine, and S-methyl-isothiourea. The rank order of inhibitor potencies was different from published results obtained with rodent inducible NOS. Parental V79 cells do not express and cannot be induced for NO synthase activity. Therefore, the genetically engineered V79 cell line is defined for the cDNA-encoded human inducible NO synthase. The new cell line may serve as a useful tool to study human inducible NO synthase. BIOCHEM PHARMACOL 52;9:1365–1374, 1996. *Copyright © 1996 Elsevier Science Inc.*

KEY WORDS. nitric oxide; nitric oxide synthase; human; inhibitors; V79 cells; heterologous expression

Nitric oxide is an important mediator in several physiological processes [1, 2]. Three isoforms of NO** synthases generate NO from L-arginine. They are described as the constitutively expressed and the inducible isoforms. The constitutively expressed endothelial isoform NOS3 controls blood pressure and platelet aggregation [3], whereas the constitutively expressed neuronal isoform NOS1 plays an important role in the modulation of neuronal transmission [4]. The activity of both constitutively expressed NO synthase isoforms is regulated receptor-dependently by variations in intracellular calcium levels. In contrast, inducible NO synthase NOS2 is active at the calcium levels in resting cells. The expression of NOS2 is induced in many tissues by proinflammatory cytokines and results in massive NO production far exceeding the range synthesized by the constitutive NOS isoforms [5]. Excess NO impairs various physiological and cellular functions. A critical effect is loss of blood pressure in septic shock. Furthermore, NO can inactivate metal-containing enzymes. This was shown for the heme-thiolate enzymes cytochromes P450, which are known to function as key enzymes in biotransformation [6, 7]. Thus, the NOS2 isoform is responsible for several adverse health effects and poses a potential target for therapeutical intervention [8, 9].

Heterologous expression of NO synthase isoforms is a useful method allowing various studies on these enzymes without the need for human tissue material. Heterologous expression of cDNAs encoding NO synthase isoforms has already been achieved for human NOS2 [10–12], for human and bovine NOS3 [13, 14], and for NOS1 from rat [15, 16].

Here, we report on stable expression of the cDNA en-

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** *Abbreviations:* AG, aminoguanidine; BSA, bovine serum albumin fraction V; CM, cytokine mix; HRP, horseradish peroxidase; IPTG, isopropyl-β-D-thiogalactoside; NAME, N^G-nitro-L-arginine methyl ester; NMA, N^G-monomethyl-L-arginine; NO, nitric acid; NO_2-Arg, N^G-nitro-L-arginine; NOS1, constitutively expressed neuronal isoform of NO synthase (ncNOS); NOS2, inducible isoform of NO synthase (iNOS); NOS3, constitutively expressed endothelial isoform of NO synthase (ecNOS); NTA, nitrilotriacetic acid; PBS, phosphate-buffered saline; SD, standard deviation; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; SMUT, S-methyl-isothiourea; 6-TG, 6-thioguanine; TGF-β1, transforming growth factor-β1; U, units.

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coding human NOS2 cloned from hepatic tissue [10] in V79 Chinese hamster cells. This cell line has characteristics making it a preferred tool for studying toxicological effects. Therefore, a V79 cell line stably expressing human NOS2 may be applied to study this enzyme and its adverse effects.

MATERIALS AND METHODS

Chemicals

L-arginine, N^G-nitro-L-arginine, *S*-methyl isothiourea and aminoguanidine were from Sigma (Deisenhofen, F.R.G.), and N^G-monomethyl-L-arginine monoacetate, N^G-nitro-L-arginine methyl-ester · HCl, and tetrahydrobiopterin came from Alexis Corp. (Läufelfingen, Switzerland).

Construction of the Eukaryotic Human hep-NOS2 Expression Unit

*Eco*RI-linked NOS2 cDNA [10] (GenBank accession #L09210) was excised from pBluescript SK− by partial *Eco*RI digestion and cloned into the *Eco*RI site of pIC19H [17]. One clone oriented with the 5′-end of the cDNA next to the *Sma*I-site and the 3′-end next to the *Cla*I site in the pIC19H multiple cloning site was selected. An ATG triplet in the 5′-noncoding region at position 29 of the cDNA was removed using a unique *Xba*I site in the cDNA at position 47 and *Eco*RI partial digestion. The resulting approximately 4.0-kb-long *Xba*I/*Eco*RI cDNA fragment, termed X/E NOS2, was cloned into the respective sites of the pIC19H multiple cloning site prepared from the dam, dcm strain GM 2167. From this strain, X/E NOS2-cDNA was prepared using the *Hind*III sites flanking the multiple cloning site in pIC19H. The 4.0-kb-long *Hind*III fragment was purified and partially digested with *Bam*HI. The latter partial restriction was allowed to proceed to virtual completeness and the residual 4.0-kb band, representing a mixture of *Hind*III/*Hind*III and *Hind*III/*Bam*HI ending fragments, was ligated into the 3.5-kb vector moiety of the expression vector pSV450 [18] from which rat CYP2B1 cDNA had been removed by restriction with *Hind*III and *Bgl*II. The integrity and correct orientation of the NOS2 expression construct were verified by restriction analysis.

Cell Culture and Cell Lines

V79MZ cells [19] were propagated in Dulbecco Vogt's modified Eagle's medium, DMEM (Seromed, Berlin, F.R.G.), supplemented with 4.5 g/L glucose, 1 mM pyruvate, and 10% fetal calf serum (Gibco, Eggenstein, F.R.G.) in the presence of 100 U/mL penicillin and 100 μg/mL streptomycin at 37°C, 7% carbon dioxide. G418-resistant cell lines were grown under identical conditions in media containing 500 μg/mL G418 sulfate.

AKN1 cells are derived from human liver cells of a normal male donor by spontaneous transformation [20, 21] and express markers for hepatocytes as well as for biliary epithelial cells. This cell line expresses NOS2 upon stimulation with a cytokine mix.

Hepatocytes from male Sprague-Dawley rats were isolated and cultured as described [7].

Transfection of the NOS2 cDNA Expression Unit into V79MZ Cells

Prior to transfection into V79MZ cells, the expression vector pSVhNOS2 was linearized by restriction at the unique *Sca*I site in the pBR322 moiety of the construct. Cotransfection along with *Eco*RI-linearized pSV2NEO DNA was performed using the calcium phosphate coprecipitation method with pSVhNOS2 and pSV2NEO DNAs at a molar ratio of 50:1. G418-resistant clones were selected in media containing 1 mg/mL G418 sulfate. Isolated colonies were screened for production of nitrite in the cell culture supernatant by the Griess reaction [22].

Induction of NOS2 by Treatment with Cytokine Mix

For transcriptional induction of NOS2 in the AKN1 cell line, human liver biopsies, and isolated rat hepatocytes, cells were treated with a mixture of 500 U/mL recombinant murine TNF-α, 5 U/mL recombinant human IL-1 (Genzyme, Cambridge, MA, U.S.A.), 100 U/mL recombinant rat IFN-γ (Gibco/Life Technologies, Long Island, NY, U.S.A.), and 10 μg/mL bacterial lipopolysaccharide from *Escherichia coli* 111:B4 (Sigma, Deisenhofen, F.R.G.). For the suppression of NO synthesis in cytokine mix-treated cultured rat hepatocytes, NMA was included in the medium at a concentration of 0.5 mM.

Preparation of Cytosolic Fractions

The cytosolic fractions from V79MZhNOS2 cells were prepared from cells grown to 70 ± 10% confluency in 15-cm-diameter tissue culture plates. Trypsinated cells were washed twice with PBS. Cells (2×10^7) were resuspended in 500 μL protease inhibitor solution containing 0.1 mM phenylmethylsulfonylfluoride, 5 μg/mL pepstatin, 5 μg/mL aprotinin, and 1 μg/mL chymostatin and lysed by three freeze/thaw cycles using liquid nitrogen and a 30°C water bath. The cellular lysate was centrifuged at 100,000 × g, 4°C, for 45 min. The cytosolic supernatant was separated and stored at −70°C until use. Protein was measured according to Bradford [23] with BSA as the standard.

Enzyme Assays

NOS activity was assessed with the Griess reaction. Cytosolic protein (100–200 μg) was assayed in a volume of 100 μL containing 2 mM NADPH, 100 nM tetrahydrobiopterin, 2 μM FAD, 3 mM reduced glutathione, 40 mM Tris/HCl, pH 7.9, and 4 mM L-arginine final concentrations. Concentrations were chosen according to v_{max} conditions. The reaction was allowed to proceed for 16 hr at 37°C.

Inhibitor studies with cytosolic preparations were carried out under the same conditions, but with exogenous L-arginine added to a final concentration of 4 μM.

NOS activity was also assayed by following the conversion of [^{3}H]-L-arginine to [^{3}H]-L-citrulline. In a total volume of 200 μL, 200 μg of cytosolic protein was incubated in a buffer containing 2 mM NADPH, 2 μM FAD, 3 mM glutathione, 100 nM tetrahydrobiopterin, 50 mM Tris, pH 7.6, and 500,000 dpm of [^{3}H]-L-arginine with a specific activity of 2.22×10^{12} TBq mmol^{-1} at 37°C. In parallel assays, 1.2 mM L-citrulline was included in the incubation. The reactions were allowed to proceed for 45 min. Then, the samples were heated to 95°C for 5 min, 1 mL of a solution containing 1 mM L-arginine and 1 mM L-citrulline was added, and the samples were centrifuged at 12,000 × *g* for 5 min. The supernatant (1000 μL) was applied to an NaOH-equilibrated ion exchange column (AG 50W-X8 resin, 200–400 mesh; BioRad Laboratories, Munich, F.R.G.). The column was rinsed with 1000 μL H_2O, and the eluates were combined. After the addition of 1.5 mL of scintillation liquid (Quickszint 212, Zinsser Analytic, Frankfurt/Main, F.R.G.), the samples were analysed by liquid scintillation counting.

For inhibition studies on expressed NOS2 in live cells, V79MZhNOS2 cells were seeded at a density of 10,000 cells per well in a 24-well plate and precultured for 24 hr in standard medium. The media were then replaced by fresh media containing various concentrations of inhibitors. Nitrite concentrations in the supernatant media were determined 48 hr later with the Griess reaction.

Expression of NOS2 Domains in E. coli

For the generation of NOS2-specific antigens, two domains of NOS2 were expressed in *E. coli*. An 891-bp-long internal *Sst*I cDNA fragment encompassing nucleotide positions 1155–2046 and an approximately 1800-bp-long *Sst*I fragment from nucleotide positions 2187 to 3668 and including parts of the 3′-noncoding region were cloned into the dephosphorylated *Sst*I site of the expression plasmid pQE 30 (Qiagen, Chatsworth, CA, U.S.A.). This vector provides an N-terminal 6× histidine tag to expressed peptides. The 1800-bp fragment was prepared from NOS2 cDNA cloned in the *Eco*RI site of the pIC19H multiple cloning site (see above), making use of the *Sst*I site in the pIC19H multiple cloning site. The recombinant vectors were transfected into the *E. coli* strain M15 [REP 4] kan^R (Qiagen). Plasmid-containing bacteria were selected on LB agar plates containing 25 μg/mL kanamycin, 150 μg/mL ampicillin, and 2% glucose at 30°C. Clones containing NOS2 cDNA fragments were identified by colony hybridization using ^{32}P-labeled NOS2 cDNA as a hybridization probe. Thirteen hybridization-positive clones for each fragment were checked for IPTG-dependent inducibility of an approximately 33-kDa protein in the case of the shorter fragment and of an approximately 55-kDa protein in the case of the longer NOS2 cDNA fragment. Five and four clones, respectively, were identified as IPTG-inducible producers of the desired peptides. One clone for each fragment, designated 891/C3 and 1481/C2, was chosen for further characterization.

The integrity of the expression constructs was verified by restriction analysis of plasmids prepared from selected amp^R, kan^S *E. coli* DH5α [24] transformants into which plasmid preparations from 891/C3 and 1481/C2 had been transfected.

Purification of His-Tagged NOS2 Protein Fragments from E. coli *Lysates*

For expression of NOS2 fragments, M15 [REP 4] transformant cells were precultured for 3–4 hr in 5 mL LB medium containing 150 μg/mL ampicillin, 25 μg/mL kanamycin, and 2% glucose to stationary phase. Cells were pelleted and resuspended in 400 mL LB medium containing 150 μg/mL ampicillin. After 30 min, IPTG was added to a final concentration of 2 mM. Cells were grown under inducing conditions for 16 hr overnight, harvested by centrifugation, and treated with 1 mg/mL lysozyme in 20 mM Tris/HCl, pH 8.0, 2 mM EDTA, for 30 min at 37°C. Lysozyme-treated cells were sonicated and the lysate centrifuged at 12,000 × *g*, 4°C, for 15 min. The particulate pellet was resuspended in inclusion body buffer containing 0.5 M NaCl, 20 mM EDTA, 100 mM Tris/HCl, pH 7.2, 1% Triton X-100, and 0.5 mM phenylmethylsulfonylfluoride. Inclusion bodies were centrifuged at 8000 × *g* for 15 min to give a white pellet. The pellet was resuspended twice in the same buffer and centrifuged again for removal of soluble *E. coli* host protein contaminants. The precipitated NOS2 peptides were solubilized in denaturing buffers containing 100 mM sodium phosphate, 10 mM Tris/HCl, pH 8.0, 5 mM 2-mercaptoethanol, and 6 M guanidine hydrochloride in the case of the 55-kDa fragment or 8 M urea in the case of the 33-kDa fragment and added to an Ni-NTA resin for batch binding of histidine-tagged peptides. Nonspecific protein was discarded by sequential washing of the column with 10 volumes of the respective buffers adjusted to pH 8.0 and 10 volumes adjusted to pH 6.3. Specifically bound peptides were eluted from the resin with the same buffers adjusted to pH 5.9. The eluates were dialysed against TE, and the precipitated peptides were collected by centrifugation. The yield was estimated by comparison of Coomassie blue staining intensities of peptide bands in SDS-PAGE, with BSA as the standard.

Immunization of Rabbits

For initial immunization and for the first booster injection 14 days later, two White New Zealand rabbits per histidine-tagged fragment were injected subcutaneously at multiple sites in 100-μL aliquots with 250 μg of purified protein suspended in 500 μL of PBS mixed to homogeneity with 500 μL of Freund's complete adjuvant. For the second booster injection 14 days later, Freund's complete adjuvant

was replaced by Freund's incomplete adjuvant. Sera were obtained 12 days after the second booster injection.

Southern and Northern Hybridization

Nucleic acids from cultured cells were prepared and processed for Southern and Northern analyses as described [25].

Western Blot and Immunodetection

For immunodetection, protein was separated by SDS-PAGE and electrophoretically transferred to a protein-binding membrane (Immobilon, Millipore, Dreieich, F.R.G.) for 1.5 hr at 1.5 mA/cm^2. Prior to 2 hr incubation with a 1000-fold dilution of one of the anti-human NOS2 sera from rabbit in PBS, 0.5% Tween 20, blots were saturated in 5% nonfat dry milk in PBS for 1 hr. Specifically bound antibodies were detected with a 1000-fold dilution of horseradish peroxidase-conjugated pig anti-rabbit IgG secondary antiserum (Dakopatts, Hamburg, F.R.G.) and enhanced chemiluminescence (ECL, Amersham, Braunschweig, F.R.G.).

Determination of Mutant Frequencies

For the determination of frequencies of 6-TG-resistant mutants in the parental V79MZ and the V79MZhNOS2 cell line, cells were seeded in 15-cm-diameter plates at a density of 10^6 cells per plate, incubated in DMEM medium containing 7 μg/mL 6-TG, and grown for 12 days. Clones were fixed with ethanol and stained with Giemsa solution for visualization. For determination of plating efficiencies, 200 cells were seeded per plate.

RESULTS

Human NOS2 cDNA was ligated to the eukaryotic expression vector pSV. The resulting recombinant expression vector is presented in Fig. 1. The linearized NOS2 expression vector was cotransfected along with the selectable neomycin phosphotransferase gene into V79MZ Chinese hamster fibroblastoid cells, and recipient cells were selected in G418-containing medium. One clone, designated V79MZhNOS2, which had the highest and most stable rate of nitrite accumulation in the supernatant medium, was chosen from 30 candidate clones for subcloning and further characterization. The doubling time of transfectant cells was prolonged to 15 hr as compared with 12 hr of the parental V79MZ cells. Nitrite production of the new V79MZhNOS2 cell line remained stable for more than a 4-month period of continuous cultivation.

Parental V79MZ cells did not produce NO under standard culture conditions or under inducing conditions in the presence of cytokines and LPS, as measured by nitrite accumulation of the medium. Nitrite levels in the medium remained at the detection limit of 1 μM after 24 hr cultivation of parental V79MZ cells.

Chromosomal integration of the NOS2 expression unit into the V79MZ genome was demonstrated by Southern blotting. The restriction fragment lengths hybridizing to a ^{32}P-labeled NOS2-cDNA probe in V79MZhNOS2 transfectants were as expected from sequence data (Fig. 2). In DNA from parental V79MZ cells, no bands were labeled using this probe under stringent hybridization conditions. For visualization of transcriptional expression of the integrated human NOS2 expression unit, total RNA from parental V79MZ cells and from transfectant cells was analysed in a Northern blot using ^{32}P-labeled NOS2-cDNA as

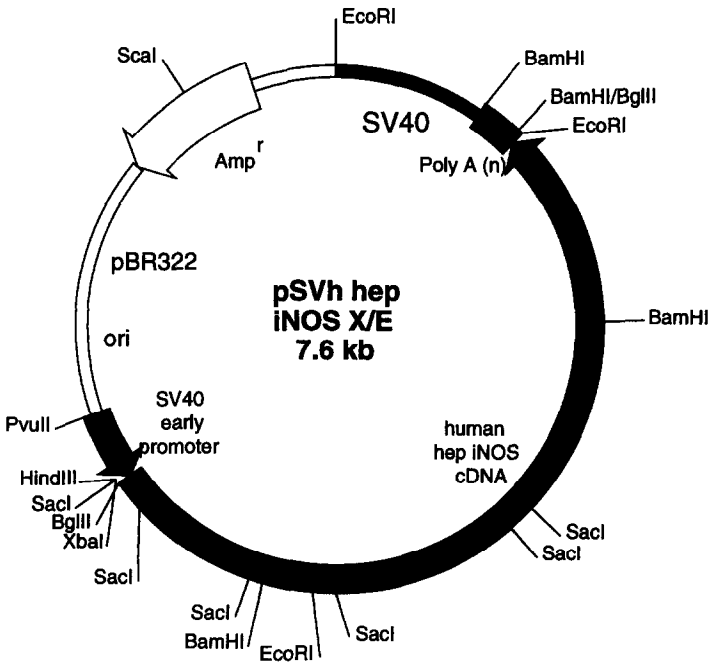

FIG. 1. Eukaryotic expression vector pSVhNOS2 X/E.

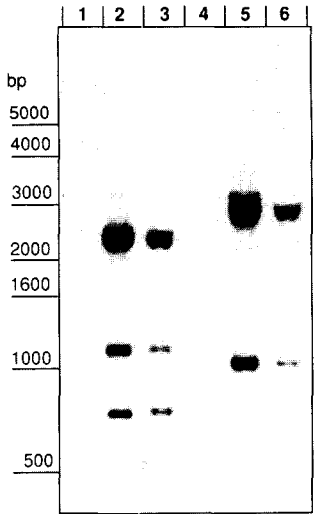

FIG. 2. Chromosomal integration of the human NOS2 cDNA expression unit into the V79MZ genome. Each sample contained 25 μg of DNA prepared from parental V79MZ (lanes 1 and 4), from V79MZhNOS2 (lanes 2 and 5), and from another independent transfectant clone (lanes 3 and 6), which was restricted with *Bam*HI and *Bgl*II (lanes 1, 2, and 3) or *Hind*III and *Eco*RI (lanes 4, 5, and 6) and separated on 0.8% agarose gel, vacuum-transferred on a Porablot Plus nucleic acid binding membrane (Macherey & Nagel) using 0.5 M NaOH as transfer buffer, and hybridized with 20 μCi of ^{32}P-labeled NOS2 cDNA hybridization probe. Exposure was for 4 hr with eightfold amplifying screen. The 1-kb DNA ladder from Gibco/BRL was used as a fragment length standard.

a probe. A single signal corresponding to an mRNA length of approximately 3.8 kb was labeled in RNA from transfectant clones but not in RNA from parental V79MZ cells (Fig. 3).

Two antisera from rabbit against human NOS2 were prepared by immunization with two peptides expressed from NOS2 cDNA fragments in *E. coli*. As depicted in Fig. 4, the shorter cDNA fragment, designated "891", corresponds to the region coding for amino acids 317–615 in the NOS2 amino acid sequence. Peptide "891" has a calculated molecular mass of 33 kDa and encompasses parts of the N-terminal hemethiolate monooxidase moiety and the calmodulin binding site located between amino acid positions 505 and 533. The "891" peptide ends immediately before the FMN-binding site belonging to the reductase moiety of NOS2. The larger cDNA fragment, designated "1481", covers the region coding for the NOS2 amino acid sequence from position 661 to the opal stop codon corresponding to codon 1154 of the complete NOS2 reading frame. The "1481" fragment has the coding capacity for a 55-kDa peptide representing nearly the complete C-terminal reductase moiety of the holoprotein except for the FMN-binding consensus at the N-terminal side of the reductase-like sequence.

Both resulting sera detected an antigen migrating with a molecular mass of 130 kDa in the V79MZhNOS2 cytosolic fraction that was not present in cytosols prepared from

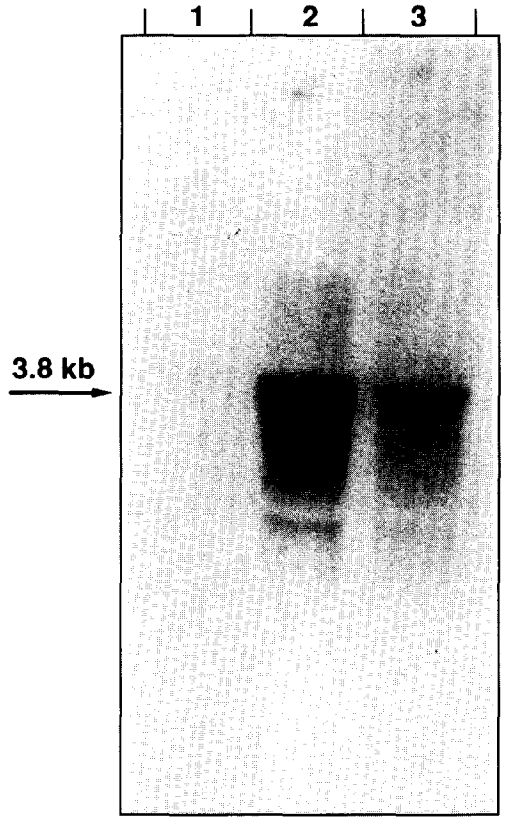

FIG. 3. Human NOS2 mRNA in V79MZhNOS2. Total glyoxylated RNA (25 μg) prepared from parental V79MZ cells (lane 1), from V79MZhNOS2 (lane 2), and from another independent NOS2 cDNA transfectant clone (lane 3) was separated on a 1.5% agarose gel with 25 mM potassium phosphate, pH 6.5, as electrophoresis buffer, vacuum-transferred to Porablot Plus hybridization membrane, and hybridized overnight at 42°C with an NOS2 cDNA probe labeled with 4 μCi ^{32}P in 50% formamide. After stringent washing, the blot was exposed for 3 days to x-ray film with eightfold amplifying screen. The 0.24–9.5-kb RNA ladder from Gibco/BRL was used as a length standard.

parental V79MZ cells. Moreover, a band of identical size was labeled to cytosols prepared from cytokine mix-treated AKN1 cells, which had been established from male human liver cells by spontaneous transformation. The AKN1 cell line is characterized by an inducible NOS activity as deter-

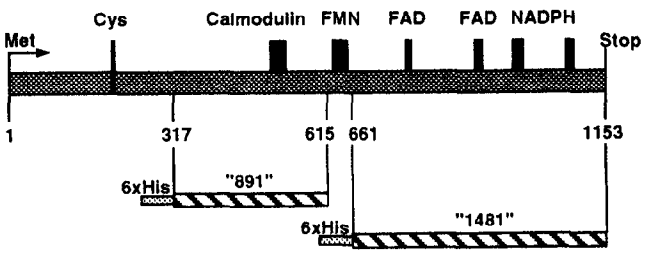

FIG. 4. Schematic presentation of the domain structure of human NOS2 and fragments "891" and "1481" expressed in *E. coli* as His-tagged peptides. Positions of cofactor binding sites and the putative heme binding cysteinyl residue are indicated. Numbering refers to the first and last amino acid position of the expressed peptides.

mined by nitrite accumulation in the medium supernatant after treatment with cytokine mix. In addition, a band with identical mobility could be induced in two independent human liver biopsies that had been treated with cytokine mix 1 day after they had been transferred into cell culture. Neither the AKN1 cell line nor the human primary hepatocytes displayed the 130-kDa band in parallel control cultures with no cytokine mix administered (Fig. 5A, B). Hence, both sera specifically recognized the inducible form of human NO synthase. The identical size of specific antigens detected in induced human tissue samples and in V79MZhNOS2 argues for the integrity of the heterologously expressed human NOS2. Human endothelial NO synthase was not recognized by the antisera (not shown). Some nonspecific binding to proteins migrating with molecular masses of 50–65 kDa in the gel did not interfere with the detection of NOS2 in the high molecular weight area in the gel. As a control, the cytosolic fractions of V79MZhNOS2 and V79MZ were analysed using a monoclonal antibody directed against the NADPH-binding region of murine macrophage NOS2 corresponding to amino acids 961–1144 at the C-terminus of the protein. As expected, this antibody detected the expressed human NOS2 in V79MZhNOS2 but not in parental cells (Fig. 5C).

In addition, the antisera "891" and "1481" were characterized with regard to cross-reactivity with the rat orthologue of NOS2. Purified rat hepatocytes were either kept in normal medium, exposed to the competitive NOS2 inhibitor NMA, exposed to cytokine mix alone, or exposed to cytokine mix in the presence of NMA. Previously, it had been shown that pretreatment with cytokine mix heavily induced NO synthesis in isolated rat hepatocytes, resulting

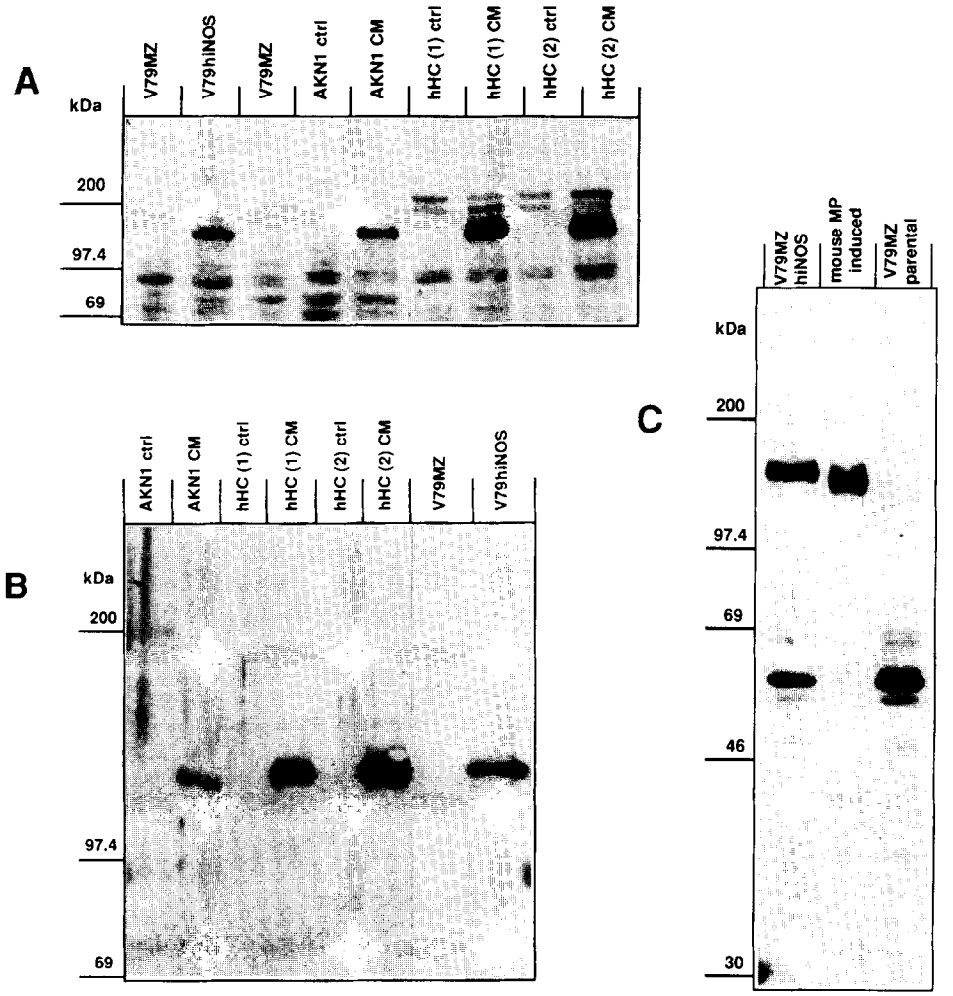

FIG. 5. Immunological detection of human NOS2 in V79MZhNOS2 (V79hiNOS) and cytokine-induced human liver cells. AKN1 indicates protein prepared from AKN1 cells. Lanes marked by hHC (1) and hHC (2) were loaded with cytosolic protein from two independent human primary hepatocyte cultures. Ctrl indicates noninduced control cultures, and CM denotes a pretreatment of cells with cytokine mix. Cytosolic protein was separated by 5%/8% discontinuous polyacrylamide gel electrophoresis and electrotransferred to Immobilon protein binding membrane (Millipore). For blot A, 40 μg protein was separated per lane and reacted with serum "891"; for blot B, 12.5 μg per lane was reacted with serum "1481" (see Fig. 4); and for blot C, 12.5 μg per lane was separated and reacted with a monoclonal antibody from mouse directed against a C-terminal fragment of mouse macrophage inducible NOS (Transduction Laboratories, U.S.A.). Protein from induced mouse macrophages was used as a control. All sera were applied at a dilution of 1:1000 in PBS, 0.5% Tween 20. Horseradish peroxidase-conjugated secondary antibodies and the ECL-Western blot kit from Amersham (Braunschweig, F.R.G.) were used for detection. Amersham's rainbow marker, 14–200-kDa, was used as a size standard.

in almost complete inactivation of cytochrome P450 1A-dependent hydroxylation reactions. Inclusion of NMA in the medium largely prevented the induced burst of NO production and, hence, for the most part the functional inactivation of P450s [7]. Both sera "891" (Fig. 6A) and "1481" (Fig. 6B) allowed the detection of an inducible band in total protein from rat hepatocytes pretreated with cytokine mix that had the same mobility in the gel as the human orthologue. Additionally administered NMA had no effect on band intensity, ruling out the possibility of an effect of NMA on the expression of NOS2 as a mechanism of suppression of NO production. In addition to the inducible band, serum "891" detected an antigen migrating just below the inducible 130-kDa band. This obviously nonspecific band had similar intensities in all four rat liver samples regardless of cytokine mix induction or inclusion of NMA in the growth medium. Serum "1481" did not recognize this band. In conclusion, both sera are suitable for the specific detection of the rat orthologue of NOS2.

Cytosols prepared from V79MZhNOS2 and parental V79MZ were analysed for their specific content of NOS2 activity by using the Griess reaction, which measures nitrite as stable product from NO. However, nitrite accounts for only 51.5 ± 3.5% of total NO produced [26]. In preliminary experiments, the influence of cell density of cultured cells on the content of NOS2 specific activity was determined. Specific NOS2 activities appeared to be inversely related to cell density, dropping substantially in cultures exceeding a confluency of 90%. To find a middle ground between specific activity and total yield, further experiments were performed using cytosolic fractions from cultures grown to approximately 70% confluency. The specific activity of these preparations was 25.1 ± 3.2 pmol mg^{-1} min^{-1} (N = 5), as

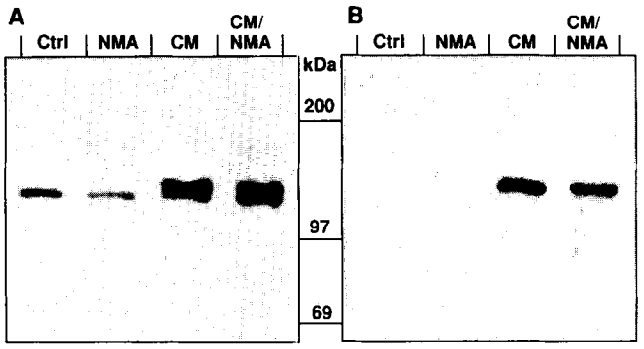

FIG. 6. Immunological detection of rat hepatic NOS2 in primary hepatocytes induced with cytokine mix. Each sample contained 30 μg of total protein from cultured rat hepatocytes maintained for 24 hr either in normal medium (ctrl), in the presence of 0.5 mM N^G-monomethyl-L-arginine (NMA), in the presence of cytokine mix (CM), or in the presence of both cytokine mix and N^G-monomethyl-L-arginine (CM/NMA). Serum "891" (blot A) and serum "1481" (blot B) were used as primary sera at a dilution of 1:1000. Bound antibody was detected using HRP-conjugated porcine anti-rabbit IgG and the ECL kit from Amersham. Positions of protein size standards are indicated in the margin.

determined by the Griess reaction. The cytosolic fraction from V79MZ parental cells had a specific NO synthase activity below 1 pmol mg^{-1} min^{-1}, as had been expected, given the lack of nitrite accumulation in medium supernatants of cultured V79MZ cells. As stated above, parental V79MZ cells do not possess any NO synthase activity.

In addition, NOS activity was checked in cytosolic preparations from V79MZhNOS2 by following the conversion of [^{3}H]-L-arginine to [^{3}H]-L-citrulline. In the absence of exogenous L-citrulline in the incubation, 13,142 dpm (N = 2; ±236 dpm deviation of single values from the mean) were converted to [^{3}H]-L-citrulline by 200 μg cytosolic protein from V79MZhNOS2 within 45 min, as compared with a background of 1589 dpm measured with preparations from V79MZ parental cells. The inclusion of 1.2 mM L-citrulline in the incubation resulted in an increase of measurable [^{3}H]-L-citrulline production to 17,712 dpm (N = 2; ±1682 dpm deviation of single values from the mean) under otherwise identical conditions. The increase in measurable [^{3}H]-L-citrulline was most likely due to the saturation of the enzymes of the urea cycle by the overshoot of unlabeled L-citrulline. Obviously, these assay conditions prevented, in part, [^{3}H]-L-citrulline synthesized by NOS2 from being degraded by the urea cycle enzymes.

NO synthesis from L-arginine involves two consecutive oxidation reactions. In the first step, L-arginine is oxidized at the guanidino group to N^G-hydroxyl-L-arginine. In the second step, N^G-hydroxy-L-arginine is further oxidatively metabolized to L-citrulline and the NO radical. NO synthases readily accept the hydroxy-intermediate as a substrate. With N^G-hydroxy-L-arginine substituted for L-arginine at concentrations of 0.5, 1, and 3 mM under otherwise v_{max} conditions, specific activities of 41.0, 46.8, and 47.3 pmol mg^{-1} min^{-1}, respectively, were determined in cytosolic preparations of V79MZhNOS2.

The V79MZhNOS2 cell line was tested as to its suitability as a screening tool for inhibitor studies. Cytosols prepared from V79MZhNOS2 were tested for specific NO synthase activity in the presence of various concentrations of inhibitors (Fig. 7). All of the inhibitors tested were efficient with the expressed human isoform, as they had been with rodent sources for the orthologous enzyme [27, 28].

In addition, the feasibility of using V79MZhNOS2 cells for inhibitor studies applying whole cells was assayed. Cells were grown in media containing various concentrations of inhibitors. Relative nitrite concentrations in the supernatant media as determined after 48 hr are depicted in Fig. 8. Except for NMA, all inhibitors impaired cell growth substantially at concentrations of 10 mM (nitrite values not shown).

V79 cells have long served as indicator cells in toxicology for mutagenicity and other toxicological endpoints. NO has been described to be mutagenic *in vitro* and in bacteria [29] and cytotoxic toward tumor cells [30], microbes, and parasites [31]. To address the question of whether a constitutive exposure to endogenous NO results

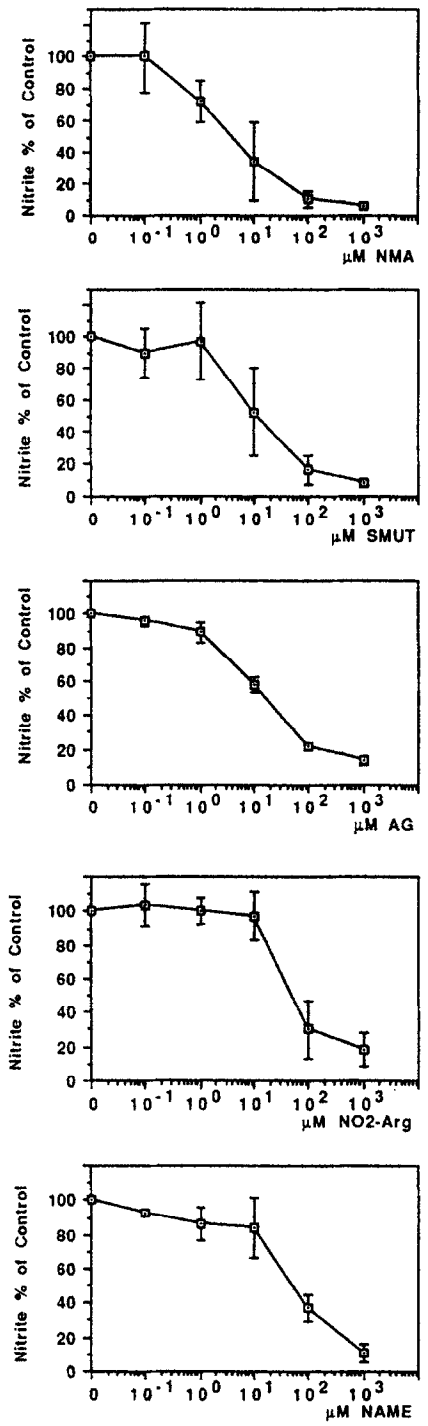

FIG. 7. Inhibition of NOS2 activity in cytosols from V79MZhNOS2. N^G-monomethyl-L-arginine (NMA), S-methyl-isothiourea (SMUT), aminoguanidine (AG), N^G-nitro-L-arginine (NO_2-Arg), and N^G-nitro-L-arginine methyl ester (NAME) were assayed for inhibition of expressed NOS2 activity at various concentrations. Assays were performed with 4 µM exogenous L-arginine introduced. Values represent the mean of four experiments, except for inhibition by AG, where two experiments were considered.

in an increased mutation rate, the frequency of 6-TG-resistant cells was compared in parental V79MZ and V79MZhNOS2 cells that had been continuously cultivated for longer than 2 months. The continuous exposure to endogenous NO in V79MZhNOS2 cells during culturing did not give rise to an increased proportion of mutated cells as compared with parental V79MZ cells. Frequencies of 6-TG-resistant mutants were 9.4 ± 3.0 for parental V79 cells and 8.4 ± 3.2 for V79MZhNOS2 cells per 10^6 cells. Obviously, moderate NO levels, as in V79MZhNOS2, were not mutagenic even with prolonged exposure.

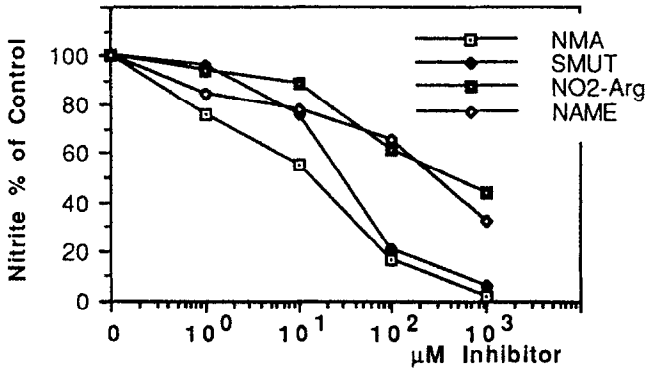

FIG. 8. Inhibition of NOS2 activity in intact V79MZhNOS2 cells. Cells were grown in medium containing 0.7 mM L-arginine in the presence of various concentrations of inhibitors for 48 hr. Confluency of cultures was approximately 90% at the end of the experiments. Values represent the mean of nitrite concentrations from two experiments. Deviation from the average value was less than 10% in all cases.

DISCUSSION

A V79 Chinese hamster cell line was genetically engineered for stable and constitutive expression of human inducible NO synthase. The new cell line expressed an authentic NOS2 protein as judged by comparison of mobilities in SDS-PAGE of NOS2-immunoreactive bands from V79MZhNOS2 and cytokine-induced NOS2 from human liver cells. Even upon treatment with cytokine mix, parental V79MZ cells did not express any NOS activity. Therefore, V79MZhNOS2 cells present a background-free host for human NOS2 activity. In cytosolic preparations from V79MZhNOS2, specific NOS2 activities of 25 pmol min^{-1} mg^{-1} were determined with L-arginine as substrate. This specific activity is roughly 1–2 orders of magnitude lower than typical specific activities obtained from fully induced cells from native tissues such as rat hepatocytes [7], mouse macrophages [32], mouse brain endothelial cells [33], or a human tumor cell line [34]. The moderate expression of the activity probably allows permanent cultivation of this cell line without substantial impairment of cell growth by the constant intracellular production of noxious NO. Nevertheless, V79MZhNOS2 cells are suitable as a source for NOS2 activity for studies on the human isoform. For most studies involving NOS2, the murine macrophage enzyme has been used as a model. Murine macrophage NOS2 displays only 80% sequence identity with the inducible human

form [10]. Therefore, species-dependent differences in sensitivity toward inhibitors can be expected between the human and murine forms. On the other hand, the identity of cDNA-deduced NOS2 sequences from different human tissues was more than 99% [10–12]. Thus, the enzyme expressed in V79MZhNOS2 is likely to be representative for the human inducible enzyme. The new cell line V79MZhNOS2 may serve as a source of human NOS2 in future studies, not only avoiding problems related to species differences but also bypassing technical and ethical problems related to limitations and availability of human NOS2 from native tissues.

In V79MZhNOS2 cells, expression of NOS2 is under the control of the constitutive SV40 Early promoter and is therefore uncoupled from inducibility. This construction is beneficial for inhibition experiments using intact cells, because induction phenomena will not interfere with NO synthase activities. The hierarchy of inhibitor potencies presented in this paper (NMA > SMUT > AG > NO_2-Arg = NAME) as determined with V79MZ-expressed human NOS2 were different from published results obtained with rodent inducible NO synthase (SMUT > NMA = AG > NO_2-Arg > NAME) [35]. This finding underlines the importance of observing species differences.

As part of the host defense system, NO has been attributed a considerable mutagenic and cytotoxic potential toward microbes and tumor cells [29, 30]. However, in the V79MZhNOS2 cell line, no increase in the frequency of 6-TG-resistant cells was observed as compared with the parental cell line. We conclude that permanent exposure to moderate concentrations of endogenous NO does not result in considerable mutagenicity.

For completion of the cellular expression system, the construction of further V79 cell lines stably expressing the constitutively expressed human NOS isoforms is currently being pursued.

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References

1. Nathan C, Nitric oxide as a secretory product of mammalian cells. *FASEB J* **6:** 3051–3064, 1992.
2. Schmidt HHHW and Walter U, NO at work. *Cell* **78:** 919–925, 1994.
3. Radomski MW, Palmer RMJ and Moncada S, The role of NO and cGMP in platelet adhesion to vascular endothelium. *Biochem Biophys Res Commun* **148:** 1482–1489, 1987.
4. Schuman EM and Madison DV, A requirement for the intercellular messenger NO in long-term potentiation. *Science* **254:** 1503–1506, 1991.
5. Nathan C and Xie Q-w, Nitric oxide synthases: Roles, tolls, and controls. *Cell* **78:** 915–918, 1994.
6. Khatsenko OG, Gross SS, Rifkind RB and Vane JR, NO is a mediator of the decrease in the cytochrome P450-dependent metabolism caused by immunostimulants. *Proc Natl Acad Sci USA* **90:** 11147–11151, 1993.
7. Stadler J, Trockfeld J, Schmalix WA, Brill T, Siewert JR, Greim HR and Doehmer J, Inhibition of cytochromes P450 1A by NO. *Proc Natl Acad Sci USA* **91:** 3559–3563, 1994.
8. Nava E, Palmer RMJ and Moncada S, Inhibition of NO synthesis in septic shock: How much is beneficial? *Lancet* **338:** 1555–1557, 1991.
9. Petros A, Bennett D and Vallance P, Effect of NO synthase inhibitors on hypotension in patients with septic shock. *Lancet* **338:** 1557–1558, 1991.
10. Geller DA, Lowenstein CJ, Shapiro RA, Nussler AK, Di Silvio M, Wang SC, Nakayama DK, Simmons RL, Snyder SH and Billiar TR, Molecular cloning and expression of inducible NO synthase from human hepatocytes. *Proc Natl Acad Sci USA* **90:** 3491–3495, 1993.
11. Charles IG, Palmer RMG, Hickery MS, Bayliss MT, Chubb AP, Hall VS, Moss DW and Moncada S, Cloning characterization, and expression of a cDNA encoding an inducible NO synthase from the human chondrocyte. *Proc Natl Acad Sci USA* **90:** 11419–11423, 1993.
12. Hokari A, Zenija M and Esumi H, Cloning and functional expression of human inducible NO synthase (NOS) cDNA from gliablastoma cell line A-172. *J Biochem* **116:** 575–581, 1994.
13. Janssens SP, Shimouchi A, Quertermous T, Bloch DB and Bloch KD, Cloning and expression of a cDNA encoding human endothelium-derived relaxing factor/NO synthase. *J Biol Chem* **267:** 14519–14522, 1992.
14. Lamas S, Marsden PA, Li GK, Tempst P and Michel T, Endothelial NO synthase: Molecular cloning and characterization of a distinct constitutive enzyme isoform. *Proc Natl Acad Sci USA* **89:** 6348–6352, 1992.
15. Bredt DS, Hwang PM, Glatt CE, Lowenstein C, Reed RR and Snyder SH, Cloned and expressed NO synthase structurally resembles cytochrome P-450 reductase. *Nature* **351:** 714–718, 1991.
16. Charles IG, Chubb A, Gill R, Clare J, Lowe PN, Holmes LS, Page M, Keeling JG, Moncada S and Riveros-Moreno V, Cloning and expression of rat neuronal NO synthase coding sequence in a baculovirus/insect cell system. *Biochem Biophys Res Commun* **196:** 1481–1489, 1993.
17. Marsh JL, Erfle M and Wykes EJ, The pIC plasmid and phage vectors with versatile cloning sites for recombinant selection by insertional inactivation. *Gene* **32:** 481–485, 1984.
18. Doehmer J, Dogra S, Friedberg T, Monier S, Adesnik M, Glatt HR and Oesch F, Stable expression of rat P-450IIB1 cDNA in Chinese hamster cells (V79) and metabolic activation of aflatoxin B_1. *Proc Natl Acad Sci USA* **85:** 5769–5773, 1988.
19. Glatt HR, Seidel A, Ribeiro O, Kirkby C, Hirom P and Oesch F, Metabolic activation to a mutagen of 3-hydroxy-trans-7,8-dihydroxy-7,8-dihydrobenzo[*a*]pyrene. *Carcinogenesis* **8:** 1621–1627, 1987.
20. Nussler AK, Vergani G, Gollin S, Dorko K, Gansauge S, Morris SM Jr, Demetris AJ, Nomoto M, Beger HG and Strom SC, Isolation and characterization of a human hepatic epithelial cell line (AKN-1) from a normal human liver. *In Vitro*, submitted.
21. de Vera ME, Shapiro RA, Nussler AK, Mudgett JS, Simmons RL, Morris SM, Billiar TR and Geller DA, Transcriptional regulation of human inducible NO synthase (NOS2) gene by cytokines: Initial analysis of the human NOS2 promoter. *Proc Natl Acad Sci USA*, **93:** 1054–1059, 1996.
22. Green LC, Wagner DA, Glogowski J, Skipper PL, Wishnok JS and Tannenbaum SR, Analysis of nitrate, nitrite, and [^{15}N]nitrate in biological fluids. *Anal Biochem* **126:** 131–138, 1982.
23. Bradford MM, A rapid and sensitive method for the quanti-

tation of microgram quantities of protein using the principle of dye binding. *Anal Biochem* **72:** 248–254, 1976.
24. Hanahan D, Studies on transformation of *Escherichia coli* with plasmids. *J Mol Biol* **166:** 557–580, 1983.
25. Schmalix WA, Mäser H, Kiefer F, Reen R, Wiebel FJ, Gonzalez F, Seidel A, Glatt H, Greim HR and Doehmer J, Stable expression of human cytochrome P450 1A1 in V79 Chinese hamster cells and metabolic activation of benzo[*a*]pyrene. *Eur J Pharmacol* **248:** 251–261, 1993.
26. Stadler J, Billiar TR, Curren RD, McIntyre LA, Georgescu HE, Simmons RL and Evans CH, Articular chondrocytes synthesize nitric oxide in response to cytokines and lipopolysaccharide. *J Immunol* **147:** 3915–3920, 1991.
27. Szabó C, Southan GJ and Thiemermann C, Beneficial effects and improved survival in rodent models of septic shock with S-methylsothiourea sulfate, a novel, potent and selective inhibitor of inducible NO synthase. *Proc Natl Acad Sci USA* **91:** 12472–12476, 1994.
28. Southan GJ, Szabó C and Thiemermann C, Isothioureas: Potent inhibitors of NO synthases with variable isoform selectivity. *Br J Pharmacol* **114:** 510–516, 1995.
29. Wink DA, Kasprzak KS, Maragos CM, Elespuru RK, Misra M, Dunams TM, Cebula TA, Koch WH, Andrews AW, Allen JS and Keefer LK, DNA deaminating ability and genotoxicity of NO and its progenitors. *Science* **254:** 1001–1002, 1991.
30. Stuehr DJ and Nathan CF, NO: A macrophage product responsible for cytostasis and respiratory inhibition in tumor target cells. *J Exp Med* **169:** 1543–1545, 1989.
31. Nathan C and Hibbs JB Jr, Role of NO synthesis in macrophage antimicrobial activity. *Curr Opin Immunol* **3:** 65–70, 1991.
32. Lambert LE, Whitten JP, Baron BM, Cheng HC, Doherty NS and McDonald IA, NO synthesis in the CNS, endothelium and macrophages differs in its sensitivity to inhibition by arginine analogues. *Life Sci* **48:** 69–75, 1991.
33. Gross SS, Jaffe EA, Levi R and Kilbourn RG, Cytokine-activated endothelial cells express an isotype of NO synthase which is tetrahydrobiopterin-dependent, calmodulin-independent and inhibited by arginine-analogs with a rank order characteristic of activated macrophages. *Biochem Biophys Res Commun* **178:** 823–829, 1991.
34. Sherman PA, Laubach VE, Reep BR and Wood ER, Purification and cDNA sequence of an inducible NO synthase from a human tumor cell line. *Biochemistry* **32:** 11600–11605, 1993.
35. Szabo C, Southan GJ, and Thiemermann C, Beneficial effect and improved survival in rodent models of septic shock with S-methylisothiourea sulfate, a potent and selective inhibitor of inducible nitric oxide synthase. *Proc Natl Acad Sci USA* **91:** 12472–12476, 1994.