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Cloned human brain nitric oxide synthase is highly expressed in skeletal muscle

Masaki Nakane[a], Harald H.H.W. Schmidt[b,*], Jennifer S. Pollock[a], Ulrich Förstermann[a,b] and Ferid Murad[a,b]

[a]*Vascular Biology, Abbott Laboratories, Abbott Park, Illinois 60064, USA* and [b]*Department of Pharmacology, Northwestern University Medical School, Chicago, Illinois 60611, USA*

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Complementary DNA clones corresponding to human brain nitric oxide (NO) synthase have been isolated. The deduced amino acid sequence revealed an overall identity with rat brain NO synthase of about 93% and contained all suggested consensus sites for binding of the co-factors. The cDNA transfected COS-1 cells showed significant NO synthase activity with the typical co-factor requirements. Unexpectedly, messenger RNA levels of this isoform of NO synthase was more abundant in human skeletal muscle than human brain. Moreover, we detected high NO synthase activity and the expressed protein in human skeletal muscle by Western blot analysis, indicating a possible novel function of NO in skeletal muscle.

Brain; Molecular cloning; Human; Nitric oxide synthase; Skeletal muscle

1. INTRODUCTION

Nitric oxide (NO) is a ubiquitous paracrine substance and can also act as an intracellular second messenger in various cells and tissues [1–3]. In brain and neuronal cells, NO is synthesized from L-arginine by NO synthase (EC 1.14.23) in a Ca^{2+}/calmodulin (CaM)-dependent manner [4–6]. NO mediates the stimulatory actions of neurotransmitters on the intracellular concentration of cyclic GMP [3]. NO has also been shown to be involved in long-term depression and long-term potentiation, suggesting the possibility of playing a vital role in memory and learning [7–11].

Recently, Bredt et al. have cloned a cDNA for rat brain NO synthase and showed a close homology with cytochrome P-450 reductase [12]. We report here the isolation, functional expression of human brain NO synthase cDNA, and evidence that NO synthase is highly expressed in human skeletal muscle.

Correspondence address: M. Nakane, D46B, AP10, Abbott Laboratories, Abbott Park, IL 60064, USA. Fax: (1) (708) 938–6107.

**Present address:* Medizinische Universitätsklinik, Klinische Forschergruppe, Josef-Schneider-Str. 2, W-8700 Würzburg, Germany.

Abbreviations: BH_4, 5,6,7,8-tetrahydrobiopterin; CaM, calmodulin; cDNA, complimentary DNA; EGTA, [ethylenebis (oxyethylenenitrilo)]tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1- piperazineethanesulfonic acid; NMA, N^G-methyl-L-arginine; NNA, N^G-nitro-L-arginine; NO, nitric oxide; PCR, polymerase chain reaction; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate; TFP, trifluoperazine.

2. MATERIALS AND METHODS

2.1. *Screening of the human cerebellum cDNA library*

Using published 32-mer oligonucleotide sequences of rat brain NO synthase [12], we performed polymerase chain reaction (PCR) on first-strand rat cerebellum cDNA and produced a 599-bp fragment. This fragment was subcloned into pBluescript SK(−) (Stratagene, La Jolla, CA) and sequenced. A human cerebellum cDNA library in λgt11 (Clontech, Palo Alto, CA) was screened using the ^{32}P-labeled fragment as a probe. The nylon filters (Hybond-N, Amersham, Arlington Hights, IL) were hybridized at 42°C for 24 h in 50 mM Tris-HCl, pH 7.5, 1 M NaCl, 10 × Denhardt's solution, 1% SDS, 10% dextran sulfate, 100 μg/ml denatured salmon sperm DNA and the ^{32}P-labeled probe, and washed at 42°C with 6 × SSC containing 1% SDS. Blots were exposed overnight to Kodak XAR film at − 80°C. Positive clones were purified, and the inserts were subcloned into the *Eco*RI site of pBluescript. Restriction fragments were subcloned into pBluescript and sequenced by the dideoxy chain termination method using Sequenase (United States Biochemicals, Cleveland, OH).

2.2. *Expression in mammalian cells*

The cDNAs of #5 and #18 clones were ligated at the unique *Bfr*I site (position 1,394) to make a cDNA that contains the full open reading frame for NO synthase. The insert was subcloned into the expression vectors, pcDL-SRa (Y. Takebe, National Institute of Health, Tokyo, Japan, [13]) and pcDNA I (Invitrogen, San Diego, CA). About 50% confluent COS-1 cells in 60-mm dishes were transfected with the complex of 3 μg DNA and 30 μg Lipofectin (Gibco-BRL, Gaithersburg, MD) in 2.5 ml of Opti-MEM (Gibco-BRL), and incubated for 5 h at 37°C in a CO_2 incubator. The medium was replaced with DMEM containing 10% serum and the cells were incubated for another 2 days. The cells were suspended in 0.1 ml of 50 mM HEPES (pH 7.4), 0.1 mM EDTA, 100 μM PMSF, 1 μM pepstatin, 1 μM leupeptin, 0.3 μM aprotinin, and freeze-thawed 3 times using liquid nitrogen. NO synthase activity in the homogenate was measured as described below.

2.3. *Northern blot analysis*

The Human multi-tissue Northern blot filter (Clontech) was hybridized at 42°C for 24 h in 50% formamide, 5 × SSPE, 10 × Denhardt's

solution, 2% SDS, 100 μg/ml salmon sperm DNA and the ^{32}P-labeled probe, and washed at 50°C for 1 h with 0.1 × SSC containing 0.1% SDS. Blots were exposed to Kodak XAR film at − 80°C for 4 days.

2.4. *Assay of NO synthase*

Human skeletal muscle tissue (45-year-old male, International Institute for the Advancement of Medicine, Exton, PA) was homogenized in 5 vols. of buffer (50 mM Tris-HCl, pH 7.5 containing 1 mM EDTA, 5 mM 2-mercaptoethanol, 10 μg/ml pepstatin, 10 μg/ml aprotinin, 10 μg/ml leupeptin and 100 μg/ml PMSF), and centrifuged at 105 000 × *g* for 60 min. The pellet was washed twice with the equal volume of the buffer containing 1 M KCl. The KCl-washed pellet was resuspended in the buffer (without KCl). NO synthase activity was measured by the conversion of [^{3}H]arginine to [^{3}H]citrulline as described [6,14]. Samples (50 μl) were incubated in 50 mM HEPES, pH 7.4 with 10 μM L-[2,3,4,5-^{3}H]arginine (9.8 GBq/mmol), 1 mM NADPH, 100 μM $CaCl_2$, 30 nM CaM, 3 μM BH_4, 1 μM FAD and 1 μM FMN in a final volume of 100 μl. The reaction was carried out for 10 min at 37°C and terminated by adding 0.5 ml of 20 mM HEPES, pH 5.5, containing 2 mM EDTA and 2 mM EGTA. The incubates were applied to 1 ml columns of Dowex AG50WX-8 (Na^+form) and eluted three times with 0.5 ml of the above buffer. All the liquid was pooled and radioactivity was determined by liquid scintillation counting. Protein was measured using the Bradford reagent [15] with bovine serum albumin as a standard.

2.5. *Western blot analysis*

The fractions (50 μg protein) were separated on 8% SDS-polyacrylamide gels and the proteins were blotted onto nitrocellulose membrane. The membrane was blocked with 6% non-fat dried milk, and subsequently incubated with the polyclonal antibody against rat brain NO synthase (6781-8) [16], or the monoclonal antibody against bovine aortic endothelial NO synthase (H32) [17]. The specific proteins were detected by enhanced chemiluminescence (ECL, Amersham).

3. RESULTS

A human cerebellum λgt11 cDNA library was screened with the PCR fragment of rat brain NO synthase at low stringency. After repeated cloning, one positive clone (#1 920), which showed a highly homologous deduced amino acid sequence with rat brain NO synthase [12], was isolated. Since the clone had only a partial coding region, the same library was re-screened using the *Bam*HI fragments from both ends of the cDNA (400 bp and 300 bp) as probes, and #5 and #18 clones were isolated and sequenced. Based on the consensus sequence for the translational start site [18], the ATG at position 430 defines the beginning of an open reading frame. The open reading frame from position 430 to position 4,728 encodes for 1,433 amino acids (Fig. 1). The calculated molecular weight of the protein was 16,1037 Da, which is consistent with that deter-

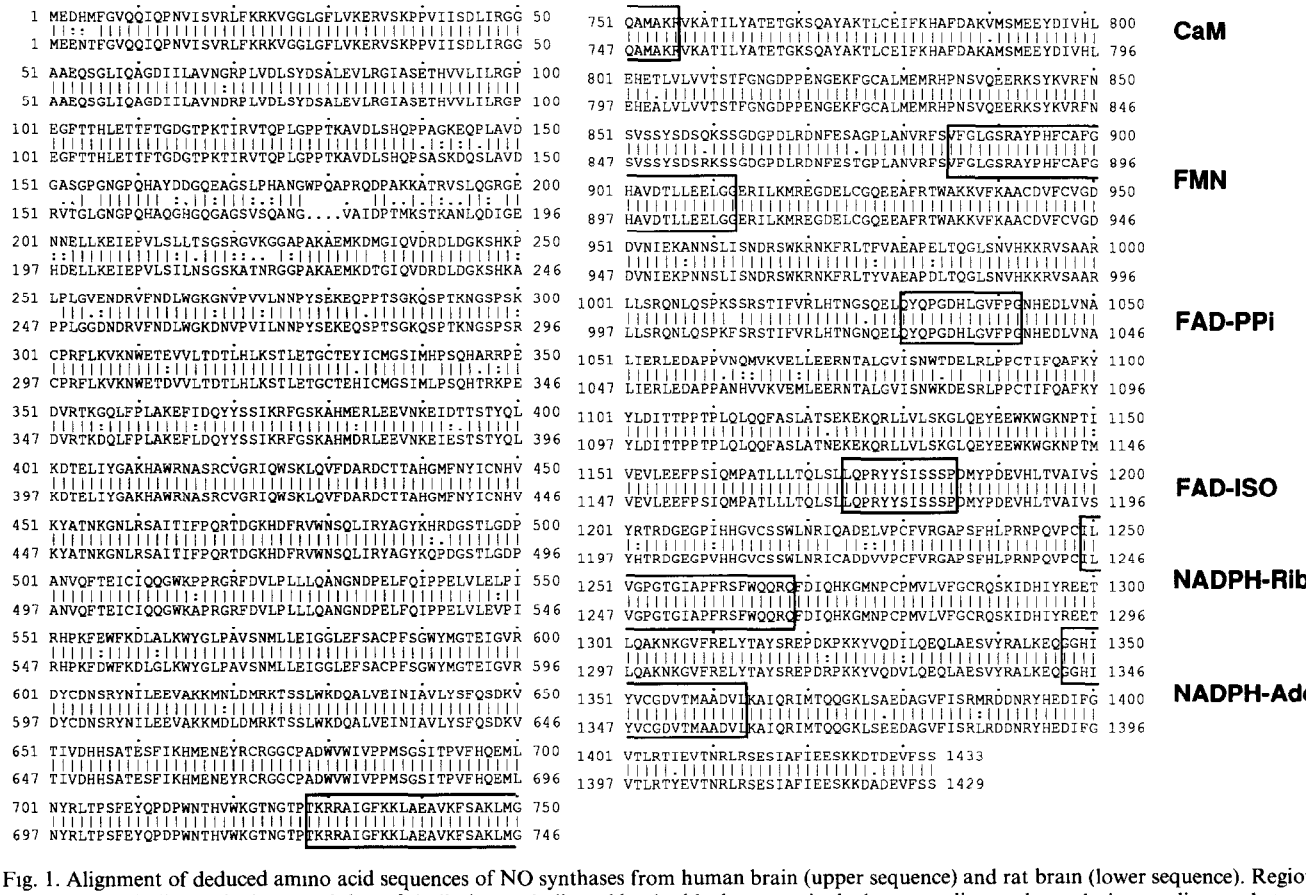

Fig. 1. Alignment of deduced amino acid sequences of NO synthases from human brain (upper sequence) and rat brain (lower sequence). Regions of identity are indicated by bars, and that of similarity are indicated by double dots or a single dot, according to the evolutionary distance between the amino acids [30]. Consensus sequences of co-factor binding regions for CaM, FMN, FAD pyrophosphate (FAD-PPi), FAD isoalloxazine (FAD-ISO), NADPH ribose (NADPH-rib), NADPH adenine (NADPH-Ade) are boxed. The nucleotide sequence has been submitted to the GenBank/EMBL Data Bank with accession number L02881

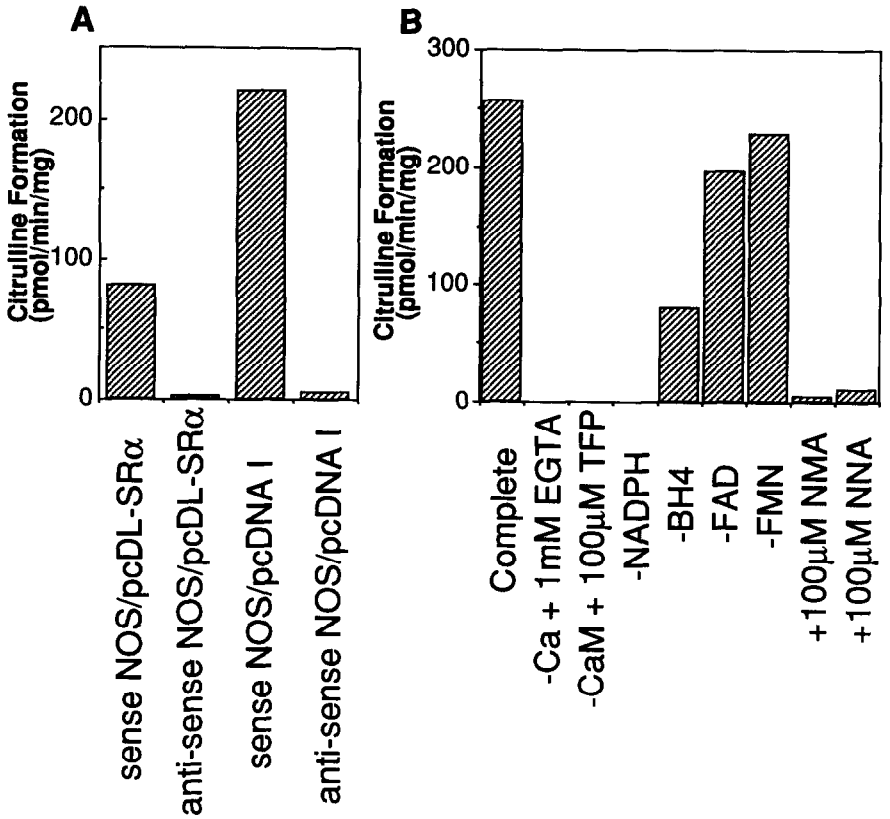

Fig. 2. Transient expression of cDNAs of NO synthase in COS-1 cells. (A) COS-1 cells were transfected with the indicated plasmids, and NO synthase activity in the homogenate was assayed as described in Materials and Methods. (B) COS-1 cells were transfected with sense NO synthase cDNA/pcDNA I, and NO synthase activity in the homogenate with various conditions as indicated was measured. Results are the means of duplicate determinations.

mined by Western blot analysis using anti-brain NO synthase [19]. Comparison of the deduced amino acid sequence of human brain NO synthase to that of rat brain enzyme revealed a high homology (93%), although the human enzyme was 4 amino acids longer than the rat enzyme (Fig. 1). The human NO synthase also contained all the suggested consensus binding sites for CaM, FAD, FMN and NADPH, and the sequences of these sites were identical with that of rat enzyme.

We subcloned the cDNA with full coding sequence into the *Eco*RI site of the pcDL-SRa expression vector [13] and the pcDNA I expression vector (human cytomegalovirus enhancer/promoter). Two days after transfection, the COS-1 cell homogenate was assayed for NO synthase activity using the conversion of L-arginine to L-citrulline (Fig. 2). Only the cells trans-

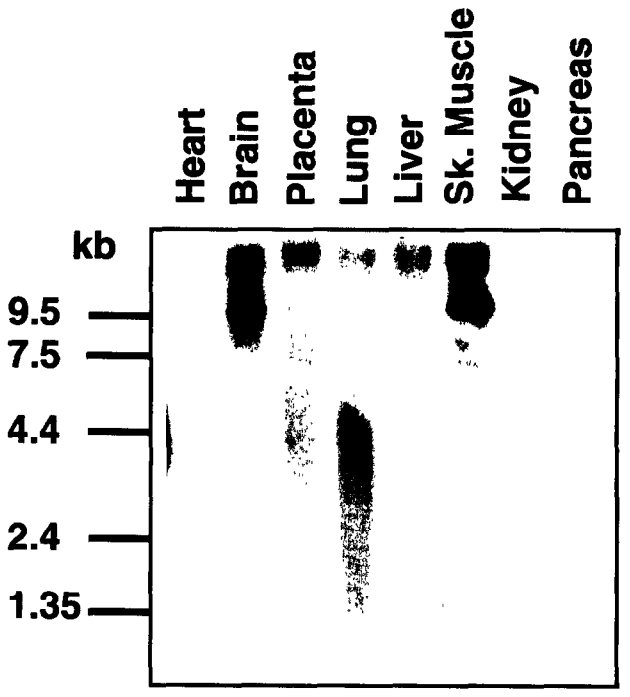

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Fig. 3. Northern blot analysis of human NO synthase type I mRNA. Poly(A)$^+$ RNAs (2 μg) from heart, brain, placenta, lung, liver, skeletal muscle, kidney, and pancreas from human were hybridized with the ^{32}P-labeled cDNA as described in Materials and Methods. RNA ladder (Gibco-BRL) was used as a size standard. Sk. Muscle, skeletal muscle.

fected with sense cDNA showed significant NO synthase activity. The activity using the pcDNA I vector was higher than that using pcDL-SRa vector (Fig. 2A). The expressed activity was dependent on Ca^{2+}, CaM, and NADPH, partially dependent on BH_4, and markedly inhibited by NMA and NNA (Fig. 2B). FAD and FMN did not significantly stimulate the activity. All the expressed NO synthase activity was in the 100 000 × g supernatant fraction, and the specific activity using pcDNA I vector was about 7-times higher than that from human cerebellum supernatant fraction (data not shown). Western blot analysis of the supernatant fraction of the transfected cells using an antibody prepared to purified rat brain enzyme [16] showed a single protein band of 160 kDa (data not shown).

Northern blot analysis of poly(A)$^+$ RNA from different human tissues of a 32-year-old male probed with ^{32}P-labeled cDNA revealed a single 10-kb mRNA for the NO synthase (Fig. 3). Levels of mRNA were more abundant in skeletal muscle than brain, lower in pancreas, and not detected in heart, placenta, lung, liver, and kidney. For skeletal muscle and brain, we repeated the experiment using a poly(A)$^+$ RNA preparation from a 15-year-old female and obtained the same results. Hybridization of a β-actin probe to the same filters showed comparable levels of β-actin mRNA in all samples, and two forms of β-actin mRNA, 2.0-kb and 1.8-kb forms, were detected in heart and skeletal muscle, which are typical characteristics of muscle tissue β-actin (data not shown).

Homogenates of human skeletal muscle tissue showed significant NO synthase activity that was higher than in human brain, whereas rat skeletal muscle failed to show the activity (Fig. 4A). Most of the NO synthase activity of human skeletal muscle was found in the supernatant of the KCl wash, indicating that it is soluble and/or loosely bound to membranes. The NO synthase activity in skeletal muscle was dependent on Ca^{2+}, CaM, NADPH, and BH_4, partially dependent on FAD and FMN, and markedly inhibited by NMA and NNA (Fig. 4B).

High levels of expression of NO synthase in human skeletal muscle was also confirmed by Western blot analysis. The antibody against rat brain NO synthase

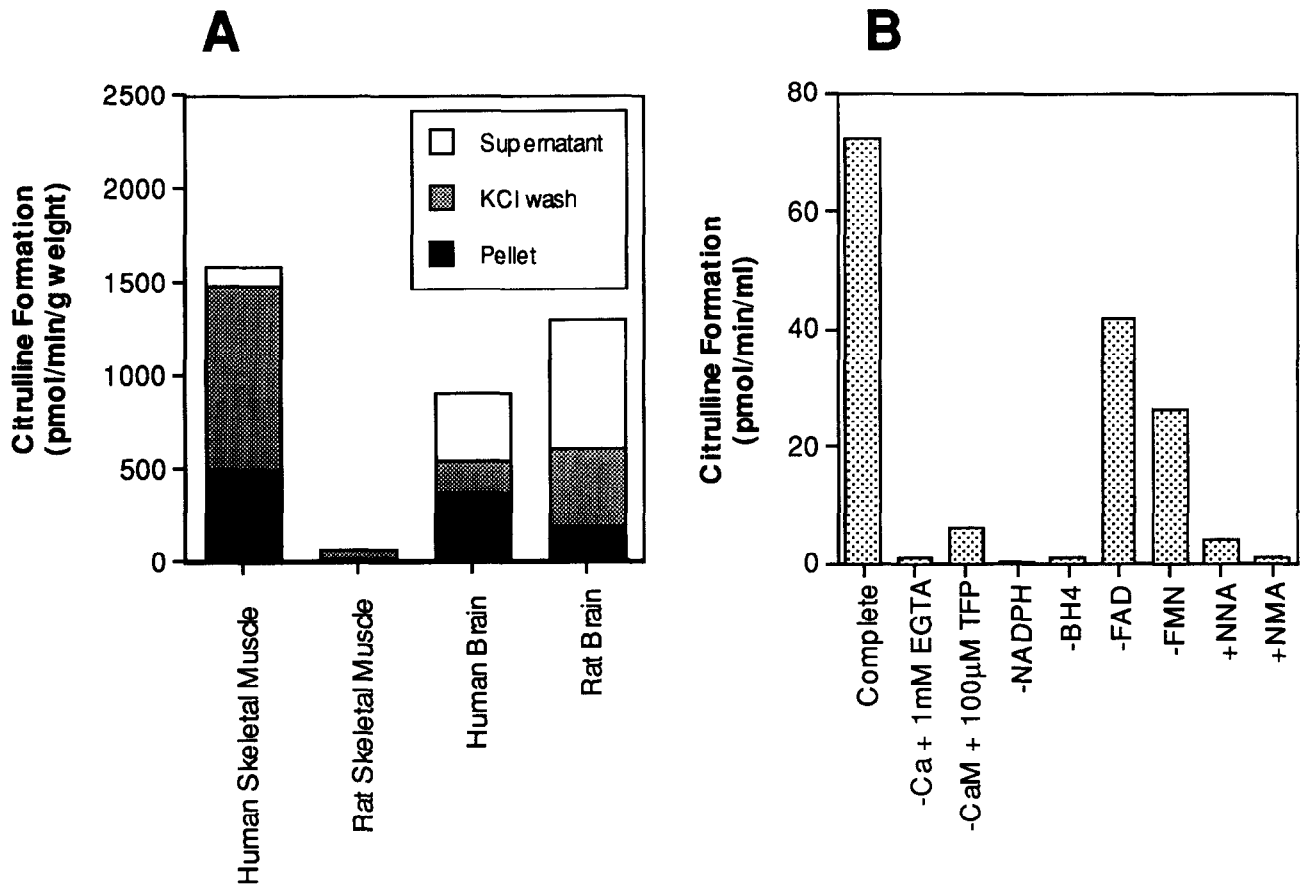

Fig. 4. NO synthase activity in skeletal muscle and brain from human and rat. (A) NO synthase activity in the supernatant, KCl wash and pellet was assayed as described in Materials and Methods. (B) NO synthase activity was measured in a homogenate of human skeletal muscle under the various conditions indicated. Results are the means of duplicate determinations.

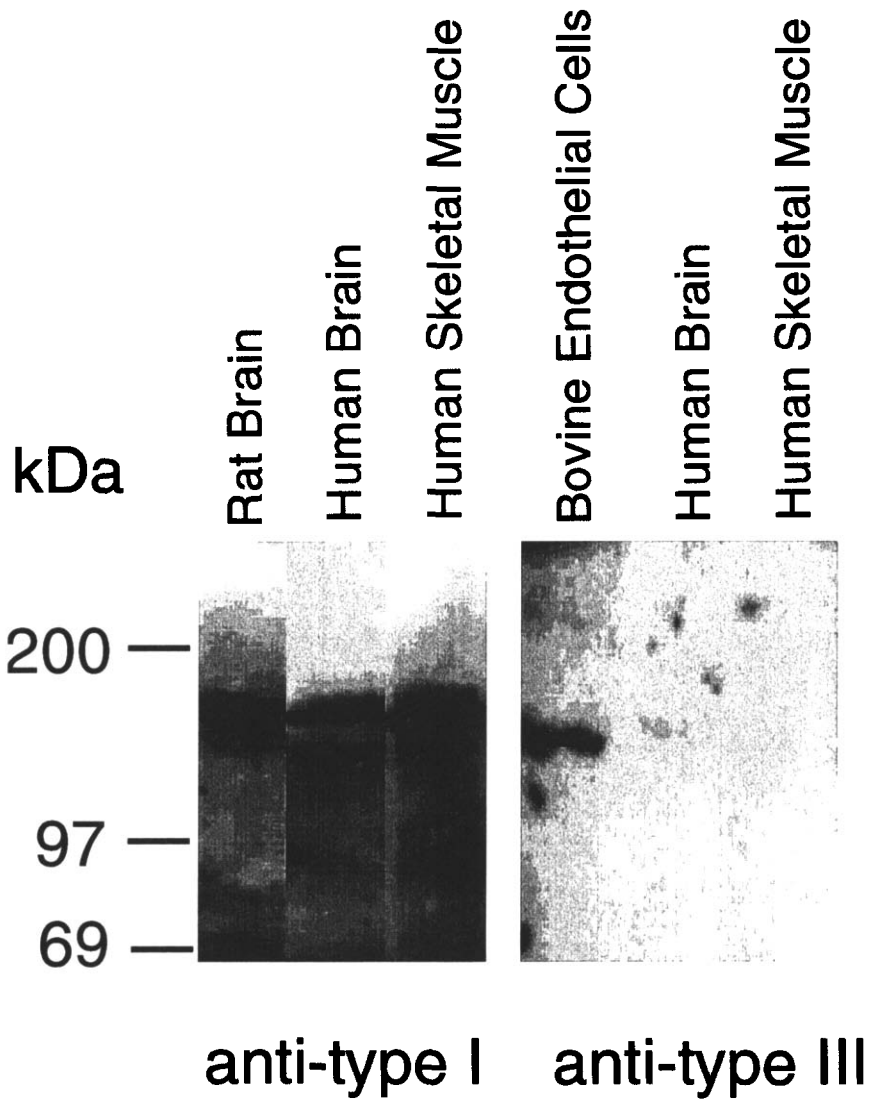

Fig. 5. Western blot analysis of NO synthase in human skeletal muscle. 50 μg of homogenate protein from human brain and skeletal muscle were separated by SDS-PAGE, transferred to nitrocellulose membrane and probed with the antibody against rat brain NO synthase (anti-type I) or with the antibody against bovine aortic endothelial NO synthase (anti-type III). Rat brain supernatant (anti-type I) or bovine aortic endothelial cells homogenate (anti-type III) were used as positive controls.

recognized a single 160 kDa protein band in human skeletal muscle (as well as human and rat brain). No protein band was detected by the antibody against endothelial NO synthase (Fig. 5).

4. DISCUSSION

NO synthase exists in multiple isoforms, some of which have been purified and characterized (type I from brain type, type II from induced macrophages, type III from endothelial cells [20]). Recently, cDNAs for three of these isoenzymes have been cloned and the structural differences between these three isoforms became apparent [12,21–25]. In the present study, we identified the cDNA clones that encode the entire sequence of human NO synthase type I from cerebellum. We found an overall identity between the human and rat brain type I enzyme of 93%. All the suggested binding sites for various co-factors were identical, and the possible phosphorylation sites by protein kinases were also highly conserved, suggesting that the two enzymes share the same mechanisms of regulation. Regulation of NO synthase type I by phosphorylation has been suggested [14,26,27]. In addition, we compared the amino acid sequence of human NO synthase type I with the amino acid sequences of the recently cloned mouse macrophage type II [21,22] and bovine and human endothelial cell type III [23–25] enzymes. The human type I enzyme showed 53% and 57% identity to type II and type III enzymes, respectively (data not shown). There is a

highly conserved region near the center (residues 384–703 in human NO synthase type I) in all the types of NO synthase, suggesting that this region could be the potential arginine recognition domain and/or the BH_4 binding domain.

As expected from the high homology with the rat brain enzyme, expressed human NO synthase type I showed properties similar to that of rat NO synthase type I. It was completely dependent on Ca^{2+}, CaM and NADPH, inhibited by NMA and NNA, and all the activity was localized in the cytosolic fraction of the COS-1 cells. It was only partially dependent on BH_4, and FAD and FMN did not stimulate the activity, probably because the expressed enzyme already contains tightly bound BH_4, FAD and FMN. Purified rat NO synthase type I contains BH_4, FAD and FMN [28].

Analysis by Northern blot showed hybridization of NO synthase cDNA to a 10-kb mRNA that is similar in size to the rat brain NO synthase type I [12] but much larger than that of NO synthases type II and type III (4.4 kb or 5–6 kb for type II [21,22] and 4.4 kb or 4.8 kb for type III [23–25]). When the filter from the Northern blot was washed in less stringent conditions, we detected faint 4.4-kb bands in all the human tissues tested, including skeletal muscle, which probably represent mRNA of endothelial type III enzyme (data not shown). We detected some mRNA for NO synthase type I in pancreas. It has been suggested that NO synthase type I is present in rat pancreatic B cells [29]. Surprisingly, more mRNA for NO synthase type I is transcribed in skeletal muscle than in brain. It is unlikely that this derives from contaminating tissue or cells (for example, neuronal or para-neuronal cells), because the poly(A)$^+$ RNA preparations from two different human sources showed the same result. In contrast, mRNA of NO synthase type I was not detected in rat skeletal muscle [12]. In addition to the mRNA, we detected high enzyme activity (Fig. 4) and the specific type I immuno-reactive protein of NO synthase in human, but not rat, skeletal muscle (Fig. 5). Most of the expressed NO synthase in human skeletal muscle seems to be type I (brain type); the mRNA is about 10 kb (Fig. 3), the expressed protein cross-reacted only with type I specific antibody, and the specific band of NO synthase in skeletal muscle migrated at a 160 kDa region similar to the rat brain NO synthase protein (Fig. 5).

Whether the human is the only species expressing high levels of type I NO synthase in skeletal muscle, and the potential function of this NO synthase, is presently being explored.

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