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Comparative Biochemistry and Physiology Part B 130 (2001) 479–491

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Nitric oxide synthase sequences in the marine fish *Stenotomus chrysops* and the sea urchin *Arbacia punctulata*, and phylogenetic analysis of nitric oxide synthase calmodulin-binding domains ☆

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Received 4 April 2001; received in revised form 17 July 2001; accepted 19 July 2001

Abstract

The phylogenetic distribution and structural diversity of the nitric oxide synthases (NOS) remain important and issues that are little understood. We present sequence information, as well as phylogenetic analysis, for three NOS cDNAs identified in two non-mammalian species: the vertebrate marine teleost fish *Stenotomus chrysops* (scup) and the invertebrate echinoderm *Arbacia punctulata* (sea urchin). Partial gene sequences containing the well-conserved calmodulin (CaM)-binding domain were amplified by RT-PCR. Identical 375-bp cDNAs were amplified from scup brain, heart, liver and spleen; this sequence shares 82% nucleic acid and 91% predicted amino acid identity with the corresponding region of human neuronal NOS. A 387-bp cDNA was amplified from sea urchin ovary and testes; this sequence shares 72% nucleic acid identity and 65% deduced amino acid identity with human neuronal NOS. A second cDNA of 381 bp was amplified from sea urchin ovary and it shares 66% nucleic acid and 57% deduced amino acid identity with the first sea urchin sequence. Together with earlier reports of neuronal and inducible NOS sequences in fish, these data indicate that multiple NOS isoforms exist in non-mammalian species. Phylogenetic analysis of these sequences confirms the conserved nature of NOS, particularly of the calmodulin-binding domains.

Keywords: Calmodulin-binding site; Echinodermata; Nitric oxide (NO); Nitric oxide synthase (NOS); Non-mammalian; Phylogenetic analysis; Scup (*Stenotomus chrysops*); Sea urchin (*Arbacia punctulata*); Teleost

Abbreviations: CaM, calmodulin; EtBr, ethidium bromide; NOS, nitric oxide synthase; PCAP, pituitary adenylate cyclase-activating polypeptide; TAE, 40 mM Tris/acetate/1 mM EDTA

☆ Sequences reported here have been submitted to the Genbank database: accession numbers: AF191749 (scup), AF191750 (Arbacia 1) and AF191751 (Arbacia 2).

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1096-4959/01/$ - see front matter
PII: S1096-4959(01)00446-8

1. Introduction

Nitric oxide (NO) is a highly reactive, labile gas produced by the enzymatic conversion of L-arginine by nitric oxide synthases (NOS). In mammals, NO mediates multiple physiological processes from cardiovascular control to neural transmission. Three NOS isoforms (neuronal, inducible and endothelial) have been cloned, sequenced and characterized from several mammalian species (Bredt et al., 1991; Marsden et al., 1992; Xie et al., 1992). These NOS isoforms are variously involved in the diverse physiological functions of NO. Rapidly emerging research results point to the importance of NOS in non-mammalian physiology as well. NOS activity has been detected in non-mammalian vertebrates (Nilsson and Soderstrom, 1997, for review), invertebrates (Martinez, 1995, for review), fungi (Werner-Felmayer et al., 1994) and bacteria (Chen and Rosazza, 1994). However, little is known about the diversity of NOS forms in non-mammalian taxa.

Of particular interest is whether the NOS diversity occurring in mammals arose early or late in vertebrate evolution. We (Cox and Stegeman, 1996) and others (Huque and Brand, 1994; Schoor and Plumb, 1994; Conte and Ottaviani, 1998; Barroso et al., 2000) have demonstrated that NOS enzyme activity occurs in fish. NADPH–diaphorase staining, a non-specific indication of NOS activity, as well as NOS immunochemical staining, suggests the occurrence of nNOS in fish (Holmqvist et al., 1994). Inducible (iNOS) (Laing et al., 1999; Saeij et al., 2000) and partial neuronal (nNOS) (Oyan et al., 2000) sequences have been identified in fish. Endothelial (eNOS) homologues have not been reported in fish, and physiological studies question whether NO functions as a vasodilator in fish (Olson and Villa, 1991; Mustafa and Agnisola, 1998).

Here, we report on the molecular identification of NOS cDNA sequences from a marine vertebrate, the teleost fish *Stenotomus chrysops* (scup), and an invertebrate, the echinoderm *Arbacia punctulata* (sea urchin). Fishes are the earliest diverging vertebrate group, while sea urchins represent a phylum, the Echinodermata, that is a sister group to the chordates (Jefferies, 1986). The sea urchin was also chosen because our earlier results suggest that NOS may be responsible for production of NO in sea urchin sperm, for which NO regulates motility (Heck et al., 1994).

We used reverse transcriptase-polymerase chain reaction (RT-PCR) with degenerate oligonucleotide primers designed against a portion of the mammalian NOS gene that codes for the calmodulin (CaM)-binding region. This region was chosen because it is highly conserved among NOS sequences to date and is a functionally important region of NOS proteins. Using this approach, we identified three distinct NOS cDNA sequences. One sequence, amplified from scup brain, heart, liver and spleen, represents a fish neuronal NOS. Two distinct sequences were amplified from sea urchin ovary and testes, indicating that multiple NOS isoforms exist in some invertebrates. Our sequence analyses also demonstrate that the NOS CaM-binding region is highly conserved across these diverse species.

2. Materials and methods

2.1. Animals

Male and female scup (*Stenotomus chrysops*) ranging in weight from 150 to 450 g were caught by angling in Vineyard Sound, MA, USA, during summer and autumn months. Fish were maintained for 1 year at 14°C in a continuously flowing seawater system and fed Purina trout chow, approximately twice per week. Fish were sacrificed by cervical transection and dissected immediately. For RNA isolation, brains, hearts, livers and spleens were pooled from three to four individuals of both sexes. Whole brains were used; atria were removed from hearts. After dissection, organs were immediately frozen in liquid nitrogen and stored at −70°C until use.

Mature sea urchins (*Arbacia punctulata*) were obtained from the Marine Biological Laboratory, Woods Hole, MA, USA. Ovaries (with eggs) and testes (with sperm) were dissected from several animals and washed with artificial seawater. Tissues were pooled for isolation of RNA, which was performed immediately.

2.2. Materials

RNA STAT-60 was purchased from Tel-Test Inc (Friendswood, TX, USA) and oligo(dT) cellu-

lose spin columns from 5 Prime → 3 Prime Inc (Boulder, CO, USA). For RT-PCR and cloning, we used the Perkin Elmer GeneAmp RNA PCR kit (Applied Biosystems, Foster City, CA, USA). The Geneclean kit was obtained from Bio 101 (La Jolla, CA, USA). The pT7Blue T-Vector, NovaBlue competent cells and T4 DNA ligase were purchased from Novagen (Madison, WI, USA) and ligation buffer and ATP from Epicentre Technologies (Madison, WI, USA).

All reagents, including agarose and molecular weight markers, were from Sigma (St. Louis, MO, USA), Pharmacia Biotechnology (Piscataway, NJ, USA), Bayou Labs (Harahan, LA, USA), or Promega (Madison, WI, USA). All reagents necessary for plasmid DNA preparation, LB broth, LB agar, ampicillin, tetracycline, lysozyme, RNase, phenol, chloroform, isoamyl alcohol, polyethylene glycol (PEG) and ethanol were supplied from Sigma or Pharmacia Biotechnology. Restriction enzymes were obtained from Promega. The Sequitherm EXCEL Long Read DNA sequencing kit-LC (Epicentre Technologies) was used with labeled M13 forward and reverse primers (LI-COR, Inc., Lincoln, NE, USA).

2.3. Cloning and sequence analysis

Two pairs of degenerate oligonucleotide primers were designed based on the sequence of a region encompassing the NOS calmodulin-binding site. The primers were designed such that the second pair targeted a sequence internal to the first pair. Table 1 gives the sequences of all primers. The upstream primer of the external pair (NOS-590F) was designed based on a partial cDNA sequence of inducible NOS cloned from trout head kidney macrophages (Grabowski et al., 1996). In designing all other primers, target regions were chosen which had a prevalence of amino acids encoded by one or two codons. In cases of amino acids encoded by multiple codons, a fish codon usage chart (Wada et al., 1990) was used to reduce degeneracy. In few cases, 4 codon possibilities were included, but degeneracy of the entire oligonucleotide was kept to a minimum. All oligonucleotides were synthesized by National Biosciences (Plymouth, MN, USA).

Selected organs were pooled from four to eight fish. Total RNA was isolated from scup organs (0.25–1.2 g) using RNA STAT-60 and from sea urchin ovaries and testes (0.5 g) using Tri reagent. Both procedures followed the manufacturer's instructions. The quality of RNA preparations was monitored by agarose gel electrophoresis on an EtBr-stained 1% (w/v) gel in 2.2 M formaldehyde, 1 × MOPS/acetate buffer, pH 7.0. Poly(A)$^+$ RNA was purified by oligo(dT) cellulose chromatography with the QuickPrep mRNA Purification Kit (Pharmacia Biotechnology) according to the manufacturer's instructions. Eluted mRNA was precipitated, washed and dissolved in water. The yield and purity of RNA samples were determined spectrophotometrically.

NOS-specific oligonucleotide primers were used in a nested RT-PCR strategy as follows: first-strand cDNA synthesis and subsequent amplification were carried out using the Gene-Amp RNA PCR kit (Perkin-Elmer) according to the manufacturer's instructions. For scup tissues, 0.15–1.5 μg of total RNA, and for sea urchin tissues, 50–250 ng of poly(A)$^+$ RNA were reverse transcribed by priming with random hexamers in a 20-μl final volume. Subsequent amplification, using primers NOS-590F and NOS-893R (each at 1 μM), was performed in the same tube in a 100-μl final volume. PCR conditions were as follows: 1 min 45 s at 95°C, followed by 40 cycles of denatur-

Table 1
Degenerate oligodeoxynucleotides used for RT-PCR amplification of cDNA coding for the NOS calmodulin-binding region in fish and sea urchin

Oligo-nucleotide	Sequence[a]	Target amino acid sequence	Amino acid number[b]	Fold degeneracy
NOS-590F	GGYTGGTACATGRGCACYGAGATYGG	GWYMSTEIG	590–598	16
NOS-693F	CCNGTBTTYCAYCAGGAGATG	PVFHQEM	693–699	48
NOS-812R	GGRTCNCCRTTSCCAAAKGT	TFGNGDP	812–818	64
NOS-893R	AAGGCRCARAASTGDGGRTA	YPHFCAF	893–899	48

[a] Y = C or T; R = A or G; S = C or G; K = G or T; D = A or G or T; B = C or G or T; and N = A or C or G or T.
[b] Amino acid number is based on the complete human neuronal cDNA sequence (Nakane et al., 1993).

ing at 95°C (15 s); annealing at 42°C (30 s); and extension at 72°C (1 min). This was followed by a 7-min extension at 72°C. A low annealing temperature was used based on previous studies, in which mammalian gene sequences were used to design oligonucleotide probes for amplification of fish cDNAs (McMahon et al., 1990). A 1-μl aliquot of this reaction was re-amplified using the internal pair of oligonucleotides NOS-693F and NOS-812R (each at 1 μM) under the same conditions, except that 35 cycles were run, and annealing was carried out at 55°C. PCR products were visualized by EtBr staining after separation on 1.5% (w/v) agarose in 1× TAE buffer. Bands of the expected size were excised and purified (Geneclean II, Bio 101). The PCR product was ligated into the pT7Blue vector (Novagen) using T4 ligase (Epicentre Technologies) and transformed into Novablue competent cells (Novagen). Plasmids from positive colonies were purified by the boiling method (Sambrook et al., 1989). Plasmids containing an appropriate size insert were identified by digesting plasmid DNA with restriction enzymes *Eco*RI and *Pst*I (Promega). Digested products were visualized by EtBr staining after separation on 1.5% agarose in 1× TAE buffer, pH 8.5. Two or three individual clones from each reaction were sequenced (both strands) by cycle sequencing (SequiTherm, Epicentre) using an automated DNA sequencer (LI-COR Inc). cDNA sequences were translated using MacVector 6.0.

2.4. Phylogenetic analysis of NOS sequences

NOS sequences used for phylogenetic analyses were retrieved from Genbank and are listed along with accession numbers in Table 2. Using CLUSTALW (MacVector 6.0), amino acid alignments of the calmodulin-binding regions were produced. These approximately 115 amino acid alignments were used to construct phylogenetic trees by two methods. For the first method, a Fitch–Margoliash minimum evolution distance tree was constructed using the programs PROTDIST and FITCH in the PHYLIP package (Felsenstein, 1993). Protein distances were determined by PROTDIST using the Dayhoff model. The distance tree was constructed using global rearrangements and 1000 random-sequence addition replicates. By the second method, a maximum parsimony tree was constructed (PAUP 3.1.1) (Swofford, 1993), using the heuristic search method. Of the 119 characters analyzed, 87 were informative sites and 16 were constant sites. For both methods, bootstrap analyses (Felsenstein, 1985) were used to assess the relative confidence of phylogenetic groupings. For the Fitch–Margoliash tree, the confidence level was determined by bootstrap

Table 2
Sources, references and accession numbers for the nitric oxide synthase sequences (ps, partial sequence)

Species	Common name	Protein	Source	Reference	Accession number
Homo sapiens	Human	nNOS	Brain	Nakane et al., 1993	L02881
Rattus rattus	Rat	nNOS	Brain	Bredt et al., 1991	X59949
Mus musculus	Mouse	nNOS	Brain	Ogura et al., 1993	D14552
Stenotomus chrysops	Scup	nNOS (ps)	Various organs	Present study	AF191749
Homo sapiens	Human	iNOS	Chondrocyte	Charles et al., 1993	X73029
Rattus norvegicus	Rat	iNOS	Hepatocyte	Wood et al., 1993	L12562
Mus musculus	Mouse	iNOS	Macrophage	Xie et al., 1992	M87039
Gallus gallus	Chicken	iNOS	Macrophage	Lin et al., 1996	U46504
Oncorhynchus mykiss	Rainbow trout	iNOS (ps)	Macrophage	Laing et al., 1999	X97013
Carassius auratus	Goldfish	iNOS (ps)	Macrophage	Laing et al., 1996	X97603
Homo sapiens	Human	eNOS	Endothelial cells	Marsden et al., 1992	M95296
Bos taurus	Bovine	eNOS	Aortic endothelial cells	Lamas et al., 1992	M89952
Sus scrofa	Pig	eNOS	Endothelial cells	Zhang et al., 1996	U59924
Drosophila melanogaster	Fruit fly	NOS	Whole organism	Regulski and Tully, 1995	U25117
Rhodnius prolixus	Blood sucking bug	NOS	Salivary gland	Yuda et al., 1996	U59389
Manduca sexta	Tobacco hornworm	NOS	Olfactory system	Nighorn et al., 1998	AF062749
Anopheles stephensi	Mosquito	NOS	Whole organism	Luckhart et al., 1998	AF053344
Lymnaea stagnalis	Snail	NOS	Neuronal cells	Korneev et al., 1998	AF012531
Arbacia punctulata	Sea urchin	NOS 1 (ps)	Testes/ovary	Present study	AF191750
Arbacia punctulata	Sea urchin	NOS 2 (ps)	Ovary	Present study	AF191751

analysis using 100 bootstrap replicates, 100 global rearrangements, and 10 random sequence addition replicates per bootstrap replicate. Confidence levels for the maximum parsimony tree were assessed using 100 bootstrap replicates.

3. Results

3.1. Amplification of NOS calmodulin-binding regions from scup and sea urchin

Using degenerate primers in RT-PCR, a 375-bp product was amplified from scup brain. Subsequently, using the same primers, an identical sequence was amplified from scup heart, liver and spleen. The nucleotide and deduced amino acid sequences are available from the Genbank database (AF191749). This scup sequence shares 82% nucleotide and 91% deduced amino acid sequence identity with the calmodulin-binding region of human neuronal NOS. A partial nNOS sequence was recently reported from salmon (*Salmo salar*) (Oyan et al., 2000) that also encompasses the calmodulin-binding domain. A comparison of the deduced amino acid sequence from scup to that of salmon shows 98% identity. These results indicate that the scup sequence is the calmodulin-binding domain of NOS, and we designate this sequence as a scup neuronal NOS.

Two distinct NOS-like cDNAs were amplified from the sea urchin tissues. Identical 387-bp products were isolated from ovary and from testes. This sequence was designated Arbacia NOS 1. Using the same primers and PCR conditions, a second product was obtained from ovary. This product was slightly smaller (381 bp) than, and clearly distinct from, Arbacia NOS 1, and was designated Arbacia NOS 2. The nucleotide and deduced amino acid sequences are available from the Genbank database (AF191750 for Arbacia 1, and AF191751 for Arbacia 2). The sea urchin nucleotide sequences are aligned in Fig. 1. Arbacia NOS 2 shares 66% nucleic acid and 57% deduced amino acid identity with Arbacia NOS 1. The Arbacia NOS 1 nucleotide sequence is 72% identical and the deduced amino acid sequence is 65% identical (81% similar) to the CaM-binding region of human neuronal NOS. At the amino acid level, Arbacia NOS 1 shares 56% identity and 74% similarity to the NOS CaM-binding region from *Drosophila* (Regulski and Tully, 1995). The deduced amino acid sequence identities for Arbacia NOS 2 are 52% with human neuronal and 45% with *Drosophila* NOS calmodulin-binding domains (Table 3).

Fig. 2 shows the alignment of deduced NOS amino acid sequences from scup and sea urchin CaM-binding regions with those from other NOS, as well as from two non-NOS, calcium/calmodulin-dependent enzymes. Although their calcium–calmodulin requirements differ, all NOS protein sequences contain the 1-5-8-14 type A calmodulin-binding motif that is characterized by the position and order of specific hydrophobic residues. This motif is common among at least 30 calcium–calmodulin-binding proteins (Rhoads and Frieberg, 1997). The scup and sea urchin sequences conform to the type A CaM-binding motif, with some exceptions for sea urchin. Arbacia NOS 1 deviates at position 14, where methionine replaces the hydrophobic residue. Arbacia NOS 2 deviates at positions 1 and 5, where serine and histidine replace the hydrophobic residues.

3.2. Phylogenetic analyses of NOS and the NOS calmodulin-binding domain

Using aligned amino acid sequences spanning the CaM-binding region, two phylogenetic trees were inferred by different methods. Similar topologies were obtained with both methods, with strong bootstrap support for the clustering of the scup NOS with mammalian neuronal NOS isoforms: 100% for the Fitch–Margoliash distance tree (Fig. 3a), and 98% for the parsimony tree (Fig. 3b). The parsimony tree is presented in order to confirm the clustering of scup NOS with mammalian neuronal NOS as shown in the Fitch–Margoliash tree. In the latter (Fig. 3a), Arbacia NOS 1 and NOS 2 sequences diverged. Arbacia NOS 1 clustered with the mammalian endothelial and neuronal isoforms. Arbacia NOS 2 clustered with inducible isoforms.

The topology of the Fitch–Margoliash tree (Fig. 3a) is identical to a tree constructed by the same method, but with full-length NOS amino acid sequences (data not shown), as well as to previous phylogenetic analyses of NOS (Hughes, 1998; Oyan et al., 2000). Four distinct clades correspond to three mammalian isoforms plus the invertebrate NOS sequences. Neuronal and endothelial sequences form a monophyletic group.

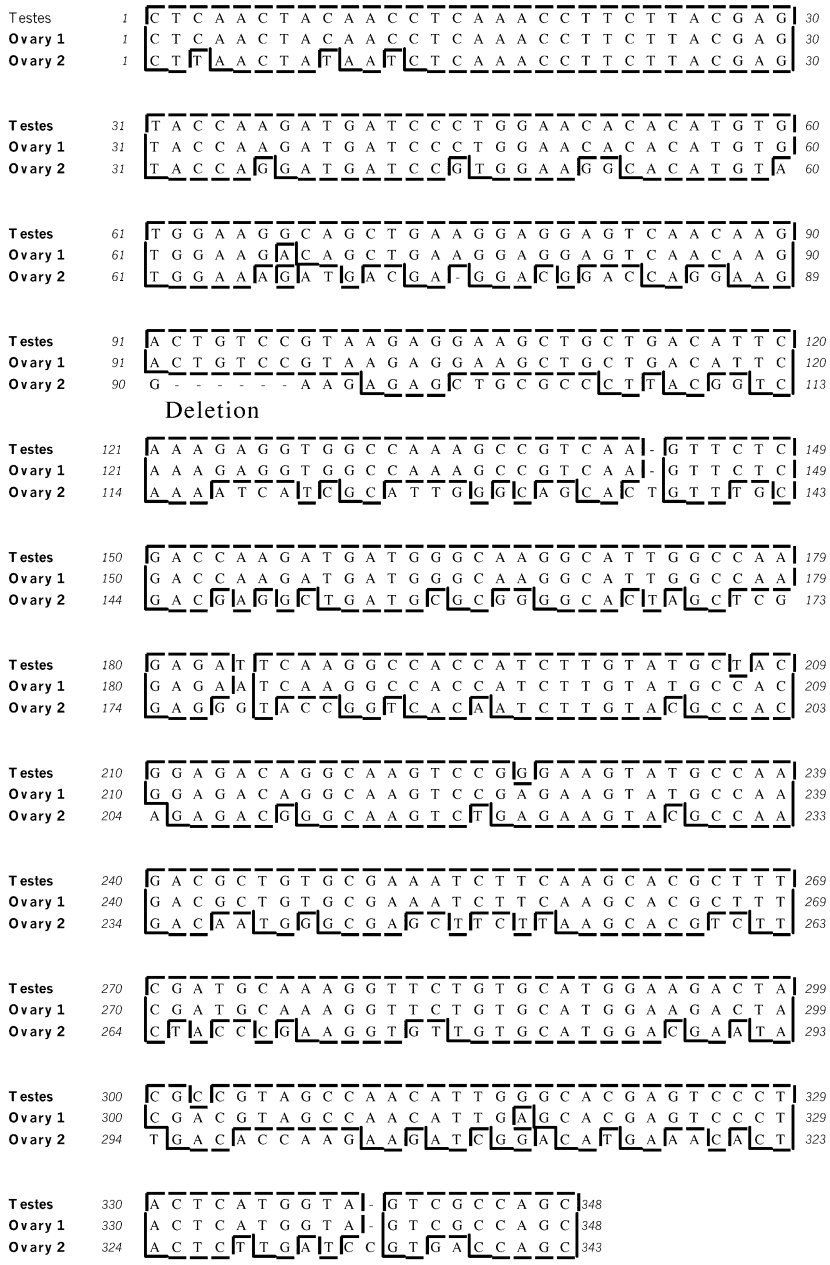

Fig. 1. Nucleotide alignment of the three sea urchin NOS RT-PCR products. Alignment of Arbacia NOS sequences amplified from testes (Testes) and ovary (Ovary 1 and Ovary 2). Identities are boxed. Region of deletion in Ovary 2 sequence is indicated.

This indicates that the best-fit Fitch–Margoliash tree constructed with sequences corresponding to CaM-binding regions is entirely representative of full-length NOS sequences, and strongly argues for the occurrence of two distinct Arbacia isoforms, even though bootstrap support is low due to the smaller amount of data analyzed.

4. Discussion

In this study, cDNA sequences coding for a calmodulin-binding domain of NOS were cloned from brain, heart, liver and spleen of the marine fish, scup. In phylogenetic analysis, this scup sequence consistently clustered with neuronal NOS

Table 3
Amino acid conservation between aligned NOS calmodulin-binding regions

	Neuronal Type I			Endothelial Type III			Inducible Type II				Invertebrate			
	Human	Mouse	Scup	Human	Bovine	Porcine	Human	Rat	Chicken	Trout	Dros	Rhod	Arbacia 1	Arbacia 2
Neuronal														
Human	100	98	91	62	61	58	51	51	51	47	54	52	65	52
Mouse		100	90	60	61	56	50	50	50	47	55	52	64	50
Scup			100	62	62	58	52	50	53	47	50	53	65	52
Endothelial														
Human				100	94	93	42	41	41	43	43	44	49	42
Bovine						90	44	41	41	45	45	45	49	42
Porcine					100	100	41	40	40	42	42	41	46	41
Inducible														
Human							100	76	67	46	41	45	43	51
Rat								100	60	46	40	42	42	43
Chicken									100	50	44	41	42	45
Trout										100	47	43	45	40
Invertebrate														
Dros[a]											100	62	56	45
Rhod[b]												100	53	45
Arbacia 1													100	57
Arbacia 2														100

Percent identities calculated using CLUSTALW (MacVector 6.0). See Table 2 for sequence accession numbers.
[a] *Drosophila melanogaster*.
[b] *Rhodnius prolixus*.

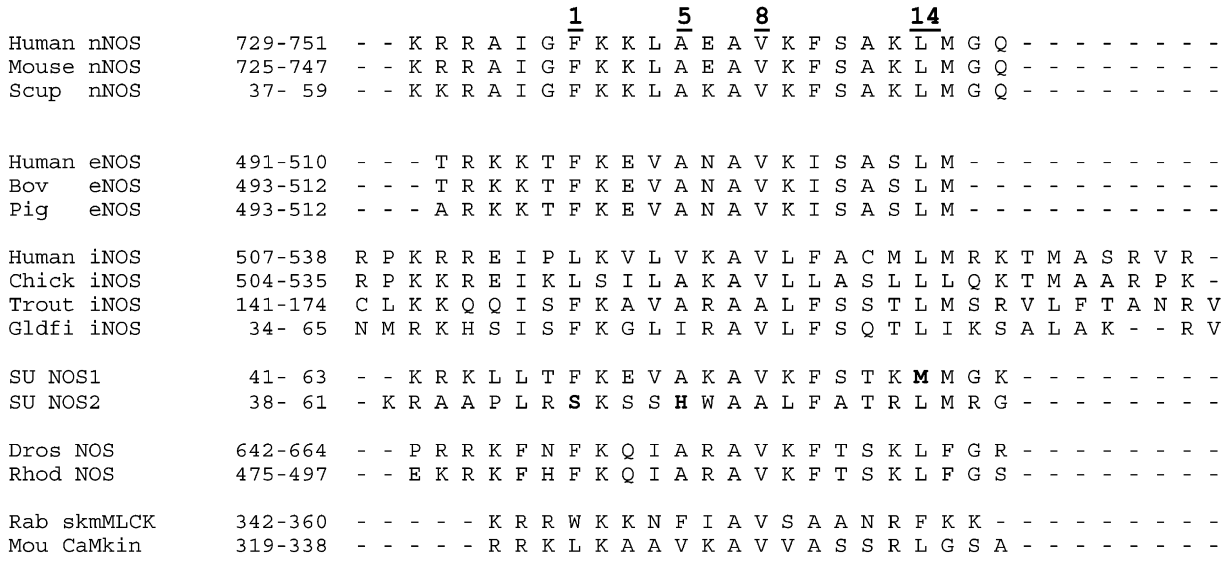

Fig. 2. Alignment of putative calmodulin-binding regions from calcium–calmodulin-dependent proteins. Amino acid residue numbers for calmodulin-binding sites are given. Alignments were performed using CLUSTAL version 1.6. Sources and accession numbers for NOS sequences are given in Table 2. Rabbit skeletal muscle myosin light chain kinase (skmLCK) is from Ikura et al. (1992). Mouse calmodulin-dependent kinase (Mou CaM-dep kinase) is from Jones et al. (1991). Abbreviations are: Chick, chicken; Gldfi, goldfish; Dros, *Drosophila*; and Rhod, *Rhodnius prolixus*. Hydrophobic residues corresponding to the Ca^{2+}-dependent calmodulin-binding 1-5-8-14 Type A motif are designated. Residues in sea urchin sequences deviating from that motif are in bold. 1-5-8-14 Type A motif is as follows: (FILVW)xxx(FAILVW)xx(FAILVW)xxxxx(FILVW).

(nNOS) isoforms, in a clade distinct from all known eNOS and iNOS sequences. Thus, we conclude that this sequence represents an nNOS. Together with the results of Laing et al. (1999) and Oyan et al. (2000), this establishes that at least two NOS isoforms, apparent homologues of nNOS and iNOS, occur in fish. Although RT-PCR does not indicate levels of expression, the results also imply that an nNOS is expressed and presumably functions in brain, liver, heart and spleen of fish.

The occurrence of nNOS in fish was predicted by studies showing NADPH–diaphorase staining and NOS immunoreactivity (Schober et al., 1993; Holmqvist et al., 1994) in brain and nerve tissues of other organs. NOS immunoreactivity in cells that innervate the fish pituitary gland (Holmqvist and Ekstrom, 1995) suggests that NO could modulate neuroendocrine functions in fish. Abundant NOS immunostaining in visual centers (Holmqvist et al., 1994) and along retinal projections in salmon (Holmqvist and Ekstrom, 1997), as well as the inhibitory effect of NO on horizontal cell responsiveness in the hybrid bass retina (McMahon and Ponomareva, 1996), point to a role of NO in visual signal transmission in fish. Other functions that may involve NOS in fish brain include regulation of blood flow. Application of acetylcholine and NO donors to fish optic lobes caused increased velocity of blood flow, a process that was blocked by application of NOS inhibitors (Hylland and Nilsson, 1995; Soderstrom et al., 1995), suggesting that NO acts as a vasodilator to regulate cerebral blood flow. However, it is not clear whether this involves nNOS or some other NOS form.

We also cloned nNOS from scup heart, but the potential role of NOS in fish heart is not known. In mammalian heart, NOS is expressed by endothelial cells, nerve and ganglion cells (Ursell and Mayes, 1995), cardiac macrophages (Buttery et al., 1994) and myocytes (Haywood et al., 1996), and NO produced in mammalian heart controls vasodilation and mediates immune function. Previous studies on the possible functions of NO in fish heart are not conclusive. The effects of L-arginine and its analogues on vasoactivity of intact trout coronary system suggested that NO mediates vasodilation in trout heart (Mustafa et al., 1997). However, application of L-arginine, NO donors and calcium ionophores to aortic rings of spiny dogfish (Evans and Gunderson, 1998) had no effect on vasodilation. Endothelial cells (Stegeman et al., 1989) and macrophages

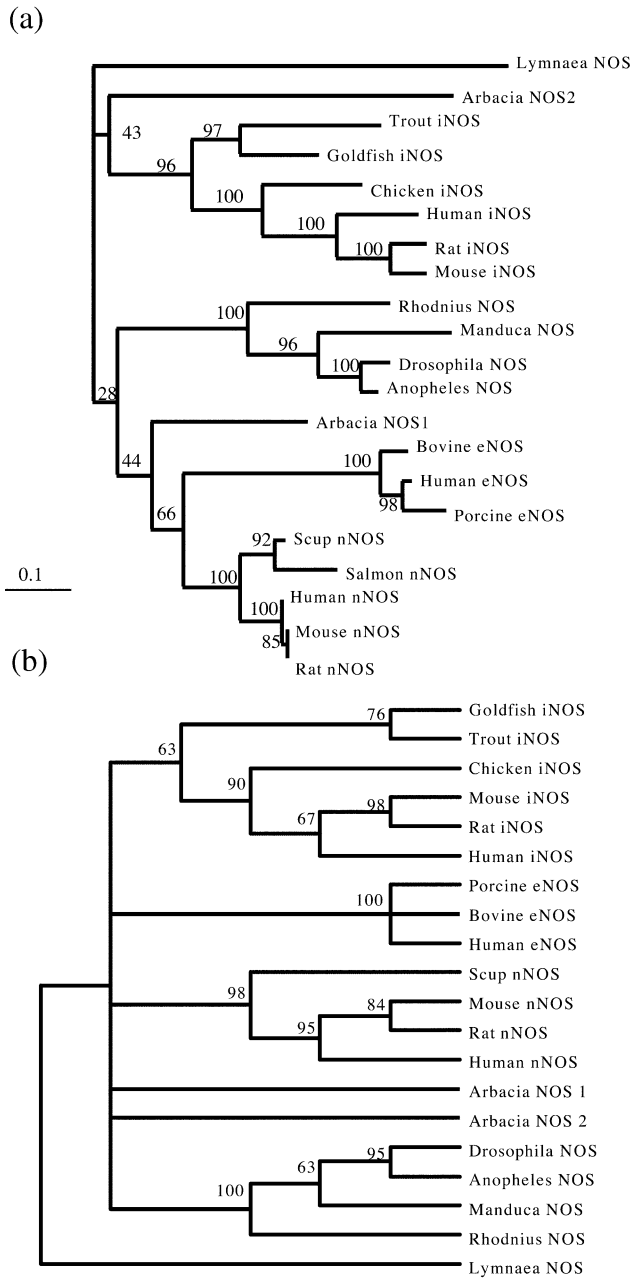

Fig. 3. Unrooted NOS phylogenetic trees. (a) Distance tree: amino acid sequences were used to infer phylogenetic trees by the minimum evolution (distance) method. The single best Fitch–Margoliash distance tree was found by Fitch (see Section 2) using the 115 amino acid sequences spanning the NOS calmodulin-binding regions. The tree found is shown, with bootstrap values (a measure of relative confidence to the grouping) superimposed. Branch lengths represent evolutionary change. (b) Parsimony tree: the 115 amino acid sequences spanning the NOS calmodulin-binding region were used to infer a phylogenetic tree by the maximum parsimony method (see Section 2). Sequence sources and accession numbers are given in Table 2.

(Nakamura and Shimozawa, 1994) are present in teleost heart, but whether these are the cells that express NOS is not known.

Our finding that nNOS is expressed in scup liver supports the observation of NOS activity in fish liver (Cox and Stegeman, 1996; Conte and Ottaviani, 1998). In mammalian liver, NO from an nNOS could regulate hepatic blood circula-

tion, indicated by diaphorase staining in nerve fibers surrounding the hepatic artery and bile ducts (Esteban et al., 1997). Similarly, a function for NO in regulation of hepatic blood flow in fish is suggested by the presence of nNOS immunoreactivity in nerve fibers of vessels and ducts in teleost liver (Esteban et al., 1998). Mammalian iNOS expression can be induced in hepatic macrophages and endothelial cells (Laskin et al., 1994), as well as in parenchymal cells (Knowles et al., 1990), and may participate in protection against pathogens. Immune function of NO in fish liver is indicated by detection of NO generation by fish liver macrophages (Neumann et al., 1995). However, as with heart, it is not known which cells in fish liver express iNOS or nNOS.

Our cloning of a neuronal NOS sequence from scup spleen is the first indication of NOS expression in fish spleen. In mammalian spleen, inducible and neuronal isoforms are expressed and appear to be counter-regulated (Cunha et al., 1994).

In part, these studies were initiated because of our observation that cytochrome P450 1A can be induced to high levels in the endothelium of fish (Stegeman et al., 1989), and the possibility that this heme protein interacts with endothelial-derived NO in fish. However, we do not know whether fish employ NO as an endothelium-derived relaxing factor (EDRF). Application of acetylcholine and NO donors to fish vessels have yielded conflicting results (Olson and Villa, 1991; Mustafa and Agnisola, 1998), leading some to suggest that endothelium-dependent relaxation mediated by NO occurs only in some fish species (Nilsson and Soderstrom, 1997). Others have speculated that the vasodilatory function of NO evolved with air-breathing capacity and/or the movement from an aquatic to a terrestrial environment (Miller and Vanhoutte, 1992). However, using cGMP levels as a measure of NOS activity, Staples et al. (1995) found no evidence for a NOS pathway in the air-breathing dipnoan lungfish, suggesting that the function of NO as a vasodilator did not necessarily co-evolve with air-breathing in fish. As yet, there have been no endothelial NOS sequences reported from fish, or from any other non-mammalian vertebrate. We identified a NOS sequence in endothelial cells cultured from the eel *Anguilla rostrata* (Cox et al., unpublished), but the isoform of this sequence is not confirmed.

The two distinct NOS cDNAs from sea urchin, Arbacia NOS 1 from ovary and testes and Arbacia NOS 2 from ovary, shared only 57% amino acid identity, implying that they represent an ancient divergence. Clustering of Arbacia NOS 1 and NOS 2 sequences with mammalian constitutive and inducible isoforms, respectively (Fig. 3a), suggests that these sea urchin genes could represent early homologues of these two NOS types. Some physiological roles of NO in invertebrates parallel those observed in mammals. The occurrence of NADPH–diaphorase staining in invertebrate nervous systems has been widely demonstrated (Martinez, 1995 for review), and effects of L-arginine application on acetylcholine release in *Aplysia* neurons (Meulemans et al., 1995) indicate a role for NO as a neurotransmitter in invertebrates. Studies in insects suggest that NO mediates olfactory and visual input, controls vasodilation (Muller, 1997, for review), regulates cell proliferation (Kuzin et al., 1996) and plays a role in cellular defense (Luckhart et al., 1998). NOS-like activity in hemocytes of horseshoe crab (Radomski et al., 1991) and snail (Franchini et al., 1995) further suggest that NO mediates cellular defense in these species. These results are consistent with the possible occurrence of nNOS- and iNOS-like functions in some invertebrates.

The physiological function(s) of an NO/NOS pathway in sea urchin remains under investigation. In other studies, we observed that NO mediates sea urchin sperm motility, which suggests a role for NOS in fertilization (Heck et al., 1994).

An analysis of the numbers of genes in each of 26 multigene families in chordate species (Iwabe et al., 1996) shows that the rate of gene duplication was higher during chordate evolution before the emergence of fishes. In many gene families, paralogous genes may then have arisen before the separation of the lines leading to fishes and tetrapods. If the diversification of NOS genes followed this pattern, then we predict early chordates and vertebrates to show the same diversity observed in mammals. The occurrence of nNOS and iNOS sequences in fish is consistent with that prediction. Further analysis of early diverging vertebrates is necessary to determine whether an eNOS orthologue occurs in fish, or in other non-mammalian species.

Thus far, only one NOS form has been identified in other invertebrates examined. Due to the late branching pattern of the Echinodermata and its classification as a sister group to the chordates,

the sea urchin may conform more to the pattern of NOS gene distribution observed in vertebrates. Identification of NOS genes from various invertebrate families could disclose the evolutionary pattern of NOS gene divergence and the emergence of different NOS subfamilies.

In summary, data presented here provide evidence for the occurrence of multiple NOS genes in fish and echinoderms, indicating the appearance of multiple forms early in chordate evolution. However, the physiological functions of an NOS/NO pathway in fish and echinoderms are not fully understood. The conserved nature of the NOS calmodulin-binding site suggests a shared function across distant phyla, and may be a target for further evaluation of NOS diversity.

Acknowledgements

We thank Drs Andrew McArthur, Sibel Karchner and Mark Hahn for technical advice and helpful discussions. Supported in part by National Institute of Health grants T32HL07501, ES 03647, ES 05022 and ES 06897.

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