Comparative Biochemistry and Physiology, Part A 166 (2013) 207–214

Contents lists available at SciVerse ScienceDirect

Comparative Biochemistry and Physiology, Part A

journal homepage: www.elsevier.com/locate/cbpa

How ubiquitous is endothelial NOS?

Fahima Syeda [a], David Hauton [a], Steven Young [a], Stuart Egginton [a,b,*]

[a] Centre for Cardiovascular Sciences, University of Birmingham, UK
[b] School of Biomedical Sciences, University of Leeds, UK

ARTICLE INFO

Article history:
Received 8 August 2012
Received in revised form 11 May 2013
Accepted 25 May 2013
Available online 30 May 2013

Keywords:
Capillaries
eNOS
Fish
Histochemistry
Muscle
Myography
Synteny

ABSTRACT

The ability to regulate vascular tone is an essential cardiovascular control mechanism, with nitric oxide (NO) assumed to be a ubiquitous smooth muscle relaxant. However, the literature contains reports of vasoconstrictor, vasodilator and no response to nitroergic stimulation in non-mammalian vertebrates. We examined functional (branchial artery myography), structural (immunohistochemistry of skeletal muscle), proteomic (Western analysis) and genomic (RT-PCR, sequence orthologues, syntenic analysis) evidence for endothelial NO synthase (NOS3) in model and non-model fish species. A variety of nitrodilators failed to elicit any changes in vascular tone, although a dilatation to exogenous cyclic GMP was noted. NOS3 antibody staining does not localise to endothelial markers in cryosections, and gives rise to non-specific staining of Western blots. Abundant NOS2 mRNA was found in all species but NOS3 was not found in any fish, while putative orthologues are not flanked by similar genes to NOS3 in humans. We conclude that NOS3 does not exist in fish, and that previous reports of its presence may reflect use of antibodies raised against mammalian epitopes.

1. Introduction

In mammals, nitric oxide (NO) is an important biological signalling molecule synthesised by one of three NO synthase (NOS; EC 1.14.13.39) isoforms: neuronal (nNOS/NOS1), inducible (iNOS/NOS2) or endothelial (eNOS/NOS3). The endothelium of blood vessels express eNOS and on activation NO diffuses to overlying vascular smooth muscle cells to stimulate soluble guanylate cyclase, and produce cyclic GMP (cGMP)-mediated vasodilatation (Moncada and Higgs, 2006). The importance of eNOS in the regulation of vascular tone followed identification of NO as an endothelium-derived relaxing factor (EDRF) (Palmer et al., 1987) and the finding that eNOS gene ablation in mice leads to hypertension and bradycardia (Shesely et al., 1996). The co-expression of eNOS and nNOS in human endothelial cells suggests that nNOS may play a supporting role. Indeed, ablation of either eNOS or nNOS elevated baseline blood pressure and hampered the murine baroreflex response (Carvalho et al., 2006).

The existence of an EDRF similar to that found in mammals has been demonstrated in a number of non-mammalian vertebrates such as birds (Hasegawa and Nishimura, 1991), frogs (Knight and Burnstock, 1996), toads (Broughton and Donald, 2002), and alligators (Skovgaard et al., 2008). However, evidence for the presence of eNOS or that NO is the EDRF in fishes is limited. Such studies have used non-specific NOS blockade using L-arginine analogues, L-NAME (L-nitro-arginine methyl ester) and L-NNA (NG-nitro-L-arginine), to demonstrate NO-dependent vasodilatation. While the vasoconstriction caused by NOS inhibitors was attributed to blockade of eNOS (Fritsche et al., 2000), neither the eNOS gene nor protein has been identified in zebrafish endothelium. To our knowledge, eNOS mRNA expression has not been studied in any fish species. Rather, immunohistochemical localisation of putative eNOS has used mammalian antibodies to demonstrate apparent eNOS expression (Fritsche et al., 2000; Ebbesson et al., 2005; Amelio et al., 2006; McNeill and Perry, 2006) although extensive literature searches and phyologenetic analyses did not yield a result in fishes (Andreakis et al., 2010; Gonzalez-Domenech and Munoz-Chapuli, 2010). Chemical denudation of the endothelium in trout conduit artery inhibited arginine- and adenosine-mediated vasodilatation and NO_2^--production, suggesting a role for EDRF in trout (Mustafa and Agnisola, 1998). However, in carp aorta vasodilatation using the calcium ionophore, A23187, is endothelium-dependent but not through the actions of NO (Park et al., 2000). Instead, endothelium-dependent vasodilatation has been shown to be through the actions of cyclooxygenase products in carp (Park et al., 2000) and spiny dogfish Evans and Gunderson (1998). In the eel, NO causes vasodilatation in the ventral aorta Evans and Harrie (2001) but vasoconstriction in the branchial circulation (Pellegrino et al., 2002), suggesting diverse roles for NO in the vasculature. In the western clawed frog, *Xenopus tropicalis*, an eNOS analogue protein has been identified in the kidney, heart and stomach tissue but is not localised to blood vessels (Trajanovska and Donald, 2011). The authors concluded that the protein is more similar to mammalian eNOS than iNOS or nNOS, and contains some, but not all, palmitoylation and myristoylation sites characteristic of mammalian eNOS (Trajanovska and Donald, 2011). Here, we provide evidence for the absence of eNOS mRNA and protein in tissues from model and

* Corresponding author at: School of Biomedical Sciences, Faculty of Biological Sciences, University of Leeds, Leeds LS2 9NQ, UK. Tel.:+44 113 343 2264; fax: +44 113 343 4272.
E-mail address: s.egginton@leeds.ac.uk (S. Egginton).

1095-6433/$ – see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.cbpa.2013.05.027

non-model fish species, zebrafish (*Danio rerio*), spotted green pufferfish (*Tetraodon nigroviridis*), common carp (*Cyprinus carpio*) and rainbow trout (*Oncorhynchus mykiss*). The ideas in this paper were first published in abstract form (Syeda et al., 2010).

2. Materials and methods

2.1. Animals and tissue collection

Rainbow trout (*Oncorhynchus mykiss*, Salmonidae) and common carp (*Cyprinus carpio* Cyprinidae), ~250 g body mass, were purchased from local farms (Leadmill trout farm, Hathersage and Ripples Waterlife, Telford, respectively) and held in recirculating, aerated, charcoal-filtered Birmingham tap water. Spotted green pufferfish (*Tetraodon nigroviridis*, Tetraodontidae) were gifted by F. Mueller and zebrafish (*Danio rerio*, Cyprinidae) by R. Bicknell (both University of Birmingham). Wistar rats (*Rattus norvegicus*) were purchased from Harlan UK Ltd (Bicester, UK). All animals were killed according to the Animals (Scientific Procedures) Act, 1986, using Home Office Schedule 1 methods. Trout, carp and rats were killed by stunning followed by transecting of the brainstem, pufferfish and zebrafish were killed by immersion in 5% (w/v) MS222. Samples were excised immediately and snap-frozen in liquid nitrogen. Human umbilical vein endothelial cell (HUVEC) RNA was gifted by P. Stone (University of Birmingham). HUVEC were isolated from umbilical cords with maternal consent, and cultured in M199, supplemented with 20% heat-inactivated fetal calf serum, 2.5 μg/mL amphotericin B, 1 μg/mL hydrocortisone, 10 ng/mL epidermal growth factor (all from Sigma-Aldrich) and 35 μg/mL gentamycin (Gibco Invitrogen Compounds) until confluent.

2.2. Antibodies and chemicals

The following antibodies were used: *anti-eNOS*, Ab1- BD Transduction Laboratories (cat#610293); Ab2- Chemicon, Tenecula, USA (cat#AB16301) and *anti-nNOS*, Ab3- Santa Cruz Biotechnology, USA (cat#SC-648). All other materials were from Sigma Aldrich, UK, unless otherwise stated.

2.3. Extraction of total RNA and reverse transcription

Total RNA was extracted using the RNeasy Mini kit (Qiagen, Valencia, CA, USA); genomic DNA contamination was removed using the RNase-free DNase set (Qiagen). Prior to RNA extraction, all samples were homogenised in lysis buffer using pre-cooled 5 mm stainless steel beads (Qiagen) and a high-speed shaker. Skeletal muscle samples were digested with Proteinase K (Qiagen) as per the manufacturer's instructions, prior to column separation of RNA. The quantity and quality of RNA were assessed using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). RNA was stored at −80 °C until further analysis.

2.4. Phylogenetic analysis

A selection of full-length sequences from species randomly selected from each mammalian subclass was assembled from Ensembl (www.ensembl.org) and the National Centre for Biotechnology Information (NCBI; www.ncbi.nlm.nih.gov) nucleotide and protein databases. Accession numbers of sequences for eNOS amino acids were as follows: *H. sapiens* [P29474], *M. mulatta* [XP_002803570], *P. troglodytes* [XR_02287], *M. musculus* [NP_032739], *R. norvegicus* [AAT99567], *B. taurus* [NP_8513380], *C. lupus familiaris* [NP_001003158], *E. caballus* [XP_001504700], *O. aries* [NP_001123373], *and S. scrofa* [NP_999460]. Accession numbers for eNOS nucleotide sequences were as follows: *H. sapiens* [NM_00603.4], *M. mulatta* [XR_012468.1], *P. troglodytes* [XR_02287], *M. musculus* [NM_008713], *R. norvegicus* [NM_021838.2], *B. taurus* [NM_181037.2], *C. lupus familiaris* [NM_001003158.1], *E. caballus* [XM_001504649], *O. aries* [NM_001129901], and *S. scrofa* [NM_2142951. The accession numbers for the amino acid and nucleotide sequences for *X. tropicalis* were XP_002943058 and NW_003164518, respectively. The phylogeny based on multiple sequence alignments was constructed by the widely-used neighbour-joining method, and phylogenetic trees created using ClustalW (Saitou and Nei, 1987; Thompson et al., 1994), to identify sequence similarity, with the branch length of the trees representing the fraction of mismatches at aligned positions. Unlike computationally-intensive maximum likelihood methods, neighbour-joining does not employ a model of sequence evolution. Instead, it uses a pairwise distance matrix to progressively cluster sequences from an initial star phylogeny; details of the clustering heuristic are given in (Saitou and Nei, 1987). This was carried out for all mammalian eNOS sequences. In addition, eNOS amino acid and nucleotide sequences for *X. tropicalis* were compared with human nNOS (NP_001191147/NM_001204218.1) and iNOS (NP_000616.3/NM_000625.4) and xenopus nNOS (ENSXETP00000048371/ENSXETG00000022354). The statistical significance of each tree branch was evaluated by bootstrapping with 1000 resamples.

2.5. Myography

Blunt dissection revealed the carp efferent branchial arteries (EBA), which were quickly removed and placed in ice-cold teleost Ringers solution (118 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 1 mM NaH_2PO_4, 2.5 mM Na_2HPO_4, 1.9 mM $NaHCO_3$, 2 mM $CaCl_2$, 5.5 mM D-glucose; pH 7.8 at 12 °C) equilibrated with air. A modified wire myograph (Multi Myograph Model 610 M; Danish Myo AB) interfaced with PowerLab (AD Instruments) allowed experiments on sections of vessel (approximately 2 mm) to be performed at the acclimation temperature. Passive tension on the vessels was gradually increased and left until constant basal tone was reached. Thereafter, pre-constriction with 50 mM KCl was used to verify vessel viability. Agonist dose–response curves were constructed using half decades on a log scale (Hill and Egginton, 2010). Attempts were made to obtain a nitrodilator response using sodium nitroprusside (SNP as sodium nitroferricyanide), 3-morpholosyndonimine (SIN-1), S-nitroso-N-acetylpenicillamine (SNAP), and bradykinin (BK), and to block basal nitrergic tone using L-NNA. A small number of cGMP (as 8-Bromoguanosine-3′,5′ cyclic monophosphate sodium) dose–response curves were obtained opportunistically. Mass specific change in tension (mN mg^{-1}) of the agonist was normalised to the KCl response (%KCl_{max}). Dilator responses were also calculated as a proportion of the pre-constriction from which the dilator was added. Dose–response curves were established and the pEC_{50} (dose at the midpoint of dilatation) was calculated using R software. StatView 5.0 (SAS Institute Inc.) was used to compare groups by ANOVA, with Fisher's PLSD post-hoc test of significance.

2.6. Synteny analysis

The location of the eNOS gene in mammals was identified using the human, mouse and rat genome assemblies in Ensembl (GRCh37, NCBIM7 and RGSC3 respectively). Syntenic regions in the zebrafish were identified using zebrafish assembly Zv8.

3. PCR

eNOS and nNOS were sought in pufferfish liver, zebrafish liver, carp red muscle and trout red muscle, using degenerate primers (eNOS: forward CCCCGGCACCGGNATHGCNCC (amino acid sequence: PGTGIAP), reverse ACAGCACCCGGTGCACYTCNSMNGC (amino acid sequence: ACEVHRVLC); nNOS: forward GCCAAGCACGCCTGGMGNAAY, reverse GTACTTGATGTGGTTGCADATR), and the validity of the PCR protocol was determined by carrying out the same assay in HUVECs using a TaqMan Gene Expression Assays primer/probe set (Applied Biosystems Inc., USA; Hs_01574659_m1). Degenerate primers were designed