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NOTE

Induction of nitric oxide synthase in channel catfish *Ictalurus punctatus* by *Edwardsiella ictaluri*

W. Peter Schoor[1], John A. Plumb[2]

[1]Environmental Research Laboratory, U.S. Environmental Protection Agency, 1 Sabine Drive, Gulf Breeze, Florida 32561, USA
[2]Department of Fisheries and Allied Aquacultures, Alabama Agricultural Experiment Station, Auburn University, Auburn, Alabama 36849, USA

ABSTRACT: Channel catfish *Ictalurus punctatus* were injected intraperitoneally with live cells of *Edwardsiella ictaluri* and held in flow-through tanks for 5 d. The head kidneys from injected and control fish were removed and the nitric oxide synthase activity measured. Activities found were 0.16 ± 0.05 nmol L-citrulline formed mg^{-1} protein min^{-1} for the injected fish, and 0.002 nmol mg^{-1} min^{-1} for the control fish.

KEY WORDS: Nitric oxide synthase · Channel catfish · *Edwardsiella ictaluri*

Enteric septicemia in catfish caused by *Edwardsiella ictaluri* (Hawke et al. 1981) has been described as the most serious infectious disease to affect the catfish industry in the United States (Vinitnantharat & Plumb 1993). Based on recent findings that nitric oxide is involved in septic shock (Moncada et al. 1991, Lancaster 1992), and is inducible in macrophages (Stuehr & Marletta 1985), attempts were made to document the induction of nitric oxide synthase (NOS) in the head kidney of channel catfish *Ictalurus punctatus* by the enterobacterium. The choice of this tissue reflects the fact that it contains large numbers of macrophages.

Two types of nitric oxide synthase have been shown to exist in mammals, a constitutive type involved in signal transmission (Ignarro 1991) and an inducible type involved in cellular defense (Moncada et al. 1991). Of the 2 types, only the constitutive form has been found in other than mammalian species. It was demonstrated by immunochemical techniques in neurons of the intestinal tract of the toad *Bufo marinus* using rabbit antibody against purified rat cerebellum NOS (Li et al. 1992).

Methods. Nine channel catfish, ranging from 420 to 560 g weight, were used in the experiment. Six fish were injected each with 3×10^6 colony-forming *Edwardsiella ictaluri* in 1 ml of sterile saline solution. Three fish served as controls, and were not sham-injected. The head kidneys from 3 exposed catfish were pooled (Fish 1 to 3); the others were analyzed individually (Fish 4, 5 and 6). Tissues from the control catfish were pooled. The head kidneys were removed 5 d after the injection (approximately 0.5 g tissue per fish) and homogenized in a glass grinder at a 3:1 ratio (w/v) with a buffer containing 40 mM Tris (pH 7.9), 0.25 M glucose, 0.1 mM phenylmethylsulfonylfluoride, 3 mM dithiothreitol, 4 µM flavine adenine dinucleotide (FAD), 5 mM L-arginine, 5 µg ml^{-1} aprotinin, 5 µg ml^{-1} pepstatin A, and 1 µg ml^{-1} chymostatin (Stuehr et al. 1991). The homogenate was centrifuged at 100 000 × *g* for 90 min at 4 °C and the supernate concentrated and washed by ultrafiltration using a 10 000 MW cut-off membrane with the same buffer, but lacking glucose and L-arginine. The supernate was frozen at –80 °C.

The NOS activities were determined in a buffer containing 40 mM HEPES [N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)] (pH 7.9), 1 mM nicotinamide adenine dinucleotide phosphate (reduced form) (NADPH), 1 mM dithiothreitol, 1 mM L-arginine, 0.1 mM tetrahydrobiopterin, 1 µM FAD, and 10 to 25 µl of enzyme in a total volume of 0.6 ml, incubated at 37 °C (Stuehr et al. 1991). Aliquots of 100 µl were taken at various times and reacted with 5 µl of a pre-column derivatization mixture containing 10 mg *o*-phthalaldehyde, 25 µl β-mercaptoethanol, and 0.5 ml buffer containg 0.4 M borate, 7 mM EDTA (ethylenediaminetetraacetic acid), and 0.1% Brij-35, pH 9.4. The mixture was allowed to react for 2 min in the dark before being chromatographed on a C-18 HPLC (high performance liquid chromatography) column. Elution

Table 1. Comparison of nitric oxide synthase activities from various sources

Enzyme source	Enzyme type	Activity[a] (nmol mg^{-1} min^{-1})	Reference
Rat cerebellum	Constitutive	0.16 (960)	Bredt & Snyder (1990)
Bovine cerebrum	Constitutive	0.074 (350)	Ohshima et al. (1992)
Rat peritoneal neutrophils	Induced	0.93 (122)	Yui et al. (1991a)
Mouse peritoneal macrophages	Induced (LPS)	2.5 (1060)	Stuehr et al. (1991)
Rat peritoneal macrophages	Induced (LPS)	2.1 (944)	Yui et al. (1991b)
Rat liver	Induced (LPS)	0.05 (223)	Evans et al. (1992)

[a] Activities are based on crude homogenate protein (first value) or on purified homogenate protein (second value, in parentheses)

conditions were: 0.4 ml min^{-1} flow rate of an 85%/15% mixture of 50 mM sodium acetate/4% acetonitrile, pH 5.85, and of 75%/25% acetonitrile/methanol. The fluorescence was measured at 254 nm and compared to that of L-arginine and L-citrulline standards. The enzymatic activity, determined from the linear portion of the activity curve, was expressed in nmol L-citrulline produced mg^{-1} protein min^{-1}. The lowest level of activity detectable under the above conditions was 1 pmol mg^{-1} min^{-1}.

An LPS- (lipopolysaccharide-) induced murine peritoneal macrophage NOS preparation was used as the methodology standard; it exhibited an activity of 5.3 nmol mg^{-1} min^{-1}. Using this material as a positive control, the assay procedure for NOS listed above showed a reproducibility of ±5%.

Results and discussion. Using the method described above for the determination of NOS activity, the following values were obtained:

0.15 nmol mg^{-1} min^{-1}	*Edwardsiella*-exposed fish (Fish 1 to 3, pooled)
0.16 ± 0.05 nmol mg^{-1} min^{-1}	*Edwardsiella*-exposed fish (Fish 4, 5 and 6)
0.002 nmol mg^{-1} min^{-1}	Control fish

Because this work reports the first NOS activity measured in a fish species, the NOS values in Table 1 are provided for comparison. It is noted that these studies used different starting materials as well as different methods for measuring NOS activity. Additional, preliminary information on the occurrence of NOS in fishes obtained from chinook salmon *Oncorhynchus tshawytscha* with natural (clinically evident) bacterial kidney disease showed an activity of approximately 2 nmol citrulline formed mg^{-1} protein min^{-1} in the head kidney; the analogous NOS value for clinically normal but possibly infected chinook salmon was 2-fold lower In the light of this information it is highly unlikely that handling the exposed catfish caused an increase in the observed NOS activity.

The NOS activity observed in the control catfish head kidneys, while very low, has been observed in peritoneal macrophages from healthy mice (Palacios et al. 1992). Knowles et al. (1990) also found background activity in control rat liver, but not rat lung tissue. The significance of this is not clear, but in the absence of the constitutive enzyme, might reflect some sub-effects level.

Conclusion. We report an approximately 80-fold increase in the NOS activity in channel catfish after treatment with *Edwardsiella ictaluri*, the first such activity to be measured in a fish species, and suggest that this occurred in direct response to the treatment. We infer from the data that the model developed for the involvement of NOS in septic shock in mammalian species also applies to fish species. Future information on a possible dose-response relationship in fish will aid greatly in evaluating the use of NOS data for indicating exposure to natural pathogens and/or their toxins.

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