

Induction of nitric oxide synthase and associated toxicity in livers of hardhead catfish, *Arius felis*,
from control and epizootic sites

W. Peter Schoor

Gulf Ecology Division, U.S. Environmental Protection Agency, 1 Sabine Drive, Gulf Breeze,
Florida 32561, USA

October 10, 1996

Introduction

Earlier work with a live channel catfish (*Ictalurus punctatus*) pathogen, *Edwardsiella ictaluri*, demonstrated the induction of nitric oxide synthase (NOS) in the head kidney, paralleling enteric septicemia (Hawke et al. 1981; Schoor and Plumb 1994). However, another study exposing hardhead catfish (*Arius felis*) to microcystin-LR failed to show the induction of NOS (Schoor et al. In prep.). Similar studies in mammalian systems have shown the involvement of the cytokines in the induction of NOS by a mechanism which may possibly have been bypassed by the pure toxin. In order to pursue these studies, the Florida Marine Research Institute, State of Florida Department of Natural Resources, St. Petersburg, Florida, was contacted with a request for fish livers from an epizootic. Dr. Jan H. Landsberg answered the request in November of 1995.

Methods

Hardhead catfish livers were received on dry-ice from the State of Florida Department of Natural Resources Laboratory in St.Petersburg, Florida, in November of 1995 and stored at -80°C. Three of the samples were from control sites and eight were from locations where catfish epizootics occurred during the sampling period.

AFL	10/23/95	#001
		#002
		#003
		#004
		#005
		#006
AFL	10/25/95	#001
		#002
AFL	10/30/95	#001
		#001
AFL	11/02/95	#001

Determination of induced nitric oxide synthase (iNOS) activity was conducted by a modification of the method reported by Schoor and Plumb (1994). Frozen tissues were weighed and minced before homogenization in a glass tissue grinder at a 2:1 (v/w) ratio with a buffer containing 40 mM Tris (pH 7.9), 0.25 M glucose, 0.1 mM phenylmethylsulfonylfluoride, 3 mM dithiothreitol, 4 μ M flavine adenine dinucleotide (FAD), 5 mM L-arginine, 5 μ g/ml aprotinin, 5 μ g/ml pepstatin A,

and 1 $\mu\text{g/ml}$ chymostatin (Stuehr et al. 1991). The homogenates were centrifuged at 9,000 $\times g$ for 60 min to yield a crude supernate (E-1), a small amount of which was passed through Dowex-50W (anionic form) to remove constitutive L-citrulline and L-arginine (E-1-1). The remaining E-1 supernate was centrifuged at 100,000 $\times g$ for 60 min before passage through Dowex-50W (E-2).

The induced enzyme activities were determined in a buffer containing 40 mM HEPES (pH 7.9), 1 mM nicotinamide adenine dinucleotide phosphate-reduced (NADPH), 1 mM dithiothreitol, 1 mM L-arginine, 0.1 mM tetrahydrobiopterin, 1 μM FAD, and 10-25 μl of enzyme in a total volume of 0.6 ml, incubated at 37°C. 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 containing 0.4 M borate, 7 mM EDTA, 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 column. Elution conditions were: 0.4 ml/min flow rate at 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, is expressed in nanomoles of L-citrulline produced per mg of protein per minute. The lowest detection limit under the above conditions was 0.1 picomole/mg/min.

Investigation of the possible presence of microcystins in the livers was accomplished using high pressure liquid chromatography and UV detection (Hewlett-Packard HPLC Series 1050 with UV-DAD). Remaining liver fractions of each sample were combined and extracted with 15 ml of methanol in a Brinckmann Polytron. The supernate was removed by centrifugation and the

precipitate was re-extracted with 15 ml methanol. The combined methanol/water extracts were extracted three-times with 5 ml portions of hexane before final evaporation to a volume of about 2 ml. No clean-up was performed before injection. A water/acetonitrile gradient was developed for the H-P ODS-Hypersil column (10 cm), starting with 100% water, going to 55% water in 18 min, and then to 0% water at 21 min and holding this mixture for 5 min to elute all nonpolar materials from the column. The MC-LR primary standard peak was confirmed by spectral comparison to the UV spectrum of MC-LR obtained with a Cary Model 118-C.

Ninety-six hour bioassay tests were performed on the liver extracts using freshly-hatched first stage zoeae of fiddler crabs (*Uca panacea*) collected from habitats maintained at EPA GED. Twenty-five ml of filtered seawater and 25 zoeae were placed into 6 cm Carolina bowls to which were added 50 μ l of the liver extract. The controls received only seawater. Dead zoeae were counted and removed twice daily.

Results

The physical appearance of three of the liver samples during the first homogenization step was quite different from all others. Samples 10/23/95: #004, #005 and #006 had a bright red precipitate, in contrast to brown or reddish-brown precipitates from the remaining samples (Table 1). These samples were later identified as controls. The purpose of the Dowex-50W treatment was to remove L-arg, which was originally added to stabilize iNOS during purification, as well as to remove constitutive L-citr. The treatment had no effect on the color of the supernates.

Induced nitric oxide synthase activity was measured in all liver samples. Initial activity measurements indicated a rapid depletion of L-arginine (L-arg) without concomitant increase in L-citrulline (L-citr), the substrate for iNOS, and its metabolite, respectively. While L-citr was present initially, no consistent increases were found during the assays. Since the disappearance of L-arg was most likely due to arginase activity, L-valine (L-val) was added to inhibit that activity (Table 2). L-val did not inhibit the iNOS activity of the positive iNOS control (murine macrophage homogenate obtained from Dennis J. Stuehr, Cleveland Clinic Foundation). Addition of L-citr to reaction mixtures containing liver homogenates showed no degradation, indicating the absence of enzymes degrading L-citr, thus allowing for the use of the appearance of L-citr as an indicator of iNOS activity. This was also found to be true for iNOS activity of head kidney homogenates from channel catfish (Schoor and Plumb 1994). The time courses for the specific iNOS activity of the three active liver preparations are shown in Tables 3, 4 and 5.

Table 6 shows the iNOS activities for all catfish liver samples and, for comparative purposes, activities from *Edwardsiella*-exposed channel catfish (*Ictalurus punctatus*), as well as the

activities from murine macrophage homogenates which were used as a positive control. No differences were found in the activities from E-1-1 and E-2 homogenates.

All liver extracts were analyzed by HPLC for the presence of microcystins and nodularins, but none were found at the detection limit, 5 ng microcystin-LR/g liver tissue. The toxicity of the extracts was assayed by exposing first-stage zoeae of fiddler crabs (*Uca panacea*) to 50 µl of aqueous extract in two separate experiments. The results are shown in Tables 7 and 8.

Discussion

When the initial liver sample homogenates were prepared for iNOS determinations, it became clear that samples 10/23/95: #004, #005 and #006 behaved quite differently than did any other liver sample. While there was little difference in the color of the supernates, the color of the precipitates was a bright red in the above samples, whereas it was mostly a muddy dark brown in the others. The brown precipitates were packed much more tightly than the red ones after centrifugation. It was learned later that the above samples came from a control site.

The time courses of the appearance of L-citrulline, the byproduct of the formation of nitric oxide from L-arginine, from samples 10/23/95: #001, #002 and #003 are shown in Tables 3, 4 and 5. The iNOS activities from those samples were calculated from regression analyses and are shown in Table 6. The above samples show an induced NOS activity about ten-fold higher than found in the controls or the other samples. There appears to be no correlation to any initial concentration of L-citrulline.

HPLC analyses of the liver extracts revealed no microcystins or nodularins, reducing the chance of involvement of algae which produce these groups of toxins. However, bioassays of the liver extracts show toxicity which parallels the iNOS activity (Tables 7 and 8). It should be noted that this assay system is under development for sediment toxicity testing and is not the most suitable bioassay system for water soluble toxins. It is remarkable that in both tests the relative mortality in samples 10/23/95: #001, #002 and #003 is close, in spite of the drastic differences in the control mortalities.

Conclusion

In the absence of more detailed information on liver histopathology, it is very difficult to associate any cause with the findings above. It is tempting to speculate that an organism is responsible which is capable of turning-on iNOS in certain hepatocytes either via a toxin, cell wall fragment (LPS) or some cytokine-related mechanism, not so much because of the induction of NOS but because of the paralleling toxicity data.

Acknowledgements

The authors thanks Dr. Jan H. Landsberg, Florida Marine Research Institute, Florida Department of Environmental Protection, for providing the catfish liver samples. Mention of trade names does not imply endorsement by the U.S. Environmental Protection Agency.

Table 1. Physical appearance of liver samples after first homogenization and ultracentrifugation at 9,000xg for 60 minutes.

Sample identification	Physical appearance	
	E-1	Precipitate
10/23/95 001	clear, amber	brown/red
002	clear, red/brown	brown/red
003	clear, brown	brown
004	clear, amber	bright red
005	clear, amber	bright red
006	clear, amber	bright red
10/25/95 001	clear, amber	brown
002	clear, amber	brown/red
10/30/95 001	clear, brown	brown
001	clear, brown	brown
11/02/95 001	clear, amber	brown/red

Table 2. Effects of addition of L-valine on the disappearance of L-arginine.

Time (min)	L-arginine (nanomoles)	L-arginine + L-valine (nanomoles)
0	305	305 ¹
60	250	310
120	215	300

¹Value normalized to 305

Table 3. Time course of induced nitric oxide synthase activity from catfish liver 10/23/95 001, E-1-1 preparation.

Time (min)	L-citrulline (picomoles)	L-arginine (nanomoles)
0	38	350
45	68	370
105	100	370
165	190	375
220	240	360
275	390	370

Table 4. Time course of induced nitric oxide synthase activity from catfish liver 10/23/95 002, E-1-1 preparation.

Time (min)	L-citrulline (picomoles)	L-arginine (nanomoles)
0	31	370
60	84	375
90	110	370
120	150	355
180	220	360
330	330	375

Table 5. Time course of induced nitric oxide synthase activity from catfish liver 10/23/95 003, E-1-1 preparation.

Time (min)	L-citrulline (picomoles)	L-arginine (nanomoles)
0	29	375
45	50	370
80	86	355
120	100	360
250	360	360
340	630	350

Table 6. Induced NOS activity in hardhead catfish, *Arius felis*, liver homogenates from various sites and comparative iNOS activities from other sources.

Liver Sample	Initial L-Citrulline (T=0) (picomoles)	iNOS Activity (E-1-1) (picomoles/mg/min)
10/23/95 001	38	4.9
002	31	6.2
003	29	4.2
004	20	0.4
005	40	0.4
006	26	0.3 ¹
10/25/95 001	19	0.3
002	12	0.4
10/30/95 001	11	0.5
11/02/95 001	26	0.7 ¹
Murine Macrophage Homog.		5,300 ² 125 ³
Channel Catfish ⁴		
Exposed		160
Control		2.0

¹ Values from E-2 fraction

² Murine macrophage homogenates, when originally received (Stuehr)

³ Murine macrophage homogenates, stored at -80°C for two years (Stuehr)

⁴ From head kidney (Schoor and Plumb 1994)

Table 7. Toxicity of liver extracts to first-stage zoeae of fiddler crabs, *Uca panacea*. Test date 6/24/96.

Liver sample	Liver extract ¹ (mg)	Relative mortality ² (Percent mortality)			
		22 hrs	29 hrs	46 hrs	53 hrs
10/23/95 001	59	0.9(52)	1.0(59)	1.4(81)	1.7(100)
002	33	1.2(39)	1.3(43)	2.1(70)	2.8(91)
003	83	0.4(30)	0.5(39)	0.6(48)	1.2(96)
004	140	0.1(17)	0.1(17)	0.7(96)	0.7(100)
005	430	0.1(52)	0.2(60)	0.2(60)	0.2(60)
006	130	0.3(32)	0.3(44)	0.4(52)	0.7(88)
10/25/95 001	130	0.3(43)	0.4(47)	0.5(70)	0.7(90)
002	140	0.1(19)	0.2(31)	0.4(58)	0.7(96)
10/30/95 001	160	0.2(24)	0.2(36)	0.4(56)	0.4(69)
11/02/95 001	170	0.2(35)	0.2(35)	0.3(52)	0.5(87)
Control		(18)	(31)	(63)	(80)

¹As used per assay; liver extract equivalent to frozen liver weight in 50 µl aqueous solution

²Percent mortality divided by relative mortality

Table 8. Toxicity of liver extracts to first-stage zoeae of fiddler crabs, *Uca panacea*. Test date 7/17/96.

Liver sample	Liver extract ¹ (mg)	Relative mortality ² (Percent mortality)	
		44 hrs	53 hrs
10/23/95 001	59	1.6(96)	1.7(100)
002	33	2.5(83)	3.0(100)
003	83	1.2(96)	1.2(100)
004	140	0.7(96)	0.7(100)
005	430	0.2(93)	0.2(93)
006	130	0.6(74)	0.7(93)
10/25/95 001	130	0.4(52)	0.7(88)
002	140	0.3(48)	0.5(74)
10/30/95 001	160	0.5(78)	0.6(100)
11/02/95 001	170	0.4(74)	0.5(81)
Control		(20)	(25)

¹As used per assay; liver extract equivalent to frozen liver weight in 50 µl of aqueous solution

²Percent mortality divided by relative mortality

References

Hawke, J.P., McWhorter, A.C., Steigerwalt, A.G., Brenner, D.J. (1981). *Edwardsiella ictaluri* sp. nov., the causative agent of enteric septicemia of catfish. Intern. J. Syst. Bact. 31: 396-400

Schoor, W.P., Plumb, J.A. (1994). Induction of nitric oxide synthase in channel catfish *Ictalurus punctatus* by *Edwardsiella ictaluri*. Dis. Aquat. Org. 19:153-155

Stuehr, D.J., Cho, H.J., Kwon, N.S., Weise M.F., Nathan, C.F. (1991). Purification and characterization of the cytokine-induced macrophage nitric oxide synthase: An FAD- and FMN-containing flavoprotein. Proc. Natl. Acad. Sci. USA. 88: 7773-7777