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SHORT COMMUNICATION

Correction of Salinity with Flavin-Containing Monooxygenase Activity but Not Cytochrome P450 Activity in the Euryhaline Fish (*Platichthys flesus*)

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ABSTRACT. To test the association between flavin-containing monooxygenases (FMOs) and osmoregulation, saltwater-adapted euryhaline flounder (*Platichthys flesus*) were statically exposed to 34 (ambient), 25, and 15 parts per thousand (‰) salinity for 1 or 2 weeks. FMO activity (thiourea *S*-oxidase) was assayed in gill and liver microsomes in *P. flesus*. Branchial FMO activity was reduced dramatically (98%) in fish exposed to a salinity of 15‰ as compared with control, while hepatic FMO activity was reduced by 60%. Reduction of FMO activity in response to reduced salinity (15‰) appeared to occur within 1 week or less in both liver and gill of the flounder. Although hepatic FMO activity continued to fall and was not detected after 2 weeks at 15‰, branchial FMO activity was still present. A dose–response relationship in FMO reduction was present in liver, but there was no difference observed between 25 and 15‰ salinity in FMO activity of flounder gill. Serum osmolality and hepatic cytochrome P450 content were unchanged by salinity. In an attempt to determine whether trimethylamine (TMA) plays a role in piscine FMO, the effect of TMA on hepatic and branchial FMO activity was examined. Intraperitoneal injections of TMA failed to induce activity. Thus, an association between osmoregulatory function and FMO expression was observed in a species of euryhaline fish, indicating that alterations by salinity may affect xenobiotic biotransformation in euryhaline animals. BIOCHEM PHARMACOL 52;5:815–818, 1996.

KEY WORDS. flavin-containing monooxygenases; osmoregulation; trimethylamine; salinity; euryhaline

FMOs§ are a polymorphic family of enzymes, found in the smooth endoplasmic reticulum of the cell, that primarily catalyze the oxidation of soft nucleophilic heteroatom-containing substrates to their respective oxides [1, 2]. Five gene families have been partially characterized in mammals, which appear to correspond to at least five unique isoforms that are differentially expressed in various tissues [3]. For example, form 2 of FMO (designated FMO2) is the predominant form in rabbit lung, whereas expression of FMO1 is greater in the liver [4, 5]. The differential expression of FMO isoforms in various tissues suggests distinct functional roles for each isoform. Although a role in the biotransformation of xenobiotics has been established for FMOs, endogenous functions have not been identified clearly for the isoforms. Thus, it has been difficult to determine how FMOs evolved into significant xenobiotic oxidases.

One reason why endogenous functions have not been assigned to FMOs is that few endogenous substrates for the isoforms have been identified [1, 2]. Early work demonstrated that FMO1 in pig liver was responsible for the oxidation of cysteamine [1]. Recent studies have shown that FMO3 in the rabbit kidney catalyzes the S-oxidation of methionine [6], as well as other cysteine conjugates [7]. Lastly, it has been reported that FMOs may be responsible for the oxidation of choline-derived TMA to TMAO. Studies in humans afflicted with "fish odor syndrome" or trimethylaminuria, have indicated a genetic deficiency in certain individuals who are unable to convert odorous TMA to the non-odorous TMAO [8, 9]. *In vitro* studies have shown that one or more forms of FMO are responsible for this reaction and that individuals with this disease also possess low FMO activity [10].

Although TMAO appears to have no known function in mammals, it serves an osmoregulatory function in marine elasmobranchs [11]. Initial studies of sharks, who possess tissue levels of TMAO as high as 70 mM, indicated that these organisms have relatively high levels of FMO activity [12]. Recently, we have shown that at least two shark and one marine teleost species possess significant FMO activity and a microsomal protein from the liver that reacts with antibodies generated against mammalian FMO1 and FMO2 [12–14]. Examination of freshwater fish species that do not possess TMA or TMAO, such as the channel catfish, showed that catfish do not have detectable FMO activity or

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§ *Abbreviations:* FMO, flavin-containing monooxygenase; TMA, trimethylamine; TMAO, *N*-oxide of TMA; and ‰, parts per thousand.

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homologous protein in liver, gill, or kidney [15]. However, anadromous freshwater species such as the rainbow trout have FMO activity in liver, kidney, and gill, as well as two homologous proteins found in each tissue [16, 17]. Recent studies with a saltwater-adapted euryhaline fish, the flounder (*Platichthys flesus*), showed that the gill possesses the highest amounts of activity, followed by the kidney, whereas the liver had negligible activity [18]. The differential expression of FMO activity and protein in the gill, which is the predominant osmoregulatory organ of this euryhaline species, and the lack of expression in TMA-deficient species were consistent with the hypothesis that piscine FMOs serve an osmoregulatory function. Consequently, the purpose of this study was to examine the role of FMO in osmoregulation by examining the effect of change in salinity on FMO expression in a euryhaline teleost.

MATERIALS AND METHODS

Animals

Sexually mature flounder (*P. flesus*, length 25–30 cm) were caught in local waters off Plymouth, U.K., and maintained in 1000-L tanks with continuous flowing seawater at 12–15° for 1 month before analysis. Four fish were sampled randomly and placed in one 40-L aquarium containing seawater at 34‰ (100% seawater) for 2 weeks. A second group of four fish were also placed in another 40-L aquarium containing seawater at 25‰ (1:1 dilution with dechlorinated tap water) for 2 weeks. Lastly, two groups of four fish were placed in two separate 40-L aquaria (four fish/container) containing seawater at 15‰ (1:4 dilution). One of these 15‰ groups was maintained for 1 week while the other was maintained for 2 weeks. As observed in previous studies with trout [16] and turbot [14], no sexual differences in branchial or hepatic FMO activity were observed between male and female flounder (data not shown); thus, data from animals of both sex were combined. Each static system was aerated and changed daily, and fish were fed a diet of fresh fish daily. Following exposure (1 or 2 weeks), fish were removed from the aquarium, blood was sampled from the caudal vein, and then the fish were euthanized. Tissues were dissected immediately and used for enzyme analyses. Blood osmolality was measured using a Wescor 5100C Vapor Pressure Osmometer.

To determine the effects of TMA on FMO expression, four mature male flounder in flow-through tanks were given intraperitoneal injections of 100 mg/kg TMA on days 0 and 2 and were killed on day 3. Control animals received an equal volume of the carrier, nanopure water. Livers were dissected on day 3 and frozen at −80° for enzyme analysis.

Chemicals

Thiocholine was the gift of Dr. Daniel Ziegler. Most of the remaining biochemical reagents, including NADPH, and phenylmethylsulfonyl fluoride (PMSF) were from the Sigma Chemical Co. Ltd., U.K. and U.S.A.

Microsomal Preparation for Enzyme Studies

All sample procedures were carried out at 4°. Dissected tissues from three individual fish were homogenized in 100 mM Tris–HCl, pH 7.6, containing 0.15 M KCl, 0.1 mM PMSF (added in a minimal volume of ethanol), using either an electrically driven Potter–Elvehjem (liver) or a Polytron (gill) homogenizer. The microsomal (100,000 *g*) pellet was resuspended in 100 mM potassium phosphate, pH 8.0, containing 20% (w/v) glycerol. This microsomal suspension was used immediately as the source of enzyme activity. FMO activity was assessed in flounder by measuring the thiourea-dependent oxidation of thiocholine [19]. Briefly, in a total volume of 1.0 mL, containing 0.1 mM potassium phosphate, pH 8.8, 0.1 to 1.0 mg of microsomal protein, 0.15 mM thiocholine, 0.1 mM NADPH, and 1.2 mM thiourea was added. After 30 min, 0.8 mL was removed, placed in a tube containing 0.1 mL of 3 M trichloroacetic acid, and centrifuged for 10 min at 10,000 *g*. The resulting supernatant was then transferred to a tube containing 1 mL of 1.0 M potassium phosphate, pH 7.5, 0.6 mL of water, and 0.05 mL of dithionitrobenzoate (DTNB) (10 mM). Incubation conditions were determined from previous studies [12, 14, 20]. The concentration of thiocholine was measured by using a millimolar absorptivity of 13.6 cm^{-1} for 5-thio-2-nitrobenzoate at 412 nm and compared against incubations that did not contain NADPH or thiourea. Hepatic cytochrome P450 content was measured by the method of Omura and Sato [21]. Protein was measured by the method of Lowry *et al.* [22].

RESULTS AND DISCUSSION

Utilizing organisms that reside in different osmoregulatory "media" (i.e. teleost fish), studies have shown that hepatic FMO activity is present primarily in saltwater fish or freshwater fish, such as the rainbow trout (*Oncorhynchus mykiss*), that are able to adapt to saltwater conditions [13]. In addition, examination of FMO activity, protein, and mRNA in the euryhaline species of flatfish used in the current study, *P. flesus*, showed that expression of FMO and its activity were higher in the osmoregulatory organs, gill and kidney, than in liver, further supporting an association between FMOs and osmoregulation [18]. Previous studies examining the euryhaline American eel and guppy found that TMA oxygenase was induced by enhanced salinity as well as intraperitoneal injections of TMA [23]. Consequently, the effect of salinity on FMO expression was examined in a euryhaline fish to test the hypothesis that FMOs have a role in osmoregulation.

To determine whether daily water changes impaired osmoregulatory function, the serum osmolality of flounder was examined after 2 weeks, prior to liver and gill dissection (Table 1). No significant differences in serum osmolality

TABLE 1. Effect of salinity on serum osmolality and hepatic P450 content in the flounder (*P. flesus*)

Salinity (‰)	Hepatic P450 content (nmol/mg)	Osmolality (mmol/kg)
Untreated		307 ± 8
34	0.090 ± 0.075	301 ± 6
25	0.076 ± 0.003	303 ± 4
15	0.071 ± 0.003	302 ± 2

Each value is the mean ± SD of four individuals.

were observed between fish held in a 1000-L container with recirculating seawater (untreated fish) and those that resided in 40-L containers receiving daily water changes and salinity treatments. Earlier studies have shown that excessive handling of fish can impair osmoregulatory function and cause temporary laboratory diuresis [24]. To prevent this type of phenomenon in this study, fish were not handled during the entire duration of exposure. Water was changed by siphon, with daily salinity measurements. Serum osmolality was unaltered by any of the salinity treatments, indicating no impairment of osmoregulatory function. To determine whether salinity reduced biochemical function in general, hepatic cytochrome P450 content was examined in flounder and was also unchanged by salinity.

As shown in earlier FMO studies with *N*,*N*-dimethylaniline, thiourea oxidase in flounder gill, although extremely variable, was significantly higher in gill than liver (Table 2). In contrast, FMO activity was found to be higher in the liver than in the kidney or gill in the stenohaline flatfish *Scophthalmus maximus* [14]. FMO activity and protein were also higher in the liver than in kidney and gill, respectively, in freshwater-raised rainbow trout [25]. Thus, hepatic FMO activity and protein appear to be relatively greater in freshwater-raised euryhaline fish than in saltwater-raised euryhaline fish. It should be noted, however, that FMO activity has only been examined in one species of saltwater-raised euryhaline fish. Clearly, more species need to be examined to see whether other species of saltwater-raised euryhaline fish have profiles similar to that of the flounder.

TABLE 2. Effect of time and salinity on hepatic and branchial FMO activity in the flounder (*P. flesus*)

Salinity (‰)	Duration	FMO activity (nmol thiourea oxidized/mg/30 min) Hepatic	Branchial
Untreated		1.35	13.6
		2.01	3.4
		2.60	23.8
34	1 week	2.12	77.2
15	1 week	0.82	1.32
		1.28	1.96
		0.36	0.20
34	2 weeks	1.29	14.8
		0.12	2.84
		2.12	76.8
25	2 weeks	0.91	0.17
		0.21	ND*
		ND	ND
		ND	†
15	2 weeks	ND	0.86
		ND	0.82
		ND	ND
		ND	0.80

Each value is the measurement of enzyme activity in an individual fish.
* ND = not detected (<10 pmol/mg/min).
† Not measured.

To determine whether a functional relationship between FMO and osmoregulation was present, saltwater-adapted flounder were housed in chambers with decreased salinity. FMO activity in both gill and liver microsomes was directly correlated with salinity exposure (Table 2). Reduction in activity appeared to occur within a 1-week period in both tissues of flounder. Preliminary studies in the euryhaline medaka showed increases in a 57 kDa branchial FMO1-like protein within 24 hr after increasing salinity.* In flounder, changes in FMO activity appeared to occur within 1 week. During salinity changes, dramatic histological and biochemical changes occur in euryhaline fish such as the up-regulation of Na^+/K^+-ATPase and the production of chloride cells [24]. How these well-characterized changes affect FMO expression and the time–course of FMO expression after altering salinity is currently being explored.

Increases in TMAO have been observed in freshwater-raised euryhaline fish as they migrate to higher salinity [26]. Thus, flounder were treated with TMA to determine whether this osmoregulatory compound had any effect on FMO activity. Treatment of flounder with various doses of TMA did not alter FMO activity in flounder (data not shown). This is consistent with previous studies in freshwater-raised rainbow trout and channel catfish, which showed that intraperitoneal injection of TMA did not change levels of FMO activity or protein [15, 17]. However, these results contrast with the studies performed by Daikoku *et al.* [23], who treated freshwater-raised eel and guppy with an intraperitoneal dose of TMA and increased salinity and observed a significant induction of hepatic TMA oxygenase and TMAO in the treated animals. Thus, regulation of FMO does not appear to be consistently controlled by TMA. Although species differences may explain this phenomenon, it is likely that other mechanisms are responsible for enzyme regulation. Examinations of the effects of osmoregulatory hormones on enzyme expression is currently underway and should provide better insight into the mechanism of FMO regulation, function, and evolution.

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