Published in final edited form as:
Toxicol Lett. 2009 June 1; 187(2): 115–118. doi:10.1016/j.toxlet.2009.02.006.

Effect of hyperosmotic conditions on flavin-containing monooxygenase activity, protein and mRNA expression in rat kidney

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Abstract

Flavin-containing monooxigenases (FMOs) are a polymorphic family of drug and pesticide metabolizing enzymes, found in the smooth endoplasmatic reticulum that catalyze the oxidation of soft nucleophilic heteroatom substances to their respective oxides. Previous studies in euryhaline fishes have indicated induction of FMO expression and activity *in vivo* under hyperosmotic conditions. In this study we evaluated the effect of hypersaline conditions in rat kidney. Male Sprague–Dawley rats were injected intraperitoneal with 3.5 M NaCl at a doses ranging from 0.3 cm^3/100 g to 0.6 cm^3/100 g in two separate treatments. Three hours after injection, FMO activities and FMO1 protein was examined in the first experiment, and the expression of FMO1 mRNA was measured in the second experiment from kidneys after treatment with NaCl. A positive significant correlation was found between FMO1 protein expression and plasma osmolarity ($p < 0.05$, $r = 0.6193$). Methyl-*p*-tolyl sulfide oxidase showed a statistically significant increase in FMO activity, and a positive correlation was observed between plasma osmolarity and production of FMO1-derived (*R*)-methyl-*p*-tolyl sulfoxide ($p < 0.05$, $r = 0.6736$). Expression of FMO1 mRNA was also positively correlated with plasma osmolality ($p < 0.05$, $r = 0.8428$). Similar to studies in fish, these results suggest that expression and activities of FMOs may be influenced by hyperosmotic conditions in the kidney of rats.

Keywords

FMO; Osmoregulation; Rat Kidney

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Conflict of interest
No conflict of interest.

1. Introduction

Flavin-containing monooxigenases (FMOs) are a polymorphic family of enzymes and are found in the smooth endoplasmatic reticulum which catalyze the oxidation of soft nucleophilic heteroatom substances to their respective oxides (Ziegler, 1988, 1990; Cashman, 2004). Some known FMO substrates include alkaloids, pesticides and pharmaceutical compounds (Ziegler, 1990; Cashman, 1995). Five families of catalytically active isoforms of FMO have been identified and classified based on amino acid sequence (Cashman, 2004; Lawton et al., 1994). These isoforms differ in tissue distribution, regulation and substrate specificity (Ziegler, 1990; Krueger and Williams, 2005).

Previous studies have demonstrated that FMOs are regulated in response to changes in diet (Katchamart et al., 2000), sex hormones (Lee et al., 1993; Ripp et al., 1999; Lattard et al., 2002a; El-Alfy and Schlenk, 2002), pregnancy (Osimitz and Kulkarni, 1982; Williams et al., 1985), cortisol (El-Alfy et al., 2002), and in disease conditions, such as diabetes mellitus (Rouer et al., 1988; Borbás et al., 2006). However, the mechanisms that regulate FMOs during these events remain unclear. Since previous studies have demonstrated changes in FMO expression during physiological events that influence hormonal and osmotic status, expression of FMO isoforms in the kidney may be significantly altered (Lee et al., 1993).

Since the kidney can significantly contribute to extrahepatic biotransformation and bioactivation of nephrotoxic xenobiotics such as haloalkane thioethers (Barshteyn and Elfarra, 2007; Elfarra, 1995), it is critical to understand the impact of physiological alterations which influence expression of FMO within the kidney. The current study evaluated the effect of hyperosmotic conditions on FMO activity as well as gene and protein expression in rat kidney.

2. Materials and methods

2.1. Animals and systemic osmotic challenge

A total of 25 adult male Sprague–Dawley rats (350–450 g) were used for this study. The experiment was run in two separate trials. Rats were individually housed in a vivarium with a 12:12 h photoperiod and maintained with *ad libitum* access to standard rat chow pellets and water until the day of sacrifice. To alter osmolality, 11 rats were injected interperitoneally (i.p.: 0.6 cm^3/100 g body weight) with 3.5 M NaCl (to produce acute dehydration). Nine control rats received 0.15 M NaCl (physiological saline control) and water was withheld until the animals were sacrificed 3.0–3.5 h later during which time plasma osmolarity has been shown to increase significantly reflecting the dehydrated state (Ludwig, 1988; Coburn et al., 2005). After injection, a blood sample from the tail was taken and plasma osmolarity was measured using a vapor pressure osmometer. Rats were subsequently killed by decapitation and kidney samples were taken immediately and frozen at −80 °C until enzymatic and immunoblot analysis. For qPCR analyses of FMO1 mRNA, a separate experiment was conducted where two animals were treated with saline control solution and three animals were treated with 0.3 cm^3/100 g of 3.5 M NaCl for the same time period as above. A lower injection volume was used to try to provide a better distribution of salinities for regression analyses. Following blood sampling and decapitation as described above, kidneys were placed in RNALater solution before freezing at −80 °C until analysis. Due to placement in RNALater, neither protein measurements nor catalytic activities were evaluated. All animals manipulations were carried out under an approved protocol by the Institutional Animal Use and Care Committee for the University of California, Riverside.

2.2. FMO activities

Rat kidney microsomes were prepared as described previously (El-Alfy and Schlenk, 2002). FMO activities were measured using methyl-*p*-tolyl sulfide (MTS) as described in Schlenk et al. (2004) and Furnes and Schlenk (2004) which was a modification of Rettie et al. (1994). A 0.25 ml reaction volume containing 400 μg of microsomal protein, 1 mM NADPH 3.3 mM $MgCl_2$, 1.0 mM MTS in a 50 mM glycine buffer pH 8.8 were incubated at 37 °C for 10 min. Previous studies had indicated that this was in the linear range for catalytic activity (Furnes and Schlenk, 2004). The reaction was stopped by the addition of 75 μl acetonitrile and centrifuged 5 min 10,000 × *g*. Supernatant was filtered with Millipore durapore (Bedford, MA) membrane and analyzed on a Regis Technologies (*R*,*R*) Whelk-01 10/100 chromasil chiral column. Samples were eluted with methanol 46% (v/v) (0–7 min) that was slowly increased to 100% (7–20 min). Purified (*R*)- and (*S*)-methyl-*p*-tolyl sulfoxides ((*R*)- and (*S*)-MTSO: Sigma–Aldrich, St. Louis, MO) were used to establish a standard curve. (*R*)- and (*S*)-enantiomers were eluted with retention times of 13.5 min and 14.5 min, respectively. Blanks omitted the addition of NADPH. Protein content of the microsomes was determined using the Bradford (1976) method. Bovine serum albumin was used as a standard.

2.3. Western blot

Microsomal proteins were separated by SDS-PAGE using a 10% polyacrylamide separating gel. Proteins were transferred to a nitrocellulose membrane using a semi-dry electrophoretic transfer cell (Biorad, Hercules, CA). Since enzyme activities indicated stereoselective formation of (*R*)-MTSO during hyperosmotic treatments, anti-guinea pig FMO1 (Yeung and Rettie, 2006) was used to measure protein expression. Although FMO2 also stereoselectively converts MTS to (*R*)-MTSO, an inactive truncated protein is expressed in rat kidney (Lattard et al., 2002b). Following incubation with the primary antibody and appropriate washing, an alkaline phosphatase-linked secondary antibody was used for detection. Bands were quantified using Gel Doc Software (Biorad, Hercules, CA).

2.4. Quantitative-PCR

Total RNA was extracted from kidney using QIAShredder (Qiagen, Valencia, CA) and RNEasy Mini RNA extraction Kit (Qiagen, Valencia, CA) following the manufacturer's instructions. One microliter of isolated total RNA was reverse transcribed with 1 U of Super Spript™ Reverse Transcriptase (Invitrogen Life Technologies, Carlsbad, CA) in the presence of random hexamers according to the manufacturer's instructions. One microliter of the reverse transcriptase reaction mixture was used for RCR amplications using primers for FMO1 (Lattard et al., 2002a). FMO1 was quantified with the SyBr-Green based qPCR method using an icycler iQ apparatus (Biorad, Hercules, CA). The standard mixture consisted of a 1:10,000 dilution of SyBr Green I (Molecular Probe), 10 mM Tris–HCl (pH 8.5), 40 mM KCl, 2 mM $MgCl_2$, 0.1 mM dNTP, 10 pmol of each primer and 2.5 U of Taq polymerase in a 30 μl reaction volume. The optimal PCR conditions for quantification were determined using melting curve analysis by heating from 55 °C to 95 °C (0.5 C for 10 s per cycle for 80 cycles) with simultaneous detection of the SyBr Green I fluorescence signal. Twenty-eight cycles with a 56 °C alignment temperature were utilized for optimal quantification as this was in the linear range for amplification of the FMO1 signal. Forward Primers were 5′-TGT CAA GGG AAG CAA AGC-3′ and reverse primers were 5′-CCT GAA TCA AAG ACT CGG C-3′. A 447 bp fragment was resolved with electrophoresis on an agarose gel (1%) stained with ethidium bromide. β-Actin was used as a housekeeping gene using primers reported elsewhere (Nishimura et al., 2005). All PCR products were sequenced at the UCR Genomic Center to verify that PCR products corresponded to amplicons of the targeted genes.

2.5. Statistical analysis

Differences between treatments were calculated using Student's *t*-test for parametric data and Mann–Whitney *U*-test for non-parametric data. Correlation between variables was calculated using the Spearman rank order method. Criterion for significance was set at $p \leq 0.05$.

3. Results

As a result of intra-peritoneal injection with 3.5 M NaCl plasma osmolarity increased from a mean of 298.77 ± 4.86 in control rats ($n = 9$) to 347.82 ± 15.06 mOsm in osmotically stimulated animals ($n = 11$). Mann–Whitney *U*-test indicated that the increase in plasma osmolarity was statistically significant with respect to control animals ($p = 0.0002$).

MTS oxidase showed a significant increase with hypersaline treatment ($p = 0.002$, Fig. 1). Kidney microsomes produced MTSO at a 70:30 ratio for (*R*) or (*S*) enantiomers, and there was no significant difference in this ratio between normal or hyperosmotic rats ($p = 0.8$). Pretreatment with lubrol reduced the formation of MTSO-(*R*) and MTSO-(*S*) 30% and 40%, respectively in hyperosmotic and normal rats (Fig. 2). There were no differences in the *R*/*S* ratio between normal or hyperosmotic animals when both were pretreated with lubrol ($p = 0.06$); but there was a statistically significant increase in the production of MTSO-(*R*) between hyperosmotic ($p = 0.01$, Fig. 2) or normal ($p = 0.01$, Fig. 2) rats with respect to lubrol pre-treatment.

Densitometric analysis of western blots showed a significant increase in FMO1 protein expression in hyperosmotic rats (Fig. 3A, $p = 0.0002$). Plasma osmolarity presented a significant positive correlation with FMO1 protein expression ($p < 0.05$, $r = 0.6193$, Fig. 3B) and with the production of (*R*)-MTSO ($p < 0.05$, $r = 0.6735$, data not shown). qPCR evaluation for FMO1 mRNA indicated a direct positive correlation with plasma osmolality ($p < 0.05$, $r = 0.8428$, Fig. 4).

4. Discussion

Given the critical physiological role of the kidney in osmoregulation of mammals and its role in extrahepatic biotransformation, FMO regulation under hyperosmotic conditions was evaluated in rat kidney. Previous studies of FMOs in rat kidney have demonstrated expression of FMO1, FMO3 and FMO5 (Lattard et al., 2002a; Krause et al., 1996; Cherrington et al., 1998). Thus, catalytic activities, mRNA and protein expression in the kidney were compared after hyperosmotic treatments in the rat. Formation of (*R*)-MTSO stereoisomer was enhanced by hyperosmotic treatments. Production of (*R*)-MTSO enantiomers is stereoselective in FMOs; for example (*R*)-MTSO formation is preferentially catalyzed by FMO1 and FMO2 (human [Sadeque et al., 1992; Furnes and Schlenk, 2004], rabbit [Rettie et al., 1994]), (*S*)-MTSO is preferentially formed by FMO5 (rabbit [Fisher et al., 1995]) and FMO3 lacks stereoselectivity for formation of MTSO (rabbit [Rettie et al., 1995]). Lubrol has been shown to be an effective inhibitor of CYP, but not FMO in microsomal incubations (Rettie et al., 1994). When MTS was used as a substrate in kidney microsomes, activities after treatment with lubrol indicated that FMOs were participating in about 60–70% of the MTS oxidation.

MTSO formation was well correlated with FMO1 protein expression in hyperosmotic rats, which also correlated with plasma osmolarity. Expression of FMO1 mRNA also correlated with hyperosmotic conditions. Up-regulation of FMO mRNA, protein and catalytic activity by hyperosmotic conditions in euryhaline fish has been previously described (El-Alfy et al., 2002; Schlenk et al., 1996; Larsen and Schlenk, 2001) and studies in rainbow trout have

demonstrated an increase in FMO activity when plasma osmolarity is augmented (Larsen and Schlenk, 2001). Treatment of isolated primary hepatocytes with NaCl and cortisol enhanced the expression of a novel FMO mRNA (Rodríguez-Fuentes et al., 2008). The similarity between piscine and mammalian FMO up-regulation in the presence of osmotic stress may indicate that FMOs play an osmoregulatory role in both systems, perhaps by promoting the formation of "compatible" organic osmolytes (such as trimethylamine N-oxide) or maintaining the redox potential through sulfhydryl metabolism. Studies are underway to identify potential mechanisms regulating FMO by hyperosmotic conditions.

In summary, hyperosmotic conditions increased FMO1 protein expression and FMO1-specific catalytic activity in rat kidneys. There was also a correlation between FMO1 mRNA, and plasma osmolarity. These results suggest that substrates activated by FMO in the kidney such as certain pesticides and thioether metabolites of halogenated hydrocarbons may be more toxic in osmotically stressed animals.

Acknowledgments

The authors would like to thank UC Mexus-CONACYT Postdoctoral Fellowship for its financial support for this project and to Dr. Ramón Lavado for his technical assistance.

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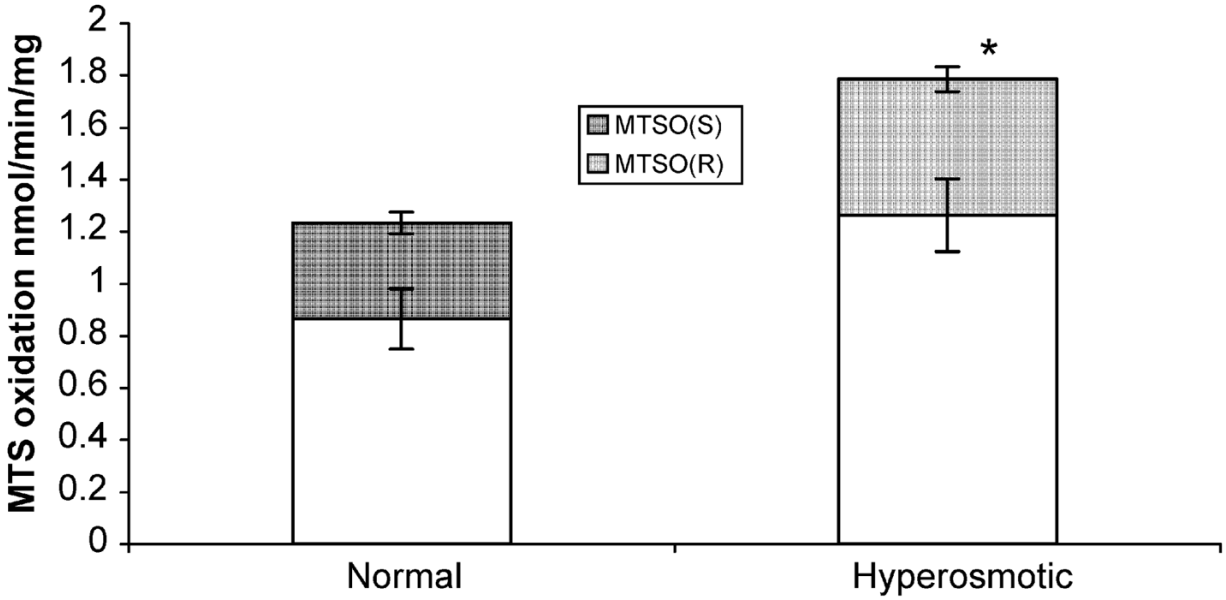

Fig. 1.

Methyl-*p*-tolyl sulfide oxidation by rat kidney microsomes. Comparison of activities in normal or hyperosmotic rats. Bars represent mean activities of (*R*) or (*S*) metabolites ± standard deviation. *Significant differences at $p \leq 0.05$.

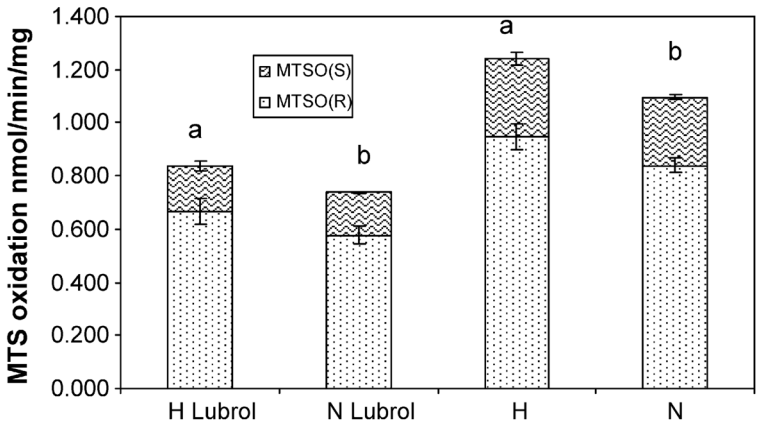

Fig. 2.

Effect of lubrol in methyl-*p*-tolyl sulfide oxidation by rat kidney microsomes. Comparison of activities between normal (N) or hyperosmotic (H) rats. Bars represent mean activities of (*R*) or (*S*) metabolites ± standard deviation. Equal letters represent significant differences between treatments at $p \leq 0.05$.

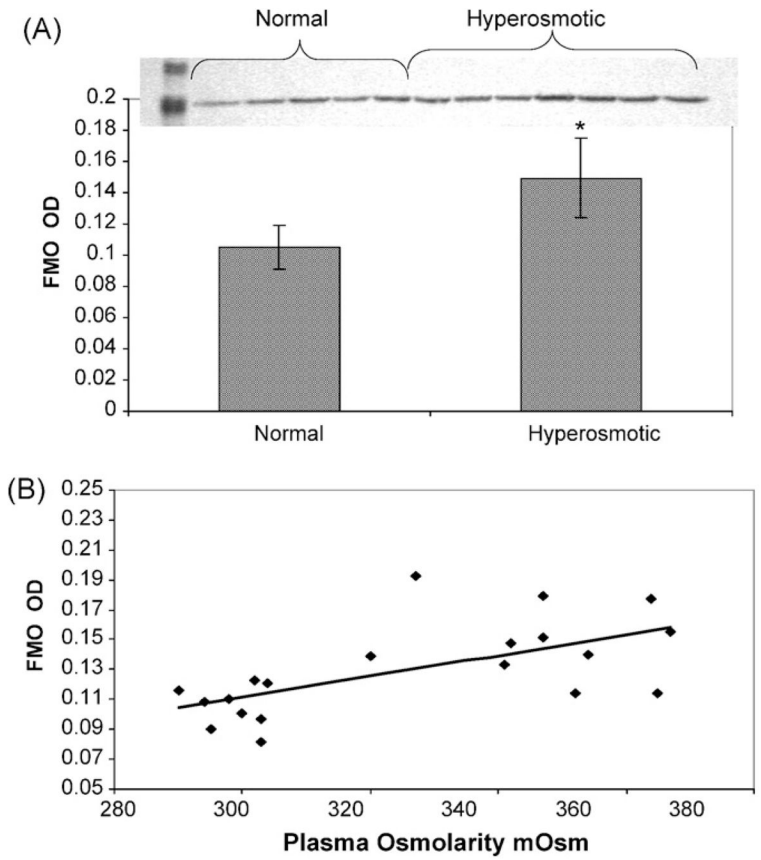

Fig. 3.

Effect of hyperosmotic conditions in rat kidney. (A) FMO1 protein expression in rat kidney microsomes in normal or hyperosmotic rats. Bars represent mean ± standard deviation. *Significant differences at $p = 0.05$. (B) FMO1 correlation between protein expression in rat kidney microsomes and plasma osmolarity.

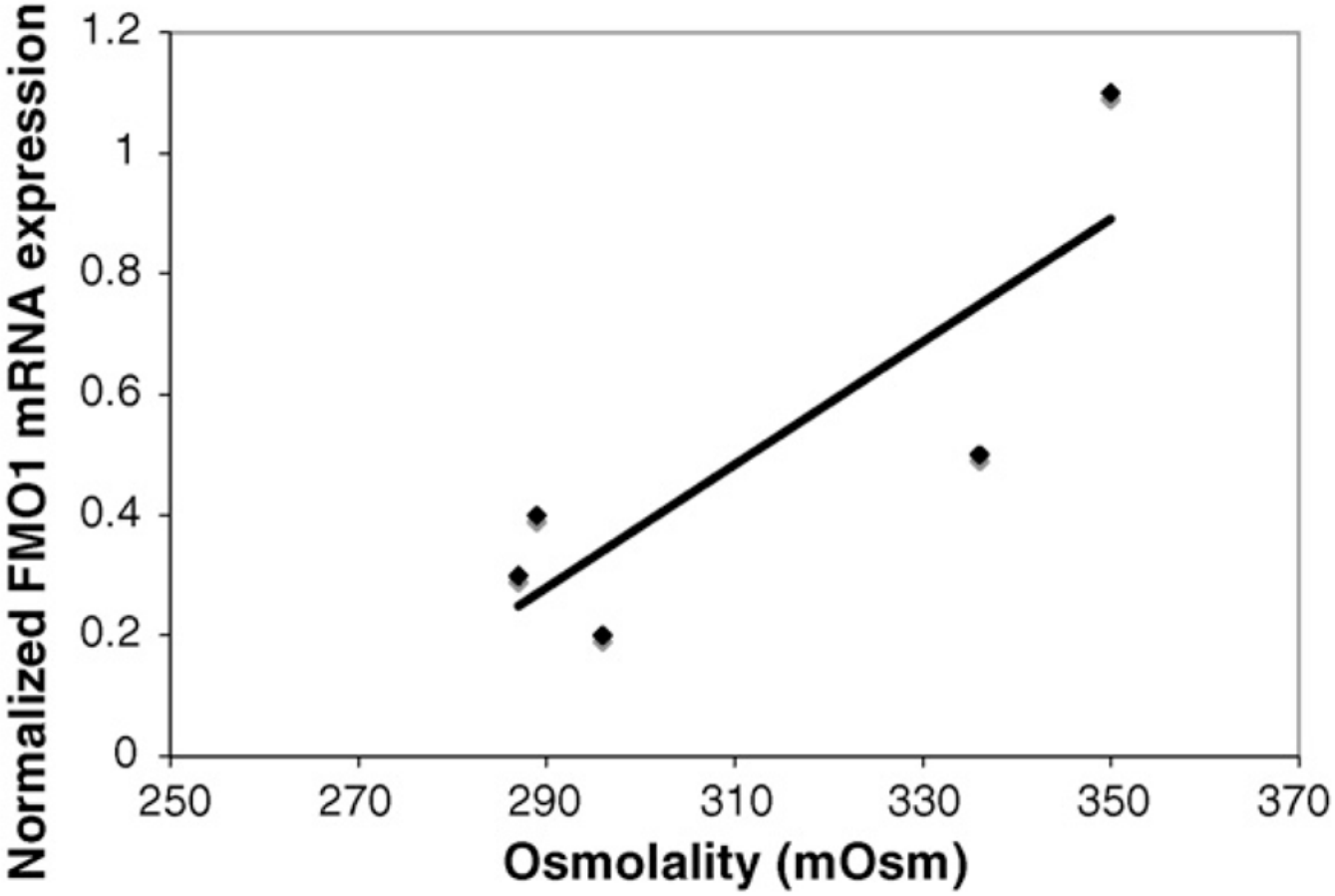

Fig. 4.
Relationships between hyperosmotic conditions and FMO1 mRNA expression in rat kidneys from animals treated with NaCl.