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Comparative Biochemistry and Physiology, Part C 140 (2005) 87–96

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# Effects of nonylphenol on hepatic testosterone metabolism and the expression of acute phase proteins in winter flounder (*Pleuronectes americanus*): Comparison to the effects of Saint John's Wort

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Received 27 August 2004; received in revised form 8 January 2005; accepted 13 January 2005

**Abstract**

4-Nonylphenol (4-NP), a major by-product of alkylphenol ethoxylates, is used in several industries and as a consequence is quite common in rivers, estuaries and other aquatic environments that receive sewage discharges or are near offshore oil platforms. 4-NP is an environmental estrogen that also binds human and rodent Pregnane X-receptor (PXR), the orphan nuclear receptor that controls the expression of several detoxication genes in mammals, including several CYP3A and CYP2B family members. These P450s preferentially hydroxylate testosterone in the 6β- and 16β-positions, respectively. In this study, the effects of 4-NP on testosterone metabolism and hepatic CYP3A induction were compared to the effects of St. John's Wort (SJW), a well established mammalian PXR agonist, in winter flounder. Male winter flounder (*Pleuronectes americanus*) were injected with 100 mg/kg/day 4-NP or 500 mg/kg/day SJW or both (S and N) every 24 h. Forty-eight hours after the initial injections, flounder were euthanized. Western blots and testosterone 6β-hydroxylation indicated that CYP3A was increased 50% by 4-NP, but was not affected by SJW. Testosterone 16β-hydroxylase activity was also significantly increased in flounder treated with 4-NP (2.8×), but not with SJW. This is not consistent with our hypothesis that both SJW and 4-NP would induce CYP3A. Subtractive hybridization was performed between control and 4-NP treated hepatic mRNA samples to isolate differentially expressed genes. Subtractive hybridization indicated that several acute phase proteins were altered by 4-NP. Quantitative real-time PCR (Q-PCR) confirmed 4-NP altered the expression of complement components C8b, cathepsin L, C-type lectin domain, FK506 binding protein 2 precursor (FKBP2) and an EST (expressed sequence tag). SJW and 4-NP treated flounder demonstrated similar induction profiles for the EST, cathepsin L and FKBP2, suggesting that SJW was at a sufficient dose to alter gene expression but not induce P450s. In conclusion, testosterone hydroxylase activity and Western blots indicate that SJW did not activate detoxication pathways in a similar manner to 4-NP.


*Keywords:* Nonylphenol; PXR; P450; CYP3A; Testosterone metabolism; Acute phase proteins; Flounder; Gene expression

## 1. Introduction

4-Nonylphenol (4-NP) is a by-product of alkylphenol ethoxylates (APEs), which can be found in many products including detergents, plastics, emulsifiers, pesticides, and industrial and consumer cleaning products (Talmage, 1994). Over one billion pounds of nonylphenol ethoxylates are produced annually, and over 450 million pounds were sold in the United States in 1990 (Talmage, 1994). Research has identified nonylphenol as the most critical metabolite of APEs because of its resistance to biodegradation, its ability to bioaccumulate, and its toxicity (Ahel et al., 1994; Tyler et al., 1998). As a consequence of their use in a variety of products, they are quite common in rivers, estuaries and other aquatic environments that receive sewage discharges or are near offshore oil platforms

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doi:10.1016/j.cca.2005.01.007

(Brendehaug et al., 1992; Isobe et al., 2001; Ying et al., 2002; Ashley et al., 2003; Jonkers et al., 2003). In the United States, nonylphenol ethoxylates have been quantified at concentrations greater than 50 μg/g in Jamaica Bay, NY (Ferguson et al., 2003), and nonylphenol has been quantified at concentrations of 12.5 μg/g in Delaware Bay, PA (Ashley et al., 2003).

4-NP is an environmental estrogen that binds both estrogen receptors (ERα and ERβ; also known as NR3A1, NR3A2), and 4-NP's estrogenicity has increased its visibility among scientists and non-scientists. However, while 4-NP treatment causes a similar gene expression profile to 17β-estradiol ($E_2$) in the uterus of the mouse, it has a different expression profile than $E_2$ in the liver indicating that 4-NP is binding and activating other receptors in the liver (Watanabe et al., 2004). Similarly, differences in the gene expression profiles in the liver of largemouth bass (*Micropterus salmoides*) treated with $E_2$ and 4-NP suggests that 4-NP has modes of action that are independent of the ER (Larkin et al., 2002).

Recently, 4-NP has been shown to bind and activate the pregnane X-receptor (PXR; also known as SXR, NR1I2) and in turn induce CYP3A. For example, 4-NP has been shown to activate the mouse PXR in transfected COS-7 cells and enhance PXR-mediated transcription through its interaction with co-activator proteins (Masuyama et al., 2000). CYP3A was induced in male wistar rats and female Sprague Dawley rats injected intrapertineal (IP) with 4-NP, but not in $E_2$ treated rats (Lee et al., 1996; Masuyama et al., 2000). Recently, 4-NP was shown to increase the interaction of the PXR with the steroid receptor co-activator-1 (SRC-1) in a yeast two-hybrid assay and induce CYP3A1 in rats (Mikamo et al., 2003). Unpublished results from our laboratory indicate that nonylphenol also activates the human PXR in transient transfection assays. 4-NP has also been shown to induce CYP3A in many of the fish species tested (Arukwe et al., 1997; Hasselberg et al., 2004a).

The PXR is an orphan nuclear receptor that acts as a master regulator of phase I and phase II enzymes involved in the detoxication and elimination of steroids, bile acids and xenobiotics. It primarily activates the transcription of CYP3A family members, but may also induce CYP2B and 2C family members in mammals (Kliewer et al., 1998; Wei et al., 2000). The ligand binding domain of the PXR demonstrates an unusually low degree of sequence identity between species, and therefore there are numerous accounts of ligands that activate the human PXR, but not the rodent PXR or zebrafish PXR (Moore et al., 2002; Tirona et al., 2004).

Saint John's Wort (SJW), a herb used as a natural anti-depressant, contains the chemical hyperforin, which binds and activates the PXR in a number of species, including chicken, pig, dog, rabbit, monkey and human (Moore et al., 2002). Hyperforin has an extremely low $EC_{50}$ for the human PXR and is a potent activator of the PXR because it has all 5 principal pharmacophore features of potent PXR ligands (Ekins and Erickson, 2002). This makes SJW an excellent positive control for PXR activation and induction of CYP3A in most species tested. However, SJW does not activate the PXR in mouse (Moore et al., 2002), and is a poor inducer of CYP3A in the rat in vivo (Bray et al., 2002), presumably because the rat PXR has a phenylalanine instead of leucine at amino acid 305 (Tirona et al., 2004). Whether SJW activates the PXR in winter flounder is unknown and the PXR has not been cloned from winter flounder.

Winter flounder (*Pleuronectes americanus*) were used to study the effects of SJW and the estuarine contaminant nonylphenol. Winter flounder are an excellent sentinel organism to study the effects of nonylphenol and other toxicants on gene expression since they are in direct contact with the sediments, ingest other sediment dwelling organisms and are in turn consumed by humans. The objectives of this research were to (1) determine if SJW and 4-NP alter the expression of CYP3A and increase testosterone metabolism in a manner consistent with PXR activation in winter flounder, and (2) identify other genes induced in winter flounder liver by 4-NP at a concentration that also increases CYP3A.

## 2. Material and methods

### 2.1. Chemicals

Technical grade 4-nonylphenol (4-NP) was obtained from Fluka Chemical Co. (Ronkonkoma, NY, USA). This grade of 4-NP is a mixture of branched side chains containing 85% para-isomers. Saint John's Wort (SJW) was obtained from Nature's Resource Products (Mission Hills, CA, USA) and contains approximately 2.3% hyperforin. SJW was methanol extracted and prepared in corn oil as described previously (Bray et al., 2002).

### 2.2. Winter flounder

All studies were carried out according to NIH guidelines for humane use of research animals and were pre-approved by the Mount Desert Island Biological Laboratory (MDIBL) Animal Care and Use Committee. Winter flounder (*P. americanus*) weighing between 190 and 360 g were captured in the Gulf of Maine and transported to Mount Desert Island Biological Laboratory (MDIBL) in Salsbury Cove, ME. Flounder were housed in 2348 gallon tanks for 1 week and then male winter flounder (3–4 per group) were transferred to 4 different 157 gallon tanks and acclimated for 24 h prior to treatment. Male winter flounder (3–4 per group) were injected IP with 500 mg/kg/day SJW, 100 mg/kg/day 4-NP, both SJW and 4-NP (S and N), or corn oil as a control once daily for 2 days. Forty-eight hours after the initial injection, flounder were euthanized with MS-222