GENE EXPRESSION PROFILES OF LARGEMOUTH BASS EXPOSED TO NONYLPHENOL AND ICI 182,780

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Abstract

The goal of this study was to observe changes in gene expression in largemouth bass following exposure to 4- nonylphenol (4-NP) and ICI 182,780 (ICI) using real-time PCR (RT-PCR) and macroarrays. The expression levels of three estrogen receptor (ER) isotypes alpha, beta, and gamma, and vitellogenin (Vtg) I as measured by RT- PCR show that following exposure to 4-NP only ER alpha and Vtg 1were significantly induced. The addition of ICI to 4-NP repressed the induction of ER alpha by 4-NP but surprisingly had no effect on Vtg I mRNA levels. By macroarray, 4 Vtgs, 2 choriogenins, aspartic protease, signal peptidase and one unidentified clone were up-regulated while two genes including transferrin and clone 50-1 were down-regulated by 4-NP. Co-exposure to 4-NP and ICI resulted in a repressed response for aspartic protease. As seen for Vtg I by RT-PCR, the up-regulation of the Vtgs and choriogenins and down-regulation of transferrin by 4-NP was not altered by ICI. An additional set of genes that were not up or down-regulated by 4-NP were depressed by ICI. It is possible that differences in message stability or the composition of response elements in gene promoters play a role in changes in gene expression observed with the ICI treatment.

Introduction

Several chemicals that are found in the environment, termed hormonally active agents (HAA), may be linked to a variety of adverse biological effects in wildlife and humans (Carlsen et al., 1993; Guillette, 1994). One synthetic chemical found in the environment that functions as weak estrogen is 4-nonylphenol (4-NP). 4-NP primarily acts by binding to the estrogen receptor (ER) and subsequently inducing transcription of downstream genes, including the vitellogenins and choriogenins (Celius et al., 1999). However, there is evidence that 4-NP also has other mechanisms of action (Masuyama et al., 2000).

The ER signaling cascade has become increasingly complex with the discovery of multiple ER isotypes namely ER alpha and ER beta in both mammals and fish (Kuiper et al., 1996; Tchoudakova et al., 1999). Additionally, a third isotype termed ER gamma has recently been shown to exist in the teleost fish, Atlantic Croaker (Hawkins et al., 2000). The specific role that each ER subtype plays in the regulation of genes has not been determined. Studies have revealed that estrogen and estrogen mimicking compounds differentially bind and activate the various ER isotypes *in vitro*, however results vary between species including fish and mammals (Gutendorf and Westendorf, 2001; Kuiper et al., 1998; Matthews et al., 2000).

To fully understand the impact of HAA including 4-NP, effects at the molecular level need to be characterized in animals, particularly those that are exposed to these compounds in the environment, including fish. The goal of this study was to measure gene expression in largemouth bass (*Micropterous salmoides*) (LMB) following exposure to 4-NP. In particular, a real-time PCR assay was developed to measure the change in expression of three LMB ER isotypes alpha, beta, gamma, and the ER regulated gene Vtg 1. We also tested the effects of the co-administration of 4-NP with the antiestrogen ICI 182,780 (ICI) on the expression of the ERs and Vtg 1. In addition, we used a gene macroarray, developed in our laboratory, to characterize the specific expression profile of other estrogen responsive genes following exposure to 4-NP and 4-NP in conjunction with ICI.

Materials and methods

Experimental Design and Sample Collection: Adult male LMB were purchased from American Sports Fish Hatchery (Montgomery, Alabama) and maintained

in fiberglass tanks at the University of Florida Aquatic Toxicology Facility as previously described (Larkin in review 2002, Bowman in review 2002). Each fish was injected intraperitoneally (IP) with either 50 mg/kg 4-NP (Fluka # 74430), the combination of 50 mg/kg 4-NP and 1.0 mg/kg ICI (Tocris Cookson Inc.), or vehicle, which consisted of ethanol and dimethylsulfoxide (DMSO, Sigma #5879). Fish were euthanized by submersion in a water bath containing 50-100 ppm MS-22 48 hours post injection and sacrificed by a sharp blow to the head followed by cervical transaction. The livers were excised, immediately flash frozen in liquid nitrogen, and stored at –80°C until RNA was isolated.

RNA Isolation: Isolation of total RNA from liver tissue was performed with the RNA Stat-60 reagent (Tel-test) as described previously (Sabo-Attwood et al., in review). For all RNA samples, the quantity and quality of total RNA was assessed by spectrophotometric readings at 260 nm and by electrophoresis through a 1% formaldahyde agarose gel stained with ethidium bromide.

***Real-Time PCR*:** Real time PCR was performed using reagents and a 5700 thermocycler purchased from Applied Biosystems. Primers and probes for the ER subtypes and Vtg 1 have been described previously (Sabo-Attwood et al., in review). Each real time PCR reaction consisted of 0.01 – 0.2 ug of reverse transcribed total RNA as template. To generate a standard curve, varying amounts of plasmid containing the specific cDNA inserts for each gene were used as template in the PCR reactions. For each gene, a 6 point standard curve encompassing a 1 x 10^6 fold range of approximately 25 – 2.5 x 10^6 copies of cDNA was constructed. Each sample was run in duplicate and normalized 18s rRNA. Both the intra-assay and inter-assay variability never exceeded 10%. Statistical differences between the treatments were determined by one way analysis of variance with Dunnets post-hoc analysis.

Amplification of cDNA to be spotted on the macro arrays: The macroarrays were prepared and printed as previously described (Larkin et al., in review). Briefly, the 132 LMB clones were PCR amplified, purified, and concentrated. Following denaturation, 20 X SSC (3M NaCl, 0.3M sodium citrate, pH 7.0) containing 0.01 mM bromophenol blue was added to the samples to yield a final concentration of 100 ng/µL cDNA template. The PCR products were robotically spotted (Biomek 2000, Beckman Coulter, Fullerton, CA) in duplicate onto neutral nylon membranes (Fisher scientific) using 100 nL pins. Membranes were UV cross-linked and stored under vacuum at room temperature until hybridization.

Labeling of RNA, hybridization, and detection: Radiolabeled probes were generated by random primer labeling of DNase treated (DNA-*free*, Ambion, Austin, TX) total RNA from LMB livers with [α-^{33}P] dATP (Strip-EZ RT, Ambion). The blots were hybridized with ultraArray hybridization buffer (Ambion) as previously described (Larkin et al., in review). The membranes were exposed to a phosphor screen (Molecular Dynamics, Piscataway, NJ) and quantitatively evaluated using a Typhoon 8600 imaging system (Molecular Dynamics). For each cDNA clone, the general background of each membrane was subtracted from the average value of the duplicate spots. The values were normalized to the average value of 12 cDNA clones specific to ribosomal genes. (Larkin et al., 2002). Genes were not included for analysis that had values less than the background value for two out of the three replicates and/or fluctuated more then two fold when aliquots of the same RNA were hybridized to blots printed at the beginning, middle, and the end of the array printing process.

Results

Measurement of ER and Vtg 1 mRNA by real-time PCR: Real-time PCR is a sensitive assay that can be used to quantitate expression levels of genes. Using this technology we designed assays to quantitate the expression of 4 genes, ERs alpha, beta, and gamma, and Vtg 1 in LMB following exposure to 4-NP and 4-NP/ICI. Using primers and probes specific to each gene we were able to differentiate between the ER isotypes with no cross reactivity (Sabo-Atwood et al., in review). Exposure of LMB to a single injection (IP) of 4-NP (50 mg/kg) significantly increased ER alpha by 80 fold ($P < 0.05$) after 48 hours when compared to controls (Figure. 1). During the same time frame, the levels of both ER beta and ER gamma decreased approximately 1.3 fold and 2.6 fold respectively, however these changes were not statistically significant from controls. When the LMB were exposed to a combination of 4-NP (50 mg/kg) and the anti-estrogen ICI (1.0 mg/kg), the levels of ER alpha increased only 4 fold over controls ($P < 0.08$), suggesting that the anti-estrogen had interfered with the activation process (Figure 1). As with the 4-NP treatment, the expression of ER beta and gamma decreased (1.9 fold) but the values did not differ significantly from controls.

Since the Vtg gene is an E_2 responsive gene that is under transcriptional control by ERs in the liver, we also determined the expression levels of Vtg 1 by real-time PCR. Exposure to 4-NP increased message levels by approximately 40 fold over controls ($P < 0.05$), however, this induction was not repressed by the addition of ICI (Figure 2).

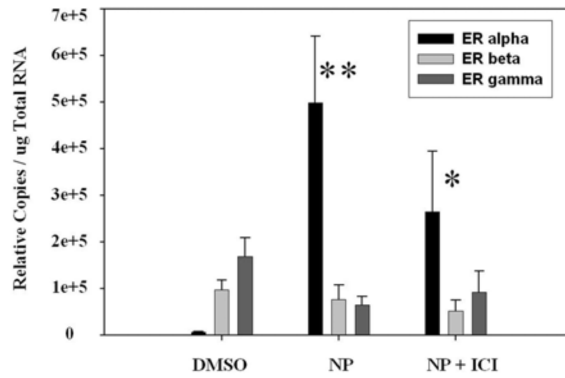

Figure 1 shows the expression of ERs alpha, beta, and gamma measured by real-time PCR. Each bar represents the mean and standard error (n = 7). Statistically significant differences between treatments and controls are marked with astericks. P < 0.05 (**), P < 0.1 (*).

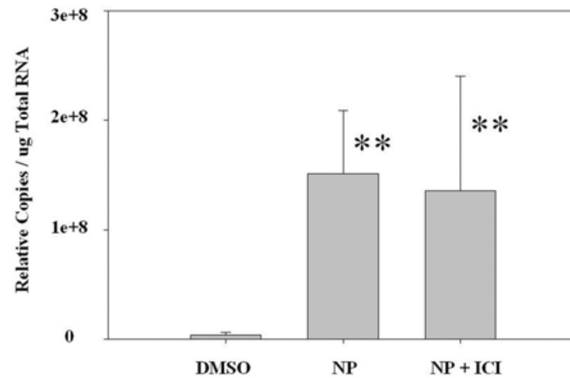

Figure 2 illustrates the expression of VTG I measured by real-time PCR. Each bar represents the mean and standard error (n = 7). Statistically significant differences between treatments and controls is marked with an asterick. P < 0.05 (**).

LMB gene array analysis: In order to further characterize the effects of 4-NP alone or in conjunction with ICI on hepatic gene regulation in LMB, we examined the expression of 132 genes, many of which are estrogen responsive, by gene arrays. Total hepatic RNA isolated from control and exposed fish was radiolabeled and hybridized to the membranes. Of the 132 genes on the array, only genes that changed by at least 3 standard deviations from the mean of the 12 ribosomal genes that were used to normalize the data are included in Figure 3. These include several that are up of down-regulated by more than 2 fold, a conservative cutoff generally used for array interpretation. Figure 3 shows the fold induction of each gene over controls for both the 4-NP and 4-NP/ICI treatments.

In the 4-NP treated fish, 9 genes were up-regulated 2 fold or greater including 4 Vtgs, choriogenin 2, choriogenin 3, aspartic protease, signal peptidase, and one unidentified clone designated 92-1. Two genes were found to be down-regulated by 4-NP including transferrin and clone 50-1. In the case of the mixture of 4-NP and ICI, the expression of one gene, aspartic protease, was reduced. In addition, the expression levels of 4 Vtgs, 2 choriogenins, and transferrin were not affected at all, but instead appeared to be expressed to the same levels as with 4-NP alone.

Genes which were reduced by the mixture and that exhibited as least a 2 fold change in expression when compared to 4-NP alone are marked with an asterisk. These genes included aspartic protease, protein disulfide isomerase, integral membrane protein, methionine sulfoxide reductase, ER gamma, glucocorticoid receptor, aldose reductase, ER beta, FK506 binding protein, and 21 unidentified clones. All of these genes except for clone 53-1 were down-regulated by the addition of ICI to 4-NP.

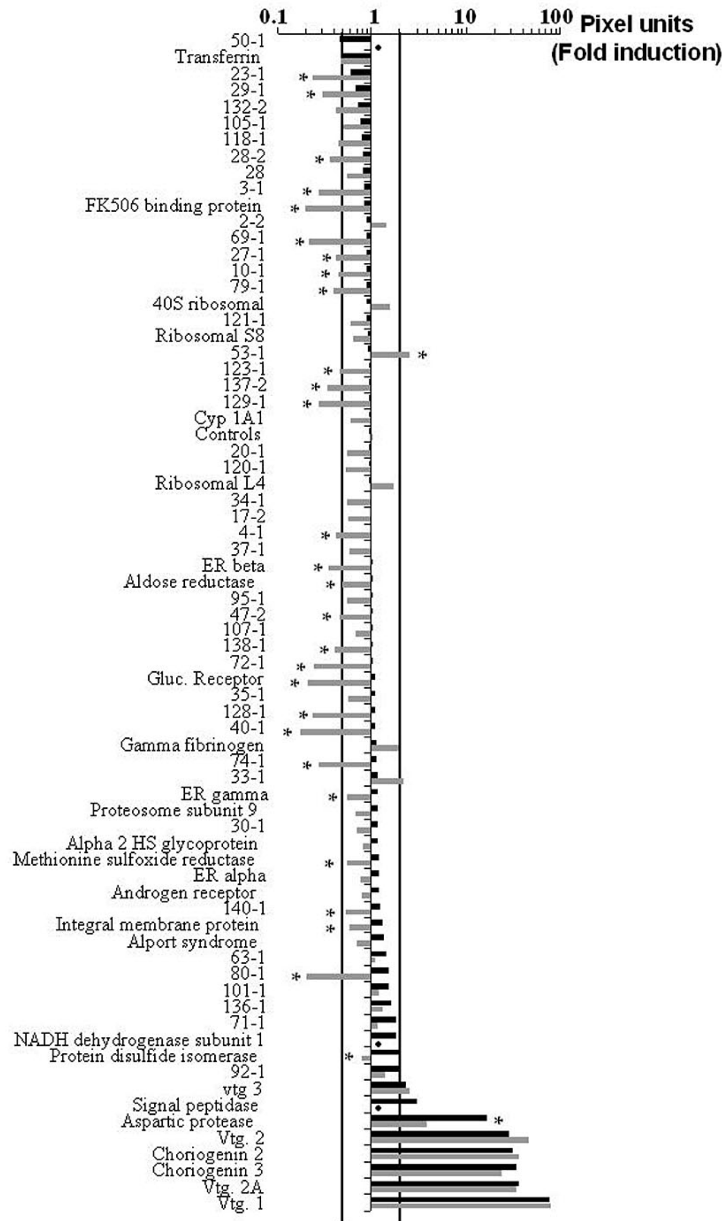
0.1
1
10
100
Pixel units
(Fold induction)
50-1
Transferrin
23-1
29-1
132-2
105-1
118-1
28-2
28
3-1
FK506 binding protein
2-2
69-1
27-1
10-1
79-1
40S ribosomal
121-1
Ribosomal S8
53-1
123-1
137-2
129-1
Cyp 1A1
Controls
20-1
120-1
Ribosomal L4
34-1
17-2
4-1
37-1
ER beta
Aldose reductase
95-1
47-2
107-1
138-1
72-1
Gluc. Receptor
35-1
128-1
40-1
Gamma fibrinogen
74-1
33-1
ER gamma
Proteosome subunit 9
30-1
Alpha 2 HS glycoprotein
Methionine sulfoxide reductase
ER alpha
Androgen receptor
140-1
Integral membrane protein
Alport syndrome
63-1
80-1
101-1
136-1
71-1
NADH dehydrogenase subunit 1
Protein disulfide isomerase
92-1
vtg 3
Signal peptidase
Aspartic protease
Vtg. 2
Choriogenin 2
Choriogenin 3
Vtg. 2A
Vtg. 1

Figure 3 shows gene expression profiles from control, 4-NP, and 4-NP/ICI reated male fish. The mean intensity values for each of the cDNA clones for 4-NP (black bar) or 4-NP/ICI (gray bar) divided by the mean intensity values of the respective cDNA clones from control fish. Genes that changed by more then two fold for the 4-NP treatment compared to the 4-NP/ICI treatment are indicated by an asterick. The black dots in panel indicate genes that were not used in the analysis (see methods section).

Discussion

The goal of this study was to observe changes in gene expression following exposure to the weak estrogen mimic 4-NP and the anti-estrogen ICI using real-time PCR and macroarrays. We picked 4 genes to monitor by real-time PCR, ERs alpha, beta, gamma, and Vtg 1. Real-time PCR is a sensitive and specific assay that allows for relative quantitation of gene expression levels with a limit of detection of 25 copies. More importantly, the ER isotypes share a great deal of sequence identity specifically in the DNA and ligand binding regions, but we were able to distinguish between the ER subtypes using this assay (Sabo-Attwood et al., in review).

Macroarrays allow researchers to simultaneously monitor the expression patterns of multiple genes from the same sample. Although not as quantitative and specific as real-time PCR, the macroarrays are an appropriate tool for rapidly screening large numbers of genes. We have constructed a LMB specific gene array containing 132 clones and have previously shown that the inter-assay variability of these arrays was minimal (Larkin et al., in review).

To determine the effect of 4-NP on the expression levels of ERs alpha, beta, and gamma *in vivo*, we measured the relative copies of each by real-time PCR. The data in Figure 1 shows only ER alpha was significantly induced, approximately by 80 fold compared to controls. The expression levels of ER beta and gamma did not significantly change. This result suggests that the three ERs differ in their regulation by estrogenic compounds. Apparently in LMB, only ER alpha is inducible by 4-NP.

We have previously shown that during the reproductive cycle of female LMB, as E_2 synthesis increases there is a strong correlation between the induction of vitellogenin and ER alpha mRNA (Sabo-Attwood et al., in review). Since ER alpha expression increased following exposure to 4-NP, we wanted to determine

if the mRNA expression levels of one vitellogenin, Vtg 1 also increased. Figure 2 shows there was a significant ($p < .05$) induction of Vtg 1 mRNA approximately 40 fold compared to controls following exposure to 4-NP. While the fold induction level is less than that for ER alpha, it actually is 3 orders of magnitude greater in copy number. This difference in induction was reported previously for LMB (Bowman et al., in review) and rainbow trout (Flouriot et al., 1996). The difference in expression response for these two genes may be due to the nature of the response elements in the promoters of these genes.

ICI has been used in numerous mammalian models but has not been well characterized in fish. Since the ICI compound is an anti-estrogen in mammalian systems we had expected that it would diminish the response observed by 4-NP for genes that were regulated via the ER pathway. Figure 1 shows that the combination of 4-NP and ICI induced ER alpha expression over controls but not to the same extent when compared to 4-NP alone. In fact, the response was approximately half that seen with the 4-NP treatment. Although the difference between ER alpha expression by the 4-NP and 4-NP/ICI treatments is not statistically significant ($p< 0.16$) the data suggests that ICI repressed the induction of ER alpha by 4-NP. This was the expected result for an anti-estrogen. The expression of ER beta and gamma did not differ significantly from controls for any of the treatments.

Since ICI diminished the induction of ER alpha we expected the expression of Vtg 1 to be repressed as well. Surprisingly, the induction of Vtg 1 message by 4-NP was not affected by the ICI treatment (Figure 2). It is possible that since we are measuring steady state levels of the mRNAs only at 48 hours by the real-time PCR assay, we missed the inhibition by ICI. Consistent with this hypothesis is the suggestion that ER alpha mRNA half-life is shorter than that of Vtg (Bowman et al., in review).

In order to determine the expression profile of multiple genes following exposure to 4-NP we hybridized radiolabeled RNA from exposed and control fish livers onto a macroarray. All of the genes that were up-regulated by 4-NP except for signal peptidase have been shown previously to be induced by E_2 (2.5 mg/kg) by this method (Larkin et al., in review). The 4 Vtg's, 2 choriogenins, and aspartic protease were up-regulated while transferrin and clone 50-1were down-regulated (Figure 3). This indicates 4-NP is acting at least in part as an estrogen mimic.

To determine the effect of the anti-estrogen ICI on gene expression, we determined the expression pattern of the same 132 genes following exposure to the combination of 4-NP and ICI by macroarray. The induction of one gene, aspartic protease was decreased by 75% by ICI, similar to the pattern observed with ER alpha by real-time PCR.

Surprisingly, a number of genes on the array including the Vtgs and choriogenins which have been well characterized as E_2 responsive genes were not affected by ICI. As shown in Figure 3, the 4 Vtgs and both choriogenins were induced to the same extent as in the 4-NP treated fish. This correlates to the data seen by real-time PCR which showed no difference in Vtg 1 induction of mRNA between the two treatments. Also, transferrin which has been shown previously by our lab to be down-regulated by exposure to E_2 in LMB and sheepshead minnows was also down regulated by 4-NP. However, like the Vtgs and choriogenins, the expression of this gene was not affected by the ICI compound. Why the induction of certain genes such as the Vtgs and choriogenins was not affected whereas the up-regulation other genes such as aspartic protease were repressed is not known. It is possible that differences in message stability or the composition of response elements in gene promoters may play a role.

There was also a set of genes that were not up or down-regulated by 4-NP, but were down-regulated by ICI. The expression of these genes were altered by at least 2 fold by ICI compared to administration of 4-NP alone. These include protein disulfide isomerase (PDI), integral membrane protein, methionine sulfoxide reductase, ER gamma, glucocorticoid receptor, aldose reductase, FK506 binding protein, and 20 unidentified clones. Both PDI and aldose reductase were shown to be upregulated in LMB by E_2 (2.5 mg/kg) previously (Larkin et al., in review). Since 4-NP is a weak estrogen the capacity of this compound to induce certain genes may be limited. The fact that ICI depressed genes that were induced by E_2 suggests that they are regulated in part by ERs. Only one gene, clone 53-1 was up-regulated by ICI. Since the effects of ICI in fish *in vivo* have not been well characterized it is difficult to speculate on the precise mechanisms involved in this genetic profile. Additional experiments are being conducted to further characterize the effects of anti-estrogens on gene expression in fish.

The change in expression levels for the ERs on the array does not exactly correspond to the levels measured by real-time PCR. Compared to genes such as the Vtgs and choriogenins, the levels of expression of the ERs is low. We have

previously shown that more inter-assay variability exists in genes with low expression compared to genes that are highly expressed. Also, since the ER cDNA clones on the array contain domains with high sequence identity between the isotypes, it is possible that cross-reactivity plays a role in the differential expression levels observed between the real-time PCR and macroarray analysis. We are confident that the real-time PCR assay specifically amplifies each ER with no cross-reactivity between isotypes.

Overall our study indicates that 4-NP acts as an estrogen mimic based on the induction of known E_2 responsive genes such as the Vtgs and choriogenins by both real-time PCR and macroarray analysis. Using real-time PCR we found that only ER alpha mRNA, not ER beta or gamma is significantly induced by 4-NP. The expression of some genes following exposure to the ICI compound was as expected which included the reduction in ER alpha and aspartic protease mRNAs as measured by real-time PCR and macroarray respectively. Interestingly the antiestrogen ICI did not affect the induction of other E_2 and 4-NP responsive genes including the Vtgs and choriogenins.

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