Dose-response relationships in gene expression profiles in a harbor seal B lymphoma cell line exposed to 17α-ethinyl estradiol

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Abstract

The determination of changes in gene expression profiles with xenobiotic dose will allow identifying biomarkers and modes of toxicant action. The harbor seal (*Phoca vitulina*) 11B7501 B lymphoma cell line was exposed to 1, 10, 100, 1000, 10,000, or 25,000 µg/L 17α-ethinyl estradiol (EE_2, the active compound of the contraceptive pill) for 24 h. Following exposure, RNA was extracted and transformed into cDNA. Transcript expression in exposed vs. control lymphocytes was analyzed *via* RT-qPCR to identify genes with altered expression. Our analysis indicates that gene expression for all but the reference gene varied with dose, suggesting that different doses induce distinct physiological responses. These findings demonstrate that RT-qPCR could be used to identify immunotoxicity and relative dose in harbor seal leukocytes.

Introduction

Harbor seals are exposed to various anthropogenic stressors in the environment due to their coastal habitat, *e.g.* chemical pollution, litter (plastics and nanoparticles), noise, and climate change.[1-3] Since exposure to persistent organic pollutants and metals has been shown to suppress the seal's immune system,[4,5] it is important to understand the relative contributions of other stressors to immunosuppression as well.

Pharmaceuticals are a compound class that has only recently been analyzed for its immunosuppressive potential in marine mammal leukocytes.[6,7] The synthetic estrogen 17α-ethinyl estradiol (EE_2), which is the active compound of the contraceptive pill, has been the focus of this study. Firstly, it is an environmental xenobiotic and frequently found in municipal effluents and surface waters.[8] EE_2 was found at concentrations up to 42 ng/L in effluent samples from Ontario,[9] up to 831 ng/L in surface water samples from the USA[10] and at concentrations up to 2.32±1.45 µg/L in plasma of free-ranging neonate bull shark (*Carcharhinus leucas*) from Florida with the latter indicating accumulative potential.[11] Secondly, EE_2 has previously affected lymphocyte proliferation and the cell cycle of the harbor seal 11B7501 B lymphoma cell line and harbor seal peripheral blood mononuclear cells (Kleinert *et al*. T lymphocyte-proliferative responses of harbor seal (*Phoca vitulina*) PBMCs exposed to pharmaceuticals *in vitro*; 2017; unpublished data).[6] Lastly, B lymphocytes and lymphomas are regulated by estrogens through estrogen receptors (ERα and ERß) in mammals.[12,13] An active ERß receptor acts as an anti-proliferative and pro-apoptotic stimulus in non-Hodgkin lymphoma in humans.[14]

The model used in this study was the harbor seal 11B7501 B lymphoma cell line. This lymphoma cell line is an ideal proxy for immunocompetence assessments in marine mammals since confounding factors like physiological state and inflammation of an individual are of no concern.

New molecular biomarkers have recently been developed to identify early biological effects using minimally invasive blood samples.[15-17] Since pharmaceuticals are designed to have a low acute toxicity, these potentially more sensitive methods might be more relevant in assessing immunotoxicity for this class of compounds and help infer underlying mechanisms of toxic action. The objectives of this study were to determine the immunomodulation and cytotoxicity of EE_2 and gene expression profiles in a harbor seal B lymphoma cell line.

Materials and Methods

The harbor seal 11B7501 B lymphoma cell line (CRL-1940 purchased from ATCC, Manassas, VA) was maintained as previously described.[18] 17α-ethinyl estradiol (EE_2) was dissolved in dimethyl sulfoxide (DMSO) (both Sigma-Aldrich, Oakville, Canada). The final concentration of DMSO in the samples was 0.1%.

For the *in vitro* exposures, 1×10^6 of cells were incubated with EE_2 in 24-well plates for 24 h. EE_2 concentrations were 0, 1, 10, 100, 1000, 10,000, or 25,000 µg/L. Concentrations ranging from 12,500 to 25,000 µg/L have previously affected the lymphocyte proliferation and cell cycle of the 11B7501 cell line.[6] We chose four additional lower concentrations to assess if RT-qPCR is a more sensitive method for detection of immunomodulation. In a parallel set of experiments the cell line was exposed to the same concentrations of EE_2 in the presence of 5 µg/mL of the mitogen lipopolysaccharide (LPS). LPS activated B lymphocytes non-specifically (induced proliferation) and was meant to simulate the cell activity for proliferation as if under pathogen exposure.

RNA was extracted using the Aurum total RNA kit (Bio-Rad, Mississauga, ON). NanoDrop-normalized (A260 nm) levels of total RNA were reverse transcribed with the iScript cDNA synthesis kit (Bio-Rad, Mississauga, ON). cDNA were used for quantitative polymerase chain reaction (qPCR) using Ssofast Evagreen supermix (Bio-Rad, Mississauga, ON). qPCR was initiated with 95°C for 30 s, followed by 40 cycles with denaturation at 95°C for 5 s, and the primer specific annealing temperature (62°C) for 15 s. Melting curves were created by denaturation at 95°C followed by 61 steps during which the temperature was increasing steadily for 0.5°C every 5 s, starting at 65°C and ending at 95°C, to exclude the measurement of non-specific PCR products and primer dimers and to determine true amplification. Results were

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Contributions: CK planned and conducted experiments, wrote manuscript; MB designed primers, planned experiments; FG supervised the project, corrected manuscript; MF provided funding and lab space.

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analyzed using the comparative Ct method. Primers are shown in Table 1.

The reference gene ß2M was evaluated for its stability with bestkeeper (stability value =0.073) and was chosen to normalize mRNA transcription levels of the genes of interest. Genes of cells exposed to EE_2 only were normalized with the ß2M 0 µg/L EE_2 negative control, whereas genes of cells exposed to EE_2 and LPS were normalized with the ß2M 0 µg/L EE_2+LPS negative control.

Results are obtained from two independent experiments with two technical replicates for each sample.

Viability assays were set up in parallel to observe if EE_2 concentrations were cytotoxic to the 11B7501 B lymphoma cell line. After 24 h, viability of cells was evaluated by adding 0.8 µg/mL propidium iodide (PI) (Sigma-Aldrich, Oakville, Canada) to the cell suspension. A FACSCalibur (Becton Dickinson, San Jose, CA, USA) with an air-cooled argon laser providing an excitation at 488 nm was used. For each sample 5000 events were acquired at a fluorescence emission of 620 nm (FL3). The cell population was electronically gated in a FSC/SSC dot plot and the fluorescence frequency distribution histogram was obtained. The percentage of dead cells was determined as the proportion of cells with an elevated fluorescence using a marker. Data collection and analysis were performed with the CellQuest Pro software (Version 4.0.1). The results were expressed in percentage of viable cells.

Differences between controls and treated groups were evaluated by one-way and two-way ANOVA followed by Dunnett's post hoc test. To evaluate correlations of inter-relationships of genes of interest a Spearman rank order correlation analysis was conducted and results are summarized in Table 3. The calculations were performed using GraphPad Prism 6 for Mac (GraphPad Software). The level of significance was set at $P \leq 0.05$.

Results and Discussion

A range of eight immune-relevant (CD9, IFNγ, LYN1, MAPKK3, SLAM, TGFß1), pollutant-associated (AHR) and stress (HSP70) biomarkers as well as a reference gene (ß2M) were selected to analyze differential gene expression in the harbor seal 11B7501 B lymphoma cell line exposed to EE_2. We limited our choice of genes to sequences that have already been validated in the harbor seal.

To obtain an overview of potential effects on the mRNA expression level of the chosen genes, we analyzed the control samples and the highest exposure concentration of EE_2 (25,000 µg/L) for the genes of interest (Figure 1A). The mRNA expression of all but one (ß2M) gene was impacted at 25,000 µg/L EE_2. Genes at this concentration were both up- and downregulated. Lymphocytes exposed to EE_2 without stimulation from LPS downregulated four out of five genes (Table 2). Lymphocytes exposed to EE_2 and the mitogen LPS were more likely to have changes in gene expression, and genes were mainly upregulated (6 of 9) (Table 2).

HSP70 protects proteins from stress by aiding protein folding. HSP70 mRNA was upregulated two-fold compared to controls upon exposure to EE_2 alone as well as EE_2 and LPS (Table 2). Since HSP70 showed the most marked change in gene expression of all genes tested, we further evaluated the remaining exposure concentrations. The analysis revealed that HSP70 was induced only at the highest concentration (Figure 1B). Previous studies in harbor seal blood demonstrated negative (cadmium, Cd) and positive (lead, Pb) correlations between trace metal concentrations in blood and HSP70 expression.[1] These trends are in accordance with previous work that demonstrated that xenobiotic exposure could lead to immunosuppression or acute and chronic inflammatory processes that could cause hypersensitivities or autoimmune diseases.[19] When assessing HSP70 in blood of free-ranging animals it has to be taken into account that capture and sampling stress might alter the physiological levels of HSP70 in these animals. HSP70 and cortisol were correlated in adult seals, and habituation (*i.e.* decreasing levels of HSP70 over

Table 1. Primers used for qPCR analysis. All primers are listed from 5' to 3'.

Gene	Description	Forward (F) & Reverse (R) Primers
Housekeeping Gene		
ß2M	Component of MHC class I molecules	F' CTA CGT GTC AGG GTT CCA T R' TGC TTT ACA CGG CAG CTA
Genes of Interest		
AHR	Regulator of cytochrome P450	F' ATA CAG AGT TGG ACC GTT TG R' AAG AAG CTC TTG GCT CTT A
CD9	Regulator of cell development, activation, growth and motility	F' TCT TTG GCT TCC TCT TGG T R' TTG GAC TTC AGC TTG TTG TA
HSP70	Aids with protein folding under heat or chemical stress	F' GCA ACG TGC TCA TCT TTG A R' AGC CTG TTG TCA AAG TCC T
IFNγ	Cytokine; activates macrophages; induces MHC class II expression	F' CAA GGC GAT AAA TGA ACT CA R' CGG CCT CGA AAC AGA TTC
LYN1	Key enzyme in regulation of cell activation in hematopoietic cells	F' CAA GGG AAG GTG CCA AAT T R' GAC CAT ACA TCA GAC TTA ATC G
MAPKK3	Phosphorylates mitogen-activated protein kinase (MAPK)	F' TTG GTG GAT TCT GTA GCC A R' AAG CCC ACA CAT CAG ACT T
SLAM	Surface receptor of activated T and B cells; enhances proliferation and IFNγ production	F' CAT GAC CCT GGA GGA GAA R' CAA GCT GCA GTT CCC ATT
TGFß1	Cytokine involved in cell growth, proliferation, differentiation and apoptosis	F' ACA CCA ACT ACT GCT TCA G R' GCA GAA GTT GGC GTG GTA

ß2M=beta-2 microglobulin; AHR=aryl hydrocarbon receptor; CD9=Cluster of differentiation 9; HSP70=70 kDa heat shock protein; IFNγ=interferon gamma; LYN1=Lck/Yes novel tyrosine kinase; MAPKK3=mitogen-activated protein kinase kinase; SLAM=signaling lymphocytic activation molecule; TGFß1=transforming growth factor beta 1

time) was observed in rehabilitation processes where seals have been handled frequently.[1,15,17,20] Moreover, effects on HSP70 expression strongly correlated with AHR expression (r=0.606) and IFNγ expression (r=0.743) (Table 3).

Interferon gamma (IFNγ) and signaling lymphocytic activation molecule (SLAM) are associated with susceptibility to mammalian infectious diseases.[21] IFNγ is a cytokine that activates macrophages and induces class II major histocompatibility complex (MHC II) expression. In our study, IFNγ expression was upregulated after exposure to 25,000 µg/L EE_2 + LPS, but not when the mitogen LPS was not present (Figure 1A, Table 2). Since the +LPS negative control was identical to the -LPS negative control, this upregulation can be attributed to a combined effect of EE_2 with LPS. Furthermore, effects on IFNγ expression displayed strong correlations with pattern changes in AHR expression (r=0.606) and HSP70 expression (r=0.743) (Table 3).

SLAM is a surface receptor on activated B and T lymphocytes. In our study, its expression was not affected by 25,000 µg/L EE_2 alone, but it was one of the few genes that was significantly downregulated when additionally exposed to LPS. It has been shown to enhance IFNγ production, and is the primary cellular receptor for *Morbillivirus*.[22] Interestingly, SLAM and IFNγ mRNA levels showed inverse trends in regulation after exposure to EE_2 and LPS. While the exposure to the EE_2 or LPS alone did not change the mRNA expression, the additional stimulus and stress of a potential pathogen (LPS) impacted the B lymphoma cell line. The increase in IFNγ can therefore be attributed to the presence of LPS, and not due to an increased production due to SLAM. The Spearman rank order correlation analysis revealed negative correlations with expression of SLAM and LYN1 (r= –0.680) as well as MAPKK3 (r= –0.568) (Table 3).

Tyrosine-protein kinase LYN1 is involved in the regulation of cell activation of hematopoietic cells. LYN1 expression was upregulated after exposure to

Table 2. Summary of significant gene expression changes in the 11B7501 B lymphoma cell line. Up- or downregulation is described as the percentage of expression relative to the negative controls of ß2M. The ß2M negative control (-EE_2/-LPS) was used to normalize genes exposed to EE_2 only, whereas the ß2M negative control (-EE_2/+LPS) was used to normalize genes exposed to EE_2 and LPS.

Genes	25 000 µg/L E_{E2}	25 000 µg/L EE_2 + 5 µg/mL LPS
AHR	↓ 81±12 %	↑ 120±13 %
CD9		↓ 59±7 %
HSP70	↑191±36 %	↑ 228±80 %
IFNγ		↑ 141±13 %
LYN1	↓ 63±14 %	↑ 142±17 %
MAPKK3		↑ 163±48 %
SLAM		↓ 63±29 %
TGFß1	↓ 62±15 %	

EE_2=17α-ethinyl estradiol; LPS=lipopolysaccharide; AHR=aryl hydrocarbon receptor; CD9=Cluster of differentiation 9; HSP70=70 kDa heat shock protein; IFNγ=interferon gamma; LYN1=Lck/Yes novel tyrosine kinase; MAPKK3=mitogen-activated protein kinase kinase; SLAM=signaling lymphocytic activation molecule; TGFß1=transforming growth factor beta 1.

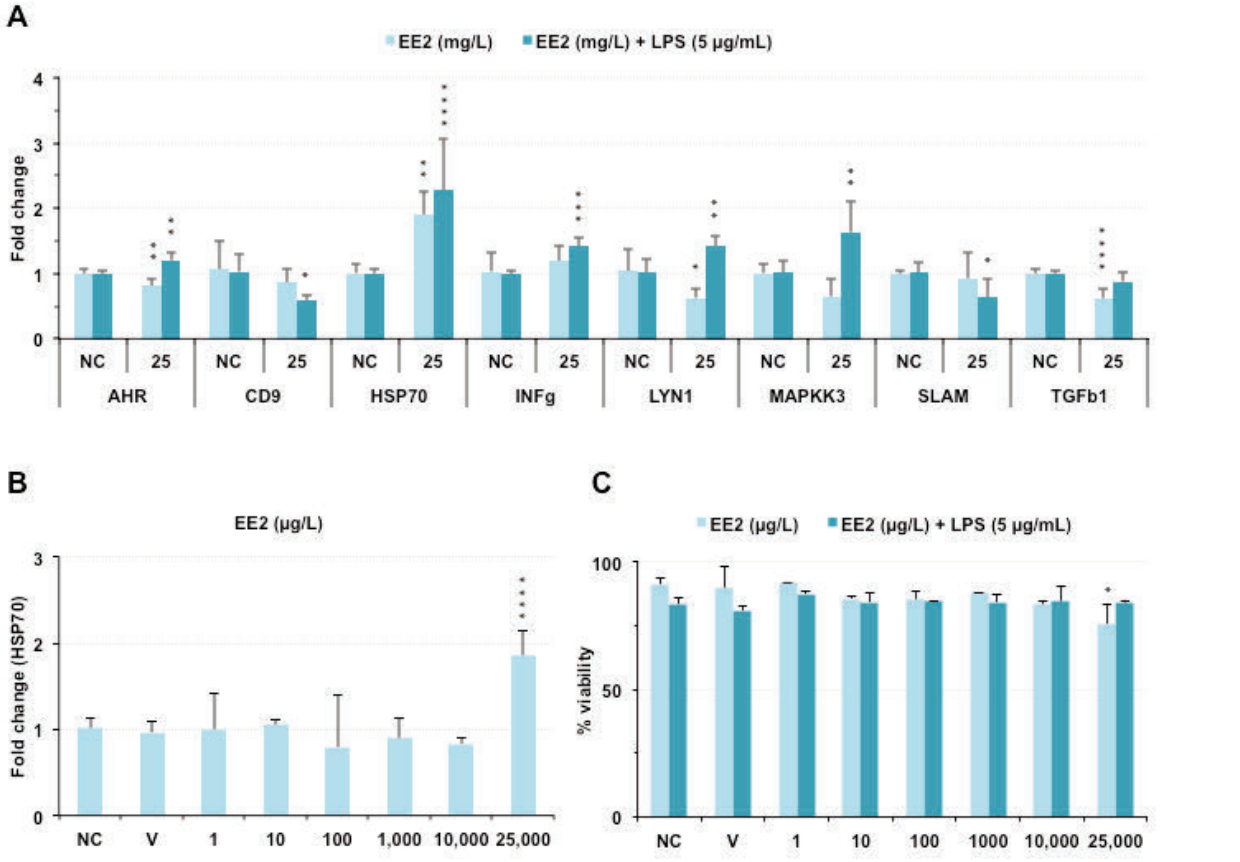

Figure 1. (A) Fold change in gene expression after 24 h exposure to 0 (NC) and 25 mg/L EE_2 as measured by qPCR. A fold change of 1 indicated no change in comparison to the reference gene ß2M. (B) Dose-response gene expression changes of HSP70 mRNA after 24 h exposure to EE_2. (C) Viability of the 11B7501 B lymphoma cell line after 24 h exposure to EE_2 and EE_2 + LPS. Results are expressed as Mean ± SD. (* p<0.05; ** p<0.01; *** p<0.001; **** p<0.0001). Abbreviations: EE_2=17α-ethinyl estradiol; LPS=lipopolysaccharide; ßACT=beta actin; YWHAZ=14-3-3 protein zeta/delta; AHR=aryl hydrocarbon receptor; CD9=Cluster of differentiation 9; HSP70=70 kDa heat shock protein; IFNγ=interferon gamma; LYN1=Lck/Yes novel tyrosine kinase; MAPKK3=mitogen-activated protein kinase kinase; SLAM=signaling lymphocytic activation molecule; TGFß1=transforming growth factor beta 1.

25,000 µg/L EE_2 + LPS, but significantly downregulated when LPS was not present (Figure 1A, Table 2). These results are in accordance with previous work demonstrating an upregulation of LYN in murine macrophages after LPS as well as IFNγ stimulation.[23] Effects on LYN1 expression very strongly correlated with AHR expression changes (r=0.809) and moderately to strongly correlated with expression changes of MAPKK3 (r=0.546) and SLAM (r=–0.680), respectively (Table 3).

Mitogen-activated protein kinase kinase 3 (MAPKK3) activates MAPK3, a protein involved in the regulation of cellular processes such as proliferation and differentiation in lymphocytes.[24] MAPKK3 mRNA was upregulated in response to 25,000 µg/L EE_2 + LPS exposure, but not when the mitogen LPS was not present (Figure 1A, Table 2). A change in the MAP kinase pathway could impact both B cell receptor or T cell receptor signaling and T cell development.[25-27] MAPKK3 expression changes correlated with expression changes in four other genes (Table 3). Moderate positive and negative correlations were observed with CD9 (r= –0.593), LYN1 (r=0.546), SLAM (r= –0.568) and TGFß1 (r=0.519).

The observed downregulation of the transmembrane protein CD9 at 25,000 µg/L EE_2 + LPS is potentially related to the inactivation of histone deacetylases by LPS.[28] The Spearman rank order correlation analysis revealed CD9 expression changes correlation only with one other gene (MAPKK3, moderate negative correlation, r= –0.593) (Table 3).

TGFß1, a cytokine involved in cell growth, proliferation, differentiation and apoptosis, is the only gene affected by EE_2 alone, but not EE_2 + LPS. A moderate correlation was observed with MAPKK3 (r=0.519) (Table 3).

As the differential gene expression patterns revealed (Figure 1A), it is crucial to test the immunotoxicity of a compound with and without stimulation from a potential pathogen like LPS, since immunotoxicity of a compound can vary quite considerably in the two exposure scenarios.

Viability of the harbor seal 11B7501 B lymphoma cell line after 24 h was 91±3% in the negative controls and 83±2% in the negative controls of cells exposed with LPS (Figure 1C). The cell line was significantly impacted only in one exposure at 25,000 µg/L EE_2 without LPS reducing it to 76±8%. It is problematic to conclude environmentally relevant effects of EE_2 on gene expression of harbor seal lymphocytes in this study, since the maximum dose tested was quite high. Furthermore, it should be investigated to what extent hormone levels in FBS might have influenced the bioavailability of EE_2 to the cells, by comparing with charcoal stripped FBS.

Quantitative PCR has rarely been utilized in toxicity testing in the marine mammal immune system,[1] while the method is more common in other species. To our knowledge this is the second study after Lehnert *et al*., 2016 that has used this method to assess the toxic mechanisms of action of gene expression in marine mammal immune cells. To date, only few mRNA sequences are known for pinnipeds in comparison to *e.g*. rodent sequences. It is therefore important to continue the effort to sequence the gene expression transcript and continue using marine mammal lymphocyte cell lines or blood samples from free-ranging animals to further the knowledge of immunotoxic action under contaminant load in marine mammals.

Conclusions

In conclusion, the observed effect of EE_2 depends on the activation status of the lymphocyte, therefore pinpointing complexity of such studies. Indeed, the present results show that two genes were expressed differently with or without LPS. Two genes reacted similarly with or without LPS. One gene was downregulated with EE_2 alone, while two genes were downregulated and three upregulated with EE_2+LPS. Correlations of gene expression changes between genes further indicate the complexity of immunotoxic mechanisms and the need for in depth studies elucidating mechanisms of toxic action. We estimate that gene expression analysis is a useful tool in marine mammal immunotoxicological research that should be further developed and used.

Research highlights

- First study to assess effect of EE_2 on gene expression profiles in harbor seal leukocytes.

Table 3. Spearman correlation coefficient hemi-matrix for inter-relationships of genes of interest. Significant correlations (p < 0.05 *, p < 0.01 **, p < 0.001 ***, p < 0.0001 **) are in bold.**

		AHR	CD9	HSP70	IFNg	LYN1	MAPKK3	SLAM	TGFb1
AHR	r	1.000							
	p	<0.0001					Strength of correlation		
CD9	r	.335	1.000				r .00 – .19 "very weak"		
	p	.204	<0.0001				r .20 – .39 "weak"		
							r .40 – .59 "moderate		
HSP70	r	.606	-.359	1.000			r .60 – .79 "strong"		
	p	.015 *	.173	<0.0001			r .80 – 1.0 "very strong"		
IFNγ	r	.606	-.015	.743	1.000				
	p	.015 *	.961	.0002 ***	<0.0001				
LYN1	r	.809	-.462	.379	.326	1.000			
	p	.0003 ***	.074	.148	.217	<0.0001			
MAPKK3	r	.337	-.593	.041	-.132	.546	1.000		
	p	.201	.017 *	.880	.619	.031 *	<0.0001		
SLAM	r	-.483	.350	-.403	-.143	-.680	-.568	1.000	
	p	.059	.183	.121	.592	.005 **	.023 *	<0.0001	
TGFß1	r	.236	.003	-.336	-.339	.483	.519	-.158	1.000
	p	.377	.994	.200	.196	.060	.041 *	.549	<0.0001

- Development and validation of mRNA primers for harbor seal.
- Gene expression of selected sequences varied in a dose-response depended manner.

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