

Environmental toxin 4-nonylphenol and autoimmune diseases: using DNA microarray to examine genetic markers of cytokine expression

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Abstract

Introduction: Adverse progression of autoimmune diseases is linked to the dysregulation of cytokines. In this regard we investigated the role of 4-nonylphenol (4-NP), as a potential contributing factor in the development of immune diseases and compared it to estrogens actions since 4-NP may work via estrogen processes.

Material and methods: The study made cytokine level expression changes in U937 cells by microarray technology coupled to RT PCR as a validating technique.

Results: It was determined that 4-NP significantly up-regulated proinflammatory cytokine expression (toll-like-receptor [TLR]-6, TLR-10, interleukin [IL]-1, IL-5, IL-6, IL-17C, IL-23A, IL-8RB, IL-receptor-associated-kinase [IRAK-2], tumor-necrosis-factor-receptor [TNFR]-5, and TNFR-10). Estrogen caused insignificant increases but the changes paralleled that of 4-NP. Simultaneously, 4-NP down-regulated the expression of anti-inflammatory cytokines (IL-4 and IL-10), while estrogen up-regulated them.

Conclusions: 4-Nonylphenol may initiate its toxic effects and pose a risk to autoimmunity-prone individuals by eliciting effects up to 4 times more potent than estrogen. Overall, exposure to 4-NP may contribute to autoimmune susceptibility and/or exacerbate existing autoimmune conditions by dysregulating normal expression of cytokines.

Key words: 4-nonylphenol (4-NP), estrogen, interleukins, cytokines, proinflammatory, environmental toxins.

Introduction

Autoimmune diseases represent the 3rd most common major illnesses in the United States. Although there are 80 different forms of known autoimmune diseases, all are characterized by a “confused” immune response that self-attacks its own cells and organs. Currently, around 23.5 million Americans (1 in 12) are affected by at least one autoimmune disease, 78% of whom are women, suggesting an estrogen involvement.

Estrogen, a major gonadal steroid that serves as a naturally occurring chemical messenger, has been shown to directly regulate the immune response, due to the presence of estrogen receptors on lymphocytes [1]. Thus, environmental factors that affect estrogen-related processes in the body may also play consequential roles in eliciting autoimmune disease [2-5]. Accordingly, evidence suggests that recently developed and now

ubiquitous industrial estrogens are possible contributors to autoimmune risk [6-9].

Xenoestrogens are man-made, endocrine-disrupting chemicals that specifically mimic estrogen-regulated processes in the body. Through environmental exposures, they alter an organism's ability to maintain homeostasis, especially those controlled by estrogen [10]. One of the fastest growing xenoestrogens worldwide is 4-nonylphenol (4-NP) [11]. 4-Nonylphenol is the biodegradation product of nonylphenol ethoxylates (NPEOs) [12]. Since NPEOs are nonionic surfactants, they are typically used to manufacture domestic liquid detergents, cosmetics, industrial cleaners, paints, and pesticides [13, 14]. After 4-NP exits wastewater plants via sewage effluents, humans are exposed to 4-NP by drinking contaminated water, breathing contaminated air, contacting tainted sediments, and/or ingesting contaminated food, plants and animals [15-18].

Proinflammatory cytokines, while initially protective, may act to worsen disease, while anti-inflammatory cytokines serve to reduce inflammation and promote healing [19]. Autoimmune diseases are triggered by a dysregulation of cytokines – *too many* proinflammatory and *too few* anti-inflammatory cytokines are expressed, causing the immune system to function uncontrollably [20, 21]. Interestingly, in 2004, Lee *et al.* demonstrated that 4-octylphenol (OP), a different xenoestrogen, significantly *inhibited* the activation of the anti-inflammatory interleukin-4 (IL-4) gene in mouse T-cells [22]. Yano *et al.* (2003) reported similar results by showing that OP inhibited the production of IL-4 in mouse spleen cells [23]. This suggests that OP, as well as other xenoestrogens, may play a negative role in the immune system by inhibiting the production of anti-inflammatory, pro-healing cytokines. In the present study we investigated the effects of 4-NP and estrogen on proinflammatory *and* anti-inflammatory cytokine gene expression using the well established human lymphocytic cell line U937 as an *in vitro* cell model. We demonstrate that exposure to 4-NP promotes proinflammatory cytokine gene expression as an estrogen mimic, but with considerably greater potency than estrogen would. Thus, 4-NP modulation of proinflammatory cytokine gene expression in lymphocytes may dysregulate the immune system to increase susceptibility to autoimmune disease and/or exacerbate existing autoimmune conditions.

Material and methods

Microarray analysis

Cell culturing

U937 histiocytic lymphocytes (ATCC, USA) were cultured in RPMI1640 media (Invitrogen, USA) in

six-well plates. U937 cells mature into lymphocytes when subjected to immune stress. The U937 cell line was selected based on its ability to be estrogen-responsive *and* express both proinflammatory and anti-inflammatory cytokine genes [24]. Cells were kept in a 37°C incubator (NAPCO) in 5% CO₂/95% air. Experimental procedures were performed under a sterile, laminar airflow hood. Cells were divided into three groups and treated accordingly: 1) control (left untreated), 2) 5 micromolar (µM) estrogen (E2), and 3) 5 µM 4-NP. These concentrations were chosen in order to compare the effects of naturally occurring estrogen to two different concentrations (high vs. low) of 4-NP. The estrogen and 4-NP solutes were obtained from Sigma-Aldrich (St. Louis, MO) and then diluted in methanol.

Isolation of total mRNA

After 48 h incubation, the cells were detached from the plate, transferred to a 15 ml tube, and pelleted via centrifugation. Pelleted cells were homogenized with RLT buffer by passing the cell lysate through a 1000 µl pipette tip. RNA from the cells was then isolated using the RNeasy Protect Mini Kit (Qiagen, Stanford, CA) and by following manufacturer's protocol. RNA was eluted with 50 µl of RNase-DNase-free water by centrifugation for 1 min at 10,000 rpm.

Applied Biosystems Expression Microarray Analysis

DNA microarray is a state of the art technique that allows scientists to examine the effect of any environmental agent on tens of thousands of gene expressions at once. The quality of the RNA was analyzed on a model 2100 bioanalyzer (Agilent, Santa Clara, CA) using a total RNA nanochip following the manufacturer's protocol. Then, using the Applied Biosystems Chemiluminescent RT-IVT Labeling Kit version 2.0 and manufacturer's protocol, Digoxigenin-UTP labeled cRNA was generated and linearly amplified from 1 µg of total mRNA. Three chips, one for each group, each used 15 µg of labeled cRNA and were hybridized at 55°C for 19 h using the Applied Biosystems Chemiluminescence Detection Kit and Applied Biosystems 1700 Chemiluminescent Microarray Analyzer following the manufacturer's protocol. Following hybridization, Applied Biosystems 1700 Expression System software was utilized to extract chemiluminescence values from each of the three microarray images. Experiment microarray showed that U937 cells expressed 14,913 genes (out of 33,155 possible genes).

Data from the four microarray whole-genome chips was analyzed using the Spotfire software from TIBCO (Palo Alto, CA). The fold changes were

measured by dividing the signals of the three variables by the control signal. The control signal served as the normalizing baseline for each of the other three chips. A fold change of +3 or more was considered significant dysregulation. Data was graphically represented via Excel.

Semi-quantitative reverse transcription-polymerase chain reaction

In order to support accuracy of the data of the microarray analysis, a conventional reverse transcription-polymerase chain reaction (RT-PCR) was performed for the interleukin-(IL)-8R β gene. This represents a conventional validation step. The IL-8R β gene was selected via random selection to ensure an un-biased decision. Primer sequences utilized were: 5' CCA GCC TGC TAT GAG GAC AT-3' (IL-8RB forward), and 5'GAA TCT CGG TGG CAT CCA GAG-3' (IL-8R β reverse). An RT-PCR was also performed for the β -actin gene which served as a reference gene to ensure accuracy of the RT-PCR and to normalize the results for the variable treatments. The primer sequences utilized were: 5' TGA CGG GGT CAC CCA CAC TGT GCC CAT CTA-3' (β -actin forward), and 5' CTA GAA GCA TTG CGG TGG ACG ATG GAG GG-3' (β -actin reverse).

Some RNA that was collected to run the microarray was saved and utilized in this RT-PCR. Total RNA concentration was standardized for RT using a spectrophotometer. Using Invitrogen reagents and manufacturer's protocol, the RT and PCR were carried out in a GeneAmp Thermocycler PCR System 9700 (PE. Applied Biosystems). The PCR products were then run on a 2% Agarose gel stained with ethidium bromide (Sigma-Aldrich) in Tris-Borate-EDTA buffer (TBE). The gel was then analyzed using a UV Transilluminator (UVP) and GelPro Analyzer Software (Media Cybernetics, Silver Spring, MD).

Lower exposures to 4-nonylphenol

Cell culturing/RNA isolation/reverse transcription-polymerase chain reaction

A six-well plate of U937 histiocytic lymphocytes was cultured in RPMI1640 media. Cells were divided into six groups, and treated accordingly: 1) control (left untreated), 2) 1 nanomolar (nM) 4-NP, 3) 3 nM 4-NP, 4) 5 nM 4-NP, 5) 10 nM 4-NP, and 6) 50 nM 4-NP. Cells were incubated for 48 h. Total RNA was isolated following above-mentioned protocol. An RT-PCR was run to analyze the effect of these extremely low, nanomolar-level concentrations of 4-NP on interleukin (IL)-8R β expression. An RT-PCR was also performed for β -actin, which served as a reference gene and normalizer for the variables. The PCR products were run on a 2% agarose gel

stained with ethidium bromide, and analyzed via GelPro Analyzer software.

Statistical analysis

Statistical analysis for the microarray trials ($n = 2$ for each gene) and the conventional RT-PCRs ($n = 3$ each) was done using an ANOVA test followed by a post-HOC Sheffe test. Values of $p < 0.05$ were considered significant.

Reagents

PCR reagents (10 \times buffer, 50 μ M MgCl₂, TAQ polymerase, custom primers) and RT reagents (Superscript enzyme, 5 \times First Strand Buffer, 0.1 M dithiothreitol (DTT), Random Primers) were purchased from Invitrogen, USA, and stored at -20° C. Phosphate-buffered saline (PBS) was also purchased from Invitrogen, USA, and stored at room temperature. Nucleotides (dNTPs) were purchased from Amershar Pharmacia Biotech, USA, and stored at -20° C. RNeasy RNA Isolation reagents and columns were purchased from Qiagen, USA. Electrophoresis-grade agarose was purchased from Fisher Biotech, USA, and was stored at room temperature. Ethidium bromide (EtBr), the gel stain, was purchased from Sigma-Aldrich, USA.

Results

U937 cells exposed to 4-NP showed an increased expression of all 123 DNA microarray-detected *pro inflammatory* cytokine genes. *Significant* up regulation ($p < 0.05$, ANOVA) was seen for: interleukin-1 (IL-1), IL-5, IL-6, IL-17C, IL-23 α , interleukin-8-receptor- β (IL-8R β), and interleukin-1-receptor-associated-kinase (IRAK2) (Figure 1). Microarray revealed that estrogen (E2) treated cells also displayed increased pro inflammatory cytokine gene expression, although insignificant compared to 4-NP's effects (Figure 1). Thus, the microarray data supports 4-NP's immunomodulatory role and also that it appears to be more toxic than naturally occurring estrogen, since it elicits effects up to 4 times greater than estrogen does.

Tumor necrosis factors are a class of pro-inflammatory cytokines that act via tumor-necrosis-factor-receptors (TNFRs) in influencing endothelial cells vascular permeability, which impacts infection sites (National Cancer Institute, 09). Autoimmune diseases have been characterized as an over-expression of TNFRs [25]. We demonstrate that 4-NP significantly up regulated the expression of TNFR-5, and TNFR-10 (Figure 2).

Toll-like receptors (TLRs) are proteins that recognize foreign structures associated with microbes and can activate immune cell responses and the production of various cytokines [26]. They usually facilitate beneficial localized inflammatory

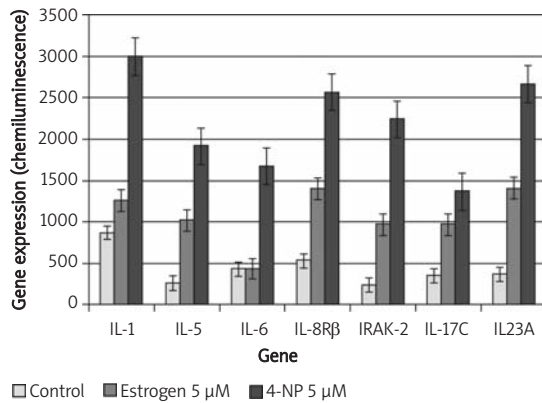


Figure 1. U937 cells treated with 5 μM 4-NP for 48 h showed significantly up regulated pro-inflammatory cytokine gene expressions, compared to control expressions, while estrogen (E2 5 μM) elicited insignificant effects. For IL-1, 4-NP caused a 3.451-fold up regulation compared to control, and 4-NP was 2.385 times more potent than E2. For IL-5, 4-NP caused a 7.291-fold up regulation compared to control, and 4-NP was 1.876 times more potent than E2. For IL-6, 4-NP caused a 5.587-fold up regulation compared to control, and 4-NP was 2.483 times more potent than E2. For IL-8RB, 4-NP caused a 4.808-fold up regulation compared to the control, and 4-NP was 1.832 times more potent than E2. For IRAK-2, 4-NP caused a 9.161-fold up regulation compared to control, and 4-NP was 2.305 times more potent than E2. For IL-17C, 4-NP caused a 3.856-fold up regulation compared to the control, and 4-NP was 1.402 times more potent than E2. For IL-23A, 4-NP caused a 7.185-fold up regulation compared to the control, and 4-NP was 1.892 times more potent than E2

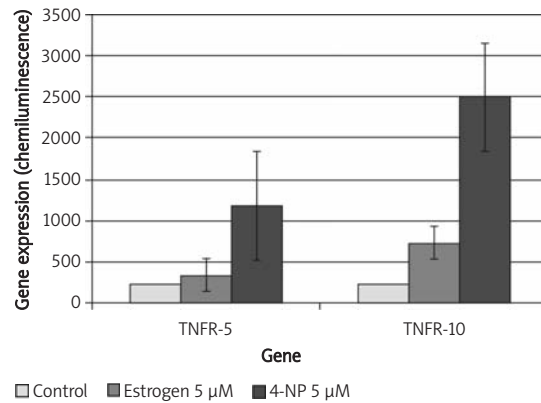


Figure 2. 4-Nonylphenol 5 μM caused significant up regulation of TNFR-5 and TNFR-10. For TNFR-5, 4-NP caused a 5.137-fold up regulation compared to the control, and 4-NP was 3.545 times more potent than E2. For TNFR-10, 4-NP caused a 10.992-fold up regulation compared to the control, and 4-NP was 3.433 times more potent than E2

increased the expression of these cytokine-signaling genes, 4-NP elicited a *greater* up regulation. These data further indicate that 4-NP could elicit more potent effects on pro inflammatory cytokine gene regulation than naturally occurring estrogen.

Additionally, 4-NP *reduced* the expression of 8 detected *anti-inflammatory* cytokine genes (Figure 4). Significant change (of at least a 3-fold change, $p < 0.05$ ANOVA) was observed with the 4-NP administration for the following cytokines: IL-4 and IL-10. Anti-inflammatory cytokine genes (i.e. IL-4) are considered pro-healing through their ability to suppress pro-inflammatory cytokine production (i.e. that of IL-1) while up regulating the expression of pro-inflammatory cytokine receptor antagonists (i.e. IL-1 receptor antagonists) [28]. Our data demonstrate that 4-NP may exacerbate autoimmunity by *suppressing* beneficial, pro-healing gene expression (Figure 4). Interestingly, E2 up regulated the same genes that 4-NP *down* regulated, suggesting that naturally occurring E2 may be beneficial in promoting a healthy presence of anti-inflammatory cytokines, while xenoestrogens may act to exacerbate disease by decreasing the expression of such pro-healing genes during immune stress.

In the “validation” RT-PCR, IL-8RB was up regulated in U937 cells (Figure 5). This data correlated to the up regulation of IL-8RB observed in the microarray experiments. An RT-PCR for the β-actin reference gene was used to normalize the results and ensure accuracy, which further supported our observations.

The Environmental Protection Agency (EPA, 2009) sets the environmental “safety” limit of 4-NP at 3.1 μg/l (or parts per billion). This is the equivalent of 3,100 nM. Since the microarrays examined the

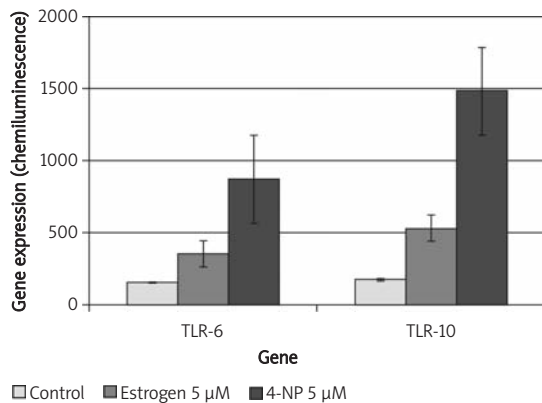


Figure 3. 4-Nonylphenol 5 μM caused significant up regulation of TLR-6 and TLR-10. For TLR-6, 4-NP caused a 5.587-fold up regulation compared to the control, and 4-NP was 2.483 times more potent than E2. For TLR-10, 4-NP caused an 8.760-fold up regulation compared to the control, and 4-NP was 2.802 times more potent than E2

responses, but *improper* activation could lead to over-production of pro-inflammatory cytokines and possibly autoimmune diseases [27]. Out of the 9 detected TLRs, 4-NP most significantly up regulated TLR-6 and TLR-10 (Figure 3). Although E2 also

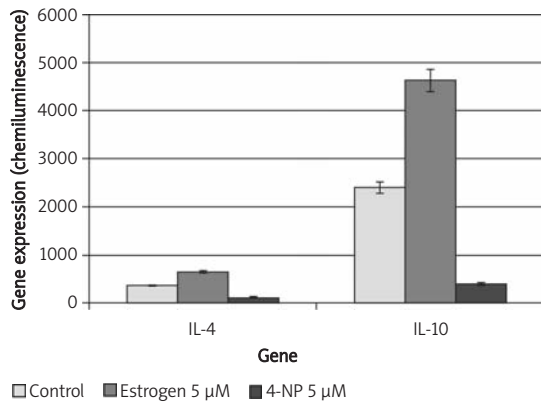


Figure 4. At the same time, 4-NP 5 μM caused significant down-regulation of anti-inflammatory cytokine genes – IL-4 and IL-10. For IL-4, 4-NP caused a significant 3.680-fold down regulation compared to control, while E2 caused an insignificant 1.176 fold up regulation compared to the control. For IL-10, 4-NP caused a significant 5.903-fold down regulation compared to the control, while E2 caused an insignificant 1.923 fold up regulation compared to the control

effect of 4-NP at relatively high, micromolar-level concentrations, an experiment was run to examine whether 4-NP could elicit effects at even lower, nanomolar (nM), concentrations. The data in Figure 6, however, shows that 4-NP is capable of eliciting effects on the immune system at 1 nM, although these effects are not statistically significant. Indeed, an up regulation of IL-8Rβ was seen even at the lowest observed concentration of 4-NP, 1 nM. This may suggest that 4-NP is capable of altering human immunity, a speculation that requires greater sample size.

Discussion

This report demonstrates that the ubiquitous environmental toxin 4-NP may be a contributing factor in the development of immune-type disease as noted by its enhancement of proinflammatory cytokine gene expression in U937 cells. In this regard it exhibits estrogenic properties. Specifically, 4-NP induced a 3.451-fold up regulation of IL-1 as compared to the controls. 4-Nonylphenol also caused a 9.161-fold up regulation of the expression of IRAK-2. Since IL-1 is an important regulator of lymphocyte differentiation and the inflammatory response, an abnormal up regulation of IL-1 and/or IRAK-2 may contribute to increased inflammation. 4-NP elicited a 7.291-fold up regulation of IL-5, stimulator of β cell growth, compared to the control. 4-Nonylphenol also induced a significant increase in the expression of IL-6, IL-8Rβ, IL-17C, and IL-23A (Figure 1). Additionally, the TNFRs were also significantly up regulated by 4-NP. Since

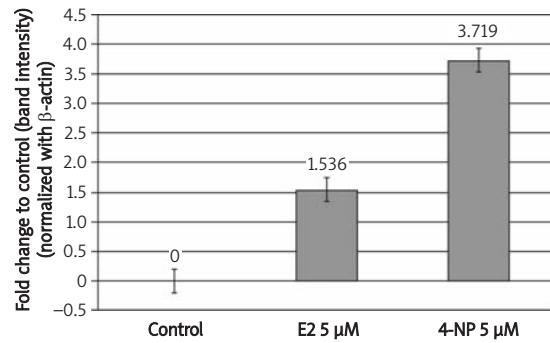


Figure 5. This figure depicts the results from the conventional RT-PCR for the IL-8Rβ gene, which served as the validation for our microarray. Fold change was obtained by dividing the photometric measure (determined by GelPro Analysis software) of each variable by the control signal. These figures were then normalized with the results of the β-actin signals. While E2 5 μM caused an insignificant up regulation of IL-8Rβ 1.536 times greater than control expression, 4-NP 5 μM caused a significant 2.555-fold up regulation compared to the control. These data correlate to the up-regulation of IL-8Rβ seen in the microarray data, thus we demonstrate that all other microarray data is accurate

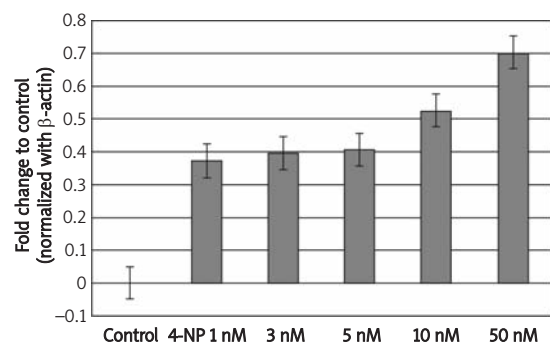


Figure 6. This graph depicts the effect of 4-NP at extremely low, nanomolar concentrations on IL-8Rβ gene expression. 4-NP caused a concentration-dependent up regulation of IL-8Rβ. Fold change was normalized with the results of the β-actin signals. At 1 nM, 4-NP caused a 0.371 fold up regulation. At 3 nM, there was a 0.395 fold up regulation. At 5 nM, there was a 0.405 fold up regulation. At 10 nM, there was a 0.526 fold up regulation. At 50 nM, there was a 0.702 fold up regulation

autoimmune diseases can be characterized by over-expression of TNFRs and TLRs [25-27], this data supports 4-NP as a contributor to increasing autoimmune disease risk.

It was also demonstrated that estrogen (E2) elicited a similar up regulation of these same pro inflammatory cytokine genes but to a lesser degree than 4-NP. 4-Nonylphenol up regulated IL-1 expression 2.385 times more than E2 did and 4-NP was 1.876 times more potent in up regulating IL-5 than E2. This trend continues for IL-6, IL-8Rβ,

IRAK-2, IL-17C, and IL-23A, where 4-NP elicits an up regulation 2.483, 2.802, 1.832, 2.305, 1.402, and 1.892 times greater than E2, respectively. This supports the hypothesis that 4-NP is more toxic than naturally occurring estrogen in regard to disturbing the immune response, because 4-NP elicits a more potent dysregulation of the proinflammatory cytokine gene expression.

Importantly, 4-NP was also shown to significantly down regulate the expression of beneficial, pro-healing, anti-inflammatory cytokine gene expression. 4-Nonylphenol elicited a 3.477-fold down regulation of IL-4. Interleukin-4 has been shown to suppress IL-1 production [26]. Thus, 4-NP may increase immune malfunction by decreasing the expression of the beneficial IL-4 gene that acts to reduce harmful, proinflammatory IL-1 production. 4-Nonylphenol also caused a 5.903 fold down regulation of the anti-inflammatory cytokine, IL-10. This gene is known to counteract inflammatory responses and is also capable of suppressing the synthesis of certain pro-inflammatory cytokines [29]. Therefore, a down regulation of this beneficial gene may lead to an increased synthesis of proinflammatory cytokines, contributing to increased autoimmune risk.

Estrogen's effect on the same anti-inflammatory cytokine genes is opposite to the effect of 4-NP. Although there is no significant up regulation by estrogen, the data is thought provoking, suggesting that industrial estrogens like 4-NP may play a more adverse role in the risk of developing immune disease than naturally occurring estrogens.

In conclusion, since the EPA sets a "safe" environmental exposure limit of 4-NP at 3.1 µg/l = 3.1 parts per billion our results in tissue culture reveal that this level have to be adjusted. For example, although the up regulation of proinflammatory IL-8Rβ with the various 4-NP administrations ranging from 1 nM ~ 50 nM is insignificant ($p > 0.05$ ANOVA, $n = 3$), it is still clear that at concentrations far below the EPA's "safe" limit, 4-NP can still up regulate the proinflammatory cytokine gene.

The current project demonstrates the following: 1) 4-NP significantly up regulates proinflammatory cytokine gene expression in U937 cells, 2) estrogen causes a similar but less marked increase in proinflammatory cytokine gene expression, 3) 4-NP may pose greater risk to the immune-prone individuals by eliciting effects up to 4 times greater than estrogen, 4) 4-NP disturbs cytokine gene expression at concentrations far below the EPA limit.

It is interesting that E2 can have varied effects, some beneficial and others detrimental [30-33]. The negative effects may be linked to abnormal regulation of nitric oxide production, release and/or

degradation. A similar story exists for endogenous morphine and its related phenomena, for example, tolerance and addiction [34-40]. In considering this hypothesis one is drawn to the ability of constitutive nitric oxide to modulate mitochondrial function [36, 41]. The full development of this hypothesis is presently under our focus.

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