Effects of Perfluorinated Compounds on GnRH Gene Expression in vitro

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Perfluorinated Chemicals: What are they?

• Fluorine-saturated hydrocarbons of variable chain length
  – May be substituted with various functional groups
    • Hydroxyl, carbonyl, carboxylic acid, etc.

• Commonly used in consumer products
  – Teflon, ScotchGard, plastics, cosmetics, paper food wrappers, popcorn bags, flame retardants, cosmetics…

• No naturally occurring perfluoro chemicals exist
  – All are man-made
Physico-chemical Properties

- Biological metabolites of fluorotelomel alcohol
- Incredibly stable fluorine-saturated carbon compounds
  - Electronegativity of fluorine strengthens C-C bonds and lends strength to C-F bonds
- Immiscible in water and organic solvents
- Become amphipathic with addition of hydrophilic substituents
  - Difficult to determine compartmentalization in the body
OK... So what?

- Ubiquitous in world water supplies
  - Found in North Pacific and Arctic Oceans

- Classed as persistent organic pollutants
  - Capable of atmospheric transport
  - Resistant to degradation by:
    - Photolysis (direct and indirect)
    - Microbial degradation
    - Biological degradation/detoxification

- Tendency to bioaccumulate and biomagnify
  - Higher concentrations in the liver and serum of predatory animals
High rates of human exposure

- Experimentally confirmed to off-gas from non-stick cookware at normal cooking temperatures (Anyone use Teflon?)
- Frequent ingestion: drinking water, paper food wrappers, treated textiles...
- Epidemiological studies have approximated general population serum concentrations
  - 33.1 ng/mL PFOS
  - 4.5 ng/mL PFOA
- Children generally carry 5-10x higher chemical load
  - in utero exposure
  - Breast milk primary source of nutrition
  - Closer proximity to treated carpets, textiles
Biological Endpoints

• Immunotoxicity
  – Cell cycle arrest and apoptosis in spleen and thymus
  – Immunoglobulin reductions (IgM, IgY)
  – Suppression of lysozyme activity
  – Disruption of innate and adaptive immune response

• Developmental toxicity
  – Decreased body weight following in utero exposure
  – Increased neonatal mortality
  – Altered nutritional status
  – Brain asymmetry
  – Delayed sexual maturity
  – Adrenal and hepatocellular hypertrophy
Oh, wait... there's more!

• Neurotoxicity
  – Decreased DNA synthesis
  – Disruptions in neuronal differentiation
    • Decreased dopamine phenotype
    • Increase acetylcholine phenotype

• Endocrine disruption
  – Decreased free thyroid hormone
  – Decreased testosterone synthesis
  – Elevated estradiol
  – Estrus cycle interruption
  – Estrogenic activity
    • Induction of oocyte formation in dosed males
    • No action on ER-α or -β
Reproductive Toxicity

- Rats dosed with PFOS
  - 0, 1, or 10 mg/kg body weight x2 weeks
  - Persistent diestrus in 58% of high dose group
  - Irregular cycles or diestrus observed in 34% of low dose group

Austin et al., *Environmental Health Perspectives*, September 2003
Hypothalamo-pituitary-gonadal Axis Regulation

• SCN stimulates release of GnRH
• GnRH tells pituitary to secrete gonadotropins — FSH and LH
• FSH and LH stimulate androgen synthesis and secretion from the gonads
• Circulating estrogen reaches the hypothalamus, turns off GnRH signal
Mammalian Reproductive Signaling

Figure 3. Metestrus

SCN → DNS → GnRH
Mammalian Reproductive Signaling

SCN → DNS → GnRH

Figure 4. Proestrus
Generation of GT1-7 GnRH Immortalized Neurons

Immortalized GnRH neurons derived by targeted tumorigenesis

3kb GnRH regulatory region  SV40 T antigen

Tumorigenic mouse expresses oncogene in hypothalamic neurons

Tumors are isolated and tumor cells cultured

Clonal hypothalamic neuronal cell lines
Generation of GnRH-MetLuc Subclone

- *Metridia longa* luciferase vector inserted into pBSK
  - *HincII*
- Firefly luciferase gene excised from GnRH-Fluc vector
  - *Xho* & *Xba*
- MetLuc excised from pBSK
  - *Xho* & *Xba*
- MetLuc inserted behind GnRH promoter
  - *Xho* & *Xba*
So, what’s luciferase good for?

- Secreteable reporter
- Light emitted from media after addition of reagent quantifies gene expression rates
- Superior method
  - No need to lyse cells
  - Measurements can be taken from same cell population
Initial testing of PFC activity on GnRH gene expression
Fold-increase of normalized PFOA and PFOS treatments over controls

- **Fold-increase over EtOH vehicle normalized values**
  - 10μM PFOA
  - 20μM PFOA
  - 10μM PFOS
  - 20μM PFOS

- **Fold-increase over estradiol control normalized values**
  - 10μM PFOA
  - 20μM PFOA
  - 10μM PFOS
  - 20μM PFOS
Net change of GnRH gene expression in cultures treated with PFOA and PFOS as compared to EtOH
Dose-response of GnRH expression following treatment of cell cultures with PFOA and PFOS
Dose-response curves generated for PFOA and PFOS treated GT1-7 cells.
Percent change in number of cells with DNA nicks/entering apoptosis vs DMSO control
## TUNEL data vs. dose response

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Fold-change over DMSO control</th>
<th>% cells in apoptotic phase compared to DMSO</th>
<th>Dose-response determined effect on GnRH gene expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMSO</td>
<td>1</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>100mM PFOA</td>
<td>1.314</td>
<td>31.4</td>
<td>Very significant down-regulation</td>
</tr>
<tr>
<td>1mM PFOA</td>
<td>1.417</td>
<td>41.7</td>
<td>Very significant up-regulation</td>
</tr>
<tr>
<td>100μM PFOA</td>
<td>0.906</td>
<td>-8.4</td>
<td>No statistically significant changes</td>
</tr>
<tr>
<td>1μM PFOA</td>
<td>1.162</td>
<td>16.2</td>
<td>Significant up-regulation</td>
</tr>
<tr>
<td>100nM PFOA</td>
<td>1.186</td>
<td>18.6</td>
<td>No statistically significant changes</td>
</tr>
<tr>
<td>50μM PFOS</td>
<td>1.073</td>
<td>7.3</td>
<td>Very significant down-regulation</td>
</tr>
<tr>
<td>500nM PFOS</td>
<td>0.990</td>
<td>-1</td>
<td>No statistically significant changes</td>
</tr>
<tr>
<td>5nM PFOS</td>
<td>0.954</td>
<td>-4.6</td>
<td>Significant up-regulation</td>
</tr>
<tr>
<td>500pM PFOS</td>
<td>0.623</td>
<td>-37.7</td>
<td>Significant up-regulation</td>
</tr>
<tr>
<td>5pM PFOS</td>
<td>0.796</td>
<td>-20.4</td>
<td>Very significant up-regulation</td>
</tr>
</tbody>
</table>
What does it all mean?

• 1 mM and 1 μM concentrations of PFOA induced up-regulation of GnRH in conjunction with higher rates of DNA nicks
  – Cells producing much more GnRH as a result of PFOA treatment

• 100 mM PFOA treated cells demonstrated strong down-regulation of GnRH expression with high rates of DNA nicks
  – Possible correlation between rates of cell death and GnRH transcription

• Low and mid-range doses of PFOA elicited no change compared to DMSO control
What does it all mean?

• 50μM PFOS treatment caused significant down-regulation of GnRH gene expression with negligible cell death
  – Presumably inhibits gene transcription
• 5nM, 500pM, and 5pM PFOS all elicited significant up-regulation of GnRH gene expression with lower rates of apoptosis in culture
  – Protective effect?
  – More GnRH because comparatively more cells present?
Some questions to ask...

- If perfluoro chemicals are capable of disregulating GnRH gene expression, are the concentrations found in the general population adequate to elicit effects?
  - 33.1 ng/mL PFOS is equivalent to 66.1nM
    - significant up-regulation observed at much lower doses
    - Flanking doses elicited very different effects
      - 500nM dose saw no changes in GnRH gene expression or cell death
      - 5nM dose saw negligible apoptosis with significant up-regulation of GnRH gene expression
  - 4.5 ng/mL PFOA is equivalent to 10.9nM
    - Closest dose tested was 100nM; no changes in GnRH gene expression observed, but notable increase in cell death (approximately 20%)
Some questions to ask...

• Do those cells that demonstrated up-regulation of GnRH transcription maintain their secretory capacity?
  – More expression
  – More protein product
  – More secretion?
And some more questions to ask...

• Experimental data shows preferential formation of ACh phenotype in PC12 cell models
  – ACh has both stimulatory and inhibitory effects on GnRH neurons (Krsmanovic et al.)
    • Possibly via action on different cholaminergic receptors
  – How would this effect GnRH gene expression in vivo?
...and a few more...

• If estrogen receptors \( \alpha \) and \( \beta \) are non-responsive to perfluoro chemicals, what mechanisms lie behind GnRH gene disregulation? (Ishisbashi et al.)
  – Studies conducted in yeast
    • Appropriate model?
    • Equivalent receptor to humans, or just a homolog?
Where to next, Captain?

- Secretion rates indeterminable via tests conducted
  - Radioimmunoassay
- In vivo studies to check for biological consequences of GnRH disregulation
  - Polycystic ovaries
  - Precocious puberty
  - Fertility issues
  - Disrupted estrus cycling (Remember the Austin study?)

Austin et al., *Environmental Health Perspectives*, September 2003
Where to next, Captain?

• Other estrogen-responsive genes contribute to estrus cycles and time-keeping
  – Daily neuronal signals from master clock in SCN stimulate GnRH neurons
  – BMAL/MOP3
  – Mechanisms still under investigation
  – Preliminary data in RORE-MetLuc cells indicative of disregulation

![Graph showing fold-increase over DMSO vehicle normalized values]
Questions?