Keynote Address

Integrating Novel Approaches to Pollution Research in Terrestrial and Marine Environments

British Society of Soil Science
&
Society of Environmental Toxicology and Chemistry-UK Branch
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Endocrine Disruptor Mechanisms: Beyond Receptor Binding

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ENDOCRINE DISRUPTION

“...an exogenous agent that interferes with the synthesis, secretion, transport, binding, action, or elimination of natural hormones in the body that are responsible for the maintenance of homeostasis, reproduction, development, and/or behavior.”

Kavlock et al., 1996. Research needs for the assessment of environmental effects of endocrine disruptors: a report of the USEPA-sponsored workshop

Zoology Dept. & National Food Safety and Toxicology Center
Michigan State University
Maintaining Homeostasis is a complex process

- Involves many signal transduction pathways
- Many alternative pathways
- Many enzymes involved
- Rate limiting steps unknown
- Cybernetic feedback loops
Hypothalamic-Pituitary-Gonadal Axis

- Hypothalamus
  - GnRH, CRH
  - ACTH
  - (Steroidogenesis)

- Pituitary
  - GnTH, LH, FSH,

- Gonads
  - DHT, T, E2

- Adrenals
Mechanisms of Endocrine Disruption

Endocrine disrupting chemicals

• At level of receptors
  – estrogen, androgen, glucocorticoid etc.

• At level of enzymes
  – induction and/or inhibition of catalytic activity
  – induction and/or inhibition of enzyme expression
ENDOCRINE DISRUPTION

• Alteration of Precursors and Substrate Pools
• Cascade Effects - General Adaptation Syndrome
• Tissue Lesions that Damage Structure and Function - Key target Endocrine glands
ENDOCRINE DISRUPTION

- The endocrine system maintains homeostasis
- Thus, anything that impairs the ability to maintain homeostasis, is by definition an endocrine disrupter
- All stresses are potentially endocrine disrupters
- General Adaptation Syndrome
- No special treatment necessary
  - All usual techniques, assumptions & uncertainties apply
ENDOCRINE DISRUPTION

• Direct - (Mimics)
  – Agonists
  – Antagonists
  – Partial Agonists

• Environmental estrogens are only one mimic

• Indirect
  • Induction of Enzymes that Directly or Indirectly Affect Hormone Concentrations

• Alteration of signal transduction pathways
Mechanism of Action for ER-Activation

**Estrogen or xenoestrogen**

**Protein Phosphorylation of ER**

**Ligand-Independent Activation**

**DNA Binding**

**mRNA**

**ER-Responsive Genes**

**Estrogenic Effects**
Endocrine Disruption

• In the United States current attention is focused primarily on compounds that can affect steroid hormones

• Reauthorization of the clean Water Act

• Food Safety Protection Act

• Endocrine Disrupter Screening and Testing Committee (EDSTAC)
  – Estrogen receptor (ER)
  – Androgen Receptor (AR)
  – Thyroid Receptor (TR)
U.S. Environmental Protection Agency
Endocrine Disruptor Screening Program
http://www.epa.gov/oscpmont/oscpendo/index.htm

Initial Sorting
↓
Priority Setting
↓
Tier 1 Screening
↓
Tier 2 Testing
Endocrine Disruptor Screening Program

Tier 1 Screening
- detect chemical substances capable of interacting with the estrogen, androgen, and thyroid hormonal systems
- combination of *in vitro* and *in vivo* assays
- weight of evidence approach will be used to determine if substance warrants further testing or placed in “HOLD” box

Tier 2 Testing
- designed to determine if substance has effects similar to naturally occurring hormones
- designed as a battery of *in vivo* testing that encompass critical life stages and processes, a broad range of doses, and administration by a relevant route of exposure
- data will be used as the basis of the dose response characterization for risk assessment purposes
Proposed Tier 1 Screening Battery

• **In Vitro Screens**
  – ER Binding / Reporter Gene Assay*
  – AR Binding / Reporter Gene Assay*
  – Steroidogenesis Assay with minced testis

• **In Vivo Screens**
  – Rodent 3-day Uterotrophic Assay (sc)
  – Rodent 20-day Pubertal Female Assay with Thyroid
  – Rodent 5-7 day Hershberger Assay
  – Frog Metamorphosis Assay
  – Fish Reproduction Screening Assay

* These assays are in the HTPS

Alternate assays have also been proposed
Proposed TIER 2 Testing Battery

• multigenerational reproduction and development studies in:
  – rodents
  – birds
  – frogs
  – fish
  – shrimp
Limitations of Screening Methods

• If used in a sequential decision process
  – False negatives
  – If negative in the binding assay, may still be positive as an endocrine disrupting compound
The proposed sequential testing provides useful information for designing additional testing, but does not allow for a sorting of compounds and does not assist in prioritizing compounds for additional testing.
Assessment of Exogenous Hormone Agonists I

- Concentration at Target Tissue
- Turnover Time in Tissue
- Available Fraction - Bound vs. Free
- Metabolism - Activation / deactivation
- Binding to receptor - Strength of Affinity
- Relative Potency - Compared to model compounds
Assessment of Exogenous Hormone Agonists II

• Exposures - Ambient Environment
  – Dietary Dose
  – Tissue Dose - Most Proximal

• Calibration of *in vitro* with *in vivo* Assays
Assessment of Exogenous Hormone Agonists III

• Compare Concentrations of Exogenous Agonist to Model compound

• Duration and Intensity of Exposure
  – half time or turnover rate
  – Concentration * Available fraction * relative potency

• Sensitivy Life Stages - Responsiveness

• Comparator = I*D
Example: Triazine Herbicides

Do not bind to ER!

Results in estrogenic effect *in vitro*

**In vitro Model System**

- Needed a flexible assay system that would allow for rapid studies of mechanisms of action
- Needed to express major enzyme systems
- Needed to be stable so results are reproducible
H295R cell line - I

• Human female adrenocortical carcinoma

• Produces many steroid hormones
  – gluco- & mineralo-corticoids, progestins, androgens & estrogens

• Expresses (inducible) steroidogenic cytochrome P450 (CYP) enzymes
  – CYP11A, CYP11B, CYP17, CYP19, CYP21
H295 & H295R Cells - II

- Human adrenocorticoid carcinoma cells
- H295R is a sub-population that forms monolayers in culture
- Physiologically have the characteristics of zonally undifferentiated human fetal adrenal cells
- Express most of the important steroid synthetic enzymes
H295 & H295R Cells - III

- The cells maintain the capacity to synthesize most of the steroid hormones characteristic of three phenotypically distinct zones of the adult adrenal cortex
  - Zona glomerulosa
  - Zona fasciculata
  - Zona reticularis
Key Enzymes

• **CYP11A** (Cholesterol side chain cleavage)
• **CYP11B1** (Steroid 11\(-\)hydroxylase)
• **CYP11B2** (Aldosterone synthetase)
• **CYP17** (Steroid 17-hydroxylase
  – also 17,20 lyase)
• **CYP19** (Aromatase)
• **CYP21B2** (Steroid 21-hydroxylase)
Steroidogenesis

- Cholesterol → Pregnenolone → 3β-hydroxysteroid dehydrogenase → Progesterone
  - 17α-hydroxylase → Glucocorticoids
  - 17-20 lyase → Androgens
  - c18-hydroxylase → Mineralocorticoids
- Progesterone → 17β-hydroxysteroid dehydrogenase → Testosterone
  - aromatase → Estradiol
- Testosterone → 5α-reductase → Dihydrotestosterone
Effects on steroidogenic enzymes

• At level of expression
  – measure mRNA levels: RT-PCR

• Effects on enzyme concentrations
  – measure catalytic activities: selective substrates

• Effects on metabolism of steroid hormones
  – measure steroid hormone concentrations
Assay for CYP19 (aromatase) activity

- ANDROGENS
  - Androstenedione
  - Testosterone
- ESTROGENS
  - Estrone
  - Estriol
  - 17β-Estradiol

CYP19 (aromatase) catalyzes the conversion of androgens to estrogens. The process involves the release of tritium and the measurement of radioactivity.
Induction of CYP19 mRNA by triazines

Amplification response ratio of CYP19/beta-actin (% of control ratio)

Control  30 µM  100 µM
DMSO  Atrazine  Simazine  Propazine  8Br-cAMP

Zoology Dept. & National Food Safety and Toxicology Center
Michigan State University
Proposed Mechanisms of Action for Triazines

- Aromatase induction / promotion
  - via protein kinase A pathway
  - via steroidogenic pathways
- Inhibition of phosphodiesterase
- Results in less conversion of c-AMP to AMP so that AMP increases
- c-AMP increases signal transduction of Protein Kinase A
- Protein kinase A increases CREB and SF-1
- Aromatase m-RNA is up-regulated such that more aromatase is formed and aromatase activity increases
Protein Kinase A Signaling Pathway

Ligands (ACTH, LH, FSH, GnTH) → Membrane bound receptors → G-protein → Adenylyl cyclase → cAMP → AMP → STAR → MIS

- Phosphodiesterase
- CREB
- SF-1
- Protein Kinase A
- Aromatase
- Cholesterol-esterase
- Cholesterol
Effects of other compounds on Aromatase activity  H295R cells

• Imidazole-type fungicides decrease aromatase activity. Competitive inhibitors
  – imazalil
  – prochloraz
  – difenoconazol
  – penconazole
  – Propiconazole
  – Diclobutrazole
  – Tricyclazole
  – Paclobutrazole
  – Nuarimol

  the structurally similar fungicide Vinclozolin increases cAMP 150%, whereas forskolin increases cAMP 300%

Cross-Talk of AhR-Mediated Processes

• Compounds that do not bind to the AhR can affect ARNT-requiring pathways, thus affecting AhR-mediated pathways without binding to the AhR
  – Example: Hypoxia-inducible factor (HIF)

Interference between AhR and Hypoxia Signaling Pathways

TCDD

TCDD

TCDD

TCDD

AhR

AhR

Hsp90

Hsp90

Hsp90

Hypoxia, CoCl₂, Dfx

HIF-1α

HIF-1α

ARNT

ARNT

ARNT

ARNT
Inhibition of AhR-Mediated Response by Dfx

Inhibition of AHR-mediated luciferase activity in H4IIE-luc cells by 10 mM Dfx (1.25 nM TCDD was used to induce the luciferase activity).
Concentration-dependent inhibition of AHR-mediated transcriptional activation by Dfx treatment on B-1 cells. Transcriptional activation induced by AHR activation was measured as relative EROD induction in B-1 cells. The relative induction was calculated against solvent control (methanol). (A) Dose-response induction of EROD activity in B-1 cells treated with 12.5 pM to 3.75 nM TCDD. (B) Inhibition of EROD induction by Dfx on B-1 cells treated with 3.75 nM TCDD (bar plot, using left y-axis). B-1 cells were pre-treated with 5 to 250 µM Dfx 6 hours before TCDD treatment. The inhibition of EROD activity by Dfx is inversely proportional to its induction of HIF-1-mediated luciferase activity (line and scatter plot, using right y-axis).
TCDD (pM) vs EROD Activity

Desferrioxamine (µM) vs Relative Luciferase Induction

A

B

Col 16 vs Col 19
Affymetrix Gene Chips

Rat Genome U34A containing ~8,000 fully-sequenced and functionally annotated genes and ESTs (partial sequence, no function characterized) on 1cm² surface
Biological Framework

Watson-Crick base pairing --- Hybridization between DNA and its complementary sequence

Probe --- cDNA fragments, oligonucleotides or ESTs that are tethered on supporting surface

Target --- free floating, radioactive or fluorescent labeled complementary sequence
Target Sample Preparation

Excise rat liver samples
Total RNA extraction

In vitro transcription to cRNA label with Biotin

ds cDNA synthesis

CRNA fragmentation

Hybridize with gene chip
GeneChip Expression Tiling Array Design

Gene Sequence

5' 3'

Multiple oligo probes

Perfect Match

Mismatch

Perfect match

Mismatch
The GeneChip Expression Assay Format

Cells → Poly (A)^+ RNA → cDNA (IVT) → Labeled transcript (heat, Mg^{2+}) → Labeled fragments

Scan → Stain → Hybridize (1-18 hours) → Wash → Hybridize
## Output and Data Analysis

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<tr>
<th>Probe Set</th>
<th>Avg Diff</th>
<th>Diff Call</th>
<th>Fold Change</th>
<th>Descriptions</th>
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Scatter Plot of Gene Chip Data (showing correlation between Samples)

A1_A2 (2 controls)

C2_A2

C1_C2 (2 PFOS treatment)

Control in vivo_in vitro

Zoology Dept. & National Food Safety and Toxicology Center
Michigan State University
Expression level of majority of genes stay constant

![Bar chart showing the number of genes with different ranges of fold change]
Hormone regulator

Lipid metabolism

CYPs

DNA polymerase alpha

testosterone 6-beta-hydroxylase (CYP3A1)

Tax gene

neuroendocrine-specific protein (RESP18)

aldehyde dehydrogenase (ALDH)

delta2-enoyl-CoA isomerase

carboxylesterase precursor

cytochrome P450 PCN1, NADPH mono-oxygenase

cytocherm P-450b, exon1

P-450(1) variant

cytochrome P-450e, exon9

cytochrome P450 2B15 gene

fold_change

C8_A7  C2_A2  C1_A1

DNA polymerase alpha
testosterone 6-beta-hydroxylase (CYP3A1)

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neuroendocrine-specific protein (RESP18)

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P-450(1) variant

cytochrome P-450e, exon9

cytochrome P450 2B15 gene

fold_change

C8_A7  C2_A2  C1_A1
Fatty Acid Metabolism Pathway

PFOS treatment vs Control
Zoom In on Fatty Acid Metabolism Pathway

Selected: AF001898_at(ALDH)

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ALDH

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Differential Display
Tissue sample → Control + exposure → Extract RNA

Reverse Transcribe to cDNA fragments

PCR

identify differently expressed gene

sub-cloning

Search Matches in Gene-bank and determine gene function

Determine Biological Significance
Suppression Subtraction Hybridization
Suppression Subtractive Hybridization

ds tester cDNA
ds driver cDNA

Tester cDNA + Adaptor A

Restriction enzyme digestion

Driver cDNA (in excess)

Tester cDNA + Adaptor B

Denature, first hybridization

Mix, Second hybridization

a, b, c, d,

e)
Suppression Subtractive Hybridization

- a, b, c, d,
- e)

Fill in the ends

- a)
- d)
- e)
- b)

PCR Amplification

No amplification

Exponential amplification

Linear amplification

No amplification
Questions ????????
Thank You

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• Web Site: http://www.msu.edu/user/giesy