Gene Expression Changes in the Immature Rat Uterus: Effects of Uterotrophic and Sub-Uterotrophic Doses of Bisphenol A

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Two independent reports of the uterotrophic activity of bisphenol A (BPA) to the immature rat appeared in 1998. One reported uterotrophic activity at and above a dose of 400 mg/kg/day BPA (Ashby and Tinwell, 1998), while the other concluded the absence of uterotrophic activity over the dose range 5–150 mg/kg/day BPA (Gould et al., 1998). Gould et al. did not evaluate doses above 150 mg/kg BPA, but the two data sets are consistent with them being separate parts of the same dose response curve to orally administered BPA (Table 1). Despite the absence of a uterotropic effect for BPA over the dose range 5–150 mg/kg BPA, Gould et al. (1998) reported non-dose-related elevations in uterine peroxidase activity and progesterone receptor (PR) levels (Table 1). Those data therefore raise the prospect of BPA affecting gene expression at dose levels insufficient to trigger uterine growth. This, in turn, raises the further question of whether uterine growth is of sufficient sensitivity to act as a sentinel for the estrogenic activity of chemicals in vivo. Therefore, the uterotrophic activity of BPA to the immature rat has been re-evaluated over the dose range 2 μg/kg–800 mg/kg/day. Expression levels of three estrogen responsive uterine genes were determined using real-time RT-PCR—namely, complement component 3, lipocalin 2, and PR. 18S rRNA and RNA polymerase II large subunit acted as control genes. Observations of gene expression were made 4 h and 72 h after the first of three daily p.o administrations of BPA. Increases in gene expression were observed over the uterotropic dose range (~200–800 mg/kg BPA). Over the dose range 2 μg/kg–20 mg/kg BPA there was no uterotropic response and no increase in gene expression. We conclude that BPA does not produce reproducible changes in gene expression in the uterus of immature rats at dose levels that are not also uterotrophic. Therefore, in the present study, the no effect level for uterotropic activity for BPA coincided with the no transcriptional effect level for uterine genes.

Key Words: bisphenol A; uterotrophic; gene expression; progesterone receptor.

MATERIALS AND METHODS

Chemicals. Bisphenol A (BPA) (purity >99%) was obtained from Aldrich (Gillingham, Dorset, U.K.). 17β-Estradiol (E2) and arachis oil (AO) were obtained from Sigma Chemicals (Poole, Dorset, U.K.).

Animals. Immature Alpk:APfSD (Wistar derived) rats were obtained from the AstraZeneca breeding unit (Alderley Park). Rats were 18–19 days old (body weights 37–45 g), on arrival and were acclimatized for one day before dosing. Animal studies were performed in accordance with the U.K. “Animals (Scientific Procedures) Act.” Rats were housed (up to five per cage) in polypropylene cages. Rat and Mouse No. 1 diet (Special Diet Services Ltd., Witham, Essex, U.K.) and water were available ad libitum. The phytoestrogen levels in the diet have been described earlier (Odum et al., 2001). Animal care and procedures were conducted according to in-house standards as described previously (Odum et al., 2001).

Uterotrophic assays. Uterine growth and gene expression following BPA treatment was investigated in three immature rat uterotrophic assays, conducted according to current OECD protocols (Kanno et al., 2003a,b). E2 was used as a reference estrogen in all studies. All compounds were given daily by gavage in AO (5 ml/kg) beginning on PND 19–20. First, a time course was conducted where BPA (800 mg/kg/day) and E2 (0.4 mg/kg/day) were administered at doses
and weighed individually, frozen in liquid N2 and stored at −70°C. Animals were killed using overexposure to halothane (Concord Pharmaceuticals, Dunmow, Essex, U.K.) followed by cervical dislocation. Uteri were removed, blotted dry, ground to a powder in liquid N2 using a dismembrator (Brownes, Reading, U.K.). The frozen uteri were pooled according to group and mow, Essex, U.K.) followed by cervical dislocation. Uteri were removed, blotted dry, ground to a powder in liquid N2 using a dismembrator (Brownes, Reading, U.K.). The frozen uteri were pooled according to group and

TABLE 1
Data Taken from Gould et al. and Tinwell et al. Tabulated Together to Illustrate the Continuity of the Uterotrophic Responses and the Unusual PR and Peroxidase Results

<table>
<thead>
<tr>
<th>Reference</th>
<th>Treatment</th>
<th>Fold increase in rat uterus wet weight (mg)</th>
<th>Fold change in peroxidase activity (activity/mg protein)</th>
<th>Fold increase in PR (fmol/uterus)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gould et al. (1998)</td>
<td>BPA 5 mg/kg</td>
<td>−1.05</td>
<td>0.33*</td>
<td>1.34*</td>
</tr>
<tr>
<td></td>
<td>BPA 10 mg/kg</td>
<td>−1.20</td>
<td>1.11</td>
<td>1.50*</td>
</tr>
<tr>
<td></td>
<td>BPA 25 mg/kg</td>
<td>−1.14</td>
<td>−1.72</td>
<td>1.32*</td>
</tr>
<tr>
<td></td>
<td>BPA 50 mg/kg</td>
<td>−1.14</td>
<td>−1.52</td>
<td>1.38*</td>
</tr>
<tr>
<td></td>
<td>BPA 100 mg/kg</td>
<td>−1.18</td>
<td>1.44*</td>
<td>1.33*</td>
</tr>
<tr>
<td></td>
<td>BPA 150 mg/kg</td>
<td>1.00</td>
<td>2.08*</td>
<td>1.76*</td>
</tr>
<tr>
<td>Ashby and Tinwell (1998)</td>
<td>BPA 400 mg/kg</td>
<td>1.31*</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>BPA 600 mg/kg</td>
<td>1.38*</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>BPA 800 mg/kg</td>
<td>2.18*</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Note. Data from Gould et al. (1998, Table 1) and Tinwell et al. (1998, Table 1). Values are fold changes over control. Statistically significant *p < 0.05

TABLE 2
Primers Used for Gene Expression Analysis by Real-Time RT-PCR

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Accession no.</th>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>Probe</th>
</tr>
</thead>
<tbody>
<tr>
<td>Progesterone receptor (PR)</td>
<td>NM022847</td>
<td>5′-GTCCTGCGCAGGCT-CAGGA-3′</td>
<td>5′-CTCCACGTCCGAC-AGG-3′</td>
<td>5′-TCCCAGAGAAAGACACAAAATCAGCA-3′</td>
</tr>
<tr>
<td>Lipocalin 2</td>
<td>NM130741</td>
<td>5′-CGGGCGAGTGTTT-GTTT-3′</td>
<td>5′-CGGCTTTTGTCTTTT-TCTGA-3′</td>
<td>5′-TCGCGCTTGCAAGGAAATGC-3′</td>
</tr>
<tr>
<td>Complement component 3 (C3)</td>
<td>NM016994</td>
<td>5′-TGATCGAGGATGTTT-CAGGG-3′</td>
<td>5′-GAAGGCGCATCCCGTC-3′</td>
<td>5′-GGCGAGTGCTCAGCGGAAA-GTGC-3′</td>
</tr>
<tr>
<td>RNA polymerase II large subunit (RPB1)</td>
<td>AA819317</td>
<td>5′-CACAGCCGACCATCC-TCAA-3′</td>
<td>5′-TGAGGGAAGATTG-CTTG-3′</td>
<td>5′-CCCCGCCCCCTGCGA-3′</td>
</tr>
</tbody>
</table>

*Sequences for the probes contained the dyes FAM (6-carboxy-fluorescein) (5′) and TAMRA (6-carboxy-tetramethyl-rhodamine) (3′).

previously shown to give a uterotrophic response after three days (Ashby and Tinwell, 1998; Odum et al., 1997). Controls received AO only. Rats (five per group) were killed 4, 8, and 24 h after a single dose of compound and 24 h after the last of three daily doses (72 h after the first dose). Second, two dose-responses to BPA (0.002–800 mg/kg/day) were conducted. E2 (0.4 mg/kg/day) was used at a single dose level, controls received AO. Rats were killed 4 h after a single dose of compound and 24 h after the last of three daily doses (72 h after the first dose) in both experiments. The first experiment contained five rats per group and the second experiment 10 rats per group, but they were otherwise identical. Animals were killed using overexposure to halothane (Concord Pharmaceuticals, Dunmow, Essex, U.K.) followed by cervical dislocation. Uteri were removed, blotted dry, and weighed individually, frozen in liquid N2 and stored at −70°C.

Real-time RT-PCR. The frozen uteri were pooled according to group and ground to a powder in liquid N2 using a dismembrator (Brownes, Reading, U.K.). Gene expression was determined by the quantitative real-time reverse transcriptase polymerase chain reaction assay (RT-PCR) on uterine RNA. Three genes regulated by estrogen were measured: PR, complement component 3 (C3) and lipocalin 2 (lipocalin). The first two contain estrogen response elements (Klinge, 1990). The third gene, lipocalin, is a secreted iron trafficking protein that mediates iron delivery to differentiating epithelial cells (reviewed in Kaplan, 2002). It was identified as being an estrogen-responsive gene by microarray analysis of E2-induced uterine growth in the immature mouse uterus (Moggs et al., 2004), and was upregulated at similar times to PR. Two genes were used for confirmation that RNA concentrations were equal in control and test samples: 18S rRNA (using commercial primers and probes supplied by Applied Biosystems, Foster City, CA) and RNA polymerase II large subunit (RPB1), which was also identified by the same microarray analysis (Moggs et al., 2004) as a gene which was unchanged after estrogen treatment. The nucleotide sequences for the primers and probes (supplied by MWG Biotech, Milton Keynes, U.K.) used in the real-time RT-PCR assays were generated by “Primer Express” software (Applied Biosystems) and are shown in Table 2.

RNA was extracted from ~100–500 mg of the powdered uteri using 2 ml Trizol reagent (Invitrogen, Paisley, U.K.) and a rRNA standard (Sigma, Poole, Dorset, U.K.). No visible degradation was observed in any sample. Contaminating DNA was removed with DNeasy-1 (DNA-free, Ambion, Austin, TX) (4 units DNease/μg RNA) using the protocol described.
by the manufacturer. The RNA samples were then diluted to 50 ng/μl and A260 was checked several times to ensure that the RNA concentrations of control and test samples were within 10% of each other. Real-time RT-PCR reactions were carried out using the Taqman EZ RT-PCR kit (from Applied Biosystems), a single tube system in which reverse transcription and the polymerase chain reaction are carried out by the same enzyme. A total reaction volume of 50 μl/incubation was used containing 50–200 ng RNA. Real-time RT-PCR was carried out on an ABI Prism 7700 Sequence Detection System (Applied Biosystems). Thermal cycling conditions were 94°C for 2 min, 60°C for 30 min followed by 40 cycles of 94°C for 15 s and 60°C for 1 min. 

Reactions were carried out in triplicate in each run, “no-template” controls were used to check for contamination. Each run contained a standard curve consisting of six points prepared from dilutions of a positive control sample for which all of the genes studied were known to be expressed. A standard curve was plotted of Ct (threshold cycle) against log RNA concentration (in arbitrary units). The control and test sample RNA concentrations were then interpolated from their Ct values and relative gene expression calculated (test divided by control). Real-time RT-PCR was carried out on 2–5 separate occasions for each gene. Gene expression was reproducible within experiments, as illustrated for PR expression (Table 3).

**SDS-PAGE and Western analysis.** SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis) and Western blotting were used to determine uterine PR contents of tissues from the first time course experiment. Aliquots (100–200 μl) of the frozen tissue were solubilized and stored as described by Williams et al. (2001). Protein contents were determined by the method of Lowry et al. (1951).

SDS-PAGE and Western blotting were performed using a “Novex” gel system with 4–12% tris-glycine gels (supplied by Invitrogen) according to the method described by Williams et al. (2001). A uterine sample prepared from a mature female rat was used as a positive control for PR as it is constitutively expressed in adult females (Cotroneo et al., 2001). Gels were loaded with 60 μg protein/lane. Loading consistency was checked by staining the gel with a general protein stain ("SimplyBlue" supplied by Invitrogen) after blotting and was acceptable in all cases. A rabbit polyclonal primary antibody to PR (sc-7208) was purchased from Santa Cruz (CA) and used at a 500× dilution. Donkey anti-rabbit IgG-HRP linked secondary antibody was purchased from Amersham Biosciences (Amerham, U.K.) and used at a 2500× dilution. Proteins were detected by chemiluminescence using the “ECL Western Blotting Analysis System” from Amersham Biosciences. PR was quantified by densitometry.

**Statistical analysis.** In the time course experiment, uterine weights were analyzed by variance and by covariance with the terminal body weights (Shirley, 1996). A representative run was selected for the expression of each gene, and that was then analyzed by variance following logarithmic transformation. Differences from control values were assessed statistically using a two-sided Student’s t-test based on the error mean square from the analysis of variance. Statistical significance of these gene expression data should be treated with caution because samples were pooled according to group; consequently, the statistical analyses reflect variability between repeat measurements of a sample, rather than representing interanimal variability.

In the two dose-response experiments, uterine weights from the two experiments were analyzed by variance and by covariance with the terminal body weights (Shirley, 1996). The data from both studies were then combined using a mixed model analysis of variance. Differences from control values were assessed statistically using a two-sided Student’s t-test based on the group experiment interaction mean square. Analyses were carried out as described in SAS (1999).

**RESULTS**

**Time Course of Uterine Growth and Gene Expression after Treatment with BPA or E2**

BPA (800 mg/kg) and E2 (0.4 mg/kg) gave 2.6-fold and 4.4-fold increases in uterine weight when administered orally to immature rats for three days (Table 4, Fig. 2) as previously described (Ashby and Tinwell, 1998; Odum et al., 1997). The time course of the responses showed that uterine weight was increased to a similar extent 4 h after a single dose of either compound, but that after 8, 24, and 72 h, an increasingly differential uterotrophic effect was seen between the two chemicals. There was no effect on body weight in any group (Table 4).

Relative changes in uterine gene expression were determined by real-time RT-PCR analysis of RNA. The statistical significance of changes in gene expression for this experiment is indicated in Table 4, but not in Figure 1 because the pooling of samples means that statistical significance for this experiment should be treated with caution. Two control genes (RPB1 and 18S rRNA) were used in order to confirm that RNA concentrations were similar across groups. RPB1 varied no more than ±1.5-fold across groups, but that of 18S rRNA increased over the time course to a maximum of 3.8-fold after 72 h (Table 4, Fig. 1). The change in 18S rRNA expression is consistent with the results of Moggs et al. (unpublished data) who showed an increase in many components of transcriptional and translational cellular machinery during E2-induced uterine growth in the mouse uterus. It was concluded that RPB1 provides a more appropriate control gene than does 18S rRNA Nevertheless the latter was included in the later experiments because it is commonly used by other workers.

The estrogen-regulated genes PR, C3, and lipocalin had different time courses of RNA expression (Table 4, Fig. 1). E2 gave maximal increases in gene expression for PR, lipocalin, and C3 of 6–14- and 1800-fold, respectively. BPA gave increases for PR, lipocalin, and C3 of 3-, 9- and 730-fold, respectively. The time courses of C3 and PR expression were similar for both chemicals. C3 was increased at 4 h, but achieved maximum expression at 72 h. As reported by Kraus and Katzenellenbogen (1993), PR RNA expression was rapid, with highest expression at 4 h and a decline to control levels by 24 h (Table 4, Fig. 1). Lipocalin expression differed marginally between BPA and E2 treatments (maximal expression at 4 h and 8 h, respectively). Based on these results gene expressions in the dose response studies were determined 4 h and 72 h after the first dose of chemical.

Western analysis of PR protein expression confirmed that the increased transcription of PR RNA was followed by translation (Fig. 2). Although a single gene encodes PR, two isoforms of the receptor (A and B) are produced under the control of distinct promoters (Graham and Clarke, 1997). Both forms of the PR receptor were increased from 4 h onwards (the point at which PR RNA expression was greatest), with maximal protein expression at 72 h (Table 4, Fig. 2). Ilenchuk and Walters (1987) reported a ratio of A/B in mature female rats of ~3. In the present study the ratio in control immature female rats was ~5–6. Cotroneo et al. (2001) also showed that PR A and B were increased in rat uterus after genistein and estradiol benzoate treatment.
### TABLE 3
Intra-experiment Reproducibility of PR Gene Expression

<table>
<thead>
<tr>
<th>BPA (mg/kg)</th>
<th>E₂ (mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.002</td>
<td>0.02</td>
</tr>
<tr>
<td><strong>Control</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Responses 4 h after a single dose</strong></td>
<td></td>
</tr>
<tr>
<td>Experiment 1</td>
<td></td>
</tr>
<tr>
<td>Run 1</td>
<td>1.00 ± 0.04</td>
</tr>
<tr>
<td>Run 2</td>
<td>1.00 ± 0.12</td>
</tr>
<tr>
<td>Run 3</td>
<td>1.00 ± 0.07</td>
</tr>
<tr>
<td>Experiment 2</td>
<td></td>
</tr>
<tr>
<td>Run 1</td>
<td>1.00 ± 0.06</td>
</tr>
<tr>
<td>Run 2</td>
<td>1.00 ± 0.09</td>
</tr>
<tr>
<td><strong>Responses 72 h after the first of three daily doses</strong></td>
<td></td>
</tr>
<tr>
<td>Experiment 1</td>
<td></td>
</tr>
<tr>
<td>Run 1</td>
<td>1.00 ± 0.03</td>
</tr>
<tr>
<td>Run 2</td>
<td>1.00 ± 0.02</td>
</tr>
<tr>
<td>Run 3</td>
<td>1.00 ± 0.21</td>
</tr>
<tr>
<td>Experiment 2</td>
<td></td>
</tr>
<tr>
<td>Run 1</td>
<td>1.00 ± 0.18</td>
</tr>
<tr>
<td>Run 2</td>
<td>1.00 ± 0.08</td>
</tr>
</tbody>
</table>

*Note.* The results for repeat assays for the two dose response experiments (Experiment 1 and Experiment 2) are shown. All values are fold changes over control expression ± SD for three replicates. Statistical significance is not shown. All samples were pooled according to group (Experiment 1, \( n = 5 \); Experiment 2, \( n = 10 \)).
TABLE 4
Time Course of Uterine Growth and Gene Expression after Treatment of Immature Rats with BPA or \( \text{E}_2 \)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>4 h</th>
<th>8 h</th>
<th>24 h</th>
<th>72 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blotted uterine weight (mg)(^a)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>23.2 ± 3.8</td>
<td>22.3 ± 1.2</td>
<td>23.1 ± 3.5</td>
<td>21.0 ± 3.3</td>
</tr>
<tr>
<td>BPA 800 mg/kg</td>
<td>35.3 ± 1.9**</td>
<td>37.9 ± 3.9**</td>
<td>45.8 ± 2.7**</td>
<td>55.4 ± 12.3**</td>
</tr>
<tr>
<td>( \text{E}_2 ) 0.4 mg/kg</td>
<td>36.8 ± 2.0**</td>
<td>42.4 ± 2.1**</td>
<td>54.2 ± 7.8**</td>
<td>93.0 ± 12.0**</td>
</tr>
<tr>
<td>PR expression (RNA)(^b)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>1.00 ± 0.07</td>
<td>1.00 ± 0.13</td>
<td>1.00 ± 0.24</td>
<td>1.00 ± 0.07</td>
</tr>
<tr>
<td>BPA 800 mg/kg</td>
<td>3.11 ± 0.18**</td>
<td>1.98 ± 0.17**</td>
<td>1.17 ± 0.09</td>
<td>1.05 ± 0.15</td>
</tr>
<tr>
<td>( \text{E}_2 ) 0.4 mg/kg</td>
<td>6.19 ± 3.11**</td>
<td>3.44 ± 0.22**</td>
<td>1.03 ± 0.15</td>
<td>1.07 ± 0.10</td>
</tr>
<tr>
<td>PR expression (A isoform) (protein)(^c)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>62.6 (1.0)</td>
<td>27.1 (1.0)</td>
<td>41.7 (1.0)</td>
<td>99.3 (1.0)</td>
</tr>
<tr>
<td>BPA 800 mg/kg</td>
<td>132.0 (1.0)</td>
<td>70.8 (1.0)</td>
<td>132.0 (1.0)</td>
<td>132.0 (1.0)</td>
</tr>
<tr>
<td>( \text{E}_2 ) 0.4 mg/kg</td>
<td>212.0 (1.0)</td>
<td>132.0 (1.0)</td>
<td>144.0 (1.0)</td>
<td>144.0 (1.0)</td>
</tr>
<tr>
<td>PR expression (B isoform) (protein)(^d)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>1.00 ± 0.19</td>
<td>1.00 ± 0.31</td>
<td>1.00 ± 0.35</td>
<td>1.00 ± 0.22</td>
</tr>
<tr>
<td>BPA 800 mg/kg</td>
<td>9.28 ± 1.86**</td>
<td>7.57 ± 0.42**</td>
<td>1.71 ± 0.43</td>
<td>1.52 ± 0.16(^e)</td>
</tr>
<tr>
<td>( \text{E}_2 ) 0.4 mg/kg</td>
<td>6.49 ± 1.46**</td>
<td>14.24 ± 4.09**</td>
<td>2.74 ± 0.77</td>
<td>−1.04 ± 0.37</td>
</tr>
<tr>
<td>Lipocalin expression (RNA)(^b)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>1.00 ± 0.19</td>
<td>1.00 ± 0.16</td>
<td>1.00 ± 0.10</td>
<td>1.00 ± 0.15</td>
</tr>
<tr>
<td>BPA 800 mg/kg</td>
<td>3.27 ± 0.89**</td>
<td>7.57 ± 0.42**</td>
<td>74.3 ± 3.49**</td>
<td>732.7 ± 71.5**</td>
</tr>
<tr>
<td>( \text{E}_2 ) 0.4 mg/kg</td>
<td>11.36 ± 0.31**</td>
<td>7.20 ± 1.13**</td>
<td>51.77 ± 9.62**</td>
<td>1786.9 ± 76.6**</td>
</tr>
<tr>
<td>C3 expression (RNA)(^b)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>1.00 ± 0.15</td>
<td>1.00 ± 0.07</td>
<td>1.00 ± 0.01</td>
<td>1.00 ± 0.13</td>
</tr>
<tr>
<td>BPA 800 mg/kg</td>
<td>1.21 ± 0.08</td>
<td>1.29 ± 0.05**</td>
<td>1.08 ± 0.10</td>
<td>1.08 ± 0.04</td>
</tr>
<tr>
<td>( \text{E}_2 ) 0.4 mg/kg</td>
<td>1.27 ± 0.11</td>
<td>1.41 ± 0.11**</td>
<td>−1.01 ± 0.04</td>
<td>−1.12 ± 0.02</td>
</tr>
<tr>
<td>RPB1 expression (RNA)(^b)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>1.00 ± 0.13</td>
<td>1.00 ± 0.14</td>
<td>1.00 ± 0.13</td>
<td>1.00 ± 0.24</td>
</tr>
<tr>
<td>BPA 800 mg/kg</td>
<td>1.15 ± 0.06</td>
<td>1.43 ± 0.53</td>
<td>3.33 ± 0.94**</td>
<td>3.88 ± 0.70**</td>
</tr>
<tr>
<td>( \text{E}_2 ) 0.4 mg/kg</td>
<td>2.72 ± 1.62</td>
<td>1.56 ± 0.10**</td>
<td>3.59 ± 0.48**</td>
<td>3.84 ± 0.54**</td>
</tr>
<tr>
<td>18S rRNA expression (RNA)(^b)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>42.8 ± 3.9</td>
<td>46.9 ± 2.7</td>
<td>44.8 ± 1.4</td>
<td>47.5 ± 3.1</td>
</tr>
<tr>
<td>BPA 800 mg/kg</td>
<td>43.7 ± 1.9</td>
<td>46.4 ± 1.5</td>
<td>45.2 ± 1.7</td>
<td>46.2 ± 4.0</td>
</tr>
<tr>
<td>( \text{E}_2 ) 0.4 mg/kg</td>
<td>44.5 ± 2.4</td>
<td>46.8 ± 1.3</td>
<td>43.6 ± 1.5</td>
<td>48.3 ± 3.3</td>
</tr>
</tbody>
</table>

\(^a\)Values are mean ± SD, \( n = 5 \).

\(^b\)Samples for analysis of RNA by real-time RT-PCR were pooled according to group (\( n = 5 \)), values are fold changes over control ± SD for three replicates taken from a representative run. PR, lipocalin, and C3 represent estrogen-responsive genes whilst RPB1 and 18S rRNA are control genes.

\(^c\)Samples for analysis of protein by Westerns were pooled according to group (\( n = 5 \)), values are densitometry results in arbitrary units, values in parentheses are fold changes over control.

\(^d\)Samples for analysis of protein by Westerns were pooled according to group (\( n = 5 \)), values shown are densitometry results in arbitrary units, values in parentheses are fold changes over control.

\(^e\)\( p < 0.05 \).

\(^{**} p < 0.01 \).

Dose-Response Studies

Administration of BPA over the dose range 0.002–800 mg/kg gave an increase in uterine weight 4 h after a single dose of between 200–800 mg/kg. Three daily administrations of the same dose-range of BPA gave increased uterine weight over the same dose range, with statistical significance observed for the doses 400–800 mg/kg (Table 5, Fig. 3). The lowest positive uterotrophic dose of BPA was 200 mg/kg (Table 5). \( \text{E}_2 \) (0.4 mg/kg) increased uterine weight at the same time points and the magnitude of the uterotrophic response was similar to that in the previous experiment (Tables 4 and 5). There were no effects on body weight after any treatment (Table 5).

Changes in uterine gene expression at the RNA level after administration of BPA to immature rats over the dose range 0.002–800 mg/kg are shown in Table 5 and Figure 3. Expression of the control gene RPB1 indicated RNA concentrations varied...
1.5-fold between all groups. PR, C3, and lipocalin showed increased RNA expression at 4 h and/or 72 h following E2 and 800 mg/kg BPA treatment. The results were similar to those obtained in the time course experiment, although the magnitude of some of the changes was different. This could be due to slight variability in the time course of gene response, or to differences in the expression of these genes in the control animals, which in turn, would affect the relative expression level.

Statistically significant changes in the expression of PR, C3, and lipocalin RNA were generally seen at doses of BPA that were also uterotrophic. PR expression was increased 3-fold 4 h after a single dose of 200–800 mg/kg BPA. Lipocalin expression was increased 3–4-fold 4 h after a single dose of 200–800 mg/kg BPA. No statistically significant changes in PR or lipocalin RNA levels were noted at doses below 200 mg/kg BPA at either time point. Changes in C3 expression are particularly interesting. At the 4 h time point an increase of 2.5-fold was seen after a single dose of 20 mg/kg BPA, rising to ~6-fold for both the 800 mg/kg dose of BPA and the E2 positive control at 4 h. However, at the 72 h time point, where C3 expression was increased >500-fold by E2, statistically significant increases in C3 expression were only observed for doses of BPA that were also statistically significant in the uterotrophic assay (400 mg/kg and 800 mg/kg BPA; 4-fold and 83-fold increases in C3 expression, respectively; Table 5, Fig. 3).

**DISCUSSION**

The uterotrophic activity of BPA over the dose range 2 μg/kg–800 mg/kg, as described here, is consistent with earlier reports (Ashby and Tinwell, 1998; Gould et al., 1998; Matthews et al., 2001). Thus, uterotrophic activity, as defined by the trophic response at 72 h, was constrained to 400 and 800 mg/kg BPA (Table 5 and Fig. 3). The expression of C3 at 72 h mirrored the uterotrophic response, precisely (Table 5 and Fig. 3). The combined uterine weights for 200 mg/kg BPA were non-significantly increased, but both of the constituent experiments had elevated uterine weights at this dose, in one case the increase was statistically significant (Table 5). It therefore seems likely that the effects seen at 200 mg/kg BPA form the start of its uterotrophic dose response. However, C3 expression, which is at its most sensitive at the 72 h time, was unequivocally negative at 200 mg/kg BPA. In conclusion, C3, the estrogen-sensitive...
The Effect of BPA on Uterine Growth and Gene Expression in Immature Rats

<table>
<thead>
<tr>
<th>BPA (mg/kg)</th>
<th>Control</th>
<th>0.002</th>
<th>0.02</th>
<th>0.2</th>
<th>2.0</th>
<th>20</th>
<th>200</th>
<th>800</th>
<th>E2 (mg/kg)</th>
</tr>
</thead>
</table>

4 h after the first of three daily doses

<table>
<thead>
<tr>
<th>BPA (mg/kg)</th>
<th>Control</th>
<th>0.002</th>
<th>0.02</th>
<th>0.2</th>
<th>2.0</th>
<th>20</th>
<th>200</th>
<th>800</th>
<th>E2 (mg/kg)</th>
</tr>
</thead>
</table>

72 h after the first of three daily doses

<table>
<thead>
<tr>
<th>BPA (mg/kg)</th>
<th>Control</th>
<th>0.002</th>
<th>0.02</th>
<th>0.2</th>
<th>2.0</th>
<th>20</th>
<th>200</th>
<th>800</th>
<th>E2 (mg/kg)</th>
</tr>
</thead>
</table>

**Note:** Values are mean ± SD, n = 5 (Experiment 1) or n = 10 (Experiment 2).
gene selected for use at 72 h, was no more sensitive than the gravimetric uterotrophic endpoint at low doses of BPA but showed greater sensitivity at higher doses of BPA (>500-fold increase seen for E<sub>2</sub>).

Immediate (4 h) increases in uterine weight are associated with imbibition of fluid. The increases seen for BPA were statistically significant between 200–800 mg/kg BPA, with an increase of similar magnitude seen for E<sub>2</sub> (Table 5). Expression

**FIG. 3.** Uterine weight and gene expression for immature rats given one dose of BPA and sampled 4 h later or three daily doses of BPA and sampled 72 h after the first dose (data from Table 5). The shaded areas indicate the dose levels at which uterine weight and gene expression increased. *Values are the combined means from two experiments. **Values are fold changes over control expression, derived from the combined means from two experiments. *p < 0.05, **p < 0.01. PR, lipocalin, and C3 represent estrogen-responsive genes whilst RPB1 and 18S rRNA are control genes.
of lipocalin and PR was increased at the same time as this im-
bition (Table 5 and Fig. 3), although a causal relationship is not
implied. In contrast, the expression of C3 at 4 h is rather perplex-
ing. The time course for this gene (Fig. 1) indicates major expres-
sion between 24–72 h, with expression levels at 72 h as high
as 1700-fold control levels. Small increases in C3 expression
(2–6 fold) at 4 h were seen for doses of BPA between 20–800 mg/kg,
and for E2 (Fig. 3). However, the early expression of C3 may not
be functionally related to the expression occurring at 72 h. For
example, the fold increase in C3 expression for 200 mg/kg
BPA and for E2 was similar (5.3 and 6.1, respectively). However,
by 72 h, these responses had diverged—the 200 mg/kg BPA CC3
expression had returned to control level, while the response to E2
had amplified to >500-fold above control. As is evident from
Figure 3, the C3 responses at 4 h occurred at the same dose levels
(200–800 mg/kg BPA) as the responses seen for PR and lipo-
calin—with the exception of the 2.5-fold increase in C3 only
seen for 20 mg/kg BPA. In conclusion, the early imbibition
response of the uterus to 200–800 mg/kg BPA at 4 h is followed
by the trophic response seen at 72 h (400–800 mg/kg). Increases
in the expression of uterine PR, lipocalin, and C3 also occur at
4 and 72 h after the same doses of BPA producing the physio-
logical changes. The only exception to this is a small increase in
C3 expression for 20 mg/kg BPA at 4 h—the biological signifi-
cance of which is uncertain.

The changes in uterine PR protein level and peroxidase activ-
ity reported by Gould et al. (1998) for non-uterotrophic doses of
BPA were all less than 2-fold (Table 1)—the marginally greater
activities seen for 100 and 150 mg/kg BPA are probably asso-
ciated with the start of the uterotrophic effect. Consequently,
there is a level of consistency between the data of Gould et al.
(1998) and the present data—i.e., minor (<2-fold) fluctuations in
gene expressions were observed in both studies.

The effects of low doses of endocrine disrupting compounds
are highly controversial (Brucker-Davis et al., 2001; Melick
et al., 2002), particularly with respect to BPA where changes
have proved difficult to replicate, for example prostate weight
increases observed at 2 and 20 μg/kg BPA (Ashby et al., 1999,
2004; Cagen et al., 1999; Nagel et al., 1997). The use of microarray
technology to identify novel genes differentially expressed by endocrine disrupting compounds, and real-time
RT-PCR to quantify gene expression, has given rise to an
increasing number of markers for endocrine disrupting activity
(e.g., Naciff et al., 2002; Rockett et al., 2002; Watanabe et al.,
2002; Waters et al., 2001). This technology has not been used
extensively so far to identify genes which may be expressed at
low dose levels, although Naciff et al. (2002) showed that BPA
below 50 mg/kg had no effect on estrogen-sensitive genes in the
developing female reproductive system of the rat. Lobenhofer
et al. (2004) have recently suggested that identification of a
NOTEL (no transcriptional effect level) for compounds
would be useful to identify doses below which transcriptional
(and therefore toxicological or pharmacological) effects do not
occur. They used this approach to demonstrate that only doses
of E2 which are physiologically relevant, and above, were cap-
able of inducing a transcriptional response.

In the present study we have attempted to determine whether
changes in estrogen responsive genes occur at levels of BPA
below those capable of inducing a uterotrophic response. We
extended the dose levels from 800 mg to 2 μg/kg BPA in order to
provide sufficient groups to detect low dose effects. Hunt and
Bowman (2004) described a model for detecting low dose effects
and stress the necessity for including sufficient groups. We also
replicated the study to demonstrate reproducibility of the
response. With the exception of changes in C3 at 20 mg/kg
BPA, uterine growth and gene expression were observed at
the same dose levels. Gene expression changes were of greater
magnitude than the uterotrophic response, but there was no
evidence of gene expression changes at low doses (below
2 mg/kg) in this study. Therefore, in the present study, the
NOEL for uterotrophic activity for BPA coincided with the
NOTEL for uterine transcriptional activity.

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