Uterine and ovarian aryl hydrocarbon receptor (AHR) and aryl hydrocarbon receptor nuclear translocator (ARNT) mRNA expression in benign and malignant gynaecological conditions

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The transcriptional regulators aryl hydrocarbon receptor (AHR) and aryl hydrocarbon receptor nuclear translocator (ARNT) modulate the transcription of genes involved in cellular differentiation and proliferation. In this study, we investigated the expression of these transcriptional regulators in the female reproductive tract. AHR and ARNT mRNA transcripts were readily detected by a ribonuclease protection assay in all reproductive tissues examined. The expression of these factors in the endometrium and myometrium did not vary during the menstrual cycle, and was not different in pre-versus post-menopausal women. However, post-menopausal women on continuous hormone replacement therapy had greater expression of AHR but not of ARNT in the endometrium and myometrium when compared with women not taking hormones. Leiomyomas expressed significantly less AHR and ARNT mRNA compared with normal myometrium. The ovaries expressed both AHR and ARNT mRNA, and expression was unaffected by age. Endometriotic ovarian cysts expressed more AHR but not more ARNT mRNA compared with healthy ovarian tissue. However, there were no changes in the expression of AHR or ARNT mRNA in ovarian cancer. In conclusion, the female reproductive tract expresses mRNA for the transcription factors AHR and ARNT, and changes in their expression at select target sites in specific pathological conditions such as endometriosis and uterine leiomyomas suggest a potential role for these factors in the pathogenesis of these conditions.

Key words: dioxin/endometriosis/ovarian cancer/transcription factors/uterine leiomyomas

Introduction

In recent years, emerging evidence has accumulated to suggest that environmental contaminants such as dioxin can act as endocrine disrupters through inappropriate modulation of target responses to hormones. Dioxin and related compounds affect cell proliferation and differentiation, promote tumour formation, and act as potent immunological, developmental and reproductive toxicants by mechanisms not dependent on cytotoxicity. These compounds produce their biological and toxicological effects by binding to the aryl hydrocarbon receptor (AHR) whose normal function has not been clearly delineated and whose endogenous ligand has not been identified (Schmidt and Bradfield, 1996; Gasiewicz, 1997).

The AHR is a ligand-activated transcription factor which exists as a heteromeric complex with two molecules of 90 kDa heat shock protein (Hsp90) and a 43 kDa protein. Binding of AHR by agonists such as dioxin initiates a series of events resulting in dissociation of Hsp90, movement into the nuclear compartment and heterodimerization with another protein, the aryl hydrocarbon receptor nuclear translocator (ARNT). The resulting AHR–ARNT complex binds to cis-acting DNA sequences (dioxin response elements) in the 5’ regulatory regions of responsive genes to modulate the rates of transcription of a number of genes, including those encoding drug-metabolizing enzymes such as CYP1A1, and growth regulating factors such as the EGF receptor, the estrogen receptor, interleukin-1β, transforming growth factor (TGF)-α and TGF-β (Schmidt and Bradfield, 1996; Gasiewicz, 1997).

Inactivation of the AHR gene by homologous recombination in mice results in a 50% decrease in the size of the liver and a decrease in the number of lymphocytes in lymphoid organs (Fernandez-Sulgnero et al., 1995). Knockout of the ARNT gene has been shown to be lethal in mice, with no embryonic development past embryonic day 9.5. ARNT-deficient embryos are markedly growth retarded with abnormal angiogenesis. Embryonic stem cell lines lacking this gene fail to respond to
hypoxic and hypoglycaemic challenges with the expected induction of glycolytic enzymes (Maltepe et al., 1997). Thus, AHR and ARNT are not only critical for cell differentiation, but also for angiogenesis and adaptive responses to hypoxia.

Human studies examining the physiology of AHR/ARNT are scant. High levels of AHR are detectable in the liver, lung, placenta and thymus (Pohjanvirta and Tuomisto, 1994). A functional aryl hydrocarbon receptor has been reported in the rat (Bhattacharyya et al., 1995) and mouse (Nesaretnam et al., 1996) uterus. Although AHR knockout mice showed no histopathological changes in uterine tissues (Schmidt and Bradfield, 1996), Safe and Krishnan have provided indirect evidence that the uterus is one of the targets of dioxin, as dioxin inhibits estrogen-induced responses including estrogen and progesterone receptor binding in rat uteri (Safe and Krishnan, 1995). In addition, intrauterine and lactational exposure to dioxin results in decreased uterine weights in female offspring during estrus and diestrus (Theobold and Peterson, 1997). Most recently Kuchenhoff et al. reported AHR immunoreactivity localized exclusively in the apical part of the cytoplasm in the epithelial cells of the human endometrial glands. Maximum immunostaining was found around the time of ovulation, with decreased expression of AHR with ageing (Kuchenhoff et al., 1999). However, the tissue distribution of ARNT throughout the reproductive tract has not been well studied. The purpose of this study was to characterize the tissue expression of AHR and ARNT mRNA during the menstrual cycle, and to examine the influence of exogenous sex hormones or the presence of fibroids on uterine AHR and ARNT mRNA expression. The expression of AHR and ARNT in ovaries and in endometriomas and ovarian cancers was also examined.

Materials and methods

Subjects

The characteristics of the subjects from whom uterine tissue was obtained for the menstrual cycle study and their indications for surgery are shown in Table I. For the menopause study, endometrial and myometrial tissues were obtained from two different groups of patients, with four patients per group. In the case of the endometrium, the mean age of patients not on hormone replacement therapy (HRT) was 69.7 ± 4.2 (mean ± SEM) years, and that of women on HRT was 62.0 ± 4.9 years. In the case of myometrium, the mean age of women not on HRT was 67.0 ± 2.0 years and those on HRT was 66.2 ± 2.9 years respectively. There were no statistical differences in the mean age of the different groups. Indications for surgery included uterine prolapse (four patients), abnormal bleeding (four patients), symptomatic leiomyomas (five patients) and chronic pain (three patients). For the study on uterine fibroids, the mean age of the subjects was 48.0 ± 4.7 years and none of the patients had received GnRH analogues or oral contraceptives. In the ovarian study comparing malignant tissue with normal and endometriotic tissues, tumour tissue was obtained from metastatic tumour implants from five patients with stage III–IV epithelial ovarian carcinomas (patients’ ages were unavailable). Patients with ovarian endometriomas had a mean age of 37 ± 1.3 years and were undergoing laparoscopy or laparotomy primarily for infertility and chronic pelvic pain. All of these patients had stage III–IV disease and had not been treated with oral contraceptives or GnRH analogues. Normal ovarian tissue, confirmed by pathology, was obtained from nine women (mean age 38 ± 3.9 years) who were undergoing total abdominal hysterectomy or oophorectomy for pelvic pain, abnormal bleeding or symptomatic myomas. They had not received any other treatments, except one woman who was on continuous HRT.

Collection of tissue samples

Tissue was obtained from women undergoing surgery for benign and malignant conditions, and stored frozen at –70°C until processed. For uterine tissues, the endometrium was dissected carefully to avoid any myometrial tissue. Cycle phase was determined by histological analysis of paraffin-embedded sections according to previously described criteria (Noyes et al., 1950). IRB approval was obtained from the Human Subjects Committee at the University of Wisconsin. Informed consent was not obtained from the patients because the study was exempt as no identifying information was used.

Ribonuclease protection assay (RPA)

Total RNA was isolated from tissues using RNA STAT-60 (Tel-Test, Friendswood, TX, USA). A 325 base pair (bp) fragment of ARNT and a 316 bp fragment of AHR were each amplified by PCR from human ARNT and AHR cDNAs (Dolwick et al., 1993) (kindly provided by Chris Bradfield, University of Wisconsin) and subcloned into PGEM-3Zf(+) (Promega, Madison, WI, USA). These plasmids, as well as pTRI-actin-125-Human internal control template (Ambion, Austin, TX, USA), were used as templates for riboprobe synthesis. Antisense RNA probes labelled with 32P-CTP were generated from the linearized DNA templates by in-vitro transcription with T7 RNA polymerase (Ambion) and were purified by polyacrylamide gel-electrophoresis. The RNase protection assays were performed using an RPA III kit (Ambion) according to the manufacturer’s instructions. Briefly, RNA samples (40 μg) were co-precipitated with 70 000 cpm of cRNA probe and allowed to hybridize at 42°C overnight in hybridization buffer. Unhybridized RNA was digested with RNase A/RNase T1 mix (for ARNT) or RNase T1 only (for AHR). Protected cRNA fragments were fractionated through 5% acrylamide/8 mol/l urea gels and analysed with a Storm 840 PhosphorImager (Molecular Dynamics, Sunnyvale, CA, USA). Semi-quantitative analysis of mRNA expression was accomplished by densitometry of the captured data with ImageQuant software (Molecular Dynamics).

Statistics

Results were analysed by paired or unpaired Student’s t-test for normally distributed data. For data that was not normally distributed, the Wilcoxon test was used for analysis of paired data and the Mann–Whitney U-test for non-paired data. Correlation analysis was done using the Pearson test. Significance was established at P < 0.05.

Results

The demographic data of the study population for the menstrual cycle study and indications for surgery in these patients are shown in Table I. There were no group differences with respect

Table I. Characteristics of subjects from whom uterine tissue was obtained

<table>
<thead>
<tr>
<th>Indications (n)</th>
<th>Proliferative phase</th>
<th>Secretory phase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean age (year) ± SEM</td>
<td>37.8 ± 1.8</td>
<td>40.3 ± 1.4</td>
</tr>
<tr>
<td>Menometrohagia/dysmenorhoea</td>
<td>11</td>
<td>11</td>
</tr>
<tr>
<td>Prolapse</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Symptomatic myomas</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Other</td>
<td>1</td>
<td>–</td>
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to the age of the studied populations. AHR and ARNT were detectable in all reproductive tissues examined, with both transcripts showing a similar pattern of expression. Relatively higher levels of AHR and ARNT transcripts were detected in the Fallopian tube and placenta, while similar lower levels of expression occurred in the endometrium, myometrium and ovary (Figures 1 and 2).

There were no differences in the expression of AHR or ARNT mRNA in the endometrium and myometrium in the proliferative phase as compared with the secretory phase of the menstrual cycle (Table II). Furthermore, there were no differences in mRNA expression of these transcription factors in pre- versus post-menopausal women (data not shown), and no correlations were found between age and the expression of AHR or ARNT in the uterus. In post-menopausal women, exogenous hormones administered as a combination of estrogen and progesterone daily induced a significant increase ($P < 0.05$, Student’s $t$-test and Mann–Whitney $U$-test) in the expression of AHR mRNA in the endometrium and myometrium. These changes were not found for ARNT mRNA (Figure 3).

The expression of AHR and ARNT mRNA in normal myometrium and uterine leiomyomas is shown in Figure 4. There was significantly lower expression of both AHR ($P < 0.05$, paired Student $t$-test) and ARNT ($P < 0.04$, Wilcoxon test) in uterine myomas when compared with normal myometrium obtained from the same patients.

Ovarian expression of AHR and ARNT was unaffected by ageing (data not shown). However, a significant increase ($P < 0.05$, Student’s $t$-test) in the expression of AHR, but not of ARNT, mRNA was found in endometriotic cysts as compared with normal ovarian tissue (Figure 5). There were no changes in the expression of AHR or ARNT mRNA in metastatic human ovarian cancer implants as compared with normal ovaries (Figure 5).

**Discussion**

Our study demonstrates that AHR and ARNT mRNAs are widely expressed in human reproductive tissues. Both transcription factors show a similar pattern of distribution. This would be expected since dimerization of the two factors is required in order to elicit a transcriptional response. The expression of AHR in reproductive tissues provides a basis by which environmental dioxin could potentially influence the function of these tissues. Other investigators have reported high levels of AHR mRNA expression in the human placenta (Dolwick et al., 1993) and endometrium (Kuchenhoff et al., 1999). To our knowledge, this is the first report on AHR and ARNT expression in conditions such as endometriosis and leiomyomas.

The expression of AHR and ARNT mRNA did not change during the menstrual cycle in the endometrium and myometrium. This is in contrast to the findings of Kuchenhoff et al. who reported higher expression of AHR protein in the secretory phase, particularly around the time of ovulation (Kuchenhoff...
Figure 2. Expression of ARNT mRNA in the female reproductive tract (top panel), and densitometric analysis of these data presented as mean \pm SEM (bottom panel).

Table II. Endometrial and myometrial AHR and ARNT mRNA expression during the menstrual cycle. Values are expressed as densitometric band intensities for AHR or ARNT relative to \( \beta \)-actin

<table>
<thead>
<tr>
<th>Tissue</th>
<th>AHR</th>
<th></th>
<th>ARNT</th>
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<tbody>
<tr>
<td></td>
<td>Mean \pm SEM</td>
<td>( n )</td>
<td>Mean \pm SEM</td>
<td>( n )</td>
</tr>
<tr>
<td>Endometrium</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Proliferative</td>
<td>0.16 \pm 0.06</td>
<td>16</td>
<td>0.014 \pm 0.002</td>
<td>9</td>
</tr>
<tr>
<td>Secretory</td>
<td>0.16 \pm 0.05</td>
<td>16</td>
<td>0.014 \pm 0.004</td>
<td>9</td>
</tr>
<tr>
<td>Myometrium</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Proliferative</td>
<td>0.027 \pm 0.007</td>
<td>7</td>
<td>0.011 \pm 0.002</td>
<td>8</td>
</tr>
<tr>
<td>Secretory</td>
<td>0.021 \pm 0.006</td>
<td>8</td>
<td>0.014 \pm 0.003</td>
<td>8</td>
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et al., 1999). These differences may be attributed to measurement of protein by a semi-quantitative technique of immunohistochemistry (Kuchenhoff et al., 1999) versus mRNA expression as determined by the quantitative technique of ribonuclease protection assay (present study). Our results suggest that endogenous steroids do not influence transcription of AHR and ARNT. However, other regulatory factors that come into play during the menstrual cycle could alter the translation of AHR. In contrast to previously reported results (Kuchenhoff et al., 1999), no correlations between age and expression of AHR or ARNT mRNA in the uterus and ovary were found. Again, differences in measurement of AHR protein versus mRNA may account for this discrepancy. Alternatively, there may be differential regulation of AHR transcription versus AHR translation.

In contrast to our data showing a lack of influence of endogenous sex steroids on AHR and ARNT mRNA levels, we found that continuous HRT up-regulated the expression of endometrial and myometrial AHR, but not of ARNT mRNA, in post-menopausal women. The discrepancy between the influence of exogenous versus endogenous sex steroids on AHR mRNA levels could be related to several factors, namely compositional differences between conjugated equine estrogen/medroxy progesterone acetate and circulating estradiol and progesterone, a lack of dynamic fluctuation in hormone levels as a result of HRT treatment, and differences in circulating levels of sex steroids in the menstrual cycle as compared with HRT. There is molecular evidence showing cross-talk between aryl hydrocarbon and estrogen-mediated signalling (Safe and Krishnan, 1995). Ligands of AHR have anti-estrogenic activity and estrogen blocks dioxin-induced accumulation of CYP1A1 mRNA and AHR-mediated activation of the CYP1A1 promoter (Kharat and Saatcioglu, 1996). The interaction of progesterone and AHR has not been explored.

The expression of both AHR and ARNT mRNA was lower in uterine myomas as compared with normal myometrial tissue from the same patients. Exposure to dioxin in utero results in
AHR and ARNT expression in the female reproductive tract

Figure 3. Expression of AHR (left side of figure) and ARNT (right side of figure) mRNA in the endometrium and myometrium of post-menopausal women not taking HRT (labelled as PM, clear bars) or post-menopausal women on continuous HRT (labelled as HRT, solid bars). Each bar represents the mean ± SEM from four specimens. *P < 0.05 compared with PM by Student’s t-test (endometrium) and Mann–Whitney U-test.

Figure 4. Expression of AHR and ARNT mRNA in normal myometrial tissue and uterine leiomyomas obtained from the same six patients. *P < 0.05 versus normal myometrium by Wilcoxon test. **P < 0.01 versus normal myometrium by Student’s paired t-test.

lowered uterine weights of the offspring (Theobold and Peterson, 1997) whereas estrogen is known to stimulate endometrial and myometrial proliferation. The significance and mechanism(s) by which down-regulation of AHR and ARNT mRNA occurs in myomatous tissue is unknown. However, based on the previously demonstrated antagonistic effects of dioxin and estrogen (Kharat and Saatcioglu, 1996), it may be speculated that higher tissue estrogen produced locally in myomas, as previously demonstrated (Sumitani et al., 2000), may down-regulate AHR and ARNT, and ligands of AHR may potentially be therapeutic for treatment of uterine fibroids.

The ovary was found to express both AHR and ARNT mRNA and their expression was not influenced by ageing. Greater expression of AHR, but not of ARNT mRNA, was found in endometriotic cysts. Dioxin exposure has been linked to the incidence of endometriosis (Rier et al., 1993), and women with this disease have higher circulating levels of dioxin (Mayani et al., 1997). The greater expression of AHR in endometriotic cysts suggests increased susceptibility to developing this disease secondary to the presence of elevated AHR expression. This appears to hold for specific tissues like the ovary but not the uterus, because when uterine expression of AHR mRNA was compared in women with and without endometriosis, no differences were found (data not shown). Bulun et al. have also found no differences in the expression of AHR mRNA in eutopic and ectopic endometrium of patients with endometriosis, whereas one of the target genes of dioxin, namely CYP1A1, was up-regulated in endometriotic tissue (Bulun et al., 2000). These investigators proposed that a potential link between dioxin and endometriosis may be through local formation of catechol estrogens or procarcinogens as a result of CYP1A1 activation (Bulun et al., 2000). The significance of high ovarian AHR expression in the pathogenesis of endometriosis remains to be determined, but
does signify the importance of the ovary as a target for dioxin and the potential action of dioxin on germ cells.

Dioxin has been shown to promote cell proliferation and tumour formation (Hankinson, 1995). One potential mechanism for the development of tumours might be greater expression of AHR in certain tissues. However, AHR transcription does not appear to play a role in ovarian or endometrial (data not shown) cancer, since the expression of both AHR and ARNT mRNA was unaltered in tumour-bearing tissue.

In summary, AHR and ARNT mRNAs are widely distributed in human female reproductive tissues. Although cyclic variation in the expression of these transcription factors was not detected, exogenous sex hormones administered in the form of HRT up-regulated the expression of AHR, but not of ARNT mRNA, in both the endometrium and myometrium. The expression of both AHR and ARNT mRNA was reduced in myometrial tissue compared with normal myometrium. Levels of AHR mRNA were higher in endometriotic cysts compared with normal ovarian tissue. Although dimerization of AHR and ARNT is necessary for transcription, these factors do not always seem to be regulated in the same fashion. These results suggest that reproductive tissues could be targets for environmental dioxin, and altered expression of these factors in uterine leiomyomas and endometriotic cysts suggests a role for these transcription factors in the pathogenesis of these common gynaecological conditions.

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References


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Figure 5. Expression of AHR and ARNT mRNA in undiseased premenopausal ovarian tissue (normal, nine specimens), endometriotic cysts (endometriosis, nine specimens) and metastatic ovarian cancer implants (cancer, five specimens). *P < 0.05 versus normal ovarian tissue by Student’s t-test.