The effect of long-term treatment with steroid hormones or tamoxifen on oestrogen receptors (α and β) in the endometrium of ovariectomized cynomolgus macaques

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Abstract

The effects of oestrogen are mediated by two specific intracellular receptors, oestrogen receptors (ER) α and β , which function as ligand-activated transcriptional regulators. Ovariectomized macaques (*Macaca fascicularis*) were used to study the regulation of ER α and ER β in the endometrium by immunohistochemistry and *in situ* hybridization after long-term hormone treatment. Animals were treated continuously for 35 months with either conjugated equine oestrogen (CEE), medroxyprogesterone acetate (MPA), combined CEE/MPA, or tamoxifen (TAM). Treatment with CEE/MPA down-regulated ER α in the superficial glands. In the superficial stroma the ER α level was lower in the CEE/MPA group than in the

CEE and MPA groups. ER β immunostaining was faint with minor variation in response to treatment, but increased in the superficial stroma after MPA treatment. The ratio of ER β /ER α increased in superficial stroma and gland after CEE/MPA treatment, and also in stroma after MPA and TAM. Cystic endometrial hyperplasia was observed in TAM-treated animals, in combination with a high level of ER α protein expression. The present data show that long-term hormone treatment affects the ER α and ER β protein levels in the endometrium. The balance between ER α and ER β seems to be important for the proliferative response to oestrogen.

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Introduction

Oestrogens have important effects on normal cell growth, differentiation and malignant progression in many reproductive tissues including the mammary gland (Henderson *et al.* 1982). Oestrogen alone or different combinations of oestrogen and progestogen are commonly used for reducing the menopausal symptoms among postmenopausal women on hormone replacement therapy (HRT), but are associated with a duration-dependent increased risk of endometrial carcinoma (Grady *et al.* 1992, 1995). In addition, others have shown that both progestogen-only contraception and continuous-combined HRT reduce endometrial cancer risk (Vihko & Isomaa 1989, Pike & Ross 2000). The anti-oestrogen tamoxifen (TAM) is the most commonly used treatment for patients with oestrogen receptor (ER)-positive breast cancers. TAM has opposite effects in different organs, acting as an oestrogen antagonist with antiproliferative effects in breast (Rose *et al.* 1985), while having agonistic effects in the endometrium (Helgason *et al.* 1982, Katzenellenbogen &

Katzenellenbogen 1996). Women receiving long-term TAM therapy have an increased risk of developing endometrial cancer (Malfetano 1990). The association between HRT or TAM treatment and endometrial cancer is still not clearly understood.

The effects of oestrogen are mediated by two specific intracellular receptors, ER α and ER β , functioning as ligand-activated transcriptional regulators. Although the affinity of ER β for oestradiol (E_2) is similar to that of ER α , they have different affinities for other oestrogen agonists and antagonists (Kuiper *et al.* 1996). The regulation of cellular responses is complex, since ERs not only mediate endocrine but also paracrine and autocrine signals (Dickson & Lippman 1987). Both *in vitro* and *in vivo* studies have suggested that human ER modulates the transcriptional activity of ER (Hall & McDonnell 1999, Weihua *et al.* 2000) and is a key determinant of cellular responses to agonists and antagonists (Hall & McDonnell 1999).

Oestrogens exert their effects in the target organs by binding to the receptors thereby modulating the expression of various proteins such as growth factors (Murphy & Ghahary 1990).

Table 1 Hormone levels in each group (means ± S.E.M.)

	OVX (n=5)	CEE (n=4)	MPA (n=5)	CEE/MPA (n=5)	TAM (n=6)
E ₂ (pg/ml)	14.7 ± 10.0	152.0 ± 31.5	3.2 ± 1.0	137.0 ± 29.5	0.8 ± 2.2
MPA (pg/ml)	0.0 ± 0.0	11.2 ± 7.4	81.7 ± 11.5	76.4 ± 10.8	30.0 ± 7.4
TAM (ng/ml)	—	—	—	—	4.5 ± 1.0

The distribution and regulation of ERα have been extensively studied in several mammalian species (Clark *et al.* 1992). A few studies have shown ERβ expression in the endometrium of monkeys (Pelletier *et al.* 1999, Critchley *et al.* 2001) and humans (Matsuzaki *et al.* 1999, Critchley *et al.* 2001, Lecce *et al.* 2001) with a cyclic change during the menstrual cycle. The data suggest that ERβ may have important roles in endometrial function in humans and nonhuman primates. However, no studies have been performed comparing regulation of ERα and ERβ in the endometrium after long-term hormone treatment.

Macaques are similar to human beings in many aspects of reproductive biology such as endometrial responses to exogenous oestrogen (Brenner & Slayden 1994), sex steroid receptor expression (Brenner *et al.* 1990) and expression of oestrogen- and progestin-regulated genes (Ace & Okulicz 1995). The aim of this study was to compare the expression of ERα and ERβ in the endometrium after long-term exposure to conjugated equine oestrogen (CEE), medroxyprogesterone acetate (MPA), CEE/MPA or TAM by using ovariectomized (OVX) animals. The results will give an indication of the effects of HRT and possible explanations for the different effects on the endometrium found after treatment with these compounds.

Materials and Methods

Animals

The 25 female adult cynomolgus macaques (*Macaca fascicularis*) used in this study were imported from Indonesia to the United States. All the macaques were 4–6 years old. They were housed in social groups of four to

eight monkeys each in a facility. Bilateral ovariectomies were performed 3 months before initiation of hormone treatment. Twenty monkeys were treated continuously with either 0.625 mg/day CEE; 2.5 mg/day MPA; the combination of CEE+MPA; or 20 mg/day TAM. The compounds were administered in the diet for 35 months at doses equivalent, on a caloric basis, to those given to women (Adams *et al.* 1997). The animal study was approved by the Association for the Advancement and Accreditation of Laboratory Animal Care and Use Committee in the US.

Hormone measurement

Serum concentrations of E₂ and MPA were determined by RIA (Wilson *et al.* 1988). The TAM level was measured by HPLC described by Langan-Fahey *et al.* (1990).

Tissue collection

Uterine tissues were collected at the end of the treatment period, when all the animals were killed. Tissues were fixed in 4% paraformaldehyde for 24 h and stored at 4 °C in 70% ethanol. Thereafter tissues were trimmed to 3 mm in thickness and embedded in paraffin.

Immunohistochemistry

Paraffin sections (5 μm) were used and a standard immunohistochemical technique (avidin–biotin–peroxidase) was carried out as described before (Wang *et al.* 1999) for immunolocalization of ERα and Ki67. A two-step indirect method (using an enzyme-labelled secondary antibody)

Figure 1 (Opposite) Immunohistochemical localization of ERα and ERβ in the endometrial stroma (Str), GE and LE. Positive immunostaining was present in the nuclei. The left column shows ERα in both superficial and basal layers of the endometrium in a representative animal from each group ((a) OVX; (d) CEE; (g) MPA; (j) CEE/MPA; and (m) TAM) at low magnification (× 100, bar=100 μm). The middle column shows ERα in the superficial or basal layer at a high magnification (× 400, bar=20 μm) ((b) OVX (superficial layer); (e) CEE (basal layer); (h) MPA (superficial layer); (k) CEE/MPA (basal layer); and (n) TAM (superficial layer)). The right column shows ERβ staining in the endometrium of a representative animal from each group ((c) OVX (superficial layer); (f) CEE (basal layer); (i) MPA (superficial layer); white arrow: positive vascular endothelial cells); (l) CEE/MPA (basal layer); and (o) TAM (superficial layer)) at a high magnification (× 400, bar=20 μm). (r) A negative control for ERβ, incubated with the ERβ antibody pre-absorbed overnight with a synthetic ER peptide. (s) A positive control of ERβ staining as shown in breast tissue from an OVX monkey. Magnification × 400 (bar=20 μm). (p and q) Ki67 immunostaining ((p) OVX, (q) CEE) at low magnification (× 100). A high magnification picture (× 400) is shown in the right corner.

was carried out for ER β immunostaining (Wang *et al.* 2001). The different immunostaining procedures for each specific antibody were performed as follows.

ER α A monoclonal mouse antihuman antibody, recognizing the A/B region, was used for detection of ER α (08-1149; Zymed Laboratories, Inc., San Francisco, CA, USA). The primary antibody was incubated overnight at 4 °C. The secondary antibody, biotinylated horse anti-mouse (BA-2000; Vector Laboratory, Burlingame, CA, USA) was incubated for 1 h, and an avidin–biotin–peroxidase complex for 1 h at room temperature (RT). Replacement of the primary antibody with an equivalent concentration of non-immune mouse IgG was used for negative controls of ER α immunostaining.

ER β A polyclonal chicken ER β (503) antibody was used for the ER β immunostaining. This antibody is directed against a hybrid between human and rat ER β . The preparation of this antibody was described by Saji *et al.* (2000).

Tissue sections were incubated with 1:500 dilution of ER β antibody overnight at 4 °C in PBS with 3% BSA. To obtain negative controls, incubations were done with the ER β antibody pre-absorbed with ER β protein (Panvera, Madison, WI, USA) (1:50 (v/v) overnight at 4 °C). After washing, sections were incubated with peroxidase-conjugated rabbit anti-chicken IgG (A-9046; Sigma Chemical Co.) for 1 h at RT.

The peroxidase substrate diaminobenzidine was used to visualize the immunostaining reaction (SK 4100; Vector Laboratory). Thereafter the procedure was as described before (Wang *et al.* 1999).

Ki67 A monoclonal mouse antihuman antibody (MM-1) was used for Ki67 immunostaining (Novocastra, Newcastle upon Tyne, UK). The procedure was the same as that for ER α (see above). Vector Red (Vector Laboratory) was used to visualize the immunostaining reaction.

Image analysis

A Leica microscope and Sony video camera (Park Ridge, NJ, USA) connected to a computer with an image analysis system (Leica Imaging System Ltd, Cambridge, UK) were used to assess semi-quantitative values from ER α and ER β immunohistochemistry. The quantification of immunostaining was performed as described previously (Wang *et al.* 1999). In short, by using colour discrimination software the total area of positively stained nuclei was measured, and expressed as a ratio of the total area of cell nuclei.

Statistics

Statistical calculations for the immunohistochemistry results were performed by ANOVA on ranks (Kruskal–Wallis test) and significances were evaluated by Dunn's test. Values with different letter designations are significantly different ($P < 0.05$).

Results

Hormone levels

The mean values for E₂, MPA and TAM in the serum of the monkeys in the different groups are shown in Table 1.

Immunohistochemistry

Immunohistochemistry showed that ER α and ER β were present in the nuclei of cells in all compartments of the endometrium (Fig. 1). Variations were found in the levels of ERs between the superficial and basal layers of the endometrium. Hormone and TAM treatment induced changes in the histological features of the endometrium, e.g. CEE induced thickening of the endometrium and increased the glandular area fraction (Fig. 1d). MPA treatment did not induce distinguishable morphological changes (Fig. 1g) as compared with controls (Fig. 1a). TAM induced cystic endometrial hyperplasia (Fig. 1m).

ER α In the OVX control group, ER α immunoreactivity was similar in the stroma of the superficial and basal layers (Fig. 1a and Fig. 2a and b). The ER α level was high in the superficial stroma in the CEE and MPA groups (Fig. 1d and g and Fig. 2a), but was significantly decreased in the CEE/MPA group (Fig. 1j) as compared with the CEE and MPA groups (Fig. 2a). In addition, the superficial stroma cells in the CEE/MPA group showed decidualization, and ER α staining was faint (Fig. 1j). The variation in the ER α level in the basal stroma was less, except in the MPA group where the ER α level was significantly higher than in the OVX controls (Fig. 2b).

In the glandular epithelium (GE), over 95% of the nuclei stained positive for ER α in the OVX and TAM groups with no difference between the superficial and basal layers (Fig. 1a, b, m, n and Fig. 2c and d). In the glands of the superficial layer a gradual decrease of ER α immunoreactivity was observed in the CEE, MPA and CEE/MPA groups (Fig. 2c), although only to a significant level in the CEE/MPA group as compared with the OVX group (Fig. 2c). In the glands of the basal layer no differences between groups were found (Fig. 2d). Almost all epithelial cells were strongly positive after TAM treatment (Fig. 1m and n and Fig. 2c–e). There was a significant difference in the luminal epithelium (LE)

between the TAM- and MPA-treated animals (Fig. 2e). No immunostaining was found in the negative controls (data not shown).

ER β Most ER β immunostaining was faint in all compartments of the endometrium and in all study groups (Fig. 1c, f, i, l, o). High immunoreactivity of ER β was observed in the mammary gland of the same animals, which was used as a positive control in this study (Fig. 1s). The image analysis results imply less variations than in ER α levels both within and between the different groups, but the ER β level in the superficial stroma after MPA treatment is significantly increased as compared with the OVX control (Fig. 1i and Fig. 3a).

It appears that overall ER β immunoreactivity was higher in the LE (Fig. 3e) as compared with the stroma and glands (Fig. 3a–d). No specific nuclear staining was found in the negative control sections after incubation with a peptide corresponding to the epitope of the ER β antibody (Fig. 1r).

The ratio of ER β /ER α immunoreactivity in the functionalis was estimated from the image analysis results and presented as means $\pm$ S.E.M. (Table 2). The results imply that the ratio was increased in superficial stroma and GE after CEE/MPA treatment, and also in stroma after MPA and TAM treatment (Table 2), but it could not be statistically verified.

Ki67 The Ki67 immunostaining in the different treatment groups is shown in Table 3. The highest level of Ki67-positive cells was found in the superficial gland after CEE treatment (Fig. 1q and Table 3). The Ki67-positive staining was very low or absent in the basal stroma and glands. There were no significant differences observed between the groups (Fig. 1p and q and Table 3).

Discussion

Epidemiological studies have shown that continuous-combined oestrogen and progesterone (P₄) replacement therapy is not associated with any increased risk of endometrial cancer (Pike & Ross 2000). This may be due to decreased endometrial cell proliferation caused by progestin in the uterus (Lane *et al.* 1986, Persson 2000). The present result and previously published data (Cline *et al.* 2001) demonstrated that 3 years of continuous oral treatment with CEE induced a marked glandular proliferation (increased Ki67 labelling) with thickening of the endometrium, and an increased glandular area fraction within the tissue. MPA reduced such proliferation and even reduced the CEE-stimulated proliferation when given in a continuous combined treatment with CEE (Cline *et al.* 2001). The decreased ER α level in the superficial GE and LE in this group implies that the MPA-antagonized epithelial proliferation could be due to

a decrease in the ER α level. ER β was found to be present in human endometrium with a decreased level in GE in the late secretory phase as compared with the other phases of the menstrual cycle (Critchley *et al.* 2001, Lecce *et al.* 2001). It was suggested that the low ER β level is associated with the declined P₄ concentration in this stage (Critchley *et al.* 2001). In the rhesus macaque endometrium during a hormonally controlled cycle (treated sequentially with E₂ or E₂+P₄ for 14 days), the pattern of ER β staining was identical to that seen in the human endometrium (Critchley *et al.* 2001). It is known that P₄ induces proliferation of stromal cells (Finn & Martin 1978). In the present study an increased immunoreactivity of ER β was observed in the superficial stroma after MPA treatment. In addition, the increased ratio of ER β /ER α in the stroma after CEE/MPA treatment, and also after MPA treatment, suggests that the balance between these two receptors might determine the oestrogen response and indicate the rate of proliferation. However, the effects from MPA treatment on ER β /ER α expression are most probably secondary to progestin-mediated PR activity.

Combined treatment with CEE and MPA, as given in this study, reduced the ER β protein in the basal stroma and glands, whereas the ER α level remained high. This suggests that ER α and ER β are regulated differently in the functionalis and basalis of the endometrium after long-term hormone treatment. This lesser effect on ERs in response to steroids in the basal layer as compared with the functional layer is in agreement with recently published results from humans and monkeys during the menstrual cycle and a hormonally controlled cycle respectively (Critchley *et al.* 2001).

Previous studies on the rat uterus did not find any dominant effects on ER β expression after short-term E₂ and/or P₄ treatment (Shughrue *et al.* 1998, Wang *et al.* 1999). In addition, the lack of trophic responses to oestrogen in female ER α knock out (α ERKO) mice might suggest that ER β plays a minor role in the rodent uterus. In immature β ERKO mice, cell proliferation in the LE of the uterus was increased and cell responsiveness to E₂ was enhanced, which suggests an anti-uterotrophic role of ER β and therefore an important role in regulating cellular proliferation in rodent uteri (Weihua *et al.* 2000). These data, together with ours, indicate that the steroid-mediated effects of the ERs in the endometrium are different after long- and short-term treatment, and also in between species and/or developmental stage (e.g. immature or mature, premenopausal or postmenopausal).

The transcriptional activity of ERs in the oestrogen signalling pathways depends on dimerization, where the ER α /ER α and ER β /ER β homodimers, or ER α /ER β heterodimers, bind directly to the classical oestrogen responsive element (ERE) in the target genes (Pettersson *et al.* 1997). Varying ratios of ER α and ER β in different

Figure 2 Image analysis scores of positive ER α immunoreactivity in superficial stroma (A), basal stroma (B), superficial glands (C), basal glands (D) and LE (E). Box and whisker plots representing the median value with 50% of all data falling within the box. The whiskers extend to the 5th and 95th percentiles. In OVX, MPA and CEE/MPA groups $n=5$, in CEE group $n=4$ and in the TAM group $n=6$. Values with different letter designations are significantly different ($P < 0.05$).

cells result in different populations of homo- and heterodimers, which could constitute a mechanism for the tissue- and cell type-specific actions of not only oestrogens, but also of anti-oestrogens (Kuiper & Gustafsson 1997, Pettersson *et al.* 1997). The presence of both ER α and

ER β in the macaque endometrium enables the formation of both homo- and heterodimers. Therefore, ER β could act as a modulator of ER α -mediated gene transcription in the uterus (Weihua *et al.* 2000), as indicated by the changes in ER β /ER α ratios.

Figure 3 Image analysis scores of positive ER β immunoreactivity in superficial stroma (a), basal stroma (b), superficial glands (c), basal glands (d) and LE (e). Box and whisker plots representing the median value with 50% of all data falling within the box. The whiskers extend to the 5th and 95th percentiles. $n=5$ in OVX, MPA and CEE/MPA groups (except LE, $n=3$ in CEE/MPA group since two samples lacked LE); $n=4$ in the CEE group; and $n=6$ in the TAM group. Values with different letter designations are significantly different ($P<0.05$).

TAM is used in breast cancer therapy and functions as an oestrogen antagonist by competing with oestrogen binding to ER, inhibiting the action of ER in breast tissue

(Jordan 1994). In the uterus, TAM acts as a partial oestrogen agonist. This tissue-specific agonistic effect may explain the increased risk of endometrial cancer that is

Table 2 Ratios of ERβ/ERα in the endometrium in the different study groups (means ± S.E.M.)

	OVX (n=5)	CEE (n=4)	MPA (n=5)	CEE/MPA (n=5)	TAM (n=6)
Cell type					
Superficial stroma	0.01 ± 0.00	0.06 ± 0.03	0.15 ± 0.04	0.17 ± 0.06	0.08 ± 0.03
Basal stroma	0.01 ± 0.00	0.01 ± 0.01	0.03 ± 0.01	0.05 ± 0.03	0.02 ± 0.00
Superficial GE	0.35 ± 0.10	0.26 ± 0.12	0.38 ± 0.10	0.84 ± 0.32	0.35 ± 0.04
Basal GE	0.19 ± 0.06	0.16 ± 0.07	0.14 ± 0.04	0.20 ± 0.08	0.17 ± 0.04
LE	0.66 ± 0.13	0.41 ± 0.14	0.61 ± 0.20	0.50 ± 0.11	0.47 ± 0.03

Table 3 Image analysis score (%) of positive Ki67 staining cells in the stroma and gland (median and range). No significant differences were observed

	OVX (n=5)	CEE (n=4)	MPA (n=5)	CEE/MPA (n=5)	TAM (n=6)
Cell type					
Superficial stroma	0 (0–1.25)	1 (1–7)	2 (0.75–7.25)	6 (2–14)	0 (0–8.75)
Basal stroma	0 (0–3.5)	0 (0–0)	0 (0–0.5)	0 (0–3)	0 (0–0)
Superficial GE	2 (0–13.75)	37 (15.5–46.5)	4 (1.5–46.5)	0 (0–12.5)	10 (3.75–19)
Basal GE	0 (0–1)	0 (0–0)	0 (0–22.75)	0 (0–1.5)	0 (0–1.75)

observed with prolonged TAM therapy (Kedar *et al.* 1994). *In vitro* studies have demonstrated that the agonistic effect of TAM depends on the cell type, ERE-promoter context and ER subtype (Watanabe *et al.* 1997), and that it is also mediated by the ER/AP-1 pathway (Webb *et al.* 1995).

ERβ can function as a transdominant inhibitor of ERα transcriptional activity at sub-saturating hormone levels and to decrease overall cellular sensitivity to E₂ *in vitro* (Hall & McDonnell 1999). The partial agonistic activity of TAM manifest via ERα was completely abolished upon coexpression of ERβ with ERα (Hall & McDonnell 1999). We speculate that a reduced repression from ERβ results in an unopposed proliferation from ERα activity, an effect that could be responsible for the increased risk of endometrial carcinoma after TAM treatment. When looking at the ratios of ERβ/ERα immunoreactivity in the different groups, TAM treatment showed an increased level only in the superficial stroma as compared to OVX. However, the regulation and relationship between the two subtypes of ER are more complicated *in vivo* than *in vitro* since there are many factors directly or indirectly involved in the endocrine system.

In conclusion, the present data further confirm that the multifunctional activities of oestrogen and progesterin in the uterus are mediated by a complex regulation of ERs (Tibbetts *et al.* 1998, Wang *et al.* 1999) in the different compartments. This is, to our knowledge, the first study on the effect of long-term hormone treatment on the expression of ERβ in primate endometrium. ERβ levels vary less than those of ERα. In addition, ERβ is distributed predominantly in the functionalis of the endometrium, while ERα is found in considerable amounts also in the basalis. The endometrial hyperplasia found after TAM treatment could be due to the increased ERα level observed in the present study. The ratio of

ERβ/ERα after the different treatments could determine the oestrogen response and indicate the rate of proliferation. Thus, ERβ might be of importance in repressing the proliferative activity of ERα. Further studies are needed to determine the importance of ERβ/ERα interactions for proliferative activity in the endometrium and possible side-effects of HRT and TAM therapy.

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