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Contrasting Effects of Tamoxifen and ICI 182 780 on Estrogen-induced Calbindin-D 9k Gene Expression in the Uterus and in Primary Culture of Myometrial Cells

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Antiestrogens have a large range of tissue- and promoter-specific actions, many of which still remain unclear, particularly in the uterus. Thus, we have analyzed the effects of two antiestrogens, tamoxifen (TAM) and ICI 182 780 (ICI) on the uterine estrogen-responsive gene calbindin-D9k (CaBP9k), in the ovariectomized rat uterus, and in primary cultures of myometrial cells. In the ovariectomized rat uterus, estradiol (E_2) or E_2 plus TAM induced CaBP9k mRNA to the same levels in 6h. Rats given TAM alone had the same mRNA concentration, but maximal induction was obtained later, 12h after injection. ICI alone did not induce CaBP9k gene expression. Rats given E_2 plus ICI had low uterine CaBP9k mRNA levels at 6–12h that became undetectable at 24h. Thus ICI has a full antagonistic effect on E_2-induced CaBP9k gene. Estradiol receptor (ER) assays showed that TAM had a partial antagonist effect, while ICI had a full antagonist effect on the ER. We also analyzed the effect of TAM and ICI on CaBP9k gene expression in primary cultures of myometrial cells. The effects were similar to those observed in whole uterus. Thus, TAM has mixed effects, being an agonist for CaBP9k gene induction, and an antagonist for ER. ICI antagonizes the effects of E_2 on the CaBP9k gene in myometrial cells and in the intact uterus, but in a way that does not involve a decrease in the cellular content of ER. Instead, it interferes with at least one of the events leading to transcriptional activation.

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INTRODUCTION

Calbindin-D9k (CaBP9k) is an intracellular calcium binding protein whose gene is strongly expressed in the rat duodenum, under the control of calcitriol [1, 2]. This gene is also estradiol-responsive in the rat uterus. CaBP9k is present in the myometrium and in the stromal cells of non-pregnant animals [3] and it appears in the epithelium of pregnant rats [4, 5]. Estradiol (E_2) is the major factor controlling the expression of the CaBP9k gene in the uterus. The CaBP9k gene is not expressed in the immature or ovariectomized rat uterus. Its expression increase after a single injection of 17β-estradiol [6]. During the estrous cycle, the CaBP9k mRNA concentration is maximal at proestrus, during the estrogen-dominated phase, and it drops at estrus to become undetectable at diestrus, when the circulating E_2 level decreases [7].

The CaBP9k gene contains an imperfect E_2 responsive element (ERE) [8, 9] that differs by only one nucleotide from the vitellogenin consensus ERE. Although this single nucleotide difference is important in the interaction between the estrogen receptor and the ERE, the CaBP9k ERE binds to the estrogen receptor [8, 9]. This ERE has been cloned in front of TK-CAT and analyzed by transfection in MCF-7 cells. It confers only a small induction of about 2-fold, but the induction is about 15-fold when it is cloned in tandem. This result suggests that the CaBP9k ERE cooperates with another *cis* element to induce this estrogenic response *in vivo*, where the CaBP9k gene is very dependent upon E_2 [9].

Non-steroidal antiestrogens, such as tamoxifen (TAM), have been used to treat breast cancer for several years [10, 11]. But these compounds have a wide

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range of effects, from complete antagonism to full agonist effect, depending on the target species, tissue, and gene [see 10 for review]. TAM is a partial antagonist for the estrogen receptor, and it is widely used clinically because of its efficacity in the treatment of hormone-dependent breast cancer. However, resistance to TAM can occur. In the uterus, TAM has frequently been described as an agonist for the estradiol receptor (ER). The uterotrophic effect of TAM has been observed in a variety of experimental systems [12, 13]. It stimulates uterine growth in the immature rat [14–16] and it induces complement component C3 mRNA in the rat uterus, as do true estrogens [17]. TAM can enhance endometrial carcinoma growth in athymic mice [12, 18]. These estrogenic effects of TAM in the uterus raise doubts about the use of TAM in breast cancer prevention.

"Pure" antiestrogens were, therefore, developed, and some, like the 7α-substituted steroids ICI 164 384 and ICI 182 780 (ICI), seem to behave as full antagonists [15, 16, 19]. They bind to the ER with high affinity [19] and completely inhibit the effects of estrogen on rat uterine growth [15, 19, 20], on the growth of MCF7 cells *in vitro*, and E_2-stimulated breast tumor and endometrial tumor growth in nude mice [12, 21]. However, these ICI drugs also have some estrogenic effects on progesterone receptor gene expression, as in the uterus of the fetal guinea pig [22], and in human endometrial Ishikawa cells [23].

Since their effects in uterine tissues remain unclear, it is important to analyze the response of uterine genes to antiestrogens. The present study examines the effects of TAM and ICI on the expression of the uterine E_2-responsive gene, CaBP9k. Two models were used. The first is the ovariectomized rat [6]. The second is a primary culture of myometrial cells isolated from the rat uterus. TAM was found to act as an E_2 agonist on CaBP9k gene expression, while ICI appears to be a full antagonist in both models. Our results also suggest that ICI inhibits CaBP9k gene expression in the rat uterus *in vivo*, in a way that does not involve a decrease in the cellular content of estrogen receptor protein. Instead, it interferes with at least one of the events leading to transcriptional activation.

MATERIALS AND METHODS

Materials

17β-Estradiol and TAM were purchased from Sigma (La Verpillère, France). ICI 182 780 was a gift from ICI-Pharmaceutical (Macclesfield, England). All radioisotopes and nylon membranes (Hybond N^+) were purchased from Amersham (Les Ulis, France). The random primed cDNA labelling kit was from Boehringer Mannheim (Meylan, France), and the ER-EIA kit was from Abbott (Rungis, France).

Animals and treatments

Mature ovariectomized female Sprague–Dawley rats (180–200 g) were obtained from Charles River (St Aubin Les Elbeuf, France). They were housed under controlled conditions of temperature and light (12h light–dark). Two weeks later, they were divided into groups of 4 rats. Group 1 was given 0.5 μg E_2/100 μl vehicle/100 g B.W, group 2 was given 100 μg TAM/100 μl vehicle/100 g B.W, group 3 was given 100 μg ICI/100 μl vehicle/100 g B.W, group 4 was given both E_2 and TAM and group 5 was given both E_2 and ICI. Control rats were given 100 μl vehicle/100 g body weight. Rats were killed by decapitation 6, 12, 24, 48, and 72 h after injection and the uterus was removed. One part was used for Northern blot analysis and the other part for ER assay.

Primary cultures of uterine myometrial cells

Immature female 21-day-old Sprague–Dawley rats (Charles River, St Aubin Les Elbeuf, France) were killed by pentobarbital injection and the uterus was rapidly excised and rinsed in sterile phosphate buffered saline (PBS). The endometrium was removed by digestion with trypsin (5mg/ml) for 1 h at 4°C. Myometrial cells were dissociated with collagenase (100μg/ml) at 37°C and collected by centrifugation at 2000 ***g*** for 10 min. They were plated out in M199 medium supplemented with 10% fetal calf serum (ATGC) and grew to confluence after 10 days in culture. The medium was then changed to M199 without phenol red, supplemented with 0.3% dextran-coated charcoal-treated donor calf serum (ICN). Hormone treatment was begun 24 h later. One group of four dishes of cells was incubated with 10^{-8}M E_2, one with 10^{-6}M TAM, one with 10^{-6}M ICI, one with E_2 plus TAM, and the last with E_2 plus ICI, for 24, 48, or 72 h. One group without any hormonal treatment served as control.

Northern analysis

Total RNA was extracted as previously described [7], and separated by electrophoresis on 1% agarose gel. In these experimental conditions, only one band was detected for the two uterine CaBP9k mRNAs [6, 9]. Total RNA was transferred by capillarity (2h) from the agarose gel to the nylon membrane using 0.05M NaOH. The membrane was washed in 2× standard saline citrate buffer (SSC) (0.15M NaCl, 0.015M Na citrate, pH 7.0) and hybridized and autoradiographed as previously reported [7]. 1A mRNA was used as an internal control [24].

The amounts of CaBP9k mRNA and 1A mRNA in each blotted RNA solution were quantified by spectrodensitometric analysis (Shimadzu Scientific Instruments, Columbia, MD) of four autoradiograms of Northern blots, corresponding to four rats or four dishes of cells for each time point. Peak areas were

directly proportional to the amount of radioactive ^{32}P cDNA bound to CaBP9k mRNA or 1A mRNA. The area for each CaBP9K mRNA concentration was compared to the areas of 1A mRNA internal control. The means and the standard deviations for each time point were calculated. Groups were compared using the Student's *t*-test when statistical significance was reached.

Preparation of cloned labeled cDNA probe

The CaBP9k [25] and 1A [24] cDNA probes were labeled with ^{32}P nucleotides by the "random-primed" procedure (Boehringer). The specific activity of the cDNA probes was about 3.10^9 cpm/μg DNA.

ER assay

Tissue were pulverized and homogenized in 10 mM Tris, 1.5mM EDTA, 5mM Na_2MoO_4, 1mM monothioglycerol. The total ER concentration was measured by immunoassay using the ER-EIA monoclonal kit according to the manufacturer's instructions (Abbott). Total proteins were estimated using the Bio-Rad protein assay (Paris, France).

RESULTS

Control of CaBP9k gene expression by E_2 and antiestrogens in the rat uterus in vivo

The results in Fig. 1 show that the antiestrogen TAM induced an increase in the concentration of CaBP9k mRNA similar to that induced by E_2. The kinetics of induction were different, since TAM induced CaBP9k gene expression maximally at 12h, 6h later than the maximal expression induced by E_2. Differences between Fig. 1(A and B) are due to experimental variations, since Fig. 1(A) shows a single Northern blot, whereas Fig.1(B) represents the results of four different blots. Standard deviations in Fig. 1(B) reflect these variations.

The antagonist effect of TAM on the E_2-induced increase in CaBP9k mRNA was tested by giving E_2 and TAM together (Fig. 1). TAM did not antagonize the E_2 stimulation of CaBP9k, and there was no synergistic increase in CaBP9k mRNA concentration in the animals given both TAM and E_2.

We also analyzed the effect on CaBP9k gene expression, of another antiestrogen, ICI 182 780 (ICI), which has been described as a "pure" antagonist

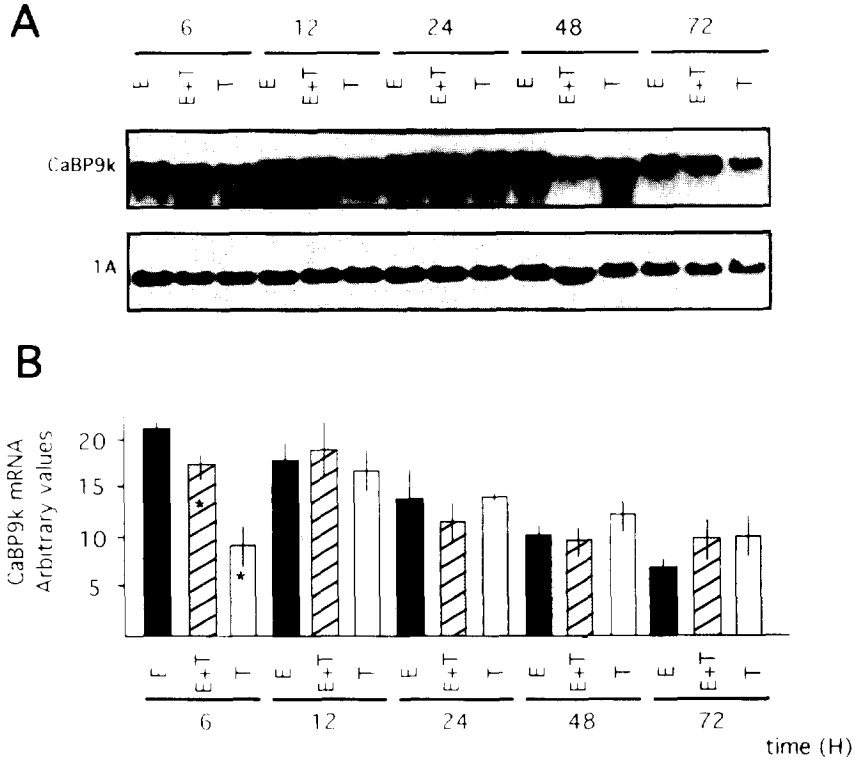

Fig. 1. Effect of TAM on CaBP9k mRNA in the uterus of ovariectomized rats. Ovariectomized rats were injected with estradiol (E), tamoxifen (T), or estradiol plus tamoxifen (E+T). Total RNA were extracted at 6, 12, 24, 48, or 72 h. Control animals were injected with vehicle alone (Ovx). CaBP9k mRNA were analyzed by Northern blot, 1A mRNA served as internal control. A single representative blot is shown (A). Four autoradiograms of Northern blots were analyzed by spectrodensitometry. These four blots correspond to four different rats for each time point. CaBP9k and 1A mRNA were scanned horizontally. (B) Each peak is the mean for four rats treated similarly. *P* was determined by Student's *t*-test: *E+T>T ($P<0.05$). Vertical bars represent the SEM.

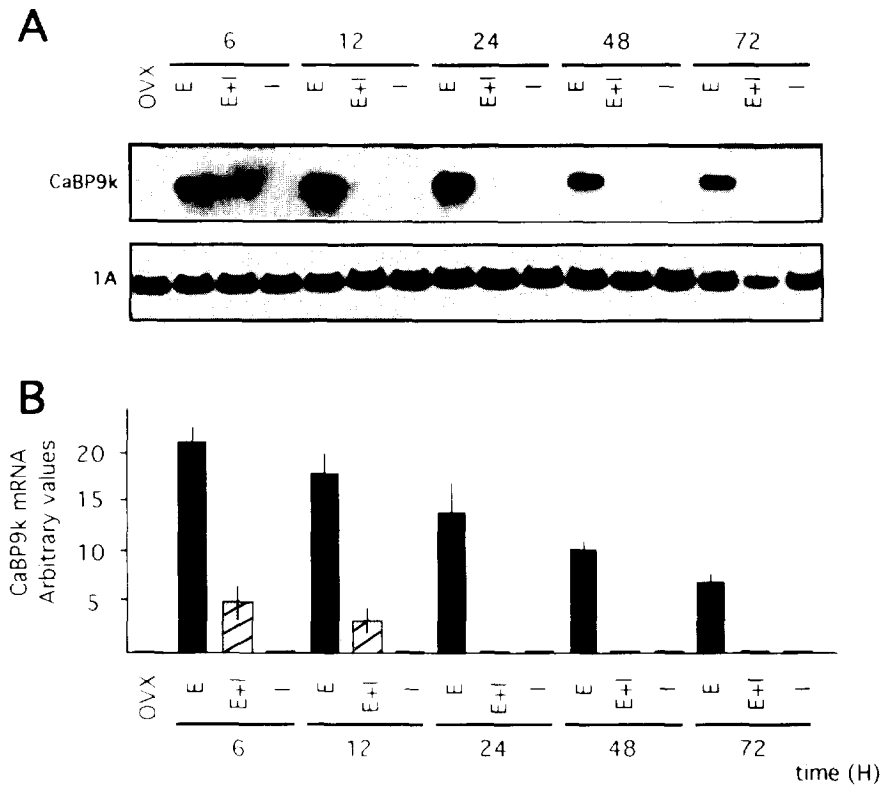

Fig. 2. Effect of ICI 182 780 on CaBP9k mRNA in the uterus of ovariectomized rats. Ovariectomized rats were injected with estradiol (E), ICI 182 780 (I), or estradiol plus ICI 182 780 (E+I). Total RNA were extracted at 6, 12, 24, 48, or 72 h. CaBP9k mRNA were analyzed by Northern blot, 1A mRNA served as internal control. A single representative blot is shown (A). Four autoradiograms of Northern blots were analyzed by spectrodensitometry. These four blots correspond to four different rats for each time point. CaBP9k and 1A mRNA were scanned horizontally. (B) Each peak is the mean for four rats treated similarly. *P* was determined by Student's *t*-test: *E+T>T (*P*<0.05). Vertical bars represent the SEM.

[20]. ICI had no effect on the uterine CaBP9k mRNA concentration when injected alone (Fig. 2). When it was injected with E_2, the expression of the CaBP9k gene was rapidly blocked, since 6h after injection of E_2 and ICI, the uterine CaBP9k mRNA concentration was only 25% of the level in the E_2-treated rats. ICI completely antagonized the E_2-induced increase in CaBP9k mRNA concentration at 24h (Fig. 2).

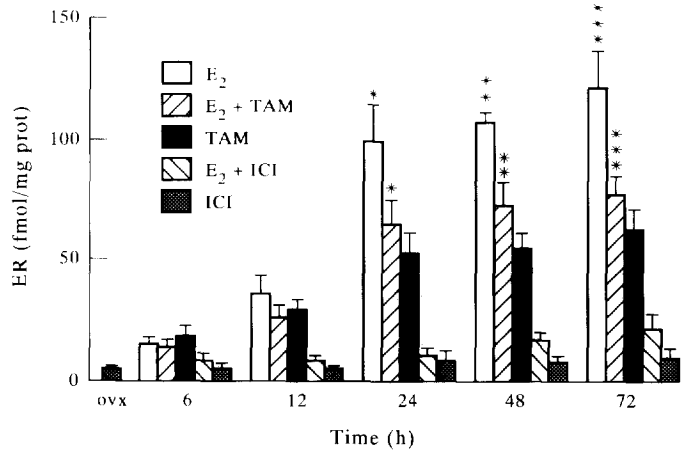

Fig. 3. Effects of TAM and ICI on total ER concentration in the uterus of ovariectomized rats. Ovariectomized rats were treated with estradiol (5 μg/kg) (E), tamoxifen (1mg/kg) (TAM), estradiol plus tamoxifen (E+TAM), ICI 182 780 (1mg/kg) (ICI) or estradiol plus ICI 182 780 (E+ICI). Animals were killed at 0 (ovx), 6, 12, 24, 48, and 72h. Uterine proteins were extracted and the total ER concentration was measured by enzyme immunoassay. Peaks represent the means for three rats treated similarly, corrected for the total protein concentration. *P* was determined by Student's *t*-test: *E>E+TAM (*P*<0.05), **E>E+TAM (*P*<0.01), ***E>E+TAM (*P*<0.01). Vertical bars represent the SEM. Ovx: ovariectomized rat.

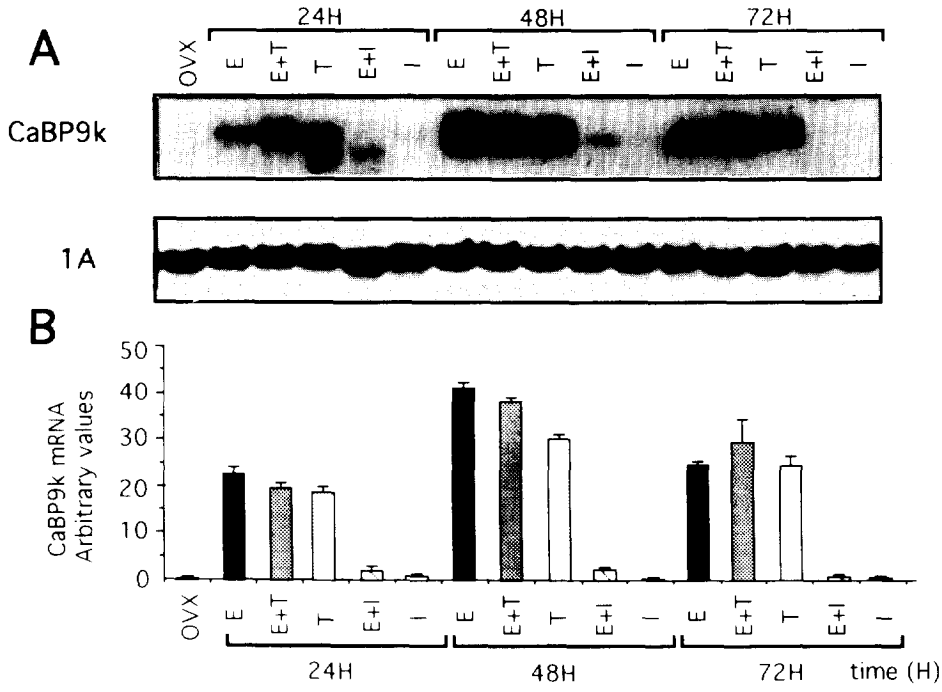

Fig. 4. Effect of TAM and ICI 182 780 on primary cultures of myometrial cells. Cells were incubated with 10^{-8}M estradiol (E), 10^{-6}M tamoxifen (TAM), estradiol plus tamoxifen (E_2+TAM), 10^{-6}M ICI 182 780 (ICI) or estradiol plus ICI 182 780 for 24, 48, or 72 h. Ovx : control cells. CaBP9k mRNA were analyzed by Northern blot. 1A mRNA served as internal control. A single representative blot is shown (A). Four Northern blots were analyzed by spectrodensitometry. These four blots correspond to four different rats for each time point. CaBP9k and 1A mRNA were scanned horizontally. Each peak represents the mean for four dishes of cells treated similarly (B). Vertical bars represent the SEM.

Since one way in which ICI could act is by reducing the half life of the estrogen receptor protein (ER) [26, 27], we measured the ER by enzyme immunoassay in the uterus of ovariectomized rat treated with E_2, TAM and ICI (Fig. 3). There was a 10-fold increase in the uterine ER 24h after giving the rats E_2, and a 5-fold increase after TAM. The effects of giving E_2 and TAM together indicate that TAM has some antagonistic effect on the E_2-induced increase in the uterine ER, since there was only a 6-fold increase in rats given E_2 plus TAM, as compared to a 10-fold increase in E_2-treated rats. ICI completely suppressed the induction of ER by E_2 without having an agonist effect itself. The uterine ER content of animals given ICI did not change and did not drop below that of ovariectomized rats, for at least 6h after the treatment (Fig. 3).

Control of CaBP9k gene expression by antiestrogens in primary cultures of myometrial cells

To analyze if the effect of the different antiestrogens were cell-specific, we have used rat primary cultures of myometrial cells in which CaBP9k gene is expressed. Northern blot analysis showed that CaBP9k mRNA were barely detectable in myometrial cells grown in E_2-free medium, as in the ovariectomized rat uterus (Figs 1 and 4). The CaBP9k mRNA concentration increased after incubation with 10^{-8}M E_2 (Fig. 4).

The effects of the two antiestrogens TAM and ICI on CaBP9k gene expression in the primary cultures of myometrial cells were similar to those obtained *in vivo* with ovariectomized rats. TAM was fully estrogenic, and induced CaBP9k mRNA concentration similar to those induced by E_2. There was no synergistic effect in cells given E_2 plus TAM. In contrast, ICI had a pure antagonist effect on the E_2-induced increase in CaBP9k mRNA (Fig. 4).

DISCUSSION

TAM was found to act as an estrogen agonist on CaBP9k gene induction, on the whole uterus as well as on myometrial cells. This is in agreement with Bruns *et al.* [4], who showed by immunoassay that TAM was a potent inducer of CaBP9k in the uterus of immature rats. Our results extend these data by showing that TAM induces an increase in uterine CaBP9k mRNA in ovariectomized rats similar to that induced by E_2, indicating that TAM acts as a full agonist on the CaBP9k gene in the uterus. The way in which estrogen and the antiestrogen act may be similar, since giving E_2 and TAM together had no synergistic effect on the CaBP9k mRNA. This is in agreement with the mode of action of TAM, which acts by binding to the E_2 receptor [11]. However, although we obtained similar CaBP9k mRNA responses to TAM and E_2, the kinetics of CaBP9k gene

induction were different. The maximal increase produced by TAM occurred 6h later than that of E_2. A similar delay has been observed for the induction of the complement component C3 mRNA [17]. Since the complement component C3 gene is expressed in the epithelial cells of the uterus, and CaBP9k is produced in the myometrium and stroma of the endometrium of non-pregnant animals, this suggests that the TAM action responsible for the delayed response peak of the C3 and CaBP9k genes is not cell-specific.

The effects of TAM on the CaBP9k gene and on the estrogen receptor gene are somewhat different. TAM has a full estrogenic effect on the CaBP9k gene, but does not induce an increase in uterine ER content to the same extent as E_2 does, and it has a partial antagonist effect on the induction of ER by E_2. These *in vivo* results are in agreement with the idea that the mixed antagonist/agonist action of TAM is due to the activation function AF1 of the estrogen receptor, and is consequently cell- and promoter-specific [28]. Our results suggest that AF1 is important for stimulating transcription after the binding of the E_2 receptor to the CaBP9k E_2-responsive element [9] in the uterus. By contrast, in the same cell, the activation of transcription for the estrogen receptor gene could depend on both AF1 and AF2 activity.

ICI 182 780 is generally believed to be a pure antagonist of E_2, devoid of any estrogenic activity [20], although there are reports that it could have an estrogenic effect on the progesterone receptor gene in isolated cells of the vagina and uterus of fetal guinea pig [22], or in human endometrial Ishikawa cells [23]. Our results also indicate that ICI is a pure estrogen antagonist. ICI alone had no estrogenic activity on the CaBP9k gene. When injected simultaneously with E_2, it dramatically reduced the E_2-induced increase in CaBP9k mRNA. The way in which ICI acts is still a matter for debate. Antihormones may interfere with E_2 at, at least, three distinct steps in the events leading to transcriptional activation. They may interfere with dissociation of the "8S" oligomer complex, or they may inhibit the binding of the receptor to the DNA responsive element, or they may interfere with the process by which steroid receptors transmit their activity to the basic transcriptional machinery [see 29 for review]. All of these possibilities have been proposed for the mechanism of action of ICI, *in vitro*. It has been found to inhibit DNA binding by interfering with receptor dimerization [30, 31], to inhibit the nucleocytoplasmic shuttling of the estrogen receptor [32], and to interfere with a later step required for receptor-mediated gene transcription [33, 34]. A reduction in the ER content has also been proposed [27, 35]: mouse uterus was found to rapidly lose estrogen receptor after ICI treatment [27]. We measured the ER content to see if we could detect any reduction in the ER after ICI treatment. The enzyme immunoassay detected no such reduction, but there may have been a rapid loss of ER before 6h. Why there should be this discrepancy between our data and those for the mouse uterus is unclear, since the doses of ICI were similar. But the methods used to assess ER level in these studies were different: one measured nuclear and cytosolic ER by exchange assay [27], whereas the other measured total ER by enzyme immunoassay. This may also explain the low level of ER we have observed in ovariectomized rat uterus as compared to previously published data [36–38]. However, it is highly unlikely that ICI acts on the estrogen receptor level in our system since ICI had an antagonist effect on E_2-induced CaBP9k mRNA at 6, 12, 24, 48 and 72h post treatment that was not correlated with any loss of ER. Thus, ICI antagonizes the estrogenic induction of the CaBP9k gene expression, probably by interfering with at least one of the events leading to transcriptional activation.

A primary culture of myometrial cells was used to compare the responses of these cells and the whole uterus. CaBP9k gene expression is under the control of E_2 in these cultures as *in vivo*. These two models showed the same response to antiestrogens. TAM is a full agonist and ICI is a strict antagonist of E_2 induction of the CaBP9k gene in both the intact uterus and in myometrial cells. Estrogenic effects of TAM have usually been reported in endometrial stroma and epithelium [11, 39] but more rarely in myometrium [40]. Our study indicates that TAM can have estrogenic effects in myometrium. Thus, our results support the view that the use of TAM in breast cancer therapy or prevention must be undertaken under strict control and that pure antiestrogens could represent an alternative to avoid the estrogenic effects of TAM in the uterus and particularly in the myometrium.

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